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Strategies for Increasing the Biohydrogen Yield in Anaerobic Fermentation of Xylose

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Abstract

The pretreatment of lignocellulosic materials to obtain cellulose generates a residual stream with hemicellulosic composition, mainly containing xylose. This C5 fraction is not directly fermentable by microorganisms traditionally used to produce ethanol. Hence, more promising alternatives for the C5 fraction have been studied, and acidogenic fermentation proves to be an attractive option for the production of biohydrogen, due to the possibility of using hemicellulose fractions and mixed anaerobic cultures. To reduce the activity of hydrogen-consuming microorganisms when mixed cultures are employed as inoculum to produce hydrogen by anaerobic fermentation, thermal pretreatment was selected. However, such pretreatment method also affects the activity of hydrogen-producing acidogenic bacteria, and strategies should be studied to enrich the inoculum for these bacteria and to increase hydrogen yields. Thus, this study evaluated the effect of some strategies on the biohydrogen production from xylose. The strategies adopted were thermal pretreatment of the sludge, maintenance of the incubation temperature at 35 °C, adaptation of the sludge by successive contacts with the xylose solution, and increasing inoculum to substrate ratio (I/S) from 1 to 2. This approach improved hydrogen yield approximately 30 times, from 0.03 to 0.93 mmol H_2/mmol xylose. However, this yield was only 56% of the theoretical value and can still be improved.

Keywords: anaerobic fermentation, hemicellulose, hydrogen, mixed culture, pretreatment, xylose

1. Introduction

The constant demand for energy and the unsustainable use of fossil fuels with their environmental problems have influenced the need to explore sustainable resources, clean sources, and lower costs. Considering these issues, the use of alternative energy produced from renewable sources becomes promising, with emphasis on hydrogen, considered the fuel of the future (Ghimire et al., 2015). From the environmental point of view, the challenge of using hydrogen as an energy vector lies in its sustainable production, which becomes attractive when combined with low-energy biological processes and the use of agricultural (Ghimire et al., 2015) and agro-industrial wastes (Urbaniec & Bakker, 2015). These materials have been widely studied as raw materials with high energy potential and value added, due to their abundance and low or no cost, thus contributing to waste management (Ratti, Delforno, Sakamoto, & Varesche, 2015).

The process of producing ethanol from sugarcane generates considerable amounts of lignocellulosic residues, such as straw and bagasse. For each ton of processed sugarcane, about 140 kg of straw are generated on a dry basis (Oliveira et al., 2013). This lignocellulosic biomass has a high energy value, and second-generation ethanol plants (2G ethanol) already use sugarcane straw and bagasse on an industrial scale to produce biofuel. However, the pretreatment of lignocellulosic materials to obtain cellulose generates a residual material with hemicellulosic composition (Sarkar, Ghosh, Bannerjee, & Aikat, 2012).

Hemicellulose, unlike cellulose, is an heterogenous, branched, and amorphous polysaccharide composed mainly of xylose (Zheng, Zhao, Xu, & Li, 2014). This hemicellulose fraction (denominated fraction C5) is not directly fermentable by microorganisms traditionally used to produce ethanol such as sucrose (sugarcane juice) or

glucose (from lignocellulosic biomass). Microorganisms with such capacity have a longer ethanol production time, making the process, which uses this fraction as a substrate, not competitive compared to the conventional process already consolidated (Sarkar et al., 2012). Thus, better alternatives for the use of the C5 fraction have been studied, such as the production of biohydrogen by anaerobic fermentation (De Sá et al., 2013; Maintinguer, Sakamoto, Adorno, & Varesche, 2015). A schematic representation of the hemicellulose fraction from sugarcane straw hydrothermal pretreatment and its conversion to H_2 via anaerobic fermentation, integrated into the production of 2G ethanol from the cellulose fraction, is shown in Figure 1.

Anaerobic fermentation proves to be an attractive option to produce biohydrogen, due to the possibility of using hemicellulose fractions and mixed anaerobic cultures. In addition to the flexibility of operating temperatures and high biohydrogen production rates, the use of mixed cultures is more advantageous when complex substrates are used (Shanmugam, Chaganti, Lalman, & Heath, 2014; Maintinguer et al., 2015). However, the main concern in the use of mixed anaerobic cultures is the loss of hydrogen produced due to hydrogen-consuming microorganisms (homoacetogenic bacteria, hydrogenotrophic methanogenic archaea, and sulfate reducing bacteria). To control this problem, several inoculum pretreatment methods can be used to select and enrich the inoculum in hydrogen-producing microorganisms. Pretreatment methods can be used to reduce the activity of H_2-consuming microorganisms when mixed cultures are employed as inoculum (Shanmugam et al., 2014).

