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Biosynthesis of polyhydroxyalkanoate copolymers from mixtures of plant oils and 3-hydroxyvalerate precursors

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Abstract

The combination of plant oils and 3-hydroxyvalerate (3HV) precursors were evaluated for the biosynthesis of polyhydroxyalkanoate (PHA) copolymers containing 3HV monomers by *Cupriavidus necator* H16. Among various mixtures of plant oils and 3HV-precursors, the mixture of palm kernel oil and sodium propionate was suitable for the biosynthesis of high concentration of PHA (6.8 g L⁻¹) containing 7 mol% of 3HV. The 3HV monomer composition can be regulated in the range of 0–23 mol% by changing culture parameters such as the initial pH, and the nitrogen source and its concentration. PHA copolymers with high weight-average molecular weights (M_w) ranging from 1,400,000 to 3,100,000 Da were successfully produced from mixtures of plant oils and 3HV-precursors. The mixture of plant oils and sodium propionate resulted in PHA copolymers with higher M_w compared to the mixture of plant oils and sodium valerate. DSC analysis on the PHA containing 3HV monomers showed the presence of two distinct melting temperature (T_m), which indicated that the PHA synthesized might be a blend of P(3HB) and P(3HB-co-3HV). Sodium propionate appears to be the better precursor of 3HV than sodium valerate. © 2008 Elsevier Ltd. All rights reserved.

Keywords: Palm kernel oil; Plant oils; Poly(3-hydroxybutyrate-co-3-hydroxyvalerate); *Cupriavidus necator*; Volatile fatty acids

1. Introduction

The use of petroleum-based synthetic plastics has led to solid waste management problems and secondary problems such as global warming, which is caused by increased carbon dioxide levels in the atmosphere during the manufacturing and disposal processes. In response to these issues, the application of biobased and biodegradable polymers as an alternative to synthetic plastics has been proposed. Thus, studies and development on biodegradable polymers, such as polyhydroxyalkanoate (PHA), have gained significant attention and popularity over the years in the US, Japan, and Germany (Sudesh and Doi, 2005).

Currently, various copolymers such as poly(3-hydroxybutyrate-co-4-hydroxybutyrate) [P(3HB-co-4HB)] (Lee et al., 2004; Amirul et al., 2008), poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] (Choi et al., 2003; Loo and Sudesh, 2007a), poly(3-hydroxybutyrate-co-hydroxyhexanoate) [P(3HB-co-3HHx)] (Qiu et al., 2006) and poly(3-hydroxybutyrate-co-3-hydroxypropionate) [P(3HB-co-3HP)] (Shimamura et al., 1994) have been successfully produced by microorganisms. Among them, P(3HB-co-3HV) copolymer has been proposed as one of the suitable substitute for synthetic thermoplastic applications due to its better physical and mechanical properties such as lower crystallinity and greater flexibility as compared to poly(3-hydroxybutyrate) [P(3HB)] (Chen et al., 2006). An early attempt to market this copolymer under the trademark BIOPOL™ was pioneered by Imperial Chemical Industries (ICI) in 1981 (Holmes, 1985). Techniques such as, fed-batch cultivation using a mixture of

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glucose and propionic acid have been used to biosynthesize a random copolymer of P(3HB-*co*-3HV) containing 3–20 mol% 3HV (Choi and Lee, 1999).

The overall cost of PHA production is expensive when compared with the production costs for petroleum-based plastics. One factor that can lead to significantly higher cost for PHA production is the cost of carbon feedstocks for the bacterial strains. Therefore, identification of inexpensive carbon sources has become an important research aim in order to synthesize PHA copolymers with a more competitive price range. Several strategies to synthesize P(3HB-*co*-3HV) copolymer from inexpensive carbon sources, as well as the feeding modes have been performed (Marangoni et al., 2002). A combination of an efficient PHA production strategy coupled with lower production costs will ensure the successful penetration of PHA into the global plastics markets.

