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THE EFFECT OF GLUCOSE ADDITION IN ACETONE-BUTANOL-ETHANOL FERMENTATION FROM PALM OIL MILL EFFLUENT BY *Clostridium Acetobutylicum* NCIMB 619

(Kesan Penambahan Glukosa dalam Fermentasi Aseton-Butanol-Etanol daripada Efluen Kilang Kelapa Sawit oleh *Clostridium Acetobutylicum* NCIMB 619)

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Abstract

This study investigates the production of acetone, butanol and ethanol (ABE) in sterilized sludge of palm oil mill effluent (POME) with the addition of glucose by *Clostridium acetobutylicum* NCIMB 619. Glucose concentrations of 5 g/L, 10 g/L and 15 g/L were used with volume percentage of 3% (v/v) and 5% (v/v) for each concentration. The highest ABE production of 9.89 g/L was observed with the addition of 10 g/L glucose with volume percentage of 5% (v/v) compared to 0.49 g/L in the control batch, with highest ABE productivity and yield, $Y_{P/S}$ of 0.14 g/L/h and 1.36 g ABE/g substrate, respectively. Providing culture with 15 g/L glucose with volume percentage of 5% (v/v) was found to reduce the ABE production with lower productivity and ABE yield. The lower production at higher sugar level might be due to sugar inhibition that occurred during the process.

Keywords: acetone-butanol-ethanol fermentation, palm oil mill effluent, *Clostridium acetobutylicum*, glucose addition

Abstrak

Kajian ini dilakukan bagi menentukan kesan penamabahan glukosa terhadap penghasilan aseton, butanol dan etanol (ABE) di dalam medium mendakan efluen kilang kelapa sawit (POME) steril oleh *Clostridium acetobutylicum* NCIMB 619. Kepekatan glukosa yang digunakan adalah 5 g/L, 10 g/L dan 15 g/L dengan peratus isipadu sebanyak 3% (v/v) dan 5% (v/v) bagi setiap kepekatan. Penghasilan ABE yang paling tinggi iaitu sebanyak 9.89 g/L diperoleh pada penambahan 10 g/L glukosa dengan peratus isipadu 5% (v/v) berbanding 0.49 g/L ABE yang diperoleh di dalam medium kawalan. Produktiviti dan hasil ABE yang diperoleh juga adalah yang tertinggi iaitu masing-masing sebanyak 0.14 g/L/j dan 1.36 g ABE/g substrat. Penambahan 15 g/L glukosa dengan peratus isipadu sebanyak 5% (v/v) didapati mengurangkan penghasilan ABE dengan produktiviti dan hasil ABE yang paling rendah disebabkan oleh perencatan gula yang berlaku semasa proses fermentasi.

Kata kunci: fermentasi aseton-butanol-etanol, efluen kilang kelapa sawit, *clostridium acetobutylicum*, penambahan glukosa

Introduction

The biological production of Acetone-Butanol-Ethanol (ABE) via fermentation have started during the First World War declined 1960s due to introduction of chemical process and higher cost of raw materials [1]. The interest in ABE fermentation was once again increases due to the rapid depletion of petroleum resources, fluctuating fuel price

as well as increase in public awareness on effects of toxic and greenhouse gases produced from fossil fuel combustion [2,3]. All these factors sparked in the attention of alternative fuels namely acetone, butanol and ethanol production through bioprocesses.

The most challenging aspects in ABE fermentation are to make the process economically feasible with zero competition with food crops to obtain raw material resources [4]. One of the solutions is to utilize industrial waste such as palm oil mill effluent (POME) that is readily available in Malaysia and able to reduce the cost of raw material. POME is an industrial waste that comes under the group of lignocellulose biomass consisting of various suspended components including cell wall, organelle and fiber, carbohydrate components which covered hemicellulose and simple sugar, nitrogenous compound including protein and amino acid and free organic acids and all these features make POME as a potential substrate for ABE fermentation [5-7].

