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ORIGINAL PAPER

Evaluation of the microbial diversity in a horizontal-flow anaerobic immobilized biomass reactor treating linear alkylbenzene sulfonate

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Abstract The purpose of this work was to assess the degradation of linear alkylbenzene sulfonate (LAS) in a horizontal-flow anaerobic immobilized biomass (HAIB) reactor. The reactor was filled with polyurethane foam where the sludge from a sanitary sewage treatment was immobilized. The hydraulic detention time (HDT) used in the experiments was of 12 h. The reactor was fed with synthetic substrate (410 mg l^{-1} of meat extract, 115 mg l^{-1} of starch, 80 mg l^{-1} of saccharose, 320 mg l^{-1} of sodium bicarbonate and 5 ml l^{-1} of salt solution) in the following stages of operation: SI—synthetic substrate, SII—synthetic substrate with 7 mg l^{-1} of LAS, SIII—synthetic substrate with 14 mg l^{-1} of LAS and SIV—synthetic substrate containing yeast extract (substituting meat extract) and 14 mg l^{-1} of LAS, without starch. At the end of the experiment (313 days) a degradation of $\sim$35% of LAS was achieved. The higher the concentration of LAS, the greater the amount of foam for its adsorption. This is necessary because the isotherm of LAS adsorption in the foam is linear for the studied concentrations (2 to 50 mg l^{-1}). Microscopic analyses of the biofilm revealed diverse microbial morphologies, while Denaturing Gradient Gel Eletrophoresis (DGGE) profiling showed variations in the population of total bacteria and sulphate-reducing bacteria (SRB). The 16S rRNA gene sequencing and phylogenetic analyses revealed that the members of the order Clostridiales were the major components of the bacterial community in the last reactor operation step.

Keywords Adsorption · Anaerobic digestion · Degradation · Linear alkylbenzene sulfonate · Polyurethane foam

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Introduction

Linear alkylbenzene sulfonate (LAS) is widely used in the surfactant formulation. LAS consist of a mixture of anionic surfactants whose surface active properties are formed by hydrophilic anionic sulfonate groups and hydrophobic alkyl chains attached to a benzene ring (Sanz et al. 2003).

Many studies about the biodegradation of LAS have shown that the surfactant is well biodegraded under a wide variety of aerobic conditions. LAS is strongly sorbed to aerobic sludge due to its hydrophobic character. The content of adsorbed LAS into the particles significantly influences their feasibility,

bioavailability and toxicity. The adsorption of LAS has been measured for a number of different types of solids, including marine sediments and soils (Garcia et al. 2002).

The non-treated wastewater with LAS can lead to the development of huge masses of foam in streams and rivers (Kertesz et al. 1994). For this reason, it is necessary to treat the wastewater before its discharge in the rivers. Further investigations about the behaviour of LAS in anaerobic conditions should be carried out.

The LAS degradation in anaerobic conditions has been recently studied. Many works have been done to confirm the complete biodegradability of LAS (Almendariz et al. 2001; Sanz et al. 2003; Mogensen et al. 2003; Lobner et al. 2005). Anaerobic degradation requires a consortium of bacteria that act on different parts of the molecule. For example, the biodegradation of LAS requires alteration of an alkyl chain, a benzene ring and a sulfonate linkage (Mogensen et al. 2003).

Stages of this degradation are still not fully defined, as its inhibitory concentration and the influence of intermediary compounds in the anaerobic microorganism growth. The knowledge about the degradation processes and the involved microbial community is not enough known. The LAS degradation is much more complex than previously speculated. LAS is not a single compound, but a typically mixture of 20 isomer compounds subterminally linked to a linear alkyl chain (Khleifat 2006).

Among various configurations of bioreactors that are used in the effluents treatment, the bench scale Upflow Anaerobic Sludge Blanket (UASB) has been the most frequently one applied in the anaerobic treatment of LAS (Almendariz et al. 2001; Sanz et al. 2003).

The biotechnological alternative related to the utilization of anaerobic reactors with biomass immobilized in polyurethane foam has been used successfully to treat several types of wastewater, e.g. water polluted containing phenol (Bolãnos et al. 2001), formaldehyde (Oliveira et al. 2004), pentachlorophenol (Saia et al. 2007) and BTEX (Cattony et al. 2005).

The main goal of this work was to evaluate the LAS removal in a horizontal anaerobic fixed-bed bioreactor inoculated with anaerobic biomass from a UASB reactor treating sanitary sewage. It was also studied the behaviour of LAS adsorption in the support material, and the characterization of the biofilm microbial community in different operation steps of the reactor using the PCR/Denaturing Gradient Gel Eletrophoresis (DGGE) technique. At the end of the operation, the microorganisms present in the biofilm were identified by 16S rRNA gene sequence analysis of a clone library.

