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Hydrogen Production by Dark Fermentation

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Alternative energy sources have been extensively studied due to the environmental concerns caused by the employment of fossil fuels. Hydrogen is considered a very clean energy source, since its combustion releases mainly water as a reaction product and also it has the advantage of having the highest energy density when compared to any other fuel. Moreover, hydrogen may be produced biologically by fermentation from renewable sources, such as effluents and agroindustrial wastes. Based on this context, this work evaluated the hydrogen production by dark fermentation, using a microbial consortium obtained from a dairy wastewater treatment plant. The fermentative process occurred in batch with a reaction volume of 75 mL using lactose (20g/L) as substrate, which was procured from whey permeate. The response hydrogen conversion was evaluated from the process variables, temperature, which ranged from 27,9 to 42°C, and magnesium sulfate concentration, which varied from 0,31 to 1,44 g/L. The response surface, which was plotted from the results obtained in the Central Composite Design pointed out for best hydrogen yield for the temperature range from 30ºC to 35ºC and $MgSO_4$ from 1.2 to 1.6 g/L. The maximum yield was 4.84 mol H_2/mol lactose.The following organic acids, acetic, lactic, butyric and propionic were produced in the fermentation.

1. Introduction

For more than a century, fossil fuel has been exploited to such an extent that it has not only induced serious environmentalproblems but also exhausted the limited fuel reserves, necessitating the quest for a clean alternative energy. H_2 is most promising in the succession of fuel evolution with severaltechnical, socio-economic and environmental benefits to its credit. It has highest energy content per unit weight of anyknown fuel (142 kJ/g). Handling of H_2 gas is safer withcomparison to other known natural gases and the mostimportant fact is that H_2 is environment friendly gas as itscombustion releases only water as the reaction product. Thesefeatures makeH_2an ideal candidate to replace the fossil fuels(Sinha and Pandey, 2011).
Even though hydrogen is the most abundant element in the earth's crust, it is hard to find it as a gas H_2. There are different methods of H_2 production and the most known techniques of production are those based on reforming reactions of hydrocarbons, such as steam reforming, CO_2 reforming and partial oxidation. Nowadays, about 40% of H_2 is produced from natural gases, 30%from heavy oil and naphtha, 18% from coal, 4% from electrolysis and about 1% from biomass(Sinha and Pandey, 2011).
The chemical methods of H_2 production are the main processes of production due mainly to the high hydrogen yield achieved in the reaction, what makes these methods more economic feasible. However, reforming of hydrocarbonsare energetically expensive since their reactions are endothermic, so these processes occur usually at high temperatures (about 800ºC). In addition, the steps of reforming releasesignificant amount of greenhouse gases, such as carbon monoxide and carbon dioxide. Also, hydrocarbons reforming uses non renewable materials and fossil feedstocks. These facts are contrary to the aim in producing an alternative clean energy source (Navarro et al., 2007).
In present scenariobiological H_2 production processes are becoming importantmainly due to two reasons: utilization of renewable energyresources and its operation at ambient temperature andatmospheric pressure(Sinha and Pandey, 2011).
There are different biological methods of H_2 production, such as photosynthetic and fermentative processes. Dark fermentation is an anaerobic digestion of organic matter and it is considered the simplest

