TESIS DOCTORAL

(COMPENDIO DE PUBLICACIONES)

“TRATAMIENTO INTEGRAL DE LOS RESIDUOS SÓLIDOS GENERADOS EN EL PROCESO DE EXTRACCIÓN DE LA HARINA DE GIRASOL MEDIANTE LA COMBINACIÓN DE DISTINTOS PRETRATAMIENTOS Y PROCESOS DE DIGESTIÓN ANAEROBIA”

Tesis Doctoral

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Sevilla, 2013

Este trabajo ha podido llevarse a cabo gracias a la financiación del proyecto CTM- 2008-05772/ Tecno del Plan Nacional de I+D+i y al Ministerio de Ciencia e Innovación (MICINN) por proporcionar financiación para el Programa de Formación de Personal Investigador FPI.

Esta Tesis Doctoral ha sido elaborada en la modalidad de "compendio de publicaciones" de acuerdo con la Normativa reguladora del régimen de tesis doctoral Acuerdo 9.1/CG 19-4-12 de la Universidad de Sevilla (BOUS núm. 3, de 23 de mayo de 2012).

Las publicaciones científicas que constituyen fundamentalmente esta Tesis Doctoral son las siguientes:

I. Influence of particle size and chemical composition on the performance and kinetics of anaerobic digestion process of sunflower oil cake in batch mode

De la Rubia, M.A., Fernández-Cegrí, V., Raposo, F., Borja, R.

(2011) Biochemical Engineering Journal, 58-59 (1), pp. 162-167.

II. Effect of hydrothermal pretreatment of sunflower oil cake on biomethane potential focusing on fibre composition

Fernández-Cegrí, V., Ángeles de la Rubia, M., Raposo, F., Borja, R.

(2012) Bioresource Technology, 123, pp. 424-429.

III. Impact of ultrasonic pretreatment under different operational conditions on the mesophilic anaerobic digestion of sunflower oil cake in batch mode

Fernández-Cegrí, V., De La Rubia, M.A., Raposo, F., Borja, R.

(2012) Ultrasonics Sonochemistry, 19 (5), pp. 1003-1010.

IV. Effects of chemical and thermochemical pretreatments on sunflower oil cake in biochemical methane potential assays

Fernández-Cegrí, V., Raposo, F., de la Rubia, M.A., Borja, R.

(2013) Journal of Chemical Technology and Biotechnology. Article in Press.

A continuación se detalla otra relación de referencias de publicaciones correspondientes al resto de artículos relacionados con el la temática de la tesis.

V. Quality improvement in determination of chemical oxygen demand in samples considered difficult to analyze, through participation in proficiency-testing schemes

Raposo, F., Fernández-Cegrí, V., De la Rubia, M.A., Borja, R., Beltrán, J., Cavinato, C., Clinckspoor, M., Demirer, G., Diamadopoulos, E., Frigon, J.C., Koubova, J., Launay, M., Méndez, R., Menin, G., Noguerol, J., Uellehdahl, H., West, S.

(2010) TrAC - Trends in Analytical Chemistry, 29 (9), pp. 1082-1091.

VI. Biochemical methane potential (BMP) of solid organic substrates: Evaluation of anaerobic biodegradability using data from an international interlaboratory study

Raposo, F., Fernández-Cegrí, V., de la Rubia, M.A., Borja, R., Béline, F., Cavinato, C., Demirer, G., Fernández, B., Fernández-Polanco, M., Frigon, J.C., Ganesh, R., Kaparaju, P., Koubova, J., Méndez, R., Menin, G., Peene, A., Scherer, P., Torrijos, M., Uellendahl, H., Wierinck, I., de Wilde, V.

(2011) Journal of Chemical Technology and Biotechnology, 86 (8), pp. 1088-1098.

VII. Feasibility of sunflower oil cake degradation with three different anaerobic consortia

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(2011) Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering, 46 (12), pp. 1409-1416.

VIII. Anaerobic digestion of solid organic substrates in batch mode: An overview relating to methane yields and experimental procedures

Raposo, F., De La Rubia, M.A., Fernández-Cegrí, V., Borja, R.

(2012) Renewable and Sustainable Energy Reviews, 16 (1), pp. 861-877.

IX. Anaerobic digestion of sunflower oil cake: a current overview

Ángeles de la Rubia, M., Fernández-Cegrí, V., Raposo, F., Borja, R.

(2013) Water Science & Technology, 62, 2. pp. 410-417.

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CAPÍTULO 1: INTRODUCCIÓN Y OBJETIVOS

1.1 INTRODUCCIÓN

El objetivo de este capítulo introductorio es describir las características y problemática de los residuos sólidos generados en el proceso de extracción de la harina de girasol, así como describir el proceso de digestión anaerobia como sistema para el aprovechamiento y tratamiento de estos residuos. Actualmente la digestión anaerobia de este tipo de sustratos se presenta como una alternativa eficaz para la conversión de subproductos o deshechos en energía limpia reutilizable. Finalmente también se especifican los objetivos de esta tesis.

1.1.1 Los residuos sólidos generados en el proceso de extracción de la harina de girasol

El girasol pertenece a la familia de las Compositae (*Asteraceas*) y al género *Helianthus*. Los científicos, en base a los restos hallados, estiman que la antigüedad de la planta de girasol se remonta a 3.000 años antes de la era cristiana.

El cultivo del girasol (*Helianthus annus L.)* tiene un alto rendimiento en semilla y la planta se adapta bien a una amplia gama de condiciones de clima y suelo. El rendimiento industrial depende de varios factores pero puede estimarse que de una tonelada de semilla con 50% de materia grasa se obtienen alrededor de 420 kg de aceite. [1]

El mercado de girasol fue el mayoritario en cuanto a producción durante la campaña 2011/12, aportando más de 5 millones de toneladas a la oferta mundial de oleaginosas. La producción mundial del aceite de girasol de las últimas tres campañas se puede observar en la siguiente tabla:

Tabla 1.1.- Producción mundial del girasol de las últimas tres campañas de recolección.

Producción de Girasol			
(mill.tn)	2011/12	2010/11	2009/2010
EU-27	8,25	7,01	7,00
Rusia	9,30	5,72	6,60
Ucrania	9,20	8,00	7,30
Argentina	3,60	3,67	2,65
China	1,70	1,71	1,96
Total Mundial	38,88	35,57	33,21

Fuente: Oil World

Este incremento en la cosecha mundial de semillas de girasol, sumado a la menor oferta de otras oleaginosas alternativas (como la soja y la colza) llevan a una mayor concentración de la demanda sobre el girasol. [2]

Los tres productos que se obtienen del proceso de obtención del aceite de girasol son los siguientes:

• ACEITE CRUDO: Es el aceite obtenido por prensado y extracción por solvente de la materia grasa contenida en la semilla.

• ACEITE REFINADO: Es el aceite que se ha sometido a procesos químicos y/o físicos de refinación para dotarlo de sabor, aroma y color adecuados para su consumo.

• HARINAS PROTEINICAS: Es la parte de la semilla que queda después de extraerle el aceite. Este producto está compuesto principalmente por proteínas, materia grasa, fibras, minerales y celulosa.

Los subproductos obtenidos del procesamiento son:

• BORRAS DE NEUTRALIZACIÓN: Provienen de la etapa de neutralización de la acidez libre del aceite crudo y están constituidas principalmente por jabones, aceite neutro y agua. Se venden tal cual o se adicionan como material graso a los pellets. También pueden destinarse a la producción de oleína o ácidos grasos.

• OLEINA: Es la materia grasa proveniente de la borra. Es sinónimo de Aceite Acido cuando su acidez alcanza el 50%.

• DESTILADOS DE DESODORIZACION: Es el material recuperado de los desodorizadores por condensación de las sustancias que se arrastran por arrastre de vapor. De allí se obtienen Tocoferoles y Esteroles, compuestos químicos muy valiosos en la industria farmacéutica y alimenticia.

• **TORTA O CÁSCARA**: Es la parte externa o pericarpio de la semilla. Se destina a calderas como combustible en la misma fábrica que las produce. También se la utiliza en camas de pollos. No se puede utilizar para alimentación directa por su alto contenido en lignina y sílice.

El nivel bajo de energía metabolizable de la torta y la poca capacidad que tienen los animales no rumiantes de utilizarla, son factores directamente relacionados con su alto contenido de fibra [3].

Por tanto, una vez obtenido el aceite de girasol, que posteriormente se refina para el consumo humano, se obtienen elevadas cantidades de harina desengrasada que contribuye el residuo mayoritario de este proceso.

La harina de girasol desengrasada presenta como componentes principales [4]: fibra (25,1%), proteína (31,2%), humedad (9,7%), cenizas (4,9%), lípidos (6,1%), polifenoles (2,1%), azúcares solubles (3.8%) y azúcares insolubles (8,0%).

Esta harina residual se ha utilizado en algunas ocasiones para la alimentación del ganado. Adicionalmente, se ha utilizado para la obtención de concentrados y aislados proteicos [5-7]. Aunque los aislados proteicos se pueden utilizar como suplemento en productos cárnicos, fórmulas infantiles, bebidas, etc. presentan dos grandes limitaciones para su aplicación en la industria alimentaria como son su baja solubilidad y su alergenicidad [8, 9].

Entre otras alternativas recientemente estudiadas para el aprovechamiento de la harina de girasol desengrasada destaca su utilización como sustrato en procesos de fermentación en estado sólido para obtener algunos productos de interés farmacéutico (Cefamicina y ácido clavulánico) y enzimas (proteasas) utilizando en este caso determinados microorganismos fúngicos, tales como *Aspergillus sp* y *Penicillium sp* [10, 11]. Aun así, las cantidades utilizadas en estas aplicaciones no resuelven el problema generado por la elevada cantidad de harina disponible.

Como puede observarse, las aplicaciones que se han sugerido para el aprovechamiento de los residuos sólidos que se generan en el proceso de obtención del aceite de girasol son muy limitadas y ninguna de ellas se ha llevado a cabo a escala industrial por lo que se justifica el estudio de otras alternativas para su reutilización y tratamiento.

Resultados obtenidos en trabajos de investigación previos han puesto de manifiesto la difícil degradabilidad anaerobia de este sustrato. Otros residuos sólidos con alto contenido en sustancias lignocelulosicas, demostraron ser difícilmente biodegradables vía anaerobia disminuyendo sensiblemente la actividad metanogénica específica de la comunidad microbiana responsable del proceso [12, 13]. Otros estudios también han demostrado que en la degradación anaerobia de residuos que presentan un elevado contenido en compuestos lignocelulosicos, tales como residuos de la fabricación de papel, bagazo y fibra de coco, e incluso purines de cerdo o residuos de ganado vacuno con un alto contenido en sólidos, la hidrólisis de la celulosa es claramente la etapa limitante de la velocidad del proceso [14-17]. La baja velocidad de hidrólisis de la celulosa debido a su estructura cristalina, la asociación de la celulosa y hemicelulosa con la lignina y la pequeña actividad de las celulasas presentes en digestores convencionales son los principales factores que influyen en la lenta degradación anaerobia de estos residuos. Estos factores determinan la necesidad de utilizar elevados tiempos de retención hidráulicos (TRH) (25-30 días) y son los responsables de la baja producción de biogás y pequeños coeficientes de rendimiento en metano observados en la digestión anaerobia de algunos subproductos y deshechos agro-industriales tales como residuos de maíz, paja de trigo, etc. y en los lodos previamente deshidratados procedentes de estaciones depuradoras de aguas residuales (EDAR) o biosólidos [18, 19].

Estudios recientes han puesto de manifiesto que determinados pretratamientos de tipo térmico, químico, termoquímico o mecánico incrementan la biodegradabilidad anaerobia, el porcentaje de sólidos eliminados y, en definitiva, la producción de biogás y metano en el proceso de digestión anaerobia de lodos deshidratados de depuradora y residuos sólidos de depuradoras convencionales que tratan efluentes

generados en la fabricación de papel, caracterizados por su elevado contenido en compuestos lignocelulósicos [12, 20-24].

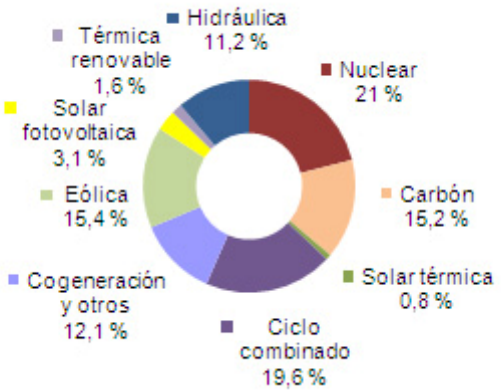
Por todo ello, teniendo en cuenta la difícil biodegradabilidad anaerobia de los residuos sólidos que se generan en el proceso de extracción del aceite de la harina de girasol, se plantea la necesidad de aplicar un tratamiento integral del mismo, mediante la combinación de determinados pretratamientos (químicos, térmicos, termoquímicos, mecánico y ultrasonidos) y procesos de digestión anaerobia (batch y semicontinuo), con vistas a la valorización energética de este residuo.

1.1.2 Aprovechamiento energético de los residuos agroindustriales

La necesidad de energía es una constatación desde el comienzo de la vida misma. Un organismo para crecer y reproducirse precisa energía, el movimiento de cualquier animal supone un gasto energético, e incluso el mismo hecho de la respiración de plantas y animales implica una acción energética. En todo lo relacionado con la vida individual o social está presente la energía.

El ser humano desde sus primeros pasos en la tierra, y a lo largo de la historia, ha sido un buscador de formas de generación de esa energía necesaria y facilitadora de una vida más confortable. Gracias al uso y conocimiento de las formas de energía ha sido capaz de cubrir necesidades básicas: luz, calor, movimiento, fuerza, y alcanzar mayores cotas de confort.

Hoy en día, la energía nuclear, la energía procedente de combustibles fósiles, la energía procedente de ciclo combinado y la energía hidráulica, satisfacen la demanda energética mundial. Datos actuales revelan que el 51,8% de la producción eléctrica de España procede de tecnologías que no emiten CO_2. (Figura 1).



Fuente: http://actualidadenergia.wordpress.com

Figura 1.1.- Producción Energética en España.

La utilización de estos recursos naturales implica, además de su cercano y progresivo agotamiento, un constante deterioro para el medio ambiente, que se manifiesta en emisiones de CO_2, NO_x, y SO_x, con el agravamiento del efecto invernadero, contaminación radioactiva y su riesgo potencial incalculable, un aumento progresivo de la desertización y la erosión y una modificación de los mayores ecosistemas mundiales con la consecuente desaparición de biodiversidad, la inmigración forzada y la generación de núcleos poblacionales aislados tendentes a la desaparición.

Estas agresiones van acompañadas de grandes obras de considerable impacto ambiental (difícilmente cuantificable) como las centrales hidroeléctricas, el sobrecalentamiento de agua en costas y ríos generado por las centrales nucleares, la creación de depósitos de elementos radiactivos, y de una gran emisión de pequeñas partículas volátiles que provocan la lluvia ácida, agravando aún más la situación del entorno: parajes naturales defoliados, ciudades con altos índices de contaminación, afecciones de salud en personas y animales, desaparición de especies animales y vegetales que no pueden seguir la aceleración de la nueva exigencia de adaptación, etc.

El futuro amenazador para nuestro entorno, aún se complica más si se tiene en cuenta que sólo un 25% de la población mundial consume el 75% de la producción energética. Este dato, además de poner de manifiesto la injusticia y desequilibrio social existente en el mundo, indica el riesgo que se está adquiriendo al exportar un modelo agotado y fracasado de países desarrollados a países en vía de desarrollo [25].

De igual modo, el cuestionamiento del modelo de desarrollo sostenido y su cambio hacia un modelo de desarrollo sostenible, implica una nueva concepción sobre la producción, el transporte y el consumo de energía.

En este modelo de desarrollo sostenible, las energías de origen renovable, son consideradas como fuentes de energía inagotables, que cuentan con la peculiaridad de ser energías limpias, definidas por las siguientes características: sus sistemas de aprovechamiento energético suponen un nulo o escaso impacto ambiental, su utilización no tiene riesgos potenciales añadidos, indirectamente suponen un enriquecimiento de los recursos naturales, la cercanía de los centros de producción energética a los lugares de consumo puede ser viable en muchas de ellas, y son una alternativa a las fuentes de energía convencionales, pudiendo generarse un proceso de sustitución paulatina de las mismas.

Por otro lado, uno de los grandes problemas que debe afrontar el mundo civilizado es el destino final de los residuos generados. En efecto, su gestión, tanto a nivel local como a nivel mundial, es vital para la preservación del hábitat humano, del medio ambiente y de la salud de la población.

La energía de la biomasa es la energía contenida en la materia orgánica y tiene diversas formas de aprovechamiento, según se trate de materia de origen animal o vegetal. Sólo en materia vegetal, se estima que se producen anualmente doscientos millones de toneladas. El principal aprovechamiento energético

de la biomasa es la combustión de la madera, que genera contaminación atmosférica y un problema indirecto de desertización y erosión, salvo que se realice una planificación forestal correcta. Los residuos o desechos orgánicos también son utilizables mediante transformaciones químicas principalmente, siendo las más conocidas las aplicaciones de digestores anaerobios para residuos orgánicos y la producción de biogás procedente de la fracción sólida de los residuos urbanos. Para el tratamiento de residuos orgánicos existe la posibilidad de implantación de sistemas de digestión anaerobia, obteniéndose productos valorizables como el biogás y fertilizantes orgánicos.

Sin embargo, la creciente innovación tecnológica de materiales y equipos está afianzando nuevos sistemas de aprovechamiento de los residuos ganaderos y forestales, y consolida un esperanzador futuro en la línea de los biocombustibles, de modo que se pueda compatibilizar una agricultura sostenible con un diseño de producción energética que respete el entorno [26].

En la actualidad la Unión Europea está fomentando la obtención y utilización de energías renovables a partir de determinados residuos agrícolas e industriales con el objetivo de alcanzar para el año 2020 un 20% del total de la energía producida a partir de estas nuevas fuentes. La gestión de los restos vegetales originados en las diversas actividades agroalimentarias ha estado inicialmente encaminada a su utilización como alimento animal bien sea de forma directa o como materia prima para la elaboración de piensos, confiriéndole de esta manera cierta revalorización, por ello estos restos vegetales están considerados como subproductos y no como residuos. Sin embargo, se pretende reconsiderar esta actuación ya que el potencial de algunos de los subproductos a los que nos estamos refiriendo es muy superior a su utilización en alimentación animal, por lo que se estarían desaprovechando oportunidades para una gestión más interesante y racional de estos residuos.

El objetivo primordial, antes de realizar acción alguna referente a la gestión de residuos y subproductos es fundamentalmente la determinación de la estimación de sus volúmenes, composición, modalidades de generación dentro de la cadena de producción agroalimentaria, distribución geográfica, etc. así como realizar un estudio prospectivo de determinación de oportunidades tecnológicas, que sustente la adopción de acciones adecuadas a cada uno de los tipos de residuo bajo principios como los de gestión integrada, tratamiento de proximidad tanto en el espacio como en el tiempo en el que son generados, valorización al máximo potencial posible y bajo el principio de jerarquía de forma que obtenga preferencia la opción de mayor calidad ambiental y de mayor valor añadido frente a otras destinadas a la simple eliminación [27].

1.1.3 La digestión anaerobia como alternativa para la valorización de residuos

La fermentación es uno de los mecanismos de degradación de biomasa más frecuentes en la Naturaleza, por el que las moléculas orgánicas complejas son descompuestas en sus componentes energéticos individuales de forma espontánea por medio de microorganismos. Cuando la fermentación transcurre en condiciones rigurosas de ausencia de oxígeno (medio anaerobio), da lugar a una mezcla de productos gaseosos (principalmente metano y dióxido de carbono), conocida como biogás y a una suspensión acuosa de materiales sólidos (lodos o fangos) en la que se encuentran los componentes difíciles de degradar, junto con el nitrógeno, el fósforo y los elementos minerales inicialmente presentes en la biomasa. Este proceso es el que se denomina digestión anaerobia.

La digestión anaerobia es un proceso biológico en el que la materia orgánica, se degrada hasta una mezcla de productos gaseosos o "biogás" (CH_4, CO_2, H_2, H_2S, etc.), y en digestato, que es una mezcla de productos minerales (N, P, K, Ca, etc.) y compuestos de difícil degradación. El biogás contiene un alto porcentaje en metano, CH_4 (entre 50-70%), por lo que es susceptible de un aprovechamiento energético mediante su combustión en motores, en turbinas o en calderas, bien sólo o mezclado con otro combustible.

El proceso controlado de digestión anaerobia es uno de los más idóneos para la reducción de emisiones de efecto invernadero, el aprovechamiento energético de los residuos orgánicos y el mantenimiento y mejora del valor fertilizante de los productos tratados.

La digestión anaerobia puede aplicarse, entre otros, a residuos ganaderos, agrícolas, así como a los residuos de las industrias de transformación de dichos productos. Entre los residuos tratados mediante este proceso se pueden citar purines, estiércol, residuos agrícolas o excedentes de cosechas, fracción sólida de los residuos sólidos urbanos (FORSU), etc. Estos residuos se pueden tratar de forma independiente o conjunta, mediante procesos de co-digestión. La digestión anaerobia también es un proceso adecuado para el tratamiento de aguas residuales de alta carga orgánica, como las generadas en muchas industrias alimentarias.

Los beneficios asociados a la digestión anaerobia son:

- Reducción significativa de malos olores,
- Mineralización (degradación completa de un compuesto a sus constituyentes minerales, en donde el carbono orgánico es oxidado hasta CO_2),
- Producción de energía renovable, si el gas se aprovecha energéticamente y sustituye a una fuente de energía fósil,

- Reducción de emisiones de gases de efecto invernadero derivadas de la reducción de emisiones incontroladas de CH_4, (que produce un efecto invernadero 20 veces superior al CO_2), y reducción del CO_2 ahorrado por sustitución de energía fósil,
- Escasa formación de lodos,
- Escasos requerimientos nutricionales (N y P) de los microorganismos implicados en el proceso.

La promoción e implantación de sistemas de producción de biogás colectivos (varias granjas), y de co-digestión (tratamiento conjunto de residuos orgánicos de diferentes orígenes en una zona geográfica, usualmente agropecuarios e industriales) permite, además, la implantación de sistemas de gestión integral de residuos orgánicos por zonas geográficas, con beneficios sociales, económicos y ambientales.

1.1.3.1 Características del biogás

El biogás es el producto gaseoso de la digestión anaerobia de compuestos orgánicos. Su composición, que depende del sustrato tratado y del tipo de tecnología utilizada, suele ser la siguiente:

50-70% de metano (CH_4).

30-40% de anhídrido carbónico (CO_2).

$\leq$ 5% de hidrógeno (H_2), ácido sulfhídrico (H_2S), y otros gases minoritarios.

Debido a su alto contenido en metano, tiene un poder calorífico algo mayor que la mitad del poder calorífico del gas natural. Un biogás con un contenido en metano del 60% tiene un poder calorífico de unas 5.500 kcal/Nm^3 (6,4 kWh/Nm^3).

1.1.3.2 Fases de la digestión anaerobia

La digestión anaerobia está caracterizada por la existencia de varias fases consecutivas diferenciadas en el proceso de degradación del sustrato (término genérico para designar, en general, el alimento de los microorganismos), interviniendo 5 grandes poblaciones de microorganismos (Figura 3). Estas poblaciones se caracterizan por estar compuestas por microorganismos de diferentes velocidades de crecimiento y diferente sensibilidad a cada compuesto intermedio como inhibidor (por ejemplo, H_2, ácido acético o amoníaco producido de la acidogénesis de aminoácidos). Esto implica que cada etapa presentará diferentes velocidades de reacción según la composición del sustrato y que el desarrollo estable del proceso global requerirá de un equilibrio que evite la acumulación de compuestos intermedios inhibidores o la acumulación de ácidos grasos volátiles (AGV), que podría producir una bajada del pH. Para la estabilidad del pH es importante el equilibrio CO_2-bicarbonato. Para hacer posible algunas reacciones es

necesaria la asociación sintrófica entre bacterias acetogénicas y arqueas metanogénicas, creando agregados de microorganismos de estas diferentes poblaciones [28].

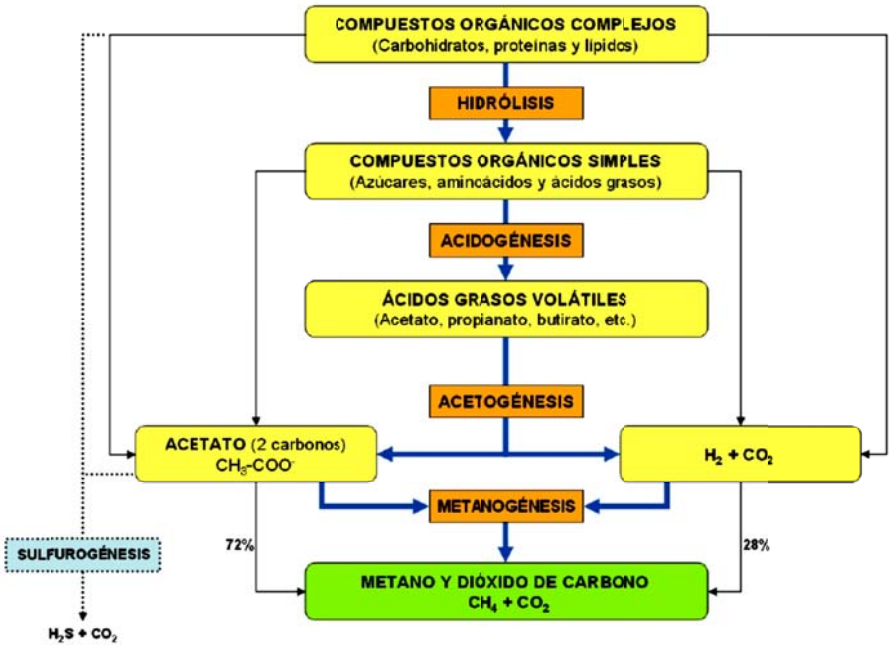


Figura 1.2.- Fases de la digestión anaerobia

La hidrólisis o licuefacción es una primera etapa y consiste en la degradación de los polímeros orgánicos complejos constituyentes de la biomasa o residuo, dando lugar a moléculas más simples.

Durante la segunda etapa, etapa acidogénica, las bacterias acidogénicas producen varios compuestos simples, que son los productos finales de su metabolismo anaerobio. En la siguiente etapa los compuestos orgánicos de bajo peso molecular y fundamentalmente los ácidos grasos volátiles (AGV) generados por las bacterias acidogénicas, son transformados en acetato, CO_2 e H_2. Finalmente en la cuarta etapa o etapa metanogénica el acetato o bien el CO_2 e H_2 producido en la etapa anterior son degradados a metano y dióxido de carbono por un grupo de microorganismos altamente especializados, denominadas metanogénicos.

En general, la velocidad del proceso está limitada por la velocidad de la etapa más lenta, la cual depende de la composición de cada residuo. Para sustratos solubles, la fase limitante acostumbra a ser la metanogénica, y para aumentar la velocidad la estrategia consiste en adoptar diseños que permitan una elevada concentración de microorganismos acetogénicos y metanogénicos en el reactor. Con esto se pueden conseguir sistemas con tiempos de retención hidráulicos del orden de días. Para residuos en los que la materia orgánica esté en forma de partículas o insolubles la hidrólisis depende entre otros factores

de la superficie de las partículas. Esta limitación hace que los tiempos de retención sean del orden de semanas, de dos a tres. Para aumentar la velocidad, una de las estrategias es el pretratamiento para disminuir el tamaño de partículas o ayudar a la solubilización (maceración, ultrasonidos, tratamiento térmico, alta presión, o combinación de altas presiones y temperaturas).

Del proceso de digestión anaerobia se obtiene además un lodo muy estable que sometido a un sencillo proceso de compostaje permitiría reciclar, no solo materia orgánica a suelos pobres en ella, sino elementos minerales contribuyendo así a una agricultura sostenible.

1.1.3.3 Variables que afectan al proceso de digestión

Las condiciones óptimas y los rangos de oscilación de las variables que afectan a la digestión anaerobia han sido estudiadas por muchos investigadores que, desgraciadamente, no se ponen de acuerdo en todos los puntos. Una razón para ello puede ser que sus estudios se han desarrollado utilizando diferentes materias primas como sustrato así como diversas metodologías y configuraciones de reactor.

La naturaleza y composición del sustrato de partida dicta el régimen del proceso pero, así y todo, existe un grupo de variables que influye ostensiblemente sobre el sistema, por lo que es necesaria su medida y control, con objeto de intentar que se produzca la digestión en las mejores condiciones posibles. Estas variables son las siguientes:

1.1.3.3.1 Temperatura

La digestión anaerobia puede llevarse a cabo en un amplio rango de temperatura (5 - 65 ºC), dentro del cual aparecen dos zonas claramente definidas, correspondientes a dos grupos diferentes de bacterias: las bacterias mesofílicas, que se desarrollan entre los 25 y los 40 ºC, y las bacterias termofílicas, que lo hacen en un rango de 40 a 65 ºC.

En general, se opera en el rango mesofílico. Como sucede en la mayoría de los procesos biológicos, la velocidad de producción de metano se duplica aproximadamente cada 15 ºC, encontrándose un óptimo de funcionamiento alrededor de los 35 ºC. Esta temperatura combina las mejores condiciones de crecimiento de las bacterias con la mayor velocidad de producción de metano.

1.1.3.3.2 pH.

El mantenimiento de un equilibrio ácido-base (valor del pH) adecuado en el transcurso de una digestión, es uno de los principales problemas que tiene el proceso, debido a la acusada influencia que tiene la acidez del medio sobre la producción de gas, habiéndose encontrado que el rango óptimo de pH es de 6,8 a 7,6. El valor del pH no sólo determina la producción total de biogás sino, lo que es más importante, su composición en metano, ya que por debajo de un pH de 6,2, la acidez existente en el digestor inhibe fuertemente la actividad de los microorganismos metanogénicos y por debajo de un pH de

4,5, la inhibición afecta también a las bacterias acidogénicas. Efectos similares se detectan a valores del pH por encima de 8,5.

1.1.3.3.3 Contenido en sólidos

El contenido en sólidos del sustrato a digerir es un factor que influye de manera considerable en el proceso anaerobio. Si la alimentación está muy diluida, los microorganismos no tienen alimento suficiente para sobrevivir. Por el contrario, una alimentación muy concentrada reduce la movilidad de las bacterias y, por tanto, la efectividad del proceso, al dificultar el acceso de aquéllas a su fuente de alimentación.

1.1.3.3.4 Nutrientes

Una célula microbiana contiene una relación de nutrientes C: N: P: S de aproximadamente 100:10:1:1. Por ello, y para que se produzca el crecimiento y la actividad microbiana, estos elementos han de estar presentes y disponibles en el medio y su ausencia o escasez pueden, de hecho, reducir la velocidad del proceso de digestión anaerobia.

1.1.3.3.5 Tóxicos

En cuanto a los posibles tóxicos, dado que la digestión anaerobia tiene etapas llevadas a cabo por microorganismos estrictamente anaerobios, la primera sustancia tóxica a citar es el oxígeno. Concentraciones elevadas de amoníaco, producidas por un exceso de nitrógeno en el sustrato a digerir también inhiben la digestión. Aunque se ha citado la necesidad en el medio de sales minerales, excesos de sales también pueden inhibir el proceso. También pueden ser tóxicas para los microorganismos diversas sustancias orgánicas. Se trata de algunos disolventes (alcoholes y ácidos de cadena larga en elevadas concentraciones), compuestos fenólicos, los pesticidas y los detergentes [29].

El proceso de digestión anaerobia para el tratamiento de residuos complejos necesita estudiarse con mayor profundidad. Ello obliga a estudiar cada residuo o materia prima individualmente, con objeto de realizar el mejor diseño posible para cada sustrato y así poder obtener máximos rendimientos energéticos y elevados niveles de degradación de materia orgánica.

1.1.4 Problemática de la digestión anaerobia de los residuos lignocelulósicos

La biodegradabilidad, entendida como la capacidad de degradar materia orgánica por la acción de agentes biológicos está relacionada principalmente con su composición química traducida como disponibilidad de carbohidratos simples (C-H-O). En la biodegradabilidad es importante tener en cuenta

el contenido de lignina (constituyente de las paredes celulares de las células fibrosas) el cuál determina la fracción biodegradable de los residuos.

Los materiales lignocelulósicos son aquellos que están formados por fibras de celulosa y hemicelulosa enlazadas mediante lignina, un polímero aromático oxigenado con un esqueleto de fenilpropano que se repite.

La celulosa es el componente más simple encontrado en el material lignocelulósico de las plantas. Está compuesto por un polímero de residuos de D- glucosa unidos por enlaces β-1,4. Las cadenas de celulosa se unen por puentes de hidrógeno intermoleculares formando agregados (microfibrillas).

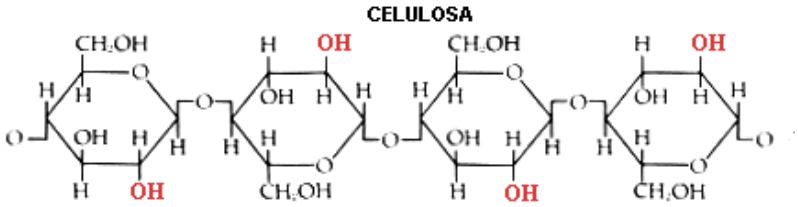


Figura 1.3.- Esquema de celulosa [30].

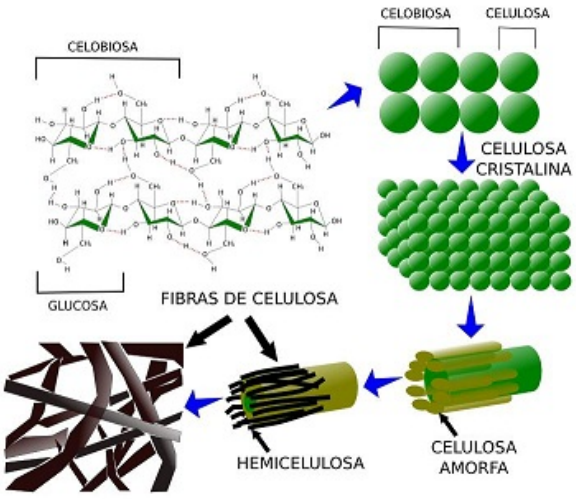


Figura 1.4.- Esquema de desglose de material lignocelulósico [31].

La celulosa puede ser hidrolizada para formar glucosa pero las cadenas de glucosa están dispuestas de una manera que permiten que se empaquen juntas formando un cristal que es impermeable al agua, consecuentemente el polímero de celulosa es insoluble y resistente a la hidrólisis.

Las hemicelulosas forman cadenas cortas y son polímeros heterogéneos que contienen tanto hexosas (azúcares de 6 carbonos como glucosa, manosa y galactosa) como pentosas (azúcares de 5

carbonos como xilosa y arabinosa). Los principales productos de la hidrólisis de la celulosa son celobiasa y glucosa, mientras que la hemicelulosa produce pentosas, hexosas y ácidos urónicos.

La lignina es un polímero complejo, tridimensional, globular, irregular, insoluble, y de alto peso molecular (>10000), formado por unidades de fenilpropano cuyos enlaces son relativamente fáciles de hidrolizar por vía química o enzimática. Esta molécula tiene diferentes tipos de uniones entre unidades aromáticas de fenilpropano. La lignina no contiene azúcares, pero encierra a la celulosa y hemicelulosa que si los contienen haciendo difícil alcanzar a estas últimas para hidrolizar sus azúcares [Figura 1.5].

En las plantas, la lignina se encuentra químicamente unida a la hemicelulosa y rodeando a las fibras compuestas por celulosa. Es responsable de la rigidez de las plantas y de sus mecanismos de resistencia al estrés. La función de la lignina es proveer un soporte estructural para la planta [32].

Este heteropolímero amorfo no es soluble en agua y ópticamente inactivo; todo esto hace que la degradación de la lignina sea muy complicada [33].

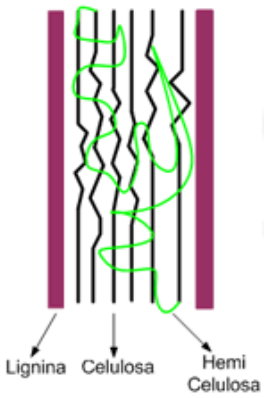


Figura 1.5.- Esquema de estructura de pared celular [32].

La deslignificación es la meta más importante para la producción de energía a partir de los materiales lignocelulósicos [34].

La lignina, por tanto, al ser un material altamente refractario a la degradación anaerobia, afectando también a la biodegradabilidad de la celulosa, de la hemicelulosa y de otros polímeros, hace que su degradación sea el proceso limitante de la velocidad de la hidrólisis y de la degradación anaerobia de determinados sustratos que contienen ese compuesto.

1.1.5 Pretratamientos para mejorar la digestibilidad anaerobia de residuos lignocelulósicos

En el caso de la utilización de materiales lignocelulosicos para la obtención de gas metano, la degradación microbiológica de la celulosa contenida en la misma no se puede conseguir de forma directa, tal como se hace en los residuos domésticos, debido a que la lignina no es directamente atacable por los microorganismos.

Trabajos previos han puesto de manifiesto que un pretratamiento resulta indispensable para conseguir una degradación apreciable en el ataque al sustrato por los microorganismos anaeróbicos. Los resultados obtenidos hasta ahora con residuos agrícolas indican que la producción de metano es baja si no se realiza un pretratamiento adecuado debido, en muchos casos, al alto contenido en lignina de los residuos utilizados (paja, tallos, etc.). Todo indica que utilizando sustratos con un menor contenido en lignina, el rendimiento aumenta considerablemente, pero estos estudios aún no están lo suficientemente desarrollados [35].

El propósito del pretratamiento es romper la mayor parte de la estructura de la lignina o disociar el complejo celulosa- lignina, reducir la cristalinidad de la celulosa y aumentar la porosidad del material. En general, los pretratamientos facilitan la liberación del carbono de la materia orgánica contenida en el sustrato, aumentan la superficie específica de la materia y solubilizan y degradan la mezcla.

En una materia prima no tratada, las fibras de celulosa, con una alta cristalinidad, se encuentran dentro de una no muy bien organizada matriz de hemicelulosa y envuelta en una pared de lignina que le da la rigidez al material lignocelulósico.

Los objetivos fundamentales del pretratamiento van encaminados a reducir el estado cristalino de la celulosa, disociar el complejo celulosa- lignina, aumentar el área superficial del material y disminuir la presencia de aquellas sustancias que dificulten la hidrolisis.

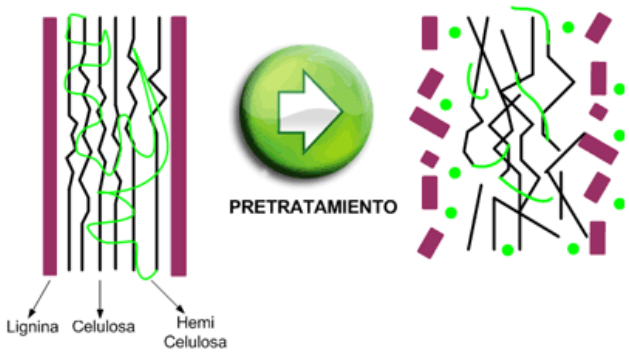


Figura 1.6.- Efecto del pretratamiento sobre la estructura de pared celular [32].

Un pretratamiento debe tener las siguientes características:

(1) Mejorar la digestibilidad de la celulosa y la hemicelulosa en la posterior hidrólisis enzimática o degradación biológica.

(2) Evitar la degradación o pérdida de carbohidratos.

(3) Evitar la formación de subproductos inhibidores para los procesos posteriores, hidrólisis enzimática y fermentación de azúcares.

Teniendo en cuenta los puntos anteriores, la literatura reporta cuatro tipos principales de pretratamientos: mecánicos, térmicos, químicos y biológicos.

1. Pretratamientos mecánicos: Con esta tecnología se trata principalmente de reducir el tamaño de partícula, aumentando así la superficie específica del material, de manera que se consiga eventualmente una mayor solubilización de la materia orgánica y una mayor biodisponibilidad de la misma. Dentro de estos los más destacados son:

a. Trituración mecánica: Molienda para reducción del tamaño de partícula a nivel de malla, tiene un efecto mínimo en los rendimientos de la hidrólisis, así como la tasa de hidrólisis de la biomasa [36].

b. Ultrasonidos: Es una técnica empleada para degradar lignina y hemicelulosa. Consiste en la aplicación de presión de sonido cíclico (ultrasonido) con una frecuencia variable para desintegrar paredes celulares. La química de la sonicación como una herramienta de tratamiento previo es bastante complejo y se compone de una combinación de cizallamiento dando lugar a las reacciones químicas con radicales libres. Durante el tratamiento con ultrasonidos se forman microburbujas a causa de aplicaciones de alta presión al material líquido, que causan colapsos violentos y altas cantidades de energía que se libera en un área pequeña.

2. Pretratamientos térmicos: El objetivo de los pretratamientos térmicos es doble. Por una parte, facilitar la degradación de algunas macromoléculas y solubilizar la materia orgánica (aumento de la biodisponibilidad) y por otra parte, y dependiendo de la temperatura y el tiempo, higienizar la materia orgánica para reducir o eliminar microorganismos indeseables. Existen diversas tecnologías que se diferencian en la forma de aplicar el calor; los pasteurizadores suelen aplicar el calor por conducción (recipientes encamisados por ejemplo), y otros métodos incluyen el uso de corrientes de vapor y/o de altas presiones. En este tipo de pretratamiento la materia prima es calentada en un rango de 100 a 200ºC, donde la hemicelulosa y posteriormente la lignina son solubilizadas [37]. Durante los procesos térmicos una parte de la hemicelulosa es hidrolizada, formándose ácidos, que pueden actuar como catalizadores para

hidrolizar la hemicelulosa [38]. Hay autores que hacen referencia a los problemas que conlleva el uso de esta opción, asociado a los requerimientos excesivos de energía para el calentamiento y enfriamiento del residuo [39]. Entre los pretratamientos térmicos se pueden destacar:

a. Explosión por vapor (Steam Explosion): La materia prima se somete a temperaturas entre 160-260ºC, mediante la inyección directa de vapor saturado, por un intervalo de tiempo entre 1 y 10 minutos. Seguidamente se lleva el producto a una rápida descompresión hasta presión atmosférica. Como resultado se obtiene biomasa con alteraciones físicas (desagregación y ruptura de las fibras), y químicas (despolimerización y rotura de enlaces) y una celulosa más accesible a la hidrólisis enzimática. Las variables a controlar en este tipo de pretratamientos son la temperatura, el tiempo de residencia, el tamaño de partícula, y la humedad [40].

b. Agua líquida a alta temperatura o hidrotermal: En este proceso se somete la biomasa o residuo al efecto de agua caliente a una temperatura entre 100 – 230ºC con tiempos controlados. El objetivo de este pretratamiento es solubilizar o separar principalmente la celulosa de la hemicelulosa para hacerla más accesible y evitar la formación de inhibidores.

3. Pretratamiento químico y termoquímico: Al igual que en el caso de los tratamientos térmicos, el objetivo de los tratamientos químicos es romper las macromoléculas poco biodegradables mediante la adición de compuestos químicos tales como ácidos o bases fuertes, o mediante otros métodos como la ozonización. Los pretratamientos químicos también pueden tener otros objetivos, como el ajuste de pH en el caso de sustratos ácidos, o el aumento de la capacidad tampón. Se puede diferenciar entre:

a. Hidrólisis ácida: Es un proceso químico que emplea ácidos para transformar las cadenas de polisacáridos que forman la biomasa (hemicelulosa y celulosa) en sus monómeros elementales. Este tipo de hidrólisis utiliza diferentes clases de ácidos: clorhídrico, sulfúrico, fosfórico, nítrico y fórmico [41]. Siendo solamente usados a nivel industrial los ácidos clorhídrico y sulfúrico. La principal reacción que ocurre durante el pretratamiento ácido es la hidrólisis de hemicelulosa, generándose especialmente xilano como glucomanano. La hidrólisis con ácido diluido ha sido probada con éxito en el pretratamiento de materiales lignocelulósicos como desechos de maíz, bagazo de caña, madera y astillas de álamo, paja de trigo y pasto. Las variables comúnmente estudiadas son la temperatura, la concentración del ácido y la razón sólido/líquido.

b. Oxidación húmeda: Un pretratamiento oxidativo consiste en la adición de un compuesto oxidante, como el peróxido de hidrógeno o ácido peracético a la biomasa o residuo, que está sumergido en el agua. Durante el pretratamiento oxidativo pueden tener lugar reacciones como sustitución electrofílica, el desplazamiento de cadenas laterales, rompimientos de vínculos de alquil, aril, éter o de núcleos aromáticos.

c. Tratamientos con ozono: El ozono ha sido utilizado para degradar la lignina y la hemicelulosa. Se lleva a cabo en condiciones de presión y temperatura ambiente aunque la celulosa es afectada.

d. Hidrólisis con álcalis: Se lleva a cabo con reactivos como NaOH, $Ca(OH)_2$, Na_2CO_3. El uso de este pretratamiento depende del contenido de lignina en el material. El mecanismo de la hidrólisis alcalina se basa en la saponificación de los enlaces de éster que atraviesan los xilanos en la hemicelulosa y otras componentes como la lignina y otra hemicelulosa. Así, el tratamiento con NaOH diluido aumenta el área superficial y disminuye el grado de polimerización y cristalinidad por la eliminación de los enlaces entre la lignina y los carbohidratos.

e. Tratamiento con solventes orgánicos: En el proceso, un compuesto orgánico acuoso se mezcla con un ácido inorgánico (HCl o H_2SO_4), este se utiliza para romper el interior de la lignina y puentes de hemicelulosa. Se emplean disolventes orgánicos como metanol, etanol, acetona, etilenglicol, trietilenglicol y alcohol tetrahidrofurfurílico. Ácidos orgánicos como oxálico, acetilsalicílico y salicílico también puede ser utilizados como catalizadores en el proceso. A temperaturas altas (por encima de 185°C), el uso de catalizadores es innecesario para la deslignificación [42].

4. Pretratamientos biológicos: Consiguen la degradación de determinados compuestos mediante la inoculación con bacterias específicas o la adición de enzimas. El ensilado se considera también un pretratamiento biológico, ya que se trata de una fermentación acido-láctica, aunque de tipo inespecífico. El objetivo principal del ensilado es la conservación del material, ya que normalmente se aplica a sustratos vegetales que se cosechan una o dos veces al año, aunque en algunos casos se consigue también un aumento de la productividad de biogás, puesto que en el proceso de ensilado se produce una hidrólisis de las macromoléculas [43].

1.2 OBJETIVOS

Esta Tesis tiene como objetivo central el tratamiento integral de los residuos sólidos generados en el proceso de extracción de la harina de girasol mediante la combinación de distintos pretratamientos y procesos de digestión anaerobia.

Por tanto, en este trabajo de investigación se persiguieron los siguientes objetivos concretos:

1. Optimización del pretratamiento mecánico, estudiando la influencia del tamaño de partícula en el nivel de degradación de compuestos lignocelulósicos.

2. Optimización del pretratamiento térmico, estudiando la influencia de la temperatura y tiempo de operación en la solubilización del residuo, para alcanzar una máxima destrucción de compuestos lignocelulósicos.

3. Optimización de los pretratamientos químico y termoquímico, estudiando distintos reactivos de tipo ácido y alcalino bien de forma individual o utilizando distintas temperaturas de trabajo para favorecer la destrucción de polímeros y compuestos complejos de elevado peso molecular.

4. Optimización del pretratamiento con ultrasonidos, estudiando la influencia de la energía específica aplicada en el grado de degradación de materia orgánica.

5. Estudio del potencial bioquímico de metano (BMP test) de cada residuo pretratado de forma individual, determinándose, mediante experimentos en régimen discontinuo, los coeficientes de rendimiento en metano, constantes específicas de velocidad aparente de los distintos sustratos pretratados, fracciones de sustratos no biodegradables, velocidades específicas de producción de metano, así como la evolución de las mismas con la concentración de sustrato para detectar posibles efectos de inhibición, etc. Tras este estudio se seleccionó el pretratamiento más idóneo, aquel que dio lugar a un sustrato más biodegradable, esto es, el que genere una máxima destrucción de compuestos lignocelulósicos, permitiendo un máximo incremento en la DQO soluble y concentración de SV solubles, conjuntamente con un máximo coeficiente de rendimiento en metano.

7. Estudio y optimización del proceso de digestión anaerobia en una etapa del residuo pretratado de forma más eficiente, mediante experimentos en régimen semicontinuo para obtener los tiempos de retención hidráulicos óptimos, así como la VCO más adecuada para obtener una máxima eficiencia de eliminación de materia orgánica, una elevada producción de biogás y alto coeficiente de rendimiento en metano. También se estudiaron algunos parámetros cinéticos del sistema, entre ellos, las velocidades máximas específicas de utilización de sustrato, constantes cinéticas, etc. Para ello se utilizaron inóculos anaerobios de distintos orígenes y características (granular y floculantes) estudiándose su influencia sobre las eficacias y cinética del proceso anaerobio.

Por tanto, el objetivo general de esta Tesis Doctoral se centra en el tratamiento integral de los residuos generados en el proceso de extracción de la harina de girasol mediante la combinación de distintos pretratamientos (térmicos, químicos, termoquímicos, mecánicos o ultrasonidos) para incrementar su biodegradabilidad y procesos de digestión anaerobia. El pretratamiento de estos residuos, dado su elevado contenido en componentes lignocelulósicos, podría permitir hacer viable y optimizar el proceso de digestión anaerobia de los mismos, mediante la utilización de un sistema que transforma la materia orgánica contenida en el residuo en biogás (biocombustible renovable).

Por ello, los objetivos específicos de esta tesis han sido evaluar previamente la eficiencia de los pretratamientos anteriores en relación con su poder para solubilizar y transformar los compuestos complejos de este residuo en otros más simples y más degradables mediante procesos de digestión anaerobia. Se han obtenido los potenciales bioquímicos de metano de cada uno de los residuos pretratados, habiéndose seleccionado para el estudio y optimización de proceso anaerobio en régimen semicontinuo, el pretratamiento que ha generado un mayor grado de degradación del sustrato y mayor coeficiente de rendimiento en metano en los ensayos BMP previos. También se han evaluado los parámetros operacionales y de control que dan lugar a una máxima estabilidad de los procesos de digestión anaerobia del residuo pretratado de forma más eficiente mediante los mencionados procesos de digestión anaerobia realizados en régimen semicontinuo.

Se pretende así aplicar un tratamiento combinado que permite obtener un biocombustible renovable de muy bajo impacto ambiental y una fase sólida de posible aplicación agrícola por su contenido en nutrientes y materia orgánica muy estabilizada.

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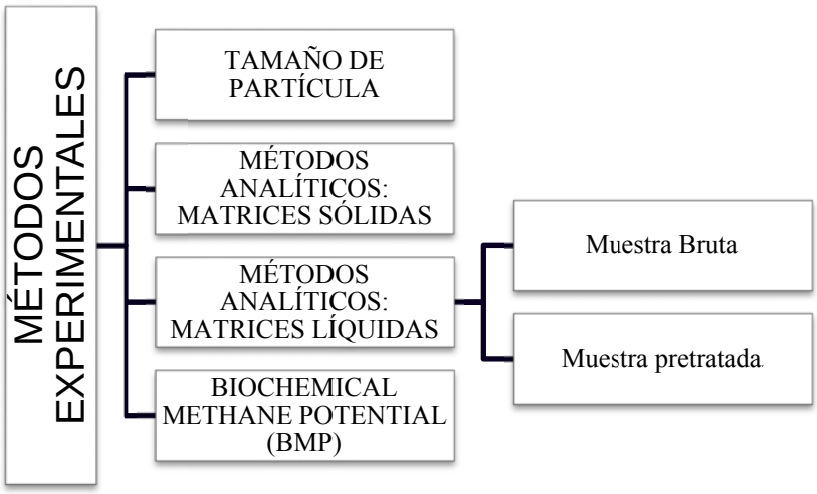
CAPÍTULO 2: MÉTODOS EXPERIMENTALES

2 MÉTODOS EXPERIMENTALES

Resumen

En este capítulo se describen las técnicas empleadas para llevar a cabo el presente trabajo. Para un mejor entendimiento de la organización del capítulo, se muestra el siguiente esquema:



Para determinar las características de un sustrato es necesario el análisis exhaustivo y detallado del mismo. De la misma forma, para el seguimiento y control del proceso de digestión anaerobia se deben analizar también los parámetros más significativos que afectan a la estabilidad y eficiencia de estos procesos.

Todos los métodos analíticos aplicados han sido validados para poder asegurar la calidad de los resultados obtenidos, mediante el uso de patrones de control, u otros medios como participación en ensayos interlaboratorios.

2.1 ESTUDIO DEL TAMAÑO DE PARTÍCULA

Antes de comenzar a detallar los métodos experimentales utilizados hay que destacar un primer trabajo que fue realizado debido a la heterogeneidad en cuanto a diámetros de partículas del sustrato objeto de estudio.

El trabajo consistió en realizar una clasificación del tamaño de partícula de la harina de girasol desengrasada (SuOC), conocer el tamaño mayoritario y estudiar si existía variación respecto a composición de las distintas fracciones encontradas. Se llevaron a cabo ensayos de potencial bioquímico

de metano (BMP) sobre el residuo de torta de girasol desengrasada de las fracciones correspondientes a los diferentes tamaños de partícula, estudiándose la influencia de su composición química en los rendimientos de metano y en la cinética del proceso.

Los rangos de tamaño de partícula que fueron evaluados correspondieron a de 0,355 a 0,55 mm (1), 0.710-1.0 mm (2) y de 1,4 a 2,0 mm (3) de diámetro de partícula.

A pesar de que en este trabajo se obtuvieron los mayores rendimientos de metano para el tamaño de partícula mayor (3), todos los estudios posteriores efectuados para alcanzar los objetivos de esta Tesis fueron realizados con el tamaño de partícula medio, debido a ser esta la fracción mayoritaria del sustrato problema y conseguir así resultados más reales para una posible aplicación a escala industrial.

A continuación, la copia del trabajo publicado con los resultados de este estudio.

Biochemical Engineering Journal 58–59 (2011) 162–167

Contents lists available at SciVerse ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Influence of particle size and chemical composition on the performance and kinetics of anaerobic digestion process of sunflower oil cake in batch mode

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ARTICLE INFO

Article history:
Received 4 April 2011
Received in revised form 7 September 2011
Accepted 10 September 2011
Available online 17 September 2011

Keywords:
Batch assay
Biochemical methane potential (BMP)
Kinetics
Lignocellulose
Particle size
Sunflower oil cake (SuOC)

ABSTRACT

Biochemical methane potential (BMP) assays of sunflower oil cake (SuOC) were carried out to research the effect of different particle sizes and their chemical composition on methane yields and kinetics. Particle size ranges of (1) 0.355–0.55 mm, (2) 0.710–1.0 mm and (3) 1.4–2.0 mm in diameter were evaluated. The highest methane yield 213 ± 8 mL CH_4 g^{-1} VS_{added} was obtained for the largest particle size analyzed (3), against 186 ± 6 mL CH_4 g^{-1} VS_{added} obtained for particles 1 and 2. This may be attributed to the different lignocellulose compositions of the various particle size ranges studied and to organic matter removals (47.2% for 3, against ~41.5% for 1 and 2). The evolution of propionic acid concentration was found to be fundamental for explaining the lowest rate of biogas production for the smallest (1) particle size studied, with a specific rate constant k of 0.45 ± 0.02 d^{-1}, while values of 0.61 ± 0.02 d^{-1} and 0.50 ± 0.01 d^{-1} were obtained for particles 2 and 3, respectively.

1. Introduction

The worldwide production of sunflower oil for 2008–2009 was 32.80 million tons with a production of around 42% of the by-product sunflower oil cake (SuOC), which means that 13.4 million tons of this waste were generated [1]. This waste has been used as animal feed as well as having other biotechnological applications [2]. However, laboratory-scale studies have recently been conducted to assess the feasibility of converting this residue into methane via conventional mesophilic digestion [2–4] or by two-stage processes [5], because its conversion to biogas is likely to be a two-part process of methane generation and residue treatment simultaneously.

A characteristic of SuOC is its high concentration of lignocellulosic material [2]. As is well known, the cellulose in the lignocellulosic polymeric form is not totally available for bacterial attack. Lignin surrounds the cellulose crystalline structure forming a 'seal' and protects the cellulose from being easily hydrolysed. Owing to the refractory structure of cellulose, one of the major problems in utilizing crop residues for stabilizing by anaerobic digestion is their low digestibility [6–8]. The anaerobic biodegradability and hence the biogas potential of a complex substrate depends on the content of biodegradable compounds: carbohydrates (including cellulose and hemicellulose), proteins and lipids [9].

It is generally accepted that hydrolysis is the rate-limiting step in the anaerobic digestion of organic vegetable solid waste. Due to the chemical and physical construction of lignocellulose, its microbial hydrolysis is a slow and difficult process. Furthermore, the surface area and particle size are important characteristics in determining its initial degradation rate [10].

The size of the feedstock should be reduced, otherwise it would result in the clogging of the digester and in the difficulty for microorganisms to digest it. A reduction in the size of the particles and the consequent enlargement of the available specific surface can support the biological process, in the event that there would be substrates with a high fibre content and low degradability, their comminution yielding an improved digester gas production [11]. This leads to a decreased amount of residues to be disposed of and to an increased quantity of useful digester gas [8].

Little research has been carried out into the effect of particle size of agricultural wastes on methane yield [12–15], and all of them were carried out by grinding, shredding, chopping or milling the residues as a physical pre-treatment. The lignocellulose structure was broken, thus enhancing the hydrolysis step. However, the lignocellulose composition of the different particle sizes can be different [16].

The influence of the substrate composition related to the different particle sizes on methane production has not previously been studied or reported in the literature. Therefore, the aim of this study was to determine the influence of particle size and chemical composition on the extent and rate of the anaerobic digestion process of SuOC. In this way, biochemical methane potential (BMP)

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doi:10.1016/j.bej.2011.09.010

tests with three different particle sizes of SuOC (0.355–0.55 mm, 0.710–1.0 mm and 1.4–2.0 mm) have been conducted. Furthermore, a first order kinetic model has been used to obtain the specific rate constants of the processes while simultaneously studying its relationship with the particle size.

2. Methods

2.1. Substrate and anaerobic inoculum

Substrate: SuOC was collected from a sunflower oil factory located near Seville (Spain). The initial particle size of SuOC is tiny (only 11% was larger than 2 mm). As the effect of particle size on anaerobic process (methane yield) will be studied, the substrate was sieved and three fractions including mean size (**2**) 0.710–1.0 mm (the most abundant one), as well as one smaller (**1**) 0.355–0.55 mm and another larger (**3**) 1.4–2.0 mm were chosen. A commercial sieve (Restch AS 200 basic) was used to shred the substrate into different particle sizes. The SuOC was classified in different particle sizes by using screen meshes.

The full composition and main features as well as the fractional composition of the fibre of these three fractions of SuOC are shown in Table 1. The main components of the three particles sizes are cellulose and protein, which represents approximately 21–25% and 24–28% of dry matter, respectively.

Inoculum: The mixed anaerobic culture used as inoculum in the three experiments carried out was collected from a municipal wastewater treatment plant (MWTP) which operates in the anaerobic stabilization of primary and waste activated sludge. The main characteristics of this digested sludge are as follows: pH 7.6 ± 0.1, $33.3 \pm 2.4\,g\,L^{-1}$ of TS, and $17.9 \pm 0.5\,g\,L^{-1}$ of VS.

2.2. Experimental design

Anaerobic digestion experiments in batch mode are useful because they can be performed quickly with simple and inexpensive equipment, and are helpful in assessing the extent to which a material can be digested. The experimental design consisted of a multiflask batch system and was fully described elsewhere [2].

The reactors, which were maintained at $35 \pm 1\,^{\circ}C$ in a temperature-controlled water bath, were initially charged with the inoculum by keeping a concentration of $15\,g\,VS\,L^{-1}$ (the volume is a function of the initial VS concentration), the inoculum to substrate ratio (ISR) was maintained in 2 (VS basis), therefore $7.5\,g\,VS\,L^{-1}$ of SuOC were added to every batch reactor, for the three experiments carried out. 25 mL of stock mineral medium solution which composition has been described elsewhere [17], were also added, and finally, distilled water was added to achieve the desirable working volume of 250 mL. Reactors were flushed with N_2 in order to achieve anaerobic conditions.

The methane released was measured by volume displacement (the carbon dioxide was removed previously by flushing the gas through a 2 N NaOH solution), and expressed at standard temperature and pressure (STP) conditions. Methane production was monitored daily and calculated by subtracting the amount of methane produced by the blank controls (endogenous tests, with the inoculum alone added) from the methane production of each fed reactor.

Every experiment consisted in 14 fed SuOC replicates, 4 blank controls (two initials and two finals) and 2 cellulose positive controls. All the experiments were run for 7–8 days, until no significant gas production was observed, (last day the production was lower than 2% of the accumulate methane produced), suggesting that biodegradation was essentially completed, as control of cellulose ($370\,mL\,CH4\,g^{-1}\,VS_{added}$) confirmed. This short period of time was sufficient to achieve maximum methane production, and can basically be explained by the high activity of the sludge and the short interval between sampling the inoculum and the start-up of the experiments (less than 72 h).

2.3. Analytical methods

Solid sample: The following parameters were assayed in the substrate: total solids (TS) and volatile solids (VS), according to the standard methods 2540B and 2540E [18], respectively; total chemical oxygen demand (CODt) was determined using the reported method proposed by Raposo et al. [19]. Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined according to van Soest et al. [6], to calculate hemicellulose (NDF-ADF), cellulose (ADF-ADL) and lignin (ADL). The total carbohydrates (including fibre and soluble sugars) were calculated by the difference between the organic matter and lipids, protein and lignin content. Total Kjeldahl nitrogen (TKN) determination was also described elsewhere [3]. Total protein was determined by multiplying TKN value by 5.5 [20]. Fat content was extracted with hexane, using a Soxhlet system [21].

Inoculum: The inoculum and digestates were characterized sampling directly. pH (using a pH-meter model Crison 20 Basic), TS and VS were determined [18].

Soluble fraction: The supernatant obtained after centrifuging the digestates for 15 min at 10,000 rpm was filtered through a filter (0.45 μm) and used to characterize the following soluble parameters: chemical oxygen demand (CODs), using the closed digestion and colorimetric standard method 5220D [18]; soluble carbohydrates were analyzed according to the colorimetric method described by Dubois et al. [22]; total alkalinity (TA), which was measured by pH titration to 4.3. Soluble ammonia nitrogen (SAN) was determined by distillation and titration according to the standard method 4500E [18]. The volatile fatty acid (VFA) concentration was performed using a gas chromatograph, as previously described elsewhere [5].

Every one or two days, two of the digesters were sacrificed and their contents analyzed (one for VS analysis, using the whole working volume of the reactor (250 mL) with the purpose of avoiding possible error and the other one for the rest of the parameters).

To assess the organic matter balance in each BMP test system as a function of volatile solid removal (VS_{rem}) the following formula was used:

$$VS_{rem}(\%) = \left[\frac{VS_{added} - (VS_{final} - VS_{final-blank})}{VS_{added}}\right] \times 100 \quad (1)$$

where VS_{added} is the amount of VS added at the beginning of the assay, VS_{final} is the amount of VS at the end of the experiment and $VS_{final\text{-}blank}$ is the difference between the amount of VS of the sample and blank control at the end of experiment.

3. Results and discussion

3.1. Methane yield vs volatile solids removal

The degradation efficiency, expressed as VS_{rem} (Eq. (1)), achieved with particle sizes of 0.355–0.5 mm (**1**), 0.710–1.0 mm (**2**) and 1.4–2.0 mm (**3**) were 41.3%, 41.9% and 47.2%, respectively. This indicates that the degradation efficiency is very similar for particle sizes less than 1 mm, and comparable to that obtained by Raposo et al. [2] for a BMP experiment of SuOC using a mix of particle sizes less than 2 mm. By contrast, the degradation efficiency of particle size 1.4–2.0 mm was higher.

In the case at hand, the increase in the available specific surface achieved with the smallest particle size, which theoretically

Table 1
Composition and features of the different particle sizes of SuOC used as substrate.[a]

	Particle size (mm)		
	0.355–0.55	0.710–1.0	1.4–2.0
Dry matter (DM) (%)	93.1 (±0.1)	93.0 (±0.1)	93.8 (±0.1)
Volatile solids (%)[b]	93.8 (±0.8)	93.0 (±0.1)	92.8 (±0.7)
Ash (%)[b]	5.8 (±0.8)	6.8(±0.1)	6.7 (±0.1)
CODt ($g O_2 g^{-1}$ TS dry basis)	1.10(±0.01)	1.24(±0.02)	1.13(±0.03)
Neutral detergent fibre (%)[b]	42.9(±1.2)	45.0(±1.1)	35.4(±0.7)
Acid detergent fibre (%)[b]	33.8(±0.8)	38.4(±0.9)	30.2(±0.6)
Acid detergent Lignin (%)[b]	10.6(±0.3)	13.3(±0.2)	9.7(±0.2)
Hemicellulose (%)[b]	9.0(±1.1)	6.6(±1.0)	5.2(±0.6)
Cellulose (%)[b]	23.3(±0.7)	25.1(±0.4)	20.5(±0.4)
Total protein (%)[b]	23.7(±0.8)	25.3(±0.8)	28.1(±0.4)
Fat content (%)[b]	1.5(±0.2)	1.6(±0.2)	1.4(±0.3)
Soluble carbohydrates (%)[b]	4.9(±0.4)	5.1(±0.2)	6.2(±0.2)
Total carbohydrates (%)[b]	58.4(±0.5)	53.0(±0.3)	54.1(±0.3)

[a] Mean values are averages of four determinations (± standard deviations).
[b] Expressed as dry matter.

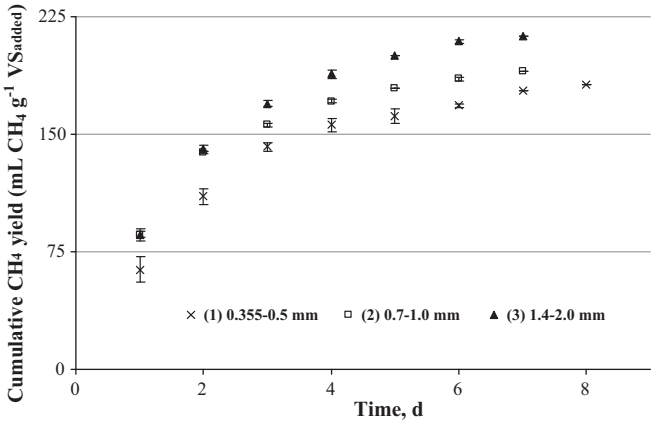

Fig. 1. Cumulative methane yield during batch anaerobic digestion of SuOC for the three different particle sizes studied.

improves the area where the microorganisms can reach and adhere, did not improve organic matter removal. This might be explained by the different chemical composition of the each fraction.

As expected [12], the highest volatile solid reduction corresponds to the highest methane yield obtained. Therefore, for particle sizes **1** and **2** the methane yields were very similar 182 ± 2 and 190 ± 4 mL CH_4 g^{-1} VS_{added}, respectively (Fig. 1). However, for particle size **3** the experimental methane yield was 213 ± 8 mL CH_4 g^{-1} VS_{added}. The differences in methane yields when varying the particle size were found to be statistically significant (*F*-test with 95% confidence limit). Therefore, an increase in the methane yield of 17% was achieved for particle size 1.4–2.0 mm as compared to particle size 0.355–0.55 mm. It seems that the enzymatic breakdown of SuOC does not increase with size reduction within the analyzed range.

Llabrés-Luengo and Mata-Álvarez [12] found increases of 4–5% of VS reduction when the particles sizes of wheat straw were reduced from 10 mm to 5 mm, and obtained an increase of only 4% in the methane yield.

Sharma et al. [13] studied 7 different kinds of raw materials to determine the effect of particle size on methane yield. The reduction from 6.0 mm to 0.088 mm meant increases in VS reduction lower than 4.5%. They also observed that for all feedstock studied, methane yield increased with decreasing particle size. However, in 5 of the 7 raw materials studied the methane yield was slightly higher for 0.40 mm than for 0.088 mm, which is in agreement with the results obtained in the present work.

Moorhead and Nordstedt [14] studied 3 different particle sizes of water hyacinth (1.6 mm, 6.4 mm and 12.7 mm) and found that the methane yields were similar for three sizes and ranged from 140 to 180 mL CH_4 g^{-1} VS_{added}, with the highest methane yield being obtained for material of 6.4 mm in size.

Although the ranges studied in the experiments carried out may seem very undersized, the reason is simple – after oil extraction, SuOC is a by-product that is small in size. So, it would be interesting to compare the results obtained with those achieved by other authors using the same size ranges. Angelidaki and Ahring [23] reported a potential increase of 4% in methane yield for macerated manure biofibres with 0.35 mm compared with fibres 2 mm in size. The methane yields obtained by Mshandete et al. [15] studying sisal fibre for particles with diameters of 2 mm and 5 mm were also very similar, 216 mL CH_4 g^{-1} VS_{added} and 205 mL CH_4 g^{-1} VS_{added}, respectively.

Izumi et al. [24] achieved higher methane yields (455 mL biogas g $CODt^{-1}$) for particle sizes of 0.7 mm than for 0.3 mm (404 mL biogas g $CODt^{-1}$) using food waste.

Therefore, in all the above-mentioned experimental studies, the biogas or methane yield was the same or slightly higher when the particle size diminished, except in the case described by Izumi et al. [24] who explained the lower biogas production to the smaller particle size, due to the fact that an accelerated hydrolysis and acidogenesis in the early stage of anaerobic digestion of food waste, resulting in accumulation of VFA.

In the case at hand, SuOC in the range 0.355–2.0 mm, the highest methane production was achieved for the largest particle size (1.4–2.0 mm). This can be explained because methane productivity not only depends on the amount of degraded volatile solids, but also on the nature (chemical composition) of the solids, because carbohydrates, proteins or fats have different methane potential [9], and their content is not uniform in the different particle size fractions, as has been stated previously by Gollakota and Meher [25].

Although grinding resulted in smaller particle sizes and consequently a higher surface area, enhancing the susceptibility of cellulose to bacterial and enzymatic attack, in this case, the highest particle size studied (1.4–2.0 mm fraction) presented the lowest NDF content (35.4 ± 0.7%) (Table 1). Therefore, the higher extent of substrate conversion of this highest particle size can be related with its higher solubility as well as the highest protein percentage (28.1 ± 0.4%), as was also stated previously by Sharma et al. [13].

The lower methane yield obtained in experiments with small and mean particle, as compared to large particle, is related with CODs as will be explained below. The lower protein content could also be the cause for the lower methane yield obtained with the particles small and mean [26].

3.2. Study of chemical control parameters

Traditionally, BMP assays focus exclusively on methane yield. Moreover, very little data is available from the literature on the evolution of chemical parameters for their comparison with the results obtained in the present experiments.

The evolution of the chemical-control parameters: VS, pH, TA, SAN, CODs, carbohydrates and total VFA (TVFA) in the digestate, has been outlined in Table 2.

pH. Methane is produced in the pH range 7.0–8.5, and the highest cellulose degradation efficiency obtained by Hu et al. [27] using ruminal microorganisms was achieved at pH 7.0–7.5. Consequently, the pH values found in the course of all experiments carried out (between 7.1 and 7.8), were not only typical values for stable mesophilic anaerobic digestion but also suitable to degrade cellulose and yield biogas.

Total alkalinity. The initial and final TA ranged from 3400 to 3920 mg $CaCO_3$ L^{-1} to 5120–5720 mg $CaCO_3$ L^{-1}, respectively. This means that the systems presented a high buffering capacity with an increase in the TA content for all cases studied, and that the particle size does not affect TA evolution.

Soluble ammonia nitrogen. SAN concentration increased noticeably for all experiments during the first two days. Over the next few days the increase was lower. As was stated before [5,28], degradation of complex organic material, including nitrogenous organic compounds during the hydrolytic step of anaerobic digestion results in the generation of ammonia. Therefore, in these first two days the hydrolytic phase occurred when the almost degradable protein was degraded and ammonia was generated. The net increase for every experiment (calculated as the difference between final and initial concentrations, taking into account the blank contribution) varied between 202 ± 32 mg N L^{-1} (large particle) and 235 ± 8 mg N L^{-1} (small particle). From these experimental results it could be concluded that the particle size ranges studied have almost no influence on the yield of the protein hydrolysis of SuOC, although the initial total protein composition was slightly higher (28.1 ± 0.4%) for the largest particle (1.4–2.0 mm) as compared to the smallest one (0.355–0.55 mm) (23.7 ± 0.8%).

Soluble carbohydrates. The initial average soluble carbohydrates concentrations for the three particle size ranges studied was 288 ± 8 mg L^{-1} (Table 2). However, at the end of the experiments, the final concentrations were 104 ± 3 mg L^{-1}, 43 ± 1 mg L^{-1} and 17 ± 5 mg L^{-1}, for small, mean and large particles, respectively. Since carbohydrates are easily utilized by anaerobic microorganisms, a low concentration of carbohydrates indicates that there was no accumulation in the anaerobic fermentation of SuOC, which occurred especially for a particle size of 1.4–2.0 mm.

CODs. The initial CODs for the blank controls were very similar for the three experiments (2300 ± 165 mg O_2 L^{-1}); however, at $t = 0$ the CODs for particle size **3** was 4718 ± 152 mg O_2 L^{-1}, against ~3800 mg O_2 L^{-1} obtained for particles **1** and **2**. These values were very revealing because the amount of CODs for a particle size of 1.4–2.0 mm was much higher than that obtained for mean and small particles, which is in agreement with the higher methane yield obtained for this particle size. This higher solubility of the largest particle size is related to initial substrate lignocellulosic composition (NDF 8–10% lower than obtained for particles **1** and **2**), evolution of carbohydrates concentration (commented above), and VFA concentration.

Volatile fatty acids. The rapid COD increase for 1.4–2.0 mm particle size assay resulted in a sharp rise in TVFA (related to a punctual low pH), which reflected the culminating moment of the hydrolytic stage, whereas the increase in TVFA for experiments 1 and 2 was lower as CODs increased.

Identification of the individual VFA formed is important, since it may provide valuable information on the metabolic pathways involved in the process [2]. As shown in Table 3, a significant amount of VFAs was produced during degradation of SuOC. The VFA distribution showed the influence of SuOC fraction on the fermentation process, and, therefore, on the composition and concentration of the different VFAs generated in the process. Acetic acid (HAc) and propionic acid (HPr) were found to be the two main VFAs for three particle sizes, especially during the first days of assay. The presence of VFA greater than i-HBu was related to the fermentation of proteins [29]. Taking into account that SuOC has a high protein content this explains their presence in the digestates. However, in all cases the individual VFA concentrations were low enough to avoid accumulation and inhibition problems. A similar VFA profile was observed in the anaerobic fermentation of maize [17].

The relevant data derived from the present study are summarized as follows:

- Particle size of 0.355–0.55 mm: the predominant VFA was HPr during the first 3 days, later the concentration of every individual fatty acid was lower than 37 mg L^{-1}. Therefore, no accumulation of VFA was observed, although the methane formation was slower due to slow HPr degradation for this particle size.
- Particle size of 0.710–1.0 mm: the highest concentration for HPr was obtained the first day ($t = 1$ d). After that, the concentration of HAc and HPr remain consistently low. The absence or very low level of HPr, i-HBu, HBu, HVa and i-HCa demonstrates that the methanogenic stage was not disturbed and the formation of methane from these intermediates was quick.
- Particle size of 1.4–2.0 mm: the predominant VFA during the first few days were HAc and HPr, followed by i-HVa and i-HBu. Scarce or no accumulation of HBu, HVa and HCa was observed in the VFA profile, whereas their respective iso-forms are difficult to convert and remained in the medium for longer periods of time, although no accumulation was observed.

3.3. Kinetic study

In order to characterize each experiment kinetically and, thus evaluate the effect of the particle size of SuOC on the methane yield, the following first-order kinetic equation for methane production can be used [30]:

$$G = G_m \times [1 - \exp(-k_0 \times t)] \quad (2)$$

where G (L) is the volume of methane gas accumulated at a given time; G_m (L) is the maximum volume accumulated at an infinite digestion time; k_0 (day^{-1}) is the specific rate constant and t (days) is the time. A similar model, that can be easily derived from Eq. (2) and has also been frequently applied to anaerobic digestion systems [31], was used to correlate the methane yield with the digestion time.

$$B = B_0 \times [1 - \exp(-k \times t)] \quad (3)$$

where B (mL CH_4 g^{-1} VS_{added}) is the cumulative methane yield, B_0 (mL CH_4 g^{-1} VS_{added}) is the maximum or ultimate methane yield of the substrate, k ($days^{-1}$) is the specific rate or apparent kinetic constant and t (days) is the time. Therefore, the ultimate methane yield gives the value when no more volume of gas from the reactor is released.

The adjustment by non-linear regression of the pairs of experimental data (B, t) using Sigmaplot software (version 9.0) allows the calculation of the apparent kinetic constant k. Table 4 lists the k values with 95% confidence limits obtained for each case studied, as well as B_0 and R^2.

The high values of the coefficient of determination, R^2 (>0.99 in all cases) and the low values of the confidence limits of the

Table 2
Evolution of chemical control parameters in the digestates at different particle sizes studied.

Experiment	Particle size (mm)	Time (d)	pH	TA ($mg\,CaCO_3\,L^{-1}$)	TVFA ($mg\,COD\,L^{-1}$)	SAN ($mg\,N\,L^{-1}$)	VS ($mg\,L^{-1}$)	CODs ($mg\,L^{-1}$)	Carbohydrate ($mg\,L^{-1}$)
		0	7.7	3920 ± 57	172 ± 2	896 ± 8	22.6	3804 ± 76	294 ± 8
		1	7.5	4760 ± 0	637 ± 3	1064 ± 16	21.6	3769 ± 10	86 ± 3
		2	7.3	4940 ± 85	525 ± 4	1137 ± 8	20.5	5054 ± 57	126 ± 2
(1)	0.355–0.55	3	7.3	5200 ± 0	399 ± 4	1182 ± 8	19.7	5108 ± 95	135 ± 3
		5	7.6	5340 ± 28	108 ± 2	1243 ± 16	19.2	5030 ± 38	133 ± 6
		6	7.6	5560 ± 0	116 ± 5	1266 ± 16	19.1	5134 ± 57	149 ± 2
		8	7.7	5720 ± 0	39 ± 3	1299 ± 16	18.3	5040 ± 38	104 ± 3
		0	7.5	3400 ± 0	194 ± 3	762 ± 0	22.2	3878 ± 102	292 ± 8
		1	7.2	4380 ± 28	384 ± 6	952 ± 0	21.0	4530 ± 76	91 ± 3
		2	7.5	4600 ± 0	63 ± 2	1014 ± 8	20.2	4207 ± 114	113 ± 2
(2)	0.710–1.0	3	7.3	4640 ± 0	57 ± 3	1042 ± 0	19.6	5081 ± 228	59 ± 5
		5	7.6	5060 ± 28	59 ± 1	1103 ± 8	19.7	4758 ± 95	50 ± 2
		6	7.6	5080 ± 57	61 ± 1	1114 ± 8	19.2	4772 ± 10	61 ± 3
		7	7.8	5120 ± 0	61 ± 2	1148 ± 8	18.8	5121 ± 133	43 ± 1
		0	7.2	3760 ± 57	182 ± 3	890 ± 24	22.0	4718 ± 172	278 ± 7
		1	7.1	4560 ± 0	1360 ± 15	1086 ± 0	20.4	5524 ± 56	32 ± 4
		2	7.5	5080 ± 0	645 ± 5	1120 ± 0	19.7	5161 ± 209	36 ± 9
(3)	1.4–2.0	3	7.7	5300 ± 28	116 ± 3	1159 ± 8	19.4	5269 ± 323	25 ± 1
		4	7.4	5280 ± 0	114 ± 4	1204 ± 8	19.0	4619 ± 38	20 ± 3
		6	7.8	5520 ± 0	112 ± 2	1243 ± 0	18.7	4798 ± 342	21 ± 2
		7	7.6	5580 ± 28	36 ± 3	1238 ± 55	18.4	5054 ± 19	17 ± 5

Table 3
Time course variations of individual VFAs in the digestate for different particle size studied. [a]

Experiment	Particle size (mm)	Time (d)	HAc ($mg\,L^{-1}$)	HPr ($mg\,L^{-1}$)	i-HBu ($mg\,L^{-1}$)	HBu ($mg\,L^{-1}$)	i-HVa ($mg\,L^{-1}$)	HVa ($mg\,L^{-1}$)	i-HCa ($mg\,L^{-1}$)
(1)	0.355–0.5	0	47	15	10	14	13	13	–
		1	110	242	30	10	27	13	–
		2	51	251	20	–	14	12	–
		3	35	146	33	10	18	13	–
		5	37	–	20	–	16	–	–
		6	34	5	25	–	13	–	–
		8	37	–	–	–	–	–	–
(2)	0.710–1.0	0	62	29	13	17	15	–	–
		1	42	163	18	–	17	12	–
		2	51	6	–	–	–	–	–
		3	47	5	–	–	–	–	–
		5	49	5	–	–	–	–	–
		6	49	6	–	–	–	–	–
		7	49	6	–	–	–	–	–
		0	46	21	13	15	13	11	–
		1	140	528	60	15	90	15	28
		2	26	291	41	13	27	12	–
(3)	1.4–2.0	3	22	7	30	–	14	–	–
		4	24	13	38	–	–	–	–
		6	32	11	34	–	–	–	–
		7	22	8	–	–	–	–	–

(–) Not detected.
[a] HCa and HEn were not detected in any samples.

Table 4
k and B_0 values with 95% confidence limits for each experiment carried out.

Experiment (particle size (mm))	k (d^{-1})	B_0 ($mL\,CH_4\,g^{-1}\,VS_{added}$)	R^2
0.355–0.55	0.45 ± 0.02	184 ± 3	0.9975
0.710–0.1	0.61 ± 0.02	189 ± 2	0.9983
1.4–2	0.50 ± 0.01	218 ± 1	0.9997

parameters obtained demonstrates how well the experimental data adapted to the model proposed.

From the results obtained it can be observed that the apparent kinetic constants of the process are related to the evolution of VFA concentration in general and HPr in particular. The highest k value ($0.61 \pm 0.02\,d^{-1}$) was obtained for the 0.710–1.0 mm particle size assay, where the HPr concentration was $29\,mg\,L^{-1}$ at $t = 0$ and $163\,mg\,L^{-1}$ at $t = 1$, decreasing rapidly to values $\leq 6\,mg\,L^{-1}$ at $t = 2$ until the end of the process. Although the highest HPr concentration was obtained at $t = 1$ during the assay of particle size 1.4–2.0 mm, at $t = 3$ the concentration dropped drastically until $7\,mg\,L^{-1}$, so the second k value ($0.50 \pm 0.01\,d^{-1}$), was obtained for this assay. Finally, the lowest k value ($0.45 \pm 0.02\,d^{-1}$) was obtained for the smallest particle size studied (0.355–0.55 mm), observing for this case at $t = 3$ days the highest value of HPr, $146\,mg\,L^{-1}$.

4. Conclusions

Batch anaerobic digestion experiments of SuOC with different particle sizes revealed that this did not affect final pH, total alkalinity, soluble ammonia nitrogen or CODs, although the largest size (1.4–2.0 mm) within the range studied (0.355–2.0 mm) made it possible to achieve the highest methane yield, 213 ± 8 mL CH_4 g^{-1} VS_{added}, when compared with particle sizes of 0.355–0.55 mm and 0.710–1.0 mm, for which 182 ± 2 and 190 ± 4 mL CH_4 g^{-1} VS_{added}, respectively, were achieved. This can be attributed to the different chemical initial composition of the different particle size fractions, which also explain the different TVFA evolution. Therefore, optimizing the size reduction of SuOC could potentially improve the methane yield of anaerobic digestion process of this substrate.

A first order kinetic model was used to obtain the specific rate constant of each size range; the slow HPr removal could explain the lowest k value (0.45 d^{-1}) obtained for the smallest particle size studied.

Acknowledgements

The authors wish to express their gratitude to the Spanish Ministry of "Ciencia and Innovación" (Project CTM 2008-05772/Tecno) for providing financial support, to the contract JAE-Doc from "Junta para la Ampliación de Estudios del CSIC" co-financed by the European Social Funds, as well as to the Program "FPI" of the above-mentioned ministry for contracting graduates.

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Las técnicas analíticas utilizadas en el desarrollo del trabajo experimental de la presente Tesis Doctoral han sido clasificadas según la matriz del analito analizado.

2.2 MÉTODOS ANALÍTICOS: MATRICES SÓLIDAS

2.2.1 Sólidos Totales (TS) y Sólidos Volátiles (VS)

La porción de muestra es secada hasta peso constante en una estufa a 105±5ºC. La diferencia de masa antes y después del proceso de secado es usada para calcular los sólidos totales o materia seca (MS) y el contenido en agua de la muestra o humedad.

Después, la muestra secada es calcinada en un horno mufla a 550±10ºC hasta quemar todo el material orgánico. La diferencia de masa antes y después del proceso de ignición es usado para calcular el contenido de solidos volátiles o sólidos orgánicos (MO) y cenizas.

Para estas determinaciones se utilizó, una estufa capaz de mantener una temperatura de 105±5ºC marca Selecta modelo Digitheat, un horno eléctrico mufla marca Heraeus modelo MR170 capaz de mantener una temperatura de 550±10ºC, crisoles de porcelana, balanza analítica marca Gram Precision Serie SV con una precisión de 1mg y desecador con agente desecante activo con indicador.

Figura 2.1.- Equipos utilizados para la determinación de ST y SV.

Estas determinaciones fueron realizadas de acuerdo a las siguientes normas Británicas:

BS EN 12880:2000. Characterization of sludges. Determination of dry residue and water content [1].

BS EN 12879:2000. Characterization of sludges. Determination of the loss of ignition of dry mass [2].

2.2.2 Demanda química de oxígeno total (CODt)

La demanda química de oxigeno es una medida indirecta del contenido de materia orgánica y compuestos oxidables en una muestra. Se define como la cantidad de oxígeno necesaria para oxidar completamente la materia orgánica y los compuestos oxidables de una determinada muestra.

Para su determinación, se digiere la muestra con exceso de dicromato potásico en un medio fuertemente ácido (H_2SO_4), durante dos horas a una temperatura de 150±5ºC. La reacción es catalizada por sulfato de plata (Ag_2SO_4) y se utiliza $HgSO_4$ para eliminar problemas de interferencias de los haluros presentes. El exceso de dicromato se valora con sal de Mohr, sulfato amónico ferroso ($Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$), usando ferroína como indicador.

Para llevar a cabo esta determinación, se utilizó un bloque de digestión de Selecta modelo Bloc Digest, y una bureta para la valoración.

Figura 2.2.- Equipos utilizados para la determinación de la CODt

La determinación se realizó de acuerdo al método propuesto por Raposo et al. (2008) [3].

Durante la ejecución de la presente tesis, el doctorando participó en el desarrollo de un ensayo de aptitud para este parámetro, el cual produzco la siguiente publicación científica:

Quality improvement in determination of chemical oxygen demand in samples considered difficult to analyze, through participation in proficiency-testing schemes

Raposo, F., Fernández-Cegrí, V., De la Rubia, M.A., Borja, R., Beltrán, J., Cavinato, C., Clinckspoor, M., Demirer, G., Diamadopoulos, E., Frigon, J.C., Koubova, J., Launay, M., Méndez, R., Menin, G., Noguerol, J., Uellehdahl, H., West, S.

(2010) TrAC - Trends in Analytical Chemistry, 29 (9), pp. 1082-1091.

Dicho estudio consistió en la realización de un ensayo intercomparativo, con la participación de 20 laboratorios tanto nacional como internacional, para evaluar la calidad del método de la determinación de la DQOt de muestras consideradas "difíciles" para analizar, es decir, muestras sólidas o muestras líquidas con altas concentraciones de sólidos en suspensión. Los resultados que se obtuvieron fueron considerados satisfactorios, mostrando un desempeño general de los laboratorios participantes aceptable.

A continuación se muestra copia de la publicación realizada:

Quality improvement in determination of chemical oxygen demand in samples considered difficult to analyze, through participation in proficiency-testing schemes

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0165-9936/$ - see front matter doi:10.1016/j.trac.2010.06.005

Chemical oxygen demand (COD) is a critical analytical parameter in waste and wastewater treatment, more specifically in anaerobic digestion, although little is known about the quality of measuring COD of anaerobic digestion samples. Proficiency testing (PT) is a powerful tool that can be used to test the performance achievable in the participants' laboratories, so we carried out a second PT of COD determination in samples considered ''difficult'' to analyze (i.e. solid samples and liquid samples with high concentrations of suspended solids). The results obtained (based on acceptable z-score values) may be considered satisfactory. When compared with the results of a previous similar scheme, the overall performance improved by around 30%, again demonstrating that analytical performance can be improved by regular participation in PT.

Keywords: Anaerobic digestion; Chemical oxygen demand (COD); Interlaboratory study; Liquid sample; Proficiency testing (PT); Solid sample; Suspended solids; Waste treatment; Wastewater treatment

1. Introduction

The performance and the control of anaerobic processes are generally assessed by monitoring different analytical parameters, including chemical oxygen demand (COD). These systems have an organic-matter content supplied by water and suspended solids from waste and biota. However, hardly anything is known about the quality of COD measurements from anaerobic-reactor samples. From a scientific point of view, it is essential to ensure that the data produced are of sufficient trueness and precision to serve as a basis for drawing meaningful conclusions about the performance of reactors and the comparative study among different laboratories.

This contribution is the third research report that deals with the analytical determination of COD using both solid and liquid samples with high concentrations of suspended solids. The first contribution looked at the proposition of a modified analytical method for COD determination [1], whereas the second focused on the first COD proficiency testing (PT) of the anaerobic digestion groups (1st COD-PT^{ADG}), compiling data from laboratories mainly specializing in anaerobic digestion [2].

The results obtained were unsatisfactory because the majority of the participating laboratories obtained inappropriate performances. This showed the difficulties that lie in determining COD in these types of sample. However the results were not surprising, because laboratories unacquainted with PT schemes invariably fail to produce satisfactory results.

There are several reasons for participating in a PT scheme:

- evaluation of the performance and continuous monitoring;
- evidence of reliable results;
- identification of problems related to the systematic nature of assays;
- the possibility of taking corrective and/or preventive measures;
- evaluation of the efficiency of internal controls;
- determination of the performance characteristics and validation of methods and technologies;
- standardization of the activities in the market; and,
- national and international recognition of assay results [3].

Despite the fact that a single result in a PT scheme simply reflects the quality of the performance of a laboratory at any given point in time and that the extrapolation from success in a PT scheme in everyday analytical work is an assumption, frequent participation in PT schemes is highly recommended and can help provide insights into the level of quality within a laboratory. Moreover, observing that another laboratory finds approximately the same measurement result from the same measurands provides analysts with great comfort and gives them self-confidence – confirmation always gives a nice feeling.

PT schemes are therefore welcome because they provide a clear, straightforward way of evaluating the accuracy (trueness and precision) of results obtained by different laboratories. The participation in PT is also considered a powerful tool for detecting and removing sources of common errors due to the lack of quality control (QC) within a laboratory.

The 2nd COD-PT^{ADG} was organized with the aim of comparing the data from both the 1st and 2nd COD-PTs and of determining if PT schemes improve the performance of participant laboratories.

2. Organization of the PT scheme

This study is the second attempt at a worldwide interlaboratory comparison of analytical COD determination using solid samples and liquid samples with high concentrations of suspended solids. These samples are considered to be difficult to analyze and are problematic in the corresponding determinations. The scheme was organized by the ''Reuse of Wastes and Wastewater Treatment Group'', of the Instituto de la Grasa (IG) of the Spanish National Research Council (CSIC).

The PT coordinator and collaborators were responsible for:

- designing the overall scheme;
- preparation, testing and distribution of selected samples;
- distribution of instructions among the participating laboratories;
- collection of data, their statistical treatment and feedback of results to participants.

This PT was carried out according to the International Harmonized Protocol for the PT of Analytical Chemistry Laboratories [4].

The PT coordinator sent invitations to participate in the 2nd COD-PT^{ADG} in June 2009. The test took place between 15 September and 15 October 2009. Each participating laboratory received four samples, together with technical guidelines on how to proceed with the measurements. A total of 20 laboratories from 13 countries agreed to participate. All the participating laboratories were highly motivated about taking part in the PT scheme, as the full return rate of data proved. All participating laboratories provided feedback, first about their own performance, and second about the general performance, all of which was reported anonymously.

3. Materials and methods

3.1. Materials

3.1.1. Description of samples. To carry out the 2nd COD-PT^{ADG}, four different samples were selected. These samples were divided into two main groups: solid samples (SS) and liquid samples with a high suspended solid concentration (LS-HSSC):

- Sample 1 (SS 1). Gelatin (Gel). Pure powder protein used as a solidifying agent in the preparation of microbiological culture media to identify proteolytic microorganisms (gelatinase producers). The gelatin used was supplied by Panreac-Spain (Code 403902).
- Sample 2 (SS 2). Sewage sludge (SewS). A sewage sludge produced by Resource Technology Corporation (USA and UK) and provided for characterization as a new certified reference material (including 19 metals as well as COD, Kjeldahl nitrogen and total phosphorus).
- Sample 3 (LS-HSSC 1). Sunflower-oil cake (SuOC). A by-product made up of the part of whole sunflower seeds that remains after oil-extraction processes. It is a heterogeneous substrate that can be broken down into three main components: a proteinaceous fraction, a lignocellulosic fraction and a soluble fraction. The sample was prepared with 5 g of raw material.
- Sample 4 (LS-HSSC 2). Mung bean (MB). The seed of *Vigna radiata*, which is native of Asia (Bangladesh, India and Pakistan). This seed is also known as green bean, green soya, and green gram. Its beans are small, ovoid in shape, green when raw and yellow when dehusked. The sample was also prepared with 5 g of raw material.

3.1.2. Preparation of samples. The suitability and the quality of the test materials distributed are fundamental for the effectiveness of a PT scheme. The two main criteria for suitable test material are that:

- it resembles, as closely as possible, the real samples with which a laboratory routinely works; and,
- variations in the composition of the samples of the test material distributed to participants are kept to the minimum [5].

The PT material was prepared by the PT coordinator. Although his working laboratory has not implemented a quality system accredited according to ISO 17025, he is very experienced in this field and has been involved in different laboratory QC systems, so all the characteristics that could affect the integrity of the test were taken into consideration, including the homogeneity and the stability of the samples.

Considering that different particle-size fractions of the solid samples dispatched would lead to a lack of homogeneity with respect to COD determination, a control of particle size was carried out by sieving the substrates selected to the desired size.

