

UNVEILING THE BLUE: INVESTIGATION OF PYOCYANIN PIGMENT PRODUCTION FROM *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

Pigments are the molecules that give color by absorbing light in the visible spectrum. Pyocyanin is a redox-active, water-soluble, blue-green, phenazine pigment produced predominantly (95%) by the bacterium *Pseudomonas aeruginosa*, which is a gram-negative, aerobic bacilli. This pigment is utilized in the fabric industry as a natural dye with chromogenic properties and in agriculture as a biocontrol agent. This research aimed to synthesize, purify, and elucidate the structure of pyocyanin from *Pseudomonas aeruginosa*. The strain of *Pseudomonas aeruginosa* was obtained and maintained on nutrient agar. Pyocyanin was screened and produced in different broth media. The extraction was performed using the chloroform dilution method, and purification was achieved through Thin Layer and Gel Permeation chromatography. Their structure was elucidated by Fourier Transform Infrared Spectroscopy, and the antibacterial action of the purified pigment was assessed using the Agar Well Diffusion Technique. The results indicated that green-pigmented colonies were produced on agar plates and in broth, the pyocyanin concentration was 4.8 µg/mL with an Rf value of 0.70, and inhibited the *Staphylococcus aureus* with a 15 mm zone of inhibition.

Keywords: Blue-green pigment, *Pseudomonas aeruginosa*, Pyocyanin purification, Thin Layer Chromatography, Fourier Transform Infrared Spectroscopy.

INTRODUCTION

Pigments are the molecules that give color by absorbing light in the visible spectrum. They are insoluble colored constituents, whereas colorants are soluble colored materials. It is expected that the pigments market value will reach between 33.2 and 49.1 billion \$ by 2027 (Grewal *et al.*, 2022). Secondary metabolites produced by microorganisms, such as pigments, support their defense and persistence (Abdelaziz *et al.*, 2023). Petro-derived synthetic colorants have achieved industrial dominance due to their high yields and low cost. However, concerns have arisen regarding their non-biodegradability, environmental toxicity, and potential carcinogenic effects (Grewal *et al.*, 2022). Industrially, synthetic pigments are applied in the production of food, drugs, dyes, and cosmetics, leading to numerous adverse effects. Pigment-producing genera include *Nocardia*, *Microbispora*, *Streptomyces*, and *Rhodococcus* (Celedón and Díaz, 2021).

Pyocyanin is a redox-active, water-soluble, blue-green, phenazine pigment produced predominantly (95%) by the bacterium *Pseudomonas aeruginosa*, which is a gram-negative, aerobic bacilli. This pigment is utilized in the fabric industry as a natural dye with chromogenic properties and in agriculture as a biocontrol mediator (Mudaliar and Bharath Prasad, 2024). The benefits of pyocyanin include its natural production, high yield using inexpensive substrates, biodegradability, and ease of downstream processing (Abdelaziz *et al.*, 2023). Pyocyanin is used in numerous biotic processes, including biofilm development, regulation of gene expression, and maintenance of bacterial cell fitness. *Pseudomonas aeruginosa* is also suspected to be involved in pathogenesis because of the expression of virulence factors after pigment production. Research has reported that pyocyanin-producing strains of *P. aeruginosa* are more multidrug-resistant compared to non-pyocyanin-producing strains, due to the presence of virulence factors (Chimi *et al.*, 2024).

P. aeruginosa is an extensive microbe that can be isolated from soil, water, humans, animals, and plants. Lipopolysaccharides, flagella, and pili are the virulence factors of *P. aeruginosa* and are responsible for secreting toxins, extracellular enzymes, and pyocyanin (Faisal *et al.*, 2024). Immunocompromised individuals and patients with chronic pulmonary ailments are susceptible to the pathogenic *P. aeruginosa* (Lew *et al.*, 2025), which causes serious nosocomial infections of the respiratory, circulatory, neurological, and urinary tracts, as well as other body

systems (Marey *et al.*, 2024). *P. aeruginosa* primarily manufactures two kinds of pigments, fluorescein and pyocyanin (yellow and blue), which give the sweet odor of grapes and the green pigmentation on culture media by synthesizing 2-aminoacetophenone during laboratory cultivation (Abdelaziz *et al.*, 2023).

