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2 **Abstract**

3 The potential of plant oils as sole carbon sources for production of P(3HB-*co*-
4 3HHx) copolymer containing a high 3HHx monomer fraction using the recombinant
5 *Cupriavidus necator* strain Re2160/pCB113 has been investigated. Various types and
6 concentrations of plant oils were evaluated for efficient conversion of P(3HB-*co*-3HHx)
7 copolymer. Crude palm kernel oil (CPKO) at a concentration of 2.5 g/L was found to be
8 most suitable for production of copolymer with a 3HHx content of approximately 70
9 mol%. The time profile of these cells was also examined in order to study the trend of
10 3HHx monomer incorporation, PHA production and PHA synthase activity. ¹H NMR and
11 ¹³C NMR analyses confirmed the presence of P(3HB-*co*-3HHx) copolymer containing a
12 high 3HHx monomer fraction, in which monomers were not randomly distributed. The
13 results of various characterization analyses revealed that the copolymers containing a
14 high 3HHx monomer fraction demonstrated soft and flexible mechanical properties.

15

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

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1 **1. Introduction**

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

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

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

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

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

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

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

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

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

5

6 **2. Materials and methods**

7 *2.1. Bacterial strain and maintenance*

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

18

19 *2.2. Carbon sources*

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

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

9

10 2.3. *Cultivation and PHA synthesis*

11 One-stage batch cultivation in shake flasks was conducted for PHA biosynthesis.
12 Two loops of bacteria (grown for 16–18 h) from an NR plate were grown for 5 h in 50
13 mL of NR medium at 30 °C and 200 rpm in order to enrich the cell mass. Approximately
14 3% (v/v) of the inoculum ($OD_{600nm} = 4.5 - 5$) was transferred into 50 mL of MM broth
15 and incubated for 48 h at 30 °C and 200 rpm for PHA accumulation. The MM was
16 prepared according to the following compositions (per liter): 3.32 g Na_2HPO_4 , 2.80 g
17 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
18 al., 1995). The trace element solution consisted of 0.22 g $CoCl_2 \cdot 6H_2O$, 9.7 g $FeCl_3$, 7.8 g
19 $CaCl_2$, 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
20 HCl (Kahar et al., 2004). The cells were harvested at the end of the 48-h cultivation
21 period. Cells were pelleted by centrifugation at 8,000 rpm and 4 °C for 5 min using a
22 KUBOTA 6500 centrifuge. Residual oil was removed by addition of approximately 20

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

7

8 2.4. *PHA synthase activity analysis*

9 Cells from culture samples at different time intervals were harvested by
10 centrifugation, and cell suspension was prepared by resuspending the cell pellet in 20
11 mM Tris-HCl (pH 8) in a ratio of 1 g of cells to 5 mL of Tris-HCl. Subsequent disruption
12 through sonication using 20 pulses (10 s) with pauses (10 s) was performed by keeping
13 cell suspension on ice. The activity of PHA synthase was determined from crude extracts
14 of sonicated cells according to the modified spectroscopic assay described previously (de
15 Roo et al., 2000; Takase et al., 2004). The total enzyme activity was determined by
16 measuring the amount of CoA released from 3HB-CoA during polymerization to P(3HB).
17 The assay mixture contained 2 mM 3HB-CoA, 40 mM potassium phosphate buffer (pH
18 7.5, 30°C), 10 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) and 1 mg/mL BSA. The
19 reaction was initiated by adding 5 µL (10 µg of total protein) of the supernatant from
20 disrupted cells into 395 µL of the above reaction mixture and the absorbance at 412 nm
21 was measured at 30 °C using a Jenway® 6505 UV/Vis Spectrophotometer. The
22 concentration of CoA released during the assay was determined (Gerngross et al., 1994)

