Appl Biochem Biotechnol (2011) 164:867–877
DOI 10.1007/s12010-011-9180-8

Biosynthesis and Characterization of Poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-4-hydroxybutyrate) Terpolymer with Various Monomer Compositions by *Cupriavidus* sp. USMAA2-4

Hema Ramachandran · Nurhezreen Md Iqbal · Coswald Stephen Sipaut · Amirul Al-Ashraf Abdullah

Received: 12 October 2010 / Accepted: 18 January 2011 /
Published online: 8 February 2011
© Springer Science+Business Media, LLC 2011

Abstract Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-4-hydroxybutyrate) [P(3HB-*co*-3HV-*co*-4HB)] terpolymer was produced using *Cupriavidus* sp. USMAA2-4 via one-step cultivation process through combination of various carbon sources such as 1,4-butanediol or γ-butyrolactone with either 1-pentanol, valeric acid, or 1-propanol. Oleic acid was added to increase the biomass production. The composition of 3HV and 4HB monomers were greatly affected by the concentration of 1,4-butanediol and 1-pentanol. Terpolymers with 3HV and 4HB molar fractions ranging from 2 to 41 mol.% and 5 to 31 mol.%, respectively, were produced by varying the concentration of carbon precursors. The thermal and mechanical properties of the terpolymers containing different proportions of the constituent monomers were characterized using gel permeation chromatography (GPC), DSC, and tensile machine. GPC analysis showed that the molecular weights (M_w) of the terpolymer produced were within the range of 346 to 1,710 kDa. The monomer compositions of 3HV and 4HB were also found to have great influences on the thermal and mechanical properties of the terpolymer P(3HB-*co*-3HV-*co*-4HB) produced.

Keywords Biosynthesis · Terpolymer · One-step cultivation · 3-Hydroxyvalerate · 4-Hydroxybutyrate

---

H. Ramachandran · N. M. Iqbal · A. A.-A. Abdullah
School of Biological Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia

A. A.-A. Abdullah (✉)
Malaysian Institute of Pharmaceuticals and Nutraceuticals, MOSTI, 11900 Bayan Lepas, Penang, Malaysia
e-mail: amirul@usm.my

C. S. Sipaut
Chemical Engineering, School of Engineering and Information Technology, Universiti Malaysia Sabah, 88999 Kota Kinabalu, Sabah, Malaysia

Introduction

In response to increasing public concern on the deleterious effects of petroleum-derived plastics in the environment, biodegradable plastics are seen as a potential solution. However, these biodegradable plastic materials must retain the desired material properties of conventional plastics [1]. Among the candidates for biodegradable plastics, polyhydroxyalkanoates (PHAs) have attracted commercial interest as plastic materials because of their remarkable similarities in physical properties with synthetic polymers such as polypropylene. In addition, extensive attention has been focused on PHAs due to their inherent biocompatibility and biodegradability which sets them apart from synthetic plastics [2]. However, a widespread applications of this high-quality material as bulk-packaging material is hampered by the high production costs [3] and inferior thermomechanical and processing properties [2].

Modification of polyesters by blending or copolymerization with other polymers will provide an inexpensive and simple means to change and regulate the properties of the polymer [4]. Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] is one of the most well-characterized PHA copolymer because of its high commercial potential [5]. According to Bluhm et al. [6], incorporation of 3HV monomer with increasing concentration will decrease the melting point of copolymer P(3HB-*co*-4HB) to a minimum value of 84 °C, thus allowing better thermal processing and even better biodegradation process because an increase in 3HV molar fraction will decrease the melting temperature without any changes in the degradation temperature. The presence of the 3HV monomer unit will also increase the flexibility [7], tensile strength, toughness, and Young's modulus of P(3HB-*co*-3HV) copolymer [8].

Homopolymer poly(4-hydroxybutyrate) [P(4HB)] has highly similar properties to those of polyethylene. It has a tensile strength that benchmarks closely to ultrahigh molecular weight polyethylene. Incorporation of 4HB monomer into 3HB can further extend the range of properties available to produce novel elastomers [9]. Besides, the degradation rate of P(3HB-*co*-4HB) copolymer is relatively higher as compared to other PHAs, which can be controlled by varying the 4HB compositions [10]. The P(3HB-*co*-4HB) copolymer has gained interest in a wide variety of medical and pharmaceutical fields such as cardiovascular, drug delivery, and tissue engineering applications [11]. The idea of producing a hybrid polymer possessing the superior and desirable physical and mechanical properties of both P(3HB-*co*-3HV) and P(3HB-*co*-4HB) has led researchers to fabricate P(3HB-*co*-3HV-*co*-4HB) terpolymer.

