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Original article

Optimization of nutritional and environmental conditions for pyocyanin production by urine isolates of *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa (*P. aeruginosa*) is a highly pathogenic bacteria involved in numerous diseases among which, are urinary tract infections (UTIs). The pyocyanin secreted as a virulence factor by this bacterium has many beneficial applications but its high cost remains an obstacle for its widespread use. In this study, a total of fifty urine isolates were identified as *P. aeruginosa*. All strains produced pyocyanin pigment with a range of 1.3–31 μg/ml. The highest producer clinical strain P21 and the standard strain PA14 were used in optimization of pyocyanin production. Among tested media, king's A fluid medium resulted in the highest yield of pyocyanin pigment followed by nutrient broth. Growth at 37 °C was superior in pyocyanin production than growth at 30 °C. Both shaking and longer incubation periods (3–4 days) improved pyocyanin production. The pyocyanin yield was indifferent upon growth of P21 at both pH 7 and pH 8. In conclusion, the optimum conditions for pyocyanin production are to use King's A fluid medium of pH 7 and incubate the inoculated medium at 37 °C with shaking at 200 rpm for a period of three to four days.

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1. Introduction

One bacterium that is commonly isolated either from natural surroundings or infected human body systems is *P. aeruginosa*. This bacterium can infect human, animals, plants and nematodes (Jander et al., 2000). The host versatility originates from the widespread of *P. aeruginosa* naturally in soil and water along with its long survival on dry surfaces which facilitates spread of infections especially nosocomial one. *P. aeruginosa* is usually detected in patients with cystic fibrosis, wounds, burns, and urinary tract infections (Mittal et al., 2009). Several virulence factors are accused for the pathogenicity of *P. aeruginosa* including enzymes, toxins and pigments (Frank, 1997). The blue-green pyocyanin pigment is a well-known phenazine pigment produced by about 95% of *P. aeruginosa* strains. It is considered a biomarker for identification of *P. aeruginosa*. Seven genes are involved in the biosynthetic pathway of pyocyanin but *phzM* and *phzS* are the principle ones in conversion of phenazine-1-carboxylic acid to pyocyanin (Mavrodi et al., 2001).

Pyocyanin was reported to have harmful effects on different body systems through formation of reactive oxygen species (ROS). The zwitterion nature beside its low molecular weight facilitate its permeation to cell membranes (Hall et al., 2016). A fact that was confirmed by detection of pyocyanin with variable concentrations in secretions and fluids associated with infections such as phlegm, ear discharge and urine (Wilson et al., 1988; Reimer, 2000). These observations indicate the high possibility of the pathophysiological role of pyocyanin in *P. aeruginosa* infections.

The ability of pyocyanin to generate ROS along with its electron transferable nature give it a wide array of applications in different fields such as medicine, agriculture, and industry with a positive impact on the environment (Venil et al., 2013). In medicine, pyocyanin was reported as anticancer for its cytotoxic effect on tumor cells (Zhao et al., 2014), and as antimicrobial against numerous pathogens which suggested its use to treat diseases caused by them (Rani et al., 2018). In agriculture, pyocyanin proved its effectiveness in improving plant growth through combating phytopathogens and decreasing toxicities resulting from chemicals used as fungicides and pesticides (Babu et al., 2015). In industry, pyocyanin can be used as a redox mediator in biosensors (Priyaja, 2012) and microbial fuel cells (Boon et al., 2008), a food

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colorant (Heer and Sharma, 2017), a preservative through its antimicrobial and antibiofilm activity against food pathogens (Rani et al., 2018), and a textile colorant (DeBritto et al., 2020).

Due to the numerous helpful applications of pyocyanin, studies must be directed to enhance its production through fermentation process with lower cost rather than its production by synthetic pathway which is very expensive. So, this study was designed to evaluate pyocyanin production from strains of *P. aeruginosa* isolated from urinary tract infection. The study selected the highest producer strain to be used in defining simple conditions for a high yield of pyocyanin pigment with clear parameters that could be conducted everywhere and thus maintain good reproducibility and economic value.

