Original Article

Optimum Conditions for Pyomelanin Production and Characterization from Local Isolates of *Pseudomonas aeruginosa*

Naz Fuad H. Khorshid, Nisreen Hadi Odaa
Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

Abstract

Background: Nowadays, there is increased interest in the biosynthesis of microbial melanin related to their numerous biological functions and applications in many fields, especially in medical fields, including immune-modulating, antimicrobial antibiotic, antiviral antivenin, anticancer, antitumor activity, and anti-biofilm activity. Pyomelanin is a hydrophobic macromolecule that is typically dark brown or black in color, formed by the oxidative polymerization of phenolic or indolic compounds. Pyomelanin is reported to be safe for consumption, thus providing a crucial strategy for biocontrol of biofilm. Furthermore, natural pyomelanin is known as a potent antioxidant, photoprotective, and free radical scavenging. **Objective:** This study focuses on the extraction and parametric optimization of melanin using *Pseudomonas aeruginosa* (*P. aeruginosa*) isolates. **Materials and Methods:** *P. aeruginosa* was isolated from various clinical sources, including urinary tract infections, burn patients, and wound infections. The isolate's identification was done by biochemical, cultural, and VITEK-2 compact system. The isolates were subjected to pyomelanin production in nutrient agar supplemented with L-tyrosine, and the potential isolate was selected and employed for melanin production using nutrient broth supplemented with L-tyrosine. The pyomelanin-producing isolates were further identified through the PCR analysis technique. Different physical parameters were used for optimum pyomelanin yield. **Results:** The potential *P. aeruginosa* isolates were further identified using 16s rRNA. Pyomelanin production was better at 2.9 µg/mL at 100 rpm. The highest pyomelanin production was 3.07 µg/mL after 72 h incubation. The optimum pH at 7 was 3.12 µg/mL. The highest pyomelanin yield at 40°C was 3.03 µg/mL, while the maximum pyomelanin was produced using 2 g/L tyrosine at 2.99 µg/mL. UV-visible (UV-Vis) spectroscopy analysis for pyomelanin pigment was at 273.5 nm. The infrared spectrum exhibited a broad absorption band around 3280.82 cm^{-1} which corresponds to the presence of -OH and N-H groups. **Conclusion:** This study provides knowledge on factors affecting pyomelanin production using bacteria in broth media supplemented with L-tyrosine.

Keywords: FT-IR spectroscopy, optimization parameters, *Pseudomonas aeruginosa*, pyomelanin, UV-Vis spectroscopy

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a Gram-negative bacterium, an environmental bacterium that can infect vulnerable patients with both acute and chronic infections.[1,2] This versatile organism is very versatile, has the capacity to build biofilms, exhibits a high level of inherent antibiotic resistance, and possesses variety of virulence factors.[3]

Also, *P. aeruginosa* bacterium is capable of producing different kinds of pigments, including pyomelanin pigment. Pyomelanin pigment is a natural black-brown, hydrophobic, and negatively charged macromolecule generated by the oxidative polymerization of phenolic or indolic compounds in bacteria, plants, animals, and fungi.[4-6] The production of pyomelanin involves many enzymatic and non-enzymatic steps, L-tyrosine is first converted by the tyrosinase catalyzer into L-3,4

Access this article online

Quick Response Code:

Website: https://journals.lww.com/mjby

DOI: 10.4103/MJBL.MJBL_770_23

Address for correspondence: Naz Fuad H. Khorshid, Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.
E-mail: nazmostafa08@gmail.com

Submission: 16-Jun-2023 **Accepted:** 10-Aug-2023 **Published:** 29-Mar-2025

How to cite this article: Khorshid NFH, Odaa NH. Optimum conditions for pyomelanin production and characterization from local isolates of *Pseudomonas aeruginosa*. Med J Babylon 2025;22:108-16.

dihydroxyphenylalanine (L-DOPA), and L-DOPA is then converted to dopachrome by the action of tyrosinase enzyme, which is finally converted to pyomelanin through several non-enzymatic oxidoreduction steps.[7,8]

Recent studies found that the function of pyomelanin in bacteria is connected to the defense against environmental stress, including UV light, free radicals, heavy metal toxicity, and hydrolytic enzymes.[9] Bacteria that produce pyomelanin are found to be more resistant to antibiotics.[10] These characteristics make pyomelanin a significant bioactive substance with numerous clinical, pharmaceutical, and agricultural applications, including the food industry for use as a natural food colorant.[5,11] Studies have also shown that pyomelanin has antimicrobial and antiviral effects, which open up new avenues for research.[7] Synthesis of pyomelanin pigment by microorganisms is eco-friendly and provides higher production outputs.[12]

Thus, the main aim of our study focuses on the optimal conditions of production, extraction, and partial purification of pyomelanin.

Materials and Methods

Identification of isolates

One hundred clinical specimens were isolated from different clinical sources from Iraqi patients' hospitals, including 45 specimens (45%) from burns patients, 40 specimens (40%) from UTI patients, and 15 specimens from wound infections. The statistical analysis system (SAS) program was used to detect the effect of different sources of *Pseudomonas* infection in this study.[13] All specimens were swabbed, inoculated, and incubated for 24 h at 37°C on MacConkey agar as a selective and differential media; for further identification, the isolates were recultured on cetrimide agar, selective, and differential media for *P. aeruginosa*, isolates were initially identified by cultural characteristics, biochemical tests, and VITEK-2 compact system (BioMerieux, France) was used in this present study.

The identification of pyomelanin producer isolates was confirmed by amplifying a fragment of 16S rRNA gene (1400 bp) using 16S Pseud-F (5′-AGGCCTAACACATGCAAGTCGA-3′) and 16S Pseud-R (5′-GGTTAGACTAGCTACTTCTGGAGC-3′). A total of 0.5 µL of each primer was added to 25 µL of Go Taq G2 Green Master Mix. Then, 1 µL of boiled bacterial extract as a DNA template was added,[14] and the volume of the PCR mixture was completed with nuclease-free water to 50 µL. The thermocycling was performed as follows: 1 cycle of 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final cycle of 72°C for 5 min. Electrophoresis was conducted at 100 V for 50 min using an agarose gel (1%) containing ethidium bromide (0.5 µg/mL) and 1X TBE buffer. UV light was used in a GelDoc-UV-Transilluminator to visualize PCR product bands (1400 bp), 100 pb DNA marker.

