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Phylogenetic characterization and quantification by Most Probable Number of the microbial communities of biomass from the Upflow Anaerobic Sludge Blanket Reactor under sulfidogenic conditions

Isabel Kimiko Sakamoto[1], Sandra Imaculada Maintinguer[2] and Maria Bernadete AmâncioVaresche[1*]

[1]Departamento de Hidráulica e Saneamento, Escola de Engenharia de São Carlos, Universidade de São Paulo, Avenida João Dagnone, 1100, 13563-120, São Carlos, São Paulo, Brazil. [2]Instituto de Pesquisa em Bioenergia, Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil. *Author for correspondence. E-mail: varesche@sc.usp.br

ABSTRACT. Granulated sludge from anaerobic reactors is constituted by the microbial consortia responsible for the degradation of different substrate present in wastewaters. This study characterized anaerobic microorganisms in a granular sludge from a Uasb reactor (Upflow Anaerobic Sludge Blanket) by Most Probable Number (MPN) technique and method of cloning and sequencing the 16S rDNA gene. The main objective of this study was to quantify and to identify the microorganisms in two different culture media containing sulfate (Posgate C [6.0 sodium lactate and 3.13 g L^{-1} sodium sulfate] and Zinder [2.24 sodium lactate and 0.96 g L^{-1} sodium sulfate]). Microorganisms quantified by Postgate C and Zinder media were: $9.30x10^{10}$ and $7.50x10^{11}$ MPN mL^{-1} for general anaerobic bacteria; $4.30x10^{8}$ and $2.10x10^{8}$ MPN mL^{-1} for sulfate reducing bacteria; and $1.20x10^{8}$ and $1.5x10^{8}$ MPN mL^{-1} for methane producing archaea, respectively. Bacteria identified by 16S rDNA gene sequencing from the MPN in both culture media were related to the phyla Firmicutes and Proteobacteria. The conditions of Postgate C culture medium favored the sulfate-reducing bacteria and the Zinder culture medium favored the general anaerobic bacteria.

Keywords: sulfate reducing bacteria; methane producing archaea; 16S rDNA gene; cloning/sequencing; upflow anaerobic sludge blanket reactor.

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Introduction

The successful use of the Upflow Anaerobic Sludge Blanket (Uasb) reactors in the treatment of wastewater has promoted the application of anaerobic technology in the treatment of industrial effluents (Del Nery, Pozzi, Damianovic, Domingues, & Zaiat, 2008).

The microbial consortia from granular sludge UASB reactor showed predominance of methanogenic archaea in previous studies by FISH (Fluorescent in situ hybridization) technique (Del Nery et al., 2008; Hirasawa, Sarti, Del Aguila, & Varesche, 2008). However, it was verified a relative abundance greater than 84% microorganisms belonging to Bacteria Domain by the large-scale sequencing technique using the Illumina MiSeq platform (Delforno et al., 2017). The methanogenesis pathways were acetoclastic, methylotrophic and hydrogenotrophic. The sulfur metabolism has been assigned to organic and inorganic sulfur assimilation, alkanesulfonate assimilation, sulfur oxidation, and sulfate reduction-associated complexes by the large-scale sequencing technique using the Illumina HiSeq platform (Delforno et al., 2017).

The study of sulfate reducing bacteria (SRB) is interesting to many industrial operations, such as shipping and oil industries. Corrosion is the main environmental hazard promoted by these microorganisms (Païssé et al., 2013; Marty et al., 2014). Moreover, they can be used for bioremediation in areas contaminated by petroleum hydrocarbons due to their metabolic versatility.

In the absence of sulfate, many SRB ferment organic acids and alcohols, producing hydrogen, acetate, and carbon dioxide, and may even rely on hydrogen- and acetate-scavenging methanogens to convert organic compounds into methane. SRB can establish two different life styles, and these can be termed as sulfidogenic and acetogenic, hydrogenogenic metabolism (Plugge, Zhang, Scholten, & Stams, 2011).

As mentioned earlier, the SRB uses a wide variety of organic compounds, which requires choosing the organic substrate for the appropriate growth of different genera in the culture media. Lactate has been used as an excellent source of organic carbon for the enrichment, isolation, cultivation and quantification of bacterial cells similar to *Desulfovibrio* sp. and *Desulfotomaculum* sp. Lactate oxidation may be incomplete or complete (Widdel, 1988).

In contrast to sulfate reducers, methanogenic archaea uses a limited number of substrates for growth. The methanogenic archaea present in granular sludge perform in the final stage of anaerobic digestion. From an ecological point of view, methanogenic metabolism depends on the presence of other microorganisms, whose anaerobic activity produces substrate for methanogenesis (Muyzer & Stams, 2008). Ozuolmez et al. (2015) verified the coexistence of an acetoclastic methanogen and an acetoclastic sulfate reducer in the presence of high sulfate concentration. This metabolic characteristic, revealed from coculture studies, brings new insight to the metabolic flexibility of methanogens and sulfate reducers residing in marine environments in response to changing environmental conditions.

The technique of cloning and sequencing the 16S rDNA gene (Sanger method) was used for the microbial characterization of anaerobic reactors used to produce biohydrogen (Botta et al., 2016; Carosia, Reis, Sakamoto, Varesche, & Silva, 2017), and to evaluate the diversity of the anaerobic bacteria present in the sediment of the reservoir (Maintinguer, Sakamoto, Adorno, & Varesche, 2015). The fluorescence *in situ* hybridization (FISH) (Sun, Hu, Sharma, Ni, & Yuan, 2014), real time PCR techniques (Stubner, 2004) and MPN-technique using radiolabeled ^{35}S-sulfate (Ingvorsen, Nielsen, & Joulian, 2003) also investigated the quantification of SRB. However, although specific and reliable, these techniques have high costs. Culture methods based on the Most Probable Number (MPN) technique have been widely used for several decades and remain the standard method for quantification of SRB (Luna, Costa, Gonçalves, & Almeida, 2008) and other microorganisms (Maintinguer, Sakamoto, Adorno, & Varesche, 2013).

The aim of this study was to perform quantitative and qualitative analysis of microbial groups present in Uasb granular sludge using two culture media with different carbon/sulfate ratio. Phylogenetic analysis of the microbial community was performed in the sample purified by dilution in multiple tubes used in quantification of sulfate reducing bacteria (SRB), methane producing archaea (MPA) and general anaerobic bacteria (GAnB) by Most Probable Number (MPN) technique. This work will collaborate with strategy of quantitative and qualitative analysis of the microbial community in sulfate environments.

