Effect of fermentation conditions on hydrogen production using *Clostridium beijerinckii* KCTC 1785

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Graduate School of Chosun University

Department of Bio Materials Engineering,

Le Nhat

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Advisor: Prof. Si Wouk Kim

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Le Nhat

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Approved by the Guidance Committee:

Professor **In Hwa Lee**
Chosun University

Professor **Jung-Heon Lee**
Chosun University

Professor **Si Wouk Kim**
Chosun University

November 2005

Graduate School of Chosun University

List of tables

List of figures

요 약

Clostridium beijerinckii KCTC 1785를 이용한 수소생산에 대한 발효 조건의 영향

Le Nhat

Advisor : Prof. Si Wouk Kim, Ph.D.

Department of Bio Materials Engineering

Graduate School of Chosun University

수소는 화석연료를 대체할 수 있는 청정 에너지이다. 이러한 수소는 주로 화석연료, 바이오매스 또는 물로부터 생산될 수 있다. 몇몇 세균들은 암발효 또는 광발효와 같은 혐기발효에 의해 유기물질로부터 수소를 생산할 수 있다고 알려져 있다. 만약 환경조건이 적절하다면 수소는 최대로 생산될 수 있다. 본 연구는 두 가지 다른 기질로부터 *Clostridium beijerinckii* KCTC 1785에 의한 수소생산의 최적 조건들을 조사 하였다. 수소생산을 위한 최적 발효 조건을 위한 인자들은 초기 pH, 온도, glucose 농도, 희석율, pH 조절, 교반 영향과 아세트산 및 뷰티르산과 같은 생산물의 영향이었다. 첫번째로 Reinforced Clostridial Medium (RCM)은 *Clostridium beijerinckii* KCTC 1785의 배양을 위한 배양배

지로 사용되었다. RCM을 이용한 균주배양의 최적조건은 초기 pH 7.0, 온도 35 °C, 최적 pH 조절은 5.5 이었으며 4%의 glucose 농도까지 성장할 수 있었다. 뷰티르산 및 아세트산과 같은 생성물의 축적량은 각각 4,000 mg/l 과 5,000 mg/l 이하였으며, 교반을 하였을 경우 보다 더 많은 수소를 생산하였다. 생산된 바이오가스 중 최고 수소함량은 발효 15시간 후 32% 이었으며, 최고 생산수율은 284.8 ml/l·h 이었다. 또한 배지성분중 yeast extract 또는 tryptose는 수소를 생산하는 동안 균체가 성장하는 반드시 필요한 성분으로 조사되었다. 두번째로 물과 혼합된 음식물쓰레기 (약 50,000 mg/l 의 COD)가 *Clostridium beijerinckii* KCTC 1785 배양을 위한 배양배지로 사용되었다. 최적 조건들은 초기 pH 7.0, 온도 40 °C, 교반속도는 150 rpm이었다. 최적 pH 조절은 5.5 이었으며 음식물쓰레기 농도는 50,000 mg/l 이었다. 약 5,500 ml 의 바이오가스가 음식물쓰레기를 36 시간 동안 발효했을 때 발생하였으며, 이 가운데 수소의 함량은 38% (2,100 ml) 이었고, 최고 수소생산수율은 87.5 ml/l·h 이었다. 이러한 결과는 *Clostridium beijerinckii* 는 수소생산을 위한 고효율 균주이며, 음식물쓰레기는 생물공정에 의해 수소를 생산 할 경우 기질로서 사용이 가능한 물질임을 나타낸다.

ABSTRACT

Hydrogen is a clean energy alternative to fossil fuels. It can be produced mainly from fossil fuels, biomass and water. From biomass some bacteria have been known to produce hydrogen from organic compounds by anaerobic fermentation. It might be dark-fermentation or photo-fermentation. The hydrogen production can be maximal if the environment condition was operation. In this study, optimal conditions of hydrogen production by *Clostridium beijerinckii* KCTC 1875 from two different sources were investigated. The parameters of optimal fermentative conditions for hydrogen production were initial pH, temperature, glucose concentration, diluting rate, pH, agitation and inhibition by product such as acetate and butyrate. First Reinforced Clostridial Medium was used as a culture medium for *Clostridium beijerinckii* KCTC 1785. The optimal conditions were initial pH 7.0, temperature 35°C, optimum pH at 5.5 and glucose concentration in the medium up to 4%. The accumulated product such as butyrate and acetate should be lower than 4,000 mg/l and 5,000 mg/l of each acid respectively, and agitation has good effect to produce more hydrogen. Maximum concentration of hydrogen in biogas produced was 32% after 15 hr and maximum hydrogen production rate was 284.8 ml/l·h. It is found that yeast extract or tryptose in the medium was essential for cells growth during hydrogen production. Secondly, food-waste (around 50,000 mg/l of COD), which was mixed with water, was sterilized and used as a culture medium for *Clostridium beijerinckii* KCTC 1785. The optimum conditions were as follows: initial pH 7.0, temperature

40℃ and agitation speed 150 rpm. The pH control was optimal at 5.5 and food waste concentration was 50,000 mg/l of COD. About 5,500 ml biogas was produced from food waste for 36 hr, and hydrogen content was 38% (2,100 ml) and maximum hydrogen production rate was 87.52 ml/l·h. This result indicates that food waste was a potential material for hydrogen production by bioprocess and *Clostridium beijerinckii* has the high effect strain to hydrogen production.

I. INTRODUCTION.

In recent years the extensive use of fuels became an important problem. Our energy requirements are almost fully provided by carbon-containing fossil sources such as oil, coal and natural gas which have been formed during many millions of years from plant biomass (about 80% of the present would's energy demand [1]). The rapid consumption of these fossil resources will bring about the depletion of fossil resources in near future. Simultaneously, it causes an accelerated release of the bound carbon as CO_2. The resulting increase of the CO_2 concentration in the earth's atmosphere is generally acknowledged as the major cause of global warming and associated climate changes. That required to researching a renewable and friendly environment energy source. Alternatively, hydrogen is a clean energy source, producing water as its only by-product when it burns. It has high-energy content thus would have great possibilities as a fuel if the production cost would be low enough. Accordingly, hydrogen is a promising alternative to fossil fuels. On the other hand, the accumulation of household garbage and waste water became a serious problem in the urbanize processing and involves a treatment process. At present hydrogen is produced mainly from fossil fuels, biomass and water by the following processes:

1) Hydrogen production from fossil fuels:

(a) Steam reforming of natural gas.

(b) Thermal cracking of natural gas.

(c) Partial oxidation of heavier than naphtha hydrocarbons.

(d) Coal gasification.

2) Hydrogen production from water:

(a) Electrolysis.

(b) Photolysis.

(c) Thermochemical process.

(d) Direct thermal decomposition or thermolysis.

(e) Biological production.

3) Hydrogen production from biomass:

Pyrolysis or gasification (which produces a mixture of gases, i.e., H_2; CH_4; CO_2; CO; N_2).

Out of the above listed processes, nearly 90% of hydrogen is produced by the reactions of natural gas or light oil fractions with steam at high temperatures (steam reforming). Coal gasification and electrolysis of water are other industrial methods for hydrogen production. These industrial methods mainly consume fossil fuel as energy source, and sometimes hydroelectricity [2, 36-39]. However, not all these processes accomplish the dual goals of waste reduction and energy production. For its, biological

hydrogen production was an auspicious way. Biological hydrogen production processes can be classified as follows:

(a) Biophotolysis of water using algae and cyanobacteria.

(b) Photodecomposition of organic compounds by photosynthetic bacteria.

(c) Fermentative hydrogen production from organic compounds.

(d) Hybrid systems using photosynthetic and fermentative bacteria.

From an environmental viewpoint, there is an urgent need for appropriate management of municipal solid wastes (MSW). Nearly 1600 million tone/year of MSW are generated worldwide with up to 43% contributed by Asia and Oceania and 28% contributed by North America and the European Union [3]. In Korea, the generation of food waste reaches 11,237 ton/day occupy up to 23.2% of municipal solid waste [4]. Food waste has high volatile solids and moisture content thus it causes decay, odor, and leachate in collection and transportation. All most cases, food waste were consolidated with other wastes, resulting in various problems such as odor emanation, vermin attraction, toxic gas emission, and groundwater contamination. Although, food wastes are by-products/residuals of food processing plants and agricultural residues, so it is rich in carbohydrate content, meaning that has a high energy reserve. Hydrogen can be produced from renewable raw materials such as organic wastes or organic materials. Many kinds of bacteria, such as green algae (*Scenedesmus obliquus, C. moewusii,* etc.) [5, 6], cyanobacteria (*Anabaena azollae, Phormidium*

valderianum, etc.) [7-20], photosynthetic bacteria (*Rhodobater sphaeroides, Halobacterium halobium*, etc.) [21-31], fermentative bacteria (*Enterobacter aerogenes, E. cloacae, Clostridium butyricum, C. beijerinckii, C. pasteurianum, C. difficile, C. sporogenes, C. welchii, C. bifermentans, C. thermolacticum, Desulfovibrio vulgaris, Magashaera elsdenii, Citrobacter intermedius, Escherichia coli, Caldicellulosiruptor saccharolyticus, Thermoanaerobacterium thermosaccharolytium, Thermoanaerobacterium sp., Desulfotomaculum geothermicum*, etc.) [19, 32-35, 44, 45, 47, 53, 58, 63, 66, 67] are known to be capable of producing hydrogen gas.