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process of obtaining biohydrogen. Fermentation occurs in the dark at room pressure and it usually shows high hydrogen production rates when compared to the photosynthetic methods (Das, 2001).
Dark fermentation may be processed using a wide range of organic compounds as substrate. Besides the use of glucose and simple carbohydrates or polymers such as starch and cellulose, dark fermentation has attracted great interest due to the possibility of using organic wastes for biohydrogen production (Sinha and Pandey, 2011). Many researchers have studied the use of agro-industrial residues from various matrices as substrate in this process (Wukovitset al. 2013; Guoet al., 2010; Kotsopouloset al., 2009; Fan et al., 2006; Vijayaraghvanand Ahmad, 2006). Considering that such residues are abundant, cheap, renewable and biodegradable, the hydrogen production using these feedstocks becomes advantageous either in economic and environmental aspects (Guoet al., 2010).
Hydrogen production via fermentation involves either facultative or strict anaerobic bacteria. The various metabolic pathwaysthat may establish can either be promoted or inhibited, dependingon the adopted operating conditions, which govern the productionof specific volatile fatty acids (VFAs) and alcohols including acetate,propionate, butyrate, lactate and ethanol. In carbohydrates fermentation, the acetate and butyrate pathways involve the production of, respectively, 4 and 2 mol of molecular hydrogen per mol ofglucose degraded. However, propionate, ethanol and lactic acid canalso be produced in mixed bacterial cultures, adversely affecting H_2production: propionate is a metabolite of a H_2consuming pathway, while ethanol and lactic acid are associated with zero H_2pathways (Guo et al., 2010).
In order to enhance H_2production, culture conditions suchas the C/N and C/P ratios, carbon sources, pH, andtemperature have been widely studied. Metal ions cansignificantly influence enzyme activities related to hydrogenproduction (Zhao et al.,2012).
Based on this context, this current work investigated the influence of magnesium sulfate concentration and temperature on the hydrogen production by dark fermentation using cheese whey permeate as substrate and carbon source and a microbial consortium as a inoculum.

2. Materials and Methods

2.1 Inoculum Preparation

The inoculum was prepared from the effluent of an up-flow anaerobic sludge blanket reactor (UASB), gently donated by a dairy located in Uberlandia-MG (Brazil). The collected anaerobic sludge consisted of a microbial consortium that was previously adapted in a synthetic medium, which composition is shown in Table 1. Lactose was the main substrate used in the fermentations and it was obtained from cheese whey permeate, which was purchased from the *Sooro Concentrado Indústria de Produtos Lácteos* Ltda company (Brazil).

Table 1- Composition of the synthetic medium used for the inoculum adaption

Nutrient	Concentration (g/L)
KH_2PO_4	3.0
K_2HPO_4	7.0
$MgSO_4$	1.0
yeast extract	3.0
meat extract	0.5
$(NH_4)_2SO_4$	1.0
Lactose	20.0

Batch fermentations were carried out in 100 mL serum vials with a working volume of 75 mL and 25 mL of headspaceas shown in Figure 1. The reaction volume was composed by the inoculum (1.6% v/v) and the synthetic medium (98.4% v/v). The medium was purged with nitrogen gas for 1 min. Afterwards, the vials were sealed with rubber septum stoppers and aluminum rings. All experiments occurred in the dark and in anaerobic condition during two days. Since it has not been observed biogas production after this time, the dark fermentation was interrupted and the biogas was analyzed. Gas production and metabolites concentration were measured as described in analytical methods.

Figure 1- Batch fermentation.

An statistical analysis based on the techniqueof a central composite design was performed in order to evaluate the influence of temperature and magnesium sulfate concentration in the hydrogen production. This methodology is ideal for sequential experimentation and allows a reasonable amount of information for testing the lack of fit while not involving an unusually large number of design points. In the CCD performed in this work, the range of the independent variables studied, temperature (X_1) and $MgSO_4$ (X_2), varied according to Table 2.

Table 2- Values of the independent variables with their respective levels used in the central composite design $2^{(2)}$.

	Level				
Variables	-α	-1	0	+1	+α
Temperature(ºC) (X_1)	27.93	30	35	40	42.07
$MgSO_4$ (g/L) (X_2)	0.314	0.48	0.88	1.28	1.446

After having the results of the composition of the gas released during the fermentation, an empiric model for the response hydrogen yield (Equation 1) was obtained through a *t of Student* hypothesis. Only parameters which index *p* of probability was lower than 5% were considered.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} x_i x_j \quad (1)$$

