Biohydrogen production by *Clostridium beijerinckii*

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Abstract

Climate change, along with the rapid depletion of petroleum and natural gas reserves, has prompted many to search for renewable and environmentally friendly energy options. Hydrogen has been identified as a possible alternative to fossil fuel energy. Biological hydrogen production from organic substrates can be achieved by a two-stage approach combining the anaerobic and photosynthetic continuous processes in series. As a first step, an investigation of the production of hydrogen from glucose by *Clostridium beijerinckii* was conducted. A study examining the effect of initial pH (range 5.7 to 6.5) and COD loading (range 1 to 3 g/L) on the specific conversion and specific hydrogen production rate has shown interaction behaviour between the two independent variables. The highest conversion of 10.3 mL H_2/(g COD/L) was achieved at pH of 6.1 and COD of 3 g/L, whereas the highest production rate of 71 mL H_2/(h*L) was measured at pH 6.3 and substrate loading of 2.5 g COD/L. In general, there appears to be a strong trend of increasing hydrogen production rate with an increase in both substrate concentration and pH. The current work focuses on the identification of soluble metabolites such as ethanol, acetate and butyrate in order to evaluate the possible shift in metabolism resulting from varying initial conditions and micro-nutrients availability. The results obtained so far, along with some preliminary experiments using industrial wastewater as substrate, indicate the possible use of such waste streams for the production of biohydrogen.

Keywords: biohydrogen, wastewater, clostridium.

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1 Introduction

Since the industrial revolution humans have been heavily dependent on fossil fuels for their energy source, and with the growing economy and globalization that dependence is only expected to intensify. In 2003, the Energy Information Administration (EIA) estimated the total world energy consumption at 420.7 quadrillion BTU, a number which is projected to increase to almost 510 quadrillion BTU by the year 2010 [1]. This continuing demand has already put an enormous strain on the available fossil fuel reserves and there is growing concern about energy supply and security. The 2003 NEB report was predicated on an assumption that fossil fuel prices would remain stable at US$22 per barrel WI (West Texas Intermediate) until 2025; in reality, WI prices for 2006-2008 were expected to triple to an average of US$65 per barrel with peaks as high as US$80 [1]. Peaks as high as US$96 were observed in 2007, reflecting a dramatic rise in price and uncertainty in market supply.

In the 2005 World Energy Report, the German Federal Institute for Geosciences and Natural Resources (BGR) estimated that more than 60% of the total potential petroleum has been extracted in North America and almost 50% in Western Europe [2]. Similarly, the total global natural gas (NG) reserves were reported at 6112 trillion cubic feet as of January 1, 2006, and the 2003 global annual NG consumption was 95 trillion cubic feet [1]; without accounting for the growth in demand, this would suggest that our resources will run out in approximately 60 years. Despite the claim by some that evolving technology will allow us to tap into currently unavailable energy sources, our present reliance on finite energy resources cannot be sustainable in the long term. Such over reliance on a very narrow portfolio of energy supply stocks has prompted serious interest in the development of sustainably produced replacement fuels.

The increasing energy consumption has not only put a strain on our raw resources, but also on our planet and its ecosystems. Incomplete combustion processes, which result from the burning of fossil fuels, produce a great amount of gases such as carbon dioxide (CO_2) and nitrogen oxides (NOx). Carbon dioxide emissions are of particular concern, since CO_2 has been identified as a green house gas (GHG). The atmospheric concentration of GHGs has been steadily rising and in 2005 the concentration of CO_2 rose to a record high of 378.9 ppm [3]. This increase has been directly linked to human activity; over 25,000 million metric tons of CO_2 were emitted in 2003 [1], a value that is projected to grow over the years. High concentrations of GHGs have resulted in a rise of the average surface temperatures as well as adverse effects on weather patterns, human and animal life.

Emission of green house gasses, along with the rapid depletion of oil and gas reserves, has prompted many to search for renewable and environmentally friendly energy alternatives. Among others, hydrogen has been identified as a possible alternative to fossil fuel energy and a worldwide investigation of hydrogen as a future energy carrier is now underway. The current hydrogen production methods can be grouped into three main

categories: fossil fuel processing which includes natural gas, coal, and biomass as the process input; electrolysis of water using conventional, renewable, or nuclear energy for the electricity source; and modern approaches which include photo-synthetic and photo-biological splitting of water [4, 5]. Hydrogen is widely recognized as an environmentally friendly energy carrier and, when generated from renewable residues, promises a substantial reduction in the overall GHG emissions.