Many agricultural and farm by-products such as whey (Ahn et al., 2001), molasses (Solaiman et al., 2006; Oliveira et al., 2007) and plant oils (Loo et al., 2005) have been considered as carbon substrates for the biosynthesis of PHA. Among these, plant oils seem to be the most suitable substrates because they are predicted to yield higher quantities of PHA and subsequently reduce the cost of production (Akiyama et al., 2003; Loo and Sudesh, 2007b). In addition, plant oils contain a much higher carbon content per weight than sugars. Studies have shown that plant oil could notably improve the biosynthesis of PHA compared to sugars, which are normally used for the PHA accumulation by various bacteria (Kahar et al., 2004). Additionally, previous studies have shown that for each gram of either glucose or plant oils, the P(3HB) yields produced from plant oils were almost twofold higher (0.8 g PHA per 1 g of plant oil) as compared to when glucose (0.3 g PHA per 1 g of glucose) was used as the sole carbon source (Akiyama et al., 2003).

In this study, a new approach to produce P(3HB-*co*-3HV) copolymer by using mixtures of various plant oils and sodium propionate has been evaluated. In addition, the 3HV-precursor feeding strategies, and variation on culture conditions that could improve the copolymer production were also studied.

2. Methods

2.1. Bacterial strain and media

The bacterial strain, *Cupriavidus necator* H16, used throughout this study was kindly provided by Prof. Y. Doi (RIKEN Institute and Tokyo Institute of Technology, Japan). *C. necator* was grown for 24 h in nutrient rich (NR) medium containing 10 g L⁻¹ meat extract, 10 g L⁻¹ peptone and 2 g L⁻¹ yeast extract at 30 °C for inoculum preparation.

2.2. Carbon sources and PHA synthesis

Palm kernel oil, crude palm oil, and palm olein that were used throughout this study as feedstocks for PHA produc-

tion were kindly provided by Acidchem International Ltd. (Malaysia) and Unitata Ltd. (Malaysia). Both crude palm oil and crude palm kernel oil are the main products from the oil palm fruit. Crude palm oil (commonly known as palm oil) is obtained from the mesocarp of the fruit while crude palm kernel oil and palm kernel oil are obtained from its endosperm (kernel). Fractionation of crude palm oil produces palm olein. Other plant oils such as olive oil, sunflower oil, cooking oil (commercially available palm olein) and coconut oil were purchased from local supermarkets and used without further purification. Each gram of plant oil contains 0.72–0.77 g of carbon. Sodium propionate and sodium valerate were added as 3HV-precursors at a predetermined time. Each gram of sodium propionate and sodium valerate consists of 0.37 g and 0.48 g of carbon, respectively.

PHA biosynthesis was carried out in one-stage cultures in shake flasks. The starter culture was grown in NR medium (50 mL medium in 250 mL flask) for 24 h at 30 °C and 1.5 mL of the culture was transferred into 50 mL of nitrogen source limited mineral salts medium (MM) (Doi et al., 1995) in 250 mL shake flasks, which were incubated at 30 °C in a rotary shaker at 200 rpm. Oils were autoclaved separately and added into the MM before inoculation. Sodium propionate or sodium valerate at a concentration of 2.5 g L⁻¹ was fed into the culture medium at 48 h and 60 h of cultivation. At the end of the cultivation (72 h), cells were harvested by centrifugation at 10,000g for 10 min. The cell pellet was then resuspended in 100 mL of hexane by vortexing and then centrifuged again at 10,000g for 5 min to remove the remaining oils. The washing step was repeated with 100 mL of distilled water before lyophilization.

2.3. Analytical procedures

In order to determine PHA content and monomer composition, approximately 7 mg of lyophilized cells were subjected to methanolysis in the presence of 15% (v/v) sulfuric acid and 85% (v/v) methanol. The resulting hydroxyacyl methyl esters were then analyzed by gas chromatography (GC) (Braunegg et al., 1978). The PHA contents were also determined gravimetrically after extraction and purification from dried cells. For this, the lyophilized cells were refluxed in chloroform at 60 °C for 4 h. After filtration, the chloroform extract was concentrated and the dissolved PHA was precipitated out in methanol.

2.3.1. Gel permeation chromatography (GPC) analysis

To calculate the weight-average molecular weight (M_w), number-average molecular weight (M_n) and polydispersity index (M_w/M_n), the polymers were subjected to gel permeation chromatography (GPC) analysis. The polymer samples were dissolved in chloroform to a final concentration of 0.7–1.1 mg mL⁻¹ and filtered through 0.45 µm Millex[®]-LH PTFE membranes (Millipore, USA) and the molecular mass data for the polyesters were obtained by GPC analysis using a Shimadzu 10A system with RID-10A refractive-

index detector with Shodex K802 M and K806M serial columns (Shimadzu, Japan). Polystyrene standards with low polydispersity were used to plot the standard curve.

