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Biosynthesis and Characterization of Polyhydroxyalkanoate Containing High 3-Hydroxyhexanoate Monomer Fraction from Crude Palm Kernel Oil by Recombinant *Cupriavidus necator*

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Abstract

The potential of plant oils as sole carbon sources for production of P(3HB-*co*-3HHx) copolymer containing a high 3HHx monomer fraction using the recombinant *Cupriavidus necator* strain Re2160/pCB113 has been investigated. Various types and concentrations of plant oils were evaluated for efficient conversion of P(3HB-*co*-3HHx) copolymer. Crude palm kernel oil (CPKO) at a concentration of 2.5 g/L was found to be most suitable for production of copolymer with a 3HHx content of approximately 70 mol%. The time profile of these cells was also examined in order to study the trend of 3HHx monomer incorporation, PHA production and PHA synthase activity. ^{1}H NMR and ^{13}C NMR analyses confirmed the presence of P(3HB-*co*-3HHx) copolymer containing a high 3HHx monomer fraction, in which monomers were not randomly distributed. The results of various characterization analyses revealed that the copolymers containing a high 3HHx monomer fraction demonstrated soft and flexible mechanical properties.

Keywords: *Cupriavidus necator*; Crude palm kernel oil; Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate); Bioplastics; Polymer characterization

1. Introduction

Polyhydroxyalkanoate (PHA) is typically present in cells in the form of hydrophobic inclusion bodies, which are produced when nutrients required for growth, such as nitrogen, magnesium, sulphur or phosphorus, are limited. Some bacteria are capable of accumulating intracellular PHA in excess of 80% (w/w) of dry cell mass. Over the past couple decades, a wide variety of bacteria have been identified as PHA producers, including, *Pseudomonas oleovorans*, *Pseudomonas putida*, *Aeromonas hydrophila*, *Cupriavidus necator,* and many others.

Most types of PHA reported in the literature are composed of (*R*)-3-hydroxyalkanoic acid monomers containing 3 to 14 carbon atoms with aliphatic, aromatic, saturated, unsaturated, straight- or branched chain side groups (Valentin and Steinbüchel, 1994). In general, most PHA that has been extensively studied has been classified into three main types, depending on the number of carbon atoms in the monomer units. PHA containing monomers consisting of 3 to 5 carbon atoms are called short chain length PHA (scl-PHA); polymer with monomers consisting of 6 to 14 carbon atoms are medium-chain-length PHA (mcl-PHA); and copolymer containing combinations of scl- and mcl-PHA monomers are referred to as mixed chain length PHA (Madison and Huisman, 1999). There are some physical differences among scl- and mcl-PHA. The mcl-PHA polymers are typically sticky, elastic, and amorphous materials, while scl-PHA are highly crystalline thermoplastic materials (Sudesh et al., 2000). Also, for scl-PHA, the monomer units can be oxidized at positions other than the third carbon, while for mcl-PHA, monomer units, with few exceptions, are typically oxidized at the third position.

P(3HB) is the most common type of PHA found in nature, and it is more crystalline than PHA copolymers, with applications normally limited to the production of thermoplastic (Sudesh et al., 2000). P(3HB-*co*-3HHx) is a type of copolymer which can be produced by some wild-type strains of bacteria, for example, *Aeromonas caviae*. PHA copolymers containing 3HB with a small amounts of other monomer units are more flexible than P(3HB) homopolymer. Unlike P(3HB), P(3HB-*co*-3HHx) is a flexible material and it shows a high degree of elongation to break (Doi et al., 1995). This copolymer is suitable to be used as a film due to its flexibility. In general, researchers have shown that the different physical and mechanical properties of the polymer (from hard crystalline polymer to elastomeric rubber) depend on the types and quantities of incorporated monomeric units (Doi et al., 1995).

Plant oils have been shown to be better carbon sources for growth and PHA accumulation than sugars for select bacteria, including *C. necator* (Kahar et al., 2004; Budde et al., 2011). Plant oils contain a higher carbon content per weight than sugars, suggesting that the theoretical yield of PHA from plant oils could be at least 2-fold higher than that from sugars (Akiyama et al., 2003).

Palm oil is a promising carbon source for microbial PHA production, and an important natural resource and commodity for Southeast Asian countries, such as Malaysia. Over the past several decades, the oil palm industry in Malaysia has grown rapidly and spurred economic growth. Malaysia has become one of the leading producers and exporters of palm oil in the world today, exporting a total of 16.7 million tonnes of palm oil into the international market in 2010. The 2010 Malaysian export of all palm oil products including palm oil, palm kernel oil, palm kernel cake, oleochemicals, biodiesel

and finished products has reached 23.1 million tonnes (MPOB, 2010). Therefore, the commercialization of PHA production in Malaysia using palm oil as the sole carbon sources is very promising.

C. necator (also known as *Ralstonia eutropha*) is a Gram-negative betaproteobacterium that is a model PHA-producing organism capable of accumulating PHA at high levels, exceeding 80% of dried cell mass. However, it can produce only P(3HB) homopolymer from simple carbon sources such as fructose. *Rhodococcus aetherivorans* is a non-spore forming, Gram-positive aerobic actinomycete that can produce PHA copolymer using sugar as the sole carbon source (Hori et al., 2009), but it has been shown to accumulate low levels of intracellular PHA (< 2wt%). *R. aetherivorans* I24 was first isolated from hydrocarbon-contaminated soil for the synthesis of indinavir sulphate, CRIXIVAN, which is a protease inhibitor used in the treatment of AIDS (Buckland et al., 1999). Budde and co-workers had successfully engineered a recombinant strain of *C. necator,* harbouring a polyhydroxyalkanoate synthase gene from *R. aetherivorans* strain I24 ($phaC2_{Ra}$), that demonstrated remarkable enhancement in P(3HB-*co*-3HHx) productivity (Budde et al., 2011).

In the present study, PHA containing as high as 70 mol% 3HHx monomer content was accumulated from this recombinant *C. necator* strain, Re2160/pCB113, using crude palm kernel oil (CPKO) as sole carbon source. The synthase of this recombinant strain was examined in order to understand the correlation between PHA synthase activity and PHA accumulation as well as 3HHx monomer compositions. In addition, extracted polymers were characterized by muclear magnetic resonance spectroscopy (NMR), gel permeation chromatography (GPC), differential scanning calorimetry (DSC),

thermogravimetric (TGA) analysis and tensile strength testing. Our work has further demonstrated the versatility of this *C. necator* P(3HB-*co*-3HHx) production strain, and we have produced and characterized biodegradable polymer suited for unique applications.

2. Materials and methods

2.1. Bacterial strain and maintenance

Recombinant *C. necator* Re2160/pCB113 was used throughout this study. This mutant strain harbors the plasmid pCB113 containing the PHA synthase of *R. aetherivorans* I24 and an enoyl-CoA hydratase (*phaJ*) gene from *Pseudomonas aeruginosa* (Budde et al., 2011). For short-term maintenance of bacteria, cells were routinely streaked onto nutrient-rich (NR) agar plates with the following composition (per liter): 10 g peptone, 10 g meat extract and 2 g yeast extract (Doi et al., 1995). For long-term storage, the bacteria were maintained in a 25% (v/v) glycerol stock solution. The glycerol stocks were prepared by addition of 12.5 mL of pure glycerol to an overnight culture of the bacterial cells in 50 mL of NR. Aliquots of 1 mL were placed in tubes and then stored at –20 °C.

