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Characterization of anticancer compound azurin from indigenous isolate *Pseudomonas aeruginosa SSj*

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Abstract: **Proteins and other products from bacteria are becoming increasingly relevant in anticancer treatment due to their high specificity and minimal side effects. Azurin is a novel anticancer protein mainly produced from *Pseudomonas aeruginosa* and some Gram negative bacteria. This study was conducted on ten *Pseudomonas* isolates from the biodiversity rich Western Ghats region of Kerala, India. Here, after confirming the presence of anticancer compounds using trypan blue exclusion assay on DLA (Daltons Lymphoma Ascites) cell lines, the *Pseudomonas aeruginosa* isolate showing the highest cytotoxic activity was chosen for further studies. The identity of the organism was confirmed by biochemical and molecular characteristics. The identified isolate has been designated as *Pseudomonas aeruginosa SSj* and the 16S rRNA partial sequence was deposited in Genbank (accession number KU821118). The presence of azurin encoding (*azu*) gene in SSj strain is confirmed using PCR amplification and its sequence is deposited in GenBank (KU821119). The partially purified azurin is studied using FTIR spectrum in comparison with azurin from *Pseudomonas aeruginosa* MTCC2453 and standard azurin (Sigma). The strain SSj is identified as potential azurin producer and a promising nominee for biomedical applications.**

Keywords: **Cancer, *Pseudomonas aeruginosa*, Bacterial protein, azurin, FTIR.**

1. INTRODUCTION

Cancer therapy using microbial products has attracted considerable interest and new microbial compounds having specific anticancer activity without side effects are constantly evaluated[1]. Researchers have been looking to engineer microorganism to produce medicinally important compounds and improve their activity using methods like protein engineering, so that cancer treatments become more efficient and inexpensive[2]. Azurin, a secondary metabolite from *Pseudomonas aeruginosa* function as donor in terminal electron transfer. Microorganisms mainly using azurin as an electron transport protein during electron transport cycle. It transports an electron between cyt c-551 and cyt oxidase in their respiration process [3]. It contains a single copper ion in A chain, which contributes to the high stability of azurin. This novel protein has a unique property to penetrate the cell membranes of cancer cells without effecting other healthy cells. Azurin is reported to have antiviral and antiparasitic activity along with anticancer activity[4]. In this study, we explore the antitumor activity of *Pseudomonas* strains from environmental samples of the Western Ghats. We intend to evaluate in vitro therapeutic efficacy of these strains in DLA cell lines by trypan blue exclusion assay and the highest activity showing strain was selected for further studies. Screening of the selected strain for the presence of azurin genes was done by PCR with specific primers. Functional groups were studied by FTIR in comparison with standard azurin.

2. MATERIALS AND METHODS

2.1 Sample collection:

Environmental samples were collected from different localities of the Western Ghats region, Kerala, a hotspot of India, were serially diluted and plated on Kings B agar. The isolates were morphologically examined for size, shape, color, pigment. Greenish mucoid colonies were selected and further identified on the basis of their biochemical characteristics. All purified isolates were stained with Gram stain and examined microscopically.

2.2 In vitro cytotoxicity assay:

Total protein from all *Pseudomonas* samples was extracted and partially purified using 70% ammonium sulfate precipitation followed by dialysis[5]. Short-term in vitro cytotoxicity of the dialysate was examined using trypan blue exclusion assay on DLA cells. The tumor cells from the peritoneal cavity of tumor-bearing mice were aspirated and washed repeatedly with PBS. Viable cells (10^6 cells in 0.1 ml) were added to tubes containing different concentrations (200µg/ml,100 µg/ml,50 µg/ml,20 µg/ml and 10 µg/ml) of partially purified azurin protein in PBS. After incubation for 3 hours at 37 ^oC, the cell suspension was mixed with 0.1ml of 1% trypan blue and kept for 2-3 minutes and loaded on a hemocytometer. Dead cells take up the blue color of trypan blue while live cells do not take up the dye. The number of stained and unstained cells were counted separately[6].The isolate showing the highest cytotoxic activity was selected and stored as glycerol stock for further studies.

$$Cytotoxicity\,(\%) = \frac{No.of\ \ dead\ cells}{No.of\ \ living\ cells + No.of\ \ dead\ cells} x100$$

