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Evaluation of Production of Hydrogen in a Batch Bioreactor using *Clostridium butyricum* DSM 2478 from Banana Peel

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Production of banana in the world was 20 million tons in 2018; this means that around 6 million tons of banana peel is converted in agricultural waste worldwide. Different methods have been evaluated for using this type of waste; one alternative use is the conversion to hydrogen by biotechnological processes. The high calorific value and clear combustion are attributes that makes hydrogen a solution to replace fossil fuels. One way to obtain hydrogen is by biological methods as fermentative pathway by bacteria. In this paper it was evaluated the production of hydrogen with *Clostridium butyricum* DSM 2478 from banana peel (BP). BP was collected and pretreated by washed, dried, grounded and sieved for the adaptation and selection of the particle size for fermentation. The fermentation was carried out at 100 ml in RCM (Reinforced Clostridial Medium) medium with the pretreated BP using initial concentrations: 2.5%, 5% and a control test (0%). The medium with 5% of BP showed the best results, obtaining 157.54 mmol H_2/l with a scale of 10 ml and a degradation percentage of 29.12% of BP. Process was scaled up in bioreactor (2.5 l). Operation conditions were temperature 37°C, pH 7, an initial concentration of BP at 5% (which was the best at laboratory scale) and agitation of 200 rpm. The hydrogen production in the bioreactor was evaluated obtaining a maximum concentration of 668.4 mmol H_2/L with a degradation percentage of 94.97% of the initial BP in the medium. These results indicate that hydrogen production through BP using *C. butyricum* is possible.

1. Introduction

Due to the high volumes of banana production worldwide, large amounts of waste have been generated (20 million tons by 2018, which represents approximately 6 million tons of waste generated) (FAO,2018) However, The BP is a sub-product obtained from *Musa cavendish* and it is a valuable source of bioactive compounds (Kadir *et al.,* 2016) and Which constitute 30%-40% of a banana fruit (Kulkarni *et al*.,2017), thus it is necessary to develop new alternatives to convert this waste generated into value-added products. Waste has high content of cellulose and hemicellulose (Mishra *et al*., 2010), so it is possible to use this waste as raw material for producing animal feed (Agneesens *et al*., 2010), organic acids (Karthikeyan and Sivakumar, 2010), bio fertilizers (Scarlett *et al.*, 20018), medical uses (Scarlett et al., 20018), and clean energies by biological processes as a good source of sugar (Kulkarni *et al*., 2016).
One alternative to obtain clean energy is hydrogen (Jusoh *et al.*, 2017), because it is considered one of the best candidates to replace fossil fuels (Seppala *et al*., 2011). The calorific value of hydrogen is 122 kJ / g, which is 2.75 times greater than that of petrochemical fuels (Classen *et al*., 1999). It also stands out for the zero environmental impact it causes during combustion because it only generates water as a byproduct (Berchem *et al.*, 2018). Therefore, continuous research on alternatives to fossil fuels reveals that one of the most suitable components to replace them is hydrogen (Jusoh *et al.*, 2017). In addition, if hydrogen is obtained by biological methods it is considered a source of clean and renewable energy (Ali *et al.,* 2017)

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An alternative to the production of hydrogen is the fermentation with bacterias of genus *Clostridium*. Different *Clostridium* species have been studied principally *C. butyricum* (Baptiste *et al.*, 2018) and *C.acetobutylicum* (Elbeshbishy *et al.*, 2011). These microorganisms have the ability to metabolize simple and complex carbohydrates that have been or not pretreated like organic solid wastes (Baptiste *et al.*, 2018). The yield reported is around 2 mol H_2 / mol glucose (Ntaikou *et al.*, 2010).
This work evaluates the biological hydrogen production by *C. butyricum* DSM 2478 from BP as a carbon source. All concentrations of these solvents, biomass, substrate consumption, hydrogen produced, and pH were monitored during fermentations at vial scale and bioreactor scale.

