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ORIGINAL RESEARCH PAPER

Characterization of an ene-reductase from *Meyerozyma guilliermondii* for asymmetric bioreduction of α,β-unsaturated compounds

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Abstract
Objectives To characterize a novel ene-reductase from *Meyerozyma guilliermondii* and achieve the ene-reductase-mediated reduction of activated C=C bonds.
Results The gene encoding an ene-reductase was cloned from *M. guilliermondii*. Sequence homology analysis showed that *Mg*ER shared the maximal amino acid sequence identity of 57 % with OYE2.6 from *Scheffersomyces stipitis*. *Mg*ER showed the highest specific activity at 30 °C and pH 7 (100 mM sodium phosphate buffer), and excellent stereoselectivities were achieved for the reduction of (*R*)-carvone and ketoisophorone. Under the reaction conditions (30 °C and pH 7.0), 150 mM (*R*)-carvone could be completely converted to (*2R,5R*)-dihydrocarvone within 22 h employing purified *Mg*ER as catalyst, resulting in a yield of 98.9 % and an optical purity of >99 % *d.e.*
Conclusion *Mg*ER was characterized as a novel ene-reductase from yeast and showed great potential for the asymmetric reduction of activated C=C bonds of α,β-unsaturated compounds.

Keywords Bioreduction · (*R*)-Carvone · Dihydrocarvone · Ene-reductase · NADPH dehydrogenase · Old yellow enzyme · α,β-Unsaturated compounds

Introduction

Asymmetric reduction of activated C=C bonds using ene-reductases (ERs, E.C. 1.3.1.x) from the 'Old Yellow Enzyme' (NADPH dehydrogenase) family of flavoproteins is receiving interest in organic chemistry, due to creation of up to two stereogenic centers in one reaction (Pei et al. 2016). Opposite to the transition-metal-catalyzed *cis*-hydrogenation, ERs reduce C=C bonds of alkenes in a *trans*-specific fashion (Jamison et al. 2006; Selvam et al. 2004). ERs are widely distributed in microorganisms (particularly in bacteria and fungi) and plants (Hall et al. 2008), ERs reduce a wide variety of substrates, such as conjugated enals, enones, imides, nitroalkenes, α,β-unsaturated nitriles, α,β-unsaturated carboxylic acids and their derivatives (esters, lactones, anhydrides), affording products with a variety of biotechnological, fine chemical and pharmaceutical applications (Chaparro-Riggers et al. 2007). However, to date, there are only a few examples of employing ERs in preparative-scale reactions, e.g. for the synthesis of optically active levodione (Kataoka et al. 2004), or citronellal (Bougioukou et al. 2010). Major limitations

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of most ERs lies in their modest activity resulting in low productivity and total turnover numbers (Durchschein et al. 2013), poor substrate tolerance precluding high substrate loading (Patterson-Orazem et al. 2014), instability (Pei et al. 2016), unsatisfied enantioselectivity (Mueller et al. 2010; Fu et al. 2013) and their dependence on NAD(P) (Chaparro-Riggers et al. 2007). Consequently, there is a great interest in searching for new ERs with improved activity, stability and stereoselectivity. Apart from conventional biocatalysts screened from nature, genome mining from the genomic database is a more effective and promising route to discover novel enzymes or identify previously untapped ER homologues (Ni et al. 2014).

In this study, an ene-reductase (*Mg*ER) from *Meyerozyma guilliermondii* ATCC6260 was identified by in silico data mining based on sequence homology. *Mg*ER was cloned, heterologously expressed and characterized for its biochemical and biocatalytic properties. Furthermore, its capability for the production of (*2R,5R*)-dihydrocarvone from (*R*)-carvone was evaluated.

Materials and methods

Materials

PCR primers were synthesized by Generay Biotech Co. Ltd. PrimeSTAR Max DNA Polymerase were purchased from TaKaRa. Restriction enzymes and T4 DNA ligase were purchased from Thermo Scientific. NADH, NADPH and $NDDP^+$ were purchased from Roche. (*R*)-carvone and other chemicals were purchased from Sigma-Aldrich.

