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By

M. E. Hernández–Rojas, S. Baéz–Pimiento and J. A. Dávila–Gómez

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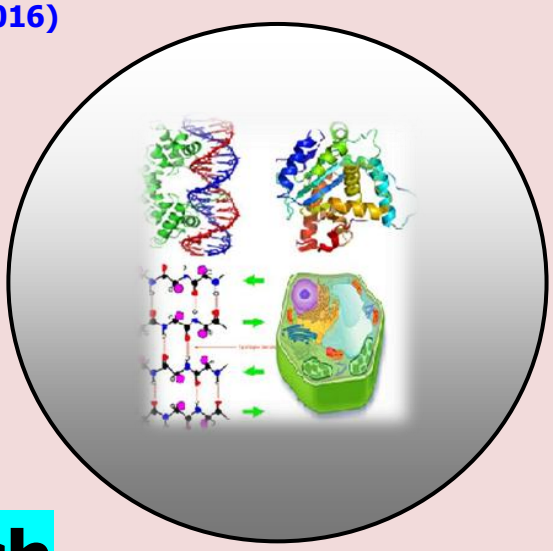

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J. A. Dávila–Gómez
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Effect of the Mixed Inocula and the Initial pH during the Dark Fermentation Process for the Production of the Hydrogen Using Molasses as Substrate

M. E. Hernández–Rojas, *S. Báez–Pimiento andJ. A. Dávila–Gómez**
Earth Ressources Department, Universidad Autonoma Metropolitana, Lerma Campus, Mexico
***Ind. Eng. Department, Universidad Nacional de Colombia, Manizales Campus, Colombia**
****Energy Department, Universidad Autonoma Metropolitana, Azcapotzalco Campus, Mexico Cy.Mexico**

ABSTRACT

In the present work, the effect of the origin of the inoculum and the heat pretreatment were evaluated in the biological production of hydrogen through the performance of a series of experiments by batches. To do so, three different mixed inocula were tested consisting of an aerobic sludge, a methanogenic anaerobic one, and an acidogenic anaerobic one. After selecting inoculum, a thermal treatment was applied to the sludge to increase the presence of hydrogen producing bacteria. Three heat treatments were tested, 70°C and 120°C per 30 min, and without treatment. About the origin of the inoculum, the methanogenic sludge produced a higher volume of hydrogen with a performance of 225mLH_2, unlike the acidogenic anaerobic sludge which produced 45 mLH_2.As a result, it was observed that the heat treatment during the hydrogen production does not present any additional effect compared to the inoculum without heat treatment. It was also studied the effect of the initial pH with values of 4, 4.5, 5, 5.5 and 6.5. The results showed that at 6.5 pH, the biogas production and the H_{max} were higher with values of 235 mL and R_m of 265 mL respectively. However, at pH 6.5, although initially it has a high production rate (22.0 mL/h), the substrate consumption and the hydrogen production are inhibited with time.
Keywords: Hydrogendark Fermentation, Conditioning of Inoculum, pH Effect, Molasses.

INTRODUCTION

Hydrogen is a renewable energy source and has a high–energy content of 122 kJ/g, which is 2.75 fold greater than traditional fossil fuels. Biological systems offer a wide variety of ways to generate renewable energy. Among them, fermentative bacteria mainly consume carbohydrates in the anaerobic digestion, generating hydrogen and organic acids of small molecular weights. The theoretical maximum hydrogen production from the fermentation of pure carbohydrates is 4 mole of H_2 by oxidized hexose by and acetic acid as a byproduct of the fermentation. In practice, the production is lower due to the production of biomass and other byproducts as butyric, propionic and alcohols. Although hydrogen production via dark fermentation by pure cultures with higher hydrogen yields (2–4 mole H_2/moleglucose) has been reported [Kumaret al., 2001],

