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An ene reductase from *Clavispora lusitaniae* for asymmetric reduction of activated alkenes

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ABSTRACT

A putative ene reductase gene from *Clavispora lusitaniae* was heterologously overexpressed in *Escherichia coli*, and the encoded protein (*Cl*ER) was purified and characterized for its biocatalytic properties. This NADPH-dependent flavoprotein was identified with reduction activities toward a diverse range of activated alkenes including conjugated enones, enals, maleimide derivative and α,β-unsaturated carboxylic esters. The purified *Cl*ER exhibited a relatively high activity of 7.3 U mg_{prot}^{-1} for ketoisophorone while a remarkable catalytic efficiency (k_{cat}/K_m = 810 s^{-1} mM^{-1}) was obtained for 2-methyl-cinnamaldehyde due to the high affinity. A series of prochiral activated alkenes were stereoselectively reduced by *Cl*ER furnishing the corresponding saturated products in up to 99% *ee*. The practical applicability of *Cl*ER was further evaluated for the production of (*R*)-levodione, a valuable chiral compound, from ketoisophorone. Using the crude enzyme of *Cl*ER and glucose dehydrogenase (GDH), 500 mM of ketoisophorone was efficiently converted to (*R*)-levodione with excellent stereoselectivity (98% *ee*) within 1 h. All these positive features demonstrate a high synthetic potential of *Cl*ER in the asymmetric reduction of activated alkenes.

1. Introduction

Biocatalysis is continuing to gain momentum and is now becoming a key component in the chemist's toolbox, with a place alongside chemo-catalysis [1]. Biocatalysts are environmentally benign and have inherent advantages of high chemo-, regio- and stereoselectivities. One class of enzymes that has received much attention and has been intensely investigated in recent years is the so-called ene reductases (ERs, E.C. 1.3.1.x), nicotinamide-dependent flavoproteins from the old yellow enzyme (OYE) family. These enzymes catalyze the stereoselective reduction of conjugated C=C bonds of structurally diverse α,β-unsaturated compounds activated by an electron withdrawing group, *e.g.* ketone, aldehyde, carboxylic acid/ester, lactone, imide, nitro and nitrile [2]. Unlike transition metal-catalyzed *syn*-hydrogenation, ER-catalyzed alkene reduction proceeds in a *trans*-specific fashion [3]. The optically active products of bioreduction represent important chiral building blocks in industrial applications. A prominent example like (*R*)-levodione, obtained by asymmetric bioreduction of ketoisophorone, is used as a precursor for the synthesis of carotenoids (*e.g.* zeaxanthin, crytoxanthin and xanthoxin) [4].

ERs are ubiquitous in nature and many of them have been isolated from microorganisms and plants [2,5] since the very early discovery of the first member (OYE1) from *Saccharomyces pastorianus* in 1933 [6]. Although ERs show some similarities concerning the substrate spectrum, the catalytic activity and stereoselectivity vary widely between homologous enzymes [7–9]. Actually, the modest reaction rate and the resulting low total turnover numbers (TTNs) of most known ERs represent a major limitation on the way toward practical preparative application [10]. Thus, there is a great interest in searching for new and robust ERs performing highly efficient reactions. Apart from conventional biocatalyst screening from soil samples or culture collections, the tremendous genomic database provides a large pool for the mining and creation of new enzymes [11].

In this study, we present an ene reductase (*Cl*ER) from *Clavispora lusitaniae* which was discovered by *in silico* data mining based on sequence homology. *Cl*ER was cloned, over-expressed and characterized for its biocatalytic properties, particularly regarding its substrate scope and stereoselectivity. Furthermore, we show its applicability for the production of (*R*)-levodione from ketoisophorone.

