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ORIGINAL RESEARCH PAPER

Cloning and characterization of three ketoreductases from soil metagenome for preparing optically active alcohols

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Abstract
Objectives To discover novel ketoreductases (KRED) from soil metagenome preparation of chiral alcohols.
Results Three putative KRED were cloned, heterologously expressed in *Eschericha coli* and characterized based on the sequence analysis of soil metagenome. All the three enzymes (KRED424, KRED432, and KRED433) had maximum activity at 55 °C and pH 7. KRED424 had a broader substrate spectrum compared with the other two. Three prochiral carbonyl compounds were used to evaluate the abilities of enantioselective reductions of the KRED. For *N*-Boc-3-pyrrolidone, all enzymes produced an (*S*)-type alcohol in enantiomeric excess (>99 % *ee*). For ethyl 2-oxo-4-phenylbutyrate, KRED424 showed a higher conversion (91.5 %) and enantioselectivity (*S*-type, >99 % *ee*) than KRED432 and KRED433. For ethyl 4-chloroacetoacetate (COBE), both of KRED424 and KRED433 completely converted 20 mM substrate and KRED433 could obtain an (*R*)-alcohol with 94 % *ee*.
Conclusions The three ketoreductases have potential in the preparation of pharmaceuticals and fine chemicals.

Keywords Asymmetric reduction · Ethyl 4-chloroacetoacetate · Ketoreductases · *N*-Boc-3-pyrrolidone · Ethyl 2-oxo-4-phenylbutyrate · Soil metagenome

Introduction

Optically active alcohols are useful chiral intermediates for the synthesis of pharmaceuticals, agricultural chemicals, and specialty materials (Schmid et al. 2001; Schoemaker et al. 2003). Among them, ethyl (*S*)-4-chloro-3-hydroxybutanoate (CHBE) is used for production of the 3-hydroxy-3-methyl glutaryl coenzyme A reductase inhibitors (statins) (Ye et al. 2011), while (*R*)-CHBE is useful for the synthesis of biologically and pharmacologically important materials: (*R*)-carnitine, (*R*)-4-amino-3-hydroxy-butayric acid, and (*R*)-4-hydroxy-2-pyrrolidone (Yamamoto et al. 2002). The chiral ketoester, enantiopure ethyl 2-hydroxy-4-phenylbutyrate, is a common precursor in the manufacture of a variety of angiotensin-converting enzyme inhibitors including benazepril, cilazapril, and enalapril (Wu et al. 2009; D'Arrigo et al. 2010). Also, (*S*)-1-

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Boc-3-hydroxypyrrolidine is a precursor in the synthesis of phosphodiesterase 10A inhibitor (Schwan et al. 2014). (*R*)-1-Boc-3-hydroxypyrrolidine is used for producing analogues of FTY720 (Gilenya) (Fransson et al. 2013).

For preparing optical active alcohols, enzyme-catalyzed biosynthesis is more attractive compared with traditional chemical synthesis because it is a highly selective and green method. It can also be performed under mild reaction conditions (Liu et al. 2014). Chiral alcohols can be obtained by ketoreductases (KRED) which include aldo–keto reductase (AKR), short-chain alcohol dehydrogenase/reductase, and medium-chain dehydrogenase families (Patel 2001; Daußmann et al. 2006). However, the application of KRED has been hampered due to limited availability of KRED with high enantioselectivity and catalytic efficiency. Therefore, discovering novel KRED is a useful approach to solve the problem.

Soil contains many microbial species, the vast majority of which are unknown and cannot be efficiently cultivated (Stoveken et al. 2015). Metagenomics approach has been widely applied to discover novel enzymes, as it could bypass any requirement for specific cultivation of the microbes. Therefore, it is an efficient way to discover novel KRED in soil metagenome. To date, many genes, such as those coding for D-amino acid oxidase, alcohol dehydrogenase and α-L-fucosidases, have been cloned from this source (Ou et al. 2015; Itoh et al. 2014; Lezyk et al. 2016).

In this study, three KREDs were cloned from a soil metagenome. They were heterologously expressed, purified, and characterized in *Eschericha coli.* The substrate spectrum of the three KRED, including aryl ketones, aliphatic ketones, ketoesters, and heterocyclic ketones, were investigated. Three important prochiral carbonyl compounds including ethyl 4-chloroacetoacetate, ethyl 2-oxo-4-phenylbutyrate and *N*-Boc-3-pyrrolidone were further chosen to evaluate the abilities of the obtained KRED in catalyzing enantioselective reduction.

Materials and methods

Materials

The soil sample was from Linyi Shandong China. The soil's metagenomics DNA was isolated by using Mo Bio Power Soil DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacture's instruction and had been sequenced and analyzed by using Metagenomic for Rapid Annotations using Subsystems Technology (MG-RAST) analysis platform. The soil metagenome DNA sequence have been deposited in the MG-RAST database with an accession number 4557667.3. *E. coli* BL21/pET28a(+) was used for expressing KRED. PCR mix and restriction enzymes were purchased from Fermentas. Ketones and chiral alcohol standard samples were purchased from Sigma-Aldrich.