mixed culture fermentation is more practical compared to pure culture process since non–sterile conditions are applied[Ren et al., 2008].However, one of the difficulties associated with hydrogen production using mixed communities is the coexistence of hydrogen–consuming microorganisms, such as methanogens. Various pretreatment methods including heat, acid, base, chloroform, sodium 2–bromoethanesulfonate (BESA), aeration and loading–shock have been conducted on the mixed inocula to enrich hydrogen–producing bacteria [O-Thong et al., 2009, Wang and Wan, 2009,]. Despite the results are inconsistent from one study to another, several studies have used heat pretreatment of the inoculum used to seed the reactor as method to inactivate or eliminate these microorganisms. The reported pretreatment conditions vary in terms of temperature and time of exposure, ranging from 70 °C to 120 °C for 15 min to 2 hours [Danko et al., 2008, Kawagoshiet al., 2005, Luo et al., 2010]. A general review shows that heat pretreatment is most widely used. Although in more recent studies it has been proposed to use the fresh inoculum without pretreatment [Luo et al., 2010].In this study we propose the evaluation of three mixed microbial cultures to produce hydrogen using the heat pretreatment to enrich the hydrogen–producing bacteria, and also determining the effect of pH on the system operation to inhibit the production of methane and its effect on yield and H_2 concentration in biogas.

MATERIAL AND METHODS

Physicochemical characterization of molasses

The medium used for this experiment was molasses from a sugar factory in the center of Mexico, without any supplement. In table 1, the composition of the molasses is presented. The analyses of concern were determined according to Standard Methods American PublicHealth Association; (1995]. These include total volatile solids (VS), pH, total nitrogen, COD andalkalinity. Sucrose was determined by the phenol-sulfuric acid method [Dubois et al., 1956].

Experimental procedures

Batch dark fermentation experiments were performed to investigate the effects of inoculum and this condition and pH on biohydrogen production from molasses. The experiments were performed in 125 mL serum bottles with 80 mL of liquid volume containing molasses and inoculum. The initial amount of biomass used in the batch experiments was approximately 2 g/L VS and the molasses concentration was 30 g/L. The initial pH of the batch experiments was adjusted to 5.5 with hydrochloric acid (HCl), the vials were sprayed with nitrogen gas for 5 min to generate anaerobic conditions. The cultures were set in a controlled incubator at 33 °C.In the first set of experiments, a total of three sources were tested as inoculum for hydrogen fermentation as it follows: activated aerobic sludge from a secondary settling tank of Municipal Wastewater Treatment Plant, anaerobic sludge from a UASB reactor of wastewater treatment plant of fruits and vegetables canning. The acidogenic sludge from a UASB pilot reactor fed with leached, operated at pH 5.5 and 35°C. The leached was obtained from a municipal landfill. In the second experiment, the inoculum was thermally pretreated, a method commonly applied for removing hydrogen–consuming microorganisms. The inoculum was incubated at 70 °C and 120°C for 30 min and also the inoculum was tested without pretreatment, the substrate concentration was constant at 30 g/L molasses. In the third set of experiments, to determine the effects of the initial pH values on hydrogen production, kinetic assays were performed with anaerobic sludge without heat treatment, by setting the initial pH with HCl at 4.0, 4.5, 5.0, 5.5 (without following control). Only to perform the experiment at 6.5, the pH was controlled with asodium hydroxide (NaOH), concentrated solution. The pH value of fermentation solution in series changed naturally with prolonged time. The SV and molasses concentration in the vials were identical to those described in the preceding paragraph.

Biogas and liquor analyses

The amount of biogas produced in each reactor was recorded by the water displacement method. The biogas contents were analyzed by a gas chromatograph (GC) (Model 580 GOW–MAC) equipped with a thermal conductivity detector and a 2m stainless column packed with Molecular Sieve Carbosphere (80/100 mesh). Injector, detector and column temperature were kept at 120°C the first two, and 140 °C respectively. Nitrogen gas was the carrier gas at a flow rate of 40 mL/min at a pressure of 40 psi.

Data Analysis

Once cumulative hydrogen production curves were obtained over the course of an entire batch experiment, a curve was modeled to the data using the following modified Gompertz equation (Eq. 1).

