MINISTRY
OF EDUCATION

VIETNAM ACADEMY OF
SCIENCE AND TECHNOLOGY

GRADUATE UNIVERSITY OF SCIENCE AND TECHNOLOGY

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RESEARCH ON CREATING A RECOMBINANT *PSEUDOMONAS AERUGINOSA* STRAIN TO ENHANCE PYOCYANIN PRODUCTION FOR APPLICATIONS IN AQUACULTURE

SUMMARY OF DOCTORAL THESIS IN APPLIED BIOLOGY

Major: Biotechnology
Code: 9 42 02 01

Hanoi - 2025

The dissertation is completed at: Graduate University of Science and Technology, Vietnam Academy Science and Technology

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The dissertation is examined by Examination Board of Graduate University of Science and Technology, Vietnam Academy of Science and Technology on … , …. , 2025

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INTRODUCTION

1. The urgency of the dissertation

Pyocyanin is a natural bioactive compound produced by the Gram-negative bacterium *Pseudomonas aeruginosa*. With its antibacterial properties, low toxicity to users, and natural degradability, pyocyanin has numerous applications in biological control, particularly in the field of aquaculture. The use of pyocyanin extracted without bacterial cell debris is an approach that can eliminate the risk of infection from *Pseudomonas aeruginosa*. Therefore, pyocyanin is a microbial bioactive compound with potential applications as an antibacterial agent to mitigate and replace the overuse of antibiotics in aquaculture. However, the yield of pyocyanin production from natural bacterial strains is still limited. Therefore, enhancing the biosynthetic capacity of pyocyanin and developing high-yield bacterial strains is an area of research interest. In this study, the enhancement of expression of key genes involved in pyocyanin synthesis in *Pseudomonas aeruginosa* was employed to create strains with high pyocyanin production efficiency. Two genes, phzM and phzS, were cloned from a natural strain of bacteria with high pyocyanin production and inserted into the vector pUCP24, under the transcriptional control of the Lac promoter. This construct was then transformed back into the selected bacterial strain to enhance the expression of these two genes. The recombinant bacterial strain was cultivated under various conditions and environments to select the optimal conditions for increasing pyocyanin production. The pyocyanin obtained from the recombinant strain has demonstrated potential applications in aquaculture through assessments of its antibacterial efficacy against various test microorganisms, including pathogenic *Vibrio* species affecting shrimp, such as *V. parahaemolyticus* and *V. harveyi*.

Based on the urgent demands of the current situation and the aforementioned approaches, the research topic titled **"Research on creating**

a recombinant *Pseudomonas aeruginosa* strain to enhance pyocyanin production for applications in aquaculture" has been conducted with specific objectives and research content as follows:

2. Research Objectives

- Isolate a *Pseudomonas aeruginosa* strain capable of producing pyocyanin and develop a recombinant strain of *Pseudomonas aeruginosa* carrying the genes *phzM* and *phzS* to enhance pyocyanin synthesis.
- Investigate methods for extracting and recovering pyocyanin and determine the characteristics of the obtained pyocyanin.
- Evaluate the antibacterial activity of pyocyanin against test microorganisms and its efficacy against pathogenic *Vibrio* species affecting shrimp, with a focus on applications in aquaculture.

3. Main research contents

- Isolation of bacterial strains capable of producing pyocyanin from aquatic products and sediments and water in aquaculture pond;
- Research on the development of recombinant *P. aeruginosa* strain PS39-phzMS carrying additional *phzM* and *phzS* genes to enhance pyocyanin synthesis;
- Investigation of methods for the extraction and purification of pyocyanin from *P. aeruginosa* PS39-phzMS;
- Study of the antibacterial properties of pyocyanin;
- Research on selection of culture conditions to optimize pyocyanin biosynthesis by *P. aeruginosa* PS39-phzMS.

CHAPTER 1. OVERVIEW

1.1. *Pseudomonas aeruginosa* and pyocyanin

Pseudomonas aeruginosa can exhibit various colony morphologies. Most colonies are flat and spread across the surface of agar, typically producing two soluble pigments: pyocyanin, which imparts a blue color to the colonies, and pyoverdin, also known as a fluorescent pigment, which gives the colonies a yellow-green hue. When *P. aeruginosa* produces both pigments simultaneously, the colonies appear blue-green. Additionally, this bacterium can produce other water-soluble pigments such as pyorubrin and pyomelanin, resulting in red or brown colonies, respectively. Pyocyanin is a secondary metabolite, a nitrogen-containing heterocyclic compound, and it is characteristically blue-green. It is produced by the Gram-negative bacterium *Pseudomonas aeruginosa*. Pyocyanin has been applied in aquaculture disease treatment to reduce antibiotic resistance in bacteria and decrease residue levels in seafood products. Research on the antibacterial properties of pyocyanin isolated from marine environments has demonstrated that nearly 90-95% of the antibacterial activity of *P. aeruginosa* strains can be attributed to pyocyanin. Pyocyanin exhibits antibacterial effects against pathogenic bacteria such as *Salmonella paratyphi, Escherichia coli*, and *Klebsiella pneumoniae*.

1.2. Transfer the gene encoding the protein involved in the pyocyanin biosynthesis process

The genes located within the locus responsible for phenazine biosynthesis have been identified and sequenced from *P. fluorescens* and *P. aeruginosa* strains. This locus comprises the PhzABCDEFG operon, which includes seven genes that play a crucial role in converting chorismic acid into PCA (phenazine-1-carboxylic acid). Both *P. aureofaciens* and *P. fluorescens* contain a single PhzABCDEFG operon. In contrast, *P. aeruginosa* possesses two operons, *Phz1* (PhzA1B1C1D1E1F1G1) and *Phz2* (PhzA2B2C2D2E2F2G2), each

encoding enzymes for PCA synthesis. This dual operon system in *P. aeruginosa* results in a more efficient PCA biosynthesis compared to other bacterial strains. Consequently, the increased PCA biosynthesis capability in *P. aeruginosa* leads to a significantly higher pyocyanin production, as PCA serves as the central precursor for pyocyanin synthesis. Pyocyanin is synthesized from the precursor PCA through two synthesis steps mediated by two enzymes encoded by the *PhzM* and *PhzS* genes. In this process, PhzM appears to catalyze the conversion of PCA to 5-methyl-PCA initially, followed by the subsequent transformation involving PhzS to synthesize pyocyanin. In *Pseudomonas aeruginosa*, the biosynthesis of pyocyanin requires the sequential involvement of both enzymes, PhzM and PhzS. This forms the basis for enhancing pyocyanin production through gene transfer methods. Developing new vector strains capable of stable replication in various bacterial strains is crucial for facilitating genetic research. The plasmid vector pUCP24, derived from pUCP18/19, is capable of carrying functional genes and replicating in both hosts, *Escherichia coli* and *Pseudomonas aeruginosa*, making it an essential tool for future genetic studies.

