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High-cell density culture of poly(lactate-*co*-3-hydroxybutyrate)-producing *Escherichia coli* by using glucose/xylose-switching fed-batch jar fermentation

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Poly(lactate-*co*-3-hydroxybutyrate) [P(LA-*co*-3HB)] is produced in engineered *Escherichia coli* harboring the genes encoding an LA-polymerizing enzyme (LPE) and monomer-supplying enzymes. In this study, high cell-density fed-batch jar fermentation was developed using xylose and/or glucose as the carbon source. Fed-batch fermentation was initially performed with 20 g/L sugar during the batch phase for 24 h, and subsequent sugar feeding from 24 to 86 h. The feeding rate was increased in a stepwise manner. When xylose alone was used for cultivation, the cells produced the polymer at 11.6 g/L, which was higher than the 4.3 g/L obtained using glucose as the sole carbon source. However, in the first 24 h the growth in the glucose culture was greater than in the xylose culture. Based on these results, glucose was used for cell growth (at the initial stage) and xylose was used for polymer production (at the feeding stage). As expected, in the glucose/xylose switching fermentation method, polymer production was significantly enhanced, eventually reaching 26.7 g/L. The enhanced polymer production obtained by using xylose was presumably due to overflow metabolism. In fact, during xylose feeding, acetic acid excretion was greater than that in case of the glucose grown culture, suggesting the channeling of the metabolic flux from acetyl-CoA towards polymer production over into the tricarboxylic acid cycle in the xylose-fed cultures. Therefore, this sequential glucose/xylose feed strategy is potentially useful for production of acetyl-CoA derived compounds in *E. coli*.

[**Key words:** Polyhydroxybutyrate; Lignocellulosic biomass; Polylactic acid; Jar fermentor; Xylose]

Poly(lactate-*co*-3-hydroxybutyrate) [P(LA-*co*-3HB)] is an artificial polyhydroxyalkanoate (PHA) copolymer having advantages over its corresponding homopolymers, P(3HB) and PLA, in terms of material properties. The copolymer was first produced in *Escherichia coli* using an evolutionarily engineered LA-polymerizing enzyme (LPE) (1). The monomer substrates lactyl-CoA (LA-CoA) and 3HB-CoA were synthesized via acetyl-CoA with the aid of the exogenous monomer-supplying enzymes propionyl-CoA transferase (PCT), β-ketothiolase (PhaA), and acetyl-CoA reductase (PhaB) (Fig. 1). As a consequence, P(LA-*co*-3HB) was produced from sugars as the sole carbon source.

Lignocellulosic biomass-derived sugars, glucose, xylose and galactose have been used for P(LA-*co*-3HB) production in *E. coli* (2). Interestingly, it has been found that the use of xylose resulted in a higher polymer production as well as a higher LA fraction in the polymer compared to glucose grown cultures (3,4). This result suggested that xylose is a potent feedstock for P(LA-*co*-3HB) production. In fact, we demonstrated that the xylose-rich woody extract obtained from dissolved pulp manufacturing can be used for producing P(LA-*co*-3HB) (2). The high titer of P(LA-*co*-3HB) from xylose motivated us to develop a jar-fermentation system for P(LA-*co*-3HB) production using xylose as the main feedstock. In addition, there are different catabolic pathways involved: pentose phosphate pathway (PPP) vs. glycolysis for xylose and glucose, respectively. Therefore, to analyze the difference in catabolic activities, the effects of the carbon source on polymer production were assessed.

Fed-batch cultivation is an important technique to achieve a high cell-density and a high volumetric productivity in a jar-fermentor (5). Fed-batch fermentation is typically performed with a specific initial concentration of non-growth inhibiting sugar followed by sugar feeding using several feed-control methods. In the previous studies on fed-batch PHA production in *E. coli*, a pH-stat feeding strategy was employed in which glucose was added in response to the sharp pH increase caused by carbon source depletion (6,7). Under the pH-stat strategy, the amount of total sugar fed during the cultivation was approximately 60 g/L. In the present study, we tested a continuous feeding method to increase the total amount of sugar fed (approximately 200 g/L). The feeding rate was increased in a stepwise manner so as to support the cell growth and polymer production and to avoid osmotic stress. As a

