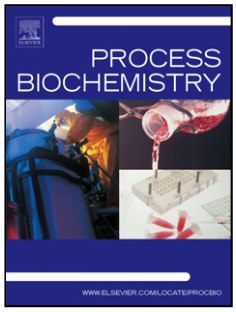


Title: Efficient bioreduction of 2-hydroxyacetophenone to (*S*)-1-phenyl-1,2-ethanediol through homologous expression of (*S*)-carbonyl reductase II in *Candida parapsilosis* CCTCC M203011

Author: Yaohui Li Rongzhen Zhang Yan Xu Rong Xiao Lei Wang Xiaotian Zhou Hongbo Liang Jiawei Jiang

PII: S1359-5113(16)30174-X
DOI: http://dx.doi.org/doi:10.1016/j.procbio.2016.05.026
Reference: PRBI 10700

To appear in: *Process Biochemistry*

Received date: 16-9-2015
Revised date: 26-5-2016
Accepted date: 26-5-2016

Please cite this article as: Li Yaohui, Zhang Rongzhen, Xu Yan, Xiao Rong, Wang Lei, Zhou Xiaotian, Liang Hongbo, Jiang Jiawei.Efficient bioreduction of 2-hydroxyacetophenone to (S)-1-phenyl-1,2-ethanediol through homologous expression of (S)-carbonyl reductase II in Candida parapsilosis CCTCC M203011.*Process Biochemistry* http://dx.doi.org/10.1016/j.procbio.2016.05.026

Efficient bioreduction of 2-hydroxyacetophenone to (*S*)-1-phenyl-1,2-ethanediol through homologous expression of (*S*)-carbonyl reductase II in *Candida parapsilosis* CCTCC M203011

Yaohui Li[a], Rongzhen Zhang[a, b, *], Yan Xu[a, b, *], Rong Xiao[c], Lei Wang[a], Xiaotian Zhou[a], Hongbo Liang[a], Jiawei Jiang[a]

[a] The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China.

[b] National Key Laboratory for Food Science, Jiangnan University, Wuxi 214122, P. R. China.

[c]Center for Advanced Biotechnology and Medicine, Rutgers University, Piscataway, NJ 08854, USA

***Corresponding author: Rongzhen Zhang, Yan Xu**

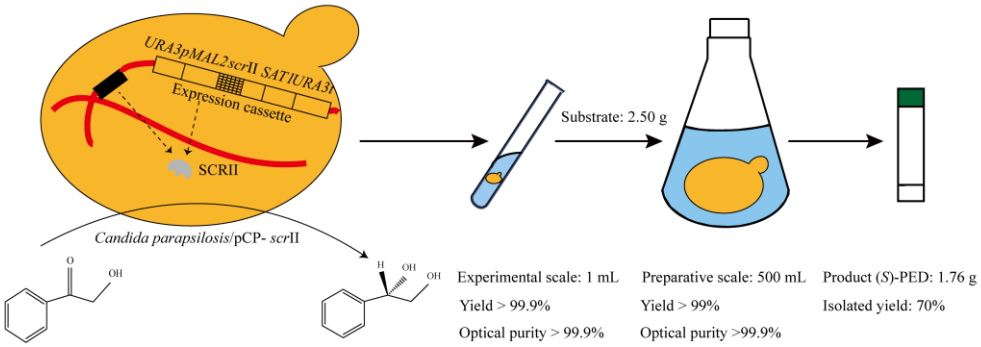
Tel: +86-510-85918201; Fax: +86-510-85864112

Email address: rzzhang@jiangnan.edu.cn; yxu@jiangnan.edu.cn

Present address: School of Biotechnology, Jiangnan University, 1800 Lihu Avenue, Wuxi, 214122, P. R. China.

Graphic abstract

The homologously expressed SCRII in *C. parapsilosis* gave good performance on asymmetric reduction of 2-HAP to (*S*)-PED.



Highlights

- Homologous expression of SCRII was successful in *C. parapsilosis*/pCP-*scr*II.
- Biotransformation efficiency of (*S*)-PED was significantly improved by *in situ* expressed SCRII.
- *C. parapsilosis*/pCP-*scr*II tolerated a wide range of temperatures and substrate concentrations.
- Large-scale of ketone reductions by *C. parapsilosis*/pCP-*scr*II was demonstrated.

Abstract

(*S*)-carbonyl reductase II (SCRII), a short-chain alcohol dehydrogenase from *Candida parapsilosis* M203011, catalyzes the bioreduction of 2-hydroxyacetophenone (2-HAP) to (*S*)-1-phenyl-1,2-ethanediol ((*S*)-PED). When SCRII was expressed in *Escherichia coli*, the biotransformation efficiency of (*S*)-PED was low. To improve its biocatalytic efficiency, the homologous expression of SCRII in *C. parapsilosis* M203011 was attempted. The *scr*II gene was cloned into an expression vector pCP carrying *MAL2* as its promoter and *SAT1* as its selection marker. Data obtained in this study showed that SCRII was successfully expressed in recombinant strain *C. parapsilosis*/pCP-*scr*II. The reductive activity toward 2-HAP exhibited about 2-fold and 6-fold increase in the cell-free extracts of *C. parapsilosis*/pCP-*scr*II than those of the wild-type and *E. coli*/pET28-SCRII. Under the optimal bioreaction conditions (pH 5.5, 35 ºC), the optical purity and yield of (*S*)-PED were both over 99.9% produced by *C. parapsilosis*/pCP-*scr*II. Additionally, 500-mL preparative scale bioreduction with efficient whole-cell process was performed, and the optical purity was over 99.9% with an isolated yield of about 70%. Our work not only demonstrated the high catalytic efficiency given by the homologous expression of SCRII in *C. parapsilosis*, but also provides an economical method for the preparation of optically pure chiral alcohols with whole-cell process.

Key words: (*S*)-carbonyl reductase II, (*S*)-1-phenyl-1,2-ethanediol, *Candida parapsilosis*, homologous expression, biocatalytic reduction

Abbreviations: 2-HAP, 2-hydroxyacetophenone; SCRII, (*S*)-carbonyl reductase II; *scr*II, (*S*)-carbonyl reductase II gene; (*S*)-PED, (*S*)-1-phenyl-1,2-ethanediol

1. Introduction

Enantiomerically pure alcohols play important roles in the synthesis of pharmaceutical agents, agrochemicals and fine chemicals [1, 2]. For example, (*S*)-1-phenyl-1,2-ethanediol (PED), a chiral block for stereoselective polymerization, can be used as an intermediate to prepare liquid crystals and chiral biphosphines [3].

Biocatalysis is an efficient and favorable way for producing chiral alcohols [4, 5]. Whole-cell biocatalysts have been successfully used for chiral synthesis in industry because of their high enantioselectivity, internal cofactor regeneration and low cost for down-stream operation [6, 7]. Many microorganisms have been applied as whole-cell biocatalysts for bioreduction processes [8, 9]. For examples, *C. parapsilosis* cells were used to reduce aromatic prochiral ketones [10]; and *Candida* spp. cells were used as an abundant biocatalytic platform for practical applications especially in reactions like selective keto-reductions, racemizations and stereoinversions. In such microorganisms, oxidoreductases are the key enzymes to catalyze chiral synthesis [11].

