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Marked improvement in the asymmetric reduction of 2-hydroxyacetophenone with mut-AcCR in a biphasic system

Ping Wei[a,b], Peng Chao[a,b], Yao-Ying Wang[a], Dong-Li Li[a], Qing-Jian Zou[a], Min-Hua Zong[b], Wen-Yong Lou[b,*]

[a] School of Biotechnology and Health Sciences, Wuyi University, Jiangmen 529020, Guangdong, China
[b] Lab of Applied Biocatalysis, School of Food Science and Engineering, South China University of Technology, Guangzhou 510640, Guangdong, China

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ABSTRACT

(*S*)-1-Phenyl-1,2-ethanediol (PED) is a vital chiral block in specialty chemical industries. The biotransformation of 2-hydroxyacetophenone (2-HAP) to (*S*)-PED was conducted successfully catalyzed with BL21(DE3)(pETDuet-*gst*-mut-*accr-gdh*) which harboring a carbonyl reductase mutant (mut-AcCR). The catalytic activity of the biocatalyst was 15.9 times higher than the recombinant cells harboring original AcCR. 250 mM 2-HAP was reduced, under optimal conditions, and the yield and space-time yield were 95.2 % and 1.89 M/d in the buffer system. Then $C_4MIMI{\cdot}PF_6$ was chosen to be the second phase to improve the catalytic efficiency, and the substrate concentration was increased to 450 mM in the $C_4MIMI{\cdot}PF_6$/buffer system. After a reaction duration of 3 h, the product yield was over 92 %, and the space-time yield increased to 2.88 M/d which was 1.52 folds higher than that in the buffer system. Also, the product *e.e.* was over 99 % consistently. Overall, the preparative scale reduction of 2-HAP (450 mM) in $C_4MIMI{\cdot}PF_6$/buffer system was conducted with promising result. The developed effective $C_4MIMI{\cdot}PF_6$/buffer system of asymmetric reduction catalyzed by BL21(DE3)(pETDuet-*gst*-mut-*accr-gdh*) will promote the preparation of the enantiomeric pureity chiral alcohol at industrial scale.

Introduction

The enantiomeric purity chiral alcohols are valuable and widely used chiral building blocks for the chemical synthesis [1–3]. Therefore, various synthetic methods have been developed to achieve optically pure chiral alcohols [4–8], among which biocatalysis represents an efficient method to produce chiral alcohols from carbonyl compounds in an environmentally friendly and sustainable process for the chemical and pharmaceutical industries [9–11]. The enantiomerically pure PED is a versatile chiral intermediate. (*R*)-PED can be used to prepare the antidepressant drug, fluoxetine. (*S*)-PED is used as a precursor for the synthesis of biphosphines, which can be used as ligands in the cationic rhodium complexes [12]. (*S*)-PED can also be used as chiral initiator for stereoselective polymerization [13,14]. Carbonyl reductase and whole-cell can be used to reduce 2-hydroxyacetophenone (2-HAP) to obtain the enantiomerically pure PED. Zhou et al. developed the biocatalytic synthesis of (*R*)-PED catalyzed by (*R*)-carbonyl reductase (RCR) from *Candida parasilosis* with 6 g/L (≈44 mmol/L) 2-HAP in the buffer system. After a reaction duration of 24 h, the product yield and the product *e.e.* were both over 99 % [15]. (*S*)-CR II from *Candida parapsilosis* can catalyze the conversion of 2-HAP to (*S*)-PED with high product *e.e.* (97.4 %) and a yield of 95.2 % after 6 h reaction [16]. Additionally, two carbonyl reductases with complementary stereoselectivity, BDHA (from *Bacillus subtilis*) and GoSCR (from *Gluconobacter oxydans*) were expressed in *E.coli* for the transformation of 2-HAP to (*S*)-PED and (*R*)-PED with outstanding stereochemical selectivity. The asymmetric reduction of about 400 mM 2-HAP could be conducted by the two *E.coli* strains to obtain optically pure PED with 99 % yield and product *e.e.* [17]. However, several limiting factor still exist in many biocatalytic processes, for example, the large additions of coenzyme [NAD(P)H], substrate toxicity to the microbial cells, high loading of the biocatalyst and the long reaction time, etc. Therefore, it is necessary to seek more oxidoreductase with excellent catalytic properties to conduct the asymmetric reduction of 2-HAP in biocompatible system. The reduction process catalyzed by the carbonyl reductase mostly requires a mass of expensive coenzyme (NADH or NADPH), and the high cost of coenzyme would be restricted the application of the carbonyl reductase for the asymmetric reduction to obtain the chiral compounds. Recently, many ways have been investigated for the regeneration of coenzyme in the catalytic processes [18–21]. In situ coenzyme

* Corresponding author.
E-mail address: wylou@scut.edu.cn (W.-Y. Lou).

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regeneration conducted by the enzyme-coupled system, which can reduce or avoid the addition of extra coenzyme and predigest the reaction process, is especially preferred for biocatalysis on account of its excellent selectivity and high-efficiency [22–24].

It has been reported many of the substrates and/or products have obviously inhibitory or toxic effects on the microbial cells in the single phase, which leads to a low productivity of the microbial cells [25–27]. Normally, a biphasic system can be used to overcome these challenges [28–30], where the buffer phase contains the microbial cells and the nonaqueous phase serves as a storage for the substrate or product.

