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Obtaining and Characterization of Mesophilic Bacterial Consortia from Tropical Sludges Applied on Biohydrogen Production

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Abstract

The biohydrogen production from different tropical sludges of biologic treatment plants was investigated in anaerobic batch reactors fed with sucrose in concentrations similar to food wastewaters. The tropical sludges tested were: I—granular sludge from a upflow anaerobic sludge blanket (UASB) reactor treating brewery wastewater; II—facultative anaerobic sludge from sedimentation pond treating domestic sewage; III—granular sludge from UASB reactor treating domestic sewage. The anaerobic batch reactors fed with 2 g L^{-1} of sucrose at pH 5.5 and 37 °C showed higher production of H$_2$ than all tropical sludges tested. The maximum yield was 2.0 mol H$_2$ mol of sucrose^{-1}. The intermediary products during fermentation were acetic and butyric acids. Therefore, the bioproduction probably followed both the acetic acid and butyric acid route. A wide diversity of hydrogen producing bacteria identified as *Clostridium* sp., *Bacillus megaterium, Staphylococcus* sp., *Bacillus subtilis and Lactobacillus* sp. was observed by phylogenetic analysis. Tropical sludges from biologic treatment plants can be applied on biohydrogen production.

Keywords Anaerobic bacteria · *Clostridium* · Denaturing gradient gel electrophoresis · *Lactobacillus* · Sucrose · 16S RNA · TOGA gas analyzer

Introduction

One of the most critical issues in biologic treatment plants of wastewaters is the sludge excess generated and it takes up about 60% of the operational costs with it management and disposal [1]. Besides the excessive cost, the disposal of sewage sludge is realized in landfills and it exacerbates the problem of management of municipal solid waste due to its high organic load and its hazardous potential. An ideal way to solve sludge-associated problems is to use it as feedstock for bioenergy and biofuels production, such as biogas generation in fermentation processes of treatment with wastewaters as carbon source.

Hydrogen gas (H$_2$) is an effective non-pollutant, cheap and renewable source of energy. Aiming this energetic potential, the hydrogen gas production increased worldwide ($\sim$6–10%). However, it is not available in nature, so methods to produce it are being studied all over the world. The use of bacteria consortia can be found in photo-fermentation, bio-photolysis and dark fermentation. Among these, the dark fermentation route does not require light or oxygen and has a high hydrogen production rate [2].

Various sources of inocula have been tested, and they had confirmed efficiency on hydrogen production, such as soil samples, sludge from domestic sewage treatment and wastewater treatment from industrial plants, among others [3]. However, these possibilities are mostly described for temperate climates. There are only a few studies about hydrogen production with tropical inocula, such as Brazil, where the annual average temperatures are around 28 °C, which is ideal

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for bacterial growth [3]. Also, there are few studies on the diversity of hydrogen producing bacteria coming from tropical sludges of biological treatment plants.

Sugars such as sucrose are present in many wastewaters from Brazilian agroindustries. Brazil is the main citrus and ethanol from sugar cane producer in the world. Annually the sugarcane production is approximately 716 million tons, which corresponds to 43% of worldwide production [4]. The area planted with sugarcane have increased from ~ 1.4 to 7 million ha, from 1960 to 2007 [5]. This kind of crop have been increasing even much more due to the low pollution power of ethanol. The Central region of São Paulo State-Brazil, which this study was carried out, is one of the most powerful ethanol producers and therefore the one of the biggest areas with sugarcane plantation [6]. The biggest company of ethanol fuel in Brazil is in the region of Ribeirão Preto and this region embrace 6 of the biggest sugarcane mills companies [7]. With these production of sugarcane and ethanol fuel we have a potential amount of sucrose being discarded with the mill's wastewater. The sucrose is removed as residual sugar in the sugarcane bagasse [4] which contributes with high potential of these kind of wastewater to be applied on hydrogen production in the future. Citrus industries from Brazil generate 8.4 million tons in 2014 [8]. Stillage—the main effluent generated from sugar cane processing industries for producing ethanol—and in other effluents from food industry of candies and soft drinks, for example [3], generate sucrose at concentrations that vary from 0.7 to 2.0 g L^{-1} [9]. However, there are few studies on hydrogen production from wastewaters with low concentrations of sucrose [10]. The hydrogen arising from these sources can be reused for energy generation. These facts encourage studies on the application of anaerobic sludges, which are used for the treatment of sewage and industrial wastewaters in biologic processes, for hydrogen generation with sucrose in concentrations about to 2.0 g L^{-1}. Many saccharides like glucose, hexoses, and polymers like starch and cellulose show different rates in hydrogen production per mole of organic substrate, depending on the fermentation route leading to carboxylic acids and/or alcohols. The acids produced are mainly lactic, acetic and butyric. The percentages of H_2 produced in these processes may vary between 60 and 90% (v/v) of the total amount of gas produced [11]. Other gases can be generated, like carbon dioxide (CO_2), methane (CH_4), hydrogen sulfide (H_2S) and oxygen (O_2) [12].

