Pretreatment on anaerobic sludge for enhancement of biohydrogen production from cassava processing wastewater

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ABSTRACT. Methods for the enrichment of an anaerobic sludge with H2-producing bacteria have been compared by using cassava processing wastewater as substrate. The sludge was submitted to three different pretreatments: 1) heat pretreatment by boiling at 98°C for 15 min., 2) heat pretreatment followed by sludge washout in a Continuous Stirring Tank Reactor (CSTR) operated at a dilution rate (D) of 0.021 h⁻¹, and 3) sludge washout as the sole enrichment method. The pretreated sludge and the sludge without pretreatment (control) were employed in the seeding of 4 batch bioreactors, in order to verify the volume and composition of the generated biogas. Maximum H2 production rates (Rm) from the pretreated sludges were estimated by the modified Gompertz model. Compared to the control, H2 production was ca. 4 times higher for the sludge submitted to the heat pretreatment only and for the sludge subjected to heat pretreatment combined with washout, and 10 times higher for washout. These findings demonstrated that the use of sludge washout as the sole sludge pretreatment method was the most effective in terms of H2 production, as compared to the heat and to the combined heat and washout pretreatments.

Keywords: mixed culture, heat pretreatment, sludge washout.

Introduction

Brazil is one of the largest world producers of cassava, whose processing for the production of flour and starch generates about 7 m³ wastewater per kg processed root. This wastewater is rich in carbohydrates, with about 5-15 and 20-50 g L⁻¹ of Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD), respectively (CEREDA, 2001). This wastewater can be treated by anaerobic processes, which produce CH4 and CO2 as the main end products (OLIVEIRA et al., 2001). However, in recent years, research on the process of anaerobic biodigestion of carbohydrate-rich wastewater has focused on converting organic material into H2 instead of CH4 and CO2 (WANG et al., 2012, MOHANAKRISHNA; MOHAN, 2013). Hydrogen production is more advantageous, because H2 combustion produces only water as byproduct, which classifies H2 as a clean fuel (WANG; WAN, 2009;
DAS; VERZIROGLU, 2008; MOHANAKRISHNA; MOHAN, 2013). Fermentative H₂ production shares common steps with the anaerobic biodigestion of organic matter, which is developed by a sequence of reactions carried out by a wide range of bacteria, responsible for hydrolysis, acetogenesis, and methanogenesis. More specifically, H₂ production shares the earlier stage of biodigestion; i.e., the hydrolysis and acetogenesis steps (VALDEZ-VASQUEZ; POGGI-VARALDO, 2009). Therefore, successful H₂ production by a mixed culture of bacteria, like an anaerobic sludge, requires inhibition of the bacteria responsible for the latest step of the anaerobic digestion; i.e., methanogenesis or inhibition of other means of H₂ consumption mediated by microorganisms that comprise the anaerobic sludge (LEANO; BABEL, 2012; ASSAWAMONGKHOLSIRI et al., 2013).

One of the most promising ways to avoid H₂-consuming reactions is to pretreat mixed cultures used as bioreactor inoculums. Such pretreatments should prevent or reduce competition between the H₂-producing and H₂-consuming bacteria (DAS; VERZIROGLU, 2008; LEANO; BABEL, 2012; ASSAWAMONGKHOLSIRI et al., 2013). Several methods have been described to enrich a mixed culture with H₂-producing bacteria, most of which involve adding chemicals to the sludge. Here, we have tested heat pretreatment, sludge washout, and a combination of both, because they do not require the addition of any chemical compounds to the mixed culture.

The effectiveness of heat to pretreat a mixed culture for biohydrogen production is based on the premise that most of the H₂-producing bacteria are spore-producing bacteria of the genus Clostridium. Therefore, heat treatment promotes sporulation of these bacteria, while restricting the development of other genera of bacteria sensitive to heat (MATHEWS; WANG, 2009; REN et al., 2009; WANG; WAN, 2009). The hydraulic retention time (HRT) is defined as the volume of the reactor (L) divided by the flow rate (L d⁻¹); it is also known as the inverse of the dilution rate (D). The continuous stirred tank reactors (CSTR) could be used to washout microorganisms by applying an HRT lower than their growth rate or a D higher than the growth rate. In this way, only microbial populations with growth rates larger than D can remain in the reactor (µmax > D). Therefore, D values higher than the methanogens specific growth rate could be used to cause washout of this kind of microorganism in a mixed culture, because the methanogens growth rates are much lower than those of H₂-producing bacteria, 0.017 h⁻¹ and 0.083 h⁻¹, respectively (VALDEZ-VASQUEZ; POGGI-VARALDO, 2009).

