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**Efficient Microbial Production of Lactate-based
Polymers Using Hemicellulose-derived Carbon Sources**

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March, 2014

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Chapter 1

General introduction

1.1 Petroleum-based plastics

Plastics are ubiquitous synthetic polymeric molecules produced from petrochemical sources that have become an indispensable part of our daily lives. Plastics are inexpensive, corrosion-resistant, lightweight, strong, and durable materials and due to the diversity of polymers and the versatility of their properties, they have been molded into a vast array of products with broad applications (Thompson et al., 2009). In consequence, the production of plastics worldwide has been increasing and is predicted to surpass 300 million tonnes in the 2010 - 2015 time period (Chen and Patel, 2012). However, these polymers are resistant against physical, chemical and microbial attack, and are often improperly discarded with the disposal by incineration leading to the production of toxic furans and dioxins thus, the escalating problems regarding plastic waste treatment (Halden, 2010). Additionally, the non-renewable petrochemical resources are predicted to rise in prices and be exhausted (Mecking, 2004). Therefore, the drawbacks regarding the petrochemical-derived plastics have necessitated the development of biodegradable biopolyesters from renewable resources as alternatives.

1.2 The biopolyesters; Polylactic acid (PLA) and polyhydroxyalkanoates (PHAs)

Among the biopolyesters that have been suggested as potential alternatives to the

petrochemical-derived plastics are polylactic acid (PLA) and polyhydroxyalkanoates (PHAs), which are a class of natural biodegradable biopolyesters produced by either chemo-bio synthesis (PLA) or accumulated by various microbes as carbon storage compounds (PHAs) (Chen, 2009; Nampoothiri et al., 2010).

PLA is a linear aliphatic polyester entirely produced from renewable resources and readily biodegradable with a huge potential as a sustainable alternative to the conventional petrochemical-based plastics such as polyethylene, polypropylene, polyethylene terephthalate and polystyrene (Carrasco et al., 2010). Fig. 1.1 shows the carbon cycling system for PLA. PLA has already found applications in packaging material (Auras et al., 2004), agricultural fields and in the medical field e.g. wound dressing, resorbable sutures and for drug delivery systems (Lasprilla et al., 2012; Rasal et al., 2010). PLA is commonly synthesized via a lactide intermediate that uses either chiral form of lactic acid or combinations produced from fermentation and is polymerized mainly by ring-opening polymerization (ROP) using catalysts such as tin or through direct condensation of lactic acid (Kricheldorf, 2001).

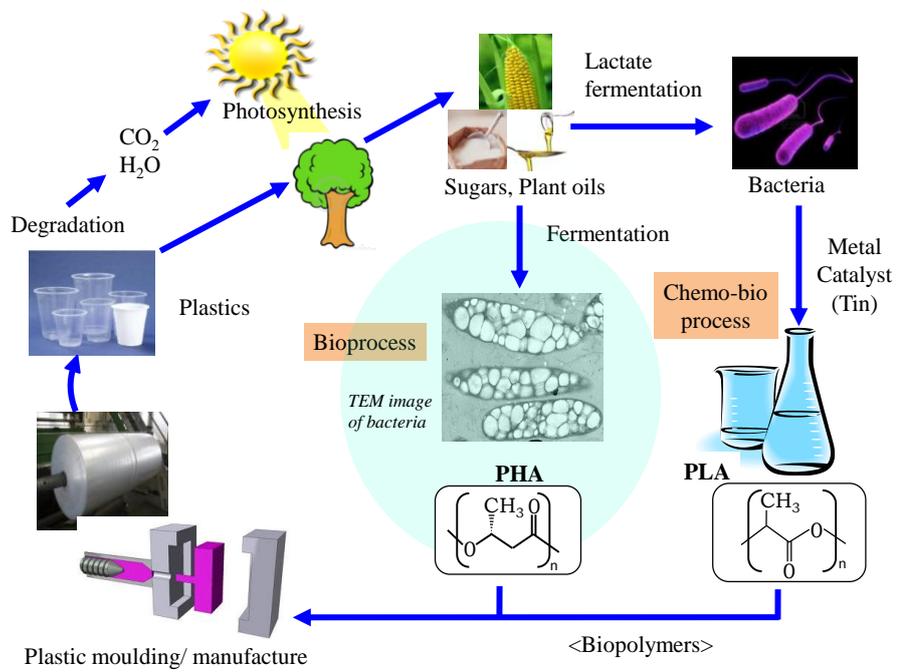


Fig. 1.1 Carbon recycling of PLA and PHAs. PLA and PHAs are biodegradable.

PHAs are the largest group of biopolyesters with over 150 different types of monomers (Steinbuchel and Lutke-Eversloh, 2003). PHAs are biodegradable (Fig. 1.1), and comprise of a big family ranging from brittle plastics to elastomers to rubbers depending on their monomer compositions. They mainly compose (*R*)-hydroxyalkanoic acids and are divided into short and medium chain length (SCL and MCL, respectively) PHAs based on carbon monomers and the microorganisms in which they are produced (Verlinden et al., 2007). SCL PHAs are C₄ – C₅ e.g. polyhydroxybutyrate [P(3HB)] and P(3HB-*co*-3-hydroxyvalerate (3HV), which are produced by such bacteria as *Ralstonia eutropha* and *Alcaligenes latus* (Hazer and Steinbuchel, 2007). MCL PHAs on the other hand consist of 6 – 14 carbon monomers (C₆ – C₁₄) and are mainly produced by Pseudomonads e.g. *Pseudomonas putida*, *P. oleovorans* and *P. aeruginosa* (Li et al., 2007; Nduko et al., 2012).

1.3 Production of PLA and PHAs

As briefly mentioned above, PLA is synthesized in a multi-step process, which is different from that of the PHAs. PLA synthesis basically involves 3 steps; (i) production of lactic acid through microbial fermentation, (ii) purification of lactic acid and preparation of its cyclic dimer (lactide) and, (iii) ring-opening polymerization of the

lactides or polymerization by polycondensation (Fig. 1.2) (Chisholm, 2010). However, such chemo- bio process of PLA synthesis involving multiple preparation and purification steps although well established, is energy-consuming and requires harmful chemical catalysts for the polymerization process. This hampers the economical and environmental impacts of the material (Corma et al., 2007).

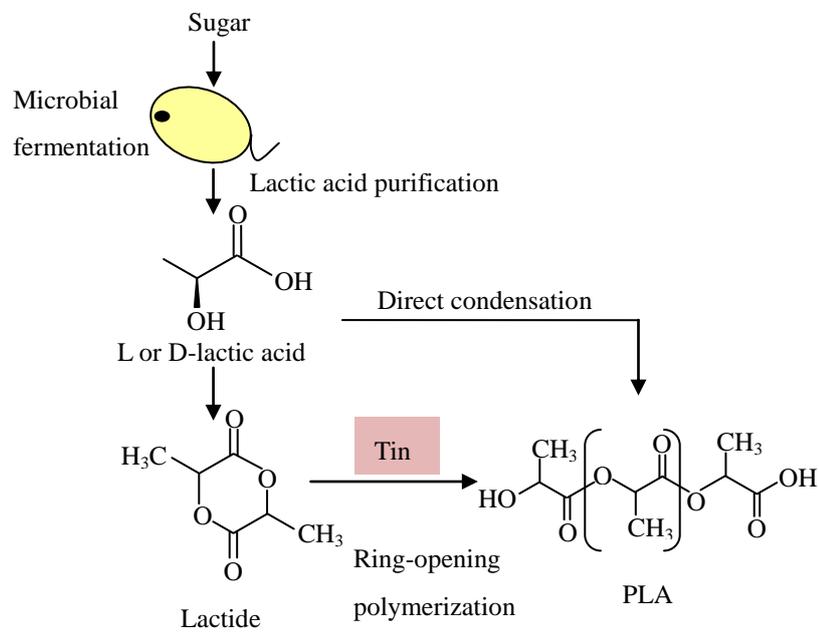


Fig. 1.2 PLA synthesis scheme. PLA is synthesized from lactic acid obtained from the fermentation of sugars

Unlike PLA, PHAs are wholly synthesized *in vivo* via a two-step process; monomer supply and polymerization and are stored as intracellular inclusions (Anderson and Dawes, 1990). Their biosynthetic pathways have been characterized in previous studies (Verlinden et al., 2007). A typical PHA is polyhydroxybutyrate [P(3HB)], whose biosynthetic pathway is shown in Fig 1.3, and is extensively investigated in *Ralstonia eutropha*. In *R. eutropha*, two molecules of acetyl-CoA are condensed to generate acetoacetyl-CoA in a reaction catalyzed by a 3-ketothiolase (PhaA). Acetoacetyl-CoA is subsequently reduced to 3-hydroxybutyryl (3HB)-CoA by NADPH-dependent acetoacetyl-CoA reductase (PhaB) and then the 3HB-CoA is polymerized by a PHA synthase (PhaC) to form P(3HB) (Verlinden et al., 2007).

The establishment of a single-pot bioprocess for PLA synthesis analogous to PHAs will overcome the need for the use of metal catalysts and purification of lactic acid; hence the cost of production will possibly be reduced. An ‘LA-polymerizing enzyme (LPE)’, which can function as an alternative to a metal catalyst would be desirable to establish such a bioprocess. The simplest strategy would have been the discovery of PLA producing microorganisms, but this has not succeeded so far (Taguchi, 2010). However, recently, a whole cell biosynthetic system for LA-based polyester production without the heavy metal catalysts was constructed in engineered bacteria (Song et al., 2012; Taguchi

et al., 2008). The system was triggered by the discovery of an LPE, which was an engineered PhaC [PhaC_{1Ps}(ST/QK)] that incorporated LA unit together with 3HB to form P[lactate (LA)-*co*-3HB] copolymer. Using this engineered LPE, copolymers and terpolymers have been produced of varying LA fraction up to over 90 mol% (Shozui et al., 2011; Song et al., 2012; Yamada et al., 2010). This process utilizes glucose (a renewable material) as a carbon source and is yet to be commercialized.

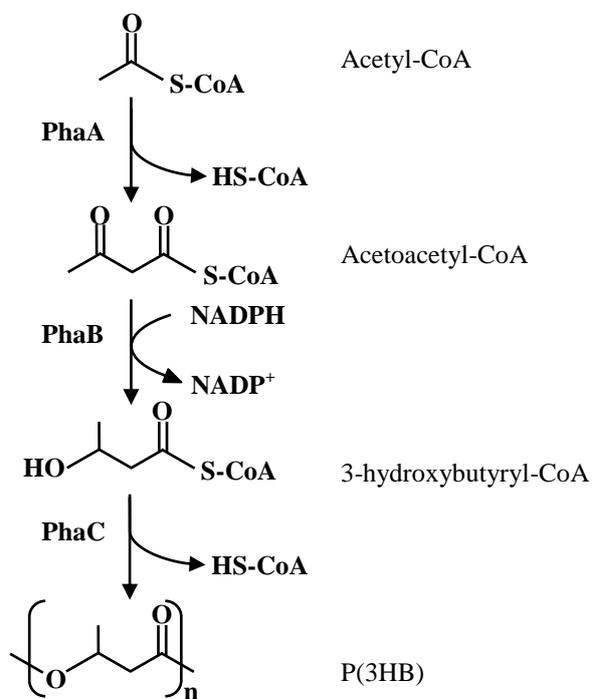


Fig. 1.3 The biosynthetic pathway for the synthesis of polyhydroxybutyrate [P(3HB)] in *Ralstonia eutropha*. PhaA, β -ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, polyhydroxyalkanoate (PHA) synthase.

1.4 Carbon sources for the production of PLA and PHAs

Although PLA and PHAs have generated significant industrial attention, they have had little success in replacing petro-chemical based plastics in commodity applications due to their high cost of production (Chen, 2009; Nampoothiri et al., 2010). In the production of PLA and PHAs, refined sugars from sugarcane, sugar beet, and starch feedstocks that are readily hydrolyzed are used as the starting carbon substrates. However, the cost of these materials is high since grain and sugar crops are expensive and they are also used as food and feeds hence, their utilization is not feasible in the long term (Nampoothiri et al., 2010; Verlinden et al., 2007). As an alternative, many research groups have attempted the use of inexpensive substrates for lactic acid and PHA production (Abdel-Rahman et al., 2011; Matsumoto et al., 2011; Yu and Stahl, 2008). The inexpensive substrates that have been attempted include; whey, lignocellulosic biomass, wastewater from olive mills, molasses, corn steep liquor, starchy wastewater and palm oil mill effluent, among others (Solaiman et al., 2006). The use of lignocellulosic biomass which is inedible, carbon rich, renewable, abundant, and inexpensive as a carbon source for P(LA-co-3HB) production is an attractive point of research as it is sustainable in creating a biorefinery for the production of the biopolyesters (Fig. 1.4).

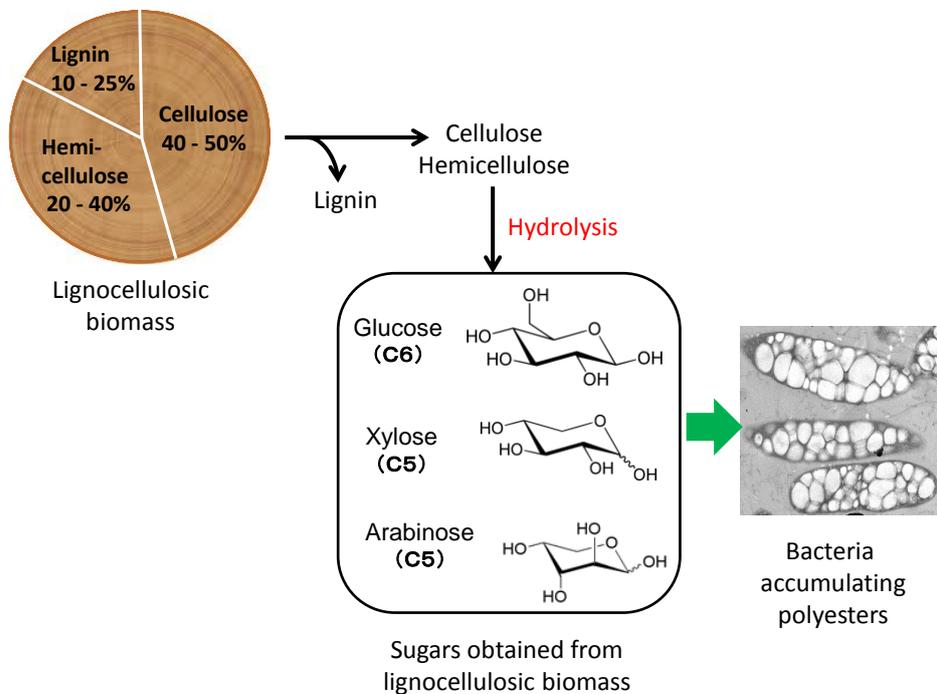


Fig. 1.4 The scheme for the establishment of a biorefinery for the production of polyesters from sugars derived from lignocellulosic biomass.

Lignocellulosic plant biomass averagely consists of 40 – 50% cellulose, 20 – 40% hemicellulose, and 10 – 25% lignin but it depends on the biomass source (Alonso et al., 2010). To utilize lignocellulosic materials for the production of value-added products, these biomasses need to be fractionated into their components: cellulose, hemicellulose and lignin. The carbohydrate components can then be hydrolyzed to obtain fermentable sugars, which could be supplied to microorganisms to produce commodity chemicals (Alonso et al., 2010). Cellulases produced mainly from *Trichoderma reesei* have been employed for cellulose hydrolysis in the production of bioethanol and a number of bioproducts (Kumar et al., 2008). On the contrary, the hemicellulose portions are underutilized. For instance, in Kraft pulping processes, hemicellulose is dissolved in black liquor along with lignin and combusted for power generation (FitzPatrick et al., 2010). Since hemicellulose has a lower heating value compared to lignin, its utilization for the production of polyesters and fuels could be more efficient (FitzPatrick et al., 2010). Hemicellulose can be recovered and subjected to treatment by methods such as acid hydrolysis, steam explosion and hot water treatment to readily obtain xylose-rich hydrolysates, which can serve as substrates for the synthesis of a number of bioproducts (FitzPatrick et al., 2010).

