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Production, Characterization, and Optimization of Pyocyanin from *Pseudomonas aeruginosa*

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ABSTRACT

Microbial pigments have gained significant scientific attention due to their biodegradability, eco-friendliness, and wide range of biological activities. This study investigated the production, characterization, and optimization of pyocyanin from Pseudomonas aeruginosa isolated from soil samples. Soil samples were collected from different locations and processed using serial dilution and standard microbiological techniques. Pigment-producing bacterial isolates were identified through morphological, biochemical, and molecular characterization. Pyocyanin production was achieved by submerged fermentation, followed by extraction using chloroform. Characterization of the extracted pigment was performed using Gas Chromatography–Mass Spectrometry (GC-MS) and Fourier Transform Infrared Spectroscopy (FTIR). Optimization of pyocyanin production was carried out using the One-Factor-At-a-Time (OFAT) approach by evaluating the effects of culture media, pH, temperature, and incubation time. Molecular analysis confirmed isolate D3 was Pseudomonas aeruginosa strain NBRC 126891 with 96% similarity. GC-MS analysis revealed Phenol, 2,4-bis(1,1-dimethylethyl) as the predominant compound, accounting for 79.66% of the peak area. FTIR analysis showed major absorption peaks at 3291 cm^{-1} and 1630 cm^{-1} corresponding to O–H and C–H stretching vibrations, respectively. Optimization studies showed that King's A medium, pH 7.0, a temperature of 35°C, and an incubation period of 48 hours were the optimal conditions for pyocyanin productionkings A with a yield of 4.2, pH of 7.0 with 4.3, temperature of 35 °C with 4.5 and incubation time of 48hrs with 4.4µg/ml. The study demonstrates that Pseudomonas aeruginosa isolated from soil can serve as an effective source of pyocyanin for potential pharmaceutical and industrial applications.

Keywords: *Pyocyanin, Pseudomonas aeruginosa, microbial pigment, optimization, GC-MS, FTIR.*

1.0. Introduction

The increasing demand for pigments in pharmaceutical, food, cosmetic, textile, and biomedical industries has intensified the search for safer and environmentally friendly alternatives to synthetic pigments. Synthetic pigments are widely used because of their stability and availability; however, many have been associated with toxicity, carcinogenicity, environmental pollution, and poor biodegradability (Joshi et al., 2023). These limitations have stimulated global interest in natural pigments derived from plants, animals, and microorganisms.

Microbial pigments have emerged as promising alternatives because they are renewable, biodegradable, and can be produced through controlled fermentation processes. Bacterial pigments are particularly attractive due to the rapid growth rate of bacteria, ease of cultivation, and their ability to produce biologically active metabolites with pharmaceutical importance (Venil et al., 2023). Several microbial pigments possess antibacterial, antioxidant, anti-inflammatory, and anticancer properties, making them valuable in biotechnology and medicine.

Among the different microbial pigments, pyocyanin is one of the most important phenazine compounds produced by *Pseudomonas aeruginosa*. Pyocyanin is a blue-green, water-soluble pigment known for its redox activity and broad biological properties. The pigment contributes significantly to the physiology and pathogenicity of *P. aeruginosa* and has demonstrated antibacterial activity against several microorganisms, especially Gram-positive bacteria such as *Staphylococcus* species (Mudaliar et al., 2024).

Pyocyanin production is influenced by several environmental and nutritional factors, including culture medium, pH, temperature, aeration, incubation time, and nutrient availability (Abdelaziz et al., 2024). Under normal laboratory conditions, pyocyanin yield is often low, thereby limiting its large-scale industrial application. Optimization of production conditions is therefore essential for improving yield, reproducibility, and commercial viability.

Recent studies have shown that pyocyanin possesses significant antimicrobial activity due to its ability to generate reactive oxygen species and disrupt microbial electron transport systems.

These properties make pyocyanin a promising candidate for pharmaceutical and biomedical applications. However, studies focusing on the production, characterization, and optimization of pyocyanin from locally isolated *Pseudomonas aeruginosa* strains remain limited. Therefore, this study aimed to isolate and characterize *Pseudomonas aeruginosa* from soil samples, produce and characterize pyocyanin, and optimize its production under different environmental conditions.

