Chapter 13

Purification of Azurin from *Pseudomonas Aeuroginosa*

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Additional information is available at the end of the chapter

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1. Introduction

The use of microorganisms and their products as possible therapeutic agents in the control of cancer begins at the latter part of the nineteenth century. The search of new drugs from microbial sources against infectious disease has been augmented when Alexander Fleming (1928) discovered penicillin [1]. The secondary metabolites from microorganisms play a vital role in developing antibiotics and chemotherapeutics [2, 3]. Several researchers have reported various anticancer molecules from different microbial sources [4]. Even though chemotherapy is efficient in enhancing patient survival with primary tumors continue to have deprived prognosis. The rapid advances in the field of antibiotics have inspired new hope that the search among biological systems will disclose a chemical agent which will exert a destructive effect upon neoplastic growth without seriously affecting normal cells. Using live or attenuated pathogenic bacteria or its metabolites in treatment of cancer excretes toxic effects among patients. Azurin, a redox protein recently fascinated biomedical researcher's immense interest as an anti cancer therapeutic agent which enters human breast cancer cells and induces apoptosis without any adverse effects in cancer patients [5]. Azurin, a secondary metabolite derived from bacterial species especially from *P. aeruginosa* function as a donor in terminal electron transfer process [6]. Azurin also termed as blue small copper proteins highly stable in nature. The presence of copper ion in the polypeptide chain contributes to the azurin stability [6-8]. Azurin reported as a potential anticancer protein against breast cancer cell lines, evoked the researchers of novel methods for enhanced synthesis of azurin has initialized. *P. aeruginosa* a common gram negative opportunistic pathogenic bacterium found naturally [9]. They are considered as facultative anaerobic grow in partial or total oxygen depletion cultural conditions. This organism can achieve anaerobic growth with nitrate as a terminal electron acceptor. *P. aeruginosa* secretes a variety of pigments, including pyocyanin, pyoverdine and pyorubin.

Previous researchers [10] adopted genetic engineering techniques and other bacterial species for purification of azurin. This study is concerned of enhanced azurin synthesis from different strains of *P. aeruginosa* with lucid homogeneity by customized methods. The growth of different *P. aeruginosa* MTCC strains 1934, 741, 2453, and 1942 for the synthesis of azurin were scrutinized for enhanced azurin synthesis. The enhanced azurin synthesis from *P. aeruginosa* strains was improved by the $CuSO_4$ and KNO_3 containing media under facultative anaerobic condition. The purification of azurin had been performed by ion-exchange and gel-filtration chromatography. High yield was reported in *P. aeruginosa* 2453 strain than other strains.

2. Materials and methods

2.1. Chemicals and reagents

Growth medium constituents were of analytical grade obtained from Hi-Media laboratories, India. The buffer ingredients were purchased from Merck Chemicals Ltd, India. Sephadex G-25, G-75, diethyl amino ethyl cellulose (DEAE) cellulose and carboxy methyl (CM) cellulose were all obtained from Sigma-Aldrich, USA. The 3, 5-dimethoxy, 4-hydroxy cinnamic acid otherwise called as sinnapinic acid a matrix-assisted laser desorption/ionization-time of flight (MALDI-ToF) was also acquired from Sigma-Aldrich. Protein concentrations were measured by Lowry's method with bovine serum albumin as standard. Standard dialysis bag with 3 kDa cutoff was purchased from Sigma-Aldrich. Powder of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), propidium iodide (PI) and dimethylsulfoxide (DMSO) solution were procured from Sigma Aldrich, India. Cell culture media and other constituents of media are purchased from Hi-media Laboratories Ltd, India. Fetal bovine serum was obtained from Invitrogen Life Technologies, USA.

2.2. Cultivation of P. aeruginosa MTCC 2453

A freeze dried culture of *P. aeruginosa* strains MTCC2453,741,1934,1942 was obtained from the Microbial type culture collection center, Chandigarh, India and was grown in a medium containing 7g yeast extract, l0g peptone, 20g of KNO3, 6.4g of KH_2PO_4, 3.6g of Na_2HPO_4 (anhydrous), 2.5g of NaCl, 5µg/ml of CuSO4 per liter. The initial pH was adjusted to 6.5 with NaOH. The strains were maintained on nutrient broth with 50% glycerol concentration and stored at -70^{o}C for further study.

Inoculums' of *P. aeruginosa* strains was prepared by inoculating a loopful of colonies in individual 100 ml conical flasks with the exact constituents of the above prescribed media and incubated at 37^{0}C, in stirring mode at 100/rev for 21 hours. These inoculums was used to seed the bulk 500ml x 4 sterile medium in 2000 liter conical flasks (separate conical flask used for all four strains) which was also kept at stirred mode (100/rev) for 21 hours at 37^{0}C [11].