Researchers from Egypt evaluated the antimicrobial, anticancer, antibiofilm, and antioxidant activities of pyocyanin from *P. aeruginosa*, isolated from both environmental and clinical sources (Shouman *et al.*, 2023). Another study examines the use of modulators (nanomaterials) to produce pyocyanin (Jabłońska *et al.*, 2022). Scientists from Iraq studied the clinically isolated *P. aeruginosa*, identified multidrug-resistant strains, assessed their antibiotic resistance, estimated pyocyanin production, and observed its effects on pathogenic bacteria (Hameed, 2024). Similarly, pyocyanin-producing *P. aeruginosa* isolated from clinical samples was identified, optimized, and characterized for its pyocyanin, with applications in agrochemical and biomedical settings (Thukkaram *et al.*, 2024). This research aimed to synthesize, purify, and elucidate the structure of pyocyanin from *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Culture Acquisition and Maintenance

The strain of *Pseudomonas aeruginosa* was obtained from the Department of Microbiology, University of Karachi, Pakistan, on nutrient agar slants. This culture was revived on nutrient agar plates (Oxoid) using the streak plate method and incubated at 37°C for 24-48 hours. After incubation, green pigmented colonies of *P. aeruginosa* appeared. This culture was maintained on nutrient agar slants at 4°C and preserved as an 80% glycerol stock at -20°C.

Screening of Pigment

The organism was cultured in nutrient broth medium in a test tube and incubated at 37°C for 24-72 hours. The growth of the organism was observed by changes in the color of the media; green media indicated that *P. aeruginosa* produced the pigment pyocyanin, as testified by Joshi *et al.* (2023) with trivial changes.

Pyocyanin Pigment Production

The *P. aeruginosa* culture was inoculated into 200 mL of King's 'B' broth medium (Composition g/200 mL: Peptone 4g, Glycerol 2 mL, MgSO₄.7H₂O 0.3g, K₂HPO₄ 0.3g, Distilled Water 200 mL) (Oves *et al.*, 2024) and 100 mL Luria-Bertani (LB) Broth medium (Composition g/100 mL: Yeast Extract 0.5g, Tryptone 1g, NaCl 0.05g, Distilled water 100 mL). King's 'B' media was placed in a shaking incubator at 35°C on 130 rpm for 72 hours. LB broth media was incubated statically at 37 °C for 3 days. After incubation, the media color changed from yellow to dark green. Pyocyanin pigment production was estimated after 3 days of incubation.

Extraction of Pyocyanin

The pyocyanin-pigmented media were centrifuged at 4000 rpm for 20 minutes, and the supernatant was filtered through Whatman filter paper no. 1 and collected in a sterile jar. The culture supernatant was extracted using a series of chloroform dilutions at a 1:2 ratio. 100 mL of CFF (Cell-free filtrate) was mixed with 200 mL of chloroform in a flask. This mixture was shaken well and kept undisturbed for 5-10 min. Two layers were separated: the aqueous layer and the blue-pigmented layer. The blue layer was re-extracted with 1 mL of 0.2 N HCL, and mixed for 10 seconds, resulting in a color change from blue to pink-red. Subsequently, 2 mL of 0.4 M Borate-NaOH (pH 10) was added to the solution, restoring the color to blue. These steps were repeated 2-3 times for re-extraction. Then the 10 mL extract is left at room temperature in petri plates overnight so that the chloroform evaporates and a powdered form of pigment is obtained. These powdered pigments were used for FTIR (Fourier Transform Infrared Spectroscopy) analysis; the remaining blue pigment was stored in a jar in a refrigerator for further use, as reported by Elbargisy (2021) with slight modifications.

