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Bioreductive preparation of ACE inhibitors precursor (*R*)-2-hydroxy-4-phenylbutanoate esters: Recent advances and future perspectives

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Abstract

Optically active (*R*)-2-hydroxy-4-phenylbutanoate esters ((*R*)-HPBE) are key precursors for the production of angiotension-converting enzyme (ACE) inhibitors, which are important prescriptive drugs for preventing the formation of angiotensin II and lowering the blood pressure. The biocatalytic asymmetric reduction of ethyl 2-oxo-4-phenylbutanoate (OPBE) to (*R*)-HPBE with carbonyl reductases has several advantageous attributes, including high enantioselectivity, mild reaction condition, high catalytic efficiency, and environmental benignity. An increasing number of OPBE reductases have been discovered owing to the drastic achievements in genomics, screening and evolution technologies, and process engineering. The potential of (*R*)-HPBE production process has also been intensively evaluated. This review covers recent progress on the bioreductive preparation of (*R*)-HPBE, especially on various screening approaches for the identification of OPBE reductases and their characterization.

Keywords: ACE inhibitors; Asymmetric reduction; Reductases; (*R*)-HPBE

Introduction

Optically active secondary alcohols, especially hydroxyl acids or hydroxyl acid esters, are important compounds for introducing chiral elements to pharmaceuticals, agricultural pesticides, and other fine chemicals [1-4]. (*R*)-2-hydroxy-4-phenylbutanoate esters (OPBE), one class of chiral alcohols, are important precursors for the production of serials of angiotensin-converting enzymes (ACE) inhibitors, generally named as pril drugs (benazepril, cilazapril, quinapril, and ramipril), possessing (*S*)-homophenylalanine moiety as pharmacophore [5-7]. ACE (Kininase II, peptidyldipeptide hydrolase, EC 3.4.15.1) could catalyze the production of vasoconstrictor angiotensin II and inactivation of vasodilator bradykinin. Whereas inhibitors of ACE could prevent the formation of angiotensin II, hence decrease the blood pressure [8,9]. Along with the economic development, the incidence of hypertension is higher and younger which may induce stroke, heart failure, heart attack, and kidney failure. The annual revenues of prescriptive antihypertensive drugs increased every year, with $45 billion in total at the end of 2011 [10]. The ACE inhibitors drugs accounted for 10% among all the antihypertensive drugs. Hence, the preparation of their key chiral precursor (*R*)-HPBE is of special and increasingly interests.

Numbers of methods have been developed for the preparation of (*R*)-HPBE, including chemical, enzymatic, and chemoenzymatic approaches. According to the starting compounds, they can be divided into (1) enantioselective resolution of *rac*-HPBE, (2) asymmetric ene reduction, (3) asymmetric reduction of ene and carbonyl groups, (4) asymmetric reduction of OPBE, (5) hydrogenolysis, (6) enantioselective hydrolysis of *rac*-hydroxynitrile, etc. (Figure 1) [11-16]. The enantioselective resolution of *rac*-HPBE and *rac*-hydroxynitrile might represent effective approaches, however, limited to maximal yield of 50% [12,17]. Asymmetric transfer hydrogenation (ATH) of ketones plays an important role in asymmetric synthesis of chiral secondary alcohols [18-22]. Enantioselective hydrogenation of ethyl 2-oxo-4-phenylbutanoate is likely one of the most economic and efficient method. Great efforts have been made to investigate the potential of this approach over the past

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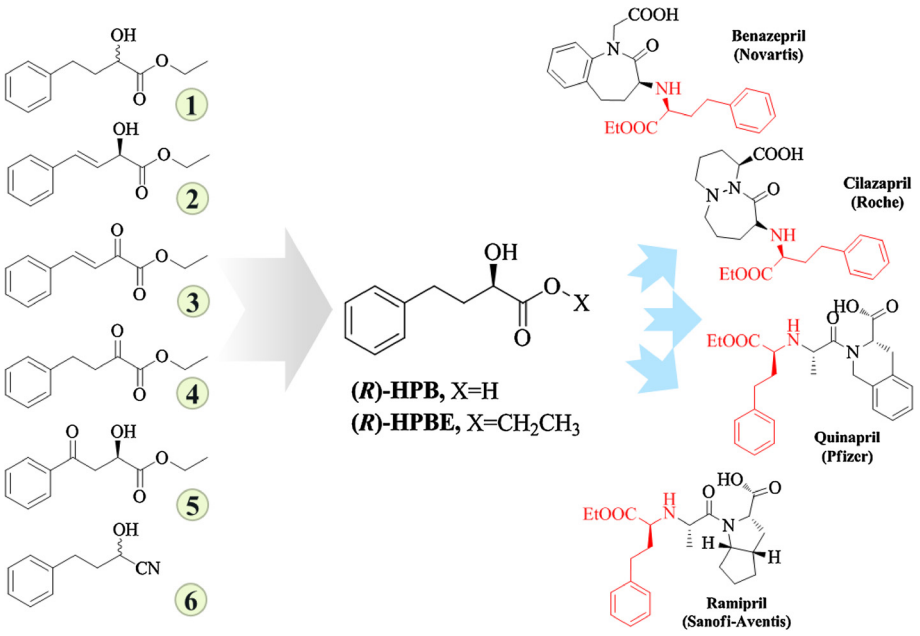

