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Efficient biosynthesis of enantiopure tolvaptan by utilizing alcohol dehydrogenase-catalyzed enantioselective reduction

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Using whole cells of *Escherichia coli* co-expressing alcohol dehydrogenase (PsADH) and formate dehydrogenase (CpFDH) in a biphasic aqueous-soybean oil system is shown to be an efficient method for the biosynthesis of enantiopure tolvaptan. In this system, (S)-tolvaptan with an optical purity of 99.5% and bioconversion efficiency of 86.1% was achieved.

Tolvaptan is the first and only oral competitive vasopressin V2 receptor antagonist that has been approved by the FDA for the treatment of clinically significant hypervolemic and euvolemic hyponatraemia.[1] Tolvaptan is currently marketed as a racemate. However, the present inventors found that an injectable depot formulation comprising an optically active tolvaptan (S- (-)- tolvaptan or R- (+)- tolvaptan) has higher metabolic stability than racemic tolvaptan in mammals for preventing or treating polycystic kidney disease. It was also found that intramuscular administration of an aqueous suspension of optically active tolvaptan produces a higher serum concentration than the racemic compound dose. Moreover, the absorption rate of optically active tolvaptan was higher than that of racemic tolvaptan in vitro.[2] To clarify the mechanism of action and the pharmacological profile of enantiopure tolvaptan, asymmetric preparation of the optically active isomer becomes particularly important.

The optically active tolvaptan is usually prepared from prochiral ketone 7-chloro-1-[2-methyl-4-[(2-methylbenzoyl) amino] benzoyl]-5-oxo-2,3,4,5-tetrahydro-1H-1-benzazepine, named as PK1, by traditional chemical synthesis (Scheme 1).[3,4] All of the strategies require the employment of an asymmetric catalytic reaction using chiral reagents, which have some drawbacks. The synthesis of chiral reagents is complex, and costly, and the compound itself is environmentally toxic. Compared to chemical synthesis, biosynthesis provides an alternative for the preparation of enantiopure tolvaptan. Only Matsubara[5,6] reported a method for enantioselective synthesis of tolvaptan metabolites by lipase-catalyzed transesterification. Although this method enables the preparation of both enantiomers of the target compounds in high enantiomeric excess (99% *e.e.*), the asymmetric reaction system usually shows a modest yield (70%), which presents a bottleneck for large-scale application. Thus, from an environmental and practical standpoint, it is necessary to develop a more effective biosynthetic approach for producing enantiopure tolvaptan.

Alcohol dehydrogenase (EC 1.1.1.1) has been demonstrated to be a valuable biocatalyst in the reduction of prochiral ketone to chiral alcohols owing to its unique advantages such as outstanding enantioselectivity, mild reaction conditions, and environmental friendliness.[7-9] Furthermore, co-expression of alcohol dehydrogenases and glucose dehydrogenase or formate dehydrogenase in a recombinant whole-cell system for regeneration of the cofactor NADPH or NADH offers another highly effective approach in asymmetric reduction.[10-12] This aspect has been extensively researched.[13,14] However, thus far, there have been no reports on the application of alcohol dehydrogenase to the synthesis of optically active tolvaptan. Therefore, we attempted the biocatalytic asymmetric preparation of tolvaptan using alcohol dehydrogenase.

In a first set of experiments, five commercially available microorganisms (*Yarrowia lipolytica* CICC 32187, *Saccharomyces cerevisiae* CICC1002, *Pichia stipitis* CICC1960, *Candida parapsilosis* CICC1973, and *Rhodosporidium toruloides* CICC32489) were compared for their potential in the preparation of tolvaptan via asymmetric reduction of PK1 in a single water phase. All five tested strains exhibited different catalytic activities (Fig. S1). One yeast species, *P. stipitis* showed more activity (12%) than the other microorganisms examined. The varied catalytic activities of these strains might be attributed to the expression of different reductases. Therefore, we decided to explore the reductase in *P. stipitis* further.