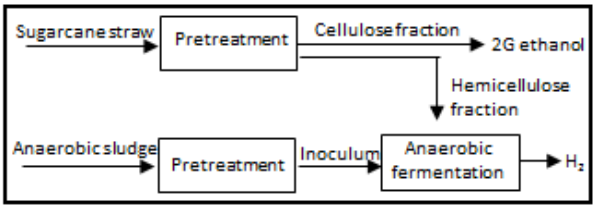

Figure 1. Integrated production of H_2 and 2G ethanol from sugarcane straw

However, such pretreatment methods also affect the activity of H_2-producing acidogenic bacteria, and it is necessary to study strategies to enrich the inoculum in these bacteria and to increase H_2 yields. Several factors influence hydrogen production, such as inoculum pretreatment method, size, and type, substrate concentration and type, inorganic nutrients concentration, temperature, initial pH, and operational conditions of the bioreactors (Wang, & Wan, 2009). Two factors that contribute to the enrichment of the initial inoculum in hydrogen producing microorganisms and consequently to the increased hydrogen yield are detailed as follows: the adaptation of the mixed culture by successive contacts with the substrate, and inoculum concentration or inoculum-substrate ratio (I/S ratio).

The concept of eco-biotechnology that employs mixed cultures to obtain bioproducts in a sustainable way is based on natural selection and competition rather than genetics or metabolic engineering. The microbial composition of an inoculum can be enriched in hydrogen producing microorganisms by selective pressure applied to the inoculum, choosing the appropriate substrate and operating conditions. The chemical and biological changes taking place in an anaerobic reactor adapting to a synthetic substrate, such as an increase in volatile suspended solids content and enzyme activities (Kleerebezem & van Loosdrecht, 2007). Repeated batch cultivation is widely used to control the feed rate of nutrients and optimize the productivity of microbial cultures. In addition, this method has the operational advantages of avoiding variation of the inoculum and thus keeping the microorganisms at high rates of growth. In this type of culture, the initial inoculum is subjected to successive contacts with the culture medium under specific incubation conditions. At regular intervals, the culture medium is removed and replaced with an equal amount of fresh medium, a part of the culture medium or the biomass being maintained in the reactor as inoculum (Liu, Zeng, & Angelidaki, 2008). Several researchers have been using this method to increase hydrogen production by mixed cultures (Liu, Zeng, & Angelidaki, 2008; Sivagurunathan, Sen, & Lin, 2014; Wong et al., 2019).

Concentrations of inoculum and substrate are especially important in anaerobic fermentation. As the isolated effect of the inoculum or substrate concentrations in the fermentation has its limits, the inoculum-substrate ratio (I/S) is more adequate. The effect of inoculum-substrate ratio (I/S) has been studied more frequently on methane production, being recommended an I/S greater than or equal to four for easily degradable substrates, where rapid accumulation of volatile fatty acids could lead to inhibition problems, and an I/S less than or equal to one for poorly degradable substrates (Holliger et al., 2016). Considering that hydrogen production is an intermediate

stage of anaerobic fermentation, it is expected that lower values of I/S ratio (e.g. 1 or 2), using carbohydrate-rich substrates, will contribute to higher hydrogen yields. The few studies that report the influence of the inoculum-substrate ratio (I/S) on fermentative hydrogen production show increased hydrogen yield by increasing the sludge addition (Argun & Dao, 2017) or decreasing the I/S ratio (Sangyoka, Reungsang, & Lin, 2016; Sun et al., 2011).

Thus, the goal of the study is to evaluate the effect of three cultivation strategies to increase the biological production of hydrogen from xylose, namely: successive contacts of the mixed culture pretreated with the substrate, gradual increase of temperature and increase of inoculum concentration or the I/S ratio.

2. Method

2.1 Pretreatment and Characterization of the C5 Fraction

The C5 fraction was obtained from the hydrothermal pretreatment of 1 kg sugarcane straw and 10 L water at 196 °C and 31 bar for 10 min. The liquid fraction obtained after vacuum filtration, designated C5 fraction, was stored under freezing for further characterization and fermentation. It was characterized (Table 1) by standard methods (APHA, 2012) and analyzed for carbohydrate, acetic acid, furfural, and hydroxymethylfurfural (HMF) content in HPLC. To estimate total carbohydrates, the C5 fraction underwent hydrolysis with 72% H_2SO_4 (v/v) at 121 °C for 1 h to convert the oligosaccharides into free monosaccharides (Sluiter et al., 2008).

2.2 Origin and Pretreatment of Inoculum

The sludge used as inoculum was collected in an anaerobic reactor, in operation at a brewery industry, characterized in terms of volatile suspended solids (61.8 g VSS/L). The sludge was subjected to alkaline (pH adjustment to 12 with 5M NaOH and maintenance for 3 h), acidic (pH adjustment to 2 with 10M HCl and maintenance for 2 h), and thermal (maintenance at 100 °C for 1 h) pretreatments. In the acclimatization pretreatment, the sludge was submitted to the same conditions of alkaline, acid, or thermal pretreatment, followed by a 24 h acclimation period at room temperature, with pH adjusted to 7.