2.2. Carbon sources

Initially, 7 types of plant oils were tested as carbon sources, including crude palm kernel oil (CPKO, Acidchem International Ltd.), jatropha oil (Sarawak, Malaysia), crude

palm oil (CPO, Acidchem International Ltd.), palm olein (Vesawit, Yee Lee Corporation Bhd., Malaysia), soybean oil (Mazola®, ACH Food Companies, Inc., Spain), corn oil (Mazola®, ACH Food Companies, Inc., Spain), and coconut oil (Parachute®, Marico Ltd., India). The concentration of oils in culture was fixed at 5 g/L. The effects of CPKO and coconut oil concentrations on the growth and PHA accumulation were further tested by varying the carbon source concentrations from 2.5 to 20.0 g/L. All carbon sources tested were autoclaved at 121 °C for 15 min prior to addition of the oil into the mineral medium (MM).

2.3. Cultivation and PHA synthesis

One-stage batch cultivation in shake flasks was conducted for PHA biosynthesis. Two loops of bacteria (grown for 16–18 h) from an NR plate were grown for 5 h in 50 mL of NR medium at 30 °C and 200 rpm in order to enrich the cell mass. Approximately 3% (v/v) of the inoculum (OD_{600nm} = 4.5 – 5) was transferred into 50 mL of MM broth and incubated for 48 h at 30 °C and 200 rpm for PHA accumulation. The MM was prepared according to the following compositions (per liter): 3.32 g Na_2HPO_4, 2.80 g KH_2PO_4, 0.54 g $(NH_2)_2CO$, 0.25 g $MgSO_4 \cdot 7H_2O$ and 1 mL trace element solution (Doi et al., 1995). The trace element solution consisted of 0.22 g $CoCl_2 \cdot 6H_2O$, 9.7 g $FeCl_3$, 7.8 g $CaCl_2$, 0.12 g $NiCl_2 \cdot 6H_2O$, 0.11 g $CrCl_3 \cdot 6H_2O$ and 0.16 g $CuSO_4 \cdot 5H_2O$ in 1 L of 0.1 N HCl (Kahar et al., 2004). The cells were harvested at the end of the 48-h cultivation period. Cells were pelleted by centrifugation at 8,000 rpm and 4 °C for 5 min using a KUBOTA 6500 centrifuge. Residual oil was removed by addition of approximately 20

mL of hexane to the cell pellet, followed by mixing by vortex and centrifugation at 8,000 rpm and 4 °C for 3 min. A final centrifugation (8,000 rpm, 4 °C for 5 min) was performed after adding 50 mL of distilled water to the pellet to remove the remaining hexane. The harvested cells were frozen at –20 °C for about 24 h prior to freeze drying for 48 h. This process was performed using LABCONCO Free Zone 4.5 L freeze dryer to remove the water from the cells in the frozen state.

2.4. PHA synthase activity analysis

Cells from culture samples at different time intervals were harvested by centrifugation, and cell suspension was prepared by resuspending the cell pellet in 20 mM Tris-HCl (pH 8) in a ratio of 1 g of cells to 5 mL of Tris-HCl. Subsequent disruption through sonication using 20 pulses (10 s) with pauses (10 s) was performed by keeping cell suspension on ice. The activity of PHA synthase was determined from crude extracts of sonicated cells according to the modified spectroscopic assay described previously (de Roo et al., 2000; Takase et al., 2004). The total enzyme activity was determined by measuring the amount of CoA released from 3HB-CoA during polymerization to P(3HB). The assay mixture contained 2 mM 3HB-CoA, 40 mM potassium phosphate buffer (pH 7.5, 30°C), 10 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) and 1 mg/mL BSA. The reaction was initiated by adding 5 µL (10 µg of total protein) of the supernatant from disrupted cells into 395 µL of the above reaction mixture and the absorbance at 412 nm was measured at 30 °C using a Jenway® 6505 UV/Vis Spectrophotometer. The concentration of CoA released during the assay was determined (Gerngross et al., 1994)

using a molar absorption coefficient of 15.6×10^3 M^{-1} cm^{-1} at 412 nm. One unit of enzyme unit (U) is defined as the amount of enzyme that catalyzed the release of 1 μmol CoA per minute.

2.5. Analytical procedures

PHA content and composition were determined by gas chromatography (GC) analysis. Approximately 20 mg of lyophilized cells were subjected to methanolysis in the presence of 15% (v/v) sulfuric acid and 85% (v/v) methanol for 140 min at 100 °C. The resulting hydroxyacyl methyl esters were then analyzed by GC (Braunegg et al., 1978). To extract PHA from lyophilized cells, approximately 3 g of freeze dried cells were mixed with 300 mL of chloroform in a ratio of 1:100 and stirred for 5 days at room temperature. The stirred solution was cooled to room temperature and filtered to remove the cell debris. The filtrate was then concentrated using a rotary evaporator before it was added, drop-wise, into vigorously stirred, cool methanol. The precipitated and purified polymer was then collected and air dried in a fume hood.

2.6. Polymer characterization

The purified and dried extracted polymer was used for various polymer characterizations. For nuclear magnetic resonance (NMR) analysis, a total of 25 mg of polymer sample was dissolved in 1 mL of deuterated chloroform ($CDCl_3$). The ^1H NMR and ^{13}C NMR spectra were measured on a Bruker AVANCE 500 (NC, USA)

spectrometer at 500 MHz at 30 °C. Tetramethylsilane (Me_4Si) was used as an internal chemical shift reference. The molecular weight was determined at 40 °C using a gel permeation chromatography (Agilent 1200 GPC) system equipped with a refractive index detector and SHODEX K-802 and K-806M columns. The samples were prepared by dissolving the extracted PHA in chloroform at a concentration of 1 mg/mL. Chloroform was used as the eluent at a flow rate of 0.8 mL/min. The weight-average molecular weight (M_w), number-average molecular weight (M_n), and polydispersity index (M_w/M_n) were determined from the elution curves obtained by this method. Calorimetric measurements (DSC) of the PHA were conducted using a Perkin Elmer Pyris 1 differential scanning calorimetry thermal analysis system in the range of –30 °C to 200 °C at a heating rate of 20 °C/min. The glass transition temperature (T_g), crystalline melting point (T_m) and enthalpy of fusion (ΔH_m) were determined from the DSC thermogram of the second scan. Thermogravimetric analysis (TGA) was performed using a Mettler-Toledo TGA/SDTA 851 thermobalance with STAR^e thermal analysis software. TGA heating of 10 mg of PHA under a nitrogen atmosphere started at 30 °C and went to 900 °C with a heating rate of 20 °C/min. The decomposition temperature (T_d) at 5% weight loss was determined. Mechanical properties were measured using Shimadzu EZTest tensile tester equipped with 500N load cell. Solution-cast films were prepared using chloroform and allowed to stand for at least 2 weeks at room temperature. Stress-strain test of solution-cast films (10 mm × 5 mm) were then performed at room temperature with a strain rate of 20 mm/min according to procedures described previously (Doi et al., 1995).

