THE PRODUCTION OF ACETONE-BUTANOL-ETHANOL FROM OIL PALM WASTES BY *Clostridium acetobutyricum* AND UTILISATION OF THE WASTEWATER FOR POLYHYDROXYBUTYRATE PRODUCTION

KANOKPHORN SANGKHARAK*; TEWAN YUNU*; NISA PAICHID* and POONSUK PRASERTSAN**

ABSTRACT

Oil palm wastes including palm oil mill effluent (POME), empty fruit bunch (EFB), palm pressed fibre (PPF) and palm kernel shell (PKS) were collected and utilised as substrate for acetone-butanol-ethanol (ABE) production. Each waste was pre-treated before use. POME was centrifuged to obtain the supernatant. EFB, PPF and PKS were pre-treated by steam explosion and hydrolysed by cellulase from Aspergillus niger. *The cultivation of* Clostridium acetobutyricum *under 180 ml of diluted POME supplemented with 10 g litre^{-1} of pre-treated EFB gave the maximum ABE and total acids at 3.8 g litre^{-1} and 2.0 g litre^{-1}, respectively. The ABE production was then recovered by distillation. The wastewater after distillation was collect and characterised. Wastewater contained organic acid profiles such as acetic acid, particularly propionic and butyric acid in the amount of 0.1-1.0 g litre^{-1}. Acetic and butyric present in wastewater can possibly be used for polyhydroxybutyrate (PHB) production. Therefore, the wastewater was utilised as substrate for PHB production by* Cupriavidus necator. *The maximum PHB (48.4% of dry cell weight, DCW) was obtained after 60 hr of cultivation. The polymer was identified by gas chromatography to be PHB when compared to commercially available product.*

Keywords: acetone, butanol, ethanol, palm oil mill effluent, palm pressed fibre, polyhydroxybutyrate.

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INTRODUCTION

Palm oil is one of the most important agricultural products for tropical countries, including Thailand. Palm oil occupies 70% of the Thai vegetable oil market and is estimated to be worth 1.5 billion USD per annum with an average growth rate of 15% during the last decade (Chavalparit *et al*., 2006). In 2003 there were 25 wet-process crude palm oil factories in Thailand, producing about 0.7 million tonnes of crude palm oil from 4 million tonnes of fresh fruit bunches (FFB). Therefore, the wastes from palm oil factories increase rapidly with the increase of palm oil production. Large amounts of solid and liquid wastes are generated by the milling process and plantation activities. The average values of waste generation rate per tonne of FFB from palm oil mill industries in Thailand were 9 x10^5 t yr^{-1} of EFB, 6x10^5

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t yr^{-1} of fibre and 2 $x10^5$ t yr^{-1} of shells (Chavalparit *et al*., 2006). Moreover, it has been estimated that 1 t of crude palm oil production requires 5-7.5 t of water in which about 50% ends up as palm oil mill effluent (POME). Normally, fibres and shells are utilised as fuel to generate steam and electricity in the palm processing mill itself. However, much is also wasted by dumping in areas adjacent to the mill, or utilised as fertiliser in the palm oil plantation. In recent years, growing attention has been paid to the production of fine chemicals from oil palm biomass. Fortunately, oil palm biomass shows potential as an industrial fermentation substrate, which could produce valuable chemical products (Sumathi *et al*., 2008).

Solvent-producing clostridia could produce acetone, butanol, and ethanol (ABE) from several biomasses such as palm oil waste (Jones and Wood, 1986). Acetic acid and butyric acid are produced first at an early stage of fermentation by *Clostridia* sp., known as the acidogenesis phase, before the cells enter solventogenesis phase to produce ABE. Hydrogen and carbon dioxide are also released as by-products throughout the glycolysis process by this microorganism (Mitchell, 1998). In addition, *Clostridia* have the ability to utilise hexose and pentose, sugar monomers released from woody materials (Singh, 1995; Ibrahim *et al*., 2012). There are many species that have been studied for acids and ABE production. *Clostridrium acetobutylicum* and *C. beijerinckii* are the main species for ABE fermentation that are normally employed for butanol production (Vandak *et al*., 1997; Liu and Yang, 2006; Lee *et al*., 2008). Several wastes from palm oil mill such as empty fruit bunch (EFB), fibre, separator sludge and POME were able to support production of ABE by *Clostridia* sp. *C. saccharoperbutylacetonicum* N1-4 without any need for mineral supplements. Besides that, enzymatic hydrolysis by cellulose prior to fermentation was found to increase the yield of butanol by 75% (from 2.47 g $litre^{-1}$ to 4.37 g $litre^{-1}$) (Mun *et al*., 1995). *Clostridium butyricum* EB6 was also employed for ABE fermentation using fermentable sugar derived from treated oil palm empty fruit bunch (OPEFB). A highest amount of ABE (3.5 g g $litre^{-1}$) was produced in a fermentation using treated OPEFB at pH 6.0 (Ibrahim *et al*., 2012). *Clostridium acetobutylicum* NCIMB 13357 grown in 90% sediment POME with initial pH 5.8 produced highest total ABE (4.0 g $litre^{-1}$) (Kalil *et al*., 2003). However, the utilisation of co-substrates from palm wastes to increase ABE production has only little information. In addition, the re-utilise of wastewater after ABE production have never been reported so far. In this study, wastewater from ABE production was utilised for polyhydroxybutyrate (PHB) production. PHB, a member of the polyhydroxyalkanoate (PHA) family, produced by various microorganisms apparently in response to conditions of physiological stress. PHB are suitable for applications in several areas such as household, industrial, pharmaceutical, medical and other application. Therefore, this research aim to investigate the possibility of ABE production from various oil palm wastes including POME, EFB, PPF and PKS by *C. acetobutylicum*. The utilisation of single and co-substrates were also determined. Afterward, the ABE was recovered and the wastewater after distillation was collected and utilised as a substrate for PHB production by *Cupriavidus necator*.

