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Citation	Polymer Degradation and Stability, 95(8), 1340-1344 https://doi.org/10.1016/j.polymdegradstab.2010.01.029
Issue Date	2010-08
Doc URL	https://hdl.handle.net/2115/47513
Type	journal article
File Information	PDS95-8_1340-1344.pdf



Establishment of a metabolic pathway to introduce the 3-hydroxyhexanoate unit into LA-based polyesters via a reverse reaction of β -oxidation in *Escherichia coli* LS5218

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ABSTRACT

New lactate (LA)-based terpolymers, P[LA-*co*-3-hydroxybutyrate (3HB)-*co*-3-hydroxyhexanoate (3HHx)]s, were produced in recombinant *Escherichia coli* LS5218 harboring three genes encoding LA-polymerizing enzyme (LPE),

propionyl-coenzyme A (CoA) transferase (PCT) and (*R*)-specific enoyl-CoA hydratase (PhaJ4). When the recombinant LS5218 was grown on glucose with the feeding of butyrate, 3HB-CoA and 3HHx-CoA were supplied, probably via reverse reactions of the β -oxidation pathway and PhaJ4. LPE copolymerized the two monomers with LA-CoA, which was generated by PCT, to yield the terpolymers. Gas chromatography analysis revealed that the terpolymers consisted of 2.7 to 34 mol% LA, 38 to 81 mol% 3HB and 17 to 33 mol% 3HHx units, which can be varied depending on the butyrate concentration fed in the medium. In addition, ^1H - ^{13}C COSY NMR analysis provided evidence for a linkage between LA and 3HHx units in the polymer.

Key words

Poly(LA-*co*-3HB-*co*-3HHx), LA-based polyester, PhaC1_{Ps}(ST/QK), Microbial cell factory, Metabolic engineering, β -oxidation pathway

1. Introduction

The discovery of a “lactate (LA)-polymerizing enzyme (LPE)”, which is an engineered polyhydroxyalkanoate (PHA) synthase to recognize the coenzyme A (CoA) ester of LA (LA-CoA) as a substrate, enabled construction of a one-step fermentation process for LA-based polyesters in recombinant bacteria with artificial metabolic pathways [1,2]. The process originates from bacterial PHA biosynthesis, in which hydroxyacyl-CoAs, most typically 3-hydroxybutyryl-CoA (3HB-CoA), are polymerized into polyesters by PHA synthase [3]. The microbial process enabled considerable change in the production of LA-based polymers compared with the conventional processes, which include fermentative production of LA and successive chemical polymerization of cyclic lactides using heavy metal catalysts [4].

The first LPE was the Ser325Thr/Gln481Lys mutant of PHA synthase 1 from *Pseudomonas* sp. 61-3 [PhaC1_{Ps}(ST/QK)] [5,6], which synthesizes P(LA-co-3HB) copolymers from two corresponding monomers, LA-CoA and 3HB-CoA, in recombinant *Escherichia coli*. Propionyl-CoA transferase (PCT) was used for converting LA to LA-CoA, while 3HB-CoA was supplied by the well-known 3HB-CoA supplying enzymes, β -ketothiolase (PhaA) and NADPH-dependent acetoacetyl-CoA reductase (PhaB). In our first report on P(LA-co-3HB) production, the LA fraction in

the copolymer was 6 mol% [1]. Thus, in the following studies, we undertook the enrichment of LA in the copolymer for the purpose of complete regulation of monomer composition, i.e. from P(3HB) to poly(lactic acid) (PLA). For this purpose, reinforcement of the LA supply in the cell has been attempted by means of anaerobic culture conditions [7] and the use of the *E. coli* LA-overproducing mutant JW0885 [8]. These approaches successfully achieved the production of LA-enriched copolymers with up to 47 mol% LA [7].

From the viewpoint of material properties, considerable interest has been focused on novel LA-based copolyesters formed in combination with other PHA constituents. Although PLAs have certain advantageous properties, such as good processability and transparency [9], they are insufficient to meet the needs of all applications. LA-based copolyesters are expected to improve the lack of elasticity of PLA by copolymerizing medium-chain-length (MCL, C₆-C₁₄) 3-hydroxyalkanoate (3HA) units with varied fractions. In this context, LA-based copolyesters should have significant potential to expand the range of applications of the polymers, and copolymer variety is very important for exploring such desirable properties.