Biological production of hydrogen is proposed as a promising avenue for the production of hydrogen because it is less energy intensive, more environmentally friendly and can be based on various substrates. Biological hydrogen production processes can be classified as follows: 1. Biophotolysis of water using algae and cyanobacteria; 2. Photodecomposition of organic compounds by photosynthetic bacteria; 3. Fermentative hydrogen production from organic compounds, and 4. Hybrid systems using photosynthetic and fermentative bacteria. Among these, the photosynthetic bacteria are favoured candidates for large-scale production due to their high substrate conversion efficiencies and their capability of producing hydrogen from a wide variety of substrates [6]. Fermentative bacteria are also favourable candidates due the lower operating costs, (when compared to photosynthetic bacteria) because there are no light requirements and growth of the microbes is simpler. Although the technical feasibility of fermentative hydrogen production from a variety of substrates has been well demonstrated in literature, the technology is still at its infancy [7]. Reviews [8, 9] demonstrate that most research work has been done using batch processes, while continuous set-ups are potentially more attractive. Previous studies on the production of biohydrogen using both fermentative and photosynthetic bacteria have also shown that research is still needed to improve the conversion efficiencies [10] and the stability of the hydrogen production [11].

Due to inherit thermodynamic limitations associated with the fermentative approach, a two-step biological hydrogen production process has been suggested. By combining the anaerobic and photosynthetic steps, higher overall substrate conversion efficiency is possible, since the photosynthetic microbes can degrade soluble metabolites from the fermentative step using sunlight to overcome the energy barrier. Recently, Kim *et al* [12] achieved conversion efficiency 30% higher than previously reported, using a two-step process which utilized *Clostridium butyricum* and *Rhodobacter sphaeroides* in the fermentative and photosynthetic degradation, respectively, from a *C. reinhardtii* biomass (mainly glucose-starch) substrate.

A long list of possible feedstocks could be considered as potential substrate for biohydrogen production. The use of various waste streams such as glycerol containing waste from the biodiesel production, non-woody residues, and agricultural products seems to be an interesting avenue. However, further improvement of the technology and conversion efficiency is still required. This paper aims to investigate the feasibility of using industrial waste water as a substrate for biological hydrogen production using a pure culture bacterium of *Clostridium beijerinckii*, with hopes of creating an economically attractive process in the long term.

2 Materials and methods

2.1 Bacterial cultivation

Pure culture of *Clostridium beijerinckii* was purchased from ATCC (#8260) and used for the duration of the study. The bacterium was pre-cultured in the recommended nutrient broth (Difco™ Reinforced Clostridial Medium) at 30°C in serum bottles inside a dark incubator shaker. The bacteria were transferred 2-3 times and cultivated for 12 hours (till stationary phase) between each transfer prior to use in the experiments in order to ensure a healthy and active culture population.

2.2 Experimental procedure

Batch hydrogen production experiments were carried out in 100 mL serum bottles with a working volume of 50 mL. The media contained a prescribed amount of glucose as well as essential growth nutrients [13] (in mg/L) 850, KH_2PO_4; 750, K_2HPO_4; 3, H_3BO_3; 200, $MgSO_4.7H_2O$; 1, $Na_2MoO_4.2H_2O$; 1, $ZnSO_4.7H_2O$; 2, $MnSO_4.4H_2O$; 0.1, $Cu(NO_3)_2.3H_2O$; 1, $CaCl_2.2H_2O$; 2, EDTA; 12, $FeSO_4.7H_2O$; 4, thiamine; 3, biotin; 5, *p*-aminobenzoic acid; 6.5, nicotinamide; 420, glutamic acid; 1, resazurin. A 0.1 M phosphate buffer was also added, in order to prevent pH decrease due to organic acid accumulation during the bacterial metabolism. The initial pH was adjusted to a desired value using 5N NaOH or 5N HCl. The media was dispensed into the bottles, which were then flushed with oxygen-free argon gas, capped with a butyl rubber stopper, sealed with an aluminium crimp and sterilized in an autoclave. In order to remove any residual oxygen in the media, prior to inoculation a reducing agent, $Na_2S.9H_2O$, was added at a 0.025% (w/w) concentration. All bottles were inoculated with 3% (v/v) *C. beijerinckii* in the stationary phase and incubated in an orbital shaker at 30°C $\pm$ 1°C and rotational speed of 180 rpm. In total, thirteen experiments were conducted each in triplicate to ensure reproducibility.

