Journal of Biotechnology 151 (2011) 94–101

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

# Efficient production of polylactic acid and its copolymers by metabolically engineered *Escherichia coli*

Yu Kyung Jung[a], Sang Yup Lee[a,b,*]

[a] *Metabolic and Biomolecular Engineering National Research Laboratory, Department of Chemical and Biomolecular Engineering (BK21 Program), Center for Systems and Synthetic Biotechnology, Institute for the BioCentury, KAIST, Daejeon 305-701, Republic of Korea*
[b] *BioProcess Engineering Research Center and Bioinformatics Research Center, KAIST, Daejeon 305-701, Republic of Korea*

ARTICLE INFO

*Article history:*
Received 17 July 2010
Received in revised form
10 November 2010
Accepted 15 November 2010

*Keywords:*
Polylactic acid
PLA
P(3HB-*co*-LA)
Metabolic engineering
*Escherichia coli*
Fed-batch culture

ABSTRACT

Polylactic acid (PLA) is one of the promising biodegradable polymers, which has been produced in a rather complicated two-step process by first producing lactic acid by fermentation followed by ring opening polymerization of lactide, a cyclic dimer of lactic acid. Recently, we reported the production of PLA and its copolymers by direct fermentation of metabolically engineered *Escherichia coli* equipped with the evolved propionate CoA-transferase and polyhydroxyalkanoate (PHA) synthase using glucose as a carbon source. When employing these initially constructed *E. coli* strains, however, it was necessary to use an inducer for the expression of the engineered genes and to feed succinate for proper cell growth. Here we report further metabolic engineering of *E. coli* strain to overcome these problems for more efficient production of PLA and its copolymers. This allowed efficient production of PLA and its copolymers without adding inducer and succinate. The finally constructed recombinant *E. coli* JLXF5 strain was able to produce P(3HB-*co*-39.6 mol% LA) having the molecular weight of 141,000 Da to 20 g $l^{-1}$ with a polymer content of 43 wt% in a chemically defined medium by the pH-stat fed-batch culture.



## 1. Introduction

Polylactic acid (PLA) and its copolymers are promising biomass-derived polymers as they possess excellent properties including biocompatibility, compostability and low toxicity to humans, making them suitable for household products, commercial plastics, and biomedical materials (Drumright et al., 2000; Mehta et al., 2005; Södergård and Stolt, 2002; Vink et al., 2003). However, the current process for PLA synthesis is not simple: fermentative production of lactic acid followed by chemical process for the ring opening polymerization of lactide, a cycic dimer derived from the dehydration of lactic acid, or solvent-based azeotrophic dehydrative condensation (Drumright et al., 2000; Mehta et al., 2005; Södergård and Stolt, 2002; Vink et al., 2003). Attempts to improve the rather stiff and brittle properties of PLA has been made by copolymerization or blending with other polymers including polyhydroxyalkanoates (PHAs) which are polyesters intracellularly synthesized by various microorganisms (Chen, 2009; Chen and Wu, 2005; Hazer and Steinbüchel, 2007; Lee, 1996a; Noda et al., 2004; Schreck and Hillmyer, 2007; Steinbüchel and Füchtenbusch, 1998). However, the existing chemical methods are not effective considering the quite complicated processes involving catalyst-assisted reaction and purification steps, availability of lactonized monomers used in copolymerization, and their costs.

PHAs are synthesized *in vivo* by PHA synthase (PhaC) which polymerizes various (D)-hydroxyacyl-CoAs generated through diverse metabolic pathways in the cell (Lee, 1996c; Steinbüchel and Füchtenbusch, 1998). Until recently, there has been no report on the production of PHAs containing lactate as a major monomer, which is most likely due to the limited substrate specificity of the natural PHA synthases (Steinbüchel and Valentin, 1995; Valentin and Steinbüchel, 1994; Yuan et al., 2001; Zhang et al., 2001). Several years ago, we reported production of unnatural PLA and lactate containing polymers by using the engineered PHA biosynthetic system (Cho et al., 2006). Indeed, it was possible to produce unnatural polymers including poly(3-hydroxybutyrate-*co*-lactate), P(3HB-*co*-LA), in recombinant bacteria expressing PHA synthase and CoA transferase such as *Clostridium propionicum* propionate CoA-transferase ($Pct_{Cp}$), which generates lactyl-CoA in the cell (Selmer et al., 2002). In a different study, Taguchi et al. (2008) also reported biosynthesis of P(3HB-*co*-LA) using a similar system. However, the lactate fraction was very low. To enhance biosynthesis of PLA and its copolymers, $Pct_{Cp}$ and *Pseudomonas* sp. MBEL 6–19 PHA synthase ($PhaC1_{Ps6-19}$) were engineered by *in vitro* mutagenesis to efficiently generate lactyl-CoA and incorporate lactyl-CoA into the polymer, respectively, which resulted in the increased production

* Corresponding author at: Department of Chemical and Biomolecular Engineering, 335 Gwahangno, Yuseong-gu, Daejeon 305-701, Republic of Korea.
Tel.: +82 42 350 3930; fax: +82 42 350 8800.
*E-mail addresses:* leesy@kaist.ac.kr, leesy@mbelmail.kaist.ac.kr (S.Y. Lee).

0168-1656/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2010.11.009

**Table 1**
*Escherichia coli* strains and plasmids used in this study.

| Strains or plasmids | Relevant characteristics[a] | Reference or source |
|---|---|---|
| Strains | | |
| XL1-Blue | *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*$^q$*ZΔM15* Tn*10* (Tet$^R$)] | Stratagene[b] |
| JLX10 | XL1-Blue *ΔackA PldhA::Ptrc Δppc ΔadhE Pacs::Ptrc* | Jung et al. (2010) |
| XB-F | *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* | This study |
| JLXF5 | XB-F *ΔlacI ΔpflB ΔfrdABCD ΔadhE PldhA::Ptrc Pacs::Ptrc* | This study |
| Plasmids | | |
| pBluescript | Ap$^R$, cloning and expression vector | Stratagene[b] |
| pPs619C1310-CpPCT540 | Ap$^R$, Promoter of the *Cupriavidus necator* PHA biosynthesis operon, *phaC1*$_{Ps6-19}$ variant (*phaC1310*$_{Ps6-19}$; E130D, S477F, Q481K), *pct*$_{Cp}$ variant (*pct540*$_{Cp}$; V193A, silent mutations: T78C, T669C, A1125G, T1158C), transcriptional terminator of the *C. necator* PHA biosynthesis operon, derivative of pBluescript II KS(+) | Yang et al., 2010 |
| pMCS104CnAB | Cm$^R$, *gntT104* promoter, *phaAB*$_{Cn}$, derivative of pBBR1MCS | Yang et al. (2010) |
| pKD46 | Ap$^R$, λ-Red recombinase expression plasmid, *ara*-inducible expression, temperature sensitive (Ts) replication | Datsenko and Wanner (2000) |
| pMloxC | Ap$^R$, lox66-Cm$^R$-lox71, derivative of pUG6 | Lee et al. (2007) |
| pJW168 | Ap$^R$, repA (Ts), pSC101-based plasmid expressing Cre-recombinase, IPTG-inducible expression | Palmeros et al. (2000) |

[a] *Abbreviations*: Ap, ampicillin; Cm, chloramphenicol; R, resistance.
[b] Stratagene Cloning System, La Jolla, CA, USA.

of P(3HB-*co*-LA) and PLA (Yang et al., 2010). Analysis of the composition, monomer sequence distribution, and stereospecificity of the polymers showed that they have the monomer units in the (D)-configuration with a random sequence (Yang et al., 2010).