The production of terpolymer P(3HB-*co*-3HV-*co*-4HB) by *Cupriavidus necator* (previously known as *Ralstonia eutropha*, *Wautersia eutropha*, and *Alcaligenes eutrophus*) using glucose and propionic acid with either 4-hydroxybutyric acid or γ-butyrolactone [6] and 4-hydroxybutyric acid with pentanoic acid [12] as carbon sources have been reported. Chanprateep and Kulpreecha [13] has also reported the production of P(3HB-*co*-3HV-*co*-4HB) terpolymer by *Alcaligenes* sp. A-04 with high 4HB content using combination of fructose, valeric acid, and 4-hydroxybutyric salt through two-step cultivation process. *Comamonas acidovorans* also able to produce P(3HB-*co*-3HV-*co*-4HB) terpolymer using 1-pentanol and 1,4-butanediol in mineral medium without the presence of nitrogen source [14].

There is a high demand of producing the biodegradable terpolymer because it has superior mechanical and thermal properties. P(3HB-*co*-3HV-*co*-4HB) [13], P(3HB-*co*-3HV-*co*-3HHx) [15], P(3HB-*co*-3HV-*co*-5HV) [16], P(3HB-*co*-3HV-*co*-4HV) [17], and P(3HB-*co*-3HV-*co*-3HHp) [18] are among the terpolymers that have been studied using various microorganisms . However, only few literature sources reported the production of P(3HB-*co*-3HV-*co*-4HB) terpolymer by *Cupriavidus* sp. using 1-pentanol and fatty acids through one-step cultivation process. Therefore, the significance aim of this study was to produce various compositions of P(3HB-*co*-3HV-*co*-4HB) terpolymer by *Cupriavidus* sp. USMAA2-4 via one-step cultivation process using combinations of various carbon sources and precursors. Also, the effects of terpolymer composition on the thermal and mechanical properties of the terpolymer were evaluated.

Materials and Methods

Microorganism

Cupriavidus sp. USMAA2-4 bacterium was cultured into the nutrient rich broth medium (grams per liter: Peptone, 10; Beef extract, 10; and Yeast extract, 2) and incubated on the orbital shaker (Certomat R & H, B.Braun, German) at 30 °C for 24 h at the rate of 200 rotations per minute (rpm) for the bacterial growth. For maintenance purpose, *Cupriavidus* sp. USMAA2-4 from the exponential growth phase was stored at −20 °C in 20% (*v/v*) glycerol.

Growth and Polymer Biosynthesis via One-Step Cultivation Process Through Shake-Flask Fermentation

Pre-cultured cells (0.02 g/l) were transferred into 50 ml of mineral salts medium (MSM) containing (grams per liter): K_2HPO_4 (5.8); KH_2PO_4 (3.7); $(NH_4)_2SO_4$ (1.1); $MgSO_4.7H_2O$ (0.2); 1.0 ml of microelements solution containing $FeSO_4.7H_2O$ (2.78); $MnCl_2.4H_2O$ (1.98); $CoSO_4.7H_2O$ (2.81); $CaCl_2.2H_2O$ (1.67); $CuCl_2.2H_2O$ (0.17); $ZnSO_4.7H_2O$ (0.29) in 0.1 M of HCl. Various fatty acids (oleic acid, palmitic acid, myristic acid, stearic acid and lauric acid), 3HV precursors (valeric acid, 1-pentanol and 1-propanol) and 4HB precursors (1,4-butanediol and γ-butyrolactone) were added to the medium to obtain different compositions of terpolymer. C/N ratio of 20 was fixed in all experiments. The flasks were incubated at 30 °C for 48 h at the rate of 200 rpm. Cell growth was monitored by measuring the optical density of the broth at 540 nm. The samples were withdrawn as whole flasks and three replicates were prepared for each culture medium. The value of 0.56 wt.%C γ-butyrolactone was equivalent to 1% (*w/v*) and was extrapolated to other carbon sources.

Analytical Procedures

PHA content and composition in the lyophilized cell materials were determined using gas chromatography (Shimadzu GC-2014, Japan). In GC analysis [19], approximately 15 mg of lyophilized cells were subjected to methanolysis in the presence of methanol and sulphuric acid [85%:15% (*v/v*)]. The reaction mixture was incubated at 100 °C for 3 h. The organic layer containing the reaction products was separated, dried over Na_2SO_4, and analyzed by GC. The molecular weight (M_w), number-average molecular weight (M_n), and polydispersity index (M_w/M_n) for each pure biopolymer sample was determined using the gel permeation chromatography (GPC; Waters 600E Multi Solvent Delivery System). The chromatography column used was PLgel MIXED C (Polymer Laboratories, Ltd., UK) with chloroform as the mobile phase at flow rate of 1.0 ml/min. A calibration curve was made based on the polystyrene samples with low polydispersity (~1.1; Polymer Laboratories, Ltd., UK) as standards. Differential scanning calorimeter (DSC) (Pyris DSC, PerkinElmer) was used to determine the thermal properties of the terpolymer. This procedure was carried out according to Vigneswari et al. [20]. The physical properties of the terpolymers were characterized using tensile machine (GOTECH A1-3000) to determine the tensile strength, Young's modulus, and elongation to break. Tensile test pieces with 4 mm width and 45 mm gauge length were cut from polymer films using steel ASTM regulation punches. The cross-head speed used was 20 mm min^{-1}.

