Malaysian Journal of Microbiology, Vol 19(3) 2023, pp. 282-290
DOI: http://dx.doi.org/10.21161/mjm.220077

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Effect of culture conditions on pyocyanin production by recombinant pyocyanin-producing strain *Pseudomonas aeruginosa* PS39-phzMS

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Received 20 September 2022; Received in revised form 31 January 2023; Accepted 23 March 2023

ABSTRACT

Aims: A suitable medium and cultivation parameters have an important role in the improvement of the production of pyocyanin pigment by *Pseudomonas aeruginosa* microorganism. The study aimed to optimize culture conditions and medium components for maximal pyocyanin production in a recombinant strain *P. aeruginosa* PS39-phzMS created in previous research. In addition, the process of extraction of pyocyanin was also investigated to select a proper applied solvent for recovering a high amount of pyocyanin as well as its quality.
Methodology and results: The pyocyanin purification has based on solvent. Among six tested solvents for extracting pyocyanin out of bacterial broth, two out of six recovered a significant amount of pyocyanin, namely dichloromethane and chloroform, in which chloroform showed a higher pyocyanin yield (25.27 ± 1.02 µg/mL) than dichloromethane (20.26 ± 0.88 µg/mL). The thin-layer chromatography (TLC) of the extracted pyocyanin illustrated a similar to pure pyocyanin with R_f of 0.72 and no mark of other impurity metabolites. The UV-Vis spectra showed a similar peak at 520 nm with pure pyocyanin and the highest peak at 274 nm. Each single culture parameter was studied for the maximal production of pyocyanin. Next, a pyocyanin-producing GM medium was modified on the base of the KingA to find the relative capacity to biosynthesize high pyocyanin yield in *P. aeruginosa* PS39-phzMS. The results showed that pyocyanin production was the highest in optimal culture conditions, at 30 °C, pH 8, 120 h and agitation of 200 rpm. In the combination of culture condition with the GM, pyocyanin was created at the highest amount of 49.57 µg/mL.
Conclusion, significance and impact of study: Based on the obtained results of the study, a pyocyanin-producing procedure was optimized, which suggests a promising application to scale-up pyocyanin production by the *P. aeruginosa* PS39-phzMS.

Keywords: Culture conditions, extraction, optimization, *Pseudomonas aeruginosa*, pyocyanin

INTRODUCTION

Pyocyanin is a secondary metabolite with a green-blue color biosynthesized by aerobic Gram-negative bacteria *Pseudomonas aeruginosa* (Vijayan *et al.*, 2006; Patil *et al.*, 2017). KingA medium was used to culture and identify pyocyanin (pigment) from the microorganism. But the pyocyanin production yield on the basal medium is relatively low (Ozdal *et al.*, 2019) and remarkedly different from the *P. aeruginosa* strains.

Pyocyanin is known for its antibacterial, antifungal activity and biocontrol agent (Sweedan, 2010; Jayaseelan *et al.*, 2014). As pyocyanin pigment is water-soluble, it has also been reported for its application in aquaculture as an effective agent against fungal diseases and the pathogen *Vibrio* sp. in aquaculture.

Medium components and culture parameters have an important role in the improvement of pyocyanin production. Several types of research have been carried out in order to increase the yield of secondary microbial metabolites via different strategies. Some authors used enhancers such as surfactants, fatty acids or organic solvents such as acetone, toluene and ethanol (Ozdal, 2019). The reasons for enhancing the activity of such compounds have been assigned to the exchange of cell membrane composition. For example, the mechanism of pigment inducing by the surfactant triton has been examined and it suggested that the surfactant enhances the unsaturation level of lipids on the cell membrane, promoting the release of intracellular pigment (Wang *et al.*, 2013). Organic solvents have been proven that they could be effective in pyocyanin production. Toluene significantly increased pyocyanin production of *P. aeruginosa* OG1 at 312%. However, not all types of solvents can be added to microbial fermentation, as some are very toxic and can kill the cell in very low amounts.

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ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 19(3) 2023, pp. 282-290
DOI: http://dx.doi.org/10.21161/mjm.220077

The study on the safety-concentration threshold is necessary for each microbial strain since the interaction of additional compounds and different microbes are unique (Ozdal, 2019).