2.3.2. Determination of PHA copolymers thermal properties

The thermal data were recorded on a Perkin–Elmer Pyris 1 differential scanning calorimeter (DSC) equipped with a liquid nitrogen-cooling accessory (Perkin–Elmer, USA). Data were collected under a nitrogen flow of 20 mL min⁻¹. Melt-quenched polyester samples (approximately 3 mg) encapsulated in aluminum pans were heated from -50 °C to 180 °C at a rate of 20 °C min⁻¹ and the heat flow curves were recorded. The observed melting temperatures were determined from the position of the endothermic peaks.

3. Results

Initially, we investigated the ability of *C. necator* to biosynthesize PHA from individual plant oils as the sole carbon sources. Table 1 clearly shows that plant oils can readily support the growth of this bacterium whereby an average of 4.4–5.6 g L⁻¹ dry cell weight was obtained. Results from GC analysis revealed that only P(3HB) homopolymer could be synthesized from plant oils (Table 1). It is also shown that plant oils are suitable carbon sources which can promote high P(3HB) accumulation up to 80 wt%.

In order to incorporate 3-hydroxyvalerate (3HV) besides 3HB, sodium propionate or sodium valerate was added into the culture medium containing plant oils. The production of PHA copolymer and dry cell weights achieved were better when the cells were cultivated in mixtures of plant oils and sodium propionate (Table 2) as compared to mixtures of plant oils and sodium valerate (Table 3). The carbon-to-nitrogen ratios (C/N given in grams per grams) for the mixtures of various plant oils with sodium valerate were in the range of 46–48. On the other hand, the C/N ratios

for the cultivation medium containing respective plant oils and sodium propionate were in the range of 42–44.

The copolymer content and dry cell weight were the highest at 90 wt% and 7.5 g L⁻¹, respectively when a mixture of palm kernel oil (Unitata) [PKO (U)] and sodium propionate was used. A combination of olive oil and sodium valerate resulted in a fairly high dry cell weight (6.1 g L⁻¹) with copolymer content of 89 wt% of the dry cell weight. The highest 3HV monomer composition (14 mol%) was achieved when olive oil was mixed with sodium valerate as the 3HV-precursor instead of sodium propionate.

In addition, the number-average molecular weight (M_n) of P(3HB-co-3HV) copolymers produced by *C. necator* from different mixtures of plant oils and 3HV-precursors were determined, and the results are shown in Tables 2 and 3. The M_n of P(3HB-co-3HV) produced from the mixture of plant oils and sodium propionate was in the range of 570,000–990,000 Da, while M_n of the polymer produced from the mixture of plant oils and sodium valerate fell within a range of 340,000–920,000 Da. The polydispersity (M_w/M_n) indices of copolymers obtained from the mixture of plant oils with sodium propionate or sodium valerate ranged from 3.1 to 4.3.

The mixture of PKO (U) and sodium propionate was used to evaluate the effect of different nitrogen sources on the biosynthesis of P(3HB-co-3HV) copolymer since the highest dry cell weight and copolymer content were achieved from this mixture. The ratio of C/N was fixed at 42. Different types of nitrogen sources generated varying copolymer yields in the range of 2.0–6.8 g L⁻¹ (Table 4). Among the various nitrogen sources tested, ammonium chloride (NH₄Cl) was found to be the best nitrogen source for the biosynthesis of P(3HB-co-3HV) copolymer. The PHA content was the highest (90 wt%) when the culture medium was enriched with NH₄Cl. Lower PHA contents were accumulated when ammonium nitrate (NH₄NO₃) and sodium nitrate (NaNO₃) were used as nitrogen sources. In addition, the molar fraction of 3HV remained nearly constant at 10–12 mol% in cultures supplemented with urea or NH₄NO₃ as the nitrogen source. NH₄Cl resulted in relatively high concentrations of 3HV fractions in the copolymer (7 mol%). Although the highest P(3HB-co-3HV) copolymer yield could be produced by using NH₄Cl, up to 6.8 g L⁻¹, urea was chosen for the subsequent experiments due to the lower cost of urea.