1.3. Factors affecting pyocyanin biosynthesis by *Pseudomonas aeruginosa*

The KingA medium is a classic environment optimized for *P. aeruginosa* to synthesize pyocyanin, as it suppresses the production of other pigments by this bacterium. Supplementing components such as the amino acid alanine and glycerol into the KingA medium has proven highly effective in enhancing pyocyanin synthesis efficiency. Some authors have applied optimization methods using statistical models to identify key factors influencing the pyocyanin synthesis process by *P. aeruginosa* and determine optimal conditions to maximize this process. The addition of solvents and surfactants has been demonstrated to increase pyocyanin production by *P.*

aeruginosa. Fermentation methods supplemented with solvents and surfactants can effectively extract secondary metabolites. Various types of supplements are used in optimizing the pigment synthesis process of *P. aeruginosa*. Besides basic environmental components like carbon and nitrogen sources, additional supplements act as inducers or enhancers for this process. Some compounds studied include vegetable oils such as olive oil, crude oil, plant extracts, or by-products from industrial processes.

CHAPTER 2. METHODS

2.1. Materials and chemicals

Samples of aquaculture water, shrimp, and fish collected from January to March 2017 in Quang Ninh, Ninh Binh, and Nam Dinh provinces served as sources for isolating bacterial strains capable of producing pyocyanin. These strains belong to the collection of the Environmental Biotechnology Department at the Institute of Biotechnology, comprising 10 strains designated as PS5, PS8, PS9, PS10, PS12, PS39, PS40, PS41, PS42, and PS43, isolated from water samples and shrimp pond sediments in Quang Ninh and Nam Dinh provinces. The bacterial strains included *Vibrio* spp.: *V. parahaemolyticus* VpKG12T1, *V. parahaemolyticus* VpST22T, *V. parahaemolyticus* VpCMT31, *V. harveyi* Vh3, and *V. alginolyticus* Val. Additionally, *Escherichia coli* strains TOP10, BL21, and Rosetta were used to assess the activity of the phzS and phzM genes in the pUCP24 vector. The pUCP24 plasmid was a gift from Professor Schweizer at the University of Florida, USA.

2.2. Microbial method

2.2.1. Method for isolating pyocyanin-producing bacteria

Aquaculture water and digestive tracts of shrimp and fish were finely ground, diluted, and cultured on KingA agar plates, then incubated at 30°C. After 24 hours, bacterial colonies exhibiting green coloration were observed and selected. These colonies were subcultured continuously to establish pure cultures.

2.2.2. Gram staining and scanning electron microscope imaging method

Gram staining was performed using the modified Hucker method. Bacterial cell morphology was observed and images were recorded under a scanning electron microscope (SEM - Hitachi S-4800, Japan).

2.2.3. Identify bacteria using biochemical methods

The method was performed according to the instructions of the manufacturer of Kit API 20NE (BioMérieux, France).

2.2.4. Identification of species-specific gene PA 956 of P. aeruginosa

Using the specific primer pair PA-F and PA-R, amplification of a 956 bp segment of the PA gene was conducted in isolated bacterial strains. These primers exhibit 100% specificity and sensitivity towards *P. aeruginosa* strains.

2.2.5. Construction of phylogenetic tree

The phylogenetic tree was built using MEGA 7 software using the Neighbor - Joining method, the boostrap value with the number of replications is 1000 times.

2.2.6. Extracellular enzyme activity assays

Culturing media and agar plates for enzyme activity assays were prepared with specific substrates for each extracellular enzyme such as amylase, protease, gelatinase, and lipase. Bacterial inoculants were streaked onto the respective media plates, which were then incubated overnight at 30°C. The experimental results were subsequently observed and recorded.

2.3. Design expression vector and gene expression

2.3.1. Isolation of total bacterial DNA

Total DNA was extracted according to the instructions of the Genomic DNA Purification Kit (Thermo Scientific).

2.3.2. Electrophoresis method

Total DNA, PCR products, plasmids, and restriction digest products are checked and identified on agarose gel with a concentration of 1-2%,

following the method described by Sambrook and colleagues.

2.3.3. Method of cloning and determining phzM and phzS gene sequences

The genes phzM and phzS of the bacterium Pseudomonas aeruginosa PS39 were amplified using the primer pairs phzM-F, phzM-R and phzS-F, phzS-R. The purified DNA of the genes phzM and phzS was ligated into the pJET1.2 vector following the instructions provided in the CloneJET™ PCR Cloning Kit (Fermentas). The recombinant plasmid obtained from E. coli cells was used for gene sequencing using the Sanger sequencing method. The obtained gene sequences of phzM and phzS were compared with other sequences of phzM and phzS genes available on GenBank to assess the degree of similarity, using the BLAST program on NCBI (https://blast.ncbi.nlm.nih.gov/BlastAlign.cgi).

