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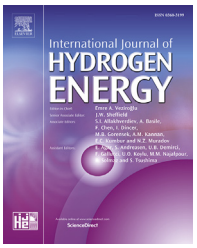

Hydrogen bioproduction with anaerobic bacteria consortium from brewery wastewater

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ABSTRACT

Biohydrogen production is a cheap and clean way to obtain hydrogen gas. In subtropical countries such as Brazil the average temperatures of 27 °C can favor the hydrogen producing bacteria growth. A mixed culture was obtained from a subtropical sludge treating brewery wastewater and anaerobic batch reactors were fed with glucose, sucrose, fructose and xylose in low concentrations (2.0, 5.0 and 10.0 g L^{-1}) at 37 °C, initial pH 5.5 and headspace with N_2 (99%) to maintain the anaerobic conditions. The inoculum was a subtropical granulated sludge from UASB (Upflow Anaerobic Sludge Blanket) reactor treating brewery wastewater. The higher H_2 yields were obtained in reactors operated with 2 and 5 g L^{-1} of fructose and they were 1.5 mol H_2 mol^{-1} of fructose and 1.3 mol H_2 mol^{-1} of sucrose, respectively. The volatile fatty acids (VFA) generated at the end of operation were, predominantly, butyric and acetic acid, indicating the favoring of the metabolic route of hydrogen generation by the consortium of anaerobic bacteria from the brewery wastewater. Biomolecular analyses revealed the predominance of hydrogen producing bacteria from *Firmicutes* phylum distributed in the families *Streptococcaceae*, *Veillonellaceae* and uncultured bacteria. These results confirm future applications of subtropical sludges with agroindustrial wastewaters containing low concentrations of sugars on hydrogen generation.

Introduction

Fossil fuels have been the dominant source of energy reaching 87% of worldwide consumption [1]. Hydrogen is an important alternative source of energy which has been studied for several researches as a renewable, sustainable energy source [2] to replace fossil fuels, such as coal, oil, and natural gas. The hydrogen gas appears as a friendly and sustainable fuel [3].

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The combustion of hydrogen gas only generates water and energy, showing the potential against the greenhouse gases which are generated in the combustion of fossil fuels [4]. The hydrogen gas can be obtained by several processes as thermochemical and electrochemical, however it is possible to point the bio production, which uses microorganisms for dark and photo fermentation, as the cheapest one.

Subtropical countries as Brazil have average temperatures of 27 °C, which may be considered ideal for growth of hydrogen producing anaerobic bacteria [5]. Among the studies that have been carried out on biohydrogen production various kinds of sludges can be cited from wastewater treatment plant [6], from swine wastewater treatment plant [7], from the treatment of stillage from sugarcane [8], among others. However, there are just a few applications with subtropical sludges on hydrogen generation by dark fermentation.

Beer is pointed as one of the most consumed beverages worldwide. It loses just for tea, milk and coffee [9]. Brazil has a large beer production, reaching 13 billion of liters of beer per year. For 1 L of beer 3–10 L of effluent is generated, which may vary with the steps followed by the production process [9]. Effluents from beer industries have been treated by biological processes, whose diversity of anaerobic microorganisms contain anaerobic bacteria capable on biogas generation, mainly hydrogen gas. Besides that, *Clostridium* sp. is known as a great biohydrogen producer [10] but the consortium of anaerobic bacteria could be in some cases greater than the isolated microorganisms. Some interactions among the microorganisms are still unknown [11] and they can contribute with high potential of microorganisms for bioconversion from sugars into biohydrogen.

The dark fermentation using wastewater with carbohydrates, mainly sugars, as feed stock have different yields for each kind of substrate presented in two metabolic routes with generations of acetic and/or butyric acids. In this sense, glucose, fructose (glucose isomer), sucrose and xylose can be converted, respectively, in 4, 4, 8 and 6 mol of H_2 per mole of carbohydrate in metabolic route of acetic acid (Eqs (1)–(3)). However, in the metabolic route of butyric acid generation can be obtained, respectively, 2, 2, 4 and 5 mol of H_2per mole of glucose, fructose (glucose isomer), xylose and sucrose (Eqs (4)–(6)), respectively [7,12,13].

$$C_6H_{12}O_6 + 2\ H_2O \rightarrow 2\ CH_3COOH + 4H_2 + 2\ CO_2 \quad (1)$$

$$C_{12}H_{22}O_{11} + 5\ H_2O \rightarrow 4\ CH_3COOH + 4\ CO_2 + 8\ H_2 \quad (2)$$

