

# Biosynthetic enhancement of single-stage Poly(3-hydroxybutyrate-co-4-hydroxybutyrate) production by manipulating the substrate mixtures

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**Abstract** Two-stage fermentation was normally employed to achieve a high poly(3-hydroxybutyrate-co-4-hydroxybutyrate) [P(3HB-co-4HB)] productivity with higher 4HB molar fraction. Here, we demonstrated single-stage fermentation method which is more industrial feasible by implementing mixed-substrate cultivation strategy. Studies on bioreactor scale show a remarkably high PHA accumulation of 73 wt%, contributing to a high PHA concentration and product yield of 8.6 g/L and 2.7 g/g, respectively. This fermentation strategy has resulted in copolymers with wider range of 4HB monomer composition, which ranges from 12 to 55 mol%. These copolymers show a broad range of weight average molecular weight ( $M_w$ ) from 119.5 to 407.0 kDa. The copolymer characteristics were found to be predominantly affected by the nature of the substrates and the mixture strategies, regardless of the 4HB monomer compositions. This was supported by the determination of copolymer randomness using <sup>13</sup>C-NMR analysis. The study warrants significantly in the copolymer scale-up and modeling at industrial level.

**Keywords** Copolymer P(3HB-co-4HB) · Molecular weight · Polymer randomness · Substrate mixtures · Single-stage cultivation

## Introduction

Microorganism tends to trigger survival mechanisms in response to the nutrient fluctuation in their natural environment. Polyhydroxyalkanoate (PHA) accumulation is one of such adaptations, which is induced at nutrient limited conditions but with an excess of carbon sources [2]. Serving as carbon and energy storage, the polymer accumulation appears as hydrophobic inclusions in the cytoplasm. PHA exhibits similar mechanical properties with synthetic plastics, such as polypropylene. PHA is also known to be completely biodegradable. In current trends, the employment of renewable and eco-friendly resources for sustainable development has become a concern among the researches. This has garnered PHA as an alternative to conventional petroleum-based plastics.

Various kinds of PHAs could be produced when different monomer units are incorporated and this depends on the substrate uptake by the microorganism. Copolymer P(3HB-co-4HB) is one of the biopolymers which has been found to be a promising biomaterial in medical and pharmaceutical applications. This is mainly due to the presence of 4-hydroxybutyrate (4HB) monomer, as it has the ability to tolerate inflammatory responses with tissues, subsequently enhancing the overall biocompatibility of these copolymers [20].

To date, several wild-type bacterial strains were found to be potential producers of copolymer P(3HB-co-4HB). This includes *Cupriavidus necator* [5, 15, 24, 26], *Alcaligenes latus* [11, 14, 26], *Comamonas acidovorans* [17,

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21, 26], *Comamonas testosteroni* [25], *Hydrogenophaga Pseudoflava* [8], *Chromobacterium* sp. [16], *Burkholderia sacchari* [6], *Cupriavidus* sp. USMAA 2-4 [7, 27], *Cupriavidus* sp. USMAA1020 [1, 12, 28], and *Cupriavidus* sp. USMAHM 13 [23].

Production of copolymer P(3HB-co-4HB) has been extensively studied in shake flasks, but not in bioreactor scale. Apart from that, copolymers with higher 4HB monomer composition were normally achieved by two-stage cultivation whereby the bacterial strains were grown first followed by transferring the cells into nitrogen-free medium to allow accumulation of PHA [7, 8, 15, 17, 21, 24, 26, 27]. In some studies, addition of stimulators, such as propionic acid and acetic acid was found to increase 4HB monomer composition. However, this has resulted in the incorporation of 3HV monomer units as well as leading to the formation of terpolymer P(3HB-co-3HV-co-4HB) [5].

