UNIVERSIDAD DE SEVILLA

FACULTAD DE FARMACIA

PRODUCCIÓN DE UN BIOFERTILIZANTE / BIOESTIMULANTE MEDIANTE UN PROCESO BIOLÓGICO / ENZIMÁTICO A PARTIR DE SUBPRODUCTOS ORGÁNICOS: VALORIZACIÓN AGRONÓMICA Y AMBIENTAL DE LODOS DE DEPURADORA Y PLUMAS DE MATADERO

TESIS DOCTORAL

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Producción de un biofertilizante / bioestimulante mediante un proceso biológico / enzimático a partir de subproductos orgánicos: Valorización agronómica y ambiental de lodos de depuradora y plumas de matadero

Memoria de Tesis Doctoral presentada por:

Bruno Rodríguez Morgado

Para optar al grado de Doctor en Bioquímica.

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La Tesis Doctoral titulada "Producción de un biofertilizante / bioestimulante mediante un proceso biológico / enzimático a partir de subproductos orgánicos: Valorización agronómica y ambiental de lodos de depuradora y plumas de matadero", realizada por el Licenciado en Bioquímica D. BRUNO RODRÍGUEZ MORGADO, para optar al grado de Doctor en Bioquímica por la Universidad de Sevilla, se presenta bajo la modalidad de "Compendio de Publicaciones"

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D. Bruno Rodríguez Morgado, Licenciado en Bioquímica por la Facultad de Biología, de la Universidad de Sevilla, ha realizado en Departamento de Bioquímica y Biología Molecular de la Facultad de Farmacia de la Universidad de Sevilla, bajo nuestra dirección, el trabajo de investigación **"Producción de un biofertilizante / bioestimulante mediante un proceso biológico / enzimático a partir de subproductos orgánicos: Valorización agronómica y ambiental de lodos de depuradora y plumas de matadero"**, y que reúne las condiciones necesarias para ser presentada para optar al grado de Doctor por la Universidad de Sevilla.

Fdo.: Dr. Juan Parrado Rubio

Fdo.: Dr. Manuel Tejada Moral

La presente Tesis Doctoral está compuesta por los siguientes artículos científicos:

1.- Proteomic analysis of enzyme production by Bacillus licheniformis using different feather wastes as the sole fermentation media.

Parrado J, Rodríguez-Morgado B, Tejada M, Hernández T, García C.

Enzyme and Microbial Technology. 2014, Vol. 57: 1-7.

Índice de Impacto: 2,322

Ranking:

Categoría: Biotechnology and Applied Microbiology. 2º cuartil (74/163)

Citas: 13

2.- Hydrolytic enzimes production by Bacillus licheniformis growth on fermentation media formulated with sewage sludge.

Inca-Torres AR, Urbina-Salazar A, Falcón-García G, Carbonero-Aguilar P, Rodríguez Morgado B, Parrado J, Bautista J.

Journal of Biotech Research. 2018, Vol. 9, 14-26.

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Citas:

3.- Obtaining biostimulant products for land application from the sewage sludge of small populations.

Tejada M, García-Martínez AM, Rodríguez-Morgado B, Carballo M, García-Antrás D, Aragón C, Parrado J.

Ecological Engineering. 2013, Vol. 50: 31– 36.

Índice de Impacto: 3,041

Ranking:

Categoría: Environmental Sciences. 1º cuartil (47/216)

Citas: 12

4.- Obtaining edaphic biostimulants/biofertilizers from sewage sludge using fermentative processes. Short-time effects on soil biochemical properties.

Rodríguez-Morgado B, Caballero P, Paneque P, Gómez I, Parrado J, Tejada M.

Environmental Technology. 2019, Vol. 40: 399-406.

Índice de Impacto: 1,666

Ranking:

Categoría: Environmental Sciences, 3º cuartil (144/242)

Citas: 0

5.- Obtaining edaphic biostimulants/biofertilizers from different sewage sludges. Effects on soil biological properties.

Rodríguez-Morgado B, Gómez I, Parrado J, García-Martínez AM, Aragón C, Tejada M.

Environmental Technology. 2015, Vol. 36: 2217–2226.

Índice de Impacto: 1,760

Ranking:

Categoría: Environmental Sciences, 3º cuartil (113/225)

Citas: 6

6.- Effects of foliar fertilization of a biostimulant obtained from chicken feathers on maize yield.

Tejada M, Rodríguez-Morgado B, Paneque P, Parrado J.

European Journal of Agronomy. 2018, Vol. 96: 54-59.

Índice de Impacto: 3,192 (2017)

Ranking:

Categoría: Agronomy, 1º cuartil (6/83) (2017)

Citas: 1

Premio a la publicación científica del mes en la ETSIA. Mayo 2018.

3er Premio a la Publicación Científica del Año 2018 en la ETSIA

7.- Accelerated degradation of PAHs using edaphic biostimulants obtained from sewage sludge and chicken feathers.

Rodríguez-Morgado B, Gómez I, Parrado J, García C, Hernández T, Tejada M

Journal of hazardous materials. 2015, Vol. 300: 235-242.

Índice de Impacto: 4,836

Ranking:

Categoría: Environmental Sciences, 1º cuartil (19/225)

Citas: 5

8.- Degradation of chlorpyrifos using different biostimulants / biofertilizers: Effects on soil biochemical properties and microbial community.

Tejada M, Rodríguez-Morgado B, Gómez I, Parrado J.

Applied Soil Ecology. 2014, Vol. 84: 158-165.

Índice de Impacto: 2,644

Ranking:

Categoría: Soil Sciences, 1º cuartil (8/34)

Citas: 15

9.- Behaviour of oxyfluorfen in soils amended with edaphic biostimulants /biofertilizers obtained from sewage sludge and chicken feathers. Effects on soil biological properties.

Rodríguez-Morgado B, Gómez I, Parrado J, Tejada M.

Environmental science and pollution research. 2014, Vol. 18: 11027-11035.

Índice de Impacto: 2,828

Ranking:

Categoría: Environmental Sciences, 1º cuartil (54/223)

Citas: 12

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RESUMEN

En la sociedad industrializada actual existen infinidad de sub-productos de origen orgánico que tradicionalmente han sido considerados directamente como residuos, o como mucho, se consideran materias de bajo valor añadido. En muchos casos estos sub-productos ocasionan graves problemas medioambientales debido a la falta de una legislación adecuada que obligue a la correcta gestión de estos materiales o a la ausencia de alternativas ecológicamente amigables.

Dos de estos sub-productos de origen orgánico constituyen las materias de partida de este trabajo. Por una parte están los lodos de depuradora, que si bien no pueden ser considerados como sub-productos de ninguna industria específica, dados el elevado volumen que se genera de ellos y su origen totalmente antropogénico, pueden ser considerados como un sub-producto de la actividad humana. Dependiendo del tipo de tratamiento que se haya realizado en las aguas residuales de partida, es posible obtener lodos con propiedades y características distintas, lo que aumenta la variabilidad de lodos existentes. Por otra parte están las plumas de ave, originadas en la industria avícola y caracterizadas por estar formadas mayoritariamente por la proteína queratina y grasas en mayor o menor medida. En ambos casos, su habitual gestión consiste en su eliminación por diversos medios, como puede ser la incineración o el almacenamiento en vertederos.

Una alternativa a los procesos de eliminación cada vez más instaurada es el reaprovechamiento como materias primas para otros procesos, aunque el valor de los productos obtenidos es relativamente bajo (ya sea económica o cualitativamente). Estos procesos de revalorización son en el caso de los lodos de depuradora el compostaje y la metanización, y en el caso de las plumas la producción de aminoácidos de uso agrícola y en menor medida el compostaje.

Aunque estos procesos de reaprovechamiento suponen un importante avance con respecto a los procesos tradicionales de eliminación, no están exentos de problemas. En el caso del compostaje, los tiempos de procesamiento son relativamente altos, la relación C/N del producto final es muy alta, el nivel de control sobre el proceso es muy bajo, si en la materia de partida existen compuestos tóxicos como los metales pesados, estos permanecen en el producto final, etc. Con respecto a la metanización, el principal problema son los rendimientos relativamente bajos y la existencia de un efluente o digestato de características similares a los lodos de depuradora que deber ser gestionado correctamente. En cuanto a los procesos de obtención de aminoácidos de uso agrícola, los procesos actuales son altamente agresivos, empleando altas temperaturas y presiones (con el consiguiente coste energético) y reactivos peligrosos tanto para el ser humano como ara el medio ambiente, como son ácidos y bases concentradas. Además, este tipo de procesamiento provoca diversas modificaciones en ciertos aminoácidos que disminuyen el valor nutricional del producto, o en casos más extremos, resultan perjudiciales tanto para plantas como para microorganismos.

En el presente trabajo se propone el uso de tecnologías biológicas como alternativas para revalorizar estos sub-productos y transformarlos en otros de alto valor añadido. Estas tecnologías se resumen en el uso de enzimas hidrolíticas o bien en el uso de microorganismos completos.

El proceso de hidrólisis enzimática consiste en usar enzimas capaces de digerir y disgregar alguna de las macromoléculas presentes en las materias primas a fin de obtener fragmentos de ellas o incluso los monómeros constituyentes. Para ello, los sub-productos orgánicos se disponen en un biorreactor donde se controlan diversos parámetros como son pH, temperatura, agitación, concentración de sustrato, concentración de enzima, etc. Una vez que las condiciones se han establecido y tras un

tiempo previamente determinado, se obtiene un producto que puede ser usado tal cual (producto hidrolizado), o bien puede sufrir una serie de procesos a fin de separar las distintas fracciones y obtener un producto final de características adecuadas para su uso final. En general, estas características finales son una alta solubilidad en agua y bajo peso molecular que permita su fácil asimilación tanto por plantas como por microorganismos, y un bajo contenido en agua para su correcta conservación.

En el caso de los procesos fermentativos, en vez de emplear una enzima, se usan microorganismos completos que producirán una batería de enzimas extracelulares que serán las encargadas de romper los componentes macromoleculares de las materias de partida. Uno de estos microorganismos con gran uso industrial para la obtención de enzimas y la digestión "in situ" es la bacteria *Bacillus licheniformis*. Esta bacteria, en presencia de un sustrato inductor es capaz de excretar al medio las enzimas necesarias para digerir dicho sustrato y poder asimilar los nutrientes que lo constituye, garantizando así su supervivencia.

Después de utilizar ambas tecnologías sobre ambos sub-productos, hemos obtenido una serie de productos caracterizados en general por tener un alto contenido en péptidos de bajo peso molecular y aminoácidos libres, ser completamente solubles (si se realiza el proceso de separación) y tener un alto potencial como bioestimulantes edáficos y vegetales.

Los estudios de bioestimulación tanto en suelos han revelado que estos productos son capaces de inducir una rápida respuesta por parte de los microorganismos del suelo, los cuales se desarrollan de forma explosiva en un plazo de tiempo relativamente corto. Como parte de esta respuesta, los microorganismos excretan una serie de enzimas destinadas a la adquisición de nutrientes como son el carbono o el

fósforo. Sin embargo, no se produce una inducción en la producción de enzimas implicadas en la adquisición de nitrógeno, ya que este elemento es aportado por los propios productos. Aun así, se observaron diferencias entre los distintos productos, siendo los de mayor contenido en péptidos de bajo peso molecular y aminoácidos libres los que presentaban mayores niveles de estimulación.

Cuando los productos bioestimulantes fueron aplicados en cultivos comerciales (como maíz), se observó cómo se producía un aumento de los nutrientes existentes en la planta, lo cual se traducía en un incremento del contenido en proteínas del grano y de los rendimientos generales del cultivo, como son la producción total, el tamaño y número de granos, etc. Sin embargo, para que se diese este efecto la aplicación debía ser foliar, no observándose estos efectos cuando la aplicación era radicular.

Por otra parte, y en vista a los resultados obtenidos en los ensayos de bioestimulación, se pasó a comprobar la capacidad de estos productos de mejorar los procesos de biorremediación de suelos contaminados con tóxicos como son los hidrocarburos aromáticos policíclicos o diversos plaguicidas. En todos los casos estudiados, se observó como la aplicación de estos productos bioestimulantes era capaz de revertir el efecto inhibitorio causado por el agente contaminante, alcanzando valores de actividad microbiana, que si bien no alcanzaban los encontrados en suelos no contaminados, superaban con creces los obtenidos en suelos contaminados sin tratar. Como consecuencia de este incremento de la actividad microbiana en general, se observó una mayor tasa de biodegradación de los xenobióticos, los cuales eran en general completamente eliminados. Sin embargo, la magnitud de este efecto dependía de las características del producto, y más concretamente, de la proporción de péptidos de bajo peso molecular y aminoácidos libres.

CAPITULO 1

PRODUCCIÓN DE BIOESTIMULANTES A PARTIR DE LODOS DE DEPURADORA Y PLUMAS DE POLLO

1. INTRODUCCIÓN

1.1. LODOS DE DEPURADORA

1.1.1. Depuración de aguas residuales

La contaminación de las aguas constituye uno de los problemas medioambientales más importantes debido, por una parte, a que los seres humanos y las actividades que estos realizan necesitan de un aporte de agua con una calidad mínima para su correcto desarrollo, y por otro lado, esta contaminación de las aguas afecta a los diversos ecosistemas vinculados a la masa acuífera afectada, deteriorándolos e incluso destruyéndolos.

Como respuesta por parte de los gobiernos, se han promulgado diversas leyes, decretos, planes, etc. tanto a nivel nacional como europeo con el objetivo de tratar las aguas residuales generadas por la actividad humana para poder proteger los sistemas ecológicos donde se viertan estas aguas, prevenir peligros para la salud humana y/u obtener un efluente que pueda ser reutilizado (Martín y col., 2006).

Una de las primeras acciones tomadas ha sido la construcción y puesta en marcha de numerosas Estaciones Depuradoras de Aguas Residuales (EDAR) por todo el territorio a fin de tratar esas aguas residuales y obtener un efluente que pueda ser vertido al medio ambiente sin riesgos ni para éste ni para el ser humano (Tchobanoglous y Burton, 1995).

1.1.2. Estaciones Depuradoras de Aguas Residuales

La entrada de una EDAR está constituida por un flujo de aguas residuales, que pueden ser urbanas o industriales, adaptándose las instalaciones a sus distintas características, mientras que los efluentes son una corriente de agua "depurada" que puede ser vertida al medio o reutilizada y unos sólidos denominados comúnmente lodos o fangos de depuradora. Estos lodos son un subproducto de desecho de las EDAR que deben ser convenientemente procesados para evitar daños a la salud pública o al medio ambiente. Por todo ello, dentro de

una EDAR se distinguen una línea de aguas destinada al tratamiento de ésta, y por el otro, una línea de lodos donde se separarán estos de la masa de agua depurada y se tratarán convenientemente (Figura 1).

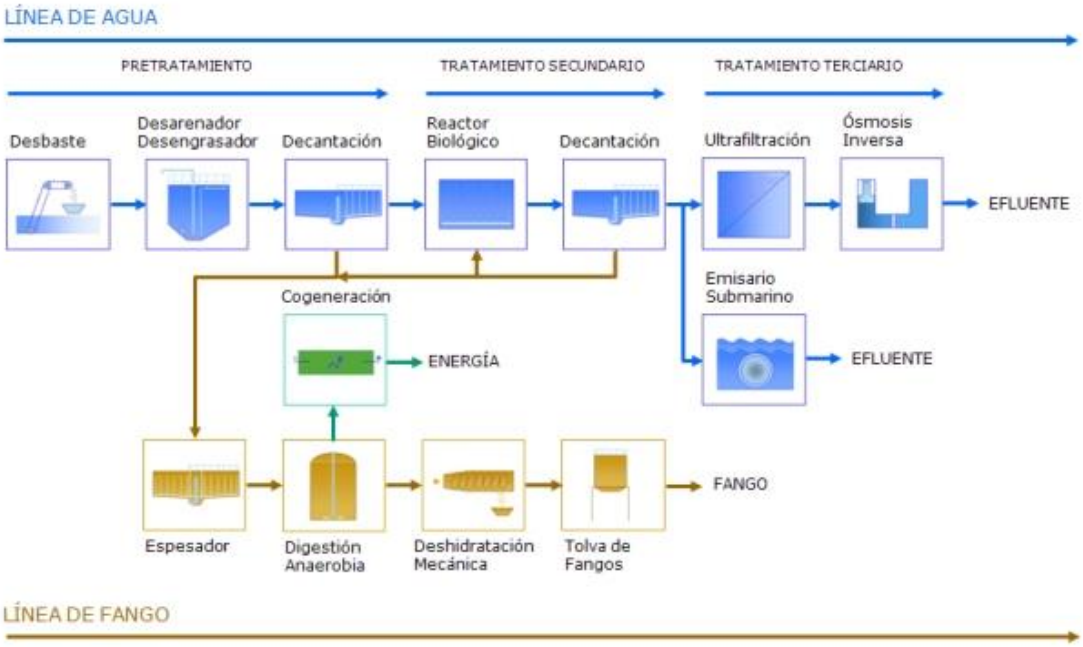

Figura 1: Esquema de una Estación Depuradora de Aguas Residuales (EDAR) convencional.

1.1.3. Línea de aguas

La línea de aguas de una EDAR consta de cuatro pasos o procesos básicos destinados a su depuración (Tchobanoglous y Burton, 1995):

Pretratamientos:

Consiste en el acondicionamiento del agua y la retirada de los sólidos en suspensión más groseros mediante rejas de desbaste, tamices, desarenadores, desengrasadores, etc. Dependiendo del tipo de influente y del destino final de las aguas residuales, estos pretratamientos pueden constituir el único proceso que sufran dichas aguas.

Tratamientos primarios:

Principalmente consisten en procesos donde se va reducir o eliminar completamente los sólidos en suspensión de las aguas residuales que hayan superado los pretratamientos previos. Algunos de estos tratamientos primarios son la sedimentación o decantación primaria, la flotación o la neutralización.

Tratamientos secundarios:

Mediante estos tratamientos se busca reducir o eliminar la contaminación orgánica en solución así como aquellos sólidos en suspensión no decantables. Estos tratamientos pueden ser de naturaleza físico-química o bien de tipo biológico.

En los tratamientos biológicos se emplean microorganismos que consumen la materia orgánica para dar nuevos microorganismos al tiempo que se favorece la sedimentación de la materia inorgánica. Esto se debe a que esos nuevos microrganismos son más densos que el agua y precipitan, pudiéndose separar entonces del agua. Según las condiciones de oxigenación del proceso se distinguen los tratamientos aerobios (presencia de oxígeno) de los anaerobios (ausencia de oxígeno).

Independientemente del si el tratamiento es aerobio o anaerobio, las distintas tecnologías aplicables pueden agruparse en tecnologías intensivas (o convencionales) que requieren de un aporte externo de energía, como son los sistemas de lodos activos, los digestores anaerobios, los reactores secuenciales o de membranas; y tecnologías extensivas (o no convencionales) que requieren de grandes superficies de trabajo como los sistemas de lagunaje, filtros verdes, filtros de turba, etc. También existen tecnologías intermedias como son los lechos bacterianos o los contactores biológicos rotativos. El uso de unas tecnologías u otras dependerá de la disponibilidad de energía de bajo coste y de superficie donde instalarlas.

Tratamientos terciarios:

Mediante estos tratamientos se busca obtener un efluente de gran calidad con vistas a un fin en particular. Son sistemas muy caros donde se van a retirar los sólidos en suspensión que no se hayan retirado con anterioridad, adsorber diversos contaminantes, eliminar iones perjudiciales, desinfectar las aguas u obtener una corriente de agua relativamente exenta de contaminantes mediante sistemas de membranas.

1.1.4. Línea de lodos

Los lodos de depuradora presentan unas características diferentes dependiendo de las aguas residuales de origen y del tratamiento al que se sometan estas en la EDAR. En una estación depuradora convencional con tratamiento biológico se generan dos tipos de lodos:

- Lodos primarios: proceden del decantador primario y lo conforman los sólidos precipitados previamente al tratamiento biológico. En su mayor parte se componen de restos fecales así como de otros desechos arrastrados por las aguas.
- Lodos secundarios o biológicos: proceden del decantador secundario y se componen fundamentalmente de la biomasa microbiana generada en el tratamiento biológico de las aguas residuales. Parte de estos lodos se recirculan en el sistema de "fangos activos" y el exceso constituye el efluente sólido del sistema (Figura 2).

Figura 2: Lodos secundarios generados por una EDAR.

Independientemente del origen de los lodos, todos se caracterizan por unos elevados contenidos de agua, materia orgánica putrescible y microorganismos patógenos. Debido a ello, los objetivos de las EDAR en cuanto al tratamiento de los lodos generados se centran en la reducción del contenido de agua, para reducir costes de transporte y mejorar la estabilidad del producto; y estabilizar la materia orgánica para evitar su putrefacción (Cieślik y col., 2015).

Los distintos procesos destinados a conseguir estos objetivos son:

- Espesamiento: se reduce el volumen de los lodos al eliminar agua de los mismos por medio de sistema de sedimentación gravitacional, flotación o centrifugación. Esta reducción del volumen es vital para reducir el volumen de lodos a tratar en los siguientes procesos, incidiendo directamente en el coste de inversión y operación de los mismos.
- Estabilización: se disminuye el contenido en materia orgánica, evitando la podredumbre de los lodos así como malos olores y la presencia de organismos

patógenos. Existen dos tipos de sistemas de estabilización, los biológicos y los químicos. En la digestión biológica los lodos sirven como sustrato para el crecimiento de otros microorganismos, pudiendo ser esta digestión aeróbica, donde en presencia de oxígeno la materia orgánica se oxida hasta un fango digerido conjuntamente con CO_2 y agua; o bien anaeróbica en ausencia de oxígeno, donde se genera también un fango digerido junto con CO_2 y metano. En la estabilización química se emplea un reactivo químico, normalmente cal para alterar las características químicas como el pH del lodo e impedir su putrefacción.

- Acondicionamiento: es una etapa previa al deshidratado de los lodos para reducir la afinidad del agua por los sólidos presentes en estos. Se puede realizar mediante la adicción de reactivos químicos o mediante tratamiento térmico, de forma que los lodos pierden parte del agua intersticial al formarse agregados mayores.
- Deshidratación: consistente en la práctica eliminación del contenido en agua de los lodos. Este proceso suele constituir la etapa final del tratamiento de los lodos en muchas depuradoras. Mediante la deshidratación se reducen los costes de transporte de los lodos al disminuir su peso y volumen, se reducen los problemas de malos olores y putrefacción y constituye un paso previo necesario en el caso de que el destino final de los lodos sea la incineración. Se puede realizar mediante sistemas de filtrado o centrifugado.
- Destino final: el destino final de los lodos de depuradora dependerá del valor que se les dé a los mismos, pudiendo almacenarse como un residuo sin valor en vertederos (Figura 3) o utilizarse como un recurso o materia prima.

Figura 3: Lodos de depuradora depositados en un vertedero.

1.1.5. Sistema de lodos activos

El sistema de lodos o fangos activos es el sistema biológico más ampliamente usado en la mayoría de las depuradoras de aguas residuales. En este proceso, una masa microbiana metabólicamente activa actúa sobre un sustrato orgánico, estabilizándolo por vía aerobia (Hreiz y col., 2015).

Este proceso se divide en dos etapas, una de aireación, donde se metaboliza y degrada la materia orgánica por acción de los microorganismos y una segunda de sedimentación donde se separan los lodos del agua depurada.

En la etapa de aireación se mezclan dentro de un reactor la materia orgánica presente en las aguas residuales con los microorganismos que la van a digerir en presencia de oxígeno suficiente para que el proceso se dé a la máxima velocidad posible. El resultado final de esta etapa es una mezcla de agua y biomasa microbiana que se ha generado tras consumir la materia orgánica disuelta en las aguas residuales. Entre los microorganismos que

normalmente componen los fangos activos se encuentran bacterias de diversos grupos, hongos, algas, protozoos, rotíferos, etc. (Xia y col., 2018)

El reactor biológico funciona en continuo, es decir, se introduce constantemente aguas residuales al mismo tiempo que se purga una mezcla de agua depurada con lodos activos en suspensión. Al mismo tiempo, se realiza un aporte de oxígeno, generalmente proveniente del aire atmosférico mediante sistemas de bombeo o por agitación vigorosa. La velocidad de entrada y salida del sistema están controladas para permitir que la materia orgánica permanezca en su interior el tiempo necesario para su completa degradación, lo que se denomina como tiempo hidráulico de retención.

Como consecuencia de las condiciones establecidas en el seno del reactor, los microorganismos se reproducen y el exceso es evacuado del mismo conjuntamente con el agua depurada. Esta mezcla pasa a la etapa de sedimentación o clarificación, donde la biomasa en suspensión sedimenta, obteniéndose una corriente de agua ya depurada y otra de lodos "activos". Para aumentar la eficiencia del sistema, parte de los lodos activos son reincorporados de nuevo al proceso, aumentando la concentración de biomasa activa en el reactor biológico y acelerando el tratamiento de las aguas residuales.

1.1.6. Gestión de los lodos de depuradora

El adecuado tratamiento y gestión de los lodos de depuradora es de vital importancia debido a que concentran muchos de los contaminantes presentes en las aguas residuales de las que proceden (Aragón, 2009). Con el objetivo de regular esta gestión, los diferentes gobiernos han promulgado una serie de leyes y directivas como son la Directiva Europea 2008/98/CE sobre residuos o la 86/278/CEE de protección del medio ambiente. Por otra parte, se han aprobado también otras nuevas directivas como la 2000/60/CE del Marco de Aguas, la 2006/7/CE de Aguas de Baño, etc. con las que se busca tratar todas, o en su mayor parte, las

aguas residuales generadas en la Unión Europea. En conjunto, la aplicación de estas directivas, así como otras relacionadas, conllevará a un incremento sustancial de la producción de lodos de depuradora al tiempo que los usos actuales de estos se ven restringidos. Esto nos lleva a la pequeña paradoja de una mayor cantidad de lodos y menos usos posibles de estos al tiempo que se exige su adecuada gestión.

La producción de lodos es un proceso continuo que requiere soluciones para su disposición final que sean flexibles, seguras, económica y medioambientalmente aceptables. La nueva normativa marca una serie de pautas a seguir, como son la disminución de la cantidad de lodos generada y su correcta caracterización, la reducción del depósito en vertederos, y la valorización de los lodos. Esta valorización de los lodos se centra actualmente en los usos agrícolas de los mismos tras ser compostados o digeridos anaeróbicamente y en la valorización energética. Sin embargo, la posibilidad de usar estos lodos dependerá de que estos no superen ciertos límites legales relacionados intrínsecamente con las propias características de los lodos, como son el contenido en metales pesados, organismos patógenos o contaminantes orgánicos. A pesar de ello, el tratamiento de los lodos de depuradora no está exento de problemas. Los procesos a los que son sometidos son caros (en algunos casos, suponen más del 50% de los costes de explotación y mantenimiento de las EDAR), complejos y no siempre tienen aceptación social (por ejemplo, la incineración de lodos) (Aragón, 2009).

En Europa Occidental, según datos de la Comisión Europea, entre el 40 - 50% de los lodos son utilizados en agricultura, si bien, está determinantemente prohibido su aplicación directa sin un tratamiento adecuado (Directiva 86/278/CEE). Además, su aplicación al terreno depende de las propias características del lodo, más concretamente, de su contenido en metales pesados, contaminantes orgánicos (emergentes) y microorganismos patógenos, lo cual imposibilita, en ocasiones, el uso de lodos tratados para este fin. Se necesitan, por tanto, otras alternativas que permitan o bien reducir la cantidad de lodos de depuradora (aplicación

del principio "prevención en origen de la contaminación") o bien valorizar dichos subproductos y obtener, a partir de ellos, productos de alto valor añadido (incluyendo su papel como enmendador de suelos agrícolas).

1.1.7. Tratamientos convencionales de los lodos de depuradora

Los tratamientos que se realizan en las Estaciones depuradoras de Aguas Residuales actualmente con el fin de tratar y valorizar los lodos son la digestión anaerobia con o sin uso energético del biogás generado, compostaje, secado y vertido, estabilización química e incineración. En algunos de estos tratamientos, el lodo se trata como una materia prima con valor económico, mientras que en otros como el vertido, se tratan como un residuo sin valor alguno.

Dentro de los tratamientos de reaprovechamiento de los lodos, una vez estabilizados y deshidratados, los más comunes y extendidos son:

- Compostaje: este tratamiento consiste en descomponer biológicamente la materia orgánica presente en la materia de partida, los lodos en este caso, gracias a la acción de bacterias y hongos principalmente. Esta descomposición conlleva la estabilización de la materia orgánica y la eliminación de patógenos gracias a las temperaturas alcanzadas durante el proceso, al tiempo que se conservan los nutrientes presentes y se obtiene un producto de aplicabilidad agronómica.

  Este proceso se compone de varias etapas, como son el pre-acondicionamiento de la materia de partida ajustando sus características, como puede ser la humedad, porosidad, composición elemental, etc., la biodegradación de la materia orgánica en un proceso termogénico que evapora agua al tiempo que elimina muchos organismos patógenos; maduración, en la que la materia orgánica más resistente a la

biodegradación es digerida y el refinado, donde se ajustan diversos parámetros para el futuro uso del producto (Bueno Márquez y col., 2008) (Figura 4).

Figura 4: Compostaje de lodos de depuradora conjuntamente con restos vegetales para mejorar su porosidad.

Las ventajas del proceso son la obtención de un producto estable e higienizado con utilidad comercial a relativo bajo coste. Los inconvenientes son las necesidades de ajustar los parámetros de entrada de la materia prima, un suministro de oxígeno adecuado, ya sea por soplantes o mediante volteo mecánico y la producción de olores durante el proceso.

- Secado térmico: consiste en la reducción en el contenido de agua de los lodos hasta aproximadamente un 10%. De esta forma, se aumenta el poder calorífico de los lodos en caso de usarse como combustible y se reduce el peso y volumen de los mismos, reduciendo los costes de transporte.

  El secado térmico se realiza con posterioridad a la etapa de deshidratación de los lodos debido al relativo alto coste. Es producto obtenido se destina fundamentalmente al

aprovechamiento térmico como combustible y al uso agronómico como en enmienda del suelo.

Entre las ventajas de este proceso están la mencionada reducción de peso y volumen, además de la higienización de los lodos o la baja necesidad de espacio para implantar esta tecnología. Como desventajas aparecen los altos coses de inversión, mantenimiento y explotación de los sistemas de secado y el hecho de que los lodos no se encuentran estabilizados, pudiendo entrar en fase de putrescencia en caso de que se humedezcan de nuevo.

- Procesos termoquímicos:
  - Incineración: Consiste en la oxidación de los lodos a alta temperatura en presencia de exceso de oxígeno. Tras este proceso se obtiene una mezcla de gases, principalmente CO_2, H_2O, N_2 además de otros en menores proporciones, y unas cenizas constituidas por la materia no combustionable. Este sistema tiene la ventaja de que el volumen de los lodos es reducido a una fracción del de partida al tiempo de que el calor generado puede ser aprovechado para la obtención de energía o en otros procesos que requieran de un aporte calorífico.

    La reducción del volumen supone una ventaja en cuanto a los costes de transporte al lugar de depósito final de las cenizas generadas, careciendo las mismas de materia orgánica putrescible ni posibles patógenos. Sin embargo, se pueden generar problemas medioambientales derivados de la emisión de gases, como son el CO_2, SO_2, NO_x, etc. (Mahamud, 2000).
  - Gasificación: consiste en la conversión de los lodos de depuradora en un gas combustible y en un residuo sólido inerte. Este proceso se realiza a temperaturas superiores a los 1000 ºC y en presencia de oxígeno, generándose una mezcla gaseosa de CO, CO_2, H_2, CH_4, H_2O, N_2 y pequeñas cantidades de hidrocarburos.

Este gas se puede utilizar como combustible de motores, turbinas, etc. El residuo producido normalmente se almacena en vertederos (Syed-Hassan y col., 2017).

- Pirolisis: es un proceso similar a la gasificación, pero se realiza a temperaturas sustancialmente inferiores y en ausencia de oxígeno. Este proceso, a diferencia de los anteriores, es endotérmico, necesitando por ello de un aporte externo de energía. Los productos obtenidos son un residuo sólido, aceite pirolítico y una mezcla gaseosa de hidrógeno, metano y monóxido de carbono principalmente. Las ventajas del proceso son la reducción de volumen, la obtención de subproductos con posible utilidad comercial y la ausencia de compuestos peligrosos (Syed-Hassan y col., 2017).

1.2. PLUMAS DE POLLO

1.2.1. Origen y funciones

Las plumas son un órgano epitelial exclusivo de la clase de las aves. Las funciones principales de estos elementos son el mantenimiento de la temperatura corporal, el vuelo, protección frente a los elementos externos, amortiguación mecánica, nado, buceo, reconocimiento, advertencia, etc. Como consecuencia de la aparición de estos órganos, las aves han sido capaces de adaptarse y colonizar nuevos nichos ecológicos a los que anteriormente no tenían acceso, como son el propio cielo, islas remotas, etc. (Stettenheim, 1972; Widelitz y col., 2007)

1.2.2. Composición

Las plumas están constituidas aproximadamente en un 90% por una única proteína, la β-queratina. Esta proteína fibrosa presenta una estructura típica en lámina β antiparalela muy rica en enlaces disulfuro, lo que le otorga una gran estabilidad frente a la degradación (Figura 5). Aunque todas las queratinas son similares, su composición aminoacídica y estructura difiere ligeramente entre los distintos grupos de animales (Stettenheim, 1972).

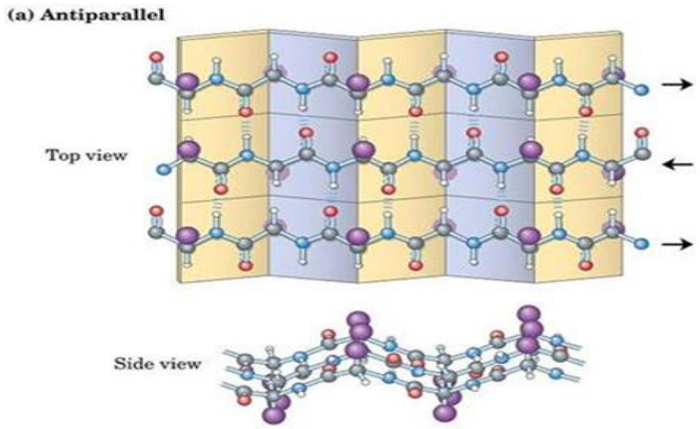

Figura 5: Estructura de lámina β antiparalela típica de la queratina.

1.2.3. Estructura y tipología de las plumas

En general, las partes de una pluma son las que se muestran en la figura 6 y son: un eje o mástil, formado por el cálamo, cañón o plumilla, la hipopluma y el raquis; y un estandarte o vexilo, compuesto por ramificaciones que parten del raquis llamadas barbas, las cuales se subdividen en barbillas, de las que parten numerosas espinas o ganchillos que unen unas barbillas con otras y estas a su vez a las barbas contiguas, formando una estructura cohesionada y semirrígida (López-Albors y col., 1999).

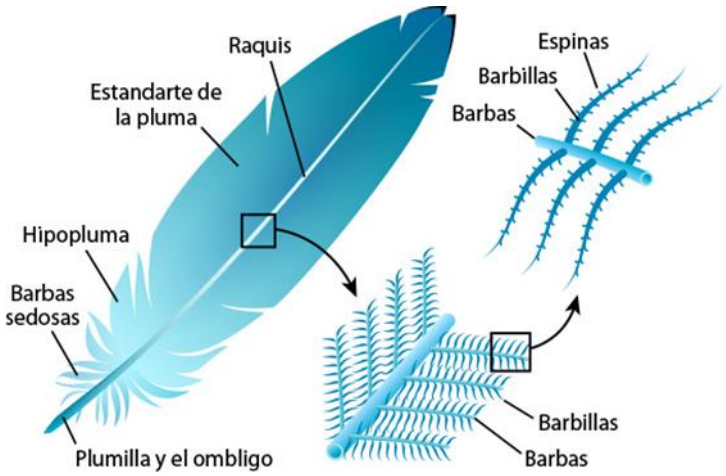

Figura 6: Partes de la pluma.

Las plumas presentan diversas formas dependiendo de las funciones que vayan a desempeñar y de su posición en el ave. Los tipos de plumas que aparecen en un ave son: plumón, cuya función es el mantenimiento de la temperatura corporal; semipluma, intermedia entre plumón y pluma de contorno; plumón polvoriento, que acumula polvo para el acicalamiento del ave; plumas de contorno, determinan la morfología del plumaje; plumas de vuelo, encargadas de permitir y dirigir el vuelo, dividiéndose en remeras o rémiges (contorno exterior del ala), timoneras o rectrices (cola) y coberteras o tectrices; cerdas, con función sensorial y filoplumas, también con función sensorial (López-Albors y col., 1999) (Figura 7).

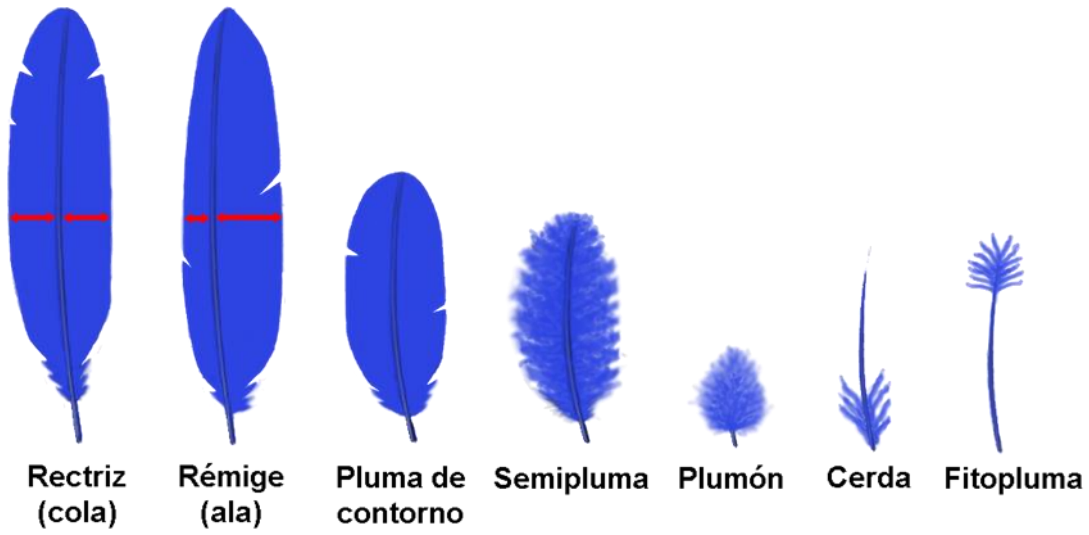

Figura 7: Diagrama comparativo de los distintos tipos de plumas

1.2.4. Problemática de las plumas

La carne de pollo es la segunda carne más consumida en el mundo después de la carne de cerdo, a lo que se suma un incremento de su consumo, sobre todo en países asiáticos. Durante el procesado de los pollos por las industrias avícolas se genera este subproducto, que supone entre un 5 y un 10% del peso corporal del ave, generando una producción mundial de plumas que ronda los 9 millones de toneladas (Reddy, 2015; Callegaro y col., 2018). Aunque inicialmente se consideraban las plumas como un residuo, actualmente se encuentran recogidas dentro de la Categoría 3 de subproductos animales. Los materiales enmarcados en dicha categoría pueden ser utilizados como materia prima para procesos como la producción de alimentos para animales distintos de los de partida, compostados o ensilados, lo cual abre su posible uso como biofertilizantes o bioestimulantes una vez procesados correctamente (Reglamento CE nº 1069/2009).

1.3. PRODUCCIÓN DE BIOFERTILIZANTES Y BIOESTIMULANTES ORGÁNICOS

Desde hace tiempo se vienen empleando de forma convencional como materias primas para la elaboración de fertilizantes y bioestimulantes orgánicos diversos sustratos de variada índole. La selección de una materia prima u otra depende en muchos casos de la composición química, la facilidad de procesado y sobre todo del precio y disponibilidad en el momento de la elección. Aunque la decisión final se toma teniendo en cuenta toda esta información y con un periodo de validez de varios años, en muchos casos, las condiciones iniciales cambian debido a agentes externos como cambios geopolíticos, crisis financieras, catástrofes ambientales, etc. o simplemente, el periodo inicial de validez a espirado y debe realizarse un estudio y valoración nuevos.

A lo largo del tiempo, muchas de las materias primas que inicialmente se empleaban en la producción de fertilizantes o bioestimulantes han incrementado su precio, disminuido su disponibilidad o reducido sus usos y aplicaciones por motivos relacionados con la legislación vigente. Esto ha obligado a la búsqueda de nuevas materias primas de bajo coste y alta disponibilidad, como son los subproductos de la industria agro-alimentaria.

Atendiendo únicamente a la composición química, muchos sustratos orgánicos, como los subproductos de la industria agro-alimentaria, resultan adecuados para aplicarlos como fertilizantes o bioestimulantes. Sin embargo, estos subproductos no pueden ser utilizados tal cual debido a una serie de problemas inherentes a su estructura, propiedades fisicoquímicas, etc. Entre estos parámetros limitantes a tener en cuenta destacan algunos como la solubilidad o distribución de pesos moleculares. Debido a ello, el uso agronómico o ambiental directo de estos sustratos orgánicos resulta inviable técnica y económicamente.

Para solventar estos problemas, los subproductos deben ser procesados de forma que se soslayen las limitaciones de los mismos. Tradicionalmente, se han empleado tratamientos de carácter físico o químico con el fin de extraer y/o modificar los componentes de interés que forman parte de esos sustratos (Prendergast, 1974). En muchos casos, estos tratamientos conllevan el empleo de condiciones extremas, como altas temperaturas, pH extremos y el uso de compuestos químicos como solventes orgánicos, agentes cáusticos, etc. Aunque estos métodos consiguen obtener el producto final requerido, en general, también se producen efectos indeseados, como la aparición de modificaciones perjudiciales en aminoácidos, residuos tóxicos, alto coste energético, etc. (Fox, 1982).

Una alternativa a los tratamientos fisicoquímicos son los tratamientos biológicos o bioquímicos, como son los procesos de hidrólisis enzimática o los procesos fermentativos.

1.3.1. Tecnología enzimática

1.3.1.1. Las enzimas

Las enzimas son catalizadores biológicos que, al igual que los catalizadores químicos, su función es aumentar la velocidad a la que tienen lugar la reacción que catalizan sin el consumo de ellos mismos. Las enzimas tienen la particularidad, en general, de ser bastante específicas con respecto al sustrato sobre el que actúan, la reacción que catalizan y el producto que se genera (Palmer y Bonner, 2011).

Aunque las enzimas se suelen denominar de forma común teniendo en cuenta el sustrato sobre el que actúan, la reacción que catalizan, o ambos, existe una nomenclatura sistemática creada por la Enzyme Commission (EC). Esta nomenclatura consiste en 4 números separados por puntos, donde el primer dígito indica una de las seis clases principales de reacciones catalizadas (Tabla 1). El segundo dígito describe la subclase de reacción en particular. El tercer dígito es común a aquellas enzimas que catalizan reacciones similares

pero no exactamente iguales, mientras que el cuarto dígito define el sustrato específico (Palmer y Bonner, 2011).

PRIMER DÍGITO	CLASE	REACCION CATALIZADA
1	Oxidorreductasas	Reacciones de oxidación/reducción
2	Transferasas	Transferencia de un átomo o grupo entre dos moléculas
3	Hidrolasas	Reacciones de hidrólisis
4	Liasas	Ruptura de una molécula (no por hidrólisis)
5	Isomerasas	Reacciones de isomerización
6	Ligasas	Unión de dos moléculas

Tabla 1. Principales clases de reacciones catalizadas por enzimas.

1.3.1.2. Hidrólisis enzimática

La hidrólisis enzimática es un tipo de reacción catalizada por una hidrolasa, donde una molécula de agua reacciona con un sustrato, de forma que se generan dos productos, uno que contiene el catión hidrógeno y el otro al anión hidroxilo, como se muestra en la ecuación 1:

Eq. 1:

$$A - B \; + \; H_2O \; \rightarrow \; A - OH \; + \; B - H$$

Este tipo de reacciones se da habitualmente en los procesos de digestión de las macromoléculas biológicas a fin de obtener los monómeros que las componen, como es el caso de las proteínas en péptidos y aminoácidos, los polisacáridos en oligosacáridos y monosacáridos, etc.

1.3.1.3. Hidrólisis de proteínas

Las proteínas son biopolímeros compuestos por cadenas lineales de aminoácidos unidos entre sí por enlaces peptídicos y que adquiere diversas estructuras tridimensionales determinadas por la propia secuencia de aminoácidos (Berg y col., 2007).

Las proteínas poseen una amplia variedad de funciones, aunque se pueden agrupar en 5 tipos principales (Tabla 2):

FUNCIÓN	DESCRIPCIÓN
Estructurales	Proporcionan estructura y soporte a las células y organismos
Enzimas	Catalizan las diversas reacciones químicas que tienen lugar en la célula u organismo
Transporte	Transportan átomos, moléculas, etc. de una parte a otra de la célula u organismo.
Mensajeros	Transmiten señales entre células, tejidos u órganos para coordinar diversos procesos
Anticuerpos	Se unen a partículas extrañas al organismo a fin de identificarlas y protegerlo.

Tabla 2: Funciones de las proteínas.

El nitrógeno es un elemento esencial para el desarrollo de cualquier célula y organismo debido a que forma parte de los aminoácidos y estos conforman las distintas proteínas existentes. Por ello, es necesario un aporte continuo de este elemento a lo largo de todo el desarrollo de cualquier ser vivo. Este aporte se puede dar en forma de sales de nitrógeno como nitratos, amonio, urea, etc. con los que aquellos organismos capaces de ello forman sus propios aminoácidos, o bien, pueden asimilar directamente los aminoácidos ya formados por otros organismos.

Dado que en general las células no pueden incorporar directamente proteínas de gran tamaño para poder asimilar sus aminoácidos, es necesario un paso previo de hidrólisis o ruptura de esas proteínas en fragmentos de menor tamaño, los cuales reciben diferentes nombres atendiendo al peso molecular que poseen (Tabla 3)

PESO MOLECULAR (Daltons)	DENOMINACIÓN
>10.000	Proteínas
7.000-10.000	Peptonas
200-7.000	Péptidos
<200	Aminoácidos

Tabla 3. Distribución de pesos moleculares de las distintas especies proteicas

Una de las formas de reducir el tamaño de las proteínas para hacerlas fácilmente disponibles para las células es la hidrólisis enzimática, que mediante la acción de enzimas hidrolíticas, hidrolasas o más específicamente proteasas o proteinasas, "rompen" los enlaces que unen los aminoácidos y liberan fragmentos proteicos de menor tamaño que la proteína inicial (Adler-Nissen, 1977; 1986).

La unión entre los distintos aminoácidos que forman las proteínas se llama enlace peptídico, el cual es un tipo de enlace covalente. Este enlace se da entre el grupo carboxilo (-COOH) de un aminoácido y el grupo amino ($-NH_2$) del siguiente (Figura 7). Este enlace posee una serie de características particulares, como el hecho de que actúa como un enlace doble, se suelen disponer en posición *trans* en vez de *cis*, o que presenta un carácter polar.

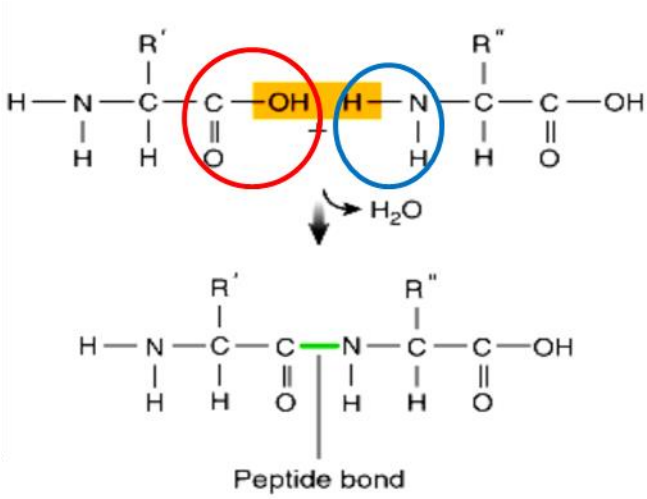

Figura 7: Formación del enlace peptídico.

La hidrólisis del enlace peptídico se da a través de varias reacciones simultaneas como son la ruptura propiamente dicha del enlace, transferencia de protones, etc. (Guadix y col., 2000; Benítez y col., 2008) (Figura 8).

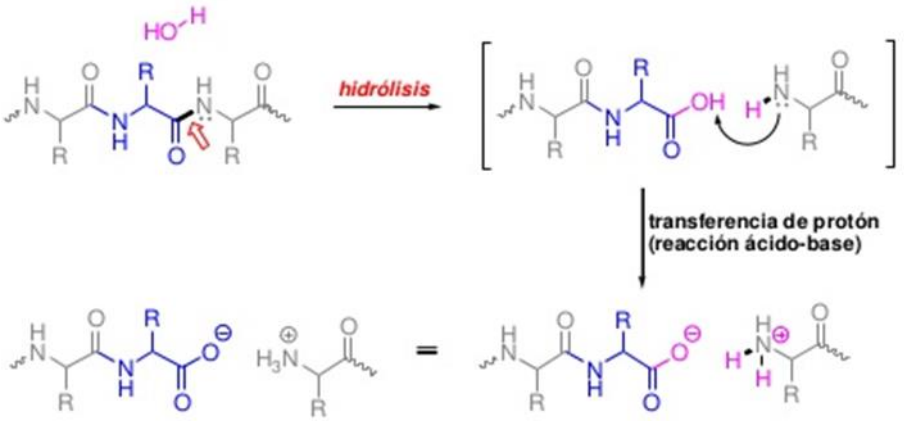

Figura 8: Hidrólisis del enlace peptídico.

Cuando esta reacción de hidrólisis está catalizada por una proteasa, el proceso tiene lugar en tres reacciones consecutivas (Adlesr-Nissen, 1986; Benítez y col., 2008):

1) Formación del complejo enzima-sustrato (proteína).
2) Ruptura del enlace peptídico y liberación de péptidos y/o aminoácidos.
3) Separación del péptido restante de la enzima tras el ataque nucleofílico de una molécula de agua.

Los grupos –COOH y -NH_2 de las proteínas presentan normalmente unos valores de pK comprendidos entre 3,1-3,6 y 7,5-7,8 respectivamente. (Steinhardt y Beychok, 1964; Rupley, 1967). De esta forma, a pH ácidos, los grupos carboxilo estarán parcialmente disociados y los amino protonados. Si la reacción de hidrólisis se da a estos pH, el pH irá aumentando rápidamente. Si, por el contrario, se trabaja a pH alcalino, los grupos carboxilo estarán totalmente disociados y los grupos amino parcialmente protonados, por lo que el valor de pH disminuirá constantemente.

1.3.1.4. Proteasas

Las proteasas son enzimas esenciales para todos los seres vivos (Gupta y col., 2002a) ya que al catalizar la hidrólisis de los enlaces peptídicos, intervienen en los procesos de reciclaje de proteínas, ya sean endógenas o exógenas (Kumar y Takagi, 1999).

Este tipo de enzimas constituye más del 50% del mercado mundial de enzimas con usos industriales, como son la producción de detergentes, alimentos, productos farmacéuticos, gestión de residuos, etc. (Rao y col., 1998; Gupta y col., 2002b).

Las proteasas se clasifican atendiendo a una serie de parámetros (Tabla 4):

PARÁMETRO	TIPO	DESCRIPCION
Acción catalítica	Endoproteasas	Hidrolizan enlaces peptídicos dentro de la cadena polipeptídica, liberando péptidos
	Exoproteasas	Hidrolizan el enlace peptídico que une el último aminoácido de la cadena, liberando aminoácidos libres
pH óptimo	Ácidas	pH óptimo entre 8 y 12
	Neutras	pH óptimo cercano a 7
	Alcalinas	pH óptimo entre 2 y 6
Elemento catalítico en el sitio activo	Aminoácidos en el sitio activo	Uno o varios aminoácidos realizan las diversas reacciones de hidrólisis, los más habituales son serina (serín proteasas), cisteína (cisteín proteasas) y aspartato.
	Metalo-proteasas (iones metálicos en el sitio activo)	Utilizan un ion metálico como el zinc como agente catalítico.
Origen	Animal	
	Vegetal	
	Bacteriana	
	Fúngica	

Figura 4: Clasificación de las proteasas.

1.3.1.5. Propiedades de los hidrolizados enzimáticos: Biofertilizantes / Bioestimulantes

Tras el proceso de hidrólisis enzimática de un sustrato proteico se obtiene un producto hidrolizado rico en péptidos de bajo peso molecular y aminoácidos libres. Estos productos tienen diversas propiedades funcionales propias de sus componentes, como son la alta solubilidad, poder emulsionante, espumante, saborizante, etc. (Jost y col., 1987; Turgeon y Gauthier, 1990; Parrado y col., 1991; 1993).

Estos hidrolizados proteicos, además de estar muy difundidos en la industria alimentaria, han recibido en los últimos años un creciente interés desde la perspectiva agronómica. Esto es debido, por una parte, a que estos productos constituyen una fuente de nitrógeno fácilmente biodisponible tanto para las plantas como para los organismos que viven en el suelo.

Por otra parte, los péptidos y aminoácidos libres son capaces de estimular el crecimiento y desarrollo de las plantas así como de las poblaciones microbianas del suelo. Además, también estimulan las respuesta a diversos tipos de estrés como son el estrés hídrico, térmico, oxidativo, salino, biótico, etc.

1.3.2. Tecnología fermentativa

Como alternativa al uso de enzimas, es posible emplear microorganismos con el objeto de producir "*in situ*" las enzimas necesarias para hidrolizar las distintas biomoléculas orgánicas y obtener de esta forma productos con capacidad biofertilizante y/o bioestimulante.

A diferencia de las enzimas, que resultan muy específicas con respecto a los sustratos y reacciones sobre las que actúan, los microorganismos, al disponer no sólo de una enzima en particular, sino de una batería de ellas, pueden actuar sobre muchos sustratos y de distintas formas simultáneamente.

Los microorganismos se han usado con diversos fines desde la antigüedad, como son los procesos de obtención del vino, la cerveza, el pan o el queso. Sin embargo, no es hasta la mitad del siglo XIX, gracias a Louis Pasteur, que no se descubre el fundamento subsecuente a dichos procesos y a otros muchos más. Este investigador identificó muchos procesos que tenían un origen microbiano y descubrió que en los procesos fermentativos, los microorganismos consumen un sustrato para producir metabolitos primarios, secundarios y productos finales.

Actualmente, y gracias al conocimiento adquirido por la humanidad, se han identificado infinidad de procesos realizados por microorganismos con algún tipo de utilidad. A esto se suman los avances en biotecnología e ingeniería genética, gracias a los cuales, es posible desarrollar nuevos procesos microbianos que no existían previamente en la naturaleza y escalarlos a un uso industrial. Este desarrollo ha permitido reducir los costes de producción de muchos productos al tiempo que simplifica los procesos empleados tradicionalmente y se reducen las emisiones de residuos, etc. Un ejemplo de ello, es la obtención de aminoácidos como el ácido glutámico o la lisina, que actualmente se lleva a cabo por vía fermentativa, en vez de la clásica vía química, obteniéndose productos isoméricamente puros (sólo se obtiene uno de los dos isómeros) a un coste mucho menor y sin generar residuos peligrosos (Najafpour, 2007).

Para llevar a cabo todos estos procesos a escala industrial, existe un sistema con prácticamente infinitas variaciones llamado biorreactor. Un biorreactor no es más que un recipiente donde se mantienen más o menos controladas una serie de condiciones ambientales como son la temperatura, el pH, la concentración de oxígeno, sustratos, etc. además de muchos otros parámetros. Dentro de dicho sistema, los microorganismos se alimentarán y multiplicarán, como consecuencia de ello, generarán el o los productos de interés, que pueden ser la propia biomasa, enzimas, o metabolitos.

1.3.2.1. Microorganismos

Los microorganismos son por definición todos aquellos organismos microscópicos que no pueden ser observados directamente por el ojo humano y que existen como células aisladas o asociadas. Además, son capaces de realizar todas sus funciones vitales sin la interacción con otras células (Madigan y col., 2015).

Dentro de los microorganismos podemos encontrar organismos pertenecientes al dominio *Prokaryota* como bacterias o arqueas, y al dominio *Eukaryota* como hongos (levaduras), algas o protozoos. La existencia de microorganismos en ambos dominios da una idea de la vasta variedad metabólica existente. Sin embargo, no todos los microorganismos pueden ser cultivados "*in vitro*" y menos aun los que poseen un uso industrial, quedando estos últimos restringidos a ciertas bacterias, levaduras y hongos filamentosos principalmente (Figura 9).

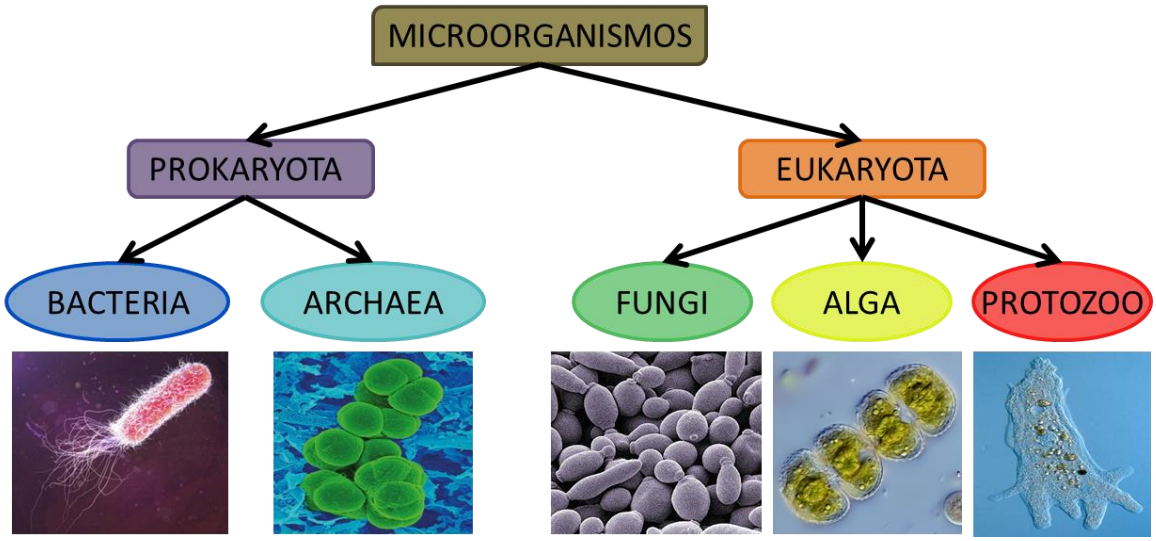

Figura 9: Taxones que incluyen microorganismos.

Los microorganismos han colonizado virtualmente todos los nichos ecológicos del planeta, y como consecuencia de ello, han desarrollado un gran número de adaptaciones morfológicas, metabólicas y fisiológicas para poder sobrevivir y desarrollarse en todos esos ambientes. Estas adaptaciones van desde cambios en el tamaño celular hasta modificaciones

en la propia química del ADN, pasando por cambios en las estrategias metabólicas, la movilidad, mecanismos de división celular, etc.

Algunas de estas adaptaciones o modificaciones de los microorganismos tienen algún tipo de interés para el ser humano, ya sea porque producen un nutriente específico, compuestos con actividad antibiótica, enzimas con usos industriales, etc. Para poder aprovechar estas características, el hombre en primer lugar ha identificado los microorganismos que las poseen, los han aislado del medio ambiente y los han reproducido en condiciones controladas. Llegado a este punto, si los rendimientos no son los esperados, existen varias posibilidades, como son optimizar el proceso "*per se*" (condiciones de cultivo principalmente), introducir cambios en la secuencia génica que mejoren el rendimiento (modificación o edición del ADN), introducir los genes implicados en otro organismo mejor adaptado a la producción industrial (organismos transgénicos), etc.

1.3.2.2. Género *Bacillus* y *Bacillus licheniformis*

El género *Bacillus* es uno de los más estudiados dentro de los procariotas gracias a su capacidad genética y tamaño relativamente grande. Este género de bacterias se encuentra de forma ubicua en todos los suelos del mundo, de donde es fácil aislar alguna de las especies que lo componen. Estas bacterias Gram positivas con forma de bastón (de ahí su nombre) son aerobios estrictos o anaerobios facultativos. Cuando las condiciones así lo requieren, estos organismos forman una endospora de localización central muy resistente a altas temperaturas y a agentes químicos. En la mayoría de las especies, presentan flagelos peritricos que les proporcionan movilidad. Este género posee varios programas genéticos que se activan en respuesta a los recursos y condiciones existentes, de forma que es capaz de sobrevivir y adaptarse a diferencia de otros organismos competidores. Además, posee la capacidad de hacerse competentes asimilar ADN del medio a fin de adaptarse mejor a este. Por otra parte,

Bacillus sp. muestra un comportamiento "social" al comunicarse las células entre sí para formar estructuras multicelulares (biofilms) donde forman distintos subtipos celulares con funciones distintas (Graumann, 2017). Estas bacterias son capaces de producir ciertas sustancias de interés industrial/agronómico. Las más estudiadas de estas sustancias son las enzimas hidrolíticas como proteasas (Bezawada y col., 2010; Drouin y col., 2008), celulasas (Saeed Al-Gheethi, 2015), amilasas (Kavitha, y col., 2013), etc.

Para poder crecer estas bacterias necesitan de una fuente de carbono y nitrógeno, las cuales se encuentran normalmente en forma de polímeros (polisacáridos, proteínas, etc.) en el medio. Con el fin de poder absorber estos nutrientes, excretan una amplia variedad de enzimas hidrolíticas, capaces de digerir dichos polímeros y hacerlos biodisponibles (Kavitha, y col., 2013).

La especie *Bacillus licheniformis*, perteneciente al género anteriormente descrito, posee una gran importancia industrial debido a que es capaz de producir extracelularmente numerosas enzimas, antibióticos y otros metabolitos. Entre los productos producidos por este organismo se encuentra un tipo de serín proteasa denominada subtilisina, la cual es de gran interés industrial y comercial. Además de esta proteasa, también excreta con usos comerciales la enzima amilasa y el antibiótico bacitracina (Rey y col., 2004; Veith y col., 2004). Una vez que *B. licheniformis* ha producido estas enzimas, es posible purificarlas a partir del medio de cultivo y emplearlas en una digestión enzimática, o bien pueden utilizarse tal cual en forma de mezcla compleja conjuntamente con los productos de la digestión (azúcares sencillos, aminoácidos, etc.).

1.3.2.3. Fermentaciones

El término "fermentación" procede del vocablo latino "*fervere*", el cual hace referencia al hecho de que los extractos de frutas (como la uva) o de granos malteados (la

cebada), al convertirse en vino o cerveza aparentemente "hierven" durante dicho proceso. Una definición más técnica de la fermentación sería "la transformación química de diversos compuestos orgánicos con la ayuda de microorganismos y enzimas" (Najafpour, 2007). Aunque a nivel estrictamente bioquímico, la fermentación es un proceso de obtención de energía por medio del catabolismo de diversos compuestos orgánicos, a nivel microbiológico e industrial, las fermentaciones son procesos donde se producen nuevos productos gracias al cultivo en masa de microorganismos (Stanbury y col., 2017).

Dentro de las fermentaciones, se pueden distinguir cinco grandes grupos que se diferencian en el producto de interés generado:

- Las que producen células microbianas (biomasa) como producto
- Las que producen enzimas
- Las que producen metabolitos
- Las que producen productos recombinantes
- Las que modifican un compuesto añadido al proceso

Biomasa microbiana

La producción de biomasa tiene dos objetivos fundamentales, por un lado la producción de levadura para su uso en procesos industriales (obtención de etanol, producción de alimentos, etc.) y por otro lado la obtención de la llamada Proteína Unicelular usada como fuente de proteínas para alimentación humana o animal.

Enzimas microbianas

Aunque las enzimas se pueden obtener de fuentes animales, vegetales o microbianas, son estas últimas las que presentan mayores ventajas gracias al avance en los procesos fermentativos (Tabla 5). La primera ventaja inherente al uso de microrganismos es su

facilidad de producción, lo cual no ocurre en el caso de los animales o las plantas. La segunda gran ventaja radica en el uso de las nuevas tecnologías de modificación del ADN, que han permitido producir enzimas de otros orígenes en microorganismos, de forma que se obtiene un producto con las características del organismo original pero producido en masa. Además, la optimización de los procesos de producción implica otros procesos como la inducción en el momento oportuno la enzima requerida, la eliminación de represores, o la posibilidad de introducir un mayor número de copias del gen en cuestión, haciendo al microorganismo superproductor de esa enzima.

