Effectiveness of Vitamin C on Biofilm formation by clinical isolates of *Pseudomonas aeruginosa*

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Declaration

It is hereby declared that

1. The thesis report submitted is our original work while completing a degree at BRAC University.
2. The thesis report does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis report does not contain material that has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. We have acknowledged all main sources of help.

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The thesis/project titled "Isolation and characterization of bacteria from burn-injured patients and detection of their antibiotic sensitivity pattern" submitted by **Syeda Sadia Akther Cynthia (ID:18326009) and Homaira Tasnim (ID: 18326005)** of Summer 2023 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Microbiology.

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Dedicated To

This research is entirely dedicated to our parents for providing us with spiritual, mental, and financial support throughout our lives.

Finally, we thank the Almighty for giving us strength, mental power, and a healthy life.

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Abstract

The experiment started with collecting samples of *Pseudomonas aeruginosa* from a clinical source of burnt patients and storing them in T1N1 media at room temperature. For confirmation of identification of the supplied organism *Pseudomonas aeruginosa,* they were grown in different selective media, and related biochemical tests were carried out for its identification. Next, the ability of the organism to form biofilm was determined. Qualitative and quantitative methods were carried out to see if our tested organism could form biofilms. After successfully forming biofilms with the tested organism, we applied ascorbic acid solutions in different concentrations to assess the effectiveness of vitamin C in reducing the ability of these microorganisms to inhibit biofilm formation. Antibiotic Susceptibility tests were also performed for the tested organism and out of six isolates used, all of them were resistant to at least seven antibiotics of three different classes. As *Pseudomonas aeruginosa* is resistant to antibiotics and there are concerns about its inefficacy of antibacterial medications, alternative ways can be considered to combat such microorganisms. In this study, it was observed that some concentration of ascorbic acid was able to reduce the ability of biofilm formation by *Pseudomonas aeruginosa*. From this study, it was found that Vitamin C at 25.6 µg/mL of concentration in the microtitre plate method and at 50 µg/ /mL concentration in the tube method could inhibit biofilm formation.

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Chapter 1: Introduction

1.1 Background

Pseudomonas aeruginosa is recognized as a major contributor to hospital-acquired infections all around the world. The persistence of prolonged infections is caused by two major virulence factors of *P. aeruginosa*: biofilm development and antimicrobial resistance (Kamali et al., 2020). Medical treatment for *P. aeruginosa* has become more challenging due to the rise of multidrug-resistant (MDR) strains (Moreira et al., 2013). Numerous antibiotics, including Beta-lactams, fluoroquinolones, tetracyclines, and aminoglycosides, have been discovered to cause *P. aeruginosa* to become resistant. An international research project called Multi-drug Resistant Organism Network sought to better understand the clinical and molecular epidemiology of Gram-negative bacteria that are carbapenem-resistant. In this work, *P aeruginosa* infections are widespread, highly lethal, and becoming more and more carbapenem resistant. For this reason, one of the three Critical Priority diseases identified by the WHO is carbapenem-resistant *P aeruginosa* (CRPA) (Reyes et al., 2023). *Pseudomonas aeruginosa* is one of the top three critical pathogens that the World Health Organization (WHO) has designated as requiring immediate research into novel treatments (Tacconelli et al., 2018). Between the nations in the Asia-Pacific region, there were considerable differences in the resistance patterns and related genes. According to a study, Carbapenem resistance (29.3%, 154/525) and gene detection (17.7%, 93/525) were most prevalent in India and carbapenemase genes were more frequently detected in Asian countries than in the Pacific region. (Lee et al., n.d.). Thus, antimicrobial resistance must be controlled by reasonable antimicrobial usage and rigorous infection prevention and control guidelines. To eradicate biofilm-associated infections, we can also use combination techniques based on the appropriate anti-pseudomonal antibiotics and anti-biofilm agents (Kamali et al., 2020). Thus, we investigated the effectiveness of ascorbic acid as an anti-biofilm agent for treating biofilm-associated infections.

1.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a gram-negative bacterium known for its opportunistic pathogenicity. It is commonly found in various environments like soil and water. Some key characteristics include its ability to produce a blue-green pigment, pyocyanin, and a fruity odor. It's highly adaptable, often causing infections in immunocompromised individuals, particularly in hospitals. *P. aeruginosa* is resistant to many antibiotics due to its intrinsic and acquired resistance mechanisms, making it challenging to treat. It causes a variety of infections, including skin, eye, and ear infections, respiratory tract, urinary tract infections, gut-derived sepsis, soft tissue infections, and bone and joint infections (Alsaffar & Mohammed, 2019). *P. aeruginosa* in biofilm states can live in hypoxic environments or other severe conditions (Qin et al.,2022). Additionally, *P. aeruginosa* infection treatments are exceedingly difficult due to its rapid mutations and adaption to gain antibiotic resistance. Furthermore, *P. aeruginosa* is one of the leading bacteria causing hospital-acquired infections, and it is commonly discovered in medical devices (ventilation) because it thrives on damp surfaces (Qin et al., 2022). *P. aeruginosa* is a common pathogen in natural environments and can cause a wide range of illnesses in people, including septicemia, urinary tract infections, burns, and respiratory infections. In the intensive care unit, it is the major contributor of ventilated, comorbid pneumonia (Yang et al., 2011). The proliferation of these bacteria among hospital staff in moist environments, however, might be a source of resistance genes. As a result, it is important to assess how hospital resources and staff affect the path that resistance genes take to spread. We can now learn a complete description of the characteristics of strains isolated from clinical and environmental wards due to genetic approaches supplemented by phenotypic assays (Czekajło-Kołodziej et al., 2006). *P. aeruginosa* is one of the diseases on the Infectious Disease Society of America's list of "ESKAPE" pathogens that represent the greatest concern to public health because of their rising prevalence and the inefficacy of current antibacterial medications (Boucher et al., 2009).

1.3 Virulence factors

P. aeruginosa may adapt to the hostile environment in hosts by secreting several virulence factors that aid in infection and disease transmission. To begin, lipopolysaccharide (LPS) is a key surface structural component that protects the external leaflet and poison host cells, and the endotoxicity of lipid A in LPS allows tissue injury, attachment, and identification by host receptors. Antibiotic tolerance and biofilm development may be linked to LPS. Second, outer membrane proteins (OMPs) help with nutrition exchange, adhesion, and antibiotic resistance. Furthermore, drug resistance caused by biofilm formation is linked to the flagellum, pili, and other adhesins. Fourth, there are six types of secretion systems, including flagella (T6SS-associated), pili (T4SS), and multi-toxin components type 3 secretion system (T3SS), all of which are involved in host colonization, adhesion, swimming, and swarming responding chemotactic signaling. Finally, exopolysaccharides such as alginate, Psl, and Pel may aid in biofilm development while inhibiting bacterial clearance.

T3SS is a complicated system that can severely limit host defense by injecting cytotoxins such as ExoU, ExoT, ExoS, and ExoY, which influence the intracellular environment, particularly by impeding phagocytosis and bacterial clearance. Through ADP ribosylation, exotoxin A (ETA) can limit host protein synthesis. Pyocyanin is also toxic to hosts, causing illness severity, tissue damage, and organ dysfunction. Moreover, lytic enzymes such as LasA and LasB elastases, alkaline protease (AprA), LipC lipases, phospholipase C, and esterase A represent a wide set of virulence factors. Furthermore, rhamnolipid-mediated lung surfactant degradation and tight junction destruction might cause direct damage to the trachea or lung epithelial cells. Additionally, siderophores (pyoverdine and pyochelin) as iron absorption mechanisms aid in bacterial survival in iron-depleted environments, hence increasing virulence strength.

Finally, antioxidant enzymes such as catalases (KatA, KatB, and KatE), alkyl hydroperoxide reductases, and superoxide dismutases counteract ROS activity in phagocyte settings to prevent clearance.