2. Materials and methods

2.1. Microorganisms

This study was approved by the research ethics committee, Faculty of Pharmacy, Mansoura University, Egypt. Urine samples were collected from patients with urinary tract infections (UTIs) admitted to Urology and Nephrology center at Mansoura university. Samples were handled aseptically, cultured in nutrient broth, then streaked on nutrient agar plates. Separated bacterial colonies were subjected to conventional identification steps including gram stain and biochemical activities (MacFaddin, 2000) and isolates identified as *P. aeruginosa* were selected for further studies. A standard strain PA14, was used as a pyocyanin producing reference strain.

2.2. Pyocyanin pigment production

The test strains were cultured overnight in King's A fluid medium. Then the optical densities of the grown cultures were adjusted to 0.257 at λ_{600} and then inoculated in the same medium at 37 °C for 48 h with shaking at 200 rpm. For each bacterial culture, five ml were taken, centrifuged, and 3 ml of chloroform are then added to the supernatant then centrifuged at 1300 rpm for 10 min on cold. The extracted pigment was transferred to a new tube containing 1 ml of 0.2 N HCl, mixed and centrifuged. The absorbance of the pink colored aqueous layer was measured at λ_{520} nm. The quantity of pyocyanin produced by each strain was calculated in micrograms by multiplying the optical density at 520 nm by 17.072 (Kurachi, 1958; Essar et al., 1990). Strain No. 21 (P21) was selected for subsequent experiments.

2.3. Factors affecting pyocyanin pigment production

All experiments done in this section were conducted typically as mentioned in Section 2.2 except for the factor under study. PA14 was used as a reference strain.

2.3.1. Effect of culture media

P21 and PA14 were tested for pyocyanin pigment production in different media; nutrient broth, Luria Bertani (LB) broth, King's A fluid medium and king's A fluid medium supplemented with cetrimide 0.03%. The amount of pyocyanin pigment produced in each medium was calculated and the medium/media achieved the highest production for both the clinical and the standard strains was/were used in the upcoming experiments.

2.3.2. Effect of shaking

P21 and PA14 were inoculated in nutrient broth and King's A fluid medium. One set of inoculated flasks was kept at static condition while the other set was agitated at 200 rpm.

2.3.3. Effect of temperature

The pyocyanin pigment production by P21 and PA14 was estimated in nutrient broth and King's A fluid medium at 30 °C and 37 °C with shaking at 200 rpm. The amount of pyocyanin pigment produced at both temperatures was calculated and the temperature achieved the highest production for both the clinical and the standard strains was applied on the forthcoming experiments.

2.3.4. Effect of pH of the medium

Only two pH values 7 and 8 were selected to study their effect on pyocyanin production where the pH of nutrient broth and king's A fluid medium was initially adjusted to the required pH prior to sterilization and culturing processes.

2.3.5. Effect of incubation period

P21 and PA14 were inoculated in nutrient broth and King's A fluid medium at 37 °C under both static and shaking conditions at 200 rpm. Five ml from each test culture were harvested after 24 h, 48 h, 72 h, and 96 h and processed as done previously to determine the amount of the pyocyanin pigment produced.

2.4. Statistical analysis

All experiments were performed in triplicates and results were expressed as the mean of three readings ± standard deviation. SPSS (version 13; SPSS Inc.) software was used to carry out statistical analysis using student's *t*-test for paired data. A probability (P) of < 0.05 was considered statistically significant.

3. Results

3.1. Microorganisms

In the present study, a total of 50 isolates from UTIs were identified as *P. aeruginosa* based on their morphological and biochemical characteristics.

3.2. Pyocyanin pigment production

All clinical strains of *P. aeruginosa* were tested for pyocyanin pigment production. The strains were divided into four categories based on concentration of produced pyocyanin, Fig. 1. Most of clinical strains (78%) produced 10 μg/ml or less of pyocyanin pigment, seven strains (14%) exceeded 10 μg/ml to a maximum of 20 μg/ml of pyocyanin while only four strains formed more than 20 μg/ml of pyocyanin. The highest pyocyanin yield (31 μg/ml) was recorded for strain No.21 (P21) which was then selected for further investigations.