2.3 Evaluation of Inoculum Pretreatment Efficiency

The efficiency of the pretreatments applied to the inoculum was evaluated by H_2 production in experiments with five replicates carried out in 50 mL penicillin flasks, containing 45 mL of a mixture of synthetic xylose solution and pretreated sludge or raw sludge (control). The xylose solution was prepared at a concentration close to that found in the hydrolysate of the hydrothermal pretreatment of sugarcane straw (9.4 g/L) and had 10 g/L of soluble chemical oxygen demand (COD), macronutrients in a proportion COD: N: P of 350: 5: 1 and micronutrients (De Sá et al., 2013). The mass of inoculum added was calculated for an initial inoculum/substrate ratio (I/S) of 1 (g VSS/g COD). The pH of the mixture was adjusted to 5.5 ± 0.1 and the flasks incubated at 35 °C for 24 h.

2.4 Evaluation of Biohydrogen Production at Different Incubation Temperatures

The experiments were conducted in 50 mL penicillin flasks, containing 45 mL of a mixture of synthetic xylose solution and pretreated sludge or raw sludge (control). The sludge used as inoculum was subjected to thermal pretreatment (maintenance at 100 °C for 1 h). The xylose solution was prepared and supplemented with macro and micronutrients as described in section 2.3. The mass of inoculum added was calculated for an initial inoculum/substrate ratio (I/S) of 1 (g VSS/g COD). The pH of the mixture was adjusted to 5.5 ± 0.1 and the flasks incubated at 35, 40, or 45 °C.

Adaptation of the sludge at different incubation temperatures was conducted by subjecting the same initial inoculum to successive exchanges of supplemented synthetic solution every 24 h and gradual increase of the incubation temperature. Fermentative media samples were collected at 24 h and analyzed to quantify xylose. Samples of the biogas produced were collected in 24 h and analyzed for H_2, CO_2, and CH_4 contents.

2.5 Evaluation of Biohydrogen Production at Different Inoculum/Substrate Ratios

In this study, the mass of inoculum added was calculated for an initial inoculum/substrate ratio (I/S) of 1 or 2 (g VSS/g COD). The experiments were conducted in 50 mL penicillin flasks, containing 45 mL of a mixture of synthetic xylose solution and pretreated sludge or raw sludge (control). The sludge used as inoculum was subjected to thermal pretreatment (maintenance at 100 °C for 1 h). The xylose solution was prepared and supplemented with macro and micronutrients as described in section 2.3. The pH of the mixture was adjusted to 5.5 ± 0.1, and the flasks incubated at 35 °C.

Adaptation of the sludge at different I/S ratios was conducted by subjecting the same initial inoculum to successive exchanges of supplemented synthetic solution every 72 h. Fermentative media samples were collected

at 72 h and analyzed to quantify organic acids and xylose. Samples of the biogas produced were collected in 24 h, 48 h, and 72 h and analyzed for H_2, CO_2, and CH_4 contents.

2.6 Analytical Methods

The C5 fraction and samples collected from the liquid phase were analyzed by high-performance liquid chromatography (HPLC *Shimadzu* LC 10 AT, Kyoto, Japan) to determine carbohydrates, volatile fatty acids, HMF, and furfural. The chromatograph had a UV-VIS detector (model SPD-10AV) connected in series with a refractive index detector (model RID-10A). The liquid samples were centrifuged at 3500 rpm for 5 min, and the supernatants were filtered through a 0.22 μm Millipore filter. The analytes were determined using an Aminex HPX-87H column (BioRad) and a deashing guard cartridge (BioRad). The column temperature was 55 °C or 65 °C (for C5 fraction), and the mobile phase was 5 mM H_2SO_4 with a flow rate of 0.6 mL/min. Carbohydrates were analyzed using a refractive index detector (RI), while volatile fatty acids, HMF, and furfural were analyzed by UV-Vis detection at 210 nm (De Sá, Moutta, Bon, Cammarota & Ferreira-Leitão, 2015).

Analysis of H_2, CO_2, and CH_4 was performed using a gas chromatograph *Shimadzu* (GC-17 A) with thermal conductivity detector (TCD) and flame ionization detector (FID). The chromatographic column used was PoraPLOT U (27.5 m × 0.53 mm × 20 μm) at 30 °C. The temperature of the injector and detectors FID and TCD was 230, 250, 230 °C, respectively. Nitrogen was used as the entrainment gas. To determine the number of H_2 moles for each sample, the quantification of gases was carried out with the aid of the ideal gas equation. Other parameters such as COD, VSS, and phosphorus were measured according to Standard Methods for the Examination of Water and Wastewater (APHA, 2012).

3. Results and Discussion

3.1 Characterization of the C5 Fraction

The chemical composition (Table 1) of hemicellulose fraction obtained from sugarcane straw is mostly pentoses sugars, with xylose the main component (60% of the total composition). However, about 67% of xylose (and 65% of total sugars) is in the form of oligosaccharides that are not directly assimilated by acidogenic fermentative bacteria. Therefore, hydrolytic bacteria are needed to hydrolyze polymeric carbohydrates in monosaccharides (Nasr et al., 2014). A 94% conversion efficiency of polymeric sugars, using experiments in batches, for monomeric sugars from different partially hydrolyzed corn cob streams was observed by Nasr et al. (2014). Both pentose and hexose sugars present in the studied hydrolyzate are a rich source of organic matter and can be assimilated by the acidogenic bacteria to produce H_2, CO_2, and various metabolites (such as acetic acid and butyric acid).