*Cupriavidus* sp. USMAA1020 was known to be a potential producer of copolymer P(3HB-co-4HB) [1]. In two-stage fermentation, the strain was reported to be able to accumulate 75 mol% 4HB monomer while only 14 mol% 4HB was achieved in single-stage cultivation by using  $\gamma$ -butyrolactone as the sole carbon source [1, 28]. It was concluded that in single-stage cultivation, the bacterium has the ability to accumulate high PHA content with lower 4HB molar fraction [1]. However, in our recent report we demonstrated that the shake-flask scale research has not only revealed its potential in improving the copolymer accumulation, but it has also resulted in the accumulation of a wide range of 4HB monomer, ranging from 7 to 70 mol% [12]. This is a breakthrough study on single-stage biosynthesis of copolymer P(3HB-co-4HB), which resulted in the increase in monomer composition by using mixed-substrate cultivation strategy.

In this work, the effect of different substrate mixtures on the copolymer production in bioreactor scale was explored. It was performed in batch fermentation using the mixtures of 4HB precursors namely  $\gamma$ -butyrolactone, 1,4-butanediol, and 1,6-hexanediol which act as substrates. The properties of the copolymers were also examined in order to provide insight into the usage of mixed substrate for the development of fermentation industries.

## Materials and methods

### Bacterial strain

A local isolate of *Cupriavidus* sp. USMAA1020 (DSM 19416) was used in this study [1]. For inoculum preparation, *Cupriavidus* sp. USMAA1020 was grown at 30 °C in nutrient-rich broth (10 g peptone, 2 g yeast extract, and 10 g beef extract in 1 L distilled water).

### Growth and copolymer P(3HB-co-4HB) accumulation through single-stage cultivation process

Inocula (0.1 g/L) were transferred into 3 L of mineral salts medium (MSM) in benchtop bioreactor (Sartorius Biostat B plus, Germany) containing 3.70 g/L  $\text{KH}_2\text{PO}_4$ , 5.80 g/L  $\text{K}_2\text{HPO}_4$ , 1.1 g/L  $(\text{NH}_4)_2\text{SO}_4$ , 0.2 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 1.0 mL/L microelements solution (2.78 g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.98 g  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 2.81 g  $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.67 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.17 g  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ , and 0.29 g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  per liter of 0.1 M HCl). The medium was added with carbon sources ( $\gamma$ -butyrolactone, 1,4-butanediol, and 1,6-hexanediol) to induce growth and PHA accumulation. The fermentation process was carried out at 30 °C by using agitation of 300 rpm, with an aeration of 1 vvm. Biomass was monitored by measuring the optical density value of the culture broth at a wavelength of 540 nm. Triplicates were carried out for each set of experiments.

### Analytical procedures

PHA content and compositions in the lyophilized cells were determined using gas chromatography (GC-2014, Shimadzu, Kyoto, Japan). Based on the standard method [4], approximately 15 mg of freeze-dried cell was subjected to methanolysis (incubation at 100 °C for 2 h and 20 min) in the presence of methanol and sulfuric acid [85:15 % (v/v)]. The organic layer which contained reaction products was dried with  $\text{Na}_2\text{SO}_4$ , and analyzed by GC. In nuclear magnetic resonance analysis, about 5 mg purified PHA was dissolved in deuterated chloroform ( $\text{CDCl}_3$ ) and subjected to the 400 MHz  $^1\text{H}$  and 300 MHz  $^{13}\text{C}$  NMR analysis at 25 °C (Bruker, Switzerland) [1]. Molecular weight data were obtained by gel permeation chromatography (GPC) using a Shimadzu LC-9A system equipped with a refractive index detector (RID-10A) and a PLgel mixed C column (Polymer Laboratories, Ltd, UK) at 40 °C. About 1.0 mg/mL sample was injected and chloroform was used as an eluent at a flow rate of 1.0 mL/min. Polystyrene was used in standard calibration and molecular weight was calculated using Mark-Houwink equation. The Mark-Houwink constant for polystyrene in chloroform was taken as  $K = 0.011$  mL/mg and  $\alpha = 0.73$ , whereas those for P(3HB) in chloroform were taken as  $K = 0.016$  mL/mg and  $\alpha = 0.76$  [19]. The mechanical test of PHA films were carried out in the form of dumbbell shapes (4 mm  $\times$  75 mm) using a Universal Testing Machine (GOTECH A1-3000 with U60 software, Taiwan) [28]. The thermal properties of the polymer films were determined using Pyris 1 differential scanning calorimetry (DSC) (Perkin Elmer, US). Samples (8–10 mg) were heated, quenched, and reheated in the temperature range of  $-50$  to 200 °C. The heating rate was 10 °C/min, whereas the cooling rate was 20 °C/min.

