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Production enhancement and chemical characterization of pyocyanin pigment extracted from *Pseudomonas aeruginosa*

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Abstract

Pyocyanin is a blue-greenish phenazine pigment produced by certain strains of *Pseudomonas aeruginosa*. This secondary product pigment is revealed to have antibacterial, anticancer, antiparasitic, antioxidant, and antibiofilm formation activity. This study investigates the effects of organic solvents, such as toluene, surfactants, and particularly triton X-100, on pyocyanin synthesis in the presence of glycerol. Moreover, the pyocyanin pigment was chemically characterised using Fourie Transform Infra-Red spectroscopy (FT-IR). The results showed the highest yield of pyocyanin was produced when glycerol 1% was the only carbon source used in the growth medium (nutrient broth). The production of pyocyanin went up to 31.1 mg/L and 13.1 mg/L when either 0.2% toluene or 0.3% triton X-100 was added to the nutrient broth along with 1% glycerol. This was compared to the control level of 6.2 mg/L. FT-IR analysis revealed that pyocyanin had a hydroxyl group (O-H), an aromatic hydrocarbon ring (C-H), aliphatic saturated hydrocarbon chains (CH, CH_2, CH_3), and a C=N bond. It seems that adding glycerol-toluene to the nutrient broth medium can help *P. aeruginosa* make more extracellular yields, such as pyocyanin. Furthermore, FT-IR analysis showed that pyocyanin is biologically safe (has no biological toxic chemical compounds), and these findings confirmed that pyocyanin could be widely used in applications such as in the therapeutic, medication, nutrition, fabric, bio-control, nanotechnology, and physicochemical industries.

Keywords: *Pseudomonas aeruginosa*, pyocyanin, glycerol, organic solvents and FT-IR.

تعزيز إنتاج وتوصيف كيميائي لصبغة البيوسيانين المستخرجة من الزائفة الزنجارية

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الخلاصة:

البيوسيانين صبغة فينازين زرقاء مخضرة تنتجها بعض سلالات من بكتريا الزائفة الزنجارية. اثبت تجريبيا أن صبغة البايوسيانين لها نشاط بايولوجي مضاد للبكتيريا ومضاد للسرطان ومضاد للطفيليات ومضاد للأكسدة ومضاد لتكوين البكتريا للاغشية الحيوية. الهدف من هذه الدراسة هو التحري عن تأثيرات المذيبات العضوية مثل التولوين، والمواد الخافضة للتوتر السطحي مثل تريتون100-X على تصنيع و انتاجية البيوسيانين بوجود الجلسرين. بالاضافة الى، توصيف و دراسة التحليل الكياوي لصبغة البيوسيانين باستخدام مطيافية تحويل فوري بالأشعة تحت الحمراء (FT-IR) . أظهرت نتائج هذه الدراسة زيادة معنوية في إنتاج

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صبغة البيوسيانين باضافة الجلسرين بتركيز 1٪ كمصدر كربوني وحيد لوسط النمو المرق المغذي. كما ارتفعت قابلية إنتاج البيوسيانين إلى 31.1 ملغم / لتر و 13.1 ملغم / لتر عند إضافة اما 0.2٪ تولوين أو 0.3٪ تريتون X-100 إلى مرق المغذيات, على التوالي, بوجود 1٪ جلسرين, بينما كان تركيز انتاجية الصبغة في عينة السيطرة 6.2 ملغم / لتر. كشف تحليل FT-IR أن البيوسيانين يحتوي على مجموعة هيدروكسيل (O-H) و مجموعة امين (NH_2) و حلقة هيدروكربون عطرية (C-H) وسلاسل هيدروكربونية مشبعة أليفاتية (CH, CH_2, CH_3) و اصرة (C = N). استنتجت هذه الدراسة, أن إضافة الجلسرين والتولوين و التريتون إلى وسط مرق المغذيات يمكن أن يساعد الزائفة الزنجارية في إنتاج المزيد من الغلات خارج الخلية، مثل البيوسيانين. علاوة على ذلك، أظهر تحليل FT-IR ان البيوسيانين آمن بايولوجيا (لا تحتوي على مركبات كيماوية سامة) و بالتالي بينت نتائج هذا البحث امكانية استخدام صبغة البيوسيانين في تطبيقات واسعة مثل الصناعات العلاجية والأدوية والتغذية وصناعة الأقمشة والتحكم البيولوجي وتكنولوجيا النانو والصناعات الفيزيائية.

1. Introduction

Pseudomonas species are Gram-negative, aerobic, curved rods that measure 0.5-1.0 µm by 1.5-5.0 µm. Through the use of one or more polar flagella, they can move. They have highly tight oxygen-based aerobic respiratory metabolism, but nitrate has occasionally been utilised as a substitute that permits anaerobic development [1]. The pathogen that is most commonly related to nosocomial pneumonia is *P. aeruginosa* [2]. It is usually connected to a chronic, fatal lung condition that is the primary cause of morbidity and mortality in people suffering from cystic fibrosis (CF), in addition to the colonisation of burn wounds with *P. aeruginosa*, whose systemic invasion can lead to lethal consequences [3, 4].

