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Author(s)	Nduko, John Masani; Matsumoto, Ken'ichiro; Ooi, Toshihiko et al.
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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

John Masani Nduko[a], Ken' ichiro Matsumoto[a, b]* Toshihiko Ooi[a, b]

and Seiichi Taguchi[a, b]*

[a]Division of Biotechnology and Macromolecular Chemistry, Graduate School of Engineering, Hokkaido University, N13-W8, Kita-ku, Sapporo 060-8628, Japan.

[b]JST, CREST, Sanbancho, Chiyoda-ku, Tokyo, Japan

*Corresponding authors:
Ken'ichiro Matsumoto, Tel & Fax: +81-11-706-6612. E-mail: mken@eng.hokudai.ac.jp
Seiichi Taguchi, Tel & Fax: +81-11-706-6610, E-mail: staguchi@eng.hokudai.ac.jp

Abstract

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

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

1. Introduction

Poly(lactate-*co*-3-hydroxybutyrate) [P(LA-*co*-3HB)] is a newly recognized member of the bacterial polyhydroxyalkanoate(PHA) family that is synthesized using a PHA synthase possessing an acquired LA-polymerizing activity (Matsumoto and Taguchi, 2010; Taguchi et al., 2008). P(LA-*co*-3HB) was produced from glucose in recombinant *Escherichia coli* expressing a Ser325Thr/Gln481Lys mutant of PHA synthase from *Pseudomonas* sp. 61-3 [PhaC1$_{Ps}$(ST/QK)] as an LA-polymerizing enzyme (LPE), together with a propionyl-CoA transferase (PCT) from *Megasphaera elsdenii* and 3HB monomer supplying enzymes [β-ketothiolase (PhaA) and NADPH-dependent acetoacetyl-CoA reductase (PhaB)] from *Ralstonia eutropha* (Taguchi et al., 2008).

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

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

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

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

host for the production of LA-based polymers. During the course of xylose utilization experiments, we unexpectedly found that xylose gave a higher LA yield and fraction in P(LA-*co*-3HB) compared to glucose. This finding prompted us to further increase the productivity and LA fraction of P(LA-*co*-3HB) from xylose by enhancing the flux towards the LA units in the polymer using an evolved LPE, PhaC1$_{Ps}$(ST/FS/QK) (Yamada et al., 2009). The effectiveness of these trials will be discussed on the basis of the findings related to the carbon yield of the polymers and excreted organic acids, thereby revealing the high yield conversion of xylose into P(LA-*co*-3HB) with high LA fraction. Thus, this paper proposes the LA-based copolymer as a new target of xylose utilization for the production of value-added materials.

2. Materials and methods

2. 1. Bacterial strains and plasmids

The Keio collection strain *E. coli* JW0885 (*pflA*$^-$) (purchased from National BioResource Project, Japan) (Baba et al., 2006), was used as a host for polymer production. The expression vector pTV118N*pctphaC1*$_{Ps}$(ST/QK)*AB,* which harbors the *pct*, *phaC1*$_{Ps}$(ST/QK), *phaA,* and *phaB* genes (Taguchi et al., 2008), was used for the production of LA-based polyesters. Additionally, the recombinant cells harboring

pTV118N*pctphaC1*$_{Ps}$(ST/FS/QK)*AB* with a Ser325Thr/Phe392Ser/Gln481Lys mutated PHA synthase gene from *Pseudomonas* sp. 61-3 (Yamada et al., 2010) were used for the production of high LA-containing polymers. The pGEM*phaC1*$_{Ps}$(ST/QK)*AB* (Takase et al., 2003) was used for P(3HB) production.

2.2 Culture conditions

2.2.1 Test-tube cultures

Recombinant *E. coli* cells harboring either pTV118N*pctphaC1*$_{Ps}$(ST/QK)*AB* or pTV118N*pctphaC1*$_{Ps}$(ST/FS/QK)*AB* were grown in 10 mL glass test-tubes on Luria-Bertani (LB) medium (1.7 mL) containing 20 g l^{-1} xylose or glucose, 100 μg l^{-1} ampicillin and 10 mM calcium pantothenate at 30°C for 48 hours for the production of P(LA-*co*-3HB). Recombinant *E. coli* cells harboring pGEM*phaC1*$_{Ps}$(ST/QK)*AB* were similarly prepared for the production of P(3HB). To test the effect of xylose concentrations on polymer yield and LA fractions, recombinant cells harboring pTV118N*pctphaC1*$_{Ps}$(ST/QK)*AB* were prepared in a similar manner with varying concentrations of xylose (5 - 100 g l^{-1}).

