VNU Journal of Science: Natural Sciences and Technology, Vol. 32, No. 1S (2016) 258-268

Effects of Environmental Parameters on Hydrogen Production of Strain *Clostridium beijerinckii* CB3 Isolated in North of Vietnam under Anaerobic Condition

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Received 02 June 2016
Revised 02 August 2016; Accepted 09 Septeber 2016

Abstract: Hydrogen is considered as an ideal substitute to fossil fuels in the energy and non-polluting characteristics. Biological hydrogen production using microorganisms is a promising method to the world's energy industry. The anaerobic, mesophilic, Gram-positive strain *Clostridium beijerinckii* CB3 (*C. beijerinckii* CB3) isolated from cattle feces in North of Vietnam has been studied to optimize the biohydrogen production in anaerobic condition. In this study, the effects of culture conditions on hydrogen production by *C. beijerinckii* CB3 were investigated in batch culture using serum bottles. Various medium components (carbon and nitrogen sources, inorganic salts) and environmental factors (initial pH, temperature of incubation), time and orbital shaker of culture were optimized for hydrogen production by *C. beijerinckii* CB3. The optimal parameters for the best growth and biohydrogen production in batch tests were incubation time 48 h, 37^{o}C, pH 8.5, and orbital shaker 200 rpm. The maximum cell growth of 1.6 in OD_{600} and biohydrogen production of 881.25 mL/L were obtained, respectively, in the medium containing 10 g/L of glucose, 10 g/L of yeast extract or 10 g/L of peptone, 480 mL/L of $NaHCO_3$, and 32 mL/L of K_2HPO_4. These results indicated that *C. beijerinckii* CB3 is a potential candidate for fermentative biohydrogen production.

Keywords: Biohydrogen production, *C. beijerinckii*, culture condition, growth, anaerobic condition.

1. Introduction

Hydrogen is recognized as a clean, renewable and promising energy alternative for the future since it is efficient and environmentally friendly. It has the highest energy content per unit weight (142 kJ/g or 61,000 Btu/lb) of all the naturally occurring fuels [1]. Hydrogen can be produced by geological and biological processes in natural environments. Among the biohydrogen processes [2] dark fermentation is considered as a promising technology for the treatment of high strength industrial wastes such as distillery

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waste. Fermentative hydrogen producers belong to anaerobic acid forming bacteria such as *Clostridium* sp. [1, 3], *Enterobacter* sp. [1] and *Bacillus* sp. [4] isolated from bioreactors and natural environments. *Clostridium* is one of the highly -effective hydrogen producers within the Firmicutes phylum, and many strains of which have been isolated and studied (Leena B et al, 2010, Wang et al., 2008; Dan An et al., 2014) [5-7]. *Clostridium beijerinckii* recently has been found to be capable of biohydrogen production [7, 8].

Previous studies [9-11] indicated that the culture conditions (e.g., substrate concentration, inoculums, pH, nitrogen source, metal ion, temperature, etc.) significantly affect on the cell growth and hydrogen production. Dan An *et al.*, [7] studied the effects of pH, temperature, xylose concentration on hydrogen yield of *Clostridium beijerinckii* YA001, the highest yield of hydrogen (2.31 mol/mol xylose) was obtained at pH of 8.0 and temperature of 40°C. Yokoi *et al.,* [12] performed a repeated batch culture using a mixed culture of *Clostidium butyricum* and *Enterobacter aerogene*, the hydrogen yield was 2.4 mol H_2 /mol glucose. Fang *et al.* [13] carried out an experiment on bio-hydrogen production by *Clostridium* sp. using rice slurry containing 5.5 g carbohydrate L^{-1}. After a 36-h acclimation period, the sludge showed maximum hydrogen production of 346 mL H_2 g^{-1} carbohydrate. Although culture conditions have been investigated widely, the optimal culture conditions for different species or strains vary.

The aim of the present study was investigated deals with the optimization of culture conditions for bio-hydrogen production using *C. beijerinckii* CB3 isolated from cattle feces. In addition, the effects of culture temperature, pH, substrate concentration, nitrogen source, inorganic salts, time and orbital shaker on hydrogen production were investigated.