Where Y is the response variable, x_i are the independent variables, β_0 is the constant of the model, β_i is the *i*-th linear coefficient, β_{ii} is the quadratic coefficient and β_{ij} is the coefficient for the *ij*-thinteraction.
Concentration of lactose and organic acids were quantified using High Performance Liquid Chromatography (HPLC). Samples were diluted with ultrapure water, filtered through a membrane (0.22µm pore size, Millipore®), and injected into a chromatographic system (Shimadzu® model LC-20A Prominence, Supelcogel® C-610H column) equipped with ultra-violet and refractive detectors. The former was used to read organic acids at a wave length of 210 nm, and the latter quantified lactose. Analyses were carried out using phosphoric acid 0.1% as carrier solution, pump flow rate at 0.5 mL/min, temperature oven at 32°C. The sample volume injected into the chromatograph was 20 µL.
The produced gas was collected in graduated syringes and its composition was determined by gas chromatography using the chromatograph Shimadzu® model GC 17A, equipped with a thermal conductivity detector (TCD) and a capillary column Carboxen® 1010 (length 30m, internal diameter 0.53 mm). The operating temperatures of the injection port, the oven, and the detector were 230, 32, and 230ºC, respectively. Argon was used as carrier gas.

3. Results and Discussion

The following empiric model (Equation 2) was obtained after a statistical analysis of the results achieved for hydrogen yield:

$$\text{Hydrogen Yield} = 4.39 - 0.47X_1 - 1.23X_1^2 + 0.63X_2 - 0.33X_1X_2 \quad (2)$$

The correlation coefficient (R^2) obtained after adjustment was 93.5 %. The negative sign accompanying the linear variable temperature indicates that an increase in the value of the variable provides a reduction in the response studied. The same analysis can be made for the positive signal that accompanies the linear variable concentrations of magnesium sulfate. This signal indicates that an increase in the studied variable has a positive effect on H_2 yield. A response surface and its contour curves were plotted from the model and they are shown in Figure 2.

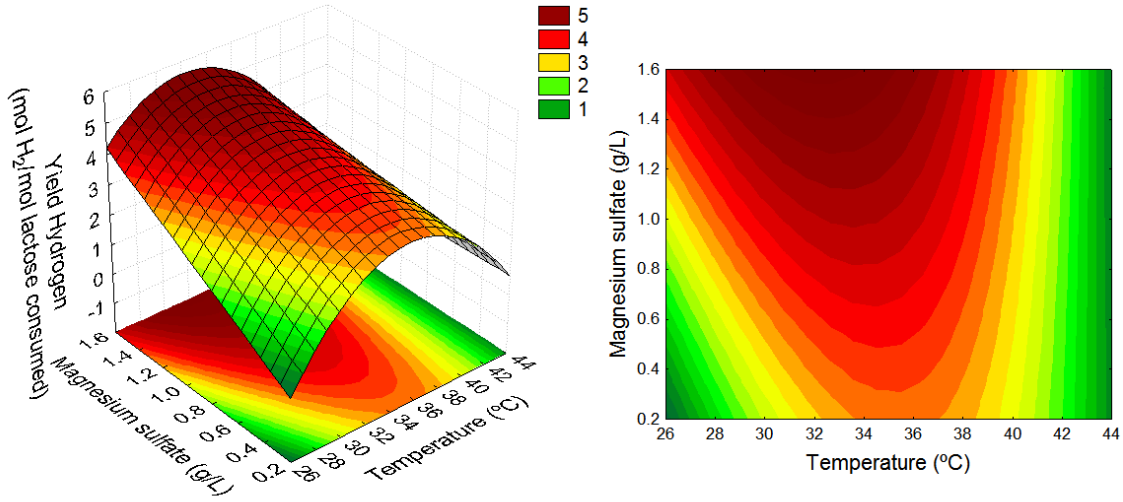

Figure 2- Response surface (a) and contour curve (b) for hydrogen productivity in relation to the variables magnesium sulfate and temperature.

It is noted from Figure 2 that the variables magnesium sulfate concentration and temperature affect the fermentation process, and highest yields were achievedfor temperatures between 30°C and 35ºC and magnesium sulfate concentrations from 1.2 to 1.6 g/L. Analysis of the fermented medium showed that different organic acids were produced during the fermentation process: acetic, lactic, butyric and propionic (data not shown). In most cases, higher hydrogen yields are reached for the metabolic pathways of substrate conversion in acetic and butyric acids.