## Results and discussion

### Effect of manipulating substrates mixture on bacterial growth and P(3HB-co-4HB) biosynthesis

This study was carried out in order to evaluate the biosynthesis of copolymer P(3HB-co-4HB) by manipulating the substrate mixture. Table 1 provides data pertaining to the final bacterial growth and PHA accumulation, as well as the fermentation performance for each batch. As can be seen, variation of substrates in mixed-substrate cultivation has significantly influenced the P(3HB-co-4HB) accumulation. Our finding revealed that the mixed-substrate cultivation strategy works well in overcoming the limitation of single-stage P(3HB-co-4HB) biosynthesis. Most of the cultivations have resulted in P(3HB-co-4HB) accumulation of more than 50 wt% of the biomass. With the product yield of more than 1.0, this result indicates that more PHA was accumulated in relative to the biomass formation. The substrate mixtures exhibited a synergistic influence in which channeling of carbon flow for PHA biosynthetic pathway was more favorable compared to the TCA cycle, which is responsible for cell growth. This trend can be obviously seen for the carbon combination of H<sub>1,4</sub> + L<sub>1,6</sub> which resulted in surprisingly high PHA accumulation of 73 wt%. A significantly higher PHA concentration of 8.6 g/L was obtained with the bacterial growth of 3.2 g/L. This has contributed to higher product yield and PHA productivity of 2.7 g/g and 0.0717 g/L/h, respectively. According to Babel and co-workers, implementation of auxiliary substrate concept can be an effective strategy in overcoming the limitation of microbial performance [3]. In their review, the efficiency of the bio-transformation to desired products was greatly enhanced due to the simultaneous utilization of the substrate mixture. The authors also reported that the sequential utilization (diauxic or polyauxic growth) of the substrate mixture can also be overcome by adopting a mixed culture strategy. Therefore, in this study, absence of diauxic growth pattern by the carbon combination of H<sub>1,4</sub> + L<sub>1,6</sub> supports the synergistic effect of the substrate mixture on the copolymer P(3HB-co-4HB) production (refer Supplementary Fig. 1). However, an extensive literature review conducted as a part of this investigation failed to reveal any studies on the P(3HB-co-4HB) accumulation of 73 wt% in single-stage cultivation.

Most of the studies reported that the usage of single-stage cultivation usually resulted in high PHA accumulation with lower bacterial growth or vice versa [15, 23]. Amongst all the substrate mixtures in this study, L<sub>γ</sub> + H<sub>1,4</sub> recorded the highest growth of 6.2 g/L. The bacterial growth recorded for this combination was 1.4-fold higher than other fermentation batches. With the highest specific