The ratio of C/N plays an important role in regulating the overall PHA biosynthesis in bacterial fermentation. In order to determine the most suitable C/N ratio, a mixture of 5 g L⁻¹ PKO (U) and 5 g L⁻¹ sodium propionate was used as the carbon source. In Fig. 1, it can be seen that 5 mM urea is the optimum concentration for the biosynthesis of P(3HB-co-3HV) by *C. necator*, whereby 90 wt% of copolymer from dry cell weight was accumulated. Fig. 1 also shows that an increase in urea concentration (a decrease of C/N ratio) significantly affected the biosynthesis of PHA copolymer and 3HV molar fractions. A

Table 1
Biosynthesis of P(3HB) from different plant oils by *C. necator*

Plant oil ^a (5 g L ⁻¹)	Total P(3HB) (g L ⁻¹)	Cell dry weight (g L ⁻¹)	P(3HB) content ^b (wt%)
Crude palm kernel oil ^c	3.4	5.0	67
Crude palm oil ^c	3.5	4.6	75
Palm kernel oil ^d	4.1	5.5	75
Palm kernel oil ^c	4.3	5.6	77
Palm olein ^c	3.6	5.2	70
Cooking oil	4.2	5.4	78
Olive oil	3.9	4.9	80
Sunflower oil	3.4	4.7	72
Coconut oil	3.3	4.4	76

3HB, 3-hydroxybutyrate.

^a Incubated for 72 h at 30 °C, initial pH 7.0, 200 rpm.

^b PHA content in freeze-dried cells.

^c Source from Unitata Ltd.

^d Source from Acidchem Ltd.

Table 2

Biosynthesis of PHA containing 3HB and 3HV monomers from a mixture of plant oils and sodium propionate^a

Plant oil (5 g L ⁻¹) + sodium propionate (5 g L ⁻¹)	C/N ratio ^b	Cell dry weight (g L ⁻¹)	PHA content ^c (wt%)	PHA composition (mol%)		Molecular weight		Thermal properties		
				3HB	3HV	M_n^d ($\times 10^5$)	M_w/M_n^e	T_g^f (°C)	T_m^g (°C)	ΔH_m^h (J g ⁻¹)
Crude palm kernel oil ⁱ	43	6.0	78	92	8	9.9	3.1	-0.3	167	41.7
Olive oil	43	6.0	78	92	8	8.5	3.1	-1.6	169	41.5
Sunflower oil	44	6.7	78	92	8	7.0	3.6	-1.2	169	49.0
Palm kernel oil ^j	42	7.3	65	93	7	7.7	3.5	-0.2	167	54.5
Palm kernel oil ⁱ	42	7.5	90	93	7	7.4	3.8	-0.8	166	64.7
Cooking oil	43	6.3	85	95	5	5.7	4.1	-0.2	169	56.8
Palm olein ⁱ	43	6.7	88	96	4	7.1	3.9	-0.5	170	59.1
Crude palm oil ⁱ	43	3.6	74	97	3	6.7	3.9	-0.9	168	59.5
Coconut oil	42	5.4	75	98	2	6.4	4.0	-0.7	168	65.2

3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate.

^a Incubated for 72 h at 30 °C, initial pH 7.0, 200 rpm. Sodium propionate was added at 48 h (2.5 g L⁻¹) and 60 h (2.5 g L⁻¹).^b C/N ratio expressed in grams per grams.^c PHA content in freeze-dried cells.^d Number-average molecular weight.^e Polydispersity index.^f Glass transition temperature.^g Melting temperature.^h Enthalpy of fusion.ⁱ Source from Unitata Ltd.^j Source from Acidchem Ltd.

Table 3

Biosynthesis of PHA containing 3HB and 3HV monomers from a mixture of plant oils and sodium valerate^a

Plant oil (5 g L ⁻¹) + sodium valerate (5 g L ⁻¹)	C/N ratio ^b	Cell dry weight (g L ⁻¹)	PHA content ^c (wt%)	PHA composition (mol%)		Molecular weight		Thermal properties		
				3HB	3HV	M_n^d ($\times 10^5$)	M_w/M_n^e	T_g^f (°C)	T_m^g (°C)	ΔH_m^h (J g ⁻¹)
Olive oil	48	6.1	89	86	14	6.6	3.8	-13, 0.6	113, 170	0.6, 43.3
Sunflower oil	48	5.2	80	86	14	6.2	4.0	-20, -0.1	170	59.8
Crude palm kernel oil ⁱ	47	4.1	64	90	10	7.0	3.7	-17, 0	168	61.7
Crude palm oil ⁱ	48	3.3	64	94	6	4.0	4.2	-8.9, -0.5	170	60.2
Palm olein ⁱ	48	3.5	68	96	4	3.5	4.1	0	165, 170	64.6
Cooking oil	48	4.3	75	97	3	3.4	4.0	-0.1	171	67.1
Coconut oil	46	6.0	78	97	3	5.5	4.3	-0.5	169	65.7
Palm kernel oil ^j	47	4.5	68	97	3	9.2	3.1	-0.5	167	67.4
Palm kernel oil ⁱ	47	5.3	69	97	3	8.4	3.2	-0.7	168	68.7