3. Results

3.1. Biosynthesis of P(3HB-co-3HHx) copolymer by C. necator strain Re2160/pCB113 from different types of plant oil

The recombinant *C. necator* Re2160/pCB113 has been shown to produce PHA with a significantly high level of 3HHx monomer fraction (25.3 mol%) when grown on palm oil as the sole carbon source (Budde et al., 2011). The current study was aimed at evaluating the effects of provision of different types of plant oils (see Materials and Methods) on the production of PHA with high 3HHx monomer fraction. Cell dry weight, PHA content, and PHA compositions were analyzed and shown in Table 1. In this experiment, growth and PHA production on CPKO or coconut oil gave unexpectedly high molar fractions of 3HHx, which were 56 mol% and 63 mol%, respectively. P(3HB-*co*-3HHx) biosynthesis on the other plant oils tested exhibited concentrations of 3HHx monomer that were similar to each other, ranging from 41mol% to 46mol%. Efficient carbon source utilization and PHA accumulation were observed using all plant oils tested, and cell dry weights and PHA contents ranged from 4.1 – 5.0 g/L and 61 – 77 wt%, respectively. Both CPKO and coconut oil, which have shown better capacity for production of elevated 3HHx concentrations in PHA copolymers, were chosen as carbon sources for further experimentation.

3.2. Biosynthesis of P(3HB-co-3HHx) copolymer by strain Re2160/pCB113 using different concentrations of CPKO or coconut oil as sole carbon source

The effect of different concentrations of CPKO on cell dry weight, PHA content and PHA compositions is shown in Table 2. A maximum 3HHx monomer fraction of 68 mol% was produced by strain Re2160/pCB113 when 2.5 g/L of CPKO was supplied. An increase in the CPKO concentrations from 5.0 g/L onwards showed no significant difference on the 3HHx monomer fraction. The cell dry weight increased to a maximum of 6.73 g/L and decreased to a minimum of 2.78 g/L as the concentration of CPKO was increased from 2.5 g/L to 20.0 g/L. At the same time, the PHA content of the cells showed an increase from 45 wt% to 87 wt% as the concentration of carbon source increased from 2.5 g/L to 20.0 g/L.

Meanwhile, 2.5 g/L of coconut oil showed the highest 3HHx monomer fraction of the copolymer, 70 mol%. The 3HHx monomer fraction of P(3HB-*co*-3HHx) decreased to 56 mol% when 5.0 g/L of coconut oil was added into the medium but increased to 62 mol% as the oil concentration increased to 20.0 g/L. The cell dry weight exhibited no significant changes as the concentration of coconut oil increased from 2.5 g/L to 20.0 g/L. The PHA content of the cells increased from 48 wt% to 79 wt% as the concentration of the supplied carbon source increased, similar to results seen in CPKO cultures.

Generally, both CPKO and coconut oil support the synthesis of PHA with similar 3HHx compositions, approximately 70 mol%, at the same concentration of carbon source, 2.5 g/L. However, the results showed that CPKO, when fed to the initial culture in the 5 – 12.5 g/L range, could support better bacterial growth when compared to coconut oil, as well as high PHA production (Table 2). *C. necator* strain Re2160/pCB113 showed high P(3HB-*co*-3HHx) copolymer productivity concomitant with better overall

growth when CPKO was used as the sole carbon source. Therefore, CPKO was selected for further experiments.

3.3. Time profile for the production of P(3HB-co-3HHx) by C. necator Re2160/pCB113

After selecting a suitable carbon source and concentration for the production of P(3HB-*co*-3HHx), a time profile of copolymer biosynthesis using supplementation of 2.5 g/L CPKO was carried out, in order to study the trend of 3HHx incorporation and overall PHA production over time. For this, cells were harvested at intervals of every 12 h. As shown in Figure 1, the PHA content of the cells did not exhibit significant differences throughout the cultivation. These data show that this recombinant strain has favorable PHA-accumulating ability, as it demonstrated high PHA content at the early stationary growth phase. There were few significant changes in cell dry weight after the first 24 h until the end of the cultivation period (72 h). The 3HHx monomer fraction of the copolymer was the highest at 12 h (70 mol%) and decreased by 24 h and then remained mostly constant over the remainder of the cultivation period.

3.4. PHA synthase activity analysis

In order to determine the activity of the recombinantly expressed PHA synthase from the *C. necator* PHA production strain, CoA release using 3HB-CoA as the substrate was examined during polymerization by cell extracts of Re2160/pCB113 (Figure 2). This strain demonstrated peak synthase activity (577 U/g protein) at 24 h of cultivation.

Activity then dropped drastically to 20 U/g protein and remained constant until 72 h of incubation period. Since only the soluble PHA synthase was measured in this experiment, the levels of granule-bound PHA synthase in the cells were not being detected. The low synthase activity measured from 36 h onwards is likely due to the presence of the majority of PHA synthase molecules in the granule-bound form, which is likely why the activities of these were not measured. Supplemental Table 1 shows the comparison of measurable PHA synthase activities among different strains at early stationary growth phase (24 – 30 h). PHA synthase of this recombinant strain showed intermediate levels of activity when compared to the synthases of wild type *C. necator* H16 (Schubert et al., 1988; Kichise et al., 1999) and *Chromobacterium* sp. USM2 harboring a high activity PHA synthase enzyme (Bhubalan et al., 2011).

3.5. Characterization of P(3HB-co-3HHx) copolymers

The extraction and characterization of copolymers from Re2160/pCB113 cells was performed in order to understand the physical, structural, and thermal properties of the PHA, prior to future application studies. P(3HB-*co*-3HHx) with five different 3HHx monomer compositions, synthesized from the previous biosynthesis experiments, were extracted and subjected to various thermal and mechanical characterizations as described in Materials and Methods. In order to further confirm the presence of high monomer fraction of 3HHx in the P(3HB-*co*-3HHx) copolymer synthesized, ^{1}H NMR analysis was carried out. The ^{1}H NMR spectrum of P(3HB-*co*-3HHx) closely resemble the spectra obtained in the literature (Bhubalan et al., 2011). The monomer fractions of each polymer were calculated based on the intensity ratio of the methyl constituents in the copolymer

from the ^{1}H spectrum. The values of the 3HHx monomer fractions obtained were slightly lower than those detected by gas chromatography (GC) analysis, with a 1 – 4 mol% difference (Supplemental Table 2). From the ^{1}H NMR spectrum, the presence of 3HHx monomer in P(3HB-*co*-3HHx) copolymers produced by strain Re2160/pCB113 from CPKO was confirmed. Figure 4 depicted the 500-MHz ^{13}C NMR spectrum of P(3HB-*co*-70% 3HHx). The ^{13}C chemical shift assignment of four peaks in the carbonyl resonances arose from different diad sequences connecting 3HB and 3HHx units: 3HB*3HB, 3HB*3HHx, 3HHx*3HB, 3HHx*3HHx. The diad sequence distribution data for two monomeric units were compared with Bernoullian statistics applicable to a statistically random copolymerization. The randomness of the copolymers was determined as a parameter, D. D is defined as $(F_{3HB*3HB}F_{3HHx*3HHx})/(F_{3HB*3HHx}F_{3HHx*3HB})$, where F_{x-y} indicates the molar fraction of the X-Y diad sequence. The sequence distributions of 3HB and 3HHx units would be considered a statistically random copolymer when the D value is close to 1, a blocky-natured copolymer when the D value more than 1 and an alternate-natured copolymer when D value smaller than 1. Since the calculated D values for 5 different 3HHx monomer fraction of copolymers were larger than 1 (Supplemental Table 2), this result suggests that monomer distribution in the samples were likely not random (Shimamura et al., 1994; Doi et al., 1995).

