Chapter III:

Purification and partial characterisation of Cytochrome P450.

3.1 Introduction

In the previous chapter, we confirmed the presence of the Cytochrome P450 monooxygenase in 35 bacterial isolates by using n-hexane as inducer; out of which two were selected for further identification using 16s rDNA sequencing. Between them, extremophile *Bacillus stratosphericus* was found better induced as monitored by CYP450 content measurement. The isolation and purification of the enzyme CYP450 was attempted for its further possible use as bioreceptor in biosensing device.

3.2 Objectives

1. Isolation and purification of the Cytochrome P450 component from *Bacillus stratosphericus.*
2. Characterisation of the purified Cytochrome P450 component.

3.3 Materials and Methodologies

3.3.1 Materials used

3.3.1.1 Chemicals used

The various chemicals used in the experiments are listed below according to 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).

3.3.1.2 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.

*3.3.2 **Methodologies***

3.3.2.1 Preparation of Cell Free Extracts

After the induction of the bacterial cells, they were harvested using a cold centrifuge followed by preparation of speheroplast as mentioned in the previous chapter under section 2.2.1.4. and 2.2.1.7.

For preparation of cell free extracts (CFE), spheroplasts were suspended in standard working (SW) buffer (50 mM Tris HCl, pH 7.4 containing 10 mM $MgSO_4$, 1 mM PMSF, 50g/l glycerol and 2g/l Tween 80). After adjusting the pH to 7.4, DNAse and RNAse (60 mg/l each) were added. The suspension was stirred at room temperature for an hour and centrifuged at 10000 g for 30 minutes. The pellets obtained after centrifugation were washed with buffer and subjected to further centrifugation at the same speed for three hours. The supernatants after each centrifugation step were pooled and filtered through membrane filter to remove particulate matters. After removing the cellular debris the filtered supernatant is transferred to ultracentrifuge tubes and centrifuged at 65,000g for 1 hour [1]. Further, the supernatant generated is concentrated by lyophilising overnight at -80 ^oC and used for further experiments.

3.3.2.2 Protein quantification by Bradford's assay

For protein quantification, a standard curve using Bovine Serum Albumin (BSA) was prepared at concentrations of 1.25, 2.5, 5, 10, 20, 30, 40 and 50 µg/mL in triplicates. The samples were added in amounts as shown in the table 3.1. After the incubation period of 5 minutes, the absorbance was subsequently taken at 595nm [2].

Table 3.1: Table for preparation of BSA standard curve

Sl. No	Protein Conc. (µg/ml)	From Stock (µl)	dH_2O (µl)	Bradford Reagent (µl)
1	0 (Blank)	0	100	200
2	1.25	2.5	97.5	200

3	2.5	5	95	200
4	5	10	90	200
5	10	20	80	200
6	20	40	60	200
7	30	60	40	200
8	40	80	20	200
9	50	100	0	200

3.3.2.3 Partial purification of the CYP450 components

The purification was done as described by Agarwal *et al* [3,4] with modifications done as per requirement. 0.5 ml of the supernatant obtained after ultracentrifugation was applied to a 7.2 ml DEAE-Cellulose column, equilibrated with 0.1 M potassium phosphate buffer containing 1 mM PMSF (pH 7). The column had a flow rate of 0.5 ml/min. The column was washed with 12 ml of 0.1 M Potassium phosphate buffer and eluted isocratically using 0.1 M, 0.2 M and 0.3 M KCl containing 1 mM PMSF. The eluted fractions were examined for its absorbance at 215 nm and 280 nm. The CYP450 content in these fractions was then measured by CO difference spectra [5]. The pooled fractions found positive for CYP450 obtained from anion exchange chromatography was loaded (500 µl) on to a 10 ml Seralose CL-6B column (0.5 × 15 cm, GE) equilibrated with three column volume of 0.1M Potassium phosphate buffer (pH 7) at a flow rate of 0.5ml/min. The CYP content in the pooled fractions was examined using CO difference spectra [5]. Finally, the homogeneity of the protein

was analysed by Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) [6].

3.3.2.4 Sample preparation for MALDI-MS

The mass spectroscopy using MALDI was done at SAIC, IIT Guwahati. For sample preparation of MALDI MS, two types of matrices were used, *viz.* alpha-Cyano-4-Hydroxycinnamic acid and Sinapinic Acid for the smaller and bigger band respectively [7]. For both the matrices, alpha-Cyano-4-Hydroxycinnamic acid and Sinapinic acid were mixed in saturation in a TA30 solvent (30:70 [v/v] acetonitrile: 0.1% TFA in water). Further for the analyses of the samples, it was mixed in a ratio of 1:1, 1:2, 1:4, 1:6, 1:12 and 1:25 with the samples.