1.4 Biofilm

Biofilms are dense cell aggregations that produce extracellular matrix components that keep the community together. The biofilm mode of growth permits cells to stay close to nutrients, facilitates genetic material exchange, and protects cells from a variety of chemical and environmental stresses, including phagocyte engulfment (Davey & O'toole, 2000). Biofilms are one of the most difficult aspects of treating *P. aeruginosa* infections. A biofilm is composed of a self-secreted matrix made up of proteins (<2%), DNA (<1%), polysaccharides (1–2%), and RNA (<1%), with water as the remainder (97%). Alginate, Psl, and Pel are the three exopolysaccharides responsible for the main components in the biofilm matrix. They carry out several biological functions, especially concerning the protection of bacterial cells from antibiotics and the human immune system (Al-Wrafy et al., 2017).

The intricate structure of the *P. aeruginosa* biofilm adds to the toxicity of this bacteria, resulting in treatment failure, escape from the immune system, and chronic infections that are difficult to eliminate. Biofilms are the underlying cause of a variety of tissue and implant-associated infections. Infections associated with biofilms encompass tooth caries, periodontitis, otitis media, chronic sinusitis, chronic wound changes, musculoskeletal infections (osteomyelitis), biliary tract infection, bacterial prostatitis, native valve endocarditis, and medical device-related infections. *Pseudomonas aeruginosa* is considered a model organism for studying biofilm formation and is the most studied microorganism concerning quorum sensing (QS) (Tuon et al., 2022). This pathogen can impact immunocompromised people due to its capacity to avoid both innate and acquired immune defenses via adhesion, colonization, and biofilm formation, as well as to create numerous virulence factors that cause substantial tissue damage. *P. aeruginosa* is one of the most prevalent pathogens in hospital environments, causing more than 50% of healthcare acquired infections (Kang et al., 2003).

Three *P. aeruginosa* gene clusters were termed cup (chaperone-usher pathway) based on their sequence relatedness to the chaperone-usher fimbrial assembly pathway in other bacteria. The *cupA* gene cluster, but not the *cupB* or *cupC* cluster, is required for biofilm formation on abiotic surfaces. In this study, it was identified a gene (*mvaT*) encoding a negative regulator of *cupA* expression. Such regulatory control was confirmed by several approaches, including *lacZ*

transcriptional fusions, Northern blotting, and transcriptional profiling using DNA microarrays (Vallet et al., 2004).

The ability of Pseudomonas aeruginosa to build biofilms, which are complex colonies of bacteria wrapped in a self-produced matrix, is well documented. Several steps are involved in the creation of a biofilm:

1. Attachment: *P. aeruginosa* cells cling to surfaces via features such as pili and flagella.
2. Colonization: At first, a few bacterial cells stick to the surface and form microcolonies.
3. Exopolysaccharide Matrix Production: Bacteria release extracellular polymeric substances (EPS) such as polysaccharides, proteins, and DNA, which form a protective matrix around microcolonies.
4. Maturation: The structure of the biofilm becomes more structured, with routes for nutrition and waste exchange.
5. Detachment: Mature biofilms can release planktonic cells into their surroundings, allowing for continued colonization.
The biofilm matrix protects the infection from external dangers such as antibiotics and the immune system, making infection treatment difficult. The matrix also improves nutritional access, which improves bacterial survival and growth.

Among the biofilm-related virulence mechanisms of *P. aeruginosa* are:
1. Antibiotic Resistance: The matrix physically prevents antibiotics from reaching the bacterial cells, and the biofilm's slow-growing or dormant bacteria are less vulnerable to antibiotics.
2. Quorum Sensing: Bacteria within the biofilm communicate by quorum sensing, which regulates their collective behavior. This can result in coordinated expression of virulence factors.
3. Iron Scavenging: *P. aeruginosa* can create siderophores, which are molecules that scavenge iron from the environment. This may provide the bacteria an advantage in biofilms.
4. Immune Response Resistance: The biofilm matrix prevents immune cells from successfully accessing and destroying bacterial cells.
Overall, *P. aeruginosa* biofilms play an important role in chronic infections because they are more durable and difficult to eliminate than free-floating bacteria.

1.5 Vitamin C / Ascorbic acid:

Vitamin C, also known as ascorbic acid, is a water-soluble essential nutrient that plays a crucial role in various biological processes. Here are some key points about vitamin C. There is considerable concern about the pandemic increase in antibiotic-resistant bacteria leading to treatment failure (Hancock and Speert, 2000). Vitamin C (ascorbic acid) represents one such alternative, and many studies have demonstrated its antibacterial action. Initially, it was thought that vitamin C's antibacterial activity was owing to its pH-lowering impact. Another study, however, demonstrated that vitamin C has significant antibacterial effects against *Streptocccus pyogenes* even under pH-neutral settings. Furthermore, vitamin C is a potent antioxidant that protects the body against free radicals and reactive oxygen species (Slade and Knox, 1950). In this study, we have taken the concentration of different citrus fruits like oranges and lemons as standard concentration to see the effectiveness of ascorbic acid in Biofilm reduction.

Chapter 2: Materials and Methods

2.0 Study design

- Sample processing and confirmation
- Biofilm formation
- Effect of Vitamin C on Biofilm

2.1 Working laboratory

All project works were performed in the Microbiology Laboratory, Department of Mathematics and Natural Sciences, BRAC University.

2.2 Sample collection

In this study, previously identified clinical isolates of *P. aeruginosa* were used from burn patients admitted to the burn and plastic surgery unit of Sheikh Hasina National Institute of Burn and Plastic Surgery, Dhaka, Bangladesh.

2.3 Confirmation of the sample

Different physical and biochemical tests were conducted to confirm *P.aerugesona.* As well as the cultural properties of the organisms were observed by using plate culture methods.

2.3.1 Media for plate culture method

- Nutrient agar
- Cetrimide agar
- Mac Conkey

In this method, Nutrient agar (NA) media was used to subculture the bacteria. Then bluish-green colonies from the nutrient agar plate were transferred to Cetrimide agar, which is the standard selective media for *P. aerugesona,* and for further confirmation, the colonies were streaked on Mac Conkey media.

Preparation of Nutrient agar :

- For the preparation of NA, the standard form was 28.0g for 1000 ml of distilled water. We prepared our required amount using it as a standard.
- Then it was boiled to dissolve properly.
- For sterilization, the media was autoclaved at 121°C for 15 minutes.
- Then it was poured into sterile petri dishes under sterile conditions and used after solidifying the agar.

Preparation of Cetrimide agar:

- For preparing this, the standard measurement was 45.3g of cetrimide agar for 1000 ml of distilled water.
- Then it was heated to dissolve the mixture properly.
- For sterilization, it was autoclaved at 121°C for 15 minutes.
- Then it was poured into sterile petri dishes.
- After solidifying the media, it was used for streaking or stored in a refrigerator for further use.

Preparation of Mac Conkey agar :

- To prepare this medium, 49.53 g of Mac Conkey agar was used for 1000 ml of distilled water.
- Then it was boiled to dissolve the agar properly.
- For sterilization, it was autoclaved at 121°C for 15 minutes.
- Then it was poured into sterile petri dishes under sterile conditions to solidify the agar before use.

2.3.2 Physical characterization :

I. **Colony morphology:** This method was used to describe the characteristics of the bacterial colony developing on agar in a Petri dish. Different bacterial colonies have different shapes, sizes, colors, and textures in agar media. Under sterile conditions in a laminar airflow cabinet, the bacterial strain was streaked on Nutrient agar, Cetrimide

agar, and Mac Conkey agar plates for this examination. Then the culture plates were incubated at 37°C for 24 hours. After incubation, cultural properties were observed and recorded.

II. **Gram staining :**

- A small amount of the bacterial isolate was smeared over clean slides after adding one drop of distilled water, and the slides were then air-dried and heated twice or three times over a flame to fix them.
- After that, crystal violet was added and kept for one minute. Then washed with distilled water.
- Then Gram's Iodine was added to the smear for one minute and washed with water.
- Then the slides were decolorized with 95% ethyl alcohol for 15 seconds.
- The slides were then counterstained for about 30 seconds with safranin and then washed with water. After being air-dried, the slides were observed under a microscope.
- Cells were then distinguished by color under the microscope. Purple for gram-positive bacteria and pink or red for gram-negative bacteria.

