

Production, Extraction, and Purification of An Extracellular Melanin Pigment from Clinically Isolated *Pseudomonas aeruginosa*

Mohammed R. Ghani*, Nisreen H. Odaa

Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

Correspondence Author: Mohammed Redha Ghani, Phone: +96 47714377682, E-mail: moh.merza93@gmail.com

ABSTRACT

Introduction: Melanin is a high-molecular weight pigment produced through the oxidative polymerization of phenolic or indolic compounds and plays a perfect role in UV-light shielding, as well as in photoprotection. Among biopolymers, melanin is unique in many aspects. This study is designed to screen Production, extraction and characterizes of an extracellular melanin pigment from clinically isolated *P. aeruginosa*.

Objective: The aim of the current study is isolation and diagnosis of *P. aeruginosa* using vitek-2 compact system and screening the ability to produce melanin and characterization of extracted melanin by UV-vis, FTIR, XRD and SEM.

Materials and methods: the samples swab inoculated on cetrimide agar as selective media and incubated aerobically for 24 hours at 37°C and used nutrient agar with nutrient broth supplement with 1% tyrosine for Screening for melanin production by *P. aeruginosa* isolates.

Results: Four isolates *P. aeruginosa* were identified out of 109 specimens have ability to produce melanin pigment under specific medium and culture conditions. According to morphological, cultural, biochemical, and VITEK-2 characteristics, isolates were identified as *P. aeruginosa*. The results showed that the isolate of burn was greatest in production of melanin. Based on its solubility in organic solvents, the black pigment was identified as melanin and structurally and functionally characterized by UV-Vis absorbance and presence of various characteristic peaks that determined by FTIR analysis, and the morphology of the pigment surface was examined using SEM and XRD spectra analysis.

Conclusion: Depending on the type of bacteria and the conditions of their culture, different melanin-producing bacteria produce different amounts of melanin. The primary factors for bacterial melanin production are L-tyrosine, energy source, pH, temperature and surface area for shaker agitation. In this study, results found that the optimum condition for melanin production by *P. aeruginosa* in pH=7.3 and the effect of L-tyrosine concentration on melanin production found that use 1g/L is the best concentration for production.

Keywords: *P. aeruginosa*, Detection, Melanin pigment, Physico-Chemical Characterization, Experimental study, University of Baghdad.

INTRODUCTION

Melanin is a biopolymer synthesized from phenolic or indolic compounds⁽¹⁾. It has the capacity to absorb a variety of electromagnetic radiation, from visible light to the X-ray region⁽²⁾. It serves as a shield against chemicals, heat, desiccation and hyperosmotic shock (heavy metals and oxidizing agents)⁽³⁾.

The most important ones are free radical scavenging and drug delivery capability⁽⁴⁾. Although melanin is required to improve microbes' capability of competing and surviving in different unfavorable conditions, such as those caused by ultraviolet rays, it is thought to be not necessary for their growth and development⁽⁵⁾.

P. aeruginosa is a motile, heterotrophic, gram-negative rod shape that measures 0.5-1.0 µm in width and 1-5 µm in length. It is facultative non-spore forming and grows using aerobic respiration, *P. aeruginosa* can survive in varying temperatures ranging from 4 to 42°C and produces flat, large, serrated, green or blue pigmented colonies giving characteristic sweetish fruity odour^(6,7). *P. aeruginosa* is a commercially valuable organism because it produces many soluble pigments like pyocyanin (blue-green), pyorubin (red), pyoverdinin (yellow-green) and pyomelanin (brown-black)⁽⁸⁾.

Melanin was first used by Berzelius to refer to dark animal pigments in 1840⁽⁹⁾. The source and mechanism of melanin synthesis depend on many factors like enzymes, polymerization pathways and building blocks⁽¹⁰⁾. It can be classified depending on chemical composition like monomer subunit structure⁽¹¹⁾.

The aim of the current study is isolation and diagnosis of *P. aeruginosa* using vitek-2 compact system and screening the ability to produce melanin and characterization of extracted melanin by UV-vis, FTIR, XRD and SEM.

MATERIALS AND METHODS

Collection and Identification of *P. aeruginosa* isolate:

From November 2021 to February 2022, 109 samples were collected. All the samples were clinically isolated from Iraqi patient's hospitals (burns patients, UTI, tracheostomy patients in ICU, endotracheal tube patients in ICU, mastoiditis, and wound infection). These samples were collected from Medical City/Burning Hospital, ICU Ghazy AL-Hariri Hospital for surgical specialties, Arabi Private Hospital, and Al Alawia Hospital. All samples swab inoculated on cetrimide agar as selective media and

incubated aerobically for 24 hours at 37°C. Isolates were initial identification by cultural characteristics, biochemical Tests, and Vitek -2 system.

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

The bacterial strains were inoculated and incubated on cetrimide agar at 37°C for 24 hr. after that, a loopful of bacterial strains was inoculated to nutrient agar and nutrient broth supplement with 1% tyrosine to detect the ability of bacteria strains to produce melanin. This method was previously described ⁽¹²⁾.

Measurement of melanin concentration:

In 1 N NaOH, synthetic melanin (Sigma Aldrich, USA) was dissolved at various concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1) g/l. A standard curve was made by using ELISA Versa Max at A₄₅₀ and used to quantify melanin by comparing the OD at 450 nm of unknown samples with a standard curve ⁽¹³⁾, NaOH was used as a blank.

Production of melanin:

Liquid medium was used for *P. aeruginosa* cultivation and melanin production according to ⁽¹²⁾.

1. Nutrient broth was dissolved in 150 ml of D.W. (500ml Erlenmeyer flask) supplement with 1% of tyrosine.
2. The pH has been fixed at 7.0.
3. The medium was sterilized by autoclave for 15 minutes at 15 lbs pressure (121°C).
4. After sterilizing, kept it for cooling, a colony of fresh culture was added to this medium and placed in a rotary shaker moving incubated at 37°C 160 rpm for 3 to 6 days until the liquid medium became darkly pigmented.