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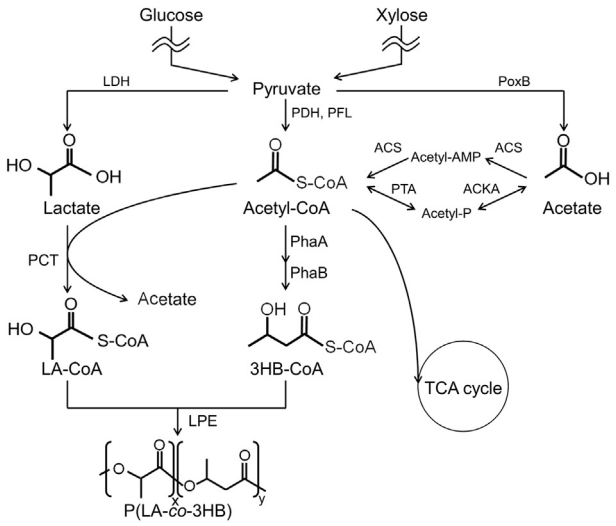

FIG. 1. Pathways for P(LA-*co*-3HB) synthesis in the recombinant *E. coli* MG1655 harboring pBBR1MCS4ΔP$_{Re}$pctC1STQKAB. PhaA, β-ketothiolase; PhaB, acetyl-CoA reductase; PCT, propionyl-CoA transferase; LPE, LA polymerizing enzyme (PhaC1$_{Ps}$[ST/QK]); LDH, lactate dehydrogenase; PoxB, pyruvate oxidase; ACS, acetyl-CoA synthetase; PTA, phosphotransacetylase; ACKA, acetate kinase; PDH, pyruvate dehydrogenase complex; PFL, pyruvate formate lyase; TCA, tricarboxylic acid.

result, a carbon feed switching strategy, which is a combination of the glucose added initially and subsequent xylose feeding, was found to be effective for P(LA-*co*-3HB) production in *E. coli*.

MATERIALS AND METHODS

Plasmid and strain for fed-batch fermentation *E. coli* MG1655, which is *lacI* positive strain, was used as the host. The expression vector pBBR1MCS4ΔP$_{Re}$pctC1STQKAB harboring the *pct*, *phaC1*$_{Ps}$(ST/QK), *phaA* and *phaB* genes regulated under the *lac* promoter was constructed as follows. The *Xba*I/*Hin*dIII fragment from pTV118N*pctphaC1*$_{Ps}$(ST/QK)*AB* (1) containing the *phaC1*$_{Ps}$(ST/QK), *phaA* and *phaB* genes was inserted into the *Xba*I/*Hin*dIII sites of pBBR1MCS4 (8) to yield pBBR1MCS4C1STQKAB. The promoter region of the *phb* operon (P$_{Re}$) in *Ralstonia eutropha* (currently designated as *Cupriavidus necator*) in pTV118N*pctphaC1*$_{Ps}$(ST/QK)*AB* was removed by digestion with *Sma*I/*Xba*I and underwent self-ligation to yield pTV118NΔP$_{Re}$*pctphaC1*$_{Ps}$(ST/QK)*AB*. The *Eco*RI fragment of the obtained plasmid containing the *pct* gene was inserted into two *Eco*RI sites of pBBR1MCS4C1STQKAB. Subsequently, the S/D sequence of the *pct* gene was inserted into the *Eco*RI site of the obtained plasmid using the oligo DNAs 5′-AATTCAGGAGGT-3′ and 5′AATTACCTCCTG-3′ via partial digestion to yield pBBR1MCS4ΔP$_{Re}$pctC1STQKAB, which was used for polymer production by the fed-batch culture.

