Isolation of Hidrogen

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ISOLATION OF HYDROGEN PRODUCING BACTERIA FROM SLUDGE OF ANAEROBIC BIOGAS REACTOR

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ABSTRACT

Hydrogen-producing bacteria can be found in the activated sludge generated from anaerobic waste treatment processes. Microbes in it consists of acetogenesis, metanogenesis and hydrogen-producing bacteria such as *Bacillus* and *Clostridium*. The purpose of this study was to isolate, characterize and identify a hydrogen-producing bacteria from sludge of anaerobic biogas reactor. Hydrogen production was analyzed by a gas chromatograph (GC-Shimadzu). Organics acid and ethanol productions were analyzed by HPLC, whereas reducing sugar was analysis by DNS method. Isolates identification was done based on the 16S rDNA gene sequences. Five isolates bacteria have been obtained from enrichment culture (culture C4) were developed using liquid minerals medium (HM medium). Based on the examination on the similarities of the isolates morphology obtained, three isolates have been selected. They were BYM1, BYM2, and BYM3. According to the sequence of their 16S-rDNA, the BYM1, BYM2, and BYM3 were identified as *Bacillus circulans* with 97% similarities. The three isolate bacteria were then designated as *Bacillus circulans* BYM1, *Bacillus circulans* BYM2 and *Bacillus circulans* BYM3. The acid production by the isolate and their combination was higher than the C4 culture. The mixed cultures *Bacillus circulans* BYW2- *Bacillus circulans* BYW3 had higher ability to produce hydrogen than the C4 culture. The fermentation by-products were ethanol, acetic acid and propionic acid, and acetic acid is the major metabolite.

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Keywords: biogas sludge, *Bacillus*, bio-hydrogen.

INTRODUCTION

Hydrogen (H_2) is considered to be a promising fuel in the future, because environmentally friendly and high calorie content (122 kJ/g), compared with the calorie content of hydrocarbons only (2-4 kJ/g). Some bacteria have the ability to convert carbohydrates to hydrogen in anaerobic conditions. Especially species of spore-forming bacteria such as Clostridium, Bacillus sp, Enterobacter sp. and some are of thermopiles bacteria. Along with hydrogen is also produced organic acids, methane and carbon

dioxide. To increase the production of hydrogen, to be assured that organic acids produced as small as possible. To improve the productivity of hydrogen can also be done by controlling the conditions of pH, temperature and HRT (Hydraulics Retention Time) in order to prevent the growth of non hydrogen bacteria culture (Cheng et al., 2011; Liu et al., 2011). Optimal hydrogen production in the fermentation on asidogensis phase at pH 5.5 and 6.5. At pH 4.5 has been to form organic acids because Clostridium acetobutylicum, Clostridium butylicum, and Clostridium beijerinkii have the ability to produce ethanol, butanol and acetone at low pH and reduces the formation of hydrogen . Another study using the alkaline conditions in the dark fermentation at pH 10 to avoid the formation of acid and propionate to inhibit the growth of non hydrogen bacteria. It is therefore very important to control the pH of media. Formation of hydrogen gas in fermentation can be carried out under the mesophyll (25-40) °C, thermofil (40-65) °C or hiperthermofil (> 80 ° C). The most favorable conditions in the mesophyll because not much need energy ((Claassen et al., 2010; Argun and Kargi, 2011; Sagnak et al., 2011). Isolation of hydrogen producing bacteria like Bacillus or Clostridium species is essential to increase the productivity of hydrogen. Therefore this study focused on isolating, characterizing and identifing new H_2-producing bacteria from sludge of anaerobic biogas reactor.

MATERIAL AND METHODS

The composition of the culture hydrogen medium (HM) according to the used by reference Stjryanto & Suwanto, 2000; Okonkin et al., 2008; Liu et al., 2010; Ozmihci et al., 2011). The composition of the media can be seen in table 1.

Tabel 1. The composition of hydrogen media (HM).

