

Optimization of Simple Sugars and Process pH for Effective Biohydrogen Production Using *Enterobacter Aerogens*: An Experimental Study

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This communication presents the potential of process parameters including simple sugars and pH for biohydrogen production using facultative anaerobic bacteria *Enterobacter aerogens*. The obtained data was fit in the modified Gompertz equation and the regression coefficient (R^2) was found in the range of 0.998 which provides a strong correlation between the experimental data and the curve fit. The study revealed that glucose is the most compatible and cost effective substrate for biohydrogen production having yield of 0.87mol H₂/mol of glucose consumed. It is also found that there is a strong possibility to use sugar based organic residues from industrial substrates and wastewater for clean energy products which are cost effective and environmental friendly.

Keywords: Biohydrogen, *Enterobacter aerogens*, Pure Sugars, pH.

Introduction

The biohydrogen production process can use a wide variety of organic substrates as feedstock like pure sugars, agricultural wastes, food processing and other industrial organic wastes, etc. However, so far most of the studies have been done at laboratory scale with the pure sugars like glucose, sucrose and starch^{1,2}. Based on the available literature, around 80% of biohydrogen production was found using pure sugars, including monosaccharides, disaccharides and polysaccharides as the feedstock. Also among them, the simple sugars have been the preferable feedstock for biohydrogen production, due to their simple structure and easily degradable qualities. However, the use of pure sugars is found to be costly for large scale biohydrogen production yet, these are the model substrates to study at the laboratory scale. During the recent decades, the fermentative biohydrogen production involving the bacterial inoculums taken from different sources including the sludge of wastewater treatment plant³, pure culture⁴ and/or mixture of more than one bacteria^{5,6} is found to be effective. However, the technology is not yet matured and an extensive research is going globally on to find out the suitable bacterial strain for fermentative hydrogen production through cost effective way. Another fact about the biohydrogen production is

that most of the studies for biohydrogen production involve the use of pure culture of bacterium *Clostridium* sp. Although the process of fermentative hydrogen production is completely anaerobic yet maintaining the anoxic conditions during the whole process is very difficult and requires expensive reducing chemicals (such as, cysteine or cysteine hydrochloride) to eliminate oxygen in the process which enhances the cost of biohydrogen production⁷. To avoid the toxicity of oxygen during the process, the use of some other facultative anaerobic bacteria could be a novel approach. These bacteria belong to the family of Enterobacteriaceae like *Enterobacter* sp. and *E.Coli* have capability to produce biohydrogen in a cost effective way viz. without the addition of expensive chemicals. The facultative anaerobe *Enterobacter aerogens* lives in the intestine of human as well as animal and grows under both aerobic and anaerobic conditions. One of its advantage is its short doubling time and consequent fast rate of hydrogen evolution^{1,2}. This article presents the experimental study of facultative anaerobic bacteria, *Enterobacter aerogens* to utilize different sugars and selected glucose as a substrate, observes the effect of process pH on the pattern of biohydrogen production, besides, the significance of the modified Gompertz equation model.

Materials and methods

The different facultative anaerobic bacteria are present in environment but the selection and

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preparation of bacterial inoculums is required for specific need to fulfil the research objectives. Thus after extensive literature survey a few of them viz. *Citrobacter* sp., *Enterobacter* sp. and *Escherichia coli* are found to be suitable for the present study. Among these only *Enterobacter aerogens* was found to be having some advantages such as, short doubling time (0.25h) and consequent fast rate of biohydrogen evolution^{8,9} as compared to other bacterial strains, while the preparation of bacterial inoculums and the details of experimental setup are given as below.

Preparation of bacterial inoculums

A bacterial culture of *Enterobacter aerogens* was brought from Microbial Technology and Culture Centre, Chandigarh (India) and two nutrient growth medium were used for the study, one for bacterial culture development and another for biohydrogen production. The selected bacterial strain were grown in medium having beef extract of 1 g/L with yeast extract of 2 g/L, Peptone of 5 g/L and NaCl of 5 g/L while the fermentation medium having (NH₄)₂SO₄ of 4.0 g/L, KH₂PO₄ of 4.0 g/L, Na₂HPO₄ of 4.0 g/L, yeast extract of 1.0 g/L; MgSO₄ of 0.20 g/L with trace element solution of 2 mL (including HCl of 1.0 mL/L, MnCl₂·4H₂O, 100 mg/L, ZnCl₂, 70 mg/L; H₃BO₃, 60 mg/L; CoCl₂·6H₂O, 200 mg/L; CuCl₂·2H₂O, 10 mg/L; NiCl₂, 20mg/L; Na₂MoO₄·2H₂O, 30 mg/L), respectively.

