Increasing in the Extraction Yield of Environmentally Friendly Antifouling Agent from *Pseudomonas Aeruginosa* MUT3 by Response Surface Methodology (RSM)

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ABSTRACT: *In the present study, the solvent-solvent extraction of phenazine 1-carboxylic acid (PCA) as an environmentally friendly antifouling agent from pseudomonas aeruginosa MUT3 culture was investigated. Accordingly, after screening the extraction ability of various solvents, the combined effects of operating parameters such as solvent type (ethyl acetate, dichloromethane, and n-hexane), solvent percent and mixing time on the PCA extraction process were analyzed using response surface methodology (RSM). As a consequence, ethyl acetate showed higher extraction yield (68%) and the optimum condition for PCA extraction were identified as 150% of solvent and 120 min mixing time. Meanwhile, the extraction yields for dichloromethane and n-hexane were measured by HPLC assay around 48.75 and 25.2%, respectively. The accuracy of the obtained model was proved by 99.90% R^2 and 99.84% Adj R^2. In addition, the disk diffusion test showed 9.2, 8 and 7.3 mm inhibition zone for ethyl acetate, dichloromethane and n-hexane, respectively. Consequently, the present study provided a great insight into the solvent-solvent extraction of antibiotics from the fermentation broth.*

KEYWORDS: *Phenazine 1- carboxylic acid; Response surface methodology; Solvent extraction; Cross current; Production yield.*

INTRODUCTION

Marine biofouling is referred to as the accumulation of bacteria, algae, plants or animals on the manmade submerged structures such as vessels and submarines. This phenomena considerably raises the fuel consumption by increasing the drag force on the ship hull (around 29%) [1-3]. Since the International Maritime Organization (IMO) banned on tributyltin (TBT) application in the marine coatings, most of the investigations have focused

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on the alternative antifouling coatings replacement by the biological and natural agents which have no negative impacts on the marine environments [4]. Biological antifouling compounds have recently been extracted from the various marine organisms such as sponge [5-8], algae [9-12], fishes [13], microorganisms [14-17], biological materials like enzymes [18-21] and etc. [22-24].

Several studies have been conducted on the application of microbial antibacterial metabolites in the marine coatings [25, 26]. Additionally, over 650 antibacterial metabolites have been extracted from marine epibiotic bacteria [27]. Among these metabolites, phenazine-1-carboxylic acid (PCA) is one of the most effective antibacterial agents which is able to be applied in the marine coatings as an antifoulant [28, 29]. Moreover, *pseudomonas* specious is one of the most appropriate candidates for PCA production [30-34].

Numerous studies have been conducted on PCA extraction from various *Pseudomonas* sp. such as *fluorescens* [35, 36], *chlororaphis* [37-39] and *aeruginosa* [32, 40-42]. *Thoo et al.* (2010), *Su et al.* (2010) and *Yuan et al.* (2008) investigated the effect of medium components on PCA production from *P. aeruginosa* [43-45]. In addition, the antibacterial activity of PCA has been studied against the different microorganisms [46].

Since the maximum amount of PCA which is able to be produced by *pseudomonas* sp. is around 6 mg/L, therefore, achieving the higher antibacterial agent the optimization of the production process is essential. The production process of each biological agent by microorganisms is divided into three main parts including fermentation, extraction and purification processes. Although numerous investigations have been conducted on the phenazine production from *pseudomonas* sp., none have studied the PCA extraction process.

Phenazine 1-carboxylic acid is able to be extracted from fermentation broth by the solvent-solvent extraction [43, 44]. *Chandaliya et al.* indicated that the solvent type, supernatant to solvent ratio, mixing time and temperature are the factors affecting the solvent-solvent extraction process [47]. *Jeganathan et al.* optimized the solvent-solvent extraction process by using Box-Behnken design [48]. Furthermore, in several studies PCA was extracted from the fermentation broth by chloroform [49], ethyl acetate [50], methylene dichloride [51], acetone [52], benzene [53].

In the present study, phenazine-1-carboxylic acid produced by *pseudomonas aeruginosa* MUT3 was studied to optimize the extraction process using Response Surface Methodology (RSM). Accordingly, extraction parameters including solvent type, mixing time and temperature were optimized in the PCA production. This study prepared a remarkable insight into the solvent-solvent extraction process of an antibacterial agent from fermentation broth.

