J. Microbiol. Biotechnol. (2001), **11**(2), 333–336

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

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Production of P(3-hydroxybutyrate-3-hydroxyvalerate) and P(3-hydroxybutyrate-4-hydroxybutyrate) Using Transformant *Alcaligenes latus* Enforcing Its Own *phb*C Gene

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Received: September 27, 2000
Accepted: January 22, 2001

Abstract An isolated *phb*C gene from *Alcaligenes latus* was reintroduced into the parent *A. latus* through the transformation process, and the effect of the amplified *phb*C gene on the biosynthesis of P(3-hydroxybutyrate-3-hydroxyvalerate) [P(3HB-3HV)] and P(3-hydroxybutyrate-4-hydroxybutyrate) [P(3HB-4HB)] in the transformant *A. latus* was investigated. The biosynthesis rate and content of the above copolymers increased up to 1.3-fold after enforcing its own *phb*C gene, and the molar fractions of 3HV and 4HB in P(3HB-3HV) and P(3HB-4HB) also changed remarkably from 35.0 to 48.0% and from 34.0 to 56.0%, respectively, showing a critical role of PHB synthase which catalyzes the polymerizing reactions between either 3HV or 4HB from precursor compounds and 3HB.

Key words: P(3HB-3HV), P(3HB-4HB), *Alcaligenes latus*, transformant, *phb*C gene, PHB synthase

Polyhydroxyalkanoate (PHA) is a biopolymer that is accumulated inside cells under unbalanced growth conditions [4, 12]. The most representative PHA is poly-β-hydroxybutyrate (PHB), which exhibits distinct physical properties similar to synthetic plastics, such as polyethylene or polypropylene. The higher crystallinity and the stiffness of PHB prevent its practical application, therefore, various copolymers of PHB have been developed to overcome the above limitations.

The most common copolymers of PHB include poly (3-hydroxybutyrate-3-hydroxyvalerate) [P(3HB-3HV)] polymerizing 3-hydroxyvalerate (3HV) with 3HB, and poly(3-hydroxybutyrate-4-hydroxybutyrate) [P(3HB-4HB)] polymerizing 4-hydroxybutyrate (4HB) with 3HB, randomly. These copolymers exhibit more desirable properties for practical processing because of their high flexibility and lower crystallinity [9, 10]. The above-mentioned physical properties of the copolymers are mainly determined by their molar fractions of 3HV and 4HB in P(3HB-3HV) and P(3HB-4HB), respectively [1].

Alcaligenes latus can accumulate PHA during its growth phase even without imposing the unbalanced growth conditions, such as the depletion of nitrogen or phosphate sources. Furthermore, it can also utilize cheap carbon sources, such as sucrose, as the substrate. Because of these reasons, *A. latus* has become recognized as a promising strain for the commercial production of PHB [3, 8, 12, 16] and its copolymers, P(3HB-3HV) and P(3HB-4HB) [2, 5].

In our previous works, the *phb*CAB operon of *R. eutropha* was isolated and then recombined in order to construct an *E. coli-R. eutropha* shuttle vector [11, 13]. The vectors were reintroduced by electroporation into the parent strain *R. eutropha* to obtain the transformant *R. eutropha* [14]. The transformants harboring cloned *phb*CAB and *phb*C genes showed a significant increase in PHA accumulation, and the monomer compositions of 3HV in P(3HB-3HV) and 4HB in P(3HB-4HB) furthermore increased noticeably in the transformant specifically harboring the cloned *phb*C gene [11]. PHB synthase was found to play a key role in determining the monomer composition in the copolymer in *R. eutropha* [6, 11].

In this work, the isolated *phb*C gene from *A. latus* was recombined into an *E. coli-A. latus* shuttle vector plasmid, and then transformed into the parent *A. latus*. The transformant *A. latus* enforcing its own *phb*C gene was cultivated to ascertain the effects of amplified PHB synthase on the biosynthesis of P(3HB-3HV) and P(3HB-4HB). The variations of the enzyme activities of the related PHA biosynthesis after transformation of *phb*C gene into *A. latus* were also measured to analyze the flux of the intermediate metabolites into the PHA biosynthesis pathway.

