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LAS DEGRADATION IN A FLUIDIZED BED REACTOR AND PHYLOGENETIC CHARACTERIZATION OF THE BIOFILM

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Abstract - A fluidized bed reactor was used to study the degradation of the surfactant linear alkylbenzene sulfonate (LAS). The reactor was inoculated with anaerobic sludge and was fed with a synthetic substrate supplemented with LAS in increasing concentrations (8.2 to 45.8 mg l^{-1}). The removal efficiency of 93% was obtained after 270 days of operation. Subsequently, 16S rRNA gene sequencing and phylogenetic analysis of the sample at the last stage of the reactor operation recovered 105 clones belonging to the domain *Bacteria*. These clones represented a variety of phyla with significant homology to Bacteroidetes (40%), Proteobacteria (42%), Verrucomicrobia (4%), Acidobacteria (3%), Firmicutes (2%), and Gemmatimonadetes (1%). A small fraction of the clones (8%) was not related to any phylum. Such phyla variety indicated the role of microbial consortia in degrading the surfactant LAS.
Keywords: Immobilized biomass; Sand; 16S rRNA, *Aeromonas*; *Novosphingobium*.

INTRODUCTION

Linear alkylbenzene sulfonate (LAS) is an anionic surfactant introduced in 1964 as the readily biodegradable replacement for highly branched AlkylBenzene Sulfonates (ABS). Since then, its consumption has increased considerably. The estimated world consumption in 2000 was of 2.5 million tons (Sanz *et al*., 2003).

LAS is one of the xenobiotic compounds frequently found in sewage and industrial wastewater. Its degradation has been widely studied in aerobic systems, and removal efficiencies of more than 97% achieved (Brunner *et al*., 1988). However, for anaerobic systems, information is scarce and has been reported only recently, mainly due to recalcitrance of LAS in those systems. Removal efficiency of about 35% was achieved in horizontal anaerobic fixed bed (Duarte *et al*., 2008), and 85% in Upflow Anaerobic Sludge Blanket (UASB) reactors (Sanz *et al*., 2003).

Regarding the microbial identification of organisms involved in LAS degradation, Lara-Martín *et al*. (2007) identified bacteria that could degrade LAS in marine sediments. They created a clone library showing high phylogenetic variety, which included members of the following classes: Alphaproteobacteria, Gamaproteobacteria (*Pseudomonas*).

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Firmicutes, Verrucomicrobia, Actinobacteria, Acidobacteria, Chloroflexi and Bacteroidetes were also detected. One of the identified clones showed 97% sequence similarity to *Desulfosarcina*. Furthermore, *Pseudomonas* was present.

In recent research, Oliveira *et al*. (2010a, 2010b) studied the anaerobic degradation of LAS in a bench scale fluidized bed reactor filled with different support materials. The authors concluded that the anaerobic fluidized bed reactor containing immobilized biomass can be considered to be a feasible reactor configuration for treating LAS. The reactor's performance filled with sand reached an LAS removal efficiency of over 98 ± 2% over the operating period, with LAS concentrations in the influent of 18.8 ± 4.2 mg l^{-1}. Regarding the molecular studies, the reactor presented wide microbial diversity, with microorganisms mainly belonging to the phyla Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria.

Thus, this work was focused on LAS degradation under anaerobic conditions in a fluidized bed reactor. Recent studies in this area have focused on the use of UASB and fixed bed reactors (Almendariz *et al*., 2001; Sanz *et al*., 2003; Duarte *et al*. 2008, Oliveira *et al*. 2009). The microbial communities from biomass present in the reactor were described by 16S rRNA gene analysis. The microbial consortium present in the biofilm was probably involved in LAS degradation.