2. Materials and methods

2.1. Cloning, expression and purification of ClER

The genomic DNA of *C. lusitaniae* (CGMCC 2.1597) was isolated using the TIANamp Bacteria DNA Kit from Tiangen (Shanghai, China) and then

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used as template for amplification of the ene reductase gene (GenBank Accession No. XM_002615435) using the primers with an *Nde*I and a *Hind*III restriction site (5′-GGAATTCCATATGGTTGCAGTAAAGCCTT-3′ and 5′-CCCAAGCTTTTACGCCTTGGTATCAATAG-3′). The PCR product was double-digested with *Nde*I and *Hind*III, and integrated into expression vector pET28a(+), which was subsequently transformed into *Escherichia coli* BL21(DE3) cells (Invitrogen). The recombinant strain was cultivated at 37 °C in LB medium containing 50 μg/mL kanamycin until the OD_{600} of the culture reached 0.6. IPTG was added as an inducer to a final concentration of 0.2 mM and the culture was incubated at 25 °C for an additional 12 h. Cells were harvested by centrifugation (13,000 × *g*, 10 min) and washed with normal saline solution (0.9% w/v). Harvested cells were resuspended in 20 mM sodium phosphate buffer (pH 7.0) and disrupted by ultrasonication for 90 times at 400 W for 4 s with 6 s of interval under ice bath. After a centrifugation step (13,000 × *g*, 20 min), the cleared crude extract was used for protein purification on ÄKTA using a HisTrap FF column (5 mL, GE Healthcare). The target protein was eluted with an increasing gradient from 10 to 500 mM of imidazole in sodium phosphate buffer. After dialyzation for desalting, the enzyme was concentrated fourfold to 2.3 mg_{prot}/mL and stored at −20 °C for further use.

2.2. Protein analysis

Protein concentration was determined using the Bradford assay. SDS–PAGE was carried out on 4–12.5% Bis–Tris gels in Tris–glycine buffer with Protein Molecular Weight Marker from TaKaRa. The native molecular weight was determined by gel filtration chromatography using a TSK gel 2000SWxl column (Tosoh) equilibrated with 100 mM sodium phosphate buffer (pH 7.0) containing 100 mM Na_2SO_4 at a flow rate of 0.4 mL/min. Protein molecular weight standards were horse heart cytochrome c (12.4 kDa), bovine carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), yeast alcohol dehydrogenase (150 kDa), and sweet potato β-amylase (200 kDa) from Sigma–Aldrich.

2.3. Determination of flavin content

Flavin coenzyme content was determined by HPLC using a reverse phase C_{18}-HPLC column. The enzyme sample was incubated for 15 min at 100 °C to release the flavin cofactor, followed by centrifugation (18,000 × *g*, 20 min) for the removal of the denatured protein. After ultrafiltration, the supernatant was applied to HPLC column and eluted with an isocratic mobile phase of methanol/H_2O (3:7, *v*/*v*). Flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) were used as standards.

2.4. Activity assay and determination of kinetic parameters

The ene reductase activity was assayed at 30 °C by measuring the oxidation of NADPH or NADH through the decrease in absorbance at 340 nm on a UVmini-1240 spectrophotometer (Shimadzu) under aerobic condition. The standard assay mixture (1 mL) was composed of 100 mM potassium phosphate buffer (pH 6.0), 2.0 mM alkene substrate, 0.15 mM NADPH, and an appropriate amount of purified enzyme. One unit of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol NADPH per minute.

Kinetic parameters for the reduction of different alkenes were determined by standard activity assay in triplicate at various substrate concentrations and a constant NADPH concentration (0.15 mM). The K_m and k_{cat} values were calculated from non-linear regression of Michaelis–Menten plots.

2.5. Effect of pH and temperature on activity

The optimum pH was determined by standard activity assay at different pH (5.0–11.0), with sodium citrate (50 mM) for a pH range from 5.0 to 6.0, sodium phosphate (50 mM) for pH range from 6.0 to 9.0, and Gly-NaOH (50 mM) for pH range from 9.0 to 11.0. The optimum temperature was determined under standard conditions at different temperatures in the range of 20–60 °C. The stability was examined by incubating the purified enzymes in the phosphate buffer (pH 7.0) at 30 °C, 40 °C and 50 °C for a varied period of time, and the residual activity was assayed as described above.