Experimental set-up

The biohydrogen production measurement during the study was done by water displacement method. Substrate with bacterial inoculums was fermented in anaerobic aspirator bottle in a batch set-up, based on the Walker *et al.*, (2009)¹⁰ with the little modification such as, the use of stirrer, gas collection port and liquid sampling port. The total volume of the batch reactor was of 1000 mL with working volume of 600 mL for the study, while 10% v/v microbial seed culture of the fermentation medium sample with the initial pH of 6.5 was kept for this study.

Analytical methods

Gas samples were collected using a gas sampling injector and a sample volume of 100-200 µmL used for each run. The hydrogen content in the produced gas was determined with Gas Chromatograph (GC 5765, Nucon India Make) equipped with thermal conductivity detector (TCD) and a stainless steel column that was 6 feet long with a ¼ inch OD and 2mm ID contained Porapak Q 100 that had a mesh range from 80–100. Nitrogen was used as a carrier gas

at a flow rate of 20 mL min⁻¹. The operational temperature of the oven, injector port and detector were kept as 70°C, 120°C and 120°C, respectively. The sugar content was determined by DNS method¹¹ and biomass growth was measured at 600 nm using UV-Vis Spectrophotometer (Perkin Elmer Lambda 35). The cumulative volume of hydrogen gas production obtained in the anaerobic reactor was modelled using the modified Gompertz equation, as given below¹²:

$$H(t) = P \exp \{ -\exp [(R_m e / P) (\lambda - t) + 1] \} \quad \dots (1)$$

Where, H(t) represents the cumulative volume of hydrogen production (mL) as a function of time, P is the gas production potential (mL), R_m is the maximum production rate (mL/h), λ is the lag time (h), t is incubation time (h) and e is the exponential constant having value of 2.718¹². The typical cumulative hydrogen production curve was nonlinearly modelled by the above equation, while other parameters viz. P, R_m and λ were estimated using non-linear curve fitting tool of Origin 8.5 as used by Xiao *et al.* (2014)¹³.

Results and Discussion

The effects of substrate and pH were observed in relation with biohydrogen production to understand the role of each parameter separately. Most of the facultative anaerobes produce biohydrogen through breakdown of glucose to pyruvate forwarded by volatile fatty acid formations during fermentation⁷, while the growth pattern of the bacteria in growth medium was analyzed after each hour for OD₆₀₀. The growth of selected bacteria viz. *Enterobacter aerogens* was started after the lag phase of 2 hours in growth media and the exponential phase ended at 24th hour. The stationary phase of the bacteria was achieved after 24th hour in 30 hours retention time as shown in Figure 1.