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

5 2.5. *Analytical procedures*

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

17 2.6. *Polymer characterization*

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

1 spectrometer at 500 MHz at 30 °C. Tetramethylsilane (Me₄Si) was used as an internal
2 chemical shift reference. The molecular weight was determined at 40 °C using a gel
3 permeation chromatography (Agilent 1200 GPC) system equipped with a refractive index
4 detector and SHODEX K-802 and K-806M columns. The samples were prepared by
5 dissolving the extracted PHA in chloroform at a concentration of 1 mg/mL. Chloroform
6 was used as the eluent at a flow rate of 0.8 mL/min. The weight-average molecular
7 weight (M_w), number-average molecular weight (M_n), and polydispersity index (M_w/M_n)
8 were determined from the elution curves obtained by this method. Calorimetric
9 measurements (DSC) of the PHA were conducted using a Perkin Elmer Pyris 1
10 differential scanning calorimetry thermal analysis system in the range of -30 °C to 200
11 °C at a heating rate of 20 °C/min. The glass transition temperature (T_g), crystalline
12 melting point (T_m) and enthalpy of fusion (ΔH_m) were determined from the DSC
13 thermogram of the second scan. Thermogravimetric analysis (TGA) was performed using
14 a Mettler-Toledo TGA/SDTA 851 thermobalance with STAR^e thermal analysis software.
15 TGA heating of 10 mg of PHA under a nitrogen atmosphere started at 30 °C and went to
16 900 °C with a heating rate of 20 °C/min. The decomposition temperature (T_d) at 5%
17 weight loss was determined. Mechanical properties were measured using Shimadzu
18 EZTest tensile tester equipped with 500N load cell. Solution-cast films were prepared
19 using chloroform and allowed to stand for at least 2 weeks at room temperature. Stress-
20 strain test of solution-cast films (10 mm × 5 mm) were then performed at room
21 temperature with a strain rate of 20 mm/min according to procedures described
22 previously (Doi et al., 1995).

23

1 **3. Results**

2 *3.1. Biosynthesis of P(3HB-co-3HHx) copolymer by C. necator strain Re2160/pCB113*
3 *from different types of plant oil*

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

19

20 *3.2. Biosynthesis of P(3HB-co-3HHx) copolymer by strain Re2160/pCB113 using*
21 *different concentrations of CPKO or coconut oil as sole carbon source*

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

10 Meanwhile, 2.5 g/L of coconut oil showed the highest 3HHx monomer fraction of
11 the copolymer, 70 mol%. The 3HHx monomer fraction of P(3HB-*co*-3HHx) decreased to
12 56 mol% when 5.0 g/L of coconut oil was added into the medium but increased to 62
13 mol% as the oil concentration increased to 20.0 g/L. The cell dry weight exhibited no
14 significant changes as the concentration of coconut oil increased from 2.5 g/L to 20.0 g/L.
15 The PHA content of the cells increased from 48 wt% to 79 wt% as the concentration of
16 the supplied carbon source increased, similar to results seen in CPKO cultures.

17 Generally, both CPKO and coconut oil support the synthesis of PHA with similar
18 3HHx compositions, approximately 70 mol%, at the same concentration of carbon
19 source, 2.5 g/L. However, the results showed that CPKO, when fed to the initial culture
20 in the 5 – 12.5 g/L range, could support better bacterial growth when compared to
21 coconut oil, as well as high PHA production (Table 2). *C. necator* strain Re2160/pCB113
22 showed high P(3HB-*co*-3HHx) copolymer productivity concomitant with better overall

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

3

4 *3.3. Time profile for the production of P(3HB-co-3HHx) by C. necator* 5 *Re2160/pCB113*

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

17

18 *3.4. PHA synthase activity analysis*

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

1 Activity then dropped drastically to 20 U/g protein and remained constant until 72 h of
2 incubation period. Since only the soluble PHA synthase was measured in this experiment,
3 the levels of granule-bound PHA synthase in the cells were not being detected. The low
4 synthase activity measured from 36 h onwards is likely due to the presence of the
5 majority of PHA synthase molecules in the granule-bound form, which is likely why the
6 activities of these were not measured. Supplemental Table 1 shows the comparison of
7 measurable PHA synthase activities among different strains at early stationary growth
8 phase (24 – 30 h). PHA synthase of this recombinant strain showed intermediate levels of
9 activity when compared to the synthases of wild type *C. necator* H16 (Schubert et al.,
10 1988; Kichise et al., 1999) and *Chromobacterium* sp. USM2 harboring a high activity
11 PHA synthase enzyme (Bhubalan et al., 2011).

