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Optimizing the Pyocyanin Extraction Via Pseudomonas Aeruginosa Through Evaluating Its Antimicrobial Impending

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ABSTRACT

Pyocyanin a blue-green pigments synthesized by the Gram-negative bacterium Pseudomonas aeruginosa. The present study aims to develop pigment-producing *P. aeruginosa*, scrutinize pyocyanin extraction, and look at its antimicrobial efficacy in opposition to pathogenic bacteria. The untainted strain of *P. aeruginosa* (ATCC-2863) be developed on cetrimide agar plates, and pigment-producing colonies were isolated. Pyocyanin extraction was performed by means of chloroform and acid-base extraction steps, with utmost production pragmatic at 72 hours of incubation. The extracted pyocyanin was evaluated for its antimicrobial efficacy via the disc diffusion method against 3 Gram-negative bacteria (*Proteus, Klebsiella*, and *Escherichia coli*) and 2 Gram-positive bacteria (*Bacillus species* and *Staphylococcus aureus*). The outcomes verified that pyocyanin showed noteworthy antibacterial efficacy against the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*, amid inhibition zones of 12 mm and 10 mm, correspondingly, at a 20 µg/ml concentration. Modest inhibitory property was also practical against the Gram-negative strain *Escherichia coli* (12 mm) and *Proteus* species (10 mm). These outcomes represents that Gram-negative bacteria are commonly a reduced amount of susceptible to pyocyanin compared to Gram-positive strains. Overall, the current research underscores the prospective of pyocyanin as a valuable antimicrobial agent amid promising uses in fields together with agriculture, medicine, and environmental science.

Keywords: Antimicrobial efficacy, Gram-positive and negative strains, *Pseudomonas aeruginosa*, Pyocyanin, Pigment extraction, Secondary metabolites

Introduction

Microorganisms create additional pigments like bacterial pigments as a by-product of their metabolism for their defense (Narsing Rao et al., 201; Saubenova et al., 2024). There are also protective mechanisms for ultraviolet radiation (Sajjad et al., 2020) as well as multifunctional factors such as signaling for gene expression (Liu and Nizet, 2009), iron (Cornelis and Dingemans, 2013) and, photosynthetic pigments (Nowicka and Kruk, 2016). Biodegradable bacterial pigments are not as costly as synthetic ones and can be used in food, pharmaceuticals, cosmetics and textiles (Tirumale & Wani, 2018). Of the pigments produced by bacteria, Pseudomonas aeruginosa synthesizes and secretes the blue-green pyocyanin as pigment extracellularly. The species name comes from the Latin term ‘verdigris’, which means ‘copper rust’, alluding to the culture coloration caused by the existence of pyocyanin and pyoverdine. The phrase pyocyanin derived from the Greek phyco- meaning ‘algae’, and kyanos which means ‘blue’, hence its

name. Alternatively, pyoverdine extracted from pyo- meaning 'pus' and verdine 'green' which aligns to its color. Devoid of pyocyanin, pyoverdine is bright yellow fluorescent. Pseudomonas aeruginosa is a Gram-negative moreover an aerobic bacterium which can grow anaerobically only in occurrence of nitrate. This bacterium is an opportunistic pathogen that is detrimental to both humans and plants, which makes it medically appropriate. *P. aeruginosa* is well-known to reveal positive reactions for citrate consumption, catalase activity, and oxidase synthesis. This species is familiar in soil, water, and other environmental surroundings. *P. aeruginosa* is proficient to nurture in both normoxic as well as hypoxic environments and can employ a wide range of organic compounds as nutrients (Reyes et al., 1981). *Pseudomonas aeruginosa* can construct the blue-green pigment pyocyanin (Jayaseelan et al., 2013), which is a phycobiliprotein (Abdelaziz et al., 2023) . Phycobiliproteins are compounds that can be kaput down into heterocyclic rings that are made by little bacteria (through side chains bonded at various points on their rings). Besides producing pyocyanin, which is blue in color, Pseudomonas aeruginosa also produces supplementary soluble pigments for instance pyoverdine, (yellow-green), pyorubin (red), and pyomelani (brown). Pyocyanin (N-methyl-1-hydroxyphenazine) is a blue-green phenazine pigment that is water soluble and is produced in large quantity by Pseudomonas aeruginosa. The majority, 90-95% of P. aeruginosa, produce active and water soluble pyocyanin which is a virulence factor and has biotechnological uses (Saleem et al., 2021).

