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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 H_2-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 H_2 production rates (R_m) from the pretreated sludges were estimated by the modified Gompertz model. Compared to the control, H_2 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 H_2 production, as compared to the heat and to the combined heat and washout pretreatments.

Keywords: mixed culture, heat pretreatment, sludge washout.

Pré-tratamento de lodo anaeróbio para o aumento da produção de bio-hidrogênio pela água residuária de processamento da mandioca

RESUMO. Foram comparados métodos para o enriquecimento de um lodo anaeróbio em bactérias produtoras de H_2 utilizando água residuária do processamento da mandioca como substrato. O lodo foi submetido a três pré-tratamentos: 1) pré-tratamento térmico a 98°C por 15 min., 2) pré-tratamento térmico seguido por lavagem do lodo em um Reator Contínuo de Mistura Completa, operado a uma vazão específica de alimentação (D) de 0,5 dia^{-1}, e 3) lavagem do lodo como único método de enriquecimento. Os lodos pré-tratados e o sem pré-tratamento foram utilizados como inóculo de reatores em batelada para verificar o volume e a composição do biogás gerado. As velocidades máximas de produção de H_2 (R_m) dos lodos foram estimadas pelo modelo de Gompertz modificado. Comparado ao controle (lodo sem tratamento), a produção de H_2 foi cerca de quatro vezes maior para o lodo submetido ao pré-tratamento térmico e para o lodo submetido ao tratamento térmico seguido de lavagem, e dez vezes maior para o lodo que sofreu apenas lavagem. Estes resultados demonstraram que a lavagem do lodo, como único método de pré-tratamento, foi o mais efetivo em termos de produção de H_2, quando comparado com o tratamento térmico e o tratamento térmico seguido da lavagem.

Palavras-chave: cultura mista, tratamento térmico, lavagem do lodo.

Introduction

Brazil is one of the largest world producers of cassava, whose processing for the production of flour and starch generates about 7 m^3 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 CH_4 and CO_2 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 H_2 instead of CH_4 and CO_2 (WANG et al., 2012, MOHANAKRISHNA; MOHAN, 2013). Hydrogen production is more advantageous, because H_2 combustion produces only water as byproduct, which classifies H_2 as a clean fuel (WANG; WAN, 2009;

DAS; VERZIROGLU, 2008; MOHANAKRISHNA; MOHAN, 2013). Fermentative H_2 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, H_2 production shares the earlier stage of biodigestion; i.e., the hydrolysis and acetogenesis steps (VALDEZ-VASQUEZ; POGGI-VARALDO, 2009). Therefore, successful H_2 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 H_2 consumption mediated by microorganisms that comprise the anaerobic sludge (LEANO; BABEL, 2012; ASSAWAMONGKHOLSIRI et al., 2013).

One of the most promising ways to avoid H_2-consuming reactions is to pretreat mixed cultures used as bioreactor inoculums. Such pretreatments should prevent or reduce competition between the H_2-producing and H_2-consuming bacteria (DAS; VERZIROGLU, 2008; LEANO; BABEL, 2012; ASSAWAMONGKHOLSIRI et al., 2013). Several methods have been described to enrich a mixed culture with H_2-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 H_2-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 ($\mu max > D$). 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 H_2-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 H_2-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 carbon source was produced under laboratory conditions, to standardize the Chemical Oxygen Demand (COD) concentration applied to the bioreactor. To produce wastewater from cassava, processing roots were peeled, washed, and cut into small pieces. Cassava was liquefied with water at a 1:1 (w w^{-1}) ratio, filtered, and settled for about 24h under refrigeration. The supernatant was removed, homogenized, and stored in 500 mL bottles in a freezer, at – 15°C. The COD was analyzed, and dilution was carried out if necessary, so that the initial COD would be around 5000 mg L^{-1}. The pH of the cassava wastewater was adjusted to 7.0 by addition of NaOH 1% (w v^{-1}). This wastewater used as substrate was enriched by addition of the following macro and micronutrients (mg L^{-1}): K_2HPO_4 63, Na_2CO_3 1000, $NaHCO_3$ 1465, NH_4Cl 435, and 1 mL L^{-1} of a micronutrient solution with the formulation (mg L^{-1}): $FeSO_4.7H_2O$ 10000, $CaCl_2.2H_2O$ 2000, $ZnSO_4.7H_2O$ 2200, $MnSO_4.4H_2O$ 500, $CuSO_4.5H_2O$ 1000, $(NH_4)_6Mo_7O_{24}.4H_2O$ 100, and $Na_2B_4O_7.10H_2O$ 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 H_2-producing bacteria were heat treatment and sludge washout, used alone and in combination. Heat treatment of the inoculum was performed in a 2L glass reactor. One liter of seed sludge containing 21.9 and 15.4 g L^{-1} of TSS and VSS, respectively, was added to the reactor containing 1 L of cassava processing wastewater with COD equal to 5000 mg L^{-1}. The reactor was placed in a bath consisting of boiling water for 15 min., to ensure a temperature of 98ºC inside the reactor. Selective washout was conducted for the heat-pretreated sludge and seed sludge without pretreatment 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 48h 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).