Quantitative Detection of Pyocyanin

The strain of *P. aeruginosa* was inoculated into test tubes containing LB broth and incubated overnight at 37 °C, then centrifuged at 4000 rpm for 15 minutes to obtain the cell-free filtrate. Chloroform was then added to the cell-free filtrate at a 1:2 ratio, and the mixture was mixed thoroughly for 10 seconds. The resulting lower blue layer was subsequently mixed with the pyocyanin pigment reagent and 0.2 N NaCl. The blue color turned reddish-pink, and absorbance was measured at 520 nm with a spectrophotometer to determine the pigment concentration using the equations reported by Dange *et al.* (2019), Elbargisy (2021), and Najafi *et al.* (2021) with minor alterations.

$$\text{Concentration of Pyocyanin Pigment} = OD_{520} \times 17.07$$

Where,

17.07 = extinction coefficient

Pigment Concentration by Rotary Evaporator

The blue extracted pyocyanin pigment was concentrated by a rotary evaporator at 40°C. After that, the chloroform solvent was removed from the pigment, and a pure, concentrated, dark blue color pigment was obtained, which was kept in a Falcon tube, covered with aluminum foil, and stored at -20°C for purification.

Pyocyanin Pigment Purification

- **Thin Layer Chromatography (TLC)**

The concentrated pyocyanin drop was positioned on the baseline drawn on the TLC plate (Composition: Mixture of 5g silica with 10 mL of distilled water, spread in microscopic slides smoothly, dried at room temperature, and baked in an oven for 1 hour at 100°C) by capillary tube drop by drop, repeated five times. The TLC plate was then positioned vertically in an air-tight glass beaker, containing a mobile phase composed of chloroform and methanol (85 mL each) mixed in a 1:1 ratio. The pigment spot was moved towards the upper end. After 3 hours, remove the plates from the container, leave them at room temperature for 24 hours to dry (Joshi *et al.*, 2023), and the Rf (Resolution factor) was measured by this formula:

$$R_f = \text{Distance traveled by solute} / \text{Distance traveled by solvent}$$

- **Gel Permeation Chromatography**

5g of G-75 gel were soaked in 500 mL of buffer (0.2 M Tris-NaCl) overnight. The gel permeation column, packed with a porous bed of the soaked G-75 gel, was prepared. The mobile phase was made up of 0.1 M Tris-NaCl buffer. 1 mL of concentrated pyocyanin pigment was loaded into the column, running the mobile phase. The flow rate was determined every 15 minutes, and the purified pigment was collected into test tubes as stated in Joshi *et al.* (2023).

Structural Elucidation

Fourier Transform Infrared Spectroscopy (FTIR) was employed to find out the functional group and chemical bond in the compound; the analysis was performed by the International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Pakistan. The powdered sample was obtained from the above extraction of pyocyanin pigment. 2 mg of powder was required for the FTIR analysis. The analysis was executed by an FTIR Spectrometer (BRUKER, Vector-22) instrument (Shouman *et al.*, 2023).

Antibacterial Activity of Purified Pigment

The Agar Well Diffusion Technique was applied to determine the antibacterial activity of pyocyanin. The test microorganisms were *Escherichia coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus*. Inoculate each culture into the fresh LB broth individually and incubated at 37°C for 24 hours. Then, 50 µl of each of the overnight cultures was spread on MH (Muller-Hinton) agar plates through the sterile glass spreader. Make the wells in culture spreading plates after 15 min with the sterile borer. Transferred 100 µl of purified pyocyanin (sample) into the wells and incubated at 37°C for 24 hours. Inhibitory zones appeared after 24 hours of incubation, indicating pyocyanin's activity against these microorganisms (Dhairh and Al-Azawi, 2022).

RESULTS AND DISCUSSIONS

Culture Acquisition and Maintenance

P. aeruginosa produced white colonies on nutrient agar slants. When this culture streaked onto agar plates, it produced green-pigmented colonies of *P. aeruginosa* after incubation for 24-48 hours at 37°C (**Fig. 1**). These pigmented colonies were used to produce pyocyanin pigment. Jamaluddin *et al.* (2024) reported similar results after 24 hours of incubation, with other studies noting bluish-green colonies of *P. aeruginosa* at 37°C on nutrient agar after the same incubation period (Anaso and Al-Hassan, 2025; Dim *et al.*, 2025). Green colonies of *P. aeruginosa* can be maintained and stored in a refrigerator for further study.