2. Materials and Methods

2.1 Collection of Soil Samples

Soil samples were collected from five different locations in Sokoto State, Nigeria, approximately 20 g of soil was collected aseptically from a depth of 1.5 cm using a sterile spatula and transferred into sterile sample containers. and transported to the Microbiology Laboratory of Sokoto State University for analysis.

2.2 Sample Processing

A fivefold serial dilution was prepared for each soil sample using sterile distilled water. About 0.1 g of each sample was transferred into the first test tube containing 9 ml sterile distilled water to obtain a dilution factor of 10^{-1}. Subsequent serial dilutions up to 10^{-5} were prepared.

2.3 Isolation of Pigment-Producing Bacteria

1ml from the serially diluted samples was cultured on nutrient agar plates and incubated at 37°C for 24 hours. Distinct colonies showing pigment production were sub-cultured repeatedly to obtain pure cultures. Pure isolates were maintained on nutrient agar slants and stored at 4°C.

2.4 Morphological and Biochemical Characterization

The isolates were characterized using standard microbiological methods including Gram staining, catalase test, oxidase test, citrate utilization test, motility test, coagulase test, methyl red test, Voges-Proskauer test, indole test, and Triple Sugar Iron (TSI) test according to standard procedures described by Bhat and Tawheed (2013).

2.5 Molecular Identification of Isolates

2.5.1 DNA Extraction

Genomic DNA was extracted using Qiagen DNA extraction kits. Pure bacterial cultures were suspended in nuclease-free water and processed according to the manufacturer's instructions.

2.5.2 Polymerase Chain Reaction (PCR)

Amplification of the 16S rRNA gene was carried out using universal primers 27F and 1492R. PCR amplification was performed using a Bio-Rad thermal cycler under optimized conditions.

2.5.3 Agarose Gel Electrophoresis

Amplified PCR products were analyzed using 1% agarose gel electrophoresis at 100 V for 35 minutes.

2.5.4 DNA Sequencing and Phylogenetic Analysis

Purified PCR products were subjected to Sanger sequencing. Obtained nucleotide sequences were compared with sequences in the NCBI database using BLASTn, while phylogenetic analysis was performed using MEGA 11 software.

2.6 Production and Extraction of Pyocyanin

Pyocyanin production was carried out using nutrient broth fermentation. Pigment-producing isolates were inoculated into sterile nutrient broth and incubated at 37°C for 48 hours. After incubation, cultures were centrifuged at 4000 rpm for 10 minutes to remove bacterial cells. Pyocyanin was extracted from the supernatant using chloroform in a 1:1 ratio. The mixture was shaken vigorously for proper extraction and filtered using Whatman No. 1 filter paper. Extracted pigment was air-dried to remove residual chloroform and stored at 10°C until further analysis(Barakat*et al.*, 2015)

2.7 Characterization of Pyocyanin

2.7.1 Gas Chromatography–Mass Spectrometry (GC-MS)

GC-MS analysis was performed using a Trace 1300 GC Ultra/Mass Spectrophotometer equipped with a TG-5MS Zebron capillary column. Helium was used as the carrier gas at a flow rate of 1 ml/min. Compound identification was achieved by comparing obtained spectra with NIST library databases(Barakat*et al.*, 2015)

2.7.2 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis was carried out to identify functional groups present in the extracted pigment. About 1 mg of the extract was mixed with potassium bromide (KBr) and analyzed using a Perkin-Elmer FTIR spectrophotometer within the range of 400–4000 cm^{-1}(Barakat*et al.*, 2015)

2.8 Optimization of Pyocyanin Production

2.8.1 Effect of Culture Media

Four different media were evaluated: nutrient broth supplemented with 10% glycerol, King's A broth, King's B broth, and peptone water. Each medium was inoculated with standardized bacterial inoculum and incubated at 35°C for 48 hours.

