Biotransformations

Deutsche Ausgabe: DOI: 10.1002/ange.201606235
Internationale Ausgabe: DOI: 10.1002/anie.201606235

Cascade Biocatalysis for Sustainable Asymmetric Synthesis: From Biobased L-Phenylalanine to High-Value Chiral Chemicals

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Abstract: *Sustainable synthesis of useful and valuable chiral fine chemicals from renewable feedstocks is highly desirable but remains challenging. Reported herein is a designed and engineered set of unique non-natural biocatalytic cascades to achieve the asymmetric synthesis of chiral epoxide, diols, hydroxy acid, and amino acid in high yield and with excellent ee values from the easily available biobased L-phenylalanine. Each of the cascades was efficiently performed in one pot by using the cells of a single recombinant strain over-expressing 4–10 different enzymes. The cascade biocatalysis approach is promising for upgrading biobased bulk chemicals to high-value chiral chemicals. In addition, combining the non-natural enzyme cascades with the natural metabolic pathway of the host strain enabled the fermentative production of the chiral fine chemicals from glucose.*

Sustainable manufacturing of chemicals from renewable feedstocks is attracting increasing attention because of the oil depletion and global climate change.[1] Significant progress has been made in chemical or enzymatic conversion[2] of biomass into bulk chemicals.[3,4] The advances of metabolic engineering and synthetic biology have enabled the fermentation of (hemi)cellulose-derived sugars to produce a variety of biobased bulk chemicals.[5] However, the fermentative production of non-natural high-value fine chemicals still faces many challenges, including the lack of efficient pathways towards the non-natural chemicals. We envision a feasible and potentially general approach for green, efficient, and sustainable production of non-natural chiral fine chemicals by developing novel types of one-pot non-natural biocatalytic cascades to convert a selected easily available biobased bulk chemical into the target fine chemicals (Scheme 1). The selected bulk chemical could be a primary cell metabolite, thus the designed enzyme cascades to convert this metabolite can be integrated with the native metabolic pathway of the host microorganism to achieve the fermentative production of high-value chiral chemicals directly from sugar.

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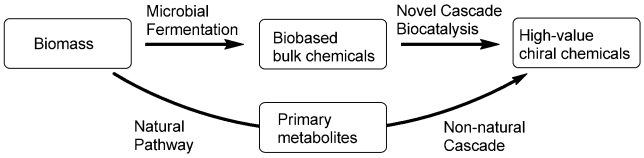
Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under http://dx.doi.org/10.1002/anie.201606235.

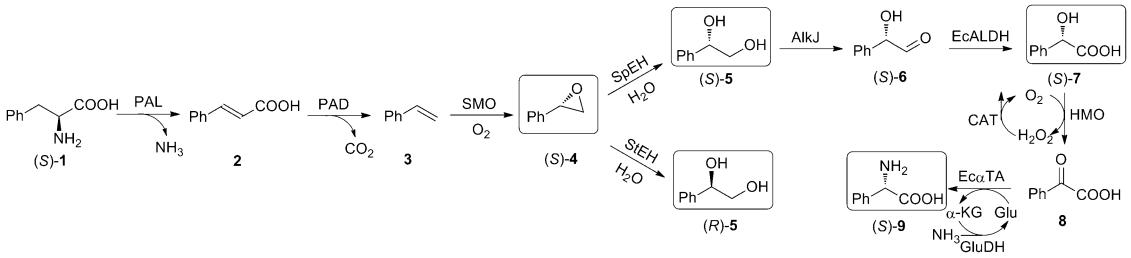


Scheme 1. General concept of sustainable synthesis of high-value non-natural chiral chemicals from biobased chemicals or biomass by using non-natural enzyme cascades.

Amino acids are attractive biobased chemicals for sustainable synthesis of chiral molecules since they are currently produced by fermentation in large amounts and at low cost.[6] One-pot chemical transformations of amino acids into chiral chemicals are still rare. In contrast, cascade biocatalysis, an emerging green tool for asymmetric synthesis with high selectivity and no intermediate separation,[7,8] could provide new asymmetric synthesis of chiral chemicals from amino acids. For instance, L-amino acids were converted into the corresponding chiral α-hydroxy acids by replacing an amino group with a hydroxy group via a two-step biocascade, which is advantageous over the chemical method.[9] Herein we report new types of cascade biocatalysis consisting of 3–8 enzymatic steps for the enantioselective conversion of L-phenylalanine [(*S*)-**1**] into chiral epoxide, diols, hydroxy acid, and amino acid (Scheme 2), for which there are no chemical counterparts; and the one-pot synthesis of these chiral compounds, which are useful and valuable synthons and pharmaceutical intermediates (see Table S1 in the Supporting Information), from L-phenylalanine or glucose by using the developed cascades.