Already, xylose has been shown as a carbon source for the production of P(3HB)

with yields of 1.7 g l⁻¹ of polymer from 20 g l⁻¹ xylose (Lee, 1998). Higher yields (4.4 g l⁻¹) were obtained with the supplementation of soybean hydrolysate. Another group produced P(3HB-*co*-3HV) copolymer at 4.2 g l⁻¹ from 22 g l⁻¹ xylose and 0.52% levulinic acid in *Burkholderia cepacia* (Keenan et al., 2004). In another attempt to obtain microorganisms capable of efficiently utilizing xylose as a carbon source for PHA synthesis, Lopes *et al.* (Lopes et al., 2009) screened for microorganisms, and only a few were able to grow on xylose and produce P(3HB) (3.9 g l⁻¹ for the best strain). This result clearly demonstrated that although xylose is a major natural sugar, its assimilation by microorganisms is not widespread, and is not a suitable substrate for PHA production, thus limiting its application for many bioprocesses. In contrast, glucose has been converted efficiently to P(3HB) (Adsul et al., 2011). Therefore, to establish a biorefinery for the production of P(LA-*co*-3HB) from lignocellulosic biomass, the utilization of the major constituent sugars of lignocellulosic biomass is essential. In effect, utilization and efficient bioconversion of xylose into P(LA-*co*-3HB) is necessary.

1.5 Aim of this thesis

The efficient utilization of xylose for microbial production of PHAs and the LA-based polyesters has potential to lower production costs. Therefore, the aim of this

study was to explore the possibility of using xylose as a carbon source to produce P(LA-*co*-3HB) copolymer in engineered *E. coli*. Furthermore, the study aimed at improving the efficiency of the biotransformation of xylose into P(LA-*co*-3HB)s, thus offering xylose and xylose-rich hemicellulose hydrolysates as potential carbon sources for biopolyester production. In addition, the use of xylose will expand the carbon substrate diversity from which the P(LA-*co*-3HB) could be produced. The study also aimed at demonstrating the use of lignocellulosic biomass for the production of PHAs.

The first chapter of this thesis is the general introduction, and chapter 2 examines the application of xylose for P(LA-*co*-3HB) polyester production. First, xylose was applied to the production of polyhydroxybutyrate [P(3HB)]. Next, xylose was employed for the production of P(LA-*co*-3HB) and its yields were compared with those of glucose. The metabolic differences between the two sugars, which accounts for differences in polymer yields and monomer compositions, are discussed. Additionally, the differences in P(3HB) and P(LA-*co*-3HB) synthesis are as well discussed. Chapter 3 of this thesis investigates the metabolic engineering strategies including xylose transporter overexpression and lactic acid overproduction to regulate the monomer composition in P(LA-*co*-3HB) and improve productivity. High polymer yields and LA fractions in P(LA-*co*-3HB) were attained and are discussed together with carbon flux

distributions during polymer synthesis. Chapter 4 of this thesis discusses the utilization of lignocellulosic biomass for the production of polymers to demonstrate their applicability as inexpensive carbon substrates. In this study, cellulose was hydrolyzed by ruthenium catalyst into glucose. However, the reaction also generated 5-hydroxymethylfurfural (5-HMF) as a by-product that was toxic to *E. coli*. To overcome this obstacle, *E. coli* strains were screened for 5-HMF tolerance. As a result, a 5-HMF-tolerant strain was obtained and applied for polymer synthesis. The strain grew on the hydrolysate and produced polymers with yields similar to those from pure glucose. The potential replacement of pure sugars by lignocellulosic biomass was demonstrated in this study. The final part of this thesis (chapter 5) is the conclusion.

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Chapter 2

Production of P(LA-*co*-3HB) polyester from xylose

2.1 Introduction

The lactate-based polymer, poly(lactate-*co*-3-hydroxybutyrate) [P(LA-*co*-3HB)] is a new member of the PHA family produced from renewable biomass in engineered microorganisms expressing LA-polymerizing enzyme (LPE) (Song et al., 2012; Taguchi et al., 2008). This copolymer is wholly biosynthesized from refined glucose by one-pot fermentation and the variation of the LA/3HB ratio in P(LA-*co*-3HB) generates polymers with different properties (Yamada et al., 2011).

For industrial production of P(LA-*co*-3HB), the use of inexpensive carbon sources is essential. Lignocellulosic biomass is an abundant and inexpensive carbon source that can replace refined sugars (Adsul et al., 2011). The carbohydrates in lignocellulosic biomass that could be employed are cellulose that mainly consist glucose and the xylose-rich hemicellulose (Weber et al., 2010). The use of cellulose is extensively studied however, the utilization of hemicellulose is also essential for the efficient bioconversion of lignocellulosic biomass into value-added chemicals and materials (Saha, 2003). Therefore, to establish a hemicellulosic hydrolysate platform for the production of biopolyesters, it is essential to design a system whereby xylose is efficiently utilized to achieve optimum polymer yields and productivities. The aim of this study was therefore to establish a platform for efficient biotransformation of xylose into biopolyesters. The

focus on xylose stems from its abundance in the inedible lignocellulosic biomass (Liu et al., 2010). Moreover, the xylose separation technologies through extraction and hydrolysis of the hemicelluloses have been well characterized (Saha, 2003). Therefore, xylose is an attractive inexpensive feedstock for the production of biopolyesters. Already, demands for multiple utilization of xylose for the production of value-added chemicals are increasing (Dien et al., 2001; Lee, 1998; Manow et al., 2012). 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.

In this study, the platform for polyester production was based on *Escherichia coli* (*E. coli*) that is easy to culture and utilizes xylose and all the lignocellulosic biomass sugars as carbon sources (Huffer et al., 2012). Furthermore, it does not have a depolymerase, hence it can accumulate polyesters with high molecular weight (Yim et al., 1996).

Xylose has been used for the production of polyhydroxybutyrate [P(3HB)]. However, when compared to glucose, it gives poor yields (Keenan et al., 2004; Lee, 1998; Silva et al., 2004). To improve P(3HB) productivity from xylose, some researchers have suggested the conversion of xylose first into lactic acid by lactic acid bacteria, then feeding the lactic acid into P(3HB) producing bacteria as a carbon substrate (Tsuge et al.,

1999). Although this strategy achieved high P(3HB) productivity, the multi-step processes involved makes the bioconversion of xylose costly. In that regard, a direct single-step process for efficient bioconversion of xylose into biopolyesters is necessary.

In this study, xylose was first applied for P(3HB) production and yields compared with those from glucose. Since lactic acid could be produced efficiently from xylose, the production of lactic acid in *E. coli* was interfaced with polyester production with prospects of improving the polymer productivity. Lactic acid could be polymerized in a scheme shown in Fig. 2.1 (Nduko et al., 2013) to produce P(LA-*co*-3HB), which has been produced from glucose in recombinant *E. coli* expressing a Ser325Thr/Gln481Lys mutant of PHA synthase [PhaC1_{Ps}(ST/QK)] from *Pseudomonas* sp. 61-3 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 *R. eutropha* (Taguchi et al., 2008). Elimination of PCT from the system leads to the synthesis of P(3HB). In this study, using the metabolic pathways illustrated in Fig. 2.1, P(LA-*co*-3HB) production in *E. coli* from xylose was attempted for the first time and efforts that enhanced lactate incorporation into P(LA-*co*-3HB) and polymer productivities were made by the use of a further evolved LPE.

2.2 Materials and methods

2.2.1 Bacterial strains and plasmids

The Keio collection mutant *E. coli* JW0885 (*pflA*), which overproduces lactic acid (purchased from National BioResource Project, Japan) (Baba et al., 2006; Zhu and Shimizu, 2005), was used as a host for the polymer production. The expression vector pTV118N*pctphaCI*_{Ps}(ST/QK)*AB* having *pct*, *phaCI*_{Ps}(ST/QK), *phaA*, and *phaB* genes (Taguchi et al., 2008) was used for the production of P(LA-*co*-3HB). Moreover, recombinant *E. coli* harboring pTV118N*pctphaCI*_{Ps}(ST/FS/QK)*AB* with a Ser325Thr/Phe392Ser/Gln481Lys mutated PHA synthase gene from *Pseudomonas* sp. 61-3 (Yamada et al., 2010) was used for the production of high LA-containing polymers. The pGEM*phaCI*_{Ps}(ST/QK)*AB* (Takase et al., 2003) was used for P(3HB) production.

2.2.2 Culture conditions

Test-tube cultures

Recombinant *E. coli* cells harboring either pTV118N*pctphaCI*_{Ps}(ST/QK)*AB* or pTV118N*pctphaCI*_{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*phaCI*_{Ps}(ST/QK)*AB* were prepared likewise for the production of P(3HB).

Shake flask cultures

To produce P(LA-*co*-3HB) in shake flasks, seed cultures of recombinant *E. coli* cells harboring pTV118N*pctphaCI*_{Ps}(ST/QK)*AB* or pTV118N*pctphaCI*_{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*phaCI*_{Ps}(ST/QK)*AB* were cultured similarly for P(3HB) production.

2.2.3 Polymer, cell growth, sugars and metabolites analysis during P(LA-*co*-3HB) and P(3HB) production

The samples from *E. coli* shake flask cultures were taken periodically during cultivation and centrifuged at 12 000 rpm for 5 min to separate cells and the supernatant. The cells were lyophilized and used for analyzing cell growth and polymer production.

Polymer analysis by HPLC

The lyophilized cells were treated with concentrated sulphuric acid (H₂SO₄) at 120 °C for 45 minutes in micro tubes with screw cap to convert intracellular 3HB units into crotonic acid or LA unit into acrylic acid for quantitation by high-performance liquid chromatography (HPLC) at 60 °C using an aminex HPX-87H ion exclusion column (7.8 mm I.D. × 300 mm; (Bio-Rad laboratories, Hercules, CA, USA). The solution was diluted with 0.014 N H₂SO₄ 10 times (or more) then filtered through a 0.20 µm PTFE membrane (Advantec, Tokyo) sieves. Ten µL of the filtered samples were then applied by Auto injector (Jasco) and eluted with 0.014 N H₂SO₄ at flow rate of 0.7 ml/min. The absorbance of crotonic acid and acrylic acid were measured at 210 nm using Jasco UV-2077 plus 4-λ intelligent UV/VIS detector. The relationship between absorbed area of detected crotonic acid/ acrylic acid and the 3HB/ LA unit contents in P(LA-co-3HB) of samples was derived from the calibration curves prepared from P(3HB) and PLA standards.

Sugar and metabolites analysis by HPLC

The concentrations of sugars, 3-hydroxybutyric acid (3HB) and acetic acid in the culture supernatants were determined by first passing the supernatant through a 0.2- μ m cellulose acetate filter. The sugars, 3HB and acetate in the medium were analyzed using HPLC (Jasco, Japan) equipped with an Aminex HPX-87H column (Bio-Rad) and an RI detector. Samples were eluted by 0.014 N H₂SO₄ at a flow rate of 0.5 mL/ min at 60 °C.

Lactic acid analysis

The concentration of lactic acid in the supernatant was estimated using a D-/L-lactic acid assay kit (Megazyme international, Ireland). The cell-free supernatant of the culture medium was prepared by centrifugation and passage through 0.2- μ m cellulose acetate filter. Lactic acid was then determined as per the manufacturer's instructions.

2.2.4 Determination of 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 sampled at intervals and washed by ice-cold phosphate buffer. The cofactors were then extracted and their concentrations were estimated using EnzyChromTM/NADP⁺/NADPH (ECNP-100) and EnzyChromTM/

NAD⁺/NADH (E2ND-100) kits (Bioassay systems, USA) as per the manufacturer's instructions.

2.2.5 Polymer extraction and analyses

The polymers were extracted from lyophilized cells with chloroform at 60°C 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°C for 3 days to prompt the precipitation, which increased polymer recovery. 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) was dissolved in 1 mL of CDCl₃ and set for ¹H NMR analysis by a broker MSL 400 spectrometer (400 MHz) and the chemical shifts were recorded in parts per million using tetramethylsilane (TMS) as an internal reference.

2.3 Results

2.3.1 P(3HB) production from xylose

The use of xylose for P(3HB) production was investigated using recombinant *E. coli* JW0885 harboring pGEM ϕ haC1_{Ps}(ST/QK)AB without the *pct* gene, whose gene product is involved in the LA-CoA generation (Fig. 2.1).

The P(3HB) yield using cells cultivated on 20 g l⁻¹ xylose was 4.1 g l⁻¹ (Table 2.1, No. 1), while the cells cultivated on 20 g l⁻¹ glucose produced 5.7 g l⁻¹ (Table 2.1, No. 2), which was consistent with previously reported results (Keenan et al., 2004; Lee, 1998), and proved the fact that although theoretically 20 g l⁻¹ of glucose or xylose is supposed to give the same amount of P(3HB) (9.6 g/L) (Eq.1) (Tyo et al., 2010; Uchino et al., 2008), glucose gives a higher P(3HB) productivity than xylose.

Eq. 1

Sugars: 20 g/L xylose (M_w : 150): 20 g/L glucose (M_w : 180)

Mole ratio: 6 : 5

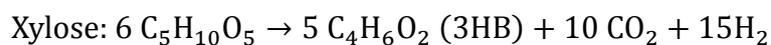
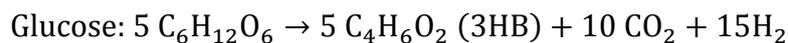


Table 2.1 P(3HB) and P(LA-co-3HB) biosynthesis from xylose and glucose in *E. coli* JW0885 expressing PhaC1_{Ps}(ST/QK)(Nos. 1 – 4); P(LA-co-3HB) production in *E. coli* JW0885 expressing PhaC1_{Ps}(ST/FS/QK) (Nos. 5 and 6).

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_{Ps}(ST/QK), phaAB</i>	xylose	7.0 ± 0.2	4.1 ± 0.3	nd	4.1 ± 0.3	0.21 ± 0.01	59 ± 4	-
2	<i>phaC1_{Ps}(ST/QK), phaAB</i>	glucose	9.3 ± 0.2	5.7 ± 0.6	nd	5.7 ± 0.6	0.29 ± 0.03	61 ± 5	-
3	<i>pct, phaC1_{Ps}(ST/QK), 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, phaC1_{Ps}(ST/QK), 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, phaC1_{Ps}(ST/FS/QK), 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, phaC1_{Ps}(ST/FS/QK), 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

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; nd, not detected. Data is average ± standard deviation of quadruplicate experiments (Nduko et al., 2013).

2.3.2 P(LA-co-3HB) production from xylose

In the next step, the biosynthesis of P(LA-co-3HB) from xylose was attempted using the recombinant *E. coli* JW0885 harboring the pTV118NpctphaC1_{Ps}(ST/QK)AB. In contrast to the P(3HB) production, 5.5 g l⁻¹ of P(34 mol% LA-co-3HB) was produced from 20 g l⁻¹ xylose (Table 2.1, No. 3). This indicated for the first time that xylose could be used for P(LA-co-3HB) synthesis. For glucose, the cells produced 6.5 g l⁻¹ of P(26 mol% LA-co-3HB) (Table 2.1, No. 4); productivity that was higher than that of xylose. Focusing on the LA incorporation, the yield of 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 for gaining 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. Thus, the higher productivity of P(LA-co-3HB) than P(3HB) is mainly due to the incorporation of LA units into the polymers. Therefore, xylose and glucose are utilized more efficiently for the production of P(LA-co-3HB) compared to P(3HB).

2.3.3 Enhancement of LA incorporation to improve polymer productivity

As described in section 2.3.2 above, the incorporation of LA to form P(LA-co-3HB) led to higher polymer productivity compared to the production of P(3HB).

Therefore, to further enhance the incorporation of LA units into the polymer thereby improving polymer productivity, a further 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) was applied for P(LA-*co*-3HB) biosynthesis from xylose. The use of PhaC1_{Ps}(ST/FS/QK) led to 2.5- and 2.3-fold increases in the LA unit yields in P(LA-*co*-3HB)s from xylose and glucose, respectively (Table 2.1, Nos. 5 and 6) relative to the LA units yields obtained by using PhaC1_{Ps}(ST/QK) (Table 2.1, Nos. 3 and 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). 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).

2.3.4 Metabolic flux analysis from xylose toward polymers

The results shown in Table 2.1 suggested xylose to be superior to glucose as a carbon source for P(LA-co-3HB) production because of the higher LA fraction and LA unit yields. To gain insights into the metabolic fluxes from xylose, 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 (Figs. 2.2 A – F) were analyzed. Formic acid and succinic acid; metabolites in *E. coli* 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 below the detection limit of 0.01 g l⁻¹ 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 (C₃) 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 (Fig. 2.2). The cell growth profiles are shown in Fig. 2.3.