2. Materials and methods

2.1. Materials and culture medium

The anaerobic, mesophilic, Gram - positive, hydrogen-producing strain *C. beijerinckii* CB3 was isolated and identified as *C. beijerinckii* CB3 in Biochemistry and Environmental microbiology Laboratory, Center for Life Science Research, VNU-University of Science (data not show).

The synthetic medium used for growth bacterial, PY medium (1 L) [5] contained 10 g glucose, 10 g peptone, 10 g yeast extract, reazurine: 1 mg, salts solution: 40 mL, H_2O 960 mL. 100 mL salts solution composed of: KH_2PO_4: 0.1 g, K_2HPO_4: 0.1 g, $NaHCO_3$: 1 g, NaCl: 0.2 g, $CaCl_2$: 0.02 g, $MgSO_4$: 0.02 g, Clarified rumen fluid 50 mL, pH = 7.0.

2.2. Experimental conditions

The PY medium was dispensed anaerobically into 15 mL anaerobic culture bottles. Prior to testing, the bottles containing 8 mL liquid medium were sealed with rubber plugs and autoclaved at 121°C for 20 min. Then, they were flushed with ultra high-pure nitrogen gas (99.999%) for 20 min. The bottles were shaken in an air bath at 150 rpm. Inoculated with 10% (w/v) colony and incubated at 35°C, pH 7.0. The hydrogen gas was collected by the gas-tight syringe after culture for 48 h. To determine the effects of culture conditions on growth and hydrogen production, parameters including time, temperature, pH value, carbon sources, substrate concentration, nitrogen source, inorganic salts, orbital shaker were alternately varied.

2.3. Analytical methods

Growth was monitored by taking the difference in absorbance at 600 nm (Labomed UV invisible double beam spectrophotometer) of sample collected after the completion of

experiment. Hydrogen gas in the headspace was sampled with a gas-tight syringe (100mL injection volume) and determined by a gas chromatograph (GC, Outlet gas Inlet N2 from gas tank) equipped with a thermal conductivity detector (TCD) and a 2 m stainless column packed with carboxen 1000, 50/80 mesh (Supelco). Volume of hydrogen was determined by the water displacement method.

3. Results and discussion

3.1. Effects of culture time to the growth and hydrogen production

The culture time of growth and hydrogen production using glucose as a substrate is given in Figure 1. Growth and hydrogen production of strain *C. beijerinckii* CB3 were observed at culture time ranging from 0 to 72 h with an optimal incubation time of 48 h.

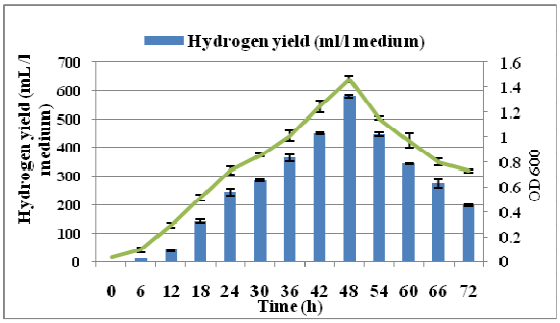

Fig 1. Effects of culture time to the growth and hydrogen production.

Figure1 showed that over time, both cell biomass and hydrogen production increased gradually. From time points 0 h to 6 h, cell biomass increased slowly, resulting the OD_{660} values of 0.032-0.094 and hydrogen yield achieved to be 0 - 10.83 mL/L medium, respectively. After 12 hours, hydrogen yield began to increase rapidly. From 12 h to 36 h hydrogen production increased rapidly from 40.38 to 365.13 mL/L medium, then increased slowly at an optimal time of 48 h. Hydrogen production and OD_{600} obtained their highest values of 581 mL/L medium and 1.469, respectively. This can be explained that, by the cells entered the rapid growth phase, cell biomass increased along with increasing metabolism, and hydrogen production increased rapidly. After 48 hours hydrogen production generated diminutively according cells entered the decline phase.

3.2. Effect of carbon source to the growth and hydrogen production

Carbohydrate is a major component in the culture medium and is one of the most important factors affecting growth and hydrogen production. The various carbohydrate substrates used in the study such as glucose, xylose, lactose, maltose, sucrose and cellulose. Carbohydrate substrates were added to the culture medium PY with the concentration of 10 g/L. The result was obtained in Figure 2.

