*Korean J. Chem. Eng.*, 32(4), 702-706 (2015)
DOI: 10.1007/s11814-014-0240-y

pISSN: 0256-1115
eISSN: 1975-7220

# Establishment of a biosynthesis pathway for (*R*)-3-hydroxyalkanoates in recombinant *Escherichia coli*

**Si Jae Park*, Seung Hwan Lee**, Young Hoon Oh**, and Sang Yup Lee***,†**

*Department of Environmental Engineering and Energy, Myongji University,
116, Myongji-ro, Cheoin-gu, Yongin, Gyeonggy 449-728, Korea
**Industrial Biochemicals Research Group, Research Center for Biobased Chemistry, Division of Convergence Chemistry,
Korea Research Institute of Chemical Technology, P. O. Box 107, 141, Gajeong-ro, Yuseong-gu, Daejeon 305-600, Korea
***Metabolic and Biomolecular Engineering National Research Laboratory,
Department of Chemical and Biomolecular Engineering (BK21 Plus Program),
Center for Systems and Synthetic Biotechnology, and Institute for the BioCentury, KAIST,
291, Daehak-ro, Yuseong-gu, Daejeon 305-701, Korea
(*Received 8 July 2014 • accepted 19 August 2014*)

**Abstract**−A biosynthetic pathway for the production of (*R*)-3-hydroxyalkanoates (R3HAs) through *in vivo* depolymerization of poly(3-hydroxyalkanoates) [P(3HAs)] was constructed in recombinant *Escherichia coli fadA* mutant WA101 by introducing the *Pseudomonas* sp. 61-3 PHA synthase gene (*phaC2*) and the *P. aeruginosa* intracellular PHA depolymerase gene (*phaZ*). When recombinant *E. coli* WA101 strain expressing the *phaC2* gene and the *phaZ* gene was cultured in Luria-Bertani (LB) medium containing 2 g/L of sodium decanoate, R3HAs could be produced to the concentration of 0.49 g/L. The mole fraction of R3HAs was 7.5 mol% of 3-hydroxybutyrate (3HB), 31.6 mol% of 3-hydroxyhexanoate (3HHx), 30 mol% of 3-hydroxyoctanoate (3HO), 29.4 mol% of 3-hydroxydecanoate (3HD), and 1.5 mol% of 3-hydroxydodecanoate (3HDD). When the *E. coli* 3-ketoacyl-ACP reductase gene (*fabG*) was overexpressed to provide more (*R*)-3-hydroxyacyl-CoA (R3HA-CoA), the concentration of R3HAs was increased up to 1.05 g/L. Also, expression of the *fabG* gene resulted in the mole fraction change of produced R3HAs, in which 3HD fraction was enriched from 29.4 mol% to 57.9 mol% with the decrease of 3HHx fraction from 31.6 mol% to 9.6 mol%. Interestingly, the only expression of the *fabG* gene in *E. coli* WA101 could produce R3HAs to 0.55 g/L, which suggests that *E. coli* might have unidentified CoA hydrolases that have substrate specificities toward R3HA-CoA. This study shows the enantiomerically pure RHAs can be efficiently produced by metabolically engineered *E. coli* with high yield.

Keywords: (*R*)-3-hydroxyalkanoate, *Escherichia coli*, Polyhydroxyalkanoate, PHA Depolymerase, *In vivo* Depolymerization

## INTRODUCTION

(*R*)-hydroxyalkanoic acids (RHAs) can be used as chiral building blocks for the chemical synthesis of optically active fine chemicals such as antibiotics, vitamins, perfumes and pheromones [1]. Since RHAs have hydroxyl and carboxyl functional groups, they are useful for further chemical modification. For example, (R)-3-hydroxybutyrate is a promising starting material for the synthesis of carbapenems, $\beta$-lactam antibiotics [1].

Chemical synthesis of RHAs is not economically feasible due to complex reactions and expensive starting materials. An alternative for chemical synthesis of RHAs can be found in polyhydroxyalkanoates (PHAs), the polyesters of hydroxyalkanoates that are synthesized in many microorganisms under unfavorable growth conditions as carbon/energy storage materials [2,3]. Since the PHA synthase, a key enzyme for the polymerization of PHAs, can accept only (*R*)-hydroxyacyl-CoA (RHA-CoAs) as substrates if HA-CoAs have asymmetric center, the monomers of PHAs are all in (*R*)-configuration. Also, due to the broad substrate specificities of the PHA synthase, RHAs containing various side (R)-pending groups can be polymerized into PHAs as monomers. To date, more than 150 different kinds of RHAs having different functional groups are known to be incorporated into PHAs [4].