Farther more, as microbial hydrogen production is concerned, studies were reported in the literature with different microbial hydrogen-producing systems. In a comparison of that processing, Table 1 indicates clearly that the rate of fermentative hydrogen production is always faster than that of the photosynthetic hydrogen production.

Table 1. Comparison of different biological hydrogen production processes.

Organisms	Raw materials	A	B	C	Major products of the process
Photosynthetic bacteria		2.2-9			H_2; CO_2; O_2 small amount of
Rhodopseudomonas capsulata	Lactate with other nitrogen source		5.3	5.3	fatty acids, biomass
Rhodopseudomonas capsulata	Fermented cow dung			0.3	
Rhodopseudomonas sp.	Vegetable starch			1.3	
Rhodopseudomonas sp	Dairy wastewater			16[b]	
Rhodopseudomonas sphaeroides	Orange processing effluent			133[b]	
Rhodopseudomonas palustris	Sugar refinery waste			1.2	
Rhodobacter sphaeroides	Lactic acid ferm. Waste			5.9	
Rhodobacter sphaeroide	Distillery wastewater			0.46	
Rhodospirillium rubrum	Organic compounds		3.0	2.5	
Mixed microorganisms[a] containing *Phormidium valderianum, Halobacterium halobium, Escherichia coli* in 1:1:1 proportion	ASNIII medium[a] devoid of combined nitrogen in TES buffer			19[c]	

Fermentative bacteria Strict anaerobe		0.16-2	---		
Clostridium butyricum	Glucose containing medium[a]		11	7.3	H_2; CO_2, high conc. of fatty acids, biomass
Facultative anaerobe *Citrobacter intermedius*	Cellulose, starch, glucose			9.5	H_2; CO_2, high conc. of fatty acids, biomass
C. freundii	Stillage		11.36	1.8	H_2; CO_2, high conc. of fatty acids, biomass
Enterobacter aerogens E82005	Sugar cane	0.25	37.03	17	H_2; CO_2, high conc. of fatty acids, biomass
Enterobacter cloacae IIT BT-08	Sucrose containing medium[a]	0.32		29.63	H_2; CO_2, high conc. of fatty acids, biomass $H_2/CO_2 = 9$

[a] Medium contains different components.

[b] Expressed as ml H_2/g Bchlorophyll-a h.

[c] Expressed in mmol/mg protein h.

A. Doubling time (h).

B. Maximum rate of H_2 production (mmol H_2/l h).

C. Maximum rate of H_2 production (mmol H_2/g dry cell h).

Dark fermentation was main process to hydrogen produce from food waste. When bacteria grow on organic substrates (heterotrophic growth), these substrates are degraded by oxidation to provide for building blocks and metabolic energy for growth. This oxidation generates electrons which need to be disposed of for maintaining electrical neutrality. In aerobic environments, oxygen is reduced and water is the product. In anaerobic or anoxic environments, other compounds need to act as electron acceptor, e.g., protons which are reduced to molecular hydrogen (H_2). Other examples of alternative electron acceptors in anaerobic environments are nitrate with nitrogen gas (N_2) as the product or sulfate with dihydrogensulfide (H_2S) as the reduced product. Even organic compounds can act as electron acceptors as e.g. in the microbial production of butanol which is done through the reduction of butyric acid. The capacity to reduce other electron acceptors than oxygen requires the presence of a specific enzyme system in the micro-organisms: hydrogen producing bacteria possess hydrogenase enzymes; nitrate reducing bacteria possess an elaborate set of enzymes catalyzing the stepwise reduction of nitrate to nitrogen etc. Even though many organic compounds enable the production of hydrogen during dark fermentation, estimations of potential yields are mostly based on hexose conversions. The theoretical yield per mole of glucose is described in the following reaction:

$$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2 \quad (67)$$

or $$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3\,CH_2\,CH_2COO^- + 2HCO_3^- + 3H^+ + 2H_2 \quad (71)$$

It mean that maximum of 4 moles of H_2 per mole of glucose can be produced. In fact, the remainder of the hydrogen in the hexose is conserved in these others products like ethanol, lactate, alanine or change mechanism, results were formed other products such as butyric acid, lactic acid, etc under non-ideal circumstances. In addition, products were produced depend on characteristic of microorganism. So that all most case hydrogen produce is less than 4 moles per mole of glucose. The understanding of the properties of hydrogen fermentation lies in an appreciation of the response to environmental changes that influence hydrogen metabolism. Researchers have started to investigate hydrogen production with anaerobic bacteria since 1980s. There are some of the relevant research used bacterial culture with simple medium such as sucrose [40-48], starch [49, 67], glucose [50-52] or lactose [53] as carbon source. Some others used waste matter such as palm oil mill, wheat straw, waste water or food waste [4, 54- 65, 68]. Das and Veziroglu [1] indicated in a recent literature survey that in contrast to photolytic production of H_2, fermentative processes have the advantages of high hydrogen production rates and of the capability to convert organic wastes in the environment into more valuable energy resources. Hydrogen fermentation of food waste is effected by several factors such as pH, retention time, temperature, carbon source, etc. It means that hydrogen fermentation of food waste can be improved by adjusting the environmental conditions. Concomitantly, to design a fermentation system which has high effect to hydrogen production, it requires the knowledge about microorganism that grow in the reactor as more as possible. Collet *et. al* [53] has reported properties during hydrogen production from lactose by

C. thermolacticum. It indicated the pH and dilution rate effects. Chen and others [45] investigated *C. butyricum*. But most of experiments that were reported mix-culture [4, 40-44, 46-52, 54-65, 68]. However, these demonstrate that pH, temperature, loading rate concomitant product release are important factors to improve hydrogen-producing bioprocess. This study was, therefore, conducted to maximize hydrogen fermentation using dark fermentation by *Clostridium beijerinckii* KCTC 1785. The optimal pH, temperature, substrate concentration, agitation effect, and inhibition effect by product were investigated.

II. MATERIALS AND METHOD

1. Strain and medium

Clostridium beijerinckii KCTC 1785 from Korean Collection of Type Culture was used in this study.

Two types of media were used, that are Reinforced Clostridial Medium (RCM) and food waste. The RCM compositions were shown in Table 2.

The other is the food waste collected from dining hall in Chosun University which was grinded by crusher. There are some different concentrations of food-waste used in this study. For optimal temperature, initial pH and agitation effect, food waste solution which was a mixture with water to 45,000 mg/l of COD was used. The others, 50,000 mg/l of COD was used for investigation effect of pH controls, and 100,000 mg/l, 70,000 mg/l, 50,000 mg/l and 40,000 mg/l were used for loading rate effect. All substrates collected were sterilized by autoclave and kept at 4°C then warmed at room temperature before using in the experiment.

To study components of RCM for the essential elements to cells growth during hydrogen production with fermentative by *C. beijerinckii*, we have used RCM component with Standard mineral base medium (SMM). Composition of SMM were shown in Table 3.

Table 2. Composition of Reinforced Clostridial Medium

Components	Amounts
Tryptose	10.0 g
Beef extract	10.0 g
Yeast extract	3.0 g
Dextrose	5.0 g
Sodium chloride	5.0 g
Soluble starch	1.0 g
Cysteine hydrochloride	0.5 g
Sodium acetate	3.0 g
(Agar)	15.0 g
Distilled water	1.0 l

Table 3. Composition of standard mineral base medium (SMM)

Components	Amounts
Na_2HPO_4	2.34 g
KH_2PO_4	6.10 g
$MgSO_4.7H_2O$	0.20 g
$CaCl_2$	0.01 g
Ferric EDTA solution[a]	0.10 ml
$ZnSO_4.7H_2O$	0.50 mg
$MnSO_4.H_2O$	0.05 mg
$CuSO_4.5H_2O$	0.10 mg
Cobalt nitrate	0.10 mg
Sodium Borate	0.10 mg
Sodium molybdate	2.00 mg
Distilled water	1.0 l

[a] The ferric EDTA solution was made by combining a solution containing 17.9 g of sodium EDTA and 3.23 g of KOH dissolved in 186 ml distilled water and a solution of $FeSO_4.7H_2O$ in 364 ml of distilled water. The mixture was bubbled overnight with air, and stored in a brown glass bottle.

2. Culture condition and experimental procedure

Subcultures were carried out by 100 ml bottles which contain 50 ml of medium. The culture was incubated at 35°C for 12 hour at least 3 times before using as inoculum (1% v/v).