2.3. Impact of copper sulphate and potassium nitrate on culture medium

All P. *aeruginosa* strains MTCC2453, 741, 1934, and 1942 are inoculated separately in a sterile medium. Impact of copper sulphate and potassium nitrate in azurin synthesis were

observed by adding different concentration of copper sulphate (1μg/ml- 5μg/ml) and potassium nitrate (5μg/l – 20μg/l) at different flasks for each concentration distinctly. The azurin protein optimization and quantification was studied in UV spectrometer (Perkin Elmer, Massachusetts, USA) at 595 nm by Bradford's method. The azurin synthesized from P. aeruginosa MTCC2453 is significantly higher than other strains [11, 12].

2.4. Extraction of cellular protein

After 21 hrs incubation, cells were harvested by centrifugation method at 13200 g for 15-20 minutes by using ultra centrifuge (Eppendorf, Hamburg, Germany). Cell pellets was collected and suspended in the appropriate volume of 0.02M potassium phosphate buffer pH 7 with protease inhibitor and kept in the ice basket for sonication. Cells were sheared by Ultra sonicator (Cole Parmer, USA) of approximately 100 ml batches of cell suspension. All batches were sonicated for 1-2 minutes at 100W. After sonication the samples was stirred vigorously and centrifuged at 10000g for 20 minutes which removes cell wall debris. The green-brown crude supernatant was stored. Resuspended the precipitate in same buffer, stirred it vigorously and centrifuged as before and the supernatant were stored with the previous extracts [11, 12].

2.5. Ammonium sulfate precipitation of proteins

The Crude (supernatant) was saturated to 45% (277g/l) by slowly adding ammonium sulfate salt at 4^0C for precipitation, kept it for overnight [5,6]. After precipitation the solution was centrifuged at 20,000g for 25 minutes [6,]. Collected the yellow supernatant saturated again to 95% by adding (NH4)2SO4 (372g/l) slowly and kept at 4^0C for overnight. The overnight precipitated solution was centrifuged at 23000g for 45 minutes. Pale supernatant was discarded. Precipitate (contains azurin) were collected and resuspended in 0.02M Potassium Phosphate buffer pH 7[11, 12].

2.6. Dialysis of the supernatant

Azurin suspended in 0.02M potassium phosphate buffer pH 7 was dialysed by standard dialyses bag purchased from Sigma-Aldrich, (Kolkata, India) having 3 kDa molecular weight cut off at 4^0C for 20 hours on the same buffer for overnight with continuous gentle stirring. Dialysis was done until the solution attains its buffer pH. The solutions were kept at 4^0C after dialysis for further purification [11, 12].

2.6.1. Purification of Azurin on Ion – Exchange chromatography

2.6.1.1. DEAE cellulose treatment

Dialysate (contains azurin) were initially treated with DEAE. 100 ml slurry of DEAE cellulose equilibrated in 0.02M potassium phosphate buffer pH 7 were treated with the

dialysate and stirred for 20-30 min at 4^0C.The suspension was centrifuged at 10,000g for 15 min. Azurin does not adsorb in the gel remains in supernatant but most of the unwanted proteins like yellow flavo proteins are removed. Supernatant was collected. DEAE cellulose precipitate was resuspended in the same buffer and again centrifuged at 10,000g for 15 minutes to remove all unattached proteins [11-13].

The supernatant after DEAE treatment was saturated to 100% (766g/l) with (NH4)2SO4 at 4^0C for overnight for precipitation. After saturation, precipitates are mixed gently and kept for centrifugation at 10000g for 10 min. supernatant was collected for dialysis at 4^0C for overnight with gentle stirring with the same before. Dialysis was continued till the solution pH attains its buffer pH [11-13].

2.6.2. Purification of Azurin on gel-filtration chromatography

2.6.2.1. Chromatography on Sephadex G-25

Sephadex G-25 beads were equilibrated in the 0.02M potassium phosphate buffer pH 7 [Parr S R et al 1976] for overnight, and tightly packed in 3cm x 25cm length glass column without any bubbles. The column was initially washed with 0.02M potassium phosphate buffer for twenty volumes of the gel packed. The Flow rate was adjusted to one minute per ml. slowly the dialysate (after DEAE treatment) was added with the eluent buffer 0.02M potassium phosphate buffer pH 7 on the column. Thirty fractions were collected at one minute interval [12-14].

2.6.2.2. Chromatography on Sephadex G-75

Sephadex G-75 beads in powder form are equilibrated in 0.01M Tris/Hcl buffer pH 7.5 for overnight. After equilibration the beads were tightly packed in a 3cm x 45cm glass column. The column was washed with same equilibrating buffer for fifty volumes of the column value. After washing with buffer one ml of the sample (fraction (a) collected from the G-25) were passaged and eluted with the same equilibrating buffer. Seventy five fractions were collected at 1 ml/6minutes flow rate [12-14].

2.6.3. Purification of Azurin on ion – Exchange (anionic) chromatography

2.6.3.1. Chromatography on CM cellulose

The CM cellulose beads from Sigma–Aldrich (Kolkata, India) were equilibrated for overnight in the ammonium acetate buffer pH 3.9 adjusting the pH by 0.05M acetic acid with 2M NH_3.After swelling, the beads were packed in a 5cm x 15cm glass column and washed for ten times of the column volume. Gently one ml of the sample added (Fraction (e) collected from G-75) over the top of the column and left it for 5-10 minutes to bind the protein inside the beads. After 10 minutes the column was eluted with ammonium acetate buffer pH 4.65 [12-14].

