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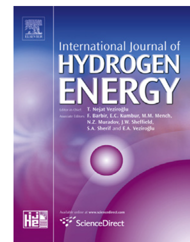

Hydrogen bioproduction with *Enterobacter* sp. isolated from brewery wastewater

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ABSTRACT

Hydrogen-producing bacterial strains were isolated from granular sludge from a UASB reactor that treats brewery wastewater. Most of the isolated strains were related to the *Enterobacter* genus through a phylogenetic analysis of the 16S rRNA sequences. The strains could use various carbon sources (sugars and glycerol) to produce hydrogen. The isolated strain, identified as *Enterobacter* sp. based on 16S rRNA gene sequencing, produced 6.8 mmol H_2 L^{-1} culture medium when growing on glucose (2.0 g L^{-1}) in anaerobic conditions at 30 °C. The main liquid metabolites were acetic acid (367 mg L^{-1}), methanol (437 mg L^{-1}) and ethanol (1101.26 mg L^{-1}), after 9 h of fermentation. The maximum hydrogen yield of 0.8 mol H_2/mol glucose was observed, indicating that *Enterobacter* sp. isolated from brewery wastewater was an efficient hydrogen-producing bacterium under mesophilic conditions.

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Introduction

A limited source of fossil fuels and environmental issues related to their combustions has driven to intensive searches for alternative energy sources worldwide. The hydrogen has been considered a potential energy carrier for the future and its production has been the focus of several researches. It can be produced through physicochemical and biological processes. One of the advantages of the biological hydrogen production is that it can occur under room pressure and temperature, and there is also the possibility of using several organic wastes as a substrate for microbial growth and biogas production [1].

Agricultural and industrial wastewaters are rich in organic matters as well as in nutrient compounds, which can benefit the bacterial growth. Therefore, the use of those wastes for

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bacterial fermentation for hydrogen production allows the production of clean renewable sources of energy and wastewater treatment, minimizing the cost of both processes [2].

Beer is one of the most consumed beverages around the world, losing only to tea, milk and coffee [3]. The brewing industry generates large amounts of highly polluting effluents (3–10 L of effluents for each liter of beer). Their compositions are variable upon to the process step (e.g. brewing and packing). For example, the effluents generated from bottle washing steps are produced in large amount; however, their organic matter concentration is lower than the brewing and filtering steps effluents, which have high BOD (Biochemical Oxygen Demand) content [4]. The global beer production from 2004 to 2014 increased about 37.26 million kiloliters. China presented the highest growth in beer production volume, followed by Brazil (about 5.42 million kiloliters) and Vietnam [5].

The conventional methods of treating brewery wastewater include physical, chemical and biological processes. The last one is based on the activity of a wide range of microorganisms, either through aerobic or anaerobic processes. Its advantages over physicochemical or chemical methods rely mainly on its high efficiency of COD (Chemical Oxygen Demand) and BOD removal and low investment cost [6].

Many studies have been developed worldwide in the past two decades with the aim of increasing hydrogen production rates and yields. Furthermore, their focus is also to look for potential solid wastes and wastewaters [7–9], which are considered ideal substrates at the economic point of view. Consortia of microorganisms from industrial sludges include anaerobic and facultative bacteria and they would be used for hydrogen production [10]. Additionally, their aim is to screen and isolate new hydrogen producing bacterial strains from natural (soil, compost, plant, animal) and artificial sources (industrial treatment plants sludge) [11,12] under mesophilic conditions [13]. Among the mesophilic anaerobes, Clostridium species have been isolated and used for several authors [14–16]. In bioreactors operated at thermophilic temperatures, Thermoanaerobacterium species have been often employed as an inoculum source [17–19]. Besides anaerobes, facultative microorganisms, such as *Enterobacter* species, can produce hydrogen by themselves [11,12,20,21] and also play an important role in co-cultures for dark fermentative hydrogen production bioreactors, by consuming oxygen and making the environment anaerobic for the strict anaerobes [22,23]. It is worth to highlight that just only few studies have been investigating the biological hydrogen production through the facultative anaerobic bacteria, such as *Enterobacter* species. These pure cultures have been tested during fermentative processes of hydrogen production with crude glycerol [24]. Strains as *Enterobacter aerogenes* HGN-2 and HT 34, isolated from sea crude oil pipelines, demonstrated the ability to produce hydrogen yielding almost 2 mol H_2/mol xylose [23]. In order to improve the hydrogen yields, Box–Behnken experimental design evaluated the performance of a newly isolated *Enterobacter* CN1 that yielded 0.6 and 2 mol H_2 per mol of glucose and xylose, respectively [25]. Based on a Plackett–Burman design there were identified important parameters (glucose, initial pH and ferric chloride) that affected the hydrogen production by *E. aerogenes* (MTCC 111). The maximum yield of 1.7 mol H_2/mol glucose was achieved using statistical design [26]. .Recently, it was demonstrated that at a certain degree the hydrogen producing *Enterobacter cloacae* augmented biogas production in anaerobic digesters [27].

