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Lactate fraction dependent mechanical properties of semitransparent poly(lactate-co-hydroxybutyrate)s produced by control of lactyl-CoA monomer fluxes in recombinant *Escherichia coli*

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ABSTRACT

In order to evaluate the mechanical properties of poly(lactate-*co*-3-hydroxybutyrate) [P(LA-*co*-3HB)] and its correlation with the LA fraction, P(LA-*co*-3HB) with a variety of LA fractions were prepared using recombinant *Escherichia coli* expressing the LA-polymerizing enzyme and monomer supplying enzymes. The LA-overproducing mutant *E. coli* JW0885 with a *pflA* gene disruption was used for the LA-enriched polymer production. The LA fraction was also varied by jar-fermentor based fine-regulation of the anaerobic status of the culture conditions, resulting in LA fractions ranging from 4 to 47 mol%. In contrary to the opaque P(3HB) film, the copolymer films attained semitransparency depending on the LA fraction. Young's modulus values of the P(LA-*co*-3HB)s (from 148 to 905 MPa) were lower than those of poly(lactic acid) (PLA) (1020 MPa) and P(3HB) (1079 MPa). In addition, the values of elongation at break of the copolymers with 29 mol% LA reached 150%. In conclusion, P(LA-*co*-3HB)s were found to be a comparatively pliable and flexible material, differing from both of the rigid homopolymers.

Keywords:

lactate-based polyester

lactate-polymerizing enzyme

poly(lactic acid)

polyhydroxyalkanoate

lactic acid

1. Introduction

The development of bio-based plastics as an alternative to petroleum-derived materials is an urgent issue because of diminishing fossil resources and the increasing carbon dioxide in the atmosphere (Dodds and Gross, 2007). Among the bio-based plastics, poly(lactic acid)s (PLAs) are one of the most valuable materials which have excellent transparency, and are currently used for packaging, containers, stationary and so forth (Auras et al., 2004; Tsuji, 2002; Tsuji, 2005). However, the relatively poor impact and heat resistance of the material are considered to be a crucial weak point blocking a wider range of applications. Thus, PLAs are an active area of research into improving their properties, for example, by the addition of plasticizers, blending with other polymers, copolymerizations and various chemical modifications, as may be seen in the large number of reports and patents (Haynes et al., 2007; Noda et al., 2004; Ozkoc and Kemalolu, 2009; Pillin et al., 2006; Younes and Cohn, 1988).

Bacterial polyhydroxyalkanoates (PHAs) are also a major class of bio-based plastic, which are intracellularly produced by the PHA synthase-catalyzed polymerization of hydroxyacyl-CoAs (Rehm and Steinbüchel, 1999). Among them, poly(3-hydroxybutyrate) [P(3HB)] is the most common PHA, and is efficiently produced from renewable carbon sources. However, in considering practical use, it has been an obstacle that P(3HB) tends to be a stiff and brittle material due to crystallinity. In addition, because of such crystallization, P(3HB) becomes an opaque. These properties have limited the range of applications of the material.

We recently established a new whole-cell system for producing lactate (LA)-based polyesters using recombinant *Escherichia coli* based on the bacterial PHA biosynthetic pathways (Taguchi et al., 2008). To meet this challenge, the discovery of a PHA synthase with activity toward lactyl-CoA (LA-CoA), which is designated LA-polymerizing enzyme (LPE), was essential. The exploration of LPE was conducted using an *in vitro* enzymatic

