Application of molecular techniques to evaluate the methanogenic archaea and anaerobic bacteria in the presence of oxygen with different COD : sulfate ratios in a UASB reactor
Application of molecular techniques to evaluate the methanogenic archaea and anaerobic bacteria in the presence of oxygen with different COD:Sulfate ratios in a UASB reactor

Julia Sumiko Hirasawa *, Arnaldo Sarti, Nora Katia Saavedra Del Aguila, Maria Bernadete A. Varesche
Laboratório de Processos Biológicos, Departamento de Hidráulica e Saneamento, Escola de Engenharia de São Carlos, Universidade de São Paulo (EESC/USP), Avenida João Dagnone, 1100 Jardim Santa Angelina, 13.563-120 São Carlos, SP, Brazil

ARTICLE INFO

Article history:
Received 8 October 2007
Received in revised form
16 June 2008
Accepted 22 June 2008
Available online 25 June 2008

Keywords:
FISH
Oxygen
Ethanol
Sulfate-reducing bacteria
Methanogenic archaea

ABSTRACT

In this paper, the microbial characteristics of the granular sludge in the presence of oxygen (3.0 ± 0.7 mg O₂ l⁻¹) were analyzed using molecular biology techniques. The granules were provided by an upflow anaerobic sludge blanket (UASB) operated over 469 days and fed with synthetic substrate. Ethanol and sulfate were added to obtain different COD/SO₄²⁻ ratios (3.0, 2.0, and 1.6). The results of fluorescent in situ hybridization (FISH) analyses showed that archaeal cells, detected by the ARCh15 probe, accounted for 77%, 84%, and 75% in the COD/SO₄²⁻ ratios (3.0, 2.0, and 1.6, respectively). Methanoseta sp. was the predominant acetoclastic archaea observed by optical microscopy and FISH analyses, and confirmed by sequencing of the excised bands of the DGGE gel with a similarity of 96%. The sulfate-reducing bacterium Desulfobibrio vulgaris subsp. vulgaris (similarity of 99%) was verified by sequencing of the DGGE band. Others identified microorganism were similar to Shewanella sp. and Desulfitobacterium hafniense, with similarities of 95% and 99%, respectively. These results confirmed that the presence of oxygen did not severely affect the metabolism of microorganisms that are commonly considered strictly anaerobic. We obtained mean efficiencies of organic matter conversion and sulfate reducing higher than 74%.

© 2008 Elsevier Ltd. All rights reserved.

1. Introduction

Sulfur compounds, in sulfate, sulfide, and sulfite forms, are commonly found in the industrial effluents of several chemical and food industries, such as tanneries, paper mills, edible oil, molasses fermentation, and potato starch processing facilities [1–3]. These industrial effluents usually present COD:SO₄²⁻ ratios ranging from 1 to 10. It is important to investigate the factors that determine the competition between sulfate-reducing bacteria (SRB) and methanogenic archaea (MA) communities in order to improve the anaerobic treatment of these wastewaters [4]. The outcome of this competition can determine the composition of the biogas produced in bioreactors [1,2]. Some strategies for evaluating the competition between SRB and MA are pH and temperature variation [5] and oxygen exposition through anaerobic sludge [6].

However, the major problem is related to the microorganisms inhibition caused by the presence of the hydrogen sulfide formed in the sulfate-reducing process. This product is toxic to anaerobic microorganisms, especially the hydrogenothrophic MA. The competition between SRB and MA for the available substrate leads to a decrease in methane production. Some conditions have been applied to minimize the sulfide toxicity problem: chemical removal of heavy metals and sulfide by precipitation [3,7], pH control to ensure high-sulfide production in the HS⁻ form [8] that is less toxic and needs MA cellular synthesis, application of oxygen-limited concentrations [6], and partial biological oxidation of sulfide to elemental sulfur [2].

Some SRB compete interspecifically for available sulfite, while others have fermentative or syntrophic capabilities [8]. In the absence of electron acceptors, the SRB are able to grow by obtaining energy from the acidogenic reactions that can use propionate, pyruvate, lactate, and ethanol as organic substrates [2,8]. Ethanol has been used specifically as a carbon source and an electron donor for biological sulfate reducing in anaerobic reactors [9].

Anaerobic high-rate reactors, including upflow anaerobic sludge blanket (UASB), have been applied widely in the treatment of sulfate-containing wastewaters. The predominance of SRB in UASB reactors with pre-adapted sludge to sulfate has been reported [10]. However, little is known about the composition of the microbial population or the performance of the reactors...
inoculated with sludge that presents reduced pre-availability of sulfate with oxygen.

The development of the molecular biology tools has contributed to the detection, quantification, and identification of the microbial communities involved in the treatment of sulfate-containing wastewaters. Cloning and sequencing of 16S rRNA gene fragments provide information about the phylogeny of the microorganisms [11]. In addition, denaturing gradient gel electrophoresis (DGGE) and fluorescent in situ hybridization (FISH) techniques are appropriate tools for comparison and quantification, respectively, of the changes in the microbial composition in different bioreactors [12].

We investigated the microbial communities involved in the sulfate reduction under three different COD/SO$_4^{2-}$/C0$_1$ ratios (3.0, 1.6, and 2.0) and in the presence of 3.0 g O$_2$/C0$_1$. The microbial characterization of the granular sludge of the UASB reactor was carried out using optical microscopy, FISH, PCR/DGGE, and sequencing of fragments of 16S rRNA genes.

2. Materials and methods

2.1. Characteristics and operation of the UASB reactor

The UASB reactor used in this study had a total volume of 10.5 l. The reactor had a prismatic geometry (12 x 12 cm in the square cross-section) and 45 cm of height in the reaction chamber. Detailed dimensions of this reactor were presented previously [13]. Fig. 1 presents the experimental schema. The experiments in the UASB reactor were carried out continuously, under mesophilic conditions (30 ± 1°C), with a hydraulic retention time (HRT) of 12 h. The reactor was fed with a synthetic substrate composed by Zinder basal medium [14], supplemented with vitamins [15,16], trace metal solutions [14], and sodium bicarbonate (10%). The medium was prepared daily with tap water at a pH of 7–8. Ethanol and sulfate were used as organic and sulfur sources, respectively. The reactor was inoculated with approximately 4 l of the granular sludge from a full-scale UASB reactor used to treat poultry slaughterhouse wastewater. This sludge presented 52 g l$^{-1}$ of total volatile solids (TVS) and 59 g l$^{-1}$ of total solids (TS). The adaptation of this granular sludge was mainly required due to higher concentration of sulfate ion in the bench-scale experiments than in the poultry slaughterhouse wastewater from the full-scale UASB reactor.