MATERIAL AND METHODS

Reactor Operation

Assays were performed in a fluidized bed reactor (FBR). The reactor was made of acrylic with a diameter of 4 cm and a height of 100 cm. It was kept in a controlled temperature chamber (30 ± 1 °C) and operated with a hydraulic retention time (HRT) of 18 h. The inoculum used in the experiment came from an UASB reactor used to treat swine wastewater at the UNESP University (Jaboticabal-SP, Brazil). The seed sludge contained 36 g L^{-1} of volatile solids (VS). The reactor was kept in a closed circuit for biomass immobilization and adaptation to synthetic substrate. In this stage, 3 L of feed medium was prepared. This feed consisted of synthetic substrate, prepared as reported by Oliveira *et al*. (2010a) (approximately 560 mg L^{-1} of COD), and anaerobic sludge (10% v/v). The six experimental stages were defined by modifications to the composition of the synthetic substrate: Phase I, synthetic substrate without LAS; Phase II, synthetic substrate with 8.2 mg L^{-1} of LAS; Phase III, synthetic substrate with 24.4 mg L^{-1} of LAS; Phase IV, synthetic substrate with 30.8 mg L^{-1} of LAS; Phase V, synthetic substrate with 38.4 mg L^{-1} of LAS and Phase VI, synthetic substrate with 45.8 mg L^{-1} of LAS.

Physicochemical Analysis

To assess the behavior of the FBR, the following parameters were measured: chemical oxygen demand (COD) and pH (APHA, 2005). Bicarbonate alkalinity (BA) in the form of $CaCO_3$ was measured as described in Dillalo 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). The final mass balance of LAS in the system was calculated to quantify LAS degradation. The amounts of LAS supplied, measured at the outlet and adsorbed by the support plus biomass in the reactor at the end of the experiment were used in the calculations.

Molecular Biology

The extraction of total DNA was performed using the phenol:chloroform-based protocol described by Griffiths *et al*. (2000). To construct a 16S rRNA gene library, amplification was performed using the bacterial primers 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, and then transformed inside *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 BLAST and RDP routines.

The DEREPLICATE program was used to define the operational taxonomic units (OTUs). The alignment

was done using ALIGNER with the default parameter settings. An evolutionary distance matrix was then calculated using the program COMPLETE LINKAGE CLUSTERING (http://pyro.cme.msu.edu/). The numbers and sequences of each OTU were determined based on an evolutionary distance of 0.05.

The sequences from the different OTUs were aligned with RDP database sequences in MEGA 5.05. The phylogenetic relationships obtained were analyzed by the Neighbor-Joining method using the MEGA 5.05 software package. Bootstrap analysis was based on 100 re-samplings.

The nucleotide sequences determined in this study and included in the phylogenetic trees have been deposited in the Genbank under the accession numbers JX561117-JX561132.

RESULTS AND DISCUSSION

Table 1 presents the results in each of the six phases of reactor's operation, such as the initial and final concentrations of organic matter, pH, bicarbonate alkalinity, total volatile acids and effluent LAS concentration, and its percentage of removal.

The percentage of organic matter removal in the first stage was similar to that found in fixed-bed reactors for LAS treatment (Oliveira *et al*. 2009). The reactors used in the previous study were fed with the same synthetic substrate and the mean value of COD removal was 87±3% for the biomass adaptation stage (Stage I).

The filtered COD values obtained in the second stage (86±7%) indicated that LAS did not affect the organic matter degradation as occurred in the study of Oliveira *et al*. (2009). The other parameters remained constant and stable over the previous steps without LAS in the influent (Table 1).

The high values of COD removal in all stages indicated that LAS did not affect the organic matter degradation. This is possibly justified because the reactor is high-rate. The reactor's configuration allowed operation with a wide range of organic matter concentration. Furthermore, the development of a mature biofilm in the adaptation stage with co-substrate (sucrose and yeast extract) favored the growth of active biomass capable of degrading LAS. In association with these possibilities, the recirculation rate must be highlighted because it favored surfactant dilution, and consequently the microbial degradation.

During the reactor's operation, the pH, Bicarbonate Alkalinity and Total Volatile Acid of the effluent remained constant with mean values of 8.0, 152 ($mgCaCO_3\ L^{-1}$) and 15 ($mgHAc\ L^{-1}$), respectively.

