Biotechnology and Bioprocess Engineering 26: 402-407 (2021)
DOI 10.1007/s12257-020-0270-8

pISSN 1226-8372
eISSN 1976-3816

RESEARCH PAPER

Efficient Production of Phenyllactic Acid by Whole-cell Biocatalysis with Cofactor Regeneration System

Wanseo Lee, Young-Tae Park, Seongah Lim, Sung Ho Yeom, Choong Jeon, Hoe-Suk Lee, and Young Joo Yeon

Received: 21 August 2020 / Revised: 10 November 2020 / Accepted: 26 November 2020

Abstract D-phenyllactic acid is a value added chemical with potential uses in wide areas of industry such as antibiotics, biopolymers, and pharmaceutical syntheses. It can be reduced from phenylpyruvic acid by various 2-hydroxy acid dehydrogenases. In this work, the 2-hydroxy acid dehydrogenase from *Oenococcus oeni* has been expressed in *Escherichia coli* whole cell along with formate dehydrogenases from two difference sources, *Candida boidinii* and *Pseudomonas* species, for regeneration of NADH cofactor. This could enhance the conversion of the product up to 78%, 3.4-fold increase from the one without cofactor regeneration, demonstrating a possibility of an efficient D-phenyllactic acid production system. Structural analysis by molecular dynamics simulation indicated the flexibility of the enzyme was lowered when the bound substrate was phenylpyruvic acid, compared to the natural substrate, pyruvate. This can be exploited to design 2-hydroxy acid dehydrogenase to increase the flexibility for phenylpyruvic acid, in order to further improve the production of D-phenyllactic acid.

Keywords: D-phenyllactic acid, 2-hydroxy acid dehydrogenase, formate dehydrogenase, cofactor regeneration

Wanseo Lee†, Seongah Lim, Sung Ho Yeom, Choong Jeon, Hoe-Suk Lee*, Young Joo Yeon*
Department of Biochemical Engineering, Gangneung-Wonju National University, Gangneung 25457, Korea
Tel: +82-33-640-2401; Fax: +82-33-641-2410
E-mail: hsl0828@snu.ac.kr
E-mail: yjyeon@gwnu.ac.kr

Young-Tae Park†
Natural Product Research Center, Korea Institute of Science and Technology, Gangneung 25451, Korea

†These authors have contributed equally.

*These are co-corresponding authors.

1. Introduction

Phenyllactic acid (2-hydroxy-3-phenylpropanoic acid, PLA) is a promising value added chemical for uses in pharmaceuticals, cosmetics, and other organic syntheses [1]. Optically pure D-PLA has antimicrobial activities against bacteria and fungi such as certain *Aspergillus*, *Staphylococcus*, and *Escherichia coli* strains, and can be used as food preservatives [2-4]. PLA is also a precursor to the drugs such as Danshensu and Englitazone used in the treatment of cardiovascular diseases and diabetes [5,6]. More recently, a possibility of utilizing the D-PLA as a substrate to polymerization has been suggested, with the resulting polymer molecular weight up to 100,000 g/mol [7,8].

D-PLA was first discovered in lactic acid bacteria, which used D-lactate dehydrogenase (D-LDH, EC. 1.1.1.28) to convert phenylpyruvic acid (PPA) to D-PLA [9-11]. Later, it was found that 5 g/L PPA was converted per g *E. coli* co-expressing a phenylpyruvate reductase from *Lactobacillus* sp. CGMCC 9967 and a glucose dehydrogenase with an addition of 1.5 equivalent glucose [5]. Structural studies with pyruvate as a primary substrate docked *in silico* or substrate analogues bound in the D-LDH crystal show that D-LDHs are allosteric dimers with the active site located in the interface between a cofactor binding and a catalytic domain [12-15]. NADH cofactor binding is a prerequisite of the enzyme's catalytic function, as it induces domain movement to bring NADH and pyruvate in proximity, and allows the hydride transfer from the cofactor to the substrate C2 atom [15]. In the meantime, the 2-oxo functional group is reduced by proton from nearby histidine, hence leading to reduction of the substrate. Computational modeling also has suggested decreasing the hydride transfer distance

