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Development of an improved phenylacetaldehyde reductase mutant by an efficient selection procedure

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Abstract Chiral alcohols are valuable as diverse chemicals and synthetic intermediate materials. Phenylacetaldehyde reductase (PAR) is an enzyme that converts a wide variety of ketones into chiral alcohols with high optical purity. When an alcohol such as 2-propanol is used as a hydrogen donor, PAR itself will also mediate the regeneration of the coenzyme NADH in situ. Perceiving a capacity for improvement, we sought to develop a PAR that is able to convert higher concentrations of substrates in the presence of high concentrations of 2-propanol. The selection procedure for mutants was re-examined and a procedure able to select an effective amino acid substitution was established. Two advantageous amino acid substitutions were successfully selected using the procedure. When high-concentration substrate conversion reaction was subjected with a mutant that integrated both the two amino acid substitutions, near-complete conversions of *m*-chlorophenacyl chloride (*m*-CPC) (2.1 mmol/ml) and ethyl 4-chloro-3-oxobutanoate (ECOB) (1.9 mmol/ml) were achieved.

Keywords Phenylacetaldehyde reductase (PAR) · *Rhodococcus* sp · Optically pure alcohols · Asymmetric reduction · Coupled NADH regeneration · Engineered enzymes · 2-Propanol

Introduction

Chiral alcohols are valuable as intermediates of various high value-added functional materials. One of the best methods for producing chiral alcohols is enzyme-catalyzed asymmetric reduction (Goldberg et al. 2007a; Goldberg et al. 2007b; Kroutil et al. 2004; Matsuda et al. 2009; Moore et al. 2007; Ni and Xu 2012). Various alcohol dehydrogenases and carbonyl reductases have been discovered and developed for production of chiral alcohols. Many of these enzymes have broad substrate specificity. The development of improved enzymes has facilitated the production of chiral alcohols.

Phenylacetaldehyde reductase (PAR) is an enzyme that converts various ketones into the corresponding alcohols (Itoh et al. 2002). PAR belongs to a medium-chain alcohol dehydrogenase family found in bacteria of the *Rhodococcus* strain ST-10 (Itoh et al. 2002; Itoh et al. 1997; Wang et al. 1999). PAR can convert diverse compounds, such as alkyl alcohols, aryl alcohols, and aromatic ketones (Itoh et al. 2012; Itoh et al. 2002; Itoh et al. 2007). In many cases, the product has very high optical purity. These characteristics promise a broad industrial application of PAR.

The advantage of PAR is its utility as an enzyme for coenzyme regeneration. PAR requires NADH as a coenzyme. When NADH is regenerated in a reaction solution, a continuous production of ketone is attained. Glucose dehydrogenases and formate dehydrogenases are often used for this purpose when other alcohol dehydrogenases are being used (De Wildeman et al. 2007). In a PAR-mediated reaction, if an alcohol different from the product is also present, NADH is regenerated by the reverse reaction (Fig. 1) (Itoh et al. 2002). 2-Propanol is inexpensive and suitable for coenzyme regeneration. Because the acetone generated by 2-propanol oxidation has a high K_m with PAR, its reduction can be disregarded in practice (Itoh et al. 2012; Itoh et al. 2002). Removing acetone from the solvent system by aeration can also be used to suppress the reverse reaction (Itoh et al. 2002).

Protein engineering of PAR to yield the Sar268 and HAR1 mutants has dramatically improved the conversion efficiency of substrates (Itoh et al. 2002; Makino et al. 2007; Makino et al. 2005). The improvements were due to multiple amino acid

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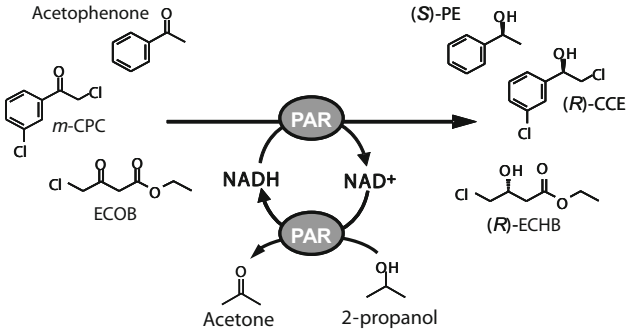