MATERIALS AND METHODS

Microorganism and Inoculum Preparation

Clostridium acetobutylicum TISTR1462 and *Cupriavidus necator* TISTR1335 were purchased from the Thai Institute of Scientific and Technological Research (Thailand) and utilised for ABE and PHA production, respectively.

The stock culture of *C. acetobutylicum* was maintained in the form of a spore suspension in 25% glycerol and frozen at -20°C. The inoculum was prepared by transferring the suspension of spores (1 ml) to 10 ml of 15% potato-glucose (PG) medium [contained 150 g $litre^{-1}$ grated fresh potato, 10 g $litre^{-1}$ glucose, 0.5 g $litre^{-1}$ $(NH_4)_2SO_4$ and 3 g $litre^{-1}$ $CaCO_3$] and heat shock for 1 min in boiling water, followed by cooling in iced water and incubation under anaerobic conditions for one to two days at 30°C (Al-Shorgani *et al*., 2012). The colony morphology and gram-staining behaviour of the inoculums was checked to ensure that the culture was pure. This initial culture was then transferred to tryptone-yeast extract-acetate medium (TYA medium) and incubated for 15-18 hr and used as the inoculum. TYA medium was used for the pre-culture consisted of 20 g $litre^{-1}$ glucose, 2 g $litre^{-1}$ yeast extract, 6 g $litre^{-1}$ tryptone, 3 g $litre^{-1}$ CH_3COONH_4, 0.3 g $litre^{-1}$ $MgSO_4 \cdot 7\ H_2O$, 0.5 g $litre^{-1}$ KH_2PO_4 and 10 mg $litre^{-1}$ $FeSO_4 \cdot 7\ H_2O$. The medium was sterilised at 121°C for 15 min.

Cupriavidus necator was utilised for the production of PHA. Starter culture of *C. necator* TISTR1335 was prepared by cultivating aerobically with shaking (150 rpm) in medium consisted of (g $litre^{-1}$): 30 glycerol, 30 casein peptone, 1 KH_2PO_4, 0.01 $CaCl_2$, pH 6.8, supplemented with 5 ml of the trace elements solution. The culture was cultivated in medium at 37°C for 24 hr. Afterward aliquots were removed to determine cell growth by measurement of optical density at 660 nm.

The Preparation of Oil Palm Wastes

Oil palm wastes including POME, EFB, PPF and PKS were kindly received from the Krabi Oil Palm Farmers Cooperatives Federation Limited (Krabi,

Thailand). Each waste was pre-treated before use. POME was centrifuged. Only supernatants were collected and diluted with distilled water in the optimal ratio at 25% dilution (Sangkharak and Prasertsan, 2012). The diluted POME was kept at -4°C to prevent the microbial degradation.

EFB, PPF and PKS were cut into small piece and sun dried for two days, then stored in a plastic bag and kept at room temperature until used. Thereafter, EFB, PPF and PKS were soaked overnight in a commercial dish washing detergent before they were washed with tap water to remove oil and dust. Then, the washed materials were dried in an oven at 60°C for 24 hr (Ibrahim *et al*., 2015). Each material of 10 g was soaked again in distilled water for 4 hr followed by the steam explosion pre-treatment. The steam explosion experiments were performed in laboratory scale equipment consisting of a modified steel autoclave with a volume of 1.2 litres. All experiments were performed with saturated steam at 1.1 bar which corresponds to around 121°C in the steam explosion equipment for 60 min.

Hydrolysis of Oil Palm Wastes

Cellulase from *Aspergillus niger* (Sigma Chemical Co., USA) was utilised. The cellulase stock was diluted in 0.1 M of phosphate buffer, pH 5.5 to give an initial β-glucosidase activity of 5.0 U ml^{-1} before it was filtered through 0.45 μm of membrane filter using vacuum pump to remove the remaining debris from the cellulase solution (Ibrahim *et al*., 2015).

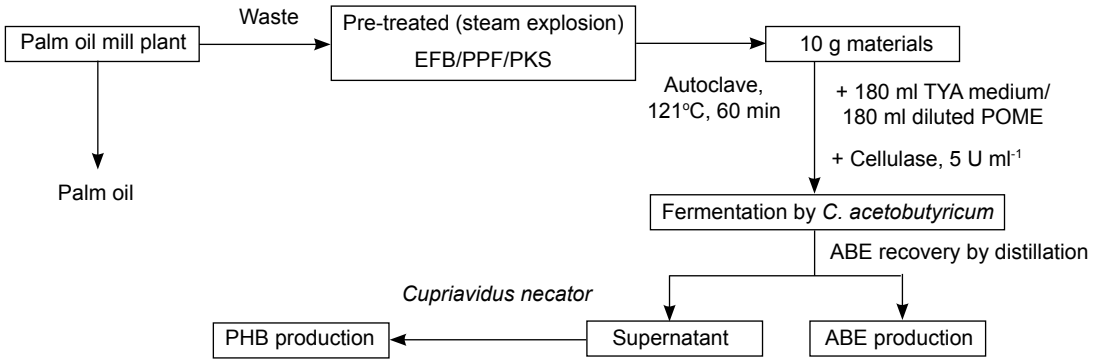
Acetone-butanol-ethanol Fermentation

Oil palm wastes were prepared and utilised for ABE fermentation as described in *Figure 1*. The ABE production was fermented in 250 ml Erlenmeyer flasks seal with cellulose caps under different medium as described in *Table 1*. The medium were prepared according to the method of Parrer *et al*. (2000) by growth of *C. acetobutylicum* TISTR1462 in a two-stage chemostat with on-line solvent removal. In addition, 5 U ml^{-1} of cellulase was added to medium (treatment number 1-7) for the saccharification of pre-treated EFB, PPF and PKS into fermentable sugar. The pH of medium was adjusted to 7.0 by an addition of 0.2 M NaOH and 0.1 M HCl prior to sterilisation (121°C for 15 min). Before inoculation, the medium was covered with nitrogen gas to maintain strict anaerobic condition. Thereafter, the process was initiated by inoculating 10 ml of prepared inoculums (with the OD_{620} set at 1.0) into the medium. The cultivation was incubated at 37°C with an agitation speed of 150 rpm for 144 hr using a shaker incubator. A 2 ml of the liquid sample from each fermentation bottle was withdrawn using a syringe and kept at -20°C prior to sample analysis.