2.6. Bioconversion of activated alkenes

The bioconversion of alkenes were performed in a reaction mixture (1.0 mL) comprising 100 mM potassium phosphate buffer (pH 6.0), 5 mM alkene substrate, 1.0 μM purified *Cl*ER, 2.5 U GDH (glucose dehydrogenase from *Bacillus subtilis* prepared as described previously [12]), 20 mM glucose and 0.5 mM $NADP^+$. All the alkene substrates were added as a 0.5 M ethanol solution (1% final ethanol conc.). The reaction mixture was shaken for 5 h at 30 °C and then extracted twice with ethyl acetate. The extract was analyzed by GC or HPLC to determine the substrate conversion and enantiomeric excess of the product.

2.7. Analytical methods

GC analysis of conversion was performed on a Shimadzu GC-2014 instrument using CP-Chirasil-DEX CB column (25 m × 0.25 mm, Varian). The enantiomeric excess of (*R*)-levodine, (2*R*,5*R*)-dihydrocarvone, (2*R*,5*S*)-dihydrocarvone, (*S*)-2-methyl-2-cyclopentanone, (*S*)-3-methyl-2-cyclopentanone and (*R*)-methyl 2-phenylpropanoate was determined by GC using CP-Chirasil-DEX-CB column as described previously [13,14]. The enantiomeric excess of (*S*)-2-methyl-hydrocinnamaldehyde, dimethyl (*R*)-2-methylsuccinate and (*R*)-3-methyl-1-phenyl-2,5-pyrrolidinedione was determined by HPLC using Chiralcel OD-H column (250 mm × 4.6 mm, Daicel) or Chiralcel OJ-H column (250 mm × 4.6 mm, Daicel) as described previously [13,15,16]. Products and their absolute configurations were identified by comparison with reference materials and/or previously published data.

2.8. Preparative synthesis of (*R*)-levodione

For the synthesis of (*R*)-levodione from ketoisophorone, the cell lysate of *E. coli* expressing *Cl*ER (1.0 g wet cells) in 10 mL potassium phosphate buffer (100 mM, pH 6.0) containing 1.0 M glucose, 0.5 mM $NADP^+$, 50 mg crude enzyme of GDH (700 U) and 500 mM ketoisophorone was incubated at 30 °C. The pH was automatically adjusted to 6.0 by titrating 1.0 M Na_2CO_3. The product was extracted twice with ethyl acetate followed by centrifugation (13,000 × *g*, 10 min) for the phase separation. The organic solution was combined, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. (*R*)-Levodione was afforded as a white solid, with an isolated yield of 80%. $[\alpha]_D^{30}$: −259.3 (*c* 1.0, MeOH) {lit. [17] $[\alpha]_D^{25}$: −269.2 (*c* 0.98, MeOH), (*R*)}.

3. Results

3.1. Identification and sequence analysis

Three hypothetical proteins from *C. lusitaniae* (C4Y835, C4Y7C4 and C4Y9K9), each annotated with an OYE-like FMN binding domain, were identified by a BLAST search using OYE1 from *S. pastorianus* (Q02899) as the template. These three proteins share high sequence identities (72%, 73% and 81%) with each other. Protein C4Y835, designated as *Cl*ER, is predicted with the biggest chance of solubility when overexpressed in *E. coli* by using online prediction programs and thus was chosen for further studies. Phylogenetic analysis of the three putative ERs from *C. lusitaniae* and 28 published OYEs (Fig. S1) indicates that *Cl*ER belongs to yeast OYE. *Cl*ER is most closely related to *Pichia stipites* OYE2.6 [18] with 56% identity. Amino acid sequence alignment of *Cl*ER with the other two putative ERs and known yeast OYEs (Fig. S2) shows high conservation in regions like the catalytic tyrosine and the residues for substrate binding.