12

13 3.5. Characterization of P(3HB-co-3HHx) copolymers

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

1 from the ^1H spectrum. The values of the 3HHx monomer fractions obtained were slightly
2 lower than those detected by gas chromatography (GC) analysis, with a 1 – 4 mol%
3 difference (Supplemental Table 2). From the ^1H NMR spectrum, the presence of 3HHx
4 monomer in P(3HB-*co*-3HHx) copolymers produced by strain Re2160/pCB113 from
5 CPKO was confirmed. Figure 4 depicted the 500-MHz ^{13}C NMR spectrum of P(3HB-*co*-
6 70% 3HHx). The ^{13}C chemical shift assignment of four peaks in the carbonyl resonances
7 arose from different diad sequences connecting 3HB and 3HHx units: 3HB*3HB,
8 3HB*3HHx, 3HHx*3HB, 3HHx*3HHx. The diad sequence distribution data for two
9 monomeric units were compared with Bernoullian statistics applicable to a statistically
10 random copolymerization. The randomness of the copolymers was determined as a
11 parameter, D. D is defined as $(F_{3\text{HB}^*3\text{HB}}F_{3\text{HHx}^*3\text{HHx}})/(F_{3\text{HB}^*3\text{HHx}}F_{3\text{HHx}^*3\text{HB}})$, where F_{x-y}
12 indicates the molar fraction of the X-Y diad sequence. The sequence distributions of 3HB
13 and 3HHx units would be considered a statistically random copolymer when the D value
14 is close to 1, a blocky-natured copolymer when the D value more than 1 and an alternate-
15 natured copolymer when D value smaller than 1. Since the calculated D values for 5
16 different 3HHx monomer fraction of copolymers were larger than 1 (Supplemental Table
17 2), this result suggests that monomer distribution in the samples were likely not random
18 (Shimamura et al., 1994; Doi et al., 1995).

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

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

4 Table 4 shows the results of PHA thermal property characterizations performed
5 using DSC and TGA. Values for DSC analysis were taken from the second heating to
6 eliminate the thermal history of the films. The T_g of the copolymers decreased from -1 °C
7 to -12 °C as the 3HHx monomer concentration increased from 32 mol% to 70 mol%.
8 This trend indicated that an increase in the average side-chain length results in the
9 decrease of the T_g value. High fractions of 3HHx monomer in the copolymer are
10 suggested to increase the amorphousness, based on the lower T_g values obtained
11 (Watanabe et al., 2001). No melting temperature (T_m) was detected for the copolymers
12 containing 56 mol%, 60 mol% and 70 mol% 3HHx monomer content. Enthalpy of fusion
13 (ΔH_m) was also not detected for these polymers during the whole analysis. This
14 demonstrated that P(3HB-*co*-3HHx) with a 3HHx monomer fraction >43 mol% was
15 highly amorphous, and crystallization did not occur in the samples studied. As measured
16 by DSC, the crystallinity of P(3HB-*co*-3HHx) decreased as higher concentrations of
17 3HHx monomer were introduced into the polymer. Determining the thermal stability of
18 polymer is important for understanding of the chemical recycling of polymer materials.
19 The measured thermal degradation temperatures (T_d) of the copolymers remained almost
20 constant (274 – 284 °C) regardless of the amount of 3HHx monomer in the polymer. The
21 overall T_d was slightly lower than that of the homopolymer P(3HB), indicating the
22 volatility of P(3HB-*co*-3HHx) is higher and the thermal stability is lower than P(3HB).

1 Tensile strength, Young's modulus and elongation to break of the copolymers
2 were tested via tensile tester analysis and data are shown in Table 5. To determine
3 suitable applications for polymers produced, it is crucial to understand the range of
4 mechanical properties preferable for these applications. The tensile strength and Young's
5 modulus of the films decreased from 7.91 MPa to 0.13 MPa and 100.96 MPa to 0.27
6 MPa, respectively, as the 3HHx monomer fraction was increased from 32 mol% to 70
7 mol%. The values of tensile strength and Young's modulus define brittleness and
8 stiffness, respectively. We thus conclude that the low tensile strength and Young's
9 modulus values of the copolymers tested here demonstrate that the introduction of 3HHx
10 monomers into the PHA tends to deliver soft and flexible copolymers. On the other hand,
11 the value of elongation to break indicates the elasticity of the polymer. In Table 5, the
12 copolymer P(3HB-co-70mol% 3HHx) showed a superior elasticity with the elongation at
13 break of 1074.60%. This value is much higher than low-density polyethylene (LDPE)
14 (700%) (Doi, 1990), a material which is often compared to P(HB-co-HHx). The high
15 percentage of 3HHx monomers in the PHA copolymers produced in this study have thus
16 greatly increased the elasticity of the copolymer.

17

18 **4. Discussion**

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

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

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

11

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

20

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

1 high 3HHx content when compared to other plant oils (Table 1). The relatively high
2 lauric acid (C12) content in CPKO and coconut oil, which comprises approximately 50%
3 of the total fatty acids, likely contributed to the results observed. It has previously been
4 reported that short chain length fatty acids (C4 – C7) were more favourable for the
5 accumulation of higher 3HHx monomer contents in PHA (Mifune et al., 2008). As is the
6 case with our recombinant *C. necator* strain, the introduction of a *phaJ* gene, encoding a
7 (*R*)-specific enoyl-CoA hydratase, and the deletion of native *phaB* genes also play
8 important roles in promoting high 3HHx content in the resulting PHA copolymer. After 3
9 cycles of β -oxidation pathway, lauric acid (C12) will be shortened to a six carbon
10 intermediate and continue for a fourth round of β -oxidation. However, the C6
11 intermediate can be converted by the action of PhaJ to (*R*)-3-hydroxyhexanoyl-CoA
12 (3HHx-CoA) and channelled to polymerization by a broad substrate specificity PHA
13 synthase. Eventually, less C4 intermediates produced from β -oxidation, coupled with less
14 3HB-CoA production resulting from a disrupted *phaCAB* operon, can lead to a high
15 3HHx monomer fraction in the synthesized PHA copolymer (Han et al., 2004).

