# Molecular detection of anticancer protein Azurin in native *Pseudomonas* isolates

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A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MATHEMATICS AND NATURAL SCIENCES, BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

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## Declaration

I hereby declare that this thesis entitled "Molecular detection of anticancer protein Azurin in native *Pseudomonas* **isolates**" is submitted by me, Fahim Sarwar, to the Department of Mathematics and Natural Sciences under the supervision and guidance of **Trosporsha Tasnim Khan** and **Nazneen Jahan** Department of Mathematics and Natural Sciences, BRAC University. I also declare that the thesis work presented here is original, and has not been submitted elsewhere for any degree or diploma.

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# Contents

# Abstract

Azurin is a potent anticancer protein which has the ability to interfere in tumor growth. The purpose of this study was to isolate azurin producing native *Pseudomonas* isolates along with the confirmation of azurin presence using molecular techniques. In this study, azurin presence was Azurin is a potent anticancer protein which has the ability to interfere in tumor growth. The purpose of this study was to isolate azurin producing native *Pseudomonas spp* along with the confirmation of azurin presence using molecular techniques. Primarily, the isolates were characterized morphologically and identified biochemically as *Pseudomonas spp.* Presence of azurin gene was tested for 25 environmental (soil) isolates using specific oligonucleotide primers (AZU-F and AZU-R) in a gradient PCR. The extracted total cellular protein was precipitated by 60% ammonium sulfate followed by dialysis and partially purified azurin using SDS-PAGE. Out of 25 isolates, 10 showed the presence of azurin gene by amplifying a single 545 bp DNA fragment in PCR. Total cellular partially purified protein extracted from 10 azurin producing isolates selected from PCR fractionated by SDS PAGE produced patterns with a wide range of discrete bands with the inclusion of 14 kDa Molecular weight. Presence of azurin gene was confirmed by PCR, and partially purified azurin protein was detected by SDS-PAGE according to specific molecular weight. Detection of high yield azurin producing microbial strains can lead us to the development of anticancer drugs.

**Keywords:**  Azurin, PCR, Anticancer protein, SDS-PAGE

# List of Tables

# List of Figures

# List of Abbreviations

| Abbreviation | Elaboration |
|---|---|
| Eph receptors | Erythropoietin-producing human hepatocellular receptors |
| DNA | Deoxyribonucleic acid |
| Caspases | Cysteine-dependent aspartate-directed proteases |
| LMIC | Low- and middle-income countries |
| CF | Cystic fibrosis |
| PCR | Polymerase Chain Reaction |
| VEGFA | Vascular endothelium growth factor A |
| SDS-PAGE | Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis |
| RT-PCR | Reverse Transcription PCR |
| FAK | Focal Adhesion Kinase |
| Temed | Tetramethylethylenediamine |
| APS | Ammonium Persulfate |
| PS | Parasporin |
| mm | Millimeter |
| μm | Micrometer |
| mg | Milligram |

# 1. Introduction

## 1.1 Overview

Cancer, a leading cause of death worldwide is most deadly in countries of all income levels. The number of cancer-related cases and deaths is expected to grow rapidly as populations grow. Low- and middle-income countries (LMIC) go through economic transition with addition of greater mechanization of transport and labor, cultural shifts in the roles of women, and increased exposure and access to international markets, eventually leading to or boasting of risk factors of regular lifestyle risk factors including tobacco use, physical inactivity, excess body weight, and reproductive patterns (Torre et al., 2015).

The subcontinent of South Asia includes Pakistan, India, Sri Lanka, Bangladesh, Nepal and Bhutan along with the population of more than 1,500 million. Asia accounts for 60% of the world population and half the global burden of cancer. The cases of cancer incident are estimated to increase from 6.1 million in 2008 to 10.6 million in 2030 (Sankaranarayanan, Ramadas, and Qiao, 2018). This incident takes place on account of aging and growing populations, lifestyle and socioeconomic changes. Bizarre amount of variations in ethnicity, sociocultural practices, human development index, habits and dietary patterns are reflected in the burden and pattern of cancer in different regions.

Bangladesh, a country with 142 million people indicating its ninth position as one of the most populous countries in the world. Among this mass population 13 to 15 lakh patients affected with cancer in Bangladesh. According to the Bangladesh Bureau of Statistics, cancer is the sixth leading cause of death. International Agency for Research on Cancer has estimated cancer-related death rates in Bangladesh to be 7.5% in 2005 and 13% in 2030. There are around 150 qualified clinical oncologists and 16 pediatric oncologists working in the different parts of the country. The available number of the hospital for regular cancer treatment is 19. 465 hospital beds are attached as indoor or daycare facilities for chemotherapy in the oncology/radiotherapy departments. There are about 15 linear accelerators, 12 Co-60 teletherapy, and 12 brachytherapy units currently available. Approximately, 56 cancer chemotherapeutic agents are obtainable in Bangladesh (Hussain, 2013).

Half a century ago, there was storytelling that a person was far more likely to die from heart disease nowadays there is an increment in the global burden of cancer (Jemal et al., 2010). This increment of cancer leads to a various number of cancer cases and deaths (Popat, McQueen and Feeley, 2013). But it is true that if the disease is diagnosed in the primary stage and treated according to diagnostics, there is a high chance of surviving. Current chemotherapeutic drugs designed for the treatment of cancer or therapies that are available to treat cancer are often toxic and prone to resistance development by the cancer cells, *Pseudomonas aeruginosa* is an organism that is able to produce or secrete protein named Azurin an anticancer agent (Lustig and Hill, 1967) that can be used as a weapon providing important insights into how a pathogen responds in the post-colonization state to disrupt other intruders for its own survival. Moreover, these molecules might find use in the pharmaceutical industry as next-generation therapeutics (Yamada et al., 2005). Azurin is a neoteric molecule for cancer treatment through nanotechnology. It is because Azurin has the ability to be a combination of other cytotoxic agents in cancer diagnosis and treatment (Fialho et al., 2007). So detection of high yield azurin producing microbial strains from environmental sources helps to design drugs for cancer-based therapies economically.

Cancer is the second leading cause of death in the world (Tarver, 2012) after cardiovascular diseases (Sudhakar, 2009). The name Cancer refers to a collection of related diseases, or Cancer is a class of diseases characterized by out-of-control cell growth. As cancer is a collection of diseases in the tissue level (Wang et al., 2018) so for this reason screening procedure is harder (Fisher, Pusztai and Swanton, 2013) because of a variety of diseases included in the collection. Human bodies are made up of billions number of cells. Among all these cells when one cell or a clump of cells divide in an uncontrolled way in which cancerous cells begin to appear. Clump cells uncontrolled growth leads to tumor formation. Cancers like leukemia or blood cancer do not form a tumor. Cancer cells can spread through organs to organs via blood circulation and lymph vessels, and new cancer cells are beginning to grow. Cancer cells growing in such procedure is known as metastasis. Over 100 different types of cancer are infecting people nowadays, and each is classified by the type of cell that is initially affected. Every type of cancer includes division of some of the body's cells and division takes place without stopping and spread into surrounding tissues.

At the beginning of the 15th century, scientists were able to develop a greater understanding of the human body. With the help of the scientific method for Galileo and Newton was able to study disease. In 1761, Giovanni Morgagni of Padua did something first to which nowadays turned out to be a   routine today – he did Autopsies was done by him to find a relation between the patient's illness and pathologic findings after death. This was resulting in the foundation for scientific oncology, the study of cancer. Scottish surgeon John Hunter (1728-1793) had a suggestion that some cancers might be curable by performing surgery. If the invasion of nearby tissue was not done by a tumor or the tumor was "moveable," he said, "There is no impropriety in removing it." After a century later the development of anesthesia development gave surgery procedure to flourish and use of classic cancer operations such as the radical mastectomy (Cancer.org, 2018).

All types of cancer share common markers and cancer cells have some unique characteristics including damaged or mutated cells, rapid multiplication, and the ability to spread throughout the body. The specific form of cancer is named and categorized according to the tissue area affected (Story et al., 2018).