Therefore, the bioaugmentation or a defined co-culture (facultative and anaerobic) can be interesting alternatives to improve several industrial biotechnological processes and, recently, it has been applied for biological hydrogen production as well [28,29]. Thus, the aim of this study was to isolate several hydrogen producing facultative bacterial strains from brewery wastewater treatment plant sludge and characterize them phylogenetically through 16S rRNA gene fragments analysis. Furthermore, it aimed to screen for potential high yield facultative strains able to grow and produce hydrogen from different carbon sources (dextrose, maltose, xylose, glucose, sucrose, fructose, lactose, starch, and glycerol), which may be present in several wastewaters.

Material and methods

Inoculum source and culture conditions

The inoculum was a granular sludge from the Upflow Anaerobic Sludge Blanket (UASB) reactor treating brewery wastewater (Araraquara – Brazil). The PYG culture media used in the experiments were prepared by the dissolution of 10 g L^{-1} the carbon source (glucose or dextrose, maltose, xylose, sucrose, glycerol, fructose, lactose, starch, all of them were added separately), peptone (5 g L^{-1}), yeast extract (5 g L^{-1}), and meat extract (5 g L^{-1}) in distilled water, prepared under anaerobic conditions, by the flush of N_2. After the preparation, the culture media were sterilized (at 121 °C for 20 min). All the experiments were carried out in a temperature controlled incubator (BOD -246/364 220V-SOLAB), kept at 37 °C.

Enrichment phase, isolation of the bacterial strains and nutritional characterization

The inoculum was transferred to a mortar with pestle in order to undo the granular arrangement and then it was submitted to the heat treatment (90 °C, for 15 min), in order to inactivate the hydrogen consumers [30]. Afterward, this biomass was cultured in PYG medium (glucose, 10 g L^{-1}; peptone, 5 g L^{-1}; yeast extract, 5 g L^{-1}, and meat extract, 5 g L^{-1}) at 37 °C, with initial pH 5.5, during 96 h. The samples from the headspace of the flasks were then analyzed and a hydrogen production was detected. Thus, the biomass was submitted to serial dilutions with PYG media and initial pH 5.5, in order to obtain the enrichment and selection of the hydrogen producing microorganisms.

The enriched culture was grown in PYG medium during 15 days and it was used as inoculum for the isolation phase. The isolation of the strains was performed in roll tubes using the same culture medium solidified with agar (2%). After the growth, single colonies were selected and picked up with strap platinum needle, and suspended in 100 μL of PYG medium, under aseptic conditions in a sterile tube (1 mL). The cellular suspension was then inoculated into serum vials (20 mL) containing PYG medium (10 mL). The vials were incubated for approximately 10 days at 37 °C without agitation. This process

was carried out with 30 colonies. The vials were incubated until the microbial growth. The hydrogen production was measured through gas chromatographic analysis from headspace samples, and the cell growth was quantified by the turbidity of medium (OD_{600}). In the vials in which hydrogen production was detected, the cells were reactivated in the PYG medium and samples were taken to microscopic and 16S rRNA gene sequencing analysis.

