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ORIGINAL PAPER

Characterization of sulfate-reducing bacteria dominated surface communities during start-up of a down-flow fluidized bed reactor

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Abstract An anaerobic down-flow fluidized bed reactor was inoculated with granular sludge and started-up with sulfate containing synthetic wastewater to promote the formation of a biofilm enriched in sulfate-reducing bacteria (SRB), to produce biogenic sulfide. The start-up was done in two stages operating the reactor in batch for 45 days followed by 85 days of continuous operation. Low-density polyethylene was used as support. The biofilm formation was followed up by biochemical and electron microscopy analyses and the composition of the community was examined by 16S rDNA sequence analysis. Maximum immobilized volatile solids (1.2 g IVS/$L_{support}$) were obtained after 14 days in batch regime. During the 85 days of continuous operation, the reactor removed up to 80% of chemical oxygen demand (COD), up to 28% of the supplied sulfate and acetate was present in the effluent. Sulfate-reducing activity determined in the biofilm with ethanol or lactate as substrate was 11.7 and 15.3 g COD/g IVS per day, respectively. These results suggested the immobilization of sulfate reducers that incompletely oxidize the substrate to acetate; the phylogenetic analysis of the cloned 16S rDNA gene sequences showed high identity to the genus *Desulfovibrio* that oxidizes the substrates incompletely. In contrast, in the granular sludge used as inoculum a considerable number of clones showed homology to *Methanobacterium* and just few clones were close to SRB. The starting-up approach allowed the enrichment of SRB within the diverse community developed over the polyethylene support.

Keywords Anaerobic activity · Biofilm · Fluidized bed reactor · Sulfate reduction · 16S rDNA

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Introduction

Biological fluidized bed reactors are based on the development of biofilms over particles that support bacterial growth, this allows to retain the biomass within the reactor and therefore to operate at short hydraulic retention times (HRT). Sulfate reduction has been carried out satisfactorily in fluidized bed reactors, in this anaerobic process sulfate is reduced to sulfide by the action of sulfate-reducing bacteria (SRB) which have the ability of coupling the oxidation of organic matter (electron donor) to the reduction of sulfate (electron acceptor) and depend on hydrolytic and fermentative bacteria that degrade complex organic matter [33].

Among the different fluidized bed reactors configurations, the down-flow fluidized bed reactor (DFFB) is an appropriate alternative for the recovery of solid products that could be formed during sulfate reduction, i.e., metal precipitates or elemental sulfur [14]. In this type of reactors a carrier support floats at the top of a liquid column and the support is fluidized by means of down-flow liquid

recirculation. After inoculation, a biofilm develops over the support that remains at the top and maintains the precipitate separated from the biomass, which does not occur in conventional fluidized bed reactors because the carrier material remains at the bottom of the reactor.

Most of the times, fluidized anaerobic reactors used for sulfate reduction are inoculated with undefined consortia of anaerobic microorganisms and thus it is desirable that a population of SRB develops in the biofilm in order to produce sulfide. Several authors had pointed out that the composition of the microbial community influences the stability and performance of anaerobic reactors, hence the importance of understanding such communities as well as the interactions within, for the effective operation and improvement of the reactor's performance [10, 11, 18]. These microbial interactions are particularly important in biofilm reactors, and are greatly influenced by the ability of microorganisms to attach to the support at the operational conditions applied to the reactor. The substrate, HRT, temperature, type of support or carrier material and source of inoculum, among others are the main conditions that influence biofilm formation [13]. For example, Silva et al. [30] evaluated the adhesion of SRB and methanogens on polyurethane foam, vegetal carbon, low-density polyethylene and alumina-based ceramics in a horizontal flow reactor, and found that in each material a different microbial equilibrium was reached, and thus the performance of the reactor was different with each support. On the other hand, the start-up phase of fluidized bed reactors dictates the type of bacteria forming the biofilm on the support; moreover, if the inoculum is granular sludge, which is a consortium of microbial populations. The biofilm developed over the carrier material becomes important in fluidized reactors; therefore, its microbial characterization would help to achieve an effective start-up, reliable operation and eventually the scale-up of the reactor.

SRB are present in three distantly related groups: Gram-positive bacteria, Gram-negative mesophilic bacteria (*Proteobacteria)* and thermophilic SRB in the archaeal domain [16]. Previous methods of understanding bacterial community structure were limited to culturing methods; the major drawback in culture-based techniques is that only 0.3% of the total cells in certain environments observed microscopically are culturable using traditional microbiological techniques in the laboratory [5]. In contrast, the use of nucleic acid techniques (culture-independent approach) based on the 16S rDNA sequences analysis, allows for the specific identification of phylogenetic groups in a particular environment [7].

The study of sulfate-reducing biofilms is taking relevance, on the one hand biofilms of SRB present in pipelines have been related to biocorrosion [25]; on the other hand, in the case of effluent treatment, biofilms of SRB have been used for the precipitation of metals [19]. In the present study the objective was to evaluate the efficiency of the sulfate-reducing biofilm developed during the start-up phase of a DFFB reactor. The determination of the specific sulfate-reducing activity and the use of molecular and biochemical methods was the approach used to characterize the occurring biofilm and contrast it from the granular sludge used as inoculum. It was expected that under the operational conditions applied a complex SRB community would develop over the support, which was confirmed by DNA analysis.

Materials and methods

DFFB reactor start-up

Inoculum

The original inoculum (granular sludge) was obtained from a full scale UASB reactor treating wastewater of paper mill (Industriewater Eerbeek B.V., Eerbeek, The Netherlands). This granular sludge was maintained under sulfate-reducing conditions in a laboratory scale UASB reactor for 135 days. The sludge contained 0.11 g volatile suspended solids (VSS) per gram of sludge (wet weight). The DFFB reactor was inoculated with 12 mL of granular sludge from the laboratory scale sulfate-reducing UASB reactor, equivalent to 1.15 g VSS; prior inoculation the granular sludge was disintegrated by passing it trough a syringe needle.

