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Full Length Research Paper

Microbial synthesis of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) copolymer by *Cupriavidus* sp. USMAA2-4 through a two step cultivation process

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A Gram negative bacterium, *Cupriavidus* sp. USMAA2-4 was isolated from a soil sample in Northern Peninsular of Malaysia and was able to synthesize polyhydroxyalkanoate containing 4-hydroxybutyrate unit when grown on γ-butyrolactone as the sole carbon source. The polyester was purified from freeze-dried cells and analyzed by nuclear magnetic resonance (NMR) spectroscopy. ^{1}H and ^{13}C NMR results confirmed the presence of 3HB and 4HB monomers. The isolated strain has the ability to synthesize poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] in a two step cultivation process on a medium containing γ-butyrolactone as the carbon source. A high fraction of 4HB monomer unit was obtained by manipulating the cell concentration, types of carbon sources and carbon source concentration in the cultivated medium. On the basis of the PHA composition, we suggest that carbon sources such as 1,6-hexanediol, 1,8-octanediol, 1,10-decanediol and 1,12-dodecanediol resulted in a skewed PHA composition. On the other hand, the molar fraction of 4HB in P(3HB-*co*-4HB) was increased significantly from 25 – 51 mol % by the higher concentration of γ-butyrolactone as the sole carbon source in the medium. The molecular weight and thermal properties of P(3HB-*co*-4HB) were revealed by gel permeation chromatography (GPC) and differential scanning calorimeter (DSC), respectively. We found that this bacterium is able to produce wide range copolymer with the number-average molecular weights (M_n) of copolymers ranging from 17 x 10^3 to 412 x 10^3 Dalton. Increase in the concentration of γ-butyrolactone lowered the molecular weight of these copolymers. Higher concentration of γ-butyrolactone also resulted in more branched polymer and consequently gave lower values for both the glass transition temperature (T_g) as well as melting temperature, T_m.

Key words: Biopolymer, polyhydroxyalkanoates, poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate), biosynthesis, characterization.

INTRODUCTION

Countless microorganisms have been isolated from the natural environment such as soil, activated sludge or even drain water. Doubtlessly, the isolated microorganisms were found as useful tools in the recent scientific era. Some of those bacteria are well-known to produce polyhydroxyalkanoates (PHAs) with different carbon sources under certain favorable culture conditions (Anderson and Dawes, 1990). PHA is a biodegradable and biocompatible thermoplastic which is an environment-friendly product, prone to biodegradation in soil, sediment, sea water or digested by depolymerase enzyme of many microorganisms (Scandola et al., 1990). PHAs exhibited various materials properties ranging from stiff, brittle to rubber-like, which makes it a close substitute for the synthetic plastic (Ojumu et al., 2004).

Wild type bacteria such as *Ralstonia eutropha* (Kim et al., 2005), *Alcaligenes latus* (Kang et al., 1995), *Comamonas acidovorans* (Lee et al., 2004), *Comamonas testosteronii* (Renner et al., 1996) and *Hydrogenophaga*

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pseudoflava (Choi et al., 1999) are able to produce the copolymer [P(3HB-*co*-4HB)] containing both the monomer 3HB and 4HB. In fact, microorganisms synthesize different polyesters, composed of various kinds of monomers depending on the fermentation conditions and the source of carbon (Anderson and Dawes, 1990). Among the various monomer constituents, the incorporation of 4HB units into the P(3HB) sequence has resulted in effective improvement of the physical properties of PHA (Doi, 1990). As reported by Takeharu (2002), the incorporation of 16 mol% of 4HB into the P(3HB) sequence has increased the 5% extension to break up to 444% with a 26 MPa tensile strength. Therefore, combining the different monomers to form copolymers, as in P(3HB-*co*-4HB), has been described as one of the most useful PHAs by Sudesh et al. (2000); it produces a family of materials with mechanical properties that can be tailored to specific needs. Interestingly, increasing of the 4HB composition in P(3HB-*co*-4HB) changes the physical property of the copolymer from high crystallinity to strong elastomer. Besides, the melting temperature (T_m) and glass transition temperature (T_g) of P(3HB-*co*-4HB) can be amended by the composition of 4HB. Both the T_m and T_g will be decreased with the increasingly 4HB composition existing in the copolymer (Saito et al., 1994). In this study, we have investigated the biosynthesis of biodegradable polyester P(3HB-*co*-4HB) using a locally isolated Gram negative bacterium, *Cupriavidus* sp. USMA A2-4. The optimization of culture conditions for P(3HB-*co*-4HB) production as well as the physical properties of the copolymer was also evaluated.