2.2.2 Shake flask cultures

To produce P(LA-*co*-3HB) in shake flasks, seed cultures of recombinant *E. coli* cells harboring pTV118N*pctphaC1*$_{Ps}$(ST/QK)*AB* or pTV118N*pctphaC1*$_{Ps}$(ST/FS/QK)*AB* were prepared using 2 mL LB medium containing 100 μg l^{-1} ampicillin in 10 mL glass test-tubes and cultured at 30°C for 12 h with reciprocal shaking at 180 rpm. One milliliter of the seed culture was then transferred into 100 mL LB medium containing 100 μg l^{-1} ampicillin, 20 g l^{-1} xylose or glucose and 10 mM calcium pantothenate in a 500 mL shake flask and cultured at 30°C for 48 h with reciprocal shaking at 120 rpm. Recombinant *E. coli* JW0885 harboring pGEM*phaC1*$_{Ps}$(ST/QK)*AB* were cultured similarly for P(3HB) production.

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

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

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

2.4 Determination of the intracellular cofactor levels during polymer production.

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

2.5 Polymer extraction and analyses

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

the precipitation, which increased the recovery of the polymer. The precipitant was dried *in vacuo* and the polymer content was calculated based on the cell dry weight. The monomer composition of P(LA-*co*-3HB) was determined by HPLC as described previously (Yamada et al., 2009). The molecular weights of the extracted polymers were determined by gel permeation chromatography (GPC, JUSCO, Japan) equipped with a Shodex GPC KF-805 column (Showa Denko K.K., Japan) using polystyrene standards (Waters, USA) for calibration (Taguchi et al., 2008). Five milligrams of the extracted P(LA-*co*-3HB) copolymer was dissolved in 1 mL of $CDCl_3$ (Wako Pure Chemical Industries ltd, Japan) and used for ^1H NMR analysis by a Bruker MSL400 spectrometer (400 MHz).

3 Results

3.1 P(LA-co-3HB) production from xylose using the metabolically engineered E. coli *JW0885*

To investigate the effect of sugars on PHA production, P(3HB) was produced in recombinant *E. coli* JW0885 harboring pGEM*phaC1*$_{Ps}$(ST/QK)*AB*. The P(3HB) yield using cells cultivated on xylose was 4.1 g l^{-1}, while 1.4-fold higher production (5.7 g l^{-1}) was obtained from glucose (Table 1, No. 1&2), which is consistent with previously

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

Next, the biosynthesis of P(LA-*co*-3HB) from xylose was attempted using the recombinant *E. coli* JW0885 harboring the pTV118N*pctphaC1*$_{Ps}$(ST/QK)*AB*. First, the effect of xylose concentration on polymer yields and LA fractions was analyzed and results are shown in Fig. S1A&B. We then chose 20 g l^{-1} for subsequent studies since this concentration gave the highest LA fraction in the copolymers. The recombinant strain cultivated on 20 g l^{-1} xylose produced 5.5 g l^{-1} of P(34 mol% LA-*co*-3HB). In contrast, this yield was lower than that obtained from 20 g l^{-1} glucose (6.5 g l^{-1}) (Table 1, No. 3&4). In this case, focusing on LA incorporation, the yield of the LA units (1.7 g l^{-1}) and the LA fraction (34 mol%) in the P(LA-*co*-3HB) obtained from xylose were higher than those from glucose (1.5 g $l^{-1}$and 26 mol% LA), indicating the effectiveness of xylose utilization in obtaining LA-enriched polyesters. For both xylose and glucose cases, the P(3HB) homopolymer yields were similar to the respective 3HB unit yields in P(LA-*co*-3HB)s (Table 1). Thus, the higher yields of P(LA-*co*-3HB) than P(3HB) were mainly due to the incorporation of LA into the polymers.