Even though it was possible to produce PLA and P(3HB-*co*-LA) containing high lactate fraction by metabolically engineered *Escherichia coli*, the polymer contents were still rather low. Thus, it was necessary to increase the metabolic fluxes at the systems level to further enhance the production of PLA and the copolymers. To do so, the metabolic pathways of *E. coli* were rationally engineered by knocking out the *ackA*, *ppc*, and *adhE* genes encoding acetate kinase, phosphoenolpyruvate carboxylase, and acetaldehyde/alcohol dehydrogenase, respectively, and by replacing the native promoters of the *ldhA* and *acs* genes encoding (D)-lactate dehydrogenase and acetyl-CoA synthetase, respectively, with the strong *trc* promoter based on *in silico* genome-scale analyses including *in silico* gene knockout simulation and flux response analysis (Jung et al., 2010). The finally engineered *E. coli* JLX10 strain allowed the most enhanced production of PLA homopolymer and P(3HB-*co*-LA) copolymers with controlled lactate fractions of greater than 50 mol%. Thus, the strategy of combined metabolic engineering and enzyme engineering made it possible to produce PLA and its copolymers by one-step direct fermentation of engineered *E. coli*.

In the above studies, however, the engineered *E. coli* strains including JLX10 strain require induction with an expensive inducer such as isopropyl β-D-1-thiogalactopyranoside (IPTG) for the expression of the genes under the *trc* promoter, and feeding of succinate for cell growth inhibited by the *ppc* gene deletion. Even though such system is suitable for the proof-of-concept type studies, it is not preferred for industrial-level production of polymers.

In this study, the metabolic pathways of *E. coli* were further engineered to overcome these problems, and consequently to achieve efficient production of PLA and lactate-containing copolymers. Although several strategies for high-level production of lactic acid in *E. coli* have been reported (Chang et al., 1999; Zhou et al., 2003; Zhu et al., 2007), the metabolic system to be developed in this study is different; enhanced generation of lactyl-CoA as well as acetyl-CoA needs to be considered, which subsequently enhances the production of lactate-containing polyesters. Thus, the metabolic pathways of *E. coli* strain expressing the evolved Pct$_{Cp}$ and PhaC1$_{Ps6-19}$, and *Cupriavidus necator* (formerly, *Ralstonia eutropha*) β-ketothiolase and acetoacetyl-CoA reductase were systematically engineered to efficiently provide lactyl-CoA without the need for IPTG induction and succinate feeding. Furthermore, the pH-stat fed-batch culture of the finally engineered *E. coli* strain was performed in a chemically defined medium.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids, and materials

The strains, plasmids, and primers used in this study are listed in Tables 1 and 2. All DNA manipulations were carried out according to standard procedures (Sambrook and Russell, 2001). All oligonucleotides were synthesized at Bioneer (Daejeon, Korea). Preparation of plasmids and DNA fragments was performed with

**Table 2**
Primers used in this study.

| Primers | Sequence (5′ → 3′) |
|---|---|
| FDpflB1 | TTACGGGCCTATAAGCCAGGCGAGATATGATCTATATCAATTTCTCATCTTAGGTGACACTATAGAACGCG |
| FDpflB2 | TATTTGGATAATCAAATATTTACTCCGTATTTGCATAAAAACCATGCGAGTTACGGGCCTATAAGCCAGG |
| RDpflB1 | TTCTTTAGTCAGCGAGTTGAAACGTACTGCGTAGCCAGATACACGGATGGTAGTGGATCTGATGGGTACC |
| RDpflB2 | TCTAATTACATAGATTGAGTGAAGGTACGAGTAATAACGTCCTGCTGCTGTTCTTTAGTCAGCGAGTTGA |
| FDfrd1 | ATTACGTGCTGCAATTGCTGCCGCGCGAGGCAAATCCGAATGCAAAAATCGTAGGTGACACTATAGAACGCG |
| FDfrd2 | GTGCAAACCTTTCAAGCCGATCTTGCCATTGTAGGCGCCGGTGGCGCGGGATTACGTGCTGCAATTGCTG |
| RDfrd1 | GACCGTAGAAAACCCATTTGCCCGCAGGTACGTGGATTTTCAGATCGTGCTAGTGGATCTGATGGGTACC |
| RDfrd2 | TTAGATTGTAACGACACCAATCAGCGTGACAACTGTCAGGATAGCAGCCAGACCGTAGAAAACCCATTTG |
| FDadhE1 | TCGAGCAGATGATTTACTAAAAAAGTTTAACATTATCAGGAGAGCATTATTAGGTGACACTATAGAACGCG |
| FDadhE2 | GATTTTCATAGGTTAAGCAAATCATCACCGCACTGACTATACTCTCGTATTCGAGCAGATGATTTACTAA |
| RDadhE1 | TGATCGGCATTGCCCAGAAGGGGCCGTTTATGTTGCCAGACAGCGCTACTTAGTGGATCTGATGGGTACC |
| RDadhE2 | GGAAGCCGTTATAGTGCCTCAGTTTAAGGATCGGTCAACTAATCCTTAACTGATCGGCATTGCCCAGAAG |
| FDlacI1 | TCAGACCGTTTCCCGCGTGGTGAACCAGGCCAGCCACGTTTCTGCGAAAATAGGTGACACTATAGAACGCG |
| FDlacI2 | GTGAAACCAGTAACGTTATACGATGTCGCAGAGTATGCCGGTGTCTCTTATCAGACCGTTTCCCGCGTGG |
| RDlacI1 | TGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTAGTGGATCTGATGGGTACC |
| RDlacI2 | CATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAAT |

Qiagen kits (Qiagen, Chatsworth, CA, USA). All other chemicals used were of analytical grade and purchased from Sigma–Aldrich (St. Louis, MO, USA). DNA sequencing was carried out using AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA, USA) on an ABI Prism 377 DNA sequencer (Perkin Elmer).

