



Title	Effectiveness of xylose utilization for high yield production of lactate-enriched P(lactate-co-3-hydroxybutyrate) using a lactate-overproducing strain of Escherichia coli and an evolved lactate-polymerizing enzyme
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Abstract

1
2 Xylose, which is a major constituent of lignocellulosic biomass, was utilized for the
3 production of poly(lactate-co-3-hydroxybutyrate) [P(LA-co-3HB)], having transparent
4 and flexible properties. The recombinant *Escherichia coli* JW0885 (*pflA*⁻) expressing
5 LA-polymerizing enzyme (LPE) and monomer supplying enzymes grown on xylose
6 produced a copolymer having a higher LA fraction (34 mol%) than that grown on
7 glucose (26 mol%). This benefit of xylose was further enhanced by combining it with
8 an evolved LPE (ST/FS/QK), achieving a copolymer production having 60 mol% LA
9 from xylose, while glucose gave a 47 mol% LA under the same condition. The overall
10 carbon yields from the sugars to the polymer were similar for xylose and glucose, but
11 the ratio of the LA and 3HB units in the copolymer was different. Notably, the
12 P(LA-co-3HB) yield from xylose (7.3 g l⁻¹) was remarkably higher than that of P(3HB)
13 (4.1 g l⁻¹), indicating P(LA-co-3HB) as a potent target for xylose utilization.

14

15 **Keywords:** Lignocellulose; bio-based plastic; Polylactic acid; microbial cell factory

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1 **1. Introduction**

2 Poly(lactate-*co*-3-hydroxybutyrate) [P(LA-*co*-3HB)] is a newly recognized member
3 of the bacterial polyhydroxyalkanoate(PHA) family that is synthesized using a PHA
4 synthase possessing an acquired LA-polymerizing activity (Matsumoto and Taguchi,
5 2010; Taguchi et al., 2008). P(LA-*co*-3HB) was produced from glucose in recombinant
6 *Escherichia coli* expressing a Ser325Thr/Gln481Lys mutant of PHA synthase from
7 *Pseudomonas* sp. 61-3 [PhaC1_{Ps}(ST/QK)] as an LA-polymerizing enzyme (LPE),
8 together with a propionyl-CoA transferase (PCT) from *Megasphaera elsdenii* and 3HB
9 monomer supplying enzymes [β -ketothiolase (PhaA) and NADPH-dependent
10 acetoacetyl-CoA reductase (PhaB)] from *Ralstonia eutropha* (Taguchi et al., 2008).

11 An attractive feature of P(LA-*co*-3HB)s is that they exhibit stretchy properties and
12 transparency depending on their LA fraction (Yamada et al., 2011). These properties are
13 distinguished from those of the transparent but rigid PLA, and the opaque and brittle
14 P(3HB) homopolymers, suggesting that P(LA-*co*-3HB)s have the potential to cover a
15 much wider range of applications. We had previously attempted to control the LA
16 fraction in the copolymer synthesized from glucose by changing the host strains and
17 modifying the cultivation conditions (Shozui et al., 2011; Song et al., 2012; Yamada et
18 al., 2009). In terms of the utilization of a variety of carbon sources derived from

1 biomass, we have explored the potential of carbon sources other than glucose that can
2 contribute to the efficient production of P(LA-co-3HB)s.

3 In the present study, we have focused on xylose, which is a major constituent of the
4 hemicellulose portion of the inedible lignocellulosic biomass (Aristidou and Penttila,
5 2000; Rubin, 2008). In addition, the biorefinery method for the preparation of xylose
6 through the extraction and hydrolyzation of hemicellulose has been reported (Agbor et
7 al., 2011; Alonso et al., 2010). A typical example of xylose utilization is the production
8 of lactic acid, which is efficiently produced from xylose with yields close to the
9 theoretical maximum (Adsul et al., 2011; Girio et al., 2010). Moreover, the demand for
10 multiple utilization of xylose in the production of value-added chemicals is steadily
11 increasing.

12 For the production of P(LA-co-3HB) from xylose, we employed the metabolically
13 engineered *E. coli* JW0885 (*pflA*⁻) as a host. The *pflA* gene product activates pyruvate
14 formate lyase (PFL) which produces formate from pyruvate (Zhu and Shimizu, 2004).
15 The deletion of *pflA* gene therefore inactivates formate formation pathway which is a
16 competing pathway of lactic acid production (Zhu and Shimizu, 2005). *E. coli* JW0885
17 constitutively overproduces lactic acid under aerobic conditions and has been shown to
18 be beneficial for P(LA-co-3HB) production (Shozui et al., 2010) hence; it was a suitable

1 host for the production of LA-based polymers. During the course of xylose utilization
2 experiments, we unexpectedly found that xylose gave a higher LA yield and fraction in
3 P(LA-*co*-3HB) compared to glucose. This finding prompted us to further increase the
4 productivity and LA fraction of P(LA-*co*-3HB) from xylose by enhancing the flux
5 towards the LA units in the polymer using an evolved LPE, PhaC1_{Ps}(ST/FS/QK)
6 (Yamada et al., 2009). The effectiveness of these trials will be discussed on the basis of
7 the findings related to the carbon yield of the polymers and excreted organic acids,
8 thereby revealing the high yield conversion of xylose into P(LA-*co*-3HB) with high LA
9 fraction. Thus, this paper proposes the LA-based copolymer as a new target of xylose
10 utilization for the production of value-added materials.

11

12 **2. Materials and methods**

13 **2.1. Bacterial strains and plasmids**

14 The Keio collection strain *E. coli* JW0885 (*pflA*⁻) (purchased from National
15 BioResource Project, Japan) (Baba et al., 2006), was used as a host for polymer
16 production. The expression vector pTV118N*pctphaC1_{Ps}*(ST/QK)*AB*, which harbors the
17 *pct*, *phaC1_{Ps}*(ST/QK), *phaA*, and *phaB* genes (Taguchi et al., 2008), was used for the
18 production of LA-based polyesters. Additionally, the recombinant cells harboring

1 pTV118N*pctphaCI*_{Ps}(ST/FS/QK)*AB* with a Ser325Thr/Phe392Ser/Gln481Lys mutated
2 PHA synthase gene from *Pseudomonas* sp. 61-3 (Yamada et al., 2010) were used for the
3 production of high LA-containing polymers. The pGEM*phaCI*_{Ps}(ST/QK)*AB* (Takase et
4 al., 2003) was used for P(3HB) production.

5

6 **2.2 Culture conditions**

7 **2.2.1 Test-tube cultures**

8 Recombinant *E. coli* cells harboring either pTV118N*pctphaCI*_{Ps}(ST/QK)*AB* or
9 pTV118N*pctphaCI*_{Ps}(ST/FS/QK)*AB* were grown in 10 mL glass test-tubes on
10 Luria-Bertani (LB) medium (1.7 mL) containing 20 g l⁻¹ xylose or glucose, 100 µg l⁻¹
11 ampicillin and 10 mM calcium pantothenate at 30°C for 48 hours for the production of
12 P(LA-*co*-3HB). Recombinant *E. coli* cells harboring pGEM*phaCI*_{Ps}(ST/QK)*AB* were
13 similarly prepared for the production of P(3HB). To test the effect of xylose
14 concentrations on polymer yield and LA fractions, recombinant cells harboring
15 pTV118N*pctphaCI*_{Ps}(ST/QK)*AB* were prepared in a similar manner with varying
16 concentrations of xylose (5 - 100 g l⁻¹).