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

To further increase the incorporation of LA units in the polymer, we applied an evolved LPE, PhaC1$_{Ps}$(ST/FS/QK), which had been shown to synthesize P(LA-*co*-3HB) with a higher LA fraction than PhaC1$_{Ps}$(ST/QK) (Yamada et al., 2010). The use of PhaC1$_{Ps}$(ST/FS/QK) led to 2.5- and 2.3-fold increases in the LA unit yield in P(LA-*co*-3HB)s from xylose and glucose, respectively (Table 1, No. 5&6) compared to PhaC1$_{Ps}$(ST/QK) (Table 1, No. 3&4). Likewise, the LA fractions of P(LA-*co*-3HB)s synthesized using PhaC1$_{Ps}$(ST/FS/QK) were significantly higher compared to those using PhaC1$_{Ps}$(ST/QK) (Table 1, No. 5&6). The xylose-specific yield of P(LA-*co*-3HB) synthesized using PhaC1$_{Ps}$(ST/FS/QK) was 0.37 g g^{-1}, which was significantly higher than the highest xylose-specific yield for P(3HB) from xylose ever reported (0.26 g g^{-1}) (da Silva et al., 2009). Moreover, the use of PhaC1$_{Ps}$(ST/FS/QK) with xylose was effective than glucose in synthesizing LA-enriched P(60 mol% LA-*co*-3HB) with high polymer yields under aerobic conditions, which was far much efficient than the system that achieved similar LA fractions in the copolymers but characterized with low polymer yields under anaerobic conditions using glucose (Yamada et al., 2010; Yamada et al., 2011).

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

The aforementioned results suggest xylose to be superior to glucose as a carbon source for LA-based polyester production because of the higher LA fraction and LA unit yields. In order to gain further insights into the metabolic fluxes, we monitored the time course of the polymer production as well as the excretion of lactic acid, acetic acid and 3-hydroxybutyric acid, which were detected as major components in the medium (Fig. 2A-F). Formic acid and succinic acid were not detected, which is consistent with previous reported results of the *pflA* mutant strain (Zhu and Shimizu, 2005). The other metabolites such as ethanol and pyruvate were also not detected hence; their contribution to carbon yield from sugars was insignificant. The results are shown as the molar amount of carbon for each compound. For example, 10 mM lactic acid (C_3) corresponds to 30 mM carbon in a molar amount, which clearly demonstrates the conversions of carbon sources into the polymers and by-products. The monitoring of polymer synthesis was important for deducing the metabolic fluxes from the sugars. The cell growth and other parameters are shown in Fig. 3.

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

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

mM xylose and 111 mM glucose, respectively, each of which corresponds to 667 mM carbon molar amount. The conversion of xylose into P(3HB) occurred mostly during the 6 - 20 h interval after inoculation. The total amount of carbon decreased during the time period, indicating that the carbons in xylose were used for cell growth (Fig. 3A) and/or emitted as CO_2. The excreted amount of lactic acid was found to be low (Fig. 2A), and the acetic acid production was trace (Fig. S3A). Xylose was completely consumed at 36 h after inoculation and the excreted lactic acid was also utilized. The production and the consumption of lactic acid from the medium were in good agreement with the pH changes in the medium (Fig. 3D). Comparing these results with the glucose culture, as expected, glucose was consumed faster than xylose (Fig. 2B), and the production of P(3HB) and lactic acid in the glucose culture were also higher. The final carbon yield from glucose to P(3HB) (331 mM) was higher than that from xylose (280 mM), whereas the theoretical maximum is 446 mM.

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

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

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

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

PhaC1$_{Ps}$(ST/QK) (Fig. 2C&D). The 3-hydroxybutyric acid levels increased up to 42 and 50 mM carbon yield for xylose and glucose (1.1 and 1.3 g l^{-1}, respectively) at the end of the culture. PCT may have transferred a CoA moiety from 3HB-CoA to acetic acid so as to release 3-hydroxybutyric acid, as previously reported (Matsumoto et al., 2012). The production of 3-hydroxybutyric acid and the synthesis and uptake of lactic acid from the media had a strong correlation of the pH of the media (Fig. 3E).

3.3.3 PhaC1$_{Ps}$ST/FS/QK channeled the carbon flux from the 3HB units to the LA units

The attempt to increase the flux toward the LA units using PhaC1$_{Ps}$(ST/FS/QK) revealed its interesting effects on the sugar consumption, cell growth and polymer synthesis (Fig. 2E&F; Fig. 3C). In the 6 - 20 h interval after inoculation, the sugar consumption rates (Fig. 2E&F) were lower than those observed during P(LA-*co*-3HB) production by PhaC1$_{Ps}$(ST/QK) over the same time period (Fig. 2C&D), that was also reflected in the slower polymer synthesis and cell growth (Fig. 3C). It should be noted that the low polymer production rate at the early stage (6 - 14 h) was partly due to the low accumulating rate of 3HB units in P(LA-*co*-3HB) (Fig. 2C&D). These phenomena allowed cells to utilize more sugars to produce LA units in the middle stage (14 - 28 h),