### 2.2. Genome engineering

Deletion of the *pflB* (encoding pyruvate formate lyase), *frdABCD* (encoding fumarate reductase), *adhE*, and *lacI* (encoding LacI transcriptional repressor) genes was performed using the one-step inactivation method using the λ-Red recombinase expression plasmid, pKD46 (Datsenko and Wanner, 2000). Recombinant *E. coli* harbouring pKD46 was cultivated at 30 °C, and the expression of λ-Red recombinase was induced by adding 10 mM L-arabinose. Then electrocompetent cells were prepared by the standard protocol (Sambrook and Russell, 2001). For the deletion of the *pflB* gene, the PCR fragment for the homologous recombination was prepared in two steps. A 1234 bp DNA fragment containing the lox71 site, the chloramphenicol resistance gene, and the lox66 site fused together was obtained by PCR using the primers FDpflB1 and RDpflB1. Plasmid pMloxC (Lee et al., 2007), which contains the chloramphenicol resistance gene flanked by lox sequences (Cre recognition target), was used as a template. Then, PCR was performed with the primers FDpflB2 and RDpflB2 using the PCR product obtained above as a template. The final PCR product was introduced by electroporation into *E. coli* harbouring pKD46, expressing the (-Red recombinase. The mutants in which gene inactivation occurred by double homologous recombination were selected on the Luria-Bertani (LB) agar plate containing 34 μg ml$^{-1}$ chloramphenicol, and subsequently screened by direct colony PCR. Deletion of the *frdABCD*, *adhE*, and *lacI* genes was performed with the following primers in the same manner as described for the *pflB* gene deletion: FDfrd1, RDfrd1, FDfrd2, and RDfrd2 for *frdABCD*, FDadhE1, RDadhE1, FDadhE2, and RDadhE2 for *adhE*, and FDlacI1, RDlacI1, FDlacI2, and RDlacI2 for *lacI*. To construct marker-free mutant strains, the antibiotic selection marker was eliminated by using a helper plasmid, pJW168 (Palmeros et al., 2000), expressing the Cre recombinase and harbouring the ampicillin resistance gene and a temperature-sensitive replication origin. The chloramphenicol resistant mutants were transformed with the pJW168, and ampicillin-resistant transformants were selected on LB agar plates containing 100 μg ml$^{-1}$ ampicillin and 1 mM IPTG (for the expression of Cre recombinase) at 30 °C. Those colonies that lost the chloramphenicol resistance were selected. Among them, positive colonies were cultivated in LB medium without antibiotic at 42 °C, and then were examined for the loss of all antibiotic resistance markers by colony PCR.

To overcome the native regulation of the *ldhA* gene expression, the native promoter of the *ldhA* gene was replaced with the *trc* promoter as described in Jung et al. (2010). Substitution of the native promoter of the *acs* gene by the *trc* promoter was performed as described in Jung et al. (2010).

### 2.3. Construction of F′ plasmid-cured E. coli XL1-Blue strain

The *E. coli* XL1-Blue strain harbours the F′ plasmid which contains the Tn10-encoded tetracycline resistance gene and the *lacIq* gene for tight regulation of *lac*-based promoters such as *tac* and *trc* promoters. The F′ plasmid was cured using the method reported by Wolfson et al. (1983) with slight modification. A single colony of XL1-Blue strain was inoculated into 25 ml test tube containing 10 ml of 1/2 Brain-heart infusion (BHI) medium supplemented with 10 μg ml$^{-1}$ tetracycline, and grown overnight at 37 °C. Cells were then diluted with sterile 0.9% NaCl, plated to determine titer, and inoculated into 250 ml flask containing 50 ml of 1/2 BHI medium without antibiotic to give a final cell concentration of *ca.* 100 CFU ml$^{-1}$. After cultivation at 37 °C for 2 h until the onset of exponential growth, cells were plated to confirm the maintenance of plasmid and determine the exact initial inoculum. One hundred microliters of the culture was inoculated into 25 ml test tube containing 10 ml 1/2 BHI medium supplemented with 50 μg ml$^{-1}$ novobiocin. After incubation at 37 °C for 18–24 h, portions of the culture were plated on LB agar, and colonies were examined for plasmid presence. A colony showing sensitivity to tetracycline due to the curing of F′ plasmid was isolated among 200 colonies. The F′ plasmid-cured *E. coli* XL1-Blue strain, XB-F, was examined for its growth characteristics and production of polymers by the introduction of the recombinant plasmid. Compared with *E. coli* XL1-Blue wild-type strain, XB-F strain did not show any difference in cell growth and polymer production. Thus, XB-F strain was used as a host strain for further metabolic engineering to produce PLA and copolymers without the need of IPTG induction.

### 2.4. Cultivation

LB medium (containing per liter: 10 g tryptone, 5 g yeast extract and 5 g NaCl) supplemented with appropriated antibiotics was used for routine cultivation and genetic manipulation of *E. coli* XL1-Blue and its derivatives. A chemically defined MR medium was used for the production of PLA and its copolymer, P(3HB-*co*-LA). The MR medium (pH 7.0) contains per l: 6.67 g $KH_2PO_4$, 4 g $(NH_4)_2HPO_4$, 0.8 g $MgSO_4{\cdot}7H_2O$, 0.8 g citric acid, and 5 ml trace metal solution. The trace metal solution contains per liter of 0.5 M HCl: 10 g $FeSO_4{\cdot}7H_2O$, 2 g $CaCl_2$, 2.2 g $ZnSO_4{\cdot}7H_2O$, 0.5 g $MnSO_4{\cdot}4H_2O$, 1 g $CuSO_4{\cdot}5H_2O$, 0.1 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, and 0.02 g $Na_2B_4O_7{\cdot}10H_2O$. Carbon source and $MgSO_4{\cdot}7H_2O$ were sterilized separately. Seed cultures were prepared in 25 ml test tubes containing 10 ml LB medium at 30 °C overnight in a rotary shaker at 220 rpm. One ml of overnight culture was inoculated into 250 ml flask containing 100 ml MR medium supplemented with 20 g l$^{-1}$ glucose, unless otherwise specified. Flask cultures were carried out at 30 °C in a rotary shaker at 220 rpm for 72 h. For the cultivation of recombinant *E. coli* JLX10 strain (Jung et al., 2010), 4 g l$^{-1}$ sodium succinate was supplemented to the medium to overcome growth limitation caused by the *ppc* gene deletion, and 1 mM IPTG was added at the $OD_{600}$ of 0.5 for the expression of the *ldhA* and *acs* genes under the *trc* promoter. In the case of recombinant *E. coli* JLXF5 strain developed in this study, neither succinate nor IPTG was added to the culture medium. When necessary, 100 μg ml$^{-1}$ ampicillin, 34 μg ml$^{-1}$ chloramphenicol, and 10 μg ml$^{-1}$ thiamine were added to the medium.

All fed-batch cultures shown in Figs. 3 and 4 were carried out in a 6.6-l jar fermentor (Bioflo 3000; New Brunswick Scientific Co., Edison, NJ) initially containing 1.6 l of MR medium plus 20 g l$^{-1}$ glucose at 30 °C. The culture pH was kept at 7.0 by the automatic addition of 28% (v/v) ammonia water. The dissolved oxygen concentration (DOC) was controlled at above 40% of air saturation by automatically changing the agitation speed and additionally supplying pure oxygen. The feeding solution contained per liter: 700 g glucose, 15 g $MgSO_4{\cdot}7H_2O$, and 250 mg thiamine. The pH-stat strategy was used for nutrient feeding (Lee, 1996b). When the pH rose to a value greater than its set point (pH 7.0) by 0.1, an appropriate volume of feeding solution was automatically added to increase the glucose concentration in the culture medium to 20 g l$^{-1}$.