Operational conditions of the UASB are described in Table 3. In period I, the granular sludge adaptation with a gradual increase in the ethanol and sulfate concentrations while maintaining a COD/SO$_4^{2-}$/C0$_1$ ratio of 3:1 in the absence of oxygen. In this period, the sulfate and ethanol concentrations were varied after the reactor had achieved stable operational conditions. These operational conditions were related to COD removal, sulfate reduction, and decrease of sludge washout in the effluent total suspended solids (TSS). Oxygen was added in the reactor influent, to the COD/SO$_4^{2-}$/C0$_1$ ratios of 3.0, 1.6, and 2.0 in subsequent periods (II, III, and IV). Finally, we studied the condition with no sulfate, but with oxygenation (V).

2.2. Oxygen gas injection system

The oxygen gas injection system was connected to the input of the UASB reactor (Fig. 1) to ensure the presence of oxygen in the feeding system. It is important to note that oxygen gas is very insoluble in water and requires special conditions to ensure high-absorption efficiency. A key feature that must be incorporated into an oxygen dissolution system is the oxygen retention time. It has been found that a retention time of approximately 100 s is necessary to optimize the absorption of pure oxygen. The U-tube contactor is one of the oxygen transfer systems with efficient dissolution of commercial oxygen and low unit energy consumption [17].

We formed a transparent flexible tube into a U-tube (diameter of 3/4 in.) for dissolution of oxygen gas into the liquid medium. The U-tube was connected to one microporose sparger (2 μm of porosity, Alltech Hastalloy) at the end of an oxygen pipeline that was also introduced into the U-tube contactor to promote the formation of microbubbles. Commercial oxygen (purity of 99%) was introduced into the U-tube by a timer-actuated solenoid valve that was set up in the intermittent form (30 min on and 15 min off). This strategy was used to avoid substrate oxidation in the tube. Although a flux meter was installed to maintain the desired oxygen concentration in the liquid medium, it was not possible to measure the flux rate. The commercial nitrogen gas was fluxed during a period of 30–40 min with a flow rate of 11 h$^{-1}$ to eliminate oxygen from the reactor feeding. Oxygen concentrations
were controlled by Horiba (OM-12) or Orion (810). Preliminary tests showed that the average oxygen concentration applied was $3.0\pm0.7\, \text{mgO}_2\,\text{l}^{-1}$, which was obtained using measurements taken every 5 min for a period of 3 h.

2.3. Analytical methods

The analyses of chemical oxygen demand (COD) as total COD and filtered COD (0.45 µm), sulfate, total dissolved sulfide (TDS), TSS, and total solids were determined according to APHA–AWWA–WPCF [18]. COD analyses were carried out in duplicate. Total volatile acids (TVA) and bicarbonate alkalinity (BA) concentrations were monitored according to the methodology described by Dillalo and Albertson [19], and adapted by Ripley et al. [20].

The biogas composition (methane (CH$_4$) and sulfide (H$_2$S)) was monitored by gas chromatography using a Porapak-Q column (2 cm × 1/4 in.–80/100 mesh) and a Gow-Mac gas chromatograph (Gow-Mac Instrument Co., Shannon, Ireland). Hydrogen was used as the gas carrier at 50 °C. These analyses were carried out in duplicate.

The oxidation–reduction potential (ORP) was monitored with an ORP electrode (DMR-CPI, Digimed, platinum electrode, Ag/AgCl reference) connected to a digital pH meter (UB-10, Denver Instruments). The ORP measures were taken from four different sampling ports of the UASB reactor: P1 (bottom of the sludge blanket), P2 (top of the sludge blanket), P5 (close to the gas separator system), and the effluent (Fig. 1). The relation between the ORP value measured against the Ag/AgCl electrode and the corresponding ORP value measured as a function of the standard hydrogen is given by the following equation [18]:

$$E_{\text{system}} = E_{\text{observed ZoBell}} - E_{\text{ZoBell ZoBell}}, \quad \text{where} \quad E_{\text{ZoBell ZoBell}} = 417\,\text{mV} \quad \text{and} \quad E_{\text{observed ZoBell}} = 224\,\text{mV at 30 °C.}$$

A vessel (50 ml) with a Teflon cover sealed with an o-ring was connected to the reactor sampling ports to measure ORP. The ORP value was monitored to verify the anaerobic/anoxic condition of the reactor.

2.4. Microbial characterization

Granules from the inoculum and from the sludge of two sampling ports (P1 and P2) of the UASB reactor were withdrawn at the end of each experimental phase and analyzed separately for microbial composition by optical microscopy, FISH, and PCR/DGGE techniques.

2.5. Microscopic examination and FISH

The granular sludge samples were crushed with a sterile mortar and pestle for use in microscopic observations of the in situ microbial community and fixation for FISH analyses. We used an Olympus BX60-FLA microscope coupled with a refrigerated digital camera (Evolution QE; Media Cybernetics Inc., USA).

One milliliter of this crushed biomass was added to antibiotic flasks containing 5 ml of phosphate buffered saline solution (PBS: 0.13 M NaCl, 7 mM Na$_2$HPO$_4$, 3 mM Na$_2$H$_2$PO$_4$; pH 7.2) and glass beads. The flasks were agitated for 20 min and 1 ml of each aliquot was transferred to a sterile microcentrifuge tube. These samples were used to quantify microorganisms by FISH.

