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INCREASED PRODUCTION OF PYOCYANIN IN RECOMBINANT *PSEUDOMONAS AERUGINOSA* PS39-phzMS STRAIN HARBORING THE pUCP24-phzMS PLASMID

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SUMMARY

PhzM and PhzS are two "core" enzymes that are necessary for conversion of phenazine-1-carboxylic acid (PCA) into pyocyanin (PYO) phenazine in Gram (-) bacterium *Pseudomonas aeruginosa*. Apparently, the raise in copy number of their genes could increase the amount of pyocyanin phenazine in the microbe. In previous research, two genes *phzM* and *phzS* originated from *Pseudomonas aeruginosa* PS39 strain had been inserted into a *Pseudomonas – Escherichia coli* shutlle vector pUCP24 to generate a plasmid pUCP24-phzMS. The obtained plasmid had been transformed into *P. aeruginosa* PS39 strain to create the recombinant *P. aeruginosa* PS39-phzMS strain. In this study, pUCP24-phzMS was sequenced to verify the polycistronic expression cassette containing both *phz*M and *phz*S genes. The results demonstrated that the recombinant plasmid comprised the *ori* of *Pseudomonas* and *E. coli,* gentamicin resistance-cassette, and polycistronic expression cassette for expression of PhzM and PhzS. In which, both genes will be transcripted together in one mRNA strand by the regulation of Lac promoter and operator. The translation from the mRNA to the corresponding proteins will be started by binding ribosome into RBS located upstream of each gene. The nucleotide sequence of these genes were completely homologous (100%) to the submitted sequences MF673740 (*phzM*) and MF770713 (*phzS)* on the NCBI GenBank database. Result on assessing the synthesis of pyocyanin in the recombinant strain with the presence of pUCP24-phzMS plasmid showed that pyocyanin concentration in the recombinant strain increased significantly over 2 times (31.22 μg/mL) more than that in the wild strain (13.47 μg/mL). The absorbance at 360 nm of PCA from the *P. aeruginosa* PS39-phzMS (OD_{367} = 0.03) strain decreased significantly compared to the one from wild type strain (OD_{367} = 0.39). Therefore, the plasmid with *phzM* and *phzS* genes was proved to improve the pyocyanin biosynthesis through a better conversion of PCA phenazine into PYO via PhzM and PhzS enzymes.

Keywords: *phzM*, *phzS*, pyocyanin, phenazine-1-carboxylic acid, recombinant plasmid, next-generation sequencing

INTRODUCTION

Pyocyanin is an exuberant blue-colored phenazine produced exclusively by 90–95% of *Pseudomonas aeruginosa* strains (Mavrodi *et al.*, 2001). The pigment with antibacterial activity against a wide range of drug-resistant bacteria and several species of foodborne pathogenic

bacteria. Also, pyocyanin exhibits antifungal activity against different plant pathogenic fungi and mycotoxigenic fungi (Marrez, Mohamad, 2020). Furthermore, pyocyanin showed antioxidant and anticancer activity against several cancer cell lines (Muller *et al*., 2009, Moayedi *et al*., 2018). The compound is a good example of a secondary metabolite, that possesses antibiotic activities. The other species do not synthesize the compound.

In *P. aeruginosa,* as reported, the biosynthesis of pyocyanin is initiated at the branching site of chorismic acid from shikimic acid. In the process, chorismic acid is converted to PCA by enzymes from the phzABCDEFG operon. Next, the *phzM* gene product, methyltransferase, performs a methylation reaction to convert PCA to 5-methyl PCA and then, is further hydroxylated by the enzyme protein, flavoprotein monooxygenase, encoded by the *phzS* gene to pyocyanin. A mutation in the phzABCDEFG operon results in a decrease in pyocyanin synthesis.

The *phzM* and *phzS* genes are two important genes found only in *P. aeruginosa* that encode two essential enzymes for the pyocyanin synthesis pathway. Mavrodi *et al* (2001) showed that the *phzM* inactivated strain haboring a phzM-carrying plasmid is capable to increase pyocyanin synthesis to a higher amount than the parent strain (Mavrodi *et al*., 2001; Sterritt *et al.*, 2018; Feghali and Na'was 2018). Therfore, PhzM and PhzS are two key enzymes involving in the biosynthesis pyocyanin of *P. aeruginosa* **(**Fig. 1**)** (Jayaseelan *et al.*, 2014).