Taking into account that the moisture content of solid-substrate samples can vary with ambient humidity, the participants were requested to report results on a dry-weight basis.

Samples 3 and 4 were two liquid samples with high concentrations of suspended solids that had to be reconstituted in-laboratory by adding 200 mL of distilled water to the spiked amount of solid content weighed into the containers. All participants were instructed to stir the samples for 1 h before COD analysis and during the sampling procedure.

3.1.3. Characterization of samples. All samples distributed were analyzed in the laboratory of the PT coordinator. Three replicates of different parameters (moisture, organic content and elemental composition) were prepared for each sample. Table 1 summarizes the main characteristics of the samples selected.

3.1.4. Homogeneity of samples. Immediately after packaging the samples, they were tested for sufficient homogeneity using the standard analytical method developed in the laboratory of the PT coordinator and used on a routine basis. To check for sufficient homogeneity, the protocol devised by Fearn and Thompson [6] was used. In accordance with their approach, three tests were carried out to estimate the corresponding experimental statistical parameters and compared with their theoretical critical values:

Table 1. Characterization of the solid samples used in the 2nd COD-PT^{ADG}

	Sample 1 *(Gel)*	Sample 2 *(SewS)*	Sample 3 *(SuOC)*	Sample 4 *(MB)*
Particle size (mm)	N.D.[a]	0.2–1	0.125–0.355	0.125–0.500
Moisture (%)	8.0 ± 0.3	10.0 ± 0.4	8.0 ± 0.4	9.0 ± 0.3
Organic content (%TS)	100.0 ± 0.1	60.3 ± 0.5	93.0 ± 0.5	97.0 ± 0.5
Chemical Composition (%-VS)				
Carbohydrates	–	N.D	55.5	72.4
Fat	–	N.D	1.1 ± 0.2	1.5 ± 0.2
Protein[b]	100	N.D	26.4 ± 0.6	23.1 ± 0.6
NDF	–	N.D	40 ± 1	5.0 ± 0.5
Elemental Analysis (%-TS)				
C	48.2 ± 0.3	32.9 ± 0.5	45.9 ± 0.6	44.6 ± 0.6
H	6.5 ± 0.3	4.5 ± 0.1	6.3 ± 0.1	6.8 ± 0.3
N	18.4 ± 0.1	4.8 ± 0.1	5.4 ± 0.4	4.4 ± 0.1
S	0.6 ± 0.1	1.4 ± 0.1	0.20 ± 0.04	0.07 ± 0.01
O	26.2 ± 0.4	16.7 ± 0.7	35.2 ± 0.8	41.1 ± 0.6
Theoretical Oxygen Demand (*ThOD*-mg $O_2 \cdot g^{-1}$ TS)	1236	956	1249	1240

[a]N.D.: Not determined.
[b]%NTK x Nitrogen-to-protein conversion factor (5.5).

Table 2. Summary of analytical procedures utilized by participating laboratories in 2nd COD-PT^{ADG}

Lab[1]	Method[2]	Digestion Reagent			Acid Reagent[3]		$HgSO_4$	Water	End Point[4]
		$K_2Cr_2O_7$			H_2SO_4-$AgSO_4$				
		Vol. (mL)	Conc. (N)	Vol. (mL)	Conc.[c] (%)	Conc.[d] (g/L)		Vol. (mL)	
1[a]	(2) OR-HCM	25	1.0	20	98	10	Yes	0	TT [g]
1[b]	(1) OR-LCM	5	0.241	15	98	10	Yes	10	TT
2	(4) CR-SM				99	10	No	No	SP[e]
3	(1) OR-LCM	20	0.5	30	98	5	Yes	10	TT
4[a]	(5) CR-KSM								SP
4[b]	(5) CR-KSM								SP
5	(2) OR-HCM	10	1.0	30	98	10	Yes	10	TT
6[a]	(2) OR-HCM	15	1.0	45	98	9.4	Yes	20	TT
6[b]	(3) CR-TM	1.5	0.21	3.5	98	10.7	Yes	0	TT
7	(2) OR-HCM	10	1.2	30	98	10	Yes	0	TT
8	(4) CR-SM	1.5	0.2148	3.5	98	10	Yes	2,5	SP
9	(2) OR-HCM	20	1.2	25	98	10	Yes	10	PT[f]
10	(2) OR-HCM	20	1.2	30	98	10	Yes	10	PT
11	(1) OR-LCM	50	0.25	50	98	10	Yes	25	TT
12	(5) CR-KSM								SP
13	(2) OR-HCM	20	1.0	30	98	10	Yes	20	TT
14	(1) OR-LCM	25	0.25	75	96	10.6	Yes	0	TT
15	(2) OR-HCM	20	1.2	30	95	10	Yes	15	PT
16	(2) OR-HCM	20	1.2	30	98	10	Yes	10	PT
17	(1) OR-LCM	0.5	0.33	2.5	95–98	26.5	Yes	2.0	SP
18	(5) CR-KSM								SP
19	(2) OR-HCM	20	1.2	30	98	10	Yes	10	TT
20	(1) OR-LCM	20	0.5	30	98	10	Yes	10	PT

[1]Type of sample: Solid Samples [a](SS) Liquid Samples with high suspended solid concentrations [b] (LS-HSSC).
[2]Analytical Method:
- Open Reflux (OR): (1) OR-LCM. Low concentration of $K_2Cr_2O_7$ (M<0.166) (2) OR-HCM. High Concentration of $K_2Cr_2O_7$ (M ⩾ 0.166)
- Closed Reflux (CR): (3) CR-TM. End-point by titration (4) CR-SM. End-point spectrophotometrically (5) CR-KSM. Kits. End-point spectrophotometrically

[3]Acid-Catalyst reagent: Concentration of H_2SO_4[c]; Concentration of $AgSO_4$[d].
[4]Visualization of end-point: spectrophotometrically (SP[e]).titration: partial and total titration (PT[f]/TT[g]).

(i) Cochran's test procedure for duplicate results or the detection of outliers by differences between pairs;
(ii) precision of the analytical method used; and,
(iii) homogeneity test or test for acceptable between-sample variance.

For this purpose, 10 randomly selected distribution units of solid substrates were analyzed in duplicate and COD values were statistically evaluated.

3.1.5. Stability of samples. Materials distributed in PT schemes must be sufficiently stable over the period in which the assigned value needs to be valid. Normally, the period in question is the interval between the preparation of the material and the deadline for the return of results (one month). The material under test should be in the packaging in which it is distributed.

To ensure that the samples used in the 2nd COD-PT^{ADG} were stable, a stability study was carried out to identify if there was reproducibility of the results with time. The stability study was carried out by applying the values of *F*, which were calculated applying the one-way analysis of variance (ANOVA) of three randomly selected distribution units from the homogenization study, and it was suggested they be kept at room temperature.

3.2. Methods

3.2.1. Analytical methods

3.2.1.1. Chemical oxygen demand. The participating laboratories were free to choose the analytical method that they considered suitable for performing the COD analysis, but were advised to analyze samples using their usual techniques. Each participating laboratory was requested to make three replicate determinations, and to report the results together with a short description of the method used. Table 2 summarizes all the experimental conditions of the analytical methods used by the participants' laboratories. The studies of homogeneity and stability were carried out by the method proposed by Raposo et al. [1].

The analytical determination of COD can be classified first into two main groups [i.e. open reflux (OR) and closed reflux (CR)], and second into five methods, with percentages of each method used by the different participants in brackets:

(1) OR, low concentration of oxidant (17.5%);
(2) OR, high concentration of oxidant (47.5%);
(3) CR, end-point by titration (2.5%);
(4) CR, end-point by spectrophotometrically determination (15%); and,
(5) CR, using kits (17.5%).

The percentages of analytical methods used for OR and CR were therefore 65% and 35%, respectively.

3.2.1.2. Other parameters. Moisture, TS-dry matter and VS-organic matter were determined according to the standard methods 2540B and 2540E-APHA, respectively [7]. Fat content was determined by extraction with hexane using a Soxhlet system [8]. Protein and elemental composition were performed in a LECO CHNS-932 combustion analyzer at 1050°C, using sulfametazine as standard substrate. Theoretical oxygen demand was calculated from the elemental composition according to ISO 10707 [9]. Fiber (neutral detergent fiber, NDF) content was obtained using the method reported by Van Soest [10]. Carbohydrate content was reported by subtraction of fat, protein and lignin contents.

3.2.2. Data treatment

The internationally recommended z-score was used as the performance criteria for participating laboratories whose results were converted into z-scores according to the following equation:

$$z\text{-}score = (X_{EV} - X_{AV})/\sigma_{PT}$$

where X_{EV} is the laboratory's experimental value, X_{AV} is the assigned value (estimation of the true value of the measurand that is used for the purpose of calculating scores), and σ_{PT} is the fitness-for-purpose-based ''standard deviation for proficiency assessment'', defined as a target value for the acceptable deviation from the assigned value.

This means that the z-score method compares the participant's deviation from the reference value with σ_{PT},

Table 3. Homogeneity of the solid substrates. Summary of the statistical parameters obtained

Sample	Test	Experimental Value	Critical Value	Result
Gel	Cochran	0.3050	0.6020	Pass
	Precision of Method	0.39	0.5	Pass
	Homogeneity	0.00011	0.00031	Pass
SewS	Cochran	0.2603	0.6020	Pass
	Precision of Method	0.44	0.5	Pass
	Homogeneity	0.00002	0.00021	Pass
SuOC	Cochran	0.2647	0.6020	Pass
	Precision of Method	0.41	0.5	Pass
	Homogeneity	0.00001	0.00034	Pass
MB	Cochran	0.2809	0.6020	Pass
	Precision of Method	0.20	0.5	Pass
	Homogeneity	0.00004	0.00020	Pass

so the assigned value and the target standard deviation have a critical influence on the calculation of z-scores and must be selected with care if they are to provide a realistic assessment of laboratory performance.

3.2.2.1. Assigned values. In the 1st COD-PTADG, the results were too widespread to be used as a reference value based on the generally used consensus approach. In this case, the assigned values were determined on the basis of *ThOD* measurements performed at the PT coordinator's working laboratory. The same criterion was used for the 2nd COD-PT^{ADG}, but, in addition, two consensus values (mean and median) based on the results from all participants were also calculated only to estimate the degree of dispersion from the assigned value. The *ThOD*-based assigned values, mean and median consensus values for Gel and SewS solid samples were: 1236, 1201 and 1224 mg O_2 g^{-1} TS and 956, 950 and 954 mg O_2 g^{-1} TS, respectively. Similarly, the values for SuOC and MB liquid samples were: 28.164, 28.828 and 29.327 g O_2 L^{-1} and 27.793, 27.791 and 28.261 g O_2 L^{-1}, respectively. Considering the data of all the

Table 4. Summary of the data reported by participating laboratories in 2nd COD-PT^{ADG}

Lab	Sample 1				Sample 2			
	EV_{Mean} (mg O_2 g^{-1} TS)	EV_{RSD} (%)	R_{Mean} (%)	R_{RSD} (%)	EV_{Mean} (mg O_2 g^{-1} TS)	EV_{RSD} (%)	R_{Mean} (%)	R_{RSD} (%)
1	1277	3	103	3	966	1	101	1
2	1190	3	96	3	970	2	101	2
3	1142	5	92	4	815	5	85	4
4	1249	6	101	6	869	5	91	4
5	1205	2	97	2	949	1	99	1
6	1035	6	84	5	792	5	83	4
7	1219	5	99	5	953	4	100	4
8	1244	3	101	3	954	2	100	2
9	889	2	72	2	638	2	67	1
10	1235	3	100	3	954	3	100	3
11	1145	1	93	1	893	1	93	1
12	1144	6	93	6	1278	2	134	3
13	1210	3	98	3	974	1	102	1
14	1255	1	102	2	950	1	99	1
15	1245	4	101	4	871	7	91	6
16	1318	2	107	2	1004	3	105	3
17	1286	6	104	6	1057	1	111	1
18	1329	6	108	6	1093	8	114	9
19	1228	1	99	1	950	1	99	1
20	1185	6	96	6	1095	7	115	7
Lab	**Sample 3**				**Sample 4**			
	EV_{Mean} (mg O_2 L^{-1})	EV_{RSD} (%)	R_{Mean} (%)	R_{RSD} (%)	EV_{Mean} (mg O_2 L^{-1})	EV_{RSD} (%)	R_{Mean} (%)	R_{RSD} (%)
1	27567	3	98	3	33570	13	121	15
2	26853	6	95	6	26042	6	94	5
3	31527	15	112	16	27323	22	98	21
4	31433	2	112	2	31767	3	114	3
5	30512	11	108	12	28543	9	103	9
6	21665	6	77	5	22470	6	81	5
7	26476	5	94	5	26194	2	94	2
8	30233	1	107	1	27647	2	99	2
9	33519	9	119	11	28948	1	104	1
10	29451	1	105	1	28207	1	101	1
11	22647	1	80	1	22350	2	80	1
12	28700	6	102	7	30433	1	109	1
13	25467	7	90	6	25900	2	93	2
14	29963	1	106	1	29838	1	107	2
15	27933	13	99	13	23767	7	86	6
16	29255	2	104	2	28314	2	102	2
17	30553	3	108	3	29749	4	107	4
18	29399	4	104	4	27603	0	99	0
19	28566	0	101	0	28418	1	102	1
20	34850	12	124	12	32730	12	118	12

	Laboratory																			
Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	1.3	-1.5	-3.0	0.4	-1.0	-6.5	-0.6	0.3	-11.2	0.0	-2.9	-3.0	-0.9	0.6	0.3	2.7	1.6	3.0	-0.2	-1.7
2	0.4	0.6	-5.9	-3.7	-0.3	-6.9	-0.1	-0.1	-13.3	-0.1	-2.6	13.5	0.7	-0.3	-3.6	2.0	4.2	5.7	-0.3	5.8
3	-0.4	-0.9	2.4	2.3	1.7	-4.6	-1.2	1.5	3.8	0.9	-3.9	0.4	-1.9	1.3	-0.2	0.8	1.7	0.9	0.3	4.7
4	4.2	-1.3	-0.3	2.9	0.5	-3.8	-1.2	-0.1	0.8	0.3	-3.9	1.9	-1.4	1.5	-2.9	0.4	1.4	-0.1	0.4	3.6

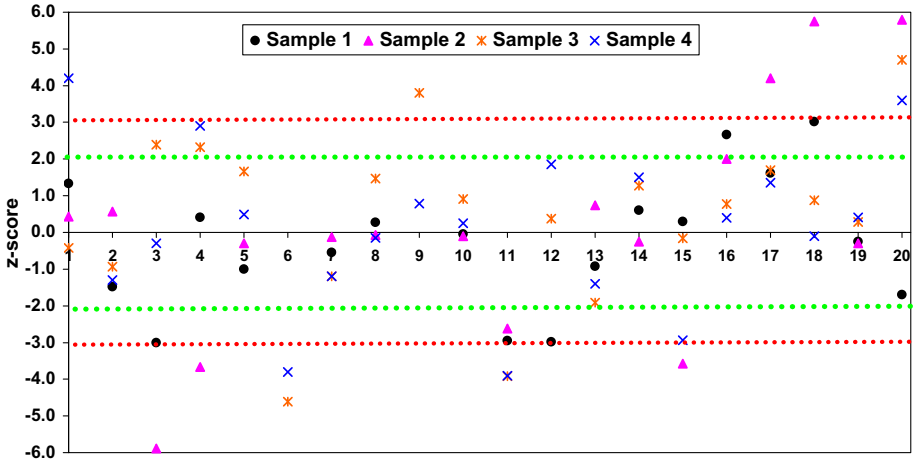

Figure 1. Overview of the z-score values obtained by laboratories participating in the 2nd COD-PT^{ADG}.

samples, it can be seen that there was a good agreement between the experimental consensus values and the theoretical assigned values.

3.2.2.2. Standard deviations for proficiency assessment. The value of σ_{PT} determines the limits of satisfactory performance in a PT scheme. It is important to note that σ_{PT} values were predefined by the PT coordinator and the criteria were communicated in advance to participating laboratories. The σ_{PT} values were determined as a percentage of the assigned value according to the appropriate form of the Horwitz equation [11], which considers the concentration level of analyte. The theoretical percentage values for GEL, SewS, SuOC and MB were 0.9%, 1.0%, 3.4% and 3.4%, respectively. However, these values were slightly modified to reflect the level of COD uncertainty in real routine work samples, so, for solid samples, the percentage was 2.5%, and for liquid samples 5.0%. These σ_{PT} values were identical to those used in the 1st COD-PTADG to prevent the different values from transferring into z-scores that could give data from different PT schemes that could not be compared.

3.2.2.3. Laboratory performance. The conventional way to evaluate the performance of each laboratory participating in a PT scheme based on z-score values was used. In the interpretation of z-scores, the following agreements were internationally made:

z-score $\leqslant \pm 2$ – satisfactory result;

z-score $> \pm 3$ – unsatisfactory result; and,

$\pm 2 >$ z-score $\leqslant \pm 3$ – doubtful result.

4. Results and discussion

4.1. Evaluation of sample-homogeneity study

Table 3 summarizes the results obtained in the statistical analysis of homogeneity data, which show that substrates selected as samples passed the statistical homogeneity tests, so they were considered homogeneous enough and suitable to be used in the PT scheme.

4.2. Evaluation of sample-stability study

The calculated F values for samples 1–4 were 0.78, 0.47, 1.72 and 2.30, respectively. All the results obtained were less than 4.96, which represents the critical F value for a confidence level of 95%. Considering that there was no significant difference between the mean values of COD determinations during the period of time established, the samples were considered stable for the study conditions.

4.3. Evaluation of laboratory performance

Table 4 summarizes the means and relative standard deviations of experimental values (EV) and recoveries (R) reported by the 20 participating laboratories. The general trend of the data reported showed that all the

Table 5. Summary of participants' results obtained for different analytical methods

Sample	Analytical Method			Average Values				Z-scores					
	Name	N^{er}	%	Mean	SD_R	RSD_R	Recovery	Z-score ⩽ ±2		±2 < Z-score ⩽ ±3		Z-score > ±3	
				(mg O_2 g^{-1} TS)	(mg O_2 g^{-1} TS)	(%)	(%)	N^{er}	%	N^{er}	%	N^{er}	%
SS-1	(1) OR-LCM	3	15	1195	56	5	97	2	67	1	33	0	0
(Gel)	(2) OR-HCM	11	55	1182	122	10	96	6	55	3	27	2	18
	OR-M	14	70	1185	109	9	96	8	57	4	29	2	14
	(3) CR-TM	0	0					0	0	0	0	0	0
	(4) CR-SM	3	15	1240	48	4	100	3	100	0	0	0	0
	(5) CR-KM	3	15	1241	93	7	100	2	67	1	33	0	0
	CR-M	6	30	1240	109	9	100	5	83	1	17	0	0
	Total	20	100	1201	100	8	97	13	65	5	25	2	10
SS-2	(1) OR-LCM	3	15	979	104	11	102	1	33	1	33	1	33
(SewS)	(2) OR-HCM	11	55	897	109	12	94	7	64	0		4	36
	OR-M	14	70	915	110	12	96	8	57	1	7	5	36
	(3) CR-TM	0	0					0	0	0	0	0	0
	(4) CR-SM	3	15	987	45	5	103	2	67	0	0	1	33
	(5) CR-KM	3	15	1080	205	19	113	0	0	0	0	3	100
	CR-M	6	30	1034	110	11	108	2	33	0	0	4	67
	Total	20	100	950	129	14	99	10	50	1	5	9	45
				(mg O_2 L^{-1})	(mg O_2 L^{-1})	(%)	(%)	N^{er}	(%)	N^{er}	(%)	N^{er}	(%)
LS-HSSC 1	(1) OR-LCM	4	20	28757	5077	18	102	2	50	0	0	2	50
(SuOC)	(2) OR-HCM	8	40	29347	2603	9	104	6	75	1	0	1	25
	OR-M	12	60	29150	3380	12	104	8	67	1	8	3	25
	(3) CR-TM	1	5	21665	0	0	77	0	0	0	0	1	100
	(4) CR-SM	3	15	29213	2050	7	104	3	100	0	0	0	0
	(5) CR-KM	4	20	29366	1502	5	104	3	75	1	25	0	0
	CR-M	8	40	28346	3204	11	101	6	75	1	12.5	1	12.5
	Total	20	100	28828	3204	11	102	14	70	2	10	4	20
LS-HSSC 2	(1) OR-LCM	4	20	29622	5105	17	105	1	25	0	0	3	75
(MB)	(2) OR-HCM	8	40	27731	1138	4	98	8	100	0	0	0	0
	OR-M	12	60	28361	2966	10	101	9	75	0	0	3	25
	(3) CR-TM	1	5	22470		0	80	0	0	0	0	1	100
	(4) CR-SM	3	15	27813	1859	7	99	3	100	0	0	0	0
	(5) CR-KM	4	20	28393	3539	12	101	2	50	2	50	0	0
	CR-M	8	40	27435	3234	12	97	5	62.5	2	25	1	12.5
	Total	20	100	27991	3027	11	99	14	70	2	10	4	20

samples were normally distributed, with a predominance of results centered on a mean value and few results in the extremes of distribution.

Fig. 1 shows an overview of all the z-scores calculated from the data reported by the participant laboratories for the four samples selected. The general impression was that the majority of reported values were satisfactory.

In addition, Table 5 summarizes participants' results obtained for the different analytical methods used. Taking into consideration the great difference in the percentages of the analytical methods used, only a relative statement could be made. However, as in the 1st COD-PT[ADG], no major differences in the results reported were due to the analytical method used.

It is interesting that 8 participating laboratories (40% of total) reported the four samples satisfactorily, with 62.5%, 25.0% and 12.5% of the data coming from OR-HCM, CR-LCM and OR-LCM, respectively.

The z-score performance of each sample was evaluated as follows:

- Sample 1 (Gel): 13 laboratories (65%) reported satisfactory results, 5 laboratories (25%) reported questionable results, and only 2 laboratories provided unsatisfactory results (10%).
- Sample 2 (SewS): Upon analysis, this sample showed poorer results than the solid sample (Sample 1). 10 laboratories (50%) reported satisfactory results, 9 laboratories (45%) reported unsatisfactory results, and 1 laboratory (5%) gave doubtful results.
- Sample 3 (SuOC): 14 laboratories (70%) reported satisfactory results, 2 laboratories (10%) reported questionable results, and 4 laboratories (20%) provided unsatisfactory results.
- Sample 4 (MB): The z-score values were identical to those reported for Sample 3 [i.e. 14 laboratories (70%) reported satisfactory results, 2 laboratories

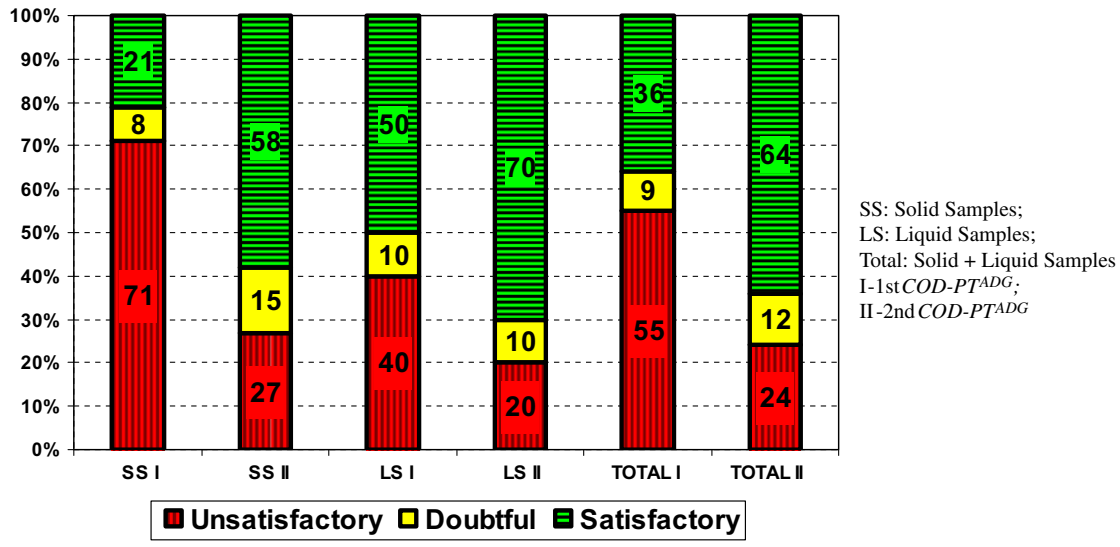

Figure 2. Comparison of z-score performance in 1st and 2nd COD-PT.

(10%) reported questionable results, and 4 laboratories (20%) provided unsatisfactory results].

The results can be outlined by the nature or characteristics of the substrate and finally grouped as total samples:

- Solid Samples: 23 z-scores (58%) were satisfactory, 11 z-scores (27%) were unsatisfactory, and 6 z-scores were doubtful (15%).
- Liquid samples with high concentrations of suspended solids: 28 z-scores (70%) were satisfactory, 8 z-scores (20%) were unsatisfactory, and 4 z-scores (10%) were doubtful.
- Total samples: 51 z-scores (64%) were satisfactory, 15 z-scores (24%) were unsatisfactory, and 14 z-scores (12%) were doubtful.

Although it is generally recognized that the analytical determination of COD samples may be ''relatively easy'' or ''relatively difficult'', it is very tempting to deduce a correlation between the type of sample analyzed and the analytical performance. For normal liquid samples (without suspended solids), the analysis of COD is considered an ''easy'' analytical determination. The results from the Aquacheck PT scheme, which ran for over 20 years, reported a percentage of acceptable results and a relative standard deviation of 91.4% and 5.8%, respectively [12]. The decrease in the overall performance of this PT scheme can be explained by considering the characteristics of the samples selected, which are potentially more difficult to analyze. However, we have no doubt that regular involvement in PT can improve the analytical performance of those laboratories taking part.

4.4. Comparisons with data from the 1st COD-PTADG

Generally, PT data are evaluated in the medium-to-long term. Although for the determination of COD in samples difficult to analyze, there have been only two PT schemes, the clear improvement in results reported could be used as ''short-term conclusions'', helping to do away with the generalized notion that solid samples and liquid samples with high concentration of suspended solids cannot be analyzed accurately, as was previously reported [13,14].

The data reported in both COD-PT schemes were summarized in terms of z-score values, and are presented in bar-chart form in Fig. 2 for graphical comparison. On the basis of the results obtained in the 2nd COD-PT^{ADG} and comparing them with the values reported in the 1st COD-PT^{ADG}, we can note that the overall performance of all participants can be considered quite satisfactory.

For solid samples, the z-scores considered unsatisfactory dropped dramatically from 71% to 27%, whilst the z-scores considered satisfactory increased from 21% to 58%. This means an improvement in the result of around 40%.

For liquid samples, the trend was also positive, with an increase in satisfactory results of around 20%.

The overall evaluation of results obtained showed that the participation in COD-PT schemes using solid samples and liquid samples with high concentrations of suspended solids improved the performance of participating laboratories by approximately 30%. This fact can be interpreted as a sign of general improvement, reinforcing the statement that the ability to produce results of acceptable quality for COD determination in ''relatively difficult'' samples seems possible.

Another indicator of the improvement in COD determination was the number of laboratories that reported the four samples satisfactorily. That 8 laboratories (40% of total) reported adequately in the 2nd PT-COD^{ADG}, compared to 2 laboratories (8% of total) in the 1st PT-COD^{ADG}, shows evident improvement.

Similar trends of overall performance improvements with participation in PT schemes were described by:

i) Whetton and Finch for some analytes of the Aquacheck PT, including COD [12];
ii) Gaunt and Whetton for analytes from alcoholic and non-alcoholic beverage industries [15];
iii) Key et al. for foods and feeds [16]; and,
iv) Earnshaw et al. for riboflavin (vitamin B_2 analysis) [5].

Nobody questions the value of PT schemes, and it is universally agreed that a well-founded laboratory must participate regularly in relevant PT. Although further research will be necessary before coming to any firm conclusion, it is foreseeable that future COD-PTs will see further potential increases in COD analytical performance, achieving satisfactory z-score values of around 90% for all the new samples distributed.

5. Conclusions

The 2nd COD-PT^{ADG} provided a valuable opportunity for evaluating the general performance of COD determination using samples considered ''difficult'' to analyze. The general performance of participating laboratories was acceptable, with 64% of the z-score values reported considered satisfactory. More significant was the improvement in results compared with the 1st COD-PT^{ADG}. Specifically, the improvement in the z-score values reported for solid samples and liquid samples with high concentrations of suspended solids was 40% and 20%, respectively. The results obtained demonstrated once more how participation in PT is successful as a way to achieve a good QC within laboratories involved in this type of chemical determinations.

Acknowledgements

The PT coordinator gratefully acknowledges the interest shown by all the participating laboratories and their excellent contribution to the 2nd COD-PT^{ADG}. The extra time-consuming task undertaken for the different analysts who participated in the study is also highly appreciated. Without their efforts, this article would not have been possible.

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2.2.3 Determinación de proteínas

Para la determinación de este parámetro se aplicó el método tradicional para el análisis de proteínas, método Kjeldahl mediante la determinación del nitrógeno orgánico. Este método fue desarrollado en 1883 por el investigador danés Johann Kjeldahl.

En esta determinación se digieren las proteínas y otros componentes orgánicos de la muestra objeto de estudio en una mezcla con ácido sulfúrico en presencia de catalizador. Muchos catalizadores son válidos, como el mercurio, cobre o el cobre/ titanio. La elección del catalizador dependerá de la dificultad de ruptura de los péptidos en la proteína de la muestra a ser analizada y problemas medioambientales asociados con la eliminación de los residuos que contiene el catalizador. El nitrógeno orgánico total se convierte mediante esta digestión en sulfato de amonio. La mezcla digerida se neutraliza con una base y se destila posteriormente. El destilado se recoge en una solución de ácido bórico. Los aniones del borato así formado se titulan con HCl (o H_2SO_4) estandarizado para determinar el nitrógeno contenido en la muestra.

En concreto para el análisis se utilizó Sulfato de Cobre penta-hidratrado ($CuSO_4 \cdot 5H_2O$) como catalizador y HCl como agente valorador.

Las reacciones que se llevan a cabo en cada fase del método son:

Digestión: Proteína(s) + H_2SO_4(c) + Catalizador(s) $\rightarrow CO_2(g) + H_2O(g) + NH_4HSO_4(ac)$

Liberación del NH_3: $NH_4HSO_4(ac) + 2NaOH(ac) \rightarrow NH_3(g) + Na_2SO_4(ac) + H_2O(g)$

Destilación: $NH_3(g) + H_2O(g) \rightarrow NH_4OH(ac)$

Recolección: $NH_4OH(ac) + H_3BO_4(ac) \rightarrow NH_4H_2BO_4 + H_2O$

Titulación: $NH_4H_2BO_4(ac) + HCI(ac) \rightarrow NH_4CI(ac) + H_3BO_4(ac)$

El método Kjeldahl ha sufrido varias modificaciones. Originalmente se utilizó permanganato de potasio para llevar a cabo el proceso de oxidación (digestión), sin embargo, los resultados no fueron satisfactorios, de manera que este reactivo se descartó. En 1885 Wilforth encontró que se podía acelerar la digestión utilizando ácido sulfúrico y añadiendo un catalizador. Gunning en 1889 propuso añadir sulfato de potasio que eleva el punto de ebullición del ácido sulfúrico utilizado en la digestión para disminuir el tiempo de la reacción [4]. Por lo tanto, el procedimiento de esta técnica es más correctamente conocido como Método Kjeldahl-Wilforth-Gunning.

El contenido total de proteínas se puede obtener de forma aproximada, multiplicando la concentración de nitrógeno orgánico, diferencia entre el nitrógeno Kjeldahl y el nitrógeno amoniacal, por el factor adecuado según la naturaleza de la muestra, para nuestro caso 5.5 [5].

El digestor utilizado fue de Selecta modelo Bloc Digest 6, con un destilador semiautomático Selecta Pronitro I.

Figura 2.2.- Equipos utilizados para la determinación de proteínas.

El método de referencia usado para realizar este análisis ha sido:
A.O.A.C. 1980. Association of Official Agricultural Chemists. Official Methods of Analysis. Washington, D.C. [6].

2.2.4 CONTENIDO EN FIBRA

La fibra es una mezcla no homogénea de varias macromoléculas. La mayoría de ellas son polisacáridos estructurales como celulosa, hemicelulosa y pectina, pero también no carbohidratos como lignina, proteínas no digeribles son componentes que pueden considerarse como constituyentes de la fibra.

Los términos más comunes utilizados, basados en las técnicas analíticas, son Fibra Neutro Detergente (NDF), Fibra Acido Detergente (ADF) y Lignina Acido Detergente (ADL). Estos métodos se basan en pasos secuenciales de tratamientos químicos para solubilizar los componentes que no son fibra y en la determinación final del residuo obtenido. Dependiendo de qué determinación se aplique, diferentes constituyentes serán determinados en los residuos. El análisis de estos compuestos ha sido realizado basándose en la determinación de fibra según Van Soest et al. [7].

Fibra neutro detergente (NDF) se define como el residuo que queda después de un tratamiento con una solución detergente neutra. En este procedimiento la muestra se somete a ebullición durante una hora con detergente neutro. La adición en este proceso de una solución enzimática de α-amilasa ayuda a degradar el posible almidón que pueda contener la muestra objeto de análisis y para la degradación de posible proteína existente en la muestra se añade sulfito sódico. El residuo es secado y calcinado. La

reducción de peso producida hasta la calcinación es el contenido de hemicelulosa, celulosa y lignina de la muestra.

La Fibra Ácido Detergente (ADF) es el residuo resultante después del tratamiento de un detergente ácido sobre el residuo obtenido de la determinación de NDF (método secuencial) o sobre la muestra directamente (método no secuencial). La muestra es sometida a ebullición con detergente ácido durante una hora y posteriormente secada y calcinada. La reducción de peso sufrida hasta la ceniza es el contenido de celulosa y lignina de la muestra.

Lignina Ácido Detergente (ADL) es definida por ser el residuo que permanece después de someter el residuo del análisis de ADF a una extracción con H_2SO_4 al 72% (w/w) para eliminar la celulosa. Posteriormente es secada y calcinada y la reducción de peso hasta la incineración es el contenido de lignina insoluble de la muestra.

La realización de estos análisis fue llevada a cabo siguiendo la norma:
Official Methods of Analysis. 1990. Association of Official Analytical Chemists, 15th Edition [6].

Para la realización de estos ensayos se utilizó el equipo Dosifiber de Selecta con crisoles con placa filtrante de tamaño de poro 2 (Φ= 60-120μm).

Figura 2.3.- Equipo utilizado para la determinación del contenido en fibra.

2.3 MÉTODOS ANALÍTICOS: MATRICES LÍQUIDAS

2.3.1 Medidas Directas

2.3.1.1 pH

El pH es la forma más común de expresar la concentración del ión hidrógeno en soluciones acuosas, y se define como el logaritmo negativo de la concentración de iones H^+ expresando la concentración de dichos iones en mol/L.

Se midió directamente sobre la muestra, de acuerdo con el método 4500B *Standard Methods for Examination of Water and Wastewater (APHA; 1998)* [8] con un electrodo de vidrio con sistema de referencia Ag/ AgCl marca y modelo Crison 52-11, conectado a un medidor de pH/mV Crison pH-Burette 24. El equipo fue calibrado con disoluciones tampón estándar CRISON de pH 7,02 y 4,01 a 20ºC. La precisión de la lectura fue de ±0,01.

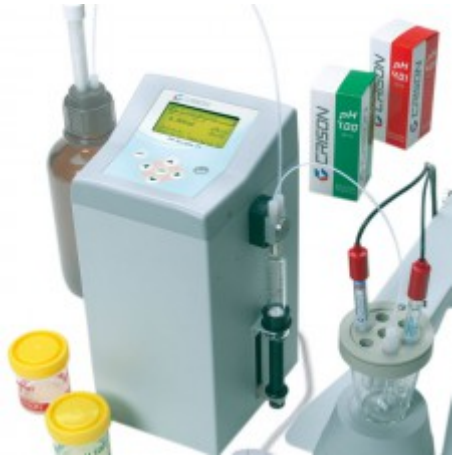

Figura 2.4.- Titrador automático.

2.3.1.2 Sólidos totales (TS) y sólidos volátiles (VS)

Se procedió siguiendo el método propuesto en Standard Methods for Examination of Water and Wastewater (APHA; 1998) [8].

2.3.1.3 Demanda química de oxígeno total (CODt)

Se analizó siguiendo el método descrito anteriormente en el apartado de matrices sólidas directamente sobre la muestra [3].

2.3.2 MEDIDAS EN MUESTRAS TRATADAS

Otras muestras líquidas fueron analizadas tras ser sometidas previamente a centrifugación a 10000 rpm y separación del sobrenadante mediante doble filtrado, en primer lugar a través de un filtro de fibra de vidrio (1,2 μm de tamaño de poro) y en segundo a través de filtro de acetato de celulosa (0,45 μm de tamaño de poro).

2.3.2.1 Demanda química de oxígeno soluble (CODs)

Se utilizó el método de reflujo cerrado *5220 C* descrito en *Standard Methods for Examination of Water and Wastewater (APHA; 1998)*[9].

El procedimiento se basa en la oxidación de la materia utilizando dicromato potásico como oxidante en presencia de ácido sulfúrico e iones plata como catalizador. La disolución acuosa se calienta bajo reflujo durante 2 h a 150 °C. Se evalúa colorimétricamente el consumo de oxígeno por medida de absorbancia a 600nm de longitud de onda. Para el cálculo de la cantidad de oxigeno disuelta se interpola la absorbancia obtenida en la recta de calibrado previamente realizada con muestras de patrón primario, ftalato ácido de potasio (KH_2PO_4), obteniendo así los mgO_2/l contenidos en la muestra.

Para la etapa de digestión se utilizó un termobloque Multiplaces de Selecta. La medición espectrofotométrica se realizó en un espectrofotómetro visible modelo Genesys 10 Vis de Thermo Electronic Corporation.

Figura 2.5.- Equipos utilizados para la determinación de CODs.

2.3.2.2 Alcalinidad parcial (PA), alcalinidad total (TA)

La alcalinidad es un parámetro indicador de la capacidad para neutralizar ácidos de una muestra y constituye la suma de todas las bases valorables. Mide la capacidad tamponante del medio aportada por sales y ácidos débiles. La alcalinidad se debe principalmente a las sales de ácidos débiles, aunque las bases débiles o fuertes también pueden contribuir, siendo el bicarbonato la forma química que más aporta a la alcalinidad.

La alcalinidad total (TA) es la capacidad del agua de neutralizar ácidos. Representa la suma de todas las bases titulables. La alcalinidad parcial (PA) corresponde a la suma de todos los hidróxidos y la mitad de los carbonatos.

Las mediciones se realizaron en base a el método *2320* de *Standard Methods for Examination of Water and Wastewater (APHA, 1998*) [9], con variaciones propuestas por Hill y Jenkins (1989) [10], realizando una valoración en dos pasos, la primera hasta pH 5,75, que se ajusta mucho mejor al valor real de alcalinidad debida al bicarbonato, y posteriormente hasta un valor de pH de 4,3. La valoración se realizó potenciometricamente con ácido sulfúrico 0.02N.

Para su determinación se utilizó el tritrador automático Crison pH-Burette 24 y un electrodo de vidrio con sistema de referencia Ag/ AgCl Crison 52-11, mostrados en la Figura 2.4.

2.3.2.3 Nitrógeno amoniacal (NH_x)

El nitrógeno amoniacal se encuentra en solución acuosa, en forma de ión amonio o como amoniaco (forma no ionizada), en función del pH de la solución y de la temperatura.

El nitrógeno amoniacal se ha analizado por el método *4500-NH_3 B* de *Standard Methods for Examination of Water and Wastewater (APHA, 1998)* [9], por destilación de la muestra, con el destilador Pronitro I de Selecta mostrado en la Figura 2.2, con una valoración con ácido clorhídrico 0.02N del destilado recogido en una disolución de ácido bórico al 2% utilizando indicador mixto como indicador.

2.3.2.4 Ácidos grasos volátiles (AGV)

Se consideran ácidos grasos volátiles los ácidos: acético propiónico, i-butírico, n-butírico, i-valérico, y n-valérico.

Fueron analizados por cromatografía de gases, utilizando un cromatógrafo marca Shimadzu, modelo GC-2010 con detector de ionización de llama (FID) a 250ºC, conectado a un inyector automático de la misma marca y un carrusel con capacidad para 20 muestras.

Las calibraciones fueron realizadas por estándar externo (Supelco®) construyendo una curva de calibración para cada componente a cuantificar.

Figura 2.5.- Cromatógrafo utilizado.

La columna usada para la separación fue una columna capilar de fase fija de Nukol (polietilenglicol modificado con ácido tereftálico) de 30 m de longitud y 25 mm de diámetro interno y 25 μm de película. Como gas portador se utilizó nitrógeno con un caudal de 42.1 mL/min y 75.5 KPa. Se utilizó aire sintético (400mL/min y 75 kPa) e hidrógeno (40mL/min y 60 kPa) como mezcla de gases para ignición de la llama.

Las características del método aplicado para la determinación fueron:

- Inyección automática de 1μL de muestra con Split 1:25 y purga de 5ml/min.
- Programación de temperaturas en el horno, con rampa de temperatura de 30ºC/min hasta 150ºC y rampa de temperatura de 15ºC/minuto hasta alcanzar 180ºC.

Se analizaron las concentraciones de ácidos: acético (C2), propiónico (C3), isobutírico (iC4), butírico (C4), isovalérico (iC5), valérico (C5), isocaproico (iC6), caproico (C6), y enántico (C7). Las muestras fueron previamente acidificadas con ácido fosfórico de concentración 1:2 (v/v) y filtradas a través de filtros de fibra de vidrio de 0.45 μm de tamaño de poro Millipore GVWP025000. Como patrón interno se usó ácido crotónico.

2.4 POTENCIAL BIOQUÍMICO DEL METANO (BMP)

La prueba o test del potencial bioquímico del metano (BMP) es un método ampliamente utilizado para estimar la cantidad de metano que puede producirse a partir de la digestión anaeróbia de residuos

orgánicos, siendo el valor teórico máximo de generación de metano de 0.35 m^3 CH_4/kg COD eliminado en condiciones estándar de presión y temperatura [11].

Esta técnica se emplea para determinar la capacidad metanogénica de un cultivo anaerobio sobre un sustrato y para evaluar la idoneidad de un determinado efluente para generar metano. Es un ensayo fundamental para el control y optimización de sistemas de tratamiento anaerobio de residuos.

Dicho ensayo, consiste en analizar la acción de un grupo de microrganismos anaerobios para transformar compuestos orgánicos en compuestos como el metano. Para ello se mide el volumen de gas generado por un cultivo a escala de laboratorio a partir de la transformación de la materia orgánica, expresada en términos de DQO, por día y por gramo de solidos volátiles añadidos. El gas generado en la digestión anaerobia está formado mayormente por metano (60- 70%), dióxido de carbono (20- 30%) y pequeñas cantidades de hidrógeno y trazas de sulfuro de hidrógeno (H_2S), amoníaco y vapor de agua. El contenido de energía del biogás está relacionado directamente con su concentración en metano, el cual tiene un contenido energético teórico de 37 MJ/m^3 (994 BTU/ft^3). Para estos ensayos se mantienen constantes algunos factores como la temperatura, pH, alcalinidad y disponibilidad de macro y micronutrientes.

Con esta herramienta es posible evaluar la actividad de la biomasa de un reactor biológico, permitiendo conocer el nivel máximo de carga orgánica que puede procesar en condiciones operativas óptimas. Por tanto la información aportada por la medición de la actividad metanogénica o BMP es fundamental para sistemas biológicos tanto a escala laboratorio como industrial.

Para asegurar la correcta realización del ensayo BMP hay que llevar a cabo el seguimiento de algunos parámetros fisicoquímicos que pueden considerarse claves en el proceso anaerobio.

Los trabajos de Owen et al. [12] y Chandler et al. [13] fueron pioneros en el desarrollo de la metodología de BMP como herramienta para la evaluación de la conversión de determinados sustratos en metano, sin embargo, aún existen diferentes protocolos a nivel mundial para esta medición, los cuales presentan diferencias en términos de la concentración de inóculo, tipo, concentración del sustrato, relación inóculo/sustrato (ISR), tipo y concentración de nutrientes, tiempo de incubación, ect. La ausencia de un método estandarizado dificulta la replicación o comparación de resultados obtenidos en diferentes estudios y limita la aplicación y difusión del ensayo BMP como herramienta de control de los procesos anaerobios.

Para cuantificar la producción de metano, existen métodos sofisticados con medición manométrica o cromatográfica o simples como el uso de mediciones volumétricas. El primero demanda equipos con específicos (ej. Sistema OXITOP® para ensayos de actividad metanogénica (AME)) mientras que el segundo solo requiere de un montaje relativamente sencillo. Teniendo en cuenta la facilidad de

implementar mediciones de BMP por el método volumétrico, el cual asegura la correcta realización del ensayo se escogió este tipo de medición.

El método volumétrico se basa en la cuantificación del volumen de metano producido mediante el uso de un agente desplazante, como el NaOH o el KOH por su propiedad de reaccionar con el CO_2 presente en el biogás, permitiendo una medición correcta del volumen de metano producido. Para garantizar la captación del CO_2 producido, el pH del NaOH debe ser superior a 12 unidades. Las reacciones que tienen lugar son las siguientes:

$$H_2O + CO_2 \rightarrow H_2CO_3$$

$$H_2CO_3 + 2NaOH \rightarrow Na_2CO_3 + 2H_2O$$

$$CO_2 + 2NaOH \rightarrow Na_2CO_3 + H_2O$$

Para llevar a cabo el ensayo BMP es necesario contar con un equipo experimental adecuado y controlar los siguientes elementos: inóculo o lodo anaerobio y cantidad de sustrato añadido (ISR), determinándose la evolución del metano acumulado en función del tiempo.

- Equipo experimental

En los ensayos descritos en la literatura se observa el uso de diferentes configuraciones de equipos de desplazamiento de líquido, siendo el usado en este trabajo, el mostrado en las Figura 2.7 y 2.8:

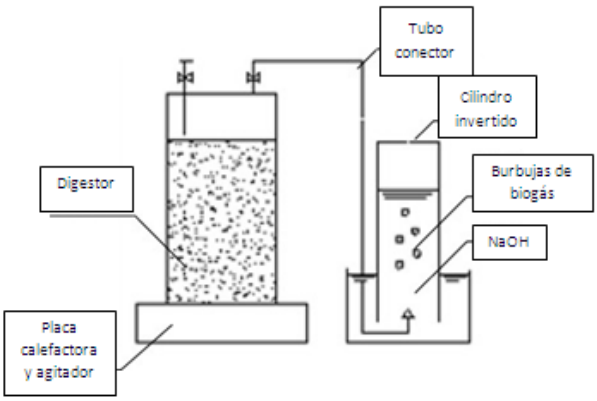

Figura 2.7.- Esquema equipo volumétrico usado.

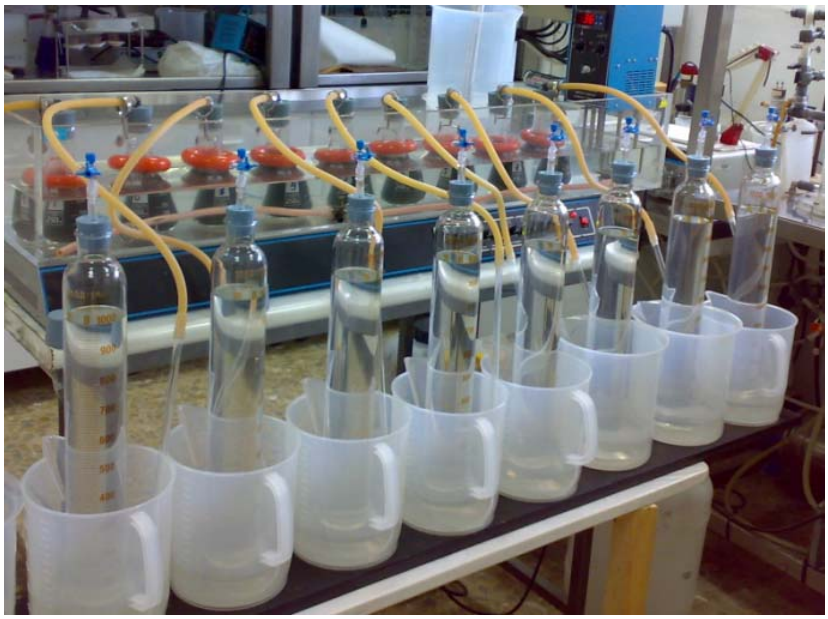

Figura 2.8.- Configuración del equipo de BMP usado.

La ubicación de los reactores en una altura superior a los depósitos de las disoluciones de NaOH evita que, en caso de ocurrir succión de NaOH por diferencia de presiones, el reactor se vea afectado.

- Inóculo

El inóculo deberá ser caracterizado previamente determinándose su contenido en sólidos (ST y SV). El volumen de lodo a adicionar se calcula considerando que la mezcla de inóculo y sustrato no debe sobrepasar el 80% del volumen útil del reactor biológico. Al inóculo se le añadió una disolución de macro y micro nutrientes descrita en Raposo et al. [14]. También se adicionó bicarbonato de sodio para regular el pH del medio y dotar al sistema de alcalinidad para asegurar la estabilidad del mismo. La presencia de oxígeno en los reactores fue eliminada burbujeando en el interior nitrógeno gaseoso. Posteriormente los reactores fueron cerrados herméticamente y se mantuvieron a una temperatura de 37ºC con agitación constante. Se mantuvo en este modo 24 horas antes de ser alimentados con el sustrato objeto de estudio en cada caso para asegurar la estabilidad del sistema.

- Control del experimento

Después de alimentar los reactores, se midió diariamente el volumen de gas generado mediante el sistema de desplazamiento de volumen con una probeta graduada invertida. Posteriormente se hicieron las correcciones de volumen necesarias para llevar los registros a condiciones normales de presión y temperatura (TPN). Se trabajó con un promedio de duplicados y con reactores blanco, sin sustrato, para sustraer la producción no debida al sustrato y con reactores control con cantidades conocidas de almidón como sustrato para controlar el correcto funcionamiento del ensayo. Los volúmenes de gas generado se

representaron gráficamente en forma acumulada en función del tiempo, obteniendo así curvas crecientes de tipo exponencial.

Durante ejecución de la presente Tesis Doctoral, se desarrollaron dos trabajos directamente relacionados con el método BMP que han sido publicados en revistas científicas, de los cuales se presentan las correspondientes copias:

- *Feasibility of sunflower oil cake degradation with three different anaerobic consortia*

Rincón, B., Del Carmen Portillo, M., González, J.M., Fernández-Cegrí, V., De La Rubia, M.A., Borja, R.

(2011) Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering, 46 (12), pp. 1409-1416.

- *Anaerobic digestion of solid organic substrates in batch mode: An overview relating to methane yields and experimental procedures*

Raposo, F., De La Rubia, M.A., Fernández-Cegrí, V., Borja, R.

(2012) Renewable and Sustainable Energy Reviews, 16 (1), pp. 861-877.

Journal of Environmental Science and Health, Part A (2011) **46**, 1409–1416

ISSN: 1093-4529 (Print); 1532-4117 (Online)
DOI: 10.1080/10934529.2011.607032

Feasibility of sunflower oil cake degradation with three different anaerobic consortia

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Sunflower oil cake (SuOC) is the solid by-product from the sunflower oil extraction process and an important pollutant waste because of its high organic content. For the anaerobic digestion of SuOC three different industrial reactors were compared as inoculum sources. This was done using a biochemical methane production (BMP) test. Inoculum I was a granular biomass from an industrial reactor treating soft-drink wastewaters. Inoculum II was a flocculent biomass from a full-scale reactor treating biosolids generated in an urban wastewater treatment plant. Inoculum III was a granular biomass from an industrial reactor treating brewery wastes. The highest kinetic constant for methane production was achieved using inoculum II. The inoculum sources were analyzed through PCR amplification of 16S rRNA genes and fingerprinting before (t = 0) and after the BMP test (t = 12 days). No significant differences were found in the bacterial community fingerprints between the beginning and the end of the experiments. The bacterial and archaeal communities of inoculum II were further analyzed. The main bacteria found in this inoculum belong to Alphaproteobacteria and Chloroflexi. Of the Archaea detected, Methanomicrobiales and Methanosarcinales made up practically the whole archaeal community. The results showed the importance of selecting an appropriate inoculum in short term processes due to the fact that the major microbial constituents in the initial consortia remained stable throughout anaerobic digestion.

Keywords: Sunflower oil cake, biochemical methane potential, microbial community, fingerprints, methane yield, kinetics.

Introduction

Sunflower oil cake (SuOC) is the solid waste generated during the sunflower seed oil extraction process. World sunflower seed production ranged between 29.1 and 31.1 million tonnes over the last few seasons.[1] As a result, large quantities of SuOC are generated every year. In Spain alone, between 4 and 5 million tonnes of this by-product are produced, giving rise to an important environmental issue.[2] Current perspectives on how to obtain high-value products from wastes involve anaerobic digestion processes for biogas generation [(a mixture of methane and carbon dioxide with a high energetic value (21.4 MJ per m^3)].

These anaerobic processes are performed by complex groups of microorganisms (Bacteria and Archaea) which coordinate the degradation of organic matter. A relatively low percentage of these microorganisms present in anaerobic digestion processes have been isolated. This lack of knowledge results sometimes in malfunctions and unexplainable failures of biogas fermenters. For these reasons, it must be analyzed in more detail.[3] Only a few studies have considered the potential influence of inoculum in anaerobic digestion systems. Moreno-Andrade and Buitrón[4] studied the influence of five different inocula on an anaerobic biodegradability test of two different substrates, one easily degradable (glucose) and the other toxic (phenol).

These authors emphasized the importance of using the appropriate inoculum to obtain satisfactory results from anaerobic processes. After testing two different inocula, granular and suspended, Pereira et al.[5] found granular inoculum to be the best option for the anaerobic treatment of synthetic oleic acid-based effluent, since the methanogenic activity of the granular inoculum was 2–7 times higher than that of the suspended biomass and was more resistant to long chain fatty acid toxicity. Foster-Carneiro et al.[6] compared six different inoculum sources for the anaerobic thermophilic digestion of the organic fraction of municipal solid wastes. Tabatabaei et al.[7] studied the importance of the microbial community, focusing on the methanogenic archaea in the anaerobic digestion of brewery wastewater, palm oil mill effluents, dairy wastes, cheese whey, dairy wastewater, pulp and paper wastewaters and olive oil mill wastewaters with respect to their dominant methanogenic population.

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Received January 20, 2011.

During the process of anaerobic digestion it is expected that the microbial communities adapt as a consequence of the growth of microorganisms under the specific conditions of digestion and the substrate treated. The dynamics of the acetoclastic methanogenic community have been evaluated under the influence of different wastewater compositions and even under inhibitory conditions.[8–10] The microbial community structure has been studied under low temperature conditions and under the influence of metal supplementation.[11–13] However, the transformations which occur in the microbial communities during the anaerobic digestion of organic wastes and methane production are still not fully understood.

It is clear that the efficiency of biogas production during the anaerobic digestion of organic residues depends on the microorganisms involved in the process. The study of these microbial communities represents an important step towards understanding and optimizing these anaerobic treatments. Thus, the aim of this work was to study the influence of the inoculum type on the anaerobic digestion of SuOC in terms of methane production. Microbial community fingerprints from the initial inoculum source and after the biochemical methane potential test (BMP) were compared, determining the major components of the communities involved in the process to achieve the best methane production kinetics.

Materials and methods

Substrate

The substrate used in this study was SuOC. Prior to the experiments, a study of the different particle sizes present in this solid waste was carried out by separation with a mechanical sieve. The most abundant size found (29.4 %) was 0.7–1.0 mm. Consequently, this size was used in the experiments. Table 1 shows the full composition and main features of the SuOC used in this study (mean values are averages of four determinations).

Inocula

Three different inoculum sources were used: a) an anaerobic granular inoculum derived from a full-scale upflow anaerobic sludge blanket (UASB) reactor treating wastewaters from a soft-drinks industry (I); b) a flocculent anaerobic inoculum from a full-scale completely stirred tank reactor (CSTR) treating biosolids from a conventional urban wastewater treatment plant (II); and c) an anaerobic granular inoculum from a UASB reactor treating brewery wastes (III). Table 2 shows the main characteristics of these three inocula. The experiments were carried out at an inoculum:substrate ratio of 2:1. An inoculum concentration of 15 g VS L^{-1} was used for each reactor.

Table 1. Characteristics of the SuOC used as substrate.

*Parameter**	*Value ± SD***
Moisture (%)	8.0 ± 0.5
Total protein (%)	31.4 ± 1.6
Fats (%)	1.7 ± 0.1
Carbohydrates (%)	58.7 ± 2.6
Hemicellulose (%)	9.2 ± 0.5
Lignin (%)	9.5 ± 0.4
Cellulose (%)	21.7 ± 1.1
TS (%)	93.4 ± 1.9
MS (%)	6.6 ± 0.1
VS (%)	86.5 ± 1.3
TCOD (g O_2 g^{-1} TS dry basis)	1.08 ± 0.04
C (%)	43.6 ± 0.3
H (%)	6.2 ± 0.1
N (%)	4.6 ± 0.6
O (%)	45.6 ± 0.5

*TS: total solids, MS: mineral solids, VS: volatile solids, TCOD: total chemical oxygen demand. **SD: standard deviation.

Reactors and operational conditions

The experiments were carried out in a thermostatized water bath (35°C) in batch mode. The reactors were stirred at 250 rpm with a magnetic stirrer. The BMP test was run by triplicate. Two controls without substrate were added in each run. A final working volume of 250 mL was used for each treatment. Methane production was measured by a NaOH solution (3N) displacement (CO_2 produced in the anaerobic process was kept in this sodium hydroxide solution).

Experimental setup

The experiment was carried out by triplicate and two control reactors with no substrate added were run for each different inoculum. The reactors were filled with 15 g VS L^{-1} of inoculum, the corresponding quantity of SuOC to reach a ratio of 2:1 inoculum to substrate, 25 mL of a 50 g $NaHCO_3$ L^{-1} solution to keep pH stable, 50 mL of nutrient solution (Table 3) and distilled water to a total volume of 250 mL. Methane production was measured for a period of 12 consecutive days.

Table 2. Characteristics and origin of the inoculum sources used in the experiments.

Sludge	*Source (reactor type)*	*Reactor volume (m^3)*	*pH*	*TS ($g\ L^{-1}$)*	*VS ($g\ L^{-1}$)*
I	UASB	450	7.4	30	25
II	CSTR	2000	7.6	43	20
III	UASB	550	7.5	83	47

TS: total solids; VS: volatile solids; UASB: upflow anaerobic sludge blanket; CSTR: continuously stirred tank reactor.

Table 3. Composition of the nutrient and trace element solutions used.

Nutrient solution composition	*Concentration (g L^{-1})*
NH_4Cl	1.4
K_2HPO_4	1.25
$MgSO_4$ H_2O	0.5
$CaCl_2$ $2H_2O$	0.05
Yeast extract	0.5
Trace element solution	5.0[a]
Trace element solution composition	*Concentration (mg L^{-1})*
$FeCl_3 4H_2O$	2000
$CoCl_2 \cdot 6H_2O$	2000
$MnCl_2$ $4H_2O$	500
$CuCl_2$ $2H_2O$	38
$ZnCl_2$	50
H_3BO_3	50
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	50
$AlCl_3$ $6H_2O$	90

Units for the trace element solution added to the nutrient solution are in mL of trace solution per L of nutrient solution (mL L^{-1}).

Analytical methods

Solids and moisture were determined according to the standard methods 2540B and 2540E.[14] Total chemical oxygen demand was determined using the solid substrate open reflux method.[15] Total protein was determined by multiplying the total Kjeldahl nitrogen (TKN) value by 6.25.[16] Fat content was extracted by a Soxhlet system using hexane (UNE-EN-ISO 659:2000). Cellulose, hemicellulose and lignin were determined by the Goering and Van Soest method.[17]

The elemental composition of the SuOC (C, N, O and H) was measured using a Leco CHNS-932 (Leco Corporation, St Joseph, MI, EEUU) elemental analyzer. For particle size selection the sunflower oil cake was sieved using a mechanical sieve (bio-meta, Retsch).

Methane production kinetics

A first-order kinetic model was used to estimate the specific rate constant according to Chen-Hashimoto Equation 1:[18]

$$B = B_o\,[1 - \exp(-k\,t)] \qquad (1)$$

where: B is the methane yield (mL CH_4 g^{-1} VS added), B_o is the ultimate or maximum methane yield, asymptote to the production curve *versus* time, k (day^{-1}) is the specific rate constant, and t is the digestion time (days). Methane yield values (B) were calculated by subtracting methane produced by the controls (inoculum only) from their corresponding treatment reactors. These differences were divided by the VS of the substrate.[18] B_o and k were calculated from the experimental data by non linear regression using Sigmaplot 9.0 (Systat Software. Inc., San Jose, CA).

Molecular characterization of microbial communities

Microbial communities, both *Archaea* and *Bacteria*, were studied by molecular fingerprinting methods complemented with cloning and sequencing for the identification of the major components of the bacterial and archaeal communities. DNA was extracted using the Nucleospin Food DNA extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations. Fragments of the 16S ribosomal RNA (16S rRNA) genes from the Bacteria and Archaea were amplified by PCR with different primer pairs. Fingerprints of the bacterial and archaeal communities were obtained by Denaturing Gradient Gel Electrophoresis (DGGE) following the method described by Muyzer et al.[19]

DNA was directly amplified by PCR using the primer pair 341F-GC (5′-CCT ACG GGA GGC AGC AG with a GC-rich tail attached to its 5′ end)[19] and 518R for the Bacteria and the primer pair 344F-GC (5′- with a GC-rich tail attached to its 5′ end) and 518R for the Archaea. Relative quantification of molecular fingerprints from pairs of community profiles was performed following the quantitative procedure described by Portillo and Gonzalez.[20] Gels obtained by DGGE were digitalized using Kodak 1D image analysis software (Kodak, New Haven, CT). The images were analyzed using the tnimage program (http://entropy.brneurosci.org/tnimage.html) applying its densitometry function. Comparisons between community fingerprints were carried out as described by Portillo and Gonzalez[20] calculating a Cramér-von Mises-type statistic through a Monte-Carlo test procedure to determine the significance of differences between microbial communities.

PCR products for 16S rRNA gene library construction were obtained with the primer pair 27F (5′-AGA GTT TGA TYM TGG CTC) and 907R (5′-CCC CGT CAA TTC ATT TGA GTT T) for the Bacteria[21] and the pair 20bF (5′-YTC CSG TTG ATC CYG CSR GA) and 1492bR (5′-GGY TAC CTT GTK WCG ACT T) for the Archaea.[22] These PCR products were purified with the PCR purification kit (JetQuick, Germany) and cloned using a TOPO-TA cloning kit (Invitrogen, Carlsbad, USA). The 16S rRNA libraries obtained were used to identify the major components of the bacterial and archaeal communities. A screening procedure based on the discrimination of clones using PCR-DGGE previously described by Gonzalez et al.[23] was applied to these libraries to identify the major DNA bands observed in DGGE analyses.

Sequence data were edited using Chromas software, version 1.45 (Technelysium, Tewantin, Australia). Homology searches from the nucleic acid sequences were performed using the Blast algorithm[24] at the NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/Blast/). Sequences were inspected for the presence of chimeras using the Ccode program as described by Gonzalez et al.[25]

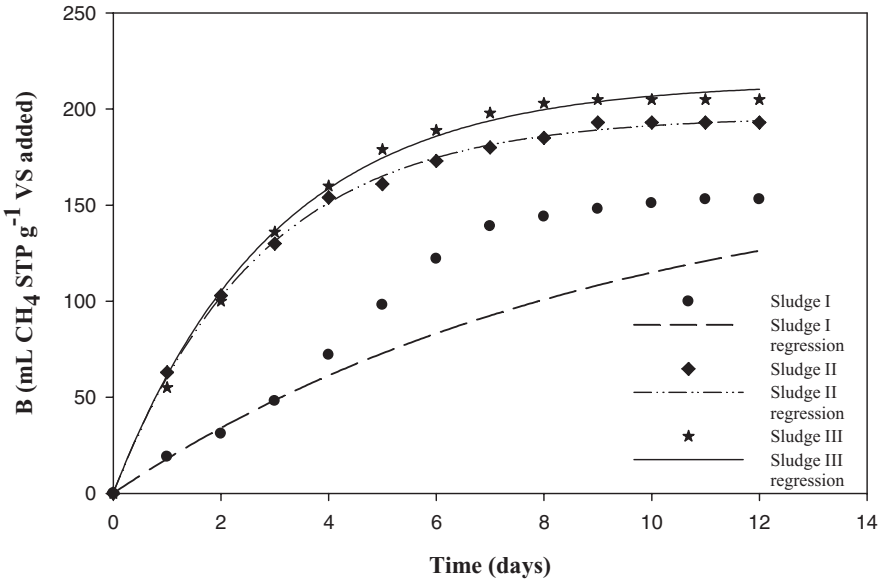

Fig. 1. Variation of the volume of methane produced per gram of VS added over time for inocula I, II and III.

Results and discussion

The volumes of methane (at standard temperature and pressure) obtained after 12 days of the BMP test for inocula II and III were higher than that obtained for inoculum I (293, 360 and 387 mL CH_4 for inocula I, II and III, respectively). Methane production for inoculum III was 7.5 % higher than for inoculum II and 31.1 % higher than for inoculum I. The experimental methane yields per gram of VS added (*B*) are shown in Figure 1. The best *B* values after 12 days were obtained for inocula II and III (193 and 205 mL CH_4 accumulated g^{-1} VS added, respectively), these yields being higher than that obtained for inoculum I (156 mL CH_4 accumulated g^{-1} VS added). The value of the methane yield for inoculum III was 6.2 % higher than for inoculum II, which in turn was 23.7 % higher than the value for inoculum I. The yield for inoculum III was 31.4 % higher than for inoculum I. Therefore, inocula II and III had similar methane yields and were both higher than for inoculum I.

The percentage of volatile solids removed was 42 % for inocula II and III and only 33 % for inoculum I. Inocula II and III from industrial reactors treating solid substrates showed better results than inoculum I from wastewater treatment. This could be attributed to the higher hydrolytic/enzymatic capacity of these inoculum sources which are used to break biosolids in urban wastewater treatment plants (inoculum II) and to treat brewery wastes (inoculum III).

The cellulose, lignin and hemicellulose structure of SuOC is complex. Cellulose is a polymer with low microbial degradability and is considered the rate-limiting substrate in the anaerobic digestion of solid wastes. [26]

In a comparative study for cellulose solubilisation in anaerobic reactors, O'Sullivan et al.[27] showed how differences in reactor configuration and operational conditions had no significant impact on the solubilisation rate of cellulose, whereas the difference in composition of the microbial communities showed a marked effect. This could be the reason why inoculum I, which had thus far been used to treat wastewaters, had given the worst results as regards methane production and kinetics for SuOC treatment. These findings should be studied in more detail.

The first-order kinetic model used to estimate the specific rate constants fit satisfactorily to the obtained experimental data (with R^2 values higher that 0.965; Fig. 1). The values obtained for k were 0.11±0.02, 0.37±0.01 and 0.34±0.01 $days^{-1}$ for inocula I, II and III, respectively (Table 4). Therefore, the specific rate constant for inoculum II was 8.8 % higher than that achieved for inoculum III and 236.4 % higher than that obtained for inoculum I.

Figures 2 and 3 show the molecular fingerprints obtained by PCR-DGGE and represent the major components of the bacterial (Fig. 2) and archaeal (Fig. 3) communities from the different inoculum sources (I, II and III) used during this study. For inoculum II, the taxonomic affiliation and the accession numbers of the closest homologue for the major components of the bacterial and archaeal communities are given in Tables 5 and 6, respectively. Comparisons of fingerprints from the bacterial and archaeal communities for the three inoculum sources used in this study (Figs. 2 [A, C and E] and 3 [G, I and K]) showed distinctive banding patterns which would indicate distinct microbial communities among the three inocula, depending on their source.

Maximum methane production was reached after 9 days for inocula II and III and after twelve days for inoculum I. After 12 days' digestion time, the bacterial communities (Fig. 2 [B, D and F]) established in the anaerobic digestion process of the SuOC, showed similar fingerprinting profiles

Table 4. Values of B_o and k obtained using the Chen-Hashimoto equation for the three sludges studied and their variation coefficients.

Sludge	R^2	$B_0 \pm SD$ (*mL* CH_4 g^{-1} *SV added*)	$k \pm SD$ ($days^{-1}$)	VC_{B0} (%)	VC_k (%)
I	0.9648	172 ± 27	0.11 ± 0.02	15.5%	25.4%
II	0.9985	196 ± 1	0.37 ± 0.01	0.6%	2.1%
III	0.9964	214 ± 2	0.34 ± 0.01	1.1%	3.6%

SD: standard deviation; VC: variation coefficient.

to those of the bacterial communities in their respective inocula (Fig. 2 [A, C and E]) before the anaerobic process. Statistical comparison of fingerprints from the initially inoculated communities and the final communities after the BMP test showed no significant differences (Table 7) in the bacterial communities from the different inoculum sources used in this study.

After the anaerobic digestion process of sunflower oil cake (Table 7), no significant differences were found in the archaeal community fingerprints between the initial inoculum (Fig. 3 [I and K]) and inocula II and III (Fig. 3 [J and L]). However, significant differences were observed between the initial inoculum (Fig. 3 [G]) and the archaeal community developed (Fig. 3 [H]) in inoculum I. Despite this change in the structure of the archaeal communities in inoculum I, the major archaeal components remained as important members of the final (after the anaerobic digestion process) communities. Changes observed in specific archaeal phylotypes in inoculum I could be the cause of a reduced performance of the process when compared to the evolution of inocula II and III, which were maintained during anaerobic digestion.

The bacterial and archaeal communities from inoculum II where the inoculum showed optimum methane kinetic parameters, was studied in further detail to identify the major components of the communities implicated in the anaerobic digestion and methane production.

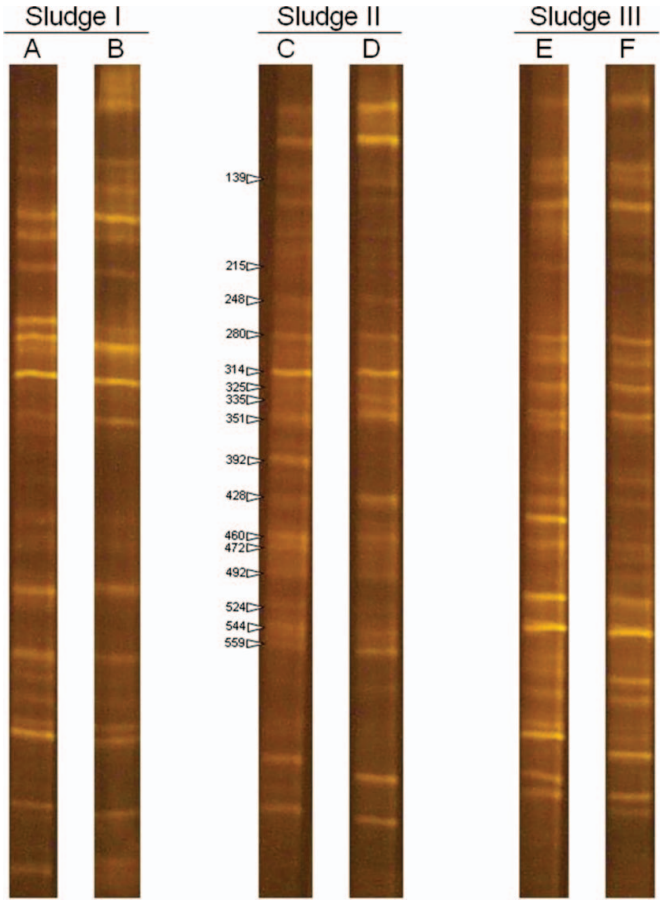

Fig. 2. Bacterial community fingerprints obtained by PCR-DGGE: (A, C, E) for the three different inoculum sources used for the initial inoculation of reactors and (B, D, F) after the BMP tests at the end of the anaerobic SuOC treatments (color figure available online).

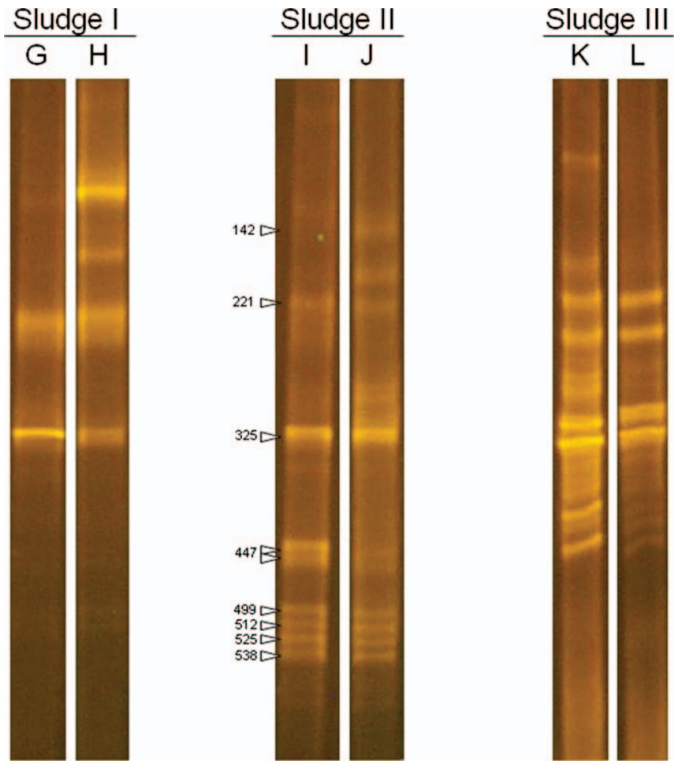

Fig. 3. Archaeal community fingerprints obtained by PCR-DGGE: (G, I, K) for the three different inoculum sources used for the initial inoculation of reactors and (H, J, L) after the BMP tests at the end of the anaerobic SuOC treatments (color figure available online).

Table 5. Accession numbers of closest homologue and proportions of the major bacterial phylotypes identified during this study determined through community fingerprinting analysis using PCR-DGGE from inoculum II.

Migration	*Taxonomic affiliation (accession no. of closest homologue)*	*Fraction inoculum**	*Fraction BMP**
139	Chloroflexi (CU926181)	3.4	3.8
215	Betaproteobacteria (GU454925)	1.9	0.8
248	Candidate Division WS6 (AF423183)	3.4	1.6
280	Chloroflexi (EF174275)	3.0	2.7
314	Chloroflexi (CU924314)	6.6	5.9
325	Actinobacteria (AY426438)	2.0	1.3
335	Alphaproteobacteria (AJ440751)	1.2	3.8
351	Alphaproteobacteria (GQ500763)	5.3	6.7
392	*Thauera*, Betaproteobacteria (DQ098974)	5.6	1.0
428	Bacteroidetes (CU922674)	2.7	6.1
460	*Paracoccus*, Alphaproteobacteria (FJ386516)	5.7	4.8
472	Chromatiales, Gammaproteobacteria (AM176837)	4.4	1.5
492	Thermoanaerobacteriales, Firmicutes (EU878332)	2.1	2.5
524	Synergistes, Synergistetes (FN436049)	2.4	1.4
544	Firmicutes (CU919983)	6.9	3.8
559	Bacteroidetes (AB330856)	2.6	5.4
	Total identified	59.2	53.1

*Percentage of total fluorescence intensity quantified from the banding pattern of PCR-DGGE analysis.

Table 6. Accession numbers of closest homologue and proportions of the major archaeal phylotypes identified during this study determined through community fingerprinting analysis using PCR-DGGE from inoculum II.

Migration	*Taxonomic affiliation (accession no. of closest homologue)*	*Fraction inoculum**	*Fraction BMP**
142	Methanosarcinales (FJ705109)	6.0	7.7
221	*Methanosaeta*, Methanosacinales (AB494241)	12.1	7.0
325	*Methanosaeta*, Methanosarcinales (FM162203)	20.5	28.8
447	Methanosarcinales (GU196156)	16.9	11.4
499	*Methanosaeta*, Methanosarcinales (EU591661)	6.4	6.3
512	Methanosarcinales (CU916012)	5.8	8.2
525	Methanomicrobiales (EU591675)	8.4	5.7
538	Methanomicrobiales (EU591675)	6.9	7.1
	Total identified	83.0	82.2

*Percentage of total fluorescence intensity quantified from the banding pattern of PCR-DGGE analysis.

Table 5 shows the proportion of the major bacterial constituents of the community in inoculum II. Alphaproteobacteria (20.6 % and 28.8 % of the total identified DNA in the inoculum and after anaerobic digestion, respectively), within the Rhodobacteraceae Family (e.g., *Paracoccus*), and Chloroflexi (22.6 % and 23.4 % of the total bacteria in the inoculum and in the community developed after anaerobic treatment, respectively) were the dominant bacterial groups. Proteobacteria, identified through members of the Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria, represented up to 40.7 % and 35 % of the identified bacteria in the inoculum and in the anaerobic digester, respectively. Other major bacterial groups identified in the community were Bacteroidetes (between 9.0 % and 21.7 % of identified bacterial phylotypes), Firmicutes (over 11 %; e.g., *Thermoanaerobacterium*), Actinobacteria (3.4 % to 2.5 %), Synergistetes (e.g., *Synergistes*) (above 2%), and Candidate Division WS6 (between 3.0 % and 5.7 % of the identified phylotypes).

The major bacterial components constituting the community of the anaerobic digestion process of sunflower oil cake coincide with the bacterial groups present in communities reported for other wastes.[22,28] Proteobacteria, Chloroflexi and Firmicutes have been reported as major components in bacterial communities during the anaerobic digestion processes of organic wastes.[22,29,30] Chloroflexi has recently been shown as a highly significant component in the transformation of complex substrates such as olive residues from oil production and this bacterial phylum is being increasingly recognized for its importance in anaerobic systems.[22,29–31] In these communities, numerous phyla, which are not well-known, such as the Bacteroidetes, Synergistetes and the Candidate Division WS6, were detected.

At present, there is limited knowledge about the metabolism of these phyla and they are generally detected only by their 16S rRNA gene sequences. Furthermore, there is little or no availability of representative cultivated microorganisms belonging to these bacterial phyla, which indicates that there is a significant portion of the bacterial community in need of further physiological research. The importance of Synergistetes, for instance, in anaerobic treatments has been highlighted in recent studies[32–33], as has the presence of Candidate Division WS6 in anaerobic

Table 7. Statistical results of the comparison between the microbial communities at the beginning (inocula) and ending of the anaerobic treatment of sunflower oil cake for the three types of inoculated sludges.

	Archaea		*Bacteria*	
Inoculated sludge	*P*	*CV (%)*	*P*	*CV (%)*
I	0.023*	0.098	0.170	0.093
II	0.188	0.081	0.211	0.079
III	0.542	0.046	0.316	0.068

P: Probability values; CV: coefficient of variation. Asterisk indicates significant differences ($P < 0.05$).

waste treatments and its relationship to methanogenic Archaea.[34]

Archaea are the microorganisms responsible for the production of methane. The archaeal communities represented by methanogenic groups constituted a critical component of the prokaryotic communities leading to methane production. Table 6 shows the proportion of the major archaeal phylotypes in inoculum II. The detected sequences from the archaeal community all corresponded to methane-producing Archaea. Different archaeal phylotypes were detected in the anaerobic digestion process of sunflower oil cake and belonged to the Methanosarcinales and Methanomicrobiales orders. The Methanosarcinales, mainly represented by different phylotypes belonging to the genus *Methanosaeta*, were the dominant methanogens, constituting over 67 % of the archaeal community.

A dominance of the methanogens Methanosarcinales and Methanomicrobiales has been previously reported as indicators of well-established methane-producing anaerobic digestion processes.[22, 35,36] These methanogens are acetoclastic methane producers and confirm the importance of this pathway in methanogenesis, as seen during the digestion of SuOC. As a consequence, a direct interaction between bacteria and archaea is envisioned, the main role of the bacterial community during this anaerobic process appeared to be the production of acetate from the polymers constituting the SuOC. This acetate is the major substrate which is directly utilized by the methanogenic archaea as the source for methane production.

Conclusions

The results obtained during this study underline the importance of using productive and active inoculum sources to initiate anaerobic digestion processes of sunflower oil cake wastes. Microbial communities showed no changes during short-term experiments (12 days). Obtaining the highest possible SuOC treatment efficiencies is a consequence of the conservation of the major components of well-established bacterial and archaeal communities during the digestion treatments. Only when an optimal inoculum is used can methane production and degradation of the processed substrate (i.e., SuOC) be maximized. A loss or reduction in specific phylotypes during the anaerobic treatments can be reflected by a diminishing efficiency both in methane production and organic load degradation.

Acknowledgments

The authors wish to express their gratitude to "Junta de Andalucía" ("Plan Andaluz de Investigación") for the financial support to groups BIO288 and AGR204, to the Spanish Ministry of "Ciencia and Innovación" (Project reference CTM2008-05772/ TECNO) and to the contracts JAE-Doc from "Junta para la Ampliación de Estudios del CSIC" co-financed by European Social Funds. The authors thank Dr. Fernando G. Fermoso for his valuable discussions during the preparation of the manuscript.

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Renewable and Sustainable Energy Reviews 16 (2011) 861–877

Contents lists available at SciVerse ScienceDirect

Renewable and Sustainable Energy Reviews

journal homepage: www.elsevier.com/locate/rser

Anaerobic digestion of solid organic substrates in batch mode: An overview relating to methane yields and experimental procedures

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ARTICLE INFO

Article history:
Received 5 April 2011
Accepted 8 September 2011

Keywords:
Anaerobic digestion
Batch tests
Biochemical methane potential (BMP)
Experimental conditions
Methane yield
Solid organic substrates

ABSTRACT

Anaerobic digestion is considered a competitive source for the production of renewable energy as far as efficiency and cost are concerned. To evaluate the anaerobic biodegradability of an organic substrate such as feedstocks, a test known as biochemical methane potential (BMP) has been commonly used. Current worldwide interest in using different organic substrates for anaerobic bioconversion is growing but there is a lack of clear references and comparability as a result of multiple factors that affect BMP determination. Several batch methods have been used to determine the methane potential. However, these technical approaches vary significantly from one reported method to the next another. In this review, the research works on the influence of different parameters of BMP determination have been discussed for critical and comparative evaluation. In addition, the extensive literature previously published dealing with BMP assays has been compiled and summarized focusing on two main subjects: firstly, methane yields of substrates, and secondly, the description of the various experimental procedures used to achieve the reported data.

Contents

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doi:10.1016/j.rser.2011.09.008

1. Introduction

Anaerobic digestion (AD) is a biochemical technological process for the treatment of organic substrates such as sewage and industrial effluents, animal manures and solid substrates (energy crops, agricultural residues and food wastes). This process has received increasing attention in recent years. It involves the degradation and stabilisation of complex organic matter by a consortium of microorganisms leading to an energy-rich biogas which can be used as renewable energy to replace fossil energy sources.

Literature shows that anaerobic digestion assays can be carried out in batch or continuous mode. Considering that continuous set-up is more laborious and time-consuming than batch tests, the latter have been more widely used. It is important to note that the batch approach can be used for three purposes: anaerobic biodegradability, inoculum activity and inhibition. These terms were defined with the aim of establishing a common terminology [1]. These three tests are based on the same principle – the measurement of biogas/methane production. However, the protocols available in the literature differ not only with regard to the method used to quantify the gas produced during the test, but also with regard to the experimental conditions adopted for incubating the inoculum. Extensive research has been carried out to study the influence of experimental conditions on the results for inoculum activity and inhibition assays. On the other hand, studies on biodegradability, of which there have been much fewer, can be placed into two main groups following the nature of the substrate:

(i) Micro-pollutants (chemical compounds and plastics). Test methods for assessing anaerobic biodegradability of chemical substances have been previously described. Some of them studied the influence of key parameters such as compound and inoculum concentrations and mineral medium composition [2–5]. Moreover, there are standards and guidelines for anaerobic testing, reviewed by Müller et al. [6].
(ii) Complex organic substrates (manures, wastewaters, sludges, solid wastes). The first report of anaerobic biodegradability assessment in batch mode was carried out by Owen et al. [7]. This test was developed to determine the biochemical methane potential (BMP). There is less research available on the influence of key parameters in BMP of organic materials.

This review will focus on the AD of solid organic substrates (SOS). Reviews have been previously published which include data on AD experiments using solid substrates in batch and continuous mode [8–11]. In spite of the reviews published, the variety of methods reported in the literature for determining BMP and the discrepancies in approaches and results obtained for each experimental procedure emphasizes the need for an extended review. The purpose of this review article is to integrate all of the anaerobic biodegradability tests in batch mode for different solid substrates which have been previously reported in the literature. The aim of this review will be threefold: firstly, the text includes extensive information about the influence of different factors affecting the BMP results, secondly, the manuscript summarizes the important energetic data of methane potential (Appendix A) and thirdly, the document gives a detailed report of the different experimental procedures used in each case described (Appendix B).

2. Factors affecting the performance of anaerobic batch tests

The general principle of all batch tests is the incubation of an inoculum containing a variety of anaerobic microorganisms in a suitable medium (water and minerals) at neutral pH and at specific temperature range (normally mesophilic or thermophilic). Substrate is added to the medium and serves as a source of carbon and energy for the microorganisms. After incubation, the degree of degradation of the substrate is assessed at pre-set time intervals to determine its extent and conversion rate. Blank controls (endogenous tests, with the inoculum alone added) are included so that the gas produced from the organic matter contained in the inoculum can be accounted for.

Certain factors have the potential to affect the biodegradability assays and, therefore, the biogas/methane production. They are detailed in the following paragraphs:

2.1. Solid organic substrates (SOS)

Raw materials can be obtained from a variety of sources. Different groups of potential sources for methane production were considered by Gunaseelan [8] such as the organic fraction of municipal solid waste (OFMSW), fruit and vegetable waste (FVW), grasses, woods, terrestrial weeds, and aquatic (marine and freshwater) biomass.

2.1.1. Characterisation

It is known that the anaerobic biodegradability of organic matter is related to its composition [12–20]. Therefore, in order to carry out a BMP assay it is essential to find out exactly what the characteristics of the substrate to be digested are.

Firstly, any uncertainty about the origin of the substrate tested should be avoided. Therefore, when dealing with plants, crops or other inhomogeneous materials, details on the part used for testing should be included. For example, the BMP tests of various components of *Jatropha curcus* ranging from 80 to 968 mL CH_4 g^{-1} VS_{added} [19]. Then, the description of the part used must be considered as a key parameter.

Secondly, the general characteristics of the substrate to be assayed should always be analyzed and the moisture, the total solids (TS) and the volatile solids (VS) should be quantified and controlled. It should be pointed out that some samples are problematic for TS and VS determination due to a possible loss of volatile organic matter during the drying process, including at low temperature or freeze-drying [21]. It is important to note that although specific methane yield on a VS basis is not a constant due to variations in organic matter composition, the VS content could be used as a primary indicator of the methane potential. It is noteworthy to mention that for energy crops and crop residues, the content and availability of VS which are able to produce methane is influenced by factors related to biomass production such as location, climate, variety, cultivation management and maturity stage at harvesting time [15,20,22,23].

Further information about the nature of VS can be assessed taking into account:

(i) Component composition. Not all VS are equal and therefore they exhibit different rates and extents of biodegradation during AD. The organic substance can be subdivided into: fats, proteins, carbohydrates and lignin. Proteins, lipids and extracted fractions of carbohydrates are usually the soluble parts, while the fibrous components represent the structural lignocellulosic content, in which case solubilization is very difficult. So, biodegradability is limited by the crystallinity of the cellulose and the lignin content [24].
(ii) Elemental composition. Another approach for characterisation involves the quantification of the content of certain elements (C, O, H, N and S). This information can be used to determine the empirical formula of the substrate.
(iii) Chemical oxygen demand (COD). This parameter is commonly used to characterize the total organic content of wastewater,

whereas it is not frequent for SOS. A simple explanation is that standardized methods are available for the measurements of COD for water and wastewater. However, COD measurements for solid substrates have been traditionally specifically adapted, where the samples have to be properly homogenized and diluted. Recently, good results were obtained using a modified method to measure the COD content of solid substrates without dilution [25]. In addition, it has been demonstrated that analytical performance in the measurement of COD of samples that are difficult to analyze, such as solid substrates and liquid samples with high suspended solid content, can be improved by regular participation in proficiency testing schemes [26]. In any case, COD is a very important analytical parameter because it is needed for modelling the energy balance of an anaerobic digester [27].

Further data on the composition of the SOS under test can be used to calculate theoretical methane yields by different approaches [28]. Although the theoretical potential provides only a basis for the quality of the substrate as a methane producer, some research estimated the methane yield without experimental work, based simply on its chemical composition [29]. However, the practical methane yield obtained in a reactor will always be lower than theoretical due to a number of factors [30]:

- Part of the organic material is often inaccessible due to binding of particles or structural organic matter.
- Some compounds are poorly degraded or not at all degraded anaerobically (e.g. lignin, peptidoglycan, etc.).
- A fraction of the substrate is used for cellular growth and maintenance. Although this portion may vary considerably depending on the operating conditions and substrates, in practice, 5–15% of COD removed can be considered typical as biomass cell factor [31,32].

2.1.2. Particle size

Particle size and the size reduction procedure may influence biodegradation results. It is generally accepted that hydrolysis is the rate-limiting step in anaerobic digestion of particulate substrates [33]. Surface area and particle size are important characteristics in determining the initial degradation rate. The size of the feedstocks should be limited, otherwise the digester may clog and it would also be difficult for microorganisms to carry out their digestion. In the case of substrates with low biodegradability, it is normally accepted that a size reduction of the particles and the resulting enlargement of the available specific surface can improve the biological process [34].

Little research has been carried out to determine the effect of particle size of solid substrates on methane yield [34–40]. The majority of results reported that methane yield was inversely proportional to particle size, but also some results reported no tangible effect on the kinetics of methane production. Since the relationship between particle size and biodegradability is not yet clarified, to allow for the results to be compared, the particle size should be comparable. A particle size of $\leq$10 mm is suggested. If the material used is difficult to reduce in size, it should be cut, broken or otherwise processed until the desirable size is achieved [41].

2.1.3. Concentration

One of the most important parameters for a batch assay design is the load of the solid substrate introduced into the digester. If the load is too low, although it limits the possibility of inhibitory effects, the microorganisms will exhibit a low metabolic activity and very low quantities of gas will be produced. If the load is too high, the biogas measurement may be more reliable but an overload situation in which intermediate volatile fatty acids (VFA) may build up, resulting in gas production inhibition.

Little detail about the influence of this parameter was found in the literature. Hansen et al. [42] described a laboratory procedure for the determination of BMP using 2% TS to more than 100 solid waste samples. On the other hand, the VDI 4630 guideline specified that the content of solids should not exceed 10% if an adequate mass transfer is to be assured [41].

2.2. Inoculum (INO)

Blok et al. [43] pointed out that even when the experimental conditions of batch test procedures can be harmonised, some variability in the results will always remain due to the biological nature of the test systems. The characteristics of microorganisms collected for use as inoculum can vary for the same treatment plant (daily or seasonal variations of flow-rate and substrate composition) and can be different from one treatment plant to another (operating conditions: organic loading rate, solid retention time, etc.).

The inoculum used for BMP assays must be fully characterized. Although subject to limitation, the easiest way to define the inoculum concentration is from the amount of volatile suspended solids (VSS). However, due to the inaccuracy of this determination in such samples, for the majority of anaerobic sludges VS are used as a measure of microorganism content. In any case, the information available for these analytical parameters is inadequate because it does not distinguish between microbial biomass and any other particulate organic material present in the reactor. This is especially evident in manures, where the inoculum VS content is mainly represented by recalcitrant lignocellulosic residues and not active microbial biomass, while in a granular sludge most of the VS consist of microbial cells [30]. Nor is it possible to determine if the microbial biomass is alive or dead.

The influence of the inoculum on the batch tests is mainly depending of six factors: origin/source, concentration, activity, preincubation, acclimation/adaptation and storage.

2.2.1. Origin/Source

The inoculum source relating to BMP tests is not uniform in the literature. Digested sludge from municipal wastewater treatment plants (MWTP), soil extracts, industrial treatment plants, rumen and animal manures have all been used. Although the use of an inoculum from such different sources may favour the environmental relevance of the tests, it is certainly not ideal for standardization [43]. On the other hand, the reproducibility of the assessment can be improved when a non-predetermined inoculum source is used [1].

Different sources could lead to different biodegradability results as a consequence of the different levels of microbial population. For a defined inoculum, the methane yield of an organic substrate is directly related to the extent of solubilization, while the degradation rate will depend on the slowest of the three steps of the anaerobic digestion process, namely hydrolysis (solubilization), acidogenesis and methanogenesis [39].

In general, digested sludge from a running biogas plant is used. The digested sludge from MWTP should offer the most suitable source of a diverse and active inoculum. This is preferable for the following reasons: (i) sewage treatment plants are found worldwide, (ii) although sewage treatment plants are different, they do have common features.

2.2.2. Concentration

Practical experience has demonstrated that the level of concentration of inoculum affects the rate of biodegradation. Normally, the higher the inoculum concentration, the faster the anaerobic conversion of the substrate, and the quicker the test will be completed.

Moreover, the concentration affects the duration of the lag period and the susceptibility of degradation due to inhibitory effects [4].

For some normalized biodegradability tests for micro-pollutants and the initial BMP procedure, the amount of inoculum used is generally expressed as a percentage of volume (10–80%). Using this unit system, the initial content of biomass is proportional to the VS content of the inoculum, whose value can range in manures and granular sludges from 2–3% to 10% VS, respectively [30]. Therefore, this criterion should be avoided because of its ambiguity. It is often more meaningful to express the concentration of inoculum in a batch assay in terms of VS.

To study the anaerobic biodegradability of micro-pollutants, a low inoculum concentration (1–3 g TS·L^{-1}) was suggested because inoculum also contributes to gas formation which can blur the results if it is relatively high in comparison with the compound being tested [3]. On the other hand, in the case of complex SOS a small amount of inoculum can lead to an overload in the process with acidification and methane production inhibition [44]. The literature survey shows that a wide range of concentration has been used up to date. The lowest value (2.1 g VS·L^{-1}) was reported by El-Mashad and Zhang [45], while the highest value (37.2 g VS·L^{-1}) was stated by Rincón et al. [46]. The VDI 4630 guideline suggested using a range of between 15 and 20 g VS·L^{-1} from seeding sludge [41].

2.2.3. Activity

Inoculum activity is one of three types of batch assays commonly used. The influence of inoculum activity was extensively researched and the results obtained were reviewed by Rozzi and Remigi [1]. Interest is still evident and a recent study carried out by Souto et al. [47] was entirely dedicated to this topic.