resulting in a higher LA units yield compared to PhaC1$_{Ps}$(ST/QK). Surprisingly, the xylose utilization and polymer synthesis in the later stage was faster than glucose, achieving a higher LA units yield from xylose. Unlike the case of PhaC1$_{Ps}$(ST/QK), 3-hydroxybutyric acid was not detected for either xylose or glucose. The change in pH was associated with lactic acid excretion and uptake (Fig. 2E; Fig. 3F). The final carbon yield of the polymers from xylose and glucose were 348 and 372 mM, respectively. Notably, the use of PhaC1$_{Ps}$(ST/FS/QK) did not greatly alter the total carbon yield of the polymer and organic acids from sugars, but remarkably increased the yield of LA units in the copolymer.

3.3.4 Nicotinamide nucleotide cofactor levels during polymer productions

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

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

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

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

To analyze the structure of the P(LA-*co*-3HB)s synthesized from xylose, the copolymers were subjected to ^{1}H NMR (Fig.S3). The results show that the polymers were random copolymers made up of LA and 3HB units, and this is in good agreement with earlier report (Yamada et al., 2009). The molecular weight (M_n) of the polymers varied in the range of 1.2 to 5.6 $\times 10^4$, depending on their LA fraction (Table 2). In particular, the P(LA-*co*-3HB)s synthesized from xylose or glucose using PhaC1$_{Ps}$(ST/FS/QK) had lower molecular weight compared to those by PhaC1$_{Ps}$ (ST/QK) (Table 2). Thus, there was an inverse relationship between the molecular weight and the LA fraction that was consistent with earlier reports (Shozui et al., 2011; Song et al., 2012; Yamada et al., 2011). This phenomenon may be due to the fact that D-lactyl-CoA is still not the preferred substrate for LPE thus causing chain termination before high molecular weight polymers are synthesized. However, studies are underway to clarify this issue. These results, taken together, indicate that the use of xylose for P(LA-*co*-3HB) production has no particular effect on the polymer structure compared to glucose, except for the higher LA fraction.

4 Discussion

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

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

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

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

To reinforce the carbon fluxes from LA-CoA to the LA units in the copolymer, we recruited an engineered LPE [$PhaC1_{Ps}$(ST/FS/QK)] that was shown to give a higher LA 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

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

5 Conclusions

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

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

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Table 1 P(LA-*co*-3HB) and P(3HB) productions from xylose and glucose in *E. coli* JW0885 expressing PhaC1$_{Ps}$(ST/QK)(No. 1- 4) and P(LA-*co*-3HB) production in *E. coli* 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	*phaC1*$_{Ps}$*(STQK), phaAB*	xylose	7.0 ±0.2	4.1 ±0.3	0	4.1 ±0.3	0.21±0.01	59 ±4	0
2	*phaC1* $_{Ps}$*(STQK), phaAB*	glucose	9.3 ±0.2	5.7 ±0.6	0	5.7 ±0.6	0.29±0.03	61 ±5	0
3	*pct, phaC1* $_{Ps}$*(STQK), phaAB*	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	*pct, phaC1* $_{Ps}$*(STQK), phaAB*	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	*pct, phaC1* $_{Ps}$*(STFSQK), phaAB*	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	*pct, phaC1* $_{Ps}$*(STFSQK), phaAB*	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

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. $Y_{Pol/C}$: Sugar specific yield, amount of polymer in g produced per g of sugar supplied for polymer production. Data is average ±standard deviation of quadruplicate experiments.

Table 2 Molecular weights of P(3HB) and P(LA-*co*-3HB) synthesized using *E. coli* 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

