STANDARISASI LUARAN HASIL PENELITIAN

PENELITIAN STRATEGIS NASIONAL

Produksi Ethanol Berbahan Dasar Hasil Dekomposisi Biomasa Tandan Kosong Kelapa Sawit oleh Mikroba Cellulolytic dan Lignocellulolytic

Tahun ke 2 dari rencana 3 tahun

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Hydrolysation of Palm Oil Empty Fruit Bunch by Using Crude Extract Enzymes of *Aspergillus niger* and Analysis of Its Hydrolizates for Bioethanol Production

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ABSTRACT

A huge amount of lignocellulosic-rich palm oil empty fruit bunch (POEFB) biomass was released during palm oil harvested. Observation showed that *Aspergillus niger* could grow well in POEFB without any nutrion added. And after 5 days cultivated at 30^{o}C the extracellular enzyme of *A. niger* was extracted using 1% NaCl. Of about 500 ml crude enzyme was concentrated by ammonium sulphate precipitation at 70% saturation. The precipitates was then dissolved to 50 ml and dialysed against 20mM acetate buffer at pH 5 to remove remaining ammonium sulphate. Four hundred milligrams of powdered POEFB was suspended in 20 ml dialysed crude enzyme for 36 hours hydrolysation at 37^{o}C and 100 rpm shaken. The hydrolyzates was then fermented using *Saccharomycess cerrevisae* at 30^{o}C for 24 hours. Colorimetric analysis at OD 580nm using QuantiChrom Kit DIET-500 showed that fermentation produced alcohol 0.88%, respectively. Suggested POEFB is an alternative biomass potential than can be used for bioethanol production efficiently.

Keywords: hydrolysation, POEFB, fermentation, bioethanol

INTRODUCTION

Indonesia as agriculture countries is well known for its potential in agriculture biomass wastes such as palm waste, sugarcane bagasse and paddy rice. At present and from forcasted production of palm oil for the year 2000–2020 [26], Indonesia is the largest producer and exporter of palm oil in the international market. In the process production of palm oil from the fruit, a huge mount of lignocellulosic material oil palm empty fruit bunch (POEFB) is generated as a waste product [6, 33], accumulated in the field, and considered to be difficult materials to digest [20] so that the utilisation of this biomass waste product is still less attention. In practice this biomass is burned in incinerators [5]. It was reported that 14.5 million tons of POEFB biomass waste is generated annually in the world and half of POEFB was produced in Indonesia [10, 30].

A bioconversion of biomass including organic waste materials to fuel is receiving interest as they are low cost, renewable and widespread in nature [28]. Researchs related to POEFB utilisation has been demonstrated for biohydrogen [2, 9], activated carbon [3], biochar [12], xylooligosaccharides [13], sugars [21], latic acid [23], biogas [29], cellulase [31], biodiesel [32] and bioethanol production [14, 19, 27]. However to convert ethanol from POEFB still limited gave low efficiency so that some researchs effort to meet POEFB conversion with high yield be done. In this paper will be explained POEFB saccharification to produce sugars by using exctracellular enzymes from *A. niger*, followed by fermentation to produce ethanol.

2 MATERIALS AND METHODS

2.1 Extracellular Enzyme Production of *A. niger* for POEFB Hydrolysation

Source extracellular enzyme was produced from one kilogram of sterilized POEFB in a 5 liter flask which inoculated with *A. niger* and incubated at 30°C for 5 days. To harvest the enzyme was done by adding 500 ml water containing 1% NaCl, shaken at room temperature for 9 hours and filtered using paper filter on funnel Buchner. Remaining debris from the filtrate was removed by centrifugation at 12000 rpm for 20 minutes. The supernatant as crude enzyme was then concentrated by ammonium sulfate at 70% saturation, dissolved and dialyzed against water for 3 days to remove the remaining ammonium sulphate, bring to 50 ml total volume with the same buffer and stored at 4°C until needed for POEFB hydrolysis.

2.2 POEFB Enzymatic Hydrolysis

One percent of powdered POEFB substrate (400 milligrams/20ml) was suspended into concentrated crude enzyme, incubated at 37°C, shaken 100 rpm, 36 hours for hydrolysis. The resulting supernatant as hydrolysate was harvested from the remaining POEFB substrate by centrifugation at 4000 rpm for 10 minutes. Hydrolysate was stored at 4°C for next sugars component analysis. This procedure was done in double.

2.3 Analysis of Sugar Component of POEFB

The reducing sugar of hydrolysate was quantified using Somogy [24] and Nelson [16] methods. For total sugars of POEFB was estimated colorimetric using phenol-sulfuric method [11]. Further analysis for sugar composition of hydrolysate was done by using Thermo Scientific Trace 1310 Gas Chromatograph (GC) using TG-225MS 15m x 0.25mm x 0.25µm column. One milliliter hydrloisate was prepared as alditol acetates [5, 25] and 0.5µl injected to GC for analysis. During analysis, the GC was setup at 190°C (5 minute hold) to 250°C at 8°C/min (5 minute hold) and carrier gas hydrogen ajusted at flow rate 45cm/sec. The hydrolysate was also analyzed by using TLC in silica gel plate (Merck, silica gel 60 F254). A Mixture (v/v) of butanol : ethanol : chloroform : amonia (4 : 7.5 : 4 : 8) was used as solvent system and 0.1% Sulfuric acid containing o.1% vanilin was employed for detection.