$$\frac{dX}{dt} = \mu \max X - DX \quad \text{or} \quad X = Xi \,.\, e^{(\mu max - D)t} \tag{1}$$

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 H_2 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).

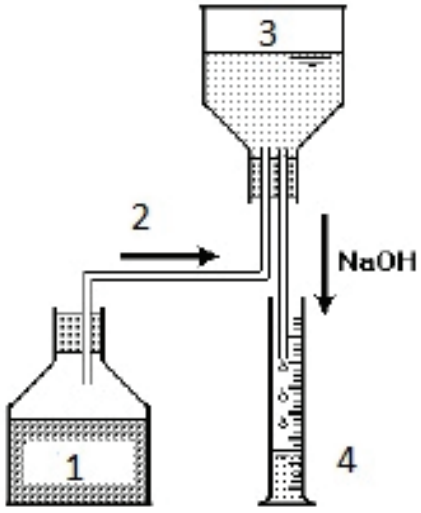

Figure 1. Batch bioreactor for H_2 production and outline of the system employed for gas capture: 1- bioreactor, 2- gas pipeline, 3- bottle containing 5% NaOH solution displaced by the generated gas, 4- NaOH uptake due to the volume of produced gas (modified from AQUINO et al., 2007).

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

$$V = \frac{(Patm - \rho.H.g).V\exp.T}{Patm.T\exp} \tag{2}$$

where:

V = volume of biogas at NCTP, P_{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_{exp} = volume of NaOH 5 % that was displaced by the generated gas,

T = temperature (K) NCTP,

T_{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 42h 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 5A 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),

$$H = A\exp\left\{-\exp\left[\frac{R_m}{A}(\lambda - t)+1\right]\right\} \quad (3)$$

where:

H (mL) is the cumulative volume of hydrogen production or the amount of H_2 produced at incubation time (t),

λ (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 (μmax) of the remaining microorganisms (H_2-producing bacteria) in the bioreactors. As shown in Figures 2a and 2b, the μmax 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 μmax 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 μmax 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 μmax of 0.172 h^{-1} using sucrose as substrate. The lower μmax 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.

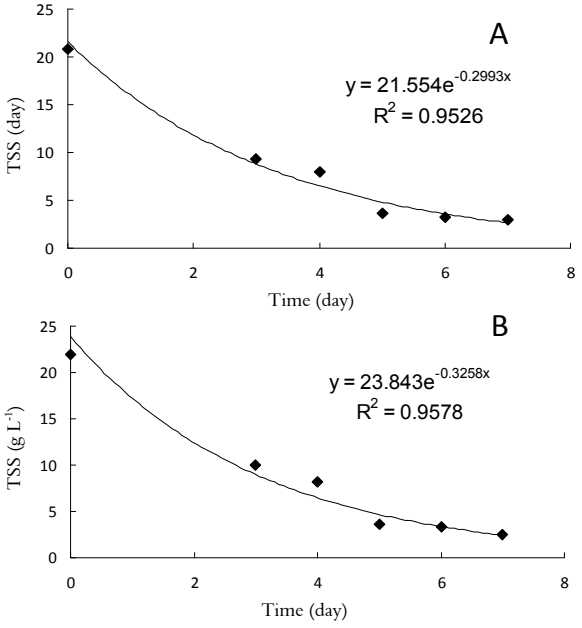

Figure 2. TSS concentrations during washout of the seed sludge (A) and the heat-pretreated sludge (B).