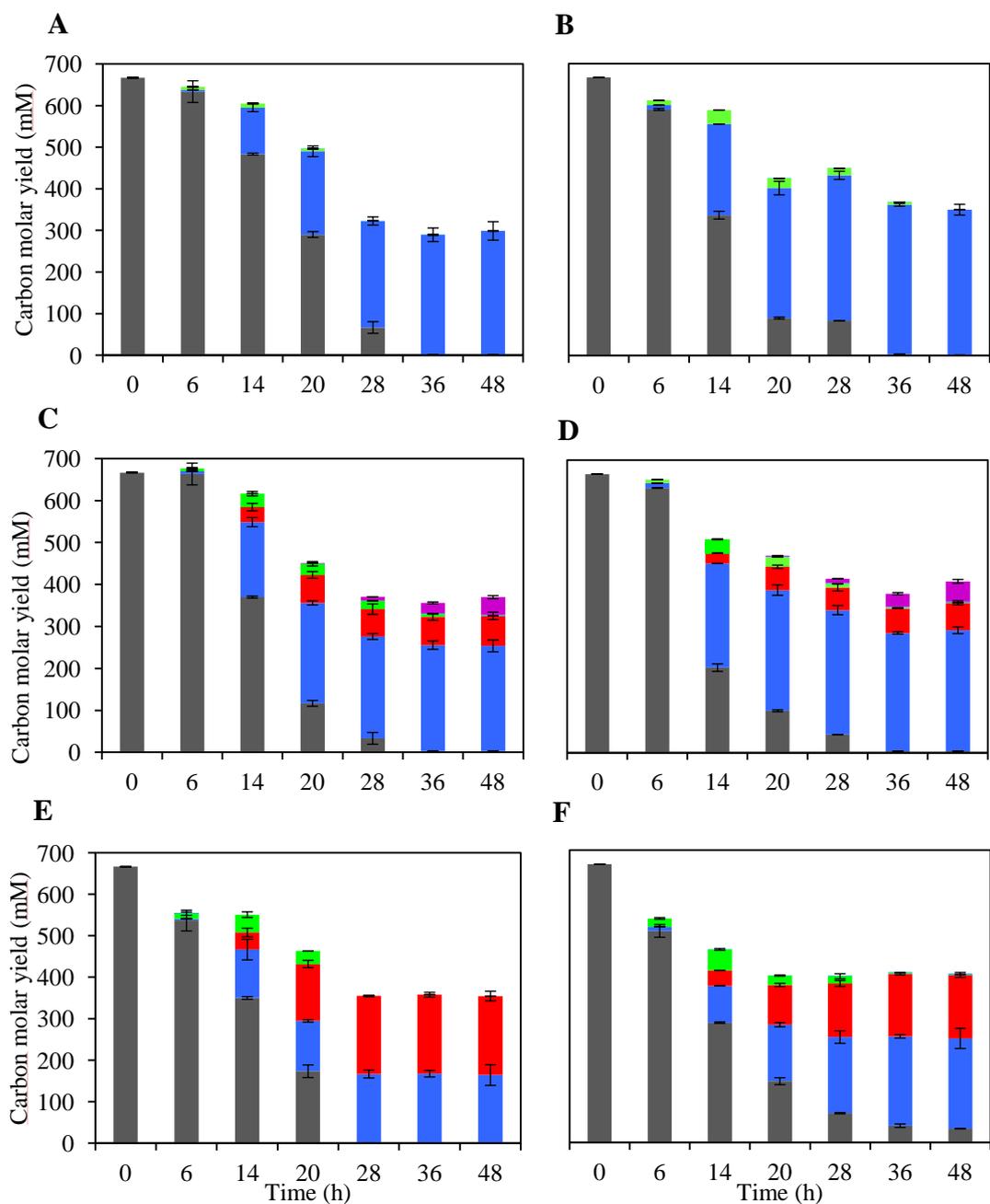


Fig. 2.2 Time course profiles of molar carbon quantity for xylose and glucose and metabolites produced in *E. coli* JW0885. Cells harboring pGEM*phaC1*_{ps}(ST/QK)*AB* (A and B), pTV118N*pctphaC1*_{ps}(ST/QK)*AB* (C and D) and pTV118N*pctphaC1*_{ps}(ST/FS/QK)*AB* (E and F) were grown on xylose (A, C and E) and glucose (B, D and F). *Gray bars*, residual sugars; *blue*, polymeric 3HB; *red*, polymeric LA; *green*, LA in the medium; *purple*, 3HB in the medium; *black*, acetic acid. The acetic acid concentrations are very small, so they are presented in a separate figure (Fig. 2.4). The data are means \pm standard deviations of three independent trials (Nduko et al., 2013).

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

The sugars supplied initially (20 g L^{-1}) were equal to 133 mM xylose and 111 mM glucose, respectively, both of which corresponds to 667 mM carbon molar quantity. During the production of P(3HB), cell growth and sugar consumptions were minimal in the first 6 h, reflecting the low polymer synthesis rates during the same time period (Figs. 2.2 A, B and Fig. 2.3 A). At 6 h, only a small amount of P(3HB) was detected, suggesting that the cells produced little polymer during the lag and early exponential growth phases. For the xylose cultures, during the 6 – 20 h interval, cell mass formation was rapid (Fig. 2.3 A) and xylose was consumed at $24 \text{ mM carbon h}^{-1}$ (Fig. 2.2 A). Over the same interval, P(3HB), a small amount of lactic acid, and trace amounts of acetic acid were produced (Figs. 2.2 A and 2.4 D). The sum of carbon decreased probably because the carbons in xylose were used for cell growth and/or emitted as carbon dioxide. For glucose, the cell mass formation and sugar consumption rate ($37 \text{ mM carbon h}^{-1}$) during 6 – 20 h period (Figs. 2.2 B and 2.3 A) was faster compared to that of xylose, and the productions of P(3HB) and lactic acid were also higher. The sugars were completely consumed at 36 h and the excreted lactic acid was also retaken. The production and uptake of lactic acid from the medium was in good agreement with the medium pH changes (Fig. 2.4 A). 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 because CO₂ is emitted during the synthesis of acetyl-CoA from pyruvate (Fig. 2.1).

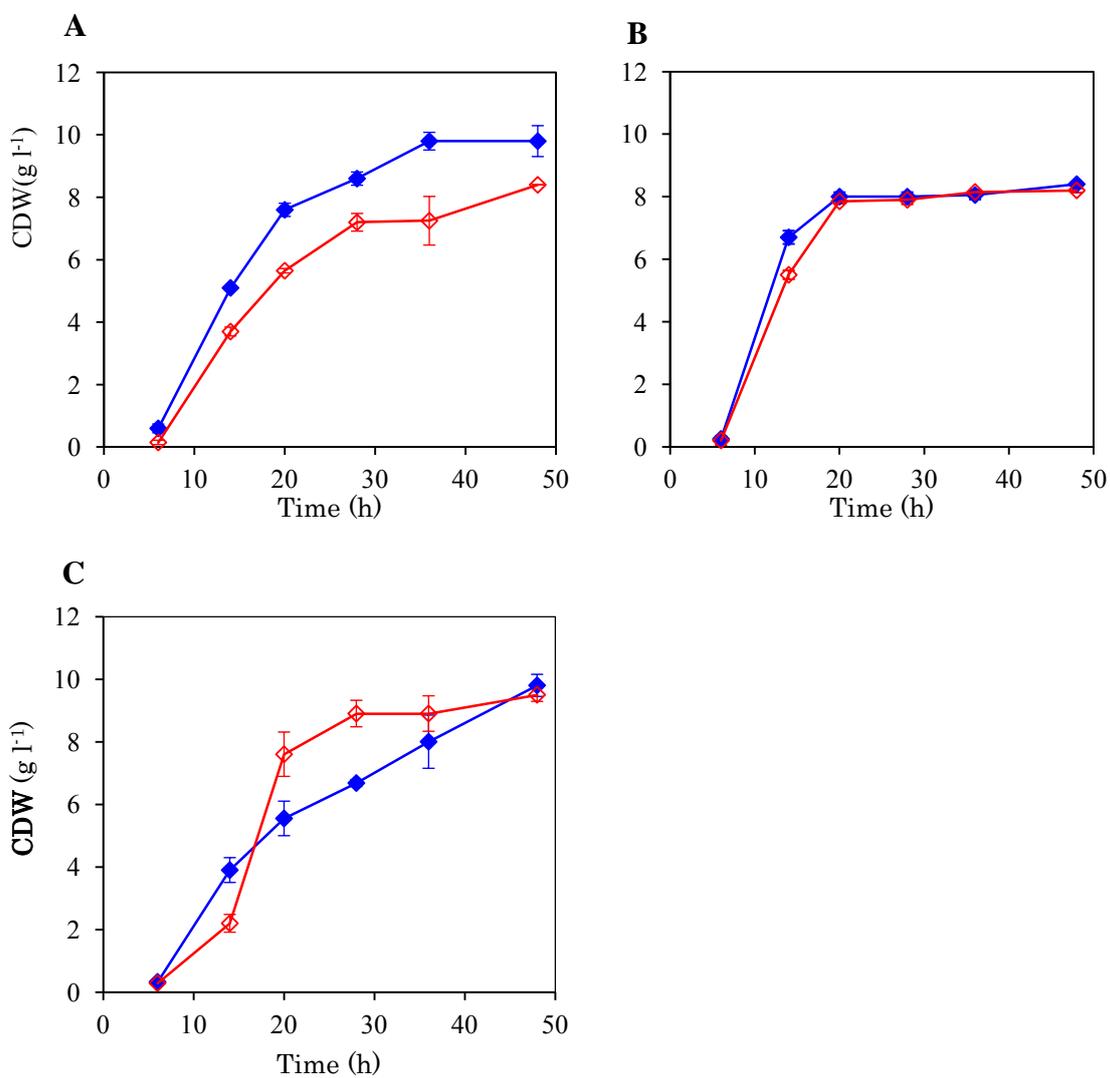


Fig. 2.3 Time profiles for cell mass formation by cells harboring pGEMphaC1_{ps}(ST/QK)AB (A), pTV118NpctphaC1_{ps}(ST/QK)AB (B) and pTV118NpctphaC1_{ps}(ST/FS/QK)AB (C) grown on xylose (*open diamonds-red color*) and glucose (*closed diamonds-blue color*).

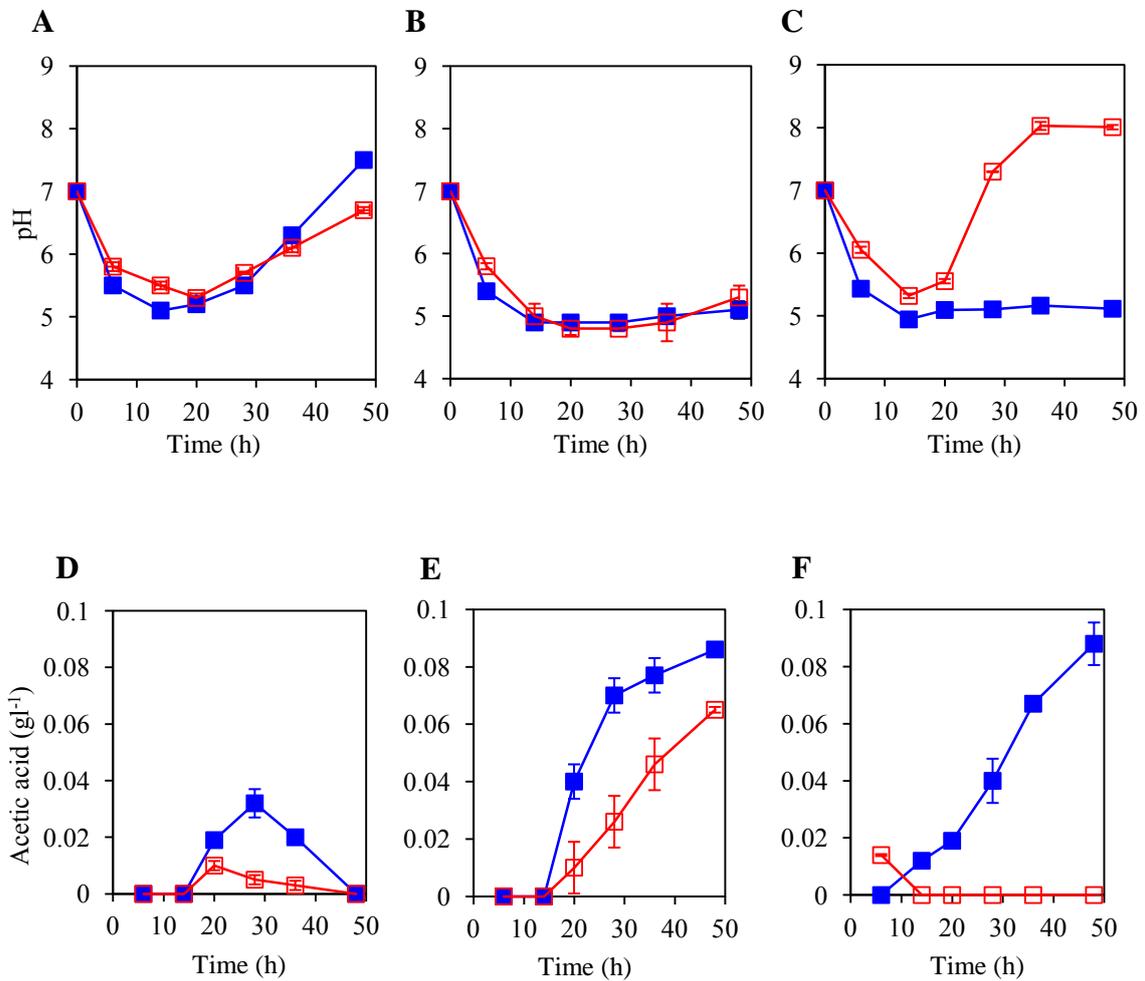


Fig. 2.4 Time profiles of pH changes (**A**, **B** and **C**) and acetic acid production (**D**, **E** and **F**) by cells harboring pGEM $phaC1_{Ps}(ST/QK)AB$ (**A** and **D**), pTV118N $pctphaC1_{Ps}(ST/QK)AB$ (**B** and **E**) and pTV118N $pctphaC1_{Ps}(ST/FS/QK)AB$ (**C** and **F**) grown on xylose (*open squares-red color*) and glucose (*closed squares-blue color*).

P(LA-*co*-3HB) production increases carbon yield from xylose

During the production of P(LA-*co*-3HB) using PhaC1_{Ps}(ST/QK), cell mass formation and sugar consumptions were minimal in the first 6 h for both glucose and xylose (Figs. 2.2 C, D and 2.3 B), similar to the cases of P(3HB) production. In contrast, during the production of P(LA-*co*-3HB) using PhaC1_{Ps}(ST/QK), in the 6 – 20 h interval; sugar consumption rates (37 and 38 mM carbon h⁻¹ for xylose and glucose, respectively) were higher (Figs. 2.2 C and D) compared to the corresponding values during P(3HB) synthesis over the same time period, and coincided with the period of rapid cell mass formation (0.55 g l⁻¹ h⁻¹ for both xylose and glucose cultures) (Fig. 2.3 B). In the same interval, vigorous polymer synthesis (19 and 20 mM carbon h⁻¹ for xylose and glucose, respectively) occurred (Figs. 2.2 C and D), which could be associated with the sugar consumptions, and were higher than corresponding values during P(3HB) production (14 and 18 mM h⁻¹ for xylose and glucose, respectively) (Figs. 2.2 A and B). At 20 h, small amounts of sugars still remained in medium (Figs. 2.2 C and D) that were depleted at 36 h however; there was no observable cell mass formation (Fig. 2.3 B) and polymer synthesis during this time period.

When P(LA-*co*-3HB) was produced by PhaC1_{Ps}(ST/QK), lactic acid was detected at 6 h then peaked at 14 h for both xylose and glucose (Figs. 2.2 C and D). In the

case of xylose, the lactic acid concentration was approximately 3-fold higher (Fig. 2.2 C) than that for P(3HB) (Fig. 2.2 A), indicating the enhanced flux towards lactic acid. The excreted lactic acid was eventually utilized by the cells (Fig. 2.2 C). However, the uptake of lactic acid 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 till the end of culturing for both xylose and glucose (Figs. 2.2 C and D).

Interestingly, 3-hydroxybutyric acid was detected in the medium at 28 – 48 h during P(LA-*co*-3HB) production from xylose and glucose using PhaC1_{Ps}(ST/QK) (Figs. 2.2 C and 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 culture. PCT may transfer a CoA moiety from 3HB-CoA to acetic acid for releasing 3-hydroxybutyric acid as previously demonstrated (Matsumoto et al., 2013). Trace amounts of acetic acid (<0.1 g l⁻¹) were detected during polymer production (Figs. 2.2 C, D and Fig. 2.4 E) and their contribution to carbon yield was low. The production of 3-hydroxybutyric acid, acetic acid and the synthesis and uptake of lactic acid from the medium had a good correlation with the medium pH (Figs. 2.2 C, D and Fig. 2.4 B and E).