Extraction and purification of melanin pigment:

The highest-produced isolate was used to produce melanin, as previously mentioned ⁽¹⁴⁾ by following steps:

1. To separate the supernatant from cells and debris, for 15 minutes, the medium was centrifuged at 8000 rpm.
2. Chloroform was mixed with the above solution to deproteinize the melanin pigment. Each tube (10) ml add (2) ml chloroform.
3. To ensure complete polymerization of melanin, 5M of NaOH was used to adjust pH to 10 and further autoclaved at 120°C for 20 minutes ⁽¹⁵⁾.
4. After autoclaved solution completed centrifuging the solution at 5000 g for 5 minutes and the supernatant was collected.
5. 5M HCl was added carefully to acidify the solution to pH 2 until melanin precipitate.
6. After the melanin precipitate by centrifuged for 20 minutes at 8000 rpm to extract crude melanin, and then

equal volumes of chloroform, ethyl acetate, and double methanol (1V:1V:2V) were added and combined.

7. To obtain melanin dissolved in this mixture, for 15 minutes, the mixture was centrifuged at 8000 rpm. and then put in a glass petri dish until solvent evaporation, 2 to 3 times this step was repeated to have powder melanin once dried, the purified preparation was kept at room temperature.

Physical and chemicals characterization of extract melanin

Chemical Analysis of extract Crude melanin Pigment:

The pigment's solubility is tested in various solvents using techniques described previously ⁽¹⁶⁾. Melanin solubility was investigated by dissolving (5mg/mL) of melanin in various inorganic and organic solutions.

UV-Visible Spectroscopic Analysis for extracted melanin:

UV-visible spectrophotometer (200–700 nm) was used to scan the extracted- melanin powder after it had been dissolved in a 0.5 M NaOH solution. 0.5 M NaOH solution served as the blank control ⁽¹⁷⁾.

Analysis fourier transform infrared spectroscopy (FTIR):

The extracted pigment was further investigated by FT-IR spectrum through using 5 ml of 3 N HCl in a sealed glass vial and kept at 100°C for 2 hours. Then melanin was ground by pressing the samples into KBr granules in 1:90 ratio, and pressed into disks under vacuum using a Spectra Lab Pelletiser. and then scanned with a resolution of 4 cm⁻¹ between 4000 and 500 cm⁻¹ by FTIR spectrophotometer (FT/IR-4100; Shimadzu-Japan) ⁽¹⁸⁾.

X-Ray Diffraction method analysis (XRD):

Extracted melanin powder was placed into 1-2 mm thick and 1cm diameter discs. The melanin pigment was scanned with an X-ray diffractometer lab XRD-600 (Shimadzu-Japan) at a wavelength 1.54056 Å⁰, at a room temperature X-ray beam with a step size of 0.02 and a scanning rate of 20/minutes. As a function of the scattering angle, the intensity of scattering was measured ⁽¹⁹⁾.

Field emission scanning electron microscopy (FE-SEM):

The dehydrated sample was dried on a slide and then coated with gold at 50 nm in a PE-5000 sputter coater (International Scientific Instrument, London). At 00 detector angle, a Scanning Electron Microscope- S-4160 (Japan) was used to view the specimen, randomly chosen fields were photographed, and well-contrast negative films were mounted and projected with a slide projector.

Ethical Consideration:

This study was approved by the Ethical Committee, Department of Biology, College of Science, University of Bagdad, Baghdad, Iraq and the Iraqi Ministry of Health [Ref.: CSEC/0122/0033].

RESULTS

Isolation and Identification of *P. aeruginosa*:

Up to 109 clinical samples were collected, and only 35(32.11%) isolates were *P. aeruginosa* were obtained from 109 clinical samples, while other 74(67.88%) represented other bacterial genera. Results show Wound specimens were 7 (30.43%), UTI specimens were 2 (14.28%), burn specimens were 15 (53.57%), tracheostomy patients in ICU specimens 5 (23.8%), endotracheal tube patients in ICU 4 (22.22%) and mastoiditis 2 (40%).

The preliminary identification of *P. aeruginosa* was carried out on MacConkey agar and cetrimide agar that appeared as a pale colony on MacConkey agar. On

nutrient agar, isolates were able to grow at 42°C and had sweat grape odor. *P. aeruginosa* is distinguished from other species by its capacity to grow at high temperatures, and some isolates have ability to produce pyocyanin pigment.

Results showed that all 35 isolates were positive for oxidase, urease, and catalase tests. IMViC test results showed that all isolates were indole, methyl red (MR) and voges-proskauer (VP) negative and had able to utilize citrate as a sole carbon source.

Screening for melanin-producing *P. aeruginosa*:

From 35 strains, four isolates, including Ps 9, 22, 29, and 51 which are identifications by Vitek -2 system, have to ability to produce a brown black diffusible pigment, as shown in **Figure 1**.

The source of the 4 isolates that produce melanin pigment were 3 from burns and one from a patient in ICU with tracheostomy.

