Chapter II:

The search of the source of Bioreceptor.

2.1.1. Introduction and objectives

Enzymes have a vast array of applications in both research and industries due to their specificity for biocatalytic transformations and operations under mild reaction conditions. Even though the source for the enzymes may vary from simple microorganisms to all the higher eukaryotes including plants and animals, the microbial enzymes have some advantages over the other sources [1]. Microbial enzymes can be produced in large volumes in short period of time, they are more stable as compared to the eukaryotic counterparts and further they are easy- to -manipulate at genetic level [2,3]. Ever since the studies on fermentation by Louis Pasteur, the search for better enzymes and the microorganism producing those enzymes have been a constant subject of inquisition in the scientific communities. In microbiology, the search for the new bacteria is termed as screening. This study focuses on the screening of microbial species which can be induced for the high production of Cytochrome P450 monooxygenase.

Cytochrome P450 monooxygenase is from a heme containing enzyme superfamily, and are found in almost every organism from simple prokaryotic bacteria to human [4-7]. In bacteria, they do not exist as constitutive enzyme and can be induced to produce in higher amounts by various inducers such as n-alkane [8], poly alicyclic aromatic (PAH) hydrocarbons [9], rifampicin [10] drugs, industrial chemicals, natural products [11] etc. The mechanism of activation of the CYP450s is due to activation of different transcription factors in presence of the particular

inducer [11]. The level of induction will vary in different bacteria producing different amounts of CYP450s.

The screening of the CYP450 is dependent on the heme-thiolate group present in the enzyme. This heme-thiolate group is responsible for the signature peak at the wavelength of 450 nm, it shows in the visible range. This peak can not only indicate the specific content but also whether the enzyme in properly folded and whether the thiolate ligand is intact. If, the thiolate ligand is catalytically functional, the heme group present within it binds with carbon monoxide (CO) and shows an absorption maxima at that peak [12]. Using this CO-Fe (II) versus Fe (II) difference spectrum, the CYP450s can be quantified in a specific and sensitive manner [13]. However, this screening method is not indicative of its capability for substrate conversion, but, this method asserts the presence of catalytically active heme-thiolate ligand.

The measurement of CYP450 contents in intact *Escherichia coli* has already been reported [12]. This method is very helpful for rapid screening of intact cells, reducing time and effort. The four main steps involved in the process are growth, induction and concentration of culture; Sodium dithionite reduction; CO-Fe (II) versus Fe (II) difference spectra measurement and data processing.

After the screening, the isolated bacteria were further optimised for CYP450 production and eventually identified using 16s rDNA technique.

2.1.2 Objectives

1. Collection of soil samples from oil contaminated sites.
2. Screening of CYP450 induced bacteria based on the CYP450 production followed by identification.
3. Optimisation of the CYP450 production.

2.2. Materials and Methodologies

2.2.1. Materials used

2.2.1.1. Chemicals used:

The various chemicals used in the experiments are listed below along with their manufacturing companies.

Ammonium chloride, Ammonium sulphate, Di- Potassium hydrogen phosphate, Magnesium sulphate heptahydrate, Potassium dihydrogen phosphate, Sodium chloride, Acetone, 1% Bromophenol Blue, Ethanol, Formic acid, Hydrochloric acid (about 35% pure), Iron (III) chloride anhydrous, 2-Propanol, Methanol, n-hexane, Manganese (II) sulphate monohydrate, Potassium Chloride, Sodium thiosulfate pentahydrate, Sodium hydroxide pellets GR, Calcium chloride dehydrate (MERCK), Luria Bertani agar, Sodium carbonate anhydrous extra pure, Sulphuric acid, Yeast extract (Himedia), Tris base, Bushnell Haass Agar, Bushnell Haass broth, Ammonium persulphate, bacteriotryptone (Himedia), glycerol and Agarose (Sigma), Calcium chloride dehydrate AR (Qualigen), Glycine, Lysozyme (SRL), QIA quick gel extraction kit (Qiagen).

Further the PCR master mix was purchased from thermo Fisher thermo scientific and the primer 27f and 1592r were purchased form Imperial life sciences (p) ltd ILS Gurgaon 122011, INDIA.

2.2.1.2.Instruments used

The instruments used in the various experiments are listed below:

Autoclave (Labtech), Biological Oxygen Monitor System YSI 5300A, Carbon Monoxide production unit (fabricated in Lab), Electronic balance (Kern), Hot water bath, Laminar air flow hood, Magnetic stirring heating plate, Micropippette (Thermo scientific) Mini centrifuge (Tarsons Spinwin) Mini incubator (Labnet), Orbital shaker cum incubator (Orbitek), pH meter, Spectrophotometer MultiScan Go (Thermo scientific), Thermal cycler (Eppendorf Mastercycler Nexus Gradient), Vortex (Tarsons Spinot), Freeze dryer, Sequencing at Eurofins ltd Banglore using ABI 3730x1 DNA analyser.