Where H is the cumulative hydrogen production (mL), $H_{max}$is the hydrogen production potential (mL), $R_m$is the maximum hydrogen production rate (mL/h), λ is the lag–phase time, t is time (h) and ***e*** is Euler number, approximately 2.718. The values of H_{max}, R_m, and λ for each batch were determined by best fitting the H_2 production data for Equation (1) using "Solver function in the Tools menu in Microsoft Excel 1995+". Modified Gompertz expression has widely be used through as back as 1932 until present by researchers[Winsor, 1932].

$$H(t) = H_{max} \cdot \exp\left\{-\exp\left[\frac{R_m \cdot e}{H_{max}}(\lambda - t) + 1\right]\right\} \quad \text{(Eq. 1)}$$

RESULTS AND DISCUSSION

Effect of the inoculum's type in the hydrogen fermentation

Several types of inocula have been used in the studies of hydrogen production via anaerobic fermentation, such as anaerobic sludge, agricultural waste, and isolated bacteria, employing different methods of inoculum's conditioning, like acid pretreatment, alkaline, temperature, among others. These studies have been developed using ideal and simple substrata like glucose, sucrose and synthetic culture media with buffer solution needed to keep the pH stable. However, in recent years studies have begun to use organic wastes. In these cases, real substrata, such as molasses, don't always exhibit this ideal behavior. Sometimes the results applied with more complex samples or actual substrata, like waste from the sugar industry are different from the obtained with pure samples; therefore to apply the scale hydrogen production it is necessary to experiment in real conditions of substrate and inoculum. In this study, experiments in batch are presented using different inocula, such as acidogenic anaerobic sludge, anaerobic sludge and activated aerobic sludge with or without thermal treatment. These were performed employing molasses as substratum at an initial concentration of 30 g/L without adding any other nutrients.

Figure 1 shows the accumulated hydrogen yield inoculated with different inocula without any pretreatment during the fermentation period. There was a remarkable difference in the hydrogen production yield among different inocula. The anaerobic sludge produced the highest volume with 227.58 mL, but surprisingly the acidogenic anaerobic sludge only produced 45.27 mL and the aerobic sludge did not produce hydrogen. With regard to the biogas production, the anaerobic sludge was also the one that produced the most, and the acidogenic and activated aerobic sludge produced a biogas composed almost completely of carbon dioxide. In relation to the presence of methane in the biogas, the yield of the methane production was gradually decreased in the anaerobic sludge until it got completely inhibited. However, for the acidogenic sludge the yield remained low and steady throughout the experiment with an average value of about 5% of methane in the biogas. The hydrogen contents in the headspace of the reactors, inoculated with anaerobic sludge, were between 0% and 55% maintaining an average of 35%. When the production of hydrogen began to be important, methane production was inhibited in the anaerobic sludge. Unlike the acidogenic sludge, the hydrogen content was less, between 0% and 20%, without inhibiting methanogenesis.

And for the aerobic sludge, the hydrogen content in the biogas was never significant as Figure 1c shows. Unlike the results reported by Watanabe [Watanabe and Yoshino, 2010], which presented a complete inhibition of methanogenesis with a leached as an inoculum, with no pretreatment used for the leachate in that study.Due to that, the acidogenic anaerobic sludge is fed with a mixture of AGV, methanogenesis precursor substrate, possibly methanogenic bacteria were enriched in the inoculum, for this reason the methanogenesis was not inhibited throughout the experiment. On the other hand, anaerobic sludge came from a reactor fed with high concentrations of sugars (fruit cannery waste), in this case, it presents a quick response in the production of H_2 and the maximum rate and hydrogen production(table 2), along with a complete inhibition of methanogenesis in the first 10 hours of culture. It would be prone to present a lack of methanogenic activities due to conditions of initial pH and high concentration of sugars in the culture medium. The table 2 summarizes the Gompertz equation constants at different inocula. The $H_{max}$and R_m coefficient values clearly show, as the inoculum source may affect the production of hydrogen, these values were 227.58 mL and 6.699 mL/H respectively. About aerobic sludge, this sludge did not present an ability to produce hydrogen, even experiments were performed several times, taking sludge samples at different periods and in all experiments the results were negatives: values of H_{max}, R_m and λ obtained were 2.74 mL, 0.20mL/h and 27.66 h, respectively. Furthermore aerobic sludge was thermally pretreated at 70°C for 30 min to induce hydrogen production but the results remained negative(results not presented).After those results, the anaerobic sludge was selected to experiment with thermally pretreated sludge at 70°C and 120 °C for 30 min.

