Original Research Article: full paper

(2023), «EUREKA: Life Sciences»
Number 4

PRODUCTION AND CHARACTERIZATION OF PYOCYANIN PIGMENT FROM *PSEUDOMONAS AERUGINOSA*

Aparna Joshi✉
Department of Biotechnology[1]
aparnashastri74@gmail.com

Rajshri Waghmare
Department of Biotechnology[1]

Mahadev Jadhav
Department of Biotechnology[1]

[1]*Deogiri College*
Station Road, Ch. Sambhajinagar (Aurangabad) Maharashtra, India, 431005

✉**Corresponding author**

Abstract

Pyocyanin, a blue-green phenazine pigment, is produced by various strains of *Pseudomonas aeruginosa* and has demonstrated diverse applications in medicine, agriculture, environmental protection, and nanotechnology. This study aimed to isolate and purify pyocyanin pigment from *Pseudomonas aeruginosa* cultures grown in different media, including succinate, glucose, King's B, and Muller-Hinton broth. The chloroform extraction method was employed to extract the pigment, resulting in the separation of two layers, with the pyocyanin-enriched pigment residing in the lower blue layer containing chloroform. Subsequently, the pigment was subjected to further purification using column chromatography on a silica gel column, employing chloroform as the elution solvent. The purified pyocyanin was verified by thin-layer chromatography (TLC), ensuring its integrity and purity. The UV-Vis spectrophotometer was then employed to characterize the purified pigments, providing valuable insights into their spectral properties. The results indicate successful isolation and purification of pyocyanin pigment from *Pseudomonas aeruginosa* cultures in various media, demonstrating the versatility of the extraction method used. The characterization through UV-Vis spectrophotometry confirms the purity of the obtained pigment, validating its potential applicability in a wide range of fields, including medicine, agriculture, environmental management, and nanotechnology for the synthesis of silver nanoparticles.

This research contributes to a better understanding of pyocyanin production in *Pseudomonas aeruginosa* and offers valuable insights into its potential applications, thereby opening new avenues for further exploration and exploitation of this valuable pigment in various industrial and scientific domains.

Keywords: *Pseudomonas aeruginosa,* Phenazyne, Pyocyanin, Cloroform, Column chromatography, TLC, UV-Visible spectrophotometer.

DOI: 10.21303/2504-5695.2023.003020

1. Introduction

Bacterial pigments, characterized by their varied colors, low molecular weight, and heterogeneous molecular structure, serve crucial roles in promoting the maintenance and persistence of producing organisms [1–3]. These secondary metabolites are involved in a multitude of functions, including photosynthesis, protection against ultraviolet radiation, iron uptake, ecological interactions with other organisms, and participation in cell density-dependent gene expression modulation signaling [4–8].

Pseudomonas aeruginosa, a Gram-negative, aerobic, rod-shaped bacterium, is ubiquitous in various environments such as soil, water, and other natural habitats [9]. One of the remarkable features of P. aeruginosa is its ability to produce a diverse range of extracellular phenazine pigments, which has drawn significant attention [10].

The pyocyanin pigment, a water-soluble compound with a blue-green color, exhibits a wide range of pharmacological effects [11]. Notably, approximately 90–95 % of *Pseudomonas aeruginosa* isolates can produce this pigment, which is commonly referred to as "blue pus" due to its association with the term pyocyaneus [12]. In media with low iron content, pyocyanin is produced abundantly and serves a significant role in iron metabolism, which is crucial for the growth of Pseudomonas aeruginosa [13]. Additionally, pyocyanin has been reported to exhibit antimicrobial activity against certain pathogenic microbial and fungal strains [14]. In *Pseudomonas aeruginosa*, the production of pigments varies among strains, with most strains producing pyocyanin, pyoverdin, or a combination of both, along with other pigments such as pyorubin and pyomelanin [15]. The pyocyanin pigment, which is based on phenazine, is of particular interest due to its ability to generate reactive oxygen species (ROS) [16]. This property becomes significant in the context of tumor cells, as they are susceptible to the reactive oxygen species produced by pyocyanin. This susceptibility arises from the interference of pyocyanin with topoisomerase I and II in eukaryotic cells [17]. Furthermore, pyocyanin finds application in biosensors as a redox compound, facilitating electron transfer between enzyme molecules and the electrode material. This characteristic makes biosensors based on pyocyanin promising for various fields, including agriculture, medicine, and environmental applications [18].