2.2.2. Methodologies

2.2.2.1. Optimisation of the fabricated carbon monoxide (CO) production unit

The CO is produced by reducing formic acid by hot concentrated sulphuric acid. Sulphuric acid removes two hydrogen atoms and one oxygen atom to form water and the one molecule of carbon monoxide. The chemical reaction depicting the production of CO is given below (Equation 1.1).

$$HCOOH(l) \xrightarrow{Hot\ concentrated\ H_2SO_4} CO(g) + H_2O(l) \qquad (1.1)$$

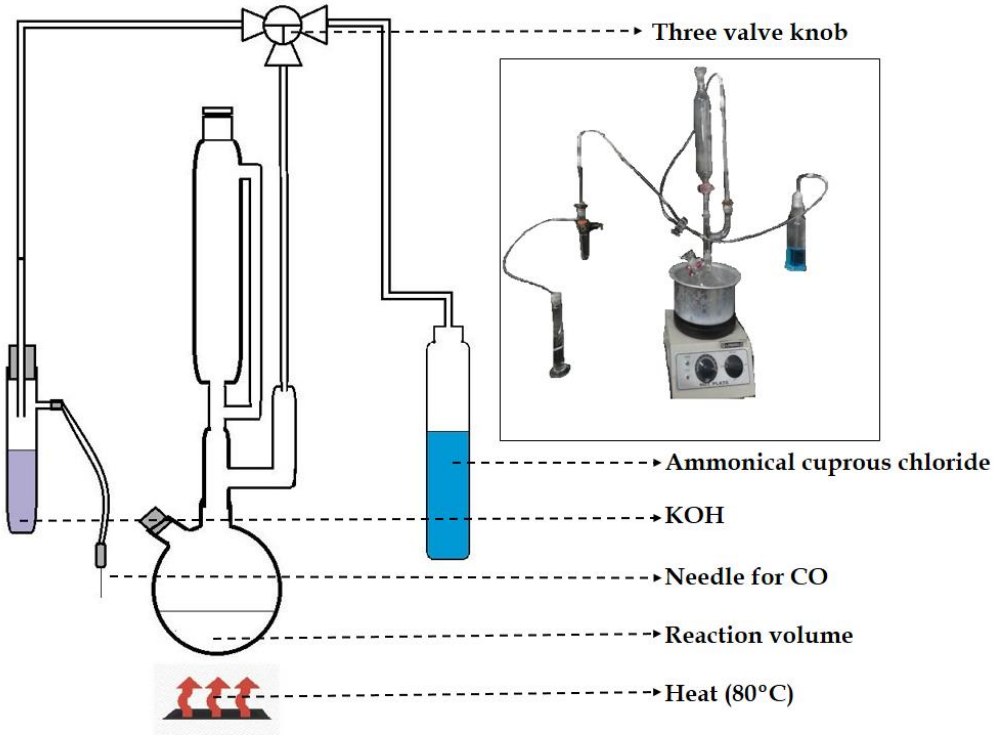


Fig. 2.1. : Schematics of the fabricated carbon monoxide production unit and the actual device in the inset. The reaction takes place in a two necked round bottom flask formic acid is poured drop wise over hot concentrated sulphuric acid. The produced CO can be directed to the either test tube with KOH or $CuSO_4$, using the three valve knob. The CO directed towards the test tube with KOH can be ejaculated via the needle. The excess CO is absorbed by blue coloured ammonical cuprous chloride solution.

2.2.2.2. Collection of soil samples and serial dilution

35 different oil contaminated soil samples were collected from four clusters of Borhola oil reserve, Jorhat, Assam using a sterile spatula from both the surface as well as 10 cm below the land surface. The collected soil samples were kept in sterile sample collection bottles and stored at 4◦C.

2.2.2.3. *Serial dilution method for isolation of hydrocarbon degrading bacteria*

For the isolation of hydrocarbon degrading bacteria, one gram of each respective samples, taking four samples at a time were homogenised with

10 ml of saline water (0.9% NaCl) and diluted upto 10^{-7} dilution. 100 µl of the diluted sample was then aseptically incubated in Bushnell Haas Agar plates and incubated at 37°C for two days.

2.2.2.4. Screening of hydrocarbon-degrading bacteria for cytochrome p450 using CO difference spectra:

As mentioned earlier, the four steps for screening are followed for each bacterial isolates as described below [12,13].

2.2.2.4.1. Growth and Induction of the bacteria

Bacterial culture from the Bushnell Haas agar plates were inoculated in 100 ml stress media (mineral salt media) with n-hexadecane (1% v/v) as the sole carbon source in 250 ml conical flasks at 37 °C (150 rpm). From this pre culture, the cultivation of the bacterial cell was performed. 2 litre of nutrient media (25 g/l) in 20 conical flasks were inoculated from the pre culture and biomass was harvested when the OD_{600} > 0.4 under shaking condition (150 rpm) for 12 hours, at 37° C. The CYP450 system was induced by using n-hexane twice, in every six hours before harvesting the cells. Biomass was harvested using a cooling centrifuge at 4 °C at 7200 g for 30 minutes in 50 ml falcon tubes. The harvested biomass was suspended in potassium phosphate buffer (20 mM, pH 7.4) and transferred to a 2 ml micro centrifuge tube followed by a centrifugation at 14000 g for 30 minutes.

2.2.2.4.2. Reduction of heme by sodium dithionite.

A few crystals of sodium dithionite were added to the bacterial suspension for complete reduction of heme moiety of cytochrome P450 and kept for incubation at 4◦ C for 20 minutes after mixing it well.