2. Materials and methods

2.1 Biomass

BP was collected in a fruit store in Bogotá, Colombia and then was washed, sun dried, grounded and sieved. The content of Carbon, Hydrogen, Oxygen, Nitrogen and Sulfur (CHONS), carbohydrates, celullose, hemicellulose, lignin, and C/N ratio, was determined. Celullose, hemicellulose and lignin were determined by Van Soest method. Finally, carbohydrates, C/N ratio were calculated by calculation as well.

2.2 Microorganism and culture medium

Clostridium butyricum DSM 2478 was used for the fermentative step. 1 ml of liquid stock culture was transferred to 80 ml RCM (Reinforced clostridia medium), Merck ® and were incubated for 24 hours at 37°C in anaerobic conditions; pH was adjusted to 7.0 with 0.1 M NaOH/HCl solutions. Culture medium was made with different initial concentrations of BP :0%, 2.5% and 5% in RCM medium. A greater amount of BP was not evaluated to avoid the difficult to degradation of the residue by bacteria. Each experiment was initiated with 1 mL of the original vial containing spores of *C. butyricum.* To create anaerobic conditions, the vials were put in anaerobic jar with anaerogen (Oxoid Microbiology Products) and resazurin (Oxoid Microbiology Products).

2.3 Fermentation vials

All fermentation processes were carried out at 37°C, with an initial biomass of 1.9 x 10^7 UFC/mL and in a glass vial with a working volume of 80 mL, under anaerobic atmosphere. Once the fermentation started, gas and liquid samples were taken every 8 h for 56 h. To analyze and determine the presence of hydrogen, the gas samples were taken by introducing commercial syringes of 3mL and sealing them. Then, samples were analyzed by gas chromatography (GC). Additionally, 1 mL of the culture was centrifuged at 14000 rpm for 10 minutes to separate the supernatant (acids an solvents) from the precipitate (BP and biomass). 1 mL of sample is used to quantify the biomass and analyze the cell growth by Neubauer chamber. The acids and solvents was determined using high performance liquid chromatography (HPLC). Once the fermentation was finished, the culture medium was separated to evaluate the degradation of the peels. For the vials the separation was made by filtration with filter paper and just was taken the finals samples. After this, the solids obtained were taken to an oven to 110°C for 24 hours. Finally, the samples were weighed and the degradation was calculated by the difference of the initial and final weight of the BP.

2.4 Fermentation in batch reactor

The bioreactor used to scale up was designed and built by Universidad Santo Tomás (Bogotá, Colombia) and. the work volume was 2.5 l. The inoculums were obtained by extracting 80 ml from preinoculum to the original and duplicate experiments. The working conditions during the fermentation were: 38 g/L of RCM, 37°C, pH: 7.00 and the best concentration of BP at laboratory scale (5%). During the fermentation at bioreactor scale both samples liquid and gas were taken every 4 h. The process was the same as the used for fermentation vials. Final degradation for the reactor was performed by vacuum separation and the samples were taken every 4 hours. Next steps were the same as vial fermentation.

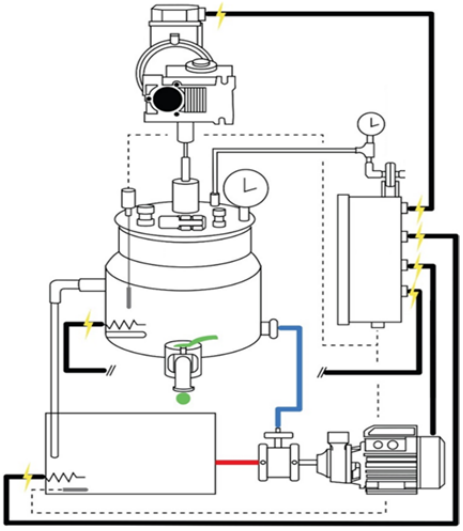

Figure 1. Bioreactor designed and build for hydrogen production.