Specifically, the novel biocatalytic cascades to synthesize five chiral molecules from L-phenylalanine [(*S*)-**1**; Scheme 2] were designed as follows. Cascade 1: (*S*)-**1** undergoes deamination/decarboxylation to styrene (**3**) by using phenylalanine ammonia lyase (PAL)[10] and phenylacrylic acid decarboxylase (PAD),[11] followed by *S*-selective epoxidation of **3** to give (*S*)-styrene oxide [(*S*)-**4**] with styrene monooxygenase (SMO).[12] Cascades 2 and 3: combining cascade 1 and regioselective hydrolysis of (*S*)-**4** with epoxide hydrolase from potato (StEH) or from *Sphingomonas* HXN-200 (SpEH)[13] gives rise to (*R*)- or (*S*)-1-phenylethane-1,2-diol [(*R*)-**5** or (*S*)-**5**]. Cascade 4: extension of cascade 3 by regioselective double oxidation of (*S*)-**5** using alcohol dehydrogenase (AlkJ) and aldehyde dehydrogenase (EcALDH)[14] produces (*S*)-mandelic acid [(*S*)-**7**]. Cascade 5: addition of oxidation–transamination to cascade 4 by using hydroxymandelate oxidase (HMO), α-transaminase (EcαTA), glutamate dehydrogenase (GluDH), and catalase (CAT),[14] forms (*S*)-phenylglycine [(*S*)-**9**].

To engineer cascade 1, AtPAL2 from *Arabidopsis thaliana*[10] was selected for the deamination of (*S*)-**1** (see Fig-



Scheme 2. New types of cascade biocatalysis for one-pot transformation of biobased L-phenylalanine [(*S*)-**1**] into (*S*)-styrene oxide [(*S*)-**4**], (*S*)- and (*R*)-1-phenylethane-1,2-diol [(*S*)-**5** and (*R*)-**5**], (*S*)-mandelic acid [(*S*)-**7**], and (*S*)-phenylglycine [(*S*)-**9**].

ure S1), and AnPAD (*fdc1* & *pad1*[11]) from *Aspergillus niger* were chosen for the decarboxylation of cinnamic acid (**2**; see Figure S2). Both AtPAL2 and AnPAD were then cloned together as PAL-PAD on compatible plasmids (see Figure S3). Combinations of PAL-PAD and SMO[12] plasmids gave *E. coli* strains (LZ01-LZ12; Table S2) for cascade 1 (see Figure S4). Similarly, combining PAL-PAD with either SMO-StEH[13] or SMO-SpEH[14] plasmids gave *E. coli* strains (LZ13-LZ24; Table S2) for cascade 2 (see Figure S5) or *E. coli* strains (LZ25-LZ36; Table S2) for cascade 3 (see Figure S6). For cascade 4, *E.coli* strains (LZ37-LZ60; Table S2) containing PAL-PAD, SMO-SpEH, and AlkJ-EcALDH[14] plasmids were constructed (see Figure S7). For cascade 5, *E. coli* strains (LZ61-LZ84; Table S2) containing PAL-PAD, SMO-SpEH, AlkJ-EcALDH, and HMO-EcαTA-GluDH-CAT[14] plasmids were engineered (see Figure S8). The best strains for the cascade biotransformations 1–5 are given in Table 1. Among them, *E. coli* (LZ76) containing four plasmids with 12 genes for cascade 5 catalyzes 8 reaction steps (Figure 1).