16

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

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

10 The time profile study demonstrates the trend of 3HHx monomer fraction
11 incorporation into P(3HB-*co*-3HHx) over time. As shown in Figure 1, the highest 3HHx
12 monomer fraction was observed at the earliest cultivation time, 12 h. This phenomenon
13 requires further study, but Budde and co-workers have proposed that the high
14 concentration of free CoA in the cell cytoplasm, which inhibits the expression of the
15 PhaA enzyme and hence decreases the rate of 3HB-CoA synthesis. As a result, more
16 3HHx monomer can thus be incorporated into P(3HB-*co*-3HHx) (Budde et al., 2011).

17

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

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

10

11 The presence of high 3HHx monomer fraction in P(3HB-*co*-3HHx) was further
12 confirmed via the ¹H NMR analysis with the assignments shown in Figure 3. ¹³C NMR
13 analysis also further revealed that the copolymer was a non-random copolymer as
14 opposed to random. The formation of a polymer blend in recombinant *C. necator*
15 Re2160/pCB113 was due mainly to the disrupted *phaCAB* operon and the insertion of
16 *phaJ* gene which facilitates the higher rate of 3HHx monomer polymerization than the
17 3HB (Budde et al., 2011).

18

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

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

7

8 In terms of thermal analysis, melting temperature (T_m) of some samples, as well
9 as enthalpy of fusion (ΔH_m), were unable to be detected from DSC analysis. As 3HHx
10 monomer is bulkier than 3HB monomer, the more frequent incorporation of the 3HHx
11 monomer into PHA has disrupted the crystallization of the copolymer and hence
12 eliminated its T_m and ΔH_m . The T_g value of the copolymers decreased from -1 °C to -12
13 °C as the average monomer side-chain length increased and thus increased the
14 amorphousness of the copolymers. The overall T_d values were slightly lower than that of
15 the homopolymer P(3HB), which is in agreement with previous findings that the T_d
16 values increase with increasing carbon number of the side chain for all the general
17 copolymers, except P(3HB-*co*-3HHx) (Wang et al., 2011).