Screening for pyomelanin-producing *Pseudomonas aeruginosa*

All *P. aeruginosa* isolate*s* were tested for pyomelanin production by inoculating a fresh *Pseudomonas* colony on a nutrient agar medium supplemented with 2% L-tyrosine.[15] Plates were then incubated for 3–4 days at 37°C and observed the brown–black color of pyomelanin. To choose the highest pyomelanin-producing isolates, all pyomelanin-producing isolates were inoculated in brain heart infusion broth and incubated at 37°C for 24 h. The optical density was measured at 600 nm to normalize the cell number, then 1 mL of overnight bacterial culture was inoculated in 100 mL flasks containing 20 mL of nutrient broth supplemented with 2% L-tyrosine (pyomelanin production broth media). Pyomelanin production broth media without tyrosine was used. The flasks were then incubated at 37°C for 3–4 days in a shaking incubator at 120 rpm until the color of the media turned brown. Subsequently, the media were centrifuged at 8000 rpm for 20 min and the supernatant. The concentration of pyomelanin in the supernatants was measured using an enzyme-linked immunosorbent assay (ELISA), (ELISA microplate reader, BioTek, USA) at 450 nm and was calculated by comparing the OD of unknown samples with a standard curve prepared according to Zhu *et al.* and Makpol *et al.*[16,17]

Optimization of agitation rate (rpm) for pyomelanin production

Various agitation speeds were tested for improved growth and optimal pyomelanin production. Four individual 100 mL conical flasks with 20 mL pyomelanin production broth media were inoculated with an overnight bacterial culture and incubated in a shaker incubator at 37°C for 72 h at 80, 100, 120, and 140 rpm. The fifth flask was incubated under static conditions.[18]

Optimization of different temperatures for pyomelanin production

This experiment has been conducted to determine the optimal incubation temperature for maximum pyomelanin production. Four different 100 mL conical flasks with 20 mL pyomelanin-producing broth medium were inoculated with 1 mL of activated bacteria and incubated at different temperatures: 25, 30, 35, 40, and 45°C, and the initial pH of each flask was adjusted to 7.0 with a shaking speed of 120 rpm for up to 72 h.[19]

Optimization of different initial pH for pyomelanin production

Pyomelanin-producing broth media with various initial pH levels were used to identify the optimal pH for

pyomelanin. The initial pH values of the media were adjusted to 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 using 0.5 M NaOH and 0.5 M HCl.[20] The production broth was incubated as described above.

Optimization of different incubation periods for pyomelanin production

The effect of the incubation period on pyomelanin production was investigated. Four different 250 mL volume flasks with 50 mL pyomelanin production broth media were inoculated with 1 mL of overnight bacterial culture and incubated at 37°C and 120 rpm for 24, 48, 72, and 96 h to determine the optimal incubation period for pigment production. At intervals of 24 h, one flask is removed and evaluated for pyomelanin production.[19]

Optimization of different L-tyrosine concentrations on pyomelanin production

Different concentrations of L-tyrosine (0,1, 1.5, 2, 2.5, and 3 g/L) were tested for optimal pyomelanin production. Four flasks with pyomelanin production broth media were inoculated with an overnight bacterial culture. All the flasks were incubated at 37°C for 72 h in a shaking incubator with a speed 120 rpm; the fifth flask lacked L-tyrosine.[18]

Production of pyomelanin pigment

Production of pyomelanin by *P. aeruginosa* was employed in pyomelanin production broth medium. Then, 1 mL of an overnight *P. aeruginosa* culture was added to 500 mL volumetric flask containing 100 mL of pyomelanin production broth medium and incubated in a shaker incubator with a shaking speed of 120 rpm at 37°C for 72 h until the broth color turned dark brown.[15]

Extraction and partial purification of pyomelanin

The isolate with the maximum pyomelanin production underwent the following procedures to extract and purification of pyomelanin according to Zhang *et al.*[21]

Solubility of partially purified pyomelanin

Pyomelanin solubility was examined by dissolving a small amount of the partially purified pigment in a variety of organic and inorganic solutions to assess the solubility of the pigment in different solvents. The solubility of the pyomelanin in distilled deionized water, ethyl acetate, chloroform, methanol 100%, ethanol 70%, chloroform, dimethyl sulfoxide (DMSO), (0.5 and 0.1 M) HCL, and (0.5 and 0.1 M) NaOH was tested.

UV-visible spectroscopy

Small amounts of pyomelanin pigment were dissolved in 0.1 M NaOH, and the alkalized pigment solution was scanned with a UV-Vis spectrophotometer (Shimadzu UV-2550, Japan) at wavelengths of 200–800 nm.

Fourier transform infrared (FT-IR) Spectroscopy analysis

The partially purified pyomelanin was mixed with potassium bromide (KBr) at a ratio 1:10, and pressed into disks under vacuum using a pellet maker, then recorded in the range of 4000–400 cm^{-1} using Thermo Fisher Scientific, USA.

Ethical approval

According to the form provided for this purpose by the Iraqi Ministry of Health, this research was subjected to ethical evaluation and was approved by the Committee of Ethical Standards in the College of Science, University of Baghdad, CSEC/1022/0129, 2022

Results

Isolation and identification of *Pseudomonas aeruginosa*

The statistical analysis of specimens shows that only 47 specimens (47%) were identified as *P. aeruginosa* obtained from 100 clinical specimens, while the other 53 specimens (53%) represented non-*Pseudomonas* genera, as shown in Table 1.

The early identification of *Pseudomonas* bacteria was done on MacConkey agar. *Pseudomonas* colonies appeared pale. On cetrimide agar, isolates are able to grow at 42°C and produce a sweet grape-like odor. Additionally, colonies appeared mucoid and could produce different pigments when they grew, and biochemical tests showed positive results for catalase and oxidase.