Figure 1 Various approaches for the synthesis of selected ACE inhibitors building blocks. (1) Enantioselective resolution of *rac*-HPBE, (2) asymmetric ene reduction, (3) asymmetric reduction of ene and carbonyl groups, (4) asymmetric reduction of OPBE, (5) hydrogenolysis, and (6) enantioselective hydrolysis of *rac*-hydroxynitrile.

few years. The highest enantioselectivity reported was 92% *ee* (*R*) using 10,11-dihydrocinchonidine (DHCd) as the chiral modified, and 94% *ee* (*R*) with the hydroxyl group in the DHCd modifier methylated. However, both cases were achieved using acetic acid as solvent, and under rigorous and commercially impractical conditions of high pressure (100 bar) and high temperature (400°C) hydrogen pretreatment of the Pt/Al_2O_3 catalyst [22]. After optimization, this process could be carried out at mild reaction condition (17°C and 5.8 bar) resulting enantioselectivity of merely 91% [23]. Therefore, seeking effective, highly enantioselective, and practical chemical approaches to (*R*)-HPBE is still of significance.

Tremendous endeavors have been committed to the preparation of (*R*)-HPBE employing biocatalytic asymmetric reduction systems, which is a reliable, scalable, and straightforward route to chiral alcohols. Compared with classic chemical processes, the asymmetric reduction of OPBE into (*R*)-HPBE, or the reduction of 2-oxo-4-phneylbutyrate (OPB) to (*R*)-2-hydroxy-4-phenylbutyrate [(*R*)-HPB] employing reductases and dehydrogenases (Figure 2) has several advantageous attributes, including high selectivity, high efficiency, mild reaction condition, and environmental friendless [24-27]. The complicated and chiral physiological structures endue the enzymes with relatively high chemo- and stereoselectivity and specificity. Various enzyme discovery and protein engineering tools have been proposed and proved, which would greatly shorten the developing period and increase the availability of robust enzymes. Several literatures on the biocatalytic asymmetric reduction for pharmaceuticals, protein engineering strategies for robust enzymes in chemical synthesis have been reviewed [28-32]. Also, various OPBE reductases have been successfully identified from natural samples or genome databases. The application potential and scalability of the biocatalytic processes have been systematically investigated. This mini-review was mainly focused on the discovery and properties of OPBE reductases. Future prospect of the application of bioreductive in preparation of optically pure (*R*)-HPBE synthesis was also discussed.

Screening of microorganisms for bioreductive preparation of (*R*)-HPBE

Due to the importance of (*R*)-HPBE as key chiral building blocks, searching for robust microorganisms has been extensively conducted as shown in Table 1. Some microorganisms, mainly yeasts, have been isolated from natural environment. Oda et al. screened 55 type culture yeasts from IFO and 499 isolated yeasts, among which two yeasts, *Rhodotorula minuta* IFO 0920 and *Candida holmii* KPY 12402, were identified with relatively high enantioselectivity. At 4.1 g L^{-1} OPBE loading, the *ee* and isolation yield could reach 90% and 58%, respectively, using *C. holmii* KPY 12402 [33,34]. *Kluyveromyces marxianus*, *Pichia pastoris*, *Pichia anomala*, and *Pichia angusta* have also been tested in the asymmetric reduction of OPBE into (*R*)-HPBE, and *P. angusta* displayed

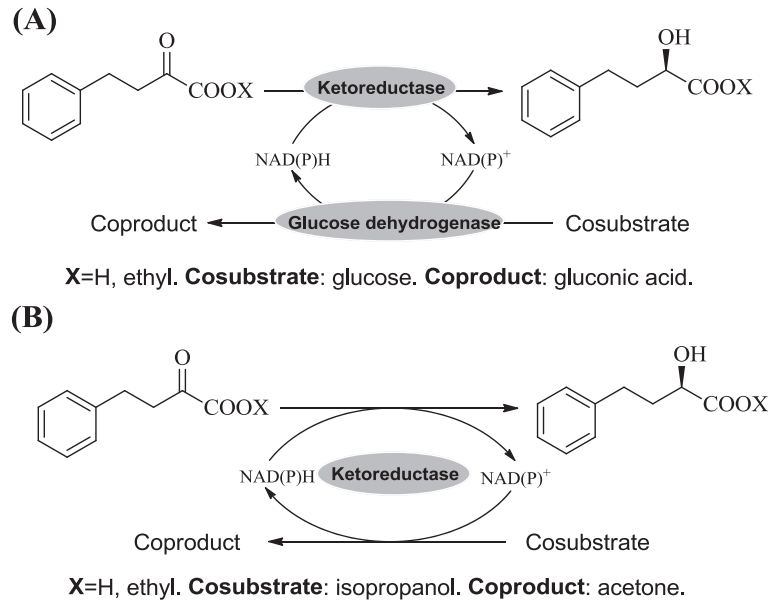

Figure 2 Asymmetric preparation of (*R*)-HPB derivatives employing oxidoreductases coupled with cofactor regeneration. (A) Enzyme-coupled system. **(B)** Substrate-coupled system.