17

18 **2.2.2 Shake flask cultures**

1 To produce P(LA-co-3HB) in shake flasks, seed cultures of recombinant *E. coli*
2 cells harboring pTV118NpctphaCI_{Ps}(ST/QK)AB or
3 pTV118NpctphaCI_{Ps}(ST/FS/QK)AB were prepared using 2 mL LB medium containing
4 100 µg l⁻¹ ampicillin in 10 mL glass test-tubes and cultured at 30°C for 12 h with
5 reciprocal shaking at 180 rpm. One milliliter of the seed culture was then transferred
6 into 100 mL LB medium containing 100 µg l⁻¹ ampicillin, 20 g l⁻¹ xylose or glucose and
7 10 mM calcium pantothenate in a 500 mL shake flask and cultured at 30°C for 48 h
8 with reciprocal shaking at 120 rpm. Recombinant *E. coli* JW0885 harboring
9 pGEMphaCI_{Ps}(ST/QK)AB were cultured similarly for P(3HB) production.

10

11 ***2.3 Analysis of cell growth, sugars and metabolites during P(LA-co-3HB) and*** 12 ***P(3HB) synthesis***

13 The samples from the *E. coli* shake flask cultures were taken periodically
14 during cultivation and centrifuged at 12 000 rpm for 5 min to separate the cells and the
15 supernatant. The cells were lyophilized and used for cell growth and polymer analysis.
16 The polymers were analyzed using gas chromatography, as described previously
17 (Shozui et al., 2010). The concentrations of the sugars, 3-hydroxybutyric acid and acetic
18 acid in the culture supernatants were determined using HPLC system equipped with a

1 refractive index detector, as described previously (Matsumoto et al., 2012). The levels
2 of formic acid, succinic acid, pyruvate and ethanol were below the detection limit of
3 0.01 g l⁻¹ throughout this study. The concentration of lactic acid in the supernatant was
4 estimated using a D-/L-lactic acid assay kit (Megazyme international, Ireland).

5

6 ***2.4 Determination of the intracellular cofactor levels during polymer production.***

7 The NADPH, NADP⁺, NADH, and NAD⁺ levels were determined as follows;
8 Recombinant *E. coli* cells cultivated in shake flasks were taken at intervals and washed
9 by ice-cold phosphate buffer. The cofactors were then extracted and the concentrations
10 were estimated using EnzyChromTM/NADP⁺/NADPH (ECNP-100) and EnzyChromTM/
11 NAD⁺/NADH (E2ND-100) kits (Bioassay systems, USA) as per the manufacturer's
12 instructions.

13

14 ***2.5 Polymer extraction and analyses***

15 The polymers were extracted from lyophilized cells with chloroform at 60°C
16 for 2 days in glass tubes with a screw-cap (Yamada et al., 2010). Cell debris was
17 removed by passing through a PTFE filter, and then a 10-fold volume of methanol was
18 added to precipitate the polymer. The mixture was incubated at 4°C for 3 days to prompt

1 the precipitation, which increased the recovery of the polymer. The precipitant was
2 dried *in vacuo* and the polymer content was calculated based on the cell dry weight. The
3 monomer composition of P(LA-*co*-3HB) was determined by HPLC as described
4 previously (Yamada et al., 2009). The molecular weights of the extracted polymers were
5 determined by gel permeation chromatography (GPC, JUSCO, Japan) equipped with a
6 Shodex GPC KF-805 column (Showa Denko K.K., Japan) using polystyrene standards
7 (Waters, USA) for calibration (Taguchi et al., 2008). Five milligrams of the extracted
8 P(LA-*co*-3HB) copolymer was dissolved in 1 mL of CDCl₃ (Wako Pure Chemical
9 Industries Ltd, Japan) and used for ¹H NMR analysis by a Bruker MSL400 spectrometer
10 (400 MHz).

11

12 **3 Results**

13 **3.1 P(LA-*co*-3HB) production from xylose using the metabolically engineered *E. coli***

14 ***JW0885***

15 To investigate the effect of sugars on PHA production, P(3HB) was produced in
16 recombinant *E. coli* JW0885 harboring pGEM*phaCl*_{Ps}(ST/QK)*AB*. The P(3HB) yield
17 using cells cultivated on xylose was 4.1 g l⁻¹, while 1.4-fold higher production (5.7 g l⁻¹)
18 was obtained from glucose (Table 1, No. 1&2), which is consistent with previously

1 reported results (da Silva et al., 2009; Keenan et al., 2004; Lee, 1998).

2 Next, the biosynthesis of P(LA-co-3HB) from xylose was attempted using the
3 recombinant *E. coli* JW0885 harboring the pTV118NpctphaC1_{Ps}(ST/QK)AB. First, the
4 effect of xylose concentration on polymer yields and LA fractions was analyzed and
5 results are shown in Fig. S1A&B. We then chose 20 g l⁻¹ for subsequent studies since
6 this concentration gave the highest LA fraction in the copolymers. The recombinant
7 strain cultivated on 20 g l⁻¹ xylose produced 5.5 g l⁻¹ of P(34 mol% LA-co-3HB). In
8 contrast, this yield was lower than that obtained from 20 g l⁻¹ glucose (6.5 g l⁻¹) (Table 1,
9 No. 3&4). In this case, focusing on LA incorporation, the yield of the LA units (1.7 g
10 l⁻¹) and the LA fraction (34 mol%) in the P(LA-co-3HB) obtained from xylose were
11 higher than those from glucose (1.5 g l⁻¹ and 26 mol% LA), indicating the effectiveness
12 of xylose utilization in obtaining LA-enriched polyesters. For both xylose and glucose
13 cases, the P(3HB) homopolymer yields were similar to the respective 3HB unit yields in
14 P(LA-co-3HB)s (Table 1). Thus, the higher yields of P(LA-co-3HB) than P(3HB) were
15 mainly due to the incorporation of LA into the polymers.

16

17 ***3.2 The evolved LPE further enhanced the yields of the polymer and LA units from***
18 ***xylose***

1 To further increase the incorporation of LA units in the polymer, we applied an
2 evolved LPE, PhaC1_{Ps}(ST/FS/QK), which had been shown to synthesize P(LA-co-3HB)
3 with a higher LA fraction than PhaC1_{Ps}(ST/QK) (Yamada et al., 2010). The use of
4 PhaC1_{Ps}(ST/FS/QK) led to 2.5- and 2.3-fold increases in the LA unit yield in
5 P(LA-co-3HB)s from xylose and glucose, respectively (Table 1, No. 5&6) compared to
6 PhaC1_{Ps}(ST/QK) (Table 1, No. 3&4). Likewise, the LA fractions of P(LA-co-3HB)s
7 synthesized using PhaC1_{Ps}(ST/FS/QK) were significantly higher compared to those
8 using PhaC1_{Ps}(ST/QK) (Table 1, No. 5&6). The xylose-specific yield of P(LA-co-3HB)
9 synthesized using PhaC1_{Ps}(ST/FS/QK) was 0.37 g g⁻¹, which was significantly higher
10 than the highest xylose-specific yield for P(3HB) from xylose ever reported (0.26 g g⁻¹)
11 (da Silva et al., 2009). Moreover, the use of PhaC1_{Ps}(ST/FS/QK) with xylose was
12 effective than glucose in synthesizing LA-enriched P(60 mol% LA-co-3HB) with high
13 polymer yields under aerobic conditions, which was far much efficient than the system
14 that achieved similar LA fractions in the copolymers but characterized with low
15 polymer yields under anaerobic conditions using glucose (Yamada et al., 2010; Yamada
16 et al., 2011).