Table 1: Values of physicochemical parameters in the fluidized bed reactor

Stage	Sample	COD_f [a] ($mg\ l^{-1}$)	pH	BA [b] ($mgCaCO_3\ l^{-1}$)	TVA [c] ($mgHAc\ l^{-1}$)	LAS ($mg\ l^{-1}$)
I 28 days	Influent Effluent Removal (%)	698±58 93±23 87±3	7.7±0.2 8.1±0.2	289±125	17±2	- - -
II 28 days	Influent Effluent Removal (%)	644±39 90±41 86±7	7.6±0.2 8.1±0.2	151±17	18±6	8.2±1.3 0.9±0.9 88±11
III 49 days	Influent Effluent Removal (%)	665±50 58±21 91±3	7.7±0.1 8.0±0.2	143±12	15±3	24.4±3.7 1.1±1.2 96±5
IV 49 days	Influent Effluent Removal (%)	608±45 42±12 93±2	7.7±0.2 8.0±0.2	152±43	12±3	30.8±4.5 2.1±2.0 93±6
V 70 days	Influent Effluent Removal (%)	639±30 43±13 93±2	7.6±0.3 8.1±0.2	146±6	15±5	38.4±5.7 2.5±1.2 93±3
VI 47 days	Influent Effluent Removal (%)	632±42 60±17 91±2	7.6±0.2 7.9±0.2	153±47	14±6	45.8±5.4 3.2±1.2 93±3

[a] COD_f: Chemical Oxygen Demand filtered
[b] BA: Bicarbonate Alkalinity
[c] TVA: Total Volatile Acid

At the end of the reactor's operation, samples of support material were removed to perform the extraction of the adsorbed LAS. The result of LAS extraction from the sand was under the detection limit of the calibration curve (0.49 mg L^{-1}). This indicates no adsorption of surfactant on the support or in the biofilm. Thus, the reactor received about 7760 mg of LAS, and 510 mg were collected in the effluent. The remaining LAS (93%) was removed from the system by the biological degradation process.

In previous research with FBRs filled with different support materials, including sand, Oliveira *et al*. (2010a) obtained results similar to this work. The feed and operating conditions of the reactors were similar, but with a fixed concentration of about 15 mg L^{-1}.of LAS. The degradation percentages were 86, 83, 98.5 and 98.2% for the reactor filled with activated charcoal, expanded clay, glass beads and sand, respectively.

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) reactors (Duarte *et al*., 2008; Oliveira *et al*., 2009). In those studies, the HAIB reactor was operated with a hydraulic retention time (HRT) of 12 h and fed with an LAS concentration of 14 mg l^{-1} during 313 and 343 days, respectively; 35% and 28% of the input LAS were degraded. In the reactor used in the present study, the percentage of degradation was constantly higher. The difference in degradation must reflect the different configurations of the systems and the microorganisms that developed inside them, because all reactors were fed with the same synthetic substrate and inoculum. Therefore, it is likely that the hydrodynamic characteristics of the system, which is a mixing reactor, combined with other parameters such as pH and alkalinity, promoted the growth of different microorganisms.

Table 2 shows the comparison of this work with others, cited above.

Identification of Microorganisms Possibly Responsible for LAS Degradation

A 16S rRNA gene library was constructed to evaluate the bacterial community composition of the biomass at the end of the reactor operation period (270 days). A total of 110 clones were obtained from the biofilm in the sand reactor bed. The clone library obtained showed a high phylogenetic diversity, with members from six phyla: 41.8% of the clones were assigned to the phylum Proteobacteria, 40% to Bacteroidetes, 3.6% to Verrucomicrobia, 2.7% to Acidobacteria, 1.8% to Firmicutes, 0.9% to Gemmatimonadetes and 0.9% to Fusobacteria. A small proportion of clones (8.2%) were not assigned to any phylum.

Figure 1 represents the clone percentages in the reactor sample related to different phylum. Although this comparison is represented as a percentage, it is not a quantitative representation of the microorganisms present in the reactor, but a qualitative one.