INDUSTRIA	USO	ENZIMA	FUENTE
Panificación	Reducción de la viscosidad de la masa, aceleración de la fermentación, aumento del volumen del pan, mejora de la suavidad de la miga y mantenimiento de la frescura.	Amilasa	Fúngica
	Mejora de la textura de la masa, reducción del tiempo de mezcla, aumento del volumen.	Proteasa	Fúngica / bacteriana
Cervecera	Maceración	Amilasa	Fúngica / bacteriana
	Turbidez	Proteasa	Fúngica / bacteriana
	Mejora de la filtración fina.	β-Glucanasa	Fúngica / bacteriana
Cereales	Alimentos para bebés, alimentos para el desayuno	Amilasa	Fúngica
Café	Fermentación de café en grano	Pectinasa	Fúngica
	Preparación de concentrados de café	Pectinasa, hemicelulasa	Fúngica
Confitería	Producción de caramelos de núcleo blando	Invertasa, pectinasa	Fúngica / bacteriana
Sirope de maíz	Producción de glucosa a partir de jarabe de maíz	Amiloglucosidasa	Fúngica
	Fabricación de jarabes de fructosa	Glucosa isomerasa	Bacteriana
Lechera	Estabilización de la leche evaporada.	Proteasa	Fúngica
	Producción de concentrados de leche entera, helados y postres congelados.	Lactasa	Levaduras
Zumos de frutas	Clarificación	Pectinasa	Fúngica
	Eliminación de oxígeno	Glucosa oxidasa	Fúngica
Lavandería	Detergentes	Proteasa, lipasa	Bacteriana

Cuero	Pelado, descarnado	Proteasa	Fúngica / bacteriana
Cárnica	Texturización	Proteasa	Fúngica
Papelera	Eliminación de ceras	Lipasa	Fúngica
Farmacéutica	Ayudas digestivas	Amilasa, proteasa	Fúngica
	Anticoagulantes	Estreptoquinasa	Bacteriana
	Análisis clínicos	Numerosas	Fúngica / bacteriana
	Biotransformación	Numerosas	Fúngica / bacteriana
Hidrolizados proteicos	Fabricación	Proteasa	Fúngica / bacteriana
Bebidas sin alcohol	Estabilización	Glucosa oxidasa, catalasa	Fúngica
Textil	Desencolado de telas	Amilasa	Bacteriana
Vegetales	Preparación de purés y sopas	Pectinasa, amilasa, celulasa	Fúngica

Tabla 5: Principales enzimas microbianas con usos industriales.

Metabolitos microbianos

Durante las distintas fases de crecimiento de un cultivo microbiano se generan una serie de compuestos, que pueden ser anabolitos, si proceden de los procesos de biosíntesis, o catabolitos si proceden de los procesos de degradación. Estos productos se llaman metabolitos primarios. Aunque estos productos son generados de forma natural en cantidades suficientes para el desarrollo del organismo, la aplicación industrial de dichos procesos requiere de la optimización de las condiciones de cultivo y la mejora del propio microorganismo, aplicando para ello los avances más novedosos en genómica, proteómica y metabolómica. Todos estos avances permiten la "sobre-producción" del metabolito en cuestión.

A diferencia de los metabolitos primarios, que se producen en general en todas las fases del crecimiento y son más o menos comunes a todos los organismos, los metabolitos secundarios son unos compuestos generados principalmente en la fase estacionaria del cultivo y sólo se producen en ciertas especies microbianas, mientras que en otras no. Estos

metabolitos secundarios proceden de otros primarios por transformación de los mismos, no siendo indispensables para el organismo productor y realizando su efecto normalmente en otro organismo distinto. Entre estos metabolitos secundarios destacan muchos antibióticos, inhibidores, moduladores, etc. (Tabla 6).

Metabolito Secundario	**Uso comercial**
Penicilina, cefalosporina, estreptomicina	Antibióticos
Bleomicina, mitomicina	Anticancerígenos
Lovastatina	Anticolesterolémico
Ciclosporina A	Inmunosupresor
Avermectinas	Antiparasitarios

Tabla 6: Principales enzimas microbianas con usos industriales.

Productos recombinantes

Muchas proteínas, como factores de crecimiento, hormonas, etc. sólo se pueden obtener de forma adecuada usando cultivos de células animales (humanas), sin embargo, esto acarrea un gran número de problemas derivados del propio cultivo de este tipo de células, ya que son muy sensibles a cambios del medio, contaminaciones, etc. Gracias a los avances en recombinación de ADN es posible expresar de forma heteróloga estas proteínas en microorganismos, lo cual permite obtener dichas proteínas a partir de microorganismos, con las ventajas de cultivo de estos últimos.

Procesos de modificación

Además de sintetizar nuevos metabolitos a partir de los nutrientes del medio, los microorganismos son capaces de transformar ciertos compuestos en otros mediante reacciones de oxidación, deshidrogenación, hidroxilación deshidratación, descarboxilación, aminación, desaminación, etc. La ventaja principal del empleo de microorganismos para realizar estas reacciones frente a la transformación química clásica es la especificidad quiral, generándose uno de los enantiómeros en concreto y no una mezcla de ambos. La principal desventaja de

los procesos fermentativos de transformación es que previamente a dicha reacción, es necesario cultivar en cantidad suficiente los microorganismos, aunque una vez producidos, se pueden inmovilizar y reutilizar en numerosas ocasiones.

1.3.2.4. Producción de proteasas

Dentro de toda la amplia variedad de enzimas hidrolíticas que el género *Bacillus* puede excretar y en particular *B. licheniformis*, destacan sobre las demás las proteasas, enzimas implicadas en los procesos de reciclaje celular y de adquisición de nutrientes del medio, de ahí que su producción sea constitutiva (Gupta y col., 2002a). Esta enzima resulta, además, de vital importancia en los procesos de digestión de la materia orgánica al constituir las proteínas un importante componente estructural de la misma, por lo que existe un creciente interés en su producción a partir de organismos sobre-productores como *B. licheniformis*.

Es conocido que la producción de estas enzimas extracelulares está unida al paso de la fase de estacionaria a la de latencia, es decir, a la esporulación, proceso regulado por la existencia de fuentes de carbono y nitrógeno en el medio (Gupta y col., 2002a). Sin embargo, ambos procesos son independientes entre sí, aunque parece ser que el desencadenante en los dos casos es la falta de nutrientes (Khan, 2000).

Una de las limitaciones a la hora de producir comercialmente proteasas a partir de microorganismos es el elevado coste de los medios de cultivo (Romero y col., 2007), constituyendo hasta el 40% del coste total de la enzima comercial (Genesse, 1997), por lo que la mayoría de los estudios actuales se centran en la reducción de estos costes mediante la búsqueda de nuevas materias primas más económicas conjuntamente con la optimización de los parámetros de producción (Reddy y col., 2007).

2. OBJETIVOS

Como consecuencia de la necesidad de crear nuevos productos con capacidad bioestimulante tanto agronómicos como ambientales, así como de buscar alternativas para revalorizar subproductos que actualmente se encuentran infrautilizados o considerados directamente como residuos, se plantean los siguientes objetivos del presente capítulo:

1. Caracterizar físico-químicamente dos subproductos de la industria agroalimentaria, como son los lodos de depuradora y las plumas de pollo.
2. Desarrollar una metodología para obtener nuevos biofertilizantes / bioestimulantes a partir de los subproductos anteriormente mencionados.
3. Producir y caracterizar nuevos productos biofertilizantes / bioestimulantes agronómicos y ambientales obtenidos a partir de lodos de depuradora y plumas de pollo.

3. ARTICULOS

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Proteomic analysis of enzyme production by *Bacillus licheniformis* using different feather wastes as the sole fermentation media

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ABSTRACT

This study evaluates the use of different types of feathers as fermentation media for enzyme production. *Bacillus licheniformis* was grown on the feathers, which lead to total biodegradation due to bacterial enzymatic hydrolytic excretion. *B. licheniformis* excretes protease and lipase activity, with feather concentration being the main parameter controlling their generation. Using a proteomic approach, the proteins excreted during fermentation were identified, and the influence of the chemical composition of the feathers on protein secretion was tested. The identified proteins are hydrolytic enzymes such as keratinase, gamma-glutamyltranspeptidase, chitosanases, and glicosidases. The diversity of proteins is related to the chemical complexity of the feathers. Understanding the composition of a hydrolytic system, when *B. licheniformis* is cultured on different feathers, may assist in utilizing such a system for producing different hydrolytic enzymes. The data indicate that proteomics can be a valuable tool for describing the physiological state of *B. licheniformis* cell populations growing on different wastes.

1. Introduction

Many bacteria belonging to the genus *Bacillus* are important organisms for research and industrial applications. They are used in many medical, pharmaceutical, agricultural, and industrial processes that take advantage of their wide range of physiological characteristics and their ability to produce enzymes and other metabolites [1,2].

Bacillus species are attractive industrial organisms for a variety of reasons, including their high growth rates leading to short fermentation cycle times, their capacity to secrete proteins into the extracellular medium, and the GRAS (generally regarded as safe) status for species, such as *Bacillus subtilis* and *Bacillus licheniformis*.

B. licheniformis is known for its ability to produce and secrete numerous hydrolytic enzymes that enable the microorganism to degrade many different substrates and to grow on a wide range of nutrient sources. It is used extensively for large-scale industrial production of exoenzymes as it can secrete large quantities of proteins of up to 20–25 g/l [1].

Alkaline serine protease (subtilisins) is one of the most important industrial enzymes excreted into the medium by strains of this specie. The annual output has been estimated at about 500 metric tonnes of pure enzyme protein [1]. Amylases [3] and the topical antibiotic bacitracin are other products that can be produced by the fermentation of *B. licheniformis* strains. The host *B. licheniformis* is also extremely important for commercial processes for heterologous exoenzymes [1].

Currently, one of the major bottlenecks for industrial enzyme producers is the high cost of enzyme production. Some 30–40% of the production cost of many industrial enzymes is estimated to come from the cost of the growth substrate [4].

The use of low-cost growth substrates for the production of industrial enzymes is expected to greatly reduce production costs. Waste materials from a wide range of agro-industrial processes may be used as the substrates for microbial growth, thereby resulting in an upgrade of the waste or in the synthesis of valuable by-products. The bulk of the wastes from agriculture or food processing are not suitable for food or animal feed [5], but microorganisms are capable of utilizing the organic matter in wastes both as a source of energy for growth and as carbon for the synthesis of cell biomass. These wastes could thus serve as inexpensive fermentation sources [5] and [6].

Feathers, which constitute up to 5–10% of total chicken weight, are an important by-product of the poultry industry. They are frequently discarded as waste and can pose an environmental problem, because they do not degrade easily. Since feathers consist primarily of keratin, they are not degradable by common proteolytic enzymes such as trypsin, pepsin, and papain in their native state. *Bacillus* was found to be able to use feathers as a primary source of energy, carbon and nitrogen [7] and [8].

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In the present study we investigated hydrolytic enzyme production by *B. licheniformis* growing on feathers as the sole organic substrate for the supply of carbon, nitrogen and energy. Secondly, we compared the protein expression of *B. licheniformis* using different types of feathers as grown substrate. Finally, proteomics analysis contributed to understanding the physiological state of the producer strain under distinct conditions.

2. Materials and methods

2.1. Microorganisms and substrates

The microorganism used in this study was *B. licheniformis* ATCC 21415, stored under freezing at −80 °C and refreshed 24 h before inoculation in LB medium (10 g tryptone, 10 g NaCl, 5 g yeast extract, and 1 L of water). Poultry feathers and LB medium were used as substrate for fermentation. Feathers were provided by two different poultry slaughterhouses: by the company TG-S.L., called TG-Feather; and another poultry industry located in Murcia (SE Spain) called MU-Feather.

2.2. Chemical determinations

Both types of feather were chemically analyzed. Macroelements were analyzed by inductively coupled plasma atomic emission spectrometry (ICP-AES) using a Fisons-ARL 3410 sequential multi-element instrument equipped with a data acquisition and control system. The standard operational conditions of this instrument can be summarized as follows: the carrier gas, coolant gas, and plasma gas is argon at 80 psi of pressure; the carrier gas flow rate is 0.8 L min^{-1}; the coolant gas flow rate is 7.5 L min^{-1}; the plasma gas flow rate is 0.8 L min^{-1}; and the integration time is 1 s. One mini-torch consumes argon gas at a radio-frequency power of 650 W.

Carbohydrates were determined according to standard AOAC methods. Lipid content was determined gravimetrically after feather extraction with hexane for 12 h in a Soxhlet extractor.

2.3. Protein molecular weight determination by size-exclusion HPLC

Molecular-mass distribution of protein in the samples was determined by size-exclusion chromatography using an ÄKTA-purifier (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), using a Superdex Peptide TM 10/300GL column (optimum separation range 0.1–7 kDa). Samples were centrifuged at 12.000 × *g* for 30 min at 4 °C to remove insolubles, and the supernatant was passed through a 0.2 μm filter and loaded into a 0.1 mL loop connected to an Äkta-purifier system. The column was equilibrated and then eluted with 0.25 M Tris–HCl buffer (pH 7.00) in isocratic mode at a flow-rate of 0.5 mL min^{-1}. Proteins/peptides were detected at 280 and 215 nm with a GE Healthcare UV900 module coupled to the column elution.

2.4. Media and fermentation operations

The LB medium was composed of 10 g tryptone, 10 g NaCl, 5 g yeast extract, and 1 L of water. After mixing, the medium was autoclaved at 121 °C for 20 min.

The media made with the different feathers were denominated. TG-0.2, TG-1 and TG-5 were composed of 0.2, 1, and 5 g of TG-Feathers per 100 mL of water, respectively. MU-0.2, MU-1 and MU-5 were composed of 0.2, 1, and 5 g of MU-Feathers per 100 mL of water, respectively.

The indicated amounts of feathers were introduced into Erlenmeyer flasks and the final volume made up to 300 mL. The feather media were inoculated with 2% inoculum of *B. licheniformis* grown in rich medium (LB) and incubated at 37 °C in an orbital shaker at 200 rpm.

2.5. Dehydrogenase activity (DHA)

Dehydrogenase activity was determined using 0.5 mL of fermentation broth and the reduction of 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride (INT) to p-iodonitrotetrazolium formazan (INTF) was measured by a modified INT assay [9]. Activity was expressed in units, where one unit corresponds to the release of 1 nmol of INTF min^{-1} mL^{-1} protein under the assay conditions.

2.6. Protease assay

Total extracellular protease activity was determined as described by Beynon and Bond [10]. Briefly, 0.5 mL of azocasein 1% (w/v) in 0.1 M phosphate buffer (pH 7) was mixed with 0.5 mL of sample. This was incubated for 10 min at 40 °C. The reaction was terminated by adding 2.5 mL of 5% (p/V) TCA solution. The reaction mixture was centrifuged, and the absorbance of the supernatant at 440 nm was measured. One unit of proteolytic activity was defined as the amount of enzyme required to produce an increase in optical density of 0.001.

2.7. Lipase assay

Total extracellular lipase activity was determined using a modification of the method described by Kilcawley [11]. In brief, 1.75 mL of buffer (0.1 M sodium phosphate pH 7; 0.15 M NaCl and 0.5% (v/v) Triton-X) was mixed with 0.25 mL of sample, previously centrifuged for 30 min at 12,000 × *g*, and 20 μL of 50 mM p-nitrophenol laurate in acetonitrile. This mixture was incubated for 30 min at 37 °C. The reaction mixture was centrifuged for 10 min at 7500 × *g*, and the absorbance of the supernatant at 400 nm was measured. Activity was quantified using the molar extinction coefficient of p-nitrophenol (14,800) at 400 nm. Activity was expressed in units, where one unit corresponds to the release of 1 nmol of p-nitrophenol h^{-1} mL^{-1} protein under the assay conditions.

2.8. Proteomic study

Samples were centrifuged at 14,000 × *g* for 20 min, and the supernatants were recovered. We centrifuged to remove contaminants such as cells and proteins in suspension. A total of 10 mL of sample was taken, and 40 mL of methanol was added and vortex mixed for about 1 min. Then 10 mL of chloroform was added and the mixture was shaken again for 1 min. Next, 30 mL of Milli-Q water was added and mixed well.

The mixture was centrifuged at 14,000 × *g* for 5 min and the supernatant was removed. Then 30 mL of methanol was added and the mixture was vortexed again for 1 min.

Samples were centrifuged at 16,000 × *g* for 5 min, the supernatant was discarded and the pellets dried in an oven. Finally, the proteins were resuspended with Milli-Q water. A total of 30 μg of protein was resuspended in 30 μL of 6 M urea, 200 mM ammonium bicarbonate and 10 mM DTT and left for 30 min at room temperature for protein reduction. Then, 10 μL of 100 mM iodoacetamide was added to protein alkylation for 30 min at room temperature in the dark. Samples were diluted with Milli-Q water until the urea concentration was below 1 M, and then trypsin (Promega) was added in a protein with an enzyme ratio of 50:1. Digestion was carried out at 37 °C overnight, and the mixture was then acidified with TFA and concentrated using a Speed C18/18 column (Applied Separations, USA). Peptides were eluted in 400 μL of 70% acetonitrile 0.1% TFA. After samples were completely dried down, they were resuspended in 15 μL of 5% acetonitrile 0.1% formic acid for tandem liquid chromatography–mass spectrometry (LC–MS) analysis.

LC–MS analysis was performed in a Surveyor HPLC system in tandem with a Finnigan LTQ mass spectrometer (ThermoFisher Scientific, USA). A total of 5 μL of sample was injected into a C18 PepMap100 μ-Precolumn Cartridge (Dionex, Netherlands) for preconcentration and washing, then resolved in a Biobasic C18 75 μm × 10 cm column (ThermoFisher Scientific, USA). Peptides were eluted with a 120-min gradient of 5% acetonitrile with 0.1% formic acid to 40% acetonitrile with 0.1% formic acid, at a nominal post-split flow rate of 250 μL min^{-1}. The LTQ was run in positive ion mode using the nanospray source. The spray voltage was set at 2 kV, and the capillary temperature was set at 170 °C. The samples were scanned in the range of 400–1500 *m*/*z* using the Full Scan mode, and Data Dependent MS/MS on the top five ions with CID was carried out with Dynamic Exclusion set to on.

The data was converted to SEQUEST format (DTA) and searched using an in-house MASCOT (Matrixscience, UK) search engine against the NCBI database (version 11/10/2007) with taxonomy restrictions set to Firmilicutes and carboximethylated cysteine as fixed modification.

3. Results and discussion

Process optimization is a topic of central importance in industrial production processes. In fermentation technology, improvements in the productivity of the microbial metabolite are achieved, in general, via the manipulation of nutritional and physical parameters and by strain improvements as the result of mutation selection.

In this study, it will be evaluated whether the use of feather waste as the culture medium affects the excretion of enzymes by *B. licheniformis* as well as the overall proteomic excretion.

3.1. Feather chemical characterization

Feathers mainly consist of protein, which is mostly keratin, a fibrillar protein insoluble in water and saline solutions, rich in sulfur and relatively resistant to degradation. Lipids and carbohydrates are secondary components. The chemical composition of feather wastes can vary slightly depending on several factors, including poultry species, age, type of feeding and the industrial processing system [12].

Table 1
Chemical composition of feathers.

Type of feather	TG-Feather	MU-Feather
Carbon (%)	46.33 ± 0.14	55.02 ± 0.17
Nitrogen (%)	14.14 ± 0.03	9.79 ± 0.07
Sulfur (%)	1.91 ± 0.02	1.11 ± 0.03
C/N relation (%)	3.28	5.62
Protein (%)	91.90 ± 0.20	63.60 ± 0.46
Carbohydrate (%)	6.50 ± 0.35	7.30 ± 0.62
Fat (%)	2.00 ± 0.15	28.10 ± 0.23

In our study, two types of feather have been used to determine the influence of their chemical compositions on protein excretion by *B. licheniformis*.

Knowledge of the different chemical compositions of both types of feathers allows us to evaluate the influence of the composition of the medium on the production of exocellular enzymes by the microorganism.

Both types of poultry feathers chosen are within the limits of chemical diversity. Table 1 shows the chemical composition of both products. TG-Feather is a classical keratinous waste with high nitrogen (14.14%) and sulfur content (1.91%) due to the fact it is mainly composed of keratin (91.90% of dry matter), with a low lipid content (2%).

MU-Feather is the other poultry waste used. This feather has a greater diversity in terms of its chemical composition than the TG-Feather. It has lower protein content, with a keratin content of 63.60%, but it has a much higher fat content, equalling approximately 30% of the total composition.

As a result of its lower protein content, the MU-Feather also has less nitrogen and sulfur content, but on the other hand, it has more carbon directly related to the fat content than do TG-Feathers.

The high lipid component is what differentiates MU feathers from TG-Feathers and from the different feathers used in the literatures [13,14,15]. Therefore, this data should be considered as a possible determinant cause of the differential growth and exocelular proteomic excretion profile of *B. licheniformis*.

3.2. The feather fermentation process

The aerobic growth of *Bacillus* using feathers as a sole source of carbon, nitrogen, and sulfur leads to the complete solubilization of keratinous material, which may be due to hydrolytic enzyme excretion. Thus, both types of feathers (MU and TG) were used at different concentrations (0.2, 1, and 5%, w/v) without any external C, N, or mineral supplementation.

In the process of fermentation, *B. licheniformis* growth led to the degradation of the feathers, which could be due to the breakdown of keratins and other biomolecules, caused by the excretion of hydrolytic enzymes by *B. licheniformis.*

Feather solubilization was inversely related to feather concentration. Thus, after 5 days of fermentation, the insoluble solid feathers disappeared at low concentrations (0.2 and 1%), meaning that keratin protein was totally hydrolyzed (100%).

However, at higher feather concentrations (5%), the solubilization process was incomplete. Furthermore, the lack of solubilization at high concentrations was also influenced by feather composition. Accordingly, the MU-Feather, with high lipid content, was less susceptible to solubilization, with a solubilization of 43%, than the TG-Feather, with a solubilization of 65%, thus 35% of the feather weight remaining water insoluble.

The different rates of solubilization linked to the fermentation media concentrations could be due to a lack of enzymatic hydrolytic cellular excretion or minimal bacterial growth, resulting in a greater amount of insoluble feather material.

Table 2
Maximum microbial activity found during the fermentation of *Bacillus licheniformis* in different media (LB and feather).

Medium		Concentration (%)	Time (h)	Microbial activity (DH units)
LB		–	18	6.980 ± 0.342
Feather	MU	0.2	–	[a]
		1	18	1.772 ± 0.186
		5	42	9.524 ± 0.274
	TG	0.2	–	[a]
		1	90	2.400 ± 0.054
		5	168	12.192 ± 0.498

The microbial activity is measured as dehydrogenase enzymatic activity (DH units).
[a] No DH activity peak was detected.

In this work, we studied the relationship between microbial growth and keratin solubilization, evaluating the microbial activity present in the fermentation medium. *Bacillus* growth was not tested in a conventional way due to biofilm formation on the surface of insoluble keratins. We therefore quantified the microbial activity by measuring dehydrogenase activity. Dehydrogenase is an intracellular enzyme related to the oxidative phosphorylation process [16] that is considered to be one of the most important indicators of overall microbial activity.

The magnitude of *Bacillus* growth was therefore assessed by measuring the level of dehydrogenase activity (Table 2). The emergence of microbial activity in LB medium was faster than in both feather media, due to the high availability of nutrients in LB (peptides, free amino acids, soluble carbohydrates, etc.). Microbial activity peaked at 18 h and then steadily declined due to a depletion of nutrients.

The maximum microbial activity peaks in the two feather media occurred at different times. In the TG-Feather (1%, w/v), this occurred at 42 h, and in the MU-Feather, at 90 h. The delay observed between the two feather media may be due to the presence of fat, which could prevent the feather surface colonization by *Bacillus*.

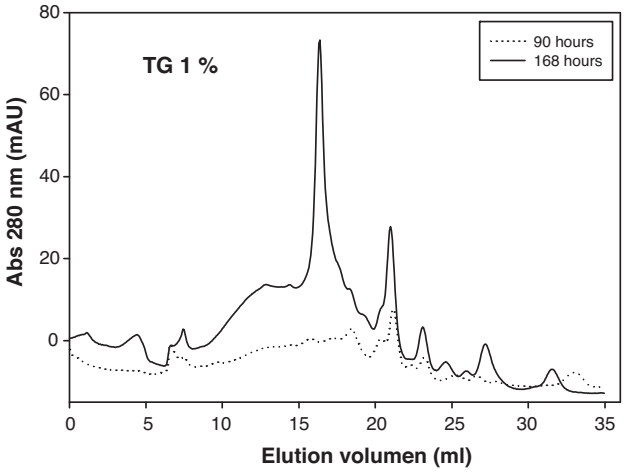
The dehydrogenase activity data were not consistent with the degree of keratin solubilization, suggesting that this process depends more on levels of hydrolytic activity than on the microbial activity. Moreover, the excretion of the main hydrolytic enzyme such as protease, is not directly related to the microbial growth, but it is inducible by environmental fermentation conditions.

Microbial activity was directly related to feather concentration. In concentrations of 0.2 and 1%, little basal activity was observed, but at higher concentrations of 5%, we observed strong microbial activity. This activity was higher in the MU-Feather than in the TG-Feather, which could be due to the nutrient composition diversity of the former.

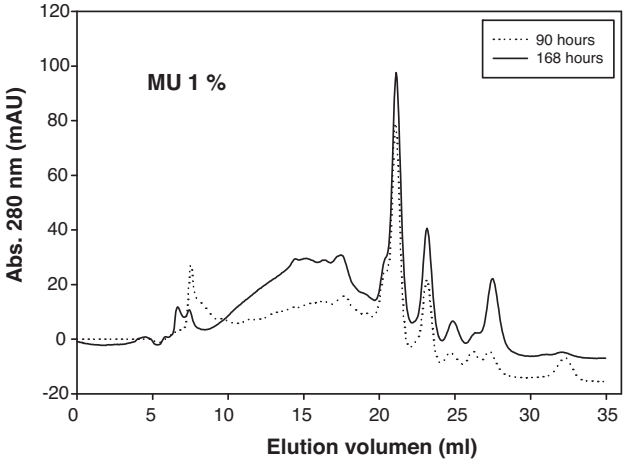
Moreover, we also studied the product conversion of keratins during the fermentation process. Fig. 1 shows the protein molecular weight distribution of soluble proteins present in the fermentation broth based on TG- and MU-Feathers (1%, w/v), evaluated by molecular exclusion chromatography. This data shows that the solubilization of the feather is connected to the conversion of keratins into peptides and amino acids.

However, a different pattern is observed in the solubilization of keratin, as is shown in Fig. 1. When we analyzed the percentage of molecular weight fractions during fermentation (90 and 168 h), soluble proteins of fermented MU-Feather showed a smaller molecular size than the TG-Feather.

The MU-Feather proteins underwent a more intense process of hydrolysis during fermentation than did the TG-Feather proteins, which is probably due to the differential expression of hydrolytic enzymes both in terms of quantity and diversity.



Molecular size (Daltons)	90 hours (%)	168 hours (%)
> 10.000	56.29	23.77
10.000 – 5.000	0.00	9.06
5.000 – 1.000	1.05	25.14
1.000 - 300	0.45	8.04
< 300	42.21	33.99



Molecular size (Daltons)	90 hours (%)	168 hours (%)
> 10.000	39.35	21.61
10.000 – 5.000	0.00	2.84
5.000 – 1.000	2.02	5.78
1.000 - 300	0.83	1.37
< 300	57.79	68.40

Fig. 1. Size-exclusion chromatography and molecular weight distribution of fermentation broth (feathers at 1%) at different fermentation times, on Superdex™ peptide 10/300GL high performance column (GE Healthcare, US). Abs: absorbance; EV: elution volume (mL).

3.3. Enzyme production

3.3.1. Proteases

Proteases form an important class of commercial and industrial enzymes. *B. licheniformis* has been described as a protease producer and is well-suited for the production of industrial enzymes [15,17,18]. The production of proteases by bacilli, using feathers, has been extensively described [8,13,19].

It is clear in our results, shown in Fig. 2, that feathers are a suitable medium for protease production. Enzyme excretion was indirectly related to the substrate concentration, and excess feather concentration inhibits the production and excretion of protease by *Bacillus*. Accordingly, high feather concentrations (>1%) adversely affected the production of protease in both types of feather, and relatively low feather concentrations (0.2–1%, w/v) resulting in maximum protease production (see Fig. 2).

In a full, rich and nutritionally balanced medium (LB medium), *Bacillus* grew quickly. In contrast, protease excretion activity was low and not directly associated with biomass production.

It is widely reported that *Bacillus* produces proteases as a response to the depletion of nutrients in the medium [5,20,21]; starvation and protease production are thus correlated. Accordingly, low levels of nutrients give the highest yield of protease, and substrate concentration is therefore the main parameter controlling protease production.

It is important to point out that feathers with greater fat content show higher protease production. It could also be assumed that protease expression could be induced by the presence of a substrate with a higher carbon/nitrogen ratio.

3.3.2. Lipase production

Lipases (E.C. 3.1.1.3) constitute a group of enzymes with the ability to hydrolyze triacylglycerols at the lipid–water interface. They

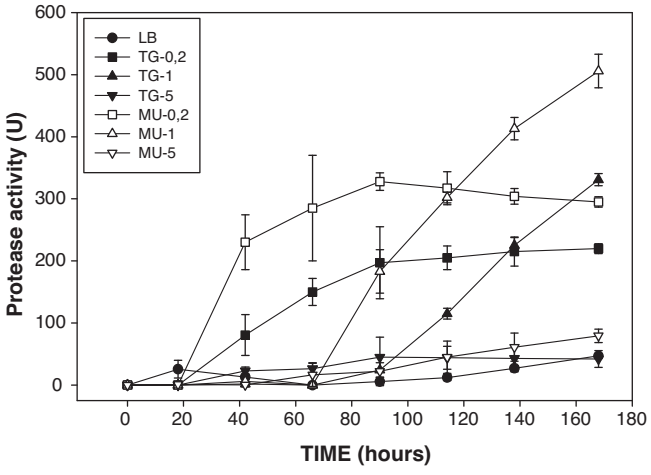


Fig. 2. Protease produced by *Bacillus licheniformis* using both types of feathers and LB medium over the course of fermentation (mean ± SD). Number of replicates: 3.

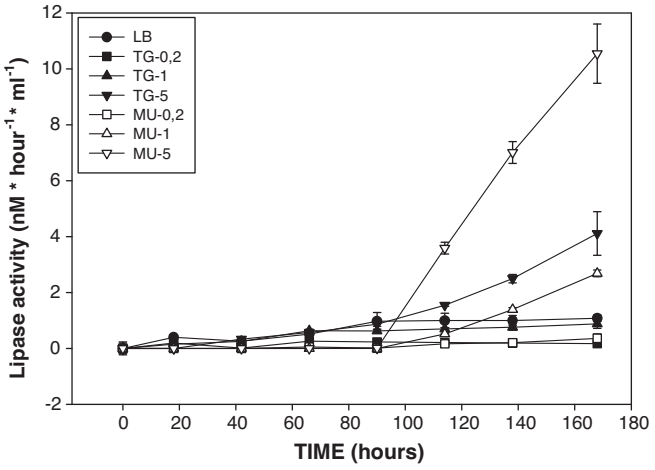


Fig. 3. Lipase produced by *Bacillus licheniformis* using both types of feathers and LB medium over the course of fermentation (mean ± SD). Number of replicates: 3.

constitute a special group of enzymes of industrial interest for use as biocatalysts for various chemical processes. Isolates of *Bacillus* species, such as *B. subtilis*, *B. pumilus* and *B. licheniformis*, have been described as lipolytic enzyme producers [22,23]. In our study on the fermentation of *B. licheniformis*, we tested the production of lipases in both types of feathers as well as in a rich medium (LB). We can thus compare the influence of the composition of the medium on the excretion of lipase enzymes.

The results show that the feathers may be an appropriate substrate for lipase production. In addition, there was a direct relationship between substrate concentration and enzyme secretion (Fig. 3). Maximum lipase secretion was obtained at the highest concentration of feathers, regardless of the type of feather used.

In LB medium, basal excretion was lower than or similar to that obtained with feathers media, having less concentration. Unlike what happened with the production of proteases, here there was a direct relationship between the concentration of the residue and the amount of secreted enzyme: so that greater concentrations enhanced the induction of the enzyme. It is noteworthy that, contrary to what happened in the measurement of protease activity, the highest index of lipase activity occurred at 5% feather concentration.

Fig. 3 clearly demonstrates that *Bacillus* growing on the MU-Feather shows higher lipase production than it does on the TG-Feather. We therefore assumed that the higher lipid content (Table 1) in the MU-Feather could induce lipase secretion.

If we compare the protease and lipase production graphics, it is remarkable that in both cases, there was greater enzyme excretion in the MU-Feather medium. It could be assumed that a higher concentration of carbon source and the synthesis of more enzymes can induce an over-expression of lipase [24,25]. Again, it is clear that the difference in feather composition influences exoenzyme excretion by *B. licheniformis*. However, it was remarkable that in LB medium, there is a basal presence of lipase production.

3.4. Proteomics: protein expression in different types of feathers

In this study, we used a proteomic approach to determine the protein cellular excretion by *B. licheniformis* when different types of feathers are used as the grown substrate (concentration of 1%) in order to characterize the secretory response compared with that in an LB medium.

In the presence of a culture medium like feathers, *B. licheniformis* is capable of excreting various kinds of enzymes in order to degrade the components of the medium and thus obtain nutrients for survival [2,26,27,28].

Most *Bacillus* sp. initiate a series of transitional responses that are designed to maintain or restore growth, including the induction of macromolecular hydrolases, such as proteases, lipases and polysaccharidases [26], under different environmental conditions. Enzymatic induction by substrate has been described in *Bacillus*, thus xylane induces the excretion of xylanases and other glycoside hydrolases [29] and substrates formulated with olive oil, which is an important inductor and source of lipids, to induce the synthesis and excretion of *Bacillus* lipases [26].

The results obtained in this study show that feathers allow for the growth of *Bacillus* and also act as a strong inducer of the secretion of hydrolytic enzymes by the microorganisms.

As previously mentioned, both types of feathers are capable of supporting *Bacillus* growth and both are good inducers of the enzymatic hydrolytic excretion.

Thus, in feather media *B. licheniformis* secreted different enzymes, including biopolymers such as proteins (keratins), fat and carbohydrates, into the extracellular medium for the purpose of degrading feathers, in order to obtain nutrients and energy.

The proteins characterized by mass spectrometry in the fermentation media after obtaining complete solubilization of the feathers are shown in Table 3.

The TG-Feather was the simplest medium used, consisting mainly of proteins (keratins 91.90%). The analysis of exocellular protein shows a relatively low diversity of proteins compared to that found in MU-Feathers and the LB fermentation media. The main protein was in the TG-Feather, a protease, keratinase (Ker A). This enzyme is typically produced from the feather-degrading bacterium *B. licheniformis*. In addition to promoting the hydrolysis of feather keratin, keratinase is capable of hydrolyzing a broad range of protein substrates and, therefore, has many potential agricultural and industrial applications.

Ggt (Gamma-glutamyltranspeptidase E.C. 2.3.2.2) was another minor protein found. This protein catalyzes the transfer of the γ-glutamyl moiety from γ-glutamyl compounds to a variety of amino acids and dipeptide acceptors [30,31]. The associated hydrolytic and glutaminase activities are used in the food industry for debittering amino acids. Ggt has also been described as assisting subtilisin during its action on keratin [32].

B. licheniformis was therefore found to secrete extracellular Ggt and Keratinase during feather degradation. Keratinase in the presence of Ggt exhibits an extended substrate spectrum as it cleaved not only α-keratin of feather but also α-keratin of hooves and nails. The complex thus exhibits better catalytic properties and can be exploited in various biotechnological applications [33].

Data also show that *B. licheniformis* expresses some of the exoenzyme genes and secretes the corresponding hydrolyzing enzymes such as glycoside hydrolase. Its excretion is due to the obtention of simple sugars from carbohydrates found in the feathers.

Besides all the proteins described, there is another protein that was strongly secreted in the three mediums, the hypothetical protein BL00275. Information about its function was found in protein databases.

We also investigated the extracellular protein pattern of *B. licheniformis* cells grown in the MU-Feather, which is a richer carbon source (55.02%) than the TG-Feather, but has a lower concentration of proteins (63.6%).

Under these conditions, *B. licheniformis* was found to secrete a greater diversity of proteins in the Mu-Feather than does the TG-Feather medium, but in both media, proteases were the main proteins excreted (data shown in Table 3). Keratinase (KerA) was the main protein produced in both the TG-Feather and the MU-Feather. In both media, we also found the associate enzyme Ggt (Gamma-glutamyltranspeptidase E.C. 2.3.2.2).

Table 3
Protein identification of extracellular proteins in *B. licheniformis* growing in different feathers, TG-Feather and CEBAS-Feather, by MASCOT (Matrixscience, UK) searching engine over NCBI database.

	Mass (Da)	Score	Queries matched	emPAI[a]	Protein name
TG-Feather	31,266	441	14	0.49	KerA [*Bacillus licheniformis*] Subtilisin precursor [*Bacillus licheniformis*]
	66,111	270	7	0.16	Glycoside hydrolase family 18 protein [*Bacillus licheniformis*]
	64,048	193	4	0.10	Gamma-glutamyltranspeptidase (Ggt) [*Bacillus licheniformis*]
	14,050	166	8	0.91	Hypothetical protein BL00275 [*Bacillus licheniformis*]
MU-Feather	31,266	640	18	1.02	KerA [*Bacillus licheniformis*] Subtilisin precursor [*Bacillus licheniformis*]
	435	468	16	0.44	Chain A, Chitosanase [*Bacillus sp.*]
	79,565	419	9	0.22	Glycoside hydrolase family protein [*Bacillus licheniformis*]
	66,111	273	5	0.10	Glycoside hydrolase family 18 protein [*Bacillus licheniformis*]
	64,048	221	3	0.10	Gamma-glutamyltranspeptidase (Ggt) [*Bacillus licheniformis*]
	3201	218	5	0.21	Flagellin [*Bacillus licheniformis*]
	85,573	184	5	0.08	Extracellular serine protease [*Bacillus licheniformis*]
	14,050	173	9	0.24	Hypothetical protein BL00275 [*Bacillus licheniformis*]
	73,549	109	2	0.04	Putative acylaminoacyl-peptidase YuxL [*Bacillus licheniformis*]
LB	33,201	910	72	7.87	Flagellin [*Bacillus licheniformis*]
	50,616	348	11	0.37	Chitosanase [*Bacillus* sp.]
	33,888	232	7	0.45	Intracellular serine protease[*Bacillus licheniformis*]
	64,048	222	6	0.22	Gamma-glutamyltranspeptidase (Ggt) [*Bacillus licheniformis*]
	22,530	187	11	0.51	Superoxide dismutase [*Bacillus licheniformis*]
	17,909	157	4	0.19	Metalloregulation DNA-binding stress protein [*Bacillus licheniformis*]
	31,266	123	5	0.11	KerA [*Bacillus licheniformis*] Subtilisin precursor [*Bacillus licheniformis*]
	24,831	122	4	0.29	Metal-dependent hydrolase [*Bacillus licheniformis*]
	14,050	166	8	0.91	Hypothetical protein BL00275 [*Bacillus licheniformis*]
	19,722	116	3	0.17	Chitosanase [*Bacillus* sp.]
	66,111	81	2	0.10	Glycoside hydrolase family 18 protein [*Bacillus licheniformis*]

[a] Exponentially modified protein abundance index.

The second most important protein produced quantitatively was a chitosanase. Chitosanases (EC 3.2.1.132) are endo-hydrolytic enzymes acting on internal glycosidic bonds within the biopolymer chains thereby releasing low molecular weight oligomers. There was no chitosan in the chemical composition of the feathers, thus the secretion of this enzyme could be due to a dual and unspecific activity of lipase.

This dual activity has been attributed to the similarity of the active sites of both enzymes chitosan and lipase [34,35]. It is known that lipases of various origins depolymerize chitosans [36,37,38]. The nonspecific activity is justified by the simplicity of the enzymatic hydrolytic mechanism and, once again, the similarity of the active sites of both enzymes [39,40].

Data also show that *B. licheniformis* expresses some of the exoenzyme genes and secretes the corresponding hydrolyzing enzymes, such as glycoside hydrolase family proteins, when a carbon source is present in the medium.

Therefore, a proteomic analysis of the fermentation broth suggests that the majority of the proteins identified tend to be exocellular enzymes. However, there were also proteins that were secreted regardless of the growth conditions, such as flagellin (Hag).

Furthermore, most of the proteases were secreted in response to all conditions, although not to the same extent. KerA was the main protease secreted by *B. licheniformis* in both by-products, but the MU-Feather induced the generation of other proteases such as extracellular serine protease and putative acylaminoacyl-peptidase.

The presence of a greater diversity of proteases leads to greater diversity in the breaking of peptide bonds, which could be the reason why the hydrolysis products found in the fermentation broth of the MU-Feather medium are smaller in size (see Fig. 1).

The growth of *B. licheniformis* on an enriched medium like LB as a standard control, showed a wide variety of secreted extracellular proteins and cell wall-associated proteins (Table 3). Few of these proteins associated with flagellum, flagellin and Hag [41,42] were secreted at baseline levels. However, the secretion of certain proteases, such as KerA and Ggt, were secreted in all types of culture media.

The present work, carried out on different kind of feathers, clearly demonstrates the influence of the chemical composition of the medium on the excretion protein profiles of the microorganism.

4. Conclusions

We have established the feasibility of using organic waste from the poultry industry as an efficient fermentation medium to produce hydrolytic enzymes as proteases and lipases. To conclude, our study shows the influence of the chemical composition of feathers on protein secretion by *B. licheniformis*. Our proteomic approach enabled us to identify all proteins secreted by *B. licheniformis* using different types of feathers as the substrate and to establish the influence of the chemical composition of the medium on enzymatic secretion.

Acknowledgements

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Hydrolytic enzymes production by *Bacillus licheniformis* growth on fermentation media formulated with sewage sludge

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In this work, the production of hydrolytic enzymes by *Bacillus licheniformis* grown in media formulated with sewage sludge as the main carbon and nitrogen source with induction by keratins has been studied. The three main types of enzymes of industrial interest produced were proteases, lipases and cellulases. The addition of an inductor, keratin in our case, improves the production of these enzymes to reach a productivity of 16.89 mU/mL·day, 0.25 mU/mL·day, and 0.51 mU/mL·day for proteases, lipases and cellulases, respectively. The secretion of proteins and enzymes into the fermentation media was studied by electrophoretic and proteomic methods, which revealed the presence of proteases, lipases, and cellulases in the fermentation media, among other excreted proteins. Our results show that the growth of *B. licheniformis* in fermentation media formulated with sewage sludge as the main carbon and nitrogen sources, supplemented with keratin from feather meal as an inductor could be used for the industrial production of these enzymes, particularly of proteases.

Keywords: Hydrolytic enzymes; *Bacillus licheniformis*; sewage sludge; proteases; lipases; cellulases.

Abbreviations: GRAS: generally regarded as safe; CDDGs: corn dried distillers grains with solubles; TCA: trichloroacetic acid; DNS: dinitrosalicylic acid; EA: enzymatic activity; p-NPG: p-nitrophenyl-β-glucopyranoside; MUB: Modified Universal Buffer; CBB: Coomassie Brilliant Blue.

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Introduction

Industrial application of enzymes began in the mid-sixteen centuries, although its stable implementation was not achieved until the mid-twenty centuries, between 1950-1960 [1]. The main industrial applications of enzymes are in the production of food, textiles and detergents, representing approximately 90% of the market volume. Other applications, such as organic synthesis, medical and pharmaceutical applications, agronomic uses, etc., are much less used [2]. However, recently, the application of enzymes in agricultural and environmental applications has experienced a significant increase, mainly due to the development of new production methods using cheap culture media derived from agricultural products and urban residues [3].

Most industrial enzymes are bacterial enzymes, although fungal enzymes have lately become increasingly important, both quantitatively and qualitatively. Bacterial enzymes produced by the genus *Bacillus* are of special interest for both research and industrial applications due to the

high rate of growth of these bacteria and their ability to secrete a high volume of enzymes into culture medium (20-25 g/L) [4]. In addition, these microorganisms are "generally regarded as safe" (GRAS). The *Bacillus* strains considered of industrial interest are the species *B. licheniformis* and *B. subtilis*.

One of the major bottlenecks for the industrial application of enzymes is the high production cost, while the cultivation medium may represent 30-40% of total production costs [5]. This has produced great interest in the development of efficient and cheap cultivation media. Among the media developed to address this issue, those derived from agroindustrial byproducts (whey, brewers grains, rice bran, defatted sunflower flour, and corn dried distillers grains with solubles (CDDGs)) and urban wastes (water and sewage sludge) are two types of promising substrates [6]. In addition to the economic benefit associated with the use of agroindustrial byproducts and urban waste, another important advantage is the resulting contribution to environment maintenance [7, 8]. In industrialized countries, the high rates of production of household and industrial liquid waste results in a high polluting effect [9, 10]. The treatment of these wastes in depuration plants generates two main products: purified water and sewage sludge. While the purification of water has received great attention, much less attention has been devoted to the management of sewage sludge, which is a dangerous source of environmental pollution if not treated properly. However, it has recently been shown that sewage sludge, when adequately processed, may serve as a useful substrate in the treatment and regeneration of soil and/or as an inexpensive cultivation media for the production of microorganisms and enzymes [11]. Sewage sludge is one of the most abundant and inexpensive substrates available for the culture of microorganisms and has practically zero cost.

Sewage sludge may be processed to modify and/or adjust its physical, chemical, and biological properties to yield acceptable material for use as fertilizer and/or culture medium for the production of substances of high added value [12, 13]. Currently, sewage sludge is transformed mainly into bio-fertilizer, while only a small portion is used for the production of high value-added products such as enzymes. The goal of this work was to study the use of sludge from water treatment plants as a cultivation medium for the production of hydrolytic enzymes (proteases, cellulases, lipases, acid phosphatase, and β-glucosidase) using *Bacillus licheniformis*.

Materials and methods

Microorganisms

Bacillus licheniformis strain 21415 provided by the American Type Culture Collection (ATCC) (Manassas, VA, USA) was used for the production of enzymes in this study. The bacteria were stored frozen at -80˚C and refreshed in LB medium (10 g/L peptone, 5 g/L yeast extract, and 10 g/L NaCl, adjusted to pH 7) 24 h before inoculation. Briefly, after thawing at 4˚C overnight, 2 mL of bacteria was inoculated into a flask containing 30 mL of LB medium at pH 7 and 37˚C, and then grown until the transmittance at 620 nm reached 20%.

Substrates: Sludge samples, medium composition, and fermentation

Sludge samples were provided by the experimental treatment plant of Carrión de los Céspedes (Sevilla, Spain) of the CENTA Foundation. Thickened sludge with a humidity of 90±1.5% was used both alone and in the preparation of cultivation medium (table 1). Culture medium was sterilized at 121˚C for 30 min prior to inoculation to eliminate pre-existing sludge bacteria. Six hundred mL sterile media was inoculated with 30 mL inoculums and grown over 14 days at 37˚C with constant agitation (150 rpm). During growth, 10 mL samples were taken each day and stored at -80˚C until use for measurement of biomass and enzymatic activities, including protease, cellulase, lipase, acid phosphatase, and β- glucosidase.

Table 1. Compositions of culture media formulations.

Media	Sludge (g)	Inductor (g) Feathers	Inoculum (mL) *B. licheniformis*
M1	600	0	0
M2	600	0	30
M3	588	12	30

Sample preparation

Samples were thawed at 4˚C and either used directly for biomass determination or centrifuged at 12,000 × g for 20 min to obtain the supernatant used for enzyme activity assays.

(1) Determination of biomass.

Biomass was determined indirectly by measuring the protein content of samples at different growth times using the Bradford method [14]. Biomass concentration was determined in µg/mL by interpolation from a standard curve plotting biomass (µg/mL) against protein concentration (µg/mL) (Figure 1).

(2) Enzymatic assays

The supernatant obtained after centrifugation was used for determination of enzyme activities.

Protease activity assay

The total extracellular protease activity was determined following the method described by Beynon and Bond [15]. Briefly, 250 µL of the sample was mixed with 250 µL of a reaction mixture containing 0.1 g azocasein and 0.2 mL of ethanol dissolved in 4.8 mL of 0.1 M phosphate buffer at pH 7. Samples were then incubated for 10 min at 40˚C. The reaction was then terminated by adding 2.5 mL of 5% (w/v) TCA solution. The reaction mixture was centrifuged at 10,000 × g for 2 min. The absorbance of the supernatant at 440 nm was measured. One unit of proteolytic activity was defined as the amount of enzyme required to produce an increase in optical density of 0.001.

Cellulase activity assay

The total cellulase activity was determined by the method described by Galindo [16], modified for application to cellulolytic enzymes. Briefly, 250 µL of a 2% soluble microcrystalline cellulose (Avicel PH-101, Sigma- Aldrich, Barcelona, Spain) solution in 0.1 M acetate buffer (pH 5) was incubated with 250 µL of the sample for 2 hours at 37˚C. The reaction was stopped by adding 1 mL of DNS reagent, and the mixture was heated to 95˚C for 10 min. The reaction mixture was then cooled to room temperature and the absorbance at 575 nm was measured. A standard curve was obtained using glucose for used in the calculation of cellulase activity. One unit of cellulase activity was defined as the amount of enzyme required to liberate 1 mg/mL of reduced sugars into the test solution.

Lipase activity assay

The total extracellular lipase activity was determined using a modified version of the method described by Kilcawley et al. [17]. Briefly, 1.75 mL of buffer (0.1 M sodium phosphate pH 7, 0.15 M NaCl, and 0.5% (v/v) Triton-X) was mixed with 0.25 mL of sample that had been previously centrifuged for 30 min at 12,000 × g, along with 20 µL of 50 mM p-nitrophenol laurate in acetonitrile. This mixture was then incubated for 30 min at 37˚C. Afterward, the mixture was cooled in an ice bath for 5 min, then centrifuged for 1 min at 9,000 × g. The supernatant absorbance at 400 nm was measured. Activity was quantified using the molar extinction coefficient of p-nitrophenol (14,800 /M·cm) at 400 nm. The activity units were defined as one unit corresponding to the release of 1 nmol of p-nitrophenol/min·mg protein under the test conditions.

β-glucosidase activity assay

The β-glucosidase activity was determined using the colorimetric method described by Eivazi and Tabatabai [18] with slight modifications. Into 250 µL of the sample, 2 mL of modified universal buffer (MUB, pH 6) and 0.5 mL of p-nitrophenyl-β-glucopyranoside 25 mM (7.53 mg/mL) were added. The mixture was then incubated at 37˚C for 30 to 60 min. The reaction was stopped by placing the mixture in an ice bath. Subsequently, 2 mL of 0.5 M NaOH was added and the solution absorbance at 400 nm was measured. The

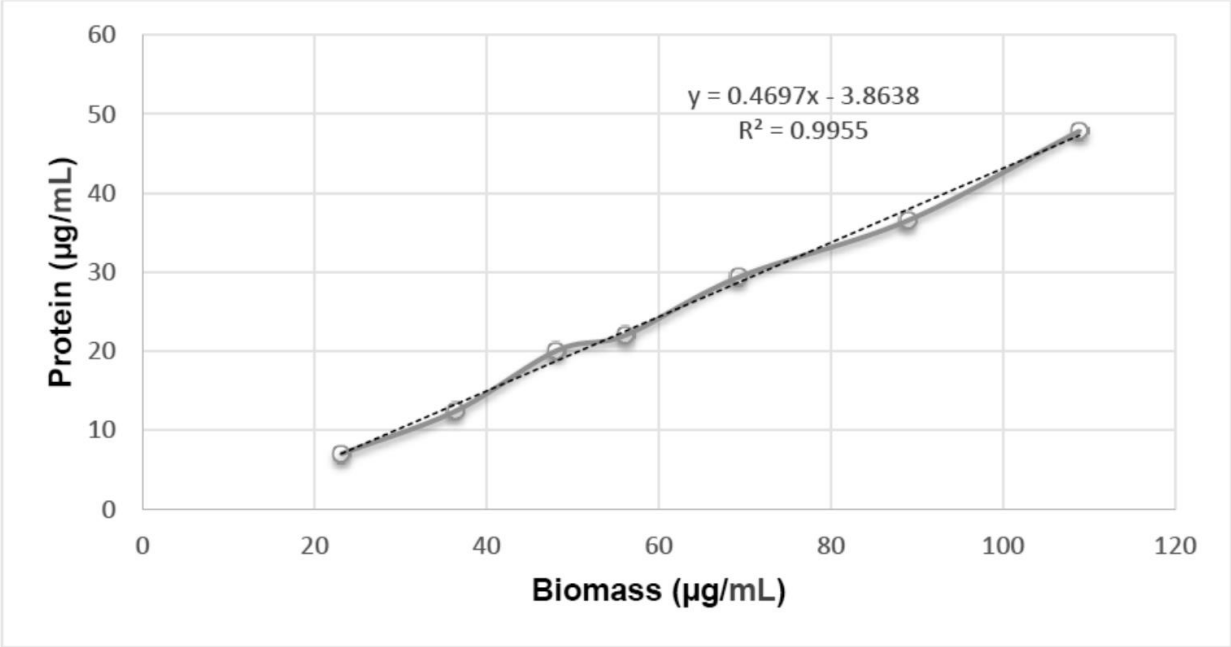

Figure 1. Standard curve of biomass versus protein concentration for *Bacillus licheniformis* grown in a defined medium.

enzymatic activity (EA) was expressed in µmol/g of sample·min and was calculated by the following formula:

$$EA = C \times V / T \times G$$

where C is the concentration of liberated p-nitrophenyl (mM); V is the sample volume (mL); T is the incubation time (min); and G is the sample weight (g). One β-glucosidase unit of activity was defined as the amount of β-glucosidase needed to release 1 nmol p-nitrophenol from p-nitrophenyl-β-glucopyranoside in 1 min under the specified conditions.

Acid phosphatase activity assay (APHA)

Acid phosphatase activity was determined by the method established by Tabatabai and Bremmer [19] with slight modifications. The procedure was initiated by creating a mixture containing 250 µL of sample, 2 mL of MUB, pH 6, and 0.5 mL of p-nitrophenyl-phosphate 25 mM (9.28 mg/mL). This mixture was incubated at 37˚C for 30-60 min. Afterward, the samples were cooled in an ice bath to stop the reaction. Then, 2 mL of 0.5M NaOH was added to the sample and the solution absorbance at 400 nm was measured. The EA was expressed in µmol/g of sample·min and was calculated by the following formula:

$$EA = C \times V / T \times G$$

where, C is the concentration of liberated p-nitrophenyl (mM); V is the sample volume (mL); T is the incubation time (min); and G is the sample weight (g). One phosphatase unit of activity was defined as to the amount of acid phosphatase needed to release 1 nmol p-nitrophenol from p-nitrophenyl-phosphate in 1 min under the specified conditions. Spectrophotometric detection of the artificial molecule p-nitrophenol manifesting yellow as a product of acid phosphatase activity was used to quantify the enzyme activity in these samples.

Protein assay

Protein concentration was quantified by the method described by Bradford, using bovine serum albumin as the protein standard [14].

SDS-PAGE protein profiling

The extracellular protein profile was investigated by SDS-PAGE analysis. After acid-acetone

precipitation of the supernatant obtained after fermentation broth centrifugation at 12,000 x g, the pellet was solubilized in sample preparation buffer (1 mM Tris–HCl, pH 6.8) containing 5% (w/v) SDS and 7% (v/v) β-mercaptoethanol. The samples were kept at room temperature for 2 h and then centrifuged at 12,000 × g for 15 min. The supernatant was collected and run on 12% SDS-PAGE gel in triplicate, with each run containing 20 µL of supernatant (approximately 50 µg of protein) and 5 µL of loading buffer (0.5 M Tris-HCl, pH 6.8, 50% (v/v) glycerol, 10% (w/v) SDS, and 5% (v/v) bromophenol blue). The samples were boiled for 5 min, centrifuged at 10,000 × g for 10 min, and cooled before being loaded on the gel. Electrophoresis was carried out at constant intensity (25 mA) using a Tris–glycine buffer system containing 0.1% SDS until the bromophenol front ran off of the gel. After completion of electrophoresis, the gels were stained with Coomassie Brilliant Blue (CBB) G-250 (Bio-Rad, Hercules, CA, USA) and/or silver staining solution (GE Healthcare, Barcelona, Spain) and digitized on a Gel Doc™ XR + Imaging system (Bio-Rad, Hercules, CA, USA).

Zymogram analysis

Proteolytic activity was detected via electrophoresis on 12% polyacrylamide gels prepared with 1% SDS and 0.1% (w/v) gelatin. After completion of electrophoresis, the gels were incubated in 0.05 M Tris–HCl buffer (pH 8.0) containing 2% (v/v) Triton X-100 at 35°C for 1 h, and then again in the same buffer without detergent at 35°C for 3 h. After CBB staining and subsequent destaining, proteolytic activity was determined from the presence of non-stained zones and bands on the stained background composed of nondigested gelatin [20].

Proteomics

A proteomics study was carried out according to the procedure described in Parrado et al. [21]. Briefly, to remove contaminants such as cells and debris in suspension, the samples were centrifuged at 12,000 × g for 20 min and the supernatants were recovered. Then, 10 µL of the supernatant was treated with 40 µL of methanol and vortex mixed for approximately 1 min. 10 µL of chloroform was added and the mixture was shaken again for 1 min before 30 µL of Milli-Q water was added and mixed well. The mixture was centrifuged at 14,000 × g for 5 min and the supernatant was removed. 30 µL of methanol was added to the pellet and the mixture was vortexed again for 1 min. Samples were centrifuged at 16,000 × g for 5 min. The supernatant was discarded, and the pellets were dried by lyophilization. The pellet proteins were resuspended in Milli-Q water. A total of 30 µg of resuspended protein was added into 30 µL of 6 M urea, 200 mM ammonium bicarbonate, 10 mM DTT and allowed to remain at room temperature for 30 min for protein reduction. 10 µL of 100 mM iodoacetamide was added to promote protein alkylation, and the samples were kept at room temperature in the dark for 30 min. Samples were then dialyzed against Milli-Q water until the urea concentration was below 0.1 M and subsequently treated with trypsin (Promega, Madison, WI, USA) in a protein-to-enzyme ratio of 50:1. Digestion was carried out at 37°C overnight. The mixture was then acidified with TFA and concentrated using a Speed C18/18 column (Applied Separations, Allentown, PA, USA). Peptides were eluted in 400 µL of 70% acetonitrile containing 0.1% TFA. After the samples were completely dried, they were resuspended in 15 µL of 5% acetonitrile containing 0.1% formic acid for use in tandem liquid chromatography–mass spectrometry (LC–MS) analysis. LC–MS analysis was performed in a Surveyor HPLC system in tandem with a Finnigan LTQ mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA). A total of 5 µL of sample was injected into a C18 PepMap100-Precolumn Cartridge (Dionex, Netherlands) for pre-concentration and washing, then resolved in a Biobasic C18 75 µm × 10 cm column (ThermoFisher Scientific, Waltham, MA, USA). Peptides were eluted at a nominal post-split flow rate of 250 L/min using a 120-min gradient of 5% acetonitrile with 0.1% formic acid to 40% acetonitrile with 0.1% formic acid. The LTQ mass spectrometer was run in positive-ion mode using the nanospray source. The spray voltage was set

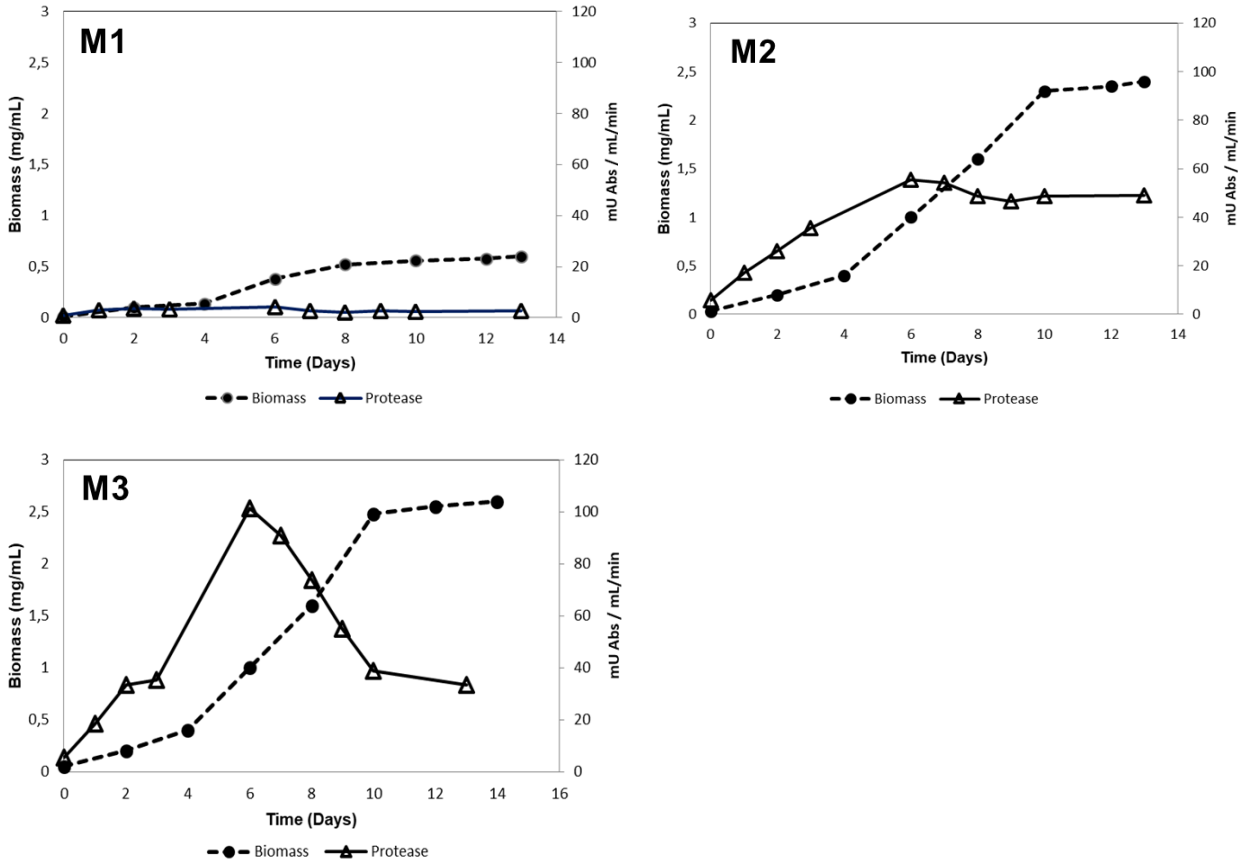

Figure 2. Biomass and protease production by *B. licheniformis* grown in media formulations M1, M2, and M3.

at 2 kV, and the capillary temperature was set to 170°C. The samples were scanned in the range of 400–1500 m/z using the full scan mode, and data-dependent MS/MS analysis with collision-induced dissociation (CID) was performed on the top five ions with dynamic exclusion. The data was converted to SEQUEST format (DTA) and compared against the NCBI database (version 11/10/2007) using an in-house MASCOT (Matrixscience, London, UK) search engine with taxonomy restrictions set to Firmicutes and with carboximethylated cysteine as a fixed modification.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). All experiments were completed in triplicate, and the mean of the three data sets is presented for each experiment. A level of significance of $\alpha < 0.05$ was used to calculate significant differences. One-way ANOVA analysis revealed that the results of the triplicate analyses for each experiment did not differ significantly.