**1.1.1 Hallmarks of Cancer**

Cancer mechanism pathway can be described by six short terms (Hanahan and Weinberg, 2000). They are-

- Self-sufficiency in growth signals

- Insensitivity to anti-growth signals

- Evading apoptosis

- Limitless replicative potential

- Sustained angiogenesis

- Tissue invasion and metastasis
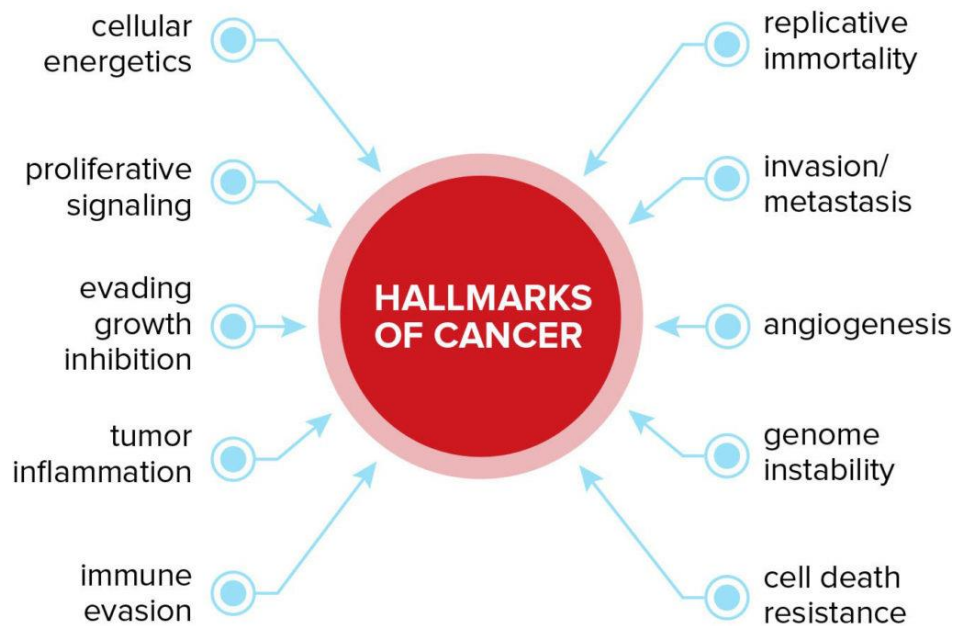
**Figure 1.1:** The Hallmarks of Cancer (Retrieved from oncosynergy.com/science)

However, after a decade there are new four terms to be added with the previous hallmarks-

- abnormal metabolic pathways

- evading the immune system,

- genome instability, and

- Inflammation (Hanahan and Weinberg, 2011).

### 1.1.2 Common types of cancer

Cancer is one of the leading cause of death worldwide, accounting for 8.8 million deaths in 2015. The most common causes of cancer death are cancers of:

- Lung (1.69 million deaths)

- Liver (788000 deaths)

- Colorectal (774000 deaths)

- Stomach (754000 deaths)

- Breast (571000 deaths)


## 1.2 Modern Day Cancer Treatment

There are many types of cancer treatment. The types of treatment received by a patient will depend on the type of cancer the particular patient have and how further it has advanced.

- Surgery- surgery is an eventual gradual processes procedure in which a surgeon removes cancer from a patient's body.

- Chemotherapy- is anti-cancer drug treatment. More than half of all people receive treatment for cancer treatment, and many different types are available. Doctors choose specific types of chemotherapy based on a number of factors, including the type of cancer diagnosed with the patient, and its advancement. (Popat, McQueen and Feeley, 2013)

- Bone Marrow/Stem Cell Transplantation- A stem cell transplant (sometimes called a bone marrow transplant) is a process of replacing diseased bone marrow with highly specialized stem cells. These specialized stem cells that can develop or has the ability to turn into healthy bone marrow (Sagar et al., 2007).

- Radiotherapy- Radiotherapy uses radiation, such as x-rays, gamma rays, electron beams or protons, to kill cancer cells or cause damage; so that they are unable to grow or multiply. It is a localized treatment meaning it normally affecting the part of the body where the radiation is targeted (Citrin, 2017).


- Immunotherapy- a biological therapy, or biotherapy that uses the immune system to fight cancer. Generally, it boosts the immune system or marks cancer cells for which immune system can find and destroy cancer cells. Therapy can be gained by various ways such as getting it by mouth as a pill, into a vein as an IV, by rubbing cream into your skin, or through a catheter directly into the bladder (Minda et al., 2016).

## 1.2.1 Side Effects of Cancer Treatment

Cancer treatments include various side effects—problems take place at the time of treatment when healthy tissues or organs are affected by the treatment. Cancer-related side effects are included in the list below (De Angelis, 2008) -

- Diarrhea
- Anemia
- Hair Loss (Alopecia)
- Infection and Neutropenia
- Delirium
- Constipation
- Edema (Swelling)
- Appetite Loss
- Bleeding and Bruising (Thrombocytopenia)
- Sexual Health Issues in Men
- Fertility Issues in Boys and Men
- Fertility Issues in Girls and Women
- Sexual Health Issues in Women
- Urinary and Bladder Problems
- Lymphedema
- Memory or Concentration Problems
- Mouth and Throat Problems
- Skin and Nail Changes
- Fatigue
- Nausea and Vomiting
- Nerve Problems (Peripheral Neuropathy)
- Pain
- Sleep Problems

**1.4** *Pseudomonas aeruginosa*

Bacteria can be carcinogenic and tumor promoters through inducing inflammation (Lax, 2005). Bacteria producing toxins can disrupt the cellular signal eventually leading to aggravation of the regulation of cell growth. Pseudomonas aeruginosa can secrete a Protein Azurin that has anticancer properties.

The genus Pseudomonas contains more than 140 s. Among these species, most are saprophytic meaning energy gaining from dead or decaying materials and 25 species are associated with humans. Responsible for opportunistic pathogen and disease-causing pseudomonads are *P aeruginosa, P fluorescens, P putida, P cepacia, P stutzeri, P maltophilia,* and *P putrefaciens*. *P mallei* and *P pseudomallei* are responsible for specific human diseases: glanders and melioidosis. *P aeruginosa* a ubiquitous free-living bacterium and generally found in most moist environments. Although it seldom causes disease in healthy individuals. However, it is mostly a threat to hospitalized patients, particularly patients with diseases such as cancer and burns (Baron, 2018). *P. aeruginosa* a common Gram-negative, rod-shaped (rod measuring 0.5 to 0.8 μm by 1.5 to 3.0 μm), asporogenous, and monoflagellated bacterium, also a member of the Gamma Proteobacteria class of Bacteria. Pseudomonas aeruginosa is responsible for causing diseases in both plants and animals and in humans, causing severe infections in immunocompromised patients with cancer and patients suffering from severe burns and cystic fibrosis (CF) (Goldberg 2000; Lyczak, Cannon and Pier 2000). *Pseudomonas aeruginosa* is an opportunistic pathogen, meaning that it has exploitation ability to break in the host defenses for initiating an infection (Lustig and Hill, 1967). *P. aeruginosa* has a cytoplasmic membrane with asymmetric phospholipid bilayer and an asymmetric outer membrane with a phospholipid inner face and a lipopolysaccharide outer layer. The outer membrane of P. aeruginosa contains numerous proteins, including lipoproteins and channels. (Remans et al. 2010).

*Pseudomonas aeruginosa* is omnipresent in soil and water, and on surfaces in contact with soil or water. It has a respiratory metabolism system and is able to grow in the absence of O2 if NO3 is available as a respiratory electron acceptor. *Pseudomonas aeruginosa* is an organism of minimal nutritional needs an example of it is being observed growing in distilled water. In the laboratory, for the growth of Pseudomonas aeruginosa requiring most simple media consists of

acetate as a source of carbon and ammonium sulfate as a source of nitrogen. The optimal growth temperature is 25°C to 37°C. It has the ability to grow at the temperature of 42°C which helps to distinguish itself from other Pseudomonas species (Stover et al., 2000; Mathee et al., 2008; Frimmersdorf et al., 2010). It does not require organic growth factors. ***Pseudomonas aeruginosa*** is able to tolerant to a wide variety of physical conditions particularly temperature. It has resistance to high concentrations of salts and dyes. Pseudomonas aeruginosa has a tendency to grow in moist environments, which is an indication of reflection of its natural existence in soil and water (Textbookofbacteriology.net, 2018). ***P. aeruginosa,*** an encoder of various numerous virulence factors. This quality of encoding virulence factors enables it to establish various human infections.