2.4 Anaerobic Fermentation

The yeast cells *S. cerreviseae* for anaerobic fermentation was preculture aerobically in 100 ml medium pH 6 in 500 ml shaker flasks, containing yeast-extract (0.3%), malt extract (0.3%), pepton (0.5%) and glucose (1%). The culture was incubated at 25°C under shaker set at 120 rpm for 24 hours, and active yeast cells were harvested by centrifugation at 4000 rpm for 5 minutes. The pellet (≈20 mg) was suspended in 1 ml sterilized water and used for source inoculum in anaerobic fermentation POEFB hydrolysate. Fermentation was done in 20 ml medium using mini fermenter at 30°C. The medium was inoculated with 2 mg cells (≈0.1ml) of source inoculum prepared above.

2.5 Analysis of Ethanol Concentration

The ethanol concentration was analysis using QuantiChrom Kit DIET-500 colorimetric method at OD 580nm. For data comparison, analysis was also analysed using GC above equipped with Trace GOLD TG-1301MS GC column. The machine was setup at 250°C with carrier gas helium at flow rate 35cm/sec.

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3 RESULTS AND DISCUSSION

3.1 Enzyme Production of *A. niger* on POEFB

Production of exctracellular enzyme of *A. niger* in POEFB medium without any nutrient added was successfully done. Evidence showed that after 3 days cultivation, *A. niger* grow well in POEFB. Much mycelium with their black spores covered whole of medium. Mean, *A. niger* certainly utilized carbon and nitrogen from POEFB for the growth. Further, we observed that after 5 days cultivation, small amount liquid was produced indicated that POEFB hydrolysed by extracellular enzyme which secreted by *A. niger* during cultivation. The exctracellular enzyme was then harvesting by 1% NaCl extraction.

3.2 Enzymatic Hydrolysis of POEFB

Hydrolysis was done without any adjusting of buffer usage as well as optimum pH and temperature. We found that this enzyme readily hydrolysed alkali extract of POEFB and maximum degree of hydrolysis was 62% after 36 hours incubation at 37°C. The longer incubation time, the more higher the degree of hydrolysis. However, thereafter 72 hours the hydrolysis is still in progress and could presumably attain the same hydrolysis at very much later time.

3.3 Determination of Sugars Component in Hydrolysate

POEFB hydrolysate was rich in monosaccharide but poor in oligosaccharides. The oligosaccharide concentration constituted less than 1% of the total sugar content. By the TLC analysis, it was clear that the hydrolysis product was predominantly monosaccharide glucose respectively as shown in the Figure 1 as follow.

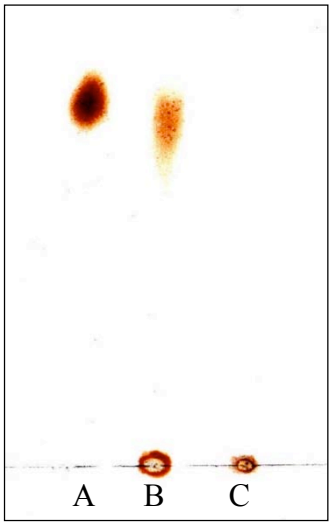

Figure 1: TLC analysis of hydrolysate ; standard glucose (A), POEFB hydrolysate (B) and POEFB before hydrolysis (C)

GC analysis revealed that POEFB-hydrolysate consisted of glucose (86.1%) while the minor are xylose (4.1%), and other monosaccharides less than 1%. GC analysis of total sugars as monosaccharides in hydrolysate 12.4 mg/ml. And GC calculation of glucose and xylose are 10.7 mg/ml and 0.51 mg/ml respectively. The capability of crude enzyme to hydrolyze POEFB and produce glucose and xylose as monosaccharide could be due to the fact that some genus *Aspergillus* secrets of various cellusases [4, 15], glucoamylase [1] and xylanase [7]. POEFB-hydrolyzate was also contain crude protein nearly 0.3% on dry-basis which was needed as a nitrogen source for fermentation.

3.4 Ethanol Production Analysis

Anaerobic fermentation to produce ethanol using POEFB hydrolysate was done directly by innoculating cells yeast of *S. cerrevisae* and without any adjusting of both pH or sugar concentration of hydrolysate at 30^{o}C for 24 hours. The maximum concentration of ethanol produced was 0.88% when analysed using colorimetric analysis. This concentration precentage also similar result when GC analysis used, that was 0.81%. Calculation ethanol production efficiency 72%.