Synthesis methods of RHAs using PHA biosynthesis pathways have been developed in three ways. The first is chemical degradation of synthesized PHAs by alcoholysis in acidic condition [5,6]. The second is the expression of the PHA biosynthesis genes without PHA synthase gene to provide RHA-CoAs in the cytoplasm [7-9]. The key challenge of this process for RHA production is how to remove CoAs from RHA-CoAs. Interestingly, *E. coli* was found to have uncharacterized CoA hydrolase activity and *E. coli* equipped with $\beta$-ketothilase (PhaA) and acetoacetyl-CoA reductase (PhaB) produced (*R*)-3-hydroxybutyrate (R3HB) from glucose [8]. Production of R3HB was enhanced by employing phosphor transbutyrylase (Ptb) and butyrate kinase (Buk) along with PhaA and PhaB, in which removal of CoA from R3HB-CoA was facilitated by Ptb and Buk [8]. Also, expression of the *phaG* gene encoding 3-hydroxy acyl carrier protein (ACP):CoA transferase in recombinant *E. coli*

†To whom correspondence should be addressed.
E-mail: leesy@kaist.ac.kr

produced medium-chain-length (MCL)-RHAs from glucose [7,9]. And the third is *in vivo* depolymerization of synthesized PHAs by expressing intracellular PHA depolymerase [10,11]. RHAs produced by the second and third ways are usually excreted into the medium.

We have developed *in vivo* depolymerization of PHAs in natural PHA producing bacteria such as *Alcaligenes latus*, *Ralstonia eutropha*, and *Pseudomonas oleovorans* for the production of short-chain-length (SCL)-RHAs consisting of 4-5 carbon atoms and MCL-RHAs consisting of 6-12 carbon atoms [10]. *In vivo* depolymerization of PHAs has also been successfully constructed in recombinant *Escherichia coli* by expressing the *R. eutropha* PHA biosynthesis genes and intracellular depolymerase gene for the production of SCL-RHAs [11].

In this study, we further extend *in vivo* depolymerization of PHAs to produce RHAs that mainly consist of MCL-R3HAs in recombinant *E. coli* by expressing the *Pseudomonas* sp. 61-3 PHA synthase gene and the *P. aeruginosa* intracellular PHA depolymerase gene.

## MATERIALS AND METHODS

### 1. Bacterial Strains, Plasmids, and Genes

All bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* XL1-Blue (*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *suppE44*, *relA1*, *l*$^-$, *lac*$^-$, *F′ [proAB lacI*$^q$ *lacZΔM15, Tn10 (tet)*$^r$*]*) (Stratagene Cloning Systems, La Jolla, CA, USA) was used for gene cloning. *E. coli* WA101 (W3110 *fadA::Km*) [12, 13] was used as host strain for the synthesis of R3HAs. Plasmids p10499613C2 for the expression of the *Pseudomonas* sp. 61-3 PHA synthase gene (*phaC2*) and pMCS104FabG for the expression of the *E. coli fabG* gene were previously described [12].

### 2. Plasmids Construction

All the DNA manipulation was performed following standard procedures [14]. PCR was performed with the PCR Thermal Cycler MP (Takara Shuzo Co., LTD., Shiga, Japan) using Expandä High Fidelity PCR System (Roche Molecular Biochemicals, Mannheim, Germany). DNA sequencing was carried out using the Bigdye terminator cycle sequencing kit (Perkin-Elmer Co., Boston, MA, USA), Taq polymerase, and ABI Prism$^{TM}$ 377 DNA sequencer (Perkin-Elmer Co.).

Plasmids and primers used in this study are listed in Tables 1 and 2, respectively. Plasmids p10499613C2 and pMCS104FabG were previously described [12]. Primers for the amplification of the *phaZ* gene were designed based on the reported sequence of *P. aeruginosa phaZ* gene [15]. A plasmid for the expression of the *phaZ* gene (p10499PhaZ) was constructed by the insertion of the PCR amplified *phaZ* gene at *Eco*RI and *Hin*dIII sites of plasmid p10499A [12]. Gene fragment containing *gntT104* promoter and the *phaZ* gene was obtained by digesting p10499PhaZ with *Eco*RV and *Ssp*I and was inserted into *Pvu*II digested pACYC184 to make pACYC104PhaZ.

