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ORIGINAL PAPER

# Enhanced production of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) copolymer with manipulated variables and its properties

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**Abstract** *Cupriavidus* sp. USMAA1020, a local isolate was able to biosynthesis poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] copolymer with various 4HB precursors as the sole carbon source. Manipulation of the culture conditions such as cell concentration, phosphate ratio and culture aeration significantly affected the synthesis of P(3HB-*co*-4HB) copolymer and 4HB composition. P(3HB-*co*-4HB) copolymer with 4HB compositions ranging from 23 to 75 mol% 4HB with various mechanical and thermal properties were successfully produced by varying the medium aeration. The physical and mechanical properties of P(3HB-*co*-4HB) copolymers were characterized by NMR spectroscopy, gel-permeation chromatography, tensile test, and differential scanning calorimetry. The number-average molecular weights ($M_n$) of copolymers ranged from $260 \times 10^3$ to $590 \times 10^3$Da, and the polydispersities ($M_w/M_n$) were between 1.8 and 3.0. Increases in the 4HB composition lowered the molecular weight of these copolymers. In addition, the increase in 4HB composition affected the randomness of copolymer, melting temperature ($T_m$), glass transition temperature ($T_g$), tensile strength, and elongation to break. Enzymatic degradation of P(3HB-*co*-4HB) films with an extracellular depolymerase from *Ochrobactrum* sp. DP5 showed that the degradation rate increased proportionally with time as the 4HB fraction increased from 17 to 50 mol% but were much lower with higher 4HB fraction. Degradation of P(3HB-*co*-4HB) films with lipase from *Chromobacterium viscosum* exhibited highest degradation rate at 75 mol% 4HB. The biocompatibility of P(3HB-*co*-4HB) copolymers were evaluated and these copolymers have been shown to support the growth and proliferation of fibroblast cells.

**Keywords** P(3HB-*co*-4HB) · Biosynthesis · Characterization · Degradation · Cytotoxicity

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## Introduction

Plastics are probably one of the most indispensable commodities in our daily life. Global dependence on these synthetic plastics is inevitable. Plastics are an essential part of almost all industries, based on their versatile qualities of strength, lightness, durability and resistance to degradation [1]. However, these very desirable properties have now caused accumulation of recalcitrant plastic waste in the environment [2]. Their persistence in our environment may cause deleterious effects on the ecosystem. In such scenario, biodegradable plastics offer the best solution to address the hazards brought about by conventional plastic [3].

Polyhydroxyalkanoate (PHA) is one of the most promising microbial polymers that is completely biodegradable, biocompatible and possess properties similar to synthetic thermoplastics [4]. Accumulation of PHAs in bacterial cells

occurs when carbon source is provided in excess, and if at least one other nutrient which is essential for growth is depleted [5]. These water insoluble polymers accumulate as discrete spherical granules in cell cytoplasm and serve as storage compounds for energy and carbon [6, 7].

Microorganisms are able to accumulate various types of PHAs in the form of homopolyesters, copolyesters, or polyester blends [8]. The properties of PHA copolymers depend strongly on the type, content, and distribution of comonomer units which comprises the polymer chains, as well as the average molecular weight and molecular weight distribution [9, 10].

Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] has been found to exhibit useful properties relative to other PHAs. P(3HB-*co*-4HB) can be tailored to achieve a variety of polymeric materials, from hard crystalline plastics to very elastic rubber by varying 4HB monomer composition [11].

This copolymer exhibits desirable mechanical properties for applications in the medical and pharmaceutical field [6, 12]. The in vivo degradation rate of P(3HB-*co*-4HB) is relatively high compared to other PHAs, and can be controlled by varying the 4HB fraction [13]. To date, P(3HB-*co*-4HB) have gained interest in a wide variety of medical field such as cardiovascular, wound healing, orthopedic, drug delivery, and tissue engineering applications [14, 15]. Thus, the prospects of synthesizing P(3HB-*co*-4HB) copolymer with a wide variety of monomer units would widen its usefulness as biomaterials.

Therefore, in this study, P(3HB-*co*-4HB) copolymers with various composition of 4HB have been biosynthesized, characterized and their rate of biodegradation was investigated. In addition, a cytotoxicity test was conducted to evaluate the biocompatibility of the synthesized copolymer. Approaches of evaluating the cell concentration and manipulating the buffering effect to increase the 4HB composition in the copolymer have been investigated.

## Methods

### Bacterial strain

The strain used in this study was *Cupriavidus* sp. USMAA1020 (DSM 19416) which was isolated from sludge samples from Lake Kulim, Malaysia as reported previously by Amirul et al. [16].

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

Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) synthesis was carried out using two-stage cultivation process. *Cupriavidus* sp. USMAA1020 was grown in nutrient broth for 20 h. The cells were then harvested by centrifugation (10,000*g*), and transferred (1 g/l) into nitrogen-free mineral salts medium as previously reported by Amirul et al. [17]. The medium was supplemented with filter-sterilized $\gamma$-butyrolactone, 1,6-hexanediol, 1,8-octanediol, 1,10-decanediol, 1,12-dodecanediol at a concentration of 0.56 wt% carbon as the sole carbon source to promote PHA synthesis. Experiments were carried out in triplicates.

### Film casting

The P(3HB-*co*-4HB) was extracted and purified from freeze-dried cells to be casted into films. For this, 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 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 pure P(3HB-*co*-4HB) films were prepared by solvent-casting technique, whereby 1 g of the copolymer was dissolved with 10 ml of chloroform and poured into a glass Petri dish (diameter of 9 cm) as a casting surface.

### Analytical procedures

The PHA content and polymer composition of lyophilized cells were determined using gas chromatography (Shimadzu GC-14B, Kyoto, Japan) and nuclear magnetic resonance (Bruker AVANCE 300; NC, USA) analyses. Approximately 15 mg of the freeze-dried cells were 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 to convert monomer units to their corresponding methyl esters. Distilled water (1 ml) was added to the cooled mixture and vortexed to enhance phase separation. The lower organic phase containing the reaction products was separated, dried over anhydrous $Na_2SO_4$, and analyzed by GC [18]. PHA content, composition, yield and $Y_{PHA/S}$ were calculated from GC analyses. PHA yield is the amount of PHA produced whereas $Y_{PHA/S}$ is the amount of PHA produced over the carbon source used.