Fig. 1 Illustration of the asymmetrical reduction reaction catalyzed by PAR. Substrates and products catalyzed by PAR and their structures are shown. When 2-propanol is present in the reaction solution, NADH can be regenerated in situ because PAR also catalyzes the oxidation of 2-propanol

substitutions introduced in both mutants (Table 1). In the presence of high concentrations of 2-propanol, the reaction efficiency of the wild-type PAR falls markedly (Itoh et al. 2002; Makino et al. 2005). Even in reaction solutions containing 2-propanol at 2 M concentration, the Sar268 and HAR1 mutants can convert various ketones into chiral alcohols with high efficiency. When Sar268 and HAR1 were used, the optical purity of the products (*R*)-2-chloro-1-(3-chlorophenyl)ethanol (CCE), (*R*)-4-chloro-3-hydroxyoxybutanoate (ECHB), and (*S*)-*N*-Boc-3-pyrrolidinol (Boc-PL) was ≥99 % enantiomeric excess (ee), the same as those produced by the wild type.

If PAR is further improved, high value-added chiral alcohols will become available at a lower cost. In previous PAR engineering, only a fraction of the possible amino acid substitutions was examined. Because improvement was readily achieved, no comprehensive search of possible amino acid substitution was undertaken. Moreover, the Sar268 and HAR1 mutants were constructed by the method of combining advantageous mutations. In view of the non-additivity of point mutations, searching for an outstanding mutation combination is not sufficient. We accordingly anticipated that further improvement of PAR would be possible.

In this study, we attempted to further improve the Sar268 and HAR1 mutants with respect to conversion of the two target compounds. (*R*)-CCE is a chiral alcohol used as an intermediate material for drugs. PAR catalyzes the conversion of *m*-chlorophenacyl chloride (*m*-CPC) to (*R*)-CCE. (*R*)-ECHB is a valuable synthetic material for compounds such as (*R*)-carnitine and similarly is converted from ethyl 4-chloro-3-oxobutanoate (ECOB) by PAR. Here we discuss the improvement of conversion efficiency in comparison with other reported efforts.

Table 1 Amino acid substitutions found in the screened clones from a mutant library of wild-type PAR (pEAR2)

Clone	Amino acid substitution(s)
Sar268[a]	E12G, D42V, K67R, L125M, S173P, A327V
HAR1[a]	A3S, I4L, E12G, D42L, K67R, L125M, S173P, A327V
1B1	E14G[b], D42V
1A3	D42N[c], V259A[b]
2A2	E12G, T180S[b]
5B1	E12G, D315N[b]
6A4	T180I[b], N230S[b]
6D7	T180I[b]
7A5	D42G[c]
7A9	D42N[c]

[a] The mutations of Sar268 (Makino et al. 2005) and HAR1 (Makino et al. 2007) are shown for reference

[b] Newly found in this study

[c] Previously examined but not promising

Materials and methods

In general, the highest quality reagents available from Wako Pure Chemical Industries (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), or Sigma-Aldrich (MO, USA) were used. Unless otherwise noted, the procedures of genetic engineering and biochemistry followed Sambrook et al. (1989). The medium for cultivation was Luria–Bertani (LB) containing 100 μg/ml ampicillin. LB medium for induction contained 100 μg/ml ampicillin, 0.4 mM IPTG, and 0.01 % (*w*/*v*) $ZnCl_2$. Lysis solution contained 50 mM MOPS–NaOH buffer (pH 7.0), 1× CelLytic B (Sigma-Aldrich), and 0.2 mg/ml hen egg lysozyme. *Escherichia coli* JM109 (*endA1*, *recA1*, *gyrA96*, *thi-1*, *hsdR17* (r_k^-, m_k^+), *relA1*, *supE44*, Δ(*lac-proAB*)/F′[*traD36*, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]) was used as a host.