After the isolation phase, a nutritional requirement screening for hydrogen generation was performed. The tests were performed in serum vials (20 mL) in duplicate, using as inoculum 20% (v/v) of each isolated strain after the reactivation step. The following carbon sources (10 g L^{-1}) were used as substrates: glucose or fructose, sucrose, xylose, starch, glycerol, dextrose, maltose, and lactose. The vials were incubated, as described previously, during 10 days. After this period, the hydrogen production was quantified (gas chromatography), as well as the cell growth (OD_{600}). Microscopic analyses were performed in order to visualize the morphologies of the isolated strains.

Kinetic tests of hydrogen production, substrate consumption and fermentation products

One of the isolated strains, identified as *Enterobacter* sp. (accession number KP893397 from NCBI database), was submitted to successive washes in a refrigerated centrifuge at 8500 rpm and 4 °C for 10 min. The pellet was re-suspended in a fresh PYG modified medium (glucose, 2 g L^{-1}; peptone, 5 g L^{-1}; yeast extract, 5 g L^{-1}, and meat extract, 5 g L^{-1}), and incubated at 37 °C, pH 7.0, during 24 h. After that, 20% (v/v) of the reactivated biomass were used as seed inoculum for hydrogen production tests. The reactors were prepared in triplicate in Duran® flasks with a total volume of 2 L and working volume of 1 L (PYG modified, described previously), capped with butyl rubber stoppers. The reactors were kept at 37 °C, without agitation, during approximately 50 h. In these experiments, it was able to quantify the hydrogen production, substrate consumption and fermentation products generated.

Chemical and chromatographic analysis

Hydrogen, methane and carbon dioxide contents in the biogas were determined simultaneously in a single gas chromatography run in a TOGA (Transformer Oil Gas Analyser system) [31], coupled with a Trace GC Ultra – Thermo Gas Chromatograph, equipped with split/splitless injectors and two detectors: thermal conductivity detector (TCD), and flame ionization detector (FID) with methanizer. The fraction containing Hydrogen, Nitrogen and Methane was analyzed by an Rt-MSieve 5Aº 30 m × 0.53 mm i.d. column. Hydrogen and Nitrogen were detected by the TCD. Methane was detected by the FID, after passing through the methanizer. When the molecular sieve column was by-passed, Carbon dioxide was eluted from the porous polymer Carboxen 1006 plot 30 m × 0,53 mm i.d column and detected by the flame ionization detector (FID), after passing through the methanizer. Argon was used as carrier gas (1.5 mL min^{-1} in splitless mode). The temperature of the FID detector was 250 °C; the TCD detector and injector were adjusted to 150 °C. The oven programming was 50 °C (4.5 min), heating 40 °C min^{-1} to 180 °C (1.5 min) and then cooling 50 °C min^{-1} to 50 °C (3.15 min). The production of H_2 was calculated considering the atmospheric pressure, and expressed as mmol H_2 L^{-1}.

Volatile fatty acid and alcohol concentrations were measured by gas chromatography using a GC 2010 (Shimatzu®), equipped with a flame ionization detector and a sample introduction system to COMBI-PAL headspace (AOC 5000 model and HP-INNOWAX column of 30 m × 0.25 mm x 0.25 μm of film thickness) [32].

The glucose concentration was determined through a colorimetric method [33,34]. The pH values and cellular growth were performed, based on the optical density at 600 nm (OD600) [35].

Microbiological analysis

DNA extraction, amplification and 16S rRNA gene fragment analysis Nucleic acid extractions were performed [36], into a direct method with glass beads and a mixture of phenol: chloroform: buffer (1:1:1 v/v) addition. A segment of the 16S rRNA gene was amplified through PCR, using the set of primers 27 forward (5′AGA GTT TGA TCM TGG CTC AG-3′) and 1110 reverse (5′-GGG TTG CGC TCG TTG-3′) [37]. The unpurified PCR products were sent to Macrogen Inc® [38] for nucleotide sequence analysis. A comparative analysis was performed using the Ribosomal Database Project (RDP) [39] and the Basic Local Alignment Search Tool (BLAST) [40] was used to search the National Center for Biotechnology Information sequence database [41]. The phylogenetic tree was developed using the software RDP-Tree Builder Version 10.0 (Neighbor treeing program). Bootstrap analyses for 1000 replicates were performed to estimate the confidence of the tree topologies. The confidence threshold adopted was 99% for the specie taxonomic level and 88% for the genus taxonomic level.