The molecular weights of the extracted polymers were analyzed by gel permeation chromatography. The results obtained indicate that the highest weight-average molecular weight (M_w) obtained, 3.47×10^5 Da, was a copolymer containing 32 mol% 3HHx monomer. Generally, P(3HB-*co*-3HHx) containing high 3HHx monomer fraction demonstrates lower molecular weight, much lower than that of P(3HB)

homopolymer produced by wild type *C. necator* H16 (Doi, 1990) The polydispersities (M_w/M_n) of the PHA copolymers tested were in the range of 1.45 to 1.75, similar to values obtained for P(3HB) (Table 3).

Table 4 shows the results of PHA thermal property characterizations performed using DSC and TGA. Values for DSC analysis were taken from the second heating to eliminate the thermal history of the films. The T_g of the copolymers decreased from −1 °C to −12 °C as the 3HHx monomer concentration increased from 32 mol% to 70 mol%. This trend indicated that an increase in the average side-chain length results in the decrease of the T_g value. High fractions of 3HHx monomer in the copolymer are suggested to increase the amorphousness, based on the lower T_g values obtained (Watanabe et al., 2001). No melting temperature (T_m) was detected for the copolymers containing 56 mol%, 60 mol% and 70 mol% 3HHx monomer content. Enthalpy of fusion (ΔH_m) was also not detected for these polymers during the whole analysis. This demonstrated that P(3HB-*co*-3HHx) with a 3HHx monomer fraction >43 mol% was highly amorphous, and crystallization did not occur in the samples studied. As measured by DSC, the crystalinity of P(3HB-*co*-3HHx) decreased as higher concentrations of 3HHx monomer were introduced into the polymer. Determining the thermal stability of polymer is important for understanding of the chemical recycling of polymer materials. The measured thermal degradation temperatures (T_d) of the copolymers remained almost constant (274 – 284 °C) regardless of the amount of 3HHx monomer in the polymer. The overall T_d was slightly lower than that of the homopolymer P(3HB), indicating the volatility of P(3HB-*co*-3HHx) is higher and the thermal stability is lower than P(3HB).

Tensile strength, Young's modulus and elongation to break of the copolymers were tested via tensile tester analysis and data are shown in Table 5. To determine suitable applications for polymers produced, it is crucial to understand the range of mechanical properties preferable for these applications. The tensile strength and Young's modulus of the films decreased from 7.91 MPa to 0.13 MPa and 100.96 MPa to 0.27 MPa, respectively, as the 3HHx monomer fraction was increased from 32 mol% to 70 mol%. The values of tensile strength and Young's modulus define brittleness and stiffness, respectively. We thus conclude that the low tensile strength and Young's modulus values of the copolymers tested here demonstrate that the introduction of 3HHx monomers into the PHA tends to deliver soft and flexible copolymers. On the other hand, the value of elongation to break indicates the elasticity of the polymer. In Table 5, the copolymer P(3HB-*co*-70mol% 3HHx) showed a superior elasticity with the elongation at break of 1074.60%. This value is much higher than low-density polyethylene (LDPE) (700%) (Doi, 1990), a material which is often compared to P(HB-*co*-HHx). The high percentage of 3HHx monomers in the PHA copolymers produced in this study have thus greatly increased the elasticity of the copolymer.

4. Discussion

A prior study showed that the engineered *C. necator* Re2160/pCB113, used also in this study, accumulated PHA with 3HHx monomer fraction of 31 mol%, which was 2-fold higher than that of the PHA produced by engineered strain containing *A. caviae* PHA synthase (15 mol%) (Budde et al., 2011). Based on these interesting findings, the PHA

biosynthesis genes of *R. aetherivorans* I24 and their respective products are worth studying in order to explore their potential in PHA production.

Synthesis and characterization of P(3HB-*co*-3HHx) containing 3HHx monomer fractions ranging from 2 to 35 mol% (Mifune et al., 2010; Ng et al., 2011) and 80 – 100 mol% (Jian et al., 2010; Wang et al., 2011) have been undertaken as stated in the literature. The characteristics of P(3HB-*co*-3HHx) containing 3HHx monomer fractions of 40 to 70 mol% have not been reported until now (Yoke Ming, please check if this statement is correct). The biosynthesis of P(3HB-*co*-3HHx) copolymer with different monomer compositions yields copolymers that have different physical and mechanical properties suitable for different commercial applications.

Plant oils have been shown to be an excellent carbon source for the biosynthesis of PHA in *C. necator*, as they contain a high number of carbon atoms per weight, contributing to robust cell growth and PHA accumulation (Akiyama et al., 2003). To date, various plant oils have been tested and proven as effective and economical carbon sources. For example, soybean oil, jatropha oil, coconut oil and corn oil used as fermentation carbon feedstocks have been reported to result in high yields of PHA. Palm oil and its derivatives are well-known as excellent carbon feedstocks for the production of PHA (Bhubalan et al., 2011; Riedel et al., 2012).

In this study, we examined different plant oils as carbon sources for facilitation of PHA biosynthesis using *C. necator* Re2160/pCB113 as the producing organism. Growth and P(3HB-*co*-3HHx) synthesis using CPKO and coconut oil exhibited a significantly

high 3HHx content when compared to other plant oils (Table 1). The relatively high lauric acid (C12) content in CPKO and coconut oil, which comprises approximately 50% of the total fatty acids, likely contributed to the results observed. It has previously been reported that short chain length fatty acids (C4 – C7) were more favourable for the accumulation of higher 3HHx monomer contents in PHA (Mifune et al., 2008). As is the case with our recombinant *C. necator* strain, the introduction of a *phaJ* gene, encoding a (*R*)-specific enoyl-CoA hydratase, and the deletion of native *phaB* genes also play important roles in promoting high 3HHx content in the resulting PHA copolymer. After 3 cycles of β-oxidation pathway, lauric acid (C12) will be shortened to a six carbon intermediate and continue for a fourth round of β-oxidation. However, the C6 intermediate can be converted by the action of PhaJ to (*R*)-3-hydroxyhexanoyl-CoA (3HHx-CoA) and channelled to polymerization by a broad substrate specificity PHA synthase. Eventually, less C4 intermediates produced from β-oxidation, coupled with less 3HB-CoA production resulting from a disrupted *phaCAB* operon, can lead to a high 3HHx monomer fraction in the synthesized PHA copolymer (Han et al., 2004).

Table 2 shows the comparison of 3HHx monomer fractions produced when different concentrations of CPKO and coconut oil were fed as the sole carbon source, respectively. CPKO was hydrolyzed by *C. necator* to produce various fatty acids such as oleic acid, linoleic acid and palmitic acid. Kahar and coworkers reported that *C. necator* can grow well on certain fatty acids such as, palmitic acid (16:0), oleic acid (18:1), and linoleic acid (18:2), but linolenic acid (18:3) does not promote optimal cell growth (Kahar et al., 2004). Growth of *C. necator* on fatty acids such as oleic acid and linoleic

acid was also confirmed in a more recent study (Riedel et al., 2012). These ideal fatty acids for growth of *C. necator* were found to be abundant in CPKO. Furthermore, linolenic acid was found only in trace amounts. Also, palm oil is more readily available in Malaysia as compared to the availability of coconut oil making it more attractive as a carbon source for PHA production. The advantage of Malaysia as the leading producer and exporter of palm oil in the world today allows resources to be allocated for PHA production from such oils, as well as commercialization and further investigations on polymer production. Generally, CPKO as the sole carbon source for PHA synthesis is feasible, sustainable, and yields more encouraging results compared to other oils.