3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate.

^a Incubated for 72 h at 30 °C, initial pH 7.0, 200 rpm. Sodium valerate was added at 48 h (2.5 g L⁻¹) and 60 h (2.5 g L⁻¹).^b C/N ratio expressed in grams per grams.^c PHA content in freeze-dried cells.^d Number-average molecular weight.^e Polydispersity index.^f Glass transition temperature.^g Melting temperature.^h Enthalpy of fusion.ⁱ Source from Unitata Ltd.^j Source from Acidchem Ltd.

sharp decrease in PHA content and cell biomass were noted when the urea concentration was increased from 10 mM to 30 mM. At 30 mM, only 29 wt% of PHA

copolymer and 5.1 g L⁻¹ cell biomass was accumulated at the end of the fermentation. Whereas at 10 mM, the amount of PHA content synthesized was more than two-

Table 4
Effect of different nitrogen sources on the biosynthesis of P(3HB-co-3HV)

Nitrogen source ^a	C/N ratio ^b	Total PHA (g L ⁻¹)	Cell dry weight (g L ⁻¹)	PHA content (wt%)	PHA composition ^c	
					3HB	3HV
NH ₄ Cl	42	6.8	7.5	90	93	7
NH ₄ NO ₃	42	5.3	6.3	84	89	11
NaNO ₃	42	4.6	6.3	73	89	11
(NH ₂) ₂ CO	42	6.3	7.1	89	90	10
NH ₄ H ₂ PO ₄	42	2.0	3.3	61	88	12

3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; NH₄Cl, ammonium chloride; NH₄NO₃, ammonium nitrate; NaNO₃, sodium nitrate; (NH₂)₂CO, urea; NH₄H₂PO₄, ammonium dihydrogen phosphate.

^a Incubated for 72 h, at 30 °C, initial pH 7.0 and shaken at 200 rpm; 2.5 g L⁻¹ of sodium propionate was added at 48 h and 60 h, respectively.

^b C/N ratio expressed in grams per grams.

^c Determined by GC.

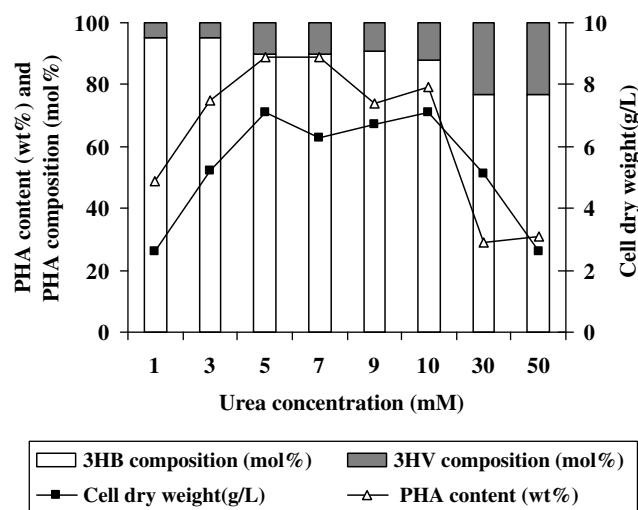


Fig. 1. The effect of urea concentration on P(3HB-co-3HV) biosynthesis.