Culture medium

The mineral medium used in the start-up and continuous operation of the reactor contained the following (mg/L): NH_4Cl 300, $MgCl_2{\cdot}6H_2O$ 120, KH_2PO_4 200, KCl 250, $CaCl_2{\cdot}2H_2O$ 15, yeast extract 20 and 1 mL of micronutrients solution [36]. This medium was supplemented in the beginning with a mixture of lactate–ethanol to enrich the SRB population; afterward ethanol was the only substrate. According to the desired chemical oxygen demand (COD), sulfate (SO_4^{2-}) was also supplemented as Na_2SO_4 to maintain a COD/SO_4^{2-} ratio of 0.6 (g/g) during reactor operation as shown in Table 1. The pH of the medium was adjusted to 6.5 with sodium bicarbonate.

Reactor start-up

The experiment was done in one Plexiglas reactor of 2.5 L of volume (inner diameter 5.5 cm, height/diameter of 16.7), a conical device served as solid–liquid separator and water level adjustor, whereas a flow equalizer reduced the

Table 1 Operational parameters of the continuous down-flow fluidized bed reactor

Period	I	II	III
Day (d)	47–65	66–76	77–131
Influent COD (g/L)	1	1	2
Ethanol:Lactate	1:1	1:0	1:0
Organic loading rate (g COD/L-d)	0.5	0.5	1
Sulfate loading rate (g SO_4^{2-}/L-d)	0.83	0.83	1.66
Recirculation flow (mL/min)	500	550	550

pulses of the peristaltic pump (Fig. 1). The carrier material consisted of 500 mL of fine ground low-density polyethylene particles (500–1,000 μm) with apparent density of 400 kg/m^3 dry weight. The reactor was filled up with culture medium, supplemented with sodium sulfate and a mixture of lactate:ethanol (3:2 COD basis) to yield 2.5 g COD/L and operated in batch mode with a recirculation flow of 500 mL/min for 45 days, the COD/SO_4^{2-} ratio was kept at 0.6 (g/g). During this period, the total liquid volume of the reactor was replenished every 48 h by fresh medium containing the amount of COD and sulfate mentioned before; the superficial velocity (U_s) was 12.6 m/h and several samples of the carrier material were withdrawn from the reactor for volatile solids determination.

Continuous operation

Once the biofilm was developed over the carrier material, the reactor was operated in continuous mode according to the operational conditions shown in Table 1 at a constant HRT of 2 days. The superficial velocity was controlled by means of the recirculation flow to maintain a bed expansion around 25% of reactor volume. COD removal and sulfate reduction were calculated as the difference between the influent and effluent COD and sulfate concentrations, respectively. Reactor was operated at ambient temperature 25 ± 3°C.

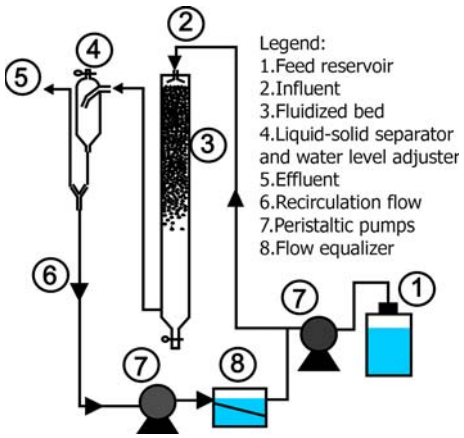

Fig. 1 Scheme of the down-flow fluidized bed reactor

Sulfate-reducing and methanogenic-specific activities

All assays were carried out in triplicate, in serum bottles sealed with rubber stoppers and aluminum crimps and were incubated in an orbital shaker (100 rpm) at 30°C.

Basal mineral medium

The basal mineral medium used for the specific activities contained (mg/L): NH_4Cl 280, $MgCl_2{\cdot}6H_2O$ 120, K_2HPO_4 600, $NaH_2PO_4{\cdot}H_2O$ 703, $CaCl_2{\cdot}2H_2O$ 6, yeast extract 20 and $NaHCO_3$ 2,000.

Sulfate-reducing specific activity (SRSA)

The sulfate-reducing specific activity was determined in 70 mL serum bottles using ethanol, lactate or acetate as individual substrates at 1 g COD/L, 1.5 g SO_4^{2-}/L was added to the basal mineral medium to obtain a COD/SO_4^{2-} ratio of 0.6 (g/g), the pH was adjusted to 6.5 with sodium bicarbonate. The bottles contained 5 mL of carrier material recently withdrawn from the reactor and 65 mL of basal mineral medium (no headspace) supplemented with sulfate and 1 g COD/L as lactate or acetate. Sulfide was determined every hour by a colorimetric method.

Methanogenic specific activity

The methanogenic activity was assayed in 60 mL serum bottles with 40 mL of basal mineral medium supplemented with acetate (1 g COD/L) and 1 mL/L of micronutrients solution [36]. The serum bottles also contained 5 mL of carrier material with the attached biofilm, recently withdrawn from the reactor. The pH was adjusted to 7.0 with sodium bicarbonate. Bottles were flushed with N_2 (99%) for 3 min. Methane production was measured every hour by displacement of a 3% NaOH solution.

The maximum specific methanogenic and sulfate-reducing activities were calculated from the slope of the cumulative methane production versus time or the sulfide concentration versus time, respectively, as the mean value of the triplicates and the immobilized volatile solids (IVS).