the best performance of 100% conversion and 81% *ee* [35]. Besides, Chadha et al. reported the preparation of (*R*)-HPBE from OPBE by using *Daucus carota* with excellent chemical yield (90%) and *ee* (99%) [36]. Chen et al. investigated the ability of 40 yeasts kept in CIOC (Chinese Institute of Organic Chemistry, Chinese Academy of Sciences) in the reduction of OPBE. Most of the candidate yeasts shown good (*S*)-preference (>99%), while *Candida boidinii* CIOC21 was proved to be (*R*)-preference. As much as 2.0 g L^{-1} OPBE could be asymmetrically reduced into (*R*)-HPBE with 99.7% *ee* and 95.1% yield [37]. Target reaction oriented enrichment and screening of microorganisms have also been carried out in our laboratory, and one yeast strain *Candida krusei* SW2026 displayed desirable *ee* (99.7%) and yield (95.1%) in aqueous phase at 2.5 g L^{-1} of OPBE [17]. The highest record of OPBE loading in microbial system was achieved with *Bacillus pumilus* Phe-C3 by He and coworkers. At 6.2 g L^{-1} of OPBE and 12 g cdw L^{-1} catalyst, 95.3% OPBE could be reduced with 97.1% *ee* [38].

Discovery and characterization of OPBE reductases

Although many wild-type microbial cells could catalyze the asymmetric reduction of OPBE into (*R*)-HPBE, their industrial applications are limited by a number of disadvantageous. The expression level and enzymatic properties of OPBE reductases are often undesirable in parent strains [28]. Additionally, there might exist several enzymes with variable or opposite stereospecificity that could result HPBE with compromised chirality [39]. And the fermentation conditions of wild-type strains are usually strict and time-consuming. Studies on the discovery and properties of isolated OPBE reductases are therefore of special interests to prompt the biocatalytic preparation of chiral (*R*)-HPBE.

Table 1 Asymmetric reduction of OPBE into (*R*)-HPBE employing microorganisms

Entry	Strains	OPBE loading [g L^{-1}]	*ee*[%]	Yield[%]	Reference
1	*Candida krusei* SW2026	2.5	99.7	95.1	[17]
2	*Bacillus pumilus* Phe-C3	6.2	97.1	91	[37]
3	*Candida boidinii* CIOC21	2	99	92	[36]
4	*Baker's yeast*	1	92	90	[38]
5	*Candida holmi*	4.1	90	58	[33]
6	*Pichia angusta*	1.4	81	99	[34]
7	*Pichia pastoris*	1	95	99	[7]

Enzyme discovery strategies

Alongside the revolution of biotechnologies, a variety of tools are available for the quick screening and identification of biocatalysts. Several comprehensive reviews discussed the discovery and engineering tools developed to obtain naturally evolved or tailor-made enzymes with enhanced properties for chemical synthesis [29,30,40]. In the case of microbial strains isolated from natural environment, enzymes could be discovered through protein purification, insertional mutation, chromosome walking, shotgun library, etc. With regard to vast amount of genome information, genome hunting and genome database mining are two direct and time-saving strategies.

Protein purification

For a long time, purification of target enzymes from wild-type strains has been the dominant approach for enzyme discovery [41]. Through several purification steps, such as ammonium sulfate precipitation, ion exchange chromatography, hydrophobic chromatography, affinity chromatography and gel chromatography, and pure or partially purified enzymes could be harvested. After characterization with protein NMR of terminal peptide and MS, the encoding gene sequence of target enzymes could be acquired. Various carbonyl reductases of different origins (microorganisms and plant tissues) have been discovered through microorganism isolation and protein purification. A robust carbonyl reductase was purified from Adzuki bean, with high enantioselectivity (>99% *ee*) and tolerance to α-chloroacetophenone (0.2 M) [42].

Insertional mutation

This strategy utilizes a proper transposon to insert randomly into the genome sequence and disturb the normal properties or phenotypes of the strains. Through screening for mutants exhibiting expected changes and sequencing, the target genes could be identified. Various novel enzymes and pathways have been discovered employing this method, such as two novel genes *pao* and *sap* from *Pseudomonas* sp. Strain HZN6, coding for 6-hydroxy-L-nicotine oxidase and $NADP^+$-dependent 3-succinoylsemialdehyde-pyridine dehydrogenase in the catabolism of nicotine [43].

Chromosome walking

Based on conserved motif of certain enzymes, chromosome walking provided a new method to identify functional enzymes [44]. A functional P450 monooxygenase from *Rhodococcus* sp. ECU0066 was identified by chromosome walking by several rounds of PCR, with capability in the enantioselective oxidation of sulfides [45].