As shown on Figure 2, strain *C. beijerinckii* CB3 could grow and produce hydrogen on all six types of carbohydrate substrates. Obtained yields ranged from 60.21 to 602.31 mL/L medium, depending on different carbon sources. Among all the substrates, glucose and lactose were found to be the best ones, supporting the highest growth and hydrogen production. Especially, the optimal growth (OD_{600} at 48 h was 1.478) and hydrogen yield (602.31 mL/L) were obtained when using glucose. The data can be explained due to the fact that glucose enters directly to the glycolysis facilitating the hydrogen production process to maintain redox balance in microbes. In a number of previous studies, glucose has been used as substrate for hydrogen production. Our results coincide with previous studies by Kapdan et al [14] and Gray et al [15]. In a similar study, Xin Zhao et al. also report a newly isolated *C. beijerinckii* RZF 1108 strain which can grow at optimal condition of pH 7.0, 35°C achieved 1.97 mol H_2/mol glucose, 2209 mL H_2/L medium [17]. In the case of

Citrobacter sp. CMC -1, H_2 production in optimal conditions (pH 6.0 and 34°C) achieved 1.82 ± 12.02 mol H_2/mol glucose [16]. Regarding the use of lactose as carbohydrate substrate, our result indicates that lactose is also found to be a favorable carbohydrate substrate to the cell growth and hydrogen production, probably because it is a disaccharide consisting of glucose and galactose.. In a study by Dan An et al., (2014), when using lactose as carbohydrate source, hydrogen production level of *C. beijerinckii* YA001 reaches 58.9 mL/g substrate [7]. Their data also indicates that maltose is an alternative suitable carbohydrate substrate for hydrogen production with achieved yield of 40.9 mL/g substrate. Regarding other substrates such as sucrose, cellulose, and xylose, our data indicates that they are not suitable carbon sources for growth and hydrogen production of *C. beijerinckii* CB3. This result can be explained by their nature or absence of metabolic enzymes in the isolated strain. Taken all the data, glucose was used as carbohydrate source in all subsequent experiments.

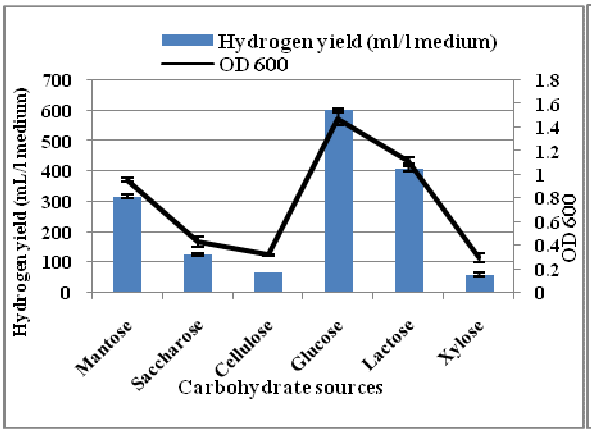

Fig.2. Effect of carbon source to the growth and hydrogen production.

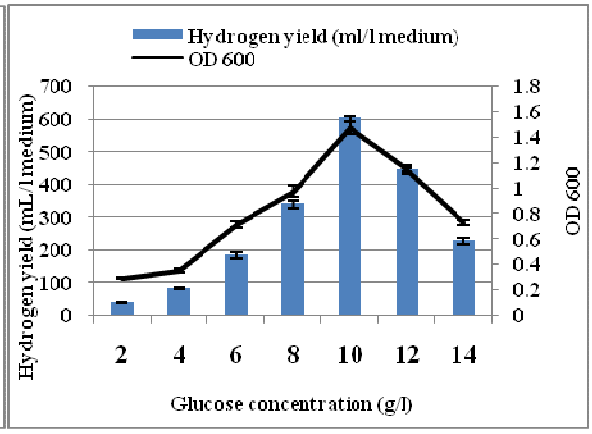

Fig.3. Effect of glucose concentration to the growth and hydrogen production.