2.3.4. Method to create recombinant plasmid pUCP24-phzMS

The vector pUCP24 was prepared by digestion with the enzyme SmaI. The resulting product after ligation was transformed into *E. coli* Top10 cells following the method described by Sambrook, and plated on LB agar supplemented with 5 µg/mL gentamycin. Subsequently, the pUCP24-phzM vector was digested with the restriction enzymes *Xba*I and *Sph*I. The *phzS* gene was then cut with *Xba*I and *Sph*I restriction enzymes. The purified fragments from these cuts were ligated together using T4 DNA ligase to create the pUCP24-phzMS vector. The resulting recombinant vector was transformed into competent *E. coli* Top10 cells for plasmid propagation and selection. Plasmids were isolated from the transformed cells and further verified by digestion with *Eco*RI and *Hin*dIII restriction enzymes to confirm the presence of phzS. Additionally, the presence of the *phzM* gene was confirmed by PCR amplification using M13F and phzM-R primers. The pUCP24-phzMS vector was sequenced using next-generation sequencing technology to validate the entire *phzMS* operon expression within the vector. The sequence of the vector after sequencing was deposited in the GenBank

database under accession number MZ399165.1.

2.3.5. Testing the expression of phzM, phzS in E. coli

In this study, to assess the expression of the gene in the pUCP24-phzMS vector, we opted for an indirect method through gene expression in E. coli. The recombinant plasmid pUCP24-phzMS was transformed into E. coli BL21 (DE3) and E. coli Rosetta for vector expression analysis. The proteins obtained were subsequently analyzed using SDS-PAGE electrophoresis on polyacrylamide gels.

2.3.6. Creation of recombinant strain P. aeruginosa PS39 carrying plasmid pUCP24-phzMS

P. aeruginosa PS39 competent cells were freshly prepared for transformation by electroporation. Transforming recombinant plasmid pUCP24-phzMS into *P. aeruginosa* PS39 cells by electroporation.

2.3.7. Checking the presence of the recombinant plasmid pUCP24-phzMS in the recombinant strain

The recovered plasmid after extraction was digested with the restriction enzymes *Eco*RI and *Hin*dIII to verify the presence of phzS and PCR amplify the *phzM* gene using the primer pair M13F and phzM-R.

2.3.8. Sequencing the pUCP24-phzMS plasmid to test the phzMS expression cassette using the next-generation sequencing system

2.3.9. Methods of preserving and cultivating strains

2.4. Method for extracting and purifying pyocyanin

2.4.1. Cultivation of P. aeruginosa strain PS39-phzMS to collect fermentation broth for pyocyanin extraction

2.4.2. Method for quantifying pyocyanin

Chloroform was added in bacteria culture (1 mL) with a 1:1 volume ratio (1 part culture: 1 part chloroform). The mixture of culture and chloroform was vigorously shaken and then centrifuged at 5000 rpm for 10 minutes. The lower chloroform phase containing pyocyanin (0.6 mL) was

transferred to a new tube. Subsequently, 0.6 mL of 0.2 M HCl was added to the sample, mixed thoroughly, and allowed to settle for phase separation. The upper aqueous phase, which is red in color, was then collected, and its absorbance was measured at 520 nm. The amount of pyocyanin was determined using the formula: pyocyanin (μg/mL) = OD520 × dilution factor × 17.072, where 17.072 is the absorption coefficient.

2.4.3. PCA extraction and quantification method

The amount of PCA in the bacterial culture medium was determined using the method developed by Mavrodi.

2.4.4. Selecting solvent to extract pyocyanin

Different organic solvents were used for extracting pyocyanin from *P. aeruginosa* PS39-phzMS cultures, including benzene, chloroform, methanol, hexane, dichloromethane, and ethyl acetate.

2.4.5. Methods for determining the purity of pyocyanin

The purity of the pyocyanin extraction fraction was assessed using thin-layer chromatography, high-performance liquid chromatography, and UV-Vis spectroscopy techniques.

2.5. Method for determining the antibacterial properties of pyocyanin

2.5.1. The antimicrobial activity test against pathogenic microorganisms and Vibrio.

2.5.2. Method for determining the minimum bactericidal concentration of pyocyanin

2.5.3. Agar plate diffusion method

2.6. Method to determine culture conditions for recombinant pyocyanin-producing strains

2.6.1. Selecting culture medium

In this study, KingA and modified KingA media were tested to select the optimal environment for pyocyanin synthesis by *P. aeruginosa* PS39-phzMS. The concentration of glutamic acid in the selective medium GM was varied to assess its impact on pyocyanin production.

2.6.2. Methods of studying the effects of culture conditions

The physical factors influencing pyocyanin biosynthesis such as pH, temperature, time, and shaking speed were studied in *P. aeruginosa* PS39-phzMS to enhance pyocyanin production.

2.7. Data analysis method

The data were analyzed using GraphPad Prism 6.01 software, employing ANOVA or t-test with $p < 0.05$ considered statistically significant. Data are presented as mean values and standard deviation (SD) in bar graphs, line graphs, or tables.

CHAPTER 3. RESULTS

3.1. Isolation and screening of pyocyanin-producing *Pseudomonas*

Among the 28 isolated strains (18 newly isolated strains and 10 strains in the collection), we identified 9 strains that exhibited green pigment on KingA medium (Figure 3.1), which turned the medium green. Pyocyanin production was quantified, with concentrations ranging from 6.01±1.2 µg/mL to 15.02±0.56 µg/mL. Strain PS39 showed the highest pyocyanin production capability, reaching 15.02±0.56 µg/mL.

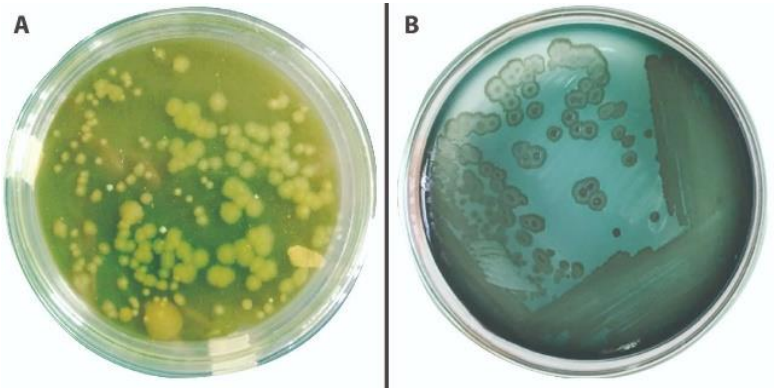


Figure 3.1. Isolation of pyocyanin-producing bacteria on KingA medium. A. Petri dish on KingA medium with digestive gland sample of shrimp collected from Ninh Binh; B. Strain PS6 isolated and purified from shrimp digestive gland sample.