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

$$C_6H_{12}O_6 + 2\ H_2O \rightarrow CH_2CH_2CH_2COOH + 2H_2 + 2\ CO_2 \quad (4)$$

$$C_{12}H_{22}O_{11} + H_2O \rightarrow 2\ CH_3CH_2CH_2COOH + 4\ CO_2 + 4H_2 \quad (5)$$

$$C_5H_{10}O_5 + 2\ H_2O \rightarrow ½\ CH_3CH_2CH_2COOH + 3\ CO_2 + 5H_2 \quad (6)$$

The sugars such as fructose, glucose, sucrose and xylose are present in agricultural, industrial and domestic wastes are rich in carbohydrate and thus offer a potential resource for dark fermentation [4] using wastewaters from Brazil which are rich in organic matter as well as in nutrient compounds, as described previously. Agricultural wastes are mainly composed of cellulose, hemicellulose and lignin [14]. Brazil is the biggest citrus producer worldwide reaching 16 million tons in 2014, which represent 33% of worldwide production. São Paulo State represents 73% of this production. However, this large production also produces large amount of wastes, reaching 8.4 million tons on orange processing in the same period. Citric wastewater contains representative concentrations of glucose and fructose (12 g L^{-1} and 4 g L^{-1}, respectively) [15]. Also in São Paulo State, Brazil, ethanol and sugar industries are one of the most important agroindustrial field, producing 23.5 billion litters of ethanol and 596 million tons of sugar in 2012 [16]. Allied with ethanol production is the vinasse production that reached 305.5 billion of litters in the same period [17] and this waste produced in agroindustrial sector can be applied on hydrogen production due to its concentration of glucose, sucrose and fructose (22–45 g L^{-1} COD) [18]. This process has to be done with some caution, mainly, because of the inhibitory compounds present in distillery wastes, which can be a barrier to the anaerobic digestion process [19]. An adjustment period is advisable to the bacteria consortia growth allied with the complex and/or inhibitory compounds. The Brazilian paper industries are important and predominant market in South America, producing 10.4 million tons in 2014 [20]. The pulp and paper industries applied pretreatments on lignocellulosic material which release carbohydrates on its wastewater [21]. The pre-treatments could include composting with of fungi aiming an efficient degradation of organic matter, which occur in nature in a very slow speed [14]. Major components of lignocellulosic hydrolysate include hexose (glucose) and pentose (xylose and trace amount of arabinose), xylose which can be used as carbon source on biohydrogen production [21,22]. Therefore, the use of these wastewaters by dark fermentation processes for hydrogen production allows the production of clean renewable sources of energy and wastewater treatment, minimizing the cost of both processes.

In this sense, the objectives of this study were to obtain a consortium of anaerobic hydrogen producing bacteria using a subtropical granulated sludge from an UASB (Upflow Anaerobic Sludge Blanket) reactor treating brewery wastewaters; to investigate hydrogen generation with a consortium of anaerobic bacteria in anaerobic batch reactors fed with low concentrations of sugar which are similar to Brazilian wastewaters found in agricultural effluents and; to identify and to characterize the consortium of anaerobic bacteria responsible for hydrogen production.

Material and methods

Inoculum

The source of inoculum was a subtropical granulated sludge from an UASB reactor treating brewery wastewater on a beer factory (Araraquara, São Paulo, Brazil) with the following characterization: BOD (mg O_2 L^{-1}) 1597; COD (mg O_2 L^{-1}) 2552; VFA (mg $CaCO_3$ L^{-1}) 473; pH 5,65. The granulated sludge was broken, separately, in a mortar and pestle and then it was

reactivated in anaerobic batch reactors containing culture medium, according described below, at 37 °C for 168 h.

Reactivation and pretreatment of the inoculum

Triplicates of anaerobic batch reactors (100 mL) were fed with culture medium (50 mL), (g L^{-1}: 10-glucose, 5-yeast extract, 5-peptone and 5-meat extract), headspace (50 mL) was filled with N_2 (99.99%) to maintain the anaerobic conditions, initial pH 7.0, capped with butyl rubber stoppers and they were sterilized (20 min, at 120 °C).

After the reactivation period the anaerobic batch reactors were opened and the sludge were submitted to a heat treatment [23]. The reactivated sludge was added into a beaker under magnetic agitation and 100 °C per 10 min. The heated sludge was cooled to the ambient temperature (25 °C) in ice bath. The inoculum was transferred to the anaerobic batch hydrogen reactors using sterile syringes.