Accession numbers of the sequences

The sequences of the isolates determined in this work were deposited in the NCBI database under the following accession numbers: KP893391, KP893392, KP893393, KP893394, KP893395, KP893396, KP893397, KP893398, KP893399, KP893400, KP893401, KP893402, KP893403 (strains from 1 to 13, respectively).

Experimental data fitting

The mean values of each anaerobic batch reactor were adjusted using Statitica® software, version 8.0, according to Equation (1). The maximum rate of hydrogen production was obtained through a non-linear sigmoidal adjust of modified Gompertz function [42].

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

where P is the hydrogen production potential (mmol H_2 L^{-1} of culture medium), Rm is the maximum hydrogen production rate (mmol H_2 L of culture medium h^{-1}), λ is the lag phase (h) of H_2 generation and e is equals to 2.718281828.

Results and discussion

The first part of the present study was a screening for hydrogen producing bacterial strains that were isolated from sludge from the brewery wastewater treatment plant. Among the 30 isolated strains, it was possible to observe H_2 generation from 13 strains. They were identified as *Enterobacter* Genus (strains 1–4 and 6–13) and uncultured bacterium from *Propionicum* Genus (strain 5) (Table 1). Rods, that are characteristic of the *Enterobacter* species, composed the observed morphology (data not shown). The most known fermentative hydrogen bacteria include species of *Enterobacter*, *Bacillus* and *Clostridium* species [43,44].

Four isolates (1, 2, 3, and 4) presented high homology 99% to *Enterobacter ludwigii*, a Gram-negative bacterium, belonging to the *Enterobacter* Genus (Fig. 1). Zhang et al. [46] identified *E. ludwigii* in DGGE analysis of the anaerobic granular sludge collected from an expanded granular sludge bed reactor (EGSB) that treated starch wastewater for biological hydrogen production with corn stover hydrolyzate. Therefore, *E. ludwigii* is probably involved in fermentation processes of hydrogen generation.

Eight isolates (6, 7, 8, 9, 10, 11, 12, and 13) were related to *Enterobacter* sp. (Fig. 1). Subudhi et al. [47] operated anaerobic batch reactors with pure cultures of *E. cloacae*, pH 7.0, at 37 °C; fed with xylose, glycerol, and cellulose. The authors ([47]) obtained maximum generation of H_2 27 mmol L^{-1}. Kumar & Das [20] operated anaerobic batch reactors inoculated with *E. cloacae* and obtained 6 mol H_2/mol of sucrose at 36 °C and pH 6.0. *E. cloacae* also produced H_2 with cellobiose (5.4 mol H_2/mol of cellobiose).

One strain (number 5) showed high similarity to uncultured bacterium belonging to the Propionicum Genus (Fig. 1), which comprises Gram-positive rod-shaped cells capable of propionic acid generation. It is mostly likely that *Enterobacter* and *Propionibacterium* were growing jointly in the brewery sludge because of their metabolic capabilities. It can be suggested that *Enterobacter* was fermenting sugars and releasing lactate in the medium that was later fermented by *Propionibacterium*. The majority of the isolated strains closely related to *Enterobacter* sp. could reflect its predominance in the composition of the sludge. Ren et al. [48] assessed the relationship between the predominance of certain bacterial species in a mixed culture and the fermentation type. The authors ([48]) found out that butyric acid and propionic acid fermentations types were dominated by *Clostridium* sp. and *Propionibacterium* sp., respectively. In another study, the large amount of *Propionibacterium* was observed after acid inoculum pretreatment [49].

Fig. 1 presents the consensus phylogenetic tree obtained with primers for Bacteria Domain from the isolates' cultures.

