Screening and bioprospecting of anaerobic consortia for biohydrogen and volatile fatty acid production in a vinasse based medium through dark fermentation

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A B S T R A C T

The use of microbial consortia and industrial effluents for the production of biohydrogen by dark fermentation is seen as a key strategy in an attempt to overcome the economic and technical drawbacks of this potential technology. Three mesophilic microbial consortia were sampled and identified. Fermentation was carried out in a vinasse-based medium supplemented with pure or complex carbon sources under different conditions of H2 partial pressure. Consortia LPBAH1 and LPBAH2 were predominantly composed of Oxalobacteraceae and Lactobacillaceae, while LPBAH3 was rich in sporulating Lactobacillaceae (> 96%). Each consortium presented specificities related to biohydrogen and VFAs production: (i) the highest biohydrogen yield was achieved with LPBAH1 (> 50% Oxalobacteraceae) in a vinasse medium supplemented with sugarcane juice (1.59 ± 0.21 molH2/molglucose); (ii) The lower H2 yields were achieved with LPBAH3, which otherwise produced the highest amount of butyric acid (up to 10 g L\(^{-1}\)); (iii) LPBAH2 presented great stability in H2 production in different conditions of H2 partial pressure.

1. Introduction

The gradual introduction of fuels with increasingly lower carbon content per unit of energy (wood – coal – oil – natural gas) results in a continuous decarbonization of the global fuel mix. This chain of lower carbon content fuel ends in Hydrogen (H\(_2\)).

Currently, the cost of H\(_2\) generated from biological processes is very high, especially due to medium cost and process sensitivity. In some bioprocesses for biohydrogen production (such as dark fermentation of organic matter) volatile fatty acids, which are platform molecules that can be used as raw material for green chemistry, are produced and accumulated in the liquid phase. The production and commercialization of these platform molecules will have a great impact on the economics of biohydrogen production technology [1], and thus deserve attention.

In the development of biohydrogen processes, two points are of great interest: (i) the use of agroindustry liquid and solid wastes as feedstock, promoting economic (and environmental) advantages; and (ii) the use of mixed cultures or consortia. The use of consortia in industrial fermentations presents some challenges, especially related to process stability, but offers less susceptibility to contamination by H\(_2\)-consuming bacteria and oxygen [2]. Moreover, a diverse microbial community is more adaptable to substrate variation (an intrinsic characteristic of (agro)industrial wastewater) due to the presence of alternative metabolic pathways capable of developing a food web where specific groups of organisms maintain a low concentration of critical intermediate products and promote the flux of carbon and electrons from the feedstock material to the desired end product [3].

With respect to the range of potential substrates which can be utilized by the broad range of hydrogen-producing bacteria, it can be stated that, at present, it is a vast and open field for further exploration. One of the major bottlenecks in developing large-scale biohydrogen technologies using waste is its availability and volume of production. Since use of bioH\(_2\) is mostly aimed at the production of energy, it can be
considered a fair value-added product, and thus has to be produced in very large quantities.

Since the last decade, complex carbon sources, such as molasses [4], food waste [5], dairy wastewater [6], mushroom waste [7], rice slurry [8], cheese whey [9], lignocellulosic materials, glycerol waste [10], vegetable waste [11] and many others have been studied and proved to be viable for the production of biohydrogen by dark fermentation.

One of the largest industries in the world is the bioethanol industry. In tropical countries, bioethanol is produced through a classic fermentation process, in which yeasts transform sugarcane juice, molasses, or a molasses-juice mixture into ethanol. At the end of fermentation, almost 100% of the sugar (sucrose) present in the culture medium is consumed by the yeast (usually a *Saccharomyces*), resulting in a liquid called wine with a yield of approx. 50%. The wine has a concentration of ethanol (% by volume) between 6 and 10° GL, which is recovered by distillation at the top of distillation columns, where the volatile substances are separated based on their different boiling points [12]. From the base of the column, the fermented broth free of ethanol, called vinasse, is removed. Vinasse contains some organic solids in suspension as well as minerals, residual sugar, and some volatile compounds.