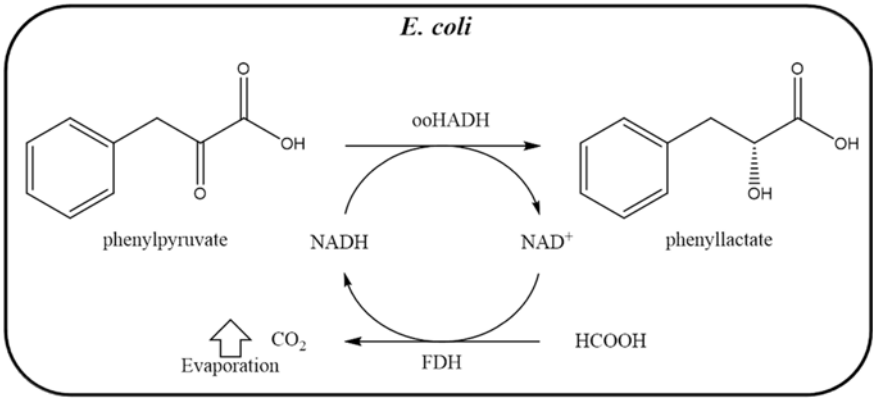

Fig. 1. Scheme for reduction of PPA to D-PLA by ooHADH coupled with formate dehydrogenase-mediated NADH regeneration.

between the cofactor and the substrate could increase the efficiency of the catalysis [16].

It is therefore inferred that increasing the available pool of NADH cofactor can increase the productivity of D-PLA. There have been many cases of co-expressing the cofactor-dependent target enzyme with cofactor regenerating enzyme to increase the productivity. Metabolic engineering of *E. coli* with NAD^+-dependent formate dehydrogenase (FDH) from *Candida boidinii* followed by the addition of formate increased the yields of NADH, lactate and succinate [17]. Whole-cell biotransformation of D-fructose to D-mannitol via *E. coli* by co-expression of a mannitol dehydrogenase along with a FDH from *Mycobacterium vaccae* increased the D-mannitol production from 0 to 15 mM [18]. In our previous study, a 2-hydroxy acid dehydrogenase from *Oenococcus oeni* (ooHADH) was able to convert 20% of phenylpyruvate to phenyllactate *in vitro* [16]. For commercial purposes, conversion must be examined in whole cells, especially because the cofactor regeneration is much cheaper. Therefore, FDHs from two different sources, *C. boidinii* (cbFDH) and *Pseudomonas* sp. (psFDH), are co-expressed with ooHADH in this work to increase the productivity of D-PLA (Fig. 1) [19]. Structural analysis using molecular dynamics simulation was also carried out to explain the activity.

2. Materials and Methods

2.1. Materials

The gene for ooDLDH (NCBI accession number MH920337) was synthesized artificially from GenScript (New Jersey, USA) after codon optimization for *E. coli* expression. A six-histidine tag-encoding sequence was added to the C-terminal of the gene. Miniprep kit for plasmid purification and Ni-NTA agarose for the affinity chromatographic purification were purchased from Qiagen (Valencia, CA, USA). Competent *E. coli* DH5α and *E. coli* BL21 (DE3) were purchased from Invitrogen (Carlsbad, CA, USA) and Novagen (Madison, WI, USA), respectively. The restriction enzymes and the In-Fusion® HD cloning kit were purchased from Thermo Scientific (Waltham, MA, USA) and TaKaRa (Mountain view, CA, USA), respectively. All other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Cloning

For the co-expression of the ooHADH and one of the FDHs, the genes need to be cloned into vectors with different antibiotic resistances for dual-selection. The ooHADH was cloned into pCDFDuet-1 vector with streptomycin resistance, while each FDH gene was cloned into pET-22b(+) with ampicillin resistance using the

Table 1. Primers used in the cloning of ooHADH, cbFDH, and psFDH

Primer name	DNA sequence (5'>3')
ooHADH For	*GTATATTAGTTAAGTATAAGAAGGAGATATACAT***ATGAAAATTTATGCGTACGGCATCCGTGATGAC**
ooHADH Rev	*TTTCTTTACCAGACTCGAG***TCAGTGGTGGTGGTGGTGGTGAAATTTGACCAGATTGCGGGTTTCTTGGC**
cbFDH For	*TTTTGTTTAACTTTAAGAAGGAGATATACAT***ATGGGCAGCAGCCATCACCATCATCACCACAGC**
cbFDH Rev	*CTTTGTTAGCAGCCGGATC***TTACTTCTTATCATGCTTTCCGTAAGCCTTGGTAACATATTCACCATTAA**
psFDH For	*TTTTGTTTAACTTTAAGAAGGAGATATACAT***ATGCATCACCATCATCACCACATGGCTAAAGTTCTG**
psFDH Rev	*CTTTGTTAGCAGCCGGATC***TTAAACTGCTTTTTTGAATTTCGCGGCTTCTTCGCTACCGCCGGTAGCGT**