2.3 Molecular characterization and phylogenetic analysis:

According to Bergey's Manual of Determinative Bacteriology [7]the chosen isolate was morphologically and biochemically identified.The 16s r RNA sequence was used to carry out BLAST with the nr database of NCBI Genbank. Based on the maximum identity score, best twenty sequences were selected and a phenogram was constructed with the selected strain. CLUSTAL W [8] was used for multiple sequence alignment and evolutionary history resolved by the neighbor-joining method [9]. The evolutionary distances and phylogenetic analysis were calculated using Kimura 2-parameter method[10] and MEGA 5[11] respectively. Bootstrap resampling analysis for 1000 replicates was performed to evaluate the confidence of tree topologies[12] and the tree generated was envisioned using FigTree v1.4.

2.4 PCR detection of azurin gene:

The presence of azurin gene (*azu*) in *Pseudomonas aeruginosa SSj* was confirmed by PCR using specific primers in comparison with standard strain. Standard strain *Pseudomonas aeruginosa* MTCC 2453 was obtained from the Microbial type culture collection center, Chandigarh, India[13]. Total genomic DNA was isolated according to modified method of Unal *et al* [14]. Azurin gene was amplified from the genome of selected bacterial strain and standard strain. The PCR was performed according to a previously described method[5].

2.5 Fourier transform infrared spectroscopy (FTIR) analysis:

The secondary structure of polypeptides and proteins are accurately studied by Infrared (IR) spectroscopy [15]. Infrared spectroscopy experiments were performed using a PerkinElmer Spectrum Two FT-IR spectrometer. FTIR spectra of protein extract from *Pseudomonas aeruginosa SSj*, was compared with azurin extracted from *Pseudomonas aeruginosa* MTCC 2453 and standard azurin (Sigma Aldrich)[18]. The curves were deconvoluted and imported into software spectral manager and a Gaussian curve fitting was performed [16].

3. RESULTS

3.1 Isolation of native *Pseudomonas* isolates:

Out of forty-two environmental samples collected from the Western Ghats region, ten native *Pseudomonas* strains were isolated. These strains were identified using conventional microbiological and biochemical techniques. Greenish mucoid colonies of *Pseudomonas* isolates were found on Kings B agar [17].

3.2 In vitro cytotoxicity assay:

Cytotoxicity activity of ten bacterial protein extracts was carried out against DLA cell line at different concentrations using trypan blue exclusion assay. Results of different concentrations of protein suspensions are tabulated in Table 1. Out of ten protein suspensions isolate A showed highest cytotoxicity effect on DLA cell lines. This particular isolate A was selected for further study. Isolates B and C have limited activity when compared with other eight strains.

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Table 1: Trypan blue exclusion assay for cytotoxicity of ten *Pseudomonas* isolates

	% of Cell death									
Con. of Extract	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
200µg/ml	85	9	10	43	37	25	33	45	71	29
100µg/ml	60	4	6	20	18	12	16	22	35	14
50µg/ml	50	0	0	12	7	8	10	11	20	7
20µg/ml	40	0	0	7	4	2	5	5	10	2
10µg/ml	28	0	0	2	0	0	0	0	8	0

3.3 Biochemical and Molecular characterization of *Pseudomonas:*

The selected *Pseudomonas* strains were identified using biochemical methods. The 16S rRNA gene sequence (Figure 1) was used for the phylogenetic analysis. The sequence of 1291 bp, deposited in Genbank(KU821118) was used for BLAST analysis. A phylogentic tree showing the relationship of the selected strain and related strains obtained from the NCBI database are presented in Figure 2. The phylogenetic analysis demonstrated close similarity with *Pseudomonas aeruginosa strain LOCK 0998* and *Pseudomonas aeruginosa strain PD 29*. From these results, the isolate was affirmed as *Pseudomonas aeruginosa* and designated with strain name *SSj*

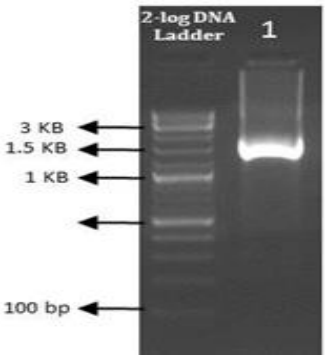

Figure 1: PCR amplification product run on agarose gel. Lane1:16S r DNA amplicon, Lane2: DNA ladder