Materials and methods

Adsorption experiments

To perform the determinations of LAS adsorption rate, it was necessary to verify the contact time to reach the equilibrium state between the polyurethane foams and LAS solution. For this purpose 0.64 g of the polyurethane foams were shaken (125 rpm and 30 ± 1°C) in flasks of 500 ml containing 200 ml of LAS solution (14 mg l^{-1}). Samples were taken at different time (15 min, 30 min, 1 h, 2.5 h, 5 h and 10 h) and the LAS concentration in the solution was determine. The experiments were performed by triplicate, used synthetic substrate (500 mg l^{-1} of yeast extract, 115 mg l^{-1} of starch, 80 mg l^{-1} of saccharose, 320 mg l^{-1} of sodium bicarbonate and 5 ml l^{-1} of salt solution) (data not shown).

With the determination of the contact time it was possible to calculate the amount of adsorbed LAS in the polyurethane foam and then estimate the necessary contact time for its saturation.

The experiments of adsorption isotherm had been carried after the assays of the contact time in the same conditions (shaken 125 rpm, temperature 30 ± 1°C). In this case, the same mass of support (0.64 g) it was placed in contact with synthetic substrate with different concentrations of surfactant. It was used concentrations of next LAS 2.6; 7.4; 20.7; 28.8; 31.9; 39.7 (mg l^{-1}) in triplicate. Samples of the solution were collected 12 h after the experiment to guarantee the saturation of the support media.

All of the adsorption experiments were carried out in aseptic conditions, with sterilization of the synthetic substrate and the polyurethane foams in autoclave for 20 min and 1 atm.

Horizontal-flow anaerobic immobilized biomass reactor

A horizontal-flow anaerobic immobilized biomass (HAIB) reactor was built of glass bore-silicate

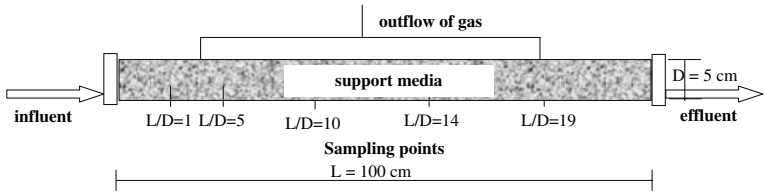

Fig. 1 Scheme of the bench-scale HAIB reactor

cylinder with a 100 cm length and 5 cm diameter, a length/diameter ratio L/D of 20, a total volume of 2,000 ml and liquid volume capacity of 800 ml (Fig. 1). The reactor contained five intermediate sampling ports along its length (L/D 1, 5, 10, 14 and 19). About 25 g of cubic polyurethane foam particles (5 mm in size and 23 kg m^{-3} apparent density) were used as the immobilized support for the microbial biomass with a bed porosity of 40%. The reactor was installed in a controlled temperature chamber 30 ± 2°C) and fed by a peristaltic pump. The anaerobic reactor was fed continuously for 313 days, with hydraulic detention time (HDT) of 12 h. The anaerobic sludge from a UASB reactor, applied in the treatment of the sanitary sewage at the University of São Paulo (São Carlos, Brazil), was used as inoculum.

Dodecylbenzene sulfonic acid sodium salt (Sigma, St. Louis, MO, USA) with 80% of purity was the anionic surfactant used in this work. The synthetic substrate used in the reactor feeding was prepared as described in the adsorption experiments. The substrate was kept refrigerated at 4°C during the feeding with mean COD of 750 mg l^{-1}. The organic loading rate applied was 0.6 gCOD l^{-1} day^{-1}.

The four experimental stages were defined by modifications in the composition of the synthetic substrate: Stage I (SI)—synthetic substrate; Stages II and III (SII and SIII)—synthetic substrate with addition of 7 and 14 mg l^{-1} of LAS, respectively; Stage (SIV)—synthetic substrate with 14 mg l^{-1} of LAS and yeast extract (500 mg l^{-1}, substituting meat extract), without starch. The values of pH varied from 7.2 to 7.5 in the influent during the experiment (Fig. 3).

Analytical methods

The HAIB reactor behaviour was accomplished by the following analyses: chemical oxygen demand (COD), sulphate concentration, solids concentration and pH (APHA 2000). Total volatile acids (TVA) as acetic acid and bicarbonate alkalinity as $CaCO_3$ was accomplished as described by Dillalo and Albertson (1961) and modified by Ripley et al. (1986). The specific concentration of acetic and propionic acids was determined by gas chromatography, using an HP 6890/FID chromatographer (flame ionization). An HP INNOWAX column was employed (30 m × 0.25 mm × 0.25 mm film thickness). Hydrogen was used as carrier gas at 2.0 ml min^{-1}.

The composition of biogas was monitored by gas chromatography, using a Gow Mac chromatographer equipped with a thermal conductivity detector (TCD) and a Porapak Q (2mx ¼ in. 80 to 100 mesh) column. Hydrogen was used as carrier gas at 60 ml min^{-1}.