Clone, expression and purification of *Mg*ER

The genomic DNA was isolated from the *M. guilliermondii* ATCC 6260 using the TIANamp Yeast DNA kit (Tiangen). Then the ene-reductase gene (GenBank Accession NO. XM_001481950) were PCR amplified with primers *Mg*ER_F_*Nde*I (5′-GGGAATTCCATATGAGCGTAAACATCAATCC-3′) and *Mg*ER_R_*Xho*I (5′-CCGCTCGAGTACCACGAGTTCTTCAGGAA-3′). For expression of the ene-reductases, the ORFs were sub-cloned into pET28a(+) (Novagen), which were transformed into *Escherichia coli* BL21(DE3) competent cells and selected on lysogeny broth (LB) plates containing 50 mg kanamycin/l.

Recombinant *E. coli* BL21 (DE3) was incubated in LB containing 50 mg kanamycin/l at 37 °C. Protein expression was induced by the addition of 0.2 mM IPTG when the OD_{600} reached 0.6–0.8; the culture was incubated at 20 °C for additional 20 h at 150 rpm. After centrifugation (12,000×*g*, 10 min) and washing with 0.9 % NaCl, the cells were resuspended in 20 mM sodium phosphate buffer (pH 7) and disrupted by ultrasonication. After centrifugation (12,000×*g*, 30 min), the crude extract was used for protein purification on AKTA using a HisTrap FF crude column (GE Healthcare). The protein was eluted with an increasing gradient from 20 to 500 mM of imidazole in sodium phosphate buffer. Proteins were evaluated using SDS-PAGE and the concentration was conducted using the Bradford assay. The purified enzyme was concentrated and stored at −20 °C for further use.

Enzyme analysis

Enzyme activity was determined by measuring the rate of NADPH oxidation at 340 nm. Standard reactions were performed in 200 µl in sodium phosphate buffer (100 mM, pH 7) at 30 °C containing 0.2 mM NADPH, 10 mM substrate (in 5 % v/v ethanol) and 5–15 µg purified protein. One unit of enzyme activity was defined as the oxidation of 1 µmol NADPH per min after removal of background absorption.

The optimal pH was determined using sodium citrate buffer (pH 3.5–5.5), sodium phosphate buffer (pH 5.5–8.5) and glycine/NaOH buffer (pH 8.5–10), all at 100 mM, using the standard assay. Each was mixed with a certain amount of enzyme and shaken at 300 rpm at ∼30 °C for 30 min before determination. The optimal temperature was determined from 25 to 45 °C. The pH stability of *Mg*ER was evaluated by holding the enzyme in buffers from pH 5 to 9 at 4 °C. Residual activity was determined using the standard assay. The thermostability of *Mg*ER was performed by incubating the enzyme in sodium phosphate buffer (100 mM, pH 7.0) at 4, 30 and 40 °C and the residual activity was assayed under standard conditions. Appropriate controls were included and experiments were conducted in triplicates.

The flavin content of *Mg*ER was released by incubating the enzyme at 100 °C for 20 min. After

removing the denatured protein by centrifugation at 13,000×*g* for 30 min at 4 °C, the supernatant was assayed by HPLC using a Extend-C18 column (4.6 × 250 mm, Aglient) and isocratic elution with methanol/H_2O (3:7, v/v) at 0.8 ml/min FMN and FAD were used as standards.

Bioconversion of α,β-unsaturated compounds

The bioreduction of prochiral compounds (**1a**, **2a**, **3a** and **8a**) (see Table 1) were performed in 100 mM sodium phosphate buffer (pH 7.0) containing 10 mM substrate (in 5 % v/v ethanol), 0.5 mM $NADP^+$, 30 mM D-glucose, 90 μg purified *Mg*ER, and 250 μg glucose dehydrogenase (GDH). The reaction was incubated at 30 °C for 12 h with shaking (200 rpm). After that, reaction mixture was extracted with ethyl acetate (1:2, v/v) containing 1 mM 1-octanol as internal standard for GC analysis.