### 1.2.2 Anticancer Protein and Peptides

Cancer is recognized as a deadly disease worldwide is responsible for disregulation of mammalian cell differentiation and growth. Present day drugs that can prevent cancer involves an adverse amount of side effects. Now a day's most common treatment of cancer is removing of tumors by surgical resection followed by radiation and chemotherapy (De Rienzo et al., 2000). Typically two types of drugs are used in the process of chemotherapy- Firstly, small molecule drugs (e.g., tyrosine kinase inhibitors) and human or humanized proteins (e.g., monoclonal antibodies). They function as one drug one target therapy. These types of drug results in various side effects in healthy cell growth along with the rapid development of resistance to these particular types of drugs (De Rienzo et al., 2004). Currently, various researches are on process to develop protein based therapeutics with no side effects and total regression of the cancerous tumor. These proteins potential anticancer agent. Azurin is one of the most studied anticancer agents. LHPP is one of the recent inventions in the prevention of liver cancer.

### 1.2.3 New anti-cancer protein LHPP

Liver tumors develop from mutated cells. Mutated cells grow and proliferate in an uncontrollable manner. Anti-cancer proteins known as tumor suppressors can prevent uncontrolled cell growth. Normally tumor suppressors in cancer cells are either damaged or function in a defective manner. The researchers led by Prof. Michael N. Hall, Biozentrum of the University of Basel

discovered a new, tumor suppressor protein LHPP. The loss of LHPP is a promoter for tumor growth and reducing the chance of survival of cancer patients (Unibas.ch, 2018).

### 1.2.4 Parasporin

Parasporin (PS), a crystal protein. Derivation of this protein is from noninsecticidal and nonhemolytic *Bascillus thurengenesis* (Kitada et al., 2006**).** This protein is also a collection of genealogically heterogeneous Cry proteins (a large number of crystalline proteins targeting a wide of insects and nematodes) normally synthesized in *Bacillus thurengenesis*. One of the strong features of this protein is cytocidal (Tendency to kill individual cells) activity which preferential for human cancer cells of various origins. Exhibition of cytocidal activities takes place when digested with proteases. Currently, this protein group is classified into four families: PS1, PS2, PS3 and PS4 (Uemori et al., 2006).

### 1.2.5 Exotoxin A

Bacterial toxins nowadays can be evaluated in various clinical trials such as potential cancer therapeutics (Kawakami et al., 2006; Kreitman, 2006, 2009). Exotoxin A from *Pseudomonas* is a bacterial toxin that has been or is currently under clinical evaluation against a variety of haematologic malignancies and solid tumors with promising results (reviewed by Becker & Benhar, 2012).

### 1.3 Azurin

Azurin, a microbial anticancer agent, mainly found in Bacteria like *Pseudomonas aeruginosa* Bordetella, or Alcaligenes bacteria. Azurin is a blue copper protein undergoing an oxidation-reduction reaction between Cu (I) and Cu (II). As it is able to perform both reactions, it can be called a redox protein.  They have higher affinity and penetrate cell membranes of the target cancer cells, reaching the target sites, than other peptides used to treat cancers. The composition of Azurin includes one alpha helix and two beta sheets. It has a molecular weight of 14 kDa with comprising of one copper atom. Intense blue color is for the presence of copper ion.  The oxidized form of Azurin is most stable, and Azurin is the most undersized copper protein. Azurin is representative of bacterial products used in the treatment of tumors (Nowak and Tarasiuk, 2012). It has the ability to defend against cancer, parasite and has anti-HIV properties related to different locales of protein (Mechanical Movement, 1846).  Azurin, a multi-targeted anticancer agent acts by three pathways (Luke, Apiyo and Wittung-Stafshede, 2005):  Inducing apoptosis

through intracellular stabilization of p53 protein (Fialho, Das Gupta and Chakrabarty, 2007), Inhibiting cell cycle progression through intracellular binding Eph receptor tyrosine kinase (Chaudhari et al., 2007) and preventing angiogenesis through inhibition of VEGFA (Vascular endothelium growth factor A) (Pasquale, 2010). Azurin raises its intracellular level by appearing to form a complex with p53. Increasing amount of p53 triggers 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 having a tumor-suppressing ability that acts as a guardian of the genome in the prevention of cancer growth and maintaining genome stability. Tumor suppressor protein p53 is the primary actor of a transcription factor and does following functions: Expression induction of genes responsible for the cell-cycle arrest, senescence and apoptosis in response to cellular stresses (Surfing the p53 network, 2000). Azurin is the first bacterial protein reported to form a complex with p53. Azurin, a small globular metalloprotein, having redox activity and having involvement in the process of bacterial denitrification process (Solomon, Baldwin and Lowery, 1992). It is an electron transferer shuttle in Pseudomonas aeruginosa and other bacteria.  In fact, studies on its anticancer activity revealed that azurin preferential entrance in the cells of breast cancer and induction apoptosis (Determination of four thiophenethylamine designer drugs in human urine by capillary electrophoresis/mass spectrometry, 2010), the halt of cell cycle arrest and angiogenesis inhibition through interaction with different cellular molecules. Azurin being capable in the induction of apoptosis in tumor cells by p53 stabilization which makes this protein suitable for being employed as an anticancer agent (Internalization of bacterial redox protein azurin in mammalian cells: entry domain and specificity, 2005). The p53 tumor suppressor has involvement in multiple central cellular processes which includes transcription, DNA repair, genomic stability, cell cycle control, and apoptosis; which not functionally activated in many human cancers (Bacterial Cupredoxin Azurin as an inducer of apoptosis and regression in human breast cancer, 2004).  Azurin forms a complex with p53 and then raising its intracellular levels (Yamada et al., 2004). Azurin functions as a redox protein in electron transfer reactions. Azurin can interfere with cell growth through multiple mechanisms. One of the mechanism includes the formation of a complex with the DNA-binding domain (DBD) of tumor suppressor protein p53 (Apiyo and Wittung-Stafshede, 2005). Stability of the complex enhances the intracellular level of the complex itself which eventually helps in taking the place of Apoptosis (Apoptosis or growth arrest: Modulation of

tumor suppressor p53's specificity by bacterial redox protein azurin, 2004). Hydrophobic patch of Azurin is responsible for interacting with DBD WHICH IS INDICATED by site-directed mutagenesis (Goto et al., 2003). Injecting azurin in xenotransplanted mice withholding melanoma and breast tumors result in regression of the significant tumor, without adverse side effects (Punj et al., 2004). The presence of azurin increases the mRNA levels of pro-apoptotic molecules with the help of tumor suppressor p53, such as the levels of BAX which by turn results in an imbalance of BCL2-BAX levels eventually enhancing cell death or growth arrest (Yamada et al., 2005).

### 1.3.1 Azurin Anticancer Mode of Action

Mode of action is an updated review that is undertaken by Bernardes et al. in 2013. P-cadherin overexpression becomes a target of Azurin in a subset of breast cancers which leads to antagonizing its pro-invasive effects.
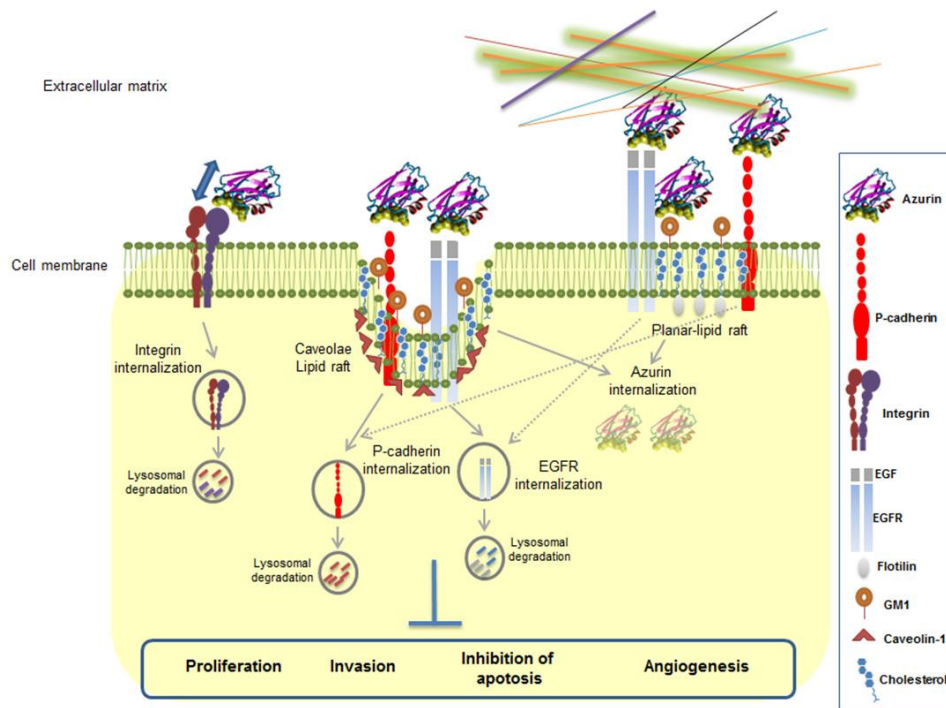


