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Scaling up Asymmetric Biocatalysis with Cofactor Regeneration by Heterologous Expression of a Supra-active Carbonyl Reductase from Candida glabrata

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A carbonyl reductase (cr) gene from *Candida glabrata* CBS138 has been cloned, over-expressed, characterised and subsequently employed in biotransformation of a prochiral keto ester (COBE) to a chiral alcohol (ethyl-4-chloro-3-hydroxybutanoate or CHBE). Using NADPH as cofactor and as substrate, the isolated enzyme (CR) exhibited a towering specific activity of 173.49 ± 6.08 $Umin^{-1}mg^{-1}$ with K_m and K_{cat} as 0.45 ± 0.02 mM and 112.77 ± 3.95 s^{-1} respectively. Unlike other proteins of this class which usually show substrate inhibition at high substrate concentration (≥ 230 mM), the CR enzyme exhibited marked velocity at substrate concentration as high as 363 mM with highest turnover number (112.77 ± 3.95 s^{-1}). This advocated utility of the enzyme in a batch reactor where maximum COBE conversion has been achieved (161.04 $g.L^{-1}$ CHBE per g of dry cell weight) compared to the reported so far (1.51~ 149 $g.L^{-1}$ CHBE per g of dry cell weight). The reaction yielded sparingly available yet greatly important (R) isomer in over 99% enantiomeric excess (e.e) with 88.30% molar bioconversion. Although numerous proteins have been investigated to accomplish the prochiral COBE to chiral CHBE bioconversion, we present our finding as a highly efficient choice for conversion of COBE into CHBE through an efficient batch reaction system.

Keywords: Enzyme Kinetics, Cofactor Regeneration, Biotransformation, Enantiomeric Excess, Binding Site

Introduction

In previous studies, many enzymes of ALR and CAR family were cloned, characterized and used in the asymmetric synthesis of (*S*)-CHBE,[1-4]and (*R*) CHBE[5], a key chiral intermediate in the enantio selective synthesis of slagenins B and C as well as total synthesis of HMG-CoA reductase inhibitors and can be converted into 1,4 dihydropyridine type blocker. Conversion of ethyl-4-chloro-3-oxobutanoate (COBE) to optically active ethyl-4-chloro-3-hydroxybutanoate (CHBE) is one of the economical ways due to the easy availability of COBE[1-5]. Although attempts have been made to augment bioconversion by either genetically manipulating the biocatalytic system with cofactor regeneration or fabricating the reaction media with single or multiple solvents, productivity has often faced shortfall due to obtaining higher reaction rate only at small substrate concentration (5~230 mM) thus eliciting a limiting batch output within the reactor[6-9]. Hence, establishment of a biocatalysis system with industrial competence has always been a prime search through the years[10-12] which has driven researchers to find out newer proteins with improved activity and higher productivity. In this context, we report the cloning, expression and purification of a carbonyl reductase from *Candida glabrata* CBS 138. In pursuit, the CR enzyme was subsequently purified and kinetically characterized. Following this, the asymmetric reduction of COBE to (*R*)-CHBE was carried out in a cofactor generating *E. coli* system expressing both CR and GDH enzymes. The monophasic and biphasic reaction yields were estimated and optical purity of the reaction product was assigned

Material and methods

Construction of CRpETDuet-I and GDHCRpETDuet-I

The *carbonyl reductase* (*cr*) (NC_006028.2) from *Candida glabrata* CBS 138 was PCR amplified with PFU DNA polymerase, subsequently digested using *Nde*-I and *Xho*-I and ligated to pETDuet-1 to obtain CRpETDuet-1. For construction of GDHCRpETDuet-I, *gdh* gene was amplified from the same of *Bacillus subtilis*, digested with *Bam* HI and *Hind*III and cloned on to the complementary site of CRpET Duet-I to construct GDHCR pET Duet-I.

Enzyme over-expression and purification

Enzyme expression and purification was done using overnight inoculums of BL21 (DE3) of correct

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individual clones in fresh Luria Bertani (LB, Bacto tryptone 10gL^{-1}, Yeast Extract 5g.L^{-1}, NaCl 10g.L^{-1}) media with IPTG (0.3 mM) at 37˚C. The cell lysate supernatant was loaded onto a Ni-His binding column (His-Gravitrap Column; GE Healthcare, USA) equilibrated with a buffer. The binding buffer contained 10 mM PPB, 20 mM imidazole, 500 mM NaCl (pH 7.2) while the protein was eluted with 10 mM PPB, 200 mM Imidazole, 500 mM NaCl (pH 7.2).