2.6.4. Characterization of Azurin (purified from P. aeruginosa MTCC2453)

2.6.4.1. Molecular weight determination by matrix-assisted laser desorption/ionization time of flight (MALDI-ToF)

The most successful method to analyze biopolymers such as, proteins, peptides, sugars and large organic molecules which are tend to be fragile and fragment when ionized by more conventional ionization methods [15]. The Fractions collected from G-25, G-75 and CM cellulose were performed MALDI for molecular weight determination. Two micro liter from each fraction of the chromatography was added with 20µl of 3, 5-Dimethoxy, 4-Hydroxy cinnamic acid otherwise called as sinnapinic acid (Sigma-Aldrich. Kolkata, India). Tiny spots were made on silver plate and kept for drying for 4-6 hours to drain the water molecules. Further spots were dried with a vacuum drier to make a crystalline molecule. After drying the samples were placed in the MALDI-ToF chamber (Voyager De pro, applied systems Illinois, USA) for analysis by using nitrogen laser at 337 nm.

2.6.5. Purification profile of Azurin ((synthesized from P. aeruginosa MTCC2453) by SDS-PAGE

Five ml of 12% resolving gel contains 1ml distilled water, 30% acryl amide, 1.5M Tris (pH 8.8), 10% SDS, 10% APS and 0.002µl TEMED for polymerization was casted in the glass slab without any bubbles and kept it for 10-15 minutes. After polymerization of the resolving gel, 3ml of stacking gel (4%) were loaded over the resolving gel which contains 0.68 ml distilled water, 30% acryl amide, 1M Tris (pH 6.8) 10% SDS,10% APS, and 0.001ml TEMED. After casting the gel, proteins purified from different chromatography were loaded with bromophenol (molecular weight marker dye) at different lanes for profiling the protein purification process.

Glass slab gel were kept in the electrophoresis tank with tank buffer (196 mM glycine, 0.1%SDS, 50mM Tris-Hcl pH 8.3 made by diluting a 10x stock solution). This setup was connected with power pack initially in 80mV to 100 mV. After running the gel up to its anode end, was removed and stained with 0.2% coomassie brilliant blue for overnight. Destained with destaining solution (45: 45: 10 – methanol: water: acetic acid) which destains the comassie blue until it reveals the bands. The bands (figure 4) were observed under UV transilluminator (Biorad, PA, USA) [16].

2.6.6. FTIR analysis

Infrared spectroscopy experiments were performed using a Nexus 870 (Thermo Nicolet Corporation, Madison, USA) spectrometer equipped with a potassium bromide (KBr) beam splitter and DTGS (deuterated triglycine sulfate) detector in the range of 3,000-4000 cm^{-1}. We recorded 32 scans per spectrum at a 2 cm^{-1} resolution for 100 µl of azurin liquid samples in 0.02 M PBS buffer (pH 7.0). We kept the same buffer as a background medium and performed all measurements at room temperature. We corrected spectra for the moisture

and carbon dioxide in the optical path. The curves were deconvoulted and imported into Omnic's peak fit software (Thermo scientific, Illinois, USA) and a Gaussian curve fitting performed [17].

3. Results

3.1. Growth of P. aeruginosa strains

The inoculated growth of *P. aeruginosa* MTCC strains 2453, 741, 1934 and 1942 under facultative anaerobic conditions, yields total dry cell protein in the range of 150- 170 g/l medium (Fig. 2.1a). We observed *P. aeruginosa* 2453 produces lesser amount of cellular proteins than other strains. The quality assay was performed after incubation for contamination of any other unwanted organisms. A unique green colour colony in nutrient agar medium was observed and hence we confirmed it as *P. aeruginosa* colonies (Fig 1.).

3.2. Effect of copper sulphate and Nitrate in azurin synthesis

Earlier studies showed that azurin production by different bacterial strains were similar to the azurin produced by *P. aeruginosa* MTCC 2453 but with more yield than previous procedures. Four strains were tested for high yield of azurin productions were *P. aeruginosa* 2453, 741, 1942, and 1934. We observed a significant increase in the yield of azurin secreted by *P. aeruginosa* 2453 than genetically engineered strains and other strains. This remarkable increase in the yield of azurin was obtained by addition of $CuSO_4$ and KNO_3 in the medium with specific facultative anaerobic cultural conditions. In contrast to earlier studies, adding both $CuSO_4$ (4-5 μg/ml) and KNO_3 (0.02 μg/ml) in the medium under facultative anaerobic conditions generate high amount of azurin (Figure 2.), rather adding either $CuSO_4$ or KNO_3 (Table 1.).