The Production of Polyhydroxyalkanoate

After 144 hr of ABE fermentation and application of the solvent removal processes, the supernatant was recovered by centrifugation (5000 rpm, 10 min) and utilised as substrate for PHB production. The broth was sterilised (121°C, 15 min) without adjust of pH and any further preparation step.

The starter culture (5%) of *C. necator* was added into the 200 ml medium (supernatant after ABE recovery) without any supplemented nutrients and cultivated on a rotary shaker (150 rpm) at 37°C for 96 hr. Samples were taken at the first 6 hr and then every 12 hr to measure for pH, growth (OD 660 nm), dry cell weight, the concentrations of PHB.



Note: PHB - polyhydroxybutyrate.
TYA - tryptone-yeast extract-acetate.

Figure 1. Experimental design for acetone-butanol-ethanol (ABE) fermentation using pre-treated empty fruit bunch (EFB), palm pressed fibre (PPF), palm kernel shell (PKS) and palm oil mill effluent (POME) as substrate.

TABLE 1. THE PRODUCTION OF ACETONE-BUTANOL-ETHANOL UNDER DIFFERENT CULTIVATION MEDIUM AFTER 144 hr OF CULTIVATION

Conditions number	Medium	Acetone (g litre^{-1})	Butanol (g litre^{-1})	Ethanol (g litre^{-1})	ABE (g litre^{-1})	Yield (g butanol/g total sugar)	Acetic acid (g litre^{-1})	Butyric acid (g litre^{-1})	Total acids (g litre^{-1})
1	180 ml diluted POME	0.1	1.5	0.1	1.7	0.05	0.8	0.8	1.6
2	180 ml TYA medium + 10 g pre-treated EFB	0.1	3.9	0.1	4.1	0.08	0.9	1.1	2.0
3	180 ml TYA medium + 10 g pre-treated PPF	0.1	3.1	0.1	3.3	0.08	0.6	0.9	1.5
4	180 ml TYA medium + 10 g pre-treated PKS	0.1	1.5	ND	1.6	0.05	0.1	0.1	0.2
5	180 ml diluted POME + 10 g pre-treated EFB	0.1	3.5	0.2	3.8	0.10	0.8	1.2	2.0
6	180 ml diluted POME + 10 g pre-treated PPF	0.1	3.0	0.1	3.2	0.10	0.5	0.9	1.4
7	180 ml diluted POME + 10 g pre-treated PKS	0.1	1.0	ND	1.1	0.05	0.1	0.1	0.2
Negative control	180 ml TYA	0.1	1.0	ND	1.1	0.03	0.8	0.9	1.7
Positive control	180 ml TYA + 40 g litre^{-1} glucose	0.2	4.1	0.2	4.5	0.07	1.0	1.2	2.2

Note: ND - not detectable. POME - palm oil mill effluent. ABE - acetone-butanol-ethanol. TYA - tryptone-yeast extract-acetate. EFB - empty fruit bunch. PPF - palm pressed fibre. PKS - palm kernel shell.

Analytical Method

The drawn samples from ABE fermentation were centrifuged at 10 000 rpm for 5 min to separate the liquid and the cells. The yield of butanol was calculated as the butanol produced divided by the total sugar utilised. The yield of products was expressed as gram butanol/gram total sugar. The productivity of ABE was calculated as ABE concentration (g litre^{-1}) divided by fermentation time (hr). The liquid was used for the determination of pH, reducing sugar, cellulase activity, solvent concentrations (ABE) and acid concentrations (acetic acid and butyric acid) while the pellets were used for the determination of cell concentrations. Changes of pH were measured using a pH meter. The concentrations of solvents and acids were determined using gas chromatography (GC) (Shimadzu, Japan) equipped with column BP20 and thermal ionisation detector (Ibrahim *et al*., 2012). The temperature of the detector and injector were maintained at 270°C and 230°C, respectively. The cell concentration was determined based on the optical density (OD) analysis measured at 620 nm using spectrophotometer (GENESYS 20, Thermo Scientific, United States) calibrated with dry cell weight (DCW) as a standard.

The cellulose and lignin amounts from oil palm wastes were estimated by the AOAC method (AOAC, 1990). The reducing sugar amounts were estimated by dinitrosalicylic acid (DNS) method (Miller, 1959) using a glucose standard calibration curve. Cellulase activity was determined by the method of Wood and Bhat (1988). One unit (U) of cellulase is defined as the amount of enzyme releases the 1 mole of glucose equivalent per min under the assay conditions.

POME was measured for pH and analysed for biological oxygen demand (BOD), chemical oxygen demand (COD), total solids, suspended solids, oil and grease as well as total nitrogen concentration (APHA, 1988). Volatile fatty acids (VFA) were identified by GC.

The polymer from *C.necator* was extracted and subjected to analysis by GC and Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR). The functional group of polymer was characterised ATR-FTIR. All IR spectra were recorded at a 2 cm^{-1} resolution, between 4000 and 400 cm^{-1} (Oliveira *et al*., 2007).