Enrichment of anaerobic bacteria consortium

Amounts (20% v/v) of the pretreated inoculum were inoculated in anaerobic batch reactors (100 mL) for 7 days, under pH 5.5 at 37 °C. Serial dilution process was done repeatedly focused on the enrichment of the H_2-producing bacteria. The pH values and cellular growth were performed, based on the optical density at 600 nm (OD_{600}) [24]. Microscopy analyses were made to monitor the morphologies of anaerobic bacteria consortium.

The consortia of anaerobic bacteria were centrifuged (9000 rpm at 4 °C for 6 min) and the cell pellets were suspended in new culture medium [25], with the following composition (g L^{-1}): 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_2PO_4 (5.36), K_2HPO_4 (1.30), $Na_2HPO_4H_2O$ (2.76) two vitamin solutions (mg L^{-1}); solution E [biotin (10.0); p-aminobenzoic acid (40.0)]; and solution F [B12 vitamin (40.0)] [9]. 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 [10]. The pH was adjusted to 5.5 with the addition of NaOH (1M) or HCl (1M). The culture medium was filtered through a previously sterilized 0.22 μm Millipore membrane. This setup was presented in a previous work [26].

Operation of anaerobic batch reactors

Anaerobic batch reactors (2 L) capped with butyl rubber stoppers were fed with sucrose, fructose, xylose and glucose (2 and 5 g L^{-1}) and operated in triplicates. One extra assay was carried out with sucrose 10 g L^{-1} at the same configurations already cited. The headspace (1 L) was purged with N_2 (99.99%) during 20 min. They were kept at 37 °C, during almost 220 h.

Chromatographic analyses

The hydrogen, methane and CO_2 gases contents in the biogas were determined simultaneously in a single gas chromatography ran in a TOGA – Transformer Oil Gas Analyzer – system, attached 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 [27]. 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 to 150 °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).

VFA and alcohol concentrations were measured 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) [28].

Analytical analyses

Carbohydrates consumption was done following the Dubois et al. [29] method adapted by Herbert et al. [30]. The measurements were performed by spectrophotometry at 490 nm (OD_{490}).

Cellular growth measurements were carried out using spectrophotometry at 600 nm (OD_{600}) according to APHA [24].

The final pH after the end of the assays were measured according to APHA [24].

Data adjustment

The experimental data were adjusted to their average values obtained from the triplicates of the anaerobic batch reactors using the Statistica® software. The maximum rate of biohydrogen production was obtained by a non-linear sigmoidal adjust of Gompertz function [31] showed below.

$$H = P \cdot \exp\{-\exp\{[(Rm \cdot e)/P](\lambda - t) + 1\}\} \quad (7)$$

Therefore, P is the hydrogen production potential (mmol L^{-1} culture), Rm is the maximum rate of hydrogen production (mmol L^{-1} culture. h^{-1}), λ is the lag phase, in hours, of H_2 generation and *e* is equal to 2,718281828.

Molecular biology analyses and sequences determination

Samples of enriched inoculum were concentrated on centrifuge at 4 °C, 9000 rpm for 10 min. The pellet obtained was washed by sterile and pH 7.4 PBS buffer solution [32] and was maintained under −20 °C. The DNA extraction was performed in chloroform phenol according to modified Griffiths et al. [33]. DNA concentration was done as follows: 1 mL of icy ethanol 95% was added and homogenized. In sequence, the sample was centrifuged (10000 rpm, 4 °C, 10 min) and the supernatant was disposed. 500 μL of icy ethanol 70% was added and centrifuged in the same conditions. The resulting supernatant was discarded, and the samples were let to dry. Then, with the samples dried, was added 50 μL of TE buffer solution and maintained at −20 °C for subsequent PCR amplification procedure. The amplification of the polymerase chain reaction

(PCR) was performed with a 27F (50 AGA GTT TGA TCM TGG CTC AG e 30) and 1000R (50 – GGG TTG CGC TCG TTG - 30) [34]. Aiming to obtain the partial bacterial 16S rRNA gene sequences, was used the 27F-1100R primers [34] to identify the bacteria population using a thermocycler Eppendorf AG-22331 Hamburg. The setup was set as follows: pre-denaturation at 94 °C for 5 min; with 30 cycles of denaturation at 94 °C for 45 s; annealing at 55 °C for 45 s; extension at 72 °C for 1 min and 45 s; final extension at 72 °C for 7 min; and cooling at 4 °C.