Considering the ethanol concentration in the wine, vinasse is generated in an average proportion of 12–15 L for each liter of alcohol produced. Because of its production rate and its chemical characteristics (see [13]), vinasse constitutes a large pollution source. It is used for the development of anaerobic microorganisms (known presence of strict/facultative anaerobes and/or absence of oxygen) were collected. From the base of the column, the fermented broth free of ethanol, called vinasse, is removed. Vinasse contains some organic solids in suspension as well as minerals, residual sugar, and some volatile compounds.

In this work, the bioprospecting, screening and identification of microbial consortia capable of producing biohydrogen in the vinasse-based medium are presented. Biohydrogen productivity, VFAs production, microbial consortia stability through time were evaluated.

## 2. Material and methods

### 2.1. Consortia sampling

Three samples from Brazilian environments with proper conditions for the development of anaerobic microorganisms (known presence of strict/facultative anaerobes and/or absence of oxygen) were collected. The samples were transferred to screw cap glass bottles with the aid of 10 mL sterile syringes (in the case of liquid samples). Their transport was conducted in closed-cell extruded polystyrene foam with dry ice. The name of the strains and the sampling sites are described in Table 1.

### 2.2. Molecular identification

The microbial identification of consortia LPB AH1, LPB AH2 and LPB AH3 was carried out at the WEMSeq Biotechnology Laboratory (Curitiba/PR – Brazil). For the sequencing and bioinformatics analyses, approximately 5 mL of each consortium sample was taken. The extraction of the total genomic DNA from the samples was performed with phenol/chloroform, followed by the PCR analysis for the V4 region of the 16S rDNA gene with 10 ng of DNA, primers 515F and 806R, in the KlenTaq system (Sigma) according to the methodology of [15]. In this work, the bioprospecting, screening and identification of microbial consortia capable of producing biohydrogen in the vinasse-based medium are presented. Biohydrogen productivity, VFAs production, microbial consortia stability through time were evaluated.

### 2.3. Medium composition, culture conditions and consortia maintenance

Vinasse composition and characterization are described in Table 2. Sucrose (VS), sugarcane juice (VSJ) or molasses (VM) were added in a vinasse medium as a carbon source at an equivalent concentration of 10 g/L of sucrose. Total carbohydrates in molasses were estimated using a refractometer, and in the sugarcane juice by using the Dubois et al. method [16].

The procedure for promoting an anaerobic culture was based on the Balch technique [17]. The removal of oxygen was achieved by boiling the medium under anoxic conditions (CO2 atmosphere). Bicarbonate (1 g/L) was added at 85 °C and Cysteine-HCl at 65 °C as reducing agents to lower the redox potential of the medium. To assure oxygen removal, Resazurin was used as an indicator (0.5 mg/L).

The experiments were carried out in 15 mL Hungate tubes, with a working volume of 6 mL, sealed with autoclavable Bakelite screw caps and rubber stoppers, and incubated at 37 °C. The medium pH was adjusted with 1 N KOH to 7.0. The cultures were maintained under these conditions for one week and then inoculated in a new medium.

Consortia maintenance was carried out by transferring the cell passages to a new fresh medium every two weeks. Monthly production of volatile fatty acids and hydrogen was carried out to evaluate consortia stability.

### 2.4. Biohydrogen production and composition analysis

Biohydrogen production in Hungate tube cultures was periodically measured using 60 mL plastic syringes. Daily gas quantification was carried out in cultures considered free of H2 partial pressure or twice a week (more precisely on the 4th and 7th day of culture) in the cultures where H2 partial pressure was allowed. Gas was collected by inserting a graduated syringe through the flask-type butyl rubber septum and purified for hydrogen content estimation, as discussed later. Purification was carried out by employing CO2 absorption in an NaOH solution [18,19]. The column consisted of a graduated cylinder filled at 50% of its volume with 2 mm glass beads to increase gas contact time with the basic solution. Gas was injected at approximately 3 mL/s through a porous stone.