Gene cloning, expression, and purification of three KREDs

Molecular cloning procedures were performed according to standard laboratory techniques. The primers used for gene cloning were as follows, KRED424: forward primer, 5′-GGATCCATGAGTACATTTTCAATTAG-3′ and reverse primer, 5′-AAGCTTAGTTTTTCAAA CTCGCC-3′ (with *Bam*HI and *Hin*dIII sites being underlined); KRED432: forward primer, 5′-GGATC-CATGAGCAATAATACGATTCAAGCTTATG-3′ and reverse primer, 5′-CTCGAGTTATTCTGCAAAGT CTGCTTTAAGCAC-3′ (with *Bam*HI and *Xho*I sites being underlined); KRED433: forward primer, 5′-GGATCCATGATCAAAGATATTTTGCTTATTCAT CTATTTTTTG-3′ and reverse primer, 5′-CTCGAGTT AGTCCCAAACTGGTGCCCATGG-3′ (with *Bam*HI and *Xho*I sites being underlined). The KRED genes were inserted into plasmid pET28a (+) and were transformed into *E. coli* BL21(DE3) for expression. Recombinant *E. coli* was cultivated in lysogeny broth containing antibiotics at 37 °C When OD_{600} reached 0.6–0.8, 0.1 mM IPTG was added. Cells were then cultured at 20 °C for 20 h. The induced cells were harvested by centrifugation (8500×*g*, 10 min). Cells were suspended in Tris/HCl buffer (100 mM, pH 8), disrupted by ultrasonication, and the cell lysate was removed by centrifugation (10,000×*g*, 30 min) at 4 °C. The resulting crude extract was purified by a Ni–NTA superflow column (1 ml, Qiagen). All purification steps were carried out at 4 °C. Protein was measured by the Bradford method.

Enzyme activity assay

Purified enzyme preparations were assayed by measuring the rate of change of NAD(P) or NAD(P)H at

340 nm over 1 min. The reaction mixtures (200 µl) contained 5 mM substrate, 100 mM phosphate buffer (pH 7), 0.25 mM NAD(P)H, and the appropriate enzyme. The reaction mixture was incubated at 55 °C for 2 min and then the enzyme activity was measured. Blanks without the enzymes were carried out for each substrate, and all the experiments were performed in triplicate. One unit enzyme activity was defined as the amount of enzyme required to catalyze the oxidation of 1 µmol NAD(P)H per minute under standard conditions (Shimizu et al. 1990).

Coenzyme dependence

The coenzyme dependence assay was conducted in a reaction mixture (200 µl) containing 5 mM *N*-Boc-3-pyrrolidone, 100 mM Tris/HCl buffer (pH 8), 0.25 mM NADH or NADPH, and the appropriate enzyme. The reaction mixture was incubated at 37 °C for 2 min and then the enzyme activity was measured at 37 °C.

Effects of temperature and pH

The optimum temperature was determined by incubating each enzyme in 100 mM Tris/HCl buffer (pH 8) for 2 min at 30–65 °C and the reaction was started by adding *N*-Boc-3-pyrrolidone as the substrate. For thermostability analysis, the enzyme was pre-incubated for 24 h at 4, 20, 30, 37, 45 or 55 °C. The residual activity was determined under standard conditions.

The optimum pH for KRED was investigated from pH 4 to 10 (see Fig. 5). Enzyme activity was measured at 55 °C with *N*-Boc-3-pyrrolione as the substrate. For pH stability, each enzyme was pre-incubated in the buffer at 4 °C for 24 h. The residual enzyme activity was assayed under standard conditions.

Effect of metal ions

Metal ions (2 mM) were added to the reaction and pre-incubated for 2 min at 37 °C. Enzyme activity was determined under standard conditions with *N*-Boc-3-pyrrolione as the substrate.

Substrate specificity and determination of kinetic parameters

Substrate specificity of each KRED was determined with 5 mM substrates under standard conditions.

Kinetic parameters of the purified enzymes were assayed by measuring the initial velocity at various concentrations of substrates (Mu et al. 2016). To determine the apparent K_m value, the substrate was from 0.5 to 16 mM with NADPH fixed at 0.25 mM. Apparent kinetic parameters of Michaelis constant (K_m) and k_{cat} were further calculated from double reciprocal Lineweaver–Burk plots. All reactions followed Michaelis–Menten-type kinetics.