assay, but no synthesis of the LA-incorporated polyester was observed with wild-type PHA synthases used (Valentin and Steinbüchel, 1994; Yuan et al., 2001; Zhang et al., 2001). The first LPE was found from a collection of PHA synthase mutants from *Pseudomonas* sp. 61-3 (Nomura and Taguchi, 2007; Taguchi and Doi, 2004) using an *in vitro* chemo-enzymatic polymerization system (Taguchi et al., 2008). In the following studies, the high degree of enantiospecificity of LPE toward D-LA-CoA was clarified by means of two experiments; an enzymatic assay using LA-CoA enantiomers (Tajima et al., 2009) and enantiomeric analysis of LA-incorporated polymer hydrolysates (Yamada et al., 2009). In contrast to its strict enantiospecificity, the LPE possesses very broad substrate specificity and can polymerize LA units along with other PHA monomeric constituents, including 3HB. Therefore, the combination of LPE with two monomer supplying pathways for LA and 3HB monomers enabled synthesis of the P(LA-co-3HB) copolymer (Fig. 1) (for detail see reviews, Matsumoto and Taguchi, 2010; Taguchi, 2010). The thermal analysis of P(LA-co-3HB)s revealed that their melting and glass transition temperatures vary depending on their LA fraction (Yamada et al., 2010). This suggested that these copolymers may have modified mechanical properties and transparency compared to PLA and P(3HB).

Thus, the aim of this study is to evaluate the mechanical properties and transparency of P(LA-co-3HB)s and their correlation with the LA fraction in the polymer. For this purpose, we attempted to produce P(LA-co-3HB)s with a variety of LA fractions on a large scale. The LA fraction in the copolymer has been reported to be regulated by two experimental strategies conducted in a shake flask; varying the monomer flux, including LA (Shozui et al., 2010a; Shozui et al., 2010b; Yamada et al., 2009) and altering the LA-incorporating activity by further mutations of the prototype LPE (Yamada et al., 2010). Here, we successfully obtained large amounts of P(LA-co-3HB)s with varied LA fractions (4 mol% to 47 mol%) by the regulation of monomer flux using an LA-overproducing *E. coli* mutant and jar-fermentor based finely regulated aerobic/anaerobic culture conditions. The thermal and mechanical

properties, and transparency of these polymers are discussed in comparison with those of PLA and P(3HB) homopolymers.

2. Material and methods

2. 1. Bacterial strain and plasmids

E. coli JW0885 (*pflA*⁻) [Keio collection (Baba et al., 2006)], which was kindly provided by the National BioResource Project (NBRP: NIG, Japan), and *E. coli* JM109 were used as hosts (Table 1). The *pflA* gene product activates pyruvate formate lyase (Zhu and Shimizu, 2005). The expression vector pTV118NpctC1STQKAB (Taguchi et al., 2008) harbors the Ser325Thr/Gln481Lys mutated PHA synthase gene from *Pseudomonas* sp. 61-3 [*phaC1_{Ps}*(STQK)] (Takase et al., 2004), β -ketothiolase (*phaA*), NADPH-dependent acetoacetyl-CoA reductase (*phaB*) from *Ralstonia eutropha* and propionyl-CoA transferase gene (*pct*) from *Megasphaera elsdenii*. The *E. coli* strains harboring pTV118NpctC1STQKAB were used for production of the LA-based polyesters.

2. 2. Jar-fermentor culture conditions

In order to produce P(LA-co-3HB) under fine-tuned aeration culture conditions, a jar-fermentor was used. Seed cultures were prepared using 2 mL Luria-Bertani (LB) medium containing 100 μ g/L ampicillin in 10 mL test tubes, which were incubated at 30°C for 14 h with reciprocal shaking at 180 rpm. One milliliter of the seed culture was transferred to 25 mL LB medium containing 100 μ g/L ampicillin in 100 mL a shake flask and incubated at 30°C for 6 h with reciprocal shaking at 130 rpm. Subsequently, 20 mL of culture were used to inoculate a laboratory-scale jar-fermentor (MD-N 31, B. E. Takasaki Co, Ltd, Tokyo, Japan) with 2 L of LB medium containing 100 μ g/L ampicillin and 0.1% adekanol LG-126 (ADEKA

Co, Tokyo, Japan), which is widely used as an antifoaming agent to facilitate large scale fermentation. In the initial stage of fermentation, cells were cultured for 16 h at 30°C, with an aeration rate of 2.0 L/min and agitation set at 300 rpm (cell growth phase). The aeration rate was then changed to 1.0 L/min, 0.5 L/min, 0.3 L/min of air or 2 L/min of nitrogen. In the case of nitrogen flow, the agitation was changed to 50 rpm. In addition, 2% glucose and 10 mM calcium pantothenate (final concentrations) were added to induce polymer production. Pantothenic acid is a known precursor for CoA which may become limiting during 3HB-CoA synthesis. The cells were cultured for 24 h to allow cells to accumulate polymers (production phase).

