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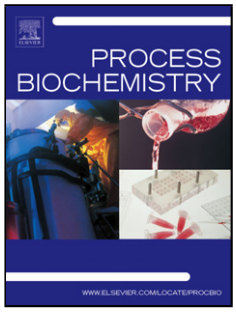

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Enzymatic preparation of optically pure t-butyl 6-chloro-(3*R*,5*S*)-dihydroxyhexanoate by a novel alcohol dehydrogenase discovered from *Klebsiella oxytoca*

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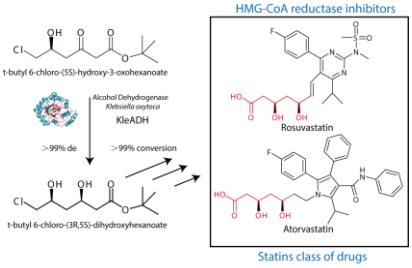
Graphical abstract



Highlights

•Discover a novel alcohol dehydrogenase (named as KleADH) from *Klebsiella oxytoca* by a genome mining method.

•First alcohol dehydrogenase from proteobacterial that could convert t-butyl 6-chloro-(5*S*)-hydroxy-3-oxohexanoate to t-butyl 6-chloro-(3*R*,5*S*)-dihydroxyhexanoate with high enantioselectivity by whole cells.

•A systematic study of several factors influencing the whole-cell catalyst activity such as temperature, pH, the effects of metal ions and organic solvent was performed.

•KleADH exhibited notable activity towards several aryl ketones with high stereoselectivity.

Abstract

Alcohol dehydrogenases can catalyze the inter-conversion of aldehydes and alcohols. The t-butyl 6-chloro-(3*R*,5*S*)-dihydroxyhexanoate is a key chiral intermediate in the synthesis of statin-type drugs such as Crestor (rosuvastatin calcium) and Lipitor (atorvastatin). Herein, a novel alcohol dehydrogenase (named as KleADH) discovered from *Klebsiella oxytoca* by a genome mining method was cloned and characterized. The KleADH was functionally overexpressed in *Escherichia coli* Rosetta (DE3) and the whole cell biocatalyst was able to convert t-butyl 6-chloro-(5*S*)-hydroxy-3-oxohexanoate to t-butyl 6-chloro-(3*R*,5*S*)-dihydroxyhexanoate with more than 99% diastereomeric excess (de) and 99% conversion in 24 h without adding any expensive cofactors. Several factors influencing the whole cell catalyst activity such as temperature, pH, the effects of metal ions and organic solvent were determined. The optimum enzyme activity was achieved at 30 °C and pH 7.0 and it was shown that 1 mM Fe^{3+} can increase the enzyme activity by 1.2 times. N-hexane/water and n-heptane/water biphasic systems can also increase the activity of KleADH. Substrate specificity studies showed that KleADH also exhibited notable activity towards several aryl ketones with high stereoselectivity. Our investigation on this novel alcohol dehydrogenase KleADH reveals a promising biocatalyst for producing chiral alcohols for preparation of valuable pharmaceuticals.

Keywords: Alcohol dehydrogenase, T-butyl 6-chloro-(3R,5S)-dihydroxyhexanoate, *Klebsiella oxytoca*, Whole-cell biocatalyst, rosuvastatin, atorvastatin

1. Introduction

Coronary heart disease is one of the most fateful diseases in the world. Past epidemiological studies have confirmed that the coronary heart disease is closely related to hypercholesterolemia[1, 2]. One widely used treatment method for

coronary heart disease was using statins class of drugs such as Crestor (rosuvastatin) and Lipitor (atorvastatin), which are good inhibitors of cholesterol synthesis enzyme. These drugs specifically inhibit the hydroxylmethylglutaryl coenzyme A (HGM-CoA) reductase which catalyzes the reductive conversion of HMG-CoA into mevalonate, an early and rate-limiting step in cholesterol biosynthesis[3-6]. By far, statins are still top-selling drugs in the world. However, chemical synthesis of the chiral side chain of statins, which is a 3,5-dihydroxyacid derivative consisting of two asymmetric centers, needs to be performed at extreme temperature (-78 °C) and produces large amount of toxic waste[7-9]. Therefore, producing statins in industrial scale is challenging and far from being economical. In contrast, biocatalytic method is advantageous and several processes have been developed for the synthesis of statin side chains[10-18]. Among them, one of the most prominent process for producing the target chiral alcohols was using alcohol dehydrogenase for the asymmetric reduction of the achiral ketones (Fig. 1). One advantage of this process is that there is no loss of substrate, unlike racemic separation using hydrolases. What is more, this biocatalytic process is more environmentally sustainable and thus more attractive for pharmaceutical manufacturing[7, 14, 16, 18].

Alcohol dehydrogenases exist widely in various kinds of microorganisms and play an important role in the process of primary and secondary metabolism[19, 20]. It could be generally divided into three categories including zinc-dependent ADHs, short-chain ADHs and iron-activated ADHs[21]. Alcohol dehydrogenases that come from *Rhodococcus erythropolis*[22], *Lactobacillus brevis*[23], *Thermobacterium brocki*[21, 24] and *Saccharomyces cerevisiae*[25, 26] have already been commercialized and widely used in the asymmetric reduction of ketones.

Generally, the exogenous addition of nicotinamide cofactors (such as NADH and NADPH) is necessary for the alcohol dehydrogenase mediated reduction reactions[12, 20]. The high cost of cofactors makes efficient cofactor regeneration a prerequisite for industrial applications[27]. One possibility to circumvent the cofactor challenge is using the whole cells as the biocatalyst which could harbor an efficient cofactor-regenerating system. Moreover, the cells can be removed from the reaction mixtures by a simple centrifugation step[11, 28]. Thus, a whole-cell catalysis system for alcohol dehydrogenases is more economical and convenient.