There were obtained hydrogen production on all carbon sources tested. The maximum cumulative hydrogen productions were (mmol H_2 L^{-1}) 8.03; 10.68; 11.22; 8.38; 14.36; 10.90; 7.09; 5.41 and 9.89 for dextrose, maltose, xylose, dextrose, glycerol, fructose, starch, lactose and glucose, respectively by different isolated strains (Table 2). During the tests, the higher hydrogen productions were observed using isolates strains with glycerol, xylose and fructose, as showed below. Subudhi et al. [47] applied heat treatment on a crude oil source with the aim of inactivating methanogenic microorganisms. The authors isolated *E. cloacae* DT-1 and used it during the operation of anaerobic batch reactors, with initial pH 7.0 at 37 °C, and different carbon sources at 10 g L^{-1}, including glycerol. They obtained lower values of hydrogen generation (6.6 mmol H_2 L^{-1}) than our results (14.4 mmol H_2 L^{-1}). Long et al. [25] obtained generation of H_2 with glucose, xylose, and sucrose as well as this study. However, it was observed higher results in anaerobic reactors fed with xylose (14.15 g of xylose L^{-1}, at 40 °C pH 7.0) than those supplemented with glucose and sucrose.

As commented previously, one of the isolated strains was used for kinetic tests *Enterobacter* sp. (KP893397), in which hydrogen production, substrate consumption and fermentation products were measured. The maximum cumulative hydrogen production was 6.8 mmol H_2 L^{-1} achieved in 8.8 h of

Table 1 – Phylogenetic affiliation of the isolated bacterial strains.

Isolated strains	Closest species in Genbank	Accession number	Similarity (%)	Base pairs	Reference
1–4	*Enterobacter ludwigii*	FR820478.2	99	1112	Unpublished
5	*Uncultered bacterium clone*	JF200891.1	99	1098	[45]
6–13	*Enterobacter* sp. KK1	GQ871449.1	99	1121	Unpublished

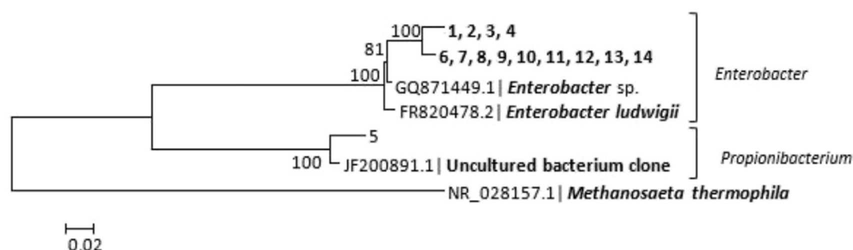


Fig. 1 – Consensus phylogenetic tree based on sequences of isolated strains. The bootstraps values indicate the repetition percentages (1000 replicate runs). *Methanosaeta thermophile* (NR 028157.1) was added as an out-group.

Table 2 – Nutritional characterization and generation of H_2 (mmol L^{-1}) with isolated strains.

Isolated strain	Dextrose	Maltose	Xylose	Sucrose	Glycerol	Fructose	Starch	Lactose	Glucose
1	4.96	10.68	11.22	6.85	5.45	9.23	4.37	4.50	9.20
2	5.81	9.79	11.01	7.78	5.35	7.05	4.52	4.37	7.12
3	8.03	7.46	8.04	–	6.69	7.56	3.41	5.41	7.10
4	6.21	6.79	6.51	8.38	4.49	10.90	3.36	2.92	9.89
5	5.41	8.17	8.18	4.15	6.70	6.91	5.20	4.60	6.80
6	5.41	4.89	6.01	5.16	3.45	5.33	–	3.54	5.45
7	6.87	14.66	5.22	4.81	2.54	7.76	2.13	3.13	7.89
8	5.11	5.10	2.89	6.56	4.48	2.44	1.68	–	3.90
9	6.79	–	5.51	–	–	–	–	–	–
10	6.03	4.58	1.16	5.09	3.57	4.32	2.21	5.41	4.12
11	3.05	5.37	4.35	4.89	14.36	4.93	2.84	2.04	4.56
12	6.01	4.65	4.95	7.31	4.28	4.84	2.16	1.81	4.69
13	4.52	7.03	3.66	3.86	7.52	6.66	7.09	2.06	6.78

(–) H_2 not detected.

fermentation in anaerobic reactors fed with glucose (2 g L^{-1}). These values were higher than the ones obtained by Lazaro et al. [8]. The authors ([8]) operated anaerobic batch reactors fed with vinasse from ethanol production (2 and 5 g COD L^{-1}) at 55 °C, initial pH 5.5, with sludge from UASB reactor, and they observed a maximum hydrogen generation of 5.0 and 5.2 mmol H_2 L^{-1}, respectively. It should be considered that cumulative hydrogen production is commonly used as unit for data comparison; however, it does not take into account the substrate consumption, which makes difficult the calculation of the real process efficiency.