RESULTS AND DISCUSSION

Acetone-butanol-ethanol Production from Palm Wastes

The cultivation was established under different medium as described in *Table 1*. In this study, 5 U ml^{-1} of cellulase was added to medium due to *C. acetobutylicum* was not able to produce enough cellulase activity (0.2 U ml^{-1}). The ABE production from each cultivation medium as presented in *Table 1*. The major components in the medium after enzyme hydrolysis are sugar and the organic acids. The quantity of initial sugar was range from 9.24-28.21 g $litre^{-1}$ depending on the substrate. Oil palm waste contains glucose as its dominant sugar followed by sucrose, fructose and galactose (data not shown). The glucose has been observed to be the most favourable substrate for butanol production by *C. acetobutylicum* (Khamaiseh *et al*., 2014). The highest ABE production was obtained from experiment number 2 consisting 180 ml TYA medium and 10 g $litre^{-1}$ pre-treated EFB which produced ABE up to 4.1 g $litre^{-1}$ (0.1 g $litre^{-1}$ acetone, 3.9 g $litre^{-1}$ butanol and 0.1 g $litre^{-1}$ ethanol) and 2.0 g $litre^{-1}$ of total acids (0.9 g $litre^{-1}$ acetic acid and 1.1 g $litre^{-1}$ butyric acid) correspond with yield of butanol at 0.08 g butanol/g total sugar. However, the highest yield of butanol (0.10 g butanol/g total sugar) was produced at 180 ml diluted POME supplemented with 10 g $litre^{-1}$ pre-treated EFB with a ABE value as high as 3.8 g $litre^{-1}$ (0.1 g $litre^{-1}$ acetone, 3.5 g $litre^{-1}$ butanol and 0.2 g $litre^{-1}$ ethanol) and 2.0 g $litre^{-1}$ of total acids (0.8 g $litre^{-1}$ acetic acid and 1.2 g $litre^{-1}$ butyric acid) (*Table 1*). Therefore, POME supplemented with pre-treated EFB is a good candidate for the ABE production. POME has great potential as a substrate for ABE fermentation because it contains a mixture of carbohydrates including starch, hemicellulose, sucrose and other carbohydrates that can be utilised by microorganisms (Al-shorgani *et al*., 2015). In addition, pre-treated EFB is a potential source of cellulose which can also serve as a promising raw material for the production of ABE. Highest cellulose (66.2%) was obtained from pre-treated EFB. The characterisation of EFB, PPF and PKS showed that EFB contained the highest cellulose (48.4%) followed by PPF (32.2%) while PKS had the lowest cellulose (10.4%). After steam explosion treatment (121°C, 60 min), the percentage of cellulose in EFB was significantly increased (*Table 2*). The use of the agro-based industrial wastes is one of the attractive strategies for economical ABE production. The utilisation of waste and/or waters, rich in organic pollutants, has a double benefit of reducing the pollutants while producing useful products. Another approach involves using raw substrates with negligible or no value (Makkar *et al*., 2011).

The ABE production of POME with addition of EFB was not significantly different from positive control. The positive control contain 180 ml TYA medium with an addition of 40 g $litre^{-1}$ glucose yield 0.2 g $litre^{-1}$ acetone, 4.1 g $litre^{-1}$ butanol and 0.2 g $litre^{-1}$ ethanol and 2.2 g $litre^{-1}$ total acids at 144 hr of cultivation. This happened due to the effect of improving the balance between the nutrients carbon and nitrogen. POME and pre-treated EFB were a suitable source for organic acids (acetic and butyric acid) and reducing sugar, respectively. POME used in this study was characterised. Low nitrogen content was observed in POME (850 ppm) and it was correlated with the presence of 150 ppm of ammonium-nitrogen. The BOD was estimated to be around 20,000 ppm. POME was acidic (pH 4.59). The organic acid profiles such as formic acid, acetic acid and propionic acid in POME were analysed and presented in the amount of 0.6, 0.1 and 0.07 g $litre^{-1}$, respectively. However, POME was pre-treated by centrifugation and diluted to 25%. From our preliminary studies it was found that *C. acetobutylicum* was able to grow in POME without sludge and produced nearly 0.8 g $litre^{-1}$ ABE after 72 hr of cultivation. In addition, it was also found that POME at 25% concentration gave better results (data not shown). The centrifugation of POME before cultivation was necessary due to the presence of sludge inhibited bacterial cell growth (Sangkharak and Prasertsan, 2012). The dilution of POME helps

TABLE 2. BIOCHEMICAL COMPOSITIONS OF OIL PALM WASTES BEFORE AND AFTER STEAM EXPLOSION TREATMENT

Samples	Cellulose (%)		Hemicellulose (%)		Lignin (%)	
	Before	After	Before	After	Before	After
Empty fruit bunch (EFB)	48.4	66.2	38.3	40.6	22.1	2.0
Palm pressed fibre (PPF)	32.2	50.8	23.8	38.8	17.2	10.4
Palm kernel shell (PKS)	10.4	12.5	20.8	22.3	50.7	48.1

to reduce oil and soluble toxic substances leaving less inhibitory POME which is more suitable for growth of Clostridia (Kalil *et al*., 2003).

The ABE production from 25% POME supplemented with EFB was compared to the production from diluted POME and TYA medium alone. However, the production of ABE from diluted POME and TYA medium were very low ABE (1.1-1.7 g litre^{-1}). It could be due to the unavailability of sugars for *Clostridium* to further convert acids to ABE (Ponthein and Cheirsilp, 2011). It was obvious that the ABE was mostly produced from glucose. The supplement of organic acid and glucose was found to enhance yield of butanol and the conversion of acids to ABE, respectively. While, the fermentation of diluted POME or TYA medium supplemented with pre-treated EFB, PPF and PKS yielded higher ABE. The production of ABE and butanol using different oil palm waste in this study were also compared with the results published by previous published articles (*Table 3*). In comparison to previously report, the production of butanol and ABE by *C. acetobutylicum* has shown a considerable increase using oil palm waste indicating the high ability of *C. acetobutylicum* for ABE production by consuming a variety of oil palm waste as substrate. However, low butanol yield and ABE production was obtained when compared with other lignocellulosic compounds due to the high concentration of hemicelluloses component in oil palm waste which hampered the consumption of glucose and mannose by *Clostridium* sp. (Shukor *et al*., 2014b).

