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Waste cooking oil as substrate for biosynthesis of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate): Turning waste into a value-added product

Hanisah Kamilah[1,3], Takeharu Tsuge[2], Tajul Aris Yang[3] and Kumar Sudesh[1*]

[1]Ecobiomaterial Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia
[2]Tsuge laboratory, Department of Innovative and Engineered Materials, Tokyo Institute of Technology, J2-47, 4259 Nagatsuta, Midori-ku, Yokohama 226-8502, Japan
[3]Food Technology Division, School of Industrial Technology, Universiti Sains Malaysia, Pulau Pinang, 11800, Penang, Malaysia
E.mail: ksudesh@usm.my

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ABSTRACT

Aims: Improper disposal of domestic wastes, such as waste cooking oil (WCO), contributes to the deterioration of the environment and may lead to health problems. In this study, we evaluated the potential of plant-based WCO as a carbon source for the commercial biosynthesis of the bio-plastics, poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate). The consumption of WCO for this purpose would mitigate their pollution of the environment at the same time.
Methodology and Results: WCO collected from several cafeterias in USM was tested as the carbon source for polyhydroxyalkanoates (PHA) production. A selection of suitable nitrogen source was first conducted in order to obtain an acceptable number of dry cell weight (DCW) and PHA content. Urea was found to be a suitable nitrogen source for the two bacterial strains used in our study, *Cupriavidus necator* H16 and its transformed mutant, *C. necator* PHB^{-}4 harboring the PHA synthase gene of *Aeromonas caviae* (PHB^{-}4/pBBREE32d13). With WCO as the sole carbon source, *C. necator* H16 yielded a relatively good dry cell weight (DCW=25.4 g/L), with 71 wt% poly(3-hydroxybutyrate) P(3HB) content. In comparison, the DCW obtained with fresh cooking oil (FCO) was 24.8 g/L. The production of poly(3 hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] from WCO by the transformant *C. necator* PHB 4 was comparable, yielding a DCW of 22.3 g/L and P(3HB-*co*-3HHx) content of 85 wt%. Lipase activities for both bacterial strains reached a maximum after 72 h of cultivation when time profile was conducted.
Conclusion, significance and impact of study: The use of WCO as a carbon source in the biosynthesis of the bio-plastic, PHA, turns a polluting domestic waste into a value-added biodegradable product. This renewable source material can thus be exploited for the low cost production of PHA.

Keywords: Waste cooking oil; Poly(3-hydroxybutyrate); Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate), *Cupriavidus necator* H16, *Cupriavidus necator* PHB^{-}4

INTRODUCTION

The increasing awareness of environmental hazards arising from plastic wastes, especially those originating from non-renewable natural resources, presents new opportunities for the development of bio-based and biodegradable polymer materials (Nikel *et al.*, 2006). Among the readily biodegradable, non-toxic and environmentally friendly materials, polyhydroxyalkanoates (PHA) have received considerable attention in recent years (Loo and Sudesh, 2007, Yunus *et al.*, 2008). Current mass production of PHA requires a cost-consuming carbon source such as sugars or plant oils. Vegetable oils such as soybean oil and palm oil are widely used as attractive carbon sources of PHA (Kahar *et al.*, 2004, Loo *et al.*, 2005). Loo and co-workers (2005) found that palm kernel oil (PKO) is an excellent carbon source for PHA production, with poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] content of 87 wt%. However, PKO is more expensive compared to other palm oil products.

In this regard, therefore, domestic waste as a cheaper carbon source is attractive for the mass production of sustainable biopolymers (Cavalheiro *et al.*, 2009). It was revealed that the utilization of agriculture wastes such as whey, wastewater from olive mills,

***Corresponding author**

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molasses, corn steep liquor, starchy wastewater and palm oil mill effluent reduces the overall production cost of PHA to as much as 40-50% (Akaraonye *et al.*, 2010). For example, Wong and Lee (1998) obtained a dry cell weight (DCW) and poly(3-hydroxbutyrate) [P(3HB)] concentration of 109.0 g/L and 50.0 g/L, respectively, from whey powder by using a recombinant *E. coli* strain GCSC 6576 harbouring a high-copy-number plasmid containing *C. necator* genes. Alternatively, Haas and co-workers (2008) found that saccharified waste potato starch can be used as an alternative carbon source as P(3HB) production, producing a total biomass of 179.0 g/L and 94.0 g/L P(3HB) concentration from a fed-batch fermentation of *Ralstonia eutropha* NCIM 5149.

Another common domestic waste with great potential as a renewable carbon resource for PHA biosynthesis is waste cooking oil (WCO). In Malaysia, 50,000 tonnes of WCO generated annually is disposed of without proper pre-treatment (Loh *et al.*, 2006). Attempts have been made to commercialize WCO as a component in animal feedstock. However, this product has been banned by the European Union (EU) since 2002 owing to the presence of harmful compounds formed in the oil during the frying process. Such compounds that could be carried over to the meat of animals can potentially enter the human food chain (Kulkarni and Dalai, 2006). This disadvantage does not arise in the use of WCO for PHA production. Its wide adoption would in fact reduce improper disposal of WCO and consequently help to maintain human health and preserve the environment at the same time (Akaraonye *et al.*, 2010).

