Title:

Computational design of stable, soluble and highly active alcohol dehydrogenase for NADPH regeneration

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Abstract:

Nicotinamide adenine dinucleotide phosphate (NADPH), as a well-known cofactor, is widely used in the most of enzymatic redox reactions, playing an important role in industrial catalysis. However, the absence of a comparable method for efficient NADP$^+$ to NADPH cofactor regeneration radically impairs efficient green chemical synthesis. Alcohol dehydrogenase (ADH) enzymes, allowing the \textit{in situ} regeneration of the redox cofactor NADPH with high specific activity and easy by-product separation process, are provided with great industrial application potential and research attention. Accordingly, herein a NADP$^+$ specific ADH from \textit{Clostridium beijerinckii} was selected to be engineered for cofactor recycle, using an automated algorithm named Protein Repair One-stop Shop (PROSS). The mutant CbADH-6M exhibited a favorable soluble and highly active expression with an activity of 46.3 U/mL, which was 16 times higher than the wild type (2.9 U/mL), and a more stable protein conformation with an enhanced thermal stability: $\Delta T_{1/2}^{60\text{min}} = +3.6^\circ\text{C}$ (temperature of 50% inactivation after incubation for 60 min). Furthermore, the activity of CbADH-6M was up-graded to 2401.8 U/mL by high cell density fermentation strategy, demonstrating its industrial potential. Finally, the superb efficiency for NADPH regeneration of the mutant enzyme was testified in the synthesis of some fine chiral aromatic alcohols coupling with another ADH from \textit{Lactobacillus kefir} (LkADH).

Keywords:

Alcohol dehydrogenase, Computational design, Solube expression, NADPH regeneration, chiral alcohols
INTRODUCTION

The ability of enzymes to operate simply in aqueous systems in a highly efficient manner makes them attractive environmentally benign synthetic reagents. However, many classes of biocatalysts are not fully exploited, and their use in the large-scale enzymatic synthesis of high added-value chemicals is often limited by the need for expensive cofactors. Typical cofactor dependent enzymes are oxidoreductases, which represent some 25% of all known enzymes (Liu & Wang, 2007), catalyzing about 30% of the biotransformations in industry (Straathof et al., 2002), and the vast majority are dependent on one of the two nicotinamide cofactors NADH or NADPH. Although this two cofactors differ only by the 2’-phosphate group that is attached to the adenine ribose in NADPH, they play a completely different function in nature. NADH is used almost exclusively for oxidative degradations that eventually lead to production of ATP, whereas NADPH is confined with few exceptions to the biosynthetic reactions (Carugo & Argos, 1997), involving a spectrum of over 300 known, repeatedly-used reaction types (Woodyer et al., 2005), e.g. C-H oxygenation (Landwehr et al., 2006), regioselective halogenation (Mori et al., 2019), Baeyer-Villiger oxidation (Schmidt et al., 2015), stereoselective reduction (Zhu & Hua, 2006) and reductive amination (Yin et al., 2020) (Fig. 1). Therefore, it is necessary to develop an efficiently applicable technique for the in situ regeneration of NADPH to fulfill “green” chemical synthesis.

Figure. 1. Biosynthetic reactions using NADPH
Currently, numerous chemical, electrochemical, photochemical and biochemical methods have been developed to regenerate NADPH (Brown et al., 2016; Fukuzumi et al., 2019; Wichmann & Vasic-Racki, 2005). Most chemical routes are hindered by cumbersome reaction conditions, low turnover number (TTN), expensive and/or toxic reagents, and/or unwanted side products, and therefore have been not preferred for commercial or preparative applications. Biochemical methods using enzyme as catalyst have been demonstrated to be more efficient and applicable (Huisman et al., 2010), including the use of glucose dehydrogenase (GDH) (Weckbecker & Hummel, 2005), glucose-6-phosphate dehydrogenase (G6PDH) (Zhang et al., 2006), alcohol dehydrogenase (ADH) (Bastos et al., 1999), phosphite dehydrogenase (PDH) (Johannes et al., 2007a), formate dehydrogenase (FDH) (Celik et al., 2014) and hydrogenase (Fan et al., 2020). GDH and G6PDH with an advantage of high specific activity (200-500 U/mg at 25-30°C) (Ding et al., 2011; Wennekes et al., 1993) suffer from high co-substrate consumption and by-product separation cost. On the contrary, PDH and FDH using cheap co-substrate to regenerate NADPH without by-product problem, possess very low specific activity (FDH, 5-20 U/mg at 25-30°C; PDH, 5 U/mg at 25°C) (Vázquez-Figueroa et al., 2007a). Additionally, because of significantly lower stability and activity, the biotechnological applications of hydrogenases are still in their infancy (Fan et al., 2020). Nevertheless, ADH with a comparable specific activity (140-170 U/mg at 25-40°C) (Peretz et al., 1997; Widdel & Wolfe, 1989) and easy by-product separation process when using isopropanol as cheap H-donor (Fig. 1), is therefore of considerable commercial interest as a catalyst for NADPH regeneration in the synthesis and/or biotransformation of valuable compounds.