2.3.3 Biochemical identification: To confirm the presence of *aeruginosa*, numerous biochemical tests were carried out following the protocol outlined in the Microbiology Laboratory Manual (Cappuccino, J.G., and Sherman, N., 1996). For the sample, several biochemical tests were performed. Such as the IMViC test (Indole production test, Methyl Red test, Voges-Proskauer test), Citrate utilization test, Triple Sugar Iron (TSI) test, and Oxidase test. The bacterial cultures were incubated at 37°C on nutrient agar plates in an incubator before the initiation of any biochemical identification.

I. **Indole production test:**

- Bacteria were inoculated into 5 ml of peptone broth.
- Then it was incubated at 37°C for 24 hours.
- After that, 5 drops of Kovac's reagent were added.

- The appearance of a ring-shaped pinkish red color on the top layer indicated the positive result. And absence of this color indicated negative results.

II. **Methyl red (MR) test:**

- Bacteria were inoculated into 5 ml of MR VP broth which contained peptone, buffers, and dextrose.
- Then it was incubated at 37°C for 24 hours.
- The mixed-acid-producing organism can produce sufficient acid within this incubation period to overcome the phosphate buffer and remain acidic.
- Then the pH of the medium was observed by adding 5 drops of MR reagent which was a methyl red indicator.
- Development of a red color was taken as MR positive and MR negative organisms would give a yellow color.

III. **Voges–Proskauer (VP) test:**

- Bacteria were inoculated into 5 ml of MR VP broth which contained peptone, dextrose and a phosphate buffer.
- Then it was incubated at 37°C for 24 hours.
- After 24 hours, 10 drops of Barritt's reagent A was added and the cultures were shaken.
- 10 drops of Barritt's reagent B were added immediately and the cultures were shaken again.
- After 15–20 minutes, the color was observed, and the results were recorded.
- The development of a red color was indicated as VP-positive, and no color development was indicated as a negative result.

IV. **Citrate utilization test :**

- A single bacterial colony was inoculated into a vial containing 3 ml of Simmon's citrate agar and incubated at 37°C for 24 hours.
- The organism that could use citrate would change the media's color during these 24 hours.
- Development of Prussian blue indicated a positive result, and no colour development indicated a negative result.

V. Triple Sugar Iron test (TSI) :

- Triple-sugar iron slants were prepared in a test tube containing dextrose, lactose, and sucrose.
- A single bacterial colony from a 24-hour incubation on a nutrient agar plate was stabbed and streaked into this test tube using a sterile needle.
- The tubes were incubated at 37°C for 24 hours and then examined.
- A yellow color in the butt indicated that the organism was capable of fermenting all three sugars, while a red color in the slant and butt indicated that the organism was a nonfermenter.
- A black color in the butt of the tube indicated H_2S production.
- CO_2 gas production was indicated by the presence of bubbles, splitting, and cracking in the medium.

VI. Oxidase test:

- Two drops of oxidase reagent (p-Aminodimethylaniline oxalate) were put into a filter paper (Whatman, 1MM).
- A toothpick was used to take one well-isolated colony from a pure 24-hour culture and rub it into the filter paper.
- Within 1 to 30 seconds, a positive reaction would change the color of the filter paper to purple.

VII. Catalase test :

- One drop of catalase test reagent, which is 3% hydrogen peroxide, was added to a sterile microscopic slide.
- Bacteria from a 24-hour pure culture were picked up using a sterile loop and placed on the reagent drop.
- An instant bubble formation indicates a positive result.

2.4 Preservation of sample

Short term stock

- A sterile vial that contained 3 ml of T1N1, or Tryptone Salt agar, was inoculated by stabbing bacterial isolates from nutrient agar plates.
- Then the vials were incubated at 37°C for 24 hours.
- After incubation, the surface of the medium was covered with 200 µl sterile paraffin oil, and the vials were stored at room temperature.

Long term stock :

- Bacterial cultures were grown in 500 µl of trypticase Soy Broth at 37°C for 6 hours in a sterile vial.
- Then 500 µl of sterile glycerol was added to the broth culture, and the vial was stored at -20°C.

2.5 Antibiotic Sensitivity Testing:

Different types of methods can be used for antibiotic sensitivity testing, but among them, the most commonly used disk diffusion method was used in this present study, which is known as the Kirby- Bauer method.

Preparation of inoculum:

- At first, a fresh nutrient media culture plate of the organism was selected to perform the test.
- Then, using a sterile loop a colony from the fresh plate was collected and dipped in the test tube containing sterile saline solution and it was mixed gently to ensure that no solid material from the colony is visible in the saline solution.
- Finally, the test tube was vortexed properly so that the suspension became homogeneous. (Labtronics; ISO 9001: 2008 Certified).

Inoculation on Muller Hinton Agar (MHA) plates:

Muller Hinton agar plates were prepared which is a non-selective, non-differential microbiological growth medium. Then, the surface of the Muller-Hinton Agar plate was lawned by a sterile cotton swab with the representative bacterial suspension prepared in physiological saline which was compared with McFarland standard 0.5 solutions. McFarland standard 0.5 solution indicates the density of 1.5×10^{8} CFU (Colony Forming Unit) per ml. However, it was made sure that the cotton swab should be swiped multiple times at different angles for the equal distribution of bacterial suspension.

Placing the antibiotic disks on MHA plates:

Antibiotic discs were placed carefully on the solidified lawned agar plate by using sterile forceps. Around 10 antibiotics were used for this study. After placing the disks, the plates were covered by the lid and were not inverted, then the plates were incubated at 37°C for 24 hours.

Measuring zone:

After the incubation period, the zone of inhibition was measured in millimeters with a ruler. The antimicrobial pattern was determined by the presence or absence of a clear zone around the antibiotic disc. Finally, antibiotic resistance patterns were categorized by the guidelines of Clinical and Laboratory Standards Institute (CLSI).

Table 01 : Antibiotic disks used for *Pseudomonas aeruginosa*

Name of antibiotics group	Name of Antibiotic	Concentration
Penicillin	Amoxicillin	30 µg
	Ampicillin	10 µg
	Amoxyclav	30 µg
Polymyxin class	Colistin sulfate	10 µg
Fluoroquinolones	Levofloxacin	5 µg
	Ciprofloxacin	5 µg
Cephalosporin	Cefixime	5 µg
	Ceftriaxone	30 µg
Aminoglycoside	Amikacin	30 µg
	Kanamycin	30 µg
	Streptomycin	10 µg
Carbanem	Meropenem	10 µg
	Imipenem	10 µg

Diaminopyrimidines	Trimethoprim	25 µg
Macrolides	Azithromycin	15 µg

2.6 Biofilm Formation

2.6.1 Qualitative techniques

Tube Method: This qualitative method was used by observing biofilm that had adhered to the tube's walls and bottom. Both tubes and vials were used to perform this process.

- Using a sterile loop, one or two colonies of the organisms to be tested were taken from the subculture plate and inoculated in 5 ml of fresh LB or Luria-Bertani broth with 1% glucose in each test tube.
- Then the tubes were incubated at room temperature for 3 days.
- After incubation, the bacterial suspensions were removed and each tube was washed with sterile saline solution (0.9% NaCl) and dried.
- Tubes were then stained with 0.1% crystal violet for 10 minutes.
- Then the tubes were washed with distilled water to remove the crystal violet that did not specifically stain the adherent bacteria.
- After that, the tubes were dried in an inverted position at room temperature.
- Then the results were observed, and the formation of a visible ring at the wall of the bottom of the tube was considered positive for biofilm formation.

- The same procedure was followed for observing biofilm formation in a vial.

2.6.2 Quantitative techniques

Microtiter Plate method: This quantitative method was used for the measurement of biofilm using the ELISA machine. This method is considered a more reliable and standard method for the detection of biofilm.