2.5 Metabolites quantification and substrate consumption

The consumption of glucose and the generation of soluble metabolites were measured by HPLC, using an Aminex HPX-87 HPLC column with a constant temperature of 65 °C and a Shimadzu RID 10A refractive index detector and 0.5 mL/min of H_2SO_4 0.003M was used as mobile phase and 10 µL of the sample to analyze. For the hydrogen determination the samples were quantified by a Shimadzu® gas Chomatography equipment, with a Porapak Q (80/100 mesh) column, thermal conductivity detector (TDC) and this conditions: colum temperatrure 90°C, TDC T°:150°C, and injector T°: 100 °C. Nitrogen UAP was used with a flow 20 mL/min. and a volume of 1 mL of sample was used to analyze.

3. Results and discussion

3.1 Biomass

The composition of BP was: %C 39.37, %H 5.67, %O 39.37, %N 1.2, %S 0.11, carbohydrates 40.36%, lignin 9.26%, cellulose 8.64%, hemicellulose 14.16%, and C/N 32.81. These results makes BP as an adequate substrate for the bacteria due to its high amount of carbon compounds which could be metabolized by the bacteria. Carbohydrates presents were similar at reported by Pathak with a different species of banana (*Musa musaceae*) of 40.24%(Kulkarni *et al.*, 2017), lignin were lower than 14% which was reported by Romero *et al.* (2015).

3.2 Fermentation vials

The results obtained by vial fermentation showed a positive increase in biomass for all vials independently from the initial concentration of BP tested as shown in figure 2. The initial concentration of biomass was 1.7 $x10^7$ cell/mL for all the test. The whole vial fermentations had a lag phase for the first 8 h. Between the 8 - 32 h, the biomass increased progressively, this behavior corresponds to the exponential phase. Comparing the speed constants between 8-32 hours, the control test (0%) showed a greater increase in biomass compared to the mediums with BP, because the exponential stage is much longer (approximately 16 h more) This behavior can be attributed to the BP added as a substrate, not limited only with the nutrients provided by the medium RCM, also with the nutrients that the BP provides. However, in the mediums with BP, the results showed the 5% concentration of BP as the best high growth because after the 48 h it is still in exponential phase while the others are in death phase. It is possible to appreciate that the substrate concentration decreased due the biomass increase. The peaks observed of the kinetic of glucose after going down are attributed to the ability of bacteria in degrading the BP, and releasing the wrapped fermentable sugars in the lignin causing a little increase in the concentration of glucose.

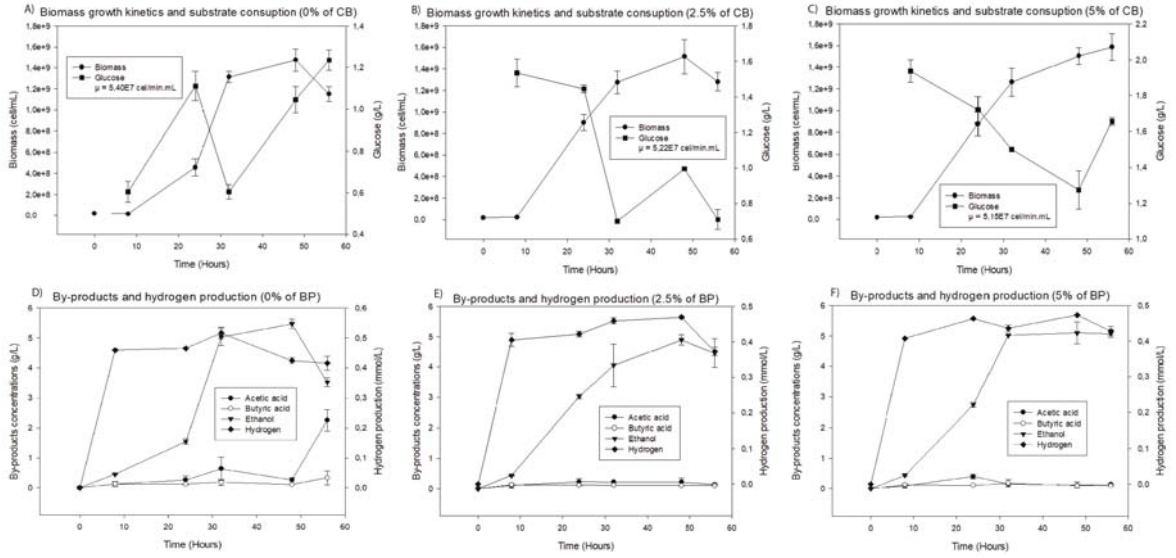

Figure 2. Biomass growth, substrate consumption and metabolites production for vials fermentations.