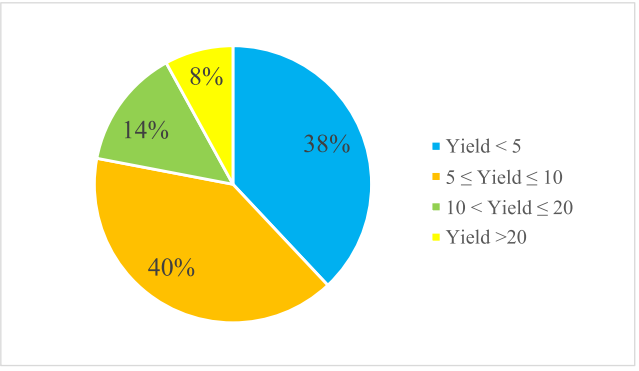

Fig. 1. Pyocyanin yield among clinical strains of *P. aeruginosa*.

3.3. Factors affecting pyocyanin pigment production

3.3.1. Effect of culture media on pyocyanin production

Table 1 shows the significant difference in pyocyanin production between the investigated culture media. The sequential order of the four tested media according to pyocyanin yield is King's A fluid medium > nutrient broth > King's A fluid medium with cetrimide 0.03% > LB broth medium, Fig. 2.

3.3.2. Effect of shaking on pyocyanin production

As shown in Fig. 3, shaking inoculated flasks caused significant increase in pyocyanin pigment yield ($p < 0.05$) compared to static condition where 0.2 and 0.3 fold increases in pyocyanin production in King's A fluid medium were recorded for P21 and PA14, respectively. Similarly, 1.2 and 0.7 fold increases in pyocyanin yield by P21 and PA14 grown in nutrient broth were observed.

3.3.3. Effect of temperature on pyocyanin production

Compared to pyocyanin yield at 37 °C, the amount of pyocyanin produced by the two tested strains at 30 °C in King's A fluid medium and nutrient broth was significantly reduced by a range of 32.5–81.4% ($p < 0.05$), Fig. 4.

3.3.4. Effect of pH on pyocyanin production

For P21, no significant difference in pyocyanin production was detected upon growth at pH 7 or pH 8 in both King's A medium and nutrient broth. On the contrary, significant decrease in pyocyanin production was observed when PA14 were grown at pH 8 either in nutrient broth or King's A fluid medium in comparison to pyocyanin yield at pH 7, Table 2.

3.3.5. Effect of incubation period on pyocyanin production

Fig. 5 shows the effect of incubation period on pyocyanin yield. In King's A fluid medium, an incubation time ranged from 24 h to 96 h resulted in a significant gradual increase in pyocyanin production that reached a comparable yield for both static and agitated cultures after 96 h of incubation, Table 3. However, the increase in pyocyanin yield in case of nutrient broth was significant in the first 48 h of incubation followed by a slight decrease or steadiness in the yield in agitated flasks, Table 4.

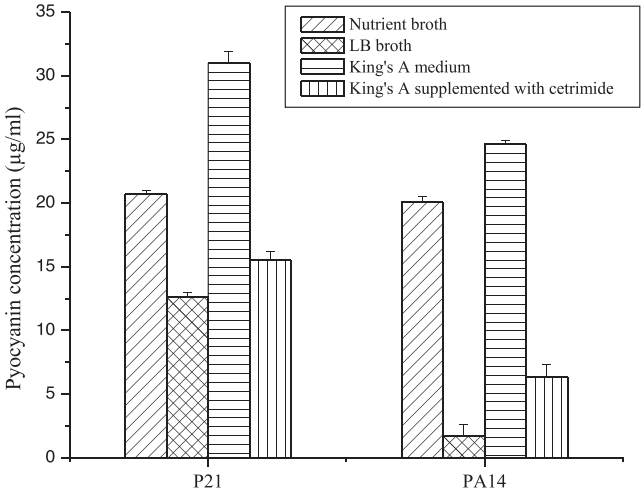

Fig. 2. Effect of media on pyocyanin production.

