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Partition separation and characterization of the polyhydroxyalkanoates synthase produced from recombinant *Escherichia coli* using an aqueous two-phase system

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Polyhydroxyalkanoates (PHAs) are renewable and biodegradable polyesters which can be synthesized either by numerous of microorganisms *in vivo* or synthase in vitro. The synthesis of PHAs in vitro requires an efficient separation for high yield of purified enzyme. The recombinant *Escherichia coli* harboring phaC gene derived from *Ralstonia eutropha* H16 was cultivated in the chemically defined medium for overexpression of synthase in the present work. The purification and characteristics of PHA synthase from clarified feedstock by using aqueous two-phase systems (ATPS) was investigated. The optimized concentration of ATPS for partitioning PHA synthase contained polyethylene glycol 6000 (30%, w/w) and potassium phosphate (8%, w/w) with 3.25 volume ratio in the absence of NaCl at pH 8.7 and 4°C. The results showed that the partition coefficient of enzyme activity and protein content are 6.07 and 0.22, respectively. The specific activity, selectivity, purification fold and recovery of $phaC_{Re}$ achieved 1.76 U mg^{-1}, 29.05, 16.23 and 95.32%, respectively. Several metal ions demonstrated a significant effect on activity of purified enzyme. The purified enzyme displayed maximum relative activity as operating condition at pH value of 7.5 and 37°C. As compared to conventional purification processes, ATPS can be a promising technique applied for rapid recovery of PHA synthase and preparation of large quantity of PHA synthase on synthesis of P(3HB) in vitro.

[**Key words:** Aqueous two-phase systems; Recombinant *Escherichia coli*; Recovery; Polyhydroxyalkanoates; PHA synthase]

Polyhydroxyalkanoates (PHAs) are known as the most fascinating and largest group of biopolyesters, and are characterized with dissimilar properties and functionalities (1). Many environmental bacteria have been found to accumulate PHAs under natural conditions (2). PHAs have drawn considerable attention from both academic and industrial circles because of their superior characteristics of biodegradability, bioabsorbability, and biocompatibility (3). Poly(3-hydroxybutyrate) [P(3HB)] is a well-known polymer and is the most widely used among the PHAs. P(3HB) can be synthesized using the enzymatic method either *in vivo* or in vitro through the polymerization of (R)-3HBCoA molecules by PHA synthases (phaC) (4–7). The synthases themselves can be divided into four classes based on their size, subunit composition, and substrate specificity (8). Several reports have been conducted on the in vitro synthesis of P(3HB) to understand the mechanisms involved, as a production method and as a technique to enhance the surface properties of other materials for specialized and novel applications (9). However, the difficulty of obtaining a high-purity PHA synthase derived from host strains by using multiple purification steps in downstream processing and a high recovery cost have become obstacles for further application of this enzyme.

Liebergesell et al. conducted two-step chromatography to separate the $phaC_{Re}$ protein from recombinant *Escherichia coli* for the synthesis of PHB in an in vitro study; however, the purification fold and yield reached only 4.4 and 34.6%, respectively (10). Gerngross et al. demonstrated that a 9.8 purification fold with 24% recovery was achieved in the purification of the PHA synthase from *Alcaligenes eutrophus* (11). A direct separation of PHA synthase by using affinity Ni-tag was performed by Qi et al., but a 1% recovery yield was found (4). Song et al. introduced a two-step operation of ammonium sulfate precipitation and the hydrophobic chromatography method to achieve a 43% yield of high-purity synthetic enzymes (12). The highest recovery yield, 44.1%, was achieved under sequential operations of ion exchange and affinity chromatography (13). However, they concluded that a low recovery of the $phaC_{Re}$ enzyme obtained from a time-consuming and complex process resulted in the preparation restriction of high-purity, high-quantity PHA synthases for its application such as in in vitro PHB polymerization (13–15).