The LAS was accomplished by HPLC using a fluorescence detector—C8 column with an eluting gradient using methanol and sodium perchlorate (0.075 mol l^{-1}), flux of 0.5 ml min^{-1} and temperature of 35°C (Duarte et al. 2006). LAS concentration was periodically measured in the liquid phase (influent and effluent). The final mass balance of LAS in the system was calculated based in the quantification of adsorbed LAS from the biomass and support media that were removed from the reactor at the end of the experiment to quantify the total solids.

The samples (triplicate) were extracted with methanol in ultrasound bath for 30 min in order to measure the adsorbed and precipitated LAS present in the biofilm. Then, the methanol samples were filtered in ionic exchange columns (SAX) and C-18 and analysed by HLPC. This protocol of extraction presented 85% of efficiency.

The toluene and benzene concentration were determined at the end the experiment, by a static headspace gas chromatographic (HSGC) method. GC was performed on a 6,869 HP gas chromatograph, with a 30 m × 0.53 mm i.d. HP-1, film thickness of 2.64 μm (cross-linked methylsilane) fused-silica capillary column, equipped with flame ionization detection (FID) systems. Hydrogen (H_2) was used as

carried gas. Injector and detector port temperatures were 250 and 300°C, respectively, oven temperature 70°C (hold 3 min), rate 4°C min^{-1} to 110°C (hold 3 min). Late-eluting compounds were removed by increasing the temperature to 220°C for 5 min. Synthetic air (300 ml min^{-1}), H_2 (30 ml min^{-1}) and N_2 (30.8 ml min^{-1}). As make-up gas were set for best fid function. The split rate was 40 (Nardi et al. 2005).

Microscopic analysis

Morphological characteristics of microorganisms immobilized in polyurethane foam matrices were monitored using phase contrast and fluorescence microscopy (Olympus BX 60—FLA with software Image Pro-Plus).

Molecular analysis

DNA extraction

The microbial biomass was retrieved from the polyurethane foam matrices by successive washing in phosphate-buffered and subsequent centrifugation to collect the cells. Then the produced pellets were kept on ice until the isolation of the DNA. The extraction of total DNA was made using the phenol : chloroform based protocol described by Griffiths et al. (2000).

PCR amplification

For the DGGE analysis, 16S rRNA gene fragments were amplified by PCR using specific primers: the primers sets 1100f and 1400r for *Archaea* Domain (Kudo et al. 1997), the primers sets 968f and 1392r for *Bacteria* Domain (Nielsen et al. 1999) and the primers sets 341f and 907r for sulphate-reducing bacteria group (SRB) (Nakagawa et al 2002). A GC-clamp (Muyzer et al. 1993) was added to the forward primers of the three primer sets. A DNA template of 2.0 µl was added to the amplification reaction, performed in accordance with the instructions of the supplier manual for Taq DNA polymerase platinum (Invitrogen®, Carlsbad, CA, USA). The PCR was performed with a System 2,400 thermocycler (Perkin-Elmer Cetus, Norwalk, CT, USA) The programmes of PCR applied for *Archaea* Domain, *Bacteria* Domain and SRB group were described by Kudo et al. (1997), Nielsen et al. (1999) and Nakagawa et al. (2002), respectively.

For 16S rRNA gene library construction, the amplification was performed using the bacterial primer set 27f and 1100r (Lane 1991). Fifty microlitres (50 µl) reaction mixtures contained 100 ng of total DNA, 2 U of *Taq* polymerase (GE Healthcare, Bucks, UK), 0.2 mM of dNTP mix and 0.4 µM of each primer, in 1× *Taq* buffer.

DGGE analysis

The amplified DNA fragments were separated by DGGE. DGGE was conducted using a Dcode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA). PCR products were applied directly onto 8% (wt/v) polyacrylamide gel in 0.5× TAE, with the linear gradient of denaturants (urea and formamide) ranging from 30 to 60%. Electrophoresis was performed at constant voltage of 75 V and temperature of 65°C during 16 h. Gels were observed in UV illumination device and photographed using the equipment Eagle Eye II (Stratagene, La Jolla, CA, USA). Gel images were worked with Eaglesight Stratagene (v3.22) software.

Cloning and sequencing of 16S rRNA gene fragments

A 16S rRNA gene library was constructed with the sludge sample that was withdrawn from the last step of the reactor operation. The products from ten independent PCR reactions were pooled, purified with GFX™ PCR DNA purification kit (GE Healthcare), and concentrated in a speed vacuum concentrator 5301 Eppendorf, A-2-VC rotor. The purified PCR product (50 ng) was ligated into pGEM-T Easy Vector (Promega, Madison, WI, USA), according to manufacturer's instructions and transformed into competent *Escherichia coli* JM109 cells. Approximately 100 clones containing inserts were randomly selected from the library for subsequent sequencing.