Table 1. Concentration of total and free sugars, inhibitors, and nutrients in the C5 fraction

Compound	Unit	Value
Xylose	g/L	3.80 (11.60)
Arabinose	g/L	0.80 (0.67)
Glucose	g/L	0.12 (1.70)
Galactose	g/L	0.35 (0.48)
Acetic acid	g/L	3.60
Furfural	g/L	0.34
Hydroxymethylfurfural	g/L	0.10
pH	--	3.5
Total Chemical Oxygen Demand	g/L	24.7
Soluble Chemical Oxygen Demand	g/L	9.5
Volatile acids	g/L	3.2
Polyphenols	g/L	2.6
Sulfate	mg/L	100
Phenols	mg/L	12
Total phosphorus	mg/L	108
Soluble phosphorus	mg/L	61
Ammoniacal nitrogen	mg/L	14

Note. values in parentheses obtained after acidic hydrolysis of the liquid fraction with sulfuric acid.

In the pretreatment of lignocellulosic biomasses, in addition to carbohydrates, by-products, such as furfural and HMF, are also generated. According to the literature they may inhibit the fermentation process by affecting cell growth or lead to a shift of H_2-producing metabolic pathways (e.g., acetate and butyrate) to non-H_2 producing

pathways (e.g., ethanol and propionate) (Monlau et al., 2014). However, the inhibition favored by these compounds is associated with their concentrations in the fermentative medium, and from 1 g/L of furfural and HMF, the inhibitory effects may be significant in the cell growth and production of H_2 (Cao et al., 2010). According to Nars et al. (2014), HMF in the range of 0.05–0.14 g/L did not impact production and H_2 yield, as well as furfural concentration of 0.21–1.09 g/L had no discernible impact on H_2 production and yield. The values found in this study from the C5 fraction obtained after the thermal pretreatment were below the values considered as inhibitory for anaerobic fermentation.

As for the acetic acid concentration in the studied hemicellulose fraction (23% of the total), hydrogen-producing bacteria could be tolerant to this component in the fermentation medium, since it is one of the main metabolites generated in acidogenic fermentations (De Sá et al., 2015; Ghimire et al., 2015).

Note that for a biological process to operate satisfactorily, the inorganic nutrients need to be available in amounts sufficient for microbial growth. Thus, the C5 fraction obtained in this study showed sufficient concentrations of phosphorus and sulfur macronutrients, without the need to supplement the fermentation medium. However, for an ideal COD: N: P (350: 5: 1) ratio, nitrogen would be insufficient, requiring the supplementation of this macronutrient (Table 1).

According to the characterization, high concentrations of phenolic compounds were identified in the hemicellulose fraction. These compounds are derived from a small amount of lignin degrading during the pretreatment of the biomass. Phenols are compounds of environmental significance because of their high toxic potential (Liang & Fang, 2010). They are present in wastewater from refineries, chemical industries, and many other activities. A number of studies have reported different concentrations tolerated by anaerobic microorganisms, ranging from 630 to 1260 mg/L (Fang, Liang, Zhang, & Liu, 2006) or 3700 mg/L (Bajaj, Gallert, & Winter, 2009).

3.2 Evaluation of Inoculum Pretreatment Efficiency

The pretreatment methods exert a strong influence on the microorganisms in the sludge, favoring the selection of different species in each condition (Wang & Yin, 2017) and consequently the production of biohydrogen. The sludge obtained after thermal pretreatment promoted higher biogas and H_2 production, than raw sludge, without pretreatment, with biogas (39.6 mL) and H_2 (16.09 mL) volumes two and ten times, respectively, greater than those obtained with the raw sludge. In the other pretreatments, except for the alkali treatment followed by acclimatization, the biogas and H_2 production was less than with the raw sludge. Thus, the hydrolytic and acidogenic fermentative bacteria that remained active after thermal pretreatment probably made better use of the xylose (Figure 2). The pH of the H_2 production media had been reduced from 5.5 to 4.1–5.2, remaining in the ideal range for acidogenic fermentative bacteria in trials with inoculum from alkaline and thermal pretreatment (4.6–4.8).

Although the xylose consumption was not evaluated at this step of the study, the amount of xylose initially available for the acidogenic fermentative bacteria (3 mmol) could generate 5 to 10 mmol of H_2, considering theoretical yields from xylose with butyric acid and acetic acid as the byproduct for mesophilic microorganisms of 1.67 and 3.33 mmol H_2/mmol xylose, respectively (Abdul et al., 2013). For example, Maintinguer et al. (2015) reported xylose consumption of 63.5% at 72 h of fermentation with H_2 yield of 0.3 mmol H_2/mmol xylose, below the theoretical at 37 °C, pH 5.5 and sediment of a water reservoir as inoculum. In contrast, Lin & Cheng (2006) obtained hydrogen yield of 1.92–2.25 mmol H_2/mmol xylose at 35 °C, pH 6-7 and with sewage sludge microflora as inoculum. In this study, the quantification of H_2 produced by the thermally pretreated inoculum in 24 h of fermentation, 0.64 mmol, also had a low H_2 yield probably because the acidogenic bacteria produced H_2 and butyrate or other metabolites that decreased the yields of H_2 (for example, ethanol and propionate).

