Degradation of detergent (linear alkylbenzene sulfonate) in an anaerobic stirred sequencing-batch reactor containing granular biomass
Degradation of detergent (linear alkylbenzene sulfonate) in an anaerobic stirred sequencing-batch reactor containing granular biomass

I.C.S. Duarte a,*, L.L. Oliveira b, M.S. Mayor b, D.Y. Okada b, M.B.A. Varesche b

a Federal University of São Carlos, Rodovia João Leme dos Santos, Km 110, Zipcode 18052-780, Sorocaba, SP, Brazil
b Laboratory of Biological Processes, Hydraulics and Sanitation Department, School of Engineering of São Carlos, University of São Paulo, Av. Trabalhador São-carlense, 400, Zipcode: 13566-590, São Carlos, SP, Brazil

A R T I C L E   I N F O

Article history:
Received 12 August 2009
Received in revised form 14 December 2009
Accepted 15 December 2009
Available online 13 January 2010

Keywords:
ASBR
Degradation
16S rRNA
Linear alkylbenzene sulfonate

A B S T R A C T

This study aimed to determine the efficiency of an anaerobic stirred sequencing-batch reactor containing granular biomass for the degradation of linear alkylbenzene sulfonate (LAS), a surfactant present in household detergent. The bioreactor was monitored for LAS concentrations in the influent, effluent and sludge, pH, chemical oxygen demand, bicarbonate alkalinity, total solids, and volatile solids. The degradation of LAS was found to be higher in the absence of co-substrates (53%) than in their presence (24–37%). Using the polymerase chain reaction and denaturing gradient gel electrophoresis (PCR/DGGE), we identified populations of microorganisms from the Bacteria and Archaea domains. The presence of methanogenic microorganisms shows that LAS did not inhibit anaerobic digestion. Sampling at the last stage of reactor operation recovered 61 clones belonging to the phylum Bacteria and 50 clones to the Archaea. These represented a variety of phyla: 34% shared significant homology with Bacteroidetes, 18% with Proteobacteria, 11% with Verrucomicrobia, 8% with Fibrobacteres, 2% with Acidobacteria, 3% with Chlorobi and Firmicutes, and 1% with Acidobacteres and Chloroflexi. A small fraction of the clones (13%) were not related to any phylum.

1. Introduction

Linear alkylbenzene sulfonate (LAS) is the most commonly used surfactant in the world. Over 80% of LAS use occurs in household detergent and as a component of liquid cleaning products (Lara-Martín et al., 2007). After use, LAS is discarded into aquatic ecosystems as discharges from both treated and untreated wastewater (León et al., 2001).

It is widely accepted that LAS is readily degradable under aerobic conditions (Haagensen et al., 2002). However, recently LAS has been reported to degrade anaerobically under certain conditions (Angelidaki et al., 2000; Sanz et al., 2003). The limited available information indicates that (a) LAS can be used as a source of sulfur by anaerobic bacteria under sulfur-limited conditions (Denger and Cook, 1999) and (b) benzenesulfonic acid and benzaldehyde may be produced as metabolites during anaerobic LAS degradation in the thermophilic temperature range.

Lara-Martín et al. (2007) studied the anaerobic degradation of LAS and identified the presence of metabolic intermediates and microorganisms involved in the process. The authors used anoxic marine sediments in laboratory tests, and they evaluated the degradation of the surfactant at concentrations of 10–50 mg L⁻¹. LAS degradation reached 79% by 165 d. Due its inhibition of microbial growth, a longer half-life for LAS degradation (~90 d) is expected for LAS concentrations up to 20 mg L⁻¹.

Among the various configurations of anaerobic reactors used in wastewater treatment, the most used in the degradation of linear alkylbenzene sulfonate is the UASB. Angelidaki et al. (2000) used a partitioned UASB (acidogenic/methanogenic) to degrade LAS at concentrations of 20 and 50 mg/L and obtained 41% efficiency. Sanz et al. (2003) also used a UASB reactor to treat LAS at 5 mg L⁻¹ with and without co-substrates; in the latter condition, the authors were able to achieve efficiencies around 85%. Lobner et al. (2005) observed that anaerobic reactors operating under the same conditions sometimes exhibited very different degradation capabilities. They found a 40–80% removal of LAS in bench-scale UASB reactors under mesophilic and thermophilic conditions.
In the present study, we investigated the anaerobic degradation of detergent in an anaerobic stirred sequencing-batch reactor (ASBR) and characterized the microbial population involved in LAS degradation.