3.3.2.5 Trypsin digestion of Protein samples

The protocol for in-gel trypsin digestion was taken up from the protocol provided by IISc Banglore. The stained gel was excised into 2-3 mm pieces and transferred to sterile microfuge tubes and washed with 500 µL of wash solution (50% acetonitrile, 50 mM ammonium bicarbonate) and incubated for 15 minutes at room temperature with slow mixing using a vortex and then the solution is discarded keeping the gel inside the tube. This process was repeated twice until the complete removal of the dye. Further, the gel was dehydrated using a 100% acetonitrile for five minutes, followed by complete removal of the acetonitrile by a pipette and air drying the gel for about 15 minutes. After completely drying the gel, 150

µL reduction solution (10 mM DTT, 100 mM ammonium bicarbonate) is added for 30 min at 55 ^{o}C, which is followed by discarding of the reduction solution and addition of 100 µL alkylation solution (50 mM iodoacetamide, 100 mM ammonium bicarbonate) for another 30 minutes at room temperature in dark. The alkylation solution was discarded and the 500 µl of wash solution is added for 15 minutes with gentle swirling. The gel is further dehydrated using 100% acetonitrile for five minutes followed by air drying of the gel in room temperature. The gel is rehydrated with 20 µl of protease digestion solution and incubated overnight at 37^{o}C.

3.3.2.6 Analysis of Data post trypsin digestion

The sample obtained after digestion were further mixed with matrix (α-Cyano-4-Hydroxycinnamic acid) and analysed using a MALDI MS. The peak values obtained for each peak were taken and searched using MASCOT database search tool in the NCBIprot database for a proper match [8,9].

3.4 Results

3.4.1 Bradford assay

Bradford assay was performed to determine the concentration of the sample. For preparation of the BSA (Bovine Serum Albumin) standard curve, the protein sample and reagents were mixed as shown in table 3.1, and the standard curve thus obtained was $y = 0.0146x + 0.0135$ with an R2 value of 0.9965

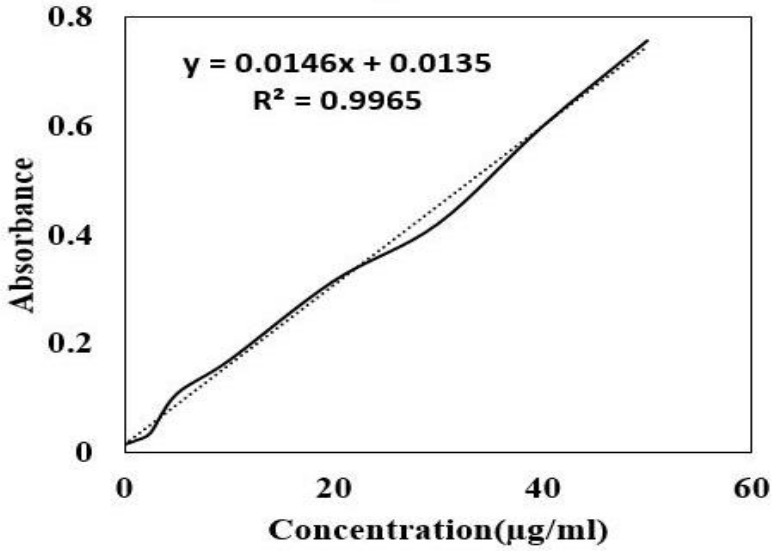


Fig. 3.1: BSA standard curve for determination of unknown protein

3.4.2 Purification of the CYP450 components

3.4.2.1 Anion exchange chromatography

Anion Exchange chromatography was done in a 7.2 ml DEAE-Cellulose column (0.5×15 cm, GE) at a elution rate of 0.5 ml/min. 42 ml fractions were collected isocratically using 0.1 M, 0.2 M and 0.3 M KCl in 0.1M