Experimental procedure:

Hydrogen production was carried out in two kinds of reactors. First, a series of 500 ml serum bottles were used and working volume was 200 ml. Conditions of hydrogen fermentation such as initial pH, temperature, organic acids or glucose concentration in the medium and agitation effect were investigated. The pH was adjusted by HCl or KOH solution. For initial pH effect, initial pH of each bottle which containing 200ml of RCM or food waste (47,000 mg/l of COD) were adjusted to pH 5.0, 6.0, 7.0 and 8.0. Then temperature was 35°C and agitation speed was 150 rpm. In case of temperature effect, a series of bottle each containing 200 ml of medium (RCM or food waste) with initial pH 7.0 were incubated at various temperatures of 25°C, 30°C, 35°C, 40°C and 45°C at 150 rpm of agitation. Experiment procedure of organic acids effects were carried out in RCM. In this part, a series of bottles which containing 200 ml of RCM and added a variety of concentrations 1000 mg/l, 2000 mg/l, 3000 mg/l, 4000 mg/l and 5000 mg/l for butyric and 2000 mg/l, 3000 mg/l, 4000 mg/l, 5000 mg/l and 6000 mg/l for acetic acid were used, and then, all the bottles were adjusted to initial pH 7.0 and incubated at temperature 35°C at 150 rpm of agitation.

To study about glucose effect, RCM medium with different glucose concentration (0%, 1%, 2%, 3%, 4%, 5%, 6% and 7%) were used. The experiment procedure is the same as organic acids effect. Secondly, to study optimum of pH control and loading rate of substrates, the fermentation (KBT Biotech, model KF 5l) was carried out with 5 l of reactor and working volume 2 l. In the reactor gas pressure was maintained from 0.18 - 0.20 atm. during fermentative. Agitation was performed at 150 rpm. The temperature was set at 35°C and 40°C for RCM and food waste, respectively. For optimum pH, the variation of pH in the reactor which containing 2 l of RCM or food waste solution with concentration about 50,000 mg/l of COD were maintained at 5.0, 5.5, 6.0, 6.5 and 7.0 during fermentative by dropping of HCl or KOH. In case of loading rate of substrate, the experiment carried out with food waste solution used as medium in a varieties of concentrations as 40,000 mg/l, 50,000 mg/l, 70,000 mg/l and 100,000 mg/l of COD. The pH was maintained at 5.5 in the reactor. All inoculations of cell or medium were performed in the vacuum anaerobic chamber (Three-Shine, SK-G002-A1).

3. Analytical methods:

- Biogas production was measured by water displacement (Fig. 1) or gas meter.

- Hydrogen analysis: Hydrogen concentration was measured by gas chromatograph. Function was follow:

Equipment: Gas chromatograph (Shimazu model GC-17A).

+ Detector: Thermal conductivity detector (TCD).

+ Column: Molecular sieve 5A.

+ Temperature of injector, detector and column temperatures: 60°C.

+ Current: 45 mA.

+ Carrier gas: Argon (45 ml/min).

- pH: pH were adjusted by few drops of HCl or KOH controlled by a pH meter (DMS Digital pH/ion meter, model DP-215M).

- Cell concentration: The cells concentration was measured by spectrophotometer (Pharmacia Biotech model Ultrospec® 2000) with wavelength 600 nm (OD_{600}).

- Glucose concentration: DNS method.

- COD: Standard method.

- Organic acids analysis: Organic acids concentration was measured by gas chromatograph. Function was followed:

Equipment: Gas chromatograph (Younglin, model M600D).

+ Detector: Flame ionization detector (FID)

+ Column: Glass column packed with 80/120, Carbopack B-DA/4% CARBOWAX 20M.

+ Temperature of injector, detector: 200°C.

+ Temperature of column was initially 135℃ for 2 min, followed by 165℃ for 1.7 min, 180℃ for 20 min, and lastly 200℃ for 15 min.

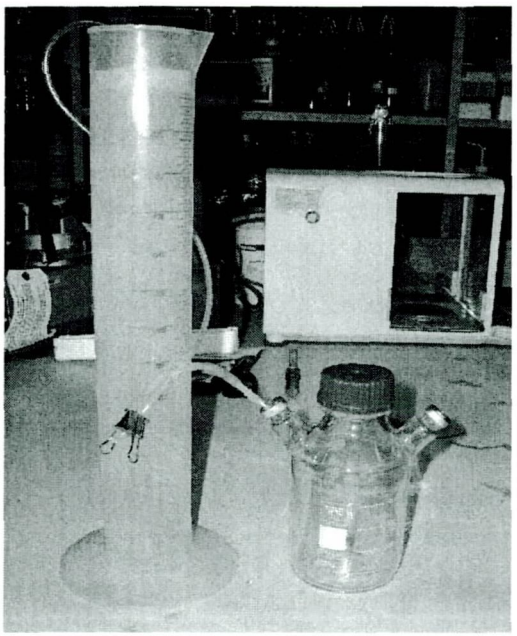

Fig. 1. Photograph of gas measurement equipment with 500 ml bottles.

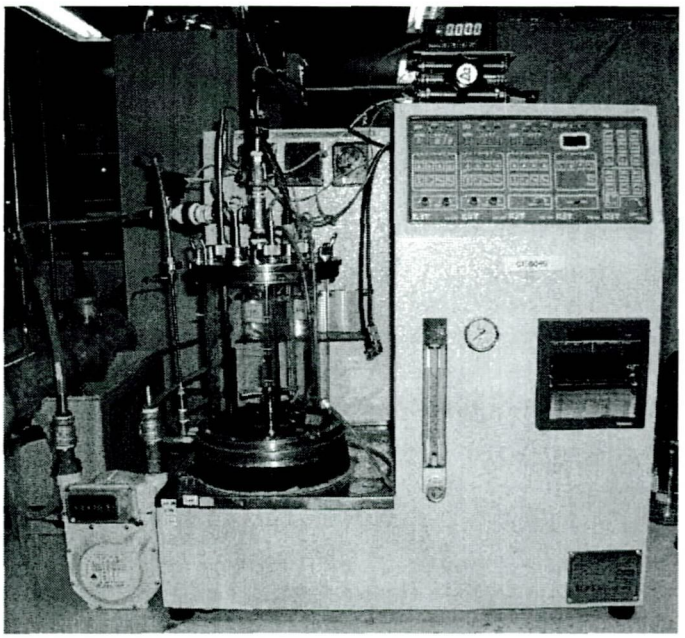

Fig. 2. Photograph of 5 l fermentor.

III. RESULT

1. Hydrogen production from Reinforced Clostridial Medium (RCM).

Hydrogen production from RCM was maximum 32% of biogas. In this part, initial pH, temperature, glucose concentration, pH control, agitation effect and inhibition effect of organic acids produce were investigated.

For optimization of medium composition we have studied the components of RCM for the essential elements to cells growth during hydrogen production with fermentative by *C. beijerinckii*. First, each component of RCM was removed however in this case there is no affection. Second, components of RCM were combined with Standard Mineral Medium (SMM). The results verified that tryptose or yeast extract were the essential compounds (Table 4). From that, we found out the optimal conditions to hydrogen production by *Clostridium beijerinckii*.

Table 4. Determination of cell growth on different medium composition

Medium composition	Growth
SMM + Glucose	-
SMM + Soluble starch	-
SMM + Glucose & Yeast extract	++
SMM + Glucose & Beef extract	-
SMM + Glucose & Tryptose	++
SMM + Glucose & Cysteine hydrochloride	-
RCM	++

(1) Effect of initial pH

A series of 500 ml serum bottles at different initial pH 6, 7 and 8 were used. Fig. 3a shows the cells growing curve. The cells were grown immediate in initial pH 6.0. When initial pH was higher, they have longer lag phase. It might be the time required to adapt to a new pH condition as the pH in the inoculum was low. For hydrogen production, at initial pH 6.0 hydrogen is produced a little higher than initial pH 7.0 (89 ml and 88 ml, respectively). However, the hydrogen production rate was highest in the initial pH 7.0. This result indicates that the optimum of initial pH was 7.0. In the optimum condition of initial pH, hydrogen was produced 87.9 ml after 24h fermentative. The hydrogen content was a maximum of 26.84% of biogas, consumption of glucose was the highest as 5405.5 mg/l. Acetic and butyric acids produced were 196.8 mg/l and 1502.1 mg/l, respectively (Fig. 3b and 3c).