Genome hunting and data mining strategies were adopted to amplify alcohol dehydrogenase genes from the genome of *P. stipitis*. In total, 7 amplified fragments were obtained and overexpressed in *E. coli* BL21 (DE3). After examination of their transformation of PK1 using whole cells of *E. coli*, one alcohol

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dehydrogenase (PsADH) was found to reduce PK1 to tolvaptan with good activity. Therefore, the alcohol dehydrogenase was chosen for further studies. The sequence of the alcohol dehydrogenase *psadh* with a length of 942 bp was obtained and characterized. The nucleotide sequence contained an open reading frame (ORF) encoding a polypeptide of 313 amino acids with a predicted molecular weight of 33.5 kDa and a predicted pI of 5.47. BLASTn results indicated that the *psadh* sequence shared 99% identity with the *sad*2 gene in *Scheffersomyces stipitis* CBS 6054 (GenBank accession no. XM_001384719.1). PsADH shared significant similarity (99%) with SADH2. However, the enzymatic activity of SADH2 has not been reported.[15] The results of homologous modelling showed that the enzyme has four monomers (Fig. S2). The purified recombinant PsADH protein was monitored by SDS-PAGE (Fig. S3a). It is indicated that PsADH was a single band with a molecular weight of 37 kDa, which is fused with 6*Histidine tags. The enzymatic assay revealed that PsADH was a coenzyme (NADH)-dependent enzyme. PsADH exhibited a specific activity of 4.7 U/mg under existence of PK1 as a substrate. The optimal temperature and pH for the activity of purified PsADH were 50°C and 5.0, respectively (Fig. S4), suggesting that PsADH had certain heat resistance and displayed moderate acid resistance.

Compared with the isolation of enzymes, preparation of whole-cell co-expression catalysts is more convenient and cost-effective. Moreover, the environment of the cell greatly favours the enzyme stability. Therefore, CpFDH from *C. parapsilosis* was used for NADH regeneration to promote PsADH-mediated reduction of PK1. Here, PsADH and CpFDH were in *E. coli* BL21 (DE3) cells in tandem, and whole cells of *E. coli* with the pG-Ps-SD-Cp-G plasmid expressing the recombinant proteins were employed as biocatalysts for the reduction of PK1. SDS-PAGE analysis of the protein extracts from these *E. coli*/pG-Ps-SD-Cp-G cells showed that both of PsADH and CpFDH enzymes are expressed correctly. It was observed in the location of PAGE gel that the position of PsADH was higher (60 kDa) than CpFDH (40 kDa) (Fig. S3b). The increased molecular weight of PsADH is due to the fusion of the GST tag (molecular weight 26 kDa). The functional expression of both enzymes was determined by measuring their activity in the whole cells of *E. coli*. To identify the optical property of the converted product by recombinant E. coli/pG-Ps-SD-Cp-G, HPLC chiral column analysis was performed. The retention time for (S)-tolvaptan and (R)-tolvaptan was determined with reference standards, as shown in Fig. 1. It was confirmed that nearly all the converted product was (S)-tolvaptan (*e.e.* >99%). The bioconversion activity toward PK1 was 42.1%. Consequently, the *E. coli*/pG-Ps-SD-Cp-G cells were chosen for further research.

To increase substrate availability and achieve a higher production titer, the biocatalytic conditions necessary for the production of tolvaptan from PK1 by the whole cells of *E. coli* BL21/pG-Ps-SD-Cp-G were optimized. First, the influence of organic solvents with different Log P values was determined in reaction mixtures for bioconversion. As shown in Table 1, solvents with Log P lower than 4 (dimethylbenzene, petroleum ether, cyclohexane, n-hexane) are not ideal for biocatalytic systems owing to the high solubility of the solvent in the water phase, as indicated by the relatively low bioconversion activity. Solvents with a Log P value higher than 4 (n-heptane, dimethicone, dibutylphthalate, soybean oil) showed both biocompatibility and certain extraction ability. It can be seen that there is a 10% conversion gap between Log P <4 and Log P ≥4. Soybean oil has the highest bioconversion of 64.4% in *E. coli*/pG-Ps-SD-Cp-G. Soybean oil mainly consists of palmic acid, $CH_3(CH_2)_{14}CO_2H$ (5%, Log P 7.1), oleic acid, $CH_3(CH_2)_{16}CO_2H$, (30%, Log P 7.7), linoleic acid, $CH_3(CH_2)_4CH{=}CHCH_2CH{-}CH(CH_2)_7CO_2H$, (60%, Log P 7.1) and stearic acid, $CH_3CH_2(CH{=}CHCH_2)_3(CH_2)_6CO_2H$, (4%, Log P 6.3). Although the composition of the soybean oil will slightly change on its source, the main ingredients are basically the same. The Log P of the soybean oil can be deduced to be greater than 6.0 from Log P of each ingredient. It is concluded that the better the compatibility of organic solvent and water, the higher the catalytic efficiency. Furthermore, soybean oil is a non-toxic, biodegradable and renewable organic solvent, which makes the future industrial production cleaner and greener. Therefore, soybean oil is considered as the best solvent in a two-phase system.