Meanwhile, other authors altered the cultivation factors to achieve a high yield (Jayaseelan *et al.*, 2014; Patil *et al.*, 2017). The medium components and cultivation conditions may affect pyocyanin production by *P. aeruginosa*. The critical factors include carbon and nitrogen sources, salt concentration, time course, oxygen concentration, pH level and temperature (Ozdal, 2019; Elbargisy, 2021). KingA medium was established by King *et al.* in the 1950s and this medium has been used as the pyocyanin-producing medium by several scientists since then (King *et al.*, 1954; Jabbar *et al.*, 2020; Gonçalves and Vasconcelos, 2021). The KingA medium has been used as a basic medium that will be altered or modified for a higher yield of pyocyanin (Mossel and Indacochea, 1971; Brodsky and Nixon, 1973). After optimization, the pyocyanin yield can be enhanced up to 18-folds, as in a study on pyocyanin production by *Pseudomonas* sp. MCC 3145 (Patil *et al.*, 2017). Phosphorus is an important element for microorganisms for metabolite synthesis, particularly for *P. aeruginosa*, which is sensitive to phosphate ions (Gonçalves and Vasconcelos, 2021). Glycerol is an important additional supplement to increase the amount of produced pyocyanin effectively. It plays as a nitrogen source (Gonçalves and Vasconcelos, 2021). Besides, industrial food waste such as malt bagasse, peanut or sesame can be used to produce bacterial pigment (Cavalcanti *et al.*, 2019; de Oliveira *et al.*, 2019). For example, pyocyanin reached 58 µg/mL with the addition of beer malt bagasse in the culture medium (de Oliveira *et al.*, 2019). DeBritto *et al.* (2020) studied the dependence of pyocyanin produced by *P. aeruginosa* and additional natural nutrients and the result showed that when KingA medium combined with other natural nutrients would raise the cell density and also increase pyocyanin yield. Soybean and sweet potato were the most effective natural nutrients when amended with KingA medium and nutrient medium, respectively (DeBritto *et al.*, 2020). Physical culture conditions also affect the secretion of pyocyanin. The crucial factors include pH, temperature, oxygen and essential ions such as Mg^{2+}, Fe^{3+}, and NH_4^+ (van Rij *et al.*, 2004; Gonçalves and Vasconcelos, 2021).

The extraction of pyocyanin from bacterial culture has been carried out mainly based on organic solvents. Many studies extracted the compound from the cultural broth by using chloroform, but another solvent, such as dichloromethane, was also useful for pyocyanin extraction (Essar *et al*., 1990; Sudhakar *et al*., 2013; Salih and Mohammed, 2017). Other reports indicated further purification of pyocyanin by precipitating in aluminum sulfate or using gel membrane filtration, ion-exchange chromatography, silica gel column, Amberlite XDA-4 column and HPLC techniques (Hassanein *et al.*, 2009; Saosoong *et al.*, 2009; Ra'oof and Latif, 2010).

P. aeruginosa PS39-phzMS is a recombinant strain constructed by wild-type *P. aeruginosa* PS39 harboring plasmid pUCP24-phzMS (Vinh *et al*., 2022). The *P. aeruginosa* PS39 was isolated from a shrimp pond and can produce pyocyanin at a relatively low level. Plasmid pUCP24-phzMS contains two genes *phzM* and *phzS*, which increase the biosynthesis of pyocyanin. The *P. aeruginosa* PS39-phzMS is promising for applying a scale-up production of pyocyanin.

In this study, we focus on the optimization of culture conditions and medium components to enhance the yield of pyocyanin production of the recombinant strain *P. aeruginosa* PS39-phzMS. Besides, pyocyanin extraction from bacterial culture broth was investigated to select the adequately applied solvent for recovering a high amount of pyocyanin. And the pyocyanin quality was characterized by thin-layer chromatography combined with UV-Vis spectrophotometry analysis.

MATERIALS AND METHODS

Microorganism, inoculum preparation and culture conditions

The P. aeruginosa PS39-phzMS used in all experiments is a recombinant strain created in the previous study by transforming recombinant plasmid pUCP24-phzMS into a wild-type *P. aeruginosa* PS39 isolate. The PS39-phzMS was maintained in glycerol 30% at –80 °C and activated by inoculating it on KingA agar plate, which was supplemented with gentamicin at a final concentration 100 µg/mL. A single fresh colony from the KingA plate was transferred into 250 mL Erlenmeyer flask containing 50 mL of KingA broth and added gentamicin as well, followed by culturing at 30 °C with shaking at 200 rpm for 24 h. The culture broth with OD_{600} of 1 was used as an inoculum for the next experiments.

The composition of KingA broth medium contains 20 g of gelatin peptone, 3 g of $MgCl_2{\cdot}6H_2O$, 10 g of K_2SO_4, 10 mL of glycerol in 1 L distilled water and the pH of the solution was adjusted to 7.2.

Pyocyanin quantitative assay

Pyocyanin was extracted from culture broth based on the method of Essar *et al.* (1990) with a minor modification. Briefly, the cells were removed by centrifuging 1 mL of bacterial culture at 5000 rpm for 10 min. The supernatant was added to an equal volume of chloroform, then mixed thoroughly and centrifuged at 5000 rpm for 10 min. After this, 0.6 mL of the lower layer was transferred into a new tube and added 0.6 mL of 0.2 N HCl. The mixture was shaken well and then kept in the tube still to separate phases. The red aqueous phase was collected for the quantitation of pyocyanin at the absorbance of 520 nm (OD_{520}).

