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Pyocyanin derived from the marine sponge-associated bacterium, *Pseudomonas aeruginosa* P1.S9, has the potential as antibacterial

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Abstract. *Mesrian DK, Astuti RI, Prastya ME, Wahyudi AT. 2024. Pyocyanin derived from the marine sponge-associated bacterium,* Pseudomonas aeruginosa *P1.S9, has the potential as antibacterial. Biodiversitas 25: 4139-4147.* Sponge-associated bacteria are a prolific source of secondary metabolites. Among them, pyocyanin-producing *Pseudomonas aeruginosa* is a subject of great interest. Pyocyanin is a blue-green pigment known for its enormous biological activity, one of the most notable being antimicrobial. Therefore, this study was performed to optimize the production, to characterize the chemical structure, and to test the antimicrobial activity of pyocyanin. As the sole isolate used, *Pseudomonas aeruginosa* P1.S9 provided a fundamental premise for pyocyanin synthesis by revealing the presence of *phz*M and *phz*S genes. The proteins generated from these genes were highly compatible with two enzymes involved in the pyocyanin production pathway. During the optimization, the maximum level of pyocyanin produced was 29.057±0.691 µg mL^{-1}. The concentration was obtained using a modified King's A medium incubated at 27°C within four days. To assess its purity, the chemical structure of pyocyanin was confirmed by several spectroscopic techniques including UV-Visible (UV-Vis), Fourier Transform Infrared (FT-IR), and Nuclear Magnetic Resonance (^{1}HNMR). All test results closely resemble purified pyocyanin compared to several prior studies. In terms of antimicrobial activity, pyocyanin was effective against ATCC strains of *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Its strongest minimum inhibitory concentration (MIC) was 62.5 µg mL^{-1} against *Bacillus subtilis*. Subsequently, the accumulation of reactive oxygen species (ROS) as a major mechanism of pyocyanin antibacterial activity has also been verified. The bacterial pathogens cells treated with pyocyanin displayed a brighter luminescence compared to the control without pyocyanin after the addition of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA). Ultimately, the present work comprehensively characterized pyocyanin's promising producer and antibacterial properties.

Keywords: Antibacterial, *Pseudomonas aeruginosa*, pyocyanin, ROS accumulation, sponge

INTRODUCTION

Antimicrobial resistance (AMR) has been a global concern due to its notable escalation over the last few years. Salam et al. (2023) have recently outlined the increasing number of AMR casualties from 1.3 million in 2019 to 5 million in 2022, and even projected to be 10 million in 2050. However, this alarming condition is not balanced by the rate of antimicrobial discovery, which has slowed down in recent decades (Claudel et al. 2020). Therefore, exploration to find more effective antimicrobial compounds still needs to be conducted.

Bioprospection of microorganisms as antimicrobial producers can be an alternative solution to combat resistance. However, several important variables need to be considered, such as the microorganism isolation source (Modolon et al. 2020). Many researchers agree that microorganism isolation sources should be directed toward extreme environments, such as deserts, polar regions, volcanoes, and the deep sea (Sayed et al. 2020). In these environments, the stresses faced by microorganisms are very diverse, including high level of pressure, temperature, salinity, toxic element, and competition. These factors are strongly thought to be the main reason for the expression of potential bioactive compounds, including antimicrobials (Giordano 2020).

Sponges are marine invertebrates that are closely associated with microorganisms in producing bioactive compounds. Sponges are sessile organisms that obtain food by filtering organic matter that passes through certain parts of their bodies. These parts are ostia (inhalant canals), spongocoel (a large cavity located in the center of the sponge), and oscula (exhalant canals) (Dahihande and Thakur 2019). In the spongocoel area, mesohyl tissues are the primary habitat of sponge-associated microorganisms. These microorganisms comprise about 40-60% of the sponge biomass, with the dominant constituents being bacteria (Rante et al. 2022). Sponge-associated bacteria are known to generate diverse bioactive substances including polyketides, alkaloids, macrolides, and pigments (Brinkmann et al. 2017; Sholekha et al. 2024).

Pyocyanin is a pigment produced by *Pseudomonas aeruginosa*. This greenish-blue compound has been widely considered as an identifier for the bacterium since it's produced by almost all strains (90-95%). The formation of pyocyanin begins with chorismic acid as the precursor, followed by several enzyme-based modifications to form pyocyanin (Goncalves and Vasconcelos 2021). In addition

to genetically-based process, the formation of pyocyanin can also be altered by environmental parameters, for instance temperature and nutrients. These factors are responsible for modulating the Quorum Sensing mechanism (QS). QS is a bacterial intercellular communication mediated by small signaling molecules to regulate gene expression, including genes involved in pyocyanin biosynthesis (Duddy and Bassler 2021).

Chemically, pyocyanin belongs to the class of phenazine compounds (heterocyclic compounds with certain side chains around the ring), with a molecular weight of 210 g mol^{-1} (Marey et al. 2024). At pH 7, pyocyanin is a zwitter-ion, a condition that allows the presence of positive and negative charges in a compound simultaneously (Jablonska et al. 2023). Beyond being distinctive features, the previously mentioned characteristics are also very important in determining the virulence aspect of pyocyanin. They facilitate pyocyanin's permeation into the target cells (Jabbar et al. 2020). Inside the cell, pyocyanin behaves as a redox-active compound and leads to the build-up of Reactive Oxygen Species (ROS). Excessive quantities of ROS induce oxidative stress and ensuing the cell death (Mudaliar and Prasad 2024). In general, this is the main mechanism of pyocyanin's biological activities, especially in terms of antimicrobials. Several investigations have reported the efficacy of pyocyanin against varieties of clinical pathogens such as *Escherichia coli*, *Klebsiella* sp., *Salmonella* sp., and *Staphylococcus aureus* (Dange et al. 2019). Our previous work has reported the antimicrobial activity of *P. aeruginosa* P1.S9 crude extract but has yet to focus on pyocyanin (Wahyudi et al. 2022). Therefore, the current study aimed to optimize the production, to chemically characterize the structure, and to test the antimicrobial activity of pyocyanin produced by *P. aeruginosa* P1.S9.

