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Extraction and Characterization of Melanin Pigment from Local Isolated *Pseudomonas aeruginosa*

Naz Fuad Hama Khorshid[1*] and Nisreen Hadi Odaa[2]
[1,2]Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.
*Corresponding Author.

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Abstract

This study shows that a clinical isolate of *Pseudomonas* spp. from different sources, like burn patients, wound infections, and UTI patients, can make melanin pigment when 2% L-tyrosine is present in both liquid and solid states. Morphological, biochemical, and VITEK-2 compact system analysis led to the identification of these bacteria as *Pseudomonas aeruginosa*. The higher melanin producer isolates Ps81, which produces melanin at a rate of 3.018933 µg/ml, has been chosen for further investigation steps. We extracted the pigment using several steps, including alkali dissolving, acid precipitation, and washing with organic solvents. The pigment particles were tested chemically and found to be acid-resistant, alkaline-soluble, and not soluble in water. They were, however, soluble in methanol 100%, ethanol 70%, and DMSO, but not in ethyl acetate or chloroform. The alkali melanin solution had a strong UV absorbance at 273.5 nm, which decreased as the wavelength got longer toward visible light and infrared. The pigment's UV-visible spectrum went from 200 to 800 nm. We further characterized the melanin pigment using FT-IR spectroscopy. The infrared spectrum of pure melanin taken from Ps 81 showed a broad absorption band around 3280.82 cm^{-1}. This means that -OH groups and N-H groups were present.

Keywords: Melanin pigments, *Pseudomonas aeruginosa*, Extraction, Purification, UV-vis spectroscopy, FT-IR spectroscopy.

1. Introduction

Pseudomonas aeruginosa is a gram-negative and environmental bacterium that can infect vulnerable patients with both acute and chronic infections. This organism is very versatile, and has the capacity to build biofilms, a high level of inherent antibiotic resistance, and a variety of virulence factors (1).Also, *P.aeruginosa* bacteria are capable of producing different kinds of pigments, including melanin. The oxidative polymerization of phenolic or indolic compounds generates melanin pigment, which is typically brown or black in color, hydrophobic, and negatively charged (2). The generation of melanin involves many enzymatic and non-enzymatic

stages . The tyrosinase catalyzer first converts L-tyrosine into L-3,4 dihydroxyphenyl alanine (L-DOPA), then the tyrosinase enzyme converts L-DOPA to dopachrome, which subsequently converts to melanin through numerous non-enzymatic oxidoreduction steps (3).

Recent studies have linked the function of melanin to the defense against environmental stressors such as UV-light, free radicals, heavy metal toxicity, and hydrolytic enzymes. Antibiotics find bacteria that produce melanin to be more resistant. These characteristics make melanin a significant bioactive substance with numerous industrial applications. Studies have also shown that melanin has antimicrobial and antiviral effects, which opens up new avenues for research (3-5).Thus, in our study, we intend to focus on the production, extraction, purification, and characterization of microbial melanin.

2. Materials and Methods

2.1. Identification of isolates and inoculated

Forty *P.aeruginosa* isolates were obtained from one hundred clinical specimens from Iraqi patients' hospitals, including burn patients, UTI patients, and wound infections. All specimen swabs were inoculated and incubated on MacConkey agar as selective and differential media for Gram-negative and enteric bacteria for 24 hrs. at 37°C in a lactose fermenter. For further identification, the isolates were re-cultured on cetrimide agar as selective and differential media for *P.aeruginosa*. The isolates were initially identified by cultural characteristics, biochemical tests, and the VITEK-2 compact system (Bio Merieux/France) used in this present study (6).

2.2. Screening for melanin production by *P.aeruginosa*

All the *Pseudomonas* isolates were tested for melanin production by inoculating fresh *Pseudomonas* colonies on nutrient agar medium supplied with 2% L-tyrosine. These plates were incubated for 3 to 4 days at 37°C and observing the brown-black color of melanin. Also, 1 ml of overnight bacterial culture inoculated on nutrient broth supplied with 2% L-tyrosine (melanin production broth media) and incubated at 37°C for 3 to 4 days in a shaker incubator with a speed 120 rpm with nutrient broth without tyrosine as a control for observing any color change, the most potent melanin-producing isolate will be chosen for further study. In order to choose the highest melanin-producing isolates, all the melanin-producing isolates were inoculated in brain-heart infusion broth and incubated at 37°C for 24 hrs. then the optical density was measured at 600 nm to normalize the cell number, then 1 ml of overnight bacterial culture inoculated in 100 ml flasks with 20 ml melanin production broth media and then incubated at 37°C for 3 to 4 days in a shaking incubator with a speed 120 rpm, until the color of the media turned brown, then the media were centrifuged at 8000 rpm for 20 minutes, and the supernatant was taken. The concentration of melanin in the supernatants was measured using an enzyme-linked immunosorbent assay (ELISA) at absorbency 450A and calculated by comparing the OD of unknown samples with a standard curve in **Figure 1** (7).