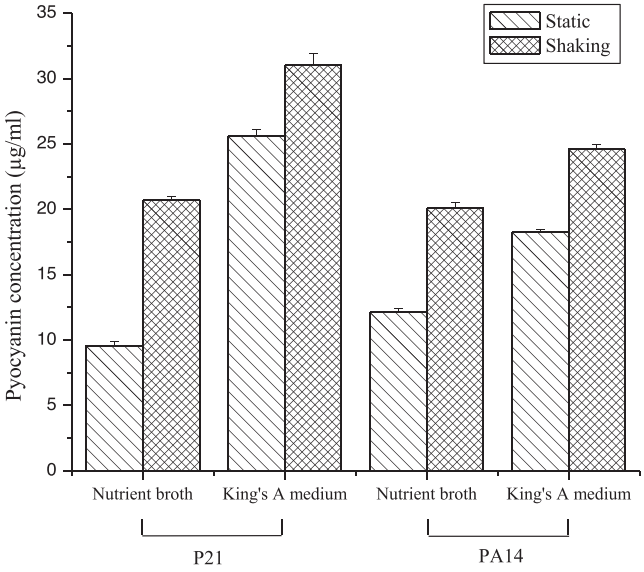

Fig. 3. Effect of shaking on pyocyanin production.

4. Discussion

P. aeruginosa is a highly resistant pathogenic bacteria that accounts for several infections among which, is urinary tract infection (Tumbarello et al., 2020). The ability of most *P. aeruginosa* strains to form biofilms and adhere to urinary catheters and urothelium makes urinary catheterized patients at high risk for developing UTIs (Mittal et al., 2009). Most of research studies have been focused on the effect of pyocyanin; a secondary metabolite secreted by *P. aeruginosa*; on human respiratory system. Lately, more studies were directed to explain pyocyanin effect on different human body systems with special concern to urinary tract (Al-Ani et al., 1986; McDermott et al., 2013).

Table 1
Mean pyocyanin concentration differences between the tested media and their *P*- values.

Strain		P21			P14		
Medium		Nutrient broth	LB broth	King's A fluid medium	Nutrient broth	LB broth	King's A fluid medium
LB broth	Mean difference	8.10000			18.36000		
	Significance level (P)	$p < 0.001$*			$P < 0.001$*		
King's A medium	Mean difference	−10.30000	−18.40000		−4.54000	−22.90000	
	Significance level (P)	$P = 0.001$	$P < 0.001$*		$P < 0.001$*	$P < 0.001$*	
King's A medium + 0.03% cetrimide	Mean difference	5.20000	−2.90000	15.50000	13.74000	−4.62000	18.28000
	Significance level (P)	$P = 0.002$*	$P = 0.004$*	$P < 0.001$*	$P = 0.001$*	$P < 0.001$*	$P < 0.001$*

* significant.

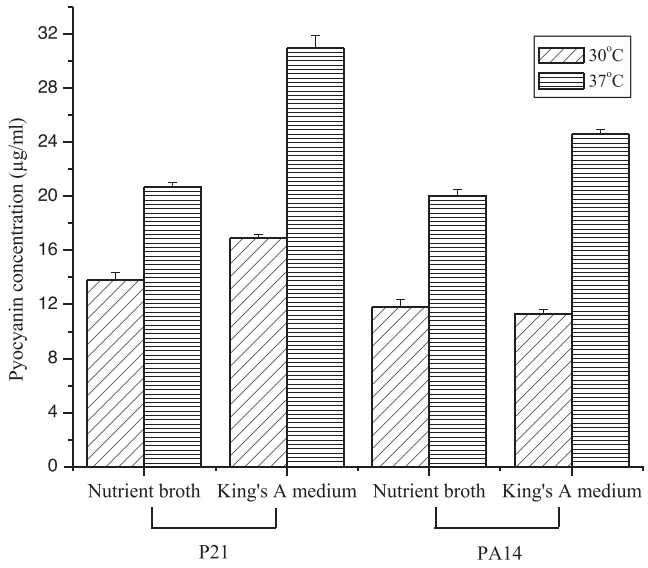

Fig. 4. Effect of temperature on pyocyanin production.

In the present study, fifty urinary isolates were identified as *P. aeruginosa*. Pyocyanin production assay showed that all *P. aeruginosa* strains were able to produce pyocyanin with variable quantities (1.3 to 31 µg/ml). A previous study had demonstrated higher yield of pyocyanin produced by *P. aeruginosa* urine isolates in comparison to that produced by *P. aeruginosa* isolated from other clinical specimens (Al-Ani et al., 1986). The variation in pyocyanin production among tested strains can be explained by the presence or absence of certain gene regulators that may have a positive or negative impact on production of bacterial secondary metabolites. QteE is an example of such regulators in *P. aeruginosa* when over expressed, it decreases homoserine lactone signals which in turn affects pyocyanin production (Liang et al., 2011; Beasley et al., 2020).