Material and methods

Sample dilution for quantification by MPN also allows the selection of microbial populations for subsequent phylogenetic analysis by cloning and sequencing. Most Probable Number (MPN) estimates were used to determine the abundance of anaerobic microorganisms in two culture media. The microorganisms collected from the Uasb were quantified in a single sample. For the phylogenetic analysis, it was used the biomass of positive dilution vials for SRB.

Quantification of microorganisms

Microorganism quantification was performed using the Uasb (Upflow Anaerobic Sludge Blanket) sludge granules for the treatment of poultry slaughterhouse wastewater (Dakar Poultry S/A, Tiête, State São Paulo, Brazil).

The granular sludge was ground with mortar and pestle. Next, the sample was transferred to an antibiotic bottle with glass beads and agitated manually at an angle of 45° for about 20 min. to homogenize and also to disrupt the granules. The dilution series used in MPN was from 10^{-1} to 10^{-15}. Firstly, 1 mL of the sample was injected into a vial containing 9 mL of an anaerobic liquid medium. Inoculated flasks (n = 3) were incubated at 30±1°C for 28 days.

The anaerobic liquid media used were: Postgate C (Postgate, 1984) and Zinder (Zinder, Cardwell, Anguish, Lee, & Koch, 1984). Table 1 lists the composition of two culture media used in the quantification of the following microorganisms: sulfate reducing bacteria (SRB), methane producing archaea (MPA) and general anaerobic bacteria (GAnB) by Most Probable Number (MPN).

Table 1. Composition of the culture media Postgate C and Zinder.

Reagents	*Postgate C (g L^{-1})	Zinder (g L^{-1})
NH_4Cl	1.0	0.5
KH_2PO_4	0.5	0.4
$MgCl_2.6H_2O$	0.1	0.1
$CaCl_2.6H_2O$	0.06	0.15
Na_2SO_4	4.5	1.12
$MgSO_4$	0.06	-
Sodium lactate	6.0	2.24
Yeast extract	1.0	-
$C_6H_5O_7Na_3$	0.3	-
Rezasurin	0.0001	0.0001
$FeSO_4$	0.073	0.073
Solution vitamins	-	(1 ml L^{-1}) (Touzel & Albagnac, 1983)
Trace metal solution	-	(10 m L^{-1}) (Zinder & Koch, 1984)
Sodium bicarbonate	-	1.0

*The pH was adjusted to 7.2 with NaOH (Ilhan-Sungur, Cansever, & Cotuk, 2007).

The media were prepared with an anaerobic gas mixture (70% N_2 and 30% CO_2). The anaerobic microorganisms require an environmental redox potential (Eh) of around -200 mV (Widdel, 1988; Yerushalmi, Alimahmoodi, & Mulligan, 2011). To achieve this redox potential, Na_2S (0.04 g L^{-1}) was used as a reducing agent in both media. The dilute solution was composed of potassium phosphate dibasic (K_2HPO_4 - 0.56 g L^{-1}) and potassium phosphate monobasic (KH_2PO_4 - 0.11 g L^{-1}).

Supplemental trace-metal and vitamins solutions were used in the composition of Zinder culture medium. The composition of the trace metal solution (g L^{-1}) used was as follow: Nitroloacetic acid, 4.5; $FeSO_4.7H_2O$, 0.556; $MnSO_4.H_2O$, 0.086; $CoCl_2.6H_2O$, 0.17; $ZnSO_4.7H_2O$, 0.21; H_3BO_3, 0.19; NiC_{l2}, 0.02; $Na_2MoO_4.2H_2O$, 0.01 (Zinder & Koch, 1984). The components of the vitamin solution (g L^{-1}) used was as follow: Biotin, 0.002; folic acid, 0.002; thiamine hydrochloride, 0.005; ridoflavin, 0.005; nicotinic acid, 0.005; D-pantothenic acid calcium, 0.005; pyridoxolhydrochlorid, 0.01; cyanocobalamine, 0.0001; lipoic acid, 0.005; p-aminobenzoic acid, 0.005 (Touzel & Albagnac, 1983).

The sulfate reducing bacteria (SRB) use the sulfate ion and convert in to sulfide under anaerobic conditions. The growth of SRB was indirectly observed by sulfide production and detected by the reaction with ferrous salt present in the medium, forming a black precipitate of FeS (Ilhan-Sungur et al., 2007). Methanogenic archaea use acetate, CO_2 and H_2 in anaerobic respiration with methane production. The growth of methanogens was confirmed indirectly through methane production. Biogas was determined by gas chromatography (Gow Mac chromatographer equipped with a thermal conductivity detector (TCD) and a Porapak Q (2 m x $^1/_4$ in 80 a 100 mesh) column. Hydrogen was used as a carrier gas at 60 mL min^{-1}. The observation of turbidity in the culture media confirmed the positive growth of GAnB. In the present study, the following steps were used to calculate NMP values: 1) Finding the combination of three consecutive dilutions with cell growth (positive); 2) Using the combination (3-1-0) to find the value in Table of NMP Indexes (American Public Health Association [Apha]; American Water Works Association [Awwa]; & WPCF, 1975); 3) Applying the value (43) found in Table of NMP Indexes (Apha et al., 1975), in the following formula.

Where: V = lowest sample dilution in the selected combinations series.

The estimation of MPN cells was performed according to the Apha method (Apha et al., 1975).

Phylogenetic analysis

Phylogenetic analysis was performed on positive dilution for SRB growth, dilution 10^{-6} and 10^{-5} for the Postgate C and Zinder media, respectively. Genomic DNA was extracted from 3-tube (positive) biomass for the growth of sulfate-reducing bacteria. Nucleic acids were extracted from 0.10 g (wet weight) of the sample using 0.3 g glass beads (150-212 µm diameter Sigma), 1.0 mL buffer PBSx 1 (13.7 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4), 1.0 mL phenol (Tris saturated), 1.0 mL chloroform. Cells were lysed by maximum vortex speed for 25 s (Kowalchuk, Bodelier, Heilig, Stephen, & Laanbroek, 1998; Griffiths, Whiteley, O'Donnell, & Bailey, 2000). Centrifugation at 6000 rpm for 10 min. at 4°C; Transfer the supernatant (± 1.0 mL) to a clean tube 2 mL; Add an equal volume of phenol (Tris saturated) and vortex briefly (± 3 s); Centrifugation at 6000 rpm for 10 min. at 4°C (Ng, Melvin, & Hobson, 1994); Transfer the supernatant (± 0.8 mL) to a clean tube 2 mL; Add an equal volume of chloroform and vortex briefly (± 3 s).