**Table 1** Bacterial cell growth and P(3HB-co-4HB) accumulation at the end of fermentation batches

| Type of carbon source | Conc. level of carbon sources <sup>i</sup> | Biomass (g/L)           | PHA content (wt%)    | PHA conc. (g/L)        | Residual biomass (g/L)  | 4HB monomer comp. (mol%) | 4HB monomer conc. (g/L) | Specific growth rate, u max (h <sup>-1</sup> ) | Product yield, Y <sub>PHX</sub> (g/g) | Productivity (g/L/h) |
|-----------------------|--|-------------------------|----------------------|------------------------|-------------------------|--------------------------|-------------------------|--|---------------------------------------|----------------------|
| γ + 1,4               | L <sub>γ</sub> + H <sub>1,4</sub>          | 13.1 ± 0.1 <sup>a</sup> | 55 ± 3 <sup>de</sup> | 7.1 ± 0.3 <sup>b</sup> | 6.2 ± 0.2 <sup>a</sup>  | 20 ± 1 <sup>e</sup>      | 1.4 ± 0.2 <sup>c</sup>  | 0.27   | 1.2                                   | 0.0592               |
|                       | M <sub>γ</sub> + M <sub>1,4</sub>          | 10.8 ± 0.1 <sup>c</sup> | 63 ± 3 <sup>b</sup>  | 6.8 ± 0.3 <sup>b</sup> | 3.9 ± 0.2 <sup>bc</sup> | 20 ± 2 <sup>e</sup>      | 1.4 ± 0.1 <sup>c</sup>  | 0.23   | 1.7                                   | 0.0567               |
|                       | H <sub>γ</sub> + L <sub>1,4</sub>          | 10.1 ± 0.1 <sup>c</sup> | 60 ± 1 <sup>c</sup>  | 6.1 ± 0.1 <sup>c</sup> | 4.0 ± 0.2 <sup>bc</sup> | 12 ± 1 <sup>f</sup>      | 0.7 ± 0.1 <sup>f</sup>  | 0.12   | 1.5                                   | 0.0508               |
| γ + 1,6               | L <sub>γ</sub> + H <sub>1,6</sub>          | 6.5 ± 0.2 <sup>e</sup>  | 45 ± 1 <sup>g</sup>  | 3.0 ± 0.2 <sup>e</sup> | 3.6 ± 0.1 <sup>de</sup> | 55 ± 2 <sup>a</sup>      | 1.7 ± 0.1 <sup>c</sup>  | 0.10   | 0.8                                   | 0.0250               |
|                       | M <sub>γ</sub> + M <sub>1,6</sub>          | 7.4 ± 0.4 <sup>d</sup>  | 53 ± 1 <sup>ef</sup> | 4.0 ± 0.1 <sup>d</sup> | 3.4 ± 0.2 <sup>ef</sup> | 26 ± 2 <sup>d</sup>      | 1.0 ± 0.1 <sup>d</sup>  | 0.15   | 1.2                                   | 0.0333               |
|                       | H <sub>γ</sub> + L <sub>1,6</sub>          | 4.8 ± 0.1 <sup>f</sup>  | 25 ± 1 <sup>h</sup>  | 1.2 ± 0.1 <sup>f</sup> | 3.6 ± 0.1 <sup>de</sup> | 35 ± 1 <sup>c</sup>      | 0.4 ± 0.1 <sup>e</sup>  | 0.15   | 0.3                                   | 0.0100               |
| 1,4 + 1,6             | L <sub>1,4</sub> + H <sub>1,6</sub>        | 8.1 ± 0.1 <sup>d</sup>  | 52 ± 3 <sup>f</sup>  | 4.2 ± 0.2 <sup>d</sup> | 3.9 ± 0.2 <sup>bc</sup> | 37 ± 1 <sup>c</sup>      | 1.6 ± 0.2 <sup>c</sup>  | 0.10   | 1.1                                   | 0.0350               |
|                       | M <sub>1,4</sub> + M <sub>1,6</sub>        | 10.1 ± 0.2 <sup>c</sup> | 57 ± 2 <sup>cd</sup> | 5.8 ± 0.2 <sup>c</sup> | 4.3 ± 0.1 <sup>b</sup>  | 40 ± 1 <sup>b</sup>      | 2.4 ± 0.1 <sup>b</sup>  | 0.14   | 1.3                                   | 0.0483               |
|                       | H <sub>1,4</sub> + L <sub>1,6</sub>        | 11.8 ± 0.1 <sup>b</sup> | 73 ± 1 <sup>a</sup>  | 8.6 ± 0.1 <sup>a</sup> | 3.2 ± 0.1 <sup>f</sup>  | 35 ± 1 <sup>c</sup>      | 3.0 ± 0.1 <sup>a</sup>  | 0.14   | 2.7                                   | 0.0717               |