2. Experimental procedure

Assays were performed in a mechanically stirred ASBR. The reactor was made of borosilicate glass with a diameter of 20 cm and a height of 16 cm. Mechanical stirring was carried out using an impeller-type turbine, with three blades measuring 14 cm. The operating temperature was 30 ± 1 °C and batches were incubated for 24 h with agitation at 50 rpm. The total wastewater volume contained in the reactor was 5 L. Assays were performed as follows: at the beginning of the cycle, the reactor was fed with 3 L of synthetic substrate for approximately 15 min, then the reaction phase was allowed to proceed for 23 h, after which the agitation was turned off and sediment was allowed to collect for 30 min; finally, at the end of the cycle, the effluent was discharged for approximately 15 min.

The inoculum used in experiment came from an upward-flow anaerobic sludge blanket reactor used to treat swine wastewater at the Estadual Paulista University (Jaboticabal-SP, Brazil). The inoculum contained 52 g L\(^{-1}\) of total solids (TS) and 43 g L\(^{-1}\) of volatile solids (VS).

The reactor was fed with a synthetic substrate composed of yeast extract (50 mg L\(^{-1}\)), starch (230 mg L\(^{-1}\)), sucrose (80 mg L\(^{-1}\)), sodium bicarbonate (400 mg L\(^{-1}\)), and 5 mL L\(^{-1}\) of saline solution (50 g L\(^{-1}\) of NaCl, 1.4 g L\(^{-1}\) of MgCl\(_2\)-H\(_2\)O, 0.9 g L\(^{-1}\) of CaCl\(_2\)-2H\(_2\)O; Duarte et al., 2007). The substrate was kept refrigerated at 4 °C during reactor feeding. The five experimental stages were defined by modifications to the composition of the synthetic substrate: Phase I, synthetic substrate without LAS; Phase II, synthetic substrate with 22 mg L\(^{-1}\) of LAS; Phase III, synthetic substrate with 500 mg L\(^{-1}\) of yeast extract; Phase IV, feeding only with detergent (22 mg L\(^{-1}\) of LAS), sodium bicarbonate, and salt in the absence of co-substrate; and Phase V, sludge digestion.

To assess the behavior of the ASBR, we measured the following parameters: chemical oxygen demand (COD), pH, and concentration of solids (APHA, 1998). Bicarbonate alkalinity (BA) in the form of CaCO\(_3\) was measured as described in Dilallo and Albertson (1961) with the modifications of Ripley et al. (1986). The LAS concentration was periodically measured in the liquid phase (influent and effluent) using high-performance liquid chromatography (HPLC) as previously described (Duarte et al., 2006). To quantify LAS degradation, the final mass balance of LAS in the system was calculated based on the amount of LAS adsorbed by the biomass in the reactor at the end of the experiment.

To measure the LAS adsorbed by and precipitated with the biomass, triplicate samples were extracted with methanol in an ultrasonic bath for 30 min and analyzed by HPLC. This extraction protocol had an efficiency of 85% (Duarte et al., 2007).

2.1. DNA extraction, polymerase chain reaction/denaturing gradient-gel electrophoresis, amplification, and library construction

The extraction of total DNA was performed using the phenol:chloroform-based protocol described by Griffiths et al. (2000). For the DGGE analysis, 16S rRNA gene fragments were amplified by PCR using specific primers: the primer pair 1100F and 1400R for the domain Archaea (Kudo et al., 1997) and the primer pair 968F and 1392r for the domain Bacteria (Nielsen et al., 1999). A GC-clamp (Muyzer et al., 1993) was added to the forward primers of the three primer pairs. The PCR programs used for the domains Archaea and Bacteria were described by Kudo et al. (1997) and Nielsen et al. (1999), respectively.

To construct a 16S rRNA gene library, amplification was performed using the bacterial primer pair 27f and 1100F (Lane, 1991). A 16S rRNA gene library was constructed from the sludge sample taken in the final stage of reactor operation. The purified PCR product was ligated into the pGEM-T Easy Vector (Promega, Madison, WI, USA) according to the manufacturer’s instructions, then transformed into Escherichia coli JM109 cells. The 16S rRNA gene inserts were amplified from the plasmid DNA of selected clones using universal M13 forward and reverse primers (Invitrogen).