The oxidoreductases are the main enzymes to reduce prochiral ketones to chiral alcohols [12]. Some oxidoreductases have been heterologously expressed to realize their catalytic functions in *Escherichia coli* [13]. However, these enzymes may face two major problems: 1) low expression levels [14]; 2) low catalytic functions. Previously, we have isolated, characterized and successfully expressed (*S*)-carbonyl reductase II (SCRII), a typically short-chain alcohol dehydrogenase from *C. parapasilosis*, in *E. coli*. We found that SCRII could catalyze 2-hydroxyacetophenone (2-HAP) to (*S*)-PED [15]. However, the recombinant *E. coli* harboring SCRII showed low enzyme activities toward the bioreduction of 2-HAP and produced (*S*)-PED with a yield of 80% [16]. Chen *et al*. [17] suggested that these deficiencies may be due to the lack of protein post-translational modifications or incorrect protein folding in the host of *E. coli*. Meanwhile,

we studied the other (*S*)-carbonyl reductase (SCR) from *C. parapsilosis*, which had a high similarity of 88.5% with SCRII in amino acid sequence [18]. The recombinant *E. coli*-SCR and *Pichia pastoris*-SCR catalyzed 2-HAP to (*S*)-PED with a yield of about 84% [19] and 89% [15, 20]. Wang *et al*. [21] reported that the homologous expression probably supplied better post-translational modification or correct protein folding, thus giving better biotransformation efficiency. In their work, the reductive activity toward xylan of the homologously expressed Xyn III in *Trichoderma reesei* was 60 times more than that of the heterologously expressed Xyn III in *E. coli*.

In this work, we attempted homologous expression of SCRII in its native host *C. parapsilosis* on reducing prochiral ketones. We have successfully integrated gene *scr*II encoding SCRII into the genome of *C. parapsilosis.* The whole-cell biotransformation process of *C. parapsilosis/*pCP-*scr*II was studied both on experimental and preparative scale. This work was to demonstrate the biotransformation efficiency given by homologous expression of SCRII in *C. parapsilosis* and intends to facilitate the preparation of optically pure chiral alcohols.

2. Materials and methods

2.1. Strains and chemicals

C. parapsilosis CCTCC M203011 was provided by China center (CCTCC, Wuhan, China). Plasmid pMD^{TM}19-T was purchased from Takara Biotechnology Co., Ltd (Dalian, China). Plasmid pCP was constructed in this lab. Nourseothricin was purchased from United States Biological Co., Inc. (*R*)-PED, (*S*)-PED and 2-HAP were bought from TCI Development Co., Ltd (Shanghai, China). Other chemicals were of all analytical grade and commercially available. The strains and plasmids used in this work are listed

in Table 1.

2.2. Gene cloning

Genomic DNA of *C. parapsilosis* was isolated by Genomic DNA Mini Preparation Kit from Takara Co., Ltd (Dalian, China). The *scr*II gene (GenBank accession No. GQ411433) was amplified from the *C. parapsilosis* genome using primers of SCRII_*Sac* I_F1 and SCRII_*Kpn* I_R1 with a His_6-tag at its N-terminus. Meanwhile, the substitutions of T to C and C to A in *scr*II at positions 525 and 528 (525 T/C and 528 C/A), using primers of Mut *Kpn* I F2 and Mut *Kpn* I R2, were designed to mutate *Kpn* I restriction site. Then his_6-*scr*II fusion gene was ligated to pMD^{TM}19-T vector to construct the recombinant plasmid T-his_6-SCRII. The primers used in this work are listed in Table 1. The restriction sites of the primers were underlined.

2.3. Construction of an expression vector pCP-*scr*II

The expression plasmid pCP-RCR with a nourseothricin-resistance marker of *SAT1* [22] and a constitutive promoter of *MAL2* [23] was constructed in this lab (data not shown). pCP-RCR and T-his_6-SCRII were both digested with *Sac* I and *Kpn* I, and the digested fragments were ligated to generate pCP-*scr*II. The recombinant plasmid pCP-*scr*II was confirmed by DNA sequencing (Fig. 1). The primers used in this work are listed in Table 1. All the restriction sites of the primers were shown in Table 1 in underlined letters.

2.4. Yeast transformation and selection

The pCP-*scr*II was linearized by *Eco*R I and then was transformed to *C. parapsilosis* cells by electroporation as described by Nosek *et al*. [24]. After

electroporation, cells were immediately resuspended in YPS medium (1.0% yeast extract, 2.0% peptone, 1 M sorbitol) and incubated at 28 ºC for 2 h before being plated on MD plates supplemented with 80 ug/mL of nourseothricin [22]. Genomic DNA of the cultured cells were isolated [25], and the transformants carrying exogenous gene *scr*II were identified using primers of SAT1_1 and URA3t_1 (Table 1), which were used to amplify a 1.0-kb fragment (*SAT 1*) from genomic DNA of recombinant *C. parapsilosis* (Fig. 1). These positive clones containing the exogenous gene *scr*II were used as biocatalysts to transform 2-HAP to (*S*)-PED under the conditions described previously with some modifications [26]. Instead of using (*RS*)-PED, we used 5 g/L 2-HAP as substrates. Then we chose one of these strains which showed the highest optical purity and yield to continue further research.

2.5. Homologous expression of SCRII in *C. parapsilosis*

C. parapsilosis/pCP-*scr*II was cultivated at 28 ºC in fermentation medium described as Nie *et al*. [14] using the wild-type *C. parapsilosis* as the control. After 12−60 h cultivation, the *C. parapsilosis*/pCP-*scr*II cells were collected at 6000×g centrifugation, washed twice with saline, and resuspended in 0.2 M phosphate buffer (pH 6.0) for cell disruption using high-pressure homogenizer (SPX, Norderstedt, Germany). The cell-free extracts containing SCRII were obtained by centrifugation at 12,000×g for 40 min at 4 ºC, and they were applied for SDS-PAGE analysis at different fermentation time.

2.6. Enzyme activity assay

The enzyme activity for the reduction of 2-HAP to (*S*)-PED by SCRII was determined with cell-free extracts by measuring the decrease in absorbance at 340 nm

resulting from the oxidation of NADPH. One unit of the enzyme activity is defined as the amount of enzyme catalyzing the formation and oxidation of 1μmol of NAD(P)H per minute under measurement condition [18]. Protein concentration was determined by Bradford reagents from Beyotime (Shanghai, China) with bovine serum albumin as a standard [27]. All the experiments were done in triplicate both for *C. parapsilosis*/pCP-*scr*II and the wild-type *C. parapsilosis*.

2.7. Biotransformation and analytical methods

The asymmetric reduction of 2-HAP to (*S*)-PED by *C. parapsilosis* was conducted as described by Nie *et al*. [26] with some modifications. After 36 h incubation, cells were collected at 6000×g centrifugation and washed twice with saline. For asymmetric reaction, 1 mL of the reaction mixture contained 0.2 M acetate (pH 4.0 to 6.0) or 0.2 M phosphate buffer (pH 6.0 to 7.0), 2-HAP (1 g/L to 30 g/L) and 10% (W/V) wet-cells of *C. parapsilosis*. The products were extracted with ethyl acetate, and the optical purity and yield were determined by HPLC on a Chiralcel OB-H column (Daicel Chemical Ind, Ltd., Japan) as described previously [26]. The optical purity and yield were calculated basing on the following equations [10]:

%e.e. (enantiomer excess)=$(C_s-C_r)/(C_s+C_r)$ ×100%, C_s is the concentration of the (*S*)-PED and C_r is the concentration of (*R*)-PED.

Yield=C_p/C_o×100%, C_p is the concentration of the (*S*)-PED and C_o is the initial substrate concentration of 2-HAP.