The extracted pyocyanin pigment 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.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 19(3) 2023, pp. 282-290
DOI: http://dx.doi.org/10.21161/mjm.220077

Table 1: The composition of the tested media.

Components (g/L)	KingA	KingA+A	KingA+G	AM	GM
Peptone (g/L)	20	20	20	-	-
K_2SO_4 (g/L)	10	10	10	10	10
$MgCl_2 \cdot 6H_2O$ (g/L)	3	3	3	3	3
Glycerol (mL/L)	10	10	10	10	10
Glutamic acid (g/L)	-	-	0.5	-	10
Alanine (g/L)	-	0.5	-	10	-
pH	7 ± 0.2	7 ± 0.2	7 ± 0.2	7 ± 0.2	7 ± 0.2

(−) indicates that the component was not added into the tested medium.

Effect of culture conditions on pyocyanin production

Physicochemical factors affecting pyocyanin pigment production such as temperature, pH, agitation speed and incubation time, were taken into account for optimum production of pyocyanin yield by *P. aeruginosa* PS39-phzMS. The KingA broth medium was used as pyocyanin-producing basal medium in the following assays. The experiments were repeated 3 times.

The effect of culture temperature on pyocyanin production was examined by incubating the inoculated flasks with 10% of inoculum at the different temperatures of 20 °C, 28 °C, 30 °C, 37 °C and 45 °C for 72 h with shaking at 200 rpm. The free cell supernatant was used for pyocyanin extraction and its concentration was determined as the above method.

To study the effect of culture medium pH on the pyocyanin production of the *P. aeruginosa* PS39-phzMS, 10% of the inoculum was respectively added into the media flasks with the pH level ranging from 6, 7, 8 and 9. The flasks were incubated at 30 °C with shaking speed at 200 rpm and sampled to quantify pyocyanin concentration at intervals of 72, 96 and 120 h of cultivation.

An optimum agitation speed of bacterial culture flasks containing 10% of the inoculum for the highest pyocyanin yield was determined by comparing obtained pyocyanin concentrations at the different shaking rates which were set at 50, 100, 150 and 200 rpm for a culture period of 72 h.

The effect of incubation time on pyocyanin production was tested by measuring pyocyanin yield in the growing broths at a 24 h-interval for six days with a shaking of 200 rpm at 30 °C.

Effect of organic nitrogen component on pyocyanin production

The KingA medium is a typical medium for the secretion of pyocyanin in *P. aeruginosa* and it contains peptone as the organic nitrogen source (Dieppois *et al.*, 2012; DeBritto *et al.*, 2020; Elbargisy *et al.*, 2021). In this research, the KingA and the modified KingA were tested to select the appropriate medium for pyocyanin production by *P. aeruginosa* PS39-phzMS. The modified KingA media included KingA+A, KingA+G, AM and GM as detailed in Table 1. These media were modified by replacing a part or entirely peptone with glutamic acid or Alanine as an organic nitrogen source. The *P. aeruginosa* PS39-phzMS was inoculated in each medium with 10% (v/v) and cultured for 72 h at 30 °C and 200 rpm. The concentration of secreted pyocyanin was used to evaluate the effect of replaced components.

Next, the concentration of glutamic acid was changed from 0.75%, 1%, 1.25% to 1.5% w/v (for GM medium) to test its effect on the production of pyocyanin.

Selection of solvent for extraction of pyocyanin

To select the appropriate solvent, benzene, chloroform, methanol, hexane, dichloromethane and ethyl acetate were used to extract pyocyanin. The cell-free supernatant obtained by centrifuging the broth culture of *P. aeruginosa* PS39-phzMS was mixed with each solvent. The ratio of added solvent and the supernatant was 1:1 (v/v). The mixture was well agitated until its blue colour appeared. The blue layer was transferred to a new tube. An equal volume of 0.2 N HCl was added, and the solution changed from blue to red. Subsequently, the red solution was neutralized by adding 1 N NaOH until the liquid showed blue color. The purity and quantity of the obtained compound was further measured by using thin-layer chromatography, UV-Vis spectroscopy technique and pyocyanin quantitative assay.

Thin-layer chromatography (TLC) and UV-Vis spectroscopy

The technique of TLC was used as described by Cheluvappa *et al*. (2014) with some modifications. In brief, the silica gel plate of 90 × 90 mm (silica gel 60 F254, Germany) was used for analyzing samples. The liquid of extracted pyocyanin was spotted at the marked line from the bottom edge of the plate of 1 cm. The plate was left to dry at room temperature and were developed by placing it in the solvent system of chloroform: methanol (1:1 v/v) in a TLC glass tank until the solvent front reached a top-marked line. The plate was removed and left to dry, examined under ultraviolet light at 254 nm and R_f values were calculated.