POME characteristics as shown in Table 1 depend on the quality of raw material and method of oil processing in palm oil mill [8]. More than 50% of water used in crude oil production eventually ended up as POME [9,10]. POME was generated from three unit processes in a palm oil mill namely sterilization of fresh palm fruit (FFB) (38%), clarification process of extracted crude oil (6%) and hydro cyclone separation (4%) [11]. Based on previous studies, ABE fermentation using POME as media can produce total solvent between 0.30 and 2.66 g/L [7,12-15].

Several studies have reported that limited glucose content in the substrate produced higher amount of organic acids compared to ABE [1]. The cell metabolic shifting to ABE production will not occur if the glucose concentration in the substrate less than 7 g/L [16,17]. Other studies also reported that fermentation with limited presence of glucose resulting in the insufficient of final product of acids to achieve the desired amount of ABE [18-20]. Therefore, the objective of this study was to investigate the effect of glucose addition in ABE production via fermentation using sterilized POME sludge by *C. acetobutylicum* NCIMB 619.

Table 1. Characteristics of palm oil mill effluent (POME) [21]

Parameter	Average	Range
pH	4.2	3.4 - 5.2
Biological oxygen demand (BOD) (g/L)	25000	10250 - 43750
Chemical oxygen demand (COD) (g/L)	51000	15000 - 100000
Total solid (g/L)	40000	11500 - 79000
Suspended solid (g/L)	18000	5000 - 54000
Volatile solid (g/L)	34000	9000 - 72000
Oil and grease (g/L)	6000	130 - 18000
Ammoniacal nitrogen (g/L)	35	4 - 80
Total nitrogen (g/L)	750	180 - 1400

Materials and Methods

Organism, maintenance and inoculum preparation

C. acetobutylicum NCIMB 619 was obtained from NCIMB Ltd (Aberdeen, UK) and the laboratory stocks were routinely maintained as spore suspensions in sterile deoxygenated Reinforced Clostridial Medium (RCM) at 37 ^{o}C. Raw POME obtained from Sime Darby's East Palm Oil Mill in Carey Island was centrifuged at 2000 rpm for 5 minutes to separate the supernatant and the sludge. Sterilization of the sludge was conducted using autoclave at 121 ^{o}C for 15 minutes.

Fermentation process and analysis

The fermentation process was carried out anaerobically in 250 mL conical flask at 37 ^{o}C for 72 hours with working volume of 170 mL without agitation and no pH correction was made. Glucose concentration of 5 g/L (POME5),

10 g/L (POME10) and 15 g/L (POME15) were used with volume percentage of 3% (v/v) and 5% (v/v) for each concentration. The glucose was added into sterilized POME sludge and deoxygenated using purified nitrogen gas for 15 minutes to achieve the anaerobic condition. Samples for analysis of pH, cell concentration, reducing sugar and solvents production were taken at regular interval. The pH values were determined using pH meter, while cell concentration and reducing sugar in the samples were determined by volatile suspended solid (VSS) and Miller methods [22], respectively. The concentrations of acetone, butanol, ethanol and organic acids were measured by gas chromatography (Eppendorf, German) that is equipped with flame ionization detector (FID) with initial operating temperature of 40^{o}C for 8 minutes and then increased to 130 ^{o}C at the rate of 4 ^{o}C/minute for 2 minutes. The temperature of injector and detector was operated at 250 ^{o}C with helium as the carrier gas.

Liquid-liquid extraction

Liquid-liquid extraction was conducted to recover ABE from the fermentation broth before the samples were injected into gas chromatography. Oleyl alcohol was used as solvent which has been reported as a potential separation solvent for ABE [23]. Oleyl alcohol was added into the sample in centrifuge tube at the ratio of 1:1 (oleyl alcohol-sample). The solution was then mixed using vortex mixer at 1600 rpm for 1 minute in order to increase the interaction between oleyl alcohol and the sample. To complete the phase separation, the solution was centrifuged at 2000 rpm for 5 minutes. Assuming the organic and aqueous phase were separated completely, all samples were diluted with 99.5% dichloromethane (DCM) at the ratio of 0.5:1 (DCM:sample). Samples from both phases were mixed by vortex mixer at 1600 rpm for 1 minute to ensure the interaction between samples and DCM occurred effectively. A complete mixing will produce two layers of liquid due to the different density. The bottom layer was taken and filtered using 0.2 µm nylon syringe filter before being transferred to sealed GC vial tubes for further analysis.