Overall, the final carbon yield of the polymers from xylose and glucose were 310 and 331 mM, respectively. This indicated that for xylose, the incorporation of LA

units into the polymer by LPE is efficient in carbon conversion from sugars to polymer compared to the production of P(3HB).

PhaC1_{Ps} (ST/FS/QK) channeled the carbon flux from 3HB units to LA units

During the P(LA-*co*-3HB) production by PhaC1_{Ps}(ST/FS/QK), the sugar consumption and cell mass formation rates revealed different profiles (Figs. 2.2 E, F and Fig. 2.3 C) compared with those expressing PhaC1_{Ps}(ST/QK). In the first 6 h, 130 and 160 mM carbon of xylose and glucose, respectively were consumed although with little cell mass formation (Figs. 2.2 E, F and Fig. 2.3 C), suggesting that *E. coli* cells could be generating other metabolites rather than polymers. In the 6 – 20 h interval, the sugar consumption rates (26 mM carbon h⁻¹ for both xylose and glucose) (Figs. 2.2 E and F) were lower than those observed during P(LA-*co*-3HB) production by PhaC1_{Ps}(ST/QK) over the same time period (Figs. 2.2 C and D) and were reflected in the slower polymer synthesis. It should be noted that the low polymer production rate at the early stage was partly due to the low accumulating rate of 3HB units in P(LA-*co*-3HB) (Figs. 2.2 E and F). On the contrary, the lactic acid concentrations increased up to 43 and 50 mM carbon yields for xylose and glucose, respectively at 14 h, suggesting that carbon flux was drawn into lactic acid production (Figs. 2.2 E and F). Subsequently, the incorporation of LA

units into the polymers increased up to 164 and 131 mM carbon yield of LA units from xylose and glucose, respectively.

At later stages of cultivation (20 – 28 h), the sugars were further consumed and surprisingly, all xylose was exhausted at 28 h, coinciding with the cessation of the cell mass formation and polymer synthesis (Figs. 2.2 E and 2.3 C). In contrast, cell mass formation from glucose continued at low rates up to the end of culture (Fig. 2.3 C). 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 and acetic acid formation (Figs. 2.2 E, F and Figs. 2.4 C, F). For the case of glucose, the pH profiles were similar to the case of P(LA-*co*-3HB) synthesis by PhaC1_{Ps}(ST/QK) (Figs. 2.4 B and C), possibly due to the formation of acetic acid in both cases.

The final carbon yield of the polymers from xylose and glucose were 348 and 372 mM, respectively. This suggested that the use of PhaC1_{Ps}(ST/FS/QK) was effective for improving the carbon yields from the sugars compared to the use of PhaC1_{Ps}(ST/QK).

2.3.5 Nicotinamide nucleotide cofactor levels during polymer productions

The results in Figs. 2.2 E and F indicated that xylose and glucose were converted into P(LA-*co*-3HB) copolymers with similar carbon yield but with a different LA/3HB

ratio. The possible reason to cause this difference could be the supply of nicotinamide cofactors, because the synthesis of lactic acid from pyruvate is NADH-dependent, whereas the 3HB-CoA supplying pathway uses NADPH (Fig. 2.1). To date, there is no information on the reducing cofactor levels during P(LA-*co*-3HB) production. Therefore, [NADPH]/[NADP⁺] and [NADH]/[NAD⁺] levels were determined during the production of P(LA-*co*-3HB) and P(3HB) (Tables 2.2 A, B and C).

During the P(3HB) production, the [NADPH]/[NADP⁺] ratios at 14 h, when the polymer synthesis rate was maximum were similar between xylose (1.0) and glucose (1.1; Table 2.2 A). Likewise, during the synthesis of P(LA-*co*-3HB) by PhaC1_{P_s}(ST/QK), there was no difference in [NADPH]/[NADP⁺] ratios for xylose (1.2) and glucose (1.2) (Table 2.2 B). In the case of the production of P(LA-*co*-3HB) using PhaC1_{P_s}(ST/FS/QK), [NADPH]/[NADP⁺] ratios for glucose (1.7) were slightly higher than those for xylose (1.2; Table 2.2 C). Overall, the [NADPH]/[NADP⁺] ratios tended to be slightly higher under the low 3HB-accumulating conditions, maybe because NADPH was consumed for synthesizing 3HB-CoA.

The [NADH]/[NAD⁺] ratios in the cells growing on glucose were higher or at similar levels to those for xylose (Tables 2.2 A – C). In particular at 14 h, the sugar consumption rates were faster for glucose than xylose that could generate more NADH

via glycolysis (Figs. 2.2 A – F). Although the higher $[\text{NADH}]/[\text{NAD}^+]$ ratio was thought to induce higher LA productivity, the LA unit yields in P(LA-*co*-3HB) synthesized from glucose were lower compared to those from xylose (Table 2.1), suggesting that the carbon fluxes toward other compounds, particularly 3HB units, influenced the production of LA units rather than the $[\text{NADH}]/[\text{NAD}^+]$ ratio.

Table 2.2 Nicotinamide nucleotide cofactor levels in *E. coli* JW0885 grown on xylose and glucose during polymer production

A. Cells expressing PhaC1_{PS}(ST/QK) for P(3HB) production

Time (h)	Sugar	NADPH	NADP ⁺	NADH	NAD ⁺	NADPH/	NADH/
		($\mu\text{M g}^{-1}\text{CDW}$)	NADP ⁺	NAD ⁺			
6	xylose	2.60±0.00	2.60±0.20	4.60±0.20	8.00±2.90	1.00±0.00	0.58±0.02
	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
14	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
	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
28	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
	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
48	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
	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

B. Cells expressing PhaC1_{PS}(ST/QK) for P(LA-co-3HB) production

Time (h)	Sugar	NADPH	NADP ⁺	NADH	NAD ⁺	NADPH/	NADH/
		($\mu\text{M g}^{-1}\text{CDW}$)	NADP ⁺	NAD ⁺			
6	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
	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
14	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
	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
28	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
	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
48	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
	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

C. Cells expressing PhaC1_{PS}(ST/FS/QK) for P(LA-co-3HB) production

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	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
	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
14	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
	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
28	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
	glucose	0.14±0.03	0.06±0.00	2.67±0.03	0.35±0.07	2.48±0.50	7.70±1.60
48	xylose	0.08±0.00	0.06±0.00	6.91±1.80	1.68±0.63	1.40±0.00	4.64±2.82
	glucose	0.09±0.04	0.05±0.00	12.24±0.30	2.87±0.13	1.76±0.82	4.27±0.30

CDW, cell dry weight

2.3.6 The monomer composition and molecular weights of the polymers produced from xylose

To confirm the structure of the P(LA-*co*-3HB)s synthesized from xylose, the copolymers were subjected to ¹H NMR (Fig. 2.5). The result indicated that the polymers were random copolymers of LA and 3HB units that were in good conformation with earlier reports (Yamada et al., 2009). The molecular weight (M_n) of the polymers varied in the range of 1.2 to 5.6×10^4 depending on their LA fractions (Table 2.3). In particular, the P(LA-*co*-3HB) synthesized from xylose or glucose using PhaC1_{Ps}(ST/FS/QK) had lower molecular weights compared to those by PhaC1_{Ps}(ST/QK) (Table 2.3). Thus, there was an inverse relationship between molecular weight and LA fraction that was consistent with earlier reported results (Shozui et al., 2011; Song et al., 2012; Yamada et al., 2011). These results indicate that the use of xylose for P(LA-*co*-3HB) production has no particular effect on the molecular weights of polymers compared to glucose, except for the higher LA fractions.

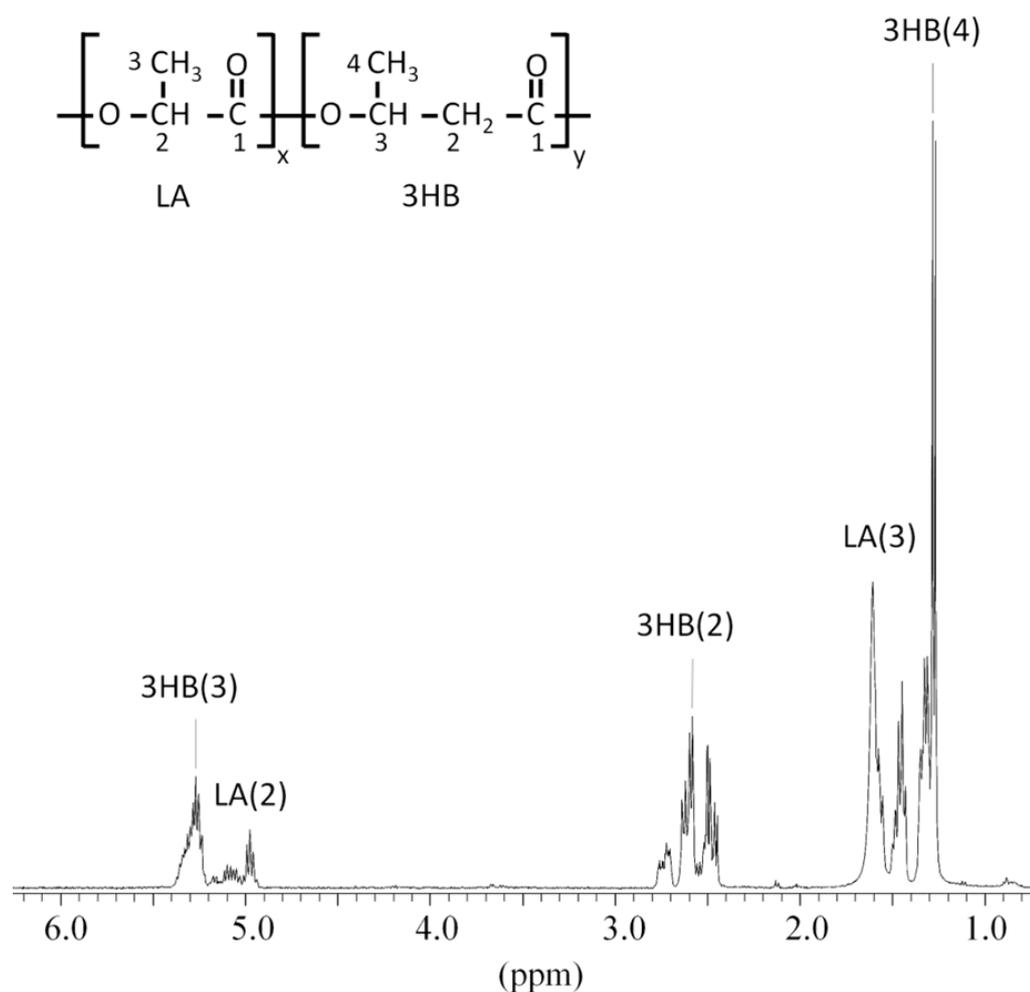


Fig. 2.5 ^1H NMR spectrum of P(34 mol% LA-*co*-3HB) synthesized by PhaC1_{Ps}(ST/QK) from xylose. The ^1H NMR chemical shifts around 1.5 and 5.0 ppm were assigned to the methyl and methine groups of the LA unit within the main chain of the copolymer as described previously (Yamada et al., 2010).

Table 2.3 Molecular weights of P(3HB) and P(LA-*co*-3HB) synthesized using *E. coli* JW0885 expressing PhaC1_{Ps}(ST/QK) (Nos. 1– 4) or PhaC1_{Ps}(ST/FS/QK) (Nos. 5 and 6).

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- <i>co</i> -3HB)	4.0	17	4.1
4	glucose	P(26 mol% LA- <i>co</i> -3HB)	4.2	33	7.8
5	xylose	P(60 mol% LA- <i>co</i> -3HB)	1.2	1.4	1.2
6	glucose	P(47 mol% LA- <i>co</i> -3HB)	1.2	1.4	1.2

The experimental numbers refer the culture conditions in Table 2.1. M_n , number-average molecular weight; M_w , weight-average molecular weight. M_w/M_n , polydispersity index.

2.4 Discussion

In this study, for the first time, the synthesis of P(LA-*co*-3HB) from xylose was demonstrated. The advantage of xylose utilization can be justified here in that the cellular content and LA fraction of the copolymer were compatible and higher, respectively, compared to the utilization of glucose. Regarding the polymer content, in contrast, glucose gave a much higher P(3HB) yield than xylose, in consistent with earlier reports (da Silva et al., 2009; Keenan et al., 2004; Lee, 1998; Silva et al., 2004; Young et al., 1994). This contrasted result between the two polymers should be ascribed to the supplying capacities of LA and 3HB units, which are generated in the pathways related to 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 would be a key factor affecting monomer fluxes channeling to the polymer, since PhaB that generates 3HB-CoA is NADPH-dependent for its activity (Tyo et al., 2010). Although [NADPH]/[NADP⁺] ratios during the polymer productions were not apparently different between the xylose and glucose (Tables 2.2 A, B and C), it has been known that the carbon flux in *E. coli* grown on xylose could be drawn into the TCA cycle to regenerate NADPH (Chin et al., 2009; Lim et al., 2002; Shi et al., 1999). Therefore, the high demand of NADPH for synthesizing 3HB-CoA may decrease the overall carbon

yield from xylose into 3HB units compared to the cases of glucose, and in contrary, the channeling of flux from 3HB to LA units can drastically increase the polymer yield from xylose.

For 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 by PCT from lactic acid and presumably acetyl-CoA as a CoA donor, generating a stoichiometric amount of acetic acid (Fig. 2.1). This reaction could 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} (Figs. 2.4 D – F) compared to typical cases for *E. coli* grown on sugars (Zhou et al., 2011). This suggests that acetic acid might be recycled intracellularly into acetyl-CoA, and therefore, the PCT-catalyzed LA-CoA supplying route may not be a limiting factor for achieving high polymer productivities.

To reinforce the carbon fluxes from LA-CoA to LA units, an engineered LPE [PhaC1_{Ps}(ST/FS/QK)] that was shown to give a higher LA fraction in a previous study was recruited (Yamada et al., 2010). The difference between the two LPEs was the polymer production at the early stage (6 – 14 h), that is, PhaC1_{Ps}(ST/QK) accumulated more 3HB units, while PhaC1_{Ps}(ST/FS/QK) incorporated relatively higher LA units into the copolymer (Figs. 2.2 C – F). This suggests a relatively higher reactivity of

PhaC1_{Ps}(ST/FS/QK) toward LA-CoA than 3HB-CoA compared to PhaC1_{Ps}(ST/QK). In addition, the use of PhaC1_{Ps}(ST/FS/QK) remarkably changed the ratio of LA units over 3HB units, while slightly increased in the overall carbon yields from sugars into polymer. Thus, LPE contributes to the relative fluxes to LA and 3HB units rather than the absolute polymer yield. Therefore, in order to further increase the LA unit yields in P(LA-*co*-3HB), the further engineering of LPE to shift substrate specificity to LA-CoA as well as the related strategies discussed by Taguchi et al. (Taguchi and Doi, 2004) and/or the suppression of 3HB-CoA supplying pathway will be necessary.

In this study, the utilization of xylose for P(LA-*co*-3HB) production demonstrated its dual merits in its quantity and quality. Although xylose utilization 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, P(LA-*co*-3HB) can be a promising target for the utilization of xylose. Another finding was the enrichment of LA units in the copolymer by changing the non-related carbon sources, because, the regulation of monomer composition in PHAs by feedstock has been only 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).

This study has demonstrated the production of P(LA-*co*-3HB) from xylose for the first time. Xylose gave a higher LA fraction (60 mol%) in P(LA-*co*-3HB) than glucose (47 mol%) and the polymer yields from either sugar were comparable. The time course analysis of polymer and organic acids synthesis acted as a reliable and facile monitoring tool of the metabolic fluxes. Since xylose forms a substantial portion of the lignocellulosic biomass and can be obtained easily as xylose-rich hemicellulose hydrolysates, this system can open avenues for the cost-effective production of P(LA-*co*-3HB)s.

2.5 References

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Chapter 3

Enhanced production of P(LA-co-3HB) from xylose in engineered

***Escherichia coli* overexpressing a galactitol transporter**

3.1 Introduction

The variation of the LA/3HB ratio in P(LA-*co*-3HB) has demonstrated the generation of polymers with properties different from those of respective homopolymers (Yamada et al., 2011). Therefore, there have been concerted efforts to regulate the monomer composition in the P(LA-*co*-3HB)s using enzyme and metabolic engineering, fermentation technology and culture media manipulation techniques (Shozui et al., 2011; Song et al., 2012; Yamada et al., 2009). During the course of these studies, it has emerged that the productivity of P(LA-*co*-3HB) having LA fraction greater than 50 mol% is relatively low ($>0.2 \text{ g l}^{-1}$); limiting the exploration of polymer properties of the LA-enriched copolymers (Shozui et al., 2011; Song et al., 2012).