- Bacteria were inoculated in 5 ml of LB broth with 1% glucose for enrichment in a falcon tube compared with 0.5 McFarland standard solution. Here, McFarland standard 0.5 solution indicates the density of 1.5×10^8 CFU (Colony Forming Unit) per ml ("Preparation of Routine Media and Reagents Used in Antimicrobial Susceptibility Testing," 2016).
- Then the falcon tubes were incubated at 37°C in a shaking incubator at 120 rpm for 24 hours.
- After incubation, a fresh 96-well microtiter plate was taken and 200 µl of enriched bacterial suspension where cell count for 0.5 McFarland is 3×10^7 CFU was placed in the wells of the microtiter plate. Fresh LB broth was used as a negative control for this test.
- The plate was then covered and incubated at room temperature for 3 days to grow and measure the biofilm.
- After the incubation period, the bacterial suspension was removed, and each well was washed three times with sterile saline solution (0.9% NaCl).
- Then 225 µl of 0.1% crystal violet solution was placed in each well and stained for 10 minutes.
- The crystal violet solution was then removed by shaking the microtiter plate. Then the plate was washed with distilled water and shaken properly. Any crystal violet that did not particularly stain the adhering bacteria was removed during this step.
- To get rid of any extra cells and dye, the microtiter dish turned upside down and vigorously tapped on a paper towel. The plate was then dried by air.
- After the plates were completely air dried, 250 µl of 30% glacial acetic acid solvent was added to each stained well to solubilize crystal violet.

- The microtiter plate was incubated at room temperature for 15 minutes.
- 250 µl of the solubilized content of each well was transferred to a fresh flat-bottomed microtiter dish after being briefly mixed by pipetting.
- Finally, the optical density (OD) was measured at a wavelength of 570 nm by using an ELISA machine.

2.7 Effect of Vitamin C on Biofilm Production

The effect of vitamin C on the biofilm-producing isolates of *P. aeruginosa* was measured by the microtiter plate method and also the tube method, which was previously mentioned. Different concentrations of filtered ascorbic acid solution were used in this test. The concentrations of ascorbic acid to be used were :

- 0.16 mg/mL
- 0.3 mg/mL
- 0.5 mg/mL
- 0.8 mg/mL
- 2.5 mg/mL

Tube method: The bacteria to be tested were inoculated in 5 mL of LB broth with different concentrations of ascorbic acid in different test tubes. By adding different concentrations of ascorbic acid, it was possible to determine the most effective concentration that could reduce or prevent biofilm formation. The concentration of the added vitamin C were 32 µl, 60 µl, 100 µl, 160 µl and 500 µl which brings the final concentration to 5.12 µg/mL,18 µg/mL,50 µg/mL,128 µg/mL, and 1250 µg/mL respectively in the tubes. Then the tubes were incubated at room

temperature for 72 hours. After incubation, the bacterial suspension was removed and stained to observe whether the ring had formed.

Microtiter plate :

- A fresh 96-well microtiter plate was taken, and 10 µl of enriched bacterial suspension where cell counts for 0.5 McFarland is 1.5×10^6 CFU/mL (because standard concentration at 0.5 McFarland is 1.5×10^8 CFU/mL) was placed in the first row of the well of the plate. Then, 232 µl of fresh LB was placed in that well. Then, 0.8 mg/mL concentration of ascorbic acid or 8 µl of ascorbic acid solution was added to the wells which brings the final concentration to 25.6 µg/mL.
- Next, in the second row, 10 µl of 0.5 McFarland compared bacterial suspension were placed in the wells. Then, 235 µl of fresh LB and 0.5 mg/mL concentration or 5 µl of ascorbic acid solution was added to those wells which brings the final concentration to 10 µg/mL.
- Similarly, in the third row, 10 µl of 0.5 McFarland compared bacterial suspension was added to the wells. Then, 237 µl of fresh LB and 3 µl of ascorbic acid solution or 0.3 mg/mL concentration of ascorbic acid were added to each well of that row which brings the final concentration to 3.6 µg/mL.
- Then, in the same way, 10 µl of 0.5 McFarland compared bacterial suspension were added in the 4th row with the 0.16 mg/mL concentration of ascorbic acid or 1.6 µl of ascorbic acid solution which brings the final concentration to 1.024 µg/mL. Also, 237.5 µl of fresh LB were added to the wells.
- Finally, 10 µl of 0.5 McFarland compared bacterial suspension was added in the fifth row with 25 µl of ascorbic acid solution or 2.5 mg/mL concentration of ascorbic acid. Also, 215 µl of fresh LB was added to each well of that row which brings the final concentration to 250 µg/mL.
- Fresh LB broth was used as a negative control and bacterial suspension without ascorbic acid solution was used as a positive control for this test.
- Then the microtiter plate was covered at room temperature for 72 hours to measure the optical density (OD) with an ELISA reader and observe the effectiveness of vitamin C.

Chapter 3 : Results

3.1 Confirmation of sample

A clinical strain was identified routinely to distinguish *Pseudomonas aeruginosa* by streaking on nutrient agar and selective media. The results of clinical strain of *P.aeruginosa* obtained from patients admitted to the burn and plastic surgery unit of Sheikh Hasina National Institute of Burn and Plastic Surgery, Dhaka, Bangladesh were confirmed to be *P. aeruginosa* by physical and biochemical characterization.

3.2 Results of physical characterization

The clinical isolate was preliminary identified by its cultural properties upon streaking on nutrient agar media and then in the selective media. *Pseudomonas* may grow in a variety of temperature ranges, but the optimum growth temperature is 37°C. Cultural characteristics include: colonial morphology (smooth mucoid) produces large, opaque, flat colonies with irregular margins and distinctively fruity odor colonies. In nutrient agar plates, it was observed that the growing colonies were identified according to pigments and odor production (grape-like odor),

After 24 hours incubation at 37°C the colonies were subcultured on cetrimide agar plates to be viewed under UV Transilluminator (WealtecCorporation, USA) to check if it glows. It was

observed that the colonies appear pigmented bluish-green with fluorescence. Additionally, the growing colonies were identified according to their ability to ferment lactose on MacConkey Agar.

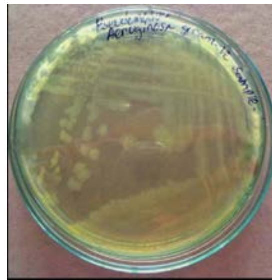


Figure 1: ***Pseudomonas*** **in nutrient agar**

Figure 2: ***Pseudomonas*** **in cetrimide agar**

Figure 3: ***Pseudomonas*** **in MacConkey agar**

Figure 4: ***Pseudomonas*** **in cetrimide agar, viewed under UV transilluminator**

3.3 Result of gram staining and cell morphology observation of 24 hours culture

Pseudomonas is a strict aerobe, motile Gram-negative bacteria, so from microscopic examination, we observed some pink rod-shaped motile arrangements of cells with each other.

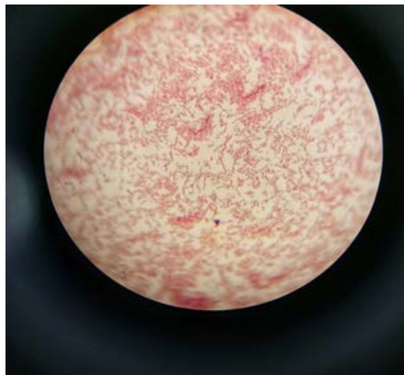


Table 02: Biochemical test results of sample bacteria and their probable identification

Organism	Biochemical tests									
	Indole production test	MR test	VP test	Citrate utilization test	TSI test				Oxidase test	Catalase test
					Slant	Butt	CO_2	H_2S		
Sample bacteria	-	-	-	+	K	K	-	-	+	+

Key: **'+'** = positive, **'-'** = negative, **'K'** = alkaline conditions

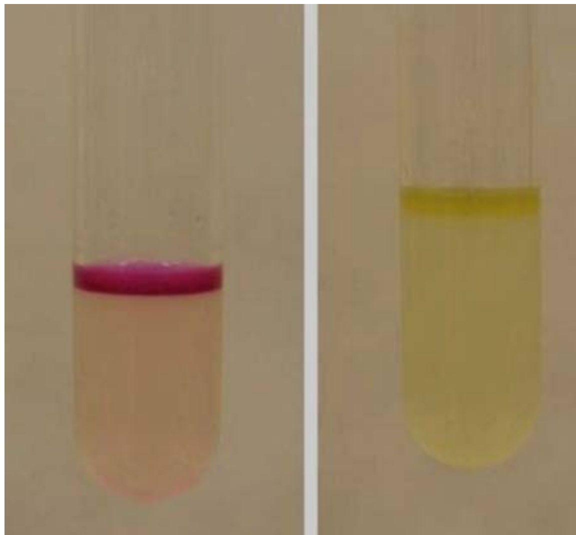


Figure 6: Result for indole production

- Colour remains yellow for the clinical strain showing negative results (right)
- Positive control (left)