3.5 Ethanol Production Analysis

After fermentation, the remaining sugar in hydrolysate also analysed by GC and we found the glucose concentration was very low with value 0.2 mg/ml. However, the xylose almost nearly same comparing with initial concetration before fermentation, that was 0.49 mg/ml respectively. This evidence proved that *S. cerrevisae* could not ferments xylose from POEFB hydrolysate, but in contrast *S. cerrevisae* definitely utilized and converted glucose to ethanol as well as for their cells growth. By these results, improvement to increase the yield of is needed. Optimizing condition both enzymatic hydrolysis [22, 30, 17] and environtment factors as well as parameters in fermentation such as feed sugar concentration [8, 18], pH of medium and temperature [8].

4 CONCLUSION

Bionconversion of POEFB biomass to bioethanol in laboratory scale was successfully done and resulting in production efficiency was 72%, respectively. Suggested POEFB is an alternative biomass potential than can be used for bioethanol production efficiently so that improvement in industrial scale must be planed. However, effort in optimizing of the process as well as progressively research incuding economically calculation must be highly considerated.

ACKNOWLEDGEMENT

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No : 06/Pan-ICBB/08/2014
Attachement :
Subject : Invitation as Speaker of the ICBB 2014

To
Dr. Kahar Muzakar, M.Sc.
Universitas Jember
Jember - Indonesia

Dear Sir,

The committee of ICBB 2014 in collaboration with the Faculty of Agriculture, University of Udayana is going to hold **the 5th International Conference on Bioscience and Biotechnology**. This year conference will also to celebrate the 47th Anniversary of the Faculty of Agriculture. Therefore it would be an honor if you are willing to share your vision or projects experiences as one of our speakers in the event. The theme for this year conference is **International Research Collaboration on Bioscience and Biotechnology for Better Achievement and Sustainability**.

The International Conference will be held on Saturday, September 20, 2014. The venue will be the Main Auditorium Hall of Post Graduate Building of Udayana University, Jl. PB Sudirman, Denpasar - Bali.

Once again, we kindly ask you to participate in the 5th International Conference on Bioscience and Biotechnology.

We thank you for your attention.

Denpasar, August 8, 2014

Sincerely yours,

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Dr. Ir. I Wayan Nuarsa, M.Sc.

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Naniek Kohdrata, SP., MLA.

Dean of Faculty of Agriculture,

Prof. Dr. Ir. I Nyoman Rai, M.Sc.

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SUGAR PRODUCTION BY DIGESTING OF OIL PALM EMPTY FRUIT BUNCH USING EXTRACELLULAR ENZYMES FROM *Aspergillus niger* AND *Trichoderma reesei* FOR ETHANOL PRODUCTION

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ABSTRACT

Extracellular enzymes which obtained from 4 days cultivation *Aspergillus niger* and *Trichoderma reesei* on solid state fermentation of oil palm empty fruit bunch (OPEFB) were used for lignocellulosic-rich OPEFB digestion. The enzymes were concentrated using 70% saturated ammonium sulphate, dialysed against 20mM acetate buffer at pH 5 and adjusted one tenth (v/v) from the initial volume with the same buffer. The concentrated enzymes were then used in hydrolysation of powdered OPEFB. Amount of 10.65 mg/ ml and 11.47 mg/ml sugars were produced when each concetrated enzyme *A. niger* and *T. reesei* mixed with2%OPEFB. These hydrolysation were done on 100 ml total volume, incubated at 37°C with 100 rpm shaken for 36 hours. Further, both hydrolyzates results were sterilised and fermented anaerobically using *Saccharomycess cerevisiae* at concentration 0.5mg/ml cells and incubated in 30°C for 24 hours. Colorimetric analysis using QuantiChrom Kit DIET-500 at OD 580nm gave results the alcohol production were 0.86% and 0.92% which were similar with Gas Chromatograph analysis that of 0.83% and 0.93%, respectively.

Keywords: extracellular enzymes, hydrolysation, fermentation

INTRODUCTION

It is forecasted, in year 2000–2020 Indonesia is the largest producer and exporter of oil palm in the international market (Sumathi *et al.,* 2008). A huge amount of lignocellulosic material oil palm empty fruit bunch (OPEFB) is generated during production of oil palm from the fruit and remained as agriculture waste product (Baharuddin *et al.,* 2013; Huzairi *et al.,* 2013). Abundant of OPEFB is accumulated in the field, and considered to be difficult materials to digest (Purwandari *et al.,* 2013) which consists of three main polymeric components, i.e., cellulose (46.7%), hemicellulose (17.9%),and lignin (4.2%) (Quintero *et al.,* 2011). Thus, OPEFB is a common waste problem in oil palm plantations. Unadventurously, they are burned resulting in air pollution and massive residues on landfills. To solve the disadvantages, several of processing methods i.e., biologically (Huzairi *et al.,* 2013; Zhang *et al.,* 2013) mechanical (Baharuddin *et al.,* 2013; Shamsudin *et al.,* 2011), chemical (Kim and Ho 2013; Rahman *et al.,* 2007) have been investigated which shift the structural and chemical compositions of OPEFB lignocellulose to sugars (Ye *et al.,* 2014) and was converted to renewable energy bioethanol (Cui *et al.,* 2014; Hoon *et al.,* 2011). Further, researchs related to OPEFB utilization, have also been developed to produce biogas (Mohamed *et al.,* 2013; O-thong *et al.,* 2013; She *et al.,* 2013), enzymes (Ariffin *et al.,* 2008; Ottenheim *et al.,* 2014), sugar and oligosaccharides derivates (Ling A *et al.,* 2014; Ottenheim *et al.,* 2014; Rahman *et al.,* 2006) and other products. However, in fact, little attention is put forward on OPEFB utilisation in terms of economic point of view. In this paper, microbial utilization of OPEFB to produce sugar using crude extract from *A.niger* and *T. reesei,* and conversion of its hydrolysates to ethanol will be reported.