MATERIALS AND METHODS

Isolation and characterization of microorganisms

Cupriavidus sp. USMAA2-4 (DSM 19379) used in this study was isolated from a soil sample collected in Sg. Pinang, Penang, Malaysia (Amirul et al., 2004). The isolation of biodegradable polyester producer was carried out by collecting samples from environment such as soil, sludge and water in Peninsular Malaysia. Samples were enriched with mineral salts medium (MSM) containing γ-butyrolactone as the sole carbon source 0.74% (v/v) but with limited nitrogen source to maintain the C/N ratio at 20. After overnight incubation, cultures were diluted in sterile distilled water and plated on solid mineral salts medium containing γ-butyrolactone and Nile red stain (5 µg/ml) (Spiekermann et al., 1999). Colonies that formed following incubation were replicated into a fresh medium. The original plate was then exposed to ultraviolet (320 nm) illumination to identify PHA producers. In addition, biochemical characterization, 16S rRNA sequencing, DNA base composition, cellular fatty acids analysis and DNA-DNA hybridization were performed to determine the identity of the isolate, USMAA2-4. For conventional biochemical characterization, bacteria were grown on nutrient agar plates for 24-48 h which were then inoculated into the test reagent. The API 20 NE (bioMerieux) was utilized according to the protocol supplied by the manufacturer. The 16S rRNA gene sequence was determined by direct sequencing of PCR-amplified 16S rDNA. Genomic DNA extraction, PCR mediated amplification of the 16S rDNA and purification of the PCR product were carried out as described previously (Rainey et al., 1996). Purified PCR products were sequenced using the CEQ™ DTCS-Quick Start Kit (Beckmann Coluter) as directed in the manufacturer's protocol. Sequence reactions were electrophoresed using the CEQ™8000 genetic analysis system. The resulting sequence data from the strain were put into the alignment editor ae2 (Maidak et al., 1999), aligned manually and compared with the representative 16S rRNA gene sequences of organisms belonging to the *β-Proteobacteria*. A phylogenetic tree based on the neighbour-joining method was constructed by using the PHYLIP package (Felsenstein, 1993).

For the determination of DNA base composition, DNA was enzymically degraded into nucleosides as described by Mesbah and coworkers (1989). For fatty acids analysis, fatty acid methyl esters were obtained by saponification, methylation and extraction using the method as described by Kuykendall et al. (1988). The fatty acid methyl esters mixtures were separated using Sherlock microbial identification system (MIS) (Microbial ID, Newark, DE 19711 U.S.A.). Peaks were automatically integrated and fatty acid names and % were calculated by the MIS standard software (microbial ID). DNA-DNA hybridization by the initial renaturation rate was carried out under consideration of the modifications described by Huss et al. (1983) using spectrophotometer (Cary 100 Bio UV/VIS).

To prepare inoculum, *Cupriavidus* sp. USMAA2-4 was grown at 30°C under aerobic condition in a nutrient broth (5 g of peptone, 2 g of yeast extract, 1 g of beef extract and 5 g of NaCl in 1 l of distilled water). For maintenance purpose, *Cupriavidus* sp. USMAA2-4 from the exponential growth phase was stored at -20°C in 20% (v/v) glycerol.

Biosynthesis of P(3HB-*co*-4HB)

P(3HB-*co*-4HB) accumulation was carried out in a two step cultivation process. The microorganisms were grown in 150 ml nutrient broth (10 g of peptone, 10 g of yeast extract, 5 g of beef extract, and 5 g of $(NH_4)_2SO_4$ in 1 l distilled water) for 20 to 22 h. The cells were then harvested by centrifugation (10000 g), washed with sterile distilled water and transferred (1 g/l) into nitrogen-free mineral salts medium (MSM), containing 3.70 g/l KH_2PO_4, 5.80 g/l K_2HPO_4, 1.1 g/l $(NH_4)_2SO_4$, 0.2 g/l $MgSO_4.7H_2O$ and 1.0 ml/l microelements solution (2.78 g/l $FeSO_4.7H_2O$, 1.98 g/l $MnCl_2.4H_2O$, and 2.81 g/l $CoSO_4.7H_2O$, 1.67 g/l $CaCl_2.2H_2O$, 0.17 g/l $CuCl_2.2H_2O$ and 0.29 g/l $ZnSO_4.7H_2O$ per liter of 0.1 M HCl). The medium was added with filter-sterilized γ-butyrolactone or other carbon sources at a concentration of 0.56 (wt.%) carbon as the sole source to promote PHA synthesis. This value evolved from using 1% (wt/v) γ-butyrolactone which corresponds to 0.56 (wt.%) carbon and was extrapolated to other carbon sources. Cell growth was monitored by measuring the optical density at 540 nm. Unless noted otherwise, all growth experiments were performed under aerobic conditions in 250 ml flasks containing 50 ml medium, on a rotary shaker (Certomat R and H, B. Braun, Germany) at 30°C and 200 rpm. Two replicates were prepared for each culture medium.