For NMR analysis, the lyophilized cells were stirred in chloroform under ambient condition for 24 h. After filtration, the chloroform extract was concentrated and the dissolved PHA was precipitated out in methanol. The purified polymer sample was dissolved in 0.5% (v/v) of deuterated chloroform ($CDCl_3$) and subjected to the 300 MHz $^{13}$C-NMR analysis (Bruker AVANCE 300; NC, USA). Tetramethylsilane ($Me_4Si$, $\delta = 0$) was used as an internal chemical shift.

The molecular weight of polyesters was analyzed by gel permeation chromatography (GPC) using a Waters 600E GPC system and Waters 410 refractive index detector with a PLgel Mixed C column (Polymer Laboratories, Ltd., UK). Chloroform was used as the eluent at a flow rate of 0.8 ml/min. The sample concentrations and injection volumes were 0.1% (w/v) and 20 µl, respectively. The operating temperature was maintained at 35°C. Polystyrene standards with a low polydispersity were used to construct a calibration curve. Number-average molecular weight ($M_n$), weight-average molecular weight ($M_w$) and polydispersity index ($M_w/M_n$) were calculated using Clarity chromatography software (Version 2.4).

Thermal characterization was determined using a differential scanning calorimeter (DSC, Pyris 1 DSC; Perkin Elmer). Samples (8–10 mg) were heated at a rate of 10°C/min from −50 to 200°C (first heating run). After the first heating run, the sample was cooled to −50°C at a rate of 20°C/min. Then, the sample was heated (10°C/min) again to 200°C (second heating run). The melting temperature ($T_m$) was determined from the DSC endotherms. The glass transition temperature ($T_g$) was taken as the midpoint of the heating capacity change.

The tensile test was carried out using a tensile testing machine (Instron 3366; USA) at a cross-head speed of 20 mm/min under ambient temperature. Tensile test pieces (5 mm width, 48 mm gauge length) were cut from polymer films using steel ASTM regulation punches. Mechanical tensile data were calculated from the stress–strain curves on an average of four specimens.

### Enzymatic degradation

Depolymerase enzyme from *Ochrobactrum* sp. (DP5) and lipase enzyme from *Chromobacterium viscosum* were used in this study. The P(3HB-*co*-4HB) films (initial weight, 0.011 g; film dimensions, 10 mm × 10 mm and thickness, 0.06 mm) at different 4HB composition was immersed in 40 ml glycine–NaOH buffer solution (pH 9.0) which contains either the depolymerase enzyme (0.05 unit) or lipase enzyme (50 unit) and 0.02% (w/v) sodium azide in sterilized conical flask. The reaction mixture was incubated at 37°C with agitation. The films were periodically removed, rinsed with water and dried to constant weight before analyses. Experiments were carried out in duplicates.

### Observation of P(3HB-*co*-4HB) films by scanning electron microscope

The P(3HB-*co*-4HB) films before and after enzymatic degradation were mounted on aluminum stumps and coated with gold. The films were observed with a scanning electron microscope (SEM, Leo Supra 50 VP Field Mission SEM; Carl-Ziess SMT, Oberkochen, Germany). For the polymer films cultured with mouse fibroblast cell (L929), the films were washed twice with phosphate buffered saline (PBS) and immersed in PBS containing 3% (v/v) glutaraldehyde (pH 7.4) for 4 h. They were then dehydrated in increasing concentrations of ethanol (30, 50, 70, 90, 95 and 100%) followed by lyophilization. They were then examined under SEM.

### In vitro cytotoxicity evaluation

The mouse fibroblast cell line (L929), was cultured in polystyrene flasks incubated at 37°C in incubator supplied with 5% $CO_2$ (Hera Cell, Heraeus). The culture medium was Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 mM L-glutamine, 10% fetal calf serum and 1% penicillin–streptomycin solution. The medium was changed every 2 days.

The mouse fibroblast cells were grown on confluence followed by detachment using trypsin. The cell suspension of $1.25 \times 10^5$ cells/ml was added to the polymer films in the Petri dish. Cell morphology was examined daily using an inverted phase contrast microscope.

The cell viability and proliferation was assayed with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay from Promega). A 20 µl of the combined MTS/PMS solution was pipetted into each well with 100 µl of cells and was incubated for another 1–4 h at 37°C in incubator supplied with 5% $CO_2$ (Hera Cell, Heraeus). The absorbance of the formazon was checked using a Microplate Reader (BioRad, Model 680, UK) at 490 nm and DMEM was used as blank. MTS/PMS assay was first performed on a directly counted L929 serial and the absorbency values were plotted against the counted cell numbers to establish a standard curve. Viable cell numbers on polymers was then determined from the standard curve based on their MTS absorbency. These films were observed under SEM for cell attachment. Experiments were carried out in replicates of four.

## Results and discussion

### Synthesis of P(3HB-*co*-4HB) copolymer with various 4HB molar fraction

Synthesis of P(3HB-*co*-4HB) was carried out through a two-stage cultivation. Table 1 shows the effect of initial cell concentration towards the copolymer P(3HB-*co*-4HB) synthesized by *Cupriavidus* sp. USMAA1020. Based on the results obtained, as the cell concentration was increased

**Table 1** The effect of cell concentration on the synthesis of copolymer P(3HB-*co*-4HB) by *Cupriavidus* sp. USMAA1020 in two-stage cultivation

| Initial cell concentration (g/l)[a] | Cell dry weight (g/l) | PHA content (wt.%)[b] | PHA composition (mol%)[a] | | PHA yield (g/l) | γ-Butyrolactone residual concentration (g/l)[c] |
|---|---|---|---|---|---|---|
| | | | 3HB | 4HB | | |
| 0.33 | 1.35 ± 0.02 | 44.5 ± 1.4 | 67 ± 1 | 33 ± 1 | 0.58 ± 0.02 | 4.9 ± 0.2 |
| 0.50 | 1.65 ± 0.02 | 45.7 ± 0.9 | 60 ± 1 | 40 ± 1 | 0.72 ± 0.02 | 4.1 ± 0.1 |
| 0.67 | 2.26 ± 0.03 | 41.5 ± 0.3 | 55 ± 1 | 45 ± 1 | 0.90 ± 0.02 | 3.7 ± 0.2 |
| 1.00 | 2.57 ± 0.04 | 43.0 ± 1.0 | 47 ± 1 | 53 ± 1 | 1.05 ± 0.03 | 3.2 ± 0.1 |
| 1.67 | 3.05 ± 0.10 | 44.7 ± 3.4 | 48 ± 1 | 52 ± 1 | 1.25 ± 0.07 | 2.5 ± 0.1 |
| 2.50 | 2.63 ± 0.06 | 25.3 ± 2.4 | 43 ± 1 | 57 ± 1 | 0.51 ± 0.06 | 1.8 ± 0.2 |