The 16S rRNA gene inserts were amplified from plasmid DNA of selected clones using the universal M13 forward and reverse primers. PCR products were checked in 1.2% agarose gels and purified as previously described for subsequent automated sequencing in the MegaBace DNA Analysis System 1000 (GE Healthcare). The sequencing was carried

out using the M13, T7 and 782 (Chun 1995) reverse primers and the *DYEnamic ET Dye Terminator Cycle Sequencing Kit* for automated MegaBace 500 system (GE Healthcare), in accordance with the recommendations of the manufacturer.

Sequence analyses

Partial 16S rRNA gene sequences (obtained from clones were assembled in a contig using the phred/Phrap/CONSED programme (Ewing et al. 1998; Godon et al. 1997). The identification was achieved by comparing the contiguous 16S rRNA gene sequences obtained (~500 bp in length) with 16S rRNA sequence data from reference and type strains, as well as environmental clones, available in the public databases Genbank (http://www.ncbi.nlm.nih.gov) and RDP (Ribosomal Database Project, WI, USA, http://www.cme.msu.edu/RDP/html/index.html), using BLASTn and RDP sequence match routines.

The sequences were aligned using CLUSTAL X programme (Thompson et al. 1997) and analysed with MEGA software Version 2.1 (Kumar et al. 2001). The evolutionary distances were derived from the sequence-pair dissimilarities, calculated as implemented in MEGA using the DNA substitution model reported by Kimura (1980). The phylogenetic reconstruction was done using the neighbour-joining (NJ) algorithm (Saitou and Nei 1987), with bootstrap values calculated from 1,000 replicate runs, using the routines included in MEGA software.

Nucleotide sequence accession numbers

The 16S rDNA partial sequences determined in this study for the environmental clones were deposited at the Genbank database under the accession numbers: EF583469 to EF583521

Results and discussion

Adsorption

The assays of the contact time achieved the equilibrium state within 4 h after the beginning of the experiment. After 10 h of the contact, the adsorbed mass of LAS remained constant in 2 mg. It was possible to conclude that the saturation was achieved. The amount of degraded LAS during this period of time was negligible because the assays were carried out with no inoculum and under aseptic conditions (data not shown).

Fytianos et al. (1998) worked with isotherms of adsorption of LAS in marine sediments had evidenced that the balance between the adsorption and desorption occurred in 4 h.

The adsorption isotherms and also the amount of LAS adsorbed per unit of adsorbent (mg g^{-1}) versus the final concentration (mg l^{-1}) are shown in Fig. 2. The mass of LAS adsorbed in the foam increased proportionally in relation the different increasing concentrations of LAS. The LAS adsorbed mass per gram of foam were 0.38 and 6.15, to initial concentrations of LAS equal to 2.6 and 39.7 mg l^{-1}, respectively.

The data obtained in this assay had not been adjusted adequately for Freundlich and Lagmuir isotherms. The optimum adjustment (to the evaluated concentration range) was achieved to the linear adsorption isotherm reported by Giles et al. (1960). The linearity shows that the higher the concentration of LAS, the greater the quantity of foam necessary to its adsorption.

HAIB reactor

The HAIB reactor was fed during 27 days with synthetic substrate without LAS. In this first experimental phase, the organic matter removal efficiency was 90% with low production of acids (<20 mg l^{-1}), alkalinity generation (320 mg l^{-1}), methane production was of 13 µmol l^{-1} and pH effluent 8.16. After

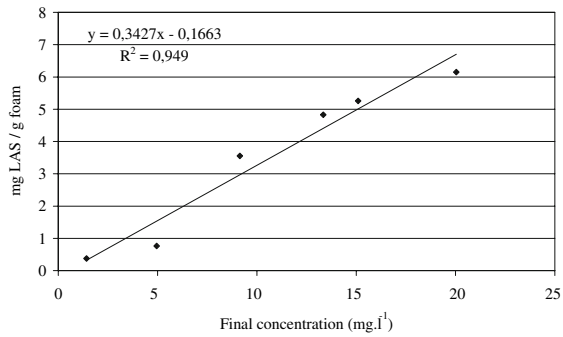

Fig. 2 Adsorption isotherm of LAS onto polyurethane foam

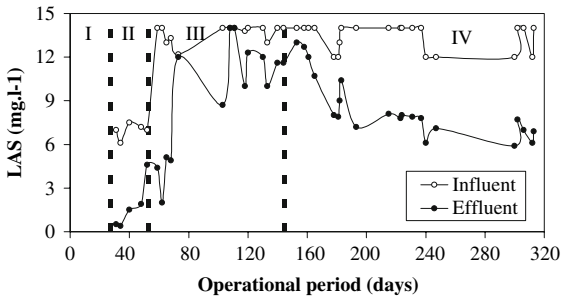

Fig. 3 Temporal variation of LAS acquired in the HAIB reactor

this period, 7 mg l^{-1} of LAS was added to the influent during 24 days (SII). The existence of LAS did not change the stability of the system regarding to the organic material removal (93%), low production of acids (12 mg l^{-1}), alkalinity was 308 mg l^{-1} and methane production was 9.23 μmol l^{-1}.

