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Enhancement of pyocyanin production by *Pseudomonas aeruginosa* via the addition of *n*-hexane as an oxygen vector

Murat Ozdal [a,*], Sumeyra Gurkok [a], Ozlem Gur Ozdal [a], Esabi Basaran Kurbanoglu [a]

[a] Department of Biology, Science Faculty, Ataturk University, 25240, Erzurum, Turkey

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ABSTRACT

Pseudomonas aeruginosa is capable of producing a wide range of industrially important biotechnological products like pigment, biosurfactants, enzymes, etc. Pyocyanin is a pigment produced by *P. aeruginosa* and widely used in many industries such as food, agriculture, pharmaceuticals and cosmetics. In order to increase the production of pyocyanin, in this study, the effects of previously untested oxygen vectors on *P. aeruginosa* OG1 pyocyanin production were studied. The oxygen vectors (*n*-hexadecane, *n*-dodecane and *n*-hexane) were compared in terms of pyocyanin production. All of the oxygen vectors were found to exhibit a stimulatory effect on pyocyanin production and the best result (11.96 mg/L) was obtained with *n*-hexane. In the next step, pyocyanin production in the presence of *n*-hexane, was optimized by response surface methodology. In addition to the *n*-hexane concentration, the optimum size of inoculum and the time of *n*-hexane addition to the fermentation liquid were investigated. Experimental design and the results were analysed by using Box-Behnken design of Response Surface Methodology (RSM). Accordingly, the optimum size of inoculum was found to be 2.07%, *n*-hexane concentration was 3.48% and the addition time was 33.93 h and at these optimum values, the amount of pyocyanin increased 2-fold (21.2 mg/L). According to these results, *n*-hexane addition as an oxygen carrier could be a useful technique in industrial production of pyocyanin.

1. Introduction

Pseudomonas aeruginosa is capable of producing a wide range of industrially important biotechnological products such as biosurfactant, enzymes and pigments. Pyocyanin is one of the pigments produced by *P. aeruginosa* and widely used in many industries such as food, pharmaceuticals and cosmetics. Pyocyanin is a nitrogen-containing heterocyclic compound with blue-green color. It has antioxidant (Laxmi and Bhat, 2016), antiprotozoal, antifungal (Patil et al., 2017), anticancer (Zhao et al., 2014) properties. In order to increase the production of pyocyanin, fermentation media as well as environmental conditions have been optimized in previous studies (Jayaseelan et al., 2014; Moayedi et al., 2017; Iiyama et al., 2017; Ozdal, 2019).

The introduction of oxygen into fermentation media constitutes one of the determinants of the cultures of aerobic microorganisms and plays an important role in the growth and economics of large-scale fermentation systems (Galaction et al., 2015). The compounds that are added to the fermentation medium and improve the oxygen transfer to microorganisms are defined as oxygen vectors (Cascaval et al., 2006). Oxygen vectors are used as an alternative to increase the oxygen solubility (Lai et al., 2012; Häusler et al., 2016). The oxygen solubility in these compounds is much higher than that of water. The main oxygen vectors tested in biotechnology are oils used as hydrocarbons, perfluorocarbons and defoaming agents (Zhu et al., 2014). Oxygen vectors do not show toxicity against microorganisms in the environment, they can be used as additional carbon and energy source. Studies have shown that dissolved oxygen is an important factor in increasing the industrial aerobic fermentation process (Häusler et al., 2016; Wei et al., 2017).

The yield of pyocyanin production by *P. aeruginosa* is very low. For this reason, optimization studies of carbon, nitrogen and mineral substances have been performed in order to increase pyocyanin production. As *P. aeruginosa* is a facultative aerobic microorganism, the presence of oxygen in fermentation medium is important in pyocyanin production (El-Fouly et al., 2015; Hassan et al., 2016; Asshifa et al., 2017). Therefore, in this study, the production of this pigment was carried out for the first time using different oxygen vectors and the optimal conditions for the production were determined by the use of response surface methodology.

* Corresponding author.
E-mail address: mozdal@atauni.edu.tr (M. Ozdal).

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Table 1
Biomass and pyocyanin production in the presence of different oxygen vectors.