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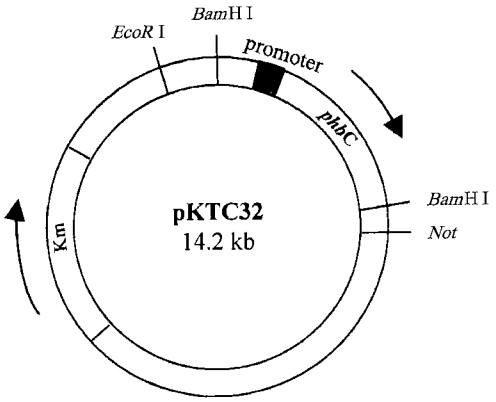

Fig. 1. Structure of the plasmid pKTC32 harboring the *phbC* gene of *A. latus*.

As shown in Fig. 1, the parent strain was *A. latus* DSM 1123 and the plasmid vector was the pKTC32 containing the cloned *phbC* gene isolated from the parent *A. latus* [16]. The plasmid harboring the *phbC* gene was transformed into *A. latus* by electroporation of the following conditions; a competent cell concentration of 10^2/ml, DNA concentration of 1.5 μg, pulse field strength of 10.0 kV/cm, and pulse time of 5 ms, thereby obtaining the transformant *A. latus* T1 harboring the cloned *phbC* gene. The parent and transformant *A. latus* T1 were cultivated in the first stage in nutrient-rich medium for 36 h for cell growth, and then the grown harvested cells were transferred to minimal medium (1.5 g KH_2PO_4/l, 9.0 g $Na_2HPO_4 \cdot 12H_2O$/l, 0.2 g $MgSO_4 \cdot 7H_2O$/l, 0.01 g $CaCl_2 \cdot 2H_2O$/l, 1.5 g $(NH_4)_2SO_4$/l, 0.1 g citric acid /l, and 1.0 ml trace mineral/l) containing 10.0 g/l of sucrose and 10.0 g/l of valeric acid as the precursor compounds to induce the biosynthesis of P(3HB-3HV), and γ-butyrolactone as the precursor for the biosynthesis of P(3HB-4HB) at the second-stage culture for 48 h.

The total cell mass was measured as dry cell weight and the residual or true cell mass was determined by subtracting either the P(3HB-3HV) or P(3HB-4HB) concentration from the total cell mass. The copolymers P(3HB-3HV) and P(3HB-4HB) were purified through extraction by hot chloroform, precipitation with hexane, and then filtration using a filter paper [2]. Finally, their concentrations were measured using a gas chromatography (Young-In Co., Seoul, Korea) equipped with a carbowax 20 M column (Hewlett-Packard Co., Palo Alto, CA, U.S.A.) [11]. The valeric acid and γ-butyrolactone concentrations were also determined by a gas chromatography as described above.

The activities of the three enzymes related to biosynthesis of P(3HB-4HB) were measured after disrupting the suspended cells in 50.0 mM phosphate buffer (pH 7.0) by ultrasonication. The activities of β-ketothiolase and acetoacetyl-CoA reductase were analyzed according to the method of Kim *et al.* [8] by measuring the absorbance of CoA and NADPH in a crude cellular extract at A_{412} and A_{340}, respectively, and the PHB synthase was determined by the modified method of Park *et al.* [15] by measuring the absorbance of CoA at A_{412}.