The purpose of this study is to synthesize LA-based polyesters comprised of MCL monomers. The MCL monomers are known to contribute to the elasticity and

flexibility of polyesters when they are copolymerized as a minor fraction with the 3HB unit [10]. As the initial step, we established a metabolic pathway for co-supply of both LA and MCL monomers in recombinant *E. coli*, which was achieved with the help of the interesting finding that butyrate can serve as a precursor of the 3-hydroxyhexanoate (3HHx) monomer. Here, the biosynthesis and structural analysis of a novel LA-based terpolymer, P(LA-*co*-3HB-*co*-3HHx), are reported.

2. Experimental

2.1. Plasmids

pTV118NpctC1STQKAB [1], which bears the *phaC*_{Ps}(STQK) gene from *Pseudomonas* sp. 61-3 [11], the *phaA* and *phaB* genes from *Ralstonia eutropha* (also known as *Cupriavidus necator*) [12] and the *pct* gene from *Megasphaera elsdenii* [13], was modified for production of LA-based polyester comprising of MCL monomers as follows. First, a *Pst*I-*Afl*III fragment of pGEM''C1ABJ4 [11] including *phaA*, *phaB*, and (*R*)-specific enoyl-CoA hydratase (*phaJ4*) [14] genes was inserted into pTV118NpctC1STQKAB, which was digested with the same restriction enzymes, to yield pTVpctC1(ST/QK)ABJ4 bearing the *pct*, *phaC*_{Ps}STQK, *phaAB*, and *phaJ4* genes. Subsequently, pTVpctC1(ST/QK)ABJ4 was digested with *Pst*I and *Nru*I, and

self-ligated after blunting treatment to eliminate the *phaAB* genes. The resultant vector was referred as pTVpctC1(ST/QK)J4.

2.2. Bacterial strain and culture condition

E. coli LS5218 [*FadR*, *atoC*(Con)] [15] was used as a host for productions of LA-based polyesters. *E. coli* JM109 [16] was used as a negative control strain. *E. coli* LS5218 harboring pTVpctC1(ST/QK)ABJ4 or pTVpctC1(ST/QK)J4 were transferred into 100 ml Luria-Bertani (LB) medium containing 2% glucose, 10 mM calcium pantothenate, 100 µg/l ampicilin and variable amounts of either sodium butyrate, sodium hexanoate (0.1, 0.25, 0.5, 0.75, 1, 1.25, and 1.5 mg/mL), or 3 mg/mL sodium dodecanoate in a 500 ml shake flask. Cells were cultivated under aerobic conditions at 30°C for 48 h on a reciprocal shaker at 120 rpm.

2.3. Polymer extraction and analyses

The intracellular polymer was extracted with chloroform and purified by precipitation with methanol as described previously [8]. Polymer content was calculated based on the weight of extracted polymer and the dry cell weight. These polymers were subjected to further analyses.

The monomer composition of the extracted polymers were determined by gas chromatography (GC) on a Shimadzu GC-2010 system equipped with Neutra Bond-1 capillary column (30 m by 0.25 mm) and a flame ionization detector as described previously [8,17]. P(3HB-*co*-3-hydroxyvalerate-*co*-3HHx) and PLA (Toyota, Japan) were used as standards. Molecular weights of extracted polymers were determined by gel permeation chromatography (GPC) (Shimadzu, Japan) equipped with TSKgel Super HZM-H (Tosoh, Japan) using polystyrene standards (Waters, USA) to calibrate [1]. The ¹H-NMR spectrum of the polymer was obtained using a Bruker MSL400 spectrometer (400 MHz) and the chemical shifts are reported in ppm with tetramethylsilane as an internal reference. The ¹H-¹³C COSY NMR spectrum was recorded using a JEOL JNM-A400II instrument (400MHz).

