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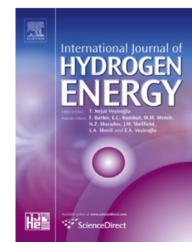

Review

Bacterial diversity from environmental sample applied to bio-hydrogen production

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ABSTRACT

Environmental from tropical climate countries as sediments in standing waters are complex habitats which are able to provide favorable living conditions for manifold microbial species. The aim of this study was to evaluate the diversity of the anaerobic bacteria present in the sediment of the reservoir and its application in biological production of hydrogen gas. The anaerobic batch reactors showed a xylose consumption of 63.5% at 72 h of operation with yield of H_2 production of 0.3 (mol H_2/mol xylose) at 37 °C, pH 5.5. Molecular biology techniques used for genomic DNA extraction, cloning, sequencing and phylogenetic analyses of the sediment sampling revealed clones similar to the phyla *Proteobacteria*, *Chloroflexi*, *Firmicutes*, *Deferribacteres*, *Fusobacteria*, *Cyanobacteria* and uncultured bacteria. The analysis of DGGE revealed changes in microbial populations from the sediment and the anaerobic consortia of bacteria from the reactors fed with xylose. Anaerobic bacteria coming from the sediment, mainly rods forming endospores from Phylum *Firmicutes* were favored by the experimental conditions imposed and they were probably involved in the biologic process of the H_2 production.
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Introduction

The search for biotechnology to obtain hydrogen production from industrial effluents can be advantageous as energy alternative. The hydrogen gas generated in the wastewater treatment by biological processes can be used as an alternative energy source and development of commercial processes to obtain hydrogen production, exploring the ability of microorganisms with modern biotechnology practices [1,2]. In

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this way, the knowledge about the hydrogen-producing microorganisms is fundamental to the development of alternative and cleaner sources of energy production.

Reservoirs and freshwater lakes are natural environments with conditions for the survival of a wide variety of microorganisms, such as aerobic and anaerobic bacteria. Sediments are the major component of a freshwater ecosystem with many complex interactions with the water body. Microorganisms living in the sediment likely play a key role in the transformation of organic matter in the cycle of nutrients and are influenced by, as well as having an influence on, the chemical composition of the surrounding environment [3]. These microorganisms may consume different carbon sources like sugars, which can be used for hydrogen production as well.

Various inoculum sources (natural soil, anaerobic digestion sludge, wastewater treating plants, domestic landfill, and others) have been tested and their biological hydrogen production efficiencies were confirmed. However, these inocula are predominantly obtained from countries with a temperate climate. There are only a few studies with hydrogen production using inoculum from tropical countries, such as Brazil [4] where the media temperatures are around 25 °C and they are ideal for the growth bacterial. In addition, there are some studies with pure cultures that were isolated from sediments but in high concentrations of substrates. Khamtib et al. (2012) [5], obtained H_2 bio-production with *Thermoanaerobacterium thermosaccharolyticum* KKU19 isolated from hot spring sediment with xylose (10 g/L), initial pH of 6.50, at 60 °C. Junghare et al. (2012) [6] isolated *Clostridium butyricum* TM-9A from an estuarine sediment and it was capable on H_2 bio-production with glucose (10 g/L) at 37 °C and initial pH of 8.0. There are no studies with mixture cultures from tropical climates. In this sense, the investigation of consortia of hydrogen producing bacteria from the sediments is necessary for the improvement application of these environmental samples for H_2 bio-production.

Biological processes are catalyzed by microorganisms in aqueous environments at ambient temperature [7]. These processes are usually carried out by different anaerobic bacteria. The characteristics of these microorganisms differ from each other with respect to environmental conditions and the metabolic process for the substrate consumption. The type of microbial culture, composition of the substrate, media composition and temperature are also important parameters affecting the hydrogen bio-production. Moreover, reactor configuration and heavy metals concentrations may be affecting the bio-hydrogen production [8].

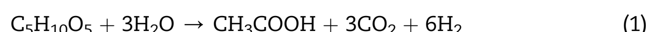
Different microorganisms participate in the biological hydrogen generation systems like green algae, cyanobacteria (or blue-green algae), photosynthetic bacteria and fermentative bacteria [2]. The Phylum *Firmicutes*, mainly *Bacillus* sp. and *Clostridium* sp. are very effective for hydrogen generation [4]. The bacteria belonging to the genus Clostridium are obligate anaerobes and spore forming organisms. Clostridia and Bacilli classes produce hydrogen gas during the exponential growth phase in batch growth. The dominant culture of these bacteria can be easily obtained by heat treatment of biological sludge [9]. The spores formed at high temperatures can be activated when required environmental conditions are provided for hydrogen gas production [10].

Fermentative hydrogen production from hexose, glucose and sucrose is well studied [11–15]. In contrast, fermentative hydrogen production of xylose (i.e., pentose) is less studied [14,16,17]. Several authors [16,18,19] have obtained high yields of biological hydrogen production from xylose, though such testing have been performed with high concentrations (10–20 g xylose/L) with additions of nutrient rich media (yeast extract, peptone, among others).

Many anaerobic organisms can produce hydrogen from carbohydrate containing organic wastes such as glucose, sucrose and xylose [4]. Several compounds such as glucose, hexose isomers and polymers as starch or cellulose, give different hydrogen yields per mole of organic substrate, depending on the fermentative pathway and on the end products formed. Diverse wastewater from agriculture, wood processing and the paper industry may contain xylose [14,16]. Waste containing simple compounds such as sugar is readily degradable, requiring low hydraulic retention time, whereas complex wastes, for example, chlorinated organic compounds, are slowly degradable and need longer hydraulic retention time for their metabolism [8].

Xylose is a common and abundant sugar obtained from the hydrolysis of lignocellulosic materials, in particular hemicelluloses [20]. The major components of lignocellulosic hydrolysate include hexose (glucose) and pentose (xylose and trace amount of arabinose) [19]. The lignin cellulosic materials may contain 35–45% of xylose in their composition [14]. Glucose and sucrose are excellent substrates for fermentative hydrogen production and they have been well studied using pure cultures and consortia of anaerobic bacteria as hydrogen producers. However, the information about anaerobic bacteria for the conversion of xylose to hydrogen is limited [21].

The equations (1) and (2) show the xylose degradation and generation of hydrogen to form two end products [4]: (1) consumption of xylose and generation of acetic acid and (2) butyric acid:



$$C_5H_{10}O_5 + 3H_2O \rightarrow CH_3COOH + 3CO_2 + 6H_2 \quad (1)$$

$$C_5H_{10}O_5 + 2H_2O \rightarrow \tfrac{1}{2}CH_3CH_2CH_2COOH + 3CO_2 + 5H_2 \quad (2)$$

Moreover, in Brazil there is a wide variety of industrial effluents containing xylose like the paper industry and lignin cellulosic materials, at concentrations close to 2 g/L. Microbial fermentation of xylose derived from such wastes is quite promising for combining alternative energy production and waste reduction.