Traditionally, activity has been limited to assessing specific methanogenic activity (SMA), but for a better identification of the quality of the inoculum used, it has been recently suggested by Angelidaki et al. [30] that activity of the different groups of microorganisms involved in the anaerobic process should be determined.

The use of different positive control substrates can be used for measuring activity and also for checking if the anaerobic biodegradation assays are performing well, for quality control purposes. These reference substrates should not ferment too quickly and should be completely biodegradable. As far as biodegradability is concerned, the experimental values should be close to the theoretical ones, because, as reported previously, only a limited percentage of substrate is not converted into biogas and utilised for cellular growth and maintenance. Partial biodegradation has on occasions been observed when positive control substrates have been tested. This could have been due to faulty experimental equipment or to inactive sludge. If the experimental equipment is shown not to be faulty, the safest course of action is to repeat the assay with fresh sludge [42]. Cellulose is the most frequent substrate used for measuring the adequate level of potential performance. However, the number of BMP research works where this substrate has been used is very low compared with the huge amount of articles on BMP assays.

Regarding to the influence of the inoculum activity into anaerobic biodegradability a few research works were reported [48,49]. It is noteworthy that Tait et al. [50] used an abiotic sludge control (inactivated inoculum) to evaluate the indigenous activities of some bedding (wheat straw and rice husks) from piggery housing.

2.2.4. Pre-incubation

Pre-incubation of sludge before feeding reduces the volume of gas produced in the blank controls and has been postulated as a mean of improving the precision with which net gas production can be measured. Recently, the use of a "degassed" inoculum has been suggested where 2–7 days of pre-digestion seemed to give an optimum decrease in background gas production with acceptable increases in both the lag and the total incubation periods [51].

The literature shows that most studies regarding this factor are for micro-pollutants. Pre-incubation has been widely recommended for testing the anaerobic biodegradability of these substrates, because in such cases it is difficult to clearly relate biogas evolution to degradation of the test compound or to distinguish the amount of biogas produced by the sludge itself [4]. On the other hand, a pre-incubation time of up to 3 weeks had no significant effect on the estimation of gas production [3].

2.2.5. Acclimation/Adaptation

The preculturing of the inoculum with a substrate leads to the induction of metabolic pathways for biodegradation, an increase of microorganism affinity for the compound and also an increase in the number of specific degraders. However, this idea of adaptation, although widely accepted by the scientific community, has not previously been reported for BMP tests, where the reported tests fit well with the philosophy of using not acclimated inocula.

2.2.6. Storage

For micro-pollutants, sludge storage had no significant effect on the extent of degradation, but the duration of lag times could be affected, and, therefore, substrates could be degraded more slowly [2]. The effect of storage on the batch biodegradability test for SOS is also scarce in the literature. Angelidaki et al. [30] suggested that fresh sludge should be used whenever possible.

2.3. Experimental conditions

2.3.1. Gas measurement systems (GMS)

Gasometric methods are the most frequently used for determining anaerobic biodegradability. In such methods, biogas/methane production can be quantified either manometrically by keeping the volume constant and measuring the pressure increase, or volumetrically by providing constant pressure conditions allowing measurement of the gas volume. Techniques for measuring the rate and volume of gas produced from anaerobic biodegradability assays include different systems such as lubricated syringes, volume displacement devices, manometers or pressure transducers, manometer assisted syringes, or low pressure flow meters. In addition, some automatic gas flow meters may be considered as mixed volumetric/manometric systems.

2.3.1.1. Volumetric methods (Vol). The first description of a volumetric measurement system for biogas production consisted in the displacement of the piston of a glass syringe with its needle being inserted into the reactor [7]. Alternatively, liquid displacement systems were proposed. In this case the biogas produced inside the reactor moved into a suitable external vessel which contained a barrier solution and displaced an equivalent volume of liquid. More recently, the Eudiometer unit was described as a more sophisticated apparatus which operated by a liquid displacement technique [52].

It is important to mention that precaution must be taken with the barrier solution used so as to avoid certain biogas components being lost. For the improvement of this measurement system, it is better to use an alkaline solution for washing the biogas, which means that the sole methane fraction can be measured directly [1,53]. Another option is to collect the biogas in a gas sampling bag with low permeability [54]. This system avoids the problem of adsorption during long periods of contact with the barrier solution, but it has the disadvantage of requiring a complementary gas meter for measuring the volume of gas collected.

2.3.1.2. Manometric methods (Man). In a manometric respirometer, the biogas produced is confined inside the bioreactor and hence generates proportional overpressure. An early manometric method was the Warburg respirometer [55]. Later, the method was improved by introducing the use of a pressure transducer to measure the gas production [56].

For this method, complementary biogas analyses are needed for calculating methane production. The major difficulty in accurately quantifying the overall gas production arises from the solubility of carbon dioxide in the digesting liquor as it is affected by pressure, pH, the ratio of headspace to liquid volume, temperature and the complex thermodynamic equilibrium established between carbon dioxide and the carbonates/bicarbonates of calcium and magnesium [4].

Recently a digital pressure transducer, called OxiTop® (WTW, Germany) and originally developed for biochemical oxygen demand (BOD) measurements, has been reported as useful for anaerobic biodegradability assays [57].

2.3.1.3. Gas chromatography (GC). Dolfing and Bloemen [58] determined the SMA of a sludge based on the GC analysis of the headspace of closed anaerobic vials. They sampled with a pressure lock syringe, which allows quantification independent of the pressure prevailing in the reactor. The volume of methane can be estimated based on the molar fraction of this gas in the headspace.

Hansen et al. [42] sampled only 10 mL of headspace gas during the full BMP test (0.2 mL every time), which represents less than 0.7% of the headspace volume, and the results were, thus, not significantly affected by the change of headspace pressure.

2.3.2. Operational conditions (OpC)

2.3.2.1. Physical operational conditions.

2.3.2.1.1. Volume. The total reactor volume used for batch tests is inversely related to the number of replicate samples that could be tested at the same time using a prefixed amount of sludge and substrate. The nature of the substrate can also influence the selection of the ideal volume, because the more homogeneous the material, the smaller the volume of reactor required to determine methane potential more accurately. The results of the extensive literature review showed that a wide range of different total volumes were utilised for anaerobic biodegradability batch assays, ranging from 0.1 to 120 L. However, the most common and useful volumes used for BMP assays are lower than 1 L.

2.3.2.1.2. Temperature. Although anaerobic biodegradation can take place within a wide range of temperatures, AD processes strongly depend on temperature. Depending upon the temperature at which the process is carried out, three temperature ranges can be differentiated: thermophilic (45–60 °C), mesophilic (20–45 °C), and psychrophilic (<20 °C) [59]. The main problem at the low temperature is the decrease in the microbial consortia activity.

The majority of data in the literature refers to experiments performed at mesophilic temperature, with only some at thermophilic temperature. The reason could be that the anaerobic digestion process is efficient enough at 35 °C and there is little to gain by increasing the operational temperature when increased costs are involved [11]. Taking into account the important influence of temperature, comparatively few studies have been carried out to relate its influence on biodegradation assays in batch mode using solid substrates [60–62].

2.3.2.1.3. Stirring. Agitation of digesters can be carried out in a number of ways: manual shaking, magnetic stirrers, orbital shaker, etc. The main factors affecting the mixing method are intensity and duration. The effect of mixing on the general performance of anaerobic digestion is contradictory. The continuous mixing of the content of the bioreactor favours contact between the substrate and the microorganisms as well as the release of biogas into the headspace, but it may also damage the structure of the flocs or granules, thereby worsening the close interaction between the different microbial populations within the agglomerate [1].

For micro-pollutants the stirring process is invariably essential to the rate of gas production, whereas it is independent of the extent of degradation [5]. On the other hand, the influence of mixing on the anaerobic biodegradability assays of SOS has never been reported in detail, although an optional device for mixing the reactors thoroughly may be useful in most cases.

2.3.2.1.4. Duration. The performance time of a batch assay can be related with the kinetics of the process. The main drawback of BMP testing is that it is very time-consuming [63]. A wide range of incubation time was reported in the literature. Owen et al. [7] advised the use of an incubation time of 30 days, which enables the complete degradation of organic substrates in most cases. Hansen et al. [42] increased the incubation time to 50 days to ensure maximum degradation of organic matter that has a lower rate of anaerobic biodegradability, although they reported that typically 80–90% of methane potential can be produced during the first 8–10 days. A high incubation time of 365 days was reported by Lopes et al. [64], 240 days by Rao et al. [65], and 155 days by Kaparaju et al. [66]. On the contrary, a shorter period of 7 days was reported in some batch tests [67,68].

2.3.2.2. Chemical operational conditions.

2.3.2.2.1. Headspace gas. Different gases have been reported in the literature to flush the reactor headspace: N_2, a mixture of N_2 and CO_2, He and air. The mixture of N_2 and CO_2 has been reported as the most commonly used gas within the headspace. Different ratios of both components (70–80% N_2 and 20–30% CO_2) can be found. The content of CO_2 is related to the buffering power of the system. No extensive research has been carried out to study the influence of CO_2 on anaerobic biodegradation in batch mode, but experimental results using only N_2 were similar when different substrates were selected [69].

More worthy of comment is the use of air as gas within the headspace. Oxygenation of the sample by exposure to air or sparging with oxygen reduces the biogas/methane production in proportion to the degree of oxygenation [70,71]. However, surprisingly the results were not different when air was used as headspace gas [69].

2.3.2.2.2. pH and alkalinity adjustment. pH is a measure of the acidity or alkalinity of the liquid content of the reactor. Most methanogenic microorganisms have an optimum pH of between 7 and 8, while the acid-forming bacteria often have a lower optimum pH [44]. If the pH of the waste to be tested is outside the optimal range, and if there is insufficient buffer capacity, the anaerobic process will be inhibited. Therefore, to avoid underestimating the methane potential, most batch tests are carried out at pH values ranging from 7.0 to 7.8. If the pH needs to be adjusted, a basic diluted solution such as NaOH or lime, or an acid solution such as HCl, could be used.

Alkalinity is the capacity to neutralize acids that provides resistance to significant rapid changes in pH. It is also known as "buffering capacity". It is the result of the presence of various compounds (mainly bicarbonate, carbonate and hydroxides). A value of 2500 mg $CaCO_3 \cdot L^{-1}$ is considered to be normal for sewage sludge. A more desirable range of 2500–5000 mg $CaCO_3 \cdot L^{-1}$ provides a higher buffering capacity for which a much larger increase in VFA can be accommodated with a minimum drop in pH [72].

The initial BMP test procedure suggested using an alkalinity of 2500 mg $CaCO_3 \cdot L^{-1}$. Later, most procedures reported for micropollutant biodegradation tests used the phosphate/biphosphate species as the sole source of alkalinity. Recently, Pabón [57] reported the inhibitory effect of the applied phosphate buffer to BMP tests.

2.3.2.2.3. Mineral medium (MM). It is well documented that all microbial-mediated processes require nutrients and trace elements (metals and vitamins) during organic biodegradation. In fact, eight inorganic nutrients: nitrogen, phosphorous, sulphur, potassium, magnesium, sodium, calcium, and iron were reported as necessary macronutrients in synthetic media [44]. In addition, some metals (chromium, cobalt, copper, manganese, molybdenum, nickel, selenium, vanadium and zinc), known as trace metals, are considered micronutrients, most of which are necessary as part of the active site of enzymes. Trace metals need to be dosed when added to the reactors so as to maintain microbial metabolism and growth [73]. The dose added must balance the requirements to support high activity, taking into consideration that above this concentration, trace metals become inhibitory or toxic [74].

Literature reports on the effect of mineral medium in batch tests are very inconsistent in this respect, because they vary from one to the other:

- For micro-pollutant biodegradation, different mineral media were compared for their effect on background gas production, lag times, and extent of degradation [2]. There was no significant effect on lag times with any of the media. However, the extent of degradation did vary.
- In a similar way, there is no general consensus on BMP tests as to whether these growth factors are readily available. A question that may arise is to what extent nutrients and trace elements are necessary depending on their content in the inoculum and the substrate used, being this aspect especially crucial when degrading mono-substrate. For instance, Pobeheim et al. [75] obtained different concentrations of macro- and micronutrients when various sludges from agricultural biogas plants were analyzed. On the other hand, some substrates were characterised before anaerobic biodegradability assays and found that they contained a balanced concentration of macro- and micronutrients necessary for anaerobic microorganisms [76,77].

It is important to note that if the mixture of inoculum-substrate lacks an important element, biodegradability could be severely affected. In this way, some research works demonstrated the positive effect of the addition of some nutrients and metals [75,78,79].

2.3.2.3. Inoculum to substrate ratio (ISR). Chudoba et al. [80] reported that one of the most important parameters in activated sludge batch testing is the initial substrate/microorganism ratio (S_o/Xo). However, the role of the influence of the ISR on anaerobic biodegradation tests is not clear. Theoretically, the methane yield should be independent of the ISR and only affect the kinetics of the process. But, experimental data demonstrated that the ISR can influence both the extent and the rate of the anaerobic biodegradation process. Unfortunately, many research works do not include the ISR used in the experimental design. It is sometimes possible to calculate the ISR with the information provided, but not when the data of the substrate and/or inoculum VS content are omitted.

Owen et al. [7] gave no detail of the ISR in their procedure, merely recommending a 20% volume of inoculum and a substrate concentration lower than 2 g COD·L^{-1}. Doing calculations, the ISR of the initial BMP procedure can be considered to be approximately 1 (VS basis). The first report dealing with the influence of ISR was published by Hashimoto [81]. He showed that the methane yield was drastically reduced at an ISR below 0.25 (VS basis) using wheat straw as substrate. The methane production rate was also found to increase as the ISR rose stepwise to 2, after which it remained relatively constant. Later, Chynoweth et al. [37] determined the effect of ISR on the biodegradation of cellulose. The extent values were similar, but the methane production rate was slightly higher for the highest ISR. In addition, imbalance was explained by the presence of higher concentrations of VFA in the assays with the lowest ISR. Consequently, they modified the ISR of the batch test to 2 (VS basis). The same conclusion about the clear influence of the ISR on anaerobic degradation was reported by other researchers using different substrates [49,62,64,68,82–84].

Finally, taking into account the potential amount of VFA produced and the possible ammonium generated, if proteinaceous matter is present, each substrate probably has the best ISR for performing the assay. However, for the harmonisation of the anaerobic biodegradation assays it is necessary to work at a high ISR value. Considering that an ISR $\geq$ 2 has never been reported as inhibitory, it could be used as the mandatory ratio for future standardized tests, as the VDI 4630 guideline suggested [41].

3. Conclusions

The BMP results compiled in this review demonstrated the lack of uniformity in the data reported, probably due to different inocula and experimental conditions utilised. BMP tests made in one laboratory should be consistent with those made elsewhere. It should be desirable that comparability are not very different with others making similar measurements. A dedicated IWA task group on anaerobic biodegradability, activity and inhibition (TG-ABAI) is working on this topic since 2002.

Acknowledgments

The authors wish to express their gratitude to the Spanish Ministry of "Ciencia and Innovación" (Project CTM 2008-05772/Tecno) and Junta de Andalucia for providing financial support, to the Program "FPI" of the above-mentioned ministry for contracting graduates, as well as to the contract JAE-Doc from "Junta para la Ampliación de Estudios del CSIC" co-financed by the European Social Funds.

Appendix A. Methane yields of solid organic substrates

Solid Organic Substrate (SOS)			Methane Yield	Reference
Name	Part	Size(mm)	(mL CH_4/g VS_{added})	
Alfalfa			210	[23]
Alfalfa	Silage		226	[23]
Apple	Fresh wastes		317	[89]
Azolla	Whole plant		132	[85]
Bagasse		< 2	77[c]	[67]
Bagasse		0.85–5		[112]
Bamboo			250[a]	[90]
Banana	Peeling		289	[89]
Banana			400[a]	[90]
Banana	Peels	2	243–322	[99]
Banana	Waste stem	10–20	81–196[a]	[107]
Banana	Peeling		374–409	[36]
Barley	Whole plant silage	20–40	375	[87]
Barley	Straw	50–100	229	[93]
Barley	Waste silage		222	[122]
Barley	Waste		20	[123]
Barley	Residue	10	271	[126]
Black locust			300	[139]
Braken			180	[57]
Bread-wholewheat			N.R.	[60]
Brewery	Grain		N.R.	[108]
Brewing draffs			385–400	[117]
Brinjal	Stalk	2	374	[99]
Brinjal	Whole fruit	2	396	[99]
Buckwheat			320	[57]
Cabbage			150[a]	[90]
Cabbage (fresh)				[91]
Cabbage	Leaves	2	309	[99]
Cabbage	Stem	2	291	[99]
Cabbage-white	Leaves		382	[143]
Cabbage-white	Leaves silage		343	[143]
Cabomba			155–160	[127]
Calotropis procera	Leaves		280	[117]
Candy-black			390	[66]
Cardboard			217	[105]
Carrot			310	[57]
Carrot	Peeling		388	[89]
Carrot	Leaves	2	241	[99]
Carrot	Petiole	2	309	[99]
Cassava	Pulp		370	[129]
Cattail			350	[129]
Cauliflower	Leaves	2	190	[99]
Cauliflower	Stem	2	331	[99]
Cauliflower	Leaves		352	[143]
Cauliflower	Leaves		341	[143]
Cellulose			404	[19]
Cellulose			370	[37]
Cellulose			379	[42]
Cellulose			345	[89]
Cellulose			356	[91]
Cellulose			419	[99]
Cellulose			356–375	[128]
Cellulose			367	[138]
Cellulose		100 mesh	373	[138]
Cellulose		100 mesh	390	[139]
Ceratopteris	Whole plant		204	[85]
Chocolate			370	[66]
Clover		< 20	140–210	[66]
Cocksfoot			325	[118]
Cocksfoot		10	308–382	[135]
Coconut	Fibres	0.85–5	N.R.	[112]
Comfrey	Tops		334	[143]
Comfrey	Tops		323	[143]
Confectionery	Raw material		320	[66]
Coriander	Leaves	2	325	[99]
Coriander	Stems	2	309	[99]
Coriander	Roots	2	283	[99]
Coriander	Whole plant	2	322	[99]
Corn stover			N.R.	[102]
Corn stover		30–60 mesh	360	[138]
Cotton	Stalks-wastes		62[a]	[78]

Solid Organic Substrate (SOS)			Methane Yield	Reference
Name	Part	Size(mm)	(mL CH_4/g VS_{added})	
Cotton	Seed hull-wastes		86[a]	[78]
Cotton	Oil cake-wastes		104[a]	[78]
Cotton	Stalks		145	[95]
Cotton	Residues		365	[126]
Crops-mixture	Silage	50	320–510	[130]
Cyperas	Whole plant	10	38	[85]
Dhub grass			205–228	[36]
Diapers			204	[105]
Faba bean	Straw		440	[131]
Fat-pork			900	[42]
Fish waste	Various		390	[120]
Food packaging			318–349	[128]
Food waste	Leachate		478	[114]
Food	Wastes		245–510	[62]
Food	Wastes		425–445	[77]
Food	Wastes		472	[91]
Food	Wastes	20 × 50	301[a]	[94]
Food	Wastes		525	[116]
Fruit and vegetable	Wastes		470	[134]
Garbage	Waste	10 × 10 × 5	395	[65]
Garden pea	Pods	2	390	[99]
Gelatine			100–150	[42]
Giant knotweed			170–270	[22]
Gliciridia	Leaves		165–180	[98]
Glucose			351	[42]
Glucose			335	[138]
Gracilaria spp.			280–400	[12]
Gracilaria tikvahiae		20–30	190–230	[100]
Grape	Stalk		116	[93]
Grape	Marc		98	[93]
Grape	Pressings	2	283	[99]
Grape	Peduncle	2	180	[99]
Grass			267	[23]
Grass			374	[23]
Grass			N.R.	[60]
Grass			388	[89]
Grass			128–144[a]	[94]
Grass			320	[134]
Grass cuttings			300	[22]
Grass hay			270–350	[66]
Grassland			128–392	[15]
Green pea	Shells	10–20	194–220[a]	[106]
Green wastes			206–357	[62]
Grey	waste		147	[105]
Hydrilla	Whole plant		81	[85]
Ipomea fistulosa	Leaves		413–429	[36]
Jatropha curcus	Leaf lamina		227	[19]
Jatropha curcus	Leaf petiole		335	[19]
Jatropha curcus	Leaf entire		224–237	[19]
Jatropha curcus	Green fruit		326	[19]
Jatropha curcus	Yellow fruit		518	[19]
Jatropha curcus	Brown fruit		469	[19]
Jatropha curcus	Fruit hull		306	[19]
Jatropha curcus	Seed testa		80	[19]
Jatropha curcus	Seed kernel		968	[19]
Jatropha curcus	Seed entire		610	[19]
Jatropha curcus	De-oiled cake		230	[19]
Jerusalem artichoke			360–370	[22]
Jerusalem artichoke	Tops		309	[143]
Jerusalem artichoke	Tops silage		301	[143]
Kitchen waste			432	[122]
Kitchen waste		1–3	370–430	[124]
Kitchen waste			450	[134]
Ladies finger	Stalk		350	[99]
Laminaria		0.8	260–280	[37]
Leather fleshing			490	[136]
Lemon	Pressings		473	[99]
Lettuce	Residues		294	[89]
Lucerne	Whole plant silage	20–40	357	[87]
Lupine			310–360	[22]
Lupine (white)		< 0.2	260	[57]
Lupine (yellow)		< 0.2	260	[57]
Macrocystis		0.8	390–410	[37]
Maize	Mixture	0.5–3	268–366	[14]
Maize			398	[15]
Maize	Whole plant		282–419	[18]

Solid Organic Substrate (SOS)			Methane Yield	Reference
Name	Part	Size(mm)	(mL CH_4/g VS_{added})	
Maize		2–4	251–349	[20]
Maize			315	[23]
Maize	Silage		364	[23]
Maize	Bran		64 (3)	[67]
Maize			250–340	[74]
Maize		2	196–233	[83]
Maize	Whole plant silage	20–40	345	[87]
Maize	Fresh whole plant	10	300–400	[88]
Maize	Whole plant silage	various	370–410	[88]
Maize	Residues		317	[93]
Maize	Stalks		229	[95]
Maize	Residues	10	363	[126]
Maize	Whole plant		378	[140]
Maize	Whole plant silage		328–418	[140]
Mandarin	Peels	2	486	[99]
Mandarin	Pressings	2	433	[99]
Mandarin	Whole rotten fruit	2	494	[99]
Mandarin	Seeds	2	732	[99]
Mango	Peels	2	370–523	[99]
Marrow kale			310–320	[22]
Meadow foxtail			310	[118]
Meat and bone meal			351–381	[142]
Meat-cooked			482	[91]
Microcystis			94–141	[84]
Millet	Bran		590	[117]
Millet	Straw		390	[117]
Mirabilis	Leaves		241	[137]
Mirabilis	Leaves		327–341	[36]
Mustard	Tops		300	[143]
Mustard	Tops silage		326	[143]
Napiergrass		0.8	190–340	[37]
Napiergrass	Lamina	2	372	[99]
Napiergrass	Sheat	2	342	[99]
Napiergrass		< 20 mesh	288	[138]
Nettle			210–420	[22]
Newspaper		shredded	92	[138]
Newsprints			58	[105]
Oat		< 20	250–260	[66]
Oat			320	[22]
OFMSW			298–573	[28]
OFMSW		0.8	200–220	[37]
OFMSW		2–50	160–250	[40]
OFMSW			495	[42]
OFMSW			353	[45]
OFMSW			230–550[d]	[64]
OFMSW			92[a]	[94]
OFMSW			60–530	[96]
OFMSW			187	[97]
OFMSW		Screw press	450	[101]
OFMSW		Disc screen	450	[101]
OFMSW		Shredding	450	[101]
OFMSW		10	157	[103]
OFMSW			50–200[a]	[111]
OFMSW			186–222	[128]
OFMSW			360	[136]
Onion	Exterior peel	2	400	[99]
Orange	Peeling		N.R.	[60]
Orange	Peeling		297	[89]
Orange			115[a]	[90]
Orange	Peel	2	455	[99]
Orange	Pressings	2	502	[99]
Orange	Waste	< 7	490	[109]
Palm Oil	Fruit bunches		370	[129]
Paper			300[a]	[90]
Paper (coated)			84[a]	[94]
Paper (newsprint)			74[a]	[94]
Paper (office)			217[a]	[94]
Paper (bag)			250	[42]
Paper (office printer)			340	[105]
Paper			84–369	[128]
Paper and cardboard			109–128	[132]
Parthenium			140–152	[82]
Pea-green	Shell	10–20	194–220[a]	[106]
Pig waste			230–620	[61]
Pineapple	Peel	2	357	[99]
Pineapple	Leafy shoot	2	355	[99]
Pineapple	Peel		400	[129]

Solid Organic Substrate (SOS)			Methane Yield	Reference
Name	Part	Size(mm)	(mL CH_4/g VS_{added})	
Pomegranate	Peels	2	312	[99]
Pomegranate	Rotten pulpy seeds	2	430	[99]
Pomegranate	Whole rotten fruit	2	342	[99]
Pomegranate	Pressings	2	420	[99]
Poplar (Populus sp)		0.8	230–320	[37]
Poplar (Populus sp)			350–420	[139]
Potato	Waste		320[c]	[54]
Potato			390	[89]
Potato	Peel	2	267	[99]
Potato	Pulp		N.R.	[108]
Potato	Pulp	3–10	332	[113]
Potato	Peel-pulp	3–10	377	[113]
Potato	Fruit water		323	[113]
Poultry slaughterhouse	Waste		550–670	[133]
Quinoa			330	[57]
Radish	Shoots	2	293–304	[99]
Rape	Straw		240	[22]
Rape	Oil seed		800–900	[42]
Rape			290	[57]
Rape	Straw		420	[131]
Rape	Tops		334	[143]
Red clover			280–300	[22]
Reed canary grass			340–430	[22]
Reed canary grass		10	253–351	[135]
Rhubarb			320–490	[22]
Rhubarb	Tops		316	[143]
Rhubarb	Tops silage		345	[143]
Rice-boil			294	[91]
Rice	Straw		347–367	[36]
Rice	Straw		347–367	[36]
Rice	Straw	50–100	195	[93]
Rice	Straw		215	[95]
Rice	Straw		270–290	[115]
Rice	Straw		340	[129]
Rosebay willow			200	[57]
Rye-winter			140–275	[15]
Rye-winter	Straw	< 2	360	[131]
Ryegrass			360	[118]
Saccharum spp.			270–310	[92]
Salvinia	Whole plant		242	[85]
Salvinia			50	[127]
Sapota	Peels	2	244	[99]
Sapota	Whole rotten fruit	2	327	[99]
Sargassum spp.			150–180	[12]
Sargassum		0.8	260–390	[37]
Scirpas	Whole plant		66	[85]
Seaweed		2–3	90–120	[125]
Sisal fibre waste		2–100	176–216	[34]
Sisal pulp			320	[120]
Sisal pulp waste	Leaf tissues + fibres		120–240	[121]
Sludge-kraft pulp mill			90[b]	[141]
Sludge-sulfite pulp mill			320[b]	[141]
Sorghum		0.8	260–390	[37]
Sorghum	Whole plant silage	20–40	362	[87]
Sorghum	Lamina	2	367	[99]
Sorghum	Sheath	2	407	[99]
Sorghum	Inflorescence + flowers	2	480	[99]
Sorghum	Inflorescence + grains	2	538	[99]
Sorghum	Roots	2	228	[99]
Sorghum		0.8	280–400	[104]
Spartina			290	[57]
Starch			348	[42]
Sugar beet			340	[22]
Sugar beet	Leaves	2	231	[99]
Sugar beet	Pulp		N.R.	[108]
Sugar beet	Pulp	3–5	430	[113]
Sugar beet	Tail	1–3	481	[113]
Sugar beet	Tops		360	[143]
Sugar beet	Tops silage		381	[143]
Sugarcane			230–300	[37]
Sugarcane	Residue	1	177	[126]
Sunflower			428–454	[15]
Sunflower	De-oiled cake	< 2	107–227	[68]
Sunflower	Whole plant silage	20–40	345	[87]
Sweet clover			290	[57]
Sweet gum			260	[139]
Sweet pea			370	[57]

Solid Organic Substrate (SOS)			Methane Yield	Reference
Name	Part	Size(mm)	(mL CH_4/g VS_{added})	
Switch grass			191–309	[119]
Sycamore			380	[139]
Tall fescue		10	296–394	[135]
Tea	Residue	10	67	[126]
Teak			270[a]	[90]
Textiles			228	[105]
Timothy		10	308–365	[135]
Timothy-clover grass			370–380	[22]
Tomato	Skins and seeds		218	[93]
Tomato	Whole rotten fruit	2	211–384	[99]
Triticale			212–286	[15]
Triticale			290	[57]
Turnip	Leaves	2	314	[99]
Ulva spp.			94–177	[13]
Ulva spp.		20–30	220–330	[100]
Utricularia	Whole plant		132	[85]
Vetch			290	[57]
Vetch–oat mixture			400–410	[22]
Water hyacinth		0.8	190–320	[37]
Water hyacinth	Whole plant	1.6–12.7	130–180	[38]
Water hyacinth			244	[95]
Water hyacinth			60–190	[127]
Water hyacinth			350	[129]
Wheat	Straw	0.088–6	227–249	[36]
Wheat	Straw	10	299–331	[81]
Wheat	Straw		267	[86]
Wheat	Straw silage		396	[86]
Wheat	Whole plant silage	< 1	276	[87]
Wheat	Straw	<1	297	[110]
Wheat	Straw	30–60 mesh	302	[138]
Wheat	Straw	<30 mesh	333	[138]
Wheat-winter			229–343	[15]
Wheat-winter		5–15	311–360	[46]
White fir		<40 mesh	42	[138]
Willow (Salix spp.)			130–300	[37]
Willow (Salix spp.)		<0.8	280–370	[139]
Winter bean			350	[57]
Winter harley			300	[57]
Wood grass		<20 mesh	291	[138]
Yard	Wastes		345	[116]
Yard	Wastes		123–209	[128]

[a] mL CH_4/ g TS_{added}.
[b] mL biogas/g VS_{added}.
[c] mL CH_4/g $VS_{removed}$.
[d] mL biogas/g $VS_{removed}$.
N.R.–not reported.

Appendix B. Description of experimental BMP procedures

Reference	INO			GMS	Physical-OpC							Chemical-OpC			ISR
	Source	VS	C_o		Capacity (L)		Temp		Mixing		TD	Gas	Adj	MM	
		(%)			TV	WV	°C	System	Type	Times	(days)		pH/Alk		VS basis
[12]	MWTP		10 (%-vol)	Vol (syringe)	0.282	0.100	35				60	N_2-CO_2 (70–30%)		Yes	1
[13]	No inoculum				30		35				64				
[14]	Energy crops	58		Vol (liq-disp)	1		38	TWB	Cont (mag bar)	10 s/10 min	45				2 (TS)
[15]				Vol (liq-disp)	1		38	TWB							
[18]	Digested material			Vol (gas meter)	20		37				50				
[19]	Manure + Veg wastes		20 (%-vol)	Vol (syringe)	0.135	0.075	35				105	N_2-CO_2 (70–30%)		Yes	2
[20]				Vol (liq-disp)	0.5	0.4	35	TWB			35	N_2			
[22]	Cow manure + Byproducts	79	13.3 (g VS/L)	Vol (liq-disp)	2	1.5	35		Batch (manually)	1/day	≈150	N_2	Yes ($NaHCO_3$)		
[23]				Vol (bag + meter)	2	1.5	35	TC			35				
[28]			20 (%-vol)	GC	2		55				50				
[34]	Sisal WW sludge	48		Vol (syringe)	1	0.6	33	Ambient room	Batch (manually)	2/day	65	N_2			0.35
[36]	Manure (cattle)			Vol (liq-disp)	5	4	37		Batch (mag bar)	2 min/3h	56		Yes [$Ca(OH)_2$]		
[37]	MWTP		20 (%-vol)		0.250	0.100	35				46	N_2-CO_2 (70–30%)		Yes	2
[38]	Various			Vol (gas meter)		55	35	TC			60		Yes ($NaHCO_3$)		
[40]	MSW-leach bed			Vol (gas meter)	220	110	38	TC	Mixer	290 rpm	20–40		Yes ($NaHCO_3$)	Yes	
[42]	Manure + Org wastes		400 (mL)	GC	2	0.5	55	TC	Batch (manually)	Ocassionally	50	N_2-CO_2 (80–20%)			2 (%-w/vol)
[45]	OFMSW	59	2.1 (g VS/L)	Man	1	0.500	35		Batch (manually)	1/day	30	He			1
[46]	MWTP	65	37.2 (g VS/L)	Vol (liq-disp)		1.5	35	TWB	Cont (stirrer)	300 rpm	96			Yes	2
[54]	MWTP	57		Vol (bag+meter)	0.5	0.3	37	TWB	Cont (shaker)	70 rpm	50	N_2-CO_2 (80–20%)			0.15–5.4
[57]	MWTP + distillery			Man (Oxytop®)	1	0.600	35	TC	Batch (manually)		40	N_2	Yes ($NaHCO_3$)	Yes	2
[60]	Paper-mill WW				1	0.600	20–40		Cont (shaker)	100 rpm	55	N_2-CO_2 (70–30%)	Yes ($NaHCO_3$)	Yes	1.4–2.1
[61]	Manure (digested)		60 (%-vol)			0.5/2	55				30–40	N_2-CO_2 (80–20%)		Yes	
[62]	MWTP meso/thermo	5652		Man	1	0.600	35 50		Batch (manually)	1/day	25	He			0.3 0.2–0.6
[64]	Rumen (bovine)				20						365				0–0.17
[65]	Manure (cattle)		15 (%-vol)	Vol (liq-disp)	3.25	2	26		Batch (manually)	1/day	240				
[66]	Manure (cow)	63	11.3 (g VS/L)		2.0	1.5	35				155	N_2-CO_2 (80–20%)			0.3–0.7
[67]	Rumen (sheep)				0.125	0. 050	39		Cont (shaker)	100 rpm	7	N_2		Yes	
[68]	Brewery (UASB)	75	15 (g VS/L)	Vol (liq-disp)	0.300	0.250	35	TWB	Cont (stirrer)	40 rpm	7	N_2	Yes ($NaHCO_3$)	Yes	0.5–3
[75]	Maize silage		15 (g VS/L)	Vol (liq-disp)	2	1	35		Batch (mag bar)	8 × 15 s/day	30			Yes	1.5
[77]	MWTP	51		Vol (liq-disp)	1	0.500	50		Batch (manually)	1/day	28	He			0.4–0.6

Reference	INO			GMS	Physical-OpC							Chemical-OpC			ISR
	Source	VS	C_o		Capacity (L)		Temp		Mixing		TD	Gas	Adj	MM	
		(%)			TV	WV	°C	System	Type	Times	(days)		pH/Alk		VS basis
[78]	MWTP			Vol (liq-disp)	0.250	0.100	35	TC			23	N_2-CO_2 (75–25%)	Yes ($NaHCO_3$)	Yes	
[81]	Manure (cattle)	60	10–90 (%-vol)	Vol (syringe)	0.119	0.050	35	TC			150	N_2			0.03–11
[82]	Manure (cattle)				2		26		Cont (mag bar)		35	N_2			
[83]	MWTP	63	15 (g VS/L)	Vol (liq-disp)		5	35	TWB	Cont (stirrer)		20	N_2	Yes ($NaHCO_3$)	Yes	1–3
[84]	Manure (cattle)			Man	0.250	0.120	35	TWB	Batch (manually)	2/day	30	N_2-CO_2 (80–20%)			0.5–2
[85]					1		37	TC			35				
[86]				Vol (liq-disp)	1		38				42				
[87]				Vol (liq-disp)	0.250		37								3 (TS)
[88]	Manure (cow)			GC	2.140	0.590	55					N_2			
[89]	Waste mixtures			Vol (liq-disp)		3.5	55	TWB			15–22	N_2-CO_2 (75–25%)		Yes	1.3–2
[90]	OFMSW		99 (%-w)		0.135	0.050	30	TC			45	N_2			
[91]	MWTP		20 (%-vol)				37	TC			28				
[92]	Manure (cattle) dung						35				100				2
[93]	Mixture (codigestion)			Vol (bag+meter)	2		40		Batch (manually)	2/day	40				2
[94]	OFMSW		30 (%-vol)	Vol (bag+meter)	2	0.8	40						Yes	Yes	
[95]	Manure (cattle) slurry			Vol (liq-disp)	2.5		35	TC			120				
[96]	Various		25 (%-w)		1.1		55	TWB			60				
[97]	Waste mixture				0.5		37		Cont (shaker)					Yes	
[98]	Manure (cattle)			Vol (syringe)	3		32		Cont (mag bar)		30				
[99]	Vegetable wastes		20 (%-vol)	Vol (syringe)	0.135	0.075	35				100	N_2-CO_2 (70–30%)		Yes	2
[100]	Seaweeds				2	1.7	32		Batch (manually)	15 sec/day	58				
[101]	Manure + Org wastes						55				50				
[102]	Rumen (goat)			Vol (liq-disp)	0.250	0.100	25–40	TC	Cont (shaker)	130 rpm	10	N_2	Yes ($NaHCO_3$)	Yes	0.3–0.8
[103]	MWTP			Vol (liq-disp)	1	0.9	30	WB			919	N_2		Yes	
[104]							35				60				
[105]	MWTP	53		Vol (bag+meter)	2	1.6	35				237	N_2-CO_2 (80–20%)	Yes ($NaHCO_3$)	Yes	
[106]	Manure (cattle)			Vol (liq-disp)	0.300	0.275	40				25–35	N_2	Yes		
[107]	Manure (cattle)			Vol (liq-disp)	0.300	0.275	40				57	N_2	Yes (NaOH)		
[108]	Effluent two-stage	60 85		Vol (gas meter)	5		35		Cont (mag bar)		37–85				0.8–2
[109]	MSW	53	10 (g VS/L)		0.120	0.060	55				122	N_2-CO_2 (80–20%)	Yes ($NaHCO_3$)		3.2
[110]	Manure (cow)			GC	0.118	0.040	55		Static		60	N_2			
[111]	MWTP		10 (%-vol)	Vol (syringe)	0.250	0.100	35				45			Yes	

Reference	INO			GMS	Physical-OpC							Chemical-OpC			ISR
	Source	VS	C_o		Capacity (L)		Temp		Mixing		TD	Gas	Adj	MM	
		(%)			TV	WV	°C	System	Type	Times	(days)		pH/Alk		VS basis
[112]	Rumen										30				
[113]	Vegetable+ Crops			Vol (liq-disp)	1		37.5	WB	Batch (mag bar)	10/30 min	28–38				3 (TS)
[114]	MWTP		20 (g VS/L)		0.500	0.250	35		Cont (shaker)	100 rpm	28	N_2	Yes ($NaHCO_3$)		10
[115]	Rice	60	3.3 (g VS/L)	Vol (liq-disp)	5	4	22		Cont (mag bar)		120	N_2	Yes (NaOH)		
[116]	Pig manure + Food waste				0.100	0.060	55				28		Yes ($NaHCO_3$)		
[117]	Manure (cattle)		8–10 (g VS/L)	Vol (syringe)	0.100	0.050	35	TWB	Batch (manually)	2/day	70	N_2	Yes (NaOH)		0.5
[118]	Digested material			Vol (gas meter)		2	35	TWB			28				
[119]	Manure (swine)			Vol (gas meter)	30	20	35	TC	recirculation	3/day (1 min)	50–60				
[120]	Sisal WW sludge	52		Vol (syringe)	1	0.6	27		Batch (manually)	2/day	25–29	N_2			0.4–20
[121]	Activated sludge	55	4.9 (g VS/L)	Vol (syringe)	0.5	0.3	37	TWB	Cont (shaker)	70 rpm	32–85	N_2-CO_2 (80–20%)	Yes ($NaHCO_3$)		0.65
[122]	Brewery (UASB)			Man	0.160		37			150 rpm	100		Yes ($NaHCO_3$)		0.43 (TS)
[123]	Brewery (UASB)			Man	0.160 120	80	37			150 rpm			Yes		0.14
[124]	Brewery (UASB)	65		Man	0.160		37			150 rpm			Yes ($NaHCO_3$)	Yes	0.74
[125]	MWTP			Vol (bag + meter)	0.5	0.3	37	TWB	Cont (shaker)	70 rpm	30	N_2-CO_2 (80–20%)	Yes ($NaHCO_3$)		1–2
[126]	Cow manure + Paper mill			Man	1	0.2	30		Shaker		30	N_2	Yes (phosphate)	Yes	1.5
[127]	Primary WAS + foodwaste			Man	0.200	0.100	38		Stirring	Sampling				Yes	
[128]	Primary WAS		20 (%-vol)	GC	0.275	0.100	35	TC			60	N_2-CO_2 (70–30%)		Yes	3–4
[129]	Manure (pig)			Vol (liq-disp)	0.120	0.065	37				90	N_2-CO_2 (70–30%)		Yes	3 (?)
[130]	Manure (cow)	77		Vol (liq-disp)	1	0.750					70–80	N_2	Yes ($NaHCO_3$)		1–2
[131]	Manure	69		GC	0.100	0.025	42	TWB	Cont (shaker)	100 rpm	67	N_2			2 (?)
[132]	MWTP			Man	1.130	0.800	35				100	N_2		Yes	0.03 0.04
[133]	MWTP	64	40–80 (%-vol)		0.118	0.050	35		Cont (shaker)		27	N_2-CO_2 (80–20%)			1.1–4.3
[134]	MWTP			Man	1.140										1.81
[135]	Manure, silage and byproducts	79	19 (g VS/L)	Vol (bag + meter)	1	0.750	35		Batch (manually)	Sampling	95	N_2	Yes ($NaHCO_3$)		1
[136]				Vol (liq-disp)	0.500	0.400	35		Batch (stirrer)	15/30 m 140 rpm	40			Yes	
[137]	Manure (cattle)	89	50 (%-vol)		1		36	TC	Batch	2/day	56				
[138]	MWTP		10 (%-vol)	Vol (syringe)	0.260	0.130	35		Cont (mechanical)	15 rpm	60–133	N_2-CO_2 (70–30%)		Yes	

Reference	INO			GMS	Physical-OpC				Mixing		TD	Chemical-OpC			ISR	
	Source	VS	C_0		Capacity (L)		Temp					Gas	Adj	MM		
		(%)			TV	WV	°C	System	Type	Times	(days)		pH/Alk			VS basis
[139]	MWTP		20 (%-vol)	Man	0.250	0.100	35	TC			60–100	N_2-CO_2 (70–30%)	Yes ($NaHCO_3$)	Yes	2	
[140]	Landfill leachate			Vol (liq-disp)	2.5	1.5	35		Batch (manually)	1/day	21	N_2				
[141]	Paper-mill WW			Man	0.160	0.100	35	TWB	Cont (shaker)	200 rpm	35	N_2/CO_2/H_2 (80-10-10%)		Yes		
[142]	MWTP		3.3 (g VS/L)	Vol (liq-disp)	1.175	0.600	35	TC	Batch (manually)	2/day	10–72	N_2	Yes (HCl/NaOH)			
[143]	Plant material			Vol (liq-disp)	3		35					25–36	Yes (NH_4OH)			

Source: MWTP-municipal wastewater treatment plant; WW: wastewater; VS: Volatile solids; C_0: Concentration (g VS/L); (%-w): % in weight; (%-vol): % in volume; GMS: gas measurement system; Vol: volumetric system; (liq-disp): liquid displacement; Man: manometric system; GC:gas chromatography; TV: Total volume; WV: working volume; Temp: Temperature; TWB: thermostatic water bath; TC: thermostatic chamber; Mixing: Cont-continuously; mag bar: magnetic bar; TD: Test duration; Adj: Adjustment of pH and/or alkalinity; MM: Mineral medium; (?): Units were not reported.

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2.5 PROYECTO ADRIL

Durante la ejecución de la presente Tesis Doctoral, el doctorando contribuyó al desarrollo y ejecución del mencionado proyecto.

El proyecto Adril (acrónimo que deriva de los nombres de "Anaerobic Digestion Research-ADR" y "Interlaboratory-IL") tiene como misión contribuir a una base de conocimiento en el campo de la digestión anaerobia y más concretamente en los parámetros analíticos de control de este proceso proporcionando apoyo a los laboratorios para la obtención de datos que se consideren aptos y que sean así comparables con los de otros laboratorios en el mismo campo.

La calidad analítica de un laboratorio, representada por el problema analítico, puede considerarse a distintos niveles:

1º. Calidad de los resultados (exactitud y representatividad)

2º. Calidad del proceso analítico (precisión, sensibilidad, coste)

3º. Calidad del trabajo y la organización del laboratorio

4º. Calidad de los materiales e instrumentos implicados en el trabajo analítico.

La Norma UNE-EN ISO/IEC 17025 (ISO, 1999) surgió como una guía genérica de referencia para aquellos laboratorios de ensayo y calibración que pretenden demostrar:

- Que operan un sistema de control de la calidad eficaz y en mejora continua.
- Que son técnicamente competentes
- Que son capaces de producir resultados de ensayo o calibración fiables.

El apartado 5.9 de la misma se refiere al aseguramiento de la calidad de los resultados de ensayos y calibraciones, concretamente indica que el laboratorio debe disponer de procedimientos de control de calidad para comprobar la validez de los resultados obtenidos. Entre los sistemas de control que se citan, aparecen dos medidas de evaluación externa de la calidad muy importantes:

1) Uso de materiales de referencia (MR)

Se define como un material o sustancia de una o más propiedades suficientemente homogéneas y bien establecidas para la calibración de un aparato, la verificación de un método de medida o la asignación de valores a materiales. Por otro lado, este material será certificado (MRC) cuando una o más propiedades tienen sus valores certificados por procedimientos técnicamente válidos y poseen una certificación y/o trazabilidad expedida por un organismo competente. Para la selección de los MRC se debe tener en cuenta su disponibilidad, coste e idoneidad para la determinación a realizar, así como el nivel de incertidumbre requerido para alcanzar el objetivo analítico deseado.

2) Participación en ejercicios o programas interlaboratorios (IL), también denominados ensayos o pruebas de intercomparación

Las comparaciones interlaboratorios comprenden la organización, realización y evaluación de ensayos del mismo material o materiales de ensayo similares por dos o más laboratorios de acuerdo con condiciones predeterminadas. Aparte de esta característica en común, los programas IL poseen características muy variadas. (Horwitz, 1993) [15]. Los programas de ensayo de aptitud (EA) (estudio del desempeño analítico del laboratorio) suelen ser los tipos de IL más comunes y quizás los más importantes. La participación en EA posibilita la comparación de los propios resultados con los obtenidos por otros laboratorios. También puede proveer de:

• Una evaluación regular, objetiva e independiente de la calidad de los análisis de rutina.

• Información comparativa acerca del método así como del desempeño del instrumento.

• Un panorama de la calidad de análisis específicos en un sector, región o país.

El Proyecto ADRIL, coordinado por el grupo de investigación “Tratamiento de Residuos” del Instituto de la Grasa (CSIC) en Sevilla, consiste en una actividad internacional con carácter de participación voluntario de un grupo importante de científicos de numerosos laboratorios diferentes que trabajan en el campo de digestión anaerobia. Este proyecto permite describir la calidad de los datos que están siendo producidos para la caracterización de las materias primas sólidas, así como de efluentes con un alto contenido en sólidos en suspensión para el seguimiento de los reactores anaerobios.

La mejor manera de avanzar en un campo en particular es la de colaborar con colegas que trabajan en áreas similares. La difusión de información a través de la literatura, seminarios o conferencias internacionales, y el intercambio de ideas durante las reuniones, son muy importantes, pero a veces no suficiente para mejorar el estado de una técnica concreta en un campo determinado. Por lo tanto, además de la transferencia de conocimiento a través de las "rutas clásicas", una tarea experimental, compartiendo experiencias en química analítica mediante el intercambio de muestras, uso de materiales de referencia comunes y la evaluación de diferentes técnicas podría ayudar con las numerosas dificultades encontradas en el trabajo diario de laboratorio.

El proyecto ha sido concebido como una serie de ejercicios de intercomparación y tiene como objetivos principales:

• La organización de programas de ensayos de aptitud (PTS). De esta manera, se establece el conocimiento global de la calidad de las mediciones químicas realizadas en ADRIL para determinar un acuerdo entre laboratorios para estas determinaciones analíticas.

• Para el intercambio de experiencias e información entre los laboratorios para desarrollar metodología analítica de una manera eficiente y mejorar las capacidades analíticas.

- La incorporación de un elemento educativo o el aprendizaje (como la función docente).

En definitiva, el objetivo de este programa IL es detectar los parámetros con mayores dificultades analíticas con vistas a una mejora de la calidad de los resultados analíticos y la consiguiente armonización de los mismos en un futuro cercano.

1.1 REFERENCIAS

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CAPÍTULO 3: DISCUSIÓN GLOBAL DE RESULTADOS

3 DISCUSION GLOBAL DE RESULTADOS

En este capítulo se resumen los resultados de los trabajos que conforman la parte principal de la presente tesis doctoral.

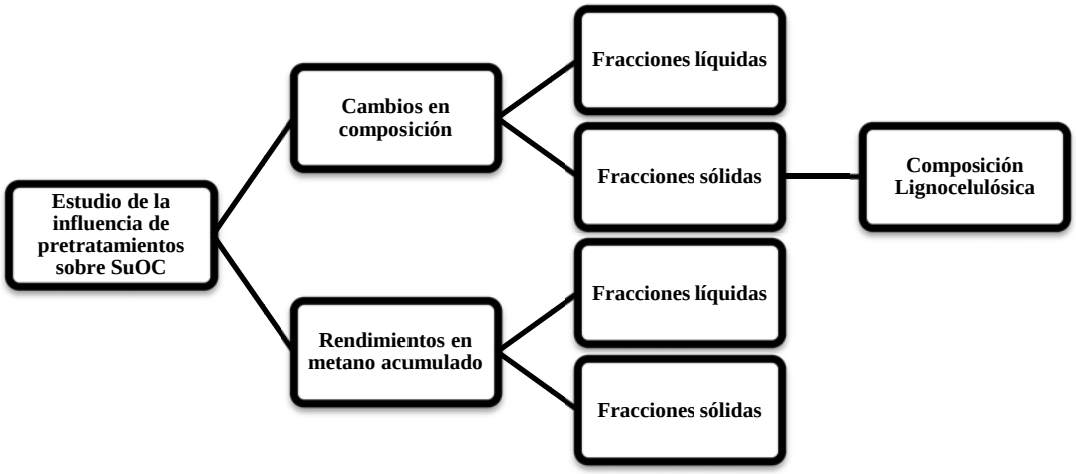
La mayor parte de estos resultados han sido publicados en revistas científicas, todas ellas incluidas en el Journal Citation Report (ICR) del Science Citation Index (SCI) con elevados índices de impacto.

La organización del capítulo consiste en un resumen de los resultados obtenidos en los distintos ensayos, mostrándose a continuación la copia exacta de la publicación científica correspondiente.

Los resultados obtenidos se han clasificado según el modo de ensayo aplicado en la experimentación: ensayos tipo "Batch" y ensayo tipo "Semicontinuo".

3.1 Ensayos modo Batch

Los ensayos tipo Batch fueron aplicados al sustrato objeto de estudio, SuOC, después de ser pretratado de diferentes formas. En todos los ensayos que se realizaron, se estudió la influencia y la eficacia de cada pretratamiento sobre el sustrato siguiendo los mismos criterios: el análisis de los cambios sufridos en su composición y los rendimientos en metano acumulado obtenidos. Estos ensayos fueron realizados sobre la fase líquida y sobre la fase sólida por separado, realizándose finalmente un análisis global de los mismos. La organización de dicho estudio se puede resumir con el siguiente esquema:



3.1.1 Pretratamiento Hidrotermal

Referencia del artículo publicado que refleja estos resultados:

Effect of hydrothermal pretreatment of sunflower oil cake on biomethane potential focusing on fibre composition.

Fernández-Cegrí, V., Ángeles de la Rubia, M., Raposo, F., Borja, R. (2012) Bioresource Technology, 123, pp. 424-429.

El objetivo de este trabajo fue estudiar el efecto del pretratamiento hidrotermal a 25, 100, 150 y 200ºC sobre la composición y el coeficiente de rendimiento en metano obtenido a partir de la harina de girasol desengrasada (sunflower oil cake, SuOC)

Durante los pretratamientos, el único factor variable fue la temperatura que se aplicó en cada ensayo. Factores como tiempos de aplicación, concentraciones, formas de llevar a cabo los experimentos, etc. se mantuvieron inalterados para evaluar sólo el efecto de la temperatura.

Los resultados obtenidos en cuanto a la composición química de las fracciones líquidas fueron proporcionales al aumento de la temperatura, es decir, en términos de CODs, NHx, TVFA e hidratos de carbono, las menores concentraciones obtenidas correspondieron al sustrato obtenido después de aplicar 25ºC como pretratamiento y los mayores para el pretratamiento a 200ºC. El incremento en la concentración de ácidos orgánicos de cadena corta provocó una disminución en el pH cuando se aumentó la temperatura.

En el caso de las fracciones sólidas, los contenidos de hemicelulosa y celulosa variaron relativamente poco después de aplicar los pretratamientos respecto al sustrato sin pretratar, a pesar de la solubilización encontrada después de los pretratamientos, resultando éstos poco ventajosos en lo que se refiere a liberación de las sustancias biodegradables que forman parte del complejo lignocelulósico.

El análisis de los rendimientos en metano acumulado, expresados como $mLCH_4/gCOD_{added}$, reveló que para la fracción sólida, los mejores resultados se obtuvieron al aplicar 25ºC de temperatura, con 114 $mLCH_4/gCOD_{added}$ respecto a 53 $mLCH_4/gCOD_{added}$, obtenidos con el sustrato sólido después de la aplicación de 200ºC, no siendo singular este resultado ya que la fracción liquida obtenida después de 25ºC fue el sustrato que presentó menos proporción de material solubilizado.

En cuanto a los rendimientos de metano acumulados obtenidos en las fracciones líquidas, los resultados más destacados se obtuvieron tras la aplicación de 100ºC de temperatura. Para temperaturas mayores de 100ºC, los bajos coeficientes de rendimiento obtenidos pueden ser explicados debido a la formación de compuestos fenólicos, furfural, ácido urónico, y otros

compuestos que pueden llegar a inhibir el crecimiento y metabolismo de los microorganismos anaerobios.

Analizando de forma global los resultados de rendimiento en metano acumulado para las dos fracciones, se considera el pretratamiento a 100ºC como óptimo, con el que se obtiene un valor medio del coeficiente de rendimiento en metano de 207 $mLCH_4/ gCOD_{added}$, siendo éste en 6.5%, 52.5%, y 41.6% superior que los obtenidos a 25 (AT), 150 y 200ºC, respectivamente.

Bioresource Technology 123 (2012) 424–429

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Effect of hydrothermal pretreatment of sunflower oil cake on biomethane potential focusing on fibre composition

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HIGHLIGHTS

- Hydrothermal pre-treatment at 100 °C is an option for improving the AD (anaerobic digestion) of SuOC (sunflower oil cake).
- $T > 100$ °C worsen the chemical composition of pre-treated SuOC, decreasing the CH_4 yield.
- The kinetic constants of the AD process of the pre-treated solid fraction are related to the lignin concentration.
- Lignin content increases with pre-treatment temperature, while the kinetic constant decreases.

ARTICLE INFO

Article history:
Received 6 January 2012
Received in revised form 30 June 2012
Accepted 30 July 2012
Available online 8 August 2012

Keywords:
Biochemical methane potential
Hydrothermal pretreatment
Lignocellulose
Sunflower oil cake

ABSTRACT

The aim of this study was to elucidate the effect of hydrothermal pretreatment at 25, 100, 150 and 200 °C on fibre composition and the biomethane potential of sunflower oil cake (SuOC). An increase in pretreatment temperature from 25 to 200 °C caused a decrease in hemicellulose content in the solid pretreated fraction from 13 to 6% while the lignin content increased by 16%. Soluble compounds also increased with temperature. Digestion of solid fractions from pretreatments at 25, 100, 150 and 200 °C in batch assays at 35 ± 1 °C resulted in methane yields of 114 ± 9, 105 ± 7, 82 ± 7 and 53 ± 8 mL CH_4 $g^{-1}COD_{added}$, respectively. The corresponding methane yields for the liquid fractions were 276 ± 6, 310 ± 4, 220 ± 15 and 247 ± 10 mL CH_4 $g^{-1}COD_{added}$, respectively. Therefore the overall methane yield was highest for SuOC pretreated at 100 °C; however, this value was only 6.5% higher than that achieved after pretreatment at 25 °C.

1. Introduction

Agro-food wastes, such as sunflower oil cake (SuOC), a by-product of sunflower oil extraction, provide an inexpensive feedstock for biological conversion to biogas by anaerobic digestion (AD) (Antonopoulou et al., 2010); however, the effectiveness of this technology in treating this kind of residue is limited because of the complex structure of lignocellulosic biomass which is resistant to AD. Although cellulose and hemicellulose can be degraded under anaerobic conditions, indigestible lignin which is connected to cellulose by hemicelluloses (Laureano-Perez et al., 2005) prevents access of enzymes to the carbohydrates (Zhu et al., 2008). Therefore, pretreatment of lignocellulosic biomass is necessary to remove lignin and to make cellulose more accessible to the enzymes that convert carbohydrate polymers into fermentable sugars (Mosier et al., 2005).

Physical, physico-chemical, chemical, and biological processes have been used for pretreatment of lignocellulosic materials (He et al., 2008; Taherzadeh and Karimi, 2008), not only for removing the inhibitory lignin complex but also for reducing cellulose crystallinity, which is a major limitation for cellulose hydrolysis (Jeihanipour et al., 2010). However, most of these studies have been carried out with the goal of producing ethanol and only a few studies have investigated biogas production (Kumar et al., 2011; Teghammar et al., 2010).

Conventional chemical pretreatment using acids or alkalis negatively impact process costs and the environment and complicate waste disposal (Bordeleau and Droste, 2011). Thus, hydrothermal treatments are promising alternatives to chemical treatments (Pérez et al., 2008).

During hydrothermal pretreatment, water under high pressure can penetrate into the biomass, hydrating cellulose and removing most of the hemicellulose and part of the lignin (Taherzadeh and Karimi, 2008; Pérez et al., 2007). At high temperatures, water lowers pH and enables release of O-acetyl, acetic and uronic acids from hemicellulose (Pérez et al., 2007). The most significant drawback of high temperatures is the formation of phenolic compounds and furan derivatives (furfural and hydroxymethylfurfural-HMF) that are undesirable because they not only represent a loss of fermentable

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http://dx.doi.org/10.1016/j.biortech.2012.07.111

sugars, but are also inhibitors of bacteria and *Archaea* (Negro et al., 2003). Therefore, conditions must be determined in each case to optimise hydrothermal pretreatments.

The objective of the present work was to optimise the hydrothermal pretreatment of SuOC for methane production and to study the effect of pretreatment temperature on fibre and chemical composition of the solid and liquid fractions. Furthermore, a first-order kinetic model was used to obtain the specific rate constants of the batch anaerobic digestion processes.

2. Methods

2.1. Hydrothermal pretreatment

Hydrothermal pretreatment of SuOC was carried out in closed 40-mL Pyrex glass cylinders kept in a thermo-reactor with temperature control. Twenty-five mL of a suspension of 20 g L^{-1} SuOC in distilled water was added to every cylinder. After hydrothermal pretreatment, the glass cylinders were cooled to ambient temperature in a water bath. A total of 20 g of SuOC were pretreated at each temperature assayed to obtain sufficient amounts of solid and liquid fractions to perform BMP assays.

The experimental settings of hydrothermal pretreatment were chosen from the best conditions obtained during a preliminary study carried out by determining SuOC solubilisation at two different concentrations (20 g L^{-1} and 40 g L^{-1}), eight temperatures (25, 50, 75, 100, 125, 150, 175 and 200 °C) and four pretreatment times (1, 2, 4 and 6 h). The aim of the experiment carried out at ambient temperature (AT) was to determine the solubilisation capability of water with no rise in temperature. A concentration of 20 g L^{-1} and digestion time of 4 h were selected on the basis of the preliminary assays.

The solubilisation of organic matter was determined to evaluate the transfer of the SuOC solid fraction to the hydrolysate. The solubilisation was expressed as a percentage, following the equation:

$$\text{Solubilisation } (\%) = (S_S - S_{S0}/S_i) \times 100 \quad (1)$$

where S_S and S_{S0} are the soluble hydrolysate concentrations expressed as grams of soluble chemical oxygen demand (CODs), measured in hydrothermally pretreated SuOC and pretreated SuOC at AT, respectively; S_i is the initial total substrate concentration (expressed as grams of total COD–COD_T), measured in untreated SuOC.

Wet material was vacuum filtered through a 0.45 μm filter to obtain a water-insoluble solid fraction and a liquid (prehydrolysate) fraction. The corresponding solid and liquid fractions after each pretreatment were subjected to separate batch anaerobic digestions by carrying out BMP tests as described by Kaparaju et al. (2009).

2.2. Raw material and inoculum used in the BMP assays

SuOC was collected from a sunflower oil factory located near Seville (Spain). The substrate was shredded into different particle sizes using a commercial sieve (Restch AS 200 basic) and different screen meshes. The most abundant particle size of this substrate (0.71–1.0 mm diameter) was selected for the hydrothermal pretreatment experiments. The composition of SuOC without pretreatment has been described elsewhere (De la Rubia et al., 2011).

The inoculum used in the BMP assays was obtained from an industrial anaerobic reactor treating brewery wastewater and operating at mesophilic (35 °C) conditions. This inoculum was selected due its high methanogenic activity as determined previously (Rincón et al., 2011). The main characteristics of this digested sludge are: pH 7.6 ± 0.1, 119 g L^{-1} of total solids (TS) and 75 g L^{-1} of volatile solids (VS).

2.3. Batch anaerobic digestions

The digesters were glass Erlenmeyer flasks with 300 mL total volume organised as a multiflask batch system.

The inoculum to substrate ratio (ISR) was 2 (VS basis for solid fraction and VS/COD basis for liquid fraction). For each flask containing 50 mL of inoculum (with a final concentration of 15 g VS L^{-1}), solid pretreated SuOC or liquid hydrolysate were added together with stock mineral medium solution and distilled water to a working volume of 250 mL. The mineral medium solution was composed of buffer, ($NaHCO_3$, 5000 mg L^{-1}); macronutrients (NH_4Cl, 280 mg L^{-1}; K_2HPO_4, 250 mg L^{-1}; $MgSO_4{\cdot}7H_20$, 100 mg L^{-1}; $CaCl_2{\cdot}2H_2O$, 10 mg L^{-1}; yeast extract, 100 mg L^{-1}) and micronutrients, ($FeCl_2{\cdot}4H_2O$, 2 mg L^{-1}; $CoCl_2{\cdot}6H_2O$, 2 mg L^{-1}; $MnCl_2{\cdot}4H_2O$, mg L^{-1}; $AlCl_3{\cdot}6H_2O$, 0.09 mg L^{-1}; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.05 mg L^{-1}; H_3BO_3, 0.05 mg L^{-1}; $ZnCl_2$, 0.05 mg L^{-1}; $CuCl_2{\cdot}2H_2O$, 0.038 mg L^{-1}. Inoculum supplemented with nutrients and distilled water were used as a blank to determine gas production by the inoculum itself. BMP tests with starch as substrate were also carried out as positive controls (Raposo et al., 2012).

The headspace of each bottle was flushed with nitrogen. The reactors were continuously stirred with magnetic bars at 300 rpm and placed in a thermostated water bath at 35 ± 1 °C. The gas released was passed through a 2 N NaOH solution to capture CO_2; the remaining gas was assumed to be methane. The digestion experiments were run for 7–8 days until the accumulated gas production remained essentially unchanged (on the last day, production was lower than 2% of the accumulated methane produced). Each experiment was performed in triplicate.

2.4. Analytical methods

TS and VS were determined according to standard methods 2540B and 2540E (APHA-AWWA-WPCF, 1998), respectively; COD_T was determined by the method described by Raposo et al. (2008). To determine the total Kjeldahl nitrogen (TKN), 1000 mg of sample was acidified with 15 mL concentrated H_2SO_4. In addition, 5 g catalyst [(Cu–Se) (1.5% $CuSO_4{\cdot}5H_2O$ + 2% Se)] was added, and the sample was digested sequentially in a thermoblock for 15 min at 150 °C, 15 min at 250 °C and 90 min at 390 °C. After cooling, the sample was diluted with 10 mL distilled water, neutralised with 12.5 N NaOH and distilled in 50 mL of indicator mix. The solution was titrated with 0.02 N H_2SO_4. Total protein was determined by multiplying the TKN value by 5.5 (Mossé, 1990). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined so as to calculate hemicellulose (NDF–ADF), cellulose (ADF–ADL) and lignin (ADL) according to van Soest et al. (1991) with slight modifications. To determine NDF, 1000 mg of dried sample was boiled in a sintered glass crucible (40–100 μm pore size) with 100 mL of a neutral detergent solution composed by 30 g L^{-1} of Sodium Dodecyl Sulphate, 18.6 g L^{-1} Ethylenediaminetetraacetic Acid Disodium Salt 2-hydrate, 6.8 g L^{-1}di-Sodium tetra-Borate 10-hydrate, 4.6 g L^{-1}di-Sodium Hidrogen Phosphate anhydrous and 10 mL L^{-1} Triethylene Glycol, together with 1 g of sodium anhydrous sulphite to remove proteins and 200 μL of α-amylase (heat-stable solution, for use in total dietary fibre assay, TDF-100A from Sigma–Aldrich), to eliminate starch, for 1 h. Neutral detergent was removed and the sample washed with 100 mL of distilled boiling water. The sample was washed with 50 mL of acetone and dried at 105 °C overnight in an oven and weighed. Corrections for residual protein and ash were made, determining the remaining content of TKN and mineral solids. ADF was determined by non-sequential fibre analysis. Dried sample (1 g) was heated with 100 mL of a solution of 2% N-acetyl-N,N,N-trimethyl ammonium bromide in 1 N H_2SO_4 to boiling for 1 h in a sintered glass crucible (40–100 μm pore size). ADF was recovered by filtration,

washed with 100 mL of distilled boiling water, followed by 50 mL of acetone. The sample was dried overnight at 105 °C and weighed. The weight was corrected for ash and protein. To determine ADL, 250 mg of sample obtained after ADF analysis was stirred for 3 h with 25 mL of H_2SO_4 (72% w/w). The sample was placed in a sintered glass crucible (40–100 μm pore size) and washed with 100 mL of distilled water and dried at 105 °C in an oven overnight and weighed. Correction for ash was made.

The inoculum and digestates were characterised by measuring: pH (using a pH-metre model Crison 20 Basic), total alkalinity (TA) by pH titration to 4.3, and TS and VS (APHA-AWWA-WPCF, 1998).

The hydrolysate obtained after each hydrothermal pretreatment as well as the digestates (centrifuged at 8000*g* for 15 min and filtered through a 0.45 μm filter) were characterised with respect to soluble chemical oxygen demand (CODs), using the closed digestion and colorimetric standard method 5220D (APHA-AWWA-WPCF, 1998); carbohydrates, according to the colorimetric method described by Dubois et al. (1956); TA, by pH titration to 4.3; ammoniacal nitrogen (NH_x), by distillation and titration according to standard method 4500E (APHA-AWWA-WPCF, 1998). The volatile fatty acid (VFA) concentration was measured using a gas chromatograph, as previously described (De la Rubia et al., 2009).

2.5. Kinetic model

To evaluate the effect of hydrothermal pretreatment of SuOC on methane yield, a first-order kinetic model, frequently applied to anaerobic digestion systems (Hashimoto, 1986), was used to correlate the methane yield with the digestion time.

$$B = B_0 \cdot [1 - exp(-k \cdot t)] \quad (2)$$

where B (mL CH_4 g^{-1} COD_{added}) is the cumulative methane yield at a time t, B_0 (mL CH_4 g^{-1} COD_{added}) is the maximum or ultimate methane yield of the substrate, k ($days^{-1}$) is the specific rate or apparent kinetic constant and t (days) is the time. Therefore, the ultimate methane yield gives the final value when no more volume of gas from the reactor is released. The adjustment by non-linear regression of the pairs of experimental data (B, t) using the Sigmaplot software (version 11.0) allowed the calculation of the apparent kinetic constant (k).

3. Results and discussion

3.1. Effect of pretreatment on solid and liquid fraction compositions

The components analysed in the solid fractions to determine the effect of temperature after hydrothermal pretreatment were cellulose, hemicellulose and lignin (Table 1). Hemicellulose remained in the solid fraction after pretreatment at AT and at 100 °C, but at temperatures above 150 °C hemicellulose was hydrolysed and passed into the liquid fraction or hydrolysate, as also observed by Hendriks and Zeeman (2009).

In spite of the solubilisation of raw material components such as hemicellulose during the pretreatment, the percentage of cellulose remained between 29 and 32% (Table 1). The increase in cellulose in the hydrothermally pretreated material was very low in comparison with that of the treated SuOC at AT (29%). Therefore, in this case, hydrothermal pretreatment does not offer any advantage since the cellulose introduced into the reactor to be digested was only slightly more available than the material treated at AT. Furthermore, the cellulose content in the solid fraction, as opposed to the untreated material, indicates a low cellulose solubilisation rate (5%) as the temperature increased.

In general, hydrothermal pretreatment at temperatures above 150 °C causes not only solubilisation of hemicellulose, but also partial solubilisation of lignin (Hendriks and Zeeman, 2009). However, in the case at hand, as lignin was expressed on a VS basis and some organic materials were removed during hydrothermal pretreatment, in the end lignin was considerably more concentrated in the treated than in the untreated material (14%) or SuOC treated at AT (17%), as it reached values of up to 33% under the most severe conditions (200 °C). In spite of its possible solubilisation, the concentration of lignin may be related to solidification and re-deposition of the lignin on the biomass surface upon cooling after severe pretreatment conditions (Liu and Wyman, 2005; Negro et al., 2003).Thus, there is no lignin removal but rather a re-allocation of lignin during these high temperature pretreatments (Kristensen et al., 2008). This fact has already been described and reported for agricultural residues such as switchgrass (Kumar et al., 2011), corn stover and wheat straw (Kaparaju and Felby, 2010), as well as for paper tube residuals (Teghammar et al., 2010) subjected to hydrothermal pretreatments.

The parameters considered in the analysis of the liquid fraction obtained after pretreatment were CODs, pH, VFA, NH_x and carbohydrates (Table 2).

As expected, an increase in temperature caused an increase in solubilisation due to enhanced removal of non-structural components. Compared with the AT pretreatment, the COD contents obtained after increasing the temperature were higher. The highest level of CODs was obtained when the most severe pretreatment conditions were used (200 °C), while the lowest levels were obtained after no rise in temperature (AT).

The pH of the liquid hydrolysate decreased from 6.2 to 4.3 when the temperature of the pretreatment increased from AT to 200 °C, which was due to the increased formation of short-chain organic acids with the rise in temperature since a concentration of 524 ± 1 mg L^{-1} of VFA, expressed as COD, was obtained after hydrothermal pretreatment at 200 °C. The increased VFA concentration was likely due to acetic acid being released from the hydrolysis of acetyl groups contained in the hemicelluloses (Kaparaju et al., 2009; Kumar et al., 2011; Pérez et al., 2007).

As previously observed by Qiao et al. (2011) after hydrothermal pretreatment of food waste, NH_x was also released during heating. With regard to soluble carbohydrates present in the liquor, an increase in severity of the treatments led to an increase in sugar

Table 1
Fibre composition of untreated sunflower oil cake (SuOC) and SuOC after hydrothermal pre-treatment at ambient temperature (AT), 100, 150 and 200 °C.

	NDF[1] (%)*	ADF[2] (%)*	ADL[3] (%)*	Hemicellulose (%)*	Cellulose (%)*
Without pretreatment	48 ± 1	41 ± 1	14 ± 0	7	27
AT	59 ± 1	46 ± 0	17 ± 0	13	29
100 °C	61 ± 0	51 ± 1	19 ± 2	10	32
150 °C	66 ± 0	59 ± 0	29 ± 1	7	30
200 °C	71 ± 1	64 ± 0	33 ± 1	7	31

[1] NDF: Neutral detergent fibre.
[2] ADF: acid detergent fibre.
[3] ADL: acid detergent lignin.
* Expressed as volatile solids (w/w).

Table 2
Characterisation of the liquid fraction obtained after hydrothermal pretreatment of sunflower oil cake at different temperatures.

Pre-treatment	CODs[1] (mg O_2 L^{-1})	pH	TVFA[2] (mg COD L^{-1})	NH_x[3] (mg N L^{-1})	Carbohydrates (mg glucose L^{-1})
AT	2291 ± 68	6.2 ± 0.2	98 ± 6	8 ± 1	10 ± 1
100 °C	3125 ± 78	5.5 ± 0.3	158 ± 10	25 ± 1	40 ± 2
150 °C	5031 ± 141	5.1 ± 0.2	280 ± 6	64 ± 2	60 ± 2
200 °C	8468 ± 171	4.3 ± 0.2	524 ± 1	137 ± 3	130 ± 4

[1] CODs: soluble chemical oxygen demand.
[2] TVFA: total volatile fatty acids.
[3] NHx: ammoniacal nitrogen.

contents (from 10 ± 1 mg glucose L^{-1} at AT to 130 ± 4 mg glucose L^{-1} at 200 °C). A similar behaviour was reported by Ruiz et al. (2011) during the hydrothermal pretreatment of wheat straw at different temperatures. With the increase in the temperature and the production of hydrolysates, Maillard reactions occurred, which are responsible for the formation of refractory dissolved organic compounds that result in a dark colour and a burnt sugar odour.

In general, hemicellulose removal from the solid fraction can explain the increase in the soluble compounds in liquid fraction after hydrothermal pretreatment at temperatures above 150 °C (Negro et al., 2003; Pérez et al., 2008).

3.2. Methane production yields of solid and liquid fractions

Specific methane production during batch assays was the response variable used to evaluate the effect of the hydrothermal pretreatment on both fractions. Fig. 1 shows the cumulative methane yield as a function of time for solid (A) and liquid (B) fractions, respectively, during the BMP tests.

The methane yields for the solid fractions were 114 ± 9, 105 ± 7, 82 ± 7 and 53 ± 8 mL CH_4 g^{-1} COD_{added} at temperatures of 25, 100, 150 and 200 °C, respectively. For the solid fractions, the highest methane yield was obtained from SuOC treated at AT. This result was expected, taking into account that at this low temperature more organic matter remained than after pretreatment at higher temperatures. When the pretreatment temperature was increased, the methane yield decreased because a higher portion of carbohydrates and soluble compounds passed into the liquid fractions (Table 2). Therefore, a lower methane yield, than that obtained by De la Rubia et al. (2011) for SuOC without pretreatment (143 ± 3 mL CH_4 g^{-1} COD_{added}), was achieved. Pretreated SuOC at 200 °C achieved a 33% lignin content after pretreatment, a high value when compared with that of the sample treated at AT, containing 17% lignin. Thus, the pretreatment increased the lignin content by 94%, while it decreased the methane yield of the solid fraction by 46%. This relationship between the amounts of methane and lignin, was also observed by Kobayashi et al. (2004) during batch anaerobic digestion of steam-exploded bamboo.

The methane yields obtained for the liquid fractions (Fig. 1B) were 276 ± 6, 310 ± 4, 220 ± 15 and 240 ± 15 mL CH_4 g^{-1} COD_{added} from fractions obtained after pretreatments at 25, 100, 150 and 200 °C, respectively. It has been reported (Hendriks and Zeeman, 2009; Negro et al., 2003; Teghammar et al., 2010) that temperatures ⩾200 °C caused the formation of phenolic compounds as

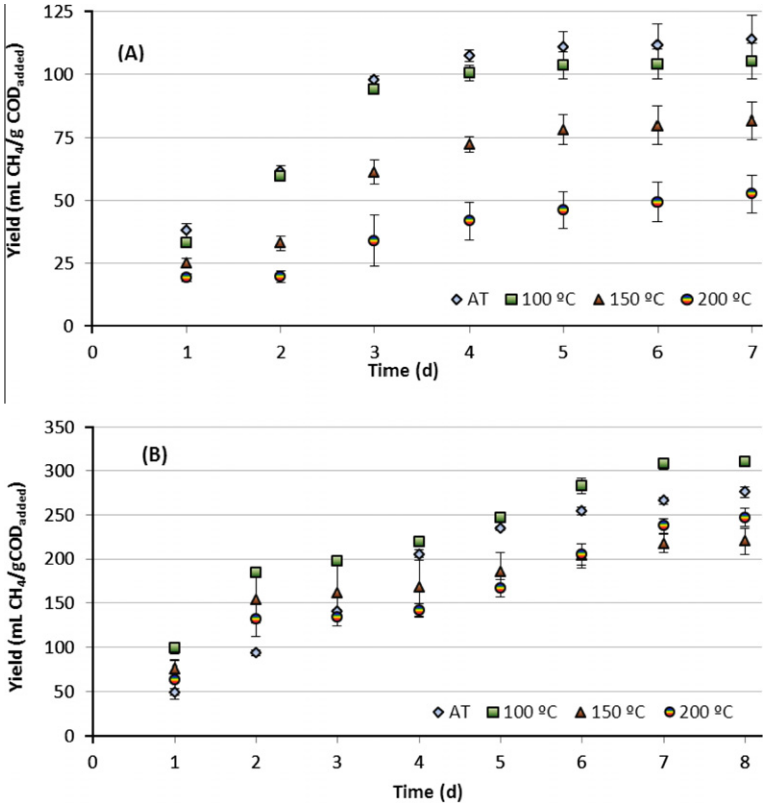

Fig. 1. Cumulative methane yield, expressed as mL CH_4/g COD_{added}, obtained during batch anaerobic digestion of hydrothermally pre-treated sunflower oil cake (A) Solid fraction; (B) Liquid fraction. Values are averages from three trials; errors bars indicate the standard deviation of the mean values ($p < 0.05$).

Table 3
Characterisation of digestates obtained after biochemical methane potential (BMP) experiments.

Assay	T[1] (°C)	CODs[2] mg O_2 L^{-1}	NH_x[3] mg N L^{-1}	TA [4] mg $CaCO_3$ L^{-1}	TVFA[5] mg COD L^{-1}
Solid fraction	AT	1593 ± 48	582 ± 11	5120 ± 10	7 ± 1
	100	1633 ± 33	582 ± 12	5120 ± 12	6 ± 1
	150	1720 ± 48	526 ± 16	4880 ± 15	6 ± 1
	200	2426 ± 66	650 ± 13	4600 ± 13	7 ± 1
Liquid fraction	AT	1882 ± 38	392 ± 8	4320 ± 11	7 ± 1
	100	2347 ± 60	526 ± 18	5160 ± 9	10 ± 1
	150	2890 ± 36	470 ± 9	5120 ± 10	8 ± 1
	200	3565 ± 107	358 ± 7	5040 ± 11	7 ± 1
Blank		1452 ± 28	336 ± 10	4320 ± 12	7 ± 1

[1] T: temperature.
[2] CODs: soluble chemical oxygen demand.
[3] NHx: ammoniacal nitrogen.
[4] TA: total alkalinity.
[5] TVFA: total volatile fatty acids.

well as furfural and HMF, which could inhibit the growth of anaerobic microorganisms. Therefore, the methane production obtained at AT was only improved for pretreatment at 100 °C.

When the methane yields from the solid and liquid fractions are combined, pretreatment at 100 °C can be considered optimal for hydrothermally pretreating SuOC before anaerobic digestion. Since, the overall or mean methane yields were 195, 207.5, 136 and 146.5 mL CH_4 g^{-1} COD_{added}, for AT, 100, 150 and 200 °C, respectively. This indicates that the value obtained at 100 °C was 6.5, 52.5 and 41.6% higher than those from SuOC pretreated at AT, 150 and 200 °C, respectively.

At the end of the BMP assays, digestates were also characterised (Table 3). Taking the final concentration values obtained for NH_x, alkalinity and VFA into account, the digestion process was carried out satisfactorily.

The highest CODs value obtained after the BMP assay of the liquid fraction at 200 °C can be explained by the formation of non-degradable or toxic compounds (Pérez et al., 2007).

3.3. Kinetic study

Table 4 lists the k values with 95% confidence, as well as the corresponding values of B_0 and R^2. The high values of the coefficient of determination R^2 (>0.94 for solid fractions and $\geqslant$0.91 for liquid fractions) and the low values of the confidence limits of the parameters obtained demonstrate the good fit of the experimental data to the proposed model.

The apparent kinetic constants of the process for the solid fractions are related to the concentration of lignin. The highest k values (0.41 ± 0.07 d^{-1} and 0.43 ± 0.08 d^{-1}) were obtained for AT as well as for 100 °C, for which the lignin concentration achieved the lowest values (around 17–19%). When the lignin content increased to 33% at 200 °C, the value of k decreased to 0.25 ± 0.06 d^{-1} showing the occurrence of an inhibitory phenomenon by lignin. A similar tendency was observed by Teghammar et al. (2010) after hydrothermal pretreatment of paper residues at 200 °C. Although the experiment carried out with the liquid fraction at 150 °C showed a kinetically more favourable value (k = 0.46 ± 0.07 d^{-1}) than that with the hydrolysate obtained after pretreatment at 100 °C, the highest B_0 was obtained for the sample originating from pretreatment at 100 °C (328 ± 21 mL CH_4 g^{-1} COD_{added}).

Table 4
Apparent kinetic constant (k) and ultimate methane yield (B_0) values with 95% confidence limits for experiments carried out with hydrothermal pretreated sunflower oil cake (solid and liquid fractions) at different temperatures.

Experiment	Temperature (°C)	k d^{-1}	B_0 mL CH_4 g^{-1} COD_{added}	R^2
Solid fraction	AT	0.41 ± 0.07	125 ± 8	0.9538
	100	0.43 ± 0.08	116 ± 8	0.9436
	150	0.29 ± 0.07	98 ± 11	0.9480
	200	0.25 ± 0.06	64 ± 9	0.9490
Liquid fraction	AT	0.24 ± 0.03	320 ± 17	0.9724
	100	0.33 ± 0.05	328 ± 21	0.9510
	150	0.46 ± 0.07	218 ± 10	0.9339
	200	0.21 ± 0.07	288 ± 49	0.9086

4. Conclusions

Hydrothermal pretreatment temperatures higher than 100 °C alter the chemical composition of the solid and liquid fractions, obtained with SuOC such that the methane yield from anaerobic digestion decreases. Therefore, hydrothermal pretreatment at 100 °C was the best option to improve the anaerobic digestion of SuOC and its methane yield, among the temperatures assayed. However, with only a 6.5% increase in yield compared to the yield which material treated at AT, it would be difficult to justify conducting this pretreatment.

The kinetic constant of the anaerobic digestion of solid fraction released after the pretreatment are related to the lignin concentration, decreasing when lignin content increases with temperature pretreatment.

Acknowledgements

The authors wish to express their gratitude to the Spanish Ministry of "Ciencia e Innovación" (Project CTM 2008-05772/Tecno) for providing financial support, to the program "FPI" of the above-mentioned Ministry for contracting graduates, as well as to the contract JAE-Doc from "Junta para la Ampliación de Estudios del CSIC", co-financed by European Social Funds.

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3.1.2 Pretratamiento Químico y Termoquímico

Referencia del artículo publicado que refleja estos resultados:

Effects of chemical and thermochemical pretreatments on sunflower oil cake in biochemical methane potential assays.

Fernández-Cegrí, V., Raposo, F., de la Rubia, M.A., Borja, R. (2013) Journal of Chemical Technology and Biotechnology. En prensa. (Aceptado el 6/08/2012). DOI: 10.1002/jctb.3922.

El objetivo de este trabajo fue estudiar el efecto de distintos pretratamientos químicos y termoquímicos sobre la composición y el coeficiente de rendimiento de metano del SuOC.

Los reactivos y tiempo de aplicación a los que fue sometido el sustrato objeto de estudio (SuOC) fueron, tanto para el pretratamiento químico como para el termoquímico: NaOH, $Ca(OH)_2$, H_2SO_4 y $NaHCO_3$ durante 4 horas, todos ellos sin agitación para evitar añadir los posibles efectos de un pretratamiento mecánico adicional. Para los tratamientos termoquímicos y se agregó el factor temperatura, calentando para ello las muestras a 75ºC.

Observando los efectos de los pretratamiento generados sobre la composición química de SuOC, se concluye que las fracciones líquidas obtenidas después de aplicar los pretratamientos con NaOH fueron las que presentaron mayores grados de solubilización en términos de CODs comparados con los obtenidos con el sustrato no tratado (Untreated). No hubo variación significativa en cuanto a las solubilidades conseguidas entre los pretratamientos químico y termoquímico con NaOH como reactivo, a diferencia de con los demás reactivos, los cuales se observaron diferencias entre los dos tipos de pretratamientos, alcanzandose mayores solubilizaciones en las condiciones termoquímicas, con mayores diferencias cuando se usa el ácido como agente químico.

En cuanto a las fracciones solidas obtenidas después de aplicar los pretratamientos, se observó una disminución significativa en el contenido de hemicelulosa excepto cuando se utilizó el bicarbonato como reactivo, con el cual los porcentajes de hemicelulosa se mantuvieron constantes respecto al sustrato sin pretratar. El pretratamiento termoquímico con H_2SO_4 fue el que alcanzó una mayor degradación de hemicelulosa, con reducciones del 84% respecto al no tratado. Junto con éste, el pretratamiento químico con NaOH permitió obtener buenos resultados con reducciones del 60% del contenido en hemicelulosa. En el caso de la celulosa, solamente el pretratamiento con cal permitió reducir el contenido relativo respecto al sustrato sin tratamiento, siendo las condiciones termoquímicas las que presentaron mayor disminución en el porcentaje de celulosa.

Es importante destacar que a pesar de que la aplicación de $Ca(OH)_2$, H_2SO_4 y $NaHCO_3$ como reactivos tanto para los pretratamientos químicos como en los termoquímicos generan niveles de solubilidad similares en las fracciones líquidas, no ocurre lo mismo al analizar el efecto de éstos sobre la fibra de las fracciones sólidas. Este hecho es debido a que cada reactivo degrada de manera diferente a los distintos componentes del sustrato objeto de estudio, generando materiales con distintas características en cada caso.

En cuanto a los efectos de estos pretratamientos sobre el rendimiento de metano acumulado, los obtenidos de las fracciones sólidas resultaron ser más bajos en la mayoría de los casos con rendimientos entre un 15 y un 48% menores que los obtenidos en la fracción sólida del sustrato sin tratar, excepto en el caso del pretratamiento químico con cal, el cual produjo un aumento del 14 % de rendimiento de metano acumulado respecto a sustrato no tratado. Sin embargo, los menores coeficientes de rendimiento fueron los producidos por el pretratamiento termoquímico con cal, en el que se observó una inhibición del proceso por acumulación de ácidos, detectados al final del ensayo una vez analizado el digestato.

Los rendimientos de metano acumulado de las fracciones líquidas obtenidas después de aplicar la cal como reactivo, presentaron resultados opuestos a los obtenidos con la fracción sólida, siendo en este caso el pretratamiento termoquímico el que obtuvo mejores resultados, con un incremento del rendimiento en metano respecto al no tratado de un 24.6%. El pretratamiento químico con bicarbonato también logró superar el rendimiento en metano acumulado producido por el sustrato no tratado en un 14%. Para esta fracción, los resultados de los pretratamientos con ácido tanto en condiciones químicas como termoquímicas, presentaron inhibiciones durante el proceso, efecto también observado en el caso del pretratamiento químico con sosa.

De manera general, no se observó un aumento destacado en el coeficiente de rendimiento en metano del sustrato objeto de estudio (SuOC) después de ser pretratado química y termoquímicamente con las condiciones estudiadas.

Research Article

Received: 10 July 2012 Accepted: 6 August 2012 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jctb.3922

Effects of chemical and thermochemical pretreatments on sunflower oil cake in biochemical methane potential assays

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Abstract

BACKGROUND: The effects of chemical and thermochemical pretreatments on the composition and anaerobic biodegradability of sunflower oil cake were studied to compare these pretreatments and to assess their effectiveness. Four reagents (lime, sodium hydroxide, sulphuric acid, and sodium bicarbonate) at concentrations of 25% (w/w) of dry weight of substrate and 20 g L^{-1} substrate concentration were used for the chemical pretreatment for 4 h. The same conditions were used for thermochemical pretreatment with heating at 75°C. After the pretreatments, the solid and liquid fractions were separated and subjected to biochemical methane potential tests.

RESULTS: The methane yields of the solid fraction obtained with lime, sodium hydroxide, sulphuric acid and bicarbonate were 130±9, 54±4, 61±6 and 88±7 mL CH_4 $g^{-1}COD_{added}$, respectively, and after thermochemical pretreatment were 26±2, 84±7, 74±7, and 77±6 mL CH_4 $g^{-1}COD_{added}$, respectively. The methane yields for liquids were 152±13, 2±0, 0±0, 249±19 mL CH_4 $g^{-1}COD_{added}$, for the chemical pre-treatment, respectively, and after the thermochemical pretreatment were 273±13, 58±5, 0±0 and 145±12 mL CH_4 $g^{-1}COD_{added}$, respectively.

CONCLUSION: Only the solid fraction obtained after the chemical pretreatment with lime gave a methane yield higher (130 mL CH_4 $g^{-1}COD_{added}$) than the obtained for the untreated solid material (114 mL CH_4 $g^{-1}COD_{add}$). No thermochemical pretreatment enhanced the methane yield of the solid or liquid fractions of the untreated material.

Keywords: anaerobic digestion; biodegradable; pre-treatment; biogas; biochemical methane potential

INTRODUCTION

Biomass is considered a worldwide valuable energy alternative to fossil fuels, because it may be converted into a variety of usable forms of energy such as heat, steam, electricity, hydrogen, biogas, and liquid transportation biofuels.[1] Anaerobic digestion (AD) is widely used as an alternative energy source because various agricultural residues and other biodegradable wastes may be subjected to the AD bioprocess to produce a methane-rich biogas that is suitable for energy production. In the industrial processing of sunflower seeds into edible oil, large quantities of solid wastes called sunflower oil cake (SuOC) are generated. SuOC is considered to be of relatively poor quality due to high concentrations of lignocellulosic compounds. The relatively poor quality of SuOC restricts the amount that can be included in feed blends for ruminant animals. Lignocellulosic biomass is difficult to degrade biologically and consists of three main biopolymers: cellulose, hemicelluloses, and lignin. In this type of substance, cellulose is physically associated with hemicelluloses, and physically and chemically associated with lignin. Lignin and hemicelluloses are intermeshed and chemically bound through covalent cross-linkages such as ester or ether linkages. The low biodegradability (BD) of lignocellulose in biogas reactors is due to lignin, which is not degradable in anaerobic environments because the extracellular enzymes require oxygen to depolymerize them. Furthermore, the hydrolysis of cellulose in lignocellulosic materials is reduced by lignin and hemicelluloses, since these components act as a protective coating, making the cellulose resistant to enzymatic digestion.[2] Due to the refractory structure of these compounds, one of the major problems in utilizing SuOC and other vegetable crop residues for methane production by AD is their low digestibility. AD and hence the methane potential of a complex substrate depends on the content of biodegradable compounds: carbohydrates (including cellulose), proteins and lipids. The AD efficiency of lignocellulosic biomass can be improved by applying several pretreatment methods. In general, the limiting step of solid waste AD is the first step, hydrolysis, where the cell wall is broken, making the organic matter inside the cell available for biological degradation. Some pretreatments have been developed in order to achieve the release of lignocellulosic material and thus accelerate the degradation process by means of waste solubilization. In order to increase methane production, the pretreatment options hydrolyse the cell wall.[3] So, it is necessary to carry out a pretreatment step to break

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the lignin seal, thus exposing the cellulose and hemicellulose to enzymatic action.[4] Pretreatment also aims to decrease the crystallinity of cellulose, increase biomass surface area and remove hemicellulose. The removal of hemicellulose increases the mean pore size of the substrate and therefore increases the probability of hydrolysing the cellulose.[4] Pretreatments should not only substantially improve the accessibility of the biomass to enzymes in the subsequent hydrolysis, but also avoid intensive carbohydrate loss or degradation during the process. Acid and alkaline pre-hydrolysis are the two most intensively studied chemical methods in the pretreatment of lignocellulosic biomass. Acid pretreatment results in disruption of covalent bonds, hydrogen bonds, and Van der Waals forces that hold together the biomass components, which, consequently causes the solubilization of hemicellulose and the reduction of cellulose crystallinity.[5] Rather than treating lignocellulose using concentrated acid, diluted acid pretreatments are normally practised at high temperatures to improve cellulose hydrolysis.[6] In contrast, alkaline pretreatment causes delignification of the biomass and makes the lignocellulose swell through saponification reactions. Unlike acid pretreatment, alkaline pretreatment has been proven effective within a wide temperature range at various chemical concentrations. Sodium hydroxide (NaOH) and lime ($Ca(OH)_2$) are the two alkaline reagents that have attracted the most attention. Several authors studied a pretreatment using NaOH and verified that the removal rate of lignin generally increased with increase in pretreatment severity. Some results show that, compared with NaOH pretreatment, the delignification capability of $Ca(OH)_2$ is much lower, which could be because divalent calcium ions from $Ca(OH)_2$ dissociation have a high affinity to lignin and can effectively crosslink lignin molecules, thus preventing them from solubilization under alkaline attack. The objective pursued by the inclusion of a pretreatment alternative is to modify the structure of complex materials with decreasing degrees of polymerization, to weaken the links of lignin to carbohydrates, and to increase the surface area of the particles that constitute the substrates.

The goal of this study was to examine the effects of chemical pretreatment on a BMP test of SuOC, with acid and basic reagent. The same conditions except for the application of simultaneous thermal energy were also analysed with the aim of comparing the effects of chemical and thermo-chemical pretreatments on the methane yield coefficients.

MATERIALS AND METHODS

Raw material

The SuOC sample used in this study was collected from a sunflower oil factory located near Seville (Spain). Prior to use, the substrate was shredded into different particle sizes using a commercial sieve (Restch AS 200 basic) and different screen meshes. In order to ensure the homogeneity of the sample, the most abundant particle size of this substrate (0.71 – 1.0 mm diameter) was selected to carry out the experiments.

The full composition and main features as well as the fractional composition of the fibre of the above-mentioned SuOC particle size selected are as follows (mean values of four determinations ± standard deviations): COD_{total}, 1.24 (±0.02) g O_2 g^{-1} TS dry basis; Total solid dry basis (TS), 93.0 (±0.1)%; volatile solids (VS) expressed as dry basis, 93.0 (±0.1)%; ash, 6.8 (±0.1)%; neutral detergent fibre (NDF), 45.0 (±1.1)%; acid detergent fibre (ADF), 38.4 (±0.9)%; acid detergent lignin (ADL), 13.3 (±0.2)%; hemicellulose, 6.6 (±1.0)%; cellulose, 25.3 (±0.4)%; total protein, 25.3 (±0.8)%; fat content, 1.6 (±0.2)%; soluble carbohydrates, 5.1 (±0.2)% and total carbohydrates (by difference), 53.0 (±0.3)% (% expressed as TS dry basis).