3.2. Cloning, protein expression and purification

*Cl*ER was heterologously expressed in *E. coli* cells with an *N*-terminal His_6-tag. The recombinant protein was mainly present in the soluble fraction and was purified by nickel affinity chromatography. The specific activity of the purified enzyme on ketoisophorone was 7.3 U mg_{prot}^{-1}, corresponding to a threefold improvement in specific activity compared to the crude extract. SDS–PAGE analysis revealed the apparent subunit molecular size of 45 kDa (Fig. 1), in line with the theoretical value of the enzyme. The native molecular weight was determined to be 79 kDa by gel-filtration chromatography, which suggests a dimer structure for *Cl*ER, same as OYE1 from *S. pastorianus* and OYE2.6 from *P. stipites* [18]. The requirement of the ene reductase for a flavin coenzyme was investigated by HPLC analysis of the heat-denatured enzyme solution. The only detected FMN indicated *Cl*ER as an FMN-containing protein.

3.3. Cofactor specificity

The purified *Cl*ER was found to catalyze the oxidation of both NADH and NADPH in the presence of molecular oxygen (4.6 and 6.5 mol of NADH/NADPH oxidized min^{-1} mol^{-1} of enzyme,

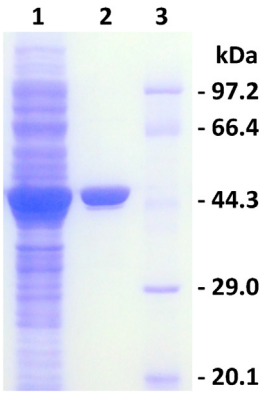


Fig. 1. SDS–PAGE analysis of the purified *Cl*ER. Lane 1, crude extract; Lane 2, purified enzyme; Lane 3, protein markers.

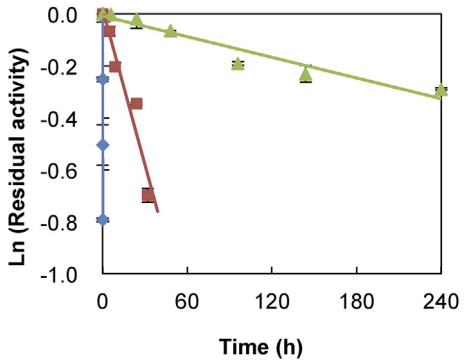


Fig. 3. Thermostability of the purified enzyme at 30 °C (▲), 40 °C (■), and 50 °C (♦). Residual activity was expressed as a percentage of the activity measured initially without any pre-incubation.

respectively), but a remarkably accelerated rate of NADPH oxidation in the presence of enones was observed (*e.g.* 655 mol of NADPH oxidized min^{-1} mol^{-1} of enzyme in the presence of ketoisophorone). When NADH rather than NADPH was used, the rate of oxidation barely increased with ketoisophorone as the substrate (6.2 mol of NADH oxidized min^{-1} mol^{-1} of enzyme), indicating NADPH as the preferred cofactor.

3.4. Dependence of enzyme activity on pH and temperature

The effects of temperature and pH on *Cl*ER were investigated by monitoring the change in enzyme activity over a range of 20–60 °C (Fig. 2**A**), and a pH range of 5.0–11.0 (Fig. 2**B**). The purified enzyme exhibited an optimal activity at 40 °C, while the enzyme activity dropped rapidly over 45 °C with only 50% relative activity at 50 °C. The activity-pH profile showed a pH optimum at 6.0–6.5 in phosphate buffer and more than 70% activity was retained between pH 5.5 and 9.0.