The time profile study demonstrates the trend of 3HHx monomer fraction incorporation into P(3HB-*co*-3HHx) over time. As shown in Figure 1, the highest 3HHx monomer fraction was observed at the earliest cultivation time, 12 h. This phenomenon requires further study, but Budde and co-workers have proposed that the high concentration of free CoA in the cell cytoplasm, which inhibits the expression of the PhaA enzyme and hence decreases the rate of 3HB-CoA synthesis. As a result, more 3HHx monomer can thus be incorporated into P(3HB-*co*-3HHx) (Budde et al., 2011).

The synthase activity of *R. aetherivorans* I24 expressed by recombinant *C. necator* Re2160/pCB113 showed an activity of 577 U/g of protein at early stationary growth phase, which is significantly higher than the native PHA synthase, $phaC_{Cn}$ (Supplemental Table 1). The correlation between the high 3HHx monomer fraction and the synthase activity measured in this work support the observation that a higher 3HHx fraction in P(3HB-*co*-3HHx) is promoted by the increased PHA synthase activity (Fukui

et al., 2001). It is believed that the granule-bound PHA synthase from *C. necator* exhibits approximately 40 times higher activity compared with the soluble PHA synthase (Gerngross and Martin, 1995). As reported, wild type *Rhodococcus* has the ability to produce scl-PHA copolymer, P(3HB-*co*-3HV) (Hori et al., 2009). However, the constructed *C. necator* strain harbouring *R. aetherivorans* I24 synthase in this study was able to synthesize the mixture of scl- and mcl-PHA copolymer, P(3HB-*co*-3HHx). From the above observations, this PHA synthase has shown broad substrate specificity towards the polymerization of scl-PHA monomers and also a mixture of scl- and mcl-PHA monomers.

The presence of high 3HHx monomer fraction in P(3HB-*co*-3HHx) was further confirmed via the ^{1}H NMR analysis with the assignments shown in Figure 3. ^{13}C NMR analysis also further revealed that the copolymer was a non-random copolymer as opposed to random. The formation of a polymer blend in recombinant *C. necator* Re2160/pCB113 was due mainly to the disrupted *phaCAB* operon and the insertion of *phaJ* gene which facilitates the higher rate of 3HHx monomer polymerization than the 3HB (Budde et al., 2011).

As reported, M_w values are more closely related to material properties than M_n. In fact, a minimum polymer M_w of 3.0×10^6 Da is required for determining material property of PHA (Iwata, 2005). The molecular mass of the PHA depends on several factors: type of PHA synthase, the availability of precursors for PHA synthesis, the availability of enzymes that hydrolyze PHA and the expression level of PHA synthases

(Rehm, 2003). A low molecular mass value will be obtained if the accumulated polyesters are polymerized by a high concentration of active PHA synthase protein in the cells (Sim et al., 1997). The low M_w obtained for this case might be due, in part, to the high expression level of PHA synthase, which comprises both soluble and granule-bound PHA synthases. The concentrations of of bulkier comonomer, 3HHx, in the copolymer may also cause a drastically decreased molecular mass.

In terms of thermal analysis, melting temperature (T_m) of some samples, as well as enthalphy of fusion (ΔH_m), were unable to be detected from DSC analysis. As 3HHx monomer is bulkier than 3HB monomer, the more frequent incorporation of the 3HHx monomer into PHA has disrupted the crystallization of the copolymer and hence eliminated its T_m and ΔH_m. The T_g value of the copolymers decreased from –1 °C to –12 °C as the average monomer side-chain length increased and thus increased the amorphousness of the copolymers. The overall T_d values were slightly lower than that of the homopolymer P(3HB), which is in agreement with previous findings that the T_d values increase with increasing carbon number of the side chain for all the general copolymers, except P(3HB-*co*-3HHx) (Wang et al., 2011).

P(3HB-*co*-70 mol% 3HHx) was determined to be a very elastic material that exhibited an elongation at break value of 1075%. However, this extremely high elastic copolymer posses a very low tensile strength and Young's modulus. This is in accordance with the observation that the copolymer is a gluey and sticky material. Based on the mechanical properties explained above, this type of material has high potential

applications as biodegradable pressure sensitive adhesives, coatings and polymer binding agents in organic- solvent-free paints (van der Walle et al., 1999; Ward et al., 2005), and a chiral pool for production of *R*-3-hydroxyhexanoic acid through its depolymerisation (Reddy et al., 2003). The properties of P(3HB-*co*-32 mol% 3HHx) closely resemble the common petroleum-based polymer, low-density polyethylene (LDPE).

Acknowledgements

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Figure captions:

Fig. 1. (A) Polymer content and monomer composition of P(HB-co-HHx) produced by *C. necator* Re2160/pCB113 grown on 2.5 g/L CPKO as the sole carbon source. (B) Total PHA and total cell dry weight of Re2160/pCB113 during a 72 h time profile experiment of cultures grown on 2.5 g/L CPKO as the sole carbon source. In (A) and (B), all data shown are the means of triplicate tests, and mean data accompanied by different alphabet letters are significantly different (Tukey's HSD test, $p < 0.05$).

Fig. 2. Specific activity of the recombinantly expressed *R. aetherivorans* I24 PhaC enzyme in *C. necator* Re2160/pCB113 cells grown in cultures with 2.5 g/L CPKO as the sole carbon source. CoA release from 3HB-CoA was measured using cell extracts as described in Materials and Methods. One unit (U) of enzyme activity is defined as the amount of enzyme required to catalyze the transformation of 1 μmol substrate per minute. Data shown are the means of triplicate tests. Mean data accompanied by different alphabet letters are significantly different (Tukey's HSD test, $p < 0.05$).

Fig.3. 500-MHz ^{1}H NMR spectrum confirming the presence of 3HHx monomer in P(3HB-*co*-70% 3HHx) copolymer produced by recombinant *C. necator* Re2160/pCB113 from CPKO.

Fig.4. 500-MHz ^{13}C NMR spectrum of P(3HB-*co*-70% 3HHx) copolymer produced by recombinant *C. necator* Re2160/pCB113 from CPKO.