MATERIALS AND METHODS

Extracellular Enzymes Production

Source extracellular enzymes were obtained from solid culture of one kilogram sterilized OPEFB in a ten liter flask which each culture was inoculated with *A. niger* and *T. reesei*. The enzymes were harvested after optimum incubation at 30°C for 5 days. Enzymes extraction

was done by adding 1000 ml water containing 1% NaCl, shaken at room temperature for 9 hours and filtered using paper filter on funnel Buchner. To remove remaining cells from the filtrate, centrifugation at 4000 rpm for 20 minutes was performed. The supernatant which containing enzymes were then concentrated by ammonium sulfate at 70% saturation, dissolved on 30 ml acetate buffer pH5. The remaining ammonium sulphate was removed through dialysis on mPES MicroKros Filter Modules C02-E010-05-S 10 KDa against the same buffer for 24 hours, and then enzyme solution brought to one tenth (v/v) from the initial volume using buffer above. The two concentrated enzymes were stored at 4ºC until needed for OPEFB hydrolysis.

Enzymatic Hydrolysis OPEFB

OPEFB hydrolysis was done by using concentrated extracellular enzymes containing 2% powdered OPEFB. Two grams of powdered OPEFB was suspended into 100 ml concentrated crude enzyme, incubated at 37ºC, shaken 100 rpm, 36 hours. Enzyme reaction was stopped by boiling at 100ºC for 10 minutes. The resulting supernatant as hydrolysate was recovered and separated from remaining OPEFB substrate by filtration on paper filter and then centrifuged at 4000 rpm for 10 minutes. Hydrolysate was stored at 4ºC for next sugar analysis. This experiment was carried out twice.

Sugar Analysis of OPEFB Hydrolysate

Total sugar of OPEFB hydrolysate was estimated by phenol-sulfuric colorimetric method (Dubois, *et al.,* 1956). The reducing sugar of hydrolysate was also measured using Somogy (Somogyi *et al.,* 1926) and Nelson (Nelson *et al.,* 1944) methods. Sugar components of hydrolysate were analysed by Gas Chromatograph (GC) Thermo Scientific Trace 1310 equipped withTG-225MS 15m x 0.25mm x 0.25µm column. GC was setup at 190°C (5 minute hold) to 250°C at 8°C/ min (5 minute hold) and carrier gas hydrogen adjusted at flow rate 45cm/sec. One milliliter OPEFB hydrolysate sample was transmethylated as alditol acetate (Arai and Murao 1978; Spiro *et al.,* 1972). An amount 0.5µl of sample was injected to GC for analysis. The hydrolysate was also analyzed by using TLC in silica gel plate (Merck, 60 F254). A mixture (v/v) of butanol : ethanol : chloroform : amonia (4 : 7.5 : 4 : 8) was used as solvent system and 0.1% sulfuric acid containing 0.1% vanilin was employed for detection.

Anaerobic Fermentation and Analysis of Ethanol Concentration

In anaerobic fermentation to produce ethanol, yeast cells *S. cerevisiae* was used. The yeast was pre-cultured aerobically in 100 ml medium pH 6 in 1 liter shaker flasks, containing yeast-extract (0.3%), malt extract (0.3%), pepton (0.5%) and glucose (1%). The culture was incubated at 25°C under shaker set at 120 rpm for 24 hours. Active yeast cells were harvested by centrifugation at 4000 rpm for 5 minutes. The pellet was suspended into sterilized water with the concentration of cells 25 mg cells/ ml and further used for source inoculum in anaerobic fermentation of OPEFB hydrolysate. Fermentation was done in 50 ml medium using mini fermenter at 30ºC, inoculated with 25 mg cells (≈1ml) of source yeast prepared above. The ethanol concentration was analysed using QuantiChrom Kit DIET-500 colorimetric method at OD 580nm. Ethanol concentration was also analysed using GC above equipped with Trace GOLD TG-1301MS GC column with sample volume 0.5µl. The GC machine was setup at 250°C with carrier gas helium at flow rate 35cm/sec. The remaining sugar component after fermentantion was analysed with the same method as described earlier at Sugar Analysis of OPEFB Hydrolysate.