In our previous study, a carbonyl reductase AcCR from *Acetobacter* sp. CCTCC M209061 was characterized, and AcCR possessed the ability to catalyze the asymmetric reduction of different prechiral compounds, including 2-HAP with excellent stereoselectivity [31]. AcCR was modified by the site-directed mutation and the specific activity of the mutant AcCR (mut-AcCR) reached 6.4 U/mg, which was 17.4-fold higher than the wild type AcCR [32]. Therefore, it would be of great interest to study the asymmetric reduction of 2-HAP catalyzed with the recombinant *E.coli* harboring mut-AcCR. In this study, to improve the catalytic efficiency of 2-HAP and achieve coenzyme recycle, the *E.coli* coexpressed mut-AcCR (I147 V/G152 L) gene and glucose dehydrogenase gene (*gdh*) were used to systematically investigate the asymmetric reduction of 2-HAP to obtain (*S*)-PED systematically. A biphasic system was introduced to further improve the substrate concentration and catalytic efficiency (Scheme 1). Moreover, the efficient biocatalytic process in the hydrophobic ionic liquid (IL)/buffer system for the 2-HAP reduction to (*S*)-PED was carried out on the preparative scale.

Material and methods

Biological and chemical materials

DH5α, BL21(DE3) and plasmids pETDuet-1 were bought from Novagen. The restricted enzymes including FastDigest BglII and FastDigest XhoI, T4 DNA Ligase, DNA and protein marker were bought from Thermo. pET-gst-*accr-gdh* and pGEX-mut-*accr* were constructed in our previous study [21,32]. The kits used to construct the recombinant plasmids were purchased from TaKaRa. 2-HAP, (*R*)-PED, (*S*)-PED and PED were bought from Aladdin. 1-butyl-3-methylimidazolium hexafluoro phosphate ($C_4MIMI{\cdot}PF_6$) was purchased from Lanzhou Institute of Chemical Physics. Ampicillin was bought from Sangon Biotech, whereas the PCR primers were synthesized by Sangon Biotech.

Medium and cultivation

Luria-Bertani (LB) medium was used to culture the recombinant *E.coli*. At first, the strains were cultured at 37 °C. Once the culture concentration met the requirement (OD_{600} = 1.2), the culture temperature was reduced to 20 °C, Subsequently, isopropyl-β-D-thiogalactoside (IPTG, final concentration 0.4 mM) was added, and continued to be cultured at 20 °C for 15 − 18 h. On completion of the culturing process, the cells were collected at 4 °C by centrifugation (8000 rpm, 5 min) and using physiological saline (0.85 %) washed three times. Further, the cells were freeze-dried, and stored at − 20 °C for later use.

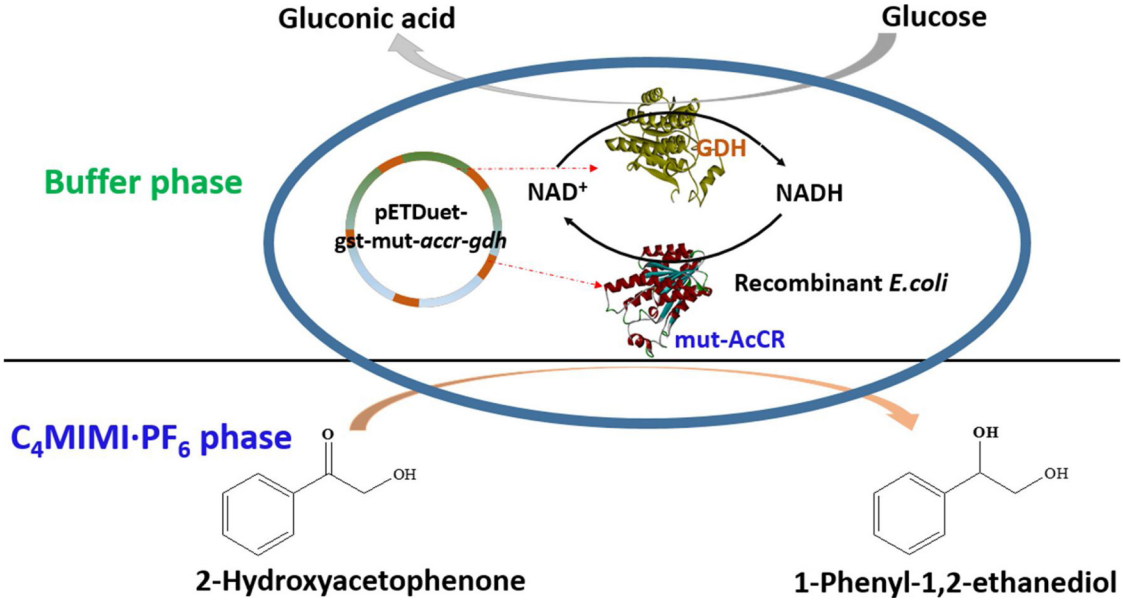
General procedure for the asymmetric reduction of 2-HAP in the buffer system

The general procedure was as follows, 4 mL citrate-phosphate buffer (pH 6.5, 200 mM) containing 100 mM 2-HAP, 200 mM glucose and 10 mg-dcw/mL BL21(DE3)(pETDuet-*gst*-mut-*accr-gdh*) formed the reaction system. The mixture was shaken at 200 rpm and 35 °C in an air-bath shaker, and the samples (20 μL × 2) were withdrawn regularly for the HPLC (Agilent 1260 Series) analysis. One sample was diluted by adding the purified water before filtering with the filter membrane (0.22 μm), followed by the HPLC analysis. Another sample was extracted with 600 μL (30 × 20 μL) ethyl acetate for the analysis of the *e.e.* value by HPLC. The pH value of the buffer system was adjusted by batch-feeding the $NaHCO_3$ powder.

Several parameters, i.e. temperature, buffer pH and concentration of substrate, glucose and cells, were studied with respect to the general procedure to efficiently synthesize (*S*)-PED from 2-HAP.