(a) (b)

Figure 1. (a) Bacterial culture medium incorporated with $CuSO_4$ and KNO_3 (b) Green colour colonies a unique characteristic of *P. aeruginosa* colonies

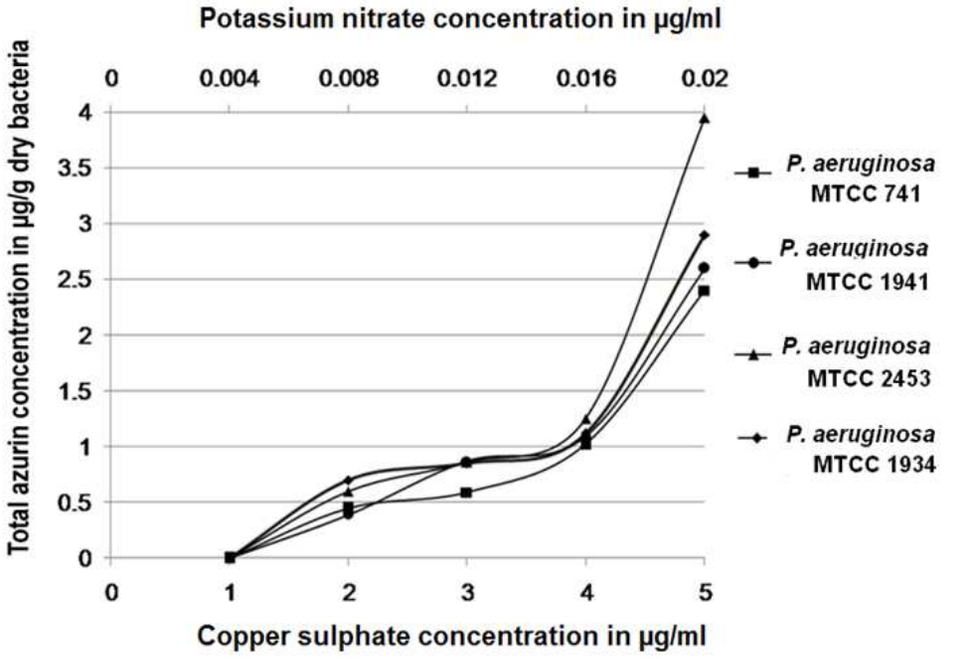


Figure 2. Quantification of azurin synthesis by different strains of *P. aeruginosa* MTCC 741, 1934, 1942 and 2453 and impact of $CuSO_4$ and KNO_3: 1-5 µg/ml range of $CuSO_4$ concentration with 0.004-0.02 µg/ml of KNO_3 was added in the culture medium to study the impact of azurin synthesis.

Purification step	*P. aeruginosa* MTCC 1934	*P. aeruginosa* MTCC 741	*P. aeruginosa* MTCC 2453	*P. aeruginosa* MTCC 1942
Total dry cell yield in g/l medium	1650	1780	**156 0**	1590
Protein concentration after 45/95 % (NH4)2SO4 precipitation (g/l)	1520/1410	1460/1550	**1105/1250**	1205/1300
Protein concentration after DEAE treatment in g/l medium	560	610	**460**	490
Protein concentration after G-25 treatment g/l.	440	485	**315**	384
Protein concentration after G-75 treatment g/l.	320	350	**295**	302
Total Azurin synthesis in mg/g dry bacteria. (CM cellulose)	2.9	2.4	**3.95**	2.6

Table 1. Azurin yield from different strains (*P. aeruginosa* MTCC 741, 1934, 1942 and 2453) in addition of 5 µg/ ml $CuSO_4$ and 0.02 µg/ml KNO_3 in the culture medium

3.3. Chromatography methods for azurin Purification

DEAE and G-25 are gel filtration columns which remove positively and negatively charged proteins respectively. The unwanted flavo proteins and positively charged proteins were removed during DEAE chromatography. The collected fractions from G-25 were quantified for protein concentration in the UV-Spectrophotometer at 280_{nm} wavelength. Azurin and other proteins more than 5 kDa were eluted immediately after void volume is plotted as graph (Figure 3.).

Peak (a) from G-25 was loaded on G-75 for further purification. The G-75 fractions were quantified for protein concentration in the UV-Spectrophotometer at 280nm wavelength. Azurin a 14 kDa protein will elute after binding in to the beads when the elution buffer elutes it. Thus, azurin and some other proteins will elute very lately, was confirmed from the OD values of the spectrometer, when plotted as graph (Figure 4). The azurin will form a thick band when passages through CM cellulose column which was eluted by ammonium acetate buffer pH 4.65. Ten fractions were collected and absorbed under UV spectrometer at 280nm wavelengths for azurin concentration (Figure 5.).