Figure 7: Result for MR test

- Colour remains yellow for the clinical strain showing negative result (right)
- Control (left)

Figure 8: Result for VP test

- Colour remains yellow for the clinical strain showing negative result (right)
- Control (left)

Figure 9: Result of Citrate utilization test

- Colour changes to bright blue from green for the clinical strain showing negative results (right)
- Control (left)

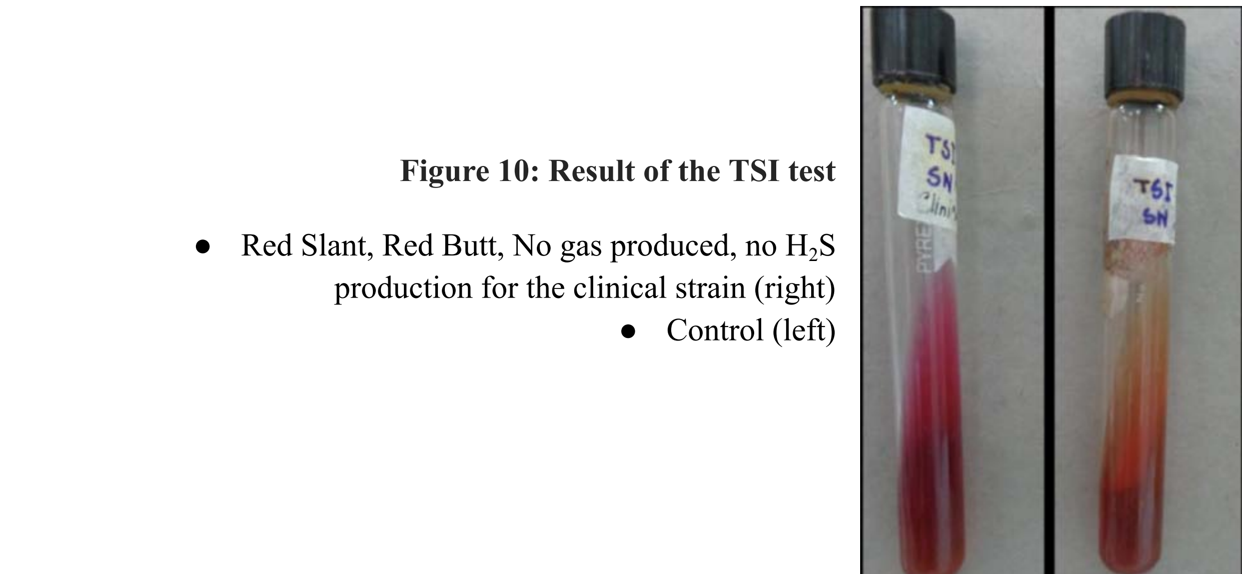
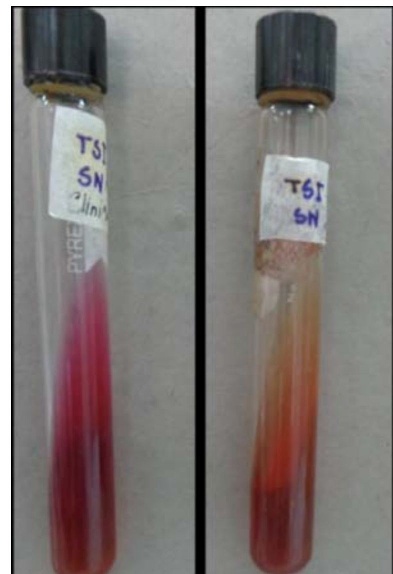


Figure 10: Result of the TSI test

- Red Slant, Red Butt, No gas produced, no H_2S production for the clinical strain (right)
- Control (left)



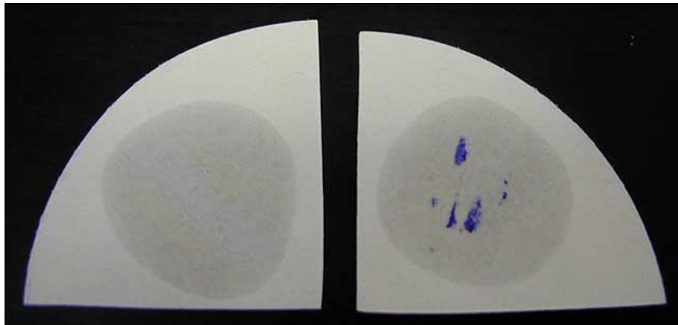


Figure 11: Result for oxidase test

- Colour changes to purple showing positive results for the clinical strain (right)
- Control (left)

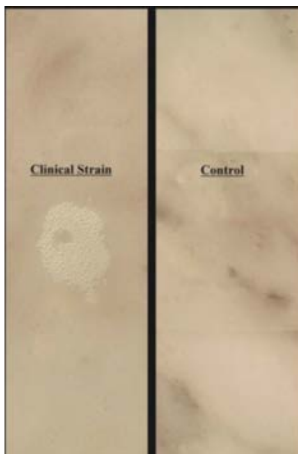


Figure 12: Result of Catalase test

- Formation of bubble detected for the clinical strain showing positive result (left)
- Control (right)

3.5 Result of antibiotic susceptibility test

Different inhibition zones were seen around the antibiotic disc. For some antibiotics, there is no inhibitory zone, so the bacteria are resistant to those antibiotics. By measuring the zone diameter of the antibiotic, the sensitivity or resistance pattern of those antibiotics against the bacteria was found.

Figure 13: Antibiotic susceptibility test for *Pseudomonas aeruginosa*

Table 03: Zone diameter interpretive standards (mm) for *Pseudomonas aeruginosa* by the guidelines of the Clinical and Laboratory Standards Institute (CLSI) are given below:

Name of antibiotics group	Antibiotics	Sensitive	Intermediate	Resistant
Penicillin	Amoxicillin (AML 30)	≥18	14-17	≤13
	Ampicillin (AMP10)	≥17	14-16	≤13
	Amoxyclav (AMC 30)	≥20	18-19	≤17
Polymyxin class	Colistin sulfate (CT10) (CL10)	≥14	12-13	≤11
Fluoroquinolones	Levofloxacin (LEV5)	≥17	14-16	≤13
	Ciprofloxacin (CIP5)	≥21	16-20	≤15
Cephalosporin	Cefixime (CFM 5)	≥19	16-18	≤15
	Ceftriaxone (CTR 30)	≥25	22-24	≤21

Aminoglycoside	Amikacin (Ak30)	≥17	15-16	≤14
	Kanamycin (K30)	≥18	14-17	≤13
	Streptomycin (S 10)	≥15	12-14	≤11
Carbanem	Meropenem (MEM 10)	≥23	20-22	≤19
	Imipenem (IMP10)	≥23	20-22	≤19
Diaminopyrimidines	Trimethoprim (COT25)	≥16	11-15	≤10
Macrolides	Azithromycin (AZM 5)	≥18	14-17	≤13

Table 04: Antibiotic sensitivity pattern of the isolated bacteria

	Used antibiotics									
Sample ID	**AMP /AML**	**AMC**	**CT/ CL**	**LEV/ CIP**	**CFM**	**AK/ KK/ S**	**IPM/ MEM**	**COT**	**CTR**	**AZM**
4766	R	S	R	R	R	S	R	R	R	R
4367	R	R	R	R	R	R	R	R	R	R
4792	R	S	R	R	R	S	R	R	R	R
4373	R	S	R	R	R	S	S	R	R	I
4780	R	R	R	R	R	R	S	R	R	S
4765	R	S	S	R	R	R	I	R	R	S