Procedure for the asymmetric reduction of 2-HAP to (S)-PED in the biphasic system

In a typical experiment, 2.0 mL of citrate-phosphate buffer (200 mM, pH 6.5) including dry cells (15 mg/mL, concentration based on the whole system, similarly hereinafter) and a predetermined quantity of glucose (glucose/2-HAP = 1.5/1) were added to a flask in duplicate, and pre-incubated at 200 rpm and 35 °C for 10 min in bath shaker. The



Scheme 1. The asymmetric reduction of 2-HAP to (*S*)-PED catalyzed by recombinant BL21(DE3)(pETDuet-*gst*-mut-*accr-gdh*) cells in the $C_4MIMI{\cdot}PF_6$/buffer system.

reaction was initiated by adding the hydrophobic phase containing 200 mM 2-HAP. Aliquots (2 × 20 μL) were taken at specified time intervals from the biphasic system. The sample from the buffer phase was diluted by adding the purified water before filtering with the 0.22 μm filter membrane, whereas the sample from the hydrophobic phase was diluted using acetonitrile or ethyl acetate for HPLC analysis. The pH value of the buffer system was adjusted by batch-feeding the $NaHCO_3$ powder.

Biocatalytic reduction of 2-HAP on the preparative scale

The biocatalytic reduction of 2-HAP on preparative scale with whole-cell BL21(DE3)(pETDuet-*gst*-mut-*accr-gdh*) was conducted by adding 1.5 g dry-cell of BL21(DE3)(pETDuet-*gst*-mut-*accr-gdh*) (15 mg-dcw/mL) and 675 mM glucose (675 mM, stage addition) in 50 mL buffer phase as well as 450 mM 2-HAP to 50 mL IL phase, followed by the execution of the reaction at 200 rpm and 35 °C. Subsequently, the samples were analyzed by HPLC. The pH value of buffer system was adjusted by batch-feeding the $NaHCO_3$ powder.

HPLC analysis

The substrate and product concentration were analyzed by HPLC (Agilent 1260 Series) with UV detection at 245 nm and 215 nm using C18 column (Waters, XBridge™ C18, 5 μm, 4.6 φ × 250 mm). The mobile phase was water/acetonitrile (3/2, v/v, 0.1 % trifluoroacetic acid in water phase) and its flow rate was 0.5 mL/min at a column temperature of 35 °C. The retention durations for 2-HAP and PED were 7.80 and 6.24 min, respectively.

The *e.e.* value of (*S*)-PED was analyzed by HPLC (Agilent 1100 Series) with UV detection at 215 nm using OB-H column (Daicel, 4.6 mm × 150 mm, 5 μm). The mobile phase was the mixture of 2-propanol and *n*-hexane (1/9, v/v) and its flow rate was 0.7 mL/min at a column temperature of 35 °C. The retention time for (*R*)-PED and (*S*)-PED was 8.51 and 10.21 min, respectively.

The initial reaction rate, yield and *e.e.* value of these reactions were calculated as described in our previous study [27].

The gluconic acid concentration was analyzed indirectly by detecting sodium gluconate, as reported previously (as the sodium gluconate concentration was equal to that of gluconic acid) [33]. $NaHCO_3$ was added to the reaction solution until the pH of the mixture was about 10. The sodium gluconate concentration was analyzed using the XDB-C18 column (Agilent, 4.6 mm × 250 mm, 5 mm) by HPLC. The mobile phase was the mixture of water, methanol and 1.0 % H_3PO_4 solution (45/ 5/ 50, v/v/v), and its flow rate was 0.6 mL/min. The retention duration of sodium gluconate was 5.7 min.

Results and discussion

Effect of coenzyme recycling on the asymmetric reaction of 2-HAP catalyzed by recombinant BL21(DE3)

The coenzyme recycling is an important influencing factor for the asymmetric reduction catalyzed by the whole-cell. As shown in Table 1, different coenzyme regenerations were investigated, and the initial reaction rate and yield were observed to be 1.6 mM/min and 62.6 %, respectively. It indicated that the substrate coupling for the coenzyme regeneration with isopropanol did not provide NADH fast enough for the reduction process. The catalytic efficiency based on the enzyme-coupled coenzyme regeneration overmatched that of the substrate coupling method. The initial reaction rate was 3.0 mM/min, which was almost 2 times higher than that of the coenzyme regenerated by isopropanol, along with an excellent yield (90.7 %). Therefore, the coexpressed strain harboring mut-AcCR and GDH gene had higher catalytic efficiency, which was chosen to be used to the follow-up study.

Table 1
Effect of coenzyme recycling on the asymmtric reaction of 2-HAP catalyzed by recombinant BL21(DE3).

Cosubstrate (200 mmol/L)	Strain	Initial reaction rate (mmol L^{-1} min^{-1})	Yield (%)	*e.e* (%)	Config-uration
Isopropanol	BL21(DE3) (pETDuet-*gst*-mut-*accr*)	1.6	62.6	> 99	S
Glucose	BL21(DE3) (pETDuet-*gst*-mut-*accr-gdh*)	3.0	90.7	> 99	S

Reaction condition: 2 mL citrate phosphate buffer (100 − 200 mM, pH 6.5) containing 100 mM 2-HAP, 200 mM glucose, 10 mg-dcw/mL cells, 35 °C, 200 rpm.

Table 2
Enzymatic activity of recombinant BL21 (DE3).

Number	Strain	AcCR activity (U/g-dw)
A	BL21(DE3) (pETDuet-*gst-accr-gdh*)	26.5 ± 0.7
B	BL21(DE3) (pETDuet-*gst*-mut-*accr-gdh*)	420.9 ± 4.8

Condition: 2 mL citrate phosphate buffer (100 − 200 mM, pH 6.5) containing 50 mM 2-HAP, 50 mM glucose, 10 mg-dcw/mL cells, 30 °C,180 rpm.

The catalytic activity of the recombinant *E. coli* harboring wild-type AcCR and mut-AcCR was analyzed for the reduction of 2-HAP. As shown in Table 2, the catalytic activity of strain A was only 26.5 ± 0.7 U/g-dcw, however the stain B exhibited an activity of 420.9 ± 4.8 U/g-dcw, which was 15.9 times as compared to the wild-type one.

