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Investigating the Bacterially Derived Anticancer Compound Azurin Isolated from Pseudomonas aeruginosa

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ABSTRACT

Pseudomonas aeruginosa **is a bacterium of considerable medical relevance as it is known for its multidrug resistance, intrinsic antibiotic resistance, and for causing nosocomial infections .Nonetheless, the proteins, and derivatives of which it produces, show great promises for their antitumor activity. The aim of this study is to isolate, extract, and identify the anticancer agent gene Azurin from the bacteria *Pseudomonas aeruginosa* for future downstream processes. *Pseudomonas aeruginosa* bacterial isolates were collected from inpatient and outpatient clinics at Mansoura University Hospitals, and 95 gram-negative bacterial isolates were cultured and incubated at 37 degrees Celsius for 24-48 hours, then 10 strains were selected for their ability to produce azurin then grown on azurin broth media. A gram staining experiment was then performed on those isolates, as well as a variety of different biochemical characterization tests. Molecular characterization was then performed on the *Pseudomonas aeruginosa* isolates using SDS-PAGE, PCR was performed using azurinspecific primers, and gel electrophoresis was performed to visualize the results. Lastly, PCR was performed again using different azurin-specific primers to confirm the presence of the azurin coding gene. The biochemical tests performed on *Pseudomonas aeruginosa* revealed negative results for the TSI, and gas production tests, but showed positive results for the urease, and citrate tests. The results of the PCR profiles after the confirmatory test showed a single major band at 545bp in all the tested isolates, and standard ATCC 15442 using the azurin-specific primers which proved the presences of the azurin-coding gene in all *Pseudomonas aeruginosa* local strains, as well as the standard strain. *Pseudomonas aeruginosa* local strains, and ATCC standard strains were identified for their ability to produce the anticancer agent gene azurin.**

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INTRODUCTION

Pseudomonas aeruginosa is an aerobic, gram-negative bacteria, commonly encapsulated, nonspore forming, and rod shaped **(Wilson & Pandey, 2023).** It is commonly known for causing nosocomial infections which are often very hard to treat due to the intrinsic resistance of the species, as well as its resistance to several antimicrobial agents **(Strateva & Yordanov, 2019).**

In the field of anticancer agents' research, bacterial proteins, as well as their derivatives have shown great potential due to their diverse modes of action, and motility. Therefore, many bacterial proteins are under studies lately to uncover their anticancer potential. Pseudomonas aeruginosa is a widely used bacteria in cancer therapies due to its great anti-tumor efficacy, and specificity **(Pang, *et al*., 2022).** This has prompted researchers to further increase their understanding of the bacteria, and thereby, study the proteins it produces.

Azurin bacteriocin is a small water-soluble protein produced by *Pseudomonas aeruginosa,* and it is known for its anti-tumor ability as it can easily penetrate the breast cancer cells, and induce apoptosis without causing harm to other surrounding normal cells **(Yamada, *et al.*, 2004, Fialho, *et al.*, 2016),** cell cycle arrest **(Chaudhari, *et al.,* 2007)** and inhibits angiogenesis **(Mehta, *et al*., 2011).** Azurin, as well as other microbial-related peptides or proteins offer promising options as therapeutic agents against different types of human cancers. This is greatly shown not only by their high affinity to the target cancer, but by their low toxicity against other host cells. Azurin has shown great specificity to enter cancer cells, interferes with multiple cellular pathways and prevents growth of cancer cell.

The aim of this study is to isolate, and extract and identify the anti-cancer agent gene Azurin from the bacteria *Pseudomonas aeruginosa* for future down stream processes.

MATERIALS AND METHODS

ISOLATION AND PURIFICATION:

GRAM-STAINING OF PSEUDOMONAS AERUGINOSA

95-gram negative bacterial isolates of *Pseudomonas aeruginosa* were taken from inpatient, and outpatient clinics at Mansoura University Hospitals. They were collected from different infected sites including wound, urine, sputum, pus, stool and blood stream