In a fluidized bed reactor operated on a smaller scale with the same support material and operating conditions for 70 days, Oliveira *et al*. (2010b) obtained the following classification as regards the Phyla: Bacteroidetes (25%), Proteobacteria (25%), Actinobacteria (12%), Deinococcus-Thermus (12%), Firmicutes (5%), Acidobacteria (3%), Anaerolineae (2%), Gemmatimonadetes (2%), Nitrospira (2%). In the present research lower microbial diversity was observed, which can be related to a potential selection of microorganisms by the higher concentration of surfactant.

Table 2: Comparative study of the efficiency of LAS removal in anaerobic reactors.

Reactor configuration	Operation period (days)	Support material	Inoculum	Input (mg)	Degradation (%)	Phylogenetic characterization (predominance)	Reference
HAIB*	313	polyurethane foam	sludge from a sanitary sewage treatment	5,782	35	Phylum Firmicutes Class Clostridia Order Clostridiales	Duarte *et al*., 2008
Anaerobic stirred sequencing-batch	122	granular biomass without support	UASB reactor treating swine wastewater	13,100	53	Phylum Bacteroidetes	Duarte *et al*., 2010b
Fluidized bed	79	glass beads sand	UASB reactor treating swine wastewater	385	98.5 98.2	Phylum Proteobacteria Phyla Proteobacteria and Bacteroidetes	Oliveira *et al*., 2010b
Fluidized bed	270	sand	UASB reactor treating swine wastewater	7,760	93	Phyla Proteobacteria and Bacteroidetes Class Sphingobacteria	this study

*Horizontal-Flow Anaerobic Immobilized Biomass

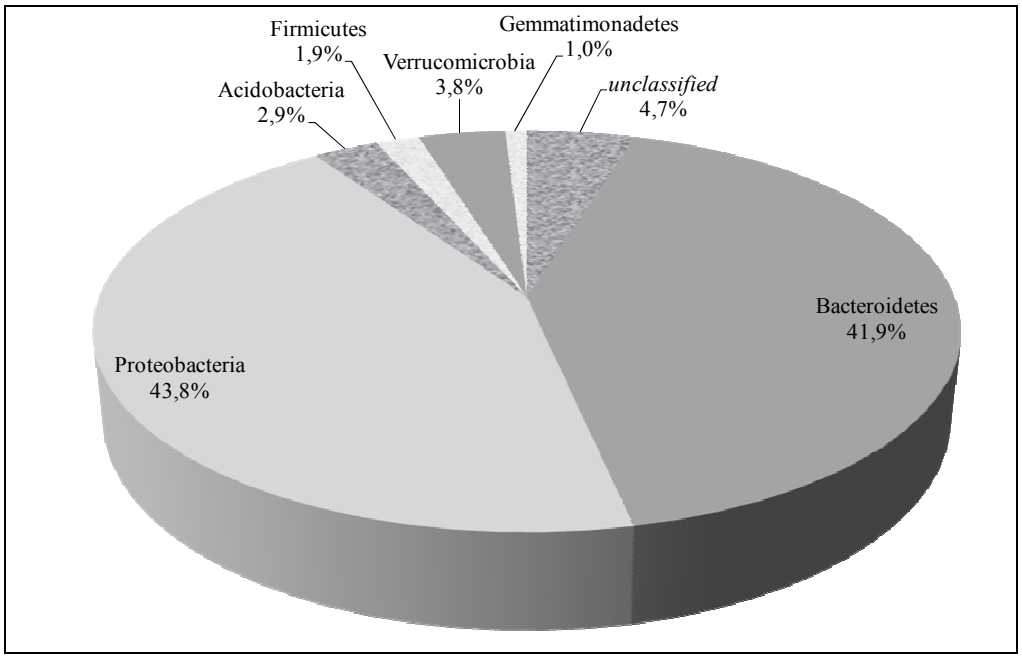

Figure 1: Phylogenetic representation of the microorganisms sampled during FBR operation