2. 3. Polymer extraction from recombinant cells

The cells were harvested by centrifugation and lyophilized, and the polymer was extracted with chloroform at 60 °C for 2 days in glass tubes with a screw-cap. Cell debris was removed by passing through a PTFE filter. Then, a 10-fold volume of hexane was added to precipitate the polymer (Doi et al., 1987, Yamada et al., 2010). The precipitant was collected on a PTFE filter and dried *in vacuo* to measure the weight of the polymer. Polymer content was calculated based on the dry cell weight. The polymer films were prepared by solution casting from their chloroform solution using glass Petri dishes as casting surfaces. Then, the cast films were aged at 3 weeks at room temperature. The films were subsequently subjected to further analyses. The transparency of the films was calculated from digital images based on a comparison of the averaged brightness of black grid using ImageJ 1.45 software (National institute of Health, USA, <http://rsb.info.nih.gov/ij>). The data is shown as a value relative to that of PLA.

2. 4. Analysis of LA-based polyesters and LA in the medium

The monomer composition of P(LA-co-3HB) were determined by HPLC as described previously (Takase et al., 2003). For the molecular weight analysis of the LA-based polyesters, the extracted polymer samples were applied to analytical gel permeation chromatography (GPC) (Shimadzu, Japan) equipped with tandem TSK gel Super HZM-H columns (Tosoh, Japan). The polystyrene standards (Waters, USA) were used for calibration (Taguchi et al., 2008). The concentration of LA in the medium was determined using a D-/L-lactic acid assay kit (BIOCON, Nagoya, Japan).

2. 5. Analysis of thermal properties

Differential scanning calorimetry (DSC) data were recorded on a Perkin-Elmer Pyris 1 using solvent-cast films (10 mg) as described previously (Taguchi et al., 2008). The solvent-cast films were encapsulated in aluminum pans and heated from -50 to 210 °C at 20 °C/min (the first heating scan). The melt samples were then followed by rapid quenching at -90 °C, and maintained at -90 °C for 5 min. They were heated from -90 to 210 °C at 20 °C/min (second heating scan). The glass-transition temperature (T_g), melting temperature (T_m), and enthalpy of fusion (ΔH_m) were determined from the second heating scan.

2.6. Mechanical property analysis

Stress-strain tests of solvent-cast films (10 mm × 5 mm) were performed at room temperature with a strain rate of 100 mm/min, according to the method described (Doi et al., 1995). The mechanical tensile data were measured by a Tensilon tester, RTC-1150A (Orientec) instrument, and calculated from such curves by MSAT0002 software using the average of three specimens.

3. Results and Discussion

3.1. Production of P(LA-co-3HB)s with various LA fractions in recombinant *E. coli*

We made an effort to produce P(LA-co-3HB)s with various LA fractions in the recombinant *E. coli* by altering monomer fluxes of LA and 3HB. Because both monomers are derived from pyruvate (Fig. 1), metabolic shift from acetyl-CoA to LA would be necessary to increase LA fraction in the copolymer. In *E. coli*, acetyl-CoA synthesis from pyruvate is catalyzed by two enzymes; pyruvate dehydrogenase complex (PDH) and pyruvate formate lyase (PFL). PDH activity is inhibited by NADH, normally elevated under anaerobic conditions (Hansen and Henning, 1966), while PFL is induced under anaerobic conditions and compensates the acetyl-CoA supply (Peng and Shimizu, 2003). Thus, the flux toward acetyl-CoA is effectively reduced in the PFL-knockout mutant grown under anaerobic culture conditions (Zhou et al., 2003). Additionally, the deletion of PFL led to the production of large amount of LA by upregulating LDH activity (Zhu and Shimizu, 2004) that would be preferable for the production of LA-enriched polymers. Therefore, we cultured *E. coli* JW0885 (*pflA*⁻) under several different aerobic and anaerobic conditions for regulating LA fraction in the copolymer, together with the standard strain JM109 for lower LA fraction.