2.2.2.4.3. Measurement of the CO-Fe (II) versus Fe (II) difference spectra and data processing.

The reduced cell suspension was transferred into a quartz cuvette and was saturated with CO (g) for 30 seconds. Spectra of Fe (II)-CO formed of the CYP-450 containing bacteria was recorded between 400 nm to 500 nm in 2 nanometre increment. The concentration of P450 was calculated for each sample by using the following formula;

$$CYP\ content = [A(CO_{Abs450} - CO_{Abs\,490}) - B(WCO_{Abs450} - WCO_{Abs490})]/9 \quad 1$$

…… (2.1)

Where A is from the absorbance spectra after passing through CO, B is from absorbance spectra from the baseline spectrum. 91 is the molar extinction coefficient (91 mM −1 cm −1). CO is carbon monoxide and WCO is without carbon monoxide.

2.2.2.5. Determination of growth curve of screened bacteria

The bacterial strains screened for CYP450 induction were grown in stressed media (MSM) containing 1 % n-Hexadecane for 48 hours at 150 rpm and 37° C till the optical density reached 0.2 to 0.6 as measured at 600 nm. The growth of the culture after that was recorded at an interval of 2 hours for up to 84 hours.

2.2.2.6. Production of CYP450 of screened bacteria

2.2.2.6.1. Production of CYP450 for screened bacteria with respect to the time

CYP450 content was also monitored along with the biomass estimation for bacterial growth pattern, at every 0 hr, 3 hrs, 6 hrs, 9 hrs, and 12 hrs of interval. CYP450 contents of the harvested cell suspension were calculated as mentioned in section 2.2.2.4.

2.2.2.6.2. Production of CYP450 for screened bacteria with respect to the pH

50 ml volume of mineral salt media was dispensed in five 250 ml of conical flasks with subsequent adjustment of their pHs at 4, 5, 6, 7, 8, and 9 by addition of 0.1N HCl and 0.1N NaOH. 1% n-hexadecane was used as the carbon source for all the preparations. A similar set of flasks containing 100 ml of nutrient broth and pH similar to MSM were inoculated from cultures of stressed media. 1 ml aliquot of *n*-hexane was added as inducer when the O.D of culture broth reached 0.2 to 0.6 at 600 nm and incubated further for 48 hours at 150 rpm and at 37^{o} C. The harvested bacterial cellmass was used for calculation of CYP content as mentioned above.

2.2.2.6.3. Production of CYP450 for screened bacteria with respect to the temperature

Three 250 ml of conical flasks were dispensed with 50 ml volume of mineral salt media each to grow them at temperatures 27^{o} C, 37^{o}C and 45^{o}C

respectively using 1% n-hexadecane as the sole carbon source. The bacterial culture thus grown were used for inoculating the flasks containing nutrient broth to be incubated at 150 rpm and at above mentioned temperatures. 1 ml *n*-hexane was added to each flask for CYP450 induction when O.D value reached between 0.2 to 0.6 at 600 nm. The bacterial cellmass of the culture was used for estimation of the amount of cytochrome P450 content as mentioned in the previous section.

2.2.2.7. Preparation of spheroplast for storage

The freshly harvested cells were suspended in a modified potassium phosphate buffer (20 mM) containing 250 g/l sucrose, 5 mM EDTA, and at pH 7.4 and lysozyme (100 mg/l) was added after adjusting the pH to 8.4 with NaOH. The suspension was stirred at room temperature for 45 minutes and pH was adjusted back to pH 7.4 and were centrifuged at 14000g for 30 minutes. The prepared spheroplasts were stored at -80 ºC

2.2.2.8. *Identification of strains using Colony PCR*

Two bacterial strains (TM140001 and TM14023) were used in this study. The strains were grown at 37°C on Luria Bertani agar (Himedia) for 12 hours. Single colonies were isolated using pure culture technique. Identification was carried out based on the 16S rDNA sequences amplified by using universal primers (forward: 27f: 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse: 1492R: 5′- GGTTACCTTGTTGTTACGACTT-3′) purchased from Imperial Life Sciences (P) Ltd, Gurgaon. A single colony was streaked out from a pure culture plate and dissolved in 0.5 M NaOH (1000 ul). The tubes

were incubated at 95°C for 10 minutes followed by centrifugation at 13,000 rpm for 10 minutes. The supernatant obtained was then used as a template. Quantification of DNA was done by measuring the absorbance at 260nm. PCR was performed in a thermal cycler (Eppendorf Mastercycler Nexus Gradient) in a final volume of 25 µl containing PCR master mix 12.5 µl, (purchased from Thermo Scientific) of Forward primer (1 µl), Reverse primer (1 µl),1 µl template and deionized water. The conditions set for amplification are :initial denaturation at 95 ° C (2 minutes), denaturation at 95°C (30 secs), annealing at 54° C (30 secs), extension at 72° C (2 minutes) and final extension at 95° C (10 mins) for a total of 30 cycles. The PCR products were resolved by electrophoresis on a 0.8 % agarose gel stained with Ethidium Bromide (EtBr). The appeared DNA bands were then excised and extracted using a QIAquick gel extraction kit purchased from Molbiogen, Guwahati. The purified PCR products obtained were then sent for sequencing to Eurofins, Bangalore. The resulting sequenced base pairs that resulted were aligned using BLAST. Multiple sequence alignment was done using CLC sequence viewer 7 (CLC Bio Qiagen, Aarhus, Denmark). The phylogenetic tree was constructed using CLC sequence viewer 7.

2.2.2.9. Optimisation study for Bacillus stratosphericus.

In order to evaluate the effect of environmental factors (pH and temperature) on CYP450 production, optimisation study was performed at different pH ranging from 6 to 10 and temperatures ranging from 25^{o}C to 45^{o}C. The experiments were performed in triplicates using 50 ml MSM

culture media in 250 ml Erlenmeyer flasks. The design and analyses of the Taguchi experiments for determing the contribution of the individual component were done using online free software Qualitek- 4 (Demo version). The CYP450 content was measured as mentioned above.