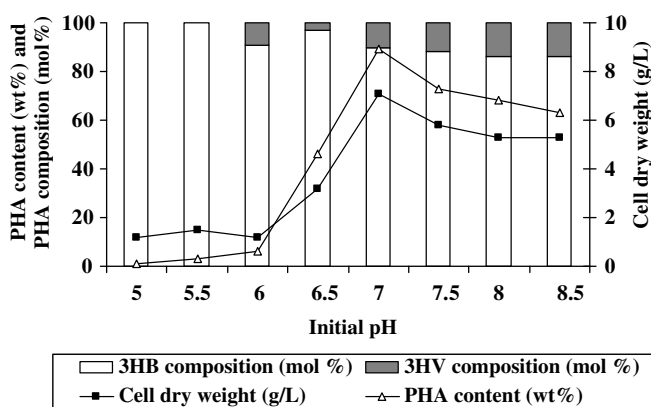


Fig. 2. The effect of different initial pH on the production of P(3HB-co-3HV) by using 5 g L⁻¹ PKO(U) and 5 g L⁻¹ sodium propionate.

fold higher (79 wt%). At the same time, it was observed that the molar fraction of 3HV increased from 5 mol% to

23 mol% depending on the urea concentration in the media.

Fig. 2 shows the effect of the initial culture medium pH on P(3HB-co-3HV) content and monomer composition. The optimum pH for the biosynthesis of P(3HB-co-3HV) copolymer by *C. necator* was 7.0. Up to 90 wt% of P(3HB-co-3HV) was successfully synthesized at pH 7.0 (Fig. 2). However, neither acidic nor alkaline conditions were favorable for the biosynthesis of this copolymer. In alkaline conditions, higher 3HV monomer content was incorporated into the PHA copolymer whereas only P(3HB) homopolymer was produced when cells were grown under acidic conditions (pH 5.0–5.5). On average, 12–14 mol% molar fraction of 3HV was synthesized under alkaline conditions (pH 7.5–8.5). Further increase in the initial pH resulted in decreased PHA content (90–63 wt%).

4. Discussion

A previous report by Fukui and Doi (1998) showed that *C. necator* H16 can biosynthesize homopolymer of P(3HB) efficiently when plant oils such as olive oil and palm oil are used as carbon feedstocks. Both *in vivo* and *in vitro* studies had proven that the PHA synthase from *C. necator* could only polymerize 3-hydroxypropionate (C3) to 3-hydroxyvalerate (C5) monomers (Yuan et al., 2001). This indicated that the PHA synthase from this bacterium exhibits narrow substrate specificity. However, other studies have demonstrated that 3-hydroxyhexanoate (3HHx) (Dennis et al., 1998), 3-hydroxyoctanoate (3HO) and 3-hydroxydodecanoate (3HHD) (Antonio et al., 2000) could be polymerized by the PHA synthase of *C. necator*. Dennis et al. found that by knocking out the gene for 3-ketothiolase (PhaA) from *C. necator*, 3HHx monomer can be incorporated by the PHA synthase. It was proposed that the absence of this enzyme (PhaA) would reduce the production of acetoacetyl-CoA (an intermediate for 3HB monomer formation) and consequently the PHA production had to rely on other intermediates from β -oxidation pathway. This would lead to the biosynthesis of PHA containing other medium carbon chain monomers (Dennis et al., 1998).

However, for the incorporation of 3HV monomer, special precursors have to be added into the culture medium. Previous studies have used glucose as the carbon feedstock for PHA production in the presence of 3HV-precursors. In this study, palm oil has been used to replace glucose as the main carbon source for the growth of cells. When sodium propionate was fed during early cultivation stage (12 h) at a concentration of 5 g L⁻¹, both cell biomass (0.2 g L⁻¹) and PHA production (13 wt%) were severely affected (results not shown). Our result is in accord with a previous finding by Byrom (1987) whereby 1 g L⁻¹ of propionic acid in the culture medium leads to inhibition of PHA biosynthesis and is further supported by a study by Ramsay et al. (1990) in which the growth and PHA production were severely suppressed in the presence of more than 3 g L⁻¹ propionic acid. This observation might be due to the fact

that sodium propionate is a volatile fatty acid and therefore is toxic to the bacterial cell (Yu et al., 2002).

Hence, implementation of a suitable feeding strategy has been proposed to reduce the toxicity effect by volatile fatty acids (Steinbüchel and Lütke-Eversloh, 2003). We have amended our experiments by adding 3HV-precursors (sodium valerate and sodium propionate) at a lower concentration (2.5 g L^{-1}) at 48 h and 60 h of cultivation. Up to 7.5 g L^{-1} dry cell weight with 90 wt% P(3HB-co-3HV) could be achieved by using the mixture of PKO (U) and sodium propionate as bacterial feedstocks for PHA production.