The PCR products were sent to Macrogen Inc® for nucleotide sequence analysis. With the resulting PCR product, the cloning and sequencing processes were performed using the pGEM Easy Vector System I for Bacteria Domain. A comparative analysis was performed using the Ribosomal Database Project (RDP – http://rdp.cme.msu.edu/), and the Basic Local Alignment Search Tool (BLAST) was used to search the National Center for Biotechnology Information sequence database (http://www.ncbi.nlm.nih.gov/BLAST/). Ninety-eight sequences within 8 OTUs were found in this study. The sequences obtained were deposited in Genbank with accessing numbers from KY866665 to KY866672. The taxonomic identification of the sequences which represents the OTU were performed using the RDP-Classifier.

Results and discussion

Reactivation and pretreatment of inoculum

The heat treatment was efficient at inactivation of methanogenic bacteria and to maintain anaerobic bacteria with rods morphology. Song et al. [35] applied different pretreatments: infrared oven for 2 h; 1:4 solid/liquid boiling for 30 min; aeration for 72 h and aeration for 72 h adding sugar; using caw wastes as source of inoculum in anaerobic batch reactors to inactivate methanogenic microorganisms in anaerobic batch reactors fed with 10 g L^{-1} of sucrose at pH 7.0, 36 ± 1 °C. The authors observed no methane generation only to heat treated inoculum, such as in the present study, with a great increasing in biohydrogen production of 273.5 mL g^{-1} of substrate. Sá et al. [36] observed morphologies of rods after heat treatment of domestic sewage, such as in present study, and they concluded the anaerobic bacteria were involved in hydrogen production observed. Kawagoshi et al. [37]obtained similar results, such as the present study, during pretreatments of: pH reduction (pH 3.0 for 18 h) and heat (100 °C for 2 h) in six different bacteria sources (aerobic activated sludge from a sewage water treatment plant, anaerobic digested sludge, soil from watermelon field, soil from kiwi grove, lake sediment and aerobic refuse compost). The authors observed the best results for biohydrogen production in reactors with heat treated inoculum previously. In the present study, the headspace of anaerobic batch reactors contained 75% H_2 in the composition of biogas after 168 h of operation and no methane was detected during the chromatography analysis with the predominance of rods and cocci in the consortium of anaerobic bacteria (data not shown). These results confirmed the efficiency of the heat treatment applied in the subtropical granular sludge.

Operation of anaerobic batch reactors

The H_2 generation in the anaerobic batch reactors fed with fructose 2.0 and 5.0 g L^{-1} had their maximum potential of generation (Table 1 and Fig. 1) of 13.8 mmol H_2 L^{-1} and 26.1 mmol H_2 L^{-1}, respectively. These results were higher than the reactors operated with glucose 2.0 and 5.0 g L^{-1} (3.4 and 4.2 mmol H_2 L^{-1}), sucrose 2.0, 5.0 g L^{-1} and 10.0 g L^{-1} (4.5, 10.3 and 13.3 mmol H_2 L^{-1}) and xylose 2.0 and 5.0 gL^{-1} (3.4 and 4.5 mmol H_2 L^{-1}). In addition, the maximum production rate in reactor with fructose (5.0 g L^{-1}) was 0.5 mmol H_2 L^{-1} h^{-1}. These results with fructose indicated that the concentrations applied were not inhibitory for the bacteria consortium. Hence, the maximum potential of H_2 generation followed a direct relation with the sugar concentrations for the reactors fed with fructose, xylose, glucose and sucrose. Subudhi et al. (2013) studied the bacterial strain *Enterobacter cloacae* DT-1 on hydrogen production using 10 g L^{-1} of glucose, pH 8.0 at 37 °C, obtaining a maximum hydrogen of 32 mmol H_2 L^{-1}. These higher hydrogen production, compared to the present work of

Table 1 – Results obtained from hydrogen producing bacteria of anaerobic batch reactors.