*Bold type, gene; Italic type, vector; Underlined, histidine tag for purification.

primers in the Table 1. In more details, the vectors were linearized with NdeI and XhoI restriction enzymes and the gene was inserted via the Gibson cloning following the In-Fusion® HD Cloning Kit protocol. The cloned plasmids were transformed into *E. coli* DH5α and selected on LB-agar plates with respective antibiotics. Whether the genes had been correctly cloned was confirmed by DNA sequencing (Cosmogenetech, Korea).

2.3. Co-expression and purification of enzymes

E. coli BL21 was transformed with ooHADH and selected on a LB-agar plate supplemented with 50 μg/mL streptomycin as a control. Co-transformation with ooHADH/psFDH or with ooHADH/cbFDH and selection on LB-agar plates supplemented with 50 μg/mL streptomycin and ampicillin was also carried out. Single colony from each plate was inoculated to 4 mL LB media with respective antibiotic concentrations and grown overnight at 37°C, 200 rpm for preculture, followed by 200 mL batch culture at the same condition for growth up to 0.4~0.6 OD_{600nm} value. 0.8 mM IPTG (isopropyl-β-D-thiogalacto-pyranoside) was added for protein induction at 20°C, 200 rpm.

The protein induced cells were harvested by centrifugation at 4°C, 1,800 × g for 20 min and stored in -80°C for further uses. To confirm protein expression, cells were lysed in 5 mL of Bugbuster® at 20°C for 20 min, followed by centrifugation (11,300 × g, 4°C, 20 min) from which the supernatant was subjected to Ni-NTA affinity chromatography. The affinity column was washed with six times the column volume of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). For elution, 1 mL elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0) was used. Each step of the purification was sampled and subjected to 12% SDS-PAGE.

2.4. Whole-cell bioconversion of D-PLA

Harvested cells were diluted in 20 mL of 50 mM phosphate buffer (pH 6.5) with 20 mM PPA and incubated for 8 h at 30°C, 200 rpm. 0, 20, 60, and 100 mM concentrations of sodium formate was added to each culture to determine the formate-dependent conversion of D-PLA, while minimizing the pH change. The reaction mixture was sampled throughout the incubation time and subjected to HPLC equipped with an Aminex HPX-87H (300 × 7.8 mm, 9 μm) column and an UV detector (210 nm). 5 mM sulfuric acid was used as a mobile phase at 0.6 mL/min.

2.5. Structural analysis

The crystal structure of D-LDH from *Lactobacillus bulgaricus* (PDB: 1J49) was used as a template to homology model the ooHADH in the Prime module of Maestro software from Schrödinger, with OPLS3e forcefield [20]. LigPrep was used to create the energy-stable configuration of pyruvate and PPA substrates for docking, with all possible ionization states [21]. Glide module was used for docking of the substrates in the enzyme structure, which includes Grid Generation to encompass the active site of ooHADH, and the Standard Precision mode of Glide to produce docking poses [16,22]. For the molecular dynamics simulation, the substrate-docked enzyme structures were prepared by creating a TIP3P solvent box encompassing each structure with OPLS2005 forcefield, and subjected to a NVT ensemble, 200 nanoseconds, at 303 K via Desmond module.