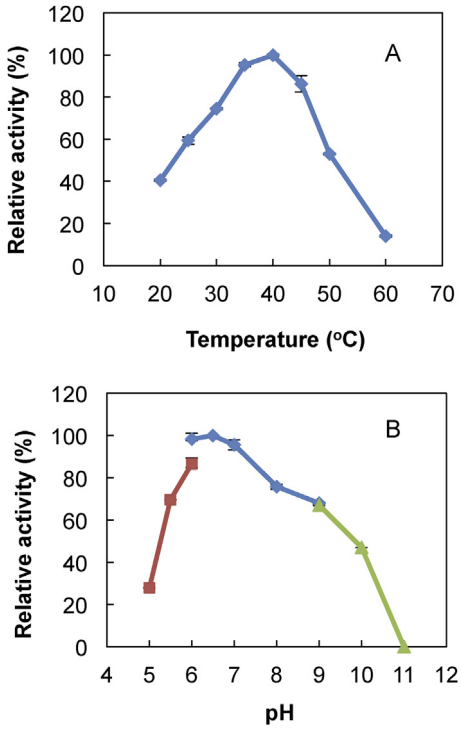


Fig. 2. Effects of pH and temperature on activity of the purified enzyme. (A) Activity-temperature profile was estimated at various temperatures (20–60 °C) in phosphate buffer (100 mM, pH 7.0) using standard assay. (B) Activity-pH profile was determined using standard assay in the following 100 mM buffer: (i) citrate (pH 5.0–6.0) (■), (ii) phosphate (pH 6.0–9.0) (♦), and (iii) Gly-NaOH (pH 9.0–11.0) (▲). Relative activity was expressed as a percentage of the maximum activity under experimental conditions.

Studies concerning the thermostability were performed at 30, 40 and 50 °C (Fig. 3). The enzyme was stable at 30 °C and 40 °C but labile at 50 °C, giving half-lives of 495 h, 36 h and 6 min at 30, 40 and 50 °C, respectively. It suggests that although high temperatures resulted in a rapid loss of enzyme activity, *Cl*ER was quite stable under mild reaction conditions.

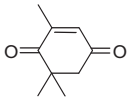
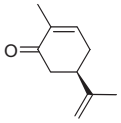
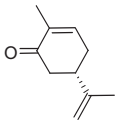
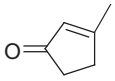
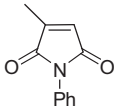
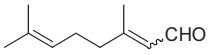
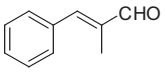
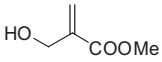
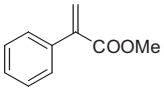
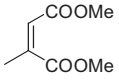
3.5. Substrate specificity and kinetic properties

The substrate specificity of *Cl*ER was assessed rapidly by using spectrophotometric assays under aerobic condition. In order to obtain a reasonably broad picture of the substrate scope, we selected a series of structurally diverse α,β-unsaturated compounds bearing a ketone, aldehyde, cyclic imide and carboxylic ester as electron-withdrawing group. As shown in Table 1, α,β-unsaturated ketones and aldehydes were suitable substrates which were readily reduced by *Cl*ER. Ketoisophorone **1a** and 2-methyl-*N*-phenylmaleimide **7a**, both bearing two activating carbonyl groups next to the C=C bond, led to high reduction rate (k_{cat}) while the highest catalytic efficiency (k_{cat}/K_m) was observed toward 2-methyl-cinnamaldehyde **9a**. *Cl*ER accepted (*R*)-carvone **2a** and (*S*)-carvone **3a** with a slight preference for the (*R*)-enantiomer. The position of the methyl substituent at the C=C bond turned out to be crucial: *Cl*ER showed a marked preference for 2-methyl cyclopentenone **5a** over the 3-methyl substituted substrate **6a**, with almost no detectable activity toward **6a**. Similar phenomenon has been observed for most ERs such as *Lycopersicon esculentum* OPR1, *B. subtilis* YqjM, *Thermoanaerobacter pseudethanolicus* TOYE which did not significantly reduce **6a** [19,20]. Like other ERs, *Cl*ER hardly reduced α,β-unsaturated mono-carboxylic esters **10a** and **11a**. Nevertheless, in the presence of an additional electron-withdrawing group, α,β-unsaturated dicarboxylic diester **12a** was well accepted by *Cl*ER.

