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# Improved synthesis of P(3HB-*co*-3HV-*co*-3HHx) terpolymers by mutant *Cupriavidus necator* using the PHA synthase gene of *Chromobacterium* sp. USM2 with high affinity towards 3HV

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ABSTRACT

Unlike polyhydroxyalkanoates (PHAs) copolymers, the controlled and efficient synthesis of PHA terpolymers from triglycerides and fatty acids are yet to be established. This study demonstrates the production of P(3HB-*co*-3HV-*co*-3HHx) terpolymer with a wide range of 3HV monomer compositions from mixtures of crude palm kernel oil and 3HV precursors using a mutant *Cupriavidus necator* PHB$^{-}$4 transformant harboring the PHA synthase gene (*phaC*) of a locally isolated *Chromobacterium* sp. USM2. The PHA synthase of *Chromobacterium* has an unusually high affinity towards 3HV monomer. P(3HB-*co*-3HV-*co*-3HHx) terpolymers with 3HV monomer composition ranging from 2 to 91 mol% were produced. Generation of 3HHx monomers was affected by the concentration and feeding time of 3HV precursor. P(3HB-*co*-24 mol% 3HV-*co*-7 mol% 3HHx) exhibited mechanical properties similar to that of common low-density polyethylene. P(3HB-*co*-3HV-*co*-3HHx) terpolymers with a wide range of 3HV molar fraction had been successfully synthesized by adding lower concentrations of 3HV precursors and using a PHA synthase with high affinity towards 3HV monomer.



## 1. Introduction

The characteristics of polyhydroxyalkanoate (PHA) bioplastic depend on the type and composition of monomeric units incorporated into its polymer backbone. The monomer composition of PHA can be controlled by employing a suitable production microorganism which has the ability to utilize a wide range of different carbon substrates and in addition, the microorganism should possess a PHA synthase with a broad range of substrate specificity. In order to achieve the above objectives, numerous genetic and fermentation techniques have been investigated and tested [1–3].

*Cupriavidus necator* is able to use a wide range of carbon substrates for growth and production of PHA. It is a robust strain that can be cultivated to high cell densities. However, the PHA synthase of the wild-type *C. necator* shows high polymerizing affinity towards only short chain length (SCL) 3–5 carbon monomers [4,5]. By using its PHA-negative mutant, *C. necator* PHB$^{-}$4 that is transformed with heterologous PHA synthase gene, PHAs with better properties can be produced. Generally, the copolymers consisting of SCL and medium chain length (MCL) 3-hydroxyalkanoates possess better thermal and physical properties compared to either SCL or MCL polymers [6]. Several wild-type bacteria have been previously investigated for the biosynthesis of P(3HB-*co*-3HV-*co*-3HHx) terpolymers from hexanoate including *Rhodospirillum rubrum* [7], *Rhodocyclus gelatinosus* [8] and *Rhodococcus* sp. [9]. Recombinant strains have also been developed for the production of P(3HB-*co*-3HV-*co*-3HHx). Park and co-workers synthesized P(3HB-*co*-3HV-*co*-3HHx) terpolymer from dodecanoic acid and odd numbered carbon fatty acids using an *Escherichia coli* transformant harboring the PHA synthase gene (*phaC*) of *Aeromonas* sp. [10]. P(3HB-*co*-3HV-*co*-3HHx) with various compositions of 3-hydroxyvalerate (3HV) [0.5–33.6 mol%] and 3-hydroxyhexanoate (3HHx) [4–22 mol%] monomers were produced. Polymer accumulations of 4–33 wt% were obtained from 0.8 to 3.6 g/L of cell biomass.

In a separate study, a *Aeromonas hydrophila* transformant harboring the monomer supplying genes of *C. necator* was used to synthesize P(3HB-*co*-3HV-*co*-3HHx) from mixtures of dodecanoic acid and propionic acid [11]. The P(3HB-*co*-3HV-*co*-3HHx) terpolymers produced contained 1.7–7.1 mol% of 3HV and 5.0–11.6 mol% of 3HHx, respectively. A total polymer accumulation of 20–37 wt% was observed in 0.4–3.4 g/L of cell dry weight (CDW).

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Recently, we reported the production of P(3HB-*co*-3HV-*co*-3HHx) terpolymer using a PHB$^{-}$4 transformant harboring the *phaC* of *Aeromonas caviae* [12]. P(3HB-*co*-3HV-*co*-3HHx) with 2–60 mol% of 3HV and 2–7 mol% of 3HHx were produced from mixtures of palm kernel oil and sodium propionate or sodium valerate. Cell biomass and PHA content were recorded in the range of 0.7–7.1 g/L and 6–80 wt%, respectively.

In order to generate polymers with a wide range of 3HV molar fraction, high amounts of precursor substrates are often required. These precursor substrates are usually odd carbon numbered volatile fatty acids with carbon numbers ranging from 3 to 13. Propionic and valeric acids or their salt forms are commonly supplemented as 3HV precursors. However, at higher concentrations these precursors are known to exert certain levels of inhibitory effect on cell metabolism [10,12]. This subsequently results in lower cell biomass and PHA accumulation. One possible way to overcome this problem is to reduce the precursor concentration and use a PHA synthase with higher affinity towards 3HV monomer. The PHA synthase of bacteria from genus *Chromobacterium* is known to have high affinity towards the synthesis of 3HV monomer [13–15]. The broad substrate specificity of the PHA synthase of *Chromobacterium violaceum* also enabled the polymerization of PHA monomers with carbon number ranging from 4 to 8 [14]. Recently, we isolated a strain of *Chromobacterium* sp. USM2 from a local environment and found that it could incorporate high amounts of 3HV (up to 98 mol%) in the presence of precursor substrates [15]. Heterologous expression of its PHA synthase (*phaC*) in *C. necator* PHB$^{-}$4 enabled the synthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] from crude palm kernel oil (CPKO) and upon the addition of sodium valerate, P(3HB-*co*-3HV-*co*-3HHx) terpolymer was generated [15].