3. Results and Discussion

3.1. Co-expression of ooHADH and FDH

The co-expressed ooHADH and FDH were purified and resolved on SDS-PAGE gel (Fig. 2). When ooHADH was co-expressed with psFDH, both proteins were produced to nearly the same quantity, clearly being resolved on the gel. However, when ooHADH was co-expressed with cbFDH, cbFDH was expressed much more than ooHADH. In both cases, the amount of ooHADH expression decreased comparable to the sole expression of ooHADH. This can affect the whole cell productivity for D-PLA, along with the difference in the cofactor regeneration capacity of the two FDHs. The k_{cat} and K_M of ooHADH were 77.8 s^{-1} and 15.6 mM for PPA, which in case for pyruvate are 674 s^{-1} and 1.12 mM, showing lower preference for PPA. However, the k_{cat} is significantly higher than the previously known phenylpyruvate reductase from *Lactobacillus* sp. CGMCC 9967, which was 47.3 s^{-1}, and the disadvantageously high K_M of ooHADH for PPA may be overcome by utilizing

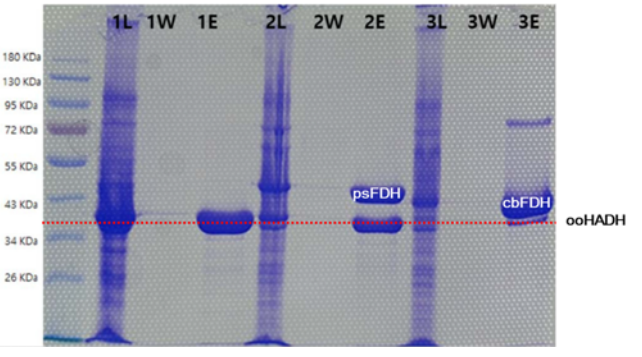

Fig. 2. Co-expression of ooHADH and FDH resolved on SDS-PAGE. Each lane number above indicates: 1, ooHADH expressed alone; 2, ooHADH and psFDH co-expressed; 3, ooHADH and cbFDH co-expressed. Each lane letter above indicates a step from the protein purification: L, cell lysate after centrifugation (11,300 × g, 4°C, 20 min) to remove debris; W, sampled from the wash step; E, sampled from the elution step.

sufficiently high concentration of the substrate. This also suggests further engineering of ooHADH is preferred to change the substrate preference and to enhance the D-PLA biosynthetic activity.

3.2. Whole cell bioconversion of D-PLA

20 mM PPA was used as a substrate in bioconversion by *E. coli* whole cell expressing ooHADH only or co-expressing FDH (Fig. 3A). The cells expressing ooHADH alone converted 35% of PPA after 8 h, and the conversions were lower for ooHADH-psFDH (31%) and ooHADH-cbFDH (20%) co-expressing cells. This is possibly due to the lowered expression level of ooHADH in the co-expression cells as shown in the SDS-PAGE (Fig. 2). However, when varying concentrations of formate were added to the co-expression cells, the conversion was greatly enhanced. Up to 100 mM of formate was added, at which further increasing formate concentration did not improve the yield. For the ooHADH and psFDH co-expressing cells, the conversion reached maximum 31, 57, 68, and 76% in 8 h with 0, 20, 60, and 100 mM formate concentration, respectively, leading to an overall 2.5-fold increase in conversion compared to the one without formate (Fig. 3B). The conversion by the ooHADH and cbFDH co-expressing cells also reached 23, 56, 76, and 78% with 0, 20, 60, and 100 mM formate concentration, respectively, a 3.4-fold increase to the one without formate (Fig. 3C). Most notably, the cbFDH co-expression was able to enhance the conversion faster as well as requiring less formate than the psFDH, reaching the maximum conversion within 4 h with 60 mM formate. This is also comparable with the 25% conversion observed *in vitro* from previous study, when 8.59 nM of purified ooHADH and 1 unit of commercial cbFDH were used to react 20 mM PPA, 1.5 mM NADH, and 30 mM sodium formate, due to the amount and stability of enzyme expressed [16]. Any further increase in reaction time or higher concentration of formate did not improve the yield significantly.