### 2.5. Analysis of organic components

The organic components of the culture medium were determined by using High Performance Liquid Chromatography (HPLC). Before injection, the samples (2 mL) were centrifuged and filtered (Millipore...
of vertebrates, is described to be related to the excretion of formate [22]. Due to the origin of this consortium (feces of fruit bats) and the presence of formate among the fermentation products O. formigenes is a candidate that plays a key role in this consortium.

Despite the representative composition of lactobacillus in the consortium, lactic acid was not detected in the fermented broth. This may be explained by the use of lactic acid as a substrate by other bacteria, resulting in no accumulation at the end of fermentation. LPB AH1 demonstrated a high capacity to produce biohydrogen in a vinasae medium. Higher yield was achieved in VS medium without partial pressure (2.1 molH₂/molglucose), statistically different from the yield achieved in VSJ medium (p = .049). This is close to the maximal hydrogen yield expected in mixed cultures [23,24] and corroborates with the observation of [25] that there might be an association between high H₂ yields and a low microbial diversity of communities.

In the VSJ medium, hydrogen production reached 1.59 ± 0.21 molH₂/molglucose, accompanied by considerable amounts of acetate and butyrate production (3.5 and 7.6 g L⁻¹ respectively), which is relevant in coupling with methane or solvent production processes. From Fig. 2, it is observed that in the VSJ medium the effect of H₂ partial pressure in H₂ production was minimum (yield reduction < 10%, p = .93). This is very interesting considering industrial applications as it facilitates process handling. Fermentation carried out on other complex media (VS and VM) presented relevant negative effects of hydrogen partial pressure.

The lower biohydrogen yield in the VM medium can be explained by the inhibitory effect of the sugar components in molasses that influence the microbial growth of bacteria, which can be observed by the lower production of volatile fatty acids in relation to other media (Figs. 2 and 3).

On the other hand, the profile of VFAs with and without H₂ pressure was very different for the four media (Fig. 2), especially for the VS medium where the composition of VFAs at the end of fermentation was completely altered. The development of acetate consumers in the VM medium was also seen. LPBAH1 presented the potential of acetate consumption probably due to the presence of Archea in the consortium [26] and butyrate production due to the presence of Lactobacilaceae [27].

3.2. LPB AH2

Metabolic analysis of the consortium LPB AH2 showed a great potential for biohydrogen production in the vinasae media (Fig. 4), reaching yields between 1.5 and 1.55 molH₂/molglucose. No statistical difference of biohydrogen yields was obtained in relation to the carbon source. The effect of hydrogen partial pressure was statistically insignificant for fermentations in sugarcane juice and the sucrose supplemented medium, which is interesting because it facilitates the management of the process on an industrial scale. On the other hand, H₂ partial pressure caused a decrease in approx. 100% of the yield in fermentations that used molasses as carbon sources.

As observed for LPBAH1, Oxalobacteraceae dominates LPBHAH2 composition (Fig. 3), but it led to the production of succinic, lactic and propionic acids. The production of lactic acid in the VSJ medium (Fig. 4) can be explained by the presence of Lactobacillus in the LPB AH2 consortium (Fig. 3). With the H₂ partial pressure reduced, this organic acid was not detected, which is in agreement with the expected effects of biohydrogen metabolism (Fig. 4).

The presence of succinic and propionic acid can be explained by the presence of lactic acid bacteria but also by the presence of Clostridium strains or other types of bacteria designated as “others”. Lactic acid bacteria were described as producing succinic and propionic acid in fish infusion broth [28], while Clostridium propionicum, for example, is a known and explored producer of propionic acid [29].

The use of molasses as a carbon source together with the maintenance of a low H₂ partial pressure environment resulted in the

0.2 μm).