Enantioselective reduction of ketones

The enantioselectivity of KRED was determined by employing NADPH regeneration system composed of *Bacillus subtilis* D-glucose dehydrogenase (BsGDH) and D-glucose. The BsGDH (GenBank accession no. WP_041340171) gene from *B. subtilis* was cloned and expressed in *E. coli* BL21/pET21a (+) in the form of intracellular protein. Both of the recombinant *E. coli* cells harboring KRED and BsGDH were freeze-dried by a vacuum freeze drier. [The drying procedure may break the recombinant cells and thus allow the cofactor and oxidized cofactor to directly interact with the KRED and BsGDH (Kramer et al. 2002)]. The reaction mixture (1 ml) consisted of 0.05 g glucose, 20 mM substrate, 1 mg BsGDH (dried cells), 0.25 mM $NADP^+$, and 7 mg KRED (dried cells). The mixture was shaken at 37 °C for 20 h. To determine the absolute configuration of chiral alcohols, each sample was extracted twice with an equivalent volume of ethyl acetate, and the organic layer was used for analysis by HPLC. Chiral CHBE was analyzed on a Chiracel OB-H column (Daicel, Japan) at 210 nm using *n*-hexane/2-propanol (90:10, v/v) as eluent at 0.8 ml/min and 25 °C. Chiral HPBE was analyzed on the same column at 210 nm using *n*-hexane/2-propanol (98:2, v/v) at 1 ml/min and 25 °C. Chiral *N*-Boc-3-pyrrolidinol was analyzed on the same column at 210 nm using *n*-hexane/2-propanol (95:5, v/v) at 0.8 ml/min and 25 °C.

Results and discussion

Gene cloning and identification of three KRED

Based on the sequence analysis result of the soil metagenome, three ORFs that were putative KRED were discovered (Table 1). Among them, KRED424 and KRED432 were annotated as alcohol

Table 1 Properties of the putative ketoreductase genes and their coding products discovered from soil metagenome

Gene	GenBank accession number	Length of ORF (bp)	Amino acid residue number of encoded protein	Theoretical MW of encoded protein (kDa)
KRED424	KU196786	1056	351	41.7
KRED432	WP_032063522	1029	342	39.8
KRED433	KU196787	903	300	37.2

dehydrogenases. KRED433 was annotated as an aldo–keto reductase. The KRED genes were cloned into pET28a(+) vector which were then introduced into *E. coli* BL21(DE3) for expression.

Protein basic Local Alignment Search Tool analysis (BLAST-P) indicated that KRED424 and KRED432 showed high identities with cinnamyl alcohol dehydrogenase (CAD) family which is a