P. aeruginosa is considered one of the most important species of commercial interest, which produces a variation of soluble pigments, including pyocyanin (blue-green), pyoveridin (yellow-green), pyorubin (red) and pyomelanin (brown) [5, 6]. Pyocyanin is a secondary blue-green metabolite, which converts red when the pH drops > 4.9, and has the capability to oxidize and reduce other molecules [7]. Carbon and nitrogen supplies, incubation, salt, oxygen, temperature, and pH are considered the major factors affecting the biological activity of the pyocyanin formation [7, 8]. Furthermore, alkaline protease escalates the synthesis of pyocyanin [10]. Pyocyanin is derived from phenazine. Phenazin is a type of extracellular secondary metabolite pigment produced by *P. aeruginosa,* which contains nitrogen ($C_{13}H_{10}N_2O$) and naturally soluble in aqueous solutions. Pyocyanin applies a diversity of biomedical activities such as anticancer, antiparasitic, antibacterial, antibiofilm, antimalarial, immunosuppressive, and antioxidant [10-12]. All these characters of the pyocyanin form it as a vital pigment to be performed in pharmaceutical, medical, nanotechnology, nutrition, fabric, physicochemical and bio-control fields [7, 13, 14]. Different supplements such as fatty acids, organic solvents, surfactants, and vegetable oils have been tried by various researchers to enhance the synthesis of microbial products. They report that these supplements lead to the change in cell membrane composition, thus enhancing the quality of the desired product [16].

Pyocyanin is used in various fields, such as medicine, agriculture, and research. However, due to its limited availability (lack of large-scale yield) and the complexity of its production process, pyocyanin can be relatively expensive in the market. In view of the prospective uses, it is necessary to develop a new technique in order to get pyocyanin at an affordable price.

Therefore, this project sought to study the effect of toluene on *P. aeruginosa* pyocyanin production and examined its composition and possible impacts. In addition, examining the functional groups and chemical structure of pyocyanin is an essential aspect of a safety investigation. It gives vital insights into potential risks, toxicity mechanisms, and the creation of safety measures to protect human health and the environment.

2. Materials and methods

2.1 Microorganism and preparation of inoculum

Out of eight *P. aeruginosa* isolates (SPA 1–8) used in the current study were obtained from postgraduate students at the Department of Biology, College of Science, University of Baghdad. These strains were isolated from wound burn patients in hospitals across Baghdad city in Iraq. Standard procedures were used to confirm the isolated identities. Bacterial isolates were stored at -20°C in nutrient broth (HiMedia, India) supplemented with 20% (v/v) glycerol (Merck). Isolates were grown on cetrimide plates at 30°C for 20 h. One colony was transferred to 10 mL of nutrient broth and cultivated at 30°C for 20 hours. 1 mL of bacterial suspension ~ (10^8 CFU/mL) was inoculated into a flask (250 mL), which contained 100 mL of nutrient broth, and cultivated at 30°C for 20 h. For pyocyanin production and extraction, the nutrient broth supplemented with either toluene or triton X-100 along with glycerol was inoculated into flasks and incubated at 30°C for 72 h with agitation at 150 rpm/min.

2.2 Bacterial strains, deoxyribonucleic acid (DNA) extraction and polymerase chain reaction (PCR) identification

The cultivation of all strains was conducted in nutrient broth by incubation at 37°C for 20 h. The growth was collected using centrifugation at 12,000 xg for 5 min. The bacterial gDNA was extracted using the EZNA Bacteria Genome Kit (Omega Bio-Tek, USA) following the guidance provided by the manufacturer. DNA concentration and purity were assessed by agarose gel electrophoresis and NanoDrop 2000c UV-Vis spectrophotometer (Thermo Fisher Scientific, USA). The extracted DNA was preserved at -20°C until it was subjected to PCR investigation.

Primer3 software was used to design the PCR primers 5’→ 3’ (FOR-16s: AGAAAGTCGGGGATCTTCGG) and (REV-16s: CTTGCGCCCATTGTCCAATA) targeting the conserved sequence of 16S rDNA. Primers with no hairpin assemblies or dimers were picked. Their specificity was first confirmed using the NCBI Blast tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The PCR mixture included 10 μL of 2 × Phusion Master Mix (Thermo Fisher Scientific, USA), 1 μL of each primer (10 μM), 50 ng of DNA template, and DNase/RNase-free H_2O to achieve a final volume of 20 μL. The process of PCR amplification was performed using a thermal cycler (Bio-Rad, USA). PCR conditions used: initial denaturation phase (98°C for 3 min), followed by 35 cycles (95°C for 30 s, 52°C for 30 s, and 72°C for 15 s), final extension step (72°C for 10 min). PCR amplicons were visualised by running on 2% agarose gel, and they made visible by staining with ethidium bromide (0.5 μg/mL) [16, 17].