In this context, this work evaluates the efficacy of heat treatment, sludge washout, and the combination of both methods to enrich a mixed culture with H₂-producing bacteria, using cassava processing wastewater as substrate.

Material and methods

The mixed culture used as seed was obtained from an upflow anaerobic sludge blanket reactor (UASB) employed to treat of swine manure, located in Concórdia, Santa Catarina State, Brazil. The seed sludge concentration is expressed as the content of total suspended solids (TSS) and volatile suspended solids (VSS).

The cassava processing wastewater used as substrate was enriched by addition of the following macro and micronutrients (mg L⁻¹): K₂HPO₄ 63, Na₂CO₃ 1000, NaHCO₃ 1465, NH₄Cl 435, and 1 mL L⁻¹ of a micronutrient solution with the formulation (mg L⁻¹): FeSO₄·7H₂O 10000, CaCl₂·2H₂O 2000, ZnSO₄·7H₂O 2200, MnSO₄·4H₂O 500, CuSO₄·5H₂O 1000, (NH₄)₆Mo₇O₂₄·4H₂O 100, and Na₂B₄O₇·10H₂O 20 (KAWAGOSHI et al., 2005).

All the chemicals used in this work were analytical grade.

The pretreatment methods employed to enrich of the sludge with H₂-producing bacteria were heat treatment and sludge washout, used alone and in combination. Heat treatment of the inoculum was performed in a 2L CSTR. The temperature was maintained at 45°C by means of a water bath; the...
sludge and the wastewater were stirred at 100 rpm with the aid of a magnetic stirrer. Anaerobic operating conditions in the reactor were guaranteed by daily flushing argon into the feeding medium and the reactor. The CSTRs were inoculated with 21.9 g L⁻¹ TSS sludge submitted to heat pretreatment sludge or 20.8 g L⁻¹ seed sludge with no heat pretreatment. During the pretreatment period, the CSTRs were operated using a Hydraulic Retention Time (HRT) of 48 h or a dilution rate (D = 1 HRT⁻¹) of 0.021 h⁻¹ (0.5 day⁻¹), which promoted sludge washout. TSS values of 2.50 and 2.98 g L⁻¹ were obtained for the heat pretreated sludge and the sludge without heat-pretreatment, respectively. Daily TSS determinations during the washout period allowed us to calculate the microorganism maximum specific growth rate (μmax) according to the biomass mass balance described in Equation 1 (KIELING et al., 2007).

$$\frac{dX}{dt} = \mu_{\text{max}} X - DX$$

or

$$X = X_i \cdot e^{\mu_{\text{max}} \cdot t}$$

(1)

where:

- Xi = initial cell concentration,
- X = cell concentration at time t,
- D = dilution rate, and
- μmax is the maximum cell specific growth rate.

To verify the efficiency of the pretreatment methods described above in terms of increasing H₂ production by the mixed culture, the pretreated sludges were used as inoculums for batch-operated bioreactors, and the quantitative and qualitative gas production was assessed. The pretreated mixed cultures were used to seed four bioreactors, which were numbered as follows: Reactor 1 (R1) - Control, non-treated sludge; Reactor 2 (R2) - sludge submitted to heat pretreatment; Reactor 3 (R3) - sludge submitted to a combination of heat and washout pretreatments; Reactor 4 (R4) – sludge submitted to washout.

The initial VSS concentration in the batch reactors was about 2000 mg L⁻¹. Then, 400 mL of cassava wastewater containing 5000 mg L⁻¹ COD as well as macro- and micronutrients were added to each reactor. The 500 mL reactors were placed in a temperature-controlled bath shaker, which kept the temperature at 45°C, and stirred at 80 rpm.