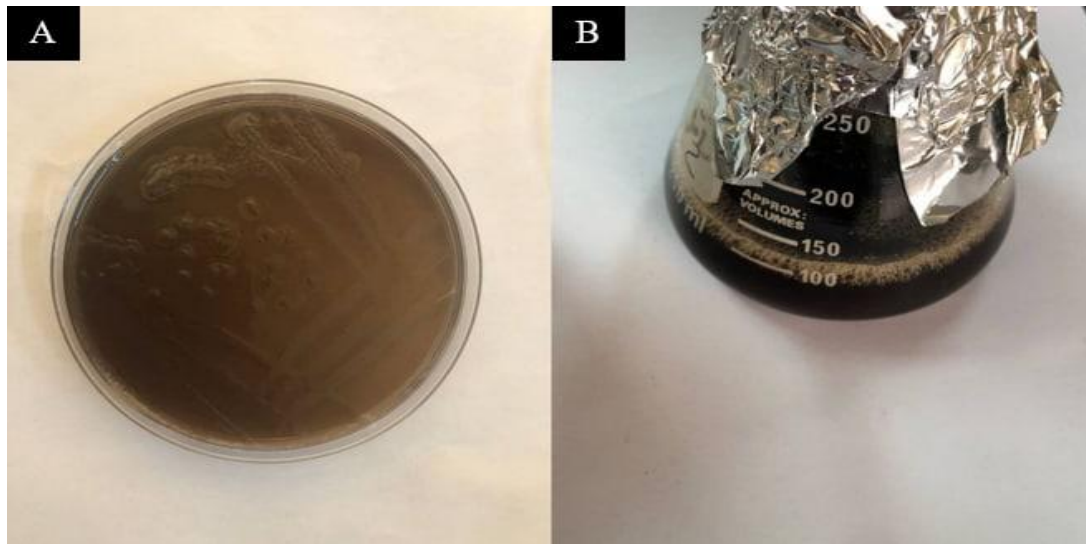


Figure 1: *P. aeruginosa* produce melanin pigment (A) on nutrient agar supplement with L-tyrosine after 4 days incubation (B) on nutrient broth supplement with L-tyrosine after 6 days shaker incubation

Extraction and purification of *P. aeruginosa* melanin pigment: The extraction was performed as described by ⁽¹⁴⁾ with slight modifications as shown in **Figure 2**. Many steps involve to having melanin powder these steps are illustrated in discussion section.

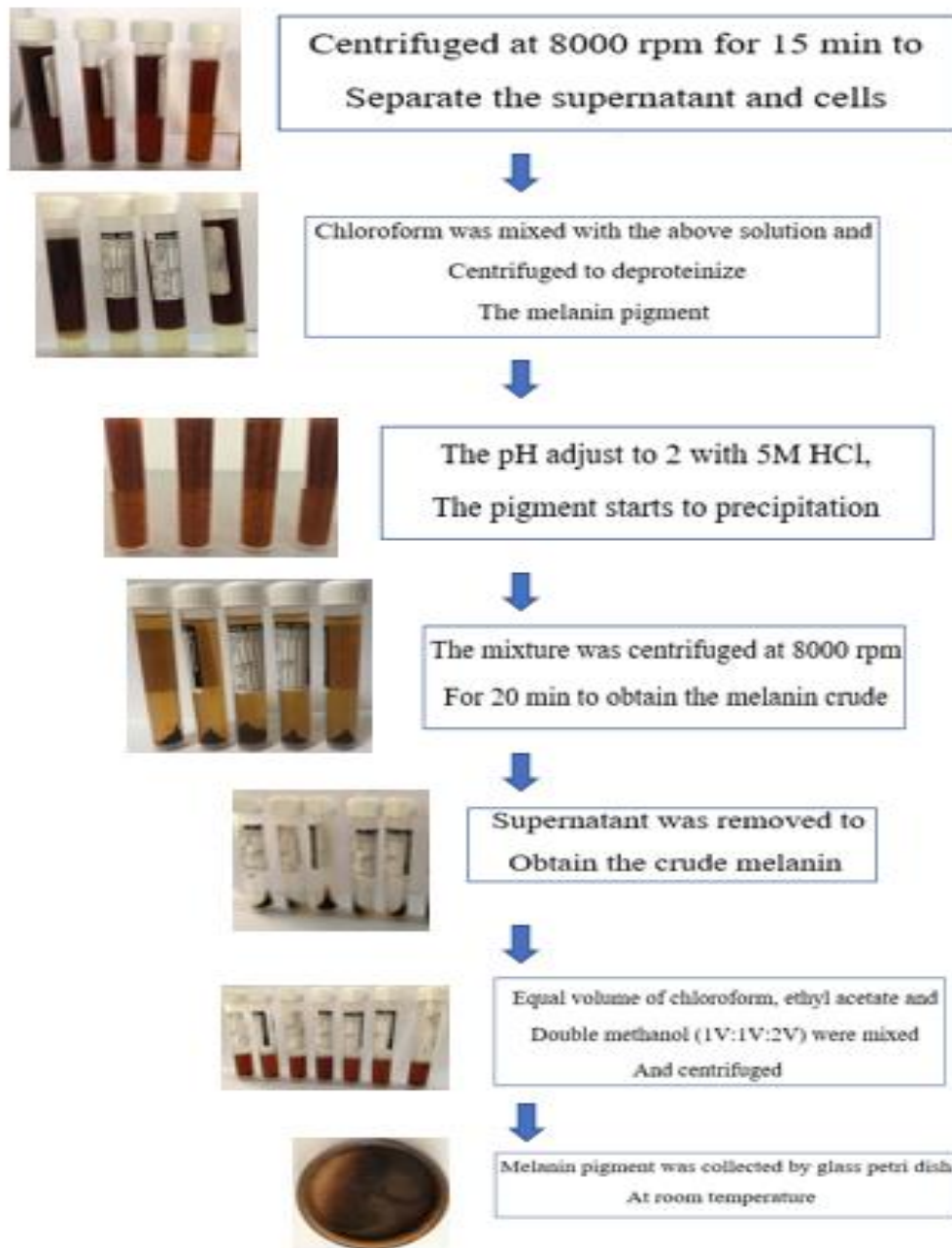


Figure 2: Extraction steps of melanin pigment.

Calculates standard melanin concentration: Melanin concentration was determined according to the formula ($Y = 1.8306X + 0.0497$) by comparing unknown samples' OD at 450 nm to a standard curve **Figure 3**. The absorbance of the centrifuged culture supernatant at 450 nm was used to quantify melanin production (**Table 1**).