3. Results

3.1. Production of P(LA-*co*-3HB-*co*-3HHx)s in *E. coli* LS5218 with the feeding of butyrate

The MCL monomers of PHA are known to be supplied from fatty acids and/or lipids via the β -oxidation pathway and (*R*)-specific enoyl-CoA hydratase (PhaJ4) [18] [19]. The mutant strain *E. coli* LS5218, which constitutively expresses the genes

involved in fatty acid uptake and the β -oxidation pathway, is commonly used for the production of MCL PHA from fatty acids. Therefore, we first examined the production of P(LA-co-3HA) by cultivating *E. coli* LS5218 harboring pTVpctC1(ST/QK)ABJ4 on glucose, with a feeding of dodecanoate. However, the cells produced only 18 wt% of P(14 mol% LA-co-3HB) and no MCL monomers were detected, suggesting that cells did not utilize dodecanoate in the presence of more preferred carbon source, glucose. However, we previously succeeded in synthesizing LA-based polyesters consisting of the 3HV unit in *E. coli* by feeding propionate together with glucose [8]. This result suggested that *E. coli* could take in short-chain fatty acids along with glucose. Therefore, we attempted to feed short-chain fatty acids, hexanoate and butyrate, which serve as precursors of the monomers, 3HHx-CoA and 3HB-CoA, through β -oxidation pathway.

The *E. coli* LS5218 harboring pTVpctC1(ST/QK)J4 were grown on glucose with a feeding of hexanoate or butyrate. When hexanoate was added, cell growth was significantly inhibited and no polymer accumulated (data not shown), suggesting that hexanoate had negative effects for both cell growth and polymer synthesis. In contrast, when butyrate was fed to the medium, cells grew normally and accumulated polymers. Unexpectedly, GC analysis revealed that the polymers consisted of the 3HHx unit along with the LA and 3HB units. The changes in monomer composition of the polymers upon

the addition of various concentrations of sodium butyrate are summarized in Table 1. The terpolymers P(LA-co-3HB-co-3HHx)s were produced when 0.25 to 1.5 mg/mL sodium butyrate was added. The highest LA fraction (34 mol%) was obtained at the sodium butyrate concentration of 1.75 mg/mL. No LA was incorporated when the sodium butyrate concentration was lower than 0.1 mg/mL (data not shown). The 3HHx fraction was increased with increasing sodium butyrate concentrations, reaching the highest value of 38 mol% upon the addition of 1.25 mg/mL of sodium butyrate. This result suggested that butyrate acts as a precursor of the 3HHx unit (see discussion for detail). The *E. coli* JM109 harboring same plasmid grown on same culture conditions did not produce any detectable polymer (data not shown), suggesting that activated β -oxidation pathway was essential to the production of the terpolymers.

The molecular weights of the generated polymers are presented in Table 1. Without the feeding of sodium butyrate, the weight-average molecular weight of P(3HB) was 3.4×10^4 . When sodium butyrate was added, the molecular weight of the P(LA-co-3HB-co-3HHx)s was decreased from 2.6×10^4 to 1.3×10^4 with increasing sodium butyrate concentrations.

3.2. NMR analyses of P(LA-co-3HB-co-3HHx)

The sample of terpolymer P(16 mol% LA-*co*-52 mol% 3HB-*co*-31 mol% 3HHx) was subjected to NMR analyses to confirm monomer incorporation as well as for analyzing the polymer sequence. ¹H-NMR, ¹³C-NMR and 2D-NMR demonstrated that the polymer had signals assigned to LA, 3HB, and 3HHx (Fig. 1, 2 and 3) [7,10], and their intensities agreed with the GC result. In the carbonyl region of ¹³C-NMR spectrum, six resonances were observed (Fig. 2). On the basis of comparison with P(3HB-*co*-3HV) [20], the peak at 169.13 ppm was assigned to the 3HB*-3HB sequence, and the peak at 169.29 ppm was assigned to 3HHx*-3HB and 3HB*-3HHx sequences. The other peaks (169.46—169.92 ppm), which were also observed for P(LA-*co*-3HB) [7], presumably corresponded to the carbonyl group of LA and possibly 3HB with adjacent LA unit. Additionally, the resonance for 3HHx*-3HHx sequence could appear in the region. Thus, these resonances (169.46—169.92 ppm) were not clearly assignable.