Phylogenetic assignment of the microorganisms in the reactor samples was achieved by comparing the ~500-bp contiguous 16S rRNA gene sequences obtained with 16S rRNA sequence data from reference and type strains and environmental clones deposited in the GenBank (http://www.ncbi.nlm.nih.gov) and RDP (Ribosomal Database Project, WI, USA, http://www.cme.msu.edu/RDP/html/index.html) public databases. Sequence matching was carried out using the BLASTn and RDP routines.

3. Results and discussion

After 21 d of operation with synthetic substrate lacking LAS (Phase I), the sludge concentration in the reactor decreased significantly from 52.5 to 13 g L\(^{-1}\) of TS and from 43.1 to 6 g L\(^{-1}\) of TVS. The decrease in the concentration was not due to the loss of suspended solids to the effluent but instead was attributed to the low concentration of organic material in the influent (149 ± 83 mg COD L\(^{-1}\)). Despite the cell death caused by the lack of organic material, COD removal was 74 ± 19%. The pH of both the influent and effluent was near 7 and there was generation of alkalinity (Table 1).

On day 22 of operation, liquid detergent was added at a concentration of 22 ± 5 mg LAS L\(^{-1}\) (Phase II). During this phase, which lasted 55 d, the TS concentration changed from 13 g L\(^{-1}\) to 149 g L\(^{-1}\) and TVS concentration from 6 to 2 g L\(^{-1}\). Immediately after the LAS was added to the liquid phase, nearly all of it was adsorbed by the sludge. Similar results were observed by Garcia et al. (2005) in batch reactors. According to these authors, the LAS adsorption by the sludge decreased the fraction available for degradation.

The addition of LAS also affected the removal of organic material. During the operation without LAS, organic-material removal was 74 ± 19%. After LAS was added, the efficiency decreased to 44 ± 14% (Table 1). During this phase (Phase II) the total mass of LAS added was 5.39 g, and 2.94 g were recovered in the effluent and 0.441 g was adsorbed or precipitated by the sludge. These numbers translate to a 37% efficiency of LAS degradation.

In order to improve the efficiency of LAS and COD removal (Phase III), yeast extract was added at a concentration of 500 mg L\(^{-1}\), which changed the concentration of organic material in the influent from 183 ± 35 mg COD L\(^{-1}\) to 771 mg COD L\(^{-1}\). In order to calculate the efficiency of LAS degradation, we measured the mass of LAS accumulated throughout the operation. Over 91 d, 6.93 g of LAS were added, 3.88 g were recovered in the effluent and 2.041 g was adsorbed or precipitated by the sludge. These numbers translate to a 37% efficiency of LAS degradation.
values of the influent (7.43) and effluent (7.35) were higher than during the other phases (Table 1).

The total LAS mass added over 122 d of operation was 13.1 g. Of that amount, 5.9 g were recovered in the effluent and only 0.21 g was adsorbed in the sludge, which indicates an efficiency of 53% for LAS degradation in the absence of co-substrates (Phase IV). This is far higher than the efficiency (24%) during Phase III when the concentration of yeast extract was increased from 50 to 500 mg L⁻¹. Thus, using influent containing LAS as the sole carbon source allowed more efficient removal of LAS.

To determine the anaerobic treatment of detergent without residual LAS adsorbed in sludge, this was referred to as digestion. After 25 d of digestion, the sludge with 0.21 g LAS degraded 96% of it. LAS may have been used as an energy or a carbon source, as the concentration of TVS remained constant (2.2 g L⁻¹), indicating that there was no cell death.

The reactor proved to be a suitable alternative for the treatment of LAS, especially when compared with the experiments carried out in horizontal anaerobic immobilized biomass (HAIB) (Duarte et al., 2007). In that work, the HAIB reactor was operated with a hydraulic retention time (HRT) of 12 h and fed with an LAS mass of 6 g over 313 d; 35% of the input LAS was degraded. In our reactor, the same percentage was obtained after only 113 d of operation with an applied mass of 10 g. This difference in degradation kinetics must reflect the different configurations of the system and the microorganisms that developed inside, as both reactors were fed with the same synthetic substrate and inoculum. Therefore, it is likely that the hydrodynamic characteristics of the reactors, which involve a mixture of flow and plug-flow, promoted the growth of different microorganisms.