TABLE 3. THE COMPARISON OF ACETONE, BUTANOL AND ETHANOL (ABE) PRODUCTION FROM VARIOUS LIGNOCELLULOSIC WASTES

Substrate	Hydrolysis method	Microorganism	Productivity (g litre^{-1} hr^{-1})	Total ABE (g litre^{-1})	Reference
Wheat straw	H_2SO_4+enzyme	*Clostridium beijerinckii* P260	0.42	25	Qureshi *et al.* (2007)
Corn fibre	H_2SO_4	*C. beijerinckii* BA101	0.10	9.3	Qureshi *et al.* (2008)
Dried distillers' grains and soluble (DDGS)	Ammonium fibre expansion+ enzyme	*C. beijerinckii* BA101	0.14	10.4	Ezeji and Blaschek (2008)
Rice bran+defatted rice bran	HCl+enzyme	*C. beijerinckii* NCIMB 8052	0.26	16.42	Lee *et al.* (2009)
Barley straw	H_2SO_4+enzyme	*C. beijerinckii* P260	0.39	26.64	Qureshi *et al.* (2010a)
Corn stover	H_2SO_4+enzyme	*C. beijerinckii* P260	0.31	26.27	Qureshi *et al.* (2010b)
Switchgrass			0.17	14.61	
Wheat bran	H_2SO_4	*C. beijerinckii* ATCC 55025	0.16	11.8	Liu *et al.* (2010)
Sugar maple wood	Hot water extraction+ H_2SO_4	*C. acetobutylicum* ATCC 824	0.15	11.0	Sun and Liu (2011)
Rice straw	H_2SO_4+enzyme	*C. acetobutylicum* MTCC 481	0.017	3.0	Ranjan and Moholkar (2011)
Cassava bagasse	Enzyme	*C. acetobutylicum* JB 200	0.62	33.87	Lu *et al.* (2012)
Maize stalk juice	NA	*C.beijerinckii* NCIMB 8052	0.30	11.5	Wang and Blaschek (2011)
Dried date fruit	No treatment	*C. acetobutylicum* NCIMB 13557	0.16	11.0	Khamaiseh *et al.* (2014)
Empty fruit bunch (EFB)	Enzyme	*C. acetobutylicum*	0.004	1.262	Noomtim and Cheirsilp (2011)
30% Palm kernel cake (PKC)	No treatment	*C. saccharoperbutylacetonicum* N1-4	0.003	0.579	Shukor *et al.* (2014a)
PKC	Acid hydrolysis	*C. saccharoperbutylacetonicum* N1-4	0.02	3.59	Shukor *et al.* (2014b)
Palm oil mill effluent (POME)	No treatment	*C. saccharoperbutylacetonicum* N1-4	0.007	2.09	Al-Shorgani *et al.* (2015)
POME+sago starch	Enzyme	*C. saccharoperbutylacetonicum* N1-4	0.10	14.38	Hipolito *et al.* (2008)
Diluted POME	Steam explosion	*C. acetobutylicum* TISTR1462	0.02	1.7	This study
EFB+TYA medium	-	-	0.05	4.1	-
Palm pressed fibre (PPF)+TYA medium	-	-	0.04	3.3	-
Palm pressed fibre (PPF)+TYA medium	-	-	0.02	1.6	-
Diluted POME+EFB	-	-	0.05	3.8	-
Diluted POME+EFB	-	-	0.04	3.2	-
Diluted POME+EFB	-	-	0.01	1.1	-

Note: ABE – acetone-butanol-ethanol. NA – not available. TYA – tryptone-yeast extract actate.

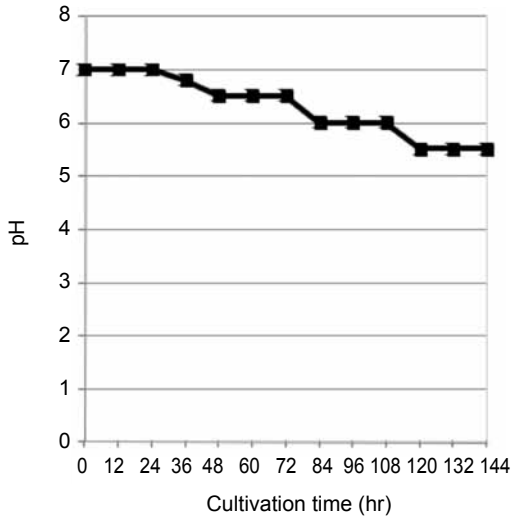
Time Course of Acetone-butanol-ethanol Production from *C. acetobutyricum* Using Diluted Palm Oil Mill Effluent Supplemented with Pre-treated Palm Oil Empty Fruit Bunch as Substrate

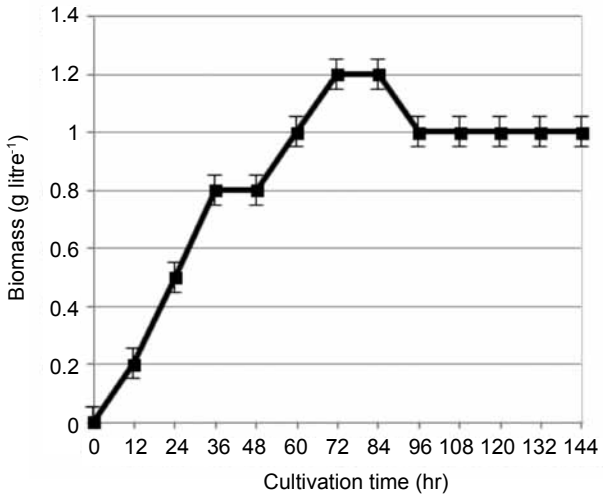
Time course on growth and ABE production by *C. acetobutyricum* in diluted POME and pre-treated EFB as carbon sources for 144 hr incubation on a shaker (60 rpm) at 37°C was obtained (*Figure 2*). During cultivation, the pH decreased to a slightly acidic pH (from 7.0 to 5.5) due to the acid production (acetic acid and butyric acid). Maximum growth was obtained after 72 hr cultivation whereas ABE production showed the same potential as cellular growth. The maximum ABE was 3.8 g litre^{-1} (0.1 g litre^{-1} acetone, 3.5 g litre^{-1} butanol and 0.2 g litre^{-1} ethanol) and 2.0 g litre^{-1} total acids (0.8 g litre^{-1} acetic acid and 1.2 g litre^{-1} butyric acid).