Table 2 summarizes the result of the fermentation experiments. The LA concentration in the medium at the time of harvest was altered 0.3 to 5.7 g/L. The highest LA level was comparable to that of P(3HB)-producing *E. coli* under anaerobic conditions (Carlson et al., 2005). Next, the polymer was extracted from the cells and subjected to HPLC analysis, revealing that P(LA-co-3HB) with 4 to 47 mol% LA had been produced (Table 2). The LA fraction in the polymers was positively correlated with the LA concentration secreted into the medium (Fig. 2A). Thus, the above mentioned strategies successfully altered the LA fraction in the copolymer, although the polymer content tended to be low under anaerobic conditions.

These results provide insight into the carbon fluxes from glucose to the polymer. In order to estimate the monomer fluxes, the amount of the LA and 3HB units incorporated into the polymer was calculated (Fig. 2B). The amount of 3HB in the polymer was inversely

correlated with the LA concentration in the medium. This result was contrast to the fact that the amount of P(3HB) produced in *E. coli* under anaerobic conditions was comparable to that of aerobic conditions (Carlson et al., 2005). This difference may be due to the lowered acetyl-CoA synthesis caused by the deletion of PFL in JW0885. Additionally, the synthesis of LA-CoA by PCT may be competitive to 3HB-CoA supply from acetyl-CoA, because acetyl-CoA presumably acts as a major CoA donor for the PCT-catalyzed reaction in *E. coli* (Fig. 1). In contrast to 3HB, the amount of LA in the polymer was as a maximum under moderately anaerobic conditions, and was relatively decreased under stringent anaerobic conditions, despite the high LA concentration in the medium (Fig. 2B). This phenomenon might be interpreted to indicate the limitation of the CoA transferring step, because the drastic decrease in acetyl-CoA (donor) level under anaerobic conditions could reduce LA-CoA supply. In addition, we previously found that presence of 3HB-CoA is necessary for the polymerization of LA-CoA, probably because 3HB-CoA acts as an initiator of the polymerization (Shozui et al., 2011; Taguchi et al., 2008). Therefore, the decrease in 3HB-CoA supply could reduce the incorporation of LA units into the polymer chain. The hypothesis is supported by the *in vitro* experiment that P(LA-co-3HB) yield decreased by adding higher amount of LA monomers (Tajima et al., 2009). The carbon yield of 3HB from glucose was 12% of the theoretical maximum under aerobic conditions (1.16 g/L), was lower than the highest carbon yield of LA (29%) at stringent anaerobic conditions, suggesting that glucose was partially consumed for cell growth under aerobic conditions. However, this estimation did not consider the generation of small amount of monomeric 3HB, which was reported to occur during P(3HB) production in recombinant *E. coli* (Carlson et al., 2005).

3. 2. Molecular weights, thermal properties and transparency of P(LA-co-3HB)s

The weight-averaged molecular weights (M_w) of the copolymers with 4 and 15 mol% LA were 720 000 and 820 000, respectively, which were comparable to those of chemically

synthesized PLAs (Table 3). With higher LA fractions (29 and 47 mol%), however, the molecular weight of polymer tended to be low (70 000 to 90 000). This result suggested that incorporation of the LA units into the polymer chain led to a reduction in molecular weight of the polymer.