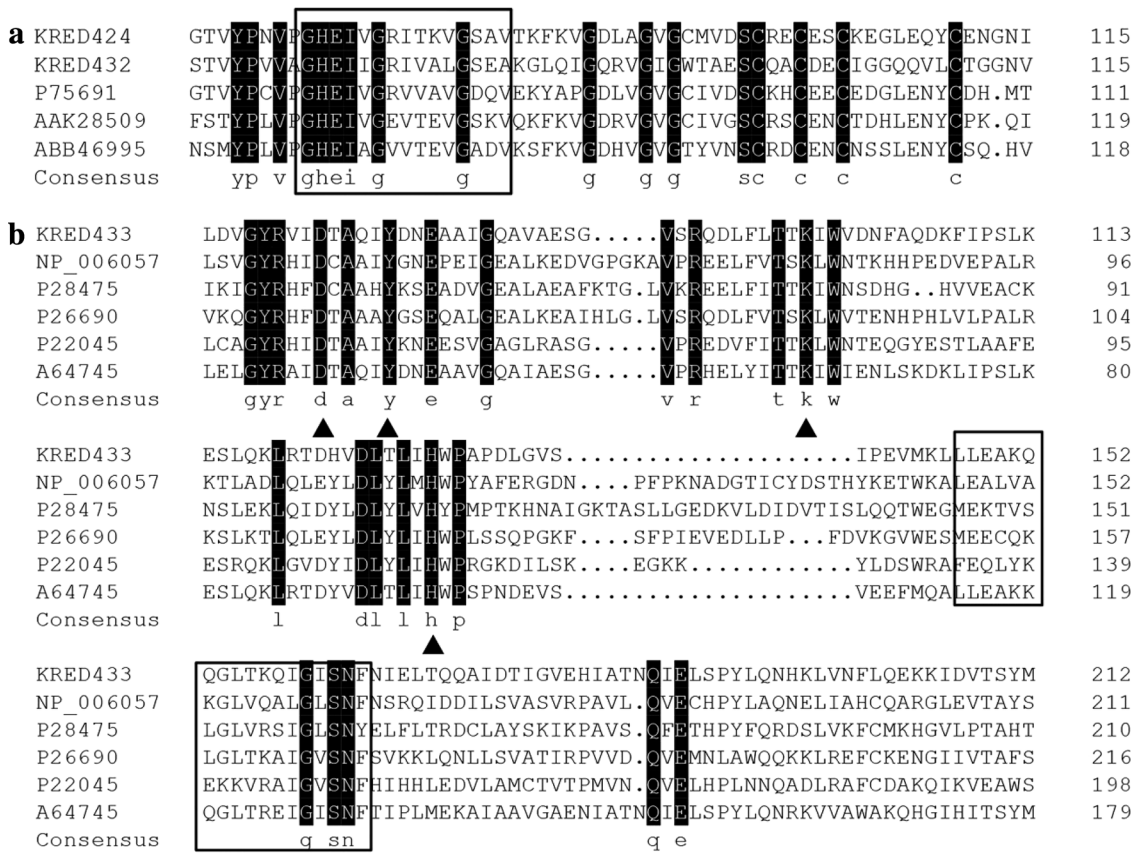

Fig. 1 Amino acid sequence alignment of putative ketoreductases and other selected ketoreductases. **a** The sequence alignment result of KRED424, KRED432, and other selected cinnamyl alcohol dehydrogenases. GenBank accession numbers were as follows: Aldehyde reductase YahK from *Escherichia coli K-12* (P75691), cinnamyl alcohol dehydrogenase from *Fragaria x ananassa* (AAK28509), and mannitol dehydrogenase from *Oryza sativa Japonica Group* (ABB46995). Conserved zinc-containing alcohol dehydrogenases signature (PROSITE accession number PS00059) was involved in the *textbox*. **b** The sequence alignment result of KRED433 and other aldo–keto reductases. GenBank accession numbers were as follows: Aldehyde reductase from *Homo sapiens* (NP_006057), $NADP^+$-dependent sorbitol-6-phosphate reductase from *Malus domestica* (P28475), 2,5-diketo-D-gluconic acid reductase B from *E. coli K-12* (A64745), NAD(P)H dependent 6′-eoxychalcone synthase from *Glycine max* (P26690), and reductase from *Leishmania major* (P22045). The key catalytic tetrad of D, Y, K and H residues were marked with *triangle*. Also, an aldo–keto reductase family signature 2 (PROSITE accession number PS00062) was involved in the *text box*. The sequences were aligned using the DNAMAN program

member of the middle-chain dehydrogenase superfamily. As showed in Fig. 1a, a conserved zinc-containing alcohol dehydrogenase signature (PROSITE accession number PS00059) could be discovered in all the five enzymes. A phylogenetic tree (Fig. 2a), constructed using the neighbor-joining method, showed that both of KRED424 and KRED432 would be the members of the CAD1 family.

BLAST-P showed that KRED433 was more similar with the AKR superfamily. As shown in Fig. 1b, these amino acid sequences shared the conservative sites of Asp^{66}, Tyr^{71}, Lys^{96}, and His^{129} (the number of amino acid residues in this article corresponded to that of KRED433), which constituted the catalytic tetrad of AKR. An AKR family signature 2 (PROSITE accession number PS00062) could be observed in all the sequences of putative enzymes. A phylogenetic tree (Fig. 2b) constructed using the neighbor-joining method showed that KRED433 was supposed to belong to the AKR3 family.

Expression and purification of KRED

The three KRED were expressed in soluble form. The recombinant enzymes were purified using a Ni–NTA affinity chromatography with appropriate imidazole elution concentration. SDS-PAGE showed distinct bands with the size consistent with the theoretically calculated molecular weight (Fig. 3).

Coenzyme dependence

To determine the coenzyme specificity of each KRED, 0.25 mM NADPH or NADH was used as the cofactor in a standard reaction mixture. All the three KRED