Results and discussion

In an attempt to find an abundant and cheap cultivation medium for the production of hydrolytic enzymes using *Bacillus licheniformis*, we present in this work the results obtained from the use of sewage sludge as the main fermentation source in the culture of *B. licheniformis*. As observed in figures 2 to 4, measurement of the protein production indicated that the microorganism grew well, although the biomass production was not high (2.76 µg/mL). Biomass estimation was carried out by protein determination using the Bradford method. As figure 1 shows, a linear relationship (R2 = 0.995) between protein concentration and

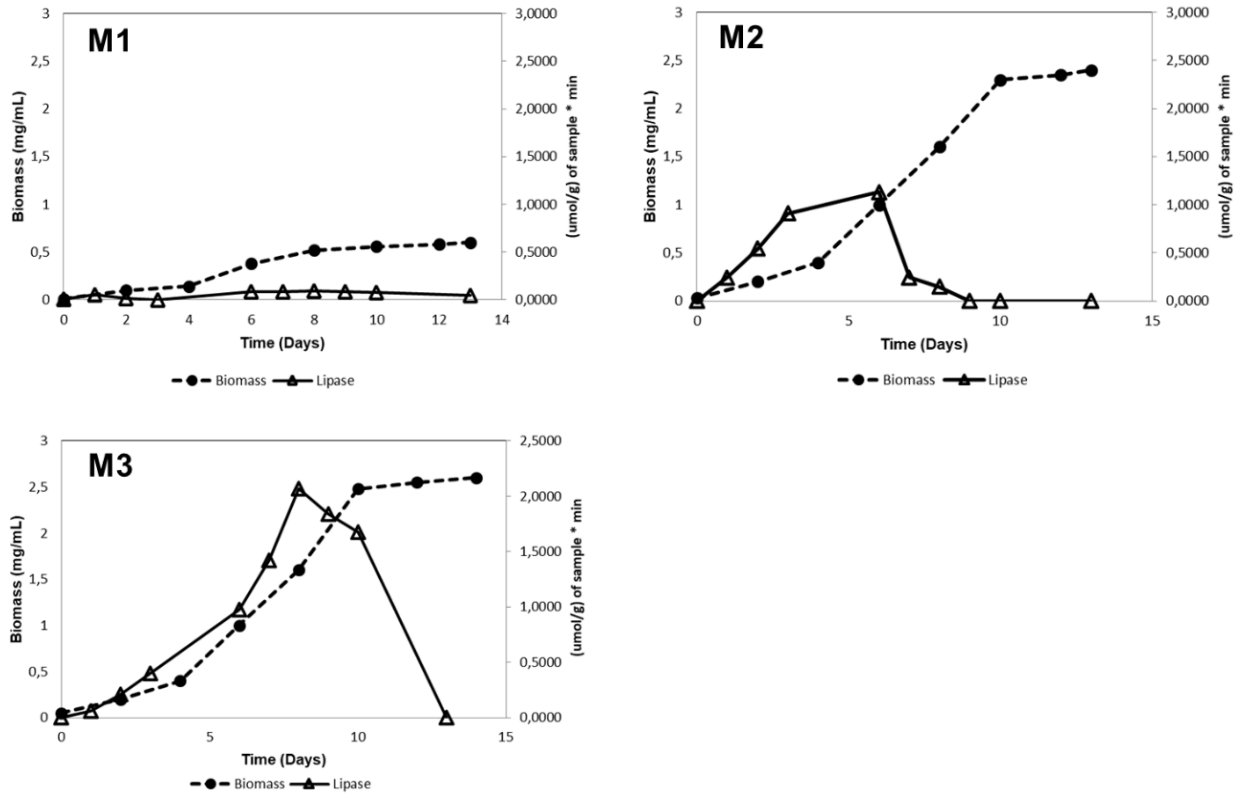

Figure 3. Biomass and lipase production by *B. licheniformis* grown in media formulations M1, M2, and M3.

dry biomass was observed, from which biomass concentration can be estimated by interpolation. Figures 2 to 4 also show hydrolase production by *B. licheniformis* grown in media M1, M2, and M3, specifically, proteases, lipases, and cellulases. Among these hydrolases, proteases are of the highest industrial interest. Proteases (EC 3.4.21-24 and 99) are industrially useful hydrolytic enzymes that cleave peptide bonds between amino acid residues and are the dominant enzyme in the worldwide market. Two-thirds of the proteases produced annually are used in the detergent industry. Microbial proteases, especially from *Bacillus* species, have traditionally comprised the predominant portion of industrial enzymes on the market. The major application of microbial proteases is in the formulation of various detergents, which constitutes a significant share of worldwide enzyme sales [22]. Many bacteria in the genus *Bacillus* excrete large amounts of enzymes into culture medium. Alkaline serine protease, one of the most important enzymes industrially, is excreted into culture medium by strains of *B. licheniformis* or *B. pumilus* [23]. These alkaline proteases (i.e., subtilisin) operate in a high alkaline pH range of 8.5-10 and are thus optimal for use in the formulation of detergents. In addition to their use in the detergents industry [24], these alkaline proteases have several other industrial applications, including in the production of foods, pharmaceuticals, leathers, and diagnostic reagents [25]. Proteases are also used for the bioconversion of chitinous materials as a waste-treatment alternative for the disposal of shellfish wastes [26, 27]. As shown in figure 2, *B. licheniformis* produces extracellular proteases during exponential growth and at the start of the stationary growth phase [28], after which point the production decreased. The analysis of the results obtained in medium M2 (without the addition of feather meal) and M3 (with added feather meal) show that the production of proteases by *B. licheniformis* is induced by the

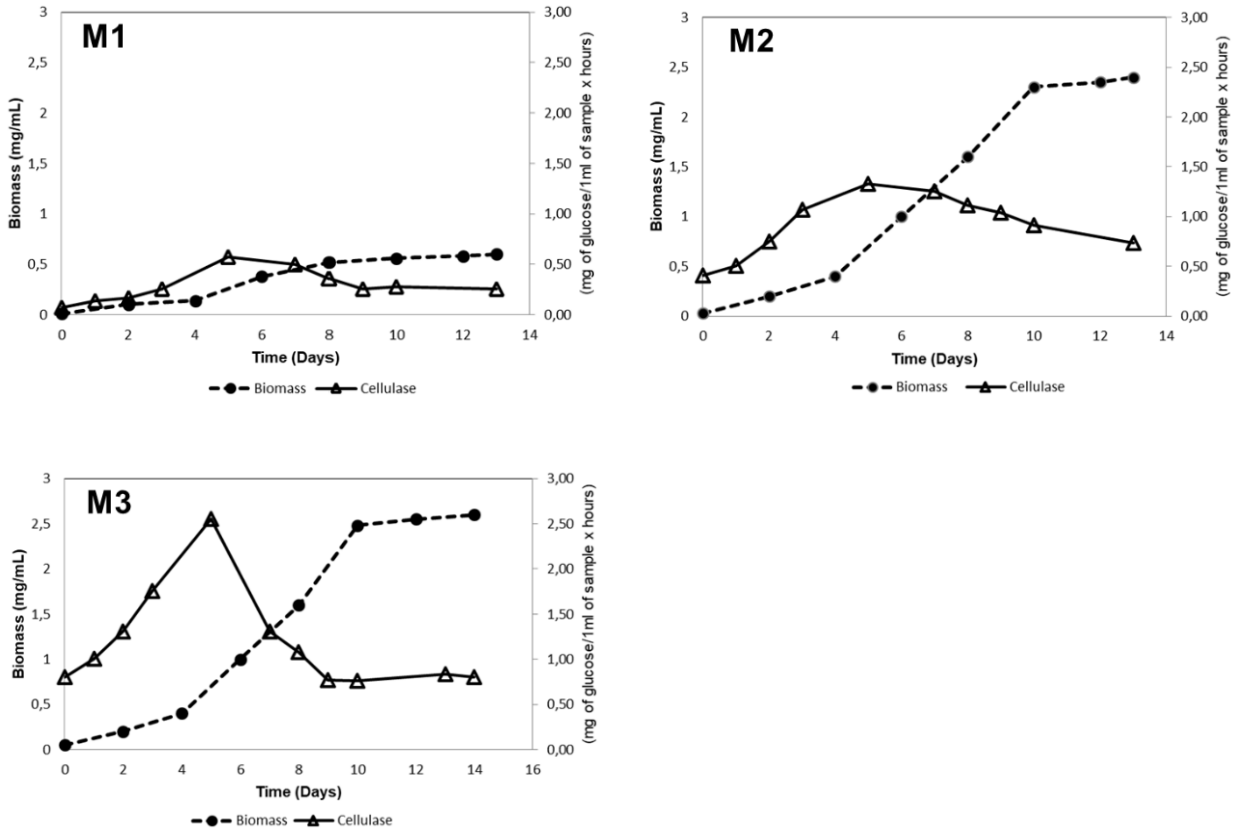

Figure 4. Biomass and cellulase production by *B. licheniformis* grown in media formulations M1, M2, and M3.

presence of feather meal. In both cases, it was also observed that the protease production activity became decreased if the stationary growth phase was prolonged. Therefore, we harvested the fermentation after 6 days of growth. The productivity at the point of harvesting was 16.89 mU/mL· day in medium M3 and 9.22 mU/mL· day in medium M2. Our results are in accordance with those previously reported by other authors [21, 28, 29, 30]. Extracellular protease production in microorganisms is highly influenced by media components, including variations in the carbon/nitrogen ratio, presence of some easily metabolizable sugars such as glucose [28, 29], and presence of metal ions [30]. In addition to these factors, several other properties such as aeration, inoculum density, pH, temperature, and incubation time were also found to affect the amount of extracellular protease produced [31, 32]. Glucose and peptone were found to be important factors in enhancing the formation of alkaline protease, specifically (figure 2). Protease production is only of industrial interest if the microorganisms that produce substantial amounts of extracellular enzymes can be grown well in an easily prepared and low-cost medium. The use of sewage sludge amended with the soluble sugar fraction of agroindustrial by-products (beer bagasse, sugar bagasse, rice bran, etc.) as fermentation media, such as media sample M2 and M3 in this study, to achieve these requirements could substantially improve the industrial production of proteases (Bautista et al., unpublished results). The recovery of proteases from fermentation broth was carried out by continuous centrifugation of the fermentation broth on a non-foaming disc centrifuge and subsequent recovery of the filtrate and discarding of the cake. The clear filtrate was then concentrated by ultrafiltration using a 50 kDa ultrafiltration membrane, and the resulting

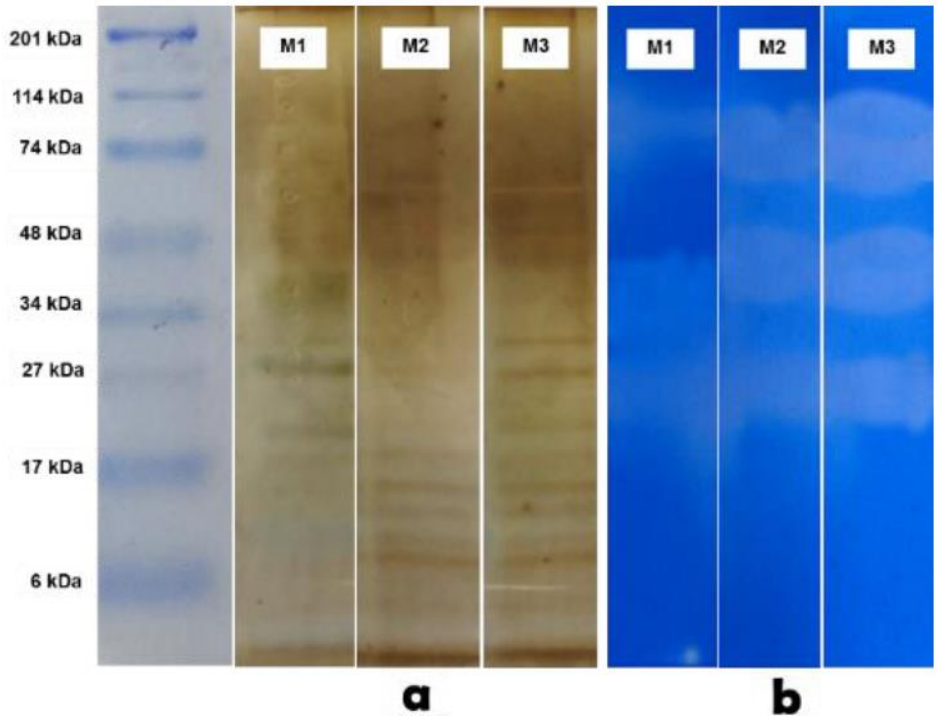

Figure 5. Electrophoretic separation by SDS-PAGE analysis of protein secreted by *B. licheniformis* grown in media formulations M1, M2, and M3. (a: silver staining; b: activity staining (zymogram)). (Molecular weight standards: Myosin 201 kDa, β-Galactosidase 114 kDa, BSA 74 kDa, Ovalbumin 48 kDa, Carbonic anhydrase 34 kDa, Soybean trypsin inhibitor 27 kDa, Lysozyme 17 kDa, Aprotinin 6 kDa).

ultraconcentrate was precipitated with the addition of 80% ethanol. The precipitate was then liophylized or dried with air at 30-40°C. Lipases are a group of enzymes of which the main function is the hydrolysis of triacylglycerol in a lipid-water interphase. This group of enzymes is of special interest in many industrial sectors, such as the detergent industry, food-industry and chemical and pharmaceutical industry [33]. *B. licheniformis* has been described as a major producer of lipases; therefore, we also studied the production of lipases by this microorganism in the media formulated with sewage sludge used in this work. Figure 3 shows the production of lipases in the three tested media formulations. These results show that the addition of feather meal also induced the production of lipases. This induction could be attributed to the relatively high fat content of the feather meal (8.4%). However, in this case an important difference was observed in lipase production by *B. licheniformis* in the two media formulations. In medium M2, the production of lipase was observed from the beginning of bacteria growth, while in medium M3, the production increased drastically after 4 days of growth. In both formulations, the lipase production level was maintained between days 5-9, after which point the lipase concentration in the medium decreased sharply, probably due to the actions of proteases. Therefore, the lipase productivity at day 8 was used to evaluate lipase production and was estimated as 0.25 mU/mL· day (figure 3). The production of enzymes that degrade cellulosic material, such as cellulases, is of great importance in the development of new biorefinery approaches to produce biofuels and high value-added products through fermentation. Therefore, the development of abundant and cheap sources of cellulose-degrading enzymes is necessary. For this reason, we also tested the production of cellulases by *B. licheniformis* in media formulated with sewage sludge. Figure 4 shows the production of

cellulases by *B. licheniformis* grown in media formulations M1, M2, and M3. The production of cellulase in medium M2 started at the beginning of bacteria growth, likely due to the presence of cellulosic materials in the sewage sludge. Cellulase production reached a maximum after 5 days of bacteria growth, with a productivity of 0.51 mU/mL· day. Surprisingly, the addition of feather meal also induced the production of cellulolytic enzymes, although this induction was less pronounced than that seen for proteases and lipases. The cause of this induction remains obscure. In addition to protease, lipase and cellulase activities, other enzyme activities, such as those of β-glucosidase and acid phosphatase, were also tested in the supernatant from the growth of *B. licheniformis* in media formulations M1, M2, and M3. No enzymatic activities beyond those of proteases, lipases, and cellulases were detected (figure 4).

Proteases, lipases and cellulase are inherently produced by *B. licheniformis*, but in the presence of feather meal, the production of these enzymes was clearly enhanced, particularly in the case of proteases and lipases [21]. Induction of the production of these enzymes with hydrolytic activities suggests that these enzymes are involved in the utilization of insoluble C- and N-sources for *B. licheniformis* survival when soluble C- and N-sources are low. Other enzymes are likely also involved in this adaptation process; therefore, we analyzed the set of proteins excreted (secretome) by *B. licheniformis* using electrophoretic and proteomic techniques.

The proteins produced by *B. licheniformis* grown in the media formulations M1, M2, and M3 were separated by SDS-PAGE analysis and are shown in figure 5. As these results show, the excretion of proteins/enzymes into the growth medium was greater in mediums M3 and M2 than in M1 as a result of the enhanced growth of *B. licheniformis*. Zymographic analysis showed that in both M2 and M3, three main protease zones can be detected—one approximately 75 kDa and other two approximately 34 and 27 kDa. In order to study the set of proteins secreted by *B. licheniformis* grown in media formulated with sewage sludge (M2 and M3), we used a proteomic approach based on the shotgun procedure. In the presence of a culture medium that includes feather meal as an inductor, *B. licheniformis* is capable of excreting various kinds of enzymes in order to degrade the components of the medium and, thus obtain nutrients required for survival [34-37] (figure 5). Most *Bacillus sp*. initiate a series of transitional responses that are designed to maintain or restore growth under different environmental conditions, including the induction of macromolecular hydrolases, such as proteases, lipases, and polysaccharidases. Enzymatic induction through addition of materials to the substrate has been described for *Bacillus*. For example, xylane addition induces the excretion of xylanases and other glycoside hydrolases [38], and substrates formulated with olive oil, an important inductor and source of lipids, induces the synthesis and excretion of lipases [34]. The results obtained in this study show that feather meal can be used as a N-source for the growth of *B. licheniformis* and can also act as a strong inducer of the secretion of hydrolytic enzymes, specifically, proteases, lipases, and to a lesser extent, cellulases, and other proteins and enzymes. The proteins and enzymes secreted by *B. licheniformis* grown in medium M3 was characterized by a proteomic approach using the shotgun procedure, and the results are shown in table 2. The analysis of exocellular proteins excreted by *B. licheniformis* in medium M3 showed a relatively low diversity of proteins compared to that found in other fermentation media, such as dextrose broth [21] (table 2). The main proteins and/or enzymes secreted were hydrolases (> 80%), and of these, the protease keratinase (KerA) is of special relevance. This enzyme is typically produced when *B. licheniformis is* grown in a media with primarily insoluble N-sources, such as keratins. In addition to promoting the hydrolysis of keratins, keratinase is capable of hydrolyzing a broad range of protein substrates and, therefore, has many potential agricultural and industrial

Table 2. Identified extracellular proteins/enzymes secreted by *Bacillus licheniformis* grown in medium M3.

Protein name	Mass (Da)	Score	Queries Matched	emPAI*
KerA, Subtilisin precursor	31,266	640	18	1.02
Chain A, Chitosanase	435	468	16	0.44
Glycoside hydrolase family 14 protein	79,565	419	9	0.22
Glycoside hydrolase family 18 protein	66,111	273	5	0.10
Gamma-glutamyltranspeptidase (Ggt)	54,048	221	3	0.10
Flagellin	3,201	218	5	0.21
Extracellular serine protease	85,573	184	5	0.08
Hypothetical protein BL00275	14,050	173	9	0.24
Putative acylaminoacyl-peptidase YuxL	73,549	109	2	0.04
Chitosanase	50,616	348	11	0.37
Intracellular serine protease	33,888	232	7	0.45
Superoxide dismutase	22,530	187	11	0.51
Metalloregulation DNA-binding stress protein	17,909	157	4	0.19
Metal-dependent hydrolase	24,831	122	2	0.10
Chitosanase	19,722	116	3	0.17

applications. Gamma-glutamyl transpeptidase (GGT, E.C. 2.3.2.2) was another minor protein found in the fermentation solutions examined. This protein catalyzes the transfer of the γ-glutamyl moiety from γ-glutamyl compounds to a variety of amino acids and dipeptide acceptors [39, 40]. The hydrolytic and glutaminase activities involved in this process are used in the food industry for debittering amino acids. GGT has also been suggested to assist subtilisin during its action on keratin [41]. *B. licheniformis* was found to secrete extracellular GGT and keratinase during keratin degradation processes. Keratinase in the presence of GGT exhibits an extended substrate spectrum, cleaving all types of keratins. The complex thus exhibits better catalytic properties when GGT is present, a characteristic which can be exploited in various biotechnological applications [42].

Conclusion

These results demonstrate the successful culture of *B. licheniformis* in a medium formulated with sewage sludge as the main source of carbon and nitrogen with added feather meal (keratin) as an inductor. This formulation is a cheap and abundant fermentation medium that can be used for the regular production of hydrolases, particularly proteases and lipases, on a large scale in relatively short culture periods of 5 and 8 days. The subsequent recovery of these enzymes through continuous centrifugation and concentration by ultrafiltration, followed by fractionation with ethanol (actually underway), could be a promising process for the preparation of products with high proteolytic and/or lipolytic activity for inclusion in enzymatic formulations for food production and agro-industrial uses.

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Obtaining biostimulant products for land application from the sewage sludge of small populations

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ABSTRACT

Large amounts of sewage sludge are generated in wastewater treatment plants during the water purification process. In small populations – defined as urban settlements with less than 2000 population equivalent – sewage sludge is normally produced in anaerobic systems (e.g. septic tanks, Imhoff tanks or anaerobic ponds) or in extended aeration systems. According to the European Directives 86/278/EEC and 2006/12/EC, sewage sludge must be treated before its final fate. Although there are various alternatives, land application of sewage sludge after its composting has been widely promoted because of its high nutrient content. However, the employment of composted sludge as a soil amendment has some limitations, including social rejection. In this paper, a hydrolytic process for obtaining an agricultural biofertiliser from sewage sludge has been assessed. The enzymatic hydrolysis of sludge produced two by-products: an insoluble paste and a nutrient-enriched liquid that constituted the fertilising product. This biofertiliser had higher contents of organic matter, proteins, potassium (K) and sulphur (S) compared to the fresh sludge. Protein content was characterised by a low molecular size, thus increasing the bioavailability of nitrogen (N). Further, high levels of enzymatic activity were detected in soil after the addition of the obtained biofertiliser.

1. Introduction

The treatment and management of residual solid waste generated during sewage treatment remains as a very important issue since the sludge concentrates all the pollutants that are removed from wastewater (Aragon et al., 2009). In order to regulate the management of these wastes, the European Union has promulgated different Directives: Directive 2006/12/EC on Waste; Council Directive 86/278/EEC on the protection of the environment, and in particular of the soil, when sewage sludge (SS) is used in agriculture. The main goals of the above-mentioned Directives have been to improve the management of SS, and optimise its agricultural application (promoting sludge recycling), and to protect the environment and, especially, the quality of the soil. The current policy of the European Commission is to provide the adequate quality of sludge to encourage its beneficial use on land and, consequently, consolidate this long-term sustainable solution to sludge disposal (Schowanek et al., 2004; Carbonell et al., 2009). Furthermore, land application is the least expensive method of SS management.

Soils are often subject to severe degradation processes, which are linked to an organic matter loss, which affects adversely the soil's fertility and increases the risks of erosion and desertification, especially in agricultural ecosystems (Fernández et al., 2007). The usual way to recover the soil's quality and its physical, chemical and biological functions is the use of SS as a resource of organic matter (Navas et al., 1998; Wong et al., 1998; Kizilkaya and Bayrakli, 2005; Pascual et al., 2007; Fernández et al., 2007). Thus, SS can be employed as an organic amendment for both agricultural purposes and the restoration of degraded areas. SS can be used as fertiliser because of its high N and P content and low C/N ratio (Boyd et al., 1980; Iakimenko et al., 1996). However, the direct use of these wastes has major drawbacks that may cause problems derived from the presence of heavy metals, pathogenic microorganisms, bad odours or phytotoxicity. The heavy metal content

Abbreviations: SS, sewage sludge; BF, biofertiliser; IP, insoluble paste; WWTP, wastewater treatment plant; DHA, dehydrogenase activity; PHA, phosphatase activity; GA, ß-glucosidase activity; INT, 2-*p*-iodo-3-nitrophenyl-5-phenyl tetrazolium chloride; INTF, iodonitrotetrazaolium formazan.

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in SS may prevent its use as biofertiliser. Therefore, an adequate stabilisation and disinfection treatment is required before application of SS on land. Composting is the main biological approach to stabilise the SS. Composts represent an important resource to maintain and restore soil fertility and are of great value nowadays, particularly in those countries where the organic matter content of the soil is low (Castaldi et al., 2008; Tejada et al., 2009). However, the composting of SS has several problems including the long time of production, the low control of the process, the low value of the final products, the presence of large size solids that avoid some kind of agronomic applications and the slow assimilation by soil microorganisms and plants (Ranalli et al., 2001).In recent years, there has been an increasing use of organic biofertilisers obtained from the hydrolysis of different organic materials (Romero et al., 2007; Parrado et al., 2008; García-Martínez et al., 2010). These products generally comprise peptides, amino acids, polysaccharides, humic substances, phytohormones, etc., that are directly absorbed by plants, thus spending a smaller amount of energy in the absorption process. This fact has a positive effect not only on growth but also in the quality and production of fruit or grain harvested. Thus, the aim of these products is not only to provide nutrition, but also to encourage and stimulate plant metabolism, stress reduction, etc. (Parrado et al., 1991, 2006, 2007, 2008). In fact, the development of new fertilisers and soil-stimulating products has become a focus of interest. Similarly, the application of these biofertilisers to the soil not only leads to an increased content of organic matter and macro- and micro-nutrients, but also a significant activation of the soil's microbial community.

In this study, a biofertiliser from the SS generated in a small wastewater treatment plant (WWTP) (serving less than 2000 population equivalent) has been obtained by applying an enzymatic hydrolysis process and, additionally, its quality and its effects on soils' biochemical properties has been assessed.

2. Materials and methods

2.1. SS sampling

SS was collected from the waste sludge line of an extended aeration unit located at the Experimental Plant at the Foundation Centre for New Water Technologies (CENTA) manages in Carrión de los Céspedes (Seville, Spain) (Fahd et al., 2007). The pilot unit was treating around 30 m^3/d of urban wastewater, which meant an F/M rate of 0.1 kg BOD_5 kg $MLVSS^{-1}$ d^{-1}. The sludge age was 20 days and the excess sludge production was estimated to be 0.35 kgVSS kg $BOD_5{}^{-1}$. This sludge was taken once at the beginning of the trial and was physically and chemically characterised as described in Section 2.3.

2.2. Enzymatic hydrolysis process

The SS was hydrolysed according to the pH-stat method (Adler-Nissen, 1977), using an endoprotease obtained by liquid fermentation of *Bacillus lichiniformis* ATCC 21415 as the hydrolytic agent. This process took place in a bioreactor operating under controlled temperature and pH, agitation and NaOH consumption (Parrado et al., 2008). The sludge hydrolysis was carried out under the following conditions: (a) substrate: 15–20% of SS; (b) solvent: water; (c) catalytic agent: subtilisin produced by the fermentation of *Bacillus lichiniformis* ATCC 21415, 0.15% (v/v) (Romero et al., 2007); (d) Enzyme concentration: 1 ml l^{-1} of substrate; (e) temperature: 55–60 °C; (f) pH: 8.5, controlled by the addition of 10 M NaOH; (g) time: 120 min. Finally, the hydrolysed product was centrifuged obtaining two by-products: the hydrolysed liquid, which constituted the biofertiliser (BF) and an insoluble paste (IP). Both products were characterised as described in Section 2.3.

2.3. Analytical characterization of SS and by-products obtained by enzymatic hydrolysis

2.3.1. Physico-chemical characterisation

For SS, as well as for the IP and the BF, the determination of organic matter was performed using the dry combustion method (MAPA, 1986). Organic matter content was determined by combustion at 550 °C for 3 h and estimated by difference after obtaining the ash percentage. Total nitrogen was determined by the Kjeldahl method (AOAC, 1990). The protein content was determined by multiplying the total nitrogen content in a protein by the conversion factor 6.25, which is applicable to most proteins. For SS and IP, macro- and micro-nutrients were determined after digestion with nitric and perchloric acid. The nutrients were measured by atomic emission spectrometry and ICP plasma-AES (Fisons-ARL 3410). For BF, the measurements of macro- and micro-nutrients were made directly on the hydrolysed product using the same equipment above mentioned.

2.3.2. Molecular weight analysis by size-exclusion chromatography

For SS and BF, the molecular weight distribution of proteins was determined by size-exclusion chromatography using an ÄKTA-purifier (GE Healthcare), according to the procedure described by Bautista et al. (1996) employing a Superdex Peptide™ 10/300GL column (optimum separation range 0.1–7 kDa). Samples were centrifuged at 13,300 × *g* for 15 min at 4 °C to remove insoluble particles, and the supernatant was filtered through a 0.2 μm filter and loaded into a 0.1 ml loop connected to an ÄKTA purifier system. The column was equilibrated and eluted with Na_2HPO_4 50 mM (pH 7.5) in isocratic mode, at a flow-rate of 0.5 ml min^{-1}. Protein standard mixture (cytochrome C, 12,500 Da; aprotinin, 6512 Da; vitamin B12, 1255 Da; cytidine, 246 Da; glycine 75 Da) was used to cover the range 100–7000 Da.

2.4. Experimental design for the soil stimulation assessment

2.4.1. Soil characterisation

In order to assess the potential use of SS and BF as an agricultural biostimulant, an experimental soil classified as Plagic Antrosol was employed (FAO, 1989). The soil was collected from the University of Seville's experimental research field, from the 0–25 cm surface layer. Soil characteristics determined are shown in Table 1. Soil pH was monitored in distilled water with a glass electrode (soil:H_2O ratio 1:10, MAPA, 1986). Soil texture was determined by the Robinson's pipette method (SSEW, 1982) and quantification and the dominant clay types were determined by X-ray diffraction. Total carbonates were measured by quantifying the CO_2 produced after HCl addition by standard methods (MAPA, 1986). Soil organic matter was determined by the method of Yeomans and Bremner (1988). Total N was determined by the Kjeldhal method (MAPA, 1986). After nitric and perchloric acid digestion, total Fe, Cu, Mn, Zn, Cd, Pb, Ni and Cr concentrations were determined by atomic absorption spectrometry, according to MAPA methods (MAPA, 1986).

2.4.2. Biostimulation experiment design

Soil microcosms were employed to investigate the effects of SS and BF on soil microbial activity. Those microcosms consisted of 300 g of dried and 2 mm-sieved soil mixed and packed into a plastic container. Soil was pre-incubated at 25 °C for 7 days at 30–40% of their water-holding capacity according to Moreno et al. (2003)

Table 1
Physico-chemical characteristics of the experimental soil expressed as mean ± standard error. Data are the mean of four samples.

pH (H_2O)	8.6 ± 0.2
CO_3^{2-} (g kg^{-1})	203 ± 12
Fine sand (g kg^{-1})	142 ± 35
Coarse sand (g kg^{-1})	387 ± 26
Silt (g kg^{-1})	242 ± 19
Clay (g kg^{-1})	229 ± 10
Clay types	Smectite: 66% Kaolinite: 20% Illite: 14%
Organic matter (g kg^{-1})	4.1 ± 0.8
Total N (g kg^{-1})	0.4 ± 0.1
Fe (mg kg^{-1})	35.8 ± 3.7
Cu (mg kg^{-1})	9.7 ± 1.3
Mn (mg kg^{-1})	11.3 ± 2.1
Zn (mg kg^{-1})	8.1 ± 1.5
Cd (mg kg^{-1})	6.5 ± 1.2
Pb (mg kg^{-1})	0.36 ± 0.11
Ni (mg kg^{-1})	2.9 ± 0.7
Cr (mg kg^{-1})	5.3 ± 0.6

prior to the experiment. After this pre-incubation period, soil samples were mixed with SS and BF at 0.2%, 0.5% and 1% (w/w) and then adjusted to a water-holding capacity of 60%. A non-amended soil was used as a control. Soil microcosms were incubated in darkness at 25 °C in an incubation chamber for 0, 1, 5, 7, 14 and 30 days. For each product and each incubation time, three soil sub-samples were taken (10 g of soil per sample). The incubation conditions were the following: (a) control: unamended soil; (b) SS 0.2: amended soil with SS at 0.2% (w/w); (c) SS 0.5: amended soil with SS at 0.5% (w/w); (d) SS 1: amended soil with SS at 1% (w/w); (e) BF 0.2: amended soil with BF at 0.2% (w/w); (f) BF 0.5: amended soil with BF at 0.5% (w/w); (g) BF 1: amended soil with SS at 1% (w/w).

2.4.3. Biochemical soil assays: enzymatic activity determinations

Dehydrogenase activity (DHA) was determined by the modified method of García et al. (1993) based on the measurement of iodonitrophenylformazan formed by the reduction of 2-*p*-iodo-3-nitrophenyl-5-phenyl tetrazolium chloride (INT) (García et al., 1993). In this procedure, 0.5 g of soil was exposed to 2 ml of 0.2% INT and 1 ml of 20 mM sodium succinate in distilled water for 30 min at room temperature in darkness. The iodonitrotetrazaolium formazan (INTF) formed was extracted with 5 ml of ethylacetate by shaking vigorously for 20 min. INTF was measured spectrophotometrically at 490 nm using a spectrophotometer (GeneQuant 1300, GE Healthcare Bio-Sciences AB, USA). Controls were prepared without substrate.Phosphatase activity (PHA) was measured using *p*-nitrophenyl phosphate disodium as substrate (Tabatabai and Bremner, 1969). Enzymatic activity was measured using 0.025 M *p*-nitrophenyl phosphate. 0.5 g of soil was incubated at 37 °C in malate buffer MUB (pH 6.5) for 30 min. 0.5 M $CaCl_2$ and 0.5 M NaOH were added to stop the reaction and to extract the product, i.e. *p*-nitrophenol. The concentration of *p*-nitrophenol was determined spectrophotometrically at 410 nm. Controls were prepared without substrate.

β-Glucosidase activity (GA) was determined using *p*-nitrophenyl-*b*-D-glucopyranoside as substrate (Masciandaro et al., 1994). Enzymatic activity was measured using 0.025 M *p*-nitrophenyl-*b*-D-glucopyranoside. 0.5 M NaOH was added to stop the reaction and extract the product, i.e. *p*-nitrophenol. The product concentrations were determined spectrophotometrically at 410 nm. Controls were prepared without substrate

Table 2
Average composition of SS, IP and BF. Different letters following the number indicate a significant difference at $p < 0.05$.

	SS	IP	BF
pH (1/10)	7.9a ± 0.2	8.2a ± 0.3	7.8a ± 0.2
Organic matter (g kg^{-1})	392a ± 13	327a ± 15	555b ± 19
Total N (g kg^{-1})	27.0b ± 1.4	15.8a ± 1.1	29.3b ± 1.6
Proteins (g kg^{-1})	168.7b ± 9.1	102.7a ± 7.1	190.4b ± 10.4

2.5. Statistical analysis

Analysis of variance (ANOVA) was performed using the Statgraphics v. 5.0 software package. Means were separated by the LSD test, considering a significance level of $p < 0.05$ throughout the study. For the ANOVA, triplicate data were used for each product and every day of incubation.

3. Results and discussion

3.1. Chemical properties of the hydrolysis products

In this study, the enzymatic hydrolysis of SS generated two by-products: IP and BF. Table 2 shows the average composition of both by-products, showing that these materials are highly bioavailable. As observed in Table 2, soluble organic matter content in the BF raised significantly ($p < 0.05$) up to 555 ± 1.6 g kg^{-1}, which represents an increase of 41.6% compared to the original SS. On the other hand, the protein content was increased in BF by 12.8%, although, the statistical analysis indicated that this increase was not significant. Moreover, there was a notable difference in both K and S content (Table 3), elements that have a great significance on the physical properties of soil since they constitute the basis of plant nutrition. To this respect, K and S contents were 96.5% and 28.4% lower, respectively, in SS than in BF. Regarding the remaining nutrients analysed, their concentration decreased in BF compared with the SS content (being increased in IP due to the precipitation process) (Table 3). The decrease in P content in BF of around 89.5% is remarkable ($p < 0.05$; Table 3). The chemical properties of the BF had a great effect on the use of BF as biofertiliser and soil stimulant. An increase in the organic matter content and in fundamental elements for plant nutrition, such as K, has a major influence in converting BF to a more advantageous product for agronomic use than the starting raw material, SS, whose characteristics depend upon the quality of sewage and type of treatment processes followed (Singh and Agrawal, 2008). SS may substitute for fertiliser but the usual way for using this residue from wastewater treatment is the composting process. The application of SS (Albiach et al., 2001)

Table 3
Elemental composition of SS, IP and BF.

	SS	IP	BF
P (g kg^{-1})	20.9a† ± 1.6	21.5a ± 1.3	2.2b ± 0.4
K (g kg^{-1})	2.9a† ± 0.3	194.2c ± 12.1	83.2b ± 15.6
S (g kg^{-1})	6.3a† ± 1.0	6.7a ± 1.1	8.8b ± 0.9
Ca (g kg^{-1})	85.7a† ± 3.3	100.5a ± 4.7	26.5b ± 1.9
Mg (g kg^{-1})	6.3a† ± 1.6	7.8b ± 1.4	5.4a ± 0.8
B (g kg^{-1})	70.8a† ± 2.6	82.1a ± 3.9	50.1b ± 2.1
Fe (g kg^{-1})	27.8a† ± 1.6	20.1a ± 2.3	0.6b ± 0.1
Cu (mg kg^{-1})	300a† ± 10.9	327a ± 11.8	33.8b ± 1.5
Mn (mg kg^{-1})	196.1ab† ± 14.8	237.3b ± 19.6	116.1a ± 12.3
Zn (mg kg^{-1})	736a† ± 16	778a ± 22	87.6b ± 2.3
Cd (mg kg^{-1})	1.4a† ± 0.3	1.5a ± 0.6	0.15b ± 0.05
Pb (mg kg^{-1})	51.2a† ± 1.9	56.7a ± 1.6	ND
Ni (mg kg^{-1})	36a† ± 8	31.9a ± 5.5	4.3b ± 0.9
Hg (mg kg^{-1})	1.6a† ± 0.3	2.2a ± 0.4	0.04b ± 0.002

†Different letters following the number indicate a significant difference at $p < 0.05$.

or compost (Tejada and González, 2003) to soil is a current environmental and agricultural practice for maintaining soil organic matter, reclaiming degraded soils and supplying plant nutrients. However, SS compost use has drawbacks such as the high amount of time required to generate compost, odour generation from composting operations (Sikora, 1998) and lack of process control which avoid SS compost bioavailability. Moreover, the low bioavailability of nutrients and the presence of toxic metals restrict its uses (Ranalli et al., 2001). In fact, the major limitation of soil application of SS compost is the total heavy metal content and their availability to the soil–plant system (Amir et al., 2005). To this regard, when comparing heavy metal contents in the product to that of the original SS, it is observed that their concentration significantly decreased ($p < 0.05$) in the fertilising soluble product and most of them were retained in the IP (Table 3). The reduction in Fe content was 97.8%, 88.7% for Cu, 40.8% for Mn, 88.1% for Zn, 89.3% for Cd, 88.1% for Ni and 97.5% for Hg. Pb concentrations in BF were not detectable. As mentioned above, the proposed hydrolytic process occurs at an alkaline pH (pH 8.5), and in those circumstances the heavy metals precipitate and are concentrated in the IP. In the environment, heavy metals have been identified as the most dangerous pollutants, so attention is increasingly being given to the potential health hazard presented by heavy metals in the environment. Heavy metals are not only biodegradable and toxic at some concentrations, but also they tend to accumulate along the food chain where man is the last link (Dudka and Miller, 1999). Bioavailability of heavy metals and their transfer to plants, groundwater, animals and, finally, to humans depends on the SS quality (Katsoyiannis and Samara, 2007). During composting, heavy metal bioavailability is dependent on several physico-chemical properties of the medium besides total metal content such as decomposition of organic matter, humic substances content and pH (Amir et al., 2005), which may complicate the composting process and the removal of heavy metal content. However, the low heavy metal content found in the BF product has important environmental implications, since the final use of BF as fertiliser will not cause dangerous damage to the ecosystem. Thus, the enzymatic hydrolytic process could constitute in an effective treatment to remove heavy metals from SS. Nevertheless, heavy metals are accumulated in the IP and, therefore, this by-product requires an adequate treatment.

Another aspect of the enzymatic hydrolysis process on SS is the protein content, which was increased in BF compared to the raw material, although without statistical significance. However, the analysis by size-exclusion chromatography of soluble protein components of SS and BF showed differences in protein solubility (Fig. 1). The distribution of the chromatogram obtained for the SS indicates that the proteins were highly insoluble. During the hydrolytic process, the protein component was broken down, and so the peptide and free amino acid content was increased until the end of the process (120 min), displaying different chromatographic signals throughout the process. As a final product, BF showed a soluble protein component characterised mainly by small molecular peptides, and even free amino acids (<10 kDa). The decrease in the molecular size indicates that N is in a form readily bioavailable to soil-living organisms, such as plants (Higgings and Payne, 1982) and microorganisms (Vasileva-Tonkova et al., 2007), since peptides are an ideal vehicle to transport N in soils, better than free amino acids (Higgings and Payne, 1982). Our results show several benefits of the molecular size of the proteins on the biostimulation process. Proteins must be hydrolysed to peptides and amino acids by extracellular proteases to be absorbed by plants and microorganism, due to the lack of carrier proteins that transfer proteins intact through cell membranes (Jan et al., 2009). Recent studies have suggested that plants are able to absorb N easily from soil in the form of amino acids, although the significance of this in the regulation of

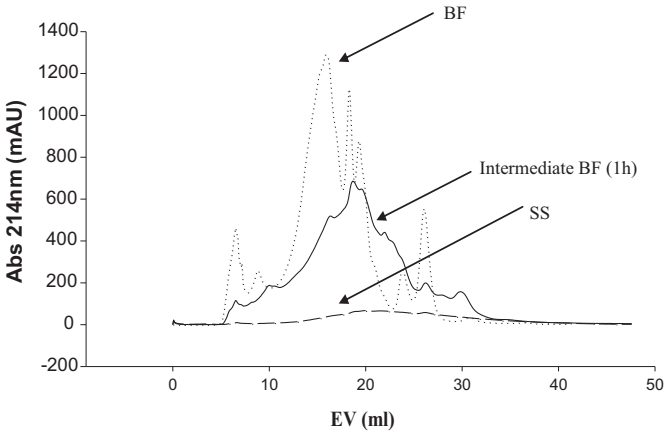

Fig. 1. Proteins' molecular weight distribution in time of SS and the hydrolysed product (BF) determined by size-exclusion chromatography (ÄKTA-purifier, GE Healthcare, employing a Superdex Peptide 10/300GL column.

productivity of plants is unknown (Jones et al., 2009). On the other hand, amino acids and small peptides can be absorbed easily by soil microbial populations through a series of membrane transporters (Jones and Hodge, 1999; Walker and Altaman, 2005). This suggests that the breakdown of proteins in soil is the bottleneck step in the N incorporation for soil microorganisms.

3.2. Biochemical soil assays

Fig. 2 shows the evolution of enzymatic activities (dehydrogenase, phosphatase and β-glucosidase) in soils amended with SS and BF in different dosages. The application of both SS and BF products to the soil caused a stimulatory effect on DHA. Land application of both products serves as a good source of microbial nutrient and the organic constituents providing beneficial soil-conditioning properties (Logan and Harrison, 1995; Singh and Agrawal, 2008). This stimulating effect was dose-dependent and changed with the type of organic material. Thus, for SS-amended soil, DHA was progressively increased until the end of the incubation period. However, in BF-amended soil, the stimulant effect was extremely marked from the first day of incubation. Moreover, as it is shown, after 5 days the values of DHA measured in BF-amended soils increased significantly compared to the control soil and those amended with SS. At that time, the increase observed was not dose-dependent and was about 35 mmol INTF g^{-1} $soil^{-1}$ min^{-1} in BF-amended soils. Over the experiment, DHA was higher in BF-amended soils compared to the SS-amended treatments. After 14 days, treatments with BF showed how DHA increased significantly up to almost 21-, 13.5- and 11-fold in BF 0.2, BF 0.5 and BF 1, respectively, when compared to SS-amended soil. Regarding the dosage effect, this appeared along the incubation period: after 14 days, the BF 1 treatment showed a 1.7- and 2-fold higher DHA than in BF 0.5 and BF 0.2 treatments, respectively. At the end of the experiment, the dose effect remained and the DHA levels were increased significantly in BF 1 treatment compared with BF 0.5 and BF 0.2 treatments. The results obtained for DHA, an intracellular enzyme involved in microbial respiratory metabolism (Masciandaro et al., 2001), demonstrate that the biostimulant capacity of SS and BF are different, being significantly higher in BF, which suggests that it is a consequence of the lower molecular size of the protein content in BF. The higher values of this enzyme in BF-amended soils suggest that the bioavailability of the organic material is significantly higher and this causes an increase in the microbial activity. The enzymatic hydrolysis process solves the problem of the N-cycle in soil: the breakdown of proteins into peptides and free amino acids (Jones et al., 2009). In fact, the

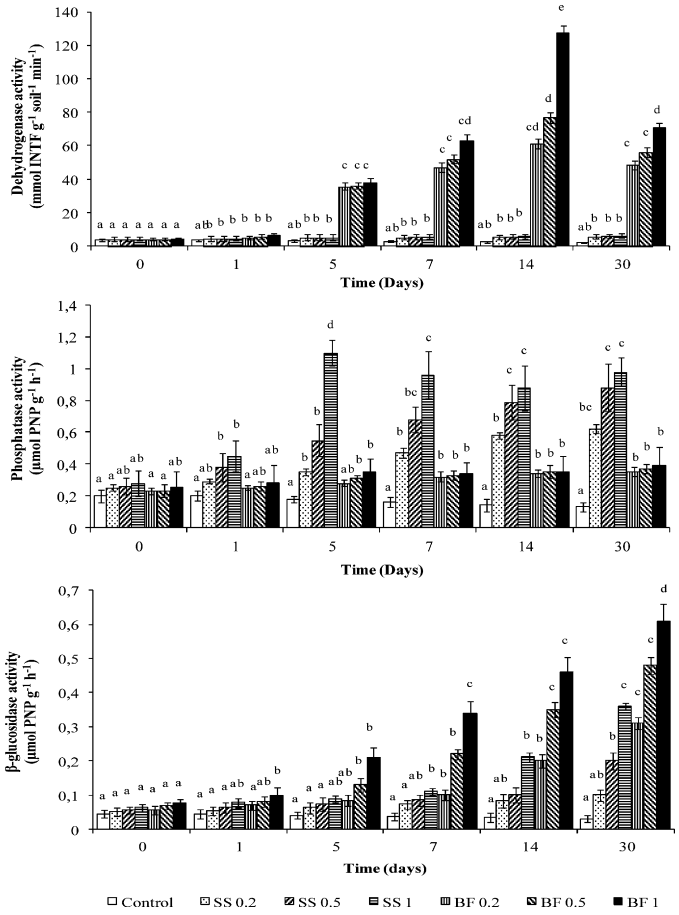

Fig. 2. Evolution of enzymatic activities (dehydrogenase, phosphatase and β-glucosidase) in soils amended with SS and BF. Data are expressed as mean values ± standard error. Columns (mean ± S.E.) followed by the same letter(s) are not significantly different ($p < 0.05$). INTF, 2-*p*-iodo-3-nitrophenyl; PNP, *p*-nitrophenol.

constituent proteins of the organic matter tend to be quite resistant to microbial attack due to physico-chemical protection provided by soil colloids (Schulten and Schnitzer, 1998) and the range of mineralisation depends on the ecosystem (Joanisse et al., 2008). This demonstrates that the proteins are not as accessible as peptides and free amino acids.

In addition, the application of SS and BF to the soil caused a stimulation of PHA. However, this stimulation was different to that detected for DHA. In fact, the stimulating effect was higher in soils amended with SS than with BF. Once again, the application rate greatly influenced the stimulation. At the end of the incubation period and when compared with treatment BF 1, PHA was up to 60.2% higher in the SS1 microcosm. Compared with the control treatment, the PHA showed a minor stimulation for the soils amended with BF compared with SS. To understand these results, it should be noted that microorganisms produce this enzyme due to the demand for P (García et al., 1994). In SS, there is a high content of P (20.9 g kg^{-1}), while in BF, the P content drastically decreases (2.2 g kg^{-1}). However, most of the P from SS was part of the insoluble paste. In BF, the existing P is in soluble form, and although its concentration is lower, it is accessible and easily absorbed.

Regarding GA activity, an increase was observed after the application of both organic amendments to the soil. This stimulation was similar to that obtained for DHA. At the end of the incubation period and compared to the SS1 treatment, GA was significantly higher (41%) in the BF1 treatment. Moreover, a dose-dependent effect was observed during the incubation time.

4. Conclusions

The hydrolysis of SS generated in WWTPs is an alternative process to composting for land application purposes.

In this study, a new enzymatic process has been designed for obtaining a new fertilising product (BF) from SS. This product is mainly characterised by its low content of heavy metals and low molecular size of proteins. The hydrolysed product obtained presents a high stability and bioavailability thus enhancing the enzymatic activities of the soil's microbial community. BF is mainly constituted by peptides and free amino acids that have a potential biostimulatory capacity, higher than SS which is mainly composed by large proteins. Thus, the results of enzymatic activities in soil suggest that this new product has a high potential for stimulating soil microorganisms, and therefore could be used for fertilisation and soil bioremediation.Unlike other processes such as composting and chemical acid hydrolysis, the enzymatic hydrolysis has important advantages in terms of both product quality and technological level. Firstly, the process is technologically controllable (type of enzyme used in the hydrolysis, reaction time, etc.), leading to a uniform product and the possible design of different types of hydrolysates. Secondly, all peptides and amino acids are conserved and there are no nutritional losses, so the quality of BF formed principally of peptides is superior to that of a mixture of free amino acids. This characteristic is very important, since free amino acids are generally unstable and hydrophobic amino acids (tryptophan, valine, isoleucine, leucine, etc.) cannot be administered via liquid due to their insolubility. In addition, the process is biological and respectful to the environment, being non-pollutant (neutral pH, no utilisation of chemical products, etc.).

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Obtaining edaphic biostimulants/biofertilizers from sewage sludge using fermentative processes. Short-time effects on soil biochemical properties

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ABSTRACT

In this manuscript, we study the manufacture and effect on soils of different edaphic biostimulants/biofertilizers (BS) obtained from sewage sludge using *Bacillus licheniformis* as biological tool. These BS consist of different combinations of organic matter, bacteria and enzymes that were subjected to several treatments. These BS were applied in soil in order to observe their influence on the biochemical properties (enzymatic activities and ergosterol content). Dehydrogenase, urease, β-glucosidase, phosphatase activities and ergosterol content were measured at different incubation days. Only dehydrogenase activity and ergosterol content were significantly stimulated after the application of BS1 and BS4. Rest of the extracellular activities were not stimulated probably because *B. licheniformis* practically has digested all organic substrates during fermentation process.

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Sewage sludge; *Bacillus licheniformis*; fermentation process; edaphic biostimulant/biofertilizer; soil biochemical properties

1. Introduction

Currently, the continuous and progressive growth in the world population as well as the awareness of the need to treat wastewater has led to an increase in the volume of sewage sludge produced [1,2]. Also, all this amount of sewage sludge requires new methods for its use and disposal [2].

Due to high content of organic matter as well as essential nutrients, application of sewage sludge to agricultural soils is of great interest in order to improve physical, chemical and biological properties of such soils and to provide essential nutrients to plants [3–6].

However, as they could often contain considerable amounts of heavy metals, organic contaminants and pathogens (e.g. helminth eggs, protozoan cysts, *Escherichia coli*, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, halogenated organic compounds, linear alkylbenzene sulfonates, bis (2-ethylhexyl) phthalate, nonylphenol ethoxylates, polychlorinated dibenzodioxines and dibenzofuranes) and may pose health risks, use of sewage sludges is not feasible [7–12]. Therefore, sewage sludge composting for its posterior use is a good solution. In addition to eliminating potentially harmful organisms, this technique helps stabilize organic matter, converting sewage sludge into a stabilized, nutrient-rich compost suitable for agricultural applications [13,14].

It is well known that composting sewage sludge entail several problems. The high moisture content and low C/N ratio are responsible for significant losses of N in the form of ammonia during composting process, thus decrease the quality of the compost obtained [15,16]. In addition, long processing time, low level of control over the process and the presence of large solids that hinder some agronomic applications must be taken into account [17]. This has led several researchers to seek alternatives for composting in order to increase the use of sewage sludge.

In previous works, Rodríguez-Morgado et al. [17] and Tejada et al. [18] obtained an edaphic biostimulant/biofertilizer (BS) from sewage sludge using proteolytic enzymes. That BS was characterized by a low concentration of heavy metals and a high concentration of low molecular-weight peptides (<300 Daltons), which can be assimilated directly by soil microorganisms, stimulating them significantly. These authors demonstrated that microbial stimulation is essential for the degradation of xenobiotic substances in soil such as pesticides and hydrocarbons [19–22].

The use of *Bacillus licheniformis* as a tool to obtain BS has been described. Furthermore, this bacteria is able to produce and secrete numerous hydrolytic enzymes that enable the microorganisms to degrade many different

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Table 1. Chemical characteristics and protein molecular-weight distribution (mean ± standard error, $n = 3$) of sewage sludge and biostimulants obtained by fermentation processes.

	SS	BS1	BS2	BS3	BS4
Dry matter (%)	5.1a[a] ± 0.4	5.3a ± 0.3	16.7b ± 1.1	5.2a ± 0.2	5.3a ± 0.3
pH	6.6a[a] ± 0.2	8.1b ± 0.1	8.3b ± 0.2	8.0b ± 0.2	8.2b ± 0.2
Organic matter (g kg^{-1})	472a[a] ± 14	470a ± 16	475a ± 18	470a ± 11	473a ± 15
N (g kg^{-1})	30.5a[a] ± 7.9	30.3a ± 7.0	21.6a ± 5.1	31.2a ± 7.1	30.0a ± 6.9
P (g kg^{-1})	12.2a[a] ± 1.4	12.0a ± 1.6	17.1a ± 1.9	12.1a ± 1.9	12.2a ± 1.5
K (g kg^{-1})	6.2a[a] ± 1.6	6.4a ± 1.2	6.2a ± 1.7	6.1a ± 1.6	6.4a ± 1.3
S (g kg^{-1})	16.6a[a] ± 1.4	16.6a ± 1.2	13.9a ± 1.1	16.9a ± 1.4	16.1a ± 1.2
Ca (g kg^{-1})	44.8a ± 4.1	43.5a ± 3.6	45.1a ± 2.7	45.1a ± 4.2	43.8a ± 3.2
Mg (g kg^{-1})	7.1a[a] ± 1.7	7.3a ± 1.4	8.1a ± 1.6	7.0a ± 1.6	7.3a ± 1.3
Fe (g kg^{-1})	18.7a[a] ± 1.5	18.9a ± 1.3	20.8a ± 1.8	19.0a ± 1.2	18.5a ± 1.3
Si (g kg^{-1})	25.5a[a] ± 2.6	25.9a ± 2.4	21.3a ± 1.7	25.9a ± 2.6	25.3a ± 2.1
Cu (mg kg^{-1})	341a[a] ± 14	344a ± 17	401a ± 15	344a ± 18	347a ± 12
Mn (mg kg^{-1})	166a[a] ± 4	160a ± 5	162a ± 5	165a ± 4	164a ± 4
Zn (mg kg^{-1})	864a[a] ± 15	874a ± 14	887a ± 22	860a ± 14	862a ± 17
Protein molecular-weight distribution (Da)					
>10,000	98.5b[a] ± 1.1	43.3a ± 1.4	ND	42.2a ± 2.1	43.9a ± 2.9
10,000–5000	0.0a[a] ± 0.0	14.7b ± 1.1	ND	15.1b ± 1.3	14.1b ± 1.6
5000–1000	1.5a[a] ± 0.8	11.5b ± 1.8	ND	12.6b ± 1.4	10.5b ± 1.4
1000–300	0.0a[a] ± 0.0	1.7b ± 0.3	ND	1.3b ± 0.2	1.2b ± 0.2
<300	0.0a[a] ± 0.0	26.7b ± 2.1	ND	28.8b ± 1.9	30.3b ± 2.2

Notes: SS: sewage sludge; BS1: biostimulant/biofertilizer 1; BS2: biostimulant/biofertilizer 2; BS3: biostimulant/biofertilizer 3; BS4: biostimulant/biofertilizer 4. ND: Not determined because the compound is insoluble.
[a]Files followed by the same letter(s) are not significantly different according to the Tukey test ($p < .05$).

substrates and grow in a wide range of nutrient sources [23]. These new products, which also act as biostimulants of soil microbial community, could be very useful in organic-xenobiotics-contaminated soils' bioremediation.

The objective of this paper was to study the influence of different BSs obtained by fermentation process from a sewage sludge on the biochemical characteristics of a Mediterranean agricultural soil.

2. Material and methods

2.1. Sampling of sewage sludge and fermentation process

The sewage sludge was supplied by the Experimental Water Treatment Plant of the Center for New Water Technologies (CENTA) located in Carrión de los Céspedes (Seville, Spain). The sludge had a maturity of 4 months and its chemical characteristics are described in Table 1. The methodology used in determination of pH, macro and micronutrients and molecular mass distribution was the same, as described in Rodriguez-Morgado et al. [17].

In order to obtain the different experimental BS, the sewage sludge was subjected to a fermentation process. For this purpose, sewage sludge was autoclaved, inoculated with *B. licheniformis* ATCC21415 (2% w/w) and incubated under agitation for 7 days at 37°C, thus obtaining what was termed as BS1 (Figure 1).

Once this BS was obtained, the following treatments were performed to obtain the rest of experimental BS:

(1) Fermented sludge was centrifuged, obtaining a liquid fraction that was discarded and a solid fraction, consisting mainly of microbial biomass generated during the process. This new product was called BS2 (Figure 2).
(2) Fermented sludge was centrifuged, obtaining a liquid fraction that was subjected to heat treatment in order to eliminate undesirable enzymes and a solid fraction. Both fractions were mixed again to obtain BS3 (Figure 3).

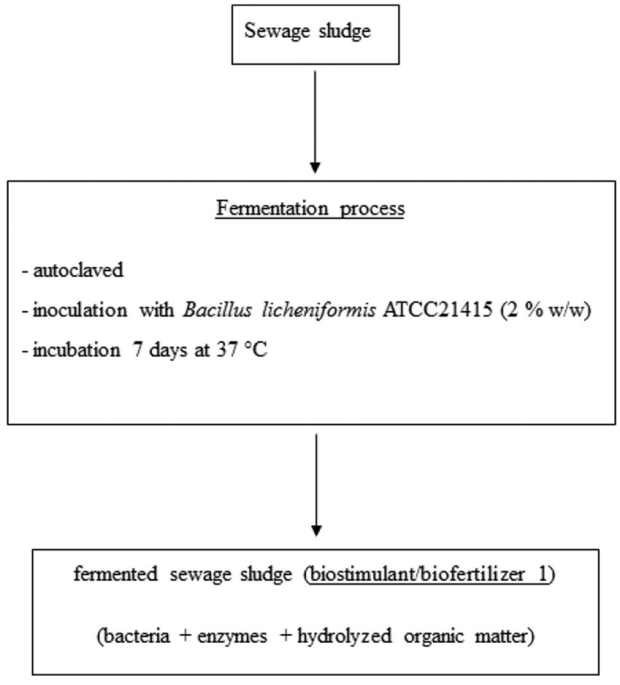

Figure 1. Process for obtaining the edaphic biostimulant/biofertilizer 1.

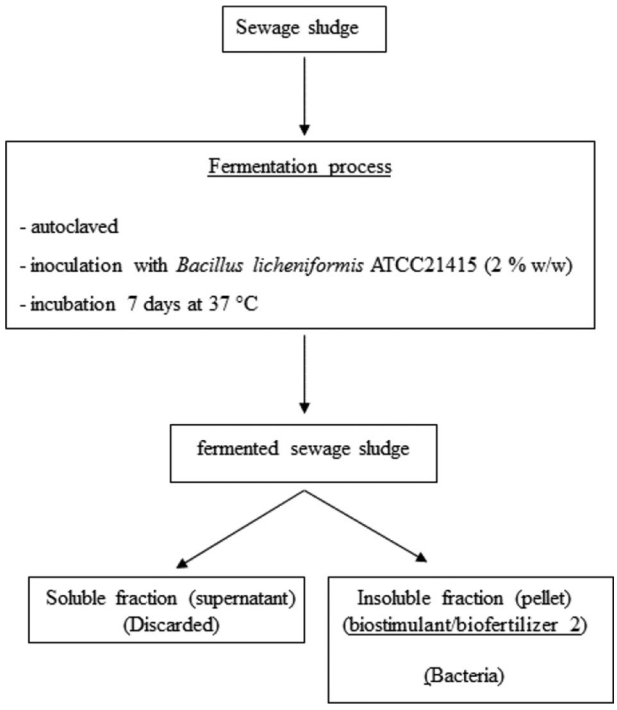

Figure 2. Process for obtaining the edaphic biostimulant/biofertilizer 2.

(3) To obtain BS4, fermented sludge was heat treated in order to destroy both enzymes and microorganisms (bacteria). BS4 is composed of organic matter only, with no biological or biochemical activity (Figure 4).

Chemical composition of these new biostimulants is shown in Table 1. As with sewage sludge, methodology used to determine all chemical parameters analysed is detailed in Rodríguez-Morgado et al. [17].

2.2. Biostimulation experiment design

Soil used in this experiment is a Calcaric Regosol [24]. Soil samples were collected at a depth of 0–25 cm. Main soil characteristics have already been reported [19] and are summarized in Table 2.

Soil microcosms were used to investigate the effects of four BS on soil biochemical properties. The microcosms consisted of 300 g grams of dry soil, sieved to 2 mm and placed in dark glass vessels.

Soil was amended with the four BSs obtained at a rate of 1% of organic matter due to the fact that the organic content of each BS was different. In this respect, soil samples were mixed with 60 g of BS1 (S + BS1 treatment), 38.6 g of BS2 (S + BS2 treatment), 60 g of BS3 (S + BS3 treatment) and 71.4 g of BS4 (S + BS4 treatment). An unamended soil was used as control (S). All experimental BSs were liquid, having been solubilized

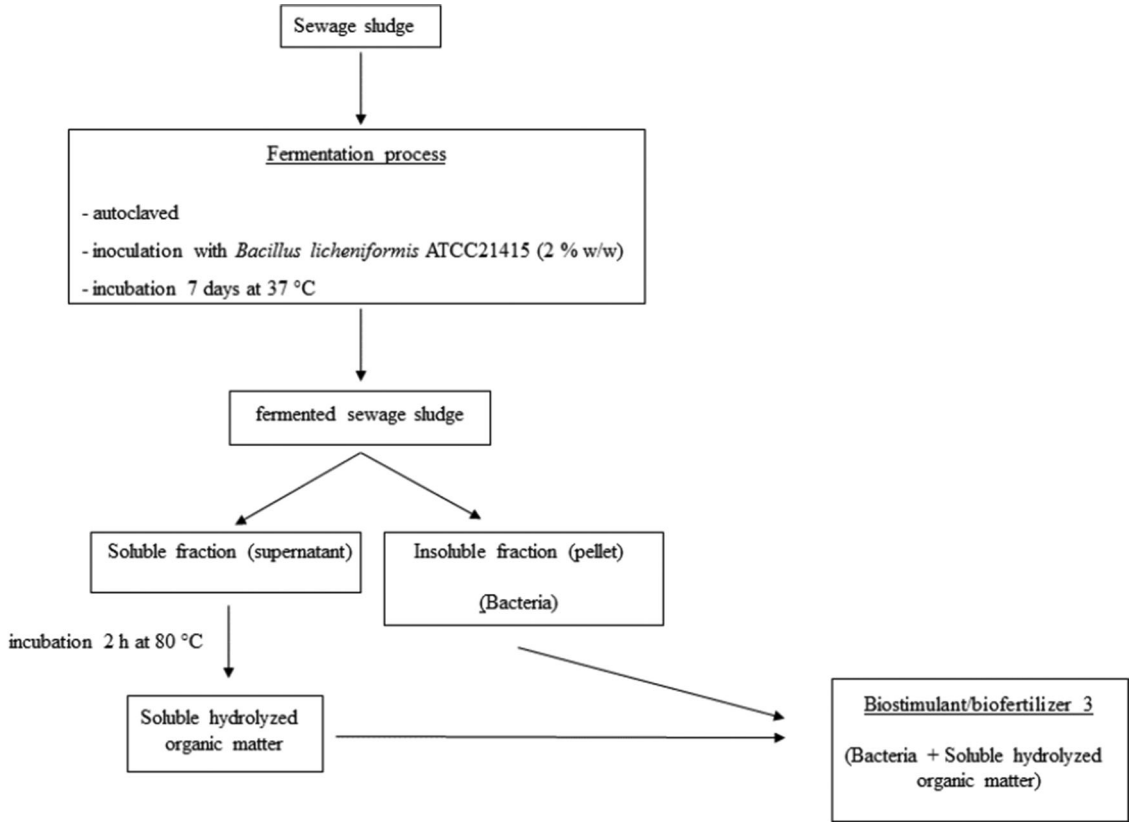

Figure 3. Process for obtaining the edaphic biostimulant/biofertilizer 3.

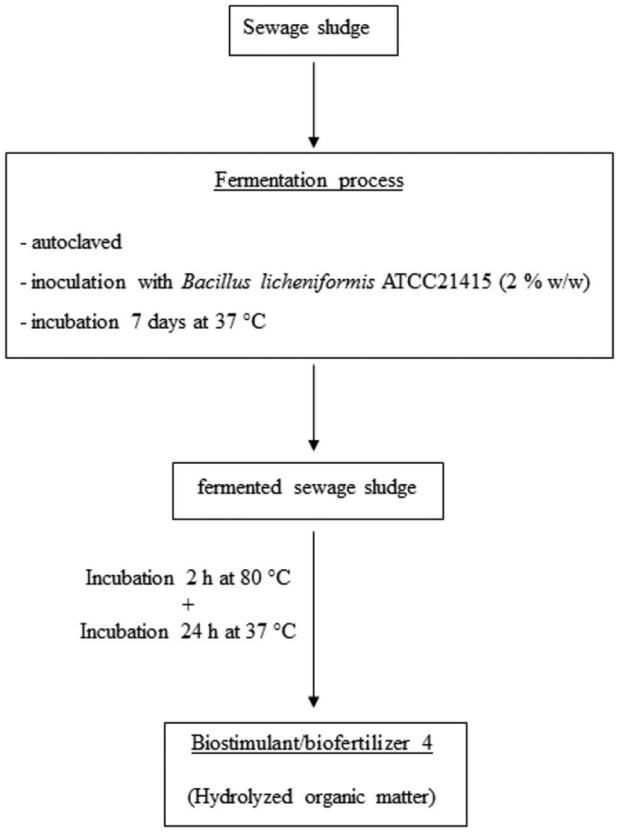

Figure 4. Process for obtaining the edaphic biostimulant/biofertilizer 4.

in distilled water prior to the application. During the experiment, distilled water was added to each soil to bring it to 60% of its water-holding capacity.