The bioreduction of (*R*)-carvone (**1a**) was carried out in 10 ml sodium phosphate buffer (100 mM, pH 7.0) containing 150 mM substrate (in 5 % v/v ethanol), 6 mg purified *Mg*ER, 1.25 mg GDH, 0.5 mM $NADP^+$ and 300 mM D-glucose. The reactions were conducted at 30 °C and the pH was automatically adjusted to 7.0 with 2 M Na_2CO_3. Samples (50 μl) were taken periodically and treated with the method mentioned above.

Analytical procedures

The conversion and *d.e.* of **1a** and **2a** were determined by GC using a DB-5 column (30 m × 0.32 mm × 0.25 μm, Aglient) as described by Yin et al. (2015). Sample was 1 ul with N_2 as carrier gas (1 ml/min) and a split ratio of 10:1, with a flame ionization detector (280 °C) and injection at 250 °C. The temperature program was: 2 min at 90 °C, 10 °C/min to 200 °C, then holding for 2 min. Retention times were: (*R*)-carvone 7.9 min, (*2R*,*5R*)-dihydrocarvone 7.3 min and (*2S*,*5R*)-dihydrocarvone 7.4 min. The product of (*R*)-carvone reduction was purified and subjected to GC–MS analysis. For GC–MS analysis, the ion source of MS was electron ionization (EI, 70 eV) with the ion source at 230 °C; GC conditions were as mentioned above.

The *e.e.* of levodione (**3b**) was determined by GC using a CP-ChiraSil-DEX CB column (25 m × 0.25 mm × 0.25 μm, Varian) with split injection of 10:1 and N_2 as carrier gas. The injection port was at 220 °C and detector was at 250 °C. The temperature programs was: 2 min at 95 °C, 5 °C/min to 110 °C and hold for 1 min, 10 °C/min to 180 °C, hold 2 min. Retention times: ketoisophorone 5.9 min, (*R*)-levodione 6.5 min and (*S*)-levodione 6.7 min. The *e.e.* of 2-methyl-hydrocinnamaldehyde (**8b**) was determined using a Chiralcel OJ-H column (250 mm × 4.6 mm, 5 μm, Daicel) with n-hexane/2-propanol (99:1 v/v) as eluting solvent. Retention times were: α-methylcinnamaldehyde 15.6 min, (*S*)-2-methyl-hydrocinnamaldehyde 13.9 min and (*R*)-2-methyl-hydrocinnamaldehyde 12.2 min. Products and their absolute configurations were identified by comparison with reference materials on chiral GC or HPLC.

Phylogenetic analysis

OYEs homologue sequences were obtained through the National Center of Biotechnology Information (NCBI). The best amino acid substitution model was estimated for tree building using the MEGA 5 software. Amino acid sequences were used to generate the distance tree by ClustalX with default settings and a distance neighbor-joining tree was then constructed by using MEGA 5.

Results and discussion

Identification and sequence homology analysis

The hypothetical protein (NCBI accession number XP_001482000.1) from *M. Guilliermondii* ATC C6260, designated as *Mg*ER, was identified by a Blast search using the amino acid sequence of *Saccharomyces pastorianus* Old yellow enzyme 1 (NADPH dehydrogenase) (OYE1; NCBI accession number Q02899) as the template. Multiple sequence alignment of *Mg*ER with other OYEs revealed highest similarity to OYE2.6 (57 % identity; 75 % similarity) (Supplementary Fig. 1). The sequence comparison also established that *Mg*ER contains the conserved substrate binding site (Thr^{34}, Trp^{111}, His^{186}, His^{189}, Tyr^{191}, Arg^{345}) and catalytic residue (Tyr^{191}) (Supplementary Fig. 1). Phylogenetic analysis of *Mg*ER and 25 published OYEs revealed that *Mg*ER belongs to yeast OYE, and *Mg*ER is most closely related to the OYE2.6 (NCBI accession number XP_001384055.1)

