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Oil Palm Empty Fruit Bunch as Alternative Substrate for Acetone–Butanol–Ethanol Production by *Clostridium butyricum* EB6

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Abstract Acetone–butanol–ethanol (ABE) production from renewable resources has been widely reported. In this study, *Clostridium butyricum* EB6 was employed for ABE fermentation using fermentable sugar derived from treated oil palm empty fruit bunch (OPEFB). A higher amount of ABE (2.61 g/l) was produced in a fermentation using treated OPEFB as the substrate when compared to a glucose based medium that produced 0.24 g/l at pH 5.5. ABE production was increased to 3.47 g/l with a yield of 0.24 g/g at pH 6.0. The fermentation using limited nitrogen concentration of 3 g/l improved the ABE yield by 64%. The study showed that OPEFB has the potential to be applied for renewable ABE production by *C. butyricum* EB6.

Keywords *Clostridium butyricum* · Acetone butanol ethanol (ABE) · Oil palm empty fruit bunch (OPEFB) · Anaerobic fermentation · Biomass

Introduction

In recent years, growing attention has been paid to the production of fine chemicals from biomass. Acetone–butanol–ethanol (ABE) fermentation by Clostridia has attracted many

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researchers to look upon the ability of this microorganism to produce multiple valuable products. These anaerobic bacteria produce acids, solvents and gasses in a complete metabolic pathway [1]. Acetic acid and butyric acid will be produced at an early stage of fermentation known as the acidogenesis phase before the cells enter solventogenesis phase to produce acetone, butanol and ethanol. Hydrogen and carbon dioxide are also released as byproducts throughout the glycolysis process by this microorganism [2].

There are many species that have been studied for acids and ABE production. *Clostridium acetobutylicum* and *C. beijerinckii* are the main species for ABE fermentation that are normally employed for butanol production [3]. *C. butyricum* and *C. tyrobutyricum* were known as butyric acid producers [4, 5] and a few studies had been done for hydrogen production [6]. In addition, Clostridia have the ability to consume hexose and pentose, sugar monomers released from woody materials including lignocellulosic biomass [7]. Thus, the possibility of utilizing lignocellulosic biomass for acids and ABE production by Clostridia could become a reality. Recently, research and development has focused on the conversion of waste material for conversion into fuel and fine chemicals. Lignocellulosic biomass generated from the agricultural industry has been chosen as the fermentation feedstock because it is an abundant, renewable, cheap and readily available material.

Lignocellulosic biomass is composed of cellulose and hemicellulose which can be converted into sugar monomers through enzymatic hydrolysis. In Malaysia, oil palm empty fruit bunch (OPEFB) generated from the milling activity of the palm oil industry is the most abundant biomass produced annually. About 17 million tons/year of OPEFB were produced in 2005, and this amount is expected to increase due to the increasing demand of palm oil [8]. However, the current application of OPEFB is limited to mulching and it is normally left over at the mill. Fortunately, this biomass has an energy availability that could be used as an energy source for the fermentation process, which could produce value added chemical products [9].

The aim of this study is to investigate the production of ABE from treated OPEFB by *C. butyricum* EB6. Previously, this strain was used to produce hydrogen from palm oil mill effluent with high productivity [6]. However, there were only few reports on utilization of oil palm biomass for acids and ABE production by *C. butyricum* and none for strain EB6. The ability of this strain to produce ABE using local biomass as substrate is presented in this paper.

Materials and Methods

Pretreatment of OPEFB

Pressed and shredded OPEFB were obtained from the Ulu Langat Palm Oil Mill, Dengkil, Selangor. Pretreatment was carried out as described by Umi Kalsom et al. [10]. OPEFB was soaked with detergent overnight and then washed to remove the remaining oil and dust before being oven dried at 60°C for 24 h. Washed OPEFB (5% w/v) was soaked in 2% NaOH for 4 h and autoclaved at 121°C for 5 min. The treated OPEFB was then washed with tap water until the pH was equivalent to pH 7 and oven dried at 60°C for 24 h.