Assays were performed in triplicate and incubated at 25 ± 1°C for 50 days. For each treatment and each incubation time, 15 g of soil were taken. Soil samples were stored in sealed polyethylene bags at 4°C for 15 days, prior to analysis of enzymatic activities.

2.3. Soil analysis

At days 3, 7, 12, 20, 30 and 45 of incubation period and for each treatment, four soil enzyme activities were measured. Dehydrogenase activity was measured as the reduction of 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride (INT) to *p*-iodonitrotetrazolium formazan (INTF), as described by García et al. [25].

Table 2. Characteristics of the experimental soil (mean ± standard error, $n = 3$).

pH (H_2O)	7.8 ± 0.2
Course sand (g kg^{-1})	479 ± 55
Fine sand (g kg^{-1})	141 ± 17
Silt (g kg^{-1})	113 ± 18
Clay (g kg^{-1})	267 ± 24
Organic matter (g kg^{-1})	16.4 ± 1.3
Total N (g kg^{-1})	0.095 ± 0.05

Samples were incubated for 30 min at room temperature. The product concentration was measured at 485 nm using a GeneQuant 1300, spectrophotometer (GE Healthcare Bio-Sciences AB, USA). Controls were prepared without substrate.

Urease activity was determined by the buffered method of Kandeler and Gerber [26], using urea as substrate. In this determination, soil was mixed with a solution of urea (0.48%) and borate buffer and incubated for 2 h at 37°C. Ammonium content of the extracts was determined by a modified indophenol-blue reaction. Controls were prepared without substrate to determine ammonium produced in the absence of added urea.

β-glycosidase activity was determined mixing 0.1 M maleate buffer and *p*-nitrophenyl-*β*-D-glucopyranoside as substrate to 0.5 g of soil. Also, controls were prepared without substrate [27].

Phosphatase activity was measured using *p*-nitrophenyl phosphate as substrate [28].

Ergosterol was extracted with ethanol for 30 min by oscillating shaking at 250 rpm [29]. Ergosterol was determined by reversed-phase HPLC with 100% methanol as mobile phase and detected at a wavelength of 282 nm.

2.4. Statistical analysis

Data were submitted to two-way analysis of variance (ANOVA) with treatment and sampling time as factors followed by Tukey significant difference as a *post hoc* test, considering a significance level of $p < .05$ throughout the study. The ANOVA was performed using the Statgraphics Plus 2.1 software package. For the ANOVA, triplicate data were used for each treatment and every day of incubation.

3. Results and discussion

3.1. Process of obtaining BSs from sewage sludge

Table 1 shows chemical characteristics and protein molecular-weight distribution of sewage sludge and BSs obtained through fermentation process.

Firstly, results indicate that there was a significant difference in the pH value between sewage sludge (SS) and the new BSs. There are several authors who emphasize that pH is a critical parameter that can affect microbial growth directly. This is due to the fact that pH can greatly affect many enzymatic and transport processes that occur in the microorganism's cell membrane. Each species of microorganism has its own optimal pH range in order to be able to grown properly in any type of media [30,31].

In the case of *Bacillus sp.*, some authors suggest that this bacteria acts optimally in alkaline pH media, since at that pH range, it is optimally able to produce and excrete different hydrolases capable of degrading organic compounds in the medium in order to obtain energy [32,33]. Therefore, this is the possible explanation of why the BSs were basalized.

Statistical analysis of analyzed macro- and micronutrients indicates that there are no significant differences between these elements between SS and BSs.

On the other hand, after fermentation process and compared to SS, BSs protein molecular mass distribution showed a significant decrease in the fraction of high molecular-weight proteins (>10,000 Daltons) and a significant increase in the low molecular-weight fraction (<300 Daltons). There were no significant differences between the different BSs analyzed.

3.2. Soil biostimulation

Since dehydrogenase activity typically occurs in all living microorganisms, several authors suggest that this activity could be used as a good indicator of soil microbial activity [25,27]. Figure 5 shows evolution of

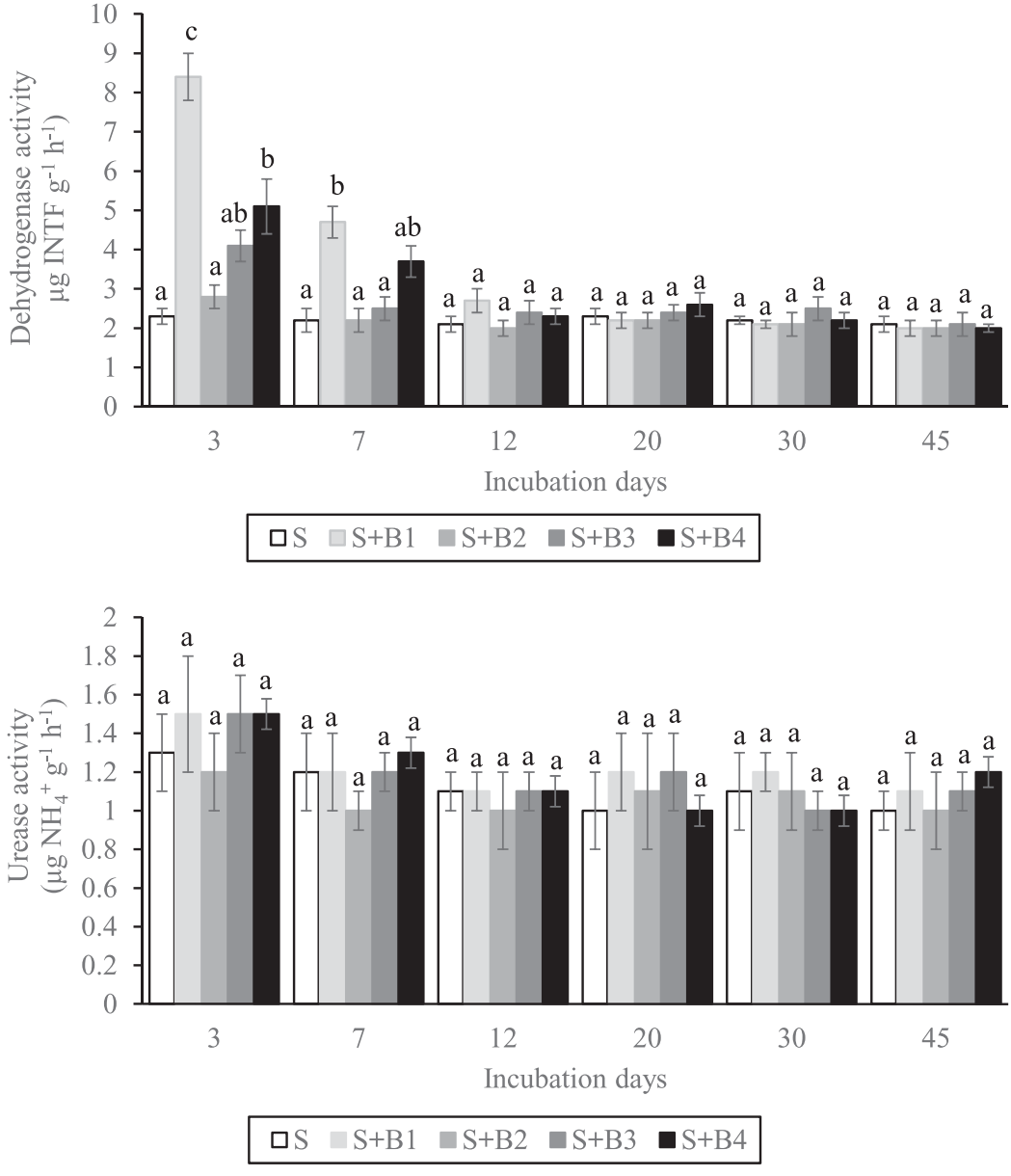

Figure 5. Evolution of dehydrogenase and urease activities (mean ± standard error, $n = 3$) in soils amended with the experimental biostimulants/biofertilizers obtained from sewage sludge during the experimental period. Columns followed by the same letter(s) are not significantly different ($p < .05$). INTF: 2-*p*-iodo-3-nitrophenyl formazan.

dehydrogenase activity during incubation time and for all experimental treatments. The results obtained indicate that this intracellular activity increased significantly, with respect to control soil, by 72.6%, 54.9%, 43.9% and 17.8% in the S + SB1, S + SB4, S + SB3 and S + SB2 treatments, respectively.

Rodríguez-Morgado et al. [17,20–22] and Tejada et al. [18,19] suggest that the major reason for this stimulation of dehydrogenase activity is the size of certain proteins that constitute or form part of organic matter. Increasing lower molecular-weight protein fraction increases stimulation of soil dehydrogenase activity. Decrease in molecular size of proteins indicates that N is more readily available to soil microorganisms. This, in turn, facilitates a better uptake of these compounds and consequently, a greater proliferation of microorganisms in soil [20]. Mooshammer et al. [34] also suggested that simple organic N compounds could be quickly utilized by microbes as energy and nutrient sources.

However, our experimental BSs show a similar protein size distribution and chemical composition. The only difference was the presence/absence of live bacteria and enzymes in these products. By adding BS1 to soil, the highest values of dehydrogenase activity were observed, possibly due to the fact that BS1 consists of organic matter + enzymes + bacteria.

Dehydrogenase activity began to decrease from day 3 of the experiment until the end of the incubation period, when it reached values similar to those of the unamended soil. These results are also in agreement with those obtained by Rodríguez-Morgado et al. [20] by applying to the soil other BSs obtained from sewage sludge, in this case by an enzymatic process. Dehydrogenase activity stimulation was, however, greater in soils amended with BS obtained by enzymatic hydrolysis than in soils amended with BS obtained by fermentation process.

Figures 5 and 6 show evolution of urease, *β*-glucosidase and phosphatase activities during incubation period. Results indicate that the application of BSs obtained by fermentation process did not stimulate enzymatic activities of soil. These results are very different from those obtained when applied BSs obtained from sewage sludge by an enzymatic hydrolysis process, which stimulated extracellular *β*-glucosidase and phosphatase activities in soil.

We think that during the manufacture of the new BS by fermentation processes, *B. licheniformis* excretes a large number of enzymes in order to obtain energy and nutrients for its development, thus degrading practically all organic compounds in the media [23]. Thus, when this type of BS is applied to the soil, the soil microorganisms do not need to excrete any extracellular enzymes to degrade organic compounds and thus obtain different nutrients necessary for their growth.

In contrast to dehydrogenase activity, at day 12 of incubation and compared with S treatment, ergosterol content in soils increased significantly by 43.3%, 36.3%, 31.5% and 28.1% in S + SB1, S + SB4, S + SB3 and S + SB2 treatments, respectively. Then, it progressively decreased to values similar to unamended soil (Figure 7) at the end of the experiment.

Several studies suggest that ergosterol, considered the most important fungal sterol, could be a sensitive indicator of soil fungal biomass [35–37]. Consequently, our results indicate that the increase of fungal biomass in amended soils depended on the chemical composition of BSs, being BS4 that exercised the greatest biostimulating effect.

Using metaproteomic and protein-stable isotope probing studies, Starke et al. [38] found that bacteria dominate fungi in the assimilation of N in short term. This is probably the reason why dehydrogenase activity showed stimulation 3 days after the beginning of experiment. When bacteria consumed BS, dehydrogenase activity decreased due to a reduction in bacterial population, probably caused by numerous opportunistic bacteria. According to Miltner et al. [39], the most abundant components of bacterial biomass are aminoacids and

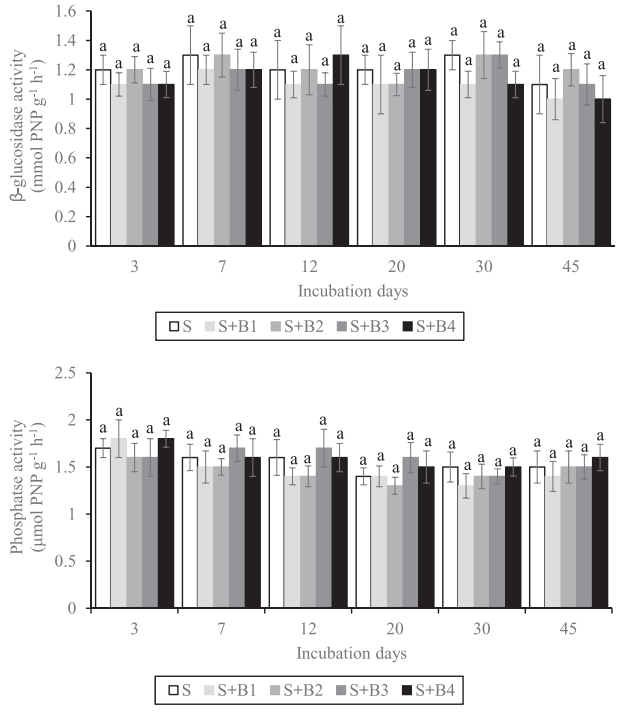

Figure 6. Evolution of *β*-glucosidase and phosphatase activities (mean ± standard error, $n = 3$) in soils amended with the experimental biostimulants/biofertilizers obtained from sewage sludge during the experimental period. Columns followed by the same letter(s) are not significantly different ($p < .05$). INTF: 2-*p*-iodo-3-nitrophenyl formazan.

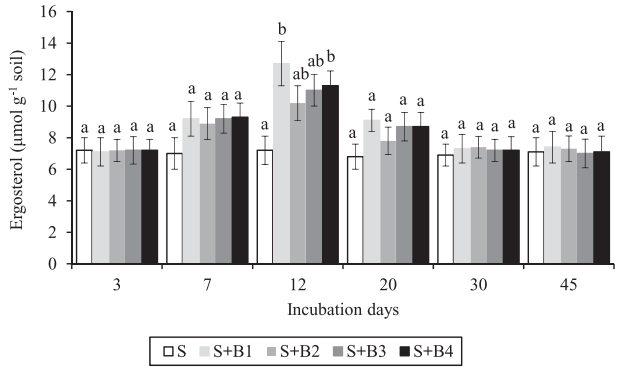

Figure 7. Evolution of ergosterol (mean ± standard error, $n = 3$) in soils amended with the experimental biostimulants/biofertilizers obtained from sewage sludge during the experimental period. Columns followed by the same letter(s) are not significantly different ($p < .05$).

proteins. These amino acids are possible sources of C and N for existing fungi, explaining why they show a maximum ergosterol content value at 12 days of incubation.

4. Conclusions

Our results suggest that new edaphic biostimulants can be obtained by treating sewage sludge by *B. licheniformis* in a fermentative process. The fundamental characteristic of these new biostimulants is their high content of low molecular-weight protein fraction, which considerably increases dehydrogenase activity when applied to soil. This decrease in the molecular size of proteins makes N assimilation by soil microorganisms very fast, which facilitates a greater proliferation of these microorganisms. However, this stimulation depends on biostimulant's chemical composition and consequently it depends on the type of the treatment employed. Rest of the extracellular enzymatic activities were not stimulated because Bacillus during fermentation process digests practically all complex organic substrates.

However, the effect of these new biostimulants obtained by the fermentation process needs further study. In order to have a deeper understanding of possible uses of these biostimulants, dosage rates as well as the treated soils' physical and chemical characteristics should be taken into account, either in agronomic studies or for the bioremediation of soils contaminated by organic xenobiotics.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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4. RESULTADOS Y DISCUSIÓN

4.1. Proteomic analysis of enzyme production by *Bacillus licheniformis* using different feather wastes as the sole fermentation media

Dado que la optimización de los procesos de producción resulta clave en la biotecnología en general y en los procesos fermentativos en particular, en este trabajo se han evaluado las plumas de pollo como medio de cultivo y de producción de enzimas para el organismo *B. licheniformis* a través de la manipulación de parámetros nutricionales y físico-químicos, estudiándose además el secretoma mediante técnicas proteómicas.

Caracterización química de las plumas

Las plumas están compuestas principalmente por queratina, una proteína fibrilar insoluble, rica en azufre y relativamente resistente a la degradación. Además de queratina, en las plumas existen otros componentes secundarios como son pequeñas cantidades de lípidos y carbohidratos.

En este estudio se han empleado dos tipos de plumas, la pluma TG, con un contenido en proteínas de más del 90% y un 2% de lípidos, y la pluma MU, con un contenido en proteínas en torno al 65% y un contenido en lípidos del 30%. Estas diferencias en el contenido en lípidos pueden ser determinantes a la hora de determinar las diferencias de crecimiento y producción extracelular observadas (Kim y col., 2001; Fakhfakh-Zouari y col., 2010; Kornillowicz-Kowalska y Bohacz, 2011).

Proceso de fermentación

Bacillus licheniformis es capaz de solubilizar en condiciones aeróbicas de ambos tipos de plumas a las concentraciones estudiadas sin el aporte de fuentes externas de carbono, nitrógeno u otros elementos. Esta solubilización es inversamente proporcional a la concentración de plumas, de forma que a los 5 días de incubación y a las concentraciones más

bajas (0,2 y 1%) la solubilización es total, no apreciándose restos sólidos de pluma. A la concentración más alta estudiada (5%), esta solubilización no fue completa, existiendo unos remanentes insolubles del 57 y 35% en las muestras MU y TG respectivamente.

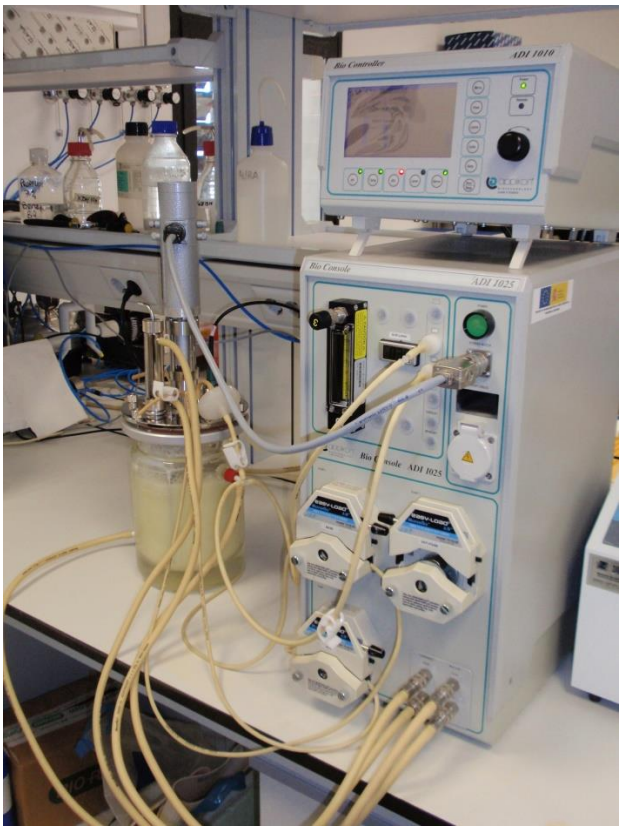

Figura 10: Cultivo de *B. licheniformis* en bioreactor de laboratorio.

Estas diferencias en las tasas de solubilización pueden ser debidas a una reducción en la excreción de enzimas o bien a un crecimiento menor de la bacteria. Con el fin de determinar si el caso se corresponde con la última hipótesis, se ha estudiado la actividad microbiana de los cultivos mediante la determinación de la actividad deshidrogenasa, enzima intracelular implicada en los procesos de fosforilación oxidativa e indicador de la actividad microbiana (Bolton y col., 1985).

Cuando *B. licheniformis* se cultiva en un medio rico (medio LB), se observa un rápido incremento de esta actividad a las 18 horas de incubación gracias a la alta disponibilidad de los nutrientes presentes. Sin embargo, cuando se cultiva en los medios compuestos por

plumas, este máximo de actividad se retrasa en las muestras TG y Mu (1%) a las 42 y 90 horas respectivamente. El retraso observado entre ambos tipos de plumas puede ser debido a la presencia de grasas que impiden la colonización superficial de la pluma por *B. licheniformis*.

La actividad deshidrogenasa no está vinculada directamente con la solubilización de la queratina, lo que sugiere que la excreción de enzimas no está relacionada con el nivel de crecimiento microbiano, pero si puede estar inducida por las condiciones de fermentación. De forma contraria a la solubilización de la pluma, la actividad microbiana está directamente relacionada con la concentración y tipo de pluma empleado, observándose los valores más altos en las muestras MU a concentraciones del 5%.

Cuando se estudió el perfil de pesos moleculares de las proteínas solubles mediante cromatografía de exclusión molecular, se observan diferencias entre las plumas MU y TG (1%). La muestra MU muestra un contenido mayor en la fracción de bajo peso molecular probablemente debido a la expresión diferencial de enzimas hidrolíticas observadas entre ambos tipos de plumas.

Producción de proteasas

Bacillus licheniformis es un microorganismo ampliamente empleado en la producción de enzimas de uso industrial principalmente proteasas (Pillai y col., 2011; Zaghloul y col., 2011), así como se ha descrito el uso de plumas como sustrato para la producción de proteasas por diversos bacilos (Wang y col., 2005; Fakhfakh-Zouari y col., 2010; Daroit y col., 2011).

La producción de proteasas por *B. licheniformis* cultivado en un medio queratinoso, al igual que la solubilización del sustrato, está inversamente relacionada con la concentración de pluma, independientemente de la pluma utilizada. A concentraciones relativamente altas (>1%), la producción de proteasas se encuentra afectada negativamente.

La excreción de proteasas por parte del genero *Bacillus* es una respuesta al agotamiento de nutrientes en el medio (Van Putten y col., 1996; Calik y col., 2002; Romero Ramírez y col., 2007), lo que explica la producción de esta enzima a bajas concentraciones de sustrato. Además, la presencia de un sustrato con una alta relación carbono / nitrógeno parece producir una mayor inducción de estas enzimas.

Producción de lipasas

Varias especies de *Bacillus*, entre ellas *B. licheniformis*, están reconocidas como productoras de lipasas, unas enzimas de uso industrial capaces de hidrolizar los triacil glicéridos (Nthangeni y col., 2001; Chen y col., 2004).

Las plumas de pollo constituyen un sustrato adecuado para la producción de estas enzimas, estando su excreción directamente relacionada con la concentración de sustrato. De esta forma, los mayores valores de actividad lipasa se han encontrado en las muestras al 5%. Aunque ambos tipos de pluma inducen esta enzima, las muestras TG presentan unos valores sensiblemente inferiores a los encontrados en las muestras MU, probablemente debido al mayor contenido en lípidos de esta última.

Comparando la producción de proteasas y lipasas, la pluma MU es la que produce una mayor inducción de ambas enzimas a causa del mayor contenido en fuentes de carbono de esta pluma (Kapoor y Gupta, 2002; Gupta y Rathi, 2004). De esta forma queda corroborado que la composición del medio afecta al secretoma de *B. licheniformis*.

Análisis proteómico

Para analizar la producción de proteínas extracelulares de *Bacillus licheniformis* cultivado en medios elaborados con plumas de pollo se han utilizado las concentraciones intermedias de sustrato (1%) así como la muestra crecida en medio rico (medio LB). En cualquiera de los medios utilizados *B. licheniformis* ha sido capaz de crecer y de excretar

diversas proteínas (Sinchaikul y col., 2002; Voigt y col., 2004; Harwood y Cranenburgh, 2007; Van Dyk y col., 2010).

Muchas especies del género *Bacillus* son capaces de producir diversas hidrolasas en respuesta a la presencia de ciertos inductores en el medio, como son los propios sustratos macromoleculares a hidrolizar (Chu y col., 2000; Sinchaikul y col., 2002). Esta inducción en la excreción de enzimas al medio extracelular tiene como objetivo la captación de nutrientes y energía.

La pluma TG induce principalmente la producción de una proteasa, la queratinasa (Ker A), probablemente debido al hecho de que esta pluma se compone en más de un 90% de proteínas (queratinas mayoritariamente). Esta enzima posee numerosos usos tanto industriales como agronómicos. Junto con la queratinasa, aunque en menor medida, se excreta la enzima Ggt o gamma-glutamil transpeptidasa (EC 2.3.2.2), que cataliza la transferencia de restos glutamilo desde diversos compuestos que lo contienen hasta una amplia variedad de aminoácidos y dipéptidos (Suzuki y col., 2003; Wu y col., 2006). Ambas enzimas se asocian para incrementar el espectro de acción de la queratinasa así como su potencial catalítico (Tiwary y Gupta, 2010a; b). Además de estas dos enzimas, *B. licheniformis* también excreta otras proteínas, como son glicosidasa a fin de obtener los azúcares existentes en forma de polisacáridos en las plumas TG, etc.

La pluma MU es capaz de inducir una mayor variedad de proteínas probablemente debido a que su composición es más compleja que la pluma TG. Aun así, la proteína más inducida en ambos casos es la queratinasa asociada a la gamma-glutamil transpeptidasa. La segunda proteína más producida por *Bacillus licheniformis* es una quitosanasa (EC 3.2.1.132), que cataliza la hidrólisis del quitosano. Dado que no existe quitosano en la composición de la pluma MU, este enzima puede aparecer debido a una actividad lipasa secundaria e inespecífica. Esta actividad es posible debido a la similitud entre los sitios activos de las

quitosanasas y las lipasas (Muzzarelli y col., 1995; Struszczyk y col., 2009). Se conocen varios casos de lipasas capaces de hidrolizar quitosano (Qin y col., 2002; Sashiwa y col., 2002; Muzzarelli y col., 2004), probablemente debido a la simplicidad y similitud de los mecanismos hidrolíticos implicados (Fu y col., 2003; Roy y col., 2003). De forma similar al medio formulado con pluma TG, la pluma MU también induce la excreción de otras hidrolasas como las glicosidasas.

Aunque tanto en las muestras TG como MU se produce la inducción de la enzima Ker A, el segundo tipo de pluma también induce la producción de otras proteasas con mecanismos de acción y especificidades distintas, lo que podría explicar el hecho de que el tamaño molecular de los péptidos en la muestra MU es menor.

Cuando *B. licheniformis* se crece en un medio rico (medio LB), se observa una amplia variedad de proteínas extracelulares y otras asociadas a la pared bacteriana, todas ellas producidas de forma basal (Antelmann y col., 2005; Voigt y col., 2005). También se detectó la presencia de Ker A y Ggt, lo que indica que estas proteínas se excretan de forma constitutiva.

4.2. Hydrolytic enzymes production by *Bacillus licheniformis* growth on fermentation media formulated with sewage sludge

Con el objetivo de encontrar un medio de cultivo económico y abundante para la producción de enzimas hidrolíticas por parte de *B. licheniformis*, hemos usado lodos de depuradora. En este medio, este organismo es capaz de crecer, aunque sin una gran producción de biomasa. Sin embargo, si se produce una importante producción de enzimas hidrolíticas extracelulares, como son proteasas, lipasas o celulasas, todas ellas de gran interés comercial e industrial.

La producción de enzimas extracelulares por microrganismos suele estar altamente influenciada por la composición del medio, por parámetros como la relación carbono / nitrógeno, presencia de fuentes de nutrientes fácilmente disponibles o iones metálicos (Kole y col., 1988; Ferrero y col., 1996; Varela y col., 1996). A esto se le unen otros factores como la oxigenación, la densidad del inóculo, pH, temperatura etc. (Nehete y col., 1985; Hameed y col., 1999).

Las proteasas son probablemente las enzimas más comercializadas del mundo, empleándose más de dos tercios de la producción en la industria de los detergentes (Gupta y col., 2002a). Este uso se debe principalmente a la existencia de las proteasas alcalinas, muchas de las cuales son excretadas por especies del género *Bacillus* (Ward, 1983). Estas proteasas alcalinas trabajan a pH entre 8,5 y 10, el cual es óptimo para muchas formulaciones de detergentes (Maurer, 2004). Otros usos de estas proteasas son la producción de alimentos, la industria farmacéutica, las curtidurías, etc. (Gupta y col., 2002b).

Bacillus licheniformis produce proteasas extracelulares durante la fase exponencial y al comienzo de la fase estacionaria (Kole y col., 1988), disminuyendo a continuación. Para aumentar los rendimientos de producción de proteasas, se ha estudiado la adicción de plumas

al medio de cultivo, produciéndose una inducción en la producción de estas hidrolasas (Ferrero y col., 1996; Varela y col., 1996, Parrado y col., 2014).

Las lipasas, enzimas que catalizan la hidrolisis de los triacil gliceroles, tienen un especial interés en industrias como la de los detergentes, alimentaria, química o farmacéutica (Patel y col., 2016). *Bacillus licheniformis*, organismo ampliamente reconocido como productor de estas enzimas, es capaz de producir lipasas cuando se cultiva en medios de fermentación formulados con lodos de depuradora. Además, la adición de plumas produce una inducción aún mayor. Esta inducción puede deberse al relativamente alto contenido en grasas de las plumas. No obstante, se observa como la producción en los medios sin suplementar empieza al inicio del cultivo, mientras que en el medio con plumas esta producción se retrasa hasta 4 días.

También se ha estudiado la producción de enzimas capaces de degradar los materiales celulósicos, como son las celulasas, ya que estas enzimas tienen un gran uso en procesos de biorrefinería como los de obtención de biocarburantes. *B. licheniformis*, reconocido también como productor de estas enzimas, es capaz de excretar celulasas cuando se cultiva en un medio con lodos de depuradora, comenzando esta producción al inicio del cultivo y alcanzando su valor máximo tras 5 días. Cuando se añade pluma de pollo al medio, también se produce una importante inducción de esta enzima, aunque se desconoce el motivo.

Aunque se determinaron otras actividades enzimáticas, como la actividad β-glucosidasa o la actividad fosfatasa ácida, no se detectaron indicios de estas. Las únicas actividades enzimáticas detectadas fueron proteasas, lipasas y celulasas, cuya producción fue inducida por la presencia de plumas de pollo en el medio de cultivo. Esto puede ser debido a que *B. licheniformis* excreta estas enzimas en respuesta a bajas concentraciones de fuentes de carbono o nitrógeno.

Cuando se estudiaron las proteínas extracelulares presentes en los distintos medios de cultivo, se observó un incremento en el número de proteínas detectadas por SDS-PAGE como consecuencia del crecimiento de *Bacillus licheniformis*, detectándose por medio de un análisis zimográfico tres proteasas distintas en las muestras M2 y M3.

Tras el análisis proteómico de las muestras, se observa como *B. licheniformis* excreta al medio diversas enzimas a fin de obtener los nutrientes necesarios para su supervivencia (Sinchaikul y col., 2002; Voigt y col., 2004; Harwood y Cranenburgh, 2007; Van Dyk y col., 2010). Entre estas enzimas se encuentran numerosas hidrolasas de macromoléculas, como proteasas, lipasas o polisacaridasas. La inducción de unas enzimas u otras depende de la presencia de compuestos inductores en el medio, como xilano, lípidos, etc. (Chu y col., 2000; Sinchaikul y col., 2002). Cuando se añaden plumas de pollo a un medio de cultivo, estas actúan como fuente de nitrógeno así como de inductor en la secreción de enzimas hidrolíticas como proteasas, lipasas, etc.

El análisis proteómico de las proteínas excretadas por *B. licheniformis* en el medio de cultivo M3 muestra que el 80% de estas eran hidrolasas, siendo predominante la queratinasa Ker A (proteasa). Esta enzima es típicamente producida por este organismo cuando se cultiva en medios con fuentes de nitrógeno insolubles como la queratina. Otra proteína identificada es la Ggt o gamma-glutamil transpeptidasa (EC 2.3.2.2), enzima que cataliza la transferencia de residuos glutamilo (Suzuki y col., 2003; Wu y col., 2006) y que se asocia a Ker A, aumentando el espectro de acción de la proteasa y mejorando su actividad catalítica. Este fenómeno tiene importantes aplicaciones industriales (Tiwary y Gupta, 2010a; b).

4.3. Obtaining biostimulant products for land application from the sewage sludge of small populations

Caracterización química de los productos

La hidrólisis enzimática de los lodos de depuradora (SS) generó dos productos: una fracción líquida y soluble (BF) y otra sólida (IP). El contenido en materia orgánica y proteínas de BF aumentó con respecto a los lodos de partida, así como algunos elementos como el potasio o el azufre, todos componentes esenciales en la nutrición vegetal. Sin embargo, el contenido de otros elementos como el fósforo disminuye en el extracto soluble, probablemente debido a procesos de precipitación.

Figura 11: Hidrólisis enzimática de lodos de depuradora.

El elevado contenido en materia orgánica y otros elementos esenciales de BF lo convierten en un producto de elevado interés para aplicaciones agronómicas con respecto a SS, cuyas características dependen del tipo de residuos y tratamientos recibidos (Singh y Agrawal, 2008). Aunque los lodos de depuradora se pueden usar como fertilizante, lo más común es compostarlos previamente. La aplicación de SS (Albiach y col., 2001) o de compost (Tejada y González, 2003) a los suelos es una técnica ampliamente utilizada en práctica agronómica y ambiental para conservar el contenido en materia orgánica de los suelos, recuperar suelos degradados y proporcionar nutrientes a las plantas. Sin embargo, el uso de los SS tiene varios inconvenientes como es el tiempo requerido para compostarlos, la generación de olores desagradables y la carencia de control sobre el proceso (Sikora, 1998). Además, hay que tener en cuenta también la baja biodisponibilidad de los nutrientes o la presencia de metales pesados que reducen sus posibles usos (Ranalli et al., 2001; Amir et al., 2005). A este respecto, se observa una disminución significativa en la concentración de metales pesados en BF con respecto al material de partida y un incremento en IP. Esto puede ser debido a que el proceso de hidrólisis tuvo lugar a un pH alcalino (pH 8,5), condiciones en las cuales los metales pesados precipitan. Estos metales no se degradan, por lo que se acumulan (Dudka y Miller, 1999) y finalmente acaban siendo transferidos al ser humano (Katsoyiannis y Samara, 2007). En el proceso de compostaje, el contenido en metales pesados depende de numerosos factores (Amir y col., 2005), sin embargo, el producto BF tiene un bajo contenido en estos elementos, lo que convierte al proceso de hidrólisis enzimática en un buen tratamiento para retirar estos metales y obtener un producto que no provoque daños ambientales.

Por otra parte, el contenido en materia orgánica de BF se aumentó con respecto a SS después del tratamiento enzimático. Al analizar el perfil cromatográfico de pesos moleculares, los SS presentan un elevado contenido en proteínas insolubles. Durante el proceso de

hidrólisis estas proteínas se rompen y el contenido en péptidos y aminoácidos libres aumenta, de ahí el alto contenido en péptidos de menos de 10 kDa. Esta disminución del tamaño molecular indica que el nitrógeno se encuentra en una forma altamente biodisponible para plantas o microorganismos (Higgings y Payne, 1982; Vasileva-Tonkova y col., 2007) al ser los péptidos un vehículo de transporte más efectivo que los aminoácidos libres (Higgings y Payne, 1982). En los suelos, las proteínas deben ser previamente hidrolizadas por proteasas extracelulares debido a que no abundan los transportadores de membrana capaces de incorporar proteínas completas (Jan y col., 2009). Sin embargo, los péptidos de bajo peso molecular y los aminoácidos libres pueden ser absorbidos fácilmente por plantas (Jones et al., 2009) y microorganismos (Jones y Hodge, 1999; Walker y Altman, 2005).

Análisis bioquímico del suelo

Tanto la aplicación de SS como de BF producen una estimulación de la actividad deshidrogenasa en los suelos tratados. Ambos productos proporcionan una fuente de nutrientes para los microorganismos del suelo así como acondicionan las propiedades de este (Logan y Harrison, 1995; Singh y Agrawal, 2008). Este efecto es dosis dependiente y varía con el tipo de materia orgánica empleada. Mientras que con SS, la actividad deshidrogenasa aumenta progresivamente, con BF este aumento se produce el primer día de incubación, y a los 5 días superan con diferencia los valores del suelo control y el enmendado con SS. En ese momento, el efecto de estimulación no es dependiente de la dosis aplicada. Tras 14 días de incubación y hasta el final del experimento, la estimulación de la actividad deshidrogenasa se hizo dosis dependiente, superando siempre a la obtenida con SS.

La actividad deshidrogenasa, implicada en el metabolismo respiratorio (Masciandaro y col., 2001), es un indicador de la capacidad bioestimulante de cada producto, la cual a su vez es mayor en BF. Esto puede ser debido al mayor contenido en materia orgánica y proteínas de bajo peso molecular de BF, lo que produce una mayor biodisponibilidad del nitrógeno. El

proceso de hidrólisis enzimática elimina la necesidad de romper las proteínas en péptidos y aminoácidos libres para poder ser absorbidas (Jones et al., 2009). De hecho, las proteínas tienen a resistir los procesos degradativos edáficos (Schulten y Schnitzer, 1998), dependiendo su degradación además del propio ecosistema (Joanisse et al., 2008).

La aplicación de SS y BF también produjo una estimulación de la actividad fosfatasa ácida, aunque el mayor efecto se observó en los suelos tratados con SS, mientras que en los suelos enmendados con BF este efecto fue menor. Esto puede ser explicado debido a que los organismos producen esta enzima en respuesta a necesidades de fósforo (García y col., 1994). Dado que el contenido de fósforo de SS es mucho mayor que el de BF y que se encuentra en formas más insolubles, mientras que el de BF está en forma soluble y disponible, los microorganismos deben producir fosfatasas a fin de solubilizar el fósforo insoluble de SS.

Con respecto a la actividad β-glucosidasa, ambos productos produjeron una estimulación similar a la observada con la actividad deshidrogenasa, siendo los valores de BF superiores a los de SS y detectándose de nuevo el efecto dosis dependiente.

4.4. Obtaining edaphic biostimulants/biofertilizers from sewage sludge using fermentative processes. Short-time effects on soil biochemical properties

Obtención de los BS

Tras el proceso fermentativo, se puede observar como los lodos de depuradora han sufrido diversos cambios, siendo los dos fundamentales la alcalinización y la disminución del tamaño molecular del componente proteico soluble de los productos fermentados con respecto a los lodos de partida.

Por una parte, cambios en el pH del medio influyen sobre los distintos organismos ya que afectan procesos enzimáticos y de transporte vitales, además de que cada especie posee un pH óptimo en el cual se desarrolla adecuadamente (Bhunia y col., 2012; Ibrahim y col., 2013). La alcalinización detectada se debe a que el microorganismo empleado en la fermentación es *Bacillus licheniformis*, una bacteria alcalófila capaz de modificar el pH del medio para adecuarlos a sus necesidades fisiológicas (Khosravi-Darani y col., 2008; Vijayalakshmi y col., 2013).

Por otro lado, la disminución del peso molecular del componente proteico soluble de los lodos es consecuencia de la actividad de diversas proteasas excretadas por este microorganismo durante su cultivo, las cuales hidrolizan las proteínas presentes en los lodos hasta péptidos de bajo peso molecular e incluso hasta aminoácidos libres.

Bioestimulación de suelos

La actividad deshidrogenasa es buen un indicador de la actividad microbiana del suelo al estar directamente vinculada con el metabolismo energético (García y col., 1993; Masciandaro y col., 1994). La aplicación de los distintos productos obtenidos a partir de lodos de depuradora provocó una significativa estimulación de esta actividad con respecto al

control. Esta estimulación fue mayor en los suelos tratados con BS1 probablemente debido a que se componen de materia orgánica soluble, enzimas hidrolíticas capaces de degradar la materia orgánica presente en el suelo y bacterias, siendo menor en el resto de suelos tratados al faltar alguno de dichos componentes en las formulaciones de los distintos productos.

El incremento de la actividad deshidrogenasa se dio en los primeros 3 días de incubación, disminuyendo a continuación a lo largo del tiempo hasta alcanzar los valores del control Esto se puede deber a que la materia orgánica y en particular el componente proteico de la misma, el cual es una fuente de nitrógeno que es un elemento limitante en el crecimiento microbiano, se encuentra hidrolizado en formas de bajo peso molecular fácilmente biodisponibles para los microorganismos del suelo, los cuales lo consumen rápidamente para su crecimiento (Rodríguez-Morgado y col., 2014, 2015a, b; Tejada y col., 2013, 2014; Mooshammer y col., 2014).

Con respecto a las actividades ureasa, β-glucosidasa y fosfatasa no se detecta bioestimulación ninguna en las muestras estudiadas. Esto puede ser debido a que durante el proceso fermentativo, las enzimas excretadas por *Bacillus licheniformis* han digerido las diferentes macromoléculas que contienen nitrógeno, azúcares y fosfato (Parrado y col., 2014). Al encontrarse estos compuestos en formas fácilmente biodisponibles, los microorganismos del suelo no necesitan excretar estas enzimas con el fin de extraer esos nutrientes del medio, por lo que no se detectan dichas actividades enzimáticas.

Finalmente, se estudió el contenido en ergosterol de las distintas muestras. Este esterol es el más abundante en los hongos y constituye un buen indicador de la presencia de biomasa fúngica (Olsson y col., 2003; Lau y col., 2006; Teste y col., 2016). Se observó una estimulación en la producción de este esterol en todas las muestras tratadas y de nuevo una mayor inducción en los suelos tratados con BS1 con respecto a los otros productos. Estos resultados se pueden explicar del mismo modo que los obtenidos con la actividad

deshidrogenasa. Sin embargo, a diferencia de la actividad enzimática, el valor máximo de ergosterol se detectó a los 12 días de incubación, probablemente debido a que el crecimiento de los hongos es más lento que el de las bacterias (Starke y col., 2016), digiriendo los cuerpos celulares de estás tras haber consumido los nutrientes aportados por los bioestimulantes (Miltner y col., 2009).

5. CONCLUSIONES

Tras haber desarrollado nuevos procesos para obtener biofertilizantes / bioestimulantes a partir de lodos de depuradora y plumas de pollo aplicando dos tecnologías distintas y después de caracterizar los productos obtenidos, se exponen a continuación las conclusiones más relevantes:

1. Es posible obtener productos biofertilizantes / bioestimulantes mediante el uso de tecnología enzimática o tecnología fermentativa a partir de subproductos agroindustriales como son los lodos de depuradora o las plumas de pollo.
2. Los productos obtenidos por ambas tecnologías son ricos en péptidos bajo peso molecular y aminoácidos libres, lo que les confiere una elevada capacidad bioestimulante.
3. Es posible crecer ciertos microorganismos, en particular la bacteria *Bacillus licheniformis*, en medios de cultivo formulados con lodos de depuradora, plumas de pollo o combinaciones de ambos.
4. Es posible producir enzimas hidrolíticas (proteasas y lipasas fundamentalmente) de forma relativamente económica empleando como organismo productor a *B. licheniformis* y como medio de cultivo lodos de depuradora, plumas de pollo o combinaciones de ambos.
5. La composición del medio de cultivo donde se crece a *Bacillus licheniformis* afecta al secretoma de este, alterando tanto cualitativa como cuantitativamente las proteínas secretadas.

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NOTAS:

CAPITULO 2

VALORIZACIÓN AGRONÓMICA DE LOS BIOESTIMULANTES OBTENIDOS A PARTIR DE LODOS DE DEPURADORA

1. INTRODUCCIÓN

1.1. FERTILIDAD EDÁFICA

Para que un suelo se considere fértil es necesario que pueda aportar las condiciones óptimas para el crecimiento y el desarrollo de las plantas y otros organismos del suelo. La fertilidad en sí misma es el resultado de la interacción entre las propiedades físico-químicas y biológicas del suelo.

Para el correcto desarrollo de cualquier cultivo, es necesario que el suelo en el que crece disponga, además de otros parámetros, de los nutrientes necesarios o en caso contrario será necesario aportarlos de forma exógena en forma de fertilizante.

Los elementos más demandados por los cultivos agrícolas son nitrógeno, fósforo y potasio, implicados en el crecimiento, floración y fructificación de las plantas. Debido a esta demanda continua, los suelos suelen presentar deficiencias de los mismos tras un tiempo. Mediante el uso de los fertilizantes se suministran estos elementos además de otros con el fin de suplir dicha deficiencia y al mismo tiempo permitir un uso más intensivo para obtener rendimientos superiores.

Actualmente, se está produciendo un consumo anual de 6.691,3 millones de toneladas de fertilizantes en todo el mundo, 5 millones de las cuales en España. (https://www.mapama.gob.es; https://datos.bancomundial.org) Además, este consumo está aumentando con una tasa de aproximadamente el 2% anual (Figura 12), lo cual genera varios problemas, como la contaminación de suelos y acuíferos por el uso indiscriminado de estos fertilizantes o la propia contaminación generada durante el proceso de producción de estos fertilizantes.

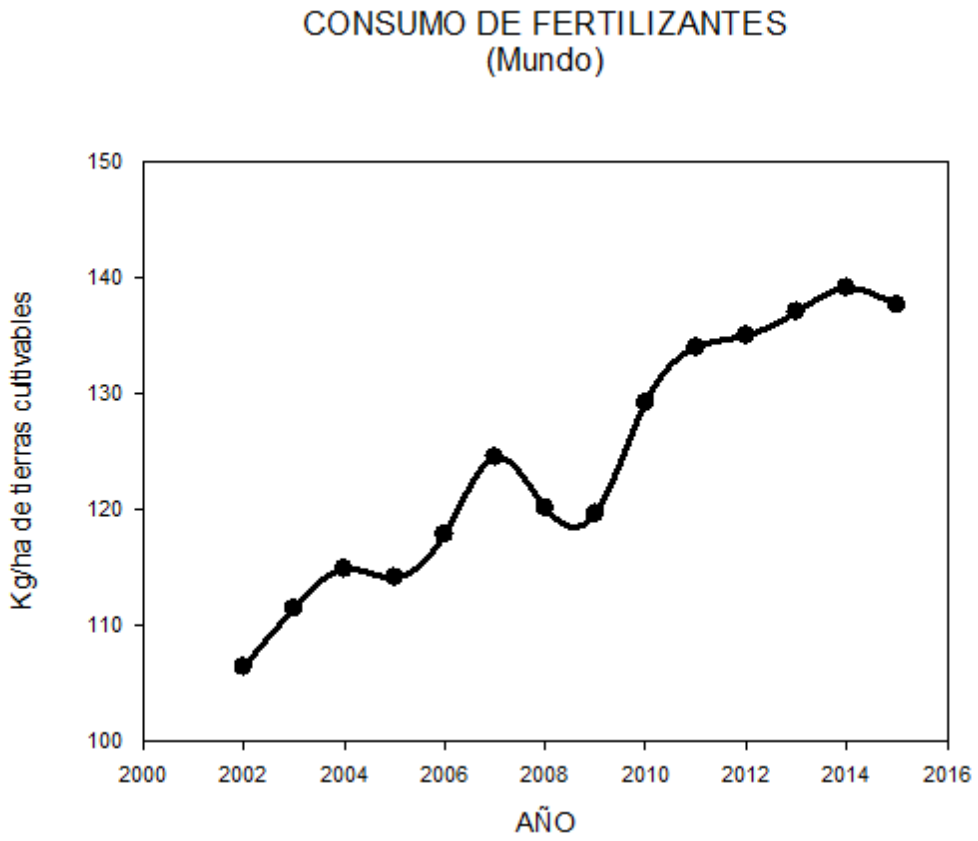

Figura 12: Consumo anual de fertilizantes (kg/ha de tierra cultivable)

1.2. BIOESTIMULANTES

Los bioestimulantes, a diferencia de los fertilizantes químicos, son compuestos o mezclas de ellos y compuestos orgánicos que tras ser aplicados provocan un incremento de las tasas de crecimiento y desarrollo de los cultivos y/o de los distintos microorganismos que habitan en el suelo (Yakhin y col., 2016). Los mecanismos por los cuales se dan los procesos de bioestimulación van desde la propia incorporación de nutrientes o microorganismos beneficiosos hasta la activación de sistemas enzimáticos críticos, producción de hormonas o directamente la activación de los microorganismos existentes. (Chen y col., 2002; Parrado y col., 2008, García-Martínez y col., 2010; du Jardin, 2015).

Sea el mecanismo que sea el que produzca este proceso de bioestimulación, su objetivo es aumentarla diversidad, número y principalmente, la actividad metabólica del cultivo, englobando en éste tanto a la planta en sí misma como al medio que la rodea, es decir, rizosfera, microorganismos superficiales, etc. (Yakhin y col., 2016).

1.3. BIOESTIMULACIÓN MICROBIANA EN SUELOS

La bioestimulación microbiana consiste en añadir al suelo que se quiere tratar nutrientes y/u oxígeno, de forma que los microorganismos nativos que se encuentran allí encuentran unas condiciones más favorables para su crecimiento, aumentando en consecuencia su población (Pankrantz, 2001). Este incremento de la población de microorganismos puede tener como objetivo el aumento de fertilidad del suelo en cuestión, o en el caso de suelos contaminados, aumentar la tasa de biodegradación del contaminante que se encuentra en el mismo.

1.4. TIPOS DE BIOESTIMULANTES

Aunque existe una gran variedad de productos clasificados como bioestimulantes, se pueden englobar en dos grupos principales, los bioestimulantes inorgánicos y los orgánicos.

1.4.1. Bioestimulantes inorgánicos

Los bioestimulantes inorgánicos o generalmente conocidos como fertilizantes inorgánicos o químicos, aportan principalmente y casi exclusivamente N, P y K además de otros micronutrientes en forma inorgánica a base de sales de dichos elementos. Estos productos presentan numerosos inconvenientes, ya que pueden tener un efecto inhibidor (Geisseler y Scow, 2014), tanto de la cantidad como de la diversidad de las poblaciones

microbianas del suelo. Además, existe una alta probabilidad de contaminación de los suelos y acuíferos (Ruberto y col., 2003).

1.4.2. Bioestimulantes orgánicos

Este tipo de bioestimulantes se caracterizan, en general, por poseer un elevado contenido en materia orgánica. Entre los compuestos químicos que los componen se encuentran biomoléculas como proteínas, azúcares, lípidos, etc. Los bioestimulantes orgánicos a su vez se subdividen en bioestimulantes orgánicos no hidrolizados y bioestimulantes orgánicos hidrolizados.

Debido a la importancia de dichos bioestimulantes orgánicos a nivel agrícola y puesto que el tema principal de esta memoria se basa en compuestos orgánicos de estas características, pasamos a la descripción más detallada de tales compuestos.

1.5. BIOESTIMULANTES ORGÁNICOS

1.5.1. Bioestimulantes orgánicos no hidrolizados

Los bioestimulantes orgánicos no hidrolizados son principalmente productos originados a partir de procesos de compostaje y vermicompostaje, tales como estiércoles, purines, etc. (Namkoong y col., 2002; Tejada y González, 2007; 2009; Aynehband y col., 2017; Goswami y col., 2017). La aplicación al suelo agrícola de este tipo de compuestos conlleva una mejora en las propiedades físicas, químicas del suelo, repercutiendo así en el crecimiento y desarrollo de los cultivos y en la producción y calidad de los cultivos (Tejada y Benítez, 2011; Cesarano y col., 2017; Cotching, 2018; Maharjana y col., 2018; Wood y Baudron, 2018).

Normalmente, la composición química de este tipo de materia orgánica en este tipo de bioestimulantes se caracteriza por estar constituida por proteínas y péptidos de alto peso molecular (Franco-Andreu, 2017). Por ello, para que la materia orgánica proporcione nutrientes esenciales a la planta, necesita un tiempo para mineralizarse (Tejada y González, 2007; 2009), por lo que hay un lapso de tiempo entre la aplicación del fertilizante y la absorción de nutrientes por parte de la planta. Este tiempo de mineralización es variable y depende principalmente de la composición química de la materia orgánica, así como de las características físico-químicas del suelo, la humedad y la temperatura (Tejada y col., 2014).

Por otra parte, también afecta a la absorción de los nutrientes la forma química en la que se encuentran, lo cual a su vez depende de las propiedades del suelo y de las reacciones químicas que se dan en el mismo (Lehmann y Kleber, 2015). Otro factor implicado es la propia dinámica de absorción de los nutrientes por parte de las plantas, la cual es bastante compleja y varía con la etapa de crecimiento del cultivo, influyendo en qué formas químicas son captadas preferentemente por la planta y cuáles no (Bindraban y col., 2015).

Además, los bioestimulantes orgánicos no hidrolizados presentan otras desventajas como son una composición menos controlada y definida, debido a sus materias primas de partida y a su proceso de producción; o composiciones desequilibradas donde ciertos elementos aparecen en exceso mientras que otros se encuentran ausentes.

1.5.2. Bioestimulantes orgánicos hidrolizados

Los bioestimulantes orgánicos hidrolizados son productos obtenidos por procesos de hidrólisis, principalmente de tipo enzimática, a partir de diversas materias

orgánicas. En este proceso de hidrólisis las biomoléculas orgánicas complejas que componen la materia prima son digeridas hasta sus componentes básicos, como son las proteínas en péptidos y aminoácidos libres, los polisacáridos en oligosacáridos y monosacáridos, etc., liberándose en este proceso otras moléculas potencialmente beneficiosas asociadas a las anteriores como ácidos húmicos, fitohormonas, etc. (Parrado y col., 2008). A diferencia de sus macromoléculas de partida, estos hidrolizados son fácilmente asimilables por los distintos microorganismos y plantas, por lo que no es necesario mineralizarlos y son totalmente solubles, lo que mejora su disponibilidad física (Ordoñez y col., 2001; Gjalakshimi y Abbasi, 2004).

En el caso de los bioestimulantes orgánicos hidrolizados ricos en péptidos y aminoácidos libres, además de aportar el elemento nitrógeno de una forma fácilmente asimilable, también tienen otros efectos beneficiosos directos como son aumentar la resistencia de las plantas frente a diferentes tipos de estrés abiótio y biótico o ser precursores de diversas fitohormonas. Por otro lado, de forma indirecta mejoran las propiedades químicas de los suelos, aumentando y estimulando las poblaciones microbianas del suelo y como consecuencia de todo esto, mejoran los rendimientos y calidad de los cultivos donde son aplicados (Yakhin y col., 2016).

1.6. LODOS DE DEPURADORA Y AGRICULTURA

Los lodos de depuradora urbana son los residuos obtenidos por concentración de la fase sólida procedente de la depuración de las aguas residuales urbanas (Kulikowska y Sindrewicz, 2018). Durante muchos años, los lodos de depuradora, debido a su alto contenido de materia orgánica y nutrientes esenciales, como N, P, S, Ca y S, se han aplicado comúnmente a los suelos agrícolas para mejorar las propiedades físicas, químicas y biológicas de dichos suelos y para proporcionar nutrientes esenciales a las

plantas, constituyendo a su vez uno de los métodos más económicos y sostenibles de eliminación de estos lodos, usándose de este modo hasta el 80% de los lodos generados en España (Pathak y col., 2009; Kang y col., 2011; Masto y col., 2012; Roig y col., 2012; Tejada y col., 2013; Ramdani y col., 2015).

Sin embargo, los lodos de depuradora también se caracterizan por tener unos elevados niveles de metales como cadmio, plomo, zinc, etc.; contaminantes orgánicos como son los compuestos orgánicos halogenados, hidrocarburos aromáticos policíclicos, bifenilos policlorados, sulfonatos de alquilbenceno lineales, bis (2-etilhexil) ftalato, etoxilatos de nonilfenol, dibenzodioxinas o dibenzofuranos; y organismos patógenos, por ejemplo, huevos de helmintos, quistes de protozoos, *Escherichia coli, Salmonella*, etc. que podrían constituir una fuente de contaminación tanto para el suelo como para las aguas subterráneas, pudiendo llegar incluso a la cadena alimentaria (Ranalli y col., 2001; Jouraiphy y col.,2005; Mosquera-Losada y col., 2010; Kulikowska y Sindrewicz, 2018).

Desde hace varios años, la Comisión Europea trata de fomentar el uso beneficioso de los lodos de depuradora en agronomía, siempre que la calidad del lodo sea compatible con los requisitos ambientales y sanitarios, evitando el uso directo salvo contadas excepciones. Para solventar los problemas propios de la aplicación directa de los lodos se requiere de un tratamiento ya sea químico, biológico o térmico para minimizar e incluso eliminar estos efectos indeseables y al mismo tiempo mejorar la eficiencia de los nutrientes una vez son aplicados al suelo (Directiva 91/271/CEE; Roig y col., 2012).

Entre los diferentes métodos de tratamiento de los lodos de depuradora, el compostaje es uno de los preferidos entre los investigadores. Durante este proceso, la masa y la humedad del lodo de aguas residuales disminuyen. Además, el compostaje

descompone la materia orgánica biodegradable y, debido al calor generado, destruye los patógenos (Figura 13) (Kulikowska y Sindrewicz, 2018).

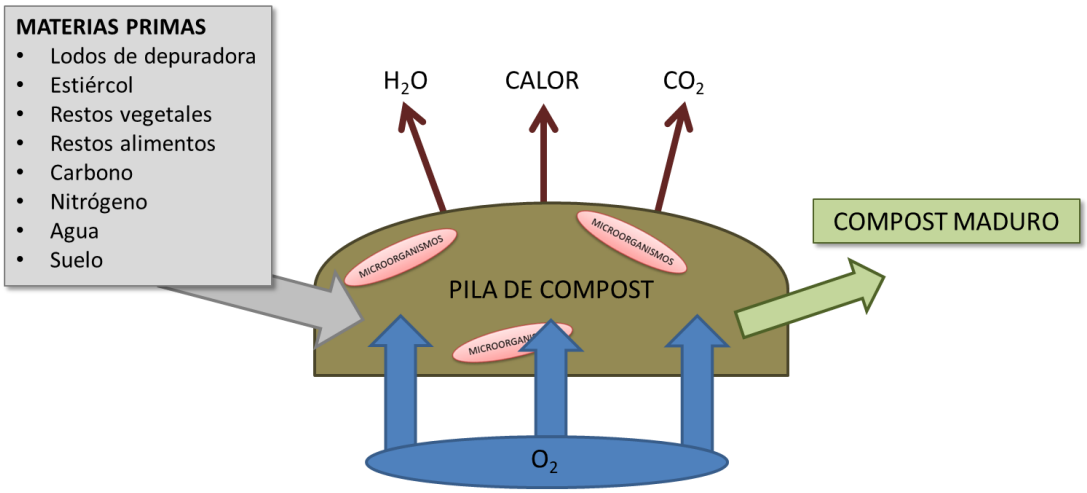

Figura 13: Esquema proceso de compostaje

El compostaje es el principal enfoque biológico para tratar los lodos de depuradora. Este proceso, además de eliminar los organismos potencialmente dañinos, principalmente patógenos, ayuda a estabilizar la materia orgánica y a reducir la disponibilidad de diversos metales tóxicos en los suelos enmendados, convirtiendo los lodos de depuradora en una materia adecuada para aplicaciones agrícolas (Kang y col., 2011).

Sin embargo, el compostaje de lodos de depuradora conlleva varios problemas. Por un lado el alto contenido de humedad y la baja relación C/N que son responsables de significativas pérdidas de Nitrógeno en forma de amoníaco durante el proceso de compostaje, lo que disminuye la calidad del compost obtenido. Además, se debe tener en cuenta el largo tiempo de procesamiento, el bajo nivel de control sobre el proceso y la presencia de sólidos grandes que dificultan algunas aplicaciones agronómicas, como puede ser la fertilización foliar o el bajo valor añadido de los productos finales (Ranalli y col., 2001).

2. OBJETIVOS

Como consecuencia de la importancia de la materia orgánica en la mejora de propiedades físicas, químicas y biológicas de los suelos, así como elemento fundamental en los planes de fertilización en el campo agrícola para no solo aumentar la productividad de las cosechas sino también de mejorar la calidad del grano o fruto recogido, los objetivos planteados en el presente capítulo se describen a continuación:

1. Estudiar la capacidad bioestimulante de los productos obtenidos a partir de lodos de depuradora sobre los microorganismos del suelo a nivel bioquímico y biológico, en particular, sobre las actividades enzimáticas vinculadas a los ciclos biogeoquímicos y sobre la diversidad microbiana. La información obtenida en este aspecto nos daría una importante idea del posible comportamiento de dichos bioestimulantes sobre la nutrición mineral de las plantas.
2. Estudiar la capacidad fertilizante del bioestimulante obtenido a partir de plumas de pollo sobre un cultivo de maíz (*Zea mays*), aplicando dicho producto por vía foliar.

3. ARTICULOS

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Obtaining edaphic biostimulants/biofertilizers from different sewage sludges. Effects on soil biological properties

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We studied the influence of six edaphic biostimulants/biofertilizers (BSs) manufactured by the pH-stat method from different sewage sludge (SS): SS1 (an anaerobic mature sludge, one year old), SS2 (an aerobic young sludge, without maturation) and SS3 (an aerobic mature sludge, four months old), not previously autoclaved (A) and autoclaved (B), by analysing their effects on soil biological properties. Soil enzymatic activities were measured at 1, 3, 5, 7, 15, 30 and 60 days of the incubation period, whereas the 16S rDNA-DGGE profiles were determined at 0, 5 and 60 days. The enzymatic activities were significantly stimulated. The highest stimulation was found in the B2 treatment followed by B3, A2, A3, B1 and A1 treatments. Increasing the number of lower molecular weight proteins in the BS enhances the stimulation of soil enzymatic activities. The application of BS caused at 5 days of the incubation period temporal variations in the soil bacterial community structure.

Keywords: edaphic biostimulant/biofertilizer; sewage sludge; soil enzymatic activities; soil bacterial community

1. Introduction

After the implementation of the Council Directive concerning urban wastewater treatment, sewage sludge (SS) production has significantly increased in the European Union.[1,2]

SS contains high levels of major nutrients, such as N and P, and is rich in organic matter and, therefore, land application is the most commonly used method worldwide and it is considered as one of the most economical methods of sludge disposal.[2–5] The application of SS to agricultural and degraded soils improves their physical, chemical and biological properties.[6,7]

However, SSs are characterized by their high level of metals, organic pollutants and pathogenic organisms that could constitute a contamination source for the soil and groundwater, and may reach the food chain.[2] The current policy of the European Commission is to encourage the beneficial use of SS on land provided that the quality of the sludge is compatible with public health and environmental requirements. Therefore, to overcome the risks incurred by the direct use of SS in soil, a treatment is required to minimize and eliminate the undesirable effects and to optimize the efficiency of the materials once applied to the soil.[8]

Composting is the main biological approach to stabilize the SS because the composting process contributes to lower the availability of metals in amended soils.[3] However, the composting of SS has several problems including the long time of production, the low control of the process, the low value of the final products, the presence of large size solids that hamper some agronomic applications and the slow assimilation by soil microorganisms and plants.[9]

In recent years, there has been an increasing use of organic biostimulants/biofertilizers (BSs) obtained from the hydrolysis of different organic materials.[10,11] These BSs, generally comprising peptides, amino acids, polysaccharides, humic acids, etc., are directly absorbed by soil microorganisms and plants which spend a small amount of energy in the absorption process.[6,11,12] Therefore, the application of these BSs to the soil leads not only to an increased content of organic matter and macro- and micronutrients, but also to a significant activation of the soil's microbial community.

Some scientists have obtained a BS from SS by an enzymatic hydrolysis process with a low content of heavy metals.[6] This aspect is of great interest due to the toxic effect exerted by these heavy metals in soil microorganisms.[13] Furthermore, the obtained protein hydrolysate is characterized by a high organic N content consisting mainly of low molecular weight peptides. Tejada et al. found that after applying the BS obtained from

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SS to the soil, the soil enzymatic activities showed a higher increase when a BS with higher content of low molecular weight proteins was applied.[8] Lowering the protein molecular size makes N more readily available to soil microorganisms. This promotes an increased proliferation of soil microorganisms.[14]

However, the chemical and/or biological behaviour of these BSs in soil depends on the chemical composition of the SS used in the manufacture of these protein hydrolyzates. In this respect, this chemical composition is different depending on whether the sludge has occurred in aerobic or anaerobic conditions. Also, the sludge maturation state may change this chemical composition. For this reason, it would be very interesting to study the behaviour in soil of different BSs obtained from sludges of different origins.

The current literature indicates that soil enzymatic activities react faster than physical variables and/or after any chemical change in the soil and, therefore, they may be useful as early indicators of the various biological changes that may occur in soil.[15] On the other hand, the number of physiological groups of bacteria is also useful when measuring structural changes in soil due to several anthropogenic factors.[12,16] Therefore, the comparison of the soil enzymatic activities and biodiversity could be useful when evaluating the impacts of the different BSs on soils.