RESULTS AND DISCUSSION

Optimization and Production of Extracellular Enzymes

Extracellular enzyme produced by *A. niger* and *T. reesei* in OPEFB medium were done without any nutrient added that has been confirmed. For

preliminary step, the optimization of enzyme extracellularproductionfromthetwospecieswere done in small scale using 10 gr OPEFB medium. Evidence revealed that in 3 days cultivation, *A. niger* and *T. reesei* grow well in OPEFB medium. Much mycelium with their black spores covered whole of OPEFB medium. Additionally, OPEFB was also contain crude protein nearly 0.3% on dry-basis which was needed as a nitrogen source for fermentation. Mean, *A. niger* and *T. reesei* certainly utilized carbon and nitrogen from OPEFB for their growth. Further, we observed that in 5 days cultivation, small amount liquid phase was produced, indicating hydrolysation process happen. Accordingly, some extracellular enzymes were released by both *A. niger* either *T. reesei* during solid state fermentation. The extracellular enzyme was then harvested by 1% NaCl extraction. The enzyme activity against 1% OPEFB alkali extract substrate was measured daily based on reducing sugar produced. Thus, incubation days of cultivation to produce extracellular enzyme released optimally can be represented as maximum of enzyme activity to produce reducing sugar against OPEFB alkali extract substrate. The activity levels of extracellular enzymes were, therefore, determined for incubation periods of 1 to 7 days as shown at Figure 1.

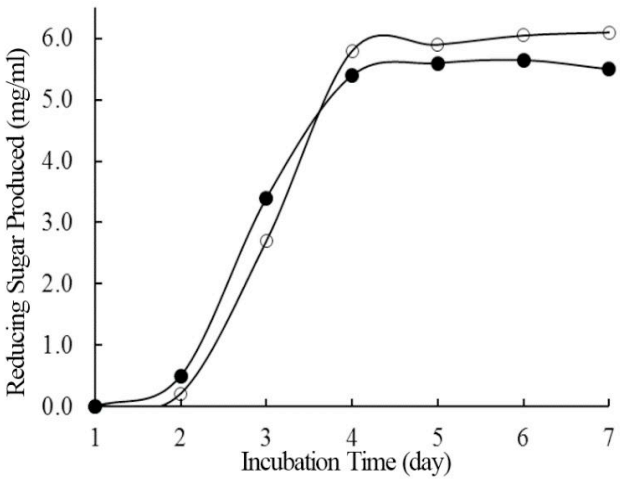

Fig 1. Optimizing of extracellular enzymes production of *A. niger* (֍) and *T. reesei* (⌘) on OPEFB.

As stated in Figure 1, extracellular enzymes of *A. niger* and *T. reesei* has highest activity to hydrolysed OPEFB alkali extract substrate when incubation time was done in 4 days. Both isolates produced reducing sugars 5.4 mg/ml and 5.8 mg/ml, respectively. Based on this result, the enzyme production was scaled up in 1 kg of OPEFB medium. The enzymes activity were also examined and gave similar results compare with previous small scale enzymes production, where the enzymes hydrolysed OPEFB alkali extract and produced 5.1 mg/ml and 5.3 mg/ml reducing sugars. It was confirmed already that genus *Aspergillus* (Alam *et al.,* 2011; Ottenheim *et al.,* 2014) and *Trichoderma* (Shafawati *et al.,* 2013; Wang Z *et al.,* 2014) release some extracellular enzymes which decomposed cellulose, hemicellulose, and lignocellulose of OPEFB.

OPEFB Hydrolysis and Sugar Analysis

Hydrolysis was carried out in 100 ml concentrated enzymes containing 2% of powdered OPEFB and incubated at 37^{o}C with 100 rpm shaken for 36 hours. The hydrolysis process by the two enzymes were measured every 4 hours. They produced optimum of reducing sugar production in between 32 and 36 hours incubation. The longer incubation time, the more higher the degree of hydrolysis. And thereafter 40 hours the hydrolysis is still in progress but not significant increasing in reducing sugar production and could presumably attain similar degree of hydrolysis at very much later time. Analysis revealed that enzymes from *A. niger* and *T. reesei* released 10.65 mg/ml and 11.47 mg/ ml reducing sugars after 36 hours hydrolysis. In addition, OPEFB consist of 66.5% total sugar (or 13.3 mg/ml when the suspension contain 2% OPEFB)measuredbyphenol-sulfuriccolorimetric method (20)so that it can be concluded that concentrated enzyme from *A. niger* and *T. reesei* digested OPEFB reach 81.7% and 89.2% degree of hydrolysis, respectively. To improve the degree of hydrolysis, optimization of hydrolysation must be done, e.g., pH, temperature and period of incubation time. In another words to make

the process more effective, suitable pretreatment and enzymatic reaction parameters need to be optimized (Hassan *et al.,* 2013).