(2) Effect of agitation

Two 500 ml serum bottles were used with initial pH adjust to 7.0. Temperature maintained was 35°C. When incubate with agitation is 150 rpm, the cells grow faster than no-agitation (Fig. 4a and 4b). Maximum hydrogen was 158.22 ml at agitation of 150rpm after 15h. Acetate and butyrate produced were 681.58 mg/l and 1831.2 mg/l, respectively, in case of agitation at 150rpm. In case of no agitation, after 15h hydrogen produced was 83 ml, and butyric acid and acetic acid were accumulated

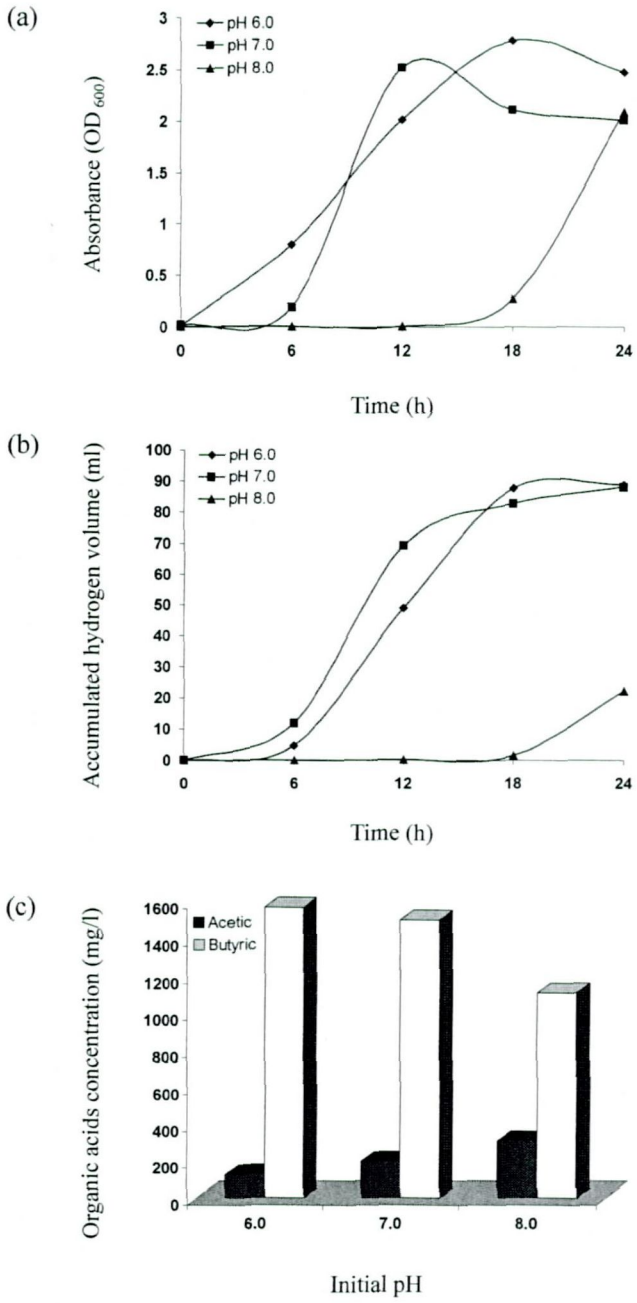

Fig. 3. Effect of initial pH on (a) cell growth, (b) hydrogen production and (c) organic acids production in RCM.

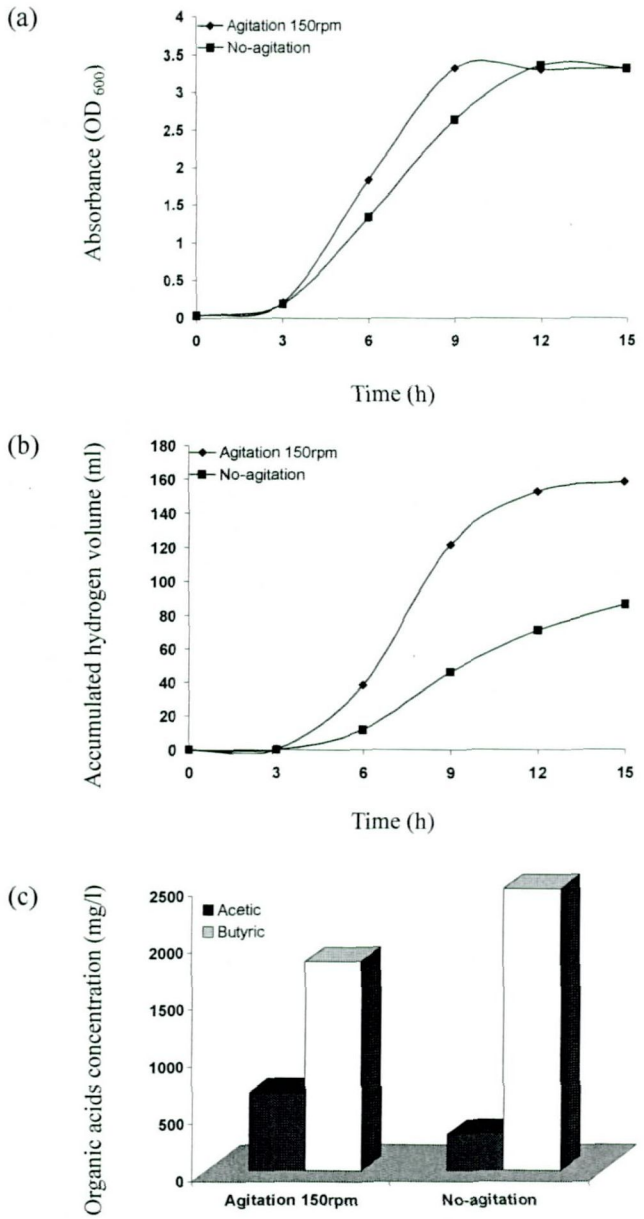

Fig. 4. Effect of agitation on (a) cell growth, (b) hydrogen production and (c) organic acids production in RCM.

2470.28 mg/l and 321.10 mg/l in the medium, respectively. It indicated that the mechanism was changed when agitation was effective (Fig. 4b and 4c).

(3) Effect of temperature

To investigate the optimal temperature, a series of 500 ml serum bottles at initial pH 7 were incubated at various temperatures of 25°C, 30°C, 35°C, 40°C and 45°C. During fermentative, the cells can grow and produce hydrogen earlier in 40°C, after that, they lose activity, resulting the cells density and hydrogen production were low (Fig. 5a and 5b). Fig 5c shows the accumulation of organic acids in the reactor. At temperature 30°C acetic acid produce was more than 35°C. But in case of temperature of 35°C much more of butyric acid was produced. Therefore, the optimum temperature was 35°C. In the optimum of temperature maximum hydrogen produced was 144.55 ml after 12h fermentative and butyric acid and acetic acid were 847.60 mg/l and 624.23 mg/l respectively. The hydrogen concentration maximum was 31.5% of biogas.

(4) Effect of glucose concentration

Glucose concentration effect was investigated with a variety of concentrations such as 0%, 1%, 2%, 3%, 4%, 5%, 6% and 7%. The results indicate that the *Clostridium beijerinckii* can be grown well up to 6% of glucose concentration in the medium (Fig. 6a). During fermentative, hydrogen and organic acids were produced at the same time. Hydrogen

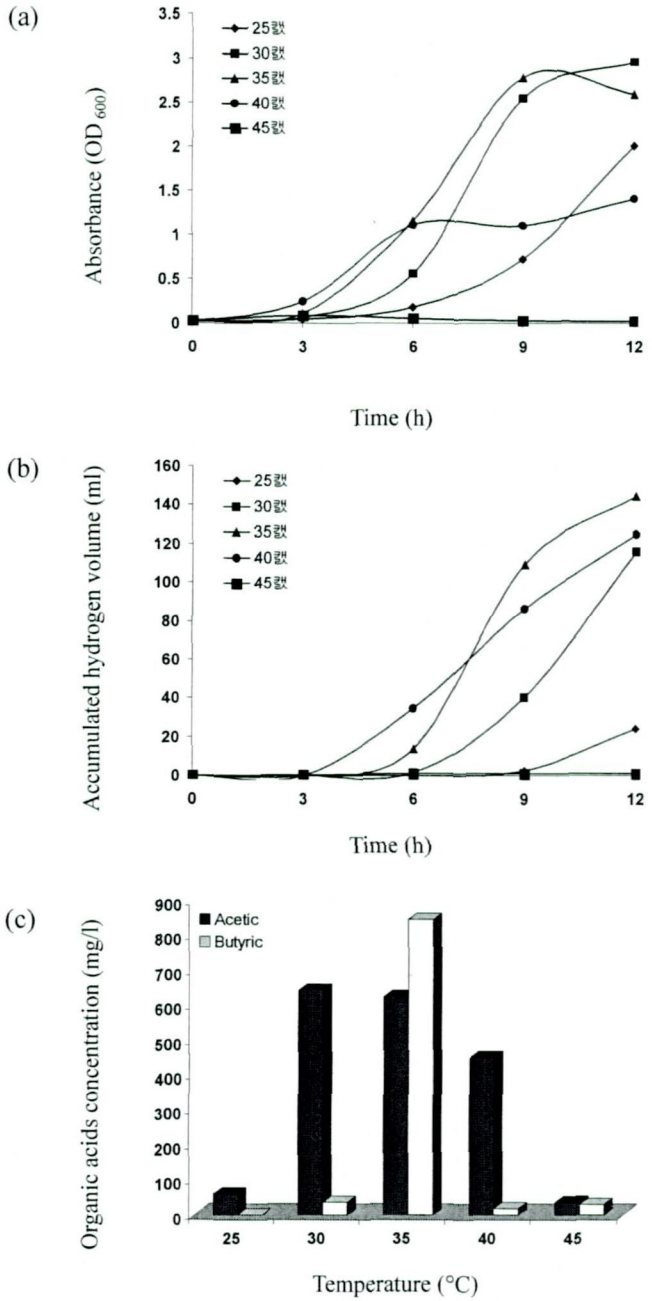

Fig. 5. Effect of temperature on (a) cell growth, (b) hydrogen production and (c) organic acids production in RCM.