The optimal pH for biotransformation of 2-HAP to (*S*)-PED using *C. parapsilosis*/pCP-*scr*II was determined by the optical purity and yield at different pH values ranging from 4.0 to 7.0. To determine the optimal bioreaction temperature, the asymmetric reductions were conducted between 20 ºC and 50 ºC [16]. The influences of

substrate concentrations ranging from 1 g/L to 30 g/L on asymmetric reduction of 2-HAP to (*S*)-PED were also detected. The peak times of 2-HAP, (*S*)-PED and (*R*)-PED are 33.0 min, 24.2 min and 19.3 min, respectively. All the measurements were done in triplicate.

2.8. Preparative scale bioreduction of 2-HAP to (*S*)-PED

The preparative scale bioreduction of 2-HAP by whole cells of *C. parapsilosis*/pCP-*scr*II was conducted under the optimized reaction conditions. The bioreaction mixtures of 500 mL 0.2 M NaAC-HAC buffer contained 50 g wet-cells of *C. parapsilosis*/pCP-*scr*II (10 g/L) and 2.5 g 2-HAP (5 g/L) shaking at 35 ºC and 200 rpm for 45 h. The products were first extracted with ethyl acetate as described previously [26]. Ethyl acetate was evaporated with a rotary evaporator and a vaccum pump system, and the dried product was applied for HPLC analysis as described previously [26]. The extracted products were purified using preparative liquid chromatography on a Chiralpak AY-H column (0.46 cm×15 cm; Daicel Chemical Ind, Ltd., Japan). The product purity was further confirmed by ^{1}H NMR analysis on a Bruker Aduance III 400 MHz spectrometer (400 MHz, $CDCl_3$) as reported recently [28].

3. Results and discussion

3.1. Screening of *C. parapsilosis*/pCP-*scr*II

To obtain homologously SCRII-expressing strains, we applied the *in-situ* expression cassette (pCP-*scr*II) to the genome of *C. parapsilosis* by deleting the gene *URA3* using primers *URA*3p_1 and *URA*3t_2 as the homologous arms to realize homologous recombination (Fig. 1).

After electroporation of *Eco*R I-linearized pCP-*scr*II, about 5 nourseothricin-resistant transformants were obtained after 60 h of recombinant-cell growth on MD agar plates. PCR fragments of *SAT1* about 1.0-kb were amplified from two of them (e.g. M-1 and M-2, Table 2), which confirmed the correct integration of gene *scr*II into the genome of *C. parapsilosis*. As shown in Table 2, the catalytic performances (e.g. enzyme activity, optical purity and yield) given by these strains were different. These differences may result from the variation of gene copy numbers in the genome of *C. parapsilosis*. As reported by Myhre *et al*. [29], it is possible to increase the gene copy numbers for further improvement of the protein expression, thus increasing the total enzyme activity. Since M-2 had better catalytic performances than M-1 (Table 2), we chose M-2 to further explore its catalytic performances (e.g. optical purity and yield).

3.2. Homologous expression of SCRII in *C. parapsilosis*

SDS-PAGE analysis of cell-free extracts from M-2 showed that protein expression reached the highest level when fermentation time was increased from 30 to 36 h (Fig. 2). Moreover, the enzyme activity of SCRII for 2-HAP reached 3.07 U/mg at 36 h (Fig. 2B). The SCRII activity was twice as that of SCRII expressed by wild-type *C. parapsilosis*, and almost 6-fold as that of SCRII expressed by recombinant *E. coli* (Table 3). These improvements probably not only caused by the increase of gene copy numbers in the genome of *C. parapsilosis* [30], but also resulted from the correct conformation and modification of SCRII in its native host [17].

We only measured the enzyme activity of cell-free extracts from *C. parapsilosis*/pCP-*scr*II while using the wild type as the control. We did not measure the specific reducing activity of purified SCRII, as the expression level of SCRII in *C. parapsilosis*/pCP-*scr*II was too low to obtain purified SCRII, even after adding His_6-tag

at its N-Terminus (data not shown). However, these results showed that *C. parapsilosis/*pCP-*scr*II can still be used as a host strain for overexpression of SCRII to explore its catalytic activities toward 2-HAP. Sapio *et al*. [31] and Wang *et al*. [21] used constitutive promoters and successfully over-expressed and purified homologously expressed proteins from their native hosts. Berkner *et al*. [32] found that inducible promoters sometimes will have a relatively positive effect on protein expression. It may be a good way to improve the expression level of SCRII by using inducible promoters, such as *CpGAL1* [33]. *C. parapsilosis* is a glucose-limited strain that relies on respiratory metabolism [34], so using glycerol instead of glucose as the main carbon resource will also be good to the protein expression [35].

3.3. Optimization of initial pH and temperature on the biotransformation of 2-HAP to (*S*)-PED by whole cells of *C. parapsilosis*/pCP-*scr*II

To improve the optical purity and yield of (*S*)-PED, we studied the effects of two crucial variables (i.e. initial buffer pH and reaction temperature) on the asymmetric reduction of 2-HAP to (*S*)-PED when using the whole cells of *C. parapsilosis/*pCP-*scr*II as the biocatalysts.

It is well known that pH plays a key role in biocatalytic processes [36]. Keinan *et al*. thought that pH affected the product enantiospecificity during asymmetric reduction of ketones by *Thermoanaerobium brockii* alcohol dehydrogenase [37]. Secundo *et al*. also reported that the SDRs showed a significant pH dependence on enantiospecificity [36]. As depicted in Fig. 3, for *C. parapsilosis/*pCP-*scr*II, with pH varying from 4.5 to 5.5, the optical purity and yield of (*S*)-PED both increased to the highest level of 99.9%. When pH continued to rise, the optical purity and yield dropped sharply. Interestingly, for the wild-type *C. parapsilosis*, the optical purity first decreased sharply to about 60%

at pH 6.0 and then increased to about 78% at pH 6.5 of 0.2 M phosphate buffer. This fluctuation probably was caused by pH inhibition on SCRII or pH activation on (*R*)-carbonyl reductase (RCR) [14]. Our study also showed that the optical purity of (*S*)-PED catalyzed by *C. parapsilosis/*pCP-*scr*II was higher than that of the wild-type strain by 36.9% at pH 6.0 of 0.2 M phosphate buffer, and the *C. parapsilosis/*pCP-*scr*II seemed to be more tolerant to pH change when it was used as biocatalyst to reduce 2-HAP to (*S*)-PED. This phenomenon was probably caused by the relatively high expression of SCRII in *C. parapsilosis/*pCP-*scr*II. Overall, the optimum pH for the bioreaction was 5.5 for both the *C. parapsilosis/*pCP-*scr*II and the wild-type strain.