Shotgun library

Compared with other strategies, shotgun library was much more common and easier to operate in discover functional enzymes. Through construction the subclones of the genome in proper fragment length, a shotgun library could be developed. Many enzymes were identified using this approach. For example, Zhang and coworkers successfully cloned a novel arylamidase encoding gene from *Paracoccus* sp. strain FLN-7 which could hydrolyze amide pesticides [46].

Genome hunting

In the case of available genome data, genome hunting could be adopted to identify the target enzymes by cloning all of the potential genes from this single genome. Through genome hunting, Ni and coworkers heterogeneously expressed 13 reductases genes from *Bacillus* sp. ECU0013. Among them, the FabG (a β-ketoacyl-ACP reductase) exhibited high efficiency in the asymmetric reduction of 620 g L^{-1} OPBE to (*S*)-HPBE, with no addition of external expensive cofactor and 99% *ee*. Although the product configuration is not the desired one, it could be converted to (*R*)-HPBE through one-step configuration inversion [47].

Genome database mining

Another effective and promising strategy for quick discovery of suitable enzymes is genome database mining for homologous protein sequences of targeted enzymes. Owing to the revolution of sequencing technology, there are 58,117 sequencing projects (including 6,649 complete projects, 23,552 permanent drafts, 26,572 incomplete projects, and 1,404 targeted projects) in genome online database as of September 2014, providing massive data on protein-coding genes [48]. The selection of reported enzymes as probe and the choice of candidate proteins in database with moderate identity are the two key factors for the successful identification of new enzymes. The sequence of several known ethyl 4-chloro-3-oxobutanate (COBE) reductases was regarded as probes to search for novel enzymes in GenBank, resulting in an excellent reductase from *Streptomyces coelicolor* out of ten candidates for the synthesis of optically pure ethyl (*S*)-4-chloro-3-hydroxybutanoate, a chiral building blocks for HMG-CoA reductase inhibitors, with a stunning high productivity of 609 g L^{-1} day^{-1} [49].

Selected OPB(E) redutases

As shown in Table 2, a number of OPBE reductases have been discovered using above mentioned strategies. Most of the OPBE reductases are NADPH-dependent. A phenylacetaldehyde reductase (PAR) was purified from *Rhodococcus* ST-10 [50]. After amino-acid sequence analysis, the recombinant PAR was expressed in *Escherichia coli* [51]. One PAR mutant (HAR-1) could asymmetrically reduce 195 g L^{-1} OPBE to (*R*)-HPBE with 99% *ee* and 89% isolation yield [52]. The shotgun library of

Table 2 Enzymatic properties of oxidoreductases for producing (*R*)-HPB derivatives

Enzyme	Source strain	Accession no	Discovery strategy	Length [aa]	Cofactor	Family[a]	Specific activity [U mg^{-1}]	K_m [mM]	V_{max} [U mg^{-1}]	Opt. temp. [°C][b]	Sub. loading [g L^{-1}]	Time [h]	*ee* [%]	TTN[c]	STY [g L^{-1} d^{-1}][d]	Reference
Ypr1p	*S. cerevisae*	AHY75323.1	Genome hunting	312	NADPH	AKR	*nd* [e]	*nd*	*nd*	*nd*	2.0	*nd*	87	*nd*	*nd*	[54]
IolS	*B. subtilis*	CAB16014.1	Database mining	310	NADPH	AKR	5.1	2.61	4.18	30	330	12	>99	32039	660	[57]
CgKR2	*C. glabrata*	CAG61069.1	Database mining	311	NADPH	AKR	2.4	0.1	18.5	45	206	6	99	*nc* [f]	700	[59]
NcCR	*Neurospora crassa*	XM_954278	Genome hunting	331	NADPH	AKR	1.6	*nd*	*nd*	40	2.0	24	80	20	2	[56]
CgKR1	*C. glabrata*	CAG58832.1	Database mining	352	NADPH	MDR	23.1	*nd*	*nd*	*nd*	412	24	98.1	*nc* [f]	358	[58]
YOL151w	*S. cerevisiae*	CAA99172.1	Genome hunting	342	NADPH	MDR	*nd*	*nd*	*nd*	*nd*	1	48	99	10	0.5	[55]
PpADH	*Paracoccus pantotrophus* DSM 11072	EU427522	Shotgun library	262	NADH	SDR	0.0043	*nd*	*nd*	*nd*	5	24	99	24.3	5	[53]
PAR	*Rhodococcus* sp. ST-10	AB190261	Purification	348	NADH	MDR	*nd*	*nd*	*nd*	*nd*	195	24	99	947	174	[52]
YiaE	*E. coli* K12	BAE77742.1	Genome hunting	324	NADPH	HAD	18	7.2	24.6	45	20	24	99	*nc* [f]	20	[60]

[a]Enzyme families, AKR aldo-keto reductase, HAD 2-hydroxyacid dehydrogenase, SDR short chain dehydrogenase/reductase, MDR medium chain dehydrogenase/reductase. [b]Optimal temperature. [d]total turnover number of cofactor. [d]space-time yield. [e]nd, not determined. [f]not calculated because no cofactor was added.