In the nature, pyocyanin is produced in *P. aeruginosa* at exceptionally low amount. To increase its production for practical application, strain improvement is essential (Priyaja, 2012).

In a previous study, two genes *phzM* and *phzS* from *P. aeruginosa* PS39 were sucessfully inserted into the shutlle vector pUCP24 to generate pUCP24-phzMS (Vinh *et al.*, 2018). The evaluation of pyocyanin biosynthesis in different colonies of this recombinant strain *P. aeruginosa* PS39-phzMS enabled to select a clone secreting higher pyocyanin yield than the original *P.aeruginosa* PS39 strain (Vinh *et al.*, 2018).

In the last two decades, next-generation sequencing (NGS) technology has been sharply developed and applied in medicine, agriculture, environment, microbiology, andbiology (Gupta, Gupta, 2020). NGS is a powerful tool for analysis of genes, whole genome, and metagenomic. In microbiology, it could help study disease and characterize function genes in bacteria (Gupta, Gupta, 2020). Millan *et al*. (2015) applied NGS for analysis of plasmids pAMBL1 and pAMBL2 from *P. aeruginosa*. The study used Geneious 7.1.7 software to process the data from PacBio system. They found that plasmids pAMBL1 and pAMBL2 contain duplication of *blaVIM-1* cassette, which could contribute to increasing the antibiotic resistance level of *P. aeruginosa*. The duplication cassette is easier to detect than before because of the development new sequencing technology and the analysis method (San Millan *et al.*, 2015).

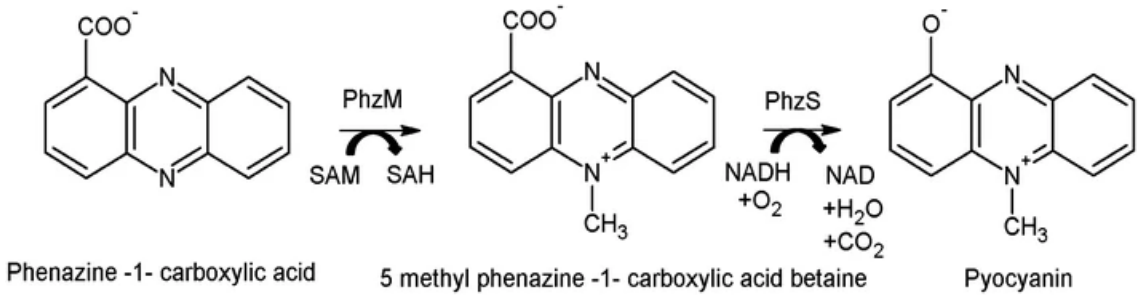


Figure 1. Pyocyanin is synthesized from phenazine-1-carboxylic acid(Jayaseelan *et al.*, 2014).

In this study, we sequenced the plasmid pUCP24-phzMS from selected colony to confirm the exist of polycistronic expression cassette *phzM-phzS* in the plasmid by Miseq system. Also, evaluation of the PCA and pyocyanin was performed based on the bacterial cell culture of the wild type strain *P. aeruginosa* PS39 and recombinant strain *P. aeruginosa* PS39-phzMS.

MATERIALS AND METHODS

Bacterial strains, media and culture conditions for metabolite production

In previous study, a recombinant strain *P. aeruginosa* PS39-phzMS was created. In brief, whole gene *phzM* (nucleotide from +1 to +1005) in the genome of *P. aeruginosa* PS39 was amplified by specific primers then inserted into pUCP24 at *Sma*I restriction site to generate pUCP24-phzM. A gene *phz*S (nucleotide from -13 to +1209, containing sequence AAGGAA for ribosome binding) was amplified from genome of *P. aeruginosa* PS39 by specific primers overhanged with *Xba*I, *Sph*I then inserted into pUCP24-phzM at the corresponding sites to obtain pUCP24-phzMS to yield a polycistronic expression cassette of both *phzM* and *phz*S (Fig. 2). At this construction, the genes *phzM* and *phz*S will be transcripted together in one mRNA strand by Lac promoter regulation. The translation of the mRNA into PhzM and PhzS will be started when ribosomes bind to RBS regions upstream of each gene.