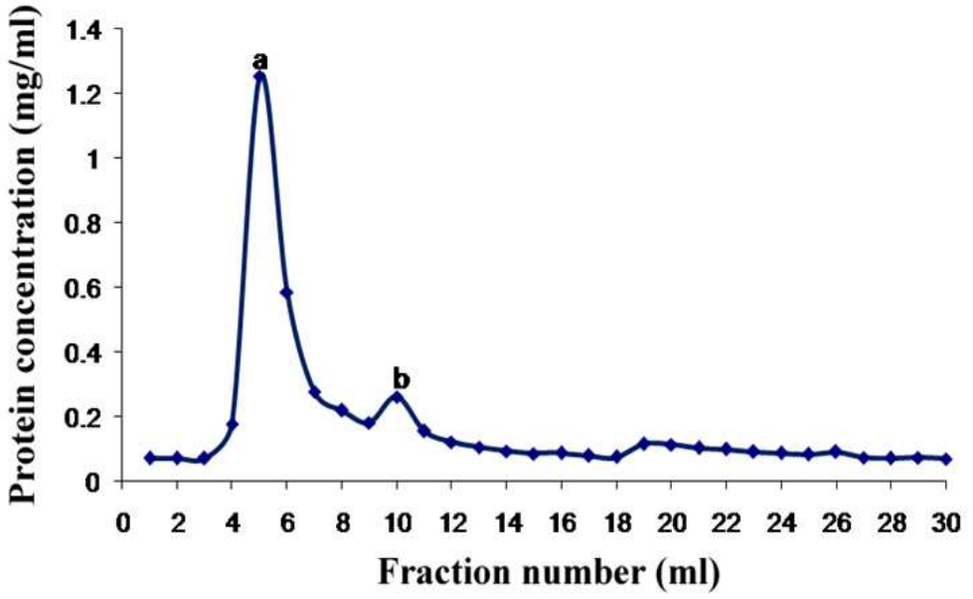


Figure 3. Elution on sephadex G-25: The active fraction from DEAE was loaded on G-25 column. Upon thirty fractions only peak (a) collected for further purification.

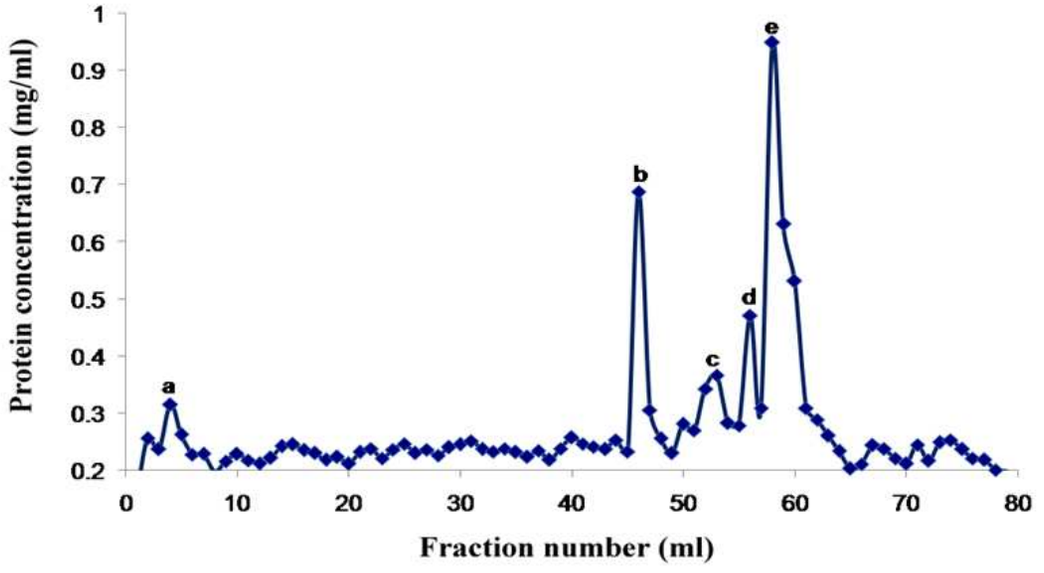
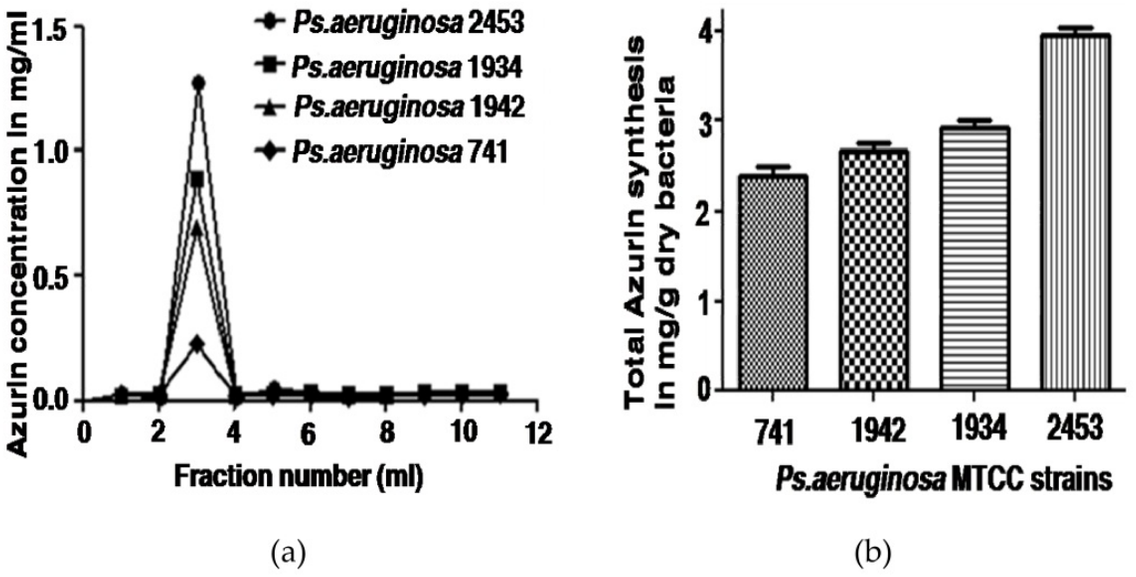


Figure 4. Elution on sephadex G-75: Fraction (a) collected from the G-25 was loaded with eluent buffer (PBS) to elute bounded proteins. Seventy five fractions were collected at 1 ml/6minutes flow rate. Peak (e) collected for further purification.

