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Production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) in recombinant *Corynebacterium glutamicum* using propionate as a precursor

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ABSTRACT

Lipopolysaccharides free P[3-hydroxybutyrate (3HB)-*co*-3-hydroxyvalerate (3HV)] production was achieved using recombinant *Corynebacterium glutamicum* harboring polyhydroxyalkanoate (PHA) biosynthetic genes from *Ralstonia eutropha*. Cells grown

on glucose with feeding of propionate as a precursor of 3HV unit accumulated 8 to 47 wt% of P(3HB-*co*-3HV). The 3HV fraction in the copolymer was varied from 0 to 28 mol% depending on the propionate concentrations.

Bacterial polyhydroxyalkanoates (PHAs) are bio-based and biodegradable polyesters that have been proposed as an alternative to petroleum-derived plastics (Doi, 1990; Rehm, 2003). PHAs remain as the spotlights of the sustainable oriented society because their renewability reduces the dependence on diminishing fossil fuels (Sudesh et al., 2000). However, the high production cost has been an obstacle to the widespread use of PHAs. In addition, lipopolysaccharides (LPS), which is often co-purified with PHAs synthesized in the Gram-negative bacteria, causes immunogenic reactions, thus limiting the application of PHAs in the medical and food container industry (Furrer et al., 2007; Lee et al., 1999; Valappil et al., 2007).

To address the problems, we previously created P(3-hydroxybutyrate) [P(3HB)] production system in recombinant *Corynebacterium glutamicum* (Jo et al., 2006; Jo et al., 2007), which is a Gram-positive bacterium, and therefore, lacks LPS. The production of LPS-free PHA has a great advantage against conventional PHA production system using

Gram-negative bacteria (Mifune et al., 2009), such as *Ralstonia eutropha* (Peoples and Sinskey, 1989). In addition, *C. glutamicum* has extensive ability in assimilating crude sugar like the farm by-product, molasses or glucose, thus represents the efficient biomass utilization. In fact, *C. glutamicum* has been used to produce amino acids, organic acids and proteins as a cost-effective bio-converter (Hermann, 2003; Kirchner and Tauch, 2003; Leuchtenberger et al., 2005). These facts are considered as advantages over other Gram-positive PHA producers, such as *Bacillus* species.

In this study, we attempted to improve the properties of PHA produced in *C. glutamicum* by introducing secondary monomeric unit, 3-hydroxyvalerate (3HV). P(3HB-*co*-3HV) copolymers have better physical and mechanical properties as compared to P(3HB) homopolymer (Doi et al., 1987). The 3HV unit in copolymers has been shown to significantly decrease crystallinity of polymers that led to a decrease in melting temperature and improvement of flexibility. The melting temperature of P(3HB-*co*-3HV) varied in the range of 170-65°C depending on 3HV fraction (Inoue, 1998). The lowered melting temperature facilitates molding of the polymer and reduces thermal degradation of polymer during processing. In addition, elongation at break is increased along with 3HV fraction, reaching up to 700% when 28 mol% 3HV was incorporated (Inoue and Yoshie, 1992).

For P(3HB-*co*-3HV) production in *C. glutamicum*, we investigated the way of feeding propionate, which was commonly used as the structurally related secondary precursor for the production of 3HV unit in the P(3HB-*co*-3HV) copolymer (Byrom, 1987; Rhie and Dennis, 1995). Propionate could be converted into 3-hydroxyvaleryl-CoA via intrinsic metabolic pathway and external PhaAB (Fig. 1). Thus, this pathway could also be applicable to P(3HB-*co*-3HV) production in *C. glutamicum*. In addition, propionate is one of the short chain organic acids that formed as unwanted by-product in the processes to convert biomass into fermentable sugars by enzymatic saccharification of the polysaccharides. Therefore, utilization of crude sugar and propionate can be cost effective and environmentally friendly method for biopolymer production. Here, P(3HB-*co*-3HV) production in *C. glutamicum* is reported as the first case for producing PHA copolymer in the strain.

Fig. 1

For the production of PHA, pPS-*phaCAB* bearing *phaCAB* genes from *R. eutropha* constructed previously (Jo et al., 2006), and pPS-*C'AB* bearing the codon optimized *phaC* gene along with wild-type *phaAB* genes, and pVC7-*AB* bearing *phaAB* genes alone constructed previously (Jo et al., 2007) were used. *C. glutamicum* ATCC13869, was kindly provided by Dr. K. Yokoyama of Ajinomoto Co. Inc..

Transformation of *C. glutamicum* was performed using electroporation as described previously (Liebl et al., 1989). A nutrient-rich medium (CM2G) was used for pre-culture, and a minimal medium (MMTG), on which *C. glutamicum* did not produce glutamate, for the main culture was used for polymer production. Kanamycin (50 µg/ml) was added to the seed-culture when needed. We previously found that antibiotics in main culture was not essential to maintain the plasmid (Jo et al., 2006). Sodium propionate with different concentrations ranging from 0 to 2% (wt./vol.) was fed as the precursor carbon substrate for the biosynthesis of 3HV monomer. The content and composition of PHA were determined using gas chromatography as described (Kato et al., 1996). For molecular weight analysis of PHAs, polymers were extracted with chloroform and precipitated by methanol prior to the gel permeation chromatography analysis as described (Kusaka et al., 1997).

