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De novo construction of multi-enzyme system for one-pot deracemization of (*R*,*S*)-1-phenyl-1,2-ethanediol by stereoinversion of (*S*)-enantiomer to the corresponding counterpart

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ABSTRACT

Deracemization via oxidoreductive stereoinversion is one of the most attractive methods for the preparation of enantiomerically pure compounds. However, available enzymatic system is yet limited for efficiently catalyzing deracemization to produce optically pure alcohol in certain configuration. Through evaluation of available stereoselective oxidoreductases on activity, selectivity, and cofactor dependency, the suitable candidates were obtained to construct the enzymatic deracemization system involving cofactor self-recycling. For deracemizing (*R*,*S*)-1-phenyl-1,2-ethanediol (PED) to (*R*)-PED, a facile one-pot system was established by combination of two stereoselective oxidoreductases, the stereospecific carbonyl reductase 1 (SCR1) and the ketoreductase (KRD). To rebalance the activities and catalytic functions of different enzymes involved in the multi-enzyme system, the reaction conditions of SCR1-catalyzed oxidation and KRD-mediated reduction were optimized, respectively. Consequently, the deracemization system involving cofactor self-recycling was built to produce (*R*)-PED with the optical purity of 95.50%e.e. and the yield of 91.62% from the corresponding racemate (1 g L^{-1}), under the optimal reaction conditions including activity ratio of SCR1/KRD 1:4 (SCR1 10 U mL^{-1} and KRD 40 U mL^{-1}), molar ratio of $NADP^+$/NADPH 3:1, 30 °C, and pH 7.0. Therefore, the developed strategy would be useful to construct the multi-enzyme deracemization system based on systematic evaluation of enzyme features.

1. Introduction

Microbial oxidoreductive systems involving stereoselective oxidoreductases have been increasingly used in catalyzing reactions of commercial interest and applied in the preparation of optically active alcohols for pharmaceuticals, agrochemicals, pheromones, and fine chemical industries [1–7]. Of the optically active alcohols as important chiral building blocks, enantiomerically pure (*R*)-1-phenyl-1,2-ethanediol (PED) generally serves as a synthon for the preparation of (*R*)-norfluoxetine, (*R*)-fluoxetine, and β-lactam antibiotics, which can be used for the treatment of psychiatric disorders and metabolic problems [8–10].

A series of bio-methods for preparation of (*R*)-PED have been explored, including stereospecific dihydroxylation of styrene catalyzed by naphthalene dioxygenase, lipase-mediated resolution of racemic PED, biocatalytic reduction of phenylglyoxal, asymmetric reduction of 2-hydroxy-1-phenylethanone by microbial cells or isolated ketoreductases, and enantioconvergent hydrolysis of racemic styrene oxide using recombinant whole cells [10–15]. However, the involvement of the carcinogenic styrene oxide inherently occurs in some strategies and the maximal yield can never exceed 50% for the approach of kinetic resolution. Regarding the methods involving bio-oxidoreduction, the reported examples mainly employed asymmetric reduction of the corresponding prochiral ketone to produce (*R*)-PED. However, reductase-involved wet cells of relatively high concentration (100–300 g L^{-1}) were generally used for the substrate of limited concentration (1–5 g L^{-1}), where a relatively long reaction time (36–48 h) was usually required for the whole-cell reaction system, and on the other hand, in general large amount of expensive coenzyme was stoichiometri-

Abbreviations: PED, 1-phenyl-1,2-ethanediol; SCR1, stereospecific carbonyl reductase 1; KRD, ketoreductase; 2-HAP, 2-hydroxyacetophenone; HPLC, high performance liquid chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; TCEP, Tris (2-carboxyethyl) phosphine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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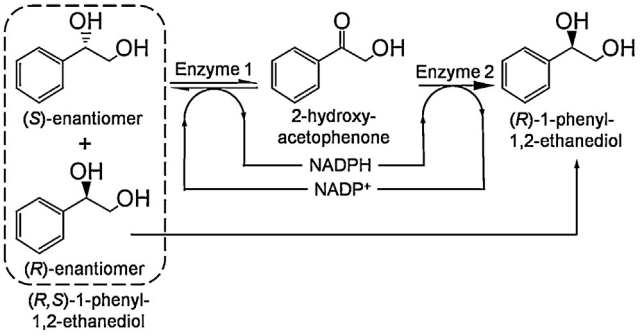

Fig. 1. Deracemization of racemic PED to (*R*)-enantiomer through selective oxidation and asymmetric reduction involving cofactor self-recycling. By enzyme 1, (*S*)-PED was oxidized to 2-HAP and $NADP^+$ was reduced to NADPH simultaneously. Subsequently, by enzyme 2, 2-HAP was reduced to (*R*)-PED and NADPH was oxidized to $NADP^+$ meanwhile.

cally in demand for the biocatalytic system using isolated enzyme [10–15]. Thus, the biocatalytic methods are yet limited for stereoselectively asymmetric synthesis of (*R*)-PED enantiomer.

As a hot topic in microbial catalysts, deracemization by microbial stereoinversion in one-pot is one of the most attractive methods for the preparation of enantiomerically pure compounds in 100% theoretical yield from racemic mixtures [16–19]. Tandem oxidation and reduction are carried out sequentially in one pot, and the key oxidoreductases involved in the oxidation-reduction procedure are of great importance. During the reaction, one enantiomer is usually oxidized to the carbonyl intermediate, while the other enantiomer remains unchanged, then the intermediate is reduced to the opposite enantiomer subsequently. Thus, this approach is more efficient because it allows complete transformation of racemate into single stereoisomeric product [20].

