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Biohydrogen Production by Local Isolate of *Clostridium butyricum:* Initial Nutrients Optimization Study

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ABSTRACT

In this study, an anaerobic mesophilic bacterial strain, namely *Clostridium butyricum* KBH1, was isolated from a natural source. This strain grew well and produced biogas with an average hydrogen concentration of 60% (v/v) in the Reinforced Clostridial Media (RCM). To study the basic nutrient requirements, three main nutrients namely peptone (Pep), yeast extracts (Yes) and glucose (Glu) were chosen as factors, using an experimental design. The experiments were run according to 2^3 Full Factorial Design, followed by the Response Surface Method (RSM). The fermentation was performed in 30 ml serum bottles with 20 ml working volume in a sterile and anaerobic condition at 37°C with 5% inoculums. The results from the Analysis of Variance (ANOVA) for the factorial design showed that all the three factors had significantly affected the gas production by the *C. butyricum*. The response surface plot of the gas production by *C. butyricum* showed that the gas production could be enhanced by increasing peptone and yeast extract concentrations up to 15 g/l and 24 g/l respectively, without showing any substrate inhibition. Meanwhile, the glucose concentration showed an optimum at the middle point (8 g/l) with possible substrate inhibition at a high concentration (12 g/l). The total biogas production could be correlated to the three factors, using the quadratic equation: Gas = 0.17 + 7.11Glu - 0.02Pep + 0.77Yes - $0.53Glu^2$ + 0.09Glu*Pep. The experimental results showed that the strain could grow well in substrate with high organic nitrogen content such as POME and might be not suitable for substrate with high sugar content due to substrate inhibition.

Keywords: Anaerobic fermentation, biohydrogen, *Clostridium*, optimization

INTRODUCTION

Pollution to the environment due to the use of conventional fuels, in conjunction with concerns on the depletion of oil reserves, necessitate the intensification of research for alternative energy sources. Amongst many alternative energy sources, hydrogen (H_2) offers tremendous potential as a clean, renewable energy. According to Levin *et al*. (2004), hydrogen has the highest gravimetric energy density of any known fuel and is compatible with electrochemical and combustion process for energy conversion without producing carbon-based emissions. Therefore, hydrogen fuel cells are considered as the main technology which makes the utilization of hydrogen energy possible (Lin *et al*., 2007).

Hydrogen may be produced by various processes such as electrolysis of water, thermo catalytic reformation of hydrogen rich compounds, and biological processes. Biological production of hydrogen (biohydrogen) using micro-organisms is an exciting new area which offers tremendous potential in using various renewable raw materials (Levin *et al*., 2005). Two main methods for biohydrogen production are possible via photo and dark fermentation. On the contrary to photolytic

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production, dark fermentation has the advantages for high hydrogen production rate, without the need for illumination and capability to utilize organic wastes. Many types of bacteria which have been reported as capable of generating hydrogen in dark fermentation such as the species of *Enterobacter* (Shin *et al*., 2007), as well as *Bacillus* and *Clostridium* (Chen *et al*., 2005).

Several studies on biohydrogen production reported that the hydrogen gas yields are determined by its fermentation pathway and the end-products such as acetic acid and butyric acid. Chen *et al*. (2006) indicated that process operating conditions such as pH, nutrient levels, mixing, levels of carbon dioxide, shock loads, metabolites in liquid phase and gas partial pressure could also affect metabolic balance and alter the hydrogen production.

The Response Surface Methodology (RSM) is widely used to study individual and interactive effects of various factors affecting the final results in any experiment. For instance, Wang *et al*. (2005) used the RSM to study the effect of pH, temperature and substrate concentration on the production of biohydrogen by mixed culture using sucrose as the main carbon source. They reported that all the factors gave a significant influence on biohydrogen production.

The commercial Reinforced Clostridial Medium (RCM) is widely used for isolation and cultivation of Clostridial strains. The RCM contains only 5 g/l glucose while the main nutrients composition are peptone (10 g/l), meat extract (10 g/l) and yeast extract (3 g/l). In this study, three main nutrients, namely Glucose, Peptone and Yeast Extract, were chosen as parameters. Peptone is rich in organic nitrogen content, while yeast extract mainly provides vitamins and amino acids necessary for growth.