Centrifugation at 6000 rpm for 10 min. at 4ºC; Transfer the supernatant (± 0.5 mL) to a clean tube 2 mL. Total nucleic acids were then precipitated from the extracted aqueous layer with 1.0 mL ice cold 95% (v v^{-1}) ethanol; Centrifugation at 6000 rpm for 10 min. at 4ºC; Discard the supernatant; Pellet nucleic acids are then washed in 0.5 mL ice cold 70% (v v^{-1}) ethanol; Centrifugation at 6000 rpm for 10 min. at 4ºC; Discard the supernatant; Dry the pellet DNA at room temperature for 24 hours; Before using, resuspend the dried pellet with 100 µl TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0); Store the DNA at -20ºC.

The amplification of the polymerase chain reaction (PCR) was performed with a bacterial domain primer set for the 16S rRNA gene, 27 forward (5'-AGAGTT TGATCCTGGCTCAG-3') and 1100 reverse (5'-AGGGTTGCGCTCGTTG-3') (Lane, 1991).

The PCR product purification was performed with an Illustra GFX PCR DNA kit and Gel Band Purification (GE Healthcare). The clone's library was pGEM®-T Easy Vector Systems (Promega), and transformed into *Escherichia coli* competent cells following the manufacturer's instructions. Approximately 100 white clones were randomly picked; these were used for the recovery of DNA fragments, used as template for PCR. The 16S rDNA inserts were amplified from plasmid DNA of selected clones using the universal M13 forward and reverse primers.

The nucleotide sequencing was performed in an ABI Prism® 310 Genetic Analyzer sequencer (Applied Biosystems, California - USA). Partial 16S rRNA sequences obtained from clones were assembled in a contig using the SeqMan – DNA-STAR (Lasergene) sequence analysis. Identification was achieved by comparing the contiguous 16S rRNA sequences obtained with 16S rRNA sequence data from reference type strains, as well as environmental clones available in the public databases GenBank (http://www.ncbi.nlm. nih.gov) and RDP (Ribosomal Database Project e Release 10; http://rdp.cme.msu.edu/) using the BLASTn. The phylogenetic tree was constructed by the Neighbor-Joining method using the software MEGA version 5.2 (Tamura et al., 2011). Bootstrap resampling analysis for 1000 replicates was performed to estimate the confidence of tree topologies. The 16 rRNA gene sequences obtained in this study were deposited at the Genbank database with the accession numbers ranging from KJ651861 to KJ651872.

Results and discussion

Quantification of anaerobic microorganisms by MPN

Cellular quantification in Postgate C and Zinder media were as follows: general anaerobic bacteria (GAnB) $9.30x10^{10}$ and $7.50x10^{11}$ MPN mL^{-1}; sulfate reducing bacteria (SRB) $4.30x10^{8}$ and $2.10x10^{8}$ MPN mL^{-1}; and methane producing archaea (MPA) $1.20x10^{8}$ and $1.5x10^{8}$ MPN mL^{-1}, respectively (Figure 1).

The MPN of SRB was slightly higher In Postgate C culture medium ($4.30 x10^{8}$ MPN mL^{-1}) than in Zinder medium ($2.10x10^{8}$ MPN mL^{-1}). SRB were favored in the first nutritional requirement (Postgate C) due to the greater amount of sodium lactate (6.0 g L^{-1}) and sulfate (3.13 g L^{-1}) when compared with the second condition (Zinder medium, sodium lactate 2.24 and sulfate 0.96 g L^{-1}). Therefore, the ratios of lactate to sulfate under these conditions were 1.92 and 2.33, respectively.

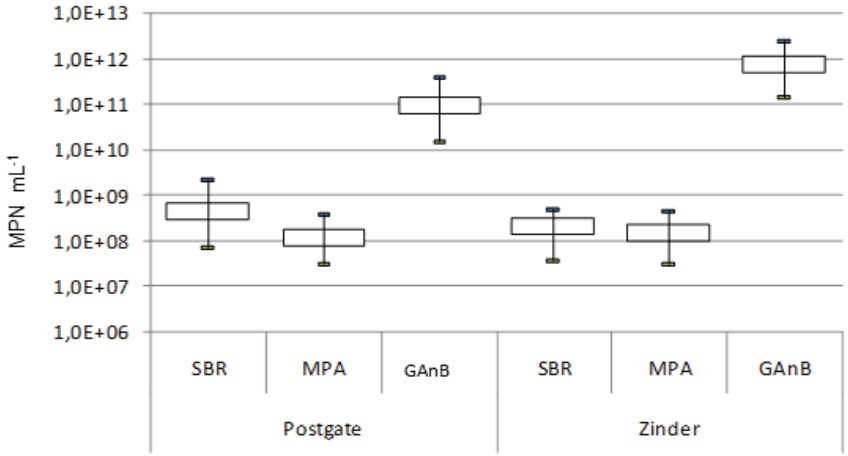

Figure 1. Most Probable Number of SRB, MPA and GAnB, in the Postgate C and Zinder media. The error bars represent the lower and upper values of the method. SRB (Sulfate Reducing Bacteria), MPA (Methane Producing Archaea); GAnB (General Anaerobic Bacteria).

Domingues, Moraes, Vazoller, and Varesche (2004) used 2 L capacity Duran® flasks containing the mineral medium (Zinder & Koch, 1984) plus lactate (1.8 g L^{-1}) and sulfate (1.1 g L^{-1}) inoculated with anaerobic granular sludge from upflow anaerobic sludge blanket (Uasb) thermophilic reactor treating vinasse. The MPN results presented a range of value of $3.0x10^{11}$ MPN mL^{-1} for SRB and $4.0x10^{2}$ MPN mL^{-1} for MPA initially and $9.0x10^{12}$ MPN mL^{-1} for SRB and $8.0x10^{2}$ MPN mL^{-1} for MPA after 55 days of operation, incubated at 55±1°C.

Ilhan-Sungur et al. (2007) conducted a count of planktonic populations by MPN technique after incubation of galvanized steel for different times with Postgate C medium and pure culture *Desulfovibrio* sp. The SRB population after 3 days of incubation reached the maximum value of $4.1x10^{9}$ MPN mL^{-1}. After 31 days of incubation, a decrease was observed to $6.5x10^{7}$ MPN mL^{-1}. The authors suggest that the SRB were responsible for the corrosion of galvanized steel.

