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Support material selection for anaerobic fluidized bed reactors by phospholipid analysis

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Abstract

Many different types of support materials are used in anaerobic fluidized bed in order to obtain high levels of biomass in the reactors. A material is usually considered the most suitable in terms of physical–chemical parameters or reactor performance, but not in terms of microbial adhesion. In this work, two commercial materials were tested as support for anaerobic biomass: perlite and sepiolite. Biofilm accumulation was estimated by two methods, attached volatile solids and lipid phosphate concentration, both determined directly on attached biofilm. The biomass adhesion on sepiolite was higher than the perlite for all the tested initial total organic carbon concentration in the reactors. We have found that biomass measurement method developed directly on attached biofilm is relevant to the amount of living cells. We also discuss the suitability of estimating attached biomass by means of direct measures on biocovered particles rather than on enrichment cultures of free cells detached from the supports.

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1. Introduction

Anaerobic digestion offers significant advantages over aerobic systems, like low energy consumption, reduced solids formation, low nutrient requirement and potential energy recovery from the methane produced [1]. However, anaerobic digestion has a low growth rate of anaerobic bacteria and requires longer hydraulic retention time to achieve satisfactory degradation when compared to aerobic treatment [2]. In the last decades, the development of various techniques like fixed or fluidized beds for the retention of anaerobic microorganisms inside the reactor has enabled to shorten the hydraulic retention time and to increase the organic loading rate.

Fluidized bed biofilm reactors are now commonly adapted to wastewater treatment [3–7]. In the field of anaerobic digestion, however, it has not been extensively used at full-scale. The lack of industrial success may result from a combination of several negative points: a high level of maintenance because of their complexity, the need for fluid recycling, hydrodynamic problems [8] or an inadequate selection of the support material for microbial adhesion [9].

The most significant variable in anaerobic digestion in a fluidized bed reactor is the selection of support medium for microbial adhesion [9]. Many materials have already been tested as anaerobic fluidized bed reactor supports. However, a material is usually considered the most suitable in terms of reactor performance, such as substrate removal rate [10] and biogas flow rate [11,12], or physical–chemical parameters, such as specific surface area, roughness, minimum fluidisation velocity or composition [6,7], not in terms of microbial adhesion [13].

Many methods have been described for biofilm biomass estimation [14] but, unfortunately, most of them have been developed and tested on free cell cultures or enrichment

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Nomenclature

ALP	attached lipid phosphate (nmol/mL$_{CARRIER}$)
d_p	particle diameter
SLP	suspended lipid phosphate (nmol/mL)
SSA	specific surface area
TOC	total organic carbon (mg/mL)
VAS	volatile attached solids (mg/mL$_{CARRIER}$)
VSS	volatile suspended solids (mg/mL)

cultures of free cells detached from the bioparticles. Total dry weight and volatile solids are widely used parameters for biofilm characterization [6,15–18]. The main disadvantages of these methods are detailed elsewhere [14] and they are mainly focused on time-consumption, low repeatability and overestimation.

Phospholipids, a cell wall component, offer many advantages over other assays for selective cellular biomass estimation in biofilms, and their determination by colorimetric methods is relatively simple, reproducible and sensitive [14]. Lipids have been widely used in environmental samples [19–21] and recently applied to aerobic and anaerobic wastewater biofilm [7,22].

In this work, we use the phospholipid analysis to determine the suitability of two materials as anaerobic fluidized bed reactor support. Phospholipid content was assumed to represent the living cells inside the biofilm. We also discuss the suitability of estimating attached biomass by means of direct measures on biocovered particles rather than on enrichment cultures of free cells detached from the supports.

2. Materials and methods

2.1. Batch reactors and influent composition

The reactors consisted of screw-cap bottles (125 mL volume) with a one-way valve for gases exit, containing 10 mL of material support, 10 mL of inoculum and 80 mL of synthetic feed.

The inoculum was obtained from an anaerobic semi-continuous stirred tank reactor of 1 L capacity. The inoculum for this work was initially digested activated sludge obtained from the anaerobic reactor of an urban wastewater treatment plant, with a concentration of 7040 mg L^{-1} of volatile suspended solids (VSS) and a concentration of 60 nmol mL^{-1} of suspended lipid phosphate (SLP), and sequentially adapted to a mixture of potassium acetate and sodium lactate. The proportion of inoculum in the mixture to start up the reactor for the experiment was 1:4 (v/v). The temperature was held at 35 °C by a thermostatic water bath, and stirred by means of a magnetic stirring bar at 100 rpm. Dark conditions were maintained by wrapping the reactors with black plastic bags.