Results and Discussion

ABE production with the addition of 3% (v/v) glucose

The summary of total ABE and organic acids production is presented in Table 2 and Figure 1 with ethanol as the major product. It also shows that the production of ABE increased as the glucose concentration increased where in the control batch, only 0.49 g/L ABE produced with a yield, $Y_{P/S}$ of 0.04 g ABE/g substrate and productivity of 0.007 g/L/h. POME15 produced the highest amount of ABE which is 3.44 g/L with the production of acetic acid and butyric acid of 123.9 g/L and 1.17 g/L, respectively. POME5 produced the lowest amount of ABE (2.84 g/L) and produced the highest amount of organic acids which is 165.7 g/L with 163.4 g/L of acetic acid and 0.34 g/L of butyric acid. All three medium produced a high concentration of acetic acid. ABE production was related to the activity of proteolysis enzyme that could affect the degradation of enzymes activity that involved in ABE fermentation [24]. The activity of proteolysis enzyme also might degrade the activity of tiolase enzyme that can change acetyl-CoA to acetoacetyl-CoA in the culture which is important in the production of acetone and butanol [25].

Table 2. Summary of ABE fermentation performance with the addition of 3% (v/v) glucose

Parameter	Glucose Concentration		
	5 g/L	10 g/L	15 g/L
Initial pH	4.26	4.27	4.30
Max. cell concentration (g/L)	63.5	59.0	58.0
Total ABE (g/L)	2.84	3.30	3.44
A:B:E ratio	0.2:0.6:9.2	0.3:0.7:9	0.3:0.9:8.8
Total organic acids (g/L)	165.7	106.0	125.1
$Y_{P/S}$ (g ABE/g substrate)	0.57	0.81	0.82
$Y_{X/S}$ (g cell/g substrate)	0.71	0.98	0.71
Productivity (g/L/h)	0.04	0.05	0.05

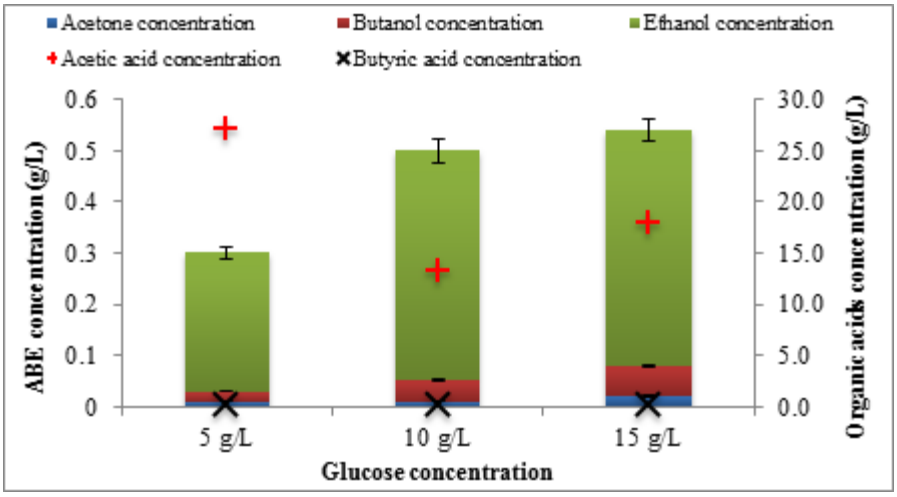

Figure 1. Maximum concentration of ABE and organic acids with the addition of 3% (v/v) glucose

ABE production with the addition of 5% (v/v) glucose

Total production of ABE and organic acids with the addition of 5% (v/v) glucose was summarized in Table 3 and Figure 2 with ethanol as the major product. For POME5 and POME10, the maximum ABE concentration of 0.50 g/L and 0.52 g/L, respectively was collected after 48 hours of fermentation while for POME10, maximum ABE concentration of 1.95 g/L was obtained after 72 hours of fermentation. POME5 produced 30.6% and 86.7% more acetone compared to POME15 and POME10.