3.6. Conversion and stereoselectivity

The stereoselectivity of *Cl*ER in the reduction of various prochiral alkenes was performed over 5 h with the GDH-mediated cofactor recycling system. As shown in Table 2, all the α-methyl substituted α,β-unsaturated ketones were efficiently reduced by *Cl*ER at high *ee* values. Although no significant activity was detectable for **6a** in spectrophotometric assays, the bioreduction assay yielded low conversion (28%) with excellent stereoselectivity (>99% *ee*). The maleimide derivative **7a** was an excellent substrate for *Cl*ER and

Table 1
Steady-state kinetics of *Cl*ER with activated alkene substrates.[a]

Substrate	Structure		Specific activity (U mg^{-1}_{prot})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Ketoisophorone		**1a**	7.3	0.40 ± 0.03	12.8 ± 0.2	32
(*R*)-Carvone		**2a**	2.2	1.0 ± 0.1	5.0 ± 0.4	5.0
(*S*)-Carvone		**3a**	1.3	1.6 ± 0.1	3.6 ± 0.1	2.2
Cyclopentenone		**4a**	3.8	0.32 ± 0.00	6.6 ± 0.2	21
2-Methyl cyclopentenone		**5a**	0.51	0.22 ± 0.03	0.93 ± 0.02	4.4
3-Methyl cyclopentenone		**6a**	0[b]	n.d.	n.d.	n.d.
2-Methyl-*N*-phenylmaleimide		**7a**	6.7	0.11 ± 0.01	11.4 ± 0.1	106
Citral		**8a**	1.5	0.070 ± 0.009	2.5 ± 0.1	36
2-Methyl-cinnamaldehyde		**9a**	3.6	0.0078 ± 0.0008	6.2 ± 0.1	810
Methyl 2-(hydroxymethyl)acrylate		**10a**	0[b]	n.d.	n.d.	n.d.
Methyl 2-phenylacrylate		**11a**	0[b]	n.d.	n.d.	n.d.
Dimethyl 2-methylmaleate		**12a**	1.8	0.25 ± 0.01	3.0 ± 0.0	12

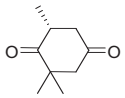
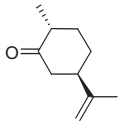
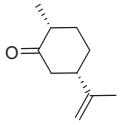
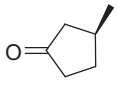
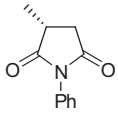
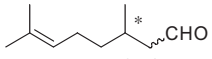
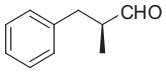
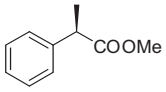
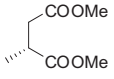
[a] Reactions (1 mL) were performed in potassium phosphate buffer (100 mM, pH 6.0) containing NADPH (0.15 mM), alkene substrate (0.005–10 mM) and *Cl*ER (0.025–0.25 μM). The reactions were followed continuously by monitoring NADPH oxidation at 340 nm for 1 min at 30 °C.
[b] No significant activity was detected by spectrophotometric assays; n.d. = not determined due to weak activity of *Cl*ER.

gave almost full conversion with a high stereopreference (>99% *ee*). Citral **8a**, an approximately 3: 2 mixture of the isomers geranial (*trans*) and neral (*cis*), was converted by the purified enzyme into citronellal (54% conversion) and no citronellol was detected. The only residual *cis*-isomer neral after 5 h suggests that *Cl*ER showed a diastereoselectivity for the reduction of the *trans*-isomer geranial. Due to the isomerization, the neral concentration was slowly reduced with the prolonged reaction time, resulting in a conversion of 78% after 24 h (data not shown). Reduction of **9a** furnished (*S*)-2-methyl-3-phenylpropanal **9b** with poor stereoselectivity (67% *ee*). The low optical purities of **9b** can be attributed to product racemization in aqueous media [13] as the saturated aldehyde **9b** was obtained in almost racemic manner after a reaction time of 24 h (data not shown). Although compounds **10a** and **11a** were hardly reduced by *Cl*ER, dimethyl 2-methylmaleate **12a** was fully converted into optically pure (*R*)-dimethyl 2-methylsuccinate **12b**.