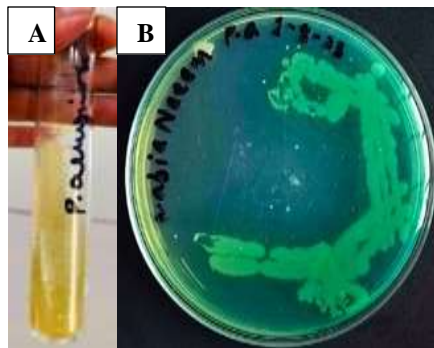


Fig. 1. *P. aeruginosa* colonies (A) White on Nutrient Slant (B) Green on a Nutrient Agar plate.

Screening & Production of Pyocyanin Pigment

Pyocyanin produced by the bacterium *P. aeruginosa* is a blue-green pigment that is a phenazine compound with several notable biological properties, including antibiotic activity, redox activity, and the ability to generate reactive oxygen species. Screening of pigment refers to the process of evaluating and selecting the pigment for various applications, which are based on their applications and properties. This step includes the examination of such factors as pigment color, tinting strength, light fastness, and chemical stability. *Pseudomonas* culture can be grown in Nutrient Broth media. During the 3 days of incubation at 37°C, it produces a greenish-blue color in the media; this change of color of the media indicates that this bacterium produces a pyocyanin pigment (**Fig. 2**). Screening for pigment production in microorganisms involves isolating and identifying strains that produce colored compounds. This process is essential in various fields, including biotechnology, pharmaceuticals, and the food industry, as microbial pigments can serve as natural colorants, antibiotics, and have other bioactive properties. Another research testified the nutrient broth for pyocyanin production and extraction at 37°C for 48-72 hours (Verma *et al.*, 2015). Comparable results in 48 hours were reported by El-Sayed *et al.* (2021).

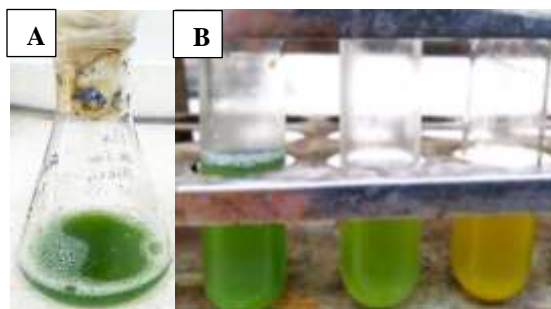


Fig. 2. Pigment production by *P. aeruginosa* (A) Green on Nutrient Broth. (B) Culture broth turned from yellowish green to bluish green (Right to left: T1: 24 Hours, T2: 48 Hours and T3: 72 Hours)

The *P. aeruginosa* culture can be inoculated into two different media for 3 days. First was King's 'B' Broth medium, which was kept in shaking condition at 35°C on 130 rpm for 72 hours; it produced light green pigment production, but in static condition at 37°C for 72 hours, LB Broth media produced dark greenish blue colored pyocyanin pigment. These results showed that LB broth produced a large amount of pyocyanin pigment as compared to the King's 'B' Broth medium (**Fig. 3**). Researchers from Italy described the production of green pyocyanin in LB Broth at 37°C for overnight under aerobic conditions (Orlandi *et al.*, 2015). In contrast, the same results were observed in King's 'A' Broth by this study in shaking conditions at 35°C on 130 rpm for 72 hours (Eltarahony *et al.*, 2025).

Extraction of Pyocyanin

Following the extraction process, a blue colored solution appeared in chloroform, which changed into a pink-red shade upon adding 0.1 N HCL. Pyocyanin dissolves in chloroform, imparting a distinct blue color. Pyocyanin, formed as a resonance hybrid of various mesomeric structures, including N-methyl-1-hydroxyphenazine. It can be present in its oxidized or reduced state. In reduced nature, the pyocyanin was labile and quickly reacted with

molecular oxygen, wine-red at acidic conditions because of the basic property of one of the nitrogen atoms, and blue at alkaline conditions. Similar results were observed by the previous research (Shouman *et al.*, 2023).