Biochemical analysis of biofilms

The biochemical composition of microbial biofilms associated with the polyethylene support and that of the granular sludge used as inoculum was assessed in terms of total cell protein and polysaccharides as previously reported [1]. Although the extracellular matrix of the biofilms

may contain proteins [17], the protein content of the biofilm can be employed as a reliable indirect indicator of biofilm cell concentration [1, 22]. After washing the biofilmed support carefully with distilled water, the attached microbial biomass was recovered by placing a known amount of support in distilled water for ultra-sound treatment for 5 min (Sonication-Vibra-Cell, Sonics Materials Inc., USA). Once the polyethylene support was removed, the released biomass was then used to determine total cellular protein using the Lowry modified method (SIGMA-Protein Assay Kit P5656) with bovine serum albumin as standard. The exopolysaccharide content was assessed using the phenol–sulfuric method with a glucose:mannose standard (1:1 ratio on a mole basis).

DNA extraction, cloning and sequencing

Samples of the granular sludge used as inoculum, the biofilm developed over the plastic support and the liquid in the reactor were collected in sterile conical tubes, after 107 days of operation. To identify a high number of species present in the consortium DNA was extracted using three different protocols. A method using liquid N_2 and sand modified from Xu et al. [35], a bead beating procedure (Ultra Clean, MoBio Laboratories, USA) following the manufacturer's instructions, and a high proteases concentration procedure modified from Wisotzkey et al. [34]. The protocols had been tested in our laboratory for Bacteria and Archaea present in soil both in moderate and extreme conditions (unpublished data). Briefly, the modified method from Wisotzkey uses 3 mL of sample which is centrifuged at 3500×*g* for 15 min and the pellet is re-suspended in EDTA (0.15 M NaCl, 0.01 M EDTA, pH 8.0). Then 10 μg/μL of lysozyme and 20 mg/mL of proteinase K are added and incubated 1 h at 37°C. Subsequently, 10% SDS is added and washes with 24:1 chloroform/isoamylic alcohol are employed. Finally, DNA is precipitated in ethanol.

PCR targeting 16S rRNA genes of all bacteria and archaea was performed using universal primers 533F (5′-GTGCCAGCMGCCGCGGTAA-3′) designed for Bacteria [9] and 1391R (5′-GACGGGCGGTGTGTRCA-3′) for Archaea [7]. All PCR mixtures (25 μL) contained: 1.25 units of Go *Taq* Flexi DNA polymerase (Promega Corp., USA), 2.5 μL *Taq* 10× buffer pH 8.5, 2.5 mM $MgCl_2$, 200 μM of each dNTP (Bioline Ltd, UK), 0.9 μM of each primer and 1 μL of DNA template. The reaction mixture was amplified using an automated Techne-Touchgene Gradient thermal cycler (Barloworld Scientific Ltd, UK) as follows: one denaturation step at 94°C for 3 min and 35 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s, extension at 72°C for 1 min, and a final extension step at 72°C for 7 min. PCR products purification was done using QIAquick Spin PCR purification kit (Qiagen Inc., USA) following the manufacturer's instructions. The size and amount of PCR products were estimated by 1% agarose gel electrophoresis stained with ethidium bromide (0.5 μg/mL) and by fluorometry, respectively.

The 16S rDNA purified amplicons of 880–900 bp were ligated into pGEM-t easy cloning vector (Promega) and transformed by thermal shock of competent *E. coli* TOP-10F'cells. At least ten transformed colonies were selected for inoculation in 3 mL LB medium containing 100 mg/L carbeniciline. After 12 h incubation at 37°C, plasmidic DNA was purified from the liquid culture by minipreps using the method of Birnboim and Dolly [8]. Plasmidic DNA was visualized by agarose gel electrophoresis to verify the size of the inserts and was quantified by fluorometry in a GENios Fluorometer (Tecan Trading AG, Switzerland). The sequencing was performed by fluorescent dye labeled dideoxynucleotides method in DNA automated sequencer ABI Prism Model 377 r (Applied Biosystems, USA) operated by CINVESTAV-Irapuato, Mexico. The obtained sequences were aligned using the Mega 4 program together with the closest 16S rDNA sequences available at the GenBank database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) using the Neighbor-Joining Method with bootstrap with 500 replicates and the Kimura 2-parameter model. The related sequences were retrieved by nucleotide BLAST (basic local alignment search tool) [2].

Nomenclature

Letter G, P or L corresponds to the origin of DNA: granular sludge, the biofilm attached to the plastic support or liquid of the reactor, respectively. The DNA extraction method was denoted as: E, for the method with liquid N_2 [35]; M, for the MoBio kit and W for the method modified from Wisotzkey et al. [34]; the number indicates the clone number.

Analytical methods

Chemical oxygen demand was determined by the closed reflux method according to standard methods [6]. Before COD determination, the sulfide present in the effluent samples was removed by adding a drop of concentrated H_2SO_4 to 15 mL of effluent, and flushing the sample during 10–15 min with air. Sulfide concentration in the reactor effluent was determined by the iodimetric method according to standard methods [6] using thiosulfate and starch as indicator. In the sulfate-reducing activities sulfide was determined by the colorimetric method described by Cord-Ruwisch [15]. Sulfate was determined according to the standard gravimetric method [6]. Acetate was determined by FID gas chromatography after acidification of the

sample with 50% HCl, the sample was filtered through a 0.22 μm mixed cellulose esters membrane syringe filter. The Agilent Technologies gas chromatograph (6890 N, Agilent Technologies Inc., USA) was fitted with a D8-FAP capillary column (Alltech Associates Inc., USA), the temperature of the injection port and the detector was 250 and 140°C, respectively. A temperature ramp was applied to the column starting at 100°C and increasing 10°C per minute up to 140°C, the carrier gas was nitrogen at 40 mL/min. VSS in the granular sludge were analyzed according to standard methods [6]. The solids attached to the support are reported as IVS per volume of support (g IVS/L_s), the biofilm was detached with successive 10 min washes using an ultrasonic bath, each 10 mL wash was analyzed for VSS according to standard methods [6]. Alkalinity was determined according to standard methods [6]. Scanning electron microscopy was used to study the development and structure of the anaerobic biofilms, biomass examination was performed in a scanning microscope (XL-30, Philips Electronics B.V., The Netherlands). The sample was mounted in an aluminum pin and sputtered with carbon. The micrographs were scanned with a retro dispersive electron detector at 10 kV.