Song and colleagues reported the utilization of WCO from corn oil to produce medium chain length PHA (*mcl*-PHA) by *Pseudomonas* sp. strain DR2. *Pseudomonas* sp. strain DR2 is capable of accumulating 37.3% *mcl*-PHA after 72 h cultivation. The *mcl*-PHA produced by *Pseudomonas* sp. strain DR2 is gluey and rubbery (Song *et al.*, 2008). The ability of various *Pseudomonas* sp. to convert oil and fats into *mcl*-PHA have been tested by Rao *et al.* (2010). Results show that the productivity of PHA production is relatively low wherein the PHA content is less than 30 wt%.

In another study, a DCW of 5.0 g/L and PHA content of 81 wt% poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] was achieved after 144 h cultivation using *Cupriavidus necator* fed with WCO (palm oil) and 1,4-butanediol. *C. necator* is an efficient microbe for PHA production, whereby the P(3HB-*co*-4HB) yield was 0.75-0.8 g/g of WCO which higher compared to glucose, 0.32-0.48 g/g of glucose. It is also capable of accumulating P(3HB) homopolymer at high levels exceeding 80 wt% of DCW (Tsuge *et al.*, 2004, Rao *et al.*, 2010). P(3HB) is the general type of PHA which has unfavourable properties such as high crystallinity and poor tensile strength (Sudesh *et al.*, 2000). The decreasing of molecular weight that caused by rapid thermal degradation have reduced the value of the homopolymer as it is near to its melting temperature (Tsuge *et al.*, 2004). By the incorporation of 3-hydroxyhexanoate (3HHx) as the second monomer results a better physical properties of copolymer and there is much interest to produce P(3HB-*co*-3HHx) because of its attractive characteristics (Doi *et al.*, 1995, Bhubalan *et al.*, 2010).

Therefore, in this study we used *C. necator* H16 and transformant *C. necator* PHB^{-}4 harboring the PHA synthase gene of *A. caviae* (PHB^{-}4/pBBREE32d13) (Tsuge *et al.*, 2004) to synthesize P(3HB) and P(3HB-*co*-3HHx) due to the efficiency in utilizing WCO as the sole carbon source. Our objective was to evaluate the potential of palm oil-based WCO as a carbon source for the commercial biosynthesis of these bio-plastics.

MATERIALS AND METHODS

Bacterial strains and maintenance

Two bacterial strains, *C. necator* H16 and *C. necator* PHB^{-}4 harboring the PHA synthase gene of *Aeromonas caviae* (PHB^{-}4/pBBREE32d13) were used in this study. The *C. necator* H16 strain was grown in a nutrient-rich (NR) medium containing (per liter): 2 g yeast extract, 10 g meat extract and 10 g peptone while the transformant *C. necator* PHB^{-}4 (Tsuge, 2002) was grown in the same medium supplemented with 50.0 mg/L kanamycin (to maintain selection pressure to retain the plasmid of the PHB^{-}4 strain). For cell maintenance, *C. necator* H16 was grown on mineral salt medium (MM) agar containing (per liter): 0.5 g NH_4Cl, 2.8 g KH_2PO_4, 3.32 g Na_2HPO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 14 g agar, 5 g fructose and 1 mL of trace elements. The trace element composition was according to Kahar's formulation (Kahar *et al.*, 2004). The transformant *C. necator* PHB^{-}4 was maintained on MM agar containing (per liter) the above formulation with additional 50.0 mg/L kanamycin. During the preparation of both NR and MM media, in liquid culture or for agar plates, the initial pH was adjusted to 7.

Carbon sources

Palm oil-based WCO was obtained from several cafeterias in Universiti Sains Malaysia (USM Penang, Malaysia). Commercial grade fresh cooking palm oil (FCO) (Brand: Vesawit) was purchased from local market. Both WCO and FCO were sterilized separately at 121 ºC for 15 min prior to their use for the biosynthesis of PHA.

One-stage cultivation of P(3HB) and P(3HB-*co*-3HHx)

PHA biosynthesis was carried out in 250 mL conical flasks at 30 ºC. In the early stage of inoculum preparation, two loops of fully-grown culture from cell maintenance (MM agar plates) were transferred into 50 mL NR medium and incubated on a rotary shaker (Lab Companion, SK-71) with agitation speed of 200 revolutions per minute (rpm). *C. necator* H16 and *C. necator* PHB^{-}4 was transferred for further step of inoculation after approximately 7 h when the optical density (OD) at 600 nm (JENWAY 6505 UV/Vis Spectrophotometer, England) reached 4 to 4.5. At this stage, 3% (v/v) of this inoculum was inoculated into 50 mL

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MM medium. For every 50 mL MM medium of transformant strain, 50.0 mg/L kanamycin was added.