Within prokaryotic cells, this pigment exhibits diverse pharmacological behavior, and its action is due to similitude to isoalloxazine, flavoproteins, FMN, and FAD. Furthermore, pyocyanin is efficient in the control of phytopathogens. There is substantial literature on the bio-processing and downstream recovery of pyocyanin for use in aquaculture. It is this phenazine-based pigment that is of unique concern since of its knack to produce reactive oxygen species (Mudaliar and Bharath Prasad, 2024). Pyocyanin has also been utilized in biosensor development, functioning as a redox mediator that facilitates electron transfer between enzyme molecules and electrode surfaces. Such pyocyanin-based biosensors are projected to find applications across agriculture, medicine, and environmental monitoring. More broadly, microbial pigments exhibit considerable clinical potential, with activities ranging from antibiotic and immunosuppressive effects to therapeutic roles in conditions such as cancer (Numan et al., 2018). Major advantages of pigment production from microorganisms include rapid and easy growth in inexpensive culture media, independence from weather conditions, and the availability of various color shades. Pyocyanin shows potential as a bio-control agent, affecting both prokaryotic and eukaryotic cells, serving as an anti-fungal molecule and in bio-sensors (Jayaseelan et al., 2013). The study aims to: i. Cultivate pigment-producing *P. aeruginosa*; ii. Investigate pyocyanin pigment extraction; iii. Explore the pigment's antimicrobial efficacy against pathogenic bacteria.

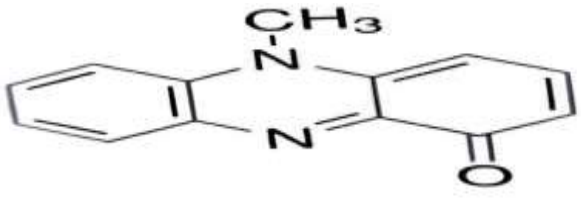

Fig- 01 Structure of Pyocyanin

Materials:

Chemicals: The required chemicals were procured from Hi-Media for the preparation of media,

extraction, and for the respective designed experiments.

Glassware: Petri plates, measuring cylinders, conical flasks, pipettes, separating funnels, centrifugation tubes, and other materials essential for the present study were procured from Borosil.

Methods:

Collection of Sample:

A pure strain of *Pseudomonas aeruginosa* (ATCC 2863) was collected from the VR Laboratory, Microbiology Department, Gulbarga University, Kalaburagi, Karnataka. **Isolation of organisms:** The pure culture was marked on top of cetrimide agar plates (M. R. W. Brown and Richards, 1965) and incubated at 37 °C for 24 hours. Pigment-producing single colonies that grew on the plates were preferred and purified using the streak plate method on the same medium. The purified colonies were subsequently sub cultured on cetrimide agar and maintained as stock cultures for further experimentation. Working stock cultures were stored at 4 °C and refreshed by subculturing every two weeks.

Cultivation of Pseudomonas aeruginosa:

The pure strain of *P. aeruginosa* was subcultured on cetrimide agar plates in support of 48 hours at 37°C and observed under UV light for the presence of the blue-green fluorescent pigment.

Pigment Extraction and Purification:

The medium was inoculated with a loopful of bacterial culture and incubated for 48 hours. The extraction procedure of the pigment was carried out individually, and among all, the optimal medium for pigment production was standardized and used for subsequent studies. A loopful of the P. aeruginosa strain from the agar plate was inoculated into 100 ml of sterilized nutrient broth and asparagine proline medium(Saleem et al.,2021)in a conical flask and incubated at 37°C at 150 rpm in an orbitalshaker for 48 hours.

Standardization of Procedure for Extraction of Pigment:

The standard extraction procedure is as follows (Cheluvappa,2014; Abdelaziz et al., 2022; and Abdelaziz et al., 2023)

1. The cultured broth from the conical flask was transferred into centrifuge tubes and centrifugation at 10,000 rpm for 20 minutes at 4 °C. The resultant supernatant was collected for pigment extraction.
2. A 24-hour culture broth was similarly transferred to a 50 mL glass centrifuge tube and centrifuged at 10,000 rpm for 20 minutes at 4 °C. The supernatant was then transferred into a 500 mL separating funnel and mixed with chloroform in a 2:1 ratio (supernatant: chloroform).
3. The mixture was shaken thoroughly and allowed to stand undisturbed for 5–10 minutes, enabling the pyocyanin fraction to partition into the chloroform phase. The blue chloroform layer, located beneath the aqueous phase, was carefully collected into a covered conical flask to protect it from light and minimize oxidation.
4. The chloroform extract was transferred into a 500 mL rotary evaporation flask and concentrated using a vacuum rotary evaporator at 40 °C.
5. A volume of 10 mL of 0.2 M HCl was added to the chloroform concentrate, and the mixture was shaken to facilitate pigment transfer into the aqueous phase, leaving behind a lemon-yellow chloroform layer.