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.

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.

time	R1	R2	R3	R4
0h	5644	5480	6349	6245
42h	4984	5205	5952	5909
COD consumed	660	275	397	336

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 H_2 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 H_2 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 H_2 volume produced in each reactor. R4, inoculated with the sludge submitted to washout only, led to higher H_2 production, reaching a total H_2 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 H_2-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 H_2-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.

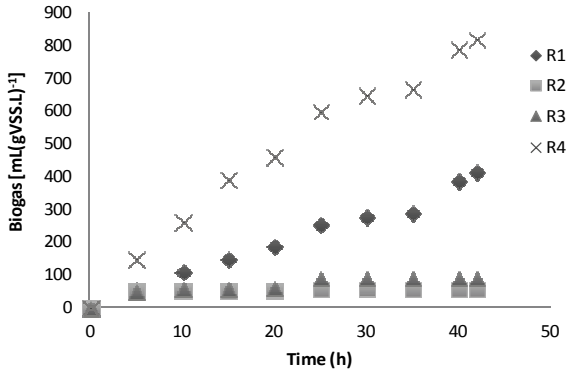

Figure 3. volume of total biogas production by the sludges submitted to different pretreatments. R1- control sludge, R2-heat-pretreated sludge, R3-heat-pretreated sludge submitted to washout, and R4-sludge submitted to washout only.

Although the D value used in this study was adequate to enrich the sludge with H_2-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 H_2 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 H_2-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 H_2-producing bacteria as compared with heat treatment. However, most literature studies aiming at H_2 production by mixed cultures from cassava derivatives have employed heat treatment as the sole method to enrich sludge with H_2-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 H_2 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 H_2 from enzymatic pretreated cassava wastewater.

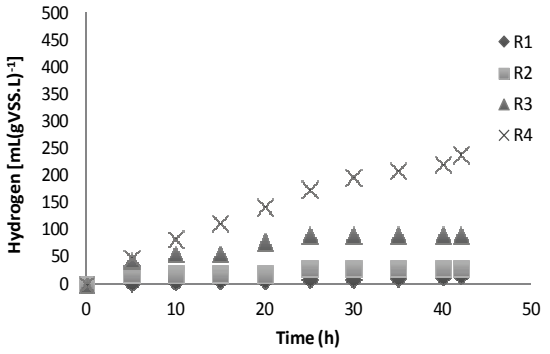

Figure 4. H_2 volume generated by the sludges submitted to different pretreatments. R1-control sludge, R2-heat-pretreated sludge, R3-heat-pretreated sludge submitted to washout, and R4-sludge submitted to washout only.

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 H_2 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 H_2 production. The aforementioned authors pointed out that applying chemical and heat pretreatment methods to mixed sludges have short-term effects on H_2 production only, and that the pretreatment methods will not affect H_2 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 H_2-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 H_2-producing bacteria. Indeed, R1 (control), which was inoculated with the sludge submitted to no pretreatment, produced only 4.2 mL of H_2. 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 H_2-producing cells. This could explain why R2 and R3, seeded with heat-treated sludge, produced a lower amount of H_2 as compared with R4, seeded with the sludge submitted to washout only. Heat treatment favors the selection of some spore-forming H_2-producing bacteria like *Clostridium*, but it also eliminates other non-spore forming H_2-producing bacteria that could contribute to H_2 production (LI; FANG, 2007).

The Gompertz model, the most widely used in the literature for modeling of biological H_2 production, was applied for estimation of the H_2 production rates and the kinetic parameters of H_2 production for the different sludges after pretreatement, using cassava processing wastewater as substrate (Table 2).

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).