The increase of hydrogen production is related to a mixture of byproducts, such as acetic acid and butyric acid; and the decrease of hydrogen production is associated to the formation of propionic acid and by products that are lesser reduced, such as alcohols and lactic acid [9].

The fermentative production of hydrogen can be facilitated by the inactivation of methanogenic microorganisms [9] since they may be present in the inocula and consume hydrogen gas during the anaerobic processes. Avoiding the methanogenic microorganisms, some actions have been studied, such as pH control, dry heat treatment, heat shock, freezing and thawing or dry heat and desiccation. Among the pretreatments that shows higher efficiency in inactivation and hydrogen production, there is the heat shock [15]. It can be an effective way to avoid hydrogen consuming microorganisms [13] and favor the development of hydrogen producing bacteria. To that end, the tropical sludges from three different biologic treatment plants were applied for the biohydrogen production with sucrose as substrate in concentrations frequently found in mill's wastewaters and domestic sewages. Most probable number (MPN) analyses were performed to estimate the total of anaerobic bacteria and hydrogen producing bacteria.

Materials and Methods

Sources of Inoculum

The inocula were from tropical sludges: I—granular sludge from a upflow anaerobic sludge blanket (UASB) reactor treating brewery wastewater (Araraquara-SP-Brazil); II—facultative anaerobic sludge from sedimentation pond treating domestic sewage (Araraquara-SP-Brazil); III—granular sludge from UASB reactor treating domestic sewage (São José do Rio Preto-SP-Brazil). The granules of sources I, II and III were broken, separately, in a mortar and pestle.

Microbial Consortium Obtained and Growth Conditions

The cellular suspensions (20% v/v) from inocula I, II and III were reactivated separately in anaerobic batch reactors (100 ml) using sterile (120 °C, 20 min) PYG medium [glucose (10.0 g L^{-1}), peptone (5.0 g L^{-1}), yeast extract (5.0 g L^{-1}), and meat extract (5.0 g L^{-1})], under N_2 100% atmosphere at 37 °C during 96 h. Afterwards, these samples were submitted to heat treatment at 90 °C for 15 min, in order to inactivate hydrogen consumers and to obtain spore-forming anaerobic bacteria [15, 16]. After that, serial dilutions on PYG media at pH 5.5 were performed and the resulting cultures were used for the inoculation of the anaerobic batch reactors.

Operation of the Anaerobic Batch Reactors

The adapted cultures were centrifuged (9000 rpm at 4 °C for 6 min) and the cell pellets were suspended in new culture media, with the following composition (g L^{-1}) [14]: Solution A [$NiSO_4 \cdot 6H_2O$ (0.50), $FeSO_4 \cdot 7H_2O$ (2.50), $FeCl_3 \cdot 6H_2O$ (0.25), $CoCl_2 \cdot 2H_2O$ (0.04)]; Solution B [$CaCl_2 \cdot 6H_2O$

(2.06)]; Solution C [SeO$_2$ (0.14)]; Solution D [KH$_2$PO$_4$ (5.36), K$_2$HPO$_4$ (1.30), Na$_2$HPO$_4$·H$_2$O (2.76) two vitamin solutions (mg L^{-1}); solution E [biotin (10.0); p-aminobenzoic acid (40.0)]; and solution F [B$_{12}$ vitamin (40.0)] [13]. The culture media contained peptone (1.0 g L^{-1}), urea (0.04 g L^{-1}) and solutions (mL^{-1}): A 2.0, B 2.0, C 2.0, D 2.0, vitamin solutions (E) 2.0, (F) 2.0 [14]. The pH was adjusted to 5.5. The culture media were filtered through a previously sterilized 0.22 μm Millipore membrane.

Anaerobic batch reactors (2 L) capped with butyl rubber stoppers were fed with sucrose (2 g L^{-1}) and operated in duplicate. The headspace (1 L) was purged with N$_2$ (99.99%) during 20 min. They were kept at 37 °C, during 187 h.

Analytical Methods

The hydrogen, methane and CO$_2$ contents in the biogas were determined simultaneously in a single gas chromatography run in a TOGA—transformer oil gas analyzer system, coupled with a Trace GC Ultra–Thermo Gas Chromatograph—equipped with split/splitless injector and two detectors: thermal conductivity detector (TCD) and flame ionization detector (FID), with methanizer [17]. The fraction containing hydrogen, nitrogen, and methane was analyzed in a Rt-MSieve 5A° 30 m × 0.53 mm i.d. column. Hydrogen and nitrogen were detected by the TCD. CO$_2$ was detected by the FID after passing through the methanizer. Argon was used as carrier gas (1.5 mL min^{-1} in splitless mode). The FID temperature was 250 °C; the TCD and injector were adjusted to150 °C. The oven programing was 50 °C (4.5 min), heating from 40 °C min^{-1} to 180 °C (1.5 min) then cooling from 50 °C min^{-1} to 50 °C (3.15 min).