<sup>a-e</sup> Data show the mean ± standard deviation of duplicates. Means with different superscripts within the same column are significantly different at P ≤ 0.05 level (Tukey test)

<sup>b</sup> γ-butyrolactone denoted by γ; 1,4-butanediol denoted by 1,4; 1,6-hexanediol denoted by 1,6

<sup>i</sup> Two carbon precursors with different concentration levels (low, medium, high) of weight percentage carbon were combined: L = 0.1725 wt% C; M = 0.345 wt% C; H = 0.5175 wt% C. Total weight percentage of carbon for all combinations was fixed at 0.69 wt% C

growth rate of  $0.27 \text{ h}^{-1}$ , this combination provides the most favorable and optimal condition for the bacterial growth. This substrate combination has also resulted in high PHA accumulation and PHA concentration (55 wt% and 7.1 g/L PHA). Therefore, the usage of substrate mixture in this study was found responsible for both the optimum growth of bacteria and PHA accumulation. The bacterial growth and P(3HB-co-4HB) accumulation obtained in this study was the highest reported so far by using mixed culture strategy.

An unusual growth pattern for the substrate mixtures of  $\gamma + 1,6$  was observed (refer Supplementary Fig. 2). This was most probably due to the utilization of mixed substrate which contributed to secondary lag and exponential phase. The sequential utilization of substrate in the media containing mixture of two carbon sources might have contributed to diauxic growth pattern [10]. However, Harder and Dijkhuizen (1982) reported that there might also be other factors contributing to diauxic growth. Therefore, in this study we describe the phenomenon as “diauxic-like” growth [10], which needed further investigation to support the hypothesis.

The mixed-substrate cultivation strategy has resulted in copolymer P(3HB-co-4HB) with various 4HB monomer compositions, from 12 to 55 mol% (Table 1). Generally, the usage of substrate mixtures of  $\gamma$ -butyrolactone and 1,4-butanediol has produced the copolymer with lower range of 4HB monomer (12–20 mol%); and the substrate mixture of 1,4-butanediol and 1,6-hexanediol produced copolymers with higher range of 4HB monomer (35–40 mol%); substrate mixture of  $\gamma$ -butyrolactone and 1,6-hexanediol produced copolymers with a wider range of 4HB monomer composition (25–55 mol%). The 4HB precursors were normally metabolized for growth and 3HB

monomer formation, resulting to only a small portion being converted to 4HB monomers. However, in this study, we observed the copolymer production with higher 4HB monomer composition in single-stage cultivation. Carbon combination of  $L_\gamma + H_{1,6}$  recorded the highest 4HB monomer composition of 55 mol%. The ability of the strain to accumulate 4HB monomer was enhanced by using mixed-substrate cultivation strategy in bioreactor scale fermentation.

### Effect of substrates mixture manipulation on P(3HB-co-4HB) copolymer characteristics

#### Mechanical and thermal properties

Based on the Table 2, the nature of the substrates and the mixture strategy were found to exert more influential effect towards the copolymer's mechanical properties in relation to the 4HB monomer composition. In comparison to copolymers produced from  $\gamma + 1,4$  (all combinations) to the copolymer produced from  $M_\gamma + M_{1,6}$ , there is not much variation in 4HB monomer composition. However, the former has resulted in copolymers with high tensile strength and Young's modulus (from 11.2 to 13.2 MPa and 72.4 to 95.9 MPa, respectively); whereas the latter has resulted in copolymers with lower tensile strength and Young's modulus (2.3 and 48.3 MPa).