The MPA values were of the same magnitude, $1.20x10^{8}$ and $1.5x10^{8}$ MPN mL^{-1} for Postgate C and Zinder, respectively. The GAnB measurement was $9.30x10^{10}$ MPN mL^{-1} for Postgate C and $7.50x10^{11}$ MPN mL^{-1} for Zinder. This higher magnitude in Zinder may be related to the presence of trace metal solutions and vitamins in the composition.

According to Muyzer and Stams (2008), most SRB are associated with methanogenic archaea in anaerobic environments. In the absence of sulfate, as SRB, it behaves like acetogenic microorganisms, using lactate and ethanol to produce substrates such as acetate, carbon dioxide and hydrogen to the methanogens. This association was observed, for example, between *Desulfovibrio desulfuricans* or *Desulfovibrio vulgaris* and methanogenic archaea. These interactions are important in maintaining the hydrogen balance in an anaerobic environment. The high partial pressure of hydrogen prevents the oxidation of compounds to more reduced products, impairing the metabolism of methane fermentation (Muyzer & Stams, 2008).

Phylogenetic analysis by cloning and partial sequencing

Sequencing of about 50 clones was performed for each sample, one from the Postgate C medium (3 tubes - dilution 10^{-6}) and the other from the Zinder medium (3 tubes - dilution 10^{-5}). The sequences containing less than 200 bp were discarded. After reviewing 100 sequences in the 'SeqMan' DNASTAR software (Lasergene sequence analysis), 74 sequences were used in the analysis. There were 39 sequences in the Postgate C medium (dilution 10^{-6}) sample and 35 sequences in the Zinder medium (dilution 10^{-5}) sample. All sequences presented similarity in the range from 95 to 99% percent (http://blast.ncbi.nlm.nih.gov).

The 39 clones from the Posgate C medium analyzed presented sequences closely related to uncultivated *Veillonella* sp. (64%), uncultivated *Desulfovibrio* sp. (21%), uncultivated *Clostridium* sp. (10%) and *Citrobacter freundii* (5%). The 35 clones from the Zinder medium analyzed showed sequences closely related to *Acinetobacter* sp. (69%), *Dechloromonas* sp. (17%), *Desulfovibrio* sp. (6%), *Clostridium* sp. (6%), *Sulfurospirillum* sp. (3%) (Table 2 and Figure 2).

Table 2. Approximation of the phylogenetic identity of the clones sequenced obtained from samples of Postgate C and Zinder media.

Medium	Clones	Access	Similarity (average %)	Microorganisms
Postgate C (39 clones)	1, 2, 4, 7, 8, 12, 15,16, 21, 22, 25, 28, 30, 32, 37, 39, 41, 46, 53-56, 62, 66, 68	DQ443905.1 EU728725.1	99	Uncultured *bacterium* *Veillonella parvula*
	13, 31, 33, 34, 36, 48, 63, 69	FJ393061.1 DQ450463.1	96	Uncultured *Desulfovibrio* sp. *Desulfovibrio desulf.*
	10, 27, 35, 71	AB513435.1 EF199998.1	95	uncultured *Clostridium* sp. *Clostridium sulfidigenes*
	6, 70	AF025365.1 EF593056.1	99	*Citrobacter freundii* Uncultured *Citrobacter* sp.
	1, 3- 8, 11, 12, 15, 25, 31, 32, 35- 39, 43, 49-52, 54	FJ976600.1 FJ975124.1	99	*Acinetobacter* sp. *Acinetobacter calcoaceticus*
Zinder (34 clones)	17, 18, 19, 27, 29, 40	AY124797.1 AF170354.1	98	*Dechloromonas* sp.
	13, 16	AM084133.1 EF055877.1	95	*Desulfovibrio* sp.
	34, 44	EU517557.1 FJ527031.1	98	*Clostridiaceae* *Clostridium* sp.
	26	AJ535704.1	97	*Sulfurospirillum* sp.

