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Molecular Detection of Azurin: A Powerful Anticancer Protein from Native *Pseudomonas* Isolates

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Authors' contributions

This work was carried out in collaboration between both authors. Author DS designed the study and wrote the protocol. Author MCS manage the literature searches, analyses of the study performed the molecular techniques and managed the experimental process. Both authors read and approved the final manuscript.

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ABSTRACT

Aim: The present study was carried out to isolate azurin producing native *Pseudomonas aeruginosa* strains and confirm its presence using molecular techniques.
Study Design: Presence of azurin detection by molecular techniques such as PCR and SDS PAGE.
Place and Duration of the Study: Department of Life Sciences, University of Calicut, Kerala, India, between September 2013 to December 2014.
Methodology: Conventional microbiological techniques were used to isolate several *Pseudomonas aeruginosa* from environmental sources. The presence of azurin gene was confirmed by PCR. The extracted total cellular protein was precipitated by 70% ammonium sulfate followed by dialysis and partially purified azurin fractionated using SDS-PAGE.
Results: Out of 10 samples selected for this study 8 shows the presence of azurin gene using specific oligonucleotide primers in a gradient PCR, amplifying a single 545 bp DNA fragment

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characteristic of azurin gene. The extracted total cellular protein analysed using SDS-Polyacrylamide gel electrophoresis, wide range of bands were present including a band corresponding to 14 KD Molecular weight in all 8 samples selected from PCR.
Conclusion: Presence of azurin gene was confirmed by PCR and partially purified protein fractionated in 15% SDS PAGE. Further study of purification and cytotoxic effect of azurin is required.

Keywords: Azurin; Pseudomonas aeruginosa; Polymerase chain reaction; SDS PAGE.

1. INTRODUCTION

Azurin is a bacterial redox protein produced mainly by *Pseudomonas aeruginosa,* is placed in periplasmic space and it gives the bacteria the ability to escape the host defense system. Azurin is composed of one alpha helix and two beta sheets which create a beta barrel motiff. It acts as an electron shuttle in *Pseudomonas aeruginosa*. It has a molecular weight of 14 kDa and contain one copper atom. The presence of copper ion gives intense blue color, a high reduction potential and a small parallel hyperfine coupling in the electron resonance spectrum. Azurin is the smallest copper proteins and oxidized form is more stable. Azurin is one of the representative bacterial products used in the treatment of tumors [1]. It has anticancer, antiparasitic and anti HIV properties associated with different regions of proteins [2]. Azurin is a multi targeted anticancer agent that acts through three pathways; induction of apoptosis through intracellular stabilization of p53 protein, inhibition of cell cycle progression through intracellular binding *Eph* receptor tyrosine kinase and prevention of angiogenesis through inhibition of VEGFA (Vascular endothelium growth factor A) [3-6]. Azurin appeared to form a complex with p53 and raising its intracellular level. The increased amount of p53 then triggered apoptosis in the cells through enhanced *Bax* formation and release of mitochondrial *Cyt c* in the cytosol. Tumor suppressor p53 is a complex multifunctional protein that acts as a guardian of the genome in preventing cancer growth and maintaining genome stability. The tumor suppressor protein p53 acts primarily as a transcription factor, inducing the expression of genes involved in cell-cycle arrest, senescence and apoptosis in response to cellular stresses [7]. Azurin is the first bacterial protein reported to form a complex with p53 [8,9].

Azurin was proficient in entering human cancer cells but was deficient in entering normal cell. The preferential entry of azurin in cancer cells have been identified to be a domain between amino acids 50 and 77 of azurin termed p28, which can be used as a vehicle to transport cargo proteins [10]. Several microorganisms are used for the development of novel therapies for treating cancer. A main disadvantage of their use has been the undesired infections caused by the bacteria. To overcome this draw back the novel approaches for anticancer therapies include the use of purified products from microbial origin such as proteins, enzymes, antibiotics and other secondary metabolites. *Pseudomonas aeruginosa* is known to secrete the protein azurin as a weapon against invaders as cancers, parasites and viruses. The production of such weapons by pathogenic bacteria could provide important insights into how a pathogen responds in the post-colonization state to impede other intruders for its own survival. Moreover, these molecules might find use in the pharmaceutical industry as next generation therapeutics [11]. It is a small globular cuprous protein, endowed with redox activity, involved in bacterial denitrification process [12].