Cultivation conditions Seed cultures were prepared in 100 mL of LB medium containing 100 mg/L ampicillin in a flask, which was incubated for 12 h at 30°C with reciprocal shaking at 120 rpm. Fed-batch cultivation was performed using a laboratory-scale jar fermentor (2 L) (TSC-M3L, Takasugi MFG. Co. Ltd., Tokyo, Japan) with an initial volume of 1 L of MR medium with the following composition (g/L): KH_2PO_4, 13.5; $(NH_4)_2HPO_4$, 4; $MgSO_4 \cdot 7H_2O$, 1.4; citric acid 1.7 (7). Ten milliliters of trace metal solution (10.0 g/L $FeSO_4 \cdot 7H_2O$; 2.0 g/L $CaCl_2$; 2.2 g/L $ZnSO_4 \cdot 7H_2O$; 0.5 g/L $MnSO_4 \cdot 4H_2O$; 1.0 g/L $CuSO_4 \cdot 5H_2O$; 0.1 g/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$; 0.02 g/L $Na_2B_4O_7 \cdot 10H_2O$ in 5M HCl) were added into 1 L of MR medium. The initial sugar concentration was 20 g/L of glucose or xylose. The pH was set at 7.0 using 4N NaOH. One hundred mg/L of ampicillin was added in an aseptic way. One hundred microliters of anti-foaming agents (ADEKA NOL LG-109, ADEKA Co., Tokyo, Japan) were added when the cultivation was started and each time foam was generated. Ten milliliters of seed culture were inoculated, then cultivation was carried out at 30°C at 700 rpm with an aeration rate of 3 L/min of air. The pH was automatically controlled at 7.0 by the addition of 4N NaOH. The feeding of the filter-sterilized solution (400 g/L glucose or xylose, 73.2 g/L $(NH_4)_2SO_4$, 11.7 g/L $MgSO_4$) was started at 24 h. The feeding rate of sugar solution (ρ = 1.167 g/cm^3) was increased in a stepwise manner; 5 g/h from 24 to 40 h, 10 g/L from 40 to 48 h, and 30 g/L from 48 to 81.6 h, as shown in Fig. S1. Culture broth (5 mL) was manually sampled every 12 h and maintained on ice until further processing.

Analysis of bacterial growth and polymer production One point five milliliters of the culture broth in triplicate was centrifuged at 13,000 × *g* at room temperature for 10 min. The supernatants were stored at −20°C until analysis. The pellets were washed twice with 800 μL of water and weighed after overnight lyophilization. The polymer was extracted from lyophilized cells with 10 mL of chloroform in closed screw cap tubes at 60°C for 2 days. The remaining cell debris was removed by passage through a PTFE filter with a pore size of 0.20 μm. Polymer was precipitated by adding 10-fold volume of methanol and weighed after overnight lyophilization to remove the solvents. The true cell weight (TCW, often described as residual biomass) was defined as the polymer weight subtracted from the cell dry weight (CDW). Specific growth rates were calculated from the plots of ln(CDW) and time using linear correlation. Growth yields were calculated from the TCW at the end of cultivation.

Sugar accumulation in the culture supernatant The glucose and xylose concentrations in the culture supernatant were quantified by high-performance liquid chromatography (HPLC). The supernatant was diluted 40 times using 15 v/v % of 0.1 M aqueous phosphoric acid in acetonitrile, and centrifuged at 13,000 × *g* at room temperature for 15 min. Ten microliters of the supernatant sample were injected into HPLC (JASCO, Tokyo, Japan) equipped with a column (Asahipak NH2P-50 4E, 250 × 4.6 mm diameter, Showa Denko K. K., Tokyo, Japan) at 47°C and eluted at a flow rate of 0.35 mL/min with 15 v/v% of 0.1 M aqueous phosphoric acid in acetonitrile. The separated sugars were detected using a refractive index (RI) detector.

Expression analysis Ten milliliters of the recombinant *E. coli* culture were harvested at different time points (12, 24, 48 and 60 h) during cultivation and disrupted in 4 mL of lysis buffer (9) by sonication. The protein concentration was measured by the Bradford protein assay (Bio-Rad Laboratories Inc., Hercules, CA, USA) and 5 μg of the crude proteins were applied to SDS-PAGE analysis (Bio-Rad). The proteins were transferred onto a PVDF membrane and LacZ was immunolabeled using the anti-β-galactosidase rabbit IgG fraction (Thermo Fisher Scientific Invitrogen, Waltham, MA, USA) of an Amersham ECL Select Western Blotting Detection Reagents kit (GE Healthcare Life Sciences, Marlborough, MA, USA), and the chemiluminescence was visualized using the ChemiDoc XRS+ System (Bio-Rad).