The copolymers produced from the substrate mixtures of  $\gamma + 1,6$  and  $1,4 + 1,6$  possessed low Young's modulus. Therefore, it could be suggested that these copolymers are more elastomeric. The 4HB monomer compositions obtained from these substrate mixtures were between 20 to 35 mol%. This finding was in parallel with the fact that this category of copolymers possessed higher flexibility [18, 20]. Martin and Williams (2003) reported that these

**Table 2** Mechanical and thermal properties of the copolymers P(3HB-co-4HB) produced

| Type of carbon source | Conc. level of carbon sources | 4HB monomer comp. (%) | Mechanical properties  |                         |                       | Thermal properties |       |       |
|-----------------------|-------------------------------|-----------------------|------------------------|-------------------------|-----------------------|--------------------|-------|-------|
|                       |                               |                       | Tensile strength (MPa) | Elongation to break (%) | Young's modulus (MPa) | $T_g$              | $T_c$ | $T_m$ |
| $\gamma + 1,4$        | $L_\gamma + H_{1,4}$          | 20                    | $11.22 \pm 1.4^a$      | $494.8 \pm 40^a$        | $75.6 \pm 8^b$        | -13                | 56    | 138   |
|                       | $M_\gamma + M_{1,4}$          | 20                    | $12.2 \pm 1.3^a$       | $353.1 \pm 25^b$        | $72.4 \pm 9^b$        | -16                | 59    | 129   |
|                       | $H_\gamma + L_{1,4}$          | 12                    | $13.2 \pm 0.8^a$       | $331.5 \pm 3^{bc0}$     | $95.9 \pm 7^a$        | -11                | 70    | 143   |
| $\gamma + 1,6$        | $L_\gamma + H_{1,6}$          | 55                    | $2.27 \pm 0.4^{bc}$    | $477.2 \pm 27^a$        | $28.4 \pm 4^d$        | -24                | N.D.  | 128   |
|                       | $M_\gamma + M_{1,6}$          | 26                    | $2.30 \pm 0.1^{bc}$    | $154.9 \pm 48^e$        | $48.3 \pm 2^c$        | -14                | 72    | 142   |
|                       | $H_\gamma + L_{1,6}$          | 35                    | $3.15 \pm 0.1^{bc}$    | $319.5 \pm 12^{bc}$     | $27.9 \pm 2^d$        | -20                | N.D.  | N.D.  |
| $1,4 + 1,6$           | $L_{1,4} + H_{1,6}$           | 37                    | $3.77 \pm 0.3^b$       | $254.0 \pm 12^{cd}$     | $30.7 \pm 5^{cd}$     | -18                | N.D.  | 114   |
|                       | $M_{1,4} + M_{1,6}$           | 40                    | N.D.                   | N.D.                    | N.D.                  | -20                | N.D.  | 84    |
|                       | $H_{1,4} + L_{1,6}$           | 35                    | $1.60 \pm 0.1^c$       | $229.7 \pm 31^{de}$     | $17.0 \pm 1^d$        | -22                | N.D.  | 98    |

Means with different superscripts within the same column are significantly different at  $P \leq 0.05$  level (Tukey test)