The results show that the PA gene has been successfully amplified from bacterial strains PS4, PS6, PS11, PS33, PS35, and PS39, with a size of approximately 1000 bp as expected (Figure 3.2). These gene segments were amplified specifically. Therefore, strains PS4, PS6, PS11, PS33, PS35, and PS39 are likely to belong to the species *P. aeruginosa*. Additionally, some isolated strains did not show a DNA band around 1 kb, such as PS2, PS3, and PS44. These strains may not belong to *P. aeruginosa* as originally thought and may require further isolation.

Based on the biochemical characteristics, strain PS39 shows similarity to the standard strain *P. aeruginosa* ATCC27853. Comparison of the 16S rRNA gene sequence of strain PS39 with other bacterial strains on GenBank reveals the highest similarity of 99.8% with the gene from Pseudomonas aeruginosa strain CNEB4 accession number MZ648186.1. Combining morphological, biochemical, and PA gene sequence characteristics for the species *P. aeruginosa*, it can be confirmed that strain PS39 belongs to the genus *P. aeruginosa* and is designated as *P. aeruginosa* PS39.

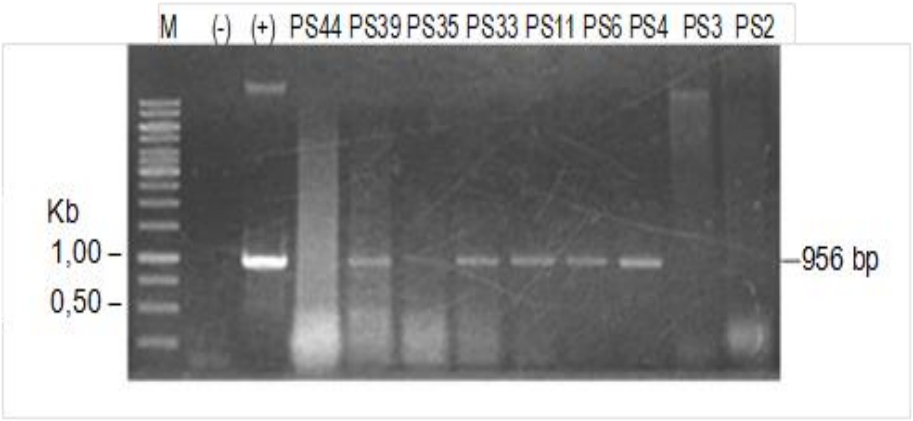


Figure 3.2. Electrophoresis analysis of PCR products of PA gene segment 956 bp of 9 high pyocianin-producing strains on 0.8% agarose gel. M: 1kb DNA ladder (GeneRular 1kb DNA ladder, ThermoFisher), (-): negative control; (+): DNA of P. aeruginosa bacterial strain (Environmental Bioremediation Biotechnology Department Collection)

3.2. Creating recombinant strain *P. aeruginosa* PS39 carrying additional copies of *phzM* and *phzS* genes in the plasmid

3.2.1. PS39 Isolation and cloning of phzM and phzS genes from P. aeruginosa PS39

The PCR products of the two genes *phzM* and *phzS* were cloned into the vector pJET1.2 to create the recombinant vectors pJET1.2-phzM and pJET1.2-phzS. The gene *phzM* has been deposited in the gene bank under accession number MF673740. The gene *phzS* has been deposited in the gene bank under accession number MF770713.

3.2.2. Designing vector pUCP24-phzMS carrying two genes phzM and phzS

To create the recombinant plasmid pUCP24-phzM, the vector pUCP24 was linearized using the restriction enzyme *Sma*I. The resulting linearized vector was purified and ligated with the *phzM* gene, which had

been previously digested and prepared with compatible ends. For the construction of the recombinant plasmid containing both genes *phzM* and *phzS* (pUCP24-phzMS), the PCR product of the phzS gene and the plasmid pUCP24-phzM were both digested with the restriction enzymes *Xba*I and *Sph*I. After purification of the digested products, they were used in a ligation reaction to insert the *phzS* gene into pUCP24-phzM using T4 DNA ligase. Following verification steps, it can be confirmed that the vector pUCP24-phzMS was successfully designed (Figure 3.13).

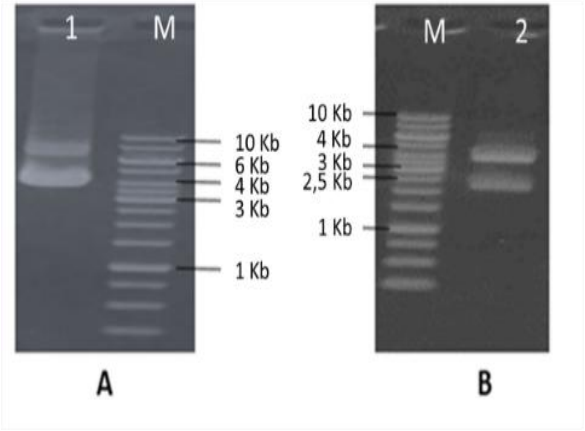
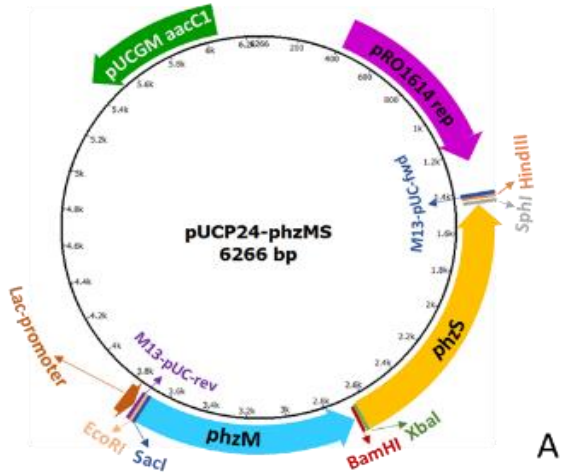


Figure 3.13. Plasmid pUCP24-phzMS was examined by electrophoresis after extraction (A) and cutting with *Eco*RI and *Hin*dIII (B) on a 0.8% agarose gel. M: DNA standard (O'GeneRuler 1Kb DNA Ladder- ThermoScientific).