Library construction

pEAR2 is a plasmid for expressing wild-type PAR, with which a C-terminal polyhistidine tag was fused (Makino et al. 2005). The random mutation libraries based on pEAR2 were newly constructed or reported ones, for which the construction procedure was the same and the Mn^{2+} concentration for error-prone PCR was 0.5 mM (Makino et al. 2005). Construction of a random mutation library based on pHAR1 was performed as follows: First, the domain containing the HAR1 gene was amplified by error-prone PCR, for which the primers were PARSQ-R0 and PARSQ-F1 and the Mn^{2+} concentration was 0.25 or 0.5 mM (Makino et al. 2007). Then some of the PCR products were used as templates and PCR was performed using KOD-plus-DNA polymerase (Toyobo, Osaka, Japan), following the manufacturer's instructions. Primers HAR_IF_R1 (5′-CTGGAGAGTCTCATATGAAA TCATTACAATAT-3′) and HAR_IF_SfiI_F1 (5′-TGGTGG TGGTGGCCGGACAGGCC-3′) were used. PCR products were purified after gel electrophoresis. Primer extension was performed using the PCR product purified as a megaprimer

(Tseng et al. 2008). The composition of the PCR reaction solution corresponds to the method for KOD-plus-DNA polymerase, to which 1 ng of pHAR1 and 2.5 μg of purified PCR product were added. The thermocycling program was 94 °C for 2 min, followed by 25 cycles of 94 °C for 30 s, 58 °C for 15 s, and 68 °C for 4 min, and a final extension for 5 min at 68 °C. The pHAR1 template was then digested with restriction enzyme *Dpn*I (Thermo Fisher Scientific, Kanagawa, Japan), and then *E. coli* JM109 was transformed. Approximately 2,000 colonies appeared at either 0.25 and 0.5 mM Mn^{2+} concentration.

Colony screening

A library was diluted, spread on cultivation medium containing 1.5 % (*w*/*v*) agarose, and left at 37 °C overnight and independent colonies were obtained. Colonies were transferred to a nylon membrane (Biodyne A, 0.45 μm, PALL, NY, USA). On the induction medium containing 1.5 % (*w*/*v*) agarose, a membrane was placed such that the transfer side faced upward. After incubation at 30 °C overnight, membranes were placed on the filter paper containing lysis solution such that colonies faced upward and were allowed to stand at room temperature for 30 min. Debris was flushed away with water and membranes were soaked in activity-staining solution containing 1 mM NAD^+, 50 mM MOPS-NaOH (pH 7.0), 200 μM 5-bromo-4-chloro-3-indolyl phosphate, 10 μM 1-methoxy PMS (Dojin Laboratories, Kumamoto, Japan), 40 mM DL-1-phenylethanol, and 20 % (*v*/*v*) *n*-propanol. They were left for 30 min at room temperature and the colonies with the deepest coloring were selected visually.

Activity measurement on microwell plates

E. coli clones were inoculated into microwells with 100 μl of induction medium. The microwell plates were rotary shaken at 1,100 rpm and 30 °C with a Deep Well Maximizer (Taitec, Saitama, Japan) for 19 h. The plate was centrifuged at 4,000 rpm for 10 min at 4 °C and the supernatants were aspirated. Lysis solutions were added at 100 μl per well, and the plate was agitated at 1,100 rpm and 30 °C for 20 min. After centrifugation at 4 °C at 4,000 rpm for 20 min, the supernatants were transferred to another plate. The supernatant (20 μl) and 180 μl of activity measurement solution were mixed, and change of absorption at 340 nm was measured with a microplate reader (Viento XS, Dainippon Sumitomo Pharma, Osaka, Japan). Compositions and final concentrations of each solution for activity measurement were as follows: The solution for acetophenone activity measurement consisted of 3 mM acetophenone, 0.27 mM NADH, 50 mM MOPS–NaOH (pH 7.0), and 10 % (*v*/*v*) *n*-propanol (Note: *n*-propanol is not catalyzed by PAR or its mutants). The solution for DL-1-phenylethanol activity measurement consisted of 40 mM DL-1-phenylethanol, 1 mM NAD^+, 50 mM MOPS–NaOH (pH 7.0), and 10 % (*v*/*v*) *n*-propanol. The solution for 2-propanol activity measurement consisted of 2-propanol, 1 mM NAD^+, and 50 mM MOPS–NaOH 10 % (*v*/*v*) (pH 7.0).