Results

Reactor performance

The reactor start-up was done in two stages, in batch mode for 45 days and afterward in continuous mode from days 46 to 131. The establishment of the biofilm in a fluidized bed reactor is of great importance; therefore, special attention was paid to the amount of attached biomass during reactor operation. The biomass attached to the support was quantified at days 14, 33, 54, 82 and 105 (Table 2). The down-flow superficial velocity was modified to obtain a bed expansion around 25% and avoid the formation of dead zones. By day 14 the biomass attached to the support amounted to 1.29 (g IVS/L_s) and the bed expansion was 22% of the reactor volume. The biofilm at this time was incipient because it detached very easily from the support, this could be observed by the unaided eye. At day 33, the biomass attached to the support was 0.8 g IVS/L_S, the reduction was due to the increment in the U_s from 10 to 15.2 m/h, and the consequent bed expansion to 26%. Just before the continuous operation (day 43), 1.15 g VSS of granular sludge was added to the reactor; with this it was expected to achieve a higher amount of biomass attached to the biofilm. At day 54 the down-flow superficial velocity was reduced to 12.6 m/h and the biomass concentration amounted to 0.94 gIVS/L_S, the bed expansion was 24% and some fractionation of the bed gradually occurred. From day 82 onward, the down-flow superficial velocity was increased to 13.9 m/h that promoted a uniform bed fluidization, the biomass decreased to 0.7 g IVS/L_S and the bed expansion amounted to 25% of reactor's volume. At day 105 the attached biomass remained almost the same, 0.76 g IVS/L_S, which indicated that the biofilm reached a mature state.

Table 2 Changes in the attached biomass and percentage of bed expansion as a function of the superficial velocity at different days of reactor operation

Time (days)	Superficial velocity (m/h)	Attached biomass (g IVS/Ls)	Bed expansion (%)
14	10	1.29	22
33	15.2	0.8	26
54	12.6	0.94	24
82	13.9	0.7	25
105	13.9	0.76	25

Continuous operation started at day 46 and lasted 84 days, with an initial organic loading rate of 0.5 g COD/L-d using ethanol and lactate as substrates (Table 1). During the first 20 days (period I), COD removal efficiency amounted to 64% and the sulfate conversion efficiency was 3.9% (Fig. 2a), these results indicated that only a small fraction of COD (6.1%) was removed by sulfate reduction. From day 66 onward the reactor was fed only with ethanol as substrate, the COD removal efficiency increased to 71.8% (day 71) and remained in this value until the end of period II (Fig. 2a). Sulfate conversion efficiency increased to 21.8% and the COD removed via sulfate reduction amounted to 30.4%.

From day 77 onward (period III) the loading rate was increased to 1 g COD/L-d at the same HRT of 2 days. At these conditions the COD removal decreased to 60%, whereas the sulfate reduction efficiency was up to 28.1% to yield a COD removal by SRB of 47%. During this period the immobilized solids concentration decreased to 0.7 g IVS/L_S compared with periods I and II, where the attached biomass amounted to 0.94 g IVS/L_S. Acetate was detected in the effluent during all the continuous operation; this was mainly due to an incomplete oxidation of lactate and ethanol, and to the participation of other metabolic groups, such as fermenters or acetogens. The acetate production during periods I and II was 0.1 g COD/L-d and in period III was 0.18 g COD/L-d.

The pH of the influent was 6.5 and the effluent pH increased to around 7.7 during periods I and II (Fig. 2b), in period III the effluent pH decreased up to 6.7. Concerning alkalinity a gradual decrease from 3,400 mg $CaCO_3$/L, at the beginning of continuous operation, to around 500 mg $CaCO_3$/L at day 77 was observed. Toward the end of the experiment the alkalinity increased to 1,125 mg $CaCO_3$/L