Figure 3 shows enlarged signals for the β-carbon of the 3HB and 3HHx units [3HB(3) and 3HHx(3)], and the α-carbon of the LA unit [LA(2)]. The cross signals in the region serve as a fingerprint for LA-based polyesters, since a pattern of alteration in the chemical shifts by the effect of the adjacent LA unit was clearly observed. As expected, the signal for 3HB(3) was divided into two peaks; (a) and (b). The

cross-signal (b) was assigned to the LA-3HB* sequence based on the comparisons with P(47 mol% LA-co-3HB) [7], which exhibited both of the peaks (a) and (b) with almost the same intensities, while P(3HB) exhibited only peak (a) [21]. The low-field shift of the cross signal is a characteristic effect of the neighboring LA unit. Because the effect of the adjacent 3HHx was small [10], both 3HB-3HB* and 3HHx-3HB* were assigned to peak (a). Similarly, the low-field shift of the signal for 3HHx(3) was observed in [peak (c) and (d)]. By analogy to the signal for 3HB(3), the peak (d) was presumed to be assigned to the LA-3HHx* sequence, and peak (c) should correspond to the 3HB-3HHx* and 3HHx-3HHx* sequences. On the other hand, the signal for LA(2) was divided into four peaks, because the α -carbon was affected by both adjacent units, which provides direct evidence for the random copolymerization of the LA with other 3-hydroxyalkanoates. The phenomenon is characteristic to the LA unit, with a short main-chain compared to the 3HB and 3HHx units, in that the effect of one of the adjacent monomers bound to the carboxyl group was negligible for the 3-hydroxyl monomers. From these results, it was concluded that the polymer was terpolymer, P(LA-co-3HB-co-3HHx).

4. Discussion

The result of this study demonstrated that a feeding of butyrate led to the incorporation of 3HHx unit in the polymers produced in *E. coli* LS5218. The phenomenon could be interpreted as a reverse reaction of the enzymes involved in the β -oxidation pathway [22], which has been proposed in the case of *Pseudomonas putida* [23]. The pathway is illustrated in Fig. 4. If the reactions by FadA and FadB were to proceed in reverse, butyryl-CoA would be converted into hexenoyl-CoA, which would then subsequently be hydrated into (*R*)-3HHx-CoA by PhaJ4. This hypothesis was further supported by the similar phenomenon in LS5218 reported by Sato et al. [24], i.e. that the cells expressing PHA synthase from *R. eutropha* and PhaJ4 produced P(3HB) from glucose. In that case, acetyl-CoA would be converted into crotonyl-CoA by the reverse reaction of FadAB and successively hydrated into (*R*)-3HB-CoA by PhaJ4. The metabolic route is basically the same reaction as the proposed pathway, but with different chain length. Although further analysis is necessary to directly demonstrate the pathway, the butyrate-feeding technique was useful for supplying the 3HHx monomer for LA-based polyesters, because the pathway was functional together with the LA-CoA supplying pathway from glucose via glycolysis and PCT.

In order to create new LA-based polyesters in combination with other PHA monomeric constituents, the ability of LPE to connect LA with other monomers is a

critical factor. In previous reports, we demonstrated that there was no bias in the sequence of P(LA-*co*-3HB) [7], namely LPE can incorporate the LA unit next to the 3HB unit and vice versa. In addition, the LA-3HV sequence was also generated by the LPE [8]. Furthermore, in this study, the presence of LA-3HHx sequence in the terpolymer was revealed by 2D-NMR analysis (Fig. 3). These results suggest that LPE has no evident limitations in incorporating various PHA monomers next to the LA unit. Basically, LPE may have inherited the broad substrate specificity towards monomer substrates present in the original enzyme, PhaC1_{Ps}. This property of LPE is preferable to synthesizing various LA-based random copolymers comprised of the abundant building blocks of the PHA family. The further development of novel LA-based polyesters incorporating natural and/or unnatural monomers will be facilitated by a coordinated combination of pathway engineering, as demonstrated in this paper, and the further directed evolution of LPE which will be published elsewhere [25].

In conclusion, the metabolic pathway for the production of the novel terpolymer, P(LA-*co*-3HB-*co*-3HHx), was established in recombinant *E. coli* LS5218. To the best of our knowledge, this is the first report of the biological production of LA-based polyester comprised of the MCL monomer. The monomer composition was variable to a certain extent by regulating the concentrations of butyrate fed into the

cultivated cells. NMR analysis of the terpolymer suggested that LPE should be capable of connecting various types of monomers. To increase the polymer content and molecular weight, reinforcement of the capacity to supply monomers to LPE could be effected by activation of the LA-CoA synthetic and β -oxidation pathways, including PhaJ4, based on either the gene dosage effect or enzyme engineering, as shown by our previous studies [26].