Key: "S"=Sensitive, **"R"**=Resistant, **"I"**=Intermediate

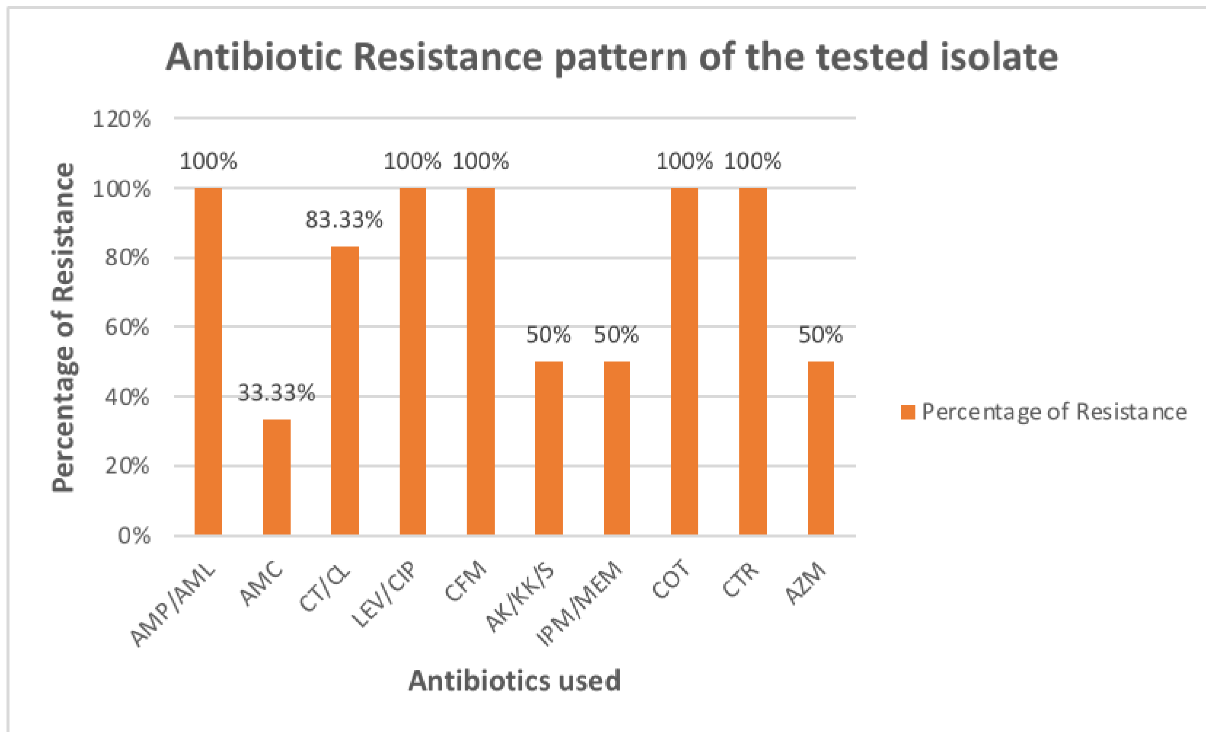


Figure 14: Percentages of antimicrobial resistance patterns in tested isolates

It was observed that the tested isolate was highly resistant (100%) to seven antibiotics which indicates its multidrug-resistant characteristics, 83.33% resistance to CT/CL, 50% resistance to AK/KK/S, IPM/MEM and AZM antibiotics, and 33.33% resistant to AMC.

3.6 Result for biofilm formation

Qualitative technique

As a qualitative measurement, we carried out the tube method to see biofilm formation adhering to the walls of tubes and vials. It was seen that there was a formation of rings at the wall and bottom of the tubes and vials used for staining. The ring formation represented a biofilm ring adhering to the surface of the tubes and vials, giving a positive result.

Figure 15: Ring formation in tube

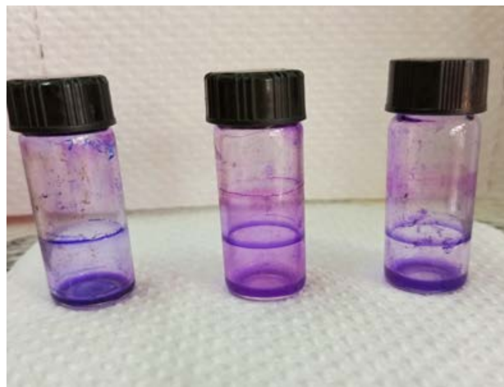


Figure 16: Ring formation in the vial

Quantitative technique

For quantitative analysis, we checked the results of growing and analyzing static biofilms by a 96-well microtiter plate biofilm assay. The static biofilms were grown with the clinical strain in the wells of the microtiter plate. The optical density was measured using an ELISA machine (Multiskan, Finland) after 3 days of incubation at room temperature to assess the bacterial attachment by measuring the staining of the adherent biomass. The optical density of the remaining crystal violet and glacial acetic acid solution in each well of the optically clear flat-bottom 96-well plate was measured by an ELISA machine at 570 nm. It was observed that in the raw solution, the optical density was higher than in the diluted solution. Biofilm formation was classified into Strong, moderate, and weak/nonbiofilm producers according to the criteria of Stepanovic *et al. (2007)*

Table 05: Average optical density value for biofilm production of *Pseudomonas aeruginosa* in a microtiter plate according to criteria of Stepanovic *et al. (2007)*

Average OD value	Biofilm formation
<0.064	Negative
0.064-0.124	Weak positive
0.124-0.248	Moderate positive
>0.248	Strong positive

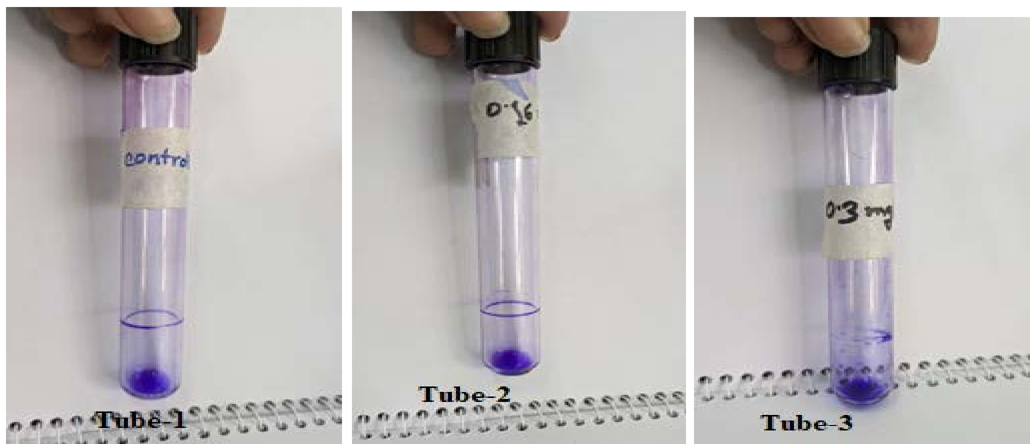
Table 06: Absorbance reading of 3 days biofilm screening by ELISA machine

No of isolates (6)	**Biofilm formation**	**Quantitative analysis, *n* (%)**
	Strong positive	6 (100%)
	Moderate positive	0(0%)
	Weak positive	0(0%)
	Negative	0 (0%)

So, Table 6. shows that all of the tested isolates could form strong biofilms in microtiter plates.

3.7 Result of the effectiveness of vitamin C on biofilm

In Tube : Ring formation was observed at the wall and bottom of the tubes and vials used for staining after the addition of different concentrations of ascorbic acid.