Effect of the critical variables on the reduction of 2-HAP to (S)-PED with BL21(DE3)(pETDuet-gst-mut-accr-gdh) cells in the buffer system

To investigate the asymmetric bioreduction of 2-HAP in detail and to achieve an improvement in the initial reaction rate and yield, a systematic study about the effect of the important variables was made.

The buffer pH plays a vital role in the biocatalytic reduction process. The buffer pH has an effect on the activity and selectivity of the biocatalyst, along with coenzyme regeneration, which have an inverse on the reduction rate [34]. Therefore, an optimum pH range needed for the reduction process. As presented in Fig. 1a, as the buffer pH increased from 5.0 to 6.0–6.5, both the reaction rate and yield were observed to enhance. As the buffer pH reached 7.0, both the initial reaction rate and yield were noted to drop. There was an insignificant change in the product *e.e.* on changing the buffer pH. Obviously, the optimal buffer pH for the reduction of 2-HAP lied in the range 6.0 − 6.5. At the pH range, the initial reaction rate was 2.97 mM/min with a yield of about 90 %.

The coenzyme regeneration relies on the oxidation of glucose, catalyzed by GDH, which can couple with the reduction reaction catalyzed by the carbonyl reductase. The oxidation process produced D-Gluconic acid, which can bring down the buffer pH of the reaction system. As shown in Fig. 1b, the buffer pH exhibited a significant drop accompanied by an increase in the D-Gluconic acid concentration during the reaction progress. Owing to the simultaneous production of D-Gluconic acid, the buffer pH decreased to ~ 4.5 after a reaction duration of 4 h. As shown in Fig. 1a, the buffer pH influenced the initial reaction rate and yield. The buffer pH was controlled by batch-feeding of the alkalescent carbonate ($NaHCO_3$) to the system. The result was remarkable as the maximum product yield reached 94.1 % after feeding $NaHCO_3$ to

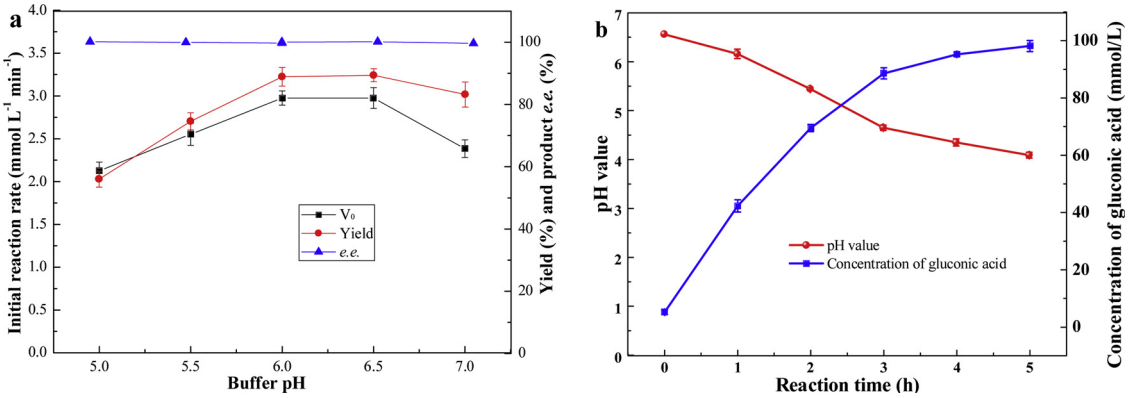


Fig. 1. Effect of buffer pH on the asymmetric reduction of 2-HAP catalyzed by recombinant BL21(DE3). Reaction condition: a) 2 mL citrate phosphate buffer (100-200 mM, pH 5.0-7.0) containing 100 mM 2-HAP, 200 mM glucose, 10 mg-dcw/mL cells, 35 ˚C, 200 rpm. b) 2 mL citrate phosphate buffer (100-200 mM, pH 6.5) containing 100 mM 2-HAP, 200 mM glucose, 10 mg-dcw/mL cells, 35 ˚C, 200 rpm.

achieve the buffer pH of 6.5.

The reaction temperature can also affect the stereoselectivity, stability and activity of a biocatalyst, as well as the reaction equilibrium. As shown in Fig. S1, the product yield was over 92 % for the reaction temperature in the range 25 − 40 ˚C. However, the initial reaction rate was slow for this temperature range (25 − 30 ˚C). The maximum initial reaction rate reached 2.97 mM/min for the reaction temperature of 35 ˚C, which was similar to that at 40 ˚C (2.93 mM/min), and the product yield was around 94 % at the two reaction temperatures. Therefore, taking the initial reaction rate and yield into account, the temperature range of 35 − 40 ˚C was confirmed to be optimal range for the bioreduction of 2-HAP.

Fig. 2a presented the effect of the substrate concentration on the reduction of 2-HAP in the buffer system. In the range of the tested concentrations, the *e.e.* value revealed was little change and was maintained above 99 %. The reaction obviously expedited as the 2-HAP concentration was increased from 100 to 200 mM, however, the yield exhibited almost no change (around 94 %), with a small decrease (90.1 %) as the concentration of 2-HAP increased to 250 mM. Further increasing the concentration led to a decrease in the initial rate and yield, probably due to the increased inhibition effect and toxicity of 2-HAP towards the cells. Therefore, the relative activities of the cells were tested to further analyze the effect of 2-HAP concentration on the recombinant cells. As shown in Fig. 2b, after incubation for 5 h in different substrate concentration, the relative activity of the recombinant cells varied markedly. Over 75 % relative activity was observed in case of the substrate concentration ≤ 200 mM, whereas 60.2 % of the equivalent value was obtained as the 2-HAP concentration reached 250 mM. Further increasing the concentration of substrate led to a significant drop in the relative activity (6.4 %–41.3 %), possibly due to the growing inhibiting effect of 2-HAP towards the cells. Therefore, a second phase could be introduced to relieve the inhibitory effect.