Acknowledgements

We thank Dr. Toshihiko Ooi in our group for useful advice. This work was supported by funding from the following sources: Grant-in-Aid for Scientific Research of Japan (no. 70216828) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to S. Taguchi), the Global Center of Excellence Program (project no. B01: Catalysis as the Basis for Innovation in Materials Science) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan and the Association for the Progress of New Chemistry, Japan (to K. Matsumoto). Pacific Edit reviewed the manuscript prior to submission.

Figure legends

Fig. 1. ^1H -NMR spectrum of P(LA-co-3HB-co-3HHx) produced in the recombinant *E. coli* LS5218 harboring the plasmid pTVpctC1(ST/QK)J4. LA, lactate; 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate.

Fig. 2. ^{13}C -NMR spectrum of P(LA-co-3HB-co-3HHx) produced in the recombinant *E. coli* LS5218 harboring the plasmid pTVpctC1(ST/QK)J4. LA, lactate; 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate.

Fig. 3. 2D-NMR (^1H - ^{13}C COSY) spectra of P(LA-co-3HB-co-3HHx) produced in the recombinant *E. coli* LS5218 harboring the plasmid pTVpctC1(ST/QK)J4. LA, lactate; 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate.

Fig. 4. Putative metabolic pathway for the synthesis of P(LA-co-3HB-co-3HHx) in recombinant *E. coli* LS5218. Dashed-arrows indicate the reverse reaction of the β -oxidation pathway. The bold letters by the arrows indicate the enzymes which are relevant for polymer synthesis, and the enzymes in the boxes were

heterologously-expressed. LDH, lactate dehydrogenase; PCT, propionyl-CoA transferase from *Megasphaera elsdenii*; PhaJ4, (*R*)-specific enoyl-CoA hydratase 4 from *Pseudomonas aeruginosa*; FadA, 3-ketoacyl-CoA thiolase; FadB, 3-hydroxyacyl-CoA dehydrogenase/(*S*)-specific enoyl-CoA hydratase; LPE, LA-polymerizing enzyme.

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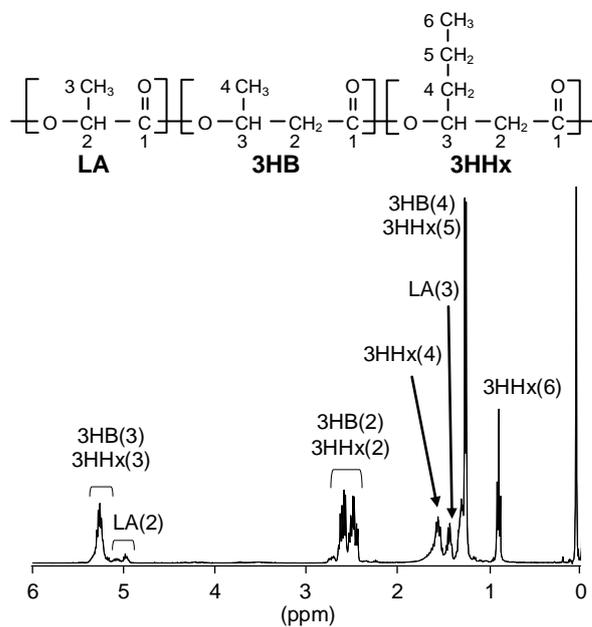


Fig. 1.