MATERIALS AND METHODS

Materials

Pseudomonas aeruginosa P1.S9 (Accession Number: PP974651) was isolated from the sponge *Hyrtios* sp. collected in Pramuka Island, Kepulauan Seribu, Jakarta, Indonesia, (5°44'46.3 S 106°36'35.7 T). This study used the bacterium for the first time as a source of pyocyanin pigments. The bacterium was routinely cultured on seawater complete medium (1 g L^{-1} yeast extract, 3 mL L^{-1} glycerol 85%, 5 g L^{-1} peptone, and 20 g L^{-1} agar, dissolved in a mixture of 250 mL distilled water and 750 mL seawater) (Rini et al. 2017). On the other hand, the microbial pathogens employed for the antimicrobial test consist of Bacillus subtilis ATCC 19659, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 15442, *Staphylococcus aureus* ATCC 6538, and *Candida albicans* ATCC 10231. They were grown routinely on Luria-Bertani (LB) medium (5 g L^{-1} yeast extract, 10 g L-1 NaCl, 10 g L-1 tryptone, and 20 g L^{-1} agar, dissolved in 1000 mL distilled water).

Bacterial characterization

P. aeruginosa P1.S9 was grown on seawater complete agar and broth. The incubation period was 96 hours at 27°C. After the incubation period, some characteristics of bacterial morphology were observed, such as colony size, elevation, margin, and pigmentation.

*phz*M and *phz*S genes analysis

The bacterium was rejuvenated in tryptic soy broth for 16-18 hours at 27°C. The detection of *phz*M and *phz*S genes began with genomic extraction using the Presto™ Mini gDNA Bacteria Kit (according to the manufacturer's instructions). The PCR mixture for each gene consists of 4 µL of each primer (10 pmol), 4 µL of genomic DNA (~100 ng), 13 µL of nuclease free-water, and 25 µL MyTaq HS Red Mix 2x. The primer used for *phz*M was *phz*M-F (5'-ATG GAG AGC GGG ATC GAC AG-3') and *phz*M-R (5'-ATG CGG GTT TCC ATC GGC AG-3') (Fazeli and Momtaz 2014), while the primer used for *phz*S was *phz*S-F (5'-TGC GCT ACA TCG ACC AGA G-3') and *phz*S-R (5'-CGG GTA CTG CAG GAT CAA CT-3') (Nowroozi et al. 2012). PCR was conducted by the following protocol: pre-denaturation (96°C, 5 min), denaturation (94°C, 30 s), annealing (60°C, 30 s), elongation (72°C, 1 min (*phz*M), 30 s (*phz*S)), and post-elongation (72°C, 4 min). Denaturation, annealing, and elongation steps were performed in 30 cycles. After PCR, amplicons were verified using agarose gel (1%, w/v) and sequenced (Apical Scientific, Malaysia). In-depth analysis for *phz*M and *phz*S proteins consists of identification using BlastX, phylogenetic tree construction using MEGA11, and domain identification using InterPro.

Optimization of pyocyanin production

Optimization was carried out in two stages. The first stage aimed to select the optimum combination of media and temperature. There are three media (King's A, King's B, and seawater complete) and two temperatures (27°C and 37°C) evaluated for their effects on pyocyanin production. Meanwhile, the second stage focus on media components, such as carbon sources (starch, glycerol, fructose, and sucrose), nitrogen sources (peptone, yeast extract, tryptone, and ammonium sulfate), and combination of metal ion-inorganic salt sources (K_2SO_4-$MgCl_2$, K_2HPO_4-$CuSO_4$, K_2HPO_4-$FeSO_4$, and NaCl-$FeSO_4$). Each treatment for both optimization stages was repeated three times. Some conditions were kept constant, such as inoculum (2% (v/v), OD_{600}=1), static condition, and incubation period of 96 hours under dark condition. After the incubation, the bacterial culture was centrifuged at 4000 rpm at 25°C for 15 minutes. The supernatant was separated and then mixed with chloroform (supernatant: chloroform, 1:1). Pyocyanin dissolved in chloroform (blue phase) was purified by the addition of 0.2 M HCl (supernatant: HCl, 5:1). The absorbance of resulting red phase was recorded at 520 nm and then multiplied by 17.072 to determine the estimated concentration of pyocyanin (Essar et al. 1990).

Production of pyocyanin

The production method refers to Feghali and Nawas (2018). First, the optimum condition in the optimization stage was used to grow *P. aeruginosa* P1.S9. After incubation, the bacterial culture was centrifuged at 4000 rpm at 25°C

for 15 minutes. Then, the supernatant was separated and mixed with chloroform (supernatant: chloroform, 1:1). Pyocyanin dissolved in chloroform (blue phase) was purified by the addition of 0.2 M HCl (supernatant: HCl, 5:1). Subsequently, the red phase then titrated with 0.2 M NaOH until the color changed from red to blue. Finally, the solution was redissolved in chloroform (ratio 1:1) before evaporated at 40°C to obtain the pyocyanin extract (Hamad et al. 2020).