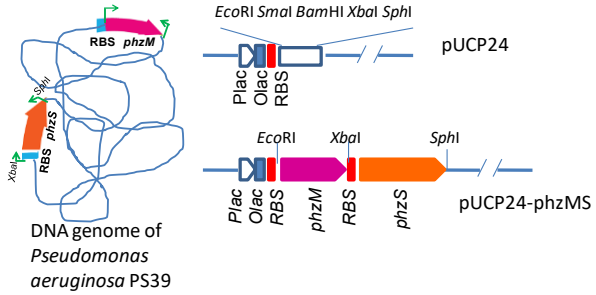


Figure 2. Design of expression vector pUC24-phzMS for expression of *phzM* and *phzS*. Green arrows indicate the position of primers for *phzM* and *phzS* genes.

The bacterial strains, *P. aeruginosa* PS39 (wild-type) and *P. aeruginosa* PS39-phzMS (recombinant strain) were cultured in KingA liquid medium composed of 20 g gelatin peptone, 3 g $MgCl_2.6H_2O$, 10 g K_2SO_4, 10 mL glycerol in 1 L medium, pH 7.2. For the recombinant strain, gentamicin was added to KingA medium at final concentration 100 µg/mL. To obtain metabolite products (PCA and pyocyanin), precultured *P. aeruginosa* PS39 or *P. aeruginosa* PS39-phzMS at 10% (v/v) was inoculated into the Erlenmeyer flask and incubated at 30ºC for 120 h with shaking frequency at 200 rpm. The culture liquid of these two strains was collected for extracting PCA and pyocyanin. In addition, the culture liquid of *P. aeruginosa* PS39-phzMS strain in KingA medium for 24 h was used as material for plasmid extraction.

Plasmid extraction and restriction endonuclease reaction

The recombinant plasmid was extracted for next-generation sequencing by using *AccuPrep*® *plasmid Mini Extraction Kit* (Bioneer, Korea) following the manufacturer's instructions. In brief, bacterial cells were collected by centrifugation at 8000 rpm for two minutes then lysed with alkaline. Then the plasmid dissolved in liquid was separated from insoluble complex of chromosomal DNA and cell debris by centrifugation at 13000 rpm, 4ºC for 10 minutes. Plasmid DNA solution was added into a column composed of silica-based membrane enalbe the plasmid DNA binding to the membrane. Thus contamination was washed off by washing buffer. Finally, plasmid DNA was eluted using elution buffer (10 mM Tris-HCl pH 8). The

plasmid pUCP24-phzMS was confirmed by restriction enzymes *Eco*RI và *Hin*dIII.

DNA sequencing by next-generation sequencing

After extraction, the recombinant plasmid was used as input to generate a paired-end library using the *Nextera XT DNA Library Prep Kit* (Illumina, USA). The procedure was followed according to the manufacturer's instructions. The average size of the amplicons was 300 bp. The sequencing was performed from both ends (150 cycles x2) by Miseq system (Illumina Inc.) at GENTIS (Genetic testing service joint stock company). Raw data was compared to theoretical plasmid pUCP24-phzMS sequence (Gene bankaccession number U07167) using BWA program (Li, Durbin, 2009). Comparison results were visualized by IGV (Robinson *et al.*, 2011).

Phenazine-1-carboxylic acid (PCA) extraction and quantification

The PCA production in bacterial cell culture was determined following the method of Mavrodi (Mavrodi *et al.*, 2001). In brief, the cell culture of strains *P. aeruginosa* PS39 and *P. aeruginosa* PS39-phzMS was centrifuged at 5000 rpm for 10 minutes. The cell-free culture supernatant (10 mL) was transferred to another flask and acidified with concentrated hydrochloric acid to achieve a pH of 2.0. The acidified supernatant was extracted with an equal volume of benzene. The organic phase was pooled and dried by evaporation. The dried pale-yellow residue was dissolved in 1 mL of 0.1 M NaOH, and the absorbance was read at optical density 367 nm against the benzene extract of acidified KingA alone, used as a blank.

Pyocyanin extraction and quantification

Pyocyanin in broth culture was extracted by two-step extraction using chloroform and HCl. The broth culture (1 mL) was centrifuged at 5000 rpm for 10 minutes to remove bacterial cells. The equal volume of chloroform was added, then mixed thoroughly before centrifugation at 5000 rpm for 10 minutes. After centrifugation, 0.6 mL of the lower layer was transferred into a new tube and added 0.6 mL of 0.2 M HCl. The mixture was shaken well then kept for a while. The top red layer was collected, and the absorbance was measured at 520 nm (Essar *et al.*, 1990). The extracted pyocyanin amount was quantified by using the formula given by Essar et al. (1990): Pyocyanin (µg/mL) = OD at 520 nm × 17.072 where 17.072 is the extinction coefficient.