In this study, a new alcohol dehydrogenase (KleADH) was discovered from *Klebsiella oxytoca* via a genome mining method. The KleADH gene was cloned and functionally overexpressed in *E. coli* Rosetta. The recombinant whole cells were found to be an excellent biocatalyst for several ketone reduction reactions. Specifically, it could be used in the enantioselective reduction of t-butyl 6-chloro-(5*S*)-hydroxy-3-oxohexanoate to t-butyl 6-chloro-(3*R*,5*S*)-dihydroxyhexanoate with more than 99% diastereomeric excess and 99% conversion. Taken together, our findings provide another efficient process for preparation of statin side chains.

2. Material and methods

2.1 Materials

K. oxytoca was purchased from China General Microbiological Culture Collection Center (CGMCC). T-butyl 6-chloro-(5*S*)-hydroxy-3-oxohexanoate were purchased from Changzhou pharmaceutical factory (China). *E. coli* DH5*α* and Rosetta (DE3) cells (TransGen Biotech, China) were used for cloning and expression, respectively. Restriction enzymes (*Eco*RI and *Xho*I), rTaq DNA polymerase and T_4 DNA ligase were purchased from Takara. The expression vector pET-28(+) was purchased from Invitrogen. The gel extraction kit was purchased from Qiagen (Germany). The plasmid extraction kit and genomic DNA purification kit were from Tiangen (China). Primers were synthesized by Sangon Biotech (Beijing, China). Kanamycin was added to the medium at a final concentration of 50 μg/mL for recombinant plasmids selection. DNA dideoxy sequencing by Ruibio Biotech (China) confirmed the identity of all plasmids. Other general chemicals were purchased from Sigma-Aldrich. All the chemicals used in the present study were in analytical grade and obtained from commercial sources.

2.2 Generation of expression constructs for KleADH

To identify a potential alcohol dehydrogenase that could be used for preparation of statin side chains, a genome mining method was applied. A Blast (*Basic Local Alignment Search Tool*) using the alcohol dehydrogenase from *S. cerevisiae* (GenBank accession: NP_010159) as a template was performed in NCBI (National Center for Biotechnology Information). A hypothetical protein (GenBank accession:

WP_064378515.1) was found from *K. oxytoca*. This gene encoding KleADH was then amplified by polymerase chain reaction (PCR) from genomic DNA of *K. oxytoca*. The forward primer KleADH-FP and reverse primer KleADH-RP (Table. 1) were utilized for PCR reactions (the *Eco*RI and *Xho*I restriction site are introduced, respectively). PCR fragments were purified by Gel Extraction Kit. Purified PCR products and pET-28a (+) were then digested with restriction endonucleases *Eco*RI and *Xho*I and ligated with T_4 DNA ligase to yield an expression plasmid containing KleADH gene (pET28-KleADH). After transforming into *E. coli* DH5*α* cells, plasmids were prepared from individual transformants and confirmed by DNA sequencing. All of the alanine scanning mutants of KleADH: Asp44Ala, Tyr49Ala, Lys74Ala and His107Ala were created by QuickChange PCR method with the template pET28-KleADH[29]. All the primers were listed in Table. 1.

2.3 Expression of KleADH in *E. coli*

E. coli Rosetta (DE3) cells were retransformed with the expression construct and cultured in 5 mL lysogeny broth (LB) medium containing kanamycin (50 μg/mL) and chloramphenicol (34 μg/mL) at 37 °C with shaking. 5 mL of the overnight culture of *E. coli* Rosetta (DE3) cells was then inoculated into 50 mL fresh LB medium supplemented with the same antibiotics and cultivated at 37 °C on a rotary shaker (220 rpm). When OD_{600} value of the culture reached 0.8–1.0, isopropyl-β-D-dthiogalactopyranoside (IPTG, final concentration of 0.1 mM) was added to induce the expression. The cultures were cultivated at 30 °C for a further 15 h and then harvested by centrifugation (6000 rpm for 10 min at 4 °C). The expression of target protein was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4 Structure analysis

Homology model of KleADH was constructed with the structure of the reductase from *Yersinia pestis* (PDB: 4MHB_A, 90% identities, 100% confidence). The 2D structure of t-butyl 6-chloro-(5S)-hydroxy-3-oxohexanoate and NADH were created using ChemDraw, minimized and saved as a pdb file. The docking experiments were

performed using Discovery Studio 2.5. Generally, 3D structures of the chiral ligands were generated by the *Build Fragment Tool* through energy minimization which is optimized by the *Prepare Ligands Protocol* in DS 2.5, and by adding force field for docking. Model of KleADH were further prepared by the *Clean Protein Tool* to remove H_2O molecular and other ions on the surface of protein, then a force field was also added to prepare the receptor. The substrate cavity was selected as the binding site by the *Define Sphere Form Selection Tool*. The NADPH was then docked into the active sites of KleADH using the *Dock Ligand Protocol*. The docking poses selected from this analysis were scored with Libdock score, where a higher score indicates stronger ligand binding affinity to the receptor. The best docking result was got by *Analyze Ligand Poses Tool*. Then the 6-chloro-(5S)-hydroxy-3-oxohexanoate structure was docked into the active sites after NADPH, all the methods are the same as above.

2.5 Optimization of reaction conditions

Optimum reaction time was determined as following. In a typical assay, 400 mg/mL wet cells were incubated with 5 mg/mL t-butyl 6-chloro-(5*S*)-hydroxy-3-oxohexanoate in 1 mL triethanolamine buffer (100 mM, pH 7.0). The reactions were carried out at 30 °C at 220 rpm for different time. Then reaction mixtures were extracted with 1 mL ethyl acetate, and analyzed by chiral GC.

To test the optimal concentration of wet recombinant cells used in the reaction, different amount of wet cells were suspended in 1 mL triethanolamine buffer containing 5 mg/mL substrates, other reaction conditions as above except the incubation time was 24 h. After reaction, the mixtures were extracted and analyzed by chiral GC.