The obtained pyocyanin solutions in 0.2 N HCl were subjected to UV-visible spectrophotometer analysis. The optical density spectrum between a wavelength of 200 and 800 nm was detected. The pure pyocyanin was taken along as standard control and the peaks of each extracted solution were recorded.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 19(3) 2023, pp. 282-290
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Statistical analysis

All tests were in triplicate. The data were analyzed by ANOVA with $p<0.05$ considered significant. Data was demonstrated by a bar graph, line graph or table where it was relevant.

RESULTS

Selection of solvents for pyocyanin extraction

Six solvents were used to extract pyocyanin from the growing bacterial culture, including benzene, hexane, methanol, dichloromethane, ethyl acetate and chloroform. Among them, benzene, hexane and ethyl acetate generated two separate layers, including no colour to light blue-green at the upper layer and dark blue-green at the lower layer (Figure 1A). Meanwhile, methanol in tube 3 was mixed entirely with broth culture and did not exhibit any phase separation. Dichloromethane and chloroform in tubes 4 and 6, respectively, created good separations in which the lower was blue and the upper layer was red-brown (Figure 1A).

In the next acidification step, two out of six tested solvents, dichloromethane and chloroform, separated the solution into two layers, yellow (lower) and red (upper) layers (Figure 1B). After being neutralized with 1 N NaOH, the extracts carried out by dichloromethane and chloroform changed into blue, which was similar to the control's colour (Figure 1C).

The result of pyocyanin quantity in the extracted solutions was presented in Table 2. As shown, the concentration of pyocyanin extracted with chloroform gave the highest extraction yield (25.27 ± 1.02 µg/mL), followed by dichloromethane (20.26 ± 0.88 µg/mL) and ethyl acetate generated the lowest (4.20 ± 0.64 µg/mL).

Table 2: Pyocyanin extraction yield (after 72 hours of cultivation) by different solvents .

No.	Solvents	Pyocyanin (µg/mL)
1	Benzene	10.50 ± 1.02
2	Hexane	11.24 ± 1.24
3	Methanol	12.42 ± 1.08
4	Dichloromethane	20.26 ± 0.88
5	Ethyl acetate	4.20 ± 0.64
6	Chloroform	25.27 ± 1.02

The purity of extracted pyocyanin

Pyocyanin extracted solutions by six tested solvents were examined for their purity by TLC technique and UV-Vis analysis. TLC chromatogram showed that, in all the lanes, blue bands appeared at the top of the plate, corresponding to a value R_f 0.72. Extraction with benzene, hexane, methanol and ethyl acetate showed brown bands at the bottom mark line of the TLC plate. Meanwhile, with dichloromethane and chloroform, the brown bands were not seen (lanes 4 and 6) nor in the control lane (Figure 2A).

The spectrum analysis of extracted pyocyanin in HCl solution showed that the solutions using dichloromethane and chloroform had similar spectra as pure pyocyanin. The highest peak in spectra of extracted pyocyanin from dichloromethane and chloroform was 274 nm. Pure pyocyanin and extracted from solvents both showed a peak at 520 nm, which was used to quantify the presence of pyocyanin (Figure 2B).

Effect of cultural parameters on pyocyanin production by *P. aeruginosa* PS39-phzMS

Temperature

The influence of temperature was determined by incubating *P. aeruginosa* PS39-phzMS culture at different temperature ranges from 20 °C to 45 °C. The obtained result indicated that the highest pyocyanin amount was seen at the temperature of 28 °C (24.04 ± 0.25 µg/mL pyocyanin) and 30 °C (24.24 ± 0.15 µg/mL pyocyanin). The yield was no significant difference between these examined two temperatures. However, the increase or decrease in temperature caused both the reduction in pyocyanin production; when the temperature reached 37 °C and 45 °C the produced pyocyanin concentration dropped to 7.26 ± 0.43 µg/mL and 3.7 ± 0.49 µg/mL, respectively (Figure 3A). Similarly, the culture temperature was set at a low level of 20 °C, pyocyanin yield was limited (3.56 ± 0.3 µg/mL). Therefore, a temperature range of 28–30 °C was considered optimal for the effective biosynthesis of pyocyanin by the *P. aeruginosa* PS39-phzMS.

Agitation speed

During fermentation, the concentration of oxygen gas (O_2) is an important factor for bacterial growth and the production of secondary metabolites. The various shaking speeds were examined at 50, 100, 150 and 200 rpm. As shown in Figure 3B, pyocyanin production was enhanced relating to agitation speed and reached the highest yield (23.97 ± 1.89 µg/mL) at 200 rpm compared to 16.66 ± 0.77 µg/mL at 100 rpm, 17.35 ± 1.4 µg/mL at 150 rpm and the lowest at 50 rpm (10.56 ± 0.27 µg/mL). So, the shaking rate at 200 rpm was the optimal speed in the tested range.