Analytical procedures

PHA content and composition in the lyophilized cell material were determined using gas chromatography (Shimadzu GC-14B) and nuclear magnetic resonance (Bruker) analyses. In GC analysis (Braunegg et al., 1978), approximately 15 mg of lyophilized cell was subjected to methanolysis in the presence of methanol and sulfuric 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. In NMR analysis, the PHA was extracted from freeze-dried cells. 1.0 g freeze-dried cells were stirred in 200 ml of chloroform for 24 h at 30°C. The extract was filtered to remove cell debris and the chloroform was concentrated to a volume of about 15 ml using a rotary

Table 1. General and nutritional characteristics of *Cupriavidus* sp. USMAA2-4.

Characteristic	Presence[a]	Utilization of substrate	Growth[b]
Cell morphology	Rods	p-hydroxy-benzoate	+
Motility	+	mesaconate	+
Flagella	Polar	Trans-aconitate	+
Gram reaction	-	D-galactose	-
Lysis by 3% KOH	+	D/L-tartrate	+
Aminopeptidase (Cerny)	+	D-fucose	-
Catalase activity	+	D-xylose	-
Oxidase activity	+	Glucose	-
ADH	-	Citrate	+
Hydrolisis of gelatin	-	Malate	+
Hydrolisis of esculin	-	Arabinose	-
NO_2 from NO_3 (24H)	-	Mannose	-
Urease	+	Mannit	-
		Adipate	+
		Caprate	+
		Gluconate	+
		Maltose	-

[a] + =Positive; - = negative.
[b] + = Growth; - = no growth.

evaporator. The concentrated solution was then added drop-wise to 150 ml of rapidly stirred methanol to precipitate the dissolved PHA. The precipitated PHA was recovered by filtration using a 0.45 µm PTFE membrane and dried overnight at room temperature. The purified PHA was dissolved in deuterated chloroform ($CDCl_3$) and subjected to the 400 MHz ^1H and 300 MHz ^{13}C NMR analyses. All molecular weight data were obtained at 35°C by using 600E GPC waters system and waters 410 refractive index detector with a PLgel Mixed C column (Polymer Laboratories, Ltd., UK). Chloroform was used as eluent at a flow rate of 0.8 ml/min and a sample concentration of 1.0 mg/ml was used. Polystyrene standards with a low polydispersity were used to construct a calibration curve. Thermal properties were determined with differential scanning calorimeter (DSC) (Pyris 1 DSC, Perkin Elmer). The melting temperature (T_m) and enthalpy of fusion (ΔH_m) were determined from the DSC endotherms. For measurement of glass transition temperature (T_g), the melt samples (10 - 15 mg) were rapidly cooled down to -50°C. They were heated from -50 to 200°C at heating rate of 20°C min/min. The T_g was taken as the midpoint of the heating capacity change.

RESULTS AND DISCUSSION

Isolation and identification of P(3HB-*co*-4HB) producer

Cupriavidus sp. USMAA2-4 was identified as possible PHA producer based on Nile red staining methods (Spikermann et al., 1999). The potential producer emitted pink fluorescence when grown on solid MSM containing Nile red and exposed to UV light. Upon cultivation in MSM containing γ-butyrolactone as the carbon source, gas chromatography analysis confirmed that the bacterium was capable of producing PHA containing 3-hydroxybutyrate (3HB) and 4-hydroxybutyrate (4HB) monomers. For further confirmation, PHA was extracted from freeze-dried cells and analyzed by NMR. Results from the analyses confirmed that *Cupriavidus* sp. USMA A2-4 was capable of producing P(3HB-*co*-4HB). The ^{13}C NMR spectroscopy of the purified PHA showed signal peaks identical to the spectrum of P(3HB-*co*-4HB) and the six ^{13}C resonances at 15 -70 ppm could be assigned to specific carbon species in the 3HB and 4HB units as previously published (Valentin and Dennis, 1997).

Morphological and biochemical tests were performed on USMAA2-4 and the results shown in Table 1. The isolated bacterium was Gram-negative, aerobic, non-spore forming, motile and rod-shaped. Further tests revealed that the USMAA2-4 does not produce indole or acid from glucose. Gelatin, aesculin and arginine are not hydrolysed. Nitrate is not reduced to nitrite. The isolate assimilates p-hydroxy-benzoate, mesaconate, trans-aconitate, D/L-tartrate, citrate, malate, adipate, caprate and gluconate but not D-galactose, D-fucose, D-xylose, glucose, arabinose, mannose, mannitol and maltose. The results of the physiological tests do not allow identifying of this strain with one of the described species within the genus *Cupriavidus.* Therefore, further identification such as 16S rRNA sequencing, DNA base composition, cellular fatty acids analysis and DNA-DNA hybridization were performed. The profile of the cellular fatty acids is typical for the genus *Cupriavidus* with the identification score of 0.262 (*C. necator*). The 16S rRNA gene of *Cupriavidus* sp. USMAA2-4 has high similarity to the 16S rRNA genes of reference strain of *C. pauculus* LMG 3413^T (98.5%) and *C. respiraculli* LMG 21510^T (98.4%). The DNA G + C

Table 2. Effect of cell concentration of *Cupriavidus* sp. USMAA2-4 on P(3HB-*co*-4HB) synthesis in a two step cultivation.