The effect of temperature on the asymmetric catalysis was determined at various temperatures ranging from 15 °C to 50 °C. The following condition was used: 250 mg/mL wet cells and 5 mg/mL t-butyl 6-chloro-(5*S*)-hydroxy-3-oxohexanoate in 1 mL triethanolamine buffer (100 mM, pH 7.0) were shaken at different temperature (220 rpm, 24 h). The effect of pH on the enzymatic activity was investigated at various pH

values ranging from 5.0 to 10.0 with a universal buffer (50 mM Tris, 50 mM boric acid, 33 mM citric acid and 50 mM Na_2PO_4, pH was adjusted by NaOH or HCl). All the reaction mixtures were extracted and analyzed by GC and the highest activity was set as 100%.

The effect of substrate concentration on the activity of KleADH was examined at different t-butyl 6-chloro-(5*S*)-hydroxy-3-oxohexanoate concentrations from 3 to 21 mM, under the optimized pH value and temperature. Other conditions are similar as above. The effects of metal ions (Ca^{2+}, Cu^{2+}, Fe^{3+}, Fe^{2+}, Zn^{2+}, Mn^{2+}, K^{+}, Ni^{2+}, Li^{+}, Sn^{2+}, Mg^{2+}, Co^{2+} and Na^{+}) on activity were studied at a final concentration of 0.1 mM, 0.5 mM and 1 mM under otherwise similar conditions. Last, the effects of organic solvent/water (v/v=1/2) biphasic systems were investigated. 8 different kinds of organic solvents including dichloromethane, toluene, methyl tert-butyl ether (MTBE), aether, ethyl acetate, isopropyl ether, n-hexane, n-hepane were tested as organic phases.

Generally, one Unit (1 U) of enzyme activity was defined as the amount of wet cells required to reduce 1 μmol t-butyl 6-chloro-(5*S*)-hydroxy-3-oxohexanoate or produce 1 μmol t-butyl 6-chloro-(3*R*,5*S*)-dihydroxyhexanoate per minute at 30 °C, pH 7.0.

2.6 Analytical methods

The optical purity (de value) of the product was determined by a SHIMADZU GC2014C Series gas chromatography (GC) equipped with a chiral G-TA column (length 10 m, Advanced Separation Technologies Co. Ltd.). The nitrogen flow-rate was 5 ml/min. A temperature of 110 °C was held for 1 min, followed by a gradient of 0.5 °C/min to 119 °C and a hold of 119 °C for 2 min, then up to 125 °C by a gradient of 2 °C/min. Temperature of the injection chamber and detector was set to 200 °C. The retention times were 16.056 min for (3*R*,5*S*) isomer, and 17.458 min for (3*S*,5*S*) isomer, respectively.

2.7 Substrate specificity

First, two different cofactors NADH and NADPH were tested using whole cells biocatalysts to see which one could improve the activity of KleADH. Reactions were conducted in 1 mL of triethanolamine buffer (100 mM, pH7.0) containing 10 mM substrates, 10 mM cofactors, and 400 mg/mL recombinant wet cells at 30 °C with vigorous shaking for 24 h. Then, a wide range of potential substrates were investigated to explore the substrate specificity of KleADH. The substrates used in this study were listed in Table. 2. Reactions were conducted in 1 mL of triethanolamine buffer (100 mM, pH7.0) containing 10 mM substrates and 400 mg/mL recombinant wet cells at 30 °C with vigorous shaking for 24 h. Then the aqueous phase was extracted by ethyl acetate and analyzed by chiral HPLC or GC[30, 31].

3. Results and discussion

3.1 Identification of KleADH from *K. oxytoca.*

Currently, although several biocatalytic processes for the preparation of statin side chains have been reported, only the α-keto reductase from *S. cerevisiae* is promising for industrial application[12]. Past research has showed that this novel NADPH-dependent α-keto reductase has strong activity on reducing *t*-butyl-6-cyano-(5*R*)-hydroxy-3-carboxylhexanoat with high enantiomeric excess (ee) and diastereomeric excess (de) values[12, 32, 33]. We initiated our study with a Blast search using the α-keto reductase from *S. cerevisiae* as a query. The position-specific iterated model was applied and the organism was limited to *K. oxytoca*. By this setting, a hypothetical reductase (KleADH, GenBank accession: WP_064378515.1) with very low identities (32%) was found from *K. oxytoca*. It should be noted that *S. cerevisiae* is an eukaryota while *K. oxytoca* belongs to prokaryote which makes this genome mining strategy risky. However, this new reductase might be a novel family of α-keto reductase and suitable to handle in *E. coli* for industrial application. Further bioinformatics studies showed that KleADH is more like a 2,5-diketo-D-gluconic acid reductase, which belongs to the short chain ADHs family. This family of enzyme could catalyze stereospecific reduction of 2,5-diketo-D-gluconate (2,5-DKG) to 2-

keto-L-gulonate. Considering the similarity between 2,5-diketo-D-gluconate and t-butyl 6-chloro-(5*S*)-hydroxy-3-oxohexanoate, this enzyme could be potential biocatalyst for the preparation of statin side chains.

3.2 Heterelogous expression of KleADH in *E. coli.*

The KleADH gene of *K. oxytoca* was amplified by PCR reactions and inserted into the IPTG inducible expression vector pET-28a (+). The vector could provide recombinant proteins with both a N terminal and a C terminal His_6-tag. After the cells containing the KleADH expression plasmids were induced overnight with the addition of IPTG, all cells were harvested by centrifugation and resuspended in 40 mL binding buffer (50 mM/L NaH_2PO_4, 300 mM/L NaCl, and 10 mM/L imidazole; pH 8.0) and sonicated. Cell lysates were clarified by ultracentrifugation and both the supernatants and pellets were analyzed by SDS-PAGE to monitor the expression of target protein. SDS-PAGE analysis showed that this potential alcohol dehydrogenase was successfully expressed in *E. coli* Rosetta with a high expression level. Even though parts of proteins are in the insoluble pellets, the majority of the expressed protein could be detected in the supernatant as a soluble form. The recombinant KleADH showed a single band of approximately 30 kDa on the SDS-PAGE gel which is corresponding to the predicted molecular mass of 32 kDa (Fig. 2).