Characterization and optimization of enzyme kinetics

Enzyme characterization and its temperature and pH dependant activity were studied. A reaction system (1ml) comprising the substrate COBE (0-4 mM); cofactor NADPH (200 µM); enzyme CR (100~113 ng) in a reaction media of 100 mM PPB was constructed to evaluate enzyme characteristics. The enzyme activity was assayed spectrophotometrically at 340 nm where a temperature of 30^{0}C was used monitoring the reaction for 120s.

Bioconversion in batch reaction

Both monophasic and biphasic reactions were carried out using 500 ml cultured cell pellets (dry cell weight 0.33 gm) over-expressing CR and GDH proteins, 18 mg $NADP^+$ and 0.1 (biphasic) ~ 0.18 (monophasic) g.L^{-1} glucose. While monophasic reaction involved only 25 ml 100 mM PPB, pH 7.5 comprising of 0.00074 mol of COBE, biphasic reaction involved 25 ml 100 mM PPB, pH 7.5 harboring the cell pellet together with 25 ml butyl actetate containing 6% (w/v) COBE.

Analyzing bio-production

Gas Chromatography (GC) was followed to determine the COBE and CHBE concentrations (GC, Agilent, model 6890) after the batch reaction using a flame ionization detector and a HP-5 capillary column (20m x 0.18 mm; film thickness 0.18 µm). The inlet temperature was set to 210ºC and temperature gradient was set as 45ºC-150 ºC with 10 ºC.min^{-1}. The detector temperature was set at 150 ºC.

Enantiomeric excess estimation

High Performance Liquid Chromatography (HPLC) technique was employed to calculate the enantiomeric excess of the formed isomer with *n*-hexane/IPA solvent system as the mobile phase (90:10) and Chiralcel OB packed column (4.6 x 250 mm, Daicel Chemical industries, Japan) at column temperature 30ºC. The mobile phase flow rate was set at 0.5 ml min^{-1}. The detection was performed at 217 nm at UV- spectrophotometric detector.

Results and Discussion

Over-expression, purification and enzyme characterization

The enzymes were over expressed by IPTG induction (Supplementary Figure S1a) as shown by