2.3 Effect of glycerol on pyocyanin production

To determine the effects and identify the optimal concentration of supplementing media with carbon sources on the efficiency of pyocyanin production, glycerol was added to the nutrient broth at 0.5, 1, and 1.5% (v/v). Before autoclaved media, the pH values were adjusted to 7.2. One isolated colony for each bacterial strain was separately inoculated into the media and incubated at 30 °C for 24 h with agitation at 150 rpm/min.

2.4 Effect of toluene and triton X-100 on pyocyanin formation in the presence of glycerol

Toluene and triton X-100 were separately supplemented to the growth (production) media at one of the following concentrations (0.1%, 0.2%, or 0.3%, v/v) post 24 h of bacterial cultivation. Both control and test flasks were made from nutrient broth without toluene or triton X-100, and it was included 1% (v/v) glycerol. For the test flasks, post 24 h incubation, either toluene or triton X-100 was added under aseptic conditions. The fermentation was conducted for an additional 48 h (for a total of 72 h fermentation time point) at 30°C with agitation at 150 rpm/min.

2.5 Measurement of pyocyanin production

The quantity of pyocyanin synthesised by *P. aeruginosa* was evaluated after 72 h of incubation. The bacterial culture was centrifuged at 5000 xg for 25 min to collect the bacterial supernatant. The absorbance (A) at 520 nm was utilised to calculate the quantity of pyocyanin existing in the supernatant. The below formula was used to estimate the quantity of pyocyanin (mg/L) = $A_{520} \times 17.072$ [19].

2.6 Pyocyanin extraction and partial purification

At a concentration of 10^8 colony forming unit/mL (CFU/mL), the strain was used to make pyocyanin for the first 72 h of incubation with shaking at 30°C with 150 rpm/min. The blue-green colour in the broth medium acted as a sign that pigment was being produced. After 72 h incubation, the culture was precipitated at 5000 xg for 25 min. The separated supernatant was then mixed in a 1:2 ratio with chloroform (Merck) to extract the blue-green pigment. A fresh test tube was filled with the newly combined solution before being treated with 0.2 M hydrochloric acid (HCl) (Merck). After that, 0.2 M NaOH was added until the colour of the solution changed from deep pink to greenish-blue. A 0.45 µm membrane filter (Fisher Scientific) was used to filter the mixture in order to get a clear, blue-greenish solution of pyocyanin, which was then extracted in chloroform once again. Drop by drop, dry petroleum ether was added until the chloroform evaporated and the formation of blue pyocyanin crystals occurred (3 mL of petroleum ether was added for every 1 mL of chloroform) [5].

2.7 FT-IR spectroscopy for pyocyanin analysis

To get the FT-IR spectrum for pyocyanin and figure out what functional groups it belongs to, a Shimadzu IRprestige-21 in transmittance mode was used. Mixing 2 mg of pyocyanin with 200 mg of KBr (Sigma-Aldrich) made 3 mm discs that were compressed. The spectrum was then changed to account for KBr background. This proceeded by inspecting the pellets in the 3500-750 cm^1 range with a resolution of 4 cm^1 and 10 scans [20]. The analysis was carried out at the Department of Chemistry/ University of Baghdad, Iraq.

2.8 Statistical analysis

The research data of this study were statically analyzed against the control sample by using graphPad (Presim version 5). The results are presented as the mean value (+/-) [(standard deviation (SD)] gained from 3 independent biological experiments. Data were analysed against the control sample, and an asterisk represents a P value of (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$), which was considered to be significant and was determined using the student t-test (two-tailed).

3. Results

3.1 Identification of 16S rDNA for P. aeruginosa

PCR amplification of the genomic DNA from the SPA 2–7 isolates of *P. aeruginosa* generated an amplicon of the anticipated size (about 192 bp). However, no PCR products were obtained for the two isolates (SPA 1 and 8). Consequently, these two isolates were excluded from further experiments or studies in this research (Figure 1).

