Study of mixed function oxidase system in *Aspergillus ochraceus* (NCIM 1146)

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Abstract *Aspergillus ochraceus* (NCIM-1146) has shown the ability to degrade cholesterol, camphor and naphthalene, when 96 h grown mycelium incubated in medium containing these organic compounds. Presence of higher level of electron transport components and biotransformation enzyme activity were observed in *Aspergillus ochraceus*, when grown in potato dextrose medium for 96 h. The enzyme activity preferred NADPH as a cofactor and shows inhibition in the presence of CO, indicating cytochrome P-450 mediated reactions. A significant increase in the levels of electron transport components and biotransformation enzyme activity were observed in presence of different inducers (viz. cholesterol, camphor, naphthalene, veratrole, phenobarbital, n-hexane, kerosene and saffola oil) when compared with mycelium incubated in same way with similar conditions for 2 min incubation. Analyses of the products of cholesterol and camphor using HPLC and GCMS confirm the degradation of these compounds.

Keywords Biotransformation enzymes · hexane oxidase · cytochrome *c* reductase · camphor · cholesterol · naphthalene · n-hexane

Introduction

The cytochrome P-450 monooxygenase system is of great catalytic versatility and widely distributed in nature being found in animals, plants and microorganisms. Cytochrome P-450 usually acts as terminal oxidase in multicomponent electron transfer chains. Cytochrome P-450s are ubiquitous enzymes and important in the oxidative, peroxidative and reductive metabolism of numerous endogenous compounds such as steroids, bile acids, fatty acids, prostaglandins, leukotrienes and foreign chemicals including drugs, environmental pollutant and natural plant products¹. In this way, by using capable microorganisms certainly contribute to keep clean environment. The presence of cytochrome P-450 enzyme systems in the various fungi has been reported (Viz. *Serratia marcescens*,² *Curvularia lunata*,³ *Cunninghamella blakesleeana*⁴ etc.) for important biotransformation reactions. Cytochrome P-450 mediated biotransformation reactions are well studied in *Cunninghamella* species viz. *C. elegans*⁵⁻⁶, *C. echinulata*⁷ and *C. bainieri*⁸.

A cytochrome P-450 linked monooxygenase catalyzed 11 α -hydroxylation of progesterone in *Aspergillus ochraceus* TS. The different classical inducers of cytochrome P-450 induce the benzo(a)pyrene hydroxylase activity to varying degree and suggests the existence of multiple forms of cytochrome P-450 in this fungus⁹⁻¹⁰.

The present paper reports the presence of standard cytochrome P-450 mediated biotransformation enzymes cytochrome *c* reductase and hexane oxidase in *Aspergillus ochraceus* (NCIM 1146), and their inducible nature by using various inducers. Present paper deals with the involvement of fungal enzymes in the degradation of cholesterol, camphor and naphthalene.

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Materials and Methods

Organism and culture conditions: Pure culture of *Aspergillus ochraceus* (NCIM-1146) was obtained from National Chemical Laboratory (NCL, Pune, India). Pure culture was maintained on potato dextrose agar slants and stored in test tubes at 4 °C and sub cultured monthly. The fungal mycelium was grown aerobically in 250 ml Erlenmeyer flask containing 100 ml of sterile media containing (g l⁻¹) peeled potatoes 200, dextrose 20, yeast extract 0.1, pH being adjusted to 5.7 and incubated at 30 °C, under static condition, for different time intervals (viz. 48, 72, 96, 120 and 144 h). Fungal growth was measured after incubation.

Preparation of cell free extract and enzyme assays: After incubation at defined time intervals mycelium was removed and cut into small pieces and homogenized with two volumes of potassium phosphate buffer (50 mM, pH 7.4) in Potter-Elvehjem type homogenizer and used for different enzyme assays. The protein content was estimated by Biuret method¹¹. The measurement of cytochrome *b5* was carried out by using Shimadzu UV-vis recording spectrophotometer (UV-160A)¹. The NADPH cytochrome *c* reductase was determined by the method of Masters et al¹² with some modification².