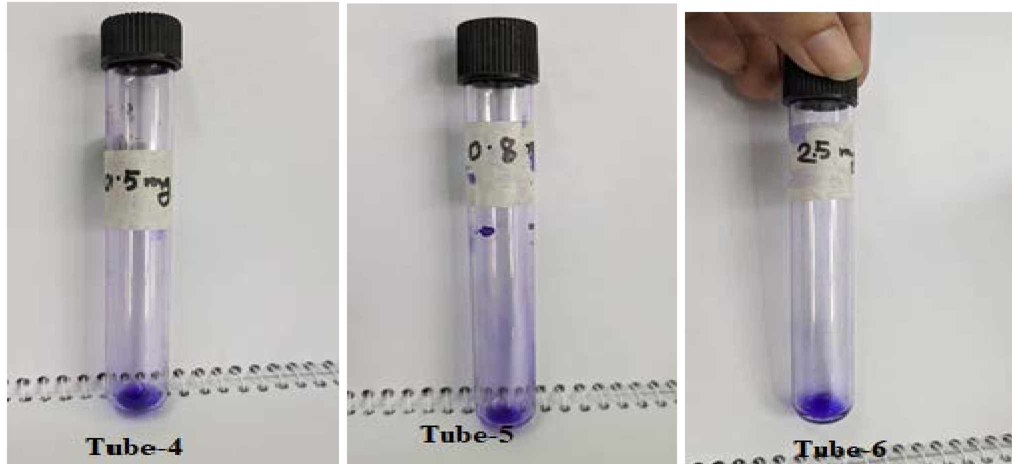


Figure 17: Result of effectiveness of Vitamin C in Tube Method

By comparing the control tube (Tube 1: 5ml bacterial suspension in LB), ring formation was observed in Tube 2 (5 mL Bacterial suspension in LB+ 32 µL of 0.16 mg/mL to bring final conc. 5.2 µg/mL), in Tube 3 (5mL Bacterial suspension in LB+ 60 µL of 0.3 mg/mL to bring final conc. 18 µg/mL), in Tube-4 (5mL Bacterial suspension in LB+ 100 µL of 0.5 mg/mL to bring final conc. 50 µg/mL), in Tube- 5(5mL Bacterial suspension in LB+ 160 µL of 0.8 mg/mL to bring final conc. 128 µg/mL) and in Tube-6 (5mL Bacterial suspension in LB+ 500 µL of 2.5 mg/mL to bring final conc. 1250 µg/mL). It shows that biofilm formation started to inhibit from Vitamin C concentration of 50 µg/mL

In microtiter plate :

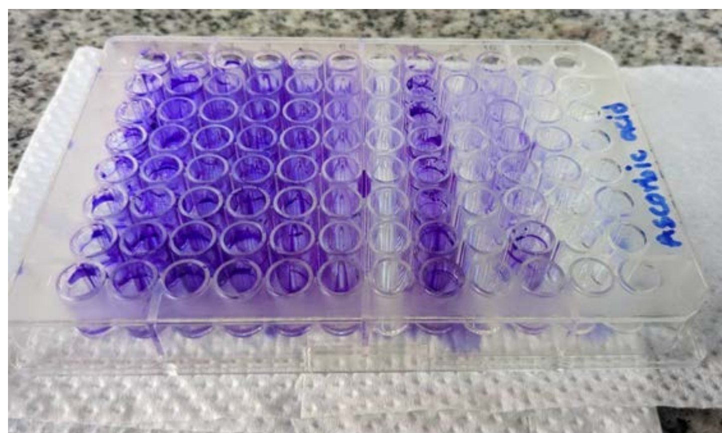


Figure 18: Measuring optical density in microtiter plate

Table 07: Optical density reading in microtiter well plate

			Optical density					
	Concentration of vitamin C	**Final Vit-C concentration in wells**	**1**	**2**	**3**	**4**	**5**	**6**
A	0.16 mg/ml	1.024 µg/ml	0.706	0.980	0.679	1.031	0.909	0.794
B	0.3 mg/ml	3.6 µg/ml	0.620	0.431	0.321	0.352	0.599	0.434
C	0.5 mg/ml	10 µg/ml	0.156	0.098	0.060	0.109	0.106	0.101
D	0.8 mg/ml	25.6 µg/ml	0.060	0.045	0.069	0.080	0.070	0.052
E	2.5 mg/ml	250 µg/ml	0.052	0.049	0.059	0.046	0.060	0.059
F	+(ve) without vitamin C	-	1.450	1.401	0.950	0.821	0.620	0.591
G	-(ve) LB solution	-	0.020	0.012	0.032	0.021	0.019	0.015

Table 08: Average optical density and measurements for the biofilms of the bacteria after adding vitamin C in different concentration

	Concentration of vitamin C (mg/mL)	Final concentration of Vitamin C (µg/mL)	Optical density	Biofilm formation
A	0.16	1.024	0.850	Strong
B	0.3	3.6	0.460	Strong
C	0.5	10	0.105	Weak
D	0.8	25.6	0.063	Negative
E	2.5	250	0.054	Negative
F	+(ve)	-	0.972	Strong

G	-(ve)	-	0.020	Negative

We used five different concentrations of vitamin C solution. After three days of incubation, drastic changes in biofilm formation were observed. It was observed that ascorbic acid solutions can inhibit the formation of biofilms. We got the most effective result from a concentration of 25.6 µg/ /mL of Vitamin C concentration in the microtitre plate method. It was also shown that the higher the concentration of ascorbic acid solution, the lower the optical density.

Previously it was observed that all the tested isolates of *Pseudomonas aeruginosa* could form strong biofilms, but after adding vitamin C, their biofilm formation ability was inhibited. Vitamin C at 25.6 µg/mL of concentration in the microtitre plate method and at 50 µg/ /mL concentration in the tube method could inhibit biofilm formation, and at a concentration of 10 µg/ml, weak biofilms were formed in the microtiter plate.

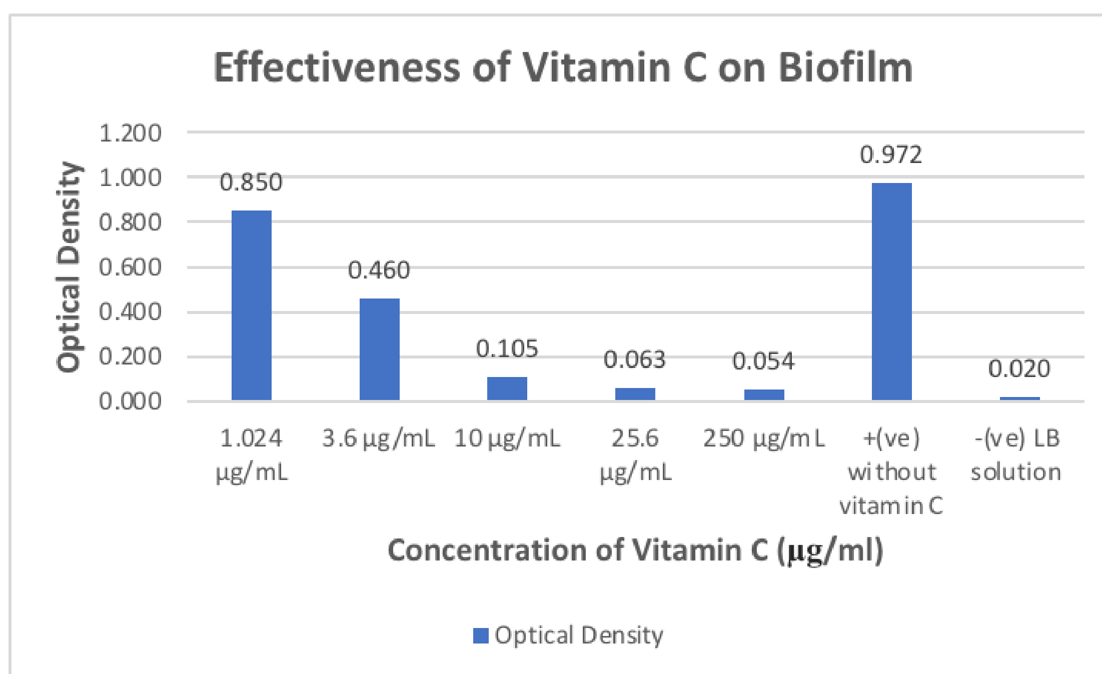


Figure 19: Bar chart showing effectiveness of Vitamin C on Biofilm reduction. It was observed that biofilm started to reduce from a concentration of 25.6 µg/ /mL of Vitamin C concentration in the microtitre plate method.