In conclusion, no hydrogen was produced when aerobic sludge was used as inoculum treated and untreated, a sludge used asa substrate with a high concentration of carbohydrate is an efficient inoculum to produce hydrogen, and we consider that this was enriched in hydrogen producing bacteria because they are accustomed to the high concentration of carbohydrates.

Effect of the heat pretreatment of inoculum

Figure 2 illustrates the cumulative hydrogen for different conditions of heat pretreatment. It is quite interesting to notice that the untreated anaerobic sludge gives the highest cumulative hydrogen production of 227.58 mL, while the heat treated sludge at 70°C gave relatively low hydrogen production with a volume of 203.44 mL, and a further heating of the inoculum at 120°C gave the lowest production of hydrogen with a volume of only 103.95 mL such as the table 3 presents. Several authors have reported the heat treatment as the most efficient method to enrich the inoculum in hydrogen producing microorganisms. However, it was reported in this study that the highest hydrogen yield was achieved with fresh inoculum and that a higher pretreatment temperature has a negative effect on the production of hydrogen and biogas. However, the heat treatment at 70 °C has improved the hydrogen production rate compared to untreated inoculum, R_m and λ were6.69 mL/h and 14.57 h for untreated sludge, and for heat treated sludge at 70°C, they were 7.60 mL/h and 14.09, respectively. These results indicate that the heat pretreatment could decrease the period of adaptation of the microorganisms to accelerate the rate of production, but the conditions in the culture medium may possibly inhibit the maximum production. Reported conditions to perform the pretreatment vary between 70 ºC and 120ºC for 30min –2h, and some authors when comparing different pretreatment methods like alkaline, acids, and thermal show that the thermal treatment is the most adaptable for the conditioning. However, they worked with pure substrata, and they did not compare the conditions of temperature and time to pretreat the inoculum. From our results we can conclude that it is not necessary a pretreatment to enrich the inoculum, whereas from a treatment of 70°C for 30 min, and without treatment, volumes of hydrogen production the same are practically obtained, and on the contrary, if the conditioning temperature is increased, it is detrimental to the production. Previous studies carried out with other substrata have shown that the different methods to inhibit methanogenesis can affect hydrogen production [Kawagoshi et al., 2005].

Although the heat pretreatment has been the most widely used method to condition the hydrogen–producing inoculum, there are conflicting reports in the literature about the effects of temperature over hydrogen production. The hydrogen yields and rates increase as the temperature increases. However, the increase of temperature can also have detrimental effects over hydrogen production. The results obtained in this study suggest that inoculum pretreatment is not always effective in enhancing hydrogen productivity, and it will depend on the particular characteristics of the inoculum. In this case, a pretreatment was not necessary, because the inoculum was accustomed to a substratum with a high concentration of carbohydrates, such as fruits and vegetables waste.

Effect of initial pH value

Accordingly, the effects of acclimation and pH on hydrogen production were investigated by subculture experiment at different pH conditions. Figure 3 shows the cumulative hydrogen amount and sucrose consumption rate for every batch culture with no pretreatment of the anaerobic sludge at each pH condition. The initial pH's of 4.0, 4.5 5.0 and 5.5, were adjusted, and 6.5 pH was constant. It is apparent from figure 3 that the pH values of fermentation molasses in the experimental time course decreased with increasing time due to the production of acids derived from accelerated metabolism of carbohydrate–rich substrata. For pH 4.0 and 4.5, the sucrose was rapidly removed within 25 h, and then it was consumed slowly due to due to a decrease in pH below 4. The hydrogen production follows the opposite tendency, due to that in the first 25 h it was produced and then this production is generated slowly, this decrease in pH inhibits both hydrogen production and substrate consumption, and consumed little substrate is converted to a biogas mainly composed of CO_2,and about 45 h, the pH reaches the value of hydrogen production inhibition, whose value is below 4 units of pH.The end of production of hydrogen is 142.58 mL and 172.49 mL for pH 4 and 4.5 respectively such as table 4 shows.