	Biomass (g/L) 48 h	Biomass (g/L) 72 h	Pyocyanin (mg/L) 48 h	Pyocyanin (mg/L) 72 h
Control	1,8	1,86	6,4	6,95
***n*-hexane**	1,3	1,44	10,9	11,96
***n*-dodecane**	2,3	2,4	6,97	7,65
***n*-hexadecane**	2,04	2,28	7,31	7,99

2. Materials and methods

2.1. Microorganism

P. aeruginosa OG1 (Accession Number: KC453990) used in this study was isolated from cockroaches living in contaminated pesticides (Ozdal et al., 2016). *P. aeruginosa* OG1 from stock culture was grown on Nutrient Agar (Difco, USA) for 24 h at 30 °C. One loop of cells grown on plates was used to inoculate 10 mL Nutrient Broth and 1 mL of bacterial suspension (OD600: 1) was used as preculture to inoculate a 250 mL flask containing 50 mL of Nutrient Broth (Difco, USA). At the end of the incubation period, bacterial suspension (OD600: 1) was prepared for inoculation.

2.2. Pyocyanin production medium

The pyocyanin production media prepared by adding 1% glycerol to Nutrient Broth was sterilized for 15 min at 121 °C. After cooling at room temperature, bacterial cell suspensions were inoculated to 50 mL of production media in 250 mL flasks and incubated in a shaker at 150 rpm, 30 °C for 72 h.

2.3. Comparison of oxygen vectors

Bacteria were incubated in production media for 24 h, after which different oxygen vectors (*n*-hexadecane, *n*-dodecane and *n*-hexane) were added at a concentration of 1% (Xu et al., 2007).

2.4. Optimization of pyocyanin production conditions

Statistical optimization of the production conditions of pyocyanin was performed by Minitab® Statistical Software Release 18 (Minitab Inc., State College, PA). The experimental design of the Response Surface Methodology (RSM) was performed using the Box-Behnken design of the program. As independent variables in the design, the concentration of the oxygen vector (1–4%), the time of addition of the oxygen vector (0–48 h) and the inoculum concentration (1–4%) were evaluated as low, medium and high at three different levels and 2 replicates (Abo-Zaid et al., 2015). In order to check the validity of the model, three replicated experiments were performed under the optimized conditions.

2.5. Determination of the pyocyanin concentration

After the fermentation period, the fermentation liquid was centrifuged at 10,000 rpm for 12 min. The supernatants (5 mL) were then mixed with 3 mL of chloroform and the samples were vortexed for 5 min. One mL of 0.2 N HCl was added to the organic phase. Samples were vortexed for 3 min and centrifuged at 13000 rpm for 5 min. After centrifugation, the red layer was collected and the optical density was measured at 520 nm. In the end, the pyocyanin concentration was determined by multiplying the A_{520} values by 17.072 and the results were expressed in mg/L (Essar et al., 1990).

2.6. Purification and characterization

At the end of the fermentation, the cells were centrifuged at 5000 rpm for 10 min, and the cell-free supernatant was extracted twice with equal volume of chloroform in the separating funnel. Extracted pigment was purified by using column chromatography on a silica gel column (30 cm length × 2 cm) and eluted by using solvent of chloroform. The blue chloroform layer was separated and purified by TLC (Silica gel 60 F254, Merck). In the TLC system, chloroform was used as an eluent (Cheluvappa, 2014). The produced pyocyanin was analysed by gas chromatography-mass spectrometry (GC-MS) using a Shimadzu QP2010 Ultra.

Table 2
Box–Behnken design of three factors (Inoculum, *n*-Hexane, and Addition time) along with the response (pyocyanin production) using response surface methodology.

Test Id	Inoculum (%)	*n*-Hexane (%)	Addition time (h)	Pyocyanin (mg/L)
1	1	1,0	24	10,20
2	3	1,0	24	8,83
3	1	4,0	24	16,09
4	3	4,0	24	16,20
5	1	2,5	0	8,97
6	3	2,5	0	9,40
7	1	2,5	48	13,50
8	3	2,5	48	14,96
9	2	1,0	0	6,12
10	2	4,0	0	10,82
11	2	1,0	48	8,80
12	2	4,0	48	18,70
13	2	2,5	24	18,90
14	2	2,5	24	18,90
15	2	2,5	24	19,00
16	1	1,0	24	9,30
17	3	1,0	24	9,36
18	1	4,0	24	16,98
19	3	4,0	24	17,20
20	1	2,5	0	9,35
21	3	2,5	0	10,00
22	1	2,5	48	14,15
23	3	2,5	48	15,30
24	2	1,0	0	6,80
25	2	4,0	0	10,48
26	2	1,0	48	9,00
27	2	4,0	48	19,21
28	2	2,5	24	18,50
29	2	2,5	24	17,80
30	2	2,5	24	19,70