A one-step purification procedure for the recombinant KleADH was then employed by using immobilized Ni-NTA affinity chromatography to purify the KleADH. SDS-PAGE analysis indicated that the purified enzyme was more than 95% pure after elution from the affinity column, however, most of the proteins directly precipitated after that and completely lost their bioactivity. To solve this problem, different factors like buffer, pH and different fusion tags were investigated, but none of them worked (data not shown). It seems that this alcohol dehydrogenase is very sensitive outside the cells, and thus, the whole cells as the biocatalyst were selected for future studies. The activity of KleADH was measured by chiral GC using t-butyl 6-chloro-(5*S*)-hydroxy-3-oxohexanoate as the first attempt. Surprisingly, whole cells of *E. coli* harboring KleADH showed a very high activity towards t-butyl 6-chloro-

(5*S*)-hydroxy-3-oxohexanoate. As shown in Fig. 3, the other isomeride t-butyl 6-chloro-(3*S*,5*S*)-dihydroxyhexanoate was not detected, showed that the diastereomeric excess was more than 99%.

3.3 Homology modeling and catalytic residues of KleADH

KleADH belongs to the aldo-keto reductase (AKR) family[34]. This family of proteins consists of a group of diverse NAD(P)H-dependent oxidoreductases that catalyze the reduction of a wide range of substrates, such as steroids, ketones and aldehydes[34]. Generally, most of the AKR family has a common $(\alpha/\beta)_8$-barrel fold with 300 amino acids in length and a similar catalytic tetrad composed of a tyrosine, a lysine, an aspartic acid and a histidine[35]. Despite these similarities, members of the AKR family often exhibit little sequence similarity. However, bioinformatic studies showed that KleADH has high levels of identity to a putative reductase from *Y. pestis* (PDB: 4MHB_A, 90% identities). The structure of this reductase was release at 2013, but none of its biochemical characterization, catalytic mechanism or any experimental data have been published. Sequence alignments showed that amino acids known to be involved in the activity of the aldo-keto reductase were generally conserved in both reductases. Hence, a Phyre2 (Protein Homology/analogY Recognition Engine V 2.0) homology model of KleADH was constructed with the structure of the reductase from *Y. pestis* (PDB: 4MHB_A, 90% identities, 100% confidence). Notably, homology to another aldo-keto reductase *Thermotoga maritima* (PDB: 1VP5_A, 58% identities, 100% confidence) was also predicted with high confidence (100%). These two modeling structures even though built on two different template are very similar to each other.

As predicted, the conserved AKR catalytic tetrad for KleADH are Asp44, Tyr49, Lys74, and His107 (shown in blue), permitting the transfer of a hydride ion from NAD(P)H to the substrate. Based on the docking studies, the NADPH directly interact with the substrate cavity formed by β1, β7, β8, α7 and α8. Compared with the known reductase structure (PDB: 1VP5) from *Thermotoga maritima* where the NADPH binds to a similar position, this docking result is quite convincing. The substrate t-

butyl 6-chloro-(5*S*)-hydroxy-3-oxohexanoate, on the other hand, could only dock into the left side of NADPH, which also directly interact with the conserved AKR catalytic tetrad. However, detailed catalytic mechanism is still need further elucidated, the docking studies could only give a limited speculation. To validate the catalytic tetrad, we performed an alanine scan for those four residues. The mutants were transformed and expressed in *E. coli* Rosetta (DE3) and assayed for the reductase activity using t-butyl 6-chloro-(5*S*)-hydroxy-3-oxohexanoate as the substrate again. As we expected, the reductase activities of all the mutants were totally lost. These results confirmed that Asp44, Tyr49, Lys74, and His107 are indeed involved in the catalytic tetrad.

3.4 Effect of incubation time and wet cell weight in aqueous system

Since KleADH could be a very promising catalyst for industrial application, we tried to further optimize the reaction condition for this biotransformation process. A time course study was first performed using 5 mg/mL substrates and 400 mg/mL wet cells. As shown in Fig. 5A, within the first 14 h, enzyme activity of KleADH was quickly increased to the highest level. Compared to the reaction for 24 h, the difference of the conversion rate is slight. Thus, the optimal incubation time was determined as 14 h. We then turned to invest the effect of wet cell weight on the biotransformation process. When 5 mg/mL of the substrate was used, the conversion increased with the increment of wet cells (Fig. 5B). Conversion reached 98% when 400 mg/mL of wet cell was used.

3.5 Effects of pH and temperature

Temperature and pH play important roles during the biotransformation process, because of their impacts on enzyme's activity and stability. The optimal temperature for KleADH were measured at different temperatures ranging from 15 °C to 50 °C using t-butyl 6-chloro-(5*S*)-hydroxy-3-oxohexanoate as substrate. The conversion rate gradually rose when temperature was elevated from 15 to 30 °C and significantly dropped when temperature further went up to 35 °C. Therefore, the optimal temperature for KleADH was determined as 30 °C (Fig. 6A).

The effect of pH on the activity of KleADH was examined in universal buffer (50 mM boric acid, 50 mM Tris, 50 mM Na_2HPO_4 and 33 mM citric acid) with pH varied from 5.0-10.0. The effect of pH on KleADH was significant. As shown in Fig. 6B, KleADH exhibited over 90% of its maximum activity in the pH 7.0 to 9.0. Below or above these range, the activity significantly dropped until it lost all the activity. Thus, the pH 7.0 was used as the optimal pH for the biotransformation process.