Table 1: *P. aeruginosa* isolates and melanin concentration.

| <i>P. aeruginosa</i> isolates | Melanin Conc. (µg/mL) |
|-------------------------------|-----------------------|
| Ps 9 | 1.188299 |
| Ps 22 | 1.082869 |
| Ps 29 | 1.05337 |
| Ps 51 | 1.272424 |

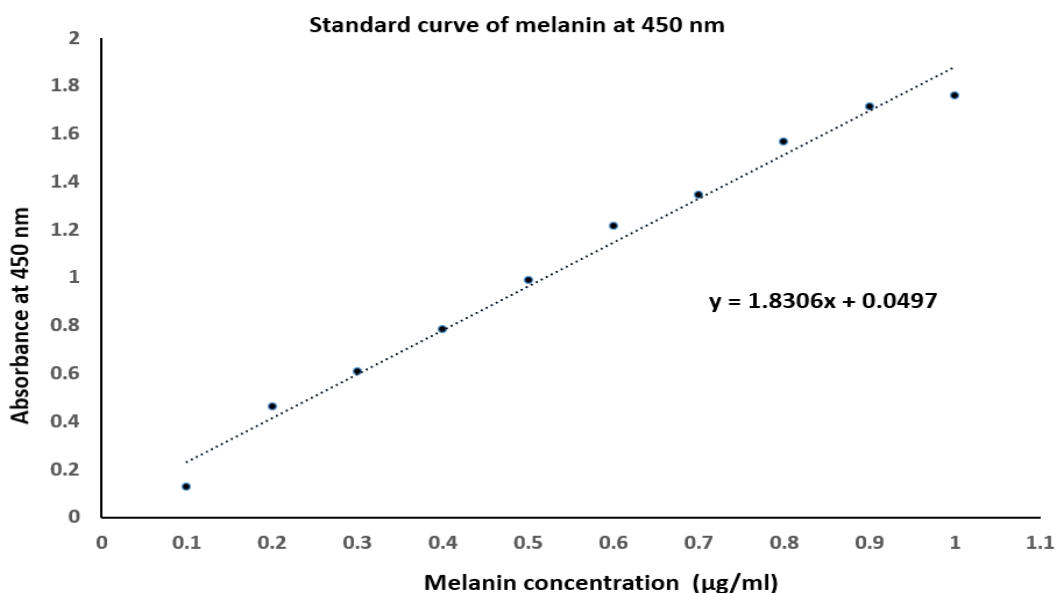


Figure 3: Standard curve for synthetic melanin.

Physical and chemicals characterization of melanin

Solubility of extract melanin: In this study, melanin was insoluble in mineral acids such as HCl, water (cold, hot) and organic solvents such as ether and chloroform but it was easily soluble in NaOH and dimethyl sulfoxide (DSMO) and precipitated in hydrochloric acid (1N HCl).

UV-Visible Spectrophotometer (UV-Vis): For the extracted pigment, the UV-visible spectrum scanned from 200 to 1000 nm and the melanin exhibited a maximum spectral peak at 274 nm which then decreased as it approached the visible region, as shown in Figure 4.

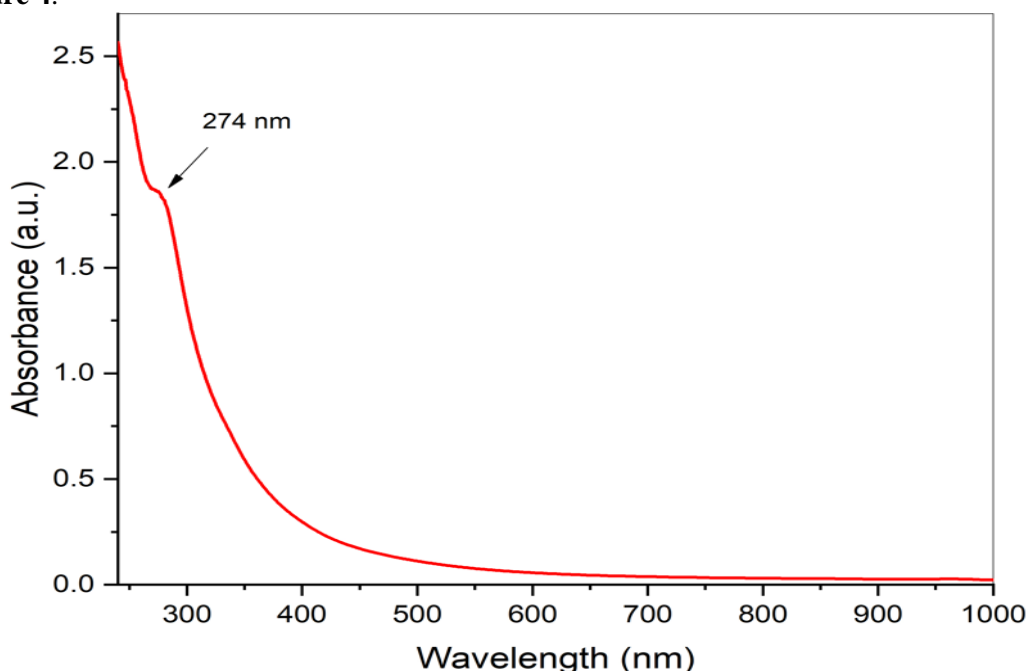


Figure 4: UV-visible absorption spectrum of extract melanin pigment.

Fourier Transform IR Analysis (FTIR): Results show FTIR absorption peak at 3362.70 cm^{-1} corresponds to the OH group. Peaks 2359.04, 2920.04, 2851.89 cm^{-1} are due to aliphatic C-H groups. The peak at 1650.38 refers to aromatic C=C conjugated with C=O and/or COO- groups and 1575.03 cm^{-1} aromatic C=C bonds. The band at 1418.46 cm^{-1} corresponds to a C-H flexion in aliphatic groups. The peak at 1258.46 cm^{-1} relates to the anhydride group (C-O). Peaks at 1089.28, 1039.37 cm^{-1} relates to C-N stretch of aliphatic amines. Peaks at 874.11, 667.52, 468.38 cm^{-1} contributes to the aromatic C-H group (Figure 5).