In this sense, the aim of this study was to investigate the microbial consortium composition from the environmental samples such as sediment from a reservoir and its application in the biological hydrogen production from sugars such as xylose at concentrations close to the industrial effluents.

Materials and methods

Environmental sample

The environmental sample was the sediment taken from Itupararanga Reservoir located in São Paulo State,

southeastern Brazil (23°36 42 S and 47°23 48 W). The storage capacity of the reservoir is 286 million m^3, and the mean theoretical residence time varies between 4 and 13 months, depending on the distribution and the intensity of rainfall. The water from the reservoir is used for the generation of hydroelectricity (discharge of approximately 16 m^3/s and installed capacity of 55 mW) to supply drinking water (2.2 m^3/s for 800,000 people), and for irrigation (0.13 m^3/s). Agriculture and native vegetation are the predominant land uses in the watershed, representing 42% and 25%, respectively, of the total area of the watershed [22]. The physical, chemical and biological parameters of the sampling site have been showed in Table 1.

Obtaining microbial consortium and growth conditions

The sediment sample was used as inoculum previously purified by means of serial dilutions for H_2 generation (10^{-5}, 10^{-7}, 10^{-10}) and then heat treated (90 °C–10 min) [23]. The pretreated inoculum (20% v/v) and the cellular purification was performed using culture medium [24] containing the nutrient solutions A, B, C and D, glucose (2 g/L), urea (40 mg/L), peptone (1 g/L) and 1 mL of vitamin solution [25], as described in Table 1. The vitamin solution was composed of p-aminobenzoic acid (40 mg/L) and biotin (10 mg/L).

Flasks were then submitted to Argon atmosphere (99.99%) for 10 min and incubated at 37 °C during 48 h, without agitation. After that, under aseptic conditions, the purified inoculum (40%) was submitted to successive washes and centrifuged under refrigeration at 8500 rpm and 3 °C for 10 min. The supernatant was discarded and the pellet was suspended in the culture medium (solutions A, B, C and D, peptone (1.0 g/L), urea (40.0 mg/L), 1.0 mL of vitamin solutions [25] and xylose (2.0 g/L)). The pH was adjusted to 5.5 to inhibit the H_2 consumers by the addition of hydrochloric acid or sodium hydroxide.

Table 1 – Physical-chemical analysis from the sampling site (Itupararanga Reservoir).

Parameter	
Weather	Sunny
Environmental temperature	29.5 °C
Water temperature	24.9 °C
Collecting sediment (m)	11
Visibility (Secchi Disk) (m)	1.4
Turbidity	<10
Water ([a])	
pH (in site)	7.72
pH (laboratory)	6.96
DO	2.1
BOD	297
COD	11.9
N–NH_3	5.0
TN	25
SO_4	<dl
S_2–	<dl
TP	126.1
chlorophyll	21.1
TS	2.0
FTS	1.6
VTS	0.5
Sediment	
TP	0.2
TOM	1.9
TN	336.0
TS	240.6
FTS	200.8
VTS	39.8

Temperature (T, °C), pH, dissolved oxygen (DO, mg/L), total solid (TS, mg/L), fix total solid (FTS, mg/L), volatile total solid (VTS, mg/L), biochemistry oxigen demand (BOD, mg O_2/L), chemistry oxigen demand (COD, mg/L), chlorophyll (μg/L), total nitrogen (TN, mg/L), ammonia nitrogen (N–NH_3, mg/L), total organic matter (TOM, %), sulfate (SO_4, mg/L), sulphide (S_2–, mg/L), turbidity (NTU – nephelometric turbidity units), total phosphorus (TP, μg/g).
[a] Water-sediment interface dl: detection limit.

Operation of the batch reactors

The inoculum comprised 20% of the total reaction volume (0.2 L) and it was previously purified in serial dilutions with xylose (2 g/L). The pH was adjusted to 5.5 with hydrochloric acid or sodium hydroxide.

The culture medium was filtered through a 0.22 μm membrane in a filtration system that was sterilized in an autoclave (121 °C, 20 min). The reactors had 4 L of reaction volume and 1 L of headspace. They were submitted to Argon atmosphere (99.99%) for 20 min after the distribution of the solutions (Table 2). The anaerobic batch reactors fed with xylose were made in duplicate and they were operated for 102 h.

After that they were then capped with butyl rubber stoppers, wrapped and kept at 37 °C, without agitation.

Chemical and chromatographic analyses

The hydrogen content in the biogas was determined by gas chromatography (GC) in a Shimadzu GC 2010, equipped with a Carboxen 1010 PLOT (30 m × 0.53 mm) column, with thermal conductivity detector and Argon as the carrier gas. The temperatures of the injector and detector were 220 °C and 230 °C,

Table 2 – Synthetic substrate composition of the anaerobic batch reactors fed xylose.

Composition	Anaerobic batch reactors
Xylose (g)	8.0 g
Urea (g)	0.08 g
Peptone (g)	1.0 g
Vitamins solution (L)	0.004 L
A Nutrient (g/L): $NiSO_4 \cdot 6H_2O$ (0.5); $FeSO_4.7H_2O$ (2.5); $FeCl_3 \cdot 6H_2O$ (0.25); $CoCl_2 \cdot 2H_2O$ (0.04)	0.008 L
B Nutrient (g/L): $CaCl_2 \cdot 6H_2O$ (2.06)	0.008 L
C Nutrient (g/L): SeO_2 (0.144)	0.008 L
D Nutrient (g/L): KH_2PO_4 (5.36); K_2HPO_4 (1.30); $Na_2HPO_4H_2O$ (2.76)	0.008 L
Purification inoculum (mL)	0.8 L
Headspace (L): Ar (100%)	1.0 L
Ultra pure water (L)	4.0 L

respectively. The temperature of the column was from 130 °C to 135 °C (5.5 min), at 46 °C/min.

Volatile fatty acid and alcohol concentrations were determined by GC using a Shimadzu GC 2010, equipped with a flame ionization detector and a headspace auto sampler COMBI-PAL (AOC 5000) and an HP-INNOWAX column (30 m × 0.25 mm × 0.25 μm of film thickness) [26].

The xylose concentration was determined by a colorimetric method [27,28]. The volatile suspended solid concentrations (VSS) and pH values were measured as described in APHA (2005) [29].

Cellular growth analysis

Cellular growth was monitored by optical density at 600 nm (OD_{600}) [15]. The cellular mass was expressed as volatile suspended solids (VSS g/L) and was calculated by equation (3):

$$VSS = 0.7436 * ABS_{600} + 0.0146 \quad (3)$$

Experimental data fitting

The experimental data were fitted to the mean values obtained from the duplicates of the reactors using Microcal Origin® 5.0 software. The maximum specific activities of hydrogen gas production were obtained by non-linear sigmoidal adjustment of the Boltzman function. The maximum xylose consumption velocity was verified by the major angular coefficient generated from the screened lines between the points representing the experimental xylose concentrations in relation to time.