A total of 44 clones were related to the phylum Bacteroidetes, with 42 belonging to Class Sphingobacteria. The cells of species in this Class are gram-negative, non-spore-forming, straight rods that have no flagella but may exhibit sliding motility. Acid is produced from carbohydrates oxidatively but not fermentatively (Yabuuchi *et al*., 1983). *Niastela* sp. (related to 3 clones with 92% similarity) and *Terrimonas* sp. (related to 12 clones with 83% similarity), belonging to this Class, were also found in another reactor filled with sand (Oliveira *et al*., 2010b). The former is Gram-negative, aerobic, non-flagellated, gliding and filamentous. It was isolated from soil cultivated with Korean ginseng (Weon, H., *et al*., 2006). The latter is a nitrogen fixator, aerobic, gram-negative and filamentous. It was isolated from garden soil (Xie and Yokota, 2006). *Sediminibacterium* sp. and *Ferruginibacter* sp. were also similar to some OTUs. *Sediminibacterium* sp. is aerobic, Gram-negative rods, phylogenetically related to the genera *Terrimonas* and *Niastella* in the phylum Bacteroidetes (Qu and Yuan, 2008). In this research, they were similar to microorganisms present in activated sludge. *Ferruginibacter* sp. is gram-negative, aerobic, non-gliding, nonfilamentous single rods (Lim *et al*., 2009). The OTUs were similar to organisms present in water supply systems, in estrogen-degrading bioreactors, in soil and sea sediments receiving agricultural discharges.

Similarly to Oliveira *et al*. (2010b), the Phylum Proteobacteria represented a significant proportion of clones obtained (46), but in the present study, the Class Betaproteobacteria (presenting bacteria highly versatile in their degradation abilities) was more effective with 27 clones. Clones classified as Alphaproteobacteria (14), Gammaproteobacteria (4) and Deltaproteobacteria (1) were also obtained, differently from Oliveira *et al*. (2010b).

Three clones were related to *Aeromonas* sp. (100% sequence homology). It has been suggested that this genus degrades surfactants. Jimenez *et al*. (1991) studied a mixed microbial community consisting of three species of *Pseudomonas* and one *Aeromonas* sp. LAS was added at a concentration of 1 mg ml^{-1} as the only source of carbon, and all species studied were able to degrade over 25% of the input LAS and convert it to CO_2. Duarte *et al*. (2010) also found clones related to *Pseudomonas* in anaerobic batch reactor treating detergent.

Another clone was identified as *Novosphingobium*, with 99% 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). Moreover, this genus was also found in the sequencing of a batch anaerobic reactor treating detergent (Duarte *et al*., 2010).

Some clones were related to the genus *Rhodobacter* (98%) and *Novispirillum* (99%) belonging to the Phylum Proteobacteria, Class Alphaproteobacteria. In general, they are aerobic, anaerobic or facultative, gram-negative microorganisms, with broad metabolic capabilities, found in sewage treatment plants (Bergey and Holt, 1994).

Some clones were related to denitrifying bacteria (*Alicycliphilus*, 99%) (Heylen *et al*., 2006) and a nitrogen-fixing bacterium isolated from rice paddy soil (*Zoogloea*, 91-99%) (Xie and Yokota, 2006). These bacteria belong to the Class *Betaproteobacteria*. A clone was related to *Azospira* sp. with 99% similarity. This organism uses aromatic compounds. It was isolated from contaminated soils and water and catabolized benzoate and other aromatic compounds anaerobically (Bergey and Holt, 1994). The presence of *Azospira* sp. in a fluidized bed reactor fed with LAS can be justified by the aromatic ring linked to the sulfonate group in the surfactant molecule.

Several members of the phylum Firmicutes (family *Clostridiales*) can use xenobiotic compounds 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 arylsulfonates (Duarte *et al*., 2010). Some clones sequenced in this study were related to *Clostridium* sp. The reactor's nutritional composition favored the growth of these cells, since both carbohydrates and amino acids (yeast extract) were available.

To illustrate the microbial diversity, the phylogenetic tree related to Phylum Proteobacteria was constructed (Figure 2). Thus, the sequences (exceeding 600 bp) were grouped into operational taxonomic units (OTUs) with more than 95% similarity. Table 3 shows the clones present in Figure 2 classified in OTUs, as well their similarity to sequences with access number in NCBI and the reference.