### 3. Culture Condition

*E. coli* XL1-Blue was cultured at 37 °C in Luria-Bertani (LB) medium (containing per liter: 10 g tryptone, 5 g yeast extract and 5 g NaCl). For the biosynthesis of R3HAs, recombinant *E. coli* WA101 strain was cultivated for 96 h in 250 ml flasks containing 100 ml of LB medium supplemented with 2 g/L of sodium decanoate (Sigma Co., St. Louis, MO, USA). Flask cultures were carried out in a rotary

**Table 1. Lists of strains and plasmids used in this study**

| Strains or plasmids | Relevant characteristics | Reference or source |
|---|---|---|
| Strains | | |
| *E. coli* XL1-Blue | *recA1, endA1, gyrA96, thi, hsdR17, suppE44, relA1, l*$^-$*, lac*$^-$*, F′ [proAB lacI*$^q$ *lacZΔM15, Tn10 (tet)*$^r$*]* | Stratagene[a] |
| *E. coli* WA101 | *E. coli* W3110 *fadA* mutant | 12, 13 |
| *P. aeruginosa* PAO1 | Wild type | KCTC[b] |
| Plasmids | | |
| p10499A | pTrc99A derivative; Ap$^r$; *gntT104* promoter | 12 |
| pACYC184 | Cm$^r$; Tc$^r$; Cloning vehicle | New England Biolabs[c] |
| p10499613C2 | p10499A derivative; *Pseudomonas* sp. 61-3 *phaC2* | 12 |
| pMCS104FabG | pBBR1MCS derivative; *gntT104* promoter, *E. coli fabG*$_{Ec}$ gene | 12 |
| p10499PhaZ | p10499A derivative; *P. aeruginosa phaZ* | This study |
| pACYC104PhaZ | pACYC184 derivative; Tc$^r$; *gntT104* promoter; *P. aeruginosa phaZ* | This study |

[a]Stratagene Cloning System, La Jolla, CA, USA
[b]Korean Collection for Type Cultures, Daejeon, Republic of Korea
[c]New England Biolabs, Beverly, MA, USA

**Table 2. List of primers used in PCR experiment using the *P. aeruginosa* chromosome as a template[a]**

| Primer | Primer sequence | Target gene |
|---|---|---|
| Primer1 | G**GAATTC**ATGCCCCAGCCCTTCGTCTTC | *phaZ* gene |
| Primer2 | CCC**AAGCTT**TAAGCCGGTCTTGGGCAGGAA | |

[a]Restriction enzyme sites are shown in bold

shaker at 250 rpm and 30 °C. Ampicillin (Ap, 50 mg/L), chloramphenicol (Cm, 34 mg/L) and tetracyclin (Tc, 10 mg/L) were added to the medium depending on the resistant marker of employed plasmid.

**4. Extraction of R3HAs in Culture Medium**

Cells were removed by centrifugation of 30 ml of culture medium at 3,000 g for 15 min. Cell pellet was used for the analysis of PHA. The culture broth was filtered and acidified to pH 2.0 with 5 N HCl and was extracted with an equal volume of ethyl acetate three times. The mixture was separated by centrifugation, and RHAs were obtained by evaporating ethyl acetate. Dried RHAs were further subjected to methanolysis in chloroform using $H_2SO_4$ and methanol for gas chromatography analysis.

**5. Analytical Procedures**

The concentrations of PHA and R3HA and their monomer compositions were determined by measuring R3HA methyl ester using gas chromatography (Agilent 6890N GC System, Agilent Technologies, Inc., CA, USA) equipped with a fused silica capillary column (Supelco SPB™-5, 30 m×0.32 mm ID 0.25 mm film, Bellefonte, PA, USA) using benzoic acid methyl ester as an internal standard [16]. Cell concentration, defined as dry cell weight (DCW) per liter of culture broth, was determined as previously described [17]. The PHA content (wt%) was defined as the percentage of the ratio of PHA concentration to cell concentration.