The synthetic feed was prepared with a mixture 2:1 of potassium acetate and sodium lactate diluted with a mineral medium (Table 1). Initial total organic carbon concentration (TOC) of the batch reactors were 600, 800 and 1000 mg L^{-1}. The inoculation was carried out under a non-oxygen gas flow, because of the strict anaerobic technique that relies on excluding all traces of oxygen from the medium during culture preparation. In this case, the culture was bubbled with nitrogen for 15 min. The flasks used had a one-way valve to allow gas to escape [23]. The batch was incubated in darkness, in an orbital shaker-incubator, New Brunswick Scientific G25, at 100 rpm and at 35 °C. All the tests were run in triplicate. Blank support-free reactors were also prepared.

2.2. Physical properties of the carrier material

Table 2 shows physical properties of the support materials used in this work. Commercially available perlite (provided by Europerlita S.A., Spain) is an expanded volcanic rock, while sepiolite (Sepitol 30/60 supplied by Tolsa S.A., Spain) is a clay material. Both materials were calcinated (550 °C, 24 h) to eliminate impurities and then washed.

2.3. Analytical methods and biomass determination

Biodegradation was followed by monitoring TOC of filtered samples. TOC was measured with a Dorhman DC-190 analyser whose operation is based on the combustion/non dispersive infrared gas analysis.

Bioparticles for phospholipid analysis and attached solids were taken on day 12 and on day 38 after starting-up the reactors. In this study, fixed biomass was measured as volatile attached solids to the carrier (VAS) and attached lipid phosphate to the carrier (ALP).

VAS were determined on washed samples of bioparticles according to [15]. A sample of biomass-laden particles (0.5 mL) was withdrawn from the reactor and gently washed with 100 mL of distilled water to remove any unattached biomass. This sample was then transferred to a weighed crucible and placed in an oven at 105 °C for 24 h to remove all unbound moisture. The crucible was reweighed and placed in a furnace at 560 °C for 1 h to burn off all the biomass, followed by a reweighing. Because perlite and sepiolite themselves showed some ignition loss, the obtained values were corrected accordingly. The biomass concentration was calculated as the difference between the two weightings divided by the total volume of support material present in the sample.

The procedure used in this study in order to determine ALP was a modification of that found in [21]. Samples of biomass-laden particles were withdrawn from the reactor and gently washed with distilled water to remove any unattached biomass. The procedure consisted of: (a) 0.5 mL of bioparticles for ALP, were added into 70 mL screw-cap test tubes. Then, 20 mL of chloroform, 20 mL of methanol and 20 mL of deionized water were added to the samples. The extraction mixture was gently shaken for 10 min and

Table 1
Composition of the mineral medium for anaerobic cultures

Solution A		Solution B	
Compound	Concentration ($g L^{-1}$)	Compound	Concentration ($g L^{-1}$)
KH_2PO_4	10.88	NH_4Cl	106
K_2HPO_4	73.12	$CaCl_2$	5.96
		$MgCl_2 \cdot 6H_2O$	10
Sterilize		Sterilize	

	Solution C		Solution D	
	Compound	Concentration ($g L^{-1}$)	Compound	Concentration ($g L^{-1}$)
Solution 1	EDTA	64	$MnCl_2 \cdot 4H_2O$	5
	KOH	56	H_3BO_3	0.5
			$ZnCl_2$	0.5
Solution 2	$SO_4Fe \cdot 7H_2O$	54.8	$CuCl_2 \cdot 2H_2O$	0.38
			$(NH_4)_6Mo_7O_4 \cdot 4H_2O$	0.08
			$CoCl_2 \cdot 6H_2O$	5
			$NiCl_2 \cdot 6H_2O$	0.5
			Na_2SeO_3	0.5
Mix solutions 1 and 2			Sterilize	

Composition for 1 L: 40 mL solution A, 10 mL solution B, 1 mL solution C and 0.1 mL solution D. All solutions were separately autoclaved before mixing.