### 3.1. Identification of microorganisms possibly responsible for LAS degradation

The microbial community in this reactor was examined by PCR/DGGE. The addition of detergent caused significant changes in the
population of microorganisms belonging to the domain Bacteria (Fig. 1a). Despite the absence of co-substrates and sludge digestion, the microbial community showed significant diversity. However, the growth of certain populations was favored during the phase of sludge digestion. The population represented by band 2 was observed only in channel C, where the feed was supplemented with more yeast extract. This band was assigned to Arcanobacterium (94%), and the population represented by band 5 was assigned to Opitutus spp. (96%) (Fig. 1a). The Opitutus spp. belonging to the phylum Verrucomicrobia are strictly anaerobic microorganisms. These microorganisms ferment carbohydrates such as mono-, di-, and polysaccharides, and they produce propionic and acetic acids (Chien et al., 2001). This population was abundant during the sludge-digestion phase.

Arcanobacterium pyogenes was detected in the inoculum and during Phases I and II of reactor operation. The increase in the concentration of yeast extract favored its growth, probably because of the vitamins and proteins present in the extract. Uncultured bacteria were observed in bands 1, 3, and 4.

The conditions of reactor operation, such as agitation and food quality, influenced the diversity of methanogenic microorganisms. This was proven by the analysis of COD, which showed reductions in the efficiency of organic-material removal during the same period. However, the inhibition of LAS on methanogenic microorganisms was temporary and reversible, as shown by the DGGE gel (Fig. 1b) and the COD analysis. Moreover, most of the population found during that phase of operation were phylogenetically related to Methanosaeta spp. (98% homology with bands 3, 5, 7, and 10), Methanospirillum spp. (90%, band 1), and Methanobacterium (96%, band 8). The other bands sequenced (2, 4, 6, 9, and 12) were assigned to uncultivated archaea.

Methanospirillum and Methanobacterium are strictly anaerobic hydrogenotrophic archaea (Bergey’s, 1994). Both genera use H₂, CO₂, and formate. Some strains use 2-propanol as a hydrogen donor for methanogenesis, while Methanosaeta uses only acetate for methanogenesis (Garcia et al., 2000). According to Chin and Janssen (2002), Opitutus terraee strain PB90-1 is a bacterium that produces formate, which, if used by Methanospirillum, maintains the production of hydrogen. Under these conditions these microorganisms live in a syntrophic relationship.

The clone library we created showed a high phylogenetic diversity, with members from nine phyla: 34% of the clones were assigned to the phylum Bacteroidetes, 18% to Proteobacteria, 11% to Verrucomicrobia, 8% to Fibrobacteres, 2% to Acidobacteria, 3% to Chlorobi and Firmicutes, and 1% to Acidobacteres and Chloroflexi. A small proportion of clones (13%) were not assigned to any phylum (Fig. 2).

Of the clones identified, two were related to Flavobacterium (98% sequence homology); they belonged to the phylum Bacteroidetes. Two clones were related to Pseudomonas spp. (94% sequence homology). It has been suggested that this genus degrades surfactants. When used, in a reactor, they have been immobilized on different support materials such as polyacrylamide gels (Thomas and White, 1991), glass (Jarabkova et al., 1999), or polyurethane foam (Roig et al., 1999). Kertesz et al. (1994) used Pseudomonas putida S-313 in a sulfate-free medium with sulfonate as the sole source of sulfur. The authors obtained evidence that this microorganism caused the desulfonation of LAS to yield the corresponding phenols. The authors speculated that under sulfur-limited growth conditions, P. putida S-313 catalyzes the degradation of aromatic sulfonate using a monooxygenase system never before seen in vitro. In a natural environment, several species of microorganisms cooperate to fully degrade LAS. Jimenez et al. (1991) studied a mixed microbial community consisting of three species of Pseudomonas and an Aeromonas sp. LAS was added at a concentration of 1 µg mL⁻¹ as the sole source of carbon, and all species studied were able to degrade over 25% of the input LAS and convert it to CO₂.

One clone was identified in the present study as Syntrophus sp., with a sequence similarity of 98%. Bacteria of this genus live in co-culture with hydrogenotrophic methanogens or with sulfate-reducing bacteria. They are anaerobic, and they metabolize alcohols, fatty acids such as aromatic acids, sugars, and hydrocarbons including methane and some amino acids (McInerney, 2007). This genus was present in our reactor and probably consumed the sucrose and amino acids in the yeast extract while living in a syntrophic relationship with the microorganisms of the Methanobacterium and Methanospirillum genera. We have also previously observed sulfate-reducing bacteria of the genera Desulfovibrio and Syntrophobacter in an HAIB reactor fed with the same synthetic substrate (Duarte et al., 2007). It should be