In the experimental phase SIII, the influent LAS concentration was raised to 14 mg l^{-1} (Fig. 3). In order to avoid any obstruction of the bed, the starch fraction was removed from the influent synthetic substrate and the meat extract was substituted by yeast extract, but maintaining the LAS concentration of 14 mg l^{-1} (SIV). After this change, it was started the fourth step (SIV) of the operation (145–313 days). The addition of the yeast extract and the absence of starch (SIV) favoured the reduction of organic matter from 456 ± 97 to 91 ± 51 mg l^{-1}, alkalinity generation (222 ± 35 to 268 ± 123 mg l^{-1}) and also the reduction of volatile acids concentration (from 151 (29 to 33 ± 9 mg l^{-1}), and methane production remained constant (9 μmol l^{-1})

The organic matter removal efficiency (filtrated COD) increased from 41 ± 14 to 84 ± 7%. The mean LAS removal increased to 20 ± 9% in the step III (144 days of operation) to 37 ± 14% in the step IV (313 days of operation).

At the end of the experiment, after extracting adsorbed LAS in the foam, it was possible to execute the global mass balance of the system (28th to 313rd day). The total applied mass of LAS to the reactor was 5,782 and 3,616 mg was verified in the effluent, being 165 mg adsorbed in the foam with biofilm. These results indicated 35% of biological degradation. It is important to emphasize that the biological degradation of LAS can also be related to the existence of 20 μg l^{-1} of benzene and toluene in the reactor effluent (end of experiment). These compounds could serve as organics substrates for these microorganisms in the period when the substrate concentration was low.

The influent sulphate concentration remained constant and equal to 10 mg l^{-1} in the IV step, probably due to sulphate concentration present in the yeast extract that contributed to the increase of its concentration in the feeding. However, the sulphate concentrations showed lesser values than 2 mg l^{-1} along the reactor.

Information concerning LAS removal is mainly related to the application of UASB reactor. Almendariz et al. (2001) used a UASB reactor compartmented in acidogenic and methanogenic units. The acidogenic reactor presented 41% of LAS degradation after 250 days of the operation. Sanz et al. (2003) also used a UASB reactor with HDT of 24 h obtaining concentration of LAS varying from 4 to 5 mg l^{-1}. After the 80th day of operation, it was verified 85% of degradation in the absence of co-substrates.

Microbial analysis

Samples of the biofilm were removed in the end of each phase operation, through the point of sampling (L/D 1, 5, 10, 14 and 19). It was noted, as much in microscopic analysis as in DGGE results, that different populations were preferentially selected with the presence of LAS in the feeding.

Fluorescent rods and morphology similar to *Methanosarcina* sp. were favoured by the surfactant during all the reactor operation. Probably such organisms used intermediate compounds resulting from LAS degradation or from remaining compounds in the synthetic substrate.

Cell morphology similar to *Methanosaeta* sp. was not observed when concentration of LAS in the influent was 14 mg l^{-1}(SIII). The methane concentration was reduced during the operation probably due to the reduction in the diversity of the methanogenic *archaea*.

The microorganisms can be considered similar to the *Methanosarcina* sp. genus due to the characteristic cubic disposition of the cocci forming sarcina and also because of the typical fluorescence that results from the presence of the coenzyme F420. Coenzyme F420 interacts with a number of different enzymes in

methanogens including hydrogenase and NADP+ reductase. F420 also plays a role in methanogenesis as the electron donor in at least one of the steps of CO_2 reduction. The oxidized form of F420 absorbs light at 420 nm and fluoresces blue–green, on reduction, the coenzyme becomes colourless. The fluorescence of F420 is a useful tool for preliminary identification of an organism as a methanogenic (Madigan et al. 1997). The similarity to the *Methanosaeta* sp. genus results from the observation of rods with flat ends that forms very long and flexible filaments which end to aggregate in characteristic bundles. In the *Methanosaeta* sp. cells, the F420 content is too low to cause visible auto fluorescence under the UV microscope (Konig and Stetter 1989).

A decrease of the number of bands in the samples taken along operation was observed in the DGGE performed to study the *Archaea* community (Fig. 4a). Three populations (*Archaea* Domain), bands 5, 6 and 7, were selected in the presence of 14 mg l^{-1} of LAS. Some bands (1–4) present in the inoculum were detected in the samples taken in the experimental phases SI, SII and SIII.

Nevertheless, one band (7) present in the inoculum was detected with a higher intensity in the sample taken at the end of operation (Fig. 4a).

According to Almendariz et al. (2001), the acidogenic bacteria are responsible for the degradation of LAS, while methanogenic *archaea* are more sensitive to LAS and do not participate in the degradation process. Complex microbial communities were observed for *Bacteria* Domain (Fig. 4b) and SRB group (Fig. 4c). Many bands (8, 9 and 10) in the gel performed to study the *Bacteria* Domain present in the inoculum were also evidenced in the last operational phase (SIV).