### 2.5. Cell growth and metabolite analysis

The concentrations of glucose and metabolites, including pyruvic acid, lactic acid, acetic acid, and 3-hydroxybutyrate (3HB), were analyzed by high-performance liquid chromatography (Varian ProStar 210; Palo Alto, CA, USA) equipped with UV/Vis (Varian

ProStar 320) and refractive index (Shodex RI-71; Tokyo, Japan) detectors. A MetaCarb 87H column (300 mm × 7.8 mm; Varian) was eluted isocratically using 0.01N $H_2SO_4$ as a mobile phase at a flow rate of 0.6 ml $min^{-1}$ and 60 °C. Cell growth was monitored by measuring the absorbance at 600 nm ($OD_{600}$) using an Ultrospec 3000 spectrophotometer (Amersham Biosciences, Uppsala, Sweden). Cell concentration defined as gram dry cell weight (DCW) per liter was routinely calculated using the pre-determined conversion factor between the $OD_{600}$ and DCW (1 $OD_{600}$ = 0.373 gDCW $l^{-1}$). In the case of the fed-batch culture shown in Fig. 4, cell concentration was determined by weighing dried cells per liter. For this, cells were collected by centrifugation at 4000 × *g* for 20 min, washed twice with distilled water, and were dried overnight at 100 °C.

### 2.6. Polymer analysis

The polymer content and the monomer composition of the polymer were determined by gas chromatography (GC; Braunegg et al., 1978). Cells were collected by centrifugation at 4000 × *g* for 20 min, washed twice with distilled water, and were dried overnight at 100 °C. About 30 mg of dried cell pellet was subjected to methanolysis with benzoic acid as an internal standard in the presence of 15% (v/v) sulfuric acid. The resulting methyl esters of constituent lactic acid and 3HB were assayed by GC according to the method reported previously (Braunegg et al., 1978). GC analysis was performed by injecting 1 μl of sample into a Agilent 6890N GC system (Agilent Technologies, Palo Alto, CA, USA) equipped with Agilent 7683 automatic injector, flame ionization detector, and a fused silica capillary column (AT$^{TM}$-Wax, 30 m, ID 0.53 mm, film thickness 1.20 μm, Alltech, Deerfield, IL, USA). The GC oven temperature was initially maintained at 80 °C for 5 min and ramped to 230 °C at 7.5 °C $min^{-1}$. And then it was increased with a gradient of 10 °C $min^{-1}$ to 260 °C and held for 5 min. Helium was used as a carrier gas. The injector and detector temperatures were maintained at 250 and 300 °C, respectively.

The molecular weight of the polymer was determined by gel permeation chromatography (GPC). Polymers were purified from the cells by the solvent extraction method (Jacquel et al., 2008). Cells accumulating polymers were harvested by centrifugation at 2500 × *g* and 4 °C, washed twice with distilled water, and lyophilized in a freeze-dryer overnight. Polymer was extracted from the lyophilized cells (*ca.* 1 g of cells) with 200 volumes of hot chloroform refluxed in a Soxhlet apparatus (Corning, Lowell, MA, USA) for 14 h. Cell debris was removed by filtration (number 2 filter, Whatman, Piscataway, NJ, USA) and solvent was evaporated. The polymer concentrate was then precipitated using 10 volumes of ice-cold methanol. The isolated polymers were dried overnight at 55 °C. Finally, the molecular weights of the polymers were determined by GPC at 40 °C using a Waters Alliance 2695 Separation Module (Waters, Milford, MA, USA) equipped with Waters 2414 RI detector and two PL Gel columns (Mixed C, 5 μm particles, 30 cm, ID 7.5 mm, Polymer Laboratories, Amherst, MA, USA). Chloroform was used as the eluent at a flow rate of 0.8 ml $min^{-1}$. Polystyrene molecular weight standards (weight average molar masses of 3,900,000, 316,500, 52,200, 31,400, 9860, 3940, 381 Da, Polymer Standard Service-USA, Silver Spring, MD, USA) with a narrow range of polydispersity (1.03–1.05) were used for calibration.

## 3. Results

### 3.1. Metabolic engineering of *E. coli* for the production of PLA and its copolymers without induction and succinate feeding

In order to use the *trc* promoter as a strong constitutive promoter without IPTG induction, the F′ plasmid which harbours the *lacIq* gene was eliminated from *E. coli* XL1-Blue. Also, the chromosomal *lacI* gene was deleted to completely remove the repressor (Fig. 1). This mutant was used as a parent strain for further metabolic engineering. To increase the intracellular pyruvic acid pool, which is a common precursor of the two main substrates, lactic acid and acetyl-CoA, for polymer synthesis, major competing pathways were blocked by chromosomal inactivation of the following genes (Fig. 1). First, the *pflB* gene was deleted to reduce the flux drain towards the formation of formic acid, ethanol, and acetic acid. Second, to block succinate synthesis, the *frdABCD* genes were deleted instead of the *ppc* gene deletion which causes succinate feeding for cell growth in a chemically defined medium. Third, the *adhE* gene was deleted to prevent the formation of ethanol from acetyl-CoA.

In order to efficiently convert the increased pyruvic acid into lactic acid, the native promoter of the *ldhA* gene was replaced with the *trc* promoter (Fig. 1) as we previously noted (Jung et al., 2010). Also, the native promoter of the *acs* gene was replaced with the *trc* promoter to increase the acetyl-CoA pool as a major CoA donor for the generation of lactyl-CoA and 3HB-CoA (Fig. 1). Even though blockage of phosphotransacetylase (Pta)-AckA pathway as a primary acetic acid forming pathway can result in the increase of

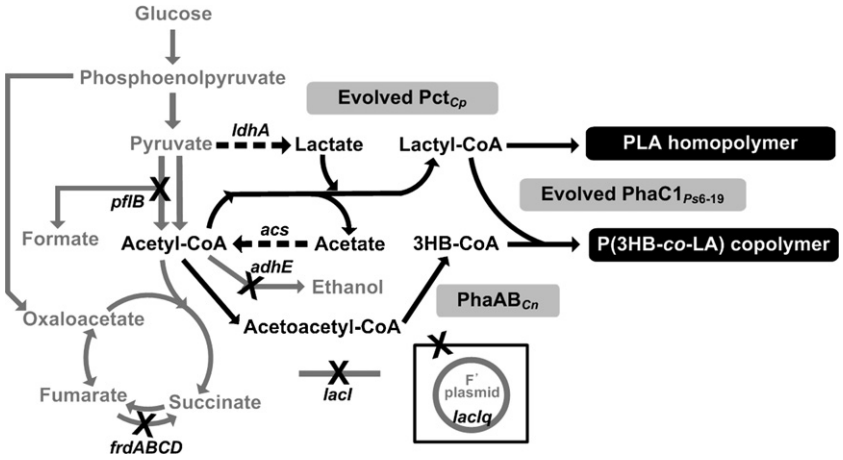


**Fig. 1.** Metabolic engineering of *Escherichia coli* XL1-Blue for efficient production of PLA polymers. The overall metabolic network is shown in gray together with the introduced metabolic pathways shown in black for the production of PLA homopolymer and P(3HB-*co*-LA) copolymer in *E. coli*. The genes with cross marks shown in black represent the chromosomal gene inactivation and the elimination of F′ plasmid shown in box, and the genes with dashed arrows shown in black represent the overexpression of the genes by chromosomal promoter replacement. The *E. coli* metabolic network shown is quite simplified to focus on the genetic manipulations performed in this study. Also, the metabolites which are directly involved in the production of PLA polymers are shown in black.