Saccharification of OPEFB

The cellulase used in this study was Celluclast 1.5 L supplied by Novozyme (Denmark) with concentrated enzyme activities equal to 400.69, 9,156.92 and 138.29 U/ml of β-glucosidase, CMCase and FPase, respectively. The enzyme activities were determined according to the method described by Wood and Bhat [11].

The saccharification process was carried out with 5% (w/v) of substrate in 50 μM of sodium acetate buffer at pH 4.8. Diluted 2% (v/v) enzyme was added to the solution for the conversation process lasting 24 h in an orbital shaking incubator (Labwit, China) with temperature of 50°C and agitation at 200 rpm. Cellulose, hemicellulose, lignin and ash contents were determined as described by Goering and Van Soest [12] using the gravimetric method. The OPEFB hydrolysate was recovered by centrifuging at 4,000 rpm for 10 min prior to ABE fermentation.

Acetone–Butanol–Ethanol Fermentation

Inoculum Preparation

Locally isolated *C. butyricum* EB6 was employed for ABE production. The stock culture was inoculated anaerobically in reinforced clostridia medium (RCM) and incubated for 48 h at 37°C using a static Memmert incubator after heat shock in a Memmert water bath for 2 min at 80°C.

Media Preparation

The basal medium consisted of (in g/l) NaCl 0.2, KH_2PO_4 0.75, K_2HPO_4 0.75, $MgSO_4$ $7H_2O$ 0.8, $MnSO_4$ $7H_2O$ 0.2, $FeSO_4$ $5H_2O$ 0.03, yeast extract 6.0, L-cysteine HCl 0.5 and different carbon sources. The pH was adjusted to pH 5.5 based on the study made by Chong et al. [6] using 2 M HCl and/or 2 M NaOH. The medium was sparged with nitrogen gas for 20 min to remove oxygen and autoclaved at 121°C for 15 min. Resazurin (0.001 g/l) was used as an indicator to detect the presence of oxygen in the media.

Fermentation

The anaerobic batch fermentation was carried out in 125-ml serum bottles (sealed with rubber stopper) containing 90 ml basal medium with 20 g/l (glucose or OPEFB hydrolysate) as carbon source. The fermentation process was started with an aseptically transfer of 10 ml inoculum with optical density of 1.0 at 680 nm into the sterilized anaerobic medium using a syringe. The fermentation was carried out at 37°C without agitation [13].

Analytical Procedures

All the samples were analyzed for their sugar residues, cell and product concentrations. Consumption of reducing sugar was determined using the DNS method described by Miller [14]. Cells concentration was determined based on the optical density (OD) method together with dry cell weight (DCW). Acids and ABE concentrations were analyzed using gas chromatography (Shimadzu, GC-17A, Japan) with column BP20 and equipped with thermal ionization detector. The standard curves of acids and ABE were plotted based on area ratio of 1% of 1-propanol to standard of acetic acid, butyric acid, acetone, butanol and ethanol, respectively.

Results and Discussion

ABE Production from Glucose-Based Medium

The acids and ABE production employing *C. butyricum* EB6 produced up to 6.60±0.21 g/l of total organic acid (4.78±0.09 g/l acetic acid and 1.82±0.12 g/l butyric acid) using glucose as