The P(3HB) biosynthesis study started with the screening of various nitrogen sources, followed by the evaluation of carbon sources (WCO or FCO). In the initial stage of nitrogen screening, the carbon source concentration was fixed at 20.0 g/L. Once the highest DCW and PHA accumulations were obtained, nitrogen content in the media was held constant while concentrations of the carbon source were varied. After incubation for 72 h at 200 rpm, the bacterial cells were harvested by centrifugation at 15,816 *g* at 4 ºC for 10 min. The cell pellets were washed with 5 mL hexane, followed by removal of the hexane with 50 mL distilled water. The mixture was re-centrifuged and the cell pellets that were recovered were then frozen at –20 ºC for 24 h prior to lyophilization. The cell-free supernatant from the initial centrifugation was used for residual oil analysis where 2 mL of the supernatant was added to 5 mL hexane. The mixture was vortexed vigorously and allowed to stand for 1 min for phase separation. Approximately 1 mL hexane was transferred onto a pre-weighed plastic plate in a fume hood for the hexane to evaporate. The weight of the residual oil after hexane evaporation was then determined.

Lipase assay

Lipase activity was measured spectrophotometrically according to a slight modification of (Kilcawley *et al.*, 2002), using *p*-nitrophenyl laurate (*p*NPL) as the substrate. A sample of 5 mM of *p*NPL was added to 10 mL of dimethyl sulphoxide (DMSO) and emulsified in 90 mL of phosphate buffer (100 mM, pH 7) containing 0.1% (w/v) polyvinyl alcohol (PVA) and 0.4% (w/v) Triton X-100. A 100 µL sample of cell-free supernatant was reacted with 1.9 mL emulsion solution and incubated at 37 ºC for 20 min before the OD measurement was taken at 410 nm using a spectrophotometer (JENWAY 6505 UV/Vis. Spectrophotometer, England). A 2 mL of emulsion solution was used as the blank.

Analytical procedure

Gas chromatography (GC) was employed to determine the intracellular PHA content and monomer composition of the biosynthesized PHA. Approximately 15 mg of lyophilized cells were subjected to methanolysis, whereby the lyophilized cells were heated for 140 min at 100 ºC in the presence of a mixture of chloroform and methanolysis (1:1) solution (Braunegg *et al.*, 1978). Methanolysis solution was prepared by mixing methanol and concentrated sulphuric acid in a ratio of 85:15 (v/v). 85 mL of methanol was initially added into a beaker and 15 mL of concentrated sulphuric acid was added drop wise into methanol solution. The mixture was stirred slowly and cooled to room temperature prior to usage and storage at 4 °C in a refrigerator. After methanolysis, the mixture was allowed to cool to room temperature before adding 2 mL distilled water and vortexed vigorously. The organic fraction (the bottom layer) was transferred into a container containing a small amount of anhydrous sodium sulfate (Na_2SO_4) to absorb moisture. An exact amount of 0.5 mL organic solution was then transferred into GC vials which contain 0.5 mL of caprylic methyl ester (CME) solution. CME was used as the internal standard for all GC analyses [Shimadzu GC-2010 (Japan)]. The injection volume of converted hydroxyacyl methyl ester samples solutions was 50 µL, with injector temperature of 270 ºC.

RESULTS AND DISCUSSION

Two potential industrial PHA producing bacteria, *C. necator* H16 and its transformant, *C. necator* PHB^{-}4 harboring PHA synthase gene from *A. caviae*, were used to evaluate the sustainability of two carbon sources, WCO and FCO, respectively. *C. necator* H16 is responsible for the production of P(3HB), while the transformant *C. necator* PHB^{-}4 is responsible for the production of P(3HB-*co*-3HHx). WCO is a substrate that is not only easily available but is also abundant and cheap compared with FCO (Akaraonye *et al.*, 2010). Both WCO and FCO in this study were derived from plant oils, and were used to observe the proficiency and competency of bacterial growth and PHA accumulation. The high carbon content in plant oils makes them a suitable feedstock for PHA production (Taguchi *et al.*, 2003). Besides, fatty acids of plant oils produce a higher theoretical yield coefficient of PHA, *viz.* 0.65-0.98 g-PHA/g-oil compared with sugars like glucose, which gives yield coefficients of 0.32-0.48 g-PHA/g-glucose (Yamane, 1992, Tsuge, 2002).

Effects of different nitrogen sources on P(3HB) and P(3HB-*co*-3HHx) biosynthesis

Prior to the evaluation of carbon sources, the various sources of nitrogen for *C. necator* culture were compared to identify appropriate nitrogen sources that enhanced the growth and PHA content of the bacterial cells. Table 1 shows the effect of various nitrogen sources on the biosynthesis of P(3HB) and P(3HB-*co*-3HHx) using 20.0 g/L of WCO as the sole carbon source. Among the six nitrogen sources tested, urea and ammonium chloride showed the highest accumulation and gross yield of PHA, with no residual oil after 72 h cultivation. Utilization of urea as the nitrogen source resulted in a lower PHA accumulation in both *C. necator* H16 (64 wt%) and its transformant *C. necator* PHB^{-}4 (61 wt%), as compared with the use of ammonium chloride as the nitrogen source. With ammonium chloride, P(3HB) and P(3HB-*co*-3HHx) accumulations were respectively 83 wt% and 79 wt% with the two strains of bacteria. This implies that ammonium chloride is preferred for high accumulation of PHA. Although high accumulation of PHA is the target of many biotechnologists, from the economical point of view