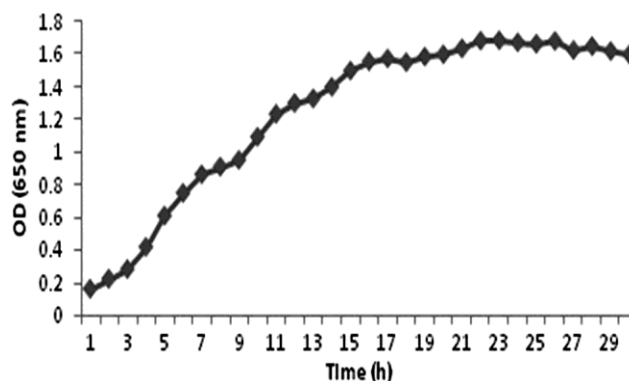


Fig.1- Growth curve of bacteria in medium

Effect of various sugars

The enteric organisms on fermentation of carbohydrates produce lactic acid, formic acid, succinic acid, ethanol and gases (CO_2 and H_2). Although, the selected bacteria showed biohydrogen production with all sugars taken in this study viz. Maltose, Sucrose L-Arbinose, Cellobiose, Glucose and Lactose yet, the rate of production with different sugar was found to be different, varying from 2.13 mol/mol to 0.33 mol/mol. In other words, the biohydrogen production was found to be the highest at 2.13 mol/mol for Cellobiose, followed by Glucose as of 0.87 mol/mol, which is around 40% of the former. On the other hand, it is found to be 0.73 mol/mol for Sucrose followed by L-Arbinose of 0.58 mol/mol, whereas, it was found to be just 0.33 mol/mol for both Lactose and Maltose, respectively. From the above results, it is found that the selected bacteria is more compatible with simple sugars in this study viz. Cellobiose (2.13 mol/mol) followed by Glucose (0.87 mol/mol) as the maximum production was achieved from Cellobiose. On the other hand, it is found to be the least compatible with Lactose and Maltose having the minimum production of 0.33 mol/mol only. Further, it seems that the present bacteria have better adaptation to Cellobiose as compared to that of the Glucose, as a result there is a sharp 40% decrease in the production of biohydrogen with the later, as can be seen clearly from the above results. The reason maybe that Cellobiose was found to be main product of cellulolysis by various bacterial strains¹⁴⁻¹⁶. Also, better yield obtained by Cellobiose is due to the phosphorylation in the Cellobiose phosphorylase. The results shown above exhibit that both Glucose and Cellobiose both have good potential for biohydrogen production with selected bacterial strain, but due to cost limitation with Cellobiose, Glucose is found to be preferable. In other words, it is found that the cost of Cellobiose, is INR 14,852 per 100 gm, while for Glucose is found to be INR 2,773 per 100 gm with the same make viz. Sigma Aldrich. Thus, the production of biohydrogen with the former is around INR 6,972/mol while with later is it around INR 3,187/mol, which is around two times expensive for Cellobiose as compared to that of Glucose. Thus keeping in mind the economics of the biohydrogen production cost/mol, Glucose was selected as a model substrate for which the experimental set-up was installed, which lasted for around 30 hours. For

glucose (substrate), the initial lag period was observed in the range of 4-5 hours for the substrate concentration at 10 g/L. The concentration of H_2 gas was found to be 35% as per gas chromatograph analysis viz. H_2 was 210 mL (600 mL water displaced) or 105 mL H_2 /g glucose (0.105 l/g glucose) i.e. 0.87 mol/mol of glucose. This low yield was due to decrease in the pH of the medium, during the fermentation. Although, the concentration of volatile fatty acid (VFA) was not measured during the study due to instrumental limitations at that time but from the literature we can say that the decrease in pH was the indication of increase in the concentration of VFA and thus biohydrogen production was somewhat inhibited¹⁷. However, the yield of biohydrogen from one mol of glucose is 1 mol from enteric bacteria and 2.3 mol from other bacteria such as, *Clostridium butyricum*¹⁸. The initial concentration of glucose was taken as 10g/L and after fermentation only 6g/l was observed in the fermented solution of the reactor. Thus around 4g/L (40%) of glucose was consumed by the bacterial organisms and 0.87 mol H_2 /mol of glucose was obtained as biohydrogen yield and the rest may have been converted to other fermentation products (succinic acid, lactic acid, acetic acid).

Effect of pH

The effect of initial pH on biohydrogen production was investigated in the process from 6.5 to 4.5, as shown in Figure 2. From the graphical representation, it is clearly seen that both biohydrogen production and yields were strongly dependent on the pH of the solution. In the initial hours of the fermentation, the gas production was moderate (pH 6.5 to 6.0) but as the time exceed to 7th hour, the gas production was found to be increasing, while with pH of 5.5 the highest gas production was observed at 12th hour of the fermentation. However, the pH below 5.5 found to unsupportive of gas production and hence, the production decreased drastically. These findings were found to be in good agreement and comparable with those reported in the literature⁹. Effect of pH on biohydrogen production was determined by fitting the cumulative biohydrogen production data in modified Gompertz equation (Table 1). Kinetic parameters such as P, Rm and λ were determined from the curve plotted between cumulative biogas production and time (Figure 3), where the data points represent the experimental data modelled non-linearly. The R^2 value (0.98) is found close to one which indicates the best fit of experimental data on curve. Lower