Table 1 Substrate spectrum of *Mg*ER

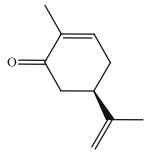
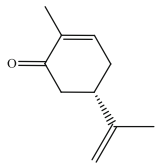
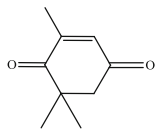
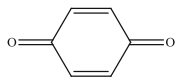
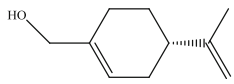
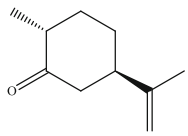
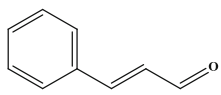
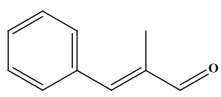
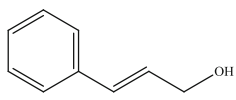
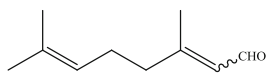
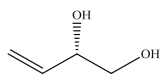
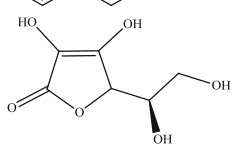
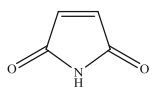
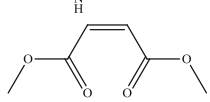
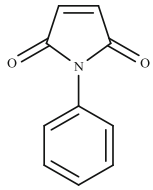
Substrate	Structure		Specific activity (U mg^{Fl})	*e.e.* (%)/config.
(*R*)-Carvone		**1a**	1.67	>99 *d.e.* (*2R,5R*)
(*S*)-Carvone		**2a**	0.22	72 *d.e.* (*2R,5S*)
Ketoisophorone		**3a**	2.69	>99 (*R*)
p-Benzoquinone		**4a**	3.21	–
(−)-Perillyl alcohol		**5a**	0.11	n.d.
(*2R,5R*)-Dihydrocarvone		**6a**	0.09	–
Cinnamaldehyde		**7a**	1.00	–
α-Methylcinnamaldehyde		**8a**	1.74	25 (*S*)
Cinnamyl alcohol		**9a**	0.42	–
Citral		**10a**	0.69	n.d.
3-Butene-1,2-diol		**11a**	0.11	–
Ascorbic acid		**12a**	0.10	n.d.
Maleimide		**13a**	7.53	–
Dimethyl maleate		**14a**	8.56	–

Table 1 continued

Substrate	Structure		Specific activity (U mg^{F1})	*e.e.* (%)/config.
N-Phenylmaleimide		**15a**	10.16	–

n.d not determined

from *Scheffersomyces stipitis* CBS 6054 with 57 % identity (Supplementary Fig. 2). The structures and catalysis properties of *S. stipitis* OYE2.6 were characterized (Pompeu et al. 2012), and it was used to produce gram-scale quantities of both (*R*)- and (*S*)-citronellal (Bougioukou et al. 2010).

Clone, protein expression and purification

*Mg*ER was heterologously expressed as a soluble protein in *E. coli* BL21 (DE3) with an *N*-terminal 6× His-tag using the vector pET28a(+). The enzyme was purified from the bacterial cell extract via a HisTrap FF crude column with Ni–NTA. As expected, a single band was observed using SDS-PAGE analysis (Fig. 1). The apparent molecular weight of about 45 kDa was in accordance with the calculated value of 47 kDa. The purified protein was a bright yellow, indicating the presence of flavin. HPLC analysis of the supernatants from the heat-denatured protein showed that *Mg*ER contained FMN as cofactor.

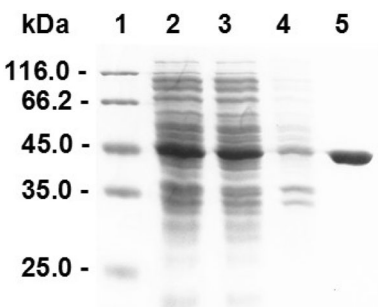


Fig. 1 SDS-PAGE analysis of the expression and purification of *Mg*ER. *Lane 1* protein molecular weight marker; *Lane 2* whole-cell of *E. coli* expressing *Mg*ER; *Lane 3* curde extract of *E. coli* expressing *Mg*ER; *Lane 4* precipitate of *E. coli* expressing *Mg*ER; *Lane 5* purified *Mg*ER

Cofactor preference

The purified *Mg*ER had activity towards (*R*)-carvone using either NADH or NADPH as the cofactor with a specific activity of 1.67 U/mg with NADPH and 0.02 U/mg with NADH, indicating NADPH as the preferred cofactor.

