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Isolation of Cytochrome P-450$_{cam}$ from Alkaliphilic bacteria*Kocuria sp. DL*

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Abstract: *Cytochrome P-450 is a terminal oxidase, involved in biotransformation of endogenous and exogenous substances. Cytochrome P-450$_{cam}$ is isozyme present in various bacteria. CYP-450$_{cam}$ was purified from alkaliphilic bacteria, Kocuria sp. DL, isolated from pristine alkaline Crater Lake, Lonar, (Maharashtra State), India. Identification of this bacterial strain was carried out by analysis of 16S rRNA gene sequence [Kocuria species DL strain]. The content of CYP-450$_{cam}$ was found to be 2.68 n mol/mg of protein in 50 h grown culture under aerobic condition. Purification of CYP-450$_{cam}$ was carried out by applying analytical procedures at 4 ºC. The K_m values for both aminopyrine and acetanilide were 0.4 and 0.25 mM, respectively. The maximal velocity V_{max} was 12.98 and 13.2 mM/mg of protein per min for aminopyrine and acetanilide, respectively. The optimal pH and temperatures were found to be 7.4 and 40 ºC, respectively. The purification fold was found to be 6. The molecular mass was found to be 48 kDa. Thermal stability of purified CYP-450$_{cam}$ is up to 40 ºC. This enzyme has shown the characteristic type-I and type-II substrate binding spectra.*

Key Words:*Alkaliphilic, Kocuria sp. DL, Cytochrome P-450$_{cam}$, Aminopyrine, Acetanilide, Lonarlake.*

I. Introduction

The cytochrome P-450 constitutes an extremely large family of hemoprotein, which catalyze the biotransformation of a wide range of endogenous and exogenous compounds. It is widely distributed in all species of mammals and bacteria and fungi. The first experimental evidence of existence of CYP-450 in rabbit liver microsomes was made in 1955 by Axelrod[1]and Brodie *et al.*[2], who identified an enzyme system in the endoplasmic reticulum of the liver, which was able to oxidize xenobiotic compounds. In 1958,Garfinkel[3] and Klingenberg[4] detected a CO binding pigment in liver microsomes, which had an absorption maximum at 450 nm.

Bacterial CYP-450 is mainly present in soluble fraction of the cell and involved in critical metabolic processes. Structural features and mechanism of action of CYP-450$_{cam}$ has been reported from *Pseudomonas putida* (CYP101)[5]and has been used as a model for many CYP-450, it was the first 3-D structure solved by X-ray crystallography. This CYP450$_{cam}$ is part of a camphor-hydroxylating catalytic cycle consisting of two electron transfer steps from putidaredoxin, a 2Fe^{-2} S cluster-containing protein cofactor. In bacteria, CYP-450, vary qualitatively and quantitatively from one species to another and even in a given species under different growth conditions[6]. Cytochrome-450$_{eryF}$ (CYP107A1)[7] from the erythromycin-metabolizing bacteria *Saccharopolyspora erythraea*[8] is responsible for the biosynthesis of the antibiotic erythromycin by C-6-hydroxylation of the macrolide 6-deoxyerythronolide Bhydroxylase[9]. CYP-450 BM3 from the soil bacterium *Bacillus megaterium* catalyzes the NADPH-dependent hydroxylation of several long-chain fatty acids at the ω–1 through ω–3 positions. Unlike almost every other known CYP [except CYP505A1, from liver cytochrome P450$_{foxy}$ (5-methoxy-N, N-diisopropyltryptamine)], it constitutes a natural fusion protein between the CYP domain and an electron donating cofactor. Thus, BM3 is potentially very useful in biotechnological applications[10-11].

