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Enoate reductases from non conventional yeasts: Bioconversion, cloning, and functional expression in *Saccharomyces cerevisiae*

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Old yellow enzymes (OYEs, EC 1.6.99.1) are flavin-dependent oxidoreductases that catalyze the stereoselective trans-hydrogenation of the double bond, representing a promising alternative to metal-based catalysis. Bioconversion of ketoisophorone (KIP) by 28 non-conventional yeasts belonging to 16 different species was investigated. Growing cells of most of the strains reduced KIP via OYE and showed high stereoselectivity, producing *R*-levodione as major product. Competition by carbonyl reductase (CR) activity was observed in several strains. The best performing yeasts belong to *Candida castellii*, *Kazachstania spencerorum* and *Kluyveromyces marxianus* exhibited yields of levodione ≥77% up to 95% e.e., and. *Candida freyschussii*, the sole strain lacking the *OYE* gene, reduced KIP only to unsaturated alcohols via CR. Nine unedited *OYE* genes were cloned, sequenced, and heterologously expressed in *Saccharomyces cerevisiae* BY4741*ΔOye2*, a mutant that showed negligible OYE and CR activities. Compared with the corresponding wild-type yeasts, growing cells of the recombinant strains bioconverted KIP with improved yields of OYE products, minor competition by CR activity, and lower enantioselectivity. In particular, resting cells of recombinant *S. cerevisae* presented the best performance in KIP bioconversion. Based on the results herein reported, selected strains of non-conventional yeasts and novel *OYE* genes can be profitably used as innovative biocatalysts in asymmetric reductions.

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1. Introduction

Biocatalysts are increasingly used in industrial chemistry, particularly for the production of chiral synthons and high-value products (Panke et al., 2004). They offer the advantage of performing reactions under mild conditions through an environmentally benign approach. Furthermore, biocatalytic exploitation of enzymes offers fine results in terms of chemo-, regio-, and stereoselectivity, producing enantiomerically pure products (Reetz, 2009).

Chemocatalysis and biocatalysis are complementary approaches for the stereoselective hydrogenations of double bonds. Asymmetric homogeneous catalysis through chiral complexes of transition metals has proved to be a powerful technology for generating pure enantiomers (Palmer and Zanotti-Gerosa, 2010). Reductases/dehydrogenases can be exploited for the stereo controlled reduction of C=C bonds. In particular, flavin-dependent enoate reductases (also called old yellow enzymes, OYE, EC 1.6.99.1) catalyze the chemo- and stereoselective hydrogenation of electron-poor alkenes. This bio-reduction can result in important chiral synthons towards synthesis of pharmaceuticals, fragrances, and chemicals (Toogood et al., 2010).

The biotechnological potential of OYEs lies on the ability to generate up to two diverse stereocenters by the stereoselective trans-hydrogenation of the C=C, which is highly demanded in asymmetric synthesis, but difficult to perform with conventional methods (Stuermer et al., 2007). OYEs can reduce a wide variety of substrates, such as conjugated enals, enones, α,β-unsaturated carboxylic acids, imides, nitroalkenes, and ynones (Williams and Bruce, 2002; Stuermer et al., 2007). Although the physiological role of OYEs still remains unknown, and their natural substrates have not been clearly identified, this family of enzymes has been object of intensive investigation in order to develop new catalysts. Several OYEs have been isolated from higher plants, bacteria, yeasts and filamentous fungi (Strassner et al., 1999; Brigé et al., 2006; Kataoka et al., 2004; Quezada et al., 2009). However, due to the increasing demand of optically pure building blocks, the scientific community is still addressing considerable efforts at identifying and developing OYE biocatalysts.