Dependence of *Mg*ER activity on pH and Temperature

Maximum enzyme activity was at pH 7 in sodium phosphate buffer with >70 % activity between pH 5.5 and 8.5 (Fig. 2a). *Mg*ER was optimally active at 30 °C in sodium phosphate buffer (Fig. 2b).

Purified *Mg*ER had half-lives of 40–180 h between pH 5 to 9 at 4 °C (Fig. 2c). The enzyme was most stable at pH 7. Its half-lives at 4, 30 and 40 °C were 180, 100 and 60 h, respectively (Fig. 2d) suggesting that *Mg*ER is stable under mild reaction conditions.

Substrate specificity and stereoselectivity

To map the substrate spectrum of *Mg*ER, enzyme activities were determined for a series of structurally diverse α,β-unsaturated compounds in the presence of NADPH, and the results are summarized in Table 1. Maleimide (**13a**), dimethyl maleate (**14a**) and *N*-phenylmaleimide (**15a**), bearing two activated carbonyl groups next to C=C bond, were well accepted substrates for *Mg*ER, while the highest specific activity was observed toward *N*-phenylmaleimide (**15a**). Ketoisophorone (**3a**), *p*-benzoquinone (**4a**), cinnamaldehyde (**7a**),α-methylcinnamaldehyde (**8a**) and citral (**10a**), bearing a carbonyl group next to C=C bond, led to moderate specific activities, while *Mg*ER showed higher activity toward vinyl ketones compared to vinyl aldehydes. *Mg*ER had higher activity toward