In a bio-catalyzed reaction, the cell concentration is of significant importance for the highly efficient completion of the bioreduction process. Therefore, the cell concentration was investigated for achieving an efficient reaction and catalyst savings, in order to achieve more economically competitive process. The initial reaction rate increased as the cell concentration was enhanced up to 20 mg-dcw/mL (V^0 = 4.91 mmol/L/min). Further increasing the concentration led to a minor growth in the initial reaction rate (Fig. S2). In addition, the product yield reached to 94.7 % for the cell concentration of 15 mg-dcw/mL with the initial reaction rate reaching to 4.22 mM/min. From an economic standpoint, the recombinant cell concentration of 15 mg-dcw/mL was sufficient for the bioreduction of 2-HAP to (*S*)-PED.

Coenzyme regeneration also plays a crucial part in the reduction process. In our study, glucose was used as cosubstrate for the coenzyme regeneration by the oxidation process catalyzed with GDH. As shown in Fig. S3, as the molar ratio of glucose and 2-HAP reached 1.5, the yield was observed to be over 94 %, and the initial reaction rate was about 4.2 mM/min. Further increase in the ratio had an insignificant effect on the corresponding value. Hence, the optimal ratio of glucose and 2-HAP was noted to be 1.5.

Apart from the inhibition of the substrate, the product may also have other negative effects in the aqueous system. Therefore, the effect

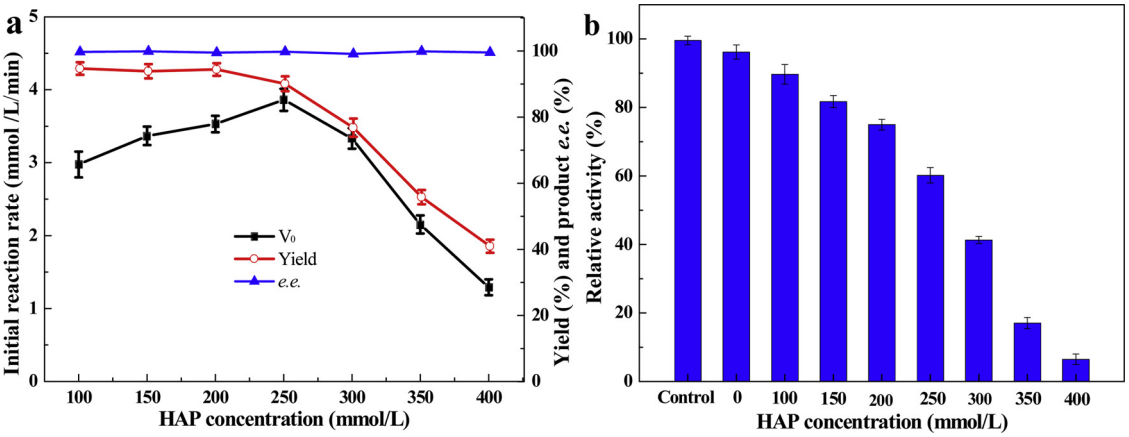


Fig. 2. Effect of substrate concentration on the asymmetric reduction of 2-HAP and the activity of recombinant BL21(DE3). Reaction condition: a) 2 mL citrate phosphate buffer (100-200 mM, pH 6.5) containing 100-400 mM 2-HAP, 200 mM glucose, 10 mg-dcw/mL cells, 35 ˚C, 200 rpm; b) 2 mL citrate phosphate buffer (100-200 mM, pH 6.5) containing 100-400 mM 2-HAP, 200 mM glucose, 10 mg-dcw/mL cells, 35 ˚C, 200 rpm, incubate for 5 h.

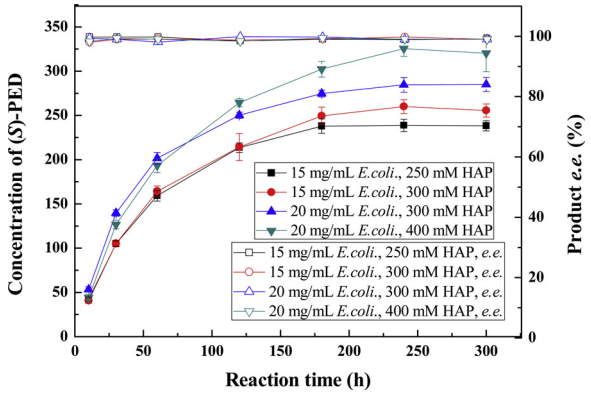


Fig. 3. Process of the asymmetric reduction of 2-HAP catalyzed by recombinant BL21(DE3). Reaction condition: 2 mL citrate phosphate buffer (100-200 mM, pH 6.5) containing 250-400 mM 2-HAP, glucose: 2-HAP = 1.5, 15 or 20 mg-dcw/mL cells, 35 °C, 200 rpm.

of product has been investigated, as presented in Fig. S4. The initial rate and yield remained essentially unchanged at different concentrations of exogenous-product. Thus, no significant inhibition was observed in the range of the investigated product concentration. The observed findings proved that the product had hardly any inhibitory effect on the asymmetric reduction, which is beneficial for establishing an efficient reduction system.