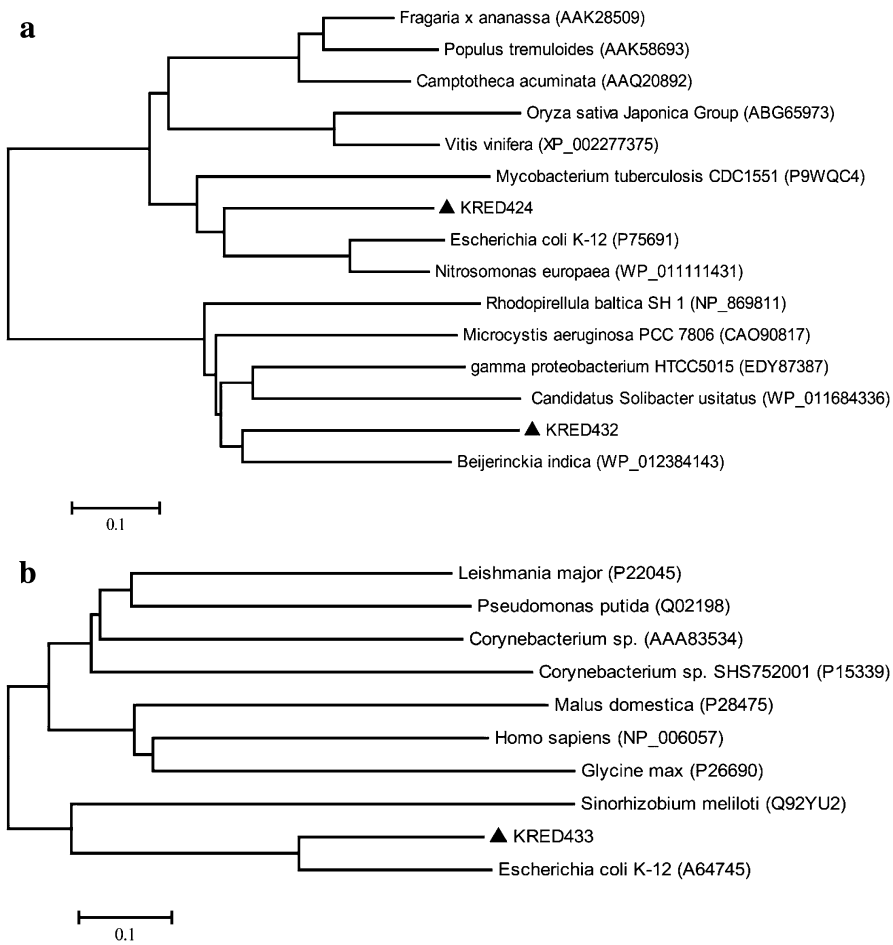

Fig. 2 Phylogenetic tree of putative ketoreductases and other selected ketoreductases. **a** The neighbour-joining phylogenetic tree of KRED424, KRED432 protein sequence, and other cinnamyl alcohol dehydrogenases. KRED424 and KRED433 were marked by *triangle*. **b** The neighbour-joining phylogenetic tree of KRED433 protein sequence and other aldo–keto reductases. KRED433 was marked by *triangle*. All the sequences used in phylogenetic analysis were from GenBank. The tree was constructed using MEGA 6 software with Jones–Taylor–Thornton model. The value of 0.1 represented the evolution distance

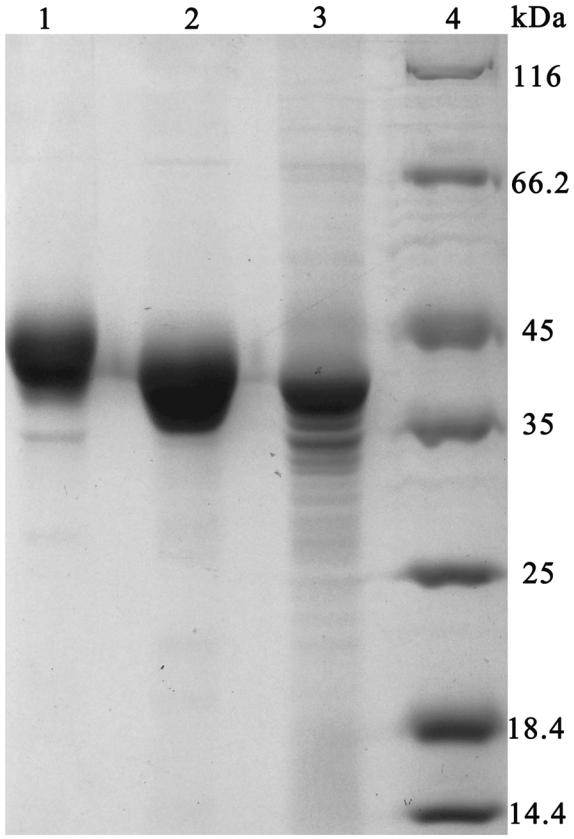

Fig. 3 SDS-PAGE analysis of the purified ketoreductases. *Lane 1*, purified KRED424; *Lane 2*, purified KRED432; *Lane 3*, purified KRED 433; *Lane 4*, molecular mass standard

showed catalytic activity with NADPH, but no apparent activity with NADH, suggesting that they were all NADPH-dependent KRED.

Effects of temperature and pH

All three KREDs had maximum activity at 55 °C and pH 7 (Fig. 4a). The KREDs retained >70 % activity from 45 to 65 °C. In contrast to KRED424 and KRED432, KRED433 was more sensitive from 55 to 65 °C. All three KRED were relatively stable at 4, 20, and 30 °C for 24 h (>60 % residue activity), and remained active (>39 %) at 37 °C for 24 h (Fig. 4b).

KRED424 and KRED432 retained activity (>50 %) from pH 5 to 9. KRED433 retained activity >50 % only from pH 6 to 8 (Fig. 5a–c). In addition, KRED432 had a broader catalytic pH than KRED424 and KRED433. KRED432 and KRED433 retained activity >70 % at pH 6 to 10 for 24 h, and showed alkali tolerance. KRED424 retained activity >70 % only from pH 7 to 9 for 24 h (Fig. 5d–f).

The three KRED showed higher optimal temperatures than Gox2036 (30 °C) from *Gluconobacter oxydans* (Liu et al. 2014) and YlCR2 (30 °C) from *Yarrowia lipolytica* (Xu et al. 2016), and had similar optimal temperature with LEAKR48 (55 °C) from *Lodderomyces elongisporus* (Ning et al. 2014). In the case of optimal pH, all the three KRED showed a higher pH than Gox2036 (pH 6), LEAKR48 (pH 6), and YICR2 (pH 5). For pH stability, the KRED were more stable in alkali condition, while Gox2036 exhibited higher stability in acidic condition, LEAKR48 had a wide range of pH stability ranging from 3 to 10.

Effect of metal ions

There was no significant ion dependency or inhibition for any of the three KREDs when tested with Li^{+}, K^{+}, Ca^{2+}, Cu^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Zr^{2+} or Fe^{3+} each at 2 mM (see Supplementary Table 1).

Substrate specificity

KREDs can asymmetrically reduce carbonyl compounds to produce optically active alcohols, differing in stereospecificities and substrate specificities (Huisman et al. 2010). To evaluate the substrate specificities of the KRED, ketones including aryl ketones, aliphatic ketones, ketoesters, and heterocyclic ketones were selected. As shown in Table 2, the three KRED had different substrate specificities. KRED424 had a broad substrate spectrum and showed activity to all of tested substrates. Ketoesters, including ethyl butyrylacetate, COBE, and ethyl bromopyruvate were its most preferred substrates. KRED433 showed activity to most of the tested substrates especially to COBE which was its best substrate (1.84 U/mg). Higher activity against ketoesters compared with the other three kinds of ketons was found with KRED433. KRED432 only showed activity to ketoesters and heterocyclic ketones, indicating a narrow substrate spectrum. Compared with Gox2036 from *G. oxydans* (Liu et al. 2014) and LEAKR48 from *L. elongisporus* (Ning et al. 2014), the three KRED showed lower activities. For example, Gox2036 (7.15 U/mg) had much higher activity than the KRED towards OPBE.