The *E. coli* (pRSF-PAL-PAD) and the best five strains for each cascade grew well in M9 medium, and the resting cells harvested at late exponential phase were used for the corresponding biotransformation in a two-phase system containing KP buffer and *n*-hexadecane (1:1). The time courses of the biotransformations are shown in Figure 2. *E. coli* (pRSF-PAL-PAD) efficiently converted 150 mM of **1** into 139 mM (14 g L^{-1}) **3** in 92% conversion at 5 hours, with no accumulation of the intermediate or byproducts (Figure 2a). In comparison, fermentative production of **3** gave a much lower concentration, even if designed micelles were used.[15]

Biotransformation of 140 mM of (*S*)-**1** with *E. coli* (LZ03) produced 125 mM (15 g L^{-1}) of (*S*)-**4** in 99% *ee* and 89% conversion at 10 hours (Figure 2b). Bioconversion of 120 mM of (*S*)-**1** with *E. coli* (LZ20) and *E. coli* (LZ26) for 10 hours

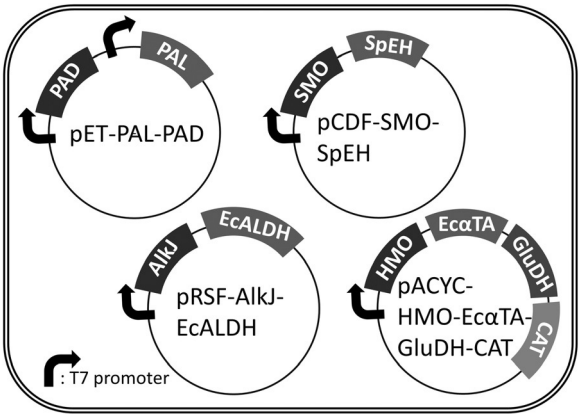


Figure 1. *E. coli* (LZ76) containing four recombinant plasmids and over-expressing 10 enzymes for converting (*S*)-**1** into (*S*)-**9**.

gave 109 mM (15 g L^{-1}) of (*R*)-**5** in 96% *ee* and 91% conversion (Figure 2c) and 107 mM (15 g L^{-1}) of (*S*)-**5** in 97% *ee* and 89% conversion, respectively (Figure 2d). Reaction of 120 mM of (*S*)-**1** with *E. coli* (LZ37) afforded 100 mM of (*S*)-**7** at 10 hours and 110 mM (17 g L^{-1}) of (*S*)-**7** at 24 hours in 99% *ee* (92% conversion; Figure 2e). In all these biotransformations, nearly no intermediates or byproducts remained in the final reaction mixtures.

Bioconversion of 40 mM of (*S*)-**1** with *E. coli* (LZ76) gave 34 mM (5.1 g L^{-1}) of (*S*)-**9** in greater than 99% *ee* and 85% conversion at 24 hours. Thus, the very challenging eight-step biocatalytic cascade was successfully achieved with a single recombinant strain coexpressing 10 enzymes. During the bioconversion, significant amounts of (*S*)-**7** accumulated before the addition of new portions of NH_3/NH_4Cl and glucose at 10 hours. Thus, the HMO-EcαTA-GluDH-CAT part of the cascade is less efficient than the PAL-PAD-SMO-SpEH-AlkJ-EcALDH part. Choosing other more active enzymes for the cascade from (*S*)-**7** to (*S*)-**9** could further improve the efficiency and productivity of cascade 5.

To further enhance the sustainability of the bioprocesses, the petro-based *n*-hexadecane was replaced by ethyl oleate (EO),[16] which could be produced from biobased oleic acid and ethanol, in

Table 1: Engineered recombinant *E. coli* strains for the designed cascade biotransformations.

Strain[a]	Recombinant plasmids[b]	Reactions	Conv. [%]
E. coli (LZ03)	pACYC-PAL-PAD, pRSF-SMO	(*S*)-**1** to (*S*)-**4**	89
E. coli (LZ20)	pET-PAL-PAD, pCDF-SMO-StEH	(*S*)-**1** to (*R*)-**5**	91
E. coli (LZ26)	pACYC-PAL-PAD, pET-SMO-SpEH	(*S*)-**1** to (*S*)-**5**	89
E. coli (LZ37)	pACYC-PAL-PAD, pCDF-SMO-SpEH, pET-AlkJ-EcALDH	(*S*)-**1** to (*S*)-**7**	92
E. coli (LZ76)	pET-PAL-PAD, pCDF-SMO-SpEH, pRSF-AlkJ-EcALDH, pACYC-HMO-EcαTA-GluDH-CAT	(*S*)-**1** to (*S*)-**9**	85

[a] *E. coli* T7 expression strains harboring the recombinant plasmids. [b] Plasmids pACYC, pCDF, pET, and pRSF were constructed on empty pACYCDuet-1, pCDFDuet-1, pETDuet-1, and pRSFDuet-1, respectively.