Organic acid accumulation in the culture supernatant The acetic acid and lactic acid concentrations in the culture supernatant were determined by HPLC. The supernatant was diluted 20 times using demineralized water and centrifuged at 13,000 × *g* for 15 min at room temperature. Ten microliters of the supernatant sample were injected into HPLC equipped with a column (Aminex HPX-87H, 300 × 7.8 mm, Bio-Rad) at 60°C and eluted at a flow rate of 0.50 mL/min of 15 v/v% of 0.1 M aqueous phosphoric acid in acetonitrile. Absorbance of the separated organic acid was measured at 210 nm.

Ammonium concentration in the culture supernatant The ammonium concentration in the supernatant was measured by using Spectroquant Ammonium Test (Merck & Co., Inc., Kenilworth, NJ, USA) according to manufacturer's instructions.

Monomeric unit composition of the polymer determined by using HPLC and NMR Polymer content was determined by HPLC as follows (10): One milliliter of 98% sulfuric acid was added to the lyophilized cells in a microtube with a screw cap. The tube was heated at 120°C for 45 min with mixing by inversion at 5, 15, 30 and 45 min. Ten microliters of the appropriately diluted supernatant were subjected to HPLC equipped with a column (Aminex HPX-87H, 300 × 7.8 mm, Bio-Rad) at 60°C and eluted with 0.014N sulfuric acid at a constant flow rate of 0.70 mL/min. Absorbance was measured at 210 nm. P(3HB) and chemically synthesized PLA were used as standards. For monomer composition analysis, 5 mg of extracted polymer were dissolved in 1 mL of deuterated chloroform ($CDCl_3$) and ^{1}H NMR was recorded using an NMR spectrometer MSL400 (Bruker) with tetramethylsilane (TMS) as the internal chemical shift standard.

RESULTS AND DISCUSSION

The *E. coli* was cultivated in a fed-batch jar fermentor using glucose (Fig. 2A) or xylose (Fig. 2B) as the sole carbon source. During the initial 24 h, the growth and polymer production of the glucose culture was higher than the ones of the xylose culture. However, the glucose culture produced almost no polymer during 24–84 h. In contrast, the polymer was continuously produced in the xylose culture, and eventually the polymer concentration as well as volumetric productivity from xylose (11.6 g/L and 0.14 g/L/h) was much greater than the ones found for glucose (4.3 g/L and 0.05 g/L/h). The μ_{max} of the glucose culture, which was calculated based on CDW, was 0.065 1/h at 12–24 h and the μ value was slightly decreased during the cultivation. The μ_{max} of the xylose culture was 0.12 1/h at 24–36 h, but the growth rate was rapidly decelerated at the later stage of cultivation. Overall, the specific growth rates observed in this study were lower than the typical values of non-PHA-producing *E. coli* (11).

Based on these cultivation results, we designed a two-phase cultivation strategy where glucose would be used for cell growth