However, the absence of industrializable ADH as an excellent NADPH regenerator radically impairs the development of green chemical industry, since almost all the research and development of NADPH dependent ADH have been applied to the synthesis of chiral alcohols (Benitez-Mateos et al., 2017; He et al., 2015; Itoh, 2014). Although some ADHs can catalyze the synthesis of product and the regeneration of cofactor simultaneously in a substrate-coupled system, the activities of the two reactions hardly coordinate, not to mention the competitive inhibition of substrates.
Moreover, advanced enzyme engineering technologies are used to improve ADH performances that are optimized to the chemical manufacturing process, such as substrate specificity, enantioselectivity and catalytic activity, no exclusively NADPH regenerating ADH was explored.

Here an ADH from *Clostridium beijerinckii* (CbADH) was identificated with excellent thermostability and high specific activity for NADPH regeneration. Aiming to improve the poor heterologous expression level of CbADH (Peretz et al., 1997), an automated structure- and sequence-based computational protein redesign, namely Protein Repair One-Stop Shop (PROSS) (Goldenzweig et al., 2016), was applied to modificate wild CbADH to obtain better mutants that could satisfy laboratory and even industrial application. Furthermore, a two-phase high cell density fermentation strategy was explored to the large-scale production of the best mutant, to demonstrate its industrial potentiality. Last but not least, we showed the enzyme’s efficacy at *in situ* NADPH regeneration in the synthesis of some fine chiral aromatic alcohols coupling with anther engineered ADH from *Lactobacillus kefir* (LkADH) (He et al., 2015).
Materials and methods

Microorganisms and plasmids

E. coli BL21 (DE3) as a host bacteria for recombinant expression was preserved by our laboratory. The plasmid pET-28a (with N-terminal His-tag fused) carrying the TgADH, CbADH, TbADH, TeADH, EhADH and LkADH gene and the primer synthesis and sequencing were all completed by Beijing Tsingke Biological Technology Company.

Culture and induction conditions

The recombinant E. coli cells were first cultured for 6-8 hours at 37℃ in 5 mL Luria-Bertani (LB) medium supplemented with 50 μg/mL kanamycin. Then transformed into flask (50 mL LB) at 37℃ until the OD600 reached 0.4–0.6. Cells were induced at 18℃ for 16h by addition of isopropyl-β-D-thiogalactopyranoside (IPTG, 0.5 mM).

Construction of PROSS mutants

The CbADH related parameters and protein crystal structure were submitted online on a dedicated web server (http://PROSS.weizmann.ac.il). The mutant proteins were constructed through whole gene synthesis, and expressed in Escherichia coli BL21. All sequences were verified by DNA sequencing at Beijing Tsingke biological technology Co.Ltd.

Enzyme assay

The induced cells were harvested by centrifugation and washed three times using deionized water. Finally, the harvested cells were resuspended in 0.1 M phosphate buffer (pH 7.5) and disrupted ultrasonically. The resultant slurry was used for enzyme assay.

The standard assay mixture (1 mL) consisted of 0.1 M phosphate buffer (pH 7.5), 50 mM isopropyl alcohol, 1 mM NADP+, and enzyme. The substrate and coenzyme
solution were incubated in a metal bath at 35℃ and 650rpm for 10 min. Once the enzyme solution was added, the reaction solution was scanned at 340nm by spectrophotometer for 60 second, and the change in absorbance was recorded to calculate the enzyme activity. One unit of enzyme activity was defined as the amount of enzyme required to catalyze the formation of 1 μmol NADPH per minute.

SDS-PAGE and protein concentration assays

The expression and purification of the recombinant ADHs were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12%) with a 5% stacking gel. The gels were stained with Coomassie Brilliant Blue G-250. Protein concentrations were determined using a Bradford protein assay kit (Quick Start™, Bio-Rad, USA).