Incubation time

The incubation time played an important role in the pyocyanin production process because the secondary metabolite is secreted in the stationary phase of bacterial growth. For the *P. aeruginosa* PS39-phzMS, pyocyanin concentration measured after 24 h of cultivation was 14.51 ± 0.52 µg/mL. The compound yield increased gradually, depending on incubation time, until 120 h, with the highest amount of 34.04 ± 0.87 µg/mL. However, a rapid decrease of obtained pyocyanin was observed afterwards (19.68 ± 0.91 µg/mL at 144 h of culture period) (Figure 3C). As a result, the recommended harvesting

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 19(3) 2023, pp. 282-290
DOI: http://dx.doi.org/10.21161/mjm.220077

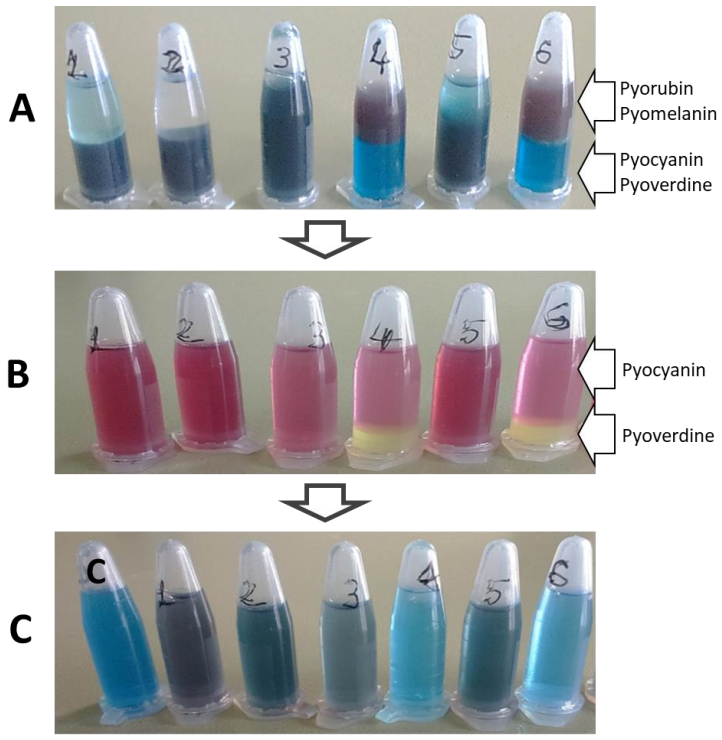

Figure 1: Selection of solvents for pyocyanin extraction process. A) Mixing the cell-free bacterial culture with different solvents; B) Acidification with 0.2 N HCl; C) Neutralization with 1 N NaOH (The used solvents were 1: benzene; 2: hexane; 3: methanol; 4: dichloromethane; 5: ethyl acetate; 6: chloroform).