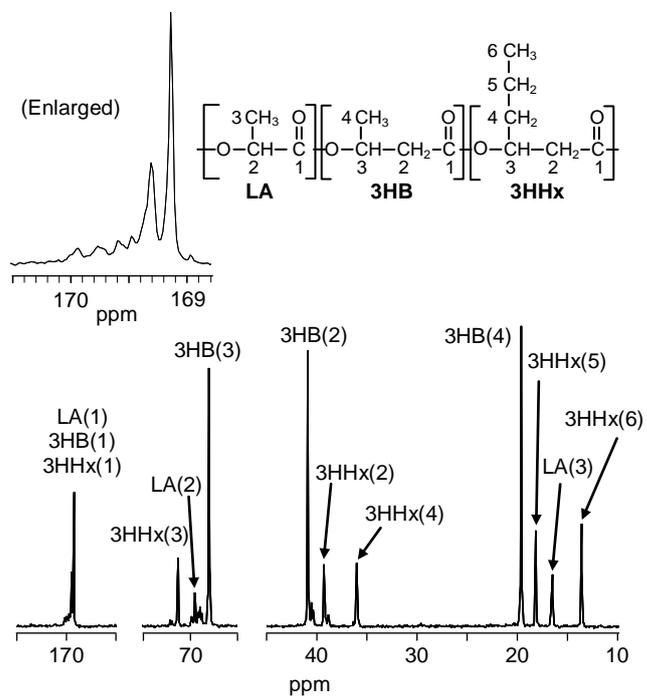


Fig. 2.

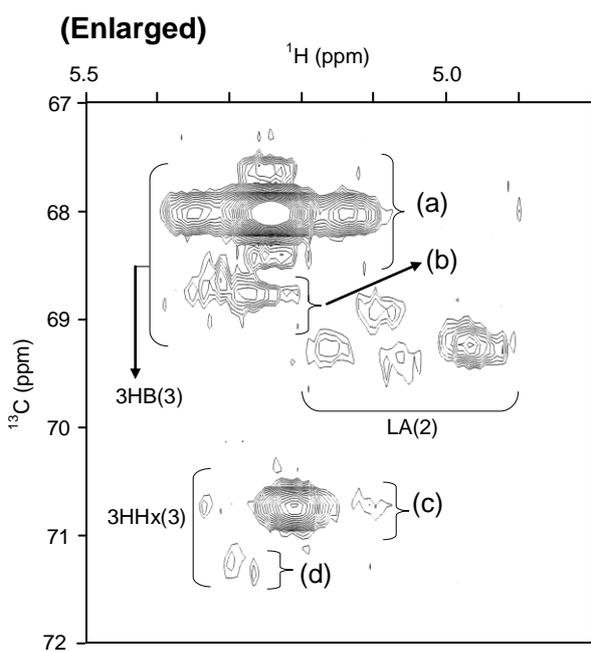
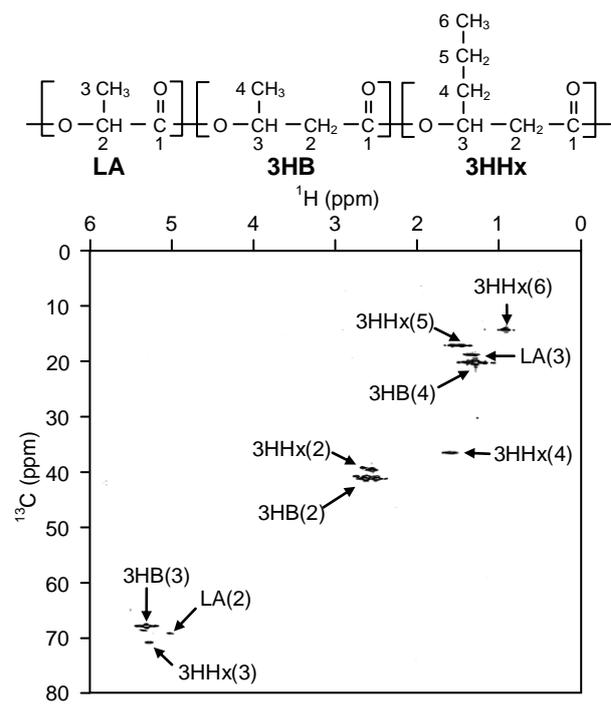


Fig. 3.