.No	Stock nutrients	Chemical formula (g/100ml)	Usage (ml/L)
1.	A	NH_4Cl (10 g), NaCl (1 g), $MgCl_2$ $6H_2O$ (1 g), $CaCl_2$ 2 H_2O (0,5 g).	10
2.	B	K_2HPO_4 3 H_2O (20 g)	2
3.	C	$NaHCO_3$ (10,4 g)	50
4;	D	$FeCl_2$ $4H_2O$ (0,2 g), H_3BO_3 (0,005 g), $ZnCl_2$ (0,005 g), $CuCl_2$ $2H_2O$ (0,0038 g), $MnCl_2$ $4H_2O$ (0,005 g), NH_4 6 Mo7 (0,005 g), $AlCl_3$ (0,005 g), $CaCl_2$ 6 H_2O (0,005 g), $NiCl_2$ $6H_2O$ (0,0092 g), EDTA (0,05 g), Na_2SeO_3 $5H_2O$ (0,01 g), HCl pekat (0,1 ml)	1
5.	E	Yeast ekstract (10 g)	10
6.	F	Na_2S (2,5 g)	10
7.	G	HCl 0,2 N	
8.	H	Glutamic acid (0,001 g), Ascorbic acid (0,0025 g), Riboflavin (0,0025 g), Citric acid (0,002 g), Folic acid (0,001 g), p-amino benzoic acid (0,001 g), Creatine (0,025 g).	1
9.		Glucose	10 g

The stock solutions media were sterilized separate at a temperature of 121° C for 20 minutes. The medium pH is adjusted to be 7 by adding 0.2 M HCl (stock G). Sludge from the anaerobic biogas reactor was wet heat treated (100°C, 2 hrs), cooled and then

mixed with hydrogen medium (HM) in the 250 ml of serum bottle. The ratio of activated sludge: media = 1:9 (v/v). Existing air in the bottle is removed by way of flushing using nitrogen gas. The enrichment culture was incubated in a shaker incubator at 65 ° C for 72 hours (C1 culture), 10% of the fermentation broth was subcultured to sterile HM medium (C2 culture). The subculturing was done three times. Hydrogen production was analyzed on C2, C3 and C4 cultures, whereas the metabolite (organic acids) and reducing sugar were analyzed on C4 culture. The C4 culture was then serially diluted in the same medium solidified by 1,5% Agar and purged with nitrogen gas to create anaerobic condition. The cultures were incubated for 72 hours. Single colonies were isolated, characterized and identification. Hydrogen production was analysed by a gas chromatograph (GC-Shimadzu). Organics acid and etanol productions were analyzed by HPLC, whereas reducing sugar was analysis by DNS method.

Identification of bacterial isolates.

Identification was based on sequences of genes encoding 16S rRNA. 16S rRNA gene was amplified by PCR from genomic DNA using the forward primer 27f with the sequence (5' - AGAGTTTGATCMTGGCTCAG-3') and reverse primer 1492r with the sequence (5' - TACGGHTACCTTGTTACGACTT-3'). PCR reactions were performed using the KIT (Pure TaqTMReady - To GoTMPCR Beads Amersham Biosciences) . The PCR program used was as follows : 95 ° C for 1 min , 30 cycles (95 ° C for 1 min , 50 ° C for 1 min and 72oC for 1.5 min) and 72oC for 10 minutes to an extension of the final product . PCR products were extracted from the gel and purified using a DNA gel extraction KIT. Purified DNA fragments used as templates for sequence analysis (sequencing). Partial DNA sequence of the 16S rDNA of the isolates were identified by entering the data analyzed DNA sequences into BLAST program at http://www.ncbi.nlm.nih.gov/BLAST/, sites used to obtain DNA sequence comparison is most similar to the data available in GenBank .