In that context, the utilization of xylose and a further evolved LPE, PhaC1_{PS}ST/FS/QK has achieved the production of P(60 mol% LA-*co*-3HB) with the productivity of 7.3 g l^{-1} from 20 g l^{-1} xylose (Chapter 2, Table 2.1) (Nduko et al., 2013), which is a critical breakthrough toward efficient production of LA-enriched polymers. In addition, since xylose can be readily extracted from the lignocellulosic biomass (Liu et al., 2010), it is a suitable substrate for cost-effective production of P(LA-*co*-3HB)s. Pursuant to that, the present study embarked on the construction of a platform for high-yield synthesis of LA-enriched P(LA-*co*-3HB) from xylose.

To meet this goal, two distinct strategies were investigated. In the earlier study (Chapter 2), a $\Delta pflA$ mutant of *E. coli* was used as a host strain that strengthened the supply of lactic acid (Fig. 3.1), which could eventually be incorporated into P(LA-co-3HB) (Shozui et al., 2010; Yamada et al., 2010). However, other gene disruptants, which have been reported to increase lactic acid production, as well as their parent strain *E. coli* BW25113 have not been evaluated for P(LA-co-3HB) synthesis from xylose. Thus, *E. coli* BW25113 and four mutants (Δpta , $\Delta ackA$, $\Delta poxB$, and Δdld), which reduces the competition for carbon flux at the pyruvate node and redirect it into lactic acid (Zhou et al., 2011) were recruited (Fig. 3.1). Secondly, the study focused on the enhancement of xylose uptake by *E. coli*. In this organism, there are two different D-xylose-specific transport systems; the XylE, a member of the major facilitator superfamily of transporters and the ATP-binding cassette (ABC) transporter encoded by the *xylFGH* operon (Hasona et al., 2004). The ATP-dependent transporter is the major xylose transporter, and could reduce the amount of ATP available to the cells. Therefore, to eliminate the possibility of ATP limitation, a non-ATP consuming galactitol permease, GatC, that has been demonstrated to transport xylose efficiently for lactic acid production (Utrilla et al., 2012) was overexpressed. In this study, efforts towards the synthesis of P(LA-co-3HB)s with high productivities and varying LA/ 3HB ratio are described.

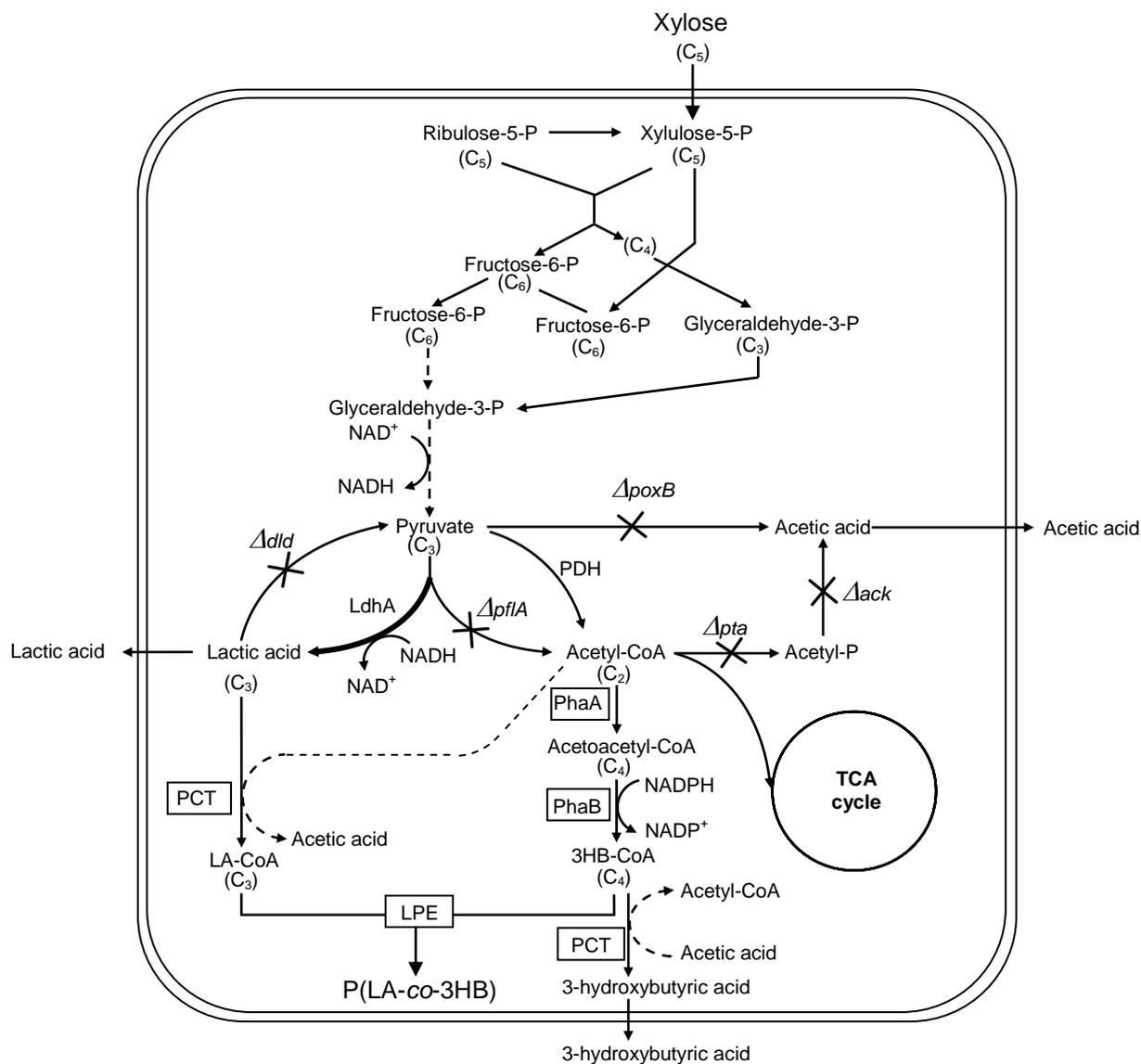


Fig. 3.1 Metabolic pathways in *E. coli* for xylose metabolism for the production of P(LA-co-3HB) and related metabolites. Deleted pathways are indicated by crosses and the genes codes for; *pflA*, pyruvate formate lyase activating enzyme; *poxB*, pyruvate oxidase; *dld*, NAD⁺-independent lactate dehydrogenase; *ackA*, acetate kinase; *pta*, phosphate acetyltransferase; PDH, pyruvate dehydrogenase complex; LdhA, Lactate dehydrogenase. The enzymes in the boxes were heterologously expressed and are involved in P(LA-co-3HB) synthesis. LPE, lactate polymerizing enzyme [PhaC1_{ps}(ST/FS/QK)]; PCT, propionyl-CoA transferase; PhaA, β -kethothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase. The dashed arrows indicate proposed pathways catalyzed by PCT (Matsumoto et al., 2013).

3.2 Materials and methods

3.2.1 Bacterial strains and plasmids

The expression vector pTV118NpctphaC1_{Ps}(ST/FS/QK)AB having *pct*, *phaC1_{Ps}(ST/FS/QK)*, *phaA* and *phaB* genes was constructed previously (Yamada et al., 2009). *E. coli* BW25113, the single-gene knockout mutants (Keio collection strains) (Table 3.1) (Baba et al., 2006) and the pCA24N*gatC* plasmid having *gatC* (Kitagawa et al., 2005) were purchased from the National BioResource Project, Japan. The construction of *E. coli* JWMB1 (Δdld and $\Delta pflA$ double mutant) is described below in section 3.2.2.

3.2.2 Genome engineering

The dual gene-knockout mutant ($\Delta pflA$ and Δdld) was created as follows; to construct marker-free mutant strain of *E. coli* JW0885 ($\Delta pflA$), the kanamycin selection marker was excised first. *E. coli* JW0885 cells were grown at 37 °C until the OD₆₀₀ was 0.4 – 0.6. One mL of the culture was aliquoted into microfuge tubes then chilled on ice for 15 minutes. After the chilling, the cells were centrifuged at 5000 rpm at 4 °C for 5 minutes then gently resuspended in 50 μ L ice-cold distilled water. The now electrocompetent cells were electroporated with 50 ng of pCP20 plasmid and

immediately 1 mL of SOC/LB medium was added to the electroporation cuvette to re-suspend the cells, then transferred into a glass test tube. Subsequently, the cells were grown at 30 °C for 1 – 3 hrs, after which 200 µL of the cell culture was each plated on LB/ampicillin and LB/kanamycin plates. The cells were grown at 30 °C for 16 – 24 hrs. The ampicillin plates will have cells harbouring pCP20 plasmid, whereas the kanamycin plate gives an idea whether the cells survives electroporation. The transformants were colony purified (cultivated) non-selectively at 43 °C (LB plates with no antibiotics). After growing the cells at 43 °C on plates, colonies were picked and plated on LB/ampicillin and LB/kanamycin. The colonies which failed to grow on both plates were kept since they had lost the kanamycin resistance gene and the temperature sensitive pCP20 plasmid. These cells were grown on LB medium then a glycerol stock was prepared.

Introduction of Δdld mutation into markerless *E. coli* JW0885

Chemically competent markerless *E. coli* JW0885 cells with kanamycin excised were transformed with pKD46 plasmid then plated on LB/ampicillin plates at 30 °C. A single colony was picked and cultivated at 30 °C to prepare a glycerol stock. The cells from the glycerol stock were cultivated on 2 mL LB/ampicillin plates at 30 °C and when the OD₆₀₀ reached 0.1, 1 mM of arabinose was added into the cultures. The cells were

then grown until the OD₆₀₀ reached 0.4 – 0.6 after which they were pelleted, washed twice by ice-cold distilled water then finally resuspended in 50 µL ice-cold distilled water.

These cells were ready for electroporation. In the meantime, PCR was carried out to amplify the kanamycin resistance gene from the pKD13 plasmid using the pair of

primers; H1P1dld;

5-CGCTATTCTAGTTTGTGATATTTTTTCGCCACCACAAGGAGTGGAAAATGATT

CCGGGGATCCGTCGACC-3 and H2P2dld;

5-GGATGGCGATACTCTGCCATCCGTAATTTTTACTCCACTTCCTGCCAGTTTGT

AGGCTGGAGCTGCTTCG-3. The kanamycin resistance gene will replace the *dld* gene

in the genome of *E. coli* as described previously (Datsenko and Wanner, 2000). After

PCR, the PCR products were electrophoresed on 1% agarose gel then purified by DNA

extraction kit (Promega, USA) and the purified DNA (0.5 µg) was transformed into

electrocompetent *E. coli* JW0885 (marker free) cells harboring pKD46 plasmid. The

transformed cells were cultivated on SOC/LB medium (No antibiotics) for 1 – 2 hrs at

37 °C (Not 30 °C since the pKD46 plasmid is now not necessary). Subsequently, 100 µL of

the cells were cultivated on LB/kanamycin plates for 24 hrs at 37 °C.

Verification of successful deletion of *dld* gene

Successful deletion of *dld* gene was confirmed by colony direct PCR using the colonies obtained from above using the verification primers; Dld F; 5'-GCCTCTTCCATGACAACAACACTGA-3' and Dld R; 5'-CCTTCCACTTCCTGCCAGTTTTT-3'. The colonies that indicated absence of *dld* gene were challenged on LB/kanamycin medium and ampicillin plates. Growth on kanamycin indicates successful incorporation of kanamycin resistance gene into the genome of *E. coli* and hence elimination of *dld*. The failure of growth on LB/ ampicillin indicates elimination of pKD46 plasmid. The mutant harboring $\Delta pflA$ and Δdld mutations was obtained and named *E. coli* JWMB1.

Table 3.1 Bacterial strains used in this study

<i>E. coli</i> strain	Relevant genotype	Reference
BW25113	<i>rrnB3ΔlacZ4887 hsdR514 Δ(araBAD)567 Δ(rhaBAD)568 rph-1</i>	(Baba et al., 2006)
JW0885	<i>ΔpflA::FRT-kan^R-FRT</i>	(Baba et al., 2006)
JW2121	<i>Δdld::FRT-kan^R-FRT</i>	(Baba et al., 2006)
JW2294	<i>ΔPTA::FRT-kan^R-FRT</i>	(Baba et al., 2006)
JW2293	<i>ΔackA::FRT-kan^R-FRT</i>	(Baba et al., 2006)
JW0855	<i>ΔpoxB::FRT-kan^R-FRT</i>	(Baba et al., 2006)
JWMB1	<i>ΔpflA,Δdld::FRT-FRT</i>	This study

3.2.3 Culture conditions

Cultivations for production and extraction of P(LA-co-3HB) polymers, lactic acid and sugar consumption measurements were carried out in 500 mL shake flasks. The pre-cultures used to inoculate the shake flask cultivations were prepared as follows. A single colony of recombinant *E. coli* cells harboring pTV118NpctphaC1_{Ps}(ST/FS/QK)AB with or without pCA24NgatC was used to inoculate 10 mL test tubes having 2 mL of LB medium supplemented with the necessary antibiotic/s (ampicillin; 100 µg l⁻¹, chloramphenicol; 30 µg l⁻¹). The tubes were cultivated at 30 °C for 12 hrs in a rotary shaker with reciprocal shaking at 180 rpm. One milliliter of the pre-culture was then transferred into 100 mL LB medium containing 100 µg l⁻¹ ampicillin (and 30 µg l⁻¹ chloramphenicol and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for pCA24NgatC-harboring transformants), 20/30/40 g l⁻¹ xylose and 10 mM calcium pantothenate in a 500 mL shake flask and cultured at 30 °C for 72 hrs with reciprocal shaking at 120 rpm. The samples from *E. coli* cultures were taken at the end of cultivation and centrifuged at 12 000 rpm for 5 min to separate the cells and the supernatant. The concentrations of sugars, lactic acids and other metabolites in the supernatant were determined by HPLC system using a refractive index detector as described in chapter 2, section 2.2.3.

3.2.4 Polymer extraction and analyses

The lyophilized cells were soaked in chloroform at room temperature for 2 days in glass flasks with constant stirring. The cell debris was separated by filtration using a 0.2 μm PTFE filter. A 10-fold volume of methanol was then added into the filtrate to precipitate the polymer as described in chapter 2, section 2.2.5. Polymer yields and monomer compositions of P(LA-*co*-3HB)s were determined by HPLC as described previously (Yamada et al., 2009). Five milligrams of the extracted P(LA-*co*-3HB) 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) with tetramethylsilane (TMS) as an internal chemical shift standard. The molecular weights of the extracted P(LA-*co*-3HB)s were estimated by gel permeation chromatography (GPC, JUSCO, Japan) equipped with a Shodex GPC KF-805 column (Showa Denko K. K., Japan) with polystyrene standards (Waters, USA) for calibration (Taguchi et al., 2008).

3.3 Results

3.3.1 Single gene knockout increases the LA fraction in P(LA-co-3HB)

The P(LA-co-3HB) yields in the cells cultivated on 20 g l⁻¹ xylose are shown in Table 3.2 (Nos. 1 – 7). For the knockout mutants, the Δpta , $\Delta ackA$, and Δdld mutants had higher polymer productivities (6.5 – 7.4 g l⁻¹) than BW25113 (6.3 g l⁻¹). Focusing on the LA fractions in P(LA-co-3HB)s, the Δpta and Δdld mutants had higher LA fractions (58 and 66 mol%, respectively) compared to BW25113 (56 mol%). Thus, these mutations as well as $\Delta pflA$ were effective for improving both or either of polymer productivity and LA fractions in the polymers. On the contrary, the $\Delta poxB$ mutant had lower LA fractions and polymer productivity than BW25113 strain, suggesting an important role of its gene product in P(LA-co-3HB) production. Since the $\Delta pflA$ and Δdld single-gene knockout mutants were found to effectively increase the P(LA-co-3HB) productivity and LA fraction (Table 3.2, Nos. 2 and 3), the two mutations were combined as described in section 3.2.2 to create a double mutant, *E. coli* JWMB1. The strain exhibited an additive effect on the increase of the LA fraction (73 mol% LA) in P(LA-co-3HB) (Table 3.2, No. 7), which was the highest value among the conditions tested in this study. However, the JWMB1 had lower polymer productivity than the other mutants.

Table 3.2 P(LA-*co*-3HB) production from xylose in engineered *E. coli* strains and the parent strain harboring pTV118N*pctphaC1*(ST/FS/QK)AB.