17

18 ***3.3 Metabolic flux analysis from xylose toward P(LA-co-3HB)***

1 The aforementioned results suggest xylose to be superior to glucose as a carbon
2 source for LA-based polyester production because of the higher LA fraction and LA unit
3 yields. In order to gain further insights into the metabolic fluxes, we monitored the time
4 course of the polymer production as well as the excretion of lactic acid, acetic acid and
5 3-hydroxybutyric acid, which were detected as major components in the medium (Fig.
6 2A-F). Formic acid and succinic acid were not detected, which is consistent with
7 previous reported results of the *pflA* mutant strain (Zhu and Shimizu, 2005). The other
8 metabolites such as ethanol and pyruvate were also not detected hence; their
9 contribution to carbon yield from sugars was insignificant. The results are shown as the
10 molar amount of carbon for each compound. For example, 10 mM lactic acid (C₃)
11 corresponds to 30 mM carbon in a molar amount, which clearly demonstrates the
12 conversions of carbon sources into the polymers and by-products. The monitoring of
13 polymer synthesis was important for deducing the metabolic fluxes from the sugars. The
14 cell growth and other parameters are shown in Fig. 3.

15

16 ***3.3.1 Glucose gave a higher yield of P(3HB) than xylose***

17 First, the P(3HB) production from xylose and glucose was monitored (Fig.
18 2A&B, Fig. 3A). The initial concentrations of the sugars (20 g l⁻¹) were equal to 133

1 mM xylose and 111 mM glucose, respectively, each of which corresponds to 667 mM
2 carbon molar amount. The conversion of xylose into P(3HB) occurred mostly during the
3 6 - 20 h interval after inoculation. The total amount of carbon decreased during the time
4 period, indicating that the carbons in xylose were used for cell growth (Fig. 3A) and/or
5 emitted as CO₂. The excreted amount of lactic acid was found to be low (Fig. 2A), and
6 the acetic acid production was trace (Fig. S3A). Xylose was completely consumed at 36
7 h after inoculation and the excreted lactic acid was also utilized. The production and the
8 consumption of lactic acid from the medium were in good agreement with the pH
9 changes in the medium (Fig. 3D). Comparing these results with the glucose culture, as
10 expected, glucose was consumed faster than xylose (Fig. 2B), and the production of
11 P(3HB) and lactic acid in the glucose culture were also higher. The final carbon yield
12 from glucose to P(3HB) (331 mM) was higher than that from xylose (280 mM),
13 whereas the theoretical maximum is 446 mM.

14

15 ***3.3.2 P(LA-co-3HB) production increased the carbon yield from xylose***

16 The introduction of LA-CoA supplying pathway into the aforementioned
17 P(3HB) producing pathway for P(LA-co-3HB) production (Fig. 1) led to drastic
18 changes in the carbon conversions (Fig. 2C&D). First, the sugar consumption rates for

1 xylose and glucose during the 6 - 20 h interval after inoculation were increased
2 compared to those of P(3HB) production, and coincided with the period of cell growth
3 (Fig. 3B). In the same interval, P(LA-co-3HB)s were synthesized more rapidly than
4 P(3HB). It should be noted that LA units yield in the copolymer synthesized from
5 xylose was higher than that from glucose. Lactic acid was detected in the supernatant at
6 6 h after inoculation then peaked at 14 h for both xylose and glucose (Fig. 2C&D). In
7 the case of xylose, the lactic acid concentrations were approximately 3-fold higher than
8 those for P(3HB) (Fig. 2A&C). The excreted lactic acid was eventually utilized (Fig.
9 2C&D). However, the uptake of lactic acid by the *E. coli* JW0885 did not contribute to
10 the yield of LA units in the P(LA-co-3HB) because the yields of LA units stagnated
11 from 20 h after inoculation till the end of culturing (Fig. 2C-F) for both xylose and
12 glucose. These results demonstrated that LPE increased the flux towards lactic acid and
13 LA units in the copolymer, and consequently accelerated the glycolytic pathway, and
14 that xylose was converted into LA units more efficiently than glucose. Overall, the final
15 carbon yield of the polymers from xylose and glucose were 310 and 331 mM,
16 respectively.

17 Interestingly, 3-hydroxybutyric acid was detected in the medium at 28 - 48 h
18 during the course of P(LA-co-3HB) production from both xylose and glucose using

1 PhaC1_{Ps}(ST/QK) (Fig. 2C&D). The 3-hydroxybutyric acid levels increased up to 42 and
2 50 mM carbon yield for xylose and glucose (1.1 and 1.3 g l⁻¹, respectively) at the end of
3 the culture. PCT may have transferred a CoA moiety from 3HB-CoA to acetic acid so
4 as to release 3-hydroxybutyric acid, as previously reported (Matsumoto et al., 2012).
5 The production of 3-hydroxybutyric acid and the synthesis and uptake of lactic acid
6 from the media had a strong correlation of the pH of the media (Fig. 3E).

7

8 ***3.3.3 PhaC1_{Ps}ST/FS/QK channeled the carbon flux from the 3HB units to the LA*** 9 ***units***

10 The attempt to increase the flux toward the LA units using PhaC1_{Ps}(ST/FS/QK)
11 revealed its interesting effects on the sugar consumption, cell growth and polymer
12 synthesis (Fig. 2E&F; Fig. 3C). In the 6 - 20 h interval after inoculation, the sugar
13 consumption rates (Fig. 2E&F) were lower than those observed during P(LA-co-3HB)
14 production by PhaC1_{Ps}(ST/QK) over the same time period (Fig. 2C&D), that was also
15 reflected in the slower polymer synthesis and cell growth (Fig. 3C). It should be noted
16 that the low polymer production rate at the early stage (6 - 14 h) was partly due to the
17 low accumulating rate of 3HB units in P(LA-co-3HB) (Fig. 2C&D). These phenomena
18 allowed cells to utilize more sugars to produce LA units in the middle stage (14 - 28 h),

1 resulting in a higher LA units yield compared to PhaC1_{Ps}(ST/QK). Surprisingly, the
2 xylose utilization and polymer synthesis in the later stage was faster than glucose,
3 achieving a higher LA units yield from xylose. Unlike the case of PhaC1_{Ps}(ST/QK),
4 3-hydroxybutyric acid was not detected for either xylose or glucose. The change in pH
5 was associated with lactic acid excretion and uptake (Fig. 2E; Fig. 3F). The final carbon
6 yield of the polymers from xylose and glucose were 348 and 372 mM, respectively.
7 Notably, the use of PhaC1_{Ps}(ST/FS/QK) did not greatly alter the total carbon yield of
8 the polymer and organic acids from sugars, but remarkably increased the yield of LA
9 units in the copolymer.