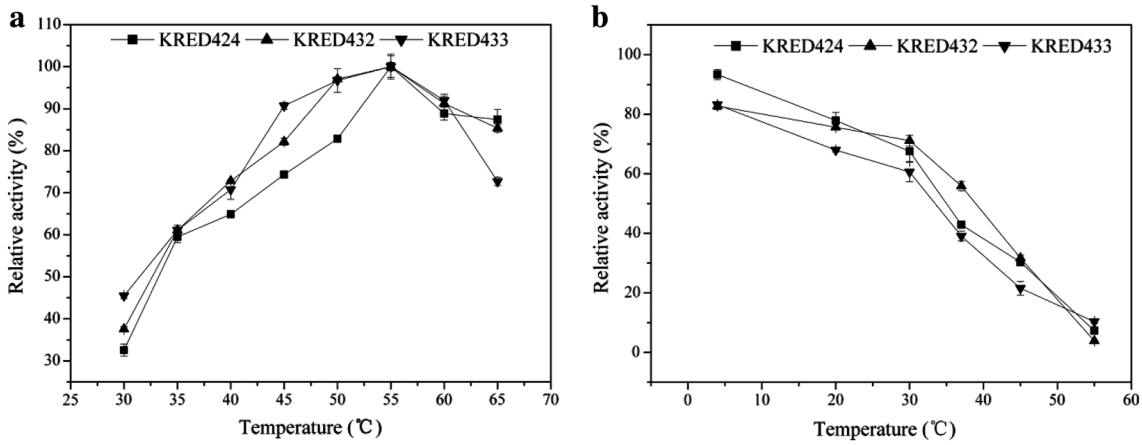

Fig. 4 Effect of temperature on the ketoreductases (KRED) activity and stability. **a** Optimum temperature of the KRED. The enzyme activity was determined with *N*-Boc-3-pyrrolidone as the substrate in 100 mM Tris/HCl buffer (pH 8) from 30 to 65 °C. Activity at 55 °C (optimum temperature) was taken to be 100 % (0.043, 0.044, and 0.048 U/mg for KRED424, 432, and 433, respectively). The optimal temperature was derived from initial enzyme activities measured within the first 2 min of reaction. **b** Thermal stability of the KRED. The purified enzymes were incubated in phosphate buffer (pH 7) at different temperatures for 24 h and the residual activity with *N*-Boc-3-pyrrolidone as substrate was measured. The activity at initial time was taken as 100 % (0.07, 0.052, and 0.066 U/mg for KRED424, 432, and 433, respectively). The experiments were done in triplicate and the *error bar* represented the percentage error in each set of reading. The *line chart* was made by Origin 8 program

Determination of kinetic parameters and enantioselective reductions of ketones

Kinetic parameters (K_m, k_{cat}, and k_{cat}/K_m) and enantioselective reductions of ketones were evaluated towards COBE, OPBE, and *N*-Boc-3-pyrrolidone which were important prochiral carbonyl compounds (Table 3). For COBE, both of KRED424 and KRED433 showed useful specificities with k_{cat}/K_m values of 94.4 and 232 $M^{-1}S^{-1}$, respectively suggesting that they had potential in preparing chiral CHBE. KRED424 had a lower K_m value (3.2 mM) than KRED433 (5.9 mM), indicating that the KRED424 could reach its maximal reactive velocity at a lower substrate concentration, which is important because high concentrations of substrate can destabilize the enzyme. For OPBE, The apparent K_m value of KRED433 was 2.9 mM, which was lowest in the three test enzymes. However, its k_{cat} was low at 0.03 s^{-1}. For *N*-Boc-3-pyrrolione, KRED432 and KRED433 had lower K_m values compared with KRED424, while KRED424 had a higher k_{cat} value than KRED432 or KRED433.

To further evaluate the abilities of enantioselective reductions of the KRED on the three important prochiral carbonyl compounds (COBE, OPBE and *N*-Boc-3-pyrrolidone), the conversion rates and absolute configurations of the corresponding chiral alcohol products were determined (Table 4). For COBE, both of KRED424 and KRED433 completely converted 20 mM substrate. KRED433 produced an (*R*)-alcohol with an *ee* of 94 % towards COBE and KRED424 showed a relatively lower stereoselectivity (*S*-type, 52.3 % *ee*). For OPBE, KRED424 showed a higher conversion (91.5 %) and stereoselectivity (*S*-type, >99 % *ee*) than KRED432 and KRED433. For *N*-Boc-3-pyrrolidone, all three KREDs produced (*S*)-type alcohols with excellent enantioselectivities (>99 % *ee*) suggesting that they have a potential to prepare chiral *N*-Boc-3-pyrrolidinol.