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Table 1: Effect of different nitrogen sources on P(3HB) and P(3HB-*co*-3HHx) biosynthesis, using WCO as the sole carbon source[1]

Nitrogen source 5 mM	Dry cell weight (g/L)	PHA content (wt%)[2]	PHA composition (mol %)		PHA yield (g/L)	Residual oil (g/L)
			3HB	3HHx		
***C. necator* H16**						
$(NH_4)_2SO_4$	2.5±0.1^{a}	52±3^{ab}	100	-	1.3±0.1^{a}	0.4
$NH_4H_2PO_4$	4.1±0.2^{bc}	52±3^{b}	100	-	2.1±0.3^{b}	0.2
$NaNO_3$	3.8±0.2^{b}	51±5^{bc}	100	-	1.9±0.1^{b}	0.3
NH_4NO_3	4.1±0.1^{c}	47±3^{a}	100	-	1.9±0.1^{b}	0.3
NH_4Cl	4.1±0.1^{bc}	83±2^{d}	100	-	3.4±0.1^{c}	0
$(NH_2)_2CO$	6.1±0.5^{d}	64±2^{c}	100	-	3.9±0.4^{d}	0
***C. necator* PHB^{-}4/pBBREE32d13**						
$(NH_4)_2SO_4$	2.4±0.1^{a}	51±2^{bc}	98	2	1.2±0.1^{a}	0.3
$NH_4H_2PO_4$	3.9±0.1^{c}	54±3^{c}	97	3	2.1±0.1^{b}	0.2
$NaNO_3$	3.6±0.1^{b}	49±3^{ab}	97	3	1.8±0.1^{b}	0.3
NH_4NO_3	4.0±0.1^{c}	46±4^{a}	96	4	1.8±0.2^{b}	0.4
NH_4Cl	4.0±0.1^{c}	79±2^{e}	97	3	3.2±0.1^{c}	0
$(NH_2)_2CO$	5.3±0.1^{d}	61±1^{d}	97	3	3.2±0.1^{d}	0

[1]The carbon source was 20.0 g/L WCO. Samples were incubated for 72 h, at 30 °C, initial pH 7.0 and shaken at 200 rpm.
[2]Polymer content in lyophilized cells.
3HB: 3-hydroxybutyrate; 3HHx: 3-hydroxyhexanoate; $(NH_4)_2SO_4$: Ammonium sulphate; $NH_4H_2PO_4$: Ammonium dihydrogen phosphate; $NaNO_3$: Sodium nitrate; NH_4NO_3: Ammonium nitrate; NH_4Cl: Ammonium chloride; $(NH_2)_2CO$: Urea. Means in the same column with different superscript are significantly different (Duncan test, $p < 0.05$)

urea is a cheap nitrogen source (RM 26.20/kg) compared with other nitrogen source such as ammonium nitrate (RM 57/kg), ammonium chloride (RM 43.70/kg) and ammonium sulphate (RM 29.50/kg). This makes the price factor essential for commercial production of cost-competitive bioplastics (Ng *et al.*, 2010). When urea was used as nitrogen source, the PHA yield was 3.9 g/L and 3.2 g/L in wild type and transformant strain, respectively.

It is also worth noting that the P(3HB-*co*-3HHx) obtained in our study has similar molar fraction of 3-hydroxyhexanoate units to that from the biosynthesis of P(3HB-*co*-3HHx) of the same transformant strain when fed with crude palm oil (Loo *et al.,* 2005). When FCO was used as the sole carbon source, it gave a similar result with the same experimental treatment (results not shown). Whilst FCO was the carbon source, the wild type strain yielded a DCW of 5.3 g/L and relatively good P(3HB) content of 65 wt% while the transformant had a P(3HB-*co*-3HHx) content of 64 wt% and a DCW of 6.0 g/L (Table 2). The yield and accumulation of P(3HB) and P(3HB-*co*-3HHx) were similar in WCO and FCO with urea used as the source of nitrogen (Results not shown). Thus, we proceeded with the selection of urea as a nitrogen source suitable for biosynthesis of P(3HB) and P(3HB-*co*-3HHx).

Effects of different concentrations of cooking oils (WCO and FCO) on P(3HB) and P(3HB-*co*-3HHx) biosynthesis

The study of WCO and FCO concentrations is summarized in Table 2. The utilization of WCO by the wild type yielded slightly higher P(3HB) as compared with FCO. The nature of the substrate used will affect the final composition of the polymer because the substrate does not only determine the PHA content but also the PHA composition (Satoh *et al.*, 1998). In the production of P(3HB) by wild type bacteria, 10.7 g/L P(3HB) was obtained with 15.0 g/L WCO while 7.2 g/L P(3HB) was obtained when the same concentration of FCO was the carbon source. Wild type H16 gave the highest P(3HB) accumulation of 72 wt% at 15.0 g/L WCO concentration. With increasing oil concentrations, the amount of acetyl-CoA generated also increases. The acetyl-CoA is directed to tricarboxylic acid cycle (TCA) or PHA biosynthesis pathway depending on the culture condition. If there is a nutrient limitation, the acetyl-CoA is directed into PHA biosynthesis pathway (Potter and Steinbuchel, 2006).