Standardization of hexane oxidase enzyme assay: Hexane oxidation assay was standardized in our laboratory. It includes incubation in 50 mM HEPES buffer (pH 7.8), containing NADPH-generating system (NADPH, 2.6 mM; Glucose 6-phosphate, 12.5 mM; Glucose 6-phosphate dehydrogenase, 4 units) and 0.5 ml homogenate (5 mg ml⁻¹ protein) in 2 ml reaction mixture. After the addition of 0.4 ml of hexane to the incubation medium as a substrate, the reaction mixture was incubated at 37 °C for 20 min and terminated the reaction by adding 1 ml of ice cold 20% trichloroacetic acid solution. The amount of acetaldehyde liberated was estimated using Nash reagent^{16,29}.

Effect of carbon source on biotransformation enzyme activities: Ninety six hour grown mycelium of *Aspergillus ochraceus* (5 g) was resuspended in incubation medium containing 50 mM potassium phosphate buffer (pH 7.4) and one of the inducer as a sole carbon, and kept under shaking condition (200 rpm) at 30 °C, for 18 h. Mycelium was removed after incubation and washed three times with 50 mM potassium phosphate buffer (pH 7.4), homogenized in two volumes of potassium phosphate buffer (50 mM, pH 7.4) in Potter-Elvehjem type homogenizer. This crude homogenate was used for enzyme assay. Since presence of these compounds interferes in the color development process, the control experiments were performed with similar conditions for 2 min incubation.

Degradation study of cholesterol, naphthalene and camphor: Ninety six hour grown mycelium of *Aspergillus ochraceus* (5 g) resuspended into incubation medium containing 50 ml of phosphate buffer saline (50 mM, pH 7.4) containing cholesterol (0.15 g), naphthalene (0.25 g) or camphor (0.20 g) as one of the carbon source. This reaction mixture was incubated, at 30 °C with shaking (200 rpm). 2 ml of this reaction mixture was extracted into 1 ml of ethyl acetate and analyzed at different time intervals by thin layer chromatography (TLC) on silica gel plate with a solvent mixture of benzene: acetone (3:1). The separated compounds on silica gel plate were visualized by keeping the plate in an iodine chamber.

Ethyl acetate extract was dried over anhydrous Na₂SO₄ and evaporated to dryness in rotary evaporator. The crystals obtained were dissolved in small volumes of methanol and the same sample was used for HPLC and GCMS analysis. HPLC analysis was carried out (waters model no. 2487) on RP-C18 guard column with dual UV- Visible detector. The mobile phase was the isocratic methanol with flow rate 1.0 ml min⁻¹. GCMS analysis was performed using a QP2010 gas chromatography coupled with mass spectrometer (Shimadzu). The ionization voltage was 70 eV. Gas chromatography was conducted in the temperature programming mode with a Restek column (0.25 mm, 60 m; XTI-5). The initial column temperature was 80 °C for 1 min, and then increased linearly at 8 °C min⁻¹ to 180 °C followed by 15 °C min⁻¹ to 270 °C, held for 2 and 7 min, respectively. The temperature of the injection port was 280 °C and the GC/MS interface was maintained at 290 °C. The helium carrier gas flow rate was 1.0 ml min⁻¹. Degradation products were identified by comparison of retention time and fragmentation pattern, as well as with mass spectra in the NIST spectral library stored in the computer software (version 1.10 beta, Shimadzu) of the GCMS.

Results and Discussion

The level of electron transport components viz cytochrome *b5*, cytochrome *c* reductase and biotransformation enzyme hexane oxidase were observed in *Aspergillus ochraceus* (NCIM 1146) during the growth in potato dextrose medium. The highest levels of all the enzyme activity of *Aspergillus ochraceus* were observed at 96 h and decreased thereafter at 120 h and further at 144 h (Table 1). Similar results were observed in case of laccase that showed age dependent production in white rot fungi viz. *Cariolus versicolor* and *Funalia trogii*¹³, *Phlebia facicularia*, *Phlebia floridensis* and *Dichomitus squalens*¹⁴ and *Bjerkandera adusta*¹⁵. Decline in the activities after 96 h cultivation time may be

Table 1 Levels of the electron transport components and hexane oxidase activities in *Aspergillus ochraceus* (NCIM 1146) during the growth in potato-dextrose medium.