Results and Discussion

Biosynthesis of P(3HB-*co*-3HV-*co*-4HB) Terpolymer Through One-Step Cultivation Process

Cupriavidus sp. USMAA2-4 was used to study the production of the terpolymer P(3HB-*co*-3HV-*co*-4HB) through one-step cultivation process. This Gram-negative bacterium was isolated from a soil samples collected in Sg. Pinang, Penang, Malaysia and capable of producing various types of biodegradable biopolymers [21]. The biopolymers were copolymers P(3HB-*co*-4HB), P(3HB-*co*-3HV) and terpolymer P(3HB-*co*-3HV-*co*-4HB) [22]. Two-step cultivation process was normally applied for the production of PHA. Previous research has reported two-step cultivation process as one of the effective method to produce PHA; however the high cost operation for this process hinders its applicability. Therefore, one-step cultivation process could be one of the ways to reduce the production cost as well as to minimize the cultivation period.

The bacterial cells were provided with 1,4-butanediol (0.25 wt.%C), 1-pentanol (0.06 wt.%C) and different fatty acids (0.25 wt.%C) to determine the effect of fatty acids as nutritional supplement on the cell growth and production of the terpolymer (Table 1). Terpolymer was analyzed using gas chromatography and the chromatogram of methyl esters of 3-hydroxybutyric acid, 3-hydroxyvaleric acid, 4-hydroxybutyric acid, and γ-butyrolactone of terpolymer P(68%3HB-*co*-9%3HV-*co*-23%4HB) was illustrated in Fig. 1.

Cupriavidus sp. USMAA2-4 was able to utilize fatty acids for growth and terpolymer production. The highest cell dry weight, 8.3 g/l was obtained when the bacterial cells were

Table 1 Effect of different fatty acids on P(3HB-*co*-3HV-*co*-4HB) terpolymer production through one-step cultivation process[a]

Carbon source[b]	Cell dry weight (g/l)	PHA concentration (g/l)	PHA composition (mol.%)[c]		
			3HB	3HV	4HB
Oleic acid	8.3±0.3	3.3±0.4	69±1	3±0	28±1
Myristic acid	5.4±1.4	1.6±0.5	68±2	9±3	23±1
Stearic acid	6.1±0.7	1.1±0.1	59±1	9±3	32±2
Palmitic acid	4.6±0.3	0.8±0.1	42±1	3±1	55±2
Lauric acid	1.2±0.2	0.01±0.1	59±5	14±1	27±6

Values are means±SD of three replications

[a] The cells were harvested after 48 h

[b] Different fatty acids (0.25 wt.%C)+1,4-butanediol (0.25 wt.%C)+1-pentanol (0.06 wt.%C)

[c] Calculated from GC analysis

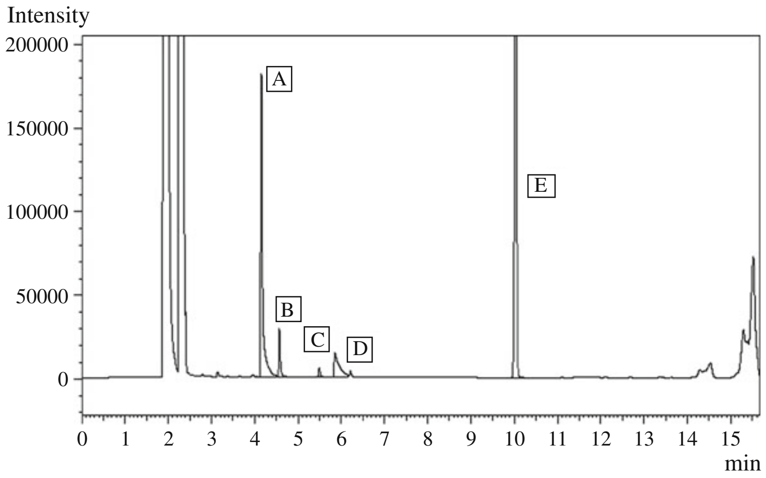

Fig. 1 Chromatogram of methyl esters of **a** 3-hydroxybutyric acid (RT, 4.1), **b** 4-butyrolactone (RT, 4.5), **c** 4-hydroxybutyric acid (RT, 5.4), **d** 3-hydroxyvaleric acid (RT: 5.7) and **e** Caprillic methyl ester (RT, 10) of terpolymer P(68%3HB-*co*-9%3HV-*co*-23%4HB) *ASterisk* RT, retention time

provided with oleic acid. Oleic acid also produced the highest 3HB molar fraction of 69 mol.% with PHA concentration of 3.3 g/l. It was found that oleic acid promoted the cell growth and enhanced the 3HB monomer production. Marangoni et al. [23] reported that oleic acid (C18:1) increased PHA production when used as a nutritional supplement. It could be suggested that the double bond of the fatty acids enhanced the enzymatic reaction of PHA accumulation. High amount of acetyl-CoA were saved because oleic acid provided precursors for C_{2n} fatty acids synthesis in the metabolic pathway of bacteria. Therefore, it enhanced the biosynthesis of P(3HB) due to the presence of more acetyl-CoA and/or NADPH [24]. Although oleic acid acted as the precursor for the synthesis of acetyl-CoA, it also has significant effect on the TCA cycle which increased the cell biomass. Through the addition of oleic acid, enhancement of both cell mass and the amount of P(3HB) were achieved. An enhanced PHA formation by oleic acid has also been observed in *R. eutropha* and recombinant *E. coli* [25].