6. The aqueous phase was separated, and 0.2 M NaOH was added dropwise until the blue coloration reappeared. Subsequently, 20 mL of dry chloroform was added to the aqueous solution and shaken vigorously until the blue pigment migrated back into the chloroform phase.

Antimicrobial efficacy of Pyocyanin:

Test microorganism:

The antibacterial activity of the pyocyanin extract was evaluated against five pathogenic bacterial species, including three Gram-negative strains (*Proteus* spp., *Klebsiella* spp., and *Escherichia coli*) and two Gram-positive strains (*Bacillus* spp. and *Staphylococcus aureus*). Stock cultures of these organisms were grown in nutrient broth at 37 °C for 9 hours and subsequently used to determine the antibiotic susceptibility of the pyocyanin pigments.

Disc diffusion technique:

The disc diffusion method was employed to assess antibacterial activity. Inocula for seeding the susceptibility medium were prepared from fresh pure cultures. Bacterial cell suspensions were prepared in sterile saline or nutrient broth by transferring a portion of fresh growth with a sterile swab or inoculating loop into the suspending medium. The turbidity of each suspension was adjusted to match the McFarland standard by visual comparison against a white background with contrasting black lines. On Mueller–Hinton agar plates, two wells were prepared—one for the control and the other for the pigment extract. For the test well, the extracted pyocyanin pigment was dissolved in 1 mL of dimethyl sulfoxide (DMSO) and labeled accordingly, serving as the positive control. The negative control well contained only DMSO. Plates were incubated at 37 °C for 24 hours, and the resulting zones of inhibition were measured (Saleem et al., 2021).

RESULTS AND DISCUSSION

Segregation and detection of Pseudomonas aeruginosa:

The segregation and recognition of *P.aeruginosa* revealed three distinct colony morphologies on the choosy medium Pseudomonas Cetrimide Agar: colonies with yellow-green pigmentation. Colonies with scant pigmentation, and colonies without pigmentation.(fig-02)

Fig-2: Culture of Pseudomonas aeruginosa on Nutrient Agar.

Pyocyanin Production, Purification, and Extraction:

Pyocyanin production in *Pseudomonas aeruginosa* is time dependent. The bacterium was cultured in

nutrient broth and incubated at 37 °C to induce pigment synthesis. Pigment production began approximately 10 hours after inoculation, with pyocyanin concentration gradually increasing between 24 and 72 hours. Extended incubation was avoided to minimize the synthesis of other pigments—such as pyomelanin (light brown), pyorubin (red-brown), and pyoverdine (green, yellow, fluorescent)—which could reduce pyocyanin yield and interfere with extraction (Meyer, 2000). Maximum production, indicated by a steady color change from light to dark green, was achieved at 72 hours. This trend aligns with the findings of Gahlout et al. (2017), who reported peak pyocyanin levels at 72 hours followed by a decline with prolonged incubation (Fig. 3).

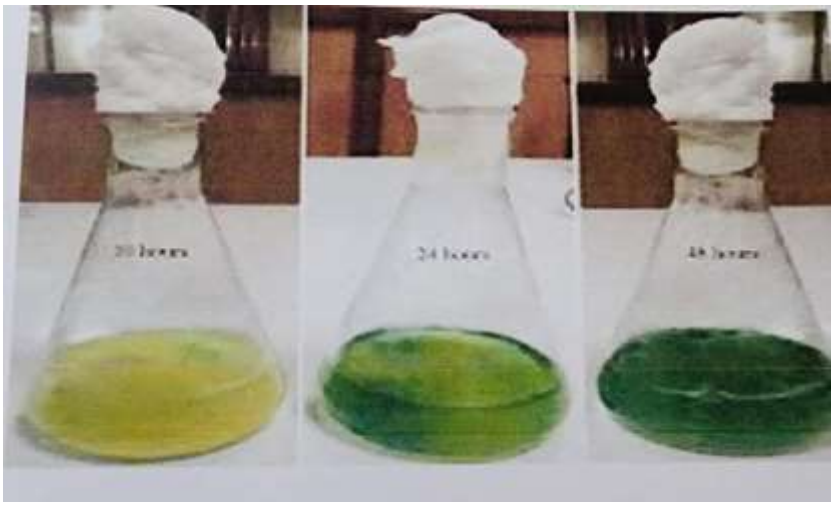

Fig-3: Incubation of selected *P.aeruginosa* strain in nutrient broth from 24to 72hrs.