Characterization of pyocyanin

Pyocyanin from *P. aeruginosa* P1.S9 was characterized using several instruments, including UV-Vis (Metertech SP-8001, United Kingdom), FT-IR (Bruker Tensor II, Germany), and ^{1}HNMR ("1" in ^{1}HNMR refers to hydrogen atomic number) (Bruker Ascend, Germany). The red phase, obtained from HCl 0.2 M addition, was observed at 200-800 nm using UV-Vis (Darwesh et al. 2019). Meanwhile, the dark blue extract of pyocyanin was used for functional group analysis using FT-IR at the wavenumber range between 4000-500 cm^{-1} (Moayedi et al. 2018). Then, the same extract was also characterized using ^{1}HNMR to elucidate the chemical structure (El-Fouly et al. 2015). All data collected from the characterization process were compared with previous studies about pyocyanin's chemical properties.

Antimicrobial test of pyocyanin using the disc diffusion method

The test microbes were inoculated into Mueller Hinton Agar for bacteria and Potato Dextrose Agar for fungi (1% (v/v), OD_{625}=0.1). Each paper disc was injected with 20 µL pyocyanin extract (1000 µg mL^{-1}) and placed on the media surface. Each treatment was repeated three times. The dishes were kept at 27°C for 24-48 hours. The positive controls used were 100 µg mL^{-1} tetracycline for bacteria and 100 µg mL^{-1} nystatin for fungi, while the negative control was DMSO 10% (v/v) (Mesrian et al. 2021).

Determination of minimum inhibitory concentration (MIC)

The MIC value was determined using the micro broth-dilution method (CLSI 2020). The microbial suspension was adjusted to reach 10^8 CFU mL^{-1} (OD_{625}=0.1). Next, a mixture of pathogen, media (Mueller Hinton Broth (MHB), and pyocyanin was made in every well of microplate until reaching the final cell concentration of pathogen at 10^6 CFU mL^{-1} and the final concentration of pyocyanin at 1000, 500, 250, 125, 62.5, 31.25, 15.625, and 7.812 µg mL^{-1}. The final volume for each treatment was 200 µL and all of them were repeated three times. Tetracycline at the same concentration as the sample was used for positive controls, while 10% DMSO was used as a negative control. The microplates were kept at 37°C for 24-48 hours. MIC is defined as the lowest concentration of extract that can suppress bacterial growth, indicated by clear media in the well.

Evaluation of reactive oxygen species (ROS) accumulation

The ROS accumulation was detected qualitatively using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Bhargava et al. 2014). The test microbes (OD_{625}=0.1) were mixed with pyocyanin at the MIC concentration and incubated at 27°C for 2 hours. The control was left without the addition of pyocyanin. After incubation, the culture was centrifuged at 8000 g for 10 minutes at 4°C. The pellet was dissolved in phosphate buffer (10mM). Then, H_2DCF-DA (5 µM) was added and the mixture was kept for 30 minutes at 27°C under dark condition. ROS will oxidize H_2DCF-DA and emit light when observed under the fluorescent microscope. Brighter luminescence indicated more ROS generation.

RESULTS AND DISCUSSION

Bacterial characterization

Colonies of *P. aeruginosa* P1.S9 were characterized by irregular margins, umbilicated elevation, and ±2-3 mm size, after four days of incubation on seawater complete agar. Additionally, noticeable pigmentation with a greenish-blue color and a pungent wine-like odour was also identified on both seawater complete agar and broth. The detailed characteristics are shown in Figure 1.

***phz*M and *phz*S genes analysis**

Partial sections of *phz*M and *phz*S genes from *P. aeruginosa* P1.S9 were successfully detected using gel electrophoresis. The band sizes were ±875 bp and ±664 bp, respectively (Figure 2). Analysis of these genes using BlastX revealed their similarity with enzymes involved in the pyocyanin production pathway. *phz*M and *phz*S showed the closest similarity with phenazine-1-carboxylate N-methyltransferase and flavin-dependent oxidoreductase, respectively. The detailed results are shown in Table 1 and Figure 3.

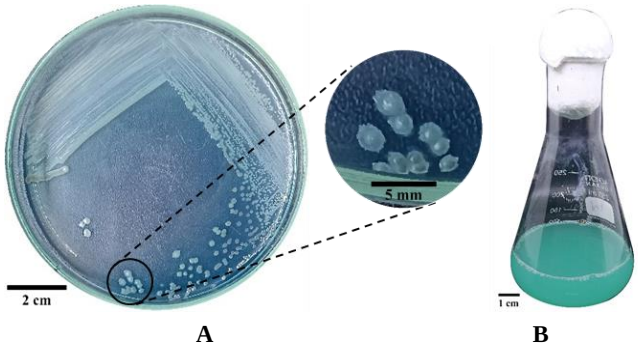


Figure 1. Morphological characteristics of *P.aeruginosa* P1.S9 after four days of incubation at 27°C. A. Colonies on seawater complete agar; B. Culture in seawater complete broth

In order to describe their activity, domains of *phz*M and *phz*S protein complete sequence were analyzed using the protein classification website InterPro. Reference sequences were obtained from RCSB Protein Data Bank (RCSB PDB) (RCSB PDB ID: 2IP2 for *phz*M and 3C96 for *phz*S). For *phz*M, the reference confirmed the presence of two domains, namely Acetylserotonin O-methyltransferase dimerization (ASMT-dimerization) (IPR031725) and O-Methyltransferase (IPR001077) (Figure 4). The protein sequence of *phz*M from *P. aeruginosa* P1.S9 had a linear results but only included full domain of O-methyltransferase and some part of the acetylserotonin O-methyltransferase dimerisation (ASMT-dimerisation). Meanwhile, for *phz*S, the reference protein contains two identical domains, namely FAD-binding (IPR002938) (Figure 5). The *phz*S protein from *P. aeruginosa* P1.S9 had some similarities with reference, although it only covered part of a FAD-binding domain.