To ensure that the design did not affect the genes and expression cassette of the genes, the plasmid pUCP24-phzMS was sequenced using a state-of-the-art gene sequencing system. The sequencing results confirmed and elucidated the positions of the phzM and phzS genes, as well as the presence of necessary elements for the expression of these two genes within the plasmid structure (Figure 3.14).



Name	Type	Value
Annotations [plasmid annotation.gb]		
inserted genes (0, 2)		
phzM	Gene	complement(2689..3720)
phzS	Gene	complement(1436..2644)
plasmid pUCP24 (0, 2)		
pRO1614 rep	Gene	411..1244
pUCGM aacC1	Gene	complement(5511..6044)
ribosome binding sites (0, 3)		
RBS Lac	RBS	complement(3732..3736)
RBS pRO	RBS	398..401
RBS pUC	RBS	complement(6053..6058)
Auto-annotations [pUCP24-phzMS - Copy.txt...		
enzyme (9, 0)		
AflI (0, 26)		
ApaLI (0, 1)		
AscI (0, 1)		
BamHI (0, 1)		
Eco53kI (0, 1)		
SacI (0, 1)		
SmaI (0, 1)		
SphI (0, 1)		
XbaI (0, 1)		
orf (0, 1)		
plasmid_features (0, 4)		
M13_forward20_primer	Misc. Feature	1405..1421
M13_pUC_fwd_primer	Misc. Feature	1390..1412
M13_pUC_rev_primer	Misc. Feature	complement(3734..3756)
lac_promoter	Misc. Feature	complement(3770..3799)

B

Figure 3.14. Annotation of recombinant plasmid pUCP24-phzMS. Diagram of pUCP24-phzMS plasmid (A) and detailed annotation of plasmid components (B) (made by UGENE v.39 software).

3.2.3. Testing the expression ability of two genes phzM and phzS in recombinant vector pUCP24-phzMS

Before introducing the plasmid into the *P. aeruginosa* PS39 strain, we conducted an activity test of the entire expression cassette by expressing

the *phzM* and *phzS* genes in *E. coli* cells. Based on the results obtained (Figure 3.16), it can be inferred that the *lac* promoter in the pUCP24-phzMS vector was active and likely synthesized PhzS and PhzM. Therefore, the structure of the pUCP24-phzMS vector can be confirmed as suitable for transformation into the *P. aeruginosa* PS39 strain.

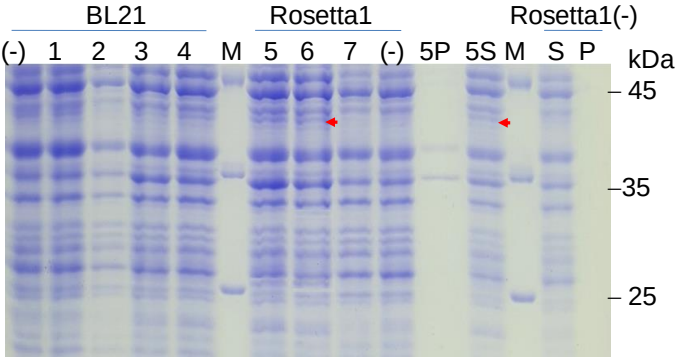


Figure 3.16. Analysis of total protein, soluble and insoluble phase proteins expressed in two strains of *E. coli* BL21 and Rosetta carrying pUCP24-phzMS.

3.2.4. Creation of recombinant P. aeruginosa PS39 strain carrying vector pUCP24-phzMS

The plasmid pUCP24-phzMS obtained from the previous experiment was used to transform the host cells of *P. aeruginosa* PS39 by electroporation. To preliminarily check for the presence of the two genes *phzM* and *phzS* in pUCP24-phzMS, the recombinant plasmids were simultaneously digested with the two restriction enzymes *Eco*RI and *Hin*dIII. The electrophoresis pattern of the restriction digestion products (Figure 3.17B) shows that the plasmid was successfully digested, producing two distinct DNA bands of approximately 2.2 kb and 4 kb when compared to the DNA ladder. This confirms that the recombinant plasmid pUCP24-phzMS was successfully transformed into the strain *P. aeruginosa* PS39, and the genetically modified strain is referred to as *P. aeruginosa* PS39-phzMS

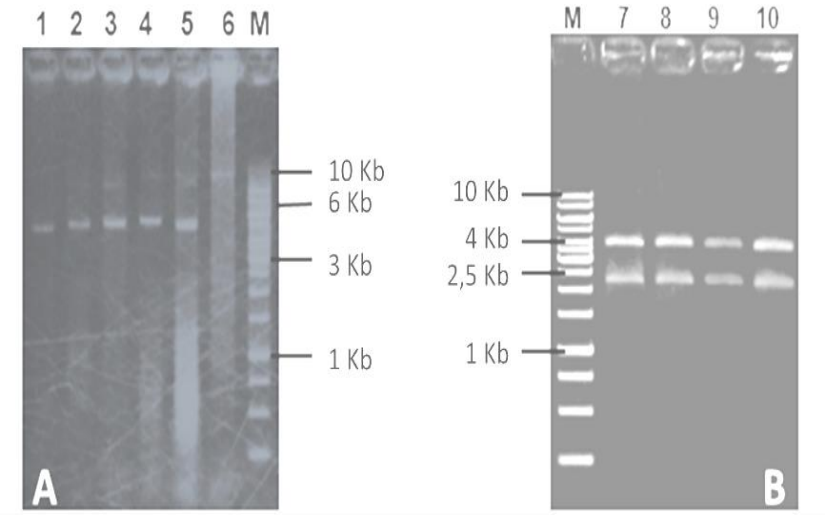


Figure 3.17. Electrophoresis to check pUCP24-phzMS cleavage product separated from recombinant P. aeruginosa PS39 on 0.8% agarose gel. M- DNA standard (O'GeneRuler 1Kb DNA Ladder- ThermoScientific); A) 1-5: Plasmid from *P. aeruginosa* PS39-phzMS recombinant clones; 6: DNA of original *P. aeruginosa* PS39; B) 7-10: pUCP24-phzMS plasmid cutting product of cell lines after transformation with restriction enzymes *Eco*RI and *Hin*dIII.