Purification of recombinant protein

The collected cells were washed and resuspended in Ni-0-native buffer (20 mM sodium phosphate, pH 7.5, containing 500 mM NaCl). Resuspended cells were disrupted by ultrasonication in an ice bath, followed by centrifugation at 12000 × g for 30 min to discard cell debris. The supernatant was loaded onto a Ni-NTA column (Thermo Scientific, USA) pre-equilibrated with Ni-0-native buffer, and the proteins were eluted by an increasing gradient of imidazole (from 50 to 250 mM). The purities of the collected fractions were analyzed by SDS-PAGE. Fractions containing the pure target protein were gathered and desalted by ultrafiltration. The purified proteins were concentrated and stored in 20% (v/v) glycerol at −80℃ until further use.

Characterization of mutant CbADH-6M

Optimum temperature and temperature stability: The temperature dependence was determined over the range 15–75℃. The mixture except for enzyme was preincubated for 10 min at a serious of temperature, and the reaction was initiated by the addition of enzyme liquor. The thermal stability was measured through determination of $T_{50}^{60}$, the
temperature where the enzyme activity is reduced to 50% of its initial activity after incubation for 60 min.

Optimum pH and pH stability: The optimum pH was determined at 35°C using different buffer systems to cover the pH scale: Phosphate buffer (pH 6.0-7.5), Tris-HCl buffer (pH 7.5-9.0), Glycine-NaOH buffer (pH 9.5-11.0), Na2HPO4 buffer (pH 11.0-13.0). The pH stability was measured by incubation of purified enzyme in buffer with different pH for 24 h at 35°C, and the residual activity was then measured by standard assay.

High-density fermentation

Single colonies were selected from the plate and incubated in 50 mL fermentation medium for primary seeds, and cultured overnight at 37°C, 200rpm. Then transferred to 500 mL fermentation medium for primary seeds and cultured at 200 rpm, 37°C for 4h. The secondary seed culture solution was inoculated in a 15 L fermentation tank under aseptic conditions, cultured at 37 °C for 10h and then cooled to 22°C for induction. The pH was controlled at 7.0 through the adjustment of ammonia, additionally the dissolved oxygen was controlled at about 30%. Feeding operation was after batch fermentation and the rate was regulated according to the fermentation parameters.

HPLC analysis and detection

The conversion of ketones and the enantiomeric excess of chiral alcohol products were detected with a normal-phase chiral column (CHIRAL PAK, ZWIX(-), 3 μm, 4 × 150 mm) using a FL-2200 HPLC system (Fuli Analytical Instrument Co., Ltd., China). The flow rate was maintained at 0.6 mL/min, with detection at 230 nm, and the column
temperature was constant at 25 °C, applying isocratic elution of n-hexane/isopropanol/trichloroacetic acid (90/10/0.1, v/v/v).
Results and discussion

Alcohol dehydrogenase library construction and activity identification

So far, NADPH-specific ADH (EC 1.1.1.2) from different sources has been reported a lot according to the enzyme database BRENDA, but the high-activity ADH is not much. Thus, an ADH library was constructed by selecting the one with specific activity exceeding about 10 U/mg protein (Table S1). However, some members of this group (though highly-active) are not appropriate as NADPH regenerator, due to their function of oxidization only primary alcohols that will be transformed to aldehydes to vitiate enzyme proteins. The others that can act also on secondary alcohols were selected to further investigate. Soluble expression levels and NADPH regenerating activities (per volume of fermentation broth) of these secondary ADHs were then tested except for MtADH (Fig. S1), which necessitates unavailable F420 as cofactor (Widdel & Wolfe, 1989). All the left six enzymes were successfully expressed in E. coli (Fig. S2) and considerable activities of TgADH, CbADH, TbADH and LkADH were obtained (Fig. 2A). Among them, TgADH exhibited a highest activity but a vulnerable stability in atmosphere, and LkADH was also demonstrated to be unstable than CbADH (Fig. 2B). CbADH showed the second highest activity (2.9 U/mL) with a soluble enzyme protein ratio of 25.1%, which was the lowest and less than a quarter of TbADH (96.9%), indicating that the specific activity of CbADH was supreme. Considering the activity and stability, CbADH was testified to be the best choice for NADPH regeneration, despite that the heterogenous expression of CbADH urgently needed to be improved.