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

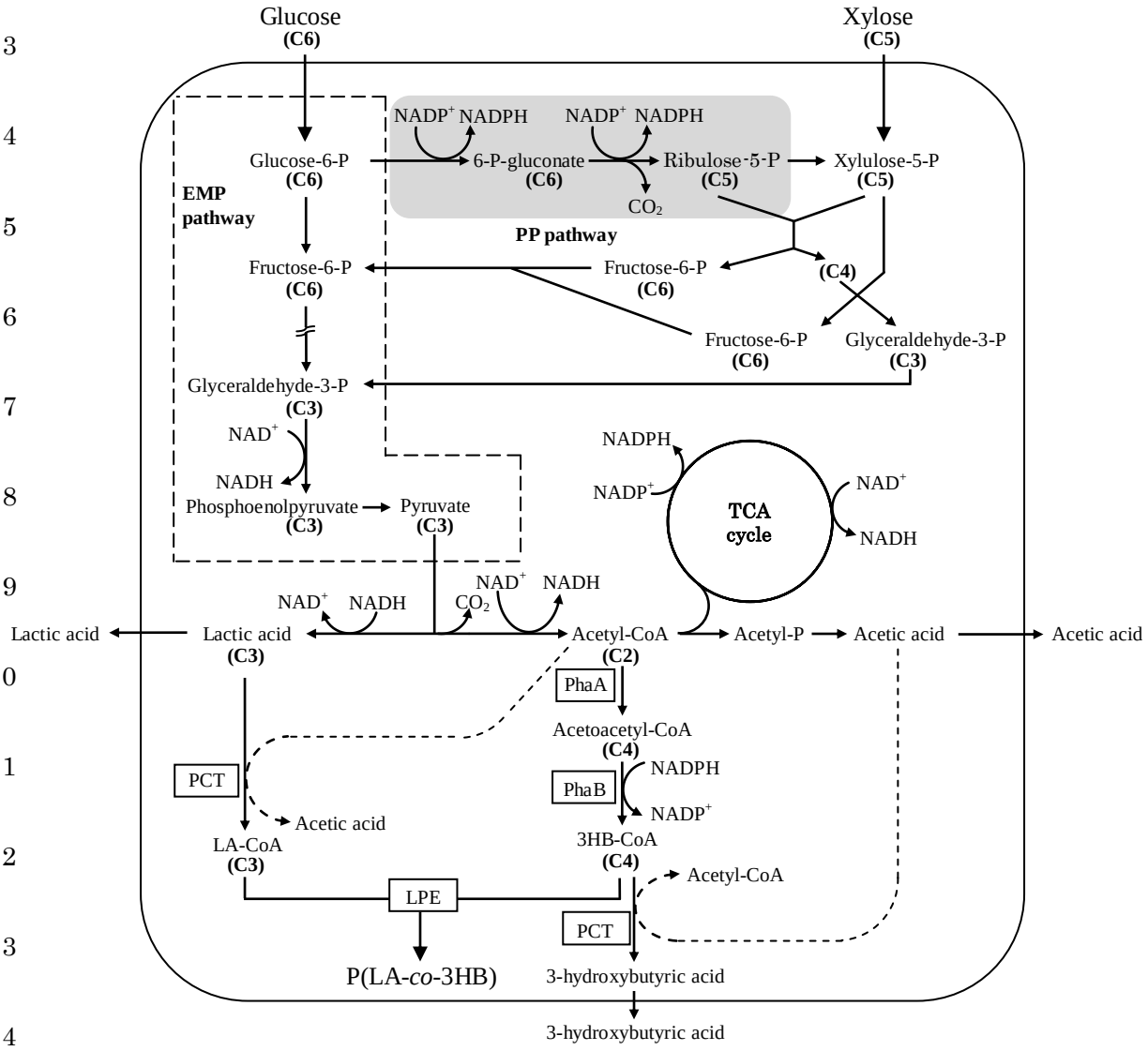


Fig. 1 Metabolic pathways in *E. coli* for xylose and glucose metabolism, and the production of P(LA-*co*-3HB) and related organic acids. EMP pathway, Embden-Meyerhof-Parnas pathway (highlighted by dashed box); PP pathway, pentose phosphate pathway (oxidative branch is highlighted in gray). The enzymes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