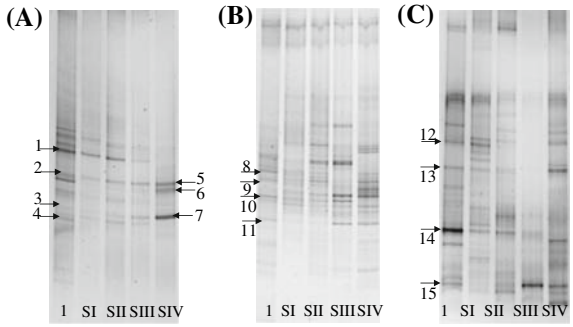

Fig. 4 DGGE-profiles (40–60%) of the inoculum and the biofilm reactor samples. Lane 1—inoculum, Lane 2—sample of biofilm obtained at the end of operation stage I, Lane 3—sample of biofilm obtained at the end of operation stage II, Lane 4—sample of biofilm obtained at the end of operation stage III, Lane 5—sample of biofilm obtained at the end of operation stage IV (**A**) *Archaea* Domain, (**B**) *Bacteria* Domain and (**C**) SRB group

Despite the low-sulphate concentration in the influent (10 mg l^{-1}), the microorganism diversity of the SRB group was maintained during the operation. Some bands (12–15) presented higher intensity with the increase of LAS concentration. Under such sulphate concentrations, SRB performed, preferentially, fermentative metabolism with the production of organic acids that were consumed by anaerobic bacteria and methanogenic *archaea*.

LAS can be used as a source of sulphur by anaerobic bacteria under sulphur limited conditions (Denger and Cook 1999). It is generally believed that sulphur is eliminated very late in the normal biodegradation pathway for LAS surfactants, following initial alkyl chain oxidation to give carboxylated intermediates, such as sulfophenylbutyrate (Kertesz et al. 1994).

For some time it has been known that sulfonates can be metabolized as the only source of carbon, energy and sulphur for aerobic bacterial growth. Studies demonstrated that sulfonate-sulphur can be assimilated for both aerobic and anaerobic bacteria (Lie et al. 1996). According to these authors certain sulfonates are used as terminal electron acceptors for anaerobic respiratory growth of various classic SRB.

A 16S rRNA gene library was performed to evaluate the bacterial community composition from the biomass at the end of the reactor operation. A total of 53 clones were obtained; the sequences of the inserts were determined and analysed using Genbank's BLASTn routine. According to the analysis, 43 sequences were related to sequences from organisms phylogenetically positioned in the phylum Firmicutes, more specifically to the class Clostridia, order Clostridiales. The sequences from the other ten clones were related to sequences from organisms belonging to the phylum Proteobacteria.

The phylogenetic tree illustrating the relationships between the clones and organisms from the class Clostridia represented in the databases included 40 sequences (Fig. 5). The majority of these clones were clustered with uncultured bacteria detected in anaer-