Cell concentration (g/l)	Dry cell weight (g/l)	PHA content (wt%)[b]	PHA concentration (g/l)	PHA composition (mol%)[b]	
				3HB	4HB
0.2	1.2	39	0.5	3HB	15
0.4	2.1	56	1.2	85	14
0.6	3.2	59	1.9	86	15
0.8	4.3	57	2.5	85	15
1.0	5.1	58	3.0	85	19

[a] The cells were harvested after 48 h. Values are means of two replications.
[b] Calculated from GC analysis.

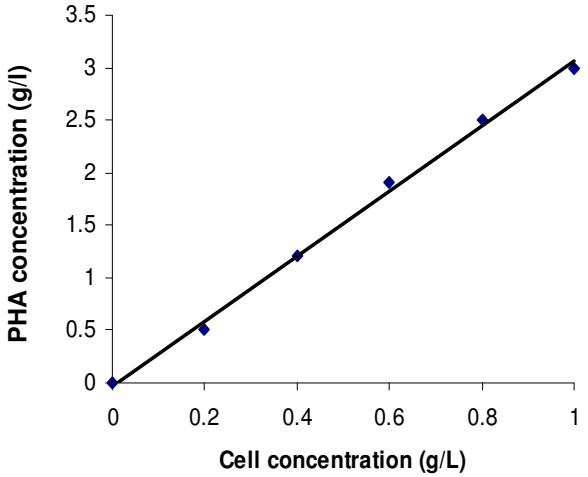

Figure 1. Relationship between cell concentration and PHA content.

contents of USMAA2-4 are 63.6%. This DNA base ratio is within the range of the genus *Cupriavidus* (63–70%) (Vandamme and Coenye, 2004). This clearly indicates that this organism belongs to the genus *Cupriavidus.* However, DNA-DNA hybridization between USMAA2-4 and the closest phylogenetic neighbours, the strain of *C. pauculus* and *C. respiraculli* showed low DNA-DNA binding values of 8 and 15%, respectively. This low value clearly demonstrates that the isolate USMAA2-4 represents a new species within the genus *Cupriavidus*.

Biosynthesis of P(3HB-*co*-4HB) through a two step cultivation process

Effect of cell concentration on P(3HB-co-4HB) synthesis

Biosynthesis of P(3HB-*co*-4HB) through a two step cultivation process was carried out by transferring cell biomass from nutrient broth into a medium which is usually nitrogen-limited or nitrogen-free (Amirul et al., 2008). The two step cultivation is widely used to produce PHAs because nutrient depletion, such as nitrogen, oxygen and other essential elements in excess carbon is favorable for the accumulation of PHAs. In this study, different cell concentrations of *Cupriavidus* sp. USMAA2-4 from nutrient broth were transferred to PHA synthesis mineral medium containing γ-butyrolactone to promote the biosynthesis of P(3HB-*co*-4HB) copolymer. Table 2 lists the results of P(3HB-*co*-4HB) synthesis by this bacterium and the accumulation of P(3HB-*co*-4HB) ranged from 39 to 59wt% of the dry cell weight. Based on this study, an increase in the cell concentration resulted in a higher PHA content and PHA concentration is directly proportional to the cell concentration, as shown in Figure 1. Meanwhile, the mol fraction of 3HB and 4HB monomers remains relatively constant regardless of cell concentration. This clearly indicates that cell concentration in two step cultivation is not the main factor that affects the 3HB and 4HB composition, whereas it may be influenced by others factors such as carbon source in PHA synthesis medium.

Effect of carbon source on P(3HB-*co*-4HB) production

In this study, a total of 7 kinds of precursor carbon sources was applied to investigate the effect on P(3HB-*co*-4HB) production. These include γ-butyrolactone, 1,4-butanediol, 4-hydroxybutyric acid, 1,6-hexanediol, 1,8-octanediol, 1,10-decanediol and 1,12-dodecanediol. Table 3 shows the result of P(3HB-*co*-4HB) biosynthesis by *Cupriavidus* sp. USMAA2-4 from the mentioned carbon sources. *Cupriavidus* sp. USMAA2-4 was able to accumulate P(3HB-*co*-4HB) from γ-butyrolactone, 4-hydroxybutyric acid and alkanediols. It was revealed that γ-butyrolactone, 4-hydroxybutyric acid and 1,4-butanediol resulted in the accumulation of 45 to 67wt% of the dry cell weight P(3HB-*co*-4HB) and 4HB content ranged from 18 to 24 mol%. Meanwhile 1, 6-hexanediol and 1,8-octanediol produced the lowest PHA content in the set, having the highest portion of 4HB units with 99 and 92