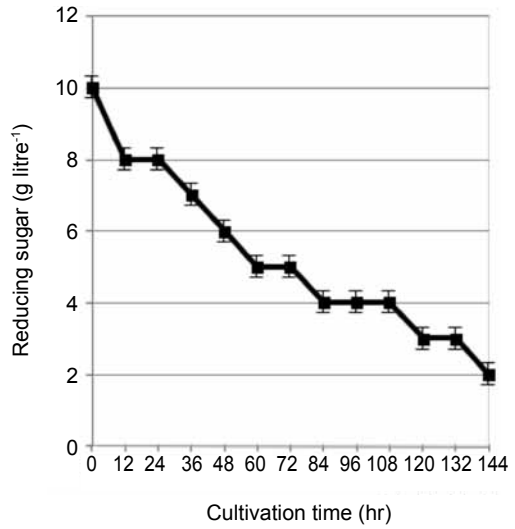
The Production of Polyhydroxyalkanoate from Wastewater after Acetone-butanol-ethanol Production

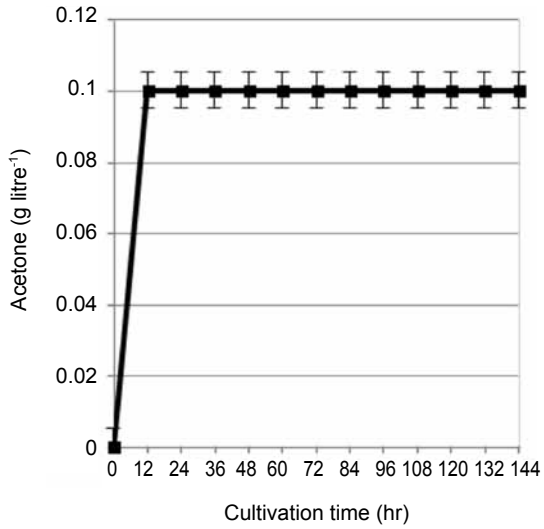
After ABE production was recovered by the distillation method, the wastewater was collected and characterised. Low nitrogen content and ammonium-nitrogen at 460 ppm and 130 ppm in supernatant were observed, respectively. The BOD, COD and oil content decreased after ABE fermentation process (*Table 4*). The pH was acidic (pH 5.5) due to the present of organic acid profiles such as acetic acid, particularly propionic and butyric acid in supernatant at the amount of 0.1-1.0 g litre^{-1}. Higher amount of organic acid detected in supernatant indicated the conversion of organic acid from nutrients in the substrate. In addition, acetic and butyric present in supernatant are possibly used for PHA production.

The production of PHB was firstly evaluated in different medium (*Table 5*). The major components in the medium are sugars and the organic acids. The quantity of sugar was range from 2.09 to 18.01 g litre^{-1} depending on the substrate. Fermentation using *C. necato*r resulted in the PHB concentration of around 0.80-3.24 g litre^{-1} with a PHB content of 31.02%-48.40% of DCW. The amount of biomass and PHB generated was strongly correlated with the initial amount of sugar. The PHB production was better in medium with higher initial sugar concentration. The highest PHB content (48.40%)