Yeasts are important sources of biotechnologically relevant enoate reductases (Raimondi et al., 2010) and can be exploited as sources of new *OYE* genes. Several biochemical and crystallographic studies described Oye1p of *Saccharomyces pastorianus* (former *Saccharomyces carlsbergensis*) and the homologous enzyme (Oye2p) of

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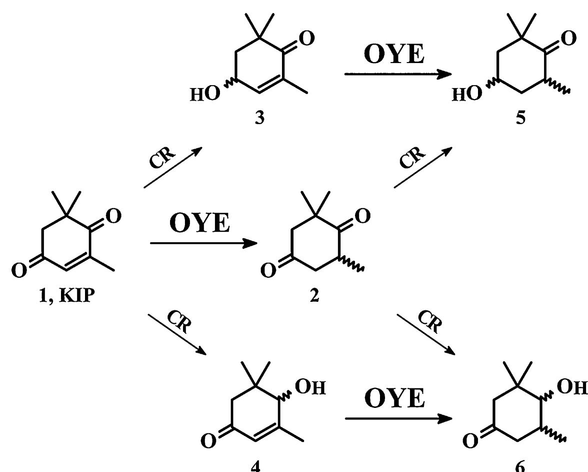

Fig. 1. KIP bioreduction in yeasts. **1** – 2,6,6-trimethylcyclohex-2-ene-1,4-dione [ketoisophorone]; **2** – 2,6,6-trimethylcyclohexane-1,4-dione [(6R)-levodione]; **3** – 4-hydroxy-2,6,6-trimethylcyclohex-2-en-1-one [(4S)-phorenol]; **4** – 4-hydroxy-3,5,5-trimethylcyclohex-2-en-1-one [hydroxyisophorone]; **5** – 4-hydroxy-2,6,6-trimethylcyclohexaone [(4R, 6R)-actinol]; **6** – 4-hydroxy-3,5,5-trimethylcyclohexanone. **OYE** – enoate reductase; **CR** – carbonyl reductase.

Saccharomyces cerevisiae (Fox and Karplus, 1994; Stott et al., 1993; Niino et al., 1995). Oye1p is a dimeric protein of around 45 kDa, with a single α/β-barrel domain. In these OYE enzymes, the reduced cofactor flavin adenine mononucleotide (FMN) mediates a net *anti*-hydrogenation of the olefinic bond, acting as a transitory reservoir of electrons that are transferred from the coenzyme NAD(P)H to the substrate (Kohli and Massey, 1998). Both molecules use the same binding site and react following a Bi Bi Ping Pong mechanism. OYEs are loosely specific, being preferably reduced by β-NADPH, but accepting also α-NAPDH and NADH as cofactors (Massey and Schopfer, 1986).

Despite the relevant interest on their catalytic properties, the exploitation of OYE enzymes has been hampered by the necessity to regenerate the reducing cofactor. This issue has been only partially overcome by whole-cell biocatalysts. The presence of competing carbonyl reductases (CRs) frequently limits the chemoselectivity by producing side-products, and hinders product yields (Buque-Taboada et al., 2005; Yoshisumi et al., 2001). Furthermore, OYEs from different sources often possess identical stereoselectivity and generate the same enantiomers or diastomers, thus resulting in a drastic limit to their application (Bougioukou and Stewart, 2008).

Enzymes with improved performances (e.g. thermal stability, activity within a broader range of pH, stereo-selectivity, activity in organic solvents, etc.) may be obtained by protein engineering or by screening among unexplored strains. Besides, the availability of broad *OYE* gene libraries facilitates the application of techniques of improvement based on direct evolution (Jäckel et al., 2008). In this perspective, non-conventional yeasts still represent an understudied source of biodiversity that deserve to be explored for the identification of *OYE* enzymes with novel catalytic performances and/or different chemo- or stereoselectivity (Raimondi et al., 2010; Goretti et al., 2011). In the present paper we analyzed in detail the reduction of the unsaturated compound 2,6,6-trimethylcyclohex-2-ene-1,4dione (ketosiophorone, KIP, **1**; Fig. 1) by 30 yeasts belonging to 7 genera and 16 species. Nine novel *OYE* genes were cloned from the above strains and compared to the gene *OYE2* of *S. cerevisiae*. In order to evaluate and compare the biocatalytic performance of the novel enzymes, the *OYE* genes were expressed in the host *S. cerevisiae* BY4741Δ*Oye2*, which presents negligible OYE activity and fails to reduce the two carbonyl groups of KIP. The utilization of the same host and the same expression cassette would lead to the similar transcription level in each recombinant strain. Therefore the varied OYE activities in the recombinant strains should be mainly due to the different coding sequences of the *OYE* variants. Therefore, any influence of the biocatalytic systems, in terms of different expression level, efficiency of substrate uptake, availability of cofactors, and presence of competing reactions, is minimized (Blank et al., 2010).