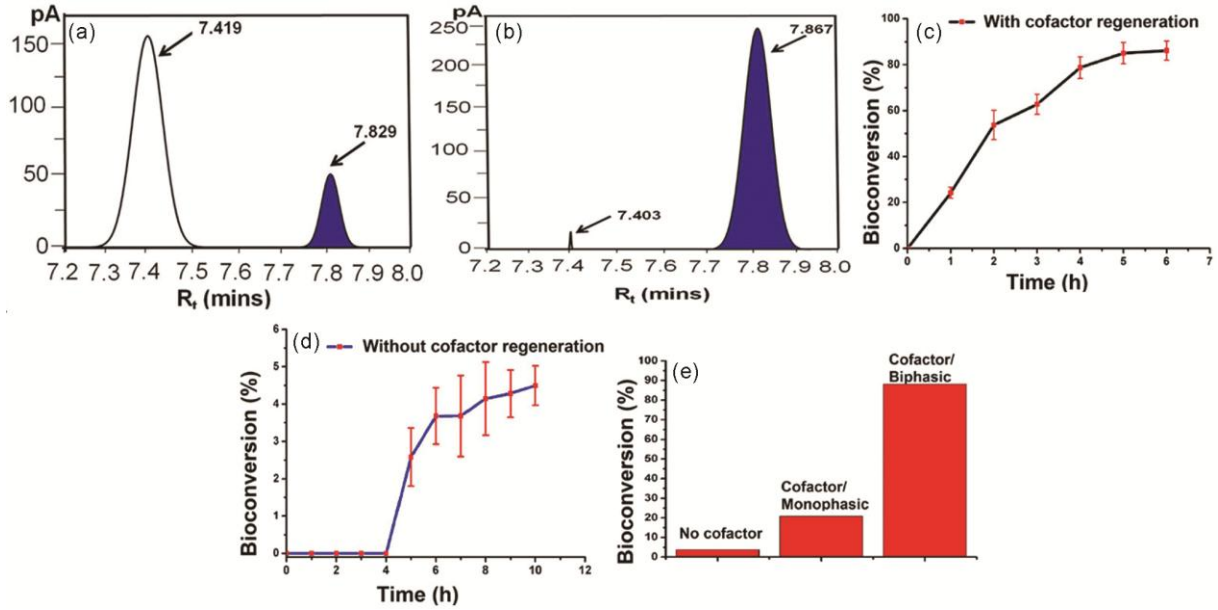

Fig.1 — (R) CHBE production profile in biotransformation reaction. (a) GC chromatograms of reaction products without cofactor regeneration and (b) GC chromatograms of reaction products with cofactor regeneration. (c) Yield profile without cofactor regeneration (d) Yield profile with cofactor regeneration (e) Comparative study of bioconversion under three reaction schemes the data points in Figure. (3c) and (3d) are the average (±standard deviation) of three independent observations (n=3)

band over 37 KDa line (CR protein, molecular weight 39 KDa) and a band corresponding 28 KDa line (GDH protein). The CR enzyme has been purified by affinity chromatography which was clearly revealed on SDS PAGE by isolated band visible after desalting (Supplementary Figure S1b). The K_m was determined in the range of 0.42 to 0.46 mM having a mean of 0.45±0.02 mM. In addition the Specific Activity was found in the range of 167.56 to 173.19 µmol of NADPH converted/min/mg of enzyme with a mean of 173.49±6.08. The mean K_{cat} was calculated as 112.77±3.95 s^{-1}. Finally the mean enzyme efficiency (K_{cat}/K_m) was elucidated as $2.5x10^{-5}$ M^{-1} s^{-1}.

Optimization of reaction condition

For pH dependent activity evaluation, the enzyme showed marked activity at pH 7.5, 7.8 and 8.2 (108.85, 109.15 and 91.4 µmol.min^{-1}.mg^{-1} respectively, Supplementary Figure S2a). Considering this, pH 7.5 has been chosen as the optimum pH for all subsequent studies. Furthermore, the enzyme showed marked stability and activity in a zone of 30 ºC ~ 40 ºC (100.0 -113.0 µmol.min^{-1}.mg^{-1} at 1mM COBE concentration, Supplementary Figure S2b).

Bioconversion

Whole cells were used for biocatalysis due to easy scale-up options by cell culture than isolated enzyme, greater stability than the latter in extracellular media and its cost-effectiveness to regenerate than that of isolated enzyme. When whole cells over-expressing the CR and GDH proteins were employed, reaction started almost immediately upon exposure to the substrate (COBE). Without cofactor regeneration, the COBE reduction proceeded very slowly and showed much lesser yield (Figure 1a) than that of with cofactor regeneration (Figure 1b). On adding 6% (w/v, 363.63 mM) COBE to an 1:1 (organic solvent volume/ buffer volume) biphasic reaction system, with cofactor regeneration, the product formation increased exponentially up to 4h and reached saturation within 6h (88.3 % bioconversion from COBE to CHBE, Figure 1c). On the contrary, without cofactor regeneration, the reaction reached saturation at around 5% bioconversion within 8-10 h (Figure 1d). Interestingly, when monophasic reaction system was used consisting of PPB buffer, cofactor regenerating GDHCRpET Duet-I plasmid produced a bioconversion of 20.80% after 24h. Figure 1e depicts a comparative portfolio of bioconversion under three aforementioned conditions. Under the stated HPLC conditions (for details, see 2. experimental methods), pure (*R*) and (*S*) - CHBE peaks got separated and were eluted at 14.945 min and 15.592 min respectively (Figure 2a). When we analyzed our reaction product we obtained only one peak at 14.907 min (Figure 2b). It implies the reaction product to be the (*R*) - isomer suggesting an enantiomeric excess > 99%. HPLC analyses were also used to check if the enantio selective product formation was really due to CR enzyme's stereo selectivity or due to any other cytosolic or extracellular reaction condition. The reaction catered by either the pure enzyme (Figure 3a) or the whole cell (Figure 3b) revealed the same product ((*R*)-CHBE) which suggested the endogeneous *(R)* - stereo specificity of the enzyme. The lesser yield of the reaction product without