The aim of the present study was to isolate, characterize and identify dark fermentative bacteria from the local sources. An initial study on the effect of nutrients concentration was done using the Response Surface Methodology (RSM). The results obtained from the RSM could then be used as a guideline to further optimize raw substrate such as Palm Oil Mill Effluent (POME). This may include amending POME with specific organic nutrients for higher biohydrogen production and simultaneous COD reduction.

MATERIALS AND METHODS

Culture of Micro-organism

Hydrogen producing bacteria was isolated from the water samples of Sungai Langat nearby UKM Bangi, in Selangor. The samples were cultured and purified using the Reinforced Clostridial Medium (RCM) (Oxoid, UK). RCM contains: glucose (5 g/l), peptone (10 g/l), yeast extract (3 g/l), meat extract (10 g/l), starch (1 g/l), NaCl (5 g/l), $NaC_2H_3O_2$ (3 g/l), Agar (0.5 g/l) and Cysteine HCl (0.5 g/l).

Samples were heat shocked at 100ºC for 5 minutes, and 2 ml was transferred into 30 ml serum bottles, containing 18 ml of liquid RCM. The bottles were incubated at 37°C overnight. Then, the cultures which produced gases were sub-cultured into another bottle. The cultures were then streaked onto the RCM agar and incubated at 37°C inside an anaerobic jar (HP00011A, Oxoid, UK). A single colony obtained from agar plate was incubated and analyzed for hydrogen production. Hydrogen producing strains were then stored at 4°C as stock cultures. The morphological identification and observations were performed using a light electron microscope (Nikon YS100, Japan). Bacteria sample were sent to Vivantis Technologies Sdn. Bhd. (Shah Alam, Selangor) for identification using the 16S/18S rRNA sequencing method.

Analytical Methods

Cell density was analyzed by measuring the optical density (OD) of the cell suspension, at a wavelength of 600 nm, using a spectrophotometer (Thermo spectronic, Model Genesys 10_{UV}).

Hydrogen and carbon dioxide were measured by gas chromatography (GC-8A, Shimadzu, Japan) with thermal conductivity detectors; TCD equipped with a stainless steel column packed with Porapak Q. Nitrogen was used as a carrier gas at a flow rate of 30 ml/min. The temperatures of the injection port, oven and detector were 100, 50 and 100ºC, respectively.

Nutrients Optimization

Glucose (Sigma-Aldrich, USA), Peptone (Bacto Peptone, Difco, UK) and Yeast Extract (Bacto Yeast Extract, Difco, UK) were chosen as factors in this study. The experiment was performed in 30 ml serum bottles containing 18 ml medium, with 2 ml inoculums. The amount of glucose, peptone and yeast extracts were varied, while the other ingredients such as NaCl (5 g/l) and $NaC_2H_3O_2$ (3 g/l) were kept constant. The concentration of glucose ranged from 4 to 12 g/l, Peptone from 12 to 24 g/l and yeast extract 5-15 g/l. The evolved gas was collected every 3 hour using a syringe.

A factorial central composite design was used to study the effect of nutrients concentration on gas production. Design expert software version 6.0 (Stat-Ease. Inc., MN, USA) was used for regression and graphical analyses of the experimental data. The optimum levels of the selected variables were obtained by solving the regression equation and analyzing the response surface contour and surface plots. The quality of the fit of quadratic model was expressed by the coefficient of determination R^2, and its statistical significance was checked using the F-test in the same program.

RESULTS AND DISCUSSION

Microbe and Growth Profile

A hydrogen producing bacteria was isolated from a small river stream of Sungai Langat, located near UKM, Bangi. It was observed as a spore former, rod shape, and gram positive bacteria. The result obtained from 16S rRNA sequencing confirmed that the bacteria were closely related to *Clostridium butyricum* species with 99% similarity.