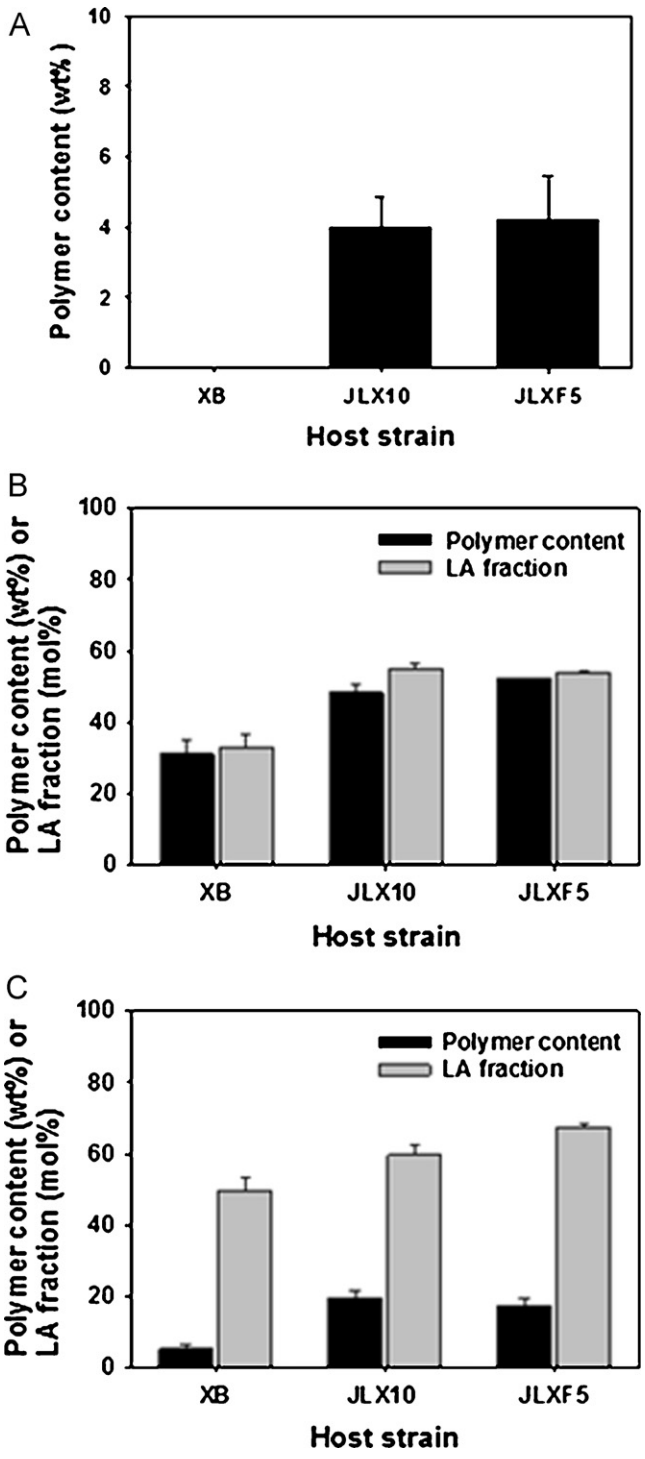


**Fig. 2.** Enhanced production of PLA homopolymer and P(3HB-*co*-LA) copolymer using recombinant *E. coli* JLXF5 strain. (A) Production of PLA homopolymers from glucose by different recombinant *E. coli* strains expressing PhaC1310$_{Ps6-19}$ and Pct540$_{Cp}$. Black bars represent the polymer content. The genotypes of the host strains employed are: XB, wild-type XL1-Blue; JLX10, XB *ΔackA PldhA::Ptrc Δppc ΔadhE Pacs::Ptrc*; JLXF5, XB-F *ΔlacI ΔpflB ΔfrdABCD ΔadhE PldhA::Ptrc Pacs::Ptrc*. For the cultivation of recombinant JLX10 strain, 4 g l$^{-1}$ sodium succinate was provided in the medium to overcome growth limitation resulted from the *ppc* gene deletion, and 1 mM IPTG was added at the OD$_{600}$ of 0.5 for the expression of the *ldhA* and *acs* genes under the *trc* promoter. (B) P(3HB-*co*-LA) copolymers produced by employing different recombinant *E. coli* strains expressing PhaC1310$_{Ps6-19}$ and Pct540$_{Cp}$ in a chemically defined MR medium supplemented with 20 g l$^{-1}$ glucose and 2 g l$^{-1}$ 3HB. Black and gray bars represent the P(3HB-*co*-LA) content and the lactate fraction in P(3HB-*co*-LA), respectively. (C) Production of P(3HB-*co*-LA) copolymers directly from glucose by different recombinant *E. coli* strains expressing PhaC1310$_{Ps6-19}$, Pct540$_{Cp}$, and PhaAB$_{Cn}$. Black and gray bars represent the P(3HB-*co*-LA) content and the lactate fraction in P(3HB-*co*-LA), respectively. The genotypes of the host strains employed and the cultivation of recombinant JLX10 strain are the same as described in (A). Results are presented as mean ± S.D. Error bars indicate S.D.

acetyl-CoA pool, the deletion of *ackA* gene in the pathway was not performed because it could retard cell growth and negatively affect the polymer production as shown in our previous study (Jung et al., 2010). Finally, the metabolically engineered *E. coli* strain, JLXF5, was constructed and used as a host strain for polymer production (Table 1).

Recombinant *E. coli* JLXF5 strain expressing the evolved Pct$_{Cp}$ and PhaC1$_{Ps6-19}$ (Fig. 1) was cultivated and its ability to produce polymers was compared with those of recombinant *E. coli* XL1-Blue and JLX10 strains expressing the same enzymes (Fig. 2). Among the various evolved enzymes Pct$_{Cp}$ and PhaC1$_{Ps6-19}$, we selected PhaC1310$_{Ps6-19}$ (PhaC1$_{Ps6-19}$ mutant having E130D, S477F, and Q481K mutaions) and Pct540$_{Cp}$ (Pct$_{Cp}$ mutant having V193A mutation and four silent nucleotide mutations of T78C, T669C, A1125G, and T1158C) (Table 1) for the production of PLA and lactate-containing copolymers because this combination allowed both relatively high polymer content and molecular weight with the increased lactate fraction in the polymer (Yang et al., 2010). The recombinant *E. coli* JLXF5 strain expressing Pct540$_{Cp}$ and PhaC1310$_{Ps6-19}$ was able to produce PLA homopolymer to a polymer content of 4.2 wt% in a medium containing 20 g l$^{-1}$ glucose, and P(3HB-*co*-52.3 mol% LA) copolymer to a polymer content of 53.9 wt% in a medium containing 20 g l$^{-1}$ glucose and 2 g l$^{-1}$ 3HB (Fig. 2A and B). In order to produce P(3HB-*co*-LA) without 3HB feeding, the *Cupriavidus necator phaA* and *phaB* genes encoding β-ketothiolase and acetoacetyl-CoA reductase, respectively, were additionally introduced (Fig. 1).

