A. PRODUCTION OF BIOHYDROGEN AND BIOACETIC ACID BY USING ISOLATES OF Bacillus circulans

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A. PRODUCTION OF BIOHYDROGEN AND BIOACETIC ACID BY USING ISOLATES OF *Bacillus circulans*

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Abstract

Biohydrogen and bioacetic acid are very important chemicals. Both of these chemicals can be produced through anaerobic fermentation by using *Bacillus circulans*. *Bacillus sp* can be isolated from biogas solid sludge by growing it in the modified media. The isolation was carried out by boiling the biogas sludge for 2 hours. After it boiled, only the spores-producing microbes would survive, such as *Bacillus sp* and *Clostridium sp*. Therefore, spores were grown in hydrogen media with glucose as a carbon source, for 72 hours, in anaerobic conditions and at 60 °C. During the growth, hydrogen produced was analyzed by using gas chromatography (GC), whereas metabolite liquid products were analyzed by using HPLC to determine the composition of organic acids and other products quantitatively. Finally, the identification of microbes producing hydrogen was conducted through the PCR test. The results of the analysis proved that the microbes matched to *Bacillus circulans* with 97% similarity. The results of GC showed that the hydrogen was produced by *Bacillus circulans*. The highest content of hydrogen was found in samples 8 and 9 (SC IV). They contained acetic acid of 7.800 ppm, propionate acid of 53 ppm. PCR test by comparing the 16S rDNA gene sequences through Blast program showed that the three isolates were most likely identified as *Bacillus circulans* (first isolate) and *Bacillus sp* (second and third isolates), with 97% similarity. *Bacillus circulans* was known as a microbe producing enzyme. Therefore, it could be concluded that this study has successfully found a species of *Bacillus circulans* with a new strain that was able to produce hydrogen and has not been previously discovered.

Key Words: *Anaerobic*; *Bacillus circulans*; *bioacetic acid; biohydrogen; PCR; thermophilic*

1. Introduction

Energy consumption is growing because of the increasing world population. The imbalance between supply and demand led researchers and industry to find and produce alternative energy from renewable sources [1].

Hydrogen is one of the renewable energy can be produced by anaerobic bacteria that grow on the substrate that is rich in carbohydrates. The fermentation process can be done in the dark conditions and can be performed at low temperature using mesophile (298-313 K), thermopile (313-338 K), extreme thermopile (338-353 K) and hiperthermophile (> 353 K) bacteria. Gas produced from the fermentation process is a mixture of H_2, CO_2, H_2S and CH_4 . Theoretically every mole of glucose can produce 4 moles of H_2. Bacillus and Clostridium are two species of bacteria that can produce spores and can be found in sludge biogas and can be obtained from anaerobic sludge processing [2].

Microbes that will be used to produce chemical products or other materials on an industrial scale has to go through the process of isolation and acclimatization. Microbial sources should be adjusted to the desired

microbial species. For example, to get the microbes that can produce hydrogen, microbial sources can be obtained from biogas sludge. As already known, hydrogen contained in the biogas. Therefore we can conclude that in the biogas reactor is expected to have a hydrogen-producing microbial. Due to in the mud is not only the hydrogen-producing microbial so isolation is necessary for one of the microbes from other. From the literature says that the hydrogen-producing microbes can form spores. This information is used as the basis of the isolation process [3]. To separate the hydrogen-producing microbes by heating the sludge to the conditions in which microbial cells that do not form spores will die. The spores then cultured in a enriched medium to support the growth of hydrogen-producing bacteria. Isolation produces a particular microbial species [4]. But does not guarantee consists of stable microorganism so the next step is acclimatization. Acclimatization is done by expanding the culture of microorganisms that have been isolated in fresh medium several times. Stability of the microorganisms tested again using the non glucose substrates such as sucrose or molasses and then in real substrate i.e. lignocelluloses substrates. It is expected that the type of substrate does not affect the product.