Bioreactor	Kinetic parameters⋆			
	A (mL)	R_m (mL h^{-1})	λ (h)	r^2
R1	4.3	0.2	18.6	0.9729
R2	13.0	2.1	0.3	0.9169
R3	15.4	2.1	0.0	0.9711
R4	44.6	3.7	0.0	0.9962

⋆(A) Potential for H_2 production, (R_m) maximum H_2 production rate, and (λ) lag phase time calculated by the modified Gompertz model, Equation (3).

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 H_2, 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 H_2. In the present work, the highest A value was 44.6 mL of H_2, 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 H_2-producing bacteria using cassava wastewater as substrate and not optimize conditions for biohydrogen production. The low H_2 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

that is poor in nutrients; optimization of macro and trace elements concentration could lead to higher H_2 production.

Regardless of the volume of produced H_2, it was possible to compare the heat treatment and sludge washout methods to enrich of a mixed culture with H_2-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 H_2 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 H_2-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 H_2-producing bacteria. The differences in H_2 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 H_2 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 H_2-producing bacteria in a continuous bioreactor, while sludge boiling is a viable method at the laboratory-scale only.

Acknowledgements

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References

APHA-American Public Health Association. **Standard methods for the examination of water and wastewater**. 19th ed. Washington, D.C.: APHA, 1995.

AQUINO, S. F.; CHERNICHARO, C. A. L.; FORESTI, E.; DOS SANTOS, M. L. F.; MONTEGGIA, L. O. Metodologias para determinação da atividade metanogênica (AME) em lodos anaeróbios. **Engenharia Sanitária Ambiental**, v. 12, n. 2, p. 192-201, 2007.

ASSAWAMONGKHOLSIRI, T.; REUNGSANG, A.; PATTRA, S. Effect of acid, heat and combined acid-heat pretreatments of anaerobic sludge on hydrogen production by anaerobic mixed cultures. **International Journal of Hydrogen Energy**, v. 38, n. 14, p. 6146-6153, 2013.

CEREDA, M. P. Caracterização dos subprodutos da industrialização da mandioca. **Manejo, uso e tratamento de subprodutos da industrialização da mandioca**, v. 4, n.1, p. 13-37, 2001.

CHEN, C. C.; LIN, C. Y.; CHANG, J. S. Kinetics of hydrogen production with continuous anaerobic cultures utilizing sucrose as the limiting substrate. **Applied Microbiology and Biotechnology**, v. 57, n. 1-2, p. 56-64, 2001.

CHEN, W. M.; TSENG, Z. J.; LEE, K. S.; CHANG, J. S. Fermentative hydrogen production with *Clostridium butyricum* CGS5 isolated from anaerobic sewage sludge. **International Journal of Hydrogen Energy**, v. 30, n. 10, p. 1063-1070, 2005.

DAS, T.; VEZIROGLU, N. Advances in biological hydrogen production processes. **International Journal of Hydrogen Energy**, v. 33, n. 21, p. 6046-6057, 2008.

HAN, S. K.; SHIN, H. S. Biohydrogen production by anaerobic fermentation of food waste. **International Journal of Hydrogen Energy**, v. 29, n. 6, p. 569-577, 2004.

KAWAGOSHI, Y.; HINO, N.; FUJIMOTO, A.; NAKAO, M.; FUJITA, Y.; SUGIMURA, S.; FURUKAWA, K. Effect of inoculum conditioning on hydrogen fermentation, pH effect on bacterial community relevant to hydrogen production. **Journal of Bioscience Bioengineering**, v. 100, n. 5, p. 524-530, 2005.

KIELING, D.; REGINATTO, V.; SCHMIDELL, W.; TRAVERS, D.; MENES, R.; SOARES, H. Sludge wash-out as strategy for Anammox process start-up. **Process Biochemistry**, v. 42, n. 12, p. 1579-1585, 2007.

KIM, J. O.; KIM, Y. H.; YEOM, S. H.; SONG, B. K.; KIM, I. H. Enhancing continuous hydrogen gas production by the addition of nitrate into an anaerobic reactor. **Process Biochemistry**, v. 41, n. 5, p. 1208-1212, 2006.