2.3. Results

2.3.2. Optimisation of the fabricated CO production unit

The fabricated device was used after ensuring the optimal production of Carbon Monoxide (CO) which was found stable at around 70 ^{o}C.

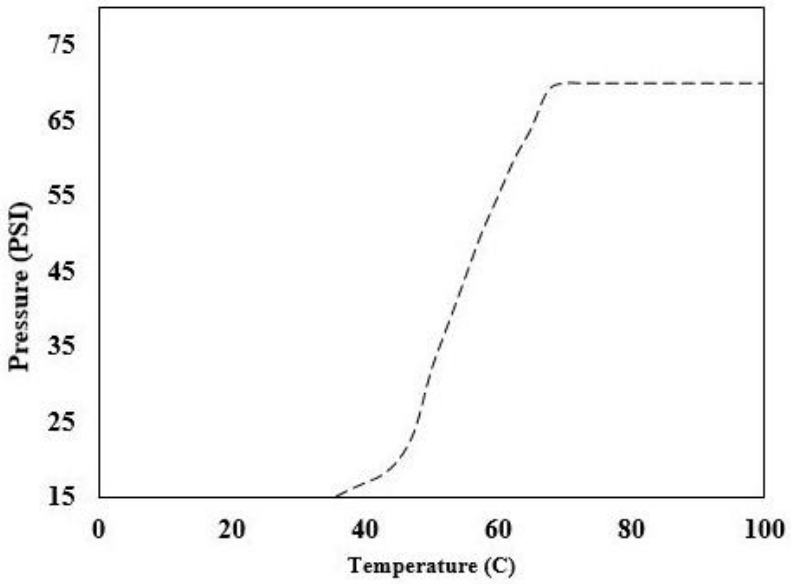


Fig.2.2. CO production unit for pressure and temperature

2.3.3. Collection of samples and morphology study

As many as thirty five samples of oil contaminated soil were collected from four different clusters of Borhola Oil Reserve, Jorhat, Assam (Table 2.1). Out of the collected samples, 72 different bacterial strains were isolated using

serial dilution technique. The colony morphologies of the isolated strains were recorded as indicated in Table 2.2.

Table 2.1: Soil samples collected according to the region of collection

Area	No. of Samples
Borhola Gr G	14
Borhola Cluster 1	6
Borhola Cluster 2	7
Borhola Cluster 3	8
Total No. of Samples	35

Fig. 2.3.: An oil contaminated drilling site in Borhola Culster 1

Table 2.2: The colony morphologies of the isolated strains

Sl. No	Form	Elevation	Margin	Colour
14001	Spindle	Convex	Entire	yellowish
14002	Circular	Convex	Entire	yellowish
14003	Circular	Convex	Entire	
14004	Puncetiform	Convex	Entire	
14005	Rhizoide	Convex	Undulate	
14006	Rhizoide	Convex	Entire	
14007	Irregular	Convex	Undulate	
14008	Irregular	Convex	Entire	
14009	Puncetiform	Flat	Entire	
14010	Puncetiform	Pulvinate	Undulate	reddish
14011	Circular	Convex	Lobate	
14012	Circular	Convex	Undulate	
14013	Rhizoide	Umbonate	Lobate	brownish
14014	Circular	Convex	Entire	brownish
14015	Circular	Convex	Entire	
14016	Irregular	Convex	Undulate	

Sl. No	Form	Elevation	Margin	Colour
14017	Irregular	Flat	Undulate	
14018	Circular	Convex	Entire	
14019	Circular	Convex	Entire	
14020	Circular	Convex	Entire	
14021	Circular	Convex	Entire	
14022	Spindle	Pulvinate	Erose	
14023	Rhizoide	Pulvinate	Undulate	
14024	Irregular	Raised	Undulate	
14025	Irregular	Raised	Undulate	
14026	Circular	Convex	Entire	
14027	Circular	Convex	Entire	
14028	Circular	Convex	Entire	
14029	Puncetiform	Pulvinate	Entire	
14030	Circular	Flat	Entire	
14031	Circular	Convex	Entire	
14032	Irregular	Convex	Entire	
14033	Circular	Convex	Entire	
14034	Circular	Convex	Erose	
14035	Circular	Convex	Entire	
14036	Circular	Pulvinate	Erose	
14037	Circular	Raised	Entire	
14038	Filamentous	Flat	Undulate	
14039	Circular	Pulvinate	Entire	reddish
14040	Circular	Flat	Entire	
14041	Circular	Convex	Entire	
14042	Filamentous	Flat	Entire	
14043	Puncetiform	Convex	Undulate	
14044	Irregular	Flat	Entire	
14045	Circular	Flat	Erose	
14046	Circular	Flat	Erose	