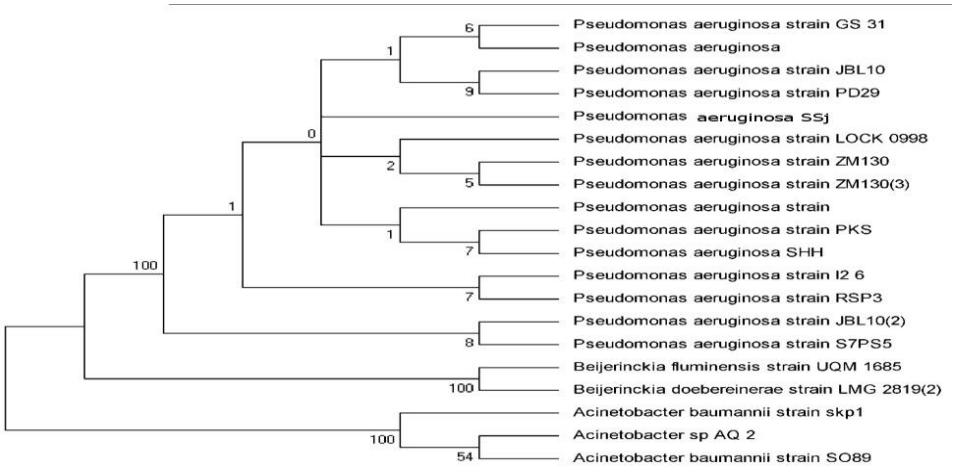

Figure 2: Phenogram of *Pseudomonas aeruginosa* SSj and closely related strains.

3.4 PCR detection of azurin gene:

Amplification of *azu* gene with azurin specific primer pair produced a 545 bp product as shown in Ethidium Bromide stained agarose gel electrophoresis. Both *Pseudomonas aeruginosa SS*j and the control strain *Pseudomonas aeruginosa* MTCC 2453 showed specific bands with 545 bp size (Figure 3). The presence of azurin gene was detected in selected isolate and *Pseudomonas aeruginosa* MTCC 2453 taken as positive control for this study.

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To confirm the gene identification results further, the *azu* gene sequence obtained was compared with sequences available in the Genbank using BLAST. The isolate showed 100% similarity with *Pseudomonas aeruginosa* strain NCGM (accession number AP01465). Partial sequence of azurin gene from *Pseudomonas aeruginosa SSj* is deposited on Genbank with accession number KU821119.

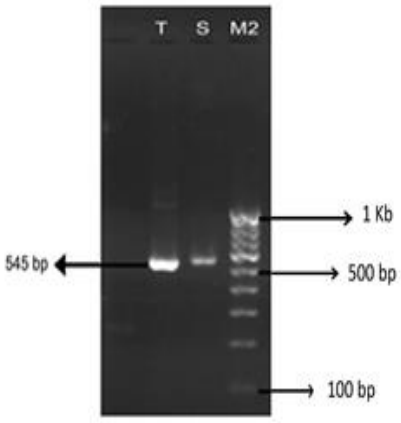

Figure 3: PCR products on agarose gel- Lane M2: DNA ladder, Lane T: *Pseudomonas aeruginosa SSj* Lane S: *Pseudomonas aeruginosa* MTCC2453.

3.5 FTIR analysis:

FTIR spectroscopy is one of the finest analysis to provide the information about the secondary structure of proteins. The principle behind the FTIR spectroscopy is detecting the particular wavelengths of radiation in the infrared region of the spectrum are absorbed by the sample**.** *Pseudomonas aeruginosa* SSj shows a peak around 1638 cm^{-1} region and *Pseudomonas aeruginosa* 2453 shows a peak around 1637cm^{-1}region, final confirmation was done in comparison with standard azurin which also showed a peak around 1641 cm^{-1} region(Figure 4) these all peaks signifies α-helix secondary structure of azurin.. The significant differences in the value of peaks in the FTIR spectrum indicate differences in the amide I band and secondary structure of azurin. This may be the first study about the functional group characteristics of azurin using standard azurin.