The oligonucleotide probes used for FISH were EUB338 (Bacteria domain) [21], ARC915 (Archaea domain) [22], SRB385 (SRB of δ-Proteobacteria) [21], and NON338 (negative control) [23]. These probes were 5′-end-labeled with rhodamine or CY3. The fixation and hybridization conditions were described previously by Araujo et al. [24]. Samples were examined with an Olympus microscope, using specific filter sets for DAPI and rhodamine or CY3. A total of 800–1000 DAPI-stained cells and their respective hybridized cells in 10–20 independent microscopic fields were counted for each probe and sample. The Image-Pro-Plus 4.5 software was used to acquire and analyze images.

2.6. PCR, DGGE, and sequencing

Microbial diversity was characterized by PCR/DGGE techniques in samples taken from the inoculum and granular sludge of the UASB reactor at the end of each operational condition. The DNA extraction of the samples was done with glass beads, phenol, and chloroform as described by Griffiths et al. [25]. The primers used for partial amplification of 16S rRNA genes corresponding to the Bacteria domain [26], Archaea domain [27], and SRB group [28] are presented in Table 1. A thermocycler (Gene Amp PCR System 2400; Perkin-Elmer Cetus, Norwalk, CT) was used for the amplification. The reaction components used for amplification by PCR were 5 µl of dNTPs (2 mM each: dATP, dCTP, dGTP, dTTP), 5 µl of 10X PCR buffer, 1.5 µl of MgCl$_2$ (50 mM), 0.5 µl of each primer (100 pmol, Invitrogen), 0.5 U AmpliTaq DNA polymerase (5 U ml$^{-1}$), 2 µl of DNA template (50–100 ng). These components were brought to 50 µl total volume with water. The cyclic parameter conditions [29] used for each microbial group by the thermocycler are described in Table 2.

The amplified product was analyzed on a 1% agarose gel and used in the DGGE [29]. The samples were transferred to gel with 10% of acrylamide–bisacrylamide, with 30–70%, 20–60%, and 40–60% of denaturants (urea and formamide) for the gel DGGE of Bacteria domain, SRB group and Archaea domain, respectively. We used an electrophoretic time of 16 h in 1X TAE buffer at 65 °C and a constant voltage (75V). The gel was stained with ethidium bromide for 20 min and displayed on a UV transilluminator.

Bands with a high intensity on each DGGE gel were excised, eluted, re-amplified, and sequenced. The bands were cut out from the DGGE gel and eluted, separately, in 20 µl of water and stored at 4 °C overnight. Eluted DNA was re-amplified using the primers without clamp GC, purified and sequenced using MegaBACE 1000 (GE Healthcare). The identification of the different microorganisms was performed using a set of sequencing reagents based on didexoxynucleotides marked with fluorochrome (dye terminators) to facilitate automatic analysis. Mold DNA and primer concentrations followed manufacturers’ recommendations. The sequencing reactions were purified by Sephadex G50 column and sequenced. Analysis was performed using Sequence Analyzer 3.0 software with basecaller Cimarron 3.12. The sequences were checked using the DNA Star program (SeqMan). These sequences were subsequently compared with other sequences previously deposited in GenBank (National Center for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov), using the Alignment Basic Local
Values with parentheses indicate maximum and minimum values and “–” indicates not observed.

### Table 2
The cyclical parameters of the thermocycler program used for the 16S rRNA gene fragment amplification

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of cycles</th>
<th>Initial denaturation</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final extension</th>
<th>Cooling (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Archaia</td>
<td>35</td>
<td>94 C for 1.30 min</td>
<td>94 C for 30 s</td>
<td>55 °C for 30 s</td>
<td>72 C for 1.30 min</td>
<td>94 C for 3 min</td>
<td>4</td>
</tr>
<tr>
<td>Bacteria</td>
<td>35</td>
<td>94 C for 5 min</td>
<td>94 C for 45 s</td>
<td>38 °C for 1 min</td>
<td>72 C for 2 min</td>
<td>72 C for 10 min</td>
<td>4</td>
</tr>
<tr>
<td>SRB</td>
<td>10</td>
<td>94 C for 5 min</td>
<td>94 C for 45 s</td>
<td>62 °C for 1 min</td>
<td>72 C for 1.30 min</td>
<td>72 C for 10 min</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>94 C for 45 s</td>
<td>57 °C for 1 min</td>
<td>72 C for 1.30 min</td>
<td>72 C for 10 min</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 3
Mean values obtained for the different operation conditions of the UASB reactor

<table>
<thead>
<tr>
<th>Variables</th>
<th>Experimental periods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>COD influent (mg l⁻¹)</td>
<td>40 ± 40</td>
</tr>
<tr>
<td>Sulfate influent (mg l⁻¹)</td>
<td>511 ± 151</td>
</tr>
<tr>
<td>COD:sulfate ratio</td>
<td>40 ± 40</td>
</tr>
<tr>
<td>Oxygen</td>
<td>Adaptation</td>
</tr>
<tr>
<td>pH influent</td>
<td>7.8 ± 0.2</td>
</tr>
<tr>
<td>pH influent removal (%)</td>
<td>76 ± 76</td>
</tr>
<tr>
<td>COD_removal (%)</td>
<td>74 ± 74</td>
</tr>
<tr>
<td>BA influent (mg CaCO₃ l⁻¹)</td>
<td>561 ± 45</td>
</tr>
<tr>
<td>BA effluent (mg CaCO₃ l⁻¹)</td>
<td>832 ± 104</td>
</tr>
</tbody>
</table>

Search Tool (BLASTn). The aligned nucleotides were used to build the consensus phylogenetic trees using neighbor-joining algorithm and Mega 3.1 software [30].

### 2.7. Granule-size distribution

Aliquots of the granule samples were collected at the end of each experimental period and transferred into a Petri dish. Images of these granules were captured with an Olympus BH2T microscope equipped with an image acquisition camera. Analysis was performed with Image Pro-Plus 4.5 software to determine the granule size distribution (mean diameter), assuming that granules are spherical in shape. Approximately, 1000 granules were counted in each sample, and these results were treated statistically by Anova one-way test (α = 0.05). This methodology was adapted from Alphenaar et al. [31].