3.6 Effects of metal ions and organic solvents

The effects of metal ions were studied at a final concentration of 0.1 mM, 0.5 mM and 1 mM. The reaction was carried out under the standard conditions, and the activity of the KleADH without the addition of metal ions was measured as a control group and set to 100%. Reaction mixtures containing 1 mM Fe^{3+} or Fe^{2+} displayed 120% and 110% activity respectively, while Cu^{2+} had a significant inhibitory effect on enzyme activity, Zn^{2+}, Ca^{2+} and Li^{+} slightly decreased enzyme activity, and others slightly promoted the activity (Fig. 7A). When we used lower concentration of metal ions, the effects were similar. However, the inhibitory effect of Cu^{2+} dropped. Based on these result, KleADH is a metal ion independent enzyme and it indeed belongs to the short chain ADHs family.

As the enzymatic reactions are usually carried out in aqueous system, the use of organic solvents as co-solvent has been proved to be useful for expanding the applicability of biocatalytic process for poor soluble substrate[36]. As shown in Fig.8, dichloromethane, toluene, aether and ethyl acetate could greatly inhibit KleADH activity at the concentration of 1/2 (v/v). Enzyme activity were partially reduced or completely lost as the logP value varies between 0.785-2.720 when ethyl acetate and toluene were used as co-solvent. On the other hand, in the relatively hydrophobic alkane solvent n-hexane and n-heptane, the enzyme activity had a great improvement up to 125% and 135% respectively.

3.7 Substrate spectra

Two different cofactors were tested for KleADH. Results showed that both could improve the bioactivity of KleADH indicating KleADH could use both cofactors. The

substrate spectrum of KleADH toward several types of ketones was examined using the whole cells biocatalyst, as shown in Table 2. The enantiomeric excess of products and conversion were determined by chiral GC or HPLC analysis. 6-chloro-(5*S*)-hydroxy-3-oxohexanoate was chosen as the standard substrate, and the specific activity of KleADH toward this substrate was set as 100%. Considering the importance of aromatic chiral alcohols as useful blocks in pharmaceutical applications, we also tested a series of acetophenone derivatives as substrates. As shown in Table 2, KleADH well accepted acetophenone with substitutions at various position on the aromatic ring with an activity range of 6.70% to 56.92% and high stereoselectivity. Details are shown in Table 2. In summary, KleADH had a broad substrate specificity, including aromatic ketones and alkane ketones, and could be a very promising biocatalyst for further industrial application.

4. Conclusion

Enzymes are able to perform chemical reactions under mild conditions with remarkable regio- and stereoselectivity[37]. Because of this feature, the number of biocatalysts (including isolated enzymes and whole cells biocatalyst) used in organic synthesis has rapidly increased during the last decades, especially for the production of chiral compounds[38]. One of the most valued biotechnological process is the production of chiral alcohols by reducing prochiral ketones using alcohol dehydrogenases. For alcohol dehydrogenases, the use of whole cells as biocatalyst is often preferred because these reactions are dependent on effective method for regeneration of the consumed cofactors. In this study, we discovered a novel alcohol dehydrogenase from *K. oxytoca* by a genome mining method. Even though we used a query from eukaryote, we could still find the target enzyme from the prokaryote source by this genome mining method. These two alcohol dehydrogenases showed very low sequence similarity but had similar catalytic activities. This example proved that the genome mining strategy is the most powerful strategy in looking for new biocatalysts again.

The dehydrogenase KleADH was cloned and functionally expressed in *E. coli* Rosetta. Soluble protein could be detected in the supernatant as an active form. KleADH showed a high activity and stereoselectivity to 6-chloro-(5S)-hydroxy-3-oxohexanoate and had different activity to other diverse aromatic ketones. The whole-cell reaction avoided exogenous addition of nicotinamide cofactors and thus is suitable for further application. The conversion and the enantiomeric selectivity can

reach more than 99% under the conditions of pH 7.0 and 30 °C. Additionally, our investigations showed that appropriate metal ions and appropriate organic solvents could enhance the activity of KleADH. Although KleADH is very sensitive out of cells, the recombinant *E. coli* whole-cell with high catalytic activity is very promising for the synthesis of various kinds of chiral alcohols, which were important intermediates for many pharmaceuticals. Specifically, this study laid the foundation for the production of optically pure t-butyl 6-chloro-(3*R*,5*S*)-dihydroxyhexanoate which is a key chiral intermediate in the synthesis of statin-type drugs.

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Figure legends

Fig. 1 Enzymatic preparation of t-butyl 6-chloro-(3R,5S)-dihydroxyhexanoate by alcohol dehydrogenase for statins class of drugs.

Fig. 2 SDS-PAGE (12%) of KleADH expressed by *E. coli* Rosetta (DE3). Lane M, the marker proteins with the relative molecular masses; Lane 1, crude protein extract of *E. coli* Rosetta/pET28-KleADH before induction; Lane 2, crude protein extract of *E. coli* Rosetta/pET28-KleADH after induction; Lane 3, insoluble fraction of crude extract of induced recombinant *E. coli* Rosetta/pET28-KleADH; Lane 4, soluble fraction of crude extract of induced recombinant *E. coli* Rosetta/pET28-KleADH.

Fig. 3 Chiral-GC analysis of the biocatalytic reaction catalyzed by KleADH. A, Chiral-GC analysis the substrate t-butyl 6-chloro-(5S)-hydroxy-3-oxohexanoate, the retention time is 11.5 min； B, Chiral-GC analysis the product t-butyl 6-chloro-(3R,5S)-dihydroxyhexanoate, the retention time is 16 min；

Fig. 4 Homology modelled three-dimensional structure of KleADH. Homology modelling of the KleADH structure was performed using a putative reductase from *Yersinia pestis* as a template. The conserved AKR catalytic tetrad was showed in marine blue. The substrate（t-butyl 6-chloro-(5S)-hydroxy-3-oxohexanoate, green stick）and NAPH (grey stick) were docking into the substrate cavity.