RESULTS AND DISCUSSION

Hydrogen production of enrichment culture

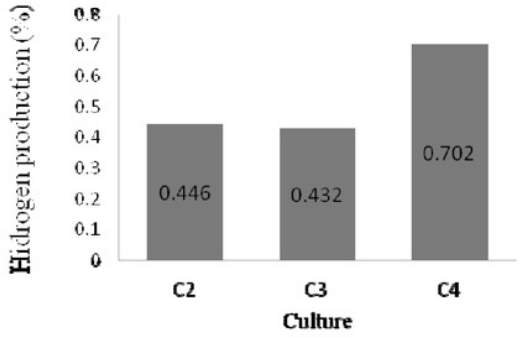

Figure 1. Hydrogen production from enrichment cultures

Figure 1 shows the H2 production of the enrichment culture C2, C3 and C4. This suggests that there are H_2 production bacteria in the culture. Among the three enrichment culture, the C4 culture had the highest production of H2, because sub culturing process resulted in the selection of the bacteria. Regardless of the subculturing conditions, the microbial communities of the the C4 culture were dominated by H_2 production bacteria. The production of H2 by the C4 culture was 0.702%, while the H2 production by the C3 culture and C2 culture was 0.432% and 0.446%, respectively.

Table 2 summarizes the acid and ethanol production, sugar utilization and pH of the C4 culture. The metabolite that were detected were acetic acid, propionic acid and ethanol.

Tabel 2. Metabolite production and pH of the C4 culture

Metabolite/reducing sugar/pH	Periode incubation (hour)	
	36	72
Acetic acid	7.73	24.970
Propionic acid	9.89	0.000
Ethanol	0.02	0.002
Reducing sugar	0.27	0.067
pH		5.500

Simultaneously, hydrogen gas was formed during a decomposition of sugar into low molecular weight organic acids such as acetate, butyrate, propionate, etc. Oh et al. (2003), also showed that during the process of the hydrogen production, the metabolites such as acetic acid, propionic acid and ethanol were formed, while the O-thong et al. (2011) found that the dominant metabolites formed are acetic acid, butyric acid and ethanol. In the same condition microbes also produce ethanol via different pathway (Koskinen, 2008). Some bacteria use Entner–Mayerhof phosphoroclastic) pathway in the oxidation of substrates, which results in the production of ethanol and hydrogen along with organic acids (e.g., acetate, butyrate, and lactate). As presented in Table 1, during periode incubation, reducing sugar is degraded into organic acids, ethanol and hydrogen. Reducing sugar decreased, while acetic acid acid increased from 7.73 mmol to 24.97 mmol. Acetic acid is the major metabolite.

Isolation, identification, and metabolite characterization.

From the C4 culture, five bacterial isolates have been obtained. Based on the examination on the similarities of the morphology of isolates obtained, three isolates have been selected. They were BYM1, BYM2, and BYM3. Each isolate had a circulair colony, opaque and cream colored, long rod-shaped cells (approximately 1x3um), gram-positive and facultatively anaerobic. According to the sequence of their 16S-rDNA, the BYM1, BYM2, and BYM3 were identified as *Bacillus circulans* with 97% similarities. The three isolate bacteria were then designated as *Bacillus circulans* BYM1, *Bacillus circulans* BYM2 and *Bacillus circulans* BYM 3.

Bacillus circulans is gram positive, occasionally curved rods, colonies are opaque, cream colored, slightly convex, growth on nutrient agar is thin and facultatively anaerobic. *B. circulans* is a typical chemoorganoheterotrophic bacterium using mono-, di- and polysaccharides and polyhydroxylic alcohols as sources of carbon, energy and

electrons. The activity of *B. Circulans* is attributed to its metabolites and their specific reactions such as acidolysis, alkalysis and complexolysis (*4*). *B circulans* are hydrolysisi of strach, acid production from such sugar, i.e. glucose, fructose, galactose, cellobiose, and sucrose. Spore are numerous in soil, may be isolated from sewage. (S.N. Groudev, Use of heterotrophic microorganisms in mineral biotechnology, *Acta Biotechnol. 7* (1987) 299–306. ABIS encyclopedia, www.tgw1916.net/Bacillus/circulans.html. There is only a few reports on H2 producing processes with *Bacillus circulans.*