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infections. They were then cultured on plates of appropriate media containing 5gm yeast extract, 10gm tryptone, 10gm sodium chloride, and one liter of distilled water (the pH was adjusted to 7). It was incubated at 37 degrees Celsius for 24-48 hours. Following that, 10 of the isolates were screened for the ability to produce azurin after growing on azurin broth media made from 5gms Sodium citrate, 4gm yeast extract, 5gm Sodium nitrate, 1gm Potassium dihydrogen phosphate, 0.5gm Magnesium Sulphate, 4gm Copper sulphate, 3gm Ferric chloride, and one liter of distilled water. The resulting colonies were taken for preparation of bacterial gram testing. This was performed by taking an inoculum of the colonies using a sterile loop, and emulsifying it in a drop of saline solution in a circular manner in the middle third of a clean glass slide and left to air dry. A drop of crystal violet dye was then added to the glass slide and after 30 seconds it was washed off. Lastly, 95% alcohol was used to wash off any violet colour and rinsed again in distilled water then the results were observed using a microscope **(Collee, *et al.,* 1996)**.

BIOCHEMICAL CHARACTERIZATION OF PSEUDOMONAS AERUGINOSA USING OXIDASE TEST, TRIPLE SUGAR IRON, UREASE, AND H2S TEST:

Different biochemical tests were then used on the *Pseudomonas aeruginosa* isolates in order to characterize them. Firstly, the Oxidase test was performed by exposing the bacterial colonies to a filter paper that had few drops of freshly prepared 1% oxidase reagent **(Collee, *et al.,* 1996).** Secondly, the TSI test was performed. The TSI media was prepared by adding 10gm Sucrose, 3gm

Beef extract, 3gm yeast extract, 15gm peptone, 5gm Protease peptone, 10gm Lactose, 1gm Glucose, 0.2gm Ferrous sulphate, 5gm Sodium chloride, 0.3gmSodium thiosulphate, 12gm Phenol red (with pH adjusted to 7). The isolated bacterial colonies were inoculated slant into the tubes with TSI agar for 18-24 hours at 37 degrees Celsius to detect the organism's ability to ferment glucose, lactose, and sucrose **(Koneman, *et al.,* 1997)**. Thirdly, the citrate utilization test was performed by taking an inoculum of the organism and placing it slant onto the surface of the agar medium which consisted of 0.2gm Magnesium sulphate, 0.2gm Ammonium dihydrogen phosphate, 0.8gm Sodium ammonium phosphate, 2gm Sodium citrate tribasic, 5gm Sodium chloride, 0.08gm Bromothymol blue, 15gm agar, and one liter distilled water. It was incubated for 24-48 hours at 38 degrees Celsius**.** Fourthly, the urease Test was

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performed by adding an inoculum of the organism onto the urea agar media which consisted of 30gm peptone, 5gm HCL, 1gm Potassium dihydrogen phosphate, 2gm phenol red (0.25%), 20gm agar, 10ml glucose (10%), 100ml urea (20%), and one liter of distilled water. They were incubated for 18 hours at 37 degrees Celsius

AERUGINOSA MOLECULAR CHARACTERIZATION

SDS-PAGE SHOWING PROTEIN BANDING PATTERNS OF PSEUDOMONAS AERUGINOSA (PREPARATION OF BACTERIAL PELLET)

The bacterial isolates were grown on 15ml of liquid nutrient media, with continuous shaking at 250rpm at 37 degrees Celsius. They were then centrifuged at 13000rmp for 5 minutes, and washed with distilled water to collect the pellets. 50µl of the cell suspension was mixed with an equal volume of treatment buffer containing 2-βmerceptoethanol (as antioxidant), and SDS (as denaturing agent). They were then boiled in a water bath for 90 seconds to solubilise, and denature the cellular proteins. Then immediately transferred to an ice-water bath. Finally, 2-3µl of the bromophenol blue tracking dye was added and kept on the ice until it is ready to be loaded onto the gel.

PREPARATION OF SDS POLYACRYLAMIDE GEL

The stacking gel was prepared first by adding monomer solution, SDS, stacking buffer, Ammonium persulphate (APS), and N-tetramethyl ethylenediamine (TEMED). Meanwhile, the separating gel consisted of monomer solution, SDS, separating buffer, APS, and TEMED. The APS, and TEMED were added last to avoid it solidifying too fast. The separating gel was added first into the closed plates, and a layer of isopropanol was added first before adding the stacking gel. The comb was introduced into the stacking gel, and the glass plates, and it was left to fully solidify for 3 hours at room temperature.