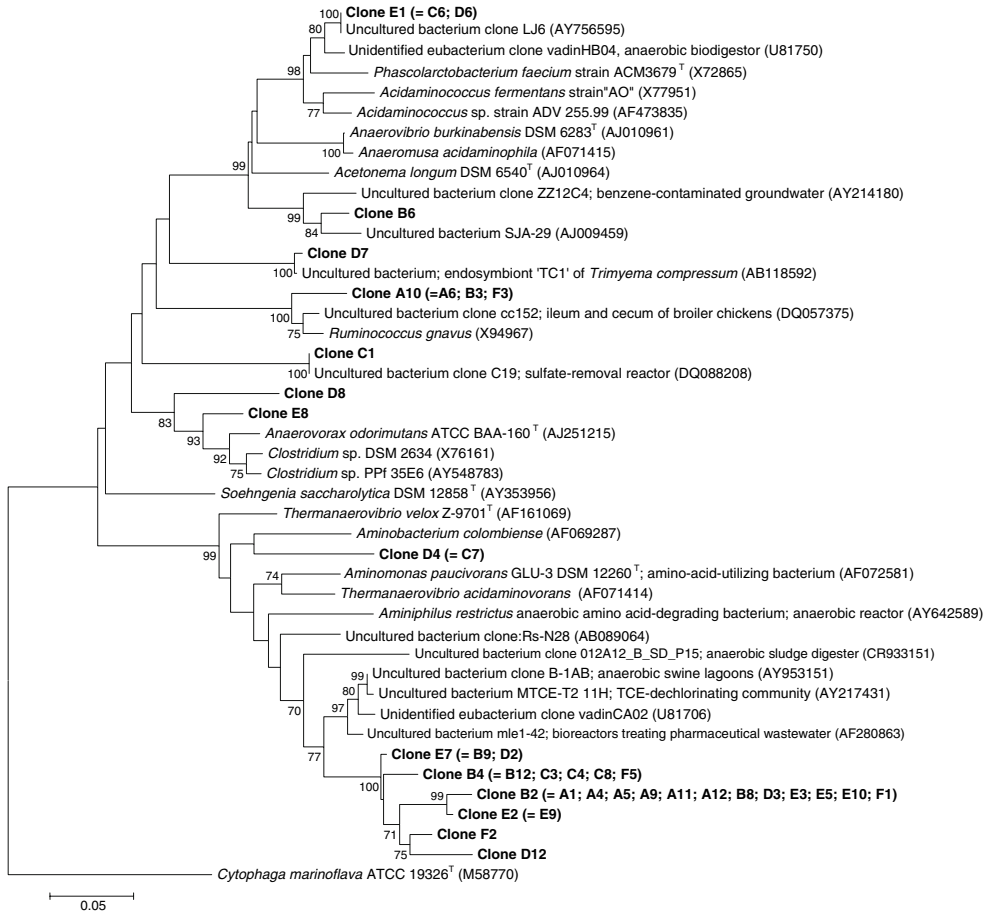

Fig. 5 Phylogenetic analysis of partial 16S rRNA gene sequences of clones derived from the bioreactor sample and related microorganisms belonging to the phylum Firmicutes. Evolutionary distances were based on Kimura 2p model and tree reconstruction on the *neighbour joining* method. Bootstrap values (1,000 replicate runs, shown as %) >70% are listed. Clones in brackets showed (97% similarity with the clone represented in the branch. Genbank accession numbers are listed after species names. *Cytophaga marinoflava* ATCC 19326^{T} was used as outgroup

obic bioreactors treating different types of wastes (e.g. Genbank accession numbers AF280863, AY953151, AY217431, AY642589 and CR933151).

These relationships were supported by high-bootstrap values and suggest that the organisms corresponding to these 16S rRNA clone sequences may represent new taxa belonging to the phylum Firmicutes. Other two clones (D8 and E8) were distantly related to *Anaerovorax odorimutans* and *Clostridium* sp., showing between 89 and 91% sequence similarity. Members of the genus *Clostridum*, most of them strictly anaerobic, produce ammonia, H_2S and large amounts of H_2, fix atmospheric nitrogen and fermenting carbohydrates. The products of their fermentation include acetic, butyric and lactic acids, ethanol, acetone, CO_2 and large amounts of H_2. Pyruvate is converted to acetate, butyrate and buthanol (Matthies et al. 2001). The nutritional composition (yeast extract) may have favoured the growth of such cells in the last phase of the reactor operation (SIV).

In all the operation stages were detected acetic acid and propionic acid. The values had oscillated from 44.1 to 14.3 mg l^{-1} for acetic acid and from 18.9 to 0.8 mg l^{-1} for the propionic acid, respectively, with 200 and 312 days of operation in the stage IV. The decrease in the concentration of acetic acid could be explained by its consumption for acetoclastic methanogenic organisms. This is in agreement with the presence of morphologies similar

to *Methanosarcina* (an acetate consuming methanogenic *archaea*) as was detected by fluorescent microscopy.

Clones E1, D6 and C6 showed 99 and 96% sequence similarity with the uncultured bacteria clones LJ6 and vadinHB04 (Genbank numbers AY756595 and U81750), respectively. These clones were clustered, with high-bootstrap values, with the more distantly related bacteria *Phascolarctobacterium faecium* (92% sequence identity) and *Acidaminococcus* sp. (90% sequence identity) (Fig. 5). Both species are characterized by using amino acids, but the second one produces acetic acid. This acid was detected during all the operation of the reactor.

The clones A10, A06, B03 and F03 showed between 92 and 93% sequence similarity with several uncultured bacteria in the BLASTn search. The phylogenetic analysis (Fig. 5) grouped these clones with the valid species *Ruminococcus gnavus* (92% sequence identity; 100% bootstrap value). The presence of bacteria related to *R. gnavus* may have probably allowed the establishment of the syntrophic relations with hydrogenotrofic archaeal cells maintaining the pH values, once the first specie uses carbohydrates and produces acids, ethanol, CO_2 and H_2.

Three clone sequences affiliated to the class Clostridia were not included in the phylogenetic analysis since they could not be properly aligned with the other 40 clone 16S rRNA gene sequences.

One of them corresponded to clone C9, which presented 96% similarity with 16S rRNA sequence of *Aminomonas paucivorans* DSM 12260^T (Genbank accession number AF02581). This bacterium was found in anaerobic ponds for dairy wastewater treatment and related with the utilization of amino acids (Baena et al. 1999). The presence of yeast extract probably favoured the growing of these microorganisms in the nutritional composition of the reactor influent. The second sequence corresponded to clone C5, which showed 94 and 92% similarity, respectively, with two uncultured bacteria found in the gut of termites, Genbank accession numbers AB234552 and AB089064. The last 16S rRNA gene sequence not included in the analysis of the Clostridia group corresponded to clone C10, which showed 98% similarity value with sequences from uncultured bacteria found in anaerobic bioreactors degrading phenol, trichlorobenzene or brewery waste (Genbank accession numbers AY261808, AJ009498 and EF515560, respectively).