Hydrogen-producing bacteria count (MPN)

Most probable number (MPN) of hydrogen producing bacteria was done in quintuplicate dilutions at the end of the anaerobic batch reactors operation. The measurements were done 10 days after incubation, by gas chromatography (hydrogen gas presence/absence), previously described and analyzed according to Standard Probability Table [29].

Microscopic analyses

Morphological characteristics of the microorganisms were monitored by phase contrast microscopy using an Olympus BX60-FLA with software Image Pro-Plus.

16S rRNA sequencing and phylogenetic analysis

The Molecular Biology analyses were made in the sediment sample before the operation of the batch anaerobic reactors. Nucleic acid extractions were performed following the procedure with a direct method with glass beads and a mixture of phenol: chloroform: buffer (1:1:1 v/v) addition [30]. A segment of the 16S rRNA gene fragments was amplified by PCR using eubacterial primers 27F (50-AGA GTT TGA TCM TGG CTC AG-30) and 1110R (50-GGG TTG CGC TCG TTG-30) [31]. For the Polymerase Chain Reaction (PCR) it was used 0.5 mL of each primer, 5 mL of dNTPs (Invitrogen), 0.5 mL of Taq DNA polymerase (Invitrogen), 5 mL of 10 × PCR buffer, 1.5 mL of $MgCl_2$ (50 mM) and approximately 100 ng of genomic DNA. The PCR was 35 cycles as follows: initial denaturation to 94 °C for 2 min, 10 cycles of denaturation at 94 °C for 1 min, annealing at 69 °C for 3.0 min, extension at 72 °C for 3.0 min, final extension at 94 °C for 10 min, and cooled at 4 °C. The PCR products were purified using a kit, Ultraclean PCR Clean-up (MoBio Laboratories, Inc), according to the manufacturer's instructions.

Samples of PCR products were cloned into the plasmid vector pGEM (Promega Easy Vector System I) according to the manufacturer's specifications. The clones were randomly selected and amplified by PCR. Nucleotide sequencing was performed on an automated ABI 310 PRISM sequencer (Dye terminator Cycle Sequencing Kit, Applied Biosystems, USA) in accordance with the manufacturer's instructions using an M13 forward primer (50-GTA AAA CGA CGG CCA G-30) [32].

The nucleotide sequences were processed and they were aligned with the Seqman program (Lasergene DNAstar package) for the removal of signals from the vector and low quality bases. The sequences aligned were compared with the 16S rRNA sequences of organisms represented in the Genbank database (http://www.ncbi.nlm.nih.gov) and Ribosomal Database Project (http://rdp.cme.msu.edu/) for a phylogenetic identity analysis.

Phylogenetic analyses of the sequences were performed using the Molecular Evolutionary Genetic Analysis 3.1 (MEGA 3.1) software [33]. Evolutionary distances were based on the Kimura model [34] and tree reconstruction, on the neighbor-joining method with bootstrap values calculated from 500 replicate runs, using the routines included in the MEGA software.

DGGE (denaturing gradient gel electrophoresis) analyses

The analysis of the microbial community were carried through the amplification of 16S rRNA fragments by polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) from the samples: (1) Itupararanga Reservoir Sediment, (2) purified cellular by serial dilutions (10^{-5}, 10^{-7}, 10^{-10}), (3) anaerobic batch reactors fed with xylose, and (4) hydrogen-producing bacteria count (MPN). The amplification of 16S rRNA fragments was performed using PCR with set primers for Bacteria Domain: 968F (5′-AACGC-GAAGAACCTTAC-3′), along with the GC clamp (5′-CGC CCG CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGGG-3′) and 1392 R (5′-GGC GGT GTG TAC AACG-3′) [35].

The amplification program of PCR was 35 cycles as follows: initial denaturation to 94 °C for 7 min, denaturation 94 °C for 0.45 min, annealing 55 °C for 0.45 min, extension 72 °C for 1.0 min, final extension 94 °C for 10 min, and cooled for 4 °C. The amplified fragments were separated on polyacrylamide gel at DGGE containing linear gradient varying from 40% to 60% of denaturant. After electrophoresis, the gel was cored with etidium bromide for 20 min. The device used for reading the banding patterns obtained in DGGE was TMIII Eagle Eye (Stratagene) under exposure to UV at 254 nm, coupled to the computer and software Eagle Sight.

The evaluation of the DGGE band patterns was done by considering the intensity, presence or absence. The distance between bands of different tests (channels) was measured by BioNumerics software. The similarities were displayed

graphically as a dendogram with similarity coefficient calculated by Pearson correlation.

Results and discussion

Operation of the batch reactors

The maximum biomass growth was observed after 66 h of operation (0.13 ABS). The maximum H_2 generation occurred after 74 h of operation (2200.5 μmol/L), according to Fig. 1.

The hydrogen yield observed at 54 h operation was 0.3 mol H_2/mol xylose (Table 3). Selembo et al. (2009) [36] obtained lower hydrogen yield than in this work (0.28 mol-H_2/mol-glycerol) with anaerobic reactors fed with glycerol (3.0 g/L), pH 6.2, at 30 °C and inoculum obtained from an environmental sample (Tomato soil – Penn State University greenhouse). Zhao et al. (2010) [37] obtained hydrogen yield close to this work (0.33 mol H_2/mol xylose) in anaerobic reactors fed with xylose (4.5 g/L), pH 6.5, at 70 °C, peptone (0.5 g/L), $FeSO_4$ (70 mg/L) with inoculum obtained from a reactor operated for H_2 production [at 70 °C, fed with xylose (2 g/L), peptone (1 g/L) and yeast extract (0.5 g/L)]. Subudhi et al. (2013) [19] obtained higher hydrogen bio-production (18 mmol H_2/L) than this work (2.2 mmol H_2/L) with *Enterobacter cloacae* DT-1 isolated from crude oil contaminated soil. However, the authors (op. cit.) made the tests with pure cultures and rich nutrient medium [peptone (2 g/L), yeast extract (2 g/L)] and higher concentrations of xylose (10 g/L).

There was no methane production in anaerobic reactors, confirming the efficiency of the heat treatment of the inoculum and the imposition of initial pH 5.5. The combination of these two factors caused the inhibition of bacteria H_2-consuming and methanogenic archaea. Xylose was not totally consumed during the operation of the anaerobic reactors. The xylose concentration at 58 h of operation was 1.27 g/L, i.e., 36.5% of their initial concentration was consumed. After 72 h of operation it was detected 0.91 g/L of xylose, i.e., 63.5% of the initial concentration were consumed. Several anaerobic bacteria for hydrogen production easily consummate sugars such as glucose and sucrose. Only few pure cultures can effectively utilize xylose for hydrogen production [19]. Probably the anaerobic consortia obtained in this study had inefficient microorganisms to make fermentation from xylose to hydrogen. Similar results of low consumption of xylose, as the present study, was verified by Lin et al. (2006) [16], with achieve only 68% of xylose removal after 120 h of operation using inoculum from domestic sewage at pH 7.0 and 35 °C in anaerobic batch reactors operated with 18.75 g xylose/L.

Table 3 – Results of the studied conditions.