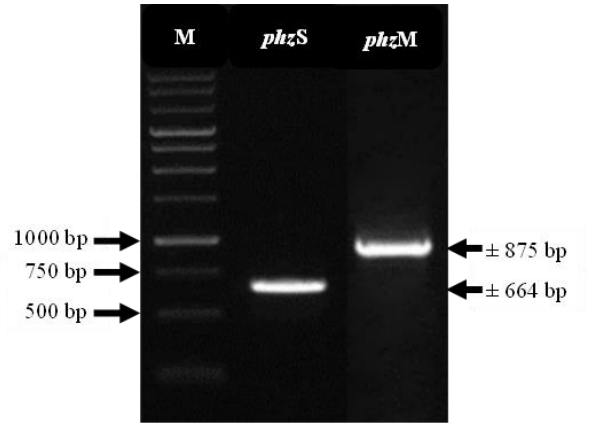


Figure 2. PCR amplification of partial *phz*M (±875 bp) and *phz*S (±664 bp) genes from *P. aeruginosa* P1.S9 detected using agarose gel electrophoresis (M: marker 1 Kb ladder)

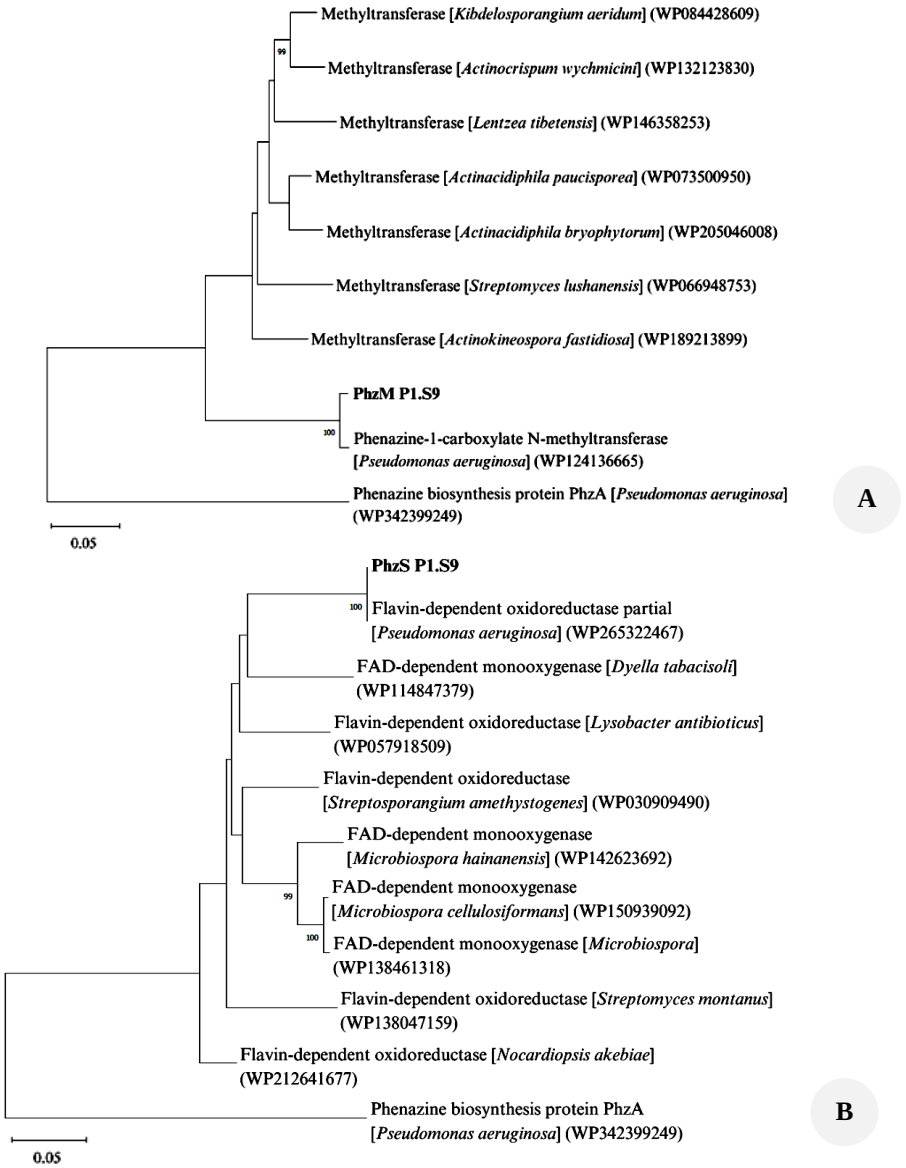


Figure 3. Phylogenetic tree of *phz*M (A) and *phz*S (B) protein compared to several related sequences obtained from BlastX analysis result. The tree was generated by neighbour-joining method with 1000× bootstrap on MEGA11. PhzA that has different function with *phz*M and *phz*S in the pyocyanin synthesis pathway was used as outgroup

Optimization of pyocyanin production

The optimization process was separated into two stages. The first stage showed the best combination of King's A medium at 27°C for pyocyanin production. The concentration was 16.179±0.517 µg mL^{-1} (Figure 6). Consequently, King's A composition was used as a reference for the second stage. This stage focused on media composition to enhance the pyocyanin production. The components tested included carbon, nitrogen, and metal ion-inorganic salt sources. Based on Figure 7, the carbon source with the highest pyocyanin production was starch (29.057±0.691 µg mL^{-1}), followed by glycerol and sucrose. Meanwhile, for nitrogen sources and metal ion-inorganic salt sources, the optimum results were still obtained by peptone and K_2SO_4-$MgCl_2$ as the essential constituents of King's A medium.