The consumption of glucose was not complete and at the end of the experiment, it was possible to notice 14.4% of the initial glucose concentration (Fig. 2). In 3.5 h of incubation, just 36% of the initial glucose concentration was degraded by *Enterobacter* sp. (KP893397). In 8.8 h, when the hydrogen production ceased, the glucose consumption percentage raised to 85.6%. Similar substrate consumption (84%) was observed by Maintinguer et al. [30] that evaluated the hydrogen production in anaerobic batch reactor fed with sucrose (1.8 g L^{-1}) by a microbial consortium of *Clostridium, Enterobacter*, and *Burkolderia* species. Higher substrate consumption (97%) was obtained in anaerobic reactors fed with xylose (1.8 g L^{-1}) [10]. Lower substrate consumption (37%) was achieved in anaerobic batch reactors fed with 50% crude glycerol from cooking oils and 50% of glycerin at initial concentration of 20 g COD L^{-1} [24]. It is mostly likely that higher substrate consumption are achieved by using simple sugars as substrates, such as glucose and sucrose. However, when residues were applied as carbon source its consumption drops dramatically due to the presence of inhibitory compounds.

Usually, the hydrogen production stops and, sometimes substrate concentration also does, due to the changes in the environmental conditions, such as pH drop, organic acids

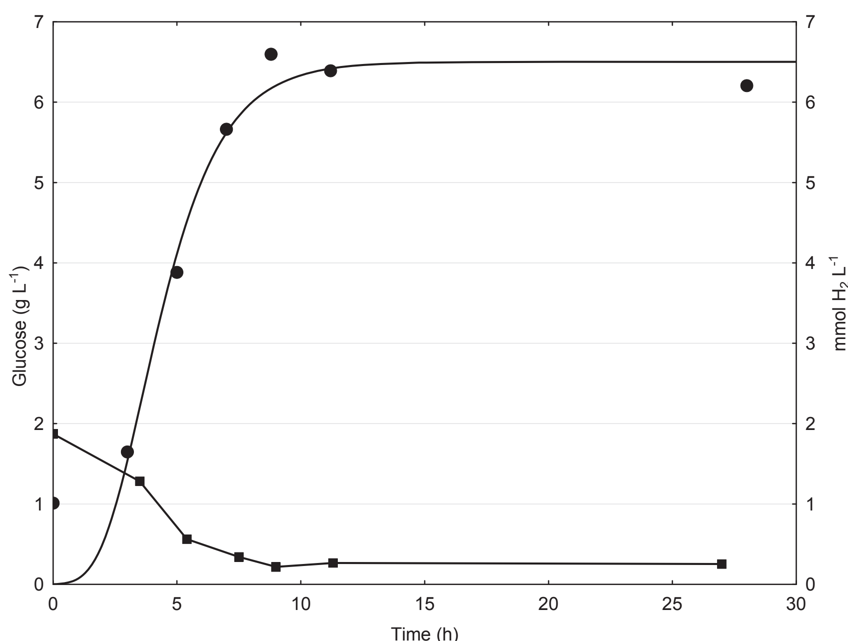


Fig. 2 – Glucose consumption (■) and cumulative hydrogen production (●) during the operation of anaerobic batch reactors inoculated with *Enterobacter* sp. strain isolated from brewery wastewater.

accumulation and higher hydrogen partial pressure. The enteric bacteria, such as *Enterobacter* species, show a mixed-acid fermentation. Lactate, ethanol, acetate can be produced beyond H_2 and CO_2. The metabolic pathway will be driven by the environmental condition, e.g. the lactate production will be favored in acidic conditions. However, the pH drop, can lead to the decrease in the hydrogen yield due to the metabolic shift. Ethanol can be also produced by enteric bacteria and the hydrogen production will be also affected. An alternative strategy for reducing the negative impact of pH drop and organic acids accumulation in the hydrogen production is a co-culture of enteric and purple non-sulfur (PNS) bacteria. This approach has been used to increase hydrogen production yield, substrate consumption and COD removal [50].

