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Pilot scale production of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) biopolymers with high molecular weight and elastomeric properties

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Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [(P(3HB-*co*-4HB)] copolymer receives attention as next generation biomaterial in medical application. However, the exploitation of the copolymer is still constrained since such copolymer has not yet successfully been performed in industrial scale production. In this work, we intended to establish pilot production system of the copolymer retaining the copolymer quality which has recently discovered to have novel characteristic from lab scale fermentation. An increase of agitation speed has significantly improved the copolymer accumulation efficiency by minimizing the utilization of substrates towards cell growth components. This is evidenced by a drastic increase of PHA content from 28 wt% to 63 wt% and PHA concentration from 3.1 g/L to 6.5 g/L but accompanied by the reduction of residual biomass from 8.0 g/L to 3.8 g/L. Besides, fermentations at lower agitation and aeration have resulted in reduced molecular weight and mechanical strength of the copolymer, suggesting the role of sufficient oxygen supply efficiency in improving the properties of the resulting copolymers. The K_La-based scale-up fermentation was performed successfully in maintaining the yield and the quality of the copolymers produced without a drastic fluctuation. This suggests that the scale-up based on the K_La values supported the fermentation system of P(3HB-*co*-4HB) copolymer production in single-stage using mixed-substrate cultivation strategy.

[**Key words:** Biopolymer; Bioreactors; Polyhydroxyalkanoate; Pilot production; P(3HB-*co*-4HB); Scale-up]

Polyhydroxyalkanoates (PHAs) are produced by microorganisms when the surrounding environment is limited of nutrients but with an excess of carbon source (1). This naturally produced polymer serve as a next generation bioplastic to replace conventional synthetic plastics, due to its attribute being biodegradable and biocompatible thermoplastic (2). Various kinds of PHA could be produced upon the incorporation of different monomer units which is heavily depending on the substrate uptake as well as the polymerizing activity by PHA synthase (3,4). The structural diversity of the monomers and compositions has resulted in PHAs with wide range of physical and thermal characteristics. Therefore, this has opened up to various opportunities for exploitation as there are many possibilities yet to be discovered and applied into various sectors from disposable goods packaging, agriculture, aquacultural to medical and biopharmaceutical industries (5). Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] copolymer is one of PHAs which receives considerable attention. The copolymer in its monomer forms of 3HB and 4HB units are naturally occurring metabolites in mammalian bodies. The 3HB monomers are ketone bodies found in human bloodstream whereas 4HB monomers are found in the extracts of brain, heart, lung, liver, kidney, and heart tissues (6). Together with its controllable biodegradability, P(3HB-*co*-4HB) is therefore possess high biocompatibility to human body and is often regarded as excellent biomaterial in tissue engineering application. To date, this copolymer has been fabricated into aminolyzed and nanofiber scaffolds incorporated with fish scale collagen peptides (7,8). Prior to fabrication of the biomaterials into scaffolds, the endotoxin removal procedures were performed. *In vitro* and *in vivo* results revealed the potential use as biomedical materials in wound dressing and tissue engineering since the scaffolds promoted better cell attachment, proliferation and higher percentage of wound closure.

The feasible application of PHAs depends on the economics and performance in industrial scale production. Meanwhile, the suitability of industrial production is evaluated based on various factors such as the safety and stability of the microorganisms, utilizable range and cost of carbon sources, the velocity (rate) of bacterial growth and PHA accumulation, achievable final cell densities and PHA contents, formation of by-products, the extractability of the intracellular PHA, and the molecular mass of the PHA produced (9). In particular to P(3HB-*co*-4HB) copolymer, the feasible single-stage fermentation for P(3HB-*co*-4HB) production has always been hampered by low copolymer yield and low 4HB monomer accumulation. However, this could be overcome by employing the mixed substrate cultivation strategy which contributed to significant copolymer accumulation in single-stage bioprocess (10,11). Based on these reports, substrate mixture of 1,4-butanediol and 1,6-hexanediol was found to be excellent in achieving higher P(3HB-*co*-4HB) productivity in single-stage fermentation. In our latest

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finding, the substrate mixture was also found capable to produce copolymer with high molecular weight and elongation at break of 927 kDa and 1637% respectively. Such characteristics has not yet been reported for the P(3HB-*co*-4HB) copolymer in so far. Therefore, this could encourage and extends the future exploration of its usage as biomaterial in various applications.