Table 1

Table 1 shows that P(3HB-*co*-3HV)s containing 0.2 to 11 mol% of 3HV were produced in the recombinant *C. glutamicum* harboring pPS-*phaCAB*. The PHA content was increased with the addition of propionate even though the 3HV fraction in the polymer was considerably low. The result could be interpreted as the carbon flux from propionate to pyruvate, which is a precursor of 3HB unit, via 2-methylcitrate pathway

(Fig. 1). The cell growth and total PHA production were reduced when 1% of sodium propionate was added (0.9 g L^{-1}). However, the tolerance of *C. glutamicum* to propionate was higher than that of *R. eutropha*, growth of which was inhibited by 0.1% of propionate (Byrom, 1987), suggesting that the thick cell wall of Gram-positive bacteria may contributed to the tolerance to short-chain fatty acids such as propionate. In addition, the toxic effect of propionate was suggested to be due to the generation of 2-methylcitrate, which was an intermediate in 2-methylcitrate cycle (Fig. 1) (Plassmeier et al., 2007). Thus, efficient conversion of propionyl-CoA into PHA may reduce the toxicity by decreasing the accumulation of 2-methylcitrate.

In order to minimize the inhibitory effect of propionate, we added propionate when cells reached to the stationary phase at 42 h. With this feeding strategy, 3HV fraction in P(3HB-*co*-3HV) was increased up to 28 mol% (Table 1). Therefore, the feeding strategy was useful for the production of P(3HB-*co*-3HV) with varied 3HV fractions in *C. glutamicum*. Molecular weights of P(3HB-*co*-3HV)s were not significantly different compared to that of P(3HB) (Table 2), suggesting that addition of propionate had little effect on the molecular weight of PHAs produced in *C. glutamicum*.

Table 2

Fig. 2

We attempted to further increase the productivity of PHA and/or 3HV fraction in

the copolymer by enhancing expressions of PHA biosynthetic genes using the dual plasmid method (Jo et al., 2007). The introduction of pPS-*C'AB* and pVC7-*AB* increased PHA content (more than twofold than single plasmid) and total PHA productivity up to 6.0 g L⁻¹ (Table 1). However, 3HV fractions in the copolymers were lowered, and amount of accumulated 3HV unit was not altered by enhancing expressions of PHA biosynthetic genes (Fig. 2), suggesting that the upstream pathway, uptake of propionate and/or conversion into propionyl-CoA, should be the rate-determining step for the incorporation of 3HV unit into polymer.

In conclusion, P(3HB-*co*-3HV) copolymer was successfully produced in the recombinant *C. glutamicum* by feeding propionate as a precursor. The maximum 3HV fraction (28 mol%) was effective to improve the physical and thermal properties of polymer as revealed previously (Inoue and Yoshie, 1992). Currently, the addition of related precursor is the only way to produce PHA copolymers in *C. glutamicum* because the strain did not produce PHA copolymers from fatty acids (unpublished result). The substrate to P(3HB-*co*-3HV) transformation efficiency was 19% for glucose and 9.6% for propionate in the case of the highest PHA production (6.0 g L⁻¹). However, the efficiency in the flask-scale fermentation could be improved by finely regulated fermentation using a bioreactor. In addition, we have reported that PHA and glutamate could be co-produced

in *C. glutamicum* by two steps cultivation technique (Jo et al., 2009). It was also known that production of P(3HB) in *C. glutamicum* increased the glutamate production (Liu et al., 2007). Thus, the combination of the copolymer production with glutamate production could further increase the cost effectiveness of the process.

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Table 1

P(3HB-*co*-3HV) productions in recombinant *C. glutamicum* harboring PHA biosynthetic genes from *R. eutropha* with supplement of propionate.

Plasmids	Sodium propionate Conc. (%)	Timing of propionate addition (h)	Cell dry weight (g L ⁻¹)	PHA content (wt%)	Total PHA (g L ⁻¹)	PHA composition (mol%)	
						3HB	3HV
pPS- <i>phaCAB</i> *	0	-	12.7±1.5	10.1±1.8	1.3	100	0
pPS- <i>phaCAB</i>	0.05	0	13.3±0.4	12.2±1.5	1.6	100	0
pPS- <i>phaCAB</i>	0.1	0	14.8±0.8	19.4±2.6	2.9	99.8	0.2
pPS- <i>phaCAB</i>	0.2	0	14.9±0.2	19.5±0.5	2.9	98	2
pPS- <i>phaCAB</i>	0.6	0	15.4±0.4	18.2±1.6	2.8	93	7
pPS- <i>phaCAB</i>	1.0	0	11.0±4.0	8.4±7.9	0.9	89	11
pPS- <i>phaCAB</i>	0.05	42	13.0±0.5	15.9±1.5	2.1	97	3
pPS- <i>phaCAB</i>	0.1	42	13.4±0.3	16.7±0.9	2.2	95	5
pPS- <i>phaCAB</i> *	0.2	42	15.7±0.5	15.3±0.7	2.4	93	7
pPS- <i>phaCAB</i>	0.6	42	14.7±0.2	25.1±0.8	3.7	78	22
pPS- <i>phaCAB</i>	1.0	42	12.0±0.1	31.0±0.7	3.7	72	28
pPS- <i>phaCAB</i> *	2.0	42	14.5±1.2	23.3±1.1	3.4	82	18
pPS- <i>C'AB</i> , pVC7- <i>AB</i>	0	-	11.6±0.7	45.0±2.6	5.2	100	0
pPS- <i>C'AB</i> , pVC7- <i>AB</i>	0.6	42	13.3±1.0	45.3±2.0	6.0	91	9
pPS- <i>C'AB</i> , pVC7- <i>AB</i>	1.0	42	12.5±0.6	47.2±2.1	5.9	88	12
pPS- <i>C'AB</i> , pVC7- <i>AB</i>	2.0	42	9.9±2.0	44.6±3.3	4.4	92	8