Screening for pyomelanin production by *Pseudomonas aeruginosa* isolates

From all *P. aeruginosa* isolates, only two isolates had the ability to produce a brown–black pigment of pyomelanin in agar media. Pyomelanin non-producing isolates appeared yellow or green, as shown in Figure 1.

Table 1: Number and percentage of *Pseudomonas aeruginosa* isolates based on the source of the specimens

Source of specimens	No. of specimens	No. of *P. aeruginosa*	%
Wound infection	15	7	46.66%
Burns	45	25	55.55%
UTI	40	15	37.5%
Total	100	47	47%
Chi-square-χ^2 (*P* value)	–	–	5.0281* (0.03982)

*($P \leq 0.05$)

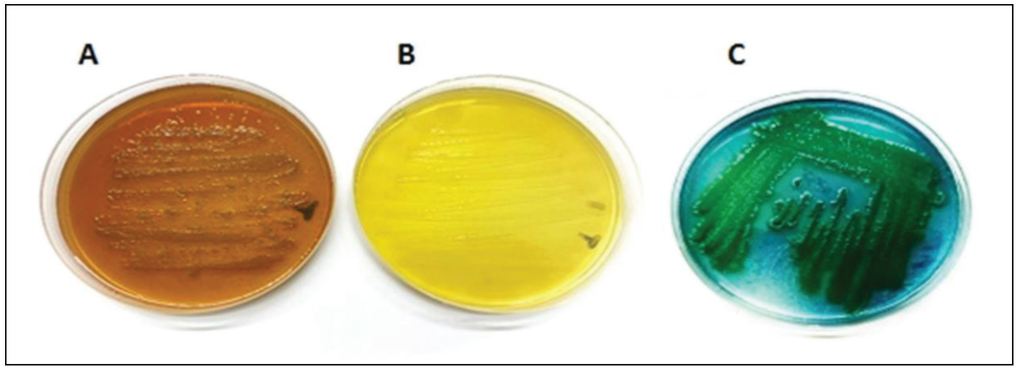

Figure 1: Screening for melanin production in agar media, (a) melanin-producing isolate, (b and c) melanin non-producing isolates

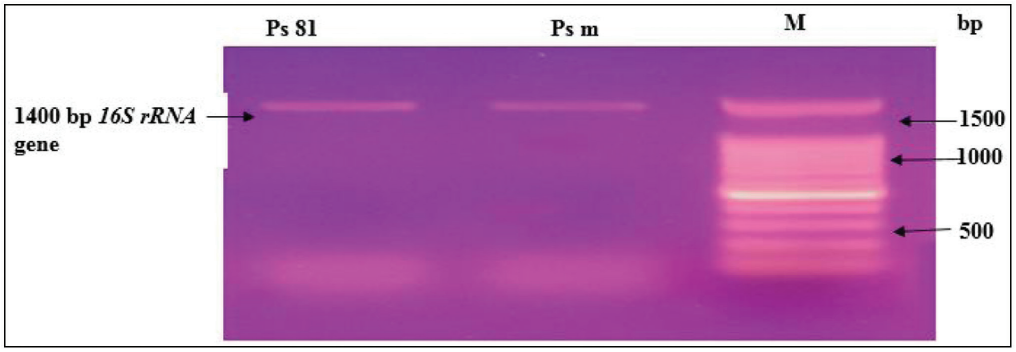

Figure 2: Gel electrophoresis of 16S rRNA gene amplification by PCR. Gel electrophoresis of PCR product showing 16S rRNA gene (1400 bp), Ps corresponds to *P. aeruginosa* isolates. M corresponds to DNA marker (100 bp)

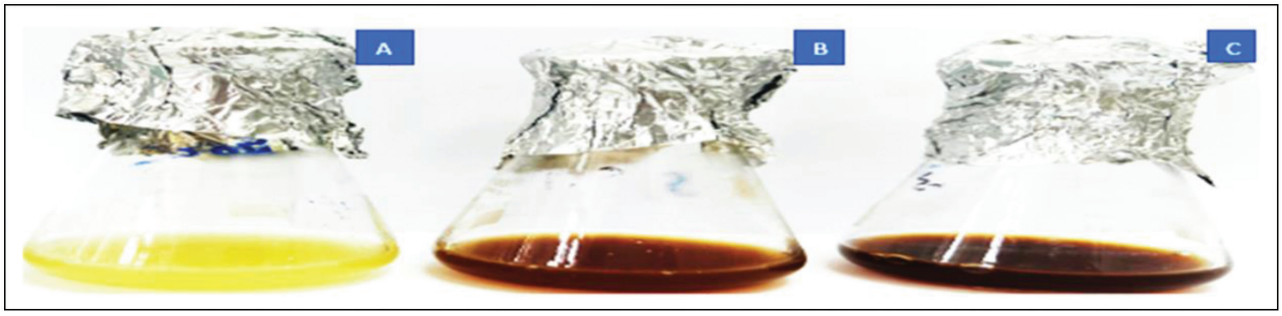

Figure 3: Screening for highest melanin-producing isolates. (a) Inoculated melanin-producing broth without tyrosine (b) Melanin-producing broth with tyrosine (c) Melanin production in Ps m isolate (d) Melanin production in Ps 81 isolate

The PCR technique analysis based on a part of the *P. aeruginosa* 16S rRNA gene (1400 pb) indicated that two *P. aeruginosa* pyomelanin producer isolates have this gene, as shown in Figure 2.

The concentration of pyomelanin produced by both Ps m and Ps 81 isolates in the production broth was 2.15 and 2.76 µg/mL, respectively. Also, the results showed that there is no pyomelanin production in the production broth without tyrosine, as shown in Figure 3.

Optimization of different agitation rates on pyomelanin production

The relationship between agitation speed and pyomelanin pigment concentration is shown in Figure 4.

Optimization of different incubation temperatures on pyomelanin production

Each bacterial species has its own characteristic range of temperature values in which it grows and reproduces. The effect of cultivation temperature on pyomelanin production is mentioned in Figure 5.

Optimization of different pH values on melanin production

pH values were tested to identify the optimal PH for pyomelanin production by Ps 81, as shown in Figure 6.