m-CPC conversion reaction

m-CPC conversion reaction and analysis were conducted following Makino et al. (2007). In brief, a colony was first inoculated into 1 ml of induction medium in a 2-ml tube, and rotary culture was performed at 30 °C and 1,800 rpm for 22 h. After centrifugation and removal of the supernatant, 50 mM MOPS–NaOH (pH 7.0) and 1 mM NAD^+ solutions were added. Then, 2-propanol in which *m*-CPC was dissolved was added. For the reaction system containing *m*-CPC at a final concentration of 1 % (*w*/*v*), 100 μl of 5 % (*w*/*v*) *m*-CPC dissolved in 2-propanol was added to 400 μl of MOPS–NAD^+ solution. For a 5 % (*w*/*v*) final concentration of *m*-CPC, 125 μl of 20 % (*w*/*v*) *m*-CPC in 2-propanol was added to 375 μl of MOPS–NAD^+ solution. For a 6.7 % (*w*/*v*) final concentration of *m*-CPC, 167 μl of 20 % (*w*/*v*) *m*-CPC in 2-propanol was added to 333 μl of MOPS–NAD^+ solution. Reaction solutions were rotary-mixed at 30 °C and 1,800 rpm for 22 h. Reaction solutions extracted with ethyl acetate were analyzed by gas chromatography equipped with a chiral column.

Site-specific mutation introduction by inverse PCR

Site-specific mutagenesis using inverse PCR followed Makino et al. (2007), where the template was pSar268, pHAR1, or their mutants. The primer sequences used for introduction of each amino acid mutation were as follows: The pair for the E14G mutant was 5′-GGAATCTCCGTGAG TCCGGG-3′ and 5′-CAAACCCGAGCCCGGTCCAG-3′. The pair for the T180S mutant was 5′-CGAGACCGCCGG AACCAATG-3′ and 5′-GCCACGTCGCTATTCAGCTC-3′. The pair for the T180I mutant was 5′-CGAGACCGCCGATA CCAATG-3′ and 5′-GCCACGTCGCTATTCAGCTC-3′. The pair for the N230S mutant was 5′-CGTCCTTGTCGGACAG AACC-3′ and 5′-CGGCCGAGAGCGTCCGCAAG-3′. The pair for the V259A mutant was 5′-CGACGCCGGCGGCA GCCATC-3′ and 5′-GATCAGACGTCACGATCGTC-3′. The pair for the D315N mutant was 5′-CGGCGTGGGCGAGG TCGATC-3′ and 5′-GCATCTTCAACATCTCGGTG-3′.

Conversion tests of high-concentration *m*-CPC and ECOB

The *E. coli* with pHAR1 or its mutant was shaken at 121 rpm and 37 °C for 22 h in LB medium containing 100 mg/ml ampicillin, 0.4 mM IPTG, and 0.01 % (*w*/*v*) $ZnCl_2$. Cells were collected by centrifugation, leaving 20 mg per tube. For *m*-CPC, 500 μl of 1 mM NAD^+, 50 mM MOPS–NaOH

(pH 7.0), and 20 % (*v*/*v*) 2-propanol and 200 mg of *m*-CPC powder were added in each tube. For ECOB, 1 mM NAD^+ and 50 mM MOPS–NaOH (pH 7.0), and 20 % (*v*/*v*) 2-propanol and 125 or 150 μl of ECOB, were added to a total of 500 μl. For the two-phase reaction, 500 μl of *n*-octane was added to the reaction solution. The reaction solution was rotary-mixed at 30 °C and 1,800 rpm for 22 h. The solution was then extracted with ethyl acetate and analyzed by gas chromatography on a chiral column. The optical purity of ECHB was analyzed by HPLC equipped with a chiral column. The GC and HPLC analyses were performed as described previously (Makino et al. 2007).

Nucleotide sequence accession number

The nucleotide sequence encoding wild-type PAR has GenBank/EMBL/DDBJ accession number AB190261 (Makino et al. 2005).