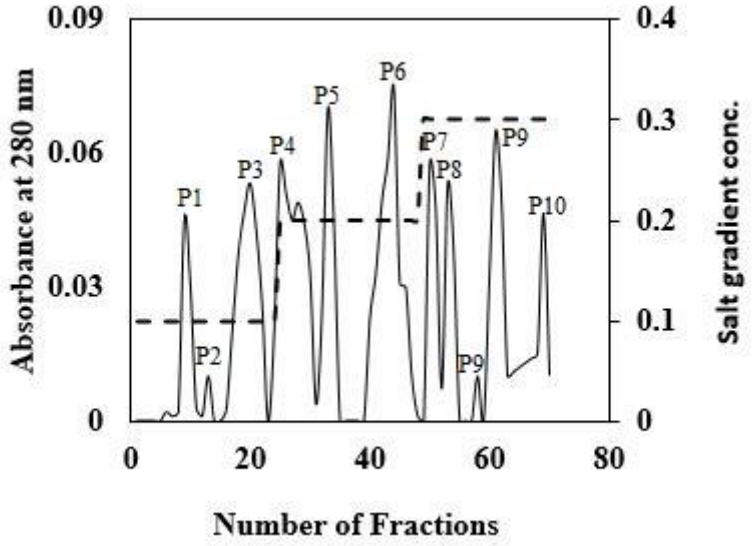


Fig. 3.2: Elution profile of fractions from anion exchange chromatography

Potassium phosphate buffer containing 1 mM PMSF. After the column run, fractions were checked for protein content by determining the optical density (OD) of the collected fractions. The O.D values when plotted showed a graph as shown in fig. 3.2. The fractions (fractions 9, 20, 25, 33, 44, 57, 60 and 74) showing peaks were assayed subsequently by CO assay [5]. And the maximal CYP450 content was shown by the pool P9 with a CYP450 content of 0.0001095 µmol/ml. This peak was further purified using gel filtration chromatography

3.4.2.2 Gel filtration chromatography

The sample purified by anion exchange chromatography was then subjected to gel filtration chromatography in a 10 ml Seralose CL-6B column (0.5 × 15 cm, GE) equilibrated with three column volume of 0.1M

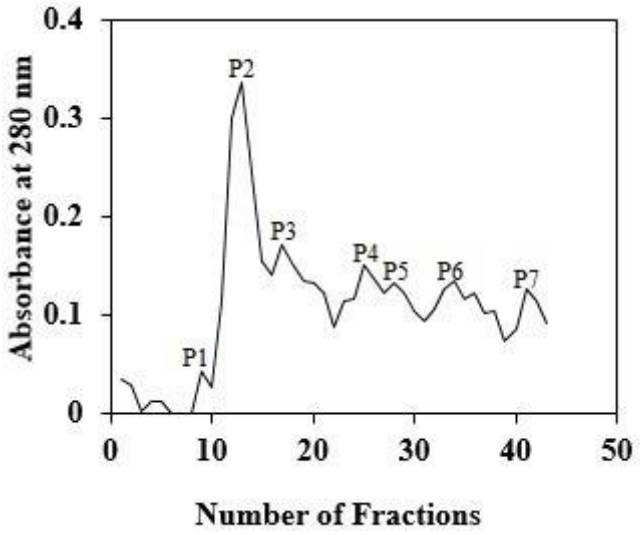


Fig. 3.3: Elution profile of gel filtration chromatography carried out using Serulose- 6 B matrix with a flow rate of 0.5 ml/min

Potassium phosphate buffer (pH 7) at a flow rate of 0.5 ml/min. 30 ml of fractions were collected and the elution profile of the fractions collected were analysed by taking their absorbance at 280 nm and the following graph was obtained (fig. 3.3). Further, when assayed for CYP content using CO difference spectra, it showed the maximal CYP450 content for the pull 13 (peak P2) having a value of 0.0001992 µmol/ml.

3.4.2.3 Preparation of purification chart

For the purification, the specific content of the CYP450 is found to be 1.3789E-08, 3.0527E-06 and 8.0113E-06 µM for Spheroplast, AEC and GFC respectively with a purification fold of 1, 221.3904 and 2.62432 respectively.

Table 3.2 : Purification chart for Cytochrome P450

Purification step	Volume (ml)	Protein (µg/ml)	CYP450 (µM)	Specific content (µM CYP450/µg protein)	Fold Purification
Spheroplast	2	2357	0.0000325	1.3789E-08	1
AEC	0.7	35.87	0.0001095	3.0527E-06	221.390491
GFC	0.5	24.865	0.0001992	8.0113E-06	2.62432808

3.4.3 SDS PAGE

Protein profile of the purified protein sample was analysed by SDS-PAGE. The protein sample was loaded both in the reducing and non-reducing condition. In both reducing and non-reducing state, two bands

were observed. The resulting SDS PAGE profile showing two bands suggested presence two different proteins associated together similar to the class II monooxygenase components namely, diflavin reductase complex and the Cytochrome P450. However no further experiments was done for confirming the nature of the second component.