Fig. 5 A. Characterizations of the KleADH. The effect of reaction time on the biocatalytic reaction. 400 mg/mL wet cells were incubated with 5 mg/mL t-butyl 6-chloro-(5*S*)-hydroxy-3-oxohexanoate in 1 mL triethanolamine buffer (100 mM, pH

7.0). KleADH activity was monitored at 30 °C at 220 rpm for different time; B. The effect of wet cell weight on the biocatalytic reaction. Different amount of wet cells were used in 1mL triethanolamine buffer containing 5 mg/mL substrates for 24 h and KleADH activity was monitored. All measurements wereperformed in triplicate.

Fig. 6 Characterizations of the KleADH. A. The effect of temperature on the biocatalytic reaction. The activity of the KleADH was measured at temperatures ranging from 15 °C to 50 °C; B. The effect of pH on the biocatalytic reaction. The activity of the KleADH was measured at pHs ranging from 5.0 to 10.0 at the optimal temperatures. All measurements wereperformed in triplicate.

Fig. 7 Characterizations of the KleADH. A. The effect of metal ions on the activity of the KleADH, Chlorates were used as the target metal ion donators. The KleADH activity was monitored at 30 °C for 14 h in 1 mL triethanolamine buffer (100 mM, pH 7.0) with 5 mg/mL substrate and 1 mM metal ions; B. The effect of organic solvents on the activity of the KleADH. KleADH activity was monitored at 30 °C for 14 h with with each organic solvent/water (v/v=1/2) biphasic systems. All measurements wereperformed in triplicate.

Fig. 1

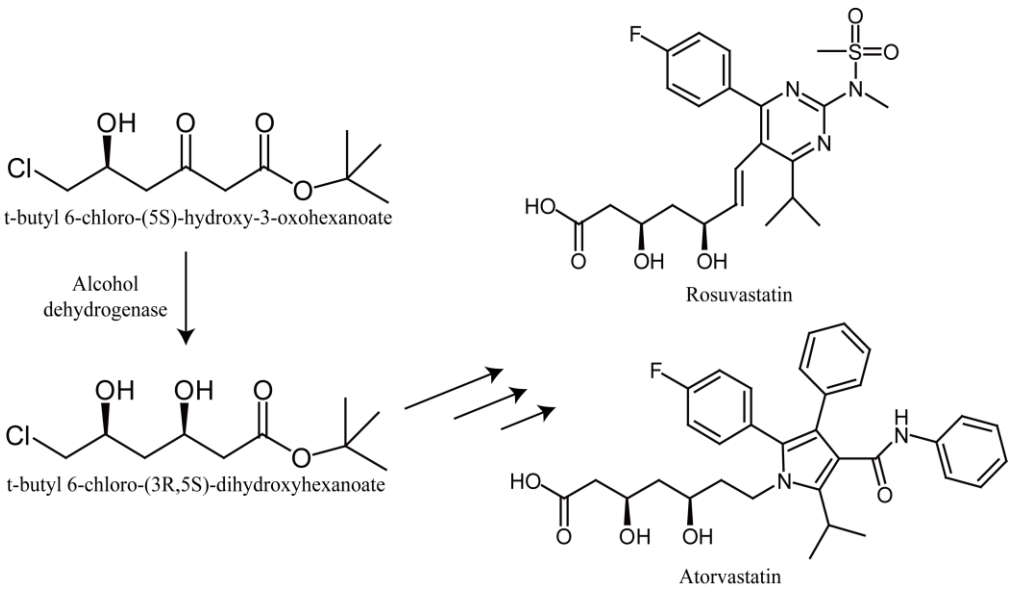
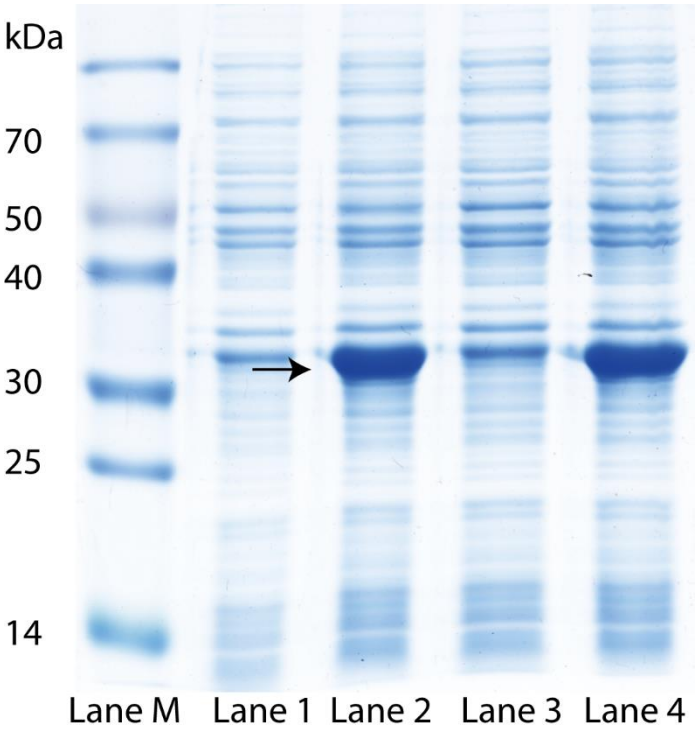