18

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

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

6

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8

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4

5

6 **Figure captions:**

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

13

14

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

22

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

26

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

29

30

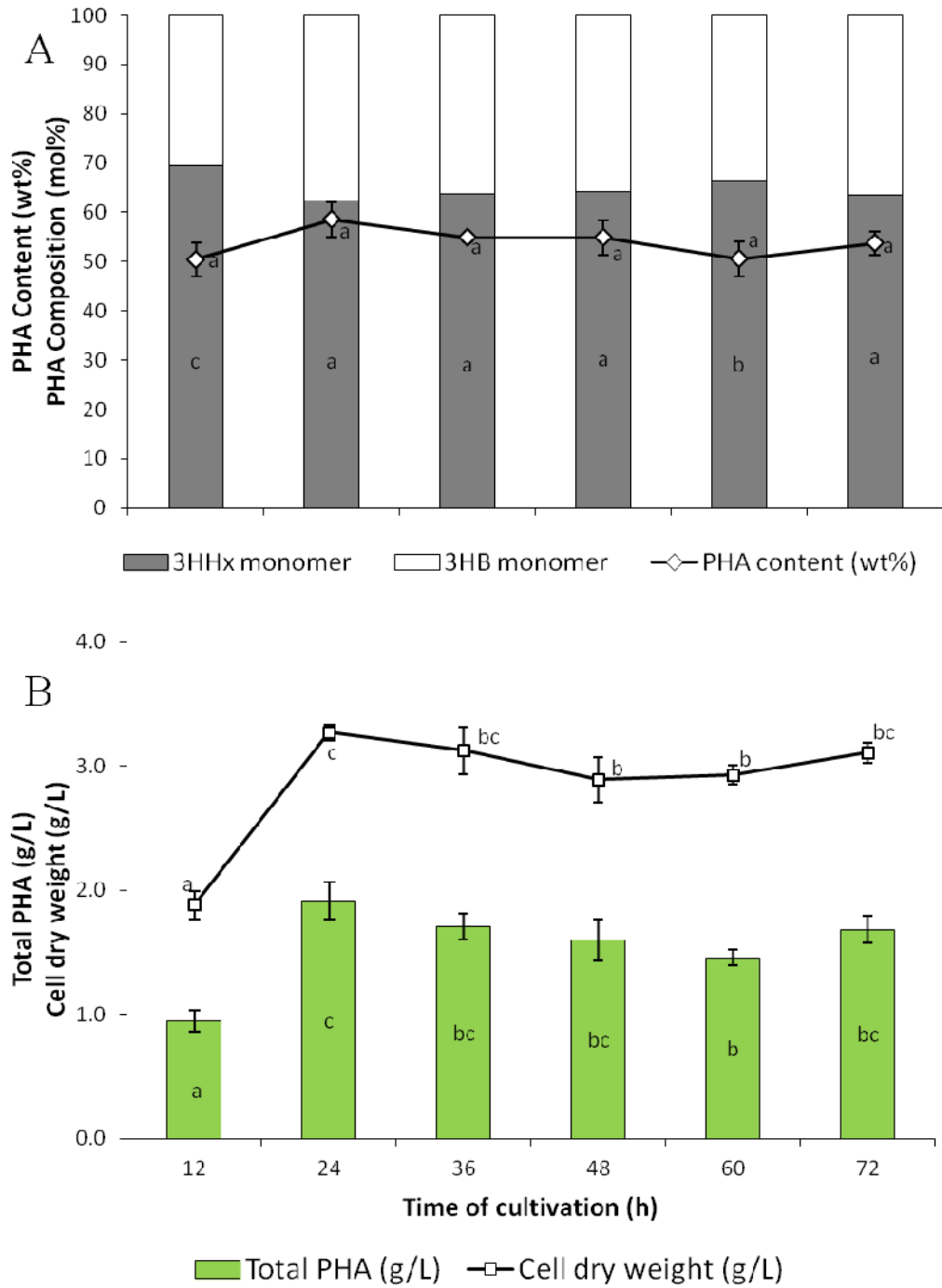


Figure 1

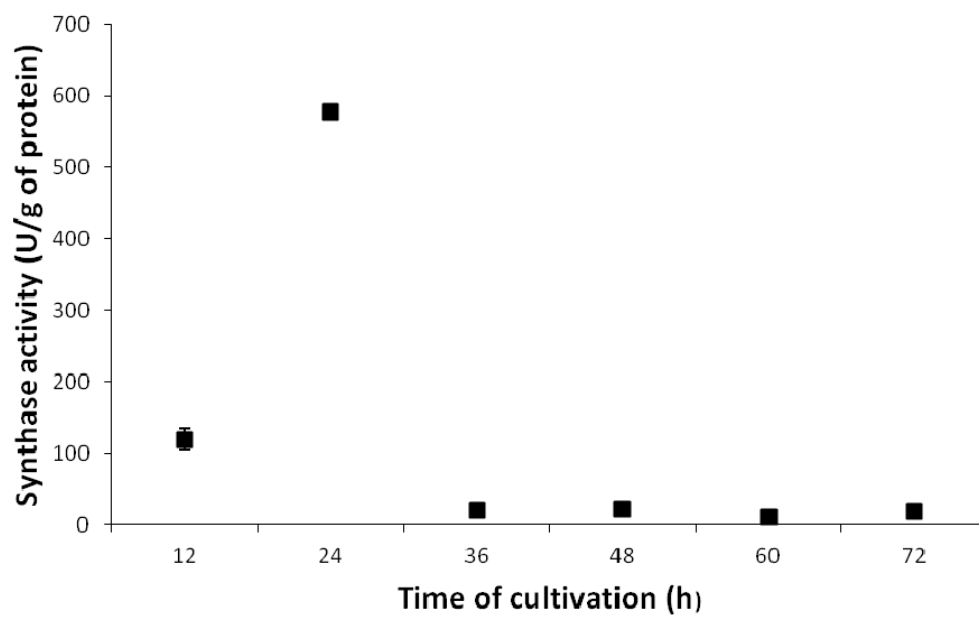
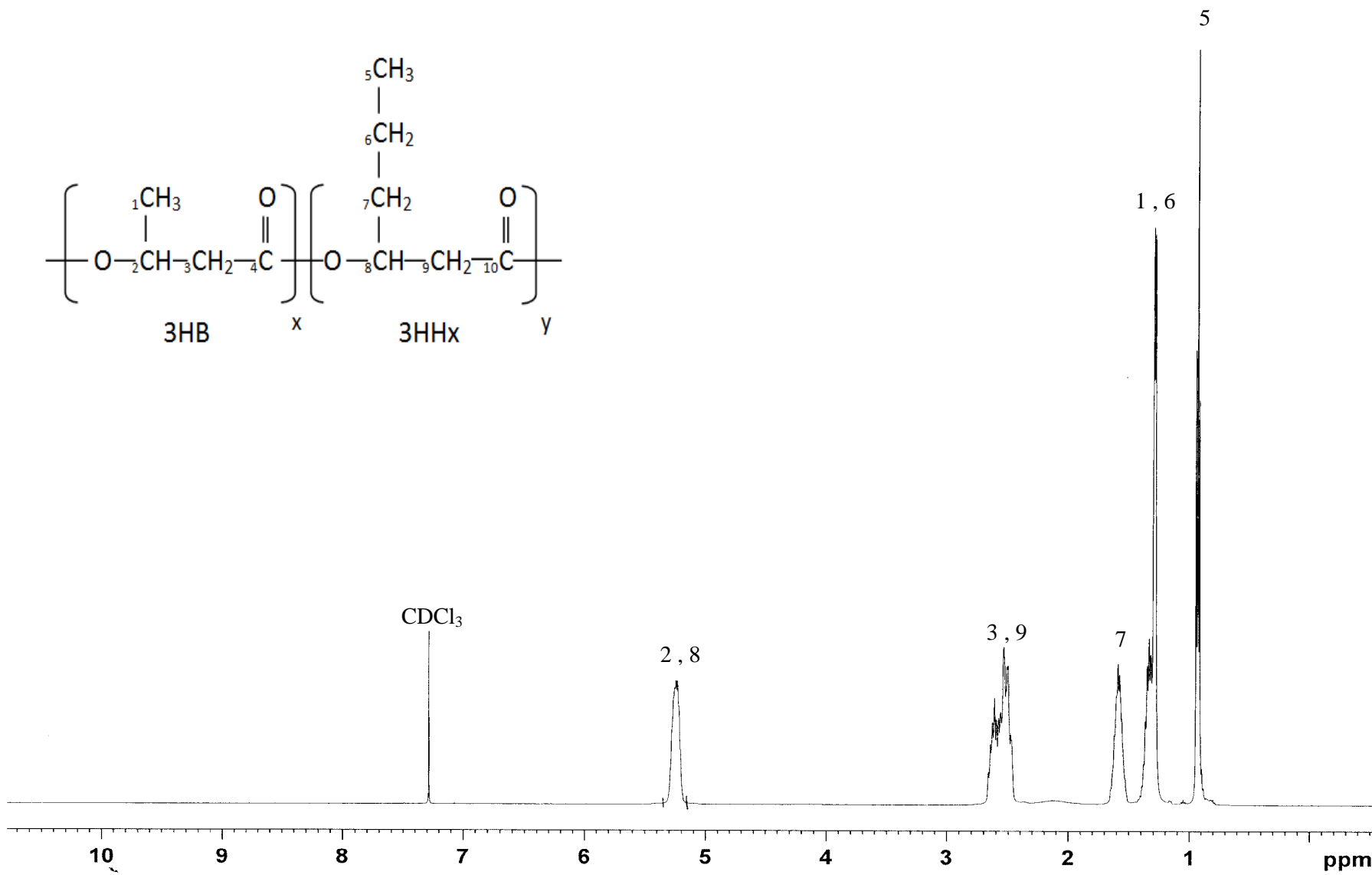
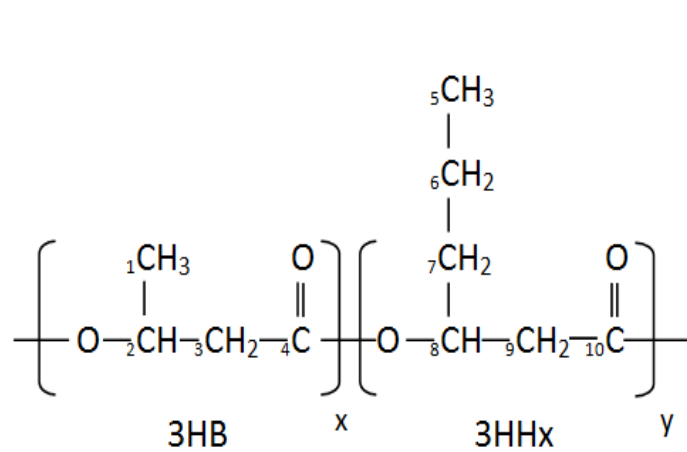