2. Material and Methods.

Isolation techniques are as follows: Sludge simmer for 30-60 minutes with the purpose of eradicating vegetative cells. In these circumstances the spores are still survive and spores will be grown in enriched media. Enriched medium composition is adapted to inflate the hydrogen-producing bacterial culture and suppress the growth of non-hydrogen bacteria. The composition of the media can be seen in Table 1. Table 1 shows the composition of the media components in one liter of media. Isolation of microbes is very important to obtain pure microbes. The process of isolation of microorganisms from biogas slurry. Can be seen in Figure 2. The first stage is to take the sludge from the bottom of the biogas reactor to obtain microorganisms that can produce gas in anaerobic conditions. Phase two boiling mud to kill vegetative cells. The spore forming microorganisms then conditioned in anaerobic conditions by bubbling nitrogen into the bottle containing the growing medium. After anaerobic condition, further incubation for 72 h at 60 ° C for 72 hours continuously to obtain the initial culture or sub-culture I (SKI). Isolation process followed by acclimatization process with the aim to obtain a stable microbial means if microbes were grown in the same media will produce the same metabolic products. Acclimatization performed three times with acclimatization time is 72 hours. Acclimatization is done by rejuvenating microbes in the new media with the same treatment. Microbial isolates named sub-culture I (SC I). Isolates were already acclimatized called sub-culture II, III and IV respectively. Sub culture IV is stable microbial. For more details, isolation, acclimatization and production of hydrogen and acetic acid using SK IV with different substrate can be seen in the flow chart below (Fig. 1).

Table 1. The composition of enriched media for hydrogen forming bacteria (MH)

No	Component	Ammount for 1 L solution
1	Pepton	4.0 g
2	L-Cystein	0.5 g
3	NaCl	3.0 g
4	$MgCl_2$	0.1 g
5	$FeCl_2$	0.1 g
6	K_2HPO_4	2.5 g
7	Liquid vitamins	10 ml
8	$MnCl_2$	0.01 g
9	$ZnCl_2$	0.05 g
10	H_3BO_3	0.01 g
11	$CaCl_2$	0.01 g
12	Na_2MoO_4	0.01 g