Optimization of incubation period on pyomelanin pigment production

Investigating the impact of incubation time involves estimating pyomelanin content every 24 h, as shown in Figure 7.

Optimization of different L-tyrosine concentrations on pyomelanin production

The results of optimizing L-tyrosine concentration on pyomelanin synthesis by Ps 81 isolate are shown in Figure 8.

Extraction and partial purification of pyomelanin

The extraction steps were performed as described by Zhang *et al.*[21] with slight modifications in the time and speed of the centrifugation process. The cultures of pyomelanin-producing broth medium were centrifuged at 8000 rpm for 15 min at 4°C to eliminate cells and other debris. After many steps of alkaline dissolving, acid precipitation, and washing by organic solvent, the partially purified pyomelanin was allowed to air dry.

Chemical and physical analysis of purified pyomelanin extract

Purified pyomelanin extracted from Ps 81 has a distinct solubility characterization, as shown in Table 2 and Figure 9.

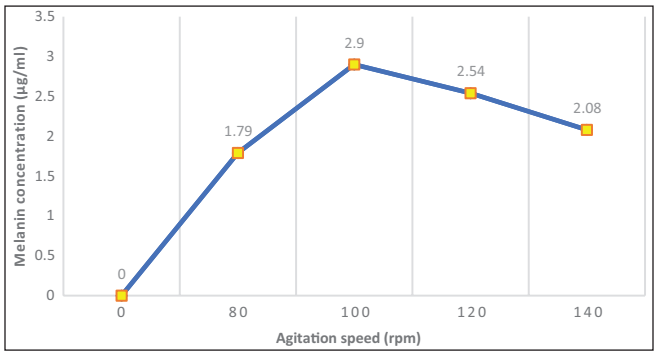

Figure 4: Effect of agitation rates on pyomelanin production

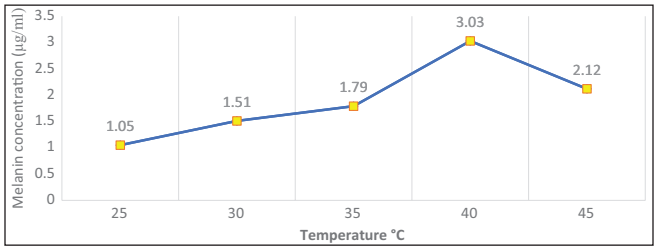

Figure 5: Effect of temperature on pyomelanin production

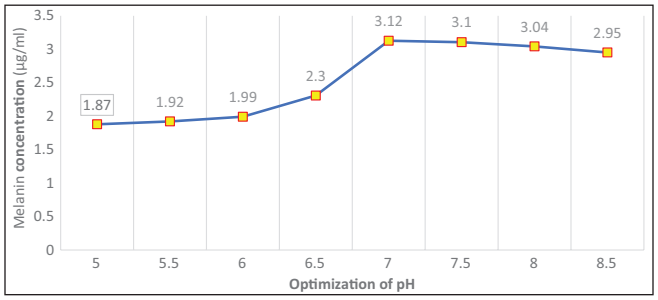

Figure 6: Effect of different pH values on melanin production

UV-visible spectroscopic analysis of purified pyomelanin

In the UV region, alkaline pyomelanin solution shows a significant optimal absorption peak at 273.5 nm that gradually fades as longer wavelengths are approached, as shown in Figure 10.

Fourier transform infrared (FT-IR) spectroscopy analysis

FT-IR analysis shows 17 peaks, as depicted in Figures 11 and 12.

Discussion

The results in Table 1 show a statistical analysis using the Chi-square test, revealing that burn wounds account for the highest rate of *Pseudomonas* infection at (55.55%), while UTIs and wounds account for 37.5 and 46.6%, respectively. The difference between the percentages was significant at $P < 0.05$. Burn wounds account for the high

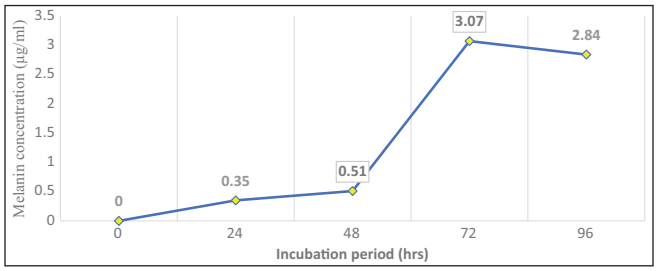

Figure 7: Effect of different incubation periods (h) on melanin pigment production

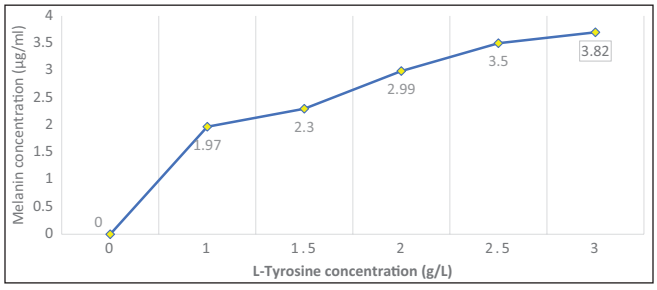

Figure 8: Effect of L-tyrosine concentration (g/L) on melanin pigment production

Table 2: Solubility test for purified pyomelanin extracted from Ps 81 isolates

Solvent	Results
5 and 0.5 M NaOH	Soluble
Methanol 100%	Soluble
Ethanol 70%	Soluble
Chloroform	Insoluble
Ethyl acetate	Insoluble
5 and 0.5 M HCl	Insoluble
Deionized Water	Insoluble
DMSO	Soluble