Figure 2



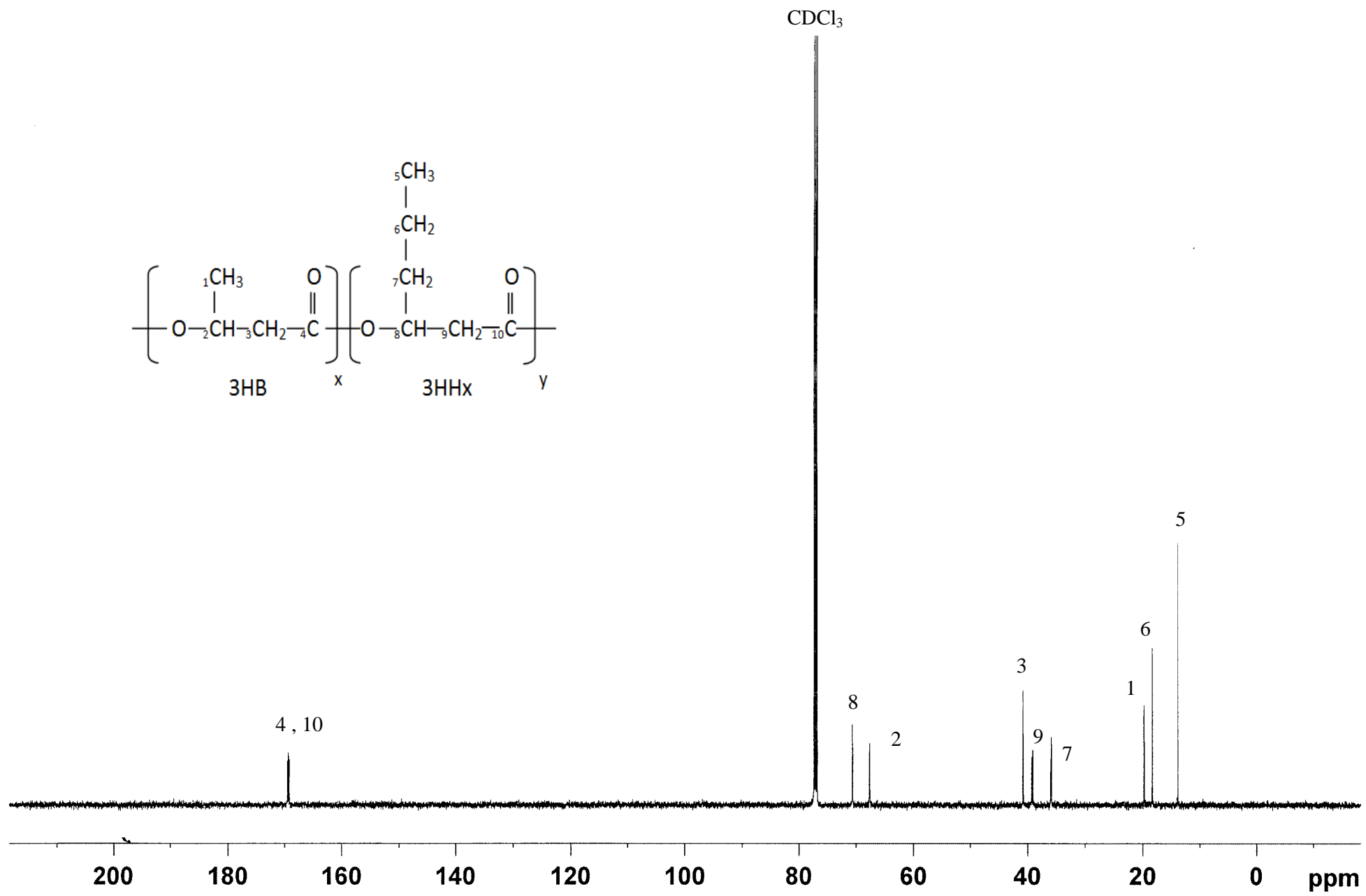
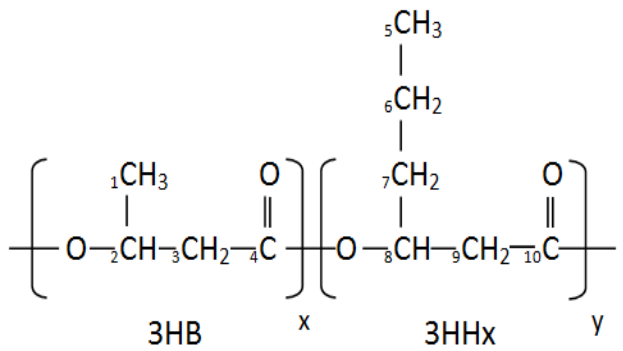


Table 1 Biosynthesis of P(3HB-*co*-3HHx) containing high 3HHx monomer fraction using different types of plant oil as the sole carbon source