Studied parameter	Fructose 2.0 g L^{-1}	Fructose 5.0 g L^{-1}	Glucose 2.0 g L^{-1}	Glucose 5.0 g L^{-1}	Sucrose 2.0 g L^{-1}	Sucrose 5.0 g L^{-1}	Sucrose 10.0 g L^{-1}	Xylose 2.0 g L^{-1}	Xylose 5.0 g L^{-1}
Operation time (h)	239	238	169	143	169	238	189	160	215
Final pH	3.7	3.5	4.5	4.5	4.4	4.4	4.3	4.2	4.3
Max. Growth (OD_{600})	0.20	0.37	0.13	0.24	0.19	0.54	0.1	0.22	0.11
[a] P (mmol H_2 L^{-1})	13.8	26.1	3.4	4.2	4.5	10.4	13.3	3.4	4.5
[a] Rm (mmol H_2 L^{-1} h^{-1})	0.1	0.5	0.2	0.3	0.2	0.1	0.2	0.2	0.04
[a] λ (h)	57.3	107.6	19.3	21.2	13.3	4.4	16.0	16.0	2.2
[a] R^2	0.990	0.999	0.994	0.996	0.995	0.986	0.944	0.999	0.973
% Sugar consumption	73.7	59.2	87.1	71.5	88.7	67.1	49	88.50	80.1
Acetic acid (mg L^{-1})	126.5	323.9	8.8	246.9	39.7	580.3	241.2	108.1	73.1
Butyric acid (mg L^{-1})	199.9	296.9	17.8	95.5	73.8	229.3	134.4	0.13	91.5
Yields (%)	33.6	37.5	10.7	5.5	10.9	11.6	14.0	4.8	2.6
moles H_2 mol^{-1} of carbohydrate	1.3	1.5	0.4	0.2	0.9	0.9	1.1	0.3	0.2

[a] Nonlinear sigmoidal adjust from modified Gompertz function – [P – Max. H_2 production (mmol H_2 L^{-1}); Rm – Max. H_2 generation rate (mmol H_2 L^{-1} h^{-1}); λ– Lag phase (h)].

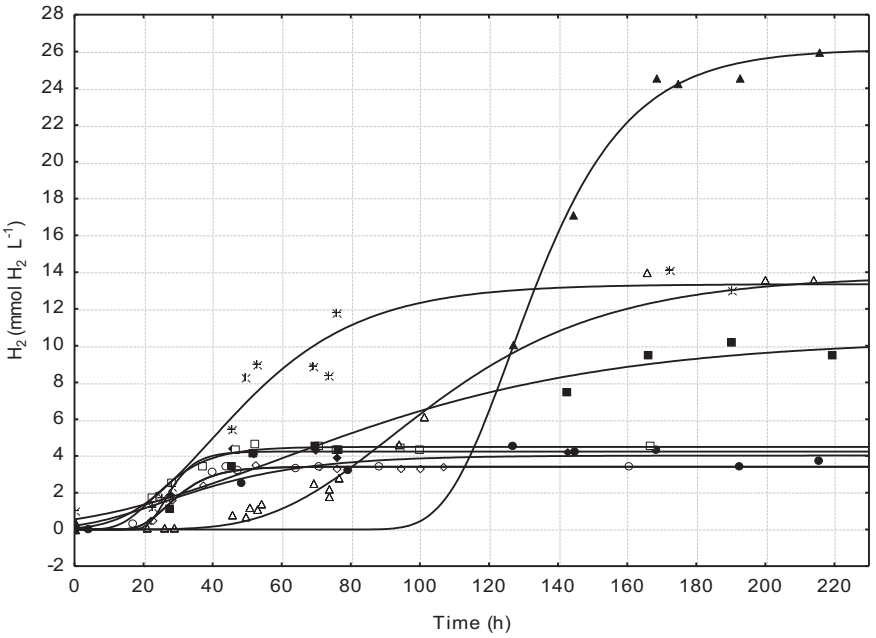

Fig. 1 – H_2 generation in headspace of anaerobic batch reactors fed with glucose [(◊) 2 g L^{-1}; (◆) 5 g L^{-1}], sucrose [(□) 2 g L^{-1}; (■) 5 g L^{-1}; (*) 10 g L^{-1}], fructose [(Δ) 2 g L^{-1}; (▲) 5 g L^{-1}] and xylose [(○) 2 g L^{-1}; (●) 5 g L^{-1}].

4.2 mmol H_2 L^{-1} with 5 g L^{-1} of glucose, was due to the higher concentration of glucose, that it could favor the increase of hydrogen production and due to the presence of pure culture, which allows to say that only this specie was present and that was responsible for high hydrogen generation, unlike the mixed culture in the present study that may contain consuming microorganisms of this biogas.

The H_2 yields (mol H_2 mol^{-1} carbohydrate) for glucose, xylose, fructose and sucrose (2.0 and 5.0 g L^{-1}) was 0.4/0.2, 0.3/0.2, 1.3/1.5 and 0.9/0.9, respectively and assay carried out with 10 g L^{-1} of sucrose and was obtained 1.11 mol H_2 mol^{-1} of sucrose (Table 1).