The aim of this paper was to study the influence of different BSs obtained from three different SS s, (1) an anaerobic mature sludge, one year old; (2) an aerobic young sludge without maturation and (3) an aerobic mature sludge, four months old, on soil, by analysing their effects on soil enzymatic activities and soil bacterial community.

2. Material and methods

2.1. SS s sampling

These SSs were provided by Experimental Plant at the Foundation Centre for New Water Technologies (CENTA). This plant of 41,000 m^2 received the wastewater from 2500 population equivalents from the municipality of Carrión de los Céspedes (Seville, Spain).

Three types of SSs were used:

(1) SS 1: an anaerobic mature sludge, one year old. Waste sludge generated in an Imhoff tank. This unit received 50 m^3 day^{-1} of raw wastewater. Settleable organic matter is accumulated at the bottom of this 30 m^3 reactor for at least 1 year under anaerobic conditions.
(2) SS 2: an aerobic young sludge, without maturation. Waste sludge from an extended aeration system with a treatment capacity of 60 m^3 day^{-1}. This fresh sludge was thickened in order to reduce the water content before its hydrolysis.
(3) SS 3: an aerobic mature sludge, four months old. Thickened sludge from a storing tank. The waste sludge obtained from the extended aeration systems was then stored in a thickening tank for 4 months in order to increase its dryness before its later treatment.

Table 1. Chemical characteristics and protein molecular weight distribution (mean ± standard error) of the three SSs (oven dry matter basis). Data are the means of three samples.

	SS 1	SS 2	SS 3
Dry matter (%)	6.4b[a] ± 0.9	2.2a ± 0.3	4.9b ± 0.7
pH	6.3a[a] ± 0.1	6.3a ± 0.2	6.6a ± 0.2
Organic matter (g kg^{-1})	238a[a] ± 15	356b ± 17	291a ± 13
N (g kg^{-1})	22.2a[a] ± 2.4	56b ± 3.6	45b ± 3.7
P (g kg^{-1})	11.8a[a] ± 1.2	26.6b ± 1.5	18.9a ± 1.7
K (g kg^{-1})	6.6a[a] ± 0.8	7.6a ± 0.5	5.5a ± 0.3
Ca (g kg^{-1})	77.1b[a] ± 10.3	31.4a ± 4.1	56.9b ± 12.1
Mg (g kg^{-1})	8.5b[a] ± 0.7	6.9a ± 0.5	8.4b ± 0.8
S (g kg^{-1})	9.1b[a] ± 1.2	6.5a ± 0.7	9.9b ± 1.5
Fe (mg kg^{-1})	15.9b[a] ± 1.6	5.4a ± 0.6	12.6b ± 1.1
Cu (mg kg^{-1})	330b[a] ± 18	160a ± 15	410b ± 25
Mn (mg kg^{-1})	160b[a] ± 16	97a ± 11	173b ± 15
Zn (mg kg^{-1})	83.4b[a] ± 15	55.7a ± 2.9	61.1a ± 3.2
Pb (mg kg^{-1})	45.1b[a] ± 4.5	14.5a ± 1.2	54.3b ± 1.8
As (mg kg^{-1})	5.0a[a] ± 0.8	3.5a ± 0.4	4.4a ± 0.6
Cd (mg kg^{-1})	2.0a[a] ± 0.4	2.2a ± 0.3	1.5a ± 0.3
Protein molecular weight distribution (Da)			
> 10,000	81.3a[a] ± 3.4	95.5a ± 3.1	98.5a ± 2.7
10,000–5000	18.7b[a] ± 2.3	0.0a ± 0.0	0.0a ± 0.0
5000–1000	0.0a[a] ± 0.0	0.0a ± 0.0	1.5b ± 0.4
1000–300	0.0a[a] ± 0.0	0.0a ± 0.0	0.0a ± 0.0
< 300	0.0a[a] ± 0.0	4.5b ± 0.9	0.0a ± 0.0

[a]Files (mean ± standard errors) followed by the same letter(s) are not significantly different according to the Tukey test ($p < .05$).

The three SSs were taken once at the beginning of the study and were physically and chemically characterized as described in Table 1. Organic matter content was determined by combustion at 550°C for 6 h. Total nitrogen was determined by the Kjeldahl method.[17] Macro- and micro-nutrients were determined after combustion and analysed by inductively coupled plasma atomic emission spectrometry (ICP-AES) using a Fisons-ARL 3410 sequential multielement instrument equipped with a data acquisition and control system. The standard operational conditions of this instrument are summarized as follows: the carrier gas, coolant gas and plasma gas are argon at 80 psi of pressure, the carrier gas flow rate is 0.8 l min^{-1}, the coolant gas flow rate is 7.5 l min^{-1}, the plasma gas flow rate is 0.8 l min^{-1} and the integration time is 1 s. One minitorch consumes argon gas at a radio-frequency power of 650 W.

The molecular mass distribution of protein in the samples was determined by size-exclusion chromatography

using an ÄKTA-purifier (GE Healthcare), using a Superdex Peptide™ 10/300GL column (optimum separation range 300–10,000 Da) (Table 1). Samples were centrifuged at 12.000 × *g* for 30 min at 4°C to remove insoluble molecules, and the supernatant was passed through a 0.2 µm filter and loaded into a 0.1 ml loop connected to an ÄKTA-purifier system. The column was equilibrated, and eluted with 0.25M Tris–HCl buffer (pH 7.0) in isocratic mode, at a flow rate of 0.5 mL min^{-1}, and proteins/peptides were detected at 280 and 215 nm with a GE Healthcare UV900 module coupled to the elution column.

2.2. *Enzymatic hydrolysis process*

Before performing the enzymatic hydrolysis process in each SS, the sludges were subjected to an autoclaving process in order to eliminate potential pathogens. Thus, six completely different sludges (three types of sludge × two autoclaving or not autoclaving process) were obtained. The hydrolysis process was carried out on every experimental sludge according to the pH-stat method,[18] using an endoprotease obtained by liquid fermentation of *Bacillus licheniformis* ATCC 21,415 as the hydrolytic agent. This process took place in a bioreactor. The conditions of this enzymatic hydrolysis process are detailed in Tejada et al.[6] However, Figure 1 shows a diagram of the enzymatic process. Thus, six types of BSs were obtained (Table 2).

Table 3 shows the chemical composition and protein molecular size distribution of the six protein hydrolysates obtained. Macro- and micro-nutrients and the size of each protein within the obtained hydrolysate were characterized by the methods described above.

2.3. *Biostimulation experiment design*

The soil used in this experiment is a Calcaric Regosol.[19] Soil samples were collected from the 0–25 cm surface layer. The main soil characteristics are shown in Table 4. Soil pH was determined in distilled water with a glass electrode (soil: H_2O ratio 1:2.5). Soil texture was determined by Robinson's pipette method.[20] Soil total N was determined by the Kjeldahl method.[21] Soil total C was determined by the method of Sims and Haby.[22] Total soil organic matter was obtained multiplying total soil organic carbon by 1.724.[21]

Table 2. Scheme of the biostimulants/biofertilizers obtained from different SSs.

SS types	SS autoclaved at 105°C	Biostimulant/biofertilizer obtained
SS 1	(−)	A1
SS 1	(+)	B1
SS 2	(−)	A2
SS 2	(+)	B2
SS 3	(−)	A3
SS 3	(+)	B3

(+): Yes.
(−): No.

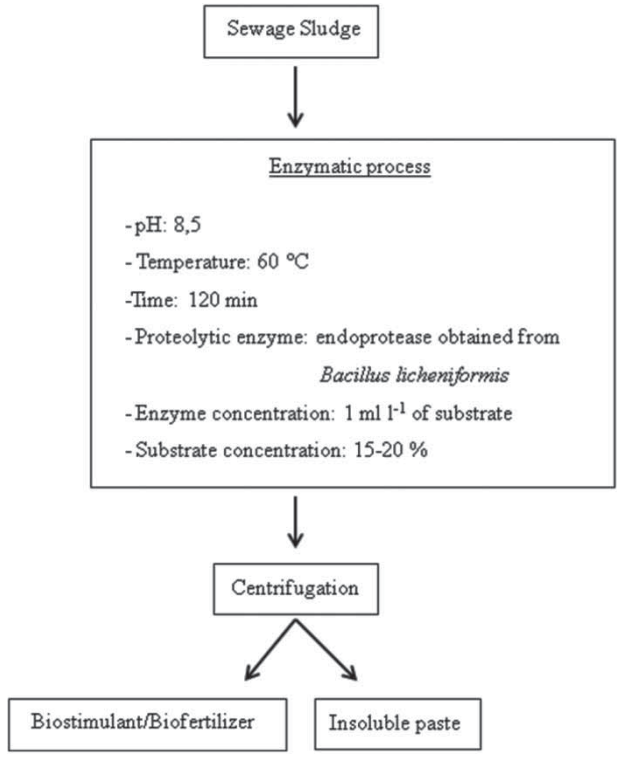

Figure 1. Enzymatic hydrolysis process used for obtaining biostimulants/biofertilizers from SSs.

Soil microcosms were used to investigate the effects of the six BSs on soil biological activity. These microcosms consisted of 500 g of dried and 2 mm sieved soil mixed and packed into a plastic container. Prior to the treatments, soil was pre-incubated at 25°C for 7 days at 30–40% of their water-holding capacity according to Tejada et al.[6].

Soil was amended with the different BSs obtained at a rate of 3 t organic matter ha^{-1}. Since the organic content of each BS was different, soil samples were mixed at different rates in order to apply to the soil the same amount of organic matter with each BS. In this respect, soil samples were mixed with B2 at a rate of 0.50%, or A1 at a rate of 0.74%, or A2 at a rate of 0.53%, or A3 at a rate of 0.59%, or B1 at a rate of 0.66% or B3 at a rate of 0.58%. An unamended soil was used as control. Distilled water was added to each soil to bring it to 60% of its water-holding capacity.

All BSs were liquid and were solubilized in distilled water before the application. Triplicate treatments were performed at 25 ± 1°C for 60 days. For each treatment and each incubation time, 20 g of soil were used. Soil samples were stored in sealed polyethylene bags at 4°C for 15 days, prior to analysis of the enzymatic activities, and at −20°C prior to soil DNA analysis.

Table 3. Chemical characteristics and protein molecular weight distribution (mean ± standard error) of the six biostimulants/biofertilizers obtained from the enzymatic hydrolysis process (oven dry matter basis). Data are the means of three samples. (A: biostimulants non-autoclaved; B: biostimulants autoclaved).

	A1	A2	A3	B1	B2	B3
Organic matter (g kg^{-1})	525a[a] ± 13	728b ± 18	640a ± 15	520a ± 19	773b ± 21	642a ± 17
N (g kg^{-1})	13.4a[a] ± 1.2	39.3b ± 2.6	33.5b ± 25	14.9a ± 1.3	34.9b ± 2.3	29.8ab ± 2.4
P (g kg^{-1})	2.4a[a] ± 0.2	2.6a ± 0.4	2.2a ± 0.2	2.7a ± 0.1	2.9a ± 0.1	2.8a ± 0.3
K (g kg^{-1})	27.1a[a] ± 2.2	25.5a ± 1.9	31.0a ± 2.8	18.5a ± 1.7	25.5a ± 1.5	26.0a ± 1.8
Ca (g kg^{-1})	88.4b[a] ± 7.2	62.6a ± 5.5	67.1a ± 4.9	88.6b ± 5.9	46.8a ± 4.2	73.6ab ± 6.6
Mg (g kg^{-1})	8.9a[a] ± 1.1	13.2a ± 1.6	8.4a ± 0.9	8.7a ± 1.5	8.6a ± 1.4	9.2a ± 1.6
S (g kg^{-1})	8.6a[a] ± 1.0	5.3a ± 1.2	8.8a ± 1.4	9.7b ± 1.2	5.9a ± 1.6	9.2ab ± 1.4
Fe (mg kg^{-1})	1.5a[a] ± 0.4	2.2a ± 0.4	1.5a ± 0.3	1.6a ± 0.5	1.8a ± 0.2	1.4a ± 0.3
Cu (mg kg^{-1})	37.5b[a] ± 4.7	19.7a ± 1.2	38.4b ± 2.2	31.6a ± 2.1	29.2a ± 2.6	32.5a ± 2.9
Mn (mg kg^{-1})	105.4b[a] ± 12.8	71.6a ± 15.1	118.2b ± 13.2	97.2b ± 4.1	44.3a ± 10.1	98.1b ± 6.2
Zn (mg kg^{-1})	0.85a[a] ± 0.13	0.72a ± 0.17	1.0a ± 0.11	0.83a ± 0.12	0.82a ± 0.14	1.1a ± 0.16
Pb (mg kg^{-1})	0.14a[a] ± 0.02	0.11a ± 0.04	0.19a ± 0.03	0.19a ± 0.03	0.12a ± 0.02	0.19a ± 0.02
As (mg kg^{-1})	0.19a[a] ± 0.04	0.13a ± 0.05	0.15a ± 0.03	0.17a ± 0.03	0.14a ± 0.02	0.16a ± 0.04
Cd (mg kg^{-1})	0.12a[a] ± 0.03	0.11a ± 0.04	0.15a ± 00.3	0.14a ± 0.04	0.16a ± 0.03	0.15a ± 0.03
Protein molecular weight distribution (Da)						
> 10,000	41.2b[a] ± 4.4	36.6ab ± 3.5	37.2ab ± 4.2	32.3a ± 2.9	21.4a ± 2.6	24.4a ± 3.2
10,000–5000	17.9b[a] ± 2.2	10.6a ± 1.4	13.3a ± 2.0	15.4b ± 2.6	7.3a ± 1.5	9.6a ± 2.4
5000–1000	9.4a[a] ± 1.6	10.9ab ± 2.0	12.7b ± 1.5	19.0b ± 3.3	5.5a ± 2.0	6.5a ± 1.1
1000–300	2.1b[a] ± 0.5	3.2b ± 0.9	1.9ab ± 0.4	2.2b ± 0.9	1.8a ± 0.4	0.7a ± 0.1
< 300	29.4a[a] ± 1.7	38.8a ± 3.1	35.0a ± 2.2	31.1a ± 3.7	64.0b ± 3.6	58.9b ± 2.8

[a]Files (mean ± standard errors) followed by the same letter(s) are not significantly different according to the Tukey test ($p < .05$).

Table 4. Characteristics of the experimental soil (mean ± standard error). Data are the means of three samples.

pH (H_2O)	7.9 ± 0.2
Coarse sand (g kg^{-1})	486 ± 49
Fine sand (g kg^{-1})	130 ± 25
Silt (g kg^{-1})	123 ± 29
Clay (g kg^{-1})	260 ± 35
Total N (g kg^{-1})	0.93 ± 0.08
Organic matter (g kg^{-1})	17 ± 1

2.4. Soil analysis

After 1, 3, 5, 7, 15, 30 and 60 days of the incubation period, and for each treatment, four soil enzyme activities were measured.

Dehydrogenase activity was measured as the reduction of 2-*p*-iodo-3-nitrophenyl 5-phenyl tetrazolium chloride to iodonitrophenyl formazan.[23] In this procedure, 0.1 g of soil was exposed to 0.2 mL of 4% INT (2-*p*-iodo-3-nitrophenyl 5-phenyl tetrazolium chloride) in distilled water for 20 h at 22°C in darkness. The iodonitrotetrazolium formazan (INTF) formed was extracted with 10 mL of a 1:1.5 mixture of ethylene chloride and acetone by shaking vigorously for 2 min. INTF was measured in a spectrophotometer at 490 nm. Controls were prepared without substrate.

Urease activity was determined using urea as substrate. [24] In this procedure, 0.5 mL of a solution of urea (0.48%) and 4 mL of borate buffer (pH 10) were added to 1 g of soil in hermetically sealed flasks, and then incubated for 2 h at 37°C. The ammonium content of the centrifuged extracts was determined by a modified indophenol-blue reaction. Controls were prepared without substrate to determine the ammonium produced in the absence of added urea.

Alkaline phosphatase activity was measured using *p*-nitrophenyl phosphate as substrate.[25] However, the incubation was at 30°C in maleate buffer (2 mL, pH 6.5) for 90 min and 0.5 mL of substrate (0.115 *p*-nitrophenyl phosphate) added to 0.5 g to soil. Controls were prepared without substrate.

β-Glucosidase activity was determined using 2 mL of 0.1 M maleate buffer (pH 6.5) and 0.5 mL of 50 mM *p*-nitrophenyl-β-D-glucopyranoside (PNG) to 0.5 g of soil. The rest of the method was the same as for the alkaline phosphatase activity.[26]

After 0, 5 and 60 days of the incubation period, and for each treatment, a direct method was used to extract DNA from soil, using an UltraClean Soil DNA Kit (Mo Bio Laboratories, Solana Beach, Calif.). Samples of 1 g of soil were processed according to the manufacturer's instructions. Aliquots of DNA were analysed on 0.7% agarose gel containing 0.5 µg ml^{-1} of ethidium bromide and quantified spectrophotometrically.

Soil DNA was amplified in a PCR Sprint thermocycler (Hybaid, Ashford, UK). Two sets of universal primers were used for bacteria rDNA. 968F-1401R eubacterial universal primers were used to amplify a 500 bp region of the 16S rDNA. PCR amplicons were analysed by denaturing gradient gel electrophoresis (DGGE). Each

PCR mixture contained 100 ng of DNA, 1 × reaction buffer supplemented with 2.5 mM $MgCl_2$, 50 pmol of each primer, 0.2 mM of each dNTP, 3 Units Taq-polimerase (Euroclone) in a final volume of 50 µL. The PCR protocol for the 16S rDNA region targeting total bacteria consisted of 3 min at 95°C followed by 40 cycles, each consisting of a denaturing step (10 s at 95°C), primer annealing (20 s at 54°C) and an extension step (40 s at 72°C); a final extension step (10 min at 72°C) was finally carried out. Amplification products together with a Low Range ladder (1000–80 bp – MBI Fermentas) were checked by electrophoresis on ethidium bromide stained with 1.5% agarose gel run at 10 V cm^{-1} in 0.5 × TBE buffer.

DGGE was carried out using the Bio-Rad Dcode system. PCR products (30 µL) were loaded into 6% polyacrylamide gel (acrylamide: bisacrylamide, 37:1) with a parallel gradient of 40% urea formamide on the top and 60% at the bottom of the gel (100% urea formamide corresponded to 7 M urea and 40% v/v formamide) and run for 16 h at 5 V cm^{-1} at 60°C in 1 × TAE buffer. DNA band(s) were visualized by staining with SYBER green (Invitrogen) and photographed using a 1D Scientific Imaging System and a Kodak DC 290 Zoom Digital Camera (New Haven, Conn.).

2.5. *Statistical analysis*

Data were submitted to two-way ANOVA with treatment and sampling time as factors followed by a Tukey significant difference as a post hoc test, considering a significance level of $p < .05$ throughout the study. The ANOVA was performed using the Statgraphics Plus 2.1 software package. For the ANOVA, triplicate data were used for each treatment and every day of incubation.

Cluster analyses of the electrophoretic profiles were performed using the Quantity One software of the Bio-Rad Gel Doc image analyser system. The similarities of the banding patterns were evaluated calculating the dice coefficients and using the unweighted pair group method with average linkage (UPGMA).[27]

3. Results

3.1. *Process of obtaining BSs from SSs*

Table 1 shows the chemical characteristics and protein molecular weight distribution of the three SSs used in the production of each experimental BS, whereas Tables 2 and 3 show the different BSs obtained for the enzymatic hydrolysis process from the three SSs previously mentioned. First, the results showed that each BS obtained had a lower heavy metal and P content than the SSs before the enzymatic hydrolysis process. On the other hand, the enzymatic hydrolysis process caused a higher number of smaller molecular size proteins. Furthermore, the results indicate that the autoclaved BS reached the highest level of low molecular weight peptides (< 300 Da). Also, the sludge type influenced the protein size. Thus, the highest number of smaller molecular weight proteins was originated in sludge type 2, followed by 3 and 1.

3.2. *Soil analysis*

Statistical analysis indicated a significant ($p < .05$) stimulation of the dehydrogenase activity during the first days after the application of both BS, mainly at the 5-day time point (Table 5). Also, these statistical differences depended on the chemical composition of the BS applied to the soil. In this respect and compared with control C, the soil dehydrogenase activity significantly increased by 93%, 92%, 91%, 90%, 90% and 90% in B2, B3, A2, A3, B1 and A1 treatments, respectively. After the first 5 days, the dehydrogenase activity began to decline gradually. At the end of the experimental period, all treatments studied had very similar values.

Soil urease activity was not stimulated after the application of the two BSs tested (Table 5). At the end of the experimental period, this enzymatic activity decreased slightly. However, the statistical analysis indicates no significant differences ($p > .05$) between these values throughout the experimental period.

The β-glucosidase activity was significantly ($p < .05$) stimulated after the application of the organic compounds to the soil, mainly in the first days of incubation (Table 6). In this respect, the highest stimulation occurred after 5 days of the experimental period, highlighting a higher stimulation in the B2 treatment followed by B3, A2, A3, B1 and A1 treatments, respectively. Compared with the C treatment, the soil β-glucosidase activity significantly increased by 43%, 36%, 31%, 29%, 29% and 28% in B2, B3, A2, A3, B1 and A1 treatments, respectively. Also, after the first 6 days, the β-glucosidase activity began to decline gradually. At the end of the experimental period all treatments studied had very similar values.

Soil phosphatase activity was also significantly ($p < .05$) stimulated after the application of all BSs to the soil (Table 6). Similar to the dehydrogenase and β-glucosidase activities, the soil phosphatase activity showed the highest stimulation at 5 days of the incubation period. Compared with the C treatment, the soil phosphatase activity significantly increased by 55%, 44%, 43%, 42%, 40% and 37% in B2, B3, A2, A3, B1 and A1 treatments, respectively. This stimulation decreased over time during the experimental period. At the end of the incubation period all experimental treatments had similar values.

Figure 2 indicates that three groups of soils can be formed. These soil groups are formed according to the sampling date and are closely related to the evolution of different enzymatic activities analysed for all experimental treatments.

Table 5. Evolution of dehydrogenase and urease activities in soils amended with the biostimulants/biofertilizers obtained from different SSs during the experimental period. Data are expressed as mean values ± standard error. Columns followed by the same letter(s) are not significantly different ($p < .05$). INTF: 2-*p*-iodo-3-nitrophenyl formazan.

	1	3	5	7	15	30	60
				Dehydrogenase activity (μg INTF g^{-1} h^{-1})			
C	2.3a ± 0.3	2.3a ± 0.3	2.1a ± 0.4	2.1a ± 0.4	1.9a ± 0.3	1.8a ± 0.4	1.6a ± 0.2
A1	8.2b ± 0.9	14.9b ± 1.3	19.6b ± 1.2	13.6b ± 1.4	5.4b ± 0.9	2.9a ± 0.2	1.7a ± 0.3
A2	12.1b ± 1.2	17.3c ± 1.5	23.3c ± 1.5	16.5b ± 1.2	6.2b ± 1.1	3.0a ± 0.4	1.8a ± 0.2
A3	10.6b ± 1.5	16.8b ± 1.2	21.8c ± 1.2	16.0b ± 1.4	6.0b ± 1.0	3.0a ± 0.4	1.6a ± 0.3
B1	9.3b ± 1.0	15.6b ± 1.1	20.6c ± 1.4	14.9b ± 1.6	5.7b ± 0.8	2.9a ± 0.3	1.7a ± 0.3
B2	15.0b ± 1.1	19.bc ± 1.4	30.5d ± 2.0	18.6b ± 1.3	6.5b ± 0.9	3.1a ± 0.3	1.8a ± 0.2
B3	14.0b ± 1.2	18.5b ± 1.5	25.9c ± 1.8	17.3b ± 1.1	6.3b ± 1.0	3.2a ± 0.4	1.8a ± 0.3
				Urease activity ($\mu g\, NH_4^+\, g^{-1}\, h^{-1}$)			
C	2.1a ± 0.3	2.1a ± 0.4	2.1a ± 0.3	2.1a ± 0.5	2.0a ± 0.5	1.9a ± 0.4	1.8a ± 0.4
A1	2.3a ± 0.3	2.5a ± 0.5	2.3a ± 0.6	2.2a ± 0.4	2.2a ± 0.6	2.1a ± 0.3	2.0a ± 0.2
A2	2.5a ± 0.4	2.5a ± 0.5	2.5a ± 0.5	2.4a ± 0.3	2.3a ± 0.4	2.3a ± 0.4	2.1a ± 0.3
A3	2.4a ± 0.5	2.5a ± 0.4	2.5a ± 0.5	2.4a ± 0.5	2.3a ± 0.5	2.2a ± 0.4	2.1a ± 0.4
B1	2.3a ± 0.5	2.4a ± 0.6	2.4a ± 0.6	2.3a ± 0.4	2.2a ± 0.4	2.1a ± 0.5	1.9a ± 0.4
B2	2.5a ± 0.6	2.5a ± 0.5	2.5a ± 0.4	2.4a ± 0.5	2.2a ± 0.4	2.1a ± 0.5	2.0a ± 0.3
B3	2.4a ± 0.5	2.5a ± 0.5	2.5a ± 0.4	2.5a ± 0.3	2.4a ± 0.6	2.2a ± 0.2	2.1a ± 0.3

Table 6. Evolution of β-glucosidase and phosphatase activities in soils amended with the biostimulants/biofertilizers obtained from different SSs during the experimental period. Data are expressed as mean values ± standard error. Columns followed by the same letter(s) are not significantly different ($p < .05$). PNP: *p*-nitrophenol.

	1	3	5	7	15	30	60
				β-Glucosidase activity (mmol PNP g^{-1} h^{-1})			
C	206a ± 14	207a ± 11	199a ± 7	200a ± 15	203a ± 11	204a ± 8	200a ± 10
A1	245a ± 16	263b ± 14	276b ± 11	258b ± 19	231a ± 13	236a ± 16	213a ± 9
A2	247b ± 19	268b ± 17	288b ± 18	256b ± 18	235a ± 15	237a ± 13	215a ± 15
A3	245a ± 10	267b ± 12	280b ± 14	255b ± 12	235a ± 18	234a ± 15	212a ± 13
B1	246b ± 21	267b ± 18	280b ± 20	269b ± 17	240a ± 13	236a ± 10	211a ± 20
B2	248b ± 15	289b ± 20	348c ± 21	298b ± 16	245a ± 15	235a ± 21	213a ± 18
B3	248b ± 17	269b ± 22	309b ± 27	275b ± 19	255a ± 14	236a ± 18	211a ± 22
				Phosphatase activity (μmol PNP g^{-1} h^{-1})			
C	14.6a ± 1.2	14.5a ± 1.4	14.5a ± 1.5	14.1a ± 1.3	13.5a ± 1.4	12.9a ± 1.8	11.9a ± 1.1
A1	19.5a ± 1.7	20.5b ± 1.4	23.0b ± 2.0	18.6a ± 1.6	18.0a ± 1.5	16.1a ± 1.2	12.0a ± 1.3
A2	20.2b ± 1.5	22.4b ± 1.6	25.6b ± 1.7	19.5a ± 1.4	18.2a ± 1.7	17.3a ± 1.6	12.6a ± 1.5
A3	19.6a ± 1.3	21.8b ± 1.4	24.9b ± 2.1	18.8a ± 1.5	18.3a ± 1.2	16.5a ± 1.7	12.5a ± 1.2
B1	19.1a ± 1.4	21.1b ± 1.5	24.1b ± 1.8	18.0a ± 1.4	17.1a ± 1.4	15.3a ± 1.4	12.4a ± 1.4
B2	20.1b ± 1.6	23.5b ± 1.5	32.4c ± 1.9	24.1b ± 1.3	19.7a ± 1.5	17.0a ± 1.4	12.5a ± 1.1
B3	20.5b ± 1.5	22.4b ± 1.6	25.9b ± 1.5	18.5a ± 1.5	17.5a ± 1.4	16.4a ± 1.5	12.1a ± 1.3

In group A, the C treatment at 0 and 5 days of incubation after beginning the incubation has initiated a similar bacterial population because the time was not long enough for any significant change in the microbial population. The A2 treatment at 5 days produced similar results. Why this happens with this treatment is not clear. It may possibly be because the treatment with the sludge may cause more favourable conditions for the emergence of new bacteria, or the possibility that these new bacteria might be brought in by the treatment.

In group B, there is a close relationship between B2 and A3 treatments, both analysed at 5 days. In comparison with two other soil groups, in group B, there is an increase in the microbial community of the soils amended with the BS. Therefore, this group of soil is directly related to the maximum soil microbial activity after addition of the experimental BS. However, no significant differences between autoclaved and non-autoclaved treatments exist, suggesting that the fact of applying the BS obtained from autoclaved SS influences the enzymatic activity of microorganisms more than causing a possible change in the soil microbial community.

Group C includes all experimental treatments for day 60, suggesting that 60 days into the experiment, the microbial populations in the organic and non-organic amended soils were very similar. These results are similar to those obtained in enzyme activities studied, which were very similar for all experimental treatments for day 60. This soil group is linked to decreased enzyme activity of the soil at the end of the incubation period.

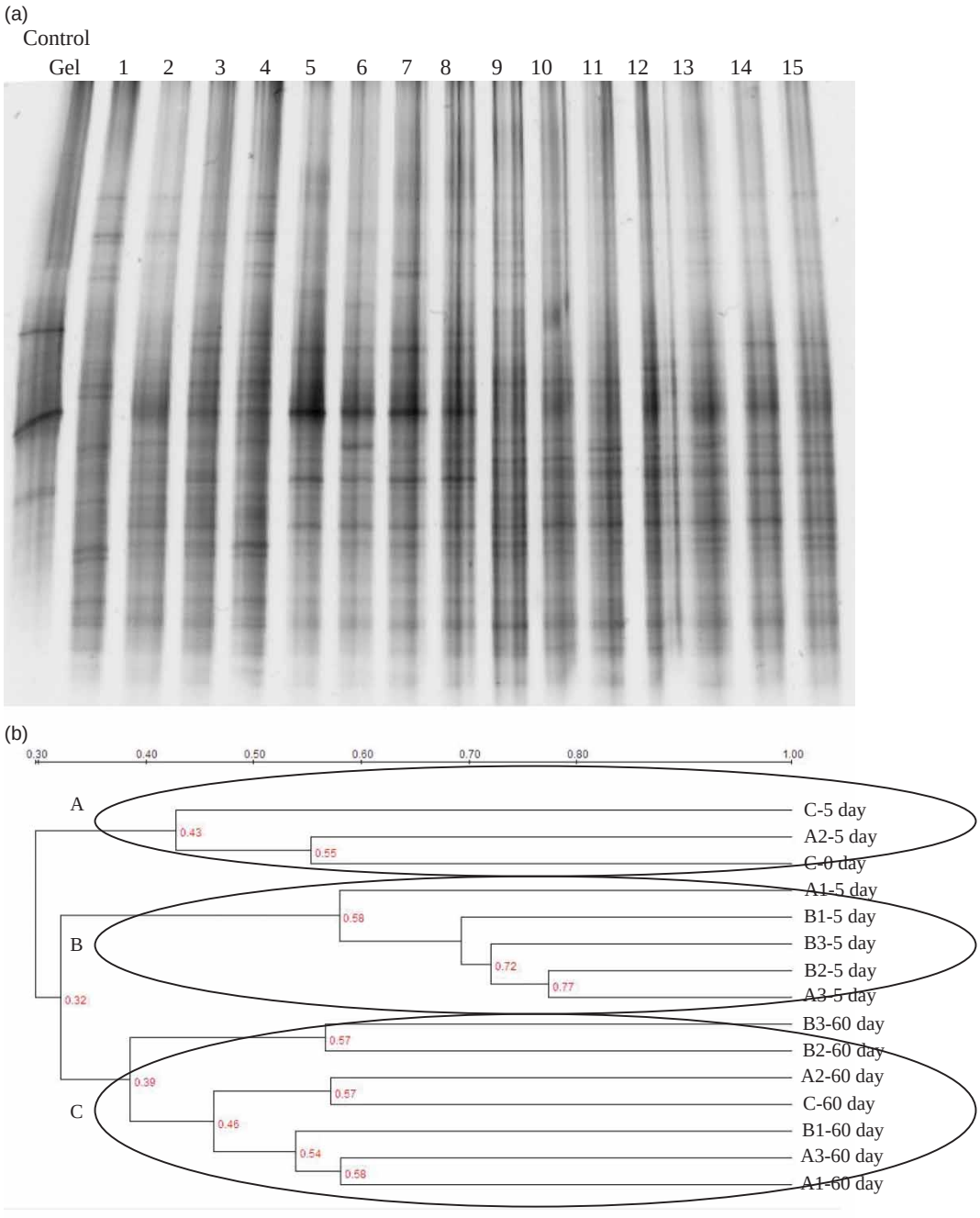

Figure 2. Genetic fingerprinting of 16S rDNA amplification fragments relative to different treatment communities: (a) DGGE and (b) cluster analysis. Lane 1: C treatment 0 days; Lane 2: C treatment 5 days; Lane 3: A1 treatment 5 days; Lane 4: A2 treatment 5 days; Lane 5: A3 treatment 5 days; Lane 6: B1 treatment 5 days; Lane 7: B2 treatment 5 days; Lane 8: B3 treatment 5 days; Lane 9: C treatment 60 days; Lane 10: A1 treatment 60 days; Lane 11: A2 treatment 60 days; Lane 12: A3 treatment 60 days; Lane 13: B1 treatment 60 days; Lane 14: B2 treatment 60 days; Lane 15: B3 treatment 60 days.

4. Discussion

Our results suggest that the application of the different BSs obtained from different SSs to the soil caused an increase in soil enzymatic activities. These results are in accord with those obtained by other authors, which suggests that the incorporation of different sources of organic matter causes an increase in the soil enzymatic activity. [12,28]

However, the way of processing the sludge used in the manufacture of BS, such as the autoclaving process prior to the enzymatic hydrolysis process, resulted in changes in the chemical composition of these enzymatic hydrolysates

and thus caused changes in the behaviour of soil enzymatic activities.

The results suggest that the autoclaving process mentioned before is very important. First, the autoclaving process favours the elimination of various pathogens, particularly *Escherichia coli*, by thermal decay and the autoclaving process can enhance the ability of enzymes to degrade proteins of higher molecular weight into others of lower molecular weight.[29] Also the maturity and type of sludge used in the enzymatic hydrolysis process showed differences in their behaviour in the soil. Thus, aerobic sludge without maturation showed a higher stimulation of soil enzymatic activities.

With respect to the enzymatic activities tested, dehydrogenase activity showed a significant stimulation in soil amended with the different BSs. This dehydrogenase activity typically occurs in all living microorganisms. For this reason, several authors suggest that the dehydrogenase activity could be used as a good indicator of the microbial activity in the soil.[26] However, the results of the dehydrogenase activity suggest that its stimulation was higher in soil amended with hydrolyzed B2, followed by B3, and then A2, A3, B1 and A1. Possibly this is a consequence of the different chemical compositions of the protein hydrolysates obtained. Accordingly, the main reason for the increased stimulation of the dehydrogenase activity is the size of certain proteins. By increasing the number of lower molecular weight proteins, the stimulation of the soil dehydrogenase activity increases. The decrease in molecular size of the protein indicates that the N is more readily available for soil microorganisms, which facilitates a greater proliferation of microorganisms in the soil.[14]

This greater assimilation of low molecular weight proteins is possibly responsible for the fact that the soil urease activity exhibits no significant stimulation after the application of the experimental BS. Since soil microorganisms obtain this N without any energy expenditure, microorganisms do not excrete any enzyme capable of degrading NH_4^+ to obtain the easily available N. These results are also in accordance with those obtained by other authors, who found that after the application to the soil of different protein hydrolysates, no stimulation was observed in the soil urease activity due to the fact that these chemical compounds were rich in low molecular weight proteins.[6,11]

These results are not in agreement with those obtained by other authors when applied to the soil organic matter of very different chemical natures (vermicomposts, green manure, etc.), constituted basically by complex proteins of a high molecular weight.[30–32] In these cases there is always a stimulation of the soil urease activity. The higher molecular weight proteins are also a source of N for microorganisms. However, not being easily assimilated by microorganisms, they need a previous process of mineralization conducted by these microorganisms, which represents energy expenditure for these microorganisms. Therefore, they excrete enzymes to degrade these higher molecular weight proteins.

Similar to dehydrogenase activity, β-glucosidase and phosphatase activities were also stimulated when the different experimental BSs were applied to the soil. These results are in agreement with those obtained by other authors, who found a significant stimulation of both extracellular enzymes after soil incorporation of different sources of organic matter.[31,32]

Cluster analysis of 16S rDNA community profiles based on a general bacterial primer pair revealed complex profiles reflecting the high diversity of the microbial community. Our results suggest that the application of different experimental BSs caused temporary variations in the soil bacterial community structure. In this respect, the bacterial community studied at five days of the experimental period was different from that obtained at the end of the experimental period.

The results obtained in the study of soil enzymatic activities indicate that the highest stimulation of these enzymatic activities is reached at five days after the start of the incubation period. Several authors suggested that the easily degradable organic compounds can be used by a wide range of organisms.[33] The presence of these compounds can lead to a rapid increase in biomass and activity and may promote the growth of copiotrophic instead of oligotrophic organisms.[34] The decomposition of this material requires enzymes that are produced by a limited number of microbial species and may increase the competitive ability of microorganisms.[33]

Once these easily degradable organic complexes have been metabolized, the structure of the soil microbial population is reestablished again. In this respect, several studies in the literature indicate that the addition of different sources of organic matter (municipal solid waste, farmyard manure, crop residues, etc.) to the soil does not cause long-term changes in the composition and diversity of bacteria in these soils.[35] Several authors suggested that this fact makes the availability of substrate a reason to find differences in the structure of the bacterial community of the soil.[33]

A primary restriction on the use of SS as a soil improver in agriculture is their content of heavy metals.[36,37] There are numerous scientific studies that show the toxic effect for soil microorganisms (activity and microbial community) after the application of SS to the soil, mainly due to the toxic effect of heavy metals present in the chemical composition of the SS.[38–41] There are currently diverse interpretations that try to explain the negative interactions between heavy metals and the soil biochemical properties. In this respect, several authors found that negative effects on the biological properties of soil contaminated by heavy metals can possibly be a consequence of a decrease in the time that substrates are available to the microorganisms,

a lower synthesis and/or liberation of the extracellular enzymes of the soil microorganisms, or the inhibition of extracellular enzymes.[42,43] Our results show that the application of different experimental BSs in soil does not cause any negative effect on the biological properties of the soil. These results suggest that the levels of heavy metals in these organic compounds were not high and did not have a toxic effect on soil microorganisms.

5. Conclusions

Our results indicated that young aerobic sludge, without maturation and previously autoclaved is the most ideal SS to get a new biostimulant/biofertilizer that causes a higher stimulation in soil enzyme activities. The autoclaving process makes high molecular weight proteins more accessible to degradation. By increasing the number of lower molecular weight proteins, stimulation of soil dehydrogenase activity increases. The decrease in molecular size of the protein results in more readily available N for soil microorganisms, which facilitates a greater proliferation of microorganisms in the soil.

However, the biostimulant effect in soils should be studied further. The quantity of these organic products and soil type are parameters that must be taken into consideration to have a deeper understanding of the action of these BSs on agronomy studies.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Effects of foliar fertilization of a biostimulant obtained from chicken feathers on maize yield

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ABSTRACT

Due to the important contribution that it makes to human nutrition, maize is one of the most widely-consumed cereals in the world. There is, therefore, high demand for fertilizers that will maintain maize production at both high yield and quality levels. The objective of this work was to study the effect of foliar fertilization using a biostimulant, obtained by enzymatic hydrolysis from chicken feathers, on the productivity and quality of maize crops (*Zea mays*, L. cv PR32W86 Pioneer), located in Trujillanos (Extremadura, Spain), over two consecutive seasons. Foliar biostimulant/biofertilizer was applied three times each season and at two rates (3.6 and 7.2 l ha^{-1}). At the higher rate and for both seasons, foliar fertilization significantly increased the leaf concentrations of macro- and micronutrients, while grain protein content and yield increased by 26% and 14%. These results suggest that the foliar use of this biostimulant could be of great interest to the farmer for improving both maize crop yield and quality.

1. Introduction

Foliar fertilization is currently a highly efficient agronomic crop fertilization technique since it favours the assimilation of the nutrients in the plant and consequently, the utilisation of the nutrients applied with the fertilizer, thus increasing crop yields and quality (Tejada and Gonzalez, 2004; Abbas and Ali, 2011; Osman et al., 2013). Since it significantly reduces the effects of groundwater contamination caused by applying inorganic fertilizers to the soil it is, moreover, a technique that contributes to sustainable, environmentally friendly agriculture (Tejada and González, 2003a; Fernández and Eichert, 2009).

In recent years, foliar fertilization has been used to apply macronutrients, micronutrients and humic substances. This results in a great number of positive effects in the plant, principally at physiological level (respiration and photosynthesis), at morphological level, (root length and leaf area index), and the yield of various crops such as rice, tomato, pepper and maize (Tejada and González, 2003a; Tejada and Gonzalez, 2004; Karakurt et al., 2009; Tejada et al., 2016).

The use of biostimulants (BS) obtained from various organic residues (carob germ, sewage sludge) by enzymatic hydrolysis processes via foliar fertilization is increasing. This is because these organic compounds are easily assimilated by crops and therefore improve crop nutrition, increasing both the productivity and the quality of the grain or fruit harvested (Parrado et al., 2008; Tejada et al., 2016).

Several authors have tested the effectiveness of a BS obtained from chicken feathers by enzymatic hydrolysis processes in the bioremediation of polluted soils with organic xenobiotics (Gómez et al., 2014; Rodríguez-Morgado et al., 2015a, 2015b). However, there are no studies concerning the use of this type of organic compound via foliar fertilisation in order to increase both crop yield and quality.

Maize (*Zea mays* L.) is one of the world's major cereal crops, ranking third in importance after wheat and rice (Lashkari et al., 2011). Most of the maize produced worldwide is used for animal feed, although it is also part of the basic diet in human nutrition, as it is a good source of starch, proteins, lipids, polyphenols, carotenoids, vitamins and dietary fibre (Nuss and Tanumihardjo, 2010; Blandino et al., 2017). Consequently, studying the response of this crop to foliar fertilization of a new BS could be of great interest to the farmer.

The main objective of this paper is to study the effect of a BS obtained from chicken feathers by enzymatic hydrolysis processes when it is applied via foliar in a corn crop, observing both maize yield and grain quality.

2. Material and methods

2.1. Site and properties of the biostimulant

The study was carried out during two consecutive experimental seasons (from April to October in 2014 and 2015) at Trujillanos,

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Table 1
Initial soil physico-chemical characteristics (mean ± standard error). Data are the means of three samples.

pH (soil:H_2O ratio 1:2.5)	7.1 ± 0.3
Electric conductivity (soil:H_2O ratio 1:5) (dS m^{-1})	0.071 ± 0.06
Coarse sand (g kg^{-1})	418 ± 21
Fine sand (g kg^{-1})	154 ± 18
Silt (g kg^{-1})	246 ± 20
Clay (g kg^{-1})	182 ± 17
Total C (g kg^{-1})	8.7 ± 1.5
Kjeldahl-N (g kg^{-1})	0.78 ± 0.13
Olsen P (mg kg^{-1})	11.0 ± 1.3
Available K (mg kg^{-1})	86.4 ± 10.7
Available Ca (mg kg^{-1})	2103 ± 21
Available Mg (mg kg^{-1})	428 ± 13
Available Fe (mg kg^{-1})	80.1 ± 7.9
Available Cu (mg kg^{-1})	4.6 ± 1.1
Available Mn (mg kg^{-1})	119 ± 22
Available Zn (mg kg^{-1})	1.8 ± 0.3

(Extremadura, Spain). The climatic characteristics of the study area are detailed in the supplemental material (Table S1) (AEMET, 2017). Total annual rainfall was 342.3 mm in 2015 and 458.4 mm in 2016. Average air temperature averaged 17.8 °C in 2015 and 17.5 °C in 2016.

The soil used was the same as that described in Tejada et al. (2016). The main soil characteristics (0–25 cm) are described in Table 1. The methodology used for determining each parameter is described in Tejada et al. (2016).

The BS used was obtained from chicken feathers by the enzymatic hydrolysis. The obtaining process is described in Rodríguez-Morgado et al. (2014). This process was carried out in a bioreactor under the following conditions: (a) substrate concentration: 10%; (b) solvent: water; (c) catalytic agent: subtilisin, 0.15% (v/v) (d) Enzymatic concentration: 1 ml l^{-1} substrate; (e) temperature: 55° C; (f) pH: 9, controlled by the addition of 10 M NaOH; (g) time: 180 min. Finally, the hydrolysed product was centrifuged obtaining the biostimulant. The organic compound's chemical composition is described in Table 2. The methodology used for determining each parameter is described in Rodríguez-Morgado et al. (2015b).

Amino acid composition was determined by reversed-phase HPLC analysis of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) derivatives, with γ-aminobutyric acid as internal standard (Table 3). The methodology used for determining each parameter of these amino acids is described in Parrado et al. (2008).

Table 2
Chemical composition (mean ± standard error) of the biostimulant used for each experimental season. Data are the means of three samples (oven wet basis).

Organic matter (g kg^{-1})	459 ± 39
Kjeldahl-N (g kg^{-1})	15.7 ± 1.9
Total carbohydrates (g kg^{-1})	69 ± 10
Total P (g kg^{-1})	29.2 ± 2.2
Total K (g kg^{-1})	1.5 ± 0.5
Total S (g kg^{-1})	18 ± 1.9
Total Ca (g kg^{-1})	110 ± 4
Total Mg (g kg^{-1})	23.9 ± 5.2
Total Fe (mg kg^{-1})	13.5 ± 2.1
Total Cu (mg kg^{-1})	2.1 ± 0.6
Total Mn (mg kg^{-1})	33.8 ± 6.2
Total Zn (mg kg^{-1})	0.59 ± 0.17
Total Ni (mg kg^{-1})	0.53 ± 0.11
Molecular weight (Da) (%)	
> 10000	23.4 ± 2.1
10000–5000	8.8 ± 1.0
5000–1000	23.2 ± 3.1
1000–300	6.9 ± 1.1
< 300	37.7 ± 3.9

Table 3
Amino acid composition (mean ± standard error) of the experimental biostimulant. Data are the means of three samples. Results are expressed as grams per 100 g of proteins.

Ala	5.0 ± 0.6	Arg	7.1 ± 0.4
Asp	10.9 ± 1.0	His	1.6 ± 0.3
Cys	ND	Ile	6.1 ± 0.7
Glu	11.8 ± 1.2	Leu	9.1 ± 0.7
Gln	15.6 ± 1.9	Val	8.9 ± 0.8
Gly	8.4 ± 0.8	Lys	2.9 ± 0.5
Pro	9.5 ± 0.6	Met	1.1 ± 0.2
Ser	10.9 ± 1.1	Phe	5.5 ± 0.6
Tyr	1.1 ± 0.2	Thr	4.2 ± 0.9

ND: not determined.

2.2. Experimental layout and treatments

For each experimental season, the experimental layout was a randomized complete block with three treatments and three replicates per treatment. Each plot size was 9 m × 7 m. The treatments were the following:

(1) A0 treatment, plots fertilized with 300 kg N ha^{-1} (as urea), 80 kg P ha^{-1} + 41.7 kg N ha^{-1} [as $(NH_4)H_2PO_4$] and 120 kg K ha^{-1} (as K_2SO_4), which is common practice in the area
(2) A1 treatment, plots fertilized with the A0 treatment mineral fertilizers and foliar fertilized with BS at a dose of 3.6 l ha^{-1}
(3) A2 treatment plots fertilized with the A0 treatment mineral fertilizers and foliar fertilized with BS at a dose of 7.2 l ha^{-1}

The doses used in the BS are those described by Tejada et al. (2016) when they applied a BS obtained from sludge and hydrolytic processes. The inorganic fertilizers were incorporated on April 13th 2015 and 18th April 2016, respectively, to a depth of 20–25 cm.

Similar to Tejada et al. (2016), BS was applied three times during the maize vegetative cycle and for each experimental season. In this regard, the BS was applied on July 13th, July 27th and August 17th during the 2015 season, and July 11th, July 25th and August 22nd during the 2016 season. Therefore, the total doses used in the experiment were 10.8 l ha^{-1} or A1 and 21.6 l ha^{-1} for A2 in each experimental season.

Maize (*Zea mays* cv PR32W86 Pioneer) was sown at a rate of 100,000 seeds ha^{-1} with 75-cm inter-row spacing. The planting dates were April 14th 2015 and April 19th 2016, respectively. Once the harvest was collected during the first experimental season, all of the residues generated were also collected. This was done to prevent these organic residues interfering with plant nutrition.

The irrigation system, irrigation time and amount of water applied to the crop were similar to that described by Tejada et al. (2016). Table 4 shows the chemical composition of the irrigation water used. Values were obtained from the arithmetic mean of 6 samples per year during each vegetative cycle of the plant.

Table 4
Chemical composition of the water used in the irrigation crop (mean ± standard error) for each experimental season. Data are the means of six samples.

pH	6.4 ± 0.2
Ca^{2+} (mg l^{-1})	96.7 ± 3.4
K^+ (mg l^{-1})	50.2 ± 3.5
Cl^- (mg l^{-1})	3.5 ± 1.6
SO_4^{2-} (mg l^{-1})	33.2 ± 4.1
HCO_3^{2-} (mg l^{-1})	314 ± 10
NO_3^- (mg l^{-1})	22.4 ± 2.1

2.3. Plant sampling and analytical determinations

In each fertilizer treatment and for each experimental season, the leaves of 10 plants located in the central area of each plot were selected. Leaf samples were collected in two stages of growth: (1) at tasselling [R1 physiological state according to Hanway scale (Ritchie et al., 1986)], occurring on August 8th 2015 and August 5th 2016; and (2) at harvest [R6 physiological state according to Hanway scale (Ritchie et al., 1986)], which took place on October 16th 2015 and October 20th 2016, by selecting the spike leaves (Tejada and González, 2003a; Tejada et al., 2016).

Before their analysis, the leaves were subjected to a washing and drying process, described in Tejada et al. (2016). Furthermore, the macro- and micronutrients in the leaves were determined according to the methods described in Tejada et al. (2016).

For each season and fertilizer treatment, all the corncobs located in each experimental plot were collected. Number of grains per corncob and crop yield (kg ha^{-1}) was determined in samples collected from each plot on October 14th 2015 and October 20th 2016, respectively.

On the other hand, protein concentration, macro- and micronutrients in the grain were determined according to the methodology described in MAPA (1986) and Tejada et al. (2016).

2.4. Statistical analysis

With the data obtained, an analysis of variance (ANOVA) was performed considering the treatment as independent variable followed by Tukey's significant difference as a post hoc test, considering a significance level of $p < 0.05$ throughout the study and using Statgraphics Plus 2.1 software package.

3. Results

Table 5 shows the macro- and micronutrient leaf contents during the maize cycle for each experimental season expressed on a dry matter basis.

Regarding N, at harvest and for both experimental season, the A2 treatment showed the highest levels of N in leaf. Compared with the A0 treatment, foliar N concentration was 14.4% and 39.1% higher in the A1 and A2 treatments for the 2015 season, whereas it was 15% and 33.3% higher in A1 and A2 treatments for the 2016 season.

In the same way, and at harvest, the A2 treatment showed the highest values of P, highlighting the effect of BS on the contents of this macronutrient in leaf. Compared with A0 treatment, leaf P concentration was 32.8% and 52.2% higher in A1 and A2 treatments for the 2015 season, whereas it was 43.5% and 51.1% higher in the A1 and A2 treatments for the 2016 season (K, Ca and Mg in leaf) are also higher in the foliar fertilized plots with A2, followed by A1, demonstrating the effect of the BS dosage on the concentration of the macronutrients analysed in maize leaf.

The micronutrients analysed in leaf show a similar evolution to the macronutrients. At harvest and for both experimental seasons, the highest values were obtained in the A2 treatment, followed by the A1 treatment. The statistical analysis showed significant differences with A2 treatment only, again highlighting the importance of the BS rate used in the experiment.

The chemical composition of the grains presented a very similar behaviour to the nutrient content in the leaves (Table 6). For both experimental seasons, there was a significant increase in macro- and micronutrients analyzed, principally when the higher BS rate was used.

Compared with the A0 treatment, grain protein concentration significantly increased by 26.5% in the A2 treatment in the first season and by 25.3% in the second (Table 7). Moreover, the number of grains per corncob significantly increased by 15% in the A2 treatment in the 2015 season and by 15.8% in the 2016 season. Finally, the higher application rate significantly increased yield by 13.4% and by 14.6% in the first and the second seasons.

Table 5
Leaf mineral nutrient content (mean ± standard error) (on a dry matter basis) after foliar application of the biostimulant during two consecutive seasons.

Treatments	N[†]	P	K	S	Ca	Mg	Fe	Cu	Mn	Zn	Ni
	(g kg^{-1})						(mg kg^{-1})				
Season 2015											
Tasseling (8th August)											
A0	17.6 ± 1.9a*	5.7 ± 1.2a	28.9 ± 2.5a	3.5 ± 1.2a	18.4 ± 2.3a	8.7 ± 1.4a	312 ± 19a	8.4 ± 1.1a	78.2 ± 6.4a	18.3 ± 1.5a	5.3 ± 1.0a
A1	22.5 ± 2.1ab	7.4 ± 1.3b	33.4 ± 2.7ab	5.4 ± 1.3b	26.2 ± 2.0ab	10.2 ± 0.9a	405 ± 24b	10.0 ± 1.4a	87.4 ± 3.9a	25.0 ± 1.9ab	6.7 ± 1.4ab
A2	28.4 ± 1.7b	9.3 ± 1.0b	37.0 ± 2.3b	6.9 ± 1.5c	32.4 ± 2.5b	17.4 ± 1.1b	489 ± 20c	16.6 ± 1.5b	117 ± 4.8b	30.2 ± 2.1b	7.8 ± 1.2b
Harvest (14th October)											
A0	9.5 ± 1.1a	4.3 ± 1.4a	16.6 ± 1.5a	3.4 ± 1.4a	16.9 ± 1.8a	7.8 ± 1.3a	126 ± 11a	6.9 ± 1.5a	63.0 ± 5.2a	17.4 ± 1.3a	4.8 ± 0.9a
A1	11.1 ± 1.8ab	6.4 ± 1.1b	21.4 ± 1.3ab	5.0 ± 1.1b	25.8 ± 2.2ab	10.9 ± 1.5a	265 ± 17b	11.0 ± 1.2ab	78.4 ± 3.5a	23.8 ± 1.6ab	6.3 ± 1.5ab
A2	15.6 ± 1.9b	9.0 ± 1.2c	26.0 ± 1.0b	6.6 ± 1.3	31.9 ± 2.0b	16.1 ± 1.2b	290 ± 13c	15.1 ± 1.1b	89.3 ± 4.2b	28.4 ± 1.1b	7.9 ± 1.0b
Season 2016											
Tasseling (5th August)											
A0	18.2 ± 2.1a	5.4 ± 1.1a	30.6 ± 1.9a	4.0 ± 1.0a	18.9 ± 1.9a	9.1 ± 1.7a	305 ± 17a	9.1 ± 1.3a	80.2 ± 7.5a	19.4 ± 1.6a	6.0 ± 1.2a
A1	24.6 ± 2.0ab	7.5 ± 1.4b	35.2 ± 2.6ab	5.7 ± 1.2b	25.7 ± 1.7a	10.7 ± 1.5a	386 ± 20b	10.4 ± 1.1a	88.9 ± 5.6a	25.9 ± 2.0ab	6.9 ± 1.1ab
A2	29.3 ± 1.9b	10.0 ± 1.2c	37.6 ± 2.5b	7.1 ± 1.1c	31.2 ± 2.3b	17.8 ± 1.3b	477 ± 25c	17.2 ± 1.5b	119 ± 5.1b	30.4 ± 2.3b	8.0 ± 1.3b
Harvest (20th October)											
A0	10.2 ± 1.4a	4.6 ± 1.0a	19.4 ± 1.6a	3.8 ± 1.1a	17.0 ± 2.0a	8.6 ± 1.5a	139 ± 13a	7.4 ± 1.1a	64.4 ± 5.9a	17.8 ± 1.4a	5.4 ± 1.3a
A1	12.0 ± 1.5ab	6.6 ± 1.2b	23.9 ± 1.8a	5.2 ± 1.2b	24.8 ± 1.7a	11.2 ± 1.3a	274 ± 19b	11.3 ± 1.0ab	77.1 ± 3.8a	23.4 ± 1.0ab	6.5 ± 1.0ab
A2	15.3 ± 1.2b	9.4 ± 1.3c	25.4 ± 1.5b	6.8 ± 1.2c	32.1 ± 1.3b	17.7 ± 1.0b	295 ± 14c	15.4 ± 1.3b	88.7 ± 4.9b	29.2 ± 1.7b	7.7 ± 1.1b

[†] Fresh matter.
* Columns (mean ± SE) followed by the same letter(s) are not significantly different ($p < 0.05$).

Table 6
Chemical analysis of the grain (mean ± standard error) (on a dry matter basis) after foliar application of the biostimulant during two consecutive seasons.

Treatments	N	P	K	S	Ca	Mg	Fe	Cu	Mn	Zn	Ni
	($g\,kg^{-1}$)						($mg\,kg^{-1}$)				
Season 2015											
A0	13.9 ± 1.5a*	1.7 ± 0.4a	3.1 ± 0.7a	0.9 ± 0.2a	1.4 ± 0.3a	0.86 ± 0.11a	10.9 ± 1.1a	3.0 ± 0.4a	9.1 ± 0.9a	10.4 ± 1.2a	0.62 ± 0.11a
A1	16.0 ± 1.1ab	2.0 ± 0.2a	3.7 ± 0.8a	1.8 ± 0.4b	2.5 ± 0.5ab	0.97 ± 0.13a	16.0 ± 1.2b	3.9 ± 0.4a	11.0 ± 1.1a	14.2 ± 1.1ab	1.0 ± 0.1b
A2	18.8 ± 1.2b	2.6 ± 0.3b	4.2 ± 0.7b	2.8 ± 0.4c	3.0 ± 0.5b	1.1 ± 0.1b	19.3 ± 1.3b	5.7 ± 0.5b	14.0 ± 1.2b	17.9 ± 1.3b	1.3 ± 0.1b
Season 2016											
A0	14.2 ± 1.2a	1.8 ± 0.3a	3.3 ± 1.0a	1.0 ± 0.2a	1.5 ± 0.3a	0.91 ± 0.17a	11.3 ± 1.4a	3.2 ± 0.5a	10.1 ± 1.1a	10.9 ± 1.5a	0.68 ± 0.12a
A1	16.3 ± 1.3ab	2.0 ± 0.3a	3.8 ± 1.1a	1.9 ± 0.3b	2.7 ± 0.5b	1.0 ± 0.1a	16.7 ± 1.3b	4.0 ± 0.6a	12.1 ± 1.3a	14.0 ± 1.2ab	1.1 ± 0.1b
A2	19.0 ± 1.4b	2.5 ± 0.2b	4.4 ± 0.8b	2.9 ± 0.4c	3.1 ± 0.4b	1.2 ± 0.1b	20.2 ± 1.4c	6.0 ± 0.5b	14.2 ± 1.4b	18.2 ± 1.2b	1.3 ± 0.1b

* Columns (mean ± SE) followed by the same letter(s) are not significantly different ($p < 0.05$).

Table 7
Grain protein content and crop yield parameters (mean ± standard error) after foliar application of the biostimulant during two consecutive seasons.

Treatments	Protein concentration ($g\,kg^{-1}$)	Number of grains per corncob	Yield ($kg\,ha^{-1}$)
Season 2015			
A0	86.8 ± 9.1a*	470 ± 31a	14118 ± 124a
A1	100.0 ± 6.6ab	516 ± 23ab	15510 ± 110b
A2	118.1 ± 7.4b	553 ± 28b	16303 ± 135b
Season 2016			
A0	88.8 ± 7.3a	474 ± 33a	14229 ± 156a
A1	101.9 ± 8.0ab	522 ± 27ab	15532 ± 120b
A2	118.8 ± 8.6b	563 ± 31b	16663 ± 144b

* Columns (mean ± SE) followed by the same letter(s) are not significantly different ($p < 0.05$).

4. Discussion

Our results suggest that there is a positive effect on the mineral nutrition of corn when a BS obtained from chicken feathers was appliedvia foliar application. These results are in agreement with those obtained by Tejada and González (2003a) and Tejada et al. (2016), who observed an increase in the plants' mineral nutrition after the application of a BS obtained from sewage sludge or from a by-product of the two-step olive oil milling process via foliar to a corn crop.

In the same way, other authors have obtained similar results after the foliar application of different organic substances and amino acids on rice, corn, tomato, pepper, cucumber, wheat, asparagus and green beans (Tejada and Gonzalez, 2003b, 2004; Yildirim, 2007; Karakurt et al., 2009; Katkat et al., 2009; Abdel-Mawgoud et al., 2011; El-Nemr et al., 2012).

This improvement in the plant mineral nutrition after the foliar fertilization of humic substances and amino acids is mainly due to an improvement in the plants' uptake of nutrients (Tejada and Gonzalez, 2003b; Tejada et al., 2016). Several studies have shown that the foliar application of humic substances increases leafcuticlepermeability, favouring the entry of ions attached to these molecules within the plant cell (Fageria et al., 2009; Çelik et al., 2010).

Numerous studies have shown the importance of amino acids in the plant's physiological activities, mainly at the cellular level. Since they are highlywater-soluble, the positive effects of applying amino acids might be due to their internal function within the cell as an osmoregulator. This increases the concentration of cellular osmotic components (Abdel-Mawgoud et al., 2011), stimulating cell growth and consequently increasing the plants' chemical composition, as well as the growth, yield and quality of the harvest (Awad et al., 2007; Abdel Aziz, 2009; Thomas et al., 2009; Abd El-Aal et al., 2010). Also and due to the chelating effect of amino acids on micronutrients, when applied together with micronutrients they facilitate the absorption and transport of these micronutrients inside the plant, since they also positively affect cell membrane permeability (Ibrahim et al., 2010).

Some authors propose different formulations of humic acids, amino acids, hydrolysed proteins, etc. as growth promoters, thus improving plant nutrition (Thomas et al., 2009). Our results confirm these judgments, since the BS, with the mixture of substances used in this experiment, favours the mineral nutrition of corn.

The increase in grain macro- and micronutrient concentrations is possibly due to the improvement in the plant's mineral nutrition. These results are in agreement with those obtained by Tejada et al. (2016), who found a significant increase in the concentration of macro- and micronutrients in corn grain when they applied the same doses of a BS obtained from sewage sludge, also composed of a mixture of humic substances, low molecular weight peptides, amino acids and macro- and micronutrients. In the same way, these data are in agreement with those obtained by other authors, who found a significant increase in

macronutrients in rice and maize grains after the foliar application of different humic substances (Tejada and Gonzalez, 2006; Osman et al., 2013).

The increase in micronutrients in grain is a consequence of the micronutrient-rich BS foliar fertilization. These results are in agreement with those obtained by other authors when applying different micronutrients via foliar to crops such as wheat, cowpea and rice (Simoglou and Dordas, 2006; Dordas, 2009; Zeidan et al., 2010; Mabesa et al., 2013; Manzeke et al., 2017). For many authors, foliar fertilization can be used to satisfy the essential micronutrient requirements in crop grains, increasing yields and the quality of production (Fang et al., 2008).

The significant increase in the concentration of micronutrients in the grain after the foliar application of the experimental biostimulant is very important. This is because, in general terms, cereal crops usually present a low concentration of such micronutrients in their grain (Cakmak, 2010).

Finally, the increase in plants' nutrient uptake may be responsible for the increase in the maize yield, highlighting again the influence of the dose of the biostimulant applied to the plant.

Many authors consider N as the essential element that directly influences the number of grains per corncob, weight of grains and, consequently, in crop yield (Osborne et al., 2002; Ma et al., 2006; Jin et al., 2012). In our experiment, the high concentration of N that was applied to the plant from the BS in the form of N or as amino acids could be responsible for this increase in the crop yields, number corncob and grains per corncob.

5. Conclusions

Foliar fertilization with biostimulants obtained from chicken feathers (rich in organic matter, low molecular weight peptides and amino acids) significantly increased the maize nutrition and, consequently, maize yield and grain quality. This increase was higher whenthe said product was applied three times during the maize vegetative cycle at a dose of 7.2 l ha^{-1}. It is, however, necessary to continue studying the behaviour of this biostimulant on crops in order to optimise both the application rates and the number of applications needed with the aim of obtainingthe maximum responses from the crops when using this compound. In the same way, it is also necessary to study the behaviour of this new organic compound on different soils, since the different physical-chemical properties of the soils can also influence the response of the crop when applying these biostimulants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.eja.2018.03.003.