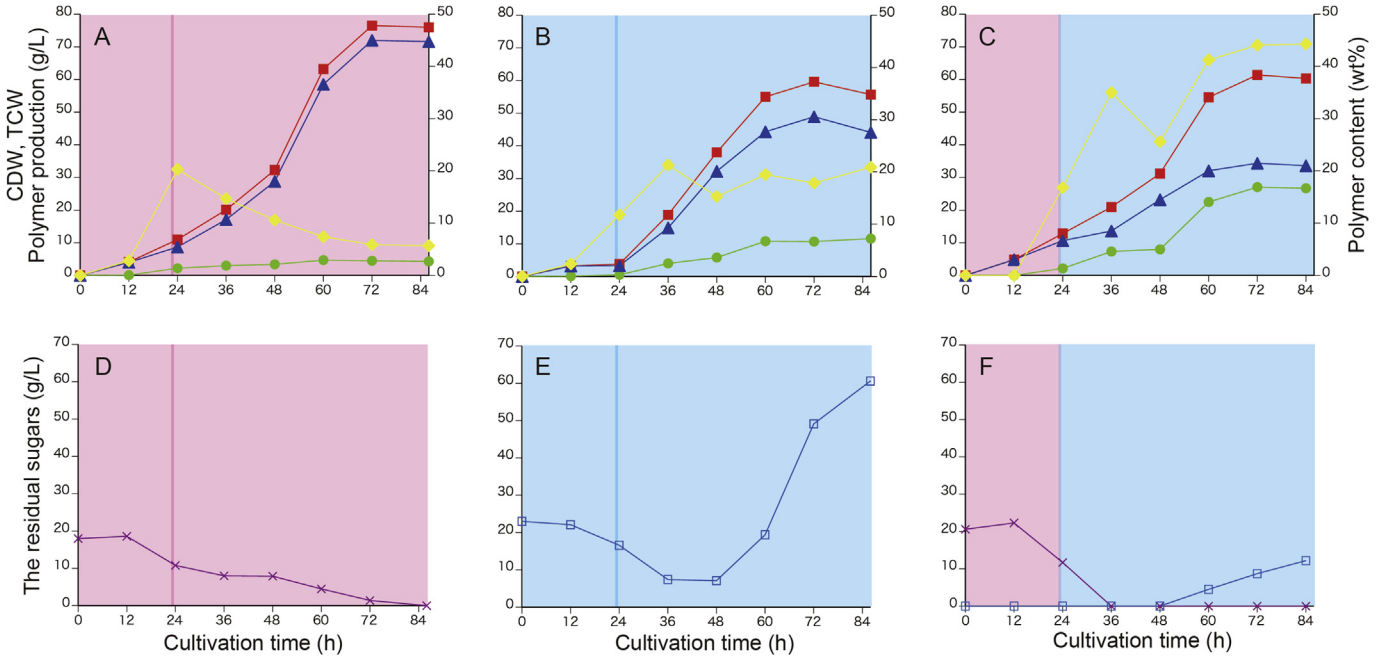

FIG. 2. Time course of microbial growth and polymer accumulation (A–C), and the residual sugars (D–F) during fed-batch fermentation under three different conditions. The initial and feeding sugars were glucose–glucose (G–G) (A, D), xylose–xylose (X–X) (B, E) and glucose–xylose (G–X) (C, F), respectively. Glucose and xylose are highlighted in pink and blue, respectively. The cell growth at the initial batch stage and polymer production at the middle-late feeding stage are shown. Closed squares, cell dry weight (CDW); circles, polymer production; triangles, true cell weight; diamonds, polymer content; crosses, glucose; and open squares, xylose. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

during the initial stage and xylose consumed for polymer production (feeding stage). By implementing the fed-batch technique, we were able to establish a glucose/xylose switching procedure in order to manage the phases of cell growth and polymer production. As expected, an improved cultivation profile was obtained, as can be seen in Fig. 2C. In the glucose/xylose culture, the CDW (60.3 g/L) and polymer production (26.7 g/L) were increased compared to the other conditions tested, as shown in Fig. 2A and B. Polymer productivity (0.32 g/L/h) was more than twice than those detected under the other conditions. In contrast, the μ_{max} value (0.048 1/h at 36-48 h) during the xylose feeding was lower than under other conditions. The concentrations of the residual sugars in the supernatant were measured as shown in Fig. 2D–F. The overall growth yield (and polymer yield) per sugar in the glucose–glucose, xylose–xylose and glucose–xylose cultures were 0.34 (0.02), 0.29 (0.08), and 0.16 (0.13) g/g, respectively. Among the sugar combinations, the glucose/xylose switching led to the highest carbon flux toward polymer production compared to glucose and xylose alone. Consequently, the cellular polymer content was significantly increased (44.3 wt%) under the switching condition.

The enhanced polymer production from xylose might be due to the high expression levels of the relevant enzymes under the *lac* promoter. The time course of LacZ expression was assayed for the three conditions tested to estimate the activity of the *lac* promoter (Fig. 3). There was no correlation between LacZ expression and polymer production, suggesting that the beneficial effect of xylose on the polymer production was not due to the expression levels of the plasmid-encoded enzymes. Thus, it is suggested that metabolic flux from the sugar to polymer production is more important. A similar result was previously reported in which P(3HB) production in *E. coli* was not correlated with the expression level of the genes involved in the polymer synthesis when glucose was used (12).