Recombinant *E. coli* JLXF5 expressing Pct540$_{Cp}$, PhaC1310$_{Ps6-19}$, and PhaAB$_{Cn}$ was able to synthesize P(3HB-*co*-67.4 mol% LA) to a polymer content of 15.2 wt% in a medium containing 20 g l$^{-1}$ glucose (Fig. 2C). In all cases, the recombinant JLXF5 strain allowed higher level production of polymers; the polymer content and lactate fraction could be increased up to 3.3-fold (Fig. 2C) and 1.6-fold (Fig. 2B), respectively, compared with those obtained with the wild-type strain expressing the same enzymes. Also, the performance of the JLXF5 strain was comparable to the most effective strain JLX10 previously constructed (Jung et al., 2010), which required succinate feeding and IPTG induction for cell growth and production of the polymers, respectively (Fig. 2).

### 3.2. Metabolic characteristics of JLXF5 strain during the production of polymers

Recombinant *E. coli* JLXF5 strain expressing Pct540$_{Cp}$ and PhaC1310$_{Ps6-19}$ was cultivated and its metabolic characteristics were compared with those of recombinant *E. coli* XL1-Blue expressing the same enzymes and also with their control strains harbouring the empty vector, pBluescript (Fig. 3). All cultures were performed under the same conditions and the culture conditions are the same as the conditions for the fed-batch culture shown in Fig. 4. The detailed conditions are described in Materials and methods. Recombinant XL1-Blue expressing Pct540$_{Cp}$ and PhaC1310$_{Ps6-19}$ showed retarded growth (cultivation time of 45 h, 3.2-fold longer) and decreased cell concentration (12.3 gDCW l$^{-1}$; 52.4% decrease) with much increased accumulation of acetic acid (13.1 g l$^{-1}$; 16.4-fold increase) compared with recombinant XL1-Blue harbouring the empty vector (Fig. 3(A) and (B)). This result would be attributed mainly to the activity of Pct540$_{Cp}$ because the expression of Pct$_{Cp}$ in *E. coli* was reported to cause growth inhibition; significant decrease of the intracellular acetyl-CoA pool by transferring CoA to carboxylate and formation of the products which might interfere with the

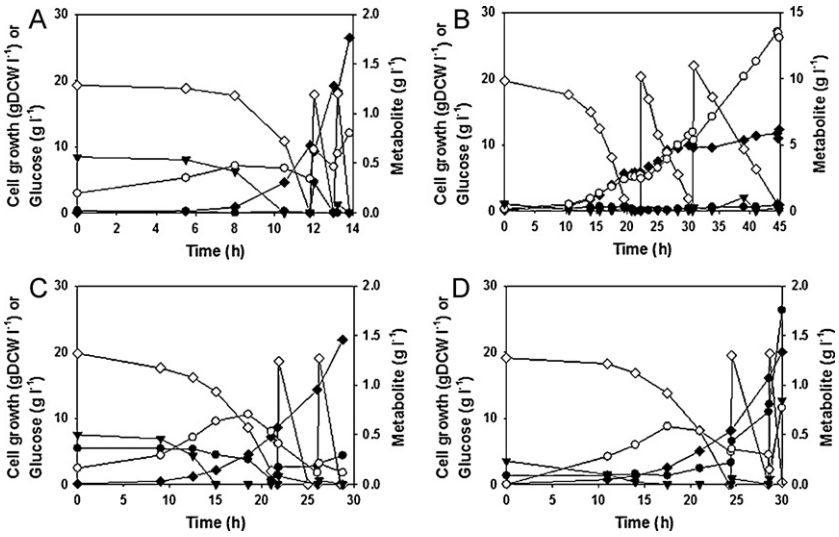


**Fig. 3.** Cultivation profiles of recombinant *E. coli* XL1-Blue and JLXF5 strains with pH-stat feeding. (A) Recombinant XL1-Blue strain harbouring pBluescript, (B) recombinant XL1-Blue strain harbouring pPs619C1310-CpPCT540, (C) recombinant JLXF5 strain harbouring pBluescript, and (D) recombinant JLXF5 strain harbouring pPs619C1310-CpPCT540. Plasmid pPs619C1310-CpPCT540 allows the expression of PhaC1310$_{Ps6-19}$ and Pct540$_{Cp}$. Symbols are: (♦) cell growth; (◊) glucose; (●) lactic acid; (○) acetic acid; (▼) pyruvic acid.

essential metabolic pathways were suggested to be the probable reasons for growth inhibition by Pct$_{Cp}$ expression in *E. coli* (Selmer et al., 2002). On the other hand, recombinant JLXF5 expressing Pct540$_{Cp}$ and PhaC1310$_{Ps6-19}$ did not show growth retardation (cultivation time of *ca.* 29 h) and reached a similar cell concentration (20–24 gDCW l$^{-1}$) compared with recombinant JLXF5 harbouring the empty vector (Fig. 3(C) and (D)). Increased accumulation of lactic acid (1.8 g l$^{-1}$; 6-fold increase) and acetic acid (0.8 g l$^{-1}$; 8-fold increase) compared with recombinant JLXF5 harbouring the empty vector was observed (Fig. 3(C) and (D)). Furthermore, at the end of the culture shown in Fig. 3(D), the recombinant JLXF5 strain was able to synthesize PLA homopolymer to a polymer content of 2.7 wt%, while the recombinant XL1-Blue strain shown in Fig. 3(B) did not synthesize PLA homopolymer. This result demonstrates that the altered metabolism in the JLXF5 strain is advantageous for the favorable functioning of the key enzymes, evolved Pct$_{Cp}$ and PhaC1$_{Ps6-19}$, and consequently enhanced the production of PLA and lactate-containing copolymers.

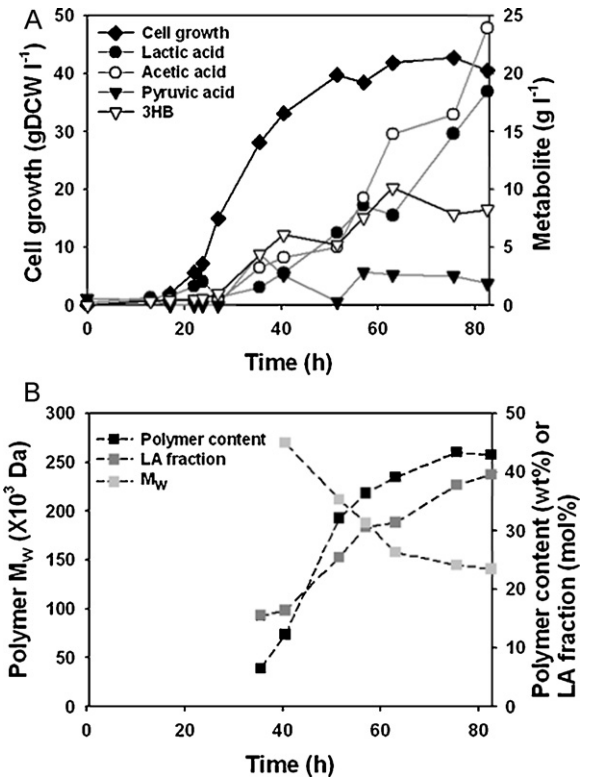


**Fig. 4.** Time profiles of polymer synthesis along with cell growth during the production of P(3HB-*co*-LA) by the pH-stat fed-batch culture of recombinant JLXF5 strain harbouring pPs619C1310-CpPCT540 and pMCS104CnAB in a chemically defined medium. (A) Time profiles of cell growth and metabolite concentrations during the fed-batch culture, and (B) time profiles of polymer content, lactate fraction, and Mw of polymer during the P(3HB-*co*-LA) production by the fed-batch culture. Plasmid pPs619C1310-CpPCT540 allows the expression of PhaC1310$_{Ps6-19}$ and Pct540$_{Cp}$, while plasmid pMCS104CnAB allows the expression of PhaAB$_{Cn}$.