The cells were harvested after 48 h. Values are mean ± SD of three replicates

[a] Cells were cultured in MSM containing 0.56% carbon (wt%) γ-butyrolactone

[b] Calculated from GC analysis

[c] Initial concentration of 10 g/l of γ-butyrolactone was used

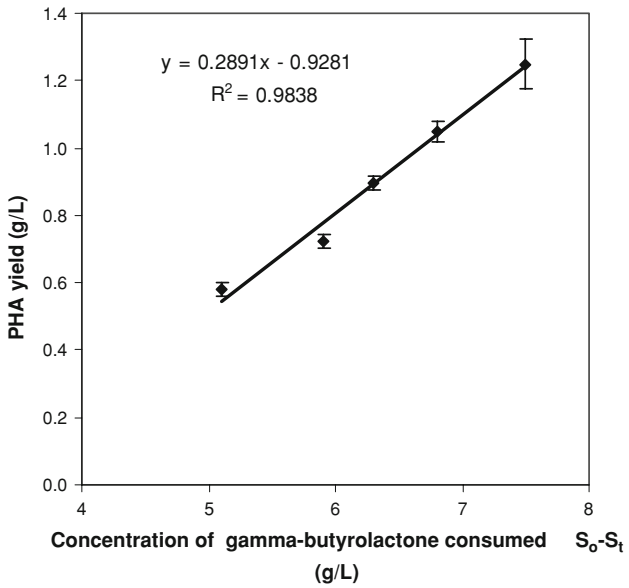


**Fig. 1** Relation between PHA yield and the concentration of the γ-butyrolactone consumed. Values are mean ± SD of three replicates

from 0.33 to 1.67 g/l, the PHA yield increased from 0.58 to 1.25 g/l and PHA content ranged from 41.5 to 45.7 wt%. However, further increase in cell concentration above 1.67 g/l resulted in a decrease in PHA content as well as PHA yield. Meanwhile, the 4HB molar fraction in the copolymer was enhanced from 33 to 57 mol%. Figure 1 shows the relation between the concentrations of the consumed γ-butyrolactone and the PHA yield. It clearly indicates that PHA yield increased proportionately with the consumed γ-butyrolactone as the cell concentration was increased up to 1.67 g/l.

*Cupriavidus* sp. USMAA1020 was found to utilize a wide range of carbon sources for the accumulation of polymer as reported previously by Amirul et al. [16]. Based on the previous study, γ-butyrolactone resulted in high molar fraction of 4HB (51 mol%) in the copolymer with an accumulation of 47.6 wt% of the dry cell weight. In this experiment, carbon sources with different alkanediols with even carbon sources exceeding four carbons such as 1,6-hexanediol, 1,8-octanediol, 1,10-decanediol and 1,12-dedocanediol were tested (Table 2). Among these carbon sources, 1,6-hexanediol resulted in the accumulation of 20.1 wt% of the copolymer and the 4HB composition was 42 mol%. On the other hand, alkanediols such as 1,8-octanediol, 1,10-decanediol, and 1,12-dedocanediol resulted in accumulation of copolymer with low compositions of 4HB units (10–13 mol%) though the PHA content ranged from 2.5 to 72.1 wt%. According to Steinbüchel and Lütke-Eversloh [19], ω-alkanediols with a greater carbon chain but an even number of carbon atoms are suitable precursor substrates for the biosynthesis of P(3HB-*co*-4HB) copolymer with high 4HB fraction. The ω-alkanediols are subjected to β-oxidation until 4HB-CoA is produced, which is then directly polymerized by PHA synthase to 4HB as it is not a chiral intermediate like 3HB-CoA [19]. However, contradicting results were observed in this study compared to the previous reports [13, 20], whereby the feeding of ω-alkanediols such as 1,10-decanediol and 1,12-dodecanediol as the sole carbon source resulted in the accumulation of low compositions of 4HB units in P(3HB-*co*-4HB) copolymer with only 10 mol%. This could be because, ω-alkanediols with greater carbon chain length, especially those with even number of carbon atoms more than 8 need not necessarily be oxidized to the ω-hydroxyfatty acid and converted to 4HB-CoA through β-oxidation cycles as stated by Steinbüchel and Lütke-Eversloh [19]. Possibly, part of the substrate could have been utilized for cell growth and conversion to 3HB-CoA as the 3HB composition obtained is relatively high.

In this experiment, the effect of various $K_2HPO_4$ to $KH_2PO_4$ ratio and its buffering capacity towards the synthesis

**Table 2** Synthesis of P(3HB-*co*-4HB) copolymer by *Cupriavidus* sp. USMAA1020 on γ-butyrolactone and alkanediols through a two-stage cultivation process

| Carbon sources[a] | Cell dry weight (g/l) | PHA content (wt%)[c] | PHA composition (mol%)[c] | |
|---|---|---|---|---|
| | | | 3HB | 4HB |
| γ-Butyrolactone[b] | 3.02 ± 0.10 | 47.6 ± 3.8 | 49 ± 1 | 51 ± 1 |
| 1,6-Hexanediol | 1.87 ± 0.06 | 20.1 ± 0.9 | 58 ± 1 | 42 ± 1 |
| 1,8-Octanediol | 1.65 ± 0.04 | 2.5 ± 0.2 | 87 ± 2 | 13 ± 2 |
| 1,10-Decanediol | 2.67 ± 0.05 | 35.9 ± 3.9 | 90 ± 1 | 10 ± 1 |
| 1,12-Dodecanediol | 4.86 ± 0.25 | 72.1 ± 1.6 | 90 ± 1 | 10 ± 1 |

The cells were harvested after 48 h; Values are mean ± SD of three replicates

[a] Cells were cultured in MSM containing 0.56% carbon (wt%)

[b] As previously reported by Amirul et al. (2008)