<sup>a-e</sup> Data show the mean  $\pm$  standard deviation of seven replicates

**Table 3** Molecular weight and dyad sequence distribution of copolymers P(3HB-co-4HB) films

| Type of carbon source | Conc. level of carbon sources | 4HB monomer composition (mol%) |                                      | Molecular weight               |                                |                    | Dyad sequence distribution |          |          |          |                                 |
|-----------------------|-------------------------------|--------------------------------|--------------------------------------|--------------------------------|--------------------------------|--------------------|----------------------------|----------|----------|----------|---------------------------------|
|                       |                               | GC-based calculation           | <sup>1</sup> H-NMR-based calculation | $M_n$ ( $\times 10^3$ ) Dalton | $M_w$ ( $\times 10^3$ ) Dalton | $M_w/M_n$          | $F_{33}$                   | $F_{34}$ | $F_{43}$ | $F_{44}$ | $D (F_{33}F_{44}/F_{34}F_{43})$ |
| $\gamma + 1,4$        | $L_\gamma + H_{1,4}$          | 20                             | 20                                   | $87 \pm 1.0^b$                 | $305 \pm 3.0^{ef}$             | $3.5 \pm 0.2^{ef}$ | 0.65                       | 0.17     | 0.15     | 0.03     | 0.61                            |
|                       | $M_\gamma + M_{1,4}$          | 20                             | 21                                   | $110 \pm 3.2^a$                | $391 \pm 2.0^b$                | $3.6 \pm 0.1^{ef}$ | 0.68                       | 0.18     | 0.13     | 0.02     | 0.61                            |
|                       | $H_\gamma + L_{1,4}$          | 12                             | 14                                   | $105.8 \pm 9^a$                | $407 \pm 2.0^a$                | $3.8 \pm 0.3^d$    | 0.61                       | 0.19     | 0.18     | 0.03     | 0.62                            |
| $\gamma + 1,6$        | $L_\gamma + H_{1,6}$          | 55                             | 52                                   | $53.9 \pm 2.0^c$               | $309.2 \pm 3.6^d$              | $5.7 \pm 0.1^{ab}$ | 0.27                       | 0.20     | 0.22     | 0.31     | 1.92                            |
|                       | $M_\gamma + M_{1,6}$          | 26                             | 22                                   | $32.5 \pm 1.6^f$               | $152.2 \pm 17^h$               | $4.7 \pm 0.1^c$    | 0.60                       | 0.18     | 0.15     | 0.06     | 1.21                            |
|                       | $H_\gamma + L_{1,6}$          | 35                             | 33                                   | $56.6 \pm 3.6^c$               | $350.6 \pm 13^c$               | $6.2 \pm 0.1^a$    | 0.46                       | 0.19     | 0.21     | 0.14     | 1.55                            |
| 1,4 + 1,6             | $L_{1,4} + H_{1,6}$           | 37                             | 33                                   | $46.6 \pm 4.1^{cd}$            | $291.9 \pm 6.2^{fg}$           | $6.3 \pm 0.6^a$    | 0.48                       | 0.19     | 0.19     | 0.14     | 1.91                            |
|                       | $M_{1,4} + M_{1,6}$           | 40                             | 35                                   | $40.0 \pm 1.6^e$               | $119.5 \pm 3.6^i$              | $3.0 \pm 0.1^g$    | 0.49                       | 0.23     | 0.21     | 0.07     | 0.74                            |
|                       | $H_{1,4} + L_{1,6}$           | 35                             | 33                                   | $53.8 \pm 9.7^c$               | $278 \pm 9.6^g$                | $5.2 \pm 0.8^{bc}$ | 0.43                       | 0.24     | 0.24     | 0.09     | 0.67                            |

Means with different superscripts within the same column are significantly different at  $P \leq 0.05$  level (Tukey test)

<sup>a-i</sup> Data show the mean  $\pm$  standard deviation of triplicates

copolymers have been used as materials in developing absorbable medical devices, such as heart valves, vascular grafts, sutures, and medical textiles products [20].

In terms of elongation at break, copolymers from the substrate mixture of  $\gamma + 1,4$  (which produced lower range of 4HB monomer compositions) possessed higher elongation at break, ranging from 331.5 to 494.8 %. However, for the copolymers produced from other substrate mixtures (which yielded copolymers which higher 4HB monomer compositions), the maximum elongation at break was only 477.2 %. This proved the absence of correlation between 4HB monomer compositions and the mechanical properties. An opposite trend was observed for the substrate mixture of  $\gamma + 1,6$ . An increase of 4HB monomer composition from 26 to 55 mol% has resulted in the increase in elongation at break from 154.9 to 477.2 %. In this case, we suggest that the 4HB monomer compositions exert some influence on the copolymer properties. It can be justified that in this study, the substrates and the mixing strategy are still the major influence on the copolymer’s physical properties.

In the case of copolymer’s thermal properties, there was no absolute trend observed for the melting temperature. Kamiya and co-workers (1989) reported that as the 4HB monomer composition increases the melting temperature decreases [13]. However, the same trend was not observed in this study; instead, the result is in line with the study carried out by Ramachandran and Amirul (2013) which observed neither apparent trend nor clear relationship between 4HB monomer composition and melting point [22]. In this study, we observed that the substrate mixture of 1,4-butanediol and 1,6-hexanediol has resulted in lower  $T_m$ . Gumel and co-workers (2012) reported that the polymers with lower range of melting temperature is highly useful

for industrial processing since lesser energy consumption is required during polymer molding or casting [9].