SEPARATING CELLULAR PROTEIN

The gel was transferred to the electrophoresis chamber after its complete polymerization and the comb was taken out. 30-40 µl of the samples were then loaded into each of the wells and the apparatus was set at 100 volts for 30 minutes. It was initially set at

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50 volutes until the protein samples had emerged from the stacking gel. Following that, the voltage was raised to 100 volts until the tracking dye had reached the bottom of the gel.

STAINING OF THE CELLULAR PROTEIN

The glass plates were removed, and the gel was emersed in Coomassie brilliant blue (R-250) stain solution for 6 hours at 37 degrees Celsius. The gel was then removed, and washed for two hours using de-staining solution I (consisting of 100ml Glacial acetic acid, 500ml methanol, and 1L distilled water), then for one hour using solution II (consisting of 100ml Glacial acetic acid, 700ml methanol, and 1L distilled water). The protein bands were examined photographically for analysis.

DETECTION OF AZURING-ENCODING GENE (TOTAL PLASMID DNA EXTRACTION)

The genes encoding for the production of azurin pigments are carried on plasmids, therefore, total plasmid DNA extraction was performed **(Sambrook, *et al*., 1989)**. The isolates were cultured on 15ml of liquid nutrient media, with continuous shaking at 250rpm at 37 degrees Celsius. They were then centrifuged at 13000rmp for 5 minutes, and washed with distilled water to collect the pellets. The pellets were left to freeze overnight at 4 degrees Celsius, then taken out in room temperature to thaw before they were suspended in 200µl of solution "A" which consisted of 10mM Tris-HCl (pH 8), 10mM EDTA (pH 8), 50mM NaCl, and (20% (w/v) sucrose, and 2mg/ml freshly prepared lysozyme. They were incubated for 45min at 37 degrees Celsius to lyses the cells. Additionally, 400µl of solution "B" which consisted of 0.2 M NaOH, and (1%) (w/v) SDS) was added to the lysate. It was shaken lightly while kept on ice for 5 minutes. Following that, 300µl of solution "C" containing potassium acetate (pH 5.5), and Glacial acetic acid was mixed well with the lysate, and kept on ice for an additional 5 minutes. it was then centrifuged for 5 minutes at 13000rpm. An equal volume of phenol-chloroform was mixed by inverting the tube repeatedly for several minutes. It was then centrifuged once more for 5 minutes at 10000rpm until an interphase layer between the cellular protein, and the rest could be observed. The upper aqueous phase was then transferred to a clean Eppendorf tube ang phenol was added two-three times until there was no protein interphase layer present. The remaining supernatant was collected and added in a new sterile Eppendorf with 0.6 volume of isopropanol for two hours.

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Lastly, the observed pellet was then centrifuged for 15 minutes at 13000rpm, and collected in 70% cold ethanol to wash it off. It was then washed again using distilled water and dried.

POLYMERASE CHAIN REACTION (PCR)

25µl reaction volume was prepared in an Eppendorf containing 1X buffer (containing 10 mM TrisHCl (pH 8.3), 50 Mm KCl, 2 mM $MgCl_2$, 250µM each of dGTP, dATP, dCTP, and dTTP, 0.5 units of Taq DNA polymerase, 100pmol of each primer AzuF, and AzuR, and the DNA template **(Mullis, *et al*., 1986).** The amplification of the DNA was carried out in the thermal cycles with an initial DNA denaturation step for 5 min at 94ºC followed by 35 cycles. Each cycle consisted of: denaturation for 1 min at 94 degrees Celsius, annealing for 45 sec at 50ºC and extension at 72 degrees Celsius for 3 min, and a final extension step for 7 min at 72 degrees Celsius.

PURIFICATION OF PCR PRODUCT ON AGAROSE GEL

The agarose gel was prepared using 0.4gm agarose, and 50ml buffer (containing 1X TBE: 0.18M Tris-borate and 0.002 M EDTA). It was added to the microwave until agarose was completely dissolved in the TBE buffer. After the gel had cooled, 3-4µl of ethidium bromide was added t the gel, then it was poured into the mold, and the comb was added. After the gel had solidified, the comb was taken out, and the TBE buffer was added to fill the chamber until it covers the gel. The sample (containing bromophenol blue, xylene cyanole, and glycerol) was loaded into the gel using a micropipette. The gel electrophoresis was run at 60 v/cm. The results were examined using UV.