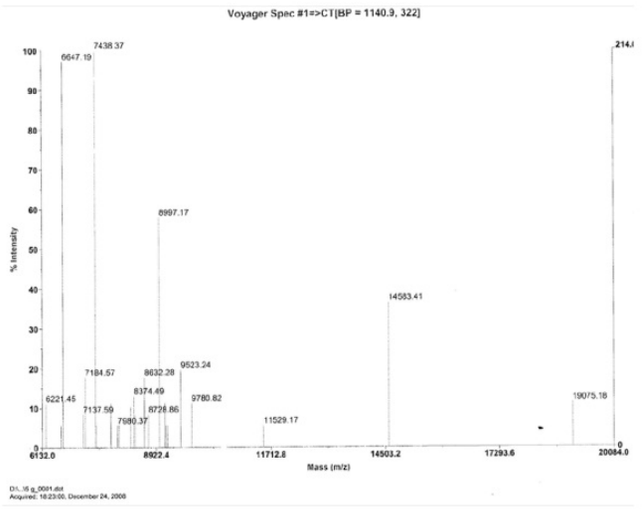


(a) (b)

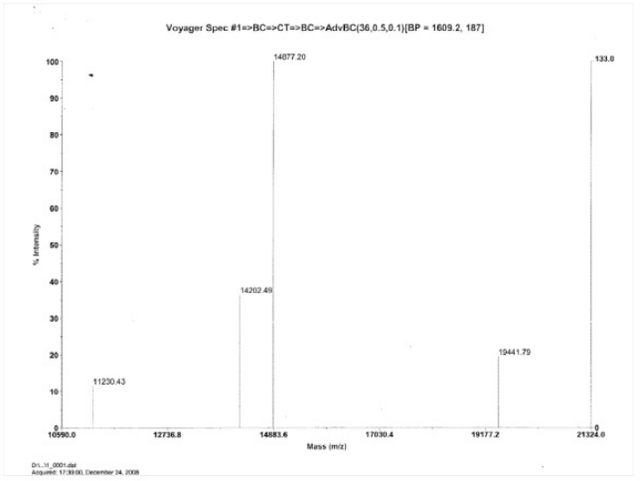
Figure 5. Azurin purified from various strains of *Pseudomonas aeruginosa*: **a** Azurin forms thick bands in CM cellulose column chromatography during their purification process. Later this was eluted by ammonium acetate buffer pH 4.65. All *Pseudomonas aeruginosa* strains, particularly *Pseudomonas aeruginosa* 2453 strain shows more significant amount of azurin production. **b** the mean of the azurin production by various *Pseudomonas.aeruginosa* strains: The production of azurin was enhanced by the copper sulphate (5μg/ml) and KNO_3 (0.02 μg/ml) containing media under facultative anaerobic condition. The bar graph shows *Pseudomonas aeruginosa* 2453 secrets more azurin than any other strains like *Pseudomonas aeruginosa* 741, 1942, 1934 strains tested.

3.4. Characterization of Azurin (Purified from P. aeruginosa MTCC2453)

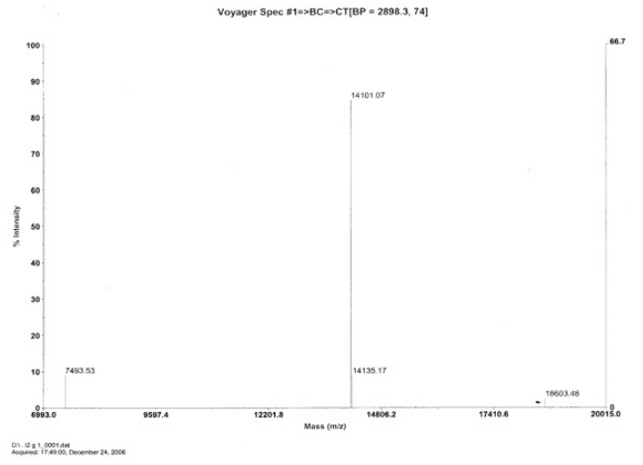
In this study we profiled our purification process at every step by MALDI-ToF (Figure 6.) and SDS-PAGE (Figure 7.) and to confirm the azurin presence in our experiments. Cellular proteins loaded in lane 2 of SDS-PAGE reveals whole cell proteins of *P. aeruginosa* MTCC 2453. Fraction (a) collected from G-25 gel filtrations were loaded in lane 3; it shows proteins above 5 kDa in SDS-PAGE which was also confirmed in MALDI-ToF results. Most unwanted proteins were deduced during G-75 gel filtration. Our SDS and MALDI results shows fraction (e) from G-75 contains azurin (14 kDa). The azurin was again purified and concentrated in CM cellulose.