Figure 2. Enzyme activity of alcohol dehydrogenase (A) and stability in atmosphere (B)
Culturing conditions optimization for CbADH expression

The most conventional and simple means to improve the heterogenous protein expression is to balance the target protein biosynthesis and the cell growth by optimizing the induction temperature and the concentration of the inducer IPTG (Donovan et al., 1996). However, the effect of this conditional optimization strategy on the expression of CbADH was not obvious. With the increase of IPTG concentration, the total enzyme activity increased slightly, but the proportion of soluble enzyme protein decreased (Fig. 3A and Fig. S4). Additionally, the induction temperature had little influence on the activity and soluble enzyme-protein ratio (Fig. 3B and Fig. S3). The maximum enzyme activity was only 3.7 U/mL with the lowest soluble target protein ratio of 16.8%, achieved at induction temperature 25°C and IPTG dosage 1 mM.

Figure 3. Effects of induction conditions on enzyme activity. (A) Induced by different IPTG concentration (B) Induced at different temperature
Improving CbADH solubility by chaperone buffering

On the other hand, chaperones assist protein folding was another frequently-used approach for overcoming the questions of low soluble expression in overexpression systems (Balbás, 2001). Therefore, five commercial chaperones were co-expressed with CbADH respectively, and the results showed that GroEL/ES preferably improved the soluble expression of CbADH with an activity of 14.1 U/mL and a soluble target protein ratio of about 59.6% (Fig. S5 and S6). Subsequently, the chaperone co-expression was optimized, results being shown in our another work (Deng et al., 2020), yet demonstrated to be defective to solve the soluble expression issue of CbADH, since the expression of chaperone would more or less occupy the resources of cells for expressing target enzyme proteins and lead to an activity plateau that cannot be surmounted.

Computational protein redesign for enhancing CbADH solubility

The gap between the high specific activity versus the extremely low expressional activity become a major obstacle to the industrial application of CbADH. Directed evolution and computational redesign of natural enzymes have proven capable of bridging equally large gaps (Musil et al., 2018). Previous studies have used site-directed mutagenesis to improve the stability of CbADH (Bogin et al., 2002; Goihberg et al., 2007), but neglected its soluble expression. Unfortunately, stability enhancements often come at the cost of reduced enzyme activity. The computational protein redesign tools offer ways to avoid this trade-off and also to solubilize the polypeptides, facilitating the purposeful adaptation of natural enzymes. Among them, Protein Repair One-Stop Shop (PROSS), an automated web-based protein stabilization platform which was proven efficacious by the solubly expression of several human proteins in bacteria with unmodified function (Goldenzweig et al., 2016), was applied here to redesign CbADH. First of all, functionally relevant key sites, including Zn$^{2+}$ and cofactor ligand binding sites, and dimer interface sites were excluded from mutation (Fig. S7 and Table S3). After three rounds of online calculations, five random mutations were selected to test
functionally. Synthetic genes encoding wild-type CbADH (CbADH-WT) and the five designs were expressed in *E. coli* BL21 (DE3), and the results was showed that mutations except CbADH-24M were solubly and actively expressed much better than the wild-type CbADH, and the soluble enzyme protein ratio and activity dramatically decreased with the number of mutation sites increased (Fig. 4A and Fig. S8), that was different from Goldenzweig’s work. What’s more, thermal stability of three mutations, CbADH-6M ($T_{1/2}^{60\text{min}}=66.8^\circ\text{C}$), CbADH-10M ($T_{1/2}^{60\text{min}}=64.9^\circ\text{C}$) and CbADH-14M ($T_{1/2}^{60\text{min}}=67.3^\circ\text{C}$) respectively, was significantly improved than that of the original enzyme ($T_{1/2}^{60\text{min}}=63.7^\circ\text{C}$) (Fig. 4B). The terrific design CbADH-6M was tested to possess an expressional activity of 46.3 U/mL, which was 15 fold higher than the nature enzyme, and a soluble enzyme protein ratio of 82.4% (Fig. 4C). Although completely soluble expression was not achieved by sequence redesign of CbADH, it is demonstrated that PROSS was an simple and applicable method for computational design of stable and soluble biocatalysts.
**Figure. 4.** Characterization of solubility and stability of PROSS mutants. (A) Enzyme activity of mutants. (B) Thermal stability of mutants at different temperatures. (C) SDS-PAGE analysis of protein expression of mutants: **Lane M:** molecular weight marker; **Lane W:** whole cell protein; **Lane S:** supernatant; **Lane P:** precipitation