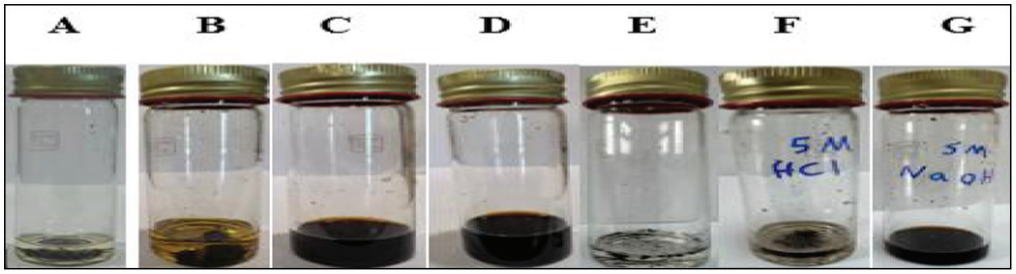

Figure 9: The solubility test of extracted melanin from Ps81. (a) Insoluble in water (b) Insoluble in ethyl acetate (c) Soluble in methanol (d) Soluble in ethanol (e) Insoluble in chloroform (f) Insoluble in HCl (g) Soluble in NaOH

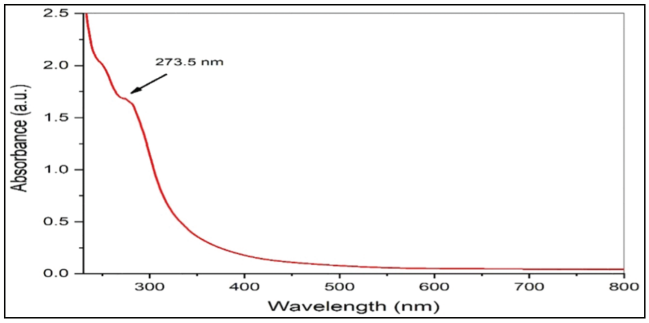

Figure 10: UV-visible absorption spectrum of partially purified extracted pyomelanin

rate of *Pseudomonas* infection (55.5%) which is due to that burn wounds are particularly susceptible sites for infection by opportunistic microorganisms such as *P. aeruginosa*, as well as the capacity of *Pseudomonas* to produce many virulence factors.[16,17] MacConkey agar, a selective and differential media, allows the growth of Gram-negative bacteria, including the genus *Pseudomonas*. Lactose and neutral red indicator are present in the media, as these bacteria are not capable of fermenting lactose and the pH of the media does not change, so the color of the colony appears pale. Similar results were obtained by Hossain *et al.* and Mahmood *et al.*[22,23]

Cetrimide agar, a selective and differential medium, contains dipotassium sulfate and magnesium chloride, which enhance the production of pyoverdine and pyocyanin pigments that work together to create distinctive green and yellow colonies of *P. aeruginosa*. It also contains quaternary ammonium, which has a bactericidal effect against Gram-positive and certain Gram-negative bacteria, including species other than *P. aeruginosa.* Colonies of *P. aeruginosa* appear colored and mucoid with a grape-like odor and are capable of growing at 42°C, with similar results reported by Kodake *et al. and* Peekate *et al.*[24,25]

Biochemical tests showed that all the *Pseudomonas* isolates were positive for the oxidase and catalase tests. The same results were obtained in a previous study.[26] Additionally, the isolates were identified by VITEK-2 system.

The compact system was similar to that mentioned by Mohammed and Zgair.[27] Only two isolates could produce a brown color within 4 days. These colonies were picked up and coded as Ps 81 and Ps m, which were isolated from burn patients. Pyomelanin non-producing isolates appeared as yellow or green colonies, as shown in Figure 1. All the isolates were positive for the catalase and oxidase tests, with similar results obtained by Gheni and Odaa.[26]

The PCR technique analysis shown in Figure 2 indicated that the two *P. aeruginosa* pyomelanin producer isolates have *P. aeruginosa* 16s RNA gene. Based on these results, the two isolates belong to *P. aeruginosa*. The Ps 81 isolate was the highest pyomelanin-producing isolate with 2.76 µg/mL, which has been selected for further steps in this study. Additionally, the results showed that there is no pyomelanin production in the broth without tyrosine, indicating that tyrosine was the substrate for pyomelanin synthesis, as shown in Figure 3A. The same result was obtained from a similar study.[4]

The optimization study's results of agitation rates are shown in Figure 4. The optimum rpm for maximum pyomelanin pigment production was at 100 rpm with 2.9 µg/mL by Ps 81, while the lower 80 rpm and higher 120 and 140 rpm resulted in 1.79, 2.54, and 2.08 µg/mL pyomelanin, respectively, because the oxygen transfer rate is directly proportional to the agitation speed.[28] Additionally, tyrosinase catalyzes the oxidation reactions, which require a certain oxygen concentration that can be maintained at 100 rpm. Different optimal agitation speeds for pyomelanin production were obtained for different microorganisms; the optimal agitation rate for *Brevibacillus invocatus* was 120 rpm, as found by Ammanagi.[19]

The maximum pyomelanin yield, 3.03 µg/mL, was obtained at 40°C, as mentioned in Figure 5. At lower and higher temperatures 25, 30, 35, and 45°C, less pyomelanin was produced, with a production of 1.05, 1.51, 1.79, and 2.12 µg/mL, respectively. Pyomelanin output was decreased because lower and higher temperatures impeded bacterial growth and enzyme activity.[29] In a previous study, the optimal temperature