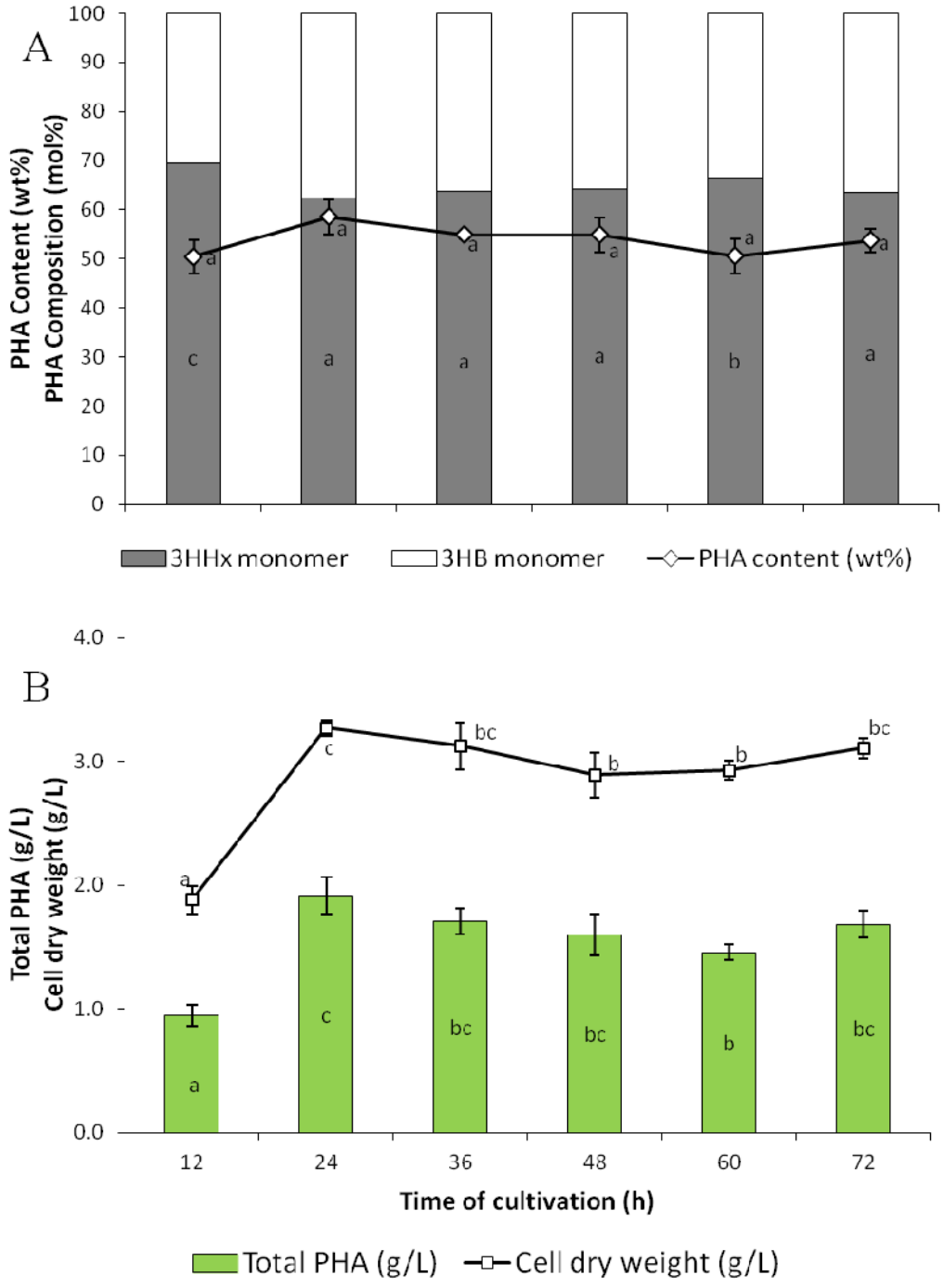

A
PHA Content (wt%)
PHA Composition (mol%)
100
90
80
70
60
50
40
30
20
10
0
c
a
a
a
b
a
a
a
a
a
a
a
3HHx monomer
3HB monomer
PHA content (wt%)
B
Total PHA (g/L)
Cell dry weight (g/L)
4.0
3.0
2.0
1.0
0.0
a
c
bc
bc
b
bc
a
c
bc
bc
b
bc
12
24
36
48
60
72
Time of cultivation (h)
Total PHA (g/L)
Cell dry weight (g/L)

Figure 1

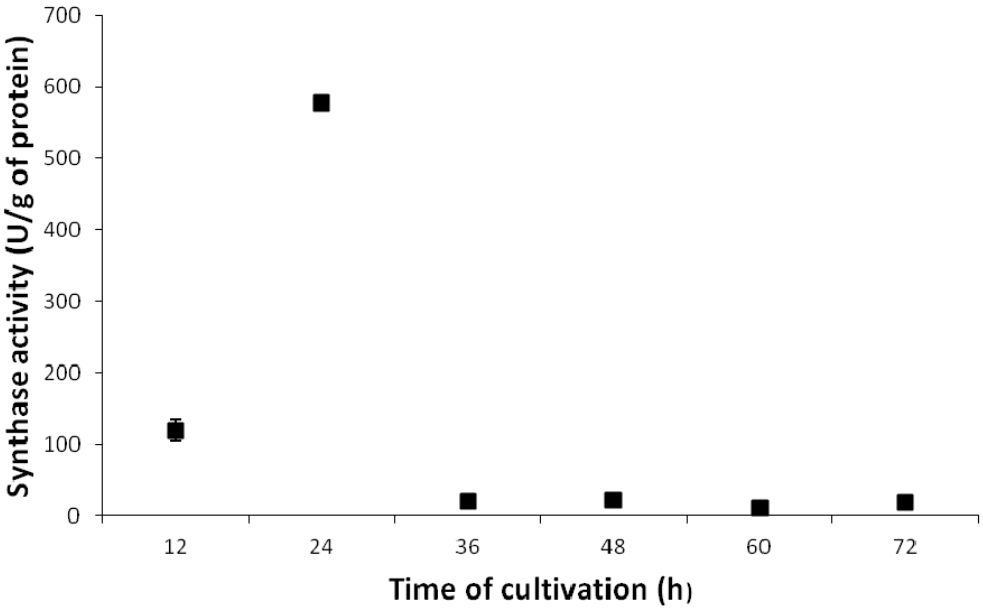
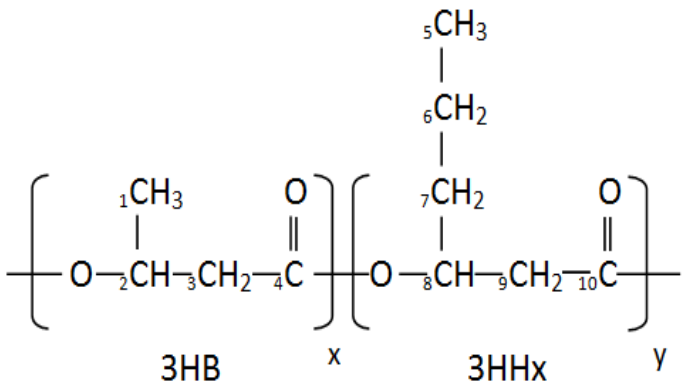

Synthase activity (U/g of protein)
700
600
500
400
300
200
100
0
12
24
36
48
60
72
Time of cultivation (h)