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

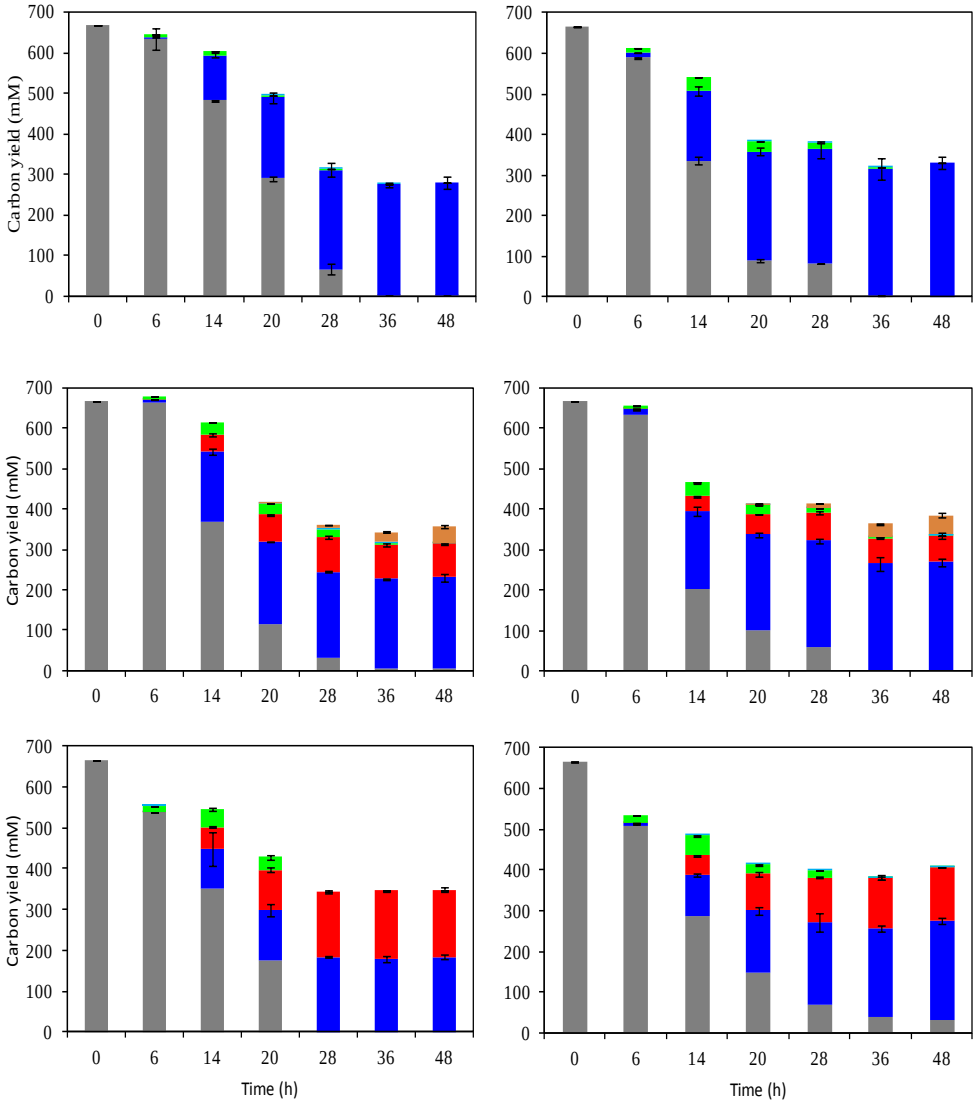


Fig. 2 Time course profiles of molar carbon amount for xylose and glucose and the metabolites produced in *E. coli* JW0885. Cells harboring pGEM*phaC1*$_{Ps}$(ST/QK)*AB* (A& B), pTV118N*pctphaC1*$_{Ps}$(ST/QK)*AB* (C&D) and pTV118N*pctphaC1*$_{Ps}$(ST/FS/QK)*AB* (E&F) were grown on 100 mL media containing xylose (A, C&E) and glucose (B, D&F) in shake flasks. Gray, residual sugars; blue,