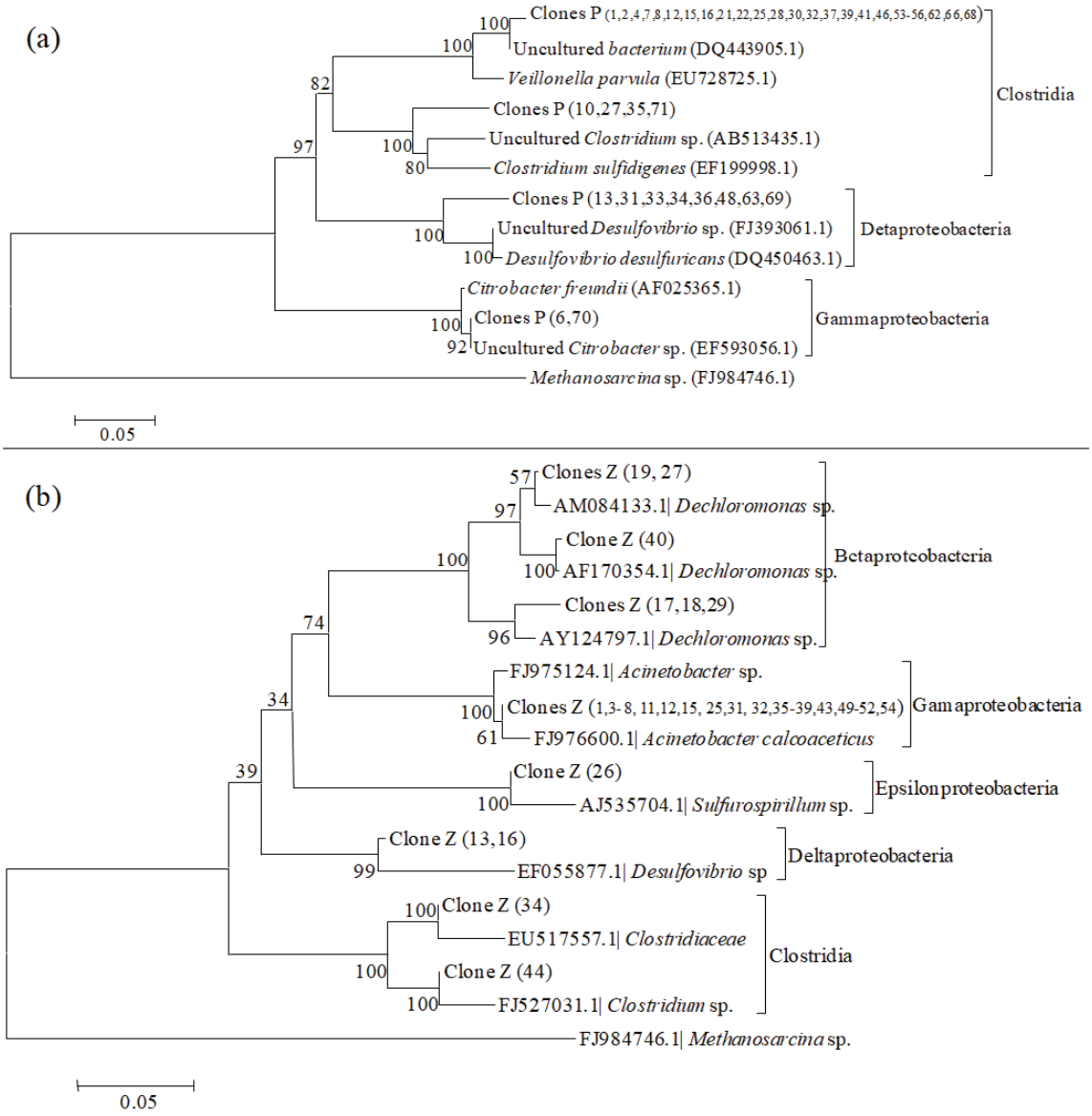

Figure 2. Phylogenetic analysis based on partial 16S rDNA sequences of clones obtained from samples of the culture media (a) Postgate C and (b) Zinder. *Methanosarcina* sp. was used as the Outgroup.

Bacteria of the genus *Veillonella* are anaerobic, Gram-negative cocci, usually occurring in pairs; grow anaerobically with lactate or pyruvate, with accompanying production of propionic and acetic acids, CO_2, and H_2, and nitrate reduction. Pyruvic, oxalacetic, malic, succinic and fumaric acid are metabolized in the stationary phase of cell growth (Carlier, 2001).

The dominant sulfidogenic communities in full-scale sulfate removal bioreactors are heterotrophic SRB belonging to the family Desulfovibrionaceae of the class Deltaproteobacteria. The genus *Desulfovibrio* represents a phylogenetically coherent group; all species incompletely oxidize lactate to acetate, but can also use hydrogen, formate and ethanol (Dar, Alfons, Stams, Kuenen, & Muyzer, 2007).

Borole, Mielenz, Vishnivetskaya, and Hamilton (2009) investigated the distribution of microbial populations in fuel cells, and identified dominant microorganisms in the consortium related to *Desulfovibrio* sp., sulfate reducing, thiosulfate, sulfite and elemental sulfur to hydrogen sulfide. SRB can metabolize lactate, ethanol, malate and formate to acetate. *Desulfovibrio* sp was identified in samples of marine sediments, industrial effluents, freshwater and intestinal tracts of animals (Borole et al., 2009).

Clostridium sp., belonging to class Clostridia, are Gram-positive anaerobic rods capable of forming endospores (Sallam & Steinbuchel, 2009). *Clostridium* sp. metabolizes carbohydrates, alcohols, amino acids, purines, steroids and other organic compounds. The members of the genus *Clostridium* are metabolically versatile, and found in soils, sea floor sediments, thermophilic environments (Rainey & Stackebrandt, 1993) and intestinal tract of animals (Lee, Romanek, & Wiegel, 2007). Lactate, however, can serve as a substrate

for other fermentation specialists, including *C. homopropionicum, C. propionicum, C. neopropionicum* and *C. lactatifermentans* exhibiting fermentation of lactate to propionic acid and acetic acid using the acrylate pathway (Wiegel, Tanner, & Rainey, 2006).

Zhao, Ren, and Wang (2008) used a reactor stirred by the agitator and continuously fed with synthetic wastewater containing sulfate and lactate at 35ºC. The authors verified that *Desulfovibrio* sp., *Desulfobulbus* sp., *Pseudomonas* sp. and *Clostridium* sp. formed a stable, dominant community structure. The present study also identified *Veillonella* sp.. According to Zhao et al. (2008), lactate could serve as an electron donor for *Desulfovibrio* sp. or it was fermented by *Clostridium* sp. and *Bacteroides* sp. to produce propionic acid or ethanol and these intermediary metabolites could be used subsequently by *Desulfobulbus* sp., *Desulfovibrio* sp. and acidotrophic SRB during the oxidation of acetate.

Citrobacter freundii are Gram-negative anaerobes belonging to the phylum Proteobacteria of the family Enterobacteriaceae and exhibit a positive H_2S characteristic. These bacteria are often found in the gastrointestinal flora of humans, but can be found in soil, water, sewage and foods. However, *Citrobacte*r sp. can use citrate as a carbon source and have the ability to accumulate uranium by building phosphate complexes. Qiu et al. (2009) isolated a strain (DMB) of sulfate reducers related to members of the genus *Citrobacter* sp., outside the group of traditional sulfate-reducing bacteria in a mining area. The authors observed that *Citrobacter* sp. can grow up faster under aerobic conditions with increasing pH, possibly due to the release of carbon dioxide, resulting from lactate oxidation. On the contrary, *Citrobacter* sp. recovered the ability to reduce sulfate to sulfide under anaerobic conditions.

Acinetobacter sp. can grow on most complex media. Nevertheless, some strains grow in defined media containing a single carbon and energy source, such as lactate or acetate, using ammonium or nitrate salts, or one of several common amino acids, as a supply of nitrogen. Frequently, amino acids such as glutamic acid or aspartic acid can serve as a single source of carbon, energy, and nitrogen in a defined mineral medium (Juni, 2001).

Heylen et al. (2006) evaluated three culture media containing nitrate and some isolates were assigned to the *Azospira, Azovibrio, Dechloromonas, Thauera*, and *Zoogloea* genera of the family Rhodocyclaceae. The majority of microorganisms were isolated in the culture media with succinate as the carbon source. *Dechloromonas* sp. are facultative anaerobic, non-spore-forming, non-fermentative, strictly respiring and can oxidize acetate in the presence of oxygen, chlorate, perchlorate or nitrate as alternative electron acceptors (Achenbach, Michaelidou, Bruce, Fryman, & Coates, 2001).

Dechloromonas agitata can use propionate, butyrate, lactate, succinate, yeast extract, fumarate and malate as alternative electron donors. Achenbach et al. (2001) observed an optimum growth with acetate (10 mM) as an electron donor and chlorate (10 mM) as an electron acceptor at 35°C, pH 7±5 and 1% NaCl.