**Molecular structure analysis of CbADH-6M**

Multiple superimposable mutations can improve the conformational stability of the protein in its natural state, allowing it to gain an advantage in the competition with other misfolded or partially folded states in folding dynamics. Six mutations, S24P, G182A, G196A, H222D, S250E and S254R, were introduced into the CbADH-6M mutant. The soluble expression of cbADH-6M was about 18.12 times higher than that of the original strain. Among the 6 mutation sites involved in CbADH-6M, four sites of 24, 222, 250 and 254 are located on the surface of the protein (**Figure. 5**). Three mutations H222D, S250E, and S254R all replace the original amino acid residues with more polar amino acid residues to enhance the surface polarity. In addition, the significant increase of $\Delta T^{60\text{min}}_{1/2}$ gained by the substitution of Pro for Gln100 in CbADH ($\Delta T^{60\text{min}}_{1/2} = +8 ^\circ C$) (Musil et al., 2018) suggested that the proline residue stabilized the protein by reducing the flexibility of a loop at this strategic region. Similarly, when S24 located on the loop on the surface of the protein was mutated to a proline residue that adopted only a limited number of conformations, the flexibility of the loop was reduced thus rigidizing the protein structure.

As to the G182 located in the internal structure of an alpha helix, it was substituted by alanine residue (J. Lo´pez-Llano et al., 2006), which is regarded as the most stabilizing residue in internal helical position, whereas glycine is the more destabilizing after proline.

Furthermore, the number of salt bridges in CbADH-6M was proved to have changed a lot. Although the Asp225-HisS222 salt bridge in the original protein CbADH-WT disappeared due to the amino acid substitution H222D. The replacement of H222D, S250E and S254R led to the formation of four new salt bridges between Arg254, Glu250, Asp254, Asp225 and Glu280, thus a salt bridge network centered on Arg254 was constituted. Moreover, the angle of the Arg80 changed, so that it was closer to the
Glu60 to form a new Salt bridge between Glu64 and Arg80. The salt bridge network centered on the Arg254 was located on the surface of the CbADH protein, which makes the one alpha helix (D222, D225 located in), another alpha helix (E250, R254 located in) and the loop (E280 located in) a closer integration. Meanwhile, the formation of salt bridge Glu64-Arg80 made the loop structures (E60 and R80 located in) more stable.

![Proteins](image)

*Figure. 5. Protein structure of CbADH-6M. (A) Proline residue located in the loop region. (B) Alanine residue located in the α-helix. (C) Alanine residue located at 196 site. (D) New salt bridge Glu64-Arg80 formed by CbADH-6M. (E) Schematic diagram of new salt bridge network centered on Arg254.*

**Large-scale production of CbADH-6M**

In two-phase high cell density fermentation, the dissolved oxygen dropped sharply in 5 h, and the agitation rate increased at the same time. The remaining fermentation parameters were shown as follow (*Figure. 6A*). Sampling was started at 9 h, and the measured OD$_{600}$ was 80.1 at 15 h, and the induction temperature was 22°C. After that, samples were taken every 2 h. During the subsequent fed fermentation, the enzyme activity and OD$_{600}$ increased steadily (*Figure. 6B*). At 36.5 h, the OD$_{600}$ reached 205.2 and the enzyme activity reached 2401.8 U/mL. From the perspective of protein gel expression, the total protein expression of samples at 19.5h, 27.5h and 36.5h continued to increase, and the soluble expression (*Figure. 6C*) was excellent with almost no
Fermentation parameter control. (B) Cell growth and CbADH-6M expression during the fermentation. (C) SDS-PAGE analysis of protein expression of samples at different time: Lane M: molecular weight marker; Lane W: whole cell protein; Lane S: supernatant; Lane P: precipitation

**Biochemical characterization of recombinant CbADH-6M**

The activity of purified CbADH-6M was measured at various temperature ranging from 15 to 75°C. As the temperature increased, the enzyme activity increased constantly,
reaching the highest at 65°C (Figure. 7A). Thermostability of the purified CbADH-6M was investigated at temperatures of 50, 60 and 70°C. It can be seen from the deactivation curve (Figure. 7B), the enzyme had half-lives ($T_{1/2}^{60\text{min}}$) of 62.4, 4.9 and 0.4h at 50, 60 and 70°C (Table 1), respectively. The optimum pH was determined by measuring the enzyme activities at different pH from 6.0-13.0 (Figure. 7C). The maximum activity was observed at pH 9.5(Glycine-NaOH). In the case of pH stability, CbADH-6M was proved to possess favorable pH stability from 6.0 to 10.0 (Figure. 7D).