2. MATERIALS AND METHODS

2.1 Sample Collection

Soil and water samples were collected from different locations of Calicut district, a part of Western Ghats of India. Samples were serially diluted and plated on Nutrient agar.

2.2 Culture Media

Nutrient agar and Kings B agar were used for this study. The samples were grown on nutrient agar plates for 24 hours at 37℃. The greenish mucoid colonies were subjected to Gram staining and then specifically selected and streaked on Kings B medium [13], which is used as a general medium for the selective isolation and pigment production of *Pseudomonas aeruginosa*. Further identification of the isolates were carried out on the basis of their biochemical characteristics [14,15].

2.3 Genomic DNA Extraction

Total genomic DNA was isolated according to modified Unal et al. method [16]. A single pure *Pseudomonas* colony was inoculated in 4 ml nutrient broth and incubated at 37℃ for 24 hours. Cells were obtained by centrifuging 4 ml of overnight broth culture at 5000 rpm for 5 min. Cells were resuspended in 10 µl lysozyme (50 mg/ml distilled water) and incubated at 37℃. After 10 min 10 µl proteinase K (20 mg/ml) solution and 500 µl 0.1 M Tris HCl (pH 7.5) buffer were added. Cell suspension was then incubated at 37℃ for 15 min and then placed in boiling water bath for 5 min. It was then centrifuged at 5000 rpm for 5 min and the supernatant was removed to another microcentrifuge tube. An equal volume of isopropanol was added, mixed well and centrifuged at 12000 rpm for 12 min for pelleting DNA. The pellet was then desalted with 200 µl of 70% ice cold ethanol and centrifuged at 3000 rpm for 3 min and tubes were inverted to dry. The pellet was then resuspended in 100 µl TE buffer (pH 8.0) (10 mM Tris Hcl (pH 7.5) and 1 mM EDTA (pH 8.0) and stored at -20℃ for further analysis.

2.4 PCR Reaction

PCR is an in vitro method for enzymatic amplification of specific DNA sequences. It uses two specific oligo nucleotide primers [17,18]. Azurin gene was amplified from the genome of isolated bacterial strains by using previously reported primers: AZU-F(5'-GCCCAAGCTTACCTAGGAGGCTGCTCCATGC TA-3') and AZU-R (5'TGAGCCCCTGTAGGCGCCCATGAAAAAGC C CGGC-3') [9] to amplify 545bp. The PCR was performed in 50 µl reaction volume containing sterile Milli Q Water (38.5 µl), 2.5 Mm DNTPs (3 µl), 3 U/ml Taq polymerase (5 µl), buffer with 15 Mm $MgCl_2$ (5 µl) and 1µl of each primer and DNA template. DNA amplification started with denaturing the template DNA at 94℃ for 5 min followed by 35 cycle of denaturation at 94℃ for 30 s, annealing at 60℃ for 45 s, extension at 72℃ for 3 min and a final extension step at 72℃ for 7 min. Amplification was carried out using different annealing temperatures (50℃ to 62℃) and time (30 s to 60 s) using thermal cycler (Biorad gradient T100^{TM}). The PCR product was analysed by agarose gel electrophoresis [19].

2.5 Agarose Gel Electrophoresis

Agarose dissolved in 1x Tris Borate EDTA buffer (pH 8.0) composed per litre (Tris base,108 g; Boric acid, 55 g; and EDTA 9.3 g) was melted to form agarose solution and while solution was cooling, Ethidium bromide (1 mg/1 ml) was added such that the final concentrate would be 0.5 µg/ml of the solution. Agarose gel of appropriate size was formed by pouring the solution into a gel casting tray and cooling it. Amplified products of reaction were mixed together and 8 µl of the amplified products were mixed with 2µl of loading dye (30% glycerol; 0.25% bromophenol blue and xylene cyanol) and added to the wells. Amplicons were electrophoresed horizontally in 1x TBE buffer (prepared freshly from 10x TBE) at 90 volts for 120 minutes. The gel was then visualized under UV in a gel documentation system (Mega Bio print 1000/26MX) and DNA bands were analyzed visually by comparing with the DNA ladder standard (Genei step up 100 bp) which was run in the last well of the gel [20].

