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Genome hunting of carbonyl reductases from *Candida glabrata* for efficient preparation of chiral secondary alcohols

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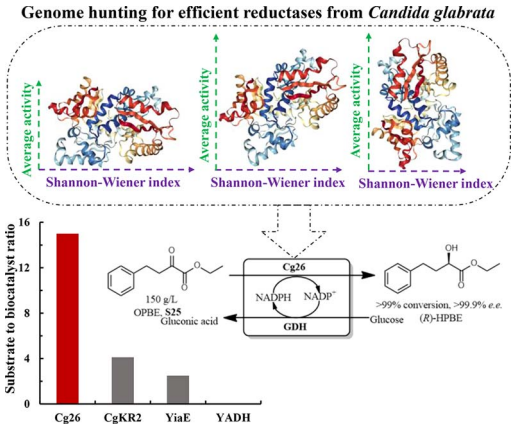
GRAPHICAL ABSTRACT



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ABSTRACT

In this work, genome hunting strategy was adopted in screening for reductases from *Candida glabrata*. A total of 37 putative reductases were successfully expressed in *E. coli* BL21(DE3). A substrate library containing 32 substrates was established for characterization of each reductase by average specific activity and Shannon-Wiener index. Among them, Cg26 was identified with the highest efficiency and wider substrate spectrum in the reduction of prochiral ketones, with average activity and Shannon-Wiener index of 8.95 $U \cdot mg^{-1}$ and 2.82. Cg26 is a member of 'extended' short chain dehydrogenase/reductase superfamily. Ni^{2+} could improve its activity. As much as 150 $g \cdot L^{-1}$ ethyl 2-oxo-4-phenylbutyrate could be completely converted by 10 $g \cdot L^{-1}$ Cg26. This study provides evidence for this newly identified Cg26 in the preparation of chiral secondary alcohols.

1. Introduction

Optically pure secondary alcohols are one class of the most important chiral compounds, and have been widely applied as building blocks in the fine chemicals, pharmaceutical, materials, agrochemicals and food industries (Zheng and Xu 2011). The efficient and economic synthesis of chiral secondary alcohols is of special interest. Various strategies have been developed for the production of single enantiomer of secondary alcohols, including chromatographic separation, enantioselective resolution or dynamic kinetic resolution, and asymmetric reduction (Zheng et al., 2017). In the view of atomic economy, product yield, operational simplicity and enantioselectivity, chemically or

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biocatalytically asymmetric hydrogenation of prochiral ketones is regarded as the most promising strategy (Goldberg et al., 2007; Matsuda et al., 2009; Huisman et al., 2010).

It is widely accepted that chemical reduction plays important role in the industrial synthesis of chiral secondary alcohols with high efficiency, however at the expense of environmental friendliness and toxicity (Kroutil et al., 2004). In addition, high enantioselectivity is often difficult to achieve by chemical routes. Biocatalytically asymmetric reduction is regarded as an alternative for chemical methods, and presents many appealing features, such as gentle reaction conditions, high activity and stereoselectivity, no need of protection and deprotection steps (Zheng et al., 2017). However, the narrow substrate profile, low substrate/product tolerance, and demand for cofactor NAD(P)$^+$ render their application less satisfactory (Itoh, 2014). As a result, there is a constant need for discovering novel biocatalysts with diverse properties, such as wide substrate scope, high activity, enantioselectivity and thermostability etc, by searching for naturally evolved enzymes, protein engineering and *de novo* design of new biocatalysts (Ni and Xu, 2012). Over the past decade, an increasing number of chemists have turned to biocatalysis in their synthetic schemes, especially in the context of great advances in genomics and protein databases, screening, and evolution technologies (Bornscheuer et al., 2012).

Genome hunting is one of the genome mining strategies for screening naturally evolved biocatalysts, and is performed by screening a genome-wide expression library of a certain microorganism with desired activity, which can be developed by overexpressing the known or putative reductases (Ni and Xu, 2012). A large number of naturally evolved reductases have been discovered through this strategy in recent years. One typical example of genome hunting is the systematic investigation on reductases from *Saccharomyces cerevisiae*, a well-known reductases producing strain. After analysis and characterization, many reductases displayed > 90% *e.e.* in the reducing of *α*- or *β*-keto esters, and the utility of this library was further demonstrated by reducing 3-oxo-3-phenylpropanenitrile to its corresponding alcohol with high enantioselectivity (Kaluzna et al., 2004). Genome hunting library of *Bacillus* sp. ECU0013, a reductase-producing strains, has also been developed and screened for useful reductases, and three versatile reductases were identified with application potential in efficient synthesis of optically active *α*- and *β*-hydroxyl esters (Ni et al., 2011). Comparative characterization of ene-reductases library of cyanobacteria revealed 9 ene-reductases with higher catalytic efficiency and enriched the toolbox for the asymmetric reduction of alkenes (Fu et al., 2013). Bioinformatics analysis based on sequence-similarity with an anti-Prelog stereospecific ADH from *Candida parapsilosis* in its whole genome returned three carbonyl reductases with reducing activity toward 2-hydroxyacetophenone (Nie et al., 2011).