3. Results

P. aeruginosa OG1 was incubated in Nutrient Broth containing 1% glycerol for 24 h and different oxygen vectors (*n*-hexadecane, *n*-dodecane and *n*-hexane) were added at a concentration of 1%. Changes in the amounts of biomass have been monitored. As a result of the addition of *n*-hexane the lowest biomass yield (1.44 g/L) was obtained and the result of the addition of *n*-dodecane resulted in the highest biomass yield (2.4 g/L). As shown in Table 1, pigment production was found to be higher than the control and other oxygen vectors when *n*-hexane was used. For this reason, *n*-hexane was chosen for optimization studies.

3.1. Optimization of independent variables: response surface methodology

Box-Behnken design was used to optimize the inoculum size (%), *n*-

Table 3
Estimation of regression coefficient for pyocyanin production.

Term	Coef	SE Coef	T-Value	P-Value	VIF
Fixed value	18,800	0,221	85,25	0,000	
Inoculum (%)	0,169	0,135	1,25	0,224	1,00
n-Hexane (%)	3,579	0,135	26,51	0,000	1,00
Addition time (h)	2,605	0,135	19,29	0,000	1,00
Inoculum (%)*Inoculum (%)	-2,534	0,199	-12,75	0,000	1,01
n-Hexane (%)*n-Hexane (%)	-3,246	0,199	-16,33	0,000	1,01
Addition time (h)*Addition time (h)	-4,312	0,199	-21,70	0,000	1,01
Inoculum (%)*n-Hexane (%)	0,205	0,191	1,07	0,296	1,00
Inoculum (%)*Addition time (h)	0,191	0,191	1,00	0,329	1,00
n-Hexane (%)*Addition time (h)	1,466	0,191	7,68	0,000	1,00

Table 4
Analysis of variance for pyocyanin production.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	561,975	62,442	214,00	0,000
Linear	3	314,026	104,675	358,74	0,000
Inoculum (%)	1	0,459	0,459	1,57	0,224
n-Hexane (%)	1	204,991	204,991	702,54	0,000
Addition time (h)	1	108,576	108,576	372,11	0,000
Square	3	230,121	76,707	262,89	0,000
Inoculum (%)*Inoculum (%)	1	47,408	47,408	162,48	0,000
n-Hexane (%)*n-Hexane (%)	1	77,820	77,820	266,70	0,000
Addition time (h)*Addition time (h)	1	137,337	137,337	470,67	0,000
2-Way Interaction	3	17,828	5,943	20,37	0,000
Inoculum (%)*n-Hexane (%)	1	0,336	0,336	1,15	0,296
Inoculum (%)*Addition time (h)	1	0,293	0,293	1,00	0,329
n-Hexane (%)*Addition time (h)	1	17,199	17,199	58,94	0,000
Error	20	5,836	0,292		
Lack-of-Fit	3	1,474	0,491	1,91	0,165
Pure Error	17	4,362	0,257		
Total	29	567,811			

hexane concentration (%) and the addition time (h) of *n*-hexane. All experiments were carried out in a 250 ml Erlenmeyer flask containing 50 ml of medium for 72 h.

The results of the experiments designed according to the Box-Behnken design are given in Table 2 by using the reaction surface regression analysis of the MINITAB 18.0 program.

The analysis given in Table 3 shows that factor with *P* values less than 0.05 have statistically significant effects on pyocyanin production. Accordingly, the time of adding hexane and oxygen vector concentration have more pronounced effects on the production of pyocyanin than the size of inoculum.

It was determined that all the quadratic effects (Inoculum, Addition time, *n*-Hexane concentration) had negative value, which means a better response is obtained at low levels of the variable. On the other hand, all the interactive effects had positive value, which indicates that the response increases if both variables change to the same level, low or high.