Table 3. Synthesis of P(3HB-*co*-4HB) by *Cupriavidus* sp. USMAA2-4 on various carbon sources through a two-step cultivation[a]

Carbon source	Dry cell weight (g/l)	PHA content (wt%)[b]	PHA concentration (g/l)	PHA composition (mol%)[b]	
				3HB	4HB
γ-butyrolactone	5.1	57	2.9	82	18
4-hydroxybutyric acid	7.3	45	3.3	76	24
1,4-butanediol	7.7	67	5.2	82	18
1,6-hexanediol	2.7	28	0.8	1	99
1,8-octanediol	1.0	9	0.1	8	92
1,10-decanediol	10.0	74	7.4	93	7
1,12-dodecanediol	9.2	64	5.9	89	11

[a] The cells were harvested after 48 h. Values are means of two replications.
[b] Calculated from GC analysis

mol%. According to Steinbüchel and Lütke-Eversloh (2003), ω-alkanediols are obviously first oxidized to the corresponding ω-hydroxy fatty acid, which is then converted into a co-enzyme A thioester and subjected to β-oxidation until 4HB-CoA occurs. In contrast, ω-alkanediols with greater carbon chain length such as 1,10-decanediol and 1,12-dodecanediol resulted in P(3HB-*co*-4HB) accumulation with low compositions of 4HB units having only 7 and 11 mol%, respectively. Our result has shown the similarity as previously reported by Kunioka et al. (1988). Obviously, ω-alkanediols with greater carbon chain length, especially those with even number of carbon atoms more than 8, seem not to necessarily oxidize to the ω-hydroxyfatty acid and to convert to 4HB-CoA through β-oxidation cycles, as stated by Steinbüchel and Lütke-Eversloh (2003). Therefore, we conclude that 1,10-decanediol and 1,12-dodecanediol might not fully oxidize as mentioned above, or the mechanism pathway of converting into 4HB-CoA could be inhibited somewhere since the PHA biosynthesis pathway of this bacterium is still under investigation.

Effect of γ-butyrolactone concentration on P(3HB-*co*-4HB) production

The production of P(3HB-*co*-4HB) was carried out under aerobic conditions in MM supplemented with either 2.5, 5.0, 7.5, 10.0, 12.5 or 15.0 g/l of γ-butyrolactone. The cultures were incubated for 48 h at 30°C. Table 4 shows the biosynthesis of P(3HB-*co*-4HB) when culture medium was supplemented with various concentration of γ-butyrolactone. PHA content decreased considerably as γ-butyrolactone concentration increased above 5.0 g/l. The PHA content appeared as the highest at 5.0 g/l γ-butyrolactone concentration. Table 4 also shows that the PHA content and 4HB molar fractions appeared as the lowest at 2.5 g/l γ-butyrolactone concentration. This may be due to inability of the insufficient carbon source to support the basic supplement for *Cupriavidus* sp. USMA A2-4. Yet, excessive carbon source presence in the culture medium may probably cause toxicity to the bacteria and hence, inhibit the growth and the production of PHA of the bacteria (Stanbury and Whitaker, 1984; Steinbüchel and Lütke Eversloh, 2003). In contrast, the mole fractions of 4HB units in the copolymer increased from 25 to 51 mol% as γ-butyrolactone concentration increased. As a precursor substrate, γ-butyrolactone is much more expensive than the simple carbon sources, and more importantly it is often also toxic. Due to the latter reason, higher concentration of the precursor carbon source in the fermentation was prohibited in our study in order to avoid toxicity occurrence in the cultures. The data from Table 4 have shown that cell dry weight and PHA content decreased with 12.5 and 15 g/l of γ-butyrolactone. Relatively, study of Doi et al. (1990) also reported that 4HB composition of copolymer P(3HB-*co*-4HB) produced by *R. eutropha* was affected by the concentration of γ-butyrolactone. Their finding showed that 4HB composition increased from 9 to 21 mol% while the concentration of γ-butyrolactone increased from 10 to 25 g/l. This was explained by Doi (1990) through the biosynthesis pathway of P (3HB-*co*-4HB) production for *R. eutropha.* When γ-butyrolactone was used as sole carbon source, 4-hydroxybutyryl-CoA was first formed and converted into 4-hydroxybutyrate. However, a small portion of 4-hydroxybutyryl-CoA was metabolized into D-3-hydroxybutyryl-CoA and then turned into 3-hydroxybutyrate through a complex pathway. P(3HB-*co*-4HB) was formed randomly when copolymerization process occurred within D-3-hydroxybutyryl-CoA and 4-hydroxybutyryl-CoA, which was driven by polyhydroxybutyryl polymerase enzyme. Therefore, the composition of 4HB in random copolyester will increase with a higher γ-butyrolactone concentration in nitrogen free medium.