The HPLC equipment comprised of Shimadzu Liquid Chromatograph equipped with an Aminex® HPX-87H 300 × 7.8 mm (Bio-Rad) column and a refractive index detector (RID-10A). The column was kept at 60 °C and 5 mM H₂SO₄ at 0.6 mL/min was used as a mobile phase. The chemical species quantified by this method were glucose, fructose, succinate, lactate, formate, acetate, propionate, and butyrate. All the chemicals were of analytical grade.

As the retention time of butyrate and ethanol are very similar, it was impossible to differentiate them with HPLC. The method used for determining ethanol content was based on the oxidation of ethanol to acetic acid by reaction with potassium dichromate in an acidic medium. The solution acquired a green color proportional to the ethanol concentration in the sample, enabling the reading on the spectrophotometer at 600 nm. The standard solution of potassium dichromate (1 L) consisted of the following components: 500 mL of distilled H₂O, 325 mL concentrated H₂SO₄ and 33.678 g of potassium dichromate.

2.6. Statistical analysis

The experimental data of biohydrogen yield obtained from the fermentations of each consortium in different conditions of hydrogen partial pressure and supplemented carbon source was subjected to Student t-tests to determine if the data was significantly different (p < .10).

3. Results and discussion

The identification of the microbial composition of consortia demonstrated a similar composition of LPBAH1 and LPBAH2 (approx. 50% of Oxalobacteraceae and 20% Lactobacillaceae). The genus Clostridiaeae was identified in the consortia, LPB AH2 and LPB AH3, while only LPB AH3 presented sporulating Lactobacillaceae (> 96%).

3.1. LPB AH1

The consortium LPB AH1 is composed of the Oxalobacteraceae (52.23% of consortia) family and the genus Lactobacillus (24.29%), while enterobacteria represented only 3% (Fig. 1).

Oxalobacteraceae are aerobic/microaerobic, facultative anaerobic and anaerobic bacteria that metabolize oxalate as a major source of carbon for growth [20]. Only a few members of the family are strict anaerobes [21], especially those belonging to the genus Oxalobacter. O. formigenes, for example, a bacterium that colonizes the large intestines...

![Image](85x73 to 241x258)

**Fig. 1.** Microbial composition of the consortium LPB AH1. The genetic analysis was based on the V4 region of the 16S rDNA gene and classification is presented at family level.

The use of molasses as a carbon source together with the maintenance of a low H₂ partial pressure environment resulted in the
exclusive production of butyrate in the liquid phase. This showed an interesting role of Lactobacilaceae in the consortium, which is described as a putative butyrate producer both under controlled and non-controlled H₂ partial pressure fermentations and/or producing butyrate precursors, such as lactate [27].

3.3. LPB AH3

When cultured in a vinasse medium, the consortium LPB AH3 showed a very high production of butyric acid for all the substrates tested. The highest amount of butyric acid (10 g L⁻¹) among all strains evaluated was produced by this consortium (in a sugarcane juice supplemented medium), but H₂ productivity was lowest because considerable amounts of propionate and ethanol were also produced (more reduced products).

Pure sucrose was the best carbon source for hydrogen production, with a significantly higher yield than that of sugarcane juice (p = .04 and p = .0005 with and without H₂ partial pressure, respectively) (Fig. 5).

The use of molasses resulted in a very low hydrogen yield (0.3 and 0.4 molH₂/molglucose with and without H₂ partial pressure, respectively) but a higher amount of propionic acid in cultures without H₂ partial pressure (approx. 14 g/L), which is probably a shift in the microbial community composition caused by molasses that deserves further analysis.

The LPBAH3 consortium presented an almost homogeneous composition of Sporolactobacilaceae (96,22%) (Fig. 6). Sporolactobacilaceae resemble Lactobacillus in metabolism, except for the capacity of the first microorganism to respire in the presence of oxygen [30], presenting a variable ability to ferment carbohydrates to lactate and other by-products, such as acetate, ethanol, CO₂, formate and succinate [31], which was observed in fermentations carried out with LPBAH3. However, Sporolactobacilaceae are not known to produce propionic acid. The role of Sporolactobacilaceae is poorly described in the literature. Fang et al. [8] and [32] detected Sporolactobacillus in consortia evaluated for hydrogen production but did not discuss its role in the microbial community.