By using the coefficients obtained from the regression analysis ($p < 0.05$), it was found that the best estimation of optimum pyocyanin production can be made according to the following model equation.

Pyocyanin (mg/L) = 18.800 + 3.579 n-Hexane (%) + 2.605 Addition time (h)-2.534 Inoculum (%)*Inoculum (%) – 3.246 n-Hexane (%)*n-Hexane (%) – 4.312 Addition time (h)*Addition time (h) + 1.466 n-Hexane (%)*Addition time (h)

When the model equation was checked in ANOVA analysis, it was seen that Quadratic model was suitable for this design. In addition, the predicted R^2 values (97.76%) and the obtained values (98.97%) were quite high and show that this was a strong model for pyocyanin production.

ANOVA (Analysis of Variance) for the production of pyocyanin is given in Table 4. The statistical significance of the regression model was evaluated by the F test. According to the results, there was no significant lack-of-fit (p: 0.165 > 0.05) in the model. This showed that the model was sufficient to predict the production of pyocyanin.

The three dimensional response surface and contour plots and the peak graphs of optimal values of variables based on the model equation are shown in Figs. 1–3, respectively. Response surface plots explain the interaction of the variables and make it possible to find the optimum level of each variable for a maximum response. The peaks in the surface lines indicate that the optimum points are within the design boundaries.

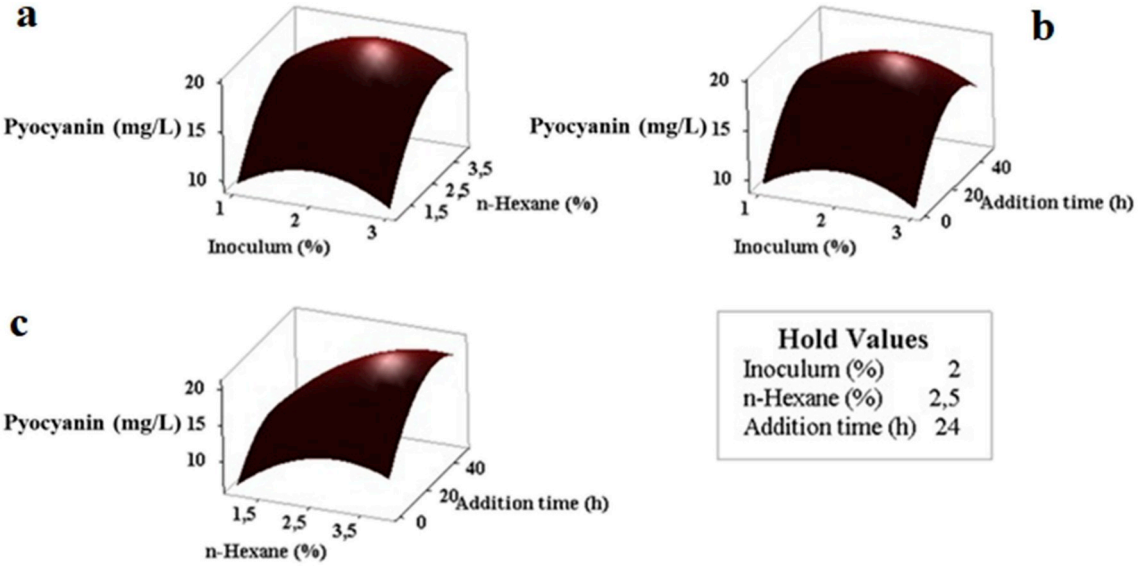

Fig. 1. Response surface plots of interactions between inoculum and *n*-hexane concentration, inoculum and addition time, *n*-hexane concentration and addition time on pyocyanin production by *P. aeruginosa* OG1.