The rising trend of DCW with cultivation time reached a plateau beyond 10.0 g/L of WCO and FCO concentrations. Further addition of WCO and FCO slightly improve the DCW. Within the range of oil concentrations for both WCO and FCO (up to 20.0 g/L), the bacteria is capable of converting the oil into P(3HB). However, above certain limit of concentrations, there are residual oils. As the result, residual oil increased with the rise in cooking oil concentration used as the substrate (Table 2). Just as the production of P(3HB) from WCO and FCO gave comparable results in the wild type *C. necator,* the production of P(3HB-*co*-3HHx) from WCO and FCO by transformant *C. necator* PHB^{-}4 also showed a similar trend. A study conducted by Fukui and Doi (1997) revealed that the transformant *C. necator* PHB^{-}4/pJRDEE32d13 harboring the PHA synthase gene from *A. caviae* was able to produce high cellular P(3HB-*co*-3HHx) content of about 80 wt% using plant oil as carbon source in shake flask experiment. Our study showed a comparable high P(3HB-*co*-3HHx) content (73 wt% using WCO by the same transformant strain). The molar fraction of 3-hexanoate at 2.5 g/L WCO feeding was 6%. Above this level, the molar fraction was reduced to 2-3%. Changes in molar fraction of 3HHx are bound to a channelling pathway of β-oxidation. Acyl-CoA from oils is degraded through cyclic β-oxidation and form enoyl-CoA intermediates of different chain length. These intermediates are then converted to (*R*)-3HA-CoA by (*R*)-specific enoyl-CoA and form (*R*)-3HA-CoA of C4 and C6. These formation will be incorporated with growing polyester chain by the assistance of PHA synthase and as the resultant, P(3HB-*co*-3HHx) is formed (Fukui and Doi, 1997). Based on the highest overall yield obtained by both bacterial strains and with both WCO and FCO, 20.0 g/L oil concentration was used for further analysis.

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Table 2: Effect of different concentrations of WCO and FCO on P(3HB) and P(3HB-*co*-3HHx) biosynthesis

	Waste Cooking Oil (WCO)						Fresh Cooking Oil (FCO)					
Oil concentration (g/L)	Dry cell weight (g/L)	PHA content (wt%)[2]	PHA composition (mol%) 3HB	PHA composition (mol%) 3HHx	PHA yield (g/L)	Residual oil (g/L)	Dry cell weight (g/L)	PHA content (wt%)[2]	PHA composition (mol%) 3HB	PHA composition (mol%) 3HHx	PHA yield (g/L)	Residual oil (g/L)
***C. necator* H16**												
2.5	2.8±0.2^{a}	19±4^{a}	100	-	0.5±0.1^{a}	0	4.2±0.3^{a}	13±3^{a}	100	-	0.6±0.1^{a}	0
5.0	6.1±0.5^{ab}	64±2^{c}	100	-	4.1±0.4^{b}	0	5.3±0.3^{b}	65±2^{b}	100	-	3.4±0.4^{b}	0
7.5	8.8±0.3^{abc}	63±1^{b}	100	-	5.6±0.1^{c}	0	8.9±0.1^{c}	62±2^{c}	100	-	5.6±0.1^{c}	0
10.0	11.3±0.3^{bc}	65±1^{bc}	100	-	7.3±0.2^{d}	0	10.6±0.8^{d}	63±2^{bc}	100	-	6.8±0.2^{de}	0
12.5	13.0±0.1^{bcd}	65±1^{bc}	100	-	8.4±0.1^{e}	1.5	11.2±1.0^{e}	66±3^{e}	100	-	7.5±0.1^{e}	1.5
15.0	15.0±0.7^{cd}	72±2^{d}	100	-	10.7±0.5^{g}	1.9	10.9±0.1^{de}	66±2^{cd}	100	-	7.2±0.5^{de}	1.9
17.5	13.7±0.3^{bcd}	63±1^{b}	100	-	8.6±0.2^{e}	3.2	10.6±0.1^{d}	64±3de	100	-	6.7±0.2^{d}	3.2
20.0	13.2±0.2^{d}	72±1^{d}	100	-	9.5±0.3^{f}	4.9	13.8±0.6^{f}	66±3^{e}	100	-	9.0±0.3^{f}	4.9
***C. necator* PHB 4/pBBREE32d13**												
2.5	2.5±0.2^{a}	21±2^{a}	94	6	0.5±0.1^{a}	0	2.5±0.1^{a}	28±5^{a}	94	6	0.7±0.1^{a}	0
5.0	5.3±0.1^{b}	61±1^{b}	97	3	3.3±0.1^{b}	0	6.0±0.5^{b}	64±2^{b}	97	3	3.9±0.4^{b}	0
7.5	9.1±0.6^{c}	65±4^{bcd}	98	2	5.9±0.5^{c}	0	7.8±0.5^{c}	67±5^{b}	98	2	7.8±0.5^{c}	0
10.0	11.5±0.2^{d}	68±2^{d}	97	3	7.7±0.4^{e}	1.1	11.2±0.2^{e}	76±4^{c}	98	2	8.3±0.1^{f}	1.7
12.5	12.6±0.4^{e}	62±1^{bc}	97	3	7.7±0.4^{e}	1.6	11.3±1.0^{f}	67±3^{b}	98	2	7.5±0.7^{e}	2.2
15.0	11.4±0.1^{d}	63±3^{cd}	97	3	7.1±0.3^{d}	3.2	10.1±0.3^{d}	67±1^{b}	97	3	6.7±0.3^{d}	3.4
17.5	11.5±0.5^{d}	63±1^{bc}	97	3	7.2±0.4^{de}	3.7	10.3±0.3^{d}	62±1^{b}	98	2	6.4±0.3^{d}	4.1
20.0	11.6±0.1^{d}	73±2^{e}	97	3	8.3±0.2^{f}	4.8	12.8±0.3^{g}	66±1^{b}	98	2	8.4±0.3^{f}	5.3

