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Broad substrate Cytochrome P450 monooxygenase activity in the cells of *Aspergillus terreus* MTCC 6324

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Abstract

Cytochrome P450 (CYP) monooxygenase activities with different category of substrates namely, alkanes, alkane derivatives, alcohols, aromatic compounds, organic solvents, and steroids were detected in the cells of *Aspergillus terreus*. High CYP specific activity was observed when methanol (5.6 ± 0.017 U mg^{-1}), acetone (7.76 ± 0.02 U mg^{-1}), dimethylsulphoxide (DMSO) (9.70 ± 0.005 U mg^{-1}), *n*-hexadecane (4.39 ± 0.02 U mg^{-1}), or *n*-octadecane (4.23 ± 0.01 U mg^{-1}) were used as substrates. Significant CYP specific activity was also detected when naphthalene (3.80 ± 0.002 U mg^{-1}) was used as substrate. The CYP catalysis of *n*-hexadecane had followed both terminal and sub terminal oxidations. The activity was localized in the cytosol of *n*-hexadecane grown cells, while, it was apparently distributed in light mitochondrial fraction and microsomal fraction of glucose grown cells. The substrate specificities of CYP present in all the locations were similar irrespective of the substrates used for the growth. Heme staining of the microsomal fraction containing CYP and other proteins in SDS–PAGE showed single heme protein band with corresponding molecular weight of 110 kDa.

Keywords: Filamentous fungi; *Aspergillus terreus*; Cytochrome P450 monooxygenase; *n*-hexadecane; Microsome

1. Introduction

Microbial monooxygenase/hydroxylase enzymes involved in the metabolism of endogenous and xenobiotic compounds have potential application in many areas such as, bioremediation of petroleum hydrocarbon contamination (Mfincnerovfi and Augustin, 1994) organic synthesis, and production of pharmaceutically and other industrially useful compounds (Beilen and Funhoff, 2005; Bistolasa et al., 2005). The microbial sources for these enzymes are mostly reported from several bacterial species (Britton, 1984), and yeasts such as *Candida* sp. (Scheller et al., 1996). Searching novel source of these enzymes is a continuing thrust with a hope of exploring enzymes of unusual catalytic properties. Research on filamentous fungi for the monooxygenases has not yet received adequate attention as revealed from the limited literature available on the area. *Fusarium oxysporum* (Nakayama and Shoun, 1994; Nakayama et al., 1996) and *Cladosporium resinae* (Goswami and Cooney, 1999) are some of the fungi only where progress is recorded to a greater extent. The filamentous fungus that belongs to the species *Aspergillus* is wide spread in nature and utilizes substrates of diverse chemical character. Since, monooxygenases are the foremost enzymes involved in the microbial metabolism of many such substrates, the fungal cells of *Aspergillus* is likely to hold enzymes such as, Cytochrome P450 (CYP) monooxygenase, with novel catalytic properties having potential industrial applications.

We report here the presence of CYP activities with broad substrate specificities in the cells of *Aspergillus terreus*. The localization of these enzymes in the sub-cellular sites during growth of the fungi in different substrates is also presented in this paper.

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2. Methods

2.1. Organism and culture conditions

The culture conditions and maintenance of *A. terreus* MTCC 6324 used in this study were described elsewhere (Kumar and Goswami, 2006).

2.2. Preparation of microsomes

The procedure for microsome preparation described earlier (Kumar and Goswami, 2006) is briefly as follows: The cell homogenate formed after disruption at 30 kpsi was first centrifuged at 10,000*g* for 10 min to pellet the undisrupted cells and nuclei. The supernatant (Sup-1) was collected and subjected to 20,000*g* for 20 min to pellet light mitochondrial fraction (LMF). The supernatant (Sup-2) collected was again centrifuged at 114,000*g* for 1 h 30 min to sediment the microsomal fraction (MF). The MF was separated and suspended in 50 mM Tris–HCl buffer (pH 8.0). The supernatant (Sup-3) formed was retained for enzymatic study. All the steps in the procedure were performed at 4 °C unless specified.