The camphor hydroxylase CYP101 from *Pseudomonas putida* has been engineered to biotransform other substrates, including halogenated aromatic compounds[12-13]. Camphor is the most universally used substrate to induce the bacterial CYP-450$_{cam}$ syntheses, which hydroxylate camphor to 5-hydroxy-camphor and form OH and H_2O molecules. Lewis *et al*, has mentioned that alkaliphiles have much higher cytochrome contents than conventional bacteria and then their own non-alkaliphilic mutants[14]. Davidson *et al*, had attempted to purify two soluble cytochromes from the alkalophile *Bacillus firmus*[15]. Yumoto *et al*, has purified and characterized two membrane-bound c-type cytochromes from a facultative alkaliphilic Bacillus[16]. A comparative studies of 10 facultative alkaliphilic bacteria was studied by Isao Yumoto 1997, two obligate alkaliphiles having relatively high total cytochrome concentrations are seen to possess considerably higher amounts of cytochrome *b* and cytochrome *c*, whereas, the amount of cytochrome *a* was not noticeably higher[17].CYP-450 119 isolated from

the thermophilic archea *Sulfolobus acidocaldarius*[18] has been used in a variety of mechanistic studies[19]. Because thermophillic enzymes evolved to function at high temperatures, they tend to function more slowly at room temperature and are therefore excellent mechanistic models.

Alkaliphiles are one of the extremophiles organisms that thrives in an extreme environment in more than one extreme, these organisms has adapted their physiological character towards alkaline pH and grow optimally or very well at pH values above 9.0 often between 10 and 12, but do not grow or grow only slowly at neutral pH values[20]. Various types of alkaliphiles have been isolated and reported. The bacteria belonging to the genera *Bacillus,Micrococcus*, *Pseudomonas*, *Streptomyces* and eukaryotes such as yeasts, *filamentous fungi*, have been isolated from a variety of environments. A number of proteolytic and hydrolytic enzymes have been isolated from the alkaliphiles that have industrial applications[21]. Additionally, microorganisms have also contributed to clean up the contamination in the environment employing their enzymes. There is enough data about CYP-450 from neutrophiles, much has to be done regarding the CYP-450 from alkaliphilic bacteria. Therefore, in present studies, the attempt has been made to isolate the alkaliphilic bacterial strains from Pristine Crater Lake of Lonar M.S., India and to purify and characterize cytochrome P-450$_{cam}$ from these strains.

II. Materials and methods

2.1. Chemicals

Sodium dithionate ($Na_2S_2O_4$), aminopyrine ($C_{13}H_{17}N_3O$), and sephadexG-75 (Sigma,USA), DEAE-cellulose (DO909-100G) from (Sigma-Aldrich, U.K), DMSO (MERCK), 500bp DNA ladder (Bangalore Genei, India) EDTA, DTT, NADPH Na_4EDTA, DTT, sodium-formate, H_2SO_4, acetanilide, ammonium sulphate, NaOH, HCl, SDS, β-mercaptoethanol, bromophenol blue, coomassie brilliant Blue R-250, and glycerol were obtained from (SRL, India), aniline, 1×TAE buffer, $MgCl_2$, dNTP mix, Taq polymerase enzyme, Protease K, DNAzol, & triton X-100(Merck, India), camphor 99.9% (Loba-chem, India), peptone, yeast extract, agar, NaCl& bovine serum albumin(HIMEDIA, India), Ethidium Bromide, and TCA from (Sd-Fine Chemicals Mumbai), R-salt was synthesized in our laboratory, All other chemicals were of the highest grade of purity and commercially available.

2.2. Isolation of microorganism and growth conditions

The *Kocuria sp. DL* strain used in this work was isolated from alkaline Crater Lake, Lonar (M.S.) India. Was grown in 1 liter bioreactor in agitating conditions, the nutrient broth medium contained the following (in grams per liter): 5.0gm yeast extract, 5.0 peptone, 5.0gm NaCl, trace elements, KH_2PO_4 170 mg, Na_2HPO_4 980 mg, $(NH_4)_2SO_4$ 100 mg, $MgSO_4$ 0.87 mg, MgO_2 0.1 mg and the pH was adjusted to 10.5. The culture media in bioreactor was inoculated with 10 ml of microorganism suspension and incubated for 48 hrs. After attaining the optical density about 1.0, a 60 mg/100 ml of camphor were added.

