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Immobilization of intracellular carbonyl reductase from *Geotrichum candidum* for the stereoselective reduction of 1-naphthyl ketone

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ABSTRACT

Different cell disintegration methods were used for the liberation of intracellular carbonyl reductase from *Geotrichum candidum*, in its active form. Solid shear (bead milling) was proved to be the best method for the extraction of the enzyme. Various solid supports were checked for the immobilization of the purified enzyme. Carbonyl reductase was immobilized on silica with an optimized protein loading of 4 mg/g support. Cross-linking with glutaraldehyde rendered the preparation more stable and suitable for use in consecutive batches. Carbonyl reductase of *G. candidum* immobilized on silica support and cross-linked by glutaraldehyde was found to be highly efficient biocatalyst formulation for the production of *S*(−)-1-(1′-naphthyl) ethanol.

1. Introduction

In recent years, development of efficient methods for the production of chiral alcohols has been a thrust area for pharmaceuticals, agrochemicals and natural substances (Wang et al., 2009; Xie et al., 2009). Asymmetric synthesis of chiral alcohols by microbial reduction of corresponding ketones is rapidly dominating over the chemical reduction or resolution of racemates (Zilbeyaz and Kurbanoglu, 2008). Carbonyl reductases are generally intracellular, low molecular weight, monomeric, cofactor (NADH/NADPH) requiring enzymes, responsible for reducing carbonyl group to optically pure chiral alcohol. However, the whole cell microbial reduction often suffers from the low productivity, low overall yield and lack of selectivity due to either insufficient chiral recognition by the enzyme or presence of various enzymes of the same function with different selectivity (Faber, 1999). The cellular membrane system itself can act as a barrier and posses the mass-transfer limitations. In the case of insoluble substrate, introduction of a co-solvent is required (Kansal and Banerjee, 2009), however, the co-solvent itself may have the denaturating effect on the enzyme activity. On the other hand, adherence of the substrate or the product or the co-solvent (or all of these) on to the cell surface causes lower yield of the product. In addition, the co-solvent itself may cause substantial damage on the cell surface rendering them unsuitable for the subsequent reuse due to the reduced cell viability. Selection of a biocatalyst with high selectivity and stereo-specificity is a prerequisite for the development of a biocatalytic process. Furthermore, immobilization of purified enzyme on suitable solid support may provide an economic and convenient alternative for the maximum exploitation of the biocatalyst, since; it stabilizes the enzyme and allows its reuse and easy separation from reaction mixture (Fressener, 2000).

S(−)-1-(1′-naphthyl) ethanol, a crucial intermediate for the lactone moiety of hydroxyl methyl glutaryl Co A (HMGCoA) inhibitors has been produced by reducing 1-acetonaphthone with *Geotrichum candidum* (Roy et al., 2003). Studies have also been carried out to enhance the carbonyl reductase production through optimization of media components and physico-chemical parameters by *G. candidum* (Bhattachartta and Banerjee, 2007). Carbonyl reductase of *G. candidum*, responsible for the stereoselective reduction of 1-acetonaphthone has already been purified and characterized (Singh et al., 2009). The purified enzyme has been found to be highly specific for the reduction of ketones containing naphthyl ring. In the present study, efforts have been made to make this enzyme suitable for the production of optically pure naphthyl ethanols with good yield and excellent enantioselectivity. Different mechanical cell disruption techniques, i.e., solid shear, liquid shear and sonication were tried for the liberation of the enzyme with high specific activity along with good yield. Various matrices were tried to immobilize the enzyme through surface adsorption. Most suitable matrix was used for the optimum enzyme loading and subsequently used for the biocatalysis.

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2. Methods

2.1. Materials

1-Acetonaphthone (methyl-1-naphthylketone) was purchased from Fluka chemical, (GMBH, Steinhein, Germany). Ethanol was obtained from Les alcohol de commerce Inc. (Brampton, Ontario L6T 3Y4). NADH and growth media components were obtained from Hi-Media Inc. (Mumbai, India). Inorganic and buffer salts were supplied by Qualigens Inc. (Mumbai, India). Amberlite resin XAD-4 and XAD-7 were purchased from Aldrich, Germany. Celite (Celite® 545 AW) was obtained from Sigma (St. Louis, MO, USA); silica gel-60 (particle size 0.040–0.063 mm) was purchased from Merck, Germany and the glass beads were available from B. Braun Biotech International, Melsungen, Germany. All other chemicals were purchased from Sigma (St. Louis, MO, USA), or otherwise mentioned.

Racemic 1-(1′-naphthyl) ethanol was prepared by the $NaBH_4$ reduction of 1-acetonaphthone. Authentic *S*(−)-1-(1′-naphthyl) ethanol was prepared (ee 95%) by the reduction of 1-acetonaphthone using baker's yeast (Type 1, Sigma, USA) as reported earlier (Roy et al., 2003).