Table 3: Sequences obtained from Genbank related to clones in the reactor, Phylum Proteobacteria

OTU	Number of Clones	Similarity (%)	Access NCBI	Description	Reference
01	2	98	AY244771	*Rhodobacter* sp. 2002-65602 16S ribosomal RNA gene, partial sequence	Drancourt, *et al*., unpublished
02	6	99	AM697216	Uncultured bacterium partial 16S rRNA gene, isolate BF0001C057	Rintala *et al*., 2008
03	2	99	AB680932	*Novispirillum itersonii* subsp. itersonii gene for 16S rRNA, partial sequence, strain: NBRC 15648	Nakagawa *et al*., unpublished
04	1	99	HM066524	Uncultured bacterium clone EDW07B003_75 16S ribosomal RNA gene, partial sequence	Gray and Engel, unpublished
05	1	99	FJ527720	*Novosphingobium subterraneum* strain F-4 16S ribosomal RNA gene, partial sequence	Liang and Li, unpublished
06	1	99	JN038823	Uncultured beta proteobacterium clone P-R36 16S ribosomal RNA gene, partial sequence	Zhang *et al*., unpublished
07	3	99	AM084015	*Alicycliphilus* sp. R-24604 16S rRNA gene, strain R-24604	Heylen *et al*., 2006
08	2	97	JF834291	Bacterium enrichment culture clone phytdeg33 16S ribosomal RNA gene, partial sequence	Dawson *et al*., unpublished
09	1	99	FJ393084	Uncultured *Azospira* sp. clone MFC-B162-C02 16S ribosomal RNA gene, partial sequence	Borole *et al*., 2009
10	4	100	JF808857	Uncultured bacterium clone R15-38 16S ribosomal RNA gene, partial sequence	Xia *et al*., unpublished
11	1	98	EF648041	Uncultured bacterium clone HB31 16S ribosomal RNA gene, partial sequence	Wang *et al*., unpublished
12	1	94	AB201044	*Zoogloea oryzae* gene for 16S rRNA, partial sequence, strain: A-4	Xie and Yokota, 2006
13	1	91	AB201044	*Zoogloea* sp. gene for 16S rRNA, partial sequence, strain: A-4	Xie and Yokota, 2006
14	4	99	AB201044	*Zoogloea oryzae* gene for 16S rRNA, partial sequence, strain: A-4	Xie and Yokota, 2006
15	1	93	JF834291	Bacterium enrichment culture clone phytdeg33 16S ribosomal RNA gene, partial sequence	Dawson *et al*., unpublished
16	2	99	AY548934	Uncultured bacterium clone 1-7 16S ribosomal RNA gene, partial sequence	Luo and Hu, unpublished
17	2	99	FM207908	Uncultured *Rhodocyclaceae* bacterium partial 16S rRNA gene, clone 408	Rotaru *et al*., 2010
18	1	99	HM921148	Uncultured bacterium clone cpw3d216 16S ribosomal RNA gene, partial sequence	White *et al*., unpublished
19	1	94	DQ315383	*Aeromonas* sp. ZL1-r 16S ribosomal RNA gene, partial sequence	Zhang *et al*., unpublished
20	2	100	AY987730	*Aeromonas schubertii* strain AE-48 16S ribosomal RNA gene, complete sequence	Jangid *et al*., unpublished