10

11 ***3.3.4 Nicotinamide nucleotide cofactor levels during polymer productions***

12 The results shown in Fig. 2E&F indicate that xylose and glucose were converted
13 into copolymers with a similar carbon yield but with different LA/3HB ratio. A possible
14 factor affecting the monomer composition might be the supply of nicotinamide
15 cofactors, because the synthesis of lactic acid from pyruvate is NADH-dependent,
16 whereas the 3HB-CoA supply pathway uses NADPH (Fig. 1). To date, there is no report
17 on the reducing cofactor levels during P(LA-co-3HB) production. Therefore, the
18 [NADPH]/[NADP⁺] and [NADH]/[NAD⁺] levels were determined during the

1 production of P(LA-*co*-3HB) and P(3HB) (Fig. 3G, H&I; the complete data is shown in
2 Table S1 A, B&C).

3 During the P(3HB) production, the [NADPH]/[NADP⁺] ratios at 14 h, the time
4 point at which the polymer synthesis rate was maximum, were similar between xylose
5 (1.0) and glucose (1.1; Fig. 3G). Likewise, during the synthesis of P(LA-*co*-3HB) by
6 PhaC1_{Ps}(ST/QK), the difference in the [NADPH]/[NADP⁺] ratios for xylose (1.2) and
7 glucose (1.2) were insignificant (Fig. 3H). In the case of the production of
8 P(LA-*co*-3HB) using PhaC1_{Ps}(ST/FS/QK), the [NADPH]/[NADP⁺] ratio for glucose
9 (1.7) was slightly higher than that for xylose (1.2; Fig. 3I). Overall, the
10 [NADPH]/[NADP⁺] ratios tended to be slightly higher under the low 3HB-accumulating
11 conditions, perhaps because NADPH was consumed for the synthesis 3HB-CoA. On the
12 other hand, the [NADH]/[NAD⁺] ratios in the cells growing on glucose were higher or
13 at similar levels to those for xylose (Fig. 2H). This result could be due to the fact that
14 the higher productions of lactic acid and LA units in the copolymer from xylose
15 consumed more NADH compared to glucose.

16

17 ***3.4 The monomer composition and molecular weight of the polymers produced from***
18 ***xylose***

1 To analyze the structure of the P(LA-*co*-3HB)s synthesized from xylose, the
2 copolymers were subjected to ¹H NMR (Fig.S3). The results show that the polymers
3 were random copolymers made up of LA and 3HB units, and this is in good agreement
4 with earlier report (Yamada et al., 2009). The molecular weight (M_n) of the polymers
5 varied in the range of 1.2 to 5.6×10^4 , depending on their LA fraction (Table 2). In
6 particular, the P(LA-*co*-3HB)s synthesized from xylose or glucose using
7 PhaC1_{PS}(ST/FS/QK) had lower molecular weight compared to those by PhaC1_{PS}
8 (ST/QK) (Table 2). Thus, there was an inverse relationship between the molecular
9 weight and the LA fraction that was consistent with earlier reports (Shozui et al., 2011;
10 Song et al., 2012; Yamada et al., 2011). This phenomenon may be due to the fact that
11 D-lactyl-CoA is still not the preferred substrate for LPE thus causing chain termination
12 before high molecular weight polymers are synthesized. However, studies are underway
13 to clarify this issue. These results, taken together, indicate that the use of xylose for
14 P(LA-*co*-3HB) production has no particular effect on the polymer structure compared to
15 glucose, except for the higher LA fraction.

16

17 ***4 Discussion***

18 In this study, it has been demonstrated for the first time that P(LA-*co*-3HB) can

1 be synthesized from xylose. The advantage of xylose utilization is justified by the fact
2 that the cellular content and LA fraction of the copolymer were compatible and higher,
3 respectively, compared to glucose utilization. However, in terms of P(3HB) content,
4 glucose gave a much higher polymer yield than xylose (da Silva et al., 2009; Keenan et
5 al., 2004; Lee, 1998; Silva et al., 2004; Young et al., 1994). This contrasting result
6 between the two polymers should be ascribed to the capacity for the supply of the LA
7 and 3HB units, which are generated in the pathways for the metabolisms of both carbon
8 sources. Considering the fact that xylose and glucose are both metabolized into pyruvate,
9 the NADPH obtained through the metabolism of the sugars (Fig. 1) would be a key
10 factor affecting the monomer fluxes channeled to the polymer, since the PhaB that
11 generates 3HB-CoA is dependent on NADPH for its activity (Chemler et al., 2010;
12 Peoples and Sinskey, 1989; Tyo et al., 2010). Although the $[NADPH]/[NADP^+]$ ratios
13 during the polymer productions were not evidently different between xylose and
14 glucose (Fig. 3G, H&I), it has been reported that the carbon flux in the *E. coli* grown on
15 xylose can be drawn into the TCA cycle to generate NADPH (Chin et al., 2009; Lim et
16 al., 2002; Shi et al., 1999). Therefore, the high demand for NADPH in synthesizing
17 3HB-CoA may decrease the overall carbon yield from xylose into the 3HB units
18 compared to the case with glucose and in contrast, the channeling of the flux from 3HB

1 to the LA units can drastically increase the polymer yield from xylose. In brief, the
2 synthesis of LA-enriched copolymers from xylose compared to glucose could be as a
3 result of a high LA units supplying rate from xylose than glucose which is presumably
4 contributed by the NADPH and NADH regeneration capacity due to different
5 metabolism routes of the two sugars (Fig. 1).

6 For the efficient production of LA-enriched polyester, the synthesis of LA-CoA is
7 also an important factor. LA-CoA is synthesized by the CoA-transferring reaction
8 carried out by the PCT from lactic acid and presumably acetyl-CoA acting as a CoA
9 donor, generating a stoichiometric amount of acetic acid (Fig.1). This reaction would be
10 expected to reduce the overall carbon flux for polymer synthesis. However, the
11 excretion of acetic acid into the medium was found to be very low; less than 0.1 g l^{-1}
12 (Fig.S3) compared to typical cases of *E. coli* grown on sugars (Shen and Liao, 2008;
13 Zhou et al., 2011). This suggests that acetic acid might be recycled intracellularly into
14 acetyl-CoA, and therefore, the PCT-catalyzed LA-CoA supply route may not be a
15 limiting factor in obtaining high polymer yields.

16 To reinforce the carbon fluxes from LA-CoA to the LA units in the copolymer, we
17 recruited an engineered LPE [PhaC1_{ps}(ST/FS/QK)] that was shown to give a higher LA
18 fraction in a previous study (Yamada et al., 2010). The difference between the two LPEs

1 was evident in the polymer production at the early stage (6 - 14 h), that is,
2 PhaC1_{Ps}(ST/QK) accumulated more 3HB units, while PhaC1_{Ps}(ST/FS/QK) incorporated
3 relatively higher LA units into the copolymer (Fig. 2). This suggests a relatively higher
4 reactivity of PhaC1_{Ps}(ST/FS/QK) toward LA-CoA than 3HB-CoA compared to the
5 reactivity of PhaC1_{Ps}(ST/QK). In addition, the use of PhaC1_{Ps}(ST/FS/QK) significantly
6 changed the ratio of the LA units over the 3HB units in the copolymers, while it slightly
7 increased the overall carbon yield from the sugars into the polymer. Thus, LPE
8 contributes to the relative flux channeling to the LA and 3HB units rather than the
9 absolute polymer yield. Therefore, in order to increase the LA unit yields in
10 P(LA-*co*-3HB), further engineering of the LPE to shift substrate specificity to LA-CoA,
11 as well as the related cases discussed by Taguchi et al. (Taguchi and Doi, 2004) and/or
12 the suppression of 3HB-CoA supply pathway, will be necessary.