2. Materials and methods

2.1. Biochemicals and reagents

All chemicals were obtained from Sigma-Aldrich (Steinheim, Germany) unless otherwise stated. Microbiological products were purchased from BD Biosciences (San Jose, CA, USA). Dream-Taq and reagents used for molecular biology procedures were supplied by Fermentas International (Burlington, Ontario, Canada).

2.2. Strains and maintenance

Most of the 30 yeasts, belonging to 16 species of 7 ascomycetous genera (Table 1), were from Industrial Yeasts Collection DBVPG (Department of Applied Biology, Perugia, Italy, www.agr.unipg.it/dbvpg). *Kazachstania exigua* L10, *Kluyveromyces marxianus* L3, and *S. cerevisiae* L12 (Dellomonaco et al., 2007) were kindly supplied by Prof. D. Matteuzzi (Dept. of Pharmaceutical Sciences, University of Bologna, Italy). *Kluyveromyces lactis* MW278-20C was described by Chen et al. (1992). To study the overexpression of *OYE* genes, two host strains derived from *S. cerevisiae* BY4741 (*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*) were obtained from EUROSCARF collection: *S. cerevisiae* YHR179W (*BY4741*, *MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, *YHR179w::kanMX4*) and *S. cerevisiae* YPL171c (*BY4741*, *MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, *YPL171c::kanMX4*), hereinafter named *S. cerevisiae* BY4741Δ*Oye2* and *S. cerevisiae* BY4741Δ*Oye3*, respectively. Yeasts were grown at 30 °C and maintained onto YPD agar slants (10 g l^{-1} yeast extract, 10 g l^{-1} peptone, 20 g l^{-1} glucose, 20 g l^{-1} agar). For plasmid maintenance, recombinant *S. cerevisiae* strains were grown in YKK synthetic medium (6.7 g l^{-1} yeast nitrogen base without amino acids, 8.5 g l^{-1} KH_2PO_4 and 3.4 g l^{-1} K_2HPO_4, 20 g l^{-1} glucose, 2 g l^{-1} drop out mix-ura; Amberg et al., 2005).

Escherichia coli DH5α (φ80*lacZΔM15*, *recA1*, *end A1*, *gyr A96*, *thi-1*, *hsd R17*, *rel A1*) was used for general cloning purposes according to standard procedures (Sambrook et al., 2001).

2.3. PRC amplification

A set of degenerate primers was designed to amplify the entire OYE coding region from yeasts genomic DNA. The primers were based on published *OYE* genes of yeasts, obtained from NCBI GenBank (http://www.ncbi.nlm.nih.gov/nucleotide/). The potential consensus regions of OYE amino acid sequences were confirmed by sequence alignment using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The primer sequences were then modified to maximize the number of *OYE* genes amplified, resulting in the primers *OYE*(f)1-deg [5′-AAG CTT ATG (C/T)CA TTT GT(A/C/T) AA(A/G) GA(C/T) TT(C/T) AAG C-3′] and *OYE*(r)1-deg [5′-AAG CTT TTA (A/C/T)T(G/T) (C/T)TT GCT CCA (A/G)CC-3′]. These primers anneal with the *S. cerevisiae OYE2* gene (GenBank accession no. L06124.1) at the positions 169–25 and 1354–1371, generating amplicons of 1215 bp. DNA amplification was performed using a thermocycler (Robocycler Gradient 96, Biorad, La Jolla, CA, USA) programmed with the following temperature profile: 94 °C for 2 min (1 cycle); 94 °C for 15 s, 54 °C for 30 s, and 72 °C for 1 min (40 cycles) and 72 °C for 7 min (1 cycle).

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