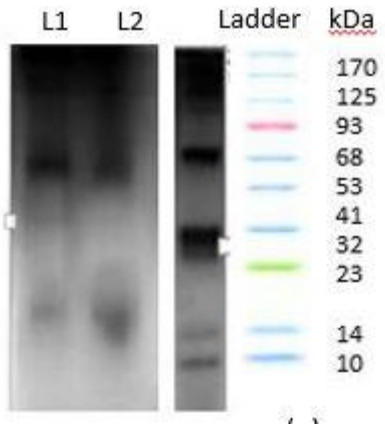


Fig. 3.4: Protein profile analysis of purified protein sample, by SDS- PAGE: L 1: Sample reduced, L2: Sample unreduced.

3.4.4 Fe Content

The purified sample was analysed by *Atomic Absorption Spectroscopy* (AAS) in order to confirm the presence of iron in the purified metalloprotein. The presence of iron was detected in purified protein apparently confirming heme-iron moiety of CYP450 component of monooxygenase complex.

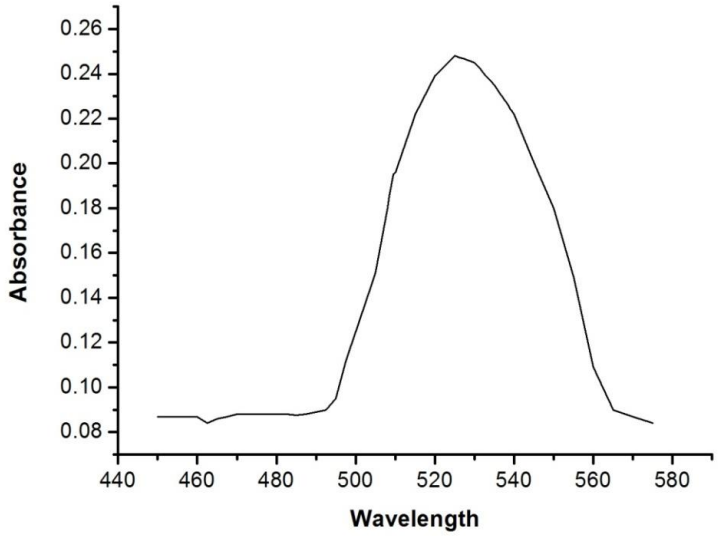
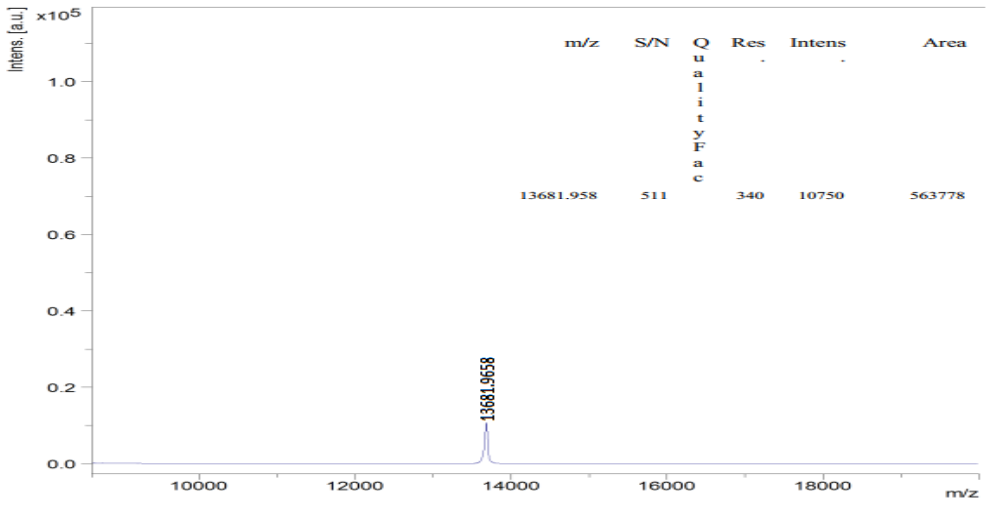
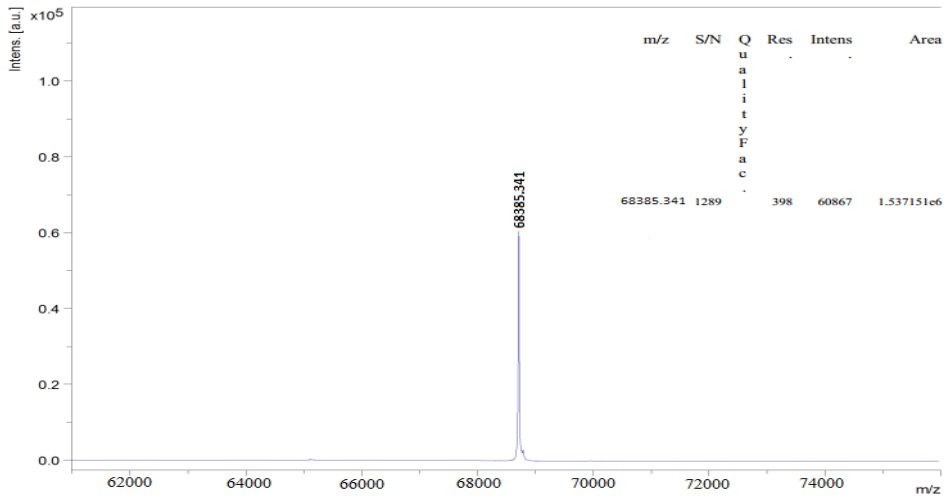