### 3.3. P(3HB-co-LA) production by fed-batch culture of recombinant E. coli JLXF5 strain

Next, fed-batch culture of the recombinant JLXF5 strain expressing Pct540$_{Cp}$, PhaC1310$_{Ps6-19}$, and PhaAB$_{Cn}$ was performed in a chemically defined medium for enhanced polymer production. The time profiles of cell growth, polymer content, lactate fraction, Mw of polymer, and metabolite concentrations are shown in Fig. 4. The DOC maintained above 40% of air saturation was changed to be above 20% when cell concentration reached 30 g l$^{-1}$. The metabolites including lactic acid, acetic acid, and 3HB accumulated up to relatively high levels of 18.5, 23.9, and 8.3 g l$^{-1}$, respectively, at the end of the culture, which demonstrated the superior metabolic ability of JLXF5 strain for the enhanced polymer production. As the lactate fraction in the polymer was increased from 16.4 to 39.6 mol% during the culture, the molecular weight of the polymer was decreased from 270,000 to 141,000 Da; this phenomenon is consistent with the results of the previous studies (Jung et al., 2010; Yang et al., 2010). In our previous study, fed-batch cultures of recombinant *E. coli* XL1-Blue expressing the same enzymes, Pct540$_{Cp}$, PhaC1310$_{Ps6-19}$, and PhaAB$_{Cn}$, as used in this study, allowed production of P(3HB-*co*-LA) copolymers containing different lactate fractions by varying the fermentation conditions (Yang et al., 2010). The properties of the synthesized copolymers including the molecular weight, thermal properties and melt viscosity can be found in our previous report (Yang et al., 2010). At the end of fed-batch culture, P(3HB-*co*-39.6 mol% LA) having

the molecular weight of 141,000 Da could be produced to a polymer concentration of 20 g $l^{-1}$ with the polymer content of 43 wt% (Fig. 4).

## 4. Discussion

In this study, *E. coli* metabolic pathways were reengineered based on the strategies employed in the previous study (Jung et al., 2010) to enhance the production of PLA and lactate containing copolymers. As a host strain for the polymer production, the metabolically engineered *E. coli* JLXF5 strain allowed production of PLA homopolymer to a content of 4.2 wt% while the wild-type strain was not able to produce PLA homopolymer (Fig. 2(A)). Also, the P(3HB-*co*-LA) content and the lactate fraction in the copolymer could be increased up to 3.3-fold and 1.6-fold, respectively, compared with those obtained with the wild-type strain (Fig. 2(B) and (C)). These results demonstrate that the JLXF5 strain has a comparable metabolic ability to JLX10 strain, but does not require succinate and inducer feeding anymore. PLA and P(3HB-*co*-LA) copolymers were accumulated as distinct inclusion granules in engineered *E. coli* cells as observed in the previous study (Jung et al., 2010). The formation of the polymer granule started at the poles of the cell and accumulated to the center of the cell, which is similar to the phenomenon observed during P(3HB) accumulation (Lee et al., 1994).

It was found that JLXF5 strain which was engineered to increase the lactic acid and acetyl-CoA pools as major substrates for polymer synthesis provides an advantageous environment for the functioning of the evolved $Pct_{Cp}$ which simultaneously consumes lactic acid and acetyl-CoA in generating lactyl-CoA (Figs. 1 and 3). During the polymer synthesis, acetic acid generated from acetyl-CoA by the evolved $Pct_{Cp}$ might be secreted to the medium while the lactyl-CoA generated from lactic acid is continuously incorporated into the PLA polymer by the evolved $PhaC1_{Ps6-19}$ (Figs. 1 and 3). Acetic acid accumulated in the medium can be reconverted to acetyl-CoA through the overexpressed Acs pathway in the JLXF5 strain, increasing intracellular acetyl-CoA pool. All of these metabolic engineering strategies relieved growth inhibition observed with the control strain overexpressing the evolved $Pct_{Cp}$ (Figs. 1 and 3). Consequently, the metabolic alteration in the JLXF5 strain positively affected the coordinated functioning of the evolved $Pct_{Cp}$ and $PhaC1_{Ps6-19}$, and subsequently enhanced the production of PLA and lactate-containing copolymers.

The time profiles of polymer synthesis along with cell growth were analyzed in detail during the production of P(3HB-*co*-LA) by the pH-stat fed-batch culture of recombinant *E. coli* JLXF5 strain expressing $PhaC1310_{Ps6-19}$, $Pct540_{Cp}$, and $PhaAB_{Cn}$ in a chemically defined medium (Fig. 4). As the polymer content and lactate fraction in the polymer increased, the molecular weight of the polymer decreased. As also observed previously (Jung et al., 2010), this phenomenon is due to that lactyl-CoA is still not a highly preferred substrate even for the engineered PHA synthase for the prolonged elongation to reach a high molecular weight (Jung et al., 2010; Yang et al., 2010). Further studies are needed to resolve this issue. Even though we were able to produce PLA and lactate-containing copolymers much more efficiently by the engineered JLXF5 strain, further studies are needed to find the optimal feeding strategy to increase the polymer concentration and productivity. Although various fermentation strategies for PHA production can be employed (Lee and Chang, 1995; Wang and Lee, 1997), the production of lactate-containing polymers is not the same as the PHA production; lactate-containing polymers require not only acetyl-CoA but also lactic acid as substrates for the polymer synthesis.

In conclusion, a metabolically engineered *E. coli* strain was developed to efficiently produce PLA and its copolymer, P(3HB-*co*-LA), from glucose without the addition of inducer or succinate by one-step fermentation process. It should be mentioned that the *E. coli* JLXF5 strain developed in this study can also be used with or without further engineering for the enhanced production of other lactate-containing copolymers because the 3HB monomer can be replaced with various hydroxyalkanoates.

## Acknowledgements

We thank Sun Ok Oh at LG Chem for her help with the GPC analysis. Also, we thank Si Jae Park at KRICT and Taek Ho Yang at LG Chem for their valuable comments during this work. This work was supported by the Korean Systems Biology Research Project (20100002164) and by the World Class University Program of the Ministry of Education, Science and Technology (MEST) through the National Research Foundation of Korea (NRF). Further support by LG Chem is appreciated.