Stolz et al. (1999) could distinguish *Sulfurospirillum barnesii* from *S. deleyianum* at optimum pH and salinity. They presented the ability to use lactate as electron donor, and the inability to use sulfite as electron acceptor. *Sulfurospirillum arsenophilurn* oxidized lactate completely to CO_2.

In the present study, metabolic pathways may be presented based on microorganisms identified and information of literature (Figure 3). *Clostridium* sp. could metabolize lactic to propionic acid and/or ethanol. *Veillonella* sp. was probably responsible for the conversion of lactic into propionic acid and acetic acid, CO_2, and H_2; *Desulfovibrio* sp. could reduce sulfate to sulfide and metabolize lactic acid and/or ethanol to acetic acid and *Citrobacter* sp. possibly performed the reduction of sulfate and used citrate as a carbon source. *Dechloromonas* sp. could metabolize propionate and acetate, while *Acinetobacter* sp. and *Sulfurospirillum* sp. possibly used lactate and acetate as energy sources. Methanogenic microorganisms were probably responsible for the conversion of acetate and H_2 into carbon dioxide and methane in the biogas, which was verified by gas chromatography (data not shown).

Comparison of culture media (Postgate C and Zinder)

MPN were observed with Postgate C and Zinder media for: GAnB $9.30x10^{10}$ and $7.50x10^{11}$ MPN mL^{-1}; SRB $4.30x10^{8}$ and $2.10x10^{8}$ MPN mL^{-1}; MPA $1.20x10^{8}$ and $1.5x10^{8}$ MPN mL^{-1}, respectively (Figure 1).

MPN of SRB was higher in the Postgate C culture medium ($4.30x10^{8}$ MPN mL^{-1}) than in Zinder culture medium ($1.20x10^{8}$ MPN mL^{-1}). The ratio of lactate to sulfate was different in the Postgate C (6.0/3.13 g L^{-1} to rates 1.92) and Zinder media (2.24/0.96 g L^{-1} to rates 2.33). In the present study, with the Postgate C medium, it was identified two genera of sulfate reducers, *Desulfovibrio* sp. and *Citrobacter* sp., whose growth was favored by the lower lactate/sulfate ratio, but with higher concentrations of carbon and sulfur sources.

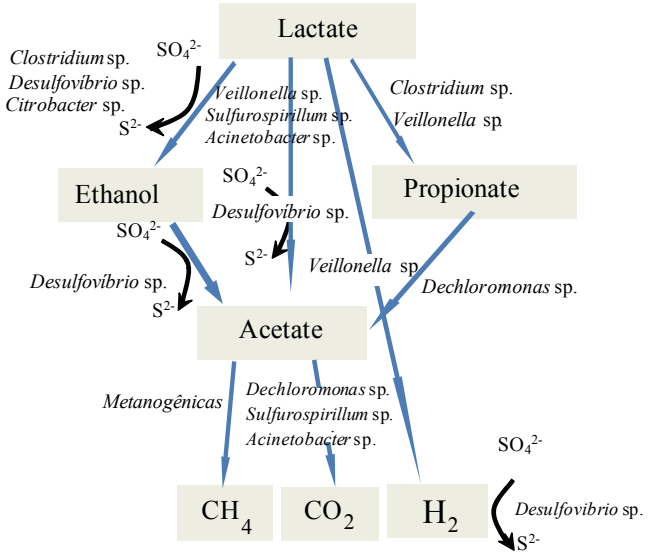

Figure 3. Possible metabolic pathways based on microorganisms identified in the literature and found in the Postagte C (dilution 10^{-6}) and Zinder (dilution 10^{-5}) culture media in this study.

Bacteria identified by sequencing the 16S rRNA gene in both culture media (Postgate C and Zinder) in the present study were related to the phyla Firmicutes and Proteobacteria (Figure 2). However, the Postgate C culture medium restricted the Delta and Gamma Proteobacteria classes, while the Zinder medium, identified representatives of the Beta, Delta, Gamma and Epsilon Proteobacteria classes. This greater diversity of microorganisms may be related to the presence of vitamins and trace metals solutions in the Zinder medium.

The microorganisms similar to *Veillonella* sp. were only identified in Postgate C culture medium (Figure 2). This may be related to the concentration of sodium lactate and yeast extract in the Postgate C medium, which were similar to the DSM (136) (Deutsche Sammlung von Mikroorganismen) medium and/or the ATCC (1760) (American Type Culture Collection) used in cultivation of *Veillonella* sp. Probably, the nutritional conditions of the Postgate C medium favored the growth of such bacteria. In the Zinder medium, the yeast extract and the sodium lactate were added at lower concentration.

The percentage of *Clostridium* sp. in the Postgate C medium was higher than in the Zinder medium. This may be related to an increased concentration of sodium lactate in the first culture medium, as *Clostridium* sp., according to Zhao et al. (2008), can ferment lactate to propionate, butyrate and acetate. In this study, *Acinetobacter* sp., *Dechloromonas* sp. and *Sulfurospirillum* sp. were identified only in the Zinder medium, probably by the constitution of the solution with trace metals and vitamins in addition to sodium lactate.

Although the quantification of microbial population by MPN revealed no significant differences, the sulfate-reducing bacteria were slightly higher in Postgate C medium and the general anaerobic bacteria were slightly higher in Zinder medium. The phylogenetic analysis indicated more diversity of Classes in the Zinder medium than in Postgate C medium.

Conclusion

The nutritional conditions of Postgate C medium favored the sulfate-reducing bacteria and the Zinder culture medium favored the general anaerobic bacteria.

The nutritional conditions of Postgate C culture medium selected microorganisms belonging to Classes Delta and Gamma Proteobacteria, while in the Zinder medium, representatives of the Classes Beta, Delta, Epsilon and Gamma Proteobacteria were identified.

The higher diversity of classes observed in Zinder medium may be related to the presence of solutions of vitamins and trace metals.

Desulfovibrio sp. was responsible for the reduction of sulfate to sulfide, with lactate as carbon source.

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References

Achenbach, L. A., Michaelidou, U., Bruce, R. A., Fryman J., & Coates, J. D. (2001). *Dechloromonas agitata* gen. nov., sp. nov. and *Dechlorosoma suillum* gen. nov., sp. nov., two novel environmentally dominant (per) chloratereducing bacteria and their phylogenetic position. *International Journal of Systematic and Evolutionary Microbiology, 51*(Pt 2), 527-533. doi: 10.1099/00207713-51-2-527

American Public Health Association [APHA].; American Water Works Association [Awwa].; & WPCF. (1975). *Standard methods for the examination of water and wastewater* (14th ed.). Washington, DC, EUA: Apha.