Results

Improvement of the screening procedure

The previous mutant selection method offered scope for improvement in efficiency (Makino et al. 2007; Makino et al. 2005). The previous method was as follows: First, colony screening to evaluate the generation of NADH accompanying 2-propanol oxidization was performed. Then, the selected clones were further screened for *m*-CPC conversion ability. Given that NADH is easily detectable by a colorimetric reaction, 2-propanol oxidization activity can be measured in high throughput. 2-Propanol is a substrate molecule that is smaller than *m*-CPC, and the reaction is not reduction but oxidization. However, it is possible to select mutants with activity under circumstances in which almost all clones have lost activity by mutation. Moreover, mutants for which activity is maintained in the presence of high concentrations of 2-propanol can be identified. High-throughput screening of the ketone reduction reaction is difficult, because analysis such as by HPLC is required.

We accordingly resolved to change the selection strategy of clones. DL-1-Phenylethanol and the acetophenone described below are an aryl alcohol and ketone to which the molecular structure is similar when *m*-CPC is used as the target substrate. We considered it more reasonable to evaluate activity using acetophenone or DL-1-phenylethanol. First, colony screening using DL-1-phenylethanol was attempted. To test the influence of a polar organic solvent, a high concentration of *n*-propanol [20 % (*v*/*v*)] was provided. The mutants of wild-type PAR, evaluated for *m*-CPC conversion ability, were utilized (Makino et al. 2005). After transferring these to a membrane and holding on the induction plate, these were evaluated by colony screening. The clones with high *m*-CPC conversion ability formed a strong color (data not shown). Thus, colony screening using DL-1-phenylethanol as a substrate was judged to be valid and was adopted.

We then investigated a procedure allowing high-throughput selection using acetophenone, DL-1-phenylethanol, or 2-propanol as a substrate after colony selection. Approximately 700 colonies obtained from the mutant library based on pEAR2 were examined, and 80 colonies with strong coloring were chosen visually. These were further cultivated on the microplate and cells were centrifuged from an equivalent amount of media. Activity was measured on the microplate using acetophenone, DL-1-phenylethanol, or 2-propanol as substrate. When DL-1-phenylethanol or acetophenone was used as substrate, a high concentration of *n*-propanol was also added. Twelve highly active clones were chosen for each substrate. The *m*-CPC conversion ability of these clones was also examined separately. The correspondence of the activity on each substrate with *m*-CPC conversion activity is shown in Fig. 2. In two clones with high conversion activity for *m*-CPC, conversion activity for DL-1-phenyl ethanol and an acetophenone was also high (Fig. 2a, b). We inferred that choosing clones that were highly active on DL-1-phenylethanol or acetophenone may yield clones with high *m*-CPC conversion activity. In practice, because reduction activity was screened, it was judged that acetophenone was more suitable. Incidentally, the 2-propanol oxidation activity of these two clones was also low (Fig. 2c). Thus, the possibility that low 2-propanol oxidation activity would be correlated with high ketone reduction activity was also expected. However, this correlation was not adopted as a selection procedure because a drop in enzyme activity caused by mutation cannot be distinguished from low activity.

Selection of the mutants by the new screening method

Based on the above results, we designed the following new selection system. An outline is shown in Fig. 3. First, activity on DL-1-phenylethanol is screened on a membrane (Fig. 3, steps 1–4). Selected clones are then further screened for acetophenone reduction activity (Fig. 3, steps 5–6). Finally, conversion of target substrates, such as *m*-CPC, is investigated for the selected clones (Fig. 3, step 7).

In order to determine whether this flow was actually effective for selection, a mutant library of wild-type PAR with a polyhistidine tag was screened. Eight clones showing improved conversion of 5 % (*w*/*v*) *m*-CPC were found. The amino acid substitutions of the eight clones are shown in Table 1. Many amino acid substitutions found previously, part of which had been verified, were discovered, supporting the effectiveness of the new screening method. Six new amino acid substitutions were also found, among which may be substitutions that improved conversion activity.