In order to ensure higher yield of PHA with minimal cost, plant oils are currently being considered as cheap and renewable feedstocks. Major commodity plant oils such as soybean oil and palm oil have been identified as possible carbon feedstock for PHA biosynthesis [12,16–18]. In this study, we further evaluated the efficiency of bioconversion of CPKO and 3HV precursors into P(3HB-*co*-3HV-*co*-3HHx) terpolymer using *C. necator* PHB$^{-}$4 transformant harboring the *phaC* of *Chromobacterium* sp. USM2 which is known for its high specificity towards the synthesis of 3HV monomer. The resulting P(3HB-*co*-3HV-*co*-3HHx) terpolymers were also characterized to determine their thermal and mechanical properties.

## 2. Materials and methods

### 2.1. Bacterial strain

*C. necator* PHB$^{-}$4 transformant harboring the PHA synthase gene of *Chromobacterium* sp. USM2 was used throughout this study. Construction of this plasmid (Fig. 1) has been described in detail elsewhere [15].

### 2.2. PHA biosynthesis

PHA biosynthesis was carried out according to the methods described previously [6]. CPKO was used as the carbon source and added during inoculation. Various concentrations of CPKO were tested in order to obtain the best cell biomass and PHA content. Sodium valerate and sodium propionate were selected as 3HV precursors and added at different concentrations and cultivation stages to initiate the production of 3HV monomer. CPKO and 3HV precursors were autoclaved separately at 121 °C for 15 min under 15 psi (103 kPa). Kanamycin at a concentration of 50 μg/mL was added for plasmid maintenance in the strain.

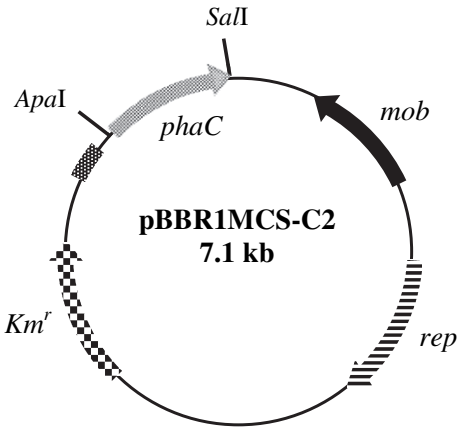


**Fig. 1.** Schematic drawing of recombinant plasmid pBBR1MCS-C2 harboring the *phaC* of *Chromobacterium* sp. USM2 used for P(3HB-*co*-3HV-*co*-3HHx) production in *C. necator* PHB$^{-}$4.

### 2.3. Analytical procedures

#### 2.3.1. Residual oil measurement

In order to measure the residual CPKO concentration in the culture broth, 2 mL of the culture broth was transferred into a screw capped test tube and mixed with 5 mL hexane. The mixture was vortexed vigorously for 1 min and then 1 mL of the hexane layer was transferred onto a pre-weighed plate and air dried in the fume cupboard until the hexane has evaporated.

#### 2.3.2. Gas chromatography (GC)

Methanolysis of the lyophilized cells in the presence of 15% (v/v) sulfuric acid and 85% (v/v) methanol was carried out prior to determination of PHA content through GC analysis [19]. The PHA content was also determined gravimetrically after extracting the polymer by refluxing lyophilized cells with chloroform for 4 h at 60 °C. The polymer solution was then purified by reprecipitation with chilled methanol. The purified polymer was then air dried in a fume cupboard.

#### 2.3.3. Gel permeation chromatography (GPC)

Average molecular weights were estimated by GPC at 40 °C, using a Shimadzu 10A GPC system and a 10A refractive index detector with Shodex K-806 M and K-802 columns. Chloroform was used as the eluent at a flow rate of 0.8 mL/min. Sample concentration of 1.0 mg/mL was used. The calibration curve was generated using polystyrene standards with a low polydispersity.

#### 2.3.4. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) data were recorded in the temperature range from 0 to 200 °C, under a nitrogen flow rate of 20 mL/min using a Shimadzu DSC-50Q instrument equipped with cooling accessory. The solution-cast films (3 mg) were encapsulated in aluminium pans and heated from 0 to 200 °C, at a heating rate of 10 °C/min (first heating scan). Samples that have been melt were then maintained at 200 °C for 1 min, proceeded by rapid quenching to −100 °C. For the second heating scan, samples were heated from −100 to 200 °C at a heating rate of 20 °C/min. Midpoint of the heat capacity change was taken as the glass transition temperature ($T_g$). The melting temperature ($T_m$) and the enthalpy of fusion ($\Delta H_m$) were determined from DSC endotherm.

#### 2.3.5. Tensile strength

Mechanical properties were measured using Shimadzu EZTest at a cross-head speed of 1 mm/min under ambient conditions.