In an organic solvent/water two-phase system, the substrate distribution in the water phase is low, which affects the bioconversion.[16] Thus, the effect of the ratio of organic and aqueous phases on bioconversion was evaluated. As shown in Fig. 2, different ratios of organic and aqueous phases significantly influenced the bioconversion. As the ratio increased, the bioconversion rate fell sharply. Engineered E. coli/pG-Ps-SD-Cp-G displayed the highest bioconversion of 85.7% when the ratio of organic phase to aqueous phase was 1:9, which greatly surpassed the bioconversion of 43.6% achieved in the aqueous phase. If the ratio of aqueous phase is increased further, the aqueous and organic phases are not easy to delaminate. The less amount of the organic solvent also results in the difficulty of separating the final product. However, if there is no organic solvent available, the suspension of the substrate directly influences the catalytic conversion. Therefore, we concluded that the ratio of 1:9 was the most efficient for bioconversion in a two-phase system. Under this condition, the optimal temperature, pH, initial concentration of PK1 in soybean oil and conversion time in this catalytic system were 37 °C, 6.0, 100 mg/mL and 36 h, respectively (Fig. S5). The highest bioconversion efficiency was 86.1%.

Based on the optimal catalytic conditions, the bioconversion was scaled up. 1.0 g of PK1 was converted in the system. After conversion and workup, 0.79 g of (S)-tolvaptan was obtained with the yield of 80.4 % and *e.e.* of 99.5%. The product was characterized in ^{1}H NMR, ^{13} CNMR, HRMS (Fig. S6) as well as HPLC. The optical rotation of (S)-tolvaptan achieved was measured as –152° (c 0.1, MeOH) at 25°C. The reported value of (S)-tolvaptan was [a]D^{26} –159°, c 0.1, MeOH.[4]

The catalytic conversion of PK1 to (S)-tolvaptan by recombinant *E. coli* whole cells (Scheme 2) was compared with other catalytic systems (Table 2). It shows that the progress of lipase-catalyzed transesterification occurs in the middle step of the synthesis of tolvaptan. The yield (52%) and *e.e.* value (93%) of the intermediate product, (S)-(+)-2 ((S)-7-Chloro-5-hydroxy-1-(p-toluenesulfonyl)-2,3,4,5-tetrahydro-1H-1-benzazepine), was relatively lower than the values obtained using the chemical synthesis method and PsADH-CpFDH combined system. Although the chemical synthesis method has a high yield (99%) and *e.e.* value (99%), and a short reaction time (3-30 h) for preparation of (S)-tolvaptan, the chiral reagents used in asymmetric catalytic reaction are of high cost and difficult to be synthesized. It runs counter to green, environmentally friendly industrial development routes. The present approach results in a moderate yield (80.4 %) with a high *e.e.* of 99.5% for the production of (S)-tolvaptan on a slightly larger scale. More importantly, the use of bioenzyme as a catalyst and the reuse of organic solvents in the catalytic system makes this method more environmentally friendly. Therefore, from the perspective of

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the environment and the economy, the biosynthesis method still holds great application prospects. Further research will be performed on enzyme modification, using random mutation and site-saturation mutagenesis for more efficient bioconversion of prochiral ketone to (S)- or (R)-tolvaptan. Moreover, the catalytic conversion system of novel nano-immobilized enzyme will be developed to enhance the synthesis of optical pure tolvaptan to meet the demand of industrial production.

Conclusions

An alcohol dehydrogenase and formate dehydrogenase co-expressing system in an engineered bacterial strain for whole-cell catalytic bioconversion in an organic solvent/water two-phase system was developed. This system achieved the bioconversion of PK1 into (S)-tolvaptan with a high optical purity and yield. This study provides a method for more efficient chiral bioconversion of prochiral ketones into valuable enantiopure alcohols in industrial applications.

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Conflicts of interest

The authors declare no conflict of interest.

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Table 1. Effects of organic solvents on the bioconversion by whole cells of *E. coli* co-expressing PsADH and CpFDH in a two-phase catalytic system.

Organic solvent	Log P	Average Bioconversion (%)
Non	-	42.1
dimethylbenzene	3.1	43.9
petroleum ether	3.4	44.0
cyclohexane	3.2	45.3
n-hexane	3.5	46.4
n-heptane	4.4	57.9
dimethicone	5.4	58.2
dibutylphthalate	5.7	60.5
soybean oil	>6	64.4

Table 2. Comparison of the three different systems for producing enantiopure tolvaptan.