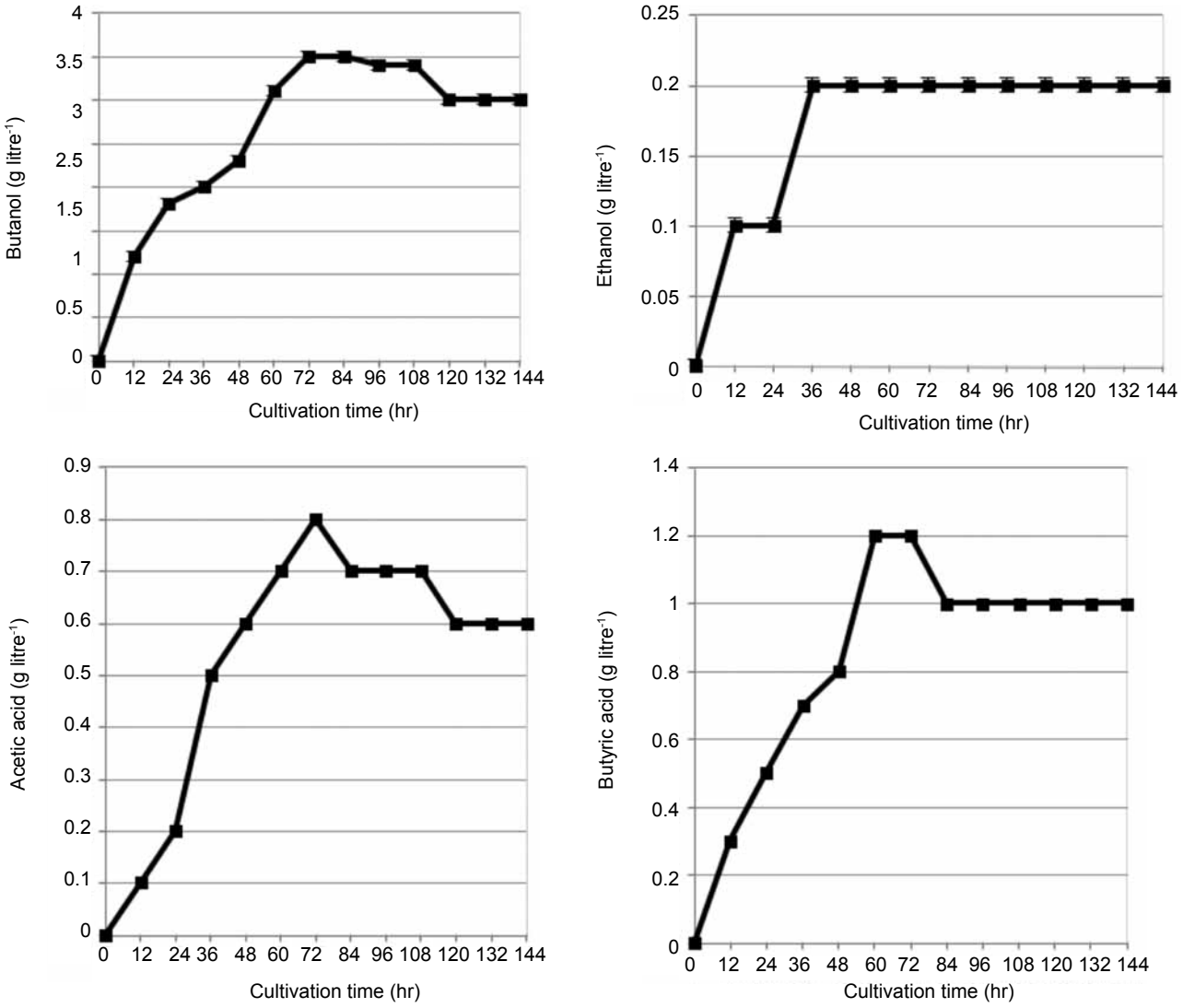


Figure 2. Time course of acetone-butanol-ethanol production from C. acetobutyricum *using diluted palm oil mill effluent supplemented with pre-treated palm oil empty fruit bunch as substrate.*

TABLE 4. CHARACTERISTICS OF PALM OIL MILL EFFLUENT (POME) BEFORE ACETONE-BUTANOL-ETHANOL (ABE) FERMENTATION AND WASTEWATER AFTER ABE RECOVERY

Compositions	Unit	POME	Wastewater after ABE recovery (%reduction)
Total nitrogen	ppm	850	460 (45.88%)
Ammonium-nitrogen	ppm	150	130 (13.33%)
Biochemical oxygen demand	ppm	20 000	15 000 (25%)
Chemical oxygen demand	ppm	80 500	75 000 (6.83%)
Total solids	mg litre^{-1}	46 060	38 800 (15.76%)
Suspended solids	mg litre^{-1}	8 354	6 514 (22.02%)
Oil	-	10 000	4 500 (55%)
pH	mg litre^{-1}	4.59	5.50
Temperature	°C	70	37
Colour	-	Brown	Brown
Acetic acid	g litre^{-1}	0.1	0.8
Propionic acid	g litre^{-1}	0.07	0.1
Butyric acid	g litre^{-1}	0	1.0

TABLE 5. THE PRODUCTION OF POLYHYDROXYBUTYRATE (PHB) UNDER DIFFERENT CULTIVATION MEDIUM AFTER ACETONE-BUTANOL-ETHANOL (ABE) RECOVERY AT 60 hr OF CULTIVATION

Conditions number	Medium after ABE recovery	Initial sugar (g litre^{-1})	Biomass (g litre^{-1})	PHB production	
				Concentration (g litre^{-1})	Content (%)
1	180 ml diluted POME	2.09±0.25	2.58±0.41	0.80±0.09	31.02
2	180 ml TYA medium + 10 g pre-treated EFB	18.01±1.01	6.72±0.52	3.24±0.14	48.25
3	180 ml TYA medium + 10 g pre-treated PPF	10.12±1.20	6.78±0.51	3.01±0.20	44.42
4	180 ml TYA medium + 10 g pre-treated PKS	3.45±0.51	6.41±0.47	2.58±0.21	40.23
5	180 ml diluted POME + 10 g pre-treated EFB	18.00±1.25	6.44±0.25	3.12±0.20	48.40
6	180 ml diluted POME + 10 g pre-treated PPF	8.45±1.24	6.24±0.50	2.75±0.19	44.10
7	180 ml diluted POME + 10 g pre-treated PKS	2.24±0.28	2.67±0.40	0.91±0.05	34.14

Note: TYA – tryptone-yeast extract- acetate. POME – palm oil mill effluent. PKS – palm kernel shell. PPF – palm pressed fibre. EFB – empty fruit bunch.

was observed in the medium with POME and pre-treated EFB. Therefore, the optimum medium was selected and scaled up into 3 litres fermentor.

The maximum PHB accumulation in *C. necato*r was reported to occur between 48-72 hr of incubation (Sangkharak and Prasertsan, 2011). Hence, *C. necator* cells grown in supernatant were harvested between 0-96 hr incubation. During cultivation, the pH increased to a slightly alkali pH (from 5.5 to 8.02) due to the depletion of carbon source and the generation of ammonia from nitrogen source consumption. Maximum growth was obtained after 60 hr cultivation whereas PHB concentration and PHB content showed the same potential as cellular growth. The maximum PHB in the cells was 48.4% of DCW and gave the PHB concentration of about 3.12±0.24 g litre^{-1} (*Figure 3*). Wastewater from ABE fermentation by *C. beijerinckii* NRRL B592 has been determined for PHA production by Parrer *et al*. (2000). After evaporation process, 7 g litre^{-1} of butyrate and 5 g litre^{-1} of acetate at pH 7.5 was added to the medium. The bacterium identified as a representative of the genus Alcaligenes (designated as *Alcaligenes* sp. G) was capable of growth up to optical densities