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

Different concentrations of oil* (g/L)	CPKO					CO				
	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 ± 0.27	45 ^a ± 1	1.3 ^a ± 0.1	32	68 ^b	2.61 ^a ± 0.24	48 ^{ab} ± 3	1.3 ^a ± 0.2	30	70 ^c
5.0	5.08 ^b ± 0.32	74 ^b ± 4	3.7 ^{bc} ± 0.2	44	56 ^a	2.71 ^a ± 0.15	63 ^{abc} ± 7	2.3 ^a ± 0.2	44	56 ^a
7.5	6.73 ^b ± 0.59	83 ^{cd} ± 3	4.4 ^{bc} ± 1.9	45	55 ^a	3.48 ^a ± 0.21	63 ^{bc} ± 3	2.2 ^a ± 0.2	44	56 ^a
10.0	6.27 ^b ± 0.55	82 ^{bcd} ± 2	5.1 ^c ± 0.3	43	57 ^a	3.44 ^a ± 0.77	61 ^{abc} ± 6	2.1 ^a ± 0.7	41	59 ^{ab}
12.5	5.41 ^b ± 0.70	81 ^{bcd} ± 5	4.3 ^{bc} ± 0.9	45	55 ^a	3.59 ^a ± 1.19	59 ^a ± 20	2.3 ^a ± 1.6	40	60 ^{ab}
15.0	2.96 ^a ± 0.10	88 ^d ± 1	2.6 ^{ab} ± 0.1	43	57 ^a	4.49 ^a ± 0.54	74 ^{cd} ± 6	3.3 ^a ± 0.3	38	62 ^b
17.5	2.97 ^a ± 0.82	79 ^{bc} ± 2	2.4 ^{ab} ± 0.6	42	58 ^a	3.93 ^a ± 0.40	75 ^{cd} ± 7	3.4 ^a ± 0.0	39	61 ^{ab}
20.0	2.78 ^a ± 0.29	87 ^{cd} ± 2	2.4 ^{ab} ± 0.2	44	56 ^a	3.95 ^a ± 0.10	79 ^d ± 2	3.3 ^a ± 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- <i>co</i> -32% 3HHx)	$3.47^c \pm 0.18$	2.24 ± 0.20	$1.55^{ab} \pm 0.06$	This study
P(3HB- <i>co</i> -43% 3HHx)	$1.17^a \pm 0.06$	0.72 ± 0.05	$1.63^{bc} \pm 0.03$	This study
P(3HB- <i>co</i> -56% 3HHx)	$1.20^a \pm 0.10$	0.82 ± 0.06	$1.45^a \pm 0.01$	This study
P(3HB- <i>co</i> -60% 3HHx)	$2.11^b \pm 0.13$	1.26 ± 0.03	$1.75^c \pm 0.07$	This study
P(3HB- <i>co</i> -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- <i>co</i> -32% 3HHx)	-1	88	N.D	278	This study
P(3HB- <i>co</i> -43% 3HHx)	-4	86	N.D	285	This study
P(3HB- <i>co</i> -56% 3HHx)	-6	N.D	N.D	274	This study
P(3HB- <i>co</i> -60% 3HHx)	-11	N.D	N.D	278	This study
P(3HB- <i>co</i> -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- <i>co</i> -32% 3HHx)	7.91 ^d ± 0.16	100.96 ^c ± 5.70	856.25 ^b ± 20.75	This study
P(3HB- <i>co</i> -43% 3HHx)	4.65 ^c ± 0.29	75.02 ^b ± 8.76	481.26 ^a ± 47.03	This study
P(3HB- <i>co</i> -56% 3HHx)	1.10 ^b ± 0.09	12.07 ^a ± 1.59	367.82 ^a ± 1.37	This study
P(3HB- <i>co</i> -60% 3HHx)	0.66 ^b ± 0.03	2.96 ^a ± 0.18	424.24 ^a ± 22.58	This study
P(3HB- <i>co</i> -70% 3HHx)	0.13 ^a ± 0.08	0.27 ^a ± 0.08	1074.60 ^b ± 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
<i>Escherichia coli</i> JM109 harboring <i>Chromobacterium</i> sp. USM2 synthase	Glucose	76	2462	(Bhubalan <i>et al.</i> , 2011)
<i>Cupriavidus necator</i> Re2160/pCB113	CPKO	59	577	This study
<i>Cupriavidus necator</i> H16	Fructose	67	180 – 327	(Schubert <i>et al.</i> , 1988; Kichise <i>et al.</i> , 1999)

CPKO, crude palm kernel oil

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

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

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

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

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