Our experimental data also show that usage of carbon substrates containing higher carbon content per weight as feedstocks could provide higher yields of PHA as compared to simple sugars such as glucose, as reported by Akiyama et al. (2003). Theoretically, a molecule of glucose or fructose, which contains six carbon atoms, is metabolized to form two acetyl-CoA and two molecules of carbon dioxide, thus leading to the formation of one 3HB monomer units. On the other hand, a molecule of linoleic acid is oxidized via the β -oxidation pathway to form nine acetyl-CoA, which likely contributes to the production of more 3HB monomer units than a mol equivalent of glucose (Akiyama et al., 2003).

In this study, we have found that a mixture of PKO and sodium propionate is suitable for the biosynthesis of P(3HB-co-3HV) copolymer in *C. necator*. Recently, we have shown the excellent ability of recombinant *C. necator* to synthesize P(3HB-co-3HHx) when PKO was utilized as the sole carbon source (Loo et al., 2005). The increased accumulation of PHA copolymers from PKO in *C. necator* is likely attributable to the presence of highly saturated fatty acids such as lauric acid ($C_{12:0}$), myristic acid ($C_{14:0}$) oleic acid ($C_{18:1}$) and trace quantity of linolenic acid ($C_{18:3}$) that make up the main composition of PKO (Loo et al., 2005). It has been demonstrated that *C. necator* poorly utilized $C_{18:3}$ compared to $C_{18:1}$ (Kahar et al., 2004). This is supported by a report from O'Leary (1962) whereby the simultaneous presence of both saturated and unsaturated fatty acids are known to cause growth inhibition in bacteria. However, the inhibitory effect of unsaturated fatty acids increases with the number of double bonds in the molecule. Since there are only trace quantities of $C_{18:3}$ in palm kernel oil, it is an excellent carbon source for the biosynthesis of P(3HB-co-3HV) copolymer when combined with 3HV-precursor such as sodium valerate or sodium propionate.

The molecular weight (M_w) of the P(3HB-co-3HV) synthesized was fairly high (1,400,000–3,100,000 Da) when mixtures of plant oils and sodium propionate were used. A similar observation was reported by Fukui and Doi (1998) whereby the (M_w) of P(3HB) was as high as 1,000,000 Da when palm oil was used as the carbon source. This suggests that palm oil is a suitable carbon source for the production of high molecular weight PHA. It should be noted that the hydrolysis of oils by lipase will produce

free fatty acids and glycerol. The presence of glycerol in the culture medium is of some concern because glycerol has been shown to reduce the molecular weight of PHA (Madden et al., 1999). However, in this study, we did not observe any such effects on the molecular weight of P(3HB-co-3HV) copolymers produced by *C. necator*.

DSC analysis on the PHA polymers obtained from the combination of different plant oils and sodium propionate showed the presence of only one melting peak (T_m), which is in the range of 166–170 °C (Table 2). On the contrary, two distinct melting peaks (T_m) were recorded for the samples extracted from the mixtures of sodium valerate with olive oil and palm olein, respectively (Table 3). The higher melting peak at 170 °C corresponds to the melting peak of P(3HB) homopolymer crystalline phase. Thus, copolymers with higher melting peaks indicate the presence of higher 3HB monomer composition. The lower melting peak at 113 °C corresponds to the P(3HB-co-3HV) copolymer containing higher 3HV monomers. This indicated that the PHA polymers produced from these carbon sources might be blends of P(3HB) and P(3HB-co-3HV). The incorporation of 3HV monomer would only take place in the presence of sodium propionate. Observation of large amounts of granules from ultrastructural studies before the addition of 3HV-precursor at 48 h (results not shown) indicates the accumulation of P(3HB) monomer. Therefore, the addition of sodium propionate at 48 h would most likely lead to the formation of blends containing P(3HB) and P(3HB-co-3HV).

The molar fraction of 3HV plays an important role in controlling the physical and mechanical properties of the copolymer. During the biosynthesis of this copolymer, 3-hydroxyvaleryl-CoA (3HV-CoA) is an intermediate to be converted into 3HV monomer. This important intermediate is synthesized from the condensation of propionyl-CoA to 3-ketovaleryl-CoA, subsequently followed by reduction to 3HV-CoA (Steinbüchel and Lütke-Eversloh, 2003). It has been shown that a wider range of 3HV monomer composition could be achieved when sodium valerate was used instead of sodium propionate (Doi, 1990). This is mainly because sodium propionate does not generate 3HV units exclusively; it is also condensed to acetyl-CoA, which is the intermediate substrate for 3HB units (Doi et al., 1987). Therefore, both 3HB and 3HV units are generated when sodium propionate is used as a 3HV precursor. Conversely, sodium valerate is metabolized via β -oxidation pathway so that it could be directly incorporated into the polymer chain without breakdown of its carbon chain length (Doi et al., 1988).