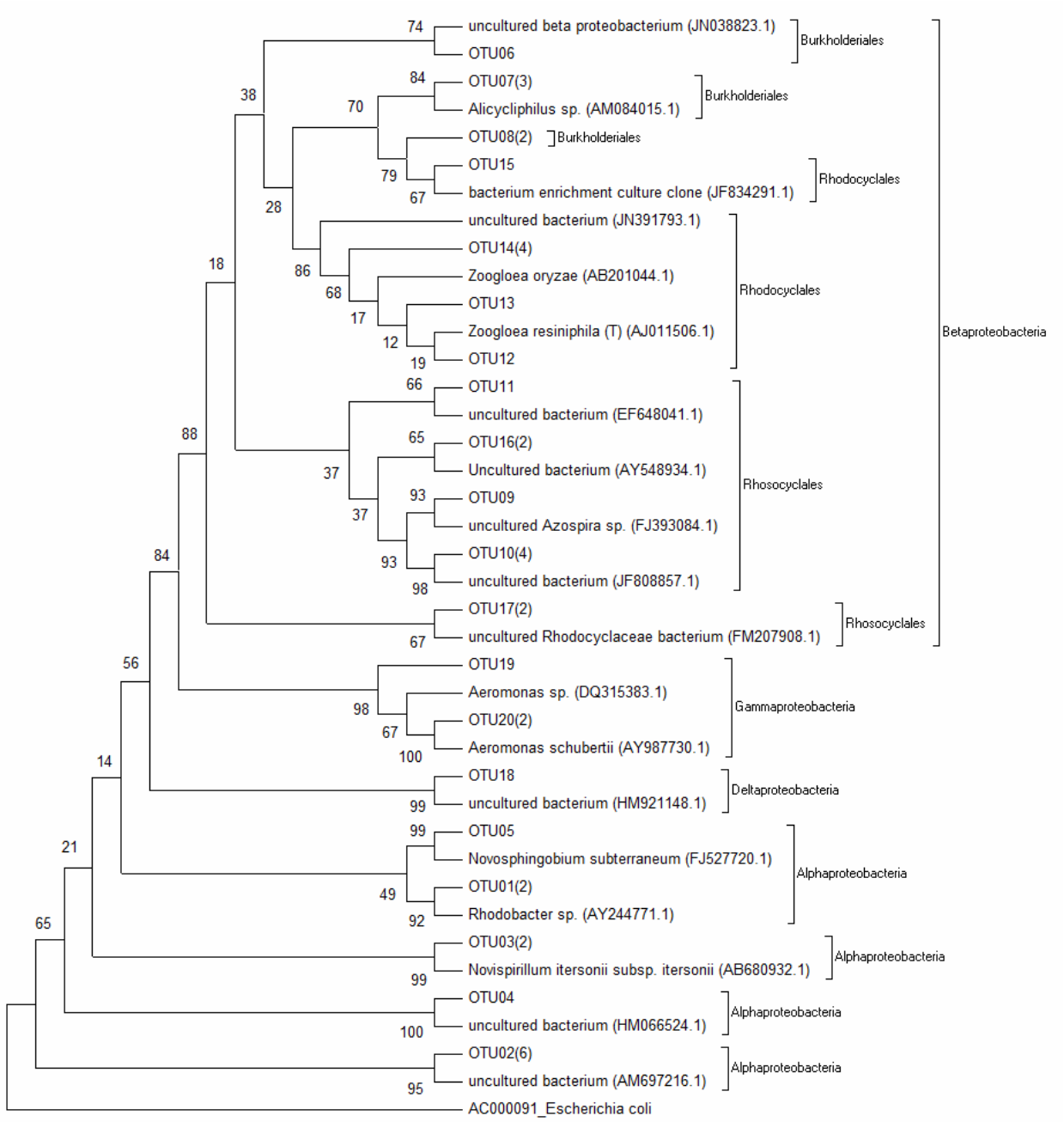

Figure 2: Phylogenetic analysis of partial 16S rRNA gene sequences of clones derived from the bioreactor sample and related microorganisms (Phylum Proteobacteria). The source of these clones and their Genbank accession numbers are listed after species names. *Eschericha coli* (AC000091) was used as an outgroup.

CONCLUSIONS

An anaerobic fluidized bed reactor with biofilm in sand was efficient for LAS removal, which was not affected by variations in the influent concentration. The COD removal remained high (above 90%) and stable under all studied conditions, even with the increase of LAS concentration.

By means of phylogenetic analyses, it was verified that the degradation of LAS occurred in the presence of a syntrophic mixed microbial community. This identification may help to elucidate the

possible bacteria present in the microbial consortium established in biofilms involved in LAS degradation. For example, *Aeromonas* spp., *Novosphingobium* spp. and *Azospira* spp. seemed to have a potential role in LAS degradation. *Aeromonas* spp. may use the molecule as a source of carbon or energy, *Novosphingobium* spp. and *Azospira* spp. may use the aromatic ring of the LAS molecule as a source of carbon and energy.

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