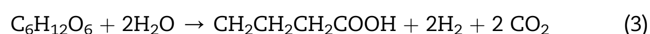
The hydrogen partial pressure is another environmental condition that has been claimed to affect the hydrogen production [50]. Mandal et al. [51] achieved an increase in the hydrogen yield from 1.9 mol to 3.9 mol H_2 mol^{-1} glucose by decreasing hydrogen partial pressure. Usually, it is done by sparging the headspace of the bioreactor with inert gases. Its main disadvantage relies on the dilution of the hydrogen that has been produced.

The cumulative hydrogen production data obtained in the kinetic test was adjusted to the modified Gompertz model in order to obtain the lag phase and maximum hydrogen production rate. The lag phase of 1.89 h was observed in the experiments. This value suggests there was not observed any inhibitory effect for the cellular growth and consequent hydrogen production (Fig. 2). Though, the imposed conditions favored the H_2 generation in the first hours of the incubation. The estimated hydrogen production potential [(P) 6.5 mmol H_2 L of culture media^{-1}] was similar to the one verified in the experiment (6.8 mmol H_2 L of culture media^{-1}). Another parameter given by the model adjustment is the maximum hydrogen production rate (Rm). Efforts have been done in order to increase both, the hydrogen yield and maximum hydrogen production rate. It was achieved 1.37 mmol H_2 L^{-1} h^{-1}.

The final pH was 6.1. It was observed the production of organic acids and alcohols. The fermentation of the glucose into organic acid justifies the pH drop at the end of the experiments. It was observed that organic acids production increased while glucose has been consumed.

Two pathways of glucose degradation can occur in fermentative processes of hydrogen gas production as follows: consumption of glucose generating acetic acid (2) and consumption of glucose generation butyric acid (3).

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



$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_2CH_2CH_2COOH + 2H_2 + 2\ CO_2 \quad (3)$$

There were higher percentages of acetic acid, ethanol and methanol during the operation of the reactors (Fig. 3). The generations of acetic acid (367.0 mg L^{-1}); butyric acid (95.1 mg L^{-1}); propionic acid (42.3 mg L^{-1}), valeric acid (879.24 mg L^{-1}), ethanol (1101.26 mg L^{-1}), and methanol (437.01 mg L^{-1}) were observed when there was a higher production of hydrogen (at 8.8 h of fermentation). Usually, a mixture of acetic, butyric, ethanol and propionic acids is produced in dark fermentation, depending on the diversity of bacterial species [52]. Higher percentages of acetic and butyric acid favor the hydrogen gas production. However, the generation of propionic acid and alcohols, as ethanol and methanol, consumes H_2 when they are produced [53] as observed in this study. Subudhi et al. [47] isolated *Enterobacter* species from crude oil contaminated soil and they were capable to obtain hydrogen production with glucose and xylose. The main intermediary products generated were acetic acid (528.0 mg L^{-1}) and ethanol (810.0 mg L^{-1}) as well as in the present study. According our results, a mixed-acid fermentation the metabolism of the *Enterobacter* species was favored to generate the mixtures of organic acids and alcohols occurred. There was observed mainly acetic acid, ethanol, and methanol.

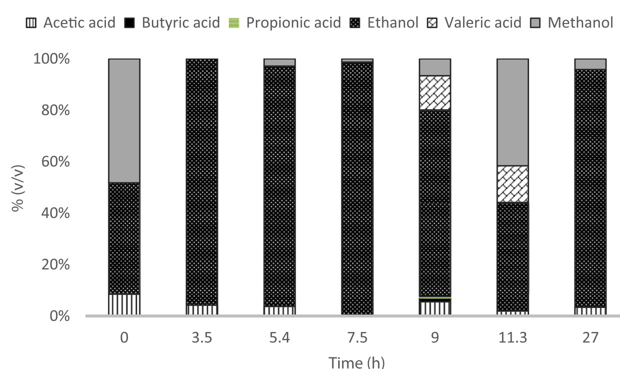


Fig. 3 – Distribution of the main fermentation products observed during the operation of anaerobic batch reactors fed with glucose (2 g L^{-1}) inoculated with *Enterobacter* sp. strain isolated from brewery wastewater.