Figure 2


1CH3
O
2CH
3CH2
4C
5CH3
6CH2
7CH2
O
8CH
9CH2
10C
3HB
x
3HHx
y

5
1 , 6
CDCl3
2 , 8
3 , 9
7
10
9
8
7
6
5
4
3
2
1
ppm

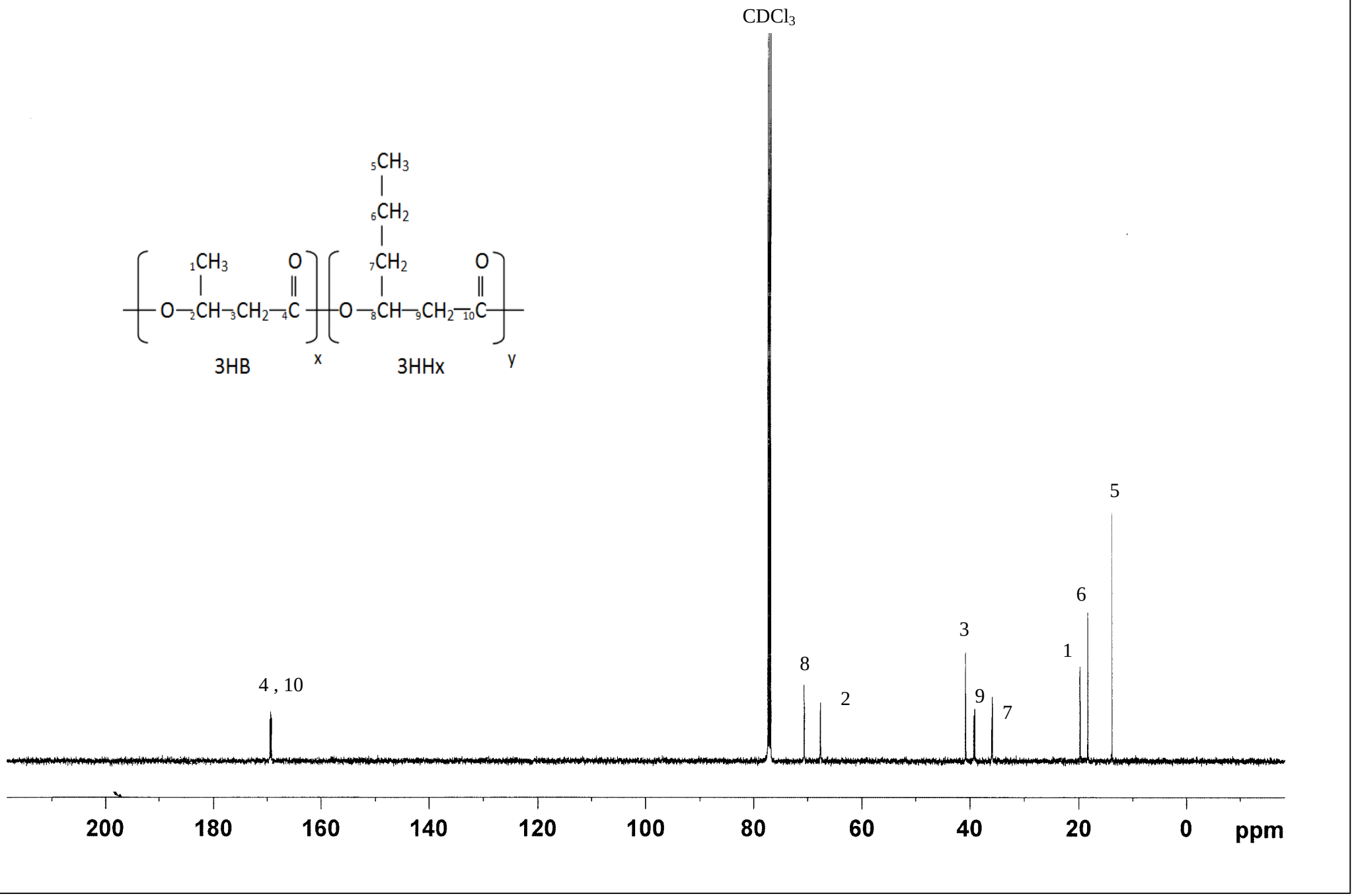

CDCl3
5CH3
6CH2
1CH3
O
7CH2
O
O2CH3CH2 4C
O8CH9CH2 10C
3HB
x
3HHx
y
4 , 10
8
2
3
9
7
1
6
5
200
180
160
140
120
100
80
60
40
20
0
ppm

Table 1 Biosynthesis of P(3HB-*co*-3HHx) containing high 3HHx monomer fraction using different types of plant oil as the sole carbon source

Carbon source[*]	Cell dry weight (g/L)[**]	PHA content (wt%)[†]	Total PHA (g/L)	Monomer composition (mol%)[‡]	
				3HB	3HHx
CPKO	4.96^{b} ± 0.29	77^{c} ± 3	3.8^{c} ± 0.2	44	56^{c}
JO	4.14^{a} ± 0.09	62^{ab} ± 3	2.5^{a} ± 0.2	59	41^{a}
CPO	4.21^{a} ± 0.30	69^{abc} ± 6	2.9^{ab} ± 0.0	59	41^{a}
PO	4.24^{a} ± 0.34	61^{a} ± 4	2.6^{ab} ± 0.4	57	43^{ab}
SBO	4.56^{ab} ± 0.11	65^{ab} ± 1	3.0^{ab} ± 0.1	55	45^{ab}
CO	4.29^{a} ± 0.14	63^{ab} ± 2	2.6^{ab} ± 0.2	54	46^{b}
CcO	4.23^{a} ± 0.19	70^{bc} ± 3	3.3^{bc} ± 0.4	38	62^{d}

CPKO, Crude Palm Kernel Oil; JO, Jatropha Oil; CPO, Crude Palm Oil; PO, Palm Olein; SBO, Soybean Oil; CO, Corn Oil; CcO, Coconut Oil; 3HB, 3-hydroxybutyrate monomer; 3HHx, 3-hydroxyhexanoate monomer

[*]Cells were cultivated in 50 mL MM supplemented with 5 g/L of respective carbon sources for 48 h at 30 °C, 200 rpm in a 250 mL flask

[**]Cell dry weight after freeze-drying

[†]PHA content and [‡]PHA compositions of the freeze-dried cells were determined by gas chromatography

Data shown are the means of triplicate tests. Mean data accompanied by different alphabet letters are significantly different (Tukey's HSD test, $p < 0.05$)

Table 2 Biosynthesis of P(3HB-*co*-3HHx) containing high 3HHx monomer fraction using CPKO or coconut oil as the sole carbon source

	CPKO					CO				
Different concentrations of oil[*] (g/L)	Cell dry weight[**] (g/L)	PHA content[†] (wt%)	Total PHA (g/L)	Monomer composition[‡] (mol%)		Cell dry weight[**] (g/L)	PHA content[†] (wt%)	Total PHA (g/L)	Monomer composition[‡] (mol%)	
				3HB	3HHx				3HB	3HHx
2.5	$2.77^{a} \pm 0.27$	$45^{a} \pm 1$	$1.3^{a} \pm 0.1$	32	68^{b}	$2.61^{a} \pm 0.24$	$48^{ab} \pm 3$	$1.3^{a} \pm 0.2$	30	70^{c}
5.0	$5.08^{b} \pm 0.32$	$74^{b} \pm 4$	$3.7^{bc} \pm 0.2$	44	56^{a}	$2.71^{a} \pm 0.15$	$63^{abc} \pm 7$	$2.3^{a} \pm 0.2$	44	56^{a}
7.5	$6.73^{b} \pm 0.59$	$83^{cd} \pm 3$	$4.4^{bc} \pm 1.9$	45	55^{a}	$3.48^{a} \pm 0.21$	$63^{bc} \pm 3$	$2.2^{a} \pm 0.2$	44	56^{a}
10.0	$6.27^{b} \pm 0.55$	$82^{bcd} \pm 2$	$5.1^{c} \pm 0.3$	43	57^{a}	$3.44^{a} \pm 0.77$	$61^{abc} \pm 6$	$2.1^{a} \pm 0.7$	41	59^{ab}
12.5	$5.41^{b} \pm 0.70$	$81^{bcd} \pm 5$	$4.3^{bc} \pm 0.9$	45	55^{a}	$3.59^{a} \pm 1.19$	$59^{a} \pm 20$	$2.3^{a} \pm 1.6$	40	60^{ab}
15.0	$2.96^{a} \pm 0.10$	$88^{d} \pm 1$	$2.6^{ab} \pm 0.1$	43	57^{a}	$4.49^{a} \pm 0.54$	$74^{cd} \pm 6$	$3.3^{a} \pm 0.3$	38	62^{b}
17.5	$2.97^{a} \pm 0.82$	$79^{bc} \pm 2$	$2.4^{ab} \pm 0.6$	42	58^{a}	$3.93^{a} \pm 0.40$	$75^{cd} \pm 7$	$3.4^{a} \pm 0.0$	39	61^{ab}
20.0	$2.78^{a} \pm 0.29$	$87^{cd} \pm 2$	$2.4^{ab} \pm 0.2$	44	56^{a}	$3.95^{a} \pm 0.10$	$79^{d} \pm 2$	$3.3^{a} \pm 0.3$	38	62^{b}