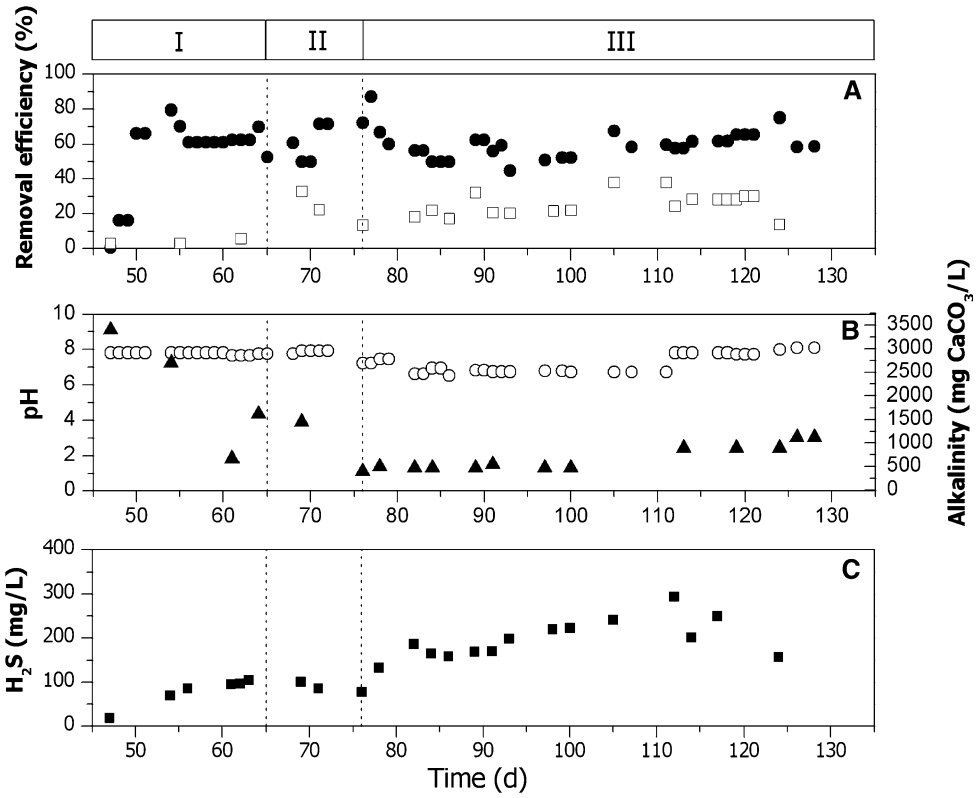

Fig. 2 Performance of the down-flow fluidized bed reactor: **a)** COD (●) and sulfate removal (□) efficiencies; **b)** pH (○) and alkalinity in mg $CaCO_3$/L (▲); **c)** sulfide concentration (mg/L)

(Fig. 2b). Sulfide increased gradually from 69 mg/L in the middle of period I up to 293 mg/L at day 112; however, at the end of the operation sulfide concentration dropped to 154 mg/L (Fig. 2c), which corresponded to the decrease in COD and sulfate removal efficiencies at day 124 of reactor operation.

Biofilm development and sulfate-reducing specific activities

The SEM images of the clean support revealed an irregular non-porous surface suitable for biofilm attachment (Fig. 3a). After 60 days of inoculation (Fig. 3b), the microbial colonization predominated in the irregular zones of the carrier and some open spaces were observed; cocci and vibrio shaped cells predominated. After 90 days the support was highly colonized (Fig. 3c) although some open spaces were still visible, the images also showed spider web-like strands of presumably exopolymeric substances responsible of bacterial adhesion. In experiments done with spherical particles it was reported a homogeneous biofilm formation over the particles [12], in contrast to this study where a non-homogenous colonization of the carrier was found mainly due to the irregular surface of the carrier.

On the other hand, the biochemical analysis of the biofilm allowed assessing, up to a certain extent, the abundance (biomass) of microbial populations attached to the support and that of the granular sludge used as inoculum. As expected, the granular sludge contained a much higher biomass (protein levels), between 50 and 500 times more abundant than those detected and associated with the polyethylene support (Table 3). In addition, different amounts of biomass were recorded in the biofilms under different nutritional regimes, the microbial communities grown in a batch fed with ethanol as sole carbon and energy source being almost two times more abundant than populations under acetate treatment, as the cellular protein and polysaccharide content showed (Table 3).

Additionally, the metabolic characteristic of the biofilm was followed by the SRSA determined at different time during reactor operation (Fig. 4). With ethanol as substrate, the SRSA increased gradually during batch operation (days 14 and 33), when the reactor was operated in continuous mode the SRSA remained fairly constant around 12 g COD-H_2S/g IVS-d. With lactate as substrate, it was observed a substantial increase in the activity from 2 g COD-H_2S/g IVS-d (day 14) to 15.32 g COD-H_2S/g IVS-d (day 33), and then a gradual decrease during continuous operation (days 54–105) possibly due to the elimination of lactate from the influent. With acetate as substrate a slight increase in the SRSA with time was found, after 14 days of inoculation its value was close to zero and reached 3 g COD-H_2S/g IVS-d at day 105, thus sulfate-reducing activity with acetate as substrate was observed. On the other hand, no methanogenic activity was detected in batch assays with acetate as substrate.

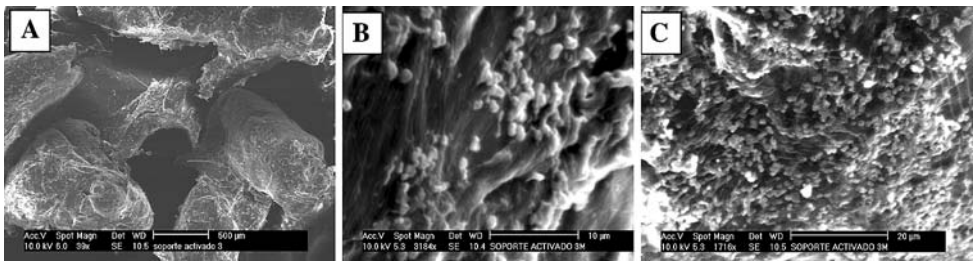

Fig. 3 Electron micrographs of the clean support **(a)** and the attached biomass after 60 **(b)** and 90 days of reactor operation **(c)**

Table 3 Biochemical characterization of the biofilm and the granular sludge

± standard deviation for triplicate measurements

Type of aggregate	Cellular protein μg/g dry weight	Polysaccharide	Protein/polysaccharide ratio
Biofilm Ethanol-fed	900 ± 224	403 ± 29	2.2 ± 0.4
Biofilm acetate-fed	517 ± 17	277 ± 29	1.9 ± 0.3
Granular sludge	46,006 ± 816	24,803 ± 125	1.8 ± 0.1

Phylogenetic diversity disclosed by analysis of 16S rRNA genes of the granular sludge (inoculum) and the biofilm

From the three DNA extraction methods, the method modified from Witzotzkey et al. [34] allowed the identification of a major number of genera, both in the granular sludge and in the biofilm; the concentration of DNA extracted from the granular sludge was 62.2 ng/μL. On the other hand using the modified method from Xu et al. [35] the DNA concentration was 56.7 ng/μL. As for the DNA extraction from the biofilm attached to the support, it was obtained almost the same concentration of DNA (50.9 and 49.6 ng/μL) using the methods modified from Xu et al. [35] and that of Wisotzkey et al. [34], respectively. The MoBio kit method rendered the lowest DNA concentration (17 ng/μL).