Pyocyanin has also been utilized as an electron shuttle in microbial fuel cells (MFCs), facilitating bacterial electron transfer toward the MFC anode [18]. Studies have shown that the addition of pyocyanin to MFCs containing *Brevibacillus sp.* PTH1 resulted in a twofold increase in the rate of electron transfer [18]. This highlights the potential of pyocyanin to enhance the performance of microbial fuel cells. Moreover, pyocyanin has the ability to form complexes with organic compounds, making it suitable for applications in organic light-emitting devices (OLEDs). These devices are gaining significance due to their advantages such as low voltage requirements, a wide range of colors, and lightweight construction. The conjugation of pyocyanin with organic compounds allows for the development of new complexes that can be utilized in the fabrication of OLEDs [18].

The principal purpose of the present study is to investigate the production and characterization of a secondary metabolite, pyocyanin from the bacterium *Pseudomonas aeruginosa*. This research aims to understand the factors influencing pyocyanin production, optimize its yield, and analyze its chemical and biological properties. This study will contribute to a deeper comprehension of *Pseudomonas aeruginosa's* bioactive compounds, potentially leading to applications in medicine, biotechnology, and environmental science.

2. Materials and Method

2. 1. Production of pigment

Various media were tested to assess the pigment production capacity of microorganisms, including King's B broth, Succinate broth, glucose broth, Glutamic acid broth, and modified MHB media. Among these, glucose broth and modified MHB broth exhibited the most promising results [19, 20]. The indicator for pigment production by microorganisms is a color change in the media to a blush green hue [14, 16, 21, 22].

2. 2. Culture Media Preparation:

To facilitate the growth and pyocyanin production of *Pseudomonas aeruginosa*, a culture medium was prepared. Various types of media were utilized, including King's B medium, Succinate broth, Glucose broth, Glutamic acid medium, Muller Hinton Broth, Nutrient medium with glycerol, and Luria-Bertani (LB) broth supplemented with appropriate additives. Additionally, a modified medium was also prepared to optimize the conditions for pyocyanin production. In the modified medium for pyocyanin production variations in sugar sources such as glucose, lactose, and maltose and their respective concentrations ranged from 1 % to 2 % alongside the standard Mueller-Hinton Broth and glycerol components.

2. 3. Observation of Colour Change

After the designated incubation period, it is crucial to carefully observe the color of each test media. The production of pyocyanin by *Pseudomonas aeruginosa* can lead to a noticeable color change in the media. Typically, pyocyanin manifests as a blush green or blue-green hue, which may become more intense with higher levels of pyocyanin production.

2. 4. Comparative Analysis

The color intensity and presence of a color change were compared among different media types to determine optimal conditions for pyocyanin production by *Pseudomonas aeruginos*a in terms of color intensity, the Glucose broth and Modified MHB broth exhibited more intense colors compared to the other media types. This suggests a higher concentration of pyocyanin pigment in these media, resulting in a more pronounced color change [23, 24].

2. 5. Pyocyanin Extraction

After an appropriate incubation period, typically ranging from 24 to 48 hours, the culture was subjected to centrifugation to separate the bacterial cells from the supernatant, which contains the pyocyanin pigment. The centrifugation process helps to concentrate the pigment in the supernatant, making it easier to collect. To extract the pyocyanin from the cell-free supernatant, a liquid-liquid extraction method using chloroform was also employed. After the appropriate incubation period, the culture was subjected to centrifugation at 8000 rpm for 10 minutes to separate the bacterial cells from the supernatant. To a separating funnel an equal volume of chloroform to the collected supernatant, in a 1:1 ratio was added, closed tightly, and Shaked vigorously for several minutes to ensure thorough mixing of the chloroform and supernatant. Subsequently, the mixture was allowed to undergo a phase separation process, facilitating the division of the solution into two distinct layers: an aqueous phase and an organic phase consisting of chloroform. The chloroform phase was then collected as it contained the extracted pyocyanin, which had been effectively separated from the aqueous phase. This method ensured the isolation of pyocyanin for further analysis and characterization [25].

2. 6. Pyocyanin Purification

The extracted pigment was purified using column chromatography on a silica gel column, followed by purification using TLC. In column chromatography, the blue chloroform layer containing the desired pigment was separated and collected. The purified pigment was then subjected to TLC using a silica gel 60 F254 stationary phase and chloroform as the eluent. This process allowed for the separation and visualization of the pigment spots, with the desired purified pigment exhibiting a distinct blue color. These purification techniques using silica gel column chromatography and TLC with chloroform as the eluent helped to isolate and refine the pigment for further analysis or application [18, 20, 25].

2. 7. Pyocyanin Quantification

After the incubation period, the liquid is subjected to centrifugation at a speed of 10,000 rpm for 12 minutes. The resulting supernatants (5 mL) were mixed with 3 mL of chloroform and vigorously vortexed for 5 minutes. Following this, 1 mL of 0.2 N HCl was added to the organic phase and vortexed for 3 minutes. The mixture was then subjected to centrifugation at 13,000 rpm for 5 minutes After centrifugation, the collected red layer, containing the pyocyanin pigment, was carefully separated. The optical density of this red layer was measured at a wavelength of 520 nm using a spectrophotometer. To determine the pyocyanin concentration, the obtained A520 values were multiplied by a conversion factor of 17.072. The results were expressed in milligrams per liter (mg/L) By following this protocol, it was possible to quantitatively assess the pyocyanin concentration in the fermentation liquid, allowing for a comprehensive understanding of pyocyanin production during the fermentation process [26].