[1] Urea concentration was held at 0.54 g/L while the carbon source was FCO in varying concentrations. Samples were incubated for 72 h, at 30 °C, initial pH 7.0, and shaken at 200 rpm.
[2] Polymer content in lyophilized cells.
3HB: 3-hydroxybutyrate; 3HHx: 3-hydroxyhexanoate. Means in the same column with different superscripts are significantly different (Duncan test, $p < 0.05$).

Effects of different concentrations of urea on P(3HB) and P(3HB-*co*-3HHx) biosynthesis

The influence of urea concentration on the DCW and PHA yield is shown in Figure 1. The highest DCW and PHA content obtained were 25.4 g/L and 71 wt% respectively when 1.08 g/L urea and 20.0 g/L WCO were fed to wild type *C. necator* H16. The total PHA yield produced by this strain was 18.0 g/L. Similar DCW (24.8 g/L) and PHA contents (72 wt%) were observed when FCO was used as the carbon source to *C. necator* H16. When transformant *C. necator* PHB^{-}4 was fed with the same concentration of WCO, the DCW and PHA content were 21.9 g/L and 85.5 wt% respectively (Figure 2). As the concentration of the urea was increased, the accumulation of PHA in the bacterial cells dropped drastically. This is because there is no more nitrogen limitation to enhance the polymer accumulation (Lee *et al.*, 1999). The carbon sources are mainly used for the cell growth because the acetyl-CoA produced from the metabolisms of the free fatty acids *via* β-oxidation pathway flows into the TCA cycle. Besides that, the increase flux of acetyl-CoA into the TCA cycle will enhance the pool of free coenzyme A (CoASH) which will inhibit the 3-ketothiolase that is the first enzyme involved in PHA biosynthesis (Mansfield *et al.*, 1995). Thus the PHA accumulated decreased when high concentration of urea used.

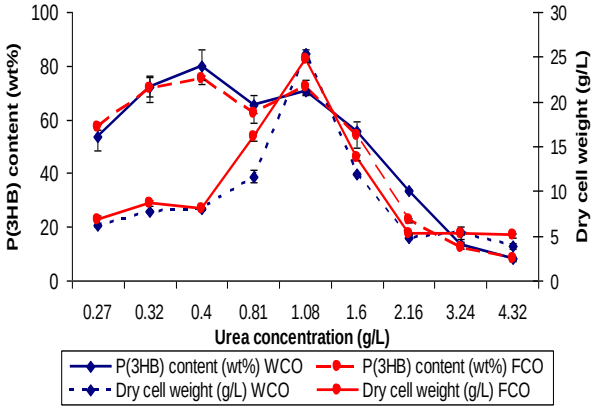

Figure 1: Effect of different concentrations of urea on P(3HB) biosynthesis by *C. necator* H16, using WCO or FCO as the sole carbon sources.
Oil concentration was 20.0 g/L. Urea, in varying concentrations, was the nitrogen source. Samples were incubated for 72 h, at 30 °C, initial pH 7.0, and shaken at 200 rpm.

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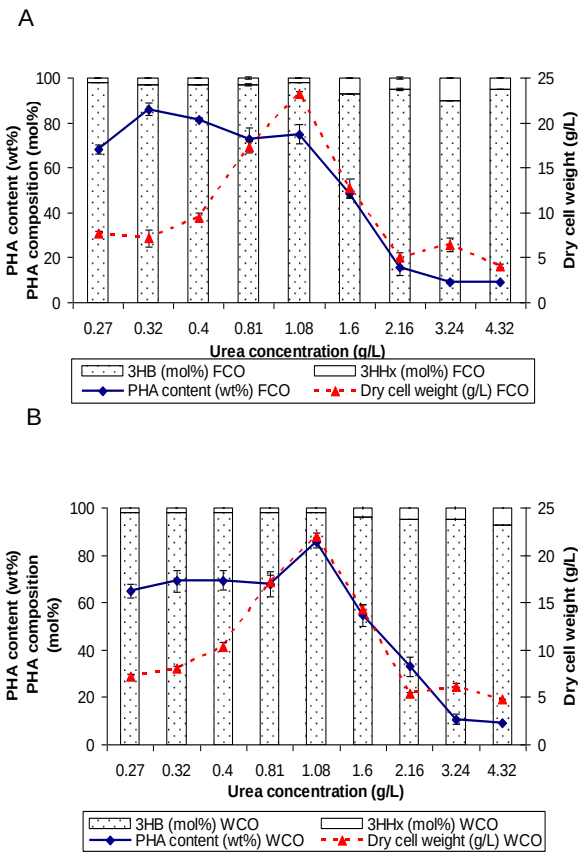

Figure 2: Effect of different concentrations of urea on P(3HB-*co*-3HHx) biosynthesis by recombinant *C. necator* PHB^{-}4/pBBREE32d13. A) FCO as the sole carbon source B) WCO as the sole carbon source.
Oil concentration was 20.0 g/L. Urea, in varying concentrations, was the nitrogen source. Samples were incubated for 72 h, at 30 °C, initial pH 7.0, and shaken at 200 rpm.