LEANO, E. P.; BABEL, S. Effects of pretreatment methods on cassava wastewater for biohydrogen production optimization. **Renewable Energy**, v. 39, n. 1, p. 339-346, 2012.

LEE, K. S.; HSU, Y. F.; LO, Y. C.; LIN, P. J.; LIN, C. Y.; CHANG, J. S. Exploring optimal environmental factors for fermentative hydrogen production from starch using mixed anaerobic microflora. **International Journal of Hydrogen Energy**, v. 33, n. 5, p. 1565-1572, 2008.

LI, C.; FANG, H. P. Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. **Critical Review in Environmental Science and Technology**, v. 37, n. 1, p. 1-39, 2007.

LUO, G.; XIE, L.; ZOU, Z.; WANG, W.; ZHOU, Q. Evaluation of pretreatment methods on mixed inoculum for both batch and continuous thermophilic biohydrogen production from cassava stillage. **Bioresource Technology**, v. 101, n. 3, p. 959-964, 2010.

MATHEWS, J.; WANG, G. Y. Metabolic pathway engineering for enhanced biohydrogen production. **International Journal of Hydrogen Energy**, v. 34, n. 17, p. 7404-7416, 2009.

MOHAN, S. V. Fermentative hydrogen production with simultaneous wastewater treatment: influence of pretreatment and system operating conditions. **Journal of Scientific and Industrial Research**, v. 67, n. 11, p. 950-961, 2008.

MOHANAKRISHNA, G.; MOHAN, S. V. Multiple process integrations for broad perspective analysis of fermentative H_2 production from wastewater treatment: Technical and environmental considerations. **Applied Energy**, v. 107, p. 244-254, 2013.

OLIVEIRA, M. A.; REIS, E. M.; NOZAKI, J. Biological treatment of wastewater from the cassava meal industry. **Environmental Research**, v. 85, n. 2, p. 177-183, 2001.

REN, N.; WANG, A.; CAO, G.; XU, J.; GAO, L. Bioconversion of lignocellulosic biomass to hydrogen: Potential and challenges. **Biotechnology Advances**, v. 27, n. 6, p. 1051-1060, 2009.

SREETHAWONG, T.; CHATSIRIWATANA, S.; RANGSUNVIGIT, P.; CHAVADEJ, S. Hydrogen production from cassava wastewater using an anaerobic sequencing batch reactor: Effects of operational parameters, COD:N ratio, and organic acid composition. **International Journal of Hydrogen Energy**, v. 35, n. 9, p. 4092-4102, 2010.

SU, H.; CHENG, J.; ZHOU, J.; SONG, W.; CEN, K. Improving hydrogen production from cassava starch by combination of dark and photo fermentation. **International Journal of Hydrogen Energy**, v. 34, n. 4, p. 1780-1786, 2009.

VALDEZ-VAZQUEZ, H. M.; POGGI-VARALDO, M. Hydrogen production by fermentative consortia. **Renewable and Sustainable Energy Reviews**, v. 13, n. 5, p. 1000-1013, 2009.

WANG, C. H.; CHANG, J. S. Continuous biohydrogen production from starch with granulated mixed bacterial microflora. **Energy and Fuels**, v. 22, p. 93-97, 2008.

WANG, J. L.; WAN, W. Factors influencing fermentative hydrogen production: A review. **International Journal of Hydrogen Energy**, v. 34, n. 2, p. 799-811, 2009.

WANG, W.; XIE, L.; LUO, G.; ZHOU, Q.; LU, Q. Optimization of biohydrogen and methane recovery within a cassava ethanol wastewater/waste integrated management system. **Bioresource Technology**, v. 120, p. 165-172, 2012.

WU, X.; WANG, X.; YANG, H.; GUO, L. A comparison of hydrogen production among three photosynthetic bacterial strains. **International Journal of Hydrogen Energy**, v. 35, n. 13, p. 7194-7199, 2010.

ZONG, W.; YU, R.; ZHANG, P.; FAN, M.; ZHOU, Z. Efficient hydrogen gas production from cassava and food waste by a two-step process of dark fermentation and photo-fermentation. **Biomass & Bioenergy**, v. 33, n. 10, p. 1458-1463, 2009.

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