Meanwhile, a decrease in the cell dry weight (4.6 g/l) and 3HB monomer (42 mol.%) was observed through the addition of palmitic acid. This finding suggested that it had little effect on the TCA cycle and P(3HB) synthesis enzymatic system which ultimately led to the production of terpolymer with highest 4HB molar fraction (55 mol.%). The highest 3HV molar fraction (14 mol.%) was obtained using combination of 1,4-butanediol and 1-pentanol with lauric acid. However, this combination also produced the lowest PHA concentration (0.01 g/l) and cell dry weight (1.2 g/l) suggesting that this fatty acid was toxic to cell metabolism and had negative effect on PHA accumulation.

The similar experiment was also conducted using γ-butyrolactone as 4HB precursor. It showed that the effect of fatty acids were same as the previous experiment (data not shown). However, the bacteria exhibited lower growth due to the toxicity of γ-butyrolactone in the culture medium [26]. Thus, 1,4-butanediol was chosen as the 4HB precursor for the subsequent experiments.

The bacterial cells were cultured into MSM containing carbon combinations of 1,4-butanediol (0.25 wt.%C) and oleic acid (0.25 wt.%C) with either 1-pentanol, 1-propanol, or valeric acid (0.06 wt.%C) to determine the effect of different HV precursors on the cell growth and terpolymer production (Table 2). Combination of carbon sources with 1-

Table 2 Effect of different HV precursors on P(3HB-co-3HV-co-4HB) terpolymer production[a]

Carbon sources[b]	Cell dry weight (g/l)	PHA concentration (g/l)	PHA composition (mol.%)[c]		
			3HB	3HV	4HB
1-Pentanol	5.3±0.6	1.6±0.2	48±3	8±1	44±4
1-Propanol	5.9±0.1	1.7±0.4	63±4	1±0	36±3
Valeric acid	4.9±0.7	1.1±0.3	56±4	3±1	41±5

Values are means±SD of three replications

[a] The cells were harvested after 48 h

[b] Different HV precursors (0.06 wt.%C)+oleic acid (0.25 wt.%C)+1,4-butanediol (0.25 wt.%C)

[c] Calculated from GC analysis

propanol produced the highest cell dry weight (5.9 g/l) but the 3HV monomer composition (1 mol.%) produced was the lowest. It could be concluded that with 1-propanol as a substrate, there was a limitation in obtaining a higher 3HV molar fraction which might be due to the lower substrate specificity of the enzyme 3-ketothialase for propionyl-CoA [27]. Besides that, the combination of carbon sources with valeric acid produced the lowest cell dry weight (4.9 g/l) and PHA concentration (1.1 g/l) which might be due to the inhibitory effect of toxic acids. Combination of carbon sources with 1-pentanol produced fairly high cell dry weight (5.3 g/l) with PHA concentration and 3HV monomer composition of 1.6 g/l and 8 mol.%, respectively. 1-Pentanol that provided to the bacteria was oxidized to valeraldehyde and subsequently to the valeric acid. Valeric acid was converted to valeryl-CoA and then to 3-hydroxyvaleryl-CoA. This monomer formed a chain of polymer through the action of PHA synthase enzyme [28].

Effect of Different Concentrations of 1,4-Butanediol and 1-Pentanol on the Terpolymer P(3HB-*co*-3HV-*co*-4HB) Production

Combination of 1,4-butanediol and 1-pentanol with oleic acid was chosen to determine the effect of different concentrations of carbon sources on the terpolymer P(3HB-*co*-3HV-*co*-4HB) production because this combination produced high cell dry weight, PHA content, and PHA concentration.

PHA concentration of the terpolymer P(3HB-*co*-3HV-*co*-4HB) produced increased gradually when the concentration of 1,4-butanediol was increased from 0.20 to 0.35 wt.%C. (Fig. 2). Furthermore, 3HB and 4HB molar fractions also gradually increased with the increasing concentration of 1,4-butanediol. 1,4-butanediol was first converted to 4-hydroxybutyryl-CoA in the cells. Then, it was metabolized to form 3-hydroxybutyryl-CoA. Both 3-hydroxybutyryl-CoA and 4-hydroxybutyryl-CoA would randomly copolymerize by action of PHA synthase [29]. Therefore, when concentration of 1,4-butanediol was increased, it enhanced the accumulation of both 3HB and 4HB compositions because the biosynthetic pathway was enhanced to produce more 3HB-CoA and 4HB-CoA. The highest cell dry weight of 9.4 g/l was obtained using 0.25 wt.%C of 1,4-butanediol. The 3HV molar fraction decreased from 10 to 5 mol.% as the concentration of 1,4-butanediol was increased.