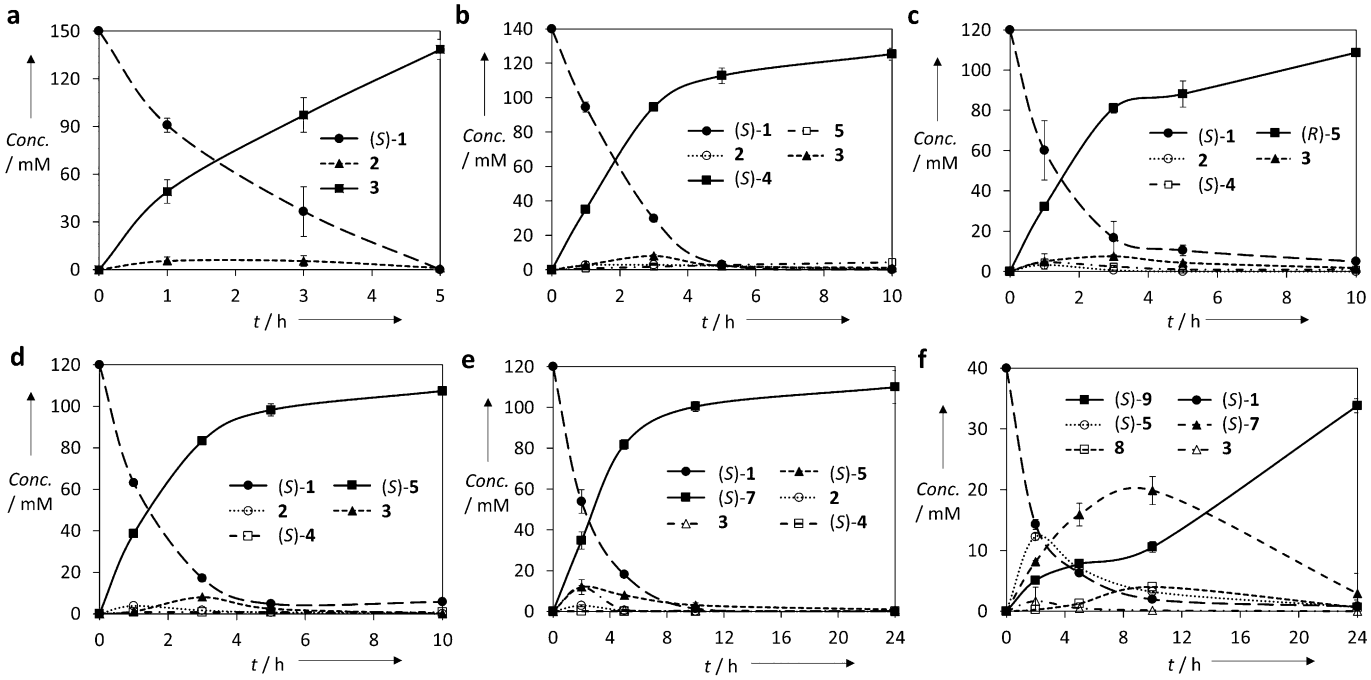


Figure 2. Time curves of cascade biotransformation of L-phenylalanine into various chemicals. a) (*S*)-**1** to **3** with *E. coli* (pRSF-PAL-PAD). b) (*S*)-**1** to (*S*)-**4** with *E. coli* (LZ03). c) (*S*)-**1** to (*R*)-**5** with *E. coli* (LZ20). d) (*S*)-**1** to (*S*)-**5** with *E. coli* (LZ26). e) (*S*)-**1** to (*S*)-**7** with *E. coli* (LZ37). f) (*S*)-**1** to (*S*)-**9** with *E. coli* (LZ76). All biotransformations were performed with resting cells (15 g cdw L^{-1}) in a two-phase system containing KP buffer (200 mM, pH 8, 0.5–2% glucose) and *n*-hexadecane (1:1) at 250 rpm and 30 °C. In the biotransformation shown in (f), 200 mM NH_3/NH_4Cl and 2% glucose was added at 10 h. Concentrations are normalized to the volume of aqueous solution. Error bars represent the standard deviation of triplicated biotransformations.

the two-phase system. As illustrated in Table S3, biotransformations of (*S*)-**1** to **3**, (*S*)-**4**, (*R*)-**5**, (*S*)-**5**, (*S*)-**7**, and (*S*)-**9**, afforded similar high conversion (84–96%) and product *ee* values (96–>99%) as those in the two-phase system using *n*-hexadecane.