13 Cell growth was shown to vary among plasmids although the cells were grown
14 under the same culture conditions (Fig. 3). This is probably due to the different
15 accumulation rates of P(LA-*co*-3HB)s and P(3HB), which contributed to the cell dry
16 weight/cell mass.

17 In this study, the dual merits of the utilization of xylose for P(LA-*co*-3HB) has
18 been demonstrated in terms of both quantity and quality. Although the utilization of

1 xylose for microbial conversions has attracted considerable research interest, the
2 conversion efficiency of xylose into PHAs has been demonstrated to be lower than that
3 of glucose. Thus, the findings in this study show that P(LA-*co*-3HB) is a promising
4 target for the utilization of xylose. Another interesting finding in this work was the
5 enrichment of the LA units in the copolymer that resulted from the changing the
6 non-related carbon sources. To the best of our knowledge, the regulation of monomer
7 composition in PHAs by feedstock has only been reported for the related carbon sources.
8 As an example, 1,3-propanediol and 1,4-butanediol were used as precursors for
9 3-hydroxypropionate and 4-hydroxybutyrate units formation, respectively (Meng et al.,
10 2012).

11

12 ***5 Conclusions***

13 The production of P(LA-*co*-3HB) from xylose has been demonstrated here for
14 the first time. Xylose gave a higher LA fraction (60 mol%) in P(LA-*co*-3HB) than
15 glucose and the polymer yield from the two sugars was comparable. A time course
16 analysis of polymer and organic acids synthesis provided a reliable and facile method of
17 monitoring the metabolic fluxes. Since xylose makes up a substantial portion of the
18 lignocellulosic biomass and can be obtained easily as xylose-rich hemicellulose

1 hydrolysates, this system is expected to open an avenue for the cost-effective production
2 of P(LA-co-3HB)s.

3

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8 Innovation in Materials Science) from the Ministry of Education, Culture, Sports,
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1 **Table 1** P(LA-co-3HB) and P(3HB) productions from xylose and glucose in *E. coli*

2 JW0885 expressing PhaC1_{Ps}(ST/QK)(No. 1- 4) and P(LA-co-3HB) production in *E. coli*

3 JW0885 expressing PhaC1_{Ps}(ST/FS/QK) (No. 5&6).

No.	relevant genes	carbon source	cell dry weight (g l ⁻¹)	polymer yield (g l ⁻¹)			Y _{Pol/C} (g g ⁻¹)	polymer content (%)	LA fraction (mol%)
				Total	LA	3HB			
1	<i>phaC1</i> _{Ps} (<i>STQK</i>), <i>phaAB</i>	xylose	7.0 ±0.2	4.1 ±0.3	0	4.1 ±0.3	0.21±0.01	59 ±4	0
2	<i>phaC1</i> _{Ps} (<i>STQK</i>), <i>phaAB</i>	glucose	9.3 ±0.2	5.7 ±0.6	0	5.7 ±0.6	0.29±0.03	61 ±5	0
3	<i>pct</i> , <i>phaC1</i> _{Ps} (<i>STQK</i>), <i>phaAB</i>	xylose	9.0 ±0.6	5.5 ±0.2	1.7±0.1	3.8 ±0.1	0.28±0.01	61 ±1	34 ±2
4	<i>pct</i> , <i>phaC1</i> _{Ps} (<i>STQK</i>), <i>phaAB</i>	glucose	10.4±0.4	6.5 ±0.6	1.5±0.1	5.0 ±0.4	0.33±0.03	62 ±5	26 ±6
5	<i>pct</i> , <i>phaC1</i> _{Ps} (<i>STFSQK</i>), <i>phaAB</i>	xylose	10.4±0.6	7.3±0.2	4.1±0.2	3.2±0.0	0.37±0.01	70±1	60±3
6	<i>pct</i> , <i>phaC1</i> _{Ps} (<i>STFSQK</i>), <i>phaAB</i>	glucose	9.7±0.4	7.9±0.6	3.4±0.1	4.5±0.1	0.39±0.03	81±5	47±2

4 Cells were grown on 1.7 mL LB medium containing 20 g l⁻¹ of either xylose or glucose at 30° C for 48 h.

5 Y_{Pol/C}: Sugar specific yield, amount of polymer in g produced per g of sugar supplied for polymer

6 production. Data is average ± standard deviation of quadruplicate experiments.

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1 **Table 2** Molecular weights of P(3HB) and P(LA-co-3HB) synthesized using *E. coli*
 2 JW0885 expressing PhaC1_{PS}(ST/QK) or PhaC1_{PS}(ST/FS/QK).

No.	carbon source	polymer	M_n ($\times 10^4$)	M_w ($\times 10^4$)	M_w/M_n
1	xylose	P(3HB)	4.6	39	8.5
2	glucose	P(3HB)	5.6	37	6.5
3	xylose	P(34 mol% LA-co-3HB)	4.0	17	4.1
4	glucose	P(26 mol% LA-co-3HB)	4.2	33	7.8
5	xylose	P(60 mol% LA-co-3HB)	1.2	1.4	1.2
6	glucose	P(47 mol% LA-co-3HB)	1.2	1.4	1.2

3 The experimental numbers refer the culture conditions in Table 1. M_n , number-average molecular weight;
 4 M_w , weight-average molecular weight. M_w/M_n , polydispersity index.

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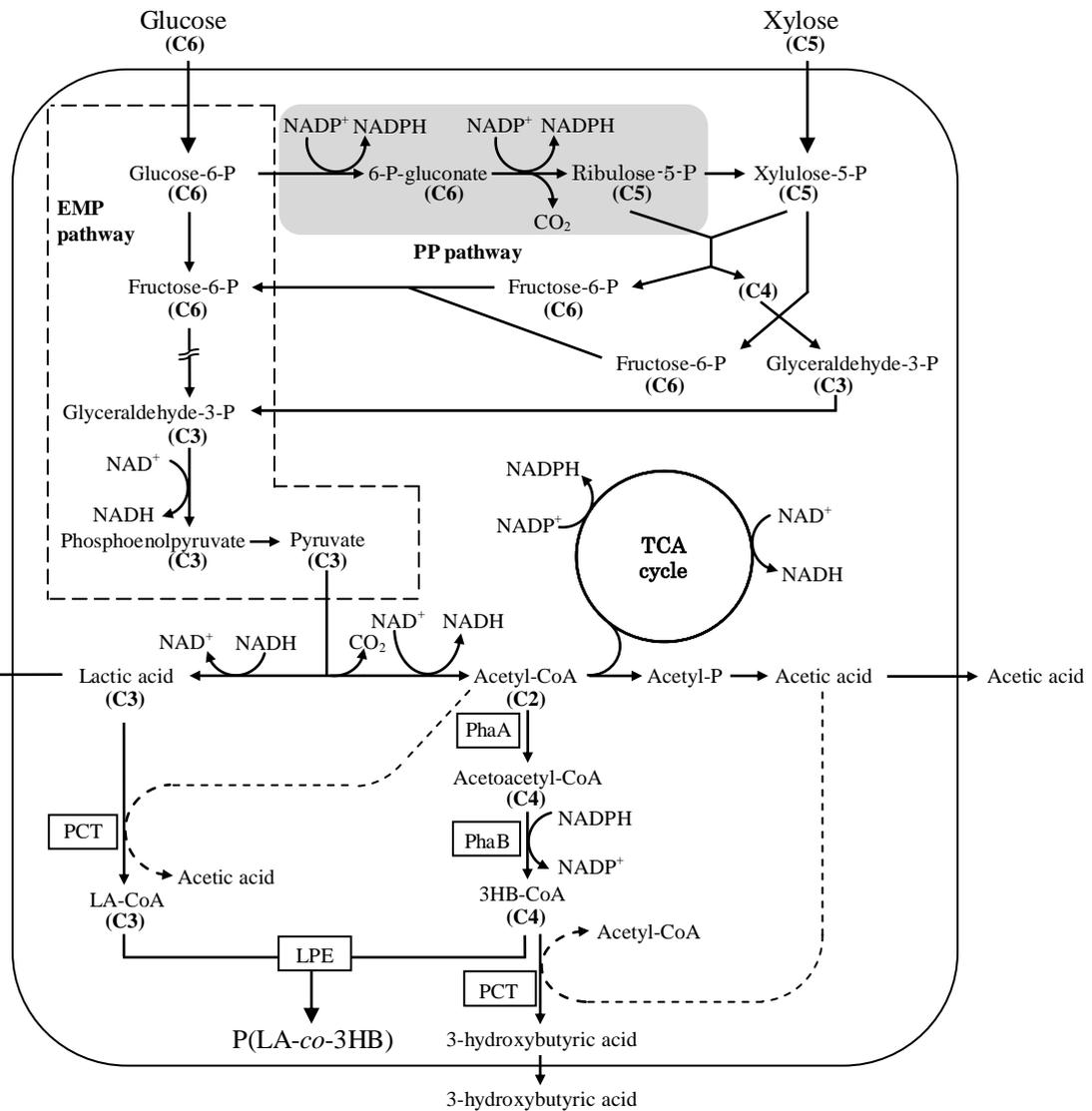