**Figure 1.2:** Mechanisms of action of Azurin against cancer cells

(Retrieved from https://medical-dictionary.thefreedictionary.com/P-cadherin)

P-cadherin, a cell-to-cell adhesion molecule with homeostatic functionality in several normal tissues. Azurin treatment decreases the levels of P-cadherin at the cell membrane. On the other hand, E-cadherin remains in an unaltered condition. In an unaltered state E-cadherin has high expression levels and with normal membrane localization. Reduction of P-cadherin concomitantly detoriates the levels of the hyper phosphorylation which results in the formation of FAK (Focal Adhesion Kinase) and Src non-receptor tyrosine kinases. These proteins regulate a wide number of signaling pathways which involved in adhesion, migration, invasion, survival, and angiogenesis linking cellular responses to environmental stimuli (Figure-1.2). Azurin exhibits a specific preference for P-cadherin and nullifying its invasive effects which indicates it might play a potential role in the treatment of breast carcinomas overexpressing this protein (Bernardes et al., 2018).

## 1.4 Objective

The objective of the thesis was to isolate *Pseudomonas spp* from the soil, extract the DNA and detect anticancer gene (Azurin) using molecular biology technique.

 - Isolation of *Pseudomonas spp.* from a soil

- Screening for anticancer protein Azurin encoding gene using DNA Gel Electrophoresis

-SDS PAGE of extracted anticancer protein

# 2. Methodology



Isolation and primary identification of target organism

↓

Genomic DNA Extraction

↓

PCR with Azurin specific marker

↓

Detection of the target gene using Gel Electrophoresis

↓

Extraction of Cellular Protein

↓

Ammonium Sulphate precipitation of proteins and Dialysis
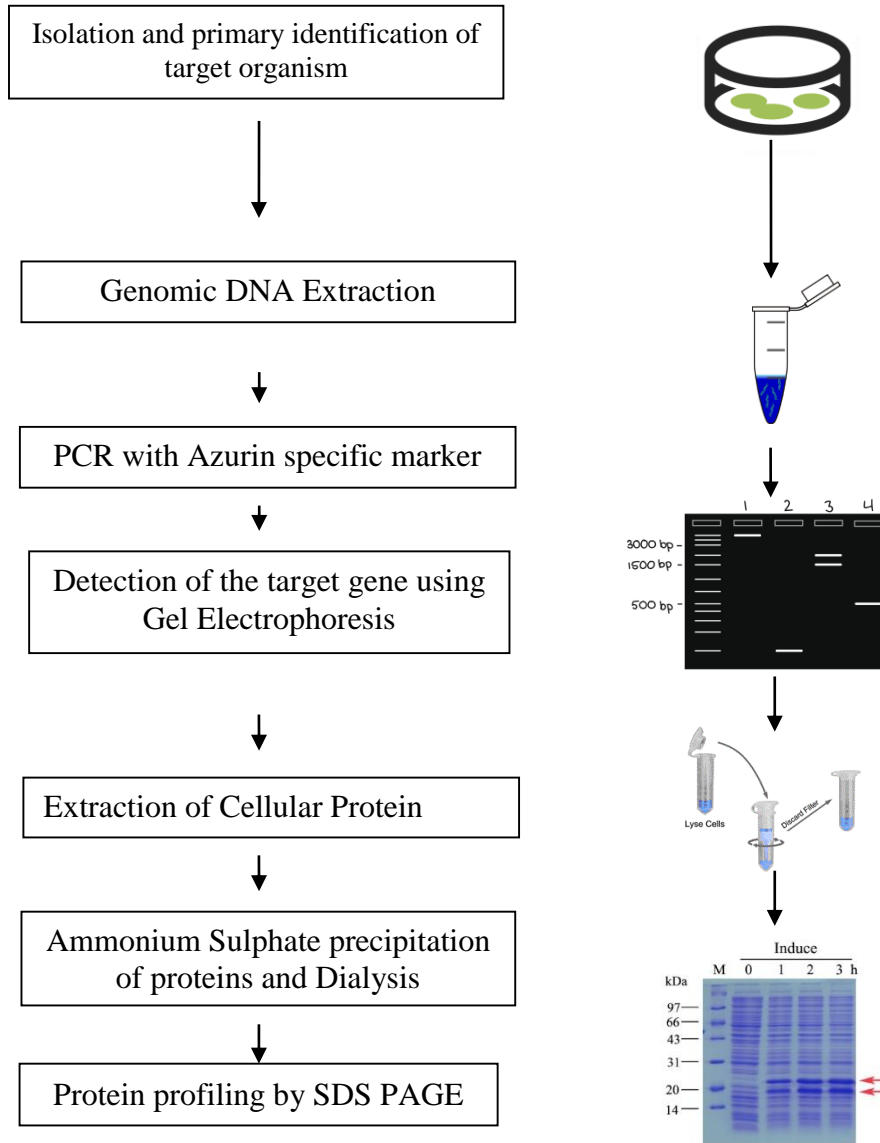
↓

Protein profiling by SDS PAGE

**Figure 2.1:** A brief outline of the methodology

## 2.1 Sample collection

Collecting Soil samples from the different location then Serial dilution of Samples and plated on Cetrimide agar.

## 2.2 Place of Study

The laboratory of the Department of Mathematics and Natural Sciences (MNS) at BRAC University.

## 2.2 Biochemical characterization of the bacteria

Several biochemical tests were carried out in order to have a presumptive identification of the potential bacteria chosen before. Most of the methods were done according to the microbiology laboratory manual (Cappuccino and Sherman, 2005).

### 2.2.1 Indole Production test

Indole production test was done to determine the ability of the bacteria to degrade the amino acid tryptophan by the enzyme tryptophanase. Tryptophan broth of 5 ml in each test tube was prepared by autoclaving at 15 psi 121∘C. Using sterile technique, small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the tubes by means of a loop inoculation method with an inoculating loop and the tubes were incubated for 48 hours at 37∘C. In order to test for indole production, 5 drops of Kovac's reagent was added directly into the tubes. (Cappuccino and Sherman, 2005)

### 2.2.2 Triple Sugar Iron Agar test

Triple sugar iron test was done to differentiate among the different groups or genera of the Enterobacteriaceae based on the ability to reduce sulfur and ferment carbohydrates. Triple sugar iron slants were prepared in the test tubes by autoclaving at 15 psi 121∘C. Using sterile technique; small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the tubes by means of a stab and streak inoculation method with an inoculating needle. The screw caps were not fully tightened and the tubes were incubated for 24 hours at 37∘C. (Cappuccino and Sherman, 2005)

### 2.2.3 Methyl red test

Methyl red test was done to determine the ability of the bacteria to oxidize glucose with the production and stabilization of high concentration of acid end products. MR-VP broth of 7 ml in

each test tubes were prepared by autoclaving at 15 psi 121∘C. Using sterile technique, small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the tubes by means of a loop inoculation method with an inoculating loop and the tubes were incubated for 24 hours at 37∘C. After 24 hours 3.5 ml from the culture tubes were transferred to clean test tubes for Voges- Proskauer test and the remaining broth were re-incubated for additional 24 hours. After 48-hour incubation 5 drops of methyl red indicator was added directly into the remaining aliquot of the culture tubes to observe the immediate development of a red colour. (Cappuccino and Sherman, 2005)

## 2.2.4 Voges Proskauer test

Voges Proskauer test was done to differentiate further among enteric organisms such as E.coli, E. aerogenes, and K. pneumoniae by determining the capability of the organisms to produce non acidic or neutral end products such as acetylmethylcarbinol. To the aliquot of MR-VP broth after 24 hour incubation, 0.6 ml (12 drops) of 5% alpha naphthol (reagent A) was added followed by 0.2 ml ( 4 drops) of 40% KOH (reagent B). The tube was gently shaked to expose the medium to atmospheric oxygen (30seconds-1 minute) and the medium was allowed to remain undisturbed for 10-15 minutes. The test was read, but not beyond, one hour following the addition of the reagents. (Cappuccino and Sherman, 2005)

## 2.2.5 Citrate utilization test

Citrate utilization test was done to differentiate among enteric organisms on the basis of their ability to ferment citrate as a sole source of carbon by the enzyme citrate permease. Simmons citrate agar slants of 2 ml in each vials were prepared by autoclaving at 15 psi 121∘C. Using sterile technique, small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the vials by means of a streak inoculation method with an inoculating needle and the vials were incubated for 48 hours at 37∘C. (Cappuccino and Sherman, 2005).