(a)



(b)



(c)

Figure 6. (a) Protein purification was assayed at each step of chromatography. Peak (a) from G-25 was analyzed in MALDI-ToF using Nitrogen laser at 337 nm, confirming the 14 kDa molecular weight of azurin.

(b) Protein purification was assayed at each step of chromatography. Peak (a) from G-25 was analyzed in MALDI-ToF using Nitrogen laser at 337 nm, confirming the 14 kDa molecular weight of azurin.
(c) Peak (e) from G-75 was analyzed in MALDI-ToF

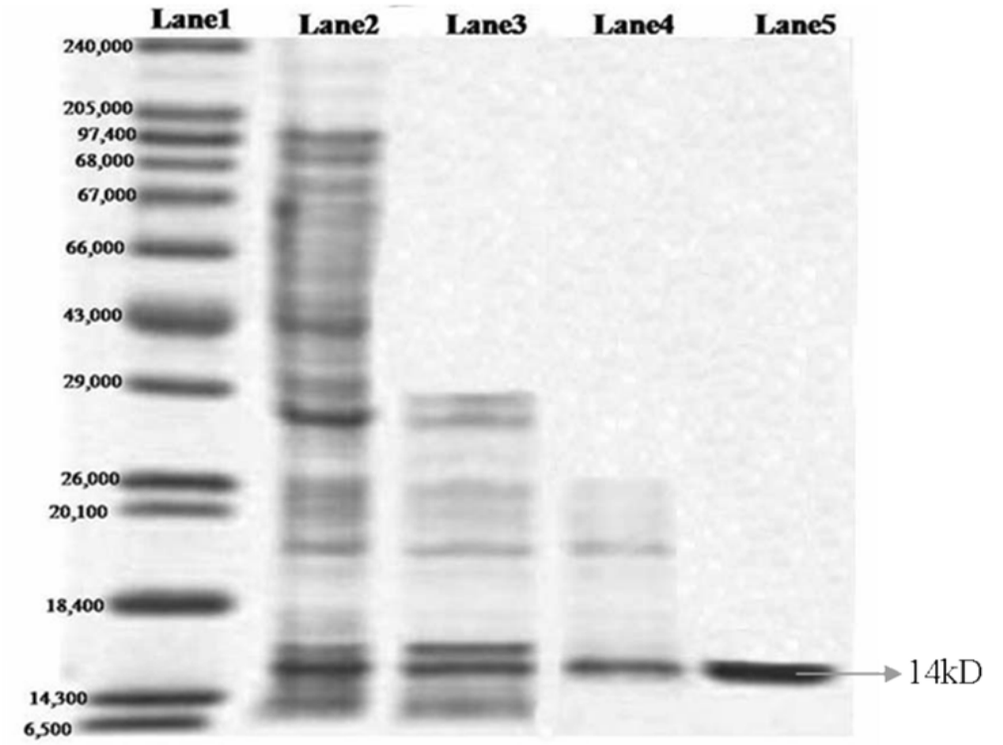


Figure 7. Protein purification profile further was confirmed by SDS-PAGE analysis: Lane 1: Molecular weight markers 6.5-240 kDa (Bangalore Gene, India), Lane 2: Total cellular proteins, Lane 3: G-25 Fraction [peak (a)], Lane 4: G-75 fraction [peak (e)], Lane 5: CM cellulose purified azurin.

3.5. FTIR analysis

The functional groups of azurin were studied using FTIR spectrum. The presence of the amide I band was indicated by the peak around 1650 cm^{-1} region, which arises primarily because of the stretching vibration of the main chain of carbonyl groups in the protein backbone coupled with the in-plane N-H bending and C-N stretching modes. Furthermore, the presence of an amide band around 1650 cm^{-1} signifies α-helix secondary structure of azurin. Azurin synthesized from all strains showed a significant shift in the amide I band with one another, indicating differences in their helix secondary structure of azurin. The most prominent among all strains is *P. aeruginosa* 2453 which showed peak around 1646.936 whereas, others showed peak around 1642.269, 1639.446, 1637.873 for *P. aeruginosa* 741, 1942, 1934 respectively (Fig. 8.). The peaks at 3695 and 3251 cm^{-1} are the amide A and B bands, respectively, which arise from a Fermi resonance between the first overtone of amide and the N-H stretching vibrations. The 1495 cm^{-1} peak refers to the amide II band, which arises because of the C-N stretching as well as the C-N-H bending motions. The 1352 peak is the amide III band, which arises predominantly because of the in-phase combination of N-H in plane bending and C-N stretching vibrations.