## RESULTS

To establish the *in vivo* depolymerization pathway in recombinant *E. coli*, we employed the *P. aeruginosa phaZ* gene encoding intracellular PHA depolymerase [15] and the *Pseudomonas* sp. 61-3 *phaC2* gene encoding SCL-MCL-PHA synthase [12,13,18]. Also, the *fadA* mutant *E. coli* WA101 was used as host strain because it has been proven to successfully provide (*R*)-3-hydroxyacyl-CoAs (R3HA-CoAs) from fatty acid [12,13]. The results of flask cultures of recombinant *E. coli* WA101 expressing various enzymes to produce RHAs are summarized in Table 3. Various plasmid sets were used to remove the potential bias effect of the presence of plasmids. Biosynthesis of RHAs was examined in two different ways: by *in vivo* depolymerization of synthesized PHA and through CoA hydrolysis of R3HA-CoAs without PHA synthesis by inherent CoA hydrolase activity of *E. coli*.

Recombinant *E. coli* WA101 harboring only the PHA synthase produced PHA to the concentration of 0.17 g/L with the PHA content of 19.4 wt%. PHA consisted of 12 mol% 3-hydroxyhexanoic acid (3HHx), 34 mol% 3-hydroxyoctanoate (3HO), and 54 mol% 3-hydroxydecanoic acid (3HD). When the PHA depolymerase was co-expressed with the PHA synthase, R3HAs were produced up to 0.49 g/L from 2 g/L of sodium decanoate along with the significant decrease of PHA content from 19.4 wt% to 4 wt%. The mole fraction of R3HAs was 7.5 mol% of 3-hydroxybutyrate (3HB), 31.6 mol% of 3-hydroxyhexanoate (3HHx), 30 mol% of 3-hydroxyoctanoate (3HO), 29.4 mol% of 3-hydroxydecanoate (3HD), and 1.5 mol% of 3-hydroxydodecanoate (3HDD). The 3HDD monomer that has two more carbons than C10 fatty acid used as carbon source was identified in R3HA mixtures, which seems to be because activation of de novo fatty acid biosynthesis pathway to synthesize 3HDD from C10 fatty acid as previously reported in several reports [12,19-25]. Interestingly, 3HB fraction in R3HAs produced by *in vivo* depolymerization of PHAs was increased up to 7.5 mol% even though very small amount of 3HB monomer around 2 mol% is generally incorporated into PHAs synthesized by the *Pseudomonas* sp. 61-3 PHA synthase II [12,13]. Amplification of FabG, 3-ketoacyl-ACP reductase, which has been confirmed to provide R3HA-CoAs by reduction of 3-ketoacyl-CoA, increased concentration of R3HAs by two-fold from 0.49 g/L to 1.05 g/L. These results clearly demonstrated that metabolic capacity of host strain to produce R3HA-CoAs is a limiting step for efficient production of R3HAs. Also, expression of the *fabG* gene resulted in the mole fraction change of produced R3HAs, in which 3HD fraction was enriched from 29.4 mol% to 57.9 mol% with the decrease of 3HHx fraction from 31.6 mol% to 9.6 mol%.

**Table 3. Results of flask cultures of recombinant *E. coli* WA101 strains for the biosynthesis of R3HAs[a]**

| Plasmids used for experiments | DCW (g/L) | PHA conc. (g/L) | PHA content (wt%) | RHA conc. (g/L) | RHA composition (mol %) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | 3HB | 3HHx | 3HO | 3HD | 3HDD |
| **With PHA biosynthesis (In vivo depolymerization)[b]** | | | | | | | | | |
| p10499613C2+pACYC814 | 0.88±0.03 | 0.17±0.02 | 19.4±0.3 | ND[c] | 0 | 0 | 0 | 0 | 0 |
| p10499613C2+pACYC104PhaZ | 0.85±0.05 | 0.03±0.01 | 4.0±0.3 | 0.49±0.10 | 7.5±1 | 31.6±2 | 30±3 | 29.4±2 | 1.5±1 |
| p10499613C2+pBBR1MCS+ pACYC104PhaZ | 0.90±0.03 | 0.02±0.01 | 2.6±0.2 | 0.31±0.05 | 4.6±1 | 28.8±2 | 31.8±3 | 33.8±2 | 1±0.5 |
| p10499613C2+pMCS104FabG+ pACYC104PhaZ | 0.93±0.04 | 0.04±0.01 | 3.8±0.2 | 1.05±0.10 | 1.7±1 | 9.6±2 | 29.8±4 | 57.9±3 | 1±0.5 |
| **Without PHA biosynthesis** | | | | | | | | | |
| pMCS104FabG | 2.13±0.02 | 0 | 0 | 0.55±0.40 | 6±1 | 35±2 | 25±2 | 33±4 | 1±1 |