Chemical and thermochemical pretreatments

The SuOC was treated with alkaline reagents lime ($Ca(OH)_2$), sodium hydroxide (NaOH), sodium bicarbonate ($NaHCO_3$) and acid reagent, sulphuric acid (H_2SO_4). The conditions of pretreatments were chosen based on a previous study carried out using different concentrations of reagents, times, and temperatures with the objective of choosing the most efficient operational conditions on this substrate. The effectiveness of the different conditions assayed was assessed through soluble chemical oxygen demand (CODs). The final conditions selected after the previous assays were 25% (w/w) of substrate of wet weight for the concentration of the reagents, 4 h hydrolysis and 20 g L^{-1} SuOC. The selected temperature for the thermochemical pretreatment was 75°C. Each suspension was treated under static conditions to avoid applying a mechanical pretreatment. The total sample was separated after pretreatment into the liquid supernatant fraction and the residual solid phase. Both fractions were separated by centrifuging the sample for 15 min at 10 000 rpm and after the liquid phase were filtered through a glass filter of 1.2 µm and then through 0.45 µm pore size filters to remove the colloidal solids and to produce the soluble pre-treated fraction. For the thermochemical pretreatment the same conditions were applied but with simultaneous heating at 75°C using a heater plate and controlling the temperature with a thermometer. In this way, it was possible to study the effect of temperature while maintaining the above mentioned experimental conditions. Subsequently, characterization of both fractions was performed: (i) liquids: pH, CODs and alkalinity; (ii) solids: total chemical oxygen demand (COD_T), total and volatile solids (TS and VS) and fibre composition.

These results were compared with the results obtained with the untreated substrate (control) prepared with the same concentration, 20 g L^{-1} , with distilled water, 4 h under static conditions and at room temperature and it was not pre-treated with any chemicals. Prior to the anaerobic biodegradability experiments, chemical supplementation of the liquid fractions was required (with H_2SO_4 98% (w/v) for the basic solutions and NaOH 50% (w/w) for the acid solutions) in order to limit the impact of pH on the system. The pH was adjusted to 7.5.

Inoculum

Granular sludge taken from an industrial anaerobic reactor, which treats wastewater from a brewing company, operating at mesophilic (35°C) conditions, was used as inoculum. This inoculum was selected due to its high methanogenic activity. The characteristics and features of the anaerobic sludge used were: pH 7.6±0.1, 85 g L^{-1} TS and 44 g L^{-1} VS.

Anaerobic digestion experiments

Anaerobic biodegradability of the pre-treated SuOC (liquid and solid fractions) obtained through different pretreatments was evaluated by biochemical methane potential (BMP).

The experimental study was carried out in a multi-batch reactor system, which consisted of nine Erlenmeyer flasks, with an effective volume of 250 mL. They were continuously stirred with magnetic bars at 300 rpm and placed in a thermostatic water bath at mesophilic temperature (35±1°C). Both fractions, solid and liquid, for each pretreatment studied, chemical and thermo-chemical,

were digested in assays performed in triplicate. Triple positive control reactors with starch as the control blanks were also carried out. All reactors were initially charged with anaerobic inoculum by maintaining a concentration of 15 g VS L^{-1} (the volume taken is a function of the initial VS concentration of the inoculum). The inoculum to substrate ratio (ISR) was maintained at 2 (VS basis) for the reactors digesting the solid fractions and at 2.5 (COD basis) for the reactors processing the liquid fractions.[7] After the pre-treated substrate was added to each reactor, 25 mL of a stock mineral medium solution were also added (the composition has been described elsewhere).[8] Finally, distilled water was added to achieve the desirable effective volume and the reactors were flushed with N_2 in order to maintain anaerobic conditions. The methane released was measured by volume displacement (carbon dioxide was previously removed by flushing the gas through a 2 N NaOH solution), and expressed at standard temperature and pressure (STP) conditions. The methane production due to biomass decay and the possible presence of residual substrate in the inoculum was subtracted by performing blank controls. The BMPs assayed were run between 7 and 10 days, until the accumulated gas production remained essentially unchanged (on the last day, production was lower than 2% of the accumulated methane produced), suggesting that biodegradation had been completed. This short period of time was sufficient to achieve maximum methane production, and can basically be explained by the high methanogenic activity of the sludge and the short interval (less than 72 h) had elapsed between inoculum sampling and the start-up of the experiments.

Analytical methods

Solid samples

The following parameters were analysed in the original solid substrate (SuOC): TS and VS, according to standard methods 2540B and 2540E, respectively.[9] CODt was determined using the method proposed by Raposo *et al*.[10] Fat content was extracted with hexane, using a Soxhlet system. Fibre analysis was done according to Van Soest *et al*.[11] using the gravimetric method with a Dosi Fiber (Selecta®) equipment and crucibles of 40–100 μm pore size. For neutral detergent fibre (NDF) heat stable α-amylase and anhydrous sodium sulphite were used.[12] The acid detergent fibre (ADF) was done non-sequentially while the acid detergent lignin (ADL) was determined sequentially. The analyses were carried out in order to calculate hemicellulose (NDF – ADF), cellulose (ADF – ADL) and lignin (ADL). The results were expressed as VS.

Soluble fractions

The soluble pre-treated fraction obtained was characterized using the following soluble parameters: CODs, using the closed digestion and colorimetric Standard Method 5220D;[9] total alkalinity (TA) measured by pH titration to 4.3; soluble ammoniacal nitrogen $(NH_x)_s$ determined by distillation and titration according to the Standard Method 4500E;[9] volatile fatty acid (VFA) concentration analysed by gas chromatography, as previously described.[13]

Inoculum and digestates

Both the inoculum and digestates were characterized by direct sampling. pH was determined using a pH-meter model Crison 20 Basic. TA, TS and VS were also analysed in these samples.

RESULTS AND DISCUSSION

In this work, the pretreatment efficiencies were evaluated with respect to the composition of SuOC and the hydrolysis yield.

Effect of pretreatments on solubilizations and fibre composition

Solubilization is just one of the indicators for evaluating the effect of the pretreatments along with the destruction of the lignocellulosic structure.[14]

Organic material solubilizations

The solubilization of organic material (OM)s, in terms of soluble chemical oxygen demand CODs, for the different pretreatments is shown in Fig. 1. The results obtained for the different pretreatments were compared with those for the untreated substrate. NaOH pretreatment produced the highest solubilization of SuOC in relation to the other pretreatments, with values of CODs of 9000 mg O_2 L^{-1}. The other reagents studied gave lower solubilization and no significant differences were observed among them, with values ranging from 3300 to 4000 mg O_2 L^{-1}. For the thermochemical pretreatment, the results of solubilization ranged between 4800 and 6000 mg O_2 L^{-1}, approximately. In the case of NaOH, there were no significant differences between the chemical and thermochemical pretreatments. However, for the rest of the reagents there was an important statistical difference between the two types of pretreatment for each reagent, especially for H_2SO_4.

The compositional changes observed after the pretreatments were carried out are summarized in Table 1. It was observed that COD_T after the pretreatments was very similar to that of the untreated material but it can be observed that the alkali reagents were more effective in degrading the total organic material than the acid reactant.

Fibre composition

With respect to fibre composition, in general it was observed that the SuOC lost significant amounts of hemicellulose after all the pretreatments except with $NaHCO_3$. The most effective pretreatment for solubilization of hemicellulose was the thermo-acid pre-treatment, with an 84% reduction with respect to the untreated material. Without temperature, again the acid, along with NaOH, were the best reagents for OM solubilization, with a 62% reduction. The relative content of cellulose and lignin increased in all cases except when $Ca(OH)_2$ was used, for which the lignin decreased noticeably by 19% in both types of pretreatments. In addition, the cellulose suffered a reduction of 18 and 11% with respect to the untreated material with and without temperature, respectively. In contrast, it is important to note the increased value in the relative content of the cellulose fraction, the component more available for hydrolysis, with increases of 25% and 46% when NaOH was used in the chemical and thermochemical pretreatments, respectively, while with $NaHCO_3$ reductions of 21 and 11% were observed with and without temperature, respectively. On the other hand, it can be confirmed that H_2SO_4 was not capable of dissolving cellulose, maintaining the same proportion as in the untreated case with this substrate. In this study, alkali pretreatment gave higher removals of lignin in comparison with other reagents, regardless of the effect of temperature. The acid pretreatment proved very effective in removing hemicellulose, in this case with a significant difference statistically, when temperature is applied, eliminating almost all the hemicellulose.

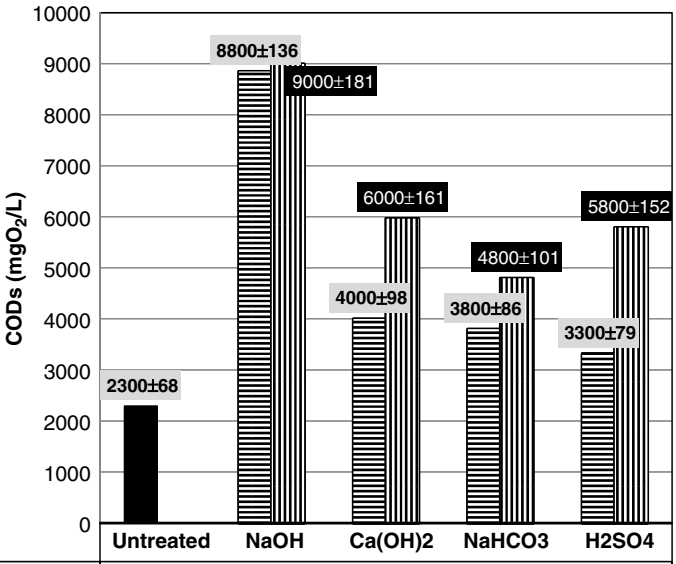

Figure 1. Effects of the different chemical and thermochemical pretreatments studied on the CODs (mg O_2 L^{-1}) of substrate tested.

Table 1. Composition of SuOC after the different chemical and thermochemical pretreatments assayed. (%, volatile solid)*

		Cellulose (%)*	Hemicellulose (%)*	Lignin (%)*	COD_T (mg g^{-1} TS)	Ash (%)	pH	Total alkalinity (mg $CaCO_3 L^{-1}$)	Ammoniacal nitrogen (mg $NH_x L^{-1}$)
Untreated		28	13	16	1.24±0.02	7.0±0.1	7.0±0.1	2.50±0.05	8±1
Chemical pretreatment	NaOH	35	5	19	1.16±0.03	18.0±0.1	12.5±0.1	5.36±0.05	62±2
	$Ca(OH)_2$	25	8	13	1.12±0.02	14.8±0.1	12.0±0.1	2.28±0.05	76±2
	H_2SO_4	28	5	16	1.26±0.04	2.0±0.1	1.3±0.1	2.23±0.05	65±1
	$NaHCO_3$	31	15	15	1.26±0.02	4.0±0.1	8.2±0.1	2.84±0.05	73±2
Thermochemical pretreatment	NaOH	41	8	19	1.18±0.03	16.3±0.1	13.4±0.1	5.48±0.05	78±4
	$Ca(OH)_2$	23	7	13	1.03±0.02	36.9±0.1	11.9±0.1	1.84±0.05	67±2
	H_2SO_4	30	2	17	1.35±0.01	1.0±0.1	1.0±0.1	2.30±0.05	67±1
	$NaHCO_3$	34	14	16	1.37±0.02	6.7±0.1	8.0±0.1	2.72±0.05	71±2

However, although the same levels of solubilization were observed with H_2SO_4, $Ca(OH)_2$ and $NaHCO_3$, these pretreatments provided many differences in fibre composition. This is because each type of reagent attacks different parts of the substrate. Chemical treatments have different effects depending on the reagent used; in the case of acid reactants, hydrolysis of the hemicellulose takes place. The alkali treatment breaks the links between lignin monomers or between lignin and polysaccharides.[15]

Xie *et al*.[16] observed that for dried grass silage pre-treated at different NaOH loading rates (1%, 2.5%, 5% and 7.5% by volatile solids and at 100°C), up to 45% of the total COD was solubilized and up to 65.6%, 36.1% and 21.2% of lignin, hemicellulose and cellulose were removed, respectively.

Rajan *et al*.[17] studied different types of alkaline agents such as NaOH, $Ca(OH)_2$ on waste activated sludge and concluded that sodium hydroxide gave better results in terms of CODs with small doses. Also, in the case of activated sludge, solubilization increases to above 46%, solubilizing the particulate material into nitrocellulose-soluble organic carbon.[18] In this case, the chemical pretreatment assay resulted in a level of solubilization of up to 46%, adding between 5 and 40 meq L^{-1} NaOH. Using NaOH, higher levels of solubilization were observed when compared with other alkalis such as $Ca(OH)_2$.

Effect of pretreatments on SuOC methane yield

For the analysis of methane yields, solid and liquid fractions were analysed independently.

Solid fraction

As shown in Fig. 2, it can be observed that pretreatment with $Ca(OH)_2$ produced the highest yield of methane, with 130.5 mL CH_4 g^{-1} COD_{added}, 14% higher than that obtained for the untreated substrate. $Ca(OH)_2$ was the unique reactant among the different reagents studied that increased the final methane yield of the solid fraction compared with the untreated material. With these results, it is logical to observe that after pretreatment the solid fraction

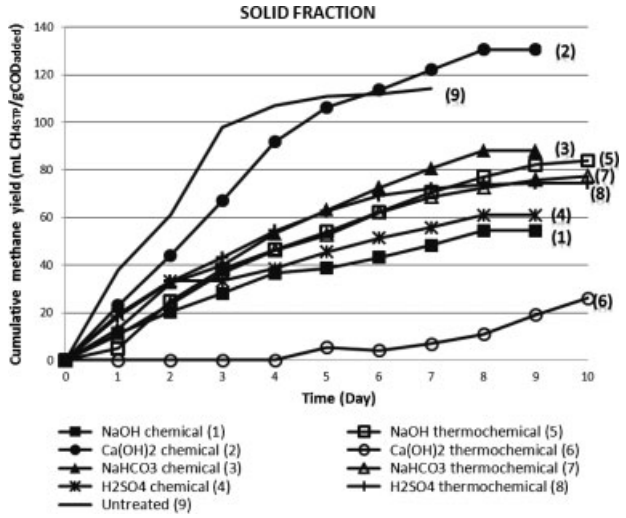

Figure 2. Cumulative methane yield (mL CH_4STP $g^{-1}COD_{added}$) for the solid fractions.

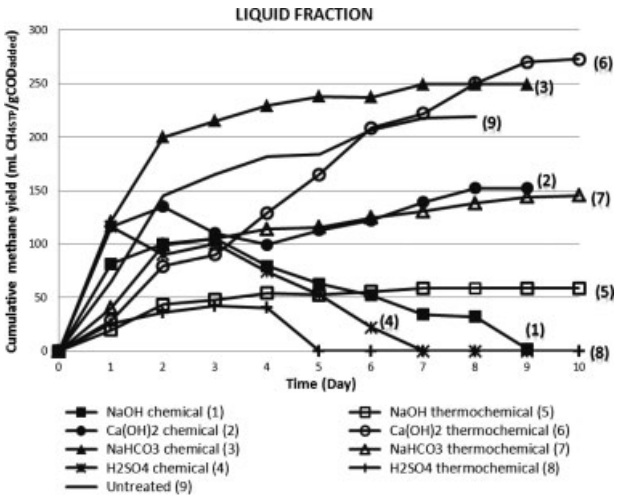

Figure 3. Cumulative methane yield (mL CH_4STP $g^{-1}COD_{added}$) for the liquid fractions.

is drained. It is important to note that the same reagent but at 75°C gave the worst result in this fraction. To justify this, Xu *et al.*[19] reported a decrease in lignin solubilization because the treatment with $Ca(OH)_2$ caused an interaction between negatively charged lignin molecules and positively charged calcium ions. This can be explained by the formation of calcium–lignin complexes at higher temperature.[20]

Inhibition in the process was observed with $Ca(OH)_2$ during thermochemical pretreatment. Acetic and isovaleric acid concentrations of 1322 and 125 mg L^{-1}, respectively, were observed in the digestates at the end of the BMP process. The rest of the pretreatments generated similar amounts of gas, with a methane yield of between 15 and 48% lower than for the untreated material. This fact confirms the exhaustion of the substrate in the solid fraction after the pretreatments.

Liquid fraction

As shown in Fig. 3, the opposite situation was found in the case of $Ca(OH)_2$. Pretreatment at higher temperature gave the best result, with methane yields reaching 24.6% higher than with the untreated material. The next highest result obtained was with $NaHCO_3$ without temperature, with 14% more methane production than for the untreated material. Regarding NaOH and H_2SO_4 with and without temperature, both presented a clear inhibition for the liquid fraction. Rice straw was pre-treated with NaOH in solid-state conditions and anaerobically digested[21] with four NaOH doses (4%, 6%, 8%, and 10%) and four loading rates (35, 50, 65, and 80 g L^{-1}). These authors observed that after pretreatment with NaOH, the biogas yield reached 3.2–28.6%, 27.3–64.5%, 30.6–57.1%, and 15.2–58.1% higher than that obtained for the untreated rice-straw at the respective loading rates. Dried grass silage was pre-treated by Xie *et al.*[16] at different NaOH loading rates (1%, 2.5%, 5% and 7.5% of volatile solids (VS) mass in grass silage) and temperatures (20°C, 60°C, 100°C and 150°C) to determine their effects on its bio-degradability in terms of the hydrolysis yield. At 100°C and for the four NaOH loadings, the BMP productions obtained were 359.5, 401.8, 449.5 and 452.5 mL CH_4 g^{-1} VS_{added}, respectively, with an improvement of 10–38.9% in comparison with the untreated substrate. Sodium hydroxide (NaOH) was used by Pang *et al.*[22] to pre-treat corn stover in a solid state at ambient temperature to improve biodegradability and anaerobic biogas production with four NaOH doses of 4%, 6%, 8%, and 10% on dry matter basis of the substrate and at four loading rates of 35, 50, 65, and 80 g L^{-1}, respectively. The results showed that 6% NaOH-treated corn stover digested at the loading rate of 65 g L^{-1} achieved 48.5% more biogas production than the untreated material.

Other researchers studied the improvement in biogas production from cattle manure with $Ca(OH)_2$ pretreatment, while observing the effects of temperature (20°C and 60°C), time (10 min, 2, and 12 h), and pH (9, 10, 11, and 12).[23] The results showed that alkaline pretreatment at 20°C did not affect biogas production, while the manure treated at 60°C produced more methane than the untreated one. The maximum improvement in methane production was achieved with a pretreatment at pH 12 for 12 h, which resulted in a methane yield of 225 mL CH_4 g^{-1} VS, which was 76% higher than that obtained from untreated manure. Results contrary to those obtained by these authors were achieved by Antonopoulou *et al.*[24] who studied the anaerobic digestion process of different sunflower residues with chemical pretreatment methods such as thermal, chemical (with NaOH and H_2SO_4 addition 2%w/v) or a combination of the two. The results obtained in this case demonstrated that the pretreatment methods tested did not enhance the methane potential of the sunflower residues.

CONCLUSIONS

The effects on composition and anaerobic biodegradability of SuOC under different chemical and thermochemical pretreatments were studied in this work. Regarding changes in composition of SuOC, it can be concluded that the pretreatments assayed did not result in increased degradation when applying additional thermal energy. Regarding the generation of methane for each solid fraction assayed, the best results were achieved with $Ca(OH)_2$ without temperature, with an increase of 25% in the methane yield compared with the untreated substrate. For the liquid fractions, the best results of methane production were reached with $Ca(OH)_2$ and temperature and $NaHCO_3$ without the effect of temperature, with increases of 37% and 11%, respectively,

compared with the untreated material. The high amounts of methane achieved with $NaHCO_3$ can be explained by the alkalinity generated when using this reagent, which favours and stabilizes the anaerobic process. In any case, it can be concluded that although the reagents used in the pretreatments changed the composition of the material, the potential toxicity of these chemical reagents in the anaerobic biomass can also affect the global anaerobic process, hindering the generation of methane from SuOC. Taking into account the overall results considering both fractions, solid and liquid, the highest methane yield was achieved with $Ca(OH)_2$ without heating, 141±11 mL CH_4 $g^{-1}COD_{added}$, but this value was lower than that obtained from the untreated material (195±7 mL CH_4 $g^{-1}COD_{added}$). The results show that the pretreatments applied to SuOC did not enhance the methane potential of this substrate.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to the Spanish Ministry of 'Ciencia e Innovación' (Project CTM 2008-05772/Tecno) for providing financial support and to the Program 'FPI' of the above-mentioned Ministry for contracting graduates.

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3.1.3 Pretratamiento con ultrasonido

Referencia del artículo publicado que refleja estos resultados:

Impact of ultrasonic pretreatment under different operational conditions on the mesophilic anaerobic digestion of sunflower oil cake in batch mode.

Fernández-Cegrí, V., De La Rubia, M.A., Raposo, F., Borja, R. (2012) Ultrasonics Sonochemistry, 19 (5), pp. 1003-1010.

En este estudio se investigó el efecto de la aplicación de ultrasonido sobre el sustrato de harina de girasol desengrasada, como pretratamiento, con el objetivo de aumentar el grado de biodegradabilidad y el coeficiente de rendimiento en metano.

Para ello, se ensayaron cinco condiciones experimentales distintas, basadas en diferentes energías específicas aplicadas sobre el sustrato. El intervalo de energía específica aplicado varió desde 24.000 kJ/kg TS (US1) a 597.600 kJ/kg TS (US5), todos ellos a frecuencia de sonicación constante de 20 kHz y potencia aplicada constante de 120 W.

Se examinó la influencia del pretratamiento con ultrasonido sobre la composición del sustrato después de cada condición ensayada, como para los anteriores pretratamientos, en función de la solubilidad en la fracción líquida. De nuevo, al igual que en el caso del pretratamiento hidrotermal, se observó un grado mayor de solubilización al aumentar la energía específica aplicada, alcanzándose valores que oscilaron entre 14 y 21% de material solubilizado cuando se aplican la menor y la mayor energía específica (US1 y US5) respectivamente. Este incremento de 1.5 veces en la solubilidad se produce cuando la energía específica aplicada aumenta en 25 veces.

En el caso de las transformaciones originadas por el pretratamiento en la composición de la fracción sólida, es destacable la reducción en el porcentaje de hemicelulosa originada al aplicar la energía específica de menor valor (24.000kJ/kg TS), disminuyendo en un 31.8% el contenido en hemicelulosa inicial del sustrato. Todas las energías específicas aplicadas redujeron el contenido relativo de lignina en torno a un 40-46%, independientemente de la cantidad de energía aplicada en cada caso.

Respecto a los efectos de las diferentes condiciones aplicadas sobre los coeficientes de rendimientos en metano, se halló para la fracción sólida, un incremento en el rendimiento de un 22% cuando se aplica la energía específica más baja (US1) en relación al valor observado cuando se utiliza la más elevada (US5). En el caso de la fracción líquida ocurrió la misma tendencia que con la fracción anterior, un aumento de un 11% aproximadamente, en este caso, para la menor energía aplicada con respecto al obtenido para la mayor energía utilizada.

Calculando el valor medio de los rendimientos en metano obtenidos a partir de ambas fracciones, se demuestra que las condiciones del primer experimento (US1) dentro del intervalo de energía específico ensayado, fueron las óptimas, incrementandose el rendimiento global en metano en un 54% con respecto al valor obtenido sin la aplicación del pretratamiento.

Ultrasonics Sonochemistry 19 (2012) 1003–1010

Contents lists available at SciVerse ScienceDirect

Ultrasonics Sonochemistry

journal homepage: www.elsevier.com/locate/ultsonch

Short Communication

Impact of ultrasonic pretreatment under different operational conditions on the mesophilic anaerobic digestion of sunflower oil cake in batch mode

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ARTICLE INFO

Article history:
Received 16 December 2011
Received in revised form 26 January 2012
Accepted 1 February 2012
Available online 9 February 2012

Keywords:
Anaerobic digestion
Ultrasonic pretreatment
Sunflower oil cake
Specific energy
Sonication time
Methane yield

ABSTRACT

In this study ultrasonic (US) pretreatment was investigated with the aim of improving the anaerobic digestion of sunflower oil cake (SuOC), the solid waste derived from the extraction process of sunflower oil. Five ultrasonic pretreatment assays were conducted at specific energy (*SE*) and sonication times in a range from 24,000 kJ/kg TS and 16.6 min (assay 1: US1) to 597,600 kJ/kg TS and 331.2 min (assay 5: US5), respectively, all operating at a constant sonication frequency (20 kHz) and ultrasonic power (120 W). As regards ultrasonic pretreatment, the working conditions of the first assay (US1) using samples of SuOC at 2% (w/v) showed to be the most appropriate in terms of both lignin and hemicellulose degradation (57.7% and 66.7%, respectively) and cellulose increase (54% increase with respect to its initial concentration). The percentage of COD solubilization increased from only 14% to 21% when *SE* was 25 times higher. Results obtained in batch anaerobic digestion experiments (biochemical methane potential – BMP – tests) conducted at 35 °C of the solid and liquid fractions released from the different ultrasonic conditions tested, indicated that for the first experiment (US1) the average ultimate methane yield obtained was 53.8% higher than that achieved for untreated SuOC. Finally, the kinetic constants of the anaerobic digestion of the solid and liquid fractions released after the ultrasonic pretreatment were virtually independent of the operation conditions assayed.

1. Introduction

In the industrial processing of sunflower seeds into edible oils, large quantities of waste called sunflower oil cake (SuOC) are generated. SuOC is the part of the whole sunflower seed which remains after the oil has been extracted. The high production level of SuOC in Spain, approximately five million tons per year, could create a significant environmental problem [1].

Anaerobic treatment processes are frequently used for the biological degradation of concentrated organic wastes leading to a stabilization of the residues because of the production of biogas, which in turn makes the process profitable. However, some doubts have been cast on the efficiency of anaerobic treatment and its process reliability because of the fact that some potential wastes for bioconversion are relatively non-biodegradable and, in addition, contain substances that are toxic to methanogenic microorganisms [1,2]. The anaerobic digestion process is conducted in four main stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis [3,4].

SuOC is characterized by its high concentration of lignocellulosic compounds [2]. As is well-known, cellulose in the ligno-cellulosic polymeric form is not totally available for bacterial attack [3]. Lignin surrounds the cellulose crystalline structure forming a seal and protects the cellulose from being easily hydrolysed. Because of the refractory structure of these compounds, one of the major problems in utilizing SuOC and other crop residues for stabilizing and for methane production by anaerobic digestion is their low digestibility. The anaerobic biodegradability and hence the methane potential of a complex substrate depends on the content of biodegradable compounds: carbohydrates (including cellulose), proteins and lipids [3].

It is generally accepted that hydrolysis is the rate-limiting step in the anaerobic digestion of organic vegetable solid waste. Owing to the chemical and physical construction of lignocellulose, its microbial hydrolysis is a slow and difficult process [3]. Previous works demonstrated the low values of the methane yield coefficient (143 mL CH_4 at standard temperature and pressure conditions, STP/g COD_{added}) achieved in Biochemical Methane Potential (BMP) tests conducted at mesophilic temperature (35 °C), using SuOC as substrate with a particle size of 0.7–1.0 mm [3]. In the same way, a considerable decrease in the methane yield from 227 to 107 mL CH_4 STP/g VS_{added} was observed when the food/microorganisms (F/M) ratio (volatile solids – VS – basis) increased from 0.3 to 2.0 during batch anaerobic digestion assays of SuOC at mesophilic temperature [1]. This proved that inhibition for substrate concentration had taken place in the anaerobic process of this waste. In addition, the anaerobic biodegradability of this substrate also decreased from 86% to 41% within the above-mentioned F/M ratio range [1].

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doi:10.1016/j.ultsonch.2012.02.001

Another recent study [5] has shown the low values of the methane yield coefficients (0.481 and 0.264 m^3/kg feedstock) obtained in BMP tests on sunflower meal and sunflower straw conducted at 35 °C. The lower value achieved for sunflower straw was attributed to the different composition of the two substrates in terms of lignin. Various pretreatment methods, such as thermal (121 °C for 60 min in a pressure cooker), chemical (through alkali or acid addition of 2% w/w H_2SO_4 or NaOH for 60 min) or a combination of the two methods (thermal–chemical) were also assayed to enhance methane productivity and yield. However, the experiments showed that the pretreatment methods did not enhance the methane potential of these residues. This was attributed to the presence of inhibitory compounds (such as furfural or hydroxymethylfurfural), which were released during the pretreatments [5].

Ultrasonic pretreatment is the application of cyclic sound pressure (ultrasound) with a variable frequency to waste activated sludge and other wastes to disintegrate sludge flocs and cell walls [4]. The chemistry of sonication as a pretreatment tool is quite complex and consists of a combination of shearing, chemical reactions with radicals, pyrolysis and combustion [6]. During sonication, microbubbles are formed because of high-pressure applications to liquid, which cause violent collapses and high amounts of energy to be released into a small area [4,6]. Consequently, because of extreme local conditions certain radicals (·OH, H·) can be formed [7]. The radical reactions can degrade volatile compounds by pyrolysis processes taking place in microbubbles [6,7].

One of the main advantages of the ultrasonic technique is that the use of external chemical agents is prevented and, therefore, an increase in the effluent volume is avoided [8].

When ultrasonication is applied to waste activated sludge (WAS), a solubilization of extracellular polymeric materials (EPS) and the cellular membranes of microorganisms takes place because of the extreme local temperatures and pressures achieved. This results in a sharp increase in soluble COD (CODs) to such an extent that sludge subjected to ultrasonic pretreatment produces CODs up to six times higher than untreated sludge, with the digesters which process the pretreated sludge producing 10–60% more biogas than conventional control digesters [4–8].

Mechanisms of ultrasonic treatment are influenced by four main factors: specific energy, ultrasonic frequency, application time and the characteristics of the substrate [7,8]. Cell disintegration is proportional to the energy supplied. High frequencies promote oxidation by radicals, whereas low frequencies promote mechanical and physical phenomena such as pressure waves. To be specific, 20–40 kHz has been reported as the optimal frequency range for achieving strong mechanical forces [9]. With complex substrates, radical performance decreases. It has been demonstrated that the degradation of excess sludge is more efficient when using low frequencies [6–9].

The aim of this work was to study the effect of different sonication operational conditions, such as time and specific energy, on the solubilization degree of SuOC at a constant frequency and an ultrasonic power of 20 kHz and 120 W, respectively. The influence of sonication working conditions on the cellulose, hemicellulose and lignin content of this substrate was also assessed. Finally, the effect of the ultrasonic pretreatments on the stability, methane yield and kinetics of the batch anaerobic digestion of pretreated SuOC was also studied.

2. Materials and methods

2.1. Substrate: sunflower oil cake (SuOC)

The sample of sunflower oil cake used in this study was collected from a sunflower oil factory located near Seville (Spain). Prior to use, the substrate was shredded into different particle sizes using a commercial sieve (Restch AS 200 basic) and different screen meshes. In order to ensure the homogeneity of the sample, the most abundant particle size of this substrate (0.71–1.0 mm diameter) was selected for carrying out the experiments.

The full composition and main features as well as the fractional composition of the fiber of the above-mentioned SuOC particle size selected are as follows (mean values of four determinations ± standard deviations): dry matter (DM) or total solids (TS), 93.8 (±0.1)%; volatile solids (VS), 93.0 (±0.1)%; ash, 6.8 (±0.1)%; total COD, 1.24 (±0.02) g O_2/g TS; neutral detergent fiber, 45 (±1.1)%; acid detergent fiber, 38.4 (±0.9)%; acid detergent lignin (ADL), 13.3 (±0.2)%; hemicellulose, 6.6 (±0.8)%; cellulose, 25.1 (±0.4)%; total protein, 25.3 (±0.8)%; fat content, 1.6 (±0.2)%; soluble carbohydrates, 5.1 (±0.2)% and total carbohydrates, 53.0 (±0.3)%. All these values are expressed as a percentage of dry matter.

A suspension of 2% w/v (20 g TS/L) of the mentioned SuOC in distilled water was used for the ultrasonic pretreatment experiments and subsequent batch anaerobic digestion assays. Therefore, the final characteristics of the SuOC sample used in the experiments were: ash, 4.3%; proteins, 24.5%; hemicellulose, 13.5%; cellulose, 28.7%; lignin, 16.8% and total COD, 1250 mg/g TS.

2.2. Ultrasonic pretreatment

The ultrasonic equipment was a Sonopuls ultrasonic homogenizer (Bandelin–Sonopuls HD 2200, Berlin, Germany). This apparatus was equipped with a KE 76 titanium tapered tip probe with a constant operating frequency of 20 kHz, 60% amplitude and 120 W of power. For each experiment, volumes of between 200 and 250 mL of sample were placed in a glass beaker and the ultrasonic probe was submerged into the sample to a depth of 2 cm. The ultrasound density and intensity were kept constant at 0.48 W/mL and 3.3 W/cm^2, respectively. The sonication times varied in a range from 16.6 to 331.2 min. The temperature of the treated samples was kept at 20 °C, while the tap water was recirculated around the beaker.

Specific energy was considered as the main variable parameter for the evaluation of the solubilization and disintegration performance of the substrate. The range of the specific energy (*SE*) varied from 24,000 kJ/kg TS (assay 1: US1) to 597,600 kJ/kg TS (assay 5: US5). *SE* (in kJ/kg TS) was calculated by using ultrasonic power (*P* in watts), ultrasonic time (*t* in seconds), sample volume (*V* in liters) and initial total solid concentration (TS_0 in g/L) according to the following equation [6,8,10,11]:

$$SE = (P \cdot t)/(V \cdot TS_0) \quad (1)$$

Table 1 summarizes the ultrasonic pretreatment conditions used in the experiments. For all ultrasonic conditions tested, the mass ratio of solid (g) to liquid (distilled water, in mL) was 2:100 (organic load: 2% w/v).

COD solubilization (*S*) after each ultrasonic pretreatment was also determined. *S* was calculated using the difference between final soluble COD (CODs) after pretreatment and initial soluble COD ($CODs_0$), as compared to the initial total COD ($CODt_0$) by using the following equation [7,10]:

$$S = (CODs - CODs_0) * 100/CODt_0 \quad (2)$$

2.3. Experimental procedure

After each ultrasonic pretreatment assay, the soluble or liquid fractions were separated from the solid fractions by centrifuging the samples for 15 min at 10,000 rpm. The corresponding solid and liquid fractions after each pretreatment were subjected to separate biochemical methane potential (BMP) tests.

Table 1
Ultrasonic pretreatment conditions on samples of SuOC at 2% (w/v).*

Assay number	Sample volume (L)	Sonication time (min)	Specific Energy (kJ/kg TS)	Ultrasound Doses (J/L)
US1	0.25	16.6	24,000	478
US2	0.23	60.6	96,000	1897
US3	0.25	133.3	192,000	3839
US4	0.25	300.0	432,000	8640
US5	0.20	331.2	597,600	11,923

* Sonication frequency: 20 kHz (constant); ultrasonic power: 120 W (constant); ultrasonic density: 0.48 W/mL (constant); ultrasonic intensity: 3.3 W/cm^2 (constant).

The anaerobic experimental study was conducted in a multi-batch reactor system, which consisted of nine Erlenmeyer flasks, with an effective volume of 250 mL. They were continuously stirred with magnetic bars at 300 rpm and placed in a thermostatic bath at mesophilic temperature (35 ± 1 °C). The reactors were initially charged with an anaerobic inoculum by maintaining a concentration of 15 g VS/L (the volume taken is a function of the initial VS concentration of the inoculum). The inoculum to substrate ratio was maintained at 2 (VS basis) for the reactors digesting the solid fractions and at 2.5 (COD basis) for the reactors processing the liquid fractions. Once the pretreated substrate was added to each reactor, 25 mL of stock mineral medium solution (whose composition has been described elsewhere) [12] were also added. Finally, distilled water was added to achieve the desirable working volume of 250 mL. The reactors were flushed with N_2 in order to achieve anaerobic conditions. Granular sludge taken from an industrial anaerobic reactor treating brewery wastewater was used as inoculum. The characteristics of this inoculum were: pH, 7.0; TS, 75 g/L and VS, 54 g/L.

The methane released was measured by volume displacement (carbon dioxide was previously removed by flushing the gas through a 2 N NaOH solution), and expressed at standard temperature and pressure (STP) conditions. Because of biomass decay and the possible presence of residual substrate in the inoculum, the methane produced was subtracted by performing blank controls. A starch control was also used for checking the BMP test performance.

All experiments took place over a 7-day period, until no significant gas production was observed (on the last day of production there was less than 1% of the accumulated methane volume), suggesting that biodegradation had been completed. This short period of time was sufficient to achieve maximum methane production, and can basically be explained by the high methanogenic activity of the sludge and the short interval (less than 72 h) which had elapsed between inoculum sampling and the start-up of the experiments.

2.4. Analytical methods

2.4.1. Solid samples

The following parameters were analyzed in the original solid substrate (SuOC): total solids (TS) and volatile solids (VS), according to standard methods 2540B and 2540E [13], respectively. To determine TS, a well-mixed sample is evaporated in a dish which has previously been weighed and dried to constant weight in an oven at a temperature of between 103 and 105 °C. The increase in weight over that of the empty dish represents the TS content [13]. Total chemical oxygen demand (COD) was determined using the reported method proposed by Raposo et al. [14]. Fat content was extracted with hexane, using a Soxhlet system [15].

To determine the total Kjeldahl nitrogen (TKN), 1000 mg of sample were acidified with 15 mL concentrated H_2SO_4. In addition, 5 g catalyst [(Cu–Se) (1.5% CuSO4·5H2O + 2% Se)] was added, and finally, the sample was digested sequentially in a thermoblock for 15 min at 150 °C, 15 min at 250 °C and 90 min at 390 °C. After cooling, the sample was diluted with 10 mL distilled water, neutralized with NaOH 12.5 N and distilled in 50 mL of solution indicator mix (H_3BO_3 at 2% w/v). The solution was titrated with H_2SO_4 0.02 N. Total protein was determined by multiplying the TKN value by 5.5 [16].

Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined in order to calculate hemicellulose (NDF–ADF), cellulose (ADF–ADL) and lignin (ADL), according to van Soest et al. [17], with slight modifications.

- To determine NDF, 1000 mg of dried sample was boiled in a sintered glass crucible (40–100 μm pore size) with 100 mL of a neutral solution of sodium dodecyl sulfate in an EDTA–borate buffer together with 1 g of sodium sulfite anhydrous (to remove proteins) and 200 μL of α-Amylase (to eliminate starch) for 1 h. Afterwards, the neutral detergent was removed and the sample washed with 100 mL of hot distilled water. Finally, the sample was washed with 50 mL of acetone and dried at 105 °C in an oven overnight and then weighed. Corrections for residual proteins and ash were made.
- ADF was determined by non-sequential fiber analysis. In this way, 1000 mg of raw dried sample were heated with 100 mL of a solution of *N*-Cetyl-*N*,*N*,*N*-trimethyl ammonium bromide (in H_2SO_4 1 N) to boiling point for 1 h in a sintered glass crucible (40–100 μm pore size). Afterwards, ADF was recovered by filtration, washed with 100 mL of hot distilled water and later with 50 mL of acetone. Finally, the sample was dried overnight at 105 °C and then weighed. Ash and proteins were also corrected during this step.
- To determine ADL, 250 mg of sample obtained after ADF analysis continued to be stirred for 3 h with 25 mL of H_2SO_4 (72% w/w). Then, the sample was placed in a sintered glass crucible (40–100 μm pore size) and washed with 100 mL of distilled water and dried at 105 °C in an oven overnight and then weighed. Correction for ash was made.

2.4.2. Soluble fractions

The supernatants obtained after centrifuging the pretreated samples and digestates for 15 min at 10,000 rpm were passed through a filter (0.45 μm) and used to characterize the following soluble parameters: soluble chemical oxygen demand (CODs), using the closed digestion and colorimetric standard method 5220D [13]; total alkalinity (TA) was measured by pH titration to 4.3. Soluble ammoniacal nitrogen (NH_x)s was determined by distillation and titration according to the standard method 4500E [13]. The volatile fatty acid (VFA) concentration was analyzed using a gas chromatograph, as previously described [18].

2.4.3. Inoculum and digestates

Both the inoculum and digestates were characterized by direct sampling. pH was determined by using a pH-meter model Crison

20 Basic. Total alkalinity (TA), TS and VS were also analyzed in these samples [13].

3. Results and discussion

3.1. Influence of the operational conditions of ultrasonic pretreatment on the characteristics and solubility of the substrate

Table 2 shows the characteristics of the substrate after the ultrasonic pretreatment under the different operational conditions tested. For the first assay (US1) the percentages of lignin and hemicellulose removals were 57.7% and 66.7%, respectively, the latter being the highest percentage of hemicellulose removed found in the different conditions assayed. This reveals the suitability of the use of a *SE* of 24,000 kJ/kg TS during ultrasonic pretreatment with a view to obtain a more appropriate substrate for anaerobic digestion.

In addition, an increase of 54% in the percentage of cellulose with respect to its initial content in the substrate was observed during the first operational conditions tested (US1), for which a cellulose content of 44.2% was achieved after pretreatment under the afore-mentioned conditions. This fact is of great importance when considering that cellulose is a more biodegradable carbohydrate than other polymers present in the waste being researched (hemicellulose and lignin). Ultrasonic treatment for obtaining cellulose nanofibers from polar wood, with high hemicellulose and lignin removals after chemical pretreatment (with a 3% potassium hydroxide solution at 80 °C for 4 h), combined with a high-intensity ultrasonication step (1200 W power for 30 min) was also reported in the literature [19].

On the other hand, low lignin contents in the ultrasonic pretreated substrate were also obtained for US2, US3 and US4 conditions, although the differences from one assay to the next were insignificant. To be specific, the initial lignin content of the substrate (16.8%) was reduced to percentages of 7.1%, 7.5%, 7.5%, 7.4% and 9.0% after assays US1, US2, US3, US4 and US5, respectively. A maximum lignin degradation percentage of 57.7% was achieved for the lowest and most reduced energetic conditions tested (US1 with a *SE* of 24,000 kJ/kg TS). Higher lignin reductions (11.4% on dry basis) were achieved during sonication of sunflower husks with the aim of accelerating the bioconversion of this substrate in biodiesel fuel production [20]. However, ultrasonic intensity used in the previously mentioned work (46 W/cm^2) [20] was much higher than that used in the present work at assay 1 operating conditions (3.3 W/cm^2).

The highest protein contents in the solid fraction were achieved during assays US1 (25.2%) and US2 (27.7%). For higher *SE* values and sonication times (assays US3, US4 and US5), protein contents were lower. A similar behavior was observed during sonication of WAS, for which an increase in the protein concentration released was observed at low *SE* [11,21]. Wang et al. also examined the release of proteins in the aqueous phase at different sonication times [22] and demonstrated that the rate of protein release from WAS was very high during the initial 20 min of sonication with polysaccharide concentration dropping after this time [21].

It can also be observed in Table 2 that ultrasonication time and *SE* had practically no effect on the total COD of the substrate because this parameter was virtually constant for all the conditions assayed, ranging between 1.28 and 1.33 g/g VS. A similar trend was also observed in the sonication of WAS, prior to being subjected to anaerobic digestion [11].

On the other hand, as can be seen in Table 2, the percentage of COD solubilization increased from 14% (US1) to 21% (US5) when the *SE* increased from 24,000 to 597,600 kJ/kg TS. Therefore, the percentage of COD solubilization was only 1.5 times higher when the *SE* was 25 times higher. Once again, this reinforces the idea of considering the first operational conditions tested as being the most suitable working requisites for carrying out the ultrasonic pretreatment of this substrate.

For comparative purposes, ultrasonic pretreatment at 20 kHz and 1 W/mL sonication density allowed for an increase in the COD solubilization percentages from 11% (control, not pretreated) to 23% for pulp sulfite mill sludges and from 1.3% (control) to 5.0% for kraft pulp mill secondary sludges [23]. For *SE* below 1000 kJ/kg, the COD solubility of WAS was low (8%). However, when the supplied energy was over the above-mentioned value, COD solubilization rose sharply to 35% for a *SE* of 15,000 kJ/kg TS [7]. A maximum COD solubilization of 15% was achieved in WAS after an ultrasonic pretreatment conducted at *SE* values in the range of 6250–9350 kJ/kg TS [24]. The effect of ultrasonication on COD solubilization was also studied for swine slurry and separated dairy manure at two power ratings (59.7 kW and 119.3 kW) and at two time settings (15 and 30 s), achieving values of up to 23% and 33%, respectively [25]. Other previous studies showed that 15 min of sonication (with a sonication frequency, power input and intensity of 24 kHz, 255 W and 4.8 W/cm^2, respectively) allowed for an increase in the initial soluble COD of WAS from 50 to 2500 mg/L [4]. However, lower COD solubilization yields (15%) were reached in WAS containing polycyclic aromatic hydrocarbons (PAH) after ultrasonic pretreatment using *SE* of 15,000 kJ/kg TS [8]. Hog manure was found to be more amenable to ultrasonication than WAS, as it took only 3000 kJ/kg TS to cause 15% more solubilization as compared to 25,000 kJ/kg TS for WAS [26]. To be specific, the maximum COD solubilization of hog manure was 27.3% at 30,000 kJ/kg TS, whereas Khanal et al. using WAS achieved 16.2% at *SE* of 66,800 kJ/kg TS [27].

3.2. Effect of ultrasonic pretreatment on methane yield

Cumulative methane productions as a function of digestion time were assessed during the BMP tests of the solid and liquid fractions obtained after the different ultrasonic pretreatment conditions conducted. It was observed during the experiments that most of the methane production and, therefore, the highest substrate utilization rates generally occurred during the first 3 days of digestion.

Table 2
Characteristics of the samples of SuOC (2% w/v, 20 g TS/L) after the ultrasonic pretreatment under different operational conditions.*

Experiment number	Ashes (%)	Proteins (%)	Hemicellulose (%)	Cellulose (%)	Lignin (%)	COD (g/g TS)	CODs (g/L)	S (%)**
US1	4.3	25.2	4.5	44.2	7.1	1.26	3.5	14
US2	3.8	27.7	8.5	39.6	7.5	1.27	4.1	17
US3	3.3	23.5	12.8	39.7	7.5	1.26	4.2	17
US4	5.6	22.6	12.3	40.9	7.4	1.25	4.8	19
US5	3.0	21.9	10.4	41.2	9.0	1.31	5.2	21

* Values are averages of five determinations: there was virtually no variation (less than 3%) between analyses.
** S (%): percentage of solubilization with respect to the total COD.

Fig. 1A and B shows the cumulative methane yield as a function of digestion time for the solid and liquid fractions obtained after the ultrasonic pretreatment performed under different operational conditions. The methane yield values were calculated for each case studied by dividing the net methane production (subtracting the blank or control methane production) at a determined time by the amount of COD added [1]. Therefore, the ultimate methane yield gives the value when no more volume of gas from the reactors is released. As can be seen, for the solid fractions the ultimate methane yield increased from 90 ± 4 to 111 ± 5 mL CH_4 STP/g COD_{added} when the *SE* decreased from 597,600 kJ/kg TS (US5) to 24,000 kJ/kg TS (US1). In the same way, for the liquid fractions, the methane yield rose again from 270 ± 13 to 330 ± 16 mL CH_4 STP/g COD_{added} when the *SE* decreased in the above-mentioned range. On the other hand, the methane yields of the solid fractions expressed as mL CH_4 STP/g VS_{added} were found to be 147 ± 7, 142 ± 7, 135 ± 6, 122 ± 6 and 110 ± 5 for assays US1, US2, US3, US4 and US5, respectively. Once again, this shows the appropriateness of US1 working conditions for carrying out ultrasonic pretreatment. Calculating the mean methane yield from the values obtained for the solid and liquid fractions at US1 operating conditions gives a value of 220 ± 11 mL CH_4 STP/g COD_{added} after ultrasonic pretreatment. This value was 53.8% higher than that obtained in BMP tests conducted with untreated SuOC under the same working conditions (143 mL CH_4 STP/g COD_{added}) [3].

In the same way, Bougrier et al. [24] showed an increase in the methane yield of WAS from 221 to 334 mL CH_4 STP/g COD_{added} after an ultrasonic pretreatment at 9350 kJ/kg TS, which was more effective than other pretreatments assayed, such as ozonation or thermal pretreatment. An increase in the methane production of 44% was also reported by Erden and Filibeli [10] for WAS previously sonicated with a *SE* of 9690 kJ/kg TS and a power density of 0.09 W/mL. Likewise, an improvement of 16% in specific biogas production was also observed after ultrasonic pretreatment of WAS with a high content in polycyclic aromatic hydrocarbons at *SE* of 11,000 kJ/kg TS, in this case in anaerobic digestion experiments conducted in continuous mode, using hydraulic retention times of 29 days [8]. Similarly, the methane potential of hog manure increased by 20.7% in comparison with unsonicated manure for an *SE* input of 30,000 kJ/kg TS [26] with a maximum increase in the methane production rate of 80.6% as compared with the untreated sample. Finally, ultrasonic pretreatment of swine slurry and separated dairy manure effluent under the above-mentioned conditions (power ratings of 59.7 kW and 119.3 kW and times of 15 and 30 s) also increased the methane yields up to 56% and 20%, respectively with respect to untreated samples [25].

3.3. Effect of ultrasonic pretreatment on chemical control parameters in BMP tests

Table 3 shows the variation of the chemical control parameters in the digestates of the solid and liquid fractions at the end of the digestion process for the different operational conditions tested during ultrasonic pretreatment.

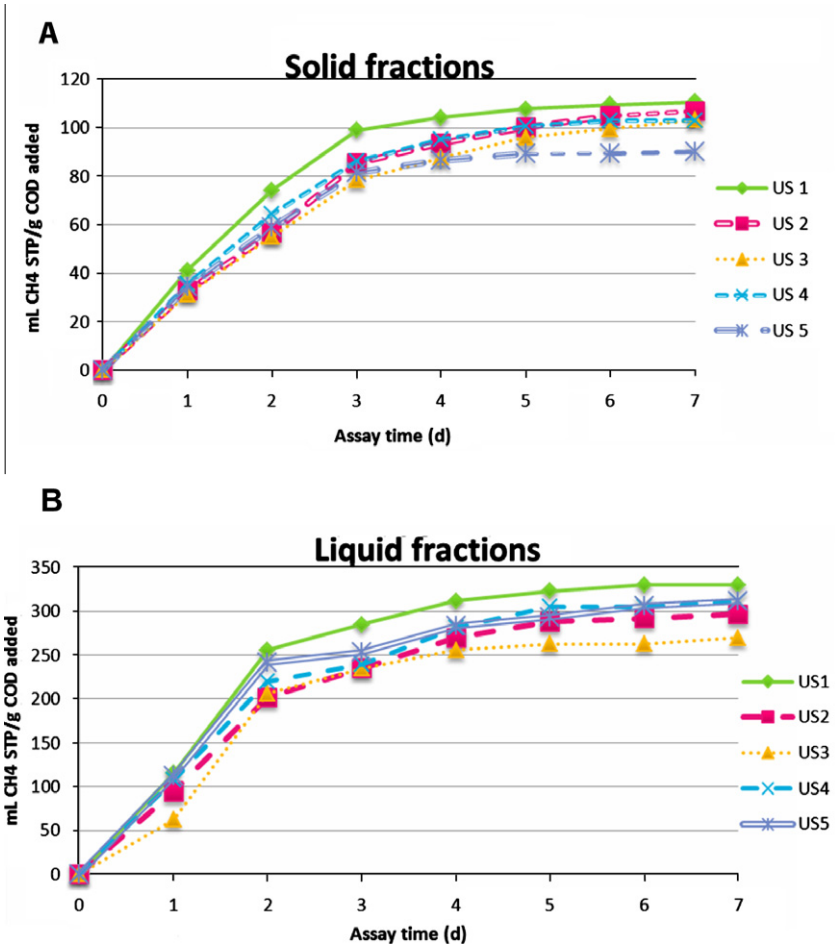

Fig. 1. Variation of the methane yield with the digestion time for both the solid (A) and liquid fractions (B) released after the ultrasonic pretreatment under the different operational conditions tested.

Table 3
Variation of the chemical control parameters (mean values ± standard deviations) during the BMP tests performed with the solid and liquid fractions obtained after the ultrasonic pretreatment.

Assay number	pH	(NH_x)s (mg/L)	CODs (mg/L)	TA (mg $CaCO_3$/L)	TVFA (mg acetic acid/L)
Solid fractions					
US1	7.6 ± 0.2	520 ± 15	1700 ± 50	4700 ± 140	5.0 ± 0.1
US2	7.6 ± 0.3	530 ± 14	2000 ± 58	4800 ± 141	11.0 ± 0.3
US3	7.6 ± 0.2	530 ± 15	2000 ± 65	4800 ± 135	12.0 ± 0.3
US4	7.5 ± 0.2	540 ± 18	1600 ± 46	4700 ± 145	12.0 ± 0.4
US5	7.5 ± 0.3	540 ± 20	1600 ± 45	4600 ± 138	13.0 ± 0.3
Liquid fractions					
US1	7.8 ± 0.3	400 ± 12	2400 ± 73	4000 ± 70	13.0 ± 0.3
US2	7.6 ± 0.2	430 ± 11	2600 ± 75	4600 ± 135	16.0 ± 0.4
US3	7.6 ± 0.2	440 ± 10	2400 ± 68	4700 ± 130	14.0 ± 0.3
US4	7.5 ± 0.3	430 ± 11	2700 ± 81	4800 ± 140	17.0 ± 0.5
US5	7.7 ± 0.2	430 ± 12	2100 ± 61	4800 ± 133	15.0 ± 0.3

Table 4
Kinetic parameters (k_0 and B_0) derived from Eq. (3) with their 95% confidence limits as well as other statistical parameters derived from the mathematical adjustment of the experimental data to the proposed model for all the conditions assayed.

Parameter	US1	US2	US3	US4	US5
Solid fractions					
k_0 ($days^{-1}$)	0.52 ± 0.05	0.37 ± 0.04	0.35 ± 0.02	0.45 ± 0.04	0.52 ± 0.05
B_0 (mL CH_4 STP/g COD_{added})	116 ± 3	118 ± 5	114 ± 3	111 ± 3	95 ± 3
R^2	0.991	0.991	0.996	0.994	0.988
Standard error of Estimate	4.10	3.91	2.36	3.11	3.84
W statistic	0.95	0.96	0.92	0.98	0.94
Liquid fractions					
Parameter	US1	US2	US3	US4	US5
k_0 ($days^{-1}$)	0.52 ± 0.06	0.46 ± 0.05	0.49 ± 0.09	0.47 ± 0.05	0.56 ± 0.07
B_0 (mL CH_4 STP/g COD_{added})	350 ± 14	316 ± 11	287 ± 22	327 ± 10	318 ± 12
R^2	0.986	0.991	0.953	0.991	0.984
Standard error of Estimate	16.25	11.03	24.33	11.60	15.12
W statistic	0.86	0.93	0.89	0.98	0.83

There was little variation in the pH: 7.5 and 7.8, values that were compatible with the normal growth of anaerobic microorganisms. This indicates that the pH was practically constant and stable during the anaerobic digestion of both the solid and liquid fractions, independently of the operational conditions used in the ultrasonic pretreatment. In addition, these pH values were within the optimum pH range (7.0–8.5) recommended for obtaining a maximum anaerobic degradation of cellulosic compounds using ruminal microorganisms [28].

Given that during anaerobic degradation, complex organic compounds are transformed into lower molecular weight compounds, soluble COD is a parameter that indicates the degradation of the substrate [29]. In the present study, the lower soluble CODs were achieved in the digestates of the samples sonicated at higher *SE* and times (US5), although no significant difference among the values achieved for the other conditions tested was observed.

The degradation of complex organic material, including nitrogenous organic compounds, results in the generation of ammonia, a compound which at certain concentrations can inhibit the anaerobic process [1]. The lower ammoniacal nitrogen concentration observed at the effluent of the liquid and solid samples for all the conditions tested did not affect the methane yield observed for these experiments.

The final values of the total volatile fatty acids (TVFA) were very low for both the solid and liquid fraction digestates, with values in the range of 5–16 mg acetic acid/L. This means that the overall anaerobic process was conducted satisfactorily and a correct balance of the process occurred [30]. In addition, the high total alkalinity (TA) values in the range from 4040 to 4800 mg $CaCO_3$/L showed the high favorable buffering capacity of the bioreactors for all conditions tested in the ultrasonic pretreatment. The experimental data obtained in this work show that a total alkalinity of about 4000 mg $CaCO_3$/L is sufficient to prevent the pH from dropping to below 7.5, independently of the working conditions of the pretreatment.

3.4. Effect of ultrasonic pretreatment on the kinetics of the anaerobic process

In order to characterize each experiment kinetically with a view to evaluate the influence of the operating conditions of the ultrasonic pretreatment on the anaerobic process and, thus facilitating a comparison, the following kinetic equation was used [3,31]:

$$B = B_0[1 - \exp(-k_0 \cdot t)] \quad (3)$$

where B is the cumulative methane yield (mL CH_4/g COD_{added}), B_0 is the maximum or ultimate methane yield of the substrate (mL CH_4/g COD_{added}), k_0 ($days^{-1}$) is the specific rate or apparent kinetic constant and t (days) is the time.

According to Eq. (3), methane yield conforms to a first-order kinetic model [31,32]. As can be seen in Fig. 1A and B for both the solid and liquid fractions, B was zero at $t = 0$, and the rate of methane yield became zero at t equal to infinite. Thus, Eq. (3) shows a good agreement with the experimental data and it seems appropriate to apply the proposed kinetic model for all conditions tested in the ultrasonic pretreatment.

The adjustment by nonlinear regression of the pairs of the experimental data (B, t) using the SigmaPlot software (version 11.0) allows the calculation of the apparent kinetic constant k_0. Table 4 lists k_0 and B_0 values with their respective 95% confidence limits for each case studied. This Table also shows the determination coefficient (R^2), the standard error of estimate and the W statistic for each case assayed.

The high values of the coefficient of determination, R^2, with values higher than 0.99 in most cases and the low values of the standard errors of estimate and confidence limits of the parameters obtained demonstrate how well the experimental data adapted to the model proposed.

As can be seen in Table 4, in general, k_0 values for the solid fractions were somewhat lower than those obtained for the liquid fractions, especially for US2 and US3 pretreatment conditions. This may be due to the fact that a part of the organic matter contained in the insoluble or solid fractions was not easily available for anaerobic microorganisms and was biodegraded more slowly than that present in the soluble or liquid fractions. This behavior was previously observed in BMP tests of WAS after sonication at *SE* lower than 3000 kJ/kg TS [8]. For the solid fractions, the highest k_0 values (0.52 $days^{-1}$) were achieved for the US1 and US5 conditions. This value was only slightly higher than those obtained for US2, US3 and US4 experiments, respectively. For the liquid fractions, the kinetic constant was virtually constant showing the independence of the kinetics of the anaerobic process with respect to the operating conditions of the ultrasonic pretreatment.

4. Conclusions

Results from this study demonstrate the suitability of ultrasonic pretreatment of SuOC for increasing the anaerobic biodegradability of this substrate and methane yield coefficient. Ultrasonic pretreatments conducted on samples of SuOC at 2% (w/v) (20 g TS/L), at *SE* ranging from 24,000 kJ/kg TS (assay US1) to 597,000 kJ/kg TS (assay US5) operating at constant sonication frequency (20 kHz) and ultrasonic power (120 W) revealed the appropriateness of the lowest conditions assayed (US1) to obtain maximum methane production and yields, both from the solid and liquid fractions released after pretreatment as compared to the other conditions assayed. Specifically, the ultimate methane yields obtained for the solid and liquid fractions (111 ± 5 and 330 ± 16 mL CH_4 STP/g COD_{added}, respectively) in US1 were higher than those obtained for the other conditions tested during pretreatment. Likewise, the mean value obtained (average of the solid and liquid fractions) in this case was 220 ± 11 mL CH_4 STP/g COD_{added}, which was 53.8% higher than that obtained for untreated SuOC.

As regards ultrasonic pretreatment, for the first condition assayed (US1) the percentages of lignin and hemicellulose removals were 57.7% and 66.7%, respectively, the latter being the highest percentage of hemicellulose removed found among the different conditions tested. Moreover, COD solubilization increased by only 7% for US5 (21%) as compared to US1 (14%), an interval for which the *SE* and sonication times were 25 and 20 times higher, respectively. This fact reveals the suitability of the ultrasonic pretreatment at an *SE* of 24,000 kJ/kg TS (US1 assay) to obtain a more appropriate substrate for anaerobic digestion.

The anaerobic digestion of the pretreated substrate under the above-mentioned conditions (US1) was very stable. The kinetic constants of the anaerobic digestion of the solid and liquid fractions released after the different pretreatments conducted were virtually independent of the working conditions of the pretreatment.

Acknowledgements

The authors wish to express their gratitude to the Spanish Ministry of "Ciencia e Innovación" (Project CTM2008-05772/TECNO) for providing financial support, to the Program "FPI" of the above-mentioned Ministry for contracting graduates, to the contract JAE-Doc from "Junta para la Ampliación de Estudios del CSIC" co-financed by the European Social Funds, as well as to the "Junta de Andalucia" for supporting long-term research activities.

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3.2 Ensayos en modo semicontinuo de SuOC previamente sonicado.

El trabajo científico correspondiente a esta parte de la tesis fue elaborado y enviado recientemente a la siguiente revista científica para su evaluación, habiéndose resuelto como revisión menor:

Performance and kinetic evaluation of the semi-continous anaerobic digestion of sunflower oil cake pretreated with ultrasound

Fernández-Cegrí, V., Raposo, F., Borja, R. (2013). Fecha de envío: 07/01/2013. Journal of Environmental Science and Health. Part A.

En este trabajo se llevó a cabo un estudio del proceso de digestión anaerobia en régimen semicontinuo del sustrato objeto de estudio (harina de girasol desengrasada- SuOC) tras ser sometido previamente a un tratamiento con ultrasonidos con una energía específica de 24.000 kJ/kg TS. El proceso anaerobio se realizó a escala de laboratorio en reactores de mezcla completa a temperatura mesofilica (35ºC).

Se utilizaron dos tipos de lodos anaerobios como inoculo, uno floculento procedente de una planta de tratamiento anaerobio de fangos activados resultantes de una EDAR (I) y otro granulado procedente de un reactor UASB que procesa vertidos de una cervecera (II).

Las eficiencias de eliminación de COD soluble oscilaron entre 67,7% y 70,1% (lodo II) y entre 61,3% y 67,7% (lodo I), operando con tiempos de retención hidráulicos (TRH) de entre 24-10 días y 24-8 días para los lodos I y II, respectivamente. Sin embargo, para HRT inferiores a 8 días y 6.7 días, equivalentes a velocidades de cargas orgánicas (OLRs) mayores de 2,62 y 3,15 g DQO / (L · d) respectivamente, se observó una brusca disminución en la eficiencia de eliminación de CODs en ambos casos.

En cualquier caso, el lodo II presentó un funcionamiento más estable y eficiente para un rango más amplio tanto de OLR_S como de HRTs, permitiendo unas condiciones de operación adecuadas a más altas OLRs (3,15 g DQO / (L·d)) y HRTs más bajos (6,7 días). Las velocidades de producción de metano alcanzadas utilizando el lodo II fueron en todos los casos superiores a los obtenidas con el lodo I. El coeficiente de rendimiento global de metano obtenido con el lodo II fue un 13% superior al alcanzado con el lodo I. Además, este valor fue 1,9 veces mayor que el rendimiento en metano obtenido para el sustrato (SuOC) sin pretratar.

Un modelo cinético de segundo orden resultó adecuado para ajustar y simular los resultados obtenidos experimentalmente. Se observó que la constante cinética obtenida con el lodo I fue 3,5 veces mayor respecto a que se alcanzó con el lodo II.

Performance and kinetic evaluation of the semi-continuous anaerobic digestion of sunflower oil cake pretreated with ultrasound

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ABSTRACT

A study of the semi-continuous anaerobic digestion of sunflower oil cake previously sonicated (at a specific energy of 24,000 kJ/kg TS, constant sonication frequency of 20 kHz and ultrasonic power of 120 W) was carried out in laboratory-scale completely stirred tank reactors at mesophilic temperature (35°C). Two anaerobic sludges were used as inoculum: a mixture of flocculant sludge (I) from a full-scale anaerobic reactor treating waste activated sludge and a granular sludge (II) from an industrial UASB reactor treating brewery wastewater. Soluble COD (CODs) removal efficiencies ranged between 67.7% and 70.1% and between 61.3% and 67.7% at hydraulic retention times (HRTs) of between 24-10 days for sludge I and 24-8 days for sludge II. However, for HRTs lower than 8 days and 6.7 days, equivalent to organic loading rates (OLRs) higher than 2.62 and 3.15 g COD/(L·d) respectively, a sudden decrease in the CODs removal efficiency was observed in both cases. In any case, sludge II allowed for a more stable and efficient operation for a wider range of both OLRs and HRTs, permitting an appropriate and reliable operation for OLRs as high as

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3.15 g COD/(L·d) and HRTs as low as 6.7 days. The methane production rates achieved with sludge II were always higher than those reached with sludge I. The overall methane yield obtained with sludge II was 13% higher than that achieved with sludge I. In addition, this value was 1.9 times higher than the methane yield obtained with untreated (non-sonicated) SuOC. A second-order kinetic model was found to be adequate to fit the experimental results obtained for the two sludges used. The kinetic constant obtained with sludge I was 3.5 times higher than that achieved with sludge II.

Keywords: Anaerobic digestion, semi-continuous mode, ultrasound, methane production rate, methane yield, kinetics.

INTRODUCTION

Anaerobic digestion (AD) is a biological process in which a complex community of microorganisms works in a stable, self-regulating steady state converting waste organic matter into a mixture of carbon dioxide and methane gases. AD of vegetable liquid, semisolid and solid wastes is an attractive alternative to other treatments. [1] Several agro-industrial residues, including wastewaters, semisolid and solid wastes can be anaerobically treated obtaining the efficient stabilization of solids as well as energy recovery. AD offers many advantages: low nutrient requirements, energy saving, generation of low quantities of sludge, excellent waste stabilization, production of biogas (methane), etc. [1]

Lignin-rich material is poorly degraded under anaerobic conditions, [2] and as a consequence the degradation of the insoluble material is the rate-limiting step in the AD of lignocellulosic wastes. [3] For this reason, the application of certain pretreatments makes the hydrolytic step easier, because the lignocellulosic material can be converted into soluble sugars. Some

pretreatments have been developed with a view to achieving the release of lignocellulosic material and thus accelerating the degradation process by means of waste solubilisation. Consequently, in these cases it is necessary to carry out a pretreatment step to break the lignin seal, thus exposing the cellulose and hemicellulose to biological degradation. Sonication has been used for many years in research laboratories to disrupt cellular matter. It has been shown to be effective at solubilizing organic matter, as well as improving biogas production. [4] Ultrasound, a mechanical pre-treatment technology, produces a pressure wave that is converted into cavitation bubbles that expand and collapse violently, which is then lost as heat. [5] Several studies have shown that substrate disintegration by sonication increased the total methane production in AD in batch mode. [6] Mechanisms of ultrasonic treatment are influenced by four main factors: specific energy, ultrasonic frequency, application time and the characteristics of the substrate. Cell disintegration is proportional to the energy supplied. High frequencies promote oxidation by radicals, whereas low frequencies promote mechanical and physical phenomena such as pressure waves. [6] Therefore, this pretreatment allows for a high level of solubilisation and a modification in substrate characteristics, which may lead to an improvement in biogas production.

Sunflower oil cake (SuOC) is the part of whole sunflower seeds which remains after the oil extraction process. It is an agro-industrial residue generated in Spain in great quantities (about 4–5 million tons per year). Because of the high production of this waste and the limited applications for its re-use or valorization, [7] controlled decomposition of this agro-waste becomes necessary. Operating a controlled anaerobic system to re-use this agro-waste also has the advantage that energy contained in the biomass of the SuOC can be recovered.

Mesophilic AD is a widespread technology where the organic loading rate (OLR) or hydraulic retention time (HTR) is the main operational variable to optimize. However, due to the chemical and physical structure of SuOC, and in particular to its high lignocellulosic content,

AD is a slow and difficult process. Previous research works on the AD of SuOC carried out at mesophilic temperature gave low organic matter removal efficiencies and methane yield coefficients (101 mL CH_4/ g COD_{added}) operating at an OLR of 2 g VS/(L·d). This process was clearly inhibited and an imbalance was observed when the OLR increased up to values of 3 g VS/(L·d). [8]

Previous research work on SuOC conducted in batch mode revealed that the pretreatment of this substrate by sonication (carried out at a specific energy of 24.000 kJ/kg TS, at a constant sonication frequency of 20 KHz and ultrasonic power of 120 W) lead to an increase in the methane yield coefficient of 53.8% as opposed to an untreated (un-sonicated) substrate. The ultrasonic pretreatment carried out at the above mentioned optimal conditions caused a reduction in the lignin and hemicellulose content of 57.7% and 66.7% respectively. [6]

Other authors studied the effect of ultrasound and hydraulic residence time during sludge hydrolysis with the aim of enhancing methane production from anaerobic digestion (AD). Waste activated sludge was ultrasonically disintegrated for hydrolysis, and it was semi-continuously fed to an anaerobic digester at various hydraulic retention times (HRTs). The results of these experiments showed that the solid and chemical oxygen demand (COD) removal efficiencies when using ultrasonically disintegrated sludge were higher during AD than the control sludge. The longer the HRT, the higher the removal efficiencies of solids and COD, while methane production increased with lower HRTs. [5]

Empirical and kinetic models of anaerobic fermentation have been widely applied to describe the process. Second-order, first-order and pseudo-first-order kinetic models have been successfully used and applied to a number of experimental data. [9]

The aim of this work was to assess the semi-continous AD process of SuOC previously treated with ultrasound. The AD process was conducted at different organic load rates (OLRs) and HRTs in order to obtain the optimal operational parameters. The effect of the variations

of these operational parameters on soluble chemical oxygen demand and methane production to improve the above-mentioned AD process was researched at laboratory-scale, using completely mixed anaerobic reactors operating at mesophilic temperature. Finally, a second-order mathematical model was used to obtain the kinetic parameters of the process.

MATERIALS AND METHODS

Raw Material

The SuOC sample used in this study was collected from a sunflower oil factory located near Seville (Spain). Prior to use, the substrate was shredded into different particle sizes using a commercial sieve (Restch AS 200 basic) and different screen meshes. In order to ensure the homogeneity of the sample, the most abundant particle size of this substrate (0.71-1.0 mm diameter) was selected for applying the ultrasonic pretreatment with a specific energy of 24.000 kJ/kg TS. The methodology and experimental procedure used to obtain the sonicated substrate was described in detail elsewhere in previous biochemical methane potential (BMP) tests of this substrate. [6]

The full composition and characteristics of the SuOC used in the experiments are as follows (mean values of four determinations ± standard deviations): total chemical oxygen demand (COD), 21 (±2) g O_2/g TS; total solids (TS), 18.3 (± 0.1) g/L; volatile solids (VS), 17.6 (±0.1) g/L; total protein, 25.3 (±0.8)%; soluble carbohydrates, 5.1 (±0.2)% and total carbohydrates (by difference), 53.0 (±0.3) % (expressed as TS dry basis).

Inocula

Two different inocula derived from two different industrial reactors were used and compared. Sludge I was a flocculent biomass from a full-scale reactor treating waste activated sludge generated in an urban wastewater treatment plant, with an average concentration of 12.0 (± 0.1) g VS/L. The second sludge, sludge II, was a granular biomass from an industrial UASB reactor treating brewery wastewater with an average concentration of 21.2 (± 0.1) g VS/L.

Semi-continuous Anaerobic Digestion Experiments: Experimental Procedure

Anaerobic biodegradability of the sonicated SuOC was evaluated by semi-continuous AD. The experiments were carried out in anaerobic stirred tank reactors with a total volume of 2.0 L and an effective working volume of 1.8 L. The reactors were hermetically sealed to maintain an anaerobic environment during the process. The reactor contents were continuously mixed with a magnetic stirrer at 250 rpm to guarantee completely mixed conditions and were placed in a thermostatic bath at a controlled mesophilic temperature of 35°C. The pH was continuously measured with an individual probe located inside the reactors. The sonicated substrate was fed daily in semi-continuous mode through the upper zone of each reactor. Prior to this, the liquid effluent was removed on a daily basis, before feeding the reactor from the upper part using a peristaltic pump to keep the volume constant. The gas produced during the fermentation was collected after passing through a distilled water–NaOH solution 3 N to remove the carbon dioxide produced during fermentation. The remaining gas produced was collected by a water displacement system. The volume of water collected was equivalent to the volume of methane produced.

The OLR was gradually increased from 0.87 to 3.50 g COD/(L·d). Five experimental runs for sludge I and six for sludge II were carried out. OLR values of: 0.87, 1.40, 1.98, 2.62, 3.15 and 3.50 g COD/(L·d) were evaluated, equivalent to HRTs of 24, 15, 10, 8, 6.7 and 6 days,

respectively. The increase in the OLR was achieved by reducing the HRT and by increasing the flow-rate fed to the reactors. Therefore, the flow-rate values used were: 0.075, 0.120, 0.170, 0.225, 0.270 and 0.300 L/d, respectively. Once steady-state conditions were achieved during each run, the daily volume of methane produced was measured. In addition, the total chemical oxygen demand (COD), the soluble chemical oxygen demand (CODs), total solids (TS), volatile solids (VS), pH, alkalinity, ammoniacal nitrogen (NH_x) and total volatile fatty acids (TVFA) were determined in the effluents obtained during each run. The samples were collected and analyzed for at least five consecutive days in duplicate reactors. The steady-state value of a given parameter was taken as the average of these consecutive measurements for that parameter when the deviations between the observed values were less than 5% in all cases. Each experiment had duration of 2–3 times the corresponding HRT.

Analytical Methods

Solid samples

TS and VS were measured according to the standard methods 2540B and 2540E, respectively.[10] Total chemical oxygen demand (COD) was determined using the reported method proposed by Raposo et al. [11]

Soluble fractions

The soluble pre-treated fractions obtained were characterized analyzing the following parameters: soluble chemical oxygen demand (CODs), using the closed digestion and colorimetric Standard Method 5220D; [10] total alkalinity (TA) was measured by pH titration

to 4.3; ammoniacal nitrogen (NH_x) by distillation and titration according to the standard method 4500E (APHA, 1998); [10] the TVFA concentration was analyzed by gas chromatography, as previously described. [12]

Inoculum and digestates

Both the inoculum and digestates were characterized by direct sampling. pH was determined using a pH-meter model Crison 20 Basic. TA, TS and VS were also analyzed in these samples.

RESULTS AND DISCUSSION

Effect of Organic Loading Rate (OLR) on the CODs Removal Efficiency

Tables 1 and 2 show the steady-state results obtained under different experimental conditions during the semi-continuous anaerobic digestion experiments of SuOC pretreated with ultrasound carried out with sludges I and II, respectively. These tables include HRT, OLR, methane production rates (q_{CH4}), pH, total COD, soluble COD (CODs), ammonia, TVFA, alkalinity and TVFA/alkalinity ratio.

The variation of the CODs removal efficiency with the HRT for the two anaerobic sludges used as inoculum is illustrated in Figure 1. In general, the percentage of CODs removed varied only slightly from 67.7% to 70.1% and from 61.3% to 67.7% at HRTs of between 24-10 days and 24-8 days for sludges I and II, respectively. However, for HRTs lower than 8 days and 6.7 days, respectively, a sudden decrease in the CODs removal efficiency was observed in both cases, achieving values of only 16.1% and almost 0% at HRTs of 6.7 days

and 6 days for inocula I and II, respectively. A maximum percentage of CODs of 77.4% was achieved for the granular sludge II at an HRT of 6.7 days, which was equivalent to an OLR of 3.15 g COD/(L·d). Consequently, it appears that the performance of the anaerobic reactors becomes virtually independent of HRT, provided that the HRT is kept above 8 and 6.7 days for sludges I and II, respectively. Below these values the performance of the reactor deteriorates sharply, especially for sludge II.

In the same way, the percentages of VS removed ranged between 66.3% and 79.7% for sludge I and between 51.8% and 62.8% for sludge II. The maximum values were achieved in both cases for the experiments corresponding to HRTs of 8 d (OLR of 2.6 g COD/(L·d) and 6.7 d (OLR of 3.15 g COD/(L·d)) for sludges I and II, respectively.

The effects of a combined pretreatment of simultaneous ultrasound (40 kHz, 50 W) and alkaline (lime, dosage of 560 mg/L) on the subsequent mesophilic anaerobic digestion of excess sludge as opposed to an untreated sludge were examined. [13] The anaerobic reactors operated at a HRT of 20 days and an OLR of 1.1 g VS/(L·d) achieving an increase in organic matter (VS) removal efficiency from 29.6% to 40.8% after this combined pretreatment. Lower organic matter (VS) removal efficiencies (35%) than those obtained in the present work were achieved in the anaerobic digestion of waste activated sludge (WAS) previously sonicated at 5000 kJ/kg TS when the anaerobic reactor operated at 10 days HRT. [14] Lower VS removal efficiency values were even obtained (24%) when the specific energy values used during the ultrasonic pretreatment were lower (3800 kJ/kg TS). [15] However, VS removal efficiencies of 44% were reached during the single anaerobic digestion of food waste previously sonicated and carried out in a CSTR reactor. [16] In addition, a similar HRT (8 days) to that used in the present work was used during the anaerobic digestion of sonicated secondary sludge with VS removal efficiencies of only 21% at ultrasonic densities in the range of 0.18-0.52 W/mL. [17]

Evolution of Operational Parameters and Process Stability

As can be seen in Tables 1 and 2, both the CODs and VS contents varied slightly with increased OLR for the two sludges used as inoculum. Specifically, the CODs was kept virtually constant ranging only from 0.9 to 1.2 g/L at OLRs of between 0.87 and 2.62 g COD/(L·d) for sludge I, and between 1.1 and 1.2 g/L at the same OLR range for sludge II. However, a sharp increase in CODs was observed when the OLR increased up to values of 3.15 and 3.50 g COD/(L·d) achieving values of 2.6 and 5.2 g/L for sludges I and II, respectively.

In the same way, the effluent TVFA achieved very low values for the two sludges used within the above-mentioned OLR ranges with extreme values of 19 and 27 mg acetic acid/L and 15 and 19 mg acetic acid/L for sludges I and II, respectively. However, for the highest OLR assayed for both sludges (3.15 and 3.50 g COD/(L·d)) a considerable increase in the TVFA concentration was achieved with values of 1615 and 2000 mg acetic acid/L respectively. This clearly shows an imbalance in the process and a destabilization and inhibition in the anaerobic process.

In addition, the buffering capacity of both systems was found to be at favorable levels with high total alkalinity values present (2.1-2.9 g $CaCO_3$/L) at OLR values in the range of 0.87-3.15 g COD/(L·d) for the two sludges used, which meant that the CODs removal efficiency and the rate of methanogenesis was not badly affected within the afore-mentioned OLR range. Therefore, the data obtained in this research indicate that a total alkalinity of between 2.1 and 2.9 g $CaCO_3$/L is adequate for preventing the pH from dropping to below 7.1 at OLRs of between 0.87 and 3.15 g COD/(L·d) for the two sludges used as inocula. The pH values in the reactors were always higher than 7.1 for HRTs and OLRs in the range of 24-8 days and 0.87-

2.62 g COD/(L·d) respectively for sludge I and for HRTs and OLRs in the range of 24-6.7 days and 0.87-3.15 g COD/(L·d) respectively for sludge II. However, the anaerobic process was slightly inhibited for sludge I at an OLR of 3.15 g COD/(L·d) (equivalent to an HRT of 6.7 days) and considerably destabilized for sludge II when the OLR achieved a value of 3.5 g COD/(L·d) (equivalent to an HRT of 6 days), OLR values for which the pH achieved values of 6.7 and 6.0, respectively. This demonstrated that sludge II lead to a better stabilization of the anaerobic process for a wider range of both OLRs and HRTs, permitting an adequate operation and a stable process for OLRs as high as 3.15 g COD/(L·d) and HRTs as low as 6.7 days. Previous semi-continuous anaerobic digestion experiments carried out with untreated (non-sonicated) SuOC using sludge II (derived from an industrial UASB reactor treating brewery wastewater) and operating with an OLR of 2 g COD/(L·d) at a HRT of 25 days showed a high imbalance in the process and a clear acidification of the reactor, which reached TVFA values of 1500 mg acetic acid/L at the above-mentioned conditions. [8] This caused a reduction of 32% in the methane yield when the OLR was increased from 2 to 3 g COD/(L·d). This underlines the importance of pretreatment with ultrasound with the aim of attaining a more stable process operating with higher OLRs and much lower HRTs than those necessary when the substrate is not previously sonicated. On the other hand, it has also been previously reported that the ultrasonication of the waste activated sludge prior to its anaerobic bioconversion provided a better buffering capacity to diminish the adverse effect of acidification caused when operating at moderate OLRs. [18]

The TVFA/alkalinity ratio can be used as a measurement of process stability: [19] when this ratio is less than 0.4-0.5 (equiv. acetic acid/equiv. $CaCO_3$) the process is considered to be operating favorably without risk of acidification. As can be observed in Figure 2, the ratio values were lower than the suggested limit value for OLRs lower than 3.15 and 3.50 g COD/(L·d) in the experiments carried out with sludges I and II, respectively. However, at the

above-mentioned OLR values, equivalent to HRT values of 6.7 and 6.0 days, respectively, a considerable increase in the TVFA/alkalinity ratio to values of 0.65 and 1.04 was observed for sludges I and II respectively, which was mainly due to considerable increases in the TVFA concentrations (1615 and 2000 mg/L as acetic acid, respectively) with simultaneous decreases in alkalinity (2.06 and 1.60 g/L as $CaCO_3$, respectively) especially for sludge II.

Methane Production Rates and Methane Yield Coefficients

The volumetric methane production rates for the two sludges used as a function of OLR are illustrated in Figure 3. As can be seen, the volume of methane produced per day increased almost linearly with OLR up to values of 2.62 and 3.15 g COD/(L·d) for sludges I and II, respectively. After these OLR values, a considerable decrease was observed. Therefore, apparently, the activity of methanogenic microorganisms was not impaired up to the above-mentioned OLR values because of the appropriate stability and adequate buffering capacities provided in the experimental systems. In all cases the methane production rate values corresponding to sludge II were always higher than those obtained with sludge I. The maximum methane production rate achieved with sludge II (0.668 L CH_4/(L·d) at an OLR of 3.15 g COD/(L·d)) was 53.6% higher than that reached with sludge I (0.435 L CH_4/(L·d) at an OLR of 2.62 g COD/(L·d)). Nevertheless, the methane production rate decreased considerably from 0.435 to 0.165 L CH_4/(L·d) when the OLR increased from 2.62 to 3.15 g COD/(L·d) for sludge I and from 0.668 to 0.167 L CH_4/(L·d) when the OLR increased from 3.15 to 3.50 g COD/(L·d) for sludge II. This decrease in the methane production at the highest OLR values might be attributed to an inhibition of the methanogens at high OLR values, which caused an increase in effluent TVFA contents and TVFA/alkalinity ratio as can be seen in Tables 1 and 2.

Other previous research has demonstrated that the maximum methane production rate achieved values of 2.1 and 1.6 L CH_4/(L·d) in the anaerobic digestion of food waste previously sonicated and untreated waste, respectively, both operating at the same experimental conditions. [16] Similarly, anaerobic digestion experiments of waste activated sludge (WAS) in continuous stirred tank reactors showed an increase in methane production of 23.4% for digesters fed with WAS pretreated with ultrasonic as opposed to a control at the effective HRT of 15 days. [15] Equally, an increase in cumulative biogas production from 472 to 640 NL after 67 days of tests was obtained using WAS previously sonicated (with an energy input of 5000 kJ/kg TS) in anaerobic digestion experiments conducted at 20 d HRT. [14] These same authors have recently reported an increase in biogas production of 30% during anaerobic digestion of WAS after sonolysis operating at OLRs from 0.7 to 2.8 g COD/(L·d). [20] Other recent studies also showed that sonolysis can significantly improve the solubilisation of the organic fraction of municipal solid waste, thus allowing higher biogas production (24% increase) from anaerobic treatment of sonicated substrates as opposed to a control (untreated). [21]

The experimental data listed in Tables 1 and 2 were used to determine the methane yield coefficients, Y_p (expressed as mL CH_4/g COD_{added}). In this way, methane yield values of 225, 213, 210 and 166 mL CH_4/g COD_{added} were obtained with sludge I for HRTs of 24, 15, 10 and 8 days, respectively, equivalent to OLRs of 0.87, 1.40, 1.98 and 2.62 g COD/(L·d), respectively. Moreover, methane yields of 257, 238, 215, 181 and 212 mL CH_4/g COD_{added} were reached with sludge II for the experiments with HRTs of 24, 15, 10, 8 and 6.7 days, respectively, equivalent to OLRs of 0.87, 1.40, 1.98, 2.62 and 3.15 g COD/(L·d), respectively. As can be observed for the same HRTs or OLRs, the methane yields achieved with sludge II were always higher than those obtained with sludge I.

On the other hand, the methane yield obtained with this substrate previously sonicated operating at an OLR of 3.15 g COD/(L·d) with sludge II (212 mL CH_4 STP/g COD_{added}) was 2.1 times higher than that obtained with the untreated (non-sonicated) substrate operating with the same sludge and a similar OLR of 3 g COD/(L·d) (101 mL CH_4/g COD_{added}). [8] However, the HRT used with the sonicated sample (6.7 days) was much lower than the necessary HRT when the untreated sample was processed (25 days). Therefore, these results demonstrate that the reactors fed with sonicated SuOC can operate more efficiently at shortened HRTs.

Figure 4 plots the pair of values: methane production, r_{CH4} (mL CH_4/d), and amount (g) of COD added per day for the two groups of experiments carried out. As can be seen, straight lines were obtained for the two sludges studied. The slope of the linear regressions allows for the calculation of the overall methane yields, the values of which were found to be 172 ± 12 and 195 ± 7 mL CH_4/g COD_{added} for sludges I and II, respectively. Thus, the overall methane yield obtained with sludge II was 13% higher than that achieved with sludge I. In addition, this value was 1.9 times higher than the methane yield obtained with untreated SuOC.

The effect of the ultrasound pretreatment on the methane yield of the anaerobic co-digestion of a mixture of dairy cattle slurry and industrial meat-processing by-products (at a ratio of 3:1, w:w) at 35°C has also been recently evaluated. [22] An increase of 11% in the methane yield was obtained when the above-mentioned mixture waste was pre-treated with ultrasound as opposed to an untreated control in reactors operating at a HRT of 21 days and OLR of 3.0 g VS/(L·d). In the same way, an increase in specific biogas production from 0.63 to 0.85 Nm^3/kg VS was reported in the anaerobic digestion of WAS after a sonication pretreatment, both anaerobic processes being conducted at an OLR of 0.7 g VS/(L·d). [23]

Kinetic Evaluation

With the aim of kinetically characterizing the semi-continuous anaerobic process studied and of evaluating the effect of HRT (or OLR) on effluent substrate concentrations and given the complexity of the substrate studied, [24; 25] multicomponent substrate kinetics was selected. Complete mixed hydraulic conditions were considered as occurring in the reactors operating with the two sludges used.

In the case of multicomponent substrate kinetics, the substrate removal rate can be expressed according to Equation 1: [24; 26; 27]

$$-dS/dt = k_{n(S)}\, X\, (S/S_0)^n \qquad (1)$$

where $-dS/dt$ is the substrate removal rate (g COD or CODs/(L·d); $k_{n(S)}$ is the reaction constant (g COD or CODs/(g VS·d)); X is the concentration of microorganisms (g VS/L); S is the concentration of substrate at any time (g COD or CODs/L) and S_0 is the initial substrate concentration (g COD or CODs/L).

Integrating the Equation 1 for $n = 1$ and $n = 2$, one obtains the most commonly existing case of multiple substrate removal kinetics. For $n = 1$, a first-order kinetic equation is obtained, Equation 2:

$$S_e = S_0 \exp\left(-k_{1(S)} X_0 HRT/S_0\right) \qquad (2)$$

where S_e is the substrate concentration of the effluent (g COD or CODs/L); $k_{1(S)}$ is the reaction constant of the first-order model (g COD or CODs/(g VS·d)); X_0 is the concentration of microorganisms in the reactor; and HRT is the hydraulic retention time (d).

The following second-order kinetic equation, Equation 3, can also be obtained by integrating the Equation 1 for $n = 2$:

$$S_e = S_0/(1 + (k_{2(S)}\, X_0\, HRT/S_0)) \qquad (3)$$

where $k_{2(S)}$ is the reaction constant of the second-order model (g COD or CODs/(g VS·d)).

Given the complexity of the substrate used, a second-order model was finally used to correlate and describe the experimental results obtained. Second-order models were also used to obtain

the kinetic parameters of the anaerobic processes when inhibition by substrate concentration or by presence of inhibitory compounds was detected such as occurs for piggery wastewater, chicken manure, textile wastewater, olive mill wastewater, two-phase olive mill solid waste, etc. [26-30]

For the determination of the kinetic parameter $k_{2(S)}$, the Equation 3 can be transformed into the following linear expression, Equation 4:

$$HRT\,[S_0/(S_0 - S_e)] = a + b\,HRT \qquad (4)$$

where the intercept, a, is equal to $S_0/k_{2(s)}\,X_0$ and the slope is b, whose value must approach the unit, where the model is valid for fitting the experimental data.

According to Equation 4, the values of the reaction constants can be obtained by the plot of the term $HRT\,[S_0/(S_0-S_e)]$ versus HRT. The validity of the application of this model was corroborated when plotting the experimental data and straight lines were obtained with a slope near the unit for the sludges studied (1.14 and 1.27) and an intercept equal to a (Figure 5). Once the value of the intercept is known, the value of the reaction constant, $k_{2(s)}$, can be easily determined. Figure 5 shows this plot, expressing the substrate concentration as CODs.

The following linear regression equation was obtained: $y = 0.801x + 1.14$ with a determination coefficient $R^2 = 0.993$ for sludge I and $y = 1.548x + 1.27$ with a $R^2 = 0.998$ for sludge II ($P \leq 0.05$). The equations obtained in both cases corroborated that a second-order model for substrate kinetics fitted adequately with the experimental results obtained. With the values of intercept (a), the corresponding value of S_0 (3.1 g CODs/L) and taking into account the average values of the biomass concentration during the experiments (X_0), the values of $k_{2(S)}$ were calculated to be: 0.32 and 0.09 g CODs/(g VS·d) for the experiments corresponding to sludges I and II, respectively. Therefore, the kinetic constant obtained with sludge I was 3.5 times higher than that achieved with sludge II.

A similar second-order kinetic constant value [0.29 g COD/(g VS·d)] to that obtained with sludge I was reported during the anaerobic acidogenic digestion of two-phase olive mill solid waste (OMSW), a very complex substrate with high concentrations of phenolic inhibitors. [28] However, higher second-order kinetic constant values were achieved during the anaerobic digestion of olive mill wastewaters (OMWs) previously treated with *Geotrichum candidum, Azotobacter chroococcum* and *Aspergillus terreus* [2.71, 4.44 and 4.58 g COD/(g VS·d), respectively]. [31] This behaviour was due to the lower phenolic content of these fermented OMWs - the lower the phenolic compound content, the lower its biotoxicity giving higher kinetic constant values in the anaerobic digestion process. [31]

With the values of the kinetic constants obtained in the present work for the two sludges used and applying equation (3), the theoretical values of the effluent substrate concentrations were calculated. Deviations lower than 10% between the theoretical and experimental values were obtained in all cases. These slight deviations between the experimental and simulated models demonstrate the suitability of the second-order kinetic model in predicting the behaviour of the microorganisms involved in the anaerobic digestion of this sonicated substrate.

CONCLUSIONS

The experimental results obtained demonstrate the stability and high performance of the semi-continuous anaerobic digestion of SuOC previously sonicated by using two different anaerobic sludges as inoculum: a mixture or flocculent sludge (I) derived from an industrial reactor treating WAS and a granular sludge (II) from an industrial UASB reactor treating brewery wastewater. CODs removals varied slightly between 67.7% and 70.1% and between 61.3% and 67.7% at HRTs of between 24-10 days and 24-8 days for sludges I and II, respectively. However, for HRTs lower than 8 and 6.7 days, respectively, a sudden decrease

in the process performance was observed in both cases. Sludge II achieved a better stabilization of the anaerobic process for a wider range of both OLRs and HRTs, allowing an adequate operation for OLRs as high as 3.15 g COD/(L·d) and HRTs as low as 6.7 days. The maximum methane production rate achieved with sludge II was 53.6% higher than that reached with sludge I. The overall methane yield obtained with sludge II was 13% higher than that achieved with sludge I. In addition, this value was 1.9 times higher than the methane yield obtained with untreated (non-sonicated) SuOC.

A second-order kinetic model (multicomponent substrate) fitted adequately with the experimental results obtained for the two sludges used. The kinetic constant obtained with sludge I was 3.5 times higher than that achieved with sludge II.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to the Spanish Ministry of “Ciencia e Innovación” (Project CTM2008-05772/TECNO) for providing financial support, to the Program “FPI” of the above-mentioned Ministry for contracting graduates, as well as to the “Junta de Andalucía” for supporting long-term research activities.

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FIGURE CAPTIONS

Figure 1. Variation in the percentage of CODs removal with HRT (days) for the two sludges used in the experiments.

Figure 2. Variation of the total volatile fatty acids (TVFA)/alkalinity ratio with the OLR for the two sludges used in the experiments.

Figure 3. Variation of the methane production rate with the OLR for the two sludges used in the experiments.

Figure 4. Variation of the daily methane production, r_{CH4}, with the amount of COD added daily to the reactors for the two sludges used in the experiments.

Figure 5. Linearization of the second-order kinetic model, according to Equation (4), to calculate the kinetic constant values ($k_{2(S)}$) for CODs.