2.3. Assay of CYP

The CYP activity was assayed by measuring the substrate dependent oxygen consumption in a biological oxygen monitor (BOM) (model 53 YSI, USA) and a Clark-type oxygen electrode with a partial modification of the method described earlier (Kumar and Goswami, 2006). Three milliliters of emulsified substrate containing 25 mM of *n*-hexadecane and Triton X-100 (0.2 mg ml^{-1} final concentration) in Tris–HCl buffer (50 mM, pH 8.0) was sonicated for 30 s and taken in each tube of BOM. Hundred microliters of NADH (0.33 mM final concentration) was added as cofactor before the addition of enzyme. A Parallel reaction containing all the components of reaction mixture except the substrate was used as control to nullify any endogenous oxygen consumption during the assay. One unit (U) of enzyme activity is the amount of enzyme that consumes 1 μmol O_2 in 1 min at 30 °C.

2.4. Assay of NCP

NADH Cytochrome P450 reductase (NCP) activity was assayed by measuring its NADH Cyt C reductase activity as described elsewhere (Imai, 1976). The rate of Cyt C reduction was calculated from the A_{550} change using an extinction coefficient ($\varepsilon = 21\ mM^{-1}\ cm^{-1}$).

2.5. Determination of substrate specificity of CYP

Substrate specificity was determined by using 25 mM of the test substrates in the reaction mixture instead of *n*-hexadecane as described earlier. In case of volatile substrates such as methanol, ethanol, propanol, acetone, benzene, toluene and benzyl alcohol, 100 mM of the test substrates were used and then the CYP activity was measured by BOM as described earlier.

2.6. Determination of pH and temperature optima and stability of CYP

In order to determine pH and temperature optima, the enzyme reactions were carried out in different test pH buffers and different test temperatures, respectively and the CYP activity was measured by BOM. For determination of pH stability, the enzyme samples were incubated in test pH buffers for minimum 30 min to maximum 96 h at 4 °C and then residual activity of CYP was determined by BOM at 30 °C in Tris–HCl buffer (50 mM, pH 8.0). Similarly, for determination of temperature stability enzyme samples were incubated at different test temperatures in circulating water bath for different time periods followed by measurement of residual activity of CYP by BOM as described earlier. Protein estimation was done following the Bradford's method using BSA as standard (Bradford, 1976).

2.7. CO difference spectra

Establishment of monooxygenase from *A. terreus* as Cyt P 450 monooxygenase was achieved by analyzing CO difference spectra following a partial modification of the original method described elsewhere (Estabrook and Werringloer, 1978). Samples of microsomes containing 1.5–2 mg of protein in 1 ml of phosphate buffer (pH 7.4) containing 20% glycerol, in a stoppered cuvette were gently sparged with carbon monoxide for 1–2 min, at which time several fine grains of solid sodium dithionite were added. Sparging was continued for 1–2 min more, and the cuvette was stoppered. Spectra (410–500 nm) were recorded on a Cary 100 UV–Vis spectrophotometer (Varian, USA) to record the maximum development of difference spectra at 450 nm.

2.8. Inhibitor study with taxifolin

Enzyme sample was incubated for 5 min with taxifolin at concentrations of 0–900 μM, before the addition to the reaction mixture containing *n*-hexadecane as substrate. The CYP activity was then measured by BOM as described earlier. A control without inhibitor was run simultaneously to calculate the effect of inhibitor concentration in terms of residual activity of the CYP.

2.9. Solubilization and reconstitution of enzyme system

Solubilization of microsomal fraction containing CYP and NCP was initiated by addition of a 10% (w/v) sodium cholate solution to give a final concentration of 0.8% (w/v) and was carried out for 30 min at 4 °C. After ultracentrifugation (100,000*g*, 60 min, 4 °C) both supernatant and pelleted fractions were checked for activities of both the