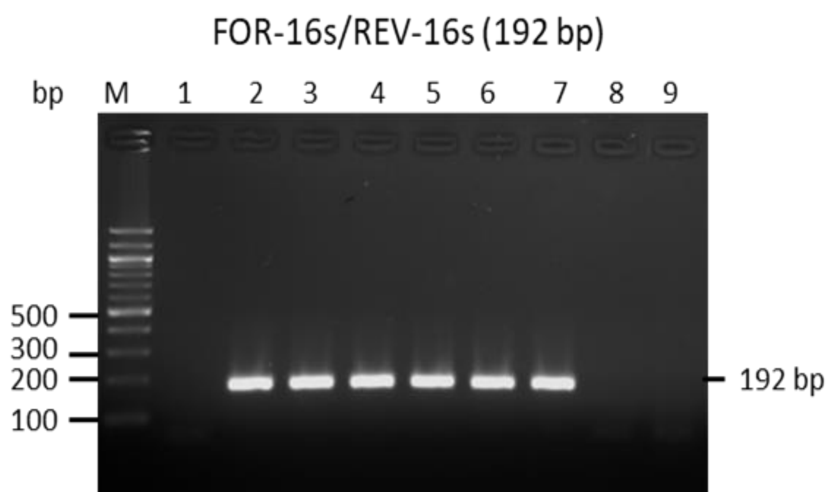

Figure 1: PCR amplicons of *P. aeruginosa* (SPA 1-8) genomic DNA samples. Lane M: 100bp DNA ladder (NEB, UK). Lane 1-8 (SPA 1-8 bacterial genomic DNA), lane 9 no template control. PCR amplicon expected size is (192 bp).

3.2 Strains for production pyocyanin and effect of glycerol on pyocyanin production

The findings of this study, which assessed the pyocyanin production capability of SAP 2–7 strains, suggested that SPA 3 isolate could be a strain worth considering. Therefore, the SPA 3 isolate was elected for the remaining experiments in this research. The inquiry was performed to evaluate the impact of glycerol on the synthesis of pyocyanin, and the findings are shown in Figure 2.

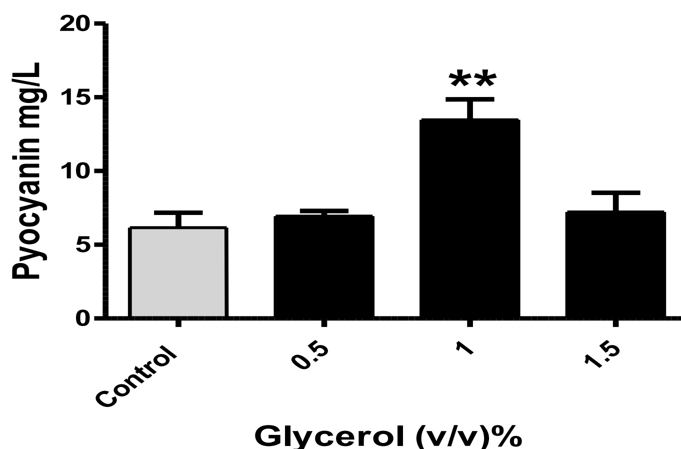

Figure 2: Effect of glycerol on the efficiency of pyocyanin production. Data were analysed against the control sample, and an asterisk represents a P value of (*p≤0.05, **p≤0.01, ***p≤0.001), which was considered to be significant and was determined using the student t-test (two-tailed).

3.3 Enhancement of pyocyanin production in presence of toluene and triton X-100

The effects of toluene and triton X-100 on pyocyanin production in *P. aeruginosa* (SPA 3) isolate were investigated by adding 0.1-0.3% (v/v) of either toluene or triton X-100 to the growth medium, which contains 1% glycerol for 72 h fermentation period time. Our result found that added of toluene at 0.2%, v/v led to the highest level of pigment formation (31.1 mg/L) (Figure 3). However, added triton X-100 at 0.3% v/v yielded the highest level of pigment formation (13.1 mg/L) (Figure 3). These outcomes propose that pyocyanin

production is greatest when toluene and triton X-100 are added to the nutrient medium supplemented with 1% glycerol. Based on these findings, toluene at 0.2 % (v/v) was chosen for further use since it produced the maximum amount of pyocyanin when supplemented to the growth medium. In general, bacteria produce pyocyanin as a secondary metabolite.

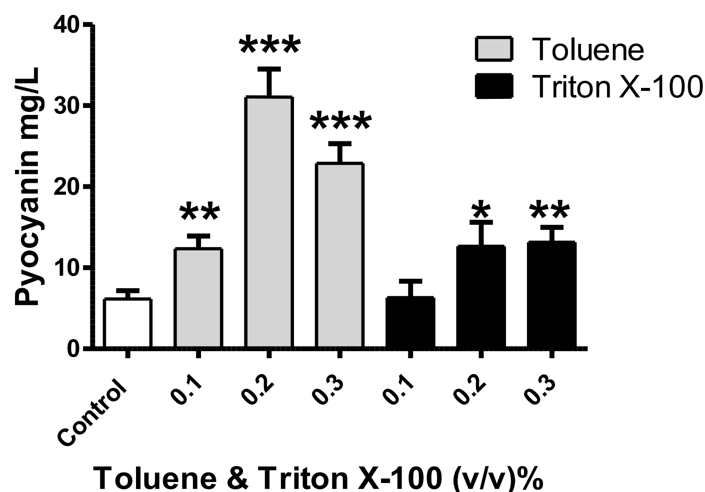

Figure 3: Effect of toluene and/or triton X-100 on pyocyanin formation. In the presence of 1% glycerol, either toluene or triton X-100 was added to the fermentation medium after 20 h post-incubation. Data were analysed against the control sample, and an asterisk represents a P value of (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$), which was considered to be significant and was determined using the student t-test (two-tailed).