Fig. 3.5: Atomic spectroscopy result showing peak at around 530 nm corresponding to iron (Fe)

3.4.5 Mass verification using MALDI MS



(a)

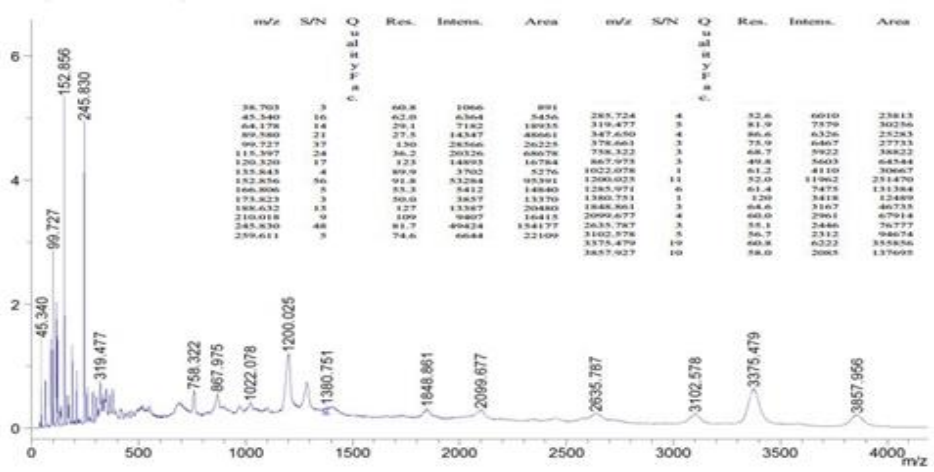


(b)

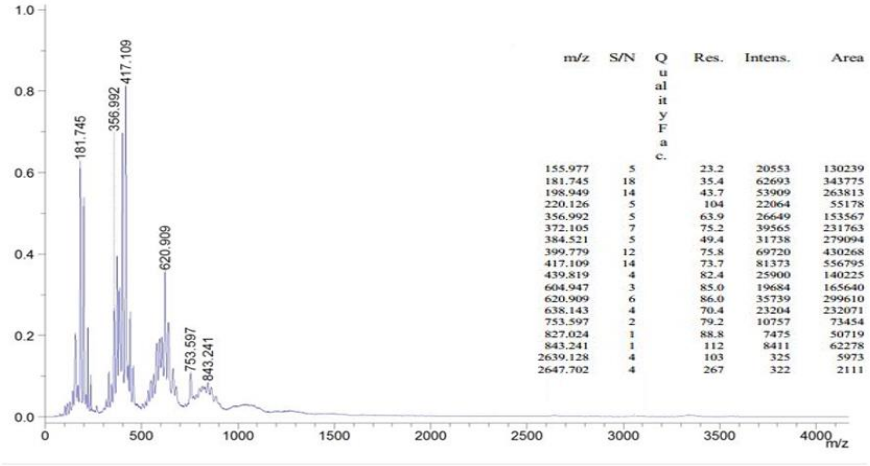
Fig. 3.6 the molecular mass were determined to be 13.6 kDa (b) and 68.1 kDa (d) respectively using MALDI MS.

The molecular mass of both the bands were determined using mass spectroscopy. The molecular mass was determined to be 13.681 kDa and 68.183 kDa for band 1 and band 2 respectively.

3.4.6 Trypsin digestion of Protein samples



(a)



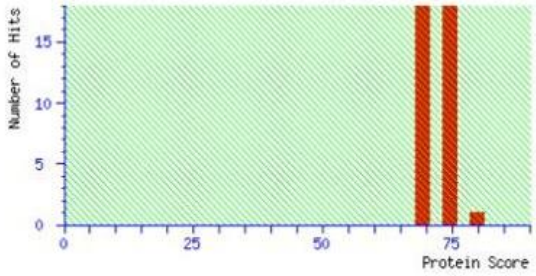
m/z	S/N	Quality Fac.	Res.	Intens.	Area
155.977	5		23.2	20553	130239
181.745	18		35.4	62693	343775
198.949	14		43.7	53909	263813
220.126	5		104	22064	55178
356.992	5		63.9	26649	153567
372.105	7		75.2	39565	231763
384.521	5		49.4	31738	279094
399.779	12		75.8	69720	430268
417.109	14		73.7	81373	556795
439.819	4		82.4	25900	140225
604.947	3		85.0	19684	165640
620.909	6		86.0	35739	299610
638.143	4		70.4	23204	232071
753.597	2		79.2	10757	73454
827.024	1		88.8	7475	50719
843.241	1		112	8411	62278
2639.128	4		103	325	5973
2647.702	4		267	322	2111