Currently, the scalability of the P(3HB-*co*-4HB) fermentation process is one of the major concerns which determines further progression of the copolymer application. The application and commercialization of this copolymer is still constrained by its large scale production. This is due to the difference of the fermentation performance affected by the fermentation volume and bioreactor geometries which influenced the resulting properties. Therefore, copolymer with desired characteristics might not be reproducible in larger scale fermentation. It is emphasized that scale up and optimization are key issues in industrial fermentation which are aimed to maintain the bioprocess reactions at homogenous and optimal conditions, to ensure consistent product yield and quality (12). According to Neubauer et al. (13), the bioprocess development which involves the transfer of the complex biological understanding of microbial system and the targeted bio-product into the engineering domain in pilot and full-scale manufacture (from natural science to engineering focus), has remained as dominant challenge faced in biotechnology industry. Therefore, it is crucial to identify and establish the most relevant scale up parameters in fermentation process to ensure the product yield and quality is kept constant as possible.

In aerobic bioprocess, oxygen is the key substrate used for microbial growth, cell maintenance and product formation (14). In most cases, the availability of dissolved oxygen is the determining factor of the carbon flux between the growth and product synthesis. Despite of that, oxygen has low solubility in aqueous media due to the presence of nutrients and ionic salts, as well as the higher rate of oxygen utilization rate by microorganisms (14,15). Therefore, sufficient supply of oxygen level is particularly crucial to obtain efficient bioprocess with high productivity (16,17). A number of studies have investigated and revealed that the impact of oxygen supply on polyhydroxyalkanoates fermentation performance varies based on the microbial strain, substrates and fermentation strategies being employed (18–27).

This work is intended to establish the pilot production of P(3HB-*co*-4HB) copolymer which retained the similar characteristics at high molecular weight and elongation at break as obtained in lab scale fermentation previously. The scale-up fermentation was performed on the basis of maintaining the K_La values cultivated at vessels with various sizes. The productivity and the characteristics of the copolymers produced at various scales were compared and examined. Prior to scale up fermentation, the effect of agitation and aeration on the copolymer production and characteristics was also examined.

MATERIALS AND METHODS

Microorganism A local isolate of *Cupriavidus* sp. USMAA1020 (DSM 19416) was used in this study (28). The inocula were prepared by growing the bacterial strain at 30°C in nutrient-rich broth (10 g peptone, 2 g yeast extract and 10 g beef extract in 1 L distilled water).

Bioreactor set-up and design of experiment The effect of aeration and agitation on P(3HB-*co*-4HB) production was performed in a Biostat C plus (Sartorius Stedim, Germany) with a vessel volume of 30 L. Meanwhile, the scale-up fermentation was carried out in Biostat D-DCU II (Sartorius Stedim, Germany) with vessel volume of 150 L. Both bioreactors were equipped with a gas flow meter, pH electrode and dissolved oxygen (DO) electrode. The bioreactor vessel was double-jacketed with a stainless steel baffle and impellers (disc-mounted flat-blade turbine).

Biosynthesis of P(3HB-*co*-4HB) copolymers was performed in single-stage fermentation whereby 0.1 g/L pre-cultured cells were transferred into MSM containing 1.1 g/L ammonium sulfate. All the fermentations were performed by using the substrate mixture of $H_{1,4} + L_{1,6}$ (H: High concentration at 0.42 wt% C; L: Low concentration at 0.14 wt% C) at the concentration of 0.56 wt% C. The effect of physical parameters was investigated in 18 L fermentation by fixing the agitation and aeration respectively at 150 rpm, 200 rpm, 250 rpm and 0.5 vvm, 0.75 vvm and 1 vvm with a total of 5 fermentation batches. Subsequently, the scale-up fermentation was carried out from 3 L to 18 L and 70 L fermentation scales. This is based on the K_La value obtained from 3 L fermentation which used 0.56 wt% C $H_{1,4} + L_{1,6}$. It is noted that the 18 L fermentation with the physical conditions of 200 rpm and 1.0 vvm has similar K_La value as obtained from 3 L fermentation; meanwhile, it is 140 rpm and 0.5 vvm for 70 L fermentation.