[a]Cells were cultivated for 96 h at 30 °C in LB medium supplemented with 2 g $l^{-1}$ of sodium decanoate. Cultures were carried out in triplicates
[b]Overall PHA monomer composition (mol%): 3HB, 2±0.5; 3HHx, 12±1; 3HO, 33±2; 3HD, 53±2
[c]ND, not detected
Abbreviation: 3HB, (*R*)-3-hydroxybutyric acid; 3HHx, (*R*)-3-hydroxyhexanoic acid; 3HO, (*R*)-3-hydroxyoctanoic acid; 3HD, (*R*)-3-hydroxydecanoic acid; 3HDD, (*R*)-3-hydroxydodecanoic acid

Interestingly, only the expression of the *fabG* gene in *E. coli* WA101 could produce R3HAs to 0.55 g/L, which means that *E. coli* might have uncharacterized CoA hydrolases that have substrate specificities toward R3HA-CoA as previously described [8] (Table 3).

## DISCUSSION

PHAs have been extensively examined for their possible application as biodegradable polymers. Microbial production of PHAs leads to semi-commercial production of PHAs with material properties suitable for everyday life (http://www.nodax.com/). Besides their applications as polymers, PHAs can be valuable sources for chiral monomers, RHAs, which are the starting materials for fine chemicals. The precursors for PHA biosynthesis are usually converted into RHA-CoAs by various enzymes including (*R*)-specific enoyl-CoA hydratase, 3-ketoacyl-CoA reductase [12,19-24) and 3-hydroxy acyl carrier protein (ACP):CoA transferase [25]. The major challenge to producing RHAs from RHA-CoAs is how to efficiently remove CoAs from RHA-CoAs. Previously, our group employed *in vivo* depolymerization of synthesized PHAs in which CoAs in RHA-CoAs are removed during polymerization and RHAs consisting of PHAs are released by intracellular PHA depolymerase, and finally excreted into medium [10]. This strategy can be widely acceptable in natural PHA producing bacteria and recombinant *E. coli* [10,11].

*In vivo* depolymerization of synthesized PHA has been successfully employed for the production of SCL- and MCL-RHAs in natural PHA producing bacteria and for SCL-RHAs in recombinant *E. coli* [10,11,26]. In this study, we further engineered *E. coli* to produce MCL enriched R3HAs by employing MCL-PHA synthase and MCL-PHA depolymerase from pseudomonads by *in vivo* depolymerization. The metabolic pathways developed in this study are described in Fig. 1.

*In vivo* depolymerization of PHAs resulted in the production of R3HAs up to 0.49 g/L and the effect of amplification of FabG was also significant in *in vivo* depolymerization process resulting in the increase of R3HA concentration to 1.05 g/L. Previously, we reported that amplification of FabG in *fadA* mutant *E. coli* strain resulted in the production of PHA enriched in 3HD monomer (approximately 90 mol%) from 2 g/L of sodium decanoate as a carbon source [12]. However, we could not observe the same effect in the production of R3HAs by *in vivo* depolymerization, in which only slight increase of mole fraction of 3HD from 34 mol% to 58 mol% was obtained by amplification of FabG. This result suggests that difference of substrate specificity of PHA synthase and PHA depolymerase might be the reason for the difference of monomer fraction in PHAs and RHAs.

Interestingly, only the expression of the *fabG* gene in *E. coli* WA101 could produce R3HAs to 0.55 g/L, which means that *E. coli* might have unidentified CoA hydrolases that have substrate specificities toward R3HA-CoAs as previously described [8] (Table 3). Since cultivation of *E. coli* WA101 in the same culture condition could not produce R3HAs, CoA hydrolases seem to have very weak hydrolysis activities towards R3HA-CoAs, which are active only in the case that metabolic pathways can sufficiently synthesize R3HA-CoAs [8].