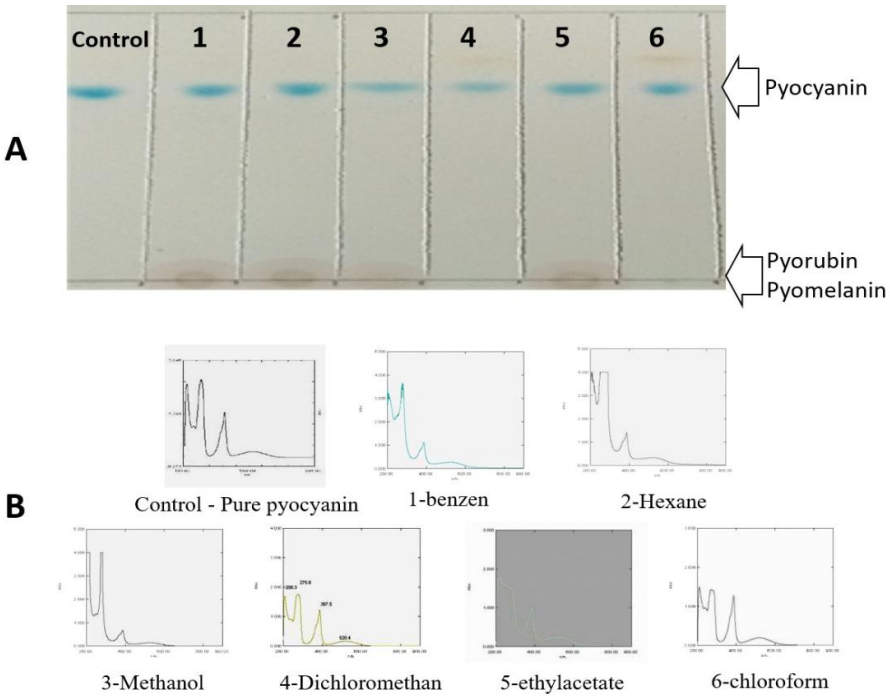

Figure 2: Purification of extracted pyocyanin using different solvents. A) TLC plate; B) UV-Vis spectra. Control - pure pyocyanin; 1 - benzene; 2 - hexane; 3 - methanol; 4 - dichloromethane; 5 - ethyl acetate; 6 - chloroform.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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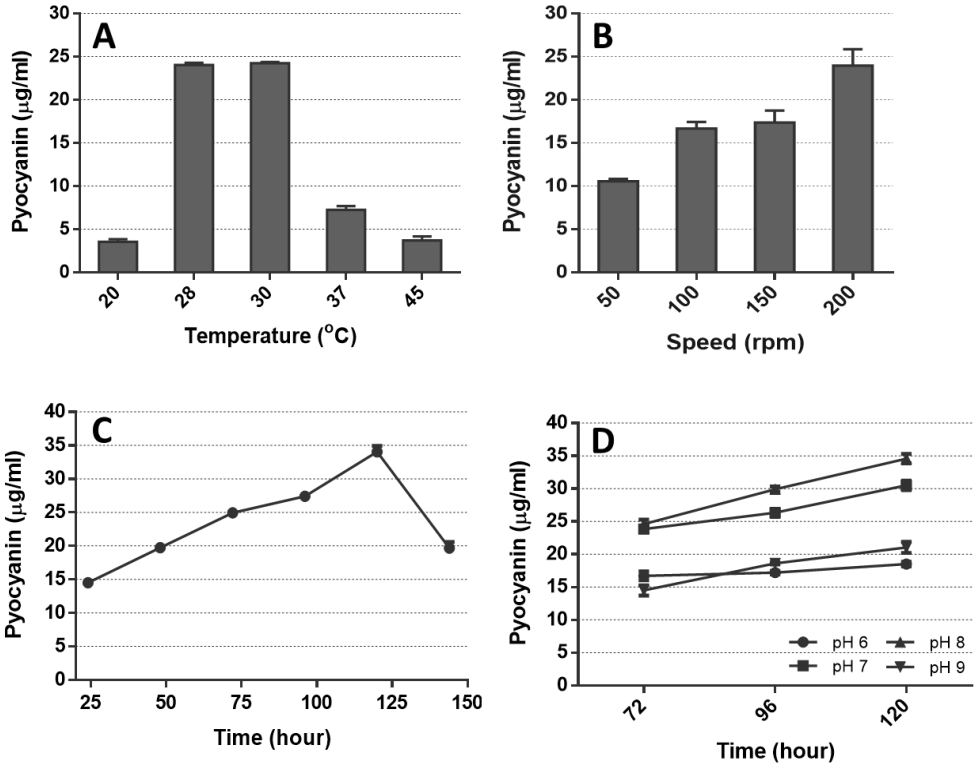

Figure 3: Effect of cultural conditions on pyocyanin production by *P. aeruginosa* PS39-phzMS. A: Effect of temperature, pyocyanin concentration displayed by bar graph with means and SD, t-test$_{28\ vs\ 30}$: p=0.3005; B: Effect of agitation speeds, ANOVA: p<0.0001; C: Effect of incubation time; D: Effect of pH, pyocyanin concentration displayed by line graph with means and SD, ANOVA: p<0.0001.

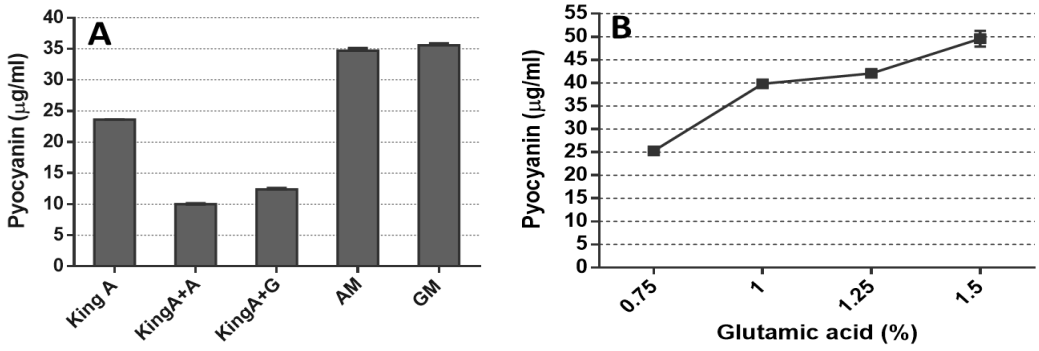

Figure 4: Effect of medium components on pyocyanin production by *P. aeruginosa* PS39-phzMS. A: Effect of additional nitrogen source, KingA: KingA medium, KingA+A: KingA + Alanine, KingA+G: KingA + Glutamic acid, AM: KingA + Alanine without peptone, GM: KingA + Glutamic acid without peptone, pyocyanin concentration displayed by bar graph with means and SD, t-test$_{AM\ vs\ GM}$: p<0.05; B: Effect of glutamic acid concentration, pyocyanin displayed by line graph with means and SD, ANOVA: p<0.0001.