Table 1. Identification of partial *phz*M and *phz*S genes from *Pseudomonas aeruginosa* P1.S9 using BlastX

Gene	Closest similarity	Percent identification (%)	Query cover (%)	E-value	Accession number
*phz*M	Phenazine-1-carboxylate N-methyltransferase (*P. aeruginosa*)	99.64	94	0.00	WP_124136665
*phz*S	Flavin-dependent oxidoreductase (*P. aeruginosa*)	99.52	94	6e-148	WP_265322467

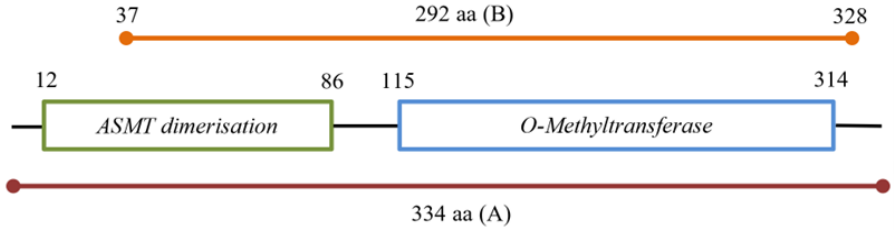


Figure 4. Predicted domains of *phz*M complete sequences (RCSB PDB ID: 2IP2, 334 amino acids) (A) and partial sequence of *phz*M from *Pseudomonas aeruginosa* P1.S9 (292 amino acids) (B)

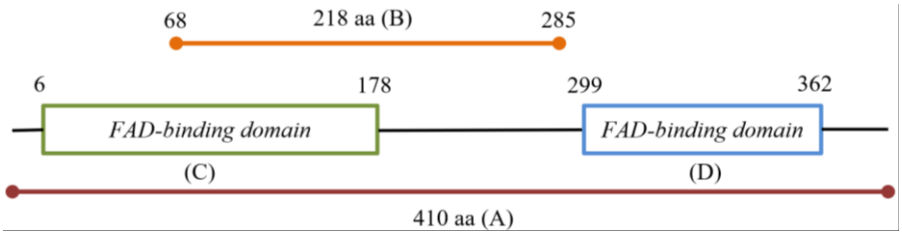


Figure 5. Predicted domains of *phz*S complete sequences (RCSB PDB ID: 3C96, 410 amino acids) (A) and partial sequence of *phz*S from *Pseudomonas aeruginosa* P1.S9 (218 amino acids) (B)

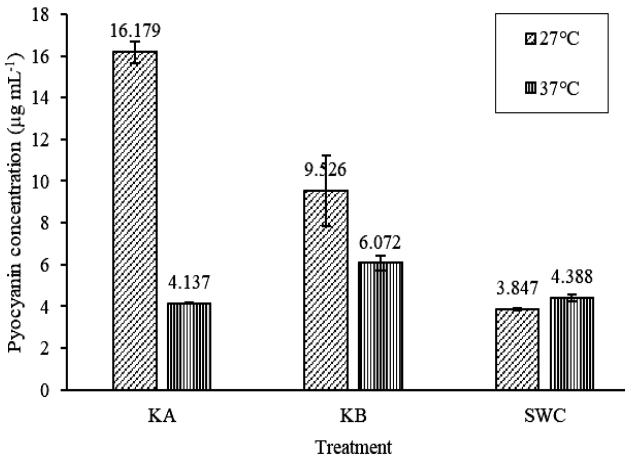


Figure 6. The effect of various media and temperatures on pyocyanin production in *Pseudomonas aeruginosa* P1.S9. Notes: KA: King's A; KB: King's B; SWC: seawater complete

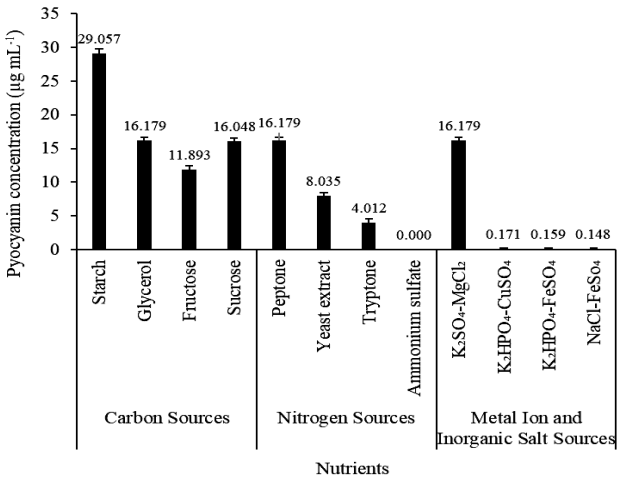


Figure 7. The effect of various media components on pyocyanin production in *Pseudomonas aeruginosa* P1.S9