[c] Calculated from GC analysis

**Table 3** The effect of different $K_2HPO_4$ to $KH_2PO_4$ ratio towards the synthesis of copolymer P(3HB-*co*-4HB) by *Cupriavidus* sp. USMAA1020 in two-stage cultivation

| $K_2HPO_4$ to $KH_2PO_4$ mol ratio[a] | Initial pH | Cell dry weight (g/l) | PHA content (wt.%)[b] | PHA composition (mol%)[b] | | PHA yield (g/l) | Final pH |
|---|---|---|---|---|---|---|---|
| | | | | 3HB | 4HB | | |
| 0 | 5.7 | 1.43 ± 0.05 | 14.6 ± 2.2 | 57 ± 1 | 43 ± 1 | 0.21 ± 0.03 | 3.3 |
| 0.15 | 6.0 | 1.93 ± 0.08 | 22.9 ± 1.6 | 71 ± 2 | 29 ± 2 | 0.44 ± 0.04 | 4.9 |
| 0.30 | 6.3 | 2.33 ± 0.04 | 28.9 ± 0.7 | 70 ± 1 | 30 ± 1 | 0.67 ± 0.02 | 5.0 |
| 0.61 | 6.6 | 2.58 ± 0.04 | 34.8 ± 0.8 | 54 ± 3 | 44 ± 3 | 0.90 ± 0.01 | 5.4 |
| 1.22 | 6.9 | 4.30 ± 0.12 | 49.5 ± 2.1 | 48 ± 4 | 52 ± 4 | 2.13 ± 0.14 | 7.0 |
| 2.45 | 7.2 | 4.64 ± 0.08 | 53.6 ± 2.1 | 46 ± 1 | 54 ± 1 | 2.49 ± 0.06 | 7.3 |
| 4.90 | 7.5 | 4.14 ± 0.07 | 46.6 ± 0.7 | 47 ± 1 | 53 ± 1 | 1.93 ± 0.03 | 7.5 |
| 9.80 | 7.8 | 3.85 ± 0.06 | 45.3 ± 1.5 | 50 ± 3 | 50 ± 3 | 1.74 ± 0.08 | 7.6 |

The cells were harvested after 48 h. Values are mean ± SD of three replicates

[a] Standard mol ratio of the source of phosphate is 1.22 based on Handerson–Hasselbach equation (3.7 g/l $KH_2PO_4$ and 5.8 g/l $K_2HPO_4$). $pK_{a2}$ value of phosphate is 6.82

[b] Calculated from GC analysis

of copolymer P(3HB-*co*-4HB) by *Cupriavidus* sp. USMAA1020 in two-stage cultivation was studied. Based on the results listed in Table 3, the PHA accumulation increased from 14.6 to 53.6 wt% as the $K_2HPO_4$ to $KH_2PO_4$ mol ratio increased from 0 to 2.45. Above this ratio, the PHA content showed a slight decrease. Similar pattern was observed in the PHA yield whereby as the $K_2HPO_4$ to $KH_2PO_4$ mol ratio increased from 0 to 2.45, PHA yield increased from 0.21 to 2.49 g/l, followed by a slight decrease upon further increase in the $K_2HPO_4$ to $KH_2PO_4$ mol. This can be due to the high concentration of phosphate [$PO_4^-$]. Although PHA content increased as the phosphate ratio increased, but above a certain extent a high concentration of phosphate [$PO_4^-$] disrupts the PHA accumulation. Meanwhile, the 4HB molar fraction ranged from 29 to 54 mol%. In addition to this, at a lower buffering capacity where the phosphate ratio ranged from 0.15 to 0.61, the final pH in the culture fluctuated. However, with the phosphate ratio above 0.61, the final pH of the culture was maintained at around pH 7. This indicates that phosphate mol ratio gives a significant effect on the microbial synthesis of PHA.

The effect of aeration towards synthesis of P(3HB-*co*-4HB) copolymer was studied by varying the culture volume over flask volume ratio. Culture aeration was found to influence the synthesis of this copolymer and the incorporation of 4HB monomer by *Cupriavidus* sp. USMAA1020. Under oxygen limiting conditions, better PHA accumulation could be achieved though limited to a certain ratio. Based on the results in Table 4, the copolymer with 4HB compositions ranging from 23 to 75 mol% 4HB were successfully produced by decreasing the culture aeration (increasing in culture volume). PHA content was increased from 35.9 to 47.5 wt% as the culture volume to flask volume was increased from 30 to 50 ml in 250 ml flask. This could be due to other limiting factor such as limited aeration in shake flask at

**Table 4** Effect of culture volume to flask volume ratio towards the synthesis of P(3HB-*co*-4HB) copolymer by *Cupriavidus* sp. USMAA1020 in two stage cultivation

| Culture volume to flask volume (ml/ml) | Dry cell weight (g/l) | PHA content (wt.%)[a] | PHA composition (mol%)[a] | | PHA yield (g/l) | Residual biomass (g/l)[b] | $Y_{X/S}$ (g/g)[c] | $Y_{PHA/S}$ (g/g)[d] |
|---|---|---|---|---|---|---|---|---|
| | | | 3HB | 4HB | | | | |
| 30/250 | 6.23 ± 0.07 | 35.9 ± 1.6 | 78 ± 2 | 23 ± 2 | 2.24 ± 0.07 | 2.80 ± 0.14 | 0.37 ± 0.01 | 0.29 ± 0.01 |
| 40/250 | 5.37 ± 0.14 | 39.3 ± 1.0 | 64 ± 1 | 36 ± 1 | 2.11 ± 0.08 | 2.06 ± 0.11 | 0.29 ± 0.01 | 0.29 ± 0.01 |
| 50/250 | 5.89 ± 0.11 | 47.5 ± 2.9 | 57 ± 1 | 43 ± 1 | 2.80 ± 0.22 | 1.89 ± 0.11 | 0.25 ± 0.01 | 0.36 ± 0.03 |
| 65/250 | 4.21 ± 0.07 | 41.7 ± 3.5 | 32 ± 1 | 68 ± 1 | 1.76 ± 0.15 | 1.26 ± 0.15 | 0.18 ± 0.02 | 0.24 ± 0.02 |
| 80/250 | 2.59 ± 0.05 | 30.2 ± 1.8 | 24 ± 1 | 75 ± 1 | 0.78 ± 0.06 | 0.61 ± 0.03 | 0.10 ± 0.01 | 0.12 ± 0.01 |

PHA content at 48 h incubation time. Values are mean ± SD of three replicates

[a] Calculated from GC analysis

[b] Calculated from GPC analysis

[c] $Y_{X/S}$ was calculated based on the dry cell weight produced from the concentration of γ-butyrolactone used