The maintenance of the recombinant plasmid in the bacterial strain through multiple subcultures is a critical factor for evaluating the stability of the recombinant strain. In this study, the recombinant strain *P. aeruginosa* PS39-phzMS was assessed for the presence of the plasmid pUCP24-phzMS continuously through 5 subcultures. The results of the plasmid digestion products from the 2nd and 5th subcultures showed that the plasmid digestion produced two DNA fragments of corresponding sizes.

3.3. Research on methods to recover and purify pyocyanin from P. aeruginosa PS39-phzMS

3.3.1. Selecting P. aeruginosa PS39-phzMS clone that produces high pyocyanin

The ability of the recombinant strain to enhance pyocyanin production, after confirming the presence and integrity of the phzM-phzS gene pair, will be evaluated through the reduction of the PCA precursor and the increase in pyocyanin levels by culturing the natural strain *P. aeruginosa* PS39 and the recombinant strain *P. aeruginosa* PS39-phzMS. The results show that the concentration of PCA extracted from the culture of the natural strain *P. aeruginosa* PS39 is 14.48 ± 1.14 µg/mL, while the PCA extracted from the culture of the recombinant strain *P. aeruginosa* PS39-phzMS is much lower (1.041 ± 0.57 µg/mL). Conversely, the concentration of pyocyanin extracted from the recombinant strain increased to 31.22 µg/mL, more than twice the concentration of pyocyanin extracted from the natural strain (13.47 µg/mL) (Figure 3.21).

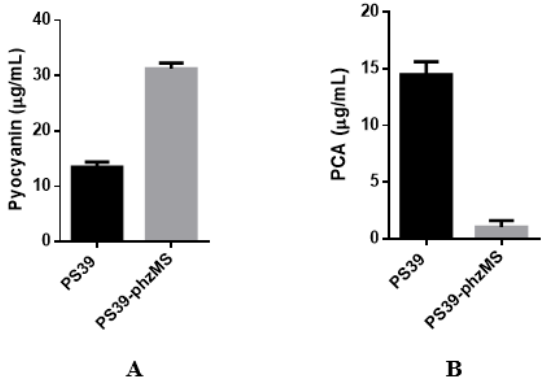


Figure 3.21. Pyocyanin (A) and PCA (B) content produced by wild-type strain *P. aeruginosa* PS39 and recombinant strain *P. aeruginosa* PS39-phzMS. PS39: *P. aeruginosa* PS39; PS39-phzMS: *P. aeruginosa* PS39-phzMS. Significant

differences were assessed by t-test analysis at $p < 0.0001$.

3.3.2. Research on extraction and purification of pyocyanin

Chloroform is the most effective solvent for extraction, with the pyocyanin yield reaching 25.27 ± 1.02 µg/mL, followed by dichloromethane at 20.26 ± 0.87 µg/mL. Therefore, chloroform is the most suitable and efficient solvent for extracting pyocyanin from the culture of *P. aeruginosa* PS39-phzMS (Figure 3.22).

The UV-Vis absorption spectrum analysis of pyocyanin in 0.2N HCl, purified from the *P. aeruginosa* PS39-phzMS strain using dichloromethane and chloroform as solvents, showed that both solvents yielded a similar level of purity as the reference pyocyanin (Figure 3.23).

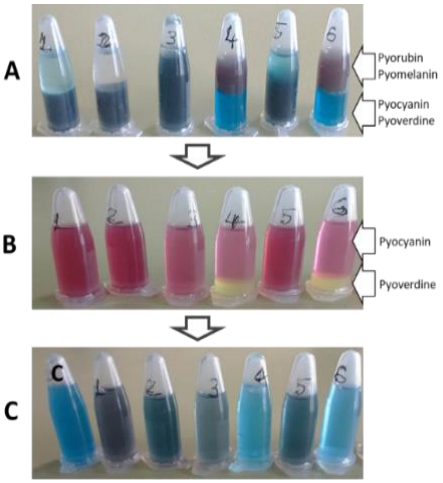


Figure 3.22. Extraction of pyocyanin from *P. aeruginosa* PS39-phzMS bacterial culture with different solvents. (A) Using different solvents to separate pyocyanin (bottom blue layer); (B) Acidification with 0.2N HCl;

(C) Neutralize with 1N NaOH. 0: PY0 pure; 1: benzene; 2: hexane; 3: methanol; 4: dichloromethane; 5: ethylacetate; 6: chloroform.

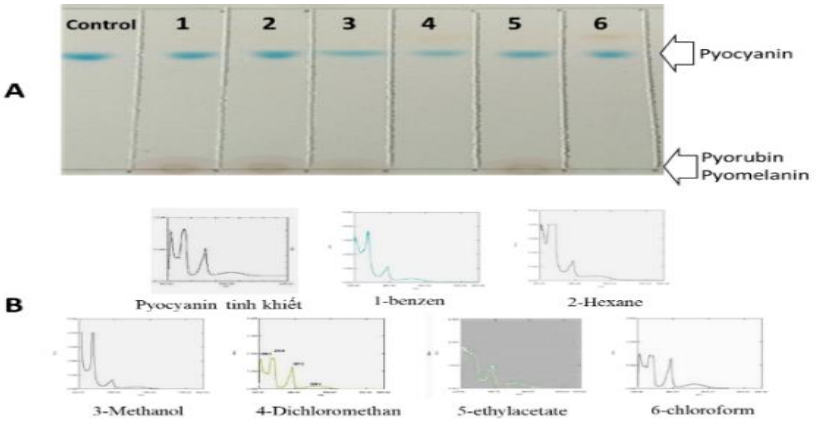


Figure 3.23. UV-Vis spectra of pyocyanin extracted using different solvents. A) TLC spectrum; B). DC: pure pyocyanin; 1: benzene; 2: hexane; 3: methanol; 4: dichlometan; 5: ethylacetate; 6: chloroform.