Production and characterization of pyocyanin

The pyocyanin extract from *P. aeruginosa* P1.S9 was dark blue in color and soluble in DMSO. The red phase's maximum absorption (resulting from addition of HCl) was 279, 389, and 522 nm (Figure 8.A). Meanwhile, the FT-IR analysis indicated the occurrence of some functional groups such as O-H (3550-3250 cm^{-1}), C-H (3100-2850 cm^{-1}), C=N (1690-1640 cm^{-1}), C=C (1625-1440 cm^{-1}), C-N (1250-1000 cm^{-1}), and C-H bend (900-680 cm^{-1}) (Figure 8.B). Finally, the ^{1}HNMR spectra confirmed the existence of several pyocyanin identifiers at 3.3-4.3 and 7-9 ppm (Figure 8.C).

Bioactivity of pyocyanin

The disc diffusion assay exhibited the inhibitory effect of pyocyanin against ATCC strain of *B. subtilis*, *E. coli*, *P. aeruginosa*, and *S. aureus* (Figure 9). The largest zone of inhibition was 14.3 mm against *S. aureus* ATCC 6538. In contrast to bacteria, the antifungal test using *C. albicans* ATCC 10231 did not obtain a significant result, as no zone of inhibition was observed. Meanwhile, pyocyanin's most potent minimum inhibitory concentration (MIC) was 62.5 µg mL^{-1} against *B. subtilis* ATCC 19659. The detailed result of MIC determination test is shown in Figure 10.

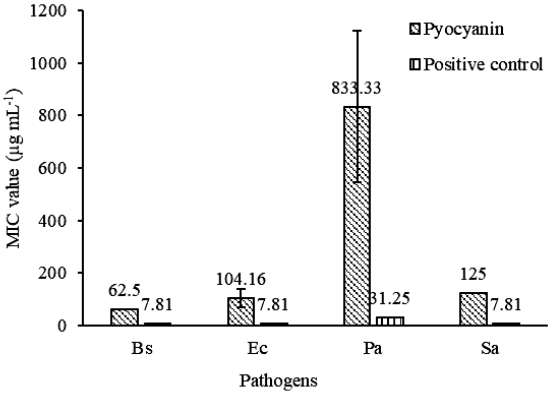
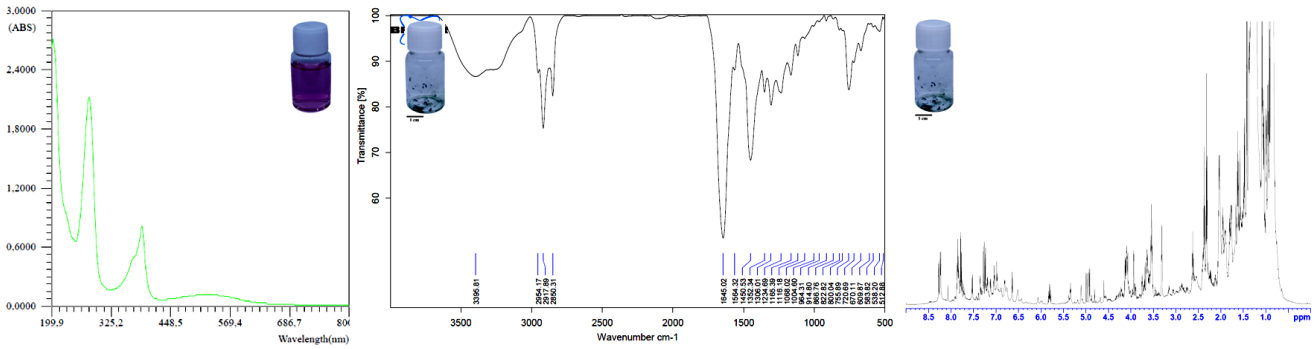


Figure 10. MIC value of pyocyanin produced by *P. aeruginosa* P1.S9 against several bacterial pathogens. Notes: Bs: *Bacillus subtilis* ATCC 19659; Ec: *Escherichia coli* ATCC 8739; Pa: *Pseudomonas aeruginosa* ATCC 15442; Sa: *Staphylococcus aureus* ATCC 6538; the positive control was tetracycline



A **B** **C**

Figure 8. Chemical characterizations of pyocyanin extract from *P. aeruginosa* P1.S9 using several spectroscopic techniques including: A. UV-Visible (UV-Vis); B. Fourier Transform Infrared (FT-IR); C. Nuclear Magnetic Resonance (^{1}HNMR)