Besides pH, temperature also plays a crucial role on enzyme activity and selectivity [38, 39]. When using *C. parapsilosis/*pCP-*scr*II as biocatalysts, the optical purity remained above 95% when reaction temperature varied from 20 ºC to 50 ºC. It reached a highest level over 99.9% at 35 ºC (Fig. 4). In contrast, the yield changed evidently as the temperature rose. It first increased at 20−35 ºC and reached a highest level over 99.9% at 35 ºC, and then decreased at 35−50 ºC, and was about 30% at 50 ºC. Particularly, when reaction temperature varied from 20 ºC to 30 ºC, the optical purity and yield at 30 ºC were both over 2% lower than those of the 25 ºC. This decrease was possibly due to the relatively high activity of RCR at 25 ºC, which led to a high percentage of (*R*)-PED in the chiral mixtures [40]. Interestingly, compared with the wild-type *C. parapsilosis* and *E.coli* BL21/pET28-SCRII [15], the *C. parapsilosis/*pCP-*scr*II cells as biocatalysts exhibited not only higher pH tolerance, but also higher reaction temperature tolerance (Fig. 4). As reported by Postmus *et al.* [42], respiratory chain efficiency had certain relationship with environmental factors, such as temperature. With the increase of catalytic enzymes of oxidoreductases in *C. parapsilosis*, such as the increase of SCRII, the respiratory chain efficiency will probably be improved, resulting in its stronger

tolerance to pH and temperature change. These results showed that the homologous expression of SCRII in *C. parapsilosis* did not change the optimal temperature of the bioreaction from 2-HAP to (*S*)-PED by *C. parapsilosis* cells, but it improved the biotransformation efficiency and pH and temperature tolerance of the whole-cell process.

3.4. Tolerance of *C. parapsilosis*/pCP-*scr*II to initial substrate concentrations of 2-HAP

Enzyme and substrate are the two basic factors in biocatalytic processes. Excess substrate concentration either results in substrate inhibition or affects the utilization of alcohol dehydrogenase for being short of cofactors, such as NADH/NAD^+ or NADPH/$NADP^+$ [43]. In Fig. 5, for both *C. parapsilosis/*pCP-*scr*II and the wild-type *C. parapsilosis*, the optical purity and yield of (*S*)-PED showed no clear variations when 2-HAP concentration was ranged from 1 g/L to 7 g/L. However, further increase in 2-HAP concentration led to an obvious drop in the maximum yield of (*S*)-PED, probably resulting from inhibition and toxicity of high substrate concentrations on *C. parapsilosis* cells. During the whole-cell process, as shown in Fig. 5, the *C. parapsilosis/*pCP-*scr*II showed better biotransformation efficiency than the wild-type *C. parapsilosis*, especially when substrate concentrations were higher than 12 g/L.

Moreover, compared with the *E. coli* BL21/pET28-SCRII, the *C. parapsilosis/*pCP-*scr*II and the wild-type *C. parapsilosis* showed stronger tolerance to substrate concentrations. As shown in Table 3, even under lower substrate concentrations (e.g. 5 g/L), the yield of (*S*)-PED produced by *C. parapsilosis/*pCP-*scr*II was about 25% higher than that of the recombinant *E. coli*. This gap probably either caused by the formation of inclusion bodies of SCRII in *E. coli* or the incorrect protein conformation from

heterologous expression [17], thus reducing the capabilities of SCRII in realizing asymmetric reduction through whole-cell process of *E. coli*.

3.5. Preparative scale bioreduction of 2-HAP to (*S*)-PED by *C. parapsilosis*/pCP-*scr*II

To determine the scalability of bioreduction of 2-HAP to (*S*)-PED by *C. parapsilosis*/pCP-*scr*II, we performed the bioreduction of 2-HAP to (*S*)-PED on a 500-mL shake-flask scale under the optimal conditions (pH 5.5, 35 ^{o}C). After 45 h reaction, the substrates of 2-HAP were almost all transformed to (*S*)-PED with an optical purity over 99.9% and a yield over 99% (Fig. 6). The product was isolated using preparative liquid chromatography, and an isolated yield of about 70% (1.76 g) was achieved. ^{1}H NMR data of the purified product was consistent with the standard sample of (*S*)-PED (Fig. 7).

As far as we know, this is the first time to successfully explore the preparative scale bioreduction of prochiral ketones by recombinant *C. parapsilosis*. The catalytic performance (i.e. the optical purity and yield) was as good as that of the 1-mL experimental scale, demonstrating that the catalytic asymmetric reduction by the *C. parapsilosis*/pCP-*scr*II can be further scaled up for industrial application. Other reports also demonstrated successful applications by whole-cell process of *C. parapsilosis* for chiral synthesis [44-46]. It is of great interest to use *C. parapsilosis* cells as the biocatalysts for efficient bioreductions of prochiral ketones. As a whole-cell process, *C. parapsilosis* contains abundant carbonyl reductases, whose biocatalytic properties are suitable for ketone reductions; it can provide a natural environment for catalytic function of carbonyl reductases [18, 47]. This work supplied an economical method for efficient chiral biosynthesis using the homologously expressed oxidordeuctase in *C. parapsilosis*.

4. Conclusions

In this study, we transformed an expression vector pCP-*scr*II into *C. parapsilosis* and realized homologous expression of SCRII in *C. parapsilosis*. To achieve higher optical purity and yield, a pH of 5.5 and a temperature of 35 ºC were found to be suitable for the biotransformation of 2-HAP to (*S*)-PED by *C. parapsilosis/*pCP-*scr*II cells, making the optical purity and yield of (*S*)-PED both over 99.9%. The preparation of (*S*)-PED was also conducted on a 500-mL preparative scale, and the biotransformation efficiency was the same as that of the 1-mL experimental scale. After the products were isolated using preparative liquid chromatography, the isolated yield of (*S*)-PED was about 70%. The homologous expression is an efficient method to enhance the acitivity of SCRII, and its potential industrial applications will be explored.

Acknowlegements

This project was supported by the National Science Foundation of China (No. 3137010 and 21336009), Jiangsu Province Basic Research Program (No. BK20131101), the Fundamental Research Funds for the Central Universities (JUSRP51409B), the Program for Advanced Talents within Six Industries of Jiangsu Province (No. 2015-SWYY-010), Chinese New Century Excellent Talents Project of Chinese Ministry of Education (NCET-13-0833), and the Program of Introducing Talents of Discipline to Universities (No. 111-2-06).

References

[1] Schoemaker HE, Mink D, Wubbolts MG. Dispelling the myths--biocatalysis in industrial synthesis. Science 2003;299:1694−1697.

[2] Matsuda T, Yamanaka R, Nakamura K. Recent progress in biocatalysis for asymmetric oxidation and reduction. Tetrahedron: Asymmetry 2009;20:513−557.

[3] Iwasaki F, Maki T, Nakashima W, Onomura O, Matsumura Y. Nonenzymatic kinetic resolution of 1,2-Diols catalyzed by an organotin compound. Org Lett 1999;1:969−972.

[4] Wohlgemuth R. Asymmetric biocatalysis with microbial enzymes and cells. Curr Opin Microbiol 2010;13:283−292.

[5] Wang SS, Nie Y, Xu Y, Zhang RZ, Ko TP, Huang CH, Chan HC, Guo RT, Xiao R. Unconserved substrate-binding sites direct the stereoselectivity of medium-chain alcohol dehydrogenase. Chem Commun 2014;50:7770−7772.

[6] Goldberg K, Schroer K, Lutz S, Liese A. Biocatalytic ketone reduction--a powerful tool for the production of chiral alcohols-part II: whole-cell reductions. Appl Microbiol Biotechnol 2007;76:249−255.

[7] Mathematik VdFf. Cloning, expression, characterization and immobilization of carbonyl reductase from *Candida parapsilosis*. Germany: RWTH Aachen University; Ph.D. thesis 2006:5−7.

[8] Vieira GAB, Araujo DMF, Lemos TLG, Mattos MC, Oliveira MCF, Melo VMM, Gonzalo G, Gotor-Fernández V, Gotor V. *Candida tropicalis* CE017: a new Brazilian enzymatic source for the bioreduction of aromatic prochiral ketones. J Braz Chem Soc 2010;21:1509−1516.