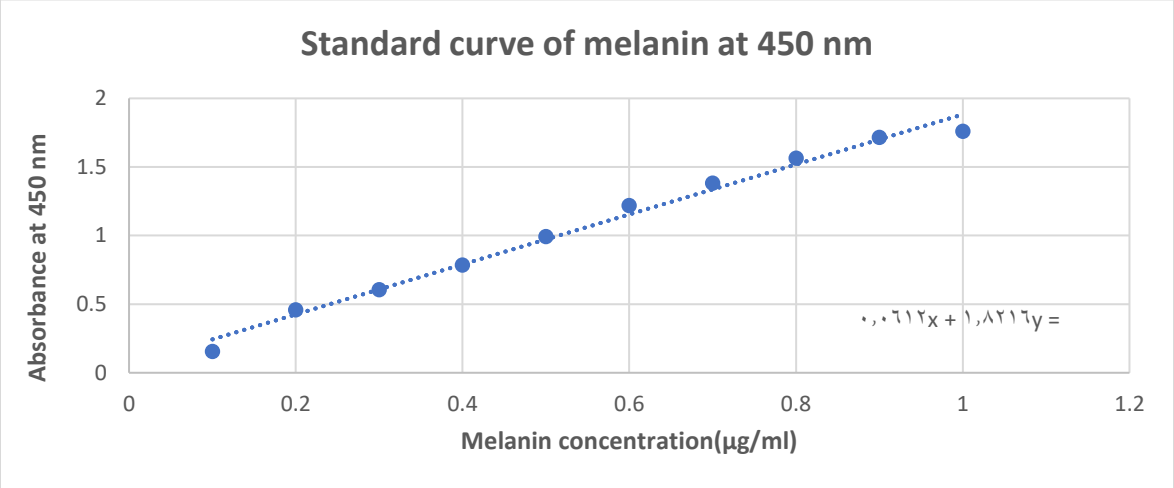

Figure 1. Standard curve for synthetic melanin.

2.3. Production of melanin pigment

Production of melanin by *P.aeruginosa* was employed in melanin production broth medium, then 1 ml of an overnight *P.aeruginosa* culture was added and incubated in a shaker incubator with a shaking speed of 120 rpm at 37°C for 72 to 96 hrs. Until the broth color turned dark brown (7).

2.4. Extraction and partial purification of melanin pigment

The isolate with the maximum melanin production underwent the following procedures according to (8) to extract and purify melanin.

1. The cultures of melanin-producing broth medium were collected into falcon tubes and centrifuged at 8000 rpm for 15 min at 4°C to eliminate other debris.
2. The cell-free supernatant was adjusted to pH 12 with 10 M NaOH to ensure complete polymerization.
3. Then the pH of the supernatant was adjusted to 2 with 5 M HCl, and the precipitated melanin was centrifuged at 10000 rpm for 20 min to remove the supernatant.
4. The collected precipitated melanin was dissolved in 10 M NaOH, then 0.2 ml of chloroform was mixed with the dissolved melanin in order to deproteinize the pigment and centrifuged at 10000 rpm for 10 min.
5. The supernatant pH was adjusted to 2 in order to precipitate the crude melanin and centrifuged again as above.
6. The partially purified melanin was obtained after it was washed twice with 0.1 ml of 100% methanol and 0.1 ml of 70% ethanol.
7. The partially purified melanin was allowed to air dry in a sterile petri dish at room temperature.

2.5. Physico-chemical characterization of partially purified melanin pigment

2.5.1. Solubility of partially purified melanin

We examined the solubility of melanin by dissolving a small amount of the partially purified pigment in various organic and inorganic solutions, as per (9, 10). The solubility of the melanin in distilled deionized water is 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.

2.5.2. UV-Visible Spectroscopy

Small amount of melanin pigment dissolved in 0.1 M NaOH, the alkalize pigment solution was scanned with UV-VIS spectrophotometer from (Shimadzu UV-2550/Japan) at wavelength 200-800 nm (10).