Many studies in the literature employ hexoses and pentoses as substrates for pure cultures of acidogenic bacteria. Although some research has used mixed cultures for xylose consumption, there is still not much information about the production of hydrogen from the hemicellulose fraction, as well as about the effect of the capacity of the hydrolytic bacteria, present in the inoculum, on H_2 yields.

Although the xylose of the hemicellulose fraction of the sugarcane straw is an attractive raw material to produce H_2 by the anaerobic fermentation, the anaerobic sludge in acidogenic bacteria needs to be enriched to obtain higher H_2 yields. One way to accomplish this is through the adaptation of acidogenic bacteria to xylose (through successive contacts of the mixed culture pretreated with the substrate), a gradual increase of temperature, and the increase of inoculum concentration (increase in the I/S ratio).

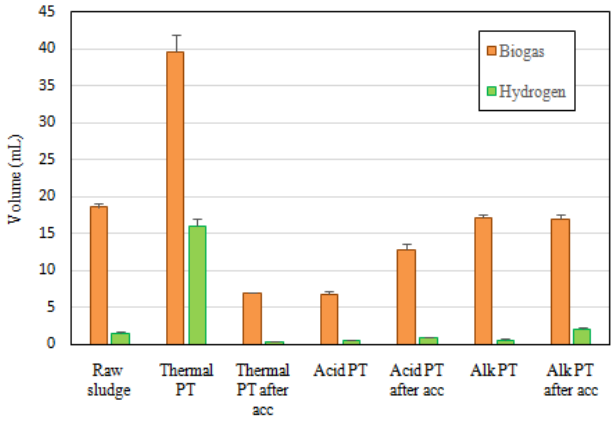

Figure 2. Biogas and H_2 volumes produced with inoculum from different pretreatments. PT = pretreatment, Alk = alkaline, acc = pretreatment followed by a 24 h acclimation period at room temperature. Average and standard deviation of five replicates

3.3 Evaluation of Biohydrogen Production

The data obtained in anaerobic fermentations conducted for 24 h with gradual increase of the incubation temperature indicate that a longer incubation time is needed for the full utilization of xylose and higher H_2 yield (Figure 3). At 35 and 40 °C, the sludge submitted to a second contact with the xylose solution obtained a higher H_2 yield, probably due to the increase of the acidogenic population in the sludge. At 45 °C, however, a decline in H_2 yield was observed in the three sludge contacts with the xylose solution, indicating that the higher temperature did not favor the increase of the population of acidogenic bacteria and even inhibited the already adapted bacteria at lower temperatures. The results indicated the temperature of 35 °C as the best for the next experiments.

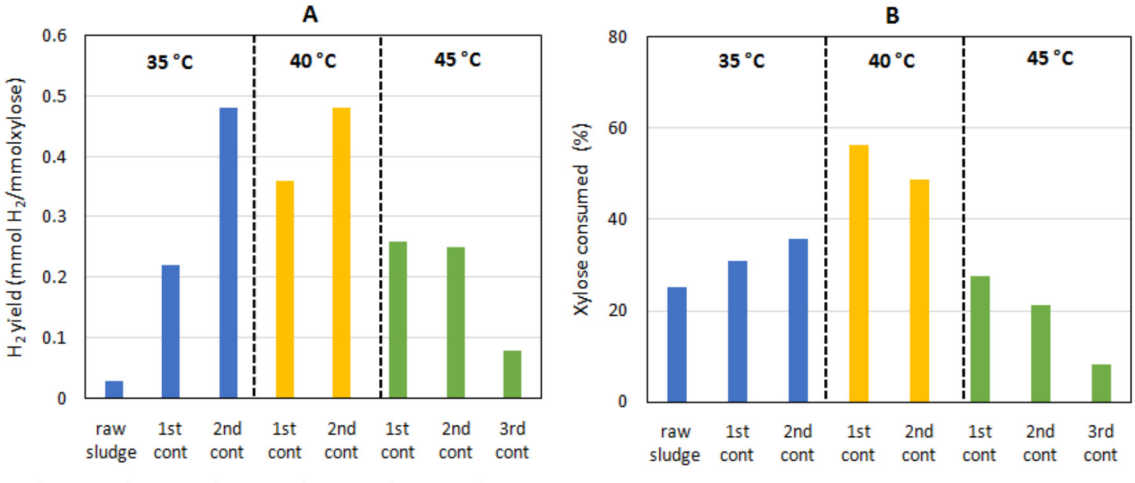

Figure 3. H_2 yield (A) and percentage of xylose consumed (B) in fermentations of 24 h with increase of temperature and successive contacts with xylose solution (9.4 g/L) supplemented with macro and micronutrients, and pH adjusted to 5.5. Initial I/S ratio 1 g VSS/g COD. Dotted lines separate the experiments conducted at 35, 40 or 45 °C