EXPERIMENTAL SECTION

Preparation of bacterial culture medium

Pseudomonas aeruginosa MUT3 was obtained from the biotechnology institute microbial bank (NCBI, Biotechnology Research Center). The strain was cultured in LB medium containing: yeast extract (3 g), peptone from casein (3 g), meat extract (5 g), glucose (12 g), K_2HPO_4 (1.5 g), KH_2PO_4 (1.5 g), NaCl (5 g) at pH 7 and with shaking at 140 rpm for 4 days at 30°C. After incubation, the mixture was centrifuged at 7500 g for 20 min to remove the bacterial cells.

Extraction of antibacterial agent

To obtain the PCA, the fermentation broth was centrifuged at 7500g for 9 min using Eppendorf centrifuge (model 5810R). Then, PCA was extracted from the supernatant by the solvent-solvent extraction using 1:0.5 ratio of solvent (50% solvent) in the separation funnel. Finally, the solvent phase which contained PCA, distilled and dried at 60 °C.

Experimental design

Central Composite Rotatable Design (CCRD) and Response Surface Methodology (RSM) were applied to optimize the extraction parameters. In addition, designing the experiments were performed by Design Expert 7 software. By the laboratory studies, the effective factors and levels for CCRD were considered as the solvent type, mixing time and solvent percent (Table 1). Moreover, the experimental response was considered as the solvent-solvent extraction yield. To identify the ability of various solvents in the PCA extraction, six solvents including; chloroform, ethyl acetate, dichloromethane, acetone, benzene, and n-hexane were studied.

Accordingly, for each sample in the optimization process, 50 mL of supernatant was mixed with a defined

Table 1: Factors and levels in the solvent-solvent extraction experimental design.

Factors	Symbol	Actual levels of coded factors				
		– 1.41	– 1	0	+ 1	+ 1.41
Mixing time (min)	X_1	11	30	75	120	138
Solvent percent (%)	X_2	30	50	100	150	170

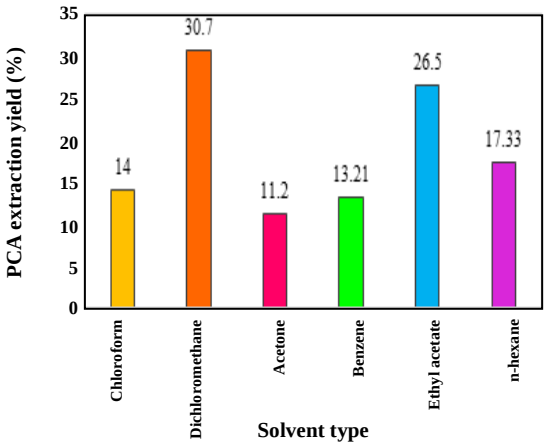

Fig. 1: The result of solvent screening in the PCA extraction.

the volume of the solvent (Table 2). After the extraction and concentration, the obtained PCA was dissolved in methanol (total volume reached to 1 mL). Finally, the antibacterial efficiency of PCA against *E. coli* DH5α was determined by the disk diffusion test according to *Owens at el.* [54].

Antibacterial extraction yield

The solvent-solvent PCA extraction yield was determined by HPLC assay. The HPLC column characteristics and the analysis conditions were according to *Mosmeri et al.* [55].

RESULTS AND DISCUSSION

For identification of the appropriate solvents for PCA extraction, different solvents were studied and the obtained antibiotic was analyzed by HPLC assay (Fig. 1). According to the results, ethyl acetate, dichloromethane, and n-hexane were identified as the solvents with the higher antibiotic extraction efficiency.

Optimization of the PCA extraction process

Process optimization of PCA extraction was conducted according to 36 experiments presented in Table 2. The experiments were designed as described by Box and Behnken [56]. Furthermore, data analysis was performed using analysis of variance (ANOVA) to identify the impacts of each parameter on the PCA extraction (Table 3).