No.	Host genotype	Xylose concentration (g l ⁻¹)	Additional plasmid	Cell dry weight (g l ⁻¹)	Polymer yield (g l ⁻¹)	Polymer content (wt%)	LA fraction (mol%)
1	Parent (BW25113)	20	-	9.5 ± 0.2	6.3 ± 0.2	67 ± 4	56 ± 5
2	$\Delta pflA$	20	-	10.4 ± 0.1	7.3 ± 0.2	61 ± 5	60 ± 2
3	Δdld	20	-	8.6 ± 0.7	6.5 ± 0.6	76 ± 1	66 ± 2
4	Δpta	20	-	10.1 ± 0.2	7.4 ± 0.4	73 ± 3	58 ± 1
5	$\Delta ackA$	20	-	9.7 ± 0.8	7.3 ± 0.4	75 ± 2	54 ± 2
6	$\Delta poxB$	20	-	8.4 ± 0.3	5.0 ± 0.4	60 ± 6	43 ± 4
7	$\Delta pflA, \Delta dld$ (JWMB1)	20	-	4.8 ± 0.3	2.8 ± 0.6	58 ± 7	73 ± 1
8	Parent	20	pCA24NgatC	9.7 ± 0.2	7.7 ± 0.3	79 ± 5	58 ± 3
9	$\Delta pflA$	20	pCA24NgatC	9.4 ± 0.2	8.3 ± 0.8	88 ± 7	67 ± 5
10	Δdld	20	pCA24NgatC	8.1 ± 0.1	6.6 ± 0.5	81 ± 6	66 ± 3
11	Δpta	20	pCA24NgatC	7.8 ± 0.6	5.0 ± 0.5	65 ± 6	57 ± 4
12	$\Delta ackA$	20	pCA24NgatC	8.4 ± 0.4	7.3 ± 0.7	87 ± 5	64 ± 3
13	$\Delta poxB$	20	pCA24NgatC	4.3 ± 0.3	0.6 ± 0.0	15 ± 1	8 ± 2
14	$\Delta pflA, \Delta dld$	20	pCA24NgatC	7.9 ± 0.5	4.4 ± 0.3	55 ± 5	70 ± 2
15	Parent	30	-	13.7 ± 0.6	10.7 ± 1.0	78 ± 5	64 ± 4
16	$\Delta pflA$	30	-	10.9 ± 0.9	9.1 ± 0.5	83 ± 0	62 ± 1
17	Δdld	30	-	11.5 ± 0.4	9.2 ± 0.9	79 ± 8	62 ± 5
18	Δpta	30	-	10.9 ± 0.4	7.8 ± 0.7	71 ± 4	63 ± 1
19	$\Delta ackA$	30	-	9.8 ± 0.4	7.7 ± 0.2	79 ± 3	69 ± 2
20	$\Delta poxB$	30	-	8.3 ± 0.6	5.9 ± 0.9	71 ± 4	63 ± 5
21	$\Delta pflA, \Delta dld$	30	-	6.7 ± 0.5	6.4 ± 0.2	96 ± 4	73 ± 1
22	Parent	30	pCA24NgatC	12.9 ± 0.4	12.0 ± 0.5	93 ± 2	63 ± 3
23	$\Delta pflA$	30	pCA24NgatC	11.4 ± 0.5	9.7 ± 0.5	85 ± 6	66 ± 6
24	Δdld	30	pCA24NgatC	8.9 ± 0.5	7.5 ± 0.9	84 ± 3	69 ± 4
25	Δpta	30	pCA24NgatC	10.0 ± 0.1	7.0 ± 0.5	70 ± 4	66 ± 1
26	$\Delta ackA$	30	pCA24NgatC	11.2 ± 0.1	9.2 ± 0.6	82 ± 6	67 ± 3
27	$\Delta poxB$	30	pCA24NgatC	5.6 ± 0.6	1.2 ± 0.4	22 ± 4	23 ± 1
28	$\Delta pflA, \Delta dld$	30	pCA24NgatC	8.4 ± 0.4	7.4 ± 0.7	88 ± 10	69 ± 5
29	Parent	40	-	15.8 ± 0.7	11.1 ± 0.8	70 ± 5	61 ± 3
30	Parent	40	pCA24NgatC	15.9 ± 0.1	14.4 ± 0.4	91 ± 3	66 ± 1

To understand the carbon flux from xylose to metabolites, the amounts of residual xylose, polymer, lactic acid (the only metabolite detected in the medium) and CO₂ are shown as the molar amount of the carbon atom, e.g. 20 g l⁻¹ xylose (C₅) corresponds to 667 mM carbon in molar amount (Fig. 3.2 A). The CO₂ amount was theoretically estimated based on the release of two CO₂ molecules upon the formation of one 3HB molecule (Matsumoto and Taguchi, 2013). Acetic acid was below the detection limit under the conditions employed. As shown in Fig. 3.2 A, at 20 g l⁻¹ xylose feeding, 121 – 428 mM carbon was captured and the remaining part probably went into cell mass. The total lactate carbon yield (LA units in the polymer and the lactic acid secreted into the medium) for the Δdld , Δpta and $\Delta ackA$ mutants was 1.1 – 1.4-fold higher compared to that of BW25113; suggesting that elimination of lactic acid formation-competing (Δdld) and by-product formation (Δpta and $\Delta ackA$) pathways is effective for enhancing the carbon flux toward lactic acid that is eventually incorporated into P(LA-co-3HB). For the JWMB1 mutant, the flux toward 3HB units was remarkably reduced resulting in the synthesis of LA-enriched polymers. From these results, the potency of improved lactic acid production in the enhancement of the LA fractions in the copolymers and improvement of polymer productivities was highlighted by select single-gene knockout mutants.

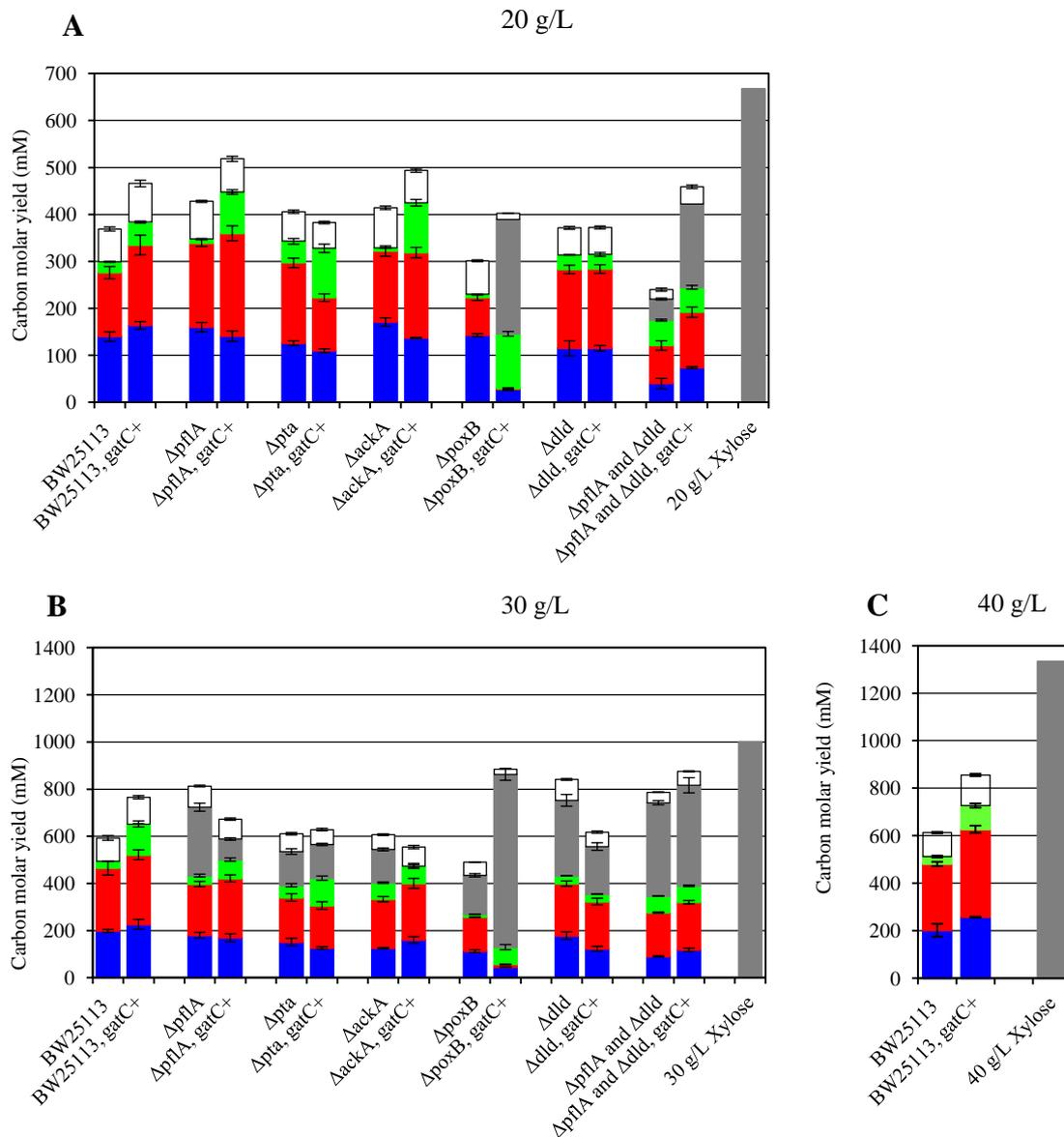


Fig. 3.2 Molar carbon amount of LA (red bars) and 3HB (blue) units in P(LA-co-3HB), lactic acid secreted into the medium (green) and residual xylose (gray) by *E. coli* BW25113 and its derivatives. The xylose bar at the end of each figure shows the initial carbon molar amount of xylose used for polymer production. The strains with ‘gatC+’ harbored pTV118NpctphaC1(ST/FS/QK)AB and pCA24NgatC. The values shown are means \pm standard deviations of triplicate experiments. The CO₂ (white bars) indicated is an estimate based on the generation of two CO₂ molecules for every 3HB unit formed. The xylose supplied were; **A**, 20 g l⁻¹ (667 mM carbon amount); **B**, 30 g l⁻¹ (1000 mM carbon amount); **C**, 40 g l⁻¹ (1333 mM carbon amount).

3.3.2 Overexpression of the xylose transporter, GatC, enhances the LA fraction and productivity of P(LA-co-3HB)

To enhance xylose consumption and potentially improve polymer productivity, xylose uptake was facilitated in *E. coli*. Therefore, *E. coli* strains harboring pCA24N*gatC* and pTV118N*pctphaC1*(ST/FS/QK)AB were cultivated for P(LA-co-3HB) production from 20 g l⁻¹ xylose. As shown in Table 3.2, Nos. 8 – 14 and Fig. 3.2 A, GatC overexpression in BW25113 increased polymer productivity (7.7 g l⁻¹) without significant change in the LA/3HB ratio. For the $\Delta pflA$, $\Delta ackA$ and JWMB1 mutants, the overexpression of GatC enhanced LA fractions in P(LA-co-3HB) together with the carbon yield of the polymers (72% of theoretical maximum for the best mutant- $\Delta pflA$) and lactic acid from xylose. Therefore, GatC was demonstrated to be a dual-useful toolkit for increasing the productivity of P(LA-co-3HB) and/or LA fraction in the polymer for BW25113 and mutants of $\Delta pflA$, $\Delta ackA$ and JWMB1. However, the beneficial effect of GatC was not observed for the Δdld mutant, and rather reduced the polymer yield and/or LA fraction for Δpta and $\Delta poxB$ mutants (Fig. 3.2 A).

3.3.3 Effects of additional xylose on P(LA-co-3HB) production

The results discussed in section 3.3.2 indicates that $\Delta pflA+gatC$ achieved the

production of 8.3 g l⁻¹ P(LA-co-3HB), which was 72% relative to the theoretical limit, suggesting that the amount of xylose fed (20 g l⁻¹) could be limiting the strains from expressing their true phenotypes i.e. high P(LA-co-3HB) yields and LA fractions. Thus, to evaluate the effect of xylose concentrations on polymers, recombinant cells were cultivated on 30 g l⁻¹ xylose for P(LA-co-3HB) production.

All the mutants and BW25113 had significant increases in the polymer productivities (1.1 – 2.3-fold) relative to polymer production at 20 g l⁻¹ (Table 3.2, Nos.15 – 21), demonstrating that sugar concentration had been the limiting factor to the polymer productivity in the aforementioned conditions. Among the tested conditions, BW25113 exhibited relatively high polymer productivity (10.7 g l⁻¹). On the other hand, JWMB1 produced P(LA-co-3HB) having the highest LA fraction (73 mol%) with the productivity of 6.4 g l⁻¹ (Table 3.2, No. 21).

Subsequently, the potential of GatC was also investigated at 30 g l⁻¹ xylose. Similar to 20 g l⁻¹ xylose feeding, GatC overexpression increased the polymer productivity for BW25113 (12%), $\Delta pflA$ (7%), $\Delta ackA$ (20%), and JWMB1 (16%) strains (Table 3.2, Nos. 22 – 28 and Fig. 3.2 B) relative to corresponding cells not expressing GatC. On the contrary, GatC overexpression adversely affected polymer productivities for the Δdld , Δpta and $\Delta poxB$ mutants. Overall, the BW25113 had higher polymer

productivity of 12.0 g l⁻¹ with 63 mol% LA than the mutants (Table 3.2, Nos. 22 – 28). With the exception of Δ *poxB* mutant, all the other mutants had insignificant changes in the LA fractions in P(LA-*co*-3HB) (Fig. 3.2 B), however these LA fractions were slightly higher than those of BW25113. The *poxB* mutation adversely affected the polymer production. This mutation has been reported to reduce growth rate of *E. coli* (Abdel-Hamid et al., 2001), which suggests that the direct conversion of pyruvate into acetate role of PoxB is essential in the growth efficiency in *E. coli*, although its physiological role is not clear.

Since the BW25113 strain fully consumed 30 g l⁻¹ xylose unlike the mutants (Fig. 3.2 B), the sugar concentration was increased to 40 g l⁻¹ for this strain. The polymer productivity was increased along with the sugar concentration (Table 3.2, No. 29). In addition, the GatC overexpression further increased the polymer productivity, where the highest polymer productivity of 14.4 g l⁻¹ with 66 mol% LA was obtained (Table 3.2, No. 30); the highest productivity for P(LA-*co*-3HB) ever reported. Overall, these results demonstrated that the overexpression of GatC was effective to the utilization of high concentration of xylose for high-yield polymer production, and that the deletions of competing pathways elevated LA fraction in the copolymer.

3.3.4 Molecular weights and NMR analysis of the polymers

The weight-averaged molecular weights (M_w) of the polymers were in the range of 34 000 - 114 000 and varied with the LA/3HB ratio (Table 3.3); consistent with earlier observations that increased LA fractions reduces the molecular weights of the P(LA-*co*-3HB)s (Yamada et al., 2011). ^1H NMR resonances of the representative copolymers in CDCl_3 were observed as multiplet peaks at δ 1.4 – 1.6 (CH_3) and δ 4.9 – 5.2 (CH) for the LA unit and at δ 1.2 – 1.4 (CH_3), δ 2.4 – 2.8 (CH_2) and δ 5.2 – 5.4 (CH) for the 3HB unit, which is consistent with the previous reports (Nduko et al., 2013; Yamada et al., 2009).

Table 3.3 Molecular weights of P(LA-co-3HB) synthesized in engineered *E. coli*

No.	Monomer composition		Molecular weights		
	(Mol%)		M_n	M_w	M_w/M_n
	LA	3HB	($\times 10^3$)	($\times 10^3$)	
1	56	44	44	89	2.0
2	60	40	15	34	2.3
3	66	34	13	36	2.8
4	58	42	22	50	2.3
5	54	46	42	114	2.7
6	43	57	46	102	2.2
7	73	27	17	38	2.2

The sample numbers corresponds to the samples Nos. 1 – 7 in Table 3.2

3.4 Discussion

Since the discovery of LPE for the synthesis of P(LA-*co*-3HB), the next objective of P(LA-*co*-3HB) production has been the enrichment of the LA fraction in the copolymer. With success, P(LA-*co*-3HB)s having LA fractions greater than 90 mol% have been synthesized (Shozui et al., 2011; Song et al., 2012). However, the copolymers having LA fractions greater than 50 mol% have been characterized by low productivity; thus limiting the exploration of their polymer properties. To address the issue of polymer productivity, xylose has been demonstrated to be effective for the synthesis of LA-enriched (60 mol%) copolymers with high productivity (7.3 g l⁻¹) (Chapter 2, Table 2.1, No. 5). In this study, efforts to exploit the advantages of xylose for the synthesis of LA-enriched P(LA-*co*-3HB) were made. For the first time, by the combination of metabolic engineering ($\Delta pflA + \Delta dld + gatC$) and use of xylose, P(LA-*co*-3HB) having LA fractions greater than 70 mol% with significantly high productivity (6.4 g l⁻¹) were successfully produced compared to previous reports; for example, LA-based polymer having 96 mol% LA with productivity of 14.8 mg l⁻¹ (Shozui et al., 2011). The *pflA* mutation eliminates formate formation and redirects the carbon flux towards lactic acid formation (Zhu and Shimizu, 2004). On the other hand, the *dld* mutation hampers lactic acid reutilization (Zhu and Shimizu, 2005). Therefore, the combination of the two

mutations had a synergistic effect in lactic acid production, resulting into the production of LA-enriched P(LA-*co*-3HB).