The ability of isolate and combination of the isolates to produce hydrogen was examined in HM medium. Hydrogen and metabolite production of isolate and their combination are summarized in Table 3. The dominated metabolite by-products was acetic acid. The acid production by the isolate and their combination culture were higher than the C4 culture. The acid production could reflect the isolate ability which has considerable effect on the hydrogen production. Given this point, the hydrogen production by the mixed culture BYW2-BYW3 was higher than the C4 culture. The hydrogen production by mixed culture BYW2-BYW3 was 0,75%, while H2 production by C4 culture was 0.70%. Because the acid production by isolate and their combination was higher, the cultures pH become lower. The hydrogen was detected only in mixed BYW2-BYW3 culture. This may be due to poor implementation of research resulting in gas leakage, because ssimultaneously the gas will be formed during the formation of organic acids.

Tabel 3. pH, H_2 and metabolite production of each isolate bacteria and their combination

Isolate and their combination	H_2 production (%)	Metabolites (mmol)		pH
		Acetic Acid	Propionic acid	
BYW1	0	75.9	0,68	4,53
BYW2	0	117.9	0,45	4,67
BYW3	0	128.6	2,15	4,55
BYW1-BYW2	0	139.0	11,94	4,50
BYW1-BYW3	0	111,0	0,77	4,63
BYW2-BYW3	0,75	123.3	1,30	5,14
BYW1-BYW2-ByYW3	0	123.8	0,77	4,61

As has been noted, hydrogen gas generated by C4 culture or mixed culture of BYW2-BYW3 were relative low. In order to induce bacteria for hydrogen production, the following items should be gathered:

1. Fermentation condition are anaerobically
2. Intensive biogas separation to retrieve hydrogen during the process. When the hydrogen production increases the reaction becomes thermodynamically unfavorable because of the increase in H_2 partial pressure (Nath and Das, 2004)
3. Adding buffer, because the medium pH usually decreases drastically due to the accumulation of organic acids as fermentation proceeds and a lower pH suppresses H_2 production significantly (Oh et al., 2003).
4. Mixed the culture with a hydrogen-production photosyntetic bacteria. Microbial H_2 production can be either photosynthetic or non-photosynthetic. In dark fermentation (non-photosynthetic), carbohydrate substrates are decomposed into

organic acids and alcohols, liberating H_2 and CO_2 in the process. Most of the microbial hydrogen production is forced by the anaerobic metabolism of pyruvate. Pyruvate biodegradation is driven by one of two enzymes (Hallenbeck & Benemann, 2002).

1). Pyruvate: formate lyase (PFL)

$$\text{Pyruvate} + \text{CoA} \xrightarrow[\text{PFL}]{} \text{acetyl-CoA} + \text{formate}$$

2). Pyruvate: ferredoxin oxido reductase (PFOR)

$$\text{Pyruvate} + \text{CoA} + 2\text{Fd (ox)} \xrightarrow[\text{PFOR}_2]{} \text{acetyl-CoA} + \text{CO} + 2\text{Fd (red)}$$

Ferredoxin is reduced, during pyruvate oxidation. The reduced ferredoxin transfers the electrons to hydrogenase enzyme which catalyzes the production reaction of molecular hydrogen.

In dark fermentation Organic acids cannot be degraded completely due to thermodynamic limitations (reaction 1), whereas the reaction of photofermentative hydrogen production is shown in reaction 2.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CO_2 + 2CH_3COOH \quad (1)$$

$$CH_3COOH + 2H_2O \rightarrow 4H_2 + 2CO_2 \quad (2)$$

As shown above, in order to achieve a complete decomposition of carbohydrate integration with photofermentation can be employed.

CONCLUSSION

Isolation of hydrogen producing bacteria have been conducted using activated sludge biogas sources. Proof through DNA tests showed that the bacteria isolated were *Bacillus citrulans* .

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