A comparison between the *C. necator* H16 and its transformant *C. necator* PHB^{-}4 in Figure 1 and Figure 2 indicated that the DCW produced from transformant *C. necator* PHB^{-}4 was slightly lower compared with *C. necator* H16 but it produced higher PHA content. In fact, our results also showed that the 3HHx monomer composition was regulated by the urea concentration. The highest 3HHx monomer composition that was obtained was 7 mol% when 4.32 g/L of urea concentration was fed to the bacterial culture. PHA yield obtained by transformant *C. necator* PHB^{-}4 was 18.3 g/L, which was similar to the PHA yield obtained from *C. necator* H16. In order to compare the results, transformant *C. necator* PHB^{-}4 was given FCO as the sole carbon source in PHA production. Up to 23.2 g/L of DCW and 75 wt% PHA contents were obtained from the experiment. The highest 3HHx monomer molar fraction was 10 mol% when fed with 3.24 g/L of urea concentration. On average, transformant *C. necator* yielded 17.4 g/L PHA when FCO was used as carbon source. Based on a consideration for employing the optimum condition for the above-discussed biosynthesis, 20.0 g/L oil (WCO and FCO) and 1.08 g/L urea were selected as the carbon and nitrogen source concentrations in a time profile study.

Time profile of P(3HB) and P(3HB-*co*-3HHx) biosynthesis using cooking oil (WCO and FCO) as the sole carbon source

In this study, we observed changes in the DCW and PHA content corresponded to time, and further selected the outstanding cultivation time in relation to the domestic wastes such as WCO. The highest growth of the bacteria cells (DCW = 25.4 g/L) and highest accumulation of PHA (71 wt%) were obtained when WCO was used as the sole carbon source for 72 h of *C. necator* H16 cultivation (**Figure 3**). The transformant strain *C. necator* PHB^{-}4 produced a DCW at 22.3 g/L and PHA content of 85 wt% at 72 h of cultivation when WCO was used (Figure 4). With FCO, it was shown that highest DCW obtained in wild type system reached 24.8 g/L with PHA accumulation of 70 wt%, while DCW of 23.3 g/L and PHA content of 75 wt% were obtained for transformant strain in the same system. The molar fraction of 3HHx varied from 2 to 5 mol% in the WCO and FCO systems.

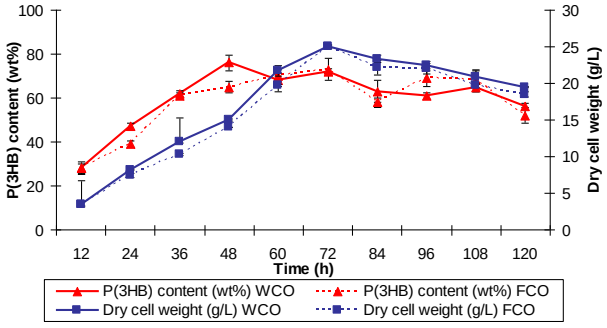

Figure 3: Time profile of P(3HB) biosynthesis by *C. necator* H16, using WCO or FCO as the sole carbon sources.
Oil concentration and urea concentration were 20.0 g/L and 1.08 g/L respectively. Samples were incubated for 72 h, at 30 °C, initial pH 7.0, and shaken at 200 rpm.

With cooking oil being the only available carbon source, bacterial lipase activity increased as the bacterial population built up. After 72 h when the maximum activity was reached, lipase activity declined gradually as the residual oil content was depleted. Similar trend was observed in another study, which indicated that *C. necator* reached the maximum activity at 96 h and depleted after the main peak. The utmost lipase production was 45 Units/mL/min when WCO was used as the carbon source (Rao *et al.*, 2010). The highest lipase activity for WCO utilised by wild type bacterium was 25 Units/mL/min at 72 h whilst a similar lipase activity (24 Units/mL/min) was observed when FCO was used. Similar lipase activities were also found in transformant strain (Figure 5). Lipase

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production by *C. necator* H16 and transformant strain *C. necator* PHB¯4 showed obvious acceleration during 36 h and 24 h, respectively.