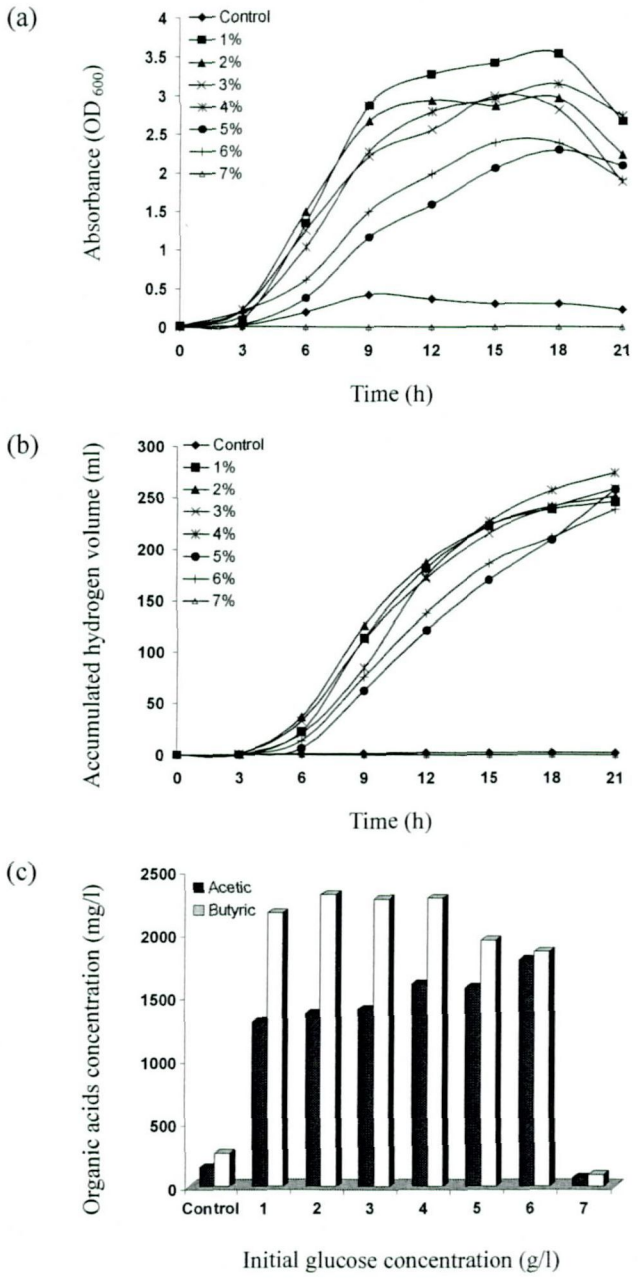

Fig. 6. Effect of glucose concentration on (a) cell growth, (b) hydrogen production and (c) organic acids production in RCM.

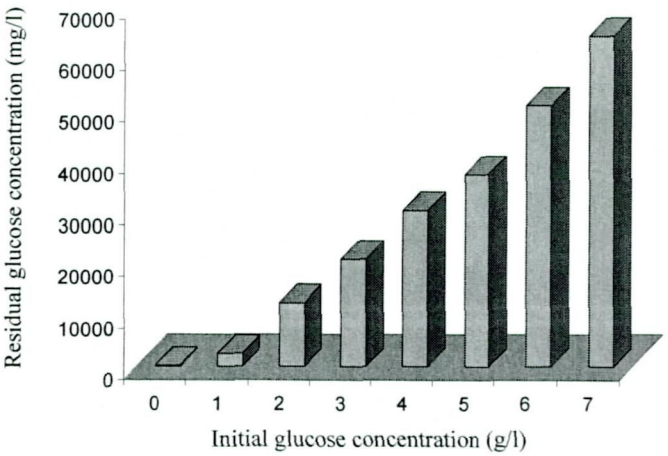

Fig. 7. Residual glucose concentration after fermentation.

production and organic acids were increased up to 4% of the glucose concentration but when it is higher, glucose became inhibitor (Fig. 6a, 6b and 6c). However, with the glucose concentration over 2%, a large amount of glucose was remained after the processing was completed by decrease of pH (Fig. 7). Consequently, optimal glucose concentration in this study was 1% (w/v).

(5) Effect of acetic acid concentration in the culture medium

One of product with large amount is acetic acid. To study the effect of acetate accumulated, acetate was added into RCM medium before fermentative with various concentrations of 2,000 mg/l, 3,000 mg/l, 4,000 mg/l, 5,000 mg/l and 6,000 mg/l. Fig. 8a and 8b show the cells density and hydrogen production during fermentative. The result indicates that if concentration is lower than 5000 mg/l, there is no effect because of butyric acid produced instead of acetic acid (Fig. 8c). If the concentration is higher than 5000 mg/l, this strain was inhibited by acetic acid. When acetic acid have affect hydrogen production was decreased in an inverse ratio with concentration of acetic acid in the reactor.

(6) Effect of butyric acid concentration in the culture medium

The main product which was produced during fermentative hydrogen was butyric acid. To study the effect of butyric acid accumulated, butyric acid with various concentrations of 1,000 mg/l, 2,000 mg/l, 3,000 mg/l, 4,000 mg/l and 5,000 mg/l were added into medium before fermentative. The

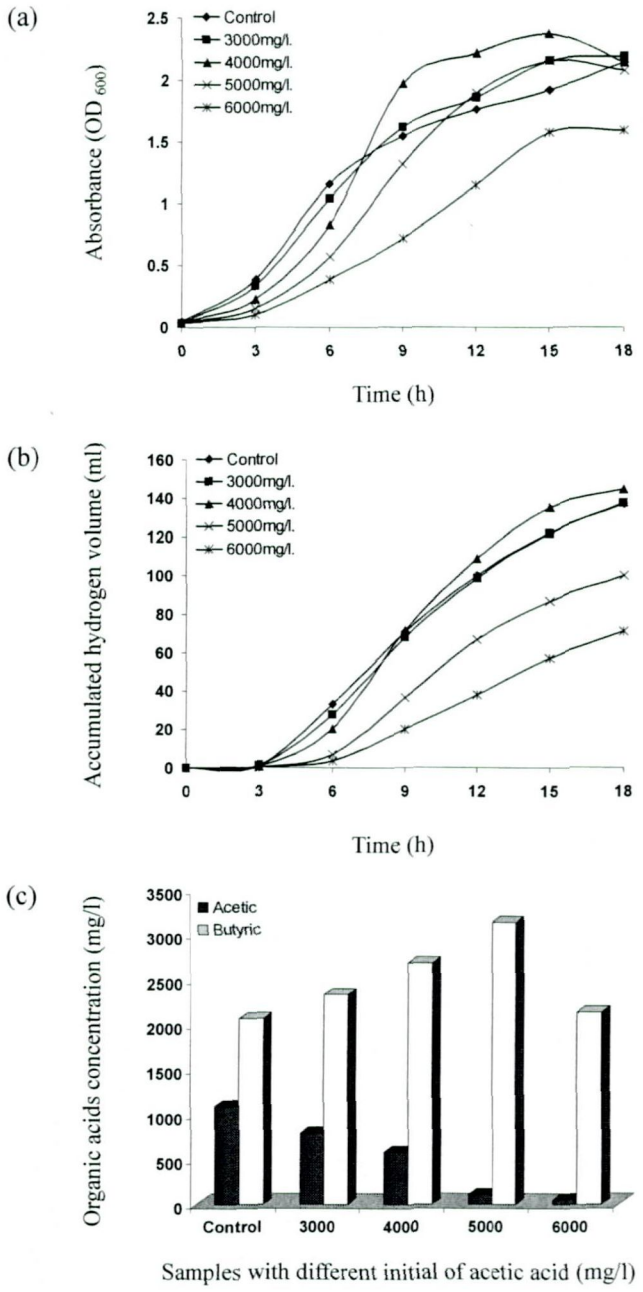

Fig. 8. Effect of acetic acid on (a) cell growth, (b) hydrogen production and (c) organic acids production in RCM.