2.3 Factorial design

In order to analyze the effect of initial glucose concentration and initial pH as well as any interactions between the two variables, a fractional factorial design was employed [14]. A nine trial design was constructed as shown in Figure 1 to

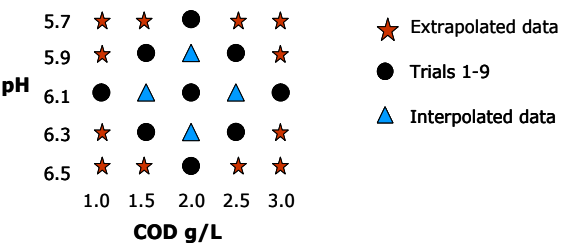

Figure 1: Fractional factorial design.

cover the area of interest. The substrate concentration varied from 1 to 3 g COD/L with the central value of 2 g COD/L and the pH varied from 5.7 to 6.5 with a central value of 6.1. Both of the ranges were based on values previously observed in a number of local industrial wastewater streams (data not shown). The pH range was modified from the original design of 4.5 - 6.5 after no growth was observed in the lower end of the range.

2.4 Analytical methods

Biogas production was periodically measured using 50, 25 and 10 mL glass syringes fitted with hypodermic needles as described by Owen *et al* [15]. The biogas was sampled from the headspace with a 2 mL gas tight syringe and analyzed for the amount of hydrogen (H_2%) using a gas chromatogram (Hewlett-Packard 5890) equipped with a thermal conductivity detector (TCD). The GC was fitted with a stainless steel molecular sieve column (6" x 1/8") and the injector, oven and detector temperatures were set at 100°C, 80 °C and 100 °C, respectively. Argon was used as the carrier gas with a flow rate of 2 mL/min. Final glucose concentrations were analyzed using a glucose (HK) assay kit (Sigma-Aldrich).

2.5 Data analysis

The cumulative hydrogen gas production curves were constructed as previously described [16] by measuring the gas composition in the headspace of the bottle and the total volume of the biogas produced at each time interval, and applying the mass balance equation (eqn 1),

$$V_{H,i} = V_{H,i-1} + C_{H,i}\left(V_{G,i} - V_{G,i-1}\right) + V_H\left(C_{H,i} - C_{H,i-1}\right) \quad (1)$$

where $V_{H,i}$ and $V_{H,i-1}$ are cumulative hydrogen gas volumes at the current *(i)* and previous *(i-1)* time interval, $V_{G,i}$ and $V_{G,i-1}$ are the total biogas volumes at the current and previous time interval, $C_{H,i}$ and $C_{H,i-1}$ are the fractions of hydrogen gas in the headspace of the bottle as determined by gas chromatography in the current and previous interval, and V_H is the total volume of headspace in the bottle.

Each of the cumulative hydrogen production curves was modeled using the modified Gompertz equation (eqn 2),

$$H(t) = H_{\max} * \exp\left\{-\exp\left[\frac{R * e}{H_{\max}}(\lambda - 1) + 1\right]\right\} \quad (2)$$

where *H(t)* is the cumulative hydrogen production (mL) during the course of the incubation time, *t* (hours), H_{max} is the hydrogen production potential (mL), *R* is the maximum production rate (mL H_2/h) and λ is the duration of the lag phase (h). This model has been commonly used to describe biological production of various gases such as methane, hydrogen and biogas in a batch set up [17-19]. The cumulative production curves were fit using Matlab 6.5 by minimizing the sum of square error (SSE). Initial estimates for the parameters (H_{max}, *R* and λ)

were selected based on visual inspection. The hydrogen production potential was normalized with respect to substrate concentration to give the conversion efficiency, P_s (mL H_2/g COD/L); the specific H_2 production rate, R_s was defined as R/V_{media} (mL H_2/h-L).