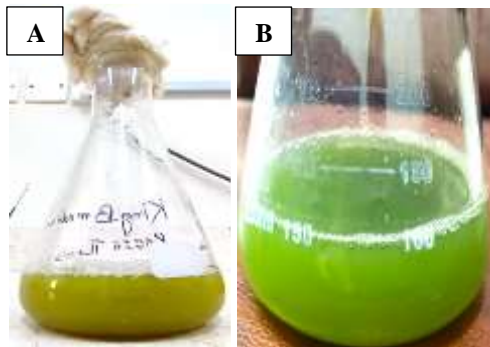


Fig. 3. Pyocyanin production (A) King's 'B' Broth (B) LB Broth

Quantitative Detection of Pyocyanin

Quantitative assay was performed to identify the concentration of pyocyanin. The value of optical density was observed as 0.2802. Applying the pigment quantitative detection equation, the amount of pyocyanin pigment reached 4.8 µg/mL. The quantitative estimation of pyocyanin pigment relied on the efficiency of pigment extraction and the measurement of light absorption at 520 nm in an acid medium. The pyocyanin pigment can be concentrated by a Rotary evaporator, which produces a dark blue color concentrated pigment (**Fig. 4**). A group of researchers from Egypt described the concentration of pyocyanin as 31.35 µg/mL by the same method (Eltarahony *et al.*, 2025). Another study reported the concentration of pyocyanin as 5.98 µg/ml by using chloroform and benzene (Mullaiselvan *et al.*, 2020).



Fig. 4. Concentrated Pyocyanin

Pyocyanin Pigment Purification

- ***Thin-Layer Chromatography***

Thin-layer chromatography purified the extracted pyocyanin pigment. In TLC, the value of distance travelled by the solute/sample is about 4.2 cm, and the distance traveled by the solvent is about 6.1cm (**Fig. 5**). These values can be applied to TLC formulae and find out the pigment resolution factor.

$$R_f = 4.2 / 6.1 = 0.70$$

The R_f values range from 0.70 to 0.81 as described by Shouman *et al.* (2023). The R_f value of 0.71 was also stated by this study (Joshi *et al.*, 2023). The single spot of purified pyocyanin with R_f value of 0.8 by TLC was reported by the Egyptian researchers (Shouman *et al.*, 2023).

- ***Gel Permeation Chromatography***

In gel permeation chromatography, we find the flow rate after every 15 minutes in each test tube. A maximum of 100 test tubes were available for collection. After 70 tubes of concentrated pigment were diluted and collected into 6 tubes. The diluted pigment in a gel permeation column can be separated, and a green ring-like structure can be formed in test tubes, which shows that pyocyanin can be purified by gel permeation chromatography. The pigment-containing test tubes were stored in the fridge for further use.

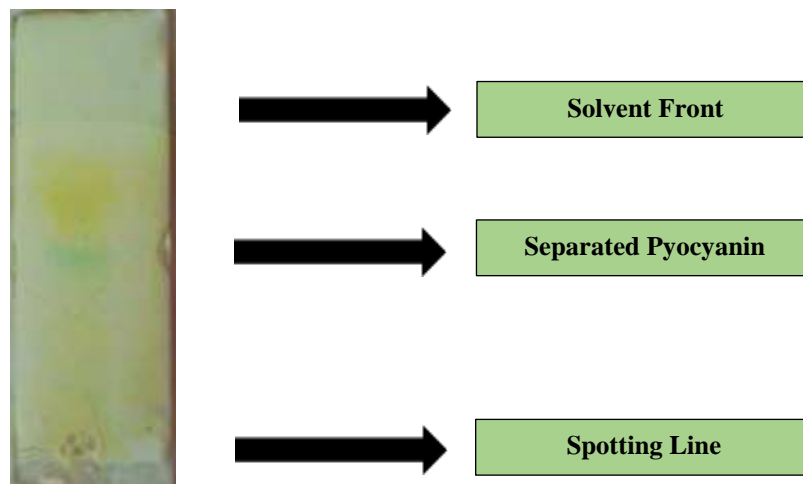


Fig. 5. Results of Pyocyanin Thin Layer Chromatography.