Much of the most recent studies on hydrogen production from organic wastes were performed under mesophilic conditions, between 30°C (Lee and Chung , 2010) and 40°C (Wang and Zhao , 2009) , or even more specifically in the range of 35-37°C (Dong et al. , 2009 ; Hong and Haiyun, 2010; Kim et al. , 2010 , 2011a , Li et al. , 2008; Zonget al. , 2009).

Thermophilic conditions are assumed to optimize the enzymatic activity of hydrogenase during fermentation byClostridia, to inhibit the activity of H_2consumers and also to suppress the growth of lactate-forming bacteria (Lay et al., 1999; Oh et al., 2004; Valdez-Vazquez et al., 2005). Kim et al. (2011b) investigated the effect of temperature in both the mesophilic and thermophilic range (35– 60ºC) on food waste fermentation in the absence of any specific inoculum, and found that the lowest and highest H_2production yields were associated to temperatures of 35 and 50ºC, respectively. Although in both cases the amount of organic acids was comparable, lactate was predominant at 35ºC while butyrate was the main volatile fatty acid component at 50ºC. Microbial analysis of the fermentation medium also indicated

that the dominating species were lactic acid bacteria at 35ºC and H_2-producers at 50ºC, thus confirming the role of temperature in dictating the nature of microbial consortium during the process.
On the other hand, however, high temperatures have also been reported to induce thermal denaturation of proteins and essential enzymes, in turn negatively affecting microbial activity (Lee et al., 2006).
Pandey et al. (2009) evaluated the effect of temperature on hydrogen production in range 27 - 40°C. The authors found that the hydrogen production increased as the temperature increased up to 30°C. After this value, higher temperatures caused a decrease in hydrogen production, which was maximum (88 mmol H_2) after 10 hours of fermentation at 30ºC.
Since magnesium ions act as cofactors for many enzymes in the glycolytic pathway, increasing its concentration in the growth medium can promote glycolysis. However, the accumulation of metabolites such as fructose diphosphate, phosphoglycerate and phosphoenolpyruvate can cause inhibition of glycolytic reactions. Hydrogen production by fermentation occurs by the transfer of electrons from the reduced ferredoxin to protons H^+ in the presence of hydrogenase enzyme. The accumulation of ferredoxin in its reduced form in the cell can occur by both pyruvate oxidation or by oxidation of NADH. Since the glycolytic pathway is inhibited, the concentration of pyruvate and NADH decreases and consequently the production of hydrogen become limited. Therefore, an optimal concentration of Mg^{2+} must be satisfied to maximize the hydrogen production and yield of the dark fermentation (Sinha and Pandey, 2011).
Several authors have reported increased production of hydrogen fermentation in fermentative process after the addition of Mg^{2+} ions. Wang et al. (2007) noted that increasing $MgCl_2.6H_2O$ concentration for the range from 10 – 200 mg/L, hydrogen production also increased from 934.9 to 2360.5 mL/L medium. GUO et al. 2009 evaluated the effects of nutritional components in the production of hydrogen using the bacteria *E. harbinense* B49 as inoculum and glucose as substrate . These authors observed that for the studied range (100 – 800 mg Mg^{2+}/L), the best hydrogen yield, which was 2.21 mol H_2/mol glucose, was achieved at 691.98 mg/L of Mg^{2+}.

4. Conclusions

The present data reports recent findings on biohydrogen production from biomass waste product by dark fermentation. A Central Composite Design was employed for determining the optimum conditions for hydrogen yield increment. The effects of the temperature and $MgSO_4$ on hydrogen yield were evaluated and the response surface obtained from the results pointed for best hydrogen yield in the temperature range of 30 to 35ºC and $MgSO_4$ concentration of 1.2 to 1.6 g/L, showing a conversion of 4.84 mol H_2/mol lactose. This clearly indicated that increases in the temperature had an effect on cell growth and then in the synthesis of the target-product. However, further investigation would provide a better understanding of cell metabolism concerning salts influence, such as magnesium sulfate, in hydrogen production. If this could be well explained, the process appears promising for a scale-up.

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