2.5.2. Fourier transform infrared (FTIR) Spectroscopy analysis

The most effective use of Fourier transform infrared (FTIR) spectroscopy is for the interpretation of structurally unidentified compounds and for determining the functional group for melanin extracted from Ps 81. The FTIR was recorded at 4,000- 400 cm-1(10) using (Thermo Fisher Scientific/USA)

3. Results

3.1. Isolation and identification of *P.aeruginosa*

Only 47 specimens (47%) were *P.aeruginosa* obtained from 100 clinical specimens, while the other 53 specimens (53%) represented non-*Pseudomonas* genera, as shown in **Table 1**.

Table 1. Number and percentage of *P.aeruginosa* isolates based on the source of the specimens.

Source of Specimens	Number of Specimens	Number of *P.aeruginosa*	Number in percentage of *P.aeruginosa*
Wound infection	15	7	46.66%
Burns	45	25	55.55%
UTI	40	15	37.5 %
Total	100	47	47%

The early identification of *Pseudomonas* bacteria was done on MacConkey agar; it is a selective and differential medium. The *pseudomonas* colony appeared pale (lactose non-fermenter), as shown in **Figure 2A**. On cetrimide agar, isolates are able to grow at 42°C and produce a sweaty grape-like odor and also have the ability to produce different pigments as they grow. Cetrimide agar is a selective and differential media. Cetride is a quaternary ammonium compound that has a bactericidal effect against certain Gram-negative organisms, including species other than *P.aeruginosa* and a wide range of Gram-positive bacteria. Dipotassium sulfate and magnesium chloride, which are present in cetrimide agar, enhance the production of pyoverdine and pyocyanin pigments, which work together to create distinctive green and yellow colonies of *Pseudomonas aeruginosa*, as shown in **Figure 2B**.

Biochemical tests showed that all the *Pseudomonas* isolates were positive to oxidase and catalase.

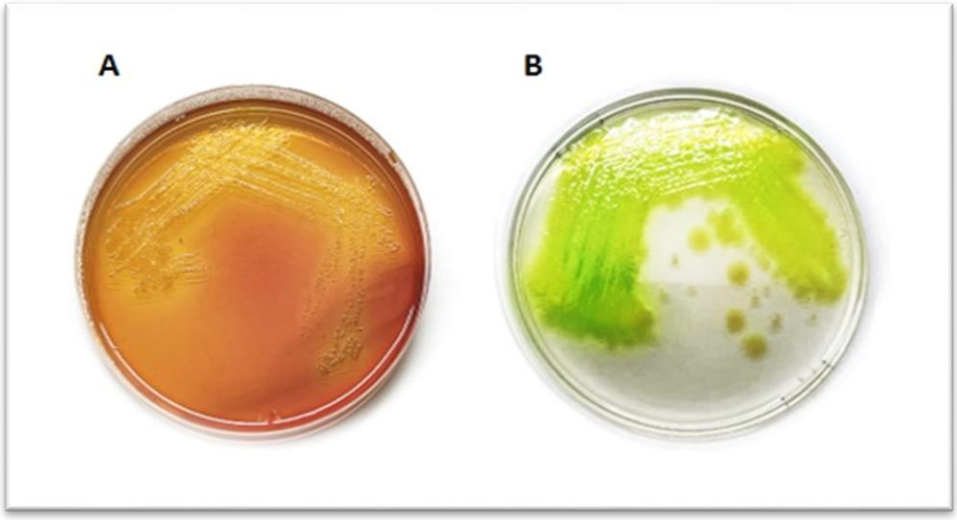

Figure 2. *P. aeruginosa*, A. on MacConkey agar, B. on cetrimide agar.

3.2. Screening for melanin production by *P.aeruginosa* isolates

From all *P.aeruginosa isolates*, only two isolates (Ps 81 and Ps m) had the ability to produce a brown-black pigment of melanin on nutrient agar with 2 % L-tyrosine as shown in **Figure 3A**. The source of these two isolates that produce melanin pigment was burns.

Figure 3. A.Melanin producing isolate, B. and C. melanin non-producing isolate.

We cultured the two melanin-producing isolates, Ps 81 and Ps m, in melanin production broth with 2% tyrosine under the same incubation conditions (initial pH 7.0, temperature 37°C, agitation speed 120 rpm). Melanin production broth without tyrosine was used as a negative control. In order to figure out how much melanin each isolate made, the absorbency of the melanin-producing broth was measured at 450 nm using an ELISA spectrophotometer. This was done after the broth was centrifuged at 8000 rpm for 15 minutes to get rid of the bacteria cells. The concentration of the melanin pigment was then calculated in µg/ml units using **Figure 1** as a guide. **Table 2** and **Figure 4** show that we selected the Ps 81 isolate for further steps of this study due to its highest melanin production.