Most of the clones analyzed from the granular sludge (original inoculum), showed sequences affiliated to *Methanobacterium* genus, whereas the bacteria attached to the plastic support showed a high homology to SRB (*Desulfovibrio* and *Desulfomicrobium*), which was in concordance with the performance of the reactor. The granular sludge (G) produced more positive clones than the samples obtained from the liquid from the reactor (L) and the biofilms attached to the plastic (P), as shown in Fig. 5, which correlates with the net accumulation of biofilm biomass showed in Table 3. The majority of the clones obtained from the granular sludge GM7, GE6, GE3, GW10, GE9, GW5, GW1, and GE2 showed high nucleotide identity to *Methanobacterium beijingense* (AY350742). Two clones, GM8 and GM1, showed 99% identity to *Methanotrix soehngenii* (X51423). Only one clone (GM5) from the granular sludge was 99% similar to *Desulfomicrobium baculatum* (AJ277896).

In the liquid of the reactor two clones (LW7, LW3) were affiliated to SRB, the closest cultured relatives were

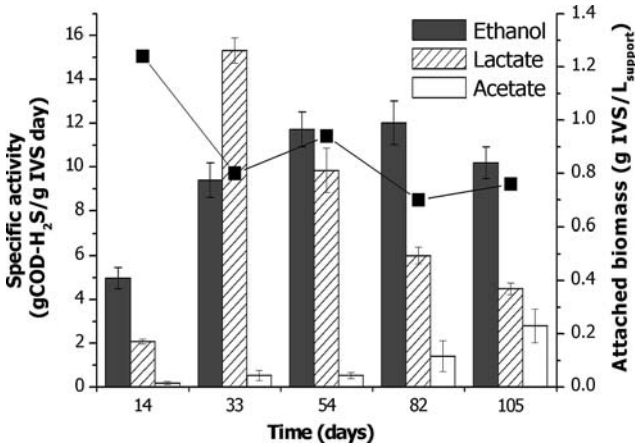

Fig. 4 Profile of the quantity of biomass adhered to the support during time of reactor operation measured as immobilized volatile solids per liter of support (■), and the specific sulfate-reducing activity of the biofilm with ethanol, lactate or acetate as substrates

Desulfomicrobium sp (AF443593) and *Desulfovibrio desulfuricans* (DQ417602), respectively. On the other hand, clone LW4 showed 99% of nucleotide identity to *Chloroflexi* (green non-sulfur bacteria, AY903656).

Within the clones obtained from the biomass attached to the plastic support it was found that most of the clones (PW9, PW10, PM4, PW7, PW3) were affiliated to *Desulfovibrio*. The clone PM9 was 99% similar to *Acinetobacter johnsonii* (EU236748), which was also found in the liquid of the reactor (clone LW6). Clone PW1 was related to *Acholeplasma polakii* (AF031479) with 98% identity, which is a fermentative bacteria found in organic wastes. Clone PM5 showed 93% homology to *Clostridium sp* (DQ852338). Some clones were related to the archaeal domain of hydrogenotrophic *Methanobacterium* (PM10, PW12, PW14 and PW17) showing 99% homology to *Methanobaterium beijingense*.

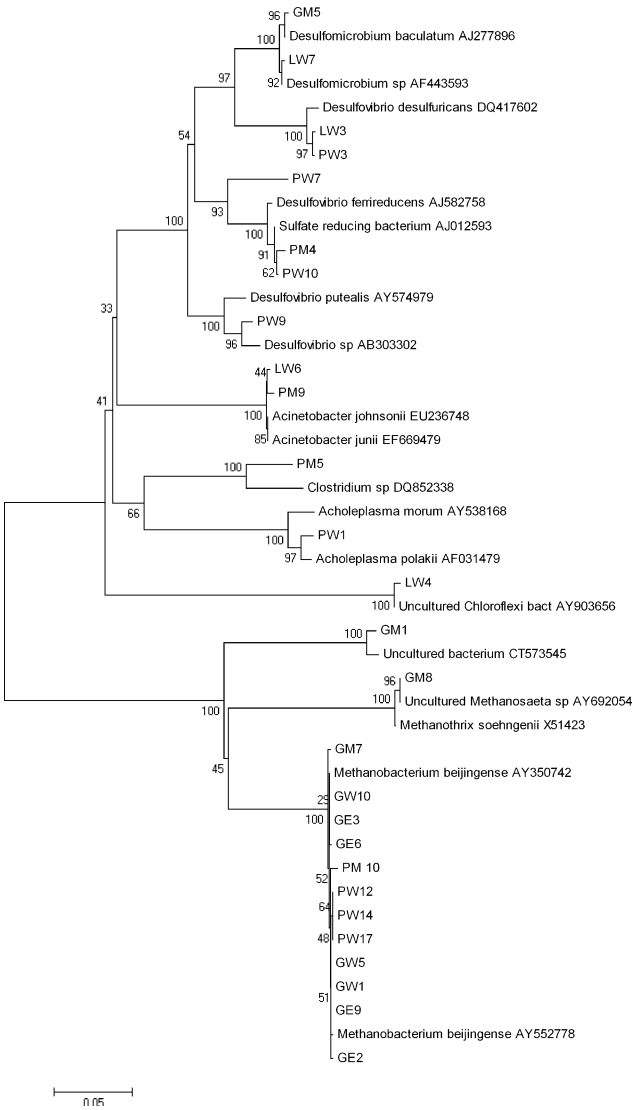

Fig. 5 Phylogenetic tree of 16S rDNA partial sequences of clones isolated from granular sludge, plastic, and liquid present in the DFFB reactor. The microorganisms with highest identity are shown in the closest branch including their GenBank accession number. The tree was generated by the Neighbor-Joining method, analyzing 890–900 bp sequences. The substitutions number per site is represented in the scale. A Bootstrap of 100 shows the highest confidence of the branch group formation. Data lower than 40 are not shown. G = granular sludge; P = Biofilm attached to the plastic support; L = Liquid of the reactor; W = Wisotzkey et al. [34]; E = Xu et al. [35]; M = MoBio; number = clone number