time of pyocyanin produced by *P. aeruginosa* PS39-phzMS in flask scale was 120 h.

pH value

The effect of pH was determined by cultivating *P. aeruginosa* PS39-phzMS in media adjusted to different pH (6, 7, 8 and 9), inoculated with 10% of inoculum, at 30 °C and 200 rpm. As a result, was seen in Figure 3D, pyocyanin production increased significantly at medium with pH 8 after 120 h of cultivation and reached maximum yield (34.6 ± 0.71 µg/mL). In contrast, at medium with pH 9, the produced pyocyanin amount (21.06 ± 0.85 µg/mL at 120 h) reduced significantly (p<0.05) compared to that of

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 19(3) 2023, pp. 282-290
DOI: http://dx.doi.org/10.21161/mjm.220077

the higher medium with pH 8 and corresponded to that of the medium with pH 6 (18.5 ± 0.34 µg/mL at 120 h).

Nitrogen sources

Four modified media based on classic KingA, were applied for pyocyanin production by *P. aeruginosa* PS39-phzMS. Pyocyanin yield was significantly different among these media, where the lowest production was found in two media supplemented with alanine and glutamic acid, namely KingA+A and KingA+G. In contrast, pyocyanin amount enhanced significantly in the other two modified media, AM and GM, where the KingA medium's peptone was entirely replaced by alanine and glutamic acid, respectively. The concentration of pyocyanin produced in AM and GM media were 34.71 ± 0.35 µg/mL and 35.57 ± 0.26 µg/mL, respectively (Figure 4A).

Alanine and glutamic acid were both suitable for pyocyanin production; however, glutamic acid showed a higher amount (t-test, p=0.0277) and was chosen for the next steps of the optimization process. The effect of glutamic acid concentration was tested within the range of 0.75 and 1.5% (w/v). The glutamic acid concentration at 0.75% did not increase pyocyanin concentration, but the higher concentration increased the pyocyanin production up to 49.57 ± 1.71 µg/mL at 1.5% glutamic acid (Figure 4B).

DISCUSSION

Pseudomonas aeruginosa attracts attention as bacterium with many applications in biotechnology and capable of producing antibacterial secondary metabolism compounds. Pyocyanin produced by these microbacteria has shown the properties of broad-spectrum antimicrobial activity, antifungal activity and pesticide degradation; moreover, pyocyanin also possesses a unique characteristic of biological control (Saha *et al.*, 2008; Babu *et al.*, 2015; Satapute and Kaliwal, 2016). Thanks to these properties, *P. aeruginosa* producing pyocyanin is applied in several fields, such as environmental remediation, the food industry and agriculture (DeBritto *et al.*, 2020). Therefore, in this study, the selection of proper solvent for extracting the compound and the characteristics of purified pyocyanin were studied. At the same time, the culture conditions were optimized for pyocyanin production by *P. aeruginosa* PS39-phzMS.

As shown by the obtained results, benzene, hexane and ethylacetate formed separated layers during the pyocyanin extraction but did not remove the pyorubin (red) and pyomelanin (brown) out of the culture broth, which contained pyocyanin. In contrast, dichloromethane and chloroform created good separations in which the top layer consisted of pyorubin and pyomelanin, and the bottom was a mixture of pyocyanin (blue) and pyoverdine (yellow) (Figure 1A). The bottom layer was green blue contained solvent and pyocyanin compound (green blue) and the top layer (red-brown) holding the other components (Figure 1A). The solvent layers which included pyocyanin were collected and acidized by adding 0.2 N HCl. Pyocyanin dissolved in HCl phase and generated pink solutions, and pyoverdine remained in solvent layers. Dichloromethane and chloroform were the appropriate solvents as they could keep pyoverdine out of pyocyanin (Figure 1B). The acid phases were neutralized using 1 N NaOH to neutral pH to yield pyocyanin solutions. Pyocyanin solutions extracted by dichloromethane and chloroform showed the typical blue color similar to pure pyocyanin (Figure 1C). Chloroform was the best solvent for pyocyanin extraction, agreeing with other studies (Cheluvappa, 2014; El-Fouly *et al.*, 2015). The pyocyanin extracted from the tested solvents had R_f of 0.72 on thin-layer chromatography. The UV-Vis of the solvent extracts showed absorbance peaks from 274 to 280 nm, which means pyocyanin produced by *P. aeruginosa* PS39-phzMS shared the same characteristics as pyocyanin in the studies of Stephen, Abdul-Hussein and others (Baron and Rowe, 1981; Ohfuji *et al.*, 2004; Sudhakar *et al.*, 2013; Abdul-Hussein and Atia, 2016; Dahah *et al.*, 2016).