2.2. Analysis of reactant and product

Qualitative and quantitative determination of bioreduction was performed on HPLC (Shimadzu 10AVP) equipped with UV detector using a C18 column (4.0 mm × 250 mm, 5 μm, Waters, Netherlands) and was monitored at 233 nm. Solvent system of acetonitrile and water (4:6) was used at a flow rate of 1 ml/min to elute the components from the column. The enantiomeric excess of the product alcohol was determined using Chiralcel OD-H column (0.46 mm × 250 mm, 5 μm, Diacel Chemical industries, NJ, USA) using solvent system of hexane and isopropanol (90:10) at a flow rate of 0.5 ml/min. The (*S*)-(−)- and (*R*)-(+)-1-(1′-naphthyl) ethanol was eluted at 17.8 and 27.5 min, respectively. Their enantiomeric excess (ee) was determined directly from the area under the curve (AUC).

2.3. Microorganism and growth

G. candidum NCIM 980 was obtained from the National Collection of Industrial Microorganism (NCIM), National Chemical Laboratory, Pune. The organism was maintained on medium containing (g/l) malt extract 3, glucose 10, yeast extract 5, peptone 5 and agar 15, whereas media containing (g/l): malt extract 3, yeast extract 3, glucose 20, peptone 5, brain–heart infusion 1.5 and meat extract 1.5 at a pH of 6.5 (adjusted with 6 N HCl or NaOH) was used as the production medium. Inoculum (20 ml) was grown for 24 h at 30 °C in a rotary shaker (200 rpm) and 2% (v/v) was transferred to the production medium. The culture was further grown for 60 h under identical condition to achieve maximum enzyme production. Cells were centrifuged at 10,000*g* for 20 min at 4 °C, washed with double distilled water (twice) and suspended in phosphate buffer (0.05 M, pH 7.0) containing 1 mM DTT and 0.1 mM PMSF to have an appropriate final cell concentration.

2.4. Liberation of carbonyl reductase from whole cells

2.4.1. Sonication

Misonix ultrasonic processor XL 2020 (NY, USA) was used to disrupt the resting cells (200 mg/ml) of *G. candidum*. Different samples (2 ml each) were sonicated for various time periods ranging from 2 to 20 min with 30 s pulse-on and 30 s pulse-off, at 120 W acoustic power. Temperature of cell suspension was maintained at 4 °C using ice bath. After sonication, samples were assayed for the enzyme activity and protein content.

2.4.2. Liquid shear

Resting cells (200 mg/ml) was disrupted at various pressures ranging from 1000 to 16,000 psi in a 20 K cell of Thermo Spectronic French Press (Rochester, USA). Temperature was maintained at 4 °C by keeping the pressure cell and cell suspension in ice bath prior to disruption. Released carbonyl reductase was assayed to determine the enzyme activity and protein content using standard assay protocols. Other related parameters of cell disruption in French press such as number of passages (1–4), flow rate of the slurry (0.5–6 ml/min) and cell concentration (50–400 mg/ml, wet cell) were also investigated.

2.4.3. Solid shear

Suspension of *G. candidum* was charged in 300 ml vessel of Multi Lab Dyno-Mill (Willy A. Bachofen Maschinenfabrik, Basel, Switzerland) with lead-free glass beads. The temperature was maintained by circulating water at 4 °C through the jacket of the vessel. Enzyme activity and protein content were determined using standard assay protocols. Various related parameters like, cell concentration (50–250 mg/ml), bead size (0.25–1.5 mm), bead volume (60–90%) and impeller speed (1000–4000 rpm) were investigated.

2.5. Enzyme assay

The disintegrated cell suspension of *G. candidum* liberated by cell disruption was centrifuged at 25,000*g* for 40 min to remove the cell debris. Enzyme activity of the supernatant was measured by the reduction of NADH absorbance at 340 nm (Badal, 2004). The assay mixture (3 ml) consisted of 300 μl cell free extract, 250 μM 1-acetonaphthone (methyl-1-naphthylketone) in phosphate buffer (0.05 M, pH 7.0). Reaction was started by adding 200 μmol NADH and the change in absorbance was measured for 10 min using UV visible spectrophotometer (Specord 50, Analyticjena, Germany). One unit (U) of enzyme activity is defined as the amount of enzyme required to oxidize 1 μmol NADH per minute at standard condition.

Enzyme activity of whole cells was determined by HPLC analysis. The reaction mixture (3 ml) contained 1-acetonaphthone (final concentration 10 mM, dissolved in ethanol) and 200 mg/ml resting cells in phosphate buffer (0.2 M, pH 7). Reaction was quenched after 10 min adding equal volume of ethyl acetate. Cells were separated by centrifugation (12,000*g*, 4 °C) from the reaction mixture, which was subsequently extracted in organic solvent, dried and analyzed.

To measure the immobilized enzyme activity, reaction was performed in phosphate buffer (0.2 M, pH 7) containing 10 mg enzyme preparation and 10 mM 1-acetonaphthone in a final volume 3 ml. The reaction was started by adding 10 mM NADH and incubated for 10 min at 30 °C. The reaction was quenched by adding equal volume of ethyl acetate. The catalyst was separated by centrifugation (12,000*g*, 4 °C) and the reaction mixture was extracted in organic phase, dried and analyzed by HPLC.

2.6. Protein determination

Protein concentration in various samples were determined by the Bradford dye binding method (Bradford, 1976) using bovine serum albumin as standard.

2.7. Screening of matrices for the enzyme immobilization

Carbonyl reductase from *G. candidum* was purified using $(NH_4)_2SO_4$ precipitation, ion exchange and hydrophobic interaction