Fig. 1 Metabolic pathways in *E. coli* for xylose and glucose metabolism, and the production of P(LA-co-3HB) and related organic acids. EMP pathway, Embden-Meyerhof-Parnas pathway (highlighted by dashed box); PP pathway, pentose phosphate pathway (oxidative branch is highlighted in gray). The enzymes in the boxes

1 were heterologously expressed and are involved in P(LA-co-3HB) synthesis. LPE,
2 lactate polymerizing enzyme [PhaC1_{Ps}(ST/QK) or PhaC1_{Ps}(ST/FS/QK)]; PCT,
3 propionyl-CoA transferase; PhaA, β -kethothiolase; PhaB, NADPH-dependent
4 acetoacetyl-CoA reductase. Removal of PCT from the pathway leads to the production
5 of P(3HB). The dashed arrows indicate proposed pathways catalyzed by PCT.

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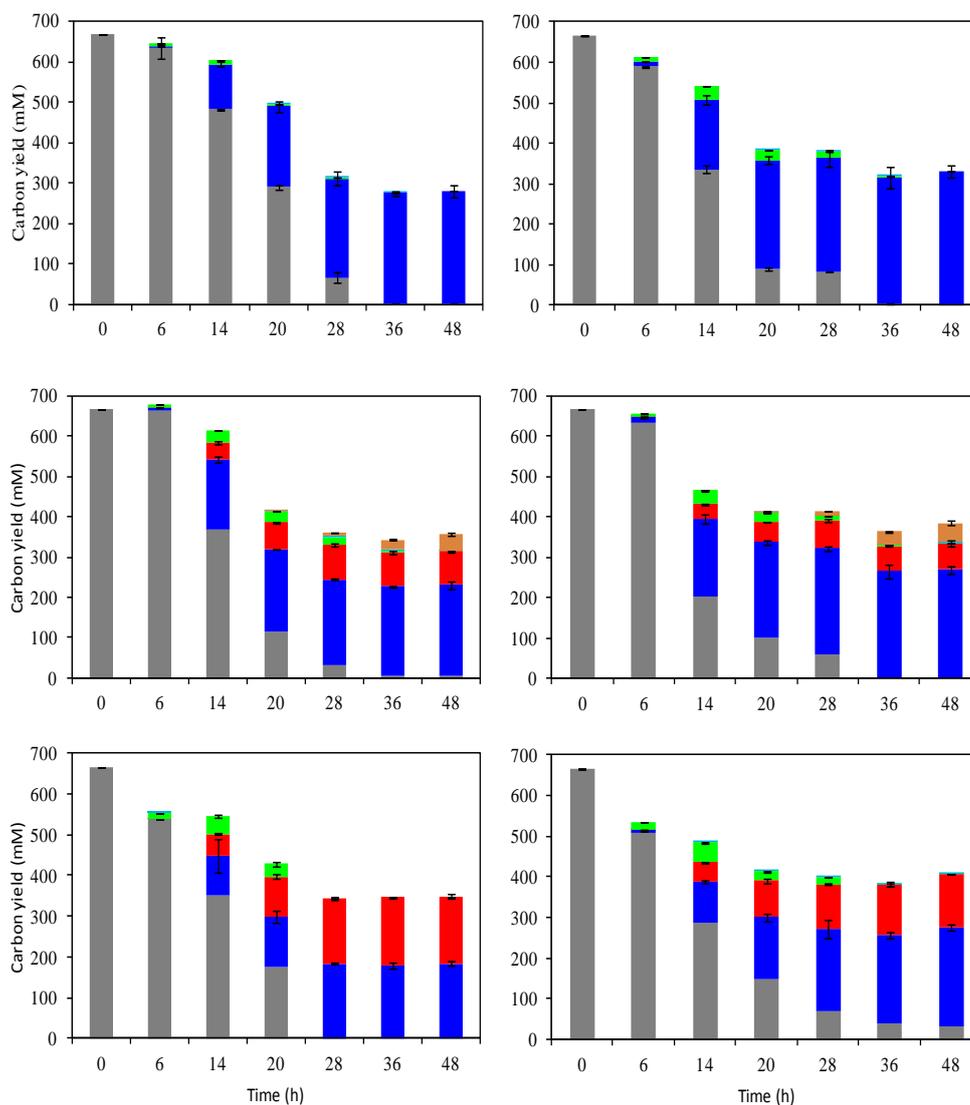
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3 **Fig. 2** Time course profiles of molar carbon amount for xylose and glucose and the
4 metabolites produced in *E. coli* JW0885. Cells harboring pGEMphaCI_{Ps}(ST/QK)AB
5 (A& B), pTV118NpctphaCI_{Ps}(ST/QK)AB (C&D) and
6 pTV118NpctphaCI_{Ps}(ST/FS/QK)AB (E&F) were grown on 100 mL media containing
7 xylose (A, C&E) and glucose (B, D&F) in shake flasks. Gray, residual sugars; blue,

1 3HB units in the polymers; red, LA units in the polymers; green, lactic acid in the
2 medium; purple, 3-hydroxybutyric acid in the medium; black, acetic acid. The acetic
3 acid concentrations were very low, so they are presented in a separate figure (Fig.S3).
4 The data are averages \pm standard deviations of three independent trials. Time 0 (zero)
5 indicates the time when the cells were inoculated.