2.8.2 Effect of pH

The effect of pH was studied using King's A medium adjusted to pH 6.0, 7.0, 8.0, and 9.0 prior to sterilization.

2.8.3 Effect of Temperature

The influence of temperature was evaluated by incubating cultures at 25°C, 30°C, 35°C, and 40°C.

2.8.4 Effect of Incubation Time

Pyocyanin production was monitored at different incubation periods of 24, 48, 72, and 96 hours.

3.0 Results and Discussion

3.3 Molecular Identification

Molecular analysis based on 16S rRNA sequencing identified isolate D3 as *Pseudomonas aeruginosa* strain NBRC 126891 with accession number PQ178302 and 96% similarity. PCR amplification showed distinct bands at approximately 900 bp, confirming successful amplification of the 16S rRNA gene.

3.4 Characterization of pyocyanin Produced by *pseudomonas aeruginosa*

3.4.1Gas Chromatography–Mass Spectrometry (GC-MS)

GC-MS analysis revealed several compounds in the extracted pigment. The major compound detected was Phenol, 2,4-bis(1,1-dimethylethyl), with a peak area of 79.66%. Other compounds identified included Bis(2-ethylhexyl) phthalate, D-limonene, Eicosane, Octasane 2-methyl, and Tritriacontane.

Table 1: Major Compounds Identified by GC-MS Analysis

Compound	Retention Time	Area (%)
Phenol, 2,4-bis(1,1-dimethylethyl)	49.73	79.66
Bis(2-ethylhexyl) phthalate	45.05	3.49
D-Limonene	12.71	1.55
Eicosane	45.71	1.27

3.5Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis of the extracted pyocyanin showed major absorption peaks at 3291 cm^{-1} and 1630 cm^{-1}, corresponding to O–H stretching and C–H stretching vibrations, respectively.

Table 2: FTIR Functional Groups Identified

Absorption peaks (cm^{-1})	Peak Description	Functional group
329	Broad and intense	OH-Stretching
1630	Sharp and moderate	C-H Stretching

3.6 Optimization of Pyocyanin Production

Optimization studies were carried out to determine the effects of different cultural and environmental parameters on pyocyanin production by the test organism. The parameters investigated included culture media, pH, temperature, and incubation time.

3.6.1 Effect of Culture Media

The effect of different culture media on pyocyanin production is presented in Figure 1.0 Pyocyanin production varied significantly depending on the type of culture medium used. Among the media tested, Kings Asupported the highest pyocyanin yield (µg/mL), whereasPeptone water recorded the lowest production **(**1.0µg/mL).

3.6.2 Effect of pH

The influence of pH on pyocyanin production is shown in Figure 2.0 Pyocyanin production increased progressive reaching maximum production at pH 7 (4.3µg/mL). Beyond this optimum pH, a decline in pyocyanin production was observed.

3.6.3 Effect of Temperature

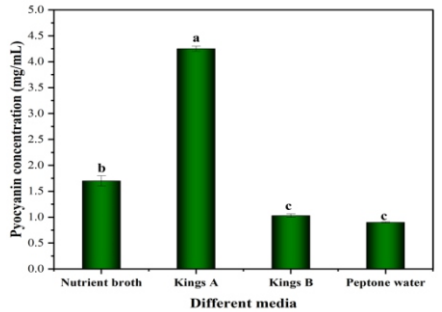
The effect of incubation temperature on pyocyanin production is presented in Figure 3.0 Pyocyanin production was observed across all temperatures tested; however, the highest production was recorded at 35°C (4.5 µg/mL), while lower yields were obtained at 45°C.

3.6.4 Effect of Incubation Time

The influence of incubation time on pyocyanin production is shown in Figure 4.0 Pyocyanin production increased with increasing incubation period, reaching a peak at 48hours (4.4 µg/mL), after which a decline was observed.

Figure 1.