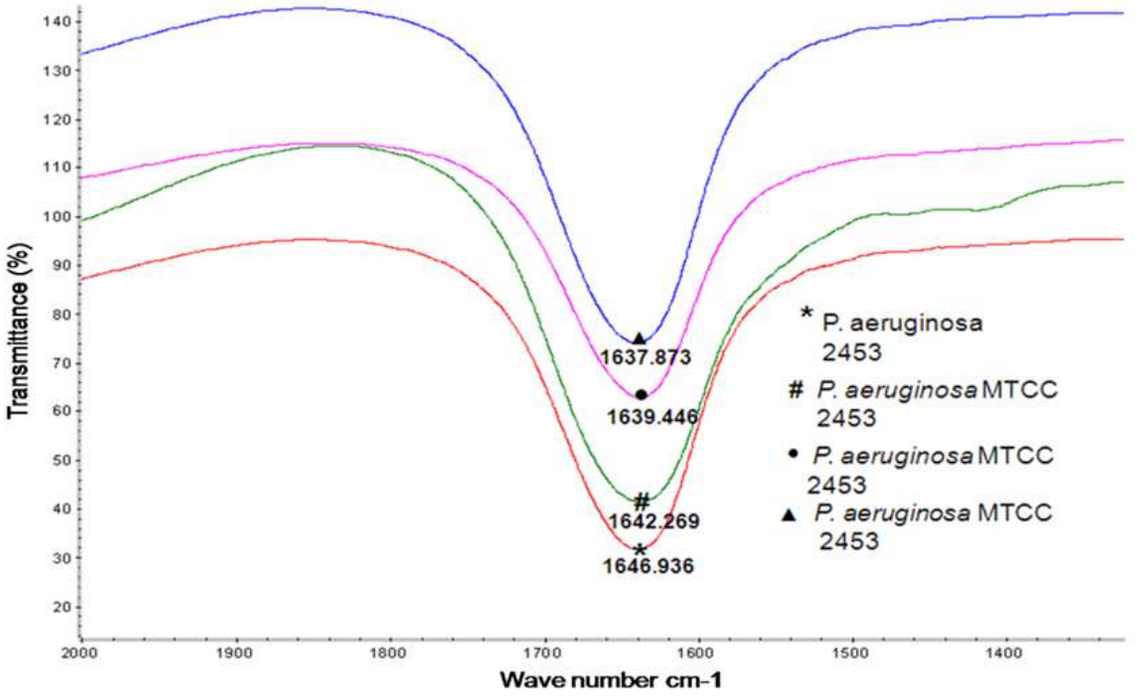


Figure 8. FTIR analysis showed peak around 1646.936 in *P. aeruginosa* 2453 whereas, others showed peak around 1642.269, 1639.446, 1637.873 for *P. aeruginosa* 741, 1942, 1934 respectively.

4. Discussion

Azurin production from *P. aeruginosa* MTCC 2453 was enhanced when 5μg/ml of copper sulphate and the potassium nitrate (0.02μg/ml) was added. At each step of the purification process protease inhibitor was added to the protein sample for inhibition of protein lyses. During dialysis most of the lower (approx) proteins up to 3 kDa molecular weight are pierced out. The retained proteins (above 3 kDa molecular weight) were washed in sephadex G-25 which serves as a desalting column and also has 3-5 kDa molecular weight fractionation range. High molecular weight more than 5 kDa proteins were eluted immediately after void volume which was revealed in SDS-PAGE and MALDI-ToF spectrometer.

The fraction collected from G-25 containing only more than 5 kDa proteins were passed through on G-75 which has 5-80 kDa fractionation range. The higher proteins above 80 kDa molecular weight elute after void volume; the remaining proteins between 6-70 kDa were bounded within the beads later eluted by the buffer. The fraction which showed 14 kDa molecular weight by analyzing in MALDI-ToF spectrometer for all the fractions (MALDI-ToF results not shown for all fractions which showed peak) were collected and again purified in CM cellulose chromatography. The fraction which showed peak in CM cellulose was again observed in MALDI-ToF spectrometer to confirm the presence of 14 kDa molecular weight of Azurin.

Our idea of adding copper in the culture medium was not only for the enhanced azurin synthesis, but to reveal the differences of azurin's stability in the secondary structure for all

P. aeruginosa strains. The FTIR investigation showed azurin has C=O (protein backbone) stretching, which is the unique nature of the amide I band. The presence of the amide band at 1650 cm^{-1} signifies the α-helix secondary structure of azurin. The significant shift among four strains synthesized azurin implies that there was a difference in their secondary structure which may be due to their physiological or genetic variations among strains. The impact of the differences in the secondary structure of azurin synthesized from all four strains tested, were also reflected in the apoptosis generation of all strains.

Abbreviations

MALDI-Matrix-Assisted Laser Desorption/Ionization, SDS-PAGE-Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis, FTIR- Fourier Transform Infrared Spectroscopy, $CuSO_4$ – copper sulphate, KNO_3 – Potassium nitrate, MTT -3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium, PI-Propidium Iodide, DMSO-dimethylsulfoxide, MALDI-ToF-Matrix-Assisted Laser Desorption/Ionization-Time of Flight, MTCC- Microbial Type Culture Collection center, CM-carboxymethyl, DEAE-Diethylaminoethyl Cellulose

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