Discussion

The operation of the reactor at low superficial liquid velocity (10 m/h) yielded a higher amount of attached biomass to the plastic support, measured as immobilized volatile solids; however, the bed expansion was only 22% of reactor volume and the formation of two zones and channels within the bed was observed. The upper part of the bed was of low fluidization where possibly a higher accumulation of biomass may occurred because of the grey color the support acquired, compared to the high fluidized zone where the support remained almost white. Concerning the channels, most probably their formation was due to the low superficial velocity that caused a low bed expansion, as was proposed by Meraz et al. [26], the bed fractionates because of the dynamic friction of the particles due to their irregular shape. Therefore the superficial liquid velocity was increased to 15.2 m/h, to achieve a uniform fluidization of the support, but this increase contributed to detach some of the biomass, there was a compromise between fluidization of the support and solids detachment that was reflected in the amount of attached biomass. Between 12.6 and 13.9 m/h no formation of channels were observed, the bed expansion amounted to 24–25% and attached biomass remained around 0.7 g IVS/L_s. Ohashi and Harada [28] and Kwok et al. [21] also found a reduction in the biomass attached to the support as the superficial velocity increased and proposed that high shear forces as result of the high superficial liquid velocities promote a thin biofilm. On the other hand, the results presented here are in accordance with the findings of Kaksonen et al. [19] who concluded that a sulfate-reducing fluidized bed reactor could not be operated at superficial velocities higher than 14 m/h due to the negative effect caused by the biofilm detachment.

Sulfate and COD removal

The increase of the sulfate-reducing efficiency in a sulfidogenic reactor has been subject of study since sulfate reduction was accepted as an alternative for sulfate removal, COD removal or both. In the present study, the objective was to promote sulfate reduction by means of selecting a sulfate-reducing population over a support. The time required to increase the COD removed by SRB from 6.1 to 47.1% was about 70 days at a COD/SO_4^{2-} ratio of 0.6, in comparison it took about 100 days to increase the COD consumed by SRB to 21% in a UASB reactor [32]. Thus the DFFBR under the conditions applied favored the enrichment of a SRB population, as was confirmed by the 16S rDNA analysis. On the other hand, in the experiment of Kaksonen et al. [19] sulfate-reducing efficiencies increased from 20 to 85% in 120 days when the HRT was lowered from 24 to 16 h with lactate, using silicate mineral as support in a fluidized bed reactor. Alvarez et al. [3] using a bed-packed reactor with pumice stone, achieved 23% of sulfate removal and 31% lactate removal at the stoichiometric COD/SO_4^{2-} ratio of 0.66 in a period of 75 days at a HRT of 4.1 days. In the present work and in the works of Kaksonen et al. [19] and Alvarez et al. [3] the inoculum was sludge previously subjected to sulfate-reducing conditions, which points out that other factors may affect the development of the sulfate-reducing biofilm besides the

origin of the inoculum, and from the information in the above mentioned works (including this) the HRT may be playing and important role. The HRT used by Kaksonen et al. [19] was less than 24 h and the sulfate-reducing efficiency was up to 85%, whereas in the present work and in the work of Alvarez et al. [3], the HRT was higher than 24 h and the sulfate reduction efficiency achieved was not over 40%. The reason why the reactor was operated at a high HRT was to enhance the time of contact between the microorganisms and the support with the expected result of increasing the biofilm development; however, from the results it seems that this approach was not appropriate in terms of achieving high sulfate-reducing efficiencies in continuous operation.

Concerning total COD removal efficiency the DFFB reactor showed a low removal (60–70%), compared to reactors in which methanogenesis also occurs. Visser et al. [32] reported that a great part of the COD was removed via methanogenesis that lead to a total COD removal efficiency of 99%, in spite of having an excess of sulfate methanogens predominated over sulfate reducers, thus the COD removed via sulfate reduction amounted only to 20%. In the present study, we did not observe methanogenic activity in the biofilm, thus most probably acetogenic bacteria besides sulfate reducers consumed the COD also. In addition, the effluent contained acetate, which points out to an incomplete oxidation of lactate or ethanol by SRB. Many authors have reported the accumulation of acetate in several types of reactors operated under sulfate-reducing conditions, and the majority agrees that acetate is the bottleneck of sulfate-reducing processes (when methanogenesis is negligible). In an upflow staged sludge bed reactor inoculated with a mixture of fully sulfidogenic and acetate-consuming granular sludge, after 138 days of continuous operation only a minor increase in the sulfate-reducing activity with acetate as substrate (13.5%) was detected in the sludge and a substantial methanogenic population remained in the reactor [24]. Moreover, no significant acetate consumption was seen in a fluidized bed reactor inoculated with a pure culture mixture in which *Desulfobacter postgatei*, a SRB that completely oxidizes the substrate to sulfide and CO_2, was present [27]. The major drawback of having acetate accumulation in a sulfate-reducing reactor due to the incomplete oxidation of the substrate is the low COD removal efficiency and alkalinity production. Alkalinity was an important parameter in this study because the immediate application of this reactor would be the treatment of an acidic synthetic wastewater (pH around 5), thus it would be necessary to have some alkalinity for buffering an acidic pH. The incomplete oxidation of ethanol to acetate does not yield CO_2, in the present study more likely due to the operational conditions applied mostly SRB that incompletely oxidized the substrate were able to growth on the support, in contrast to granular sludge reactors where the population change is slow and an important fraction of the COD is still consumed via methanogenesis. Lens et al. [24] reported for a staged bed reactor, in which a fraction of the inoculum was acetate-consuming sulfate-reducing granular sludge, that around 20–30% of the COD was consumed via methanogenesis, thus the total COD removal efficiency reported by them is as high as 95%.