Table 1.Composition of the normal Molasses (% w/w)

TKN	**0.2–2.8**
TS	**78–85**
Sucre	**48–58**
TOC	**28–34**

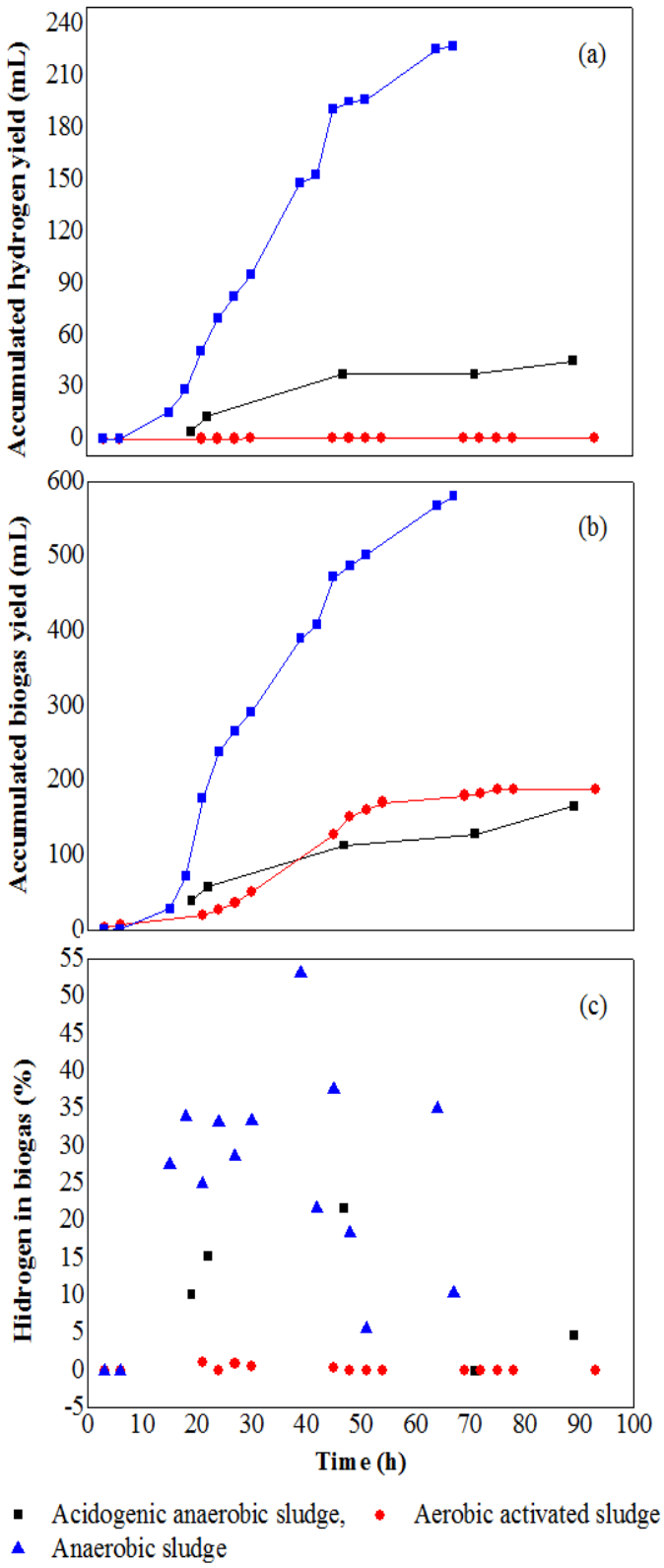

Fig 1. Accumulated (a) hydrogen production and (b) biogas production without pretreatment (c) Hydrogen concentration in biogas.