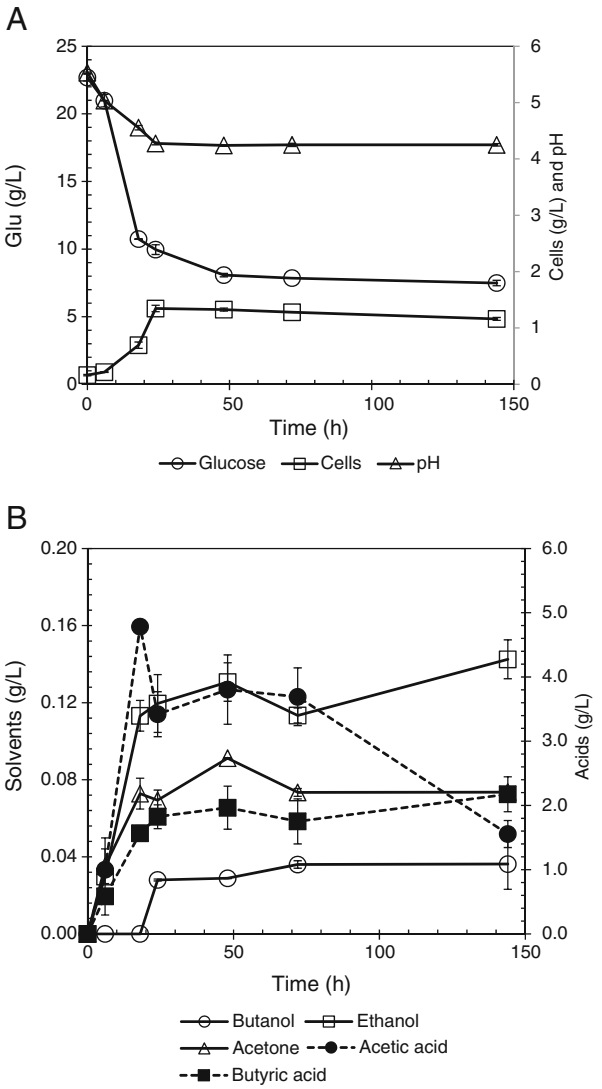

Fig. 1 ABE production by *C. butyricum* EB6 using a glucose based medium. **a** Profile of cells growth (*open square*), glucose consumption (*open circle*) and pH (*open triangle*). **b** Profile of butanol production (*open circle*), ethanol production (*open square*), acetone production (*open triangle*), acetic acid production (*filled circle*) and butyric acid production (*filled square*)

carbon source as shown in Fig. 1. The acids were mainly produced within 24 h of fermentation, decreasing the pH to 4.26. The acetic acid formed was decreased to 1.55±0.21 g/l after 144 h while the butyric acid was not consumed. In the metabolic pathway of ABE fermentation, the acids formed were used as co-substrates and inducers for the production of ABE [15, 16]. The acidogenesis phase of this fermentation occurred during the first 24 h prior to solventogenesis phase for ABE production. This situation was observed when 1.34±0.06 g/l of cells were produced, but they stopped growing after 24 h, entering the stationary phase and small amounts of ABE started to be produced. The total glucose consumption was 15.17±0.20 g/l, and the cells did not consume all the available glucose, leaving over 7.85±0.20 g/l even after 144 h of fermentation. Thus, it was assumed that cell growth was inhibited by the formation of acids in the system. Liu and Yang [4] reported that the growth of *C. tyrobutyricum* wild type was

inhibited in the presence of 19.42 g/l of butyric acid. Acid inhibition was also reported by Green et al. [17] on the ABE fermentation by *C. acetobutylicum.*

This strain, however, did not produce higher levels of ABE even when higher acid concentration was formed. The total ABE accumulated in this fermentation was 0.24± 0.00 g/l including 0.09±0.00 g/l acetone, 0.04±0.00 g/l butanol and 0.11±0.01 g/l ethanol after 72 h. Hydrogen gas was also produced by this strain with a yield of 0.948 ml/ml reported by Chong et al. [6]. The ABE started to be produced after 24 h in which the fermentation condition was not favorable to cell growth due to acids production at an early stage. Thus, low ABE production occurred in the system. Overall, this fermentation yielded 0.33 g/g of acid and only 0.012 g/g of ABE. A similar acid yield was reported by Vandak et al. [5] using *C. butyricum* S2, whereby lower ABE was produced by this strain with a high accumulation of butyric acid.

ABE Production from Treated OPEFB Hydrolysate

A production of ABE was conducted using treated OPEFB hydrolysate as the carbon source. The hydrolysis was performed by Celluclast 1.5 L with the enzyme activity described in "Saccharification of OPEFB" section. The treated OPEFB hydrolysate contained 10.7 g/l glucose, 8.3 g/l xylose, 2.9 g/l arabinose and lower concentrations of galactose and mannose with net total reducing sugars (TRS) obtained at 32.2 g/l. Approximately 20 g/l of the TRS was used in this fermentation to be comparable with the fermentation using 20 g/l glucose based medium mentioned above. The results obtained are shown in Table 1 at initial pH 5.5. It showed that higher ABE was produced from OPEFB hydrolysate compared to glucose. Approximately 2.61±0.35 g/l of total ABE was produced, which was 10× higher than the ABE production from glucose-based medium. The high concentration of xylose in the OPEFB hydrolysate affected the production of acids and ABE. Qureshi et al. [18] reported that higher ABE production was observed in fermentation using xylose as compared to glucose. It should be noted that, *Clostridium* sp. has the ability to consume both pentose and hexose sugars [3].