The pipes for gas collection were coupled to the reactors and to a gas measurement system consisting of an inverted flask containing a NaOH 5% (w v⁻¹) solution and a flask to determine of the volume displaced by the produced gas, as represented in Figure 1 and modified from Aquino et al. (2007).

Figure 1. Batch bioreactor for H₂ production and outline of the system employed for gas capture: 1- bioreactor, 2- gas pipeline, 3- bottle containing 5% NaOH solution displaced by the generated gas, 4- NaOH uptake due to the volume of produced gas (modified from AQUINO et al., 2007).

The volume of produced biogas was corrected by Equation 2, shown below:

$$V = \frac{(P_{\text{atm}} - \rho \cdot H \cdot g) \cdot V_{\text{exp}} \cdot T}{P_{\text{atm}} \cdot T_{\text{exp}}}$$

(2)

where:

- V = volume of biogas at NCTP, P_{\text{atm}} = atmospheric pressure,
- ρ = NaOH 5% (w v⁻¹) density,
- H = distance between NaOH outlet in the Duran Flask and the collector flask,
- g = gravity constant,
- V_{\text{exp}} = volume of NaOH 5 % that was displaced by the generated gas,
- T = temperature (K) NCTP,
- T_{\text{exp}} = experimental temperature.

Volume gas was measured as described above. TSS and VSS, COD, and pH analyses were performed at the beginning and at the end of the experiment. The produced gas was collected from the headspace of each reactor after 42 h of experiment and analyzed by gas chromatography.

TSS and VSS were assayed according to the Standard Methods for Examination of Water and Wastewater (APHA, 1995). COD was determined by means of the closed reflux method using non-filtered samples, according to a previously described procedure. Experiments were carried out on a spectrophotometer (Hitachi U-1800 spectrophotometer, Japan), using absorbance values obtained at 600 nm. The pH was measured potentiometrically.
The produced gases were qualitatively determined by gas chromatography (GC), and the detector temperature was 100°C. Chromatographic analysis was carried out in a GC 35 gas chromatograph equipped with a thermal conductivity detector (TCD) (HAN; SHIN, 2004). The column consisted of molecular sieve 5Å measuring 2 m x 4.7 mm, and the argon carrier gas flow was kept at 30 mL min.\(^{-1}\). The temperatures of the injector, column, and detector were 80, 50, and 100°C, respectively.

The kinetic data on biohydrogen production from the pretreated inoculums and control (not submitted to pretreatment) were obtained by following the modified Gompertz equation (Equation 3),

\[
H = A \exp \left[ \exp \left( \frac{R_m}{A} (\lambda - t) + 1 \right) \right]
\]  

(Equation 3)

where:

- \(H\) (mL) is the cumulative volume of hydrogen production or the amount of \(H_2\) produced at incubation time \(t\),
- \(\lambda\) (h) denotes the lag time required for the beginning of the exponential hydrogen production,
- \(A\) (mL) is the maximum potential hydrogen production, \(R_m\) (mL h\(^{-1}\)) is the maximum hydrogen production rate. The parameter values were estimated with the aid of the software OriginPro 7.5 using a Newtonian algorithm.

Results and discussion

The seed sludge (non-pretreated) and the heat-pretreated sludge were submitted to sludge washout using a continuous process and a dilution rate (D) high enough to promote the washout of non-\(H_2\)-producing bacteria and methanogens. Figure 2 presents the TSS concentration results obtained during seven days of washout of the seed sludge (Figure 2a) and the washout of heat-pretreated sludge (Figure 2b). The TSS concentration during the washout decreased from 20.8 to 2.98 g L\(^{-1}\) and from 21.9 to 2.5 g L\(^{-1}\) for the non-pretreated sludge (Figure 2a) and the sludge submitted to heat pretreatment (Figure 2b), respectively.