Aqueous two-phase systems (ATPSs) consist of two liquid phases that are immiscible beyond a critical concentration (16). The systems are formed by mixing two mutually incompatible polymers or one polymer and an inorganic salt. ATPSs appeal as excellent tools for the partitioning of targeted proteins from feedstock (17–19). Biomolecule partitioning in an ATPS is a complex function of various factors, including the polymer molecular weight, the concentration of polymer and salt, the pH values, the

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temperatures of the system, the net charge, and so on. This technique offers many advantages, including a short processing time, low material cost, low energy consumption, good resolution, a high yield, and a relatively high capacity (20,21). Moreover, the system can be easily scaled up and applied in the downstream process. The purpose of this study was to investigate the feasibility of using an ATPS to separate PHA synthase from clarified recombinant *E. coli* feedstock. The partition behavior of PHA synthase was studied to optimize recovery. Furthermore, the optimal activity of the purified enzyme was estimated in respect to temperature, pH values, and trace metal elements.

MATERIALS AND METHODS

Cultivation of recombinant *E. coli* and feedstock preparation The microorganism and plasmids employed in this study are listed in Table 1. The *E. coli* strains JM109 and BL21(DE3) were used as the cloning host and expression host, respectively. The chemically defined medium (glucose, 10 g L^{-1}; Na_2SO_4, 2 g L^{-1}; $(NH_4)_2SO_4$, 2.7 g L^{-1}; NH_4Cl, 0.5 g L^{-1}; K_2HPO_4, 12 g L^{-1}; NaH_2PO_4, 3.5 g L^{-1}; $(NH_4)_2$-H-citrate, 1.0 g L^{-1}; yeast extract, 1 g L^{-1}; thiamine, 0.01 g L^{-1}; 1 M $MgSO_4$, 2 mL L^{-1}; and the trace element solution, 2 mL L^{-1}; pH 7) containing ampicillin (Ap, 100 μg mL^{-1}) was introduced for recombinant cell cultivation (22).

The *E. coli* BL21(DE3) cell-harboring pET-15b::$phaC_{Re}$ was cultivated to an optical density of 600 nm (OD_{600}) equals 0.6 to 0.8 at 37°C, and 200 rpm. Isopropyl-β-D-(−)-thiogalactopyranoside (IPTG) was subsequently induced to a final concentration at 0.5 mM. The cells were further cultured at 15°C, at 200 rpm for 12 h for enzyme overexpression. The harvested cells were resuspended with phosphate buffered saline (PBS) at pH 7.5, and expressed synthase was released by introducing sonication (XL2000, Microson, USA). The clarified feedstock was collected from the supernatant derived from a further centrifugation step, and was subjected to $phaC_{Re}$ purification in the ATPS.

Analysis of PHA synthase activity The PHA synthase activity was assayed according to the method described by Satoh et al. (23). The PHA synthase activity was measured by the CoA released during the polymerization reaction of 3HB-CoA in a reaction buffer (100 mM sodium phosphate; pH 7.5). The assay mixture (a total volume of 0.4 mL) contained 100 mM sodium phosphate (pH 7.5), 1 mM 3HBCoA, 5% (v/v) glycerol, and $phaC_{Re}$. The reaction was initiated with the addition of $phaC_{Re}$, and then at defined time points the aliquots (45 μL) were mixed with 90 μL of 5% (w/v) trichloroacetic acid (TCA) to terminate the reaction. After removal of the precipitated protein by centrifugation, 120 μL of the supernatant was added to 680 μL of a solution containing 0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in 0.5 M potassium phosphate (pH 7.5), and incubated for 10 min at 30°C. Subsequently, the absorbance was measured at 412 nm. The concentration of CoA was determined by measuring the absorbance at 412 nm and correlating with a molar absorption coefficient of 13,600 M^{-1} cm^{-1}. One unit (U) was defined as the amount of enzymes that catalyzed the generation of 1 μmol of CoA in 1 min.

Determination of protein concentrations and gel analysis of the protein profile The total protein concentrations of the cell extracts were determined according to the method described by Bradford using bovine serum albumin (BSA) as a protein standard. The dye reagent (Bio-Rad, USA) was composed by diluting one part concentrated dye with four parts deionized water. One milliliter of the diluted dye reagent was added to the 1.5 mL test tube, and the 20 μL protein sample was pipetted into a 1.5 mL test tube. Protein solutions are generally mixed and assayed in triplicate to yield a mean value. The percentage deviation was within an average error of less than 5%. Absorbance at 595 nm was measured after 5 min of reaction time. The results were expressed relative to a calibration plot derived from the assay of the standard BSA protein (24). The purity of the enzyme solution was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis with a full-range rainbow molecular weight marker (GE Healthcare, USA). The solution preparation and procedure were performed according to the methods that have been described previously (25).