[9] Ni Y, Su Y, Li H, Zhou J, Sun Z. Scalable biocatalytic synthesis of optically pure

ethyl (*R*)-2-hydroxy-4-phenylbutyrate using a recombinant *E. coli* with high catalyst yield. J Biotechnol 2013;168:493−498.

[10] Mu XQ, Xu Y, Nie Y, Ouyang J, Sun ZH. *Candida parapsilosis* CCTCC M203011 and the optimization of fermentation medium for stereoinversion of (*S*)-1-phenyl-1,2-ethanediol. Process Biochem 2005;40:2345−2350.

[11] Gamenara D, Maria PD. *Candida spp*. redox machineries: an ample biocatalytic platform for practical applications and academic insights. Biotechnol Adv 2009;27:278−285.

[12] Forrest GL, Gonzalez B. Carbonyl reductase. Chemico Biological Interaction 2000;129:21−40.

[13] Bornscheuer UT, Huisman GW, Kazlauskas RJ, Lutz S, Moore JC, Robins K. Engineering the third wave of biocatalysis. Nature 2012;485:185−194.

[14] Nie Y, Xu Y, Yang M, Mu XQ. A novel NADH-dependent carbonyl reductase with unusual stereoselectivity for (*R*)-specific reduction from an (*S*)-1-phenyl-1,2-ethanediol-producing micro-organism: purification and characterization. Lett Appl Microbiol 2007;44:555−562.

[15] Zhang RZ, Geng YW, Xu Y, Zhang WC, Wang SS, Xiao R. Carbonyl reductase SCRII from *Candida parapsilosis* catalyzes anti-Prelog reaction to (*S*)-1-phenyl-1,2-ethanediol with absolute stereochemical selectivity. Bioresource Technol 2011;102:483−489.

[16] Geng YW, Zhang RZ, Xu Y, Wang SS, Sha C, Xiao R. Coexpression of a carbonyl reductase and glucose 6-phosphate dehydrogenase in *Pichia pastoris* improves the production of (*S*)-1-phenyl-1,2-ethanediol. Biocatal Biotransfor 2011;29:172−178.

[17] Chen CY, Oppermann H, Hitzeman RA. Homologous versus heterologous gene expression in the yeast, *Saccharomyces cerevisiae*. Nuclear Acids Research

1984;12:8951−8970.

[18] Nie Y, Xiao R, Xu Y, Montelione GT. Novel anti-Prelog stereospecific carbonyl reductases from *Candida parapsilosis* for asymmetric reduction of prochiral ketones. Org Biomol Chem 2011;9:4070−4078.

[19] Zhang RZ, Xu Y, Sun Y, Zhang WC, Xiao R. Ser67Asp and His68Asp substitutions in *Candida parapsilosis* carbonyl reductase alter the coenzyme specificity and enantioselectivity of ketone reduction. Appl Environ Microbiol 2009;75:2176−2183.

[20] Zhang RZ, Xu Y, Xiao R, Wang L, Zhang BT. Optimized expression of (*S*)-carbonyl reductase in *Pichia pastoris* for efficient production of (*S*)-1-phenyl-1, 2-ethanediol. J Basic Microb 2014;54:873−879.

[21] Wang J, Zeng D, Mai G, Liu G, Yu S. Homologous constitutive expression of Xyn III in *Trichoderma reesei* QM9414 and its characterization. Folia Microbiol 2014;59:229−233.

[22] Reuß O, Vik A, Kolter R, Morschhauser J. The *SAT1* flipper, an optimized tool for gene disruption in *Candida albicans*. Gene 2004;341:119−127.

[23] Ding C, Butler G. Development of a gene knockout system in *Candida parapsilosis* reveals a conserved role for BCR1 in biofilm formation. Eukaryot Cell 2007;6:1310−1319.

[24] Zemanova J, Nosek J, Tomaska L. High-efficiency transformation of the pathogenic yeast *Candida parapsilosis*. Curr Genet 2004;45:183−186.

[25] Wang HY, Zhang XJ, Zhao LP, Xu Y. Analysis and comparison of the bacterial community in fermented grains during the fermentation for two different styles of Chinese liquor. J Ind Microbiol Biot 2008;35:603−609.

[26] Nie Y, Xu Y, Mu XQ. Highly enantioselective conversion of racemic 1-Phenyl-1,2-

ethanediol by stereoinversion involving a novel cofactor-dependent oxidoreduction system of *Candida parapsilosis* CCTCC M203011. Org Process Res Dev 2004;8:246−251.

[27] Bradford. MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the Principle of Protein-Dye Binding. Anal Biochem 1976;72:248−254.[1] Schoemaker HE, Mink D, Wubbolts MG. Dispelling the myths--biocatalysis in industrial synthesis. Science 2003;299:1694-1697.

[28] Frank O, Kreissl JK, Daschner A, Hofmann T. Accurate determination of reference materials and natural isolates by means of quantitative ^{1}H NMR spectroscopy. J AGR FOOD CHEM 2014;62:2506-2515.

[29] Myhre S, Lingjaerde OC, Hennessy BT, Aure MR, Carey MS, Alsner J, Tramm T, Overgaard J, Mills GB, Borresen-Dale AL, Sorlie T. Influence of DNA copy number and mRNA levels on the expression of breast cancer related proteins. Mol Oncol 2013;7:704−718.

[30] Chino A, Makanae K, Moriya H. Relationships between cell cycle regulator gene copy numbers and protein expression levels in *Schizosaccharomyces pombe*. PloS one 2013;8:1−8.

[31] Sapio ML, Hilleringmann M, Barocchi MA, Moschioni M. A novel strategy to over-express and purify homologous proteins from *Streptococcus pneumoniae*. J Biotechnol 2012;157:279−286.

[32] Berkner S, Wlodkowski A, Albers SV, Lipps G. Inducible and constitutive promoters for genetic systems in *Sulfolobus acidocaldarius*. Extremophiles 2010;14:249−259.

[33] Kosa P, Gavenciakova B, Nosek J. Development of a set of plasmid vectors for genetic manipulations of the pathogenic yeast *Candida parapsilosis*. Gene

2007;396:338−345.

[34] Rogers PJ. Efficiency of oxidative phosphorylation in continuous cultures of *Candida parapsilosis*. J Bacteriol 1977;130:521−523.

[35] Nosek J, Holesova Z, Kosa P, Gacser A, Tomaska L. Biology and genetics of the pathogenic yeast *Candida parapsilosis*. Curr Genet 2009;55:497−509.

[36] Secundo F, Phillips RS. Effects of pH on enantiospecificity of alcohol dehydrogenases from *Thermoanaerobacter ethanolicus* and horse liver. Enzyme Microb Technol 1996;19:487−492.

[37] Keinan E, Hafeli EK, Kamal K. Seth, Lamed R. Thermostable enzymes in organic synthesis. 2. Asymmetric reduction of ketones with alcohol dehydrogenase from *Thermoanaerobium brockii*. J Am Chem Soc 1986;108:162−169.

[38] Pham VT, Phillips RS. Effects of substrate structure and temperature on the stereospecificity of secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus*. J Am Chem Soc 1989;112:3629−3632.

[39] Phillips RS. Temperature modulation of the stereochemistry of enzymatic catalysis: prospects for exploitation. Trends in Biotechnol 1996;14:13−16.

[40] Nie Y, Xu Y, Wang HY, Xu N, Xiao R, Sun ZH. Complementary selectivity to (*S*)-1-phenyl-1,2-ethanediol-forming *Candida parapsilosis* by expressing its carbonyl reductase in *Escherichia coli* for (*R*)-specific reduction of 2-hydroxyacetophenone. Biocatal Biotransfor 2008;26:210−219.