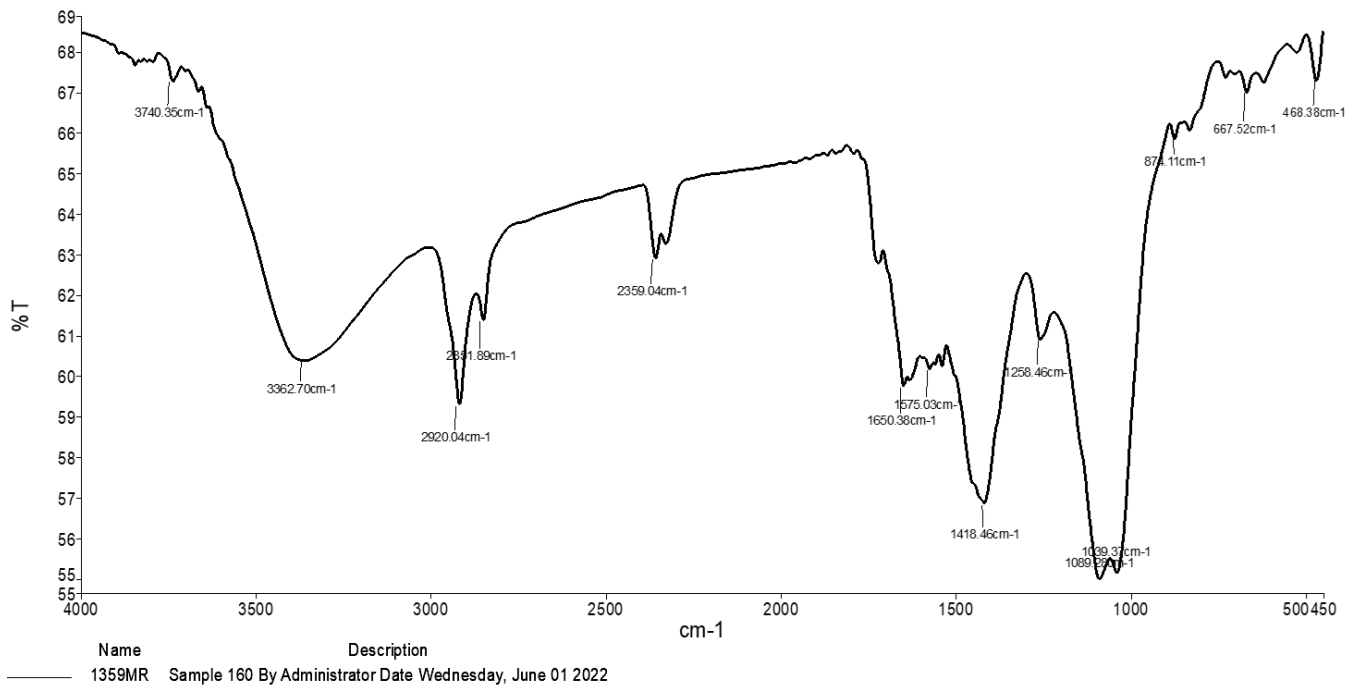


Figure 5: FTIR extract melanin pigment.

Field emission scanning electron microscopy (FE-SEM): General view of *P. aeruginosa* melanin aggregates of various sizes. as in figure 6 images of the fragment's surfaces at higher magnifications demonstrated that they are spherical-like structures and are formed by aggregates of granules of different size with irregular shape and these granules' surface is not smooth.