Aqueous two-phase systems: phase diagram and construction The binodal curves were estimated using the cloud point method, as described by Albertsson (16). The tie-line length (TLL) describes the compositions of the two phases, which are in equilibrium, and was calculated as follows:

$$\text{Tie}-\text{line length} = \sqrt{(\Delta W_T - \Delta W_B)^2_{PEG} + (\Delta W_T - \Delta W_B)^2_{Phosphate}} \quad (1)$$

where $(\Delta W)_{PEG}$ and $(\Delta W)_{Phosphate}$ are the differences between the PEG and phosphate concentration, respectively, in the two phases. The concentration of PEG and salts was analyzed using the refractive index and conductivity measurement, respectively, according the method described by Hatti-Kaul (19).

The ATPSs were constructed by successively adding polyethylene glycol with molecular masses of 6000 or 8000 g mol^{-1} [Sigma; 50% (w/w) stock solution] and dibasic/monobasic potassium phosphate [Sigma; 40% (w/w) stock solution] to yield the appropriate weight percent system at the desired pH. The solution was mixed according to the binodal partition diagrams. The phase systems were prepared in 15 mL graduated centrifuge tubes by weighing the 80% (w/w) stock solution and 20% (w/w) clarified feedstock. Distilled water was then added to each system to obtain a final mass of 10 g. The centrifuge tubes were mixed for 30 min using a laboratory mixer to achieve effective mixing between the phase-forming chemicals and proteins. Each tube was then centrifuged at 3000 ×*g* for 3 min to accelerate phase separation. The volumes of the top and bottom phases were measured, and samples were drawn from each phase and suitably diluted before determining the $phaC_{Re}$ activity assay and total protein concentration.

Determination of the partition coefficient, purification factor, phase volume, and recovery yield The partition coefficient is defined as the enzyme activity or protein concentration in the top phase divided by the correspondent value in the bottom phase, as shown in Eqs. 2 and 3.

$$K_E = \frac{A_T}{A_B} \quad (2)$$

$$K_P = \frac{C_T}{C_B} \quad (3)$$

where A_T and A_B are the enzyme activity (U) in the top and bottom phases, respectively. The C_T and C_B are the total protein concentrations (mg mL^{-1}) of the top and bottom phases, respectively. Selectivity (S) was defined as the ratio of the $phaC_{Re}$ enzyme partition coefficient (K_E) to the protein partition coefficient (K_P) (as shown in Eq. 4).

$$S = \frac{K_E}{K_P} \quad (4)$$

Specific activity (SA) was defined as the ratio between the enzyme activity (U) in the phase sample and the total protein concentration (mg) (Eq. 5).

$$\text{SA}\left(\text{U mg}^{-1}\right) = \frac{\text{Enzyme activity}(U)}{\text{Protein(mg)}} \quad (5)$$

To evaluate the purification process, the enzyme SA (expressed in units per milligram of protein), the purification factor (PF), and the enzyme recovery yield in the top phase (R_T, %) and bottom phase (R_B, %) were also calculated according to the given equations (Eqs. 6–9), which have been well described by Porto et al. (26).

$$\text{PF} = \frac{\text{SA}}{\text{SA}_i} \quad (6)$$

TABLE 1. Bacterial strains and plasmid used in this study.

Strain or plasmid	Description	Source or reference
Strain		
E. coli JM109	*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, Δ(lac-proAB⁺)/F′[traD36, proAB, lacI, lacZΔM15]*	Toyobo
E. coli BL21(DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm* (DE3)	Invitrogen
Plasmid		
pET-15b	N-terminus His-tagged fusion protein expression vector, Ap	Novagen pET System Manual, 10th ed.
pBHR68	pBluescript SK-derivative, containing *R. eutropha* H16 PHB operon	Spiekermann et al. (22)
pET-15b:$phaC_{Re}$	pET-15b derivative; phaC from *R. eutropha* H16	This study