Extraction and Purification of Pyocyanin:

The extraction method adopted in this research relied on the redox properties of pyocyanin and its unique color shift under acidic conditions, a characteristic observed only in the pure pigment. These properties result in distinct solubility behaviors: in its reduced state, pyocyanin is more soluble in aqueous solvents, whereas in its oxidized state it exhibits greater solubility in organic solvents. This dual solubility enables effective purification through pH adjustment combined with water–chloroform extraction (Feghali and Nawas, 2018a). Accordingly, the blue-green intracellular pigment was extracted using chloroform via sequential acid–base extraction steps (Fig. 4a, 4b) to maximize the yield of pure pyocyanin. The pH-dependent solubility shift, along with the observed color change of the chloroform extract from blue to deep pink upon the addition of 0.2 M HCl, confirmed the successful extraction of pure pyocyanin, consistent with previous reports (Raof and Latif, 2010; Fouly et al., 2015; Raouia et al.,2020).

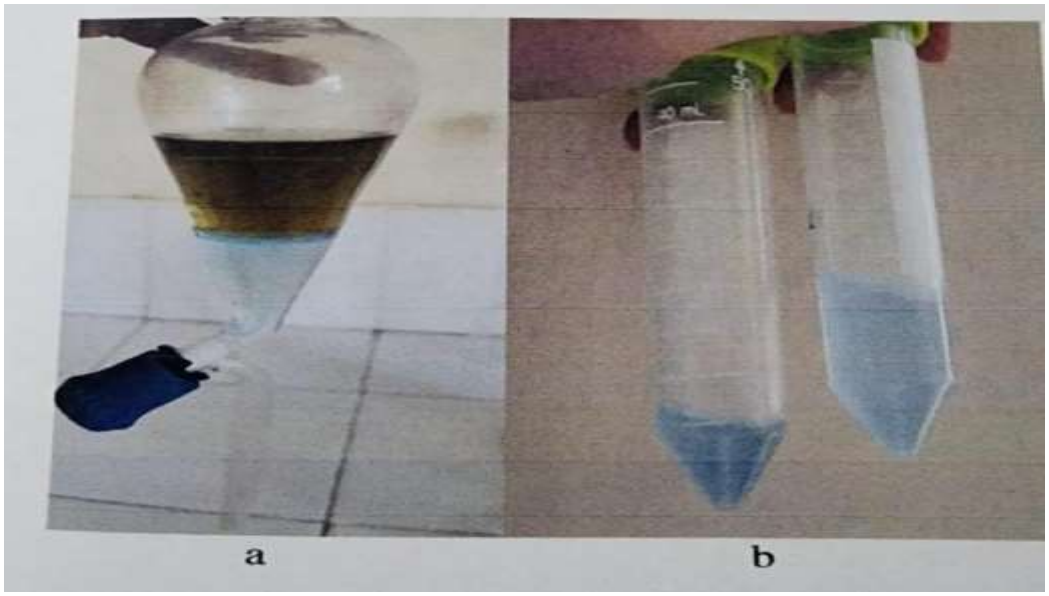

Fig 4a. Pigment extraction from 72hrs culture of P.aeruginosa b. Purified pyocyanin after acid/base extraction method.

Antimicrobial Activities of Pyocyanin:

Pyocyanin, a growth-associated pigment produced by Pseudomonas aeruginosa, plays a critical role in its survival within competitive microbial environments. One of its mechanisms of action involves disrupting the electron transport chain of susceptible microorganisms. In this study, the antibacterial activity of the extracted pyocyanin was confirmed using the agar well diffusion method against four Gram-negative and two Gram-positive bacteria species. As shown in Fig. 5A and 5B, pyocyanin displayed notable antibacterial activity against the Gram-positive strains Bacillus subtilis and Staphylococcus aureus, producing inhibition zones of 12 mm and 10 mm, respectively, at a concentration of 20 µg/mL. At the same concentration, moderate inhibitory effects were observed aligned with the Gram-negative strains Escherichia coli (12 mm) and Proteus spp. (10 mm). These results are consistent with previous studies (Devnath et al., 2017; Darwesh et al., 2019; Hamad et al., 2020), which reported higher sensitivity of Gram-positive bacteria to pyocyanin compared to Gram-negative bacteria.