From the results of the solvent selection survey for extracting pyocyanin from bacterial culture, chloroform was chosen for use in subsequent studies and in the development of the pyocyanin extraction and purification process (Figure 3.24).

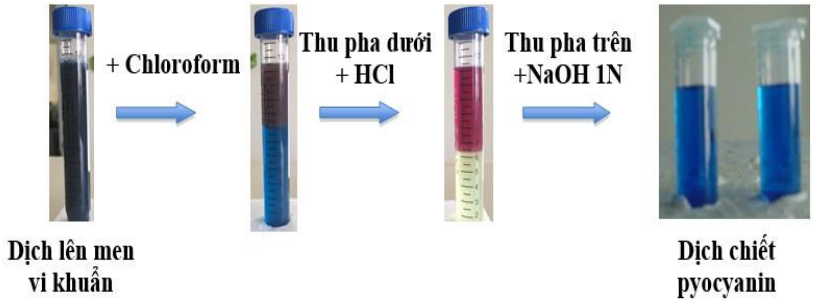


Figure 3.24. Illustration of steps to extract pyocyanin from the culture medium of *P. aeruginosa* PS39-phzMS

3.3.3. Evaluating the purity of pyocyanin

UV-Vis spectral analysis of pyocyanin in HCl solution shows that pyocyanin extracted using dichloromethane and chloroform has a spectrum similar to that of pure pyocyanin. Furthermore, high-resolution mass spectrometry (HRMS) analysis of pyocyanin indicates that the molecular weight of the compound is M = 210, corresponding to the molecular formula C13H10N2O of pyocyanin.

3.4. Antibacterial properties of pyocyanin

3.4.1. Resistance to tested microorganisms of pyocyanin

The results show that the growth of the tested microorganisms, including Gram-positive bacteria (*E. faecalis* ATCC 29212, *S. aureus* ATCC 25923, *B. cereus* ATCC 13245) and Gram-negative bacteria (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. enterica* ATCC 13076), was completely inhibited at a minimum inhibitory concentration (MIC) of 25 µg/mL. For yeast, the minimum inhibitory concentration was 6.25 µg/mL.

3.4.2. Evaluation of resistance to Vibrio spp. pyocyanin causes disease in shrimp

The strains causing acute hepatopancreatic necrosis disease in shrimp, *V. parahaemolyticus* (VpKG12T1, VpST22T, VpCMT31), were completely inhibited at a minimum concentration of 12.5 µg/mL. The two strains, *V. harveyi* Vh3 and *V. alginolyticus* Val, were inhibited at a concentration of 17.5 µg/mL. The minimum bactericidal concentration (MBC) required to achieve a 99.9% reduction for *VpCMT31*, *VpKG12T1*, and *VpST22T* was 20 µg/mL; and for *Vh3* and *Val*, it was 25 µg/mL.

Pyocyanin shows strong inhibitory effects against various *Vibrio spp.*, including *V. parahaemolyticus* strains VpKG12T1, VpST22T, and VpCMT31, which are pathogens affecting shrimp. In disk diffusion assays, there was not much difference observed among the strains at concentrations

ranging from 6.5 to 13 µg/disk. However, at a concentration of 26 µg/disk, the zone of inhibition noticeably increased, reaching approximately 25–32 mm in diameter (Figure 3.29).

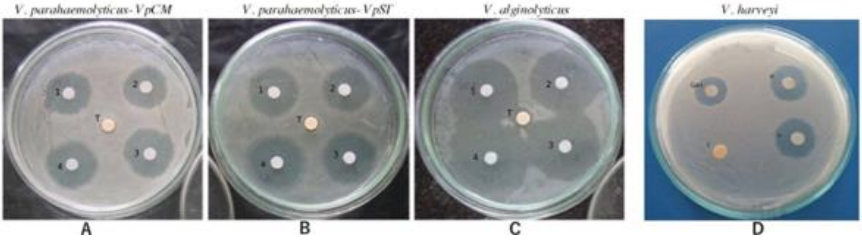


Figure 3.29. Illustrating the antimicrobial efficacy of pyocyanin against pathogenic *Vibrio spp.* in shrimp: *V. parahaemolyticus* VpCMT3 (A), *V. parahaemolyticus* VpST22T (B), *V. alginolyticus* Val (C) và *V. harveyi* Vh3 (D). T – Tetracycline; Gen – Gentamycin; a, b, 1,2,3 và 4 – The corresponding concentrations of pyocyanin0,406; 0,8125; 3,25; 6,5; 13; 26 µg/paper plug;

3.5. Research on suitable culture conditions for pyocyanin proliferation from *P. aeruginosa* PS39-phzMS

3.5.1. Effects of physicochemical factors on pyocyanin biosynthesis by P. aeruginosa PS39-phzMS

The factors such as temperature, pH, incubation time, and shaking speed were studied to optimize the production of pyocyanin from *Pseudomonas aeruginosa* PS39-phzMS, harboring the recombinant phz genes. The results revealed that the optimal conditions for highest pyocyanin yield were pH 8, temperature of 30°C, shaking speed of 200 rpm, and an incubation period of 120 hours in KingA medium supplemented with 1.5% glutamic acid.

3.5.2. Selecting suitable nutritional sources for pyocyanin biosynthesis

Among the five culture media tested during development, two, AM and GM, exhibited intense greenish-blue pyocyanin pigment production. These media are variants of KingA where peptone is replaced with alanine and glutamic acid, respectively. The synthesized pyocyanin concentrations

on AM and GM media were 34.71 ± 0.35 µg/mL and 35.57 ± 0.26 µg/mL, respectively. This data is presented in Figure 3.34.