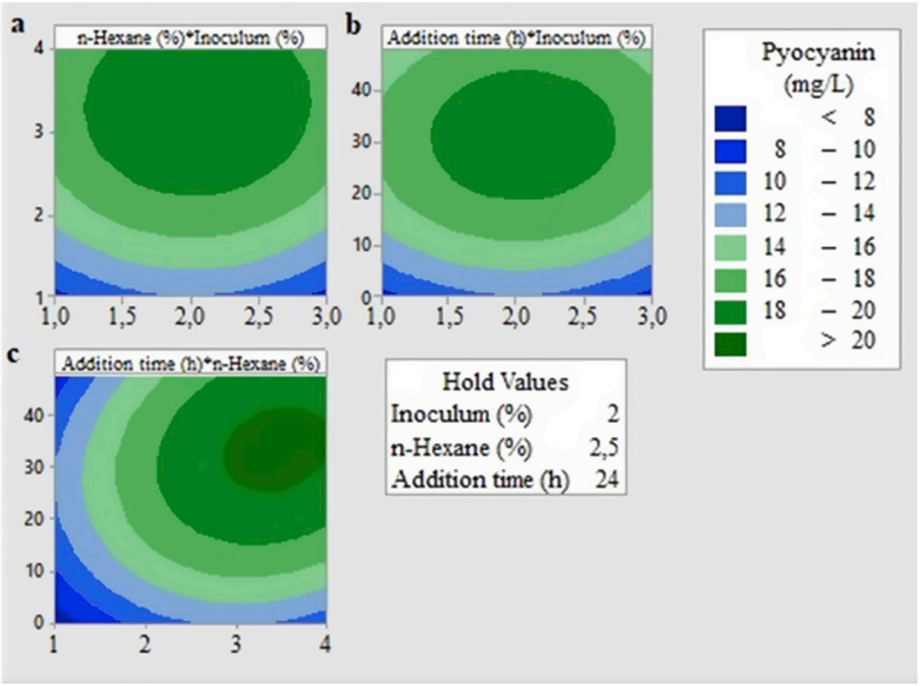

Fig. 2. Contour plots of interactions between *n*-hexane concentration and inoculum (a), addition time and inoculum (b) addition time and *n*-hexane concentration (c) on pyocyanin production by *P. aeruginosa* OG1.

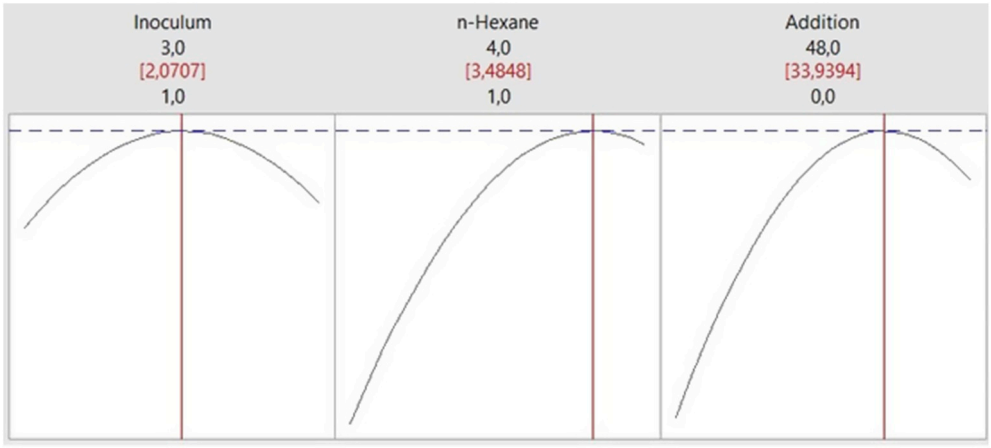

Fig. 3. Optimal values of inoculum, *n*-Hexane concentration and addition time for maximal pyocyanin production by *P. aeruginosa* OG1.

All surface graphics showed that increasing the levels of each of the three variables was beneficial to a certain level for the production of pyocyanin. The surface and contour plots (Figs. 1 and 2) showed that the inoculum, *n*-hexane concentration and addition time reached 2 (%), 2.5 (%) and 24 (h), respectively, and the pyocyanin production was maximized (18 mg/l). It was determined that higher increases in variables did not affect the production or had a negative effect on it.

3.2. Checking the validity of the model

The optimal values of the variables were found to be 2.07% for inoculum size, 3.48% for *n*-hexane concentration and 33.93 h for addition time using Minitab v18's "response optimizer (Fig. 3). When compared to the control medium, two-fold (21.2 mg/L) increase in pyocyanin production was obtained at optimum conditions.