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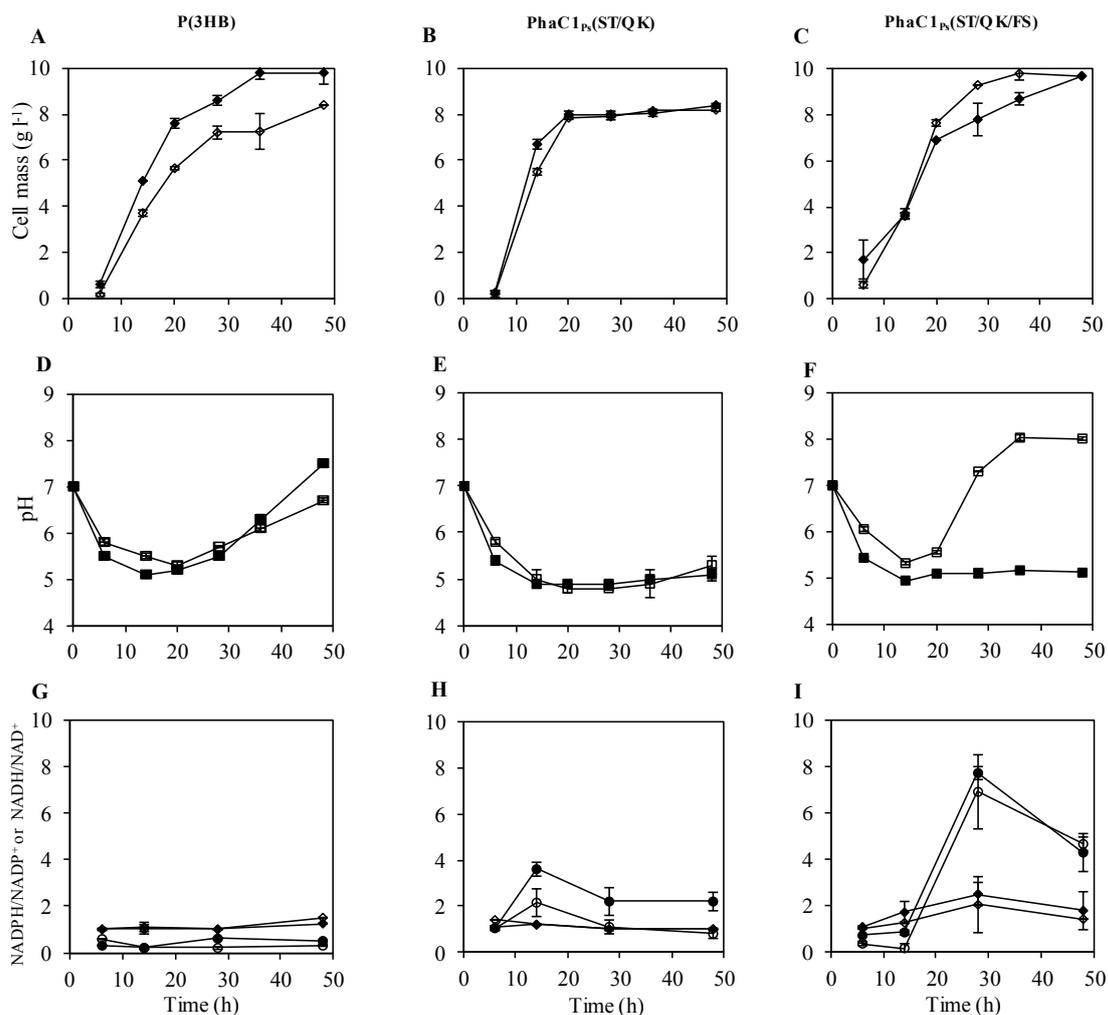
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2 **Fig. 3** Time course profiles of *E. coli* JW0885 expressing PhaC1_{Ps}(ST/QK) for P(3HB)

3 synthesis (A, D&G), PhaC1_{Ps}(ST/QK) and PCT for P(LA-co-3HB) synthesis (B, E&H)

4 or (PhaC1_{Ps}(ST/FS/QK) and PCT for P(LA-co-3HB) synthesis (C, F&I) from xylose

5 (open symbols) or glucose (closed symbols). A, B&C; cell mass (g l⁻¹). D, E&F;

6 medium pH. G, H&I; NADPH/NADP⁺ (diamond symbols) and NADH/NAD⁺ ratios

7 (circles). The data shown are averages ± standard deviations of three independent trials.

8 Time 0 (zero) indicates the time when the cells were inoculated.

1 **Supplementary**

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3 **Table S1 A** Cofactor levels ($\mu\text{M g}^{-1}_{\text{CDW}}$) and ratios during the production of P(3HB)
 4 from glucose and xylose by *E. coli* JW0885 cells expressing PhaC1_{Ps}(ST/QK).

time (h)	Sugar	NADPH ($\mu\text{M g}^{-1}_{\text{CDW}}$)	NADP ⁺ ($\mu\text{M g}^{-1}_{\text{CDW}}$)	NADH ($\mu\text{M g}^{-1}_{\text{CDW}}$)	NAD ⁺ ($\mu\text{M g}^{-1}_{\text{CDW}}$)	NADPH/ NADP ⁺	NADH/ NAD ⁺
6	glucose	2.40±0.03	2.50±0.60	2.80±0.50	9.60±2.50	1.00±0.05	0.30±0.02
	xylose	2.60±0.30	2.60±0.20	4.60±0.20	8.00±2.90	1.00±0.00	0.58±0.02
14	glucose	1.10±0.20	1.00±0.01	0.96±0.12	5.00±0.60	1.10±0.20	0.20±0.05
	xylose	1.50±0.03	1.50±0.10	1.44±0.40	7.20±1.60	1.00±0.20	0.20±0.05
28	glucose	0.60±0.03	0.60±0.03	0.74±0.05	1.20±0.20	1.00±0.01	0.60±0.05
	xylose	0.70±0.01	0.70±0.01	0.18±0.02	0.90±0.10	1.00±0.03	0.20±0.06
48	glucose	0.75±0.06	0.60±0.05	0.33±0.07	0.70±0.20	1.25±0.00	0.50±0.04
	xylose	0.75±0.07	0.50±0.03	0.07±0.03	0.24±0.10	1.50±0.01	0.30±0.03

5 The standard deviations are from duplicate measurements

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7 **S1B** Cofactor levels ($\mu\text{M g}^{-1}_{\text{CDW}}$) and ratios during the production of P(LA-co-3HB)
 8 from glucose and xylose by *E. coli* JW0885 cells expressing PhaC1_{Ps}(ST/QK).

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time (h)	Sugar	NADPH ($\mu\text{M g}^{-1}_{\text{CDW}}$)	NADP ⁺ ($\mu\text{M g}^{-1}_{\text{CDW}}$)	NADH ($\mu\text{M g}^{-1}_{\text{CDW}}$)	NAD ⁺ ($\mu\text{M g}^{-1}_{\text{CDW}}$)	NADPH/ NADP ⁺	NADH/ NAD ⁺
6	glucose	2.00±0.00	1.80±0.05	2.10±0.20	2.00±0.30	1.10±0.03	1.05±0.10
	xylose	2.50±0.02	1.80±0.05	0.70±0.10	2.00±0.40	1.40±0.01	0.35±0.10
14	glucose	1.00±0.07	0.85±0.08	5.60±0.50	1.55±0.20	1.20±0.03	3.60±0.30
	xylose	1.10±0.20	0.90±0.10	5.80±0.60	2.60±0.80	1.20±0.01	2.15±0.60
28	glucose	0.70±0.02	0.70±0.02	0.65±0.15	0.30±0.15	1.00±0.06	2.20±0.60
	xylose	0.70±0.00	0.70±0.00	0.75±0.00	0.60±0.20	1.00±0.00	1.10±0.30
48	glucose	0.74±0.02	0.70±0.02	0.22±0.08	0.10±0.00	1.00±0.05	2.20±0.40
	xylose	0.74±0.03	0.70±0.03	0.25±0.11	0.30±0.06	1.00±0.04	0.80±0.20

10 The standard deviations are from duplicate measurements

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2 **S1C** Cofactor levels ($\mu\text{M g}^{-1}_{\text{CDW}}$) and ratios during the production of P(LA-co-3HB)
3 from glucose and xylose by *E. coli* JW0885 cells expressing PhaC1_{PS}(ST/FS/QK).