The high cost of pyocyanin production artificially stands behind appearance of many researches that aimed to optimize nutritional and environmental conditions required for its production (Abo-Zaid et al., 2015; El-Fouly et al., 2015; Sadeghnia et al., 2017; Ozdal, 2019).

The current study evaluated number of factors that were reported previously to affect pyocyanin production. For media effect, results indicated that King's A fluid medium is the best medium to use for production in comparison to nutrient broth, LB broth and King's A medium supplemented with cetrimide. This finding can be explained by the large impact of carbon and nitrogen sources in the growth medium on the amount of pyocyanin produced (Young, 1947; Chang and Blackwood, 1969; Jayaseelan et al., 2014). The same result was reported in former studies (Devnath et al., 2017a, 2017b; DeBritto et al., 2020). As known, King's A fluid medium comprises important nutritive sources besides inorganic salts namely peptone, glycerol, $MgCl_2$, and K_2SO_4 in contrast to low energy sources media such as nutrient broth. These variations in media composition largely affect the bacterial growth yield and accordingly, the yield of pyocyanin pigment (Devnath et al., 2017a, 2017b). Peptone has proved high nutritive value originated from its amino acids content which enhances secondary metabolites production either by induction of important biosynthetic enzymes or by rising the amount of limiting precursors (Demain, 1998). Glycerol also was recited by several studies to be the most essential carbon source that augments both *P. aeruginosa* growth yield and pyocyanin formation (El-Fouly et al., 2015; Özcan and Kahraman, 2015).

In the current study, pyocyanin production was enhanced upon shaking inoculated flasks rather that incubation statically. This may be due to the effect of shaking in increasing aeration of the medium which is consistent with the aerobic nature of *P. aeruginosa* (Rani et al., 2018).

Regarding effect of pH on pyocyanin production, the selection of limited pH values (7 and 8) in this study is based on the previous published data which suggested that this pH range is the most suitable for *P. aeruginosa* growth (Sankaralingam et al., 2014). In addition, highest pyocyanin production has been linked to maximum growth yield which in turn affected by pH of the growth medium (Barakat et al., 2015). The results showed no significant difference in pyocyanin yield upon growth of the clinical strain P21 at pH 7 and pH 8 in both media under study. For P14, Pyocyanin production significantly increased at pH 7 compared to pH 8. This result coincides with Kurachi's earlier study which reported that a pH range of 7.4 to 8.4 is optimal for pyocyanin production (Kurachi, 1958).

Pyocyanin production reached its maximum at 37 °C for P21 and PA14 when grown either in nutrient broth or King's A fluid medium. This finding agrees with Rani et al., who tested a wide range of temperatures (25 °C to 50 °C) confirming that 37 °C is the optimum temperature for pyocyanin production (Rani et al., 2018). Same findings were reported earlier in 1958 and explained by reduction of growth rate at reduced temperature and conversion of preformed pigments into brownish blue before full production of pyocyanin pigment (Kurachi, 1958).

To study the effect of incubation period, pyocyanin production was estimated through 4 successive days with 24 h interval. The recorded data revealed that there was a gradual significant increase in pyocyanin yield from day 1 to day 4 in King's A fluid medium. On the contrary, in nutrient broth a non-significant decrease or steadiness in pyocyanin yield was recorded for P21 (shaking), PA14 (shaking) after day 2 (48 h) and P21 (static) after day 3 (72 h). However, shaking significantly improved pyocyanin yield within the first two days in both media compared to static conditions. This can be explained by the obvious effect of the nutritional components concentration on the optimum period to get highest yield of pyocyanin pigment in addition to the possibility of the gradual oxidation of the formed pyocyanin after longer incubation periods (Kurachi, 1958). These findings are in accordance with a published study by Rani et al., who noticed a gradual increase in pyocyanin yield up to 72 h of incubation, followed by a slight decrease in the yield (Rani et al., 2018).