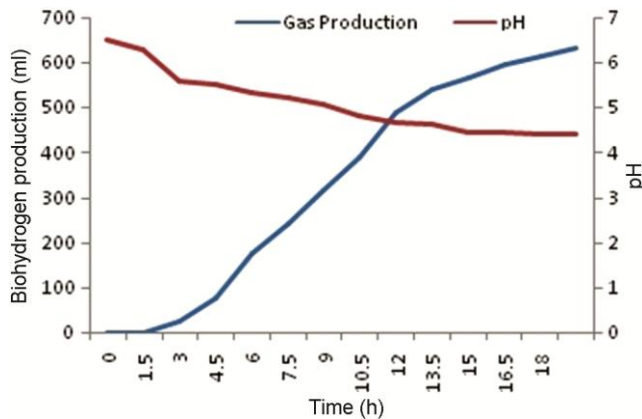


Fig.2- Substrate consumption and reduction in pH during process

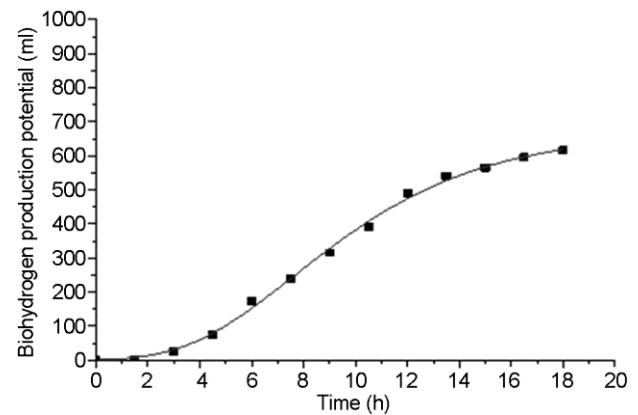


Fig.3- Typical cumulative biohydrogen production curve fitted by the modified Gompertz equation with glucose as substrate

Table 1 - Comparative review of kinetic parameters as obtained by modified Gompertz equation

Organism	Substrate used	Optimal pH	P (ml)	Rm (ml/h)	λ (h)	Reference
<i>Clostridium butyricum</i>	Sucrose	5.5	-	-	-	[21]
<i>Clostridium butyricum</i> CWBI1009	Glucose	5.1	-	-	-	[22]
<i>Caldimonas taiwanensis</i> On1	Starch	7.5	-	-	-	[23]
<i>Clostridium</i> sp. YM1	Glucose	6.5	-	-	-	[24]
<i>E. cloacae</i> IIT-BT 08	Glucose	6.5	889.38	13.97	4.04	[25]
<i>R. sphaeroides</i> 0.U.001	Glucose	-	615.65	16.15	14.3	[26]
<i>E. cloacae</i> IIT-BT 08	Glucose	pH 6.5 (Initial)	343.7	29.8	25	[27]
<i>E. cloacae</i> IIT-BT 08	Glucose	pH 6.5 (Regulated)	796.1	72.1	25	[27]
<i>E. cloacae</i> IIT-BT 08	Glucose supplemented with Na+	5.5 (Initial)	560.2	47.1	8.6	[27]
<i>Enterobacter aerogens</i>	Glucose	5.5	670	36.7	7.6	This study

biohydrogen production at high pH (6.5, 6.3) may be attributed to solvent toxicity due to lower reaction rate of hydrogenase enzyme responsible for H_2 production. From the results, it is also observed that the acidic condition in the reactors favours the H_2 production, however, process pH of 4.6 in the mechanism onwards seem to decrease in production trend. This may be due to the metabolic activities of *Enterobacter aerogens* which could not be maintained at low pH ranges, resulting inhibition in H_2 production. Extreme high and low pH values affect yield of hydrogen production during the fermentation process²⁷⁻³¹. A low pH during fermentation process limits the bacterial growth and biohydrogen production process, which is obtained due to the formation of weak acid such as acetic acid, succinic acid and lactic acid. These acids are the major by product of Genus *Enterobacter* in biohydrogen production process³². In another study, significant biohydrogen production by *Enterobacter aerogens*, was obtained even at pH 4.0 in a continuous reactor, hence it can be concluded that, in batch mode biohydrogen production process pH control is needed to obtain stable hydrogen production. Finally, the

results obtained in this particular study are more general and found to be close to those obtained by earlier works²⁶⁻²⁷. The biohydrogen yield and value of kinetic parameters found in present study is close to that obtained by Thong *et al.* (2011)³³. Similarly, the optimum pH and substrate concentration is found to be similar to those obtained by Nath *et al.* (2006)²⁶. Hence, the results obtained by earlier workers given in the literature can be directly derived from the present paper as special cases depending on the condition of optimum pH, substrate composition and concentration.

Conclusion

This communication highlights the importance of substrate composition (simple sugar i.e. glucose) with process pH at the regular interval for the rate of biohydrogen production using *E. aerogens*. From the above study, it can be concluded that the selected pure bacterial strain of *Enterobacter aerogens* shows the feasibility to produce biohydrogen from various simple sugars and it is most compatible with glucose. It has also been observed and concluded that process pH of 5.5 at 12th hour gave the best results as

compared to the extreme high and low ranges in the orderly mechanism of the biohydrogen production. This finding was significant with the modified Gompertz equation model. The experimental results also showed that pure bacterial strain, *Enterobacter aerogens* utilizes glucose and conversion into biohydrogen and organic acids strongly dependent on the bacterial isolate in comparison with other researcher's data. Thus, there is a possibility to use glucose and glucose based organic substrate materials, residues and wastewater from industries for the biohydrogen production, as clean energy source for sustainable development.

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