Fig. 1 shows the typical time course profile of pH, growth and gas production of *C. butyricum*. The time course profile is similar as reported by other researchers such as Chen *et al*. (2005) and Pan *et al*. (2007). The differences in terms of yields are due to the different media used and operating condition. The biomass growth, during the exponential phase, was accompanied by pH drop, due to the accumulation of acetic and butyric acid liquid product. The composition of hydrogen, from the GC analysis in a cumulative form, was in the average of 60% v/v and the remaining was CO_2.

Effect of Nutrients Concentration

The present study aimed to see the effects of nutrient concentration on the growth of *C. butyricum* and biohydrogen production. The *Clostridium* species have two different metabolic pathways for the production of H_2 from carbohydrates fermentation; these are acidogenesis which produces mainly organic acids like acetate and butyrate, and solventogenesis which generates solvents such as acetone, and ethanol. Thus, when environmental conditions are favourable, *Clostridium* is able to modify their metabolism to any of these pathways. A pH decrease could therefore be used to induce the shift to solventogenesis along with H_2 production decline. Cheng *et al*. (2002) studied the use of peptone and found that it avoided the abrupt pH drops in the system and allowed for further exploration of organic acids and pH effects on H_2 production.

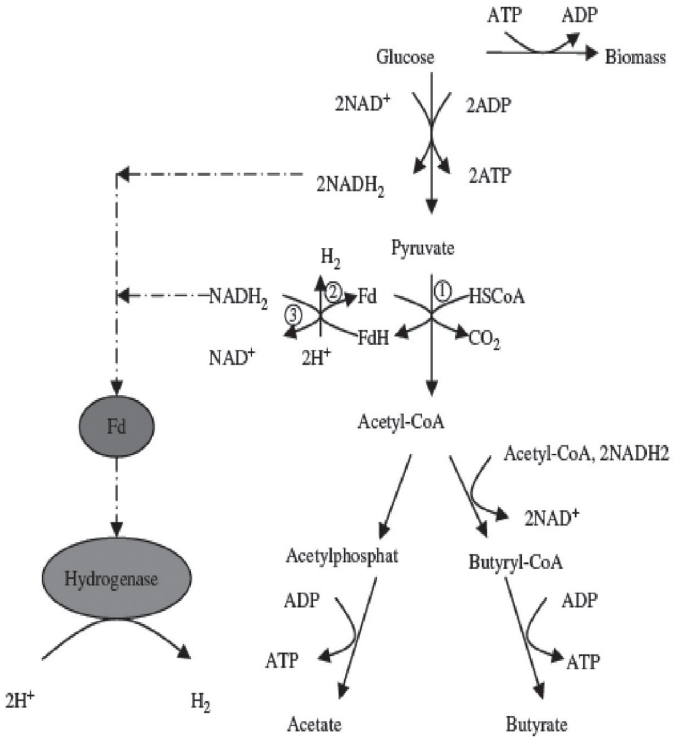

Fig. 1: Metabolic pathway of glucose by Clostridium butyricum *under anaerobic conditions. 1 Pyruvate: ferredoxin oxidoreductase (PFOR); 2 Hydrogenase; 3 NADH: ferredoxin oxidoreductase (Chen* et al., *2006)*

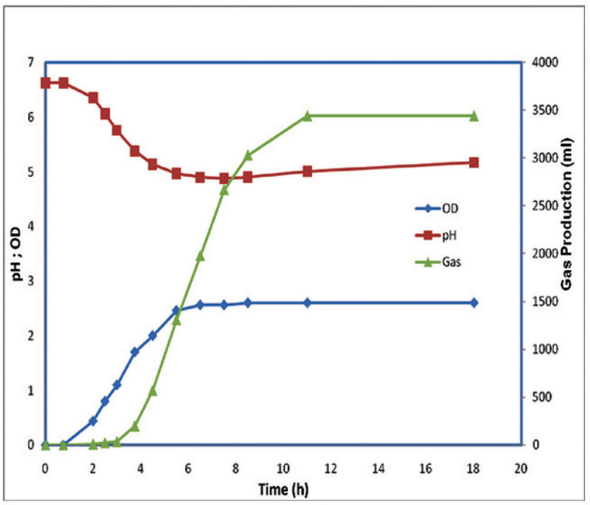

Fig. 2: Typical time course profile for pH, OD and gas production of C. butyricum *growth on RCM with 1.5L working volume*