Sl. No	Form	Elevation	Margin	Colour
14047	Circular	Convex	Erose	
14048	Circular	Convex	Entire	
14049	Puncetiform	Convex	Erose	
14050	Rhizoide	Flat	Entire	
14051	Rhizoide	Convex	Entire	
14052	Irregular	Convex	Entire	
14053	Irregular	Convex	Entire	
14054	Puncetiform	Convex	Undulate	
14055	Puncetiform	Pulvinate	Lobate	
14056	Circular	Pulvinate	Lobate	
14057	Circular	Raised	Erose	
14058	Rhizoide	Raised	Entire	
14059	Circular	Convex	Entire	
14060	Circular	Convex	Entire	
14061	Irregular	Convex	Entire	
14062	Irregular	Pulvinate	Entire	reddish
14063	Circular	Flat	Erose	
14064	Circular	Convex	Erose	
14065	Circular	Convex	Undulate	
14066	Circular	Convex	Entire	
14067	Spindle	Convex	Entire	
14068	Rhizoide	Convex	Entire	
14069	Irregular	Pulvinate	Entire	
14070	Irregular	Raised	Undulate	yellowish
14071	Circular	Flat	Entire	
14072	Circular	Pulvinate	Lobate	

2.3.4. Screening for CYP450 induced bacteria

Those hydrocarbon degrading bacteria, which survived on the Bushnell Hass Agar were transferred to Minimal Salt media (Potassium dihydrogen phosphate (2%), Di-Potassium hydrogen phosphate (0.5%), Ammonium Sulphate (3%), Sodium Chloride (0.01%), Iron(II) sulfate heptahydrate (0.001%), Magnesium sulfate heptahydrate (0.02%), Calcium chloride he xahydrate (0.001%), Manganese sulfate monohydrate (0.0002%), Yeast extract (0.03%); % in (w/v) and 1% n-hexadecane (v/v)) with n-hexadecane as the sole carbon source, and n-hexane as CYP450 inducer. Based on the results of CYP450 content measurement, 35 strains were selected for further screening and out of which four had shown significantly higher values of CYP450 content. The strains were denominated as TM14001, TM14007, TM14023 and TM14030. In this study, two of those bacteria namely TM14001 and TM14023 with a CYP450 content of 4.109 and 2.955 µmol were selected for further studies.

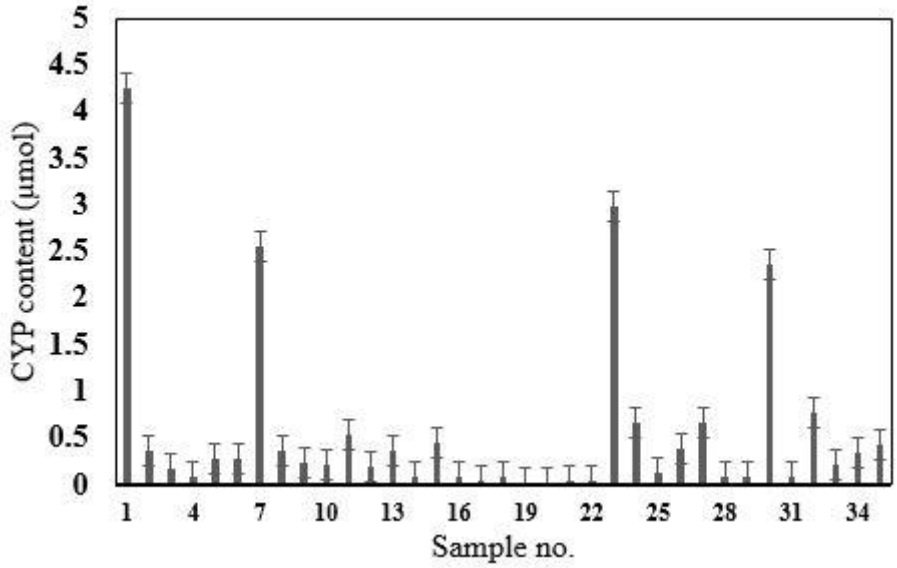


Fig. 2.4. CYP450 contents of the various samples

2.3.5. Isolation and growth curve of the selected bacteria

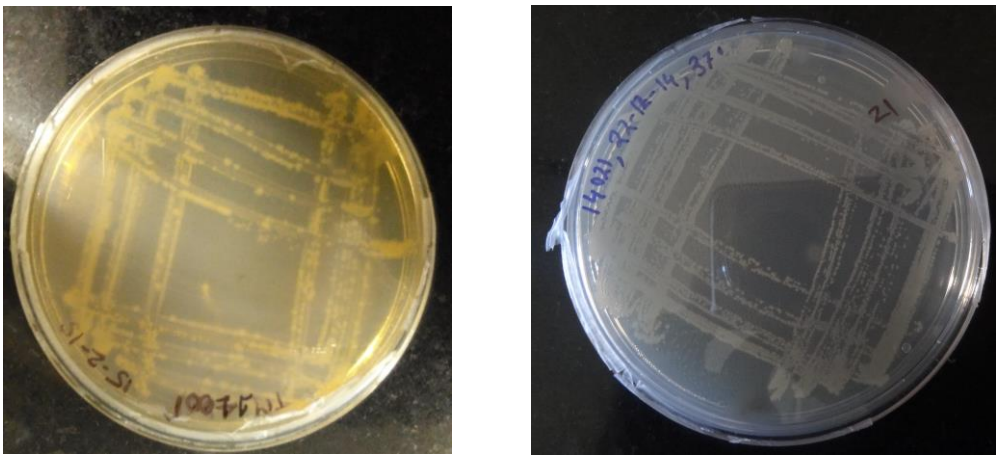


Fig 2.5. : Isolated strains grown on a streaking plates, (a) TM14001 and (b) TM14021.