3. Results and Discussion

3. 1. Production of pigment in different broths

The cultures of Pseudomonas aeruginosa grown on the selected broth medium exhibited the production of a blue-green pigment, resembling pyocyanin. This pigment was evident from the noticeable change in the color of the broth, which turned into a blue-green shade, indicating the production of pyocyanin (**Fig. 1**). The appearance of the pigment commenced after 24 hours of incubation and progressively intensified until reaching its peak at around 72 hours (**Fig. 2**).

a *b*

Fig. 1. Change in color of glucose broth: *a* – before pigment production; *b* – after pigment production

a *b*

Fig. 2. Change in color of modified medium: *a* – before pigment production; *b* – after pigment production

3. 2. Purification and characterization of pyocyanin

At the end of incubation, the cells were centrifuged at 8000 rpm for 10 min and the cell-free Supernatant was extracted twice with an equal volume of chloroform in the separating funnel (**Fig. 3**). The Blue chloroform layer was separated and purified by column chromatography and TLC. The presence of pyocyanin Pigment was confirmed as the corresponding TLC Rf value was found to be 0.71 (**Fig. 4**). The Pigment on column chromatography yielded one single fraction of Light blue color. It was then Eluted with Chloroform and methanol (**Fig. 5**).

Fig. 3. Separation of pigment by chloroform

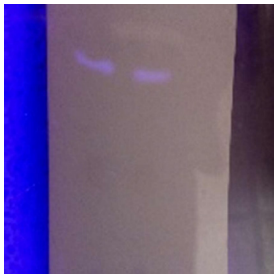


Fig. 4. Pigment separation by TLC

a *b*

Fig. 5. Pigment after separation: *a* – pigment dissolved in chloroform(blue); *b* – pigment dissolved in 0.2 N HCl(red)

3. 3. Quantification of pyocyanin

Pyocyanin quantification assay is based on the pyocyanin's measuring color at 520 nm in the acidic Phase and using 0.2 M HCI used as a blank. The pigment yield was expressed in micrograms (µg) of pyocyanin produced per milliliters (ml) of solution (µg/ml) by multiplying absorbance by 17.072 the extinction coefficient. The Modified MHB medium amended with glucose yielded the maximum Pyocyanin followed by the glucose broth medium. The maximum production of pyocyanin was Observed in MHB with a concentration of 14.850 ug per ml-1 and in glucose broth with a Concentration of 1.877 ug per ml-1.

3. 4. UV spectroscopy analysis of pigment

The absorbance spectra of pyocyanin were measured from 200–700 nm wavelength (**Fig. 6**). 0.2 N HCL as a blank was used and the absorbance peak of standard pyocyanin was eluted at 382 and 521 nm. Similarly, the absorbance peaks of pyocyanin obtained from MHB media were 214,278,387 and 516 nm, and for pyocyanin obtained from glucose broth, the peaks were obtained at 246,288 and 376 nm.