Volatile acid and alcohols, which were present in the residual media at the end of the assay, were separated by gas chromatography, using a Shimadzu gas chromatograph (GC model 2010), equipped with a flame ionization detector, a COMBI-PAL headspace auto-sampler system (AOC 5000), and a HP-INNOWAX column (30 m × 0.25 mm × 0.25 μm of film thickness) [18].

Sucrose concentrations were determined by a colorimetric method [19, 20].

The adjustments of pH were made with the addition of hydrochloric acid or sodium hydroxide diluted solutions. The pH measurements were made at the beginning and at the end of the assays [20].

Cellular Growth

The cellular growth was monitored by optical density at 600 nm (OD600), using a spectrophotometer UV–VIS BIO-SPEC SP-220 [21].

Bacteria Counting by Most Probable Number (MPN)

Most probable number (MPN) technique was performed to estimate the total of anaerobic bacteria and hydrogen producing bacteria.

The MPN were made in quintuplicate dilutions at the end of the operation of the anaerobic batch reactors. The measurements were conducted 168 h after incubation, by gas chromatography (hydrogen gas presence/absence), previously described and analyzed according to standard probability table [34].

Molecular Biology Analysis

At the end of the anaerobic reactors operation, the microbial community of samples from inocula I, II and III were analyzed using denaturing gradient gel electrophoresis (DGGE). The DNA extraction was made with phenol and chloroform [22]. The 16S rDNA gene was amplified through polymerase chain reaction (PCR) using 1968FGC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3′)–1401GC (5′-CGG TGT GTA CAA GAC CC-3′) primers for Bacteria Domain [23]. The amplification program of genomic DNA fragments for PCR were 30 cycles, using the following method: initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 0.45 min, annealing at 55 °C for 0.45 min, extension at 72 °C for 1.0 min, final extension at 94 °C for 7 min, and cooling to 4 °C [24].

The amplified fragments were separated by electrophoresis on polyacrylamide gel at DGGE on TAE (Tris-Acetate-EDTA) buffer at 75 V and 65 °C for 16 h on polyacrylamide gel containing linear gradient that varies from 45 to 65% denaturant. After electrophoresis, the gel was stained with ethidium bromide for 20 min and read in a TMIII Eagle Eye (Stratagene) under exposure to UV at 254 nm, coupled to a computer and software Eagle Sight. The gel bands were cut out and amplified with the same set primer, however without the GC clamp. The program of PCR procedure was: initial denaturation at 95 °C for 7 min.; denaturation at 95 °C for 45 s; annealing at 56 °C for 45 s; extension at 72 °C for 1 min; final extension at 72 °C for 10 min and cooling at 4 °C.

Nucleotide sequence analyses were performed by Macrogen Inc® [25]. Then they were grouped in OTU (Operational Taxonomic Unit) and compared to the National Center for Biotechnology Information (NCBI) database.

The construction of the DGGE dendrogram was performed using BioNumerics' software package (Applied Biomath, Belgium) and the similarity coefficients were calculated from the densitometric curves of the scanned DGGE profiles, using the Jaccard correlation.

Shannon-Wiener diversity indexes were calculated based on the bands' intensities on the gel tracks. The peaks heights were evaluated in the densitometric curves, according to the Eq. (1).

$$H = - \sum \left(P_i \ln \left(P_i \right) \right) \tag{1}$$

where H is the diversity index and P_i is the importance probability of the bands in a lane ($P_i = n_i/N$, where n_i is the height of an individual peak and N is the sum of all peaks heights in the densitometric curves) [26].

Experimental Data Adjustment

The cumulative H_2 production in the duplicates of anaerobic batch reactors was kinetically simulated by Statistica® software, version 8. The maximum rate of hydrogen production was obtained through a non-linear sigmoidal adjust of the modified Gompertz function [27] according to the Eq. (2).

$$H = P. \exp \left\{ - \exp \left[\frac{Rm \cdot e}{P} (\lambda - t) + 1 \right] \right\} \tag{2}$$

where H is the cumulative H_2 production (mmol), P is the H_2 production potential (mmol H_2 L^{-1} of culture), Rm is the maximum H_2 production rate (mmol H_2 L h^{-1}), λ is the lag phase time (h), and e is 2.718281828. The values of P, Rm, and λ for each batch experiment were estimated using the nonlinear estimation function in Statistica 8.0 software.