TABLE 1
Result for the 2^3 full factorial experiments

Run	Factor 1 A: Glucose, g/L	Factor 2 B: Peptone, g/L	Factor 3 C: Yeast, g/L	Response 1 R1 Gas (ml)
1	4	15	5	*31*
2	4	15	15	*35*
3	4	25	5	33
4	4	25	15	*37*
5	12	15	5	*33*
6	12	15	15	*45*
7	12	25	5	*44*
8	12	25	15	*53*

TABLE 2
Effect list and model selection

	Term	Effect	% Contribution
Model	A	9.75	46.05
Model	B	5.75	16.02
Model	C	7.25	25.46
Model	AB	3.75	6.81
Model	AC	3.25	5.12
Error	BC	-0.75	0.27
Error	ABC	-0.75	0.27

Table 1 shows the results for the 2^3 full factorial designs. The analysis of this experimental design yielded seven model terms consisting of three main effects (A = glucose, B = peptone and C = yeast), four interactions (AB, AC, BC & ABC) and one intercept. Table 2 illustrates the model terms which were selected based on the effect or percentage of contribution. Meanwhile, the ANOVA of the selected factorial model showed that the model and all the model terms are significant.

Response Surface Method

The main goal of the response surface analysis was to find the optimum combination of variables in order to maximize the response. The result of the replicate experiments at the middle point is shown in Table 3. Meanwhile, the purpose of the repetition at the middle point was to measure the consistency of the experimental result. Thus, to complete the RSM, experiments were done at the star point, as depicted in Table 4. The first three runs of the star point were used to measure the effect of nutrient concentration deficiency, while the next three runs measured the effect of excessive nutrient concentration.

TABLE 3
Repetition at middle point

Run	Factor 1 A: Glucose, g/L	Factor 2 B: Peptone, g/L	Factor 3 C: Yeast, g/L	Response 1 R1 Gas (ml)
9	8	20	10	*45*
10	8	20	10	*44*
11	8	20	10	*45*
12	8	20	10	*46*
13	8	20	10	*45*
14	8	20	10	*45*

TABLE 4
Star point

Run	Factor 1 A: Glucose, g/L	Factor 2 B: Peptone, g/L	Factor 3 C: Yeast, g/L	Response 1 R1 Gas (ml)
15	1.27	20	10	22
16	8	11.59	10	37
17	8	20	1.59	37
18	14.73	20	10	17
19	8	28.41	10	53
20	8	20	18.41	51

The RSM for the three factors would yield a quadratic model with nine model terms. In this study, however, the model was reduced to only five most significant model terms. After estimating the coefficients of the quadratic model, the amount of the total biogas could be predicted using the following quadratic equation:

$$Gas = 0.17 + 7.11Glu-0.02Pep+0.77Yes-0.53Glu^2 + 0.09Glu*Pep. \quad (1)$$

The three dimensional plots are based on Eq. (1), with one variable kept constant at its optimum level, and varying the other two variables within the experimental range. *Fig. 3* shows the response surface plot of the effect of glucose, peptone and yeast to biogas production by *C. butyricum*. The plot also demonstrates that the production of gas could be enhanced by increasing peptone and yeast extract concentrations up to 15 g/l and 24 g/l respectively, without showing any substrate inhibition. Meanwhile, the concentration of glucose was shown as optimum at the middle point (8 g/l), with possible substrate inhibition at high concentration (12 g/l). The substrate inhibition by glucose was likely due to the high osmotic pressure at higher glucose concentration.

It is crucial to highlight that peptone provides the bacteria with a readily available organic nitrogen source. Peptone and Yeast Extract also contains sources for minerals such as iron. The production of biological hydrogen is also dependent on the activity of hydrogenases, which are iron-containing enzymes directly responsible for the formation of hydrogen.