3.3. Effect of glucose concentration to the growth and hydrogen production

Glucose was added to the culture media with the concentrations: 2, 4, 6, 8, 10, 12 or 14 g/L. Investigation of glucose concentration to the growth and hydrogen production after 48h was shown in Figure 3.

As can be seen on Figure 3, glucose concentrations affected hydrogen production and growth of strain *C. beijerinckii* CB3. With the increase of glucose supplementation from 2 g/L to 10 g/L, the cell growth and hydrogen yield increased, from 0.289 to 1.475 OD_{600} values and 37.82 to 600.70 mL/L respectively. When low glucose concentration of 2 g/L, the strain used quickly, then low hydrogen production achieved 37.82 mL/L medium. When glucose was added to the medium with increasing concentrations, it was used as a direct substrate source to the cell growth and hydrogen production, cell biomass and hydrogen yield increased rapidly and achieved a maximum of 600.70 mL/L medium and OD_{600} value 1.475 after 48h at the concentration of glucose was 10 g/L and then decreased. Cell growth and hydrogen production were slowed when the glucose concentration was higher than 10 g/L. The use of too much glucose bacteria did not consume off to raise wasteful. At the same time substrate utilization and final pH decreased with increasing glucose

concentration, and some substrate remained when the glucose concentration was over 10 g/L can cause decreasing emissions produced when glucose levels rise. This observation was coincided with previous studies. The results of Dang Thi Yen et al., (2013), also resulted optimum substrate of 10 g/L glucose by strain Tr2 [18] and some other species such as *Clostridium saccharoperbutylacetonicum* N1-4 (Alalayah et al) [19]. In the research of Xin Zhao et al., a maximum hydrogen production achieved at glucose concentration 9 g/L by the strain *C. beijerinkii* RZF 1108 [17].

Since concentrations of glucose using for growth and *formation* of hydrogen *of C. beijerinckii* CB3's (10g/L) is lower than that of *Clostridium* sp. str. 6A-5 and C. *butyricum* EB6 (16 g/L [20] and 15.7 g/L [21] respectively), *C. beijerinckii* CB3 will help save materials and input costs if it is used.

3.4. Effect of nitrogen source to the growth and hydrogen production

Nitrogen is a very important component in growth and development of bacteria. In laboratory scale, investigation of the growth and hydrogen production of *C. beijerinckii* CB3 through the ability to consume nitrogen sources such as organic nitrogen: peptone (P), yeast extract (Y), meat extract (M), inorganic nitrogen (NH_4Cl, NH_4NO_3) aimed to search for a suitable nitrogen source for cultivation to reach the highest hydrogen production.

Nitrogen sources were added to the basic medium PY (BM) with the concentration of 10 g/L: BM+P (basic medium and peptone), BM+Y (basic medium and yeast extract), BM+M (basic medium and meat extract), BM + PY (basic medium and both peptone, yeast extract), BM + NH_4Cl (basic medium and NH_4Cl), BM+ NH_4NO_3 (basic medium and NH_4NO_3). The result was obtained and shown in Figure 4, indicating that the strain can consume 4 types of nitrogen sources including BM+Y, BM+P, BM+M, and BM+PY among the 6 tested nitrogen sources.

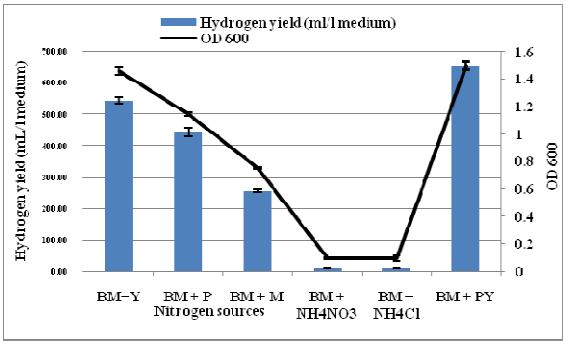

Fig.4. Effect of nitrogen sources to the growth and hydrogen production.