We adjusted the exponential equations (see Equation 1) to the experimental TSS data, to estimate the maximum specific growth rate (\(\mu_{max}\)) of the remaining microorganisms (\(H_2\)-producing bacteria) in the bioreactors. As shown in Figures 2a and 2b, the \(\mu_{max}\) for the cells that remained after washout and after washout and heat pretreatment were very similar: 0.178 day\(^{-1}\) (0.007 h\(^{-1}\)) and 0.201 day\(^{-1}\) (0.008 h\(^{-1}\)), respectively. These \(\mu_{max}\) values were low as compared with values described in the literature for \(H_2\)-producing bacteria. For the pure culture of a \(H_2\)-producing Clostridium butyricum, values of \(\mu_{max}\) varied between 0.48 and 0.77 h\(^{-1}\) from sucrose-based medium depending on nutrient composition, pH, and carbon substrate concentration (CHEN et al., 2005). For mixed cultures Chen et al. (2001) estimated a \(\mu_{max}\) of 0.172 h\(^{-1}\) using sucrose as substrate. The lower \(\mu_{max}\) found in our work could be due to the cassava wastewater used as substrate. The sludge was not adapted to this kind of substrate, so the growth rate was very poor compared with the growth rate in other substrates.

Table 1 lists the results for COD in the reactors inoculated with sludge submitted to different pretreatments and the seed sludge (control), at the beginning (0h) and end (42h) of the experiments.

![Figure 2. TSS concentrations during washout of the seed sludge (A) and the heat-pretreated sludge (B).](image)

Table 1. COD concentration (mg L\(^{-1}\)) and consumption in bioreactors 1 to 4 at the beginning (0h) and end (42h) of the batch experiment for \(H_2\) production.

<table>
<thead>
<tr>
<th>Time</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td>5644</td>
<td>5480</td>
<td>6349</td>
<td>6245</td>
</tr>
<tr>
<td>42h</td>
<td>4984</td>
<td>5205</td>
<td>5952</td>
<td>5909</td>
</tr>
</tbody>
</table>

R1- reactor seeded with sludge without pretreatment (control), R2- reactor seeded with heat pretreated sludge, R3- reactor seeded with heat-pretreated sludge submitted to washout, R4- reactor seeded with sludge submitted to washout only.

The COD values varied from 6349 to 5480 mg L\(^{-1}\) at the start of the batch test (Table 1). This variation probably refers to the COD content of the sludge...
released after the different pretreatments, because the cassava processing wastewater used as substrate was the same in all the reactors. The COD consumption ranged between 275 and 660 mg L⁻¹. The highest consumption was observed in the case of the bioreactors containing the control sludge; i.e., the non-pretreated sludge, the one containing methanogens. The lower COD consumption for all the pretreated sludges stemmed from organic acids generation as a byproduct of H₂ formation by a mixed culture, instead of methane and carbon dioxide production from complete carbohydrates anaerobic degradation.

The pH values (not shown) remained virtually unchanged during the experiment; i.e., at around 7.02 (± 0.17), probably because of the high concentration of buffering substances added to the medium. The optimum pH range for H₂ production is 5.2 - 7.0 in the case of hydrogen conversion from carbohydrates, so the pH value that was maintained in the reactor during the experiment was adequate (LI; FANG, 2007).

Figure 3 depicts the total volume of biogas produced by the seed sludge (control) and by sludges remaining after pretreatments. The results show that heat pretreatment (R2 and R3) reduced the capacity of total biogas production, as compared with the control sludge (R1). The intense heat treatment applied to the sludges used in reactors R2 and R3 probably prevented the growth of most of the microorganisms in the sludges, which could be the reason for the low biogas production (less than the amount achieved with the control – R1). However, the sludge submitted to washout only (R4) favored total biogas production, which was 50% larger as compared with the control (R1).

The biogas generated by the sludge in the bioreactors was analyzed for its composition. The composition was constant during the whole period of the experiment (42h). Figure 4 presents the H₂ volume produced in each reactor. R4, inoculated with the sludge submitted to washout only, led to higher H₂ production, reaching a total H₂ volume that was about 70% higher than those achieved with R2 (heat-pretreated sludge) and R3 (heat-pretreated sludge submitted to washout). The maximum specific growth rates of methanogenic archaea and H₂-producing bacteria were ca. 0.018 h⁻¹ and 0.083 h⁻¹, respectively (VALDEZ-VASQUEZ; POGGI-VARALDO, 2009). Therefore, dilution rates higher than 0.018 h⁻¹ and lower than 0.083 h⁻¹ are recommended to wash out methanogens from a mixed culture that should be enriched with H₂-producing bacteria.