[41] Jin X, Liu B, Ni Z, Wu Q, Lin X. A novel control of enzymatic enantioselectivity through the racemic temperature influenced by reaction media. Enzyme Microb Technol 2011;48:454−457.

[42] Postmus J, Tuzun I, Bekker M, Muller WH, Mattos MJT, Brul S, Smits GJ. Dynamic regulation of mitochondrial respiratory chain efficiency in *Saccharomyces*

cerevisiae. Microbiology 2011;157:3500−3511.

[43] Fossati E, Polentini F, Carrea G, Riva S. Exploitation of the alcohol dehydrogenase-acetone NADP-regeneration system for the enzymatic preparative-scale production of 12-ketochenodeoxycholic acid. Biotechnol Bioeng 2006;93:1216−1220.

[44] Matsuyama A, Yamamoto H, Kobayashi Y. Practical application of recombinant whole-cell biocatalysts for the manufacturing of pharmaceutical intermediates such as chiral alcohols. Org Process Res Dev 2002;6:558−561.

[45] Zhang BB, Lou WY, Chen WJ, Zong MH. Efficient asymmetric reduction of 4 (Trimethylsilyl)-3-Butyn-2-One by *Candida parapsilosis* cells in an ionic liquid-containing system. Plos one 2012;7:1−9.

[46] Venkataraman S, Chadha A. Biocatalytic deracemisation of aliphatic beta-hydroxy esters: improving the enantioselectivity by optimisation of reaction parameters. J Ind Microbiol Biot 2015;42:173−180.

[47] Kataoka M, Delacruz-Hidalgo AR, Akond MA, Sakuradani E, Kita K, Shimizu S. Gene cloning and overexpression of two conjugated polyketone reductases, novel aldo-keto reductase family enzymes, of *Candida parapsilosis*. Appl Microbiol Biot 2004;64:359−366.

Figure captions

Fig. 1. Construction of an expression vector pCP-*scr*II for *C. parapsilosis*

Restriction sites are unique. E, *Eco*R I; B, *Bgl* II; S, *Sac* I; K, *Kpn* I; P, *Pst* I; N, *Not* I; BH, *Bam*H I; H, *Hin*d III.

Fig. 2. Optimization of fermentation time for *C. parapsilosis*. (A) SDS-PAGE analysis of SCRII expression in *C. parapsilosis*/pCP-*scr*II. Lane CK, the culture of wild type at 36 h; (B) The enzyme activities of cell-free extracts from *C. parapsilosis* at different fermentation time.

Fig. 3. Effects of pH on asymmetric reduction of 2-HAP to (*S*)-PED by *C. parapsilosis.* Yield (□) and optical purity (○) of (*S*)-PED from 2-HAP catalyzed by *C. parapsilosis/*pCP-*scr*II; Yield (■) and optical purity (●) of (*S*)-PED from 2-HAP catalyzed by the wild-type strain;

Biotransformation conditions: 1 mL 0.2 M NaAC-HAC buffer (pH 4.0−6.0) or 0.2 M phosphate buffer (pH 6.0−7.0) containing 5 g/L 2-HAP and 100 g/L wet cells was shaking at 200 rpm and 35 ºC for 45 h.

Fig. 4. Effects of temperatures on asymmetric reduction of 2-HAP to (*S*)-PED by *C. parapsilosis.* Yield (□) and optical purity (○) of (*S*)-PED from 2-HAP catalyzed by *C. parapsilosis/*pCP-*scr*II; Yield (■) and optical purity (●) of (*S*)-PED from 2-HAP catalyzed by the wild-type strain;

Biotransformation conditions: 1 mL 0.2 M NaAC-HAC buffer (buffer pH 5.5) containing 5 g/L 2-HAP and 100 g/L wet cells was shaking at 200 rpm and various reaction temperatures (20−50 ºC) for 45 h.

Fig. 5. Effects of substrate concentrations on asymmetric reduction of 2-HAP to (*S*)-PED by *C. parapsilosis.* Yield (□) and optical purity (○) of (*S*)-PED from 2-HAP catalyzed by *C. parapsilosis/*pCP-*scr*II; Yield (■) and optical purity (●) of (*S*)-PED from

2-HAP catalyzed by the wild-type strain;

Biotransformation conditions: 1 mL 0.2 M NaAC-HAC buffer (buffer pH 5.5) containing 100 g/L wet cells was shaking at 200 rpm and 35 ºC with various concentrations of 2-HAP (1−30 g/L) for 45 h.

Fig. 6. Preparative scale bioreduction of 2-HAP to (*S*)-PED by *C. parapsilosis*/pCP-*scr*II. (A) Retention times of standard samples: 2-HAP, 33.0 min; (*R*)-PED, 19.3 min; (*S*)-PED, 24.2 min; (B) Bioreaction products from whole-cell catalysis of *C. parapsilosis*/pCP-*scr*II monitored at UV 215 nm.

Fig.7. ^{1}H NMR analysis of (*S*)-PED from 2-HAP reduced by *C. parapsilosis*/pCP-*scr*II (A) ^{1}H NMR analysis of the standard sample; (B) ^{1}H NMR analysis of the purified product.

Notes: The samples were purified using preparative liquid chromatography. The purified product was applied for ^{1}H NMR analysis on a Bruker Aduance III 400 MHz spectrometer (400 MHz, $CDCl_3$).