FTIR Analysis

The pyocyanin's functional group was assessed by FTIR analysis. The FTIR spectrum of pyocyanin pigment is given in (Fig. 6). The peak at 2958.69 indicates the presence of a CH bond, while the peak at 1718.49 shows the presence of a C=O carbonyl group. The peak at 1641.11 shows a C=N bond, 1543.92 shows a C-C bond, and the peak at 1405.48 shows the presence of a CH₃-CH bond of an Alkyl group. In contrast, the researchers from Egypt reported the FTIR results of clinically isolated *P. aeruginosa* pyocyanin as the CH bond bands appeared in between 3000-2900, C=N bands were observed in between 1590-1600 cm⁻¹, and the bond of CH₃-CH Alkyl group was observed in the range of 1380-1400 cm⁻¹ (Shouman *et al.*, 2023). Another study indicated the presence of CH bonds at the peak of 2365.26 cm⁻¹, C-O bond at the peak of 1252.54 cm⁻¹, C=N bond at the peak of 1633.41 cm⁻¹, and the C-O bond at the peak of 1252.54 cm⁻¹ (Hamad *et al.*, 2020).

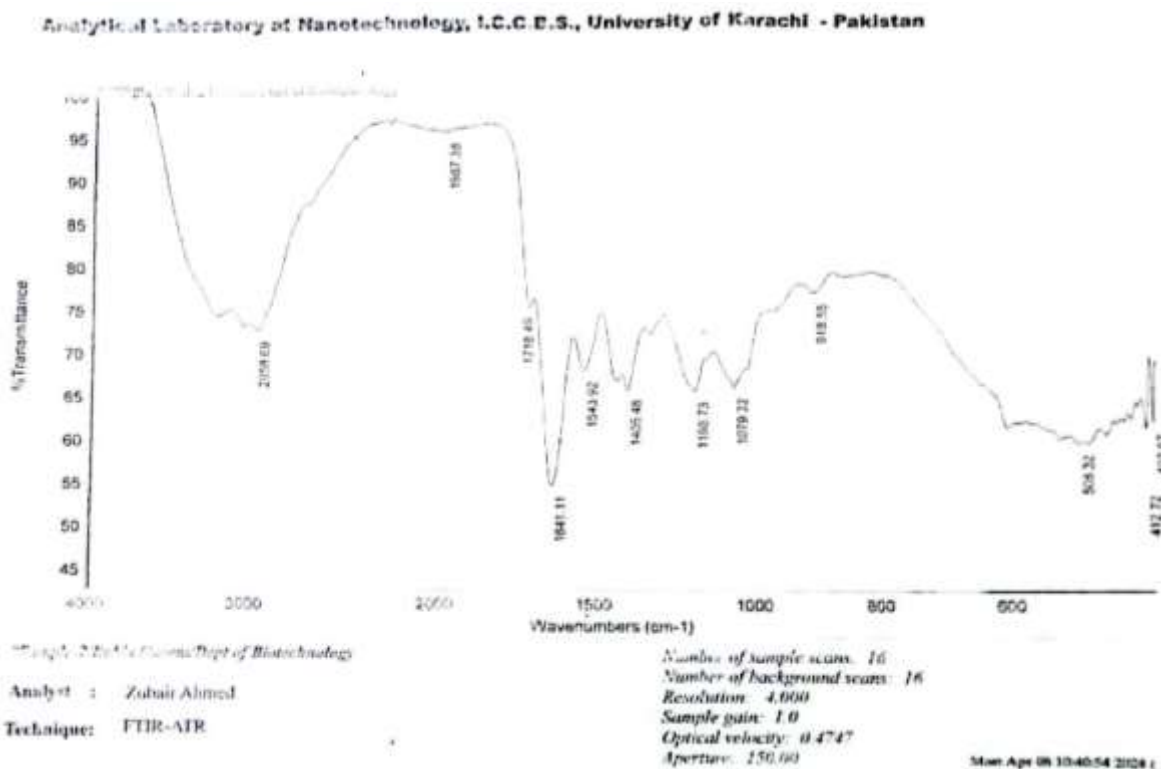


Fig. 6. Fourier Transform Infrared Spectroscopy Analysis

Antibacterial Activity of Purified Pigment

In comparison to the Gram-negative bacteria, it was noted that pyocyanin significantly inhibits the Gram-positive bacteria (Table 1). *P. aeruginosa* produces the pyocyanin pigment that inhibits *E. coli* bacteria and shows inhibition zones against this bacterium. The antibacterial activity was heat-stable and could be extracted into chloroform. It can be concluded that numerous multidrug-resistant (MDR) pathogens are inhibited by the pyocyanin and may be used topically in susceptible cases. A research group from Turkey performed the antimicrobial activity of purified pyocyanin by agar well diffusion method and described that this pigment inhibits the *Bacillus* sp., *Escherichia coli*, and *Candida* sp., with the 31 mm, 12 mm, and 15 mm zone of inhibition (Özyürek *et al.*, 2016). Another study tested the antimicrobial activity of pyocyanin by agar well diffusion method using different concentration ranges from 20-50 µL of pigment stock formulated in chloroform (3.2 µg/mL). These results represented the zone of inhibition as *Alternaria* sp., with 17 mm zone of inhibition in 40 µL of pigment, *Klebsiella* sp., with 16 mm zone of inhibition in 40 µL of pigment, *Salmonella paratyphi* with 14 mm zone of inhibition in 50 µL of pigment, and in *E. coli* with 7 mm zone of inhibition in 50 µL of pigment (Wamik, 2018). In contrast, the antimicrobial activity of purified pyocyanin was determined by the disc diffusion method (one disc of filter paper holding 10 µL of pyocyanin), incubated at 37 °C for 24 hours. The results indicated that the pyocyanin notably inhibited the *Salmonella* sp. and *Klebsiella* sp. *Proteus* sp. and *E. coli*, but the highest zone was observed in *Salmonella* sp. (Dange *et al.*, 2019).