The thermal properties of P(LA-*co*-3HB)s, PLA and P(3HB) were analyzed using a DSC (Table 3). PLA and P(3HB) had different glass transition temperatures (T_g) (61°C and -7°C, respectively), which contributed to the transparent and opaque properties of these polymers, respectively (Doi et al., 1995; Tsuji, 2002). P(LA-*co*-3HB)s exhibited a range of T_g values (-6 to 34°C) depending on their LA fractions. The copolymers with the higher LA fraction tended to have higher T_g values. The same tendency was observed in the case of the P(LA-*co*-3HB)s produced by means of shake flask culture (Yamada et al., 2010). In the case of the copolymers having greater than 15 mol% LA, two T_g values were observed. The lower T_g (-9 to -8 °C) is likely due to the presence of the 3HB-rich segment in the copolymer.

The melting temperature (T_m) and melting enthalpy (ΔH) of P(LA-*co*-3HB)s, in particular in the copolymers with 29 to 47 mol% LA, were lower than those of the P(3HB) and PLA homopolymers. The lowered T_m suggests that the packing of the polymer chains in the crystal is looser than P(3HB), and the lowered ΔH indicates a decrease in the amount of the crystal. Therefore, the result suggests that the crystallization of the copolymer is inhibited by the copolymerization of the LA and 3HB units. Similar phenomena have been observed in short-chain-length/medium-chain-length PHA copolymers, such as P[3HB-*co*-3-hydroxyalkanoate (3HA)], the crystallinity of which decreased along with an increase in the 3HA fraction (Doi et al., 1995).

The P(LA-*co*-3HB) films are shown in Fig. 3 as a comparison of their relative transparency. The P(3HB) film shrank and became opaque under the conditions employed, whereas PLA formed a completely clear film. In the case of P(LA-*co*-3HB)s, the copolymers formed semitransparent films depending on their LA fractions. The transparency of the

copolymer films were significantly increased when the LA fraction was higher than 15 mol%, suggesting that the crystallinity of the polymer was effectively lowered by the incorporation of 15 mol% LA units. The result was consistent with the lowered T_m and ΔH of the copolymers with 15 to 47 mol% LA. The reduction in crystallinity was accounted for by the T_g values of the copolymers, which were near or higher than the room temperature (Table 3), because crystallization of polymer occurs at temperatures higher than T_g .

3.3. Mechanical properties of P(LA-co-3HB)s

Table 3 shows the mechanical properties of P(LA-co-3HB)s. Young's modulus for the copolymers (148 to 905 MPa) was lower than for the PLA (1020 MPa) and P(3HB) (1079 MPa) homopolymers, and showed a decreasing tendency along with increases in the LA fraction. The tensile strength of P(LA-co-3HB)s exhibited the same tendency. In addition, the elongation at break of the copolymers was higher than that for homopolymers, and reached 150% at 29 mol% LA. These results indicate that P(LA-co-3HB)s are a more pliable and stretchy material than the homopolymers. This is consistent with their thermal properties, which indicates a lowered crystallinity of the polymers. Because the lack of impact resistance of PLA and P(3HB) has been considered a weak point, the copolymer prepared here is a more potent materials covering a wider range of applications.

The pliable and stretching properties of the P(LA-co-3HB)s were found to be similar to those of P(3HB-co-3HA) copolymers (Matsusaki et al., 2000). However, it should be noted that P(LA-co-3HB)s are distinguished from P(3HB-co-3HA)s by their glass transition temperature. The relatively high T_g of P(LA-co-3HB)s contributes to the semitransparency and long-term stability of the material.