![Fig. 1. Profiles of DGGE bands amplified using primers specific to (a) the Bacteria domain or (b) the Archaea domain. (A) Inoculum, (B) Phase I of reactor operation without LAS, (C) Phase II of operation with 22 mg L⁻¹ of LAS, (D) Phase IV of operation without co-substrates, and (E) Phase V featuring sludge digestion. Numbered bands were excised, reamplified, and sequenced. Closest phylogenetic matches: (a) lane 1, uncultured bacteria; lane 2, Arcanobacterium; lanes 3 and 4, uncultured bacteria; lane 5, Opitutus spp. (b) lane 1, Methanospirillum; lanes 2, 4, 6, 9, 11, and 12, uncultured archaea; lanes 3, 5, 7, and 10, Methanosaeta.](image-url)
emphasized that the substrate did not contain sulfate. These bacteria may have used the sulfate moiety of the LAS and maintained a sulfur-assimilating metabolism.

Other clones were identified as Novosphingobium, with a 98% sequence similarity. This genus may also have contributed to LAS degradation in the present study, as it has been shown to use the aromatic ring of LAS as a carbon source (Sohn et al., 2004).

Other clones were related to Dechloromonas sp. with a 97% sequence similarity. These are thought to be the only species that can oxidize benzene in the absence of oxygen. These species can also oxidize aromatic compounds, such as toluene, benzoate, and chloro-benzoate. We also noted the presence of toluene in the effluent from an HAIB reactor used to treat LAS in a previous study (Duarte et al., 2007). This genus probably contributed significantly to the degradation of LAS observed in the present study by acting on the aromatic ring of the molecule.

Six clones were identified as Opitutus spp., with a sequence similarity of 98%. This genus was also identified in the DGGE bands. One clone showed extremely high sequence similarity to a Turicibacter sp. (99%). These bacteria are rod-shaped, strictly anaerobic, non-endospore forming, chemioorganotrophic, and fermentative; they produce lactate as the only product of fermentation. Their optimum temperature and pH for growth are 37 °C and 7.5, respectively (Bosshard et al., 2002).

Several members of the phylum Firmicutes (family, Clostridiales) can use compound xenobiotics as electron acceptors. Therefore, these should be considered as possibly being responsible for the anaerobic degradation of LAS; in fact, several species of the order Clostridia can desulfonate alkyl- and arylsulfonate. One such species is Clostridium beijerinckii strain EV4 (Chien, 2005). Clostridium pasteurianum strain DSM 12136 uses the sulfonate group of 4-toluensulfonate as a source of sulfur (Cao et al., 2003). Lara-Martín et al. (2007) showed that Clostridiales, bacteria from the family Sedimentibacter, can degrade LAS under anaerobic conditions.

Lara-Martín et al. (2007) also identified bacteria that may degrade LAS in marine sediments. They created a clone library showing high phylogenetic variety, which included members of the classes Alphaproteobacteria and Gamaproteobacteria (Pseudomonas); Firmicutes, Verrucomicrobia, Actinobacteria, Acidobacteria, Chloroflexi, and Bacteroidetes were also detected. One of the clones identified was found to show 97% sequence similarity to Desulfoarcina. Furthermore, Pseudomonas was found to be present.

According to Chien (2005) and Denger and Cook (1997), to date only a few prokaryotes have been described that can desulfonate the aromatic ring of the LAS molecule. In the work of Lara-Martín et al. (2007) with marine sediments, one clone showed 94% sequence similarity with Sulforhopolus singaporensis, a sulfate-reducing bacteria that ferments taurine (2-aminoethanesulfonate) and that generating sulfide (Lie et al., 1999).

4. Conclusions

The ASBR reactor can be used in the treatment of detergent-contaminated wastewaters. The degradation of LAS was higher in the absence of co-substrates. When the system was operated under oxygen-depleted conditions, the degradation efficiency reached 53%.

Phylogenetic analysis revealed that the degradation of LAS occurred in the presence of a mixed microbial community living syntrophically. The identification of bacteria present in the ASBR under anaerobic conditions may help to determine which microorganisms contributed to the degradation of LAS. Pseudomonas spp. may use the molecule as a source of carbon or energy, the sulfate-reducing Syntrophus spp. may use the sulfur of the sodium sulfite group, and Novosphingobium spp. and Dechloromonas spp. may use the aromatic ring of the LAS molecule as a source of carbon and energy.

Acknowledgments

The authors gratefully acknowledge the grants received from Fapesp and CNPq.

References


Bergey’s Manual of Determinative Bacteriology, nineth ed., 1994 Editors Williams & Wilkins, Baltimore, USA.