Borole, A. P., Mielenz, J. R., Vishnivetskaya, T. A., & Hamilton, C. Y. (2009). Controlling accumulation of fermentation inhibitors in biorefinery recycle water using microbial fuel cells. *Biotech Biofuels, 2*(7), 1754-6834. doi: 10.1186/1754-6834-2-7

Botta, L. S., Ratti, R. P., Sakamoto, I. K., Ramos, L. R., Silva, E. L., & Varesche, M. B. A. (2016). Bioconversion of waste office paper to hydrogen using pretreated rumen fluid inoculum. *Bioprocess and Biosystems Engineering, 39*(12), 1887-1897. doi: 10.1007/s00449-016-1663-0

Carlier, J. P. (2001). Genus I: veillonella. The proteobacteria. Part C the Alpha-Beta, Delta, and Epsilon. In D. R. Boone, & R. W. Castenholz (Ed.)*, Bergey's manual of systematic bacteriology* (2nd ed., vol. 2; p. 1059-1064). New York, NY: Springer.

Carosia, M. F., Reis, C. M., Sakamoto, I. K., Varesche, M. B. A., & Silva, E. L. (2017). Influence of C/P and C/N ratios and microbial characterization in hydrogen and ethanol production in an anaerobic fluidized bed reactor. *International Journal of Hydrogen Energy, 42*(15), 9600-9610. doi: 0.1016/j.ijhydene.2017.01.127

Dar, S. A., Alfons, J. M., Stams, A. J. M., Kuenen, J. G., & Muyzer, G. (2007). Co-existence of physiologically similar sulfate-reducing bacteria in a full-scale sulfidogenic bioreactor fed with a single organic electron donor. *Applied Microbiology and Biotechnology, 75*(6),1463-1472. doi: 10.1007/s00253-007-0968-

Del Nery, V., Pozzi, E., Damianovic, M. H. R. Z., Domingues, M. R., & Zaiat, M. (2008). Granules characteristics in the vertical profile of a full-scale upflow anaerobic sludge blanket reactor treating poultry slaughterhouse wastewater. *Bioresource Technology, 99*(6), 2018-2024. doi: 10.1016/j.biortech.2007.03.019

Delforno, T. P., Júnior, L., Vieira, G., Noronha, M. F., Sakamoto, I. K., Varesche, M. B. A., & Oliveira, V. M. (2017). Microbial diversity of a full-scale UASB reactor applied to poultry slaughterhouse wastewater treatment: integration of 16S rRNA gene amplicon and shotgun metagenomic sequencing. *MicrobiologyOpen, 6*(3), e00443. doi: 10.1002/mbo3.443

Domingues, M. R., Moraes, E. M., Vazoller, R. F., & Varesche, M. B. A. (2004). Analysis of Microbial Community in Biofilms and Planktonic Cells of Anaerobic Thermophilic Reactors. *Brazilian Archives of Biology and Technology, 47*(5), 122-132. doi: 10.1590/S1516-89132001000500001

Griffiths, R. I., Whiteley, A. S., O'Donnell, A. G., & Bailey, M. J. (2000). Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA and rRNA-based microbial community composition. *Applied and Environmental Microbiology, 66*(12), 5488-5491. doi: 10.1128/aem.66.12.5488-5491.2000

Heylen, K., Vanparys, B., Wittebolle, L., Verstraete, W., Boon, N., & Vos, P. (2006). Cultivation of denitrifying bacteria: optimization of isolation conditions and diversity study. *Applied and Environmental Microbiology, 72*(4), 2637-2643. doi: 10.1128/AEM.72.4.2637-2643.2006

Hirasawa, J. S., Sarti, A., Del Aguila, N. K. S., & Varesche, M. B. A. (2008). Ecology/environmental microbiology 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. *Anaerobe, 14*(4), 209-218. doi: 10.1016/j.anaerobe.2008.06.003

Ilhan-Sungur, E., Cansever, N., & Cotuk, A. (2007). Microbial corrosion of galvanized steel by a freshwater strain of sulphate reducing bacteria (*Desulfovibrio* sp.). *Corrosion Science, 49*(3), 1097-1109. doi: 10.1016/j.corsci.2006.05.050

Ingvorsen, K., Nielsen, M. Y., & Joulian, C. (2003). Kinetics of bacterial sulfate reduction in an activated sludge plant. *FEMS Microbiology Ecology, 46*(2), 129-137. doi: 10.1016/S0168-6496(03)00209-5

Juni, E. (2001). *Acinetobacter* sp. In D. R. Boone, & R. W. Castenholz (Ed.)*, Bergey's manual of systematic bacteriology* (2nd ed., vol. 2; p. 425-437). New York, NY: Springer.

Kowalchuk, G. A., Bodelier, P. L. E., Heilig, G. H. J., Stephen, J. R., & Laanbroek, H. J. (1998). Community analysis of ammonia-oxidising bacteria, in relation to oxygen availability in soils and root-oxygenated sediments, using PCR, DGGE and oligonucleotide probe hybridisation. *FEMS Microbiology Ecology, 27*(4), 339-350. doi: 10.1111/j.1574-6941.1998.tb00550.x

Lane, D. J. (1991). 16S/23S rRNA sequencing in nucleic acid techniques. In E. Stackenbrandt, & M. Goodfellow (Ed.)*, Bacterial systematics* (p. 115-148). New York, NY: John Wiley and Sons, Inc.