Table 1. Antibacterial Activity of Pyocyanin.

Tested Microorganisms	Zone of Inhibition (mm)
<i>Escherichia coli</i>	14
<i>Klebsiella pneumoniae</i>	14
<i>Staphylococcus aureus</i>	15

The limitations of this research include the following: Only one strain of *P. aeruginosa* was used, which does not capture variability in pyocyanin production among different strains. The synthesis occurred solely through lab-scale fermentation, without considering industrial parameters. FTIR was the only technique employed for structural elucidation, lacking more advanced methods like mass spectrometry or NMR. Additionally, only three bacterial species were tested for antibacterial activity, which may not adequately represent pyocyanin's antimicrobial spectrum. Lastly, the study did not address the stability, toxicity, or potential industrial applications of pyocyanin. Future recommendations include utilizing pyocyanin in industrial applications as a natural colorant and biocontrol agent. Additionally, further exploration of its antimicrobial mechanisms is essential. Efforts should focus on producing stable formulations for industrial use, improving their production through genetic engineering, and comparing them with other microbial pigments to broaden their applications.

CONCLUSION

This study magnificently synthesized, purified, and characterized pyocyanin structurally, which is a blue-green, redox-active pigment mainly produced by *P. aeruginosa*. The high purity of pyocyanin was achieved through extraction using the chloroform dilution method and purification via Thin Layer and Gel Permeation Chromatography. The chemical structure was confirmed using Fourier Transform Infrared Spectroscopy. The purified pyocyanin unveiled outstanding antibacterial activity against *Staphylococcus aureus*, showing its bioactive potential. The findings of this research validate the pyocyanin's multifunctionality as an eco-friendly colorant for fabrics, a biocontrol mediator in agronomy, and a probable pharmacological compound owing to its antimicrobial properties. By showcasing a systematic approach to the production and characterization of pigment, this study offers treasured insights into biotechnology and industrial microbiology, emphasizing pyocyanin's potential for varied industrial, agronomic, and medicinal applications.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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