3.6.1 Effect of Culture Media on pyocyanin production by pseudomonas aeruginosa isolated from soil

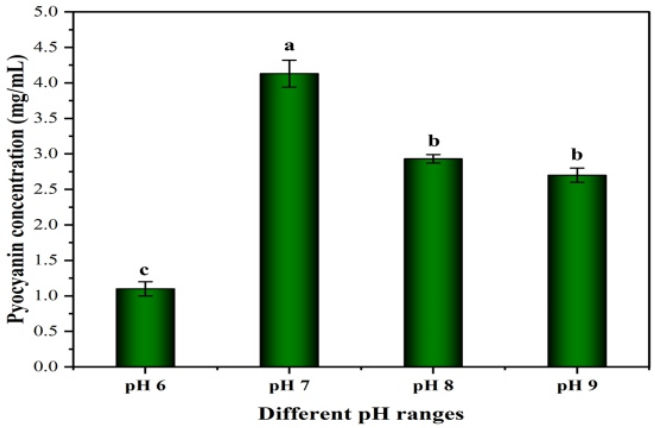


Effect Media on pyocyanin production from *pseudomonas aeruginosa*

➢ The bars bearing the same small letters (abc)are not statistically different at > 0.05.

Figure 2.

3.6.2 Effect of pH on pyocyanin production by pseudomonas aeruginosa isolated from soil

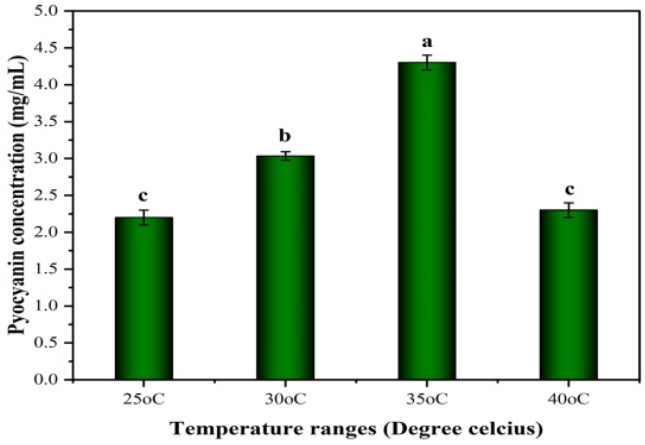


Effect of pH on pyocyanin production from *pseudomonas aeruginosa*

➢ The bars bearing the same small letters (abc)are not statistically different at > 0.05.

Figure 3.

3.6.3 Effect of Temperature on pyocyanin production by pseudomonas aeruginosa isolated from soil

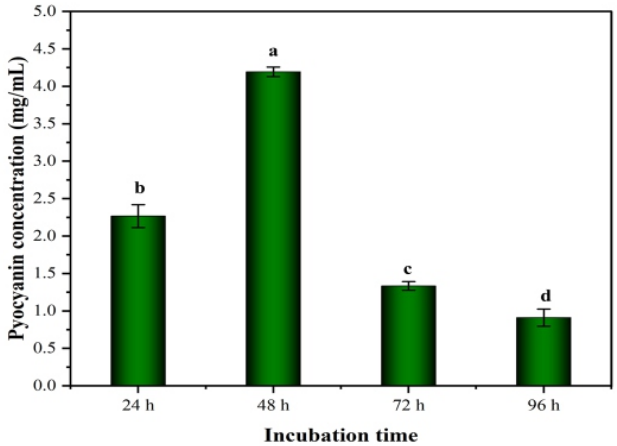


Effect of Temperature on pyocyanin production from *pseudomonas aeruginosa*

❖ The bars bearing the same small letters (abc)are not statistically different at > 0.05.

Figure 4.