On the other hand, upon the hydrolysis of palm kernel oil by extracellular lipase, free fatty acids ranging from C_6 to $C_{18:1}$ are released into the medium. The pK_a values of these fatty acids are relatively close (4.84–4.89). In fact, these pK_a values are almost similar to those of the 3HV-precursors, propionate and valerate, 4.86 and 4.84, respectively. Therefore, all these fatty acids are transported into the cells freely in an undissociated form (non-ionic

diffusion) to form 3HB and 3HV monomers (Salmond et al., 1984). Based on the results obtained (Fig. 2), the polymerization of this copolymer might be affected by the different initial pH of the culture medium.

In addition to carbon sources, selection of an appropriate nitrogen source is also a crucial factor to maximize the biosynthesis of PHA. Nitrogen limitation has been largely used to promote the biosynthesis of PHA (Doi, 1990). The ratio of C/N is important in the biosynthesis of PHA especially in a one-stage culture because the accumulation of acetyl-CoA is determined by the amount of nitrogen present in the medium (Du et al., 2001). The C/N ratios of 20–40 are widely used for PHA biosynthesis (Tian et al., 2000; Amirul et al., 2008). In a recent study, the biosynthesis of 4HB copolymer from γ -butyrolactone as the sole carbon source was not affected when the C/N ratio was increased from 20 to 40 (Amirul et al., 2008). Kato et al. (1996) had reported an interesting finding whereby the PHA biosynthesis and cell growth of *Pseudomonas* sp. 61-3 showed different trends even though the C/N ratio has been fixed at 50. It was found that better cell growth and PHA accumulation were achieved in medium supplied with higher proportions of carbon and nitrogen sources (Kato et al., 1996). This indirectly suggested that C/N ratio is not the only crucial factor for PHA biosynthesis.

In addition, the biosynthesis of PHA from acetyl-CoA is inhibited in the presence of nitrogen sources in native PHA producing bacteria (Du et al., 2001). In this study, 3HB monomers are mainly generated from acetyl-CoA when plant oils were fed as the main carbon feed stocks. In the presence of excess nitrogen source (urea), acetyl-CoA (mainly generated from the degradation of fatty acids) is channeled into the TCA cycle for energy generation and the formation of amino acids (Doi et al., 1988). The reaction of citrate synthase resulted in high free CoASH, thus slowing down the condensation of 3-ketothiolase to acetoacetyl-CoA, which is an intermediate for PHA biosynthesis.

In this study, when the concentration of urea was increased to 50 mM, accumulation of PHA copolymer up to 30 wt% could still be observed. Mansfield et al. (1995) found that the level of CoASH increased to maximum during exponential growth phase. Although the CoASH has reached the maximum level, small amounts of P(3HB) were detected, indicating that 3-ketothiolase could not be totally inhibited by CoASH.

It was also noted that the 3HV molar fractions were higher with an increase in urea concentrations. This might be due to the disruption of the availability of intracellular acetyl-CoA for 3HB monomers generation in higher urea concentration, in which acetyl-CoA is primarily channeled towards the cell growth. In a separate study, we have observed that the biosynthesis of P(3HB) from palm acid oil (PAO) was totally inhibited when the urea concentrations were above 30 mM. (Kek et al., in press). Therefore, the increase of 3HV molar fraction in the culture medium with high urea concentrations is most likely due to the reduced 3HB monomer generation.

5. Conclusion

This study has shown that *C. necator* could utilize a mixture of palm kernel oil and sodium propionate in the presence of urea to synthesize high contents of P(3HB-co-3HV) copolymer. In addition, this study has also demonstrated the potential of using palm oil as a biobased renewable carbon source for the large scale production of P(3HB-co-3HV) copolymers. Further studies are necessary to determine efficient feeding strategies to add 3HV-precursors during the onset of PHA biosynthesis without any adverse effects on cell growth.

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