3.7. Biotransformation of ketoisophorone

The asymmetric reduction of ketoisophorone **1a** is of special interest, since the corresponding product (*R*)-levodione **1b** represents an important chiral intermediate for carotenoide synthesis [4]. Thus, ketoisophorone reduction was conducted to evaluate the practical potential of the new enzyme. Using recombinant *E. coli* expressing *Cl*ER (subjected to ultrasonic disruption) coupled with GDH-mediated NADPH regeneration, as much as 500 mM of ketoisophorone was smoothly reduced to (*R*)-levodione, resulting in a product yield of 97% and an optical purity of 98% *ee* within 1 h.

Table 2
Reduction of various activated alkene substrates by *Cl*ER.[a]

Product		Conv. (%)	*ee* (%)
	**1b**	>99	96 (*R*)
	**2b**	>99	>99 *de* (2*R*,5*R*)
	**3b**	>99	93 *de* (2*R*,5*S*)
	**5b**	>99	93 (*S*)
	**6b**	28	>99 (*S*)
	**7b**	>99	>99 (*R*)
	**8b**	54	n.d.
	**9b**	>99	67 (*S*)
	**11b**	2	>99 (*R*)
	**12b**	>99	>99 (*R*)

[a] Reaction conditions: alkene (5 mM, except for **6a** and **11a** of 2 mM), *Cl*ER (1 μM), GDH (2.5 U), $NADP^+$ (0.5 mM), potassium phosphate buffer (100 mM, pH 6.0), 30 °C, 5 h; n.d. = not determined.

After normal workup, the desired saturated product was obtained with an isolated yield of 80%.

4. Discussion

ER-mediated stereoselective reduction of activated alkenes has attracted increasing interest due to the exquisite selectivity and environmental compatibility. In the current work, we present a recombinant ene reductase from *C. lusitaniae* (*Cl*ER) capable of reducing activated C=C bonds. *Cl*ER that can be assigned to yeast OYE accepts a large variety of activated alkenes including enones, enals, cyclic imides and α,β-unsaturated dicarboxylic diesters. Depending on the substrate type, remarkable difference in reaction rates was observed. Enzyme activities of *Cl*ER toward ketoisophorone and (*R*)-carvone were compared with known ERs from the literature [7,8,13,20–26] in Table 3. It can be seen that activities of *Cl*ER were comparable or higher than that of ERs described in the literature. It is noteworthy that the activity assay was conducted under aerobic conditions, indicating the stability of *Cl*ER in presence of oxygen, which is favorable for practical application.

Stereoselectivity is crucial for the evaluation of a catalyst in case of asymmetric synthesis. *Cl*ER was subjected to the reduction of a series of prochiral activated alkenes, furnishing the corresponding enantiomeric alkanes in high to moderate optical purities (67–99% *ee*). The stereoselectivity depended on nature of substituents and inferior stereoselectivity was only observed for reaction products with stereogenic centers at Cα position which are prone to racemization in aqueous medium [13]. In the case of (*R*)-levodione, although the optical purity tends to decrease quickly on prolonged reaction, the *ee* value of 96% after a reaction of 5 h indicated high stereoselectivity of *Cl*ER in the reduction of ketoisophorone. Due to the minimal product racemization in the reduction of (*R*)-carvone, the enantiomeric excess of the corresponding product can more aptly reflect the catalytic performance of the biocatalysts. The *Cl*ER-catalyzed reduction of (*R*)-carvone afforded (2*R*,5*R*)-dihydrocarvone with excellent stereoselectivity (99% *de*) in comparison with some known ERs from the literature [8,13,19–21,27] (Table 3). It is worth mentioning that (*R*)-levodione is a key intermediate for carotenoids and (2*R*,5*R*)-dihydrocarvone

Table 3
Comparison of *Cl*ER with other ERs regarding specific activity and stereoselecitvity toward ketoisophorone and (*R*)-carvone.