Process curves for the biocatalytic asymmetric reductions of prochiral 2-HAP

The process curves were monitored at different concentration of 2-HAP and biocatalyst. Under optimized conditions, the reduction process at the substrate concentrations from 250 to 400 mM with 15–20 mg-dcw/mL cells as catalyst has been presented in Fig. 3. The (*S*)-PED concentration reached 237.9 mM in case of the reduction of 250 mM 2-HAP catalyzed by 15 mg-dcw/mL BL21(DE3)(pETDuet-*gst*-mut-*accr-gdh*) lyophilized cells for 3 h. The space-time yield and yield were 1.89 M/d and 95.2 %, respectively. As the substrate concentration increased to 300 mM, the maximum concentration of (*S*)-PED was noted to be 255.9 mM after a reacted reaction duration of 4 h. The yield decreased to 86.6 % compared with the yield when 2-HAP was 250 mM. Further increase in the concentration of the cells revealed that the 300 mM substrate could be reduced by 20 mg-dcw/mL cells. 284.6 mM (*S*)-PED was obtained after reaction duration off 4 h, and the corresponding yield was 94.9 %. As the concentration of 2-HAP was enhanced to 400 mM, the final yield of reduction reached 81.4 % after a reaction period of 4 h. Therefore, 15 mg-dcw/mL and 20 mg-dcw/mL cells could reduce 250 mM and 300 mM 2-HAP with the final yield over 94 %, respectively.

The catalytic activity and efficiency improved markedly as compared to the recombinant strain containing the wild-type AcCR. The *E.coli* BL21(DE3)(pETDuet-*gst-accr-gdh*) had been constructed in our previous study [21]. The catalytic activity of this strain towards 2-HAP was only 26.5 U/g-dw. Using this strain to catalyze the reduction of 2-HAP with a substrate concentration of 100 mM, the maximum yield could only reach 62.5 % after 8 h, and the space-time yield was 187.5 mM/d. On the other hand, the catalytic activity of BL21(DE3)(pETDuet-*gst*-mut-*accr-gdh*) was noted to be 420.9 U/g-dw. As shown in Fig. 3, at a substrate concentration was 250 mM, the maximum yield reached 95.2 % after 3 h reaction. Overall the catalytic efficiency (space-time yield) improved 10.1 times.

Evaluation of various biphasic systems for the asymmetric reduction of 2-HAP catalyzed with BL21(DE3)(pETDuet-gst-mut-accr-gdh)

The inhibition of substrate was unavoidable during the biocatalytic reduction in buffer system. Therefore, a biphasic system consisting of organic solvent or ionic liquid (IL) and buffer was studied to enhance the efficiency of the reduction process. Various studies have revealed that the effect of different organic solvents and ILs on the bioreduction process varies extensively [9,29,35]. In this study, two organic solvents and one hydrophobic IL were chosen to analyze their effects on the reduction of 2-HAP catalyzed with BL21(DE3)(pETDuet-*gst*-mut-*accr-gdh*). The initial reaction rates had a minor decrease in the different biphasic systems as compared to that in the buffer system (Table 3). However, the yields after a reaction duration of 3 h were noted to be higher than that in the buffer system for the substrate concentration of 300 mM and 15 mg-dcw/mL cells as catalyst. The maximum yield of 95.1 % was attained for the $C_4MIMI{\cdot}PF_6$/buffer system. Additionally, the product *e.e.* values exhibited no change in different biphasic systems which were consistently noted to be over 99 %.

Table 3
Effect of different biphasic system on the asymmetric reduction of 2-HAP catalyzed by recombinant BL21(DE3).

Reaction system	Initial reaction rate (mmol L^{-1} min^{-1})	Reaction time (h)	Yield (%)	*e.e.* (%)
Buffer	4.2	3	82.2	> 99
Dibutyl Phthalate/ Buffer	3.7	3	91.1	> 99
Ethyl Laurate/ Buffer	3.2	3	88.8	> 99
$C_4MIMI{\cdot}PF_6$/ Buffer	4.1	3	95.1	> 99

Reaction conditions: Buffer system: 2 mL citrate phosphate buffer (100 − 200 mM, pH 6.5) containing 300 mM 2-HAP, glucose: 2-HAP = 1.5, 15 mg-dcw/mL cells, 35 °C, 200 rpm.
Biphasic system: 2 mL hydrophobic media containing 600 mM 2-HAP (final concentration 300 mM), 2 mL citrate phosphate buffer (100 − 200 mM, pH 6.5) containing glucose: 2-HAP = 1.5 (final concentration 450 mM), 30 mg-dcw/mL cells (final concentration 15 mg-dcw/mL), 35 °C, 200 rpm.

The hydrophobic phase always acts as a “storage” medium for the substrate and/or product. The hydrophobic phase in the biphasic system can extract the hydrophobic substrate or product, thus, reducing the concentration of the substrate or product in the buffer phase. It reduces the toxicity and inhibition of the substrate or product to the microbial cells during the reaction [36,37]. Therefore, the partition coefficients in the hydrophobic and buffer phases were tested to estimate the storing ability. As shown in Table 4, the partition coefficients of 2-HAP and PED varied in different biphasic systems, and the hydrophobic interaction of 2-HAP was higher than that of PED. The product had almost no inhibition effect on the reduction process (Fig. S4). Hence, the allocation of the substrate is vital for relieving the substrate inhibition. As shown in Table 4, the partition coefficient of 2-HAP was 13.4 in the $C_4MIMI{\cdot}PF_6$/buffer system, which was noted to be the best in the investigated systems and indicated strong extraction of the IL to 2-HAP.

The extraction of the hydrophobic solvent may lead to the inactivation of the microbial cells [38]. For the biocatalytic reduction taking place in the biphasic system, the catalytic performance of the whole-cell is strongly associated with the type of the hydrophobic phase. The relative catalytic activity of the whole-cell after incubation at 35 °C for 5 h in different systems has been investigated in the presence or absence of the substrate. As illustrated in Fig. 4, the catalytic

Table 4
Partition coefficients of 2-HAP and PED between biphasic systems.