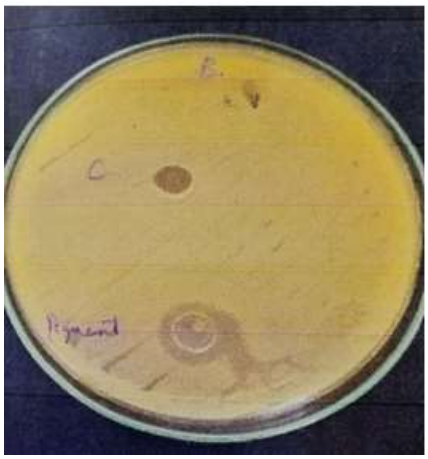

Fig-5: Anti-bacterial efficacy of pyocyanin aligned with clinical pathogen bacteria.

Conclusion:

The current research inattentive on isolating pigmented bacteria and evaluating their pigment synthesizing potential, with *Pseudomonas aeruginosa* preferred for its knack to generate the decidedly water-soluble blue-green pigment pyocyanin. Pigment extraction was performed with chloroform, subsequently purification with 0.2 M HCl and 0.2 M NaOH. The rising consumer stipulate for natural food additives ambitious by concerns in excess of synthetic alternatives underscores the significance of microbial pigments, which recommend scalability and convenient making compared to plant based sources. The extracted pyocyanin exhibited prominent antibacterial activity against numerous clinical pathogens, in conjunction with antioxidant and anti-biofilm properties, even at low concentrations. These attributes emphasize its impending as a natural food-grade colorant and preservative for scheming food borne pathogens. Conversely, prior to saleable application, widespread toxicological assessments, together with organo-leptic and oral toxicity tests, are crucial to make sure safety and regulatory compliance. In conclusion, pyocyanin from *P. aeruginosa* shows strong assure as a multifunctional, natural substitute to synthetic food colors combining vivacious coloration with bioactive properties valuable for food safeguarding.

REFERENCE

1. Chadni, Z., Rahaman, M. H., Jerin, I., Hoque, K. M. F., & Reza, M. A. (2017). Extraction and optimisation of red pigment production as secondary metabolites from Talaromyces verruculosus and its potential use in textile industries. *Mycology*, *8*(1), 48-57.
2. Narsing Rao, M. P., Xiao, M., & Li, W. J. (2017). Fungal and bacterial pigments: secondary metabolites with wide applications. *Frontiers in microbiology*, *8*, 1113.
3. Saubenova, M., Rapoport, A., Venkatachalam, M., Dufossé, L., Yermekbay, Z., & Oleinikova, Y. (2024). Production of carotenoids by microorganisms. *Fermentation*, *10*(10), 502.
4. Ramesh, C., Vinithkumar, N. V., Kirubagaran, R., Venil, C. K., & Dufossé, L. (2019). Multifaceted applications of microbial pigments: current knowledge, challenges and future directions for public health implications. *Microorganisms*, *7*(7), 186.
5. Sajjad, W., Din, G., Rafiq, M., Iqbal, A., Khan, S., Zada, S., ... & Kang, S. (2020). Pigment production by cold-adapted bacteria and fungi: colorful tale of cryosphere with wide range applications. *Extremophiles*, *24*(4), 447-473.
6. Liu, G. Y., & Nizet, V. (2009). Color me bad: microbial pigments as virulence factors. *Trends in microbiology*, *17*(9), 406-413.
7. Cornelis, P., & Dingemans, J. (2013). Pseudomonas aeruginosa adapts its iron uptake strategies in function of the type of infections. *Frontiers in cellular and infection microbiology*, *3*, 75.
8. Nowicka, B., & Kruk, J. (2016). Powered by light: Phototrophy and photosynthesis in prokaryotes and its evolution. *Microbiological Research*, *186*, 99-118.
9. Reyes, E. A., Bale, M. J., Cannon, W. H., & Matsen, J. M. (1981). Identification of Pseudomonas aeruginosa by pyocyanin production on Tech agar. *Journal of clinical microbiology*, *13*(3), 456-458.
10. Jayaseelan, S., Ramaswamy, D., & Dharmaraj, S. (2014). Pyocyanin: production, applications, challenges and new insights. *World Journal of Microbiology and Biotechnology*, *30*(4), 1159-1168.
11. Abdelaziz, A. A., Kamer, A. M. A., Al-Monofy, K. B., & Al-Madboly, L. A. (2023). Pseudomonas aeruginosa's greenish-blue pigment pyocyanin: its production and biological activities. *Microbial Cell Factories*, *22*(1), 110.