## 2.2.6 MIU (Motility- Indole- Urease) test

MIU test was done to simultaneously determine the ability of the bacteria to produce indole, check motility and degrade urea by means of the enzyme urease. MIU media was prepared by autoclaving at 15 psi 121∘C. the media was cooled to about 50-55∘C and 100ml of urea glucose solution was added aseptically to 900 ml base medium. After that, 6ml solution was transferred to each sterile test tube and allowed to form a semi solid medium. Using sterile technique, small

amount of the experimental bacteria from 24-hours old pure culture was inoculated into the tubes by means of a stab inoculation method with an inoculating needle and the tubes were then incubated for 24 hours at 37∘C. (Cappuccino and Sherman, 2005).

### 2.2.7 Nitrate reduction test

Nitrate reduction test was done to determine the ability or inability of the bacteria to reduce nitrate ($NO_3-$) to nitrite ($NO_2-$) or beyond the nitrite stage using anaerobic respiration by the enzyme nitrate reductase. Nitrate broth of 6 ml in each test tubes were prepared by autoclaving at 15 psi 121∘C. Using sterile technique, small amount of the experimental bacteria from 24-hour pure culture was inoculated into the tubes by means of a loop inoculation method with an inoculating loop and the tubes were incubated for 24 to 48 hours at 37∘C. After incubation, 5 drops of reagent A and 5 drops of reagent B was added to each broth. If there was no red colour development, a small amount of zinc was added to each broth. (Cappuccino and Sherman, 2005) Note: Caution was maintained during the use of powdered zinc since it is hazardous.

### 2.2.8 Catalase test

Catalase test was done to determine the ability of the bacteria to degrade hydrogen peroxide by producing the enzyme catalase. A microscopic slide was placed inside a petri dish. Using a sterile inoculating loop, a small amount of bacteria from 24-hour pure culture was placed onto the microscopic slide. 1 drop of 3% $H_2O_2$ was placed onto the organism on the microscopic slide using a dropper and observed for immediate bubble formation. (Cappuccino and Sherman, 2005)

### 2.2.9 Oxidase test

The oxidase test detects bacteria that produce cytochrome c oxidase, which is an enzyme of the bacterial transport system. All aerobic bacteria are oxidase positive. In positive cases, a deep blue or purple stain appears within 5-10 seconds. Organisms such as Pseudomonas, Campylobacter are oxidase positive organisms. In this procedure, Kovacs Oxidase Reagent was used. Its composition is 1% tetra-methyl-p-phenylenediamine dihydrochloride, in water (Cappuccino and Sherman, 2005).

### 2.2.10 Gelatin hydrolysis test

Gelatin hydrolysis test was done to detect the ability of the bacteria to produce gelatinase. All the ingredients of the nutrient gelatin medium were mixed and gently heated to dissolve. Three

milliliter from the media was dispensed in glass vials. The glass vials with the medium were then autoclaved at 121◦C, 15 psi. The tubed medium was allowed to cool in an upright position before use. Using sterile technique, a heavy inoculum of 24-hour old culture bacteria was stab inoculated into the tubes with an inoculating needle. The glass vials were then incubated at 37◦C and observed up to for 1 week. (Cappuccino and Sherman, 2005)

### 2.2.11 Starch hydrolysis test

Starch hydrolysis test was done to determine the ability of the bacteria to hydrolyze starch with the enzyme amylase. Starch agar was prepared and autoclaved at 121◦C, 15 psi. The media was then dispensed into sterile plates while liquid and left for a while to solidify. Using sterile technique, a starch agar plate was streaked by picking a loopful colony of 24-hours old pure culture with an inoculating loop by means of streak plate method. The plates were then incubated at 37◦C for 48 hours and the hydrolysis was observed using gram's iodine. (Cappuccino and Sherman, 2005)

### 2.2.12 Casein hydrolysis test

Casein hydrolysis test was done to determine the ability of the bacteria to produce the enzyme caseases and hydrolyze casein thereby. Distilled water and agar solution was taken in separate conical flasks and both were autoclaved at 121◦C, 15 psi. Skim milk powder was then added to the autoclaved distilled water aseptically and boiled for 1 minute to dissolve completely. After that, the milk solution was mixed with agar solution. The media was added into sterile plates while liquid and left for a while to solidify. Using sterile technique, a milk agar plate was streaked by picking a loopful colony of 24-hour old pure culture with an inoculating loop by means of streak plate method. The plates were then incubated at 37◦C for 24 hours. (Cappuccino and Sherman, 2005)

### 2.2.13 Blood agar test

Blood agar test was done to determine the hemolytic capability of the bacteria by producing hemolysins and thereby lyse red blood cells. Blood agar base was prepared in a conical flask and autoclaved at 121◦C, 15 psi. The nutrient agar medium was allowed to cool at 45-50◦C and 5% (vol/vol) sterile defibrinated sheep blood that had been warmed to room temperature was added and gently mixed avoiding air bubbles. The media was then dispensed into sterile plates while liquid and left for a while to solidify. Using sterile technique, a blood agar plate was streaked by

picking a loopful colony of 24-hour old pure culture with an inoculating loop by means of streak plate method. The plates were then incubated at 37◦C for 24 hours. After incubation, the plates were observed for gamma, beta and alpha hemolysis. (Cappuccino and Sherman, 2005)

### 2.2.14 Eosin methylene blue agar test

This test was done to select and isolate Gram negative organisms, and coliforms, and to differentiate among the family of Enterobacteriaceae. The main use of this test was to isolate fecal coliforms and to detect for fecal contamination. Using sterile technique, an EMB agar plate was streaked by picking a loopful colony of 24-hour old pure culture with an inoculating loop by means of streak plate method. The plates were then incubated at 37◦C for 24-48 hours. Slow growing species may require a day or two of additional growth. (Cappuccino and Sherman, 2005)

### 2.3 Preparation of Cetrimide agar

Cetrimide powder was taken in a sterile conical flask. After that pouring 900ml of distilled water and boil until it dissolved. The Cetrimide agar was put aside for a few times to cool down or the temperature in the manner so it can be plated in Petri dishes. Cetrimide agar is known to be a selective media containing cetrimide that promotes the growth of Pseudomonas spp., turning the media blue-green due to the increased production of pyocyanin and fluorescein, the pigments present in Pseudomonas spp. This particular media was used to selectively isolate Pseudomonas spp., in the case of mixed cultures. A loopful of 24 hours old sample isolates were aseptically streaked onto autoclaved Cetrimide agar and observed after 24 hours of incubation at 37oC.

### 2.3.1 Growth on Cetrimide agar

Samples were streaked in the Petri dishes with cetrimide agar. After plating it was incubated for 24 hours at a temperature of 37 ℃.

### 2.4 Genomic DNA extraction

### 2.4.1 Genomic DNA extraction (Boiling method)

One loop of a bacterial sample was inoculated in a microcentrifuge tube with a volume of 2ml containing Luria-Bertani broth. Tubes were autoclaved for 24 hours at 121 ℃. Tubes ware then allowed for incubation at 37 ℃ for 24 hours. All the above steps were done for all xx number of

samples.  Microcentrifuge tubes containing bacterial cultures were put into a water bath machine with duration of 15 minutes and temperature at 95 ℃. Tubes were then -20 ℃ for 20 minutes. After these shock treatment tubes were centrifuged at 13000 rpm for 5 minutes.