Surprisingly, lactic acid was not detected in the fermented medium, which may be caused due to its consumption by bacteria from the Clostridiaceae family [33–36].

3.4. Overall analysis

The bioprospecting of biohydrogen and VFAs producing consortia is an interesting tool for the development of high-yield processes since it allows the adaptation of a heterogeneous microbial community in the feedstock.
Fig. 4. Metabolic products of the cultivation of the consortium LPB AH2 in a vinasse medium containing different carbon sources with and without H₂ partial pressure; results are the average of 5 analyses and VFAs concentration is shown in g L⁻¹ and biohydrogen (mol H₂/mol glucose).

Fig. 5. Metabolic products of the cultivation of the consortium LPB AH3 in a vinasse medium containing different carbon sources with and without H₂ partial pressure; results are the average of five analyses and VFAs concentration is shown in g L⁻¹ and biohydrogen (mol H₂/mol glucose).
Among the consortia evaluated, LPB AH1 (sampled from feces of fruit bats) and LPB AH2 (sampled from the liquid waste of a dairy farm) showed the highest yields of biohydrogen production and both have higher microbial biodiversity in comparison to LPB AH3, which presented a very homogeneous composition. In terms of VFAs, each of the three consortia presented its own particularities, but all presented at least one condition where platform molecules were produced with interesting profiles and/or quantities. The generation of VFA (amounts and types) was in agreement with the hydrogen yield achieved for each consortium in specific conditions.

When comparing hydrogen yields with the literature (usage of complex media), it was observed that the results achieved for consortia LPB AH1 and LPB AH2 lie in the average productivity for mesophilic microorganisms [37], which is 1.46 molH2/molglucose. Distillery effluents are such a potential feedstock due to their chemical characteristics [e.g. Table 2] and volume of production and there are many studies on their use for biohydrogen production [38-41]. However very few studies are focused on the use of sugarcane vinasse. Because the composition of the liquid effluents from distilleries using different raw materials (agave, sugarcane juice, beet, corn) varies greatly, discussion will focus on the microbial community and production of hydrogen specifically from sugarcane vinasse.

The data on biohydrogen production from sugarcane vinasse in comparison to the present study is summarized in Table 3. Most studies on sugarcane vinasse bioprocessing for H2 production focus on vinasse concentration and operation mode. Vinasse concentration is described as influencing hydrogen yield in most studies (Table 3), except [42], which showed minimal influence of substrate concentration on microbial community composition (in this case composed of Clostridiaceae, 73%, and Ruminococcaceae, 26%). These indicate that the inoculation source and active microbial community play an important role in process development.

A previous study [42] using a mesophilic consortium from an anaerobic digester resulted in a higher hydrogen yield of 0.826 molH2/molglucose (considering 1 g of glucose = 1.07 gCOD) at 20% vinasse (diluted in water – no supplementation of organic carbon). The low hydrogen yield achieved in the referred study is a consequence of a low content of fermentable sugars in vinasse, which reflects the necessity of organic carbon supplementation.

Another study [43] achieved a 10 fold increase in hydrogen yield by supplementing vinasse with glucose. A maximum yield of 3.54 molH2/molglucose was achieved with a mixture of 75% glucose and 25% sugarcane vinasse in a continuous fluidized bed reactor, where bacteria from Prevotellaceae (55%) and Megaplasma (28%) predominated. The low abundance of the Clostridia bacterium (3%) observed was similar to the LPB AH2 (10%) and LPB AH3 (3%) for this study, although Clostridium is a common microorganism involved in H2 production from complex waste.