3.3. Purification and characterization of pyocyanin

At the end of fermentation, the cells were centrifuged at 5000 rpm for 10 min and the cell-free supernatant was extracted twice with equal volume of chloroform in the separating funnel. The blue chloroform layer was separated and purified by TLC (Fig. 4). The GC-MS spectrum of the synthesized pyocyanin was shown in Fig. 5. The molecular mass of the synthesized pyocyanin was m/z 209, which corresponds to that of pyocyanin ($C_{13}H_{10}N_2O$).

4. Discussion

The various bacteria such as *Serratia*, *Streptomyces*, *Arthrobacter*, *Bacillus* and *Pseudomonas* have the capability to produce different pigments (Kurbanoglu et al., 2015; Ozdal et al., 2017a, 2017b).

The pyocyanin is a water-soluble, nitrogen-containing heterocyclic compound with blue green color. Since it is a secondary metabolite, the

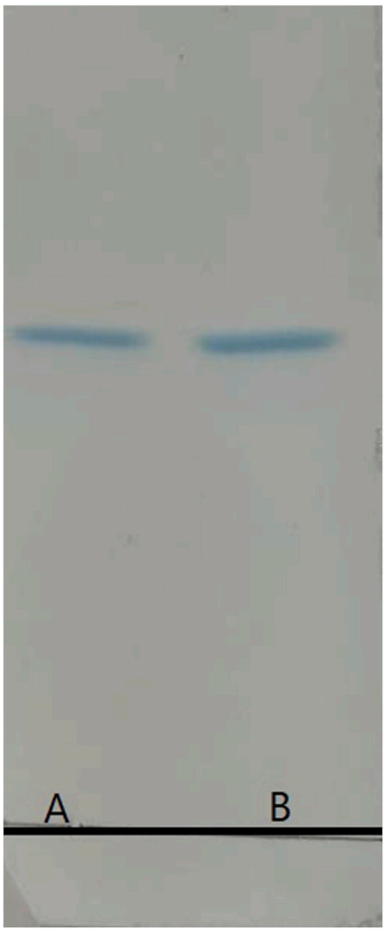

Fig. 4. TLC profiles of pure pyocyanin (A) and produced pyocyanin (B).

production is observed mainly in the stationary phase of the microbial growth (Özcan and Kahraman, 2015; Agrawal and Chauhan, 2016). The highest yields were obtained when glycerol was used as the carbon source (Özcan and Kahraman, 2015). When glucose is used, it causes catabolic repression because the pH of the medium decreases (Magnus et al., 2017). For this reason, glycerol was added to Nutrient broth medium in this study. In their study, El-Fouly et al. (2015) produced 9.3 mg/L pyocyanin with *P. aeruginosa* R1 and 5.8 mg/L pyocyanin with *P. aeruginosa* U3 in the Nutrient broth medium supplemented with glycerol.

Pyocyanin production is also affected by the nutrient components (carbon, nitrogen, iron, magnesium, potassium) and environmental conditions (pH, temperature, agitation rate) of the fermentation (Abo--Zaid et al., 2015; El-Fouly et al., 2015). Shen et al. (2014) reported the addition of biosurfactant of sophorolipid to the medium, which resulted in the increased production of pyocyanin from 7.03 mg/L to 14.81 mg/L. In the King's B medium, *P. aeruginosa* OSh1 produced 26 mg/L pyocyanin at the end of 72 h (Barakat et al., 2015). The initial inoculum concentration is also known to affect the production of pyocyanin. At the end of this study, the optimum inoculum concentration was determined as 2.07%. Similar result (2%) was previously reported in another study (Abo-Zaid et al., 2015).

Oxygen vectors have been used in the production of different microbial pigments. Lycopene and beta-carotene production by *Blakeslea trispora* was increased 2.1 and 1.8 times, respectively, with the addition of 1% (v/v) *n*-dodecane to fermentation cultures (Xu et al., 2007). The addition of *n*-hexadecane (9%, v/v) to fermentation cultures of *Phaffia rhodozyma*, a yeast, was reported to significantly increase oxygen transfer in the culture and thus increased carotenoid production by 58% (Liu and Wu, 2006). In this study, with the addition of 3.48% *n*-hexane, a 2-fold increase in the production of pyocyanin pigment was determined. The time to add oxygen vectors to the fermentation medium is also an important parameter (Liu and Wu, 2006; Mou et al., 2015). In this study, the optimum time for *n*-hexane addition was determined as 33.93 h.