Results and Discussion

The gas chromatography analysis confirmed the absence of methane in the headspace of all the batch reactors. Therefore, the heat treatment applied was efficient for the inactivation of methanogenic microorganisms, which are responsible for the hydrogen consumption during the anaerobic metabolism. Wang et al. [28] conducted assays with two different strains of *Clostridum perfringens* (W11, W12) and obtained 1.5 mol H_2 mol hexose^{-1} after heat treatment of anaerobic cultures. Subudhi et al. [29] applied heat treatment on a crude oil source to isolate hydrogen producing bacteria and inactivate methanogenic microorganisms. They isolated *E. cloacae* DT-1 and used it in anaerobic batch reactors fed with 10 g L^{-1} of glycerol at pH 7.0 and 37 °C, obtaining 6.6 mmol H_2 L^{-1}. The present study and the authors cited have carried out heat treatment and the H_2 yields for this study were, in mol H_2 mol of sucrose^{-1}, (I) 1.0, (II) 1,3 and (III) 2.0, which were higher than presented by the authors cited. It suggests the heat treatment is an efficient pretreatment to inactivate the methanogen microorganisms.

In this work, the maximum cellular growth (ABS$_{600}$) from the inocula were (I) 0.2, (II) 0.2, and (III) 0.5, during 31, 98

and 53 h of operation, respectively. The highest hydrogen production was obtained from inoculum III (granular sludge from UASB reactor treating domestic sewage), 8.1 mmol H_2 L^{-1} and these results were higher than Subuthi et al. [29] with *E. cloacae* DT-1, described previously. Inocula I (granular sludge from a UASB treating brewery wastewater) and II (facultative anaerobic sludge from sedimentation pond treating domestic sewage) produced 4.5 and 4.7 mmol H_2 L^{-1} respectively (Table 1). The yields expressed in mol H_2 mol sucrose^{-1}, were 1.0, 1.3, and 2.0 for I, II, and III, respectively. The results from inoculum III were higher than the 1.4 mol H_2 mol of sugar^{-1} obtained by Subudhi et al. [29]. This fact could be the evidence that in anaerobic bacteria consortia occur some interaction that favor the H_2 production process. It also shows the advantages to use and carry out studies using real anaerobic bacteria consortia.

The lag phase on hydrogen generation was observed during the assays. Inoculum III showed a lag phase of 21.3 h, with maximum hydrogen production after 60 h. However, the reactors that operated with inocula I and II showed lag phases of 13.3 and 17.8 h, with maximum H_2 generation at 52 and 160 h, respectively (Fig. 1). The maximum H_2 production rates were 0.2, 0.1 and 0.4 mmol H_2 L^{-1} h^{-1} for inocula I, II, and III, respectively.

Inoculum III demonstrated the best efficiency on hydrogen generation (2.0 mol H_2 mol sucrose^{-1}), when compared to inocula I and II. Maintinguer et al. [9] obtained a lower efficiency (1.2 mol H_2 mol consumed sucrose^{-1}), when using the granulated sludge of a UASB reactor from swine wastewaters, previously heat treated, using anaerobic batch reactors fed with 1.8 g sucrose L^{-1}, pH 5.5 at 37 °C, during 240 h of operation. This comparison shows that the sludge generating potential is possible to be deeply related to the waste that the sludge treats in the source.

The amounts of organic acids produced were acetic acid (39.7, 42.8 and 173.3 mg L^{-1}) and butyric acid (73.8, 157.0 and 234.0 mg L^{-1}) from inocula I, II, and III, respectively (Fig. 2). Comparable results were obtained by Liu et al. [30] during experiments on H_2 production. They observed that the two main products of fermentation were acetic and butyric acids during the operation of anaerobic batch reactors fed with synthetic wastewater containing 5 g of cellulose

Table 1 Kinetic parameters of H_2 production for different Tropical sludges tested in anaerobic batch reactors fed with 2 g L^{-1} of sucrose

Tropical sludges	P (mmol H_2 L^{-1})	Rm (mmol H_2 L h^{-1})	λ (h)	R^2
(I) Brewery	4.5	0.18	13.3	0.994646
(II) Facultative anaerobic sludge	4.7	0.05	17.8	0.979170
(III) Domestic sewage	8.1	0.39	21.3	0.993191