Paracoccus pantotrophus DSM 11072 was developed to search for enzyme with OPBE reduction activity, resulting in the novel enzyme PpADH with 99% *ee* (*R*) at 5 g L^{-1} OPBE [53]. Through genome hunting, Ypr1p and YOL151w were identified from *Saccharomyces cerevisiae* with 87% and 99% *ee* (*R*), respectively [54,55]. NcCR was cloned from *Neurospora crassa* with 1.6 U mg^{-1} specific activity and 80% *ee* (*R*) [56]. Employing genome database mining strategy, three OPBE reductases (IolS, CgKR1, and CgKR2) were discovered from genome database [57-59]. Both CgKR1 and CgKR2 were cloned from *Candida glabrata*, with specific activities of 23.1 and 2.4 U mg^{-1} and *ee* values of 98.7% and 99%, respectively. CgKR1 was stable enough to tolerate as high as 412 g L^{-1} OPBE, while CgKR2 could completely reduce 206 g L^{-1} OPBE into (*R*)-HPBE (>99% *ee*) with 700 g L^{-1} day^{-1} space-time yield. In our previous study, IolS could tolerate 330 g L^{-1} OPBE with 99% *ee* (*R*). The specific activity of IolS was 5.1 U mg^{-1} [57]. In addition, (*R*)-2-hydroxy-4-phenylbutyrate ((*R*)-HPB) is also a precursor for ACE inhibitors and could be synthesized through stereospecific reduction of 2-oxo-4-phenylbutyrate (OPB) as illustrated in Figure 1. One hydroxy acid dehydrogenase (YiaE) gene was cloned from *E. coli* K12, which could asymmetrically reduce 20 g L^{-1} OPB into its corresponding hydroxy acid, (*R*)-HPB, in >99% *ee* within 24 h. The specific activity was determined to be 20 U mg^{-1}, the optimum temperature was 45°C [60].

Table 2 Enzymatic properties of oxidoreductases for producing (*R*)-HPBE from OPBE.

Classification of OPB(E) reductases

According to the amino acid sequence and metal-ion dependency, the carbonyl reductases could be divided into short-chain dehydrogenase/reductase (SDR), medium-chain dehydrogenase/reductase (MDR), long-chain dehydrogenase/reductase (LDR), and aldo-keto reductase (AKR) [61-63]. Key motif search in online databases (SDR, http://www.sdr-enzymes.org/; MDR, http://www.bioinfo.ifm.liu.se/services/mdr/; AKR, http://www.med.upenn.edu/akr/) reveals that PpADH belongs to SDR family, CgKR1, YOL151w, and PAR belong to MDR family, while Ypr1p, CgKR2, NcCR, and IolS are members of AKR. PAR is the only zinc-dependent carbonyl reductase. Tyr-Lys-Ser, Cys-His-Asp, and His-Tyr-Lys are common catalytic triads for SDR, MDR, and AKR, respectively [21,64,65]. Reductase YiaE from *E. coli* with reduction activity in the conversion of OPB into (*R*)-HPB was classified into 2-hydroxyacid dehydrogenase family (Table 2).

Regarding to crystal structure information of the OPBE reductases, protein crystal structure of IolS from *Bacillus subtilis* is the only one deposited in the PDB database (PDB accession no. 1PYF) [65]. In the structure of IolS, the most common fold, $(\beta/\alpha)_8$ barrel motif, seems to form a barrel conformation. The cofactor $(NAD(P)^+/NAD(P)H)$ is bound at the C-terminal of internal β-barrel, extending in an extended conformation from the center of the barrel with no intra-molecular hydrogen bonds. Besides to the conserved folding, the catalytic tyrosine at 58 sites acts as an acid in the reduction reaction. The acidity is enhanced through a hydrogen bond with the lysine of catalytic triad, which also in turn forms a salt bridge with the aspartate. In the apo form, the hydrogen bond between catalytic Tyr58 and Lys84 was 3.25 Å. When $NADP^+$ binds, the distance between the lysine ζ-amino group and the tyrosine hydroxyl is extended to 4.26 Å, which causes the disruption of this hydrogen bond due to a shift of Tyr58 towards the $NADP^+$. Two additional amino acids Asp51 and Lys84, which complete the catalytic triad by activating the phenolic proton on the tyrosine, also present in IolS. The interactions between both residues in the holo conformation are mediated by hydrogen bonds with a water molecule.

Since three other OPBE reductases (Ypr1p, CgKR2, and NcCR) are also potential AKR members, the amino acid sequence alignment of them is shown in Figure 3. Four AKR members shared similar backbones, although the sequence identity is relatively low (<50%). The catalytic tyrosine and aspartate are quite conserved in all four OPBE reductases, while the lysines are located around the conserved site of IolS. Most of the AKR are NADP-dependent and share the similar loop at the C-terminal, with 2'-phosphate tightly bound via three salt bridges with Arg227, Arg282, and Lys214 and a hydrogen bond with the side-chain of Gln286.