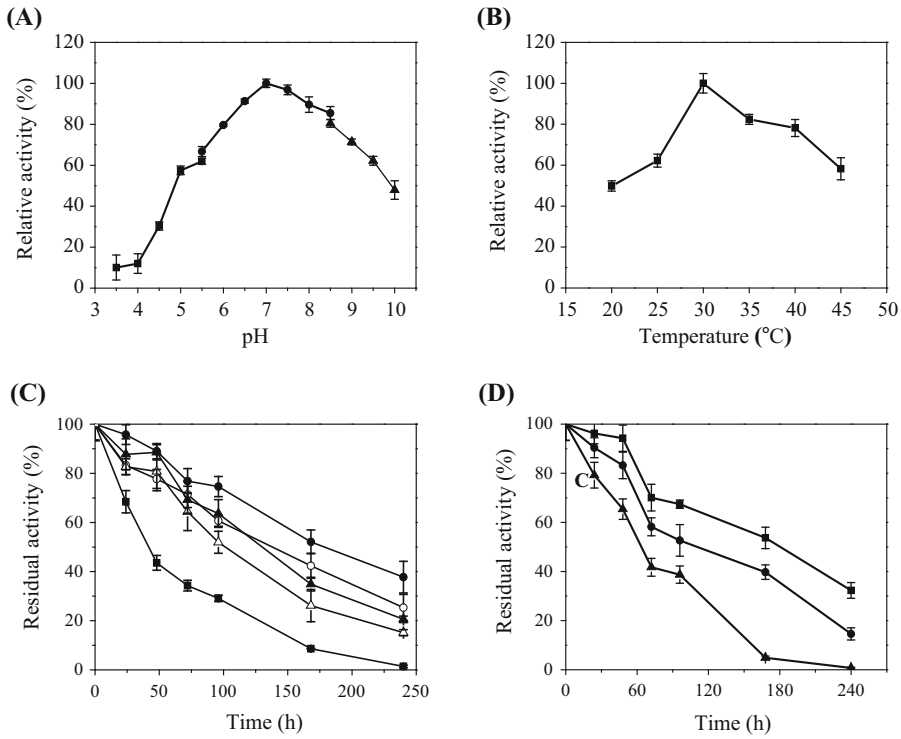


Fig. 2 **a** Effect of pH on activity of *Mg*ER: *filled square* sodium citrate buffer; *filled circle* sodium phosphate buffer; *filled triangle* glycine/NaOH buffer. **b** Effect of temperature on activity of *Mg*ER. **c** pH stability of *Mg*ER: *filled square* pH 5.0; *open circle* pH 6.0; *filled circle* pH 7.0; *filled triangle* pH 8.0; *open triangle* pH 9.0. **d** Thermo stability of *Mg*ER: *filled square* 4 °C; *filled circle* 30 °C; *filled triangle* 40 °C. Under the experimental conditions, the relative activity was expressed as a percentage of the maximum activity (100 % activity = 1.67 U/mg). *Error bars* represent the standard error of three replicates

α-methylcinnamaldehyde (**8a**) compared with cinnamaldehyde (**7a**) due to the position of the methyl substituted at the C=C bond. The existence of hydroxyl group on the substrates had negative influence on the activity of *Mg*ER, and quite low activities were detected for (−)-perillyl alcohol (**5a**), cinnamyl alcohol (**9a**), 3-butene-1,2-diol (**11a**) and ascorbic acid (**12a**). *Mg*ER accepted (*R*)-carvone (**1a**) and (*S*)-carvone (**2a**) with a marked preference for the (*R*)-enantiomer, and the corresponding product (*2R,5R*)-dihydrocarvone (**6a**) was hardly reduced by *Mg*ER.

The stereoselectivity of *Mg*ER was measured with four exemplarily chosen α,β-unsaturated aldehyde and ketones. As shown in Table 1, (*R*)-carvone (**1a**) was reduced to (*2R,5R*)-dihydrocarvone by *Mg*ER with excellent stereoselectivity (>99 % *d.e.*), while the reduction of (*S*)-carvone (**2a**) resulted in the (*2R,5S*)-dihydrocarvone with low stereoselectivity (72 % *d.e.*). Ketoisophorone (**3a**) was a good substrate for *Mg*ER, yielding the product (*R*)-levodione with a high stereopreference (>99 % *e.e.*). For α-methylcinnamaldehyde (8a), *Mg*ER exhibited poor stereoselectivity (25 % *e.e.*, for *S*-isomer).

Biotransformation of (*R*)-carvone

The asymmetric reduction of (*R*)-carvone to (*2R,5R*)-dihydrocarvone is of special interest since the product is an important chiral intermediate for valuable antimalarial drugs (dispiro-1,2,4,5-tetraoxanes) and shape memory polyesters (Dong et al. 2010; Lowe et al. 2009). In addition, due to the presence of

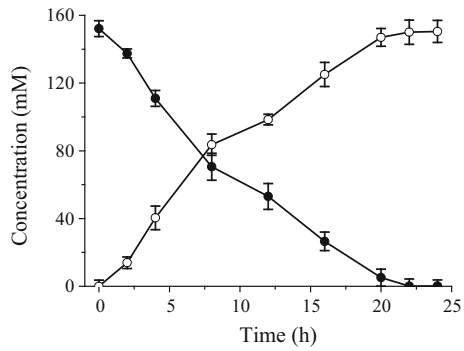


Fig. 3 Asymmetric bioreduction of (*R*)-carvone. *filled circle* (*R*)-carvone; *open circle* (*2R,5R*)-dihydrocarvone. *Error bars* represent the standard error of three parallel samples

Table 2 Comparision of *Mg*ER with other ERs for asymmetric reduction of (*R*)-carvone