In the combination of the production medium GM and the optimized culture conditions of pH 8, at 30 °C under shaking at 200 rpm for 120 h of cultivation, the yield of pyocyanin production rose to 49.57 ± 1.71 µg/mL (Figure 4B). The quantity was 159% higher than that of pyocyanin obtained before optimization of pH 7.2, at 30 °C with the agitated condition at 200 rpm for 120 h of cultivation in KingA medium (Vinh *et al.*, 2022). Abo-Zaid *et al*. (2015) optimized the pyocyanin production of *P. aeruginosa* isolate JY21 and proposed that the optimal conditions for JY21 were pH 8, 30 °C, which is similar to *P. aeruginosa* PS39-phzMS. But the optimal harvesting time was different; 72 h was the best time course for *P. aeruginosa* isolate JY21, while a more extended time was required for *P. aeruginosa* PS39-phzMS (Abo-Zaid *et al.*, 2015). The nature of these pyocyanin-producing isolates could explain the difference as they belong to a similar species but are different strains, leading to distinguishing growing characteristics and pyocyanin production time course. Culture conditions differ from *P. aeruginosa* strains for pyocyanin production; for example, *P. aeruginosa* studied by Liang *et al.* (2011) grew at pH 7 and 30 °C with a maximum yield of 10 µg/mL. Similar results showed that *P. aeruginosa* strains PA14 produce pyocyanin at pH 7 and 30 °C (Das *et al.*, 2013). The optimal pH is different from *P. aeruginosa* PS39-phzMS; however, the optimal temperatures are similar to this study.

The improved medium GM, in which peptone was replaced by glutamate 1.5%, showed a significant difference in pyocyanin production compared to the original KingA medium. The data confirmed the relationship between the nitrogen source and the pyocyanin production yield of *P. aeruginosa* strain. The trend was in line with other experimental studies that changed the nitrogen source to improve pyocyanin concentration. Peptone was replaced with additional nitrogen and nutrient sources such as soybean, corn and peanut, among which soybean showed an advantage as it increased pyocyanin concentration three times higher than peanut (DeBritto *et al.*, 2020). The stirring speed was

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

the essential factor in the reactor fermentation for *P. aeruginosa* PS39-phzMS; the optimal agitation speed was 200 rpm. This yield makes *P. aeruginosa* PS39-phzMS is a suitable candidate for the application scale. The ideal component of pyocyanin-producing medium for *P. aeruginosa* PS39-phzMS is 10 g/L K_2SO_4; 3 g/L $MgCl_2$; 10 mL/L glycerol and 15 g/L glutamic acid. At pH 8 and 30 °C, after 120 h of cultivation in GM medium with 1.5% glutamic acid, combined with an optimal speed of 200 rpm, the pyocyanin reached its highest at 49.57 µg/mL, and it was approximately 3.7 and 1.6 folds more elevated than the wild-type *P. aeruginosa* PS39 and the recombinant *P. aeruginosa* PS39-phzMS, respectively (Vinh *et al.*, 2021). The data indicated that alanine and glutamic acid could be the suitable alternative for nitrogen sources for *P. aeruginosa* PS39-phzMS to produce a high amount of pyocyanin.

P. aeruginosa PS39-phzMS and its pyocyanin possess high value in the aquaculture industry since they exhibited their antibacterial and antifungal activity. Notably, they inhibited *Vibrio* sp., causing diseases in shrimp such as *V. parahaemolyticus*, *V. harveyi* and *V. alginolyticus* (Vinh *et al.*, 2018; Vinh *et al.*, 2020). However, there are currently limited publications on pyocyanin's ability to resist *Vibrio* bacteria in aquatic products, especially *Vibrio*-causing disease in shrimp. Therefore, this study provided more fundamental knowledge on generating a high amount of pyocyanin through bacterial fermentation and extraction by chloroform with the outlook of industrial application in aquaculture, specifically shrimp farming.

CONCLUSION

This study optimized culture conditions and medium components for producing maximal pyocyanin yield of the recombinant strain *P. aeruginosa* PS39-phzMS. Fermentation of PS39-phzMS in pH 8 GM medium with shaking at 200 rpm, 30 °C and incubation time of 120 h produced the highest (49.57 µg/mL) pyocyanin amount. In addition, extraction of pyocyanin by chloroform recovered the highest amount of pyocyanin and its quality.

ACKNOWLEDGEMENTS

This study was granted by project no. 10/2017 belonging to Agricultural - Aquacultural Biotechnology Program, Ministry of Agriculture and Rural Development, Vietnam.

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