[d] $Y_{PHA/S}$ was calculated based on the PHA produced from the of γ-butyrolactone used

**Table 5** Molecular weights, thermal and mechanical properties of P(3HB-*co*-4HB) copolymer synthesized by *Cupriavidus* sp. USMAA1020

| P(3HB-*co*-4HB) copolymer | $M_n$ ($\times 10^3$)[a] Da | $M_w/M_n$ | Glass transition temp.[b] ($T_g$) °C | Melting temp.[b] ($T_m$) °C | Tensile strength (MPa)[d] | Elongation to break (%)[d] | $D$[e] |
|---|---|---|---|---|---|---|---|
| 23 mol% 4HB | 590 ± 9 | 1.8 ± 0.1 | −7 | 152 | 13 ± 2 | 626 ± 31 | 1.0 |
| 36 mol% 4HB | 540 ± 3 | 1.9 ± 0.1 | −11 | 164 | 4 ± 1 | 400 ± 6 | 2.9 |
| 45 mol% 4HB | 410 ± 7 | 2.0 ± 0.1 | −16 (−40)[e] | 162 | 3 ± 1 | 268 ± 15 | 1.1 |
| 66 mol% 4HB | 320 ± 19 | 2.5 ± 0.1 | −43 (−13)[e] | 163 (47)[c] | 9 ± 1 | 446 ± 26 | 8.7 |
| 75 mol% 4HB | 260 ± 2 | 3.0 ± 0.2 | −45 | 51 (157)[c] | 16 ± 2 | 526 ± 43 | 19.8 |

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

[a] Calculated from GPC analysis

[b] Calculated from DSC analysis

[c] Minor peak

[d] Determined using Instron 3366 Tensile Machine

[e] Calculated from $^{13}$C-NMR analysis (carbon carbonyl resonance), $D = (F_{33}F_{44})/(F_{34}F_{43})$

constant speed [21]. Similar pattern was observed in PHA yield and $Y_{PHA/S}$. Upon further decrease in aeration whereby culture volume to flask volume was increased above 50 ml, PHA accumulation decreased as well as PHA yield and $Y_{PHA/S}$. However, upon increasing the culture volume from 30 to 80 ml 4HB molar fraction increased from 23 mol% to 75 mol%. The highest 4HB molar fraction of 75 mol% was achieved with 80 ml culture volume but at a low PHA content of 30 wt%. On the other hand, increasing the culture volume from 30 to 80 ml, decreased the residual biomass from 2.8 to 0.61 g/l. The depletion of residual cell biomass can be attributed to the limited aeration when the culture volume was increased. From these results, it was concluded that the 4HB composition could be successfully increased by decreasing the culture aeration. Hein et al. [22] reported that the recombinant *Escherichia coli* produced P(3HB-*co*-4HB) copolymer instead of P(4HB) homopolymer when the culture was well aerated. Thus, the author concluded that an increase in the culture volume or decrease in aeration favored the incorporation 4HB monomer. Similar observation was also reported by Lee et al. [23] with *Comamonas acidovorans*, whereby 4HB composition as high as 50 mol% was achieved with a culture volume of 300 ml in a 500 ml flask. This could be a useful way to regulate 4HB molar fraction in the P(3HB-*co*-4HB) copolymer in a cost effective method.

## Molecular weights, thermal and mechanical properties of P(3HB-*co*-4HB)

The copolymers P(3HB-*co*-4HB) with the 4HB composition ranging from 23 to 75 mol% which were synthesized previously based on the culture volume over flask volume ratio were characterized. Properties of these copolymers were listed in Table 5. Based on the results, the $M_n$ values of the copolymer decreased from 590 to 260 kDa with the

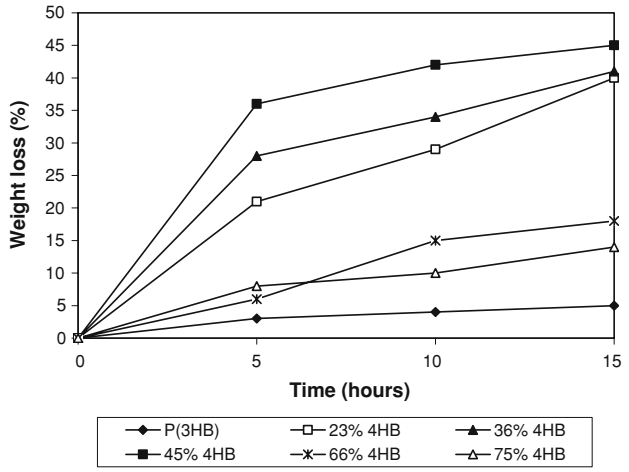


**Fig. 2** Enzymatic degradation profiles of P(3HB-*co*-4HB) films in an aqueous solution of depolymerase from *Ochrobactrum* sp. Values are mean of two replicates

increasing 4HB fraction, while polydispersity ($M_w/M_n$) increased from 1.8 to 3.0. As the increase in 4HB monomer fraction occurred concurrently with the decrease in the $M_n$ values, it can be concluded that the copolymers with higher composition of 4HB synthesized in this study have lower molecular weight.

The glass transition temperature ($T_g$) of P(3HB-*co*-4HB) decreased from −7 to −45°C as the 4HB fraction increased from 23 to 75 mol%. Similar finding were reported in previous studies [24, 25]. However, the melting temperature ($T_m$) ranged from 51 to 164°C. Copolymers with 66 and 75 mol% 4HB had two series of melting points with minor peaks at 47 and 157°C respectively. These minor peaks implies that these copolymers are mixtures of two copolymers containing lower and higher 4HB composition. Again, similar observations were reported previously [24, 25].

The tensile strength of P(3HB-*co*-4HB) films containing 23–45 mol% 4HB decreased from 13 to 3 MPa with an increase in 4HB fraction. But, the tensile strength of the films with 66 and 75 mol% 4HB fraction increased from 9 to 16 MPa. Same pattern was observed in the elongation to break whereby, 4HB fraction from 23 to 45 mol% showed a decrease in elongation to break from 626 to 268% followed by an increase from 446 to 526% as the 4HB molar fraction of the copolymers increased from 45 to 75 mol%. This indicates that the copolymer P(3HB-*co*-4HB) exhibits a wide range of material properties, ranging from plastics to elastic rubbers depending on the composition.