Also, 3HB fraction in R3HAs produced by *in vivo* depolymerization was increased up to 7.5 mol% even though very small amount of 3HB monomer lower than 2 mol% is generally incorporated into PHAs by *Pseudomonas* sp. 61-3 PHA synthase II in *fad* mutant *E. coli* strains from fatty acid as a carbon source [12,13]. This might be due to the removal of CoA from 3HB-CoA, the possible intermediate of fatty acid $\beta$-oxidation pathway, by uncharacterized CoA hydrolases of *E. coli*, which are active towards R3HB-CoA when R3HB-CoA become available for these CoA hydrolases by the weakened PHA biosynthesis.

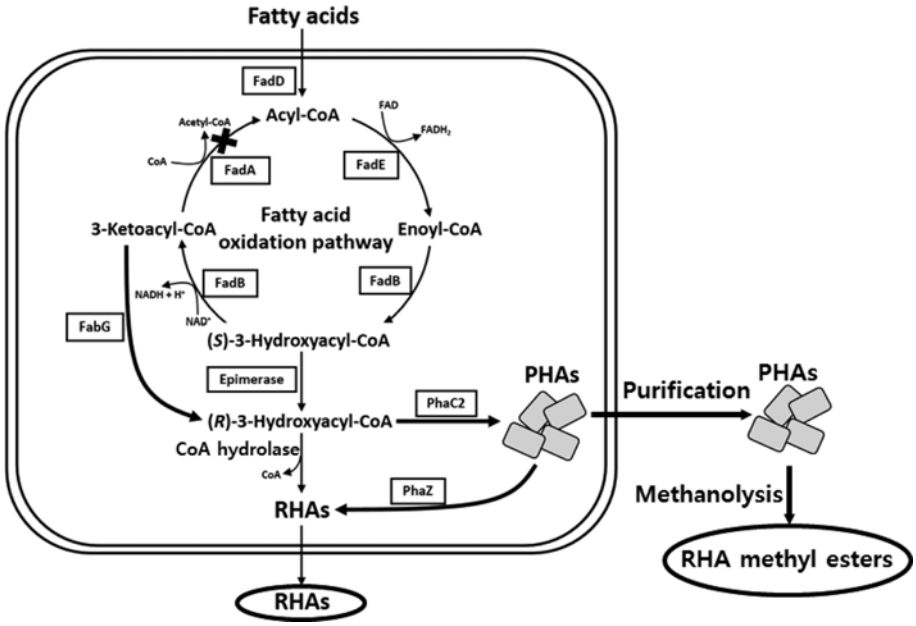


**Fig. 1. Metabolic pathway developed for the production of (*R*)-hydroxyalkanoates in recombinant *E. coli*.**

MCL-RHAs produced from *in vivo* depolymerization of PHAs are usually produced as the mixtures of RHAs having different carbon numbers as shown in this study. Thus, purification of each RHA from RHA mixtures should be carried out for further application of RHAs. It has previously been reported that R3HA methyl esters produced by methanolysis of PHAs have successfully been purified by distillation [27].

Since PHAs consisting of various monomers are used as the source for the production of RHAs by in vivo depolymerization or methanolysis, purification of each RHA from the mixtures of RHAs would not be needed if MCL-PHAs consisting of one specific monomer could be synthesized and used for the preparation of RHAs. Recently, metabolic engineering strategies for the production of homo-MCL-PHAs have been developed in recombinant Pseudomonas strains [28]. Development of in vivo depolymerization pathway in recombinant microorganisms that produce homo-MCL-PHAs can provide a novel pathway for the production of homo MCL-RHAs.

## CONCLUSION

We have produced R3HAs from *in vivo* depolymerization pathway of synthesized PHAs and directly from R3HA-CoAs by increasing metabolic capacity of *E. coli* to provide more R3HA-CoAs that are used up for inherent CoA hydrolases of *E. coli*. Increasing metabolic flux to provide more R3HA-CoAs available for R3HA synthesis is important to achieve efficient production of R3HAs. *In vivo* depolymerization strategy developed in this study can be a useful source to synthesize valuable chiral chemicals by simple fermentation.

## ACKNOWLEDGEMENTS

This work was supported by the Technology Development Program to Solve Climate Changes (Systems Metabolic Engineering for Biorefineries) from the Ministry of Science, ICT and Future Planning (MSIP) through the National Research Foundation (NRF) of Korea (NRF-2012-C1AAA001-2012M1A2A2026556). Further support from Basic Science Research Program through NRF funded by the Ministry of Education (NRF-2013R1A1A2058379) is appreciated.

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