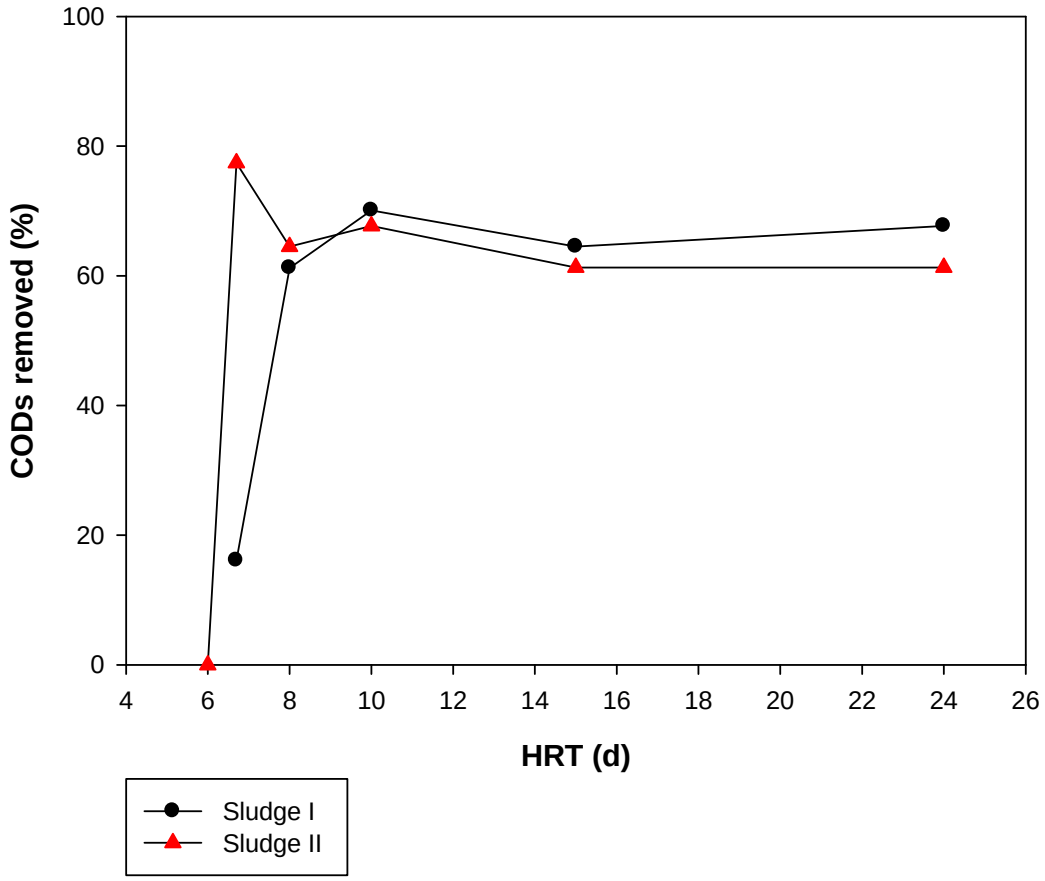
100
80
60
40
20
0
CODs removed (%)
4
6
8
10
12
14
16
18
20
22
24
26
HRT (d)
Sludge I
Sludge II

Fig. 1

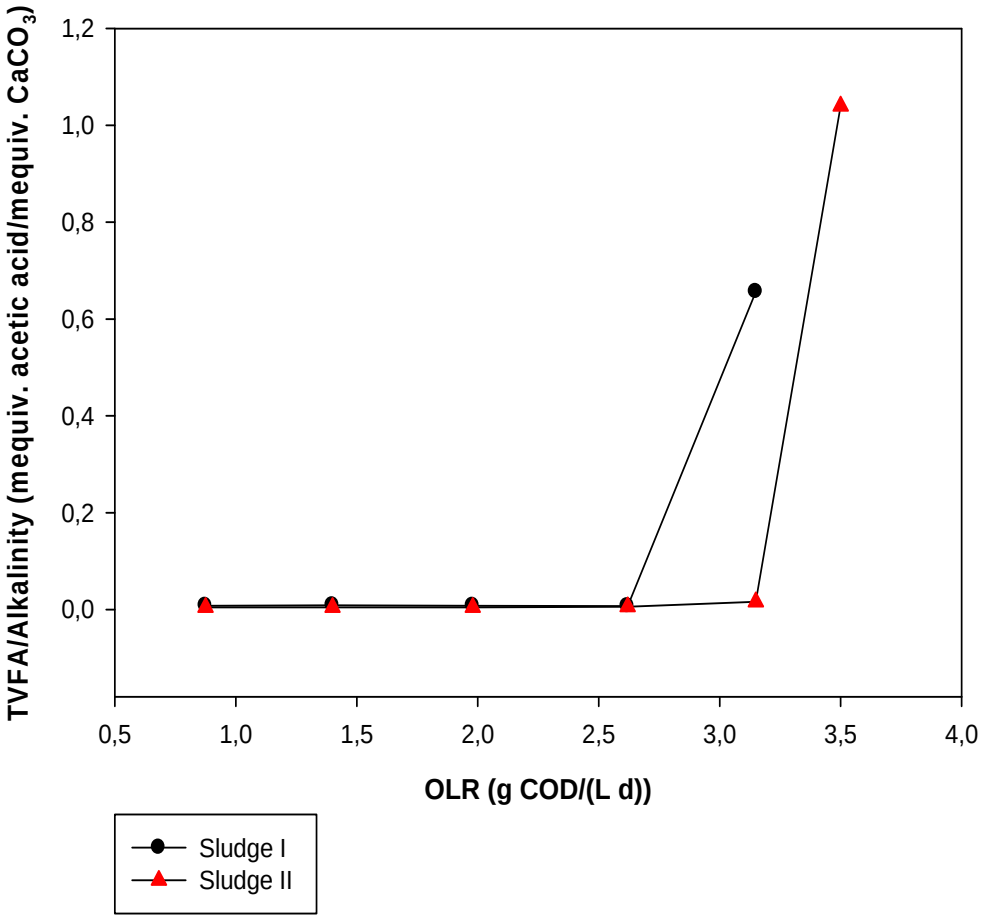
TVFA/Alkalinity (mequiv. acetic acid/mequiv. CaCO3)
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0,5
1,0
1,5
2,0
2,5
3,0
3,5
4,0
OLR (g COD/(L d))
Sludge I
Sludge II

Fig. 2

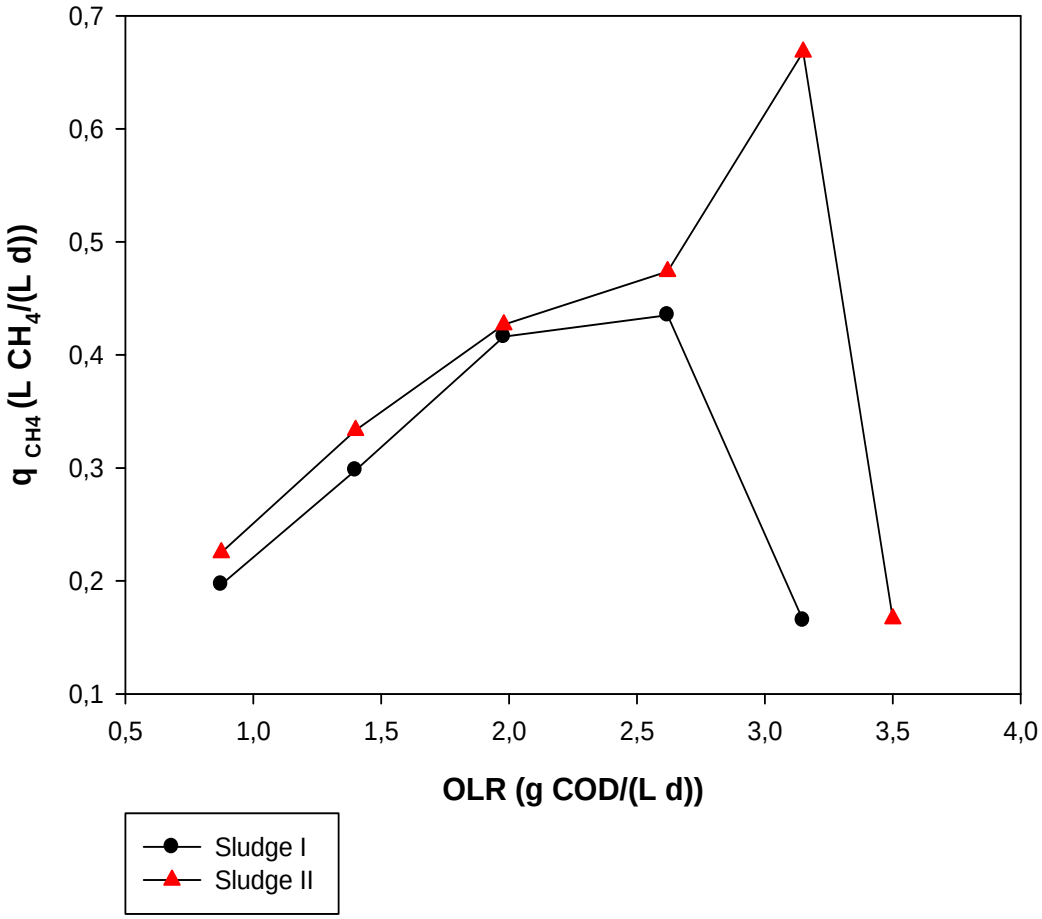
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,5
1,0
1,5
2,0
2,5
3,0
3,5
4,0
q CH4 (L CH4/(L d))
OLR (g COD/(L d))
Sludge I
Sludge II

Fig. 3

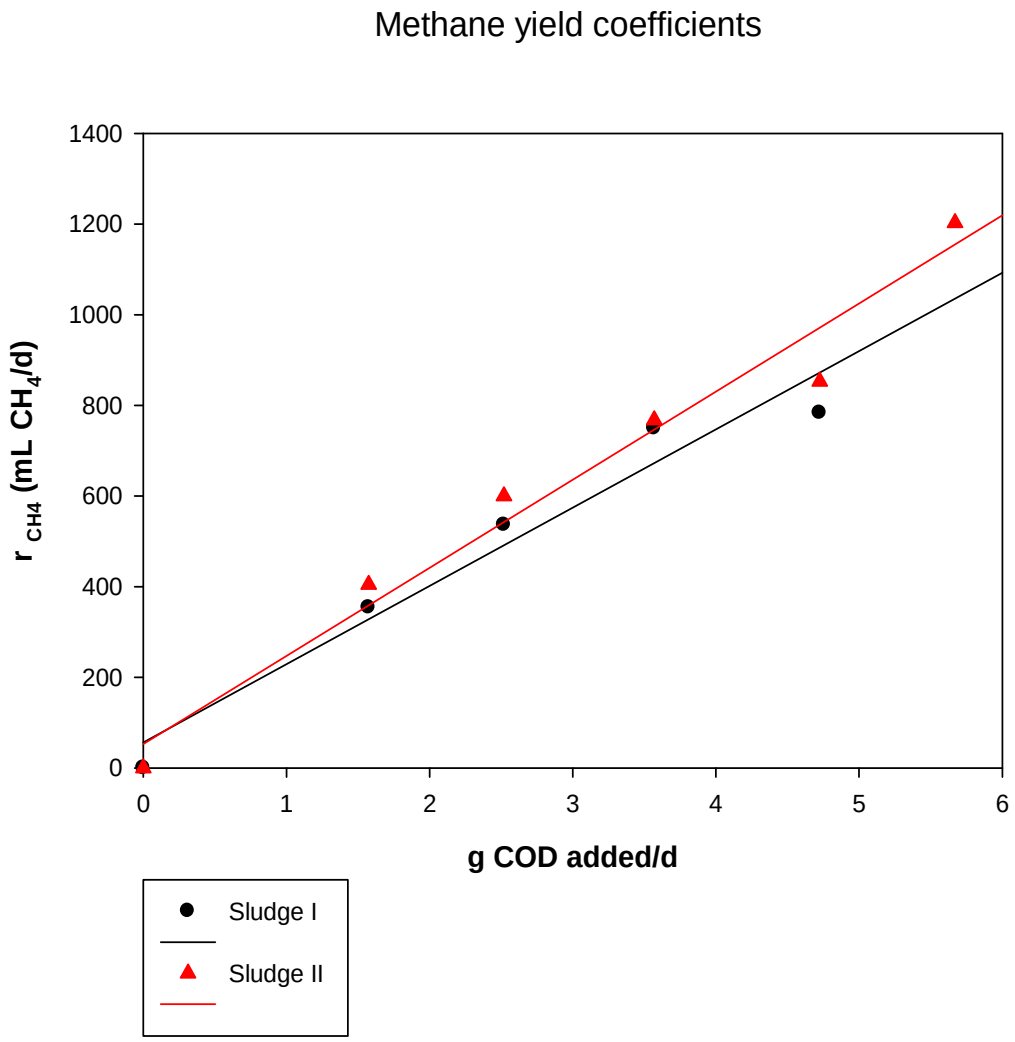
Methane yield coefficients
1400
1200
1000
800
600
400
200
0
r CH4 (mL CH4/d)
0
1
2
3
4
5
6
g COD added/d
Sludge I
Sludge II

Fig. 4

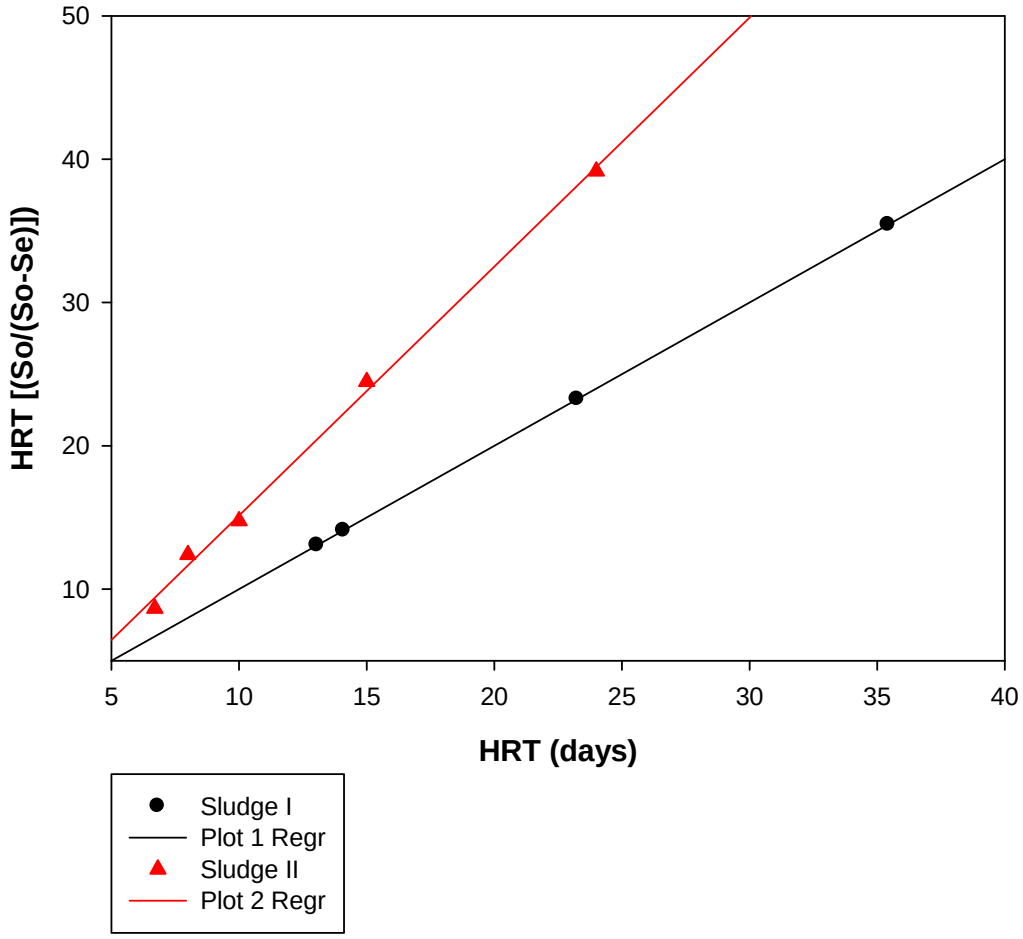
50
40
30
20
10
HRT [(So/(So-Se)])
5
10
15
20
25
30
35
40
HRT (days)
Sludge I
Plot 1 Regr
Sludge II
Plot 2 Regr

Fig. 5

Table 1. Steady-state results under different experimental conditions for the anaerobic digestion experiments of SuOC pretreated with ultrasound conducted with sludge I.

SLUDGE I					
Experiment number	**1**	**2**	**3**	**4**	**5**
OPERATIONAL CONDITIONS					
OLR (g COD/(L·d))	0.87	1.4	1.98	2.62	3.15
HRT (days)	24	15	10	8	6.7
Flow-rate (L/day)	0.075	0.12	0.17	0.225	0.27
STEADY-STATE CONDITIONS*					
r_{CH_4} **(L CH_4/ day)**	0.35	0.54	0.75	0.78	0.30
pH	7.8	7.2	7.4	7.6	6.3
TVFA (mg acetic acid/L)	26	27	25	19	1615
COD (g/L)	18.7	31.1	40.5	42.8	51.6
Soluble COD (g/L)	1.0	1.1	0.9	1.2	2.6
TS (g/L)	20.2	28.3	37.1	37.0	43.0
VS (g/L)	6.6	5.8	7.5	6.1	6.0
Alkalinity (g $CaCO_3$/L)	2.64	2.63	2.61	2.31	2.06
Ammonia (g NH_x/L)	0.54	0.56	0.58	0.54	0.59
TVFA/Alkalinity	0.008	0.009	0.008	0.007	0.656
q_{CH_4} **(L CH_4/(L·d))**	0.197	0.298	0.416	0.435	0.165

* Values are the averages of five determinations taken over five days in duplicate reactors after the steady-state conditions had been reached. The differences between the observed values were less than 5% in all cases.

Table 2. Steady-state results under different experimental conditions for the anaerobic digestion experiments of SuOC pretreated with ultrasound conducted with sludge II.

SLUDGE II						
Experiment number	**1**	**2**	**3**	**4**	**5**	**6**
OPERATIONAL CONDITIONS						
OLR (g COD/(L·d))	0.87	1.4	1.98	2.62	3.15	3.5
HRT (days)	24	15	10	8	6.7	6
Flow rate (L/day)	0.075	0.12	0.17	0.225	0.27	0.30
STEADY-STATE CONDITIONS*						
r_{CH_4} **(L CH_4/day)**	0.41	0.60	0.77	0.85	1.20	0.30
pH	7.8	7.1	7.3	7.6	7.5	6.0
TVFA (mg acetic acid/L)	19	16	15	17	49	2000
COD (g/L)	26.9	39.9	42.7	47.9	48.0	48.0
Soluble COD (g/L)	1.2	1.2	1.0	1.1	0.7	5.2
TS (g/L)	43.1	51.8	50.9	56.0	50.1	49.6
VS (g/L)	22.3	22.5	19.0	21.9	17.8	14.2
Alkalinity (g $CaCO_3$/L)	2.91	2.88	2.70	2.44	2.60	1.60
Ammonia (g NH_x/L)	0.59	0.63	0.59	0.57	0.57	0.57
TVFA/Alkalinity	0.005	0.005	0.005	0.006	0.016	1.04
q_{CH_4} **(L CH_4/(L·d))**	0.225	0.333	0.427	0.474	0.668	0.167

*Values are the averages of five determinations taken over five days in duplicate reactors after the steady-state conditions had been reached. The differences between the observed values were less than 5% in all cases.

3.3 Resumen de ensayos aplicados sobre SuOC: Revisión

Desde el año 2005 el grupo de investigación, al cual pertenece el doctorando, *Unidad de Procesos Industriales y Medioambiente* del Instituto de la Grasa (CSIC), ha estado estudiando la estabilización anaerobia de la harina de girasol desengrasada (SuOC).

Durante estos años se han llevado a cabo experimentos tanto en modo discontinuo (batch) como semicontinuo (fed-batch) en una y dos fases.

El sustrato SuOC fue seleccionado debido a que, dada su composición lignocelulósica, presenta dificultades a la hora de exponerlo a procesos de digestión anaerobia.

Después de aplicar sobre SuOC ensayos tipo semicontinuo, y considerando la baja biodegradabilidad obtenida durante estos experimentos, se llevaron a cabo pretratamientos previos a los procesos de digestión anaerobia para intentar aumentar el coeficiente de rendimiento de metano.

Este trabajo de revisión fue publicado antes de completar la última fase de experimentación de la presente tesis, el ensayo de tipo semicontinuo.

Los resultados más relevantes obtenidos durante los experimentos llevados a cabo sobre el sustrato objeto de estudio, SuOC, son resumidos en esta publicación.

Referencia del artículo que plasma estos resultados:

Anaerobic digestion of sunflower oil cake: a current overview

Ángeles de la Rubia, M., Fernández-Cegrí, V., Raposo, F., Borja, R. (2013) Water Science & Technology, 62, 2. pp. 410-417.

Anaerobic digestion of sunflower oil cake: a current overview

M. A. De la Rubia, V. Fernández-Cegrí, F. Raposo and R. Borja

ABSTRACT

Due to the chemical and physical structure of a lignocellulosic biomass, its anaerobic digestion (AD) is a slow and difficult process. In this paper, the results obtained from a batch biochemical methane potential (BMP) test and fed-batch mesophilic AD assays of sunflower oil cake (SuOC) are presented. Taking into account the low digestibility shown during one-stage experiments the methane yield decreased considerably after increasing the organic loading rate (OLR) from 2 to 3 g VS L^{-1} d^{-1}, SuOC was subjected to a two-stage AD process (hydrolytic-acidogenic and methanogenic stages), in two separate reactors operating in series where the methanogenic stage became acidified (with >1,600 mg acetic acid L^{-1}) at an OLR as low as 2 g VS L^{-1} d^{-1}. More recently, BMP assays were carried out after mechanical, thermal, and ultrasonic pre-treatments to determine the best option on the basis of the methane yield obtained.

Key words | batch assay, biochemical methane potential (BMP), fed-batch assays, pre-treatment, sunflower oil cake (SuOC), two-stage anaerobic digestion

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INTRODUCTION

Sunflower oil cake (SuOC) is the part of whole sunflower seeds which remains after the oil extraction process. It is an agro-industrial residue generated in Spain in great quantities (about 4–5 million tons per year). This extracted flour is mainly composed of fibre and protein (Vioque *et al.* 2001).

This flour has been generally used for cattle feed (Szabo *et al.* 2001; Torrijos *et al.* 2008); nevertheless it represents one of the reservoirs of proteins with major potential for the food industry (Vioque *et al.* 2001). Other applications for the extracted sunflower flour have been in the preparation of antibiotics (Kota & Sridhar 1999) and some enzymes (Proteases) (Pandey *et al.* 2000). Nevertheless, the scarce and limited applications of the different methods of re-use of these wastes and their high production justify the study of other processes or alternatives that enable their utilization and reuse.

Anaerobic digestion (AD) is the most effective process for the treatment and stabilization of organic wastes such as SuOC, offering the advantage of a net energy gain by producing methane. Moreover, the AD process can be improved by means of a process in two stages (De la Rubia *et al.* 2009), as the stability of the global process remains awkward when imbalances take place between the activity of the groups of microorganisms that carry out the first phase of hydrolysis of the high molecular weight compounds and acidification of the resulting monomers (acidogenic stage) and those that, in the second phase, metabolize the acids formed to methane (methanogenic stage). On the other hand, owing to the refractory structure of the lignocellulosic biomass the efficiency of AD to treat agriculture residues is limited. Although cellulose and hemicellulose can be degraded under anaerobic conditions, lignin (undegradable in biogas processes) prevents enzyme accessibility to cellulose (Zhu *et al.* 2008). While hemicellulose serves as a connection between the lignin and the cellulose fibres and gives the whole cellulose–hemicellulose–lignin network more rigidity. Therefore, only a low fraction of lignocellulosic biomass can be converted into biogas.

Hence, the pre-treatment of the lignocellulosic biomass is crucial to remove lignin and hemicellulose and make cellulose more accessible to the enzymes that convert carbohydrate polymers into fermentable sugars (Mosier *et al.* 2005; Pérez *et al.* 2007) and, therefore, to increase the biogas potential. Some physical, physico-chemical, chemical, and biological processes have been used for the

doi: 10.2166/wst.2012.586

pre-treatment of lignocellulosic materials, not only to remove the inhibitory lignin complex but also to reduce cellulose crystallinity, which is a major limit for cellulose hydrolysis (Jeihanipour *et al*. 2010).

Since 2005, the 'Reuse of Wastes and Wastewater Treatment Group', of the Instituto de la Grasa (IG) of the Spanish National Research Council (CSIC) has been studying the anaerobic stabilization of SuOC. During these years batch and fed-batch (one and two stage) experiments have been carried out. Recently, a combination of thermal, mechanical and ultrasonic pre-treatments and batch anaerobic assays has been assessed. Finally, the best option (ultrasound pre-treatment) has been chosen to study a combined ultrasound pre-treatment and one-stage AD of SuOC, which is currently being carried out. In this paper the most relevant results obtained during the above-mentioned experiments are summarized.

MATERIAL AND METHODS

Raw material

SuOC was collected from a sunflower oil factory located near Seville (Spain). Prior to using the substrate, it was sieved to give a fraction with a particle size lower than 2 mm (around 90% of the total particles of the SuOC had this size). The full composition and main features of the SuOC used have been described elsewhere (Raposo *et al*. 2008a).

Inocula

Two kinds of inocula were used in the different assays conducted.

Granular sludge (GS) was taken from an industrial upflow anaerobic sludge blanket (UASB) reactor which treats brewery wastewater. The main characteristics of this anaerobic sludge were: pH, 7.6 ± 0.1; total solids (TS), 60 ± 3 g L^{-1}; volatile solids (VS), 45 ± 2 g L^{-1}.

Sewage sludge (SS), a mixed anaerobic culture, was collected from a municipal wastewater treatment plant which operates in the anaerobic stabilization of primary and waste activated sludge. The main characteristics of this digested sludge were: pH, 7.6 ± 0.1; 33 ± 2 g L^{-1} of TS, and 18 ± 1 g L^{-1} of VS.

Experimental design

The experiments carried out have been summarized in Table 1 and/or in the following list:

1st: Biochemical methane potential (BMP) using SuOC as a substrate and GS as inoculum. The effect of inoculum to substrate ratio (ISR), expressed as VS basis, was studied in this set of experiments.

2nd: One-stage fed-batch experiments using SuOC as a substrate and the two previous inocula described (GS and SS). Organic loading rates (OLRs) of 1, 2 and 3 g VS L^{-1} d^{-1} were assayed.

3rd: Hydrolytic-acidogenic (H-A) fed-batch experiments using SuOC as a substrate and the inoculum GS. Six

Table 1 | Anaerobic digestion experiments conducted with SuOC as substrate without pre-treatment

Experiment								
					Two stages			
BMP	One-stage fed-batch[a]		Hydrolytic-Acidogenic[b]		Hydrolytic-Acidogenic		Methanogenic	
ISR	OLR g VS L^{-1} d^{-1}	HRT d	OLR g VS L^{-1} d^{-1}	HRT d	OLR g VS L^{-1} d^{-1}	HRT d	OLR g VS L^{-1} d^{-1}	HRT d
0.5	1	25	4	8, 10, 12, 15	6	10	1	42
0.8	2		5				1.5	28
1	3		6				2	21
1.5			7				2.5	16
2			8		8	10	1	33
3			9				1.5	22
							2	16

[a]Experiments were developed at different OLR but at the same HRT.
[b]Every OLR (4, 5, 6, 7, 8, 9) was assayed at every HRT (8, 10, 12, 15).

different OLRs from 4 to 9 g VS L^{-1} d^{-1} and four hydraulic retention times (HRTs) of 8, 10, 12 and 15 days were studied.

4th: Two-stage (H-A and methanogenic) fed-batch experiments using SuOC as a substrate and the two inocula previously described. After optimizing the H-A stage (OLR of 6 and 8 g VS L^{-1} d^{-1} and HRT of 10 days) the methanogenic reactors were fed with the effluent obtained in the first stage. OLRs of 1, 1.5, 2 and 2.5 g VS L^{-1} d^{-1} were assayed in this second methanogenic stage.

5th: BMP using pre-treated SuOC as a substrate and the two inocula mentioned above. The following pre-treatments were assayed:

- Mechanical (sieve): The SuOC $\leq$ 2 mm was sieved and three different fractions: 0.355–0.55 mm, 0.71–1.0 mm, and 1.4–2.0 mm were chosen to be assayed.
- Thermal: A 2% (w/v) SuOC suspension was treated for 4 h at ambient temperature (AT), 100, 150 and 200 °C.
- Ultrasound: A 2% (w/v) SuOC suspension was treated with an ultrasound frequency of 20 kHz and a supplied power of 120 W. Five specific energies (SE) were supplied, ranging from 24,000 to 597,600 kJ kg TS^{-1} and obtained by increasing the operation time.

Equipment

BMP assays

The experimental design consisted of a multiflask batch system which was fully described elsewhere (Raposo *et al.* 2008a). The reactors, which were maintained at 35 ± 1 °C in a temperature-controlled water bath, were initially charged with the inoculum by keeping a concentration of 15 g VS L^{-1}. The ISR was maintained at 2, except in the experiments to study the effect of ISR. A stock mineral medium solution whose composition has been described elsewhere (Raposo *et al.* 2006) was also added, and finally distilled water was added to achieve the desirable working volume of 250 mL. Reactors were flushed with N_2 in order to achieve and maintain anaerobic conditions.

The methane released was measured by volume displacement (the carbon dioxide was removed previously by flushing the gas through a 2N NaOH solution), and expressed at standard temperature and pressure conditions. Methane production was monitored daily and calculated by subtracting the amount of methane produced by the blank controls (endogenous tests, with the inoculum alone added) from the methane production of each fed reactor.

All the experiments were run for 7–8 days, until no significant gas production was observed, suggesting that biodegradation was essentially completed, as a control of cellulose (~310 mL CH_4 g^{-1} COD_{added}) also confirmed. Each experimental setup was performed in triplicate.

Fed-batch assays

Experiments were carried out in four completely mixed glass digesters, each one with a total volume of 2.5 L and a working volume of 2 L. The reactors were mixed using magnetic bars and an adjustable stirrer at 700 rpm. The digesters, maintained at 35 ± 1 °C in a temperature-controlled water bath, were started with an inoculum concentration of 17 g VS L^{-1}. Nitrogen gas was used and sparged to maintain anaerobic conditions before starting the experiments and after each feed.

Analytical methods

The chemical compositions of the raw material, inocula and digestates were determined:

- *Raw material*: The following parameters were analysed in the substrate: TS and VS, according to the Standard Methods 2540B and 2540E (APHA 1998), respectively; total chemical oxygen demand (CODt) was determined using the method proposed by Raposo *et al.* (2008b). Total Kjeldahl nitrogen (TKN) determination was also described elsewhere (Raposo *et al.* 2009).
- *Inocula*: The inocula and digestates were characterized by direct sampling. The pH (using a pH meter model Crison 20 Basic), TS and VS were determined (APHA 1998).
- *Soluble fraction*: The supernatant obtained after centrifuging the inocula and digestates for 15 min at 10,000 rpm was filtered (0.45 μm) and used to characterize the following parameters: (i) soluble chemical oxygen demand (CODs), using the closed digestion and colorimetric Standard Method 5220D (APHA 1998); (ii) total alkalinity, which was measured by pH titration to 4.3; (iii) soluble ammonia nitrogen determined by distillation and titration according to the standard method 4500E (APHA 1998); and (iv) the volatile fatty acids (VFA) concentration determined using a gas

chromatograph, as previously described elsewhere (De la Rubia *et al*. 2009).

RESULTS AND DISCUSSION

One stage

BMP assays of untreated SuOC

In order to determine the BMP of SuOC, the influence of ISRs and the evolution and variation of the chemical control parameters of the process with digestion time, different batch assays were conducted.

The results from this study suggest that SuOC is a potential substrate for AD. Batch experiments carried out at mesophilic temperatures and at ISRs of 3.0, 2.0, 1.5, 1.0, 0.8 and 0.5 demonstrated that the ultimate methane yield decreased considerably from 193 ± 19 mL CH_4 g^{-1} COD_{added} to 91 ± 9 mL CH_4 g^{-1} COD_{added} when the ISR decreased from 3.0 to 0.5, showing a marked influence of this parameter on the methane yield. However, the net VS removed only varied from 42 to 36% when the ISR decreased from 3.0 to 0.5. A considerable increase in CODs due mainly to an accumulation of VFA in the digestates was observed at ISRs of 0.5 and 0.8, which demonstrated a clear imbalance of the process, typical of stress on methanogenic microorganisms. The lower the ISRs, the greater the accumulation of the longer chain VFA, and only the ISRs of 2 and 3 were allowed to obtain digestates with no residual VFA at the end of the digestion time, as can be seen in Figure 1. Therefore, on the basis of the results obtained in the BMP test, an ISR over 2.0 is suggested and recommended in order to prevent acidification and an imbalance of the AD process of this substrate (VDI 4630 2006; Raposo *et al*. 2008a, 2012).

Fed-batch anaerobic digestion of SuOC

Once it was determined that SuOC was a potential substrate for AD, fed-batch anaerobic experiments at OLRs of 1, 2 and 3 g VS L^{-1} d^{-1} and HRT of 25 days were carried out. After the start-up step, the reactors were subjected to a programmed steady-state operation, using the mentioned OLRs. The attainment of the steady-state was verified after a period equivalent to 2–3 times the HRT by checking whether constant effluent characteristic values (TS, VS, COD and VFA levels) were achieved. The sampling during each steady-state period was performed for five consecutive days.

Taking into account the results obtained during this study, shown in Table 2, it can be stated that the activity of acidogenic microorganisms exceeded the activity of the methanogenic organisms when the OLR was increased from 2 to 3 g VS L^{-1} d^{-1}, because VFA were accumulated and reached values higher than 1,500 mg acetic acid L^{-1}. The reactor was overloaded: to be specific, the methane yield diminished from 149 ± 5 mL CH_4 g^{-1} COD_{added} to 101 ± 5 mL CH_4 g^{-1} COD_{added} when OLR was increased from 2 to 3 g VS L^{-1} d^{-1}. Because acidification occurred, the feeding was stopped before reaching a total imbalance of the process.

As Demirer & Chen (2005) stated, conventional one-stage digestion was not an effective system for wastes containing high solid concentrations, as SuOC.

Two stages

Acidogenic microorganisms and the methanogens constitute two very different groups in terms of their

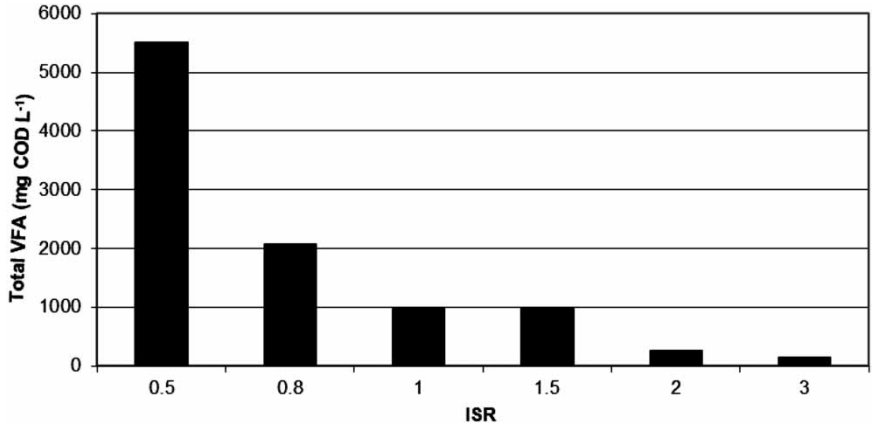

Figure 1 | Total volatile fatty acid concentration evolution with inoculum substrate ratio increase, after biochemical methane potential assays. Values are averages from three trials.

Table 2 | Methane yield and total VFA (TVFA) concentration for each experiment conducted in one and two stages[a]

One stage			Two stages						
				Methanogenic I			Methanogenic II		
OLR g VS L^{-1} d^{-1}	CH_4 mL g^{-1} COD_{added}	TVFA mg C_2 L^{-1}	OLR VS L^{-1} d^{-1}	HRT d	CH_4 mL g^{-1} COD_{added}	TVFA mg C_2 L^{-1}	HRT d	CH_4 mL g^{-1} COD_{added}	TVFA mg C_2 L^{-1}
1	136 ± 8	214 ± 27	1	45	109 ± 13	652 ± 131	36	117 ± 4	524 ± 54
			1.5	28	141 ± 6	342 ± 43	22	141 ± 6	340 ± 52
2	149 ± 5	585 ± 87	2	21	149 ± 8	607 ± 67	16	95 ± 1	1,620 ± 41
			2.5	16	48 ± 7	5,215 ± 52	–	–	–
3	101 ± 5	1,566 ± 195							

[a]Average values from five trials ± standard deviation of the mean values ($p < 0.05$).

growth kinetics, requirements for nutrients, optimum pH and capacity to support and maintain their ideal conditions before situations of overloading or 'stress' occur. Moreover, it is generally accepted that hydrolysis is the rate-limiting step in the AD of vegetable solid waste. On this basis, a process carried out in two stages can optimize the operative conditions of every step and give major stability to the global process.

By means of these experiments the suitable values of the HRT and OLR, which resulted in maximum efficiencies of elimination of organic matter accompanied with a maximum production of VFA in the first reactor and maximum methane yield coefficients in the second, were obtained.

A relevant feature of the two-stage AD approach is that when a high solid containing waste is introduced into the first stage, it is liquefied along with acidification.

Hydrolytic-acidogenic stage

In this study the effect of the variations of HRT and OLR on CODs and VFA production to improve the H-A step of the AD of SuOC was studied (De la Rubia *et al*. 2009).

During the mesophilic acidogenic fermentation of SuOC, variations in the HRT did not affect the COD solubilization of this substrate within the HRT range (15–8 days) studied. Variations in OLR affected the organic matter liquefaction slightly, with the highest value (30.1%) being reached at an HRT of 10 days and an OLR of 8 g VS L^{-1} d^{-1}. The organic matter liquefaction or hydrolysis yield can be defined by the following equation:

$$\text{Hydrolysis yield} = \frac{S_S}{S_I} \times 100$$

where S_I is the initial total substrate concentration (calculated by means of the quotient: (CODt g SuOC)/(volume related to the corresponding HRT) where CODt is the COD concentration of solid substrate: 1.1 g COD g^{-1} TS) and S_S is the soluble output COD.

The acidification yield increased with an OLR of up to 6 g VS L^{-1} d^{-1}, the highest value (83.8%) being achieved for an HRT of 10 days and an OLR of 6 g VS L^{-1} d^{-1}. However, higher OLR produced a decrease in the acidification yield, probably due to the fact that the acidogenic bacteria could have been affected and inhibited at the highest OLR studied.

Methanogenic stage

The effluents obtained under the optima OLR (6 g VS L^{-1} d^{-1}) and HRTs (8 and 10 days) of the H-A stage were treated in the methanogenic reactors to determine the optimum operational parameters. With the effluent of reactor H-AI, operated at an OLR of 6 g VS L^{-1} d^{-1} and HRT of 8 days, the methanogenic reactor MI was fed. Four different OLRs were assayed for this second stage: 1, 1.5, 2 and 2.5 g VS L^{-1} d^{-1}, at HRTs of 45, 28, 21 and 16 days, respectively, as can be seen in Table 2. The reactor MII was fed with the effluent of H-AII (operated at OLR of 6 g VS L^{-1} d^{-1} and 10 days of HRT); with this reactor three OLRs and HRTs were used: 1, 1.5 and 2 g VS L^{-1} d^{-1}, and 36, 22 and 16 days, respectively.

The best results were obtained when the methanogenic reactors were operated at HRT between 21 and 28 days, and OLR of 1.5 and 2 g VS L^{-1} d^{-1} (Table 2). At an HRT of 16 days, the methanogenic activity was clearly inhibited. This was shown by the methane yield drop, for both methanogenic reactors, and the high VFA concentration achieved, which varied between 1,600 and 5,200 mg acetic acid L^{-1}, for OLR of 2 and 2.5 g VS L^{-1} d^{-1}, respectively.

Consequently, neither the one-stage nor the two-stage mesophilic AD processes were able to efficiently degrade SuOC at an OLR higher than 2 g VS L^{-1} d^{-1}.

Pre-treatments

Pre-treatments are frequently used to facilitate the methane production by overcoming the limitation of hydrolysis, which includes the solubilization and biodegradation of hemicellulosic and lignin fractions of the substrates. Taking into account the above-stated difficulty of SuOC to be anaerobically degraded, combinations of mechanical, thermal, and ultrasonic pre-treatments and AD processes in batch mode were assessed.

To evaluate the efficiency of the above-mentioned pre-treatments, with the aim of achieving a maximum solubilization level by comparing their capacity for converting the complex organic compounds present in the waste into simpler compounds that can be easily biodegradable by AD processes, BMP experiments were carried out.

Thermal and ultrasound pre-treatments involve the addition of water to the substrate to be pre-treated; therefore after pre-treatments of SuOC two fractions are obtained: a water-insoluble solid fraction and a liquid fraction. Both of these fractions were separated and evaluated individually. The results are compared in Table 3.

Mechanical pre-treatment

Batch AD experiments of SuOC with different particle sizes (0.355–0.55, 0.71–1.0 and 1.4–2.0 mm) revealed that this parameter affects methane yield. In this way, the largest size (1.4–2.0 mm) within the range studied (0.355–2.0 mm) resulted in the highest methane yield, 175 ± 7 mL CH_4 g^{-1} COD_{added}, when compared with particle sizes of 0.355–0.55 and 0.71–1.0 mm, for which 143 ± 3 and 155 ± 2 mL CH_4 g^{-1} COD_{added}, respectively, were reached. This could be attributed to the different initial chemical composition of the different fractions (De la Rubia *et al.* 2011). Therefore, optimizing the size reduction of SuOC could potentially improve the methane yield of the AD process of this substrate.

Thermal pre-treatment

- Solid fraction: The highest methane production was obtained for SuOC pre-treated at AT (114 ± 9 mL CH_4 g^{-1} COD_{added}). This is because at this low temperature some soluble compounds still remained in the solid fraction, which can be degraded during BMP assays. The lowest methane yield was obtained at 200 °C (53 ± 8 mL CH_4 g^{-1} COD_{added}). Therefore, the higher the temperature applied, the lower the methane yield obtained for this fraction.
- Liquid fraction: In this case the best results were obtained at 100 °C (310 ± 4 mL CH_4 g^{-1} COD_{added}). The sample treated at AT resulted in 276 ± 6 mL CH_4 g^{-1} COD_{added}, while at 150 and 200 °C the methane yield decreased to 220 ± 15 and 247 ± 10 mL CH_4 g^{-1} COD_{added}, respectively. Hence, temperatures above 150 °C produced the formation of non-degradable or toxic compounds, which brought about a potential inhibition for the growth of bacteria and *Archaea* due to their lethal nature.

From the results obtained it can be stated that 100 °C is the best temperature to thermally pre-treat SuOC before AD.

Ultrasound pre-treatment

- Solid fraction: SuOC pre-treated by ultrasound obtained the highest methane production of 111 mL CH_4 g^{-1} COD_{added} for an SE of 24,000 kJ kg^{-1} TS. A higher SE brought about a lower methane yield.

Table 3 | Ultimate CH_4 yield obtained after the different pre-treatments studied

Pre-treatment						
Mechanical – particle size (mm)		**0.355–0.55**	**0.71–1.0**	**1.4–2.0**		
mL CH_4 g^{-1} COD_{added}		143 ± 3	155 ± 2	175 ± 7		
Thermal (°C)	**Fraction**	**AT**	**100 °C**	**150 °C**	**200 °C**	
mL CH_4 g^{-1} COD_{added}	Solid	114 ± 9	105 ± 7	82 ± 7	53 ± 8	
	Liquid	276 ± 6	310 ± 4	220 ± 15	247 ± 10	
Ultrasound (kJ kg^{-1} TS)	**Fraction**	**SE-1**	**SE-2**	**SE-3**	**SE-4**	**SE-5**
mL CH_4 g^{-1} COD_{added}	Solid	111 ± 5	107 ± 4	103 ± 4	103 ± 5	90 ± 4
	Liquid	330 ± 16	297 ± 8	270 ± 10	312 ± 11	312 ± 13

*Average values are from three trials ± standard deviation of the mean values ($p < 0.05$).

- Liquid fraction: the methane yield obtained for this fraction ranged between 270 ± 13 mL CH_4 g^{-1} COD_{added} (for SE of 597,600 kJ kg^{-1} TS) and 330 ± 16 mL CH_4 g^{-1} COD_{added} (for SE of 24,000 kJ kg^{-1} TS), showing that an increase in the ultrasound time did not improve the solubilization of compounds which are not easily degraded.

The final values of the TVFA were very low for both the solid and liquid fraction digestates after the three pre-treatments studied, with values in the range of 5–16 mg acetic acid L^{-1}. This means that the overall anaerobic process was conducted satisfactorily and a correct balance of the process occurred. Moreover, results from the ultrasound study, and when compared with the other two pre-treatments studied, demonstrate the suitability of the ultrasonic pre-treatment of SuOC for increasing the anaerobic biodegradability of this substrate and methane yield coefficient.

The different pre-treatments used may promote methane production because the AD of SuOC without pre-treatment is a slow and difficult process which becomes acidified at a low OLR, even when the H-A and methanogenic stages are separated in two different reactors that operate in series.

Conclusions and recommendations

Although the results obtained after BMP assays suggest that SuOC was a potential substrate for AD, neither the one-stage nor the two-stage mesophilic AD process was able to efficiently degrade SuOC at an OLR higher than 2 g VS L^{-1} d^{-1}.

A temperature of 100 °C for thermal pre-treatment and an SE of 24,000 kJ kg^{-1} TS for ultrasound pre-treatment were the best conditions among those assayed, obtaining similar mean methane yields (average of the solid and liquid fractions): 208 and 220 mL CH_4 g^{-1} COD_{added}, for thermal (100 °C) and ultrasound (24,000 kJ kg^{-1} TS) pre-treatment, respectively. The energetic cost necessary to treat SuOC by thermal pre-treatment (4 h at 100 °C) is much higher than that needed for ultrasound, where only 16 min and 120 W of power are necessary. Therefore, these ultrasound conditions were chosen to conduct fed-batch experiments with pre-treated SuOC.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to the Spanish Ministry of 'Ciencia e Innovación' (Project CTM 2008–05772/Tecno) for providing financial support, to the contract JAE-Doc from 'Junta para la Ampliación de Estudios del CSIC', co-financed by European Social Funds, as well as to the Program 'FPI' of the above-mentioned Ministry for contracting graduates.

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First received 29 February 2012; accepted in revised form 31 August 2012

CAPÍTULO 4: CONCLUSIONES GENERALES

4 CONCLUSIONES GENERALES

En este capítulo se detallan las conclusiones generales obtenidas de los trabajos que forman parte del cuerpo principal de esta tesis.

[1]. El estudio relativo a la influencia del tamaño de partícula de la harina de girasol desengrasada reveló diferencias significativas sobre aspectos como la composición química y en definitiva sobre los coeficientes de rendimiento en metano acumulado de las distintas fracciones encontradas en ensayos anaerobios tipo batch. Los diámetros de partícula examinados fueron (1) 0.355-0.55mm, (2) 0.710-1.0 mm y (3) 1.4-2.0 mm. En cuanto a sus composiciones químicas, no hubo variación apreciable en la parte soluble, pero si en las fracciones sólidas, encontrando una mayor proporción de los diferentes componentes del complejo lignocelulósico en la fracción de tamaño de partícula media (2), siendo la fracción de mayor tamaño la que presentó concentraciones inferiores. Estas diferencias en composición se manifestaron en los rendimientos en metano obtenidos tras realizar los ensayos de BMP, resultando que la fracción de mayor tamaño de partícula generó el rendimiento más elevado, un 14.5% superior en relación a los tamaños de partícula (1) y (2), los cuales no mostraron diferencias significativas entre ellos.

[2]. Pretratar hidrotermalmente la harina de girasol desengrasada (SuOC) a temperaturas superiores a 100ºC alteran la composición química de la misma disminuyendo el rendimiento en metano acumulado respecto al que se obtiene con el sustrato sin pretratar. Se concluye que el pretratamiento hidrotermal a 100ºC resulta ser la mejor opción para mejorar el rendimiento en metano acumulado en el proceso de digestión anaerobia de SuOC cuando se aplica este pretratamiento en un rango de temperaturas comprendido entre 25 y 200ºC. Sin embargo, cabe destacar que el aumento en el coeficiente de rendimiento en metano del sustrato objeto de estudio con respecto al sustrato sin pretratar es de sólo un 6,5%, por lo que no compensa, desde el punto de vista energético, la realización de este pretratamiento.

[3]. Los efectos sobre la composición y la biodegradabilidad anaerobia de SuOC de distintos pretratamientos químicos y termoquímicos demostraron ser poco eficaces en cuanto a la degradación y solubilización de la composición del sustrato objeto de estudio. Tampoco se obtuvieron diferencias significativas sobre la composición del sustrato cuando se utiliza una temperatura de 75ºC a la hora de comparar pretratamientos químicos o termoquímicos. En cuanto a la generación de metano de las fracciones sólidas ensayadas, los mejores resultados se obtuvieron utilizando $Ca(OH)_2$ como reactivo, con un incremento de un 25% en el rendimiento de metano obtenido con respecto el sustrato no tratado. En el

caso de las fracciones líquidas, los resultados más destacables en cuanto a la mejoría en la producción de metano respecto al sustrato sin pretratar, fueron alcanzados al usar $Ca(OH)_2$ a 75ºC y $NaHCO_3$, obteniéndose incrementos del 37% y 11%, respectivamente. No obstante, si se analizan los resultados de forma global, es decir, los rendimientos de las fracciones sólidas y líquidas en su conjunto, se concluye que la aplicación de estos pretratamientos, tanto químicos como termoquímicos, no mejoran significativamente el rendimiento de metano del sustrato sin pretratamiento.

[4]. Los resultados obtenidos de la utilización de ultrasonidos como pretratamiento, sobre el sustrato objeto de estudio, demostraron la idoneidad del uso de energía específica (SE) de 24.000 kJ / kg TS para pretratatar SuOC, lográndose un aumento sobre la biodegradabilidad anaerobia y sobre el coeficiente de rendimiento de metano con respecto al sustrato sin pretratar. Un aumento del 53.8% en el coeficiente de rendimiento en metano global obtenido en ensayos en régimen discontinuo, pone de manifiesto la adecuación del pretratamiento ultrasónico con una SE de 24.000 kJ / kg TS para obtener un sustrato con condiciones más apropiadas para su digestibilidad mediante procesos anaerobios.

[5]. Los resultados experimentales obtenidos demuestran la estabilidad y el alto rendimiento de la digestión anaerobia en régimen semicontinuo de SuOC previamente sonicado con una SE de 24.000 kJ/kg TS usando dos lodos anaerobios diferentes como inóculo: un lodo tipo floculento (I) y otro tipo granular (II). las eliminaciones de CODs variaron ligeramente entre 67,7% y 70,1% y entre 61,3% y 67,7% con tiempos de retenciones hidráulicos (HRT) de entre 24-10 días y 24-8 días para los lodos I y II, respectivamente. Sin embargo, para HRT inferiores a 8 y 6,7 días se observó una disminución repentina en el rendimiento del proceso en ambos casos. El lodo II logró una mejor estabilización del proceso anaerobio para un rango más amplio de OLRs y HRTs, lo que permite un funcionamiento adecuado para OLRs tan alta como 3,15 g DQO / (L · d) y TRH tan baja como 6,7 días. La velocidad máxima de producción de metano alcanzada con el lodo II fue 53,6% más alta que la obtenida con el lodo I. El rendimiento global de metano obtenido con el lodo II fue un 13% mayor que el conseguido con el lodo I. Además, este valor resultó ser 1,9 veces superior al rendimiento en metano obtenido con el sustrato no tratado con energía ultrasónica.

COPIA COMPLETA DE LAS PUBLICACIONES

Reference Type: Journal biotech
Author: V. Fernández-Cegrí, F. Raposo, M. A. de la Rubia and R. Borja
Year: 2013
Title: Effects of chemical and thermochemical pretreatments on sunflower oil cake in biochemical methane potential assays
Journal: Journal of Chemical Technology and Biotechnology
Short Title: Effects of chemical and thermochemical pretreatments on sunflower oil cake in biochemical methane potential assays
ISSN: 02682575 (ISSN)
DOI: 10.1002/jctb.3922
Notes: Article in Press
Author Address: Instituto de la Grasa Consejo Superior de Investigaciones Científicas (CSIC) Seville Spain

Research Article

Received: 10 July 2012 Accepted: 6 August 2012 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jctb.3922

Effects of chemical and thermochemical pretreatments on sunflower oil cake in biochemical methane potential assays

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Abstract

BACKGROUND: The effects of chemical and thermochemical pretreatments on the composition and anaerobic biodegradability of sunflower oil cake were studied to compare these pretreatments and to assess their effectiveness. Four reagents (lime, sodium hydroxide, sulphuric acid, and sodium bicarbonate) at concentrations of 25% (w/w) of dry weight of substrate and 20 g L^{-1} substrate concentration were used for the chemical pretreatment for 4 h. The same conditions were used for thermochemical pretreatment with heating at 75°C. After the pretreatments, the solid and liquid fractions were separated and subjected to biochemical methane potential tests.

RESULTS: The methane yields of the solid fraction obtained with lime, sodium hydroxide, sulphuric acid and bicarbonate were 130±9, 54±4, 61±6 and 88±7 mL CH_4 $g^{-1}COD_{added}$, respectively, and after thermochemical pretreatment were 26±2, 84±7, 74±7, and 77±6 mL CH_4 $g^{-1}COD_{added}$, respectively. The methane yields for liquids were 152±13, 2±0, 0±0, 249±19 mL CH_4 $g^{-1}COD_{added}$, for the chemical pre-treatment, respectively, and after the thermochemical pretreatment were 273±13, 58±5, 0±0 and 145±12 mL CH_4 $g^{-1}COD_{added}$, respectively.

CONCLUSION: Only the solid fraction obtained after the chemical pretreatment with lime gave a methane yield higher (130 mL CH_4 $g^{-1}COD_{added}$) than the obtained for the untreated solid material (114 mL CH_4 $g^{-1}COD_{add}$). No thermochemical pretreatment enhanced the methane yield of the solid or liquid fractions of the untreated material.

Keywords: anaerobic digestion; biodegradable; pre-treatment; biogas; biochemical methane potential

INTRODUCTION

Biomass is considered a worldwide valuable energy alternative to fossil fuels, because it may be converted into a variety of usable forms of energy such as heat, steam, electricity, hydrogen, biogas, and liquid transportation biofuels.[1] Anaerobic digestion (AD) is widely used as an alternative energy source because various agricultural residues and other biodegradable wastes may be subjected to the AD bioprocess to produce a methane-rich biogas that is suitable for energy production. In the industrial processing of sunflower seeds into edible oil, large quantities of solid wastes called sunflower oil cake (SuOC) are generated. SuOC is considered to be of relatively poor quality due to high concentrations of lignocellulosic compounds. The relatively poor quality of SuOC restricts the amount that can be included in feed blends for ruminant animals. Lignocellulosic biomass is difficult to degrade biologically and consists of three main biopolymers: cellulose, hemicelluloses, and lignin. In this type of substance, cellulose is physically associated with hemicelluloses, and physically and chemically associated with lignin. Lignin and hemicelluloses are intermeshed and chemically bound through covalent cross-linkages such as ester or ether linkages. The low biodegradability (BD) of lignocellulose in biogas reactors is due to lignin, which is not degradable in anaerobic environments because the extracellular enzymes require oxygen to depolymerize them. Furthermore, the hydrolysis of cellulose in lignocellulosic materials is reduced by lignin and hemicelluloses, since these components act as a protective coating, making the cellulose resistant to enzymatic digestion.[2] Due to the refractory structure of these compounds, one of the major problems in utilizing SuOC and other vegetable crop residues for methane production by AD is their low digestibility. AD and hence the methane potential of a complex substrate depends on the content of biodegradable compounds: carbohydrates (including cellulose), proteins and lipids. The AD efficiency of lignocellulosic biomass can be improved by applying several pretreatment methods. In general, the limiting step of solid waste AD is the first step, hydrolysis, where the cell wall is broken, making the organic matter inside the cell available for biological degradation. Some pretreatments have been developed in order to achieve the release of lignocellulosic material and thus accelerate the degradation process by means of waste solubilization. In order to increase methane production, the pretreatment options hydrolyse the cell wall.[3] So, it is necessary to carry out a pretreatment step to break

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the lignin seal, thus exposing the cellulose and hemicellulose to enzymatic action.[4] Pretreatment also aims to decrease the crystallinity of cellulose, increase biomass surface area and remove hemicellulose. The removal of hemicellulose increases the mean pore size of the substrate and therefore increases the probability of hydrolysing the cellulose.[4] Pretreatments should not only substantially improve the accessibility of the biomass to enzymes in the subsequent hydrolysis, but also avoid intensive carbohydrate loss or degradation during the process. Acid and alkaline pre-hydrolysis are the two most intensively studied chemical methods in the pretreatment of lignocellulosic biomass. Acid pretreatment results in disruption of covalent bonds, hydrogen bonds, and Van der Waals forces that hold together the biomass components, which, consequently causes the solubilization of hemicellulose and the reduction of cellulose crystallinity.[5] Rather than treating lignocellulose using concentrated acid, diluted acid pretreatments are normally practised at high temperatures to improve cellulose hydrolysis.[6] In contrast, alkaline pretreatment causes delignification of the biomass and makes the lignocellulose swell through saponification reactions. Unlike acid pretreatment, alkaline pretreatment has been proven effective within a wide temperature range at various chemical concentrations. Sodium hydroxide (NaOH) and lime ($Ca(OH)_2$) are the two alkaline reagents that have attracted the most attention. Several authors studied a pretreatment using NaOH and verified that the removal rate of lignin generally increased with increase in pretreatment severity. Some results show that, compared with NaOH pretreatment, the delignification capability of $Ca(OH)_2$ is much lower, which could be because divalent calcium ions from $Ca(OH)_2$ dissociation have a high affinity to lignin and can effectively crosslink lignin molecules, thus preventing them from solubilization under alkaline attack. The objective pursued by the inclusion of a pretreatment alternative is to modify the structure of complex materials with decreasing degrees of polymerization, to weaken the links of lignin to carbohydrates, and to increase the surface area of the particles that constitute the substrates.

The goal of this study was to examine the effects of chemical pretreatment on a BMP test of SuOC, with acid and basic reagent. The same conditions except for the application of simultaneous thermal energy were also analysed with the aim of comparing the effects of chemical and thermo-chemical pretreatments on the methane yield coefficients.

MATERIALS AND METHODS

Raw material

The SuOC sample used in this study was collected from a sunflower oil factory located near Seville (Spain). Prior to use, the substrate was shredded into different particle sizes using a commercial sieve (Restch AS 200 basic) and different screen meshes. In order to ensure the homogeneity of the sample, the most abundant particle size of this substrate (0.71 – 1.0 mm diameter) was selected to carry out the experiments.

The full composition and main features as well as the fractional composition of the fibre of the above-mentioned SuOC particle size selected are as follows (mean values of four determinations ± standard deviations): COD_{total}, 1.24 (±0.02) g O_2 g^{-1} TS dry basis; Total solid dry basis (TS), 93.0 (±0.1)%; volatile solids (VS) expressed as dry basis, 93.0 (±0.1)%; ash, 6.8 (±0.1)%; neutral detergent fibre (NDF), 45.0 (±1.1)%; acid detergent fibre (ADF), 38.4 (±0.9)%; acid detergent lignin (ADL), 13.3 (±0.2)%; hemicellulose, 6.6 (±1.0)%; cellulose, 25.3 (±0.4)%; total protein, 25.3 (±0.8)%; fat content, 1.6 (±0.2)%; soluble carbohydrates, 5.1 (±0.2)% and total carbohydrates (by difference), 53.0 (±0.3)% (% expressed as TS dry basis).

Chemical and thermochemical pretreatments

The SuOC was treated with alkaline reagents lime ($Ca(OH)_2$), sodium hydroxide (NaOH), sodium bicarbonate ($NaHCO_3$) and acid reagent, sulphuric acid (H_2SO_4). The conditions of pretreatments were chosen based on a previous study carried out using different concentrations of reagents, times, and temperatures with the objective of choosing the most efficient operational conditions on this substrate. The effectiveness of the different conditions assayed was assessed through soluble chemical oxygen demand (CODs). The final conditions selected after the previous assays were 25% (w/w) of substrate of wet weight for the concentration of the reagents, 4 h hydrolysis and 20 g L^{-1} SuOC. The selected temperature for the thermochemical pretreatment was 75°C. Each suspension was treated under static conditions to avoid applying a mechanical pretreatment. The total sample was separated after pretreatment into the liquid supernatant fraction and the residual solid phase. Both fractions were separated by centrifuging the sample for 15 min at 10 000 rpm and after the liquid phase were filtered through a glass filter of 1.2 µm and then through 0.45 µm pore size filters to remove the colloidal solids and to produce the soluble pre-treated fraction. For the thermochemical pretreatment the same conditions were applied but with simultaneous heating at 75°C using a heater plate and controlling the temperature with a thermometer. In this way, it was possible to study the effect of temperature while maintaining the above mentioned experimental conditions. Subsequently, characterization of both fractions was performed: (i) liquids: pH, CODs and alkalinity; (ii) solids: total chemical oxygen demand (COD_T), total and volatile solids (TS and VS) and fibre composition.

These results were compared with the results obtained with the untreated substrate (control) prepared with the same concentration, 20 g L^{-1} , with distilled water, 4 h under static conditions and at room temperature and it was not pre-treated with any chemicals. Prior to the anaerobic biodegradability experiments, chemical supplementation of the liquid fractions was required (with H_2SO_4 98% (w/v) for the basic solutions and NaOH 50% (w/w) for the acid solutions) in order to limit the impact of pH on the system. The pH was adjusted to 7.5.

Inoculum

Granular sludge taken from an industrial anaerobic reactor, which treats wastewater from a brewing company, operating at mesophilic (35°C) conditions, was used as inoculum. This inoculum was selected due to its high methanogenic activity. The characteristics and features of the anaerobic sludge used were: pH 7.6±0.1, 85 g L^{-1} TS and 44 g L^{-1} VS.

Anaerobic digestion experiments

Anaerobic biodegradability of the pre-treated SuOC (liquid and solid fractions) obtained through different pretreatments was evaluated by biochemical methane potential (BMP).

The experimental study was carried out in a multi-batch reactor system, which consisted of nine Erlenmeyer flasks, with an effective volume of 250 mL. They were continuously stirred with magnetic bars at 300 rpm and placed in a thermostatic water bath at mesophilic temperature (35±1°C). Both fractions, solid and liquid, for each pretreatment studied, chemical and thermo-chemical,

were digested in assays performed in triplicate. Triple positive control reactors with starch as the control blanks were also carried out. All reactors were initially charged with anaerobic inoculum by maintaining a concentration of 15 g VS L^{-1} (the volume taken is a function of the initial VS concentration of the inoculum). The inoculum to substrate ratio (ISR) was maintained at 2 (VS basis) for the reactors digesting the solid fractions and at 2.5 (COD basis) for the reactors processing the liquid fractions.[7] After the pre-treated substrate was added to each reactor, 25 mL of a stock mineral medium solution were also added (the composition has been described elsewhere).[8] Finally, distilled water was added to achieve the desirable effective volume and the reactors were flushed with N_2 in order to maintain anaerobic conditions. The methane released was measured by volume displacement (carbon dioxide was previously removed by flushing the gas through a 2 N NaOH solution), and expressed at standard temperature and pressure (STP) conditions. The methane production due to biomass decay and the possible presence of residual substrate in the inoculum was subtracted by performing blank controls. The BMPs assayed were run between 7 and 10 days, until the accumulated gas production remained essentially unchanged (on the last day, production was lower than 2% of the accumulated methane produced), suggesting that biodegradation had been completed. This short period of time was sufficient to achieve maximum methane production, and can basically be explained by the high methanogenic activity of the sludge and the short interval (less than 72 h) had elapsed between inoculum sampling and the start-up of the experiments.

Analytical methods

Solid samples

The following parameters were analysed in the original solid substrate (SuOC): TS and VS, according to standard methods 2540B and 2540E, respectively.[9] CODt was determined using the method proposed by Raposo *et al*.[10] Fat content was extracted with hexane, using a Soxhlet system. Fibre analysis was done according to Van Soest *et al*.[11] using the gravimetric method with a Dosi Fiber (Selecta®) equipment and crucibles of 40–100 µm pore size. For neutral detergent fibre (NDF) heat stable α-amylase and anhydrous sodium sulphite were used.[12] The acid detergent fibre (ADF) was done non-sequentially while the acid detergent lignin (ADL) was determined sequentially. The analyses were carried out in order to calculate hemicellulose (NDF – ADF), cellulose (ADF – ADL) and lignin (ADL). The results were expressed as VS.

Soluble fractions

The soluble pre-treated fraction obtained was characterized using the following soluble parameters: CODs, using the closed digestion and colorimetric Standard Method 5220D;[9] total alkalinity (TA) measured by pH titration to 4.3; soluble ammoniacal nitrogen $(NH_x)_s$ determined by distillation and titration according to the Standard Method 4500E;[9] volatile fatty acid (VFA) concentration analysed by gas chromatography, as previously described.[13]

Inoculum and digestates

Both the inoculum and digestates were characterized by direct sampling. pH was determined using a pH-meter model Crison 20 Basic. TA, TS and VS were also analysed in these samples.

RESULTS AND DISCUSSION

In this work, the pretreatment efficiencies were evaluated with respect to the composition of SuOC and the hydrolysis yield.

Effect of pretreatments on solubilizations and fibre composition

Solubilization is just one of the indicators for evaluating the effect of the pretreatments along with the destruction of the lignocellulosic structure.[14]

Organic material solubilizations

The solubilization of organic material (OM)s, in terms of soluble chemical oxygen demand CODs, for the different pretreatments is shown in Fig. 1. The results obtained for the different pretreatments were compared with those for the untreated substrate. NaOH pretreatment produced the highest solubilization of SuOC in relation to the other pretreatments, with values of CODs of 9000 mg O_2 L^{-1}. The other reagents studied gave lower solubilization and no significant differences were observed among them, with values ranging from 3300 to 4000 mg O_2 L^{-1}. For the thermochemical pretreatment, the results of solubilization ranged between 4800 and 6000 mg O_2 L^{-1}, approximately. In the case of NaOH, there were no significant differences between the chemical and thermochemical pretreatments. However, for the rest of the reagents there was an important statistical difference between the two types of pretreatment for each reagent, especially for H_2SO_4.

The compositional changes observed after the pretreatments were carried out are summarized in Table 1. It was observed that COD_T after the pretreatments was very similar to that of the untreated material but it can be observed that the alkali reagents were more effective in degrading the total organic material than the acid reactant.

Fibre composition

With respect to fibre composition, in general it was observed that the SuOC lost significant amounts of hemicellulose after all the pretreatments except with $NaHCO_3$. The most effective pretreatment for solubilization of hemicellulose was the thermo-acid pre-treatment, with an 84% reduction with respect to the untreated material. Without temperature, again the acid, along with NaOH, were the best reagents for OM solubilization, with a 62% reduction. The relative content of cellulose and lignin increased in all cases except when $Ca(OH)_2$ was used, for which the lignin decreased noticeably by 19% in both types of pretreatments. In addition, the cellulose suffered a reduction of 18 and 11% with respect to the untreated material with and without temperature, respectively. In contrast, it is important to note the increased value in the relative content of the cellulose fraction, the component more available for hydrolysis, with increases of 25% and 46% when NaOH was used in the chemical and thermochemical pretreatments, respectively, while with $NaHCO_3$ reductions of 21 and 11% were observed with and without temperature, respectively. On the other hand, it can be confirmed that H_2SO_4 was not capable of dissolving cellulose, maintaining the same proportion as in the untreated case with this substrate. In this study, alkali pretreatment gave higher removals of lignin in comparison with other reagents, regardless of the effect of temperature. The acid pretreatment proved very effective in removing hemicellulose, in this case with a significant difference statistically, when temperature is applied, eliminating almost all the hemicellulose.

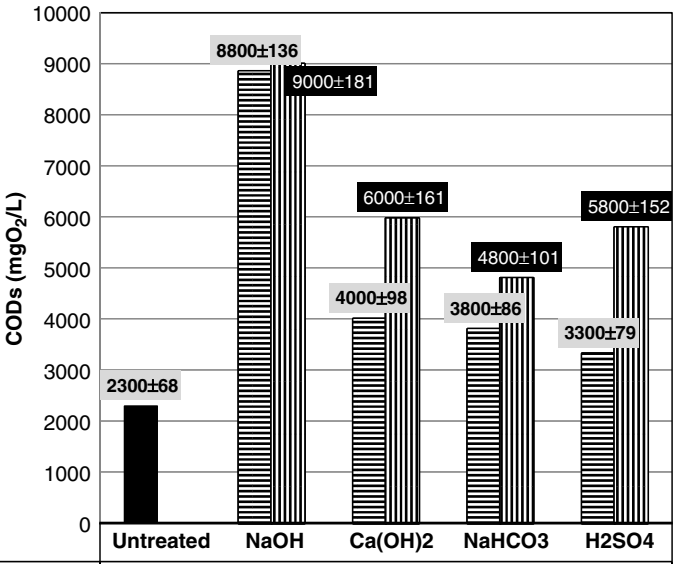

Figure 1. Effects of the different chemical and thermochemical pretreatments studied on the CODs (mg O_2 L^{-1}) of substrate tested.

Table 1. Composition of SuOC after the different chemical and thermochemical pretreatments assayed. (%, volatile solid)*

		Cellulose (%)*	Hemicellulose (%)*	Lignin (%)*	COD_T (mg g^{-1} TS)	Ash (%)	pH	Total alkalinity (mg $CaCO_3 L^{-1}$)	Ammoniacal nitrogen (mg $NH_x L^{-1}$)
Untreated		28	13	16	1.24±0.02	7.0±0.1	7.0±0.1	2.50±0.05	8±1
Chemical pretreatment	NaOH	35	5	19	1.16±0.03	18.0±0.1	12.5±0.1	5.36±0.05	62±2
	$Ca(OH)_2$	25	8	13	1.12±0.02	14.8±0.1	12.0±0.1	2.28±0.05	76±2
	H_2SO_4	28	5	16	1.26±0.04	2.0±0.1	1.3±0.1	2.23±0.05	65±1
	$NaHCO_3$	31	15	15	1.26±0.02	4.0±0.1	8.2±0.1	2.84±0.05	73±2
Thermochemical pretreatment	NaOH	41	8	19	1.18±0.03	16.3±0.1	13.4±0.1	5.48±0.05	78±4
	$Ca(OH)_2$	23	7	13	1.03±0.02	36.9±0.1	11.9±0.1	1.84±0.05	67±2
	H_2SO_4	30	2	17	1.35±0.01	1.0±0.1	1.0±0.1	2.30±0.05	67±1
	$NaHCO_3$	34	14	16	1.37±0.02	6.7±0.1	8.0±0.1	2.72±0.05	71±2

However, although the same levels of solubilization were observed with H_2SO_4, $Ca(OH)_2$ and $NaHCO_3$, these pretreatments provided many differences in fibre composition. This is because each type of reagent attacks different parts of the substrate. Chemical treatments have different effects depending on the reagent used; in the case of acid reactants, hydrolysis of the hemicellulose takes place. The alkali treatment breaks the links between lignin monomers or between lignin and polysaccharides.[15]

Xie *et al*.[16] observed that for dried grass silage pre-treated at different NaOH loading rates (1%, 2.5%, 5% and 7.5% by volatile solids and at 100°C), up to 45% of the total COD was solubilized and up to 65.6%, 36.1% and 21.2% of lignin, hemicellulose and cellulose were removed, respectively.

Rajan *et al*.[17] studied different types of alkaline agents such as NaOH, $Ca(OH)_2$ on waste activated sludge and concluded that sodium hydroxide gave better results in terms of CODs with small doses. Also, in the case of activated sludge, solubilization increases to above 46%, solubilizing the particulate material into nitrocellulose-soluble organic carbon.[18] In this case, the chemical pretreatment assay resulted in a level of solubilization of up to 46%, adding between 5 and 40 meq L^{-1} NaOH. Using NaOH, higher levels of solubilization were observed when compared with other alkalis such as $Ca(OH)_2$.

Effect of pretreatments on SuOC methane yield

For the analysis of methane yields, solid and liquid fractions were analysed independently.

Solid fraction

As shown in Fig. 2, it can be observed that pretreatment with $Ca(OH)_2$ produced the highest yield of methane, with 130.5 mL CH_4 g^{-1} COD_{added}, 14% higher than that obtained for the untreated substrate. $Ca(OH)_2$ was the unique reactant among the different reagents studied that increased the final methane yield of the solid fraction compared with the untreated material. With these results, it is logical to observe that after pretreatment the solid fraction

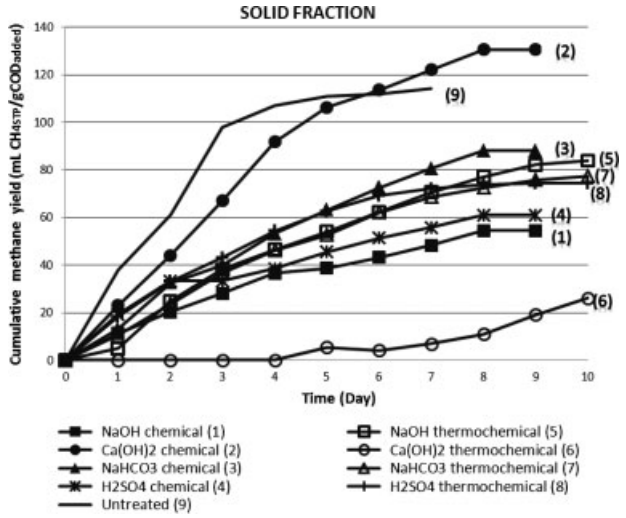

Figure 2. Cumulative methane yield (mL CH_4STP $g^{-1}COD_{added}$) for the solid fractions.

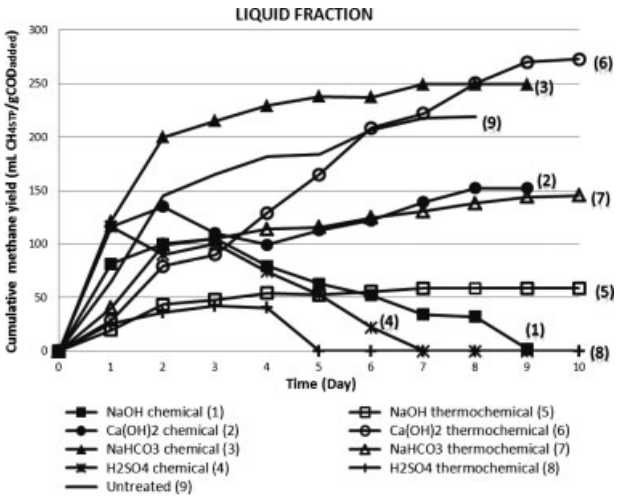

Figure 3. Cumulative methane yield (mL CH_4STP $g^{-1}COD_{added}$) for the liquid fractions.

is drained. It is important to note that the same reagent but at 75°C gave the worst result in this fraction. To justify this, Xu *et al.*[19] reported a decrease in lignin solubilization because the treatment with $Ca(OH)_2$ caused an interaction between negatively charged lignin molecules and positively charged calcium ions. This can be explained by the formation of calcium–lignin complexes at higher temperature.[20]

Inhibition in the process was observed with $Ca(OH)_2$ during thermochemical pretreatment. Acetic and isovaleric acid concentrations of 1322 and 125 mg L^{-1}, respectively, were observed in the digestates at the end of the BMP process. The rest of the pretreatments generated similar amounts of gas, with a methane yield of between 15 and 48% lower than for the untreated material. This fact confirms the exhaustion of the substrate in the solid fraction after the pretreatments.

Liquid fraction

As shown in Fig. 3, the opposite situation was found in the case of $Ca(OH)_2$. Pretreatment at higher temperature gave the best result, with methane yields reaching 24.6% higher than with the untreated material. The next highest result obtained was with $NaHCO_3$ without temperature, with 14% more methane production than for the untreated material. Regarding NaOH and H_2SO_4 with and without temperature, both presented a clear inhibition for the liquid fraction. Rice straw was pre-treated with NaOH in solid-state conditions and anaerobically digested[21] with four NaOH doses (4%, 6%, 8%, and 10%) and four loading rates (35, 50, 65, and 80 g L^{-1}). These authors observed that after pretreatment with NaOH, the biogas yield reached 3.2–28.6%, 27.3–64.5%, 30.6–57.1%, and 15.2–58.1% higher than that obtained for the untreated rice-straw at the respective loading rates. Dried grass silage was pre-treated by Xie *et al.*[16] at different NaOH loading rates (1%, 2.5%, 5% and 7.5% of volatile solids (VS) mass in grass silage) and temperatures (20°C, 60°C, 100°C and 150°C) to determine their effects on its bio-degradability in terms of the hydrolysis yield. At 100°C and for the four NaOH loadings, the BMP productions obtained were 359.5, 401.8, 449.5 and 452.5 mL CH_4 g^{-1} VS_{added}, respectively, with an improvement of 10–38.9% in comparison with the untreated substrate. Sodium hydroxide (NaOH) was used by Pang *et al.*[22] to pre-treat corn stover in a solid state at ambient temperature to improve biodegradability and anaerobic biogas production with four NaOH doses of 4%, 6%, 8%, and 10% on dry matter basis of the substrate and at four loading rates of 35, 50, 65, and 80 g L^{-1}, respectively. The results showed that 6% NaOH-treated corn stover digested at the loading rate of 65 g L^{-1} achieved 48.5% more biogas production than the untreated material.

Other researchers studied the improvement in biogas production from cattle manure with $Ca(OH)_2$ pretreatment, while observing the effects of temperature (20°C and 60°C), time (10 min, 2, and 12 h), and pH (9, 10, 11, and 12).[23] The results showed that alkaline pretreatment at 20°C did not affect biogas production, while the manure treated at 60°C produced more methane than the untreated one. The maximum improvement in methane production was achieved with a pretreatment at pH 12 for 12 h, which resulted in a methane yield of 225 mL CH_4 g^{-1} VS, which was 76% higher than that obtained from untreated manure. Results contrary to those obtained by these authors were achieved by Antonopoulou *et al.*[24] who studied the anaerobic digestion process of different sunflower residues with chemical pretreatment methods such as thermal, chemical (with NaOH and H_2SO_4 addition 2%w/v) or a combination of the two. The results obtained in this case demonstrated that the pretreatment methods tested did not enhance the methane potential of the sunflower residues.

CONCLUSIONS

The effects on composition and anaerobic biodegradability of SuOC under different chemical and thermochemical pretreatments were studied in this work. Regarding changes in composition of SuOC, it can be concluded that the pretreatments assayed did not result in increased degradation when applying additional thermal energy. Regarding the generation of methane for each solid fraction assayed, the best results were achieved with $Ca(OH)_2$ without temperature, with an increase of 25% in the methane yield compared with the untreated substrate. For the liquid fractions, the best results of methane production were reached with $Ca(OH)_2$ and temperature and $NaHCO_3$ without the effect of temperature, with increases of 37% and 11%, respectively,

compared with the untreated material. The high amounts of methane achieved with $NaHCO_3$ can be explained by the alkalinity generated when using this reagent, which favours and stabilizes the anaerobic process. In any case, it can be concluded that although the reagents used in the pretreatments changed the composition of the material, the potential toxicity of these chemical reagents in the anaerobic biomass can also affect the global anaerobic process, hindering the generation of methane from SuOC. Taking into account the overall results considering both fractions, solid and liquid, the highest methane yield was achieved with $Ca(OH)_2$ without heating, 141±11 mL CH_4 $g^{-1}COD_{added}$, but this value was lower than that obtained from the untreated material (195±7 mL CH_4 $g^{-1}COD_{added}$). The results show that the pretreatments applied to SuOC did not enhance the methane potential of this substrate.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to the Spanish Ministry of 'Ciencia e Innovación' (Project CTM 2008-05772/Tecno) for providing financial support and to the Program 'FPI' of the above-mentioned Ministry for contracting graduates.

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Reference Type: Journal biotech
Author: V. Fernández-Cegrí, M. Ángeles de la Rubia, F. Raposo and R. Borja
Year: 2012
Title: Effect of hydrothermal pretreatment of sunflower oil cake on biomethane potential focusing on fibre composition
Journal: Bioresource Technology
Volume: 123
Pages: 424-429
Short Title: Effect of hydrothermal pretreatment of sunflower oil cake on biomethane potential focusing on fibre composition
ISSN: 09608524 (ISSN)
DOI: 10.1016/j.biortech.2012.07.111
Author Address: Instituto de la Grasa, Consejo Superior de Investigaciones Científicas (CSIC), Avenida Padre García Tejero, 4, 41012 Seville, Spain

Bioresource Technology 123 (2012) 424–429

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Effect of hydrothermal pretreatment of sunflower oil cake on biomethane potential focusing on fibre composition

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HIGHLIGHTS

- Hydrothermal pre-treatment at 100 °C is an option for improving the AD (anaerobic digestion) of SuOC (sunflower oil cake).
- $T > 100$ °C worsen the chemical composition of pre-treated SuOC, decreasing the CH_4 yield.
- The kinetic constants of the AD process of the pre-treated solid fraction are related to the lignin concentration.
- Lignin content increases with pre-treatment temperature, while the kinetic constant decreases.

ARTICLE INFO

Article history:
Received 6 January 2012
Received in revised form 30 June 2012
Accepted 30 July 2012
Available online 8 August 2012

Keywords:
Biochemical methane potential
Hydrothermal pretreatment
Lignocellulose
Sunflower oil cake

ABSTRACT

The aim of this study was to elucidate the effect of hydrothermal pretreatment at 25, 100, 150 and 200 °C on fibre composition and the biomethane potential of sunflower oil cake (SuOC). An increase in pretreatment temperature from 25 to 200 °C caused a decrease in hemicellulose content in the solid pretreated fraction from 13 to 6% while the lignin content increased by 16%. Soluble compounds also increased with temperature. Digestion of solid fractions from pretreatments at 25, 100, 150 and 200 °C in batch assays at 35 ± 1 °C resulted in methane yields of 114 ± 9, 105 ± 7, 82 ± 7 and 53 ± 8 mL CH_4 $g^{-1}COD_{added}$, respectively. The corresponding methane yields for the liquid fractions were 276 ± 6, 310 ± 4, 220 ± 15 and 247 ± 10 mL CH_4 $g^{-1}COD_{added}$, respectively. Therefore the overall methane yield was highest for SuOC pretreated at 100 °C; however, this value was only 6.5% higher than that achieved after pretreatment at 25 °C.

1. Introduction

Agro-food wastes, such as sunflower oil cake (SuOC), a by-product of sunflower oil extraction, provide an inexpensive feedstock for biological conversion to biogas by anaerobic digestion (AD) (Antonopoulou et al., 2010); however, the effectiveness of this technology in treating this kind of residue is limited because of the complex structure of lignocellulosic biomass which is resistant to AD. Although cellulose and hemicellulose can be degraded under anaerobic conditions, indigestible lignin which is connected to cellulose by hemicelluloses (Laureano-Perez et al., 2005) prevents access of enzymes to the carbohydrates (Zhu et al., 2008). Therefore, pretreatment of lignocellulosic biomass is necessary to remove lignin and to make cellulose more accessible to the enzymes that convert carbohydrate polymers into fermentable sugars (Mosier et al., 2005).

Physical, physico-chemical, chemical, and biological processes have been used for pretreatment of lignocellulosic materials (He et al., 2008; Taherzadeh and Karimi, 2008), not only for removing the inhibitory lignin complex but also for reducing cellulose crystallinity, which is a major limitation for cellulose hydrolysis (Jeihanipour et al., 2010). However, most of these studies have been carried out with the goal of producing ethanol and only a few studies have investigated biogas production (Kumar et al., 2011; Teghammar et al., 2010).

Conventional chemical pretreatment using acids or alkalis negatively impact process costs and the environment and complicate waste disposal (Bordeleau and Droste, 2011). Thus, hydrothermal treatments are promising alternatives to chemical treatments (Pérez et al., 2008).

During hydrothermal pretreatment, water under high pressure can penetrate into the biomass, hydrating cellulose and removing most of the hemicellulose and part of the lignin (Taherzadeh and Karimi, 2008; Pérez et al., 2007). At high temperatures, water lowers pH and enables release of O-acetyl, acetic and uronic acids from hemicellulose (Pérez et al., 2007). The most significant drawback of high temperatures is the formation of phenolic compounds and furan derivatives (furfural and hydroxymethylfurfural-HMF) that are undesirable because they not only represent a loss of fermentable

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http://dx.doi.org/10.1016/j.biortech.2012.07.111

sugars, but are also inhibitors of bacteria and *Archaea* (Negro et al., 2003). Therefore, conditions must be determined in each case to optimise hydrothermal pretreatments.

The objective of the present work was to optimise the hydrothermal pretreatment of SuOC for methane production and to study the effect of pretreatment temperature on fibre and chemical composition of the solid and liquid fractions. Furthermore, a first-order kinetic model was used to obtain the specific rate constants of the batch anaerobic digestion processes.

2. Methods

2.1. Hydrothermal pretreatment

Hydrothermal pretreatment of SuOC was carried out in closed 40-mL Pyrex glass cylinders kept in a thermo-reactor with temperature control. Twenty-five mL of a suspension of 20 g L^{-1} SuOC in distilled water was added to every cylinder. After hydrothermal pretreatment, the glass cylinders were cooled to ambient temperature in a water bath. A total of 20 g of SuOC were pretreated at each temperature assayed to obtain sufficient amounts of solid and liquid fractions to perform BMP assays.

The experimental settings of hydrothermal pretreatment were chosen from the best conditions obtained during a preliminary study carried out by determining SuOC solubilisation at two different concentrations (20 g L^{-1} and 40 g L^{-1}), eight temperatures (25, 50, 75, 100, 125, 150, 175 and 200 °C) and four pretreatment times (1, 2, 4 and 6 h). The aim of the experiment carried out at ambient temperature (AT) was to determine the solubilisation capability of water with no rise in temperature. A concentration of 20 g L^{-1} and digestion time of 4 h were selected on the basis of the preliminary assays.

The solubilisation of organic matter was determined to evaluate the transfer of the SuOC solid fraction to the hydrolysate. The solubilisation was expressed as a percentage, following the equation:

$$\text{Solubilisation } (\%) = (S_S - S_{S0}/S_i) \times 100 \quad (1)$$

where S_S and S_{S0} are the soluble hydrolysate concentrations expressed as grams of soluble chemical oxygen demand (CODs), measured in hydrothermally pretreated SuOC and pretreated SuOC at AT, respectively; S_i is the initial total substrate concentration (expressed as grams of total COD–COD_T), measured in untreated SuOC.

Wet material was vacuum filtered through a 0.45 μm filter to obtain a water-insoluble solid fraction and a liquid (prehydrolysate) fraction. The corresponding solid and liquid fractions after each pretreatment were subjected to separate batch anaerobic digestions by carrying out BMP tests as described by Kaparaju et al. (2009).

2.2. Raw material and inoculum used in the BMP assays

SuOC was collected from a sunflower oil factory located near Seville (Spain). The substrate was shredded into different particle sizes using a commercial sieve (Restch AS 200 basic) and different screen meshes. The most abundant particle size of this substrate (0.71–1.0 mm diameter) was selected for the hydrothermal pretreatment experiments. The composition of SuOC without pretreatment has been described elsewhere (De la Rubia et al., 2011).

The inoculum used in the BMP assays was obtained from an industrial anaerobic reactor treating brewery wastewater and operating at mesophilic (35 °C) conditions. This inoculum was selected due its high methanogenic activity as determined previously (Rincón et al., 2011). The main characteristics of this digested sludge are: pH 7.6 ± 0.1, 119 g L^{-1} of total solids (TS) and 75 g L^{-1} of volatile solids (VS).

2.3. Batch anaerobic digestions

The digesters were glass Erlenmeyer flasks with 300 mL total volume organised as a multiflask batch system.

The inoculum to substrate ratio (ISR) was 2 (VS basis for solid fraction and VS/COD basis for liquid fraction). For each flask containing 50 mL of inoculum (with a final concentration of 15 g VS L^{-1}), solid pretreated SuOC or liquid hydrolysate were added together with stock mineral medium solution and distilled water to a working volume of 250 mL. The mineral medium solution was composed of buffer, ($NaHCO_3$, 5000 mg L^{-1}); macronutrients (NH_4Cl, 280 mg L^{-1}; K_2HPO_4, 250 mg L^{-1}; $MgSO_4{\cdot}7H_20$, 100 mg L^{-1}; $CaCl_2{\cdot}2H_2O$, 10 mg L^{-1}; yeast extract, 100 mg L^{-1}) and micronutrients, ($FeCl_2{\cdot}4H_2O$, 2 mg L^{-1}; $CoCl_2{\cdot}6H_2O$, 2 mg L^{-1}; $MnCl_2{\cdot}4H_2O$, mg L^{-1}; $AlCl_3{\cdot}6H_2O$, 0.09 mg L^{-1}; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.05 mg L^{-1}; H_3BO_3, 0.05 mg L^{-1}; $ZnCl_2$, 0.05 mg L^{-1}; $CuCl_2{\cdot}2H_2O$, 0.038 mg L^{-1}. Inoculum supplemented with nutrients and distilled water were used as a blank to determine gas production by the inoculum itself. BMP tests with starch as substrate were also carried out as positive controls (Raposo et al., 2012).

The headspace of each bottle was flushed with nitrogen. The reactors were continuously stirred with magnetic bars at 300 rpm and placed in a thermostated water bath at 35 ± 1 °C. The gas released was passed through a 2 N NaOH solution to capture CO_2; the remaining gas was assumed to be methane. The digestion experiments were run for 7–8 days until the accumulated gas production remained essentially unchanged (on the last day, production was lower than 2% of the accumulated methane produced). Each experiment was performed in triplicate.

2.4. Analytical methods

TS and VS were determined according to standard methods 2540B and 2540E (APHA-AWWA-WPCF, 1998), respectively; COD_T was determined by the method described by Raposo et al. (2008). To determine the total Kjeldahl nitrogen (TKN), 1000 mg of sample was acidified with 15 mL concentrated H_2SO_4. In addition, 5 g catalyst [(Cu–Se) (1.5% $CuSO_4{\cdot}5H_2O$ + 2% Se)] was added, and the sample was digested sequentially in a thermoblock for 15 min at 150 °C, 15 min at 250 °C and 90 min at 390 °C. After cooling, the sample was diluted with 10 mL distilled water, neutralised with 12.5 N NaOH and distilled in 50 mL of indicator mix. The solution was titrated with 0.02 N H_2SO_4. Total protein was determined by multiplying the TKN value by 5.5 (Mossé, 1990). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined so as to calculate hemicellulose (NDF–ADF), cellulose (ADF–ADL) and lignin (ADL) according to van Soest et al. (1991) with slight modifications. To determine NDF, 1000 mg of dried sample was boiled in a sintered glass crucible (40–100 μm pore size) with 100 mL of a neutral detergent solution composed by 30 g L^{-1} of Sodium Dodecyl Sulphate, 18.6 g L^{-1} Ethylenediaminetetraacetic Acid Disodium Salt 2-hydrate, 6.8 g L^{-1}di-Sodium tetra-Borate 10-hydrate, 4.6 g L^{-1}di-Sodium Hidrogen Phosphate anhydrous and 10 mL L^{-1} Triethylene Glycol, together with 1 g of sodium anhydrous sulphite to remove proteins and 200 μL of α-amylase (heat-stable solution, for use in total dietary fibre assay, TDF-100A from Sigma–Aldrich), to eliminate starch, for 1 h. Neutral detergent was removed and the sample washed with 100 mL of distilled boiling water. The sample was washed with 50 mL of acetone and dried at 105 °C overnight in an oven and weighed. Corrections for residual protein and ash were made, determining the remaining content of TKN and mineral solids. ADF was determined by non-sequential fibre analysis. Dried sample (1 g) was heated with 100 mL of a solution of 2% N-acetyl-N,N,N-trimethyl ammonium bromide in 1 N H_2SO_4 to boiling for 1 h in a sintered glass crucible (40–100 μm pore size). ADF was recovered by filtration,

washed with 100 mL of distilled boiling water, followed by 50 mL of acetone. The sample was dried overnight at 105 °C and weighed. The weight was corrected for ash and protein. To determine ADL, 250 mg of sample obtained after ADF analysis was stirred for 3 h with 25 mL of H_2SO_4 (72% w/w). The sample was placed in a sintered glass crucible (40–100 μm pore size) and washed with 100 mL of distilled water and dried at 105 °C in an oven overnight and weighed. Correction for ash was made.

The inoculum and digestates were characterised by measuring: pH (using a pH-metre model Crison 20 Basic), total alkalinity (TA) by pH titration to 4.3, and TS and VS (APHA-AWWA-WPCF, 1998).

The hydrolysate obtained after each hydrothermal pretreatment as well as the digestates (centrifuged at 8000*g* for 15 min and filtered through a 0.45 μm filter) were characterised with respect to soluble chemical oxygen demand (CODs), using the closed digestion and colorimetric standard method 5220D (APHA-AWWA-WPCF, 1998); carbohydrates, according to the colorimetric method described by Dubois et al. (1956); TA, by pH titration to 4.3; ammoniacal nitrogen (NH_x), by distillation and titration according to standard method 4500E (APHA-AWWA-WPCF, 1998). The volatile fatty acid (VFA) concentration was measured using a gas chromatograph, as previously described (De la Rubia et al., 2009).

2.5. Kinetic model

To evaluate the effect of hydrothermal pretreatment of SuOC on methane yield, a first-order kinetic model, frequently applied to anaerobic digestion systems (Hashimoto, 1986), was used to correlate the methane yield with the digestion time.

$$B = B_0 \cdot [1 - exp(-k \cdot t)] \quad (2)$$

where B (mL CH_4 g^{-1} COD_{added}) is the cumulative methane yield at a time t, B_0 (mL CH_4 g^{-1} COD_{added}) is the maximum or ultimate methane yield of the substrate, k ($days^{-1}$) is the specific rate or apparent kinetic constant and t (days) is the time. Therefore, the ultimate methane yield gives the final value when no more volume of gas from the reactor is released. The adjustment by non-linear regression of the pairs of experimental data (B, t) using the Sigmaplot software (version 11.0) allowed the calculation of the apparent kinetic constant (k).

3. Results and discussion

3.1. Effect of pretreatment on solid and liquid fraction compositions

The components analysed in the solid fractions to determine the effect of temperature after hydrothermal pretreatment were cellulose, hemicellulose and lignin (Table 1). Hemicellulose remained in the solid fraction after pretreatment at AT and at 100 °C, but at temperatures above 150 °C hemicellulose was hydrolysed and passed into the liquid fraction or hydrolysate, as also observed by Hendriks and Zeeman (2009).