Parameters	Time of growth (h) ^a				
	48	72	96	120	144
Cell yield (g l ⁻¹) (Wet weight)	3.05 ± 0.34	4.63 ± 0.04	6.38 ± 0.04	7.18 ± 0.01	7.93 ± 0.04
Protein content (mg g ⁻¹)	19.50 ± 0.83	23.33 ± 0.42	30.00 ± 0.51	40.50 ± 0.34	43.16 ± 0.54
Cytochrome b ₅ (nmol(mg protein) ⁻¹)	ND	0.02 ± 0.003	0.04 ± 0.005	0.02 ± 0.005	ND
NADPH-cytochrome c reductase ^b	0.74 ± 0.01	1.98 ± 0.05	2.43 ± 0.04	1.19 ± 0.03	0.55 ± 0.01
Hexane oxidase ^c	4.28 ± 0.06	4.59 ± 0.13	5.77 ± 0.06	4.53 ± 0.10	3.61 ± 0.03

^a Cells grown in potato dextrose medium^b nmol cytochrome c reduced min⁻¹ (mg protein)⁻¹^c μmol of acetaldehyde liberated min⁻¹ (mg protein)⁻¹

ND: not in detected range. Values are mean of three experiments ± SEM

Table 2 Effect of different components of the assay conditions on hexane oxidase activity.

Enzyme * (mg ml ⁻¹)	Specific activity ^a	Hexane in (ml) #	Specific activity ^a	Temperature (°C) ∞	Specific activity ^a
1.0	6.62 ± 0.15	0.1	4.63 ± 0.10	30	5.44 ± 0.09
2.0	6.62 ± 0.15	0.2	5.65 ± 0.28	35	7.16 ± 0.16
3.0	7.14 ± 0.07	0.3	7.60 ± 0.03	37	10.62 ± 0.30
4.0	8.67 ± 0.02	0.4	10.90 ± 0.08	40	8.57 ± 0.09
5.0	11.58 ± 0.12	0.5	7.95 ± 0.18	45	7.41 ± 0.05
7.5	8.16 ± 0.09	0.75	6.62 ± 0.15	50	6.28 ± 0.01
10.0	7.60 ± 0.03	1.0	3.97 ± 0.09	55	4.81 ± 0.18

^a μmol of acetaldehyde liberated min⁻¹ (mg protein)⁻¹

* Activity at 37°C with 0.4 ml hexane concentration (2 ml assay mixture)

Activity at 37°C with (5 mg/ml) enzyme concentration

∞ Activity with 0.4 ml hexane and (5 mg/ml) enzyme concentration

because of autolysis, limiting concentration of nutrients in medium or production of secondary metabolites.

We have standardized hexane oxidase enzyme assay, in which hexane (pure hydrocarbon) is used as a substrate, in our laboratory. The amount of acetaldehyde liberated was determined colorimetrically using Nash reagent¹⁶ (Table 2). Nash reagent have shown colour development only with acetaldehyde among other metabolites. The formation of acetaldehyde in reaction mixture and presence of monooxygenase enzymes in *Aspergillus ochraceus* supports this phenomenon¹⁷ have examined in *Nocardioide* sp. CF8 at physiological and genetic level, which can utilize alkanes ranging in the chain length from C₂ to C₁₆.

A number of bacteria can utilize gaseous and liquid alkanes as growth substrates. There are reports on degradation of short carbon chain (C₈–C₁₆)¹⁸ to very long carbon chain (C₄₄) hydrocarbons¹⁹. The bacterial species of *Acinetobacter*²⁰, *Pseudomonas*¹⁸, *Flavobacterium*, *Bacillus*²¹, *Alcaligenes*, *Burkholderia*, *Arthrobacter*²² etc. were few of the well-known degraders of aliphatic compounds ranging from (C₆–C₂₀).

A significant decrease in NADPH cytochrome c reductase (50%) and hexane oxidase (48%) in presence of carbon

monoxide and NADPH requirement as a cofactor indicates cytochrome P-450 mediated reactions. Presence of NADH in assay media also showed the marker enzyme activity, however, the magnitude of the activity was found to be low when compared with the presence of NADPH as a cofactor. Synergic effect in the activity was observed in the presence of both the cofactors. Results indicate that NADPH is preferred cofactor for these types of reactions. Autoclaved cells could not show any of the enzymatic activity indicating enzymatic nature of the reaction (Table 3).