To investigate the characteristic of the copolymer P(3HB-3HV) biosynthesis of the transformant *A. latus* enforcing its own cloned *phbC* gene, the parent and transformant cells were cultivated for cell growth at the first stage for 36 h, and the above-harvested cells were then recultivated at the second stage in minimal medium containing 10.0 g/l of sucrose and 10.0 g/l of valeric acid as precursor compounds, at the initial cell concentrations of 3.0 g/l. Figure 2 compares the cell growth (A), the P(3HB-3HV) content (B), and the 3HV molar fraction (C) of the parent and transformant *A. latus*. The total cell mass slightly decreased in transformant *A. latus* while the biosynthesis rate and content of P(3HB-3HV) of the transformant *A. latus* increased about 1.3-fold compared with the parent

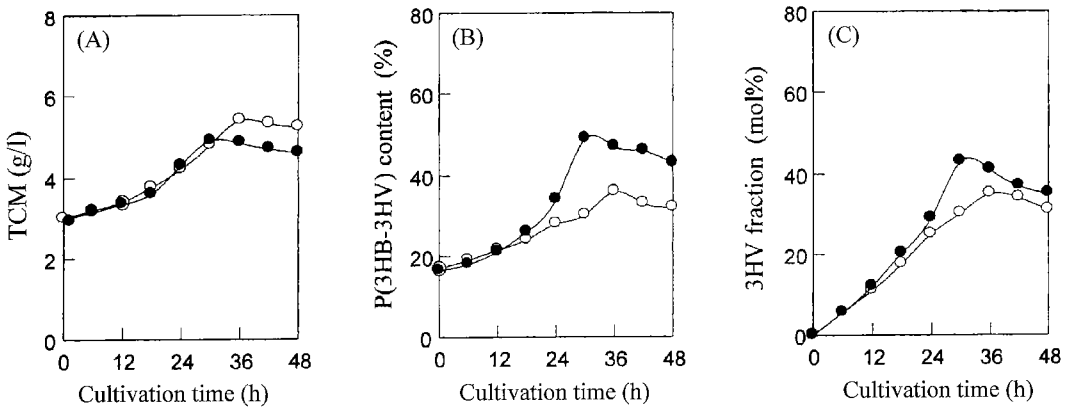

Fig. 2. Comparison of cell growth (A), P(3HB-3HV) content (B), and 3HV molar fraction (C) of parent and transformant *A. latus*.
The cells were cultivated in nutrient-rich medium for 36 h to produce cell growth at the first stage. In the second stage, the grown cells were harvested and then transferred to minimal medium containing 10.0 g/l of sucrose and 10.0 g/l of valeric acid as the precursor compounds for 48 h at 30°C. ○: parent *A. latus*; ●: transformant *A. latus*.

Table 1. Comparison of total cell mass, P(3HB-4HB) concentration, P(3HB-4HB) content, and 4HB fraction between parent and transformant *A. latus* using γ-butyrolactone as a precursor.

	Parent *A. latus*	Transformant *A. latus*
TCM (g/l)	5.2	4.8
P(3HB-4HB) concentration (g/l)	1.9	2.1
P(3HB-4HB) content (%)	36.0	43.7
4HB fraction (mol%)	34.0	56.0

The cells were cultivated in nutrient-rich medium for 36 h to produce cell growth at the first stage. In the second stage, the grown cells were harvested and then transferred to minimal medium containing 10.0 g/l of sucrose and 10.0 g/l of γ-butyrolactone as the precursor compounds for 48 h at 30°C.

strain, increasing from 0.04 to 0.06 g/l-h and 33.0 to 42.0%, respectively. In particular, the molar fraction of 3HV in P(3HB-3HV) was significantly influenced by the enforced PHB synthase, being enhanced from 35.0 mol% in the parent strain to 48.0 mol% in the transformant *A. latus*.

The effect of the amplified PHB synthase on the biosynthesis of another copolymer P(3HB-4HB) was also investigated after cultivating the parent strain and transformant *A. latus* in the second-stage minimal medium using 10.0 g/l of sucrose for 48 h, but using 10.0 g/l of γ-butyrolactone as a precursor for the 4HB fraction instead of valeric acid for 3HV. The effects of the amplified PHB synthase on the total cell mass, concentration, and content of P(3HB-4HB), and 4HB molar fraction in P(3HB-4HB) are summarized in Table 1. Similar to P(3HB-3HV), the biosynthesis rate also increased and the maximum P(3HB-4HB) content changed from 33.0 to 45.0%. In particular, the amplified PHB synthase remarkably influenced the molar fraction of 4HB in P(3HB-4HB), increasing about 1.6-fold from 34.0 to 56.0 mol%.