There were observed sugar consumptions in all anaerobic batch reactors (Fig. 2). The reactors fed with xylose 2.0 g L^{-1} and glucose 2.0 g L^{-1} presented higher sugar consumption compared to the other reactors (Fig. 2). The percentages of consumption were 88.5% for xylose and 87.1% for glucose during 160 and 169 h of operation, respectively. Maintinguer et al. [13] obtained 97% of consumption of sugar in reactors fed with xylose (1848 mg L^{-1}) with slaughterhouse sludge as inoculum source. The authors obtained 0.2 mol H_2 mol^{-1} of xylose. This result of H_2 yield was lower than the present study in reactor fed with 2 g L^{-1} of xylose (0.3 mol H_2 mol^{-1} of carbohydrate), even with a higher consumption of xylose (97%).Maintinguer et al. [7] obtained 0.3 mol H_2 mol^{-1} of sucrose with 70.3% of consumption of carbon source in reactors fed with 4128 mg L^{-1} of sucrose. The sugar consumption obtained by the authors can be related due to the concentration

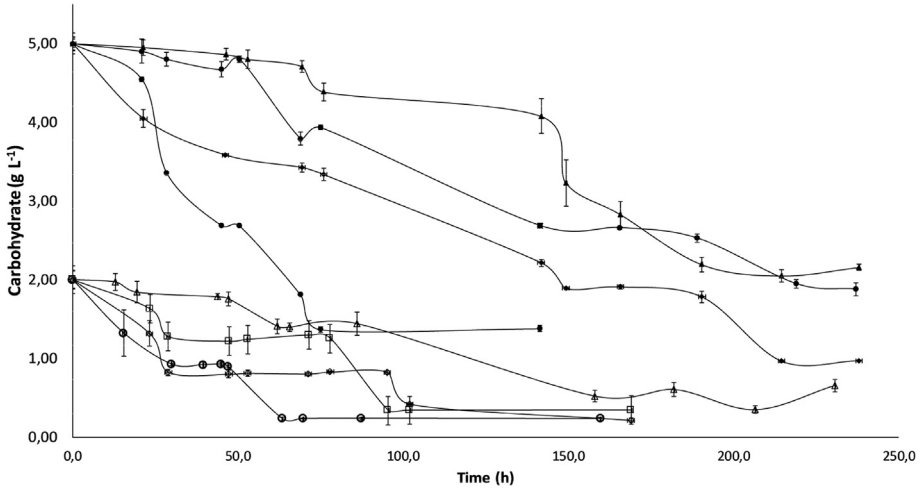

Fig. 2 – Sugar consumptions during the operation of anaerobic batch reactors fed with: glucose [(◊) 2 g L^{-1}; (◆) 5 g L^{-1}], sucrose [(□) 2 g L^{-1}; (■) 5 g L^{-1}], fructose [(Δ) 2 g L^{-1}; (▲) 5 g L^{-1}] and xylose [(○) 2 g L^{-1}; (●) 5 g L^{-1}].

of sugar in the reactor, which was the highest tested and it could have acted as inhibitor for hydrogen gas production. In the present study (67% -Sucrose 5 g L^{-1}) it was obtained great values for sugar consumption close to 70%, pointing to the absence of inhibition by concentration of substrates.

The best condition in this study on H_2 generation was in the reactor fed with fructose 5 g L^{-1}, obtaining a H_2 yield of 1.5 mol H_2 mol^{-1} fructose. The maximum rates of production in mmol H_2 L^{-1} h^{-1} doubled from assays operated with 5.0 g L^{-1} fed with fructose, glucose and xylose compared to the same substrates in 2.0 g L^{-1} (Table 1). According to Wang & Wei [38], the biohydrogen production follows a direct relationship with the increasing of the concentration of carbohydrates in the medium reaching a maximum point, which could act as inhibitory parameter on it.

The final pH was directly related to acetic and butyric acid generation at the end of the operation (Table 1). Reactors fed with fructose (2.0 and 5.0 g L^{-1}) were operated for 239 h and showed the higher decay of pH, 3.7 and 3.5, respectively, which were close to the result found by Plangklang et al. [2](3.8) Hence, higher concentrations of VFAs were observed (Volatile Fatty Acids), mainly acetic acid (126.5 and 323.9 mg L^{-1}) and butyric acid (199.9 and 296.9 mg L^{-1}) for these reactors. Plangklang et al. [2] operated a continuous reactor at pH 6.5, fed with sugarcane juice (3.2 g fructose L^{-1}) which was inoculated *Clostridium butyricum* immobilized in sugarcane bagasse and they obtained acetic and butyric acid generations (905 ± 0.14 mg L^{-1} and 5268 ± 0.54 mg L^{-1}, respectively) at the end of the operation. The authors have obtained yields of generation as 1.3 mol H_2 mol^{-1} hexose, which were lower than the present study in reactors fed with 5.0 g L^{-1} of fructose (1.5 mol H_2 mol^{-1}fructose) and generations of acetic acid (323.9 mg L^{-1}).