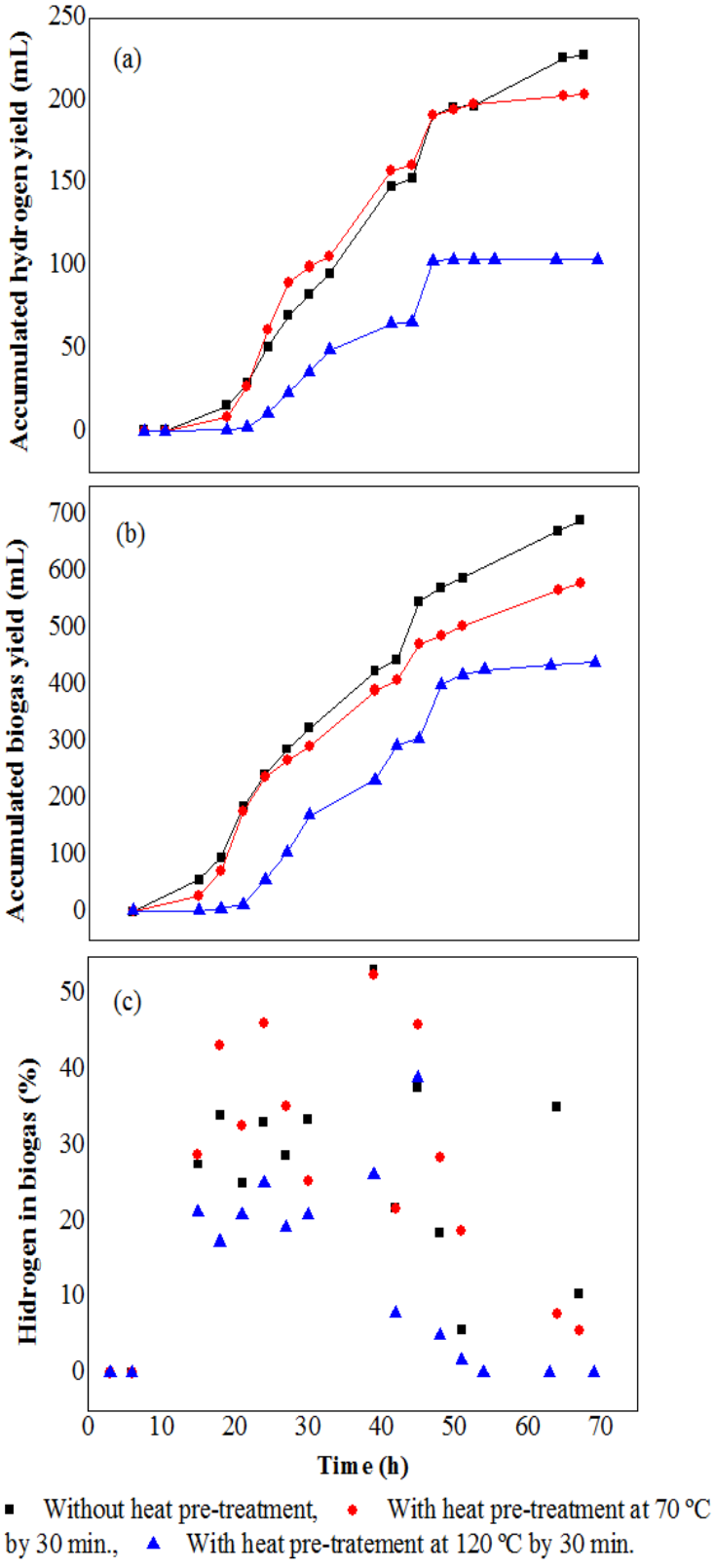

Fig 2. Accumulated (a) hydrogen production and (b) biogas production by heat pretreatment (c) Hydrogen concentration in biogas.