13	$CoCl_2$ 6 H_2O	0.2 g
14	AlK $(SO_4)_2$	0.01 g
15	$NiCl_2$ 6 H_2O	0.01 g

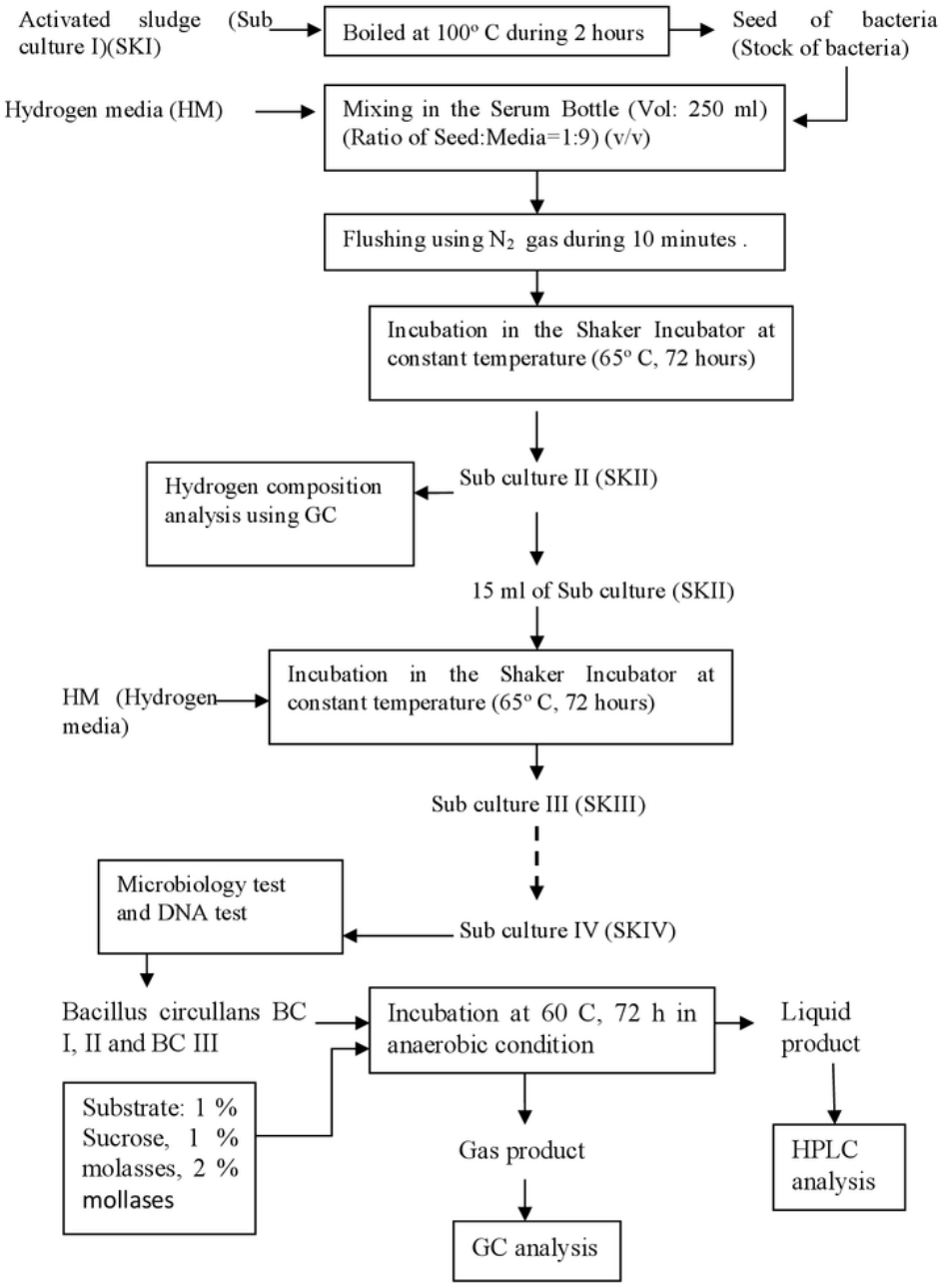

Fig. 1. Process flow diagram of hydrogen and acetic acid –production.

The result of DNA analyzed using PCR techniques SK IV is a new strain of Bacillus circullans with 97% similarity. There were three strains based on DNA sequences where all three are Bacillus circullans. Three strains of Bacillus circullans named BC I, BC II, and BC III. The next step is to choose one of the three most superior strains to produce hydrogen and acetic acid using the substrate sucrose 1%, molasses 2% and lignocelluloses. Production of hydrogen and acetic acid carried out in anaerobic conditions at 60 ° C and incubation time of 72 hours. The research method using a random system with three replications. Table 2 shows the encryption system used.

Fig. 2. The process of isolation of microorganisms from biogas slurry

Table 2. The research method of hydrogen production and acetic acid using Bacillus circullans in different substrates.

Inoculant	Substrate			Replication
	Sucrose 1%	Molasses 1%	Molasses 2%	
BC I	BC I-S11	BCI-M11	BCI-M21	1
	BC I-S12	BCI-M12	BCI-M22	2
	BC I-S13	BCI-M13	BCI-M23	3
BCII	BCII- S11	BCII- M11	BCII- M21	1
	BCII- S12	BCII- M12	BCII- M22	2
	BCII- S13	BCII- M13	BCII- M23	3
BCIII	BCIII- S11	BCIII- M11	BCIII- M21	1
	BCIII- S12	BCIII- M12	BCIII- M22	2
	BCIII- S13	BCIII- M13	BCIII- M23	3
BC(I+III)	BC(I+III)- S11	BC(I+III)- M11	BC(I+III)- M21	1
	BC(I+III)- S12	BC(I+III)- M12	BC(I+III)- M22	2
	BC(I+III)- S13	BC(I+III)- M13	BC(I+III)- M23	3
BC(I+II)	BC(I+II)- S11	BC(I+II)- M11	BC(I+II)- M21	1
	BC(I+II)- S12	BC(I+II)- M12	BC(I+II)- M22	2
	BC(I+II) -S13	BC(I+II)- M13	BC(I+II)- M23	3
BC(II+III)	BC(II+III)- S11	BC(II+III)- M11	BC(II+III)- M21	1
	BC(II+III)- S12	BC(II+III)- M12	BC(II+III)- M22	2
	BC(II+III)- S13	BC(II+III)- M13	BC(II+III)- M23	3
BC(I +II+III)	BC(I +II+III)-S11	BC(I +II+III)-M11	BC(I +II+III)-M21	1
	BC(I +II+III)-S12	BC(I +II+III)-M12	BC(I +II+III)-M22	2
	BC(I +II+III)-S13	BC(I +II+III)-M13	BC(I +II+III)-M23	3