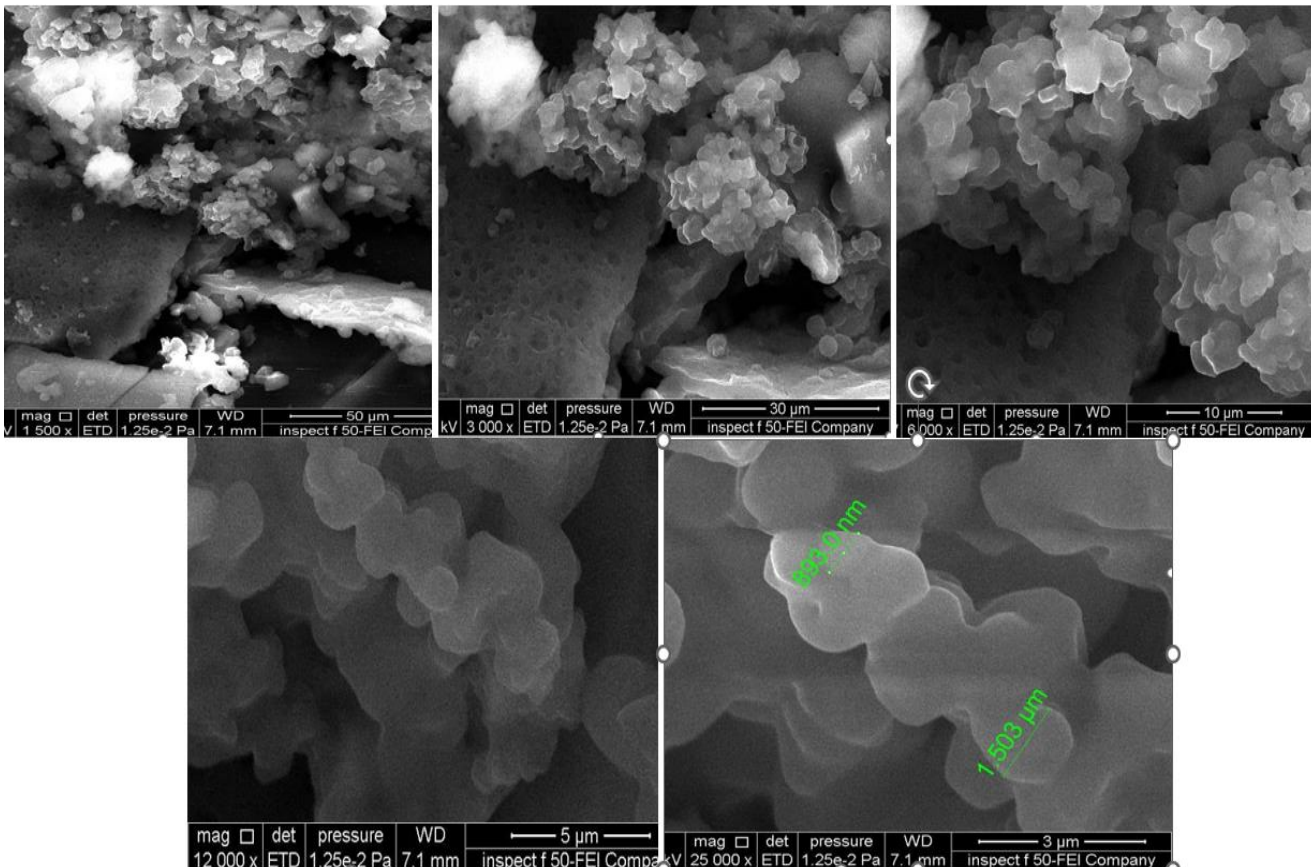


Figure 6: Field emission scanning electron microscopy (FE-SEM) image of extract melanin pigment (1500X) (3000X) (6000X) (12000X) (25000X).

X-ray Diffraction (XRD):

Broad features known as non-Bragg features were produced by the amorphous compound in the diffraction spectrum, which prevented coherent scattering from repeating and regular structures as seen in crystals. The dry melanin XRD spectrum fig 7 shows a broad diffraction peak ($2\theta=15-25^\circ$). Such broad peaks indicate non-Bragg features and show a variety of random orientations of structural elements. Amorphous nature is also distinguished by broad peaks.

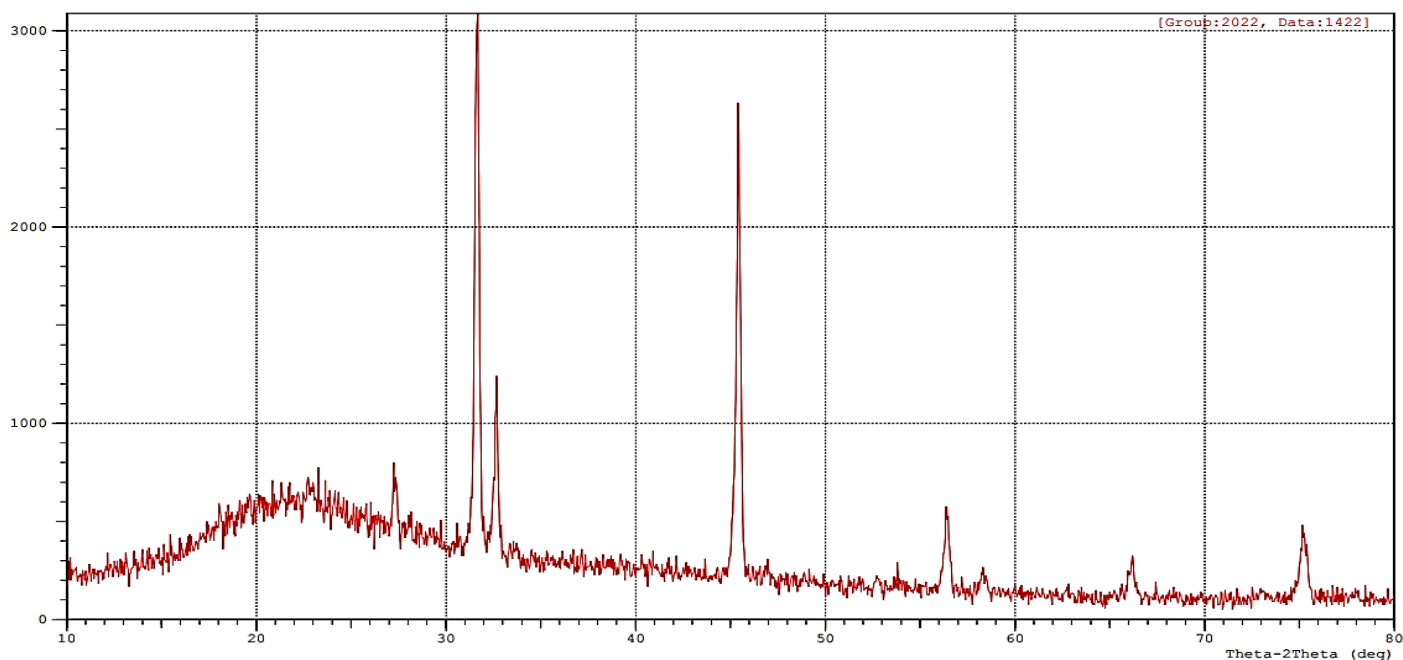


Figure 7: XRD analysis of *P. aeruginosa* melanin pigment.

DISCUSSION

Only 35 (32.11%) isolates were *P. aeruginosa* were obtained from 109 clinical samples while other 74 (67.88%) represented other bacterial genera. these isolates were first identification by Cultural Characteristics that appear as pale colony on MacConkey agar and ability to produce pyocyanin pigment similar as mentioned by ⁽²⁰⁾. The biochemical tests are similar to those described previously by ⁽²¹⁾. The screening was carried out in nutrient broth supplement with L-tyrosine as shown in the figure 1 to choose the higher isolate melanin production this media was used by ⁽²²⁾. Only 4 isolates have ability to produce melanin pigment. Results showed in Table 1 that the 4 isolates (Ps 9, 22, 29, and 51) have the ability to produce melanin pigment but the isolate (Ps 51) gave the highest melanin production (1.272424) $\mu\text{g/mL}$ thus it was selected for further steps of this study ⁽²³⁾.