## References

Braunegg, G., Sonnleitner, B., Lafferty, R.M., 1978. A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. Eur. J. Appl. Microbiol. Biotechnol. 6, 29–37.
Chang, D.E., Jung, H.C., Rhee, J.S., Pan, J.G., 1999. Homofermentative production of D- or L-lactate in metabolically engineered *Escherichia coli* RR1. Appl. Environ. Microbiol. 65, 1384–1389.
Chen, G.Q., 2009. A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. Chem. Soc. Rev. 38, 2434–2446.
Chen, G.Q., Wu, Q., 2005. The application of polyhydroxyalkanoates as tissue engineering materials. Biomaterials 26, 6565–6578.
Cho, J.H., Park, S.J., Lee, S.Y., Jung, Y.K., 2006. Cells or plants that can produce polylactate or its copolymers and to a method for preparing polylactate or its copolymers using the same patent WO/2006/126796.
Datsenko, K.A., Wanner, B.L., 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U.S.A. 97, 6640–6645.
Drumright, R.E., Gruber, P.R., Henton, D.E., 2000. Polylactic acid technology. Adv. Mater. 12, 1841–1846.
Hazer, B., Steinbüchel, A., 2007. Increased diversification of polyhydroxyalkanoates by modification reactions for industrial and medical applications. Appl. Microbiol. Biotechnol. 74, 1–12.
Jacquel, N., Lo, C.W., Wei, Y.H., Wu, H.S., Wang, S.S., 2008. Isolation and purification of bacterial poly(3-hydroxyalkanoates). Biochem. Eng. J. 39, 15–27.
Jung, Y.K., Kim, T.Y., Park, S.J., Lee, S.Y., 2010. Metabolic engineering of *Escherichia coli* for the production of PLA and its copolymers. Biotechnol. Bioeng. 105, 161–171.
Lee, K.H., Park, J.H., Kim, T.Y., Kim, H.U., Lee, S.Y., 2007. Systems metabolic engineering of *Escherichia coli* for L-threonine production. Mol. Syst. Biol. 3, 149.
Lee, S.Y., 1996a. Bacterial polyhydroxyalkanoates. Biotechnol. Bioeng. 49, 1–14.
Lee, S.Y., 1996b. High cell-density culture of *Escherichia coli*. Trends Biotechnol. 14, 98–105.
Lee, S.Y., 1996c. Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria. Trends Biotechnol. 14, 431–438.
Lee, S.Y., Chang, H.N., 1995. Production of poly(3-hydroxybutyric acid) by recombinant *Escherichia coli* strains: genetic and fermentation studies. Can. J. Microbiol. 41, 207–215.
Lee, S.Y., Lee, K.M., Chan, H.N., Steinbüchel, A., 1994. Comparison of recombinant *Escherichia coli* strains for synthesis and accumulation of poly-(3-hydroxybutyric acid) and morphological changes. Biotechnol. Bioeng. 44, 1337–1347.
Mehta, R., Kumar, V., Bhunia, H., Upadhyay, S.N., 2005. Synthesis of poly (lactic acid): a review. J. Macromol. Sci. Polym. Rev. 45, 325–349.
Noda, I., Satkowski, M.M., Dowrey, A.E., Marcott, C., 2004. Polymer alloys of Nodax copolymers and poly(lactic acid). Macromol. Biosci. 4, 269–275.
Palmeros, B., Wild, J., Szybalski, W., Le Borgne, S., Hernandez-Chavez, G., Gosset, G., Valle, F., Bolivar, F., 2000. A family of removable cassettes designed to obtain antibiotic-resistance-free genomic modifications of *Escherichia coli* and other bacteria. Gene 247, 255–264.
Södergård, A., Stolt, M., 2002. Properties of lactic acid based polymers and their correlation with composition. Prog. Polym. Sci. 27, 1123–1163.
Sambrook, J., Russell, D.W., 2001. Molecular Cloning—A Laboratory Manual, 3rd ed. Cold Spring Harbor Labratory Press, New York.
Schreck, K.M., Hillmyer, M.A., 2007. Block copolymers and melt blends of polylactide with Nodax™ microbial polyesters: preparation and mechanical properties. J. Biotechnol. 132, 287–295.
Selmer, T., Willanzheimer, A., Hetzel, M., 2002. Propionate CoA-transferase from *Clostridium propionicum*, Cloning of gene and identification of glutamate 324 at the active site. Eur. J. Biochem. 269, 372–380.
Steinbüchel, A., Füchtenbusch, B., 1998. Bacterial and other biological systems for polyester production. Trends Biotechnol. 16, 419–427.
Steinbüchel, A., Valentin, H.E., 1995. Diversity of bacterial polyhydroxyalkanoic acids. FEMS Microbiol. Lett. 128, 219–228.

Taguchi, S., Yamada, M., Matsumoto, K., Tajima, K., Satoh, Y., Munekata, M., Ohno, K., Kohda, K., Shimamura, T., Kambe, H., Obata, S., 2008. A microbial factory for lactate-based polyesters using a lactate-polymerizing enzyme. Proc. Natl. Acad. Sci. U.S.A. 105, 17323–17327.

Valentin, H.E., Steinbüchel, A., 1994. Application of enzymatically synthesized short-chain-length hydroxy fatty acid coenzyme A thioesters for assay of polyhydroxyalkanoic acid synthases. Appl. Microbiol. Biotechnol. 40, 699–709.

Vink, E.T.H., Rabago, K.R., Glassner, D., Gruber, P.R., 2003. Applications of life cycle assessment to NatureWorks™ polylactide (PLA) production. Polym. Degrad. Stab. 80, 403–419.

Wang, F., Lee, S.Y., 1997. Production of poly(3-hydroxybutyrate) by fed-batch culture of filamentation-suppressed recombinant *Escherichia coli*. Appl. Environ. Microbiol. 63, 4765–4769.

Wolfson, J.S., Hooper, D.C., Swartz, M.N., Swartz, M.D., McHugh, G.L., 1983. Novobiocin-induced elimination of F′lac and mini-F plasmids from *Escherichia coli*. J. Bacteriol. 156, 1165–1170.

Yang, T.H., Kim, T.W., Kang, H.Y., Lee, S.-H., Lee, E.J., Lim, S.-C., Oh, S.O., Song, A.-J., Park, S.J., Lee, S.Y., 2010. Biosynthesis of polylactic acid and its copolymers using evolved propionate CoA transferase and PHA synthase. Biotechnol. Bioeng. 105, 150–160.

Yuan, W., Jia, Y., Tian, J., Snell, K.D., Müh, U., Sinskey, A.J., Lambalot, R.H., Walsh, C.T., Stubbe, J.A., 2001. Class I and III polyhydroxyalkanoate synthases from *Ralstonia eutropha* and *Allochromatium vinosum*: characterization and substrate specificity studies. Arch. Biochem. Biophys. 394, 87–98.

Zhang, S., Kamachi, M., Takagi, Y., Lenz, R.W., Goodwin, S., 2001. Comparative study of the relationship between monomer structure and reactivity for two polyhydroxyalkanoate synthases. Appl. Microbiol. Biotechnol. 56, 131–136.

Zhou, S., Causey, T.B., Hasona, A., Shanmugam, K.T., Ingram, L.O., 2003. Production of optically pure D-lactic acid in mineral salts medium by metabolically engineered *Escherichia coli* W3110. Appl. Environ. Microbiol. 69, 399–407.

Zhu, Y., Eiteman, M.A., DeWitt, K., Altman, E., 2007. Homolactate fermentation by metabolically engineered *Escherichia coli* strains. Appl. Environ. Microbiol. 73, 456–464.