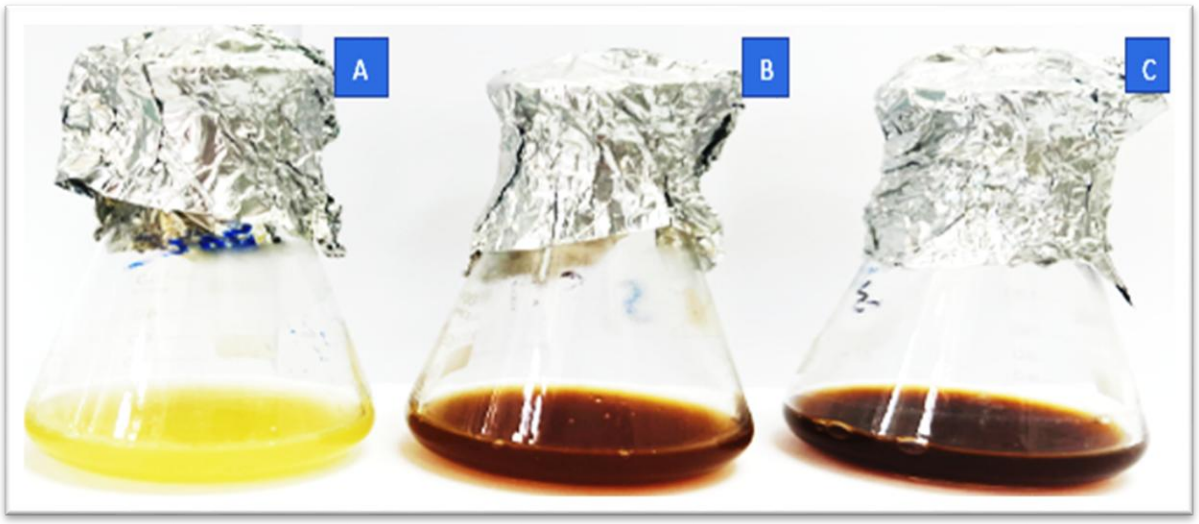

Figure 4. A. Negative control, B. Ps m isolate. C. Ps 81 isolate.

Table 2. The concentrations of melanin produce from *P.aeruginosa* isolates.

Melanin producing *P. aeruginosa* isolate	**Melanin Conc. (µg/ml)**
Ps 81	3.018933
Ps m	2.596065

3.3. Extraction and partial purification of melanin pigments

The extraction steps were performed as described by (8) with slight modification, as shown in **Figure 5**.

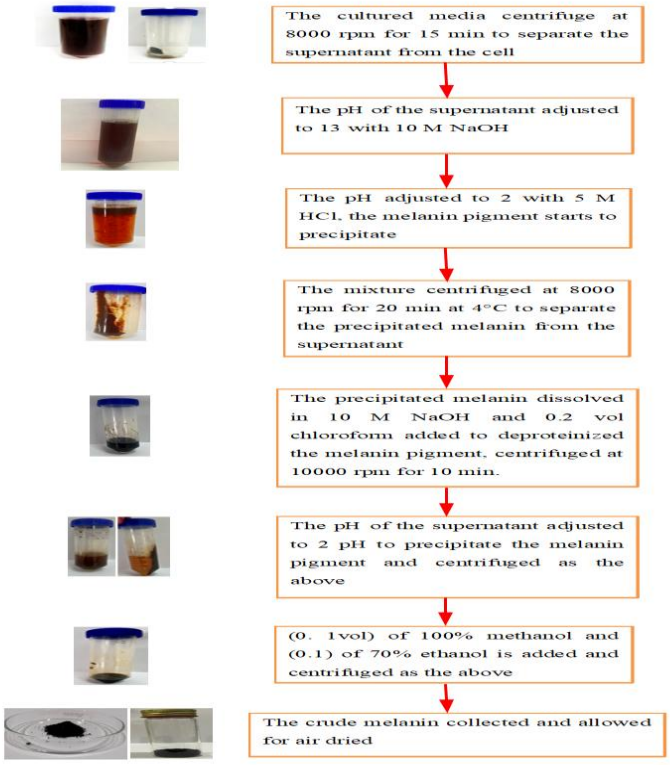

Figure 5. A. Negative control, B. Ps m isolate. C. Ps 81 isolate.