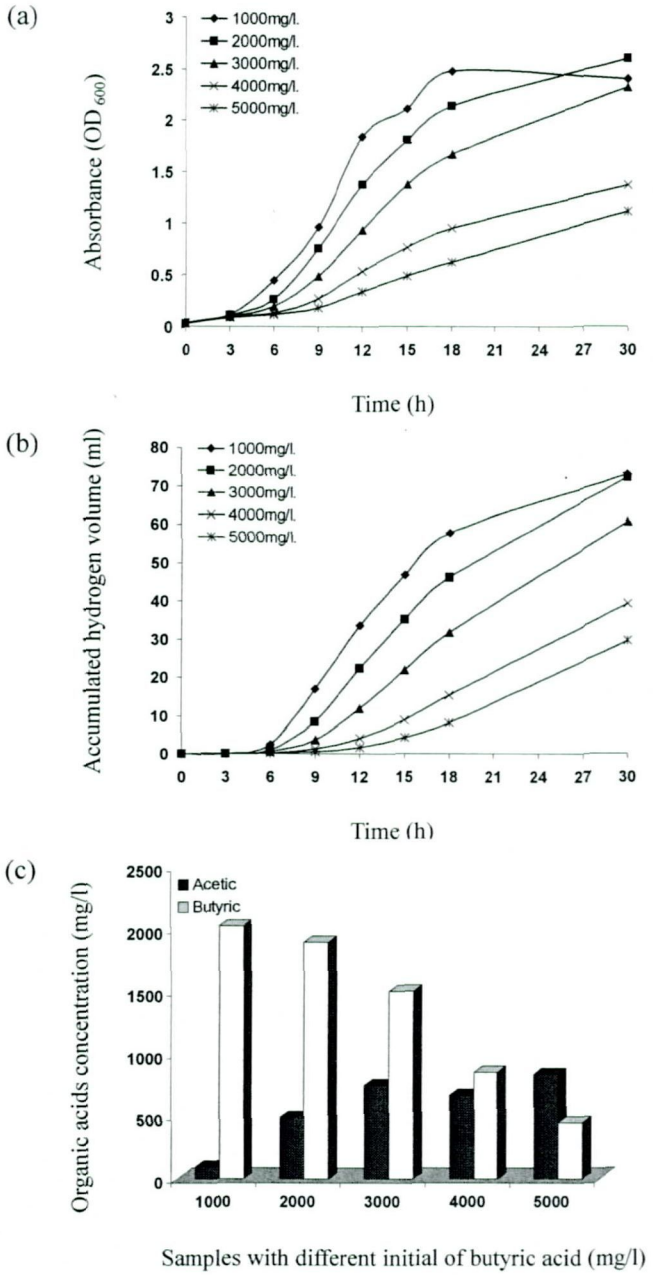

Fig. 9. Effect of butyric acid on (a) cell growth, (b) hydrogen production and (c) organic acids production in RCM.

results indicate that if concentration is lower than 4000 mg/l, there are low affected, and if the concentration is higher than 4000 mg/l, this strain was strong inhibited growth by accumulation of butyric acid whereas hydrogen production was strong inhibited at 3000 mg/l (Fig. 9a and 9b). Fig. 9c shows the accumulation of acetic and butyric acids. It suggests that acetic acid was main organic acid produce when the concentration of butyric acid in the medium is higher than 3,000 mg/l.

(7) Effect of pH control

The pH was an important factor during fermentation, to optimize the pH for hydrogen production. A variety of pH with 5.0, 5.5, 6.0, 6.5 and 7.0 were controlled. Fig. 10a and 10b show the cells density and hydrogen produced in each pH respectively. Hydrogen production was highest at pH 5.5 (Fig. 10b). With pH 5.0 and pH 7.0, a longer lag phase was required, and the cells growth is fastest at pH 6.5. Fig. 10c shows the organic acids produced. It demonstrates that acetic acid production was increased at the high pH whereas butyric acid production was increased at the low pH. Total organic acids were highest at pH 5.5. The result indicates that optimum pH for hydrogen production by *C. beijeirinckii* is 5.5. In the optimum conditions, maximum hydrogen produce is 1471.55 ml after 15h. Organic acids produced and accumulated maximum are butyric and acetic acids with concentrations of 1871.867 mg/l and 930.688 mg/l, respectively. Glucose was used about 95% (5966.7 mg/l).

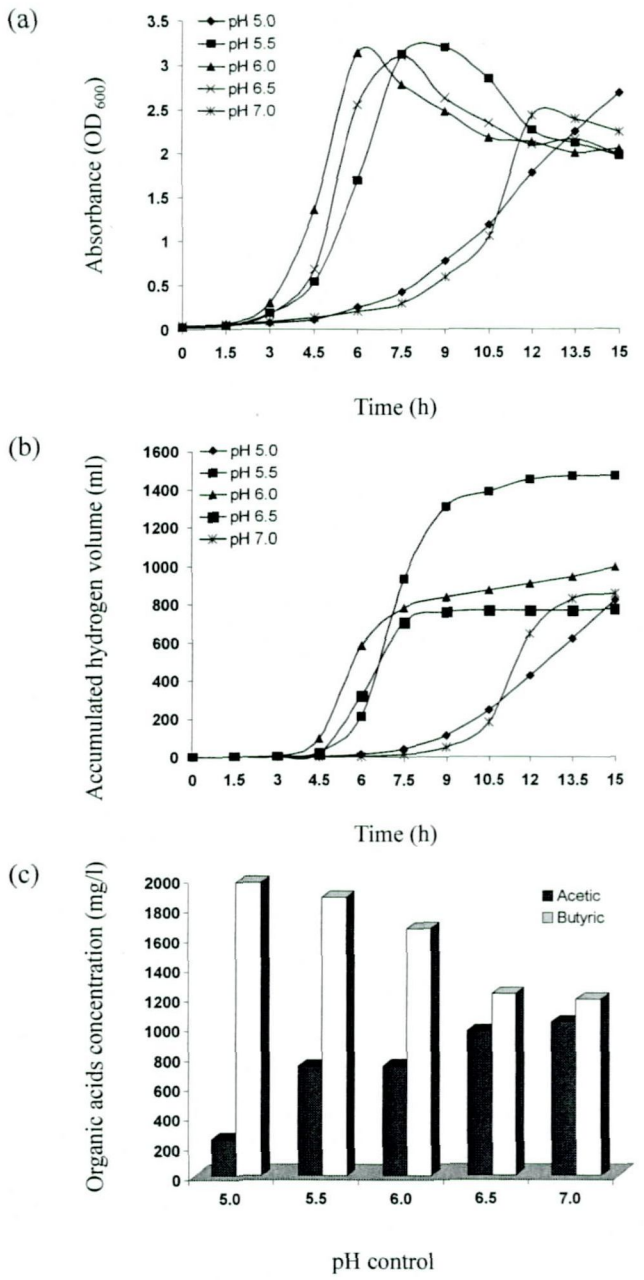

Fig. 10. Effect of pH control on (a) cell growth, (b) hydrogen production and (c) organic acids production during fermentation using 5 l fermentor in RCM.

2. Hydrogen production from food-waste:

Hydrogen production from food-waste was a maximum of 37.82% of biogas. In this part, initial pH, temperature, food-waste concentration, pH control, and agitation effects were investigated.

Clostridium beijerinckii can grow on food-waste as a substrate, but it needs time to adapt to this condition. We have adapted by culture the strain in the medium with a mix of RCM and food waste solution (around 50,000 mg/l of COD). After adaptation, we used food waste as a substrate for further experiments.

(1) Agitation effect

Two 500 ml serum bottles were used with initial pH adjust to 7.0. Temperature was maintained at 35°C. Fig. 11a shows the hydrogen production when incubate with agitation of 150 rpm. The hydrogen produce and hydrogen production rate are higher than without agitation. The results demonstrate that agitation has good effect to hydrogen production from food waste by *Clostridium beijerinckii*. In the condition with agitation of 150 rpm, maximum hydrogen produced was 254.7 ml after 24h and main organic acids produced were acetate and butyrate with concentrations of 1284.978 mg/l and 3882.999 mg/l, respectively (Fig. 11b). The maximum hydrogen content was 37.7% of biogas. This result is in accordance with the studies in RCM.

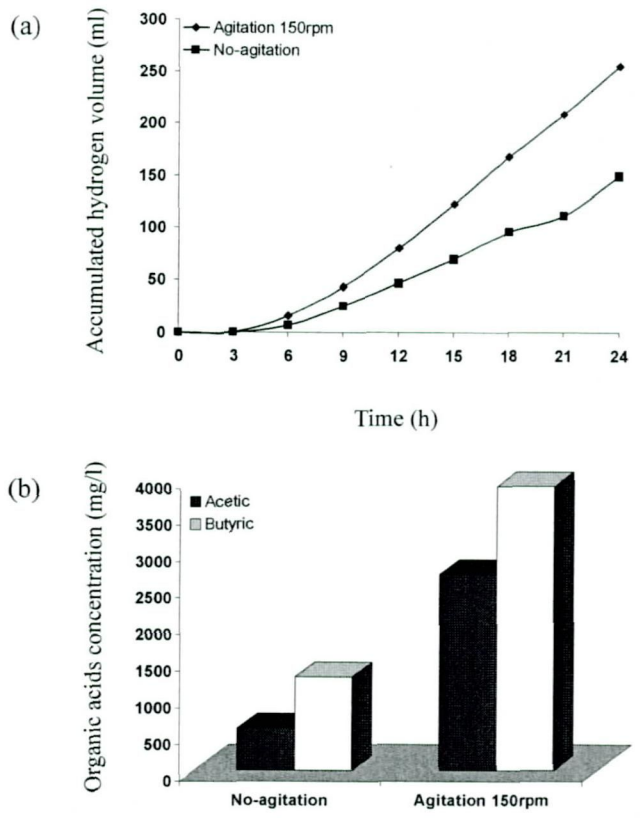

Fig. 11. Effect of agitation on (a) hydrogen production and (b) organic acids production using food waste.