However, for bioredox-based deracemization, most of the examples were merely applied to the whole-cell system, involving the inherent disadvantage on substance transfer. Furthermore, the reaction direction and product configuration of deracemization by whole-cell system is usually fixed due to the existence of intracellular functional enzymes involved in deracemization. It is generally not feasible to produce desired chiral alcohol of certain configuration by using available cells, especially wild-type cells from nature. Thus it is obviously not easy to control the reaction process of deracemization by *in vitro* manipulating the *in vivo* enzymes. Otherwise, the catalytic system using crude enzymes generally involves other enzymes acting to the substrate and the coenzyme, which may lead to the interference and competition effects toward the target reaction. Therefore, artificial construction of multi-enzyme system in purified form would be reasonable and practicable to solve these problems, although the reported one-pot deracemization systems with coupled oxidoreductases are yet limited so far.

On the other hand, the practical applications of the key oxidoreductases can be quite challenging since they require expensive cofactors, such as NAD(H) or NADP(H) [21,22]. Cofactor regeneration would be essential to overcome such oxidoreductive reaction limitations. However, cofactor recycling can usually be achieved by adding coupled enzyme acting to co-substrate for cofactor regeneration, such as formate dehydrogenase, glucose dehydrogenase, and alcohol dehydrogenases [23–27]. In the one-pot deracemization, there are both oxidation and reduction catalyzed by oxidoreductases. If the cofactor couple NAD^+/NADH or $NADP^+$/NADPH is required alone and the two reactions have matchable reaction rate, it would be possible to build a cofactor self-recycling system between the two oxidoreductases (Fig. 1).

In this work, in order to obtain the suitable candidates for construction of the cofactor self-recycling deracemization system, we investigated the catalytic properties of 20 stereoselective oxidoreductases [28–42]. By the systematic evaluation of these enzymes and optimization of the catalytic conditions, NADPH-dependent stereospecific carbonyl reductase 1 (SCR1) and ketoreductase (KRD) were selected to be responsible for the tandem oxidation and reaction in deracemization, respectively. Then the multi-enzyme system containing SCR1 and KRD was achieved for deracemization of racemic PED to give optically active product of (*R*)-PED.

Table 1
Enzymes used in the work and their sources.

Enzyme	GenBank ID	Source	Reference
ADHR	AY267012	*Lactobacillus kefiri* DSM 20587	[41]
C1	AB084515	*Candida parapsilosis* IFO 0708	[42]
C2	AB084516	*C. parapsilosis* IFO 0708	[42]
CR2	AB183149	*Kluyveromyces marxianus* AKU 4588	[36]
CR4	E59061	*K. aestuarii* DC 6752	[39]
KRD	AF178079	*Zygosaccharomyces rouxii* ATCC14462	[35]
OYE	AB126227	*K. marxianus* AKU 4588	[37]
RCR	DQ295067	*C. parapsilosis* CCTCC M203011	[33]
SCR	DQ675534	*C. parapsilosis* CCTCC M203011	[34]
SCR1	FJ939565	*C. parapsilosis* CCTCC M203011	[34]
SCR3	FJ939564	*C. parapsilosis* CCTCC M203011	[34]
S1	AB036927	*C. magnoliae* AKU 464	[32]
CPAR1	JX512911	*C. parapsilosis* CCTCC M203011	[28]
CPAR2	JX512912	*C. parapsilosis* CCTCC M203011	[28]
CPAR3	JX512913	*C. parapsilosis* CCTCC M203011	[28]
CPAR4	JX512915	*C. parapsilosis* CCTCC M203011	[28]
CPAR5	JX512916	*C. parapsilosis* CCTCC M203011	[28]
CPAR6	JX512917	*C. parapsilosis* CCTCC M203011	[28]
CPAR7	JX512918	*C. parapsilosis* CCTCC M203011	[28]
CPAR8	JX512919	*C. parapsilosis* CCTCC M203011	[28]

2. Materials and methods

2.1. Materials

The cofactors including NAD(P)H and $NAD(P)^+$, 2-hydroxyacetophenone (2-HAP), (*R*)-PED and (*S*)-PED were purchased from Sigma-Aldrich (St. Louis, USA). Hexane and isopropanol used for high performance liquid chromatography (HPLC) were of chromatographic grade from Sigma-Aldrich (St. Louis, USA). All other used chemicals were purchased from local suppliers and were of analytical grade.

2.2. Microorganisms and culture conditions

All recombinant strains expressing the enzymes used in this study were constructed previously in our laboratory (Table 1). The recombinant strains were cultivated overnight in 4 mL LB liquid medium supplemented with 100 μg mL^{-1} ampicillin at 37 °C and 200 rpm. Then the culture was inoculated into a 250-ml Erlenmeyer flask containing 50 mL fresh LB medium with 100 μg mL^{-1} ampicillin. When the $OD_{600\,nm}$ increased to the level between 0.6 and 0.8, the expressions of target recombinant proteins were subjected to the optimization of the following conditions, where IPTG (0.1, 0.5, and 1.0 mM) or lactose (2%, 4%, and 6%) was added as inducer and the culture was incubated under different temperatures (17, 20, 25, 30, and 35 °C) at 200 rpm for additional 12 h. The yield of target protein was evaluated by calculating the amount of purified protein obtained from the corresponding culture broth.

2.3. Purification of recombinant enzymes

The recombinant cells were cultivated under the optimum expression conditions and then harvested by centrifugation and washed twice with physiological saline. The bacterial pellet was resuspended in the binding buffer (20 mM Tris-HCl, pH 7.0, 0.3 M