As shown at Table 1, phenol sulfuric acid analysis showed that the total sugar of OPEFB hydrolysates were 10.86 mg/ml and 11.87 mg/ ml respectively when hydrolysis of OPEFB were done by concentrated enzymes from *A. niger* and *T. reesei*. However, GC analysis revealed that OPEFB hydrolysates contain much sugar as monosaccharides, that of10.65 mg/ml and 11.47 mg/ml. Means, OPEFB which hydrolysed by both concentrated enzymes from *A.niger* and *T. reesei* produced sugar rich hydrolysates as monosaccharides but poor in oligosaccharides. The monosaccharides concentration in hydrolysates were 98.1% and 96.6%. TLC analysis displayed that the hydrolysis product was primarily monosaccharide glucose spot respectively (figure not shown). The ability of both extracellular enzymes from *A. niger* and *T. reesei* to release monosaccharides from complex polysaccharides due to that species could produce broad spectrum of extracellular enzymes. The capability of crude enzyme to hydrolyze OPEFB and produce glucose and xylose as monosaccharide could be due to the fact that some genus *Aspergillus* and *Trichoderma* produce of various enzymes such as cellusases, glucoamylase and xylanase (Chen *et al.,* 2014; Chandra *et al.,* 2009; Krijgsheld *et al.,* 2013; Perrone *et al.,* 2007).

Analysis of Anaerobic Fermentation

Fermentation to produce ethanol was done using OPEFB hydrolysate as medium without any adjusting of either pH or sugar concentration. Further, both hydrolyzates results were sterilised and fermented anaerobically using *S. cerevisiae* at concentration 0.5mg/ml cells and incubated in 30ºC for 24 hours. Colorimetric analysis using QuantiChrom Kit DIET-500 at OD 580nm gave results the alcohol production were 0.86% and 0.92% which were similar with Gas Chromatograph analysis that of 0.83% and 0.93%. The remaining sugar in hydrolysate after fermentation as monosaccharides was also analysed by GC. After 24 hours fermentation, glucose concentration were very low with concentration value 0.12 mg/ml and 0.18 mg/ml. But, the xylose almost nearly same comparing with initial concentration, that of 0.53 mg/ml and 0.71 mg/ml, correspondingly. This evidence demonstrated that *S. cerevisiae* difficult ferments monosaccharide xylose from OPEFB hydrolysate, similar result as reported by Sudiyani *et. al*. 2013. But in contrast *S. cerevisiae* definitely utilized and converted glucose to ethanol. By these results, improvement hydrolysis OPEFB must be done which expected to increase the yield of sugar as monosachharides in OPEFB. Adjusting and optimizing environment factors in hydrolysis and as well as parameters in fermentation such as sugar concentration, pH of medium and temperature are also needed (Ferreira *et al.,* 2009; Shill *et al.,* 2012; Viell *et al.,* 2013).

Tabel 1. Sugar Analysis of OPEFB Hydrolysates

Hydrolysate analysis	Sugar concentration (mg/ml) in OPEFB hydrolysis using concentrated enzyme *A. niger* (A) and *T. reesei* (B)
	A
Gas Chromatograph Analysis (Monosaccharides component as alditol acetates)	
- Glucose	8.81
- Xylose	1.11
- Others	0.73
Total monosaccharides	**10.65**
Phenol Sulfuric Acid Analysis	
Total Sugar	**10.86**

CONCLUSION

In this study, bioconversion of potential cheap material of OPEFB to ethanol was successfully done. And it is necessary to develop more efficient method with the target to improve the ethanol yield as well as for profitability reason process. Enzymatic hydrolysis and fermentation process must be evaluated in order obtain high ethanol yield. Xylose as pentose sugar has not been consumed by *S. cereviseae*. Therefore, assessment of other pentose-consuming microorganisms with the aim to completely ferment the sugars released in OPEFB hydrolysates must be investigated.

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Phosphate Solubilizing Bacteria Adaptive to Vinasse

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Abstract. Microorganisms identified as phosphate solubilizing bacteria (PSB) adaptive to vinasse were successfully screened from sugarcane soil from an agriculatural estate in Jatiroto. By conducting a screening on Pikovskaya's agar medium (PAM), we found that five different isolates were detected as PSB (pvk-5a, pvk-5b, pvk-6b, pvk-7a, and pvk-8a). Of the five isolates only three could be grown and were found to be adaptive to vinasse based medium without any nutrients added (pvk-5a, pvk-5b and pvk-7a). The three isolates were characterized as coccus and Gram negative with no endospores detected. We suggest that these three isolates can be used as biofertilizer agent to support organic farming.

Keywords: *bacillus; biofertilizer; Gram negative; PSB; vinasse.*

1 Introduction

Soil microorganisms known as phosphate solubilizing bacteria (PSB) are found in nature within the plant rhizosphere area. This type of microorganism has the ability to solubilize phosphate sediment [1]. In field application, PSB are often used as biofertilizer agent to increase the efficiency of phosphate fertilizers [2-4]. The availability of PSB in soil may elevate the phosphate uptake by the plant and improve the yield of the crop [5]. As for cultivation, PSB can be grown on medium containing $Ca_3(PO_4)_2$, $FePO_4$, $AlPO_4$, rock phosphate and any other inorganic phosphate mineral [6]. However, to produce and grow large quantities of PSB as biofertilizer using this medium, the potential high cost due to the material used in the production process of PSB must be considered. To overcome this problem, material substitution which may reduce the production cost of PSB must be investigated. Gómez and Rodríguez found in a previous study that a huge amount of residual organic-rich vinasse was released during ethanol production from molasses [7]. From one liter of ethanol produced, around 13-30 l of vinasse was released as waste. Also, Jiang *et al.* has reported that direct application of vinasse to agricultural land can increase the productivity of plants [3]. Based on the economic potential of vinasse corroborated by these studies, we decided to further investigate vinasse as a PSB production medium. Addition of PSB to vinasse was considered to be a logical approach in this research to extend the quality of vinasse as organic

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fertilizer. Therefore we focused this study on screening PSB microorganisms that are adaptive to vinasse. Furthermore, this research was expected to justify that, in turn, the vinasse can be utilized as a cheap raw material for PSB production.