(2) Initial pH effect

A series of 500 ml serum bottles at different initial pH 5.0, 6.0, 7.0 and 8.0 were used. Fig. 12.a shows the hydrogen produced. It was denoted that there is no difference in all initial pH in the lag phase and this data was different from RCM. It might be caused by characteristic of food waste. After that, the data indicates that the optimum of initial pH for hydrogen production was 7.0. In the optimum condition of initial pH, hydrogen was produced 231.07 ml after 24hr fermentative. The hydrogen content was maximum of 35.8% of biogas. Acetic and butyric acids produced were 1030.8 mg/l and 3545.5 mg/l, respectively (Fig. 12b).

(3) Temperature effect

To investigate the optimal temperature, a series of 500 ml serum bottles at initial pH 7.0 were incubated at various temperature 25°C, 30°C, 35°C, 40°C and 45°C. Fig. 13a shows the accumulation of hydrogen produce from food waste in different temperatures. In this study, hydrogen was produced highest at 40°C. At 45°C hydrogen production was low, meaning that, at 45°C temperature *Clostridium beijerinckii* was inhibited. Maximum acetic and butyric acids produced were 1285.0 mg/l and 3883.0 mg/l, respectively, at 40°C (Fig. 13b). Therefore, in this study, the optimal temperature for hydrogen production was 40°C. At this condition maximum hydrogen produced was 271.93 ml after 24hr fermentative and the hydrogen concentration maximum was 35.8% of biogas. Nevertheless,

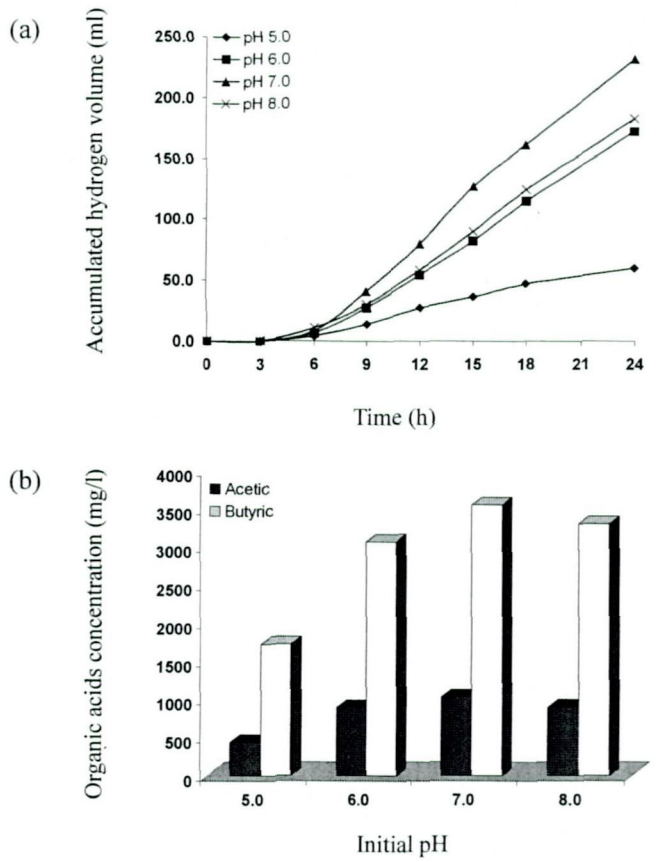

Fig. 12. Effect of initial pH on (a) hydrogen production and (b) organic acids production using food waste.

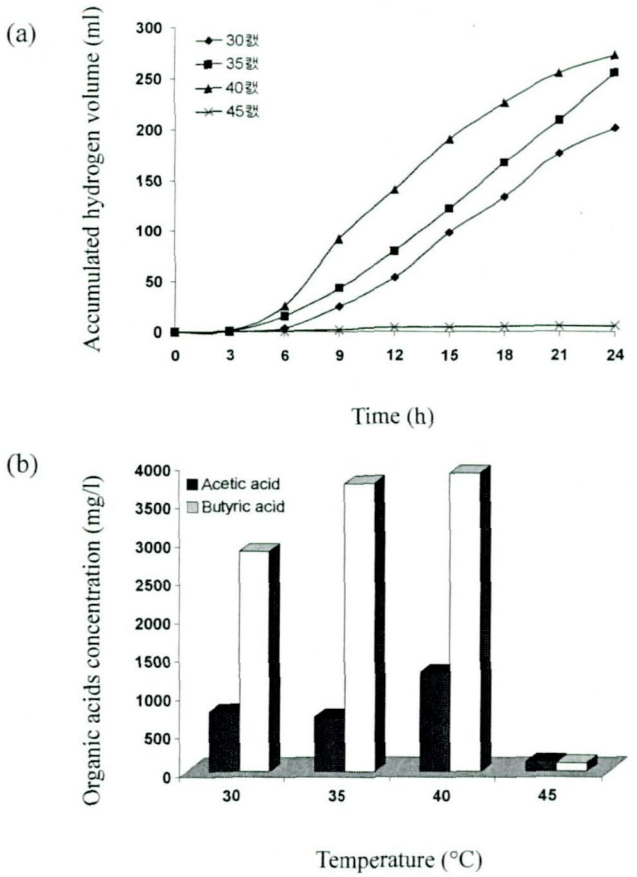

Fig. 13. Effect of temperature on (a) hydrogen production and (b) organic acids production using food waste.

at 35°C hydrogen was produced a quantity similar to at 40°C and the hydrogen production rate was stable. It suggest that in this experiment hydrogen production was highest in 40°C because of pH in the reactor was depleted by the accumulation of organic acids. It means that in case of batch culture without control pH, the optimum temperature was 40°C. However, we concern that in case of continues systems the cells activities could be lost at 40°C and the optimal temperature should be lower.

(4) pH control effect

To study optimum of pH for hydrogen production from food-waste, a variety of pH at 5.0, 5.5, 6.0 and 6.5 were controlled during fermentative at 40°C and agitation of 150 rpm. Fig. 14a shows the hydrogen production in different pH conditions. The result indicates that optimum pH for hydrogen production by *C. beijeirinckii* was 5.5. In this condition after 30hr, maximum hydrogen produce was 2138.3 ml. The hydrogen concentration produce was a maximum of 38% of biogas. In Fig. 14b the value of organic acids produce indicate that butyric acid produce more when the pH is more acidic and acetic acid produce more when pH is more alkaline. Maximum of acetic and butyric acids were 1208.18 mg/l and 3619.11 mg/l at pH 5.5. This result was in agreement with the results of RCM.

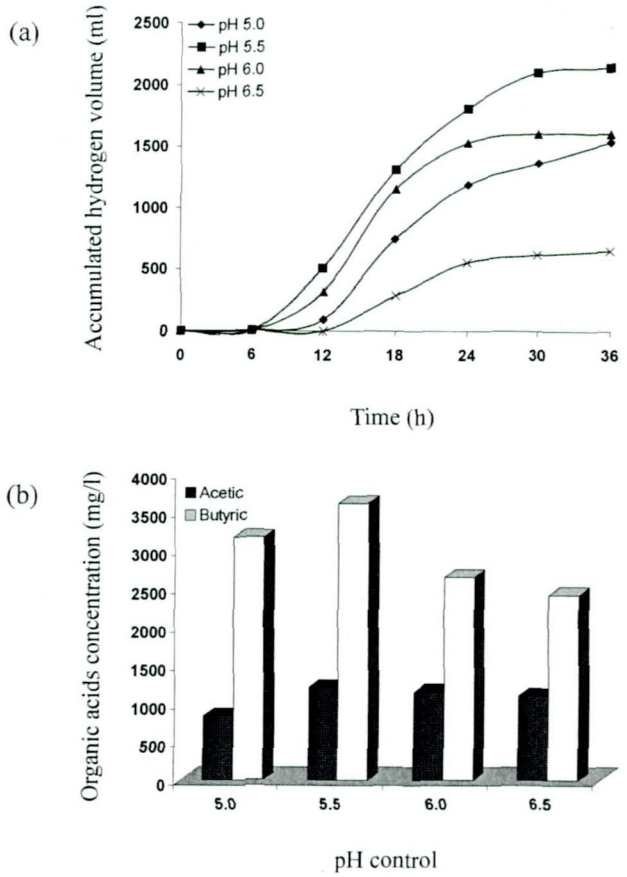

Fig. 14. Effect of pH control on (a) hydrogen production and (b) organic acids production using food waste.