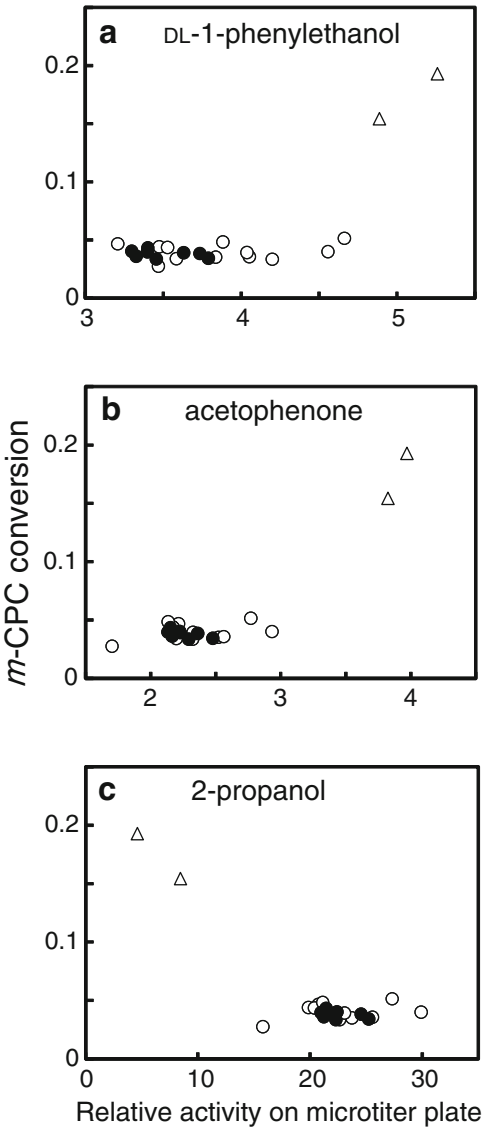

Fig. 2 Enzyme activity and *m*-CPC conversion activity of clones. The activity measured on the microplate with each substrate is shown on a *horizontal axis*. The conversion rate of 1 % (*w*/*v*) *m*-CPC is shown on a *vertical axis*. The conversion rate of *m*-CPC is derived from GC analysis by the ratio of the peak area of (*R*)-CCE to the sum of the areas of *m*-CPC and (*R*)-CCE. (Note: *m*-CPC and products other than (*R*)-CCE were not detected by the analysis.) The *closed circle* indicates eight separate colonies of *Escherichia coli* with pEAR2. The *open marker* (*circle* or *triangle*) indicates the 12 mutants that showed the highest activity on each substrate, and the two clones with the highest *m*-CPC conversion are indicated as *open triangle*. The substrates analyzed with the microplate are DL-1-phenylethanol (**a**), acetophenone (**b**), and 2-propanol (**c**)

With the aim of validating amino acid substitution, each amino acid was individually introduced into the Sar268 mutant and *m*-CPC conversion activity was examined. In the reaction solution containing 5 % (*w*/*v*) *m*-CPC, only the T180I mutation improved conversion activity, showing 1.3 times the activity of Sar268.

Because it was now evident that the selection system worked, a mutation library of HAR1 was constructed and screened similarly. The conversion test was performed with 6.7 % (*w*/*v*) *m*-CPC. As a result, the outstanding mutant 1D5 was selected. This mutant had a P173S amino acid substitution. It also had a synonymous mutation, of which the 351th cytosine was replaced with thymine. P173S was the back mutation to the wild-type from the mutation already introduced into the HAR1 and Sar268 mutants. The HAR1 mutant was constructed by simple integration of advantageous mutations (Makino et al. 2007). The back mutation to the wild-type from P173 may have become advantageous owing to the interaction between amino acid substitutions. This mutant was accordingly used for the subsequent validation.

The conversion test of *m*-CPC and ECOB with a combination mutant

Combinations of the discovered mutations were introduced into pHAR1. The 1D5 mutant and pHAR1 into which T180I was introduced showed *m*-CPC conversion activity higher than that of pHAR1 (Fig. 4). However, activity was the highest in the mutant that integrated both the mutations. This integrated mutant is called HAR11 hereafter and the plasmid encoding it is called pHAR11.

In view of these results, the ability of combination mutants to convert high-concentration *m*-CPC and ECOB was investigated. First, 200 mg of *m*-CPC powder was added to 0.5 ml of reaction solution, and conversion to (*R*)-CCE was investigated. Whereas pHAR1 gave 88.4 % yield (by GC peak area), pHAR11 gave 98.2 % yield. The optical purity of (*R*)-CCE produced by each was >99 % ee. The conversion to (*R*)-ECHB of a 500-μl reaction solution containing 125 μl of ECOB was also examined. The molar yields were 76.7 % with pHAR1 and 99.0 % with pHAR11. The optical purity of produced (*R*)-ECHB was >99 % ee with either mutant. No by-product was formed with both substrates, as confirmed by GC analysis employing internal standards.