3.6.4 Effect of Incubation Time on pyocyanin production from pseudomonas aeruginosa isolated from soil



Effect of Incubation time on pyocyanin production from *pseudomonas aeruginosa*

❖ The bars bearing the same small letters (abc)are not statistically different at > 0.05

4.0 Discussion

4.1 Molecular Identification of bacterialstrain

The molecular characterization of isolate D3 identified the organism as *Pseudomonas aeruginosa strain NBRC 126891* with 96% sequence similarity based on 16S rRNA gene analysis. PCR amplification produced a distinct band of approximately 900 bp, confirming successful amplification of the target gene. The successful identification of the isolate as *P. aeruginosa* may be attributed to the high specificity and reliability of 16S rRNA gene sequencing for bacterial classification. The 16S rRNA gene contains both conserved and variable regions that allow accurate differentiation of bacterial species. The observed result is expected because *P. aeruginosa* is a common soil inhabitant and possesses remarkable adaptability to diverse environmental conditions. Its metabolic versatility enables it to survive and proliferate in nutrient-variable environments such as soil.

This finding agrees with the reports of Abubakar and Maphosa (2024) and Yahaya *et al.* (2024), who demonstrated that 16S rRNA sequencing is an effective method for the identification and confirmation of bacterial isolates. Similarly, several studies have reported the recovery of *P. aeruginosa* from soil environments due to its ecological adaptability. The identification of the isolate confirms that soil can serve as a valuable reservoir of pyocyanin-producing *P. aeruginosa*. This finding supports the potential exploitation of environmental isolates for industrial-scale production of bioactive pigments.

4.2 GC–MS Characterization of Pyocyanin produced by *pseudomonas aeruginosa*

GC–MS analysis revealed the presence of several compounds within the extracted pigment, with Phenol, 2,4-bis(1,1-dimethylethyl) being the predominant compound, accounting for 79.66% of the total peak area. Other compounds detected included Bis(2-ethylhexyl) phthalate, D-limonene, and Eicosane. The predominance of Phenol, 2,4-bis(1,1-dimethylethyl) suggests that the extracted pigment contains bioactive phenolic constituents that may contribute to its biological properties. The presence of multiple compounds indicates that the extraction process recovered not only pyocyanin but also associated secondary metabolites produced by the organism. This result may be due to the ability of *P. aeruginosa* to synthesize a variety of secondary metabolites during active growth. Such metabolites are often produced as survival mechanisms, enabling the organism to compete with other microorganisms in its natural environment.

The finding is consistent with the observations of Hamad (2020), who reported that microbial pigment extracts frequently contain several bioactive compounds detectable by GC–MS analysis. Similar studies have also shown that phenolic compounds are commonly associated with microbial pigments and contribute significantly to their antimicrobial and antioxidant activities. The presence of bioactive compounds in the extract suggests potential applications in pharmaceutical, biomedical, and biotechnological industries. These compounds may enhance the antimicrobial effectiveness of pyocyanin-based productsHamad (2020).

4.3 FTIR Characterization of Pyocyanin produced by pseudomonas aeruginosa

FTIR analysis revealed major absorption peaks at 3291 cm^{-1} and 1630 cm^{-1}, corresponding to O–H stretching and C–H stretching vibrations, respectively.

The detection of these absorption bands indicates the presence of important functional groups within the extracted pigment. These functional groups are characteristic of many biologically active organic compounds and confirm the chemical complexity of the extract. The broad O–H stretching peak may be associated with hydroxyl-containing compounds, while the peak observed around 1630 cm^{-1} reflects the presence of carbon-containing functional groups that contribute to the structural stability of the pigment molecule. This observation is in agreement with the findings of DeBritto*et al*. (2020), who reported similar FTIR absorption patterns in microbial pigment extracts. Comparable studies have also identified hydroxyl and carbon-related functional groups as important constituents of phenazine-derived pigments. The FTIR results provide evidence of the chemical integrity of the extracted pigment and support its identification as a biologically active metabolite. The presence of these functional groups may contribute to the antimicrobial and redox properties associated with pyocyanin.DeBritto*et al*. (2020),