PCR CONFIRMATORY TEST FOR THE DETECTION OF THE GENE ENCODES FOR AZURIN

The PCR experiment was repeated, however, primers; P2 (which is specific for promoter 2 region), no.3 (bases 504-484), azu-R (bases 952-918) were used respectively. To further confirm the presence of the gene encodes for azurin in the three selected local *P. aeuroginosa* isolates.

RESULTS

ISOLATION AND PURIFICATION:

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Ninety-five Gram Negative *Pseudomonas aeruginosa* isolates **(Figure 1)**, taken from inpatient, and outpatient clinics at Mansoura University Hospitals. They were collected from different infected sites: wound, urine, sputum, pus, stool and blood stream infections. Ten of them were screened for production of azurin in comparison to the standard *Pseudomonas aeruginosa* strain; ATCC 15442. All isolates belonged to genus *Pseudomonas* with no major distinction among all of them, which was confirmed using both gram staining **(Figure 1)**, and a variety of biochemical tests. **(Table 1).** All stained negatively with Gram's stain and at the biochemical level they were negative with triple sugar iron and hydrogen sulfide production tests and positive with oxidase, citrate utilizing and urease tests **(Table 1, Fig.2 A, and B).** Triple sugar iron (TSI) contains three sugars: glucose (0.1%), lactose (1.0%) and sucrose (1.0%), when any of the three sugars is fermented, an acid is produced and sometimes gas may be trapped to the agar causing pushing up, the turning of phenol red (indicator) into yellow is an indication of acid production by the respective sugar. Generally, bacteria ferments sucrose or lactose once the glucose has been consumed. The data illustrates that all *Pseudomonas aeruginosa* negative results in this test. While all isolates were citrate test positive because it able to use citrate as the sole source of carbon and energy, the usual color change from green (neutral) to blue (alkaline). Urea hydrolysis to ammonia by *Pseudomonas aeruginosa* will overcome the indicator (phenol red 0.25%) in the medium and change it from yellow to pink **(Table 1)**.

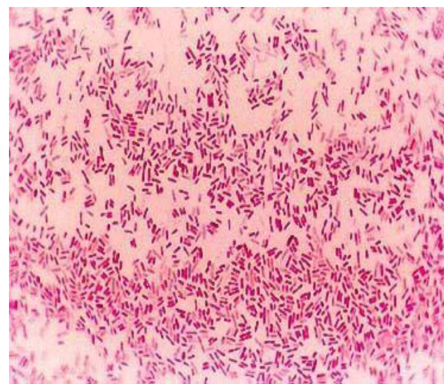

Figure 1: *Pseudomonas aeruginosa* after Gram's stain; red rods.

Table (1): Biochemical characterization of *Pseudomonas aeruginosa* local isolates

	Number	Triple sugar iron			

Genus of bacterial isolates		Slant	Butt		Urease test	Citrate test	Oxidase test
		Sucrose,lactose	Dextrose	Gas			
Pseudomonas aeruginosa	95	-	-	-	+	+	+

Fig (2 A): *Pseudomonas aeruginosa* biochemical reactions: Urease test (+, pink), Citrate test (+, blue) and TSI (-) and production (-), agar slants.

Fig (2 B): Typical biochemical tests for identification of *P. aeruginosa*; from left to right: Urease, Citrate and TSI, control gas

From all the isolates inoculated on azurin broth differential media only ten isolates (assigned numbers: 5-8-9-12-16-24-39-43-I-II) which were able to produce azurin (blue-green), while the standard strain (ATCC 15442, flask no.57) was negative **(Figure 3)**.

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Figure (3): Selected number of *Pseudomonas aeruginosa* local isolates (no. I, 5, 9, II) produced blue green color in azurin broth; while the standard strain ATCC 15442 (57) produced no azurin; creamy color.