Discussion

The efficient PAR mutant HAR11, which converts *m*-CPC into (*R*)-CCE and ECOB into (*R*)-ECHB, was developed. Stereoselectivity was not reduced in the newly developed mutant. The very high optical purity of the products simplifies the separation of optical isomers. Reactivity under high concentrations of 2-propanol was also satisfactory. This property is advantageous for regeneration of NADH in situ, promotion of substrate dissolution, and implementation of cell permeability. The production of (*R*)-CCE or (*R*)-ECHB with high optical purity can thus be accomplished more cheaply and efficiently.

A high-throughput mutant selection system employing a series of screens has been constructed. By the use of the new

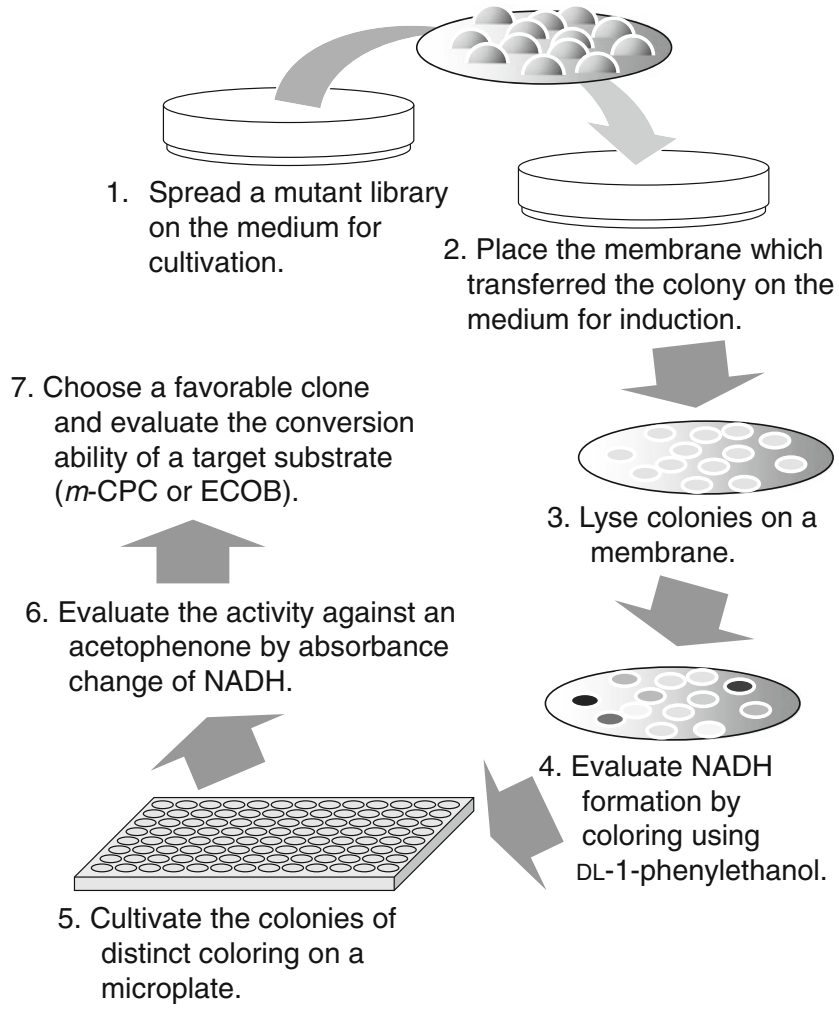

Fig. 3 Schematic illustration of the new selection procedure

membrane screening method, the number of candidate clones can be reduced. This reduction permitted evaluation on a microplate exploiting the correlation with *m*-CPC conversion and left GC or HPLC analyses as reasonably insignificant bottlenecks. In this manner, we achieved the selection of active clones with higher throughput and higher accuracy. In a previous report, a library based on pEAR2 was selected, 320 clones were examined for the *m*-CPC conversion reaction, and four improved mutants were obtained (Makino et al. 2005). In this report, 62 mutants were examined for the *m*-CPC conversion reaction and eight improved mutants were successfully obtained from the same library. In addition, from a library based on pHAR1, eight mutants were examined for the *m*-CPC conversion reaction and one improved mutant was obtained. Thus, the time-consuming *m*-CPC conversion assay was reduced, leading to an increase in screening efficiency.