In spite of the solubilisation of raw material components such as hemicellulose during the pretreatment, the percentage of cellulose remained between 29 and 32% (Table 1). The increase in cellulose in the hydrothermally pretreated material was very low in comparison with that of the treated SuOC at AT (29%). Therefore, in this case, hydrothermal pretreatment does not offer any advantage since the cellulose introduced into the reactor to be digested was only slightly more available than the material treated at AT. Furthermore, the cellulose content in the solid fraction, as opposed to the untreated material, indicates a low cellulose solubilisation rate (5%) as the temperature increased.

In general, hydrothermal pretreatment at temperatures above 150 °C causes not only solubilisation of hemicellulose, but also partial solubilisation of lignin (Hendriks and Zeeman, 2009). However, in the case at hand, as lignin was expressed on a VS basis and some organic materials were removed during hydrothermal pretreatment, in the end lignin was considerably more concentrated in the treated than in the untreated material (14%) or SuOC treated at AT (17%), as it reached values of up to 33% under the most severe conditions (200 °C). In spite of its possible solubilisation, the concentration of lignin may be related to solidification and re-deposition of the lignin on the biomass surface upon cooling after severe pretreatment conditions (Liu and Wyman, 2005; Negro et al., 2003).Thus, there is no lignin removal but rather a re-allocation of lignin during these high temperature pretreatments (Kristensen et al., 2008). This fact has already been described and reported for agricultural residues such as switchgrass (Kumar et al., 2011), corn stover and wheat straw (Kaparaju and Felby, 2010), as well as for paper tube residuals (Teghammar et al., 2010) subjected to hydrothermal pretreatments.

The parameters considered in the analysis of the liquid fraction obtained after pretreatment were CODs, pH, VFA, NH_x and carbohydrates (Table 2).

As expected, an increase in temperature caused an increase in solubilisation due to enhanced removal of non-structural components. Compared with the AT pretreatment, the COD contents obtained after increasing the temperature were higher. The highest level of CODs was obtained when the most severe pretreatment conditions were used (200 °C), while the lowest levels were obtained after no rise in temperature (AT).

The pH of the liquid hydrolysate decreased from 6.2 to 4.3 when the temperature of the pretreatment increased from AT to 200 °C, which was due to the increased formation of short-chain organic acids with the rise in temperature since a concentration of 524 ± 1 mg L^{-1} of VFA, expressed as COD, was obtained after hydrothermal pretreatment at 200 °C. The increased VFA concentration was likely due to acetic acid being released from the hydrolysis of acetyl groups contained in the hemicelluloses (Kaparaju et al., 2009; Kumar et al., 2011; Pérez et al., 2007).

As previously observed by Qiao et al. (2011) after hydrothermal pretreatment of food waste, NH_x was also released during heating. With regard to soluble carbohydrates present in the liquor, an increase in severity of the treatments led to an increase in sugar

Table 1
Fibre composition of untreated sunflower oil cake (SuOC) and SuOC after hydrothermal pre-treatment at ambient temperature (AT), 100, 150 and 200 °C.

	NDF[1] (%)*	ADF[2] (%)*	ADL[3] (%)*	Hemicellulose (%)*	Cellulose (%)*
Without pretreatment	48 ± 1	41 ± 1	14 ± 0	7	27
AT	59 ± 1	46 ± 0	17 ± 0	13	29
100 °C	61 ± 0	51 ± 1	19 ± 2	10	32
150 °C	66 ± 0	59 ± 0	29 ± 1	7	30
200 °C	71 ± 1	64 ± 0	33 ± 1	7	31

[1] NDF: Neutral detergent fibre.
[2] ADF: acid detergent fibre.
[3] ADL: acid detergent lignin.
* Expressed as volatile solids (w/w).

Table 2
Characterisation of the liquid fraction obtained after hydrothermal pretreatment of sunflower oil cake at different temperatures.

Pre-treatment	CODs[1] (mg O_2 L^{-1})	pH	TVFA[2] (mg COD L^{-1})	NH_x[3] (mg N L^{-1})	Carbohydrates (mg glucose L^{-1})
AT	2291 ± 68	6.2 ± 0.2	98 ± 6	8 ± 1	10 ± 1
100 °C	3125 ± 78	5.5 ± 0.3	158 ± 10	25 ± 1	40 ± 2
150 °C	5031 ± 141	5.1 ± 0.2	280 ± 6	64 ± 2	60 ± 2
200 °C	8468 ± 171	4.3 ± 0.2	524 ± 1	137 ± 3	130 ± 4

[1] CODs: soluble chemical oxygen demand.
[2] TVFA: total volatile fatty acids.
[3] NHx: ammoniacal nitrogen.

contents (from 10 ± 1 mg glucose L^{-1} at AT to 130 ± 4 mg glucose L^{-1} at 200 °C). A similar behaviour was reported by Ruiz et al. (2011) during the hydrothermal pretreatment of wheat straw at different temperatures. With the increase in the temperature and the production of hydrolysates, Maillard reactions occurred, which are responsible for the formation of refractory dissolved organic compounds that result in a dark colour and a burnt sugar odour.

In general, hemicellulose removal from the solid fraction can explain the increase in the soluble compounds in liquid fraction after hydrothermal pretreatment at temperatures above 150 °C (Negro et al., 2003; Pérez et al., 2008).

3.2. Methane production yields of solid and liquid fractions

Specific methane production during batch assays was the response variable used to evaluate the effect of the hydrothermal pretreatment on both fractions. Fig. 1 shows the cumulative methane yield as a function of time for solid (A) and liquid (B) fractions, respectively, during the BMP tests.

The methane yields for the solid fractions were 114 ± 9, 105 ± 7, 82 ± 7 and 53 ± 8 mL CH_4 g^{-1} COD_{added} at temperatures of 25, 100, 150 and 200 °C, respectively. For the solid fractions, the highest methane yield was obtained from SuOC treated at AT. This result was expected, taking into account that at this low temperature more organic matter remained than after pretreatment at higher temperatures. When the pretreatment temperature was increased, the methane yield decreased because a higher portion of carbohydrates and soluble compounds passed into the liquid fractions (Table 2). Therefore, a lower methane yield, than that obtained by De la Rubia et al. (2011) for SuOC without pretreatment (143 ± 3 mL CH_4 g^{-1} COD_{added}), was achieved. Pretreated SuOC at 200 °C achieved a 33% lignin content after pretreatment, a high value when compared with that of the sample treated at AT, containing 17% lignin. Thus, the pretreatment increased the lignin content by 94%, while it decreased the methane yield of the solid fraction by 46%. This relationship between the amounts of methane and lignin, was also observed by Kobayashi et al. (2004) during batch anaerobic digestion of steam-exploded bamboo.

The methane yields obtained for the liquid fractions (Fig. 1B) were 276 ± 6, 310 ± 4, 220 ± 15 and 240 ± 15 mL CH_4 g^{-1} COD_{added} from fractions obtained after pretreatments at 25, 100, 150 and 200 °C, respectively. It has been reported (Hendriks and Zeeman, 2009; Negro et al., 2003; Teghammar et al., 2010) that temperatures ⩾200 °C caused the formation of phenolic compounds as

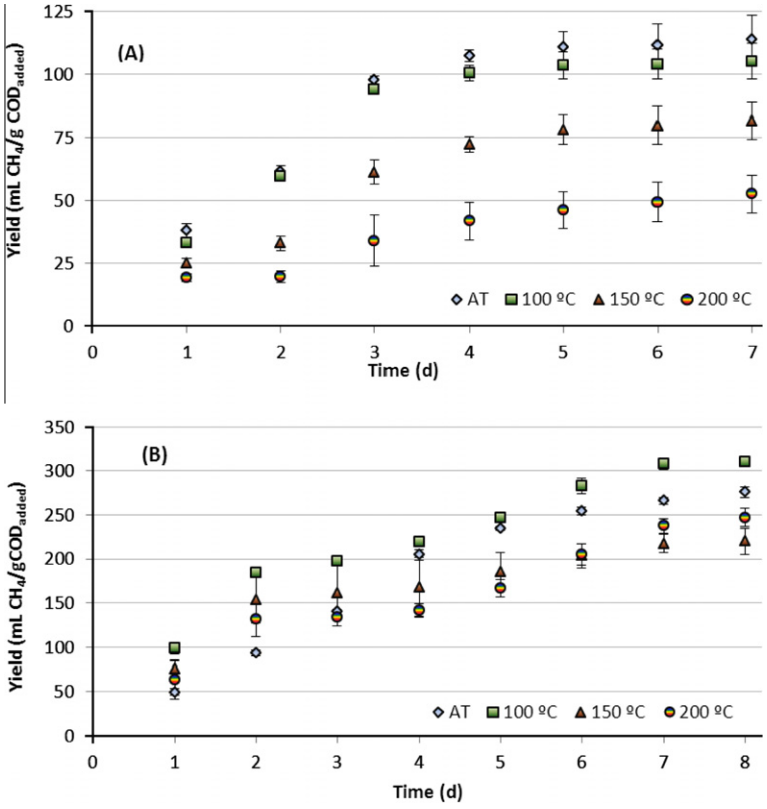

Fig. 1. Cumulative methane yield, expressed as mL CH_4/g COD_{added}, obtained during batch anaerobic digestion of hydrothermally pre-treated sunflower oil cake (A) Solid fraction; (B) Liquid fraction. Values are averages from three trials; errors bars indicate the standard deviation of the mean values ($p < 0.05$).

Table 3
Characterisation of digestates obtained after biochemical methane potential (BMP) experiments.

Assay	T[1] (°C)	CODs[2] mg O_2 L^{-1}	NH_x[3] mg N L^{-1}	TA [4] mg $CaCO_3$ L^{-1}	TVFA[5] mg COD L^{-1}
Solid fraction	AT	1593 ± 48	582 ± 11	5120 ± 10	7 ± 1
	100	1633 ± 33	582 ± 12	5120 ± 12	6 ± 1
	150	1720 ± 48	526 ± 16	4880 ± 15	6 ± 1
	200	2426 ± 66	650 ± 13	4600 ± 13	7 ± 1
Liquid fraction	AT	1882 ± 38	392 ± 8	4320 ± 11	7 ± 1
	100	2347 ± 60	526 ± 18	5160 ± 9	10 ± 1
	150	2890 ± 36	470 ± 9	5120 ± 10	8 ± 1
	200	3565 ± 107	358 ± 7	5040 ± 11	7 ± 1
Blank		1452 ± 28	336 ± 10	4320 ± 12	7 ± 1

[1] T: temperature.
[2] CODs: soluble chemical oxygen demand.
[3] NHx: ammoniacal nitrogen.
[4] TA: total alkalinity.
[5] TVFA: total volatile fatty acids.

well as furfural and HMF, which could inhibit the growth of anaerobic microorganisms. Therefore, the methane production obtained at AT was only improved for pretreatment at 100 °C.

When the methane yields from the solid and liquid fractions are combined, pretreatment at 100 °C can be considered optimal for hydrothermally pretreating SuOC before anaerobic digestion. Since, the overall or mean methane yields were 195, 207.5, 136 and 146.5 mL CH_4 g^{-1} COD_{added}, for AT, 100, 150 and 200 °C, respectively. This indicates that the value obtained at 100 °C was 6.5, 52.5 and 41.6% higher than those from SuOC pretreated at AT, 150 and 200 °C, respectively.

At the end of the BMP assays, digestates were also characterised (Table 3). Taking the final concentration values obtained for NH_x, alkalinity and VFA into account, the digestion process was carried out satisfactorily.

The highest CODs value obtained after the BMP assay of the liquid fraction at 200 °C can be explained by the formation of non-degradable or toxic compounds (Pérez et al., 2007).

3.3. Kinetic study

Table 4 lists the k values with 95% confidence, as well as the corresponding values of B_0 and R^2. The high values of the coefficient of determination R^2 (>0.94 for solid fractions and $\geqslant$0.91 for liquid fractions) and the low values of the confidence limits of the parameters obtained demonstrate the good fit of the experimental data to the proposed model.

The apparent kinetic constants of the process for the solid fractions are related to the concentration of lignin. The highest k values (0.41 ± 0.07 d^{-1} and 0.43 ± 0.08 d^{-1}) were obtained for AT as well as for 100 °C, for which the lignin concentration achieved the lowest values (around 17–19%). When the lignin content increased to 33% at 200 °C, the value of k decreased to 0.25 ± 0.06 d^{-1} showing the occurrence of an inhibitory phenomenon by lignin. A similar tendency was observed by Teghammar et al. (2010) after hydrothermal pretreatment of paper residues at 200 °C. Although the experiment carried out with the liquid fraction at 150 °C showed a kinetically more favourable value (k = 0.46 ± 0.07 d^{-1}) than that with the hydrolysate obtained after pretreatment at 100 °C, the highest B_0 was obtained for the sample originating from pretreatment at 100 °C (328 ± 21 mL CH_4 g^{-1} COD_{added}).

Table 4
Apparent kinetic constant (k) and ultimate methane yield (B_0) values with 95% confidence limits for experiments carried out with hydrothermal pretreated sunflower oil cake (solid and liquid fractions) at different temperatures.

Experiment	Temperature (°C)	k d^{-1}	B_0 mL CH_4 g^{-1} COD_{added}	R^2
Solid fraction	AT	0.41 ± 0.07	125 ± 8	0.9538
	100	0.43 ± 0.08	116 ± 8	0.9436
	150	0.29 ± 0.07	98 ± 11	0.9480
	200	0.25 ± 0.06	64 ± 9	0.9490
Liquid fraction	AT	0.24 ± 0.03	320 ± 17	0.9724
	100	0.33 ± 0.05	328 ± 21	0.9510
	150	0.46 ± 0.07	218 ± 10	0.9339
	200	0.21 ± 0.07	288 ± 49	0.9086

4. Conclusions

Hydrothermal pretreatment temperatures higher than 100 °C alter the chemical composition of the solid and liquid fractions, obtained with SuOC such that the methane yield from anaerobic digestion decreases. Therefore, hydrothermal pretreatment at 100 °C was the best option to improve the anaerobic digestion of SuOC and its methane yield, among the temperatures assayed. However, with only a 6.5% increase in yield compared to the yield which material treated at AT, it would be difficult to justify conducting this pretreatment.

The kinetic constant of the anaerobic digestion of solid fraction released after the pretreatment are related to the lignin concentration, decreasing when lignin content increases with temperature pretreatment.

Acknowledgements

The authors wish to express their gratitude to the Spanish Ministry of "Ciencia e Innovación" (Project CTM 2008-05772/Tecno) for providing financial support, to the program "FPI" of the above-mentioned Ministry for contracting graduates, as well as to the contract JAE-Doc from "Junta para la Ampliación de Estudios del CSIC", co-financed by European Social Funds.

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Reference Type: Journal biotech
Author: V. Fernández-Cegrí, M. A. De La Rubia, F. Raposo and R. Borja
Year: 2012
Title: Impact of ultrasonic pretreatment under different operational conditions on the mesophilic anaerobic digestion of sunflower oil cake in batch mode
Journal: Ultrasonics Sonochemistry
Volume: 19
Issue: 5
Pages: 1003-1010
Short Title: Impact of ultrasonic pretreatment under different operational conditions on the mesophilic anaerobic digestion of sunflower oil cake in batch mode
ISSN: 13504177 (ISSN)
DOI: 10.1016/j.ultsonch.2012.02.001
Author Address: Instituto de la Grasa (CSIC), Avda. Padre García Tejero, 4, 41012 Sevilla, Spain

Ultrasonics Sonochemistry 19 (2012) 1003–1010

Contents lists available at SciVerse ScienceDirect

Ultrasonics Sonochemistry

journal homepage: www.elsevier.com/locate/ultsonch

Short Communication

Impact of ultrasonic pretreatment under different operational conditions on the mesophilic anaerobic digestion of sunflower oil cake in batch mode

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ARTICLE INFO

Article history:
Received 16 December 2011
Received in revised form 26 January 2012
Accepted 1 February 2012
Available online 9 February 2012

Keywords:
Anaerobic digestion
Ultrasonic pretreatment
Sunflower oil cake
Specific energy
Sonication time
Methane yield

ABSTRACT

In this study ultrasonic (US) pretreatment was investigated with the aim of improving the anaerobic digestion of sunflower oil cake (SuOC), the solid waste derived from the extraction process of sunflower oil. Five ultrasonic pretreatment assays were conducted at specific energy (*SE*) and sonication times in a range from 24,000 kJ/kg TS and 16.6 min (assay 1: US1) to 597,600 kJ/kg TS and 331.2 min (assay 5: US5), respectively, all operating at a constant sonication frequency (20 kHz) and ultrasonic power (120 W). As regards ultrasonic pretreatment, the working conditions of the first assay (US1) using samples of SuOC at 2% (w/v) showed to be the most appropriate in terms of both lignin and hemicellulose degradation (57.7% and 66.7%, respectively) and cellulose increase (54% increase with respect to its initial concentration). The percentage of COD solubilization increased from only 14% to 21% when *SE* was 25 times higher. Results obtained in batch anaerobic digestion experiments (biochemical methane potential – BMP – tests) conducted at 35 °C of the solid and liquid fractions released from the different ultrasonic conditions tested, indicated that for the first experiment (US1) the average ultimate methane yield obtained was 53.8% higher than that achieved for untreated SuOC. Finally, the kinetic constants of the anaerobic digestion of the solid and liquid fractions released after the ultrasonic pretreatment were virtually independent of the operation conditions assayed.

1. Introduction

In the industrial processing of sunflower seeds into edible oils, large quantities of waste called sunflower oil cake (SuOC) are generated. SuOC is the part of the whole sunflower seed which remains after the oil has been extracted. The high production level of SuOC in Spain, approximately five million tons per year, could create a significant environmental problem [1].

Anaerobic treatment processes are frequently used for the biological degradation of concentrated organic wastes leading to a stabilization of the residues because of the production of biogas, which in turn makes the process profitable. However, some doubts have been cast on the efficiency of anaerobic treatment and its process reliability because of the fact that some potential wastes for bioconversion are relatively non-biodegradable and, in addition, contain substances that are toxic to methanogenic microorganisms [1,2]. The anaerobic digestion process is conducted in four main stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis [3,4].

SuOC is characterized by its high concentration of lignocellulosic compounds [2]. As is well-known, cellulose in the ligno-cellulosic polymeric form is not totally available for bacterial attack [3]. Lignin surrounds the cellulose crystalline structure forming a seal and protects the cellulose from being easily hydrolysed. Because of the refractory structure of these compounds, one of the major problems in utilizing SuOC and other crop residues for stabilizing and for methane production by anaerobic digestion is their low digestibility. The anaerobic biodegradability and hence the methane potential of a complex substrate depends on the content of biodegradable compounds: carbohydrates (including cellulose), proteins and lipids [3].

It is generally accepted that hydrolysis is the rate-limiting step in the anaerobic digestion of organic vegetable solid waste. Owing to the chemical and physical construction of lignocellulose, its microbial hydrolysis is a slow and difficult process [3]. Previous works demonstrated the low values of the methane yield coefficient (143 mL CH_4 at standard temperature and pressure conditions, STP/g COD_{added}) achieved in Biochemical Methane Potential (BMP) tests conducted at mesophilic temperature (35 °C), using SuOC as substrate with a particle size of 0.7–1.0 mm [3]. In the same way, a considerable decrease in the methane yield from 227 to 107 mL CH_4 STP/g VS_{added} was observed when the food/microorganisms (F/M) ratio (volatile solids – VS – basis) increased from 0.3 to 2.0 during batch anaerobic digestion assays of SuOC at mesophilic temperature [1]. This proved that inhibition for substrate concentration had taken place in the anaerobic process of this waste. In addition, the anaerobic biodegradability of this substrate also decreased from 86% to 41% within the above-mentioned F/M ratio range [1].

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doi:10.1016/j.ultsonch.2012.02.001

Another recent study [5] has shown the low values of the methane yield coefficients (0.481 and 0.264 m^3/kg feedstock) obtained in BMP tests on sunflower meal and sunflower straw conducted at 35 °C. The lower value achieved for sunflower straw was attributed to the different composition of the two substrates in terms of lignin. Various pretreatment methods, such as thermal (121 °C for 60 min in a pressure cooker), chemical (through alkali or acid addition of 2% w/w H_2SO_4 or NaOH for 60 min) or a combination of the two methods (thermal–chemical) were also assayed to enhance methane productivity and yield. However, the experiments showed that the pretreatment methods did not enhance the methane potential of these residues. This was attributed to the presence of inhibitory compounds (such as furfural or hydroxymethylfurfural), which were released during the pretreatments [5].

Ultrasonic pretreatment is the application of cyclic sound pressure (ultrasound) with a variable frequency to waste activated sludge and other wastes to disintegrate sludge flocs and cell walls [4]. The chemistry of sonication as a pretreatment tool is quite complex and consists of a combination of shearing, chemical reactions with radicals, pyrolysis and combustion [6]. During sonication, microbubbles are formed because of high-pressure applications to liquid, which cause violent collapses and high amounts of energy to be released into a small area [4,6]. Consequently, because of extreme local conditions certain radicals (·OH, H·) can be formed [7]. The radical reactions can degrade volatile compounds by pyrolysis processes taking place in microbubbles [6,7].

One of the main advantages of the ultrasonic technique is that the use of external chemical agents is prevented and, therefore, an increase in the effluent volume is avoided [8].

When ultrasonication is applied to waste activated sludge (WAS), a solubilization of extracellular polymeric materials (EPS) and the cellular membranes of microorganisms takes place because of the extreme local temperatures and pressures achieved. This results in a sharp increase in soluble COD (CODs) to such an extent that sludge subjected to ultrasonic pretreatment produces CODs up to six times higher than untreated sludge, with the digesters which process the pretreated sludge producing 10–60% more biogas than conventional control digesters [4–8].

Mechanisms of ultrasonic treatment are influenced by four main factors: specific energy, ultrasonic frequency, application time and the characteristics of the substrate [7,8]. Cell disintegration is proportional to the energy supplied. High frequencies promote oxidation by radicals, whereas low frequencies promote mechanical and physical phenomena such as pressure waves. To be specific, 20–40 kHz has been reported as the optimal frequency range for achieving strong mechanical forces [9]. With complex substrates, radical performance decreases. It has been demonstrated that the degradation of excess sludge is more efficient when using low frequencies [6–9].

The aim of this work was to study the effect of different sonication operational conditions, such as time and specific energy, on the solubilization degree of SuOC at a constant frequency and an ultrasonic power of 20 kHz and 120 W, respectively. The influence of sonication working conditions on the cellulose, hemicellulose and lignin content of this substrate was also assessed. Finally, the effect of the ultrasonic pretreatments on the stability, methane yield and kinetics of the batch anaerobic digestion of pretreated SuOC was also studied.

2. Materials and methods

2.1. Substrate: sunflower oil cake (SuOC)

The sample of sunflower oil cake used in this study was collected from a sunflower oil factory located near Seville (Spain). Prior to use, the substrate was shredded into different particle sizes using a commercial sieve (Restch AS 200 basic) and different screen meshes. In order to ensure the homogeneity of the sample, the most abundant particle size of this substrate (0.71–1.0 mm diameter) was selected for carrying out the experiments.

The full composition and main features as well as the fractional composition of the fiber of the above-mentioned SuOC particle size selected are as follows (mean values of four determinations ± standard deviations): dry matter (DM) or total solids (TS), 93.8 (±0.1)%; volatile solids (VS), 93.0 (±0.1)%; ash, 6.8 (±0.1)%; total COD, 1.24 (±0.02) g O_2/g TS; neutral detergent fiber, 45 (±1.1)%; acid detergent fiber, 38.4 (±0.9)%; acid detergent lignin (ADL), 13.3 (±0.2)%; hemicellulose, 6.6 (±0.8)%; cellulose, 25.1 (±0.4)%; total protein, 25.3 (±0.8)%; fat content, 1.6 (±0.2)%; soluble carbohydrates, 5.1 (±0.2)% and total carbohydrates, 53.0 (±0.3)%. All these values are expressed as a percentage of dry matter.

A suspension of 2% w/v (20 g TS/L) of the mentioned SuOC in distilled water was used for the ultrasonic pretreatment experiments and subsequent batch anaerobic digestion assays. Therefore, the final characteristics of the SuOC sample used in the experiments were: ash, 4.3%; proteins, 24.5%; hemicellulose, 13.5%; cellulose, 28.7%; lignin, 16.8% and total COD, 1250 mg/g TS.

2.2. Ultrasonic pretreatment

The ultrasonic equipment was a Sonopuls ultrasonic homogenizer (Bandelin–Sonopuls HD 2200, Berlin, Germany). This apparatus was equipped with a KE 76 titanium tapered tip probe with a constant operating frequency of 20 kHz, 60% amplitude and 120 W of power. For each experiment, volumes of between 200 and 250 mL of sample were placed in a glass beaker and the ultrasonic probe was submerged into the sample to a depth of 2 cm. The ultrasound density and intensity were kept constant at 0.48 W/mL and 3.3 W/cm^2, respectively. The sonication times varied in a range from 16.6 to 331.2 min. The temperature of the treated samples was kept at 20 °C, while the tap water was recirculated around the beaker.

Specific energy was considered as the main variable parameter for the evaluation of the solubilization and disintegration performance of the substrate. The range of the specific energy (*SE*) varied from 24,000 kJ/kg TS (assay 1: US1) to 597,600 kJ/kg TS (assay 5: US5). *SE* (in kJ/kg TS) was calculated by using ultrasonic power (*P* in watts), ultrasonic time (*t* in seconds), sample volume (*V* in liters) and initial total solid concentration (TS_0 in g/L) according to the following equation [6,8,10,11]:

$$SE = (P \cdot t)/(V \cdot TS_0) \quad (1)$$

Table 1 summarizes the ultrasonic pretreatment conditions used in the experiments. For all ultrasonic conditions tested, the mass ratio of solid (g) to liquid (distilled water, in mL) was 2:100 (organic load: 2% w/v).

COD solubilization (*S*) after each ultrasonic pretreatment was also determined. *S* was calculated using the difference between final soluble COD (CODs) after pretreatment and initial soluble COD ($CODs_0$), as compared to the initial total COD ($CODt_0$) by using the following equation [7,10]:

$$S = (\mathrm{CODs} - \mathrm{CODs}_0) * 100/\mathrm{CODt}_0 \quad (2)$$

2.3. Experimental procedure

After each ultrasonic pretreatment assay, the soluble or liquid fractions were separated from the solid fractions by centrifuging the samples for 15 min at 10,000 rpm. The corresponding solid and liquid fractions after each pretreatment were subjected to separate biochemical methane potential (BMP) tests.

Table 1
Ultrasonic pretreatment conditions on samples of SuOC at 2% (w/v).*

Assay number	Sample volume (L)	Sonication time (min)	Specific Energy (kJ/kg TS)	Ultrasound Doses (J/L)
US1	0.25	16.6	24,000	478
US2	0.23	60.6	96,000	1897
US3	0.25	133.3	192,000	3839
US4	0.25	300.0	432,000	8640
US5	0.20	331.2	597,600	11,923

* Sonication frequency: 20 kHz (constant); ultrasonic power: 120 W (constant); ultrasonic density: 0.48 W/mL (constant); ultrasonic intensity: 3.3 W/cm^2 (constant).

The anaerobic experimental study was conducted in a multi-batch reactor system, which consisted of nine Erlenmeyer flasks, with an effective volume of 250 mL. They were continuously stirred with magnetic bars at 300 rpm and placed in a thermostatic bath at mesophilic temperature (35 ± 1 °C). The reactors were initially charged with an anaerobic inoculum by maintaining a concentration of 15 g VS/L (the volume taken is a function of the initial VS concentration of the inoculum). The inoculum to substrate ratio was maintained at 2 (VS basis) for the reactors digesting the solid fractions and at 2.5 (COD basis) for the reactors processing the liquid fractions. Once the pretreated substrate was added to each reactor, 25 mL of stock mineral medium solution (whose composition has been described elsewhere) [12] were also added. Finally, distilled water was added to achieve the desirable working volume of 250 mL. The reactors were flushed with N_2 in order to achieve anaerobic conditions. Granular sludge taken from an industrial anaerobic reactor treating brewery wastewater was used as inoculum. The characteristics of this inoculum were: pH, 7.0; TS, 75 g/L and VS, 54 g/L.

The methane released was measured by volume displacement (carbon dioxide was previously removed by flushing the gas through a 2 N NaOH solution), and expressed at standard temperature and pressure (STP) conditions. Because of biomass decay and the possible presence of residual substrate in the inoculum, the methane produced was subtracted by performing blank controls. A starch control was also used for checking the BMP test performance.

All experiments took place over a 7-day period, until no significant gas production was observed (on the last day of production there was less than 1% of the accumulated methane volume), suggesting that biodegradation had been completed. This short period of time was sufficient to achieve maximum methane production, and can basically be explained by the high methanogenic activity of the sludge and the short interval (less than 72 h) which had elapsed between inoculum sampling and the start-up of the experiments.

2.4. Analytical methods

2.4.1. Solid samples

The following parameters were analyzed in the original solid substrate (SuOC): total solids (TS) and volatile solids (VS), according to standard methods 2540B and 2540E [13], respectively. To determine TS, a well-mixed sample is evaporated in a dish which has previously been weighed and dried to constant weight in an oven at a temperature of between 103 and 105 °C. The increase in weight over that of the empty dish represents the TS content [13]. Total chemical oxygen demand (COD) was determined using the reported method proposed by Raposo et al. [14]. Fat content was extracted with hexane, using a Soxhlet system [15].

To determine the total Kjeldahl nitrogen (TKN), 1000 mg of sample were acidified with 15 mL concentrated H_2SO_4. In addition, 5 g catalyst [(Cu–Se) (1.5% CuSO4·5H2O + 2% Se)] was added, and finally, the sample was digested sequentially in a thermoblock for 15 min at 150 °C, 15 min at 250 °C and 90 min at 390 °C. After cooling, the sample was diluted with 10 mL distilled water, neutralized with NaOH 12.5 N and distilled in 50 mL of solution indicator mix (H_3BO_3 at 2% w/v). The solution was titrated with H_2SO_4 0.02 N. Total protein was determined by multiplying the TKN value by 5.5 [16].

Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined in order to calculate hemicellulose (NDF–ADF), cellulose (ADF–ADL) and lignin (ADL), according to van Soest et al. [17], with slight modifications.

- To determine NDF, 1000 mg of dried sample was boiled in a sintered glass crucible (40–100 μm pore size) with 100 mL of a neutral solution of sodium dodecyl sulfate in an EDTA–borate buffer together with 1 g of sodium sulfite anhydrous (to remove proteins) and 200 μL of α-Amylase (to eliminate starch) for 1 h. Afterwards, the neutral detergent was removed and the sample washed with 100 mL of hot distilled water. Finally, the sample was washed with 50 mL of acetone and dried at 105 °C in an oven overnight and then weighed. Corrections for residual proteins and ash were made.
- ADF was determined by non-sequential fiber analysis. In this way, 1000 mg of raw dried sample were heated with 100 mL of a solution of *N*-Cetyl-*N*,*N*,*N*-trimethyl ammonium bromide (in H_2SO_4 1 N) to boiling point for 1 h in a sintered glass crucible (40–100 μm pore size). Afterwards, ADF was recovered by filtration, washed with 100 mL of hot distilled water and later with 50 mL of acetone. Finally, the sample was dried overnight at 105 °C and then weighed. Ash and proteins were also corrected during this step.
- To determine ADL, 250 mg of sample obtained after ADF analysis continued to be stirred for 3 h with 25 mL of H_2SO_4 (72% w/w). Then, the sample was placed in a sintered glass crucible (40–100 μm pore size) and washed with 100 mL of distilled water and dried at 105 °C in an oven overnight and then weighed. Correction for ash was made.

2.4.2. Soluble fractions

The supernatants obtained after centrifuging the pretreated samples and digestates for 15 min at 10,000 rpm were passed through a filter (0.45 μm) and used to characterize the following soluble parameters: soluble chemical oxygen demand (CODs), using the closed digestion and colorimetric standard method 5220D [13]; total alkalinity (TA) was measured by pH titration to 4.3. Soluble ammoniacal nitrogen (NH_x)s was determined by distillation and titration according to the standard method 4500E [13]. The volatile fatty acid (VFA) concentration was analyzed using a gas chromatograph, as previously described [18].

2.4.3. Inoculum and digestates

Both the inoculum and digestates were characterized by direct sampling. pH was determined by using a pH-meter model Crison

20 Basic. Total alkalinity (TA), TS and VS were also analyzed in these samples [13].

3. Results and discussion

3.1. Influence of the operational conditions of ultrasonic pretreatment on the characteristics and solubility of the substrate

Table 2 shows the characteristics of the substrate after the ultrasonic pretreatment under the different operational conditions tested. For the first assay (US1) the percentages of lignin and hemicellulose removals were 57.7% and 66.7%, respectively, the latter being the highest percentage of hemicellulose removed found in the different conditions assayed. This reveals the suitability of the use of a *SE* of 24,000 kJ/kg TS during ultrasonic pretreatment with a view to obtain a more appropriate substrate for anaerobic digestion.

In addition, an increase of 54% in the percentage of cellulose with respect to its initial content in the substrate was observed during the first operational conditions tested (US1), for which a cellulose content of 44.2% was achieved after pretreatment under the afore-mentioned conditions. This fact is of great importance when considering that cellulose is a more biodegradable carbohydrate than other polymers present in the waste being researched (hemicellulose and lignin). Ultrasonic treatment for obtaining cellulose nanofibers from polar wood, with high hemicellulose and lignin removals after chemical pretreatment (with a 3% potassium hydroxide solution at 80 °C for 4 h), combined with a high-intensity ultrasonication step (1200 W power for 30 min) was also reported in the literature [19].

On the other hand, low lignin contents in the ultrasonic pretreated substrate were also obtained for US2, US3 and US4 conditions, although the differences from one assay to the next were insignificant. To be specific, the initial lignin content of the substrate (16.8%) was reduced to percentages of 7.1%, 7.5%, 7.5%, 7.4% and 9.0% after assays US1, US2, US3, US4 and US5, respectively. A maximum lignin degradation percentage of 57.7% was achieved for the lowest and most reduced energetic conditions tested (US1 with a *SE* of 24,000 kJ/kg TS). Higher lignin reductions (11.4% on dry basis) were achieved during sonication of sunflower husks with the aim of accelerating the bioconversion of this substrate in biodiesel fuel production [20]. However, ultrasonic intensity used in the previously mentioned work (46 W/cm^2) [20] was much higher than that used in the present work at assay 1 operating conditions (3.3 W/cm^2).

The highest protein contents in the solid fraction were achieved during assays US1 (25.2%) and US2 (27.7%). For higher *SE* values and sonication times (assays US3, US4 and US5), protein contents were lower. A similar behavior was observed during sonication of WAS, for which an increase in the protein concentration released was observed at low *SE* [11,21]. Wang et al. also examined the release of proteins in the aqueous phase at different sonication times [22] and demonstrated that the rate of protein release from WAS was very high during the initial 20 min of sonication with polysaccharide concentration dropping after this time [21].

It can also be observed in Table 2 that ultrasonication time and *SE* had practically no effect on the total COD of the substrate because this parameter was virtually constant for all the conditions assayed, ranging between 1.28 and 1.33 g/g VS. A similar trend was also observed in the sonication of WAS, prior to being subjected to anaerobic digestion [11].

On the other hand, as can be seen in Table 2, the percentage of COD solubilization increased from 14% (US1) to 21% (US5) when the *SE* increased from 24,000 to 597,600 kJ/kg TS. Therefore, the percentage of COD solubilization was only 1.5 times higher when the *SE* was 25 times higher. Once again, this reinforces the idea of considering the first operational conditions tested as being the most suitable working requisites for carrying out the ultrasonic pretreatment of this substrate.

For comparative purposes, ultrasonic pretreatment at 20 kHz and 1 W/mL sonication density allowed for an increase in the COD solubilization percentages from 11% (control, not pretreated) to 23% for pulp sulfite mill sludges and from 1.3% (control) to 5.0% for kraft pulp mill secondary sludges [23]. For *SE* below 1000 kJ/kg, the COD solubility of WAS was low (8%). However, when the supplied energy was over the above-mentioned value, COD solubilization rose sharply to 35% for a *SE* of 15,000 kJ/kg TS [7]. A maximum COD solubilization of 15% was achieved in WAS after an ultrasonic pretreatment conducted at *SE* values in the range of 6250–9350 kJ/kg TS [24]. The effect of ultrasonication on COD solubilization was also studied for swine slurry and separated dairy manure at two power ratings (59.7 kW and 119.3 kW) and at two time settings (15 and 30 s), achieving values of up to 23% and 33%, respectively [25]. Other previous studies showed that 15 min of sonication (with a sonication frequency, power input and intensity of 24 kHz, 255 W and 4.8 W/cm^2, respectively) allowed for an increase in the initial soluble COD of WAS from 50 to 2500 mg/L [4]. However, lower COD solubilization yields (15%) were reached in WAS containing polycyclic aromatic hydrocarbons (PAH) after ultrasonic pretreatment using *SE* of 15,000 kJ/kg TS [8]. Hog manure was found to be more amenable to ultrasonication than WAS, as it took only 3000 kJ/kg TS to cause 15% more solubilization as compared to 25,000 kJ/kg TS for WAS [26]. To be specific, the maximum COD solubilization of hog manure was 27.3% at 30,000 kJ/kg TS, whereas Khanal et al. using WAS achieved 16.2% at *SE* of 66,800 kJ/kg TS [27].

3.2. Effect of ultrasonic pretreatment on methane yield

Cumulative methane productions as a function of digestion time were assessed during the BMP tests of the solid and liquid fractions obtained after the different ultrasonic pretreatment conditions conducted. It was observed during the experiments that most of the methane production and, therefore, the highest substrate utilization rates generally occurred during the first 3 days of digestion.

Table 2
Characteristics of the samples of SuOC (2% w/v, 20 g TS/L) after the ultrasonic pretreatment under different operational conditions.*

Experiment number	Ashes (%)	Proteins (%)	Hemicellulose (%)	Cellulose (%)	Lignin (%)	COD (g/g TS)	CODs (g/L)	S (%)**
US1	4.3	25.2	4.5	44.2	7.1	1.26	3.5	14
US2	3.8	27.7	8.5	39.6	7.5	1.27	4.1	17
US3	3.3	23.5	12.8	39.7	7.5	1.26	4.2	17
US4	5.6	22.6	12.3	40.9	7.4	1.25	4.8	19
US5	3.0	21.9	10.4	41.2	9.0	1.31	5.2	21

* Values are averages of five determinations: there was virtually no variation (less than 3%) between analyses.
** S (%): percentage of solubilization with respect to the total COD.

Fig. 1A and B shows the cumulative methane yield as a function of digestion time for the solid and liquid fractions obtained after the ultrasonic pretreatment performed under different operational conditions. The methane yield values were calculated for each case studied by dividing the net methane production (subtracting the blank or control methane production) at a determined time by the amount of COD added [1]. Therefore, the ultimate methane yield gives the value when no more volume of gas from the reactors is released. As can be seen, for the solid fractions the ultimate methane yield increased from 90 ± 4 to 111 ± 5 mL CH_4 STP/g COD_{added} when the *SE* decreased from 597,600 kJ/kg TS (US5) to 24,000 kJ/kg TS (US1). In the same way, for the liquid fractions, the methane yield rose again from 270 ± 13 to 330 ± 16 mL CH_4 STP/g COD_{added} when the *SE* decreased in the above-mentioned range. On the other hand, the methane yields of the solid fractions expressed as mL CH_4 STP/g VS_{added} were found to be 147 ± 7, 142 ± 7, 135 ± 6, 122 ± 6 and 110 ± 5 for assays US1, US2, US3, US4 and US5, respectively. Once again, this shows the appropriateness of US1 working conditions for carrying out ultrasonic pretreatment. Calculating the mean methane yield from the values obtained for the solid and liquid fractions at US1 operating conditions gives a value of 220 ± 11 mL CH_4 STP/g COD_{added} after ultrasonic pretreatment. This value was 53.8% higher than that obtained in BMP tests conducted with untreated SuOC under the same working conditions (143 mL CH_4 STP/g COD_{added}) [3].

In the same way, Bougrier et al. [24] showed an increase in the methane yield of WAS from 221 to 334 mL CH_4 STP/g COD_{added} after an ultrasonic pretreatment at 9350 kJ/kg TS, which was more effective than other pretreatments assayed, such as ozonation or thermal pretreatment. An increase in the methane production of 44% was also reported by Erden and Filibeli [10] for WAS previously sonicated with a *SE* of 9690 kJ/kg TS and a power density of 0.09 W/mL. Likewise, an improvement of 16% in specific biogas production was also observed after ultrasonic pretreatment of WAS with a high content in polycyclic aromatic hydrocarbons at *SE* of 11,000 kJ/kg TS, in this case in anaerobic digestion experiments conducted in continuous mode, using hydraulic retention times of 29 days [8]. Similarly, the methane potential of hog manure increased by 20.7% in comparison with unsonicated manure for an *SE* input of 30,000 kJ/kg TS [26] with a maximum increase in the methane production rate of 80.6% as compared with the untreated sample. Finally, ultrasonic pretreatment of swine slurry and separated dairy manure effluent under the above-mentioned conditions (power ratings of 59.7 kW and 119.3 kW and times of 15 and 30 s) also increased the methane yields up to 56% and 20%, respectively with respect to untreated samples [25].

3.3. Effect of ultrasonic pretreatment on chemical control parameters in BMP tests

Table 3 shows the variation of the chemical control parameters in the digestates of the solid and liquid fractions at the end of the digestion process for the different operational conditions tested during ultrasonic pretreatment.

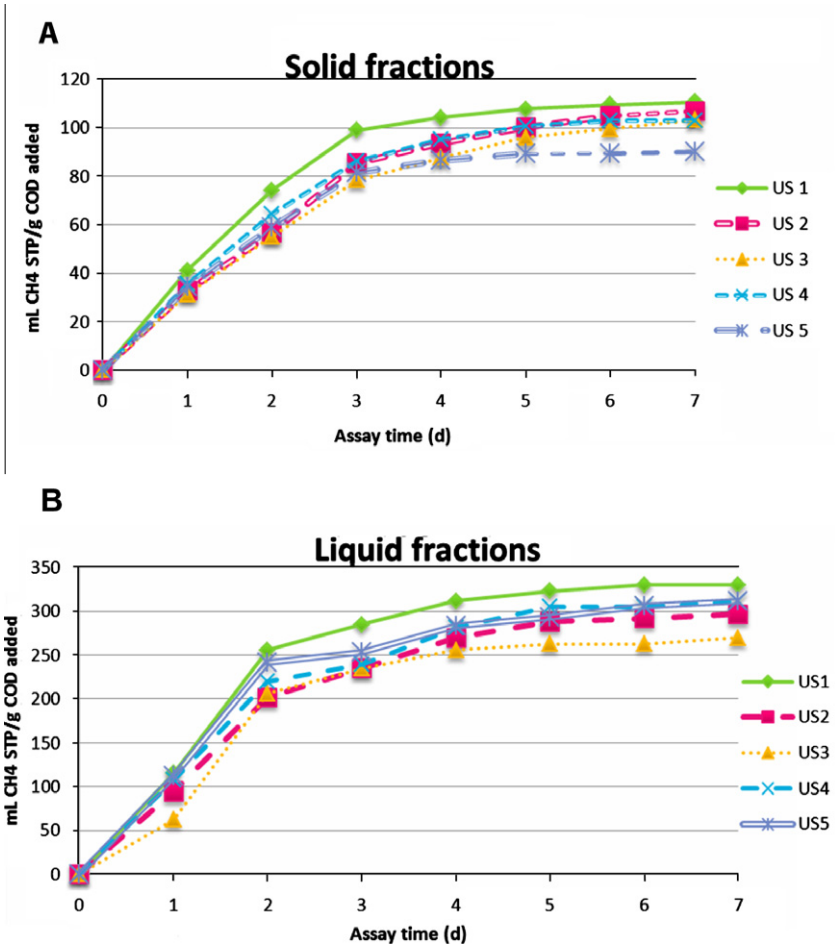

Fig. 1. Variation of the methane yield with the digestion time for both the solid (A) and liquid fractions (B) released after the ultrasonic pretreatment under the different operational conditions tested.

Table 3
Variation of the chemical control parameters (mean values ± standard deviations) during the BMP tests performed with the solid and liquid fractions obtained after the ultrasonic pretreatment.

Assay number	pH	(NH_x)s (mg/L)	CODs (mg/L)	TA (mg $CaCO_3$/L)	TVFA (mg acetic acid/L)
Solid fractions					
US1	7.6 ± 0.2	520 ± 15	1700 ± 50	4700 ± 140	5.0 ± 0.1
US2	7.6 ± 0.3	530 ± 14	2000 ± 58	4800 ± 141	11.0 ± 0.3
US3	7.6 ± 0.2	530 ± 15	2000 ± 65	4800 ± 135	12.0 ± 0.3
US4	7.5 ± 0.2	540 ± 18	1600 ± 46	4700 ± 145	12.0 ± 0.4
US5	7.5 ± 0.3	540 ± 20	1600 ± 45	4600 ± 138	13.0 ± 0.3
Liquid fractions					
US1	7.8 ± 0.3	400 ± 12	2400 ± 73	4000 ± 70	13.0 ± 0.3
US2	7.6 ± 0.2	430 ± 11	2600 ± 75	4600 ± 135	16.0 ± 0.4
US3	7.6 ± 0.2	440 ± 10	2400 ± 68	4700 ± 130	14.0 ± 0.3
US4	7.5 ± 0.3	430 ± 11	2700 ± 81	4800 ± 140	17.0 ± 0.5
US5	7.7 ± 0.2	430 ± 12	2100 ± 61	4800 ± 133	15.0 ± 0.3

Table 4
Kinetic parameters (k_0 and B_0) derived from Eq. (3) with their 95% confidence limits as well as other statistical parameters derived from the mathematical adjustment of the experimental data to the proposed model for all the conditions assayed.

Parameter	US1	US2	US3	US4	US5
Solid fractions					
k_0 ($days^{-1}$)	0.52 ± 0.05	0.37 ± 0.04	0.35 ± 0.02	0.45 ± 0.04	0.52 ± 0.05
B_0 (mL CH_4 STP/g COD_{added})	116 ± 3	118 ± 5	114 ± 3	111 ± 3	95 ± 3
R^2	0.991	0.991	0.996	0.994	0.988
Standard error of Estimate	4.10	3.91	2.36	3.11	3.84
W statistic	0.95	0.96	0.92	0.98	0.94
Liquid fractions					
Parameter	US1	US2	US3	US4	US5
k_0 ($days^{-1}$)	0.52 ± 0.06	0.46 ± 0.05	0.49 ± 0.09	0.47 ± 0.05	0.56 ± 0.07
B_0 (mL CH_4 STP/g COD_{added})	350 ± 14	316 ± 11	287 ± 22	327 ± 10	318 ± 12
R^2	0.986	0.991	0.953	0.991	0.984
Standard error of Estimate	16.25	11.03	24.33	11.60	15.12
W statistic	0.86	0.93	0.89	0.98	0.83

There was little variation in the pH: 7.5 and 7.8, values that were compatible with the normal growth of anaerobic microorganisms. This indicates that the pH was practically constant and stable during the anaerobic digestion of both the solid and liquid fractions, independently of the operational conditions used in the ultrasonic pretreatment. In addition, these pH values were within the optimum pH range (7.0–8.5) recommended for obtaining a maximum anaerobic degradation of cellulosic compounds using ruminal microorganisms [28].

Given that during anaerobic degradation, complex organic compounds are transformed into lower molecular weight compounds, soluble COD is a parameter that indicates the degradation of the substrate [29]. In the present study, the lower soluble CODs were achieved in the digestates of the samples sonicated at higher *SE* and times (US5), although no significant difference among the values achieved for the other conditions tested was observed.

The degradation of complex organic material, including nitrogenous organic compounds, results in the generation of ammonia, a compound which at certain concentrations can inhibit the anaerobic process [1]. The lower ammoniacal nitrogen concentration observed at the effluent of the liquid and solid samples for all the conditions tested did not affect the methane yield observed for these experiments.

The final values of the total volatile fatty acids (TVFA) were very low for both the solid and liquid fraction digestates, with values in the range of 5–16 mg acetic acid/L. This means that the overall anaerobic process was conducted satisfactorily and a correct balance of the process occurred [30]. In addition, the high total alkalinity (TA) values in the range from 4040 to 4800 mg $CaCO_3$/L showed the high favorable buffering capacity of the bioreactors for all conditions tested in the ultrasonic pretreatment. The experimental data obtained in this work show that a total alkalinity of about 4000 mg $CaCO_3$/L is sufficient to prevent the pH from dropping to below 7.5, independently of the working conditions of the pretreatment.

3.4. Effect of ultrasonic pretreatment on the kinetics of the anaerobic process

In order to characterize each experiment kinetically with a view to evaluate the influence of the operating conditions of the ultrasonic pretreatment on the anaerobic process and, thus facilitating a comparison, the following kinetic equation was used [3,31]:

$$B = B_0[1 - \exp(-k_0 \cdot t)] \quad (3)$$

where B is the cumulative methane yield (mL CH_4/g COD_{added}), B_0 is the maximum or ultimate methane yield of the substrate (mL CH_4/g COD_{added}), k_0 ($days^{-1}$) is the specific rate or apparent kinetic constant and t (days) is the time.

According to Eq. (3), methane yield conforms to a first-order kinetic model [31,32]. As can be seen in Fig. 1A and B for both the solid and liquid fractions, B was zero at $t = 0$, and the rate of methane yield became zero at t equal to infinite. Thus, Eq. (3) shows a good agreement with the experimental data and it seems appropriate to apply the proposed kinetic model for all conditions tested in the ultrasonic pretreatment.

The adjustment by nonlinear regression of the pairs of the experimental data (B, t) using the SigmaPlot software (version 11.0) allows the calculation of the apparent kinetic constant k_0. Table 4 lists k_0 and B_0 values with their respective 95% confidence limits for each case studied. This Table also shows the determination coefficient (R^2), the standard error of estimate and the W statistic for each case assayed.

The high values of the coefficient of determination, R^2, with values higher than 0.99 in most cases and the low values of the standard errors of estimate and confidence limits of the parameters obtained demonstrate how well the experimental data adapted to the model proposed.

As can be seen in Table 4, in general, k_0 values for the solid fractions were somewhat lower than those obtained for the liquid fractions, especially for US2 and US3 pretreatment conditions. This may be due to the fact that a part of the organic matter contained in the insoluble or solid fractions was not easily available for anaerobic microorganisms and was biodegraded more slowly than that present in the soluble or liquid fractions. This behavior was previously observed in BMP tests of WAS after sonication at *SE* lower than 3000 kJ/kg TS [8]. For the solid fractions, the highest k_0 values (0.52 $days^{-1}$) were achieved for the US1 and US5 conditions. This value was only slightly higher than those obtained for US2, US3 and US4 experiments, respectively. For the liquid fractions, the kinetic constant was virtually constant showing the independence of the kinetics of the anaerobic process with respect to the operating conditions of the ultrasonic pretreatment.

4. Conclusions

Results from this study demonstrate the suitability of ultrasonic pretreatment of SuOC for increasing the anaerobic biodegradability of this substrate and methane yield coefficient. Ultrasonic pretreatments conducted on samples of SuOC at 2% (w/v) (20 g TS/L), at *SE* ranging from 24,000 kJ/kg TS (assay US1) to 597,000 kJ/kg TS (assay US5) operating at constant sonication frequency (20 kHz) and ultrasonic power (120 W) revealed the appropriateness of the lowest conditions assayed (US1) to obtain maximum methane production and yields, both from the solid and liquid fractions released after pretreatment as compared to the other conditions assayed. Specifically, the ultimate methane yields obtained for the solid and liquid fractions (111 ± 5 and 330 ± 16 mL CH_4 STP/g COD_{added}, respectively) in US1 were higher than those obtained for the other conditions tested during pretreatment. Likewise, the mean value obtained (average of the solid and liquid fractions) in this case was 220 ± 11 mL CH_4 STP/g COD_{added}, which was 53.8% higher than that obtained for untreated SuOC.

As regards ultrasonic pretreatment, for the first condition assayed (US1) the percentages of lignin and hemicellulose removals were 57.7% and 66.7%, respectively, the latter being the highest percentage of hemicellulose removed found among the different conditions tested. Moreover, COD solubilization increased by only 7% for US5 (21%) as compared to US1 (14%), an interval for which the *SE* and sonication times were 25 and 20 times higher, respectively. This fact reveals the suitability of the ultrasonic pretreatment at an *SE* of 24,000 kJ/kg TS (US1 assay) to obtain a more appropriate substrate for anaerobic digestion.

The anaerobic digestion of the pretreated substrate under the above-mentioned conditions (US1) was very stable. The kinetic constants of the anaerobic digestion of the solid and liquid fractions released after the different pretreatments conducted were virtually independent of the working conditions of the pretreatment.

Acknowledgements

The authors wish to express their gratitude to the Spanish Ministry of "Ciencia e Innovación" (Project CTM2008-05772/TECNO) for providing financial support, to the Program "FPI" of the above-mentioned Ministry for contracting graduates, to the contract JAE-Doc from "Junta para la Ampliación de Estudios del CSIC" co-financed by the European Social Funds, as well as to the "Junta de Andalucia" for supporting long-term research activities.

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Reference Type: Journal biotech
Author: M. A. De la Rubia, V. Fernández-Cegrí, F. Raposo and R. Borja
Year: 2011
Title: Influence of particle size and chemical composition on the performance and kinetics of anaerobic digestion process of sunflower oil cake in batch mode
Journal: Biochemical Engineering Journal
Volume: 58-59
Issue: 1
Pages: 162-167
Short Title: Influence of particle size and chemical composition on the performance and kinetics of anaerobic digestion process of sunflower oil cake in batch mode
ISSN: 1369703X (ISSN)
DOI: 10.1016/j.bej.2011.09.010
Notes: Cited By (since 1996): 3
Author Address: Instituto de la Grasa, Consejo Superior de Investigaciones Científicas (CSIC), Avenida Padre García Tejero, 4. 41012 Seville, Spain

Biochemical Engineering Journal 58–59 (2011) 162–167

Contents lists available at SciVerse ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Influence of particle size and chemical composition on the performance and kinetics of anaerobic digestion process of sunflower oil cake in batch mode

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ARTICLE INFO

Article history:
Received 4 April 2011
Received in revised form 7 September 2011
Accepted 10 September 2011
Available online 17 September 2011

Keywords:
Batch assay
Biochemical methane potential (BMP)
Kinetics
Lignocellulose
Particle size
Sunflower oil cake (SuOC)

ABSTRACT

Biochemical methane potential (BMP) assays of sunflower oil cake (SuOC) were carried out to research the effect of different particle sizes and their chemical composition on methane yields and kinetics. Particle size ranges of (1) 0.355–0.55 mm, (2) 0.710–1.0 mm and (3) 1.4–2.0 mm in diameter were evaluated. The highest methane yield 213 ± 8 mL CH_4 g^{-1} VS_{added} was obtained for the largest particle size analyzed (3), against 186 ± 6 mL CH_4 g^{-1} VS_{added} obtained for particles 1 and 2. This may be attributed to the different lignocellulose compositions of the various particle size ranges studied and to organic matter removals (47.2% for 3, against ~41.5% for 1 and 2). The evolution of propionic acid concentration was found to be fundamental for explaining the lowest rate of biogas production for the smallest (1) particle size studied, with a specific rate constant k of 0.45 ± 0.02 d^{-1}, while values of 0.61 ± 0.02 d^{-1} and 0.50 ± 0.01 d^{-1} were obtained for particles 2 and 3, respectively.

1. Introduction

The worldwide production of sunflower oil for 2008–2009 was 32.80 million tons with a production of around 42% of the by-product sunflower oil cake (SuOC), which means that 13.4 million tons of this waste were generated [1]. This waste has been used as animal feed as well as having other biotechnological applications [2]. However, laboratory-scale studies have recently been conducted to assess the feasibility of converting this residue into methane via conventional mesophilic digestion [2–4] or by two-stage processes [5], because its conversion to biogas is likely to be a two-part process of methane generation and residue treatment simultaneously.

A characteristic of SuOC is its high concentration of lignocellulosic material [2]. As is well known, the cellulose in the lignocellulosic polymeric form is not totally available for bacterial attack. Lignin surrounds the cellulose crystalline structure forming a 'seal' and protects the cellulose from being easily hydrolysed. Owing to the refractory structure of cellulose, one of the major problems in utilizing crop residues for stabilizing by anaerobic digestion is their low digestibility [6–8]. The anaerobic biodegradability and hence the biogas potential of a complex substrate depends on the content of biodegradable compounds: carbohydrates (including cellulose and hemicellulose), proteins and lipids [9].

It is generally accepted that hydrolysis is the rate-limiting step in the anaerobic digestion of organic vegetable solid waste. Due to the chemical and physical construction of lignocellulose, its microbial hydrolysis is a slow and difficult process. Furthermore, the surface area and particle size are important characteristics in determining its initial degradation rate [10].

The size of the feedstock should be reduced, otherwise it would result in the clogging of the digester and in the difficulty for microorganisms to digest it. A reduction in the size of the particles and the consequent enlargement of the available specific surface can support the biological process, in the event that there would be substrates with a high fibre content and low degradability, their comminution yielding an improved digester gas production [11]. This leads to a decreased amount of residues to be disposed of and to an increased quantity of useful digester gas [8].

Little research has been carried out into the effect of particle size of agricultural wastes on methane yield [12–15], and all of them were carried out by grinding, shredding, chopping or milling the residues as a physical pre-treatment. The lignocellulose structure was broken, thus enhancing the hydrolysis step. However, the lignocellulose composition of the different particle sizes can be different [16].

The influence of the substrate composition related to the different particle sizes on methane production has not previously been studied or reported in the literature. Therefore, the aim of this study was to determine the influence of particle size and chemical composition on the extent and rate of the anaerobic digestion process of SuOC. In this way, biochemical methane potential (BMP)

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1369-703X/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bej.2011.09.010

tests with three different particle sizes of SuOC (0.355–0.55 mm, 0.710–1.0 mm and 1.4–2.0 mm) have been conducted. Furthermore, a first order kinetic model has been used to obtain the specific rate constants of the processes while simultaneously studying its relationship with the particle size.

2. Methods

2.1. Substrate and anaerobic inoculum

Substrate: SuOC was collected from a sunflower oil factory located near Seville (Spain). The initial particle size of SuOC is tiny (only 11% was larger than 2 mm). As the effect of particle size on anaerobic process (methane yield) will be studied, the substrate was sieved and three fractions including mean size (**2**) 0.710–1.0 mm (the most abundant one), as well as one smaller (**1**) 0.355–0.55 mm and another larger (**3**) 1.4–2.0 mm were chosen. A commercial sieve (Restch AS 200 basic) was used to shred the substrate into different particle sizes. The SuOC was classified in different particle sizes by using screen meshes.

The full composition and main features as well as the fractional composition of the fibre of these three fractions of SuOC are shown in Table 1. The main components of the three particles sizes are cellulose and protein, which represents approximately 21–25% and 24–28% of dry matter, respectively.

Inoculum: The mixed anaerobic culture used as inoculum in the three experiments carried out was collected from a municipal wastewater treatment plant (MWTP) which operates in the anaerobic stabilization of primary and waste activated sludge. The main characteristics of this digested sludge are as follows: pH 7.6 ± 0.1, $33.3 \pm 2.4\,g\,L^{-1}$ of TS, and $17.9 \pm 0.5\,g\,L^{-1}$ of VS.

2.2. Experimental design

Anaerobic digestion experiments in batch mode are useful because they can be performed quickly with simple and inexpensive equipment, and are helpful in assessing the extent to which a material can be digested. The experimental design consisted of a multiflask batch system and was fully described elsewhere [2].

The reactors, which were maintained at $35 \pm 1\,^{\circ}C$ in a temperature-controlled water bath, were initially charged with the inoculum by keeping a concentration of $15\,g\,VS\,L^{-1}$ (the volume is a function of the initial VS concentration), the inoculum to substrate ratio (ISR) was maintained in 2 (VS basis), therefore $7.5\,g\,VS\,L^{-1}$ of SuOC were added to every batch reactor, for the three experiments carried out. 25 mL of stock mineral medium solution which composition has been described elsewhere [17], were also added, and finally, distilled water was added to achieve the desirable working volume of 250 mL. Reactors were flushed with N_2 in order to achieve anaerobic conditions.

The methane released was measured by volume displacement (the carbon dioxide was removed previously by flushing the gas through a 2 N NaOH solution), and expressed at standard temperature and pressure (STP) conditions. Methane production was monitored daily and calculated by subtracting the amount of methane produced by the blank controls (endogenous tests, with the inoculum alone added) from the methane production of each fed reactor.

Every experiment consisted in 14 fed SuOC replicates, 4 blank controls (two initials and two finals) and 2 cellulose positive controls. All the experiments were run for 7–8 days, until no significant gas production was observed, (last day the production was lower than 2% of the accumulate methane produced), suggesting that biodegradation was essentially completed, as control of cellulose ($370\,mL\,CH4\,g^{-1}\,VS_{added}$) confirmed. This short period of time was sufficient to achieve maximum methane production, and can basically be explained by the high activity of the sludge and the short interval between sampling the inoculum and the start-up of the experiments (less than 72 h).

2.3. Analytical methods

Solid sample: The following parameters were assayed in the substrate: total solids (TS) and volatile solids (VS), according to the standard methods 2540B and 2540E [18], respectively; total chemical oxygen demand (CODt) was determined using the reported method proposed by Raposo et al. [19]. Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined according to van Soest et al. [6], to calculate hemicellulose (NDF-ADF), cellulose (ADF-ADL) and lignin (ADL). The total carbohydrates (including fibre and soluble sugars) were calculated by the difference between the organic matter and lipids, protein and lignin content. Total Kjeldahl nitrogen (TKN) determination was also described elsewhere [3]. Total protein was determined by multiplying TKN value by 5.5 [20]. Fat content was extracted with hexane, using a Soxhlet system [21].

Inoculum: The inoculum and digestates were characterized sampling directly. pH (using a pH-meter model Crison 20 Basic), TS and VS were determined [18].

Soluble fraction: The supernatant obtained after centrifuging the digestates for 15 min at 10,000 rpm was filtered through a filter (0.45 μm) and used to characterize the following soluble parameters: chemical oxygen demand (CODs), using the closed digestion and colorimetric standard method 5220D [18]; soluble carbohydrates were analyzed according to the colorimetric method described by Dubois et al. [22]; total alkalinity (TA), which was measured by pH titration to 4.3. Soluble ammonia nitrogen (SAN) was determined by distillation and titration according to the standard method 4500E [18]. The volatile fatty acid (VFA) concentration was performed using a gas chromatograph, as previously described elsewhere [5].

Every one or two days, two of the digesters were sacrificed and their contents analyzed (one for VS analysis, using the whole working volume of the reactor (250 mL) with the purpose of avoiding possible error and the other one for the rest of the parameters).

To assess the organic matter balance in each BMP test system as a function of volatile solid removal (VS_{rem}) the following formula was used:

$$VS_{rem}(\%) = \left[\frac{VS_{added} - (VS_{final} - VS_{final-blank})}{VS_{added}}\right] \times 100 \quad (1)$$

where VS_{added} is the amount of VS added at the beginning of the assay, VS_{final} is the amount of VS at the end of the experiment and $VS_{final\text{-}blank}$ is the difference between the amount of VS of the sample and blank control at the end of experiment.

3. Results and discussion

3.1. Methane yield vs volatile solids removal

The degradation efficiency, expressed as VS_{rem} (Eq. (1)), achieved with particle sizes of 0.355–0.5 mm (**1**), 0.710–1.0 mm (**2**) and 1.4–2.0 mm (**3**) were 41.3%, 41.9% and 47.2%, respectively. This indicates that the degradation efficiency is very similar for particle sizes less than 1 mm, and comparable to that obtained by Raposo et al. [2] for a BMP experiment of SuOC using a mix of particle sizes less than 2 mm. By contrast, the degradation efficiency of particle size 1.4–2.0 mm was higher.

In the case at hand, the increase in the available specific surface achieved with the smallest particle size, which theoretically

Table 1
Composition and features of the different particle sizes of SuOC used as substrate.[a]

	Particle size (mm)		
	0.355–0.55	0.710–1.0	1.4–2.0
Dry matter (DM) (%)	93.1 (±0.1)	93.0 (±0.1)	93.8 (±0.1)
Volatile solids (%)[b]	93.8 (±0.8)	93.0 (±0.1)	92.8 (±0.7)
Ash (%)[b]	5.8 (±0.8)	6.8(±0.1)	6.7 (±0.1)
CODt ($g O_2 g^{-1}$ TS dry basis)	1.10(±0.01)	1.24(±0.02)	1.13(±0.03)
Neutral detergent fibre (%)[b]	42.9(±1.2)	45.0(±1.1)	35.4(±0.7)
Acid detergent fibre (%)[b]	33.8(±0.8)	38.4(±0.9)	30.2(±0.6)
Acid detergent Lignin (%)[b]	10.6(±0.3)	13.3(±0.2)	9.7(±0.2)
Hemicellulose (%)[b]	9.0(±1.1)	6.6(±1.0)	5.2(±0.6)
Cellulose (%)[b]	23.3(±0.7)	25.1(±0.4)	20.5(±0.4)
Total protein (%)[b]	23.7(±0.8)	25.3(±0.8)	28.1(±0.4)
Fat content (%)[b]	1.5(±0.2)	1.6(±0.2)	1.4(±0.3)
Soluble carbohydrates (%)[b]	4.9(±0.4)	5.1(±0.2)	6.2(±0.2)
Total carbohydrates (%)[b]	58.4(±0.5)	53.0(±0.3)	54.1(±0.3)

[a] Mean values are averages of four determinations (± standard deviations).
[b] Expressed as dry matter.

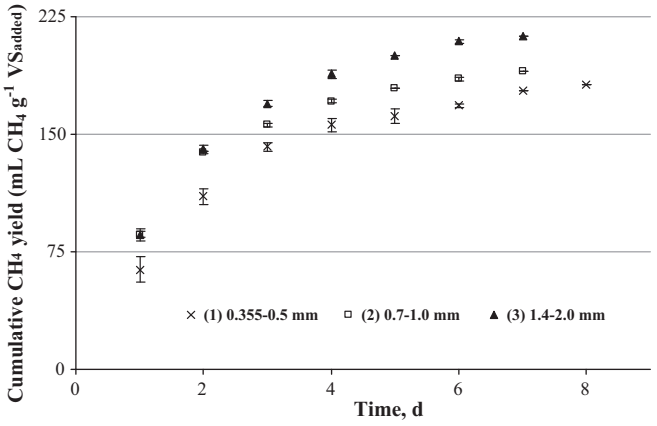

Fig. 1. Cumulative methane yield during batch anaerobic digestion of SuOC for the three different particle sizes studied.

improves the area where the microorganisms can reach and adhere, did not improve organic matter removal. This might be explained by the different chemical composition of the each fraction.

As expected [12], the highest volatile solid reduction corresponds to the highest methane yield obtained. Therefore, for particle sizes **1** and **2** the methane yields were very similar 182 ± 2 and 190 ± 4 mL CH_4 g^{-1} VS_{added}, respectively (Fig. 1). However, for particle size **3** the experimental methane yield was 213 ± 8 mL CH_4 g^{-1} VS_{added}. The differences in methane yields when varying the particle size were found to be statistically significant (*F*-test with 95% confidence limit). Therefore, an increase in the methane yield of 17% was achieved for particle size 1.4–2.0 mm as compared to particle size 0.355–0.55 mm. It seems that the enzymatic breakdown of SuOC does not increase with size reduction within the analyzed range.

Llabrés-Luengo and Mata-Álvarez [12] found increases of 4–5% of VS reduction when the particles sizes of wheat straw were reduced from 10 mm to 5 mm, and obtained an increase of only 4% in the methane yield.

Sharma et al. [13] studied 7 different kinds of raw materials to determine the effect of particle size on methane yield. The reduction from 6.0 mm to 0.088 mm meant increases in VS reduction lower than 4.5%. They also observed that for all feedstock studied, methane yield increased with decreasing particle size. However, in 5 of the 7 raw materials studied the methane yield was slightly higher for 0.40 mm than for 0.088 mm, which is in agreement with the results obtained in the present work.

Moorhead and Nordstedt [14] studied 3 different particle sizes of water hyacinth (1.6 mm, 6.4 mm and 12.7 mm) and found that the methane yields were similar for three sizes and ranged from 140 to 180 mL CH_4 g^{-1} VS_{added}, with the highest methane yield being obtained for material of 6.4 mm in size.

Although the ranges studied in the experiments carried out may seem very undersized, the reason is simple – after oil extraction, SuOC is a by-product that is small in size. So, it would be interesting to compare the results obtained with those achieved by other authors using the same size ranges. Angelidaki and Ahring [23] reported a potential increase of 4% in methane yield for macerated manure biofibres with 0.35 mm compared with fibres 2 mm in size. The methane yields obtained by Mshandete et al. [15] studying sisal fibre for particles with diameters of 2 mm and 5 mm were also very similar, 216 mL CH_4 g^{-1} VS_{added} and 205 mL CH_4 g^{-1} VS_{added}, respectively.

Izumi et al. [24] achieved higher methane yields (455 mL biogas g $CODt^{-1}$) for particle sizes of 0.7 mm than for 0.3 mm (404 mL biogas g $CODt^{-1}$) using food waste.

Therefore, in all the above-mentioned experimental studies, the biogas or methane yield was the same or slightly higher when the particle size diminished, except in the case described by Izumi et al. [24] who explained the lower biogas production to the smaller particle size, due to the fact that an accelerated hydrolysis and acidogenesis in the early stage of anaerobic digestion of food waste, resulting in accumulation of VFA.

In the case at hand, SuOC in the range 0.355–2.0 mm, the highest methane production was achieved for the largest particle size (1.4–2.0 mm). This can be explained because methane productivity not only depends on the amount of degraded volatile solids, but also on the nature (chemical composition) of the solids, because carbohydrates, proteins or fats have different methane potential [9], and their content is not uniform in the different particle size fractions, as has been stated previously by Gollakota and Meher [25].

Although grinding resulted in smaller particle sizes and consequently a higher surface area, enhancing the susceptibility of cellulose to bacterial and enzymatic attack, in this case, the highest particle size studied (1.4–2.0 mm fraction) presented the lowest NDF content (35.4 ± 0.7%) (Table 1). Therefore, the higher extent of substrate conversion of this highest particle size can be related with its higher solubility as well as the highest protein percentage (28.1 ± 0.4%), as was also stated previously by Sharma et al. [13].

The lower methane yield obtained in experiments with small and mean particle, as compared to large particle, is related with CODs as will be explained below. The lower protein content could also be the cause for the lower methane yield obtained with the particles small and mean [26].

3.2. Study of chemical control parameters

Traditionally, BMP assays focus exclusively on methane yield. Moreover, very little data is available from the literature on the evolution of chemical parameters for their comparison with the results obtained in the present experiments.

The evolution of the chemical-control parameters: VS, pH, TA, SAN, CODs, carbohydrates and total VFA (TVFA) in the digestate, has been outlined in Table 2.

pH. Methane is produced in the pH range 7.0–8.5, and the highest cellulose degradation efficiency obtained by Hu et al. [27] using ruminal microorganisms was achieved at pH 7.0–7.5. Consequently, the pH values found in the course of all experiments carried out (between 7.1 and 7.8), were not only typical values for stable mesophilic anaerobic digestion but also suitable to degrade cellulose and yield biogas.

Total alkalinity. The initial and final TA ranged from 3400 to 3920 mg $CaCO_3$ L^{-1} to 5120–5720 mg $CaCO_3$ L^{-1}, respectively. This means that the systems presented a high buffering capacity with an increase in the TA content for all cases studied, and that the particle size does not affect TA evolution.

Soluble ammonia nitrogen. SAN concentration increased noticeably for all experiments during the first two days. Over the next few days the increase was lower. As was stated before [5,28], degradation of complex organic material, including nitrogenous organic compounds during the hydrolytic step of anaerobic digestion results in the generation of ammonia. Therefore, in these first two days the hydrolytic phase occurred when the almost degradable protein was degraded and ammonia was generated. The net increase for every experiment (calculated as the difference between final and initial concentrations, taking into account the blank contribution) varied between 202 ± 32 mg N L^{-1} (large particle) and 235 ± 8 mg N L^{-1} (small particle). From these experimental results it could be concluded that the particle size ranges studied have almost no influence on the yield of the protein hydrolysis of SuOC, although the initial total protein composition was slightly higher (28.1 ± 0.4%) for the largest particle (1.4–2.0 mm) as compared to the smallest one (0.355–0.55 mm) (23.7 ± 0.8%).

Soluble carbohydrates. The initial average soluble carbohydrates concentrations for the three particle size ranges studied was 288 ± 8 mg L^{-1} (Table 2). However, at the end of the experiments, the final concentrations were 104 ± 3 mg L^{-1}, 43 ± 1 mg L^{-1} and 17 ± 5 mg L^{-1}, for small, mean and large particles, respectively. Since carbohydrates are easily utilized by anaerobic microorganisms, a low concentration of carbohydrates indicates that there was no accumulation in the anaerobic fermentation of SuOC, which occurred especially for a particle size of 1.4–2.0 mm.

CODs. The initial CODs for the blank controls were very similar for the three experiments (2300 ± 165 mg O_2 L^{-1}); however, at $t = 0$ the CODs for particle size **3** was 4718 ± 152 mg O_2 L^{-1}, against ~3800 mg O_2 L^{-1} obtained for particles **1** and **2**. These values were very revealing because the amount of CODs for a particle size of 1.4–2.0 mm was much higher than that obtained for mean and small particles, which is in agreement with the higher methane yield obtained for this particle size. This higher solubility of the largest particle size is related to initial substrate lignocellulosic composition (NDF 8–10% lower than obtained for particles **1** and **2**), evolution of carbohydrates concentration (commented above), and VFA concentration.

Volatile fatty acids. The rapid COD increase for 1.4–2.0 mm particle size assay resulted in a sharp rise in TVFA (related to a punctual low pH), which reflected the culminating moment of the hydrolytic stage, whereas the increase in TVFA for experiments 1 and 2 was lower as CODs increased.

Identification of the individual VFA formed is important, since it may provide valuable information on the metabolic pathways involved in the process [2]. As shown in Table 3, a significant amount of VFAs was produced during degradation of SuOC. The VFA distribution showed the influence of SuOC fraction on the fermentation process, and, therefore, on the composition and concentration of the different VFAs generated in the process. Acetic acid (HAc) and propionic acid (HPr) were found to be the two main VFAs for three particle sizes, especially during the first days of assay. The presence of VFA greater than i-HBu was related to the fermentation of proteins [29]. Taking into account that SuOC has a high protein content this explains their presence in the digestates. However, in all cases the individual VFA concentrations were low enough to avoid accumulation and inhibition problems. A similar VFA profile was observed in the anaerobic fermentation of maize [17].

The relevant data derived from the present study are summarized as follows:

- Particle size of 0.355–0.55 mm: the predominant VFA was HPr during the first 3 days, later the concentration of every individual fatty acid was lower than 37 mg L^{-1}. Therefore, no accumulation of VFA was observed, although the methane formation was slower due to slow HPr degradation for this particle size.
- Particle size of 0.710–1.0 mm: the highest concentration for HPr was obtained the first day ($t = 1$ d). After that, the concentration of HAc and HPr remain consistently low. The absence or very low level of HPr, i-HBu, HBu, HVa and i-HCa demonstrates that the methanogenic stage was not disturbed and the formation of methane from these intermediates was quick.
- Particle size of 1.4–2.0 mm: the predominant VFA during the first few days were HAc and HPr, followed by i-HVa and i-HBu. Scarce or no accumulation of HBu, HVa and HCa was observed in the VFA profile, whereas their respective iso-forms are difficult to convert and remained in the medium for longer periods of time, although no accumulation was observed.

3.3. Kinetic study

In order to characterize each experiment kinetically and, thus evaluate the effect of the particle size of SuOC on the methane yield, the following first-order kinetic equation for methane production can be used [30]:

$$G = G_m \times [1 - \exp(-k_0 \times t)] \quad (2)$$

where G (L) is the volume of methane gas accumulated at a given time; G_m (L) is the maximum volume accumulated at an infinite digestion time; k_0 (day^{-1}) is the specific rate constant and t (days) is the time. A similar model, that can be easily derived from Eq. (2) and has also been frequently applied to anaerobic digestion systems [31], was used to correlate the methane yield with the digestion time.

$$B = B_0 \times [1 - \exp(-k \times t)] \quad (3)$$

where B (mL CH_4 g^{-1} VS_{added}) is the cumulative methane yield, B_0 (mL CH_4 g^{-1} VS_{added}) is the maximum or ultimate methane yield of the substrate, k ($days^{-1}$) is the specific rate or apparent kinetic constant and t (days) is the time. Therefore, the ultimate methane yield gives the value when no more volume of gas from the reactor is released.

The adjustment by non-linear regression of the pairs of experimental data (B, t) using Sigmaplot software (version 9.0) allows the calculation of the apparent kinetic constant k. Table 4 lists the k values with 95% confidence limits obtained for each case studied, as well as B_0 and R^2.

The high values of the coefficient of determination, R^2 (>0.99 in all cases) and the low values of the confidence limits of the

Table 2
Evolution of chemical control parameters in the digestates at different particle sizes studied.

Experiment	Particle size (mm)	Time (d)	pH	TA ($mg\,CaCO_3\,L^{-1}$)	TVFA ($mg\,COD\,L^{-1}$)	SAN ($mg\,N\,L^{-1}$)	VS ($mg\,L^{-1}$)	CODs ($mg\,L^{-1}$)	Carbohydrate ($mg\,L^{-1}$)
		0	7.7	3920 ± 57	172 ± 2	896 ± 8	22.6	3804 ± 76	294 ± 8
		1	7.5	4760 ± 0	637 ± 3	1064 ± 16	21.6	3769 ± 10	86 ± 3
		2	7.3	4940 ± 85	525 ± 4	1137 ± 8	20.5	5054 ± 57	126 ± 2
(1)	0.355–0.55	3	7.3	5200 ± 0	399 ± 4	1182 ± 8	19.7	5108 ± 95	135 ± 3
		5	7.6	5340 ± 28	108 ± 2	1243 ± 16	19.2	5030 ± 38	133 ± 6
		6	7.6	5560 ± 0	116 ± 5	1266 ± 16	19.1	5134 ± 57	149 ± 2
		8	7.7	5720 ± 0	39 ± 3	1299 ± 16	18.3	5040 ± 38	104 ± 3
		0	7.5	3400 ± 0	194 ± 3	762 ± 0	22.2	3878 ± 102	292 ± 8
		1	7.2	4380 ± 28	384 ± 6	952 ± 0	21.0	4530 ± 76	91 ± 3
		2	7.5	4600 ± 0	63 ± 2	1014 ± 8	20.2	4207 ± 114	113 ± 2
(2)	0.710–1.0	3	7.3	4640 ± 0	57 ± 3	1042 ± 0	19.6	5081 ± 228	59 ± 5
		5	7.6	5060 ± 28	59 ± 1	1103 ± 8	19.7	4758 ± 95	50 ± 2
		6	7.6	5080 ± 57	61 ± 1	1114 ± 8	19.2	4772 ± 10	61 ± 3
		7	7.8	5120 ± 0	61 ± 2	1148 ± 8	18.8	5121 ± 133	43 ± 1
		0	7.2	3760 ± 57	182 ± 3	890 ± 24	22.0	4718 ± 172	278 ± 7
		1	7.1	4560 ± 0	1360 ± 15	1086 ± 0	20.4	5524 ± 56	32 ± 4
		2	7.5	5080 ± 0	645 ± 5	1120 ± 0	19.7	5161 ± 209	36 ± 9
(3)	1.4–2.0	3	7.7	5300 ± 28	116 ± 3	1159 ± 8	19.4	5269 ± 323	25 ± 1
		4	7.4	5280 ± 0	114 ± 4	1204 ± 8	19.0	4619 ± 38	20 ± 3
		6	7.8	5520 ± 0	112 ± 2	1243 ± 0	18.7	4798 ± 342	21 ± 2
		7	7.6	5580 ± 28	36 ± 3	1238 ± 55	18.4	5054 ± 19	17 ± 5

Table 3
Time course variations of individual VFAs in the digestate for different particle size studied. [a]

Experiment	Particle size (mm)	Time (d)	HAc ($mg\,L^{-1}$)	HPr ($mg\,L^{-1}$)	i-HBu ($mg\,L^{-1}$)	HBu ($mg\,L^{-1}$)	i-HVa ($mg\,L^{-1}$)	HVa ($mg\,L^{-1}$)	i-HCa ($mg\,L^{-1}$)
(1)	0.355–0.5	0	47	15	10	14	13	13	–
		1	110	242	30	10	27	13	–
		2	51	251	20	–	14	12	–
		3	35	146	33	10	18	13	–
		5	37	–	20	–	16	–	–
		6	34	5	25	–	13	–	–
		8	37	–	–	–	–	–	–
(2)	0.710–1.0	0	62	29	13	17	15	–	–
		1	42	163	18	–	17	12	–
		2	51	6	–	–	–	–	–
		3	47	5	–	–	–	–	–
		5	49	5	–	–	–	–	–
		6	49	6	–	–	–	–	–
		7	49	6	–	–	–	–	–
		0	46	21	13	15	13	11	–
		1	140	528	60	15	90	15	28
		2	26	291	41	13	27	12	–
(3)	1.4–2.0	3	22	7	30	–	14	–	–
		4	24	13	38	–	–	–	–
		6	32	11	34	–	–	–	–
		7	22	8	–	–	–	–	–

(–) Not detected.
[a] HCa and HEn were not detected in any samples.

Table 4
k and B_0 values with 95% confidence limits for each experiment carried out.

Experiment (particle size (mm))	k (d^{-1})	B_0 ($mL\,CH_4\,g^{-1}\,VS_{added}$)	R^2
0.355–0.55	0.45 ± 0.02	184 ± 3	0.9975
0.710–0.1	0.61 ± 0.02	189 ± 2	0.9983
1.4–2	0.50 ± 0.01	218 ± 1	0.9997

parameters obtained demonstrates how well the experimental data adapted to the model proposed.

From the results obtained it can be observed that the apparent kinetic constants of the process are related to the evolution of VFA concentration in general and HPr in particular. The highest k value ($0.61 \pm 0.02\,d^{-1}$) was obtained for the 0.710–1.0 mm particle size assay, where the HPr concentration was $29\,mg\,L^{-1}$ at $t = 0$ and $163\,mg\,L^{-1}$ at $t = 1$, decreasing rapidly to values $\leq 6\,mg\,L^{-1}$ at $t = 2$ until the end of the process. Although the highest HPr concentration was obtained at $t = 1$ during the assay of particle size 1.4–2.0 mm, at $t = 3$ the concentration dropped drastically until $7\,mg\,L^{-1}$, so the second k value ($0.50 \pm 0.01\,d^{-1}$), was obtained for this assay. Finally, the lowest k value ($0.45 \pm 0.02\,d^{-1}$) was obtained for the smallest particle size studied (0.355–0.55 mm), observing for this case at $t = 3$ days the highest value of HPr, $146\,mg\,L^{-1}$.

4. Conclusions

Batch anaerobic digestion experiments of SuOC with different particle sizes revealed that this did not affect final pH, total alkalinity, soluble ammonia nitrogen or CODs, although the largest size (1.4–2.0 mm) within the range studied (0.355–2.0 mm) made it possible to achieve the highest methane yield, 213 ± 8 mL CH_4 g^{-1} VS_{added}, when compared with particle sizes of 0.355–0.55 mm and 0.710–1.0 mm, for which 182 ± 2 and 190 ± 4 mL CH_4 g^{-1} VS_{added}, respectively, were achieved. This can be attributed to the different chemical initial composition of the different particle size fractions, which also explain the different TVFA evolution. Therefore, optimizing the size reduction of SuOC could potentially improve the methane yield of anaerobic digestion process of this substrate.

A first order kinetic model was used to obtain the specific rate constant of each size range; the slow HPr removal could explain the lowest k value (0.45 d^{-1}) obtained for the smallest particle size studied.

Acknowledgements

The authors wish to express their gratitude to the Spanish Ministry of "Ciencia and Innovación" (Project CTM 2008-05772/Tecno) for providing financial support, to the contract JAE-Doc from "Junta para la Ampliación de Estudios del CSIC" co-financed by the European Social Funds, as well as to the Program "FPI" of the above-mentioned ministry for contracting graduates.

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Reference Type: Journal biotech
Author: M. A. De La Rubia, V. Fernández-Cegrí, F. Raposo and R. Borja
Year: 2013
Title: Anaerobic digestion of solid organic substrates in batch mode: An overview relating to methane yields and experimental procedures
Journal: Water Science & Technology
Volume: 67
Issue: 2
Pages: 410-417
Short Title: Anaerobic digestion of sunflower oil cake: a current overview
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Anaerobic digestion of sunflower oil cake: a current overview

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ABSTRACT

Due to the chemical and physical structure of a lignocellulosic biomass, its anaerobic digestion (AD) is a slow and difficult process. In this paper, the results obtained from a batch biochemical methane potential (BMP) test and fed-batch mesophilic AD assays of sunflower oil cake (SuOC) are presented. Taking into account the low digestibility shown during one-stage experiments the methane yield decreased considerably after increasing the organic loading rate (OLR) from 2 to 3 g VS L^{-1} d^{-1}, SuOC was subjected to a two-stage AD process (hydrolytic-acidogenic and methanogenic stages), in two separate reactors operating in series where the methanogenic stage became acidified (with >1,600 mg acetic acid L^{-1}) at an OLR as low as 2 g VS L^{-1} d^{-1}. More recently, BMP assays were carried out after mechanical, thermal, and ultrasonic pre-treatments to determine the best option on the basis of the methane yield obtained.

Key words | batch assay, biochemical methane potential (BMP), fed-batch assays, pre-treatment, sunflower oil cake (SuOC), two-stage anaerobic digestion

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INTRODUCTION

Sunflower oil cake (SuOC) is the part of whole sunflower seeds which remains after the oil extraction process. It is an agro-industrial residue generated in Spain in great quantities (about 4–5 million tons per year). This extracted flour is mainly composed of fibre and protein (Vioque *et al.* 2001).

This flour has been generally used for cattle feed (Szabo *et al.* 2001; Torrijos *et al.* 2008); nevertheless it represents one of the reservoirs of proteins with major potential for the food industry (Vioque *et al.* 2001). Other applications for the extracted sunflower flour have been in the preparation of antibiotics (Kota & Sridhar 1999) and some enzymes (Proteases) (Pandey *et al.* 2000). Nevertheless, the scarce and limited applications of the different methods of re-use of these wastes and their high production justify the study of other processes or alternatives that enable their utilization and reuse.

Anaerobic digestion (AD) is the most effective process for the treatment and stabilization of organic wastes such as SuOC, offering the advantage of a net energy gain by producing methane. Moreover, the AD process can be improved by means of a process in two stages (De la Rubia *et al.* 2009), as the stability of the global process remains awkward when imbalances take place between the activity of the groups of microorganisms that carry out the first phase of hydrolysis of the high molecular weight compounds and acidification of the resulting monomers (acidogenic stage) and those that, in the second phase, metabolize the acids formed to methane (methanogenic stage). On the other hand, owing to the refractory structure of the lignocellulosic biomass the efficiency of AD to treat agriculture residues is limited. Although cellulose and hemicellulose can be degraded under anaerobic conditions, lignin (undegradable in biogas processes) prevents enzyme accessibility to cellulose (Zhu *et al.* 2008). While hemicellulose serves as a connection between the lignin and the cellulose fibres and gives the whole cellulose–hemicellulose–lignin network more rigidity. Therefore, only a low fraction of lignocellulosic biomass can be converted into biogas.

Hence, the pre-treatment of the lignocellulosic biomass is crucial to remove lignin and hemicellulose and make cellulose more accessible to the enzymes that convert carbohydrate polymers into fermentable sugars (Mosier *et al.* 2005; Pérez *et al.* 2007) and, therefore, to increase the biogas potential. Some physical, physico-chemical, chemical, and biological processes have been used for the

doi: 10.2166/wst.2012.586

pre-treatment of lignocellulosic materials, not only to remove the inhibitory lignin complex but also to reduce cellulose crystallinity, which is a major limit for cellulose hydrolysis (Jeihanipour *et al.* 2010).

Since 2005, the 'Reuse of Wastes and Wastewater Treatment Group', of the Instituto de la Grasa (IG) of the Spanish National Research Council (CSIC) has been studying the anaerobic stabilization of SuOC. During these years batch and fed-batch (one and two stage) experiments have been carried out. Recently, a combination of thermal, mechanical and ultrasonic pre-treatments and batch anaerobic assays has been assessed. Finally, the best option (ultrasound pre-treatment) has been chosen to study a combined ultrasound pre-treatment and one-stage AD of SuOC, which is currently being carried out. In this paper the most relevant results obtained during the above-mentioned experiments are summarized.

MATERIAL AND METHODS

Raw material

SuOC was collected from a sunflower oil factory located near Seville (Spain). Prior to using the substrate, it was sieved to give a fraction with a particle size lower than 2 mm (around 90% of the total particles of the SuOC had this size). The full composition and main features of the SuOC used have been described elsewhere (Raposo *et al.* 2008a).

Inocula

Two kinds of inocula were used in the different assays conducted.

Granular sludge (GS) was taken from an industrial upflow anaerobic sludge blanket (UASB) reactor which treats brewery wastewater. The main characteristics of this anaerobic sludge were: pH, 7.6 ± 0.1; total solids (TS), 60 ± 3 g L^{-1}; volatile solids (VS), 45 ± 2 g L^{-1}.

Sewage sludge (SS), a mixed anaerobic culture, was collected from a municipal wastewater treatment plant which operates in the anaerobic stabilization of primary and waste activated sludge. The main characteristics of this digested sludge were: pH, 7.6 ± 0.1; 33 ± 2 g L^{-1} of TS, and 18 ± 1 g L^{-1} of VS.

Experimental design

The experiments carried out have been summarized in Table 1 and/or in the following list:

1st: Biochemical methane potential (BMP) using SuOC as a substrate and GS as inoculum. The effect of inoculum to substrate ratio (ISR), expressed as VS basis, was studied in this set of experiments.

2nd: One-stage fed-batch experiments using SuOC as a substrate and the two previous inocula described (GS and SS). Organic loading rates (OLRs) of 1, 2 and 3 g VS L^{-1} d^{-1} were assayed.

3rd: Hydrolytic-acidogenic (H-A) fed-batch experiments using SuOC as a substrate and the inoculum GS. Six

Table 1 | Anaerobic digestion experiments conducted with SuOC as substrate without pre-treatment

Experiment								
					Two stages			
BMP	One-stage fed-batch[a]		Hydrolytic-Acidogenic[b]		Hydrolytic-Acidogenic		Methanogenic	
ISR	OLR g VS L^{-1} d^{-1}	HRT d	OLR g VS L^{-1} d^{-1}	HRT d	OLR g VS L^{-1} d^{-1}	HRT d	OLR g VS L^{-1} d^{-1}	HRT d
0.5	1	25	4	8, 10, 12, 15	6	10	1	42
0.8	2		5				1.5	28
1	3		6				2	21
1.5			7				2.5	16
2			8		8	10	1	33
3			9				1.5	22
							2	16

[a]Experiments were developed at different OLR but at the same HRT.
[b]Every OLR (4, 5, 6, 7, 8, 9) was assayed at every HRT (8, 10, 12, 15).

different OLRs from 4 to 9 g VS L^{-1} d^{-1} and four hydraulic retention times (HRTs) of 8, 10, 12 and 15 days were studied.

4th: Two-stage (H-A and methanogenic) fed-batch experiments using SuOC as a substrate and the two inocula previously described. After optimizing the H-A stage (OLR of 6 and 8 g VS L^{-1} d^{-1} and HRT of 10 days) the methanogenic reactors were fed with the effluent obtained in the first stage. OLRs of 1, 1.5, 2 and 2.5 g VS L^{-1} d^{-1} were assayed in this second methanogenic stage.

5th: BMP using pre-treated SuOC as a substrate and the two inocula mentioned above. The following pre-treatments were assayed:

- Mechanical (sieve): The SuOC $\leq$ 2 mm was sieved and three different fractions: 0.355–0.55 mm, 0.71–1.0 mm, and 1.4–2.0 mm were chosen to be assayed.
- Thermal: A 2% (w/v) SuOC suspension was treated for 4 h at ambient temperature (AT), 100, 150 and 200 °C.
- Ultrasound: A 2% (w/v) SuOC suspension was treated with an ultrasound frequency of 20 kHz and a supplied power of 120 W. Five specific energies (SE) were supplied, ranging from 24,000 to 597,600 kJ kg TS^{-1} and obtained by increasing the operation time.

Equipment

BMP assays

The experimental design consisted of a multiflask batch system which was fully described elsewhere (Raposo *et al.* 2008a). The reactors, which were maintained at 35 ± 1 °C in a temperature-controlled water bath, were initially charged with the inoculum by keeping a concentration of 15 g VS L^{-1}. The ISR was maintained at 2, except in the experiments to study the effect of ISR. A stock mineral medium solution whose composition has been described elsewhere (Raposo *et al.* 2006) was also added, and finally distilled water was added to achieve the desirable working volume of 250 mL. Reactors were flushed with N_2 in order to achieve and maintain anaerobic conditions.

The methane released was measured by volume displacement (the carbon dioxide was removed previously by flushing the gas through a 2N NaOH solution), and expressed at standard temperature and pressure conditions. Methane production was monitored daily and calculated by subtracting the amount of methane produced by the blank controls (endogenous tests, with the inoculum alone added) from the methane production of each fed reactor.

All the experiments were run for 7–8 days, until no significant gas production was observed, suggesting that biodegradation was essentially completed, as a control of cellulose (~310 mL CH_4 g^{-1} COD_{added}) also confirmed. Each experimental setup was performed in triplicate.

Fed-batch assays

Experiments were carried out in four completely mixed glass digesters, each one with a total volume of 2.5 L and a working volume of 2 L. The reactors were mixed using magnetic bars and an adjustable stirrer at 700 rpm. The digesters, maintained at 35 ± 1 °C in a temperature-controlled water bath, were started with an inoculum concentration of 17 g VS L^{-1}. Nitrogen gas was used and sparged to maintain anaerobic conditions before starting the experiments and after each feed.

Analytical methods

The chemical compositions of the raw material, inocula and digestates were determined:

- *Raw material*: The following parameters were analysed in the substrate: TS and VS, according to the Standard Methods 2540B and 2540E (APHA 1998), respectively; total chemical oxygen demand (CODt) was determined using the method proposed by Raposo *et al.* (2008b). Total Kjeldahl nitrogen (TKN) determination was also described elsewhere (Raposo *et al.* 2009).
- *Inocula*: The inocula and digestates were characterized by direct sampling. The pH (using a pH meter model Crison 20 Basic), TS and VS were determined (APHA 1998).
- *Soluble fraction*: The supernatant obtained after centrifuging the inocula and digestates for 15 min at 10,000 rpm was filtered (0.45 μm) and used to characterize the following parameters: (i) soluble chemical oxygen demand (CODs), using the closed digestion and colorimetric Standard Method 5220D (APHA 1998); (ii) total alkalinity, which was measured by pH titration to 4.3; (iii) soluble ammonia nitrogen determined by distillation and titration according to the standard method 4500E (APHA 1998); and (iv) the volatile fatty acids (VFA) concentration determined using a gas

chromatograph, as previously described elsewhere (De la Rubia *et al*. 2009).