(b)

Fig. 3.7: (a), (b): trypsin digestion profile of both the bands along with the peptide congeners.

After trypsin digestion of Protein samples the bigger band showed 31 peptide congeners and the smaller band showed 18 peptide congeners.

3.4.7 Analysis of Data post trypsin digestion



Database: NCBIprot 20180429 (152462470 sequences; 55858910152)

Top score : 80 for AU)14604.1, cytochrome P450 *[Bacillus megaterium]*

(a)

Protein sequence coverage: 35%

Matched peptides shown in ***bold red***.

```
  1 MITLPVIQGP SSYKLTGHLQ KFRENPLGFL ENLTQYGEIA TFRVAHKRFY
 51 VTRDPQLIKD VVITNSKAFQ KIKLTHMFKT LLGEEMLWTD EALYMGPIQP
101 SQLKQHLTCN KEAIAKIIEK HTGTWEEGQL RTIVKDIRQI VIAVLLQLVF
151 GISIEDKDKI DYVQALMRKK EKLGKIYIRL PLHQPDSDEQ LEQLLFERVQ
201 MRVHNKTEGN DLLQYILNSC GEDSDEREIY EQLNSIFLSM YEMITHVCSW
251 SIHLLSQNTR EHLQLHKEIQ AYASGESLST KNLTYMRKII AESMRLYPPL
301 WLFGRQARED IQIDGYSIKK GEIMLISPYM MHRHEDYFLE PSEFLPDRFE
351 KGGSIDVPSY MYMPLGIEHQ AERGMDYITE IVTIFLSEMT KRFLFQLTKS
401 ESITPMAGVM LNMKEELNVN VHKVHTQS
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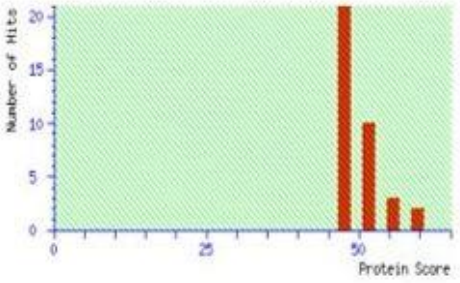
(b)



Database: NCBIprot 20180429 (152462470 sequences; 55858910152 residues)
Top score : 60 for WP_1067077219.1, transcrtional regulatory protein PhoB *[Nitrosomonous sp.]*

(c)

Protein sequence coverage: 37%

Matched peptides shown in ***bold red***.