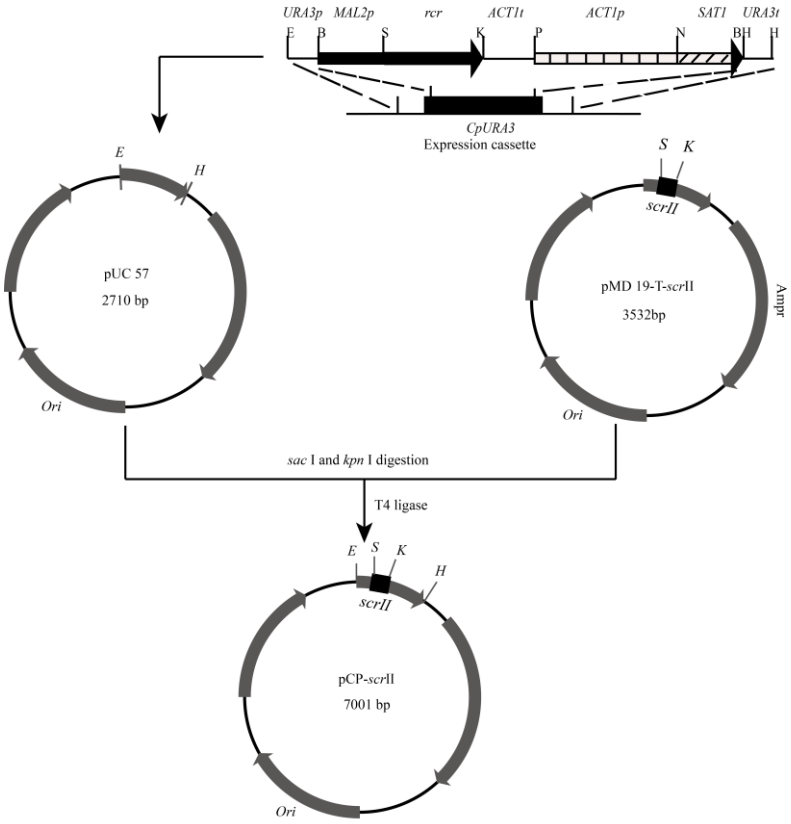


Fig. 1. Construction of an expression vector pCP-*scr*II for *C. parapsilosis*

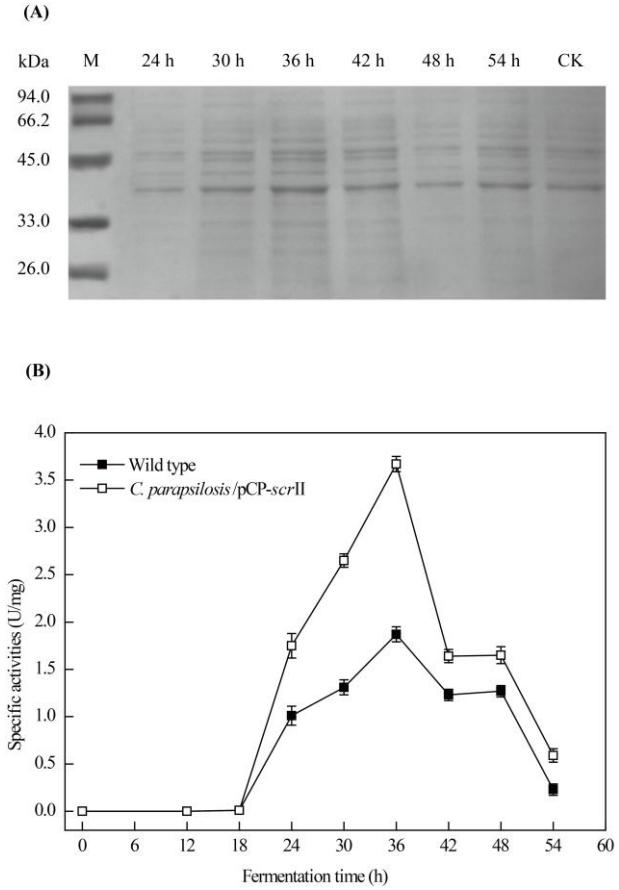


Fig. 2. Optimization of fermentation time for *C. parapsilosis*.

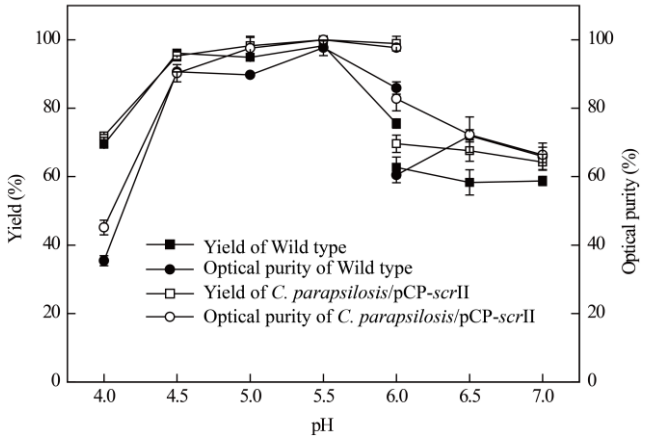


Fig. 3. Effects of pH on asymmetric reduction of 2-HAP to (*S*)-PED by *C. parapsilosis*.

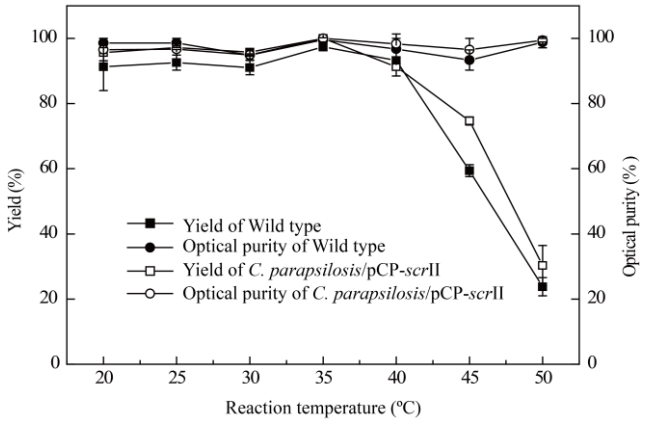


Fig. 4. Effects of temperatures on asymmetric reduction of 2-HAP to (*S*)-PED by *C. parapsilosis.*

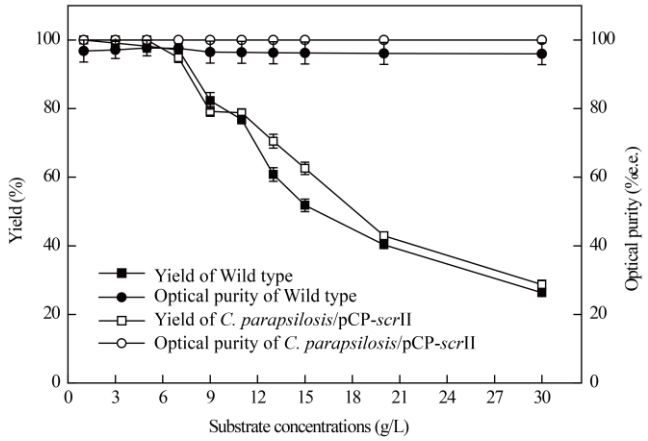


Fig. 5. Effects of substrate concentrations on asymmetric reduction of 2-HAP to (*S*)-PED by *C. parapsilosis.*

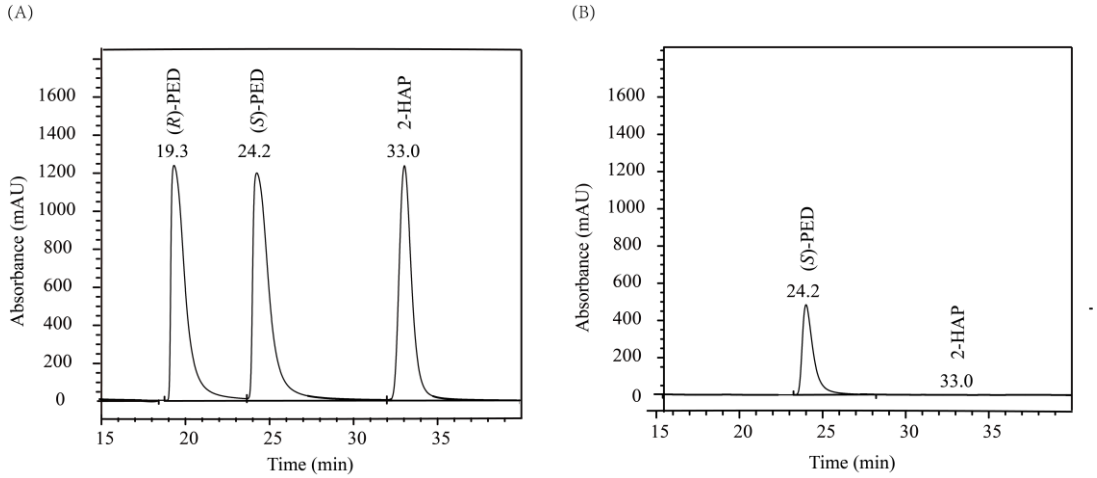


Fig. 6. Preparative scale bioreduction of 2-HAP to (*S*)-PED by *C. parapsilosis*/pCP-*scr*II.