The ten clones affiliated to the phylum Proteobacteria were related to organisms belonging to the class Alphaproteobacteria. According to the BLASTn search, nine proteobacteria clones (A03, A07, B01, B05, B07, B10, D10, E06 and F04) presented between 96 and 99% sequence similarity values to 16S rRNA sequences of several strains of *Pleomorphomonas oryzae*, including the type strain. The phylogenetic analysis confirmed the Genbank search results, recovering these clones in a tight cluster with *P. oryzae*, supported by a high-bootstrap value (Fig. 6). One clone (E04) showed 97% sequence similarity with the 16S rRNA sequence of *Azospirillum doebereinerae* and this relationship was confirmed by the phylogenetic analysis (Fig. 6).

Conclusions

The adsorption of LAS in foam is linear. This means that the higher the LAS concentration, the greater the amount of foam necessary for its adsorption.

The full-scale application of a HAIB reactor is feasible for the bioremediation of waters contaminated with LAS, since the construction of such a type of reactor is simple.

The LAS was degraded in the HAIB reactor filled with polyurethane foam without interference in the organic matter removal. With 108 days of operation, it was considered that polyurethane foams attained the saturation of LAS, beginning the biological degradation of the surfactant.

PCR/DGGE technique applied for the evaluation of the microbial community in the reactor indicated that microorganisms from the *Archaea* Domain were more sensitive to the surfactant, while the microorganisms belonging to the SRB group predominated in the biofilm of the reactor, even at low-sulphate concentrations.

The construction of a 16S rRNA gene library for the *Bacteria* Domain followed by sequencing and phylogenetic analyses allowed characterizing successfully the bacterial community diversity present in a sample of the reactor biofilm in the last operation stage. The results revealed that the predominant components were associated with the phylum Firmicutes, class Clostridia. These data corroborated the values of the mass balance in the reactor, since the metabolism of the microorganisms belonging to this

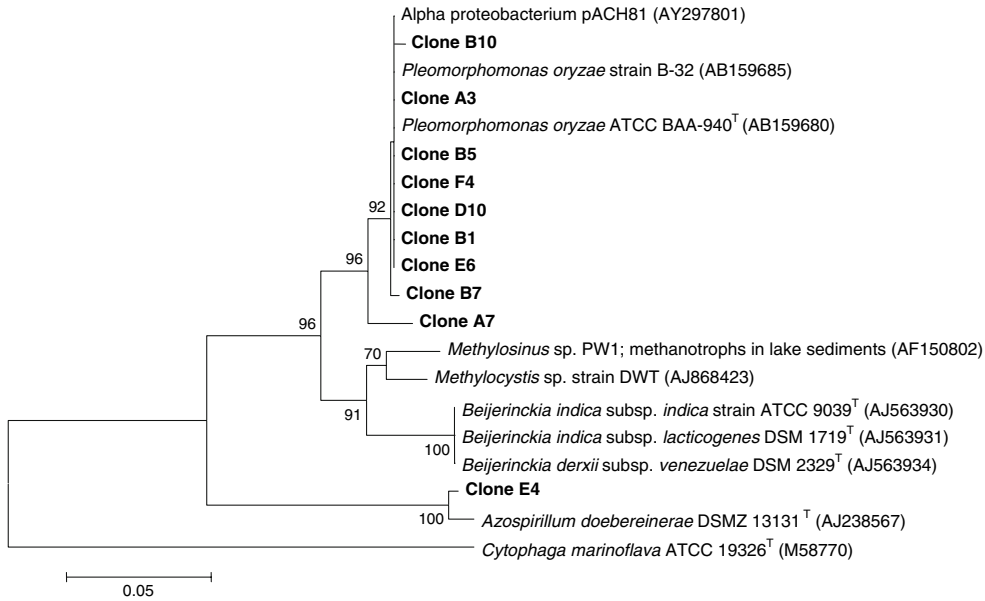

Fig. 6 Phylogenetic analysis of partial 16S rRNA gene sequences of clones derived from the bioreactor sample and related microorganisms belonging to the Phylum Proteobacteria. Evolutionary distances were based on Kimura 2p model and tree reconstruction on the *neighbour joining* method. Bootstrap values (1,000 replicate runs, shown as %) >70% are listed. GenBank accession numbers are listed after species names. *Cytophaga marinoflava* ATCC 19326^T was used as outgroup

taxonomic group could explain the presence of certain compounds in the reactor in the last operation stage and the possible establishment of cooperative and also competitive relationships with other microbial groups in the biofilm.

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