RESULTS AND DISCUSSION

Recombinant plasmid extraction

In the previous study, *phzM* and *phzS* genes were cloned into plasmid pUCP24 of 4035 bp and transformed into *P. aeruginosa* PS39 wild strain (Vinh *et al.*, 2018). The isolated recombinant plasmid from the recombinant strain was analyzed by electrophoresis on 1% agarose gel (Fig.3A) and restriction enzymes *Eco*RI and *Hin*dIII (Fig. 3B).

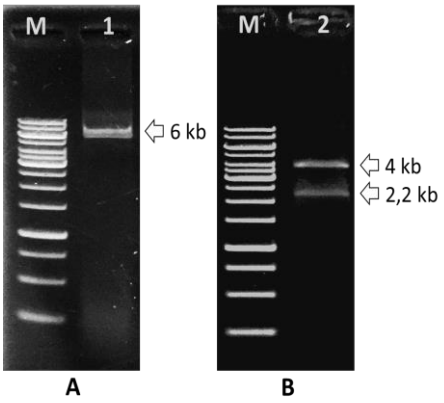


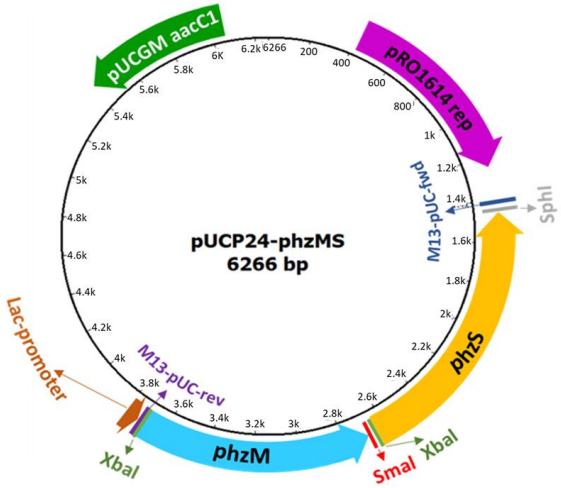
Figure 3. Electrophoresis analysis of pUCP24-phzMS (A) and digested products of pUCP24-phzMS with *Eco*RI and *Hin*dIII on agarose 1% (B). M: 1 kb DNA ladder (Thermo Scientific);.

The results (Fig. 3**)** showed that the cloned DNA in the vector had the right size of 2.2 kb composed of *phz*M and *phz*S genes (Fig. 2). The plasmid pUCP24-phzMS was then sequenced by the next-generation sequencing method.

Recombinant plasmid sequencing

The plasmid DNA library was established by Nextera XT DNA Library Prep Kit and then sequenced by Illumina next generation

sequencer Miseq system. Sequencing data was qualified and analyzed using Miseq Reporter and IGV softwares. The plasmid pUCP24-phzMS sequence was submitted to NCBI GenBank with submission ID2468488 (Supplementary data).



A.

Name	Type	Value
Annotations [plasmid annotation.gb]		
inserted genes (0, 2)		
phzM	Gene	complement(2689..3693)
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)
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 4. Annotation of the recombinant plasmid pUCP24-phzMS. A construction (A) and detail annotating components (B) of the plasmid (by UGENE program - version 39).

Plasmid pUCP24 is a shutlle vector genetically constructed, based on vector pMB1 (ColE1), with the length of 4035 bp, for the aim to replicate in both *Pseudomonas* and *E. coli* (West *et al*., 1994). It consists of (i) a nucleotide sequence from pRO1614, encoding a putative origin of replication (*ori*), that allows it stable maintenance in *Pseudomonas*, (ii) a gene *aacC1* from pUCGM responses for gentamicin antibiotic resistance; (iii) the restriction sites of the MCS (*Eco*RI, *Sac*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I, *Sac*I, *Pst*I, *Sph*I, and *Hin*dIII) located in *lacZα* gene (GenBank accession number U07167).