Among the 4 nitrogen sources which the strain could use, yeast extract was most suitable nitrogen source for optimal hydrogen production level of 544.01 mL/L medium and bacterial growth with OD_{600} of 1.456. Beside, peptone and meat extract also provided high hydrogen yield with 443.01 mL/L and 254.89 mL/L medium, respectively. Hydrogen yields by using these organic nitrogen sources were much higher than those by using inorganic nitrogen sources such as NH_4Cl and NH_4NO_3 (10.05 and 10.67 mL/L medium). This data can be explained by the fact that yeast extract, peptone, and meat extract are organic nitrogen source, which bacteria prefer to use than the inorganic ones such as NH_4Cl and NH_4NO_3. In comparison of yeast extract to other inorganic nitrogen sources, its total amino-nitrogen content is about 5% higher than in peptone and meat extract, as well as its peptides and free amino acids can be easily incorporated into proteins or transformed into other cellular nitrogenous constituents. Thus the highest bacterial growth and hydrogen production level obtained in case of yeast extract is understandable. The studies of Ferchichi et al (2005) [22] and Dang Thi Yen et al [18] also show that yeast extract is a suitable carbon source to the growth and hydrogen production of bacteria in the anaerobic condition. In their data, the combination of two nitrogen sources peptone and yeast extract provides the highest hydrogen yield and OD_{600} of 654.91 mL/L medium and 1.492, respectively. The result also

coincides with previous studies of Leena et al (2009) [5].

3.5. Effect of culture pH to the growth and hydrogen production

pH is an important factor affecting hydrogen fermentation because it affects metabolism and hydrogenase activity. The rapidly decreasing of pH value in culture medium is the main reason leading to low growth and hydrogen production during culturing. Therefore, the initial pH becomes a key factor to affect directly metabolism and hydrogen production of studied bacterial strains [20]. We tested various pH values of culture medium, ranging from 5.0 to 9.5 ((adjusted by 1 mol/L HCl or 1 mol/L NaOH solution) to determine the optimal value pH for the growth and hydrogen production, and the result was obtained and shown in Figure 5.

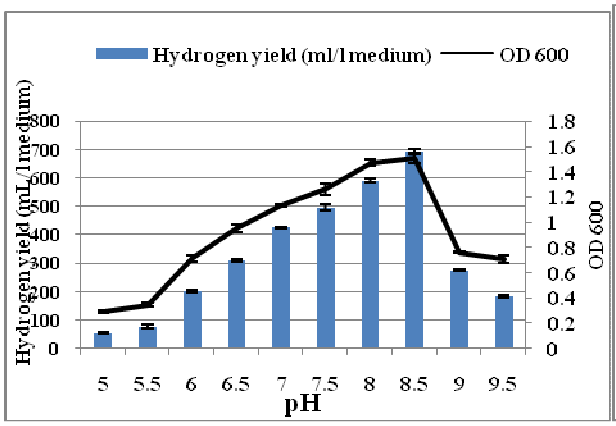

Fig.5. Effect of culture pH on the growth and hydrogen production.

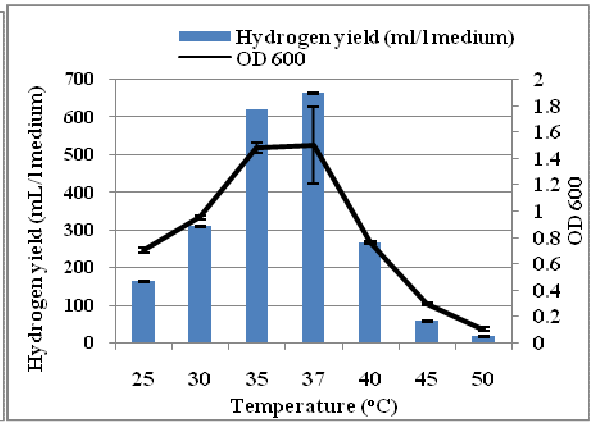

Fig.6. Effect of temperature on the growth and hydrogen production.