MOLECULAR CHARACTERIZATION

SDS-PAGE SHOWING PROTEIN BANDING PATTERNS OF PSEUDOMONAS AERUGINOSA (PREPARATION OF BACTERIAL PELLET)

To exemplify the identity of the local isolates, the total cellular proteins were fractionated by SDS-PAGE. All 11 isolates showed bands, and the molecular sizes of their bands ranged from 10-260 kDa. All the lanes appear to be similar except few bands; i.e. the high molecular weight Protein bands above 260 kDa are absent only in lane 9; isolates I. Combining the whole cellular protein patterns of the 11 isolates a dendrogram was constructed using the simple matching rules of the numerical analysis to differentiate between these closely related bacterial isolates.

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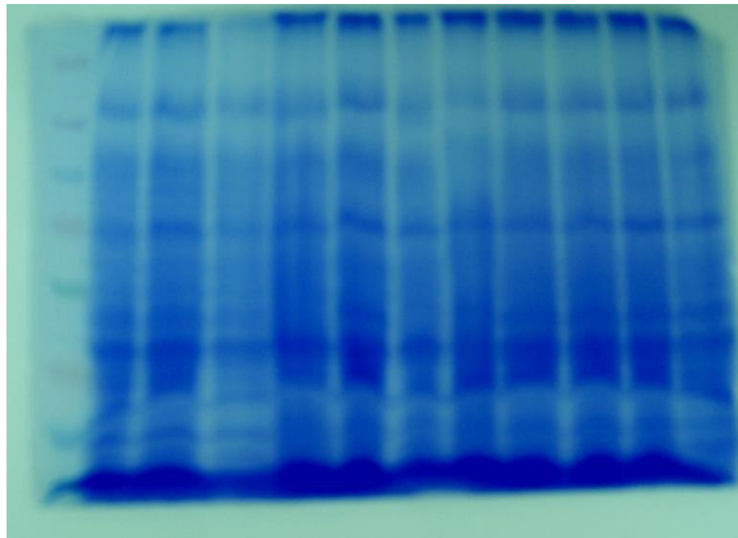

M 56 **39**

I 12 24 9
43

5 II 8 16

260

135

95

72

52

42

34

26

17 KDa

Figure (4): SDS–PAGE protein profiles of *Pseudomonas aerouginosa*. Lane 1: isolates 43, lane 2: isolates 16, lane 3: isolates 8, lane 4: isolates II, lane 5: isolates 5, lane 6: isolates 9, lane 7: isolates 24, lane 8: isolates 12, lane 9: isolates I, lane 10: isolates 39, lane 11: standard isolates 56 and lane M: protein molecular weight marker.

DETECTION OF AZURING-ENCODING GENE (TOTAL PLASMID DNA EXTRACTION)

Purified DNA of bacterial colonies was amplified used PCR technique, with azurinspecific primers; AzuF, and AzuR to detect the presence or absence of gene encode for azurin production. The gene encoding for azurin was detected in 10 selected local isolates of *P. aeuroginosa* and the ATCC standard strain, 43,16, 8, II, 5, 9, 24, 12, I, 39 and 56, respectively. The PCR profiles produced a single major band (545bp) in all tested isolates,

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including the standard one (ATCC 15442), with the specific forward and reverse azurin specific primers. This

diagnostic DNA fragment suggested the presence of the azurin-encoding gene in all local *P. aerouginosa* and the standard strain. **(Figure 4)**.

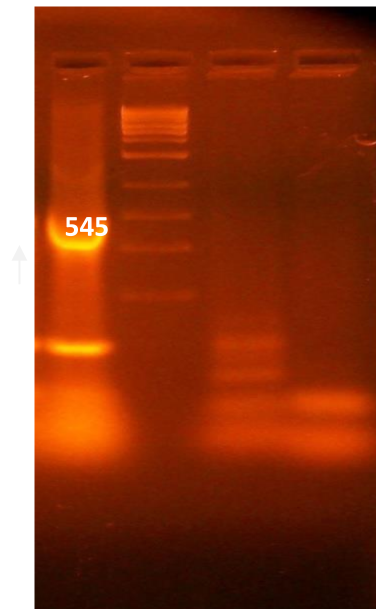

Figure 5: Local isolate no.9 (lane 9 37 degrees Celsius) showing a band at 545 bp.