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time (h)	Sugar	NADPH ($\mu\text{M g}^{-1}_{\text{CDW}}$)	NADP ⁺ ($\mu\text{M g}^{-1}_{\text{CDW}}$)	NADH ($\mu\text{M g}^{-1}_{\text{CDW}}$)	NAD ⁺ ($\mu\text{M g}^{-1}_{\text{CDW}}$)	NADPH/ NADP ⁺	NADH/ NAD ⁺
6	glucose	1.86±0.12	1.75±0.18	3.05±0.07	4.49±0.70	1.06±0.04	0.69±0.09
	xylose	1.98±0.06	1.98±0.01	2.72±2.66	7.84±0.27	1.00±0.04	0.34±0.33
14	glucose	0.24±0.04	0.14±0.02	0.69±0.27	0.82±0.12	1.69±0.47	0.82±0.20
	xylose	0.17±0.02	0.14±0.02	0.37±0.06	3.63±2.32	1.24±0.03	0.14±0.10
28	glucose	0.14±0.03	0.06±0.00	2.67±0.03	0.35±0.07	2.48±0.5	7.70±1.60
	xylose	0.13±0.08	0.06±0.00	3.02±0.44	0.44±0.05	2.02±1.21	6.89±0.28
48	glucose	0.09±0.04	0.05±0.00	12.24±0.3	2.87±0.13	1.76±0.82	4.27±0.30
	xylose	0.08±0.00	0.06±0.00	6.91±1.80	1.68±0.63	1.4±0.00	4.64±2.82

5 The standard deviations are from duplicate measurements

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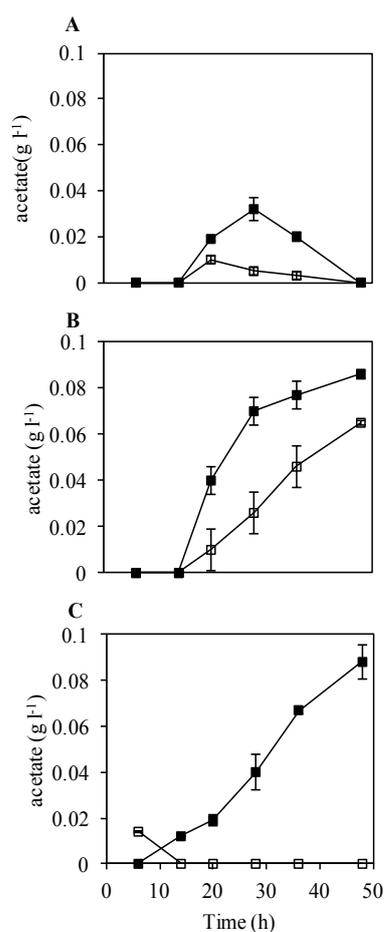
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2 **Fig. S1** Time course profiles of acetic acid (g l⁻¹) produced during P(3HB) synthesis (A)

3 and P(LA-co-3HB) production by PhaC1_{Ps}(ST/QK) and PCT (B) or

4 PhaC1_{Ps}(ST/FS/QK) and PCT (C). The data shown are means \pm standard deviations of

5 quadruplicate cultures of *E. coli* JW0885.

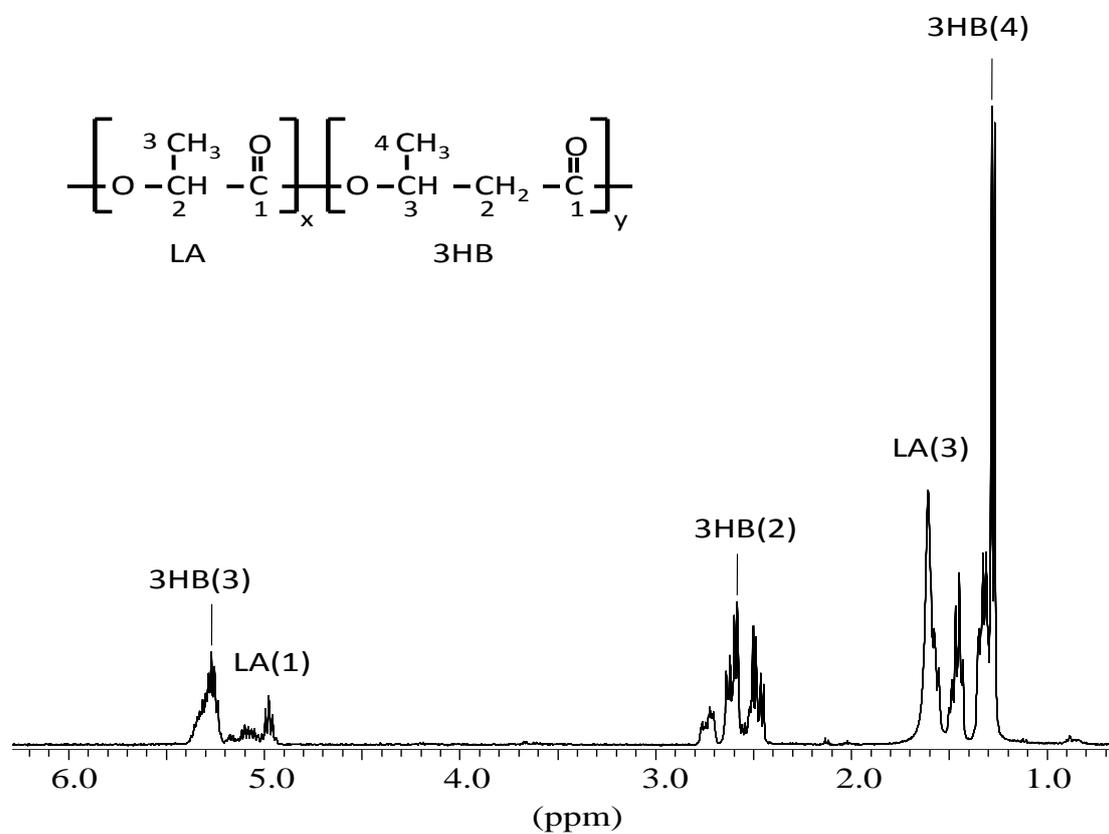
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3 **Fig. S2** ¹H NMR spectra of P(34 mol% LA-co-3HB) produced from xylose. The ¹H
4 NMR chemical shifts around 1.5 and 5.0 ppm were assigned to the methyl and methine
5 groups of the LA unit within the main chain of the copolymer as described previously
6 (Yamada et al., 2010).

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