The proposed model for ethyl acetate (y_1) dichloromethane (y_2) and n-hexane (y_3) was obtained as below:

$$y_1 = -2.61251 + 0.16673\,X_1 + 0.57798 \times X_2 - 2.95556 \times 10^{-4} X_1 X_2 - 1.34813 \times 10^{-3} X_2^2 \quad (1)$$

$$y_2 = +15.62715 - 0.13670 X_1 + 0.44176 X_2 + 5.88889 \times 10^{-4} X_1 X_2 - 1.34813 \times 10^{-3} X_2^2 \quad (2)$$

$$y_3 = +3.13144 + 2.87648 \times 10^{-3} X_1 + 0.32939 X_2 + 1.88889 \times 10^{-4} X_1 X_2 - 1.34813 \times 10^{-3} X_2^2 \quad (3)$$

As indicated in Table 3, the probability value (*P*-value) showed an appropriate agreement with the proposed model. Therefore, the accuracy of the model was proved according to Pazouki *et al.* (2017), *Goleij et al.* (2017), *Hajiaghaee et al.* (2017) and *Mosmeri et al.* (2017) which showed a *P*-value <0.0001 [57-60]. *The multiple regressions* coefficients through the *least squares* method were created for the response variables. The coefficients predicted the quadratic polynomial models based on the significant coefficients presented in Table 3.

The predicted and actual PCA extraction yield is illustrated in Fig. 2a. It shows the validity of the obtained models which are given in equations (1-3), because approximately all the points were close to the line 45°. In addition, the probability of the residuals proved the reliability of the models (Fig. 2b).

Effect of solvent percent on the extraction yield

The effect of solvent percent in the extraction process was investigated for each solvent (Fig. 3). The results

Table 2: The designed experiments based on the central composite rotatable design.

Run	Mixing time (min)	Solvent to supernatant (%)	Solvent type	Extraction yield (%)
	X_1	X_2		
1	75.00	30	dichloromethane	18
2	75.00	100	dichloromethane	41
3	75.00	30	ethyl acetate	25
4	30	150	dichloromethane	51
5	120	150	n-hexane	26
6	75	170	dichloromethane	48.56
7	11	100	dichloromethane	45
8	75	30	n-hexane	12.6
9	138	100	n-hexane	25
10	30	50	dichloromethane	32
11	30	150	ethyl acetate	57
12	120	150	ethyl acetate	68.34
13	75	100	dichloromethane	40
14	75	100	dichloromethane	40.3
15	11	100	n-hexane	22.5
16	75	100	dichloromethane	39.78
17	30	50	ethyl acetate	27
18	75	100	n-hexane	24.1
19	138	100	ethyl acetate	61
20	120	50	ethyl acetate	41
21	120	150	dichloromethane	45.3
22	120	50	n-hexan	18
23	30	50	n-hexan	16.7
24	75	100	ethyl acetate	52
25	75	100	ethyl acetate	52.2
26	75	100	n-hexan	24
27	75	100	ethyl acetate	52
28	138	100	dichloromethane	37
29	75	170	ethyl acetate	65.4
30	75	100	n-hexan	24.4
31	11	100	ethyl acetate	44
32	30	150	n-hexan	23
33	75	170	n-hexan	23.4
34	75	100	n-hexan	24
35	120	50	dichloromethane	21
36	75	100	ethyl acetate	52

Table 3: Analysis of variance of the parameters in PCA extraction process.

Source	Sum of Squares	df	Mean Square	F Value	p-value (Prob > F)	
Model	8002.62	12	666.89	1873.22	< 0.0001	significant
A- mixing time	35.54	1	35.54	99.82	< 0.0001	
B- solvent percent	2215.03	1	2215.03	6221.84	< 0.0001	
C- solvent type	4672.29	2	2336.14	6562.04	< 0.0001	
AB	1.57	1	1.57	4.41	0.0469	
AC	375.05	2	187.53	526.75	< 0.0001	
BC	468.02	2	234.01	657.31	< 0.0001	
B^2	227.18	1	227.18	11.16	0.0004	
ABC	7.94	2	3.97			
Residual	8.19	23	0.36			
Lack of Fit	7.30	14	0.52			