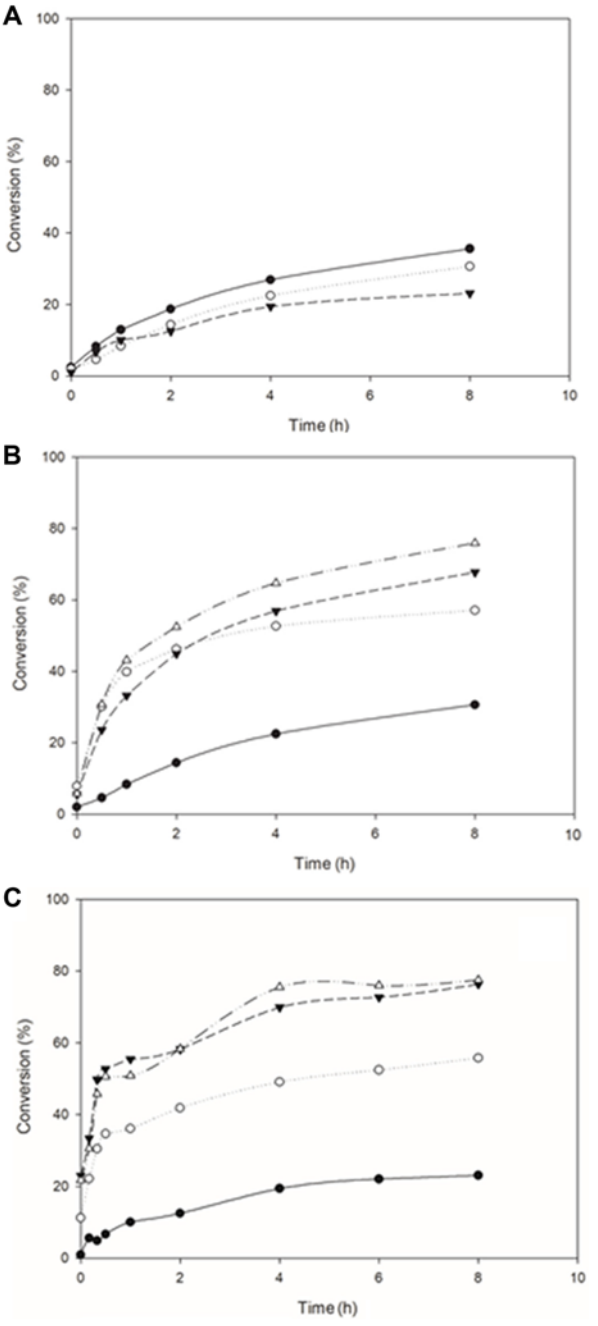

Fig. 3. *Escherichia coli* whole-cell bioconversion for D-PLA. (A) Conversion of 20 mM PPA to D-PLA by ooHADH alone or with FDH co-expression without formate. Closed circle, ooHADH; open circle, ooHADH and psFDH co-expressed; closed triangle, ooHADH and cbFDH co-expressed. (B) Conversion of 20 mM PPA by ooHADH and psFDH. (C) Conversion of 20 mM PPA by ooHADH and cbFDH. For (B) and (C): Closed circle, no formate; open circle, 20 mM formate; closed triangle, 60 mM formate; open triangle, 100 mM formate.

3.3. Molecular dynamics simulation

From the previous docking study on ooHADH, bound pyruvate had a Gibbs free energy of binding (ΔG_{bind}) of -3.32 kcal/mol, whereas that of PPA had a ΔG_{bind} of -5.31 kcal/mol [16]. This contradicted the 120-fold higher activity for pyruvate than PPA found in ooHADH, especially the 14-fold lower K_M value for pyruvate than PPA. The k_{cat} was also 8.7-fold higher for pyruvate. The contradiction could be explained by running molecular dynamics simulation on the structures of ooHADH docked with pyruvate or PPA (Fig. 4). The overall RMSD value for ooHADH-pyruvate averaged at 5 Å, but that for ooHADH-PPA was at 4 Å, indicating higher flexibility of the pyruvate bound enzyme structure. It is a general recognition that the increased flexibility of an enzyme structure is correlated with its higher catalytic activity, because the higher flexibility

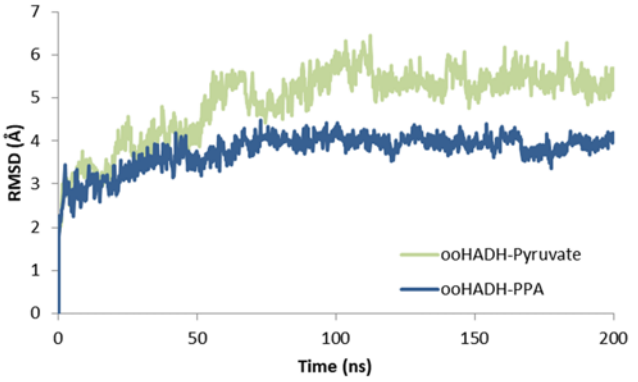

Fig. 4. RMSD values from molecular dynamics simulation of ooHADH-pyruvate and ooHADH-PPA structures.