### 3. Results and discussion

#### 3.1. Performance of the UASB reactor

Table 3 presents the results of the UASB reactor performance. The mean removal efficiencies of organic matter (COD_total) were 77% in I (adaptation, with no oxygen) and 76%, 74%, 77%, and 82% in the periods with oxygen (II, III, IV, and V), respectively. The organic matter measured as filtered COD (COD_filtered) was equal to 83%, 81%, and 77% in I, II, and III, respectively. COD_filtered values did not vary drastically from COD_total values, probably due to the reduced presence of the TSS in the reactor effluent (< 64 mg l⁻¹).

Therefore, the determination of COD_filtered was not carried out in IV and V. Mean COD removal efficiencies higher than 70% were obtained even in the presence of oxygen.

Anaerobic process stability of the UASB reactor was accomplished by the TVA and BA concentrations. Effluent BA was higher than the influent concentration during reactor operation. This fact is related to the lowest TVA concentrations determined in the effluent. However, effective consumption of the generated TVA occurred in the anaerobic process (Table 3). The means of BA concentrations (as calcium bicarbonate, CaCO₃) were 511 (max: 651; min: 151 mg l⁻¹) in the influent and 848 mg l⁻¹ (max: 1157; min: 510 mg l⁻¹) in the effluent. The influent and effluent TVA concentrations (as acetic acid, HAc) achieved mean values of 180 (max: 524; min: 91 mg l⁻¹) and 124 mg l⁻¹ (max: 199; min: 93 mg l⁻¹), respectively. These data showed a stable system.

The influent and effluent TVA concentrations (as acetic acid, HAc) achieved mean values of 180 and 124 mg Hc l⁻¹, respectively. These data showed a stable system. The pH values in the reactor effluent remained relatively constant and equal to 7.5, even in the presence of oxygen. Zitomer and Shrouf [32] observed that the completely stirred anaerobic reactors recover their initial pH in the presence of 1 g O₂LR l⁻¹ and 0.1 g O₂LR l⁻¹ on the 34 and 28 days of operation, respectively. Recovery of the pH level did not occur with the strictly anaerobic reactor even after 52 days of operation. Problems with pH alterations were not specifically observed in this study. However, the system was adequate for operation under anaerobic conditions and presented buffer capacity.

The sulfate reducing in I (adaptation, with no oxygen) presented the mean sulfate removal efficiency of 89% for the influent concentrations that varied from 157 to 345 mg l⁻¹. In
periods II, III, and IV, which were operated with oxygen, the sulfate reducing data presented some variations. However, the sulfate removal achieved 92%, 74%, and 81% in II, III, and IV, respectively (Table 3), from the influent concentrations of 370, 758, and 729 mg l⁻¹, respectively. Lower mean sulfate removal efficiencies of 74% occurred in III, probably due to the organic matter limitation in the reactor feed in terms of the influent sulfate concentration of 758 mg SO₄²⁻ l⁻¹ and 1158 mg COD l⁻¹. The sulfate removal increased to 81% when the organic matter concentration was increased to 1448 mg COD l⁻¹, with an unaltered sulfate concentration (729 mg SO₄²⁻ l⁻¹) in IV. These results support the occurrence of the lack of the organic substrate in the sulfate reducing process in III.

We verified that the methane (CH₄) and hydrogen sulfide (H₂S) concentrations were similar in all the experimental periods, even after oxygen application. The mean CH₄ concentration was equal to 31.9 μmol CH₄ ml⁻¹ in I (with no oxygen). This value was lower than those observed in II and III (34.9 and 35.2 μmol CH₄ ml⁻¹), with oxygen, and COD/SO₄²⁻ ratios of 3.0 and 1.6, respectively. Other values were equal to 31.3 and 30.8 μmol CH₄ ml⁻¹ in IV and V, respectively (Table 3). These results indicated that the oxygen concentration applied (3.0 mg l⁻¹) did not inhibit CH₄ production. Methane production in the presence of oxygen in an anaerobic batch reactor treating synthetic wastewater containing sucrose was reported previously [32]. We did not observe inhibition in the CH₄ production in relation to the initial control condition represented by I due to the predominance of MA cells in the inoculum.

Lower removal of COD_total and sulfate (both equal to 74%) occurred, in spite of the slightly higher CH₄ concentration in III (COD/SO₄²⁻ ratio of 1.6) compared with the other periods. In this period, higher H₂S concentrations in biogas (1.8 μmol ml⁻¹) and TDS (H₂S, HS⁻, and S²⁻; 96 mg l⁻¹) were obtained (Table 3). The pH value was equal to 7.6 in the effluent of this period. Under this condition, we found more than 80% of TDS as the HS⁻ form with a concentration of 79 mg l⁻¹.

Among the ionized species (HS⁻ and S²⁻) and non-ionized species (H₂S), only the latter is able to pass through the cellular membrane and corresponds to the more toxic form [33]. The HS⁻ form is utilized in the cellular synthesis of the anaerobic microorganisms [3]. The mean H₂S concentration in the biogas in the adaptation period (I), with no oxygen, was equal to 1.3 μmol ml⁻¹. In the presence of oxygen, these concentrations were 1.3 and 1.8 μmol H₂S ml⁻¹ in the biogas of II and IV, respectively. Anaerobic reactors with a high capacity for biomass retention, such as UASB reactors and biological filters, can tolerate high-sulfide levels of approximately 170 mg H₂S l⁻¹ [33]. Moreover, studies carried out under mesophilic and thermophilic conditions showed that granular sludge is less inhibited by H₂S than suspended sludges at low and neutral pH [2]. In this study, however, SRB and AM were not inhibited by the applied sulfate concentrations in which TDS concentrations varied from 44 to 96 mg l⁻¹ in the liquid medium (Table 3).

The ratios between the produced sulfide and the quantity of removed sulfate were equal to 0.18, 0.20, 0.17, and 0.16 during the four experimental periods (I, II, III, and IV). However, these results were lower than the theoretical ratio of 0.33 (S²⁻/SO₄²⁻) because part of the sulfide present in the liquid medium of the reactor was converted to elemental sulfur (S⁰) (biologically oxidized). The presence of S⁰ was inferred from a yellowish precipitate that was observed in the reactor interior and in the effluent. The presence of this material in the S⁰ form (93.5%) was confirmed by EDX analysis (results not shown). Partial biological oxidation of sulfide to S⁰ in the presence of low-oxygen concentrations is a known alternative to the bioconversion of sulfur compounds [2].