Fig. 1 Cumulative H_2 production (mmol H_2 L^{-1}) from Tropical sludges I, II, and III during the operation of anaerobic batch reactors fed with sucrose 2 g L^{-1}. (*I*) granular sludge from a UASB reactor treating brewery wastewater; (*II*) facultative anaerobic sludge from sedimentation pond treating domestic sewage; (*III*) granular sludge from UASB reactor treating domestic sewage
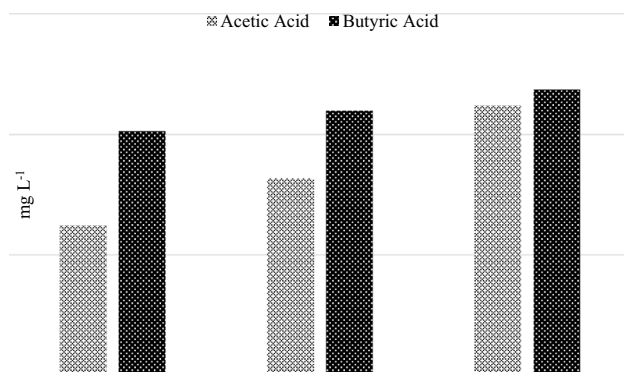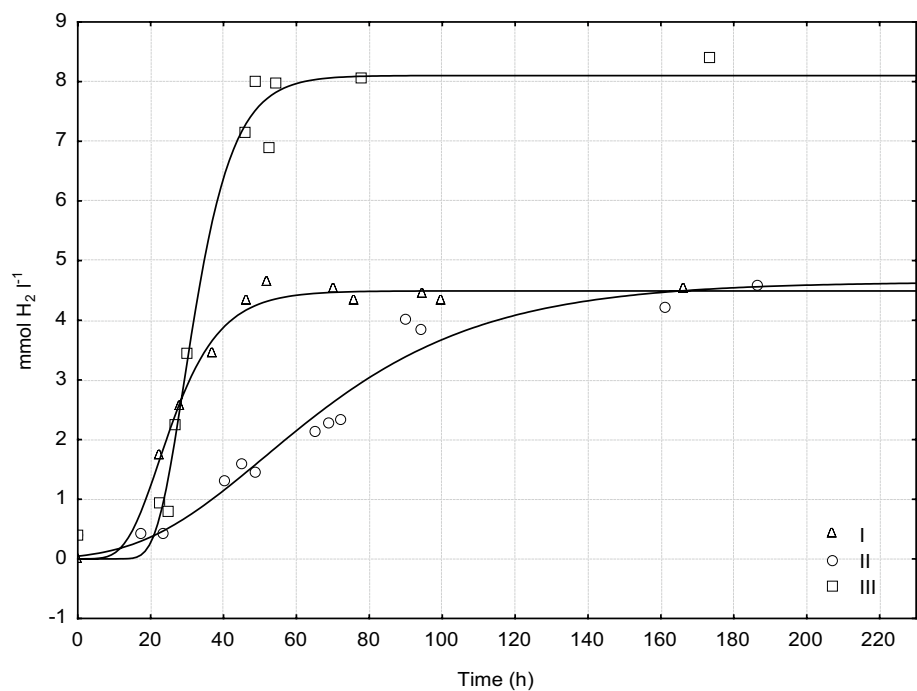

Fig. 2 Organic acids generated (mg L^{-1}) at the end of the operation of anaerobic batch reactors fed with sucrose (2 g L^{-1}) from tropical sludges (I) granular sludge from a UASB reactor treating brewery wastewater; (II) facultative anaerobic sludge from sedimentation pond treating domestic sewage; (III) granular sludge from UASB reactor treating domestic sewage

L^{-1} at 55 °C with pH varying from 5.5 to 8.5. Generally, the increasing in operating time during the hydrogen bioproduction provides an increasing in amounts of fatty acids [28, 31]. Wang et al. [28] conducted assays with two different strains of *Clostridum perfringens* (W11, W12) in reactors fed with sucrose (10 g L^{-1}) for 13 h, pH 7.0, at 37 °C. The authors obtained the following amounts of fatty acids (mg L^{-1}): acetic acid (1.680; 1.610) and butyric acid (2.280; 2.600) for W11, and W12, respectively. The yields of hydrogen were 1.5 and 1.6 mol H_2 mol sucrose^{-1} for W11, and W12, respectively. Therefore, the bacterial consortia from

the present study showed better results than the obtained by Wang et al. [28] with isolated bacteria that theoretically should show higher efficiencies. Jame et al. [31] performed a hydrogen production assay using *Clostridium* sp. fed with glucose (5 g L^{-1}) for 19 h and obtained 4.2 mmol of acetic acid L^{-1} and 2.7 mmol of butyric acid L^{-1}. Both assays conducted by Wang et al. [28] and Jame et al. [31] showed low acid production, probably due to their low operation times. As a result, Jame et al. [31] obtained 4.1 mmol H_2 L^{-1} with glucose as substrate. Therefore, these values obtained are lower than ours from inocula I, II, and III: 4.5, 4.7, and 8.1 mmol H_2 L^{-1}, respectively. In this regard, the higher production of VFAs seems to be involved in the higher biohydrogen production when compared to the authors with lower results. It can be explained with the stochiometric equations of the H_2 production from the VFAs found in these studies.

The final pH at the end of operation of the anaerobic batch reactors were 4.4, 3.5, and 3.4, from inocula I, II, and III, respectively. These results confirmed the decrease in pH was due to production of volatile fatty acids, like butyric and acetic acids, generated during the operation of the anaerobic batch reactors which deplete the buffering capacity of the medium, resulting in a lower final pH [32].