2.6 Extraction of Cellular Protein

Azurin producing *Pseudomonas* strains were inoculated separately in medium containing per litre peptone:10 g, K_2HPO_4:0.75 g, $MgSO_4$:0.75 g and $CUSO_4$:0.005 g. After 21 hours incubated cells were harvested by centrifugation at 12000 rpm for 15 min by using cold centrifuge (REMI C-24 PLUS). Cell pellets were collected and suspended in the appropriate volume of 0.02 M Potassium phosphate buffer at pH 7 and kept in the basket containing ice cubes for sonication. Cells were sonicated for 45 s at 100 W using Ultrasonicator (Sonics Vibra cell). After sonication, the suspension was stirred vigorously and centrifuged at 10000rpm for 15 min, which removes cell wall debris. The crude supernatant was stored at 4℃ [21,22].

2.6.1 Ammonium sulphate precipitation of proteins

The crude supernatant was saturated to 70% by slowly adding ammonium sulphate salt at 4℃ for precipitation, kept it for overnight [21,22]. After precipitation the solution was centrifuged at 20000 rpm for 25 min, and the pale supernatant was discarded. The precipitate was collected and resuspended in 0.02 M Pottasium phosphate buffer pH 7.

2.6.2 Dialysis

Azurin containing precipitate suspended in 0.02M potassium buffer pH 7 was dialysed by standard dialysis bag (HIMEDIA LA 395) having 12 KDa MW cut off at 4℃ for 20 hours on the same

buffer for overnight with continuous gentle stirring. Dialysis was done until the solution attains its buffer pH. The solution was kept at 4℃ after dialysis for further purification [21,22].

2.7 Protein Profile by SDS Page

Five ml of 15% resolving gel containing 1.4 ml distilled water, 30% Acryl amide, 1.5M Tris (pH 8.8), 10% SDS, 10% APS and 0.002 µl TEMED for polymeraization was casted in the glass slab without any bubble and kept it for 10-15 min. After polymeraization of the resolving gel, 3 ml 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% APS, and 0.001 ml TEMED. After casting the gel, partially purified protein sample were loaded with standard protein molecular weight marker (SRL BIOLIT^{TM}Low Range 3-40 KDa) at different lanes for profiling the protein.

Glass slab gel was kept in the electrophoresis tank with tank buffer. This set up was connected with power pack initially in 80 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 coomassie blue until it reveals the bands. The bands were observed under white light transilluminator [23,24].

3. RESULTS

3.1 Isolation and Cultural Characteristics of *Pseudomonas* Isolates

The present study revealed 10 native strains of *Pseudomonas were* isolated from environmental sources. The strains were identified using conventional microbiological and biochemical techniques.

3.2 Genomic DNA Extraction

The full amount of DNA obtained in this study (Fig. 1) refers to the successful method of DNA extracted from 10 *Pseudomonas* isolates which depend on using the protocol of modified Unal et al. method.

3.3 Results of PCR Reactions

PCR was used to amplify the azurin gene to detect azurin producing *Pseudomonas aeruginosa* samples. Altenative conditions for PCR amplification were employed using gradient PCR which include various annealing temperatures and time and 60℃ at 45 s was found to be more specific for annealing. Standard amplification with the primer pair resulted in a 545 bp band as seen in Ethidium Bromide stained agarose gel electrophoresis (Fig. 2). The azurin gene presence detected in 8 isolates out of 10 samples taken for this study.

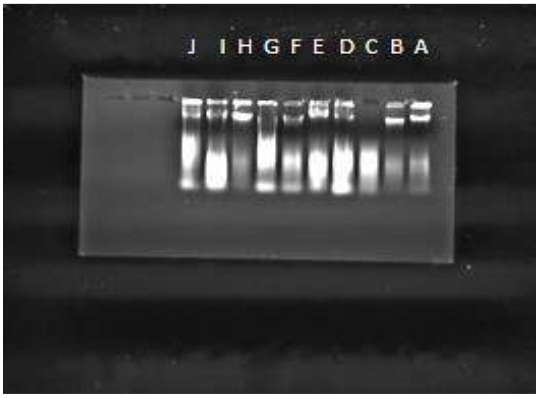

Fig. 1. Total DNA extraction from *Pseudomonas* isolates loaded on 1% agarose gel (run at 100v for 90 minutes)