3.4 FT-IR analysis of pyocyanin pigment

FT-IR test was used to detect the active chemical groups of the pyocyanin pigment generated by the SPA 3 strain, as shown in Figure 4. The existence of the following active chemical groups: hydroxyl group (O-H) at wave number rate 3134.11cm^{-1}, aromatic hydrocarbon rings (C-H), and aliphatic saturated hydrocarbon chains (CH, CH_2, CH_3) at wave number rate 2921.96 cm^{-1} remarkable the tested pyocyanin. The pyocyanin pigment, which is represented by the peak of 1629.47 cm^{-1}, is the only one with C=N link. As a result of stretching vibrations of C=C in the aromatic ring, FT-IR spectrum showed aromatic hydrocarbon at the ranges of 1500-1400 cm^{-1} and 1600-1585 cm^{-1}.

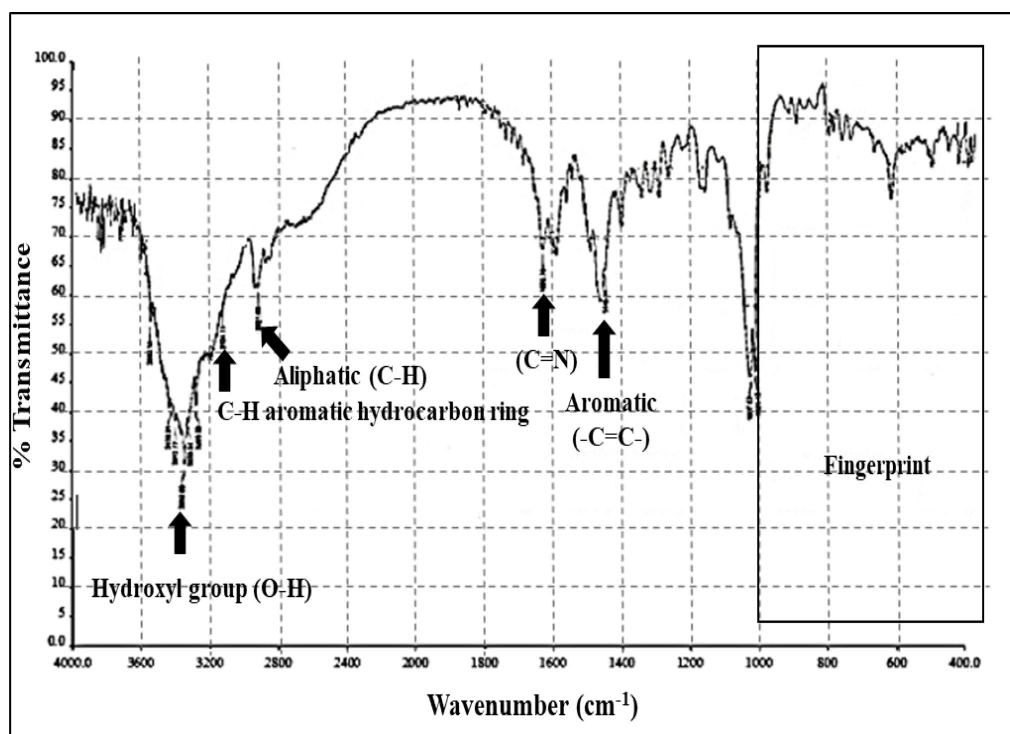

Figure 4: FT-IR analysis of extracted pyocyanin pigment.

4. Discussion

In this study, *P. aeruginosa* was identified using an endpoint PCR test by targeting the 16S rDNA. Six out of eight isolates were identified as *P. aeruginosa* strains. Differentiating between the major phyla of bacteria may be achieved by comparing their 16S rRNA gene sequences. These sequences also allow for the categorization of strains at several levels, including species and subspecies [21].

In order to enhance the formation of pyocyanin pigment, the inquiry was performed to evaluate the impact of glycerol on the synthesis of pyocyanin, and the findings are shown in Figure 2. Based on these findings, it can be concluded that a 1% concentration of glycerol is an appropriate carbon source for maximising the efficiency of pyocyanin yielding. Other studies have reported glycerol enhances and increases the production of pyocyanin pigment because it is a source of carbon [20, 21]. The exact process through which glycerol enhances pyocyanin production in *P. aeruginosa* may comprise a combination of metabolic processes, genes transcription, oxidative stress response, and quorum-sensing regulatory systems [24].
The second part of the current study was to study the enhancement of pyocyanin production in the presence of toluene and triton X-100**.** It's well knowledge that secondary metabolites are produced towards the conclusion of the growth cycle or very close to the stationary phase [25]. The various optimal fermentation periods that have been described in the literature may be a result of the various strain types, fermentation medium compositions, and culture conditions [26]. According to Ozcan and Kahraman, and Murat, *P. aeruginosa* NRRL B-771 and OG1, respectively, produced the most pyocyanin after 72 h incubation [7, 25]. When introduced directly to the growing medium, several organic solvents, including benzene, xylene, and toluene are very poisonous and kill the majority of microorganisms at low doses [28, 29]. OprF, the major porin-forming protein in *P. aeruginosa* outer membrane, allows hydrophilic solutions to flow through and reach the periplasm. Toluene cannot cross the outer membrane in toluene resistance mutants where OprF has been deleted [30]. Several research groups have found ways to significantly boost enzyme synthesis. Alkaline protease synthesis by *Streptomyces clavuligerus* was found to be greatly boosted by the addition of some organic