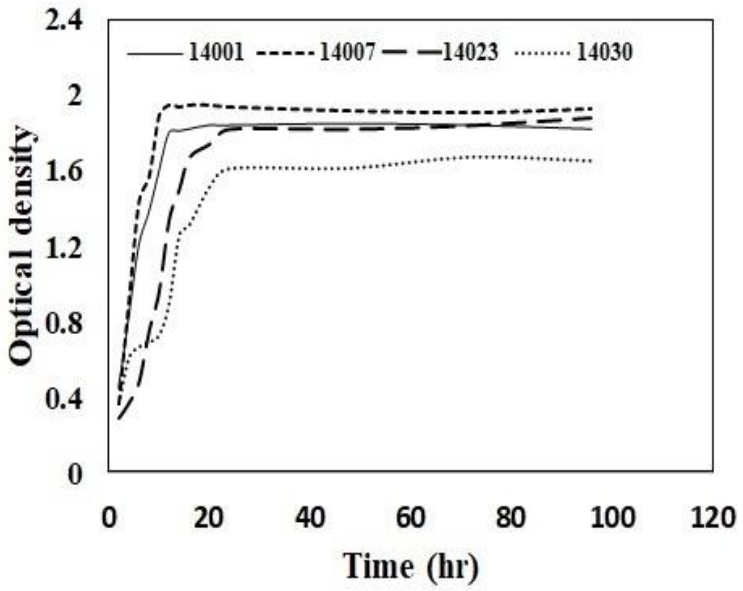


Fig. 2.6. Growth curve for the bacterial sample TM14001, TM14007, TM14023, and TM14030.

Isolated bacterial strains were streaked on the culture plate for storage and future use. Further, the growth curves showed a similar pattern of attaining a stationary phase at around 15-20 hours and which continued till 84th hour of growth (Fig. 1.6).

2.3.6. Role of pH and temperature on the growth

The pH range taken for the CYP450 induction study was from pH 4 to 9. It was observed that there was a gradual increase in CYP450 induction from 4-7 pH, beyond which the decline in the CYP450 induction was prominent. The pH optimization study as shown in fig 1.7, clearly indicated that induction at pH 7, the TM14001 and TM14023 were capable of producing 0.056 μmole and 0.384 μmole respectively at their maximum levels.

In this study, it was observed that among the temperature range of 27°C to 45°C, the highest enzyme synthesis occured at 37°C for both the strains. This shows that the bacterial strains of TM14001 and TM14023 are temperature dependent for the enzyme production (fig 1.8). The percentage increase in CYP450 synthesis from temperature 27ºC to 37ºC was observed to be 105.24 % and 80.4 % for bacterial strains of TM14001 and TM14023 respectively.