The ORP values were obtained in the periods with oxygen (II, III, and IV) in the UASB. Significant alterations of ORP values were not observed during the reactor operation in different samples: P1 and P2 (bottom and top parts of the sludge blanket), P5 (close to the gas separator system), and the effluent. The mean ORP values were −203, −208, and −215 mV in II, III, and IV, respectively (Table 3). According to Widdel [34], a favorable ORP for sulfate reducing should vary from +200 to −200 mV in aerobic and anaerobic microchirns, respectively. The ORP values, necessary for the development of MA community, are approximately −200 mV in natural habitats and vary from −330 to −400 mV in controlled pure and mixed cultures. Therefore, the observed ORP values were favorable for sulfidogenesis and methanogenesis in the UASB.

### 3.2. Microbial community analyses

Fig. 2 presents morphologies of rods, oval-rods, cocci, and filaments observed under optical microscopy with phase contrast. These morphologies did not vary significantly in the granular sludge samples of the UASB reactor in all periods studied. Fluorescent rods (hydrogenothrophic) as well as cells similar to Methanoseta sp. and Methanosarcina sp. (acetoclastic) were observed among the MA. These microorganisms are generally considered more sensitive to oxygen; however, the thickness and the granules’ architecture represented a physical barrier to oxygen diffusion, creating segregated zones with low-oxygen concentrations in their central region [35].

**Fig. 2.** Microorganism morphologies in the granular sludge samples of the UASB reactor with 3.0 ± 0.7 mg O₂ l⁻¹: (a) diversity, (b) Methanoseta-like cells in P1 port and COD:sulfate ratio of 2.0, and (c) oval rods in P1 port and COD:sulfate of 1.6 (1600 x).
The mean values of P1 and P2 samples accounted for 68%, 77%, 75%, 84%, and 87% for the MA community in I, II, III, IV, and V, respectively. These results showed that the syntrophic relations between facultative bacteria and MA were favored in the presence of oxygen (II, III, and IV). Specifically, the COD/\(\text{SO}_4^{2-}\) ratio of 2.0 (1448 mg COD l\(^{-1}\), 729 mg \(\text{SO}_4^{2-}\) l\(^{-1}\), and 3.0 mg O\(_2\) l\(^{-1}\)) accounted for 84% of these MA microorganisms in relation to the total quantified cells. It is also important to highlight that MA were mainly favored in the absence of sulfate in V. FISH results were in accordance with the \(\text{CH}_4\) production that resulted in values equal to 31.9, 34.9, 35.2, 31.3, and 30.8 \(\mu\text{mol ml}^{-1}\) in I, II, III, IV, and V, respectively.

When the sludge blanket mixing is low in lab-scale anaerobic reactors, facultative bacteria could be present near the inlet at the bottom of the reactor and uptake the dissolved oxygen present in the influent. Furthermore, channeling/dead zones may also occur, and oxygen contact time and gas–liquid mass transfer are low. Low upward liquid velocity and a low-gas production could also lead to an increase in the external mass transfer resistance. Finally, if facultative bacteria are present at the granule surface, oxygen consumption and diffusional limitations could prevent inhibition of obligate anaerobic bacteria located at the inner core. Therefore, mixing patterns and mass transfer phenomena could contribute to the results that are presented for reactor performance in the presence of oxygen. According to Kato et al. [36], anaerobic granular sludges presented higher tolerance to oxygen (2–23 mg O\(_2\) l\(^{-1}\)) in the presence of ethanol in the liquid medium that led to \(\text{CH}_4\) production.

The mean percentages in the P1 and P2 samples of bacteria detected by the EUB338 probe accounted for 37%, 23%, 26%, 16%, and 13% in I, II, III, IV, and V, respectively (Fig. 3). These results showed that the imposed nutritional conditions in the experiment probably were restrictive to maintain the growth of 38% of bacteria from the inoculum. This community gradually decreased as a function of the operation time, probably because it was damaged by the reduced diversity of the organic compounds that provided a selective metabolic group able to use ethanol in the presence of oxygen leading to acetate acid production.

Concomitantly, aceticogenic bacteria used ethanol and produced acetic acid and hydrogen. Moreover, the available energy produced as a result of the ethanol oxidation is dependent on a final electron acceptor (present in the anaerobic system) or, in a last analysis, on the hydrogenotrophic bacteria predominant in the reactor. Thus, the SRB hydrogen-utilizers should lead to the ethanol degradation towards sulfidogenesis.

Fig. 3. Microbial community composition determined by FISH in the inoculum and the granular sludge samples taken from P1 and P2 sampling ports in the respective experimental periods of reactor operation represented by relative abundance values of cell members of the domains Bacteria (EUB338) and Archaea (ARC915), the sum of these two domains (EUB338+ARC915), and SRB Group of \(\delta\)-Proteobacteria (SRB385). Error bars are standard error.

MA cells use final compounds, such as hydrogen, acetate, carbon dioxide, methanol, and methylamines [37]. However, under studied conditions, the consortium formed by aerobic and facultative bacteria present in the bottom (P1) of the UASB reactor should promote the production of suitable intermediary compounds for growth and maintenance of the MA. The presence of these microbial consortia should have been more significant in P1 than P2 once the oxygen gas was added in the inlet of the UASB reactor. However, it was not possible to observe considerable differences in the percentages of the Bacteria domain in these two sampling points in the temporal analyses carried out in the UASB reactor.

The mean percentages of P1 and P2 of SRB detected by the SRB385 probe accounted for 11%, 10%, 15%, 9%, and 8% of the granular sludge samples in I, II, III, IV, and V, respectively. A COD/\(\text{SO}_4^{2-}\) ratio of 1.6 promoted the major growth of SRB (15%) in the presence of oxygen (Fig. 3). These values were similar to those observed for the inoculum (15%). However, the SRB were favored by the major influence sulfate concentration in relation to the COD/\(\text{SO}_4^{2-}\) ratios of 3.0 and 2.0 in this condition (III).