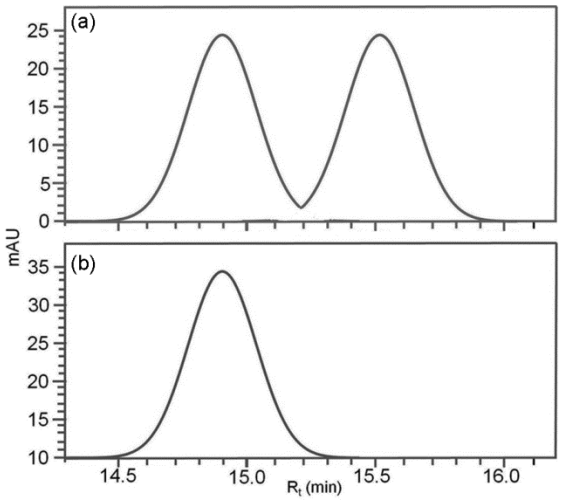

Fig.2 — Enantiomeric excess (e.e) determination of reaction product through HPLC. (a) Separation of standard racemic mixture of CHBE (b) Analysis of reaction product

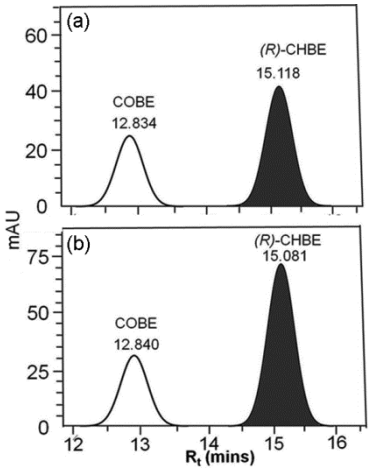

Fig.3 — HPLC chromatogram of reaction product by (a) Pure enzyme and (b) whole cell

cofactor regeneration (as shown by Figure1c and 1d) is easily explained by the lack of hydrogen supply for the reduction of COBE to CHBE, which could have been supplied from NADPH to get converted into $NADP^+$. The reaction yield reached highest at 6% (363.63 mM) COBE producing 88.3% bioconversion; may be at this concentration, the cytosolic concentration of COBE generated maximum reaction velocity (V_{max}). The high V_{max} corroborates high substrate-product conversion per unit time, the lower K_m signifies achieving half V_{max} with low substrate concentration which further indicates high enzyme-substrate binding affinity. In addition, high K_{cat} indicates high catalytic efficiency of the enzyme. Thus, we propose, this enzyme-substrate interaction has been favourable to enhance the catalytic output of the bioconversion system. The reduction in the relative yield within the monophasic system might be due to low aqueous solubility of COBE as well as partial hydrolysis of the substrate in aqueous phase. It is noteworthy to mention that although several accounts are there related to the production of (*R*)-CHBE from COBE, certain limitations have been reported in the previous works. On increasing the substrate concentration, the authors reported marked decrease in productivity thereby indicating substrate inhibition of the enzyme (supplementary Figure. S3a). Thus, scaled up production of (*R*)-CHBE in these systems require either a heavy reactor or multiple batch reactions which is both time and cost intensive. In contrast, in a single batch of 363.63 mM COBE biotransformation, our enzyme exhibited more than 88% bioconversion and 99% e.e. which surpassed commercially viability of all those previous reaction systems. Previous works on COBE bio reduction reported CHBE yield from 1.51 to 149 $g.L^{-1}$ DCW^{-1} (DCW= Dry Cell Weight) without special engineering of the biocatalytic system. Even in last few years with novel discoveries of enzyme and special engineering of biocatalytic media, 0.166 – 49.8 gL^{-1} CHBE DCW^{-1} has been achieved[13-18] only report acknowledging the yield of 265.5 gL^{-1} CHBE DCW^{-1} in 2015[16]. In comparison, our biocatalytic device yielded CHBE production 161.04 $g.L^{-1}$ DCW^{-1} (supplementary Figure S3b) which is comparable or higher than those reported so far. Furthermore, the uniqueness of the enzyme (GRAVY:- 0.359) suggests its hydrophilicity thereby advocating its applicability in wide range of biocatalysis. Hence, whole cell biocatalysis using heterologous expression of Carbonyl Reductase from *Candida glabrata* CBS138 can be justified as an alternative route of (*R*)-CHBE production with improved batch productivity, high substrate turnover per batch, and with a high cost and time effectiveness of the reaction.

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