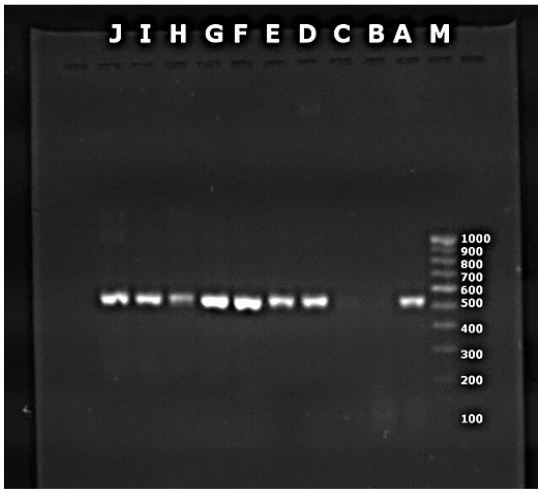

Fig. 2. Agarose gel electrophoresis of PCR product (545 bp) loaded on 2% agarose gel and run at 100 V for 120 minutes. (M=100 bp DNA ladder and A-J were 10 *Pseudomonas* isolates)

3.4 Total Cellular Protein Analysis

Total cellular partially purified protein extracted from 8 azurin producing isolates were fractionated by SDS PAGE produce patterns with wide range of discrete bands with molecular masses ranged from 6-100K Da. All the lines

appeared to be homologous except in few bands. A thick band approximately corresponding to 14 KD Molecular weight was present in all 8 azurin producing isolates.

4. DISCUSSION

Secondary metabolites produced by microorganisms play a vital role in developing new chemotherapeutics. Current chemotherapeutic drugs designed for the treatment of cancer are often toxic and prone to resistance development by the cancer cells, leading to multi-drug resistant tumors. Azurin has the ability to interfere in tumor growth at multiple points of attack to prevent resistance development, such protein weapons demonstrate a broad range of activity including anti-viral and anti-parasitic activity. It also prevents induction of precancerous lesion formation triggered by a potent carcinogen [25]. Zaborina et al. [1] reported azurin cytotoxic and apoptosis-inducing activities towards murine macrophage cell line J774. Azurin exhibit cytotoxic activity against liquid and solid tumors [26]. The studies on detection of azurin producing strains from environmental sources are infrequent. Azurin is too expensive to obtain readily [27].

Out of 10 *Pseudomonas aeruginosa* taken for this study B and C are considered as azurin negative strains. Some *Pseudomonas aeruginosa* strains with mutations or deletions in the azurin gene will not produce azurin. Some researchers reported that different strains of *Pseudomonas aeruginosa* have varying ability for azurin production due to their physiological variation [28] Yehiya et al. [29] reported that out of 95 *Pseudomonas aeruginosa* samples taken for their study only 10 samples were found to be azurin producing strains. Azurin detection is mainly carried out by genomic test. SDS PAGE and Western blotting are another methods [30]. N terminal sequence determination [31] is yet another method. These techniques demand high degree of purity of azurin but genomic tests are very efficient and rapid, so PCR is considered as a primary detection technique for large number of samples. Azurin gene in most bacteria are chromosomally encoded. Mostly PCR is efficient to determine the presence or absence of azurin.

In this study gradient PCR was used which help to provide different temperatures and time combinations for annealing simultaneously. We tried 50℃ to 62° C with different time (30 s to 60 s) combinations and 60℃ for 45 s was found to