Parameters	
Xylose consumption (%)	65
VSS (g/L)	1.5
ABS (600 nm)	0.13
pH (experiment end)	4.1
Operation time (h)	102
Intermediary products (mg/L)	
Acetic acid	97.5
Ethanol	39.2
Isobutyric acid	66.0
Iso-butanol	21.5
n-Butanol	22.5
Maximum generation H_2 (μmol H_2/L)	2200.5
Period (h)	78
Maximum specific xylose consumption (μmol xylose/L·h)	190
Period (h)	50
Maximum H_2 specific production rate (μmol H_2/g VSS·h)	16.0
Period (h)	54
Hydrogen yield (%)	5
mol H_2/mol xylose	0.3
Period (h)	78

The final value of pH for the experiment was 4.1 (Table 3). The decrease in pH was due to the production of volatile fatty acids, which depletes the buffering capacity of the medium resulting in low final pH [38].

The products generated were hydrogen gas, ethanol, n-butanol, acetic and isobutyric acids (Table 3). The maximum theoretical hydrogen production provides acetic acid as an end product of fermentation [39]. In practice, however, the high production of hydrogen is associated with a mixture of fermentation products such as acetic and butyric acid, while low hydrogen production is associated with the formation of propionic acid and less alcohols and lactic acid [40]. In the present study it was observed the generation of acetic acid. We did not detect generation of propionic acid, i.e., the metabolic pathway for the generation of H_2 was favored in the anaerobic reactors. In a previous study [4] we observed butyric acid (310.7 mg/L) and acetic acid (856.8 mg/L) generation associated with hydrogen bio-production (2106.8 μmol H_2/g·VSS·h) in an anaerobic reactor fed with xylose (1.8 g/L) and inoculated with granular sludge from an UASB reactor treating swine wastewater. In this study the hydrogen bio-production (2200 μmol H_2/g·VSS·h) were close to that obtained by the authors (op. cit.). Therefore, the sediment of the reservoir is a promising inoculum for the biological generation of hydrogen.

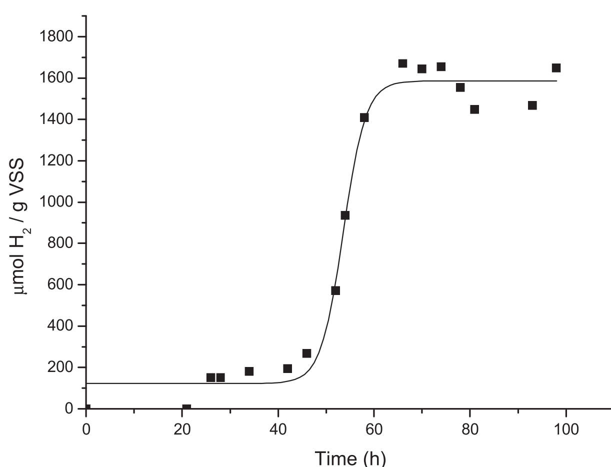


Fig. 1 – Cumulative H_2 production profile in anaerobic batch reactors fed with xylose (2 g/L).

Microbiologic analyses

The MPN of hydrogen producing bacteria at the end of the operation of the reactors revealed 4.3×10^2 MPN/mL (3.3×10^5 cells/g TVS). Castelló et al. (2009) [18] obtained values higher than in this study (>2.4×10^{11} MNP/mL and 9.0×10^{11} MNP/mL in 69 and 129 days, respectively) with an inoculum from an UASB reactor fed with cheese whey (67.0 COD g/L) at 30 °C, pH 5.0. However, the authors (op. cit.) made the tests with rich nutrient medium [tryptone (5 g/L), yeast extract (5 g/L) and beef extract (5 g/L)] and higher concentrations of substrate [glucose (10 g/L)].

The predominant morphology was Gram positive rods and rods with endospores. This may be related to the conditions imposed, i.e., pH and heat treatment of the inoculum. This evidence may suggest the selection of endospore-forming bacteria. The heat treatment was necessary to inhibit microorganisms that consume hydrogen gas and, therefore, are undesirable for the hydrogen producing process. Nevertheless, they are present in lake sediments, environments where the reduced levels of oxygen favor the persistence of such populations. Moreover, the microorganisms are sensitive to acidic pH [40] because they grow favorably at pH 6.0 to 8.0.

Oh et al. (2003) [41] used heat treatment of the inoculum and obtained high percentages of hydrogen (57%–72%) at 30 h of operation in batch reactors fed with glucose (3.0 COD g/L). Chen et al. (2005) [42] worked with heat treated inoculum from hydrogen producing reactors (CSTR, fixed bed, fluidized bed and sludge pellets) in batch anaerobic reactors fed with sucrose (5–30 g COD/L) and basal medium, yeast extract (1 g/L), pH 6.5, at 37 °C. Microscopic examination revealed the presence of rods and rods with endospores. Fang et al. (2006) [43] observed rods in anaerobic batch reactors with heat treated inoculum from a local municipal wastewater treatment plant fed with the wastewater treatment of rice (5.5 g carbohydrate/L) and yeast extract (50 mg/L).

Molecular biology analyses

The sediment sampling showed the presence of fifty-six clones and they were identified as belonging to the phyla *Proteobacteria*, *Firmicutes*, *Chloroflexi*, *Actinobacteria*, *Cyanobacteria*, *Fusobacteria*, *Deferribacteres* and uncultured bacteria. These results confirmed the high bacterial diversity in reservoir sediment samples. Im et al. (2012) [44] obtained lower diversity than the present study. The authors (op. cit.) observed that the bacterial community structure of inoculum from food waste and sewage sludge was mainly comprised of *Proteobacteria* (50%), *Bacteroidetes* (25%) and other phyla whose abundances are less than 4%, including *Firmicutes* (3.7%).

Forty per cent of the clones were identified as belonging to the phylum *Proteobacteria*. They showed similarities to bacteria from saline sediments [45,46], sediment reservoirs [47], which live under conditions aerobic [48], anaerobic [49] and facultative anaerobic [50] free-living, responsible for nitrogen fixation in roots of submerged plants [51]. In addition, several authors have reported a presence of the Phylum *Proteobacteria* on molecular analyses from anaerobic reactors operated to hydrogen bio-production [4,52–54]. These bacteria identified were probably involved in the fermentation process from the sediment.

Nineteen per cent of the clones obtained were belonging to the Phylum *Chloroflexi*. It consists of Gram-negative, facultative anaerobic [49], filamentous, green non-sulfur, which obtains energy through photosynthesis. They can grow on nutritive media containing sugars (glucose, mannose, xylose) and pyruvate. During the operation of the batch anaerobic reactors we observed the generation of acetate from xylose. May be the species of bacteria belonging to the Phylum *Chloroflexi* were involved in the biologic process of acetate fermentation. Furthermore, these clones showed similarities to bacteria acetate consuming [55].