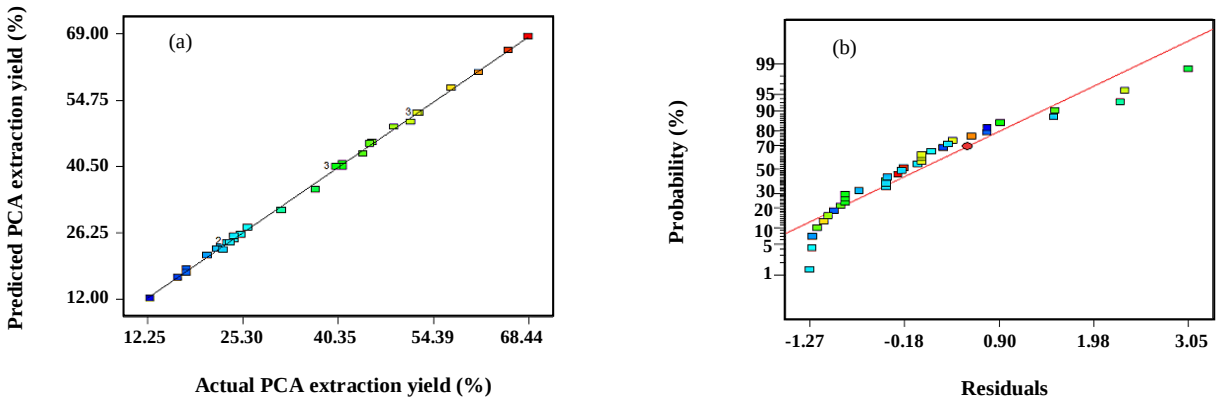

Fig. 2: (a) the comparison of the actual and predicted PCA extraction yield and (b) the probability (%) of residuals.

indicated that by raising the solvent content (from 50 to 150%), the extraction yield was increased significantly for ethyl acetate and dichloromethane around 27.29% and 24.28%, respectively. Meanwhile, it showed a slight increasing for n- hexane. *Wani et al.* were also showed that a remarkable role for solvent ratio in extraction process [61].

Effect of mixing time on the extraction yield

Mixing time was identified as an effective parameter in the antibiotic extraction process. As showed in Fig. 4, increasing in mixing time from 30 to 120 min, raised the extraction yield by ethyl acetate from 57% to more than 68%. Meanwhile, it indicated a negligible impact on the PCA extraction by dichloromethane and n-hexane. The considerable impact of mixing time on the extraction process was also proved by *Spingo et al.* [62]. Considering all the responses, the results demonstrated that PCA extraction potential using n-hexane did not notably vary with solvent percent and mixing time.

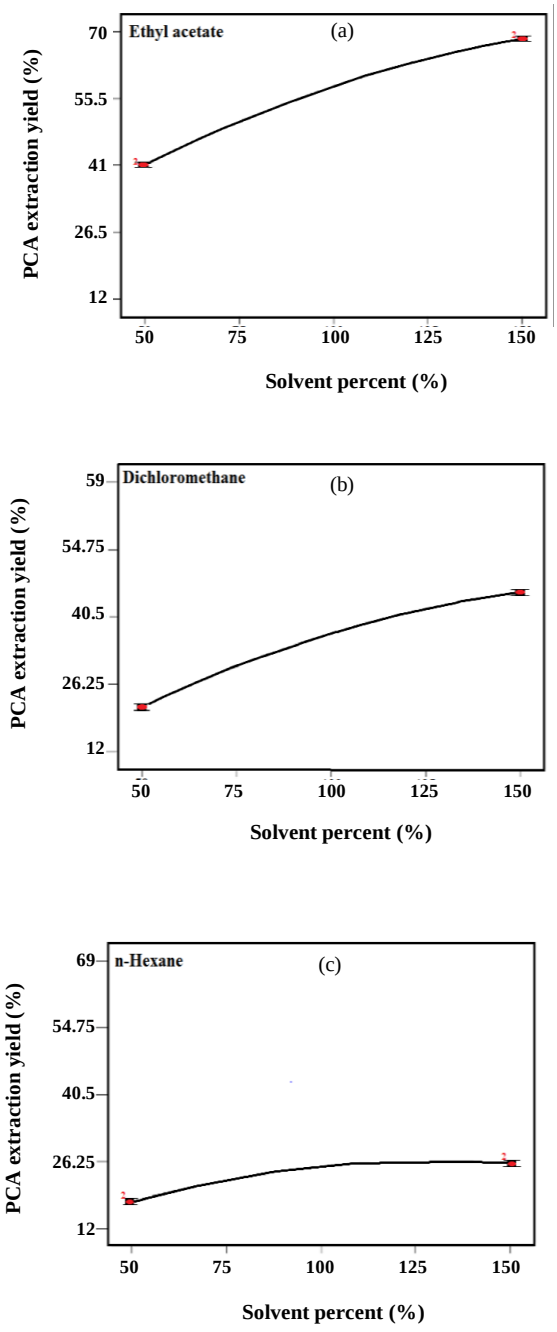

Fig. 3: (a) the comparison of the actual and predicted PCA extraction yield and (b) the probability (%) of residuals.