3. Results and Discussion

3.1. pH Measurement on inocculation *Bacillus circulans* in Sucrose and Molasses Substrate

Table 3 shows the pH measurement results of liquid fermentation medium by using *Bacillus circulans* (BC). This process used sucrose and molasses as substrates.

Table 3. pH measurement results on the product of liquid fermentation.

Inoculant	Sucrose 1%	s 1%	Molasses 2%	Repeatation
BC1	5,56	5,29	5,42	1
	5,30	5,14	5,26	2
	5,85	5,47	4,49	3
BC2	5,56	5,37	5,22	1
	5,53	4,95	4,67	2
	4,81	5,36	5,05	3
BC3	5,23	5,26	5,10	1
	5,61	5,18	4,55	2
	5,45	5,20	4,92	3
BC I+II	5,78	5,46	5,03	1
	5,53	5,31	4,94	2
	5,43	5,02	4,53	3
BCI+III	5,70	5,61	5,07	1
	5,48	5,20	4,63	2
	6	5,34	4,97	3
BCII+III	5,57	4,81	5,17	1
	5,59	5,45	5,14	2
	5,78	5,38	5,59	3
BC I+II+III	5,33	4,70	4,91	1
	6,14	4,80	4,61	2
	5,43	4,94	4,96	3

As shown in Table 3, pH of fermentation products were different from the initial pH. Intial pH was adjusted at 7.5, whereas the average final pH was measured at 5 – 5.5. This phenomenon proved that fermentation process produced organic acids. Based on the products observed through isolation process, these organic acids comprised acetic acid, propionic acid, and ethanol, in which acetic acid had the highest content compared to propionic acid and ethanol. In addition, the longer fermentation resulted in the higher acetic acid content. As with previous studies in producing hydrogen from Clostridium, the anaerobic fermentation phase (dark fermentasig) the result is hydrogen gas and organic acids in which more organic acids than hydrogen [5][6]. To improve the productivity of hydrogen, fermentation continues in the next stage of (photo fermentation) using photosynthetic microorganisms. The study of hydrogen production using organic acids using photosynthesis mikroorgnisme will be done in subsequent studies.

Table 4. Hasil analisis produk cair menggunakan HPLC dan pH meter

No.	Sample code	Acquisition (ppm)		pH
		Acetic acid	Propionic acid	
1	BCI-M23	4,526.40	50.00	4,49
2	BC(II+III)- M22	6,761.58	57.66	4,63
3	BCII- M22	7,020	33.18	4,67
4	BCIII- M22	7.020.94	153.56	4,55
5	BC(I +II+III)-M22	7,225.84	55.44	4,61

From Table 4 it can be concluded that the process of fermentation produces organic acid. This is evidenced by a decrease in pH of the medium. Decrease in pH is not the same depending on the strain of Bacillus circullans and also depends on the type of substrate. 2% molasses was the best substrate among the three substrates used. While the best strain is a strain mixture of BC (I + II + III). Type of substrate affects the production of organic acids can be explained by the level of oxidation of the substrate. Molasses is the liquid that contains high sugar in the form of monosaccharide. The most preferred by microorganisms compared to the disaccharide (sucrose). The content of monosaccharide accelerate the adaptation time in the growth of microorganisms. At the same fermentation conditions, the concentration of monosaccharide will result in higher metabolic products. Since the product is an organic acid, the pH can indicate the amount of total organic acids. As shown in Table 4. Acid content in the fermentation medium is approaching 2%. It can be concluded that Bacillus circullans can also produce acetic acid in addition to produce hydrogen. The productivity of acetic acid can be increased by increasing the concentration of sugar in the medium. This can be done in a continuous fermentation process.