Fig. 2



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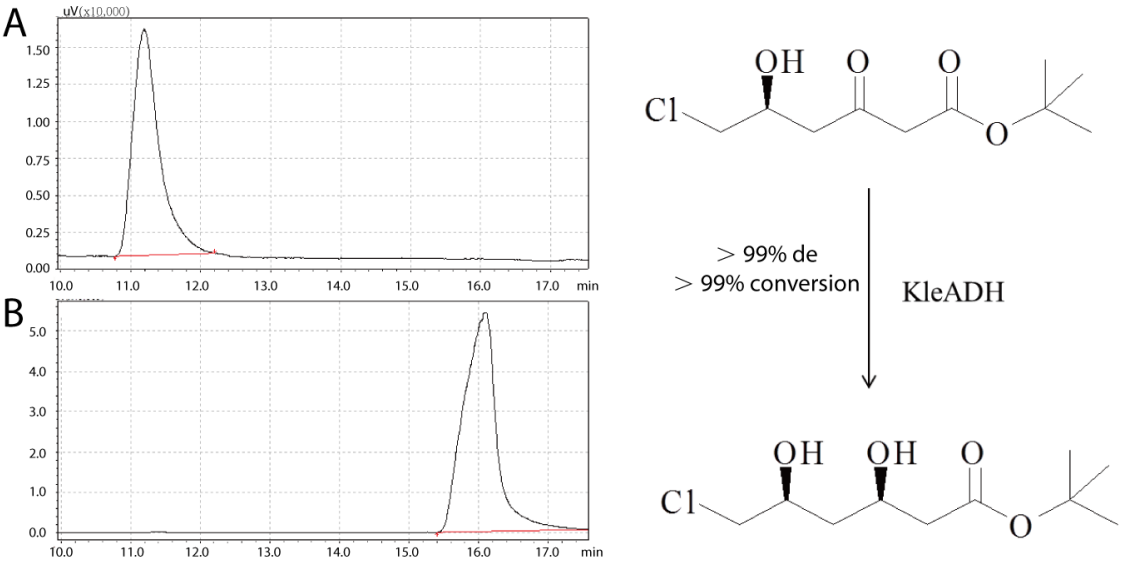

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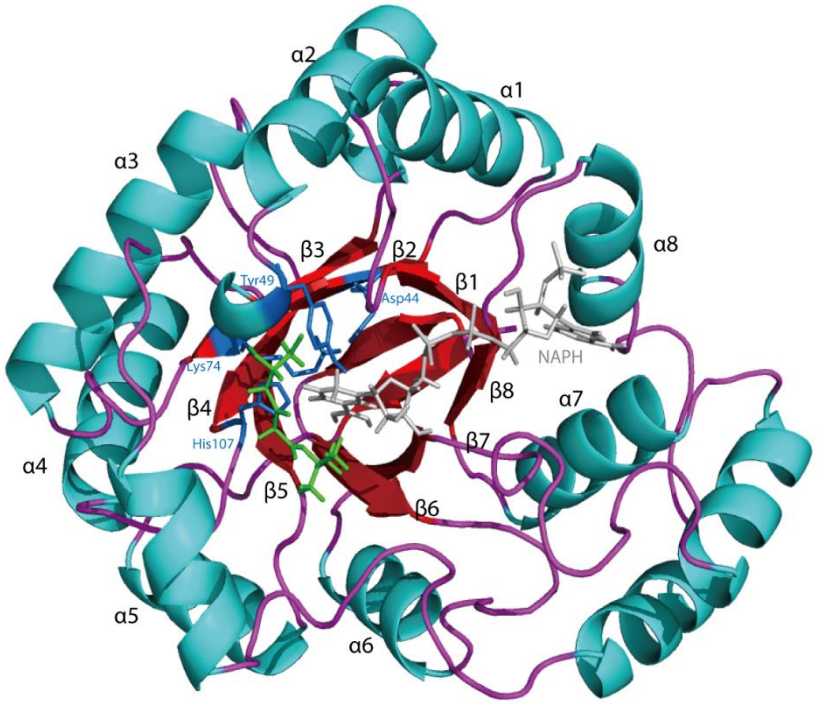

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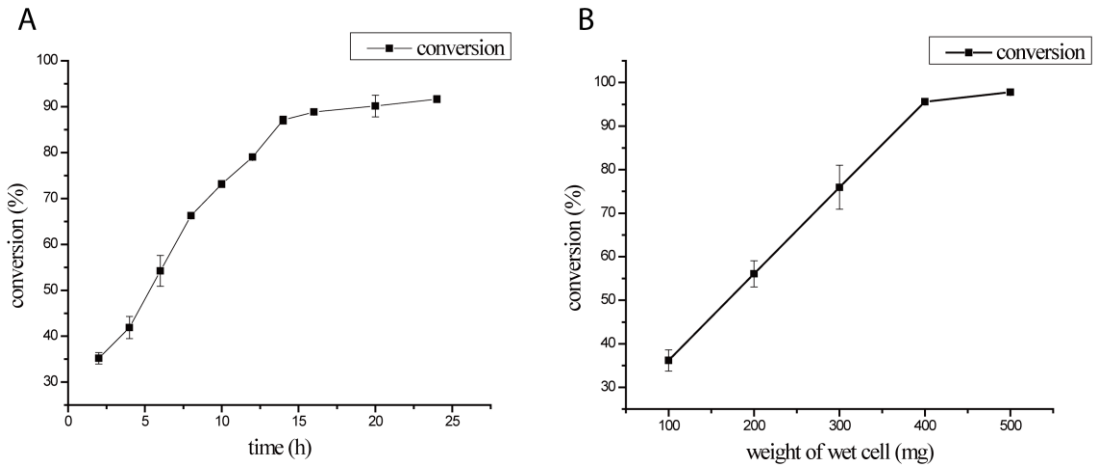