The maximum hydrogen yield from glucose was 0.8 mol H_2/mol of glucose consumed. These results did not represent 100% of conversion confirming that the metabolic routes of *Enterobacter* sp. were not only to acetic and butyric acids generation. It can be seen that hydrogen yields vary significantly among the literature data (Table 3). Nakashimada et al. [54] evaluated the hydrogen production by *E. aerogenes* HU-101 from different carbon sources and found out a yield of 1.97 mmol H_2/g glucose. Ren et al. [55] assessed the hydrogen production of *E. aerogenes* from monomeric sugars such as xylose, mannose and galactose, and got higher yields (over 2 mol H_2/mol substrate). Zhao et al. [56] have gotten similar hydrogen yields by using a wild type *E. aerogenes* IAM1183 strain and its single and double mutants. Harun et al. [11] observed 1.8 mol H_2/mol glucose by *E. cloacae*. Long et al. [25] observed a hydrogen yield of 0.6 mol H_2/mol of glucose when using *Enterobacter* species isolated from water samples in anaerobic reactors fed with glucose (19.8 g L^{-1}) during 33 h of operation. Argun et al. [52] obtained a lower hydrogen yield (0.4 mol H_2/mol of glucose) with wheat powder (2.5 g L^{-1}) in anaerobic batch reactors with a pH of 7.0, at 30 °C, with heat-treated acidogenic-anaerobic sludge rather than the one used in this study. Based on the best results reported, the authors applied higher concentrations of substrates than the present study. New tests need to be made with the isolates to be improved the hydrogen yields. In addition, it can be done by

Table 3 – Comparison of hydrogen yields presented by different strains and substrates.

Microorganisms	Substrate cultivation	Hydrogen yields	pH T (°C)	Reference
E. cloacae KBH3	Glucose 10 g L^{-1}	1.8 mol H_2/mol glucose	7.0 30 °C	[11]
Enterobacter sp.	Glucose 19.8 g L^{-1}	0.6 mol H_2/mol glucose	40 °C	[25]
Acidogenic consortia of anaerobic bacteria	Wheat powder 2.5 g L^{-1}	0.4 mol H_2/mol glucose	7.0 30 °C	[52]
E. aerogenes HU-101	Glucose 10 g L^{-1}	0.33 mol H_2/mol glucose	37 °C	[54]
E. aerogenes IAM 1183	Xylose 5 g L^{-1} Galactose 10 g L^{-1}	2.2 mol H_2/mol xylose 2.35 mol H_2/mol galactose	37 °C	[55]
E. aerogenes IAM1183 *E. aerogenes* IAM1183-B (ΔhycA) *E. aerogenes* IAM1183-O (ΔhybO) *E. aerogenes* IAM1183-AO (ΔhycA/ΔhybO)	Glucose 71.7 mM	1.16 mol H_2/mol glucose 1.20 mol H_2/mol glucose 0.27 mol H_2/mol glucose 1.36 mol H_2/mol glucose	6.9 37 °C	[56]
Enterobacter sp.	Glucose 2.0 g L^{-1}	0.8 mol H_2/mol glucose	7.0 37 °C	This study

keep screening for potential high rate and yield hydrogen producers and by using design of experiments to find out the best culture conditions.

Conclusions

- This study demonstrates the ability of *Enterobacter* sp. phylogenetic closely related strains, isolated from brewery wastewater, for hydrogen bioproduction at mesophilic condition. They were able to utilize different carbon sources, including sugars, starch and glycerol with high efficiencies on hydrogen generation. One of the them, growing in a glucose based medium (2 g L^{-1}), showed a hydrogen yield of 0.8 mol H_2 mol^{-1} glucose.
- The isolation of new facultative anaerobic hydrogen-producing strains, such as *Enterobacter* sp., is of great interest, particularly at industrial scale, because it plays an important role in co-cultures (consisting of facultative and strict anaerobes) for dark fermentative hydrogen production. Furthermore, the wide substrate versatility, make them potential candidates for fermentative hydrogen production from diverse wastewaters, containing sugars and glycerol, which is desired from the environmental and economic point of view.

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