Table 3. Solubility test for purified melanin 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
Water	Insoluble
DMSO	Soluble

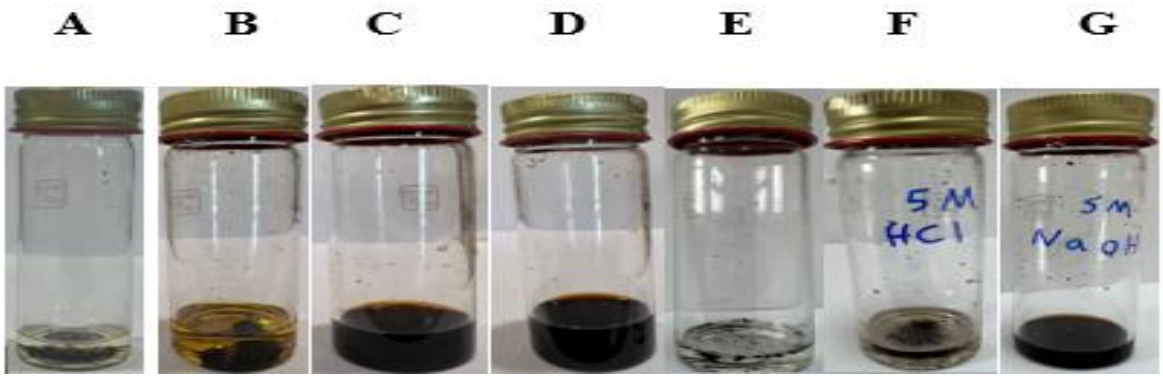

Figure 6. A. The solubility test for purified extracted melanin from Ps 81 isolates. A. Solubility in water. B. Solubility in ethyl acetate. C. Solubility in methanol. D. Solubility in ethanol. E. Solubility in chloroform. F. solubility in HCl. G. Solubility in NaOH.

3.4.2. UV-Visible Spectroscopic Analysis of partially purified melanin

In the UV region, alkaline melanin solution shows a significant optimal absorption peak at 273.5 nm that gradually fades as longer wavelengths are approached, as shown in **Figure 7.**

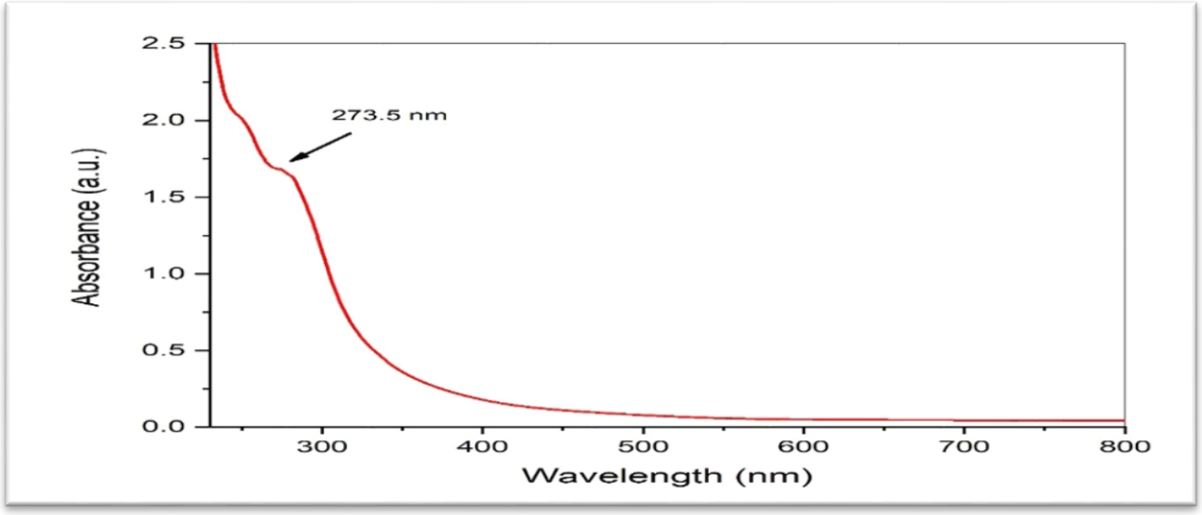

Figure 7. A. UV-visible absorption spectrum of purified extracted melanin.