3.2 Hydrogen Content in Gas Product of Fermentation by Using *Bacillus circulans*

The results of gas chromatography (GC) analysis showed that hydrogen content in gas product of fermentation was very low. It was observed on almost all single inoculants. Mixture of inoculants BC (II+III) produced the highest hydrogen product with the content of 0.075%. Based on the fact, it could be concluded that *Bacillus circulans* could produced hydrogen when it worked in mix culture (or cooperated) with other BC strain in the mixed culture.

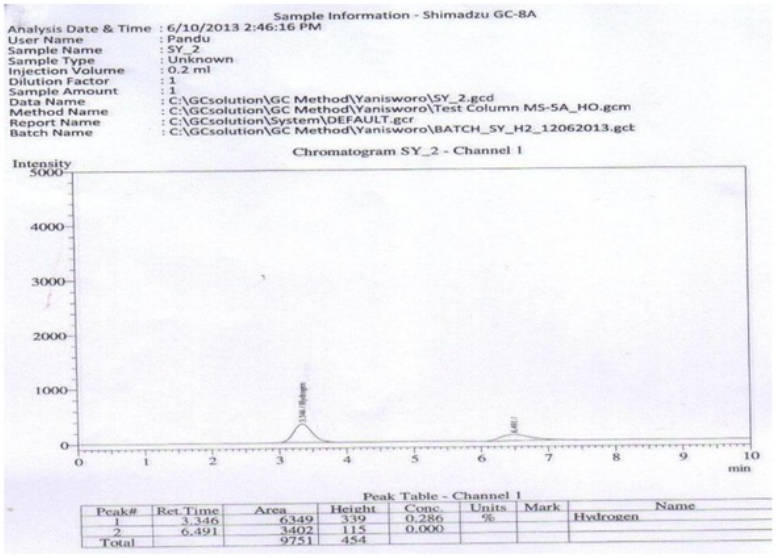

Fig. 3. The results of GC analysis. Peak seen is showing the peak of hydrogen.

Hydrogen produced by the BC I + II 0.075% (very low) because the fermentation is done in a very low concentration substrate. The results of GC analysis for all treatments are not shown but is almost equally under 1%. This is because more produce acetic acid, in the liquid products. In the anaerobic metabolic pathway, a secondary metabolite product more than the product of primary metabolites. Secondary metabolites are the gas and liquid. The product gas is a mixture of methane, carbon dioxide and hydrogen. In the biogas process, at an early stage approximately the first 2 weeks at most gas composition is carbon dioxide. The composition of the low methane. After 2 weeks the composition of reduced carbon dioxide and methane increases. Anaerobic process takes a long time to produce gas. In this study, the fermentation time was only 3 days, the possibility of the gas composition is the most carbon dioxide and methane to hydrogen low due to the incubation time is just 3 days so that the carbon dioxide in the product is dominated by CO_2 . While low hydrogen and methane. It can be concluded that in order to produce gas in large quantities, it can take a long fermentation and is expected to contain a lot of hydrogen gas composition.

There are many strains of Bacillus circullans that have been found by previous researchers. Bacillus circullans DZ 100 found by (Benkiar et al, 2013) [7] as a producer of protease enzyme. Bacillus circullans also found as a producer of biosurfactant [8][9]. Other strain can produce xylanase enzyme [10], lipase enzyme [11], polyhydrxy alcanoat (PHA) [12] PHB [13] and others. But it has not been found circullans bacillus that produces hydrogen. It was concluded that Bacillus circullans found from this research is a new strain of Bacillus circullans.

4. Conclusion.

From the description above it can be concluded that:

1. Bacillus circullans can produce hydrogen and acetic acid.
2. The best results for the hydrogen is mixed culture of BC (I + II), while for acetic acid is mixed culture of BC (I + II + III).
3. Bacillus circullans produce low hydrogen content due to the fermentation time just 72 hours. Therefore, this research needs to continue to examine the best fermentation time.

4. There has never been found (Bacillus circullans) that produce hydrogen. Some strains of Bacillus ciecullans produce cellulase enzymes, xylanase, poly hydroxy alkanoic (PHA), poly hydroxy butanoic (PHB) etc. So it can conclude that Bacillus were found from this study is a new strain of Bacillus circullans.

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