The SRB group is divided into [37] group I (non-acetate oxidizers), which includes the Desulfovibrio sp., Desulfobulbus sp., and Desulfotomaculum sp.; and group II (acetate oxidizers), which includes Desulfobacter sp., Desulfbacterium sp., Desulfovarcina sp., and Desulfococcus sp. The group I sulfate reducers utilize lactate, pyruvate, ethanol or certain fatty acids as electron donors, reducing the part of sulfate to hydrogen sulfide. The genera in group II are specialists in the oxidation of fatty acids, mainly acetate, reducing sulfate to sulfide. Although both groups were probably present in the reactor, the SRB of group II were not favored due to the competition with acetoclastic MA that predominated during the operational period.

The nature of the methanogenesis shows the importance of the microbial interactions that prevent the accumulation of organic acids and alcohols in the fermentation reaction. The posterior degradation of the acids will also depend on the hydrogen presence as measured by bacteria that produce and utilize this gas. Ethanol degradation to acetic acid is thermodynamically favorable, and acetic acid oxidation reaction mediated by SRB is thermodynamically more favorable than acetoclastic methanogenesis [33]. In this study, we verified the inverse functional process for the predominance of the acetoclastic MA (\(\text{Methano-}\)saeta sp.) that is a specialist in acetate utilization.

O’Reilly and Colleran [38] also observed great diversity in microorganisms of the granular sludge in the inoculum of a UASB
reactor used to treat the wastewater from citric acid processing. They observed cocci, microcolonies of *Methanosaeta* sp., present as rods and filaments, long and thin filaments (possibly *Methanobacterium bryantii* or *M. formicicum*) and *Methanosarcina*-like aggregates in the granule surface. For the COD/SO$_4^{2-}$ ratio of 4, the predominant microorganisms were curved rods (similar to *Desulfovibrio* sp.), as well as bulb-shaped coccobacilli, resembling *Desulfobulbus*-type species. The authors still noted that this diversity remained at the COD/SO$_4^{2-}$ ratio of 2 but was predominantly *Desulfovibrio* sp. (for higher sulfate concentration in the influent), as determined by FISH. They also observed *Methanosaeta* sp. by scanning electronic microscopy (SEM). In this study, we observed higher growth of SRB in the presence of oxygen, as determined by FISH. They also observed aggregates in the granule surface. For the COD/SO$_4^{2-}$ ratio of 4, the predominant microorganisms were curved rods (similar to *Desulfovibrio* sp.), as well as bulb-shaped coccobacilli, resembling *Desulfobulbus*-type species. The authors still noted that this diversity remained at the COD/SO$_4^{2-}$ ratio of 2 but was predominantly *Desulfovibrio* sp. (for higher sulfate concentration in the influent), as determined by FISH. They also observed *Methanosaeta* sp. by scanning electronic microscopy (SEM). In this study, we observed higher growth of SRB in the presence of higher sulfate concentrations in the influent (758 ± 124 mg l$^{-1}$) for the COD/SO$_4^{2-}$ ratio of 1.6.

Fig. 4 presents DGGE profiles obtained after DNA amplification by PCR of the inoculum and samples withdrawn from the UASB. Several bands of the gels were cut and submitted for amplification, but only bands indicated with enumerated arrows were sequenced with success. These bands presented favorable amplification results for the posterior sequencing.

Table 4 describes the information obtained from the sequencing of the excised bands, with identified microorganism names, GenBank databases access number, similarity, and references. The consensus phylogenetic trees were constructed based on these sequences for *Bacteria* and *Archaea* domains, and for the SRB group (Fig. 5). We concluded that oxygen did not significantly affect the communities in terms of microbial diversity. This conclusion was drawn because all the bands of the inoculum (lane 1) were present in the samples obtained at different periods, as well as times when the sulfur source was extinguished but ethanol existed in the influent composition (lanes 8 and 9).

The sequenced bands (E1, E2, and E3) were observed in all lanes (Fig. 4a). The sequences were similar to *Shewanella* sp. (band E1) and uncultured bacterium (band E2), both with similarity of 95% and *Desulfotobacterium hafniense* with similarity of 99% (band E3). *Shewanella* sp. is very versatile and able to use diverse compounds as electron acceptors. This microorganism can use Fe (III), Mn (IV), S$_6$, S$_2$O$_3^{2-}$, NO$_3^-$, NO$_2^-$, and other compounds in the presence of oxygen [39]. However, *Shewanella* sp. was involved in the cycle of sulfur compounds, probably using the $S^-$ that was identified in the sludge blanket of the UASB reactor. Uncultured bacterium (AY945891) was previously identified from the secondary sedimentation tank of wastewater from a coking and chemical treatment plant (Shangai, China) that was studied in a denitrifying quinoline-removal bioreactor [40]. *D. hafniense* (AP008230) were previously isolated from an environment contaminated with dechlorinate tetrachloroethene (PCE) [41]. Suyama et al. [42] observed that *D. hafniense* were capable of reducing either sulfate or nitrate when pyruvate was the electron donor. The authors did not observe microbial growth in media with succinate, citrate, malate, acetate, glutamate, alanine, methanol, ethanol, glucose, fructose, lactose, and maltose. Moreover, oxygen completely inhibited the growth of *D. hafniense*. However, the band used to identify this microorganism was observed in all samples from the UASB reactor (Fig. 4a). This observation suggests the presence of a consortia of aerobic and facultative bacteria (oxygen consumers), in addition to the possible presence of other subproducts of anaerobic degradations that occurred in the presence of the sulfate and ethanol that supported maintenance of *D. hafniense* in the UASB reactor.