CPKO, Crude Palm Kernel Oil; CO, Coconut Oil; 3HB, 3-hydroxybutyrate monomer; 3HHx, 3-hydroxyhexanoate monomer

[*]Cells were cultivated in 50 mL MM supplemented with different concentrations of carbon sources for 48 h at 30 °C, 200 rpm in a 250 mL flask

[**]Cell dry weight after freeze-drying

[†]PHA content and [‡]PHA compositions of the freeze-dried cells were determined by gas chromatography

Data shown are the means of triplicate tests. Mean data accompanied by different alphabet letters are significantly different (Tukey's HSD test, $p < 0.05$)

Table 3 Gel permeation chromatography (GPC) analysis of P(3HB-*co*-3HHx) copolymer containing high 3HHx monomer fraction

Sample	M_w (10^5 Da)	M_n (10^5 Da)	M_w/M_n	Reference
P(3HB)	9.00-14.00	1.66-7.37	1.7-2.9	(Doi, 1990)
P(3HB-*co*-32% 3HHx)	$3.47^{c} \pm 0.18$	2.24 ± 0.20	$1.55^{ab} \pm 0.06$	This study
P(3HB-*co*-43% 3HHx)	$1.17^{a} \pm 0.06$	0.72 ± 0.05	$1.63^{bc} \pm 0.03$	This study
P(3HB-*co*-56% 3HHx)	$1.20^{a} \pm 0.10$	0.82 ± 0.06	$1.45^{a} \pm 0.01$	This study
P(3HB-*co*-60% 3HHx)	$2.11^{b} \pm 0.13$	1.26 ± 0.03	$1.75^{c} \pm 0.07$	This study
P(3HB-*co*-70% 3HHx)	$2.27^{b} \pm 0.28$	1.37 ± 0.16	$1.66^{bc} \pm 0.03$	This study

M_w, weight-average molecular weight; M_n, number-average molecular weight; M_w/M_n, polydispersity index

Data shown are the means of triplicate tests. Mean data accompanied by different alphabet letters are significantly different (Tukey's HSD test, $p < 0.05$)

Table 4 Thermal properties of P(3HB-*co*-3HHx) copolymer containing high 3HHx monomer fraction

Sample	T_g * (°C)	T_m * (°C)	ΔH_m ** (Jg^{-1})	T_d * (°C)	Reference
P(3HB)	4	180	60 – 80	287	(Doi, 1990)
P(3HB-*co*-32% 3HHx)	-1	88	N.D	278	This study
P(3HB-*co*-43% 3HHx)	-4	86	N.D	285	This study
P(3HB-*co*-56% 3HHx)	-6	N.D	N.D	274	This study
P(3HB-*co*-60% 3HHx)	-11	N.D	N.D	278	This study
P(3HB-*co*-70% 3HHx)	-12	N.D	N.D	278	This study

T_g, glass transition temperature; T_m, melting temperature; T_d, decomposition temperature; ΔH_m, enthalpy of fusion; N.D, not detected

*Measured by differential scanning calorimetry (DSC)

**Measured by thermogravimetric (TGA) analysis. Temperature was measured at 5% weight loss

Table 5 Mechanical properties of P(3HB-*co*-3HHx) containing high 3HHx monomer fraction as measured by tensile tester

Sample	Tensile Strength (MPa)	Young's Modulus (MPa)	Elongation to break (%)	Reference
P(3HB)	43	3.5	5	(Doi, 1990)
P(3HB-*co*-32% 3HHx)	$7.91^{d} \pm 0.16$	$100.96^{c} \pm 5.70$	$856.25^{b} \pm 20.75$	This study
P(3HB-*co*-43% 3HHx)	$4.65^{c} \pm 0.29$	$75.02^{b} \pm 8.76$	$481.26^{a} \pm 47.03$	This study
P(3HB-*co*-56% 3HHx)	$1.10^{b} \pm 0.09$	$12.07^{a} \pm 1.59$	$367.82^{a} \pm 1.37$	This study
P(3HB-*co*-60% 3HHx)	$0.66^{b} \pm 0.03$	$2.96^{a} \pm 0.18$	$424.24^{a} \pm 22.58$	This study
P(3HB-*co*-70% 3HHx)	$0.13^{a} \pm 0.08$	$0.27^{a} \pm 0.08$	$1074.60^{b} \pm 158.11$	This study
LDPE	15.2 − 78.6	50 − 100	700	(Doi, 1990)

LDPE, low-density polyethylene

Data shown are the means of triplicate tests. Mean data accompanied by different alphabet letters are significantly different (Tukey's HSD test, $p < 0.05$)

Supplemental Table 1 Comparison of PHA synthase activity among different strains at early stationary growth phase (24 − 30 hr).

Strain	Carbon source	PHA content (wt%)	Synthase activity (U/g of protein)	Reference
Escherichia coli JM109 harboring *Chromobacterium* sp. USM2 synthase	Glucose	76	2462	(Bhubalan *et al.*, 2011)
Cupriavidus necator Re2160/pCB113	CPKO	59	577	This study
Cupriavidus necator H16	Fructose	67	180 – 327	(Schubert *et al.*, 1988; Kichise *et al.*, 1999)

CPKO, crude palm kernel oil

Supplemental Table 2 Monomer fraction of 3HHx and randomness of the P(3HB-*co*-3HHx) copolymers analyzed using ^{1}H and ^{13}C NMR spectroscopy respectively.

Sample	Monomer composition* (mol%)		D**
	3HB	3HHx	
P(3HB-*co*-32% 3HHx)	68 (71)	32 (29)	2.31
P(3HB-*co*-43% 3HHx)	57 (60)	43 (40)	2.00
P(3HB-*co*-56% 3HHx)	44 (48)	56 (52)	2.15
P(3HB-*co*-60% 3HHx)	40 (42)	60 (58)	2.35
P(3HB-*co*-70% 3HHx)	30 (31)	70 (69)	1.37

3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; D, randomness

*Values in parentheses were determined by ^{1}H NMR spectra, for comparison with values obtained by GC (no parentheses)

**Randomness of the copolymer was determined by ^{13}C NMR spectra