2.3. Identification of the microorganism by 16S rRNA method

The isolated bacteria strain was grown aerobically on nutrient agar. DNA was isolated by using DNAzol method[22]. DNA solubilization, supernatant was precipitated by using chilled absolute ethanol (Omnis, Jebsen&Jessen, GmbH, Germany), Extracted DNA was subjected to 16s rRNA PCR.Nucleotide data of 16s r RNA gene of the isolated alkaliphilic was deposited to NCBI nucleotide database.

2.4. DNA sequencing, BLAST and Phylogeny

PCR product was sequenced by Sanger's Dideoxy chain[23] termination method and the phylogenic analysis was constructed by using a Neighbor-Joining (NJ) method[24] in MEGA 4.1 software[25-26].

2.5. Cytochrome P-450$_{cam}$ Purification

2.5.1 Crude cell extract preparation:

Cell mass from culture was collected by centrifugation at 10,000×g for 30 min at 4ºC in a DupontSorvall RC-5B refrigerated centrifuge, washed with physiological saline and two times with 0.1M NaH_2PO_4 buffer, (pH 7.4). The pellets were suspended in 50 ml of the same buffer containing 0.1 mM EDTA, 0.1mM DTT, 1 mM lysozyme and 20% glycerol (v/v). Cells were disrupted in the cold condition by sonication three times lasting for 30 second with 1 minute interval and 70% outputs using a Sartorius Lab Sonic. Cells debris was removed by centrifugation at 10,000×g for 30 min at 4ºC in the refrigerated centrifuge. The supernatant obtained constitutes the crude bacterial extract was used for further studies.

2.5.2. Estimation of protein:

Concentration of protein at each step of purification was checked by Lowry's method[27] using BSA as a standard.

2.6. Assay of Cytochrome P-450$_{cam}$ content:

The content of CYP-450$_{cam}$ was determined spectrophotometrically at room temperature using a UV-visible spectrophotometer, JascoV630, by the method of Omura and Sato (1964)[28]. Total P450 content were determined by the CO bound minus oxidized UV-VIS spectra with $\Delta_{450-500nm}$ = 91 mM^{-1} x1000.

2.7. Puri cation of CYP-450$_{cam}$

The Cytochrome P0450$_{cam}$ was purified by a five-steps procedure carried out at 4 ºC.

2.7.1. Ammonium sulfate precipitation

Solid ammonium sulfate was added to the crude extract over a period of 4 h in cold condition with constant stirring to get the 30% saturation. The resulting precipitate was separated by centrifugation, and the supernatant further saturated up to 80% with solid $(NH_4)_2SO_4$[29]. The resulting precipitate was collected by centrifugation at 15,000 rpm for 30 min in a cold centrifuge. The precipitate was dissolved in a minimal volume of 100 mM phosphate buffer, pH 7.4. The protein solution was dialyzed against the same buffer for 36 hrs with repeated changes.

2.7.2. Ion-exchange chromatography

After dialysis, the solution was loaded on pre-equilibrated DEAE–cellulose column (2 cm×30 cm) the column was thoroughly washed with equilibrating 0.1 M phosphate buffer pH 7.4 containing, 0.1 mM EDTA, 0.1 mM DTT, and 20% glycerol (v/v). The protein was eluted with a linear gradient of NaCl (0–500 mM) in a 0.1 M phosphate buffer. Fractions of 3 ml were collected, those fractions showed higher content of CYP-450$_{cam}$ were pooled and reverse dialyzed in sucrose in cold condition to reduce the volume and dialyzed against same buffer for 36 h to remove the salt content, then was keep for reveres dialysis to concentrate the proteins.

2.7.3. Calcium phosphate gel Negative Adsorption:

Was prepared on lab according to the methods of Keilin and Hartree[30].