During the fermentative process the main products obtained were butyric acid, acetate acid and, ethanol according to liquid chromatography. As shown in figure 2, the metabolites obtained are the typical of *C. butyricum* as reported by Jauregui M. *et al* (2017), Zhang, H. *et al* (2009), and Beckers, L. (2010). The decrease in pH is related with the acidogenic phase of *Clostridium* in which it produces acetic acid and butyric acid. These compounds are important for the hydrogen production according to the metabolic pathway because their presence implies that *Clostidium* produce hydrogen. The amount produced of ethanol is greater than the acids, this happened due the fermentation reached the solvetogenic phase (Fonseca *et al.*, 2016), where the acids were converted in ethanol. This would suggest that the fermentation time could be less with the objective of improving the acids production and so hydrogen production.

The results shown in figure 2 exposed that is possible to produce hydrogen with *C. butyricum* and BP as substrate. There is not a significant difference in the hydrogen concentration between the control and experimental tests; the maximum hydrogen concentration obtained at 0% BP was 172. 31 mmol/L after 32 h while the maximum concentration for 2.5% BP and 5% BP were 156.61 mmol/L and157.54 mmol/L, respectively after 48 h. This concentration was lower than the reported by Jauregui M. *et al* (2017) where the maximum concentration was 430 mmol/L with glycerol as substrate at the same scale. Similarly, the concentrations obtained in this paper were higher than those of *Bernal, M. et al* (2013), where the maximum production occurred after 20 h and was approximately 11.5 mmol H_2/L. This result is due to differences in the substrate of the culture medium, because these data were obtained from glycerol.

The degradation of the BP was better at 5% BP than at 2.5% BP, while the percentage of degradation were 29.12±1.73% and 7.72±2.88% respectively. Those percentages were lower due to the presence of lignin in the substrate employed which obstructed the degradation by the bacteria. The concentration of 5% BP was selected as the best concentration to be scaled in the bioreactor due to the degradation obtained and the biomass growth at this concentration.

3.3 Fermentation in batch reactor

The hydrogen production was evaluated in a batch bioreactor with a working volume of 2.5L at 5% BP, according with the last results obtained. In the figure 3 are shown the biomass growth in the batch reactor with an initial cell concentration of $2.72x10^7$ cell/mL to begin the fermentation. the Lag phase is almost null and the increase is continuous until 24 h where the growth was maximum ($1.16 x10^9$ cell/mL) and then the early death phase. It is appreciated that the substrate increases the concentration between 4-8 h, this may be due to the fact that at this time, the greatest release of sugars happened thanks to the degradation of the BP by the strain, increasing the concentration of glucose in the medium. Then the continuous decrease of the substrate concentration due to the biomass increase over time.