ABE production by *Clostridium* sp. had been investigated by many researchers (Table 2). *C. acetobutylicum* is most commonly used for ABE fermentation, after its discovery in the late 19th century. It has been widely used due to the capability of this strain to produce high level of butanol at 8.4 and 7.9 g/l from sago and xylose, respectively [13, 19]. Recently, *C. beijerinckii* had been noted for ABE fermentation as this strain was also found to be a good butanol producer with net butanol production of 6.4 and 6.1 g/l derived from sulfuric acid corn fiber hydrolysate (SACFH) and glucose, respectively [18]. *C. butyricum*, however, has not yet identified as a good ABE producer but in this experiment, good ABE yield was observed at pH 6.0. In addition to the fermentation by *C. butyricum*, acid production was found to be high in this experiment when compared to the study of Phowan et al. [20].

Effect of Initial pH

The ABE production from OPEFB hydrolysate was further investigated. Initial pH values of 5.0, 5.5, 6.0 and 6.5 were tested in this experiment to determine the suitable initial pH for ABE production. Higher amounts of ABE were produced at pH 6.0 with final concentration equal to 3.47±0.91 g/l and yield at 0.24±0.06 g/g. Many studies used pH 6.0 to 6.8 for ABE fermentation to generate enough acids and cells for ABE production as shown in Table 2. Although acids were required for ABE production, a high acids concentration was not conducive to cell growth. It was believed that the minimum amount of acid required to

Table 1 ABE production from OPEFB hydrolysate by *C. butyricum* EB6 after 72 h of fermentation

Initial pH	Final pH	Sugar consumed (g/l)	Cell conc. (g/l)	Acids (g/l)		Total acid (g/l)	ABE (g/l)			Total ABE (g/l)	ABE yield (g/g)
				Acetic acid	Butyric acid		Acetone	Butanol	Ethanol		
5.5	4.65	12.61±0.93	0.98±0.11	2.65±0.39	3.37±0.24	6.02±0.63	0.69±0.10	0.68±0.06	1.24±0.19	2.61±0.35	0.21±0.03
6.0	4.68	14.35±0.75	1.22±0.15	3.81±0.99	3.01±0.17	6.82±1.16	0.91±0.54	0.85±0.06	1.71±0.31	3.47±0.91	0.24±0.06
6.5	4.70	17.85±1.12	1.24±0.09	7.42±0.36	5.63±0.25	13.05±0.61	0.41±0.58	0.2±0.04	1.98±0.54	2.59±1.16	0.15±0.06

No growth observed in pH 5.0

Data presented are the average of at least duplicate experiments with standard deviation

Table 2 Comparison of ABE production by *C. butyricum* EB6 to those of other *Clostridium* sp.

Microorganism	Carbon source	pH	Temp. (°C)	Acid and ABE (g/l)					References
				Acetic acid	Butyric acid	Acetone	Butanol	Ethanol	
C. acetobutylicum P262	30 g/l sago	6.0	35	0.38	1.14	2.4	8.4	0.38	Madihah et al. [13]
C. acetobutylicum ATCC824	60 g/l xylose	6.8	37	2.0	2.0	2.0	7.9	1.8	Zhijie and Shijie [20]
C. butyricum TISTR 103 with *E. aerogenes* TISTR 1468	20 g/l glucose from cassava pulp hydrolysate	6.0	36	1.2	5.9	na	0.8	0.2	Phowan et al. [21]
		7.0	36	1.1	4.8	na	0.3	0.4	
C. beijerinckii BA101	46.3 g/l treated corn fiber (SACFH)	6.8	35	na	na	2.7	6.4	0.2	Qureshi et al. [18]
	25 g/l glucose	6.8	35	na	2.0	3.9	6.1	0.4	
C. butyricum EB6	20 g/l glucose	5.5	37	4.8	1.8	0.1	0.04	0.13	This work
C. butyricum EB6	20 g/l OPEFB hydrolysate	6.0	37	3.81	3.01	0.91	0.85	1.71	This work