solvents such as xylene benzene, butanol (0.1%, v/v), and acetone (0.3%, v/v) [28, 29]. Pyocyanin is made from the amino acids alanine, glutamic acid, proline, leucine, and isoleucine, which are metabolised by the enzyme protease by means of organic solvents. In addition, pyocyanin formation may be enhanced by alkaline protease [10].
Furthermore, the FT-IR test was used to detect the active chemical groups of the pyocyanin pigment generated by the SPA 3 strain. Our data found that the extracted pyocyanin is safe (does not have any of known toxic chemical structure as a part of the chemical active groups), as shown in Figure 4. Published data supporting the representation of the pyocyanin pigment by FT-IR analysis and indicating its presence as the predominant molecule in the extract were supported by the FT-IR analysis peaks, and the bonds discovered at these peaks as follows: hydroxyl group (OH) (3300–3600), amino group (NH_2) (3200–3400), aromatic hydrocarbon ring (C–H) (3100–3200), aliphatic saturated hydrocarbon chains (CH, CH_2, CH_3) (2850–2980), C=N bond (1585–1600), aromatic double bonds (–C=C–) (1500–1600), carbonyl group (–C=O) (1700–1750), cyano group (C≡N) (2000–2250), and acetylenic group (C≡C) (1700–1750) [12, 30-32]. The absence of potentially harmful cyano (C≡N) (2000-2250) and acetylenic (C≡C) groups served as proof of safety. Most of the above-mentioned functional groups were earlier well-known in a research that confirmed the standard pyocyanin FT-IR spectrum [35].

5. Conclusions

Based on prior researches, it has been shown that pyocyanin has antimicrobial effects on fungi, bacteria, and protozoa. Pyocyanin, the main phenazine pigment connected to *P. aeruginosa* and renowned for its significant antibacterial, antioxidant, and anticancer activities, is produced by 90–95% of these strains. In order to improve pyocyanin manufacturing utilising organic solvents like toluene, we have leaned on earlier research. These investigations show that the generation and yield of pigments are greatly increased by the addition of 0.2% toluene, 1% glycerol, and chloroform. Pyocyanin's safety has also been established by FT-IR analysis, paving the way for additional research into its antibacterial and anticancer effects utilising pathogenic bacteria, cell lines and animal models. Its hypothesis that both amine (-NH_2) and hydroxyl (-OH) play a crucial role in pyocyanin's biological action via increasing the redox cycling and interaction with cell targets, making it a potent compound works as an antibacterial and anticancer agents.

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Conflict of interest

The authors have no conflicts of interest regarding this investigation**.**

References

[1] G. W. Lau, D. J. Hassett, H. Ran, and F. Kong, "The role of pyocyanin in *Pseudomonas aeruginosa* infection," *Trends in Molecular Medicine*, vol. 10, no. 12, pp. 599–606, 2004, doi: 10.1016/j.molmed.2004.10.002.

[2] T. Horan, D. Culver, W. Jarvis, G. Emori, S. Banerjee, W. Martone, and C. Thornsberry, "Pathogens causing nosocomial infections preliminary data from the national nosocomial infections surveillance system," *Antimicrobic Newsletter*, vol. 5, no. 9, pp. 65–67, 1988, doi: 10.1016/0738-1751(88)90027-5.

[3] R. B. Fick, "Pathogenesis of the Pseudomonas Lung Lesion in Cystic Fibrosis," *Chest*, vol. 96, no. 1, pp. 158–164, 1989, doi: 10.1378/chest.96.1.158.

[4] M. K. Belba, E. Y. Petrela, and A. G. Belba, "Epidemiology of infections in a Burn Unit, Albania," *Burns*, vol. 39, no. 7, pp. 1456–1467, 2013, doi: 10.1016/j.burns.2013.03.013.

[5] P. A. R. El Feghali and T. Nawas, “Extraction and purification of pyocyanin: a simpler and more reliable method,” *MOJ Toxicology*, vol. 4, no. 6, 2018, doi: 10.15406/mojt.2018.04.00139.

[6] N. F. H. Khorshid and N. H. Odaa, “Optimum Conditions for Pyomelanin Production and Characterization from Local Isolates of *Pseudomonas aeruginosa*,” *Medical Journal of Babylon*, vol. 22, no. 1, pp. 108–116, 2025, doi: 10.4103/MJBL.MJBL_770_23.