The variations in the activities of the three enzymes, related to the biosynthesis of P(3HB-4HB), β-ketothiolase (A), acetoacetyl-CoA reductase (B), and PHB synthase (C), of the transformant *A. latus* harboring its own *phb*C gene were monitored during the second-stage cultivation using γ-butyrolactone as the precursor to explain the above phenomena. As shown in Fig. 3, the β-ketothiolase and acetoacetyl-CoA reductase activities remained almost constant compared to the parent strain. On the other hand, the specific activity of PHB synthase in transformant *A. latus* was induced from 3.4 to 4.8 units/mg protein, corresponding to an approximately 1.3-fold increment.

In our previous works [6, 7], we have reported that the biosynthesis rate and the content of PHA in *R. eutropha* were closely associated with β-ketothiolase and acetoacetyl-CoA reductase activity levels and that the molar fractions of 3HV and 4HB in the copolymers of PHB were closely related with PHB synthase. The enhanced molar fractions of 3HV in P(3HB-3HV) and 4HB in P(3HB-4HB) in the transformant *A. latus* could most likely be explained in a similar way.

The physical properties of the above copolymers are mainly determined by the molar fractions of 3HV and 4HB. The molar fractions of 3HV and 4HB can be controlled either by modification of the cultivation methods or by developing new strains. Therefore, the construction of a transformant by reintroducing its own cloned *phb*C gene seems to be an effective strain development method to achieve overproduction of the accessible forms of PHA in *A. latus*.

Acknowledgment

This work was supported by HAN project from the Ministry of Science and Technology of the Republic of Korea and the LG Chemistry Co. Ltd.

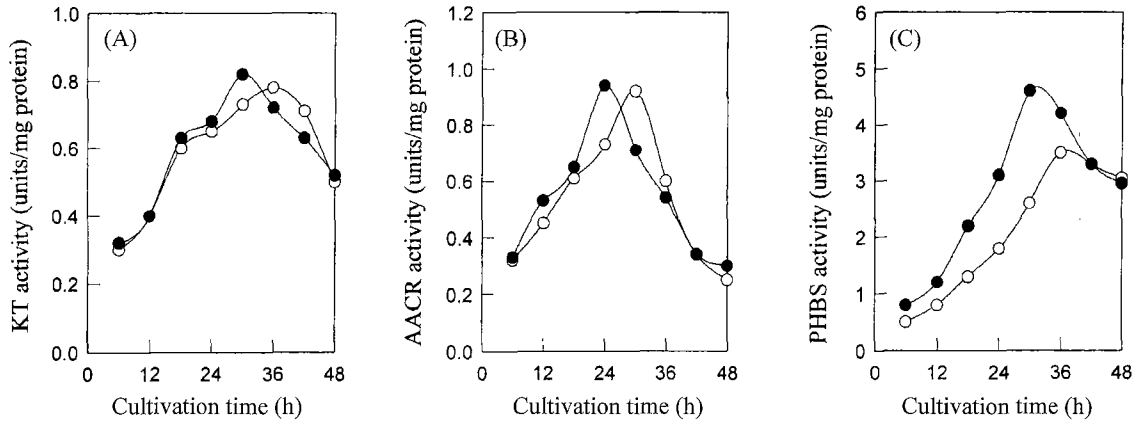

Fig. 3. Variation of specific activities of β-ketothiolase (A), acetoacetyl-CoA reductase (B), and PHB synthase (C) by transformation of the cloned *phb*C gene into *A. latus*.

The cells were cultivated in nutrient-rich medium for 36 h to produce cell growth at the first stage. In the second stage, the grown cells were harvested and then transferred to minimal medium containing 10.0 g/l of sucrose and 10.0 g/l of γ-butyrolactone as the precursor compounds for 48 h at 30°C. The specific enzyme activity was expressed as a μmol of the product or substrate per min per mg of protein. ○: parent *A. latus*; ●: transformant *A. latus*.

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