3.4.3. Fourier Transform infrared spectroscopy Analysis (FTIR)

The most effective use of Fourier transform infrared (FTIR) spectroscopy is for the interpretation of structurally unidentified compounds and for determining the functional group for melanin extracted from Ps 81. FTIR analysis shows 17 peaks, as shown in **Figure 8.**

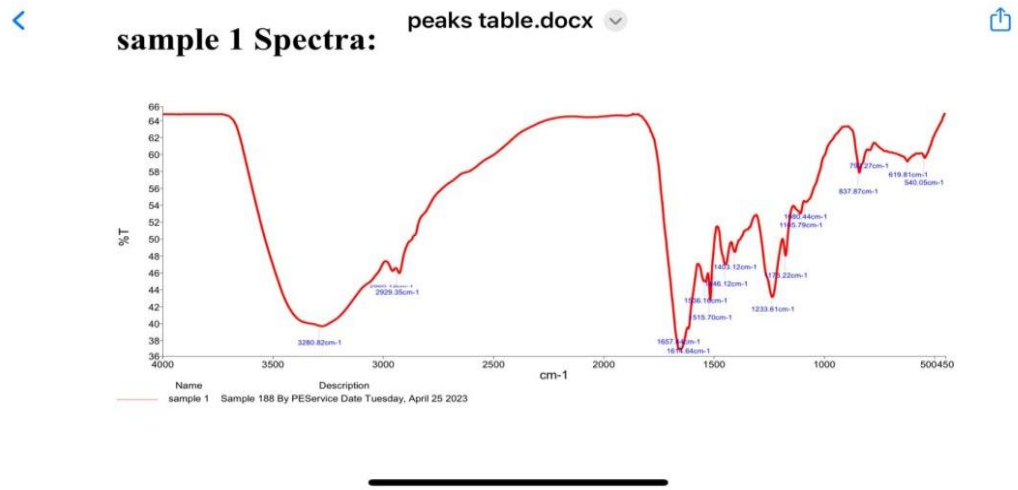

Figure 8. FTIR analysis for purified extracted melanin.

4. Discussion

We collected 100 clinical samples from various sources, such as UTI patients, wound infection patients, and burn patients. We directly inoculated all sample swabs on MacConkey agar. Only 47 isolated (47%) from 100 specimens were identified as *P.aeruginosa*, while the other 53 samples (53%) represented non-*Pseudomonas* genera, as shown in **Table 1**. These isolates manifest as pale colonies on MacConkey agar, a lactose non-fermenter, as reported by (11). They can grow at 42 °C on cetrimide agar, producing green and blue colonies with a sweet grape odor, as demonstrated in **Figure 2B**. These results are consistent with those reported by (12, 13). All the isolates were positive to catalase and oxidase tests similar to those mentioned by (14, 15, 16). Also, the isolates identified by the VITEK-2compact system are similar to those mentioned by (17).

All of the isolates have undergone melanin production screening, as shown in **Figure 3**, but only two isolates (Ps81 and Psm) from burn patients have the ability to produce melanin, as shown in **Figures 4A** and **B**, as used by (7). The results showed the isolate Ps m gave 2.596065 µg/ml of melanin, whereas the isolate Ps 81 gave the maximum melanin pigment concentration (3.018933 µg/ml), as shown in **Table 2** and **Figure 4.** The extraction and purification of melanin pigment was done by two major steps: alkaline dissolving and acid precipitation, as shown in **Figure 5** as used by (8) with little modification. **Table 3** illustrates the solubility of purified melanin in various organic and inorganic solvents. We found that the purified melanin was insoluble in water and some organic solvents like chloroform and ethyl acetate, and it precipitated when dissolved in acidic solutions like (0.5 and 5) M HCl. These results aligned with previous research (18). However, it dissolved easily in other organic solvents like methanol 100%, ethanol 70%, and DMSO. These findings align with previous research (9), (19) and (20), which found that the melanin dissolves easily in alkaline solutions like NaOH, precipitates by HCl, and is insoluble in water. The solubility of melanin in methanol and ethanol, as well as the insolubility in chloroform and ethyl acetate, were agreed with by (21), (22) and (23). The UV-visible spectroscopic analysis of partially purified melanin reveals a maximum absorption in the UV-region at 273.5 nm, as illustrated in **Figure 5**. This absorption then declines towards the visible and infrared regions, a characteristic property of melanin. When identifying and classifying melanin, people frequently utilize the slopes of linear plots as crucial criteria, as they are unique to melanin (24-26). The

infrared spectrum of partially purified melanin extracted from Ps 81 exhibited Infrared spectrum of partially purified melanin extracted from Ps 81 showed a broad absorption band around 3280.82 cm^{-1}, which was caused by the presence of -OH groups and N-H groups. There were also small bands at 2929.35 and 2960.12 cm^{-1} that were caused by the C-H stretch of alkanes groups, peaks at 1657.44 cm^{-1} and 1614.64 cm^{-1} that were caused by aromatic C=C bonds with either C=O or COO- groups, peaks at 1515.70 and 1536.16 cm^{-1} that were caused by aromatic C=C bonds, peaks at 1446.12 and 1403.12 cm^{-1} caused by C-H bending in aliphatic groups, a peak at 1233.61 cm^{-1} that was caused by anhydride group C-O, and two peaks at 1173.22 and 1105.79 cm^{-1} that were caused by aliphatic amine C-N. Sult closely aligns with the findings reported by (18). Peaks at 837.87, 792.27, 619.81, and 540.05 cm^{-1} referred to aromatic C-H groups. These outcomes correspond to the findings of (2, 9, 27).