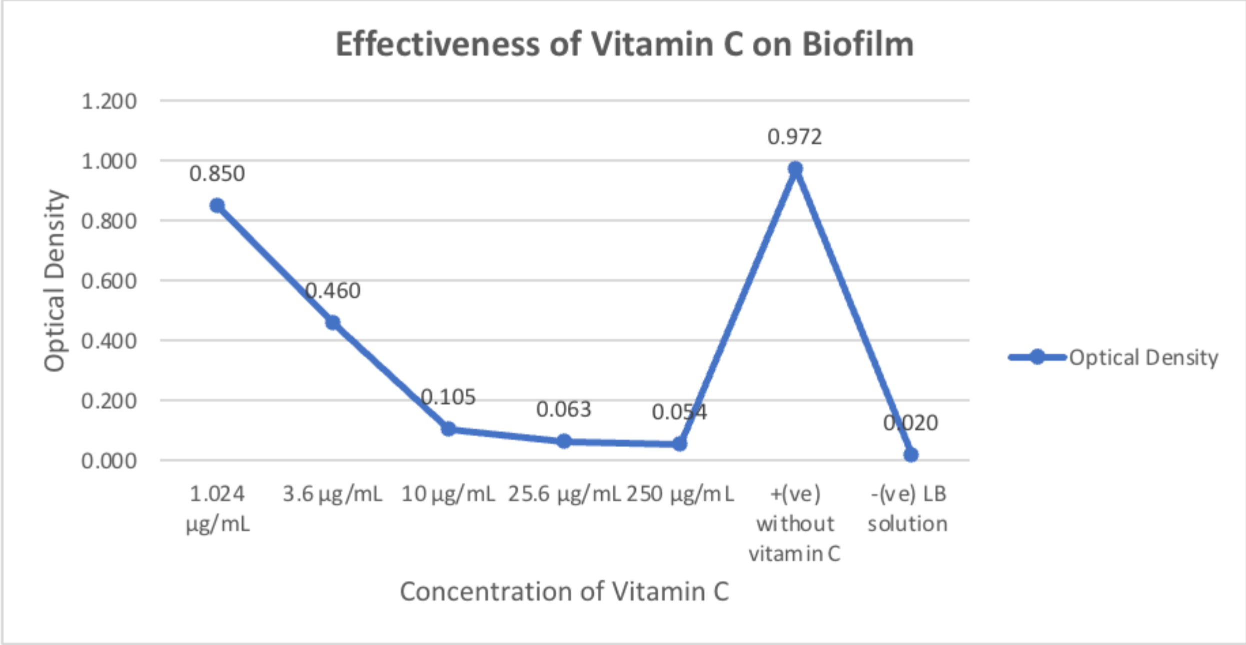


Figure 20: Graph showing the higher the concentration of vitamin C the lower the optical density

Chapter 4 : Discussions

Discussion:

This experiment was started with the clinical isolates of *P. aeruginosa*, which were collected from burn patients. The biofilm sources were chosen from the clinical isolates of *Pseudomonas aeruginosa* because burn patients are more vulnerable to getting nosocomial infections from *Pseudomonas aeruginosa*, and this bacteria forms biofilms more frequently, which can be difficult to treat. Patients suffering from severe burns are more susceptible to infections caused by *Pseudomonas* species (Pruitt, 1974). Burn victims frequently get nosocomial infections, in which multidrug-resistant bacteria enter the body and cause sepsis or death. *Pseudomonas aeruginosa* is known as the most resistant organism to nosocomial infections, which cause 10–11% of all nosocomial infections (Labovská, 2020). According to the National Institutes of Health, 75% of all microbial infections in humans are related to biofilms (Miquel et al., 2016). As confirmed in this current study, opportunistic human infections that build biofilms like *P. aeruginosa* are becoming more and more resistant to various antibiotics. As it is known, this key

opportunistic pathogen *P.aeruginosa* is characterized by its high-level multiple antibiotic resistance and biofilm formation ability (Zang et al., 2013). Treatment of these infectious diseases has become more difficult as a result of the steady rise in the population of immunosuppressed patients and the ability of bacteria to quickly evolve and adapt antibacterial agents. As a result of careless and incorrect antibiotic use, resistant bacteria have increasingly replaced sensitive ones (Hellinger WC, 2000). This is especially true for the opportunistic pathogen *P. aeruginosa*, which has the ability to be multidrug-resistant. In this experiment, we use various concentrations of ascorbic acid solution to test how well it affects the biofilm formed by *Pseudomonas aeruginosa.* We found that a certain concentration of ascorbic acid solution can inhibit the production of biofilm.

In order to confirm our target organism, the samples were streaked on nutrient agar and selective media, and the cultural properties of the organism were observed and recorded. Brown colonies on MacConkey agar and luminous colonies in cetrimide agar under UV light were used to confirm the presence of *P. aeruginosa* in the selective media. For further confirmation that the strain was *P. aeruginosa*, various physical and biochemical tests were performed, and it was assured that all the tests gave the exact results that are standard for *P. aeruginosa*. When conducting a physical test such as gram staining, pink rods were seen under a light microscope with an oil immersion at a magnification of 100x, indicating that the organism was gram negative. Furthermore, biochemical tests such as the IMViC test, citrate utilization test, triple sugar iron (TSI) test, oxidase test, and catalase test were performed to confirm that the sample was *pseudomonas aeruginosa.* Then an antibiotic susceptibility test was performed to see the resistance pattern of the tested organism. It was observed that the tested isolate was resistant to seven antibiotics, which indicates its multidrug-resistant characteristics. So, most of the antibiotics were not able to inhibit the infection caused by the bacteria, which was alarming information. The production of biofilms was then monitored using the tube method and a 96-well microtiter plate. The biofilm was stained with crystal violet after three days. Then biofilm production was quantified by measuring the optical density of the stained biofilm at 570 nm with an ELISA machine. The optical density is directly proportional to the biofilm formation, which was measured using the ELISA device. All of the tested *P. aeruginosa* strains from the clinical isolates were able to produce strong biofilm, as shown by the ELISA machine's absorbance reading after three days of biofilm screening. Following observation of our target species' ability

to form biofilms, various concentrations of vitamin C were applied to examine how the biofilm would respond. The vitamin C concentrations of different citrus fruits were used as standards in this test. We had used five different concentrations of ascorbic acid solution. After three days of incubation, drastic changes in biofilm formation were observed. It was observed that ascorbic acid solutions can inhibit the formation of biofilms. We got the most effective result from a concentration of 25.6 μg/ /mL of Vitamin C concentration in the microtitre plate method. Vitamin C at 25.6 μg/mL of concentration in the microtitre plate method and at 50 μg/ /mL concentration in the tube method could inhibit biofilm formation, and at a concentration of 10 μg/ml, weak biofilms were formed in the microtiter plate.

Biofilm can protect microorganisms from harsh environmental conditions such as extreme temperature and pH, high salinity and pressure, poor nutrients, antibiotics, etc., by acting as a barrier (Yin et el., 2019;20). Therefore, it may be challenging to cure an infection brought on by biofilms. In our experiment, these biofilm-forming strains of *P. aeruginosa* can be a great threat to the human community as they can be multidrug resistant and can also cause nosocomial infections. Certainly, biofilms grown in microtiter dishes successfully carry the properties of a mature biofilm, such as antibiotic tolerance and resistance to immune system effectors, etc (O'Toole, 2011). In this experiment, vitamin C showed excellent results as an anti-biofilm agent. However, further studies are needed before generalizing the concept of the effectiveness of ascorbic acid. Also, future research on ascorbic acid-antimicrobial interactions is required to control multidrug-resistant bacteria as well as biofilms. It would be great if vitamin C could prevent or treat the infection caused by multidrug-resistant biofilms.

Conclusion: In conclusion, we can clearly see that the bacterial strains isolated from the burn patients can form biofilms. Additionally, we are aware that the microbes contained within a biofilm are protected by an extracellular polymeric matrix that can serve as a barrier and be resistant to various unfriendly environments, including sanitizers, antibiotics, and other hygienic conditions. Therefore, using antimicrobial medications to treat or suppress biofilms is difficult work. We have gotten an effective result by using an ascorbic acid solution for inhibiting the growth of biofilm formation. Therefore, more research should be done to determine whether

vitamin C in various quantities can be helpful against bacteria that are multidrug-resistant or the biofilms those bacteria produce. In today's environment, where antibiotic resistance is the biggest danger to global health, a positive result from the study would be a major accomplishment.

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