RESULTS AND DISCUSSION

One stage

BMP assays of untreated SuOC

In order to determine the BMP of SuOC, the influence of ISRs and the evolution and variation of the chemical control parameters of the process with digestion time, different batch assays were conducted.

The results from this study suggest that SuOC is a potential substrate for AD. Batch experiments carried out at mesophilic temperatures and at ISRs of 3.0, 2.0, 1.5, 1.0, 0.8 and 0.5 demonstrated that the ultimate methane yield decreased considerably from 193 ± 19 mL CH_4 g^{-1} COD_{added} to 91 ± 9 mL CH_4 g^{-1} COD_{added} when the ISR decreased from 3.0 to 0.5, showing a marked influence of this parameter on the methane yield. However, the net VS removed only varied from 42 to 36% when the ISR decreased from 3.0 to 0.5. A considerable increase in CODs due mainly to an accumulation of VFA in the digestates was observed at ISRs of 0.5 and 0.8, which demonstrated a clear imbalance of the process, typical of stress on methanogenic microorganisms. The lower the ISRs, the greater the accumulation of the longer chain VFA, and only the ISRs of 2 and 3 were allowed to obtain digestates with no residual VFA at the end of the digestion time, as can be seen in Figure 1. Therefore, on the basis of the results obtained in the BMP test, an ISR over 2.0 is suggested and recommended in order to prevent acidification and an imbalance of the AD process of this substrate (VDI 4630 2006; Raposo *et al*. 2008a, 2012).

Fed-batch anaerobic digestion of SuOC

Once it was determined that SuOC was a potential substrate for AD, fed-batch anaerobic experiments at OLRs of 1, 2 and 3 g VS L^{-1} d^{-1} and HRT of 25 days were carried out. After the start-up step, the reactors were subjected to a programmed steady-state operation, using the mentioned OLRs. The attainment of the steady-state was verified after a period equivalent to 2–3 times the HRT by checking whether constant effluent characteristic values (TS, VS, COD and VFA levels) were achieved. The sampling during each steady-state period was performed for five consecutive days.

Taking into account the results obtained during this study, shown in Table 2, it can be stated that the activity of acidogenic microorganisms exceeded the activity of the methanogenic organisms when the OLR was increased from 2 to 3 g VS L^{-1} d^{-1}, because VFA were accumulated and reached values higher than 1,500 mg acetic acid L^{-1}. The reactor was overloaded: to be specific, the methane yield diminished from 149 ± 5 mL CH_4 g^{-1} COD_{added} to 101 ± 5 mL CH_4 g^{-1} COD_{added} when OLR was increased from 2 to 3 g VS L^{-1} d^{-1}. Because acidification occurred, the feeding was stopped before reaching a total imbalance of the process.

As Demirer & Chen (2005) stated, conventional one-stage digestion was not an effective system for wastes containing high solid concentrations, as SuOC.

Two stages

Acidogenic microorganisms and the methanogens constitute two very different groups in terms of their

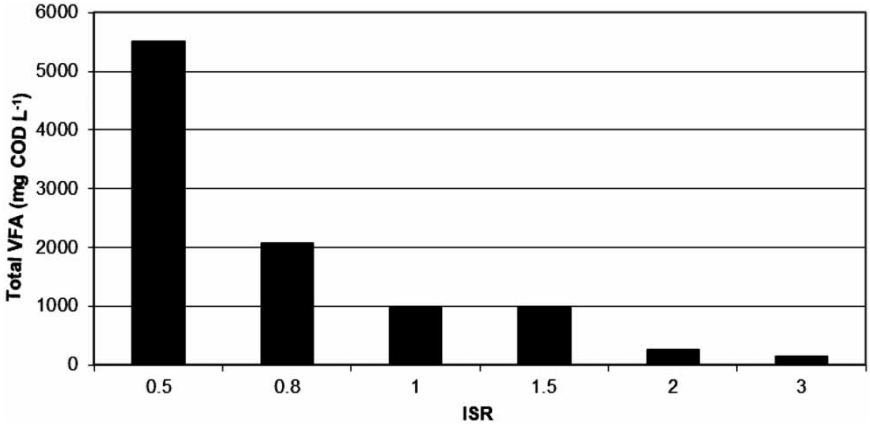

Figure 1 | Total volatile fatty acid concentration evolution with inoculum substrate ratio increase, after biochemical methane potential assays. Values are averages from three trials.

Table 2 | Methane yield and total VFA (TVFA) concentration for each experiment conducted in one and two stages[a]

One stage			Two stages						
				Methanogenic I			Methanogenic II		
OLR g VS L^{-1} d^{-1}	CH_4 mL g^{-1} COD_{added}	TVFA mg C_2 L^{-1}	OLR VS L^{-1} d^{-1}	HRT d	CH_4 mL g^{-1} COD_{added}	TVFA mg C_2 L^{-1}	HRT d	CH_4 mL g^{-1} COD_{added}	TVFA mg C_2 L^{-1}
1	136 ± 8	214 ± 27	1	45	109 ± 13	652 ± 131	36	117 ± 4	524 ± 54
			1.5	28	141 ± 6	342 ± 43	22	141 ± 6	340 ± 52
2	149 ± 5	585 ± 87	2	21	149 ± 8	607 ± 67	16	95 ± 1	1,620 ± 41
			2.5	16	48 ± 7	5,215 ± 52	–	–	–
3	101 ± 5	1,566 ± 195							

[a]Average values from five trials ± standard deviation of the mean values ($p < 0.05$).

growth kinetics, requirements for nutrients, optimum pH and capacity to support and maintain their ideal conditions before situations of overloading or 'stress' occur. Moreover, it is generally accepted that hydrolysis is the rate-limiting step in the AD of vegetable solid waste. On this basis, a process carried out in two stages can optimize the operative conditions of every step and give major stability to the global process.

By means of these experiments the suitable values of the HRT and OLR, which resulted in maximum efficiencies of elimination of organic matter accompanied with a maximum production of VFA in the first reactor and maximum methane yield coefficients in the second, were obtained.

A relevant feature of the two-stage AD approach is that when a high solid containing waste is introduced into the first stage, it is liquefied along with acidification.

Hydrolytic-acidogenic stage

In this study the effect of the variations of HRT and OLR on CODs and VFA production to improve the H-A step of the AD of SuOC was studied (De la Rubia *et al.* 2009).

During the mesophilic acidogenic fermentation of SuOC, variations in the HRT did not affect the COD solubilization of this substrate within the HRT range (15–8 days) studied. Variations in OLR affected the organic matter liquefaction slightly, with the highest value (30.1%) being reached at an HRT of 10 days and an OLR of 8 g VS L^{-1} d^{-1}. The organic matter liquefaction or hydrolysis yield can be defined by the following equation:

$$\text{Hydrolysis yield} = \frac{S_S}{S_I} \times 100$$

where S_I is the initial total substrate concentration (calculated by means of the quotient: (CODt g SuOC)/(volume related to the corresponding HRT) where CODt is the COD concentration of solid substrate: 1.1 g COD g^{-1} TS) and S_S is the soluble output COD.

The acidification yield increased with an OLR of up to 6 g VS L^{-1} d^{-1}, the highest value (83.8%) being achieved for an HRT of 10 days and an OLR of 6 g VS L^{-1} d^{-1}. However, higher OLR produced a decrease in the acidification yield, probably due to the fact that the acidogenic bacteria could have been affected and inhibited at the highest OLR studied.

Methanogenic stage

The effluents obtained under the optima OLR (6 g VS L^{-1} d^{-1}) and HRTs (8 and 10 days) of the H-A stage were treated in the methanogenic reactors to determine the optimum operational parameters. With the effluent of reactor H-AI, operated at an OLR of 6 g VS L^{-1} d^{-1} and HRT of 8 days, the methanogenic reactor MI was fed. Four different OLRs were assayed for this second stage: 1, 1.5, 2 and 2.5 g VS L^{-1} d^{-1}, at HRTs of 45, 28, 21 and 16 days, respectively, as can be seen in Table 2. The reactor MII was fed with the effluent of H-AII (operated at OLR of 6 g VS L^{-1} d^{-1} and 10 days of HRT); with this reactor three OLRs and HRTs were used: 1, 1.5 and 2 g VS L^{-1} d^{-1}, and 36, 22 and 16 days, respectively.

The best results were obtained when the methanogenic reactors were operated at HRT between 21 and 28 days, and OLR of 1.5 and 2 g VS L^{-1} d^{-1} (Table 2). At an HRT of 16 days, the methanogenic activity was clearly inhibited. This was shown by the methane yield drop, for both methanogenic reactors, and the high VFA concentration achieved, which varied between 1,600 and 5,200 mg acetic acid L^{-1}, for OLR of 2 and 2.5 g VS L^{-1} d^{-1}, respectively.

Consequently, neither the one-stage nor the two-stage mesophilic AD processes were able to efficiently degrade SuOC at an OLR higher than 2 g VS L^{-1} d^{-1}.

Pre-treatments

Pre-treatments are frequently used to facilitate the methane production by overcoming the limitation of hydrolysis, which includes the solubilization and biodegradation of hemicellulosic and lignin fractions of the substrates. Taking into account the above-stated difficulty of SuOC to be anaerobically degraded, combinations of mechanical, thermal, and ultrasonic pre-treatments and AD processes in batch mode were assessed.

To evaluate the efficiency of the above-mentioned pre-treatments, with the aim of achieving a maximum solubilization level by comparing their capacity for converting the complex organic compounds present in the waste into simpler compounds that can be easily biodegradable by AD processes, BMP experiments were carried out.

Thermal and ultrasound pre-treatments involve the addition of water to the substrate to be pre-treated; therefore after pre-treatments of SuOC two fractions are obtained: a water-insoluble solid fraction and a liquid fraction. Both of these fractions were separated and evaluated individually. The results are compared in Table 3.

Mechanical pre-treatment

Batch AD experiments of SuOC with different particle sizes (0.355–0.55, 0.71–1.0 and 1.4–2.0 mm) revealed that this parameter affects methane yield. In this way, the largest size (1.4–2.0 mm) within the range studied (0.355–2.0 mm) resulted in the highest methane yield, 175 ± 7 mL CH_4 g^{-1} COD_{added}, when compared with particle sizes of 0.355–0.55 and 0.71–1.0 mm, for which 143 ± 3 and 155 ± 2 mL CH_4 g^{-1} COD_{added}, respectively, were reached. This could be attributed to the different initial chemical composition of the different fractions (De la Rubia *et al.* 2011). Therefore, optimizing the size reduction of SuOC could potentially improve the methane yield of the AD process of this substrate.

Thermal pre-treatment

- Solid fraction: The highest methane production was obtained for SuOC pre-treated at AT (114 ± 9 mL CH_4 g^{-1} COD_{added}). This is because at this low temperature some soluble compounds still remained in the solid fraction, which can be degraded during BMP assays. The lowest methane yield was obtained at 200 °C (53 ± 8 mL CH_4 g^{-1} COD_{added}). Therefore, the higher the temperature applied, the lower the methane yield obtained for this fraction.
- Liquid fraction: In this case the best results were obtained at 100 °C (310 ± 4 mL CH_4 g^{-1} COD_{added}). The sample treated at AT resulted in 276 ± 6 mL CH_4 g^{-1} COD_{added}, while at 150 and 200 °C the methane yield decreased to 220 ± 15 and 247 ± 10 mL CH_4 g^{-1} COD_{added}, respectively. Hence, temperatures above 150 °C produced the formation of non-degradable or toxic compounds, which brought about a potential inhibition for the growth of bacteria and *Archaea* due to their lethal nature.

From the results obtained it can be stated that 100 °C is the best temperature to thermally pre-treat SuOC before AD.

Ultrasound pre-treatment

- Solid fraction: SuOC pre-treated by ultrasound obtained the highest methane production of 111 mL CH_4 g^{-1} COD_{added} for an SE of 24,000 kJ kg^{-1} TS. A higher SE brought about a lower methane yield.

Table 3 | Ultimate CH_4 yield obtained after the different pre-treatments studied

Pre-treatment						
Mechanical – particle size (mm)		**0.355–0.55**	**0.71–1.0**	**1.4–2.0**		
mL CH_4 g^{-1} COD_{added}		143 ± 3	155 ± 2	175 ± 7		
Thermal (°C)	**Fraction**	**AT**	**100 °C**	**150 °C**	**200 °C**	
mL CH_4 g^{-1} COD_{added}	Solid	114 ± 9	105 ± 7	82 ± 7	53 ± 8	
	Liquid	276 ± 6	310 ± 4	220 ± 15	247 ± 10	
Ultrasound (kJ kg^{-1} TS)	**Fraction**	**SE-1**	**SE-2**	**SE-3**	**SE-4**	**SE-5**
mL CH_4 g^{-1} COD_{added}	Solid	111 ± 5	107 ± 4	103 ± 4	103 ± 5	90 ± 4
	Liquid	330 ± 16	297 ± 8	270 ± 10	312 ± 11	312 ± 13

*Average values are from three trials ± standard deviation of the mean values ($p < 0.05$).

- Liquid fraction: the methane yield obtained for this fraction ranged between 270 ± 13 mL CH_4 g^{-1} COD_{added} (for SE of 597,600 kJ kg^{-1} TS) and 330 ± 16 mL CH_4 g^{-1} COD_{added} (for SE of 24,000 kJ kg^{-1} TS), showing that an increase in the ultrasound time did not improve the solubilization of compounds which are not easily degraded.

The final values of the TVFA were very low for both the solid and liquid fraction digestates after the three pre-treatments studied, with values in the range of 5–16 mg acetic acid L^{-1}. This means that the overall anaerobic process was conducted satisfactorily and a correct balance of the process occurred. Moreover, results from the ultrasound study, and when compared with the other two pre-treatments studied, demonstrate the suitability of the ultrasonic pre-treatment of SuOC for increasing the anaerobic biodegradability of this substrate and methane yield coefficient.

The different pre-treatments used may promote methane production because the AD of SuOC without pre-treatment is a slow and difficult process which becomes acidified at a low OLR, even when the H-A and methanogenic stages are separated in two different reactors that operate in series.

Conclusions and recommendations

Although the results obtained after BMP assays suggest that SuOC was a potential substrate for AD, neither the one-stage nor the two-stage mesophilic AD process was able to efficiently degrade SuOC at an OLR higher than 2 g VS L^{-1} d^{-1}.

A temperature of 100 °C for thermal pre-treatment and an SE of 24,000 kJ kg^{-1} TS for ultrasound pre-treatment were the best conditions among those assayed, obtaining similar mean methane yields (average of the solid and liquid fractions): 208 and 220 mL CH_4 g^{-1} COD_{added}, for thermal (100 °C) and ultrasound (24,000 kJ kg^{-1} TS) pre-treatment, respectively. The energetic cost necessary to treat SuOC by thermal pre-treatment (4 h at 100 °C) is much higher than that needed for ultrasound, where only 16 min and 120 W of power are necessary. Therefore, these ultrasound conditions were chosen to conduct fed-batch experiments with pre-treated SuOC.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to the Spanish Ministry of 'Ciencia e Innovación' (Project CTM 2008–05772/Tecno) for providing financial support, to the contract JAE-Doc from 'Junta para la Ampliación de Estudios del CSIC', co-financed by European Social Funds, as well as to the Program 'FPI' of the above-mentioned Ministry for contracting graduates.

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First received 29 February 2012; accepted in revised form 31 August 2012

Reference Type: Journal biotech
Author: F. Raposo, M. A. De La Rubia, V. Fernández-Cegrí and R. Borja
Year: 2012
Title: Anaerobic digestion of solid organic substrates in batch mode: An overview relating to methane yields and experimental procedures
Journal: Renewable and Sustainable Energy Reviews
Volume: 16
Issue: 1
Pages: 861-877
Short Title: Anaerobic digestion of solid organic substrates in batch mode: An overview relating to methane yields and experimental procedures
ISSN: 13640321 (ISSN)
DOI: 10.1016/j.rser.2011.09.008
Notes: Cited By (since 1996): 7
Author Address: Consejo Superior de Investigaciones Científicas-Instituto de la Grasa (CSIC-IG), Avenida Padre García Tejero 4, 41012 Seville, Spain

Renewable and Sustainable Energy Reviews 16 (2011) 861–877

Contents lists available at SciVerse ScienceDirect

Renewable and Sustainable Energy Reviews

journal homepage: www.elsevier.com/locate/rser

Anaerobic digestion of solid organic substrates in batch mode: An overview relating to methane yields and experimental procedures

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ARTICLE INFO

Article history:
Received 5 April 2011
Accepted 8 September 2011

Keywords:
Anaerobic digestion
Batch tests
Biochemical methane potential (BMP)
Experimental conditions
Methane yield
Solid organic substrates

ABSTRACT

Anaerobic digestion is considered a competitive source for the production of renewable energy as far as efficiency and cost are concerned. To evaluate the anaerobic biodegradability of an organic substrate such as feedstocks, a test known as biochemical methane potential (BMP) has been commonly used. Current worldwide interest in using different organic substrates for anaerobic bioconversion is growing but there is a lack of clear references and comparability as a result of multiple factors that affect BMP determination. Several batch methods have been used to determine the methane potential. However, these technical approaches vary significantly from one reported method to the next another. In this review, the research works on the influence of different parameters of BMP determination have been discussed for critical and comparative evaluation. In addition, the extensive literature previously published dealing with BMP assays has been compiled and summarized focusing on two main subjects: firstly, methane yields of substrates, and secondly, the description of the various experimental procedures used to achieve the reported data.

Contents

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doi:10.1016/j.rser.2011.09.008

1. Introduction

Anaerobic digestion (AD) is a biochemical technological process for the treatment of organic substrates such as sewage and industrial effluents, animal manures and solid substrates (energy crops, agricultural residues and food wastes). This process has received increasing attention in recent years. It involves the degradation and stabilisation of complex organic matter by a consortium of microorganisms leading to an energy-rich biogas which can be used as renewable energy to replace fossil energy sources.

Literature shows that anaerobic digestion assays can be carried out in batch or continuous mode. Considering that continuous set-up is more laborious and time-consuming than batch tests, the latter have been more widely used. It is important to note that the batch approach can be used for three purposes: anaerobic biodegradability, inoculum activity and inhibition. These terms were defined with the aim of establishing a common terminology [1]. These three tests are based on the same principle – the measurement of biogas/methane production. However, the protocols available in the literature differ not only with regard to the method used to quantify the gas produced during the test, but also with regard to the experimental conditions adopted for incubating the inoculum. Extensive research has been carried out to study the influence of experimental conditions on the results for inoculum activity and inhibition assays. On the other hand, studies on biodegradability, of which there have been much fewer, can be placed into two main groups following the nature of the substrate:

(i) Micro-pollutants (chemical compounds and plastics). Test methods for assessing anaerobic biodegradability of chemical substances have been previously described. Some of them studied the influence of key parameters such as compound and inoculum concentrations and mineral medium composition [2–5]. Moreover, there are standards and guidelines for anaerobic testing, reviewed by Müller et al. [6].
(ii) Complex organic substrates (manures, wastewaters, sludges, solid wastes). The first report of anaerobic biodegradability assessment in batch mode was carried out by Owen et al. [7]. This test was developed to determine the biochemical methane potential (BMP). There is less research available on the influence of key parameters in BMP of organic materials.

This review will focus on the AD of solid organic substrates (SOS). Reviews have been previously published which include data on AD experiments using solid substrates in batch and continuous mode [8–11]. In spite of the reviews published, the variety of methods reported in the literature for determining BMP and the discrepancies in approaches and results obtained for each experimental procedure emphasizes the need for an extended review. The purpose of this review article is to integrate all of the anaerobic biodegradability tests in batch mode for different solid substrates which have been previously reported in the literature. The aim of this review will be threefold: firstly, the text includes extensive information about the influence of different factors affecting the BMP results, secondly, the manuscript summarizes the important energetic data of methane potential (Appendix A) and thirdly, the document gives a detailed report of the different experimental procedures used in each case described (Appendix B).

2. Factors affecting the performance of anaerobic batch tests

The general principle of all batch tests is the incubation of an inoculum containing a variety of anaerobic microorganisms in a suitable medium (water and minerals) at neutral pH and at specific temperature range (normally mesophilic or thermophilic). Substrate is added to the medium and serves as a source of carbon and energy for the microorganisms. After incubation, the degree of degradation of the substrate is assessed at pre-set time intervals to determine its extent and conversion rate. Blank controls (endogenous tests, with the inoculum alone added) are included so that the gas produced from the organic matter contained in the inoculum can be accounted for.

Certain factors have the potential to affect the biodegradability assays and, therefore, the biogas/methane production. They are detailed in the following paragraphs:

2.1. Solid organic substrates (SOS)

Raw materials can be obtained from a variety of sources. Different groups of potential sources for methane production were considered by Gunaseelan [8] such as the organic fraction of municipal solid waste (OFMSW), fruit and vegetable waste (FVW), grasses, woods, terrestrial weeds, and aquatic (marine and freshwater) biomass.

2.1.1. Characterisation

It is known that the anaerobic biodegradability of organic matter is related to its composition [12–20]. Therefore, in order to carry out a BMP assay it is essential to find out exactly what the characteristics of the substrate to be digested are.

Firstly, any uncertainty about the origin of the substrate tested should be avoided. Therefore, when dealing with plants, crops or other inhomogeneous materials, details on the part used for testing should be included. For example, the BMP tests of various components of *Jatropha curcus* ranging from 80 to 968 mL CH_4 g^{-1} VS_{added} [19]. Then, the description of the part used must be considered as a key parameter.

Secondly, the general characteristics of the substrate to be assayed should always be analyzed and the moisture, the total solids (TS) and the volatile solids (VS) should be quantified and controlled. It should be pointed out that some samples are problematic for TS and VS determination due to a possible loss of volatile organic matter during the drying process, including at low temperature or freeze-drying [21]. It is important to note that although specific methane yield on a VS basis is not a constant due to variations in organic matter composition, the VS content could be used as a primary indicator of the methane potential. It is noteworthy to mention that for energy crops and crop residues, the content and availability of VS which are able to produce methane is influenced by factors related to biomass production such as location, climate, variety, cultivation management and maturity stage at harvesting time [15,20,22,23].

Further information about the nature of VS can be assessed taking into account:

(i) Component composition. Not all VS are equal and therefore they exhibit different rates and extents of biodegradation during AD. The organic substance can be subdivided into: fats, proteins, carbohydrates and lignin. Proteins, lipids and extracted fractions of carbohydrates are usually the soluble parts, while the fibrous components represent the structural lignocellulosic content, in which case solubilization is very difficult. So, biodegradability is limited by the crystallinity of the cellulose and the lignin content [24].
(ii) Elemental composition. Another approach for characterisation involves the quantification of the content of certain elements (C, O, H, N and S). This information can be used to determine the empirical formula of the substrate.
(iii) Chemical oxygen demand (COD). This parameter is commonly used to characterize the total organic content of wastewater,

whereas it is not frequent for SOS. A simple explanation is that standardized methods are available for the measurements of COD for water and wastewater. However, COD measurements for solid substrates have been traditionally specifically adapted, where the samples have to be properly homogenized and diluted. Recently, good results were obtained using a modified method to measure the COD content of solid substrates without dilution [25]. In addition, it has been demonstrated that analytical performance in the measurement of COD of samples that are difficult to analyze, such as solid substrates and liquid samples with high suspended solid content, can be improved by regular participation in proficiency testing schemes [26]. In any case, COD is a very important analytical parameter because it is needed for modelling the energy balance of an anaerobic digester [27].

Further data on the composition of the SOS under test can be used to calculate theoretical methane yields by different approaches [28]. Although the theoretical potential provides only a basis for the quality of the substrate as a methane producer, some research estimated the methane yield without experimental work, based simply on its chemical composition [29]. However, the practical methane yield obtained in a reactor will always be lower than theoretical due to a number of factors [30]:

- Part of the organic material is often inaccessible due to binding of particles or structural organic matter.
- Some compounds are poorly degraded or not at all degraded anaerobically (e.g. lignin, peptidoglycan, etc.).
- A fraction of the substrate is used for cellular growth and maintenance. Although this portion may vary considerably depending on the operating conditions and substrates, in practice, 5–15% of COD removed can be considered typical as biomass cell factor [31,32].

2.1.2. Particle size

Particle size and the size reduction procedure may influence biodegradation results. It is generally accepted that hydrolysis is the rate-limiting step in anaerobic digestion of particulate substrates [33]. Surface area and particle size are important characteristics in determining the initial degradation rate. The size of the feedstocks should be limited, otherwise the digester may clog and it would also be difficult for microorganisms to carry out their digestion. In the case of substrates with low biodegradability, it is normally accepted that a size reduction of the particles and the resulting enlargement of the available specific surface can improve the biological process [34].

Little research has been carried out to determine the effect of particle size of solid substrates on methane yield [34–40]. The majority of results reported that methane yield was inversely proportional to particle size, but also some results reported no tangible effect on the kinetics of methane production. Since the relationship between particle size and biodegradability is not yet clarified, to allow for the results to be compared, the particle size should be comparable. A particle size of $\leq$10 mm is suggested. If the material used is difficult to reduce in size, it should be cut, broken or otherwise processed until the desirable size is achieved [41].

2.1.3. Concentration

One of the most important parameters for a batch assay design is the load of the solid substrate introduced into the digester. If the load is too low, although it limits the possibility of inhibitory effects, the microorganisms will exhibit a low metabolic activity and very low quantities of gas will be produced. If the load is too high, the biogas measurement may be more reliable but an overload situation in which intermediate volatile fatty acids (VFA) may build up, resulting in gas production inhibition.

Little detail about the influence of this parameter was found in the literature. Hansen et al. [42] described a laboratory procedure for the determination of BMP using 2% TS to more than 100 solid waste samples. On the other hand, the VDI 4630 guideline specified that the content of solids should not exceed 10% if an adequate mass transfer is to be assured [41].

2.2. Inoculum (INO)

Blok et al. [43] pointed out that even when the experimental conditions of batch test procedures can be harmonised, some variability in the results will always remain due to the biological nature of the test systems. The characteristics of microorganisms collected for use as inoculum can vary for the same treatment plant (daily or seasonal variations of flow-rate and substrate composition) and can be different from one treatment plant to another (operating conditions: organic loading rate, solid retention time, etc.).

The inoculum used for BMP assays must be fully characterized. Although subject to limitation, the easiest way to define the inoculum concentration is from the amount of volatile suspended solids (VSS). However, due to the inaccuracy of this determination in such samples, for the majority of anaerobic sludges VS are used as a measure of microorganism content. In any case, the information available for these analytical parameters is inadequate because it does not distinguish between microbial biomass and any other particulate organic material present in the reactor. This is especially evident in manures, where the inoculum VS content is mainly represented by recalcitrant lignocellulosic residues and not active microbial biomass, while in a granular sludge most of the VS consist of microbial cells [30]. Nor is it possible to determine if the microbial biomass is alive or dead.

The influence of the inoculum on the batch tests is mainly depending of six factors: origin/source, concentration, activity, preincubation, acclimation/adaptation and storage.

2.2.1. Origin/Source

The inoculum source relating to BMP tests is not uniform in the literature. Digested sludge from municipal wastewater treatment plants (MWTP), soil extracts, industrial treatment plants, rumen and animal manures have all been used. Although the use of an inoculum from such different sources may favour the environmental relevance of the tests, it is certainly not ideal for standardization [43]. On the other hand, the reproducibility of the assessment can be improved when a non-predetermined inoculum source is used [1].

Different sources could lead to different biodegradability results as a consequence of the different levels of microbial population. For a defined inoculum, the methane yield of an organic substrate is directly related to the extent of solubilization, while the degradation rate will depend on the slowest of the three steps of the anaerobic digestion process, namely hydrolysis (solubilization), acidogenesis and methanogenesis [39].

In general, digested sludge from a running biogas plant is used. The digested sludge from MWTP should offer the most suitable source of a diverse and active inoculum. This is preferable for the following reasons: (i) sewage treatment plants are found worldwide, (ii) although sewage treatment plants are different, they do have common features.

2.2.2. Concentration

Practical experience has demonstrated that the level of concentration of inoculum affects the rate of biodegradation. Normally, the higher the inoculum concentration, the faster the anaerobic conversion of the substrate, and the quicker the test will be completed.

Moreover, the concentration affects the duration of the lag period and the susceptibility of degradation due to inhibitory effects [4].

For some normalized biodegradability tests for micro-pollutants and the initial BMP procedure, the amount of inoculum used is generally expressed as a percentage of volume (10–80%). Using this unit system, the initial content of biomass is proportional to the VS content of the inoculum, whose value can range in manures and granular sludges from 2–3% to 10% VS, respectively [30]. Therefore, this criterion should be avoided because of its ambiguity. It is often more meaningful to express the concentration of inoculum in a batch assay in terms of VS.

To study the anaerobic biodegradability of micro-pollutants, a low inoculum concentration (1–3 g TS·L^{-1}) was suggested because inoculum also contributes to gas formation which can blur the results if it is relatively high in comparison with the compound being tested [3]. On the other hand, in the case of complex SOS a small amount of inoculum can lead to an overload in the process with acidification and methane production inhibition [44]. The literature survey shows that a wide range of concentration has been used up to date. The lowest value (2.1 g VS·L^{-1}) was reported by El-Mashad and Zhang [45], while the highest value (37.2 g VS·L^{-1}) was stated by Rincón et al. [46]. The VDI 4630 guideline suggested using a range of between 15 and 20 g VS·L^{-1} from seeding sludge [41].

2.2.3. Activity

Inoculum activity is one of three types of batch assays commonly used. The influence of inoculum activity was extensively researched and the results obtained were reviewed by Rozzi and Remigi [1]. Interest is still evident and a recent study carried out by Souto et al. [47] was entirely dedicated to this topic.

Traditionally, activity has been limited to assessing specific methanogenic activity (SMA), but for a better identification of the quality of the inoculum used, it has been recently suggested by Angelidaki et al. [30] that activity of the different groups of microorganisms involved in the anaerobic process should be determined.

The use of different positive control substrates can be used for measuring activity and also for checking if the anaerobic biodegradation assays are performing well, for quality control purposes. These reference substrates should not ferment too quickly and should be completely biodegradable. As far as biodegradability is concerned, the experimental values should be close to the theoretical ones, because, as reported previously, only a limited percentage of substrate is not converted into biogas and utilised for cellular growth and maintenance. Partial biodegradation has on occasions been observed when positive control substrates have been tested. This could have been due to faulty experimental equipment or to inactive sludge. If the experimental equipment is shown not to be faulty, the safest course of action is to repeat the assay with fresh sludge [42]. Cellulose is the most frequent substrate used for measuring the adequate level of potential performance. However, the number of BMP research works where this substrate has been used is very low compared with the huge amount of articles on BMP assays.

Regarding to the influence of the inoculum activity into anaerobic biodegradability a few research works were reported [48,49]. It is noteworthy that Tait et al. [50] used an abiotic sludge control (inactivated inoculum) to evaluate the indigenous activities of some bedding (wheat straw and rice husks) from piggery housing.

2.2.4. Pre-incubation

Pre-incubation of sludge before feeding reduces the volume of gas produced in the blank controls and has been postulated as a mean of improving the precision with which net gas production can be measured. Recently, the use of a “degassed” inoculum has been suggested where 2–7 days of pre-digestion seemed to give an optimum decrease in background gas production with acceptable increases in both the lag and the total incubation periods [51].

The literature shows that most studies regarding this factor are for micro-pollutants. Pre-incubation has been widely recommended for testing the anaerobic biodegradability of these substrates, because in such cases it is difficult to clearly relate biogas evolution to degradation of the test compound or to distinguish the amount of biogas produced by the sludge itself [4]. On the other hand, a pre-incubation time of up to 3 weeks had no significant effect on the estimation of gas production [3].

2.2.5. Acclimation/Adaptation

The preculturing of the inoculum with a substrate leads to the induction of metabolic pathways for biodegradation, an increase of microorganism affinity for the compound and also an increase in the number of specific degraders. However, this idea of adaptation, although widely accepted by the scientific community, has not previously been reported for BMP tests, where the reported tests fit well with the philosophy of using not acclimated inocula.

2.2.6. Storage

For micro-pollutants, sludge storage had no significant effect on the extent of degradation, but the duration of lag times could be affected, and, therefore, substrates could be degraded more slowly [2]. The effect of storage on the batch biodegradability test for SOS is also scarce in the literature. Angelidaki et al. [30] suggested that fresh sludge should be used whenever possible.

2.3. Experimental conditions

2.3.1. Gas measurement systems (GMS)

Gasometric methods are the most frequently used for determining anaerobic biodegradability. In such methods, biogas/methane production can be quantified either manometrically by keeping the volume constant and measuring the pressure increase, or volumetrically by providing constant pressure conditions allowing measurement of the gas volume. Techniques for measuring the rate and volume of gas produced from anaerobic biodegradability assays include different systems such as lubricated syringes, volume displacement devices, manometers or pressure transducers, manometer assisted syringes, or low pressure flow meters. In addition, some automatic gas flow meters may be considered as mixed volumetric/manometric systems.

2.3.1.1. Volumetric methods (Vol). The first description of a volumetric measurement system for biogas production consisted in the displacement of the piston of a glass syringe with its needle being inserted into the reactor [7]. Alternatively, liquid displacement systems were proposed. In this case the biogas produced inside the reactor moved into a suitable external vessel which contained a barrier solution and displaced an equivalent volume of liquid. More recently, the Eudiometer unit was described as a more sophisticated apparatus which operated by a liquid displacement technique [52].

It is important to mention that precaution must be taken with the barrier solution used so as to avoid certain biogas components being lost. For the improvement of this measurement system, it is better to use an alkaline solution for washing the biogas, which means that the sole methane fraction can be measured directly [1,53]. Another option is to collect the biogas in a gas sampling bag with low permeability [54]. This system avoids the problem of adsorption during long periods of contact with the barrier solution, but it has the disadvantage of requiring a complementary gas meter for measuring the volume of gas collected.

2.3.1.2. Manometric methods (Man). In a manometric respirometer, the biogas produced is confined inside the bioreactor and hence generates proportional overpressure. An early manometric method was the Warburg respirometer [55]. Later, the method was improved by introducing the use of a pressure transducer to measure the gas production [56].

For this method, complementary biogas analyses are needed for calculating methane production. The major difficulty in accurately quantifying the overall gas production arises from the solubility of carbon dioxide in the digesting liquor as it is affected by pressure, pH, the ratio of headspace to liquid volume, temperature and the complex thermodynamic equilibrium established between carbon dioxide and the carbonates/bicarbonates of calcium and magnesium [4].

Recently a digital pressure transducer, called OxiTop® (WTW, Germany) and originally developed for biochemical oxygen demand (BOD) measurements, has been reported as useful for anaerobic biodegradability assays [57].

2.3.1.3. Gas chromatography (GC). Dolfing and Bloemen [58] determined the SMA of a sludge based on the GC analysis of the headspace of closed anaerobic vials. They sampled with a pressure lock syringe, which allows quantification independent of the pressure prevailing in the reactor. The volume of methane can be estimated based on the molar fraction of this gas in the headspace.

Hansen et al. [42] sampled only 10 mL of headspace gas during the full BMP test (0.2 mL every time), which represents less than 0.7% of the headspace volume, and the results were, thus, not significantly affected by the change of headspace pressure.

2.3.2. Operational conditions (OpC)

2.3.2.1. Physical operational conditions.

2.3.2.1.1. Volume. The total reactor volume used for batch tests is inversely related to the number of replicate samples that could be tested at the same time using a prefixed amount of sludge and substrate. The nature of the substrate can also influence the selection of the ideal volume, because the more homogeneous the material, the smaller the volume of reactor required to determine methane potential more accurately. The results of the extensive literature review showed that a wide range of different total volumes were utilised for anaerobic biodegradability batch assays, ranging from 0.1 to 120 L. However, the most common and useful volumes used for BMP assays are lower than 1 L.

2.3.2.1.2. Temperature. Although anaerobic biodegradation can take place within a wide range of temperatures, AD processes strongly depend on temperature. Depending upon the temperature at which the process is carried out, three temperature ranges can be differentiated: thermophilic (45–60 °C), mesophilic (20–45 °C), and psychrophilic (<20 °C) [59]. The main problem at the low temperature is the decrease in the microbial consortia activity.

The majority of data in the literature refers to experiments performed at mesophilic temperature, with only some at thermophilic temperature. The reason could be that the anaerobic digestion process is efficient enough at 35 °C and there is little to gain by increasing the operational temperature when increased costs are involved [11]. Taking into account the important influence of temperature, comparatively few studies have been carried out to relate its influence on biodegradation assays in batch mode using solid substrates [60–62].

2.3.2.1.3. Stirring. Agitation of digesters can be carried out in a number of ways: manual shaking, magnetic stirrers, orbital shaker, etc. The main factors affecting the mixing method are intensity and duration. The effect of mixing on the general performance of anaerobic digestion is contradictory. The continuous mixing of the content of the bioreactor favours contact between the substrate and the microorganisms as well as the release of biogas into the headspace, but it may also damage the structure of the flocs or granules, thereby worsening the close interaction between the different microbial populations within the agglomerate [1].

For micro-pollutants the stirring process is invariably essential to the rate of gas production, whereas it is independent of the extent of degradation [5]. On the other hand, the influence of mixing on the anaerobic biodegradability assays of SOS has never been reported in detail, although an optional device for mixing the reactors thoroughly may be useful in most cases.

2.3.2.1.4. Duration. The performance time of a batch assay can be related with the kinetics of the process. The main drawback of BMP testing is that it is very time-consuming [63]. A wide range of incubation time was reported in the literature. Owen et al. [7] advised the use of an incubation time of 30 days, which enables the complete degradation of organic substrates in most cases. Hansen et al. [42] increased the incubation time to 50 days to ensure maximum degradation of organic matter that has a lower rate of anaerobic biodegradability, although they reported that typically 80–90% of methane potential can be produced during the first 8–10 days. A high incubation time of 365 days was reported by Lopes et al. [64], 240 days by Rao et al. [65], and 155 days by Kaparaju et al. [66]. On the contrary, a shorter period of 7 days was reported in some batch tests [67,68].

2.3.2.2. Chemical operational conditions.

2.3.2.2.1. Headspace gas. Different gases have been reported in the literature to flush the reactor headspace: N_2, a mixture of N_2 and CO_2, He and air. The mixture of N_2 and CO_2 has been reported as the most commonly used gas within the headspace. Different ratios of both components (70–80% N_2 and 20–30% CO_2) can be found. The content of CO_2 is related to the buffering power of the system. No extensive research has been carried out to study the influence of CO_2 on anaerobic biodegradation in batch mode, but experimental results using only N_2 were similar when different substrates were selected [69].

More worthy of comment is the use of air as gas within the headspace. Oxygenation of the sample by exposure to air or sparging with oxygen reduces the biogas/methane production in proportion to the degree of oxygenation [70,71]. However, surprisingly the results were not different when air was used as headspace gas [69].

2.3.2.2.2. pH and alkalinity adjustment. pH is a measure of the acidity or alkalinity of the liquid content of the reactor. Most methanogenic microorganisms have an optimum pH of between 7 and 8, while the acid-forming bacteria often have a lower optimum pH [44]. If the pH of the waste to be tested is outside the optimal range, and if there is insufficient buffer capacity, the anaerobic process will be inhibited. Therefore, to avoid underestimating the methane potential, most batch tests are carried out at pH values ranging from 7.0 to 7.8. If the pH needs to be adjusted, a basic diluted solution such as NaOH or lime, or an acid solution such as HCl, could be used.

Alkalinity is the capacity to neutralize acids that provides resistance to significant rapid changes in pH. It is also known as "buffering capacity". It is the result of the presence of various compounds (mainly bicarbonate, carbonate and hydroxides). A value of 2500 mg $CaCO_3 \cdot L^{-1}$ is considered to be normal for sewage sludge. A more desirable range of 2500–5000 mg $CaCO_3 \cdot L^{-1}$ provides a higher buffering capacity for which a much larger increase in VFA can be accommodated with a minimum drop in pH [72].

The initial BMP test procedure suggested using an alkalinity of 2500 mg $CaCO_3 \cdot L^{-1}$. Later, most procedures reported for micropollutant biodegradation tests used the phosphate/biphosphate species as the sole source of alkalinity. Recently, Pabón [57] reported the inhibitory effect of the applied phosphate buffer to BMP tests.

2.3.2.2.3. Mineral medium (MM). It is well documented that all microbial-mediated processes require nutrients and trace elements (metals and vitamins) during organic biodegradation. In fact, eight inorganic nutrients: nitrogen, phosphorous, sulphur, potassium, magnesium, sodium, calcium, and iron were reported as necessary macronutrients in synthetic media [44]. In addition, some metals (chromium, cobalt, copper, manganese, molybdenum, nickel, selenium, vanadium and zinc), known as trace metals, are considered micronutrients, most of which are necessary as part of the active site of enzymes. Trace metals need to be dosed when added to the reactors so as to maintain microbial metabolism and growth [73]. The dose added must balance the requirements to support high activity, taking into consideration that above this concentration, trace metals become inhibitory or toxic [74].

Literature reports on the effect of mineral medium in batch tests are very inconsistent in this respect, because they vary from one to the other:

- For micro-pollutant biodegradation, different mineral media were compared for their effect on background gas production, lag times, and extent of degradation [2]. There was no significant effect on lag times with any of the media. However, the extent of degradation did vary.
- In a similar way, there is no general consensus on BMP tests as to whether these growth factors are readily available. A question that may arise is to what extent nutrients and trace elements are necessary depending on their content in the inoculum and the substrate used, being this aspect especially crucial when degrading mono-substrate. For instance, Pobeheim et al. [75] obtained different concentrations of macro- and micronutrients when various sludges from agricultural biogas plants were analyzed. On the other hand, some substrates were characterised before anaerobic biodegradability assays and found that they contained a balanced concentration of macro- and micronutrients necessary for anaerobic microorganisms [76,77].

It is important to note that if the mixture of inoculum-substrate lacks an important element, biodegradability could be severely affected. In this way, some research works demonstrated the positive effect of the addition of some nutrients and metals [75,78,79].

2.3.2.3. Inoculum to substrate ratio (ISR). Chudoba et al. [80] reported that one of the most important parameters in activated sludge batch testing is the initial substrate/microorganism ratio (S_o/Xo). However, the role of the influence of the ISR on anaerobic biodegradation tests is not clear. Theoretically, the methane yield should be independent of the ISR and only affect the kinetics of the process. But, experimental data demonstrated that the ISR can influence both the extent and the rate of the anaerobic biodegradation process. Unfortunately, many research works do not include the ISR used in the experimental design. It is sometimes possible to calculate the ISR with the information provided, but not when the data of the substrate and/or inoculum VS content are omitted.

Owen et al. [7] gave no detail of the ISR in their procedure, merely recommending a 20% volume of inoculum and a substrate concentration lower than 2 g COD·L^{-1}. Doing calculations, the ISR of the initial BMP procedure can be considered to be approximately 1 (VS basis). The first report dealing with the influence of ISR was published by Hashimoto [81]. He showed that the methane yield was drastically reduced at an ISR below 0.25 (VS basis) using wheat straw as substrate. The methane production rate was also found to increase as the ISR rose stepwise to 2, after which it remained relatively constant. Later, Chynoweth et al. [37] determined the effect of ISR on the biodegradation of cellulose. The extent values were similar, but the methane production rate was slightly higher for the highest ISR. In addition, imbalance was explained by the presence of higher concentrations of VFA in the assays with the lowest ISR. Consequently, they modified the ISR of the batch test to 2 (VS basis). The same conclusion about the clear influence of the ISR on anaerobic degradation was reported by other researchers using different substrates [49,62,64,68,82–84].

Finally, taking into account the potential amount of VFA produced and the possible ammonium generated, if proteinaceous matter is present, each substrate probably has the best ISR for performing the assay. However, for the harmonisation of the anaerobic biodegradation assays it is necessary to work at a high ISR value. Considering that an ISR ≥ 2 has never been reported as inhibitory, it could be used as the mandatory ratio for future standardized tests, as the VDI 4630 guideline suggested [41].

3. Conclusions

The BMP results compiled in this review demonstrated the lack of uniformity in the data reported, probably due to different inocula and experimental conditions utilised. BMP tests made in one laboratory should be consistent with those made elsewhere. It should be desirable that comparability are not very different with others making similar measurements. A dedicated IWA task group on anaerobic biodegradability, activity and inhibition (TG-ABAI) is working on this topic since 2002.

Acknowledgments

The authors wish to express their gratitude to the Spanish Ministry of "Ciencia and Innovación" (Project CTM 2008-05772/Tecno) and Junta de Andalucia for providing financial support, to the Program "FPI" of the above-mentioned ministry for contracting graduates, as well as to the contract JAE-Doc from "Junta para la Ampliación de Estudios del CSIC" co-financed by the European Social Funds.

Appendix A. Methane yields of solid organic substrates

Solid Organic Substrate (SOS)			Methane Yield	Reference
Name	Part	Size(mm)	(mL CH_4/g VS_{added})	
Alfalfa			210	[23]
Alfalfa	Silage		226	[23]
Apple	Fresh wastes		317	[89]
Azolla	Whole plant		132	[85]
Bagasse		< 2	77[c]	[67]
Bagasse		0.85–5		[112]
Bamboo			250[a]	[90]
Banana	Peeling		289	[89]
Banana			400[a]	[90]
Banana	Peels	2	243–322	[99]
Banana	Waste stem	10–20	81–196[a]	[107]
Banana	Peeling		374–409	[36]
Barley	Whole plant silage	20–40	375	[87]
Barley	Straw	50–100	229	[93]
Barley	Waste silage		222	[122]
Barley	Waste		20	[123]
Barley	Residue	10	271	[126]
Black locust			300	[139]
Braken			180	[57]
Bread-wholewheat			N.R.	[60]
Brewery	Grain		N.R.	[108]
Brewing draffs			385–400	[117]
Brinjal	Stalk	2	374	[99]
Brinjal	Whole fruit	2	396	[99]
Buckwheat			320	[57]
Cabbage			150[a]	[90]
Cabbage (fresh)				[91]
Cabbage	Leaves	2	309	[99]
Cabbage	Stem	2	291	[99]
Cabbage-white	Leaves		382	[143]
Cabbage-white	Leaves silage		343	[143]
Cabomba			155–160	[127]
Calotropis procera	Leaves		280	[117]
Candy-black			390	[66]
Cardboard			217	[105]
Carrot			310	[57]
Carrot	Peeling		388	[89]
Carrot	Leaves	2	241	[99]
Carrot	Petiole	2	309	[99]
Cassava	Pulp		370	[129]
Cattail			350	[129]
Cauliflower	Leaves	2	190	[99]
Cauliflower	Stem	2	331	[99]
Cauliflower	Leaves		352	[143]
Cauliflower	Leaves		341	[143]
Cellulose			404	[19]
Cellulose			370	[37]
Cellulose			379	[42]
Cellulose			345	[89]
Cellulose			356	[91]
Cellulose			419	[99]
Cellulose			356–375	[128]
Cellulose			367	[138]
Cellulose		100 mesh	373	[138]
Cellulose		100 mesh	390	[139]
Ceratopteris	Whole plant		204	[85]
Chocolate			370	[66]
Clover		< 20	140–210	[66]
Cocksfoot			325	[118]
Cocksfoot		10	308–382	[135]
Coconut	Fibres	0.85–5	N.R.	[112]
Comfrey	Tops		334	[143]
Comfrey	Tops		323	[143]
Confectionery	Raw material		320	[66]
Coriander	Leaves	2	325	[99]
Coriander	Stems	2	309	[99]
Coriander	Roots	2	283	[99]
Coriander	Whole plant	2	322	[99]
Corn stover			N.R.	[102]
Corn stover		30–60 mesh	360	[138]
Cotton	Stalks-wastes		62[a]	[78]

Solid Organic Substrate (SOS)			Methane Yield	Reference
Name	Part	Size(mm)	(mL CH_4/g VS_{added})	
Cotton	Seed hull-wastes		86[a]	[78]
Cotton	Oil cake-wastes		104[a]	[78]
Cotton	Stalks		145	[95]
Cotton	Residues		365	[126]
Crops-mixture	Silage	50	320–510	[130]
Cyperas	Whole plant	10	38	[85]
Dhub grass			205–228	[36]
Diapers			204	[105]
Faba bean	Straw		440	[131]
Fat-pork			900	[42]
Fish waste	Various		390	[120]
Food packaging			318–349	[128]
Food waste	Leachate		478	[114]
Food	Wastes		245–510	[62]
Food	Wastes		425–445	[77]
Food	Wastes		472	[91]
Food	Wastes	20 × 50	301[a]	[94]
Food	Wastes		525	[116]
Fruit and vegetable	Wastes		470	[134]
Garbage	Waste	10 × 10 × 5	395	[65]
Garden pea	Pods	2	390	[99]
Gelatine			100–150	[42]
Giant knotweed			170–270	[22]
Gliciridia	Leaves		165–180	[98]
Glucose			351	[42]
Glucose			335	[138]
Gracilaria spp.			280–400	[12]
Gracilaria tikvahiae		20–30	190–230	[100]
Grape	Stalk		116	[93]
Grape	Marc		98	[93]
Grape	Pressings	2	283	[99]
Grape	Peduncle	2	180	[99]
Grass			267	[23]
Grass			374	[23]
Grass			N.R.	[60]
Grass			388	[89]
Grass			128–144[a]	[94]
Grass			320	[134]
Grass cuttings			300	[22]
Grass hay			270–350	[66]
Grassland			128–392	[15]
Green pea	Shells	10–20	194–220[a]	[106]
Green wastes			206–357	[62]
Grey	waste		147	[105]
Hydrilla	Whole plant		81	[85]
Ipomea fistulosa	Leaves		413–429	[36]
Jatropha curcus	Leaf lamina		227	[19]
Jatropha curcus	Leaf petiole		335	[19]
Jatropha curcus	Leaf entire		224–237	[19]
Jatropha curcus	Green fruit		326	[19]
Jatropha curcus	Yellow fruit		518	[19]
Jatropha curcus	Brown fruit		469	[19]
Jatropha curcus	Fruit hull		306	[19]
Jatropha curcus	Seed testa		80	[19]
Jatropha curcus	Seed kernel		968	[19]
Jatropha curcus	Seed entire		610	[19]
Jatropha curcus	De-oiled cake		230	[19]
Jerusalem artichoke			360–370	[22]
Jerusalem artichoke	Tops		309	[143]
Jerusalem artichoke	Tops silage		301	[143]
Kitchen waste			432	[122]
Kitchen waste		1–3	370–430	[124]
Kitchen waste			450	[134]
Ladies finger	Stalk		350	[99]
Laminaria		0.8	260–280	[37]
Leather fleshing			490	[136]
Lemon	Pressings		473	[99]
Lettuce	Residues		294	[89]
Lucerne	Whole plant silage	20–40	357	[87]
Lupine			310–360	[22]
Lupine (white)		< 0.2	260	[57]
Lupine (yellow)		< 0.2	260	[57]
Macrocystis		0.8	390–410	[37]
Maize	Mixture	0.5–3	268–366	[14]
Maize			398	[15]
Maize	Whole plant		282–419	[18]

Solid Organic Substrate (SOS)			Methane Yield	Reference
Name	Part	Size(mm)	(mL CH_4/g VS_{added})	
Maize		2–4	251–349	[20]
Maize			315	[23]
Maize	Silage		364	[23]
Maize	Bran		64 (3)	[67]
Maize			250–340	[74]
Maize		2	196–233	[83]
Maize	Whole plant silage	20–40	345	[87]
Maize	Fresh whole plant	10	300–400	[88]
Maize	Whole plant silage	various	370–410	[88]
Maize	Residues		317	[93]
Maize	Stalks		229	[95]
Maize	Residues	10	363	[126]
Maize	Whole plant		378	[140]
Maize	Whole plant silage		328–418	[140]
Mandarin	Peels	2	486	[99]
Mandarin	Pressings	2	433	[99]
Mandarin	Whole rotten fruit	2	494	[99]
Mandarin	Seeds	2	732	[99]
Mango	Peels	2	370–523	[99]
Marrow kale			310–320	[22]
Meadow foxtail			310	[118]
Meat and bone meal			351–381	[142]
Meat-cooked			482	[91]
Microcystis			94–141	[84]
Millet	Bran		590	[117]
Millet	Straw		390	[117]
Mirabilis	Leaves		241	[137]
Mirabilis	Leaves		327–341	[36]
Mustard	Tops		300	[143]
Mustard	Tops silage		326	[143]
Napiergrass		0.8	190–340	[37]
Napiergrass	Lamina	2	372	[99]
Napiergrass	Sheat	2	342	[99]
Napiergrass		< 20 mesh	288	[138]
Nettle			210–420	[22]
Newspaper		shredded	92	[138]
Newsprints			58	[105]
Oat		< 20	250–260	[66]
Oat			320	[22]
OFMSW			298–573	[28]
OFMSW		0.8	200–220	[37]
OFMSW		2–50	160–250	[40]
OFMSW			495	[42]
OFMSW			353	[45]
OFMSW			230–550[d]	[64]
OFMSW			92[a]	[94]
OFMSW			60–530	[96]
OFMSW			187	[97]
OFMSW		Screw press	450	[101]
OFMSW		Disc screen	450	[101]
OFMSW		Shredding	450	[101]
OFMSW		10	157	[103]
OFMSW			50–200[a]	[111]
OFMSW			186–222	[128]
OFMSW			360	[136]
Onion	Exterior peel	2	400	[99]
Orange	Peeling		N.R.	[60]
Orange	Peeling		297	[89]
Orange			115[a]	[90]
Orange	Peel	2	455	[99]
Orange	Pressings	2	502	[99]
Orange	Waste	< 7	490	[109]
Palm Oil	Fruit bunches		370	[129]
Paper			300[a]	[90]
Paper (coated)			84[a]	[94]
Paper (newsprint)			74[a]	[94]
Paper (office)			217[a]	[94]
Paper (bag)			250	[42]
Paper (office printer)			340	[105]
Paper			84–369	[128]
Paper and cardboard			109–128	[132]
Parthenium			140–152	[82]
Pea-green	Shell	10–20	194–220[a]	[106]
Pig waste			230–620	[61]
Pineapple	Peel	2	357	[99]
Pineapple	Leafy shoot	2	355	[99]
Pineapple	Peel		400	[129]

Solid Organic Substrate (SOS)			Methane Yield	Reference
Name	Part	Size(mm)	(mL CH_4/g VS_{added})	
Pomegranate	Peels	2	312	[99]
Pomegranate	Rotten pulpy seeds	2	430	[99]
Pomegranate	Whole rotten fruit	2	342	[99]
Pomegranate	Pressings	2	420	[99]
Poplar (Populus sp)		0.8	230–320	[37]
Poplar (Populus sp)			350–420	[139]
Potato	Waste		320[c]	[54]
Potato			390	[89]
Potato	Peel	2	267	[99]
Potato	Pulp		N.R.	[108]
Potato	Pulp	3–10	332	[113]
Potato	Peel-pulp	3–10	377	[113]
Potato	Fruit water		323	[113]
Poultry slaughterhouse	Waste		550–670	[133]
Quinoa			330	[57]
Radish	Shoots	2	293–304	[99]
Rape	Straw		240	[22]
Rape	Oil seed		800–900	[42]
Rape			290	[57]
Rape	Straw		420	[131]
Rape	Tops		334	[143]
Red clover			280–300	[22]
Reed canary grass			340–430	[22]
Reed canary grass		10	253–351	[135]
Rhubarb			320–490	[22]
Rhubarb	Tops		316	[143]
Rhubarb	Tops silage		345	[143]
Rice-boil			294	[91]
Rice	Straw		347–367	[36]
Rice	Straw		347–367	[36]
Rice	Straw	50–100	195	[93]
Rice	Straw		215	[95]
Rice	Straw		270–290	[115]
Rice	Straw		340	[129]
Rosebay willow			200	[57]
Rye-winter			140–275	[15]
Rye-winter	Straw	< 2	360	[131]
Ryegrass			360	[118]
Saccharum spp.			270–310	[92]
Salvinia	Whole plant		242	[85]
Salvinia			50	[127]
Sapota	Peels	2	244	[99]
Sapota	Whole rotten fruit	2	327	[99]
Sargassum spp.			150–180	[12]
Sargassum		0.8	260–390	[37]
Scirpas	Whole plant		66	[85]
Seaweed		2–3	90–120	[125]
Sisal fibre waste		2–100	176–216	[34]
Sisal pulp			320	[120]
Sisal pulp waste	Leaf tissues + fibres		120–240	[121]
Sludge-kraft pulp mill			90[b]	[141]
Sludge-sulfite pulp mill			320[b]	[141]
Sorghum		0.8	260–390	[37]
Sorghum	Whole plant silage	20–40	362	[87]
Sorghum	Lamina	2	367	[99]
Sorghum	Sheath	2	407	[99]
Sorghum	Inflorescence + flowers	2	480	[99]
Sorghum	Inflorescence + grains	2	538	[99]
Sorghum	Roots	2	228	[99]
Sorghum		0.8	280–400	[104]
Spartina			290	[57]
Starch			348	[42]
Sugar beet			340	[22]
Sugar beet	Leaves	2	231	[99]
Sugar beet	Pulp		N.R.	[108]
Sugar beet	Pulp	3–5	430	[113]
Sugar beet	Tail	1–3	481	[113]
Sugar beet	Tops		360	[143]
Sugar beet	Tops silage		381	[143]
Sugarcane			230–300	[37]
Sugarcane	Residue	1	177	[126]
Sunflower			428–454	[15]
Sunflower	De-oiled cake	< 2	107–227	[68]
Sunflower	Whole plant silage	20–40	345	[87]
Sweet clover			290	[57]
Sweet gum			260	[139]
Sweet pea			370	[57]

Solid Organic Substrate (SOS)			Methane Yield	Reference
Name	Part	Size(mm)	(mL CH_4/g VS_{added})	
Switch grass			191–309	[119]
Sycamore			380	[139]
Tall fescue		10	296–394	[135]
Tea	Residue	10	67	[126]
Teak			270[a]	[90]
Textiles			228	[105]
Timothy		10	308–365	[135]
Timothy-clover grass			370–380	[22]
Tomato	Skins and seeds		218	[93]
Tomato	Whole rotten fruit	2	211–384	[99]
Triticale			212–286	[15]
Triticale			290	[57]
Turnip	Leaves	2	314	[99]
Ulva spp.			94–177	[13]
Ulva spp.		20–30	220–330	[100]
Utricularia	Whole plant		132	[85]
Vetch			290	[57]
Vetch–oat mixture			400–410	[22]
Water hyacinth		0.8	190–320	[37]
Water hyacinth	Whole plant	1.6–12.7	130–180	[38]
Water hyacinth			244	[95]
Water hyacinth			60–190	[127]
Water hyacinth			350	[129]
Wheat	Straw	0.088–6	227–249	[36]
Wheat	Straw	10	299–331	[81]
Wheat	Straw		267	[86]
Wheat	Straw silage		396	[86]
Wheat	Whole plant silage	< 1	276	[87]
Wheat	Straw	<1	297	[110]
Wheat	Straw	30–60 mesh	302	[138]
Wheat	Straw	<30 mesh	333	[138]
Wheat-winter			229–343	[15]
Wheat-winter		5–15	311–360	[46]
White fir		<40 mesh	42	[138]
Willow (Salix spp.)			130–300	[37]
Willow (Salix spp.)		<0.8	280–370	[139]
Winter bean			350	[57]
Winter harley			300	[57]
Wood grass		<20 mesh	291	[138]
Yard	Wastes		345	[116]
Yard	Wastes		123–209	[128]

[a] mL CH_4/ g TS_{added}.
[b] mL biogas/g VS_{added}.
[c] mL CH_4/g $VS_{removed}$.
[d] mL biogas/g $VS_{removed}$.
N.R.–not reported.

Appendix B. Description of experimental BMP procedures

Reference	INO			GMS	Physical-OpC							Chemical-OpC			ISR
	Source	VS	C_o		Capacity (L)		Temp		Mixing		TD	Gas	Adj	MM	
		(%)			TV	WV	°C	System	Type	Times	(days)		pH/Alk		VS basis
[12]	MWTP		10 (%-vol)	Vol (syringe)	0.282	0.100	35				60	N_2-CO_2 (70–30%)		Yes	1
[13]	No inoculum				30		35				64				
[14]	Energy crops	58		Vol (liq-disp)	1		38	TWB	Cont (mag bar)	10 s/10 min	45				2 (TS)
[15]				Vol (liq-disp)	1		38	TWB							
[18]	Digested material			Vol (gas meter)	20		37				50				
[19]	Manure + Veg wastes		20 (%-vol)	Vol (syringe)	0.135	0.075	35				105	N_2-CO_2 (70–30%)		Yes	2
[20]				Vol (liq-disp)	0.5	0.4	35	TWB			35	N_2			
[22]	Cow manure + Byproducts	79	13.3 (g VS/L)	Vol (liq-disp)	2	1.5	35		Batch (manually)	1/day	≈150	N_2	Yes ($NaHCO_3$)		
[23]				Vol (bag + meter)	2	1.5	35	TC			35				
[28]			20 (%-vol)	GC	2		55				50				
[34]	Sisal WW sludge	48		Vol (syringe)	1	0.6	33	Ambient room	Batch (manually)	2/day	65	N_2			0.35
[36]	Manure (cattle)			Vol (liq-disp)	5	4	37		Batch (mag bar)	2 min/3h	56		Yes [$Ca(OH)_2$]		
[37]	MWTP		20 (%-vol)		0.250	0.100	35				46	N_2-CO_2 (70–30%)		Yes	2
[38]	Various			Vol (gas meter)		55	35	TC			60		Yes ($NaHCO_3$)		
[40]	MSW-leach bed			Vol (gas meter)	220	110	38	TC	Mixer	290 rpm	20–40		Yes ($NaHCO_3$)	Yes	
[42]	Manure + Org wastes		400 (mL)	GC	2	0.5	55	TC	Batch (manually)	Ocassionally	50	N_2-CO_2 (80–20%)			2 (%-w/vol)
[45]	OFMSW	59	2.1 (g VS/L)	Man	1	0.500	35		Batch (manually)	1/day	30	He			1
[46]	MWTP	65	37.2 (g VS/L)	Vol (liq-disp)		1.5	35	TWB	Cont (stirrer)	300 rpm	96			Yes	2
[54]	MWTP	57		Vol (bag+meter)	0.5	0.3	37	TWB	Cont (shaker)	70 rpm	50	N_2-CO_2 (80–20%)			0.15–5.4
[57]	MWTP + distillery			Man (Oxytop®)	1	0.600	35	TC	Batch (manually)		40	N_2	Yes ($NaHCO_3$)	Yes	2
[60]	Paper-mill WW				1	0.600	20–40		Cont (shaker)	100 rpm	55	N_2-CO_2 (70–30%)	Yes ($NaHCO_3$)	Yes	1.4–2.1
[61]	Manure (digested)		60 (%-vol)			0.5/2	55				30–40	N_2-CO_2 (80–20%)		Yes	
[62]	MWTP meso/thermo	5652		Man	1	0.600	35 50		Batch (manually)	1/day	25	He			0.3 0.2–0.6
[64]	Rumen (bovine)				20						365				0–0.17
[65]	Manure (cattle)		15 (%-vol)	Vol (liq-disp)	3.25	2	26		Batch (manually)	1/day	240				
[66]	Manure (cow)	63	11.3 (g VS/L)		2.0	1.5	35				155	N_2-CO_2 (80–20%)			0.3–0.7
[67]	Rumen (sheep)				0.125	0. 050	39		Cont (shaker)	100 rpm	7	N_2		Yes	
[68]	Brewery (UASB)	75	15 (g VS/L)	Vol (liq-disp)	0.300	0.250	35	TWB	Cont (stirrer)	40 rpm	7	N_2	Yes ($NaHCO_3$)	Yes	0.5–3
[75]	Maize silage		15 (g VS/L)	Vol (liq-disp)	2	1	35		Batch (mag bar)	8 × 15 s/day	30			Yes	1.5
[77]	MWTP	51		Vol (liq-disp)	1	0.500	50		Batch (manually)	1/day	28	He			0.4–0.6

Reference	INO			GMS	Physical-OpC							Chemical-OpC			ISR
	Source	VS	C_o		Capacity (L)		Temp		Mixing		TD	Gas	Adj	MM	
		(%)			TV	WV	°C	System	Type	Times	(days)		pH/Alk		VS basis
[78]	MWTP			Vol (liq-disp)	0.250	0.100	35	TC			23	N_2-CO_2 (75–25%)	Yes ($NaHCO_3$)	Yes	
[81]	Manure (cattle)	60	10–90 (%-vol)	Vol (syringe)	0.119	0.050	35	TC			150	N_2			0.03–11
[82]	Manure (cattle)				2		26		Cont (mag bar)		35	N_2			
[83]	MWTP	63	15 (g VS/L)	Vol (liq-disp)		5	35	TWB	Cont (stirrer)		20	N_2	Yes ($NaHCO_3$)	Yes	1–3
[84]	Manure (cattle)			Man	0.250	0.120	35	TWB	Batch (manually)	2/day	30	N_2-CO_2 (80–20%)			0.5–2
[85]					1		37	TC			35				
[86]				Vol (liq-disp)	1		38				42				
[87]				Vol (liq-disp)	0.250		37								3 (TS)
[88]	Manure (cow)			GC	2.140	0.590	55					N_2			
[89]	Waste mixtures			Vol (liq-disp)		3.5	55	TWB			15–22	N_2-CO_2 (75–25%)		Yes	1.3–2
[90]	OFMSW		99 (%-w)		0.135	0.050	30	TC			45	N_2			
[91]	MWTP		20 (%-vol)				37	TC			28				
[92]	Manure (cattle) dung						35				100				2
[93]	Mixture (codigestion)			Vol (bag+meter)	2		40		Batch (manually)	2/day	40				2
[94]	OFMSW		30 (%-vol)	Vol (bag+meter)	2	0.8	40						Yes	Yes	
[95]	Manure (cattle) slurry			Vol (liq-disp)	2.5		35	TC			120				
[96]	Various		25 (%-w)		1.1		55	TWB			60				
[97]	Waste mixture				0.5		37		Cont (shaker)					Yes	
[98]	Manure (cattle)			Vol (syringe)	3		32		Cont (mag bar)		30				
[99]	Vegetable wastes		20 (%-vol)	Vol (syringe)	0.135	0.075	35				100	N_2-CO_2 (70–30%)		Yes	2
[100]	Seaweeds				2	1.7	32		Batch (manually)	15 sec/day	58				
[101]	Manure + Org wastes						55				50				
[102]	Rumen (goat)			Vol (liq-disp)	0.250	0.100	25–40	TC	Cont (shaker)	130 rpm	10	N_2	Yes ($NaHCO_3$)	Yes	0.3–0.8
[103]	MWTP			Vol (liq-disp)	1	0.9	30	WB			919	N_2		Yes	
[104]							35				60				
[105]	MWTP	53		Vol (bag+meter)	2	1.6	35				237	N_2-CO_2 (80–20%)	Yes ($NaHCO_3$)	Yes	
[106]	Manure (cattle)			Vol (liq-disp)	0.300	0.275	40				25–35	N_2	Yes		
[107]	Manure (cattle)			Vol (liq-disp)	0.300	0.275	40				57	N_2	Yes (NaOH)		
[108]	Effluent two-stage	60 85		Vol (gas meter)	5		35		Cont (mag bar)		37–85				0.8–2
[109]	MSW	53	10 (g VS/L)		0.120	0.060	55				122	N_2-CO_2 (80–20%)	Yes ($NaHCO_3$)		3.2
[110]	Manure (cow)			GC	0.118	0.040	55		Static		60	N_2			
[111]	MWTP		10 (%-vol)	Vol (syringe)	0.250	0.100	35				45			Yes	

Reference	INO			GMS	Physical-OpC							Chemical-OpC			ISR
	Source	VS	C_o		Capacity (L)		Temp		Mixing		TD	Gas	Adj	MM	
		(%)			TV	WV	°C	System	Type	Times	(days)		pH/Alk		VS basis
[112]	Rumen										30				
[113]	Vegetable+ Crops			Vol (liq-disp)	1		37.5	WB	Batch (mag bar)	10/30 min	28–38				3 (TS)
[114]	MWTP		20 (g VS/L)		0.500	0.250	35		Cont (shaker)	100 rpm	28	N_2	Yes ($NaHCO_3$)		10
[115]	Rice	60	3.3 (g VS/L)	Vol (liq-disp)	5	4	22		Cont (mag bar)		120	N_2	Yes (NaOH)		
[116]	Pig manure + Food waste				0.100	0.060	55				28		Yes ($NaHCO_3$)		
[117]	Manure (cattle)		8–10 (g VS/L)	Vol (syringe)	0.100	0.050	35	TWB	Batch (manually)	2/day	70	N_2	Yes (NaOH)		0.5
[118]	Digested material			Vol (gas meter)		2	35	TWB			28				
[119]	Manure (swine)			Vol (gas meter)	30	20	35	TC	recirculation	3/day (1 min)	50–60				
[120]	Sisal WW sludge	52		Vol (syringe)	1	0.6	27		Batch (manually)	2/day	25–29	N_2			0.4–20
[121]	Activated sludge	55	4.9 (g VS/L)	Vol (syringe)	0.5	0.3	37	TWB	Cont (shaker)	70 rpm	32–85	N_2-CO_2 (80–20%)	Yes ($NaHCO_3$)		0.65
[122]	Brewery (UASB)			Man	0.160		37			150 rpm	100		Yes ($NaHCO_3$)		0.43 (TS)
[123]	Brewery (UASB)			Man	0.160 120	80	37			150 rpm			Yes		0.14
[124]	Brewery (UASB)	65		Man	0.160		37			150 rpm			Yes ($NaHCO_3$)	Yes	0.74
[125]	MWTP			Vol (bag + meter)	0.5	0.3	37	TWB	Cont (shaker)	70 rpm	30	N_2-CO_2 (80–20%)	Yes ($NaHCO_3$)		1–2
[126]	Cow manure + Paper mill			Man	1	0.2	30		Shaker		30	N_2	Yes (phosphate)	Yes	1.5
[127]	Primary WAS + foodwaste			Man	0.200	0.100	38		Stirring	Sampling				Yes	
[128]	Primary WAS		20 (%-vol)	GC	0.275	0.100	35	TC			60	N_2-CO_2 (70–30%)		Yes	3–4
[129]	Manure (pig)			Vol (liq-disp)	0.120	0.065	37				90	N_2-CO_2 (70–30%)		Yes	3 (?)
[130]	Manure (cow)	77		Vol (liq-disp)	1	0.750					70–80	N_2	Yes ($NaHCO_3$)		1–2
[131]	Manure	69		GC	0.100	0.025	42	TWB	Cont (shaker)	100 rpm	67	N_2			2 (?)
[132]	MWTP			Man	1.130	0.800	35				100	N_2		Yes	0.03 0.04
[133]	MWTP	64	40–80 (%-vol)		0.118	0.050	35		Cont (shaker)		27	N_2-CO_2 (80–20%)			1.1–4.3
[134]	MWTP			Man	1.140										1.81
[135]	Manure, silage and byproducts	79	19 (g VS/L)	Vol (bag + meter)	1	0.750	35		Batch (manually)	Sampling	95	N_2	Yes ($NaHCO_3$)		1
[136]				Vol (liq-disp)	0.500	0.400	35		Batch (stirrer)	15/30 m 140 rpm	40			Yes	
[137]	Manure (cattle)	89	50 (%-vol)		1		36	TC	Batch	2/day	56				
[138]	MWTP		10 (%-vol)	Vol (syringe)	0.260	0.130	35		Cont (mechanical)	15 rpm	60–133	N_2-CO_2 (70–30%)		Yes	

Reference	INO			GMS	Physical-OpC						Chemical-OpC			ISR
	Source	VS	C_0		Capacity (L)		Temp		Mixing		TD	Gas	Adj	MM	
		(%)			TV	WV	°C	System	Type	Times	(days)		pH/Alk		VS basis
[139]	MWTP		20 (%-vol)	Man	0.250	0.100	35	TC			60–100	N_2-CO_2 (70–30%)	Yes ($NaHCO_3$)	Yes	2
[140]	Landfill leachate			Vol (liq-disp)	2.5	1.5	35		Batch (manually)	1/day	21	N_2			
[141]	Paper-mill WW			Man	0.160	0.100	35	TWB	Cont (shaker)	200 rpm	35	N_2/CO_2/H_2 (80-10-10%)		Yes	
[142]	MWTP		3.3 (g VS/L)	Vol (liq-disp)	1.175	0.600	35	TC	Batch (manually)	2/day	10–72	N_2	Yes (HCl/NaOH)		
[143]	Plant material			Vol (liq-disp)	3		35					25–36	Yes (NH_4OH)		

Source: MWTP-municipal wastewater treatment plant; WW: wastewater; VS: Volatile solids; C_0: Concentration (g VS/L); (%-w): % in weight; (%-vol): % in volume; GMS: gas measurement system; Vol: volumetric system; (liq-disp): liquid displacement; Man: manometric system; GC:gas chromatography; TV: Total volume; WV: working volume; Temp: Temperature; TWB: thermostatic water bath; TC: thermostatic chamber; Mixing: Cont-continuously; mag bar: magnetic bar; TD: Test duration; Adj: Adjustment of pH and/or alkalinity; MM: Mineral medium; (?): Units were not reported.

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Reference Type: Journal biotech
Author: B. Rincón, M. Del Carmen Portillo, J. M. González, V. Fernández-Cegrí, M. A. De La Rubia and R. Borja
Year: 2011
Title: Feasibility of sunflower oil cake degradation with three different anaerobic consortia
Journal: Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering
Volume: 46
Issue: 12
Pages: 1409-1416
Short Title: Feasibility of sunflower oil cake degradation with three different anaerobic consortia
ISSN: 10934529 (ISSN)
DOI: 10.1080/10934529.2011.607032
Notes: Cited By (since 1996): 1
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Journal of Environmental Science and Health, Part A (2011) **46**, 1409–1416

ISSN: 1093-4529 (Print); 1532-4117 (Online)
DOI: 10.1080/10934529.2011.607032

Feasibility of sunflower oil cake degradation with three different anaerobic consortia

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Sunflower oil cake (SuOC) is the solid by-product from the sunflower oil extraction process and an important pollutant waste because of its high organic content. For the anaerobic digestion of SuOC three different industrial reactors were compared as inoculum sources. This was done using a biochemical methane production (BMP) test. Inoculum I was a granular biomass from an industrial reactor treating soft-drink wastewaters. Inoculum II was a flocculent biomass from a full-scale reactor treating biosolids generated in an urban wastewater treatment plant. Inoculum III was a granular biomass from an industrial reactor treating brewery wastes. The highest kinetic constant for methane production was achieved using inoculum II. The inoculum sources were analyzed through PCR amplification of 16S rRNA genes and fingerprinting before (t = 0) and after the BMP test (t = 12 days). No significant differences were found in the bacterial community fingerprints between the beginning and the end of the experiments. The bacterial and archaeal communities of inoculum II were further analyzed. The main bacteria found in this inoculum belong to Alphaproteobacteria and Chloroflexi. Of the Archaea detected, Methanomicrobiales and Methanosarcinales made up practically the whole archaeal community. The results showed the importance of selecting an appropriate inoculum in short term processes due to the fact that the major microbial constituents in the initial consortia remained stable throughout anaerobic digestion.

Keywords: Sunflower oil cake, biochemical methane potential, microbial community, fingerprints, methane yield, kinetics.

Introduction

Sunflower oil cake (SuOC) is the solid waste generated during the sunflower seed oil extraction process. World sunflower seed production ranged between 29.1 and 31.1 million tonnes over the last few seasons.[1] As a result, large quantities of SuOC are generated every year. In Spain alone, between 4 and 5 million tonnes of this by-product are produced, giving rise to an important environmental issue.[2] Current perspectives on how to obtain high-value products from wastes involve anaerobic digestion processes for biogas generation [(a mixture of methane and carbon dioxide with a high energetic value (21.4 MJ per m^3)].

These anaerobic processes are performed by complex groups of microorganisms (Bacteria and Archaea) which coordinate the degradation of organic matter. A relatively low percentage of these microorganisms present in anaerobic digestion processes have been isolated. This lack of knowledge results sometimes in malfunctions and unexplainable failures of biogas fermenters. For these reasons, it must be analyzed in more detail.[3] Only a few studies have considered the potential influence of inoculum in anaerobic digestion systems. Moreno-Andrade and Buitrón[4] studied the influence of five different inocula on an anaerobic biodegradability test of two different substrates, one easily degradable (glucose) and the other toxic (phenol).

These authors emphasized the importance of using the appropriate inoculum to obtain satisfactory results from anaerobic processes. After testing two different inocula, granular and suspended, Pereira et al.[5] found granular inoculum to be the best option for the anaerobic treatment of synthetic oleic acid-based effluent, since the methanogenic activity of the granular inoculum was 2–7 times higher than that of the suspended biomass and was more resistant to long chain fatty acid toxicity. Foster-Carneiro et al.[6] compared six different inoculum sources for the anaerobic thermophilic digestion of the organic fraction of municipal solid wastes. Tabatabaei et al.[7] studied the importance of the microbial community, focusing on the methanogenic archaea in the anaerobic digestion of brewery wastewater, palm oil mill effluents, dairy wastes, cheese whey, dairy wastewater, pulp and paper wastewaters and olive oil mill wastewaters with respect to their dominant methanogenic population.

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Received January 20, 2011.

During the process of anaerobic digestion it is expected that the microbial communities adapt as a consequence of the growth of microorganisms under the specific conditions of digestion and the substrate treated. The dynamics of the acetoclastic methanogenic community have been evaluated under the influence of different wastewater compositions and even under inhibitory conditions.[8–10] The microbial community structure has been studied under low temperature conditions and under the influence of metal supplementation.[11–13] However, the transformations which occur in the microbial communities during the anaerobic digestion of organic wastes and methane production are still not fully understood.

It is clear that the efficiency of biogas production during the anaerobic digestion of organic residues depends on the microorganisms involved in the process. The study of these microbial communities represents an important step towards understanding and optimizing these anaerobic treatments. Thus, the aim of this work was to study the influence of the inoculum type on the anaerobic digestion of SuOC in terms of methane production. Microbial community fingerprints from the initial inoculum source and after the biochemical methane potential test (BMP) were compared, determining the major components of the communities involved in the process to achieve the best methane production kinetics.

Materials and methods

Substrate

The substrate used in this study was SuOC. Prior to the experiments, a study of the different particle sizes present in this solid waste was carried out by separation with a mechanical sieve. The most abundant size found (29.4 %) was 0.7–1.0 mm. Consequently, this size was used in the experiments. Table 1 shows the full composition and main features of the SuOC used in this study (mean values are averages of four determinations).

Inocula

Three different inoculum sources were used: a) an anaerobic granular inoculum derived from a full-scale upflow anaerobic sludge blanket (UASB) reactor treating wastewaters from a soft-drinks industry (I); b) a flocculent anaerobic inoculum from a full-scale completely stirred tank reactor (CSTR) treating biosolids from a conventional urban wastewater treatment plant (II); and c) an anaerobic granular inoculum from a UASB reactor treating brewery wastes (III). Table 2 shows the main characteristics of these three inocula. The experiments were carried out at an inoculum:substrate ratio of 2:1. An inoculum concentration of 15 g VS L^{-1} was used for each reactor.

Table 1. Characteristics of the SuOC used as substrate.

*Parameter**	*Value ± SD***
Moisture (%)	8.0 ± 0.5
Total protein (%)	31.4 ± 1.6
Fats (%)	1.7 ± 0.1
Carbohydrates (%)	58.7 ± 2.6
Hemicellulose (%)	9.2 ± 0.5
Lignin (%)	9.5 ± 0.4
Cellulose (%)	21.7 ± 1.1
TS (%)	93.4 ± 1.9
MS (%)	6.6 ± 0.1
VS (%)	86.5 ± 1.3
TCOD (g O_2 g^{-1} TS dry basis)	1.08 ± 0.04
C (%)	43.6 ± 0.3
H (%)	6.2 ± 0.1
N (%)	4.6 ± 0.6
O (%)	45.6 ± 0.5

*TS: total solids, MS: mineral solids, VS: volatile solids, TCOD: total chemical oxygen demand. **SD: standard deviation.

Reactors and operational conditions

The experiments were carried out in a thermostatized water bath (35°C) in batch mode. The reactors were stirred at 250 rpm with a magnetic stirrer. The BMP test was run by triplicate. Two controls without substrate were added in each run. A final working volume of 250 mL was used for each treatment. Methane production was measured by a NaOH solution (3N) displacement (CO_2 produced in the anaerobic process was kept in this sodium hydroxide solution).

Experimental setup

The experiment was carried out by triplicate and two control reactors with no substrate added were run for each different inoculum. The reactors were filled with 15 g VS L^{-1} of inoculum, the corresponding quantity of SuOC to reach a ratio of 2:1 inoculum to substrate, 25 mL of a 50 g $NaHCO_3$ L^{-1} solution to keep pH stable, 50 mL of nutrient solution (Table 3) and distilled water to a total volume of 250 mL. Methane production was measured for a period of 12 consecutive days.

Table 2. Characteristics and origin of the inoculum sources used in the experiments.

Sludge	*Source (reactor type)*	*Reactor volume (m^3)*	*pH*	*TS ($g\ L^{-1}$)*	*VS ($g\ L^{-1}$)*
I	UASB	450	7.4	30	25
II	CSTR	2000	7.6	43	20
III	UASB	550	7.5	83	47

TS: total solids; VS: volatile solids; UASB: upflow anaerobic sludge blanket; CSTR: continuously stirred tank reactor.

Table 3. Composition of the nutrient and trace element solutions used.

Nutrient solution composition	*Concentration (g L^{-1})*
NH_4Cl	1.4
K_2HPO_4	1.25
$MgSO_4$ H_2O	0.5
$CaCl_2$ $2H_2O$	0.05
Yeast extract	0.5
Trace element solution	5.0[a]
Trace element solution composition	*Concentration (mg L^{-1})*
$FeCl_3 4H_2O$	2000
$CoCl_2 \cdot 6H_2O$	2000
$MnCl_2$ $4H_2O$	500
$CuCl_2$ $2H_2O$	38
$ZnCl_2$	50
H_3BO_3	50
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	50
$AlCl_3$ $6H_2O$	90

Units for the trace element solution added to the nutrient solution are in mL of trace solution per L of nutrient solution (mL L^{-1}).

Analytical methods

Solids and moisture were determined according to the standard methods 2540B and 2540E.[14] Total chemical oxygen demand was determined using the solid substrate open reflux method.[15] Total protein was determined by multiplying the total Kjeldahl nitrogen (TKN) value by 6.25.[16] Fat content was extracted by a Soxhlet system using hexane (UNE-EN-ISO 659:2000). Cellulose, hemicellulose and lignin were determined by the Goering and Van Soest method.[17]

The elemental composition of the SuOC (C, N, O and H) was measured using a Leco CHNS-932 (Leco Corporation, St Joseph, MI, EEUU) elemental analyzer. For particle size selection the sunflower oil cake was sieved using a mechanical sieve (bio-meta, Retsch).

Methane production kinetics

A first-order kinetic model was used to estimate the specific rate constant according to Chen-Hashimoto Equation 1:[18]

$$B = B_o\,[1 - \exp(-k\,t)] \qquad (1)$$

where: B is the methane yield (mL CH_4 g^{-1} VS added), B_o is the ultimate or maximum methane yield, asymptote to the production curve *versus* time, k (day^{-1}) is the specific rate constant, and t is the digestion time (days). Methane yield values (B) were calculated by subtracting methane produced by the controls (inoculum only) from their corresponding treatment reactors. These differences were divided by the VS of the substrate.[18] B_o and k were calculated from the experimental data by non linear regression using Sigmaplot 9.0 (Systat Software. Inc., San Jose, CA).

Molecular characterization of microbial communities

Microbial communities, both *Archaea* and *Bacteria*, were studied by molecular fingerprinting methods complemented with cloning and sequencing for the identification of the major components of the bacterial and archaeal communities. DNA was extracted using the Nucleospin Food DNA extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations. Fragments of the 16S ribosomal RNA (16S rRNA) genes from the Bacteria and Archaea were amplified by PCR with different primer pairs. Fingerprints of the bacterial and archaeal communities were obtained by Denaturing Gradient Gel Electrophoresis (DGGE) following the method described by Muyzer et al.[19]

DNA was directly amplified by PCR using the primer pair 341F-GC (5′-CCT ACG GGA GGC AGC AG with a GC-rich tail attached to its 5′ end)[19] and 518R for the Bacteria and the primer pair 344F-GC (5′- with a GC-rich tail attached to its 5′ end) and 518R for the Archaea. Relative quantification of molecular fingerprints from pairs of community profiles was performed following the quantitative procedure described by Portillo and Gonzalez.[20] Gels obtained by DGGE were digitalized using Kodak 1D image analysis software (Kodak, New Haven, CT). The images were analyzed using the tnimage program (http://entropy.brneurosci.org/tnimage.html) applying its densitometry function. Comparisons between community fingerprints were carried out as described by Portillo and Gonzalez[20] calculating a Cramér-von Mises-type statistic through a Monte-Carlo test procedure to determine the significance of differences between microbial communities.

PCR products for 16S rRNA gene library construction were obtained with the primer pair 27F (5′-AGA GTT TGA TYM TGG CTC) and 907R (5′-CCC CGT CAA TTC ATT TGA GTT T) for the Bacteria[21] and the pair 20bF (5′-YTC CSG TTG ATC CYG CSR GA) and 1492bR (5′-GGY TAC CTT GTK WCG ACT T) for the Archaea.[22] These PCR products were purified with the PCR purification kit (JetQuick, Germany) and cloned using a TOPO-TA cloning kit (Invitrogen, Carlsbad, USA). The 16S rRNA libraries obtained were used to identify the major components of the bacterial and archaeal communities. A screening procedure based on the discrimination of clones using PCR-DGGE previously described by Gonzalez et al.[23] was applied to these libraries to identify the major DNA bands observed in DGGE analyses.

Sequence data were edited using Chromas software, version 1.45 (Technelysium, Tewantin, Australia). Homology searches from the nucleic acid sequences were performed using the Blast algorithm[24] at the NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/Blast/). Sequences were inspected for the presence of chimeras using the Ccode program as described by Gonzalez et al.[25]

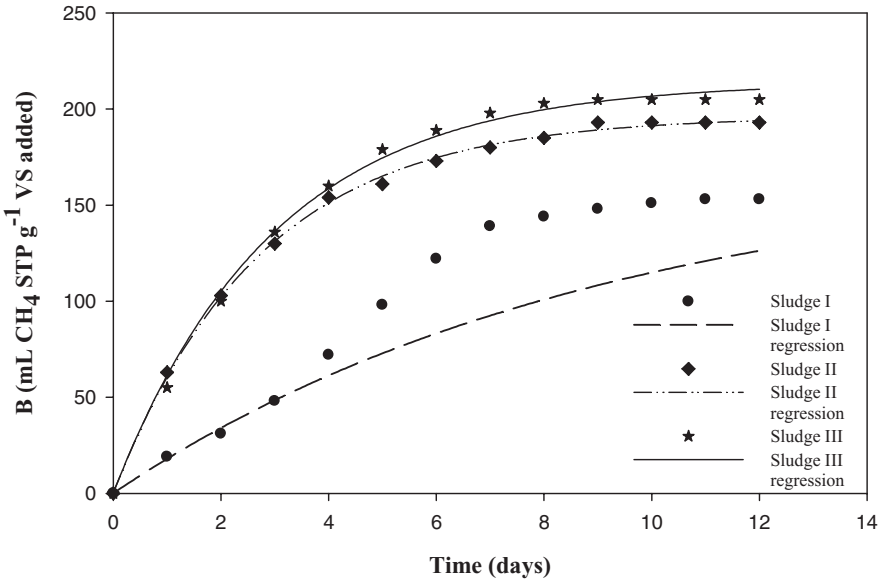

Fig. 1. Variation of the volume of methane produced per gram of VS added over time for inocula I, II and III.

Results and discussion

The volumes of methane (at standard temperature and pressure) obtained after 12 days of the BMP test for inocula II and III were higher than that obtained for inoculum I (293, 360 and 387 mL CH_4 for inocula I, II and III, respectively). Methane production for inoculum III was 7.5 % higher than for inoculum II and 31.1 % higher than for inoculum I. The experimental methane yields per gram of VS added (*B*) are shown in Figure 1. The best *B* values after 12 days were obtained for inocula II and III (193 and 205 mL CH_4 accumulated g^{-1} VS added, respectively), these yields being higher than that obtained for inoculum I (156 mL CH_4 accumulated g^{-1} VS added). The value of the methane yield for inoculum III was 6.2 % higher than for inoculum II, which in turn was 23.7 % higher than the value for inoculum I. The yield for inoculum III was 31.4 % higher than for inoculum I. Therefore, inocula II and III had similar methane yields and were both higher than for inoculum I.

The percentage of volatile solids removed was 42 % for inocula II and III and only 33 % for inoculum I. Inocula II and III from industrial reactors treating solid substrates showed better results than inoculum I from wastewater treatment. This could be attributed to the higher hydrolytic/enzymatic capacity of these inoculum sources which are used to break biosolids in urban wastewater treatment plants (inoculum II) and to treat brewery wastes (inoculum III).

The cellulose, lignin and hemicellulose structure of SuOC is complex. Cellulose is a polymer with low microbial degradability and is considered the rate-limiting substrate in the anaerobic digestion of solid wastes. [26]

In a comparative study for cellulose solubilisation in anaerobic reactors, O'Sullivan et al.[27] showed how differences in reactor configuration and operational conditions had no significant impact on the solubilisation rate of cellulose, whereas the difference in composition of the microbial communities showed a marked effect. This could be the reason why inoculum I, which had thus far been used to treat wastewaters, had given the worst results as regards methane production and kinetics for SuOC treatment. These findings should be studied in more detail.

The first-order kinetic model used to estimate the specific rate constants fit satisfactorily to the obtained experimental data (with R^2 values higher that 0.965; Fig. 1). The values obtained for *k* were 0.11±0.02, 0.37±0.01 and 0.34±0.01 $days^{-1}$ for inocula I, II and III, respectively (Table 4). Therefore, the specific rate constant for inoculum II was 8.8 % higher than that achieved for inoculum III and 236.4 % higher than that obtained for inoculum I.

Figures 2 and 3 show the molecular fingerprints obtained by PCR-DGGE and represent the major components of the bacterial (Fig. 2) and archaeal (Fig. 3) communities from the different inoculum sources (I, II and III) used during this study. For inoculum II, the taxonomic affiliation and the accession numbers of the closest homologue for the major components of the bacterial and archaeal communities are given in Tables 5 and 6, respectively. Comparisons of fingerprints from the bacterial and archaeal communities for the three inoculum sources used in this study (Figs. 2 [A, C and E] and 3 [G, I and K]) showed distinctive banding patterns which would indicate distinct microbial communities among the three inocula, depending on their source.

Maximum methane production was reached after 9 days for inocula II and III and after twelve days for inoculum I. After 12 days' digestion time, the bacterial communities (Fig. 2 [B, D and F]) established in the anaerobic digestion process of the SuOC, showed similar fingerprinting profiles

Table 4. Values of B_o and k obtained using the Chen-Hashimoto equation for the three sludges studied and their variation coefficients.

Sludge	R^2	$B_0 \pm SD$ (*mL* CH_4 g^{-1} *SV added*)	$k \pm SD$ ($days^{-1}$)	VC_{B0} (%)	VC_k (%)
I	0.9648	172 ± 27	0.11 ± 0.02	15.5%	25.4%
II	0.9985	196 ± 1	0.37 ± 0.01	0.6%	2.1%
III	0.9964	214 ± 2	0.34 ± 0.01	1.1%	3.6%

SD: standard deviation; VC: variation coefficient.

to those of the bacterial communities in their respective inocula (Fig. 2 [A, C and E]) before the anaerobic process. Statistical comparison of fingerprints from the initially inoculated communities and the final communities after the BMP test showed no significant differences (Table 7) in the bacterial communities from the different inoculum sources used in this study.

After the anaerobic digestion process of sunflower oil cake (Table 7), no significant differences were found in the archaeal community fingerprints between the initial inoculum (Fig. 3 [I and K]) and inocula II and III (Fig. 3 [J and L]). However, significant differences were observed between the initial inoculum (Fig. 3 [G]) and the archaeal community developed (Fig. 3 [H]) in inoculum I. Despite this change in the structure of the archaeal communities in inoculum I, the major archaeal components remained as important members of the final (after the anaerobic digestion process) communities. Changes observed in specific archaeal phylotypes in inoculum I could be the cause of a reduced performance of the process when compared to the evolution of inocula II and III, which were maintained during anaerobic digestion.

The bacterial and archaeal communities from inoculum II where the inoculum showed optimum methane kinetic parameters, was studied in further detail to identify the major components of the communities implicated in the anaerobic digestion and methane production.

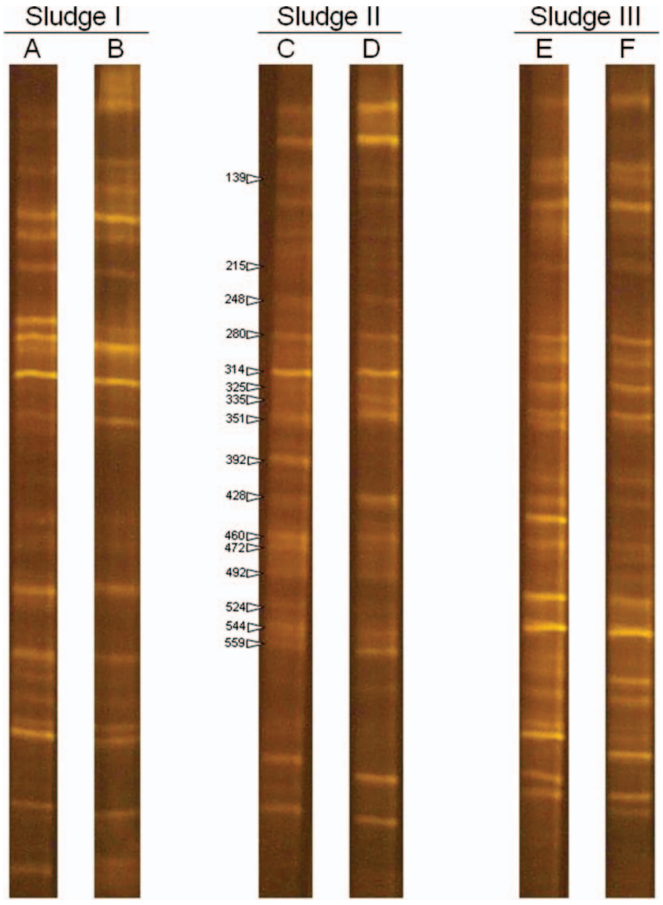

Fig. 2. Bacterial community fingerprints obtained by PCR-DGGE: (A, C, E) for the three different inoculum sources used for the initial inoculation of reactors and (B, D, F) after the BMP tests at the end of the anaerobic SuOC treatments (color figure available online).