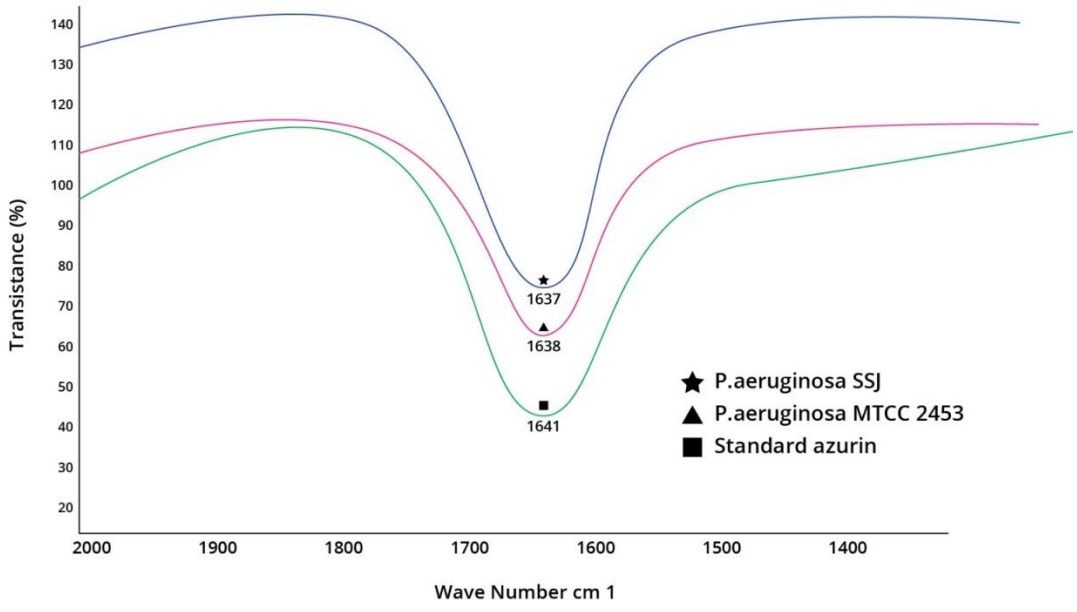

Figure 4: FTIR analysis results ■ Standard azurin (1641 cm^{-1}), ★ *Pseudomonas aeruginosa SSj* (1637 cm^{-1}) and ▲*Pseudomonas aeruginosa* MTCC 2453 (1638cm^{-1}).

4. DISCUSSION

The effective discovery of new drugs with minimal side effect and high potentiality was directed through isolation of new bioactive compounds from microbes and testing its efficacy by both invitro and invivo studies. Azurin's cytotoxicity is only for specific cancer cells and does not affect healthy cells and it has fewer toxic side effects. So it will prove a harmless treatment for patients and prevent them from risky anomalies. The second most vital feature of azurin is that, tremendous number of therapeutic substance can link to azurin molecule,because of its ability to acts as a cargo protein[18].

Using morphological, biochemical and molecular tests the azurin harboring strains were identified as *Pseudomonas aeruginosa*. Trypan blue exclusion assay was done to assess the cytotoxicity measurement of ten strains, and the best strain showed 85% of cell death on DLA cell line. This strain was characterized and designated as *Pseudomonas aeruginosa SSj* (acc KU821118) on the basis of 16S rRNA sequencing. The partial gene sequence of azurin was deposited in GenBank with accession number KU821119. It shows 100% similarity to several already existing strains.

Functional groups were studied by FTIR, and compared with azurin extracted *Pseudomonas aeruginosa* MTCC2453 and standard azurin. All these three samples showed peak around 1650 cm^{-1} confirming the amide I band of azurin. The FTIR studies of four different *Pseudomonas aeruginosa* strains were done previously by Sankar *et al*. using *Pseudomonas aeruginosa* MTCC 2453 as a control strain and results showed, azurin has C=O (protein backbone) stretching, which is the unique nature of the amide I band[13]. Other reliable reports regarding the use of standard azurin for functional group studies are not available. Current studies reveal that chemical modification of azurin protein helps to develop an oral formulation for treating and preventing the onset of cancer [19]. The efficacy of the isolate can be further improved by genetic modifications and make it a prospective candidate for medical applications.

5. CONCLUSIONS

The studies on detection of azurin producing strains from environmental sources are infrequent. This study provides evidence for the existence of azurin harboring *Pseudomonas* strains in the Western Ghats of India. A novel strain, *Pseudomonas aeruginosa* SSj, which produced promising levels of active azurin was successfully isolated and characterized in this study.

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