2.7.4. Molecular exclusion chromatography

The reversed dialyzed preparation was then applied to a Sephadex G-75 column (2 cm×30 cm) at 4 ºC and equilibrated with 0.1 M phosphate buffer pH 7.4 containing, 0.1 mM EDTA, 0.1 mM DTT, and 20% glycerol (v/v). The enzyme was eluted with a flow rate of 10 ml/h. Fractions of 3ml were collected and those showed higher CYP-450$_{cam}$ content were pooled together.

2.8. Characterization of purified Cytochrome P-450$_{cam}$

2.8.1. SDS–PAGE gel electrophoresis

SDS-polyacrylamide gel electrophoresis [SDS–PAGE] was performed with 5% stacking and 12% resolving polyacrylamide gel according to the method of Laemmli[31].

2.8.2. Substrate binding studies:

Binding of type-I substrate (aminopyrine) and type-II substrate (acetanilide) was carried out according to the procedure of Schenkman *et al*[32].

2.8.3. Catalytic activity of purified CYP-450$_{cam}$

The ability of cytochrome P-450$_{cam}$ to catalyze the *N*-demethylation of aminopyrine and hydroxylation of Acetanilide was examined by (Schenkman 1967)[32].

2.8.4. Determination of optimal pH and temperature of purified CYP-450$_{cam}$

The influence of pH on the CYP-450$_{cam}$ activity was studied over a wide range of pH (from 3.6 to 10.7) using a mixture of different buffers adjusted to the same ionic strength 100 mM (Tris-HCl, sodium phosphate, and sodium acetate). Effect of temperature on activity of CYP-450$_{cam}$ was studied by conducting the CYP-450$_{cam}$ assay at temperature range from 10 to 90ºC using JASCO V-630 spectrophotometer with temperature adaptor ETC-717, the activity was determined as described in section 2.7. The obtained data were plotted against the effect of temperature on activity and stability with time.

2.8.5. Determination of thermal stability

The enzyme samples were pre-incubated with 100 mM phosphate buffer (pH 7.4) at different temperatures between 10 and 80 ºC for 30 min and then brought to room temperature. At every interval temperature time the enzyme activity was measured as indicated in Section 2.7. & 2.9.3.

III. Results

3.1. Isolation and identification of bacterial strain *Kocuria sp. DL*.

The isolated alkaliphic species is a gram positive curved rod with high GC containing bacteria. The alkaliphilic strain *Kocuriasp DL*, was found to have a higher ability to degrade the camphor under aerobic conditions and was grown aerobically at 37ºC for 50 h. The red pigmented colonies from pure culture were used for bacterial DNA isolation. The 16S rRNA gene was amplified by PCR using the conserved region primers in the 16S ribosomal RNA gene, and its nucleotide sequence was determined by Sanger's method[23]. From BLAST analysis, it was found that the 16S rRNA gene sequence of red pigmented alkaline bacteria was 100 per cent identical to sequences of *Kocuriarosea* strains. Also, pair-wise distance computation derived by maximum composite like Neighbor-Joining (NJ) method[24] in MEGA 4.1 software[25,26] suggests very close relation of *Kocuria species DL* with *Kocuriarosea* species within *Kocuria* genus as supported by phylogenetic analysis of species within *Kocuria* genus. Accession number received for deposited nucleotide data and is available under the Gen Bank (GenBank ID:HM439779) more details are in supported supplementary data.

3.2. Purification of CYP-450$_{cam}$ from *Kocuria sp. DL*.

Table-1 summaries the purification of CYP-450$_{cam}$. A total amount of protein 159 mg was obtained from crude extract of disrupted cells of *Kocuriasp. DL*(20 g wet weight). During the purification process approximately 50% of other proteins were eliminated during ammonium sulfate precipitation. The concentrated enzyme solution after dialysis was applied on DEAE-cellulose column followed by calcium phosphate gel and then to Sephadex G-75 yielding a purification fold of approximately 6.0 giving 2.68 n mol/mg of CYTP-450 (Fig.1.A). Molecular weight of CYP-450$_{cam}$was estimated by SDS–PAGE with molecular marker proteins and was found to be 48 kDa (Fig.1.B). The Purification and characterization of the enzymes was studies well with triplicate sets.