Table 2
Perlite and sepiolite physical properties (SSA, specific surface area; d_p, particle diameter)

	Apparent density ($kg m^{-3}$)	Density ($kg m^{-3}$)	SSA ($m^2 m^{-3}$)	d_p (μm)	Shape
Perlite	154	213	6199	968	Granules
Sepiolite	575	2300	5.5×10^{8} [a]	250–600	Granules

[a] BET-N_2 SSA measurement.

allowed to stand up to complete phase separation. (b) To facilitate recovery of the chloroform, the aqueous (upper) phase was aspirated from the test tubes with the aid of a vacuum pump and subsamples of 5 mL of the chloroform layer were transferred into 10 mL screw-cap test tubes. At this point, lipids can be stored at −20 °C. (c) The chloroform was removed under a stream of nitrogen, and phosphate was liberated from lipids by adding 2.7 mL of a potassium persulfate solution (5 g added to 100 mL of 0.36N H_2SO_4) and the sealed test tubes were heated in an oven at 105 °C for 1 h. (d) Phosphate release by persulfate digestion was determined by adding 0.6 mL of an ammonium molybdate solution [2.5% of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 5.72N H_2SO_4 allowed to stand for 10 min] and 2.7 mL of a malachite green solution (0.111% polyvinyl alcohol dissolved in water at 80 °C is allowed to cool, and 0.011% malachite green is then added and allowed to stand for 30 min). (e) The absorbance at 610 nm was then read using a spectrophotometer (Beckman DU®640). The concentrations of phosphate were calculated by using the regression line from a standard curve obtained by digesting 10, 20, 40, 60, 80, 100 and 150 μL of a 1 mM glycerol–phosphate solution.

In the phospholipid analysis, solvents for lipid extraction were of high quality, lipid standard [DL-(phosphatidylethanolamine, dipalmitoyl (C16:0)], calcium glycerol phosphate and malachite green were of reagent quality (Sigma) and polyvinyl alcohol was 98% hydrolyzed (average molecular weight, 13,000–23,000; Aldrich Chemical Co., Inc.). Glassware was washed with phosphate-free detergent, rinsed five times with tap water and 2–3 times with deionized water, and air-dried. Glassware was rinsed with chloroform just before used. Potassium persulfate solution must be replaced monthly.

3. Results and discussion

Fig. 1 shows the attached biomass concentration expressed as volatile solids (plots B1–C1) and phospholipids (plots B2–C2) per unit volume of support. Data were obtained on the 12th and 38th days of incubation. Average value of TOC removal at day 12 was 89.7% in the perlite reactors and 96.3% in the sepiolite reactors.

A comparison between biomass concentration at day 12, expressed as lipid phosphate per unit volume of support, shows that sepiolite carried an amount of biomass 3.0, 2.4 and 1.8 times higher than perlite, at the initial TOC of 600, 800 and 1000 $mg L^{-1}$, respectively (plots B2–C2). Similar results are obtained if the biomass concentration is expressed as volatile solids per unit volume of support: sepiolite had an amount of biomass 2.7, 3.2 and 1.8 times higher than perlite, at the initial TOC of 600, 800 and 1000 $mg L^{-1}$, respectively (plots B1–C1). The higher specific surface area of the sepiolite material could be the reason for the higher biomass concentration on this support, if we compared it with the perlite support (5.5×10^{8} $m^2 m^{-3}$ against 6199 $m^2 m^{-3}$,