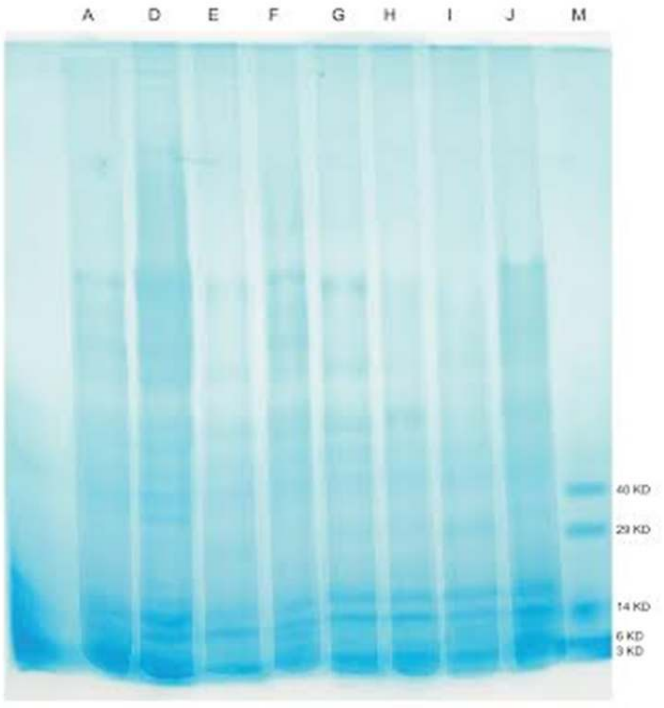

Fig. 3. Protein profile of *Pseudomonas* isolates. Lane M contains Low Range molecular weight marker. Lane A-J were crude protein extracts from 8 *Pseudomonas* isolates

be more specific for annealing, it form a single band specific to 545 bp on agarose gel electrophoresis. Azurin is a novel molecule for cancer treatment through nanotechnology because of azurin is used combination with other cytotoxic agents in cancer diagnosis and treatment [32]. So detection of high yield azurin producing microbial strains from environmental sources helps to design drugs for cancer based therapies economically.

5. CONCLUSION

In this study *Pseudomonas* strains were isolated from Calicut district, a part of Western ghats, India. Here researchers using primers of azurin encoding gene *azu* and detect its presence in all isolated samples by extracting whole genome with gradient PCR, and the presence of azurin was detected in 8 out of 10 strains. Extraction and partial purification of azurin was done and protein profile analysed by SDS PAGE.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Zaborina O, et al. Secreted product of a nonmucoid *pseudomonas aeruginosa* strain induce two modes of macrophage killing: External-ATP-dependent, P2Z-receptor-mediated necrosis and ATP-independent, caspase-mediated apoptosis. Microbiology. 2000;146(pt 10):2521-2530.
2. Fialho AM, Das Gupta TK, Chakrabarty AM. Promiscuous drugs from pathogenic bacteria in the post-antibiotics era. In: Sleator R, Hill C, eds. Patho-Biotechnology. Austin. TX: Landes Bioscience. 2008;145-162.
3. Apiyo D, Wittung-Stafshede P. Unique complex between bacterial azurin and tumor suppressor protein p53. Biochemical and Biophysical Research Communication. 2005;332:965-968.
4. Arsenio MF, Tapas KD, Ananda MC. Designing promiscuous drugs? Look at what nature made. Lett. Drug Design Discov. 2007b;4(1):40-43.
5. Choudhari A. Cupridoxin-cancer inter relationship: Azurin binding with EphB2, interference in EphB2 tyrosine phosphorylation, and inhibition of cancer growth. Biochemistry. 2007;46:1799-1810.
6. Pasquale EB Eph receptors and ephrins in cancer: Bidirectional signaling and beyond. Nature Reviews. Cancer. 2010;10:165-180.
7. Vogelstein B, Lane DP, Levine AJ. Surfing the P53 network. Nature. 2000;408:307-310.
8. Mervat SM, Said AF, Howayada MM. Azurin as antitumor protein and its effect on the cancer cell lines. Journal of Biological Sciences. 2010;2:396-401.
9. Yamada TY, Hiraoka M, Ikehata K, Kimbara BS, Avener TK, Das Gupta, Chakrabarty AM. Apoptosis or growth arrest: Modulation of tumor suppressor p53's specificity by bacterial redox protein azurin. Proc. Natl. Acad. Sci. USA. 2004; 101:4770-4775.
10. Mehta RR, Hawthorne M, Peng X. A 28-amino-acid peptide fragment of the cupredoxin azurin prevents carcinogen-induced mouse mammary lesions. Cancer Prev Res. 2010;3:1351–1360.
11. Yamada T, Fialho AM, Punj V, Bratescu L, Das Gupta TK, Chakrabarty AM. Internalization of bacterial redox protein azurin in mammalian cells: Entry domain and specificity. Cell Microbiol. 2005;7: 1418-31.
12. Edward IS, Michael JB, Michael DL. Electronic structures of active sites in copper proteins: Contributions to reactivity. Chem. Rev. 1992;92(4):521-542.
13. King EO, Ward MK, Raney DE. Two simple media for the demonstration of pyocyanine and fluorescein. J. Lab. Clin. Med. 1954;44:301-307.
14. Cappuccino J, Sherman Microbiology a laboratory manual for isolation, cultivation