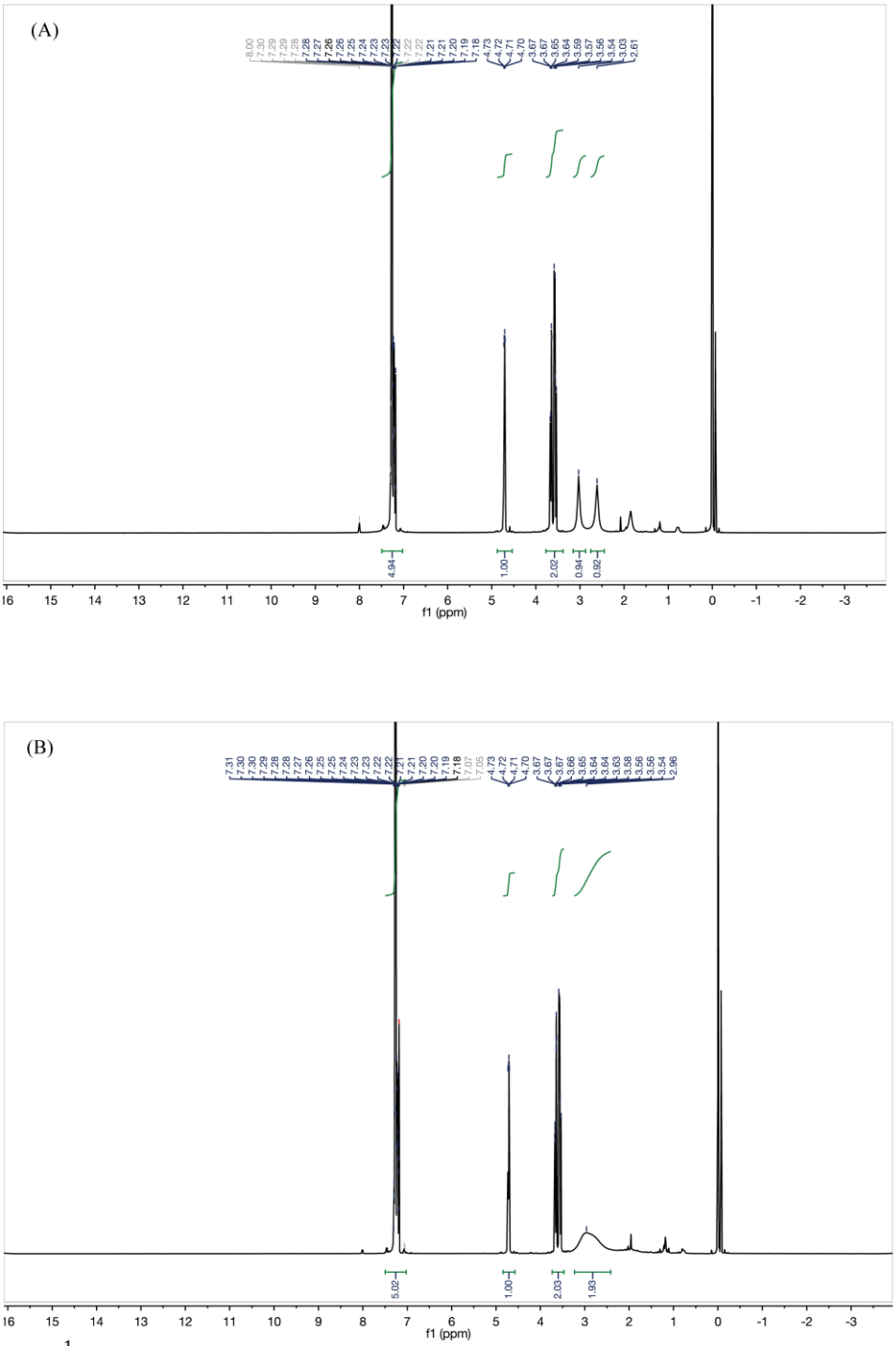


Fig. 7. ^{1}H NMR analysis of (*S*)-PED from 2-HAP reduced by *C. parapsilosis*/pCP-*scr*II.

Table 1. Strains, plasmids and synthetic oligonucleotides used in this study

Strains, plasmids and primers	Characteristics[a]	Source
Strains		
E. coli JM 109/ T-his$_6$-SCRII	*E. coli* JM 109 harboring T-his$_6$-SCRII	This work
C. parapsilosis	The donor of gene *scr*II	China center
C. parapsilosis/pCP-*scr*II	*C. parapsilosis* harboring heterologous gene *scr*II	This work
Plasmids		
pCP-RCR	The donor of vector pCP	This lab[b]
pMD19-T	Cloning plasmid, 2.7 kb, Amp^r	Takara Co.
T-his$_6$-SCRII	his$_6$-SCRII gene on pMD19-T, 3.5 kb	This work
pCP-*scr*II	*scr*II gene on pCP vector, 7.0 kb	This work
Primers	Sequence (5'→3')	
SCRII_*Sac* I_F1	ttcgggagctcatgcaccaccaccaccaccacggcgaaatcg aatcttatt (*Sac* I)	
SCRII_*Kpn* I_R1	cggggtaccctatggacaagtgtaaccacca (*Kpn* I)	
SAT1_1	cgataacggtgcggccgcatgaaaatttcggtga (*Not* I)	
URA3t_1	gatgacgcctaaggatccgaatatttaatcat (*Bam*H I)	
Mut_*Kpn* I_F2	atgtcgggcacaattgttaatgt	
Mut_*Kpn* I_R2	Acattaacaattgtgcccgacat	

[a]All the restriction sites of the primers were shown in table 1 in underlined letters.

[b]The plasmid pCP-RCR was constructed in this lab and the data are not shown and will be published.

Table 2. Reductive activities of cell-free extracts and asymmetric reductions of 2-HAP to (*S*)-PED by different *C. parapsilosis* strains.

Strains	Enzyme activities (U/mg)[a]	Optical purity (%e.e.)[c]	Yield (%)[c]
Wild-type *C. parapsilosis*	1.27±0.05	60.5±0.9	62.7±0.8
M-1[b]	0.89±0.04	77.3±1.1	63.7±1.0
M-2[b]	3.07±0.03	82.8±0.9	69.6±0.7

[a] Enzyme activities (U/mg) were measured at pH 6.0 of 0.2 M phosphate buffer.

[b] Recombinant *C. parapsilosis* strains containing exogenous gene *scr*II were selected from MD medium.

[c] Asymmetric reduction conditions: 1 mL 0.2 M phospate buffer pH 6.0, 100 g/L wet cells, 200 rpm, 35 ºC and 5 g/L 2-HAP for 45 h.

Table 3. Reductive activities of cell-free extracts and asymmetric reductions of 2-HAP to (*S*)-PED by different hosts under the optimized conditions.

Strains/Plasmids	Enzyme activities (U/mg)[a]	Optical purity (% e.e.)[b]	Yield (%)[b]	References
Wild-type *C. parapsilosis*	1.27±0.05	98.3±1.0	97.7±0.9	in this study
C. parapsilosis/pCP-*scr*II	3.07±0.03	> 99.9	> 99.9	in this study
E.coli BL21/pET28-SCRII	0.52±0.01	> 99.0	80.3±1.3	[16]

[a] Enzyme acitivities (U/mg) were measured at pH 6.0 of 0.2 M phosphate buffer.

[b] Asymmetric reduction conditions: 1 mL 0.2 M NaAC-HAC buffer (buffer pH 5.5), 100 g/L wet cells, 200 rpm, 35 ºC and 5 g/L 2-HAP for 45 h.