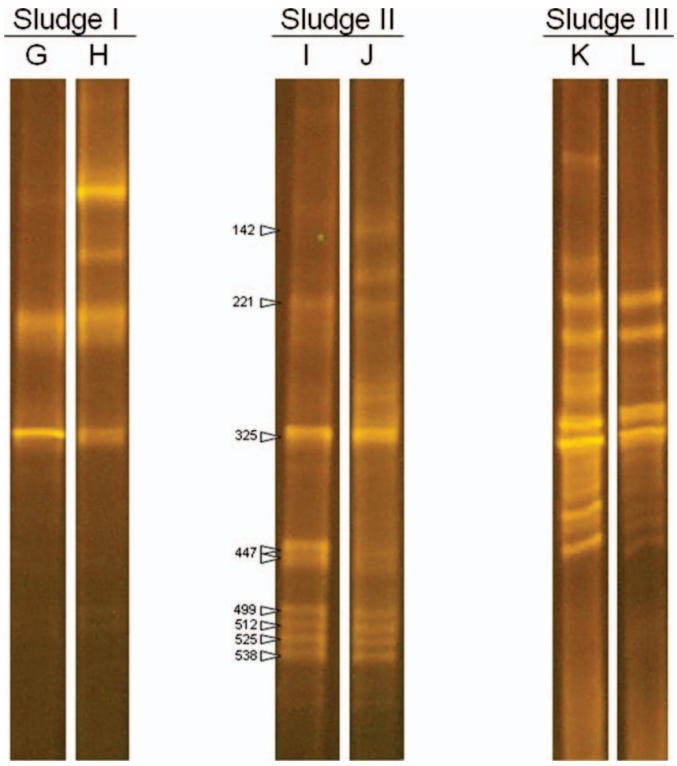

Fig. 3. Archaeal community fingerprints obtained by PCR-DGGE: (G, I, K) for the three different inoculum sources used for the initial inoculation of reactors and (H, J, L) after the BMP tests at the end of the anaerobic SuOC treatments (color figure available online).

Table 5. Accession numbers of closest homologue and proportions of the major bacterial phylotypes identified during this study determined through community fingerprinting analysis using PCR-DGGE from inoculum II.

Migration	*Taxonomic affiliation (accession no. of closest homologue)*	*Fraction inoculum**	*Fraction BMP**
139	Chloroflexi (CU926181)	3.4	3.8
215	Betaproteobacteria (GU454925)	1.9	0.8
248	Candidate Division WS6 (AF423183)	3.4	1.6
280	Chloroflexi (EF174275)	3.0	2.7
314	Chloroflexi (CU924314)	6.6	5.9
325	Actinobacteria (AY426438)	2.0	1.3
335	Alphaproteobacteria (AJ440751)	1.2	3.8
351	Alphaproteobacteria (GQ500763)	5.3	6.7
392	*Thauera*, Betaproteobacteria (DQ098974)	5.6	1.0
428	Bacteroidetes (CU922674)	2.7	6.1
460	*Paracoccus*, Alphaproteobacteria (FJ386516)	5.7	4.8
472	Chromatiales, Gammaproteobacteria (AM176837)	4.4	1.5
492	Thermoanaerobacteriales, Firmicutes (EU878332)	2.1	2.5
524	Synergistes, Synergistetes (FN436049)	2.4	1.4
544	Firmicutes (CU919983)	6.9	3.8
559	Bacteroidetes (AB330856)	2.6	5.4
	Total identified	59.2	53.1

*Percentage of total fluorescence intensity quantified from the banding pattern of PCR-DGGE analysis.

Table 6. Accession numbers of closest homologue and proportions of the major archaeal phylotypes identified during this study determined through community fingerprinting analysis using PCR-DGGE from inoculum II.

Migration	*Taxonomic affiliation (accession no. of closest homologue)*	*Fraction inoculum**	*Fraction BMP**
142	Methanosarcinales (FJ705109)	6.0	7.7
221	*Methanosaeta*, Methanosacinales (AB494241)	12.1	7.0
325	*Methanosaeta*, Methanosarcinales (FM162203)	20.5	28.8
447	Methanosarcinales (GU196156)	16.9	11.4
499	*Methanosaeta*, Methanosarcinales (EU591661)	6.4	6.3
512	Methanosarcinales (CU916012)	5.8	8.2
525	Methanomicrobiales (EU591675)	8.4	5.7
538	Methanomicrobiales (EU591675)	6.9	7.1
	Total identified	83.0	82.2

*Percentage of total fluorescence intensity quantified from the banding pattern of PCR-DGGE analysis.

Table 5 shows the proportion of the major bacterial constituents of the community in inoculum II. Alphaproteobacteria (20.6 % and 28.8 % of the total identified DNA in the inoculum and after anaerobic digestion, respectively), within the Rhodobacteraceae Family (e.g., *Paracoccus*), and Chloroflexi (22.6 % and 23.4 % of the total bacteria in the inoculum and in the community developed after anaerobic treatment, respectively) were the dominant bacterial groups. Proteobacteria, identified through members of the Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria, represented up to 40.7 % and 35 % of the identified bacteria in the inoculum and in the anaerobic digester, respectively. Other major bacterial groups identified in the community were Bacteroidetes (between 9.0 % and 21.7 % of identified bacterial phylotypes), Firmicutes (over 11 %; e.g., *Thermoanaerobacterium*), Actinobacteria (3.4 % to 2.5 %), Synergistetes (e.g., *Synergistes*) (above 2%), and Candidate Division WS6 (between 3.0 % and 5.7 % of the identified phylotypes).

The major bacterial components constituting the community of the anaerobic digestion process of sunflower oil cake coincide with the bacterial groups present in communities reported for other wastes.[22,28] Proteobacteria, Chloroflexi and Firmicutes have been reported as major components in bacterial communities during the anaerobic digestion processes of organic wastes.[22,29,30] Chloroflexi has recently been shown as a highly significant component in the transformation of complex substrates such as olive residues from oil production and this bacterial phylum is being increasingly recognized for its importance in anaerobic systems.[22,29–31] In these communities, numerous phyla, which are not well-known, such as the Bacteroidetes, Synergistetes and the Candidate Division WS6, were detected.

At present, there is limited knowledge about the metabolism of these phyla and they are generally detected only by their 16S rRNA gene sequences. Furthermore, there is little or no availability of representative cultivated microorganisms belonging to these bacterial phyla, which indicates that there is a significant portion of the bacterial community in need of further physiological research. The importance of Synergistetes, for instance, in anaerobic treatments has been highlighted in recent studies[32–33], as has the presence of Candidate Division WS6 in anaerobic

Table 7. Statistical results of the comparison between the microbial communities at the beginning (inocula) and ending of the anaerobic treatment of sunflower oil cake for the three types of inoculated sludges.

	Archaea		*Bacteria*	
Inoculated sludge	*P*	*CV (%)*	*P*	*CV (%)*
I	0.023*	0.098	0.170	0.093
II	0.188	0.081	0.211	0.079
III	0.542	0.046	0.316	0.068

P: Probability values; CV: coefficient of variation. Asterisk indicates significant differences ($P < 0.05$).

waste treatments and its relationship to methanogenic Archaea.[34]

Archaea are the microorganisms responsible for the production of methane. The archaeal communities represented by methanogenic groups constituted a critical component of the prokaryotic communities leading to methane production. Table 6 shows the proportion of the major archaeal phylotypes in inoculum II. The detected sequences from the archaeal community all corresponded to methane-producing Archaea. Different archaeal phylotypes were detected in the anaerobic digestion process of sunflower oil cake and belonged to the Methanosarcinales and Methanomicrobiales orders. The Methanosarcinales, mainly represented by different phylotypes belonging to the genus *Methanosaeta*, were the dominant methanogens, constituting over 67 % of the archaeal community.

A dominance of the methanogens Methanosarcinales and Methanomicrobiales has been previously reported as indicators of well-established methane-producing anaerobic digestion processes.[22, 35,36] These methanogens are acetoclastic methane producers and confirm the importance of this pathway in methanogenesis, as seen during the digestion of SuOC. As a consequence, a direct interaction between bacteria and archaea is envisioned, the main role of the bacterial community during this anaerobic process appeared to be the production of acetate from the polymers constituting the SuOC. This acetate is the major substrate which is directly utilized by the methanogenic archaea as the source for methane production.

Conclusions

The results obtained during this study underline the importance of using productive and active inoculum sources to initiate anaerobic digestion processes of sunflower oil cake wastes. Microbial communities showed no changes during short-term experiments (12 days). Obtaining the highest possible SuOC treatment efficiencies is a consequence of the conservation of the major components of well-established bacterial and archaeal communities during the digestion treatments. Only when an optimal inoculum is used can methane production and degradation of the processed substrate (i.e., SuOC) be maximized. A loss or reduction in specific phylotypes during the anaerobic treatments can be reflected by a diminishing efficiency both in methane production and organic load degradation.

Acknowledgments

The authors wish to express their gratitude to "Junta de Andalucía" ("Plan Andaluz de Investigación") for the financial support to groups BIO288 and AGR204, to the Spanish Ministry of "Ciencia and Innovación" (Project reference CTM2008-05772/ TECNO) and to the contracts JAE-Doc from "Junta para la Ampliación de Estudios del CSIC" co-financed by European Social Funds. The authors thank Dr. Fernando G. Fermoso for his valuable discussions during the preparation of the manuscript.

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Reference Type: Journal biotech
Author: F. Raposo, V. Fernández-Cegrí, M. A. de la Rubia, R. Borja, F. Béline, C. Cavinato, G. Demirer, B. Fernández, M. Fernández-Polanco, J. C. Frigon, R. Ganesh, P. Kaparaju, J. Koubova, R. Méndez, G. Menin, A. Peene, P. Scherer, M. Torrijos, H. Uellendahl, I. Wierinck and V. de Wilde
Year: 2011
Title: Biochemical methane potential (BMP) of solid organic substrates: Evaluation of anaerobic biodegradability using data from an international interlaboratory study
Journal: Journal of Chemical Technology and Biotechnology
Volume: 86
Issue: 8
Pages: 1088-1098
Short Title: Biochemical methane potential (BMP) of solid organic substrates: Evaluation of anaerobic biodegradability using data from an international interlaboratory study
ISSN: 02682575 (ISSN)
DOI: 10.1002/jctb.2622
Notes: Cited By (since 1996): 6
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Research Article

Received: 13 January 2011 Revised: 25 February 2011 Accepted: 28 February 2011 Published online in Wiley Online Library: 12 April 2011

(wileyonlinelibrary.com) DOI 10.1002/jctb.2622

Biochemical methane potential (BMP) of solid organic substrates: evaluation of anaerobic biodegradability using data from an international interlaboratory study

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Abstract

BACKGROUND: This paper describes results obtained for different participating research groups in an interlaboratory study related to biochemical methane potential (BMP). In this research work, all experimental conditions influencing the test such as inoculum, substrate characteristics and experimental conditions were investigated. The study was performed using four substrates: three positive control substrates (starch, cellulose and gelatine), and one raw biomass material (mung bean) at two different inoculum to substrate ratios (ISR).

RESULTS: The average methane yields for starch, cellulose, gelatine and mung bean at ISR of 2 and 1 were 350 ± 33, 350 ± 29, 380 ± 42, 370 ± 36 and 370 ± 35 mL CH_4 g^{-1} VS_{added}, respectively. The percentages of biotransformation of these substrates into methane were 85 ± 8, 85 ± 7, 88 ± 9, 85 ± 8 and 85 ± 8%, respectively. On the other hand, the first-order rate constants obtained from the experimental data were 0.24 ± 0.14, 0.23 ± 0.15, 0.27 ± 0.13, 0.31 ± 0.17 and 0.23 ± 0.13 d^{-1}, respectively.

CONCLUSION: The influence of inocula and experimental factors was nearly insignificant with respect to the extents of the anaerobic biodegradation, while the rates differed significantly according to the experimental approaches.

Keywords: anaerobic digestion; biodegradable; biomass; bioreactors; environmental biotechnology; reactor optimization

INTRODUCTION

Biochemical methane potential (BMP) is a procedure developed to determine the methane production of a given organic substrate during its anaerobic decomposition. The BMP assay has proved to be a relatively simple and reliable method to obtain the extent and rate of organic matter conversion to methane.[1] The information provided by BMP is valuable when evaluating potential substrates and for optimizing the design and functioning of an anaerobic digester. Literature related to BMP assays is extensive, showing that this test has been used to evaluate a wide variety of substrates.[2,3] Interest in recent years has increased as can be demonstrated by the wide range of research papers dealing with BMP assays. In addition, several batch methods have been utilized for measuring methane potentials, but unfortunately there is no standard protocol for carrying out the determination.[4] Consequently, methane yields reported in the literature have limited comparability and cannot be precise because of possible differences in the experimental protocol used for the assay. There are many factors that may influence the anaerobic biodegradability of organic materials, and some of these factors are, at present, only poorly understood and frequently not described in the procedure. Recently a new

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proposed protocol for BMP testing has been published, where some basic guidelines for a common procedure are given.[5]

On the other hand, very scarce information was found in the literature relating to similar research work. Only one interlaboratory study (in which 21 laboratories participated) has previously been published.[6] Unfortunately, this interlaboratory study was designed from a more restricted point of view, using two organic substrates (palmitic acid and poliethylenglicol 400) as micro-pollutant (concentration 50 mg C L^{-1}) and a complex gas measurement system (headspace pressure in conjunction with inorganic carbon determination).

Therefore, the purpose of this research work was to collect and compile results obtained in the BMP interlaboratory study using different solid organic substrates with the aim of providing an extensive database for BMP extent and rates in relation to the experimental conditions selected.

EXPERIMENTAL

The approach of the BMP test is simple. An organic substrate is mixed with an anaerobic inoculum in defined operating conditions, and the gas evolved is quantified by a specific measurement system until gas production is virtually ceased. However, the protocols available in the literature are very different. The full description of factors influencing the results of the BMP test, such as inoculum, substrate and experimental conditions (Table 1), was considered as mandatory information to be reported by participating laboratories. For this interlaboratory study, as the substrates were the same for all participants, their effect can be disregarded as a source of uncertainty in the final results.

Organization of the interlaboratory study

The interlaboratory study was organized by the Spanish National Research Council (CSIC) through the Instituto de la Grasa, specifically by the 'Water and Wastewater Treatment' group. The interlaboratory study coordinator and collaborators were responsible for designing the scheme, the preparation of test materials, the production and distribution of instructions and test material among the participating laboratories, the collection and statistical analysis of the data obtained, and feedback of the results to all participants (anonymously to guarantee confidentiality).

Each participating laboratory received a full set of samples, together with basic technical guidelines about how to proceed with the measurements; participating laboratories were free to select the inoculum and virtually free to choose the experimental conditions. In this interlaboratory study, 19 laboratories reported data, including two having results that were not appropriate for comparison purposes. The number assigned to each participating laboratory was given in random order to guarantee confidentiality of the results obtained.

Table 1. Factors affecting the BMP assays

I. Inoculum
I.1. Origin I.2. Characterization: pH, TS, VS, TSS, VSS I.3. Amount (g) and concentration (g VS L^{-1}) at start-up of the experiment I.4. Activity I.5. Time from sampling to starting test (days)
II. Substrate
II.1. Type (part and particle size) II.2. Characterization: moisture, TS, VS, TKN, organic fraction composition, atomic or elemental composition, fiber composition II.3. Amount (g) and concentration (g VS L^{-1}) at start-up of the experiment
III. Experimental conditions
III. 1. Quantification of gas III.1.1. Measurement system (MS) (a) Manometric (Man), by pressure (p) (b) Volumetric (Vol), by water displacement (wd) or gas counter (gc) (c) Gas chromatography (GC) III.1.2. Type of gas (Type): Biogas (Bg) or Methane (Me) III.1.3. Biogas composition (BgC): Yes, by GC analysis (Com)/No, CH_4 directly (Di)
III.2. Operational conditions III.2.1. Physicals (a) Reactor capacity: Working volume (W_{VOL}) and Total volume (T_{VOL}) (b)Temperature (T): Range: Mesophilic - 35 °C/Thermophilic - 55 °C System: Thermostatic water bath (TWB) or chamber (TC) (c) Stirring (St): Manual (Ma)/Automatic (Au) and Continuous (C)/Batch (B) If automatic: Magnetic bar (mb)/Shaker (sh) If batch: times/day (d) Time (t): Pre-incubation (Prel-t) and test duration (TD-t) III.2.2. Chemicals (a) Headspace gas (G_{hs}) (b) pH/alkalinity adjustement (pH/Alk Adj): If yes, chemical reagent and concentration at start-up of the experiment (c) Mineral medium (MM): If yes, chemical composition and concentration at start-up of the experiment III. 2.3. Inoculum to substrate ratio (ISR)

MATERIALS

Inocula

An important factor which cannot easily be standardized is the source of the sludge used as inoculum and its state of acclimation and adaptation to a test material.[7] Given the microbial diversity typically encountered among most groups of microorganism forming the anaerobic inocula, the use of a standard inoculum is simply unrealistic. Most previous protocols have been promulgated using anaerobic sludge from municipal wastewater treatment plants (MWTP), owing to the metabolically active microbial assemblages and to the fact that it is easily available. In the present interlaboratory study no suggestions were made about the inoculum to be used. In addition, two participating laboratories (numbers 2 and 4) used three different sources of microbial biomass to carry out the BMP test. Table 2 summarizes the main characteristics of inocula used:

- Origin/source: different sludges from operating anaerobic reactors were selected as microbial biomass. MWTP was mainly used as inoculum source (12); followed by biowaste, manure and brewery sources (2), and finally sludges from the wastewater treatment of soft drink, potato, vinasses, paper mill and agrofood industries were selected in minor proportion (1).

Table 2. Characteristics of the inocula used by participating laboratories

Laboratory	Origin/Source	pH	TS (g L^{-1})	VS (g L^{-1})	VS/TS (%)	C_o (gVS L^{-1})	Time from sampling (d)
1	Manure fed-Industry	7.8	57.9	37.8	65	37.8	30
2.1	Thermophilic biowaste (dry)	7.9	215.0	113.0	53	56.5	15
2.2	Thermophilic biowaste (wet)	8.0	66.9	39.3	59	39.3	8
2.3	MWTP	7.7	44.4	24.3	55	24.3	6
3	MWTP	7.8	21.6	12.4	57	10.4	19
4.1	Soft drink industry	7.4	30.0	25.0	84	15.0	4
4.2	Brewery industry	7.4	83.0	47.0	56	15.0	4
4.3	MWTP	7.6	43.0	20.0	48	15.0	4
5	MWTP	ND	24.8	12.1	49	10.0	4
6	Manure fed-Lab	8.0	58.0	39.0	67	11.7	6
7	Potato industry	7.8	15.0	6.3	42	5.5	10
8	MWTP	6.8	24.2	16.4	68	11.5	2
9	MWTP	6.8	25.0	13.8	55	13.7	6
10	Distillery vinasses industry	ND	ND	ND	ND	5.0	7–14
11	Brewery industry	7.3	39.4	33.9	86	10.0	60
12	MWTP	7.8	25.0	15.0	60	7.3	11
13	Paper mill industry	ND	136.0	102.0	75	8.5	Unknown
14	MWTP	7.3	24.2	13.5	56	3.1	1
15	Agrofood industry	8.2	117.0	97.0	83	20.0	150
16	MWTP	7.2	95.0	42.0	44	10.0	20
17	MWTP	7.4	50.0	30.7	61	30.0	7
18	MWTP	7.4	18.2	13.6	75	13.6	1
19	MWTP	7.4	27.5	16.2	59	8.1	1

MWTP: Municipal wastewater treatment plant.
ND: Not determined.

Table 3. Characterization of substrates used

	Starch/Cellulose	Gelatine	Mung bean
Moisture (%)	10/3	8	9
TS (%)	90/97	92	91
VS (%-TS)	99/100	100	97.0
Elemental (%-TS)			
C	44.5*/44.0**	48.2	44.7
H	6.2*/6.0**	6.5	6.8
N	–	18.4	4.4
S	–	0.6	–
O	49.3*/50.0**	26.2	41.1
Empirical formulae	$C_6H_{10}O_5$* $C_{366}H_{595}O_{313}$**	$C_{402}H_{648}O_{164}N_{131}S_2$	$C_{372}H_{670}O_{257}N_{32}$
ThOD (g O_2/g TS)	1.184*/1.158**	1.236	1.240
COD (g O_2/g TS)	1.145*/1.164**	1.246	1.225

* Using theoretical values.
** Using experimental values.

- pH: the values ranged from 6.8 to 8.2, in all cases to achieve an initial pH value between 7.0 and 7.8.
- Total solids (TS), Volatile solids (VS) and VS/TS: the solid content ranged from 15.0 gTS L^{-1} to 215.0 gTS L^{-1}, while the organic content ranged from 6.3 gVS L^{-1} to 113.0 gVS L^{-1}. VS/TS ranged from 42% to 86%.
- Concentration in BMP test at the start-up of the experiment (C_o): the initial concentration of cellular biomass ranged from 3.1 gVS L^{-1} to 56.5 gVS L^{-1}. The average value was 13.5 gVS L^{-1}.
- Time elapsed from sampling: the range was also wide, ranging from 1 d to 150 d. The average value was 19 d.

Substrates

Substrates selected for this interlaboratory study were characterized according to their relevant substance-specific properties and suitability for biodegradability (Table 3). Two main groups of substrates have been used for this research:

(i) Positive control substrates
- Starch soluble from potato (Sigma-Aldrich) to measure the amylase activity.
- Avicel® PH-101 cellulose (Sigma Aldrich) to measure the cellulase activity.

Table 4. Summary of overall experimental conditions reported by laboratories participants*

		Physicals								Chemicals			
		Capacity (L)		T (°C)		Stirring		time (d)			pH/Alk Adj		
LAB	MS	W_{VOL}	T_{VOL}					PreI-t	TD-t	G_{hs}			MM
1	GC	0.025	0.117	38	TC	No		10	35	N_2-CO_2	No		No
2	Vol-wd	0.500	2.000	52 37	TC	No		7	13	N_2-O_2	No		No
3	Vol-wd	0.080	0.120	36	TC	Ma-B		0.04	35	N_2	Yes	$NaHCO_3$ (0.4 g L^{-1})	Yes
4	Vol-wd	0.250	0.300	37	TWB	Au-C	mb	1	13	N_2	Yes	$NaHCO_3$ (5 g L^{-1})	Yes
5	GC	0.500	1.200	37	TC	Au-C	sh	1	35	N_2	Yes	$NaHCO_3$	Yes
6	Man-p	0.100	0.330	38	TC	Ma-B		0	31	N_2-CO_2	No		Yes
7	Vol-wd	0.250	0.300	35	TC	Ma-B		0	20	N_2	No		No
8	Vol-wd	0.700	1.000	35	TWB	Ma-B		2	20	N_2-O_2	No		No
9	Man-p	0.500	0.600	35	TC	Au-C	sh	5	28	He	Yes	$NaHCO_3$ (5 g L^{-1})	Yes
10	Vol-wd	0.400	0.500	35	TC	Au-C	sh	0	30	N_2	Yes	$NaHCO_3$ (2.6 g L^{-1})	Yes
11	Man-p	0.375	0.500	35	TWB	Au-C	sh	2	87	N_2	Yes	$NaHCO_3$ (5 g L^{-1})	Yes
12	Vol-wd	0.700	1.000	35	TC	Ma-B		11	22	N_2	No		No
13	Man-p	0.200	1.000	35	TC	Au-C	sh	0	20	N_2	No		Yes
14	Man-p	0.400	1.165	35	TC	Au-C	mb	18	20	N_2	No		Yes
15	Vol-wd	0.100	0.500	35	TWB	Au-C	sh	0	40	N_2-CO_2	No		Yes
16	Vol-wd	0.150	0.250	36	TWB	Au-C	sh	0.5	38	N_2	Yes	$NaHCO_3$ (6 g L^{-1})	Yes
17	Vol-gc	0.600	1.100	41	TC	No		0	24	N_2-O_2	No		Yes
18**	Vol-gc	0.100	0.125	37	TWB	Ma-B		0	66	N_2	No		Yes
19**	Vol-wd	0.750	1.000	35	TC	Ma-B		1	24	N_2	Yes	$CaCO_3$ (1.24g L^{-1})	No

* The information about terminology selected is included in Table 1.
** Data not considered for comparative purpose.

- Gelatine to bacteriological use (Panreac) as protein **substrate** to measure the proteinase activity.

(ii) Biomass material

The seed of the plant *Vigna radiata* known as mung bean (MB) was selected as biomass sample owing to its biodegradable nature and the novelty, because it had not previously been used (according to the literature) as substrate for BMP assays. The seeds were ground and sieved and used in powder form. The particle size of the material used in this assay ranged from 0.125 mm to 0.500 mm. Its organic composition (dry basis) includes mainly carbohydrates (72.4%) and protein (23.1%), with a low content of fat (1.5%). In addition the substrate presented low fiber content (5% of neutral detergent fiber-NDF and 4% of acid detergent fiber-ADF) and no lignin.

Experimental conditions

For this interlaboratory study, full details of experimental procedures such as gas measurement systems and operational conditions (physical, chemical and inoculum to substrate ratio – ISR) were reported by the participating laboratories and are compiled in Table 4.

Gas measurement systems

Gasometrical methods are the ones most frequently used for determining anaerobic biodegradability. In such methods, biogas/methane production can be quantified either manometrically or volumetrically. Also a gas chromatography (GC) technique can be used for this purpose.

For this interlaboratory study volumetric methods were used most (63%), followed by manometric methods (26.3%) and finally by GC methods (10.5%). Furthermore, all the participants based their biogas composition on GC analysis, except one laboratory (number 4) which measured the methane directly after CO_2 removal by flushing the biogas through NaOH 2N solution.

Physical operational conditions

- Reactor capacity: a wide range of working volumes (W_{VOL}) was used, varying from 25 mL to 750 mL. The most often used capacities were 100 mL and 500 mL (three times each).
- Temperature: most participants used the mesophilic range, with temperature ranging from 35 °C to 41 °C. Exceptionally, one participant (lab number 2) also used a thermophilic temperature range (52 °C).
- Stirring: agitation of digesters can be carried out in a number of ways including manual shaking, magnetic stirrers, orbital

shaking, etc. Also, the main factors affecting mixing strategy are the intensity and the duration. In this interlaboratory study, three participants used a static system, seven participants mixed manually and nine participants mixed using automatic devices.
- Time: the duration of the BMP ranged from 13 d to 87 d, with average value was 32 d.

Chemical experimental conditions
- Headspace gas (G_{hs}): different gases were reported as components of the headspace, such as N_2, N_2-CO_2 mixtures, air (N_2-O_2) and He. In this interlaboratory study, pure N_2 was the most widely used headspace gas (63%).
- pH/alkalinity adjustment (pH/Alk Adj): batch tests must be carried out at pH values ranging from 7.0 to 7.8. The alkalinity controls the capacity of the system to neutralize acids and provides resistance to significant and rapid changes in pH; it is also known as 'buffering capacity'. A value of 2500 mg $CaCO_3$ L^{-1} is considered to be normal for sewage sludge. A more desirable range of 2500–5000 mg $CaCO_3$ L^{-1} provides a buffering capacity for which a much larger increase in VFA can be accommodated with a minimum drop in pH.[8]
  In this interlaboratory study, 7 of 17 participants (41%) that reported appropriate data used different concentrations of $NaHCO_3$ to increase the buffer capacity of the system.
- Mineral medium (MM): it is well documented that all microbial-mediated processes require nutrients and trace elements (metals and vitamins) during organic biodegradation.[9] However, it is not clear if under the normal conditions of a BMP test sufficient nutrients are available from the sludge and/or organic substrate, or if additional supplements are necessary. In fact, 12 participants (71%) that reported appropriate data used different MM solutions to increase the performance of the test. Full details about the different minerals and concentrations were provided by participating laboratories, although these are not included in this manuscript.

Inoculum to substrate ratio (ISR)

Chudoba *et al*. clearly stated that ISR is one of the most important parameters in batch tests.[10] Unfortunately, many research papers do not report the ISR used in the experimental design. In addition, the units used (TS, VS or COD basis) must be clearly stated. In this interlaboratory study this parameter was considered crucial and it was fixed in advance by the interlaboratory study coordinator (VS basis). BMP determinations were established by highlighting the importance of using an adequate ISR to control the biodegradation process. The ISR can be low or high. Previous research work suggested the use of high ISR, ≥ 2.[1,11,12] Following the earlier suggested value, in this interlaboratory study an ISR of 2 was used for starch and cellulose. Taking into account that ammonia is an inhibitor of the anaerobic digestion process, the organic load for pure protein substrate (gelatine) was decreased to achieve an ISR of 3. For MB, two ISRs (2 and 1) were used to study the influence of this parameter on the BMP results.

Operational procedure

The operational procedure used in this interlaboratory study included six runs; three runs to evaluate the activity of the different inocula used and as quality control of the BMP tests; and two runs to determine the methane potential of mung bean, including the influence of ISR on the results. In addition, a blank control run was mandatory to consider the influence of background biogas production. Following the recommendations of various protocols related to BMP, triplicate determinations were carried out to evaluate the BMP tests. This is because the assay is a biological determination using inoculum from different sources (varying quality) and because the test material should also be relatively heterogeneous.

Theoretical methane potential (BMP_{Th})

The theoretical methane potential is widely used to predict the methane production of a specific organic substrate. It is frequently expressed as mL CH_4 at standard temperature and pressure (STP) conditions per amount of organic material added (VS or COD basis), although it can also be expressed per organic material removed. In the present research, the selected units used for expressing the methane potential were mainly mL CH_4 g^{-1} VS_{added}. There are different ways to calculate this parameter:

(i) Traditionally BMP_{Th} has been calculated when the atomic (AtC) or the organic fraction compositions (OFC) are known:[9]
- BMP_{ThAtC} or $B_{o-ThAtC}$. Empirical formulae ($C_aH_bO_cN_dS_e$) can be designed from experimental elemental analysis determination. Assuming the total stoichiometric conversion of the organic matter to methane and carbon dioxide using Buswell's equation the methane yield can be calculated:[13]

$$B_{o-ThAtC} = \frac{[(a/2)+(b/8)-(c/4)]\cdot 22\,400}{(12a+b+16c)} \quad (1)$$

  However, when proteins are present, ammonia and H_2S are released and must be taken into consideration using Boyle's equation:[14]

$$B_{o-ThAtC} = \frac{[(a/2)+(b/8)-(c/4)-(3d/8)-(e/4)]\cdot 22\,400}{(12a+b+16c+14d+32e)} \quad (2)$$

- BMP_{ThOFC} or $B_{o-ThOFC}$. If the organic fraction composition (lipids, proteins, and carbohydrates) is known, methane yield can be estimated using the following general equation:

$$B_{o-ThOFC} = 415\cdot\%Carbohydrates + 496\cdot\%Proteins + 1014\cdot\%Lipid \quad (3)$$

  where the different fractions must be quantified by analytical composition measurements of the organic matter. The coefficients in this equation are derived from stoichiometric conversion of model compounds representing average formulae for carbohydrates ($C_6H_{10}O_5$), proteins ($C_5H_7O_2N$) and lipids ($C_{57}H_{104}O_6$).[9]
  Recently, some authors have proposed more sophisticated multiple regression models to predict the methane yield of organic matter from their chemical composition.[15–17]

(ii) COD analysis permits the calculation of BMP_{Th}. Theoretically, 0.350 L of methane at STP or 0.395 L at 35 °C and 1 atm can be obtained from 1 g COD removed (COD_{rem}).
- BMP_{ThCOD} or $B_{o-ThCOD}$. Unfortunately, directly measuring the COD of a solid waste is often thought to produce erroneous results.[18] However, a new recent interlaboratory test showed that the participation in proficiency tests hugely improved the precision and truth of results obtained.[19] Moreover, COD is necessary for real reactor design, helping to normalize the results independently of

VS fraction composition.[20] To calculate the methane yield, the following equation can be applied:

$$B_{o-ThCOD} = VS_{added} \cdot (g\ COD/g\ VS) \cdot 350 \quad (4)$$

- BMP_{ThOD} *or* B_{o-ThOD}. The calculation of the theoretical oxygen demand (*ThOD*) based on atomic composition provides an attractive and easy alternative for obtaining the organic strength of some solid substrates. The empirical formula can also be used to calculate the estimated organic content, applying the following simple equation:[11]

$$ThOD(g\ O_2 \cdot g^{-1} VS) = \frac{[(2a) + (b/2) - c - (3d/2)] \cdot 16}{(12a + b + 16c + 14d)} \quad (5)$$

However, in this work *ThOD* has been calculated following the procedure suggested by ISO/DIS 10 707.[21] Independently of how *ThOD* is calculated, the methane yield can be obtained by applying:

$$B_{0-ThOD} = VS_{added} \cdot (g\ ThOD/g\ VS) \cdot 350 \quad (6)$$

Experimental methane potential (BMP_{Exp})

The major disadvantage of the BMP test is the duration of the assays and the fact that it does not provide short-term results. Because of the time necessary to perform a BMP test, it would be better if methane yield could be predicted by any of the earlier proposed methods. However, experimental assays are necessary to accurately check the real methane potential of the organic materials. Two experimental methane potentials can be used:

(i) BMP_{ExpCAL} or B_{o-Exp}. This value is calculated (CAL) by dividing the net methane production under STP conditions by the weight of the sample added (VS or COD basis).

(ii) BMP_{ExpKIN} *or* B_o. This derived value is defined as the ultimate methane yield or maximum value at infinite digestion time. It can be calculated by applying one of the different forms of the first-order kinetic (KIN) model, which is a simple and useful model that has been frequently applied to anaerobic digestion systems. However, this model does not predict the conditions for maximum biological activity and system failure. The basic equation is:

$$dS/dt = -k \cdot S \quad (7)$$

where k is the first-order kinetic constant (time^{-1}), t is the digestion time and S represents the biodegradable substrate concentration. As S is a difficult parameter to measure, it is preferable to derive the model by using the measurement of gas, which is much easier to determine:

$$B = B_o \cdot [1 - exp(-k \cdot t)] \quad (8)$$

where B ($mLCH_4\ g^{-1}$ VS) is the cumulative methane yield, B_o ($mLCH_4\ g^{-1}$ VS) is the maximum or ultimate methane yield of the substrate, k (days^{-1}) is the first-order rate constant and t (d) is the time.

The results from the experimental methane yields can be fitted to monophasic or biphasic curves. The former have been recommended because only when the accumulation of intermediary compounds during anaerobic digestion is negligible can methane production be related to hydrolysis rate.[22] The model is usually used to determine the extent and rate of biodegradation.

It is important to note that in the present research work B_o was not used in further analysis; however, when B_o differs from B_{o-Exp} by more than 10%, the kinetic model cannot be used to explain the data obtained because then, experimental data does not fit the proposed model (Equation (8)), and k is not valid.

Biodegradability based in methane yield (BD_{CH4})

The experimental methane yield can be used to calculate the level of anaerobic biodegradability under the defined test conditions in comparison with its theoretical value, as follows:

$$BD_{CH4}(\%) = (B_{o-Exp}/B_{o-Th}) \cdot 100 \quad (9)$$

When the anaerobic biodegradability of the organic material is calculated from the methane conversion efficiency according to the above equation, it can be considered that the main organic matter removed is converted into methane, but some defined amount of the organic matter is used for growth of the microorganisms and to maintain cellular metabolism. This amount cannot be measured directly but needs to be estimated. It is known from practical experience that about 5–15% of the organic matter removed is consumed in the generation of new microbial biomass.[23–25] However, Scherer *et al*.[26] obtained a lower value (3%) in batch assays of spent grains from breweries by measuring DNA. This means that to find the real degree of biodegradation, the value obtained from experimental data should be increased by the value of this cellular yield.

On the other hand, considering the biodegradability nature of the substrates utilized for this interlaboratory test, the results reported with BD_{CH4} <70% (methane production basis) were considered as outliers or not valid data.

Analytical methods

Standard environmental and feedstuff analytical procedures were used to characterize the inocula and substrates. These analyses were performed in duplicate or triplicate and included the following parameters:

- Moisture, TS-dry matter and VS-organic matter were determined according to the APHA Standard Methods 2540B and 2540E.[27]
- Total chemical oxygen demand (CODt) was determined using the reported method proposed by Raposo *et al*.[28]
- The fat content was extracted from a dried sample with hexane, using a Soxhlet system.[29]
- The total protein content was determined by multiplying the difference between total Kjeldahl nitrogen (TKN) and ammonia by 5.5. To determine TKN the procedure reported by Raposo *et al*. was used.[12] Ammonia was determined according to the APHA Standard Methods 4500B and E.[27]
- The total carbohydrates (including fibre and soluble sugars) were calculated by the difference between the organic matter and lipids, protein and lignin content.
- Fiber analysis (NDF, ADF, and acid detergent lignin-ADL) was carried out according to Van Soest.[30]
- Elemental composition (C, H, O, N, S) of the samples was performed in a LECO CHNS-932 combustion analyzer at 1050 °C, using sulphametazine as standard substrate.

Statistical analysis

Methane yields were reported as the average of replicate samples. Average values and corresponding standard deviations of B_o and

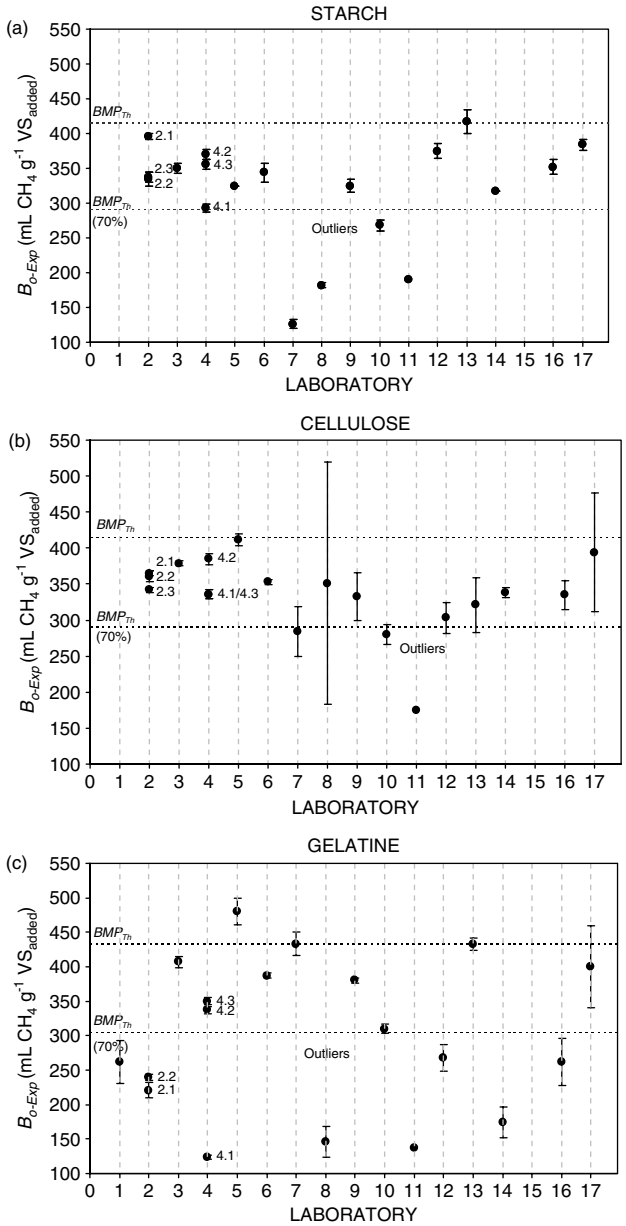

Figure 1. Methane yield reported by participants using solid positive substrates: (a) starch; (b) cellulose; (c) gelatine.

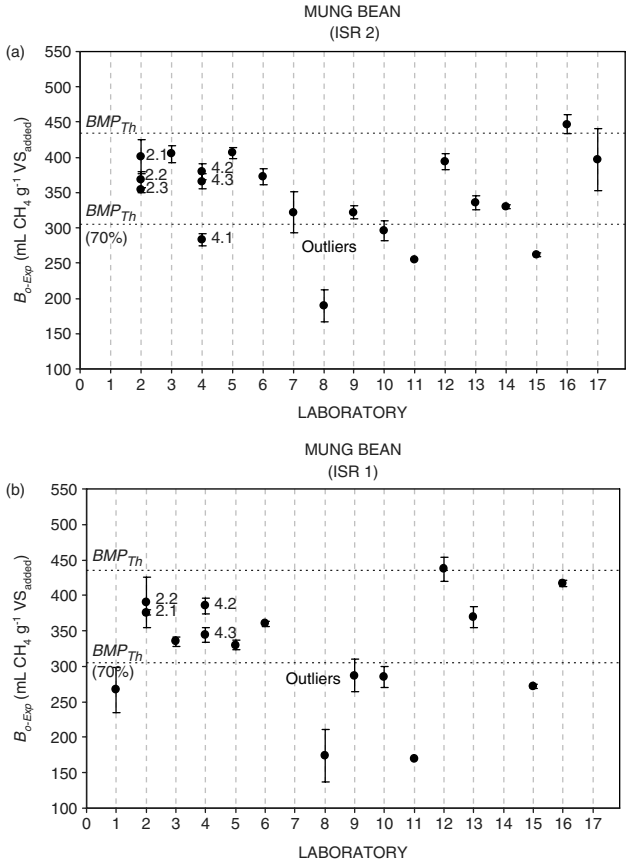

Figure 2. Methane yield reported by participants using mung bean as substrate: (a) Mung bean (ISR of 2); (b) Mung bean (ISR of 1).

k were calculated using the computer software Sigma-Plot version 9.0 by a non-linear regression method. The BMP results were compared using significance tests at a probability of significance level $P \leq 0.05$.

RESULTS AND DISCUSSION

BMP extent: specific methane yield and biodegradability

Figures 1 and 2 show the data reported by participating laboratories, including detailed information about theoretical values and valid data excluding outliers. Table 5 summarizes the results of methane production obtained during the course of experiments for each substrate, including methane yield, and the associate methane conversion efficiency or anaerobic biodegradability. This table can be evaluated considering two approaches: all the data or only data without outliers. Results excluding outliers improved the performance of the test. As a general trend, the results from valid data proportioned higher values of methane yield, precision (lower reproducibility relative standard deviation – RSD_R) and anaerobic biodegradability. It is important to note that the average precision for all the substrates assayed was around 10%. This is better than the 25% reported by the previous interlaboratory test.[6]

Starch

The theoretical methane yield (B_{o-ThOD}) calculated from the elemental composition was 414 $mLCH_4$ g^{-1} VS. The experimental methane yields (B_{o-Exp}) reported at the end of assays were substantially different, ranging from 126 ± 6 $mLCH_4$ g^{-1} VS_{added} to 417 ± 15 $mLCH_4$ g^{-1} VS_{added}, with an average value of 320 ± 77 $mLCH_4$ g^{-1} VS_{added}. When this value is compared with the stoichiometric methane yield, the BD_{CH4} was 77 ± 19%. However, when outliers (four) were deleted, the reported value was more precise. The B_{o-Exp} value ranged from 293 ± 6 $mLCH_4$ g^{-1} VS_{added} to 417 ± 15 $mLCH_4$ g^{-1} VS_{added}, with an average value of 350 ± 33 mL CH_4 $g^{-1}VS_{added}$. which assumed higher values of precision (RSD_R 9%) and BD_{CH4} (85 ± 8%). Assuming that this substrate can be fully degraded, the average amount of organic matter used for the growth of new cells and for cell metabolism calculated by subtraction was around 15%.

Literature data related to anaerobic biodegradability of starch is scarce. Hansen *et al.*[3] studied the repeatability and reproducibility of BMP tests on the basis of seven series of triplicates using a thermophilic sludge treating mainly manure mixed with other

Table 5. BMP extent: summary of the overall results obtained by participating labs

Substrate	Theoretical ($mL\ CH_4\ g^{-1}VS_{added}$)	Total data Mean±SD ($mL\ CH_4\ g^{-1}VS_{added}$)	Total data RSD_R (%)	Total data BD_{CH4} (%)	Selected data ($B_{o-Exp} \geq 70\%\ B_{o-ThOD}$) Mean±SD ($mL\ CH_4\ g^{-1}VS_{added}$)	Selected data RSD_R (%)	Selected data BD_{CH4} (%)
Starch (ISR 2)	414	320 ± 77	24	77 ± 19	350 ± 33	9	85 ± 8
Cellulose (ISR 2)	414	340 ± 52	15	82 ± 13	350 ± 29	8	85 ± 7
Gelatine (ISR 3)	433	300 ± 110	37	69 ± 26	380 ± 42	11	88 ± 9
Mung Bean (ISR 2)	434	340 ± 63	18	78 ± 15	370 ± 36	10	85 ± 8
Mung Bean (ISR 1)	434	330 ± 78	24	76 ± 18	370 ± 35	9	85 ± 8

SD: Standard Deviation
RSD_R: Reproducibility Relative Standard Deviation.

organic wastes. They reported a similar methane yield value of 348 $mLCH_4\ g^{-1}\ VS_{added}$.

Cellulose

The value of B_{o-ThOD} for this carbohydrate was of the same order of magnitude as that calculated for starch. The experimental data reported were also similar for both carbohydrates. The B_{o-Exp} values reported at the end of assays were more precise although also substantially different, ranging from 175 ± 6 $mLCH_4\ g^{-1}\ VS_{added}$ to 412 ± 8 $mLCH_4\ g^{-1}\ VS_{added}$, with an average value of 340 ± 52 $mLCH_4\ g^{-1}\ VS_{added}$. When this value is compared with the stoichiometric methane yield, the BD_{CH4} was 82 ± 13%. However, when outliers (three) were deleted the values of B_{o-Exp} ranged from 303–412 $mLCH_4\ g^{-1}\ VS_{added}$, with an average value of 350 ± 29 $mLCH_4\ g^{-1}\ VS_{added}$, which assumed a higher precision (RSD_R 8%) and BD_{CH4} (85 ± 7%). Similarly to the earlier substrate, the average amount of organic matter used to form new cells and cell metabolism was also around 15%.

Cellulose has frequently been used as a BMP reference substrate, and similar methane yields have been reported.[1,3,25,31,32]

Gelatine

The value of B_{o-ThOD} for this proteinaceous substrate calculated from the elemental composition was 433 $mLCH_4\ g^{-1}$ VS. The B_{o-Exp} values reported at the end of assays were varied, ranging from 124 ± 3 $mLCH_4\ g^{-1}\ VS_{added}$ to 480 ± 19 $mLCH_4\ g^{-1}\ VS_{added}$, with an average value of 300 ± 110 $mLCH_4\ g^{-1}\ VS_{added}$. When this value is compared with the stoichiometric methane yield, the BD_{CH4} is 69±26%. This low biodegradability can be explained considering that degradation of the protein should be inhibited due to the accumulation of intermediates (VFA and free ammonia).[9] Hansen *et al.*[3] reported the same problem of inhibition when gelatine was selected as proteinaceous substrate for anaerobic digestion. However, when outliers (nine) were deleted, the reported value was more precise (RSD_R 11%), ranging from 310 ± 6 $mLCH_4\ g^{-1}\ VS_{added}$ to 433 ± 17 $mLCH_4\ g^{-1}\ VS_{added}$, with an average value of 380 ± 42 $mLCH_4\ g^{-1}\ VS_{added}$, which assumed higher BD_{CH4} (88 ± 9%).

In this case, the average amount of organic matter used to form new cells and cell metabolism should be around 12%.

Mung bean

The theoretical methane yield values for MB using both methods (ThOD and ThOFC) ranged from 434 $mLCH_4\ g^{-1}\ VS_{added}$ to 443 $mLCH_4\ g^{-1}\ VS_{added}$, respectively. Results from $B_{o-ThOFC}$ deviated more from the rest of the theoretical values, as was previously reported.[33] In this interlaboratory study and for comparison purposes, the value of B_{o-ThOD} was considered to be the theoretical methane yield.

The B_{o-Exp} values reported at the end of assays for ISR 2 and 1 ranged from 189 ± 23 $mLCH_4\ g^{-1}\ VS_{added}$ to 447 ± 13 $mLCH_4\ g^{-1}\ VS_{added}$ and from 170 ± 6 $mLCH_4\ g^{-1}\ VS_{added}$ to 437 ± 17 $mLCH_4\ g^{-1}\ VS_{added}$, with average values of 340 ± 63 $mLCH_4\ g^{-1}\ VS_{added}$ and 330 ± 78 $mLCH_4\ g^{-1}\ VS_{added}$, respectively. When these average values are compared with the stoichiometric methane yield, the BD_{CH4} for ISR 2 and 1 were 78 ± 15% and 76 ± 18%, respectively. However, when outliers (five and six) were deleted, the B_{o-Exp} for ISR of 2 and 1 ranged from 322 ± 9 mL $CH_4 \cdot\ g^{-1}\ VS_{added}$ to 447 ± 11 mL $CH_4\ g^{-1}\ VS_{added}$ and from 330 ± 12 mL $CH_4\ g^{-1}\ VS_{added}$ to 437 ± 11 mL $CH_4\ g^{-1}\ VS_{added}$, with average values of 370 ± 36 mL $CH_4\ g^{-1}\ VS_{added}$ and 370 ± 35 mL $CH_4\ g^{-1}\ VS_{added}$, respectively. These similar average values proportioned the same value of BD_{CH4} (85 ± 8%). Following the same criterion of fully biodegradable substrates, the average amount of organic matter used to form new cells and cell metabolism was around 15%.

For this substrate it is important to note that:

- The experimental values of BMP were similar for both ISRs, and therefore, the methane yield was not at all dependent on the ISR.
- The results of methane and cellular yields were in agreement with the expected values, considering, on one hand the previous values reported for carbohydrates and proteinaceous substrates, and on the other hand the organic fraction composition of MB in terms of carbohydrates and protein and no lignin content.

Table 6. BMP rate: summary of overall results obtained by participating labs

	Total data		Selected data ($B_{o-Exp} \geq 70\%\ B_{o-ThOD}$) and ($0.9-1.1 \cdot B_o \approx B_{o-Exp}$)	
Substrate	K (d^{-1})	RSD_R (%)	k (d^{-1})	RSD_R (%)
Starch (ISR 2)	0.24 ± 0.15	63	0.24 ± 0.14	58
Cellulose (ISR 2)	0.21 ± 0.14	67	0.23 ± 0.15	65
Gelatine (ISR 3)	0.34 ± 0.23	68	0.27 ± 0.13	48
Mung Bean (ISR 2)	0.30 ± 0.17	57	0.31 ± 0.17	55
Mung Bean (ISR 1)	0.21 ± 0.13	62	0.23 ± 0.13	56

RSD_R: Reproducibility Relative Standard Deviation.

BMP rate: first-order rate constant (*k*)

Kinetic studies are also useful to understand the mechanism of anaerobic biodegradation, including inhibition of the process. Conventionally, the rate of the anaerobic digestion process can be evaluated using the methane production values from BMP data.

Table 6 shows the values corresponding to *k*. As a general trend, this parameter showed very low precision (RSD_R of 55–70%), and this parameter was only slightly affected by deletion of invalid data. Regarding the outliers, two conditions ($BD_{CH4} \geq 70\%$ and $0.9-1.1\ B_o \approx B_{o-Exp}$) were considered as criteria to select valid data. The number of full outliers was 5, 5, 10, 7 and 8 for starch, cellulose, gelatine, MB 2 and MB 1, respectively.

The highest rates of methane production were reported by the participating laboratory which used thermophilic sludges. The kinetic constant of methane production from selected substrates ranged from 0.2–0.3 d^{-1}. The data obtained in this study were higher than the values of 0.016–0.125 d^{-1} reported by Gunaseelan, using more than fifty fruits and vegetable wastes as substrates.[2]

Starch, cellulose and gelatine

The use of the raw experimental data for starch, cellulose and gelatine proportioned average rate constants of 0.24 ± 0.15 d^{-1}, 0.21 ± 0.14 d^{-1} and 0.34 ± 0.23 d^{-1}, respectively. When using only the selected experimental data (removing outliers) the values were 0.24 ± 0.14 d^{-1}, 0.23 ± 0.15 d^{-1} and 0.27 ± 0.13 d^{-1}, respectively. As expected, the average values for both carbohydrates were very similar. On the other hand, the average value for gelatine was slightly higher, probably due to the higher ISR selected for this substrate to avoid inhibition by accumulation of intermediate compounds.

Previous research work carried out using cellulose as reference substrate proportioned a wide range of values: $0.14-0.18 \pm 0.02$ d^{-1}, 0.039 ± 0.04 d^{-1}, 0.247 ± 0.020 d^{-1} and $0.090-0.145 \pm 0.015$ d^{-1}.[1,2,25,31]

Mung bean

The use of the raw experimental data for ISR 2 and 1 proportioned two different average rate constant values of 0.30 ± 0.17 d^{-1} and 0.21 ± 0.13 d^{-1}, respectively. When using only the selected experimental data, the values were 0.31 ± 0.17 d^{-1} and 0.23 ± 0.13 d^{-1}, respectively. As can be seen, for this substrate the rate constant was affected by the ISR. The lower ISR showed an inhibition phenomenon with increase in the substrate concentration, achieving a decrease in rate constant of around 26%. It can be concluded that for future harmonization of results working at high ISR is the way to obtain reproducible kinetic constants.

BMP results: influence of different factors

In this first BMP interlaboratory study, it was not possible for all the experiments to be designed by factorial planning to enable further analysis of the results obtained. Therefore, the main objective of this interlaboratory test was not to evaluate the influence of experimental factors on the BMP results. However, the results reported have been assessed in a way enabling a qualitative description of the different experimental factors affecting the anaerobic biodegradability and the final results obtained.

Influence of inoculum

Theoretically, this factor is one of the most important for the BMP test, with a clear influence on the results obtained. The results reported were analysed in terms of three different characteristics of the inocula utilized: concentration, time from sampling and source.

(1) Concentration. Practical experience has demonstrated that the level of inoculum concentration affects the rate of biodegradation. Normally, the higher the inoculum concentration, the faster the anaerobic conversion of the substrate will occur, and the quicker the test will be completed. However, in this interlaboratory study the concentration of microorganisms was adjusted considering the concentration of the organic substrates until the desired ISR was reached. Below this ISR, the extents and rates of BMP reported by different participants showed high variability, which were totally independent of the inoculum concentration.

(2) Time elapsed from sampling. The effect of sludge storage on the BMP test is not well reported in the literature. For micropollutant compounds, sludge storage had no significant effect on

the extent of degradation, but the duration of lag times could be affected, and, therefore, substrates could be degraded more slowly.[34]

Based on reported data no clear statements can be made about the influence of this factor on BMP test extent and rate.

(3) Source. Different sources of inoculum could lead to different biodegradability extent and rate values as a consequence of the different levels of microbial population and diversity.[35,36] To evaluate this factor, the results reported for the different participants in the interlaboratory study were classified into two sets of data, one from MWTPs and one from other sources. There was no significant difference in either of the parameters evaluated, the extent and the rate of the BMP test.

Influence of experimental factors

The results were also analysed considering the physical and chemical operating conditions selected.

(4) Working volume. The total volume of the reactor used for batch tests is inversely related to the number of replicate samples that could be tested at the same time using a fixed amount of sludge and substrate. The nature of the substrate can also influence the selection of the ideal volume, because the more homogeneous the material, the smaller the volume of reactor required to determine methane potential more accurately.

In this interlaboratory study, the influence of working volume on BMP extent and rate was totally insignificant.

(5) Temperature. Methane can be formed over a wide range of temperatures; however, anaerobic digestion processes depend strongly on temperature. The majority of data in the literature related to BMP assays refers to experiments performed at mesophilic temperatures, with only a few at thermophilic temperatures.

To study the influence of this parameter, the results reported by the participating laboratory using mesophilic and thermophilic sludges were utilized. The methane yields obtained were not significantly different between thermophilic wet and mesophilic sludges, while the values from the thermophilic dry sludge were slightly higher. In contrast, the rate constants of thermophilic sludges were very similar and both differed significantly from the rate constants of mesophilic sludges.

Previously, Veeken and Hamelers studied the anaerobic biodegradability of six selected components of biowaste as a function of temperatures in the mesophilic range (20 °C, 30 °C and 40 °C). They reported that the extent of anaerobic biodegradability did not depend on temperature, while the rate constants increased at higher temperatures.[22]

(6) Stirring. The influence of mixing on the BMP test has not been reported previously. The stirring process is essential for the rate of gas production, whereas it is independent of the extent of degradation.[37]

The results reported for the different participants were classified into two sets of data, one for continuous automatic stirring and one for the rest (static and manual stirring). Methane yields achieved in this interlaboratory study were comparable independently of the mixing. On the other hand, values of rate constant for the substrates selected were inconsistent, sometimes equal, sometimes higher in a stirred system and sometimes higher in static and manually stirred systems. The same lack of concrete relationship between mixing and anaerobic biodegradability was reported previously when using livestock wastes as substrate.[38]

(7) Headspace gas. No previous research work has been carried out to study the influence of headspace gas on anaerobic biodegradation in batch mode. The experimental results obtained using pure N_2 were not significantly different from those obtained with other gases.

(8) pH/Alkalinity adjustment and MM used. Results reported can be evaluated only from a restricted point of view of additional buffer/MM addition or no addition, and methane yields and rates of methane production were very similar. To analyse the influence of these factors with total accuracy, the initial pH and total alkalinity concentration, and the composition and concentration of nutrients existing throughout the BMP test system, must be obtained and reported by participating laboratories.

CONCLUSIONS

The results obtained during this interlaboratory study enabled the following conclusions to be drawn regarding the BMP test:

- Most of the BMP yield results reported by the participants were satisfactory, with a low number of outliers except for gelatine.
- The influence of inocula and experimental factors on the extents of anaerobic biodegradation were almost insignificant, while the rates differed significantly according to the experimental approaches.
- The precision (RSD_R) of the data reported for BMP extents and rates were around 10% and 55–70%, respectively.
- The ISR is a critical factor for the BMP test, with crucial influence on the kinetics, and variable influence on the yield of the BMP test depending on the biodegradable nature of the substrate.

ACKNOWLEDGEMENTS

The coordinator of this first BMP interlaboratory study gratefully acknowledges the interest shown by all the participating laboratories and their excellent contribution. The extra time-consuming tasks undertaken by the different researchers who participated in the interlaboratory study is also highly appreciated. Without their efforts this article would not have been possible.

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Reference Type: Journal biotech
Author: F. Raposo, V. Fernández-Cegrí, M. A. De la Rubia, R. Borja, J. Beltrán, C. Cavinato, M. Clinckspoor, G. Demirer, E. Diamadopoulos, J. C. Frigon, J. Koubova, M. Launay, R. Méndez, G. Menin, J. Noguerol, H. Uellehdahl and S. West
Year: 2010
Title: Quality improvement in determination of chemical oxygen demand in samples considered difficult to analyze, through participation in proficiency-testing schemes
Journal: TrAC - Trends in Analytical Chemistry
Volume: 29
Issue: 9
Pages: 1082-1091
Short Title: Quality improvement in determination of chemical oxygen demand in samples considered difficult to analyze, through participation in proficiency-testing schemes
ISSN: 01659936 (ISSN)
DOI: 10.1016/j.trac.2010.06.005
Notes: Cited By (since 1996): 7
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Quality improvement in determination of chemical oxygen demand in samples considered difficult to analyze, through participation in proficiency-testing schemes

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0165-9936/$ - see front matter doi:10.1016/j.trac.2010.06.005

Chemical oxygen demand (COD) is a critical analytical parameter in waste and wastewater treatment, more specifically in anaerobic digestion, although little is known about the quality of measuring COD of anaerobic digestion samples. Proficiency testing (PT) is a powerful tool that can be used to test the performance achievable in the participants' laboratories, so we carried out a second PT of COD determination in samples considered ''difficult'' to analyze (i.e. solid samples and liquid samples with high concentrations of suspended solids). The results obtained (based on acceptable z-score values) may be considered satisfactory. When compared with the results of a previous similar scheme, the overall performance improved by around 30%, again demonstrating that analytical performance can be improved by regular participation in PT.

Keywords: Anaerobic digestion; Chemical oxygen demand (COD); Interlaboratory study; Liquid sample; Proficiency testing (PT); Solid sample; Suspended solids; Waste treatment; Wastewater treatment

1. Introduction

The performance and the control of anaerobic processes are generally assessed by monitoring different analytical parameters, including chemical oxygen demand (COD). These systems have an organic-matter content supplied by water and suspended solids from waste and biota. However, hardly anything is known about the quality of COD measurements from anaerobic-reactor samples. From a scientific point of view, it is essential to ensure that the data produced are of sufficient trueness and precision to serve as a basis for drawing meaningful conclusions about the performance of reactors and the comparative study among different laboratories.

This contribution is the third research report that deals with the analytical determination of COD using both solid and liquid samples with high concentrations of suspended solids. The first contribution looked at the proposition of a modified analytical method for COD determination [1], whereas the second focused on the first COD proficiency testing (PT) of the anaerobic digestion groups (1st COD-PT^{ADG}), compiling data from laboratories mainly specializing in anaerobic digestion [2].

The results obtained were unsatisfactory because the majority of the participating laboratories obtained inappropriate performances. This showed the difficulties that lie in determining COD in these types of sample. However the results were not surprising, because laboratories unacquainted with PT schemes invariably fail to produce satisfactory results.

There are several reasons for participating in a PT scheme:

- evaluation of the performance and continuous monitoring;
- evidence of reliable results;
- identification of problems related to the systematic nature of assays;
- the possibility of taking corrective and/or preventive measures;
- evaluation of the efficiency of internal controls;
- determination of the performance characteristics and validation of methods and technologies;
- standardization of the activities in the market; and,
- national and international recognition of assay results [3].

Despite the fact that a single result in a PT scheme simply reflects the quality of the performance of a laboratory at any given point in time and that the extrapolation from success in a PT scheme in everyday analytical work is an assumption, frequent participation in PT schemes is highly recommended and can help provide insights into the level of quality within a laboratory. Moreover, observing that another laboratory finds approximately the same measurement result from the same measurands provides analysts with great comfort and gives them self-confidence – confirmation always gives a nice feeling.

PT schemes are therefore welcome because they provide a clear, straightforward way of evaluating the accuracy (trueness and precision) of results obtained by different laboratories. The participation in PT is also considered a powerful tool for detecting and removing sources of common errors due to the lack of quality control (QC) within a laboratory.

The 2nd COD-PT^{ADG} was organized with the aim of comparing the data from both the 1st and 2nd COD-PTs and of determining if PT schemes improve the performance of participant laboratories.

2. Organization of the PT scheme

This study is the second attempt at a worldwide interlaboratory comparison of analytical COD determination using solid samples and liquid samples with high concentrations of suspended solids. These samples are considered to be difficult to analyze and are problematic in the corresponding determinations. The scheme was organized by the ''Reuse of Wastes and Wastewater Treatment Group'', of the Instituto de la Grasa (IG) of the Spanish National Research Council (CSIC).

The PT coordinator and collaborators were responsible for:

- designing the overall scheme;
- preparation, testing and distribution of selected samples;
- distribution of instructions among the participating laboratories;
- collection of data, their statistical treatment and feedback of results to participants.

This PT was carried out according to the International Harmonized Protocol for the PT of Analytical Chemistry Laboratories [4].

The PT coordinator sent invitations to participate in the 2nd COD-PT^{ADG} in June 2009. The test took place between 15 September and 15 October 2009. Each participating laboratory received four samples, together with technical guidelines on how to proceed with the measurements. A total of 20 laboratories from 13 countries agreed to participate. All the participating laboratories were highly motivated about taking part in the PT scheme, as the full return rate of data proved. All participating laboratories provided feedback, first about their own performance, and second about the general performance, all of which was reported anonymously.

3. Materials and methods

3.1. Materials

3.1.1. Description of samples. To carry out the 2nd COD-PT^{ADG}, four different samples were selected. These samples were divided into two main groups: solid samples (SS) and liquid samples with a high suspended solid concentration (LS-HSSC):

- Sample 1 (SS 1). Gelatin (Gel). Pure powder protein used as a solidifying agent in the preparation of microbiological culture media to identify proteolytic microorganisms (gelatinase producers). The gelatin used was supplied by Panreac-Spain (Code 403902).
- Sample 2 (SS 2). Sewage sludge (SewS). A sewage sludge produced by Resource Technology Corporation (USA and UK) and provided for characterization as a new certified reference material (including 19 metals as well as COD, Kjeldahl nitrogen and total phosphorus).
- Sample 3 (LS-HSSC 1). Sunflower-oil cake (SuOC). A by-product made up of the part of whole sunflower seeds that remains after oil-extraction processes. It is a heterogeneous substrate that can be broken down into three main components: a proteinaceous fraction, a lignocellulosic fraction and a soluble fraction. The sample was prepared with 5 g of raw material.
- Sample 4 (LS-HSSC 2). Mung bean (MB). The seed of *Vigna radiata*, which is native of Asia (Bangladesh, India and Pakistan). This seed is also known as green bean, green soya, and green gram. Its beans are small, ovoid in shape, green when raw and yellow when dehusked. The sample was also prepared with 5 g of raw material.

3.1.2. Preparation of samples. The suitability and the quality of the test materials distributed are fundamental for the effectiveness of a PT scheme. The two main criteria for suitable test material are that:

- it resembles, as closely as possible, the real samples with which a laboratory routinely works; and,
- variations in the composition of the samples of the test material distributed to participants are kept to the minimum [5].

The PT material was prepared by the PT coordinator. Although his working laboratory has not implemented a quality system accredited according to ISO 17025, he is very experienced in this field and has been involved in different laboratory QC systems, so all the characteristics that could affect the integrity of the test were taken into consideration, including the homogeneity and the stability of the samples.

Considering that different particle-size fractions of the solid samples dispatched would lead to a lack of homogeneity with respect to COD determination, a control of particle size was carried out by sieving the substrates selected to the desired size.

Taking into account that the moisture content of solid-substrate samples can vary with ambient humidity, the participants were requested to report results on a dry-weight basis.

Samples 3 and 4 were two liquid samples with high concentrations of suspended solids that had to be reconstituted in-laboratory by adding 200 mL of distilled water to the spiked amount of solid content weighed into the containers. All participants were instructed to stir the samples for 1 h before COD analysis and during the sampling procedure.

3.1.3. Characterization of samples. All samples distributed were analyzed in the laboratory of the PT coordinator. Three replicates of different parameters (moisture, organic content and elemental composition) were prepared for each sample. Table 1 summarizes the main characteristics of the samples selected.

3.1.4. Homogeneity of samples. Immediately after packaging the samples, they were tested for sufficient homogeneity using the standard analytical method developed in the laboratory of the PT coordinator and used on a routine basis. To check for sufficient homogeneity, the protocol devised by Fearn and Thompson [6] was used. In accordance with their approach, three tests were carried out to estimate the corresponding experimental statistical parameters and compared with their theoretical critical values:

Table 1. Characterization of the solid samples used in the 2nd COD-PT^{ADG}

	Sample 1 *(Gel)*	Sample 2 *(SewS)*	Sample 3 *(SuOC)*	Sample 4 *(MB)*
Particle size (mm)	N.D.[a]	0.2–1	0.125–0.355	0.125–0.500
Moisture (%)	8.0 ± 0.3	10.0 ± 0.4	8.0 ± 0.4	9.0 ± 0.3
Organic content (%TS)	100.0 ± 0.1	60.3 ± 0.5	93.0 ± 0.5	97.0 ± 0.5
Chemical Composition (%-VS)				
Carbohydrates	–	N.D	55.5	72.4
Fat	–	N.D	1.1 ± 0.2	1.5 ± 0.2
Protein[b]	100	N.D	26.4 ± 0.6	23.1 ± 0.6
NDF	–	N.D	40 ± 1	5.0 ± 0.5
Elemental Analysis (%-TS)				
C	48.2 ± 0.3	32.9 ± 0.5	45.9 ± 0.6	44.6 ± 0.6
H	6.5 ± 0.3	4.5 ± 0.1	6.3 ± 0.1	6.8 ± 0.3
N	18.4 ± 0.1	4.8 ± 0.1	5.4 ± 0.4	4.4 ± 0.1
S	0.6 ± 0.1	1.4 ± 0.1	0.20 ± 0.04	0.07 ± 0.01
O	26.2 ± 0.4	16.7 ± 0.7	35.2 ± 0.8	41.1 ± 0.6
Theoretical Oxygen Demand (*ThOD*-mg $O_2 \cdot g^{-1}$ TS)	1236	956	1249	1240

[a]N.D.: Not determined.
[b]%NTK x Nitrogen-to-protein conversion factor (5.5).

Table 2. Summary of analytical procedures utilized by participating laboratories in 2nd COD-PT^{ADG}

Lab[1]	Method[2]	Digestion Reagent			Acid Reagent[3]		$HgSO_4$	Water	End Point[4]
		$K_2Cr_2O_7$			H_2SO_4-$AgSO_4$				
		Vol. (mL)	Conc. (N)	Vol. (mL)	Conc.[c] (%)	Conc.[d] (g/L)		Vol. (mL)	
1[a]	(2) OR-HCM	25	1.0	20	98	10	Yes	0	TT [g]
1[b]	(1) OR-LCM	5	0.241	15	98	10	Yes	10	TT
2	(4) CR-SM				99	10	No	No	SP[e]
3	(1) OR-LCM	20	0.5	30	98	5	Yes	10	TT
4[a]	(5) CR-KSM								SP
4[b]	(5) CR-KSM								SP
5	(2) OR-HCM	10	1.0	30	98	10	Yes	10	TT
6[a]	(2) OR-HCM	15	1.0	45	98	9.4	Yes	20	TT
6[b]	(3) CR-TM	1.5	0.21	3.5	98	10.7	Yes	0	TT
7	(2) OR-HCM	10	1.2	30	98	10	Yes	0	TT
8	(4) CR-SM	1.5	0.2148	3.5	98	10	Yes	2,5	SP
9	(2) OR-HCM	20	1.2	25	98	10	Yes	10	PT[f]
10	(2) OR-HCM	20	1.2	30	98	10	Yes	10	PT
11	(1) OR-LCM	50	0.25	50	98	10	Yes	25	TT
12	(5) CR-KSM								SP
13	(2) OR-HCM	20	1.0	30	98	10	Yes	20	TT
14	(1) OR-LCM	25	0.25	75	96	10.6	Yes	0	TT
15	(2) OR-HCM	20	1.2	30	95	10	Yes	15	PT
16	(2) OR-HCM	20	1.2	30	98	10	Yes	10	PT
17	(1) OR-LCM	0.5	0.33	2.5	95–98	26.5	Yes	2.0	SP
18	(5) CR-KSM								SP
19	(2) OR-HCM	20	1.2	30	98	10	Yes	10	TT
20	(1) OR-LCM	20	0.5	30	98	10	Yes	10	PT

[1]Type of sample: Solid Samples [a](SS) Liquid Samples with high suspended solid concentrations [b] (LS-HSSC).
[2]Analytical Method:
- Open Reflux (OR): (1) OR-LCM. Low concentration of $K_2Cr_2O_7$ (M<0.166) (2) OR-HCM. High Concentration of $K_2Cr_2O_7$ (M $\geqslant$ 0.166)
- Closed Reflux (CR): (3) CR-TM. End-point by titration (4) CR-SM. End-point spectrophotometrically (5) CR-KSM. Kits. End-point spectrophotometrically

[3]Acid-Catalyst reagent: Concentration of H_2SO_4[c]; Concentration of $AgSO_4$[d].
[4]Visualization of end-point: spectrophotometrically (SP[e]).titration: partial and total titration (PT[f]/TT[g]).

(i) Cochran's test procedure for duplicate results or the detection of outliers by differences between pairs;
(ii) precision of the analytical method used; and,
(iii) homogeneity test or test for acceptable between-sample variance.

For this purpose, 10 randomly selected distribution units of solid substrates were analyzed in duplicate and COD values were statistically evaluated.

3.1.5. Stability of samples. Materials distributed in PT schemes must be sufficiently stable over the period in which the assigned value needs to be valid. Normally, the period in question is the interval between the preparation of the material and the deadline for the return of results (one month). The material under test should be in the packaging in which it is distributed.

To ensure that the samples used in the 2nd COD-PT^{ADG} were stable, a stability study was carried out to identify if there was reproducibility of the results with time. The stability study was carried out by applying the values of F, which were calculated applying the one-way analysis of variance (ANOVA) of three randomly selected distribution units from the homogenization study, and it was suggested they be kept at room temperature.

3.2. Methods

3.2.1. Analytical methods

3.2.1.1. Chemical oxygen demand. The participating laboratories were free to choose the analytical method that they considered suitable for performing the COD analysis, but were advised to analyze samples using their usual techniques. Each participating laboratory was requested to make three replicate determinations, and to report the results together with a short description of the method used. Table 2 summarizes all the experimental conditions of the analytical methods used by the participants' laboratories. The studies of homogeneity and stability were carried out by the method proposed by Raposo et al. [1].

The analytical determination of COD can be classified first into two main groups [i.e. open reflux (OR) and closed reflux (CR)], and second into five methods, with percentages of each method used by the different participants in brackets:

(1) OR, low concentration of oxidant (17.5%);
(2) OR, high concentration of oxidant (47.5%);
(3) CR, end-point by titration (2.5%);
(4) CR, end-point by spectrophotometrically determination (15%); and,
(5) CR, using kits (17.5%).

The percentages of analytical methods used for OR and CR were therefore 65% and 35%, respectively.

3.2.1.2. Other parameters. Moisture, TS-dry matter and VS-organic matter were determined according to the standard methods 2540B and 2540E-APHA, respectively [7]. Fat content was determined by extraction with hexane using a Soxhlet system [8]. Protein and elemental composition were performed in a LECO CHNS-932 combustion analyzer at 1050°C, using sulfametazine as standard substrate. Theoretical oxygen demand was calculated from the elemental composition according to ISO 10707 [9]. Fiber (neutral detergent fiber, NDF) content was obtained using the method reported by Van Soest [10]. Carbohydrate content was reported by subtraction of fat, protein and lignin contents.

3.2.2. Data treatment

The internationally recommended z-score was used as the performance criteria for participating laboratories whose results were converted into z-scores according to the following equation:

$$z\text{-}score = (X_{EV} - X_{AV})/\sigma_{PT}$$

where X_{EV} is the laboratory's experimental value, X_{AV} is the assigned value (estimation of the true value of the measurand that is used for the purpose of calculating scores), and σ_{PT} is the fitness-for-purpose-based ''standard deviation for proficiency assessment'', defined as a target value for the acceptable deviation from the assigned value.

This means that the z-score method compares the participant's deviation from the reference value with σ_{PT},

Table 3. Homogeneity of the solid substrates. Summary of the statistical parameters obtained

Sample	Test	Experimental Value	Critical Value	Result
Gel	Cochran	0.3050	0.6020	Pass
	Precision of Method	0.39	0.5	Pass
	Homogeneity	0.00011	0.00031	Pass
SewS	Cochran	0.2603	0.6020	Pass
	Precision of Method	0.44	0.5	Pass
	Homogeneity	0.00002	0.00021	Pass
SuOC	Cochran	0.2647	0.6020	Pass
	Precision of Method	0.41	0.5	Pass
	Homogeneity	0.00001	0.00034	Pass
MB	Cochran	0.2809	0.6020	Pass
	Precision of Method	0.20	0.5	Pass
	Homogeneity	0.00004	0.00020	Pass

so the assigned value and the target standard deviation have a critical influence on the calculation of z-scores and must be selected with care if they are to provide a realistic assessment of laboratory performance.

3.2.2.1. Assigned values. In the 1st COD-PTADG, the results were too widespread to be used as a reference value based on the generally used consensus approach. In this case, the assigned values were determined on the basis of *ThOD* measurements performed at the PT coordinator's working laboratory. The same criterion was used for the 2nd COD-PT^{ADG}, but, in addition, two consensus values (mean and median) based on the results from all participants were also calculated only to estimate the degree of dispersion from the assigned value. The *ThOD*-based assigned values, mean and median consensus values for Gel and SewS solid samples were: 1236, 1201 and 1224 mg O_2 g^{-1} TS and 956, 950 and 954 mg O_2 g^{-1} TS, respectively. Similarly, the values for SuOC and MB liquid samples were: 28.164, 28.828 and 29.327 g O_2 L^{-1} and 27.793, 27.791 and 28.261 g O_2 L^{-1}, respectively. Considering the data of all the

Table 4. Summary of the data reported by participating laboratories in 2nd COD-PT^{ADG}

Lab	Sample 1				Sample 2			
	EV_{Mean} (mg O_2 g^{-1} TS)	EV_{RSD} (%)	R_{Mean} (%)	R_{RSD} (%)	EV_{Mean} (mg O_2 g^{-1} TS)	EV_{RSD} (%)	R_{Mean} (%)	R_{RSD} (%)
1	1277	3	103	3	966	1	101	1
2	1190	3	96	3	970	2	101	2
3	1142	5	92	4	815	5	85	4
4	1249	6	101	6	869	5	91	4
5	1205	2	97	2	949	1	99	1
6	1035	6	84	5	792	5	83	4
7	1219	5	99	5	953	4	100	4
8	1244	3	101	3	954	2	100	2
9	889	2	72	2	638	2	67	1
10	1235	3	100	3	954	3	100	3
11	1145	1	93	1	893	1	93	1
12	1144	6	93	6	1278	2	134	3
13	1210	3	98	3	974	1	102	1
14	1255	1	102	2	950	1	99	1
15	1245	4	101	4	871	7	91	6
16	1318	2	107	2	1004	3	105	3
17	1286	6	104	6	1057	1	111	1
18	1329	6	108	6	1093	8	114	9
19	1228	1	99	1	950	1	99	1
20	1185	6	96	6	1095	7	115	7
Lab	**Sample 3**				**Sample 4**			
	EV_{Mean} (mg O_2 L^{-1})	EV_{RSD} (%)	R_{Mean} (%)	R_{RSD} (%)	EV_{Mean} (mg O_2 L^{-1})	EV_{RSD} (%)	R_{Mean} (%)	R_{RSD} (%)
1	27567	3	98	3	33570	13	121	15
2	26853	6	95	6	26042	6	94	5
3	31527	15	112	16	27323	22	98	21
4	31433	2	112	2	31767	3	114	3
5	30512	11	108	12	28543	9	103	9
6	21665	6	77	5	22470	6	81	5
7	26476	5	94	5	26194	2	94	2
8	30233	1	107	1	27647	2	99	2
9	33519	9	119	11	28948	1	104	1
10	29451	1	105	1	28207	1	101	1
11	22647	1	80	1	22350	2	80	1
12	28700	6	102	7	30433	1	109	1
13	25467	7	90	6	25900	2	93	2
14	29963	1	106	1	29838	1	107	2
15	27933	13	99	13	23767	7	86	6
16	29255	2	104	2	28314	2	102	2
17	30553	3	108	3	29749	4	107	4
18	29399	4	104	4	27603	0	99	0
19	28566	0	101	0	28418	1	102	1
20	34850	12	124	12	32730	12	118	12

	Laboratory																			
Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	1.3	-1.5	-3.0	0.4	-1.0	-6.5	-0.6	0.3	-11.2	0.0	-2.9	-3.0	-0.9	0.6	0.3	2.7	1.6	3.0	-0.2	-1.7
2	0.4	0.6	-5.9	-3.7	-0.3	-6.9	-0.1	-0.1	-13.3	-0.1	-2.6	13.5	0.7	-0.3	-3.6	2.0	4.2	5.7	-0.3	5.8
3	-0.4	-0.9	2.4	2.3	1.7	-4.6	-1.2	1.5	3.8	0.9	-3.9	0.4	-1.9	1.3	-0.2	0.8	1.7	0.9	0.3	4.7
4	4.2	-1.3	-0.3	2.9	0.5	-3.8	-1.2	-0.1	0.8	0.3	-3.9	1.9	-1.4	1.5	-2.9	0.4	1.4	-0.1	0.4	3.6

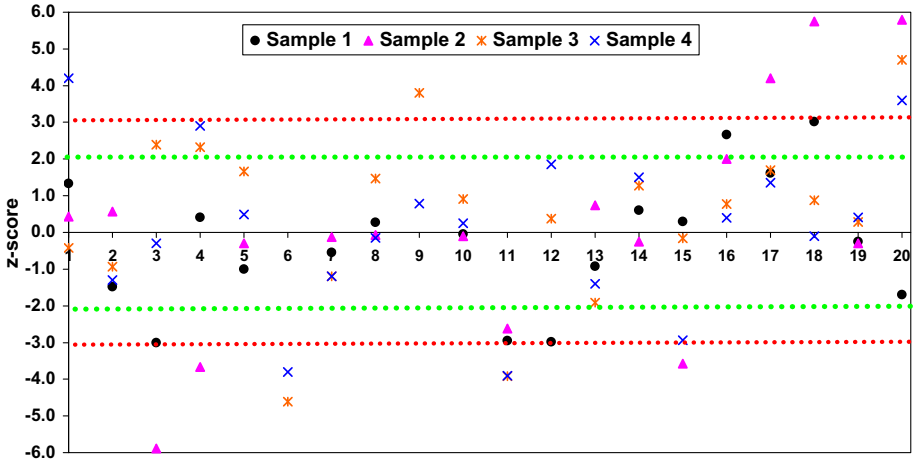

Figure 1. Overview of the z-score values obtained by laboratories participating in the 2nd COD-PT^{ADG}.

samples, it can be seen that there was a good agreement between the experimental consensus values and the theoretical assigned values.

3.2.2.2. Standard deviations for proficiency assessment. The value of σ_{PT} determines the limits of satisfactory performance in a PT scheme. It is important to note that σ_{PT} values were predefined by the PT coordinator and the criteria were communicated in advance to participating laboratories. The σ_{PT} values were determined as a percentage of the assigned value according to the appropriate form of the Horwitz equation [11], which considers the concentration level of analyte. The theoretical percentage values for GEL, SewS, SuOC and MB were 0.9%, 1.0%, 3.4% and 3.4%, respectively. However, these values were slightly modified to reflect the level of COD uncertainty in real routine work samples, so, for solid samples, the percentage was 2.5%, and for liquid samples 5.0%. These σ_{PT} values were identical to those used in the 1st COD-PTADG to prevent the different values from transferring into z-scores that could give data from different PT schemes that could not be compared.

3.2.2.3. Laboratory performance. The conventional way to evaluate the performance of each laboratory participating in a PT scheme based on z-score values was used. In the interpretation of z-scores, the following agreements were internationally made:

z-score $\leqslant \pm 2$ – satisfactory result;

z-score $> \pm 3$ – unsatisfactory result; and,

$\pm 2 >$ z-score $\leqslant \pm 3$ – doubtful result.

4. Results and discussion

4.1. Evaluation of sample-homogeneity study

Table 3 summarizes the results obtained in the statistical analysis of homogeneity data, which show that substrates selected as samples passed the statistical homogeneity tests, so they were considered homogeneous enough and suitable to be used in the PT scheme.

4.2. Evaluation of sample-stability study

The calculated F values for samples 1–4 were 0.78, 0.47, 1.72 and 2.30, respectively. All the results obtained were less than 4.96, which represents the critical F value for a confidence level of 95%. Considering that there was no significant difference between the mean values of COD determinations during the period of time established, the samples were considered stable for the study conditions.

4.3. Evaluation of laboratory performance

Table 4 summarizes the means and relative standard deviations of experimental values (EV) and recoveries (R) reported by the 20 participating laboratories. The general trend of the data reported showed that all the

Table 5. Summary of participants' results obtained for different analytical methods

Sample	Analytical Method			Average Values				Z-scores					
	Name	N^{er}	%	Mean	SD_R	RSD_R	Recovery	Z-score ⩽ ±2		±2 < Z-score ⩽ ±3		Z-score > ±3	
				(mg O_2 g^{-1} TS)	(mg O_2 g^{-1} TS)	(%)	(%)	N^{er}	%	N^{er}	%	N^{er}	%
SS-1	(1) OR-LCM	3	15	1195	56	5	97	2	67	1	33	0	0
(Gel)	(2) OR-HCM	11	55	1182	122	10	96	6	55	3	27	2	18
	OR-M	14	70	1185	109	9	96	8	57	4	29	2	14
	(3) CR-TM	0	0					0	0	0	0	0	0
	(4) CR-SM	3	15	1240	48	4	100	3	100	0	0	0	0
	(5) CR-KM	3	15	1241	93	7	100	2	67	1	33	0	0
	CR-M	6	30	1240	109	9	100	5	83	1	17	0	0
	Total	20	100	1201	100	8	97	13	65	5	25	2	10
SS-2	(1) OR-LCM	3	15	979	104	11	102	1	33	1	33	1	33
(SewS)	(2) OR-HCM	11	55	897	109	12	94	7	64	0		4	36
	OR-M	14	70	915	110	12	96	8	57	1	7	5	36
	(3) CR-TM	0	0					0	0	0	0	0	0
	(4) CR-SM	3	15	987	45	5	103	2	67	0	0	1	33
	(5) CR-KM	3	15	1080	205	19	113	0	0	0	0	3	100
	CR-M	6	30	1034	110	11	108	2	33	0	0	4	67
	Total	20	100	950	129	14	99	10	50	1	5	9	45
				(mg O_2 L^{-1})	(mg O_2 L^{-1})	(%)	(%)	N^{er}	(%)	N^{er}	(%)	N^{er}	(%)
LS-HSSC 1	(1) OR-LCM	4	20	28757	5077	18	102	2	50	0	0	2	50
(SuOC)	(2) OR-HCM	8	40	29347	2603	9	104	6	75	1	0	1	25
	OR-M	12	60	29150	3380	12	104	8	67	1	8	3	25
	(3) CR-TM	1	5	21665	0	0	77	0	0	0	0	1	100
	(4) CR-SM	3	15	29213	2050	7	104	3	100	0	0	0	0
	(5) CR-KM	4	20	29366	1502	5	104	3	75	1	25	0	0
	CR-M	8	40	28346	3204	11	101	6	75	1	12.5	1	12.5
	Total	20	100	28828	3204	11	102	14	70	2	10	4	20
LS-HSSC 2	(1) OR-LCM	4	20	29622	5105	17	105	1	25	0	0	3	75
(MB)	(2) OR-HCM	8	40	27731	1138	4	98	8	100	0	0	0	0
	OR-M	12	60	28361	2966	10	101	9	75	0	0	3	25
	(3) CR-TM	1	5	22470		0	80	0	0	0	0	1	100
	(4) CR-SM	3	15	27813	1859	7	99	3	100	0	0	0	0
	(5) CR-KM	4	20	28393	3539	12	101	2	50	2	50	0	0
	CR-M	8	40	27435	3234	12	97	5	62.5	2	25	1	12.5
	Total	20	100	27991	3027	11	99	14	70	2	10	4	20

samples were normally distributed, with a predominance of results centered on a mean value and few results in the extremes of distribution.

Fig. 1 shows an overview of all the z-scores calculated from the data reported by the participant laboratories for the four samples selected. The general impression was that the majority of reported values were satisfactory.

In addition, Table 5 summarizes participants' results obtained for the different analytical methods used. Taking into consideration the great difference in the percentages of the analytical methods used, only a relative statement could be made. However, as in the 1st COD-PT[ADG], no major differences in the results reported were due to the analytical method used.

It is interesting that 8 participating laboratories (40% of total) reported the four samples satisfactorily, with 62.5%, 25.0% and 12.5% of the data coming from OR-HCM, CR-LCM and OR-LCM, respectively.

The z-score performance of each sample was evaluated as follows:

- Sample 1 (Gel): 13 laboratories (65%) reported satisfactory results, 5 laboratories (25%) reported questionable results, and only 2 laboratories provided unsatisfactory results (10%).
- Sample 2 (SewS): Upon analysis, this sample showed poorer results than the solid sample (Sample 1). 10 laboratories (50%) reported satisfactory results, 9 laboratories (45%) reported unsatisfactory results, and 1 laboratory (5%) gave doubtful results.
- Sample 3 (SuOC): 14 laboratories (70%) reported satisfactory results, 2 laboratories (10%) reported questionable results, and 4 laboratories (20%) provided unsatisfactory results.
- Sample 4 (MB): The z-score values were identical to those reported for Sample 3 [i.e. 14 laboratories (70%) reported satisfactory results, 2 laboratories

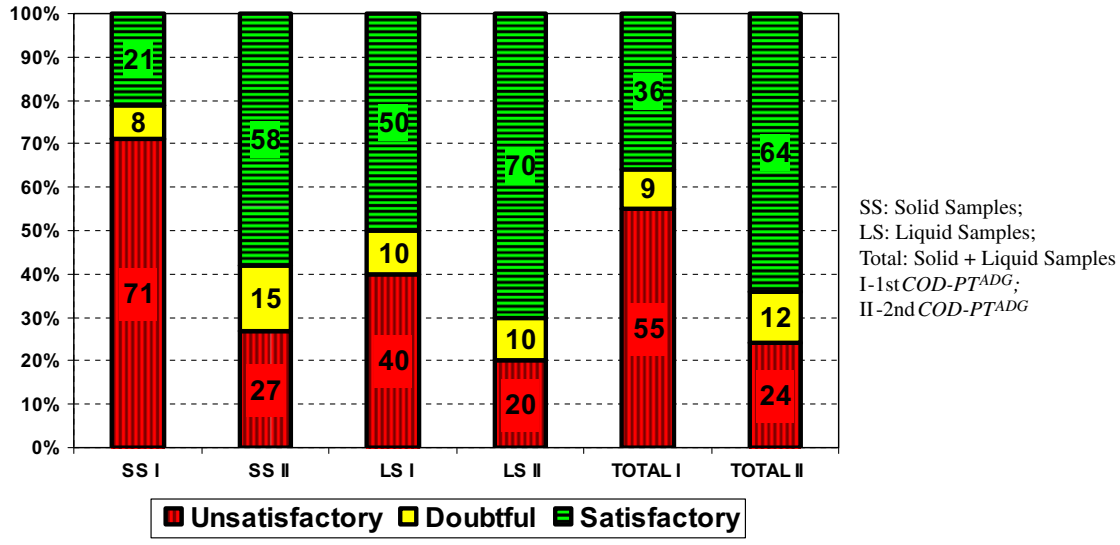

Figure 2. Comparison of z-score performance in 1st and 2nd COD-PT.

(10%) reported questionable results, and 4 laboratories (20%) provided unsatisfactory results].

The results can be outlined by the nature or characteristics of the substrate and finally grouped as total samples:

- Solid Samples: 23 z-scores (58%) were satisfactory, 11 z-scores (27%) were unsatisfactory, and 6 z-scores were doubtful (15%).
- Liquid samples with high concentrations of suspended solids: 28 z-scores (70%) were satisfactory, 8 z-scores (20%) were unsatisfactory, and 4 z-scores (10%) were doubtful.
- Total samples: 51 z-scores (64%) were satisfactory, 15 z-scores (24%) were unsatisfactory, and 14 z-scores (12%) were doubtful.

Although it is generally recognized that the analytical determination of COD samples may be ''relatively easy'' or ''relatively difficult'', it is very tempting to deduce a correlation between the type of sample analyzed and the analytical performance. For normal liquid samples (without suspended solids), the analysis of COD is considered an ''easy'' analytical determination. The results from the Aquacheck PT scheme, which ran for over 20 years, reported a percentage of acceptable results and a relative standard deviation of 91.4% and 5.8%, respectively [12]. The decrease in the overall performance of this PT scheme can be explained by considering the characteristics of the samples selected, which are potentially more difficult to analyze. However, we have no doubt that regular involvement in PT can improve the analytical performance of those laboratories taking part.

4.4. Comparisons with data from the 1st COD-PTADG

Generally, PT data are evaluated in the medium-to-long term. Although for the determination of COD in samples difficult to analyze, there have been only two PT schemes, the clear improvement in results reported could be used as ''short-term conclusions'', helping to do away with the generalized notion that solid samples and liquid samples with high concentration of suspended solids cannot be analyzed accurately, as was previously reported [13,14].

The data reported in both COD-PT schemes were summarized in terms of z-score values, and are presented in bar-chart form in Fig. 2 for graphical comparison. On the basis of the results obtained in the 2nd COD-PT^{ADG} and comparing them with the values reported in the 1st COD-PT^{ADG}, we can note that the overall performance of all participants can be considered quite satisfactory.

For solid samples, the z-scores considered unsatisfactory dropped dramatically from 71% to 27%, whilst the z-scores considered satisfactory increased from 21% to 58%. This means an improvement in the result of around 40%.

For liquid samples, the trend was also positive, with an increase in satisfactory results of around 20%.

The overall evaluation of results obtained showed that the participation in COD-PT schemes using solid samples and liquid samples with high concentrations of suspended solids improved the performance of participating laboratories by approximately 30%. This fact can be interpreted as a sign of general improvement, reinforcing the statement that the ability to produce results of acceptable quality for COD determination in ''relatively difficult'' samples seems possible.

Another indicator of the improvement in COD determination was the number of laboratories that reported the four samples satisfactorily. That 8 laboratories (40% of total) reported adequately in the 2nd PT-COD^{ADG}, compared to 2 laboratories (8% of total) in the 1st PT-COD^{ADG}, shows evident improvement.

Similar trends of overall performance improvements with participation in PT schemes were described by:

i) Whetton and Finch for some analytes of the Aquacheck PT, including COD [12];
ii) Gaunt and Whetton for analytes from alcoholic and non-alcoholic beverage industries [15];
iii) Key et al. for foods and feeds [16]; and,
iv) Earnshaw et al. for riboflavin (vitamin B_2 analysis) [5].

Nobody questions the value of PT schemes, and it is universally agreed that a well-founded laboratory must participate regularly in relevant PT. Although further research will be necessary before coming to any firm conclusion, it is foreseeable that future COD-PTs will see further potential increases in COD analytical performance, achieving satisfactory z-score values of around 90% for all the new samples distributed.

5. Conclusions

The 2nd COD-PT^{ADG} provided a valuable opportunity for evaluating the general performance of COD determination using samples considered ''difficult'' to analyze. The general performance of participating laboratories was acceptable, with 64% of the z-score values reported considered satisfactory. More significant was the improvement in results compared with the 1st COD-PT^{ADG}. Specifically, the improvement in the z-score values reported for solid samples and liquid samples with high concentrations of suspended solids was 40% and 20%, respectively. The results obtained demonstrated once more how participation in PT is successful as a way to achieve a good QC within laboratories involved in this type of chemical determinations.

Acknowledgements

The PT coordinator gratefully acknowledges the interest shown by all the participating laboratories and their excellent contribution to the 2nd COD-PT^{ADG}. The extra time-consuming task undertaken for the different analysts who participated in the study is also highly appreciated. Without their efforts, this article would not have been possible.

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