```
  1 MKQVLITLMI LFSTSTLAEG QLTKNKQLVI DFYTDVVLAE DATHIDKYLG
 51 PRYIQHNPMV ADGKEGLRAL LRSLPKRDKS AGPSGEIVRV IAEGDLVVLH
101 VKSYHWPQPN GGAIVDIFRV ENGKIVEHWD VIQAIPEQAK NTNTMF
```

(d)

Fig. 3.8: (a), (c) Mascot score histogram. Where protein score is –log(P), where P is the probability that observed match is a random event. Protein greater than 93 are significant ($p<0.05$) for the big and small band respectively. (b), (d) the protein sequence covered with the matched peptides shown in red for the big and small band respectively.

For de novo sequencing, the m/z values for the peptide peaks obtained for each peak were taken and searched using MASCOT database search tool in the NCBIprot database for a proper match. The bigger band, showed an amino acid sequence with 428 residues with a top score of 80 for

AU14604.1 for a cytochrome P450 of *Bacillus megaterium*. The calculated isoelectric point was found to be 6.26. Whereas, the smaller band had a top score of 60 for WP_1067077219.1, for a transcriptional regulatory protein PhoB of *Nitrosomonous sp*. Further, for the n terminal sequence we can consider from the 1st amino acid of both the sequences.

3.5 Discussions

For the purification of the enzyme cytochrome P450 and its components, mainly two methods are in use, namely, affinity chromatography [10,11] and ion exchange chromatography [12,13]. Which may be followed by a gel filtration chromatography [12]. Even though the method involving affinity chromatography is highly effective, it is not very suitable for larger quantity of protein due to disproportionate cost of the affinity matrices [14]. Commercial anion exchangers such as DEAE cellulose [15], Toyopearl DEAE 650M, DEAE 650S [14] etc. are in use for purification of CYP450. Due to application based nature of the work, a bigger quantity of enzyme is preferred and hence the above mentioned purification steps were performed.

The presence of two bands in the purified fraction of ion exchange chromatography as indicated in the SDS-PAGE analyses are apparently, suggestive of two partner components of monooxygenase system. Such system falls under class II family of monooxygenases more common in mammals. In mammalian liver microsomes, the prevalent components of

Class II system are diflavin reductase and CYP450 [16]. Bacteria usually contain class I system having three components i.e. cytochrome P450, ferredoxin and ferredoxin reductase [17,18]. In absence of detailed further characterisation of the other components, such claim needs further investigation. However, the limited investigations carried out in the present study and the band pattern resulting from the SDS-PAGE analysis were indicative of a bacterial system having resemblance of class II monooxygenases not usual to bacterial monooxygenases. The first reported bacterial class II redox system was found in *Bacillus megaterium* for a 119 kDa protein [19]. However, the enzyme lacked the membrane anchors, typically found in mammalian CYP450s. Even though it is not very common, the entire bacterial cytochrome P450 family "CYP102" belongs to class II system [20].

The two main methods for protein sequencing are Edman degradation using a protein sequencer and Mass spectroscopy. Even though determination of N terminal end using Edman degradation sequencing have been a valuable tool for identification over the last three decades, mass spectroscopy has also become one of most the prominent tool for sequencing of protein due to versatility of instrument, accuracy and superior information [21]. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) in association of a scoring algorithm has widely been used for determining the sequence data of a digested protein [22]. In this study, the trypsin digested polypeptide congeners were

analysed using a computer program, Mascot. After the database search (NCBIprot), using the mentioned tool, the nearest hit was for a cytochrome P450 of *Bacillus megaterium*. The mascot programme gives a score based on probability of matching for the sample sequence with a matching sequence in database. Higher the similarity with the database sequence, more is the Mascot score. However, for this study, the top score was 80, which is below the minimum significance score of 93. One of the signature motifs present in the K helix of the CYP450 is EXXR motif [23], which is present in the derived sequence from 292nd to 295th amino acid. This suggests that the protein of interest may be distantly related to the above-mentioned cytochrome P450 of *Bacillus megaterium*.

3.6 Conclusions

1. The specific content of the CYP was found to be 1.3789E-08, 3.0527E-06 and 8.0113E-06 μM for Spheroplast, AEC and GFC respectively.
2. SDS-PAGE analysis of the purified protein sample showed the presence of two bands, one between 68 kDa and 53 kDa, and the other between 10 kDa and 25 kDa, suggesting that the purified protein belongs to the class II monooxygenase system.
3. The detection of iron in the purified component in Atomic Absorption Spectroscopy (AAS) analysis indicated its metalloprotein nature.

4. The SDS-PAGE analysis of the purified protein indicated molecular masses of 13.681 kDa and 68.183 kDa for band 1 and band 2 respectively.