3 Results and discussion

In order to assess the effect of substrate concentration and initial pH on the conversion efficiency and the specific production rate of hydrogen, the data obtained from the Gompertz modelling was graphed in two three-dimensional plots. A second-order polynomial regression was conducted in order to interpolate/extrapolate results to cover the entire region of interest as outlined in Figure 1. The main objective of the regression was to enhance visual understanding of the types of trends that exist within the matrix.

As shall be shown in Section 3.3, the extrapolated regions turned out to be of particular interest upon visual inspection. Four additional experiments, covering the corner points of the matrix, were carried out in order to validate these initial results. The revised fractional factorial design can be seen in Figure 2.

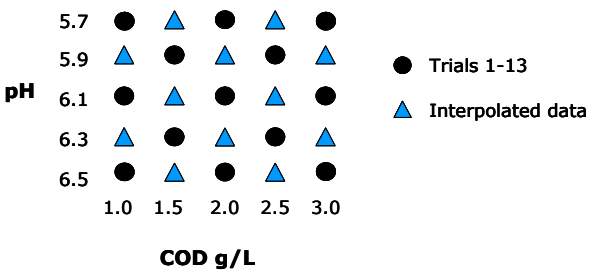

Figure 2: Revised fractional factorial design.

By eliminating extrapolated regions from the design a more concrete conclusion can be made with regards to the location of the area where initial pH and substrate concentration provide the most promising operational conditions.

3.1 Specific hydrogen production potential

The specific hydrogen production potential, or the conversion efficiency, fitted based on the original design, is plotted in Figure 3a. It is clear from the data that COD loading and initial pH play a role on the yield. The trends indicate that higher conversion is achieved at higher glucose loading and a mid-range pH in the vicinity of 6.0; the highest P_s of 10.3 mL H_2/g (COD/L) was measured at substrate concentration of 3 g COD/L and pH of 6.1. Both high and low-end pH appears to be unfavourable, but this effect is secondary to that of substrate concentration where the difference between the highest and lowest expected value is almost double.

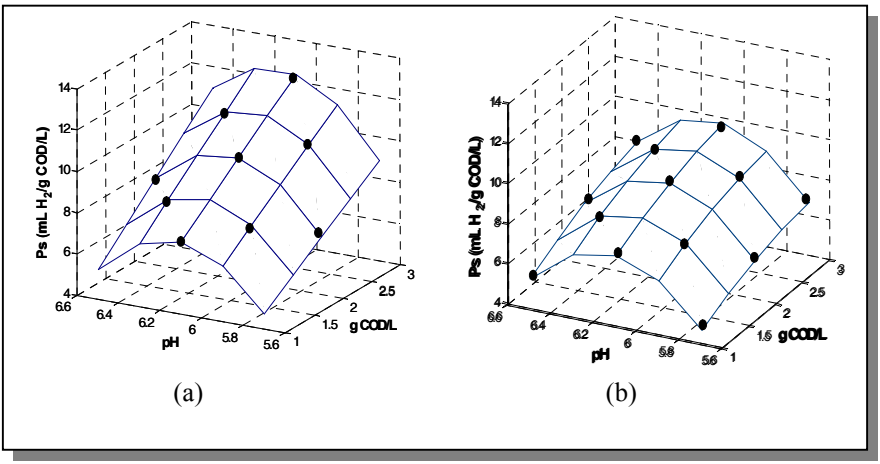

Figure 3: Specific hydrogen production potential (a) based on the fractional factorial design (9 trials); (b) based on the revisited design (13 trials). The marked points indicate the sum of measured values and the residual of the regression.

When the extra four trials (corner points) were added to the matrix, however, changes were observed in the regression of the response variable, P_s (see Figure 3b). In general, the trends discussed above remain intact, though their degree does not appear to be as pronounced. Specifically, increasing substrate concentration appears to play a smaller role on the yield than initially anticipated.