Enzyme	Ketoisophorone		(*R*)-Carvone	
	Specific activity (U mg^{-1}_{prot})[a]	Stereoselectivity (*ee* %)	Specific activity (U mg^{-1}_{prot})[a]	Stereoselectivity (*de* %)
*Syn*ER[b]	1.16	17 (*R*)	0.61	98 (2*R*,5*R*)
*Anabaena*ER3[c]	0.56	24 (*R*)	1.09	97 (2*R*,5*R*)
*Acaryo*ER1[c]	2.00	17 (*R*)	0.56	97 (2*R*,5*R*)
KYE1[d]	1.33	n.a.	0.73	n.a.
XenA[d]	1.84	2.2 (*S*)	0	n.a.
*Yers*ER[d]	10.11	n.a.	2.54	n.a.
EnR[e]	0.855	99 (*R*)	n.a.	n.a.
TOYE[f]	0.008	26 (*R*)	0.88	95 (2*R*,5*R*)
PETNR[g]	8.28	95 (*R*)	n.a.	95 (2*R*,5*R*)
OYE[h]	0.072	98 (*R*)	0.021	97 (2*R*,5*R*)
CYE[i]	0.041	>99 (*R*)	0.023	n.a.
***Cl*ER**[j]	**7.3**	**96 (*R*)**	**2.2**	**99 (2*R*,5*R*)**

n.a. = not available.
[a] Specific activities were presented with NADPH as cofactor.
[b] *Syn*ER from *Synechococcus* sp. [21].
[c] AnabaenaER3 from *Anabaena variabilis*, AcaryoER1 from *Acaryochloris marina* [8].
[d] KYE1 from *Kluyveromyces lactis*, XenA from *Pseudomonas putida*, YersER from *Yersinia bercovieri* [7,22].
[e] EnR from *Gluconobacter oxydans* [23].
[f] TOYE from *Thermoanaerobacter pseudethanolicus* [20].
[g] PETNR from *Enterobacter cloacae* [13].
[h] CYE from *Candida macedoniensis* [24,25].
[i] OYE1 from *Saccharomyces pastorianus* [19,26,27].
[j] This study.

is useful for synthesis of shape memory polyesters and antimalarial drugs [28,29].

The commercial interest in ERs has been derived from their capability of producing industrially relevant chiral compounds. However, past efforts have mainly focused on characterizing ERs by substrate screening studies on a small scale at low substrate loading (typically 1–10 mM). To date, there are only a few examples of employing ERs in preparative-scale reactions, *e.g.* for the synthesis of optically active levodione [25], citronellal [30] and dimethyl 2-methylsuccinate [15]. In order to improve the applicability of the new enzyme for the asymmetric synthesis of value-added targets, *Cl*ER-catalyzed ketoisophorone reduction at a substrate loading of 500 mM was conducted. A conversion of 97% was achieved within 1 h, giving (*R*)-levodione in 98% *ee*. The rapid reaction rate contributed to a higher productivity of 60 g L^{-1} h^{-1} in comparison with a known example (18 g L^{-1} h^{-1}) [25]. Based on a conservative estimation of the *Cl*ER expression level (15 mg g_{WCW}^{-1}), the TTN of *Cl*ER was around 30,000, higher than the general level of 10^3–10^4 [2,10].

In conclusion, a recombinant ene reductase (*Cl*ER) from *C. lusitaniae* was identified as a synthetically useful biocatalyst for the asymmetric reduction of activated C=C bonds. Given the relatively high activity and stereoselectivity, *Cl*ER seems a very promising catalyst, specifically for the synthesis of (*R*)-levodione. Further evaluation of this enzyme for the reduction of other activated alkenes bearing different functional groups and improvement of enzyme activity and/or thermostability *via* biocatalyst immobilization and protein engineering is under progress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.enzmictec.2013.12.016.

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