The bacteria count of the samples revealed higher values for total anaerobic bacteria than for H_2 producing bacteria (Table 2). Sá et al. [33] obtained lower values of total anaerobic bacteria (2.3E + 2 MPN 100 mL^{-1}) from domestic sewage sludge, pretreated on batch reactors fed with sucrose (10 g L^{-1}) compared with the present study. Maintinguer et al. [24] observed lower counts of H_2

Table 2 Bacteria count (MPN) with different tropical sludges during the operation of anaerobic batch reactors

MPN 100 mL^{-1}

Tropical sludges	Total anaerobic bacteria	H$_2$ producing bacteria
I—Granular sludge from a UASB reactor treating brewery wastewater	2.1E+10	2.4E+6
II—Facultative anaerobic sludge from sedimentation pond treating domestic sewage	>4.6E+11	3.0E+2
III—Granular sludge from UASB reactor treating domestic sewage	>1.1E+10	>1.1E+10

producing bacteria with inocula from reservoir sediment previously adapted to hydrogen production with xylose as carbon source (4.3 MPN 100 mL^{-1}). Our results (Table 2) are close to the obtained by Castelló et al. [34] (>2.4E+11 MNP mL^{-1} and 9.0E+11 MNP mL^{-1} during 69 and 129 days, respectively) with inocula from an UASB reactor fed with cheese whey (67.0 COD g L^{-1}), at 30 °C, pH 5.0. This amount of total anaerobic bacteria could be involved in the high potential of this consortia. For bioH$_2$ production interactions between microorganisms are extremally important and until now little is known. However, these interactions could contribute to cooperative work among bacteria in the consortia.

Microscopic analysis during the operation of the reactors showed the predominance of Gram-positive rods (data not shown), that are the characteristic morphologies of hydrogen producing bacteria, such as *Clostridium* and *Bacillus* species [35].

The bacterial diversity of the tropical sludges I, II, and III showed differences (Fig. 3a). The similarity index ranged between 20 and 40% among the inocula, showing a heterogeneity of the anaerobic bacteria from the tropical sludges pretreated and during the operation of the reactors fed with sucrose.

The Shannon-Wiener diversity indices (H Index) were higher for the inoculum II with pretreatment (2.42), and inoculum III with pretreatment (2.35) (Fig. 3a) when compared to the inoculum I with pretreatment (0.78). These differences could be related to the characteristics of the sanitary sewage (inocula II and III) compared to the industrial waste (inoculum I), which generally contains a lower range of carbon sources and some compounds from industrial processes that could be toxic to certain populations of microorganisms.

The decreasing of the bacterial diversity was observed between pretreated inocula [samples 3 (0.78), 1 (2.42), and 5 (2.35)] and reactivated inocula [samples 4 (0.66), 2

Fig. 3 DGGE analyses with Set Primer Bacteria Domain (968FGC–1392R): **a** UPGMA Dendogram based on similarity coefficient using the Jaccard Correlation; and **b** cut bands from DGGE gel for the samples [(*1*) tropical sludge II pretreated (pH 5.5 and heat treatment); (*2*) tropical sludge II reactivated with sucrose; (*3*) tropical sludge I pretreated (pH 5.5 + heat treatment); (*4*) tropical sludge I reactivated with sucrose; (*5*) tropical sludge III pretreated (pH 5.5 and heat treatment); (*6*) tropical sludge III reactivated with sucrose]
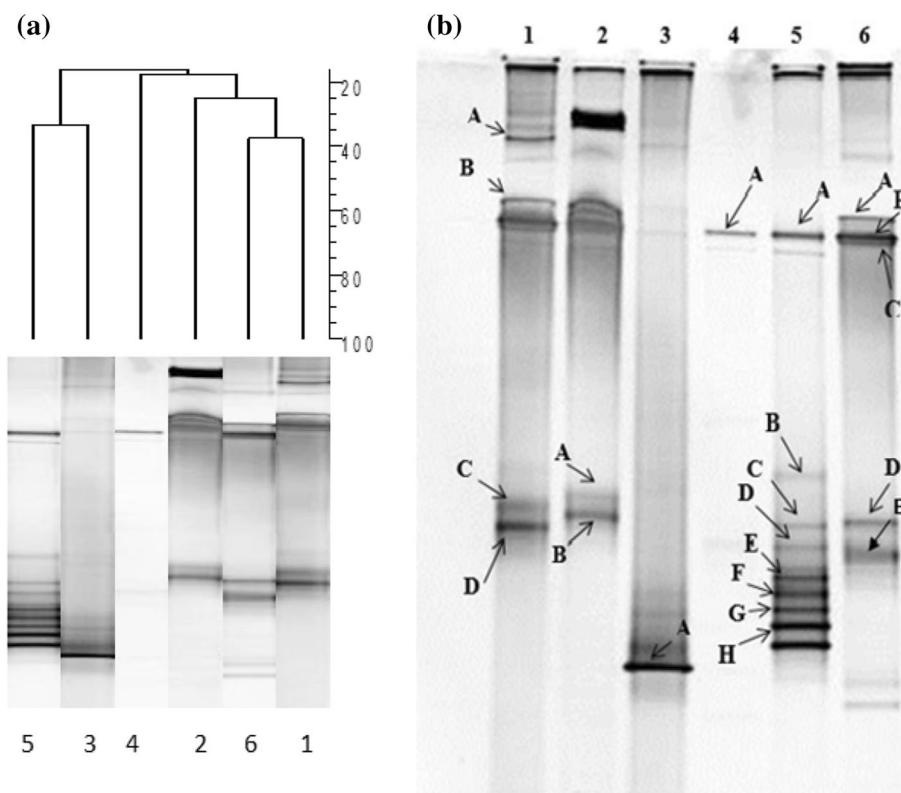
(2.15), and 6 (2.10)]. The experimental conditions imposed, selected the populations from the samples 3, 1, and 5, which were involved in the H_2 production (samples 4, 2, and 6). Abreu et al. [26] observed a decrease of the diversity during the operation of anaerobic reactors applied to H_2 generation. Ratti et al. [36] observed a difference in bands' number and pattern between the original inoculum and after the pretreatment in anaerobic batch reactors fed with 2 and 4 g L^{-1} of glucose, applied to biohydrogen production. The authors concluded that the operating conditions imposed the inactivation of non-resistant bacteria, as it was observed in the present study.