As shown on Figure 5, pH substantially influenced on the growth and hydrogen production of *C. beijerinckii* CB3. The strain could grow and produce hydrogen at pH ranging from pH 5.0 to 9.5 with hydrogen production achieved 54.50 to 691.13 mL/L medium. When the initial pH increased from 5.0 to 8.5, the hydrogen yield and cell biomass also increased and reached a maximum at pH 8.5, the highest growth and hydrogen yield achieved 691.13 mL/L medium with the OD_{600} value of 1.507 and then decreased gradually when pH was increased further. In this study, the optimum pH for hydrogen production using *C. beijerinckii* CB3 was 8.5. The optimum pH was slightly higher than the pH reported in previous literatures. In the report by Taguchi et al (1996 b), by adjusting to optimal pH 6.5, *Clostridium* sp. strain no.2 can grow well in glucose as carbon source and produce hydrogen yield 2.4 mol / mol substrate [23]. Pan et al. report that the optimum pHs for hydrogen production by *C. beijerinckii* Fanp3 are between 6.47 and 6.98 with glucose as carbon source and using 150 mmol/L phosphate as buffer [8]. Dan et al (2014) reported that low phosphate concentrations shows weaker capacity on maintaining pH during fermentation process [7]. In this study, the optimal pH value was 8.0 when using xylose as carbon source by *C. beijerinckii* YA001.

3.6. Effect of temperature to the growth and hydrogen production

We then further determined the optimal temperature to the growth and hydrogen production. The effect of culture temperature on

hydrogen production was investigated by varying the temperature between 25°C and 50°C. The result was obtained and shown in Figure 6. The growth and hydrogen production of the strain were increasing at temperatures from 25° C to 50°C with archived levels ranging from 15.91 to 662.22 mL/L medium, respectively. The optimal temperature was 37°C, which allowed maximal hydrogen production and OD_{600} achieved values of 662.42 mL/L medium and 1.498, respectively. The hydrogen production and growth then decreased vs increasing temperature more than 37°C. Our obtained result coincides with previous studies of Wang et al. (2003) when studying the hydrogen production of *C. bifermentans* on glucose substrate [6], and of Leena B et al. when studying the hydrogen production of *Clostridium* sp. DMHC-10 with achieved yield of 3.35 mol H_2/ mol glucose at temperature of 37°C [5]. In another study, Xin Zhao et al., (2011) show that the optimum temperature to the hydrogen production by *C. beijerinckii* RZF 1108 is 35°C [17]. When temperature increased from 45°C to 50°C (suitable temperature to the growth of many thermophilic bacteria), *C. beijerinckii* CB3 exhibit very low growth and hydrogen production. This is probably due to increase in denaturation rate of enzymes (ferredoxin oxidoreductase, phosphate acetyltransferase, acetate kinase and hydrogenase) responsible for the fermentative hydrogen production process when the temperature exceeded the critical point [24].

3.7. Effect of $NaHCO_3$ to the growth and hydrogen production

Inorganic elements such as P, K, Mg, Ca, Na etc. are considered to play an important role in the growth and hydrogen production of strain *C. beijerinckii* CB3. In this study, the impact of these elements on the growth and hydrogen production of strain was investigated by determining the optimal concentration of inorganic substances such as $NaHCO_3$ and K_2HPO_4.

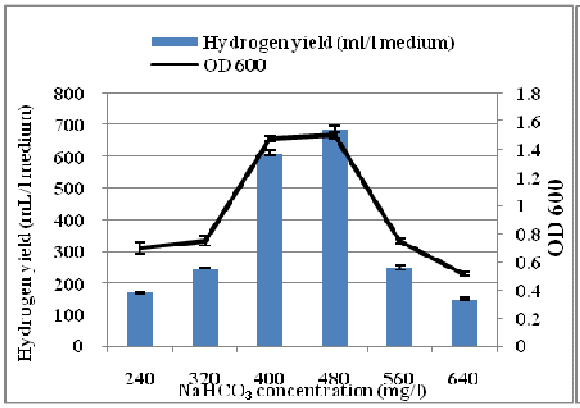

Fig.7. Effect of $NaHCO_3$ concentration to the growth and hydrogen production.

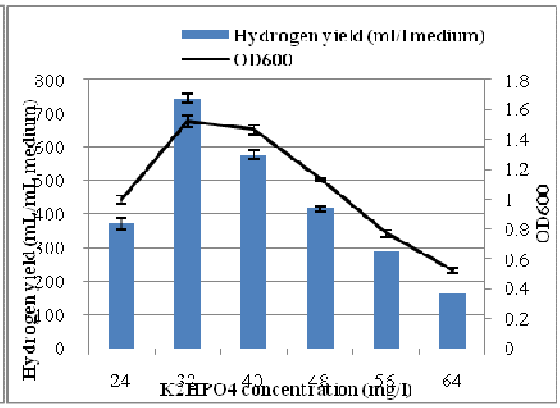

Fig.8. Effect of K_2HPO_4 concentration to the growth and hydrogen production.