Figure 4A illustrates that the strategies of thermal pretreatment, increase of the ratio I/S, and sludge adaptation are correct, because these considerably improved H_2 yield compared to sludge without treatment (control) at 35 °C. In the condition of thermal pretreatment, I/S 2, 3rd contact, and 35 °C, the total consumption of xylose, production of 2.82 mmol H_2, and yield of 0.93 mmol H_2/mmol xylose was obtained in 72 h of fermentation.

The intermediate metabolites formed during the anaerobic fermentation were acetic, butyric, and propionic acids, with the butyric acid present in the highest concentration in both conditions used I/S 1 or I/S 2. These data

(Figure 4B) indicate that butyric type acidogenesis was the dominant route in fermentation. Hydrogen yields would reach higher values, if the anaerobic fermentation was of the acetic acid type. Although the H_2 yield increased 30-fold: from 0.03 (with crude sludge at 35 °C) to 0.93 mmol H_2/mmol xylose (best result obtained for pretreated sludge), the value obtained is still below theoretical (1.67 mmol H_2/mmol xylose with butyric acid as by-product). These results may be related to the need for longer adaptation time of the sludge, which would provide better selection of the acidogenic populations and consequent enrichment of the medium with such microorganisms. Yields below the theoretical value were also reported by other authors when the major metabolic pathway was not acetic acid type, as 1.24 mmol H_2/mmol pentoses (Abdul et al., 2013) and 0.80 mmol H_2/mmol xylose (Zhao, Karakashev, Lu, Wang & Angelidaki, 2010). It should be noted that no methane production occurred in the sludge thermally pretreated in all the evaluated tests, indicating that the thermal pretreatment was efficient to inhibit the methanogenic archaea and feasible for the selection of acidogenic bacteria.

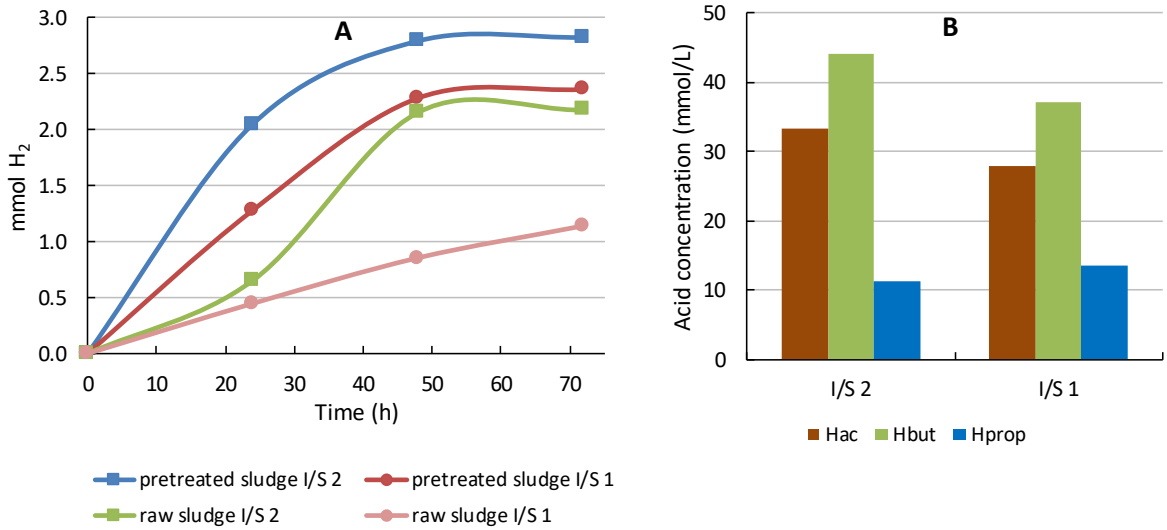

Figure 4. H_2 production during fermentation (A) and acid concentrations in 72 h of fermentation (B) with different I/S ratio and three successive contacts of the pretreated inoculum with xylose solution at 35 °C

4. Conclusion

The results of the present research indicate that the C5 fraction obtained from the thermal pretreatment of sugarcane straw is an attractive raw material to produce H_2 by anaerobic fermentation. In addition to xylose as the major sugar, it presented low concentrations of possible inhibitory compounds (furfural and HMF). A better understanding of the effects of the 65% of total sugars in the form of oligosaccharides on the potential of hydrogen production can be obtained from the processes of hydrolysis and acidogenic fermentation by mixed cultures.