3.3. Spectral Properties:

The major form of CYP-450$_{cam}$ obtained from camphor induced culture showed CO-reduced absorbance maxima at 452 nm, while the oxidized showed absorbance maxima at 414 nm and the dithionate reduced showed absorbance at 412 nm. The absorbance spectrum of the purified CYP-450$_{cam}$ from *Kocuriasp. DL*is shown in (Fig.2).

3.4. Substrate binding spectra:

Purified CYP-450$_{cam}$ has shown typical type-I and type-II substrate binding spectra with aminopyrine and acetanilide respectively (Fig.3.A and B) spectrum of type-I with a slight shift in peak at ± 384 nm and a trough at 418 nm, with an isosbestic point at 401 nm, as binding of the ligand is increased K_s values was (26.5). Acetanilide, a type-II substrate (Fig.3.B) exhibited its characteristic peak at 425 nm and trough at 389 nm with an isosbestic point at 408 nm, the K_s value was (103).

3.5. Catalytic activity:

The purified CYP-450$_{cam}$ has efficiently catalyzed the *N*-demethylation of aminopyrine and *p*-hydroxylation of acetanilide.

3.6. Effect of pH and temperature

The effect of pH on the activity of drug metabolizing enzymes was also examined. The maximum activity was at pH 7.4 for both aminopyrine-*N*-demethylase and acetanilide-hydroxylase. (Fig.4.A).Similarly the effect of temperature on the activity of these enzymes was also studied. The maximum activity was observed at 40 ºC. The CYP-450$_{cam}$ was stable up to 45 ºCand then the activity was decreased with increase in the temperature. (Fig.4.B).

3.7. Kinetic studies

To obtain the K_m, V_{max} and K_{cat}values, aminopyrine-*N*-demethylase and acetanilide-hydroxylase activities was measured by varying the concentrations of the substrates aminopyrine and acetanilide. In case of aminopyrine the values of K_m and V_{max}, were 0.4 n mol and 12.98 n mol and K_{cat} ± 9.19 S^{-1}. And in case of acetanilide were found to be 0.25 n mol, 13.2 n mol&K_{cat} ± 8.35 S^{-1}, respectively (Table-2).

IV. Discussion

The report describes the identification of alkaline bacterial strain *Kocuria sp. DL* by 16s rRNA gene sequence, which is very closely related to *Kocuriaroseastrains*. The alkaliphilicbacterium was grown in alkaline nutrient broth and the purification and characterization of the CYP-450$_{cam}$ was studied well with triplicate sets. The purification of CYP-450$_{cam}$ was difficult because of low content of CYP-450$_{cam}$ but it was easily soluble

with sodium cholate. Purified CYP-450$_{cam}$ has shown the efficient catalytic and spectral properties. The purified sample contained 2.68 n mol/mg of CYP-450$_{cam}$ which comparatively with other species are little more[33-34]. The monomeric molecular weight was found to be 48 kDa. The re-activation of CYP-420 to CYP-450 was difficult till dialyzed with a phosphate buffer pH 7.4 containing 20% glycerol for 30hrs. The Purified CYP-450$_{cam}$ has exhibited thermal stability similar to CYP119 from *Sulfolobussolfataricus*[35] which grows in volcanic springs, and *Thermobifidafusca*[36]unlike other for example CYP102A1 monooxygenase that require a thermo-stable analog of the reductase domain from *Bacillus megaterium*[36-37]. CYP450$_{cam}$, heme containing protein from *Kocuriasp. DL*, is expected to be one of the most versatile enzymes in nature and could efficiently catalyze hundreds of different substrates through a variety of difficult biotransformations.