In this work, the dilution rate (D) applied during the sludge washout was 0.021 h⁻¹, which corresponded to an HRT of 48h.

![Figure 3. volume of total biogas production by the sludges submitted to different pretreatments. R1- control sludge, R2-heat-pretreated sludge, R3-heat-pretreated sludge submitted to washout, and R4-sludge submitted to washout only.](image)

Although the D value used in this study was adequate to enrich the sludge with H₂-producing bacteria, it was very close to the growth rate of methanogenic archaea (0.018 h⁻¹). Indeed, several authors have employed higher D values (lower HRT) during reactors operation for H₂ production. For example, the optimal HRT for a reported CSTR was 12h (D = 0.083 h⁻¹) using cassava starch as substrate, while the optimal HRT described for a CSTR fed with glucose was 8.34 h (D = 0.12 h⁻¹) (WANG; CHANG, 2008; WU et al., 2010). Chen et al. (2001) revealed that operation at D of 0.075-0.167 h⁻¹ was preferable to enrich a mixed culture with H₂-producing bacteria. The possible reason for this wide range of values is the difference among these studies in terms of inoculum, substrate, and studied HRT range (WANG; WAN, 2009). According to the above mentioned authors, the D value we used here (0.021 h⁻¹) could be higher, to promote better bacterial selection.

Results of our work evidence that sludge washout is a more appropriate strategy to enrich a mixed culture with H₂-producing bacteria as compared with heat treatment. However, most literature studies aiming at H₂ production by mixed cultures from cassava derivatives have employed heat treatment as the sole method to enrich sludge with H₂-producing bacteria, regardless of the inoculum source (LEE et al., 2008; WANG; CHANG, 2008; SU et al., 2009; ZONG et al., 2009; SREETHAWONG et al., 2010; ASSAWAMONGKHOLSIRI et al., 2013). Wu et al. (2010) reported that an anaerobic sludge obtained from an anaerobic pond pretreated by boiling at 95°C for 15 min., eradicated methane-producing archaea. Zong et al. (2009) boiled a cattle dung compost for 15 min. and employed it to produce H₂ from cassava starch. Wang and Chang (2008) heat treated a sludge from municipal wastewater treatment.
(70°C for 1h) to eliminate the methanogenic activity, and used it to seed a bioreactor fed with cassava starch, to produce biohydrogen. Leano and Babel (2012) used anaerobic seed sludge subjected to heat pretreatment at 105°C for 90 min., to produce H₂ from enzymatic pretreated cassava wastewater.

Growing evidence has shown that sludge heat treatment alone does not ensure the long-term repression of methanogens (Kim et al., 2006). Kim et al. (2006) applied chemical and heat pretreatments to an anaerobic sludge, to check whether more efficient H₂ production occurred. After this first step, performed in batch reactors, the authors used the pretreated sludge to seed reactors operated in the continuous mode (CSTR) with an HRT of 3 day. In this condition, the anaerobic sludge that had not received any pretreatment furnished the highest H₂ production. The aforementioned authors pointed out that applying chemical and heat pretreatment methods to mixed sludges have short-term effects on H₂ production only, and that the pretreatment methods will not affect H₂ production after a steady-state is achieved in the long-term operation (Luo et al., 2010). Therefore, the advantage of washing out sludge as compared with heat treating inoculum when enriching the sludge with H₂-producing bacteria is that, in addition to being more efficient, the washout method could be continuously used to operate a CSTR with low HRT for a long operation time. Sludge boiling is a punctual method viable at the laboratory-scale only.