3.2 Specific hydrogen production rate

The specific hydrogen production rate obtained from the nine-trial data is shown in Figure 4a. The data indicates that both initial pH and glucose concentration have noticeable effects on the rate of hydrogen production, and also that there is interaction between these two parameters. At low substrate loadings, the largest R_s appears to be located in the middle of the pH range, in the vicinity of 6.1. As the glucose concentration increases, however, the high-end pH values seem to favour greater hydrogen production rates. Low pH gives poor R_s across the entire substrate concentration span. This is somewhat expected, as similar trends have been reported in other works [17, 19]; low pH tends to have an inhibitory effect on the bacteria causing a longer lag phase and lower rate of production.
Based on Figure 4a, the largest R_s appears to be at high initial pH and substrate concentration, a region of the plot that is mostly extrapolated. As a result, conducting experiments at the corners of the matrix became crucial prior to drawing final conclusions regarding the location of highest hydrogen production rate. The new surface plot can be seen in Figure 4b. In general the shape, as well as the scale of the graph, remained unchanged, with slight alterations to the

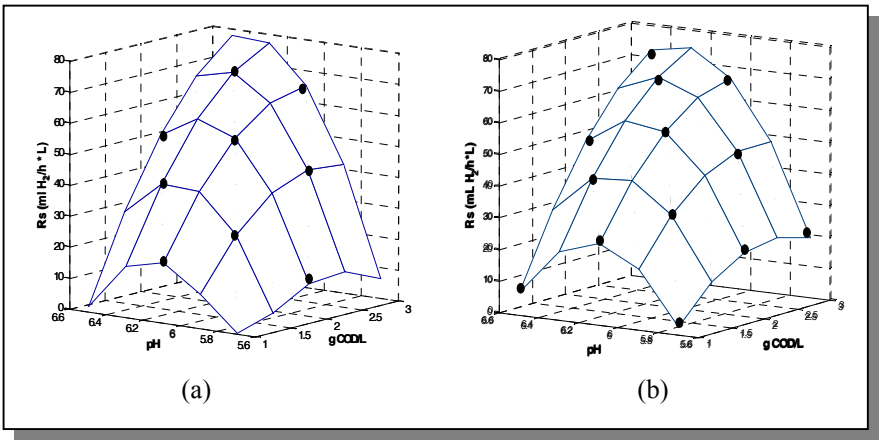

Figure 4: Specific hydrogen production rate (a) based on the initial fractional factorial design (9 trials); (b) based on the revised design (13 trials). The marked points indicate the sum of measured values and the residual of the regression.

extrapolated areas. The highest R_s of 71 mL H_2/(h*L) was measured at pH 6.3 and substrate loading of 2.5 g COD/L, and the region around these parameter values seems to consistently produce similarly high rates.

In practice, any treatment/conversion process strives for high yield and fast rate but often, such as in this case, the most promising operating conditions are different for each variable of interest. When this occurs, a compromise must be made. In this system, out of the two response variables, P_s and R_s, the rate is of more interest since continuous biogas systems operate more optimally at low to mid-range hydraulic retention times [20, 21] meaning that maximum conversion is hardly ever achieved. For this reason, more effort should be devoted to maximizing the rate of hydrogen production with partial sacrifice to the conversion efficiency.

4 Future work

Current work is focusing on the identification and quantification of the soluble metabolites from the batch experiments such as ethanol, acetate and butyrate in order to evaluate the possible shift in metabolism with varying operating conditions. Future work will involve characterization of industrial wastewater samples, testing their potential as a substrate for biohydrogen production, as well as set-up of a semi-continuous biohydrogen production process.