Interestingly, the copolymer with 23 mol% 4HB exhibited higher elongation to break (626%), tensile strength (13 MPa) and melting temperature (152°C). This suggest that, a flexible P(3HB-*co*-4HB) copolymer with better properties could be attained by incorporating a low fraction of 4HB monomer. Randomness of copolymer was determined by values from expanded $^{13}$C-NMR carbon carbonyl resonance spectrum. Based on the results in Table 5, the *D*-values of the copolymers with 4HB composition of 23–45 mol% were in the range of 1.0–2.9, suggesting that these copolymers are close to a random distribution. While the copolymers with 66 and 75 mol% of 4HB where the *D*-values are 8.7 and 19.8 indicates blend copolymers which is similar to the findings reported by Mitomo et al. [24]. Therefore, it may be concluded that this bacterium is able

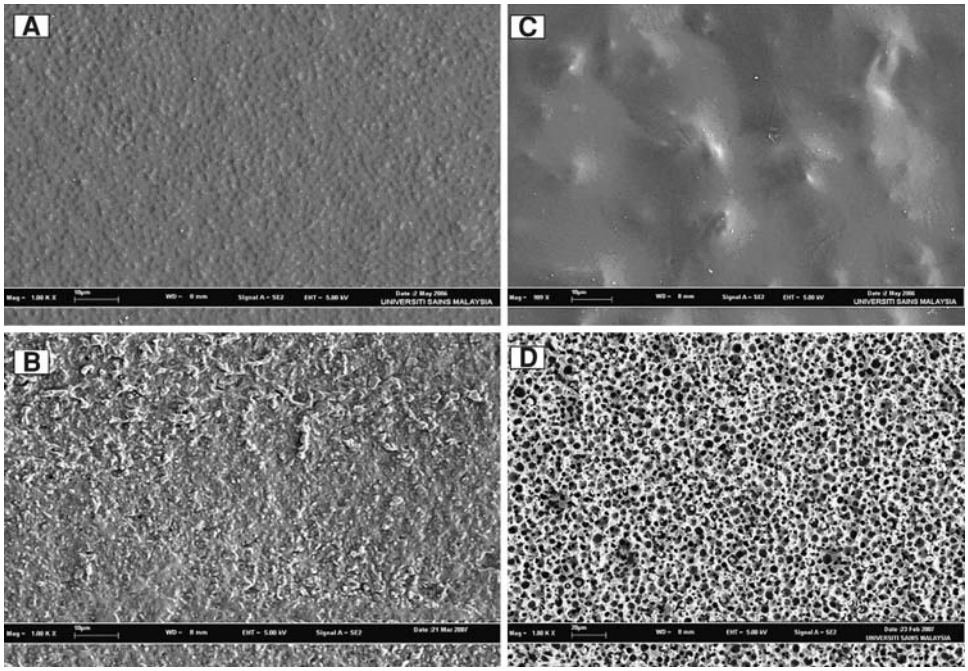


**Fig. 3** SEM micrographs of P(3HB-*co*-4HB) films before and after depolymerase enzyme erosion. **a** P(3HB-*co*-45%4HB) before degradation, **b** P(3HB-*co*-45%4HB) after degradation, **c** P(3HB-*co*-75%4HB) before degradation, **d** P(3HB-*co*-75%4HB) after degradation

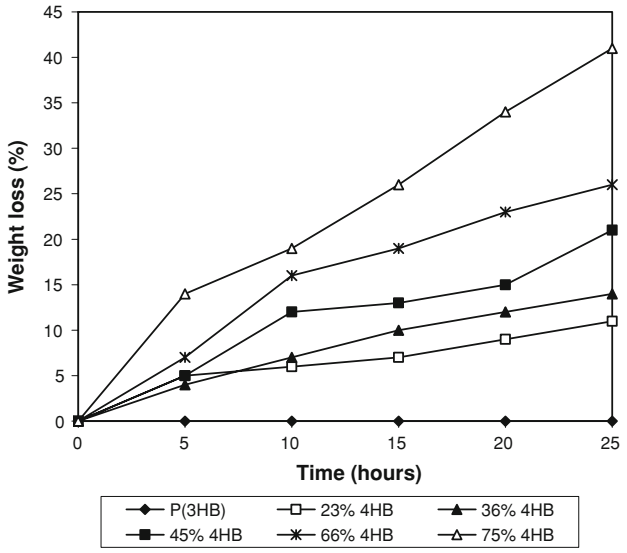


**Fig. 4** Enzymatic degradation profiles of P(3HB-*co*-4HB) films in an aqueous solution of lipase from *Chromobacterium viscosum*. Values are mean of two replicates

to produce both random copolymers and blend which is dependent on the composition of 4HB units.

Enzymatic degradation profiles of P(3HB-*co*-4HB) films

Degradation of P(3HB-*co*-4HB) was carried out using depolymerase and lipase enzyme. Figure 2 shows the enzymatic degradation (erosion) profiles of P(3HB-*co*-4HB) films as a function of time with an extracellular depolymerase enzyme from *Ochrobactrum* sp. DP5. The film erosion increased proportionally with time for all samples. The degradation rate of P(3HB-*co*-4HB) films by depolymerase enzyme increased as the 4HB fraction increased from 17 to 50 mol%. This can be due to the decrease in crystallinity with an increase in the 4HB content as previously described by Saito and Doi [26]. However, the degradation rate of copolymers with higher 4HB fraction of 66 and 75 mol% were much lower. This is because when the composition of 4HB is high, the poly(4HB) sequences crystallize and decreases the degradation. A similar trend was also noticed by Mitomo et al. [24], whereby as the 4HB composition in the copolymer increased to a certain extent, the rate of degradation decreases. Based on the result, the rate of degradation of homopolymer P(3HB) was slow due to its high crystallinity (60%) and it is similar as reported by Kumagai et al. [27]. Figure 3 shows SEM micrograph of P(3HB-*co*-4HB) films before and after depolymerase enzyme erosion. The surface of copolymer with 45 mol% 4HB was more eroded compared to copolymer with 75 mol% 4HB.