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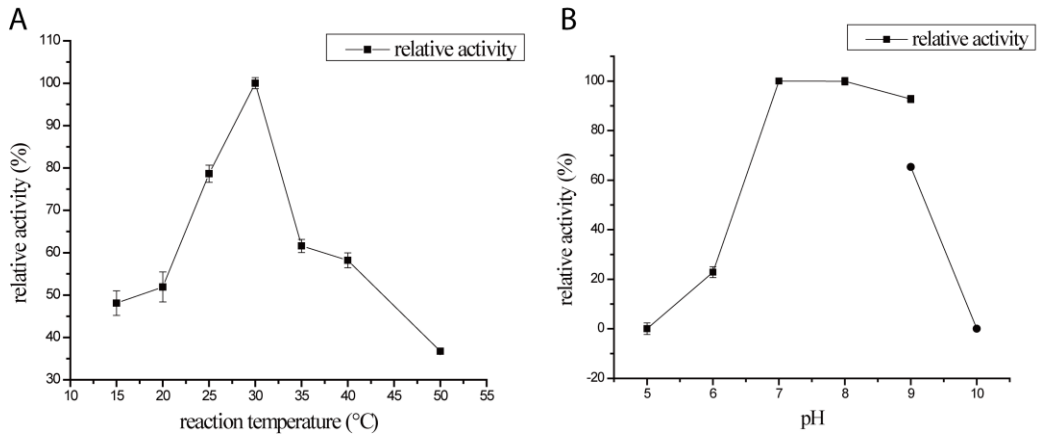

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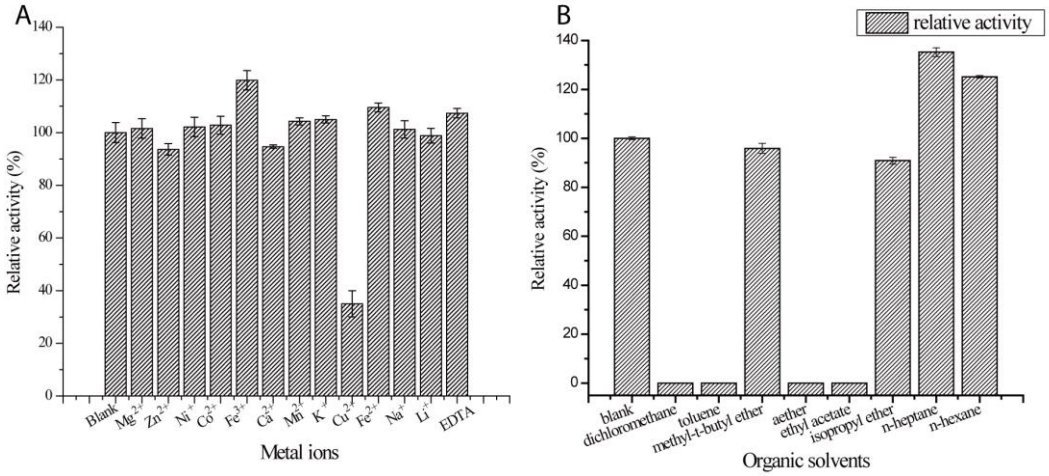
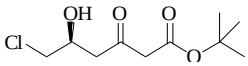
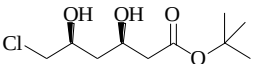
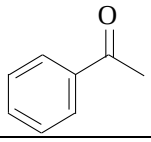
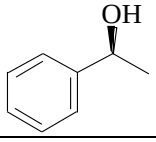
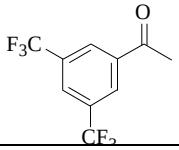
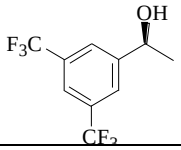
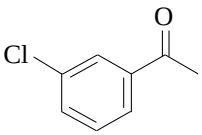
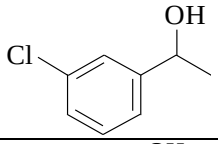
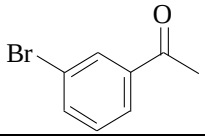
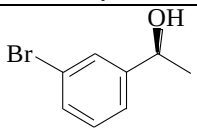
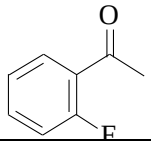
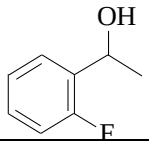
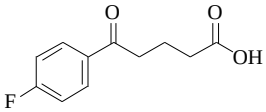
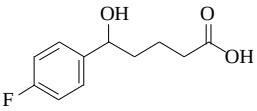
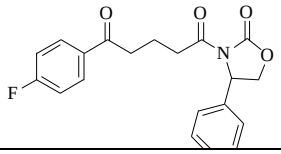
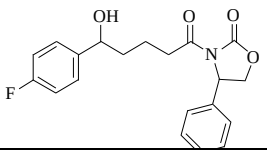


Table. 1 Primers used in this study

Name	DNA sequence (5'-3')	Description
KleADH-FP	CCGGAATTCATGCAAACTGTAAAACTG	KleADH amplification upstream
KleADH-RP	CCGCTCGAGAACATCAAGTTTGC	KleADH amplification downstream
Asp44Ala-FP	GATACGGGATACCGCCTGATCGCGACCGCAGCGT	Asp44Ala mutation upstream
Asp44Ala-RP	CATTCTGGTAAGACGCTGCGGTCGCGATCAGGCG	Asp44Ala mutation downstream
Tyr49Ala-FP	CGCCTGATCGATACCGCAGCGTCTGCGCAGAATG	Tyr49Ala mutation upstream
Tyr49Ala-RP	ATTCCCGACCTGGGTTTCATTCTGCGCAGACGCTGC	Tyr49Ala mutation downstream
Lys74Ala-FP	GTAACGAGTTCTTTGTAACGACCGCGCTGTGGCT	Lys74Ala mutation upstream
Lys74Ala-RP	AATTCGTATCCTGCAGCCACAGCGCGGTCGTTAC	Lys74Ala mutation downstream
His107Ala-FP	ATTACGTTGACCTTTATCTGATTGCGCAACCTTAC	His107Ala mutation upstream
His107Ala-RP	CCATGGACATCGCCGTAAGGTTGCGCAATCAGAT	His107Ala mutation downstream

Table. 2. Substrate specificity of KleADH

NO.	substrate	product	relative activity	e.e or d.e
1			100%	＞99.5%
2			24.23%	＞99.5%
3			25.49%	78.07%
4			6.70%	＞99.5%
5			5.12%	＞99.5%
6			56.92%	＞99.5%
7			n. d.	n. d.
8			n. d.	n. d.