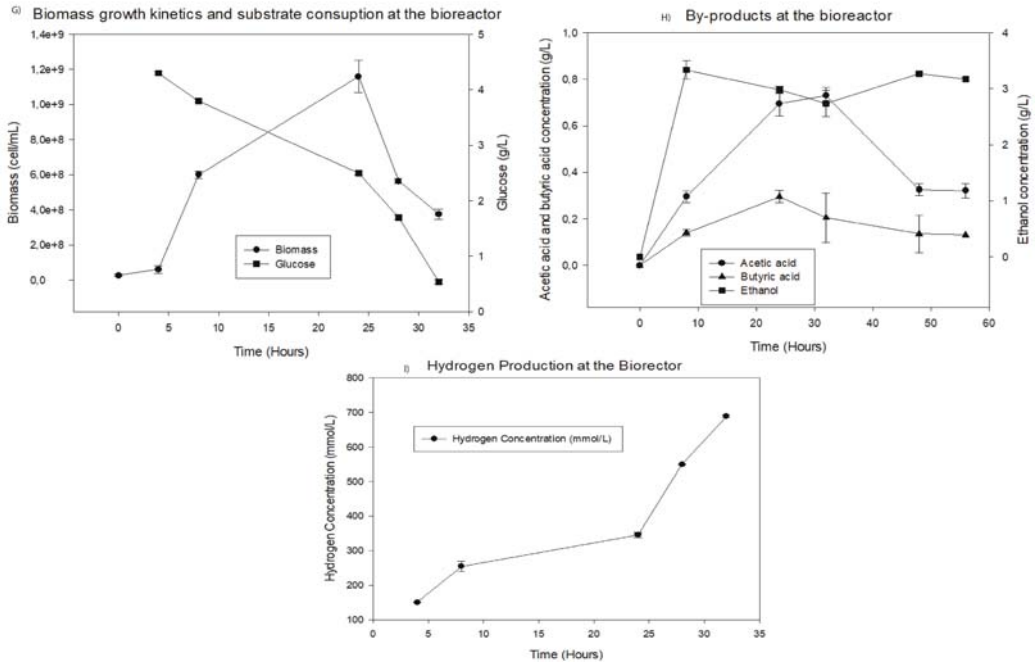

Figure 3. Biomass growth, substrate consumption and metabolites production for batch reactor.

In the fermentation, it is possible to see the production of acetic acid and butyric acid, which are products of the fermentation of *C. butyricum* according to the metabolic pathway for the production of hydrogen. Higher acetic acid production is obtained, even compared with the produced in vials fermentation, this suggesting that the metabolic pathway of *C. butyricum* was by the acetate production pathway (Yin and Wang, 2017). The concentrations produced of butyric acid and acetic acid are higher than those reported by Yin, Y. et al (2017), where the production from glucose, the same microorganism, and work volume of 150 mL is approximately 0.036 g/L and 0.034 g/L respectively, while in the present paper 0.295 g/L was obtained of butyric acid and 0.695 g/L of acetic acid. The presence of these metabolites causes acidification of the medium; therefore, the continuous decrease of the pH value causes the inhibition of bacterial metabolism for the production of hydrogen. At the figure 3 is showing the by-products concentration obtained during the fermentation in the bioreactor.

With the objective of evaluate the production of hydrogen in the reactor, the kinetics is shown in Figure 3. The production of hydrogen in the fermentation occurs from 4 h after inoculation and has a constant production until after 28 h, where the production of hydrogen increases significantly with 668.4 mmol H_2/L. This result confirmed that agitation improving the mass transfer into the reactor, so the diffusional effect could not affect the growth of *C. butyricum* and hydrogen production. It is difficult to compare this result with other authors because the design of the reactor, used in this paper, is not reported in other papers. This increment in the hydrogen concentration is due to a decrease in the BP concentration. On the other hand, in this scale ethanol is other product obtained during fermentation process, so BP could be produced two kinds of biofuels using *C. butyricum*.

The degradation of the banana with the batch reactor is better than the degradation in the vials fermentation. In the first 4 hours, the degradation of the shell is low, with a degradation percentage of 2.47%, reason for which the bacteria begin the adaptation for the consumption of this substrate. Then, the progressive degradation of the peel occurs between 4 and 8 h, which is consistent with the time of the growth phase of the bacteria. Finally, the final percentage of BP degraded was 94.97%. This showed the ability of bacteria to adapt in the culture medium.

Conclusions

The effect of the initial concentration of banana peel (2.5% and 5%) on hydrogen production was evaluated and compared with a control essay (0%) initially at vial scale. The results don't show a significant difference in terms of hydrogen production and BP degradation but the best results were obtained in the culture medium with 5% BP concentration, obtaining a maximum concentration of 157.54 mmol H_2 /L and a degradation percentage of 29.12 ± 1.73% of the BP from bacteria. The degradation of BP was scaled up to bioreactor level

with 5% BP of initial concentration. The maximum concentration obtained was 668.4 mmol H_2 / L with a percentage of BP degradation of 94.97%. These results are better than those obtained at the fermentative vials, which is why it is attributed to the agitation factor on the batch reactor. The results suggest that hydrogen production through BP using bacteria of the species *C. butyricum* is possible.

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