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4. RESULTADOS Y DISCUSIÓN

4.1. Obtaining edaphic biostimulants / biofertilizers from different sewage sludges. Effects on soil biological properties

El procesado de los distintos lodos de depuradora mediante hidrólisis enzimática tiene una serie de claros efectos, siendo los dos principales la disminución del tamaño molecular del componente proteico soluble de los lodos (Adler-Nissen, 1986) y la disminución significativa en el contenido en metales pesados de todas las muestras (Carlson-Ekvall y Morrison, 1997; Wong y col., 2000; Villar y García Jr., 2003; Tejada y González, 2007; Torri y Lavado, 2008; Tejada y col., 2014). La proteasa empleada digiere las proteínas existente en los lodos fragmentándolas en unidades de menor peso molecular llamadas péptidos e incluso en los monómeros constituyentes, aminoácidos libres. Tanto los péptidos como los aminoácidos libre son mucho más biodisponibles para los microorganismos que las proteínas de partida, favoreciendo su crecimiento y actividad. Por otra parte, tras el centrifugado de los lodos posterior al proceso de hidrólisis enzimática, gran parte de los metales pesados permanecen insolubles, pudiendo separarse del componente soluble rico en materia orgánica, péptidos y aminoácidos libres. Esto supone una ventaja de gran importancia ya que permite aumentar las dosis de aplicación de los distintos productos sin riesgos de causar una contaminación del medio por metales pesados.

El tratamiento con autoclave, que tiene como objetivo inicial reducir y/o eliminar la carga microbiológica patógena tiene otros efectos sobre los lodos, como son la solubilización de parte de la materia orgánica y la desnaturalización de las proteínas (Lang y Smith, 2008). Esto causa un aumento en el contenido de materia orgánica de la fracción soluble al destruirse las estructuras celulares de la biomasa que compone los

lodos al tiempo que se desnaturalizan las proteínas existentes, lo que las hace más susceptibles al ataque proteolítica de las enzimas empleadas en el proceso de hidrólisis.

Todos los productos usados provocaron un incremento significativo de la actividad deshidrogenasa con respecto al control, dándose éste en los primeros 5 días del ensayo, disminuyendo a continuación gradualmente hasta el final del experimento. La actividad deshidrogenasa está relacionada directamente con el crecimiento y actividad microbianas, lo que sugiere que los distintos productos actúan como fuente de energía y nutrientes de los microorganismos edáficos, incrementando estos su población (Masciandaro y col., 1994). Esto puede ser debido al bajo peso molecular del componente proteico de los productos, los que los convierte en una fuente más biodisponible de carbono y nitrógeno (Vasileva-Tonkova y col., 2007), que es un elemento limitante en el crecimiento microbiano.

La actividad ureasa no se estimuló en ningún momento del ensayo. Esto se podría deber al incremento de la biodisponibilidad del componente proteico aportado por los distintos productos. Al estar más disponible esta fuente de nitrógeno, los microorganismos del suelo no tienen la necesidad de excretar enzimas con las cuales procurarse un suministro de este elemento (García-Martínez y col., 2010; Tejada y col., 2013).

Las actividades β-glucosidasa y fosfatasa también fueron estimuladas significativamente siguiendo un patrón idéntico al de la actividad deshidrogenasa, aunque en menor medida. Esta estimulación concuerda con otros estudios donde un incremento de la actividad deshidrogenasa va a acompañado de un aumento de las actividades β-glucosidasa y fosfatasa (Tejada y col., 2008; 2010).

El análisis de clusters del ADNr 16S nos agrupa las muestras en tres grupos atendiendo principalmente al momento del experimento en el que se tomó la muestra. Se puede ver un primer grupo correspondiente a los controles a tiempo 0 y 5, debido probablemente a que en el suelo control sin enmienda tarda más tiempo en producirse una diferencia entre las poblaciones bacterianas. En el segundo grupo aparecen todas las muestras de suelos tratados, a excepción de A2, a los días de incubación. Esto indica que los distintos productos causan un cambio significativo en las poblaciones microbianas (Hu y col., 1999; Marschner y col., 2003), lo que se corresponde con el incremento de actividad detectado mediante la actividad deshidrogenasa. Hacia el final del experimento todas las muestras, incluidos los controles, se agrupan en un mismo grupo. Esto nos indica que la estructura de las poblaciones microbianas se ha restablecido, probablemente debido a que los nutrientes aportados por los distintos productos han sido consumidos, permaneciendo únicamente aquellos capaces de sobrevivir en condiciones limitantes (Marschner y col., 2003; Stark y col., 2008).

4.2. Effects of foliar fertilization of a biostimulant obtained from chicken feathers on maize yield

En primer lugar, es necesario destacar que en un trabajo anterior (Tejada y col., 2016) observaron que tras la aplicación de un bioestimulante obtenido a partir de lodos de depuradora mediante procesos enzimáticos directamente al suelo en un cultivo de maíz (*Zea mays*, L.), no se detectaron diferencias significativas en la concentración de macro y microelementos en el suelo, hojas o granos con respecto al control. Así mismo, tampoco se observaron diferencias en la concentración de proteínas del grano, el número de granos por mazorca o el rendimiento. Esto puede ser debido al carácter hidrolizado del bioestimulante que hace a sus nutrientes muy biodisponibles. Este aumento en la biodisponibilidad favorece el desarrollo de los microorganismos, que rápidamente consumen esos nutrientes, no observándose efectos a medio-largo plazo en el cultivo (Tejada y González, 2006; Gómez y col., 2014).

Figura 14: Transporte del biofertilizante de plumas al campo de experimentación.

En consecuencia, se hizo un estudio del comportamiento de un bioestimulante obtenido a partir de plumas de pollo por procesos de hidrólisis enzimática en la nutrición de un cultivo de maíz (*Zea mays*, L.) por vía foliar, descartando su aplicación en suelo.

Figura 15: Preparación del biofertilizante de lodos de depuradora para su uso en fertilización foliar.

Los resultados obtenidos en este estudio sugieren que Se ha observado como la aplicación foliar de un bioestimulante obtenido por hidrólisis enzimática de plumas de pollo es capaz aumentar el contenido en nitrógeno y micronutrientes tanto en las hojas como en el grano en un cultivo de maíz (*Zea mays*). Aunque el aumento en el contenido de N se podría explicar debido al hecho de que el bioestimulante empleado es una fuente nitrogenada en forma de péptidos de bajo peso molecular y aminoácidos libres, ambos de alta biodisponibilidad (Tejada y González 2003, 2004; Yildirim 2007; Karakurt y col., 2009; Katkat y col., 2009; Abdel-Mawgoud y col., 2011; El-Nemr y col., 2012), no es posible explicar del mismo modo el incremento en la concentración del resto de elementos. Una explicación plausible de este hecho es que los péptidos de

bajo peso molecular y los aminoácidos libres aplicados por vía foliar actúan mejorando el estado fisiológico de las plantas, ya sea incrementado la adquisición de nutrientes, estimulando el crecimiento celular, actuando como osmo-protectores, etc. (Awad y col., 2007; Abdel Aziz, 2009; Thomas y col., 2009; Abd El-Aal y col., 2010; Ibrahim y col., 2010; Abdel-Mawgoud y col., 2011).

Figura 16: Seguimiento del cultivo de maíz tras su fertilización en suelo y foliar con el biofertilizante de plumas de pollo experimental.

Esta mejora general del estado nutricional y fisiológico de la planta es la responsable del incremento en otros parámetros de tipo nutritivo y comercial del cultivo (Simoglou y Dordas 2006; Fang y col., 2008; Dordas 2009; Zeidan y col., 2010; Mabesa y col., 2013; Manzeke y col., 2017;). Por una parte, el aumento en la concentración de nitrógeno es relación directa a un aumento en el contenido proteico de los granos, lo que incrementa sustancialmente su valor nutricional. A esto hay que añadir el aumento significativo en la concentración de ciertos micronutrientes de interés como el fósforo, el potasio o el azufre, el cual, por ejemplo, triplica su concentración en el tratamiento con la dosis más alta de bioestimulante. Por otra parte se mejoran los parámetros comerciales como son el número de granos por mazorca y el rendimiento general del cultivo, que aumentan entre un 10 y un 15% (Osborne y col., 2002; Ma y col., 2006; Jin y col., 2012). Este incremento es muy sustancial teniendo en cuenta la relativa poca cantidad de bioestimulante aplicado y su coste.

5. CONCLUSIONES

De los estudios de la capacidad bioestimulante en suelo y fertilidad agronómica de los nuevos bioestimulantes obtenidos a partir de lodo de depuradora y plumas de pollo, se exponen a continuación las conclusiones más relevantes:

1. Capacidad bioestimulante en suelo

   1.1. Los biofertilizantes de lodos de depuradora obtenidos por un proceso de hidrólisis enzimática son capaces de estimular todas las actividades enzimáticas del suelo estudiadas salvo la actividad ureasa, debido a que el propio producto es una fuente de nitrógeno fácilmente disponible.

   1.2. El producto hidrolizado proveniente de lodos de depuradora aeróbicos sin madurar, que presenta un mayor contenido en el componente proteico de bajo peso molecular, es el que posee una mayor capacidad bioestimulante edáfica.

   1.3. El tratamiento de autoclavado es fundamental al mejorar el proceso a nivel de rendimiento y cualitativamente al facilitar la digestión del componente proteico de los lodos por parte de las enzimas proteolíticas.

2. Estudios agronómicos

   2.1. La aplicación foliar de un bioestimulante obtenido por hidrólisis enzimática de plumas de pollo en un cultivo de maíz produjo un incremento significativo en la nutrición de la planta y consecuentemente en la calidad y rendimiento del grano. Al igual que el punto anterior, esto sugiere el uso foliar de este tipo de compuestos para mejorar la producción y calidad de los cultivos.

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NOTAS:

CAPITULO 3

VALORIZACIÓN AMBIENTAL DE LOS BIOESTIMULANTES OBTENIDOS A PARTIR DE LODOS DE DEPURADORA

1. INTRODUCCIÓN

1.1. CONTAMINACIÓN. SUELOS CONTAMINADOS

Según la RAE, la contaminación es la "alteración nociva de la pureza o las condiciones normales de una cosa o un medio por agentes químicos o físicos" (Diccionario RAE, 2017). Por lo tanto, podríamos definir la contaminación ambiental como la presencia de agentes físicos, químicos o biológicos en un medio de forma que produzca un efecto nocivo sobre el mismo. En general, cuando se habla de contaminación ambiental, se refiere principalmente a la contaminación atmosférica, hídrica o de los suelos.

Por otra parte, la salud de un suelo se define como la capacidad del mismo para sustentar la productividad biológica, potenciar la calidad de otros compartimentos como la atmósfera y la hidrosfera y mantener la salud de las plantas, delos animales y de los seres humanos (Doran y Safley, 1997).Cuando esta capacidad se ve afectada por la presencia de un agente externo se dice que el suelo está contaminado.

Un suelo contaminado queda definido por la legislación española como aquel cuyas características han sido alteradas negativamente por la presencia de componentes químicos de carácter peligroso procedentes de la actividad humana, en concentración tal que comporte un riesgo inaceptable para la salud humana o el medio ambiente, de acuerdo con los criterios y estándares que se determinen por el Gobierno, y así se haya declarado mediante resolución expresa (Ley 22/2011, de 28 de julio, de Residuos y suelos contaminados y en el Real Decreto 9/2005, de 14 de enero).

En la Unión Europea, se estima que la cantidad de sitios potencialmente contaminados supera los 2,5 millones (Panagos y col., 2013).Por otra parte, existe una baja percepción social del problema, la cual se ve reflejada en el hecho de que aún hay países dentro de la Unión Europea que no comunican a los organismos gubernamentales

pertinentes los datos referentes a los suelos contaminados dentro de sus territorios nacionales (Tabla 7). Ambos factores en conjunto hacen que los suelos contaminados constituyan una amenaza a escala global para la salud medioambiental y humana.

	PCS y CS identificados	PCS estimados	CS estimados	Total
Países	33	12	11	38
Superficie estudiada (km²)	4,460,305	1,552,984	833,188	5,772,075
Superficie estudiada (%)	77.3	26.9	14.4	
PCS	1,169,649	739,968		2,553,000
CS	127,475		32,601	342,000

Tabla 7: Sitios identificados y estimados como contaminados (CS) o potencialmente contaminados (PCS) en la Unión Europea (Panagos y col., 2013).

1.2. TIPOS y CAUSAS DE CONTAMINACIÓN EN SUELOS

1.2.1. Tipos de contaminantes

Aunque existe una amplia variedad de contaminantes, se pueden agrupar en dos grandes grupos, los inorgánicos y los orgánicos.

Los contaminantes inorgánicos incluyen metales (Cd, Cr, Cu, Hg, Mn, Ni, Pb, V o Zn), metaloides (As, Bo o Sb), no metales (Se), actínidos (U) y halógenos (I y F). Aunque algunos de estos elementos son necesarios en bajas dosis para el desarrollo de la vida, a altas dosis son tóxicos, mientras que otros son nocivos a cualquier concentración (Hooda, 2010). Estos elementos se denominan como Elementos Potencialmente Tóxicos o PTEs, de sus siglas en inglés. Aunque los PTEs son sustancias naturales presentes en el planeta desde sus orígenes y algunos de ellos aparecen de forma natural a elevadas concentraciones en determinados lugares, suele ser

la acción del hombre la que origina muchos de los casos de contaminación por estos elementos.

Figura 17: Imagen de suelo contaminado por metales pesados como consecuencia al vertido tóxico de Aznalcóllar

Otros contaminantes inorgánicos de importancia son los radionucleidos, los cuales emiten distintos tipos de radicaciones nocivas. Aunque estos elementos también aparecen de forma natural sin causar perjuicio alguno, es la acción del hombre sobre ellos la que origina los diversos casos de contaminación por radionucleidos. Es necesario incluir también en este grupo ciertos compuestos que en un principio no son tóxicos pero que resultan peligrosos por la gran cantidad empleada, como son los nitratos y fosfatos (Cachada y col., 2018).

Cuando nos referimos a contaminantes orgánicos, hablamos de moléculas en cuya estructura contienen carbono, incluyendo pesticidas, hidrocarburos, PAHs, PCBs, dibenzo-p-dioxinas policloradas (PCDDs), dibenzofuranos policlorados (PCDF), bifenilos polibromados (PBBs), éteres de difenilo polibromados (PBDEs), surfactantes o productos farmacéuticos (Figuras XX).

Figura 18: Suelos contaminados por hidrocarburos

Figuras 19: Contaminación de suelos por pesticidas

Dentro de estos grupos hay infinidad de compuestos distintos que difieren entre ellos en sus propiedades, como polaridad, solubilidad o volatilidad, lo que origina una variedad aún mayor de efectos y toxicidad en el medioambiente y los organismos. Además, dependiendo de su estructura y estabilidad molecular, muchos de estos compuestos poseen una relativamente larga vida media en el medio, es decir, permanecen mucho tiempo en el mismo (Walker y col., 2001). Los xenobióticos son compuestos orgánicos cuya estructura es infrecuente o directamente no existe de forma natural en el medio ambiente, estando todos ellos sintetizados por el hombre en origen. El potencial contaminante de estos compuestos radica en esas estructuras no habituales difíciles de asimilar que afectan al metabolismo de los seres vivos y a su gran persistencia en el medio ambiente (Castillo y col., 2005),

Aunque muchos de estos productos ya han sido prohibidos en numerosos países, otros continúan siendo producidos y utilizados. Además, su número sigue creciendo año tras año y ampliando sus posibles usos, lo cual, sumado a cambios introducidos en sus estructuras que afecta a sus propiedades fisicoquímicas dificultan en gran medida su detección y control (Cachada y col., 2018).

1.2.2. Origen de las contaminaciones

El origen de los contaminantes puede ser o bien de tipo natural, o bien de tipo antropogénico. Ciertos fenómenos naturales pueden ser la causa de la aparición de casos de contaminación, como la actividad volcánica o incendios forestales, además de poder estar presentes de forma continuada esos contaminantes en ciertos afloramientos minerales. Sin embargo, la mayoría de los casos de contaminación tienen un origen humano, ya sea accidental o intencionado. Las actividades que causan más fenómenos de contaminación intencionados son principalmente la minería, fundición, eliminación de desechos, combustión de combustibles fósiles, industrias en general, aplicación de agroquímicos o las aguas residuales. Los casos de contaminación accidental se deben principalmente a inundaciones, fugas o derrames accidentales y accidentes nucleares (Cachada y col., 2018).

Las fuentes de contaminación antrópica más antiguas que existen son las derivadas de los procesos de eliminación de residuos y la minería, que tienen en algunos casos más de 2.000 años de antigüedad. Hoy en día, sin embargo, la mayor fuente de contaminación que está causando problemas a nivel mundial, especialmente en los países en vías de desarrollo, son los llamado e-residuos o basura electrónica, (FAO, 2015). Algunos de los contaminantes generados por estos e-residuos son metales como Hg, Pb, Cd, o Cr, PBBs, PBDEs y PCBs (UNEP, 2005; Tansel, 2017).

La contaminación atmosférica constituye otra fuente de importancia de agentes contaminantes de los suelos, ya sea por deposición seca o húmeda de las partículas transportadas por el aire (Liu y col., 2010; Cachada y col., 2013). Gran parte de estas emisiones proceden de la quema de combustibles fósiles, como las centrales térmicas o el tráfico de vehículos. Estos aerosoles y gases son desplazados largas distancias desde su origen y se componen principalmente de metales como V, Ni, Hg, Se, Sn, Cu, Pb o Zn, así como de PAHs (Biasioli y Ajmone-Marsan, 2007)

Los suelos agronómicos también son una fuente de contaminantes debido a la aplicación indiscriminada de agroquímicos y de lodos de depuración (Arunakumara y col., 2013). Sin embargo, la posible contaminación originada por la aplicación de lodos de depuradora depende del propio origen de esos lodos y de los tratamientos que hayan recibidos previamente a su aplicación en el suelo (Braguglia y col., 2015; Leschber, 2006; Gawlik y Bidoglio, 2006).

1.3. DINÁMICA DE LOS CONTAMINANTES EN EL SUELO

Diversos factores y procesos bióticos y abióticos afectan a la dinámica de los contaminantes en el suelo. Estos factores incluyen las propiedades del suelo, como mineralogía, contenido en materia orgánica, pH, humedad, etc.; las propiedades químicas del contaminante; la actividad biológica presente y otros factores ambientales como la temperatura. Además, estos factores pueden cambiar con el tiempo, de forma que los posibles destinos de esos contaminantes también cambian con ellos (Reid y col., 2000).

Los mecanismos más comunes e importantes a los que está sujeta la dinámica de los contaminantes en el suelo son la volatilización, la lixiviación, la degradación, la

bioacumulación y el secuestro (Figura 22). Debido a que un mismo contaminante puede estar afectado por uno o más mecanismos y a que el compuesto puede desplazarse entre los distintos compartimentos del suelo (suelo, agua y aire), el estudio de su dinámica en el suelo se hace muy complejo (Cachada y col., 2018).

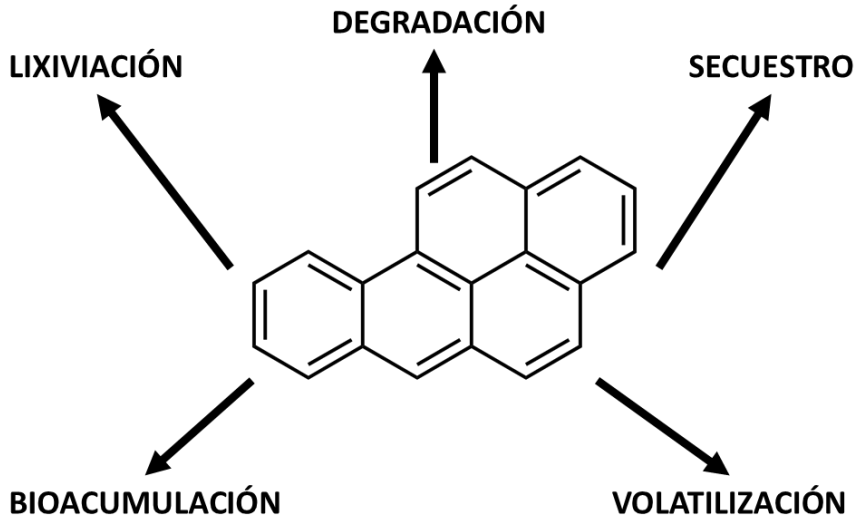

Figura 20: Posibles destinos y comportamientos de un contaminante orgánico hidrofóbico modelo (fenantreno) en el suelo.

Muchos de los contaminantes más persistentes acaban retenidos en los suelos debido a que son incorporados en medios más estables temporalmente, lo que se denomina secuestro. Este secuestro es en muchos casos un proceso irreversible debido a que el compartimento de destino es poco accesible o directamente inaccesible (Yang y col., 2001; Ehlers y Luthy, 2003). Los factores que más afectan a este proceso son las propiedades del suelo como contenido en materiales adsorbentes, tamaño de poro, estructura, etc. (Luthy y col., 1997).

Por otra parte, muchos compuestos son degradados o transformados, ya sea por procesos físicos, químicos o biológicos. Entre los procesos más comunes que suceden en el suelo aparecen reacciones químicas de hidrólisis, oxido-reducciones, isomerizaciones, etc., y procesos biológicos como la biodegradación y la

bioacumulación (Cachada y col., 2018). En algunas ocasiones, las transformaciones que sufren los contaminantes dan lugar a otros compuestos más tóxicos. (DellaGreca y col., 2014; Abdel-Shafy y Mansour, 2016; Chibwe y col., 2017).

La biodisponibilidad de los contaminantes depende de diversos factores relacionados con las características y propiedades del propio contaminante, el suelo donde se encuentre y los organismos a los que afecte. Estos factores se reflejan en la tabla 8 (Cipullo y col., 2018).

TIPO DE FACTOR	**FACTOR**	**EJEMPLOS**
Físico-químicos	Propiedades del contaminante	Estado redox, forma química, peso molecular, polaridad, hidrofobicidad, etc.
	Propiedades del suelo	pH, estado redox, mineralogía, contenido en materia orgánica, humedad, textura, granulometría, capacidad de intercambio iónico, etc.
Biológicos	Captación	Concentración del contaminante, especie biológica, ruta de captación, etc.
	Bioacumulación, bioconcentración y biotransformación	Contenido en lípidos, metabolismo, fase de crecimiento, edad, etc.
Otros	Secuestro	Adsorción, transformación, volatilización, etc.
	Interacción con otros contaminantes	Interacciones metal-metal, metal-compuesto orgánico, compuesto orgánico- compuesto orgánico, etc.

Tabla 8: Factores que afectan a la biodisponibilidad.

1.4. BIORREMEDIACIÓN DE SUELOS CONTAMINADOS

Los suelos contaminados se han tratado convencionalmente mediante técnicas físico-químicas que en muchos casos implicaban además el aislamiento del suelo contaminado para su eficaz procesamiento. Estas técnicas, si bien consiguen eliminar los compuestos contaminantes del suelo, provocan una serie de alteraciones en el suelo, principalmente debidas a la pérdida de estructura del mismo y a la propia agresividad de los tratamientos utilizados (Lombi, y Hamon, 2005)

Como alternativa a los tratamientos físico-químicos, desde mediados del siglo XX comenzaron a emplearse técnicas de carácter biológico para tratar diversos tipos de contaminación, acuñándose el término Biorremediación. La biorremediación es una técnica de descontaminación consistente en el empleo de seres vivos o parte de ellos (enzimas) para tratar un compuesto contaminante por diversos medios, ya sea por absorción, inmovilización, degradación de dicho compuesto, etc. (Alexander, 1999). Una de las principales ventajas de estas técnicas biológicas es que en muchos casos se pueden emplear "in situ", lo que supone un ahorro económico al no ser necesario extraer el suelo contaminado al mismo tiempo que se reducen los efectos sobre la estructura del suelo (Ulrici, 2000; Exner, 1994; Klein, 2000; Viñas, 2005).

Dentro de la biorremediación se engloban numerosas técnicas, las cuales se definen por el efecto que causan sobre el contaminante, quien realiza esa función y cómo la realizan. Uno de los procesos más importante es la biodegradación, consistente este en la eliminación de los compuestos contaminantes mediante la digestión, asimilación y metabolización de los mismos, llevado a cabo por bacterias, hongos, protozoos y otros organismos (García-Martínez, 2009).

Sin embargo, no todo son ventajas al emplear las técnicas de biorremediación. La más importante de todas es el tiempo necesario para que los niveles de

contaminación se reduzcan a unos valores tolerables. Además, se pueden dar casos en los que la biodegradación del compuesto xenobiótico origine un metabolito con un mayor potencial contaminante (Alexander, 1999; Alexander, 2000; Viñas, 2005). Por ello es necesario un estudio previo profundo que permita prever y solucionar todos estos hándicaps en el proceso de descontaminación.

1.5. BIODEGRADACIÓN MICROBIANA DE XENOBIÓTICOS

Muchos microorganismos que viven en el suelo son capaces de degradarlos compuestos xenobióticos gracias a que poseen la maquinaria enzimática necesaria para metabolizar compuestos naturales de estructura similar, y gracias a esa similitud pueden ser transformados para dar finalmente dióxido de carbono y agua. Gracias a esta capacidad, la biodegradación es una potente herramienta capaz de reducir y eliminar la contaminación de suelos por compuestos orgánicos.

La biodegradabilidad de un compuesto en un suelo dado se encuentra afectada por diversos parámetros:

- Estructura molecular del compuesto, su concentración y biodisponibilidad. Aquellos compuestos con estructuras químicas más similares a las naturales, en concentraciones no letales y accesibles para los microorganismos serán más fácilmente degradados que aquellos que no.
- Presencia en términos cualitativos y cuantitativos de los microorganismos que posean la capacidad biodegradativa del compuesto en cuestión, es decir, deben estar presentes en el suelo y en cantidad suficiente aquellos microrganismos que pueden consumir ese compuesto.

- Factores ambientales como pH, temperatura, humedad del suelo, presencia de otros compuestos o la presencia de nutrientes disponibles.

Aunque las propiedades del compuesto (estructura, concentración y biodisponibilidad) y de la microbiota del suelo son parámetros críticos sobre la biodegradación de ese compuesto, los factores ambientales influyen enormemente sobre la dinámica de este proceso. Por un lado, los microorganismos poseen rangos óptimos de crecimiento, fuera de los cuales, los distintos procesos que realizan, incluyendo la biodegradación, se ralentizan. Por otra parte, esos factores pueden también afectar a la biodisponibilidad del compuesto xenobiótico, que en los casos más graves pueden incluso no ser detectados por los microrganismos que lo van a degradar (Semple y col., 2004).

Aunque no existen unas condiciones óptimas universales que sirvan para todos los casos, en general, los pHs situados entre 6-8 y las temperaturas entre 20-30ºC suelen ser óptimos para los procesos biodegradativos en suelos (Alexander, 1999). En esos rangos se alcanza un compromiso entre el crecimiento y actividad microbianos por un lado y la biodisponibilidad del contaminante por otro. Otro factor ambiental que afecta al proceso de biodegradación es la presencia de otros compuestos como sales, surfactantes, coenzimas, etc., así como el contenido en agua, los cuales pueden afectar tanto favorable como desfavorablemente al proceso (Alexander, 1999; Menn y col., 2000).

Con respecto a la disponibilidad de nutrientes, algunos elementos como el nitrógeno o el fósforo se encuentran en el suelo a concentraciones limitantes, lo que suponen un hándicap en el desarrollo y crecimiento de los microorganismos del suelo. La adición de fuentes de N y P inorgánicas, en general, suelen aumentar el crecimiento microbiano y la tasa de biodegradación de los compuestos xenobióticos (Dott y col.,

1995; Breedveld y Sparrevik, 2001; Chaineau y col., 2003). Esto es debido a que esta adicción incrementa el ratio entre Carbono y otros elementos, lo que favorece el crecimiento de la masa microbiana, la cual a su vez se ve inducida a utilizar el compuesto contaminante como fuente de C, incrementando la tasa de biodegradación del compuesto.

Sin embargo, aunque la adición de fuentes inorgánicas de N y P al suelo es beneficiosa para los procesos de biodegradación, existen estudios que describen el efecto contrario (Morgan y Watkinson, 1992). Esto puede ser debido a que si se aporta un elemento de forma exógena y dicho elemento se encuentra presente en la estructura del compuesto a tratar, los microorganismos tenderán a usar aquél que sea más fácil de metabolizar, dejando sin degradar el contaminante cuya estructura es en general mucho más compleja (García-Martínez y col. 2010b).

Por todo ello, una estrategia para favorecer la biodegradación de compuestos contaminantes en suelos es modificar alguno o varios de los factores ambientales que afectan a este proceso (Zaffar Hashmi y col. 2017).

1.6. BIOESTIMULACIÓN MICROBIANA EN SUELOS

La bioestimulación microbiana consiste en añadir al suelo que se quiere tratar nutrientes y/u oxígeno, de forma que los microorganismos nativos que se encuentran allí encuentran unas condiciones más favorables para su crecimiento, aumentando en consecuencia su población (Pankrantz, 2001). Este incremento de la población de microorganismos puede tener como objetivo el aumento de fertilidad del suelo en cuestión, o en el caso de suelos contaminados, aumentar la tasa de biodegradación del contaminante que se encuentra en el mismo.

1.7. TIPOS DE BIOESTIMULANTES PARA BIORREMEDIACIÓN DE SUELOS CONTAMINADOS

Los bioestimulantes usados en procesos de biorremediación de suelos contaminados no difieren de los empleados en procesos de bioestimulación en general. Las ventajas e inconvenientes de cada tipo de bioestimulante también se aplican a los procesos de biorremediación.

Sin embargo, en los procesos de biorremediación es necesario realizar un estudio más exhaustivo tanto del medio como del contaminante a tratar para poder obtener los mejores resultados. Esto es debido a que estos compuestos pueden interaccionar con el contaminante de diversas formas, lo cual puede modificar su biodisponibilidad y biodegradabilidad, o bien pueden afectar a los propios microorganismos alterando la tasa de biodegradación del contaminante, en ambos casos este efecto puede ser tanto positivo como negativo (Katayama y col. 2010).

1.8. LODOS DE DEPURADORA Y BIORREMEDIACIÓN

La aplicación de residuos orgánicos a la tierra, como lodos de depuradora, estiércol animal, residuos sólidos urbanos, vermicompost, etc., se ha empleado comúnmente como herramienta en los procesos de biorremediación de suelos contaminados por xenobióticos. Este efecto se basa principalmente en la adsorción de los xenobióticos por parte de la materia orgánica, reduciendo sus efectos tóxicos, y por otro lado en la estimulación microbiana que esta materia orgánica ejerce sobre los microorganismos, acelerando la degradación de los compuestos contaminantes.

Debido al alto contenido en materia orgánica y nutrientes esenciales de los lodos de depuradora, se han empleado como enmienda para tratar los suelos contaminados por xenobióticos tras haber sido procesados por medio de compostaje. Sin embargo, hay

que tener en cuenta que también pueden poseer altos niveles de metales pesados, contaminantes orgánicos, organismos patógenos, etc. Lo cual podría provocar que no solo no se eliminase el contaminante preexistente, sino que empeorara la situación. Por ello, es necesario desarrollar alternativas con las que tratar los lodos de depuradora mediante las cuales se solventen los problemas inherentes a los mismos al tiempo que se conserva su potencial biorremediador o incluso se mejore.

2. OBJETIVOS

Como consecuencia de la importancia de la materia orgánica en los procesos de biorremediación de suelos contaminados por xenobióticos orgánicos, bien por plaguicidas o bien por hidrocarburos aromáticos policíclicos, el objetivo planteado en el presente capítulo será:

1. Estudiar la capacidad biorremediadora microbiana en suelos contaminados por distintos xenobióticos orgánicos (oxifluorfeno, fenantreno, pireno y benzopireno) enmendados con los productos obtenidos a partir de lodos de depuradora y plumas de pollo a nivel bioquímico, biológico y químico, en particular, sobre las actividades enzimáticas vinculadas a los ciclos biogeoquímicos, la diversidad microbiana y la evolución de los distintos contaminantes estudiados.

3. ARTICULOS

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Accelerated degradation of PAHs using edaphic biostimulants obtained from sewage sludge and chicken feathers

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HIGHLIGHTS

- The number of aromatic rings in the PAHs is closely related to the soil toxicity.
- The application of organic wastes decreased the toxic action of PAHs.
- The low molecular weight protein of wastes increased the degradation of PAHs.

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ABSTRACT

We studied in the laboratory the bioremediation effects over a 100-day period of three edaphic biostimulants (BS) obtained from sewage sludge (SS) and from two different types of chicken feathers (CF1 and CF2), in a soil polluted with three polycyclic aromatic hydrocarbons (PAH) (phenanthrene, Phe; pyrene, Py; and benzo(*a*)pyrene, BaP), at a concentration of 100 mg kg^{-1} soil. We determined their effects on enzymatic activities and on soil microbial community. Those BS with larger amounts of proteins and a higher proportion of peptides (<300 daltons), exerted a greater stimulation on the soil biochemical properties and microbial community, possibly because low molecular weight proteins can be easily assimilated by soil microorganisms. The soil dehydrogenase, urease, β-glucosidase and phosphatase activities and microbial community decreased in PAH-polluted soil. This decrease was more pronounced in soils contaminated with BaP than with Py and Phe. The application of the BS to PAH-polluted soils decreased the inhibition of the soil biological properties, principally at 7 days into the experiment. This decrease was more pronounced in soils contaminated with BaP than with Py and Phe and was higher in polluted soils amended with CF2, followed by SS and CF1, respectively.

1. Introduction

The technique of applying organic matter to soils contaminated with polycyclic aromatic hydrocarbons (PAHs) is widespread among scientists and engineers. It is a good environmental strategy which aims to eliminate or reduce the negative effects that these chemicals cause on soil microorganisms [1–5].

Because the microbial degradation is the most important process for these pollutants, for some authors this second role of organic matter has a greater importance than the first in the bioremediation of PAHs-polluted soils [6–8]. Organic matter mineralization releases nutrients continuously or intermittently over a period of time and therefore has been applied to PAH-contaminated soils to stimulate and maintain indigenous biodegradation rates. It is, however, a slow process that depends on several factors such as the PAH type, soil microorganisms tolerant to this PAH, and the quantity and chemical composition of the M.O. added to soil [4,5,9].

In recent years there has been increasing use of organic fertilizers or edaphic biostimulants (BS), obtained by hydrolysis from various organic materials, in the bioremediation of soils contaminated with organic xenobiotics such as PAHs and plaguicides. Unlike other sources of organic matter, these organic compounds

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Table 1
Characteristics of the experimental soil (mean ± standard error). Data are the means of three samples.

pH (H_2O)	7.9 ± 0.2
Coarse sand (g kg^{-1})	486 ± 49
Fine sand (g kg^{-1})	130 ± 25
Silt (g kg^{-1})	123 ± 29
Clay (g kg^{-1})	260 ± 35
Total N (g kg^{-1})	0.93 ± 0.08
Organic matter (g kg^{-1})	17 ± 1

are usually constituted by low-molecular-weight proteins and amino acids. This aspect is very important, since these compounds can be used directly by the soil microorganisms and therefore accelerates degradation of xenobiotic in soil. These products are also characterized by high polysaccharides content, and humic-like molecules that stimulate soil microorganisms, and thus, promote the degradation of the xenobiotic in soil [5,10–13].

Recently, the use of BS obtained from sewage sludge and chicken feathers has become very common in the degradation of plaguicides, mainly oxyfluorfen and chlorpyrifos [11,13], with a notable increase in the degradation of the above xenobiotics after the applying both BS to the polluted soils.Since nostudies have been reported using these BS on PAH-contaminated soil, we hypothesize that both protein hydrolysates can be very useful in remedying different PAH-contaminated soils.

The objective of this study, therefore, was to investigate, under laboratory conditions, the influence of BS obtained from sewage sludge and chicken feathers in soils polluted with different PAH andits effect on soil biochemical properties and microbial community.

2. Materials and methods

2.1. Soil, biostimulants and PAH characteristics

The soil used in this experiment is a Calcaric Regosol [14]. The main soil characteristics are reported elsewhere [11,13], and summarized in Tables 1 and 2. The methodology used to the determine the physical and chemical parameters in soil and organic wastes is also described in Tejada et al. [11] and Rodriguez-Morgado et al. [13].

Three edaphic BS were used: (1) SS, derived from sewage sludge; (2) CF1, derived from chicken feathers provided by a poultry industry located in Murcia (Spain); and (3) CF2, derived from chicken feathers provided by a local poultry slaughterhouse (TG-SL) located in Seville (Spain). All BS were obtained by enzymatic hydrolysis. Sewage sludge and both feathers were hydrolysed according to the pH-stat method [15], using an endoprotease obtained by liquid fermentation of *Bacillus licheniformis* ATCC 21415 as the hydrolytic agent in a bioreactor operating under controlled temperature and pH, agitation and NaOH consumption [16]. The enzymatic hydrolysis process is detailed in Rodriguez-Morgado et al. [13].

Three hydrocarbons were utilized to artificially contaminate the soil: (1) phenanthrene (Phe), a PAH consisting of three fused benzene rings; (2) pyrene (Py), a PAH consisting of three fused benzene rings; and (3) benzo(*a*)pyrene (BaP), a PAH consisting of five fused benzene rings.

Table 2
Chemical composition (mean ± standard error) of the three experimental edaphic biostimulants. Data are the means of three samples. Rows followed by the same letter(s) are not significantly different ($p > 0.05$).

	SS	CF1	CF2
Organic matter (g kg^{-1})	773b ± 21	550a ± 39	463a ± 48
N-Kjeldhal (g kg^{-1})	34.9c ± 2.3	9.8b ± 2.7	14.1a ± 1.6
Total carbohydrates (g kg^{-1})	42b ± 19	5.6a ± 1.6	65c ± 11
P (g kg^{-1})	2.9a ± 0.1	73c ± 18	27b ± 8
S (g kg^{-1})	5.9a ± 1.6	11b ± 3	19b ± 4
Fat (g kg^{-1})	18a ± 3	11a ± 2	20a ± 2
Protein molecular weight (daltons)			
>10,000	21.4a ± 2.6	21.6a ± 1.7	23.8a ± 2.4
10000–5000	7.3b ± 1.5	2.8a ± 0.8	9.1b ± 1.1
5000–1000	5.5a ± 2.0	5.8a ± 1.1	25.1b ± 2.6
1000–300	1.8b ± 0.4	1.4b ± 0.3	8.0b ± 1.5
<300	64.0b ± 3.6	68.4b ± 4.4	34.0a ± 3.1

2.2. Incubation procedure

Prior to being treated, seven hundred grams of soil were pre-incubated at 25 °C for 7 days at 30–40% of their water-holding capacity, according to Tejada [17]. After this pre-incubation period, soil samples were mixed with Phe Py or BaP, respectively. According to Mueller and Shann [18] and Smith et al. [19], each PAH was dissolved in acetone and added to soil at 100 mg kg^{-1}soil. Acetone was also added to the non-polluted soils (control treatment). Each treatment was mixed thoroughly and allowed to evaporate two days in a fume hood. During this period, the soil was mixed at intervals to ensure homogeneous distribution of the PAHS in each treatment. Subsequently, the three experimental BS was added to the soil. Soil samples were mixed with CF1 and CF2 to a concentration of 1%, 1.3% and 1.6%, respectively, in order to apply the same amount of organic matter with each BS to the soil. According to Rodriguez-Morgado et al. [13], all biostimulants were liquid and were solubilized in distilled water (500 l ha^{-1}) before applying. An unamended and non-polluted soil was used as control. The incubation treatments are described in Table 3.

Triplicate treatments were kept in semi-closed microcosms at 20 ± 2 °C for 100 days, respectively.

2.3. Soil PAH determinations

The Phe and Py were extracted from 5 g samples of soil. These samples were introduced into a 50 ml centrifuge tube with 4 ml of deionized water and shaken for 1 min, after which, 10 ml of acetonitrile were added to the tube and the mix was shaken for a further 30 s. Finally, a QuEChERS dSPE Phenomenex ref. KSO-8912 extraction kit was added to the centrifuge tube. It was vigorously shaken for one minute and then centrifuged for 5 min at 4000 rpm [20]. The extracts were passed through a 0.45-μm syringe filter and the filtered extracts were concentrated to 1 ml. They were then analyzed by gas chromatograph equipped with autosampler, programmable splitless inlet temperature of deactivated fused silica pre-column of intermediate polarity (1–3 m × 0.25–0.32 mm internal diameter), connected by connector to a fused silica column with 5% phenylmethylsilicone (5MS type) and dimensions 30 m × 0.25 mm × 0.1 μm, for mass spectrometry detector. Analysis conditions were as follows:

Autosampler: Wash the syringe with hexane; volume injected: 2 μl; injection speed: 1.0 μl s^{-1}; washing time of the syringe: 20 s; pause: 2 s; shooting speed: μl s^{-1}

Injector program: Initial temperature: 70 °C; ramp: hold for 0.1 min, up to 300 °C–200 °C min^{-1}, keep up to 40 min

Gas chromatograph (separation): Temperature of the transfer line 280 °C; initial temperature: 70 °C; ramp: hold for 1.5 min, up to 300 °C at 5 °C min^{-1}, hold 4 min.

Flow of carrier gas (He): 1.5 ml min^{-1} measured at baseline.

Mass detector (purchase): Acquisition time: 45 min; delay in the filament/multiplier: 2.50 min.

The extraction of BaP from soil was performed using the Song et al. [21] method. A sample of 1.5 g of soil was put in a 15-ml Pyrex tube and10 ml acetone was added, shaken on a vortex and sonicated for 20 min. Subsequently, the soil sample was centrifuged

Table 3
Experimental treatments.

1. C control soil, non-polluted soil and non-organically amended
2. Phe soil polluted with phenanthrene and non-organically mended
3. Py, soil polluted with pyrene and non-organically amended
4. BaP, soil polluted with benzo(*a*)pyrene and non-organically amended
5. SS, non-polluted soil and amended with SS
6. CF1, non-polluted soil and amended with CF1
7. CF2, non-polluted soil and amended with CF2
8. SS+Phe soil polluted with phenanthrene and amended with SS
9. SS+Py, soil polluted with pyrene and amended with SS
10. SS+BaP, soil polluted with benzo(*a*)pyrene and amended with SS
11. CF1+Phe soil polluted with phenanthrene and amended with CF1
12. CF1+Py, soil polluted with pyrene and amended with CF1
13. CF1+BaP, soil polluted with benzo(*a*)pyrene and amended with CF1
14. CF2+Phe soil polluted with phenanthrene and amended with CF2
15. CF2+Py, soil polluted with pyrene and amended with CF2
16. CF2+BaP, soil polluted with benzo(*a*)pyrene and amended with CF2

at 13700 × *g* for 15 min, the supernatant was added to 20 ml glass flasks and the acetone used to extract BaP was left to evaporate. The same procedure was repeated again twice and the extracts were added to a 20-ml flask. The extracts were passed through a 0.45-μm syringe filter. The methodology used to measure the BaP is described in Tejada et al. [5].

2.4. Soil biological determinations

The activity levels of four soil enzymes for each treatment were measured at days 1, 5, 7, 12, 20, 40, 70 and 100 during the incubation period. Dehydrogenase activity was measured as the reduction of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl) 5-phenyl tetrazoliumchloride to iodonitrophenylformazan [22]. Urease activity was determined by the buffered method of Kandeler and Gerber [23], using urea as the substrate. β-glucosidase activity was determined using *p*-nitrophenyl-β-D-glucopyranoside as the substrate [24]. Phosphatase activity was measured using *p*-nitrophenyl phosphate as the substrate [25].

For each treatment, phospholipids were extracted at days 5, 12, 40 and 100 during the incubation period, (three replicates per treatment) using a chloroform–methanol extraction method based on Bligh and Dyer [26]. Phospholipids were transformed by alkaline methanolysis into fatty acid methyl esters (FAMEs), which were quantified by a gas chromatograph (TRACE GC Ultra, Thermo Scientific) fitted with a 60-m capillary column (BPX70 60 m × 0.25 mm ID × 0.25 μm film), using helium as carrier gas. The initial temperature was 150 °C for 0.5 min and it was increased to 180 °C at 2 °C min^{-1} and then to 240 °C at 4 °C min^{-1}. The Gram+ representative fatty acids were: i15:0, a15:0, i16:0, and i17:0 and the Gram− specific fatty acids were: 18:1u9c, 18:1u9t, cy17:0, and cy19:0. The fatty acids i15:0, a15:0, 15:0, i16:0, i17:0, 18:1u9c, 18:1u9t, cy17:0, and cy19:0 were chosen to represent the biomass of bacterial community [27–29]. The fatty acid 18:2u6 was taken to indicate the fungal biomass [30–32]. All results are given in nmol g^{-1}.

For each treatment and each experimental day, 20 g of soil was taken. Soil samples were stored in sealed polyethylene bags at 4 °C for 15 days, prior to analysis of the enzymatic activities and at −20 °C prior to phospholipid analysis [11,13].

2.5. Statistical analysis

Data were submitted to two-way ANOVA with treatment and sampling time as factors followed by Tukey's significant difference as a post hoc test, considering a significance level of $p < 0.05$ throughout the study. The ANOVA was performed using the Statgraphics Plus 2.1 software package. For the ANOVA, triplicate data were used for each treatment and for every experimental day.

3. Results

3.1. Evolution of PAH in soils

In non-organically amended soils, the degradation of Phe was higher than Py and BaP (Fig. 1). At the end of the experimental period, Phe was degraded to 49.6% of what had initially been added, followed by degradation levels of 31.9% and 12.3% for Py and BaP, respectively.

The application of the different BS to contaminated soil increased Phe Py and BaP degradation. However, this degradation depended on the BS type applied to the polluted soils. In this respect and at the end of the experiment, the Phe had decreased by 70.5%, 63.2% and 59.5% when CF1, SS and CF2 had been added to the polluted soils. The Py had decreased by 59.45%, 50.6% and 47.2% when CF1, SS and CF2 had been added to the polluted soils. Finally, the BaP had decreased by 45.1%, 39% and 31.4% when CF1, SS and CF2 had been added to the polluted soils.

3.2. Soil biological analysis

After applying the three experimental BS, the statistical analysis indicated a significant ($p < 0.05$) stimulation of dehydrogenase activity during the first days, mainly at 7 days after the beginning of the experiment (Table 4). In this respect, and compared to the control treatment, soil dehydrogenase activity increased significantly by 91.1%, 90.3% and 85.6% in the CF1, SS and CF2 treatments, respectively. After the first 7 days, the dehydrogenase activity began to decline gradually. At the end of the experimental period all treatments studied had very similar values.

Applying the three experimental PAHs to soil showed a significant decrease in dehydrogenase activity (Table 4). At the end of the experimental period and compared with the control treatment, dehydrogenase activity decreased significantly ($p < 0.05$) by56%, 73.6% and 87.2% in Phe Py and BaP polluted soils, respectively.

Seven days after applying the three BS to PAH-contaminated soils, the dehydrogenase activity showed a minor decrease (Table 5). This decrease was higher CF2-amended polluted soils, followed by SS and CF1, respectively. With respect to the experimental PAHs, this decrease was higher in organic soils polluted with BaP, followed by Py and Phe, respectively. From day 7 of the beginning the incubation until the end of the experiment, the dehydrogenase activity decreased gradually. However, this decrease was less significant in organically-polluted soils amended with the three experimental BS. Again, this decrease was higher in polluted soils and amended with CF2, followed by SS and CF1, respectively.

Unlike the dehydrogenase activity, the soil urease activity was not stimulated after the application of the three BS studied (Table 5). Similar to the dehydrogenase activity, when the PAHs were applied to the unamended soil, there was a significant ($p < 0.05$) decrease in this enzyme activity throughout the experimental period. At the end of the experimental period and compared with the control treatment, dehydrogenase activity decreased significantly ($p < 0.05$) by 53.5%, 69% and 86% in soils polluted with Phe Py and BaP, respectively. These same results were also observed when the three BS studied were applied to the soil polluted with the different PAHs.

Similar to the dehydrogenase activity, the β-glucosidase activity was also stimulated in organically-amended soils, mainly 7 days after the beginning of the experiment (Tables 6 and 7). Again, this stimulation ($p < 0.05$) was significantly higher in the CF2 treatment than in the SS and CF1 treatments (14.5% and 33.9%, respectively).

The response of β-glucosidase activity to the application of the three experimental PAHs to soil was very similar to other enzyme activities studied (Table 6). Thus, an inhibition of this enzyme in the soils with PAHs was observed. This inhibition was higher in soils

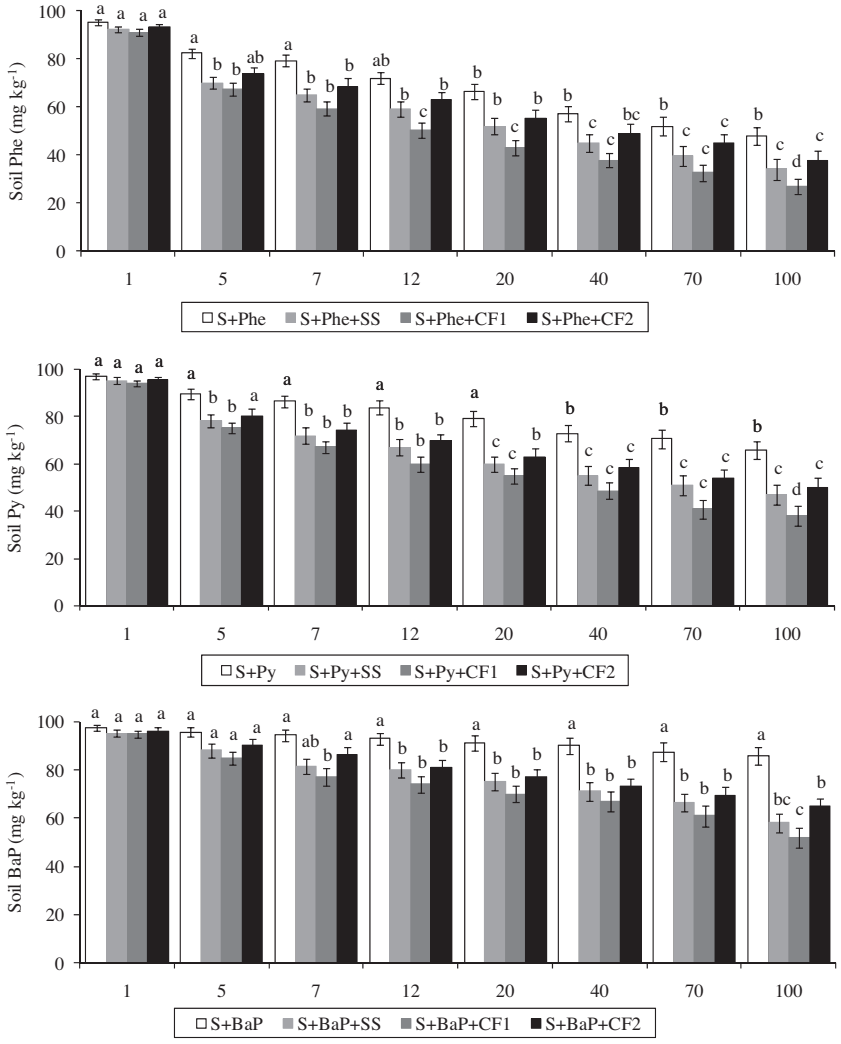

Fig. 1. Evolution of phenanthrene (Phe), pyrene (Py) and benzo(*a*)pyrene (BaP) (mean ± standard error) in soils during the experimental period. Data are the means of three samples. Columns followed by the same letter(s) are not significantly different ($p > 0.05$).

polluted with BaP, followed by Py and Phe respectively. Applying the BS to polluted soils also decreased the inhibition of this enzymatic activity. Seven days after the beginning the experiment, this decrease was higher in polluted soil amended with CF1, followed by SS and CF2, respectively. Again, the type of PAH with which the soil was contaminated influenced in this decrease; the highest decrease was always more pronounced in soils contaminated with Phe followed by Py and BaP, respectively. Compared with the unamended

Table 4
Evolution of dehydrogenase activity (μg INTF g^{-1} h^{-1}) in soils amended with the experimental edaphic biostimulants and polluted with phenanthrene (Phe), pyrene (Py) and benzo(*a*)pyrene (BaP) during the experimental period. Data are expressed as mean values ± standard error. Columns followed by the same letter(s) are not significantly different ($p > 0.05$). INTF: 2-*p*-iodo-3-nitrophenyl formazan.

	Incubation days							
	1	5	7	12	20	40	70	100
C	2.8c ± 0.5	2.6c ± 0.3	2.7c ± 0.3	2.6c ± 0.2	2.5c ± 0.4	2.5c ± 0.4	2.5c ± 0.3	2.5c ± 0.4
Phe	2.7c ± 0.4	2.4c ± 0.3	2.2c ± 0.3	2.0c ± 0.4	1.8b ± 0.2	1.5b ± 0.3	1.3b ± 0.2	1.1b ± 0.2
Py	2.4c ± 0.4	2.0c ± 0.3	1.8b ± 0.3	1.4b ± 0.3	1.1b ± 0.2	0.89b ± 0.17	0.78b ± 0.16	0.66a ± 0.13
BaP	2.0c ± 0.3	1.7b ± 0.2	1.4b ± 0.2	1.0b ± 0.2	0.89b ± 0.15	0.68a ± 0.11	0.43a ± 0.1	0.32a ± 0.09
SS	6.6d ± 1.2	15.4e ± 1.3	27.9f ± 2.4	20.9f ± 1.9	8.1d ± 1.5	3.9c ± 0.6	3.2c ± 0.6	2.5c ± 0.4
CF1	7.4d ± 1.1	17.0f ± 1.2	30.5f ± 2.4	21.2f ± 1.8	10.6e ± 1.9	4.2d ± 1.1	3.5c ± 1.3	2.7c ± 0.8
CF2	4.8d ± 1.3	14.7e ± 1.8	18.7f ± 1.6	14.0e ± 2.0	7.2d ± 1.1	3.4c ± 0.5	3.0c ± 0.5	2.6c ± 0.4
SS+Phe	6.1d ± 1.1	11.1e ± 1.4	24.9f ± 2.1	16.6e ± 1.8	2.9c ± 0.9	1.9c ± 0.5	1.7b ± 0.3	1.7b ± 0.4
SS+Py	5.8d ± 1.2	10.0e ± 1.7	22.6f ± 2.4	13.9e ± 1.4	3.4c ± 0.9	1.7b ± 0.3	1.4b ± 0.2	1.0b ± 0.2
SS+BaP	4.1d ± 1.1	8.9e ± 1.1	15.6e ± 1.9	10.4e ± 1.9	2.2c ± 0.8	1.4b ± 0.4	0.95b ± 0.18	0.81b ± 0.11
CF1+Phe	6.5d ± 1.3	12.2e ± 1.6	26.3f ± 2.3	18.7f ± 2.0	3.3c ± 1.1	2.2c ± 0.5	1.6b ± 0.3	1.8b ± 0.3
CF1+Phy	6.0d ± 1.0	11.1e ± 1.3	23.1f ± 2.6	15.0e ± 2.1	2.7c ± 0.7	1.8b ± 0.5	1.5b ± 0.3	1.2b ± 0.2
CF1+BaP	3.9c ± 1.1	8.0d ± 1.2	14.5e ± 1.9	9.2e ± 1.9	2.0c ± 0.4	1.2b ± 0.3	0.83b ± 0.20	0.71b ± 0.17
CF2+Phe	4.0d ± 1.3	8.9e ± 1.4	14.1e ± 1.1	9.3e ± 1.0	2.5c ± 0.3	1.7b ± 0.4	1.5b ± 0.3	1.4b ± 0.3
CF2+Phy	3.7c ± 1.2	7.7d ± 1.3	9.8e ± 1.4	8.2d ± 1.7	2.0c ± 0.4	1.1b ± 0.4	0.92b ± 0.15	0.83b ± 0.14
CF2+BaP	3.2c ± 1.1	6.4d ± 1.1	8.0d ± 1.5	7.0d ± 1.8	1.6b ± 0.3	0.89b ± 0.19	0.72b ± 0.18	0.59b ± 0.13

Table 5
Evolution of urease activity ($\mu g\, NH_4^+\, g^{-1}\, h^{-1}$) in soils amended with the experimental edaphic biostimulants and polluted with phenanthrene (Phe), pyrene (Py) and benzo(*a*)pyrene (BaP) during the experimental period. Data are expressed as mean values ± standard error. Columns followed by the same letter(s) are not significantly different ($p > 0.05$).

	Incubation days							
	1	5	7	12	20	40	70	100
C	2.4c ± 0.5	2.2c ± 0.3	2.2c ± 0.4	2.1c ± 0.3	2.2c ± 0.2	2.0c ± 0.4	2.1c ± 0.3	2.0c ± 0.3
Phe	2.3c ± 0.3	1.9c ± 0.3	1.7c ± 0.3	1.6c ± 0.4	1.5c ± 0.3	1.3b ± 0.3	1.2b ± 0.1	0.93b ± 0.14
Py	2.0c ± 0.4	1.5c ± 0.3	1.2b ± 0.1	0.98b ± 0.19	0.88b ± 0.20	0.79 ± 0.15	0.68b ± 0.19	0.62b ± 0.18
BaP	1.8c ± 0.4	1.1b ± 0.2	0.88b ± 0.15	0.71b ± 0.18	0.56a ± 0.11	0.44a ± 0.12	0.39a ± 0.10	0.28a ± 0.05
SS	2.2c ± 0.4	2.0c ± 0.3	1.9c ± 0.2	2.4c ± 0.4	2.5c ± 0.5	2.3c ± 0.5	2.0c ± 0.4	2.2c ± 0.4
CF1	2.2c ± 0.3	2.1c ± 0.2	2.0c ± 0.4	2.3c ± 0.4	2.2c ± 0.4	2.1c ± 0.4	2.0c ± 0.3	2.4c ± 0.5
CF2	2.4c ± 0.5	2.1c ± 0.3	2.1c ± 0.4	2.1c ± 0.3	2.2c ± 0.3	2.2c ± 0.4	2.1c ± 0.3	2.2c ± 0.4
SS+Phe	2.2c ± 0.4	2.0c ± 0.3	1.8c ± 0.3	1.5c ± 0.2	1.3b ± 0.2	1.2b ± 0.3	1.1b ± 0.2	0.89b ± 0.14
SS+Phy	1.9c ± 0.5	1.5b ± 0.2	1.1b ± 0.2	0.94b ± 0.18	0.81b ± 0.11	0.75b ± 0.15	0.69b ± 0.14	0.59a ± 0.18
SS+BaP	1.7c ± 0.3	1.2b ± 0.2	0.84b ± 0.16	0.69b ± 0.15	0.55a ± 0.11	0.46a ± 0.12	0.37a ± 0.08	0.25a ± 0.09
CF1+Phe	2.2c ± 0.4	1.9c ± 0.3	1.8c ± 0.3	1.5c ± 0.3	1.2b ± 0.1	1.3b ± 0.2	1.0b ± 0.1	0.91b ± 0.17
CF1+Phy	1.9c ± 0.3	1.6c ± 0.3	1.2b ± 0.2	1.0b ± 0.1	0.87b ± 0.12	0.77b ± 0.14	0.70b ± 0.15	0.64b ± 0.11
CF1+BaP	1.7c ± 0.4	1.1b ± 0.1	0.83b ± 0.20	0.74b ± 0.16	0.58a ± 0.17	0.41a ± 0.15	0.37a ± 0.06	0.26a ± 0.08
CF2+Phe	2.1c ± 0.2	2.0c ± 0.4	1.7c ± 0.3	1.6c ± 0.2	1.3b ± 0.2	1.2b ± 0.1	1.0b ± 0.1	0.94b ± 0.14
CF2+Phy	2.0c ± 0.4	1.6c ± 0.2	1.3b ± 0.3	1.0b ± 0.2	0.79b ± 0.18	0.72b ± 0.16	0.65b ± 0.16	0.61b ± 0.15
CF2+BaP	1.8c ± 0.3	1.2b ± 0.2	0.81b ± 0.15	0.74b ± 0.11	0.54a ± 0.13	0.41a ± 0.18	0.34a ± 0.09	0.25a ± 0.05

polluted soils and at the end of the incubation period, the soil β-glucosidase activity increased significantly ($p < 0.05$). As before, this increase was higher in polluted soils amended with CF2, followed by SS and CF1, respectively.

The soil phosphatase activity was also significantly ($p < 0.05$) stimulated after the application of both BS to the soil (Table 6). Similar to the dehydrogenase and β-glucosidase activities, the soil phosphatase activity showed a higher stimulation 7 days after beginning the experiment. Again, this stimulation was higher in soils amended with CF2, followed by SS and CF1, respectively. This stimulation decreased as the experimental period progressed. At the end of the experimental period all experimental treatments had similar values. Also, the application of PAHs to the soil inhibited this enzymatic activity during the experimental period. This inhibition was higher in soils polluted with BaP, followed by Py and Phe respectively. Similar to the enzymes studied, when BS was applied to the soil with PAHs, the inhibition of phosphatase activity decreased. This decrease was higher in the polluted soils amended with CF2, followed by SS and CF1, respectively.

The application of the three experimental BS increased the soil bacteria and fungi population (Tables 8–10). Similar to the results for enzymatic activities, this increase was greatest in the CF1 treatment than in the SS and CF treatments, respectively. Again, and in the organic treatments, the highest population of bacteria and fungi was found 7 days of beginning the experiment. These populations decreased during the experimental period. At the end of the experiment, the bacteria population was similar to that found in the control treatment. When the three experimental PAHs were applied to the soil, the soil microbial community decreased significantly ($p < 0.05$). This decrease was more pronounced in BaP-contaminated soils than in those contaminated with Py and Phe, respectively. Similar to the results of the enzymatic activities studied, the application of the BS to the PAHs-polluted soils decreased the inhibition in the growth of the microbial community. Again, this decrease was higher in the polluted soils amended with CF2, followed by SS and CF1, respectively.

4. Discussion

According to Tejada et al. [5], Zhang et al. [33], González et al. [34] and González-Paredes et al. [35], our results indicate that applying PAHs to soil induce adverse effects on biological properties. This toxicity was higher in BaP-contaminated soils, followed by Py and Phe suggesting that the number of aromatic hydrocarbon rings influences the toxicity caused on the soil's biological

Table 6
Evolution of β-glucosidase activity (mmol PNP $g^{-1}\, h^{-1}$) in soils amended with the experimental edaphic biostimulants and polluted with phenanthrene (Phe), pyrene (Py) and benzo(*a*)pyrene (BaP) during the experimental period. Data are expressed as mean values ± standard error. Columns followed by the same letter(s) are not significantly different ($p > 0.05$). PNP: *p*-nitrophenol.