and culture characterization of microorganisms. Antimicrob. Agents Chemother. 2001;44:938-942.
15. Koneman EW, Allen SD, Janda WM, Schreckenberger PC, winn WC. The Enterobacteriaceae, in Koneman EW, Allen SD, Janda WM, Schreckenberger PC, winn WC. eds. color Atlas and textbook of Diagnostic Microbiology (5ed). Lippincott, philadolphia. 1997;171-252.
16. Unal S. Detection of methicillin-resistant staphylococci by using the polymerase chain reaction. Journal of Clinical Microbiology. 1992;30:1685-1691.
17. Yamada T, Goto M, Punj V, Zaborina O, Chen ML, Kimbara K, Majumdar D, Cunningham E, Das Gupta TK, Chakrabarty AM. Bacterial redox protein azurin, tumor suppressor protein p53, and regression of cancer. Proc. Natl. Acad. Sci. USA. 2002;99:14098-14103.
18. Mullis K. The unusual origin of the polymerase chain reaction. Scientific American. 1990;262:56–61.
19. Arvidsson RH, Nordling M, Lundberg LG. The azurin gene from *Pseudomonas aeruginosa*. Cloning and characterization. Eur. J. Biochem. 1989;179:195–200.
20. Sambrook J, Russell DW. Molecular cloning: A laboratory manual, Edn.3rd. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y; 2001.
21. Parr SR, Barber D, Greenwood C, Phillips BW, Melling JA. Purification procedure for the soluble cytochrome oxidase and some other respiratory proteins from *Pseudomonas aeruginosa*. Biochemical Journal. 1976;157:423-430.
22. Sutherland IW. The production of azurin and similar proteinsm. Archly fiir Mikrobiologie. 1966;54:350-357.
23. Weber K, Osborn M. The reliability of molecular weight determinations by dodecyl sulfate polyacrylamide gel electrophoresis. The Journal of Biological Chemistry. 1969;244:4406-4412.
24. Laemmli UK. Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature. 1970;227:680-685.
25. Tangri S, Ishioka GY, Huang X, Sidney J, Southwood S, Fikes J, Sette A. Structural features of peptide an a logs of human histocompatibility leukocyte antigen class I epitopes that are more potent and immunogenic than wild type peptide. J. Exp. Med. 2001;194(6):833-846.
26. Kwan JM, Fialho AM, Kundu M, Thomas J, Hong CS, Das Gupta TK, et al. Bacterial proteins as potential drugs in the treatment of leukemia. Leuk Res. 2009;33:1392–1399.
27. Ye Z1, Peng H, Fang Y,Feng J, Yang DS, The construction of the eukaryotic expression plasmid pcDNA3.1/azurin and the increased apoptosis of U2OS cells transfected with it. Cellular & Molecular Biology Letters. 2007;12:407-421.
28. Ramachandran S, Sarkar S, Mazumadar A, Mandal M. Azurin synthesis from *Pseudomonas Aeruginosa* MTCC 2453, properties, induction of reactive oxygen species, and p53 stimulated apoptosis in breast carcinoma cells. J Cancer Sci Ther. 2011;3:104-111.
29. Yehia A Osman, Douaa R El-Deep, Sadia A Younis. Azurin: A powerful anticancer from a local *Pseudomonas aeruginosa* Isolate. J Am Sci. 2013;9(12):755-764.
30. Vasu punj, Rachna Sharma, Oiga Zaborin, Chakrabarty AM. Energy-Generating enzymes of bukholderia cepacia and their interactions with macrophages. J Bacteriol. 2003;185:3167-3178.
31. Ambler RP, Brown LH. The amino acid sequence of *Pseudomonas* fluorescence azurin. Biochem. J. 1967;104(3):784-825.
32. Arsenio MF, Fred JS, Tapas KD, Ananda MC. Beyond host-pathogen interactions: Microbial defense strategy in the host environment. Curr. Opin. Biotechnol. 2007; 18(3):279-286.

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