The total length of recombinant plasmid was 6266 bp and the sequence's annotation was demonstrated in Fig. 4 and Supplementary Data. Sequence analysis indicated that the *phzM* and *phzS* genes were inserted in the correct direction of *lac* operon. The inserted *phzM* gene contained

a complete sequence of 1005 bp spanning nucleotides 2689 to 3693, with its CDS (coding sequence) of 1005 bp including start and stop codons at nucleotide numbers 3691-3693 and 2689-2691, respectively. Ribosome binding site for this gene was located at nucleotides 3732-3736 originated from pUCP24. Next, the length of *phzS* gene was determined to be 1209 bp spanning nucleotides 1436 to 2644, with its CDS of 1209 bp including start and stop codons at nucleotide numbers 2642-2644 and 1434-1436, respectively. Ribosome binding site for this gene was located at nucleotides 2652-2655 originated from *phzS* gene from *P. aeruginosa* PS39.

Moreover, the sequences of *phzM* and *phzS* here were identical to parental genes as in the previous study, which was submitted to NCBI GenBank data with the accession numbers MF673740 (*phzM*) and MF770713 (*phzS*) (Vinh *et al*., 2018). The obtained data confirmed and clarified the location and the functional capability of co-overexpression genes *phzM* and *phzS*.

Effect of recombinant plasmid pUCP24-phzMS on phenazine-1-carboxylic acid and pyocyanin production

The bacterial culture broths of recombinant and wild strain, with and without the plasmid pUCP24-phzMS, respectively, were used to extract PCA (yellow color) and pyocyanin (blue color). The results were presented on Fig. 5 and Fig. 6.

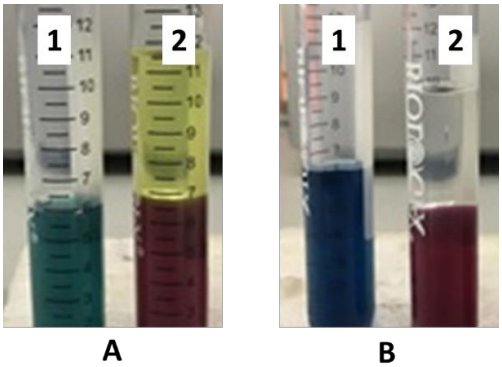


Figure 5. PCA and pyocyanin extracts from wild strain *P. aeruginosa* PS39 (A) and recombinant strain *P. aeruginosa* PS39-phzMS (B). 1: Pyocyanin (blue); 2: top yellow solvent layer of PCA.

Fig. 5A and 5B demonstrated the color difference of pyocyanin and PCA produced in wild and recombinant strains. By observing with the bare eyes, we can qualify the efficiency of recombinant strain or the recombinant plasmid in synthesis of pyocyanin and PCA. Pyocyanin in wild strain (Fig. 5A**,** tube 1) presented lighter blue color than the recombinant's pyocyanin (Fig. 5B**,** tube 1), which means the pyocyanin concentration of the wild strain was lower. Moreover, the top solvent layer of PCA extract was obviously different between two strains, while the recombinant one showed transparent solvent (Fig. 5B, tube 2), the wild one was yellow color on the top layer of the tube (Fig. 5A, tube 2). This exhibition meant that in the wild sample, the PCA remained with a significant amount not completely converted into pyocyanin, the contrast was true for the recombinant.

Further, the analysis of these extracts was conducted to quantify the concentration of pyocyanin and of PCA at the absorbance at 367 nm (Fig. 6).

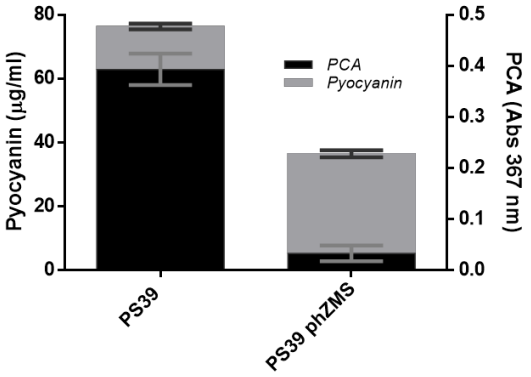


Figure 6. PCA and pyocyanin produced by wild type *P. aeruginosa* PS39 and recombinant strain *P. aeruginosa* PS39-phzMS. PS39: *P. aeruginosa* PS39; PS39-phzMS: *P. aeruginosa* PS39-phzMS. The significant difference was evaluated by t-test analysis with $p < 0.0001$.