	Incubation days							
	1	5	7	12	20	40	70	100
C	1.4c ± 0.3	1.4c ± 0.2	1.4c ± 0.3	1.3c ± 0.1	1.4c ± 0.3	1.2c ± 0.2	1.1c ± 0.2	1.1c ± 0.1
Phe	1.3c ± 0.3	1.1c ± 0.2	0.97b ± 0.19	0.88b ± 0.12	0.79b ± 0.14	0.65b ± 0.17	0.57b ± 0.11	0.49b ± 0.13
Py	1.1c ± 0.2	0.90b ± 0.19	0.79b ± 0.16	0.68b ± 0.10	0.60b ± 0.17	0.51b ± 0.13	0.44b ± 0.12	0.31a ± 0.14
BaP	0.98b ± 0.18	0.83b ± 0.15	0.68b ± 0.13	0.53b ± 0.10	0.46b ± 0.13	0.33a ± 0.10	0.26a ± 0.11	0.15a ± 0.08
SS	2.3c ± 0.8	3.5d ± 0.8	5.3e ± 1.1.	4.5d ± 0.9	3.3d ± 0.6	2.7d ± 0.4	1.4c ± 0.4	1.2c ± 0.2
CF1	2.2c ± 0.6	3.8d ± 0.6	6.2e ± 1.1	5.3e ± 1.0	3.7d ± 0.5	2.9d ± 0.6	1.6c ± 0.3	1.2c ± 0.2
CF2	2.2c ± 0.6	2.9d ± 0.6	4.1d ± 1.0	3.0d ± 0.6	2.6d ± 0.5	2.0c ± 0.4	1.3c ± 0.3	1.0b ± 0.2
SS+Phe	2.1c ± 0.3	3.0d ± 0.5	4.1d ± 0.5	3.5d ± 0.9	1.7c ± 0.3	1.0b ± 0.1	0.94b ± 0.09	0.86 ± 0.07
SS+Py	2.0c ± 0.6	2.9d ± 0.4	3.9d ± 0.7	3.0d ± 0.7	1.2c ± 0.3	0.85b ± 0.07	0.68b ± 0.08	0.50b ± 0.08
SS+BaP	1.6c ± 0.4	2.2c ± 0.6	3.0d ± 0.4	2.6d ± 0.4	0.91b ± 0.06	0.66b ± 0.08	0.47b ± 0.08	0.26a ± 0.05
CF1+Phe	2.4d ± 0.3	3.3d ± 0.8	4.7d ± 0.9	3.8d ± 0.8	2.0c ± 0.4	1.5c ± 0.3	1.2c ± 0.2	0.92b ± 0.08
CF1+Phy	2.2c ± 0.5	2.9d ± 0.6	4.2d ± 0.6	3.4d ± 0.5	1.5c ± 0.3	1.0b ± 0.1	0.76b ± 0.05	0.56b ± 0.05
CF1+BaP	1.7c ± 0.2	2.5d ± 0.4	3.5d ± 0.6	3.0d ± 0.9	1.4c ± 0.2	0.88b ± 0.05	0.60b ± 0.07	0.32a ± 0.04
CF2+Phe	1.7c ± 0.4	2.6d ± 0.3	3.2d ± 0.7	2.8d ± 0.7	1.4c ± 0.3	0.95b ± 0.06	0.83b ± 0.05	0.71b ± 0.08
CF2+Phy	1.7c ± 0.4	2.5d ± 0.4	3.7d ± 0.4	2.9d ± 0.6	1.1c ± 0.2	0.85b ± 0.09	0.62b ± 0.04	0.42b ± 0.04
CF2+BaP	1.6c ± 0.3	2.0c ± 0.5	2.6d ± 0.5	2.1c ± 0.4	0.84b ± 0.08	0.58b ± 0.04	0.39a ± 0.03	0.23a ± 0.03

Table 7
Evolution of phosphatase activity (μmol PNP g^{-1} h^{-1}) in soils amended with the experimental edaphic biostimulants and polluted with phenanthrene (Phe), pyrene (Py) and benzo(*a*) pyrene (BaP) during the experimental period. Data are expressed as mean values ± standard error. Columns followed by the same letter(s) are not significantly different ($p > 0.05$). PNP: *p*-nitrophenol.

	Incubation days							
	1	5	7	12	20	40	70	100
C	3.6c ± 1.3	3.6c ± 1.1	3.5c ± 1.4	3.5c ± 1.2	3.5c ± 1.4	3.4c ± 1.7	3.4c ± 1.3	3.3c ± 1.2
Phe	3.4c ± 1.1	3.0c ± 1.4	2.8b ± 0.9	2.5b ± 1.0	2.3b ± 0.8	1.9b ± 0.5	1.7b ± 0.5	1.4b ± 0.3
Py	3.0c ± 1.1	2.5b ± 1.3	2.3b ± 1.1	1.9b ± 0.7	1.7b ± 0.4	1.4b ± 0.2	1.1a ± 0.2	0.82a ± 0.21
BaP	2.6b ± 1.2	2.1b ± 1.3	1.9b ± 0.7	1.5b ± 0.7	1.1a ± 0.2	0.83a ± 0.19	0.59a ± 0.14	0.46a ± 0.17
SS	4.8c ± 1.3	19.1e ± 2.3	34.0e ± 2.9	20.1e ± 1.5	18.6e ± 1.9	6.9c ± 1.4	3.5c ± 1.2	3.2c ± 1.3
CF1	5.6c ± 1.5	23.9e ± 2.1	37.6f ± 2.7	26.2e ± 1.6	19.1e ± 1.1	7.4d ± 1.6	3.6c ± 1.5	3.2c ± 1.1
CF2	4.2c ± 1.4	18.7d ± 2.7	24.9e ± 2.1	15.8d ± 2.2	13.5d ± 2.1	5.2c ± 1.1	3.5c ± 1.5	3.1c ± 1.1
SS+Phe	4.5c ± 1.3	7.9d ± 1.9	18.3e ± 2.5	12.1d ± 1.8	8.6d ± 1.1	3.9c ± 1.3	2.8b ± 0.9	2.3b ± 0.8
SS+Py	4.3c ± 1.6	7.1d ± 2.1	14.9d ± 2.0	9.9d ± 1.3	7.1d ± 1.3	3.0c ± 1.1	2.2b ± 0.7	1.5b ± 0.4
SS+BaP	2.9c ± 0.8	6.4c ± 1.1	11.8d ± 2.2	7.9d ± 1.1	6.5c ± 0.9	2.4b ± 0.7	1.3b ± 0.2	0.82a ± 0.06
CF1+Phe	4.8c ± 1.1	8.3d ± 1.6	20.2d ± 2.4	14.8d ± 1.9	8.8d ± 1.6	4.2c ± 1.6	3.1c ± 1.0	2.5b ± 0.9
CF1+Phy	4.5c ± 1.5	7.7d ± 2.0	17.6d ± 2.8	10.6d ± 1.5	7.4d ± 1.2	3.1c ± 1.1	2.3b ± 0.9	1.5b ± 0.2
CF1+BaP	3.1c ± 1.1	6.7c ± 1.9	12.8d ± 1.9	8.4d ± 1.3	6.7c ± 1.3	2.6b ± 0.8	1.5b ± 0.3	0.91a ± 0.07
CF2+Phe	3.1c ± 1.3	6.4c ± 1.5	13.7d ± 2.0	8.9d ± 1.7	4.7c ± 1.5	3.0c ± 1.0	2.4b ± 0.9	1.9b ± 0.2
CF2+Phy	2.8b ± 0.6	6.1c ± 1.1	12.8d ± 2.1	7.9d ± 1.3	3.9c ± 1.2	2.5b ± 0.7	1.6b ± 0.3	1.0a ± 0.1
CF2+BaP	2.6b ± 0.8	5.4c ± 1.3	10.3d ± 2.4	6.8c ± 1.8	4.2c ± 1.6	1.8b ± 0.3	0.95a ± 0.08	0.76a ± 0.08

properties. These results are in agreement with those obtained of Wammer et al. [36] and Toledo et al. [37].

This toxic effect directly affects the inhibition of most soil microbial populations, with some remaining microbial groups that are able to adapt and grow in the presence of these organic compounds [5,38]. Tolerant microorganisms may utilize PAHs as source of carbon and energy for their growth and development, transforming and degrading these organic compounds into less toxic

Table 8
Evolution of bacterial Gram+, bacterial Gram−, total bacterial and fungal PLFAs (nmol g^{-1}) in soils amended with the experimental edaphic biostimulants and polluted with phenanthrene (Phe) during the experimental period. Data are expressed as mean values ± standard error. Columns followed by the same letter(s) are not significantly different ($p > 0.05$).

	Incubation days			
	5	12	40	100
Bacterial Gram+ PLFA				
C	2.8b ± 0.9	2.6b ± 0.7	2.8b ± 0.7	2.7b ± 0.8
Phe	2.6b ± 0.7	2.1b ± 0.5	1.5ab ± 0.4	1.1a ± 0.2
SS	19.7c ± 1.4	38.0d ± 2.7	3.9b ± 0.9	2.6b ± 0.4
CF1	21.9c ± 1.8	39.6d ± 2.5	4.4b ± 1.1	2.6b ± 0.6
CF2	16.6c ± 1.4	35.8d ± 3.2	3.1b ± 0.6	2.7b ± 0.5
SS+Phe	17.0c ± 1.1	29.8d ± 2.7	2.2b ± 0.3	1.6ab ± 0.3
CF1+Phe	18.6c ± 1.3	33.3d ± 3.1	2.4b ± 0.5	1.8ab ± 0.3
CF2+Phe	14.9c ± 1.2	24.1d ± 2.6	2.0b ± 0.5	1.4a ± 0.2
Bacterial Gram- PLFA				
C	1.2b ± 0.3	1.1b ± 0.2	1.2b ± 0.2	1.0b ± 0.2
Phe	1.1b ± 0.4	0.89ab ± 0.11	0.68a ± 0.12	0.49a ± 0.07
SS	3.9c ± 1.0	7.3d ± 2.2	1.8b ± 0.5	1.2b ± 0.2
CF1	4.3c ± 1.2	7.9d ± 2.5	2.1b ± 0.3	1.1b ± 0.2
CF2	3.1c ± 0.9	6.3d ± 2.0	1.5b ± 0.4	1.1b ± 0.3
SS+Phe	2.6c ± 0.9	6.0d ± 1.2	0.91ab ± 0.13	0.68a ± 0.10
CF1+Phe	2.9c ± 0.7	6.3d ± 1.5	1.0b ± 0.1	0.73a ± 0.08
CF2+Phe	2.1b ± 0.4	5.5c ± 1.1	0.82a ± 0.15	0.60a ± 0.08
Total bacterial PLFA				
C	4.0b ± 1.1	3.7b ± 1.2	4.0b ± 1.1	3.7b ± 1.1
Phe	3.7b ± 1.2	3.0a ± 0.7	2.2a ± 0.6	1.6a ± 0.4
SS	23.6c ± 2.5	45.1d ± 4.6	5.7b ± 1.2	3.8b ± 0.5
CF1	26.2c ± 3.1	47.5d ± 4.8	6.5b ± 1.5	3.7b ± 0.9
CF2	19.7c ± 2.4	42.1d ± 5.1	4.6b ± 1.1	3.8b ± 1.0
SS+Phe	19.6c ± 2.2	35.8d ± 3.8	3.1b ± 0.5	2.3a ± 0.5
CF1+Phe	21.5c ± 1.9	39.6d ± 4.5	3.4b ± 0.7	2.5a ± 0.5
CF2+Phe	17.0c ± 1.7	29.6cd ± 3.6	2.8a ± 0.8	2.0a ± 0.3
Fungal PLFA				
C	0.53b ± 0.12	0.48b ± 0.10	0.49b ± 0.11	0.51b ± 0.08
Phe	0.47b ± 0.15	0.37a ± 0.09	0.28a ± 0.08	0.23a ± 0.09
SS	0.92b ± 0.19	1.9c ± 0.4	1.1b ± 0.1	0.54b ± 0.09
CF1	0.96b ± 0.15	2.1c ± 0.2	1.2b ± 0.1	0.55b ± 0.08
CF2	0.77b ± 0.12	1.6c ± 0.3	0.95c ± 0.13	0.48b ± 0.07
SS+Phe	0.66b ± 0.11	1.5c ± 0.3	0.40a ± 0.11	0.35a ± 0.12
CF1+Phe	0.69b ± 0.10	1.7c ± 0.3	0.43a ± 0.08	0.39a ± 0.12
CF2+Phe	0.60b ± 0.13	1.3c ± 0.2	0.36a ± 0.07	0.27a ± 0.10

Table 9
Evolution of bacterial Gram+, bacterial Gram−, total bacterial and fungal PLFAs (nmol g^{-1}) in soils amended with the experimental edaphic biostimulants and polluted with pyrene (Py) during the experimental period. Data are expressed as mean values ± standard error. Columns followed by the same letter(s) are not significantly different ($p > 0.05$).

	Incubation days			
	5	12	40	100
Bacterial Gram+ PLFA				
C	2.8b ± 0.9	2.6b ± 0.7	2.8b ± 0.7	2.7b ± 0.8
Py	2.1b ± 0.7	1.4a ± 0.4	0.96a ± 0.18	0.74a ± 0.11
SS	19.7c ± 1.4	38.0d ± 2.7	3.9b ± 0.9	2.6b ± 0.4
CF1	21.9c ± 1.8	39.6d ± 2.5	4.4b ± 1.1	2.6b ± 0.6
CF2	16.6c ± 1.4	35.8d ± 3.2	3.1b ± 0.6	2.7b ± 0.5
SS+Py	12.0c ± 1.5	31.4d ± 3.3	1.9b ± 0.6	1.1a ± 0.2
CF1+Py	14.9c ± 1.1	33.9d ± 2.4	2.1b ± 0.6	1.3a ± 0.2
CF2+Py	11.1c ± 1.3	28.6d ± 3.0	1.7ab ± 0.4	0.89a ± 0.15
Bacterial Gram− PLFA				
C	1.2b ± 0.3	1.1b ± 0.2	1.2b ± 0.2	1.0b ± 0.2
Py	0.92b ± 0.15	0.62a ± 0.17	0.43a ± 0.11	0.30a ± 0.10
SS	3.9d ± 1.0	7.3d ± 2.2	1.8c ± 0.5	1.2b ± 0.2
CF1	4.3d ± 1.2	7.9e ± 2.5	2.1c ± 0.3	1.1b ± 0.2
CF2	3.1c ± 0.9	6.3d ± 2.0	1.5b ± 0.4	1.1b ± 0.3
SS+Py	2.5c ± 0.8	6.4d ± 1.6	0.83b ± 0.15	0.43a ± 0.11
CF1+Py	2.9c ± 0.9	6.9d ± 2.1	0.91b ± 0.13	0.47a ± 0.16
CF2+Py	2.2c ± 0.6	5.2d ± 1.5	0.74b ± 0.16	0.34a ± 0.09
Total bacterial PLFA				
C	4.0b ± 1.1	3.7b ± 1.2	4.0b ± 1.1	3.7b ± 1.1
Py	3.0b ± 0.9	2.0a ± 0.6	1.4a ± 0.4	1.0a ± 0.2
SS	23.6c ± 2.5	45.1d ± 4.6	5.7b ± 1.2	3.8b ± 0.5
CF1	26.2c ± 3.1	47.5d ± 4.8	6.5b ± 1.5	3.7b ± 0.9
CF2	19.7c ± 2.4	42.1d ± 5.1	4.6b ± 1.1	3.8b ± 1.0
SS+Py	14.5c ± 2.4	37.8d ± 4.0	2.7b ± 0.9	1.5a ± 0.3
CF1+Py	17.8c ± 2.0	40.8d ± 3.3	3.0b ± 0.8	1.8a ± 0.4
CF2+Py	13.3c ± 2.0	33.8d ± 4.4	2.4ab ± 0.6	1.2a ± 0.2
Fungal PLFA				
C	0.53b ± 0.12	0.48b ± 0.10	0.49b ± 0.11	0.51b ± 0.08
Py	0.43b ± 0.15	0.29b ± 0.11	0.18a ± 0.07	0.12a ± 0.08
SS	0.92c ± 0.19	1.9c ± 0.4	1.1c ± 0.1	0.54b ± 0.09
CF1	0.96c ± 0.15	2.1d ± 0.2	1.2c ± 0.1	0.55b ± 0.08
CF2	0.77c ± 0.12	1.6c ± 0.3	0.95c ± 0.13	0.48b ± 0.07
SS+Py	0.57bc ± 0.09	1.6c ± 0.2	0.32b ± 0.08	0.22a ± 0.07
CF1+Py	0.65c ± 0.10	1.9c ± 0.3	0.35b ± 0.07	0.26b ± 0.07
CF2+Py	0.50b ± 0.09	1.3c ± 0.2	0.28b ± 0.07	0.18a ± 0.04

Table 10
Evolution of bacterial Gram+, bacterial Gram−, total bacterial and fungal PLFAs (nmol g^{-1}) in soils amended with the experimental edaphic biostimulants and polluted with benzo(*a*)pyrene (BaP) during the experimental period. Data are expressed as mean values ± standard error. Columns followed by the same letter(s) are not significantly different ($p > 0.05$).

	Incubation days			
	5	12	40	100
Bacterial Gram+ PLFA				
C	2.8c ± 0.9	2.6c ± 0.7	2.8c ± 0.7	2.7c ± 0.8
BaP	1.9c ± 0.7	1.1b ± 0.4	0.75ab ± 0.16	0.33a ± 0.09
SS	19.7e ± 1.4	38.0f ± 2.7	3.9c ± 0.9	2.6c ± 0.4
CF1	21.9f ± 1.8	39.6f ± 2.5	4.4d ± 1.1	2.6c ± 0.6
CF2	16.6e ± 1.4	35.8f ± 3.2	3.1c ± 0.6	2.7c ± 0.5
SS+BaP	11.4e ± 1.1	13.9e ± 1.4	1.6b ± 0.3	0.86b ± 0.15
CF1+BaP	13.1e ± 1.5	14.6e ± 1-3	1.9b ± 0.2	0.93b ± 0.13
CF2+BaP	9.0d ± 1.3	10.8e ± 1.1	1.0b ± 0.2	0.64a ± 0.13
Bacterial Gram− PLFA				
C	1.2c ± 0.3	1.1c ± 0.2	1.2c ± 0.2	1.0c ± 0.2
BaP	0.80c ± 0.15	0.43b ± 0.11	0.32b ± 0.07	0.12a ± 0.04
SS	3.9d ± 1.0	7.3e ± 2.2	1.8d ± 0.5	1.2c ± 0.2
CF1	4.3e ± 1.2	7.9e ± 2.5	2.1d ± 0.3	1.1c ± 0.2
CF2	3.1d ± 0.9	6.3e ± 2.0	1.5c ± 0.4	1.1c ± 0.3
SS+BaP	2.3d ± 0.3	5.5e ± 1.5	0.63b ± 0.12	0.37b ± 0.09
CF1+BaP	2.7d ± 0.4	6.0e ± 1.3	0.69b ± 0.16	0.48b ± 0.10
CF2+BaP	1.7c ± 0.3	4.3e ± 1.3	0.45b ± 0.11	0.26a ± 0.08
Total bacterial PLFA				
C	4.0c ± 1.1	3.7c ± 1.2	4.0c ± 1.1	3.7c ± 1.1
BaP	2.7b ± 0.8	1.5b ± 0.4	1.1b ± 0.2	0.45a ± 0.11
SS	23.6e ± 2.5	45.1f ± 4.6	5.7d ± 1.2	3.8c ± 0.5
CF1	26.2ef ± 3.1	47.5f ± 4.8	6.5d ± 1.5	3.7c ± 0.9
CF2	19.7e ± 2.4	42.1f ± 5.1	4.6d ± 1.1	3.8c ± 1.0
SS+BaP	13.7e ± 1.5	19.4e ± 3.0	2.2c ± 0.6	1.2b ± 0.3
CF1+BaP	15.8e ± 2.0	20.6e ± 2.7	2.6c ± 0.4	1.4b ± 0.3
CF2+BaP	10.7e ± 1.5	15.1e ± 2.3	1.5b ± 0.4	0.9a ± 0.2
Fungal PLFA				
C	0.53c ± 0.12	0.48c ± 0.10	0.49c ± 0.11	0.51c ± 0.08
BaP	0.37b ± 0.06	0.20b ± 0.06	0.12a ± 0.09	0.07a ± 0.02
SS	0.92d ± 0.19	1.9de ± 0.4	1.1d ± 0.1	0.54c ± 0.09
CF1	0.96d ± 0.15	2.1e ± 0.2	1.2d ± 0.1	0.55c ± 0.08
CF2	0.77c ± 0.12	1.6d ± 0.3	0.95d ± 0.13	0.48c ± 0.07
SS+BaP	0.53c ± 0.11	0.88c ± 0.20	0.37b ± 0.15	0.18b ± 0.05
CF1+BaP	0.60c ± 0.14	0.94d ± 0.21	0.43c ± 0.14	0.25b ± 0.06
CF2+BaP	0.44c ± 0.12	0.61c ± 0.13	0.25b ± 0.09	0.13a ± 0.03

compounds [38]. Therefore, decreasing the PAH-tolerant microbial population, also decreases the soil's enzymatic properties. This is possibly the reason why, in our experiment, the persistence of the BaP in non-amended soils was higher than in those with Py and Phe respectively. In addition, in the biodegradation of PAHs, not only toxicity but other abiotic factors such as pH, solubility (bioavailability), etc., are important factors for biodegradation efficiency. Phe solubility, and thus bioavailability, is much higher than those of Py and BaP, accelerating their degradation [39].

The application of the three experimental BS into PAH-polluted soils caused a decrease in the contents of Phe Py and BaP in soil and, therefore, a decrease in the toxicity of these PAHs in the soil's enzymatic properties and microbial community. These results are in agreement with those obtained by Tejada et al. [5], who observed a significant decrease in BaP in soil after application of three BS (wheat condensed distillers soluble, hydrolyzed poultry feathers; and rice bran extract). According to above authors, the percentage of proteins and peptides of low molecular weight (<300 daltons) plays an essential role in the bioremediation of BaP-polluted soils, since these peptides can be absorbed directly by soil microorganisms, thus increasing the stimulation of the soil microbial community and, therefore, PAH degradation in soil.

Numerous studies pointed to the role of organic matter for sorption processes of PAHs, probably due to the functional groups of humic substances, such as carboxyl, phenolic, alcohol, and carbonyl, causing a decrease on toxicity of these PAHS [4,40,41]. However, Tejada et al. [5] suggest that the content of low molecular weight peptides of organic matter as the limiting chemical component in the bioremediation of soils contaminated by PAHs. In this respect, comparing our results with those obtained by Tejada and Masciandaro [4], BaP degradation was higher in polluted soils amended with the three experimental BS than with other sources of organic matter such as the organic fraction of municipal solid waste and poultry manure, rich in humic acids. Also, Gomez et al. [12] in a study of bioremediation of soils polluted with the oxyfluorfen herbicide using different sources of organic matter also found that the content of low-molecular-weight proteins is more limiting than humic acids content in organic matter.

In our experiment, and after applying the three experimental BS to the contaminated soil, the higher microbial stimulation occurred 7 days after initiating the incubation of the soil amended with CF1, followed by SS and CF2, due to the higher number of peptides and proteins of low molecular weight (<300 daltons). Similarly, the minor chemical complexity of the Phe is possibly responsible for its higher degradation speed in soil than Py and BaP, respectively. This leads to the fact that the highest degradation percentage occurs at day 7 after the beginning of the experiment. The stimulation of the PAH-tolerant bacteria are responsible for this high percentage of PAH degradation in the first days after applying the BS.

As the BS is degraded and consumed, the microbial population decreases. However, it is highly probable that, due to the decrease of PAH in soil, microorganisms that were previously intolerant to existing PAH concentrations, are now present in the soil. This increases the PAH degradation level and thus significantly reduces the negative effects on the soil's biochemical properties and its microbial community.

5. Conclusions

It can be concluded that the application of BS decreased the toxic action of PAH on soil biological properties. However, this effect depended on the chemical composition of the BS that was applied to the soil. When CF1 was applied to PAH-polluted soils, a greater decrease in the inhibition of the soil's biological properties occurred. This is probably due to their higher content of lower molecular weight peptides which are easily assimilated by soil microorganisms.

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Degradation of chlorpyrifos using different biostimulants/biofertilizers: Effects on soil biochemical properties and microbial community

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ABSTRACT

In this manuscript we conducted a laboratory investigation over a 120-day period studying the effect of three biostimulants/biofertilizers (BS), in a Calcaric Regosol soil, polluted with chlorpyrifos insecticide at a rate of 5 L ha^{-1} (manufactures rate recommended). The BS were manufactured by the pH-stat method, from two different types of chicken feathers (CF1 and CF2) and from sewage sludge (SS). We determined their effects on enzymatic activities and the structure of the soil microbial community by analyzing phospholipid fatty acids (PLFAs). The BS that contained higher amounts of proteins and a higher proportion of peptides, under 0.3 kDa, exerted a greater stimulation on the dehydrogenase, β-glucosidase, phosphate and arylsulfatase activities, possibly because low molecular weight proteins can be easily assimilated by soil microorganisms. The soil urease activity was not stimulated because these chemical compounds were rich in low molecular weight proteins. Soil biological parameters decreased in insecticide-polluted soil. The application of the BS in chlorpyrifos-polluted soils decreased the inhibition of the soil enzymatic activities and biodiversity, principally at 10 days into the experiment. However, this inhibition decrease was higher when CF2 was applied to soil, followed by SS and CF1, respectively. This suggested that the application of BS with higher amounts of proteins and a higher proportion of peptides under 0.3 kDa is more beneficial for remediation of soils polluted with chlorpyrifos.

1. Introduction

Chlorpyrifos [$C_9H_{11}Cl_3NO_3PS$ or *O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate] is a broad-spectrum organophosphorus insecticide that is widely used for insect pest control in agriculture and for soil and foliar treatments in different crops (Korade and Fulekar, 2009; Tejada et al., 2011; Zhang et al., 2012). However, due to its intensive use and its inappropriate application, chlorpyrifos is of environmental concern as it is toxic and can cause a high contamination risk to soil and groundwater (Korade and Fulekar, 2009). Therefore, the remediation of chlorpyrifos-contaminated soils, is required in order to mitigate the hazardous effects of this insecticide.

In soil, microbes are an important biological component of the soil ecosystem and play vital roles in soil fertility through their participation in nutrient cycling and organic matter degradation (Miltner et al., 2004; Wichern et al., 2007). Consequently, a toxic effect of chlorpyrifos on soil microorganisms would be of public concern (Tejada et al., 2011). The measurement of microbial parameters, such as enzyme activities and the microbial community, may provide information on presence and the activity of viable microorganisms as well as on the effects of chlorpyrifos on soil metabolic activity. Such measurements may serve as a good index of the impact of pollution on soil health and can provide information of the resistance and dynamics of chlorpyrifos in soils (Zhang et al., 2010; Tejada et al., 2011). Subsequently, the comparison of the soil enzymatic activities and biodiversity could be helpful when evaluating the impacts of chlorpyrifos on soils.

Organic amendments play an important role in enhancing the soil fertility and microbial activity (Saviozzi et al., 1999; Fernández et al., 2009), therefore, they may also decrease the inhibitory effects of chlorpyrifos on soil microbes. In the last year, several authors have used different sources of organic matter such as agro-residues (coconut husk, peat mass, peanut shell and rice husk),

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municipal solid waste, cow manure, biogas slurry, spent mushroom compost and vermicompost, etc., in order to accelerate the degradation of chlorpyrifos in soil (Romyen et al., 2007; Tejada et al., 2011; Kadian et al., 2012).

Generally, these organic compounds contain a higher protein content of high molecular weight and therefore, the microorganisms need to employ a large amount of energy to degrade these organics. Very slowly over time, this causes the degradation of the pesticide, by soil microorganisms. Therefore, by obtaining an organic product with a high content of low molecular weight proteins which are fast and easily assimilated by soil microorganisms, without high energy consumption, this could accelerate the degradation of the contaminant in soil (Tejada et al., 2010).

In the recent years, there has been an increasing use of hydrolysates organic biostimulants/biofertilizers (BS) obtained from different organic materials by hydrolysis reactions (Parrado et al., 2008; García-Martínez et al., 2010a,b). These products are characterized by a high content of low molecular weight proteins. This aspect is of great interest, as these small proteins may be directly assimilated by soil microorganisms with lower energy expenditure. In this respect, Tejada et al. (2010) observed a rapid MCPA herbicide degradation in soils amended with different BS such as wheat condensed distillers solubles enzymatic hydrolizate, carob germ extract and rice bran enzymatic extract. These authors concluded that the molecular size of the proteins that constitute these organic materials were a critical parameter in the rapid degradation in soil MCPA. This rapid degradation of the herbicide in soil could alleviate the environmental problems caused by pesticides. The authors also emphasized that besides these low molecular weight proteins, these products are also characterized by a high content of polysaccharides, and humic-like molecules that stimulate soil microorganisms, and thus, could promote the degradation of the xenobiotic in soil.

We hypothesize that both protein hydrolysates can be very useful in the remediation of chlorpyrifos-contaminated soils. This aspect is of great environmental interest, since no studies have been reported using different BS to remediate chlorpyrifos-contaminated soil. For this reason, the objective of this study was to investigate, under laboratory conditions, the influence of different BS in a chlorpyrifos-polluted soil and its effect on soil biological properties.

2. Material and methods

2.1. Soil, BS and insecticide characteristics

The soil used in this experiment is a Calcaric Regosol (FAO, 1989). Soil samples were collected from the 0–25 cm surface layer. The main soil characteristics are shown in Table 1. Soil pH was determined in distilled water with a glass electrode (soil:H_2O ratio 1:2.5 w/v). Soil texture was determined by Robinson's pipette method (Avery and Bascomb, 1982). N-Kjeldahl was determined by the MAPA (1986) method. Soil organic-C was determined by the method of Yeomans and Bremner (1988).

The insecticide used in this experiment was chlorpyrifos. The commercial formulation Senator® 48 (48% chlorpyrifos) was purchased from Bayer CropScience (Madrid, Spain). The recommended dose for soil application of chlorpyrifos is 5 L ha^{-1} which, according to Giménez et al. (2004), caused toxic effects on soil enzyme activities.

Three BS were used: (1) BS derived from sewage sludge (SS) by enzymatic hydrolysis process, and (2) two BS derived from chicken feathers (CF1 and CF2) also obtained by enzymatic hydrolysis. The differences between CF1 and CF2 are a consequence of the different origin of this organic material. Sewage sludge and both feathers were hydrolysed according to the pH-stat method (Adler-Nissen, 1977), using an endoprotease obtained by liquid fermentation of *Bacillus licheniformis* ATCC 21415 as the hydrolytic agent in a bioreactor operating under controlled temperature and pH, agitation and NaOH consumption (Parrado et al., 2008).

The BSs were chemically analyzed (Table 2). Organic matter content was determined by combustion at 550 °C for 6 h. Phosphorus and sulfur were determined after combustion and analyzed by an inductively coupled plasma atomic emission spectrometry (ICP-AES) using a Fisons-ARL 3410 sequential multielement instrument equipped with a data acquisition and control system. Summarized standard operational conditions of this instrument are: argon, the carrier, coolant, and plasma gas at 80 psi of pressure, the carrier gas flow rate is 0.8 L min^{-1}, the coolant gas flow rate is 7.5 L min^{-1}, the plasma gas flow rate is 0.8 L min^{-1}, and the integration time is 1 s. One mini-torch consumes argon gas at a radio-frequency power of 650 W. Crude fat was determined gravimetrically after extraction with hexane for 12 h in a soxhlet extractor (Clemente et al., 1997). Total nitrogen was determined by the Kjeldahl method (AOAC, 1990).

The molecular mass distribution of protein in the samples was determined by size-exclusion chromatography using an ÄKTA-purifier (GE Healthcare) and a Superdex Peptide™ 10/300GL column (optimum separation range 300–10,000 Da) (Table 3). Samples were centrifuged at 12,000 × *g* for 30 min at 4 °C to remove insoluble molecules; the supernatant was passed through a 0.2 μm filter and loaded into a 0.1 mL loop connected to an ÄKTA-purifier system. The column was equilibrated, and eluted with 0.25 M Tris–HCl buffer (pH 7.0) in isocratic mode, at a flow-rate of 0.5 mL min^{-1}, and proteins/peptides were detected at 280 and 215 nm with a GE Healthcare UV900 module coupled to the column elution.

2.2. Biostimulation experiment design

Five hundred grams of soil were pre-incubated at 25 °C for 7 days at 30–40% of their water-holding capacity according to Tejada (2009), prior to the treatments. After this pre-incubation period, soil samples were mixed with chlorpyrifos.

Three days after applying insecticide to soil, the three BS were also applied to the soil. Soil samples were mixed with SS at a rate of 0.50%, or CF1 at a rate of 0.8% or CF2 at a rate of 0.65%, in order to applying to the soil the same amount of organic matter with each

Table 1
Characteristics of the experimental soil (mean ± standard error). Data are the means of three samples.

pH (H_2O)	7.9 ± 0.2
Coarse sand (g kg^{-1})	486 ± 49
Fine sand (g kg^{-1})	130 ± 25
Silt (g kg^{-1})	123 ± 29
Clay (g kg^{-1})	260 ± 35
N-Kjeldahl (g kg^{-1})	0.93 ± 0.08
Organic C (g kg^{-1})	17 ± 1

Table 2
Chemical composition of the three biostimulants/biofertilizers. Data are the means of three samples. Rows (mean ± S.E.) followed by the same letter(s) are not significantly different ($p > 0.05$).

	SS	CF1	CF2
Organic matter (g kg^{-1})	773b ± 21	463a ± 48	550a ± 39
N-Kjeldhal (g kg^{-1})	34.9c ± 2.3	14.1b ± 1.6	9.8a ± 2.7
Total carbohydrates (g kg^{-1})	42a ± 19	65a ± 11	73a ± 18
Total P (g kg^{-1})	2.9a ± 0.1	27c ± 8	11b ± 3
Total S (g kg^{-1})	5.9a ± 1.6	19b ± 4	11b ± 2
Fat (g kg^{-1})	18a ± 3	20a ± 2	281b ± 10

Table 3
Molecular weight distribution of the three biostimulants/biofertilizers. Data are the means of three samples. Columns (mean ± S.E.) followed by the same letter(s) are not significantly different ($p > 0.05$).

Molecular weight (Da)	SS	CF1	CF2
>10,000	21.4a ± 2.6	23.8a ± 2.4	21.6a ± 1.7
10000–5000	7.3b ± 1.5	9.1b ± 1.1	2.8a ± 0.8
5000–1000	5.5a ± 2.0	25.1b ± 2.6	5.8a ± 1.1
1000–300	1.8a ± 0.4	8.0b ± 1.5	1.4a ± 0.3
<300	64.0b ± 3.6	34.0a ± 3.1	68.4b ± 4.4

BS. The BSs were liquid and were suspended in distilled water before the application. An unamended soil was used as the control. Distilled water was added to each soil to bring it to 60% of its water-holding capacity. The incubation treatments are detailed as follows:

1. C, control soil, no organic amendments and no pollutants.
2. I, soil polluted with chlorpyrifos and no organic amendments.
3. SS, soil non-polluted and amended with SS.
4. CF1, soil non-polluted and amended with CF1.
5. CF2, soil non-polluted and amended with CF2.
6. SS + I, soil polluted with chlorpyrifos and amended with SS.
7. CF1 + I, soil polluted with chlorpyrifos and amended with CF1.
8. CF2 + I, soil polluted with chlorpyrifos and amended with CF2.

Triplicate treatments were kept in semi-closed microcosms at 25 ± 1 °C for 120 days.

2.3. Soil analysis

The activity levels of five soil enzymes for each treatment were measured at days 3, 6, 10, 30, 60, 90 and 120 during the incubation period. Dehydrogenase activity was measured as the reduction of 2-*p*-iodo-3-nitrophenyl 5-phenyl tetrazolium chloride to iodo nitrophenyl formazan (Tejada, 2009). Urease activity was determined by the buffered method of Kandeler and Gerber (1988), using urea as the substrate. β-glucosidase activity was determined using *p*-nitrophenyl-β-D-glucopyranoside as the substrate (Masciandaro et al., 1994). Phosphatase activity was measured using *p*-nitrophenyl phosphate as the substrate (Tabatabai and Bremner, 1969). Arylsulfatase activity was determined using *p*-nitrophenyl sulfate as substrate (Tabatabai and Bremner, 1970).

Phospholipids were extracted at days 3, 10, 60 and 120 during the incubation period for each treatment, (three replicates per treatment) using a chloroform–methanol extraction based on Bligh and Dyer (1959). They were fractioned and quantified using the procedure described by Frostegard et al. (1993a,b) and Bardgett et al. (1996). Twenty-six separated fatty acid methyl esters were identified using gas chromatography and a flame ionization detector. The phospholipids were transformed by alkaline methanolysis into fatty acid methyl esters (FAMEs), which were quantified with a gas chromatograph (GC/FID, AutoSystem XL Gas Chromatograph, Varian Saturno 2000) fitted with a 50-m capillary column, using helium as the carrier gas. The injector temperature was 260 °C, the flame ionization detector temperature was 280 °C, and the initial temperature was 70 °C (for 2 min); it was increased to 160 °C at 30 °C min^{-1} and then to 280 °C at 3 °C min^{-1}.

To estimate the various proportions of the main taxa in the samples according to the phospholipid fatty acids (PLFAs), the biomarkers i15:0, a15:0, i16:0, 16:1ω7c, 17:0, i17:0, cy17:0, 18:1ω9c, and cy19:0 were used to represent bacterial biomass (bacPLFA) (Frostegard et al., 1993a,b; Bardgett et al., 1996) and 18:2ω6 (fungPLFA) was taken to indicate fungal biomass (Federle et al., 1986). All results are given in nmol g^{-1}.

For each treatment and each incubation time, 20 g of soil were taken. Soil samples were stored in sealed polyethylene bags at −20 °C and the soil analyses were carried out immediately during the first 15 days after collection.

2.4. Chlorpyrifos determination in soil

The extraction of chlorpyrifos from soil was realized using the Menon et al. (2004) method. The methodology used in the extraction and measurement of chlorpyrifos in soil is described in Tejada et al. (2011).

2.5. Statistical analysis

Data were submitted to two-way ANOVA with treatment and sampling time as factors followed by Tukey's significant difference as a post hoc test, considering a significance level of $p < 0.05$

Table 4
Evolution of dehydrogenase and urease activities in soils amended with the biostimulants/biofertilizers and polluted with chlorpyrifos during the experimental period. Data are expressed as mean values ± standard error. Columns followed by the same letter(s) are not significantly different ($p > 0.05$). INTF: 2-*p*-iodo-3-nitrophenyl formazan.

	Dehydrogenase activity (μg INTF g^{-1} h^{-1})						
	3	6	10	30	60	90	120
C	2.3b ± 0.4	2.4b ± 0.4	2.3b ± 0.5	2.2b ± 0.4	2.3b ± 0.5	2.1b ± 0.4	2.1b ± 0.5
I	1.7a ± 0.3	1.5a ± 0.2	1.2a ± 0.3	1.3a ± 0.3	1.6a ± 0.4	1.8a ± 0.3	2.0 ab ± 0.4
SS		13.3d ± 2.2	22.7d ± 3.4	5.8c ± 1.4	2.9b ± 0.7	2.2b ± 0.4	2.2b ± 0.2
CF1		8.1c ± 1.7	16.9d ± 1.9	4.7c ± 1.1	2.4b ± 0.8	2.2b ± 0.5	2.1b ± 0.3
CF2		19.6d ± 2.5	26.3e ± 3.1	7.3c ± 1.6	3.1b ± 0.8	2.3b ± 0.4	2.2b ± 0.3
SS + I		8.8c ± 1.7	15.6d ± 1.6	3.9b ± 1.1	2.3b ± 0.3	2.3b ± 0.4	2.2b ± 0.4
CF1 + I		5.9c ± 1.9	10.4d ± 1.2	3.1b ± 1.1	2.2b ± 0.4	2.4b ± 0.3	2.1b ± 0.3
CF2 + I		10.4d ± 1.5	19.1d ± 1.8	4.7c ± 1.2	2.2b ± 0.3	2.3b ± 0.4	2.3b ± 0.3
	Urease activity (μg NH_4^+ g^{-1} h^{-1})						
	3	6	10	30	60	90	120
C	1.8b ± 0.3	1.9b ± 0.3	1.8b ± 0.2	1.8b ± 0.3	1.7b ± 0.3	1.7b ± 0.2	1.6b ± 0.3
I	1.1a ± 0.2	0.8a ± 0.2	0.6a ± 0.2	0.7a ± 0.3	0.9a ± 0.2	1.4a ± 0.3	1.6b ± 0.4
SS		1.8b ± 0.4	1.9b ± 0.3	1.8b ± 0.2	1.8b ± 0.3	1.7b ± 0.3	1.7b ± 0.4
CF1		1.9b ± 0.4	1.8b ± 0.3	1.8b ± 0.4	1.7b ± 0.4	1.8b ± 0.4	1.7b ± 0.3
CF2		1.9b ± 0.3	1.8b ± 0.2	1.7b ± 0.4	1.8b ± 0.2	1.7b ± 0.3	1.6b ± 0.3
SS + I		0.8a ± 0.2	0.7a ± 0.2	0.6a ± 0.1	0.8a ± 0.2	1.3a ± 0.2	1.5b ± 0.2
CF1 + I		0.8a ± 0.3	0.6a ± 0.2	0.6a ± 0.2	0.8a ± 0.3	1.4a ± 0.3	1.6b ± 0.3
CF2 + I		0.9a ± 0.2	0.5a ± 0.1	0.6a ± 0.1	0.7a ± 0.3	1.3a ± 0.3	1.6b ± 0.4

throughout the study. The ANOVA was performed using the Statgraphics Plus 2.1 software package. For the ANOVA, triplicate data were used for each treatment and for each day of incubation.

3. Results

3.1. Enzyme activity in soil

Statistical analysis indicated a significant ($p < 0.05$) stimulation of the dehydrogenase activity during the first day after the application of the three BS, peaking 10 days after the beginning of the experiment (Table 4). In this respect, and compared to the control, the soil dehydrogenase activity significantly increased ($p < 0.05$) by 86.1%, 90.2% and 92.6% in the CF1, SS and CF2 treatments, respectively. After the first 10 days, the dehydrogenase activity began to decline gradually. At the end of the experimental period all treatments studied had very similar values.

The application of chlorpyrifos in soil showed a significant decrease in dehydrogenase activity (Table 4). This decrease was maximal at 10 days of applying the insecticide in soil (47% compared to the control treatment). This decline was diminished over the experimental period and we observed a decrease of the dehydrogenase activity at 90 days of applying the chlorpyrifos in soil (47.8% compared to the control treatment). This decline was diminished over the experimental period and we observed a significant ($p < 0.05$) decrease of the dehydrogenase activity up to 90 days of applying the chlorpyrifos in soil (47.8% compared to the control treatment). At the end of the experimental period, the dehydrogenase activity in I treatment was similar to C treatment.

The application of BS in chlorpyrifos contaminated soils caused a minor decrease in dehydrogenase activity (Table 4). In this respect, at 10 days of applying the insecticide in the soil, and compared to that CF1 treatment, the dehydrogenase activity decreased 38.5% in the CF1 + I treatment. Compared to SS treatment, the dehydrogenase activity decreased 31.2% in the SS + I treatment, and compared to CF2 treatment, the dehydrogenase activity decreased 27.4% in the CF2 + I treatment. At 30 days of the incubation period, this decrease in soil dehydrogenase activity was diminished progressively over the experimental period, noting that in soils amended with the three BS, this activity showed similar values to those of amended soil without chlorpyrifos.

Unlike the dehydrogenase activity, the soil urease activity was not stimulated after the application of the BS studied, throughout the experimental period (Table 4). Similar to the dehydrogenase activity, when chlorpyrifos was applied to the unamended soil, there was a significant ($p < 0.05$) decrease of this enzyme activity until 90 days of the experiment. At the end of the incubation period, similar results were obtained for C and I treatments. These same results were also observed when the three BS studied were applied to the soil contaminated by the insecticide.

The β-glucosidase activity was also stimulated in organically amended soils, mainly at 10 days from the beginning of the experiment (Table 5). Again, this stimulation ($p < 0.05$) was significantly higher in the CF2 and SS treatments, followed by CF1 treatment. The application of chlorpyrifos to the soils inhibited the β-glucosidase activity during the first 90 days of the experimental period. At the end of the incubation period, similar results were obtained for C and I treatments (Table 5). The application of the BS in the insecticide-polluted soil also showed a similar response to the other enzymatic activities studied. In this respect, the application of BS to the soil decreased the inhibition of this enzymatic activity. At 10 days of applying the insecticide in the soil, and compared to that CF1 treatment, the β-glucosidase activity decreased 31.8% in the CF1 + I treatment. Compared to SS treatment, the β-glucosidase activity decreased 26.4% in the SS + I treatment, and compared to CF2 treatment, the β-glucosidase activity decreased 23.1% in the CF2 + I treatment. Again, at 30 days of the incubation period, this decrease in soil β-glucosidase activity was progressively reduced during the experimental period, noting that in soils amended with the three BS, this activity showed similar values than the amended soil without chlorpyrifos.

The soil phosphatase activity was also significantly ($p < 0.05$) stimulated after the application of the three BS to the soil (Table 5). Similar to the dehydrogenase and β-glucosidase activities, the soil phosphatase activity showed a higher stimulation at 10 days after beginning the experiment. This stimulation decreased as the experimental period progressed and at the end of the incubation period all experimental treatments had similar values. Also, chlorpyrifos applied to the soil inhibited this enzymatic activity during the experimental period. This inhibition was maximal at 10

Table 5
Evolution of β-glucosidase and phosphatase activities in soils amended with the biostimulants/biofertilizers and polluted with chlorpyrifos during the experimental period. Data are expressed as mean values ± standard error. Columns followed by the same letter(s) are not significantly different ($p > 0.05$). PNP: *p*-nitrophenol.

	β-glucosidase activity (mmol PNP g^{-1} h^{-1})						
	3	6	10	30	60	90	120
C	1.4b ± 0.2	1.4b ± 0.3	1.3b ± 0.2	1.3b ± 0.1	1.3b ± 0.2	1.2b ± 0.2	1.1b ± 0.2
I	0.7a ± 0.2	0.5a ± 0.2	0.4a ± 0.1	0.5a ± 0.1	0.6a ± 0.2	0.8a ± 0.2	1.0b ± 0.1
SS		4.4c ± 0.8	5.3d ± 0.9	3.6c ± 0.4	1.6b ± 0.3	1.3b ± 0.2	1.2b± 0.2
CF1		2.9c ± 0.6	4.4c ± 0.6	3.0c ± 0.4	1.5b ± 0.3	1.2b ± 0.2	1.2b ± 0.3
CF2		5.0d ± 0.8	6.5d ± 1.1	4.1c ± 0.6	1.6b ± 0.4	1.3b ± 0.3	1.1b ± 0.2
SS + I		3.0c ± 0.9	3.9c ± 0.7	3.1c ± 0.6	1.4b ± 0.3	1.3b ± 0.3	1.2b ± 0.3
CF1 + I		2.3bc ± 0.6	3.0c ± 0.8	2.8c ± 0.4	1.5b ± 0.3	1.4b ± 0.3	1.2b ± 0.2
CF2 + I		3.4c ± 0.8	5.0c ± 1.2	3.7c ± 0.8	1.4b ± 0.2	1.3b ± 0.3	1.2b ± 0.2
	Phosphatase activity (μmol PNP g^{-1} h^{-1})						
	3	6	10	30	60	90	120
C	3.5b ± 0.9	3.4b ± 1.1	3.4b ± 0.9	3.2b ± 0.9	3.3b ± 1.0	3.1b ± 0.9	3.1b ± 0.8
I	2.6a ± 0.8	2.2a ± 0.5	1.9a ± 0.7	1.9a ± 0.8	2.2a ± 0.7	2.6a ± 0.8	3.1b ± 0.9
SS		7.6c ± 0.7	25.4d ± 2.1	8.1c ± 1.1	3.6b ± 0.9	3.4b ± 0.7	3.2b ± 1.0
CF1		6.2c ± 0.9	22.6d ± 1.8	6.9c ± 0.9	3.5b ± 0.8	3.3b ± 1.0	3.1b ± 0.9
CF2		8.9c ± 1.0	31.6e ± 2.9	9.2c ± 1.3	3.6b ± 0.7	3.5b ± 1.0	3.1b ± 0.7
SS + I		5.6c ± 0.9	16.9d ± 1.4	4.6bc ± 1.0	3.2b ± 0.9	3.3b ± 0.9	3.2b ± 0.9
CF1 + I		4.9bc ± 0.9	14.6c ± 1.3	4.0b ± 0.7	3.3b ± 1.0	3.2b ± 0.8	3.2b ± 1.1
CF2 + I		6.8c ± 1.0	22.8d ± 1.5	5.4c ± 1.1	3.4b ± 0.9	3.3b ± 0.9	3.3b ± 1.1

days of applying the insecticide in soil (44.1% compared to the control treatment) and diminished over the experimental period. Similar to the enzymes studied, when applying BS in insecticide-polluted soil, the inhibition of phosphatase activity decreased. In this respect, at 10 days of applying the insecticide in the soil, and compared to that CF1 treatment, the phosphatase activity decreased 35.4% in the CF1 + I treatment. Compared to SS treatment, the phosphatase activity decreased 33.5% in the SS + I treatment, and compared to CF2 treatment, the phosphatase activity decreased 27.8% in the CF2 + I treatment. At 30 days from the beginning of the incubation period, this decrease in soil phosphatase activity was progressively reduced during the experimental period.

The arylsulphatase activity was also stimulated in organically amended soils, mainly at 10 days from when beginning the experiment (Table 6). In this respect, and compared to the control, the soil arylsulphatase activity significantly increased by 62.3%, 74.6% and 83.1% in the CF1, SS and CF2 treatments, respectively. After the first 10 days, the arylsulphatase activity began to gradually decline. At the end of the experimental period all treatments studied had very similar values.

The application of chlorpyrifos to the soils inhibited the arylsulphatase activity during the first 90 days of the experimental period, presenting a maximum inhibition at 10 days from when starting the experiment (70%). At the end of the incubation period, similar results were obtained for C and I treatments (Table 6). The application of the BS in the insecticide-polluted soil also showed a similar response to the other enzymatic activities studied. In this respect, the application of BS to the soil decreased the inhibition of the arylsulphatase activity. At 10 days of applying the insecticide to the soil, and compared to that CF1 treatment, the arylsulphatase activity decreased 29.3% in the CF1 + I treatment. Compared to SS treatment, the arylsulphatase activity decreased 22.8% in the SS + I treatment, and compared to CF2 treatment, the arylsulphatase activity decreased 17.8% in the CF2 + I treatment. Again, at 30 days of the incubation period, the decrease in soil arylsulphatase activity was progressively reduced during the experimental period, noting that in soils amended with the three BS, this activity showed similar values than the amended soil without insecticide.

3.2. Soil microbial community

The application of the BS increased the soil bacteria and fungi population (Table 7). Similar to the results of the enzymatic activities, this increase was higher in the CF2 treatment than in the SS and CF1 treatments, respectively. Again and in the organic treatments, 10 days after the beginning of the incubation, the highest population of bacteria and fungi were found. These populations were decreasing during the experimental period. At the end of the experiment, the bacteria population was similar to the one in the control treatment.

Table 7
Evolution of bacterial Gram$^+$, bacterial Gram$^-$, Total bacterial and fungal PLFAs (nmol g^{-1}) during the experimental period. Data are the means of three samples. Columns (mean ± S.E.) followed by the same letter(s) are not significantly different ($p > 0.05$).

	bacGram$^+$	bacGram$^-$	Total bacterial PLFA	Fungal PLFA
C (3d)	13.0b ± 1.2	1.3b ± 0.4	14.2b ± 1.4	1.0b ± 0.2
C (10d)	13.4b ± 1.4	1.2b ± 0.3	14.6b ± 1.4	0.9b ± 0.2
C (60d)	12.8b ± 1.4	1.2b ± 0.4	14.0b ± 1.3	1.0b ± 0.1
C (120d)	13.0b ± 1.3	1.3b ± 0.4	14.3b ± 1.3	1.0b ± 0.3
I (3d)	7.7a ± 1.3	0.8a ± 0.2	8.5a ± 0.9	0.7a ± 0.1
I (10d)	6.8a ± 0.9	0.6a ± 0.2	7.4a ± 1.0	0.6a ± 0.1
I (60d)	8.4ab ± 1.1	0.9a ± 0.3	9.3a ± 1.0	0.8ab ± 0.2
I (120d)	13.1b ± 1.3	1.4b ± 0.3	14.5b ± 1.1	1.0b ± 0.2
SS (10d)	35.4d ± 2.4	4.1d ± 0.9	39.5d ± 2.9	1.8c ± 0.3
SS (60d)	13.0b ± 1.8	1.3b ± 0.4	14.3b ± 1.9	1.0b ± 0.1
SS (120d)	12.9b ± 1.2	1.3b ± 0.3	14.2b ± 1.3	1.0b ± 0.3
CF1 (10d)	28.6d ± 1.9	3.9d ± 1.1	32.5c ± 2.5	1.6c ± 0.4
CF1 (60d)	13.4b ± 1.1	1.2b ± 0.3	14.6b ± 0.9	0.9b ± 0.1
CF1 (1200d)	13.0b ± 1.3	1.4b ± 0.5	14.4b ± 1.2	1.0b ± 0.2
CF2 (10d)	41.1e ± 2.5	5.0e ± 1.2	46.1e ± 3.3	2.0d ± 0.4
CF2 (60d)	12.9b ± 1.6	1.3b ± 0.4	14.2b ± 1.7	1.0b ± 0.2
CF2 (120d)	13.1b ± 1.1	1.2b ± 0.4	14.3b ± 1.3	0.9b ± 0.2
SS + I (10d)	24.5c ± 1.8	2.9d ± 0.5	27.4c ± 1.9	1.4c ± 0.3
SS + I (60d)	16.3b ± 1.0	1.9c ± 0.3	17.2b ± 1.1	1.2b ± 0.2
SS + I (120d)	13.1b ± 1.4	1.2b ± 0.2	14.3b ± 1.3	0.9b ± 0.1
CF1 + I (10d)	18.4c ± 1.5	2.4d ± 0.3	20.8c ± 1.6	1.2b ± 0.3
CF1 + I (60d)	14.4b ± 1.1	1.6b ± 0.4	16.0b ± 1.3	1.2b ± 0.2
CF1 + I (120d)	13.0b ± 1.3	1.2b ± 0.2	14.2b ± 1.4	1.0b ± 0.2
CF2 + I (10d)	29.4c ± 2.0	3.7d ± 0.6	33.1d ± 2.1	1.6c ± 0.4
CF2 + I (60d)	19.4c ± 1.1	2.0c ± 0.4	21.4c ± 1.4	1.3b ± 0.2
CF2 + I (120d)	12.8b ± 1.4	1.3b ± 0.1	14.1b ± 1.4	1.0b ± 0.2

When the chlorpyrifos was applied to the soil at 10 days into the experiment, the total bacterial and fungal population significantly ($p < 0.05$) decreased. Compared to the control soil, in the chlorpyrifos-polluted soils, the bacGram$^+$ population, decreased 49.2%, the bacGram$^-$ population decreased 50.0% and fungal population decreased 33.3% (Table 7). At the end of the incubation period, similar results were obtained for C and I treatments.

The application of BS in insecticide-polluted soils decreased the inhibition of the bacterial and fungal population. At 10 days of applying the insecticide in the soil, and compared to that CF1 treatment the inhibition of the total bacterial PLFA and fungal PLFA decreased 36.0% and 25.0%, respectively in the CF1 + I treatment. Compared to SS treatment, the inhibition of the total bacterial PLFA and fungal PLFA decreased 30.6% and 22.2%, respectively in the SS + I treatment, and compared to CF2 treatment, the inhibition of the total bacterial PLFA and fungal

Table 6
Evolution of arylsulphatase activity in soils amended with the biostimulants/biofertilizers and polluted with chlorpyrifos during the experimental period. Data are expressed as mean values ± standard error. Columns followed by the same letter(s) are not significantly different ($p > 0.05$). PNP: *p*-nitrophenol.

	Arylsulphatase activity (μmol PNP g^{-1} h^{-1})						
	3	6	10	30	60	90	120
C	2.0b ± 0.4	1.8b ± 0.4	2.0b ±n0.5	1.8b ± 0.3	1.7b ± 0.4	1.6b ± 0.4	1.6b ± 0.3
I	1.1a ± 0.2	0.8a ± 0.2	0.6a ± 0.1	0.7a ± 0.2	0.9a ± 0.2	1.1a ± 0.3	1.5b ± 0.5
SS		4.9c ± 1.1	7.9d ± 1.3	5.2c ± 1.0	2.2b ± 0.8	1.7b ± 0.3	1.7b ± 0.4
CF1		3.3bc ± 1.0	5.3c ± 1.2	4.4c ± 0.9	2.0b ± 0.9	1.6b ± 0.4	1.7b ± 0.5
CF2		6.2c ± 1.2	11.8e ± 1.5	7.0d ± 1.1	2.1b ± 0.8	1.5b ± 0.3	1.8b ± 0.4
SS + I		3.3bc ± 1.0	6.1c ± 1.2	4.1c ± 0.8	1.9b ± 0.5	1.5b ± 0.4	1.5b ± 0.4
CF1 + I		2.9b ± 1.1	4.1c ± 1.1	3.4bc ± 0.9	2.0b ± 0.7	1.6b ± 0.3	1.6b ± 0.5
CF2 + I		4.5c ± 1.0	9.7d ± 1.4	5.2c ± 1.1	1.9b ± 0.4	1.5b ± 0.3	1.6b ± 0.4

PLFA decreased 28.2% and 20.0%, respectively in the CF2 + I treatment. Again, at 30 days of the incubation period, the inhibition in soil total bacterial and fungal PLFA was progressively reduced during the experimental period, noting that in soils amended with the three BS, this activity showed similar values than the amended soil without insecticide.

3.3. Fate of chlorpyrifos in soil

Fig. 1 shows the evolution of chlorpyrifos in the experimental soil during the incubation period. The results show a progressive degradation of the insecticide in the soil throughout the experiment. This degradation was faster when different BSs were applied to soil. At 60 days of the incubation period, the degradation of chlorpyrifos in organic amended and polluted soils was practically complete, whereas the concentration of insecticide in non-organic amended and polluted soils was higher. However, this decrease depended on the BS type applied to the soil. The results indicate a lower concentration of insecticide in contaminated and amended soils with CF2 and SS, followed by CF1.

4. Discussion

Our results indicated that chlorpyrifos has a toxic effect on the soil enzymatic activity and microbial diversity. These results are in agreement with those obtained by Menon et al. (2004), who found that nitrogen mineralization in the loamy sand and sandy loam was significantly inhibited after chlorpyrifos application. Shan et al. (2006) also indicated that soil bacterial, fungal, and actinomycete populations were inhibited by chlorpyrifos at a concentration of 10 mg kg^{-1}. Tejada et al. (2011) found a decrease in soil enzymatic activities in soils polluted with chlorpyrifos at a rate of 5 L ha^{-1}. Kadian et al. (2012) found that the microbial activity (measured as dehydrogenase activity) was inhibited by chlorpyrifos at a concentration of 10 mg kg^{-1}. However, contrary results have been reported by Singh et al. (2003), where the overall metabolic diversity and evenness of soil microorganisms were minorly affected in soil after chlorpyrifos application at a concentration of 10 mg kg^{-1}. Also, Dutta et al. (2010) found that when chlorpyrifos was added to the soil at a field rate of 0.5 mg kg^{-1}, soil microbial biomass was increased and fluorescein diacetate hydrolysis was not negatively affected, whereas at a higher dosage (50 mg kg^{-1}) fluorescein diacetate hydrolysis decreased significantly.

In recent years, soil contaminated with chlorpyrifos has been treated with various organic waste such as municipal solid waste, cow manure, spent mushroom compost, vermicompost, composted sewage sludge and residues from olive oil production, in order to minimize the toxic effects of this insecticide (Tejada et al., 2011; Kadian et al., 2012; Rojas et al., 2013). The adsorption of pesticides by organic matter is one of the processes governing the solubility and assimilation of pesticides in the environment. This adsorption may cause a higher decrease of insecticide in the soil solution, and therefore, decreases the chlorpyrifos availability and toxicity (Tejada et al., 2011). Also, the application of diverse organic wastes in pesticides-polluted soils causes stimulation in the soil microbial activity, and therefore can accelerate the degradation of pesticide in soil (Gan et al., 1998; Perucci et al., 2000). It is necessary that the organic material must be mineralized to available forms. This degradation is usually carried out by microorganisms which are tolerant to the concentration of the pesticide present in soil. Generally, organic wastes applied to the soil for this purpose are characterized by high molecular weight proteins, which represent significant energy expenditure for these microorganisms to degrade these organics. Therefore, the degradation of these organic materials needs a certain amount of time. This causes the degradation of xenobiotics in soil is much more slow.

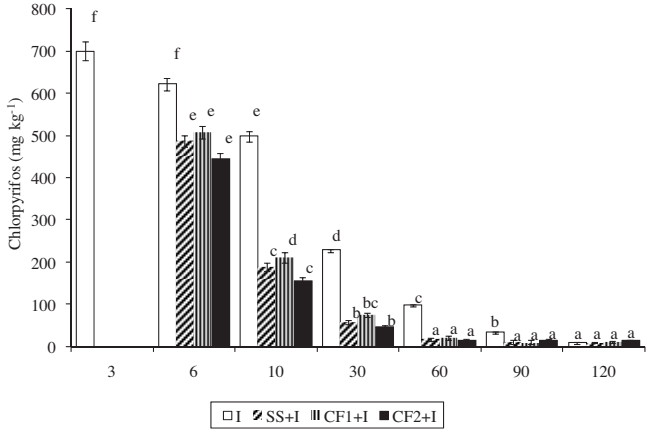

Fig. 1. Evolution of chlorpyrifos (mean ± S.E.) in soils during the experimental period. Data are the means of three samples. Columns (mean ± S.E.) followed by the same letter(s) are not significantly different ($p > 0.05$).

In our experiment, the organic compounds used are characterized by a high content of low molecular weight proteins (<300 Da). The application of these organic compounds stimulated rapidly in a very short-time period the soil microorganisms. These results are in agreement with those by Parrado et al. (2008) and García-Martínez et al. (2010a,b), who found that after applying different BSs obtained from carob germ, wheat condensed distillers and rice brain to the soil, the microbial activity increased over a very short-time period because these organic products contained a higher proportion of low molecular weight proteins. Those lower in protein molecular size indicate that the N is in a more readily available form for the soil microorganisms. This aspect promotes a rapid increased proliferation of soil microorganisms (Vasileva-Tonkova et al., 2007).

For this reason, the soil dehydrogenase, β-glucosidase, phosphatase and arylsulphatase activities were rapidly stimulated in a very short period of time. Similarly, the higher stimulation in the CF2 treatment, followed by SS and CF1 treatments, respectively, is due to the difference in the content of lower molecular weight proteins of the BS. By increasing the content of lower molecular weight proteins, the soil microbial stimulation is faster. This greater assimilation of low molecular weight proteins is possibly responsible for the fact that the soil urease activity exhibits no significant stimulation after the application of the BSs. Since soil microorganisms can obtain this N without any energy expenditure, microorganisms do not need to excrete any enzyme to obtain this easily available N. These results also agree with those obtained by García-Martínez et al. (2010a,b), who found that after the application of different protein hydrolysates to the soil, no stimulation was observed in the soil urease activity due to the fact that these chemical compounds were rich in low molecular weight proteins.

The studied BSs have high protein of low molecular weight that are easily assimilated by microorganisms in the soil. This is the reason why inhibition of the enzyme activities tested, except for the urease activity, decline sharply in the early days of incubation. Coinciding with the results obtained by Tejada et al. (2010) to apply different BS obtained from wheat condensed distillers soluble, carob germ enzymatic and rice bran extract in a soil contaminated by herbicide MCPA, BSs stimulate soil microorganisms and therefore favor and accelerate degradation of the xenobiotic compound in the soil. At 60 days of applying the BSs in the contaminated soil, the soil insecticide virtually disappeared. Tejada et al. (2011) using the same dose of chlorpyrifos in a soil amended

with a municipal solid waste and a cow manure observed that the content of insecticide lasted at least 90 days (maximum time study).

With respect to the soil microbial diversity, Marschner et al. (2003) found that the degradation of organic matter requires enzymes that are produced by a limited number of microbial species and may increase the competitive ability of microorganisms. It is very probable that this is the reason that microbial biodiversity of soils amended with the BSs would increase in the first days of the experimental period. Once these easily degradable organic complexes have been metabolized, the structure of the soil microbial population is reestablished again.

The application of chlorpyrifos, negatively affects soil microbial community composition. These results agree with those obtained when other insecticides are applied to the soil such as cypermethrin, monocrotophos, carbofuran, endosulfan and imidacloprid, which all had adverse effects on the total number of soil bacteria in the soil (Rangaswamy and Venkateswarlu, 1992; Ahmed and Ahmad, 2006). Possibly the microorganisms detected by PLFA analysis are those that are resistant or tolerant to the dose of insecticide applied to the soil. The application of the BSs in insecticide-polluted soil decreases inhibition of this microbial diversity. We think that by decreasing the toxic effects of chlorpyrifos in soil, soil microbial population will continue reestablishing.

5. Conclusions

The chlorpyrifos insecticide caused a negative effect on soil enzymatic activities and soil microorganisms diversity. The application of biostimulants/biofertilizers with a higher content of lower molecular weight peptides decreases the toxic action of chlorpyrifos on soil biological properties. By increasing the content of lower molecular weight proteins in biostimulants/biofertilizers, the soil microbial stimulation is faster. Therefore, the application of biostimulants/biofertilizers with higher amounts of proteins and a higher proportion of peptides under 0.3 kDa is more beneficial for remediation of soils polluted with chlorpyrifos.

However, the biostimulant/biofertilizer effect in chlorpyrifos polluted-soils should be studied further. The dosage of insecticide, dosage of biostimulant/biofertilizer, and soil type are parameters that must be taken into consideration to have a deeper understanding of the action of biostimulants/biofertilizers on the biology of the chlorpyrifos polluted soils.