Twenty one per cent of the clones obtained belong to the Phylum *Firmicutes* and they showed similarities to those found in anaerobic environments, mainly in depths of lakes and reservoirs [56], in samples of sediment landfill [57], soil [58], rice fields [59] and in samples from soil with agricultural activities [60]. They are Gram-negative and Gram-positive bacteria, endospore-forming, mainly belonging to the classes *Bacilli* and *Clostridia*. These bacteria have versatile metabolism [15] and they can be found in marine sediments of lakes and reservoirs, as it was observed in this study. Junghare et al. (2012) [6] isolated *C. butyricum* TM-9A – mesophilic alkaline tolerant fermentative bacteria from estuarine sediment samples. The authors (op. cit.) confirmed the bio-hydrogen production with xylose (10 g/L), pH 8.0, at 37 °C in batch anaerobic reactors with the isolates. Khamtib et al. (2012) [5] observed hydrogen bio-production with *Thermoanaerobacterium thermosaccharolyticum* KKU19, a thermophilic hydrogen producing bacteria isolated from hot spring sediments from Thailand. According to the authors (op. cit.) xylose (10 g/L) was the preferred carbon source while peptone (2 g/L) was the preferred organic nitrogen source. Wirth et al. (2012) [61] studied the anaerobic digestion in continuously stirred tank reactors fed with maize silage (68% TS) added to pig manure slurry by metagenomic analysis of biogas-producing microbial communities. According to the authors (op. cit.) members of the phylum *Firmicutes* played the most important role in the fermentation process of the organic matter decomposition. In the present study it was found predominantly rods, rods with endospores and Gram-positive during the heat treatment for H_2 bio-production and the operation of the anaerobic reactors, in which it was added glucose and xylose, the carbon sources preferred by these bacteria for fermentation. They are present in sediments, have the ability to ferment xylose and produce H_2, ethanol, acetic and butyric acids [17], as it was observed in this study. Probably the Gram-positive and endospore microorganisms observed during the heat treatment and the operation of the anaerobic reactors belong to the Phylum *Firmicutes*, coming from the sample of sediment and they were involved in the fermentation process of generation of H_2.

The other phyla (*Actinobacteria*, *Cyanobacteria*, *Deferribacteres* and *Fusobacteria*) and uncultured bacteria were detected in the sediment, but in smaller percentages (values less than five per cent). The phylum *Actinobacteria* is composed of filamentous bacteria, Gram-positive, facultative anaerobic and they have the capacity of producing bio-hydrogen [61]. Members of the phylum *Actinobacteria* are found in natural environmental [61] such as sediment. The phylum

Cyanobacteria including cocci, rods, filaments and fix nitrogen are commonly found in sediments [62] and wetland sediment [63]. In the sediment of this work it was verified the presence of roots and decomposing plant material originated from the forest around the reservoir. The phyla *Fusobacteria* and *Deferribacteres* are composed of anaerobic bacteria commonly found in aquatic environments, especially sediments. The environmental conditions of the sediment, the reduced concentrations of oxygen and the available organic matter probably favored the maintenance of such populations. In environmental samples like sediments is commonly observed the predominance of sequences related to uncultured bacteria [64], as described above. Therefore, it was expected to obtain similar sequences to these bacteria.

The roles of Gram-negative microorganisms identified in the present study (*Proteobacteria*, *Chloroflexi*, *Actinobacteria*, *Cyanobacteria*, *Fusobacteria* and *Deferribacteres* Phyla) focus on promoting a strict anaerobic environment and by breaking down complex substrates in the sediment reservoir to survive the Gram-Positive bacteria such as *Firmicutes* Phyla. The hydrogen production may involve a bacterial community of functional members [65] such as it was observed in this study.

Three consensus phylogenetic trees were constructed with primers for the Bacteria domain from the cloning and sequencing of the sample sediment used in the anaerobic reactors fed with xylose for the production of hydrogen gas. The coefficients of similarity observed between the clones and the NCBI database ranged from 95 to 99% and indicated the presence of phylogenetically-related species, based on the evaluation of partial sequences of the 16S rRNA (Figs. 2–4).

Fig. 5 shows the Dendrogram analysis of DGGE band patterns. There were differences among bands profile of the samples. The similarity index ranged between 38% and 98% among the samples. The Itupararanga Reservoir sediment (sample 1) showed 38% similarity with the other samples (2, 3 and 4). The purified sample (sample 2) showed 68% similarity with the anaerobic reactors fed with xylose (sample 3) and the Hydrogen-producing bacteria count (sample 4). Samples from the anaerobic reactors fed xylose (sample 3) and the Hydrogen-producing bacteria count (sample 4) showed 98% similarity. Two bands ("a" and "b") are predominant in samples 3 and 4. It is possible that these bands represent microbial populations which were favored by the operating conditions imposed and they were probably involved in the H_2 generation.

Zhao et al. (2010) [37] operated anaerobic reactors fed with xylose (2–5 g/L), peptone (5 g/L) and yeast extract (1 g/L), pH 5.5 for hydrogen production and observed changes in the bands profile suggesting of the microbial selection, as observed the present study. The similarity index ranged from 58% to 87% between the inoculum and tests performed.

Table 4 shows a comparison of the present study with previous studies regarding to the hydrogen yield and the configuration of the reactors as well as the pH, temperature, source of carbon and genetic affiliation of the bacteria involved in the biologic process. In the present study the yield of the production of H_2 observed was nearly to those of batch reactors operated under thermophilic conditions [37]. Lin et al. (2008) [66] and Lo et al. (2008) [67] operated batch reactors fed with xylose and they observed higher H_2 yield than in this study (0.7 mol xylose/mol H_2). However, the authors (op. cit.)

Fig. 2 – Consensus phylogenetic tree of the Phylum *Proteobacteria* based on the sequences clones of the sediment sample with primers for the Bacteria Domain. The bootstrap values indicate the repetition percentages (500 replicate runs). GenBank accession numbers are listed after the names of the species.

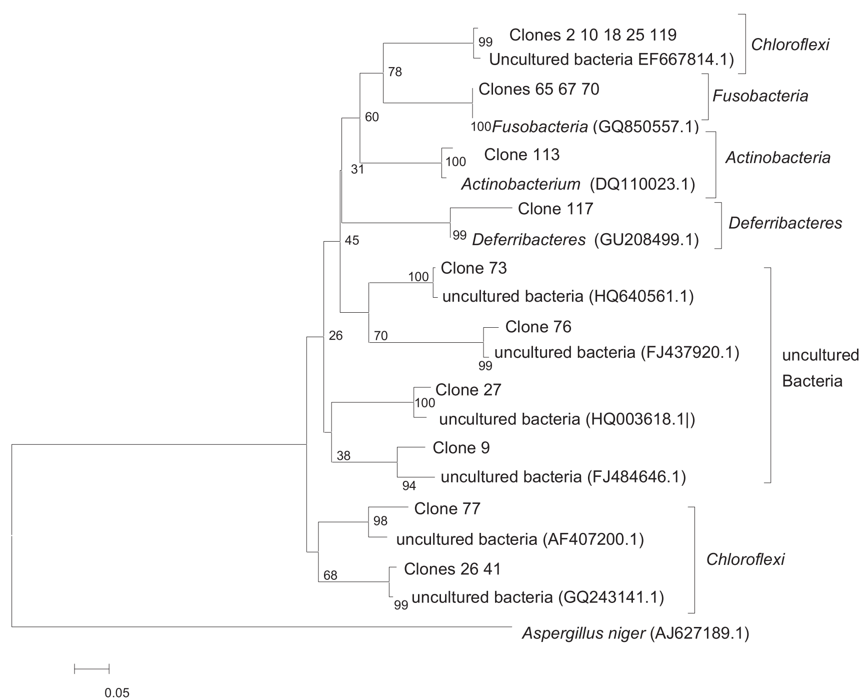


Fig. 3 – Consensus phylogenetic tree of the Phyla *Actinobacteria, Chloroflexi*, *Deferribacteres, Fusobacteria* and uncultured Bacteria based on the sequences of clones from the sediment sample with primers for the Bacteria Domain. The bootstrap values indicate the repetition percentages (500 replicate runs). GenBank accession numbers are listed after the names of the species.