In this study, we observed that the CYP-450$_{cam}$ from *Kocuria sp. DL* has hydroxylated camphor to 5-hydroxy-camphor and form OH and H_2O molecules. Stability of CYP-450$_{cam}$ to achieve maximum activity at neutral (around pH 7.4) and half at alkaline pH suggest that the cell internal pH is at slight alkaline condition although the pH of the growth medium was above pH 10.0, this enzyme can tolerate the temperature up to 60 ºC with 50% of initial activity and further increase in temperature showed a decrease in activity, hence we concluded that the CYP-450$_{cam}$ is thermo stable. Type-I and type-II spectra properties changes are small, and they resemble those obtained from other sources of CYP-450[32]. The purified CYP-450$_{cam}$ has efficiently catalyzed the *N*-demethylation of aminopyrine and *p*-hydroxylation of acetanilide. The activities of aminopyrine-*N*-demethylase and acetanilide-*p*-hydroxylase were found to be 56 n mol/mg/min and 83 n mol/mg/min of product, respectively.

CYP-450$_{cam}$ showed a significant activity for aminopyrine and acetanilide. Acetanilide was found to be the best substrate for the enzyme. This is the first report on CYP-450$_{cam}$ from alkaliphilic bacteria; the isolated enzyme could contribute a great development for the drug synthesis under the biotechnology aspect.

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Table-1. Summary of purification of CYP-450$_{cam}$ from alkaliphilic*Kocuriasp DL*.

Fractionation Steps involved	Protein (mg)	CYP-450$_{cam}$ content (n mole/mg)	Purification fold	Aminopyrine-*N*-demethylase[#]	Acetanilide *p*-Hydroxylase*
Sonication	159.0	0.444	1	34.312	48.127
$(NH_4)_2SO_4$ ppt.	83.3	0.69	1.55	40.64	55.31
DEAE cellulose column	46.25	0.892	2	47.4	68.32
Calcium phosphate gel	19.53	1.355	3.05	52.34	79.4
Sephadex G-75 column	8.3	2.68	6	56	83

[#]nmol of formaldehyde formed /min/mg of protein, ***** n mol of *p*-hydroxyl acetanilide /min/mg of protein

Table-2. Kinetic properties of CYP-450$_{cam}$monooxygenase

Substrate	K_m (μ mol.ml^{-1})	V_{max} (n mol. mg^{-1} protein min^{-1})	K_{cat} (S^{-1})
Aminopyrine	0.4	12.98	± 9.19
Acetanilide	0.25	13.2	± 8.35

Fig. 1

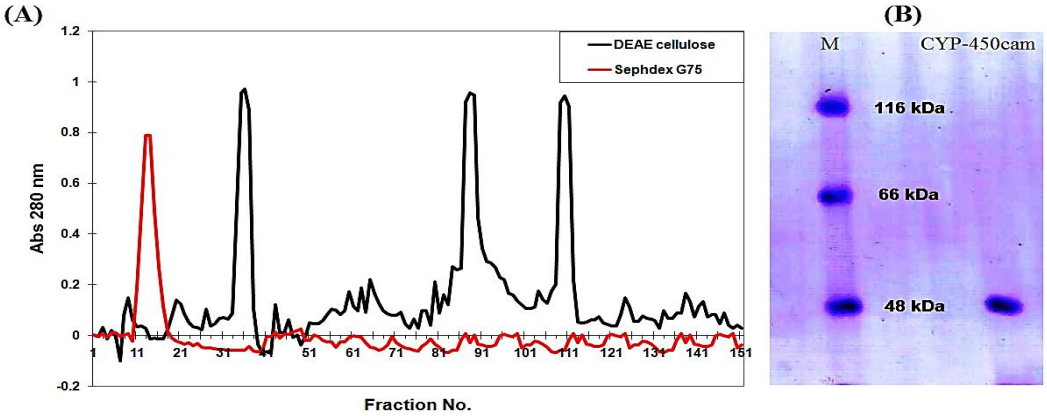

Fig. 1. **A.** Elution pattern from DEAE cellulose (Black), and Molecular exclusion Sephadex G-75 (Red) column chromatography.**B.** SDS–PAGE pattern of purified CYTP-450cam. Lane M: Protein Molecular Markers. Gel was stained with Gel was stained with 1% Coomassie brilliant blue R-250 staining.