5. Both the purified components are further subjected to de novo sequencing using MALDI MS. For the 68.1 kDa band, the predicted sequence showed a top score of 80, hitting a cytochrome P450 of *Bacillus megaterium*. Whereas the smaller band showed similarities with a transcriptional regulatory protein with a lesser significance.
6. The limited experimental analyses of the purified components done by using SDS-PAGE and Mass Spectrospy indicated its proximity to the class II monooxygenase system.

Bibliography

[1] Melancon, M., Elcombe, C., Vodicnik, M., and Lech, J (1981). Induction of cytochromes P450 and mixed-function oxidase activity by polychlorinated biphenyls and β-naphthoflavone in carp (Cyprinus carpio). *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology*, 69(2): 219-226.

[2] Kruger, N. J (2009). The Bradford method for protein quantitation. In, *The protein protocols handbook*, of, pages 17-24. Springer.

[3] Phillips, I. R., Shephard, E. A., and de Montellano, P. R. O (1998). *Cytochrome P450 protocols*. Springer.

[4] Agrawal, V. and Miller, W. L (2013). P450 oxidoreductase: Genotyping, expression, purification of recombinant protein, and activity assessments of wild-type and mutant protein. In, *Cytochrome P450 Protocols*, of, pages 225-237. Springer.

[5] Omura, T. and Sato, R (1964). The carbon monoxide-binding pigment of liver microsomes I. Evidence for its hemoprotein nature. *Journal of Biological Chemistry*, 239(7): 2370-2378.

[6] Hames, B. D. *Gel electrophoresis of proteins: a practical approach*. OUP Oxford, 1998.

[7] Nordhoff, E., Schürenberg, M., Thiele, G., Lübbert, C., Kloeppel, K.-D., Theiss, D., Lehrach, H., and Gobom, J (2003). Sample preparation protocols for MALDI-MS of peptides and oligonucleotides using prestructured sample supports. *International Journal of Mass Spectrometry*, 226(1): 163-180.

[8] Yergey, A. L., Coorssen, J. R., Backlund, P. S., Blank, P. S., Humphrey, G. A., Zimmerberg, J., Campbell, J. M., and Vestal, M. L (2002). De novo sequencing of peptides using MALDI/TOF-TOF. *Journal of the American Society for Mass Spectrometry*, 13(7): 784-791.

[9] Zhang, W. and Chait, B. T (2000). ProFound: an expert system for protein identification using mass spectrometric peptide mapping information. *Analytical Chemistry*, 72(11): 2482-2489.

[10] Black, S. D., Linger, M. H., Freck, L. C., Kazemi, S., and Galbraith, J. A (1994). Affinity Isolation and Characterization of Cytochrome-P450-102 (BM-3) from Barbiturate-Induced Bacillus megaterium. *Archives of biochemistry and biophysics*, 310(1): 126-133.

[11] Gillam, E. M., Guo, Z. Y., Martin, M. V., Jenkins, C. M., and Guengerich, F. P (1995). Expression of cytochrome P450 2D6 in Escherichia coli, purification, and spectral and catalytic characterization. *Archives of biochemistry and biophysics*, 319(2): 540-550.

[12] Munro, A. W., Daff, S., Coggins, J. R., Lindsay, J. G., and Chapman, S. K (1996). Probing electron transfer in flavocytochrome P-450 BM3 and its component domains. *European journal of biochemistry*, 239(2): 403-409.

[13] Klein, M. L. and Fulco, A (1993). Critical residues involved in FMN binding and catalytic activity in cytochrome P450BM-3. *Journal of Biological Chemistry*, 268(10): 7553-7561.

[14] Schwaneberg, U., Sprauer, A., Schmidt-Dannert, C., and Schmid, R (1999). P450 monooxygenase in biotechnology: I. Single-step, large-scale purification method for cytochrome P450 BM-3 by anion-exchange chromatography. *Journal of Chromatography A*, 848(1-2): 149-159.

[15] Yang, Y., Zhang, D., and Cerniglia, C. E (1997). Purification and characterization of a cytosolic cytochrome P450 from the yeast Trichosporon cutaneum. *FEMS microbiology letters*, 154(2): 347-353.

[16] Wang, M., Roberts, D. L., Paschke, R., Shea, T. M., Masters, B. S. S., and Kim, J.-J. P (1997). Three-dimensional structure of NADPH–cytochrome P450 reductase: prototype for FMN-and FAD-containing enzymes. *Proceedings of the National Academy of Sciences*, 94(16): 8411-8416.

[17] Roberts, G. A., Grogan, G., Greter, A., Flitsch, S. L., and Turner, N. J (2002). Identification of a New Class of Cytochrome P450 from a Rhodococcus sp. *Journal of bacteriology*, 184(14): 3898-3908.

[18] Maier, T., Förster, H.-H., Asperger, O., and Hahn, U (2001). Molecular characterization of the 56-kDa CYP153 from Acinetobacter sp. EB104. *Biochemical and biophysical research communications*, 286(3): 652-658.

[19] Narhi, L. O. and Fulco, A. J (1987). Identification and characterization of two functional domains in cytochrome P-450BM-3, a catalytically self-sufficient monooxygenase induced by barbiturates in Bacillus megaterium. *Journal of Biological Chemistry*, 262(14): 6683-6690.

[20] Munro, A. W., Girvan, H. M., and McLean, K. J (2007). Cytochrome P450–redox partner fusion enzymes. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 1770(3): 345-359.

[21] Domon, B. and Aebersold, R (2006). Mass spectrometry and protein analysis. *Science*, 312(5771): 212-217.

[22] Perkins, D. N., Pappin, D. J., Creasy, D. M., and Cottrell, J. S (1999). Probability-based protein identification by searching sequence databases using mass spectrometry data. *ELECTROPHORESIS: An International Journal*, 20(18): 3551-3567.

[23] Syed, K. and Mashele, S. S (2014). Comparative analysis of P450 signature motifs EXXR and CXG in the large and diverse kingdom of fungi: identification of evolutionarily conserved amino acid patterns characteristic of P450 family. *PLoS One*, 9(4): e95616.