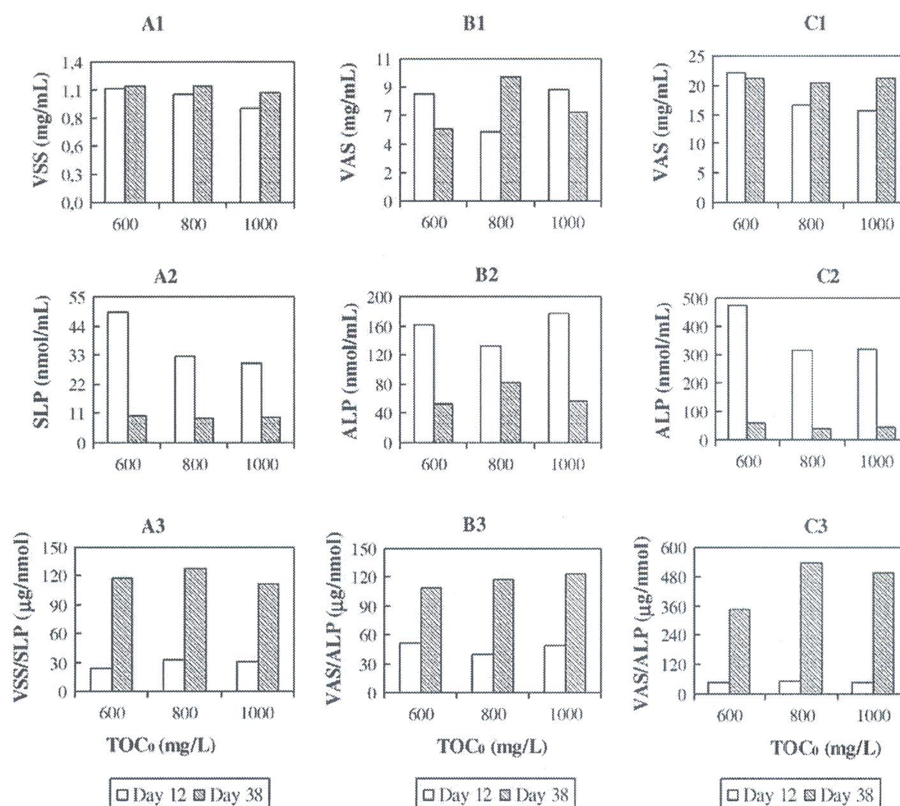

Fig. 1. Biomass concentration expressed as volatile solids (1), phospholipids (2) and conversion factors from lipid phosphate concentration to living cell weight (3). (A) Free-support reactors, (B) perlite reactors, (C) sepiolite reactors.

respectively). Together with other factors, as clay–bacteria interaction and clay-flocculating properties, this fact has been pointed out previously by other authors [24,25].

Data obtained the day 38 after starting-up the reactors (when microorganisms have been exposed 26 days under conditions of non-carbon source available) show that phospholipid content dropped down while volatile solids remained relatively constant.

The volatile attached solids concentrations into the perlite reactors, determined on the day 12 and 38 of incubation, gave a mean value at the three essayed TOC of $7.4\,\mathrm{mg_{VAS}\,mL^{-1}_{CARRIER}}$. However, the attached lipid phosphate concentration decreased 67.9, 61.5 and 68.5% at the initial TOC of 600, 800 and $1000\,\mathrm{mg\,L^{-1}}$, respectively (plots B1, B2).

The volatile attached solids concentrations into the sepiolite reactors, measured on day 38 of incubation, gave a mean value at the three tested TOC of $20.9\,\mathrm{mg_{VAS}\,mL^{-1}_{CARRIER}}$, while on day 12 that mean value was $18.1\,\mathrm{mg_{VAS}\,mL^{-1}_{CARRIER}}$. However, the attached lipid phosphate concentration decreased 87.2, 87.8 and 86.7% at the initial TOC of 600, 800 and $1000\,\mathrm{mg\,L^{-1}}$, respectively (plots C1, C2).

An important fact is that in three support-free reactors the phospholipid content also dropped down while volatile solids remained relatively constant the day 38, after almost 4 weeks under conditions of non carbon source available (average value of TOC removal at day 12 was 87.3%). The volatile suspended solids concentration in the support-free reactors, measured on day 12 and on day 38 of incubation, gave a mean value at the three essayed TOC of $1.0\,\mathrm{mg\,mL^{-1}}$. However, the suspended lipid phosphate concentration decreased 80.2, 72.2 and 67.2% at the initial TOC of 600, 800 and $1000\,\mathrm{mg\,L^{-1}}$, respectively (plots A1, A2).

In this study, total biomass amount was measured in terms of volatile solids concentration and lipid phosphate concentration. The objective in measuring lipid phosphate concentration was to find the living cell biomass into the reactors.

The main disadvantage of the volatile solids determination is that its estimation includes not only active microorganisms, but also inert mass, exopolymers and absorbed organic matter on flocs and biofilms. Phospholipids, present on bacterial membrane up to 90–98%, do not form part of cell reserves and are easily degraded during bacteria lysis [19]. Therefore, their estimation only includes living biomass. When relation between these two measures is calculated (VSS/SLP or VAS/ALP), an estimation of living biomass with regard to total biomass is obtained.