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References

[1] Energy Outlook 2006; Energy Information Administration, Online. http://www.eia.doe.gov/oiaf/aeo/
[2] World Energy Report 2005; RWE, Online www.rwe.com
[3] Shein, K.A., ed., State of the Climate in 2005. *Bulletin of the American Meteorological Society*. **87**: p. S1–S102, 2006.
[4] McHugh, K., *Hydrogen Production Methods*. MPR Associates Inc., 2005.
[5] Duwe, M., *Hydrogen technology overview*. Climate Action Network Europe, 2003.
[6] Koku, H., I. Erlogu, U. Gunduz, M. Yucel and L. Turker, Kinetics of biological hydrogen production by the photosynthetic bacterium Rhodobacter sphaeroides O.U. 001. *International Journal of Hydrogen Energy*, **28(4)**, pp. 381–388, 2003.
[7] Hawkes, F.R., R. Dinsdale, D.L. Hawkes and I. Hussy, Sustainable fermentative hydrogen production: challenges for process optimisation. *International Journal of Hydrogen Energy*, **27(11-12)**, pp. 1339–1347, 2002.
[8] Hallenbeck, P.C. and J.R. Benemann, Biological hydrogen production; fundamentals and limiting processes. *International Journal of Hydrogen Energy*, **27(11-12)**, pp. 1185–1193, 2002.
[9] Kapdan, I.K. and F. Kargi, Bio-hydrogen production from waste materials. *Enzyme and Microbial Technology*, **38(5)**, pp. 569–582, 2006.
[10] Yetis, M., U. Gunduz, I. Eroglu, M. Yucel, and L. Turker, Photoproduction of hydrogen from sugar refinery wastewater by Rhodobacter sphaeroides O.U. 001. *International Journal of Hydrogen Energy*, **25(11)**, pp. 1035–1041, 2000.
[11] Yu, H., Z. Zhu, W. Hu and H. Zhang, Hydrogen production from rice winery wastewater in an upflow anaerobic reactor by using mixed anaerobic cultures. *International Journal of Hydrogen Energy*, **27(11-12)**, pp. 1359–1365, 2002.
[12] Kim, M.-S., J-S. Baek, Y-S. Yun, S. Jun Sim, S. Park and S-C. Kim, Hydrogen production from Chlamydomonas reinhardtii biomass using a two-step conversion process: Anaerobic conversion and photosynthetic fermentation. *International Journal of Hydrogen Energy*, **31(6)**, pp. 812–816, 2006.
[13] Fang, H.H.P., H. Zhu, and T. Zhang, Phototrophic hydrogen production from glucose by pure and co-cultures of Clostridium butyricum and Rhodobacter sphaeroides. *International Journal of Hydrogen Energy*, **31(15)**, pp. 2223–2230, 2006.
[14] Box, G.E.P.H., Hunter, W. G., Hunter, J. S., *Statistics for experimenters: an introduction to design, data analysis, and model building*, New York: Wiley, 1978.

[15] Owen, W.F., D.C Stuckey, J.B. Healy, L.Y. Young, and P.L. McCarty, Bioassay for monitoring biochemical methane potential and anaerobic toxicity. *Water Research*, **13(6)**, pp. 485–492, 1979.
[16] Oh, S.E., S. VanGinkel, and B.E. Logan, The Relative Effectiveness of pH Control and Heat Treatment for Enhancing Biohydrogen Gas Production. *Environmental Science and. Technology*, **37(22)**, pp. 5186–5190, 2003.
[17] Sung, S., L. Raskin, T. Duangmanee, S. Padmasiri, and J.J Simmons, Hydrogen production by anaerobic microbial communities exposed to repeated heat treatments. *Proceedings of the 2002 U.S. DOE Hydrogen Program Review*, 2002.
[18] Shin, H.-S., S.-H. Kim, and B.-C. Paik, Characteristics of hydrogen production from food waste and waste activated sludge. *Journal of Water and Environment Technology*, **1(2)**, pp. 177–187, 2003.
[19] Khanal, S.K., W.H. Chen, L. Li, and S. Sung, Biological hydrogen production: effects of pH and intermediate products. *International Journal of Hydrogen Energy*, **29(11)**, pp. 1123–1131, 2004.
[20] Chen, W.-H., S-Y. Chen, S. Khanal and S. Sung, Kinetic study of biological hydrogen production by anaerobic fermentation. *International Journal of Hydrogen Energy*, **31(15)**, pp. 2170–2178, 2006.
[21] Cheong, D.-Y., C.L. Hansen, and D.K. Stevens, Production of bio-hydrogen by mesophilic anaerobic fermentation in an acid-phase sequencing batch reactor. *Biotechnology and Bioengineering*, **96(3)**, pp. 421–432, 2007.