12. Blankenfeldt, W., & Parsons, J. F. (2014). The structural biology of phenazine biosynthesis. *Current opinion in structural biology*, *29*, 26-33.
13. Saleem, H., Mazhar, S., Syed, Q., Javed, M. Q., & Adnan, A. (2021). Bio-characterization of food grade pyocyanin bio-pigment extracted from chromogenic Pseudomonas species found in Pakistani native flora. *Arabian Journal of Chemistry*, *14*(3), 103005.
14. Mudaliar, S. B., & Bharath Prasad, A. S. (2024). A biomedical perspective of pyocyanin from Pseudomonas aeruginosa: its applications and challenges. *World Journal of Microbiology and Biotechnology*, *40*(3), 90.
15. Numan, M., Bashir, S., Mumtaz, R., Tayyab, S., Rehman, N. U., Khan, A. L., ... & Al-Harrasi, A. (2018). Therapeutic applications of bacterial pigments: a review of current status and future opportunities. *3 Biotech*, *8*(4), 207.
16. Brown, V. I., & Lowbury, E. J. L. (1965). Use of an improved cetrimide agar medium and other culture methods for Pseudomonas aeruginosa. *Journal of clinical pathology*, *18*(6), 752-756.
17. Abdelaziz, A. A., Kamer, A. M. A., Al-Monofy, K. B., & Al-Madboly, L. A. (2022). A purified and lyophilized Pseudomonas aeruginosa derived pyocyanin induces promising apoptotic and necrotic activities against MCF-7 human breast adenocarcinoma. *Microbial Cell Factories*, *21*(1), 262.
18. Cheluvappa, R. (2014). Standardized chemical synthesis of Pseudomonas aeruginosa pyocyanin. *MethodsX*, *1*, 67-73.
19. Meyer, J. M. (2000). Pyoverdines: pigments, siderophores and potential taxonomic markers of fluorescent Pseudomonas species. *Archives of microbiology*, *174*(3), 135-142.
20. Gahlout, M., Prajapati, H., Chauhan, P., Patel, N., & Solanki, D. (2017). Isolation and screening of pyocyanin producing Pseudomonas spp. from soil. *Int J Adv Res Biol Sci*, *4*(4), 147-152.
21. Nawas, T., & Abou Raji El Feghali, P. (2018). Extraction and purification of pyocyanin.
22. Raoof, W. M., & Latif, I. A. R. (2010). In vitro study of the swarming phenomena and antimicrobial activity of pyocyanin produced by Pseudomonas aeruginosa isolated from different human infections. *Eur J Sci Res*, *47*(3), 405.
23. El-Fouly, M. Z., Sharaf, A. M., Shahin, A. A. M., El-Bialy, H. A., & Omara, A. M. A. (2015). Biosynthesis of pyocyanin pigment by Pseudomonas aeruginosa. *Journal of Radiation Research and Applied Sciences*, *8*(1), 36-48.
24. Raouia, H., Hamida, B., Khadidja, A., Ahmed, L., & Abdelwaheb, C. (2020). Effect of static magnetic field (200 mT) on biofilm formation in Pseudomonas aeruginosa. *Archives of microbiology*, *202*(1), 77-83.
25. Devnath, P., Uddin, M., & Ahamed, K. (2017). Md. Hossain F, Manchur TMA Extraction, Purification and Characterization of pyocyanin produced by Pseudomonas aeruginosa and evaluation for its antimicrobial activity. *Int. Res. J. Biological Sci*, *6*, 1-9.
26. Darwesh, O. M., Barakat, K. M., Mattar, M. Z., Sabae, S. Z., & Hassan, S. H. (2019). Production of antimicrobial blue green pigment Pyocyanin by marine Pseudomonas aeruginosa. *Biointerface Res. Appl. Chem*, *9*(5), 4334-4339.
27. Hamad, M. N., Marrez, D. A., & El-Sherbieny, S. M. (2020). Toxicity evaluation and antimicrobial activity of purified pyocyanin from Pseudomonas aeruginosa. *Biointerface Res Appl Chem*, *10*(6), 6974