2 Material and Method

Soil samples as PSB source were randomly collected in 5 locations within 7.2 h from sugarcane soil from an agricultural estate in Jatiroto, East Java, which was treated with vinasse as fertilizer. All sampling was done in duplicate. Twenty five grams of soil sample at every location was directly suspended in 225 ml of sterilized water containing 0.85% NaCl and stored at 4°C as PSB screening source.

The suspended samples were then diluted from 10-1 to 10-8 [8], plated to Pikovskaya's agar medium (PAM) and incubated at 30°C for isolation and the screening process. The presence of PSB in the PAM medium is indicated by a clear zone around colonies, grown after 48 hours of incubation. To obtain a single species, inoculation was repeated by picking up one loop of a single colony, which was directly plated in the same medium. The single colony of PSB isolates was stored at 4°C for further analysis.

The index activity of all PSB isolates was assayed using the same medium, grown and observed after incubation for 72 h at 30°C. The index activity of the PSB was then calculated by comparing the ratio between clear-zone and colony diameter [9]. All isolates of high index activity were selected for further analysis.

The selected PSB isolates were also characterized and evaluated based on colony forming units on the PAM. Macroscopical observation involved colony shape, surface, elevation, edge, concentric and radial line, and growth of colonies. Microscopical observation was based on Gram staining and endospore detection [10].

Selection of PSB adaptive to vinasse was carried out by culturing of all potent isolates on diluted vinasse medium without any nutrients added. Distillated water was used for dilution. Dilution factors (v/v = vinasse/water) used were 1:2 and 1:3). All cultures were observed and the total colony number was counted, expressed as colony form units (CFU). As control, Pikovskaya's liquid medium (PLM) was used. The selection was completed in 72 hours, after which the total colony was periodically monitored (every 4 hours).

3 Results and Discussion

Nine species of bacteria were isolated from sugarcane soil from an agricultural estate in Jatiroto. Among them only five isolates were identified as PSB (pvk-5a, pvk-5b, pvk-6b, pvk-7a and pvk-8a) as shown at Figure 1 below.

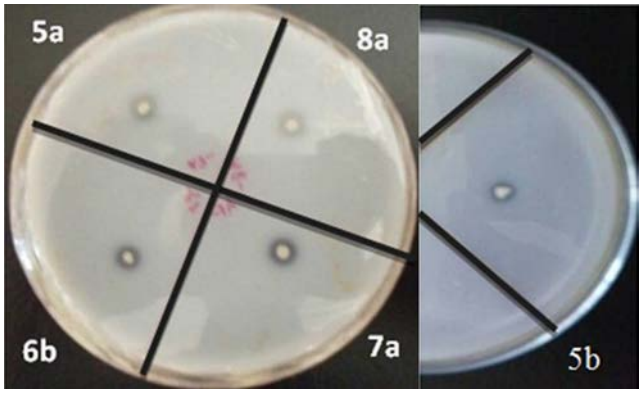

Figure 1 Five different isolates (pvk-5a, pvk-5b, pvk-6b, pvk-7a and pvk-8a) that produced a clear zone around the CFU in PAM after 72 hours incubation at 30°C were detected as PSB.

The presence of a clear zone around the colony indicated that five isolates secreted an extracellular enzyme hydrolyzed organic acid and released Ca^{++} from $Ca_3(PO_4)_2$ through which their hydroxyl and carboxyl groups chelate the cations bound to phosphate, thereby converting it into soluble form. In addition, H_2PO_4 is released to the clear-zone area from other insoluble organic or inorganic phosphate substances. The organic acid released involves citric acid, glutamic, succinate, lactate, oxalate, glyco-oxalate, malate, phumarate, tartarate or alpha-cetoglutaric acid [11,4]. It has also been reported that the principal mechanism of mineral phosphate solubilization is hydrolysis process of organic phosphorus in soil by some organic acids and acid phosphatases, secreted by PSB [6,12].

Table 1 Summary of activity index of PSB isolates after 72 hours of incubation.

Isolate	Activity Index
pvk-5a	2,01
pvk-5b	1,52
pvk-6b	1,01
pvk-7a	1,65
pvk-8a	1,58

hours) if vinasse with a dilution factor of 1:2 was used. However, all isolates could not grow if vinasse with a dilution factor of 1:3 was used.