4. Conclusion

In this study, we succeeded in producing P(LA-co-3HB)s with a varied LA fraction using large-scale cultivation under regulated aerobic/anaerobic culture conditions and an LA-overproducing *E. coli* mutant. The analysis of amounts of polymeric LA and 3HB suggested that acetyl-CoA level should be a key for determining the monomer fluxes, as well as the property of LPE. It should be noted that the microbially synthesized P(LA-co-3HB)s were a pliable and stretchy semitransparent material. The results of this study indicate the potential of highly enantiopure P(LA-co-3HB)s as a new type of biopolymer having properties of particular value, such as good transparency of the PLAs and flexibility of the PHA copolymers. Moreover, we were able to determine the effectiveness of enzyme-metabolic engineering strategies by evaluating the polymer properties in detail. Such polymer analysis should be of value for the molecular design of biopolymers and the methods of their production. The goal in the next stage is to cover the full range of the LA fraction in the copolymers and then to investigate their mechanical properties. In addition, by taking advantage of the broad substrate specificity of LPE, we have created a metabolic pathway for introducing 3-hydroxyvalerate and 3-hydroxyhexanoate units into LA-based polyesters in *E. coli* (Shozui et al., 2010a, 2010b; Shozui et al., 2011). The introduction of these monomers with longer side chains is expected to further improve the mechanical properties of the material.

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FIGURE LEGENDS

Fig. 1. Design of a biosynthetic pathway for the LA-based polyester in *Escherichia coli* and its LA-overproducing mutant, JW0885. LDH, lactate dehydrogenase; PFL, pyruvate-formate lyase; PDH, pyruvate dehydrogenase complex; PCT, propionyl-CoA transferase from *Megasphaera elsdenii*; PhaA, β -ketothiolase from *R. eutropha*; PhaB, NADPH-dependent acetoacetyl-CoA reductase from *R. eutropha*; LPE, LA-polymerizing enzyme from *Pseudomonas* sp. 61-3. LDH and PFL are inherent enzymes in *E. coli*. *E. coli* JW0885 is a *pflA* gene-disrupted strain (Baba et al., 2006; Zhu and Shimizu, 2005). Dotted arrow indicates a possible conversion reaction from acetyl-CoA to LA-CoA catalyzed by PCT.

Fig. 2. (A) Correlation between the concentration of LA in the supernatant of the culture medium and LA molar fraction of P(LA-co-3HB)s. (B) Correlation between the concentration of LA in the medium and the amount of polymeric LA (diamond) and 3HB (circle) monomers.

Fig. 3. Solvent-cast films of P(LA-*co*-3HB)s with the different LA fractions. The relative transparency of the films was calculated using ImageJ.

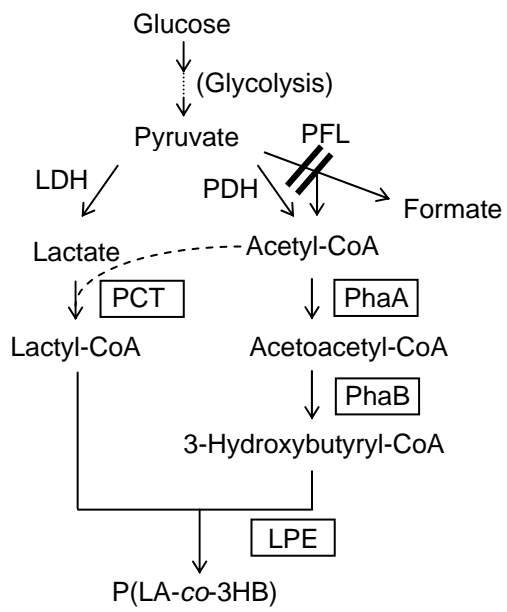


Fig. 1 Yamada et al.