means the enzyme can adapt the substrate binding mode for catalytic mechanism more frequently, with higher chances of optimized active site configuration [23]. Furthermore, 1 Å difference in RMSD of overall enzyme structure can lead to much greater motion in the active site, accounting for the inverted substrate preference between the experimental data and the docking result, in which docking does not reflect dynamic motion of the enzyme but only sample a stationary state. The production of D-PLA by ooHADH can therefore be enhanced by re-designing the ooHADH-PPA structure to this end, in combination with the FDH-cofactor regeneration system.

4. Conclusion

The conversion of PPA to D-PLA by ooHADH-expressing *E. coli* whole cell could be enhanced up to 3.4-fold by introducing a cofactor regeneration system, consisting of co-expressed FDH and formate added. Although the expression level of ooHADH was decreased due to the FDH co-expression, replenishing NADH could overcome the drawback. Furthermore, the structural information on the decreased flexibility of ooHADH-PPA compared to ooHADH-pyruvate can be exploited to re-design the enzyme for a higher flexibility, covering the decreased expression level with a higher activity. The development of an efficient synthetic process for D-PLA is expected to contribute to the production of various value added chemicals.

Acknowledgements

This research was supported by the Technology Development Program to Solve Climate Changes of the National Research Foundation (NRF) funded by the Ministry of Science and ICT (Grant Number: 2017M1A2A2087630).
This study was supported by 2017 Academic Research Support Program in Gangneung-Wonju National University.
The authors declare no conflict of interest.
No ethical approval and no informed consent required.

Nomenclature

Enzymes

cbFDH:	Formate dehydrogenase from *Candida boidinii*
psFDH:	Formate dehydrogenase from *Pseudomonas species*
ooHADH:	2-hydroxy acid dehydrogenase from *Oenococcus oeni*
D-PLA:	D-phenyllactic acid
PPA:	Phenylpyruvic acid
NADH:	Nicotinamide Adenine Dinucleotide, reduced form
k_{cat}:	Catalytic turnover number (unit: s^{-1})
K_M:	Michaelis-Menten constant (unit: mM)
ΔG_{bind}:	Gibbs free energy of binding between the enzyme and the substrate (unit: kcal mol^{-1})

References

1. Miltenberger, K. (2000) Hydroxycarboxylic acids, aliphatic. *Ullmann's encyclopedia of industrial chemistry*. Wiley-VCH Verlag GmbH & Co. KGaA: Gersthofen, Germany.
2. Chen, L., Y. Bai, T. P. Fan, X. Zheng, and Y. Cai (2017) Characterization of a D-lactate dehydrogenase from *Lactobacillus fermentum* JN248 with high phenylpyruvate reductive activity. *J. Food Sci*. 82: 2269-2275.
3. Lavermicocca, P., F. Valerio, A. Evidente, S. Lazzaroni, A. Corsetti, and M. Gobbetti (2000) Purification and characterization of novel antifungal compounds from the sourdough *Lactobacillus plantarum* strain 21B. *Appl. Environ. Microbiol*. 66: 4084-4090.
4. Valerio, F., M. Favilla, P. De Bellis, A. Sisto, S. De Candia, and P. Lavermicocca (2009) Antifungal activity of strains of lactic acid bacteria isolated from a semolina ecosystem against *Penicillium roqueforti*, *Aspergillus niger* and *Endomyces fibuliger* contaminating bakery products. *Syst. Appl. Microbiol*. 32: 438-448.
5. Xu, G. C., L. L. Zhang, and Y. Ni (2016) Enzymatic preparation of D-phenyllactic acid at high space-time yield with a novel phenylpyruvate reductase identified from *Lactobacillus* sp. Cgmcc 9967. *J. Biotechnol*. 222: 29-37.
6. Zhou, L., Z. Zuo, and M. S. S. Chow (2005) Danshen: An overview of its chemistry, pharmacology, pharmacokinetics, and clinical use. *J. Clin. Pharmacol*. 45: 1345-1359.
7. Nguyen, H. D., X. Jin, D. Kaneko, and T. Kaneko (2011) Syntheses of high molecular weight poly(L-phenyllactic acid)s by a direct polycondensation in the presence of stable lewis acids. *Chem. Lett*. 40: 584-585.
8. Sohn, Y. J., H. T. Kim, S. Y. Jo, H. M. Song, K. A. Baritugo, J. Pyo, J. Choi, J. C. Joo, and S. J. Park (2020) Recent advances in systems metabolic engineering strategies for the production of biopolymers. *Biotechnol. Bioprocess Eng*. 25: 848-861.