Other than that, POME5 also produced 0.60 g/L butanol which is two folds higher compared to POME15 that only produced 0.26 g/L and 45.8% more butanol than that in POME10. At the same time, POME10 produced the highest concentration of ethanol which is 9.44 g/L while POME5 and POME15 produced 2.87 g/L and 1.88 g/L ethanol, respectively. Total ABE of 9.89 g/L produced in POME10 was the highest with total organic acids of 40.05 g/L. POME15 produced the lowest concentration of ABE (2.82 g/L) with organic acids concentration of 6.83 g/L.

Table 3. Summary of ABE fermentation performance with the addition of 5% (v/v) glucose

Parameter	**Glucose Concentration**		
	5 g/L	**10 g/L**	**15 g/L**
Initial pH	4.30	4.48	4.31
Max. cell concentration (g/L)	63.5	59.0	62.3
Total ABE (g/L)	4.45	9.89	2.82
A:B:E ratio	2.2:1.3:6.5	0.13:0.33:9.5	2.4:0.9:6.7
Total organic acids (g/L)	0.14	40.05	6.83
$Y_{P/S}$ (g ABE/g substrate)	0.62	1.36	0.28
$Y_{X/S}$ (g cell/g substrate)	0.84	0.76	1.63
Productivity (g/L/h)	0.06	0.14	0.04

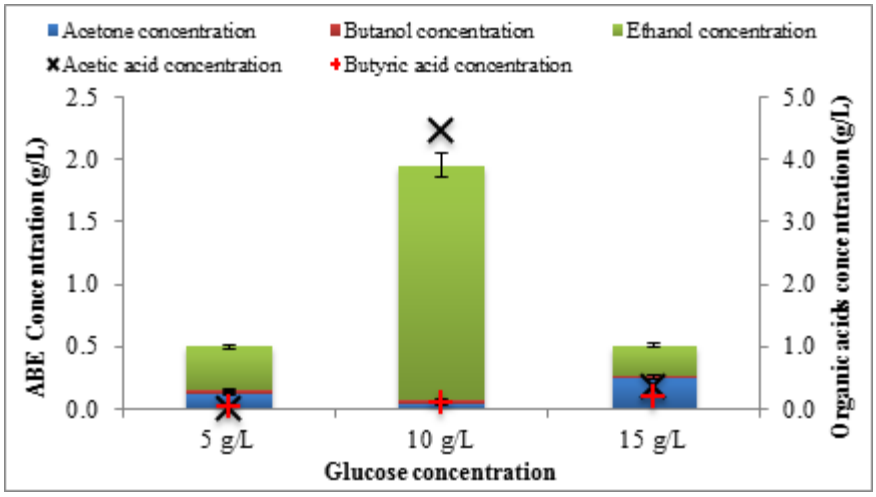

Figure 2. Maximum concentration of ABE and organic acids with the addition of 5% (v/v) glucose

Comparison of ABE fermentation performance with the addition of 3% (v/v) and 5% (v/v) glucose

Based on Figure 3, it was found that with the addition of 3% (v/v) glucose, enhanced the ABE production where in control batch, only 0.49 g/L ABE was produced with yield, $Y_{P/S}$ of 0.04 g ABE/g substrate and productivity of 0.007 g/L/h (Data was not shown). POME5 produced 2.84 g/L ABE with yield, $Y_{P/S}$ of 0.57 g ABE/g substrate. POME10 and POME15 produced 3.30 g/L ABE with yield, $Y_{P/S}$ of 0.81 g ABE/g substrate and 3.44 g/L ABE with yield, $Y_{P/S}$ of 0.82 g ABE/g substrate, respectively.