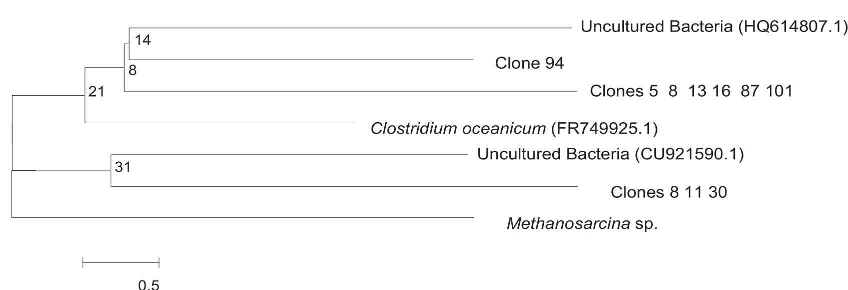


Fig. 4 – Consensus phylogenetic tree of the Phylum *Firmicutes* based on the sequences of clones from the sediment sample with primers for the Bacteria Domain. The bootstrap values indicate the repetition percentages (500 replicate runs). GenBank accession numbers are listed after the names of the species.

worked with higher concentrations of xylose (18.75 and 20.0 g/L, respectively). Furthermore, the phylogenetic analyses identified the predominance of bacteria belonging to the phylum *Firmicutes* as the main responsible for the hydrogen bio-production [4,37,65,66]. These evidences indicate that the phylum *Firmicutes* was probably involved in the biological processes of generating hydrogen in the conditions studied.

The experimental results as the generation of acetic acid and butyric acid showed that the microorganisms arising from the sediment had metabolic function similar to the bacterial species that generate H_2. The inoculum coming from the reservoir sediment heat-treated and further enriched, showed ability to generate hydrogen from xylose under the conditions studied.

This fact revealed that the bacterial diversity present in the environmental sample contributed to the metabolic processes

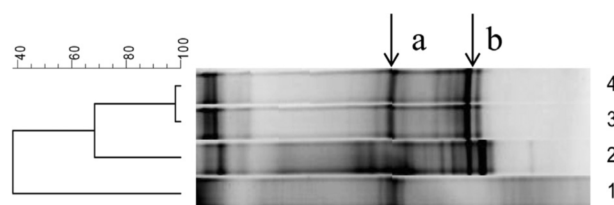


Fig. 5 – UPGMA Dendogram based on Similarity Coefficient using the Pearson Correlation from the DGGE Analysis with Set Primer Bacteria Domain (968FGC – 1392R): (1) Itupararanga Sediment Reservoir, (2) Sample of Purified Cells by Serial Dilutions (10^{-5}, 10^{-7}, 10^{-10}), (3) Anaerobic Batch Reactors Fed Xylose, and (4) Hydrogen-producing Bacteria Count (MPN).

Table 4 – Comparative study on the hydrogen fermentative production in anaerobic batch reactors fed with xylose.

Xylose	pH temperature	Inoculum	H_2/C source (mol/mol)	Phylum	Reference
1.8 g/L	5.5 37 °C	UASB reactor	0.6	*Proteobacteria* *Firmicutes*	[4]
4.5 g/L	6.5 70 °C	H_2-producing bioreactor	0.3	*Firmicutes*	[37]
18.75 g/L	7.1 35 °C	sewage treatment plant	0.7	*Firmicutes*	[66]
20.0 g/L	7.5 37 °C	*C. butyricum* CGS5	0.7	*Firmicutes*	[67]
2.0 g/L	5.5 37 °C	90 °C 10′ Sediment reservoir	0.3	*Firmicutes*	This study

involved in the degradation of xylose and H_2 generation [11], as well as the inoculum of sludge from treatment plants sewage [16], sludge from water treatment plants [23,66], activated sludge systems [68], manure [11], pure cultures from sediments [5,6,21], among others.

Therefore, in this work, with reactors fed with xylose, peptone and initial pH 5.5 it was obtained Gram positive rods and endospore formers, which probably were involved in the generation of H_2.

Conclusions

The environmental sample such as the sediment from a reservoir presented high bacterial diversity. The microorganisms from the sampling site were identified as belonging to the phyla *Proteobacteria*, *Firmicutes*, *Chloroflexi*, *Actinobacteria*, *Cyanobacteria*, *Fusobacteria*, *Deferribacteres* and uncultured bacteria.

There was no methane production in anaerobic reactors, confirming the efficiency of the heat treatment of the inoculum and the imposition of initial pH 5.5. The combination of these two factors caused the inhibition of bacteria H_2-consuming and methanogenic archaea.

The H_2 production was directly proportional to the mixture of the end products generated, which were mainly ethanol, n-butanol, acetic, isobutyric and butyric acids.

The sediment of the reservoir was an effective inoculum to enrich anaerobic microbial community capable of generating hydrogen through xylose consumption. The biological production of hydrogen gas was due to the microorganism consortia from the sediment reservoir and they were present in the studied conditions.

The concentrations of xylose used did not inhibit microbial growth and hydrogen production. This study enables future tests with environmental samples and wastewater with xylose applied to hydrogen bio production.