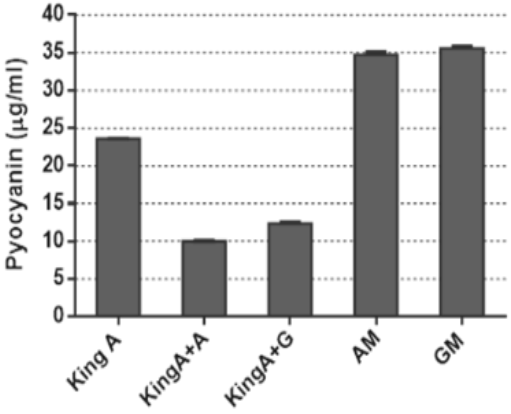


Figure 3.34. The influence of environmental components on pyocyanin biosynthesis in *Pseudomonas aeruginosa* PS39-phzMS. KingA: KingA medium; KingA+A: KingA + Alanine; KingA+G: KingA + Glutamic acid; AM: KingA + Alanine without peptone; GM: KingA + Glutamic acid without peptone. Pyocyanin concentrations are presented as mean values with standard deviations, $t\text{-test}_{AM\ vs\ GM}$: $p<0{,}05$.

At higher concentrations of glutamic acid exceeding 1%, there is an observed gradual increase in pyocyanin production. Specifically, at a glutamic acid concentration of 1.5%, the highest pyocyanin production capacity was achieved at 49.57 ± 1.71 µg/mL.

The optimized composition of the pyocyanin-producing medium for *P. aeruginosa* PS39-phzMS includes K2SO4 at 10 g/L, MgCl2·H2O at 3 g/L, glycerol at 10 mL/L, gentamicin at 200 µg/mL, and glutamic acid at 15 g/L. Under conditions of pH 8 and 30°C, after 120 hours of cultivation, pyocyanin reached its peak production, approximately three times higher than that observed in the natural strain *P. aeruginosa* PS39.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

1. Eighteen strains were isolated and screened, yielding nine bacterial strains capable of producing pyocyanin ranging from 6.01±1.2 µg/mL to 15.02±0.56 µg/mL, sourced from collected samples and strain collections at the Environmental Bioremediation Laboratory, Institute of Biotechnology. Among these, strain PS39, identified as belonging to the species *P. aeruginosa*, exhibited the highest pyocyanin synthesis at 15.02±0.56 µg/mL.
2. The vector pUCP24-phzMS had successfully designed containing two genes, *phzM* (1000 bp, MF673740) and *phzS* (1200 bp, MF770713), isolated from the natural strain PS39 and fully sequenced using the latest generation sequencing system under accession number MZ399165.1. The functionality of the lac operon carrying both *phzM* and *phzS* genes in the pUCP24-phzMS vector was assessed in *E. coli* cells. Following this, the pUCP24-phzMS vector was effectively introduced into the natural strain PS39, resulting in the recombinant strain *P. aeruginosa* PS39-phzMS, which exhibited enhanced pyocyanin synthesis.
3. Pyocyanin was successfully extracted from the culture medium of the recombinant bacterial strain and the natural strain using chloroform solvent with a volume ratio of 1 solvent: 1 culture medium. The pyocyanin product obtained (either in solution or powder form) exhibits a characteristic blue-green color. It possesses UV-Vis spectral properties similar to pure pyocyanin, with a high-resolution mass spectrum corresponding to the molecular formula of pyocyanin ($C_{13}H_{10}N_2O$). The purity of the product reaches 97% as determined by HPLC analysis.
4. Pyocyanin extracted from the culture broth of the recombinant strain *P. aeruginosa* PS39-phzMS demonstrates inhibitory and bactericidal effects against *Vibrio* spp. pathogens in shrimp at concentrations ranging

from 12.5 to 17.5 µg/mL and 20 to 25 µg/mL, respectively, depending on the strain. Pyocyanin also exhibits antimicrobial activity against tested microorganisms such as *B. cereus* ATCC 13245, *E. faecalis* ATCC 29212, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *S. enterica* ATCC 13076, *P. aeruginosa* ATCC 27853 at a minimum inhibitory concentration (MIC) of 25 µg/mL, and against *C. albicans* ATCC 10231 at 6.25 µg/mL

5. Identified GM medium (pH 8) and suitable culture conditions including optimal temperature of 30°C, shaking speed of 200 rpm for 120 hours for the highest pyocyanin production by recombinant strain *P. aeruginosa* PS39-phzMS reaching a maximum yield of 49.57±1.71 µg/mL.

RECOMMENDATIONS

1. Optimizing the large-scale fermentation process of *Pseudomonas aeruginosa* PS39-phzMS to produce a significant quantity of pyocyanin for practical testing and application.
2. Expanding the antibacterial spectrum study of pyocyanin focusing on pathogens affecting agricultural crops and aquaculture species.

NOVAL CONTRIBUTIONS OF THE DISSERTATION

1. The gene has been successfully transferred into the natural strain to create the recombinant bacterium *Pseudomonas aeruginosa* PS39-phzMS carrying the plasmid pUCP24-phzMS, enhancing pyocyanin production compared to the natural strain.
2. Suitable culture conditions and medium have been selected to cultivate the recombinant strain for high-yield pyocyanin production.

LIST OF PUBLICATIONS

1. Vinh, N. Q., N. C. Thuan, N. H. Uyen, Trang, N. H., & Loi, N. T. T. (2018). Tạo chủng tái tổ hợp *Pseudomonas aeruginosa* PS39 mang gen *phzM* và *phzS* tăng tổng hợp pyocyanin. Hội nghị khoa học Công nghệ Sinh học toàn quốc, 14-20

2. Vinh, N. Q., Thuan, N. C., Uyen, N. H., & T., L. N. T. (2022). Increased production of pyocyanin in recombinant pseudomonas aeruginosa ps39-phzms strain harboring plasmid PUCP24-phzms. Journal of biotechnology 20(1), 135-142.

3. Vinh Quang Nguyen, Uyen Hoang Nguyen, Thuan Chi Nguyen, Anh T.N Dao, Loi Thi Thanh Nguyen (2023). Effect of culture conditions on pyocyanin production by recombinant pyocyanin-producing strain *Pseudomonas aeruginosa* PS39-phzMS. Malaysian Journal of Microbiology 19(3), 282-290

4. DNA sequences registered on NCBI GenBank

- The sequence of *phzM* gene is registered on NCBI GenBank under accession number MF673740.
- The sequence of *phzS* gene is registered on NCBI GenBank under accession number MF770713.
- The sequence of plasmid pUCP24-phzMS is registered on NCBI GenBank under accession number MZ399165.1.