Bioprocess engineering for improved efficiency

For a biocatalytic process, several parameters are critical to evaluate the efficiency, including space-time yield, total turnover number (TTN) of cofactor or reductase, productivity, etc. However, hydrophobic OPBE could cause the deactivation of enzymes and often requires longer reaction time to achieve a higher conversion rate [57]. Consequently, the efficiency is usually at low level. Various process engineering strategies have been developed to solve the problems and improve the efficiency in the preparation of chiral secondary alcohols employing reductases.

Cofactor regeneration

To achieve complete conversion of OPBE by carbonyl reductases, stoichiometric amount of reducing cofactors is usually required. The high cost of cofactors is one of the main hurdles for the wide application of bioreductive preparation. Introduction of proper cofactor cycling systems could reduce the addition of expensive cofactors and promote reaction towards the synthesis of product. Numerous cofactor regeneration systems have been

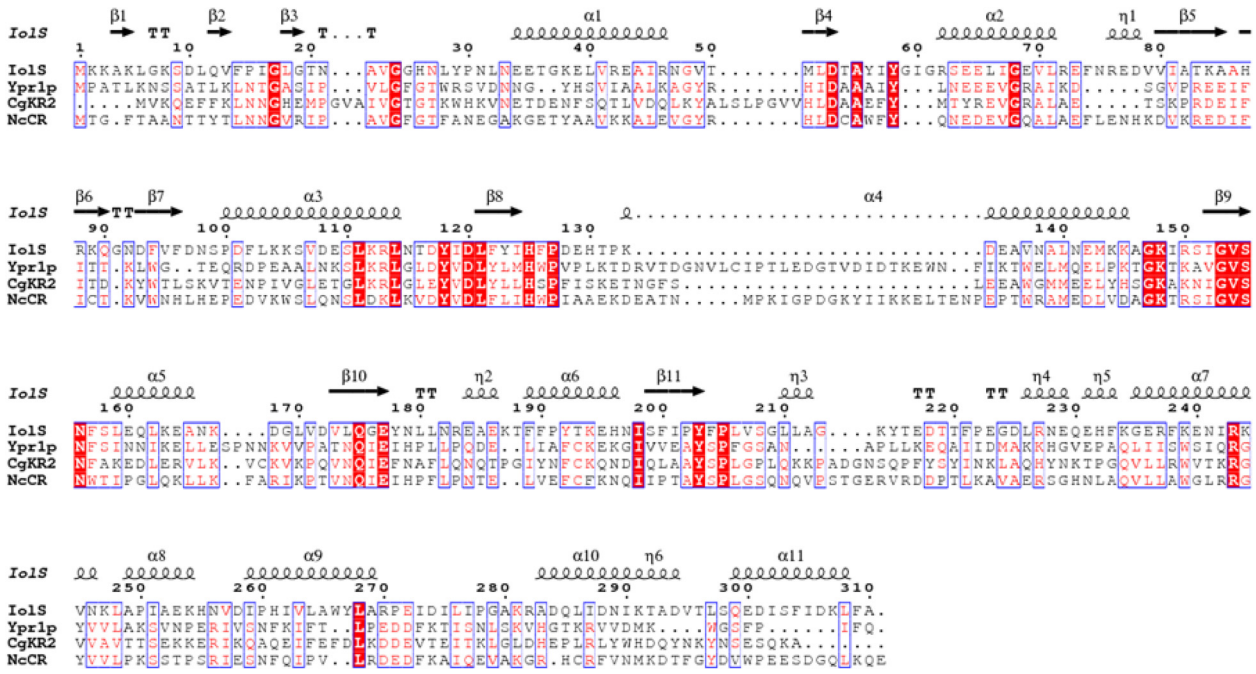

Figure 3 Amino acid sequence alignment of selected OPBE reductases belonging to AKR family using ESPript tool. *B. subtilis* (IolS) (CAB16014.1), *S. cerevisiae* (Ypr1p) (AHY75323.1), *C. glabrata* (CgKR2) (CAG61069.1), and *Neurospora crassa* (NcCR) (XM_954278). Secondary structure elements of IolS (PDB No. 1PYF) are indicated above the sequence with arrows for *β*-strands and cynlinders for *α*-helices [66].

developed and systematical summarized in recent reviews [67-69]. In general, there are three approaches to regenerate cofactors, including substrate-coupled, enzyme-coupled, and reaction-internal regeneration accomplished by enzymatic, chemical, photochemical, or electrochemical reagents. Several principles need to be considered in order to select an appropriate regeneration system as following:

1. Catalysts as well as cosubstrates should be commercially available or easily producible.
2. Product formation should be thermodynamically as well as kinetically favored and has no interference with the enzymes or with the subsequent isolation of desired product.
3. Regeneration should be practical and inexpensive and stable over a long period of time.