Figure 4 exhibits the degradation profile of P(3HB-*co*-4HB) films with lipase from *Chromobacterium viscosum*. In contrast to depolymerase enzyme, the erosion rate of films eroded by lipase increased with the increasing 4HB fraction. The copolymer with the highest 4HB composition (75 mol%) degraded the fastest. Figure 5 shows SEM micrograph of P(3HB-*co*-4HB) films before and after being degraded by lipase enzyme. The film of P(3HB)

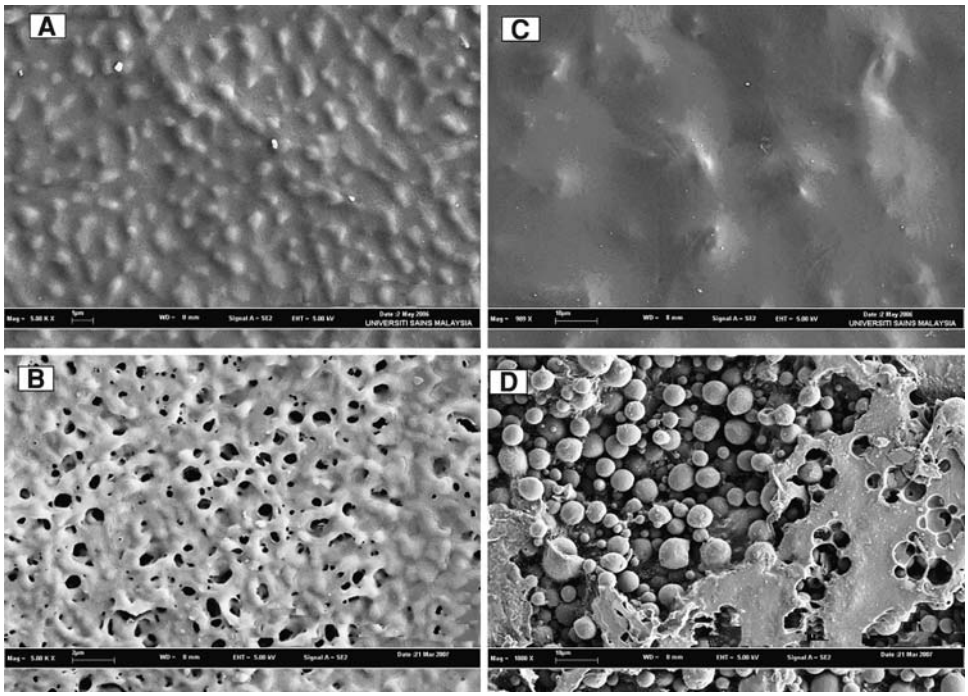


**Fig. 5** SEM micrographs of P(3HB-*co*-4HB) films before and after lipase enzyme erosion. **a** P(3HB-*co*-36%4HB) before degradation, **b** P(3HB-*co*-36%4HB) after degradation, **c** P(3HB-*co*-75%4HB) before degradation, **d** P(3HB-*co*-75%4HB) after degradation (round particles are undegraded polymer consist mainly of 3HB monomers)

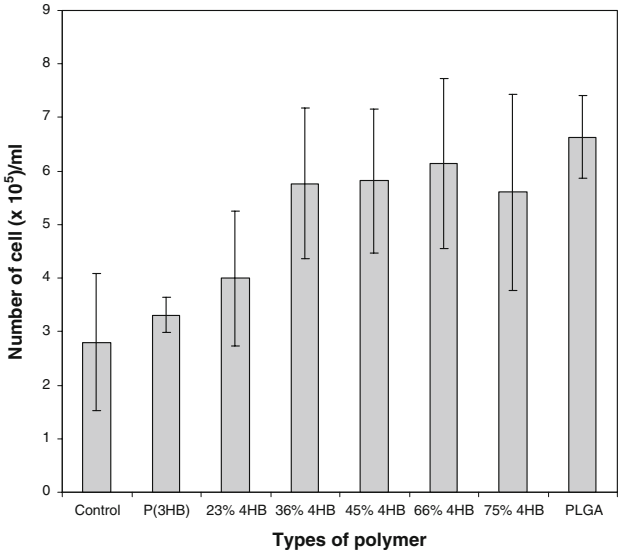


**Fig. 6** Comparison of cell viability on various P(3HB-*co*-4HB) copolymer, PLGA and P(3HB). Values are mean ± SD for five replicates

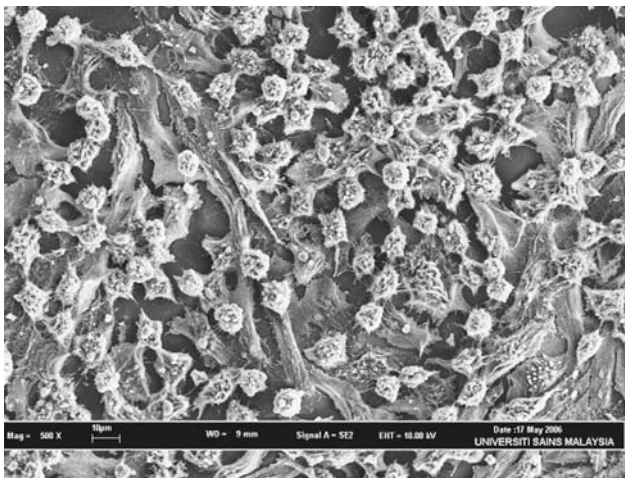


**Fig. 7** SEM micrographs of the P(3HB-*co*-75mol%4HB) copolymer film with growth of fibroblast cells (L929)

homopolyester was not eroded by the lipase. A similar trend was reported by Saito et al. [13] and Mitomo et al. [24]. The functional differences of these two enzymes can be attributed to the enzymes functional structures and biodegradation mechanism [26].

## Biocompatibility evaluation on the P(3HB-*co*-4HB) copolymer

Cytotoxicity of P(3HB-*co*-4HB) copolymer was tested using the copolymer with 4HB composition ranging from 23 to 75 mol%. Figure 6 shows the comparison of cell growth on various P(3HB-*co*-4HB) copolymer ranging from 23 to 75 mol% and poly(lactic-*co*-glycolic acid) (PLGA) films. Results showed that cell viability on P(3HB-*co*-4HB) films were two times higher compared to the medium without copolymer films and those containing P(3HB) homopolymer films. Cell viability on the 36–75 mol% 4HB copolymer films was as significant as in PLGA films (positive control). The results were similar to those reported previously by Yang et al. [15] and Chee et al. [14]. The increased number of cells on the various copolymer films demonstrated that proliferation occurred on all tested P(3HB-*co*-4HB) films. Growth of fibroblast cells (L929) on P(3HB-*co*-75mol%4HB) films is shown in Fig. 7. The result of the present study have shown that various P(-*co*-4HB) copolymers synthesized by *Cupriavidus* sp. USMAA1020 using $\gamma$-butyrolactone as the carbon substrate could support cell growth, indicating a promising application of these copolymers as biomaterials.