9. Yu, S., H. Jiang, B. Jiang, and W. Mu (2012) Characterization of D-lactate dehydrogenase producing D-3-phenyllactic acid from *Pediococcus pentosaceus*. *Biosci*. *Biotechnol*. *Biochem*. 76: 853-855.
10. Jia, J., W. Mu, T. Zhang, and B. Jiang (2010) Bioconversion of phenylpyruvate to phenyllactate: Gene cloning, expression, and enzymatic characterization of D- and L1-lactate dehydrogenases from *Lactobacillus plantarum* SK002. *Appl. Biochem. Biotechnol*. 162: 242-251.
11. Mu, W., S. Yu, L. Zhu, T. Zhang, and B. Jiang (2012) Recent research on 3-phenyllactic acid, a broad-spectrum antimicrobial compound. *Appl. Microbiol. Biotechnol*. 95: 1155-1163.
12. Zheng, Z., B. Sheng, C. Gao, H. Zhang, T. Qin, C. Ma, and P. Xu (2013) Highly stereoselective biosynthesis of (R)-alpha-hydroxy carboxylic acids through rationally re-designed mutation of d-lactate dehydrogenase. *Sci. Rep*. 3: 3401.
13. Wang, M., L. Zhu, X. Xu, L. Wang, R. Yin, and B. Yu (2016) Efficient production of enantiomerically pure D-phenyllactate from phenylpyruvate by structure-guided design of an engineered D-lactate dehydrogenase. *Appl. Microbiol. Biotechnol*. 100: 7471-7478.
14. Zheng, Z., C. Ma, C. Gao, F. Li, J. Qin, H. Zhang, K. Wang, and P. Xu (2011) Efficient conversion of phenylpyruvic acid to phenyllactic acid by using whole cells of *Bacillus coagulans* sdm. *PLoS One*. 6: e19030.
15. Razeto, A., S. Kochhar, H. Hottinger, M. Dauter, K. S. Wilson, and V. S. Lamzin (2002) Domain closure, substrate specificity and catalysis of D-lactate dehydrogenase from *Lactobacillus bulgaricus*. *J. Mol. Biol*. 318: 109-119.
16. Lee, H. S., J. Park, Y. J. Yoo, and Y. J. Yeon (2019) A novel D-2-hydroxy acid dehydrogenase with high substrate preference for phenylpyruvate originating from lactic acid bacteria: Structural analysis on the substrate specificity. *Enzyme Microb. Technol.* 125: 37-44.
17. Berrios-Rivera, S. J., G. N. Bennett, and K. Y. San (2002) Metabolic engineering of *Escherichia coli*: Increase of nadh availability by overexpressing an nad(+)-dependent formate dehydrogenase. *Metab. Eng*. 4: 217-229.
18. Kaup, B., S. Bringer-Meyer, and H. Sahm (2004) Metabolic engineering of *Escherichia coli*: Construction of an efficient biocatalyst for d-mannitol formation in a whole-cell biotransformation. *Appl. Microbiol. Biotechnol*. 64: 333-339.
19. Sathesh-Prabu, C., K. S. Shin, G. H. Kwak, S. K. Jung, and S. K. Lee (2019) Microbial production of fatty acid via metabolic engineering and synthetic biology. *Biotechnol. Bioprocess Eng*. 24: 23-40.
20. Schrödinger Release 2018-4 Prime, Schrödinger, LLC, New York, NY, USA.
21. Schrödinger Release 2018-4 Ligprep, Schrödinger, LLC, New York, NY, USA.
22. Schrödinger Release 2018-4 Glide, Schrödinger, LLC, New York, NY, USA.
23. Mukherjee, J. and M. N. Gupta (2015) Increasing importance of protein flexibility in designing biocatalytic processes. *Biotechnol. Rep. (Amst)*. 6: 119-123.