In the view of all these factors, the feasibility of glucose dehydrogenase, formate dehydrogenase, and isopropanol-coupled ketoreductase technologies have been proven. Formate dehydrogenase, however, exhibited low recycling efficiency on $NADP^+$. Glucose dehydrogenase and isopropanol-coupled systems have been applied in the enantioselective synthesis of (*R*)-HPB derivatives. By using glucose dehydrogenases from *B. subtilis*, the TTN of $NADP^+$ could reach as much as 32,039 in the asymmetric reduction of 330 g L^{-1} OPBE employing IolS from *B. subtilis* under assistance of 0.05 mM $NADP^+$ (Figure 2A). PAR from *Rhodococcus* sp. ST-10 is a reductase with isopropanol oxidation activity. Itoh and coworkers adopted the substrate-coupled strategy in the preparation of (*R*)-HPBE with PAR (Figure 2B). Almost 195 g L^{-1} OPBE was completely reduced, and the TTN of NAD^+ was 947. Isopropanol could not only work as cosubstrate, but also as cosolvent to increase the solubility of OPBE.

Solvent engineering

Solvents constitute the microenvironment around the biocatalysts. By introducing organic solvents, ionic liquids, supercritical fluids, and glycerol into the biocatalytic reduction systems, not only the toxicity of substrate/product to hydrophilic biocatalysts could be alleviated, but also their spontaneously hydrolysis and volatilization could be relieved. Among them, organic/aqueous biphasic system was the most common used approach as illustrated in Figure 4A. The key of this system was to find a good suitable and biocompatibility solvents, which could balance the influence on the activity and enantioselectivity of enzymes and the partition behavior of substrate and product. *C. krusei* SW2026 was proved to display 99.7% *ee* ((*R*)-HPBE) and 95.1% yield at 2.5 g L^{-1} OPBE in aqueous system [17]. As substrate loading increased to 20 g L^{-1}, the *ee* and yield were reduced to 87.5% and 45.8%, respectively, which was

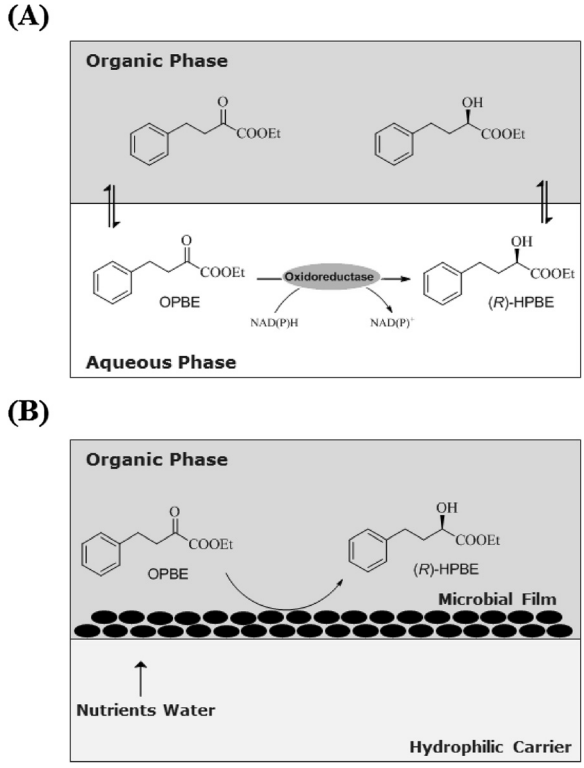

Figure 4 Scheme of bioreductive preparation of (*R*)-HPBE in organic solvent/aqueous biphasic system (A) and an interface bioreactor (B).

caused by the higher amount of toxic OPBE. Based on the solvent polarity, ten organic solvents with different Log*P* values ranging from 0.28 to 7.7 were selected. Dibutyl phthalate (Log*P* = 5.4) showed the highest biocompatibility and proper partition coefficient for OPBE and HPBE. Compared with aqueous system, less time was required to reach higher conversion, resulting in 95.0% *ee* and 92.5% isolation yield. This dibutyl phthalate/aqueous biphasic system could not only migrate the substrate toxicity effect from enzymes/cells, but also force the reaction equilibrium towards the reduction of OPBE by accumulating (*R*)-HPBE in the organic phase.

Substrate feeding

To realize a high substrate loading, substrate feeding at batches is an effective strategy to reduce the of substrate inhibition. A much higher catalytic efficiency could be achieved by substrate-feeding operation. In our studies using 1-L aqueous/octanol biphasic system, 30 g OPBE was fed once per hour for ten times (330 g in total). After 12 h of reaction, substrate conversion rate of >99% and space-time yield of 660 g L^{-1} day^{-1} were obtained at 330 g L^{-1} OPBE [57]. The substrate loading was 1.6 times higher than that of the highest record reported so for. Additionally, a S/C ratio of 31.7 was attained, ranking the highest catalyst yield in the asymmetric reduction of OPBE, which was 0.02–4.12 as reported [59].