In order to find out the effect of different inducers on electron transport components and biotransformation enzymes, we have incubated potato-dextrose grown mycelium (96 h) of *Aspergillus ochraceus* (5 g) into incubation medium (50 ml of 50 mM potassium phosphate buffer saline, pH 7.4) containing one of the inducer (viz. cholesterol, camphor, naphthalene, veratrole, phenobarbital, n-hexane, kerosene and saffola oil) as a sole source of carbon and kept under shaking conditions for 18 h (200 rpm) at 30 °C to induce the enzyme system. Cholesterol and camphor are poor inducers; however naphthalene, phenobarbital and veratrole show moderate induction in biotransformation

enzyme activities. n-hexane, kerosene and saffola oil found to be prominent inducers of hexane oxidase and cytochrome *c* reductase, respectively.

Peptone-glucose grown cells of *Serratia marcescens* NCIM 5115 also showed the presence of biotransformation

Table 3 Cofactor requirement and criteria for cytochrome P-450 mediated reactions for hexane oxidase and cytochrome *c* reductase activities in 96 h grown *Aspergillus ochraceus* (NCIM 1146).

	NADPH-cytochrome <i>c</i> reductase ^a	Hexane oxidase ^b
I		
Without generating system	No Activity	No activity
NADPH generating system	2.43 ± 0.04	3.10 ± 0.03
NADH generating system	1.95 ± 0.04	2.25 ± 0.04*
NADPH + NADH	2.74 ± 0.05*	3.92 ± 0.05*
II		
96 h grown mycelium	2.43 ± 0.04	3.10 ± 0.03
Autoclaved mycelium	No Activity	No Activity
Activity in presence of carbon monoxide	1.23 ± 0.04	1.61 ± 0.03*

^a nmol cytochrome *c* reduced min⁻¹ (mg protein)⁻¹

^b μmol of acetaldehyde liberated min⁻¹ (mg protein)⁻¹

* Significantly different from control (assay containing NADPH generating system and 96 h grown mycelium) at P<0.001 by One way analysis of variance and Tukey-Kramer multiple comparisons test.

enzymes which are inducible². The presence of camphor induced mixed function oxygenase has been reported in soil bacterium *Pseudomonas putida*²³ and induced by barbiturate in *Bacillus megaterium*²⁴, steroid 11-β-hydroxylase is also found in fungi, *Cochliobolus lunatus* and *Curvularia lunata* as inducible enzyme²⁵⁻²⁶. The transfer of cells of *Candida maltosa* from a glucose medium to an n-alkane medium had resulted in the induction of various enzymes such as cytochrome P-450 (P-450_{alk}) and dramatically increases the endoplasmic reticulum and peroxisomes²⁷.

Two or three distinct spots were observed on TLC plate after 48 h incubation by using intact mycelium (Table 4) indicates involvement of *Aspergillus ochraceus* in degradation of cholesterol, camphor and naphthalene by intact as well as immobilized mycelium. *Aspergillus ochraceus* also has ability to decolorize various industrial dyes and involvement in PAH degradation²⁸⁻²⁹. HPLC chromatogram of extracted sample after complete biodegradation confirmed the degradation of camphor and cholesterol into several metabolites. Control camphor showed major peak at retention time 3.156 min and minor peak at 3.607 min, however metabolites showed peaks at retention time 3.47, 3.70, 4.03 and 4.68 min. Control cholesterol showed major peak at retention time 2.802 min and minor peak at 3.613 min, however metabolites obtained showed peaks at retention time 2.630, 2.764 and 2.962 min. GCMS analysis was carried out to investigate the metabolite formed during the biodegradation process. The GCMS analysis of camphor showed peak at retention time 11.33 min (152 m/z and 52.76 area (%)). We have proposed the

Table 4 Effect of carbon source on hexane oxidase and cytochrome *c* reductase activities in *Aspergillus ochraceus* (NCIM 1146).

Carbon source	Hexane oxidase ⁱ		Cytochrome <i>c</i> reductase ^j	
	Control ^k	Test ^l	Control ^k	Test ^l
Cholesterol ^a	0.80 ± 0.10	0.93 ± 0.07	0.24 ± 0.01	0.28 ± 0.02
Camphor ^b	0.72 ± 0.03	0.95 ± 0.02*	0.25 ± 0.01	0.35 ± 0.01*
Naphthalene ^c	2.31 ± 0.25	4.41 ± 0.47*	0.29 ± 0.01	0.58 ± 0.01*
Veratrole ^d	3.30 ± 0.24	5.29 ± 0.14*	0.41 ± 0.02	0.73 ± 0.01*
Phenobarbital ^e	1.68 ± 0.05	3.39 ± 0.10*	0.41 ± 0.02	0.86 ± 0.05*
n-Hexane ^f	1.44 ± 0.11	4.50 ± 0.24*	0.35 ± 0.01	0.80 ± 0.01*
Kerosene ^g	1.42 ± 0.03	2.14 ± 0.05*	0.40 ± 0.01	0.85 ± 0.01*
Saffola oil ^h	1.69 ± 0.06	2.07 ± 0.03	0.34 ± 0.03	0.59 ± 0.05*