As demonstrated in chapter 2, Table 2.1, P(LA-*co*-3HB) is more efficiently produced than P(3HB). To interpret this result, it is worth to emphasize that one LA unit (C₃) is generated from one pyruvate molecule (C₃), while one 3HB unit (C₄) is synthesized using two pyruvate molecules (C₃ × 2) and losing two carbons as CO₂ in the process (Matsumoto and Taguchi, 2013). This stoichiometry influences the high-yield production of LA-enriched P(LA-*co*-3HB), namely, the P(LA-*co*-3HB) with higher LA fraction is supposed to be produced with higher carbon yield. However, it is also true that LPE requires 3HB-CoA for the incorporation of LA-CoA, thus the inverse relationship between LA fraction and polymer productivity has been observed. The requirement of 3HB-CoA for the synthesis of LA-based polymers has been interpreted as a role of 3HB-CoA as a priming unit of polymerization (Matsumoto and Taguchi, 2010). Therefore, the productivity of P(LA-*co*-3HB) and LA fraction in the polymer could be determined by the interactivity of the two factors.

The mutants examined in this study had been reported to increase the lactic acid productivity (Zhou et al., 2011). In fact, some of the mutants were shown to increase the accumulation of LA units and the LA fractions in the polymer i.e. the part of carbon flux

for 3HB units was channeled toward LA units, suggesting that the mutations causes redistribution of the carbon flux. It should be noted that the carbon yield (the carbon amount of polymer / the consumed xylose) of certain mutants was higher than that of BW25113, although the absolute productivity was lower, where limitation of polymer production was observed with high xylose feeding. This fact suggested that the blockage of the bypass pathway reduced the futile flux of carbon. All the strains had residual lactic acid in the supernatant not incorporated as LA units in the polymer (Fig. 3.2 B), suggesting that the polymerization step would be a bottleneck, possibly due to the LA-incorporating capacity of LPE. The results demonstrated that the productivities of lactic acid and P(LA-co-3HB) were closely related but not directly linked. Furthermore, although the *poxB* mutation has been shown to improve lactic acid production (Zhou et al., 2011), it had poor polymer yields. This suggests that the simple metabolic engineering toward enhanced lactic acid production would not always be useful for the production of LA-based polymers.

In terms of polymer productivity, BW25113 was shown to be the best among the strains tested. The BW25113 strain fully consumed xylose even with high concentration feeding, but a large portion of the consumed carbon was not distributed to cell mass formation (Figs. 3.2 B, C and Table 3.2), suggesting that some carbon was lost in

BW25113. However, although BW25113 seemed to waste the part of carbon source, this weakness was improved by the introduction of the *gatC* gene. In fact, the beneficial effect of GatC on the overall carbon yield was particularly apparent in BW25113; supporting the hypothesis that some xylose was being lost in BW25113.

In conclusion, in this study metabolic engineering approaches were employed to achieve high productivity of LA-enriched P(LA-co-3HB)s from xylose, which were further enhanced by the overexpression of a galactitol transporter, GatC. The deletions of some of the competing pathways ($\Delta pflA$, Δdld , Δpta , $\Delta ackA$ and $\Delta pflA + \Delta dld$) increased the LA fraction in the copolymer, but at the expense of the productivity. Considering xylose as a major constituent of lignocellulosic biomass that could be readily obtained, the platform established here could be a potent avenue for efficient utilization of xylose. Moreover, the achievements of this study will facilitate the exploration of the material properties and potential applications of the LA-enriched polymers.

3.5 References

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Chapter 4

Production of polyhydroxyalkanoates from lignocellulosic biomass

hydrolysate in *Escherichia coli*

4.1 Introduction

PHAs are polymeric materials synthesized from renewable materials with potential as an alternative to the conventional petroleum-derived plastics (Verlinden et al., 2007). However, the commercial production of PHAs such as P(3HB), P[3HB-*co*-3-hydroxyvalerate (3HV)] and P(LA-*co*-3HB) is hampered by the relatively high production cost, largely contributed by the carbon sources used. Approximately 40 – 50% of the total production cost of PHAs has been attributed to the carbon sources (Castilho et al., 2009; Van-Thuoc et al., 2008).

To overcome this challenge, several groups have attempted the production of PHAs from lignocellulosic biomass-derived sugars as inexpensive carbon substrates (Matsumoto et al., 2011; Silva et al., 2004; Yu and Stahl, 2008). These studies have demonstrated lignocellulosic biomass hydrolysate as potential inexpensive carbon substitutes for the production of PHAs. Additionally, on the basis of value addition on the use of these low-value resources, the use of lignocellulosic biomass for PHA production is an attractive venture. Lignocellulosic biomass constitutes cellulose (40 – 50%), hemicellulose (20 – 40%) and lignin (10 – 25%) (FitzPatrick et al., 2010). The cellulose portion consists of mainly glucose units whereas, hemicellulose is a heteropolymer mainly composed of xylose and other minor sugars such as arabinose, galactose, mannose and glucose (Rubin, 2008). To utilize lignocellulosic materials for the production of

PHAs, they have to be fractionated into their components (French, 2009). The cellulose and hemicellulose are then hydrolyzed to obtain constituent sugars, which could be supplied to microorganisms to produce PHAs (Fig. 4.1).

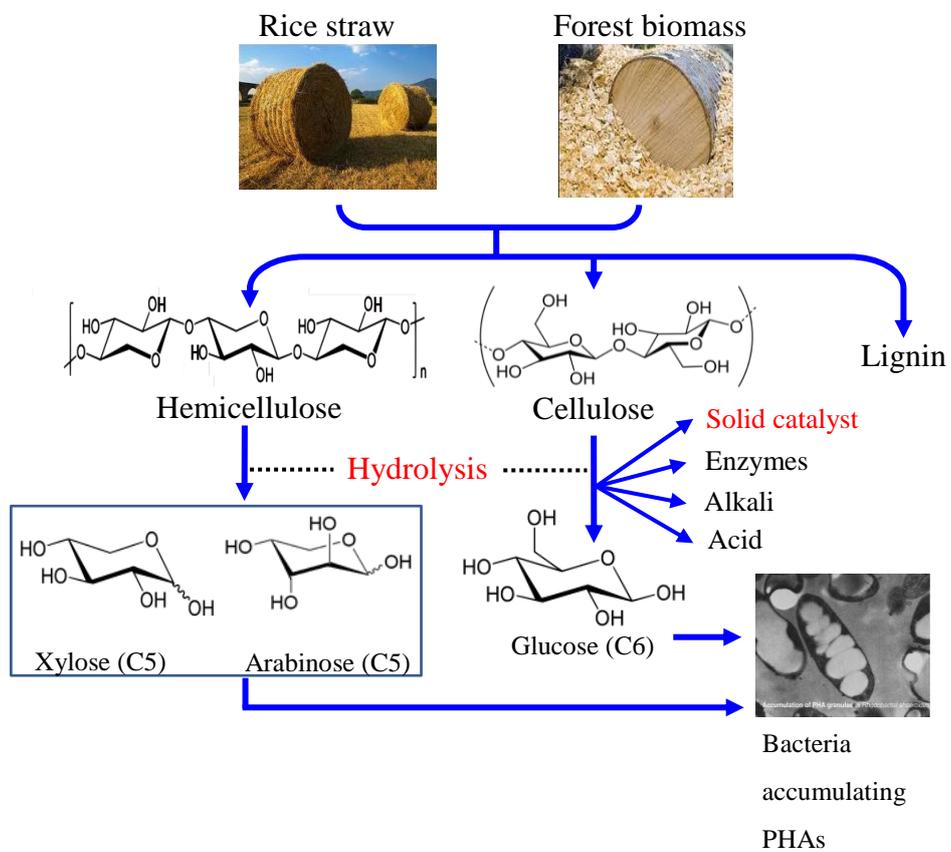


Fig. 4.1 Scheme of the utilization of lignocellulosic biomass. The biomass is fractionated into its component constituents, cellulose, hemicellulose and lignin. Cellulose and hemicellulose are then hydrolyzed into sugars that could be fed to microorganisms to produce a variety of polyhydroxyalkanoates (PHAs).

Biomass hydrolysis is the main impediment to a more widespread utilization of these renewable resources. Current technologies for the hydrolysis of lignocellulosic biomass begin with pretreatment, whereby the lignocellulosics are treated by chemical and/or physical means (Carvalho et al., 2008; Sun and Cheng, 2002). The pretreatment removes hemicellulose and lignin so that cellulose can be accessible by cellulases/chemical catalysts. Cellulases produced mainly from *Trichoderma reesei* have been extensively employed for cellulose hydrolysis in the production of a repertoire of chemicals and materials (Dashtban et al., 2009; Zhang, 2008). The enzymes are environmentally friendly but they are expensive and have slow reaction rates. Cellulose can also be hydrolyzed by dilute acids/alkali however this method generates neutralization waste material that pollutes the environment, causes corrosion of equipments and cannot be recycled (Rinaldi and Schuth, 2010; Van Dyk and Pletschke, 2012). To find a better alternative method for cellulose hydrolysis, supported ruthenium catalysts that can be recycled have been used to hydrolyze cellulose (Kobayashi et al., 2010) into glucose that has been applied for the production of P(3HB) (Matsumoto et al., 2011).

On the other hand, the hemicellulose portion is a potential by-product currently underutilized especially in the paper making industry, where the hemicelluloses are

dissolved in black liquor along with lignin and combusted for power generation (Liu et al., 2010). Since hemicellulose has a lower heating value compared to lignin, its utilization for the production of polyesters and fuels could be more efficient. Hemicellulose can be subjected to pretreatment by methods such as acid hydrolysis, steam explosion, enzyme hydrolysis and hot water treatment to readily obtain xylose-rich hydrolysates, which can serve as substrates for the synthesis of a number of bioproducts (Saha, 2003). In particular, as described in chapter 2 and 3, the effectiveness of xylose over glucose for the high-yield production of P(LA-co-3HB) with high LA fractions has been demonstrated. Therefore, the substitution of xylose with xylose-rich hemicellulose hydrolysates can potentially reduce the cost of production of P(LA-co-3HB) significantly, enhancing their cost-competitiveness against the chemically synthesized petrochemical-based plastics.

In this study, towards the substitution of pure sugars with lignocellulosic biomass-derived sugars, cellulose was hydrolyzed by the recyclable ruthenium (Ru) catalyst, resulting into fermentable sugars. However, in the hydrolysis reaction, 5-hydroxymethylfurfural (5-HMF), which was toxic to *E. coli* was formed as a by-product. The screening of *E. coli* strains for 5-HMF tolerance identified *E. coli* LS5218 as resistant against 5-HMF. Besides the 5-HMF tolerance, the *E. coli* LS5218 is well characterized for the production of P(3HB-co-3HV). To test its application, herein,

the production of P(3HB-*co*-3HV) and P(3HB) from Ru-catalyzed cellulose hydrolysate in a 5-HMF resistant *E. coli* strain is reported, demonstrating the applicability of lignocellulosic biomass as an inexpensive carbon substrate for PHA production.

4.2 Materials and methods

4.2.1 Cellulose hydrolysis

The cellulose used in this study was commercially sourced. The cellulose hydrolysis procedure was as previously described (Matsumoto et al., 2011). In brief, 2.59 g of cellulose was mixed with Ru/ γ -Al₂O₃ catalyst and 20 mL of water then heated in a reactor at 215 °C. The hydrolysate was cooled down and prepared for component analysis by HPLC as previously described (Matsumoto et al., 2011). The pH of the cellulose hydrolysate was adjusted to pH 7 by 1 N NaOH then the hydrolysate was used to prepare medium containing 1 wt% glucose for polymer production.

4.2.2 Screening of 5-HMF-tolerant *E. coli* strain

The cellulose hydrolysate obtained in this study had been previously demonstrated to constitute 5-HMF that was toxic to the *E. coli* JM109 cells, hindering growth and polymer synthesis (Matsumoto et al., 2011). To circumvent this obstacle, five *E. coli* strains were investigated for 5-HMF resistance (Table 4.1). The strains *E. coli* DH5 α and *E. coli* JM109 (Takara, Japan) are laboratory strains widely used in genetic and molecular studies. *E. coli* LS5218 constitutively expresses the enzymes involved in the utilization of fatty acids and used in the production of PHA copolymers (Shozui et al., 2010; Slater et

al., 1992) and *E. coli* JW2978, Keio collection mutant that has been shown to have improved resistance against 5-HMF. *E. coli* BL21 represented *E. coli* B strain. Detailed relevant genotypes are shown in Table 4.1.

All the strains were cultivated at 37°C overnight on LB medium then aliquots of the overnight cultures were transferred (1:100 dilution) into fresh LB medium supplemented with variable concentrations of 5-HMF (0, 2.0, 2.5, 3.0 and 4.0 g l⁻¹) and cultured at 37 °C. Growth and 5-HMF resistance were determined by measuring OD₆₀₀ of the medium.

Table 4.1 *E. coli* strains screened for tolerance against 5-HMF

<i>E. coli</i> strain	Relevant genotype	Reference
JM109	endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB ⁺ Δ(lac-proAB) e14- [F' traD36 proAB ⁺ lacI ^q lacZΔM15] hsdR17(r _K ⁻ m _K ⁺)	(Yanischperron et al., 1985)
DH5α	F endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(r _K ⁻ m _K ⁺), λ-	(Grant et al., 1990)
LS5218	<i>fadR</i> , <i>atoC</i> (<i>con</i>)	(Spratt et al., 1981)
JW2978	Δ <i>yqhD</i>	(Baba et al., 2006; Miller et al., 2010)
BL21	F- <i>dcm ompT hsdS</i> (r _B ⁻ m _B ⁻) <i>gal</i> [malB ⁺] _{K-12} (λ ^S)	(Studier and Moffatt, 1986)

4.2.3 P(3HB-*co*-3HV) and P(3HB) production from cellulose hydrolysate

Among the strains screened, the production of P(3HB-*co*-3HV) and P(3HB) was carried out in *E. coli* LS5218 harboring pGEM*phaCAB* plasmid (Taguchi et al., 2002), which bears *phaC*, *phaA* and *phaB* genes from *R. eutropha* encoding P(3HB) biosynthetic enzymes [PHA synthase (PhaC), β -ketothiolase (PhaA) and acetoacetyl-CoA reductase (PhaC)]. The cells were cultivated in 2 mL LB medium supplemented with 100 μ g/L of ampicillin at 30°C for 12 h and harvested by centrifugation then resuspended in fresh LB medium containing 100 μ g/L of ampicillin, cellulose hydrolysate giving glucose concentration of 1.0% (w/v) and variable concentrations of propionate (0, 5, 10, 20, and 40 mM) as a precursor to 3HV (Fig. 4.2) in a total volume of 2 mL each. Cultivation of cells without propionate was for the production of P(3HB). Cultures having the same concentration of analytical grade glucose were prepared likewise as controls. Subsequently, the cells were cultivated at 30°C for 60 h, harvested by centrifugation and lyophilized. The polymers accumulated in the cells were extracted with chloroform and applied to gas chromatography/mass spectrometry (GC/MS) analysis as described by Arai et al. (Arai et al., 2002).

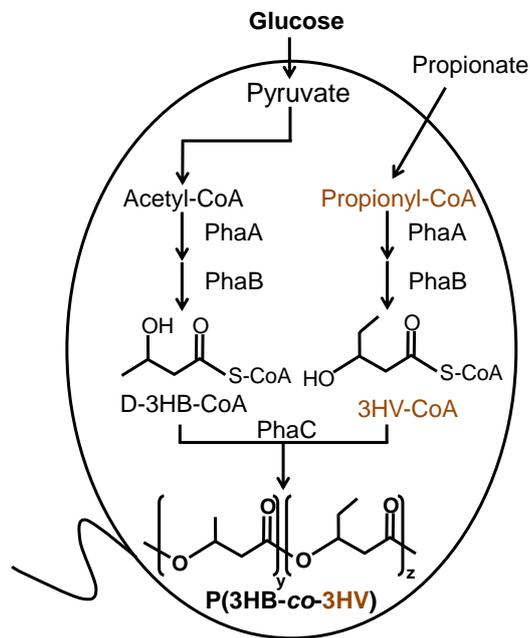


Fig.4.2 Metabolic pathway for the synthesis of P(3HB-co-3HV). Omitting propionate leads to the synthesis of P(3HB) only (Wong et al., 2008).