na not analyzed or not mentioned in the report

induce the ABE production was 0.88 g/l of butyric acid and 0.60 g/l of acetic acid [21]. However, the exact concentration remains unknown due to the complicated metabolic mechanism involved in ABE production by Clostridia. Similar to the fermentation using a glucose-based medium, this fermentation was also inhibited by formation of acids whereby only 63.05% sugar was consumed with 20 g/l initial sugar supplemented to the system. At all pH conditions tested, ethanol dominated in the total ABE production at 1.24±0.19, 1.71±0.31 and 1.98±0.54 g/l of ethanol at pH 5.5, 6.0 and 6.5, respectively. Relatively high ethanol production by *C. butyricum* was also reported by Wiegel [22]. In contrast, ABE fermentation employing *C. acetobutylicum* or *C. beijerinckii* produced acetone–butanol–ethanol in the ratio of 3:6:1 [3, 23] with a higher amount of butanol generated. The complex metabolic pathway of Clostridia may be a reason for this phenomenon. They are classified as heterotrophic producers that produce multiple types of byproducts. In addition, butanol production required high redox potential [2], and thus it needed a sufficient amount of inducer together with optimum condition for the conversion of pyruvate into butanol. However, the metabolic consequence for the *C. butyricum* used in this study has not been studied yet.

It was found that elevated of pH value enhanced cell growth and acids production as well as sugar consumption. In this experiment, 7.42±0.36 g/l of acetic acid and 5.63±0.025 g/l of butyric acid equivalent to 13.05 g/l of total acid with yield of 0.73±0.03 g/g achieved after 72 h of fermentation at pH 6.5 (Table 1). A Higher butyric acid production of 43 g/l was reported by Liu et al. [24] using a *C. tyrobutyricum* mutant, but the yield obtained was 0.4 g/g. The extensive growth and high acids formation by *C. butyricum* EB6 in this study might be due to the sufficient amount of nutrient, suitable pH condition and appropriate carbon source derived from OPEFB hydrolysate. The pH dropped to 4.70 when the cells produced acid rapidly. Vandak et al. [5] also obtained a higher level of acid formation at this pH condition however their acid yield was lower compared to this study. The remaining sugar was 3.24 g/l with 89.25% of sugar consumption indicating a good fermentation process.

At pH 5.0, no growth was observed resulting in a very low concentration or no acids and ABE being produced. The final pH was around 4.65–4.70 in all the fermentations showing that the cells produced acids to decrease the pH to below 4.80 for the solventogenesis phase to take place [25]. Many Clostridia species are neutrophiles and a few species can tolerate pH conditions as low as pH 3.9. Nevertheless, most species could not initiate growth at a very low pH [26].

Effect of Nitrogen

The effect of nitrogen concentrations on the production of ABE had not been well studied especially on the new isolate like *C. butyricum* EB6. However, it was assumed that the ABE fermentation in which the product formation is greatly influenced by cells concentration [27]. In this study, nitrogen concentrations of 0, 3, 6, 9 and 12 g/l represented by N0, N3, N6, N9 and N12, respectively, gave various effects on ABE formation in the system (Table 3). The highest ABE level was found at N6 with total ABE of 2.61±0.35 g/l corresponding to a total yield 0.21±0.02 g/g. Yusof et al. [28] obtained only 0.066 g/g of total ABE yield in a fermentation by *C. acetobutylicum* NCIMB 619 using rich RCM medium. The high ABE production at this condition might be due to the suitable carbon/nitrogen ratio as reported by Madihah et al. [29].