There are reports that KRED can reduce COBE and OPBE to the corresponding chiral alcohols, such as LEAKR48, from *L. elongisporus* (Ning et al. 2014) and Gox2036 from *G. oxydans* (Chen et al. 2014). For COBE, compared with KRED433 (*R*-type, 94 % *ee*), LEAKR48 had a lower stereoselectivity (*R*-type, 70 % *ee*). LEAKR48 (0.76 mM) also had a much lower K_m than KRED433 (5.9 mM). For OPBE, Gox2036 produced an (*R*)-alcohol with an *ee* of 52.7 %, while KRED433 showed a higher *ee* of 94 % with similar type. KRED424 and KRED432 produced (*S*)-alcohols. Gox2036 showed a lower K_m

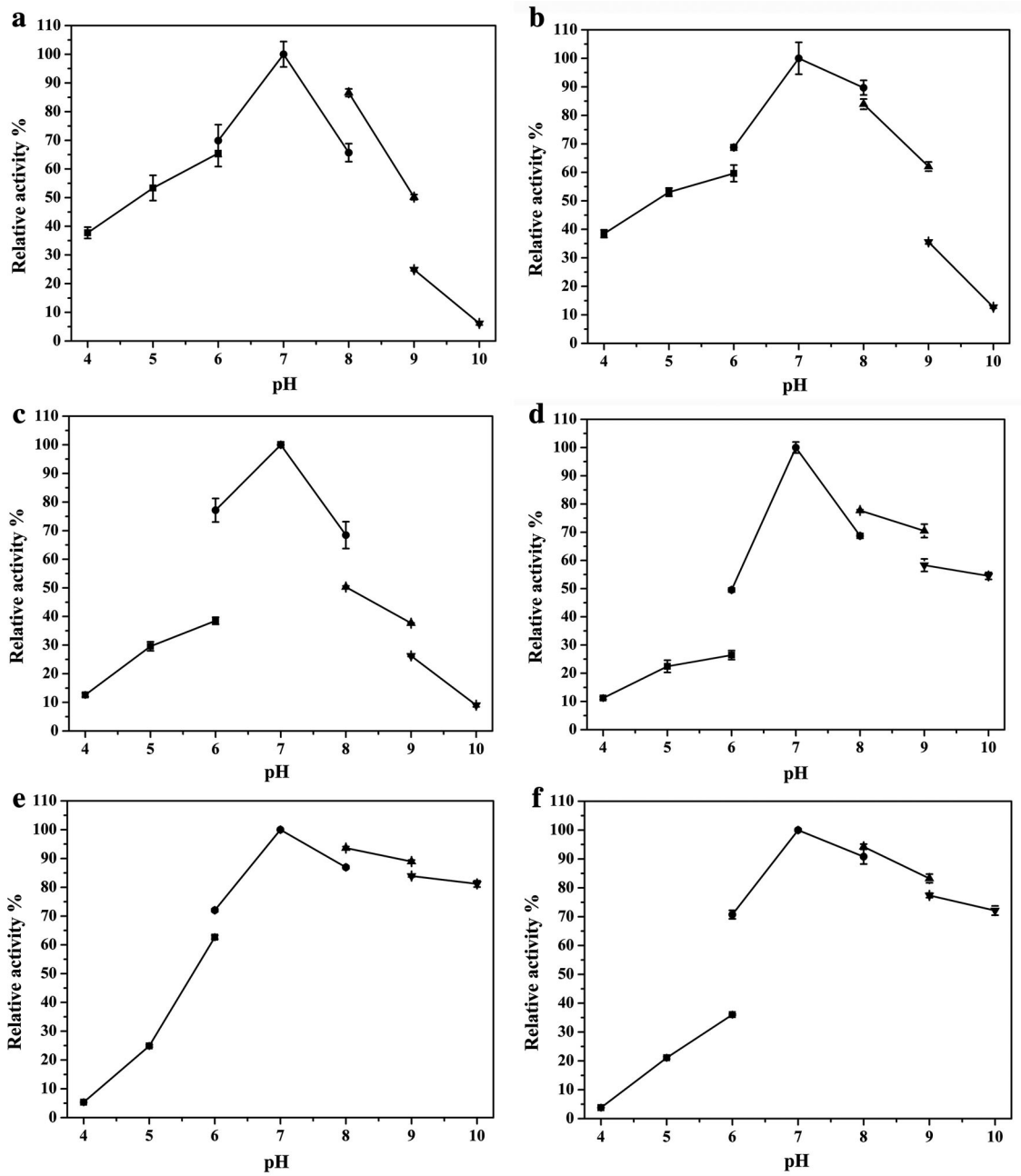

Fig. 5 Effect of pH on the ketoreductases (KRED) activity and stability. **a, b, c** Optimum pH of KRED424, KRED432, and KRED433, respectively. The enzyme activity was determined with *N*-Boc-3-pyrrolidone as the substrate at pH ranging from 4 to 10 at 55 °C. The activity at pH 7 (optimum pH) was taken to be 100 % (0.069, 0.059, 0.063 U/mg for KRED424, 432, and 433 respectively). **d, e, f** pH stability of KRED424, KRED432, and KRED433, respectively. The purified enzymes were incubated at 4 °C and different pH for 24 h. The activity at pH 7 was taken as 100 % (0.058, 0.062, and 0.057 U/mg for KKRED424, 432, and 433, respectively). The activity was measured in the following 100 mM buffers: sodium citrate/citric acid buffer (pH 4–6, 100 mM, *square*), sodium phosphate buffer (pH 6–8, 100 mM, *circle*), Tris/HCl buffer (pH 8–9, 100 mM, *triangle*), glycine/NaOH buffer (pH 9–10, 100 mM, *inverted triangle*). The experiments were done in triplicate and the *error bar* represented the percentage error in each set of reading. The *line chart* was made by Origin 8 program

Table 2 Substrate spectrum of the ketoreductases

Substrate[a]	Activity[b] (U/mg)		
	KRED424	KRED432	KRED433
Acetophenone	0.009 ± 0	NMA[c]	NMA
2′-Chloroacetophenone	0.039 ± 0.001	NMA	0.035 ± 0.001
2′-Bromoacetophenone	0.008 ± 0	NMA	0.021 ± 0
p-Nitroacetophenone	0.038 ± 0.001	NMA	NMA
4-Methoxyphenacyl chloride	0.022 ± 0.001	NMA	NMA
2-Octanone	0.005 ± 0	NMA	0.003 ± 0.000
3-Pentanone	0.002 ± 0	NMA	0.002 ± 0
Ethyl butyrylacetate	0.399 ± 0.004	NMA	NMA
Methyl-4-chloroacetoacetate	0.185 ± 0.012	NMA	0.116 ± 0.003
Ethyl 4-chloroacetoacetate	0.428 ± 0.016	NMA	1.837 ± 0.032
Ethyl-2-chloroacetoacetate	0.026 ± 0.001	0.013 ± 0	0.010 ± 0
Pyruvic acid methyl ester	0.018 ± 0	NMA	0.166 ± 0.012
Ethyl pyruvate	0.052 ± 0	NMA	0.097 ± 0.001
Ethyl bromopyruvate	0.805 ± 0.018	0.006 ± 0	0.057 ± 0.001