Molecular weight and thermal properties of P(3HB-*co*-4HB)

In our study, it was found that the concentration of carbon source has an impact on the molecular weight of P(3HB-

Table 4. Synthesis of P (3HB-*co*-4HB) by *Cupriavidus* sp. USMAA2-4[a] on different concentrations of γ-butyrolactone through a two-step cultivation[a].

γ-butyrolactone	Dry cell weight (g/l)	PHA content (w%)[b]	PHA concentration (g/l)	PHA composition (mol%)[b]	
				3HB	4HB
2.5	2.1	18	0.4	75	25
5.0	3.7	42	1.6	70	30
7.5	3.3	34	1.1	69	31
10.0	2.9	30	0.9	68	32
12.5	2.2	20	0.4	53	47
15.0	1.7	19	0.3	49	51

[a] The cells were harvested after 48 hours. Values are means of two replications.
[b] Calculated from GC analysis.

Table 5. Molecular weight of P(3HB-*co*-4HB) produced by *Cupriavidus* sp. USMAA2-4[a] on different concentrations of γ-butyrolactone.

γ-butyrolactone (g/l)	PHA composition[a] (mol%)		Molecular weight[b]		
	3HB	4HB	M_n x 10^3 (Da)	M_w x 10^3 (Da)	M_w/M_n
2.5	76	24	412	566	1.4
5.0	71	29	306	464	1.5
7.5	70	30	361	622	1.7
10.0	69	31	253	440	1.7
12.5	57	43	52	70	1.3
15.0	51	49	17	24	1.4

[a] Calculated from GC analysis. Values are means of two replications.
[b] Determined by GPC.

co-4HB). Types and concentration of carbon sources were one of the important factors influencing the PHA molecular weight (Anderson et al., 1992; Taidi et al., 1994). Table 5 shows the number-average molecular weight (M_n), weight-average molecular weight (M_w) and polydispersities (M_w/M_n) for P(3HB-*co*-4HB) copolymer produced by *Cupriavidus* sp. USMAA2-4. The value of M_n decreased from 412,000 to 17,000 Datons when γ-butyrolactone concentration increased from 2.5 to 15.0 g/l. The polydispersities were in the range of 1.3 to 1.7. Therefore, the concentration of γ-butyrolactone affected the production and molecular weight of P(3HB-*co*-4HB) produced by *Cupriavidus* sp. USMAA2-4. In fact, 4HB composition in copolymer P(3HB-*co*-4HB) may not be the main deplorably factor in affecting the molecular weight of the copolymer studied. According to Doi and coworkers (1995), copolymer P(3HB-*co*-4HB) produced from *R. eutropha*, fed with 2 different carbon sources, was showing the altered molecular weight with the analogous figure of 4HB composition. Nevertheless, the figures might be diverse with different PHA producer. For example, *R. eutropha* capable of producing high molecular weight PHA, with more than 10^6 Da but *Pseudomonas* sp. used to produce PHA with molecular weight less than 10^6 Da, respectively (Brandl et al., 1991; Doi, 1990). Co-polymer P(3HB-*co*-4HB) with a wider range of molecular weight was successfully produced from our locally isolated bacteria compared to *C. acidovorans* which produces PHA with molecular weight within 40, 000 to 90 000 Da (Saito et al., 1994).

Melting temperature (T_m) and glass transition temperature (T_g) are the two important physical features for polymers' family to determine their properties comercially (Scandola et al., 1990). T_g is referred to as the temperature where polymer properties transit from elastic to a glass form and it is a reversible transition (Young and Lovell, 1991). Table 6 summarizes the compositions and thermal properties of P(3HB-*co*-4HB) copolyesters produced by *Cupriavidus* sp. USMAA2-4. The higher composition of 4HB resulted in decrease of glass transition temperature (T_g) and ΔH_m for copolymer P(3HB-*co*-4HB) was produced from *Cupriavidus* sp. USMAA2-4. On the other hand, the T_m results remained invariable within the range of 152 to 172°C and yet, not influenced by the 4HB composition. This circumstance was explained in the study of Mitomo et al. (2001). Besides, T_m and T_g could be affected by the physical and chemical properties of the polymer itself. Polymer with more branches will give the lower values for both the temperature but the values will be increased if the polymers with a polar side-chain or

Table 6. Thermal properties of P(3HB-*co*-4HB) produced by *Cupriavidus* sp. USMAA 2-4.