![Fig. 4. Profile of DGGE gels of the amplified PCR products with primers for (a) Bacteria domain (968FGC and 1392R), (b) SRB group (341FGC and 907R), and (c) Archaea domain (1100FGC and 1400R), for samples of inoculum and sludge blanket of the UASB reactor operated in COD:sulfate ratios of 3.0, 1.6, and 2.0 and with no sulfate in the presence of oxygen. (1: inoculum; 2: P1 COD:sulfate of 3.0; 3: P2 COD:sulfate of 3.0; 4: P1 COD:sulfate of 1.6; 5: P2 COD:sulfate of 1.6; 6: P1 COD:sulfate of 2.0; 7: P2 COD:sulfate of 2.0; 8: P1 with no sulfate; 9: P2 with no sulfate).](image-url)

### Table 4

<table>
<thead>
<tr>
<th>Group</th>
<th>Microorganism</th>
<th>Access number</th>
<th>Similarity (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td><em>Shewanella</em> sp.</td>
<td>CP000444</td>
<td>95</td>
<td>Copeland et al. (2006, not published)</td>
</tr>
<tr>
<td></td>
<td>Uncultured bacterium</td>
<td>AM945891</td>
<td>95</td>
<td>[40]</td>
</tr>
<tr>
<td></td>
<td><em>Desulfotobacterium hafniense</em></td>
<td>AP008230</td>
<td>99</td>
<td>[41]</td>
</tr>
<tr>
<td><strong>SRB</strong></td>
<td>Uncultured eubacterium</td>
<td>AF005594</td>
<td>94</td>
<td>Copeland et al. (2007, not published)</td>
</tr>
<tr>
<td></td>
<td><em>Desulfovibrio vulgaris subsp. vulgaris</em></td>
<td>CP000527</td>
<td>99</td>
<td>[47]</td>
</tr>
<tr>
<td></td>
<td>Uncultured bacterium</td>
<td>DQ191727</td>
<td>94</td>
<td>Enright et al. (2005, not published)</td>
</tr>
<tr>
<td><strong>Archaea</strong></td>
<td>Uncultured Methanoseta sp.</td>
<td>AB288519</td>
<td>96</td>
<td>Ishii and Watanabe (2006, not published)</td>
</tr>
<tr>
<td></td>
<td>Uncultured archaeon</td>
<td>DQ478742</td>
<td>97</td>
<td>[12]</td>
</tr>
<tr>
<td></td>
<td>Uncultured archaeon</td>
<td>AM570646</td>
<td>99</td>
<td>[52]</td>
</tr>
</tbody>
</table>
DGGE profiles related to the SRB diversity showed that the B1, B2, and B3 bands were present in all samples (Fig. 4b). However, some alterations occurred in the SRB communities, as shown in the first two bands of lane 1 (inoculum) and lane 2 (P1, COD/SO$_4^{2-}$ ratio of 3.0, with oxygen) that did not appear in the other conditions. A considerable difference was observed in the SRB communities that predominated in the assays, specifically the communities that were present in the central part of the gel, with the exception of lane 7 (P2, COD/SO$_4^{2-}$ ratio of 2.0, with oxygen). The SRB populations were probably prejudiced by the nutritional conditions applied to the system, or they could have occurred because of some experimental error in this analysis. FISH analyses also did not show significant differences in SRB percentages, with 9 ± 0.8% in this condition. The PCR/DGGE method allows observation of populations in concentrations higher than 10$^4$ cells ml$^{-1}$ [43]. In this way, the populations remained in the sludge blanket in this nutritional condition, but with lower values that were not detected by this technique.

SRB sequences had 99% similarity to Desulfovibrio vulgaris subsp. vulgaris (band B2), and two uncultured species, registered as eubacterium (band B1) and bacterium (band B3), both had 94% similarity (Fig. 4b; Table 4). Desulfovibrio sp., commonly found in wastewater treatment systems, is very versatile and capable of adaptation in oxygen, forming aggregates or migrating to anoxic zones, or they may alternatively use oxygen as an electron acceptor [44,45]. An interesting feature of SRB is their capacity to perform acetogenic oxidation in syntrophy with hydrogenotrophic methanogens, as described in cocultures with Desulfovibrio sp. using lactate and ethanol [3,35]. Furthermore, SRB can reduce oxygen using the same organic substrates applied in the sulfate reduction, such as hydrogen, lactate, alcohols, acetate, and others, and oxidize reduced sulfur compounds to hydrogen sulfide in the aerobic respiration [46]. It was possible to conclude that this population was present in all the nutritional conditions (Fig. 4b) due to its metabolic adaptability.

Uncultured eubacterium (AF050594) was previously studied in the evaluation of microbial diversity in an aquifer contaminated with hydrocarbon and chlorinated solvent and undergoing intrinsic bioremediation [47]. Moreover, uncultured bacterium (DQ191727) was identified in a study of microbial community dynamics of psychrophilic anaerobic bioreactors treating pharmaceutical wastewater that contained solvent (Enright et al., 2005, unpublished).

The band B3 represented phylogeny closely related to Desulfofobacter sp. It was also similar to the morphology observed by optical microscopy (Fig. 2c) and FISH. Laanbroek [48] noted that Desulfofobacter sp. was less competitive in ethanol and sulfate than Desulfovibrio sp. and Desulfobulbus sp.; on the other hand, Desulfofobacter sp. presented the highest maximum specific growth rate among these three sulfate reducers in the presence of excess ethanol and sulfate. However, Desulfofobacter sp. has the particular ability to use acetate in the presence of sulfate [35]. Desulfofobacter sp. is strictly anaerobic and presents a type of respiratory metabolism with sulfate or other oxidized sulfur compounds as the final electron acceptors, which are reduced to H$_2$S. The optimal growth temperature and pH are 28–32°C and 6.2–8.5, respectively. Most strains require NaCl and MgCl$_2$. Desulfofobacter sp. is chemoorganotrophic and use acetate or other simple organic compounds as carbon sources and also as electron donors for anaerobic respiration, which they completely oxidized into CO$_2$. Other strains, such as D. postgatei use only acetate as the electron donor and carbon source; however, other strains can also use ethanol and/or lactate [15].

DGGE profiles for the Archaea domain showed the predominance of three species in the inoculum that remained in the
evaluated operational periods of the reactor (Fig. 4c), and these microorganisms were observed in all the samples. The band A1 had a similarity of 96% with the uncultured species of *Methanosaeta* sp. These cells were identified previously in a characterization study of competitive electron flow between the electricity generation and the methane formation in a microbial fuel cell (Ishii and Watanabe, 2006, unpublished). Pender et al. [49] also reported the predominance of *Methanosaeta* sp. in both the presence and absence of sulfate in hybrid anaerobic reactors. They are aceticlastic methanogens that play an important role in the formation and maintenance of stable granules after some perturbations in the system [50]. Omil et al. [5] also verified by means of FISH (40–95%), the predominance of *Methanosaeta* sp. in an UASB reactor fed with acetate and sulfate after 24 h of air exposure.