Considering the conditions tested, inoculum from thermal pretreatment was the best option for H_2 production providing a H_2 volume 10 times higher than raw sludge, without pretreatment. In the fermentations with thermally pretreated sludge and xylose as the only substrate, higher H_2 yields were obtained after successive contacts of the sludge with the substrate at 35 °C, I/S ratio of 2, and initial pH 5.5. The strategies adopted improved the H_2 yield by approximately 30 times. However, this yield was only 56% of the theoretical value and can still be improved.

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References

Abdul, P. M., Jahim, J. M., Harun, S., Markom, M., Hassan, O., Mohammad, A. W., & Asis, A. J. (2013). Biohydrogen production from pentose-rich oil palm empty fruit bunch molasses: A first trial. *International Journal of Hydrogen Energy, 38*, 15693-15699. https://doi.org/10.1016/j.ijhydene.2013.05.050

APHA. American Public Health Association. (2012). *Standard methods for examination of water and wastewater*. Washington, DC: American Public Health Association.

Argun, H., & Dao, S. (2017). Bio-hydrogen production from waste peach pulp by dark fermentation: Effect of inoculum addition. *International Journal of Hydrogen Energy, 42*, 2569-2574. https://doi.org/10.1016/j.ijhydene.2016.06.225

Bajaj, M., Gallert, C., & Winter, J. (2009). Treatment of phenolic wastewater in an anaerobic fixed bed reactor (AFBR) – recovery after shock loading. *Journal of Hazardous Materials, 162*, 1330-1339. https://doi:10.1016/j.jhazmat.2008.06.027

Cao, G. L., Ren, N. Q., Wang, A. J., Guo, W. Q., Xu, J. F., & Liu, B. F. (2010). Effect of lignocellulose-derived inhibitors on growth and hydrogen production by *Thermoanaerobacterium thermosaccharolyticum* W16. *International Journal of Hydrogen Energy, 35*, 13475-13480. https://doi:10.1016/j.ijhydene.2009.11.127

De Sá, L. R. V., Cammarota, M. C., Oliveira, T. C., Oliveira, E. M. M., Matos, A., & Ferreira-Leitão, V. S. (2013). Pentoses, hexoses and glycerin as substrates for biohydrogen production: An approach for Brazilian biofuel integration. *International Journal of Hydrogen Energy, 38*, 2986-2997. https://doi.org/10.1016/j.ijhydene.2012.12.103

De Sá, L. R. V., Moutta, R. O., Bon, E. P. S., Cammarota, M. C., & Ferreira-Leitão, V. S. (2015). Fermentative biohydrogen production using hemicellulose fractions: Analytical validation for C5 and C6-sugars, acids and inhibitors by HPLC. *International Journal of Hydrogen Energy, 40*, 13888-13900. http://dx.doi.org/10.1016/j.ijhydene.2015.08.014

Fang, H. H. P., Liang, D. W., Zhang, T., & Liu, Y. (2006). Anaerobic treatment of phenol in wastewater under thermophilic condition. *Water Research, 40*, 427-434. https://doi.org/10.1016/j.watres.2005.11.025

Ghimire, A., Frunzo, L., Pirozzi, F., Trably, E., Escudie, R., Lens, P. N. L., & Esposito, G. (2015). A review on dark fermentative biohydrogen production from organic biomass: Process parameters and use of by-products. *Applied Energy, 144*, 73-95. http://dx.doi.org/10.1016/j.apenergy.2015.01.045

Holliger, C., Alves, M., Andrade, D., Angelidaki, I., Astals, S., Baier, U.,… & Wierinck, I. (2016). Towards a standardization of biomethane potential tests. *Water Science and Technology, 74*, 2515-2522. https://doi.org/10.2166/wst.2016.336

Kleerebezem, R., & van Loosdrecht, M. C. (2007). Mixed culture biotechnology for bioenergy production. *Current Opinion in Biotechnology, 18*, 207-212. https://doi.org/10.1016/j.copbio.2007.05.001

Liang, D., & Fang, H. H. P. (2010). Anaerobic treatment of phenolic wastewaters. In: Fang, Herbert H. P. Environmental anaerobic technology: applications and new developments (pp. 185-205). London, Imperial College Press.

Lin, C. Y., & Cheng, C. H. (2006). Fermentative hydrogen production from xylose using anaerobic mixed microflora. *International Journal of Hydrogen Energy, 31*, 832-840. https://doi.org/10.1016/j.ijhydene.2005.08.010

Liu, D. W., Zeng, R. J., & Angelidaki, I. (2008). Enrichment and adaptation of extreme-thermophilic (70 °C) hydrogen producing bacteria to organic household solid waste by repeated batch cultivation. *International Journal of Hydrogen Energy, 33*, 6492-6497. https://doi.org/10.1016/j.ijhydene.2008.08.014

Maintinguer, S. I., Sakamoto, I. K., Adorno, M. A. T., & Varesche, M. B. A. (2015). Bacterial diversity from environmental sample applied to bio-hydrogen production. *International Journal of Hydrogen Energy, 40*, 3180-3190. https://doi.org/10.1016/j.ijhydene.2014.12.118