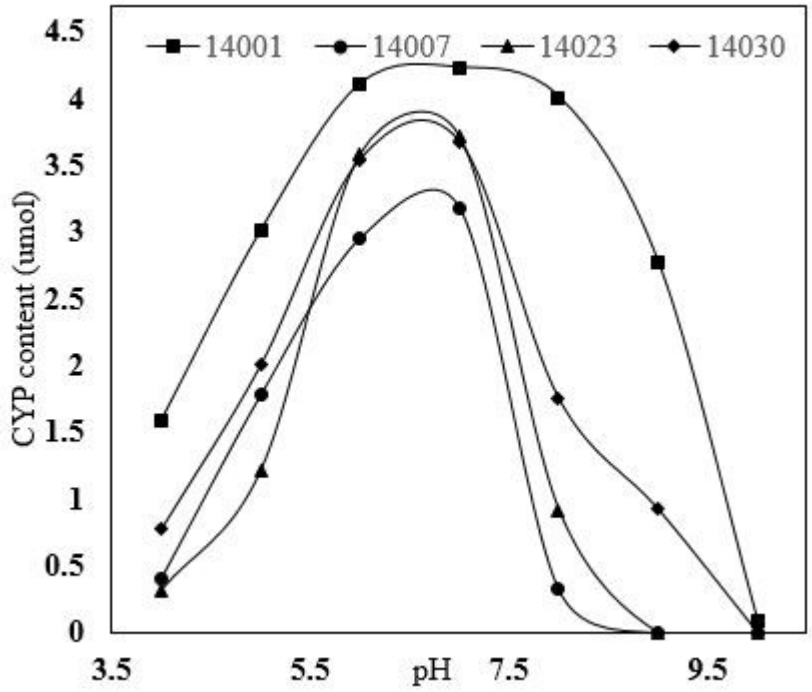


Fig. 2.7: Role of pH in CYP450 production

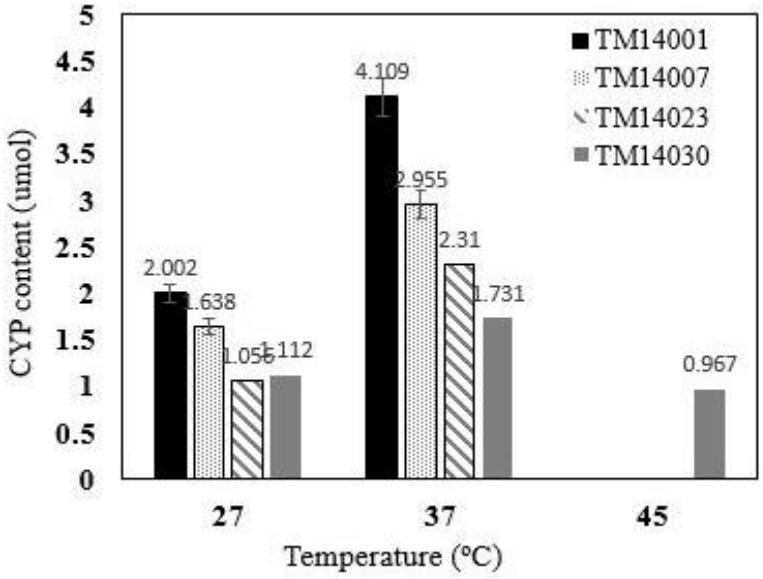
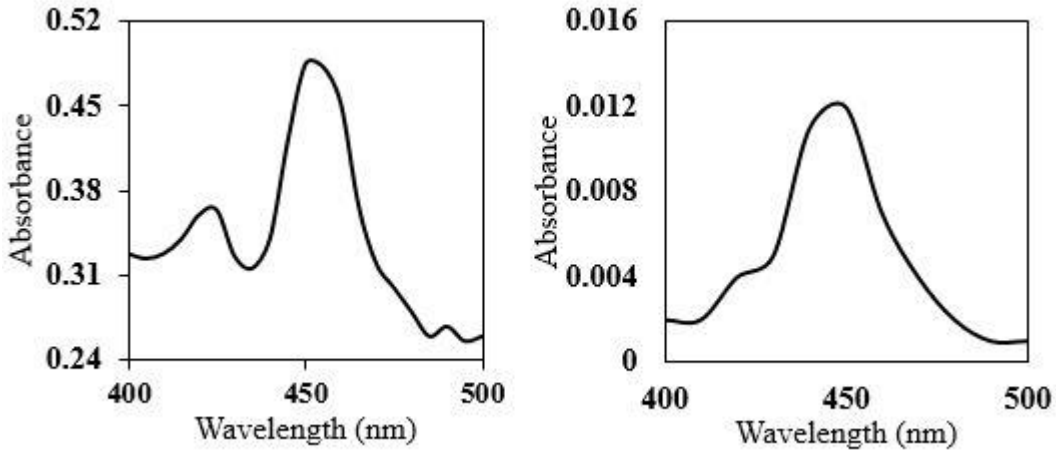


Fig 2.8: Role of temperature for CYP450 production

2.3.7. Soret Peak

The soret peak is the characteristic peak of CYP450, observed at a wave length of 450 nm. In this study, the harvested cells were screened in the wavelength range of 400 to 500 nm and the characteristic peak was observed



(a) (b)

Fig. 2.9 (a,b): The characteristic Soret peaks shown by bacteria TM14001 and TM14023

for both the bacteria. Further, the additional small hill at 420 nm was observed due to the inactive CYP450s.

2.3.8. Colony PCR for 16S rDNA sequencing of isolated bacteria

For both the microbial isolates, the genomic DNA extraction for colony PCR was effective with 260/280 ratio of >1.5 for amplification of 16S rRNA gene (Figure 2.10). After removing the anomalies for both the isolates of 16S rRNA sequence (using Pintail 1.1), the length of the sequences (in basepairs) were found to be 1002 bp for TM14001, and 1091 bp for TM14023 respectively. Further, after phylogenetic tree analyses through CLC sequence viewer 7.7, the isolated bacterial strains were identified to be *Bacillus pumilus* and *Bacillus stratosphericus* for TM14001 and TM140023 respectively (fig. 2.11).

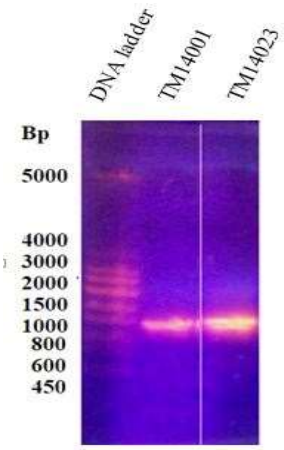
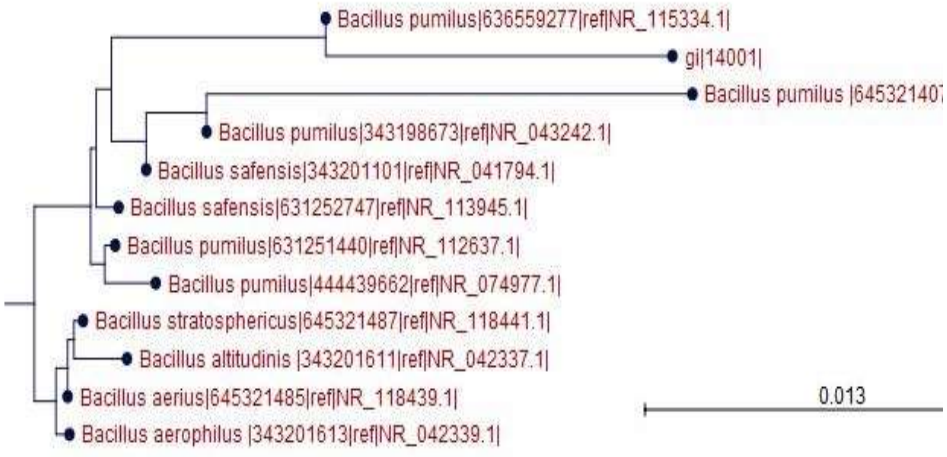
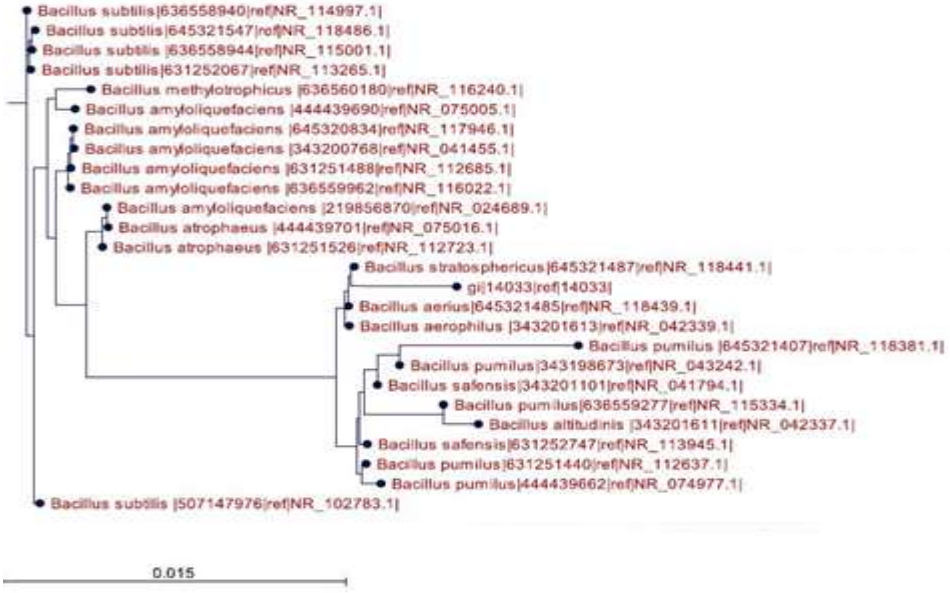