A comparison of the conversion factors of perlite and sepiolite samples after 12 days of incubation shows a mean value of $48.15\,\mathrm{\mu g_{VAS}\,nmol^{-1}}$ (plots B3–C3). This average factor is,

approximately, 10 times higher that those reported by other authors, 4.785 g-cell $nmol^{-1}$ [21] and 4.81 μg_{TS} $nmol^{-1}$ [22]. It should be mentioned that, in both works, conversion factors were obtained by enrichment cultures of free-living cells, so extracellular polymeric substances (EPS) could appear at lower levels. In the present work, the conversion factor was almost two times lower in free cells from support-free reactors, 29.25 μg_{VSS} $nmol^{-1}$ (plot A3).

Average TOC removal at day 12 was 87.3% in the support-free reactors, 89.7% in the perlite reactors and 96.3% in the sepiolite reactors. Therefore, on day 38 of incubation, microorganisms have been exposed 26 days under conditions of non-carbon source available, which is known as biomass stabilization process. At that day, living biomass decreased while volatile solids remained relatively constant (plots A1–A2; B1–B2; C1–C2). Because of that, conversion factors calculated on day 38 of incubation were higher than those calculated on day 12 of incubation (plots A3–C3).

The average conversion factors on day 38 of incubation were 118.7 μg_{VSS} $nmol^{-1}$ in the support-free reactors, 116.9 μg_{VAS} $nmol^{-1}$ in the perlite reactors and 459.3 μg_{VAS} $nmol^{-1}$ in the sepiolite reactors. In other words, when living biomass is stabilized, there are still volatile solids in suspension and within the biofilm.

Biomass consists of a biodegradable fraction and an inert fraction, which cannot be biologically degraded. This inert fraction is constituted by dead-end products and rest of extracellular matrix [26–28]. This could explain that the higher conversion factors were those obtained for attached biomass in the support reactors, since exopolymer matrix is more abundant in biofilms, up to 80% of volatile solids [29]. Therefore, conversion factors obtained from enrichment cultures of free cells detached from the biofilms seem not suitable in any condition.

The limitations of selective enrichments are also reported elsewhere [14,30,31]. Main criticism is that they involve cell reproduction and only some of the total active cells are susceptible to regrowth. In addition, the reproducibility of results depends on incubation conditions, biomass type (free cultures or cells from biofilms) and the choice of nutritive medium.

Other authors have found that measurement method developed directly on attached biofilm is relevant to the amount of living cells. Cao and Alaerts reported for the specific oxygen activity of biomass (expressed as g O_2/g biomass day) of aerobic biofilm that was almost two times higher in the suspended biomass than within the biofilm [32]. The EPS accumulation was assumed by the authors to be the main reason of that difference.

Arnaiz et al. [33] proved that biomass measurement by phospholipids analysis developed directly on attached biofilm is relevant not only of the amount to living cells, but to the active biomass. These authors, working with an anaerobic inverse turbulent bed reactor treating wine distillery wastewater, found that the comparison of the measured concentration of volatile attached solids with the estimated biomass concentration by means of phospholipids analysis indicated that extremely high active biomass concentrations could be maintained in the system. In this work, initial biomass concentration of bioparticles was 48.3 mg_{VAS} $mL_{CARRIER}^{-1}$ and 165.3 $nmol_{ALP}$ $mL_{CARRIER}^{-1}$ and the reactor processed 15.8 kg_{COD} m^3 day^{-1}. At the end of the period of study, final biomass concentration was 23.4 mg_{VAS} $mL_{CARRIER}^{-1}$ and 30.7 $nmol_{ALP}$ $mL_{CARRIER}^{-1}$ and the reactor processed an average of 28.1 kg_{COD} m^3 day^{-1}. It meant that a biomass concentration, almost 50% reduced in terms of VAS but 80% reduced in terms of ALP, almost duplicated the organic matter treated.

4. Conclusion

This work compared two types of commercial materials as support for anaerobic fluidized systems: perlite and sepiolite. The biomass adhesion in sepiolite was higher than the adhesion on the perlite in terms of volatile solids and lipid phosphate concentration.

The determination of lipid phosphate concentration directly on attached biofilm has been revealed as a good method for direct biofilm estimation and it could be used to compare the microbial adhesion on support materials under anaerobic conditions, in order to select the most adequate for fluidized bed reactors.

Phospholipid analysis could be a good tool to study the relationship between the active biomass growth onto the solid carrier and their influence on overall performance in continuously feeding fluidized bed reactor.

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