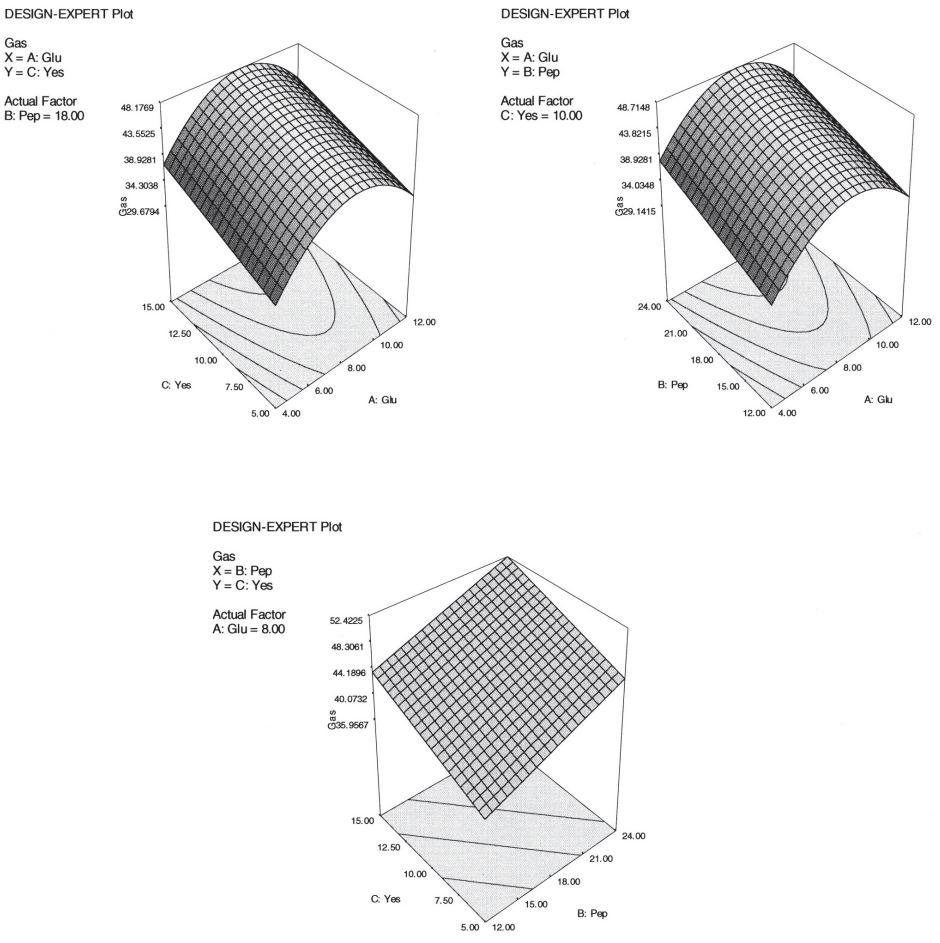

Fig. 3: Model graphs of the effect of glucose, peptone and yeast extract to biogas production

From the results of this study, the *C. butyricum* was shown to grow better on high concentration of organic nitrogen content. This information may be used to improve the yield of culture on substrate already high on organic content, such as Palm Oil Mill Effluent (POME). Thong *et al*. (2007) studied the thermophilic H_2 production bacteria and found that organic nitrogen amended medium improved the H_2 production, compared to inorganic nitrogen amended medium.

CONCLUSIONS

An anaerobic mesophilic bacterial strain with high production rate and yield of H_2 known as *Clostridium butyricum* KBH1was isolated. The results from the Response Surface Methodology (RSM) showed that peptone and yeast extracts are the limiting nutrients while glucose exhibits a substrate inhibition at high concentration. Peptone and yeast extracts provide sources for organic nitrogen, amino acids, vitamin and other growth factors. This bacterium is suitable for the production of biohydrogen using substrate with high organic loading. This optimization experiment was able to show the nutrients requirements for the growth and biohydrogen production of the isolated strain.

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