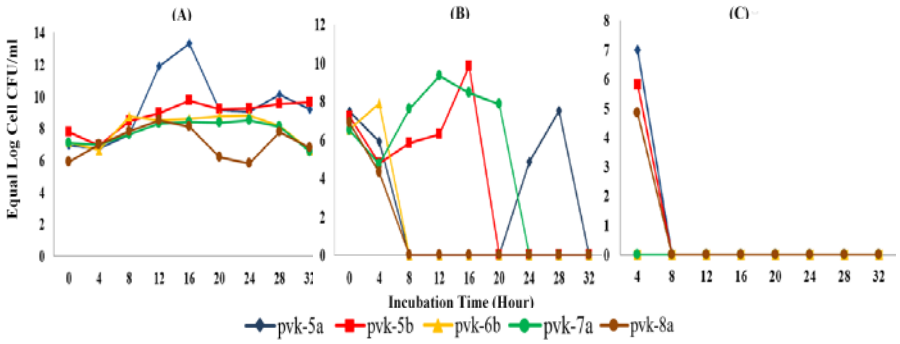

Figure 2 Growth pattern of PSB after 32 hours incubation in Pikovskaya's liquid medium (A), vinasse medium diluted 1:2 (B) and vinasse medium diluted 1:3 (C), and without any minerals or nutrients added.

Some researches have been done related to isolation and characterization of PSB [4,6], including their efficiency to produce and release phosphate in soil [12]. Researchers expect to get beneficial PSB microorganisms for the sustainable use of phosphates in agriculture [12,13]. However, techniques to increase or multiply PSB in soil have received less attention. As described in this research, vinasse has been elucidated as a resource for PSB cultivation medium. Evalution of PSB growth in vinasse against pH, temperature, and other parameters as well should still be further evaluated. Then, a PSB-rich vinasse product is expected that can be utilized on agricultural land as a cheap biofertilizer.

4 Conclusion

Five bacterial isolates of soil treated with vinasse from an agricultural estate were identified as PSB. Among them only three isolates were able to adapt and grow in vinasse liquid medium with a 1:2 dilution factor (pvk-5a, pvk-5b and pvk-7a). The three isolates were characterized as coccoid bacteria, Gram negative with no endospores detected. According to the results, there is strong evidence that the three adaptive PSB isolates had the ability to solubilize both mineral and organic phosphates. The vinasse itself is suited to be used as cheap medium source for PSB production, which may be utilised to support organic farming. This may elevate the phosphate uptake of plants and therefore increase the crop yield significantly.

As shown in Table 1, the ability of each isolate to secrete inorganic extracelllular acid resulted in different activity indexes. The isolate pvk-5a produced the highest activity index (2.01) followed by pvk-7a, pvk-8a, pvk-5b and pvk-6b respectively after 72 hours of incubation at 32°C.

Macroscopical and microscopical observation revealed that all PSB isolates showed different colony shapes when grown in PAM. However, there were only few differences concerning the elevation and edge of the colonies. All isolates consisted of similar yellowish white colored colonies with the entire colony being opaque with a concentric line while grown on the surface of the medium. Microscopical observation of all isolates showed coccus type bacteria, no Gram staining, no endospores and non motil, as described in Table 2. Based on these observations, all isolates can be classified into the same genus as *Bacillus sp*.

Table 2 Macroscopical and microscopical observation of PSB.

No	Characteristic of PSB isolate	Isolate				
		pvk-5a	**pvk-5b**	**pvk-6b**	**pvk-7a**	**pvk-8a**
1	Colony shape					
	Plate culture	Irregular	Curled	Irregular	Irregular	Curled
	Slant culture	Echinulated	Echinulated	Echinulated	Echinulated	Echinulated
	Straight culture	Beaded	Beaded	Echinulated	Beaded	Beaded
2	Surface	Unshine	Unshine	Unshine	Unshine	Unshine
3	Elevation	RCBE	RCBE	RCBE	Umbonated	Umbonated
4	Edge colony	Undulate	Crenate	Crenate	Undulate	Undulate
5	Entire	Opaque	Opaque	Opaque	Opaque	Opaque
6	Colony color	Yellowish white	White milk	Yellowish white	White milk	Yellowish white
7	Growth	Surface	Surface	Surface	Surface	Surface
8	Colony liner	Concentric	Concentric	Concentric	Concentric	Concentric
9	Cell shape	Coccus	Coccus	Coccus	Coccus	Coccus
10	Gram character	Negative	Negative	Negative	Negative	Negative
11	Endospores	-	-	-	-	-

The adaptation ability of PSB isolates that grow in vinasse based medium without any nutrients added was observed through proliferation, as shown in Figure 2. For this purpose, PLM was used as control. The investigation resulted in only three isolates that were able to grow on vinasse containing medium (pvk-5a, pvk-5b and pvk-7a). These three isolates seemed to be adaptive PSB utilizing carbon and nitrogen sources as well as macro and micro nutrients from vinasse for their growth, although they gave optimum population growth at different incubation times. Optimum growth for pvk-5b and pvk-7a was at 12-16 hours of incubation, whereas pvk-5a needed a longer incubation time (24

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