(5) Food waste concentration effect

Food-waste concentration has effect on hydrogen production. The optimal of food-waste concentration was investigated in this study. Although, when the food-waste concentration is low, the hydrogen produced decrease, however, much higher concentration of food-waste is also inhibitor of hydrogen production. To know the optimal of food waste concentration, various concentrations of 40,000 mg/l, 50,000 mg/l, 70,000 mg/l and 100,000 mg/l of COD were used. Fig. 15a and 15b show the hydrogen accumulated and organic acids produced, respectively. In case of 100,000 mg/l of COD, the hydrogen production by *Clostridium beijerinckii* was inhibited completely, after 36hr acetic acid was produced, but there is no hydrogen was detected. At concentration of 70,000 mg/l of COD, the inhibit affect weakly and 40,000 mg/l of COD concentration of substrate was low, therefore, hydrogen production was low. This study demonstrates that the optimum concentration of food-waste for hydrogen production is around 50,000 mg/l of COD. In this condition maximum concentration and hydrogen accumulated were 1003.0 ml like 30% of biogas after 36hr. The accumulations of organic acids were 616.38 mg/l and 1452.07 mg/l, respectively.

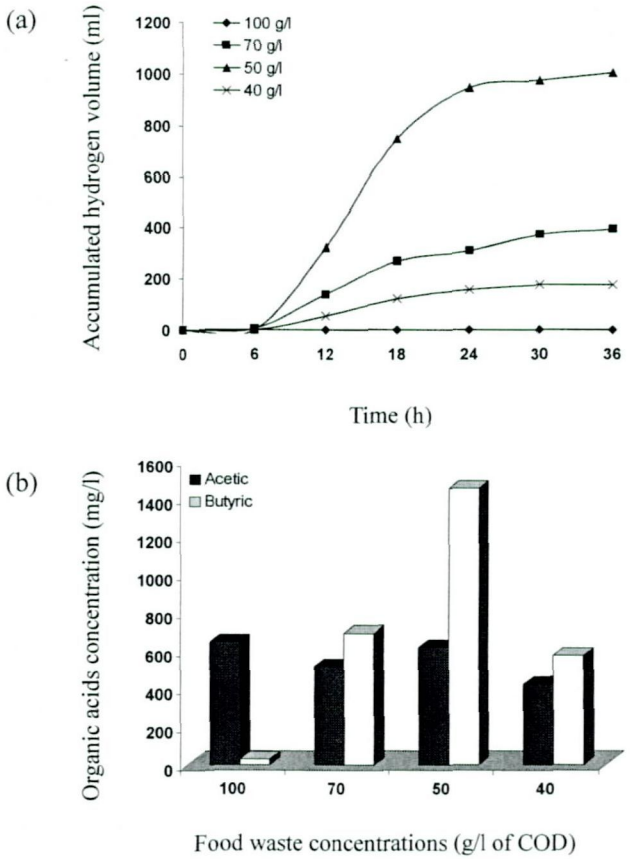

Fig. 15. Effect of food waste concentration (g/l of COD) on (a) hydrogen production and (b) organic acids production using food waste.

IV. DISCUSSION.

The *Clostridium beijerinckii* is an efficient strain to produce hydrogen during fermentative, and concurred to other researches [66, 67]. This study was considered to be optimal to produce hydrogen from two sources, the RCM and food waste.

For hydrogen production from RCM, the optimum conditions were pH 5.5, agitation 150 rpm, and temperature at 35°C. In this condition, hydrogen production was 1470 ml (32% of biogas produced) from 2 l of medium. In case of hydrogen production from food-waste, the optimum conditions were pH 5.5, agitation 150 rpm, and temperature at 40°C. The hydrogen production was 2,100 ml from 2 l with concentration around 50,000 mg/l of COD. Optimal temperature was different between RCM and food waste may be due to food waste characterize. Therefore, the optimum temperature for hydrogen production should be from 35°C to 40°C. This optimal temperature was corresponded by others reports which was informed by Taguchi *et. al* with *Clostridium beijerinckii*, strain AM21B [66, 69].

Lee *et. al* concluded that the initial pH decrease rapidly and produce hydrogen [44]. It means that hydrogen and organic acids produce simultaneously, then pH decrease by accumulation of organic acids. The effect of initial pH was reported by Khanal *et. al*, and also, it indicated that initial pH was an important factor in hydrogen production. Environmental

condition changes caused by the rapid depletion of pH might have resulted in a metabolic alteration, and subsequent inhibition of hydrogen production. In this experiment the optimum of initial pH to hydrogen production by mix culture from sucrose and starch was 5.5 [42]. The hydrogen fermentative process inhibition may be caused by the mechanism changed. When pH reached to limit point in the reactor, the solventogenesis processing was occurred instead of acidogenesis processing. Accordingly hydrogen wasn't produced any more. Collet *et. al* were found that the pH became more alkaline, the proportion of hydrogen in the gas phase more increased [53]. It conform our study that during fermentative, initial pH was decease rapidly caused by accumulation of organic acids product. Resulting hydrogen production decrease and pause before fermentative of substrate complete. In addition, when initial pH was more alkaline, acetic acid can be produce more, whereas butyric acid can be produce more when initial pH more acidic in condition of initial pH lower than 8.0 (Fig. 2 and 11). Therefore, the proportion of hydrogen has been changed.

In this study agitation has good effect to hydrogen production, when agitation was affected the mechanism of fermentative will change. Therefore, acetic acid was produced more than non-affected and hydrogen can produce more.

Van Ginkel and Logan concluded that glucose concentration has a greater effect on H_2 yields than the HRT. They also indicated that glucose itself is

not expected to inhibit hydrogen production and the H_2 yields were inversely related to the glucose feeding rate [70]. In this research, *Clostridium beijerinckii* can grow and produce hydrogen well in medium with glucose concentration up to 4%. In this study, the optimum condition of glucose concentration was 1% because initial pH decrease rapidly so that a large amount of glucose residual after fermentation complete (Fig. 5). It means that if we can control pH, hydrogen production can be improved.

For inhibition effect of product Van Niel *et. al* reported that the acetic acid has inhibition affect to hydrogen production by *Caldicellulosiruptor saccharolyticus* [47]. In this study the inhibit effect of organic acids such as butyrate and acetate were investigated also, both have inhibition effect to hydrogen production by *Clostridium beijerinckii*. The inhibition effect occurs when acetate or butyrate accumulated in the reactor is more than 5,000 mg/l of acetate and 3,000 mg/l of butyrate, respectively, and hydrogen production decease proportional to organic acids content in the medium (Fig. 6, 7).

This research also indicated that pH was an important factor in hydrogen production processing. With pH variation of 5.0, 5.5, 6.0, 6.5 and 7.0, organic acids produce was change. Acetic acid production was increased in the higher pH whereas butyric acid production was increased in the lower pH (Fig. 8, 12). This was different from the result obtained by Khanal *et. al* when they found with pH ranges of 5.5 –5.7, the HAc/HBu ratio was found

in the same range of 3–4 [42]. While using continuous monitoring of pH and hydrogen production during hydrogen fermentation, it is possible to determine the actual pH values that yield maximum hydrogen. This pH data could be employed in developing continuous flow reactor system for the maximum hydrogen yield. The result is in close agreement with the earlier work [42] where pH of 5.5 was deemed the optimum pH for hydrogen production.

In spite of same food waste concentration, hydrogen production potential was irregularity. It has been indicates that the food waste was different components and it is an important factor in bio-hydrogen processing.

It is reported that optimal condition to hydrogen production by *Clostridium beijerinckii* also demonstrate that strain was an efficient microorganism which can produces hydrogen from biomass and suggest an efficient way to treat food waste.

V. CONCLUSION.

1 The fermentative hydrogen production by *C. beijerinckii* could be increased through effectively controlling environmental factors.

2. Yeast extract or tryptose was essential for cells growth during hydrogen production from RCM.

3. Agitation was necessary for higher production of hydrogen. The optimum conditions for hydrogen production in RCM were as follows: initial pH 7.0, temperature 35°C, and glucose concentration 1% (w/v). The medium pH should be managed at 5.5 and the accumulated concentrations of butyrate and acetate should be lower than 3,000 mg/l and 5,000 mg/l, respectively. Maximum concentration of hydrogen in biogas was 32% and maximum hydrogen production rate was 284.8 ml/l·h.

4. Hydrogen could be produced from food waste. The optimum conditions were as follows: initial pH 7.0, temperature 40°C, pH should be managed at 5.5, and food waste concentration 50,000 mg/l of COD. About 5,500 ml biogas was produced from food waste during 36hr, and hydrogen content was 38% (2,100 ml). Maximum hydrogen production rate was 87.52 ml/l·h. This result indicates that food waste was a potential material for hydrogen production.

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