Effect of different concentrations of carbon sources was further carried out using 1-pentanol (Fig. 3). An increase in the cell dry weight from 6.9 to 9.4 g/l was observed when the concentration of 1-pentanol was increased from 0.03 to 0.06 wt.%C. However, further

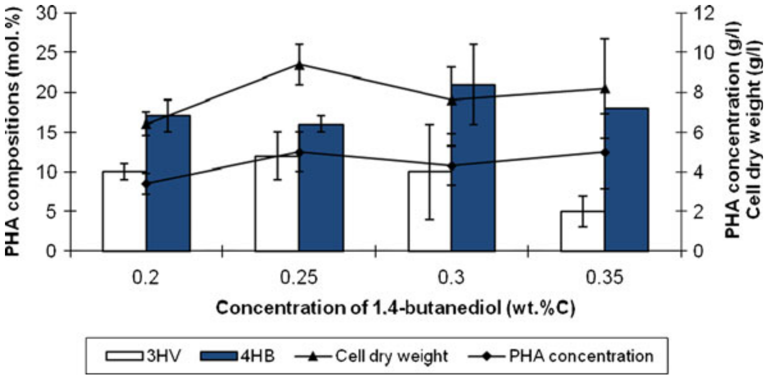

Fig. 2 Effect of different concentrations of 1,4-butanediol on the P(3HB-*co*-3HV-*co*-4HB) terpolymer production. Concentration of oleic acid and 1-pentanol were fixed at 0.25 and 0.06 wt.%C, respectively. The cells were harvested after 48 h. Values are means±SD of three replications

increasing the concentration of 1-pentanol caused the cell dry weight decreased which might be due to the inhibitory effect of 1-pentanol. The PHA concentration and composition of 3HB and 4HB also decreased when the concentration of 1-pentanol was increased. However, the molar fraction of 3HV monomer increased with the increasing concentration of 1-pentanol. Kunioka et al. [12] had proven that 3HV composition of the terpolymer P(3HB-*co*-3HV-*co*-4HB) produced by *C. necator* using pentanoic acid increased when the concentration of pentanoic acid was increased.

Biosynthesis and Characterization of P(3HB-*co*-3HV-*co*-4HB) Terpolymer by *Cupriavidus* sp. USMAA2-4

Cupriavidus sp. USMAA2-4 was cultured into MSM containing two different combinations of carbon sources with the manipulated concentration of 1-pentanol to produce various compositions of P(3HB-*co*-3HV-*co*-4HB) terpolymer (Table 3).

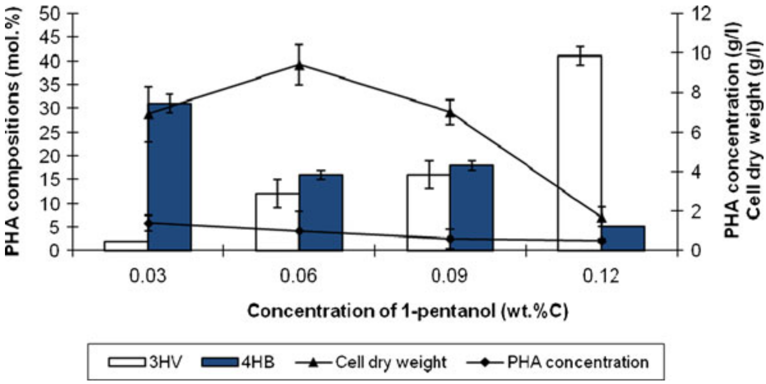

Fig. 3 Effect of different concentrations of 1-pentanol on the P(3HB-*co*-3HV-*co*-4HB) terpolymer production. Concentration of both oleic acid and 1,4-butanediol were fixed at 0.25 wt.%C. The cells were harvested after 48 h. Values are means±SD of three replications

Table 3 Production of various monomer compositions of the P(3HB-*co*-3HV-*co*-4HB) terpolymer through one-stage cultivation process[a]

Concentration of carbon sources (wt.%C)[b]		PHA composition (mol.%)[c]		
1-Pentanol	Oleic acid	3HB	3HV	4HB
0.06	0.25	73	8	19
0.09	0.25	64	32	4
1-Pentanol	Palmitic acid	3HB	3HV	4HB
0.06	0.25	63	4	33
0.09	0.25	49	18	33

[a] The cells were harvested after 48 h

[b] 1,4-Butanediol (fixed at 0.25 wt.%C) was added as 4HB precursor

[c] Calculated from GC analysis. Values are means of duplicates

Analysis through GPC was carried out to determine the molecular weight (M_w) and number-average molecular weight (M_n) of the extracted terpolymer P(3HB-*co*-3HV-*co*-4HB) (Table 4). It was found that an increase in the molar fraction of 4HB monomer was coupled with an increase in the molecular weight and number-average molecular weight of the P(3HB-*co*-3HV-*co*-4HB) terpolymer produced. Terpolymer P(49%3HB-*co*-18%3HV-*co*-33%4HB) showed the highest molecular weight of 1,710 kDa.