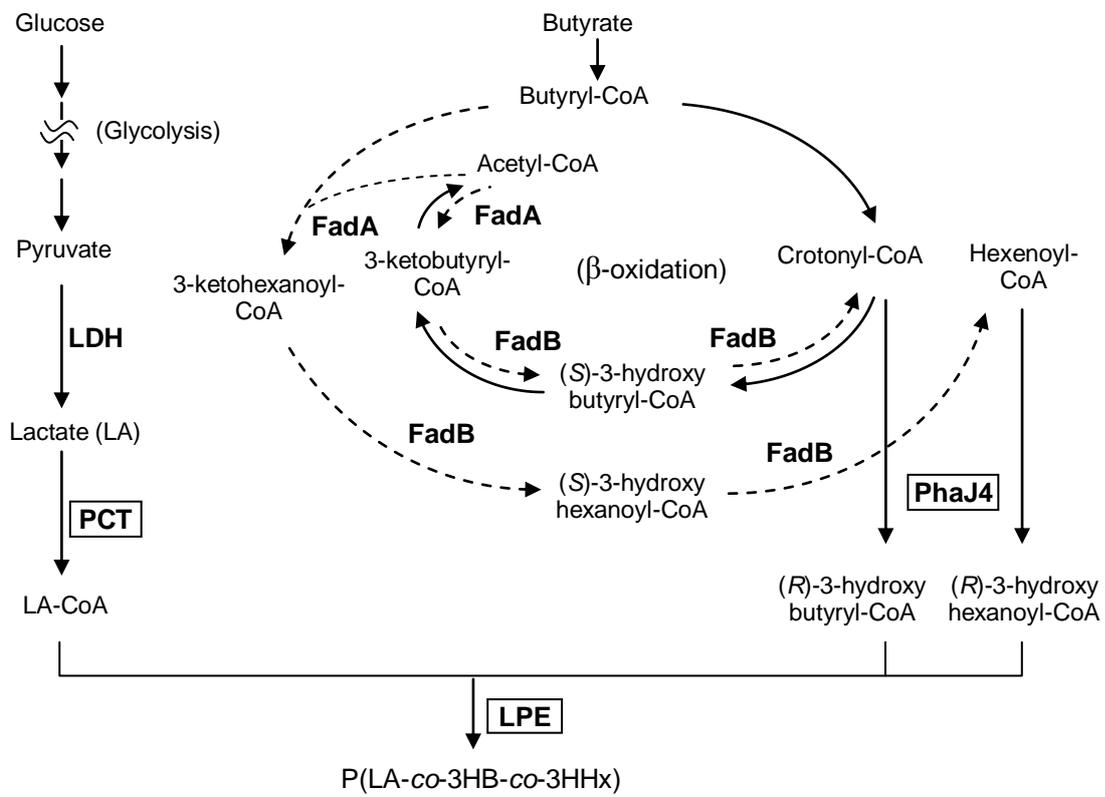


Fig. 4.

Table 1 Production of LA-based polyesters in recombinant *E. coli* LS5218 harboring pTVpctC1(ST/QK)J4 with feeding of butyrate

Sodium Butyrate Conc. (mg/mL)	Cell Dry Weight (g/L)	Polymer Content (wt%) ^a	Monomer Composition (mol%) ^b			Molecular Weights ^c		
			LA	3HB	3HHx	M_n ($\times 10^3$)	M_w ($\times 10^3$)	M_w/M_n
0	4.6 ± 0.1	2.0 ± 0.6	0	100 ± 0	0	24	34	1.4
0.25	4.5 ± 0.1	2.2 ± 0.3	2.7 ± 0.3	81 ± 11	17 ± 12	15	26	1.8
0.5	4.5 ± 0.4	3.8 ± 0.5	12 ± 4	58 ± 6	30 ± 8	12	20	1.7
0.75	4.3 ± 0.3	1.6 ± 0.5	34 ± 8	38 ± 4	28 ± 4	8.7	14	1.6
1	4.6 ± 0.1	0.9 ± 0.5	29 ± 1	40 ± 1	31 ± 2	8.1	13	1.6
1.25	4.6 ± 0.1	0.6 ± 0.3	13 ± 1	49 ± 2	38 ± 1	8.3	13	1.6
1.5	4.7 ± 0.1	0.6 ± 0.1	17 ± 1	50 ± 2	33 ± 2	8.3	13	1.6

^a Polymer content was determined based on dry cell weight and the weight of the extracted polymer. ^b Monomer composition was determined by GC analysis. LA, lactate; 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate. Polymer content and monomer composition are presented as the average of three independent experiments. ^c M_n , number-average molecular weight; M_w , weight-average molecular weight; M_w/M_n , polydispersity index.