Table 2
Effect of pH on pyocyanin production.

Strains	Pyocyanin concentration (µg/ml) Nutrient broth		Significance level	Pyocyanin concentration (µg/ml) King's A fluid medium		Significance level
	pH 7	pH 8		pH 7	pH 8	
P21	20.70 ± 0.31	19.80 ± 0.7	$P = 0.057$	31.00 ± 0.92	35.25 ± 0.19	$P = 0.01$
PA14	20.06 ± 0.43	13.70 ± 0.9	$P = 0.002$*	24.60 ± 0.34	19.60 ± 0.67	$P = 0.001$*

* Significant.

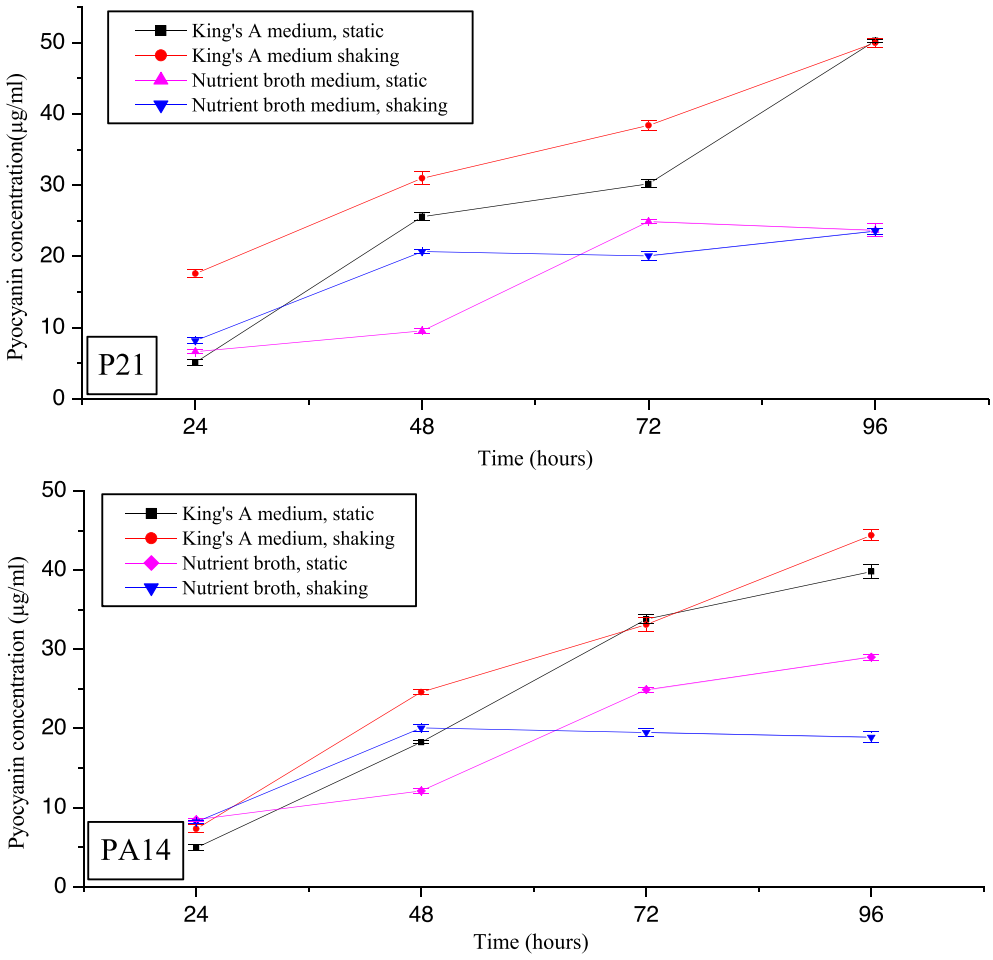

Fig. 5. Effect of incubation time along with shaking on pyocyanin production.

5. Conclusion

P. aeruginosa is a main cause of urinary tract infections especially hospital acquired type. All strains of *P. aeruginosa* secreted the pyocyanin pigment in variable concentrations, this pigment is highly valuable in different life aspects and so optimizing conditions to enhance its production is a necessity. The current study defined nutritional and environmental conditions for pyocyanin formation using PA14 and one clinical isolate that proved highest production of the pyocyanin pigment. The favorable production conditions are inoculation of King's A fluid medium previously adjusted at pH7, and incubation at 37 °C with shaking at 200 rpm for a period of three to four days.