P(3HB-*co*-4HB)[a]	Melting Temperature T_m (℃)[b]	Glass Transition Temperature T_g (℃)[b]	ΔH_m (cal/g)[c]
P(3HB-*co*-0%4HB)[c]	171.8	7.7	83.0
P(3HB-*co*-22%4HB)	152.2	-8.6	41.2
P(3HB-*co*-30%4HB)	160.2	-16.4	19.4
P(3HB-*co*-45%4HB)	161	-17.0	16.1

[a] Calculated from GC analysis. Values are means of two replications.
[b]:Calculated from DSC analysis.
[c] Data from Doi (1990).

more steric force are found in the polymer (Young and Lovell, 1991). According to Doi and coworkers (1992), T_m, T_g and ΔH_m for homopolymer P(3HB) are 177, 4℃ and 20.8 cal/g; 54 -50℃ and 11 cal/g for homopolymer P(4HB). It was noted that higher composition of 4HB in a homopolymer resulted in lower value of T_g and it decreased the ΔH_m unit in copolymer, such as P(3HB-*co*-4HB) when it was found in a higher composition in a co-polymer. This may due to the devastation of P(3HB)'s crystal lattice by the 4HB units of polymer (Kunioka et al., 1989).

Conclusion

From our study, a locally isolated novel species in *Cupriavidus* sp. USMAA2-4 bacterium was capable of producing copolymer P(3HB-*co*-4HB) from related carbon sources through a two step cultivation process. Cell concentration resulted in a higher PHA content but the 3HB and 4HB compositions remained constant. Carbon source and its concentrations were detected to be one of the factors alternating the PHA compositions. *Cupriavidus* sp. USMAA2-4 was found to be capable of synthesizing different compositions of 3HB and 4HB molar fractions when added with various carbon sources such as γ-butyrolactone, 1,4-butanediol, 4-hydroxybutyric acid, 1,6-hexanediol, 1,8-octanediol, 1,10-decanediol and 1,12-dodecanediol. A wide range of 4HB compositions in P(3HB-*co*-4HB) copolymer with biodegradable and biocompatible features were potentially applied in the medical and pharmaceutical fields. This study also found that higher concentration of γ-butyrolactone produced co-polymer with higher 4HB molar fractions but lower number-average molecular weight. In addition, the higher composition of 4HB resulted in decreased values of T_g and ΔH_m for copolymer P(3HB-*co*-4HB) produced from *Cupriavidus* sp. USMAA2-4.

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REFERENCES

Amirul AA, Tay BY, Chang CW, Azizan MNM, Majid, MIA, Sudesh K (2004). Biosynthesis and characterization of poly (3-hydroxybutyrate-*co*-4-hydroxybutyrate) produced by *Ralstonia* sp. isolated from soil. Biosci. J. 15(2): 125-135.

Amirul AA, Yahya ARM, Sudesh K, Azizan MNM, Majid MIA (2008). Biosynthesis of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) co-polymer by *Cupriavidus* sp. USMAA1020 isolated from Lake Kulim, Malaysia. Bioresour.Technol. 9: 4903-4909.

Anderson AJ, Dawes EA (1990). Occurence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. 54(4): 450-72.

Anderson AJ, William DR, Taidi B, Dawes EA, Ewing DF (1992). Studies on copolyester synthesis by *Rhodococcus rubber* and factors influency the molecular mass of polyhydroxybutyrate accumulated by *Methylobacterium extorquens* and *Alcaligenes eutrophus*. FEMS. Microbiol. Rev. 03: 93-102.

Brandl H, Gross RA, Lenz RW, Lloyd R, Fuller RC (1991). The accumulation of poly(3-hydroxyalkanoates) in *Rhodobacter sphaeroides*. Arch. Microbiol. 155(4): 337-340.

Braunegg G, Sonnleitner B, Lafferty RM (1978). A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in bacterial biomass. Eur. J. Appl .Microbiol. Biotechnol. 6: 29-37

Choi MH, Yoon SC, Lenz RW (1999).Production of poly(3-hydroxybutyric acid-co-4-hydroxybutyric acid) and poly(4-hydroxybutyric acid) without subsequent degradation by *Hydrogenophaga pseudoflava*. Appl. Environ. Microbiol. 65: 1570-1577.

Doi Y, Kanesawa Y, Tanashi N, Kumagai Y (1992). Biodegradation of Microbial polyesters in the marine environment. Polym. Degrad. Stab. 36: 173-177.

Doi Y (1990).Microbial Polyesters. Wiley-VCH Publishers, New York.

Doi Y, Kitamura S, Abe H (1995) Microbial synthesis and characterization of poly 3-hydroxybutyrate-*co*-3-hydroxyhexanoate). Macromolecules, 28: 822-4828.

Doi Y, Segawa A, Kunioka M (1990). Biosynthesis and characterization of poly (3-hydroxybutyrate-*co*-4-hydroxybutyrate) in *Alcaligenes eutrophus*. Int. J. Biol. Macromol. 12: 106-111.

Felsenstein J (1993). PHYLIP (Phylogeny Inference Package), Department of Genetics, University of Washington, Seattle.