Enzyme	Substrate concentration (mM)	Cofactor	Conv (%)	*d.e.* (%)/config.	Species
LyngbyaER1	5	NADH	99	98 (*R,R*)	*Lyngbya* sp. PCC 8106
NospuncER1	5	NADH	99	98 (*R,R*)	*Nostoc punctiforme* PCC 73102
*Cl*ER	5	NADPH	>99	>99 (*R,R*)	*Clavispora lusitaniae*
SynER	5	NADH	>99	98 (*R,R*)	*Synechococcus* sp. PCC 7942
PETNR	10	NADH	95	94 (*R,R*)	*Enterobacer cloacae*
NEMR	10	NADH	97	92 (*R,R*)	*Escherichia coli* JM109
EBP1	10	NADH	11	95 (*R,R*)	*Candida albicans*
*Ts*ER	10	NADH	>99	>99 (*R,R*)	*Thermus scotoductus*
LacER	100	NADH	>99	98 (*R,R*)	*Lactobacillus casei*
*Mg*ER	150	NADPH	>99	>99 (*R,R*)	This study

multiple C=C and C=O double bonds, at least ten different products could be generated via hydrogenation. Asymmetric reduction of (*R*)-carvone to the single product is still a challenge in organic synthesis. Thus, reduction (*R*)-carvone was conducted to evaluate the practical potential of the new enzyme *Mg*ER. Using purified *Mg*ER coupled with GDH-mediated NADPH regeneration, 150 mM (*R*)-carvone was completely reduced to (*2R,5R*)-dihydrocarvone within 22 h, resulting in a product yield of 98.9 % and an optical purity of >99 % *d.e.* (Fig. 3). The product was purified and GC–MS profile of (*2R,5R*)-dihydrocarvone was as follows: GC-EI-MS m/z (M^+ 152 for $C_{10}H_{16}O$) 137, 109, 95, 81, 67, 55 (Supplementary Fig. 3).

To date, only a few examples of employing ERs in bioreduction of (*R*)-carvone for production of (*2R,5R*)-dihydrocarvone have been reported and, in most cases, the substrate loads were quite low, typically less than 10 mM, such as LyngbyaER1 (Fu et al. 2013), NospuncER1 (Fu et al. 2013), *Cl*ER (Ni et al. 2014), SynER (Fu et al. 2012), PETNR (Mueller et al. 2010), NEMR (Mueller et al. 2010), *Ts*ER (Paul et al. 2013) and EBP1 (Mueller et al. 2010) (Table 2). In addition, the enantiopurity of product was unsatisfactory by PETNR, NEMR and EBP1. An enoate reductase (LacER) from *Lactobacillus casei* converted 100 mM (*R*)-carvone into (*2R,5R*)-dihydrocarvone with 99 % conversion and 98 % *d.e.* (Gao et al. 2012). In this work, *Mg*ER could catalyze the reduction of (*R*)-carvone at a substrate load of 150 mM with high yield of 98.9 % and *d.e.* value of >99 %. Both the product titer and stereoselectivity of *Mg*ER were outstanding as compared to the reported ERs from the literatures, making *Mg*ER very competitive and promising for practical synthesis of (*2R,5R*)-dihydrocarvone.

In conclusion, a novel ene-reductase (*Mg*ER) from *M. guilliermondii* ATCC 6260 was identified for asymmetric bioreduction of activated C=C bonds of α,β-unsaturated compounds. *Mg*ER had activities from 0.1 to 10.6 U/mg protein towards all the tested enones, maleimide derivatives, vinyl aldehydes and alcohols. Among them, excellent stereoselectivities were achieved in the reduction of (*R*)-carvone and ketoisophorone. In particular, up to 150 mM (*R*)-carvone could be asymmetrically reduced by purified *Mg*ER with full conversion, 98.9 % yield and >99 % *d.e.* indicating that *Mg*ER is a competitive catalyst for synthesis of (*2R,5R*)-dihydrocarvone considering stereoselectivity, yield and final product concentration.

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Supporting information Supplementary Figure 1—Multiple sequence alignment of *Mg*ER with the other nine Old Yellow Enzymes. Substrate binding sites are indicated.

Supplementary Figure 2—Phylogenetic relationship of amino acid sequences of *Mg*ER to other Old Yellow Enyzmes with known function.

Supplementary Figure 3—GC-MS spectrum of (*2R*,*5R*)-dihydrocarvone prepared by *Mg*ER. GC-EI-MS m/z (M^+ 152 for $C_{10}H_{16}O$) 137, 109, 95, 81, 67, 55.

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