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

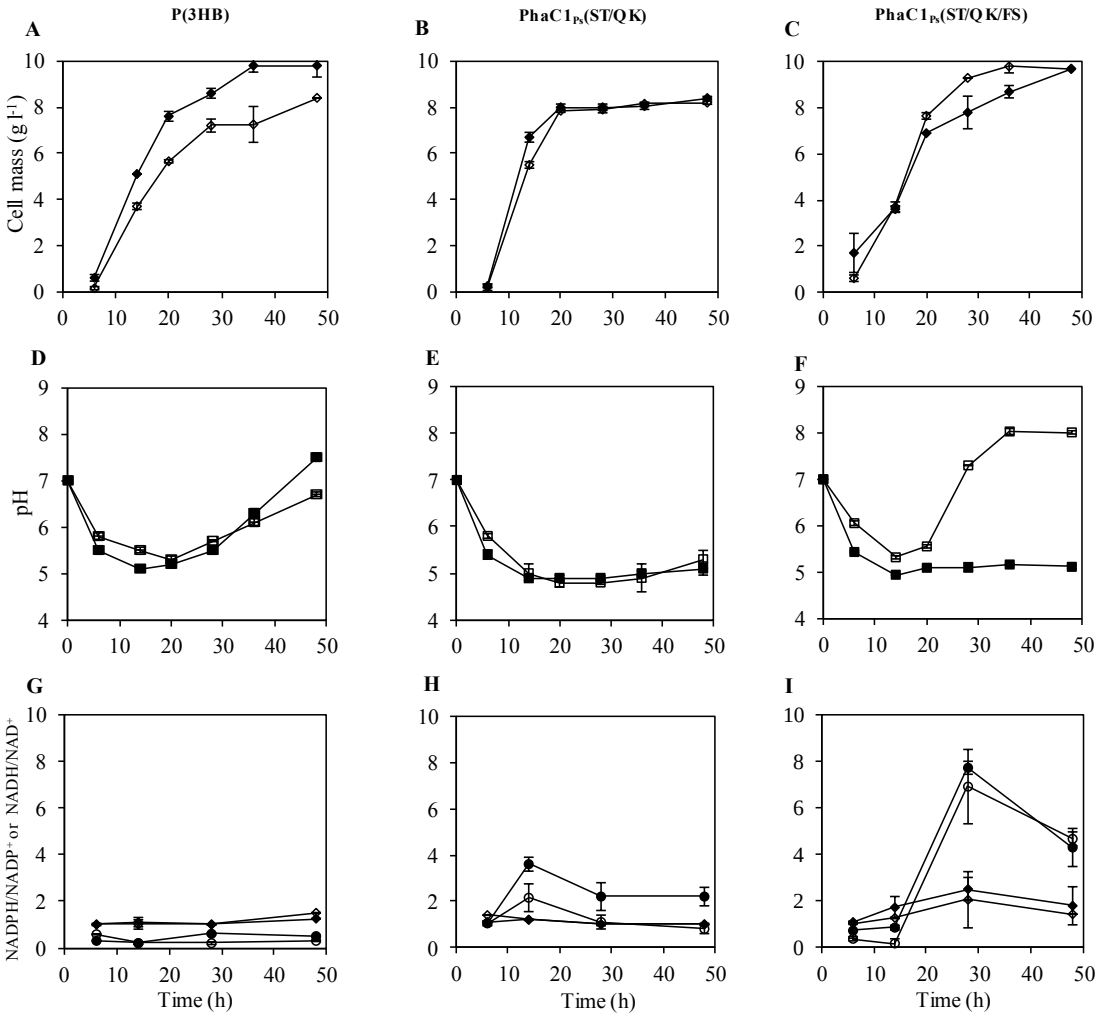


Fig. 3 Time course profiles of *E. coli* JW0885 expressing PhaC1$_{Ps}$(ST/QK) for P(3HB) synthesis (A, D&G), PhaC1$_{Ps}$(ST/QK) and PCT for P(LA-*co*-3HB) synthesis (B, E&H) or (PhaC1$_{Ps}$(ST/FS/QK) and PCT for P(LA-*co*-3HB) synthesis (C, F&I) from xylose (open symbols) or glucose (closed symbols). A, B&C; cell mass (g l^{-1}). D, E&F; medium pH. G, H&I; NADPH/$NADP^+$ (diamond symbols) and NADH/NAD^+ ratios (circles). The data shown are averages ± standard deviations of three independent trials. Time 0 (zero) indicates the time when the cells were inoculated.

1 **Supplementary**

2

3 **Table S1 A** Cofactor levels (μM g^{-1}_{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 (μM g^{-1}_{CDW})	$NADP^+$ (μM g^{-1}_{CDW})	NADH (μM g^{-1}_{CDW})	NAD^+ (μM g^{-1}_{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

6

7 **S1B** Cofactor levels (μM g^{-1}_{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).

9

time (h)	Sugar	NADPH (μM g^{-1}_{CDW})	$NADP^+$ (μM g^{-1}_{CDW})	NADH (μM g^{-1}_{CDW})	NAD^+ (μM g^{-1}_{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

11

12

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

time (h)	Sugar	NADPH (μM g^{-1}_{CDW})	$NADP^+$ (μM g^{-1}_{CDW})	NADH (μM g^{-1}_{CDW})	NAD^+ (μM g^{-1}_{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