The phylogenetic characterization beyond the bands cut, showed similarity to species like *Burkholderia, Clostridium* sp., *Staphylococcus, Streptococcus, Bacillus e Lactobacillus* (Fig. 3b; Table 3).

Clostridium sp. (OTU 1) is an anaerobic bacterium recognized as H_2 producer [37]. Song et al. [1] isolated *Clostridium* sp. from cellulosic biomass using anaerobic batch reactors at 36 °C and obtained a maximum production of 92.9 mL H_2 g^{-1} of biogas. This species demonstrates higher efficiencies for hydrogen production from the cellulosic biomass. During the operation of the anaerobic batch reactors morphologies of gram-positive bacilli and endospores (data not shown) that are characteristic of *Clostridium* sp. were observed.

Staphylococcus sp. (OTU 2) is a facultative anaerobic bacterium with a spherical shape, gram-positive, that has optimal stability temperature between 30 and 37 °C. Microscopic analysis showed the presence of these morphologies (data not shown). Probably the experimental conditions favored the presence of *Staphylococcus* species in sample (2).

Burkholderia species (OTU 3) were present in bacterial population from brewery wastewater (sample I). These species of microorganisms are found in soil samples, mainly involved in N_2-fixing [38] and can be found in granular sludges from anaerobic reactors and after their pretreatments [9, 39].Terrazas-Hoyos et al. [40] tested pure cultures of *Burkholderia unamae* and *Burkholderia*

tropica on hydrogen generation and they obtained the best results at pH 6.0, 35 °C in anaerobic batch reactors fed with a mix of sucrose, succinic acid and enzymatic cofactors such as cysteine, sodium molybdate and ferrous sulfate. These two bacteria are known as N_2-fixing and in the mechanism performed by nitrogenase enzyme, 1 mol of H_2 is produced for 1 mol of N_2 fixed [39]. Maintinguer et al. [9] tested hydrogen bioproduction with granular sludge from UASB reactor heat-treated previously in anaerobic batch reactors fed with sucrose (0.5–4.8 g L^{-1}) and they identified *Clostridium, Enterobacter* and *Burkholderia* species during the operation. Etchebehere et al. [41] affirm that *Burkholderia* species are producers of volatile fatty acids, and can be found in no treated sludge, so is possible to connect their presence with some variation in the heat pretreatment. The sludge from sample I (Brewery) was heat treated in an open beaker, therefore, under aerobic conditions. So is possible to conclude that the pretreatment applied in the present study was effective against methanogenic production, but some aerobic bacteria such as *Burkholderia* species remained present in the sludge. These species could also be involved in the biologic processes of volatile fatty acids generation [41] observed in the present study contributing to the decreasing the pH and, probably, had influence on the lower H_2 bioproduction for sample I. However, Terrazas-Hoyos et al. [40] tested pure cultures of *Burkholderia* species (*B. unamae* and *B. Tropica*) and confirm the production of biohydrogen for these species. So, probably, bacteria which belong to *Burkholderia* genus identified in this study were involved in the volatile fatty acids generation [41] and in the biohydrogen production [40].