Lee, Y.-J., Romanek, C. S., & Wiegel, J. (2007). *Clostridium aciditolerans* sp. Nov., an acid-tolerant spore-forming anaerobic bacterium from constructed wetland sediment. *International Journal of Systematic and Evolutionary Microbiology, 57*(Pt 2), 311-315. doi: 10.1099/ijs.0.64583-0

Luna, A. S., Costa, A. C. A., Gonçalves, M. M. M., & Almeida, K. Y. M. (2008). The effects of surfactants on the estimation of bacterial density in petroleum samples. *Applied Biochemistry and Biotechnology, 147* (1-3), 77-84. doi: 10.1007/s12010-007-8094-y

Maintinguer, S. I., Sakamoto, I. K., Adorno, M. A. T., & Varesche, M. B. A. (2013). Evaluation of the microbial diversity of denitrifying bacteria in batch reactor. *Brazilian Journal of Chemical Engineering, 30*(3), 457-465. doi: 10.1590/S0104-66322013000300004

Maintinguer, S. I., Sakamoto, I. K., Adorno, M. A. T., & Varesche, M. B. A. (2015). Bacterial diversity from environmental sample applied to bio-hydrogen production. *International Journal of Hydrogen Energy, 40*(8), 3180-3190. doi: 10.1016/j.ijhydene.2014.12.118

Marty, F., Gueuné, H., Malard, E., Sánchez-Amaya, J. M., Sjögren, L., Abbas, B., ... Muyzer, G. (2014). Identification of key factors in Accelerated Low Water Corrosion through experimental simulation of tidal conditions: influence of stimulated indigenous microbiota. *Biofouling, 30*(3), 281-297. doi: 10.1080/08927014.2013.864758

Muyzer, G., & Stams, A. J. M. (2008). The ecology and biotechnology of sulphate-reducing bacteria. *Nature Reviews|Microbiol, 6*(6), 441-454. doi: 10.1038/nrmicro1892

Ng, A., Melvin, W. T., & Hobson, P. N. (1994). Identification of anaerobic digester bacteria using a polymerase chain reaction method. *Bioresource Technology, 47*(1), 73-80. doi: 10.1016/0960-8524(94)90031-0

Ozuolmez, D., Na, H., Lever, M. A., Kjeldsen, K. U., Jørgensen, B. B., & Plugge, C. M. (2015). Methanogenic archaea and sulfate reducing bacteria co-cultured on acetate: teamwork or coexistence? *Frontiers in Microbiology, 6*, 492. doi: 10.3389/fmicb.2015.00492

Païssé, S., Ghiglione, J. F., Marty, F., Abbas, B., Gueuné, H., Amaya, J. M., ... Quillet, L. (2013). Sulfate-reducing bacteria inhabiting natural corrosion deposits from marine steel structures. *Applied Microbiology and Biotechnology, 97*(16), 7493-7504. doi: 10.1007/s00253-012-4464-7

Plugge, C. M., Zhang, W., Scholten, J. C., & Stams, A. J. (2011). Metabolic flexibility of sulfate-reducing bacteria. *Frontiers in Microbiology*, *2*, 81. doi: 10.3389/fmicb.2011.00081

Postgate, J. R. (1984). *The sulphate reducing bacteria* (2nd ed.). Cambridge, GB: Cambridge University Press.

Qiu, R., Zhao, B., Liu, J., Huang, X., Li, Q., Brewer, E., ... Shia, N. (2009). Sulfate reduction and copper precipitation by a *Citrobacter* sp. isolated from a mining area. *Journal of Hazardous Materials, 164*(2-3), 1310-1315. doi: 10.1016/j.jhazmat.2008.09.039

Rainey, F. A., & Stackebrandt, E. (1993). 16S rDNA analysis reveals phylogenetic diversity among the polysaccharolytic clostridia. *FEMS Microbiology Letters, 113*(2), 125-128. doi: 10.1111/j.1574-6968.1993.tb06501.x

Sallam, A., & Steinbuchel, A. (2009). *Clostridium sulfidigenes* sp. nov., a mesophilic, proteolytic, thiosulfate- and sulfur-reducing bacterium isolated from pond sediment. *International Journal of Systematic and Evolutionary Microbiology, 59*(Pt 7), 1661-1665. doi: 10.1099/ijs.0.004986-0

Stolz, J. F., Ellis, D. J., Blum, J. S., Ahmann, D., Lovley, D. R., & Oremland, R. S. (1999). *Sulfurospirillum barnesii* sp. nov. and *Sulfurospirillum arsenophilum* sp. nov., new members of the *Sulfurospirillum* clade of the € *Proteobacteria. International Journal of Systematic and Evolutionary Microbiology, 49*(Pt 3), 1177-1180. doi: 10.1099/00207713-49-3-1177

Stubner, S. (2004). Quantification of Gram-negative sulphate-reducing bacteria in rice field soil by 16S rRNA gene-targeted real-time PCR. *Journal of Microbiological Methods, 57*(2), 219-230. doi: 10.1016/j.mimet.2004.01.008

Sun, J., Hu, S., Sharma, K. R., Ni, B. J., & Yuan, Z. (2014). Stratified microbial structure and activity in sulfide-and methane-producing anaerobic sewer biofilms. *Applied and Environmental Microbiology, 80*(22), 7042-7052. doi: 10.1128/AEM.02146-14

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution, 28*(10), 2731-2739. doi: 10.1093/molbev/msr121

Touzel, J. P., & Albagnac, G. (1983). Isolation and characterization of *Methanococcus mazei* strain MC_3. *FEMS Microbiology Letters, 16*(2-3), 241-245. doi: 10.1111/j.1574-6968.1983.tb00295.x

Widdel, F. (1988). Microbiology and ecology of sulfate and sulfur-reducing bacteria. In A. J. B. Zehnder (Ed.), *Biology of anaerobic microorganisms* (p. 469-585). New York, NY: John Wiley and Sons, Inc.

Wiegel, J., Tanner, R., & Rainey, F. A. (2006). An introduction to the family Clostridiaceae in Prokaryotes. In M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer, & E. Stackebrandt (Eds.), *The prokaryotes* (p. 654-678). New York, NY: Springer.

Yerushalmi, L., Alimahmoodi, M., & Mulligan, C. N. (2011). Performance evaluation of the BioCAST technology: a new multi-zone wastewater treatment system. *Water Science & Technology, 64*(10), 1967-1972. doi: 10.2166/wst.2011.776

Zhao, Y., Ren, N., & Wang, A. (2008). Contributions of fermentative acidogenic bacteria and sulfate-reducing bacteria to lactate degradation and sulfate reduction. *Chemosphere, 72*(2), 233-242. doi: 10.1016/j.chemosphere.2008.01.046

Zinder, S. H., & Koch, M. (1984). Non-aceticlastic methanogenesis from acetate oxidation by a thermophilic syntrophic coculture. *Archives of Microbiology, 138*(3), 263-272. doi: 10.1007/BF00402133

Zinder, S. H., Cardwell, S. C., Anguish, T., Lee, M., & Koch, M. (1984). Methanogenesis in a thermofilic (58°C) anaerobic digestor. *Methanothrix* sp. as an important acetoclastic methanogen. *Applied and Environmental Microbiology, 47*(4), 796-807.