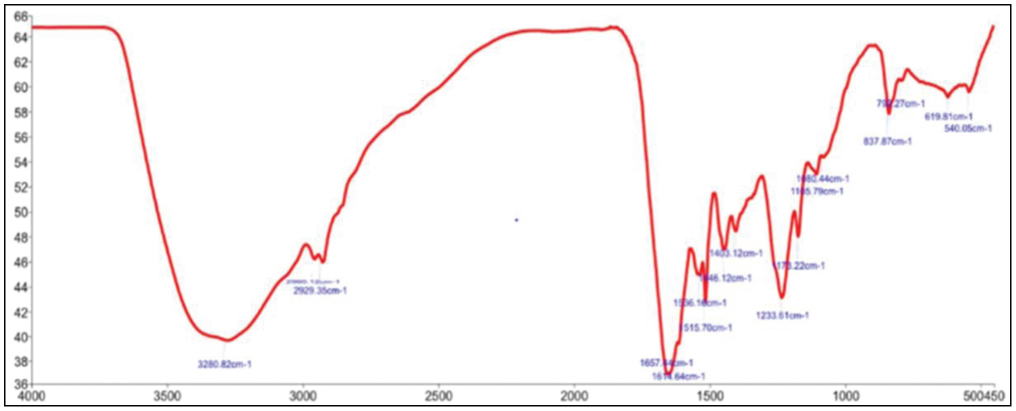

Figure 11: FT-IR analysis of partially purified extracted pyomelanin

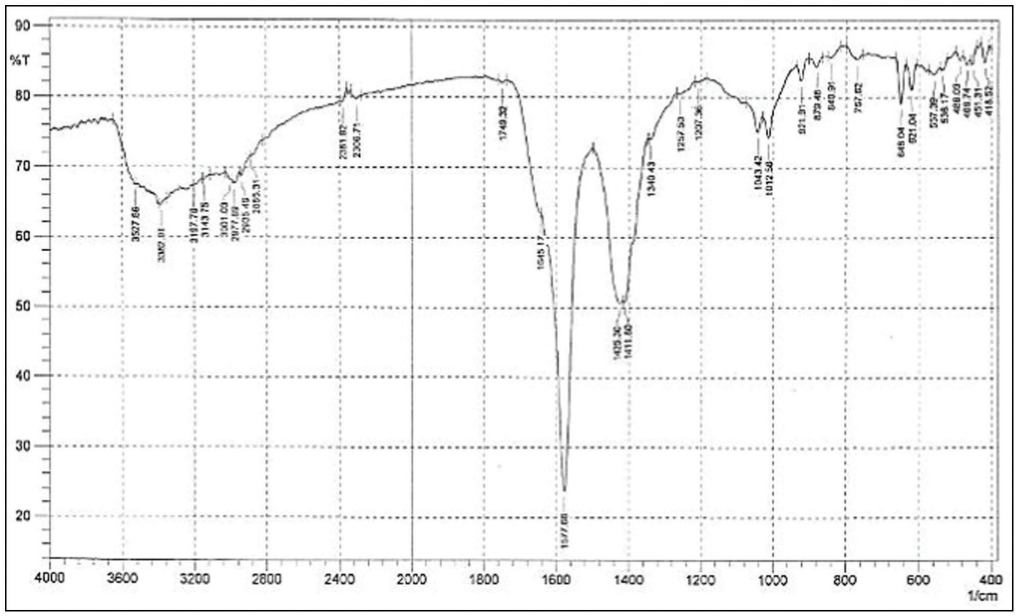

Figure 12: FT-IR spectroscopy for synthetic pyomelanin

for pyomelanin pigment production was reported for *Aspergillus oryzae* and *Klebsiella sp.* GSK as 30°C and 37°C, respectively.[18,30]

Based on the findings in Figure 6, it can be inferred that the values 7.0 and 7.5 were the ideal starting pH values for encouraging the production of pyomelanin pigment, with pyomelanin concentrations of 3.12 and 3.1 µg/mL, respectively. Since 7.5 is the ideal pH for *P. aeruginosa* growth, while 7.0 and 7.5 were the optimum pH values for the formation of secondary metabolites (pyomelanin),[20] it was mentioned that the maximum growth pH was7.5, and pH values lower than this showed a reduction in *Pseudomonas* growth. The maximum pyomelanin was obtained at 7.0 and 7.5. The same optimum pH value of 7.0 was recorded for both actinomycetes and *Pseudomonas stutzeri*.[28]

According to the findings in Figure 7, the level of the yields increased after 24 h at the stationary phase of the bacteria as secondary metabolites were produced it can be assumed that the maximum value of pyomelanin pigment was in 72 h, with 3.07 µg/mL. Eventually, the pyomelanin productivity declines with a longer incubation period. Different bacterial species possess different stationaryphases ; in *Aspergillus oryzae,* the maximum pyomelanin production was at the 7-days of incubation.[18]

As shown in Figure 8, the maximum pyomelanin produced was at 3% g/L of L-tyrosine with 3.82 µg/mL. The lowest pyomelanin yield was observed at tyrosine concentrations of 1 and 1.5 g/L, with 1.97 and 2.3 µg/mL, respectively. The moderate pyomelanin concentrations were observed at 2 and 2.5 g/L L-tyrosine concentrations, with 2.99 and 3.5 µg/mL of pyomelanin pigment, respectively. No pyomelanin synthesis was observed in the flask lacking L-tyrosine, indicating that tyrosine was the substrate for pyomelanin synthesis. Furthermore, the findings

indicated that 2 g/L of L-tyrosine was acceptable because it was proportional to both the amount of pigment produced and the incubation period. On the other hand, an increase in tyrosine concentration of more than 2 g/L led to the appearance of crystal particles in the broth, which inversely affected the extraction process and became more complicated.[31] In a previous optimization study for *Aspergillus oryzae*, the maximum L-tyrosine concentration was at 0.25% with pyomelanin production at 4.974 µg/mL,[18] and it was at 2 g/L for *Klebsiella pneumoniae*.[31]

The amount of pyomelanin yielded from culturing pyomelanin-producing isolate Ps 81 in the production media under optimum conditions (2% L-tyrosine, initial pH 7.0, incubated at 40°C with an agitation rate of 100 rpm, for 72 h) was 3.96 µg/mL.

After centrifuging the pyomelanin-producing cultured media, the pH of the supernatant was adjusted to 12 with 10M NaOH to prevent the formation of melanoidins, then to pH 2 with 5 HCl to precipitate the pyomelanin and centrifuged to remove the supernatant. The pyomelanin was re-dissolved with 10 M NaOH and mixed with 0.2 mL of chloroform to deproteinize the pyomelanin pigment. The mixture was centrifuged again, and the supernatant was adjusted to pH 2 and centrifuged once more to obtain the pyomelanin, which was then washed with 100% and 70% and allowed to air dry.