*Molecular weight distribution and polymer randomness*

The copolymers produced from the substrate mixture of  $\gamma + 1,4$  were found to result in higher number average molecular weight,  $M_n$  (87.0 to 110.0 kDa), as compared to  $\gamma + 1,6$  and 1,4 + 1,6 (32.5 to 53.9 kDa) [Table 3]. This is also true for the weight average molecular weight,  $M_w$ . Substrate mixture of  $\gamma + 1,4$  produced copolymers with higher molecular weight and this can be correlated with the higher content of 3HB monomer composition obtained for this combination. In other words, our findings suggest that different substrate combinations and concentrations exerted more influence on the molecular weight. This assertion is supported by our previous report in shake-flask fermentation [12]. However, in the case of the substrate mixture of  $\gamma + 1,6$  and 1,4 + 1,6, when both the carbon sources in the substrate mixtures were supplied at medium concentration level ( $M_\gamma + M_{1,6}$  and  $M_{1,4} + M_{1,6}$ ), the  $M_w$  and  $M_n$  values were the lowest as compared to the combination of different concentration levels. In this study, the number average molecular weight,  $M_n$ , of the copolymers produced ranged from 40.0 to 110.0 kDa. More specifically, these  $M_n$  values were slightly higher than the P(3HB-co-4HB) produced by Ramachandran and Amirul (2013) in single-stage fermentation, whereby the  $M_n$  of the copolymers ranged from 21.0 to 40 kDa [23].

We also determined copolymer randomness, as shown in Table 3. Generally, the alternating polymer is characterized by  $D < 1$ , compared to  $D \approx 1$  for a typical random copolymer. According to Kamiya and co-workers (1989),

copolymers with  $D$  values in the 0.67–1.5 range are still considered random copolymers, and those with  $D > 1.5$  should be classified as a mixture of random copolymers (blend) [13]. The copolymers with much greater  $D$  values are deemed block copolymers in nature. Randomness of the copolymer produced in this study varied with the substrate mixtures used. For example, substrate mixture of  $\gamma + 1,4$  has resulted in alternating copolymers, as evidenced by the  $D$  values of 0.61 and 0.62. For the case of  $\gamma + 1,6$ , P(3HB-*co*-26 % 4HB) produced from  $M_\gamma + M_{1,6}$  is considered as typical random copolymer ( $D = 1.21$ ); whereas P(3HB-*co*-55 % 4HB) and P(3HB-*co*-35 % 4HB) produced, respectively, from  $L_\gamma + H_{1,6}$  and  $H_\gamma + L_{1,6}$  are blend copolymers. The  $D$  value was found to increase with the increase of 4HB monomer compositions. When the carbon combination of 1,4 + 1,6 was examined, the results revealed that the copolymer randomness was not affected, irrespective of the 4HB monomer compositions. The  $D$  values ranged from 0.67 to 1.91, although the 4HB monomer composition produced was almost identical (33 to 35 mol% 4HB).

## Conclusion

The work has successfully validated the applicability of substrate mixture cultivation strategy in the bioreactor scale for copolymer P(3HB-*co*-4HB) production. By employing the benefits of substrate mixtures, this has not only proven to have a significant enhancement of copolymer accumulation, up to a maximum PHA content of 73 wt%; but has also contributed to the production of copolymers with wider range of 4HB monomer composition, from 12 mol% to as high as 55 mol%. This is an achievement that has never been reported for single-stage fermentation process. Upon conducting extensive characterization of the copolymers produced, the results revealed that the copolymer characteristics and qualities are dominantly affected by the nature of the substrates and the substrates combination strategy, regardless of the 4HB monomer compositions. In conclusion, the work has further confirmed the feasibility of the substrate mixture cultivation strategy as a successful platform for the production of copolymer P(3HB-*co*-4HB) in bioreactor scale.

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