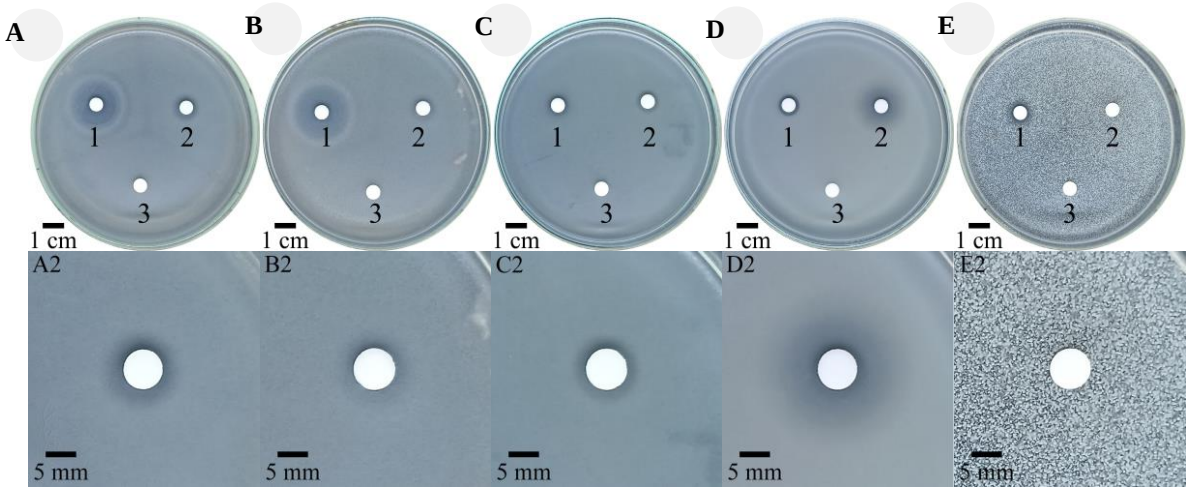


Figure 9. Antimicrobial assay of pyocyanin (1000 µg mL^{-1}) against: A. *Bacillus subtilis* ATCC 19659; B. *Pseudomonas aeruginosa* ATCC 15442; C. *Escherichia coli* ATCC 8739; D. *Staphylococcus aureus* ATCC 6538; E. *Candida albicans* ATCC 10231, 1: The positive control was tetracycline 100 µg mL^{-1} for bacteria and nystatin 100 µg mL^{-1} for Fungi; 2: Pyocyanin sample (A2-E2 images was zoom version from each petri dish); 3: The negative control was DMSO 10%

Evaluation of reactive oxygen species (ROS) accumulation

ROS accumulation was successfully investigated as a major mechanism of pyocyanin antibacterial activity. Brighter fluorescence by bacterial pathogens cells was obtained after addition of pyocyanin compared to controls without the pigment. Figure 11 depicted the detail results for each treatment.

Discussion

In this study, *P. aeruginosa* P1.S9 was used as a single isolate. The bacterium exhibited several features to affirm its capability of generating pyocyanin, two of them were the vibrant blue-green pigmentation and pungent grape-fruity aroma. Although very helpful for the initial identification, morphological characteristics of a bacterium could not stand as the only reason for secondary metabolites production. Therefore, genetically-based verification was also conducted by detection and in-depth analysis of *phz*M and *phz*S genes. The *phz*M and *phz*S genes are essential in the pyocyanin production pathway (Dong et al. 2020). *phz*M gene will be translated into phenazine-1-carboxylate N-methyltransferase, which acts on the methylation of phenazine-1-carboxylic acid to produce 5-methylphenazine-1-carboxylic acid betaine (MPCBA).

Phenazine-1-carboxylate N-methyltransferase contains two domains, Acetylserotonin O-methyltransferase dimerization, which mainly works to form a dimer structure and O-Methyltransferase that works to bind S-adenosyl methionine as a methyl donor (Botros et al. 2013; Qiu et al. 2021). Afterward, *phz*S gene will be translated into flavin-dependent oxidoreductase that responsible for hydroxylation of MPCBA to generate pyocyanin. Flavin-dependent oxidoreductase contained two identical FAD-binding domains. FAD (flavin adenine dinucleotide) is an important coenzyme that acts for accepting and carrying electrons in hydroxylation process (Amjad et al. 2021).

Besides the genetic factor, pyocyanin production is also influenced by environmental conditions such as temperature and nutrient. Both of them contribute to iniating the quorum sensing mechanism (QS). As mentioned previously, QS is a bacterial intercellular communication mediated by small signaling molecules to regulate gene expression (Duddy and Bassler 2021). In this case, *phz*M and *phz*S genes activation is influenced by QS.

In two stages of pyocyanin optimization, temperatures and nutrients were used as treatment. The highest pyocyanin obtained was 29.057±0.691 µg mL^{-1} using a combination of modified Kings A medium (starch as carbon source), incubated at 27°C. This amount exceeded reports from Narayani et al. (2021) (±13 µg mL^{-1}) and Dange et al. (2019) (±6 µg mL^{-1}). Besides the pyocyanin concentration, the optimum condition of carbon source and temperature was also very interesting to be discussed.

Abdelaziz et al. (2022) found cetrimide as the best carbon source for pyocyanin production, while Gahlout et al. (2021) reported differently with mannitol as the best. Surprisingly, starch was the best carbon source compared to glycerol, fructose, and sucrose in this study. This unique characteristic of *P. aeruginosa* P1.S9 may open an opportunity for using a low-cost medium for pyocyanin production, such as potato, corn, and cassava (Bandara and Arunakumara 2020). Similar to carbon source, the optimum growth temperature of *P. aeruginosa*-producing pyocyanin also can be varied from 4-42°C (Diggle and Whiteley 2020). The fact that our study found a *P. aeruginosa* strain, which produces the highest amount of pyocyanin at 27°C (room temperature), can be valuable for industry. It can lower energy consumption and increase pyocyanin production efficiently (Sullivan and Spencer 2022).