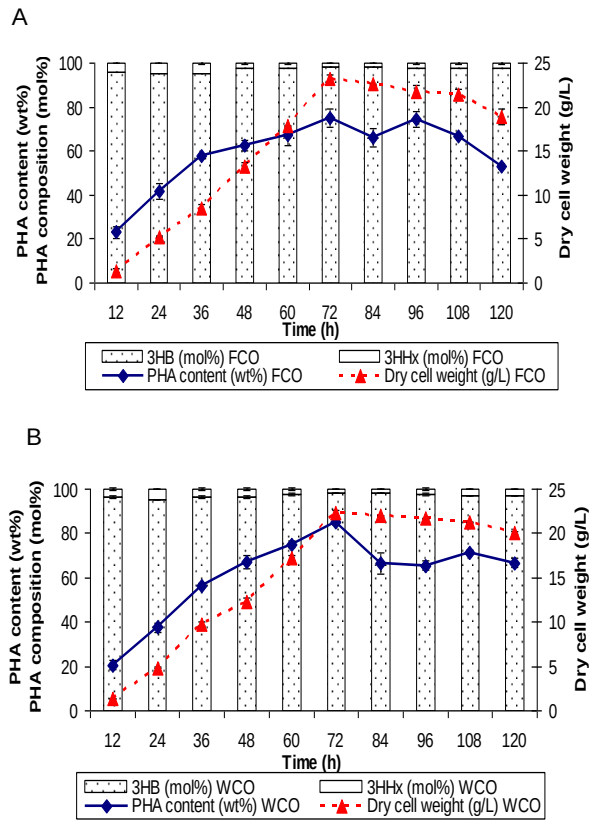

Figure 4: Time profile of P(3HB-*co*-3HHx) biosynthesis by recombinant *C. necator* PHB 4/pBBREE32d13. A) FCO as the sole carbon source B) WCO as the sole carbon source.
Oil concentration and urea concentration were 20.0 g/L and 1.08 g/L respectively. Samples were incubated for 72 h, at 30 °C, initial pH 7.0, and shaken at 200 rpm.

However, Ng and co-workers found out that lipase activity was actively produced at 18 h by using *C. necator* H16 fed with jatropha oil (Ng *et al.*, 2010). Presence of compounds such as fatty acids, triglycerides and surfactants influence the initiation of lipase secretion (Kulkarni and Gadre, 1999, Boekema *et al.*, 2007). With increasing incubation period, residual oil for both carbon sources decreased. The occurrence of maximum lipase activity after 72 h of bacterial cultivation corresponded with the time of maximum DCW and PHA production (Figure 3 and Figure 4).

CONCLUSION

C. necator H16 and *C. necator* PHB¯4 are efficient PHA producers, consuming cooking oil as the carbon source compared to other strains and manage to produced PHA content of 70-80 wt%. Production of P(3HB-co-3HHx) by using WCO is comparable to that of P(3HB-*co*-3HHx) obtained by using PKO as the carbon source which are 85 wt% and 87 wt%, respectively. It can be concluded that the growth and accumulation of PHA by bacterial cells utilizing WCO or FCO are comparable. As a cheaper replacement for FCO, WCO has been proven to be an excellent carbon source for P(3HB) and P(3HB-*co*-3HHx) biosynthesis. This has opened up the possibility of re-cycling WCO for the low-cost production of PHA.

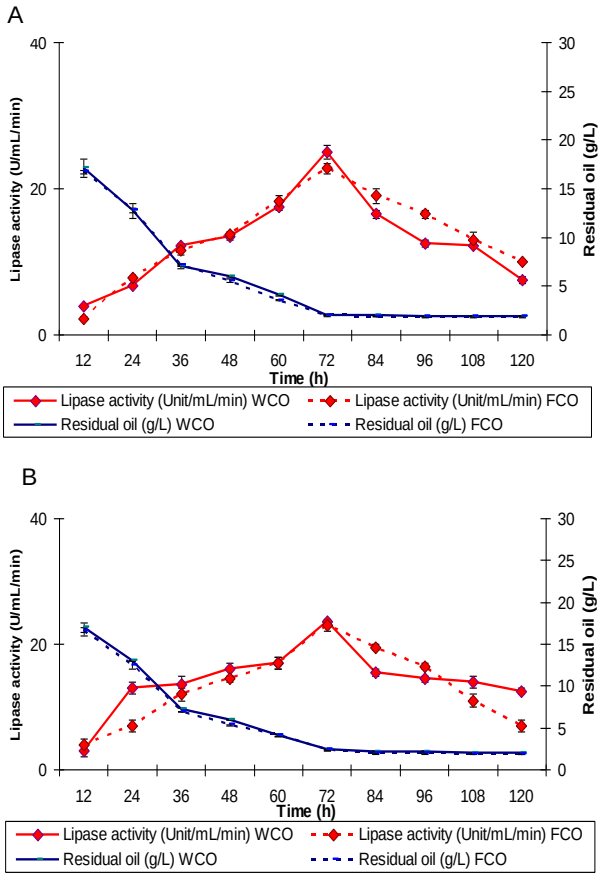

Figure 5: Lipase activity and residual oil content associated with P(3HB) biosynthesis by using FCO or WCO as the sole carbon sources. A) *C. necator* H16 B) *C. necator* PHB 4/pBBREE32d13
Oil concentration and urea concentration were 20.0 g/L and 1.08 g/L respectively. Samples were incubated for 72 h, at 30 °C, initial pH 7.0, and shaken at 200 rpm.

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