Ethyl 2-oxo-4-phenylbutyrate	0.026 ± 0.001	0.018 ± 0.001	0.036 ± 0.002
N-Boc-3-pyrrolidone	0.069 ± 0.001	0.059 ± 0	0.063 ± 0.001
2-Acetylpyridine	0.021 ± 0	NMA	NMA

[a] Each substrate was at 5 mM

[b] The activity of the enzymes was determined by the standard enzyme assay. The experiments were done in triplicate and the error bar represented the standard error of the mean

[c] No measurable activity

Table 3 Kinetic parameters of the ketoreductases

Enzyme	COBE			OPBE			*N*-Boc-3-pyrrolidone		
	K_m^a (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	K_{mc} (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
KRED424	3.2	0.31	94.4	8.9	0.03	2.9	6.2	0.06	9.3
KRED432	NMA[b]	NMA	NMA	7	0.02	2.4	3.4	0.05	14
KRED433	5.9	1.37	233	2.9	0.03	10.7	3.2	0.05	15.8

[a] To determine the apparent K_m value, a range of substrate concentration from 0.5 to 16 mM was assayed with a fixed concentration of NADPH at 0.25 mM. Apparent kinetic parameters of Michaelis constant (K_m) and k_{cat} were further calculated from double reciprocal Lineweaver–Burk plots. The activity of the enzymes was determined by the standard enzyme assay

[b] No measurable activity

Table 4 Asymmetric reduction of three ketones catalyzed by the ketoreductases

Substrate[a]	KRED424		KRED432		KRED433	
	con. (%)	*ee* (%)	*con.* (%)	*ee* (%)	*con.* (%)	*ee* (%)
N-Boc-3-pyrrolidone	96.9	100 (*S*)	99	99 (*S*)	100	100 (*S*)
COBE	100	52.3 (*S*)	NMA[b]	NMA	100	93.9 (*R*)
OPBE	91.5	99.4 (*S*)	75.9	77.1 (*S*)	86.1	74.8 (*R*)

[a] Concentration of ethyl 4-chloroacetoacetate (COBE), ethyl 2-oxo-4-phenylbutyrate (OPBE), and *N*-Boc-3-pyrrolidone was 20 mM

[b] No measurable activity

value (1.5 mM) than the KRED. KRED424 had a broad substrate spectrum and showed an excellent enantioselectivity towards OPBE and *N*-Boc-3-pyrrolidone indicating its application potential for the synthesis of pharmaceutical intermediates and fine chemicals.

Conclusion

Three ketoreductases (KREDs) from soil metagenome were cloned and expressed in *E. coli* BL21(DE3). All KREDs were stable above pH 7. KRED424 showed a broader substrate range than KRED432 and KRED433. Three KREDs have practical applications for the production of pharmaceuticals and fine chemicals.

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Supporting Information Supplementary Table 1—Effect of metal ions on the activity of the ketoreductases.

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