Bacillus megaterium was related to OTU 4, gram-positive rods belonging to the *Bacillus* genus. It has several metabolic genes that may be useful in bioremediation [42]. These microorganisms have the capability of dissolving natural phosphorus compounds from soil. The gram-positive rods observed in this study could be *Bacillus megaterium*, which are often involved in the fermentation process of hydrogen production.

Table 3 Phylogenetic characterization of bands and subsequent operational taxonomic units (OTUs) clustering from DGGE analyses

OTU	Bands	Inoculum	Affiliation	Access	Similarity (%)	Base-pairs
1	1a, 1b, 1c, 1d, 6a, 6b, 6c	II (ph 5.5 and heat treatment); III reactivated	*Clostridium* sp.	JN688047.1	99	450
2	2a, 2b	II reactivated	*Staphylococcus* sp.	KF646741.1	99	445
3	3a	I (pH 5.5 and heat treatment)	*Burkholderia* sp.KBS0801	JQ437592.1	99	449
4	4a 5a	I reactivated; III (pH 5.5 and heat treatment)	*Bacillus megaterium*	JQ833485.1	94	205
5	5b, 5c, 5d, 5e, 5f, 5 g, 5 h, 6d	III (pH 5.5 and heat treatment); III reactivated	*Bacillus subtilis 131*	JQ795145.1	99	431
6	6e	III reactivated	*Lactobacillus* sp.JCM 7747	AB911486.1	98	450

Table 4 Comparative study on efficiency of hydrogen production in anaerobic batch reactors

Seed sludge	pH T	Substrate (g L^{-1})	Yield mol H$_2$ mol substrate^{-1}	Metabolites generating	Identified bacteria	Ref.
Soil	pH 7.0 37 °C	Sucrose (10)	1.5	Lactic acid Acetic acid Butyric acid	*Clostridium perfringens*	[24]
Crude oil	pH 7.0 37 °C	Glucose (10)	1.4	Acetic acid Ethanol	*Enterobacter cloacae*	[25]
Activated sludge	pH 7.0 37 °C	Glycerol (5)	1.3	Formic acid Malic acid Citric acid Acetic acid Propionic acid	*Clostridium* sp.	[27]
I II III	pH 5.5 37 °C	Sucrose (2)	1.0 1.3 2.0	Acetic acid Butyric acid	*Clostridium* sp. *Staphylococcus* sp. *Burkholderia* sp. *Bacillus megaterium* *Bacillus subtilis* *Lactobacillus* sp.	Present study

The OTU 5 was related to *Bacillus subtilis*, as reported by Mohanapriya et al. [2], who isolated that species from contaminated soil. They operated reactors fed with glucose (1 g L^{-1}) during 24 h at 37 °C with hydrogen generations of 1.5 and 3.3 mol H$_2$ mol glucose^{-1}. The authors concluded that the Bacilli class was important in hydrogen production, coherent with the findings reported in the present study which has found Bacilli class bacteria in the morphology analyses.

The OTU 6 was related to the Lactobacillus Genus, which was reported by Kawagoshi et al. [35]. It can contribute adversely to the production of hydrogen since it produces lactate and consumes hydrogen during the fermentative process.

The H$_2$ yield for inoculum III was slightly higher than the ones observed in the literature (Table 4), even though all authors cited had used isolated bacteria and pure cultures. Additionally, our results on hydrogen yields with anaerobic bacteria consortia were higher than the ones in the literature (Table 4) with pure cultures, which theoretically, could show better hydrogen productions. Furthermore, some metabolites generated in the cited papers were mostly lactic acid and ethanol, which may consume H$_2$ for their formation, resulting in lower yields of hydrogen production, as observed. The best results obtained with mesophilic consortia of anaerobic bacteria could explain the inocula from tropical sludges, such as hydrogen producing bacteria, have ideal conditions of surviving and producing hydrogen, as observed.

Conclusions

The use of TOGA—Transformer Oil Gas Analyzer System—that analyses hydrogen, methane and CO$_2$ in a single chromatographic run, proved to be an efficient tool and can be applied for rapid and simultaneous determination of hydrogen, methane and CO$_2$ in anaerobic fermentation systems.

The heat treatment applied on tropical sludges used in this study was effective in inactivating the methanogen microorganisms that consume H$_2$.

The results obtained suggest the role of anaerobic bacteria consortia, mainly *Clostridium, Burkholderia* and *Bacillus* species which could be found in sludges used in this study for biological production of H$_2$.

The application of tropical sludges from biologic treatment plants' wastewater in hydrogen producing anaerobic reactors suggest it uses a sustainable alternative as feedstock for bioenergy and biofuels production.

The H$_2$ production obtained from tropical sludges opens the possibility to explore the abundant sugars present in waste food industries and agroindustrial effluents, to promote the integrated development of this technology in a complementary way in regions with similar conditions of tropical countries.

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