Based on the result presented on Fig. 6, OD_{367} value of the PCA extracted from the culture of *P. aeruginosa* PS39 was 13 times (OD_{367} = 0.39±0.03) higher than the one of recombinant strain PS39-phzMS (OD_{367} =

0.03±0.02). In contrast, the pyocyanin yield from the recombinant strain's extract (31.22 μg/mL) was produced 2 times higher than the one from wild-type (13.47 μg/mL). As a results, the wild-type may have synthesized simultaneously both pigments (PCA and pyocyanin) and when extracted in benzene, the retaining PCA fraction was clearly observed (top yellow layer) (Fig. 5A, tube 2). For the recombinant strain, the additional presence of *phzM* and *phzS* inserted coding genes may have elevated concentration of PhzM and PhzS to enhance the convertion of PCA to pyocyanin. So, the transfer of *phzM* and *phzS* genes into the wild-type was an effective way to increase conversion of PCA into pyocyanin.

Our results showed that the simultaneously addition of *phzM* and *phzS* genes in *P. aeruginosa* had resulted in the enhancement of the biosynthesis of pyocyanin through the PCA conversion. Priyaja achieved a similar result when the author transferred the pyocyanin synthetic gene cluster (more than 10 kb) including the phz-containing operon, *phzM* and *phzS* genes of strain *P. aeruginosa* MCCB117 using pUCP24 vector into *P. aeruginosa* MCCB117 itself (Priyaja, 2012). The pyocyanin yield of recombinant strain was twice as high as that of the natural strain (Priyaja, 2012). In Mavrodi's study, a recombinant bacterial strain with the co-expression of *phzM* and *phzS* genes showed the same efficiency of pyocyanin production. Askitosari *et al*. also reported that the genetically engineered *P. putida* KT2440 synthesized significantly amount of pyocyanin by recombination of two plasmids, one plasmid harboring *phzM* and another carrying *phz*S. Besides, the PCA amount in the strain was reduced significantly about 90%. Moreover, the study also indicated that *phzM* and *phzS* gene cluster from different *P. aeruginosa* strains had the different effect on enhancing pyocyanin production (Askitosari *et al*., 2019). Thus, the performance of the recombinant pUCP24-phzMS plasmid obviously had positive impact on pyocyanin synthesis of *P. aeruginosa* PS39-phzMS, which resulted in low PCA production. PCA is a precursor compound of PYO, 1-hydroxyphenazine (1-OH-PHZ), and phenazine-1-carboxamide (PCN), which are synthesized by the products of *phzM* and *phzS* genes cluster, *phzS*, *phzH*, respectively (Mavrodi *et al*., 2001). Mavrodi *et al* also proved that transformation of the PCA-producing *P. fluorescens* 2-79 strain with *phzM* and *phzS* genes triggered synthesis of large amounts of PYO. In addition, the *E. coli* JM109 strain expressing *phzM* and *phzS* genes efficiently converted exogenously supplied PCA to PYO (Mavrodi *et al*., 2001).

Other approaches have been investigated to increase pyocyanin production. For example, Wang *et al.* (2020) created mutant *P. aeruginosa* YTΔrpoS in which the gene *RpoS* was missing (Murakami *et al*., 2005, Wang *et al*., 2020). *P. aeruginosa* YTΔrpoS was able to double the amount of PCA, and the pyocyanin concentration was increased 4 times compared to that of the wild-type. The author found that the absence of *RpoS* gene caused the increase of *phzM* gene expression; as a result, pyocyanin was increasing (Wang *et al*., 2020).

CONCLUSION

In this study, NGS was applied for plasmid sequencing of *P. aeruginosa* PS39-phzMS. The recombinant plasmid construction with inserted complete sequence of *phzM* and *phzS* genes was confirmed. The pyocyanin concentration in recombinant strain increased significantly over 2 times (31.22 μg/mL) higher than in the wild strain (13.47 μg/mL) whereas the PCA absorbance of the *P. aeruginosa* PS39-phzMS (OD_{367} = 0.03) decreased significantly compared to the *P. aruginosa* PS39 (OD_{367} = 0.39).

Therefore, the plasmid with *phzM* and *phzS* genes may improve the pyocyanin biosynthesis through a higher conversion of PCA phenazine into pyocyanin via PhzM and PhzS enzymes. Our data are the evidences for the benefit of overexpression genes *phzM* and *phzS* in biosynthesis of PYO in *P. aeruginosa.*

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