### 2.4.2 Genomic DNA extraction (Phenol Chloroform method)

Transferring 2ml of the overnight bacterial culture (grown in LB medium) to a 2ml Eppendorf tube and centrifuge at max speed for 1min to pellet the cells. Discarding the supernatant (Removing as much of the supernatant as without disturbing the cell pellet). Then Resuspension of the cell pellet in 600 µl lysis buffer and vortex to complete resuspension of cell pellet. Incubation for 1 hour at 37 °C.  Addition of an equal volume of phenol/chloroform and mix well by inverting the tube until the phases are thoroughly mixed. Spinning at max speed for 5 min at RT (all spins are performed at room temperature). There is a white layer (protein layer) in the aqueous: phenol/chloroform interface. Carefully transferring the upper aqueous phase to a new tube by using 1 ml pipet. To remove phenol, adding an equal volume of chloroform to the aqueous layer. Again, mixing well by inverting the tube. Spinning at max speed for 5 min. Removing the aqueous layer to a new tube. To precipitate the DNA, adding 2.5 or 3 volume of cold 200 proof ethanol (storing ethanol at - 20 °C freezer) and mixing gently (DNA precipitation can be visible). Incubating the tube at -20 °C for 30 min or more. Spinning at max speed for 15 min at 4 °C. Discarding the supernatant and rinse the DNA pellet with 1 ml of 70% ethanol (stored at RT).  Spinning at max speed for 2 min. Carefully discarding the supernatant and air-dry the DNA pellet (tilting the tube a little bit on a paper towel). To be faster, drying the tube at 37 °C incubator.  Resuspending DNA in TE buffer

### 2.4.3 Genomic DNA extraction (Using lysozyme)

Inoculating A single pure Pseudomonas colony in 4 ml nutrient broth. Incubating at 37°C for 24 hours. Obtaining Cells by centrifuging in 4 ml of overnight broth culture at 5000 rpm for 5 min. Resuspension of Cells in 10 µl lysozyme (50 mg/ml of distilled water) and incubating at 37°C. After 10 min adding 10 µl proteinase K (20 mg/ml) solution and 500 µl 0.1 M Tris HCl (pH 7.5) buffer. Incubating Cell suspension at 37°C for 15 min and then placed in a boiling water bath for 5 min. Then centrifuge the cell suspension at 5000 rpm for 5 min, and the supernatant was

removed to another micro centrifuge tube. Addition of equal volume of isopropanol, mixed well and centrifuged at 12000 rpm for 12 min for pelleting DNA. Desalting the pellet with 200 µl of 70% ice-cold ethanol and centrifuged at 3000 rpm for 3 min and tubes were inverted to dry. Resuspending the 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°C for further analysis.

## 2.5 PCR

An in vitro technique for enzymatic amplification of specific DNA sequences. It uses two specific oligonucleotide primers. Azurin gene was amplified from the genome of isolated bacterial strains by using primers: AZU-F (5'GCCCAAGCTTACCTAGGAGGCTGCTCCATGC TA-3') and AZU-R (5'TGAGCCCCTGTAGGCGCCCATGAAAAAGC C CGGC-3') 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 MgCl2 (5 µl) and 1µl of each primer and DNA template. DNA amplification started with denaturing the template DNA at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 45 s, extension at 72°C for 3 min and a final extension step at 72°C for 7 min. Amplification was carried out using different annealing temperatures (50°C to 62°C) and time (30 s to 60 s) using thermal cycler (Biorad gradient T100TM). The PCR product was analyzed by agarose gel electrophoresis.

## 2.6 Agarose Gel Electrophoresis

Agarose dissolved in EDTA buffer (pH 8.0) was melted to form agarose solution, and while the 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 the reaction were mixed together, and eight 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.

## 2.7 Extraction of Cellular Protein

Azurin producing Pseudomonas strains were inoculated separately in Luria-Bertani broth. After 21 hours incubated cells were harvested by centrifugation at 12000 rpm for 15 min by using a cold centrifuge. Centrifuge machine was kept in a fridge for cold centrifugation (REMI C24 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 120 s at 10 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°C.

### 2.7.1 Ammonium sulfate precipitation of proteins

The crude supernatant was saturated to 70% by slowly adding ammonium sulfate salt at 4°C for precipitation, kept it for overnight. After precipitation, The solution was centrifuged two times at 9000 rpm for 12 min, and the pale supernatant was discarded. The precipitate was collected and resuspended in 0.02 M Potassium phosphate buffer pH 7.

### 2.7.2 Dialysis

Suspending Azurin containing precipitate suspended in 0.02M potassium buffer pH 7. Dialysis is done by standard dialysis bag (HIMEDIA LA 395) having 12 kDa MW cut off at 4°C for 20 hours on the same buffer for overnight with continuous gentle stirring. Doing dialysis until the solution attains its buffer pH. The solution was kept at 4°C after dialysis for further purification

## 2.8 Protein Profile by SDS Page

Casting 5 ml of 15% resolving gel in the glass slab without any bubble and kept it for 10-15 min. After polymerization of the resolving gel, loading 3 ml of stacking gel (4%) over the resolving

gel. After casting the gel, loading partially purified protein sample with standard protein molecular weight marker (SRL BIOLITTMLow Range 3-40 kDa) at different lanes for profiling the protein. Keeping glass slab gel in the electrophoresis tank with tank buffer. Connecting this set up was with power pack initially in 80 mV. After running the gel up to its anode end, removing and staining 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.

# 3. Results

**3.1 Agarose Gel Electrophoresis:**

PCR was used for amplification of Azurin gene to detect Azurin producing *Pseudomonas aeruginosa*
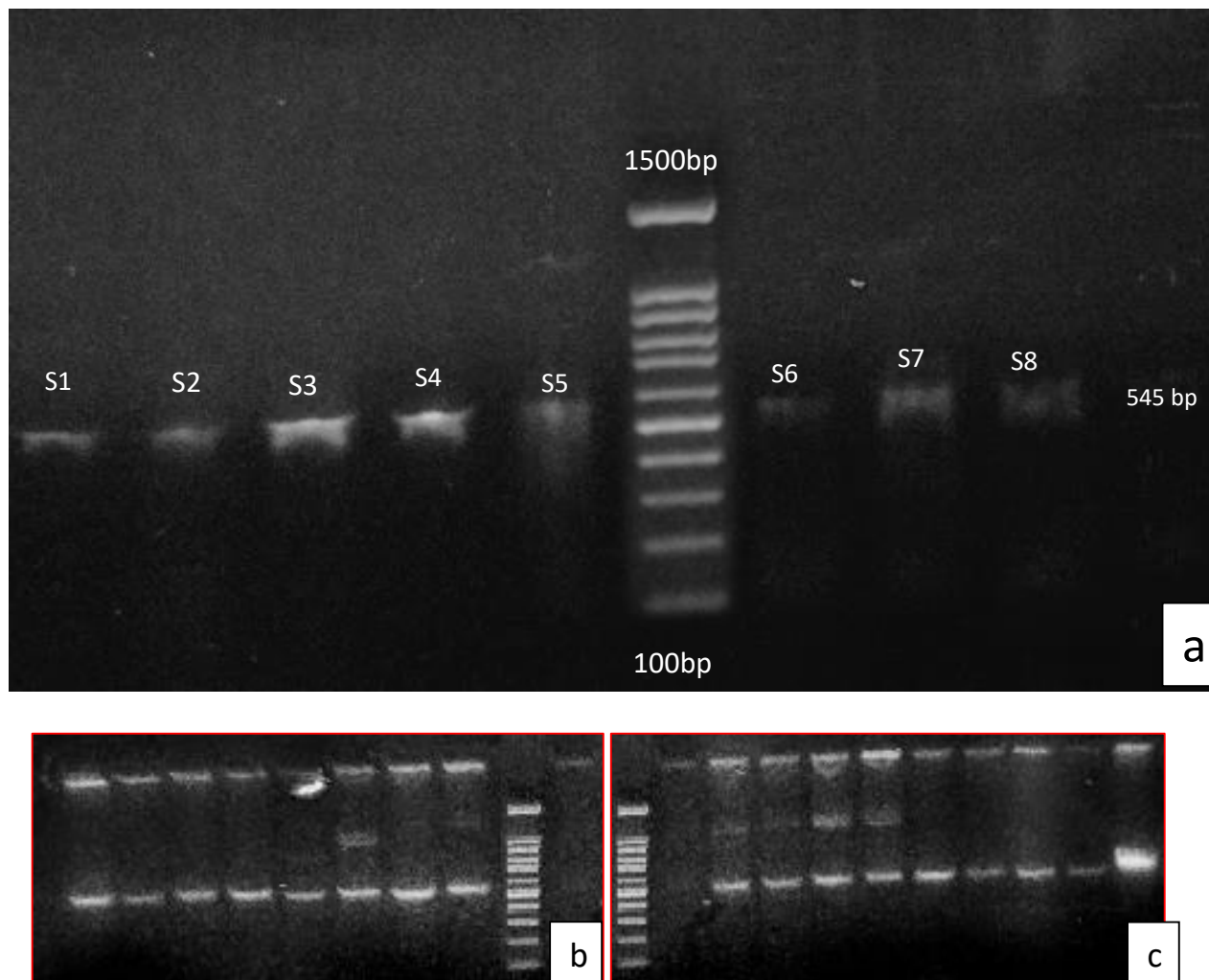


**Figure 3.1:** Agarose gel electrophoresis of PCR product (545 bp) loaded into 2% agarose gel and run at 100 V for 120 minutes.