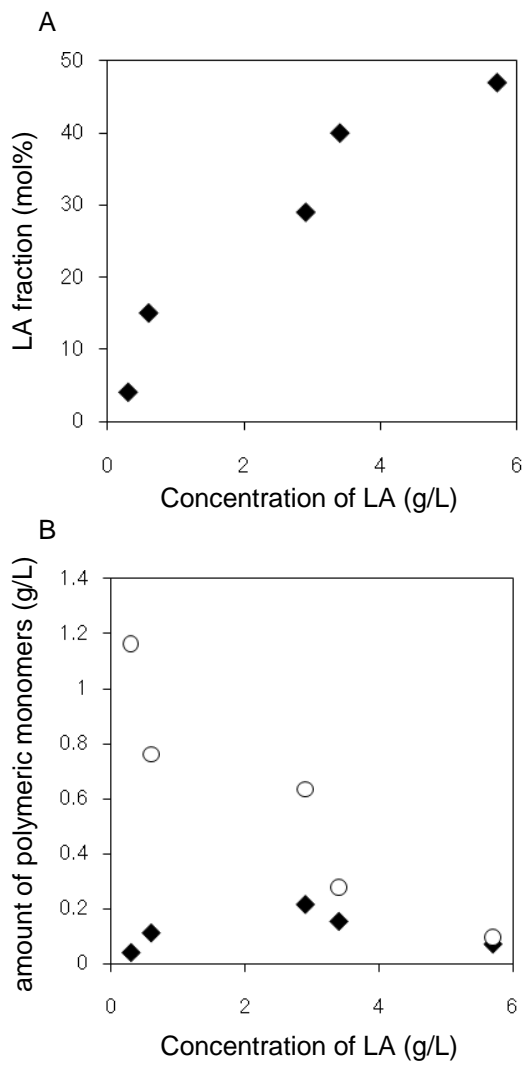


Fig. 2 Yamada et al.

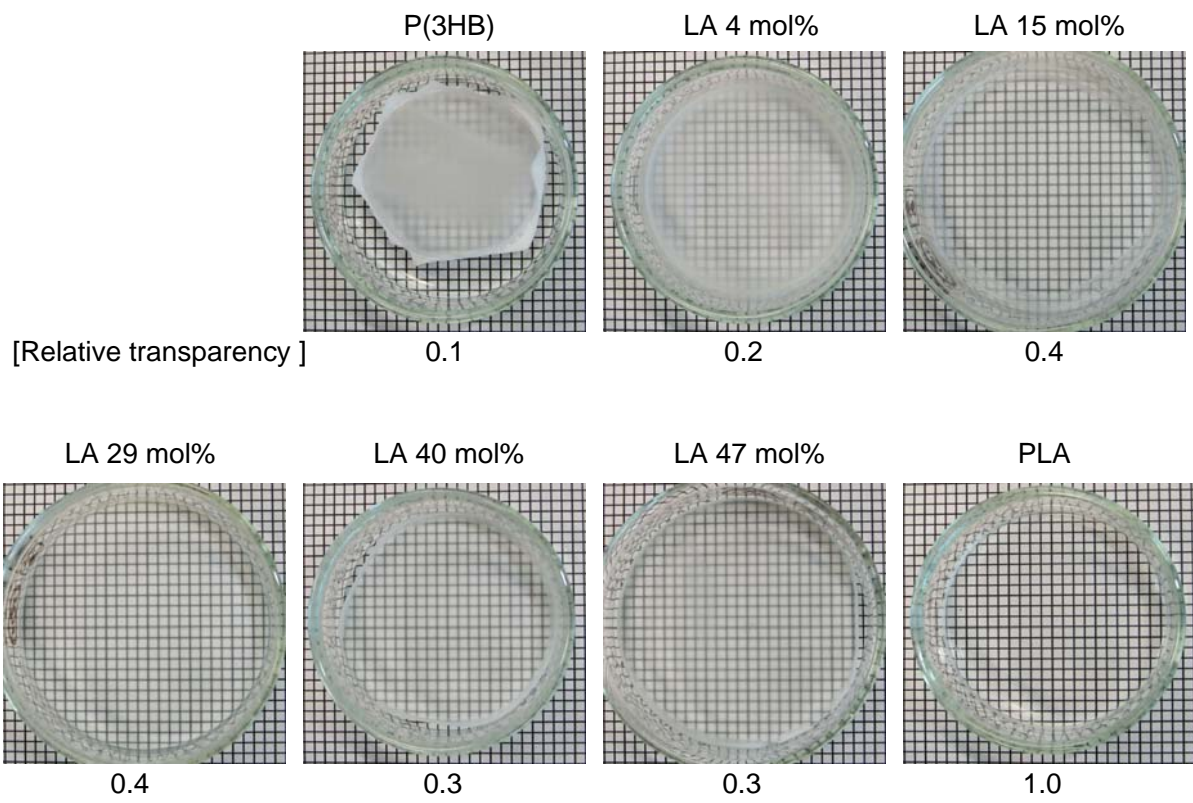


Fig. 3 Yamada et al.