$NaHCO_3$ is one of the major components in salts solution of the basic culture medium. Therefore, in this study, an appropriate concentration of $NaHCO_3$ was investigated for to archive a maximum hydrogen production. For this purpose, $NaHCO_3$ was added to the culture medium with increasing concentrations of 240, 320, 400, 480, 560, and 640 mg /L. The

result was obtained and shown in Figure 7. In details, hydrogen production and growth increased with increasing $NaHCO_3$ concentrations (from 240 to 480 mL/L) and hydrogen production levels achieved from 170.04 to 687.04 mL/L medium. The optimal $NaHCO_3$ concentration was 480 mL/L, at which both hydrogen production and OD_{600} achieved their highest values of 687.04 mL/L medium and 1.503, respectively. By increasing further $NaHCO_3$ concentration, hydrogen production and OD_{600} was gradually decreased. Therefore, 480 mg/L is the most suitable $NaHCO_3$ concentration for growth and hydrogen production of *C. beijerinckii* CB3 at 150 rpm, 37°C, glucose as carbohydrate source.

3.8. Effect of K_2HPO_4 to the growth and hydrogen production

K_2HPO_4 is also one of the major components in salts solution of the basic culture medium. The salt play an important role in maintaining pH and osmotic pressure of the organism, may resulting in maintaining the initial pH to affect greatly to the growth and hydrogen production of research strain. Therefore, in this study, we performed a similar optimization of K_2HPO_4 concentration like optimization of $NaHCO_3$. K_2HPO_4 was added to the culture medium at final concentrations of 24, 32, 40, 48, 56, 64 mg/L. The result was obtained and shown in Figure 8. Hydrogen production and growth increased with increasing K_2HPO_4 concentration (from 24 to 32 mL/L) and hydrogen production achieved 371.08 to 743.67 mL/L medium with an optimal K_2HPO_4 concentration of 32 mL/L. Both hydrogen production and OD_{600} achieved their highest values 743.67 mL/L medium and 1.518, respectively and then decreased gradually when K_2HPO_4 concentration was increased further. Therefore, K_2HPO_4 concentration of 32 mg /L is suitable for growth and hydrogen production of *C. beijerinckii* CB3 at 150 rpm, 37°C, glucose as carbohydrate source. This result shows that *C. beijerinckii* CB3 needs less K_2HPO_4 salt than *Thermotoga neapolitana* in previous studies [25].

3.9. Effect of orbital shaker to the growth and hydrogen production

Orbital shaker has been known to effect the cell growth and hydrogen production. Upon shaking, nutrients is circulated within a culture flask, enabling bacteria growth and production of hydrogen at higher level as well as to avoid bacterial settlement on the flask bottom, which would result in cell death from the lack of nutrient availability. Also, shaking prevents bacterial clumps or biofilm formation, ensuring prolific bacterial reproduction. However, if shaking rate is too high, it can create shear which can damage bacterial cells [26]. Thus, effect of orbital shaking on hydrogen production was investigated by varying the orbital shaking rate between 50 rpm and 400 rpm. The result was obtained and shown in Figure 9.

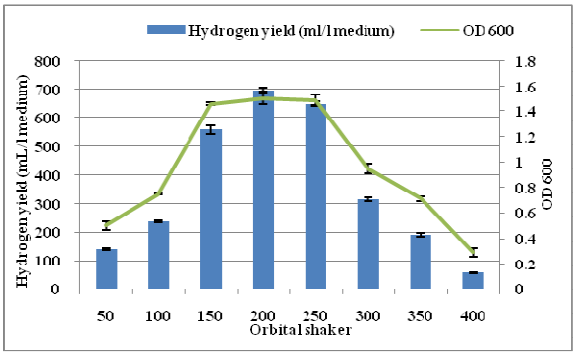

Fig. 9. Effect of orbital shaker to tgrowth and hydrogen production.