Due to the lack of information concerning the establishment of "mature" biofilms in the type of support that was used, the reactor was empirically operated in batch mode for 45 days. In the first days of operation the first change in the support was appreciable by the unaided eye, the color of the support changed from white to grey and electronic microscope observations confirmed the presence of bacteria over the support, although the support coverage was not uniform and the biofilm detached very easily from the support.

Concerning the biochemical analysis, the differences in the levels of biomass may be explained in terms of the nutritional value of ethanol as better substrate than acetate to support biofilm communities under these conditions. The net accumulation of biofilm biomass on a support results from microbial growth and detachment due to hydrodynamic stress in the reactor and cell death [31]. The cellular protein/polysaccharide ratio provides a preliminary assessment of the physiological state of the surface-associated microorganisms within these biofilms and aggregates, as it relates to bioadhesion [1]. The results (Table 3) showed a remarkable steady ratio around 2, irrespective of the amount of biomass, indicating that there was not a concomitant increase due to the nutritional regime (ethanol vs. acetate). This ratio has been shown to increase in denitrifying biofilms, as a function of phosphorous enrichment [4], suggesting that limitation of nutrients may stimulate polysaccharide biosynthesis as adaptive strategy within the community.

From the results of the activity assays, it is clear that SRB were present within the microorganisms forming the biofilm (Fig. 4). The early biofilm developed 14 days after inoculation showed higher activity with ethanol than with lactate and almost not sulfate-reducing activity was detected with acetate as substrate. The decrease observed in the SRSA with lactate as substrate could be due to the substrate change in the feed, because after day 66 the reactor was not fed with lactate anymore. The utilization of acetate is the limiting step in sulfate-reducing reactors [24] that is why the SRSA was determined also with acetate as substrate. It was interesting to find out that the SRSA with acetate had a substantial increase about 150% in 90 days, compared with the increase of the SRSA observed with acetate in a granular sludge reactor which was 13.5% in 138 days of reactor operation [24]; hence the DFFB reactor

favored the development of an acetotrophic sulfate-reducing population. The SRSA determined at day 105 with ethanol (10 g COD-H_2S/g IVS-d) was in accordance to the high sulfate removal (Fig. 2a), and the high sulfide concentration (Fig. 2c), observed in the reactor in period III of continuous operation. The high values of the SRSA resulted from the low concentration of IVS, and suggest that the DFFBR could achieve high organic loads with relatively low biomass content within the reactor, manly due to the sulfate-reducing characteristic of the biofilm. Moreover, we did not observe methanogenic activity during reactor operation, and this fact most probably allowed the development of the sulfate-reducing activity with acetate as substrate. On the other hand, the absence of methanogenic activity pointed out that with the start-up conditions mainly SRB were selected in the biofilm. Here is where the results of the phylogenetic analysis become important.

The analysis of the 16S rDNA confirmed the presence in the biofilm of mesophilic Gram-negative SRB within the delta subclass of *Proteobacteria*. The genus *Desulfovibrio* comprises sulfate reducers that can oxidize lactate and ethanol incompletely to acetate. *D. desulfuricans* is considered the type species of the genus and is widely found in several habitats including anaerobic reactors. All the SRB found in the clones are able to reduce sulfate and oxidize incompletely their substrates to acetate, which correlates with the acetate found in the effluent of the reactor, most probably these species participated actively in the process. It is notable the large number of archaeal sequences retrieved from the granular sludge used as inoculum whereas in the biofilm, sequences clustering with SRB were more abundant (Fig. 5). The methanogens found in the granular sludge represented two main groups, one is the acetoclastic *Methanosaeta* (*Methanothrix*) and the other is the hydrogen/formate-scavenging *Methanobacterium*, both methanogens are mesophilic and are commonly found in granular sludge of methanogenic bioreactors [29]. Some clones obtained from the biofilm DNA were associated to *Methanobacterium beijingense*, which uses hydrogen and CO_2 for methanogenesis, and it is not capable of using acetate, explaining the accumulation of acetate in the effluent.

A diversity of other non-sulfate-reducing facultative and obligate anaerobes were detected in the biofilm, supporting the model of the symbiotic/commensal nature of carbon and energy fluxes in a consortium, and may suggest the physiological capacity for a wide range of biotransformations by the stable microbial mixture attained in the biofilm. For instance, *Chloroflexi* is a class of Eubacteria that produce energy through photosynthesis, is facultative aerobic but does not produce oxygen during photosynthesis and the carbon fixation method is by photoheterotrophy, sequences associated with this phylum were also found in sulfate-reducing biofilms [20] and in granular sludge from UASB reactors [23]. Bacteria of the genus *Clostridium* are capable to use a variety of substrates under anaerobic conditions, and in the anaerobic digestion process some species may be considered as acetogens which may also explain the COD consumption with no sulfate reduction.

In conclusion, the starting-up approach used to enrich a SRB population resulted in the selection of a diverse community and although the community present in the biofilm showed high sulfate-reducing activities with ethanol, lactate and even acetate in batch tests, the community was not capable of reducing sulfate more than 30% or oxidize COD more than 70% in continuous mode. Future analyses of the bacterial load by real time PCR of the genera above mentioned, together with the physico-chemical follow-up during reactor operation, may help to better understand the specific role of each species within the consortium and their contribution on the ongoing processes within the reactor.

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