[7] H. M. Hassan and I. Fridovich, “Mechanism of the antibiotic action pyocyanine,” *Journal of Bacteriology*, vol. 141, no. 1, pp. 156–163, 1980, doi: 10.1128/jb.141.1.156-163.1980.

[8] M. Ozdal, “A new strategy for the efficient production of pyocyanin, a versatile pigment, in *Pseudomonas aeruginosa* OG1 via toluene addition,” *3 Biotech*, vol. 9, no. 10, p. 374, 2019, doi: 10.1007/s13205-019-1907-1.

[9] S. Patil, M. Nikam, H. Patil, T. Anokhina, V. Kochetkov, and A. Chaudhari, “Bioactive pigment production by *Pseudomonas* spp. MCC 3145: Statistical media optimization, biochemical characterization, fungicidal and DNA intercalation-based cytostatic activity,” *Process Biochemistry*, vol. 58, pp. 298–305, 2017, doi: 10.1016/j.procbio.2017.05.003.

[10] K. Iiyama, E. Takahashi, J. M. Lee, H. Mon, M. Morishita, T. Kusakabe, and C. Yasunaga-Aoki, “Alkaline protease contributes to pyocyanin production in *Pseudomonas aeruginosa*,” *FEMS Microbiology Letters*, vol. 364, no. 7, 2017, doi: 10.1093/femsle/fnx051.

[11] Diaa A Marrez and Haitham SM, “Biological activity and applications of pyocyanin produced by *Pseudomonas aeruginosa*,” *Open Access Journal of Biomedical Science*, vol. 1, no. 4, pp. 140–144, 2020, doi: 10.38125/oajbs.000133.

[12] A. Moayedi, J. Nowroozi, and A. Akhavan Sepahy, “Cytotoxic effect of pyocyanin on human pancreatic cancer cell line (Panc-1),” *Iranian Journal of Basic Medical Sciences*, vol. 21, no. 8, pp. 794–799, 2018, doi: 10.22038/ijbms.2018.27865.6799.

[13] M. A. Marey, R. Abozahra, N. A. El-Nikhely, M. F. Kamal, S. M. Abdelhamid, and M. A. El-Kholy, “Transforming microbial pigment into therapeutic revelation: extraction and characterization of pyocyanin from *Pseudomonas aeruginosa* and its therapeutic potential as an antibacterial and anticancer agent,” *Microbial Cell Factories*, vol. 23, no. 1, p. 174, 2024, doi: 10.1186/s12934-024-02438-6.

[14] A. Kumar, K. Dhar, S. S. Kanwar, and P. K. Arora, “Lipase catalysis in organic solvents: advantages and applications,” *Biological Procedures Online*, vol. 18, no. 1, p. 2, 2016, doi: 10.1186/s12575-016-0033-2.

[15] Y. Sardessai and S. Bhosle, “Tolerance of bacteria to organic solvents,” *Research in Microbiology*, vol. 153, no. 5, pp. 263–268, 2002, doi: 10.1016/S0923-2508(02)01319-0.

[16] X. Y. Lei, M. Y. Zhang, Y. J. Ma, and J. W. Wang, “Transcriptomic responses involved in enhanced production of hypocrellin A by addition of Triton X-100 in submerged cultures of Shiraia bambusicola,” *Journal of Industrial Microbiology and Biotechnology*, vol. 44, no. 10, pp. 1415–1429, 2017, doi: 10.1007/s10295-017-1965-5.

[17] A. Nubgan, “The deubiquitylase-ubiquitin-specific protease 4 absence in HeLa cells leads to a reduction in Semliki Forest virus replication,” *Human Gene*, p. 201170, 2023, doi: 10.1016/J.HUMGEN.2023.201170.

[18] A. Nubgan, “Genetic and Phenotypic Variations in Phenylthiocarbamide Bitter Taste Receptors in Iraqi Population,” *Iraqi Journal of Science*, vol. 64, no. 10, pp. 5030–5041, 2023, doi: 10.24996/ijs.2023.64.10.12.

[19] D. W. Essar, L. Eberly, A. Hadero, and I. P. Crawford, “Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications,” *Journal of Bacteriology*, vol. 172, no. 2, pp. 884–900, 1990, doi: 10.1128/jb.172.2.884-900.1990.

[20] R. K. Suryawanshi, C. D. Patil, H. P. Borase, B. K. Salunke, and S. V. Patil, “Studies on Production and Biological Potential of Prodigiosin by *Serratia marcescens*,” *Applied Biochemistry and Biotechnology*, vol. 173, no. 5, pp. 1209–1221, 2014, doi: 10.1007/s12010-014-0921-3.