5. Conclusion

Pseudomonas aeruginosa has the ability to produce melanin in the presence of tyrosine. Melanin pigment was soluble in methanol, ethanol, and NaOH and insoluble in water, chloroform, and ethyl acetate. The maximum absorption peak was at 273.5 nm in the UV region and decreased toward visible light and infrared. The FT-IR study of partially purified melanin shows a broad absorption band around 3280.82 cm^{-1}, which is caused by the -OH group and N-H groups. There are also small bands at 2929.35 and 2960.12 cm^{-1} that are caused by the C-H stretch of alkane groups. These bands helped researchers figure out the structure of melanin pigment.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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Ethical Clearance

This study was ethically approved according to the reference number CSEC/1022/0129 by the ethical committee of the collage of science, university of Baghdad

References

1. Ketelboeter L, Potharla VS, Bardy SL. NTBC treatment of the pyomelanogenic *Pseudomonas aeruginosa* clinical isolate PA1111 inhibits pigment production and increases sensitivity to oxidative stress. Curr Microbiol. 2014;69(3):343–8. https://doi.org/10.1007/s00284-014-0583-8.
2. El-Naggar NEA, El-Ewasy SM. Bioproduction, characterization, anticancer and antioxidant activities of extracellular melanin pigment produced by newly isolated microbial cell factories Streptomyces Glaucescens NEAE-H. Sci Rep. 2017;7:42129. https://doi.org/10.1038/srep42129.
3. Ammanagi AI, Shivasharana, Krishnaveni, Badiger AS, Ramaraj VK, Srinath, Karthik Y. A biotechnological approach to optimization and production of melanin by Brevibacillus invocatus strain IBA, under submerged fermentation. Biomed (Trivandrum). 2022;42(2):318–24.

https://doi.org/10.51248/.v42i2.1315.

4. Agunbiade M, Le Roes-Hill M. Application of bacterial tyrosinases in organic synthesis. World J Microbiol Biotechnol. 2022;38(1):2. https://doi.org/10.1007/s11274-021-03200-6.
5. Kamarudheen N, Naushad T, Rao KVB. Biosynthesis, characterization and antagonistic applications of extracellular melanin pigment from marine Nocardiopsis sps. Indian J Pharm Educ. 2019;53(Suppl 2):s112–20. https://doi.org/10.5530/ijper.53.2s.55.
6. Mohammed HA, Zgair AK. Detection of quorum sensing genes of *Pseudomonas aeruginosa* isolated from different areas in Iraq. Iraqi J Sci. 2022;63(11):4665–73. https://doi.org/10.24996/ijs.2022.63.11.5.
7. Surwase SN, Jadhav SB, Phugare SS, Jadhav JP. Optimization of melanin production by Brevundimonas sp. SGJ using response surface methodology. 3 Biotech. 2013;3(3):187–94. https://doi.org/10.1007/s13205-012-0082-4.
8. Zhang J, Cai J, Deng Y, Chen Y, Ren G. Characterization of melanin produced by a wild-type strain of Bacillus cereus. Front Biol China. 2007;2(1):26–9. https://doi.org/10.1007/s11515-007-0004-8.
9. Noman AE, Al-Barha NS, Chen F. Characterization of physicochemical properties of melanin produced by Gluconobacter oxydans FBFS 97. Fermentation. 2022;8(11):574. https://doi.org/10.3390/fermentation8110574.
10. Sajjan S, Kulkarni G, Yaligara V, Kyoung L, Karegoudar TB. Purification and physiochemical characterization of melanin pigment from Klebsiella sp. GSK. J Microbiol Biotechnol. 2010;20(11):1513–20. https://doi.org/10.4014/jmb.1002.02006.
11. Hossain M, *Saha S, Rahman M, Singha J, Mamun A. Isolation, identification and antibiogram study of Pseudomonas aeruginosa* from cattle in Bangladesh. J Vet Adv. 2013;3(4):180. https://doi.org/10.5455/jva.20130717123841.
12. 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(5):407–13. https://doi.org/10.1002/jobm.200310264.
13. Sulaiman SD, Abdulhasan GA. Curcumin as efflux pump inhibitor agent for enhancement treatment against multidrug-resistant *Pseudomonas aeruginosa* isolates. Iraqi J Sci. 2020;61(1):59–67. https://doi.org/10.24996/ijs.2020.61.1.6.
14. Su SS, Lae KZW, Ngwe H. Isolation and identification of *Pseudomonas aeruginosa* from the clinical soil. Univ Yangon Res J. 2018;8:271–5.
15. Gheni MR, Odaa NH. The antimicrobial activity of melanin-mediated synthesis of silver nanoparticles. Egypt J Hosp Med. 2023;90(1):3383–94. https://doi.org/10.21608/ejhm.2023.291442.
16. Wellinghausen N, Köthe J, Wirths B, Sigge A, Poppert S. Superiority of molecular techniques for identification of gram-negative, oxidase-positive rods, including morphologically nontypical *Pseudomonas aeruginosa*, from patients with cystic fibrosis. J Clin Microbiol. 2005;43(8):4070–5. https://doi.org/10.1128/JCM.43.8.4070-4075.2005.
17. Dwivedi HP, Franklin S, Chandrasekaran S, Garner O, Traczewski MM, Beasley D, Pincus DH. Multicenter Clinical Evaluation of Vitek 2 Meropenem-Vaborbactam for Susceptibility Testing of Enterobacterales and *Pseudomonas aeruginosa*. J Clin Microbiol. 2022;60(1):e01610-21. https://doi.org/10.1128/jcm.01610-21.
18. 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 Appl Sci. 2016;5(10):910–9. https://doi.org/10.20546/ijcmas.2016.510.098.
19. Pralea IE, Moldovan RC, Petrache AM, Ilieș M, Hegheș SC, Ielciu I, Nicoară R, Moldovan M, Ene M, Radu M. From extraction to advanced analytical methods: The challenges of melanin analysis. Int J Mol Sci. 2019;20(16):3943. https://doi.org/10.3390/ijms20163943.