Media	Partition coefficients between the two phases (hydrophobic/buffer)	
	2-HAP	PED
Dibutyl phthalate/buffer	6.33	0.65
Ethyl laurate/buffer	2.73	0.23
$C_4MIMI{\cdot}PF_6$/buffer	13.4	0.26

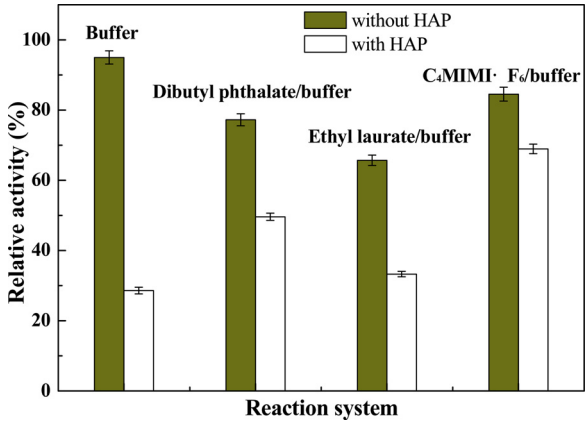


Fig. 4. Effect on the activity of recombinant BL21(DE3) in different systems. Reaction condition: 2 mL hydrophobic media containing 600 mM 2-HAP (final concentration 300 mM), 2 mL citrate phosphate buffer (100-200 mM, pH 6.5) containing 30 mg-dcw/mL cells (final concentration 15 mg-dcw/mL), glucose: 2-HAP = 1.5, 35 °C, 200 rpm, incubate for 5 h. The buffer system was as control.

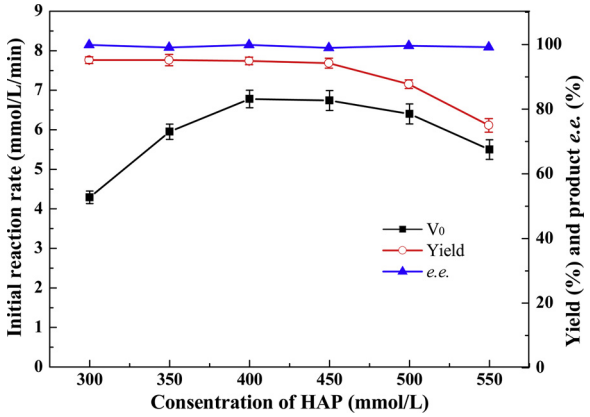


Fig. 5. Effect of substrate concentration on the asymmetric reduction catalyzed by whole-cells in the $C_4MIMI{\cdot}PF_6$/buffer system. Reaction condition: 2 mL $C_4MIMI{\cdot}PF_6$ containing 900 mM 2-HAP (final concentration 450 mM), 2 mL citrate phosphate buffer (100-200 mM, pH 6.5) containing glucose:2-HAP = 1.5, 30 mg-dcw/mL cells (final concentration 15 mg-dcw/mL), 35 °C, 200 rpm.

activity of BL21(DE3)(pETDuet-*gst*-mut-*accr-gdh*) cells was lower in the tested biphasic systems as compared to the buffer system in the absence of 2-HAP, indicating that the tested hydrophobic solvents partly damaged the BL21(DE3)(pETDuet-*gst*-mut-*accr-gdh*) cells. The relative catalytic activities were observed to vary in the studied biphasic systems, of which $C_4MIMI{\cdot}PF_6$ exhibited the satisfactory biocompatibility with the microbial cells and the best relative catalytic activity (84.5 %). Furthermore, in the presence of 2-HAP (300 mM), the relative catalytic activity of the recombinant cells after incubation for 5 h declined in the tested systems as compared to the system without 2-HAP, which was attributed to the toxicity of 2-HAP to the BL21(DE3)(pETDuet-*gst*-mut-*accr-gdh*) cells. Thus, in the presence of 2-HAP, the highest relative catalytic activity (70 %) of the recombinant cells was observed in $C_4MIMI{\cdot}PF_6$/buffer system. This result agreed with the best initial reaction rate, yield and partition coefficient achieved in the case of $C_4MIMI{\cdot}PF_6$-based system. Therefore, $C_4MIMI{\cdot}PF_6$ was chosen to be the second phase in the biphasic system for the reduction of 2-HAP.

Effect of 2-HAP concentration on the asymmetric reduction catalyzed with whole-cell in the $C_4MIMI{\cdot}PF_6$/buffer system

The concentration of 2-HAP reflects the catalytic efficiency of the reduction process catalyzed with BL21(DE3)(pETDuet-*gst*-mut-*accr-gdh*) in the $C_4MIMI{\cdot}PF_6$/buffer system. Fig. 5 described the evidently influence of 2-HAP concentration on the reduction process in the $C_4MIMI{\cdot}PF_6$/buffer system. As the 2-HAP concentration changed from 300 to 450 mM, the reaction rate accelerated obviously, while the yields (over 92 %) and the *e.e.* (> 99 %) displayed an insignificant change. The initial reaction rate reached 6.77 − 6.74 mM/min for the concentration ranging from 400 to 450 mM and the yield was over 92 % after a reaction duration of 3 h, and the space-time yield was 2.88 M/d, which was 1.52 times higher than that in buffer system. Further improving the concentration of 2-HAP from 450 to 550 mM caused a minor drop in the initial reaction rate, probably due to the growing inhibition effect of 2-HAP on BL21(DE3)(pETDuet-*gst*-mut-*accr-gdh*). Also, the yield decreased to 86.1 %-74.0 % with unchanged *e.e.* value. Consequently, the most suitable 2-HAP concentration was identified to be 450 mM in the $C_4MIMI{\cdot}PF_6$/buffer system, which was 1.8 times higher than that in the buffer system.

