B. 28

ISBN 978 - 979 - 18768 - 5 - 8

Proceedings

The Second
International Conference on Green Agro-Industry
Resource Management for Sustainable Future
August 4-6, 2015

Published by:
FACULTY OF AGRICULTURE
UNIVERSITAS PEMBANGUNAN NASIONAL "VETERAN"
YOGYAKARTA

Supported by :

Proceedings

The Second

International Conference on Green Agro-Industry (ICGAI)

"Resource Management for Sustainable Future"

Conference is held on 4 – 6 August 2015
hosted by Faculty of Agriculture, UPN "Veteran"
Yogyakarta, Indonesia

Proceedings

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Preface

Bismilahirrahmanirrahim, Assalamu'alaikum wa rahmatulahi wa barokatuh.

Praise be to Allah who has bestowed His grace, so that the event can take place smoothly.

The Honourable Rector UPN "Veteran" Yogyakarta, The Honourable Head of Agriculture Office of Yogyakarta province, the Honourable invited speakers, Distinguished Guests, Distinguished Participants, Ladies and Gentlemen,

On behalf of The International Conference on Green Agro-Industry Organizing Committees, I am pleased and honoured to welcome all of the participants to the Second International Conference on Green Agro-Industry at Mustika Sheraton Hotel, Yogyakarta, Indonesia from 4-6 August 2015. This conference is hosted by the Faculty of Agriculture Universitas Pembangunan Nasional "Veteran", Yogyakarta, Indonesia and this event would not have been possible without the support of its global partners: Tokyo University of Agriculture and Technology, Japan, Murray State University, USA, Universiti Malaysia Sarawak, Malaysia, University of Colombo, Sri Lanka, University of Western Sydney, Australia, Royal Melbourne Institute of Technology, Australia, Tongji University, China, and Gadjah Mada University, Yogyakarta, Indonesia.

Ladies and gentlemen,

The theme of the Second International Conference on Green Agro-Industry is "Green Agro-Industry: Resource Management for Sustainable Future". Agro-industry is important not only because it can transform raw agricultural materials into value added products while generating income and employment, but it is important in the bigger picture because it contributes to the overall economic development in both developed and developing countries. In the context of trade, agro-industry provides significant impact to Indonesia's export. The government is targeting exports of the agro industry to grow up to 29% amounting to USD 40 billion this year, from USD 31 billion in 2014.

As we are all well aware, the resources available to support the development of agro-industry is not unlimited, therefore, it is crucial for us to manage the resources that we have carefully. Recently, there has been an increased pressure on agro-industries to shift to more resource-efficient and low-carbon production processes as part of the global efforts to sustain growth, conserve resources and slow down the pace of climate change. To provide a sustainable future, the development of agro-industry should not merely aim for high profit, but it should also be environmentally friendly and socially sustainable.

In furtherance of this ideal, this conference is organized with the hopes of achieving three things. First, it is held to foster and support the development of highly productive methods and technologies for the various segments of the agro-industries. Second, it is designed to provide a forum for the presentation, discussion and debate of state-of-the-art and emerging technologies in the field of agro-based industry and any issues related

to sustainable agro-industry. Third it aims to promote interaction and communication among researchers, observers and practitioners to discuss and discover solutions to the problems related to the development of the agro-industry and how it can further improve welfare.

Topics of interest for the conference are divided into four major categories, namely: **Economics, Social and Business; Agronomy; Soil and Land Management; Agricultural engineering.** Our keynote speaker Prof. Lilik Soetiarso from Universitas Gadjah Mada, Yogyakarta, Indonesia will present a keynote speech entitled "*The Role of Bio-system Engineering in Green Agro-Industry*". Other invited speakers from a broad range of backgrounds including leading industry and academic experts will provide insights into sustainable agro-industry from various perspectives. In addition, the supporting papers from the participants will also enrich and liven the discussions related to the development of sustainable agro-industry.

On behalf of ICGAI Committee I would like to apologize that due to unforeseen circumstances three of our invited speakers: Assoc. Prof. Shiva Muthaly (RMIT University, Australia); Prof. (Rev). Wimalaratana (University of Colombo, Sri Lanka); and Assoc. Prof. Ping Fang (Tongji University, China) were unable to attend this conference. I am sorry for this inconvenience.

Finally, we would like to express our gratitude to the Rector UPN "Veteran", Yogyakarta for the financial support, the Dean of the Faculty of Agriculture for hosting this event, and the Scientific and Steering Committee. We would also like to convey our utmost gratitude to the keynote speaker Prof Lilik Soetiarso (Universitas Gadjah Mada, Yogyakarta), the invited speakers Prof. Sakae Shibusawa (Tokyo University of Agriculture and Technology, Japan, Mr. Marc Vanacht, MBA/ML (President, AG Business Consultants, St Louis, USA);, Mr. Jeewan Jyoti Bhagat (Managing Director-STM Projects Ltd, India); Dr. R.P. Singh (Associate Agronomist and Sugarcane Advisor for STM Projects Limited, Prof. Iin Handayani (Murray State University, USA); Dr. Partoyo (UPN "Veteran" Yogyakarta, Indonesia) as well as all the participants for their contribution in making this conference a success. We wish to also thank the sponsors of this event: PT. Bank BNI, Bank BPD, Bank BRI and Bupati Kabupaten Wonosobo, for their contribution in making this conference possible. Finally, as the Chairperson, I would like to convey my highest appreciation to the members of the organizing committee whose relentless hard work and dedication made this conference a great success.

Thank you and I wish everyone a fruitful and pleasant day ahead.

Wassalamu'alaikum wa rahmatulahi wa barokatuh

Yogyakarta, August 4, 2015

Dr. R.R. Rukmowati Brotodjojo

ICGAI Chairperson

Agriculture Engineering

Any Other Topics related to Agro-Industry

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.

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, Enterobakter 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 begun 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; Oztekin 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	$NaHClO_3$ (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), Riboplavin (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

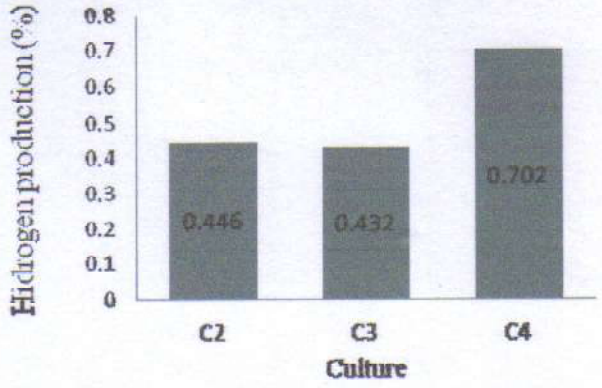

Figure 1. Hydrogen production from enrichment cultures

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, galactoses, 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)

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

2). Pyruvate: ferredoxin oxido reductase (PFOR)

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