Parameters	Chemical System[3,4]	Lipase-catalyzed system[6]	PsADH-CpFDH combined system
Substract	PK1	(±)-2[a]	PK1
Concentration (g/mL)	0.892	0.1	0.1
Product	tolvaptan	(S)-(+)-2[b]	tolvaptan
T (h)	3-30	40	36
Yields (%)	63-99	52	80.4
e.e. (%)	81-99 (*S*)	93 (*S*)	99.5 (*S*)

a: 7-chloro-1-tosyl-2,3,4,5-tetrahydro-1H-benzo[b]azepin-5-ol, key intermediate.
b: (S)-7-Chloro-5-hydroxy-1-(p-toluenesulfonyl)-2,3,4,5-tetrahydro-1H-1-benzazepine, intermediate product of (S)-tolvaptan.

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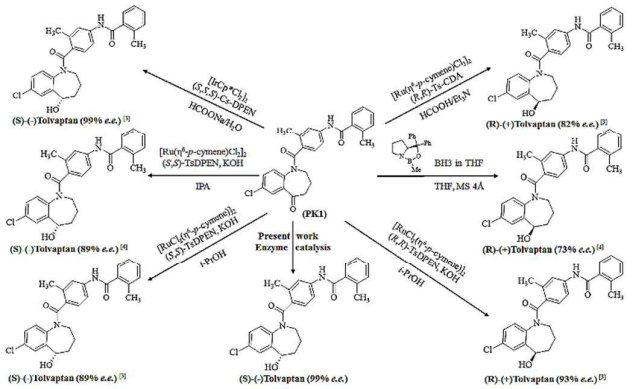
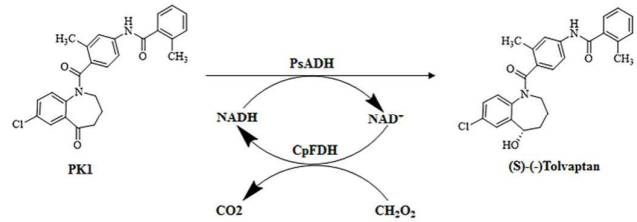


Scheme 1 Synthetic routes from PK1 to enantiopure tolvaptan.



Scheme 2 Biosynthetic pathway for the production of tolvaptan from PK1.

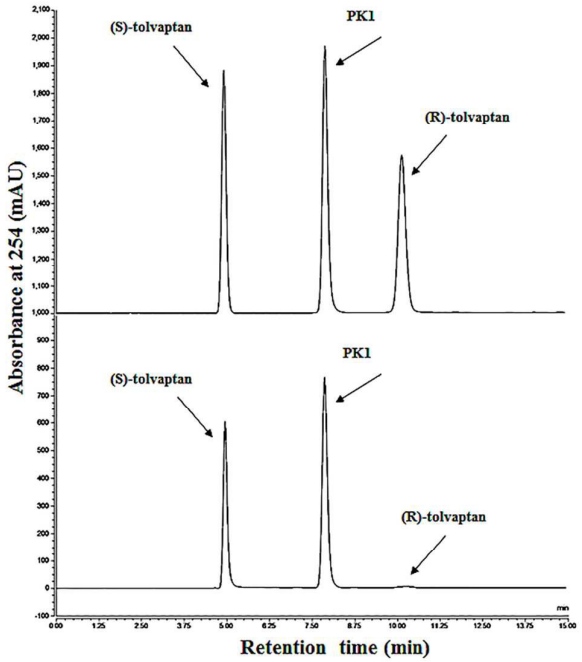


Figure 1 Retention time at HPLC trace with chiral column for substrate PK1, (S)-tolvaptan and (R)-tolvaptan. (a) PK1, (S)-tolvaptan and (R)-tolvaptan standard. (b) Co-culture with PK1 bioconversion by recombinant *E. coli*.

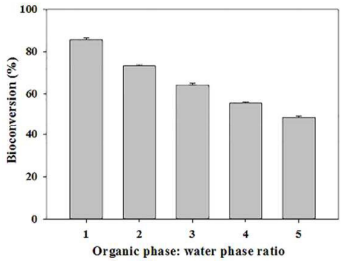


Figure 2 Effect of different ratios of organic phase: water phase on the whole-cell conversion of PK1. Numbers 1-5 represent the organic phase: water phase ratios 1:9, 1:4, 1:3, 1:2.3, and 1:1.5, respectively.