The reactors fed with glucose (5.0 g L^{-1}) and sucrose (5.0 g L^{-1}) presented generations of acetic acid (246.9 and 580.3 mg L^{-1}, respectively) with yields of 0.9 mol H_2 mol^{-1} of glucose and 0.2 mol H_2 mol^{-1} of sucrose, respectively (Table 1).These higher concentrations for acetic acid could be explained due to the homoacetogenic bacteria that consume H_2 to generate acetic acid [39], according to Eq. (8) [40], where 4 mol of hydrogen are consumed for acetic acid production. The Gibbs energy shows that the reaction can occur spontaneously. Therefore, the presence of these bacteria in the pretreated sludge can be pointed as responsible for reactors fed with glucose, fructose and sucrose 5.0 g L^{-1} to present higher concentrations of acetic acid. Homoacetogenic bacteria are recognized as spore-forming, such as *Clostridium* species [40], and, therefore, resistant to heat treatment that was applied in this study.

$$2HCO_3^- + 4H_2 + H^+ \rightarrow CH_3COO^- + H_2O \quad \Delta G^0 = -105\ kJ \qquad (8)$$

The results obtained in the present study were compared to the literature (Table 2). Fang et al. [6] operated a continuous batch reactor fed with 7.0 g L^{-1} of glucose at 36 °C and pH 5.5 with 6 h of retention time for over 60 days and the results obtained (1.1 mol H_2 mol^{-1} glucose) were similar to the present study for the reactor fed with sucrose 10 g L^{-1}. Subudhi et al. [22] operated batch reactors fed with 10 g L^{-1} of glucose for 24 h at 37 °C and pH 7.0 and obtained higher results (1.4 mol H_2 mol^{-1} of glucose) than the present study. However, the authors applied pH 7,0 that is ideal for growth of anaerobic hydrogen producers. Plangklang et al. [2] operated anaerobic batch reactors fed with 3.2 g L^{-1} of fructose at 37 °C for 24 h in an orbital shaker at 150 rpm at pH 6.5 and obtained lower yields (1.3 mol H_2 mol $^{-1}$ fructose) than the present study (1.5 mol H_2 mol $^{-1}$ fructose). Maintinguer et al. [7] operated an anaerobic batch reactor fed with 4.2 g L^{-1} sucrose for 222 h, at pH 5.5, at 37 °C and obtained lower yields (0.3 mol H_2 mol $^{-1}$ of sucrose) than the present study (1.0 mol H_2 mol^{-1} sucrose). The present study obtained similar and better results than the authors already cited for reactors with fructose (5 g L^{-1}) and sucrose (5 and 10 g L^{-1}).

Bioinformatics

Were obtained 200 clones from the anaerobic bacteria consortium, resulting in 96 sequences from molecular biology analysis. These sequences were grouped in 8 Operational Taxonomic Unit (OTU) and they have revealed belonging to *Firmicutes* phylum: *Veillonella* sp., *Streptococcus* sp. and uncultured bacteria (Table 3).

Most of the sequences (71%) were affiliated as *Veillonella* sp. which are anaerobic gram-negative cocci no spore forming. They can be present in anaerobic H_2 producing bioreactors [41–44] degrading glucose or lactate to generate acetic, propionic and lactic acids [42]. In addition, *Veillonellaceae* family has a potential for hydrogen production being reported as lactic acid consumers [44–46]. Rosa et al. [41] obtained similarity with *Veillonellaceae* family as the present study during the operation anaerobic fluidized bed reactor (AFBR) with pig breeding and poultry wastes fed with cheese whey wastewater (5 g L^{-1})and they obtained similar yields of 1.33 mol H_2

Table 2 – Comparison of hydrogen yields presented by mesophilic conditions.

Authors	Substrate	Conditions	mol H_2 mol^{-1} carbohydrate
[19]	Glucose 10 g L^{-1}	Batch – 37 °C	1.4
[6]	Sucrose 4.2 g L^{-1}	Batch – 37 °C	0.3
[5]	Glucose 7 g L^{-1}	Continuous Batch – 36 °C	1.1
[2]	Fructose 3.2 g L^{-1}	Batch – 37 °C	1.3
The present study	Fructose 5 g L^{-1}	Batch – 37 °C	1.5
	Sucrose 5 g L^{-1}		1.0
	Sucrose 10 g L^{-1}		1.1

Table 3 – Phylogenetic distribution from anaerobic bacteria consortium.