^{a-h} 96 h grown mycelium incubated for 18 h in incubation medium containing along with one of the carbon source ^a (2.5 g l⁻¹) of cholesterol, ^b (2.5 g l⁻¹) of camphor, ^c (1 g l⁻¹) of naphthalene, ^d (10 ml l⁻¹) of veratrole, ^e (2.5 g l⁻¹) of phenobarbital, ^f (20 ml l⁻¹) of n-hexane, ^g (20 ml l⁻¹) of kerosene, ^h (20 ml l⁻¹) of saffola oil.

ⁱ μmol of acetaldehyde liberated min⁻¹ (mg protein)⁻¹

^j nmol cytochrome *c* reduced min⁻¹ (mg protein)⁻¹

^k Mycelium incubated with carbon source for 2 min.

^l Mycelium incubated with carbon source for 18 hrs.

Significantly different from control at *P<0.001, **P<0.05 by One way analysis of variance and Tukey-Kramer multiple comparisons test.

Table 5 Degradation of different organic compounds using, 96 h grown intact mycelium of *Aspergillus ochraceus* (NCIM 1146).

Compound	Hours of incubation	Rf value
Cholesterol ^a	0	0.96 (Cholesterol)
	48	0.96 (Cholesterol) 0.78 (product)
Camphor ^b	0	0.93 (Camphor)
	48	0.93 (Camphor) 0.69 (product) 0.53 (product)
Naphthalene ^c	0	0.95 (Naphthalene)
	48	0.95 (Naphthalene) 0.66 (product)

Intact mycelium of 96 h grown *Aspergillus ochraceus* (NCIM-1146) were incubated in incubation medium containing ^a 0.15 g of cholesterol dissolved in 5 ml ethyl acetate, ^b 0.2 g of camphor dissolved in 5 ml ethyl acetate, ^c 0.25 g of naphthalene dissolved in 4 ml ethyl acetate in 50 ml potassium phosphate buffer saline (50 mM, pH 7.4). After 48h of incubation two distinct spots were observed on TLC plate.

* solvent system used – Benzene: Acetone (3:1)

degradation mechanism of camphor on the basis of GCMS analysis. The oxidative cleavage of cyclopentane ring leads to formation of 1-methyl cyclohexan-2-one [22.105 min, 111 m/z and 10.74 area (%)] and 2-methyl propane [23.075 min, 57 m/z and 34.31 area (%)]. Demethylation of 1-methyl cyclohexan-2-one lead to formation of cyclohexanone [20.400 min, 98 m/z and 23.35 area (%)]. The GCMS analysis of cholesterol showed peak at 17.300 min (386 m/z and 50.03 area (%)). The oxidative cleavage of cyclopentane ring leads to formation of phenanthrenol [18.65 min, 206 m/z and 16.60 area (%)], and further degradation into phenanthrene [22.11 min, 191 m/z and 27.67 area (%)]. However, we could not identify degradation products of naphthalene using these methods and conditions; hence it is matter of further investigation.

Results from this study indicate the presence of cytochrome b5, cytochrome *c* reductase and hexane oxidase in *Aspergillus ochraceus* which are inducible. Naphthalene, phenobarbital and veratrole show moderate induction whereas n- hexane, kerosene and saffola oil found to be prominent inducers of hexane oxidase and cytochrome *c* reductase, respectively. Requirement of NADPH as a cofactor and inhibition with CO indicates cytochrome P-450 mediated reactions. After 48 h incubation two or three distinct spots were observed on TLC plate indicates involvement of *Aspergillus ochraceus* in the degradation of cholesterol, naphthalene and camphor. HPLC and GCMS analysis of extracted products also confirms the degradation of camphor and cholesterol. This could be useful in developing

microbiological process for the decomposition of environmental pollutants.

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