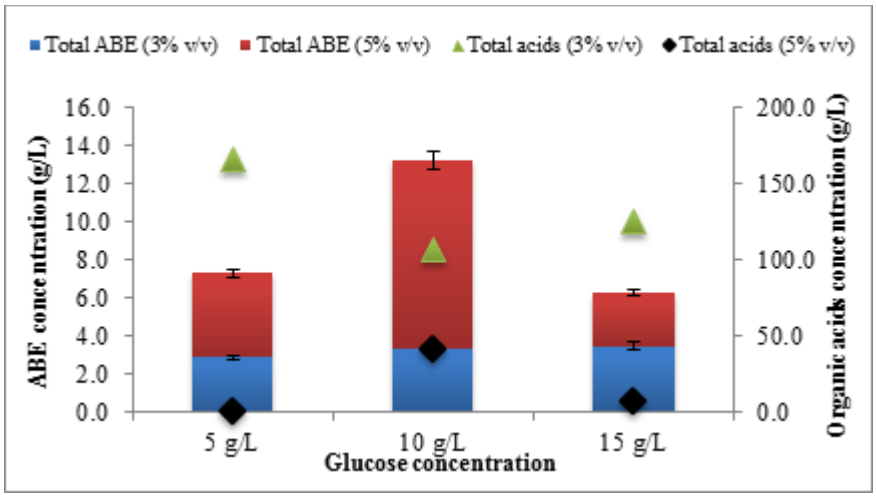

Figure 3. Comparison of ABE performances with the addition of glucose

However, ABE production was decreased at 15 g/L glucose with the addition of 5% (v/v) where high level of sugar in the medium was found to inhibit the production of ABE [26]. ABE fermentation using a high carbon source concentration may be controlled by the mass transfer rate, where high concentration carbon source could increase the viscosity of the culture and reduced the rate of mass transfer altogether [27].

Other than that, POME10 produced the highest ABE concentration which is 9.89 g/L with yield, $Y_{P/S}$ of 1.36 g ABE/g substrate while POME5 and POME15 produced 4.45 g/L and 2.82 g/L ABE, respectively where POME15 produced the lowest yield, $Y_{P/S}$ of 0.28 g ABE/g substrate. It was also found that the addition of 3% (v/v) glucose produced the highest amount of organic acids that might be due to the acid crash that occurred when the un-dissociated acids in the culture was more than 57-60 mmol/L [28]. Furthermore, the results obtained from this study are comparable to previous reports as shown in Table 4.

Table 4. Comparison of ABE fermentation performances

Species	**Substrate**	**Glucose Content (g/L)**	**ABE Concentration (g/L)**	**Yield, $Y_{P/S}$ (g ABE/g substrate)**	**Productivity (g/L/h)**	**Reference**
C. acetobutylicum NCIMB 619	Raw POME	3.40	2.05	0.90	0.04	[15]
C. beijerinckii BA101	Treated corn fiber	23.60	9.30	nd.	nd.	[29]
C. acetobutylicum ATCC 824	Spoilage date palm	36.20	10.42	nd.	0.14	[30]
C. beijerinckii ATCC 55025	Wheat bran	21.30	11.80	0.32	0.16	[31]
C. acetobutylicum NCIMB 619	Sterilized POME	15.82	9.89	1.36	0.14	This study

*nd. – not determine

Conclusion

The addition of glucose in sterilized POME media was found to increase the ABE production. Based on the results of this study, the production of ABE and organic acids is higher as the glucose concentration increasing using 3% (v/v). However, for the glucose addition of 5% (v/v), the high level of sugar present in the culture was found to inhibit the ABE production. Glucose concentration of 10 g/L produced the highest concentration of ABE (9.89 g/L) with product yield, $Y_{P/S}$ of 1.36 g ABE/g substrate while glucose concentration of 15 g/L produced the lowest concentration of ABE and product yield, $Y_{P/S}$ with 2.82 g/L and 0.28 g ABE/g substrate, respectively.

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