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REFERENCES

[1] Wu TY, Hay JXW, Kong LB, Juan JC, Jahim JM. Recent advances in reuse of waste material as substrate to produce biohydrogen by purple non-sulfur (PNS) bacteria. Renew Sustain Energy Rev 2012;16:3117–22.
[2] Hay JXW, Wu TY, Juan JC, Jahim JM. Biohydrogen production through photo fermentation or dark fermentation using waste as a substrate: overview, economics, and future prospects of hydrogen usage. Biofuels Bioprod Biorefining 2013;7:334–52.
[3] Roske K, Roske I, Uhlmann D. Characterization of the bacterial population and chemistry in the bottom sediment of a laterally subdivided drinking water reservoir system. Limnologica 2008;38:367–77.
[4] Maintinguer SI, Fernandes BS, Saavedra N, Duarte I, Adorno MAT, Varesche MB. Fermentative hydrogen production with xylose by *Clostridium* and *Klebsiella* species in anaerobic batch reactors. Int J Hydrogen Energy 2011;36:13508–17.
[5] Khamtib S, Reungsang A. Biohydrogen production from xylose by *Thermoanaerobacterium thermosaccharolyticum* KKU19 isolated from hot spring sediment. Int J Hydrogen Energy 2012;37:12219–28.
[6] Junghare M, Subudhi S, Lal B. Improvement of hydrogen production under decreased partial pressure by newly isolated alkaline tolerant anaerobe, *Clostridium butyricum* TM-9A: optimization of process parameters. Int J Hydrogen Energy 2012;37:3160–8.
[7] Veziroglu TN, Das D. Advances in biological hydrogen production process. Int J Hydrogen Energy 2008;33:6046–57.
[8] Mudhoo A, Kumar S. Effects of heavy metals as stress factors on anaerobic digestion processes and biogas production from biomass. Int J Environ Sci Technol 2013;10:1383–98.
[9] Wong YM, Wu TY, Juan JC. A review of sustainable hydrogen production using seed sludge via dark fermentation. Renew Sustain Energy Rev 2014;34:471–82.
[10] Kapdan IK, Kargi F. Bio-hydrogen production from waste materials. Enzyme Microb Technol 2006;38:569–82.
[11] Fan Y, Li C, Lay JJ, Hou H, Zhang G. Optimization of initial substrate and pH levels for germination of sporing hydrogen-producing anaerobes in cow dung compost. Bioresour Technol 2004;91:189–93.
[12] Zhang Y, Liu G, Shen J. Hydrogen production in batch culture of mixed bacteria with sucrose under different iron concentrations. Int J Hydrogen Energy 2005;30:855–60.
[13] Zhang H, Bruns MA, Logan BE. Biological hydrogen production by *Clostridium acetobutyricum* in an unsaturated flow reactor. Water Res 2006;40:728–34.
[14] Wu SY, Lin CY, Lee KS, Hung CH, Chang JS, Lin PJ, et al. Dark fermentative hydrogen production from xylose in different

bioreactors using sewage sludge microflora. Int J Hydrogen Energy 2008;22:113–9.
[15] Maintinguer SI, Fernandes BS, Duarte IC, Saavedra NK, Adorno MT, Varesche MB. Fermentative hydrogen production by microbial consortium. Int J Hydrogen Energy 2008;33:4309–17.
[16] Lin CY, Cheng CH. Fermentative hydrogen production from xylose using anaerobic mixed microflora. Int J Hydrogen Energy 2006;31:832–40.
[17] Maintinguer SI, Sakamoto IK, Oliveira LL, Lazaro CZ, Varesche MBA. Hydrogen bio-production with xylose from tropical climate environmental sample. In: Sarin Amit, editor. Essays on environmental studies, 127–138; 2012. p. 328. ISBN: 978-960-9549-75-2.
[18] Castelló E, Santos CG, Ilesias T, Paolino G, Wenzel J, Borzacconi L, et al. Feasibility of bio-hydrogen production from cheese whey using a UASB reactor: links between microbiol community and reactor performance. Int J Hydrogen Energy 2009;34:5674–82.
[19] Subudhi S, Nayak T, Kumar NR, Vijayananth P, La B. Impact of regulated pH on proto scale hydrogen production from xylose by an alkaline tolerant novel bacterial strain, *Enterobacter cloacae* DT-1. Int J Hydrogen Energy 2013;38:2728–37.
[20] Tan HT, Dykes GA, Wu TY, Siow LF. Enhanced xylose recovery from oil palm empty fruit bunch by efficient acid hydrolysis. Appl Biochem Biotechnol 2013;170:1602–13.
[21] An D, Qing Li, Wang X, Yang H, Guo L. Characterization on hydrogen production performance of a newly isolated *Clostridium beijerinckii* YA001 using xylose. Int J Hydrogen Energy 2014;39:19928–36.
[22] Cunha DGF, Ogura AP, Calijuri MDC. Nutrient reference concentrations and trophic state boundaries in subtropical reservoirs. Water Sci Technol 2012;65:1461–7.
[23] Kim DH, Han SK, Kim SH, Shin HS. Effect of gás sparging on continuous fermentative hydrogen production. Int J Hydrogen Energy 2006;31:2158–69.
[24] Del Nery V. Use of anaerobic sludge immobilized in gel in the study of the departure of reactors with upflow sludge blanket. Thesis (Masters). Engineering School of São Carlos, São Paulo University; 1987 ["In portuguese"].
[25] Widdel F, Pfennig N. Genus desulfobacter. Bergey's Man Syst Bacteriol 1984;2:1599.
[26] Adorno MAT, Hirasawa JS, Varesche MBA. Development and validation of two methods to quantify volatile acids (C2-C6) by GC/FID: headspace (automatic and manual) and liquid-liquid extraction (LLE). Am J Anal Chem 2014;5:406–14.
[27] Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28:350–6.
[28] Herbert D, Philipps OS, Strang RE. Carbohydrate analysis. Methods Enzymol 1971:265–77. SB.
[29] APHA AWWA WEF. Standard methods for the examination of water and wastewater. 21th ed. American Public Health Association; American Water Works Association, Water Environment Federation; 2005 (Centennial Edition).
[30] Griffiths RI, Whiteley AS, O'Donnell AG. Rapid method for coextration of DNA and RNA from natural environments for analysis of ribosomal DNA and rRNA-based microbial community composition. Appl Environ Microbiol 2000;66:5488–91.
[31] Lane DJ. 16S/23S rRNA sequencing. In: Stackebandt E, editor. Nucleic acid techniques in bacterial systematics; 1991. p. 115–75.
[32] Messing J. New M13 vectors for cloning. Meth Enzymol 1983;101:20–78.
[33] Kumar S, Tamura K, Jakobsen IB, Nei M. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Briefings Bioinform 2004;5:150–63.
[34] Kimura M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 1980;16:111–20.
[35] Nielsen AT, Liu WT, Filipe C, Grady L, Molin S, Stahl DA. Identification of a novel group of bacteria in sludge from a deteriorated biological phosphorus removal reactor. Appl Environ Microbiol 1999;65:1251–8.
[36] Selembo PA, Perez JM, Lloyd WA, Logan BE. Enhanced hydrogen and 1,3-propanediol production from glycerol by fermentation using mixed cultures. Biotechnol Bioeng 2009;104(6).
[37] Zhao C, Karakashev D, Lu W, Wang H, Angelidaki I. Xylose fermentation to biofuels (hydrogen and ethanol) by extreme thermophilic (70 °C) mixed culture. Int J Hydrogen Energy 2010;35:3415–22.
[38] Khanal SK, Chen W-H, Li L, Sung S. Biological hydrogen production: effects and intermediate products. Int J Hydrogen Energy 2004;29:1123–31.
[39] Das D, Veziroglu TN. Hydrogen production by biological processes: a survey of literature. Int J Hydrogen Energy 2001;26:13–28.
[40] Tabatabaei M, Rahim RA, Abdullah N, Wright AD, Shirai Y, Sakai K, et al. Importance of the metanogenic archaea populations in anaerobic wastewater treatments. Process Biochem 2010;45:1214–25.
[41] Oh SE, Ginkel SV, Logan BE. The relative effectiveness of pH control and heat treatment for enhancing bio-hydrogen gas production. Environ Sci Technol 2003;37:5186–90.
[42] Chen WM, Tseng ZJ, Lee KS, Chang JS. Fermentative hydrogen production with *Clostridium butyricum* CGS5 isolated from anaerobic sewage sludge. Int J Hydrogen Energy 2005;30:1063–70.
[43] Fang HHP, Li C, Zhang T. Acidophilic bio-hydrogen production from rice slurry. Int J Hydrogen Energy 2006;31:683–92.
[44] Im W-T, Kim D-H, Kim K-H, Kim M-S. Bacterial community analyses by pyrosequencing in dark fermentative H_2-producing reactor using organic wastes as a feedstock. Int J Hydrogen Energy 2012;37:8330–7.
[45] Ferrer M, Guazzaroni ME, Richter M, García Salamanca A, Yarza P, Suárez-Suárez A, et al. Taxonomic and functional metagenomic profiling of the microbial community in the anoxic sediment of a sub-saline shallow lake (Laguna de Carrizo, Central Spain). Environ Microbiol 2011;62(4):824–37.
[46] Tamminen M, Karkman A, Corander J, Paulin L, Virta M. Differences in bacterial community composition in Baltic Sea sediment in response to fish farming. Aquaculture 2011;313:15–23.
[47] Shao K, Gao G, Qin B, Tang X, Wang Y, Chi K, et al. Comparing sediment bacterial communities in the macrophyte-dominated and algae-dominated areas of eutrophic Lake Taihu, China. Can J Microbiol 2011;57:263–72.
[48] Kwon S, Kim T-S, Yu GH, Jung J-H, Park H-D. Bacterial community composition and diversity of a full-scale. Integrated fixed-film activated sludge system as investigated by pyrosequencing. J Microbiol Biotechnol 2010;20:1717–23.
[49] Xing W, Zhao Y, Zuo J. Microbial activity and community structure in a lake sediment used for psychrophilic anaerobic wastewater treatment. J Appl Microbiol 2010;109:1829–37.
[50] Kormas KA, Vardaka E, Goun MM, Petridou VKE, Gkelis S, Neofitou C. Molecular detection of potentially toxic cyanobacteria and their associated bacteria in lake water column and sediment. World J Microbiol Biotechnol 2010;26:1473–82.
[51] Lu Y, Rosencrantz D, Liesack W, Conrad R. Structure and activity of bacterial community inhabiting rice roots and the rhizosphere. Environ Microbiol 2006;8:1351–60.