It is worth mentioning that the results of the solubility analysis, as shown in Table 2 and Figure 9, revealed that the extracted pigment is insoluble in water and some organic solvents such as chloroform and ethyl acetate, and precipitated by HCl. It was readily soluble in NaOH and some organic solvents, including 100% ethanol, 70% ethanol, and DMSO. These results were consistent with those of Noman *et al.* and Pralea *et al.*[32,33]

It is worth mentioning that the nature of the pyomelanin pigment was further confirmed by its specific UV-Vis spectral properties, as shown in Figure 10. The maximum absorption peak was at 273.5 nm in the UV region and decreased toward the visible and infrared regions. The UV-Vis spectrum ranged from 200 to 800 nm, with the absorption peak declining toward the visible region, which is a characteristic property of melanin.[34] The complex conjugated molecules in the pyomelanin structure, which absorb and scatter UV-light photons, are thought to be the cause of the pyomelanin strong UV-light absorption.[35] Natural pyomelanin from different sources possesses different maximum UV-Vis absorption peaks, such as in *Bacillus safensis*, the *extracted pyomelanin* peak was between 200 and 300 nm and diminished toward visible light,[36] whereas *Pseudomonas* otitidis had a maximum absorption peak in the range of 215–350 nm,[37] and purified pyomelanin extracted from *Actinoalloteichus* sp. MA-32 at 300 nm.[38]

The most effective use of Fourier transform infrared (FT-IR) spectroscopy is for the interpretation of structurally unidentified compounds and for determining the functional groups in pyomelanin extracted from Ps 81. The infrared spectrum of partially purified pyomelanin extracted from Ps 81 and synthetic melanin showed a high degree of similarity, as depicted in Figure 11. Pyomelanin exhibited a broad absorption band around 3280.82 cm^{-1} due to the presence of -OH group and N–H groups, with small bands at 2929.35 and 2960.12 cm^{-1} due to alkane group C–H stretching. Peaks at 1657.44 cm^{-1} and 1614.64 cm^{-1} indicated aromatic C = C conjugation with either C = O or COO-groups, while peaks at 1515.70 cm^{-1} and 1536.16 cm^{-1} were attributed to aromatic C = C bonds.

The peaks at 1446.12 cm^{-1} and 1403.12 cm^{-1} were due to C–H bending in the aliphatic group, while the peak at 1233.61 cm^{-1} revealed an anhydride group C–O. The two peaks at 1173.22 cm^{-1} and 1105.79 cm^{-1} were assigned to aliphatic amine C–N. The peak at 1080 referred to C–O in phenols or carboxylic groups. Peaks at 837.87, 792.27, 619.81, and 540.05 cm^{-1} were related to the aromatic C–H group. The extracted pigment showed a high degree of resemblance in major absorption peaks with synthetic pyomelanin, confirming that the produced pigment is pyomelanin. These outcomes correspond to the findings in previous studies.[4,6,32,38]

Conclusion

It is inferred from this study that *P. aeruginosa* Ps 81, isolated from burns, has the potential for pyomelanin production in the presence of L-tyrosine as a precursor. Pyomelanin has been successfully extracted and characterized. Optimization parameters for cultural production observed in broth, aimed at increasing the yield of pyomelanin pigment, were found to be in the enhanced presence of 2 g/L of precursor and the pyomelanin production at an alkaline pH value of the media in a shaking statement, and there was no pyomelanin production in static states.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Valentini M, Gonzalez D, Mavridou DA, Filloux A. Lifestyle transitions and adaptive pathogenesis of *Pseudomonas aeruginosa*. Curr Opin Microbiol 2018;41:15-20.
2. Sulaiman SD, Abdulhasan GA. Curcumin as efflux pump inhibitor agent for enhancement treatment against multidrug resistant *Pseudomonas aeruginosa* isolates. Iraqi J Sci 2020;61:59-67.
3. Ketelboeter M, Potharla Y, Bardy L. NTBC treatment of the pyomelanogenic *Pseudomonas aeruginosa* clinical isolate PA1111