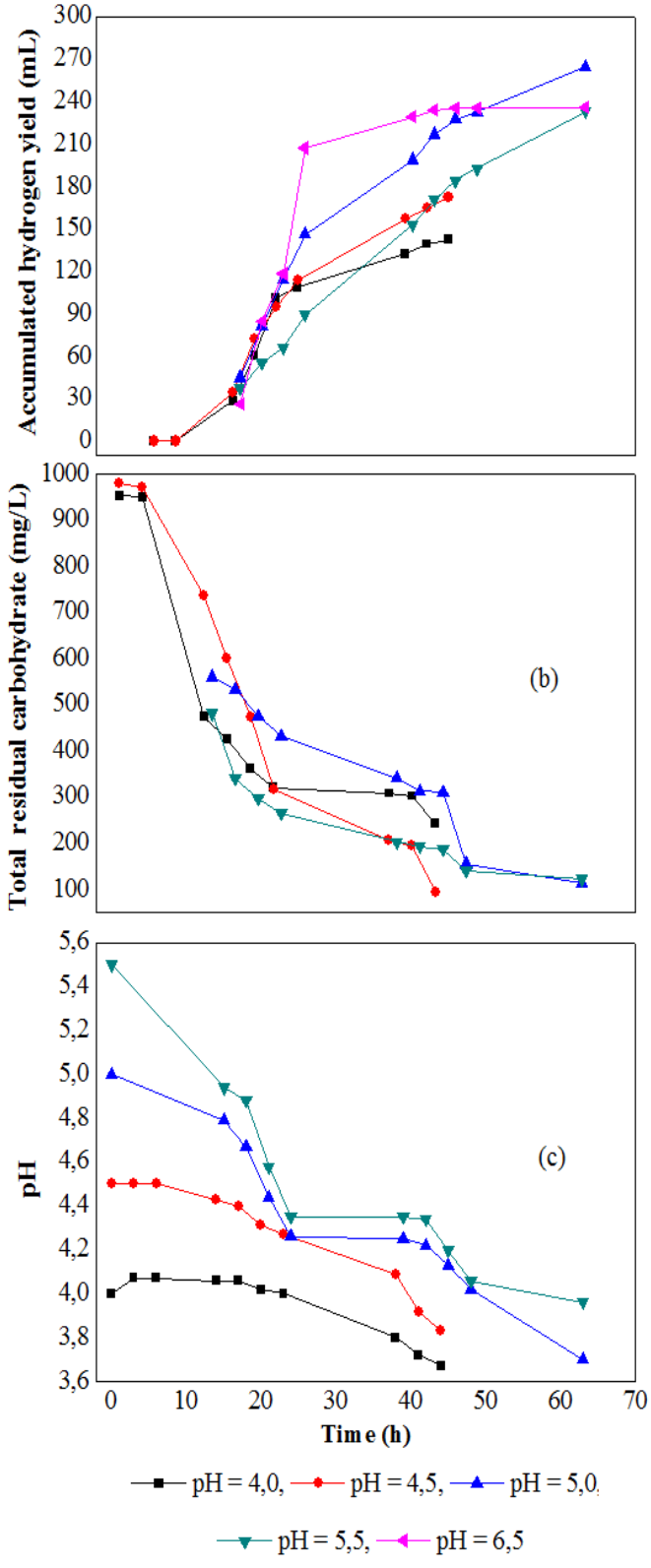

Fig 3.a) Biogas production in every batch culture with no pretreatment anaerobic sludge at different conditions.The initial pH was not controlled, except pH 6.5 was controlled through the experiment. (b) carbohydrate consumption through time. (c) Evolution of pH.

Table 2.Gompertz equation constants for different inoculum. Initial pH=5.5, initial molasses=30 g/L.

Inoculum	H_{max} (mL)	R_m (mLh^{-1})	λ (h)
Aerobic activated sludge	2.74	0.20	27.66
Acidogenic anaerobic sludge	45.27	1.22	13.58
Anaerobic sludge	227.58	6.69	14.57

Table. 3. Gompertz equation constants for different inoculum. Initial pH=5.5, initial molasses=30 g/L.