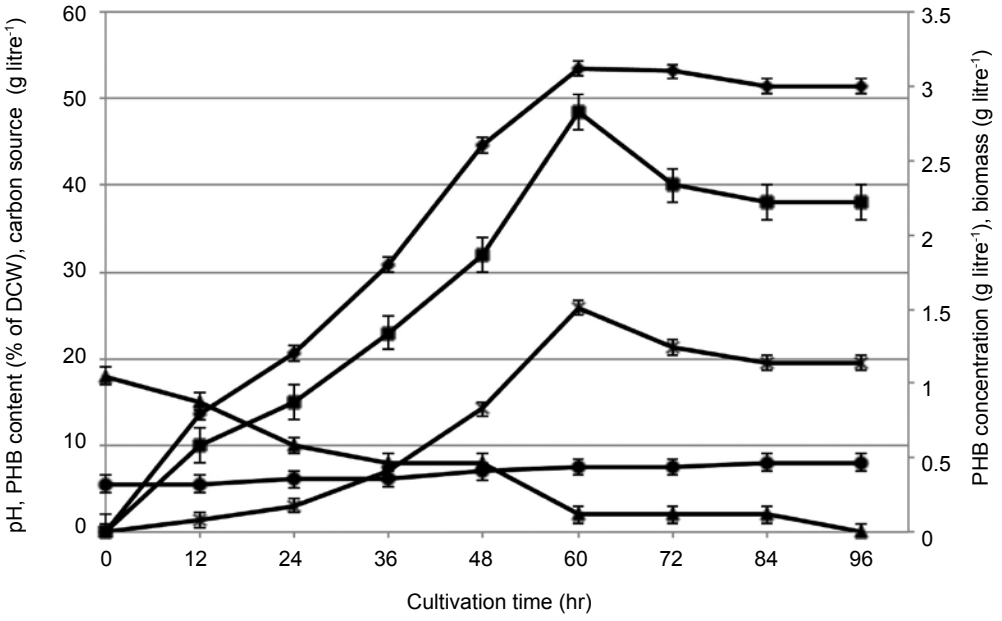


*Figure 3. Time course of pH (●), biomass (g litre^{-1}, ♦), polyhydroxybutyrate (PHB) concentration (g litre^{-1}, *), PHB content [% of dry cell weight (DCW), ■] and carbon source (g litre^{-1} , ▲) from wastewater after acetone-butanol-ethanol (ABE) production.*

ranging from 8 to 20 and simultaneously of PHA-accumulation up to 40% of DCW. In comparison to previously report, the production of PHA by *C. necator* has shown a considerable increase using ABE wastewater indicating the high ability of *C. necator* for PHA production by consuming ABE wastewater as substrate.

To specifically determine the composition of the isolated polymer, the freeze dried cell material and the commercial PHB were subjected to esterification. The propyl esters formed were analysed by GC and benzoic acid propyl ester was used as the internal standard. The peak corresponding to propyl ester of 3-hydroxybutyric acid was observed in the gas chromatogram (retention time 8.5 min) for PHB from *C. necator*. This indicated that the polymer accumulated by *C. necator* was PHB. The structure of PHB was confirmed by ATR-FTIR compared with commercial PHB. The ATR-FTIR spectra for PHB samples extracted from *C. necator* after 60 hr of cultivation was compared with the spectrum obtained from commercial PHB (*Figure 4*). The FTIR spectra obtained for extracted PHB presented almost identical peak positioning when compared to the spectrum obtained from commercial PHB. The most prominent marker band for the identification of PHB is the ester carbonyl band at 1700-1725 cm^{-1}. The bands present at 1178 cm^{-1}, 1228 cm^{-1} and 1263 cm^{-1} are bands sensitive to crystallinity and are characteristic of C-O-C. The spectra were found to be identical, which confirms the extracted polymer as PHB.

CONCLUSION

The maximum ABE was 3.8 g $litre^{-1}$ and 2.0 g $litre^{-1}$ total acids was obtained from the cultivation of *C. acetobutyricum* under 180 ml of diluted POME with 10 g $litre^{-1}$ of pre-treated. The supernatant after ABE removal was collected for PHB production. The maximum PHB in the cells was 48.4% of DCW and gave the PHB concentration of about 3.12±0.24 g $litre^{-1}$ after 60 hr of cultivation. During processing in the palm oil mill more than 70% (by weight) of the processed biomass were left over as oil palm waste. Therefore, utilisation of palm wastes for ABE and PHB fermentation as described in this study showed the great possibility to reduce wastes from palm oil process. However, the development of simply process using less chemical and technology will be the next experiment to make this method appears to be a realistic goal for the future.

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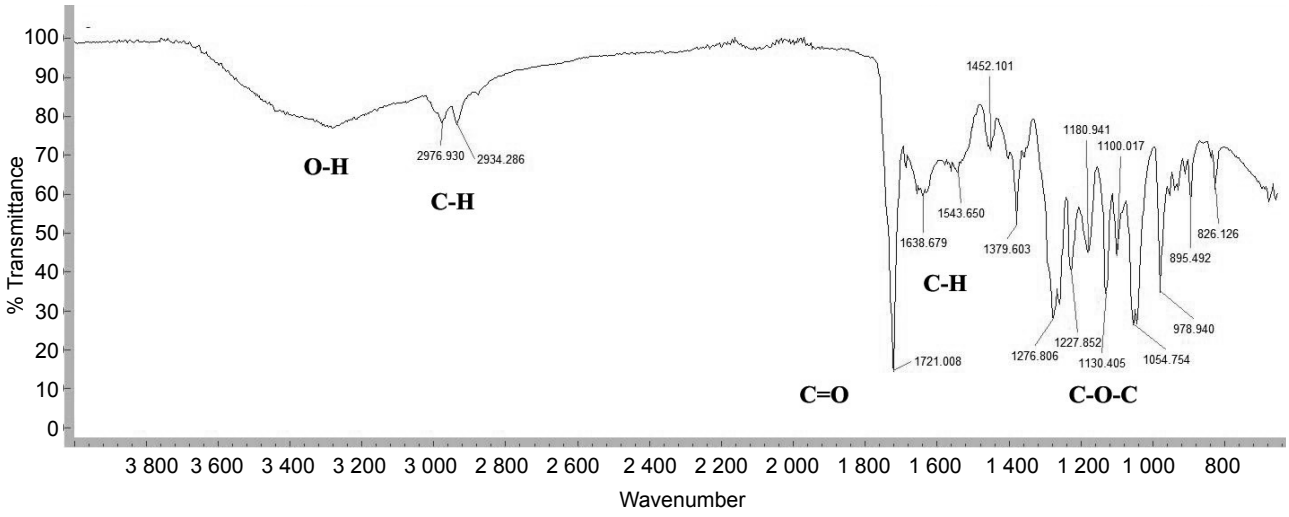

Figure 4. Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) spectra of polyhydroxybutyrate (PHB) extracted from C. necator *after 96 hr of cultivation.*

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