Sudesh et al. [30] reported that the molecular weight of PHA was determined by the activity of PHA synthase. The activity of PHA synthase was marked highly with four carbon substrates [31]. 1,4-Butanediol used in this experiment is a four carbon substrate. Therefore, it could be suggested that a significant increase in the activity of PHA synthase was achieved because PHA synthase favored the metabolism of 4HB precursor due to its substrate specificity towards the four carbon substrate which led to the improved production of 4HB monomer composition and molecular weight of the terpolymer.

Table 4 Molecular weight of the terpolymer P(3HB-*co*-3HV-*co*-4HB) produced by *Cupriavidus* sp. USMAA2-4 compared with other copolymers and terpolymers

Polymers	M_n (kDa)[a]	M_w (kDa)[a]	M_w/M_n
P(3HB)[b]	782	–	1.8
P(3HB-*co*-11%4HB)[b]	223	–	2.5
P(3HB-*co*-82%4HB)[b]	129	–	2.4
P(3HB-*co*-45%3HV)[b]	400	–	2.4
P(3HB-*co*-71%3HV)[b]	254	–	2.0
P(11%3HB -*co*-23%3HV-*co*-66%4HB) [b]	190	662	3.5
P(10%3HB-*co*-6%3HV-*co*-84%4HB) [b]	63	177	2.8
P(73%3HB-*co*-8%3HV-*co*-19%4HB)[c]	320	819	2.6
P(64%3HB-*co*-32%3HV-*co*-4%4HB)[c]	101	346	3.4
P(63%3HB-*co*-4%3HV-*co*-33%4HB)[c]	747	1457	2.0
P(49%3HB-*co*-18%3HV-*co*-33%4HB)[c]	1025	1710	1.7

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

[a] Calculated from GPC analysis

[b] Chanprateep and Kulpreecha [13]

[c] Terpolymer produced by *Cupriavidus* sp. USMAA2-4

Table 5 Thermal and mechanical properties of the terpolymer P(3HB-*co*-3HV-*co*-4HB) produced by *Cupriavidus* sp. USMAA2-4 compared with other copolymers and terpolymers

Polymers	T_g[a] (°C)	T_m[b] (°C)	Young's modulus (MPa)[c]	Tensile strength (MPa)[c]	Elongation to break (%)[c]
P(3HB)[d]	10	177	3500	40	0.4
P(3HB-*co*-10%4HB)[d]	–	160	–	24	242
P(3HB-*co*-64%4HB)[d]	–	–	30	17	591
P(3HB-*co*-9%3HV)[d]	–	–	1620	190	37
P(3HB-*co*-24%3HV)[d]	−6	138	1370	70	30
P(11%3HB-*co*-23%3HV-*co*-66%4HB) [d]	−17.7	91.8	392	9	5
P(10%3HB-*co*-6%3HV-*co*-84%4HB) [d]	−47.1	54.3	118	9	300
P(73%3HB-*co*-8%3HV-*co*-19%4HB)[e]	−10	131	102	12	316
P(64%3HB-*co*-32%3HV-*co*-4%4HB)[e]	−5	ND	138	18	19
P(63%3HB-*co*-4%3HV-*co*-33%4HB)[e]	−14	ND	101	9	937
P(49%3HB-*co*-18%3HV-*co*-33%4HB)[e]	−16	ND	34	2	554

ND not detected

[a] Glass transition temperature determined using DSC

[b] Melting temperature determined using DSC

[c] Determined using Tensile machine

[d] Chanprateep and Kulpreecha [13]

[e] Terpolymers produced by *Cupriavidus* sp. USMAA2-4

General information on the thermal and mechanical properties was required in the selection of materials for diversity applications. Incorporation of 3HV and 4HB monomer decreased both T_g and T_m of P(3HB). The T_g of the P(3HB-*co*-3HV-*co*-4HB) terpolymer produced decreased from −5 to −16 °C when the 4HB molar fractions increased from 4 to 33 mol.% (Table 5). According to the previous research, the T_g of the terpolymer P(3HB-*co*-3HV-*co*-4HB) produced by *Alcaligenes* sp. A-04 decreased from −13.7 to −51.6 °C when the 4HB molar fractions increased from 50 to 93 mol.% [13]. The overall composition of the polymer is an important factor to determine the melting point temperature (T_m). T_m was also influenced by the microstructure of the polymer [32]. It was reported that homopolymer P(3HB) has high melting temperature (175 °C) and degrading temperature of 185 °C [8]. Therefore, P(73% 3HB-*co*-8%3HV-*co*-19%4HB) terpolymer which has the highest molar fraction of 3HB exhibited the highest melting temperature of 131 °C. Whereas, for the other P(3HB-*co*-3HV-*co*-4HB) terpolymer produced which has lower 3HB molar fraction, the melting temperature could not be detected because increase in the comonomer fraction decreased the melting temperature below the threshold value. This allowed the thermal processing of the terpolymer without thermal degradation which enabled easier processing of the materials [8].