Cells were grown on MMTG medium containing various concentrations of sodium propionate at 30°C for 72 h. 3HB: 3-hydroxybutyrate; 3HV: 3-hydroxyvalerate. Data are average of three trials. * Culture conditions used for molecular weight analysis.

Table 2

Molecular weight of P(3HB) and P(3HB-*co*-3HV)s produced in recombinant *C. glutamicum*.

3HV fraction (mol%)	Molecular weight		
	M_n ($\times 10^5$)	M_w ($\times 10^5$)	M_w / M_n
0	3.4	4.7	1.3
9	4.0	5.1	1.2
15	4.0	5.2	1.3

Cells were cultivated with the conditions as indicated by asterisk in Table 1.

Figure captions:

Fig. 1. PHA biosynthetic pathway and 2-methylcitrate cycle in *C. glutamicum*. PhaA: β -ketothiolase; PhaB: NADPH-dependent acetoacetyl-CoA reductase; PhaC: PHA synthase; 3HB-CoA: 3-hydroxybutyryl-CoA; propionyl-P: propionyl phosphate.

Fig. 2. Amounts of 3HV unit in the polymers accumulated in the recombinant *C. glutamicum*. Gray: cells harboring pPS-CAB (control strain), white: cells harboring pPSCAB and pVC7AB (overexpressing strain). Data are the averages \pm standard deviations of triplicate cultures.

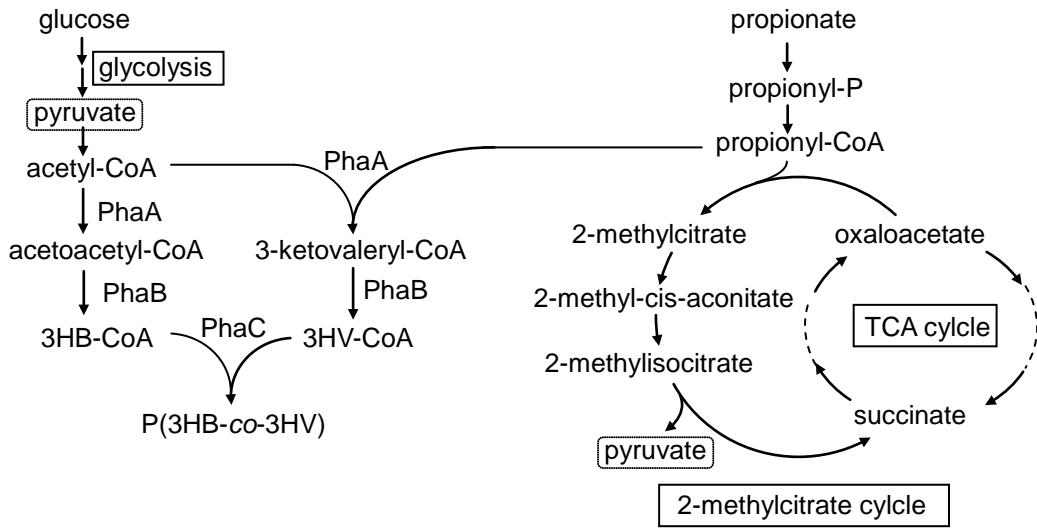


Figure 1

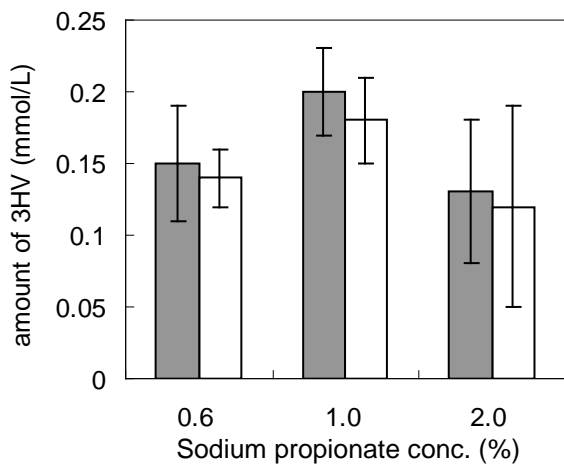


Figure 2