4.4 Effect of Culture Media on Pyocyanin Production

Among the culture media evaluated, King's A medium produced the highest pyocyanin yield (4.2 µg/mL), whereas peptone water supported the lowest production (1.0 µg/mL). The superior performance of King's A medium may be attributed to its nutrient composition, which is specifically designed to enhance pigment production in *P. aeruginosa*. The medium contains ingredients that favor phenazine biosynthesis and support optimal bacterial growth. This result occurred because pyocyanin synthesis is highly dependent on nutrient availability. Adequate carbon and nitrogen sources stimulate metabolic pathways involved in secondary metabolite production, whereas nutrient-limited media may restrict pigment synthesis. The present finding agrees with the report of Darwesh*et al.* (2024), who observed enhanced pyocyanin production in King's A medium compared with other conventional media. Similar studies have consistently identified King's A medium as one of the most effective media for pyocyanin production. The implication is that medium selection is a critical factor for maximizing pyocyanin yield. The use of King's A medium may improve productivity and reduce production costs during large-scale fermentation.

4.5 Effect of pH on Pyocyanin Production

Pyocyanin production increased progressively with increasing pH and reached its maximum value of 4.3 µg/mL at pH 7.0. Production declined when the pH exceeded this optimum value. The observed optimum at pH 7.0 indicates that neutral conditions favor both bacterial growth and pyocyanin biosynthesis. Enzymatic reactions involved in pigment production function most efficiently within a narrow pH range.

The decline observed at higher pH values may be due to reduced enzyme activity and disruption of cellular metabolic processes. Extreme pH conditions can affect membrane integrity, nutrient transport, and overall microbial physiology. This finding is consistent with the work of Abdelaziz et al. (2024), who reported maximum pyocyanin production under near-neutral pH conditions. Similar studies have shown that pH values outside the optimum range negatively affect phenazine biosynthesis. The implication is that maintaining a neutral pH during fermentation is essential for maximizing pyocyanin production and achieving consistent yields in industrial applications.

4.6 Effect of Temperature on Pyocyanin Production

Pyocyanin production occurred at all temperatures tested, with the highest yield (4.5 µg/mL) recorded at 35°C. Lower yields were observed at temperatures above and below this optimum. The optimum temperature of 35°C reflects favorable physiological conditions for bacterial growth and metabolic activity. At this temperature, enzymes involved in pyocyanin biosynthesis operate efficiently, leading to increased pigment production.

The reduced production at higher temperatures may result from thermal stress and enzyme denaturation, whereas lower temperatures may slow metabolic reactions and bacterial growth. This result agrees with Bhattacharya et al. (2025), who reported enhanced phenazine production at temperatures between 30°C and 35°C. Similar observations have been reported for other *P. aeruginosa* strains, indicating that moderate temperatures are optimal for pyocyanin synthesis. The implication is that temperature control is a crucial factor during fermentation. Maintaining cultures at approximately 35°C can significantly improve pyocyanin yield and process efficiency.

4.7 Effect of Incubation Time on Pyocyanin Production

Pyocyanin production increased with incubation time and reached a maximum yield of 4.4 µg/mL after 48 hours. Beyond this period, pigment production declined. The increase in production up to 48 hours indicates that pyocyanin synthesis is associated with the active growth phase of the organism. During this period, bacterial metabolism is highly active, promoting secondary metabolite production. The decline after 48 hours may be due to nutrient depletion, accumulation of toxic metabolic by-products, or degradation of the pigment. Extended incubation can also lead to reduced bacterial viability and metabolic activity. This observation is consistent with the findings of Dubrowska et al. (2024), who reported maximum pyocyanin accumulation during the late exponential and early stationary phases of growth, followed by a gradual decline during prolonged incubation. The implication is that harvesting cultures after 48 hours would maximize pyocyanin recovery while minimizing production losses. This information is valuable for designing efficient fermentation processes for industrial production.

5. Conclusion

The study demonstrated that soil-derived *Pseudomonas aeruginosa* is a promising producer of pyocyanin. Molecular characterization confirmed the identity of the isolate, while GC–MS and FTIR analyses verified the presence of bioactive compounds and functional groups associated with the pigment. Optimization experiments established King's A medium, pH 7.0, a temperature of 35°C, and an incubation period of 48 hours as the most favorable conditions for pyocyanin production. These findings provide a foundation for improving pyocyanin yield and support its potential application in pharmaceutical, biomedical, and industrial sectors.

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