[21] P. Tripathi, G. Banerjee, M. K. Gupta, S. Saxena, and P. W. Ramteke, “Assessment of phylogenetic affiliation using 16S rRNA gene sequence analysis for *Pseudomonas aeruginosa* in patients of lower respiratory tract infection.,” *The Indian journal of medical research*, vol. 138, no. 4, pp. 557–9, 2013, [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/24434264

[22] M. Z. El-Fouly, A. M. Sharaf, A. A. M. Shahin, H. A. El-Bialy, and A. M. A. Omara,

"Biosynthesis of pyocyanin pigment by *Pseudomonas aeruginosa*," *Journal of Radiation Research and Applied Sciences*, vol. 8, no. 1, pp. 36–48, 2015, doi: 10.1016/j.jrras.2014.10.007.

[23] G. A. Abo-Zaid, E. E. Wagih, S. M. Matar, N. A. Ashmawy, and E. E. Hafez, "Optimization of pyocyanin production from *Pseudomonas aeruginosa* JY21 using statistical experimental designs," *International Journal of ChemTech Research*, vol. 8, no. 9, pp. 137–148, 2015.

[24] W. S. da Cruz Nizer, V. Inkovskiy, Z. Versey, N. Strempel, E. Cassol, and J. Overhage, "Oxidative Stress Response in *Pseudomonas aeruginosa*," *Pathogens*, vol. 10, no. 9, p. 1187, 2021, doi: 10.3390/pathogens10091187.

[25] E. B. Kurbanoglu, M. Ozdal, O. G. Ozdal, and O. F. Algur, "Enhanced production of prodigiosin by *Serratia marcescens* MO-1 using ram horn peptone," *Brazilian Journal of Microbiology*, vol. 46, no. 2, pp. 631–637, 2015, doi: 10.1590/S1517-838246246220131143.

[26] I. Poblete-Castro, C. Wittmann, and P. I. Nikel, "Biochemistry, genetics and biotechnology of glycerol utilization in *Pseudomonas* species," *Microbial Biotechnology*, vol. 13, no. 1, pp. 32–53, 2020, doi: 10.1111/1751-7915.13400.

[27] Özcan D; Kahraman H., "Pyocyanin production in the presence of calcium ion in *Pseudomonas aeruginosa* and recombinant bacteria," *Turkish Journal of Science and Technology*, vol. 10, no. 1, pp. 13–19, 2015.

[28] C. Pacífico, P. Fernandes, and C. C. C. R. de Carvalho, "Mycobacterial Response to Organic Solvents and Possible Implications on Cross-Resistance With Antimicrobial Agents," *Frontiers in Microbiology*, vol. 9, 2018, doi: 10.3389/fmicb.2018.00961.

[29] M. N. Abed and H. M. Rasheed, "Effect of some sugars on carbapenemase gene expression in *Pseudomonas aeruginosa,*" *Plant Archives*, vol. 20, no. 1, pp. 180–186, 2023.

[30] L. Li, T. Komatsu, A. Inoue, and K. Horikoshi, "A Toluene-tolerant Mutant of *Pseudomonas aeruginosa* Lacking the Outer Membrane Protein F," *Bioscience, Biotechnology, and Biochemistry*, vol. 59, no. 12, pp. 2358–2359, 1995, doi: 10.1271/bbb.59.2358.

[31] J. T. Thumar and S. P. Singh, "Organic solvent tolerance of an alkaline protease from salt-tolerant alkaliphilic Streptomyces clavuligerus strain Mit-1," *Journal of Industrial Microbiology & Biotechnology*, vol. 36, no. 2, pp. 211–218, 2009, doi: 10.1007/s10295-008-0487-6.

[32] W. Hoidy, A. Nubgan, and M. Alsaadi, "Development of a T-ARMS-PCR Assay for Detecting Genetic Polymorphism in the Catalase (rs7943316) Gene in the Iraqi Population with Breast Cancer," *Asian Pacific Journal of Cancer Prevention*, vol. 24, no. 9, pp. 3283–3289, 2023, doi: 10.31557/APJCP.2023.24.9.3283.

[33] M. T. Koyun, S. Sirin, S. A. Erdem, and B. Aslim, "Pyocyanin Isolated from *Pseudomonas aeruginosa*: Characterization, Biological Activity and Its Role in Cancer and Neurodegenerative Diseases," *Brazilian Archives of Biology and Technology*, vol. 65, 2022, doi: 10.1590/1678-4324-2022210651.

[34] M. Laxmi and S. G. Bhat, "Characterization of pyocyanin with radical scavenging and antibiofilm properties isolated from *Pseudomonas aeruginosa* strain BTRY1," *3 Biotech*, vol. 6, no. 1, p. 27, 2016, doi: 10.1007/s13205-015-0350-1.

[35] S. DeBritto, T. D. Gajbar, P. Satapute, L. Sundaram, R. Y. Lakshmikantha, S. Jogaiah, and S. Ito, "Isolation and characterization of nutrient dependent pyocyanin from *Pseudomonas aeruginosa* and its dye and agrochemical properties," *Scientific Reports*, vol. 10, no. 1, p. 1542, 2020, doi: 10.1038/s41598-020-58335-6.