OTU	N° of sequences	Affiliation	Access	Phylum	Similarity (%)	N° of base pairs
1	65	*Veillonella* sp.	JN695643.1	Firmicutes	99	1230
2	4	*Veillonella* sp.	HQ616396.1	Firmicutes	99	1269
3	2	Uncultured	HM811978.1	Firmicutes	93	1238
4	2	Uncultured	JQ470836.1	Firmicutes	95	1201
5	1	Uncultured	JQ470836.1	Firmicutes	97	1268
6	13	*Streptococcus parasanguinis*	NR_074109.1	Firmicutes	99	1210
7	8	Uncultured	JF114361.1	Firmicutes	99	1236
8	3	*Streptococcus salivarius*	AB680534.1	Firmicutes	99	1271

mol^{-1} lactose; with generations of ethanol, acetic and butyric acids as the present study. Luo et al. [43] identified *Veillonellaceae* family bacteria in anaerobic reactors fed with glucose as substrate and they obtained 2.8 mol H_2 mol^{-1} glucose. Shida et al. [42] also observed similarity to *Veillonellaceae* family in a reactor fed with glucose (2 g L^{-1}) with yields of 1.9 mol H_2 mol^{-1} glucose. *Veillonellaceae* family can tolerate high concentrations of organic acids and can be considered as helpers in the bioH_2 reactors, stabilizing the process among the mix culture [44]. In the present study high generations of organic fatty acids were observed, according to what was described previously, and it can confirm *Veillonellaceae* family identified were involved in biologic process of H_2.

About 16% of the sequences distributed in OTUs 6 and 8 have shown phylogenetic similarity to *Streptococcus* sp. Hung et al. [11] observed hydrogen gas production in anaerobic reactors with a mix culture of *Clostridium* sp. and *Streptococcus* sp., in anaerobic granules. According to Davila-Vazques et al. [47] and Fang et al. [6] the existence of *Streptococcaceae* family in reactors while hydrogen producing is rising could occur, however, this evidence was not confirmed for hydrogen production. Wu et al. [48] has operated a sequencing batch reactor (SBR) with immobilized sludge in heat pretreated silicon (100 °C for 20 min) fed with sucrose (8.9–35.6 g L^{-1}) and they obtained 3.5 mol H_2 mol^{-1} sucrose. After pretreatment of the inoculum similarity with *Clostridium* sp., *Klebsiella* sp. and *Streptococcus* sp. were observed by biomolecular analyses. Fang et al. [6] observed *Clostridiaceae, Enterobacteriaceae* and 3.1% of *Streptococcus bovis* in continuous flow stirred-tank reactor (CSTR) fed with glucose at pH 5.5, 36 °C, 6.6 h of hydraulic retention, generating H_2. Linet al. [49]obtained yields of 1.1 mol H_2 mol^{-1} hexose and similarity with *Clostridium butyricum*, *Clostridium pasteurianum*, *Klebsiella pneumoniae*, *Streptococcus* sp. and *Pseudomonas* sp in anaerobic batch reactors from starch (20 g COD L^{-1}), at 35 °C, pH 5.5 with inoculum from a biologic treatment of paper industry. In the present study, morphologies of cocci chain such as *Streptococcus* sp were observed. However, *Streptococcus* sp. are found in anaerobic bioreactors H_2 producing [50] and they can coexist with *Clostridium* sp, after heat-treatment of inocula to degrade glucose and sucrose [6,11,47] as observed in the present study.

Only 13% of the sequences were affiliated as uncultured bacteria. Probably the Gram-positive rods spore forming observed in the microscopic analysis during the operation of anaerobic reactors were affiliated as uncultured bacteria from *Firmicutes* Phylum.

The diversity of anaerobic bacteria from a subtropical sludge verified in this study reinforces the viability of its applicability on biohydrogen producing under mesophilic conditions.

Conclusions

The heat treatment applied to the inoculum was effective in inactivating hydrogen consumers such as methanogenic archaea.

Fructose showed a high potential of biohydrogen generation.

The biological production of H_2 was due to the anaerobic bacteria consortium from the subtropical brewery sludge identified as *Firmicutes* Phylum, mainly *Veillonella* sp. *Streptococcus* sp. and uncultured bacteria.

The sludges from subtropical countries such as Brazil treating brewery wastes can be applied on biohydrogen production using wastewaters with low concentrations of sugars.

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