**Figure. 7.** Biochemical characterization of recombinant CbADH-6M (A) Optimum temperature. (B) Thermal stability at 50,60,70°C. (C) Optimum pH. (D) pH stability from 6.0-10.0

**Table 1** Thermal stability of the recombinant CbADH-6M.

| Temperature (°C) | Half-life of activity (h) |
|------------------|--------------------------|
| 50               | 62.4                     |
| 60               | 4.9                      |
| 70               | 0.4                      |
Regeneration of NADPH using recombinant CbADH-6M

To explore the potential of CbADH at in situ NADPH regeneration, an engineered ADH from Lactobacillus kefir LkADH (He et al., 2015) coupled with CbADH was employed in the synthesis of some fine chiral aromatic alcohols (Figure. 8A). As can be seen in the reduction reaction for the synthesis of (S)-1-phenylethanol, if only LkADH was used, the reaction rate was slow, and the conversion rate (Figure. 8B) hardly reached 100%. Once CbADH was added for the coenzyme cycle, the catalytic rate greatly accelerated. Additionally, in contrast to the reaction system only catalyzed by LkADH, the conversion rate of LkADH & CbADH reaction reached 100% within 6 hours. Not only in the production of (S)-1-phenylethanol, but also in the synthesis of other important chiral aromatic alcohols (Table 2), LkADH & CbADH exhibited excellent stereoselectivity with an ee value over 99% and the conversion rate of reaction was mostly above 99% much higher than LkADH only, indicating the great potential for industrial application.
**Figure 8.** The reduction reaction using CbADH-6M as coenzyme regeneration agent. (A) Schematic diagram of the coenzyme cycle. (B) Conversion rate of acetophenone in the reduction reaction.

**Table 2** Substrate spectrum of the recombinant CbADH-6M.

| Entry | Substrate | Relative activity (%) | Product | ee (%) | Conversion rate (%) |
|-------|-----------|-----------------------|---------|--------|---------------------|
| 1     | LkADH     | 100                   | (S) >99 | 94.6   | 99.5                |
| 2     | LkADH     | 70                    | (S) >99 | 80.3   | 99.5                |
| 3     | LkADH     | 92                    | (S) >99 | 92.5   | 99.5                |
| 4     | LkADH     | 84                    | (S) >99 | 86.1   | 99.5                |
| 5     | LkADH     | 59                    | (S) >99 | 76.1   | 90.8                |
Conclusions

CbADH with better stability and higher specific activity was screened for NADPH regeneration, In this work, a computational protein design named PROSS was applied for the soluble modification of target enzyme. The multipoint mutant CbADH-6M displayed a superb solubility, which was 18 times that of the wild type. Meanwhile the enzyme activity of CbADH-6M reached 46.3 U/mL (wild type 2.9 U/mL). In addition, the multipoint superimposing effect improves the conformational stability of the protein, as well as the thermal stability and pH stability. When coupling CbADH with LkADH, the catalytic rate of the reduction reaction was greatly improved and the conversion rate was significantly higher than that of the LkADH reaction with 99% conversion. The ee value of the product in the final reaction mixture was > 99%, showing strict stereoselectivity. This ADH mutant appears to an attractive biocatalyst for the asymmetric synthesis of chiral alcohols.

Availability of data and materials

The data and materials in this work are available from the corresponding author on reasonable request.

Abbreviations

ADH: alcohol dehydrogenase;
CbADH: an alcohol dehydrogenase from Clostridium beijerinckii;
LkADH: an alcohol dehydrogenase from Lactobacillus kefir;
WT: wild-type;
HPLC: high-performance liquid chromatography.

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Author’s contribution
YLR designed the concept of this study. XJL, DT and ZHS carried out the experimental procedures. XJL and ZHS analyzed the result and drafted the manuscript. YHR provided ideas for writing. WZY and ZHY provided experimental help. WJP and XG helped revise writing. All authors read and approved the final manuscript.

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Availability of data and materials
The data and materials in this work are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors approved the consent for publishing the manuscript to bioresources and bioprocessing.

Competing interests
The authors declare that they have no competing interests.
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