## Conclusion

In this study, a locally isolated bacterium, *Cupriavidus* sp. USMAA1020 was capable of accumulating P(3HB-*co*-4HB) copolymers from various 4HB precursor as the sole carbon source. The P(3HB-*co*-4HB) copolymers exhibit different mechanical and thermal properties depending on the 4HB monomer composition. The degradation of these copolymers was tested in the presence of depolymerase and lipase enzyme. The rate of degradation by depolymerase enzyme decreased as the 4HB composition in the copolymer increased. However, the copolymer with higher 4HB composition degraded the fastest with the lipase enzyme. Based on the positive results obtained from cytotoxicity test, the copolymers synthesized by *Cupriavidus* sp. USMAA1020 could be tailored into various biomaterials.

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## References

1. Ojumu TV, Yu J, Solomon BO (2004) Production of polyhydroxyalkanoates, biodegradable polymer. Afr J Biotechnol 3(1):18–24
2. Sudesh K, Doi Y (2000) Molecular design and biosynthesis of biodegradable polyesters. Polym Adv Technol 11:865–872. doi:10.1002/1099-1581(200008/12)11:8/12<865::AID-PAT34>3.0.CO;2-Z
3. Khanna S, Srivastava AK (2005) Recent advances in microbial polyhydroxyalkanoates. Process Biochem 40(2):607–619. doi:10.1016/j.procbio.2004.01.053
4. Doi Y (1990) Microbial polyesters. Wiley-VCH, New York
5. Anderson AJ, Dawes EA (1990) Occurrence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalkanoates. Microbiol Rev 54(4):450–472

6. Sudesh K, Abe H, Doi Y (2000) Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. Prog Polym Sci 25:1503–1555. doi:10.1016/S0079-6700(00)00035-6
7. Reddy CS, Ghai R, Rashmi Kalia VC (2003) Polyhydroxyalkanoates: an overview. Bioresour Technol 87(2):137–146. doi:10.1016/S0960-8524(02)00212-2
8. Bhatt R, Shah D, Patel KC, Trivedi U (2008) PHA-rubber blends: synthesis, characterization and biodegradation. Bioresour Technol 99:4615–4620. doi:10.1016/j.biortech.2007.06.054
9. Wang YJ, Hua FL, Tsang YF, Chan SY, Sin SN, Chua H, Yu PHF, Ren NQ (2007) Synthesis of PHAs from waste under various C:N ratios. Bioresour Technol 98:1690–1693. doi:10.1016/j.biortech.2006.05.039
10. Chanprateep S, Katakura Y, Visetkoop S, Shimizu H, Kulpreecha S, Shioya S (2008) Characterization of new isolated Ralstonia eutropha strain A-04 and kinetic study of biodegradable copolyester poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) production. J Ind Microbiol Biotechnol (in press)
11. Martin DP, Williams SF (2003) Medical applications of poly-4-hydroxybutyrate: a strong flexible absorbable biomaterial. Biochem Eng J 16:97–105. doi:10.1016/S1369-703X(03)00040-8
12. Zinn M, Witholt B, Egli T (2001) Occurrence, synthesis and medical application of bacterial polyhydroxyalkanoate. Adv Drug Deliv Rev 53:5–21. doi:10.1016/S0169-409X(01)00218-6
13. Saito Y, Nakamura S, Hiramitsu M, Doi Y (1996) Microbial synthesis and properties of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate). Polym Int 39(3):169–174. doi:10.1002/(SICI)1097-0126(199603)39:3<169::AID-PI453>3.0.CO;2-Z
14. Chee JW, Amirul AA, Tengku Muhammad TS, Majid MIA, Mansor SM (2008) The influence of copolymer ratio and drug loading on the biocompatibility of P(3HB-co-4HB) synthesized by Cupriavidus sp. (USMAA2–4). Biochem Eng J 38(3):314–318. doi:10.1016/j.bej.2007.07.018
15. Yang XS, Zhao K, Chen GQ (2002) Effect of surface treatment on the biocompatibility of microbial polyhydroxyalkanoates. Biomaterials 23:1391–1397. doi:10.1016/S0142-9612(01)00260-5
16. Amirul AA, Yahya ARM, Sudesh K, Azizan MNM, Majid MIA (2008) Biosynthesis of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) copolymer by *Cupriavidus* sp. USMAA1020 isolated from Lake Kulim, Malaysia. Bioresour Technol 99:4903–4909. doi:10.1016/j.biortech.2007.09.040
17. 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. J Biosci 15(2):125–135
18. 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. doi:10.1007/BF00500854
19. Steinbuchel A, Lütke-Eversloh T (2003) Metabolic engineering and pathway construction for biotechnological production of relevant polyhydroxyalkanoates in microorganisms. Biochem Eng J 16:81–96. doi:10.1016/S1369-703X(03)00036-6
20. Kunioka M, Nakamura Y, Doi Y (1988) New bacterial copolyester produced from *Alcaligenes eutrophus* from organic acids. Polym Commun (Guildf) 29:174–176
21. Shuler LM, Kargi F (1992) Bioprocess engineering, basic concepts. Prentice Hall, Upper Saddle River
22. Hein S, Sohling B, Gottschalk G, Steinbuchel A (1997) Biosynthesis of poly(4-hydroxybutyric acid) by recombinant strains of *Escherichia coli*. FEMS Microbiol Lett 153:411–418
23. 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 Degrad Stab 84:129–134. doi:10.1016/j.polymdegradstab.2003.10.003
24. Mitomo H, Hsieh WC, Nishiwaki K, Kasuya K, Doi Y (2001) Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) produced by *Comamonas acidovorans*. Polymer (Guildf) 42(8):3455–3461. doi:10.1016/S0032-3861(00)00678-9
25. Nakamura S, Doi Y (1992) Microbial synthesis and characterization of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate). Macromolecules 25(17):4237–4241. doi:10.1021/ma00043a001
26. Saito Y, Doi Y (1994) Microbial synthesis and properties of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) in *Comamonas acidovorans*. Int J Biol Macromol 16:99–104. doi:10.1016/0141-8130(94)90022-1
27. Kumagai Y, Kanesawa Y, Doi Y (1992) Enzymatic degradation of microbial poly(3-hydroxybutyrate) films. Macromol Chem 193:53–57. doi:10.1002/macp.1992.021930105