Fig. 2

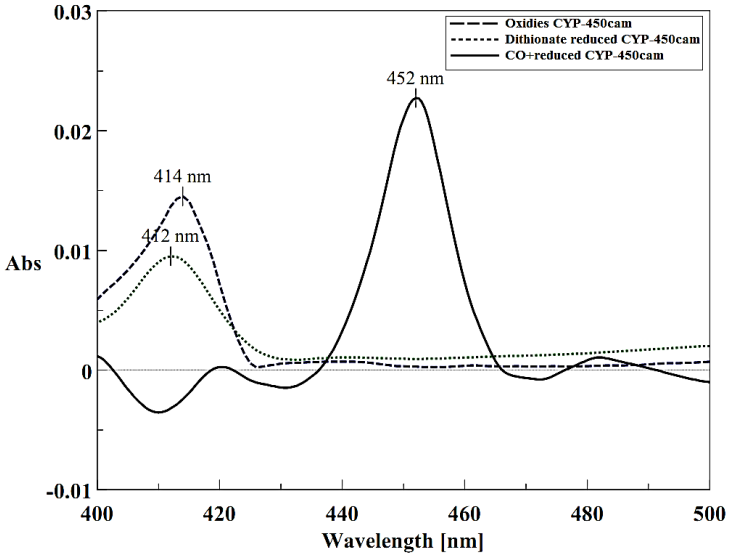

Fig. 2. Absolute absorbance spectra of purified CYP-450$_{cam}$ from *Kocuria sp. DL.*, CO + reduce Cytochrome P-450$_{cam}$ (**black**) oxidized CYP450$_{cam}$ form at 414nm (Blue dot **…..**), sodium dithionate reduce 412nm green dash (- - - -).