The extraction was done by two basic steps acid precipitation and centrifugation as shown in Figure 2. Following incubation, the medium was centrifuged at 8000 rpm for 15 minutes to separate cells from the supernatant. Firstly, to ensure that melanin is completely polymerized, with 5M NaOH, the pH of the supernatant

was adjusted to 10 to prevent the formation of melanoidins and filtered by Millipore filter 0.2 μm to get rid of cell debris. After that alkaline melanin mixture was adjusted to pH 2 with 5M HCl this step was used to precipitate melanin and centrifugation at 8000 rpm for 20 min, and then the pellet was collected. After that pellet was washed with an equal volume of chloroform, ethyl acetate and double methanol (1V:1V:2V) three times to remove proteins, carbohydrate and lipids, last step is washing step with deionized water, a rotary evaporator was used to concentrate the partially purified melanin and the pigment powder was then gathered, this procedure used by ⁽²²⁾. Since the solubility of melanin is distinctive, solubility is the most common stage for the characterization of melanin

Extract melanin was insoluble in water, mineral acids such as HCl and organic solvents such as an ether and chloroform but it was easily soluble in NaOH and dimethyl sulfoxide (DSMO). Whereas a similar study reported they were soluble in alkaline (pH 10) and dimethyl sulfoxide but were insoluble in ethanol, acetone and chloroform ⁽²⁴⁻²⁶⁾.

Extract melanin absorbs maximum spectral peak at 274 nm as shown in Figure 4. Melanin absorbs UV light due to the presence of complex conjugated molecular

structures as mentioned by ⁽¹¹⁾. A similar study reported maximum UV-Vis absorption peaks in the melanin from different sources varied such as *Pleurotus cystidiosus* melanin exhibited maximum absorption peak at 280 nm ⁽²⁷⁾, whereas another study reported the highest absorption peak at 220 nm and 244 nm, respectively, in the visible region ⁽²⁸⁾. FT-IR spectroscopy provides detailed information about an organic compound (main functional groups). The pigmentation procedure extraction and purification may cause some differences in the FT-IR spectra. FT-IR test is useful for determining the types of chemical bonds (functional groups) and can thus be used to clarify some components of an unknown mixture. The results of peaks as in **Figure 5** comparable in the functional group region with ⁽²⁹⁻³¹⁾. The image for (FE-SEM) as in **Figure 6** were shown in previous studies ^(32,33) which take the form of tiny granules with irregular or spherical shapes or filamentous projections with a porous structure. Melanin's X-ray diffraction pattern demonstrates that it lacks crystalline structure as ⁽³⁴⁾.

CONCLUSION

In this study *P. aeruginosa* isolated from clinical Iraqi patients' hospitals, only 4 isolates produced melanin pigment. *P. aeruginosa* was identified in this study as a melanin-producing bacterium through growth in nutrient agar and nutrient broth that contained L-tyrosine and the ability to produce melanin. All experiments were conducted in 500 ml conical flasks containing 100 ml of medium less than 25% of the total volume of the flask to increase the surface area (**Figure 1**). The maximum production UV-vis was observed in 274 nm (**Figure 4**), and functional groups were analyzed by FTIR (**Figure 5**) that when compared with previous studies have a high degree of similarity in main absorption peaks confirming that the pigment produced is melanin. In addition to these properties UV, FTIR and melanin's X-ray diffraction pattern exhibits a lack of crystalline structure.

Depending on the type of bacteria and the conditions of their culture, different melanin-producing bacteria produce different amounts of melanin. The primary factors for bacterial melanin production are L-tyrosine, energy source, pH, temperature and surface area for shaker agitation. In this study, results found that the optimum condition for melanin production by *P. aeruginosa* in pH =7.3 and the effect of L-tyrosine concentration on melanin production found that use 1g/L is the best concentration for production. After six days, maximum melanin production was noted at 37°C and 180 rpm of shaker agitation, and with a temperature increase of up to 45°C, there was a gradual and uniform decrease in growth and melanin production. Bacterial melanin extraction of *P. aeruginosa* is simple and can produce melanin from low-cost sources.

REFERENCES

1. **Berthelot C, Zegeye A, Gaber D et al. (2020):** Unravelling the role of melanin in cd and zn tolerance and accumulation of three dark septate endophytic species. *Microorganisms*, 8(4):537.
2. **Ganesh Kumar C, Sahu N, Narender Reddy G et al. (2013):** Production of melanin pigment from *Pseudomonas stutzeri* isolated from red seaweed *Hypnea musciformis*. *Letters in Applied Microbiology*, 57(4):295-302.
3. **Azman A, Mawang C, Abubakar S et al. (2018):** Bacterial pigments: The bioactivities and as an alternative for therapeutic applications. *Natural Product Communications*, 13(12):1747-54.
4. **Büngeler A, Hämisch B, Strube O et al. (2017):** The supramolecular buildup of eumelanin: Structures, mechanisms, controllability. *International Journal of Molecular Sciences*, 18(9):1901.
5. **Zerrad A, Anissi J, Ghanam J et al. (2014):** Antioxidant and antimicrobial activities of melanin produced by a *Pseudomonas balearica* strain. *Journal of Biotechnology Letters*, 5(1):87-94.
6. **Diggle S, Whiteley M (2020):** Microbe profile: *Pseudomonas aeruginosa*: Opportunistic pathogen and lab rat. *Microbiology (United Kingdom)*, 166(1):30-3.
7. **Katoch S (2020):** *Pseudomonas aeruginosa* Infection in African Grey Parrot and its AntibioGram Study. *Journal of Animal Research*, 10(5):849-52.
8. **DeBritto S, Gajbar T, Satapute P et al. (2020):** Isolation and characterization of nutrient dependent pyocyanin from *Pseudomonas aeruginosa* and its dye and agrochemical properties. *Scientific Reports*, 10(1):1542.
9. **Meng Z, Oh S (2021):** Antioxidant and antimelanogenic activities of kimchi-derived *Limosilactobacillus fermentum* JNU532 in B16F10 melanoma cells. *Journal of Microbiology and Biotechnology*, 31(7):990-8.
10. **Choi K (2021):** Bioprocess of Microbial Melanin Production and Isolation. *Frontiers in Bioengineering and Biotechnology*, 9:765110.
11. **Pralea I, Moldovan R, Petrache A et al. (2019):** From extraction to advanced analytical methods: The challenges of melanin analysis. *International Journal of Molecular Sciences*, 20(16):3943.
12. **Surwase S, Jadhav S, Phugare S et al. (2013):** Optimization of melanin production by *Brevundimonas* sp. SGJ using response surface methodology. *3 Biotech*, 3(3):187-94.
13. **Makpol S, Arifin N, Ismail Z et al. (2009):** Modulation of melanin synthesis and its gene expression in skin melanocytes by palm tocotrienol rich fraction. *African Journal of Biochemistry Research*, 3(12):385-92.
14. **Roy S, Rhim J (2022):** New insight into melanin for food packaging and biotechnology applications. *Critical Reviews in Food Science and Nutrition*, 62(17):4629-55.
15. **El-Batal A, Al Tamie M (2016):** Optimization of melanin production by *Aspergillus oryzae* and incorporation into