Candida glabrata, previously known as *Torulopsis glabrata*, is a haploid yeast of the genus Candida and regarded as an important industrial microorganism, with potential in fermentation for acetoin (Li et al., 2015), pyruvate (Li et al., 2001; Otto et al., 2011), malate (Chen et al., 2013) and fumarate (Chen et al., 2015) etc. *C. glabrata* have been proved to be efficient in the asymmetric reduction of 2-oxo-4-phenylbutanate (OPBE) (Zhang et al., 2009), and is also a reductase-producing strain. Three reductases have been identified, including *Cg*KR1 for ethyl (*R*)-*o*-chloromandelate (Ma et al., 2012), *Cg*KR2 for ethyl (*R*)-2-hydroxy-4-phenylbutyrate (HPBE) (Shen et al., 2012) and *Cg*CR for (*R*)-halohydrins (Xu et al., 2015). We thus supposed to introduce the screening criteria of height and breadth in substrate spectrum in order to screening the genome hunting library of *C. glabrata* for reductases with wide substrate spectrum, high activity and enantioselectivity. The genome hunting library of *C. glabrata* containing all putative reductases were developed and comparatively studied by average activity and Shannon-Wiener index toward substrate library containing 32 substrates (Fig. 1). The enzymatic properties and application potentials of the best reductase in the preparation of chiral secondary alcohols were also investigated.

2. Material and methods

2.1. Chemical reagents, plasmid and strain

All prochiral ketones, aldehyde and alkene substrates for reductases were purchased from Aladdin Co. (Shanghai). Cofactors NAD(P)$^+$ and NAD(P)H were bought from Bontac Bio-engineering Co. (Shenzhen). Other reagents were purchased from Sinopharm Co. (Shanghai) unless otherwise stated. Plasmid pET28a and *Escherichia coli* BL21(DE3) were stored in our lab.

2.2. Cloning and expression of reductases from Candida glabrata

Genomic DNA of *C. glabrata* was extracted using column genomic DNA extraction kit GK0122 (Shanghai Generay Biotech Co.) and used as template. Fifty-five putative reductase coding genes with length at range of 600–1500 bp were amplified using KOD polymerase (Toyobo Life Science Co., Shanghai) and primers listed in E-supplement from genomic DNA of *C. glabrata*. Vector pET28a was double digested with *Bam*H I and *Sal* I. The resultant PCR products were ligated into the linearized pET28a using Exnase II (Vazyme Co., Nanjing). The recombinant plasmids were transferred into *E. coli* BL21(DE3) and verified by colony PCR and sequencing. Heterogeneous expression of the reductases were performed by inoculated the recombinant *E. coli* BL21(DE3) in LB medium with 50 μg·mL^{-1} kanamycin and cultured at 37 °C and 180 rpm, and 0.2 mM isopropyl-β-D-thiogalactoside (IPTG) was supplemented when the OD_{600} reached 0.6–0.8 and further cultivated at 25 °C and 180 rpm for 6 h. The cells were collected by centrifuge at 8000 × *g* and 4 °C for 5 min, washed twice with physiological saline, re-suspended with phosphate sodium buffer (pH 7.0, 100 mM) and disrupted twice by high pressure homogenization at 600–800 bar (AH-BASICI, ATS Engineering Inc, Shanghai). The supernatant was obtained by centrifuge at 8000 × *g* and 4 °C for 20 min. SDS-PAGE analysis was performed to examine the expression level of all the recombinant reductases (E-supplement).

2.3. Activity assay

Standard enzyme activity assay protocol was spectrophotometrically performed by monitoring the absorbance changes of NADH or NADPH at 340 nm. The reaction mixture consisted of 2 mM substrate, 0.5 mM NADH or NADPH in 190 μL PBS buffer (pH 7.0, 100 mM) and 10 μL enzyme solution with appropriated concentration at 30 °C. The molar extinction coefficient of NADH or NADPH was 6220 L·mol^{-1}·cm^{-1}. One unit of reductase activity was defined as the amount of reductase that catalyzed the oxidation of 1 μmol NADH or NADPH per minute under above mentioned condition. Protein concentration was determined using Bradford method with BSA as standard protein. The specific activity of reductases was calculated according to Eq. (1).

$$\text{Activity (U/mL)}\frac{\Delta OD_{340} \times V \times 10^3}{6220 \times l}\text{, Specific activity (U/mg)} = \frac{\text{Activity (U/mL)}}{\text{Protein concentration (mg/mL)}} \quad (1)$$

where ΔOD_{340} is the absorbance changes at 340 nm per minute, V is the reaction volume (mL), l is the optical path length (cm).

2.4. Shannon-Wiener index

The Shannon-Wiener index was used as a diversity index in the statistic and adopted to describe the breadth of substrate spectrum of enzymes (Liu et al., 2013). The Shannon-Wiener index was calculated according to Eq. (2).