Fig 2.10. Colony PCR amplified product of 16S rRNA for isolated bacteria TM14001 and TM14023



2.11. (a)



(b)

Fig. 2.11.(a,b): Evolutionary relationships of TM14001 and TM14023 bacterial species based on 16S rRNA sequencing report.

2.3.9. Tagushi Experiment for *Bacillus Stratosphericus*

Temperature and pH were determined by Tagushi experiments using Qualitek 4 software, which confirms their minimum contribution for the expression of CYP450. It showed that the pH did not have any role on the production of CYP450. However, temperature had some significance during the production of CYP450. While running the ANOVA, the optimal conditions with respect to pH and temperature for *Bacillus Stratosphericus* were found to be pH 9 and 27^{o}C.

Col # / Factor	DOF (f)	Sum of Sqrs. (S)	Variance (V)	F - Ratio (F)	Pure Sum (S')	Percent P(%)
1 pH	2	.003	.001	.583	0	0
2 Temperature	2	.008	.004	1.349	.002	8.898
Other/Error	4	.012	.003			91.102
Total:	8	.025				100.00%

Fig. 2.12. ANOVA of main effects of pH and Temperature.

Column # / Factor	Level Description	Level	Contribution
1 pH	9	3	.016
2 Temperature	27	1	.029
Total Contribution From All Factors.....			.044
Current Grand Average Of Performance...			.073
Expected Result At Optimum Condition...			.118

Fig. 2.13. Optimum condition and performance

2.4. Discussions

The CYP450 content of a particular bacterial strain varies based on the environmental conditions of growth. In order to quantify the total amount of P450 in cells, the P450 heme must be exclusively in the ferrous state. In lysed cells or subcellular fractions such as microsomes or in purified protein, the CYP450 heme is generally in the oxidized (ferric) form, although some ferrous P450s have been detected in cell lysates. For the isolated ferric form, brief contact with sodium dithionite is sufficient to render all the CYP450s heme ferrous and be able to bind to CO [14]. For whole-cell P450 measurements, a longer incubation time with sodium dithionite is necessary in order to allow a plateau in dithionite response. A recent study has shown that P450s in whole cells are largely reduced. Thus,

the amount of P450 detected are not always increased by dithionite incubation [14,15].

The higher CYP450 content in the extremophile, *Bacillus stratosphericus* may suggests extended role of CYP450 in extreme environmental conditions which can be a useful attribute if used as biological component for developing CYP450 based sensor in terms of stability of the enzyme in the artificial environment. .

2.5. Conclusions

1. Out of the total 72 isolates, 35 bacterial strain showed detection of cytochrome P450 content as measured by CO difference spectrum. The bacterial strains TM14001 and TM14023 were screened for further studies based on the CYP450 induction by *n*-hexane.

2. It has been found that the CYP450 content was recorded maximum of 0.131 µmole, at pH 7 and temperature 37^{o} C in the interval of 9-12 hours.

3. The CYP450 content of TM14001, does not vary substantially with respect to pH changes in the range of 4 to 9. However, sample TM14023 has a narrower range in this respect having optimum content at pH around 7. For both the strains, TM14001 and TM140023 the CYP450 contents were maximum at 37^{o} C and recorded to be 0.0241 µmole and 0.0496 µmole respectively. At lower

temperature i.e. at 27°C, the CYP content values goes down substantially as a function of growth.

4. Spheroplast preparation resulted in significant reduction of CYP450 content as compared to the intact bacterial cells which were recorded to be 47.5% and 26.7% for bacterial strains TM14001 and TM14023 respectively

5. The phylogenetic trees predict that the sample TM14001 belongs to *Bacillus pumilus* having 99% identity and TM14023 is *Bacillus stratosphericus* having 98.75% identity.

6. The Tagushi experiment followed by ANOVA indicated that the optimal pH and temperature for *Bacillus Stratosphericus* were 9 and at 27^{o}C respectively.

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