inhibits pigment production and increases sensitivity to oxidative stress. Curr Microbiol 2014;69:343-8.
4. El-Naggar NE, El-Ewasy SM. Bioproduction, characterization, anticancer and antioxidant activities of extracellular pyomelanin pigment produced by newly isolated microbial cell factories Streptomyces glaucescens NEAE-H. Sci Rep 2017;7:42129.
5. Zou Y, Ma K. Screening of *Auricularia auricula* strains for strong production ability of pyomelanin pigments. Food Sci Technol 2017;38:41-4.
6. Mahmood HM, Mohammed AK, Flayyih MT. Purification and physiochemical characterization of pyopyomelanin pigment produced from local *Pseudomonas aeruginosa* isolates. WJPR 2015;10:289-99.
7. Singh S, Nimse SB, Mathew DE, Dhimmar A, Sahastrabudhe H, Gajjar A, *et al*. Microbial pyomelanin: Recent advances in biosynthesis, extraction, characterization, and applications. Biotechnol Adv 2021;53:107773.
8. Mahmood HM, Mohammed AK, Fleih MT. Cytotoxic effect of pyomelanin pigment produced from local *Pseudomonas aeruginosa* isolates on different cell lines using MTT assay. Iraqi J Biotechnol 2016;15:46-52.
9. Nosanchuk JD, Casadevall A. Impact of pyomelanin on microbial virulence and clinical resistance to antimicrobial compounds. Antimicrob Agents Chemother 2006;50:3519-28.
10. Płonka P, Grabacka M. Pyomelanin synthesis in microorganisms: Biotechnological and medical aspects. Acta Biochim Pol 2006;53:429-43.
11. Kamarudheen N, Naushad T, Rao KV. Biosynthesis, characterization, and antagonistic applications of extracellular pyomelanin pigment from marine *Nocardiopsis* Sps. Ind J Pharm Educ Res 2019;53:112-20.
12. Koyanagi T, Katayama T, Suzuki H, Nakazawa H, Yokozeki K, Kumagai H. Effective production of 3, 4-dihydroxyphenyl-L-alanine (L-DOPA) with Erwinia herbicola cells carrying a mutant transcriptional regulator TyrR. J Biotechnol 2005;115:303-6.
13. SAS. Statistical Analysis System, User's Guide. 6th ed. N.C., USA: SAS. Inst. Inc. Cary; 2018.
14. Al-Maslookhi H. Structure, Function, and Engineering of Biotechnologically Important Proteins (Doctoral dissertation, Cardiff University, UK); 2019.
15. Surwase SN, Jadhav SB, Phugare SS, Jadhav JP. Optimization of pyomelanin production by *Brevundimonas sp.* SGJ using response surface methodology. 3 Biotech 2013;3:187-94.
16. Zhu L, Chu Y, Zhang B, Yuan X, Wang K, Liu Z, *et al*. Creation of an industrial *Bacillus thuringiensis* strain with high pyomelanin production and UV tolerance by gene editing. Front Microbiol 2022;13:913715.
17. Makpol S, Jam FA, Rahim NA, Khor SC, Ismail Z, Yusof YA, *et al*. Comparable down-regulation of TYR, TYRP1 and TYRP2 genes and inhibition of melanogenesis by tyrostat, tocotrienol-rich fraction and tocopherol in human skin melanocytes improves skin pigmentation. Clin Ter 2014;165:e39-45.
18. El-Batal AI, Al Tamie MS. Optimization of pyomelanin production by *Aspergillus oryzae* and incorporation into silver nanoparticles. Der Pharm Lett 2016;8:315-3.
19. Ammanagi AI, Shivasharana CT, Krishnaveni R, Badiger AS, Ramaraj VK, Srinath BS, *et al*. A biotechnological approach to optimization and production of pyomelanin by *Brevibacillus invocatus* strain IBA, under submerged fermentation. Biomedicine 2022;42:318-24.
20. Charyulu EM, Gnanamani A. Condition stabilization for *Pseudomonas aeruginosa* MTCC 5210 to yield high titers of extra cellular antimicrobial secondary metabolite using response surface methodology. Curr Res Bacteriol 2010;3:197-213.
21. Zhang J, Cai J, Deng Y, Chen Y, Ren G. Characterization of pyomelanin produced by a wild-type strain of *Bacillus cereus*. Front Biol China 2007;2:26-9.
22. Hossain MG, Saha S, Rahman MM, Singha JK, Mamun AA. Isolation, identification and antibiogram study of *Pseudomonas aeruginosa* from cattle in Bangladesh. J Vet Adv 2013;3:180-5.
23. Mahmood HM, Nasir GA, Ibraheem QA. Relationship between pigments production and biofilm formation from local *Pseudomonas aeruginosa* isolates. Iraqi J Agric Sci 2020;51:1412-9.
24. Kodaka H, Iwata M, Yumoto S, Kashitani F. Evaluation of a new agar medium containing Cetrimide, Kanamycin and Nalidixic acid for isolation and enhancement of pigment production of *Pseudomonas aeruginosa* in clinical samples. J Basic Microbiol 2003;43:407-13.
25. Peekate LP, BatomBari S, Nice P. Research article comparing the efficacy of Kings B, cetrimide, and chloramphenicol nutrient agar medium in the isolation of *Pseudomonas* species. Asian J Biol Sci 2018;11:145-51.
26. Gheni MR, Odaa NH. The antimicrobial activity of pyomelanin-mediated synthesis of silver nanoparticles. Egypt J Hosp Med 2023;90:3383-94.
27. Mohammed HA, Zgair AK. Detection of quorum sensing genes of *Pseudomonas aeruginosa* isolated from different areas in Iraq. Iraqi J Sci 2022;30:4665-73.
28. Thaira H. Bioprocess Development and Optimization of Pyomelanin from *Pseudomonas stutzeri* (Doctoral dissertation). Surathkal, Mangaluru: National Institute of Technology; 2018.
29. Anjaneya O, Souche SY, Santoshkumar M, Karegoudar TB. Decolorization of sulfonated azo dye Metanil Yellow by newly isolated bacterial strains: *Bacillus sp.* strain AK1 and *Lysinibacillus sp.* strain AK2. J Hazard Mater 2011;190:351-8.
30. Sajjan S, Kulkarni G, Yaligara V, Kyoung L, Karegoudar TB. Purification and physiochemical characterization of pyomelanin pigment from *Klebsiella sp*. GSK J Microbiol Biotechnol 2010;20:1513-20.
31. Saud HM, Alaubydi MA. Production, extraction and partial purification of melanin pigment from pathogenic *Klebsiella pneumoniae* HM isolated from clinical samples. Int J Curr Microbiol App Sci 2016;5:910-9.
32. Noman AE, Al-Barha NS, Chen F. Characterization of physicochemical properties of pyomelanin produced by Gluconobacter oxydans FBFS 97. Fermentation 2022;8:574.
33. Pralea IE, Moldovan RC, Petrache AM, Ilieș M, Hegheș SC, Ielciu I, *et al*. From extraction to advanced analytical methods: The challenges of pyomelanin analysis. Int J Mol Sci 2019;20:3943.
34. Marín-Sanhueza C, Echeverría-Vega A, Gomez A, Cabrera-Barjas G, Romero R, Banerjee A. Stress dependent biofilm formation and bioactive melanin pigment production by a thermophilic Bacillus species from chilean hot spring. Polymers 2022;14:680.
35. Al Khatib M, Harir M, Costa J, Baratto MC, Schiavo I, Trabalzini L, *et al*. Spectroscopic characterization of natural pyomelanin from a *Streptomyces cyaneofuscatus* strain and comparison with pyomelanin enzymatically synthesized by tyrosinase and laccase. Molecules 2018;23:1916.
36. Tarangini K, Mishra S. Production of pyomelanin by soil microbial isolate on fruit waste extract: Two step optimizations of key parameters. Biotechnol Rep 2014;4:139-46.
37. Deepthi SS, Reddy MK, Mishra N, Agsar D. Melanin production by *Pseudomonas sp.* and *in silico* comparative analysis of tyrosinase gene sequences. BioTechnologia 2021;102:411-24.
38. Manivasagan P, Venkatesan J, Senthilkumar K, Sivakumar K, Kim SK. Isolation and characterization of biologically active pyomelanin from *Actinoalloteichus sp.* MA-32. Int J Biol Macromol 2013;58:263-74.