4.3 Results

4.3.1 Composition of cellulose hydrolysate

The major products of the cellulose hydrolysate determined by HPLC analysis are summarized in Table 4.2. Glucose was the major product followed by soluble oligosaccharides (dimer – octamer). Other sugars were fructose, mannose and levoglucosan. Furfural and 5-HMF were also detected in the hydrolysate as shown in Table 4.2 (Matsumoto et al., 2011).

Table 4.2 Composition of cellulose hydrolysate generated by Ru-catalyst

Conc. of cellulose (%)	Yield of products (%)						Conv. of Cellulose (%)
	Glucose	Oligo saccharides	Other sugars	5-HMF	Furfural	Unknown	
13	1.39	0.64	0.40	0.13	0.08	0.62	25

Conc., concentration; Conv., conversion

4.3.2 Screening for 5-HMF tolerance

Prior to the production of P(3HB-*co*-3HV) from cellulose hydrolysate, *E. coli* strains were screened for 5-HMF tolerance. The OD₆₀₀ of the *E. coli* strains after 22 hours of culturing at different concentrations of 5-HMF is shown in Fig. 4.3 A. When 2.0 g l⁻¹ of 5-HMF was added into the growth medium, all the strains had a less than 5% growth inhibition relative to the respective cells growing in the absence of 5-HMF. When the concentration of 5-HMF was increased to 2.5 g l⁻¹, all the strains grew but with some inhibition. At 3.0 g l⁻¹ of 5-HMF, *E. coli* LS5218 and JW2978 had a less than 20% growth inhibition, whereas strains BL21, DH5 α and JM109 had over 80% growth inhibition (Fig. 4.3 A).

The time course of the cells grown in the presence of 3.0 g l⁻¹ 5-HMF is shown in Fig. 4.3 B. Although *E. coli* LS5218 and JW2978 had longer lag phases compared to the *E. coli* JM109 control (Fig. 4.3 B, *closed diamonds*), they had a less than 20% growth inhibition, demonstrating their superior 5-HMF resistance compared with the other strains. Therefore, in this study, *E. coli* LS5218 was selected for P(3HB-*co*-3HV) production from Ru-catalyzed cellulose hydrolysate, because in addition to its superior 5-HMF resistance, it has also been demonstrated to synthesize various 3HB-based copolymers such as P(3HB-*co*-3HV) (Slater et al., 1992) and P(3HB-*co*-3HA)s (Shozui et al., 2010).

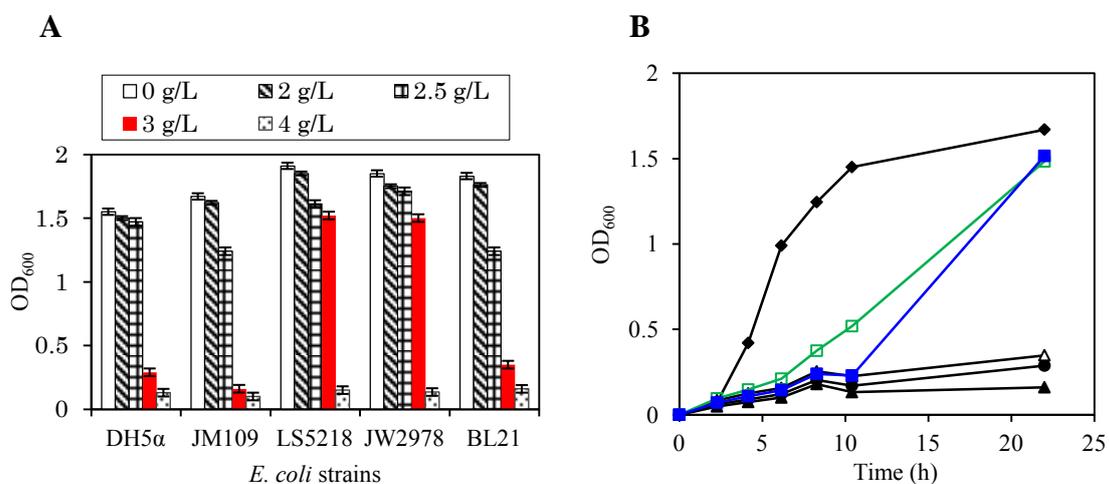


Fig. 4.3 (A) OD₆₀₀ of *E. coli* strains grown on LB medium containing different concentrations of 5-HMF after 22 hours. Symbols: □, absence of 5-HMF; ▨, 2 g/L; ▩, 2.5 g/L; ■, 3.0 g/L; ▧, 4 g/L. (B) Time course of growth of *E. coli* strains on LB medium in the presence of 3 g/L of 5-HMF. Closed diamonds, JM109 cells growing in the absence of 5-HMF (positive control); closed circles, DH5α; closed triangles, JM109; Blue closed squares, LS5218; Green open squares, JW2978; open triangles, BL21.

4.3.3 P(3HB-*co*-3HV) and P(3HB) production from cellulose hydrolysate

To investigate the application of Ru-catalyzed cellulose hydrolysate as a substrate for PHA production, the *E. coli* LS5218 cells were grown on cellulose hydrolysate and propionate, which is a precursor for supplying the 3HV monomer (Wong et al., 2008). Table 4.3 summarizes the cell dry weight, P(3HB) and P(3HB-*co*-3HV) yields from both cellulose hydrolysate and analytical grade glucose in *E. coli* LS5218. Growth and polymer production from cellulose hydrolysate (at 1 wt% glucose concentration) indicates that *E. coli* LS5218 was resistant against the inhibitors present in the hydrolysate, unlike *E. coli* JM109 cells previously reported (Matsumoto et al., 2011). In the absence of propionate, the P(3HB) content and polymer concentration from the cellulose hydrolysate were 59 wt% and 3.3 g l⁻¹, respectively. These results were similar to those obtained from analytical grade glucose; 58 wt% and 3.4 g l⁻¹ for P(3HB) content and concentration, respectively. When propionate was added, the 3HV fraction in the polymer ranged between 5.6 – 40 mol% depending on propionate concentration in the culture medium (Table 4.3). In all cases, analytical grade glucose had slightly higher 3HV fraction than cellulose hydrolysate. In contrast, cellulose hydrolysate had higher polymer yield (g l⁻¹) than analytical grade glucose probably due to the presence of other sugars such as mannose and fructose that could be metabolized for polymer production. There

was a general trend where cell weight (g l^{-1}) and polymer yield (g l^{-1}) decreased with increase in propionate concentrations due to toxicity of propionate to the cells. This phenomenon had been previously reported (Slater et al., 1992).

Table 4.3 The growth and biosynthesis of P(3HB-*co*-3HV) and P(3HB) by *E. coli* LS5218 from cellulose hydrolysate and propionate.

Propio- nate conc. (mM)	Cellulose hydrolysate				Analytical grade glucose			
	Cell dry weight (g l ⁻¹)	Polymer yield (g l ⁻¹)	Polymer content (wt%)	3HV fraction (mol%)	Cell dry weight (g l ⁻¹)	Polymer yield (g l ⁻¹)	Polymer content (wt%)	3HV fraction (mol%)
0	5.6 ±0.4	3.3 ±0.2	59	0	5.9 ±0.3	3.4 ±0.3	58	0
5	4.2 ±0.6	2.1 ±0.4	50	5.6 ±1.6	3.7 ±0.6	1.7 ±0.3	46	6.8 ±2.5
10	3.3 ±0.2	1.5 ±0.4	45	9.8±3.8	3.1 ±0.5	1.2 ±0.2	39	13 ±2
20	2.7 ±0.3	0.4 ±0.1	15	28 ±3	2.5 ±0.5	0.2 ±0	8	30 ±3
40	2.7 ±0.1	0.2 ±0	7	40 ±4	2.5 ±0.4	0.2 ±0	8	42 ±2

conc., concentration; 3HV, 3-hydroxyvalerate. The standard deviations are from duplicate measurements. Data from;(Nduko et al., 2012).

4.4 Discussion

The substitution of refined sugars with the inexpensive lignocellulosic biomass for the production of PHAs has potential to reduce the cost of producing these polymers. To substitute refined sugars with lignocellulosic biomass hydrolysates, in this study, cellulose was successfully hydrolyzed into glucose and other components (Table 4.2) and the hydrolysate was applied for P(3HB-*co*-3HV) and P(3HB) production. Previously, this hydrolysis reaction had been found to generate the toxic by-products; 5-HMF and furfural, which hinders cell growth and polymer synthesis (Matsumoto et al., 2011; Mills et al., 2009). There are several strategies that could be used to overcome hydrolysate toxicity such as detoxification, process design, strain development and addition of some compounds into the medium to protect cells (Almeida et al., 2009). However, all these additional steps add cost to the PHA production system. In this study, the screening of *E. coli* strains identified strains that were relatively resistant against 5-HMF, the main compound contributing to toxicity, hence allowing the direct application of the hydrolysate without prior treatments for PHA production. The *E. coli* LS5218 was found to be resistant against 5-HMF (Nduko et al., 2012). This strain has mutations in the *atoC* and *fadR* genes, whose products play roles in the transcriptional and regulation of the fatty acid metabolism (Mills et al., 2009). The *atoC* and *fadR* mutations allow the strain

to utilize 2-furoic acid, an intermediate of 5-HMF and furfural metabolism (Koopman et al., 2010). Therefore, *E. coli* LS5218 could utilize 5-HMF as a carbon source hence the conferment of 5-HMF resistance.

The 5-HMF resistant *E. coli* LS5218 cells were recruited for P(3HB-*co*-3HV) and P(3HB) production. The cells were able to grow and produce P(3HB) from the cellulose hydrolysate, indicating that *E. coli* LS5218 was resistant against 5-HMF compared to the *E. coli* JM109 cells that were previously unable to grow under the same conditions (Matsumoto et al., 2011). Comparing the cell and polymer yields, the cells cultivated on cellulose hydrolysate had comparable polymer yields to those of cells growing on pure glucose. These results indicate that cellulose hydrolysates could replace pure glucose as substrates for the growth and production of PHAs without detoxification steps, which have been reported to be essential in other similar studies (Pan et al., 2012; Silva et al., 2004).

Addition of propionate was found to result into the production of P(3HB-*co*-3HV) with the 3HV ratios varying with the concentration of propionate supplemented. These 3HV fractions provide copolymers with altered thermal and physical properties with potential as materials for a wide range of applications compared to P(3HB) homopolymer. The P(3HB-*co*-3HV) yields from cellulose hydrolysate and pure glucose were similar,

though propionate seemed to be toxic to the cells as also previously reported (Slater et al., 1992). However, cell and polymer yield could be increased in a fermenter, where the propionate feeding, sugar concentration and other factors could be controlled.

In conclusion, the application of cellulosic biomass for the production of PHAs has been demonstrated. Cellulose was hydrolyzed by a recyclable Ru solid catalyst into fermentable sugars that were used for the production of polymers with yields similar to pure sugars in a 5-HMF resistant *E. coli* strain. In addition, supplementation of propionate as a co-substrate led to the synthesis of P(3HB-*co*-3HV), thus diversifies the variety of products that could be produced from lignocellulosic biomass. The study demonstrated the possibility of the use of lignocellulosic biomass instead of the use of pure sugars for PHA production. However, for the development of an economically viable process to produce PHAs from biomass, the utilization of xylose-rich hemicellulose for the production of PHAs such as P(LA-*co*-3HB) is essential.

4.5 References

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Chapter 5

Conclusion

Poly(lactic acid-*co*-3-hydroxybutyrate) [P(LA-*co*-3HB)] with varying LA and 3HB units is a new PHA member that is produced from renewable resources with the potential to serve as an alternative to the petrochemical-derived plastics. For industrial scale-up, the production of this copolymer faces the challenge of high production cost, mainly due to the high cost of glucose derived from foodstocks such as sugarcane, corn starch and sugar beets. To reduce the production cost, the use of the inexpensive lignocellulosic biomass is inevitable. Lignocellulosic biomass is mainly composed of glucose and xylose as the major sugars. The use of glucose for the production of PHAs has been demonstrated to be efficient whereas, the use of xylose is inefficient. However to minimize the cost of producing PHAs from lignocellulosic biomasses, efficient utilization of both sugars is essential. In this study, methods for the efficient utilization of xylose were developed and enhanced to obtain high productivities of P(LA-*co*-3HB). In chapter 2 of this study, the utilization of xylose for the production of P(LA-*co*-3HB) was evaluated, and compared with that of P(3HB) production. It was demonstrated that the polymerization of lactic acid (LA) to form P(LA-*co*-3HB) contributed to the improvement of polymer yields from xylose. To further enhance polymer productivity, an LPE with superior ability to polymerize LA was recruited and indeed, it led to improved LA fractions in P(LA-*co*-3HB) and overall polymer yields. In addition, xylose was found

to be superior to glucose in giving higher LA fractions in P(LA-co-3HB). Since copolymer properties vary with monomer composition, xylose presented a new platform for the production of P(LA-co-3HB)s with LA fractions >50 mol%, thus enabling the exploration of material properties of P(LA-co-3HB)s, which was impossible with the use of glucose.

In chapter 3, metabolic engineering strategies to maximize the lactic acid flux were carried out. Single- and a double-gene knockout mutants along with their parent strain of *E. coli* were recruited for P(LA-co-3HB) production. The use of the knockout mutants was found to be effective in improving LA fractions (up to 73 mol%)/ yields in P(LA-co-3HB) (8.3 g l⁻¹). The uptake of xylose by *E. coli* is ATP-dependent and thus to eliminate the risk of ATP limiting polymer yields, an ATP-independent galactitol transporter, GatC, which transports xylose as well was overexpressed in *E. coli* during P(LA-co-3HB) production. Overexpression of this transporter was found to be effective in improving polymer yields due to reduced ATP-dependent uptake of xylose. The *E. coli* strains were cultivated at higher xylose concentrations and productivity of P(LA-co-3HB) was found to increase along with xylose concentrations (up to 14.4 g l⁻¹ of copolymer was produced, which is the highest P(LA-co-3HB) productivity ever reported).

In chapter 4, the possibility of substituting refined sugars with lignocellulosic

hydrolysates was explored. Cellulose was hydrolyzed by ruthenium catalyst into mainly glucose and other compounds. However, the hydrolysis reaction also generated 5-HMF, which was toxic to *E. coli*. The screening of *E. coli* strains identified a 5-HMF resistant strain (LS5218) and it was found to effectively produce P(3HB) and P(LA-*co*-3HV) from the cellulose hydrolysate, with similar polymer yields as the pure glucose. These experiments demonstrated that lignocellulosic biomass materials can serve as alternative carbon sources for the production of PHAs.

The aforementioned approaches for high yield production of P(LA-*co*-3HB), P(3HB) and P(3HB-*co*-3HV) in combination with the utilization of lignocellulosic biomass have a potential for the establishment of a biorefinery for the production of a variety of PHAs. Therefore, the studies in this thesis are worthwhile and contribute to the establishment of a system that can be scaled-up with potentially low operating costs, hence improving the competitiveness of PHAs against the petrochemical-derived plastics.

Future Work

In chapter 2 and 3, the production of P(LA-*co*-3HB) was discussed and the enhancement of LA fractions in the polymers was achieved through metabolic engineering strategies. However, in most cases during the production of P(LA-*co*-3HB), it was found that the molecular weights of the polymers decreased with the increase in

LA fractions in P(LA-*co*-3HB). To further advance this platform to the industrial level, further studies for the improvement of the molecular weights of P(LA-*co*-3HB) are required. In addition, the polymers synthesized in this study had LA fractions of 73 mol% as the highest, therefore further studies towards the full synthesis of P(LA-*co*-3HB) with 73 – 100 mol% LA at high productivities is an attractive study topic. Moreover, further studies are necessary to determine the properties and applications of P(LA-*co*-3HB)s and commercialize them.

In an attempt to utilize lignocellulosic biomass for the production of PHAs, in chapter 4, the use of cellulose hydrolysate for PHA production was demonstrated. Therefore, for the full utilization of the biomass, the use of hemicellulose for the production of PHAs should be attempted. In particular, the production of P(LA-*co*-3HB) from hemicellulose hydrolysate is an attractive venture in the efforts towards the establishment of a robust system that can be scaled up for the creation of a biorefinery for the production of P(LA-*co*-3HB)s.

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