Extracellular metabolites such as acetic acid, lactic acid and ammonium were monitored to investigate the mechanism of the carbon source effect on the metabolic fluxes toward the polymer (Fig. 4A–C). In the glucose-fed culture, extracellular metabolites were hardly detected (Fig. 4A), and moreover, the glucose concentration in the feeding stage was lower than 10 g/L (Fig. 2D), which could result in low polymer production. It is known that *E. coli* accumulates PHA only when a potent carbon source is available as well as natural PHA-producing bacteria. It was previously reported that there is a clear positive correlation between the glucose concentration and P(3HB) accumulation in *E. coli* (13). This phenomenon is due to the increase in the intracellular acetyl-CoA pool depending on glucose concentration in the medium (14). In previous studies on PHA production in *E. coli*, the glucose concentration was spiked by using a pH-stat feeding strategy that may induce the polymer accumulation (6).

In the xylose-fed cultures (xylose/xylose and glucose/xylose, Fig. 4B and C), acetate (2.7–13.9 g/L) was produced along with an increase of the residual xylose, as shown in Fig. 2E and F. The acetate production by *E. coli* under aerobic conditions is known as overflow metabolism (15). The acetate excretion is not a reflection of metabolite overflow of the central metabolism, but physiologically advantageous to facilitate a faster growth rate (16). The overflow metabolism is triggered by downregulation of acetyl-CoA

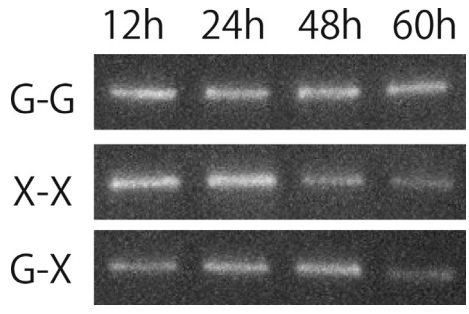

FIG. 3. The time course of LacZ expression in the crude proteins of the recombinant *E. coli* cells during fed-batch fermentation. SDS-PAGE analysis of the crude proteins is shown in Fig. S2. X, xylose culture; G, glucose culture; G–X, glucose–xylose culture.

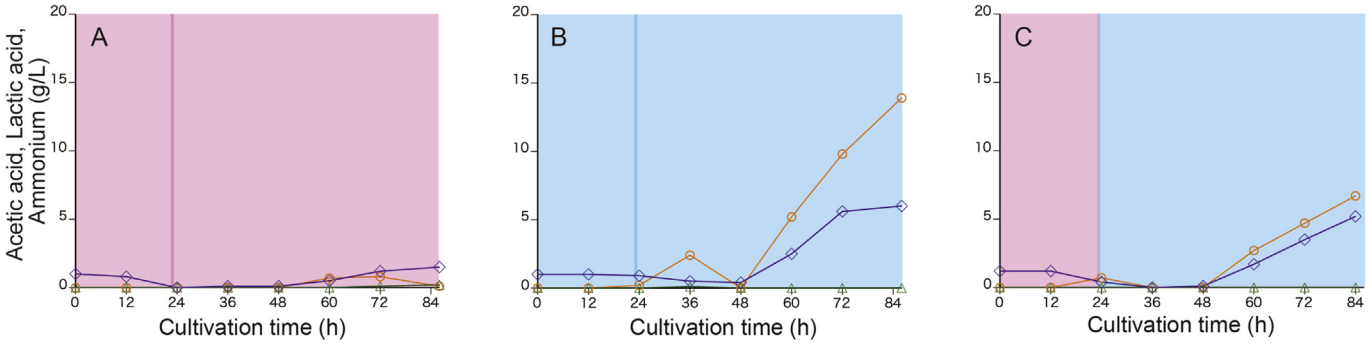

FIG. 4. Time course of extracellular metabolites during fed-batch fermentation under three different conditions. The culture conditions are (A) G–G, (B) X–X, and (C) G–X. Circles, acetic acid; open triangles, lactic acid; and open diamonds, ammonium.

synthetase (17). During overflow metabolism, the carbon flux from acetyl-CoA is channeled to acetate rather than the TCA cycle for respiration. This limitation of the flux to TCA cycle may induce polymer accumulation in the xylose-fed cultures. These results indicated that overflow metabolism plays an important role in the result that xylose was efficiently converted into both microbial biomass and PHA production, whereas glucose was mostly used for growth of biomass. In fact, xylose tended to be used for polymer production compared to glucose as previously reported in the poly(4-hydroxbutyrate)-producing *E. coli* (18).