Other processes

To alleviate the toxicity of hydrophobic OPBE/HPBE, interface bioreactors have been established. It is a biotransformation device between a hydrophilic carrier and a hydrophobic organic solvent as shown in Figure 4 [34]. In this system, the biocatalyst was immobilized on the carrier surface between the hydrophilic and hydrophobic solvents. Interestingly, the recycling of cofactors could proceed smoothly, and the product separation was relatively easier than that for aqueous systems.

The smart control of OPBE concentration in the reaction medium could also be achieved by adsorbing the substrate on hydrophobic resins (Figure 4B). D'Arrigo and coworkers proved that XAD™ 1180, a polystyrenic adsorbent selected among several Amberlite resins, was effective in controlling the OPBE amount presented in water phase. OPBE (1 g L^{-1}) could be reduced asymmetrically employing *Pichia pastoris* with 99% conversion and 95% *ee* (*R*) [7]. Continuous batch reactor (CBR) was established combining the biotransformation and OPBE/HPBE adsorption. In the CBR unit, the bioreduction of

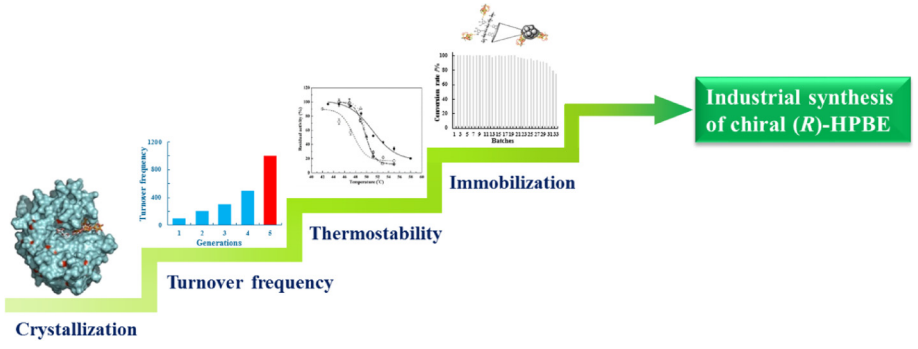

Figure 5 Further perspectives on the engineering of OPB(E) reductases for industrial synthesis of (*R*)-HPBE.

OPBE was carried out for 48 h at a substrate-feeding flow rate of 1 mL min^{-1}, resulting 95.1% *ee* (*R*) and conversion rate of 25%.

Prospects

Biocatalytic preparation of chiral alcohol is gaining momentum. Due to its high enantioselectivity, bioreductive preparation of chiral (*R*)-HPBE has been regarded as one of the most promising approaches. There are enormous difference between natural environment (i. e., cytosolic conditions) and industrial environment. Free enzymes might face hash environment, such as molar scale reactants and increased hydrophobicity instead of millimolar reagents and physiological aqueous environment. To become an efficient tool in large-scale synthesis of (*R*)-HPBE, OPBE reductases should display the following properties: (1) ≥100 g L^{-1} substrate loading, (2) ≤5 g L^{-1} biocatalyst loading (≥20 substrate to catalyst ratio), (3) ≥98% conversion rate, (4) ≤24 h reaction time, and (5) ≥99% *ee* [21,28]. Although several biocatalytic reduction systems could reach high substrate loading, enantioselectivity, and conversion, the biocatalyst loadings are still at relatively high and the S/C ratio is relatively low, which would hinder the product separation procedure.

Aiming at the industrial synthesis of (*R*)-HPBE, the biocatalytic procedure should be further engineered (Figure 5). In PDB database, extremely limited information on the crystal structure of OPBE reductases is available. The protein structure could broaden the knowledge of biocatalytic mechanism and provides guidance for further protein engineering. The catalytic efficiency (turnover frequency, k_{cat}) also remained to be improved to decrease the catalyst loading. As a robust industrial biocatalyst, thermostability and operational stability should also be high enough to tolerate rigorous reaction conditions. As shown in Table 2, most of the OPBE reductases are mesophilic enzymes, with optimum temperature of less than 45°C. Since the oxidoreduction reaction is cofactor-dependent, the cofactor engineering of bioreduction systems would fasten the reaction and decrease the addition of expensive cofactors. Importantly, reuse of the biocatalysts through proper immobilization strategies could also reduce the cost and simplify the product separation from reaction mixture.

Conclusion

Biocatalytic stereoselective reduction for (R)-2-hydroxy-4-phenylbutanate esters is gaining momentum. Various OPBE reductaes have been identified and some of them have indicated promising potential for practical manufacture of (R)-HPBE. The discovery strategies for OPBE reductases and bioprocessing engineering strategies have been reviewed. Further development of bioreductive preparation of (R)-HPBE will require the continued mining and designing industrially useful enzymes and elaboration of process engineering.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GCX wrote this manuscript. YN contributed general advice and edited the manuscript. Both authors read and approved the final manuscript.

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