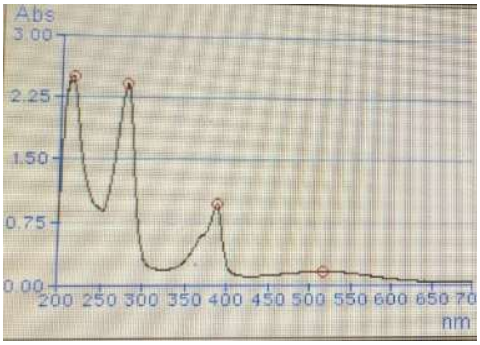
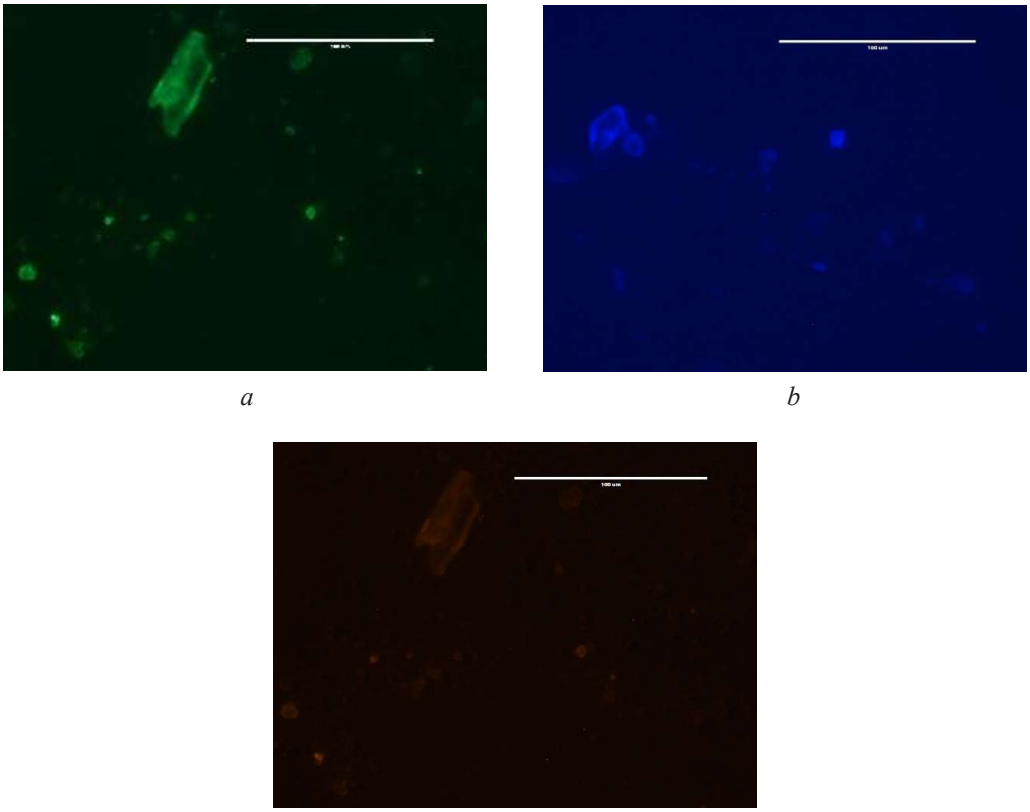


Fig. 6. UV absorption spectra of pyocyanin

The fluorescence property of the purified Pyocyanin pigment was traced under the Inverted Fluorescence microscope (Invitrogen TM EVOSTM Digital Inverted Fluorescence microscope). Microscopic images show fluorescent pigment in green, blue and red light respectively (**Fig. 7**).



a *b*

c

Fig. 7. Microscopic images of pyocyanin showing fluorescence: *a* – fluorescence in green light; *b* – fluorescence in blue light; *c* – fluorescence in red light

4. Conclusions

The present study was focused to produce and characterize the pyocyanin pigment derived from *Pseudomonas aeruginosa*. The research demonstrated that active cultures of *P. aeruginosa*, cultivated in a specific broth medium, successfully generated a distinctive blue-green pigment reminiscent of pyocyanin. This pigment formation was evidenced by a noticeable change in the color of the broth, which transformed into a vivid blue-green shade.

The pyocyanin pigment was isolated by a simple centrifugation technique to separate cell debris and subsequent liquid-liquid extraction using chloroform. This extraction procedure enabled the successful separation of the pyocyanin pigment from the cell-free supernatant. Further purification steps, including column chromatography and thin-layer chromatography (TLC), were employed to refine and isolate the pigment. The chromatographic techniques yielded a single fraction with a light blue color, indicating successful purification.

The purified pyocyanin pigment was characterized using a spectrophotometric technique which allowed the measurement of specific properties and absorbance spectra of the pigment. The combination of these findings sheds light on the production, isolation, and properties of pyocyanin derived from Pseudomonas aeruginosa.

In a broader context, this research provides valuable insights into the production and characterization of the pyocyanin pigment, which can be a vital component in understanding the biology of *Pseudomonas aeruginosa*. The results have implications for potential applications of the pigment and its role within *P. aeruginosa*. Furthermore, this study serves as a foundational step for future investigations into the biological functions and therapeutic possibilities of pyocyanin, thereby opening doors for further exploration in the realm of microbial pigments.

Conflict of interest

The authors had declared that they have no conflict of interest with respect to this work, as per financial, personal, authorship, or any other, that may affect the study and its results presented in this paper.

Financing

The study was conducted without any financial support.

Data availability

The Manuscript has data included as electronic supplementary material.

Acknowledgments

The Authors, Aparna Jayawant Joshi, Rajshri Narendra Waghmare, Mahadev Asaram Jadhav acknowledge Prof. Ashok V. Tejankar, Principal, Deogiri College, Aurangabad, for his kind support, consistent cooperation, and guidance.

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Received date 10.05.2023
Accepted date 18.07.2023
Published date 31.07.2023

How to cite: *Joshi, A., Waghmare, R., Jadhav, M. (2023). Production and characterization of pyocyanin pigment from Pseudomonas aeruginosa. EUREKA: Life Sciences, 4, 3–11, doi: http://doi.org/10.21303/2504-5695.2023.003020*