However, at all the conditions applied, N3 gave a better ABE yield of 0.39 g/g showing that the carbon source consumed was mainly transformed into ABE. This yield was

Table 3 Effect of nitrogen concentrations on ABE production from OPEFB by *C. butyricum* EB6 after 72 h of fermentation

	Nitrogen conc. (g/l)			
	3	6	9	12
Final pH	5.03±0.11	4.65±0.17	4.74±0.12	4.73±0.09
Sugar consumption (g/l)	3.73±0.00	12.61±0.93	13.77±0.41	15.17±0.03
Cell growth				
Cell conc. (g/l)	0.68±0.01	0.98±0.11	1.42±0.07	1.43±0.04
Cell yield (g/g)	0.26±0.00	0.10±0.00	0.10±0.00	0.09±0.00
Acid production				
Acetic acid conc. (g/l)	0.68±0.00	2.65±0.39	1.92±0.75	4.23±0.74
Acetic acid yield (g/g)	0.18±0.00	0.21±0.03	0.14±0.05	0.28±0.05
Butyric acid conc. (g/l)	0.94±0.00	3.37±0.24	2.45±0.14	3.05±0.06
Butyric acid yield (g/g)	0.25±0.00	0.36±0.02	0.18±0.01	0.20±0.00
Total acid (g/l)	1.62±0.00	6.02±0.63	4.37±0.89	7.28±0.80
Total acid yield (g/g)	0.43±0.00	0.65±0.05	0.32±0.06	0.48±0.05
ABE production				
Acetone conc. (g/l)	0.53±0.00	0.69±0.10	0.37±0.52	0.68±0.14
Acetone yield (g/g)	0.14±0.00	0.05±0.01	0.03±0.04	0.04±0.01
Butanol conc. (g/l)	nd	0.68±0.06	0.35±0.01	nd
Butanol yield (g/g)	–	0.05±0.00	0.03±0.00	–
Ethanol conc. (g/l)	0.91±0.00	1.24±0.19	1.32±0.25	1.41±0.16
Ethanol yield (g/g)	0.24±0.00	0.10±0.01	0.10±0.02	0.09±0.01
Total ABE (g/l)	1.44±0.00	2.61±0.35	2.04±0.78	2.09±0.30
Total ABE yield (g/g)	0.39±0.00	0.21±0.03	0.15±0.06	0.14±0.02

No growth observed in nitrogen 0 g/l

Data presented are the average of at least duplicate experiments with standard deviation

nd not detected

comparable with the ABE fermentations by *C. acetobutylicum* and *C. beijerinckii* from previous studies [18, 30, 31]. The limitation of nitrogen in the medium enhanced the yield of ABE. A similar result was obtained by Kanchanatawee and Maddox [32] when excess nutrient caused the fermentation to be acidogenic rather than solventogenic.

In cell generation, increasing nitrogen concentration enhanced cell production but the number of cells did not affect the production of ABE especially butanol. Nitrogen limitation and excess carbon source in butanol fermentation was required for high ABE production [29]. Excess nitrogen enhances cell multiplication, while more carbon will be consumed at the same time resulting in a low conversion to carbon based products including ABE. Thus, a suitable nitrogen concentration is required for the production of ABE. In this experiment, increases of nitrogen concentration enhanced cell formation from 0.68±0.01 g/l at N3 to 1.43±0.04 g/l at N12. However, the biomass yield was found to be higher at N3 (0.18±0.00 g/g) while other nitrogen concentrations had similar biomass yield (0.10±0.00 g/g). ABE fermentation by *C. beijerinckii* BA101 had cell yield in the range of 0.06–0.07 g/g but a higher ABE production was obtained [18].

Conclusions

Higher ABE yield was obtained from treated OPEFB when compared to using a glucose based medium with a net ABE concentration obtained of 2.61±0.35 g/l. A higher acid level of 13.05±0.61 g/l was produced at pH 6.5, and a higher ABE level was obtained at pH 6.0 with a concentration of 3.47±0.91 g/l. An inhibitory effect on cell growth was observed when a high acid concentration between 5 and 13 g/l was accumulated in the system. Limitation of nitrogen concentration improved the ABE yield by 64% when using 3 g/l of nitrogen compared to medium supplemented with 12 g/l of nitrogen. In this study, ABE fermentation from treated OPEFB was successfully produced by *C. butyricum* EB6.

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