SRB (k, of 102 mg acetate l\(^{-1}\)) have a higher acetate affinity than MA (k, of 116 mg acetate l\(^{-1}\)) [51]. Though SRB should have a higher growth rate in the presence of acetate [2], the competition between SRB and MA depends on other factors, such as, sulfate concentration, pH, and temperature. The aceticlastic MA, mainly *Methanosaeta* sp., was more favored in relation to SRB, in the presence of oxygen, in this study.

Other sequenced bands of MA referred to two-uncultured archaeon (bands A2 and A3), with similarities of 97% and 99%, respectively. Uncultured archaeon (DQ478742) associated with the order *Methanomicrobiales* was previously characterized in a study of microbial diversity of methanogenic granules from a full-scale UASB reactor treating brewery wastewater [13]. According to Grabowski et al. [52], uncultured archaeon (AY570646) was phylogenetically similar to *Methanathrix* (X16932), with 99% similarity in a study of microbial diversity in the production of wastewater in a biodegraded oil reservoir at low temperatures (18–20 °C). *Methanosaeta concili* are mesophilic acetotrophic organisms that probably consumed the acetate provided from ethanol degradation, maintaining high efficiencies in the COD removal and low-effluent acetate concentrations (results not shown). The growth of these strictly acetate-utilizing organisms is more favored than that of other aceticlastic MA (*Methanosarcina* sp.) in low levels of acetate, due to the former’s low K\(_s\) value for this substrate [53]. Bands A2 and A3 showed phylogeny similar to that of band A1, which presented similarity to uncultured *Methanosaeta* sp. (AB288619) (Fig. 5c).

Granules with diameters between 2 and 3 mm accounted for 76%. Once 1–2 mm granules were present in significant percentages (17%), the formation of new granules probably occurred during the reactor operation. The biomass was not monitored, but it was visually observed daily and did not show a decrease during the assays, remaining around 3.5 l of sludge in the reactor. Granules of 3–4 mm resulted in 0.7–14%. The diminishment of the granule sizes was noted for period III (COD/SO\(_4^2-\) ratio of 1.6, with oxygen) with a reduction in the percentages of the 2–3 mm granules and an increase in the percentages of 1–2 mm granules. However, the Anova (\(\alpha = 0.05\)) test did not indicate a significant difference in the variation of the granule size in the reactor profile in the evaluated operational period.

Different morphological types of granules reflect diverse steps in the development of granular sludge according to Diaz et al. [12]. They observed that “young” granules that were smaller, black in color, and compact correspond to active cells and are generally gram-negative bacteria (mainly \(\alpha\)-Proteobacteria) and MA such as *Methanospirillum* sp. and *Methanosarina* sp. They verified the higher occurrence of gray granules with a spherical or ellipsoidal shape that were predominantly gram-positive bacteria and MA that were *Methanosaeta*-like. “Old” granules were brown and larger in size, and had amorphous structures with empty areas in the central part and channels that penetrate to the interior of the granules. *Methanosaeta* sp. plays an important role in this granulation stage and three types of granules were observed in this study, with the dominant type having a gray color and cells similar to *Methanosaeta* sp.

The intermittent application of limited oxygen quantities supported the maintenance of CH\(_4\) production with no inhibition of sulfate reduction, the dampening of sulfide toxicity to anaerobic microorganisms (with HS\(^-\) production at pH > 7) and the formation of the S\(^-\) due mainly to microbial consortia that were established in the granules.

Moreover, the hydrodynamic assay of the pulse type using sodium chloride (NaCl) as conductivity electric indicator (tracer) was carried out. It proved that the reactor presented a complete-mix system (results not presented). However, the inoculum was initially well adapted in the UASB reactor, with a gradual increase in ethanol and sulfate concentrations. This adaptation probably provided favorable conditions for the growth of microorganisms, showing that the oxygen applied did not cause negative impacts. Another factor that could have contributed to this fact is that the addition of oxygen to the reactor feed was performed in the continuous mode, not beginning again the system at each operation period evaluated. This strategy could have propitiated the presence of microorganisms that were well adapted to the submitted conditions. It is also important to state that the granules have well-structured characteristics, which propitiate a barrier against the antagonist conditions for the microorganisms in the culture medium.

4. Conclusions

Microbial analyses of the granular sludge of the UASB reactor in the presence of 3.0 ± 0.7 mg O\(_2\) l\(^{-1}\) demonstrated the predominance of the MA community, specifically of *Methanosaeta*-like cells. The bacterial community presented a higher diversity, as was observed in the DGGE band profile. The COD:Sulfate ratio of 1.6 indicated a slight advantage in the SRB growth in the presence of oxygen with a predominance of microorganisms similar to *Desulfobacter* sp.

The applied oxygen concentration did not affect the efficiency of the UASB reactor operation in the evaluated conditions in terms of COD removal, methane concentration, or sulfate reduction. The mean removal efficiencies of COD\(_{total}\) and COD\(_{filtered}\) varied from 76–82% to 77–81%, respectively. The sulfate reduction varied from 74% to 92% in the studied periods in the presence of oxygen.

The decrease of the granule sizes (approximately 10%) observed at the end of the operation should be attributed to the shearing effects caused by the extensive operational period of the reactor (469 days). The statistical method did not indicate a considerable difference in the variation of the granule sizes in the reactor profile. The granules with diameters varying from 2 to 3 mm prevailed in the granular sludge of the UASB reactor.

The intermittent application of oxygen inhibits neither the methanogenesis nor the sulfate reduction. It was also possible to dampen the toxicity by sulfide because of the HS\(^-\) form production and the elemental sulfur formation.

Acknowledgments

This research was supported by the Brazilian research funding institution Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; Grants 2002/12985-7 and 2001/05489-0).

References