Comparing the obtained results with the previously reported findings for the synthesis of PED is vital for gaining further insights. The results of the biocatalytic reduction of 2-HAP to PED in different systems catalyzed with various biocatalysts were summarized in Table 5. With respect to *C.parapsilosis*/pCP-scrII, the product yield and *e.e.* value reached 99.9 %, however, the optimal 2-HAP concentration was about 36 mM only [39]. The recombinant *E.coli* (2, 3-butanediol dehydrogenase from *Bacillus subtilis*) could catalyze the reduction of about 390 mM 2-HAP with the yield and *e.e.* value of (*R*)-PED reaching 99 % [17]. The carbonyl reductase YaCRII from *Yarrowia lipolytica* and E228S/SCRII from *Candida parapsilosis* were constructed in *E. coli* and *S. cerevisiae* AN120 osw2Δ, respectively [40,41]. The two recombinant strains could catalyze higher concentrations of 2-HAP (145 − 200 mM) with excellent stereoselectivity, however, did not exhibit a high product yield (83.7 % & 88 %, respectively). Overall, as evident in Table 5, the asymmetric reduction of 2-HAP to (*S*)-PED catalyzed with BL21(DE3)(pETDuet-*gst*-mut-*accr-gdh*) could be conducted at satisfactory substrate concentration both in buffer and $C_4MIMIP^{\cdot}F_6$/buffer biphasic systems with promising performance.

Preparative scale asymmetric reduction of 2-HAP in the $C_4MIMI{\cdot}PF_6$/buffer biphasic system

The biotransformation was conducted on a 100-mL scale to ascertain the scalability of the 2-HAP reduction to (*S*)-PED catalyzed with BL21(DE3)(pETDuet-gst-mut-*accr-gdh*). HPLC analysis was used to monitor the reaction process and the results were shown in Fig. 6. 450 mM substrate was used to investigate the preparative scale. 407.3 mM (*S*)-PED could be achieved after a reaction period of 3 h, with space-time yield of 2.71 M/d. The yield and *e.e.* value were 90.5 % and > 99 %. Further prolongation of the reaction time to 4 h led to the (*S*)-PED concentration of 414.6 mM with a yield of 92.1 %. Overall, the *e.e.* value was satisfactory (> 99.0 %) during the whole catalytic process. Hence, the recombinant BL21(DE3)(pETDuet-*gst*-mut-*accr-gdh*) cell-catalyzed asymmetric reduction of 2-HAP to (*S*)-HAP on a preparative scale in the $C_4MIMI{\cdot}PF_6$/buffer system was confirmed to be promising and competitive.

Conclusions

In this study, the asymmetric reduction of 2-HAP to (*S*)-PED catalyzed with BL21(DE3)(pETDuet-*gst*-mut-*accr-gdh*) has been investigated in buffer and biphasic systems systematically, and the results were satisfactory. A highly efficient $C_4MIMI{\cdot}PF_6$/buffer system has been identified for the asymmetric reduction of 2-HAP to synthesize chiral alcohol (*S*)-PED. Using this system, 450 mM 2-HAP could be reduced in 3 h with encouraging product yield and *e.e.* value. The synthesis of the

Table 5
Comparison of the PED synthesis catalyzed by different biocatalysts.

Catalyst	Catalyst dosage	Substrate concentration	Reaction system	Product	Yield	*e.e.*	Reference
C.parapsilosis/ pCP-scrII	100 g/L wet cells	5g/L ≈36 mM	Buffer	(*S*)-PED	99.9 %	99.9 %	[39]
E. coli (BDHA-GDH)	30 g /L dry cells	54 g/L ≈390 mM	Buffer	(*R*)-PED	99 %	99 %	[17]
BL21(DE3)/ pET-28a-yacrII	50 g/L wet cells	20 g/L ≈145 mM	Dibutyl phthalate/ buffer	(*S*)-PED	83.7 %	99.9 %	[40]
S. cerevisiae osw2Δ(E228S-GDH)	50 g/L wet cells	200 mM	Ethyl acetate/ bufer	(*R*)-PED	88 %	99 %	[41]
BL21(DE3)(pETDuet-*gst*-mut-*accr-gdh*)	20 g/L dry cells	300 mM	Buffer	(*S*)-PED	95 %	> 99 %	This study
BL21(DE3)(pETDuet-*gst*-mut-*accr-gdh*)	15 g/L dry cells	450 mM	$[C_4MIMI]$•PF_6/buffer	(*S*)-PED	92.7 %	> 99 %	This study

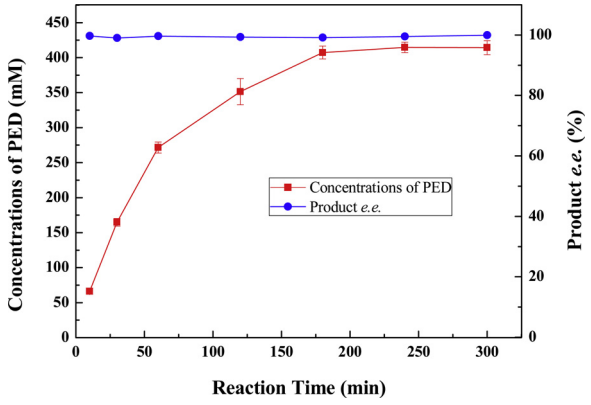


Fig. 6. Process of asymmetric reduction of 2-HAP catalyzed by whole-cells in the C_4MIMI·PF_6/buffer system. Reaction condition: 50 mL C_4MIMI·PF_6 containing 900 mM 2-HAP (final concentration 450 mM), 50 mL citrate phosphate buffer (100-200 mM, pH 6.5) containing glucose: 2-HAP = 1.5, 30 mg-dcw/mL cells (final concentration 15 mg-dcw/mL), 35 °C, 200 rpm.

enantiomerically pure (*S*)-PED on a 100-mL preparative scale could also be successfully performed via the asymmetric bioreduction of 2-HAP catalyzed with BL21(DE3)(pETDuet-*gst*-mut-*accr-gdh*) cells in C_4MIMI·PF_6/buffer system, which indicating a strong prospect of the whole-cell catalytic system for industrial application.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.mcat.2020.110903.

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