A key finding in the present study is that the glucose/xylose switching culture enhanced polymer production more than xylose culture. In the glucose–xylose culture, the flux of overflow metabolism seemed to be channeled to the polymer accumulation rather than acetate secretion. The reason for this phenomenon is currently unknown. It has been recently proposed that overflow metabolism in *E. coli* results from efficient proteome allocation into glycolytic pathway and TCA cycle, which means that when more proteins are required for the glycolytic pathway, proteins for the TCA cycle were diminished and overflow metabolism occurs (19). Therefore, the different catabolic pathways for xylose and glucose, the PPP and glycolysis, respectively, could affect the overflow metabolism and consequently the polymer production. Further studies will be needed to elucidate the role of overflow metabolism when the PPP is dominant.

The obtained polymer contained 5 mol% LA at the end of cultivation in the glucose/xylose culture. The LA fraction was higher than that obtained by the glucose and xylose cultures alone (Table 1), but lower than the value previously obtained (16 mol%) from recombinant *E. coli* BW25113 grown on LB medium containing xylose (2). MG1655 also produced a copolymer with similar LA fraction (18 mol %) on LB medium containing xylose (Table S1), indicating that effective factors influencing LA fraction is the culture medium and conditions rather than *E. coli* strains. The low LA incorporation in the polymer obtained in the present study (Table 1) was partly due to the low lactic acid production, because lactic acid was not detected in the supernatant under any of the conditions (Fig. 4A–C). There are several metabolic pathways for lactic acid production from sugars, including homo and hetero lactic acid fermentation, which have different redox potentials (20). Therefore, lactic acid production might be elevated by changing oxygen supply during cultivation. The molecular weight of the polymers (Table 1) was slightly lower than that obtained by flask-scale batch culture ($M_w = 4.8 \times 10^5$) (1). In addition, the polymer molecular weights from the xylose–xylose and glucose–xylose experiments were slightly lower than the glucose–glucose. The contributing factors to the molecular weight differences are elusive at present.

TABLE 1. Analysis of P(LA-*co*-3HB)s produced in fed-batch fermentation of the recombinant *E. coli*.[a]

Culture conditions	Polymer production (g/L)	Monomer composition (LA mol%)[b]	Molecular weight[c]		Polydispersity index
			M_n ($\times 10^4$)	M_w ($\times 10^5$)	
G–G	4.3	0.9	4.4	2.3	5.1
X–X	11.6	3.0	4.5	1.5	3.3
G–X	26.7	4.9	2.8	1.6	5.7

[a] *E. coli* MG1655 harboring pBBR1MCS4ΔP$_{Re}$pctC1STQKAB.
[b] Determined by ^{1}H NMR.
[c] Determined by gel permeation chromatography.

In conclusion, we propose a glucose/xylose switching procedure for polymer production by the fed-batch method. Numerous fed-batch fermentations of *E. coli* for valuable chemicals using mixtures of glucose and xylose derived from lignocellulosic biomass for carbon sources have been reported that have often resulted in a low consumption of xylose due to carbon catabolite repression (21). In this study, the glucose/xylose switching fed-batch fermentation method enabled *E. coli* to efficiently utilize xylose without any catabolite repression and to achieved 26.7 g/L of 5 mol% LA-based PHA, which is greater than the previous fed-batch fermentation of LA containing PHA at 20 g/L polymer production (6). The polymer yield per sugar consumed in glucose–xylose culture was 0.13 g/g, which was lower than the previously reported P(LA-*co*-3HB) yield per xylose in flask cultures, 0.2–0.4 g/g (3). These results indicated that further investigation toward higher polymer production such as optimization of the culture conditions and improved metabolic engineering (4) will be needed. Finally, we suggest that the increase in PHA production is attributed to a metabolic flux shift leading to an increase in the acetyl-CoA supply for polymer production through the overflow metabolism of xylose. Therefore, the glucose–xylose switching strategy is potentially useful for the *E. coli* production of acetyl-CoA derived compounds from lignocellulosic biomass.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2018.11.006.

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