However, the results obtained in this work suggest that heat treatment effectively enriched the sludge with H₂-producing bacteria. Indeed, R1 (control), which was inoculated with the sludge submitted to no pretreatment, produced only 4.2 mL of H₂. This value was about 4 times lower than the amount produced by R2 (heat-pretreated) and R3 (heat-pretreated and submitted to washout), and 10 times lower than that achieved with R4 (submitted to washout only). The heat treatment conditions used in the present study may also prevent growth of H₂-producing cells. This could explain why R2 and R3, seeded with heat-treated sludge, produced a lower amount of H₂ as compared with R4, seeded with the sludge submitted to washout only. Heat treatment favors the selection of some spore-forming H₂-producing bacteria like Clostridium, but it also eliminates other non-spore forming H₂-producing bacteria that could contribute to H₂ production (Li; Fang, 2007).

We compared the kinetic parameters listed in Table 2 with those from other literature studies that used similar substrates and kinetic model. According to Su et al. (2009), the raw, gelatinized, and hydrolyzed cassava starch to produce biohydrogen led to A values lying between 617 and 2137 mL of H₂, depending on the substrate concentration (Su et al., 2009). Under mesophilic conditions, the cassava starch as substrate gave A values varying between 1441 and 2970 mL of H₂. In the present work, the highest A value was 44.6 mL of H₂, obtained for the sludge submitted to washout only (R4). This value is low if compared with the values achieved by the above mentioned authors (Lee et al., 2008). However, the aim of our work was compare methods to enrich sludge with H₂-producing bacteria using cassava wastewater as substrate and not optimize conditions for biohydrogen production. The low H₂ production can be related to the substrate, which was a real cassava wastewater in our case, and not cassava starch or pretreated cassava starch as described in former papers (Lee et al., 2008; Su et al., 2009; Leano; Babel, 2012). Furthermore, cassava wastewater is a feedstock.

Table 2. Kinetic parameters estimated by the Gompertz model for the non-treated sludge (R1), the heat-pretreated sludge (R2), the heat-pretreated sludge submitted to washout (R3), and the sludge submitted to washout only (R4).

<table>
<thead>
<tr>
<th>Bioreactor</th>
<th>Kinetic parameters*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A (mL)</td>
</tr>
<tr>
<td>R1</td>
<td>4.3</td>
</tr>
<tr>
<td>R2</td>
<td>15.0</td>
</tr>
<tr>
<td>R3</td>
<td>15.4</td>
</tr>
<tr>
<td>R4</td>
<td>44.6</td>
</tr>
</tbody>
</table>

*(A) Potential for H₂ production, (Rm) maximum H₂ production rate, and (λ) lag phase time calculated by the modified Gompertz model, Equation (3).
that is poor in nutrients; optimization of macro and trace elements concentration could lead to higher H2 production.

Regardless of the volume of produced H2, it was possible to compare the heat treatment and sludge washout methods to enrich a mixed culture with H2-producing bacteria. The results demonstrated that sludge washout is the most appropriate strategy for the inoculum sludge employed herein, using cassava wastewater as substrate. In a literature review, the need to pretreat mixed cultures for H2 production has been pointed out; it has been noted that the effectiveness of each method depends on the nature of the inoculums and the substrate, and on how the bioreactor is operated (MOHAN, 2008). The effect caused by pretreating the inoculum might not last long, but in most cases it seems to reduce the time required for the H2-producing reactors startup.

For all these reasons, sludge washout can be considered a promising sludge pretreatment method that could be continuously used to operate the CSTR with low HRT, as long as bioreactors are equipped with mechanisms to retain the selected biomass.

Conclusion

In this work the pretreatment methods applied to the mixed culture effectively enrich the culture with H2-producing bacteria. The differences in H2 volume produced by the pretreated sludges enabled comparison of the pretreatment methods. In the case of the sludge employed as inoculum in this study and using cassava processing wastewater as substrate, washout is the most appropriate method when it comes to making the sludge a better H2 producer.

The advantage of the sludge washout method without heat treatment is a very attractive result, since the method is much simpler and cheaper to carry out on an industrial scale: it does not require additional energy, because it dismisses high temperatures. Furthermore, by applying an appropriate dilution rate, the sludge washout approach can be used as a permanent method to enrich cultures with H2-producing bacteria in a continuous bioreactor, while sludge boiling is a viable method at the laboratory-scale only.

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