Declaration of Competing Interest

The author declares that there is no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 3
Mean pyocyanin concentration differences between the investigated incubation periods with or without shaking in King'A medium.

King's fluid medium			P21						PA14					
			Shaking			Static			Shaking			Static		
			24 h	48 h	72 h	48 h	72 h	96 h	24 h	48 h	72 h	48 h	72 h	96 h
Static	24 h	Mean difference Significance level (*P*)	−12.48000 *P* < 0.001*			−20.49000 *P* < 0.001*	−25.08000 *P* < 0.001*	−45.18000 *P* < 0.001*	−2.39000 *P* = 0.001*			−13.32000 *P* < 0.001*	−28.85000 *P* < 0.001*	−34.85000 *P* < 0.001*
	48 h	Mean difference Significance level (*P*)		−5.39000 *P* = 0.002*			−4.59000 *P* < 0.001*	−24.69000 *P* < 0.001*		−6.33000 *P* < 0.001*			−15.53000 *P* < 0.001*	−21.53000 *P* < 0.001*
	72 h	Mean difference Significance level (*P*)						−20.10000 *P* < 0.001*						−6.00000 *P* < 0.001*
Shaking	48 h	Mean difference Significance level (*P*)	−13.40000 *P* < 0.001*						−17.26000 *P* < 0.001*					
	72 h	Mean difference Significance level (*P*)	−20.80000 *P* < 0.001*	−7.40000 *P* < 0.001*			−8.20000 *P* < 0.001*		−25.76000 *P* < 0.001*	−8.50000 *P* = 0.001*			0.70000 *P* = 0.070	
	96 h	Mean difference Significance level (*P*)	−32.40000 *P* < 0.001*	−19.00000 *P* < 0.001*	−11.60000 *P* < 0.001*			0.30000 *P* = 0.314	−37.06000 *P* < 0.001*	−19.80000 *P* < 0.001*	−11.30000 *P* < 0.001*			−4.6000 *P* < 0.001*

* Significant.

Table 4
Mean pyocyanin concentration differences between the investigated incubation periods with or without shaking in nutrient broth medium.

Nutrient Broth medium			P21						PA14					
			Shaking			Static			Shaking			Static		
			24 h	48 h	72 h	48 h	72 h	96 h	24 h	48 h	72 h	48 h	72 h	96 h
Static	24 h	Mean difference Significance level (*P*)	−1.53000 *P* = 0.001*			−2.90000 *P* < 0.001*	−18.24000 *P* < 0.001*	−17.04000 *P* < 0.001*	0.30000 *P* = 0.009*			−3.62000 *P* < 0.001*	−16.40000 *P* < 0.001*	−20.50000 *P* < 0.001*
	48 h	Mean difference Significance level (*P*)		−11.14000 *P* < 0.001*			−15.34000 *P* < 0.001*	−14.14000 *P* = 0.001*		−7.94000 *P* < 0.001*			−12.78000 *P* < 0.001*	−16.88000 *P* < 0.001*
	72 h	Mean difference						1.20000						−4.10000
		Significance level (*P*)						*P* = 0.097						*P* < 0.001*
Shaking	48 h	Mean difference	−12.51000						−11.86000					
		Significance level (*P*)	*P* < 0.001*						*P* < 0.001*					
	72 h	Mean difference	−11.91000	0.60000			4.80000		−11.30000	0.56000			5.40000	
		Significance level (*P*)	*P* < 0.001*	*P* = 0.178			*P* = 0.005*		*P* < 0.001*	*P* = 0.015			*P* = 0.001*	
	96 h	Mean difference	−15.37000	−2.86000	−3.46000			0.14000	−10.70000	1.16000	0.60000			10.10000
		Significance level (*P*)	*P* < 0.001*	*P* < 0.001*	*P* = 0.005*			*P* = 0.692	*P* = 0.001*	*P* = 0.015	*P* = 0.015			*P* < 0.001*

* Significant.

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