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RESEARCH ARTICLE

Behaviour of oxyfluorfen in soils amended with edaphic biostimulants/biofertilizers obtained from sewage sludge and chicken feathers. Effects on soil biological properties

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Abstract We studied the behaviour of oxyfluorfen herbicide at a rate of 4 l ha^{-1} on biological properties of a Calcaric Regosol amended with two edaphic biostimulants/biofertilizers (SS, derived from sewage sludge; and CF, derived from chicken feathers). Oxyfluorfen was surface broadcast on 11 March 2013. Two days after application of oxyfluorfen to soil, both biostimulants/biofertilizers (BS) were also applied to the soil. An unamended soil without oxyfluorfen was used as control. For 2, 4, 7, 9, 20, 30, 60, 90 and 120 days of the application of herbicide to the soil and for each treatment, the soil dehydrogenase, urease, β-glucosidase and phosphatase activities were measured. For 2, 7, 30 and 120 days of the application of herbicide to the soil and for each treatment, soil microbial community was determined. The application of both BS to soil without the herbicide increased the enzymatic activities and soil biodiversity, mainly at 7 days of beginning the experiment. However, this stimulation was higher in the soil amended with SS than for CF. The application of herbicide in organic-amended soils decreased the inhibition of soil enzymatic activities and soil biodiversity. Possibly, the low-molecular-weight protein content easily assimilated by soil microorganisms is responsible for less inhibition of these soil biological properties.

Keywords Oxyfluorfen · Edaphic biostimulant/biofertilizer · Sewage sludge · Chicken feathers · Soil enzymatic activities · Soil microbial community

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Introduction

Land application of organic waste, such as animal manure, municipal solid waste, sewage sludge and vermicomposts, is a common environmental measure in bioremediation of soils contaminated by xenobiotics. This effect is based on the adsorption of xenobiotic in organic matter by reducing their toxic effects, and the microbial stimulation that exerted the organic matter aspect makes xenobiotic accelerate degradation (Moreno et al. 2003; Dolaptsoglou et al. 2007; Delgado-Moreno and Peña 2009; Tejada et al. 2011).

However, that these organic products to activate soil microorganisms, the organic compounds need to be degraded into simpler, easily assimilated forms requiting a great expenditure of energy by soil microorganisms. Furthermore, this degradation depends on soil factors as the chemical composition of the organic material applied to soil (Tejada et al. 2010a; Tejada and Benitez 2011).

In the recent years, there has been increasing use of hydrolysate organic biostimulants/biofertilizers (BS) obtained from different organic materials by hydrolysis reactions (Romero et al. 2007; Parrado et al. 2008; García-Martínez et al. 2010a, 2010b). These BS, generally comprising peptides, amino acids, polysaccharides, humic acids and phytohormones, are directly absorbed by soil microorganisms and plants which spend a smaller amount of energy in the absorption process. Therefore, the application of these BS to the soil not only leads to an increased content of organic matter and macro- and micro-nutrients but also a significant activation of the soil's microbial community. For this reason, the development of new BS has become the focus of interest in research.

The current modern society is evolving towards an ideal of sustained agricultural production on three basic pillars: obtaining food quality, food safety and environmental friendliness. Therefore, it is increasingly restricted the use of herbicides, because these chemicals often cause significant levels of contamination in both soil and groundwater (Guzzella et al. 2006; Vervliet-Scheebaum et al. 2010; Lanctôt et al. 2013).

Olive farming is one of the most important in the Mediterranean, particularly in Spain, and is located predominantly in Andalusia. In recent years, there have been repeated events of herbicide contamination in soils cultivated with olive and in aquifers near these crops, which has caused some public alarm. This has led to certain herbicides, such as atrazine, simazine, diuron and terbuthylazine, before they were widely used in olive cultivation and be replaced by other less soluble herbicides. Therefore, it is increasingly common to use herbicides such as oxyfluorfen for weed control in the cultivation of olive trees.

Oxyfluorfen [2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl) benzene] is a diphenyl ether herbicide with residual activity and contact, is selective and is more readily absorbed by the leaves, especially by the roots of the buds, with little translocation.

However, despite that in recent years, oxyfluorfen is widely used, and there is no information about the toxic effects of this herbicide on the soil activity and microbial population.

No studies have been reported using different BS obtained from sewage sludge and chicken feathers to remediate oxyfluorfen-contaminated soil. For this reason, the objective of this study was to investigate the influence of different BS in the behaviour of oxyfluorfen and its influence on soil biological properties.

Material and methods

Soil, organic wastes and herbicide characteristics

The study was conducted from March to June 2013 near Seville City (Andalusia, Spain). The soil used in this experiment is a Calcaric Regosol (FAO 1989). Soil samples were collected from the 0–25-cm surface layer. The main soil characteristics are shown in Table 1. Soil pH was determined in distilled water with a glass electrode (soil/H_2O ratio 1:2.5). Soil texture was determined by Robinson's pipette method (SSEW 1982). Total N was determined by the Kjeldahl method (MAPA 1986). Soil organic matter was determined by the method of Yeomans and Bremner (1988).

The herbicide used in this experiment was the oxyfluorfen. The commercial formulation Fenfen (24 % p v^{-1}, 240 g l^{-1}) was purchased from Lainco, S.A. (Spain). The rate applied to the soil was 4 l ha^{-1} (recommended application rate).

Table 1 Characteristics of the experimental soil (mean ± standard error)

pH (H_2O)	7.9±0.2
Coarse sand (g kg^{-1})	486±49
Fine sand (g kg^{-1})	130±25
Silt (g kg^{-1})	123±29
Clay (g kg^{-1})	260±35
Total N (g kg^{-1})	0.93±0.08
Organic matter (g kg^{-1})	17±1

Data are the means of three samples

Two edaphic BS were used: (1) BS derived from sewage sludge (SS) by enzymatic hydrolysis process and (2) BS derived from chicken feathers (CF) also obtained by enzymatic hydrolysis. Figure 1 shows schematically how both protein hydrolysates were obtained.

Both types of BS were chemically analysed (Table 2). Organic matter content was determined by combustion at 550 °C for 6 h. Phosphorus and sulphur were determined after combustion and analysed by inductively coupled plasma atomic emission spectrometry (ICP-AES) using a Fisons-ARL 3410 sequential multielement instrument equipped with a data acquisition and control system. The standard operational conditions of this instrument are summarized as follows: The carrier gas, coolant gas and plasma gas are argon at 80 psi of pressure, the carrier gas flow rate is 0.8 l min^{-1}, the coolant gas flow rate is 7.5 l min^{-1}, the plasma gas flow rate is 0.8 l min^{-1}, and the integration time is 1 s. One mini-torch consumes argon gas at a radio frequency power of 650 W. Crude fat was determined gravimetrically after extraction with hexane for 12 h in a Soxhlet extractor (Clemente et al. 1997). Total nitrogen was determined by the Kjeldahl method (AOAC 1990).

The molecular mass distribution of protein in the samples was determined by size-exclusion chromatography using an ÄKTApurifier (GE Healthcare), using a Superdex Peptide™ 10/300 GL column (optimum separation range 0.1–7 kDa) (Table 3). Samples were centrifuged at 12,000×*g* for 30 min at 4 °C to remove insoluble molecules, and the supernatant was passed through a 0.2-μm filter and loaded into a 0.1-ml loop connected to the ÄKTApurifier system. The column was equilibrated and eluted with 0.25 M Tris–HCl buffer (pH 7.0) in isocratic mode, at a flow rate of 0.5 ml min^{-1}, and proteins/peptides were detected at 280 and 215 nm with a GE Healthcare UV900 module coupled to the column elution.

Experimental layout

The experimental layout was a randomized, complete block design with six treatments and three replicates per treatment. The plot size was 4 m×3 m. Oxyfluorfen was surface broadcast on 11 March 2013. Two days after application of oxyfluorfen to soil, both BS were also applied to the soil. Soil

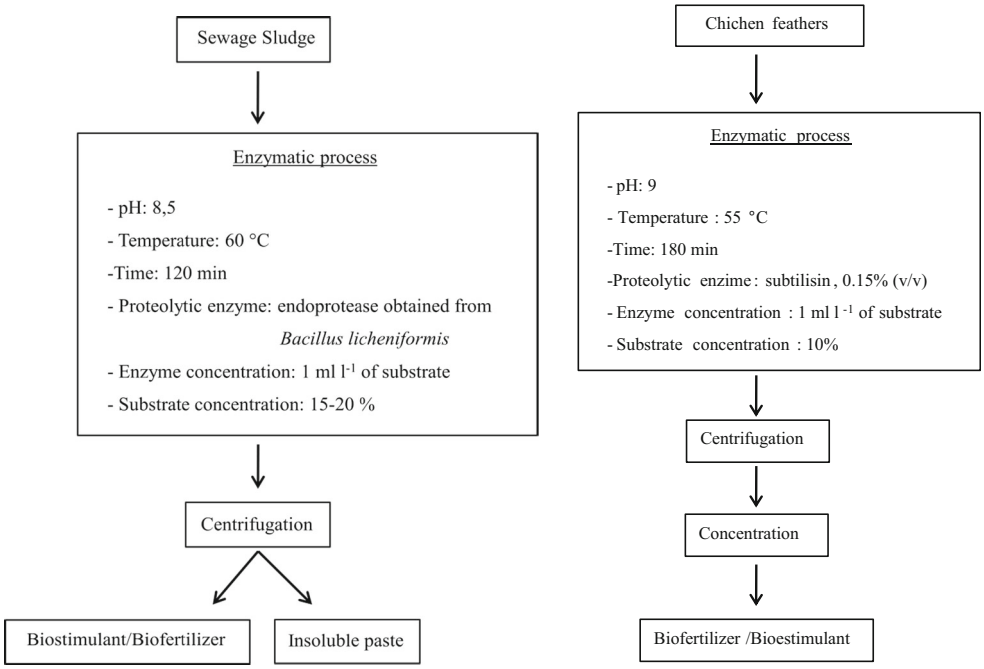

Fig. 1 Enzymatic hydrolysis process used for obtaining edaphic biostimulants/ biofertilizers from sewage sludge and chicken feathers

samples were mixed with 6.6 l of SS (5 t organic matter (OM) ha^{-1}) or 10.8 l of CF (5 t OM ha^{-1}), applying to the soil the same amount of organic matter with each BS. Both BS were liquid and were solubilized in water (500 l ha^{-1}) before the application. An unamended soil was used as control. The treatments are detailed as follows:

1. C, control soil, soil non-organic amended and without oxyfluorfen
2. H, soil with oxyfluorfen and non-organic amended
3. SS, soil without oxyfluorfen and amended with SS
4. CF, soil without oxyfluorfen and amended with CF
5. SS+H, soil with oxyfluorfen and amended with SS
6. CF+H, soil with oxyfluorfen and amended with CF

Table 2 Chemical composition of both edaphic biofertilizers/ biostimulants

	Biostimulants/biofertilizers	
	Sewage sludge	Chicken feathers
Density (g ml^{-1})	1.18a±0.04	1.20a±0.06
Organic matter (g kg^{-1})	773b±21	463a±48
N-Kjeldahl (g kg^{-1})	34.9b±2.3	14.1a±1.6
Total carbohydrates (g kg^{-1})	42a±19	65b±11
P (g kg^{-1})	2.9a±0.1	27b±8
S (g kg^{-1})	5.9a±1.6	19b±4
Fat (g kg^{-1})	18a±3	20a±2

Data are the means of three samples. Rows (mean ± S.E.) followed by the same letter(s) are not significantly different ($p>0.05$)

Soil analysis

For 2, 4, 7, 9, 20, 30, 60, 90 and 120 days of the application of herbicide to the soil and for each treatment, the activity levels of four soil enzymes were measured. Dehydrogenase activity was measured as the reduction of 2-*p*-iodo-3-nitrophenyl-5-phenyl tetrazolium chloride to iodonitrophenyl formazan (García et al. 1993). Urease activity was determined by the buffered method of Kandeler and Gerber (1988), using urea as substrate. The β-glucosidase activity was determined using *p*-nitrophenyl-β-D-glucopyranoside as substrate (Masciandaro et al. 1994). Phosphatase activity was measured using *p*-nitrophenyl phosphate as substrate (Tabatabai and Bremner 1969).

For 2, 7, 30 and 120 days of the application of herbicide to the soil and for each treatment, phospholipids were extracted (three replicates per treatment) using a chloroform–methanol

Table 3 Molecular weight distribution of both edaphic biostimulants/ biofertilizers

Molecular weight (Da)	Biostimulant/biofertilizer	
	Sewage sludge	Chicken feathers
>10000	21.4a±2.6	23.8a±2.4
10,000–5,000	7.3a±1.5	9.1a±1.1
5,000–1,000	5.5a±2.0	25.1b±2.6
1,000–300	1.8a±0.4	8.0b±1.5
<300	64.0b±3.6	34.0a±3.1

Data are the means of three samples. Columns (mean ± S.E.) followed by the same letter(s) are not significantly different ($p>0.05$)

extraction based on Bligh and Dyer (1959) and fractionated and quantified using the procedure described by Frostegard et al. (1993a) and Bardgett et al. (1996). Twenty-six separated fatty acid methyl esters were identified using gas chromatography and a flame ionization detector. The phospholipids were transformed by alkaline methanolysis into fatty acid methyl esters (FAMEs), which were quantified with a gas chromatograph (GC/FID, AutoSystem XL Gas Chromatograph, Varian Saturno 2000) fitted with a 50-m capillary column, using helium as the carrier gas. The injector temperature was 260 °C, the flame ionization detector temperature was 280 °C and the initial temperature was 70 °C (for 2 min); it was increased to 160 °C at 30 °C min^{-1} and then to 280 °C at 3 °C min^{-1}.

To estimate the various proportions of the main taxa in the samples according to the phospholipid fatty acids (PLFAs), the biomarkers i15:0, a15:0, i16:0, 16:1ω7c, 17:0, i17:0, cy17:0, 18:1ω9c and cy19:0 were used to represent bacterial biomass (bacPLFA) (Frostegard et al. 1993b; Bardgett et al. 1996) and 18:2ω6 (fungPLFA) was taken to indicate fungal biomass (Federle et al. 1986). The ratio of bacPLFA to fungPLFA (bacPLFA/fungPLFA) represents the ratio between bacterial and fungal biomass (Bardgett et al. 1996). The $gram^+$-specific fatty acids i15:0, a15:0, i16:0 and i17:0 and the $gram^-$-specific fatty acids cy17:0, 18:1ω9c and cy19:0 were taken as a measure of the ratio of the $gram^+$ and $gram^-$ bacterial biomass ($gram^+/gram^-$). All results are given in nanomole per gram.

For each treatment and each experimental day, 20 g of soil was taken. Soil samples were stored in sealed polyethylene bags at 4 °C for 15 days, prior to analysis of the enzymatic activities (Tejada et al. 2011), and at −20 °C prior to phospholipid analysis (Wu et al. 2011).

Oxyfluorfen determination in soil

The extraction of oxyfluorfen from soil was realized using the Anastassiades et al. (2003) method. Oxyfluorfen was extracted with a mixture of triphenyl phosphate and acetonitrile (1:25). Once the supernatant had been shaken and centrifuged, magnesium sulphate was added to it and it was stirred and centrifuged again. The supernatant was concentrated, and the dried residue was recomposed with 1 ml of cyclohexane/ethyl acetate (9:1).

Oxyfluorfen was determined using a tandem mass spectrometer and electron impact, where the chromatographic conditions were as follows: carrier gas, He at 1 ml min^{-1}; initial injector temperature, 70 °C for 0.50 min and 310 to 100 °C min^{-1} for 10 min; column, 30 m×0.25 mm ID; initial temperature of the column oven, 70 °C for 3.5 min and 180 to 35 °C min^{-1} and 300 °C at 10 °C min^{-1} for 5 min; temperature detector, Trap to 250 °C, Manifold to 60 °C and Xfer Line to 280 °C; injection volume, 5 μl.

The MS/MS parameters were the following: excitation storage level (m z^{-1}), 100; CID, 3.5 V; parent ion (m z^{-1}), 300; quantification ion (m z^{-1}), 223. The precursor ion was excited using a resonant waveform.

Statistical analysis

Data were submitted to two-way ANOVA with treatment and sampling time as factors followed by Tukey's significant difference as a post hoc test, considering a significance level of $p<0.05$ throughout the study. The ANOVA was performed using the Statgraphics Plus 2.1 software package. For the ANOVA, triplicate data were used for each treatment and every experimental day.

Results

Evolution of soil biological properties

Statistical analysis indicated a significant ($p<0.05$) stimulation of the dehydrogenase activity during the first days after the application of both BS, mainly at 7 days after the beginning of the experiment (Table 4). In this respect, and compared to the control, the soil dehydrogenase activity significantly increased by 92.1 and 88.2 % in the SS and CF treatments, respectively. After the first 7 days, the dehydrogenase activity began to decline gradually. At the end of the experimental period, all treatments studied had very similar values.

The application of oxyfluorfen in soil showed a significant decrease in dehydrogenase activity (Table 4). At the end of the experimental period and compared with the control treatment, dehydrogenase activity significantly ($p<0.05$) decreased by 38.1 %.

The application of both BS in soils with oxyfluorfen caused a minor decrease in dehydrogenase activity (Table 4). In this respect, at 7 days after applying the herbicide in the soil, and compared to that SS treatment, it decreased the dehydrogenase activity in the treatment by 66.6 % SS+H. Compared to the CF treatment, the dehydrogenase activity decreased by 57.4 % in the CF+H treatment. At 30 days after the beginning of the experiment, this decrease in soil dehydrogenase activity was progressively reduced during the experimental period, noting that in soils amended with both BS, this activity showed similar values than the amended soil without oxyfluorfen.

Unlike the dehydrogenase activity, the soil urease activity was not stimulated after the application of the two BS studied (Table 4). At the end of the experimental period, the soil urease activity decreased slightly. However, the statistical analysis shows no significant differences ($p>0.05$) between

Table 4 Evolution of dehydrogenase and urease activities in soils amended with the edaphic biostimulants/biofertilizers and with oxyfluorfen during the experimental period

Dehydrogenase activity (μg INTF g^{-1} h^{-1})									
	2	4	7	9	20	30	60	90	120
C	2.3±0.5	2.4±0.4	2.4±0.5	2.3±0.5	2.2±0.6	2.2b±0.4	2.3b±0.5	2.1b±0.4	2.1b±0.5
H	1.3a±0.2	1.4a±0.3	1.3a±0.3	1.3a±0.4	1.4a±0.2	1.3a±0.4	1.2a±0.2	1.3a±0.2	1.3a±0.3
SS		16.0±1.2	30.5e±2.4	20.2d±1.8	10.6±1.9	4.2b±1.1	3.5b±1.3	2.7b±0.8	2.2b±0.2
CF		14.7c±1.4	20.4d±1.6	17.3d±1.2	7.1±1.1	3.1b±0.6	3.3b±0.8	2.6b±0.5	2.3b±0.3
SS+H		11.9c±1.1	10.2c±1.2	11.4c±1.1	6.9±1.0	4.2b±0.9	3.6b±0.4	2.8b±0.3	2.2b±0.4
CF+H		7.8c±1.1	8.7c±1.5	5.1c±0.9	4.4b±0.8	3.4b±0.6	3.2b±0.5	2.6b±0.5	2.1b±0.4
Urease activity (μg NH_4^+ g^{-1} h^{-1})									
C	2.1b±0.5	1.9b±0.3	1.9b±0.4	1.8b±0.3	1.8b±0.2	1.8b±0.4	1.9b±0.3	1.9b±0.3	1.9b±0.4
H	1.2a±0.3	1.1a±0.4	1.1a±0.2	0.9a±0.3	0.9a±0.1	1.0a±0.2	1.1a±0.4	1.1a±0.2	1.0a±0.3
SS		1.8b±0.3	1.9b±0.3	1.8b±0.2	1.7b±0.2	1.7b±0.3	1.8b±0.4	1.8b±0.3	1.7b±0.2
CF		1.8b±0.5	2.1b±0.4	2.2b±0.4	1.8b±0.4	1.8b±0.3	1.9b±0.2	1.8b±0.4	1.8b±0.4
SS+H		1.0a±0.2	1.1a±0.3	1.0a±0.2	0.9a±0.1	0.9a±0.1	1.0a±0.1	0.9a±0.2	1.0a±0.2
CF+H		1.1a±0.3	1.0a±0.2	1.1a±0.3	0.9a±0.1	0.9a±0.2	0.9a±0.1	1.0a±0.2	0.9a±0.1

Data are expressed as mean values ± standard error. Columns followed by the same letter(s) are not significantly different ($p>0.05$)

INTF 2-*p*-iodo-3-nitrophenyl formazan, *SS* sewage sludge, *CF* chicken feathers

these values throughout the experimental period. Similar to the dehydrogenase activity, when oxyfluorfen was applied to the unamended soil, there was a significant ($p<0.05$) decrease of this enzyme activity throughout the experimental period. These same results were also observed when the two BS studied were applied to the soil with the herbicide.

Similar to the dehydrogenase activity, the β-glucosidase activity was also stimulated in organically amended soils, mainly at 7 days after the beginning of the experiment (Table 5). Again, this stimulation ($p<0.05$) was significantly higher in the SS treatment than in the CF treatment (24.4 %).

The response of the β-glucosidase activity to the application of oxyfluorfen in soil was very similar to other enzyme activities studied (Table 5). Thus, it was observed an inhibition of this enzyme in the soils with oxyfluorfen. The application of both BS in the soil with herbicide also showed a similar response to the other enzymatic activities studied. In this respect, the application of BS to the soil decreased the inhibition of this enzymatic activity. At 7 days of beginning the experiment, this decrease was higher in the SS+H treatment than the CF+H treatment (9.7 %). At 30 days after the beginning of the experimental period, the decrease in soil β-glucosidase activity was progressively reduced, noting that in soils amended with both BS, this activity showed similar values than the amended soil without oxyfluorfen.

The soil phosphatase activity was also significantly ($p<0.05$) stimulated after the application of both BS to the soil (Table 5). Similar to the dehydrogenase and β-glucosidase activities, the soil phosphatase activity showed a higher stimulation at 7 days after the beginning of the experiment. In this respect, and compared to the C treatment, soil phosphatase activity significantly increased by 89.7 and 82.7 % in the SS and CF treatments, respectively. This stimulation decreased as the experimental period progressed. At the end of the experimental period, all experimental treatments had similar values. Also, oxyfluorfen applying to the soil inhibited this enzymatic activity during the experimental period. Similar to the enzymes studied, when BS was applied in the soil with herbicide, the inhibition of phosphatase activity decreased. This decrease was higher in the SS+H treatment than in the CF+H treatment.

The application of both BS increased the soil bacteria and fungi population (Table 6). Similar to the results of the enzymatic activities, this increase was higher in the SS treatment than in the CF treatment. Again and in the organic treatments, at 7 days of beginning the experiment, the highest population of bacteria and fungi was found. These populations were decreasing during the experimental period. At the end of the experiment, the bacteria population was similar to the one in the control treatment. The bacgram$^+$/gram$^-$and bacPLFA/fungPLFA rates increased in all organic treatments, also indicating the variability in the biodiversity of these soils during the first days of the experiment (Table 4). At the end of the experimental period, these rates showed similar values to those obtained in the control treatment.

When the herbicide was applied to the soil, the total bacterial population significantly ($p<0.05$) decreased while the fungal population decreased slightly. However, and with

Table 5 Evolution of β-glucosidase and phosphatase activities in soils amended with the edaphic biostimulants/biofertilizers and with oxyfluorfen during the experimental period

β-glucosidase activity (mmol PNP g^{-1} h^{-1})									
	2	4	7	9	20	30	60	90	120
C	1.4b±0.3	1.4b±0.2	1.4b±0.3	1.3b±0.1	1.2b±0.3	1.2b±0.2	1.1b±0.2	1.1b±0.1	1.0b±0.2
H	0.5a±0.1	0.5a±0.1	0.5a±0.1	0.6a±0.2	0.7a±0.1	0.6a±0.1	0.7a±0.2	0.6a±0.1	0.6a±0.1
SS		3.5c±0.4	5.3d±0.7	4.5c±0.9	3.3c±0.6	2.7b±0.4	1.9b±0.4	1.2b±0.2	1.1b±0.3
CF		2.9c±0.4	4.1c±0.9	3.3c±0.4	2.6c±0.7	2.0b±0.4	1.3b±0.3	1.0b±0.2	1.0b±0.2
SS+H		2.0b±0.3	3.1c±0.6	2.5b±0.5	2.0b±0.4	1.5b±0.2	1.2b±0.2	1.1b±0.2	1.0b±0.2
CF+H		1.6b±0.2	2.8c±0.4	1.8b±0.3	1.4b±0.3	1.3b±0.2	1.2b±0.2	1.0b±0.1	1.0b±0.1
Phosphatase activity (μmol PNP g^{-1} h^{-1})									
C	3.6b±1.3	3.6b±1.1	3.5b±1.4	3.5b±1.2	3.5b±1.4	3.4b±1.7	3.4b±1.3	3.3b±1.2	3.2b±1.4
H	2.3a±0.5	2.0a±0.9	1.8a±0.6	2.0a±0.5	1.8a±0.3	1.7a±0.2	1.8a±0.4	1.7a±0.3	1.8a±0.5
SS		19.1d±2.3	34.0e±2.9	26.2d±1.5	19.1d±1.9	8.0c±1.4	4.6b±1.2	4.2b±1.3	3.0b±1.1
CF		18.7d±2.8	28.1d±3.3	20.4d±2.1	13.5c±1.1	6.9b±1.3	4.5b±1.5	4.4b±1.4	3.2b±0.9
SS+H		13.2c±1.7	20.2d±3.1	12.1c±1.9	8.4c±1.5	6.3b±1.3	4.2b±1.1	3.8b±1.1	3.1b±1.2
CF+H		10.3c±1.3	14.8c±2.2	9.6c±1.4	6.1b±1.2	4.8b±1.6	3.7b±0.9	3.6b±0.8	3.2b±1.0

Data are expressed as mean values ± standard error. Columns followed by the same letter(s) are not significantly different ($p>0.05$)

PNP p-nitrophenol, *SS* sewage sludge, *CF* chicken feathers

respect to the control treatment, no significant ($p>0.05$) differences were found in bacgram$^+$/gram$^-$ and bacPLFA/fungPLFA rates (Table 4). The application of BS to soils with oxyfluorfen caused a minor decrease in the bacteria and fungi populations. This decrease was higher when the BS applied to the soil was SS.

Table 6 Evolution of bacterial gram$^+$, bacterial gram$^-$, total bacterial and fungal PLFAs (nmol g^{-1}), gram$^+$/gram$^-$ and bacteria/fungi during the experimental period

	bacgram$^+$	bacgram$^-$	Total bacterial PLFA	Fungal PLFA	bacgram$^+$/bacgram$^-$	bacPLFA/fungPLFA
C (2 days)	13.1b±2.1	1.4b±0.2	14.7±1.8	0.9a±0.1	8.2a±1.1	16.3a±1.4
C (7 days)	12.8b±1.3	1.4b±0.4	14.2±1.4	0.9a±0.1	9.1a±1.0	15.8a±1.5
C (30 days)	13.0b±1.4	1.3b±0.2	14.5±1.1	0.8a±0.1	10.0a±1.5	18.1a±1.2
C (120 days)	14.0b±1.4	1.5b±0.3	15.5±1.5	0.9a±0.1	9.3a±1.2	17.2a±1.3
H (7 days)	8.8a±0.6	0.9a±0.2	9.7±1.1	0.7a±0.1	9.8a±1.3	13.9a±1.4
H (30 days)	9.3a±0.9	0.9a±0.2	10.2±1.1	0.6a±0.2	10.3a±1.2	17.0a±1.3
H (120 days)	8.9a±0.7	1.0a±0.3	9.9±1.2	0.6a±0.1	8.9a±1.2	16.5a±1.3
SS (7 days)	43.4d±2.6	4.6c±1.1	88.0±3.4	2.1c±0.3	9.4a±1.5	41.9c±3.9
SS (30 days)	16.3b±1.5	1.6b±0.4	17.9±1.5	1.2b±0.2	10.2a±1.2	14.9a±1.7
SS (120 days)	14.4b±1.5	1.4b±0.2	15.8±1.4	1.0a±0.2	10.3a±1.5	15.8a±1.3
CF (7 days)	34.0c±2.1	3.2b±0.9	37.2±2.6	1.6b±0.2	10.6a±1.3	23.2b±2.6
CF (30 days)	15.1b±1.2	1.5b±0.3	16.6±1.3	1.0a±0.2	10.1a±1.3	16.8a±1.4
CF (120 days)	14.6b±1.3	1.5b±0.2	15.1±1.2	0.9a±0.1	9.7a±1.2	16.8a±1.5
SS+H (7 days)	30.3c±2.8	3.7bc±1.5	34.0±3.1	1.5b±0.4	8.2a±1.3	22.7b±2.3
SS+H (30 days)	15.2b±1.1	1.5b±0.2	16.1±1.2	1.1a±0.2	10.1a±1.3	14.6a±1.6
SS+H (120 days)	13.8b±1.3	1.5b±0.3	14.3±1.3	1.0a±0.2	9.2a±1.2	14.3a±1.5
CF+H (7 days)	21.1bc±1.6	2.2b±1.2	43.3±3.4	1.3b±0.3	9.6a±1.3	33.3b±3.4
CF+H (30 days)	14.0b±1.2	1.4b±0.2	15.4±1.1	1.0a±0.2	10.0a±1.2	15.4a±1.3
CF+H (120 days)	13.2b±1.1	1.3b±0.2	14.5±1.2	1.0a±0.1	10.1a±1.3	14.5a±1.1

Data are the means of three samples. Columns (mean ± S.E.) followed by the same letter(s) are not significantly different ($p>0.05$)

PLFA phospholipid fatty acid, *SS* sewage sludge, *CF* chicken feathers

Evolution of oxyfluorfen in soil

Figure 2 shows the evolution of oxyfluorfen in the experimental soil. The application of both BS to soil decreased the soil oxyfluorfen concentration. However, this decrease depended on the BS type applied to the soil. Comparing both the SS+H and CF+H treatments, the values suggest a more rapid degradation of the herbicide when the SS was applied to the soil with oxyfluorfen.

Discussion

Our results indicated that the oxyfluorfen herbicide caused a toxic effect on soil enzymatic activity and soil diversity. These results are in agreement with those obtained by García-Orenes et al. (2010) who observed an important decrease in the biochemical properties of an agricultural soil after the application of 1.5 kg ha^{-1} oxyfluorfen to the soil. Sheeba Singh et al. (2011) observed the toxic effect of this herbicide on soil microorganisms, especially in different soil cyanobacterias. Also, Nadiger et al. (2013) highlight the toxic effect on microorganisms when applied oxyfluorfen in agricultural soils cropped with maize. According to Renella et al. (2005), the decrease in the biological properties of soils contaminated by xenobiotics may be a consequence of a decrease in the release of extracellular enzymes from soil microorganisms or an inhibition of these extracellular enzymes.

However, there are also authors who found positive effects when they applied oxyfluorfen to the soil. Das et al. (2003) and Das and Debnath (2006) indicate that the application of this herbicide in soil caused an increase in both the population and microbial activity, because this chemical acted as a source of carbon and nutrients for the microorganisms of the soil. Also, El Hussein et al. (2012) found that the application of oxyfluorfen to soil at low concentrations causes a positive effect on microbial growth. However, these authors suggested that the effect of oxyfluorfen on soil microorganisms depends on both the pesticide concentration and the soil physicochemical characteristics (texture, organic matter, etc.).

The results obtained show a high persistence of oxyfluorfen in soil. These results are in agreement with those obtained by other authors. Baruah and Mishra (1986) indicate that oxyfluorfen has a long persistence in soil (half-life of 72 to 160 days) in natural conditions. Ying and Williams (2000) found a persistence time of 119 days of this herbicide in soil. Also, Mantzos et al. (2014) found that oxyfluorfen soil dissipation is better described by first-order kinetics with half-life ranging between 45 and 52.9 days.

Our results suggest that the application of both BS caused an increase in the soil microbial activity. These results are in agreement with those obtained by García-Martínez et al. (2010a, b), Parrado et al. (2008) and Tejada et al. (2010b, 2013) who indicate that the incorporation of different BS obtained from wheat condensed distiller soluble, carob germ enzymatic, rice bran extract and SS causes a soil microbial activity stimulation. Since soil microorganisms degrade the soil organic matter through the production of a wide variety of extracellular enzymes, the application of both BS explained the increase in these enzymatic activities.

Dehydrogenase activity showed a significant stimulation in soils amended with both BS. Dehydrogenase activity is an intracellular enzyme, and therefore, it is present in all intact, viable microbial cells (García et al. 1993). Dehydrogenase activity suggests that its stimulation was higher in the soil amended with BS SS than in the one amended with BS CF. Possibly, this fact is a consequence of the different chemical composition of the protein hydrolysates obtained. We believe that the main reason for the increased stimulation of the dehydrogenase activity is the size of the proteins present in both hydrolysates. The BS SS has a higher content of low-molecular-weight proteins (<300 Da) than the BS CF. These results agree with those obtained by Tejada et al. (2013).

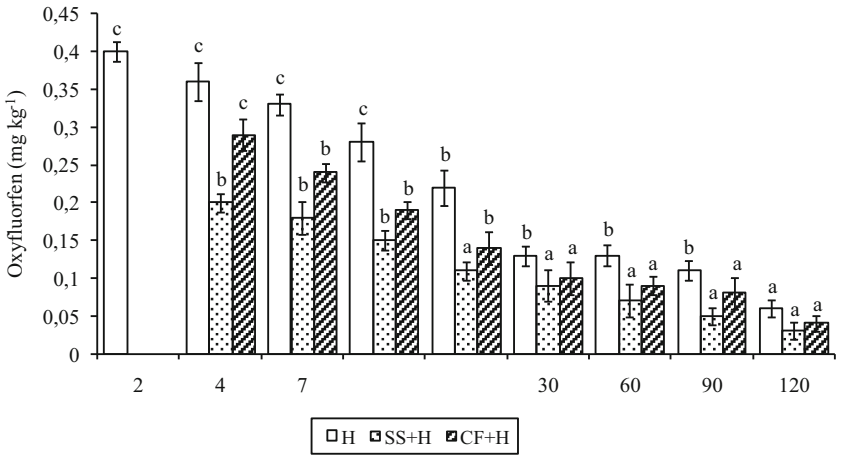

Fig. 2 Evolution of oxyfluorfen (mean ± S.E.) in soils during the experimental period. Data are the means of three samples. Columns (mean ± S.E.) followed by the same *letter*(s) are not significantly different ($p>0.05$)

These authors found that after applying the BS obtained from SS to the soil, the dehydrogenase activity showed a higher increase when the BS contained a higher proportion of low-molecular-weight proteins. The decrease in protein molecular size indicates that N is in a more readily available form for soil microorganisms. This aspect promotes an increased proliferation of soil microorganisms (Vasileva-Tonkova et al. 2007).

This greater assimilation of low-molecular-weight proteins is possibly responsible for the fact that the soil urease activity exhibits no significant stimulation after the application of both protein hydrolysates. Since soil microorganisms can obtain this N without any energy expenditure, microorganisms do not need to excrete any enzyme to obtain this easily available N. These results also agree with those obtained by García-Martínez et al. (2010a, b) and Tejada et al. (2013), who found that after the application to the soil of different protein hydrolysates, no stimulation was observed in the soil urease activity due to the fact that these chemical compounds were rich in low-molecular-weight proteins.

The soil β-glucosidase activity showed similar values to those obtained for the dehydrogenase activity, highlighting the positive influence of BS applied to the soil in the stimulation of this enzymatic activity, at least during the first days of the experimental period. These results agree with those obtained by García-Martínez et al. (2010a, b), Parrado et al. (2008) and Tejada et al. (2010b, 2013), who observed a significant stimulation of this enzymatic activity after the application of different organic compounds to the soil.

Soil phosphatase activity was also stimulated after the application of both BS. The results indicate that this stimulation was dependent on the amount and chemical composition of the BS applied to the soil. The highest values of this enzymatic activity were found in the soil amended with the SS. These results suggest that most of the P applied by the feather usually has an organic origin, because this stimulation occurs when soil microorganisms may not find an easily available soil inorganic P and therefore need to excrete this phosphatase.

With respect to the soil microbial diversity, Marschner et al. (2003) found that the degradation of organic matter requires enzymes that are produced by a limited number of microbial species and may increase the competitive ability of microorganisms. It is very probably that this is the reason that microbial biodiversity of soils amended with both BS would increase in the first days of the experimental period. Once these easily degradable organic complexes have been metabolized, the structure of the soil microbial population is reestablished again. Also, these authors note that the availability of substrate is a reason to find differences in the structure of the soil bacterial community.

Our results indicate that the application of both BS significantly stimulated the soil microbial activity. Therefore, when BS was added in soils with oxyfluorfen, a less inhibition of the enzymatic activities studied occured. Furthermore, the toxic effect of the herbicide in soil was considerably reduced. Coinciding with the results obtained by Tejada et al. (2010b) to apply different BS obtained from wheat condensed distiller soluble, carob germ enzymatic and rice bran extract in a soil with the MCPA herbicide, BS stimulate soil microorganisms and therefore favour and accelerate degradation of the xenobiotic compound in the soil. This degradation was faster in the soil amended with SS than with CF, probably due to higher stimulation of soil microorganisms in the soil treated with SS.

Conclusions

It can be concluded that the oxyfluorfen herbicide caused a negative effect on soil enzymatic activities and microbial diversity. The application of BS decreased the toxic action of oxyfluorfen on soil biological properties. However, this effect depended on the chemical composition of the BS applied to the soil. Comparing both the BS, when SS was applied in soils with herbicide occurred a higher decrease in the inhibition of the soil biological properties, probably due to their higher content of lower molecular weight peptides, easily assimilated by soil microorganisms.

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4. RESULTADOS Y DISCUSIÓN

4.1. Accelerated degradation of PAHs using edaphic biostimulants obtained from sewage sludge and chicken feathers

Los diferentes PAH estudiados se degradaron en los suelos no enmendados en mayor o menor medida dependiendo de la toxicidad, biodisponibilidad y recalcitrancia de cada compuesto, que a su vez depende de la complejidad de su estructura química. El fenantreno (Phe), que en su estructura química dispone de tres anillos aromáticos es menos tóxico, soluble y fácil de degradar que el pireno (Py) y el bezo (α) pireno (BaP) que poseen 4 y 5 anillos aromáticos respectivamente (Wammer y col. 2005; Toledo y col. 2006; Simarro y col. 2012;).

Tras aplicar los distintos bioestimulantes a los suelos contaminados con PAHs, se produjo un incremento en la degradación de los contaminantes. Esto puede ser debido a que los propios bioestimulante, además de poder aumentar la biodisponibilidad del contaminante al actuar como surfactantes, adsorbiendo diferentes compuestos, etc., favorecen el crecimiento y actividad de aquellos microorganismos tolerante al contaminante en cuestión, empleándolo como fuente de carbono y energía (Kästner y Richnow, 2001; Tejada y col., 2008; Tejada y Masciandaro, 2011) . A medida que la concentración del xenobiótico disminuye, otros microorganismos pueden desarrollarse y continuar degradándolo, hasta que finalmente desaparece (Peixoto y col., 2011). Sin embargo, la tasa de degradación depende del contaminante y del bioestimulante estudiados. Aquellos contaminantes más complejos tardaron más tiempo en ser degradados, y los bioestimulantes con un contenido superior en péptidos de bajo peso molecular (>300 Da) y aminoácidos libres resultaron más efectivos en el proceso biodegradativo (Tejada y col., 2011a).

La aplicación de los distintos PAHs al suelo provocó una disminución significativa en la actividad deshidrogenasa con respecto al control, atendiendo siempre a la toxicidad de cada uno. Esto es debido a que dicha toxicidad es la causante de la inhibición del crecimiento y actividad de las poblaciones microbianas presentes en el suelo y en consecuencia de las diferentes actividades enzimáticas. Por otra parte, los 3 bioestimulantes estudiados indujeron una rápida y sustancial estimulación de esta actividad en los primeros 7 días del ensayo, decayendo a continuación hasta el final del experimento.

Tras la aplicación de los bioestimulantes a los suelos contaminados, se observa como la actividad deshidrogenasa se encuentra estimulada con respecto al control en los primeros 7 días de incubación. Sin embargo, esta estimulación es siempre menor que la que se observa cuando únicamente está presente el bioestimulante. En este caso, los resultados siguen el mismo patrón que el observado con anterioridad con respecto a la toxicidad del contaminante y a la composición del bioestimulante empleado.

Las actividades β-glucosidasa y fosfatasa fueron también fueron estimuladas tras la aplicación de los distintos bioestimulantes e inhibidas por los PAHs, siguiendo una dinámica similar a la mostrada por la actividad deshidrogenasa y dándose el máximo de actividad a los 7 días del ensayo. De nuevo, el grado de inducción o inhibición depende del contaminante y del bioestimulantes estudiados en cada caso.

Por otra parte, la actividad ureasa fue inhibida en los suelos tratados con los PAHs siguiendo el mismo patrón observado con respecto al contaminante empleado. Sin embargo, ninguno de los bioestimulantes estudiados fue capaz de inducir esta actividad enzimática ni de revertir los efectos causados por los xenobióticos. Esto es debido a que los bioestimulantes, ricos en formas biodisponibles de nitrógeno inhiben la

excreción de esta enzima vinculada a la adquisición de este nutriente por parte de los microorganismos (García-Martínez y col. 2010a; b).

Con respecto al estudio de las poblaciones de bacterias y hongos, estas sufrieron unos aumentos y descensos similares a los observados en las actividades deshidrogenasa, β-glucosidasa y fosfatasa, donde de nuevo, la toxicidad del contaminante y el contenido en péptidos de bajo peso molecular explican los resultados obtenidos.

4.2. Degradation of chlorpyrifos using different biostimulants/biofertilizers: Effects on soil biochemical properties and microbial community

Tras aplicar el insecticida clorpirifos, se detectó una disminución significativa en la actividad deshidrogenasa del suelo tratado, siendo máxima a los 10 días de aplicarlo. Esta inhibición se fue reduciendo gradualmente a lo largo de todo el periodo experimental hasta alcanzar valores similares al control al final del ensayo. Esto se debe a que el clorpirifos tiene un efecto tóxico sobre las actividades enzimáticas del suelo y la diversidad microbiana. (Menon y col., 2004; Shan y col., 2006; Tejada y col., 2011b; Kadian y col., 2012). Sin embargo, dentro de la población microbiana del suelo existen organismos capaces de degradar al insecticida incluso a la dosis aplicada, la cual está reconocida por tener dicho efecto tóxico, disminuyendo la concentración del mismo y permitiendo el desarrollo de otros microrganismos menos tolerantes y así sucesivamente hasta que finalmente el insecticida desaparece del medio. Cuando el contaminante desaparece, las poblaciones microbianas se restablecen de forma similar a la observada en los suelos control.

Se ha observado como los tres bioestimulantes estudiados son capaces de estimular enormemente la actividad deshidrogenasa del suelo, dándose el máximo de estimulación a los 10 días del inicio del ensayo. Esto se puede deber al elevado contenido en péptidos de bajo peso molecular (>300 Da) y aminoácidos libres de los bioestimulante. Estos péptidos y aminoácidos libres constituyen una fuente de nitrógeno altamente biodisponible, el cual suele ser un elemento limitante en el crecimiento de los microorganismos edáficos (Parrado y col., 2008; García-Martínez y col., 2010a, b). Esta alta disponibilidad es también la causante de que esa inducción se produzca en los primeros 10 días, durante los cuales los nutrientes aportados por los distintos productos

son consumidos activamente (Vasileva-Tonkova y col., 2007). Los diferentes contenidos en esa fracción de bajo peso molecular del componente proteico de los bioestimulantes es el responsable de los diferentes niveles de estimulación de la actividad deshidrogenasa, siendo mayor la inducción enzimática cuando la concentración es mayor, es decir, CF2 produce una mayor inducción que SS y CF1 respectivamente.

Cuando se aplicaron los bioestimulantes en suelo previamente contaminados con clorpirifos, se observó una reversión del efecto causado por el insecticida sobre la actividad deshidrogenasa, alcanzándose el valor máximo a los 10 días de la aplicación. Sin embargo, esta reversión en ningún caso alcanzó los valores detectados en los suelos enmendados con los bioestimulantes en ausencia del xenobiótico. Una vez alcanzado el valor máximo de actividad, esta fue decayendo gradualmente hasta alcanzar valores similares al control al final del experimento. Al igual que en los suelos tratados sin contaminar, la concentración de la fracción de bajo peso molecular del componente proteico de los productos usados es la responsable del grado en que es revertida la inhibición sobre la actividad deshidrogenasa, siendo de mayor a menor la reversión en los suelos tratados con CF2, SS y CF1 respectivamente. Esto se debe a que los bioestimulantes favorecen el crecimiento y actividad de las poblaciones microbianas del suelo, enmascarando el efecto tóxico del contaminante, el cual, también se ha descrito que puede ser adsorbido por la materia orgánica, lo que disminuye su biodisponibilidad y por tanto su potencial nocivo (García-Martínez y col., 2010a,b; Tejada y col.,2011b).

Con respecto a otras actividades enzimáticas del suelo vinculadas a la adquisición de nutrientes por parte de los microorganismos del suelo, como son las actividades β-glucosidasa (captación de azúcares, carbono), fosfatasa (captación de P) o arilsulfatasa (captación de azufre) se observó un efecto similar al observado con la

actividad deshidrogenasa. En todos los casos, el clorpirifos inhibió estas actividades enzimáticas con respecto al control, del mismo modo que los distintos BS las estimularon. Tanto los valores máximos de actividad como de inhibición de la misma se produjeron a los 10 días de incubación, para a continuación decaer gradualmente hasta alcanzar valores similares al control al final del ensayo.

Este comportamiento se debe a que la estimulación o inhibición de estas actividades enzimáticas está directamente relacionada con la actividad microbiana reflejada en la actividad deshidrogenasa, la cual a su vez es una consecuencia de la adicción de materia orgánica biodisponible al suelo (Gan y col., 1998; Perucci y col., 2000). Cuando la actividad deshidrogenasa aumenta significa que las poblaciones microbianas crecen y/o aumentan su actividad, o justamente lo contrario cuando decae. Esto es así porque en respuesta a las necesidades nutricionales, los microorganismos excretan enzimas con las que poder captar los nutrientes necesarios para su actividad y multiplicación, reduciéndose cuando no es necesario, de ahí esa vinculación entre las actividades β- glucosidasa, fosfatasa y arilsulfatasa con la actividad deshidrogenasa (García-Martínez y col., 2010a, b).

Sin embargo, la actividad ureasa no fue estimulada cuando se añadieron los BS al suelo, mientras que la adicción del insecticida si produjo un efecto inhibitorio sobre esta actividad. La disminución de la actividad en presencia del contaminante se puede explicar del mismo modo que el resto de actividades enzimáticas estudiadas, es decir, una disminución de la actividad y tamaño de las poblaciones microbianas conlleva una disminución en la producción de enzimas destinadas a la captación de nutrientes. Cuando se enmendaron los suelos con los BS, no se produjo una estimulación de esta actividad a pesar de que si se produjo una estimulación de los microorganismos. Esto es debido a que estos productos ofrecen una fuente rica y fácilmente disponible de

nitrógeno por lo que los microorganismos no tienen la necesidad de excretar una enzima destinada a su captación a partir de otras fuentes más complejas (García-Martínez y col., 2010a, b).

Con respecto a las comunidades microbianas del suelo, se observó como el insecticida tuvo un efecto inhibitorio sobre las mismas (Rangaswamy y Venkateswarlu, 1992; Ahmed y Ahmad, 2006), al mismo tiempo que los BS tuvieron un efecto inductor similar al observado con las actividades enzimáticas determinadas anteriormente. Se comprueba de nuevo como los bioestimulantes con un mayor contenido en la fracción proteica de bajo peso molecular producen la mayor inducción en las poblaciones microbianas (Marschner y col., 2003). Cuando se estudiaron los PLFA en suelos enmendados con los BS previamente contaminados con clorpirifos, se observa como estos productos son capaces de revertir de nuevo el efecto causado por el insecticida de forma similar a la observada con las actividades enzimáticas. En todos los casos, después de que los nutrientes aportados por los bioestimulantes son consumidos, las poblaciones se restablecieron gradualmente para finalmente volver a ser similares a las observadas en la muestra control.

Después de aplicar clorpirifos al suelo se observa como su concentración va disminuyendo gradualmente en todas las muestras. Sin embargo, esta desaparición se encuentra acelerada en las muestras enmendadas con los diferentes bioestimulantes. Esto se debe a que la actividad general y biodegradativa en particular de los microorganismos se encuentra estimulada, al tiempo que la biodisponibilidad del contaminante es menor que en los suelos no enmendados, lo que favorece el desarrollo de microorganismos que normalmente a esa dosis de insecticida se encontrarían inhibidos. La suma de ambos factores es la causante de esta degradación acelerada del contaminante. Al igual que con las actividades enzimáticas o la estructura de las

poblaciones microbianas, el BS con mayor contenido en péptidos de bajo peso molecular es el que induce una mayor tasa de biodegradación del insecticida.

4.3. Behaviour of oxyfluorfen in soils amended with edaphic biostimulants / biofertilizers obtained from sewage sludge and chicken feathers. Effects on soil biological properties

El oxifluorfeno es un herbicida por contacto con actividad residual y alta persistencia que actúa inhibiendo la ruta de síntesis de la clorofila y citocromos y provocando una acumulación de especies reactivas de oxígeno (Kunert, 1984, Lee y col. 2000). Debido a su mecanismo de acción, resulta tóxico tanto para las plantas como para los microorganismos del suelo. Tras su aplicación al suelo se observa como permanece en el mismo un largo periodo de tiempo (Baruah y Mishra, 1986; Ying y Williams, 2000; Mantzos y col., 2014), aunque su concentración va disminuyendo progresivamente, señal de que probablemente está siendo degradado por organismos del suelo tolerantes al mismo. Cuando se enmendaron suelos contaminados por oxifluorfeno con los bioestimulantes estudiados, se observó un aumento en la tasa de desaparición de este compuesto. Esto puede estar debido a la bioestimulación de las poblaciones microbianas del suelo y/o al aumento de la biodisponibilidad del herbicida, que es altamente insoluble en agua, pudiendo actuar los BS como surfactantes (Mantzos y col., 2014; Tripathy y col. 2018). Las diferencias entre un bioestimulante y el otro se deben a la diferente composición de cada uno, siendo SS el que induce la mayor tasa de eliminación del oxifluorfeno.

Cuando se estudió la actividad deshidrogenasa en suelos enmendados con ambos BS, se observó cómo se producía una rápida y significativa inducción de esta actividad, siendo mayor en el caso de SS que en el de CF. Este incremento en la actividad deshidrogenasa es un indicativo de que las poblaciones microbianas del suelo han aumentado en número y actividad como consecuencia de este aporte de nutrientes

(Parrado y col., 2008; García-Martínez y col., 2010a, b; Tejada y col., 2010b, 2013). Además, el relativo alto contenido en péptidos de bajo peso molecular (>300 Da) y aminoácidos libres de los productos causa un efecto de bioestimulación microbiana al aportar una fuente de nitrógeno, cuya concentración en el suelo es normalmente limitante, fácilmente biodisponible (Tejada y col., 2013). Las diferencias observadas entre ambos bioestimulantes se deben a la diferente composición de cada uno y probablemente en particular al diferente contenido en el componente proteico de bajo peso molecular (Vasileva-Tonkova y col., 2007).

Cuando se aplicó el herbicida al suelo se detectó una inhibición en la actividad deshidrogenasa del mismo. Esto se debe al efecto causado por el oxifluorfeno al inhibir una ruta fundamental del metabolismo. Esta inhibición se mantuvo durante todo el periodo experimental, indicativo de la persistencia de este compuesto.

Por otra parte, cuando se aplicaron los bioestimulantes en suelos contaminados con oxifluorfeno se observó una reversión de la inhibición de la actividad deshidrogenasa causada por el herbicida. Los mayores valores de actividad deshidrogenada detectados se dieron a los 7 días de iniciado el ensayo, siendo superiores en los suelos tratados con SS que en los enmendados con CF. Una vez alcanzados los valores máximos de actividad, esta decayó gradualmente hasta alcanzar valores similares a los del suelo sin contaminar. El hecho de que los valores observados sean superiores para SS que para CF puede ser debido de nuevo a que el contenido en péptidos de bajo peso molecular (>300 Da) y aminoácidos libres es superior en el primero que en el segundo.

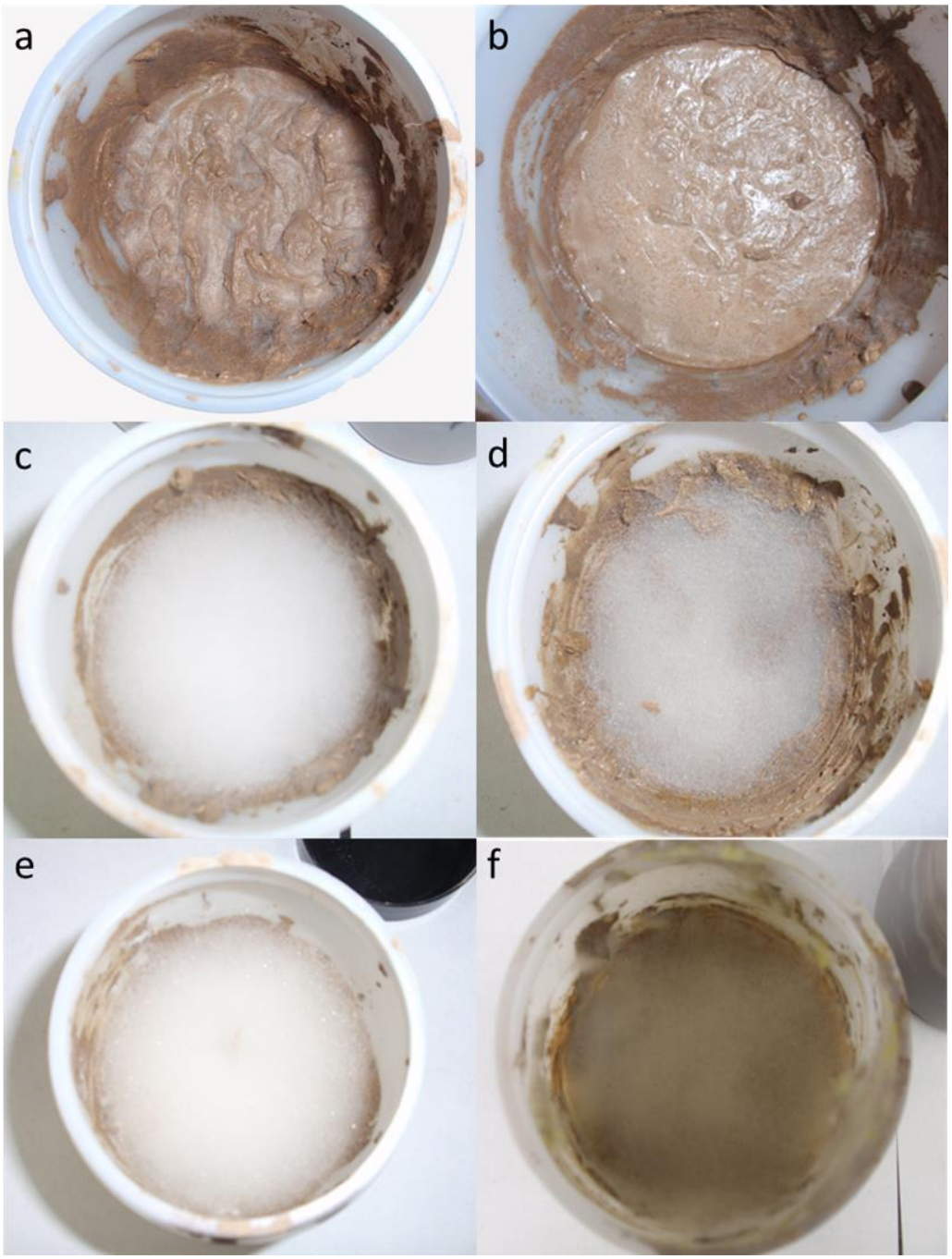

Figura 21: Microcosmos a los 7 días de incubación. a) C; b) H; c) SS; d) SS+H; e) CF; f) CF+H.

La reversión del efecto del contaminante sobre la actividad deshidrogenasa se debe en parte a que los bioestimulantes inducen en gran medida el crecimiento y actividad de las poblaciones microbianas del suelo, ignorando aparentemente el efecto tóxico del oxifluorfeno, y por otra parte, los BS actúan aumentando la biodisponibilidad del herbicida para los organismos degradadores y reducen su concentración aparente al

actuar adsorbiéndolo gracias a su contenido en materia orgánica. Gracias a ello, los organismos tolerantes son capaces de degradar más eficientemente el contaminante, permitiendo que rápidamente otros organismos menos resistentes a este compuesto también puedan desarrollarse y continuar degradando el oxifluorfeno.

De forma similar a la actividad deshidrogenasa, las actividades β-glucosidasa y fosfatasa se vieron estimuladas significativamente en los suelos enmendados con los bioestimulantes con respecto al suelo control. Del mismo modo, se inhibieron en los suelos contaminados con oxifluorfeno. Esta inducción e inhibición son consecuencia directa del aumento o disminución de las poblaciones microbianas y de su actividad, reflejada en la actividad deshidrogenasa. Cuando las poblaciones de microorganismos aumentan su tamaño o su actividad, requieren de un mayor aporte de nutrientes, principalmente a partir de compuestos ya mineralizados, o en los casos donde no haya formas de los mismos fácilmente disponibles, por mineralización de la materia orgánica (Marschner y col., 2003). De este modo, la materia orgánica aportada por los bioestimulantes actúa como inductor de la producción y secreción de estas enzimas hidrolíticas, que en primer lugar hidrolizan los compuestos orgánicos complejos en unidades estructurales menores y a continuación los mineralizan en forma de iones fácilmente translocables al interior celular.

Debido a la vinculación entre las enzimas β-glucosidasa y fosfatasa con la actividad deshidrogenasa (indicativo de la actividad microbiana), estás siguen una dinámica similar. Los valores máximos para ambas enzimas se detectaron a los 7 días de iniciarse el experimento de bioestimulación, decayendo posteriormente hasta valores similares al suelo sin contaminar, mientras que la inhibición de las mismas en suelos contaminados con el herbicida se mantuvo durante todo el ensayo.

Cuando se añadieron los bioestimulantes estudiados a un suelo contaminado con oxifluorfeno, de nuevo se observa una reversión del efecto tóxico del herbicida similar a la observada con la actividad deshidrogenasa. Ambas actividades enzimáticas alcanzan sus valores máximos a los 7 días, decayendo gradualmente hasta los valores del suelo control hacia el final del ensayo. En todos los suelos ensayados, SS siempre presenta unos valores supriores a los de CF. Esto puede ser debido de nuevo al mayor contenido en péptidos de bajo peso molecular y aminoácidos libres del primero con respecto al segundo.

Con respecto a la actividad ureasa, enzima implicada en la mineralización de nitrógeno, ninguno de los BS estudiados indujo una estimulación a la misma. Esto puede ser debido a que el componente proteico de bajo peso molecular de los bioestimulantes es una fuente de nitrógeno altamente biodisponible, por lo que los microorganismos no necesitan gastar energía en producir enzimas para su adquisición, incorporando directamente al interior celular esos compuestos nitrogenados (García-Martínez y col., 2010a, b; Tejada y col., 2013). Por otra parte, en los suelos contaminados con oxifluorfeno si se observó una inhibición de esta actividad, debido al efecto tóxico del xenobiótico, independiente de la presencia o no de los bioestimulantes.

La aplicación de ambos bioestimulantes al suelo incrementó significativamente las poblaciones de bacterias y hongos, siendo mayor el efecto causado por SS que el de CF y alcanzando los valores máximos a los 7 días de incubación. De nuevo, el efecto tóxico del oxifluorfeno causó una disminución de las poblaciones microbianas de acuerdo a lo observado con la actividad deshidrogenasa, efecto que se mantuvo hasta el final del experimento.

Cuando se añadieron los BS a suelos contaminados con el herbicida se observó un incremento de las poblaciones microbianas, aunque siempre menor al observado en

los suelos enmendados sin contaminar. Hacia el final del ensayo, los valores de todos los suelos enmendados fueron similares al control, lo que sugiere que las poblaciones microbianas se restablecieron de forma parecida al suelo sin contaminar ni enmendar.

En todos los suelos estudiados se observaron menores variaciones en las poblaciones de hongos que en las bacterianas. Esto se puede deber a que las bacterias poseen una crecimiento y capacidad de respuesta mucho más acelerado que los hongos, los cuales, al tardar más tiempo en desarrollarse, probablemente no puedan beneficiarse de los nutrientes aportados por los bioestimulantes o verse afectados por el efecto tóxico del oxifluorfeno.

5. CONCLUSIONES

De los estudios de la capacidad biorremediadora de suelos contaminados por diferentes xenobióticos orgánicos (plaguicidas como oxifluorfeno y clorpirifos e hidrocarburos aromáticos policíclicos como fenantreno, pireno y benzopireno) mediante el uso de los nuevos bioestimulantes obtenidos a partir de lodo de depuradora y plumas de pollo, se exponen a continuación las conclusiones más relevantes:

1. La aplicación al suelo de los xenobióticos orgánicos anteriormente descritos causó efectos tóxicos en la actividad bioquímica del suelo. En el caso de los hidrocarburos aromáticos policíclicos, cuanto mayor es el número de anillos aromáticos presente en su estructura química, mayor es la toxicidad causada sobre dichos microorganismos del suelo.
2. La aplicación de los bioestimulantes aceleró la degradación de los xenobióticos en suelo y en consecuencia disminuye los efectos tóxicos de los mismos sobre los microorganismos de los suelos. Los microorganismos tolerantes a dichos tóxicos, que utilizan estos contaminantes como fuente de energía y degradándolos en el medio, también utilizan a los bioestimulantes como fuente de energía y nutrientes, lo cual hace aumentar su proliferación en el suelo, aumentando consecuentemente la velocidad de degradación de los tóxicos.
3. Este efecto biorremediador de los nuevos bioestimulantes depende de la composición química de los mismos, y en concreto, de la distribución de pesos moleculares del componente proteico de estos productos orgánicos. En este sentido, la mayor asimilación por los microorganismos del suelo de péptidos de bajo peso molecular y aminoácidos libres podría ser responsable de la mayor degradación de los xenobióticos orgánicos en el suelo, debido a que son absorbidos de forma muy rápida y sin gasto de energía por los microorganismos edáficos.

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NOTAS:

CONCLUSIONES FINALES

Las conclusiones finales de esta tesis doctoral se exponen a continuación:

1. Tanto los lodos de depuradora como las plumas de ave pueden ser usados como sustratos económicos para el cultivo de *Bacillus licheniformis* y la obtención de enzimas hidrolíticas a partir de este microorganismo o como materia prima de partida para la producción de bioestimulantes.
2. El secretoma de *B. licheniformis* se ve afectado tanto cualitativa como cuantitativamente por la composición del medio de cultivo.
3. Los productos bioestimulantes obtenidos a partir de lodos de depuradora y plumas de ave, ya sea por procesos de hidrólisis enzimática o por procesos fermentativos, se caracterizan por ser ricos en péptidos bajo peso molecular y aminoácidos libres, además de tener una elevada solubilidad en agua y una alta biodisponibilidad.
4. El origen y los tratamientos que sufran los lodos de depuradora previamente al proceso de hidrólisis enzimática afectan a la proporción de péptidos de bajo peso molecular y aminoácidos libres del producto, y en consecuencia, a su potencial bioestimulante edáfico.
5. Los bioestimulantes obtenidos por hidrólisis enzimática de lodos de depuradora son capaces de estimular las poblaciones microbianas y actividades enzimáticas del suelo, a excepción de la actividad ureasa. Esta actividad no se estimula ya que el propio producto es una fuente de nitrógeno y esta enzima es excretada con el fin de captar este elemento.
6. La aplicación foliar de un bioestimulante obtenido por hidrólisis enzimática de lodos de depuradora produce mejoras en el cultivo del maíz como son aumentos de la concentración de proteínas en el grano o de los rendimientos generales del cultivo. Estos efectos no se observan tras la aplicación edáfica del producto.

7. La aplicación foliar de un bioestimulante obtenido por hidrólisis enzimática de plumas de ave produce mejoras en el cultivo del maíz como son incrementos en la calidad del grano o en los rendimientos generales del cultivo.
8. Los suelos contaminados por los compuestos xenobióticos estudiados presentaron una disminución de todas las actividades enzimáticas determinadas. El grado de complejidad del contaminante incrementa su efecto tóxico en el suelo.
9. Se observó un incremento de la velocidad de degradación de los compuestos xenobióticos en el suelo tras la aplicación de los productos bioestimulantes. Este aumento se debe al incremento poblacional causado por el producto bioestimulante, permitiendo a los organismos tolerantes al compuesto tóxico degradarlo.
10. El efecto biorremediador de los productos bioestimulantes depende de la composición y características de estos, en particular, del contenido en péptidos de bajo peso molecular y aminoácidos libres.