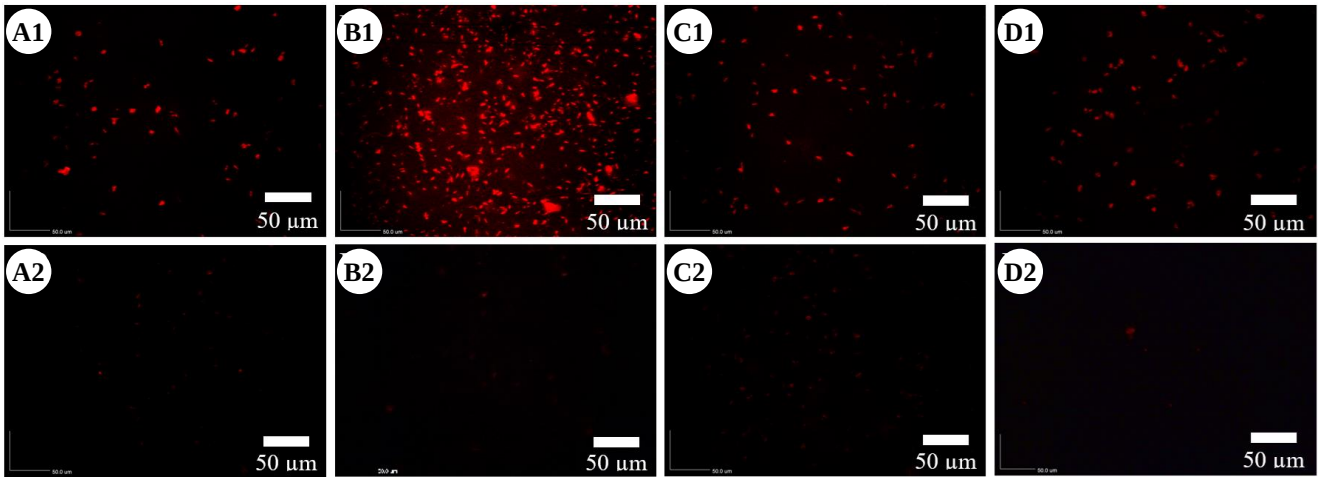


Figure 11. ROS accumulation assay of A. *Bacillus subtilis* ATCC 19659; B. *Escherichia coli* ATCC 8739; C. *Pseudomonas aeruginosa* ATCC 15442; D. *Staphylococcus aureus* ATCC 6538; A1-D1: treated with pyocyanin; A2-D2: control without pyocyanin

Pyocyanin extracted from *P. aeruginosa* P1.S9 showed a paste-texture characteristic and dark-blue color. According to the UV-Vis spectrophotometer, the maximum absorption wavelengths from the red phase were 279, 389, and 522 nm, consistent with the pyocyanin extract from *P. aeruginosa* Osh1 studied by Darwesh et al. (2019), with the maximum absorption at 280, 385, and 525 nm. Next, FT-IR characterization also showed several bonds according to pyocyanin standards (Moayedi et al. 2018). These bonds were O-H (3550-3250 cm^{-1}), C-H (3100-2850 cm^{-1}), C=N (1690-1640 cm^{-1}), C=C (1625-1440 cm^{-1}), C-N (1250-1000 cm^{-1}), and C-H bend (900-680 cm^{-1}). Then, the analysis of ^{1}HNMR resonance spectra showed areas in the range of 3.3-4.3 and 7-9 ppm as the pyocyanin identifier. These areas indicate the presence of methyl-nitrogen bond and aromatic rings, respectively (El-Fouly et al. 2015). Based on these positive characterization results, we confidently defined the extract as a pyocyanin.

Pyocyanin is a major virulence factor of *P. aeruginosa*. The pigment easily across the cell membrane with its low molecular weight and zwitter-ion characteristic. Inside the cell, pyocyanin induces oxidative stress by raising the amount of several ROS, such as hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot-}$). Consequently, the DNA will be damaged, several important enzymes production wil be inhibited, and membrane formation will be disrupted (Mudaliar and Prasad 2024). To overcome auto-poisoning, the pyocyanin producing-*P. aeruginosa* upregulate the production of detoxifying enzymes. *P. aeruginosa* produce more superoxide dismutase, catalase, and Fe-S cluster to replace the previous one damaged by ROS accumulation (Meirelles and Newman 2018).

In this research, the ROS accumulation has been successfully evaluated using 2',7'-dichlorodihydrofluorescein diacetate, which automatically confirms the extract purity as pyocyanin and its potential as an antimicrobial agent. At 1000 µg mL^{-1}, pyocyanin was effective against several bacterial pathogens such as *S. aureus* ATCC 6538, *B. subtilis* ATCC 19659, *E. coli* ATCC 8739, and *P. aeruginosa* ATCC 15442. The zone of inhibition and MIC value varied from 9.67-14.33 mm and 62.50-833.33 µg mL^{-1}, respectively. The results were in tight accordance with Marey et al. (2024) (MIC value of pyocyanin against *Bacillus*, *Staphylococcus*, and *Streptococcus* species ranged from 31.25-125 µg mL^{-1}) and Shouman et al. (2023) (MIC value of pyocyanin against *S. aureus, Streptococcus pyogens*, and *Streptococcus agalactiae* were ranging from 100-150 µg mL^{-1}). In contrast to bacteria, there was no zone of inhibition against *C. albicans* ATCC 10231. The result was similar to a report by Abuohashish and El-Sharkawy (2023). Allegedly, it was caused by the presence of the AINT1 gene, resulting in a more vigorous resistance level in *C. albicans* (Khudhair et al. 2021).

In conclusion, *P. aeruginosa* P1.S9 was molecularly proven to be a promising producer of pyocyanin. The extract has been characterized by several instruments, and the results closely resemble several prior studies on pyocyanin's chemical properties. In terms of antimicrobial activity, the pyocyanin was effective against various bacterial pathogens via ROS accumulation.

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