Preparative biotransformations were carried out with the resting cells of the corresponding strains in KP buffer/EO (10:1) except for the production of (*S*)-**4**. The products were purified by solvent extraction, crystallization, precipitation, or flash chromatography. As shown in Table 2, (*S*)-**4**, (*R*)-**5**, (*S*)-**5**, (*S*)-**7**, and (*S*)-**9** were isolated in 376–1082 mg (62–78% yield) with excellent *ee* values (96–>99%).

The five recombinant strains contain the natural pathway from glucose to L-phenylalanine and the engineered non-natural pathway from L-phenylalanine to the corresponding chiral chemicals (*S*)-**4**, (*R*)-**5**, (*S*)-**5**, (*S*)-**7**, and (*S*)-**9**. These strains grew in M9 medium supplemented with glucose, and (*S*)-**4**, (*R*)-**5**, (*S*)-**5**, (*S*)-**7**, and (*S*)-**9** were directly produced in the culture medium at 0.7–1.6 mM (see Table S4). In comparison, the product concentrations are lower than those from the cascade biotransformations of (*S*)-**1**. Nevertheless, it opens a new opportunity for sustainable production of the non-natural chiral fine chemicals by fermentation via integrating the non-natural pathway from a primary metabolite towards the target non-natural product. We are currently working on the improvement of the productivity by engineering the cells using synthetic biology tools. At this moment, the production of the chiral fine chemicals from the biobased chemical by cascade biotransformations is much more practical. In general, this approach is also much simpler and more flexible for the sustainable production of chiral fine chemicals with versatile structures and production volumes.

Table 2: Preparation of (*S*)-**4**, (*R*)-**5**, (*S*)-**5**, (*S*)-**7**, and (*S*)-**9** by cascade biotransformations of L-phenylalanine [(*S*)-**1**]

Catalyst[a]	Subs. conc. [mM]	Prod.	Yield[b] [mg]	[%]	*ee* [%][c]
E. coli (LZ03)[d]	100	(*S*)-**4**	926	77	99
E. coli (LZ20)	100	(*R*)-**5**	975	71	96
E. coli (LZ26)	100	(*S*)-**5**	1082	78	97
E. coli (LZ37)	100	(*S*)-**7**	1058	70	99
E. coli (LZ76)	40	(*S*)-**9**	376	62	>99

[a] Reaction was performed in 100 mL KP buffer (200 mM, pH 8, 0.5–2% glucose) and 10 mL ethyl oleate with resting cells (20 g cdw L^{-1}) at 250 rpm and 30 °C for 24 h. [b] Yield of isolated product. [c] Determined by chiral HPLC analysis. [d] 100 mL *n*-hexadecane was used instead of 10 mL ethyl oleate.

In summary, we designed and constructed novel types of non-natural biocatalytic cascades for one-pot enantioselective conversion of biobased L-phenylalanine [(*S*)-**1**] into a number of high-value chiral fine chemicals, with no chemical counterparts. The engineering of non-natural biocatalytic cascades with up to 8 steps of enzymatic reactions, 10 enzymes, and 12 genes was also successfully demonstrated, thus serving as a showcase of developing highly efficient and complex multienzyme cascades. Cascade biotransformations with the engineered whole-cell biocatalysts produced the target chiral epoxide (*S*)-**4**, diols (*R*)- and (*S*)-**5**, hydroxy acid (*S*)-**7**, and

amino acid (*S*)-**9** in 85–91 % conversion, 62–78 % yield, and 96–>99 % *ee* from (*S*)-**1**. The developed types of cascades could be applicable or modified for the conversion of other natural amino acids into the corresponding chiral fine chemicals. Moreover, the developed enzyme cascades to convert (*S*)-**1**, a primary cell metabolite, were easily combined with the natural pathway of a host microorganism, thus giving rise to the fermentative production of the chiral fine chemicals directly from glucose.

Acknowledgments

This work was supported by the Science & Engineering Research Council of A*STAR, Singapore, through a research grant (Project No. 1021010026) and the National University of Singapore through Synthetic Biology for Clinical and Technological Innovation (SynCTI) program.

Keywords: amino acids · asymmetric synthesis · biocatalysis · biomass · enzymes

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 11647–11650
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Received: June 27, 2016
Published online: August 11, 2016