It was found that incorporation of the 3HV and 4HB monomer into 3HB monomer strongly affected the thermal properties of the terpolymer produced because the T_m of P(3HB-*co*-3HV-*co*-4HB) was different as compared to those of P(3HB-*co*-3HV) and P(3HB-*co*-4HB) copolymers having similar 3HV and 4HB molar fraction units. For example, 138 °C T_m of the copolymer P(3HB-*co*-24%3HV) was higher than 91.8 °C T_m of the terpolymer P(11%3HB-*co*-23%3HV-*co*-66%4HB) [13].

Mechanical properties of the biopolymer such as tensile strength, Young's modulus, and elongation to break are important in the selection of materials for various applications. The presence of 3HV molar fraction increased the tensile strength and Young's modulus of the terpolymer. Whereas, 4HB molar fraction was found to increase the elasticity (elongation to break) but it reduced the toughness of the terpolymer P(3HB-*co*-3HV-*co*-4HB) produced [13]. The terpolymer P(64%3HB-*co*-32%3HV-*co*-4%4HB) which has the highest 3HV molar fraction showed the highest tensile strength (18 MPa) and Young's modulus (138 MPa; Table 5). However, it has the lowest elongation to break (19%) due to its low 4HB molar fraction. Terpolymer P(63%3HB-*co*-4%3HV-*co*-33%4HB) with high 4HB molar fraction showed very high elongation to break (937%) similar to elongation to break of homopolymer P(4HB) which was almost 1,000% [10]. Saito and Doi [33] has also reported that elongation to break of P(3HB-*co*-4HB) increased from 5% to 1320% as 4HB molar fraction increased from 0 to 82 mol.%. Also, this terpolymer P(63%3HB-*co*-4%3HV-*co*-33%4HB) has higher tensile strength (9 MPa). This was probably due to the incorporation of 4% of 3HV monomer, albeit low but still showed improvement on the tensile strength properties. However, the terpolymer P(49%3HB-*co*-18%3HV-c*o*-33%4HB) with high molar fraction of 3HV and 4HB showed the lowest tensile strength (2 MPa) and Young's modulus (34 MPa) with the elongation to break of 554%. Although the molar fraction of 3HV monomer was high but it could be concluded that its effect seemed to be hindered by the effect of higher 4HB molar fraction.

The mechanical properties of the terpolymers produced were compared with petrochemically derived plastics (Table 6) according to ASTM: D882-91 test [34]. The terpolymer P (64%3HB-*co*-32%3HV-*co*-4%4HB) exhibited Young's modulus of 138 MPa and tensile strength of 18 MPa similar to that of plastic bag low-density polyethylene (LDPE; 156 MPa) and plastic bag high-density polyethylene (HDPE; 19 MPa), respectively. The terpolymer P(73%3HB-*co*-8%3HV-*co*-19%4HB) has tensile strength (12 MPa) similar to plastic bag (LDPE; 13 MPa). Plastic bag (HDPE; 576%) has similar elongation to break with that of the terpolymer P(49%3HB-*co*-18%3HV-*co*-33%4HB) (554%).

Conclusions

In summary, bacterium *Cupriavidus* sp. USMAA2-4 was capable of producing the terpolymer P(3HB-*co*-3HV-*co*-4HB) with various monomer compositions through one-stage cultivation process when provided with different combinations of carbon sources and precursors. The monomer compositions of the terpolymer were also found to have great influences on the thermal and mechanical properties of the terpolymer. The terpolymers produced have superior thermal and mechanical properties over copolymers. Incorporation of both 3HV and 4HB monomers into the P(3HB) chain had resulted in the formation of terpolymer P(3HB-*co*-3HV-*co*-4HB) having desirable traits of both copolymers. Terpolymer P(63%3HB-*co*-4%3HV-*co*-33%4HB) has high Young's modulus and also elongation to break up to 937% which are suitable for various medical applications such as sutures, cardiovascular stents, and vascular grafts.

Table 6 Mechanical properties of the commercial petrochemically derived plastics according to ASTM: D882-91 test

Plastic type	Young's modulus (MPa)	Tensile strength (MPa)	Elongation to break (%)
Plastic bag (LDPE)	156	13	126
UV degradable bag	674	24	384
Plastic bag (HDPE)	640	19	576
Plastic bag (Polypropylene)	590	27	435

Acknowledgement The authors acknowledged the research grant provided by MOSTI, Malaysia (Science Fund, 02-01-05-SF0363), USM Science Fellowship awarded to Hema (RU: 1001/441/29301/CIPS/AUPE001), and National Science Fellowship awarded to Nurhezreen (M/0004/01/2009/BIO) that has resulted in this article.