Huss VAR, Festl H, Schleifer KH (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. Syst. Appl. Microbiol. 4: 184-192.

Kang CK, Kusaka S, Doi Y (1995). Structure and properties of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) produced by *Alcaligenes latus*. Biotechnol. Lett. 17: 583-588.

Kim JS, Lee BH, Kim BS (2005). Production of Poly(3-hydroxy-butyrate-*co*-4-hydroxybutyrate) by *Ralstonia eutropha*. Biochem. Eng. 23: 169-174.

Kunioka M, Kawaguchi Y, Doi Y (1989). Production of biodegradable copolyesters of 3-hydroxybutyrate and 4-hydroxybutyrate by *Alcaligenes eutrophus*. Appl. Microbiol. Biotechnol. 30(6): 569-573.

Kunioka M, Nakamura Y, Doi Y (1988). New bacterial copolyesters produced in *Alcaligenes eutrophus* from organic acids. Polym.

Commun. 29: 174-176.
Kuykendall LD, Roy MA, O'Neill JJ, Devine TE (1988). Fatty acids, antibiotic resistance, and deoxyribonucleic acid homology groups of Bradyrhizobium japonicum. Int. J. Syst. Bacteriol. 38: 358-361.
Lee WH, Azizan MNM, Sudesh K (2004). Effects of culture conditions on the composition of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) synthesized by *Comamonas acidovorans*. Polym. Deg. Stab. 84: 129-134.
Maidak BL, Cole JR, Parker CTJ, Garrity GM, Larsen N, Li B, Lilburn TG, McCaughey MJ, Olsen GJ, Overbeek R, Pramanik S, Schmidt TM, Tiedje JM, Woese CR (1999). A new version of the RDP (Ribosomal Database Project). Nucl. Acids. Res. 27:171-173.
Mesbah M, Premachandran U, Whitman W (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high performance liquid chromatography Int. J. Syst. Bact. 39: 159-167.
Mitomo H, Hsieh WC, Nishiwaki K, Kasuya K, Doi Y (2001). Poly (3-hydroxybutyrate-*co*-4-hydroxybutyrate) produced by *Comamonas acidovorans.* Polymerisation, 42(8): 3455-3461.
Ojumu TV, Yu J, Solomon BO (2004). Production of polyhydroxy-alkanoates, a bacterial biodegradable polymer. Afr. J. Biotech. 3: 18-24.
Rainey FA, Ward-Rainey N, Kroppenstedt RM, Stackebrandt E (1996). The genus Nocardiopsis represents a phylogenetically coherent taxon and a distinct actinomycete lineage; proposal of Nocardiopsaceaefam. Nov. Int. J. Sys. Bacteriol. 46: 1088-1092.
Renner G, Pongratz K, Braunegg G (1996). Production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) by *Comamonas testosteronii A3*. Food Technol. Biotechnol. 34: 91-95.
Saito Y, Nakamura S, Hiramitsu M, Doi Y (1996). Microbial synthesis and properties of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate). Polym. Int. 39(3): 169-74.
Scandola M, Ceccorulli G, Doi Y (1990). Viscoelastic relaxations and thermal properties of bacterial poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) and poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate). Int. J. Biol. Macromol. 12(2): 112-117.
Spiekermann P, Rehm BH, Kalscheuer R, Baumeister D, Steinbuchel A (1999). A sensitive, viable-colony staining method using Nile Red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. Arch. Microbiol. 171(2): 73-80.
Stanbury PF, Whitaker A (1984). The principles of fermentation technology. Oxford Pergamon Ltd.
Steinbüchel A, Lütke-Eversloh T (2003). Metabolic engineering and pathway construction for biotechnological production of relevant polyhydroxyalkanoates in microorganisms. Biochem. Eng. J. 16: 81-96.
Sudesh K, Abe H, Doi Y (2000). Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. Prog. Polym. Sci. 25: 1503-1555.
Taidi B, Anderson AJ, Dawes EA, Byrom D (1994). Effect of carbon source and concentration on the molecular mass of poly (3-hydroxybutyrate) produced by *Methylobacterium extorquens* and *Alcaligenes eutrophus*. Appl. Microbiol. Biotechnol. 40(6): 786-790.
Takeharu T (2002). Metabolic improvements and use of inexpensive carbon sources in microbial production of polyhydroxyalkanoates. J. Biosci. Bioeng. 94(6): 579-584.
Valentin HE, Dennis D (1997). Production of poly (3-hudroxybutyrate-*co*-4-hydroxybutyrate) in recombinant *Escherichia coli* grown in glucose. J. Biotech. 58: 33-38.
Vandamme P, Coenye T (2004). Taxonomy of the genus *Cupriavidus*: a tale of lost and found. Int. J. Syst. Evol. Microbiol .54: 2285-2289.
Young RJ, Lovell PA (1991). Introduction to polymers. Chapman & Hall Publisher, London.