[52] Shida GM, Sader LT, Cavalcante EL, Sakamoto IK, Maintinguer SI, Saavedra NK, et al. Performance and composition of bacterial communities in anaerobic fluidized bed reactors for hydrogen production: effects of organic loading rate and alkalinity. Int J Hydrogen Energy 2012;37:16925–34.
[53] Long CN, Cui JJ, Liu ZT, Liu Y, Long MN, Hu Z. Statistical optimization of fermentative hydrogen production from xylose by newly isolated *Enterobacter* sp. CN1. Int J Hydrogen Energy 2010;35:6657–64.
[54] Niu K, Zhang X, Tan WS, Zhu ML. Characteristics of fermentative hydrogen production with *Klebsiella pneumoniae* ECU-15 isolated from anaerobic sewage sludge. Int J Hydrogen Energy 2010;35:71–80.
[55] Schwarz JIK, Lueders T, Eckert W, Conrad R. Identification of acetate-utilizing bacteria and archaea in methanogenic profundal sediments of Lake Kinneret (Israel) by stable isotope probing of rRNA. Environ Microbiol 2007;9:223–37.
[56] Simonato F, Gomez-Pereira P, Fuchs BF. Bacterioplankton diversity and community composition in the Southern Lagoon of Venice. Syst Appl Microbiol 2010:33128–38.
[57] Liu J, Wu W, Chen C, Sun F, Chen Y. Prokaryotic diversity, composition structure, and phylogenetic analysis of microbial communities in leachate sediment ecosystems. Appl Microbiol Biotechnol 2011;91:1659–75.
[58] Jangid K, Williams M, Franzluebbers AJ, Sanderlin JS, Reeves JH, Jenkins MB, et al. Whitman WB relative impacts of land-use, management intensity and fertilization upon soil microbial community structure in agricultural systems. Soil Biol Biochem 2008;40:2843–53.
[59] Escoffier S, Cayola J-L, Olliviera B, Patelb BKC, Fardeaua M-L, Thomasa P, et al. Identification of thiosulfate- and sulfur-reducing bacteria unableto reduce sulfate in ricefield soils. Eur J Soil Biol 2001;37:145–56.
[60] Furlong MA, Singleton DR, Coleman DC, Whitman WB. Molecular and culture-based analyses of prokaryotic communities from an agricultural soil and the burrows and casts of the earthworm *Lumbricus rubellus*. Appl Environ Microbiol 2002:1265–79.
[61] Wirth R, Kovacs E, Maroti G, Bagi Z, Rakhely G, Kovacs K. Characterization of a biogas-producing microbial community by short-read next generation DNA sequencing. Biotechnol Biofuels 2012;5:41.
[62] Edmonds J, Weston NB, Nathaniel B, Samantha B, Xiaozhen Joye, Moran Mou Mary Ann. Microbial community response to seawater amendment in low-salinity tidal sediments. Microbiol Aquatic Syst 2009;58:558–68.
[63] Weber KA, Urrutia MM, Churchill PF, Kukkadap RK, Roden EE. Anaerobic redox cycling of iron by freshwater sediment microorganisms. Environ Microbiol 2006;8:100–13.
[64] Ye W, Liu X, Lin S, Tan J, Pan J, Li D, et al. The vertical distribution of bacterial and archaeal communities in the water and sediment of Lake Taihu. FEMS Microbiol Ecol 2009;70:263–76.
[65] Hung C-H, Chang Y-T, Chang Y-J. Roles of microorganisms other than *Clostridium* and *Enterobacter* in anaerobic fermentative bio-hydrogen production systems – a review. Bioresour Technol 2011;102:8437–44.
[66] Lin CY, Wu CC, Hung CH. Temperature effects on fermentative hydrogen production from xylose using mixed anaerobic cultures. Int J Hydrogen Energy 2008;33:43–50.
[67] Lo YC, Chen WM, Hung CH, Chen SD, Chang JS. Dark H_2 fermentation from sucrose and xylose using H_2-producing indigenous bacteria: feasibility and kinetic studies. Water Res 2008;42:827–42.
[68] Ueno Y, Haruta S, Ishii M, Igarashi Y. Microbial community in anaerobic hydrogen-producing microflora enriched from sludge compost. Appl Microbiol Biotechnol 2001;57:555–62.