- silver nanoparticles. *Der Pharmacia Lettre*, 8(2):315-33.
17. **Guo J, Rao Z, Yang T et al. (2014):** High-level production of melanin by a novel isolate of *Streptomyces kathirae*. *FEMS Microbiol Lett.*, 357:85-9.
 18. Mahmood H, Mohammed A, Flayyih M (2015): Purification and Physicochemical Characterization of World Journal of Pharmaceutical Research. *World Journal of Pharmaceutical Research*, (10):289-99.
 19. **Marín-Sanhueza C, Echeverría-Vega A, Gómez A et al. (2022):** Stress Dependent Biofilm Formation and Bioactive Melanin Pigment Production by a Thermophilic *Bacillus* Species from Chilean Hot Spring. *Polymers*, 14(4):680.
 20. **Sajjan S, Anjaneya O, Kulkarni G et al. (2013):** Properties and functions of melanin pigment from *Klebsiella* sp. GSK. *Korean Journal of Microbiology and Biotechnology*, 41(1):60-9.
 21. **Al-Sheikhly M, Musleh L, Al-Mathkhury H (2019):** Assessment of *pelA*-carried *Pseudomonas aeruginosa* isolates in respect to biofilm formation. *Iraqi Journal of Science*, 60(6):1180-7.
 22. **Tawakol M, Nabil M, Reda M (2018):** Molecular Studies on Some Virulence Factors of *Pseudomonas Aeruginosa* Isolated From Chickens As a Biofilm Forming Bacteria. *Assiut Veterinary Medical Journal*, 64(159):43-51.
 23. **Vijayan V, Jasmin C, Anas A et al. (2017):** Sponge-Associated Bacteria Produce Non-cytotoxic Melanin Which Protects Animal Cells from Photo-Toxicity. *Applied Biochemistry and Biotechnology*, 183(1):396-411.
 24. **Zhu L, Chu Y, Zhang B et al. (2022):** Creation of an Industrial *Bacillus thuringiensis* Strain with High Melanin Production and UV Tolerance by Gene Editing. *Frontiers in Microbiology*, 13:913715.
 25. **Kamarudheen N, Naushad T, Rao K (2019):** Biosynthesis, characterization and antagonistic applications of extracellular melanin pigment from marine *nocardioopsis* sps. *Indian Journal of Pharmaceutical Education and Research*, 53(2):S112-S120.
 26. **Drewnowska J, Zambrzycka M, Kalska-Szostko B et al. (2015):** Melanin-like pigment synthesis by soil *Bacillus weihenstephanensis* isolates from Northeastern Poland. *PLoS ONE*, 10(4):e0125428.
 27. **Agodi A, Stefani S, Corsaro C et al. (1996):** Study of a melanic pigment of *Proteus mirabilis*. *Research in Microbiology*, 147(3):167-74.
 28. **Selvakumar P, Rajasekar S, Periasamy K et al. (2008):** Isolation and characterization of melanin pigment from *Pleurotus cystidiosus* (telomorph of *Antromycopsis macrocarpa*). *World Journal of Microbiology and Biotechnology*, 24(10):2125-31.
 29. **Barretto D and Vootla S (2020):** Biological activities of melanin pigment extracted from *Bombyx mori* gut-associated yeast *Cryptococcus rajasthanensis* KY627764. *World Journal of Microbiology and Biotechnology*, 36(10):159.
 30. **Loi J, Yi T S, Pinsa A et al. (2020):** Pyomelanin Production From a Marine Isolate of *Acinetobacter* Spp. *International Journal of Current Microbiology and Applied Sciences*, 9(6):2250-9.
 31. **Silva C, Santos A, Salazar R et al. (2019):** Evaluation of dye sensitized solar cells based on a pigment obtained from Antarctic *Streptomyces fildesensis*. *Solar Energy*, 181:379-85.
 32. **El-Naggar N, El-Ewasy S (2017):** Bioproduction, characterization, anticancer and antioxidant activities of extracellular melanin pigment produced by newly isolated microbial cell factories *Streptomyces glaucescens* NEAE-H. *Scientific Reports*, 7:42129.
 33. **Mekala L, Mohammed M, Chinthalapati S et al. (2019):** Pyomelanin production: Insights into the incomplete aerobic L-phenylalanine catabolism of a photosynthetic bacterium, *Rubrivivax benzoatilyticus* JA2. *Int J Biol Macromol.*, 126:755-64.
 34. **Singh S, Nimse S, Mathew D et al. (2021):** Microbial melanin: Recent advances in biosynthesis, extraction, characterization, and applications. *Biotechnology Advances*, 53:107773.
 35. **Joshi M, Patil A, Adivarekar R (2021):** Characterization of brown-black pigment isolated from soil bacteria, *Beijerinckia fluminensis*. *BioRxiv Microbiology*. Available at: http://biorxiv.org/cgi/content/short/2021.07.21.453201v1?rss=1&utm_source=researcher_app&utm_medium=referral&utm_campaign=RESR_MRKT_Researcher_inbound.