20. Aghajanyan AE, Hambardzumyan AA, Minasyan EV, Tsaturyan AH, Paloyan AM, Avetisyan SV, Saghyan AS. Development of the technology for producing water-soluble melanin from waste of vinary production and the study of its physicochemical properties. Eur Food Res Technol. 2022;248(2):485–95. https://doi.org/10.1007/s00217-021-03843-6.
21. Bronze-Uhle ES, Batagin-Neto A, Xavier PH, Fernandes NI, De Azevedo ER, Graeff CF. Synthesis and characterization of melanin in DMSO. J Mol Struct. 2013;1047:102-108. https://doi.org/10.1016/j.molstruc.2013.04.058.
22. Zerrad A, Anissi J, Ghanam J, Sendide K, El Hassouni M. Antioxidant and antimicrobial activities of melanin produced by a *Pseudomonas balearica* strain. J Biotechnol Lett. 2014;5(2):87–94.
23. Singh S, Nimse SB, Mathew DE, Dhimmar A, Sahastrabudhe H, Gajjar A, Shinde PB. Microbial melanin: Recent advances in biosynthesis, extraction, characterization, and applications. Biotechnol Adv. 2021;53:107773. https://doi.org/10.1016/j.biotechadv.2021.107773.
24. Al Khatib M, Harir M, Costa J, Baratto MC, Schiavo I, Trabalzini L, Pollini S, Rossolini GM, Basosi R, Pogni R. Spectroscopic characterization of natural melanin from a Streptomyces cyaneofuscatus strain and comparison with melanin enzymatically synthesized by tyrosinase and laccase. Molecules. 2018;23(8):1916. https://doi.org/10.3390/molecules23081916.
25. Suthar M, Dufossé L, Singh SK. The enigmatic world of fungal melanin: a comprehensive review. J Fungi. 2023;9(9):891. https://doi.org/10.3390/jof9090891.
26. Roy S, Wu J, Cao J, Disu J, Bharadwaj S, Meinert-Spyker E, Wood S. Exploring the impact and influence of melanin on frequency-domain near-infrared spectroscopy measurements. J Biomed Opt. 2024;29(S3):S33310-S33310. https://doi.org/10.1117/1.JBO.29.S3.S33310.
27. Manivasagan P, Venkatesan J, Senthilkumar K, Sivakumar K, Kim SK. Isolation and characterization of biologically active melanin from Actinoalloteichus sp. MA-32. Int J Biol Macromol. 2013;58:263–74. https://doi.org/10.1016/j.ijbiomac.2013.04.041.