PCR CONFIRMATORY TEST FOR THE DETECTION OF THE GENE ENCODES FOR AZURIN

To confirm beyond doubts the presence of the gene encodes for azurin, three different primers were used: P2 (specific for promoter 2 region), no.3 (bases 504-484) and azu-R (bases 952-918). The primer pair P2 and azu-R produced ~632 bp characteristic of azurin

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encoding gene in the three local isolates of *P. aeuroginosa*: 12*, 9* and 43*, while, primer pair no.3 and azu-R produced a characteristic ~ 450bp DNA fragment in the three local isolates (9, 12 and 43) and the ATCC standard strain ; 56 **(Figure 6).** The 632 bp DNA fragment represents the full length of the azurin encoding gene from the promoter P2 to the termination codon.

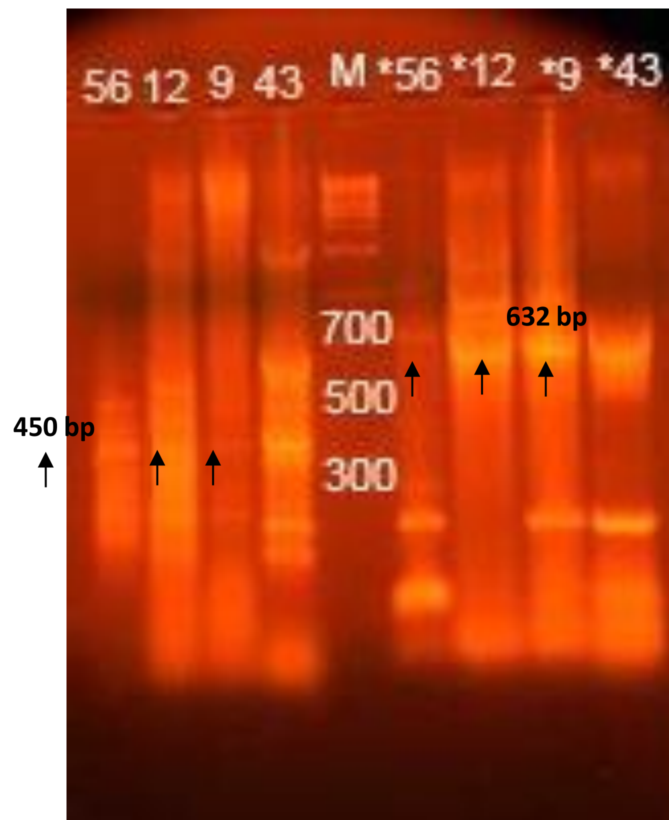

Figure (6): Two DNA bands at 444, and 632 bp for the four *P. aeuroginosa*: 56, 12, 9 and 43 and 56*, 12*, 9* and 43*

DISCUSSION

Over the past few decades, the exploration of bacterial proteins as potential anticancer agents has gained considerable attention. One example of that is azurin protein which is isolated from the bacterium *Pseudomonas aeruginosa*. It has emerged as a promising candidate due to its unique mechanism of action and selective cytotoxicity towards cancer cells. Ever since the first incidence of the identification of azurin, it was recognized for its redox properties, and early on it was known for its interaction with the tumour suppressor P53. Ongoing studied constantly aim to further out understanding of the azurin protein, and all its possible applications.

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Currently, there are many efforts applied by researchers to utilize the knowledge we have about the bacterial-based anticancer agent gene azurin. Many studies were conducted with the purpose of furthering our knowledge on the applications of azurin, but also on the modification of azurin after its subsequent isolation. Furthermore, extensive experiments are done to test the gene's reaction against various cancer cell lines to understand how they work naturally, to further understand how we can modify it in the future.

The aim of this study is to isolate, extract, and identify the anti-cancer agent gene Azurin from the bacteria *Pseudomonas aeruginosa* for future downstream processes.

This study further adds onto the knowledge surrounding the azurin coding gene by identifying it from the tested isolates, and standard ATCC 15442 using the azurin-specific primers which proved the presences of the azurin-coding gene in all *Pseudomonas aeruginosa* local strains, as well as the standard strain.

Similarly to my study, which used AzuR, and AzuF primers to amplify the azurin coding gene, a study conducted by **(Sereena & Sebastian, 2020),** were able to successfully amplify the azurin coding gene from *pseudomonas aeruginosa'*s genomic DNA. Nonetheless, they used primers specific to a different set of base pairs than the ones used in this current study. Furthermore, they also further cloned the azurin coding gene in an expression vector, and E.coli strains as the expression host.

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