The sampling of the fermentation was carried out at the interval of 6 h. This enabled the monitoring of the time course bacterial growth and PHA accumulation of each fermentation process. The fermentation broth was harvested by centrifugation of the fermentation broth at 4°C, 10,000 ×*g* for 15 min. Supernatant was discarded and the cells were washed twice with distilled water. The cells were subjected to freeze dry process prior to gas chromatography (GC) analysis.

Determination of volumetric mass transfer coefficient, K_La The rate of oxygen transfer from the bubble air to the fermentation medium can be described by following equation:

$$\frac{dC}{dt} = K_La\left(C^* - C\right) \quad (1)$$

where dC/dt stands for the change of dissolved oxygen concentration over a time period (mmol O_2 dm^{-3} h^{-1}); K_La is the volumetric mass transfer coefficient (cm h^{-1}); C^* is the saturated dissolved oxygen concentration (mmol O_2 dm^{-3}) and C represents the concentration of the dissolved oxygen in fermentation broth at a particular time point (mmol O_2 dm^{-3}).

The K_La measurement was performed based on gassing-out technique which set the polarographic oxygen electrode at zero oxygen level by sparging nitrogen gas into the fermentation broth (29). This is followed by monitoring the time-course dissolved oxygen concentration as the agitation and aeration chosen based on the experimental design was resumed. The K_La value was calculated based on the slope of the straight line obtained from the plot of $\ln(C^* - C)$ versus time.

Analytical methods The biomass concentration was measured turbidimetrically at 540 nm using Genesys 20 UV–vis spectrophotometer (ThermoSpectronic, USA). The quantification of P(3HB-*co*-4HB) content and monomer compositions was performed using gas chromatography (Shimadzu GC-2014, Shimadzu, Japan) based on the method proposed by Braunegg et al. (30). There was approximately 15 mg of lyophilized cells 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 Na_2SO_4, and analyzed by GC using a Fused Silica Capillary Column (Supelco SPB-1 column, 30 m × 0.25 mm × 0.25 μm film thickness).

Characterization of P(3HB-*co*-4HB) copolymers The intracellular P(3HB-*co*-4HB) was extracted by stirring the lyophilized cells in chloroform in the ratio of 1 g–200 mL for 48 h (28). The mixture was filtered to remove cell debris and the chloroform containing the dissolved copolymer was concentrated at 50°C using Eyela rotary vacuum evaporator N–N series (Tokyo Rikakikai Co., Japan). The copolymer was precipitated by adding the concentrated solution into stirring chilled methanol. The copolymer was filtered off and dried at room temperature.

The molecular weight of the extracted P(3HB-*co*-4HB) was determined using gel permeation chromatography (GPC) using Shimadzu LC-9A system equipped qith a refractive index detector (RID-10A) at 40°C. The samples were injected at the concentration of 1.0 mg/mL at a flow rate of 1.0 mL/min in which the chloroform was used as effluent. The weight-average molecular weight, number-average molecular weight, and polydispersity index were calculated using the Mark–Houwink equation:

$$[\eta] = KM^{\alpha} \quad (2)$$

where η represents intrinsic viscosity, K and α are Mark–Houwink constant and M is the molecular weight in g/mol. Polystyrene was used in standard calibration ($K = 0.011$ mL/mg and $\alpha = 0.73$) and the Mark–Houwink constant was corrected suited to PHA with the constant taken as $K = 0.016$ mL/mg and $\alpha = 0.76$ (31).

The mechanical strength of the copolymer films was tested in the form of dumbbell shapes (4 mm width × 75 mm length) using a Universal Testing Machine (Gotech A1-3000 with U60 software, Gotech, Taiwan). Seven replicates were prepared for each film and the test was analyzed (ASTM:D882-91). The thermal properties of the copolymer were determined using Pyris 1 differential scanning calorimetry (DSC) (Perkin Elmer, USA). The films (8–10 mg) were subjected to heating from −50°C to 200 °C at the rate of 10 °C/min. Meanwhile, the copolymer randomness was determined by dissolving the samples in deuterated chloroform and analyzed using 700 MHz ^{13}C qNMR analysis (Bruker, Switzerland).

RESULTS AND DISCUSSION

Effect of agitation and aeration on single-stage fermentation of P(3HB-*co*-4HB) Previously, it was proven that the mixed-substrate cultivation strategy could enhance the P(3HB-*co*-4HB)

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