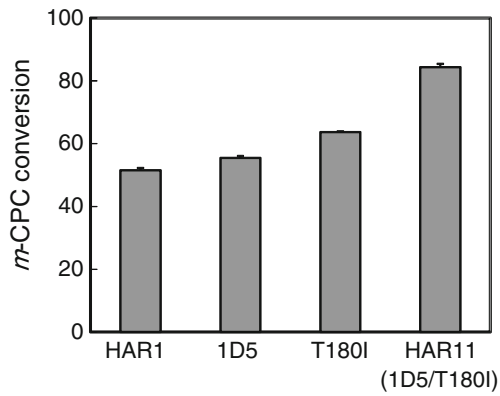

Fig. 4 *m*-CPC conversion ability of combinatorial mutants. 1D5 is a clone of the pHAR1 mutant. T180I corresponds to a HAR1 clone with a T180I amino acid substitution. 1D5/T180I, also named as HAR11, denotes a 1D5 mutant into which the T180I amino acid substitution was introduced. The conversion rate of 5 % (*w*/*v*) *m*-CPC is indicated on a *vertical axis*. Calculation of the conversion rate is the same as for Fig. 2

The detailed mechanism underlying *m*-CPC conversion ability by acetophenone in preliminary screening is unknown. Numerous preliminary screening using the *m*-CPC itself has been attempted separately. However, correlation was not observed in the result of the activity measurement in a microwell plate and a high-concentration *m*-CPC conversion test (data not shown). On the other hand, by this selection system, there was a tendency that the highly active clones by preliminary

screening using acetophenone had also shown high conversion of concentrated *m*-CPC (data not shown). In addition, the same amino acid substitutions as in a previous selection without preliminary screening were found again by this selection system (Makino et al. 2005). Therefore, although it could not necessarily explain rationally from the substrate structures, the constructed selection procedure was found to be valid.

Although only activity on *m*-CPC was screened, conversion activity on ECOB was also excellent. The introduced mutation appears to have had little or no influence on substrate specificity. Although an extensive conversion test using 30 mg of cell content was employed in the previous study, the cell content was reduced to 20 mg in the present study (Makino et al. 2007). Still, extensive conversion was achieved; 400 mg (2.1 mmol) of *m*-CPC per milliliter reaction solution can be converted almost completely by pHAR11. To our knowledge, a conversion of this level of completeness is not found elsewhere. Kataoka et al. reported the production of 268 mg/ml (91.7 % ee) ECOB in the organic phase using the enzyme from *Suillus salmonicolor* in a two-phase system (Kataoka et al. 1999). We previously reported the conversion of a maximum of 140 mg/ml (>99 % ee) using pHAR1 in a two-phase system. When we attempted conversion without adding the *n*-octane organic phase, the conversion rate remained very high and the conversion profile was hardly changed by the absence of the organic phase (data not shown). Systems that do not require an organic phase afford desirable advantages in efficiency, cost, and environmental load. From 150 μl of ECOB, (*R*)-ECHB of 341 mg (2.04 mmol) per milliliter was formed at a molar conversion yield of 92.4 and >99 % ee. Although this yield was inferior, from 125 μl of ECOB, (*R*)-ECHB of 319 mg (1.92 mmol) per milliliter was formed at 99.0 % of the molar conversion rate. In this case, purification processes would be simplified by the nearly complete conversion. In general, a system for efficient production of (*R*)-ECHB has been developed.

In conclusion, a low-cost and efficient bioprocess has been rendered feasible by the development of a novel mutant. Exploiting the broad substrate specificity of the mutant can be a promising application in the production of other chiral alcohols. We are now investigating other practical uses for the mutant.

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Conflict of interest The authors declare that they have no conflict of interest.

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