The standard deviations are from duplicate measurements

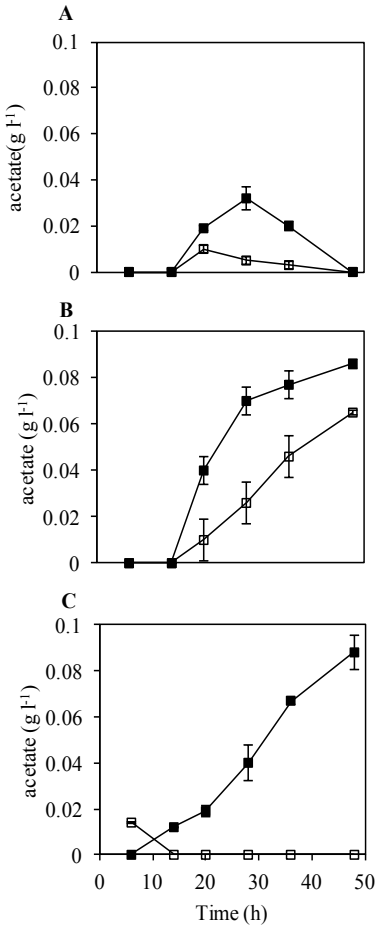


Fig. S1 Time course profiles of acetic acid (g l^{-1}) produced during P(3HB) synthesis (A) and P(LA-*co*-3HB) production by PhaC1$_{Ps}$(ST/QK) and PCT (B) or PhaC1$_{Ps}$(ST/FS/QK) and PCT (C). The data shown are means ± standard deviations of quadruplicate cultures of *E. coli* JW0885.

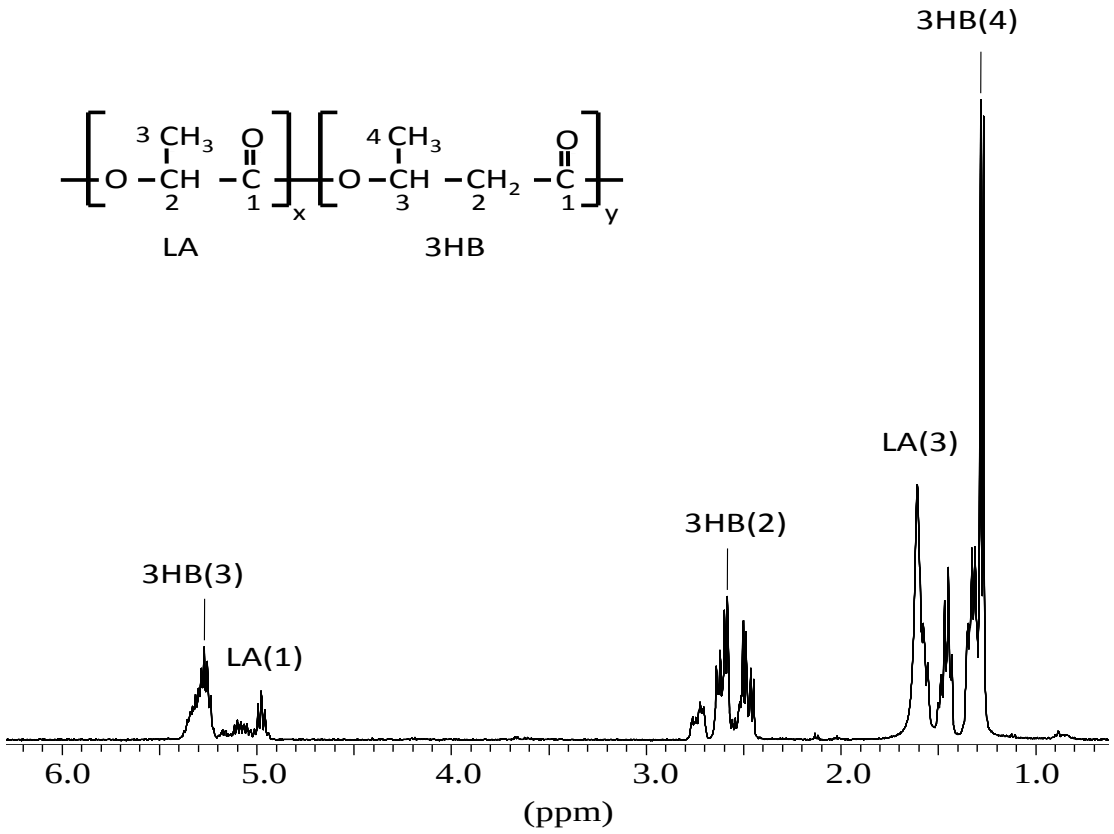


Fig. S2 ^{1}H NMR spectra of P(34 mol% LA-*co*-3HB) produced from xylose. The ^{1}H NMR chemical shifts around 1.5 and 5.0 ppm were assigned to the methyl and methine groups of the LA unit within the main chain of the copolymer as described previously (Yamada et al., 2010).