Different culture operation modes were evaluated by Albanez et al. [44] in a medium composed of 33% molasses + 67% sugarcane vinasse (6 gCOD/l), concluding that a batch mode resulted in higher H2 yields (0.759 molH2/molcarbohydrate) than a fed-batch. It must be considered, however, that sugarcane vinasse is produced in continuous mode and in huge volumes, virtually preventing industrial batch mode operation of hydrogen production processes. Another study [45] describes the use of a continuous operation anaerobic packed bed reactor in biohydrogen production using in natura (pure) sugarcane vinasse, reaching 1.4 molH2/moltotalcarbohydrates but failing in achieving process stability.

Biohydrogen yields as high as 1.5–1.6 molH2/molglucose in pure vinasse media supplemented with complex organic carbon sources, achieved in LPBAH1 and LPBAH2, have not been described in the scientific literature to date, which reinforces the enormous potential of LPBAH1 and LPBAH2 for biohydrogen and VFAs production since medium and culture conditions were not optimized.

4. Conclusion

In this work, the adaptation of three consortia producing biohydrogen and VFAs in an organic carbon supplemented vinasse based medium was successfully achieved. The different behavior of each consortium in the proposed media reinforces the importance of bio-prospecting for new consortia and screening new strains for production of biohydrogen and VFAs. In agroindustry based economies, huge volumes of waste is generated and should be reprocessed to avoid environmental issues and promote technological and economic development. Dark fermentation is an alternative to valorize the enormous volume of vinasse that is produced within bioethanol industries in the form of platform molecules and biohydrogen. Knowing the microbial

![Fig. 6. Microbial composition of the consortium LPB AH3. Genetic analysis was based on the V4 region of the 16S rDNA gene and classification is presented at family level.](Image 73x584 to 253x737)

Table 3

Hydrogen yields from sugarcane vinasse processing achieved by different studies. Inoculum, operation mode and media composition were the operational parameters compared.

<table>
<thead>
<tr>
<th>Inoculum source</th>
<th>operation mode</th>
<th>molH2/molglucose</th>
<th>Medium</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UASB reactor of a poultry slaughterhouse</td>
<td>batch</td>
<td>0.83</td>
<td>20% diluted sugarcane vinasse</td>
<td>[42]</td>
</tr>
<tr>
<td>Anaerobic reactor of a poultry slaughterhouse</td>
<td>batch</td>
<td>0.76</td>
<td>33% molasses + 67% sugarcane vinasse</td>
<td>[44]</td>
</tr>
<tr>
<td>Natural wastewater fermentation</td>
<td>continuous packed bed reactor</td>
<td>1.40</td>
<td>100% sugarcane vinasse</td>
<td>[45]</td>
</tr>
<tr>
<td>Anaerobic sludge for swine manure treatment reactor</td>
<td>continuous fluidized bed</td>
<td>3.54</td>
<td>25% sugarcane vinasse + 75% glucose</td>
<td>[43]</td>
</tr>
<tr>
<td>LPB AH1</td>
<td>batch</td>
<td>2.10</td>
<td>100% sugarcane vinasse + 10 g/L sucrose</td>
<td>this study</td>
</tr>
<tr>
<td>LPB AH1</td>
<td>batch</td>
<td>1.59</td>
<td>100% sugarcane vinasse + 10 g/L sucrose for 100% sugarcane vinasse + 10 g/L sucrose from</td>
<td>this study</td>
</tr>
<tr>
<td>LPB AH2</td>
<td>batch</td>
<td>1.50–1.60</td>
<td>100% sugarcane vinasse + 10 g/L organic carbon source*</td>
<td>this study</td>
</tr>
<tr>
<td>LPB AH3</td>
<td>batch</td>
<td>1.15</td>
<td>100% sugarcane vinasse + 10 g/L sucrose</td>
<td>this study</td>
</tr>
<tr>
<td>LPB AH3</td>
<td>batch</td>
<td>0.97</td>
<td>100% sugarcane vinasse + 10 g/L sucrose for 100% sugarcane vinasse + 10 g/L sucrose from</td>
<td>this study</td>
</tr>
</tbody>
</table>

* No statistical difference was observed using sugarcane molasses, sugarcane juice and sucrose.