Inoculum	H_{max} (mL)	R_m(mLh^{-1})	λ (h)
Without heat	227.58	6.69	14.57
Heat pre–treatment 70°C	203.44	7.60	14.09
Heat pre–treatment 120°C	103.95	4.29	19.25

Table 4.Gompertz equation constants for different initial pH. Initial molasses= 30 g/L

pH	H_{max} (mL)	R_m (mLh^{-1})	λ (h)
4.0	142.58	11.48	11.49
4.5	172.49	9.02	9.61
5.0	264.46	7.55	6.96
5.5	232.94	5.80	9.17
6.5	235.71	22.02	14.42

It is important to note that the production rate of hydrogen is 11.4mL/h and 9.02 mL/h to pH 4.0 and 4.5 respectively, these coefficients are higher than for the maximum production of hydrogen (at pH 5.0 with H_{max} of 264.46mL and R_m of 7.55 mL), indicating that the continuous hydrogen production can be maximized if the constant pH between 4.0 and 4.5 is maintained. In this study the H_{max} batch was low due to inhibition of production due to the decrease in pH below 4.0, but in a system where a continuous reactor operates at pH 4.0 could have a positive effect. For 5.0 and 5.5 initial pH, the carbohydrate consumption occurred in the first 25 hours after consumption was slowly but continues gradually, and at the same time the hydrogen production continuous producing a maximum volume of 265 ml and 232 ml, respectively, until reaching the limit value of pH (below 4.0)after of 69 h. Curiously for adjusted 6.5 pH, the maximum value of hydrogen production (236 ml) was reached in a short time, about 25 h, after that the biogas production increased indicated a slow biological activity, and not towards H_2 production, given that the biogas was formed almost by $CO_2$and 5% of methane. Moreover, we observed that the after 25 h the substrate consumption was limited, which indicated that the decrease in the production of hydrogen after 50 h, even at pH 6.0, was due to the decrease in carbohydrates utilization, such figure 3b shows. This decrease in the concentration of hydrogen in the biogas with pH adjusted culture has been reported by Li[2008]. They reported inhibition in the hydrogen production with addition of NaOH to control the pH at 7. They explain an inhibitory effect of NaOH in the production of hydrogen and recommend pH adjustment with bicarbonate because it gives a better effect as a buffer. In table 4the Gompertz equation constants for different initial pH, and based on the best experimental results obtained of maximum H_2 production rate (22.02 mL/h) and the maximal potential of production (235.71 mL), hence, the pH 6.5 could be optimal operational condition when a complex substrate, as molasses, is used on batch experiments. But for continuous production, the pH 4.0 to 4.5 may be a better option because it has a higher H_{max}. If the pH is controlled at this value, the hydrogen production and the consumption substrate could continue. At pH 6.5, although initially has a high production rate (22.0 mL/h), the substrate consumption and the hydrogen production is inhibited with time.

CONCLUSIONS

From these results, we have demonstrated that depending on the source of inoculum the pretreatment is not necessary. We also demonstrate that an excessive thermal pretreatment may generally affect the bacterial flora, including the hydrogen producing bacteria. Certainly, a sludge that hasbeen in contact with a high concentration of carbohydrates is suitable as inoculum for the production of hydrogen without previous pretreatment. Working at a pH near neutrality is not beneficial for the production of hydrogen, because the accelerated metabolism of carbohydrate consumption and subsequent production of acid compounds require constant adjustment of pH with NaOH, which causes negative effect to produce a biogas composed almost exclusively of CO_2 and inhibit the substrate consumption. Then, continuous hydrogen production could be competitive if a constant pH is maintained between 4.0 and 4.5.

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Corresponding author: Dr. M. E. Hernández–Rojas, Earth Ressources Department, Universidad Autonoma Metropolitana, Lerma Campus, Mexico

Email: mariahrojas@hotmail.com