Table 1

Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characterization	Source of reference
Strains		
<i>E. coli</i> JM109	endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB+ Δ(lac-proAB)	TakaRa
<i>E. coli</i> JW0885	e14- [F' traD36 proAB+ lacIq lacZΔM15] hsdR17(rK ⁻ mK ⁺) ΔpflA Lactate over-producing mutant, <i>pyruvate</i> <i>fomete lyase</i> gene disruption mutant of <i>E. coli</i> K-12	Baba et al., 2006 Zhu and Shimuzu, 2005
Plasmids		
pTV118N	Amp ^r ; <i>lac</i> POZ'; cloning vector	TakaRa
pTV118NpctC1STQKAB	pTV118N derivative; <i>lac</i> promoter, <i>pct</i> , <i>phb</i> _{Re} promoter, <i>phaC1</i> _{Ps} <i>STQK</i> , <i>phaA</i> _{Re} , <i>phaB</i> _{Re}	Taguchi et al., 2008

Table 2 Production of P(LA-co-3HB) by recombinant *E. coli* under various aeration conditions^a

Strain	Aeration condition ^a (L/min)	Concentration of LA in the medium ^b (g/L)	Dry cells weight (g/L)	Polymer content ^c (wt%)	Monomer composition ^c (mol%)	
					LA	3HB
JM109	1.0 (air)	0.3	4.3	28	4	96
JM109	0.5 (air)	0.6	3.8	23	15	85
JW0885	0.5 (air)	2.9	3.7	23	29	71
JW0885	0.3 (air)	3.4	2.7	16	40	60
JW0885 ^d	1.0 (N ₂)	5.7	2.4	7	47	53

^a The cells harboring pTV118NpctC1STQKAB; ^b In the initial culture conditions, culture was grown at 30°C with an aeration rate of 2 L/min for 16 h. After cell growth, the aeration rate was reduced or changed to nitrogen. The cells were cultured for 24 h to allow cells to accumulate polymers; ^c P(LA-co-3HB) content and monomer composition were determined by HPLC. LA, lactate; 3HB, 3-hydroxybutyrate; ^d Data from Yamada et al. (2010).

Table 3 Thermal and mechanical properties of P(LA-*co*-3HB)s with various LA fractions.

Monomer composition ^a (mol%)		Molecular weights ^b		Mechanical properties ^c			Thermal properties ^d		
LA	3HB	M_w ($\times 10^{-4}$)	M_w/M_n	Tensile strength (MPa)	Young's modulus (MPa)	Elongation at break (%)	T_g (°C)	T_m (°C)	ΔH_m (cal/g)
0	100 ^e	70	2.3	19±1	1079±215	9±1	-7	159, 176	1.6, 9.9
4	96	74	4.6	30±4	905±136	7±1	-6	160, 174	1.8, 5.4
15	85	82	2.4	10±0	194±5	75±2	-9, 19	149, 167	0.6, 3.2
29	71	9	2.2	7±7	154±5	156±34	-8, 25	141, 158	0.2, 0.9
40	60	7	3.5	6±0	148±10	64±7	-8, 30	140, 156	0.3, 1.4
47	53	7	2.3	7±2	153±15	84±20	-8, 34	140, 157	0.4, 1.9
100 ^f	0	20		52±2	1020	2	60	153	2.2

^a Determined by HPLC; ^b M_w , weight-averaged molecular weight; M_w/M_n , polydispersity. ^c The values are the averages from at least three independent measurements; ^d T_g , glass-transition temperature; T_m , melting temperature; ΔH_m , enthalpy of fusion. ^e P(3HB) was purchased from Mitsubishi gas chemical (Tokyo, Japan); ^f Data from Zaman et al. (2011). PLA was chemically synthesized.