Standard amplification with primer pair AZU-f AND AZU-R resulted in a 545 bp band as seen in Ethidium Bromide stained agarose gel electrophoresis. The azurin gene presence detected in 10 (a and c) out of 45 isolates from soil for this study. To compare, 10 clinical pseudomonas isolates (b) were also screened for the presence of azurin.

## 3.2 SDS-PAGE

Partially Purified Protein extracted from azurin gene positive *Pseudomonas* isolates were fractionated by SDS-PAGE. Eventually resulted in production of patterns with wide range of discrete bands with molecular masses ranged from 6-100 KDa.
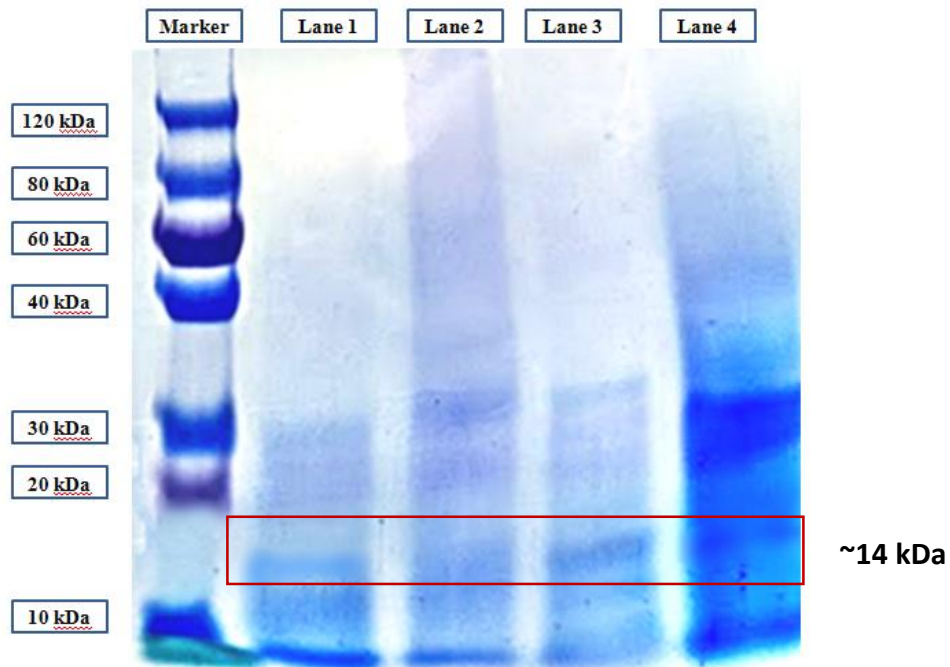


**Figure 3.2:** Protein Profiling and Analysis of Supernatant along with the Lysate preparation of *Pseudomonas aeruginosa.* Isolates selected from PCR fractionated by SDS PAGE produced patterns with wide range of discrete bands with inclusion of 14 kDa Molecular weight.

A dense band approximately corresponding to molecular weight of 14 kDa was prominently present in 3 isolates.

### 3.3 Biochemical Test

From all the sources, 45 isolates were assumed to be *Pseudomonas aeruginosa* based on their growth on selective agar media. For more confirmation few sets of biochemical tests were performed.

### 3.3.1 Catalase Test

In a sterile glass slide a small amount of organism was placed. Before placing the organism the slide needed to be dry and clean. Then a drop of $H_2O_2$ was placed. After a while bubbling indicated that the test is catalase positive.

**Table 3.3.1: Biochemical Test Results**

| Test | Result |
|------|--------|
| Catalase | Positive (+ve) |
| Oxidase | Positive (+ve) |
| Citrate | Positive (+ve) |
| Methyl Red | Negative (-ve) |
| VP | Negative (-ve) |
| Gelatin Hydrolysis | Positive (+ve) |
| EMB | Negative (-ve) |
| Blood Agar | Negative (-ve) |
| Nitrate Reduction | Negative (-ve) |
| Casein Hydrolysis | Negative (-ve) |
| Motility | Negative (-ve) |
| Indole | Negative (-ve) |
| Urease | Negative (-ve) |

### 3.3.2 Oxidase Test

Addition of Kovacs Reagent made a color change (developing deep blue color) indicated the test to be positive.

### 3.3.3 Citrate Test

The color change from green to blue along the slant indicated the test to be positive.

### 3.3.4 Indole Test

The test was negative as the organisms showed yellow color.

### 3.3.5 Methyl Red

After addition of methyl red organism's showed yellow color, indicating the test was negative.

### 3.3.6 Voges–Proskauer (VP) Test

In this particular test if the the isolates were observed in change of color to pink red the test was said to be positive. But the isolates showed no color change indicating the test to be negative.

### 3.3.7 Gelatin Hydrolysis

Liquefaction of the media indicated the test to be positive.

### 3.3.8 Urease Test

Positive test would indicate bright magenta color but the isolates used for this test did not show any color or color change indicating the test to be negative.

# 4. Discussion

In past centuries there has been an observation about a phenomenon of spontaneous regression of tumors associated with bacterial infections (Felgner et al., 2016). During the late 1890 American physician Coley reported about a treatment process based on this phenomenon (Coley et al., 1891). A relationship between bacterial infection and cancer regression was observed. The observation led him to discover a killed bacterial vaccine for cancer, known as "Coley's toxin" (Felgner et al., 2016), Which eventually gave direction in the development of new therapeutic anticancer modalities based on the use of live bacteria and their purified products including bacterial toxins, proteins, peptides, and enzymes. Recently, a number of bacterial proteins and peptides have been described to exert an anticancer activity at a preclinical level toward diverse types of cancer cells (Chakrabarty, Bernardes, and Fialho, 2014). Bacteriocins have shown possibilities to be an active therapeutic agent and their biochemical properties have been studied; their antineoplastic capability has also identified after its discovery in the late 1970s by using crude bacteriocin preparation toxic to mammalian cells.

Complex network and varied pathways by which cancer cells grow and inhibition of their own cell-arrest leading near to an impossible procedure for developing and designing and effective drugs which will be able to prevent the emergence of tumors or check their rapid growth (Lu et al., 2006) along without any side effects to take place. Pseudomonas aeruginosa a gram-negative pathogenic bacteria has the ability to secrete an anticancer protein Azurin. The protein Azurin can be used as a weapon to invade cancers, parasites, and viruses. Weapon produced from a pathogenic bacteria might be able to provide an insight into the process of pathogenic response in the post-colonization state to impede other intruders for its own survival. For this reason, molecules like Azurin can be used in the pharmaceutical industry as next-generation therapeutics (Yamada et al., 2005).