References

1. Reddy, C. S. K., Ghai, R., & Rashmi, K. V. C. (2003). *Bioresource Technology, 87*, 137–146.
2. Akaraonye, E., Keshavarz, T., & Roy, I. (2010). *Journal of Chemical Technology and Biotechnology, 85*, 732–743.
3. Zinn, M., Witholt, B., & Egli, T. (2001). *Advanced Drug Delivery Reviews, 53*, 5–21.
4. Braunegg, G., Lefebvre, G., & Genser, K. F. (1998). *Journal of Biotechnology, 65*, 127–161.
5. Bhubalan, K., Lee, W.-H., Loo, C.-Y., Yamamoto, T., Tsuge, T., Doi, Y., et al. (2008). *Polymer Degradation and Stability, 93*, 17–23.
6. Bluhm, T. L., Hamer, G. K., Marchessault, R. H., Fyfe, C. A., & Veregin, R. P. (1986). *Macromolecules, 19*, 2871–2876.
7. Madden, L. A., Anderson, A. J., Asrar, J., Berger, P., & Garrett, P. (2000). *Polymer, 41*, 3499–3505.
8. Ojumu, T. V., Yu, J., & Solomon, B. O. (2004). *African Journal of Biotechnology, 3*, 18–24.
9. Martin, D. P., & Williams, S. F. (2003). *Biochemical Engineering Journal, 16*, 97–105.
10. Saito, Y., Nakamura, S., Hiramitsu, M., & Doi, Y. (1996). *Polymer International, 39*, 169–174.
11. Chee, J. W., Amirul, A. A., Tengku Muhammad, T. S., Majid, M. I. A., & Mansor, S. M. (2008). *Biochemical Engineering Journal, 38*, 314–318.
12. Kunioka, M., Nakamura, S., & Doi, Y. (1988). *Polymer Communication, 29*, 174–176.
13. Chanprateep, S., & Kulpreecha, S. (2006). *Journal of Bioscience and Bioengineering, 101*, 51–56.
14. Kimura, H., Yoshida, Y., & Doi, Y. (1992). *Biotechnological Letters, 14*, 445–450.
15. Ye, H.-M., Wang, Z., Wang, H.-H., Chen, G.-Q., & Xu, J. (2010). *Polymer, 51*, 6037–6046.
16. Doi, Y., Tamaki, A., Kunioka, M., & Soga, K. (1987). *Macromolecular Chemistry Rapid Communication, 8*, 631–635.
17. Valentin, H. E., & Steinbuchel, A. (1995). *Journal of Environmental Polymer Degradation, 3*, 169–175.
18. Fukui, T., Kichise, T., Yoshida, Y., & Doi, Y. (1997). *Biotechnology Letters, 19*, 1093–1097.
19. Braunegg, G., Sonnleitner, B., & Lafferty, R. M. (1978). *European Journal of Applied Microbiology and Biotechnology, 6*, 29–37.
20. Vigneswari, S., Vijaya, S., Majid, M. I. A., Sudesh, K., Sipaut, C. S., Azizan, M. N. M., et al. (2009). *Journal of Industrial Microbiology & Biotechnology, 36*, 547–556.
21. Amirul, A. A., Tay, B. Y., Chang, C. W., Azizan, M. N. M., Majid, M. I. A., & Sudesh, K. (2004). *Jurnal Biosains, 15*, 125–135.
22. Amirul, A. A., Syairah, S. N., Yahya, A. R. M., Azizan, M. N. M., & Majid, M. I. A. (2008). *World Journal of Microbiology and Biotechnology, 24*, 1327–1332.
23. Marangoni, C., Furigo, A., Jr., & de Aragao, G. M. F. (2000). *Biotechnology Letters, 22*, 1635–1638.
24. Lo, K., Chua, H., Lawford, H., Lo, W., & Yu, P. (2005). *Applied Biochemistry and Biotechnology, 122*, 575–580.
25. Lee, S. Y., Kang, S. H., & Choi, C. Y. (1995). *Journal of Fermentation and Bioengineering, 79*, 328–334.
26. Steinbuchel, A., & Lütke-Eversloh, T. (2003). *Biochemical Engineering Journal, 16*, 81–96.
27. Haywood, G. W., Anderson, A. J., Chu, L., & Dawes, E. A. (1988). *FEMS Microbiology Letters, 52*, 91–96.
28. Majid, M. I. A. (1988). *Ph.D.Thesis, University of Bath, United Kingdom*.
29. Bernd, H. A. R., & Steinbuchel, A. (2002). In Y. Doi & A. Steinbüchel (Eds.), *Biopolymers, vol. 3a: PHA Synthase* (pp. 173–215). Weinheim: Wiley-VCH.
30. Sudesh, K., Abe, H., & Doi, Y. (2000). *Progress in Polymer Science, 25*, 1503–1555.
31. Haywood, G. W., Anderson, A. J., & Dawes, E. A. (1989). *FEMS Microbiology Letters, 57*, 1–6.
32. Dai, Y., Lambert, L., Yuan, Z., & Keller, J. (2008). *Journal of Biotechnology, 134*, 137–145.
33. Saito, Y., & Doi, Y. (1994). *International Journal of Biological Macromolecules, 16*, 99–104.
34. American Society for Testing and Materials (ASTM) (1991). Test methods for tensile properties of thin plastic sheeting, D882-91. In: Annual Book of ASTM Standards, American Society for Testing and Materials, Philadelphia, pp. 182-190.