As can be seen on Figure 9, hydrogen production and growth increased with increasing orbital shaking rate (from 50 to 200 rpm), resulting in hydrogen production achieved 142.16 to 696.99 mL/L medium with an optimal orbital shaker of 200 rpm. Both hydrogen production and OD_{600} achieved their highest values 696.99 mL/L medium and 1.501, respectively and then decreased gradually when orbital shaker was increased further. Strain

grew and produced hydrogen at highest level at orbital shaking rate of 200 rpm. Shaking at lower or higher speeds inhibited cell growth. Our data is close to the data reported by Dwierra, et al. (2000), in which the optimal rate is 250 rpm in case of culturing *C. paraputrificum* M-21 strain [27].

3.10. Hydrogen production under optimal conditions

Combining all the optimized conditions including 10 g/L of glucose, initial pH of 8.5, 37^0C, $NaHCO_3$ concentration of 480 mL/L, concentration K_2HPO_4 of 32 mL/L, orbital shaker of 200 rpm, time 48h, yeast extract and peptone as favorable nitrogen sources, we obtained hydrogen production of 881.25 mL H_2/L medium and OD_{600} of 1.594. The hydrogen yields of C. *beijerinckii* CB3 was comparable to that obtained by other Clostridia [5, 7, 17, 19].

4. Conclusion

The growth and biohydrogen production by *C. beijerinckii* CB3 was optimum at pH 8.5, 37^oC, $NaHCO_3$ 480 mL/L, K_2HPO_4 32 mL/L, orbital shaker of 200rpm, time 48h, glucose 10g/L, yeast extract and peptone as favorable nitrogen sources for cell growth and hydrogen production. The maximal hydrogen yield and OD_{600} was 881.25 mL/L medium and 1.594, respectively. In conclusion, the strain is potential for production of hydrogen using a variety of carbon and nitrogen sources.

Acknowledgements

This research is funded by Vietnam National University, Hanoi (VNU) under project number QG.16.03.

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Ảnh hưởng của các yếu tố môi trường đến khả năng tạo khí hydro của chủng vi khuẩn *Clostridium beijerinckii* CB3 phân lập ở Miền Bắc Việt Nam trong điều kiện kị khí

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Tóm tắt: Hydro được coi như là một sự thay thế lý tưởng cho các loại nhiên liệu hóa thạch và không gây ô nhiễm môi trường. Sản xuất hydro sinh học sử dụng vi sinh vật là một phương pháp đầy hứa hẹn cho ngành công nghiệp năng lượng thế giới. Chủng vi khuẩn kị khí, ưa nhiệt, Gram dương

Clostridium beijerinckii CB3 (*C. beijerinckii* CB3) được phân lập từ phân gia súc ở Miền Bắc Việt Nam có khả năng sản xuất hydro trong điều kiện ki khí. Trong nghiên cứu này, ảnh hưởng của các điều kiện nuôi cấy trên sản xuất hydro bởi chủng *C. beijerinckii* CB3 đã được nghiên cứu trong nuôi cấy mẻ. Các thành phần của môi trường (nguồn cacbon và nitơ, muối vô cơ) và các yếu tố môi trường (pH ban đầu, nhiệt độ), thời gian và tốc độ lắc đã được tối ưu hóa cho sản xuất hydro bởi chủng *C. beijerinckii* CB3. Các thông số tối ưu cho sản xuất hydro sinh học trong các thử nghiệm gồm: thời gian nuôi cấy 48h, glucose 10g/L, cao nấm men và pepton là nguồn nitơ thích hợp, $NaHCO_3$ 480mL/L, K_2HPO_4 32 mL/L, nhiệt độ 37^0C, pH 8.5, tốc độ lắc 200rpm. Sản lượng hydro và giá trị OD_{600} tối đa đạt được lần lượt là 881.25 mL/L môi trường và 1.594. Những kết quả này cho thấy C. *beijerinckii* CB3 là một sinh vật tiềm năng cho lên men sản xuất hydro.

*Từ khóa: S*ản xuất hydro sinh học, điều kiện nuôi cấy, *C. beijerinckii* CB3, sinh trưởng, điều kiện kị khí.