Monlau, F., Sambusiti, C., Barakat, A., Quéméneur, M., Trably, E., Steyer, J-P., & Carrere, H. (2014). Do furanic and phenolic compounds of lignocellulosic and algae biomass hydrolyzate inhibit anaerobic mixed cultures? A comprehensive review. *Biotechnology Advances, 32*, 934-951. https://doi.org/10.1016/j.biotechadv.2014.04.007

Nasr, N., Gupta, M., Elbeshbishy, E., Hafez, H., El Naggar, M. H., & Nakhla, G. (2014). Biohydrogen production from pretreated corn cobs. *International Journal of Hydrogen Energy, 39*, 19921 19927. https://doi:dx.doi.org/10.1016/j.ijhydene.2014.10.004

Oliveira, F. M., Pinheiro, I. O., Souto-Maior, A. M., Martin, C., Gonçalves, A. R., & Rocha, G. J. (2013). Industrial-scale steam explosion pretreatment of sugarcane straw for enzymatic hydrolysis of cellulose for

production of second generation ethanol and value-added products. *Bioresource Technology, 130*, 168-173. https://doi.org/10.1016/j.biortech.2012.12.030

Ratti, R. P., Delforno, T. P., Sakamoto, I. K., & Varesche, M. B. A. (2015). Thermophilic hydrogen production from sugarcane bagasse pretreated by steam explosion and alkaline delignification. *International Journal of Hydrogen Energy, 40*, 6296-6306. https://doi.org/10.1016/j.ijhydene.2015.03.067

Sangyoka, S., Reungsang, A., & Lin, C.-Y. (2016). Optimization of biohydrogen production from sugarcane bagasse by mixed cultures using a statistical method. *Sustainable Environment Research, 26*, 235-242. https://dx.doi.org/10.1016/j.serj.2016.05.001

Sarkar, N., Ghosh, S. K., Bannerjee, S., & Aikat, K. (2012). Bioethanol production from agricultural wastes: an overview. *Renewable Energy, 37*, 19-27. https://doi.org/10.1016/j.renene.2011.06.045

Shanmugam, S. R., Chaganti, S. R., Lalman, J. A., & Heath, D. D. (2014). Statistical optimization of conditions for minimum H_2 consumption in mixed anaerobic cultures: Effect on homoacetogenesis and methanogenesis. *International Journal of Hydrogen Energy, 39*, 15433-15445. https://doi.org/10.1016/j.ijhydene.2014.07.143

Sivagurunathan, P., Sen, B., & Lin, C. Y. (2014). Batch fermentative hydrogen production by enriched mixed culture: combination strategy and their microbial composition. *Journal of Bioscience and Bioengineering, 117*, 222-228. https://doi.org/10.1016/j.jbiosc.2013.07.015

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., & Templeton, D. (2008). Determination of sugars, and degradation products in liquid fraction process samples. Technical Report NREL/TP-510-42623 (pp. 1-11). https://www.nrel.gov/docs/gen/fy08/42623.pdf

Sun, J., Yuan, X., Shi, X., Chu, C., Guo, R., & Kong, H. (2011). Fermentation of Chlorella sp. for anaerobic bio-hydrogen production: influences of inoculum–substrate ratio, volatile fatty acids and NADH. *Bioresource Technology, 102*, 10480-10485. https://doi.org/10.1016/j.biortech.2011.09.016

Urbaniec, K., & Bakker, R. R. (2015). Biomass residues as raw material for dark hydrogen fermentation - A review. *International Journal of Hydrogen Energy, 40*, 3648-3658. https://doi.org/10.1016/j.ijhydene.2015.01.073

Wang, J., & Yin, Y. (2017). Enrichment of hydrogen-producing microorganisms. In Biohydrogen Production from Organic Wastes (pp. 69-121). Springer, Singapore. https://doi.org/10.1007/978-981-10-4675-9

Wang, J., & Wan, W. (2009). Experimental design methods for fermentative hydrogen production: a review. *International Journal of Hydrogen Energy, 34*, 235-244. https://doi.org/10.1016/j.ijhydene.2008.10.008

Wong, Y. M., Show, P. L., Wu, T. Y., Leong, H. Y., Ibrahim, S., & Juan, J. C. (2019). Production of bio-hydrogen from dairy wastewater using pretreated landfill leachate sludge as an inoculum. *Journal of Bioscience and Bioengineering, 127*, 150-159. https://doi.org/10.1016/j.jbiosc.2018.07.012

Zhao, C., Karakashev, D., Lu, W., Wang, H., & Angelidaki, I. (2010). Xylose fermentation to biofuels (hydrogen and ethanol) by extreme thermophilic (70 °C) mixed culture. *International Journal of Hydrogen Energy, 35*, 3415-3422. https://doi.org/10.1016/j.ijhydene.2010.01.082

Zheng, Y., Zhao, J., Xu, F., & Li, Y. (2014). Pretreatment of lignocellulosic biomass for enhanced biogas production. *Progress in Energy and Combustion Science, 42*, 35-53. https://doi.org/10.1016/j.pecs.2014.01.001