The addition of oxygen vectors to the fermentation medium resulted in increased production of many valuable microbial products. It was reported that the addition of *n*-dodecane (5%) to fermentation medium increased the production of hyaluronic acid (polysaccharide) 3.6 times by *Streptococcus zooepidemicus* (Liu et al., 2009). In the production of L-asparaginase enzyme with *Bacillus brevis*, it was determined that enzyme production increased 36% as a result of the addition of liquid paraffin (6%) as oxygen vector (Narta et al., 2011). It has been reported that the product yield of Epsilon-polylysine (ε-PL) increased by 31.6% as a result of the addition of *n*-dodecane as an oxygen vector to the environment of *Streptomyces albulus* (Xu et al., 2015). It was determined that citric acid production by *Yarrowia lipolytica* increased 25.4% with the addition of oleic acid (2%) as an oxygen vector (Liu et al., 2016). Validamycin A (antibiotic) production by *Streptomyces hygroscopicus* 5008 was found to be increased by 31% as a result of the addition of liquid paraffin as oxygen vector (Feng et al., 2016). It was reported that spinosad (bio-pesticide) production by *Saccharopolyspora spinosa* increased 44.2% as a result of the addition of *n*-dodecane (0.5%) to medium (Lu et al., 2017). The results obtained from the present study showed that

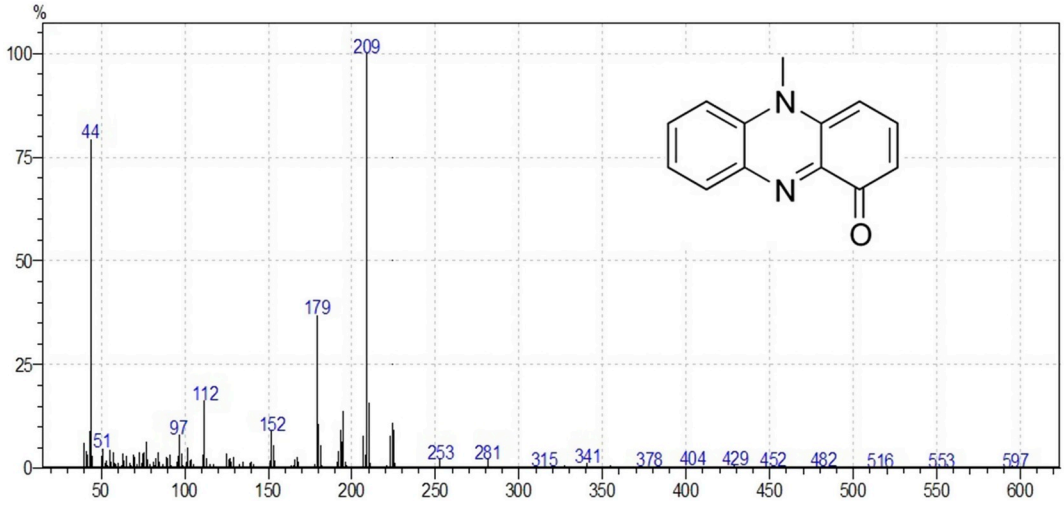

Fig. 5. GC/MS mass spectrum and the structural formula of the pyocyanin produced by *P. aeruginosa* OG1.

oxygen vectors have significant effects on the production of pyocyanin pigment (Fig. 2), and production optimization was improved with statistical optimization studies and a two-fold increase in production was achieved.

5. Conclusion

Oxygen vectors have proved to be useful in pigment production by increasing the oxygen transfer in the fermentation culture, obviously. In the present study, *n*-hexadecane, *n*-dodecane and *n*-hexane were tested as oxygen vectors for pyocyanin production. Among them, *n*-hexane was chosen as the best oxygen vector and used in the optimization studies. The optimal levels and interactions of inoculum, addition time and *n*-hexane concentration for pyocyanin production were determined by statistical analysis provided by BBD of RSM effectively and a reliably. Under the optimized conditions (2.07% inoculum, 33.93 h addition time and 3.48% *n*-hexane concentration), the yield of pyocyanin improved and 2-fold (21.2 mg/L) increase was achieved.

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