Early researches show that the synthesis of blue copper protein azurin from different microbial sources, mainly from Pseudomonas aeruginosa. Secondary metabolites such as Azurin secreted by microorganisms like Pseudomonas aeruginosa play a vital role in developing new chemotherapeutics. Present day chemotherapeutic drugs that used for the treatment of cancer are often toxic and prone to resistance development by the cancer cells eventually leading to multi-

drug resistant tumors. Azurin's ability of interference with or regression of tumor growth at multiple points of attack might be able in the prevention of resistance development. An attribute of such protein can be used as a weapon to elevate a broad range of ways like anti-viral and anti-parasitic activity. Azurin can prevent induction of precancerous lesion formation. A potent carcinogen can trigger the precancerous lesion formation (Tangri et al., 2001). *P. aeruginosa* enters human melanoma and breast cancer cells and triggers apoptotic cell death. Death sequence of cancerous cells takes place by forming a complex with the well-known tumor suppressor protein p53. The stable form of p53 protein along with activation of caspases induces apoptosis in cancer cells. The p53 protein stops typically cells that are damaged from reproducing. This protein also leads them to commit apoptosis, but a majority of cancer cells have damaged or missing p53 (Vijgenboom, Busch and Canters, 1997). Now, they demonstrate that a smaller, 28 amino-acid fragment of azurin also enters cancer cells selectively, but not in any of the healthy cells tested. This small molecule could potentially be used as a vehicle for cancer-targeted chemotherapy (Nieddu et al., 2010). Azurin exhibits cytotoxic activity against liquid and solid tumors (Kwan et al., 2009) and inhibits the growth of cancer cells. Inhibition of cancer cells can be done by interfering in the signaling pathways and angiogenesis (Arsenio et al., 2007). The studies on the detection of Azurin producing strains from environmental sources are infrequent. Azurin is too expensive to obtain readily (Ye et al., 2007). Azurin and its peptide p28 are able to penetrate cancer cells faster normal cells. The process is entirely dependent on cholesterol since its depletion significantly reduced its cellular penetration (Lulla et al., 2016). Preclinical evaluation of pharmacokinetics, metabolism, and toxicity of azurin-p28 established it as non-immunogenic and non-toxic in mice and non-human primates (Jia et al., 2010). Several international patents have been issued to cover the use of azurin-p28 in cancer therapies (M. Fialho, Bernardes and M. Chakrabarty, 2012), and azurin has shown significant activity, as well as enhancement of the activity of other drugs (Bernardes et al., 2016). Recent studies have shown that p28 in combination with lower concentrations of DNA-damaging drugs like doxorubicin, dacarbazine, temozolamide, and antimitotic agents such as paclitaxel and docetaxel, increased their cytotoxicity by stabilizing the tumor-suppressor protein p53. Taken together, these results highlight a new approach to maximize the efficacy of chemotherapeutic agents while reducing dose-related toxicity (Yamada, Das Gupta, and Beattie, 2016).

The mechanism mediating cell entry of azurin and its derived peptide has been studied. It is known that such entry is not dependent on membrane-bound glycosaminoglycans nor on clathrins. However, it is possible that N-glycosylated proteins may have a role at least in the initial steps of recognition and the depletion of cholesterol from the membrane significantly inhibited the penetration of p28 (~60%), suggesting an involvement of the caveolae-mediated endocytic route (Taylor et al., 2009). Once inside the cancer cells, the apo and holo forms of azurin are similar in their effects, supporting a copper-independent mechanism of action (Goto et al., 2003).

# 5. Conclusion

In this current era Cancer is one of the leading causes of death in the world. Development in the field of medicine and pharmacy results in the making of efficient anticancer drugs. In the study of this thesis, the anticancer protein Azurin was isolated on the basis of bands showing in the process of gel electrophoresis of PCR product and SDS-PAGE of partially purified Azurin protein that was isolated from *Pseudomonas aeruginosa*. For further confirmation of anticancer activities in Azurin can be done by inserting the proteins in cancerous cells and mRNA expression of Azurin from *Pseudomonas aeruginosa*. Modern-day Cancer treatment such as surgery, chemotherapy, etc. functions on the basis of one drug one target. Besides, treatments like these do not assure total regression of tumor or cancer cell growth. In addition these treatments always keep a place opened for cancer resistance meaning if a cancer patient is given any of these treatments there might be future possibility for that patient to be resistant for the drugs used for the treatments, indicating if the patient is again attacked by cancer the drugs required for the treatments will not work in future. In this particular thesis work, Azurin was isolated from Pseudomonas aeruginosa. Before isolation of Azurin DNA was extracted from the bacteria. Isolated samples of DNA was put to PCR method for Gel Electrophoresis. For PCR AZU-F and AZU-R were used as primers. Gel electrophoresis was done with the PCR product, and under UV ray we observed the band at 545 bp. Then Azurin was extracted. After extraction of Azurin partial purification was done by Ammonium Sulphate precipitation of the protein followed by dialysis. After Dialysis SDS-PAGE was performed and the band was observed at 14 kDa. For further expression of Azurin gene purified form of Azurin strain can be sequenced. The sequenced strain is to be analyzed for similarities to other known sequences found in the GenBank database using BLAST program of the NCBI database. Sequences which will be found with the highest similarity to our products will be extracted from the database and alignment will be done accordingly. Cloning of azurin gene in pGEM®-T easy vector. After that, the azurin/pGEM construct introduced into the Mach1™- T1® E. coli competent cells. Blue/White Screening is to be done for detecting the recombinant product (Recombinant Bacteria). Alternately purified Azurin strain can be cloned into pVAX vector which will eventually form pVAX-Azurin plasmid. A cloned sequence can be evaluated by DNA sequencing. Azurin expression for anticancer activity can be done by Western Blotting and reverse transcription PCR (RT-PCR)

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**Media composition**

The following Media were used during the study. All components were autoclaved at 121°C, 15 psi for 15 minutes unless mentioned otherwise

**Nutrient Agar**

| Component | Amount (g/L) |
|---|---|
| Peptone | 5.0 |
| Sodium chloride | 5.0 |
| Beef extract | 3.0 |
| Agar | 15.0 |
| Final pH | 7.0 |

**Nutrient Broth**

| Component | Amount (g/L) |
|---|---|
| Nutrient Broth | 13.02 |

**Luria Bertani Broth**

| Component | Amount (g/L) |
|---|---|
| Tryptone | 10.0 |
| Yeast extract | 05.0 |
| Sodium chloride | 10.0 |

**Cetrimide Agar**

| Component | Amount (g/L) |
| --- | --- |
| Gelatin peptone | 20.0 |
| Magnesium Chloride | 1.40 |
| Potassium Sulphate | 10.00 |
| Cetrimide | 0.30 |
| Agar | 15.00 |
| Final ph | 7.2 ± 0.2 |

**Reagents**

| | |
|---|---|
| TBE buffer (1x) | 5.4 g Tris-HCl<br>2.75 g Boric acid<br>2ml 0.5M EDTA<br>Adjusting volume with distilled water<br>pH: 8.0 |
| TE buffer | 10 mMTris-Cl (pH 8.0)<br>1 mM EDTA (pH 8.0) |
| LB medium | LB medium |
| Lysis buffer | (10 ml) 9.34 ml TE buffer<br>600 μl of 10% SDS<br>60 μl of proteinase K (20 mg ml-1) |

**SDS Gel (Stacking Gel 5%)**

| Gel Thickness | 1.5 MM |
|---|---|
| Gel Volume | 5 ml |
| Acrylamide/Bis | 0.8 ml |
| 0.625 M Tris/HCL Ph 6.8 (ml) | 1.0 ml |
| 0.5% SDS (ml) | 1.0 ml |
| Dist. $H_2O$ (ml) | 2.2 ml |
| TEMED (µl) | 6.0 µl |
| 10% APS (µl) | 26 µl |

**SDS Gel (Running Gel 10%)**

| Gel Thickness | 1.5 MM |
|---|---|
| Gel Volume | 5 ml |
| Acrylamide/Bis | 3.5 ml |
| 1.88 M Tris/HCL Ph 6.8 (ml) | 2.1 ml |
| 0.5% SDS (ml) | 2.1 ml |
| Dist. $H_2O$ (ml) | 2.8 ml |
| TEMED (µl) | 18.0 µl |
| 10% APS (µl) | 106.0 µl |

## Appendix-III

### Instruments

| Instrument | Company |
|---|---|
| Autoclave | SAARC |
| Freeze (-20°C) | Siemens |
| Incubator | SAARC |
| Micropipette (10-100 μL) | Eppendorf, Germany |
| Micropipette (100-1000 μL) | Eppendorf, Germany |
| pH meter, Model: E-201-C | Shanghai Ruosuaa Technology company, China |
| Pipette (5 mL, ten mL) | Eppendorf, Germany |
| Weighing balance | ADAM Weighing balance EQUIPMENT™, United Kingdom |
| Vortex Mixture | VWR International |
| Shaking Incubator, Model: WIS-20R | Daihan Scientific, Korea |
| Water Bath WiseBath® | Wisd Laboratory Instruments DAIHAN Scientific Co., Ltd Made in Korea |
| Table Top Centrifuge | Model: DSC-200A-2 Digisystem Laboratory Instruments Inc. Made in Taiwan |
| Electronic Balance | RADWAG Wagi ELEktroniczne Model: WTB 200 |
| Magnetic Stirrer, Model: JSHS-180 | JSR, Korea |
| pH Meter: pHep Tester | Hanna Instruments, Romania |