Fig. 3

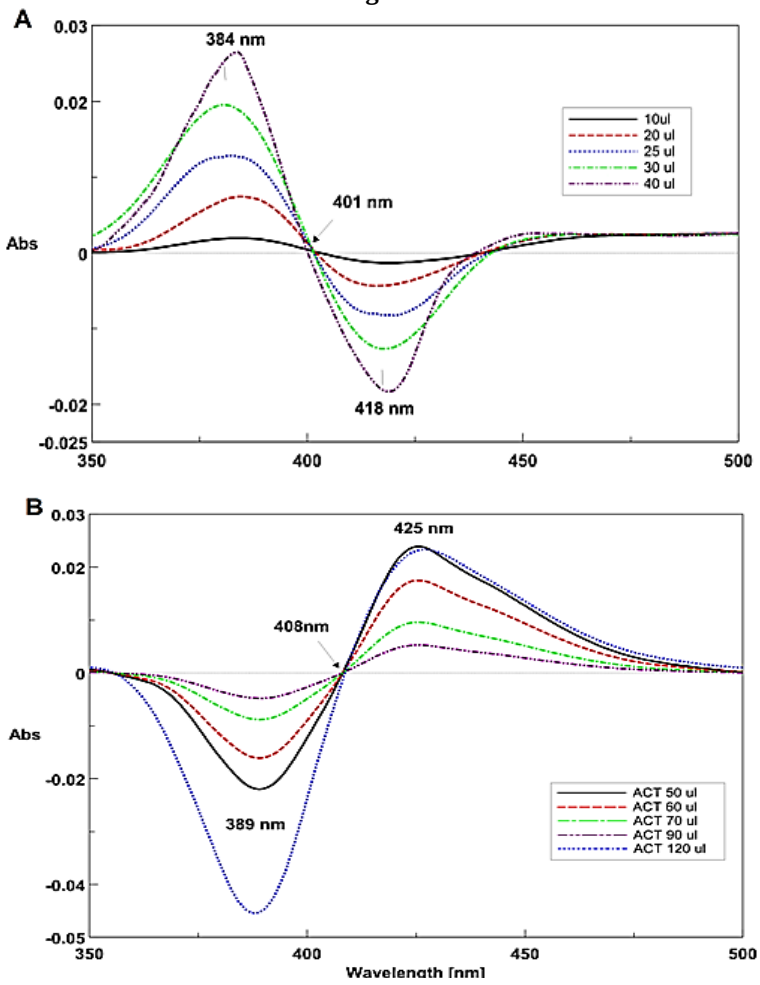

Fig. 3 A. Effectof aminopyrine on absorption spectrum of purified CYTP-450$_{cam}$ showing Type-I substrate binding Spectra from 10-40 µl with isosbestic point 401 nm, **B**. Type II substrate binding Spectra of acetanilide 50-110 µl with isosbestic point at 408 nm.

Fig. 4

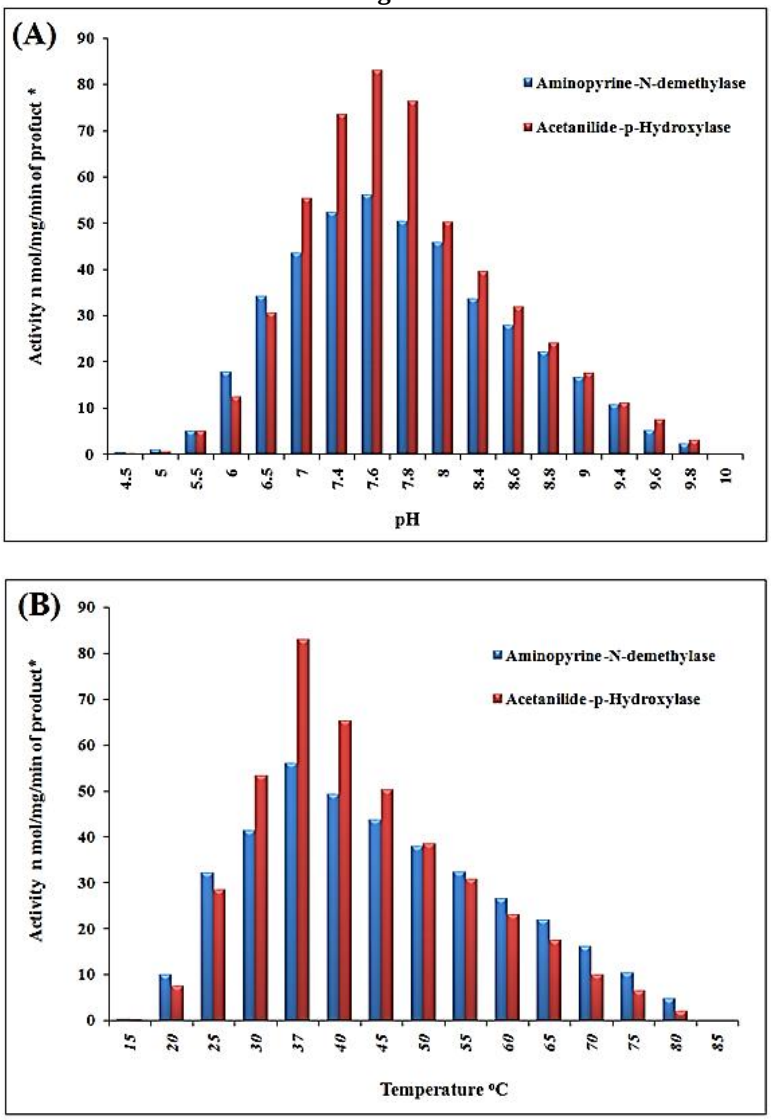

Fig. 4 A. Effect of pH and **B.** Temperature on aminopyrine-*N*-demethylase and acetanilide-*p*-hydroxylase activity of CYTP-450$_{cam}$**,** on the production of n mol of formaldehyde and *p*-hydroxyl acetanilide/min/mg of protein.