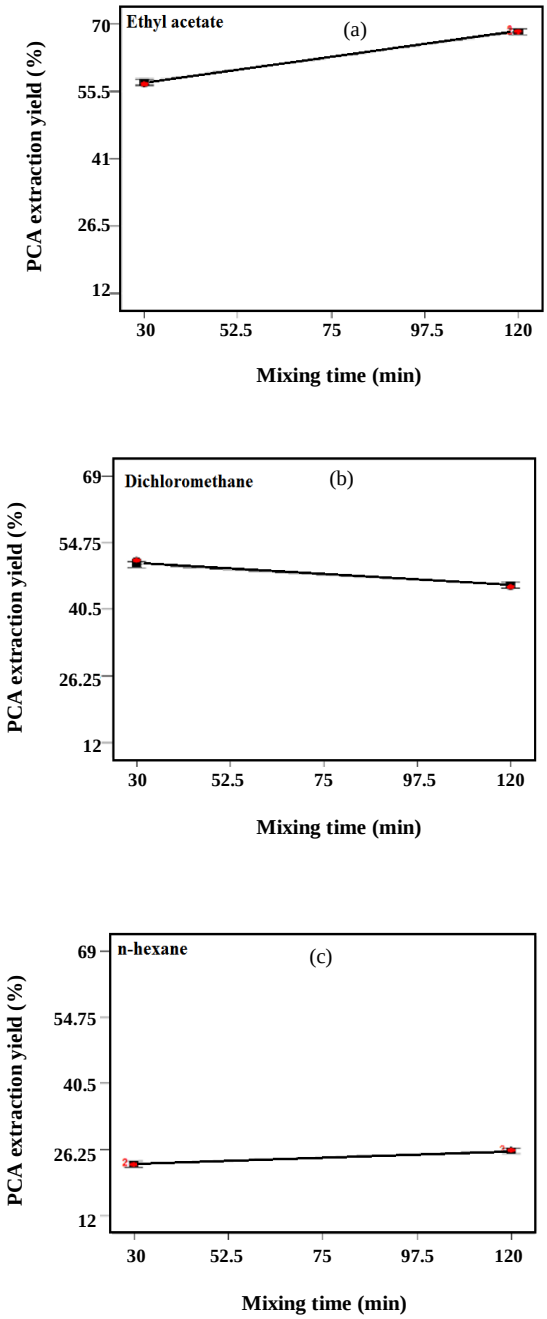

Fig. 4: Effect of mixing time on the inhibition zone at 150% solvent ratio. a) Ethyl acetate, b) n- hexane and c) dichloromethane.

Effect of various solvents on PCA extraction

The solvent type showed a considerable impact on the response of the designed experiments. For 120 min mixing time using 100% solvent, the effect of solvent type on the inhibition zone for PCA extraction is shown in Fig. 5. The results indicated that ethyl acetate and dichloromethane were able to extract around 63 and 48% of the total PCA from the fermentation broth, respectively. Meanwhile, n-hexane showed a week performance in the extraction yield (approximately 24.5%).

To determine the impact of each variable on the experimental response, the graphical analysis method was applied.

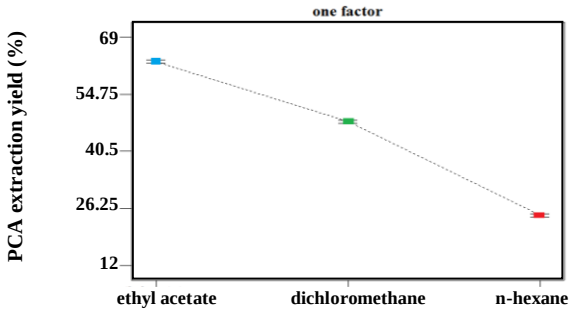

Fig. 5: Effect of solvents on extraction process at 100% solvent percent and 120 min mixing time.

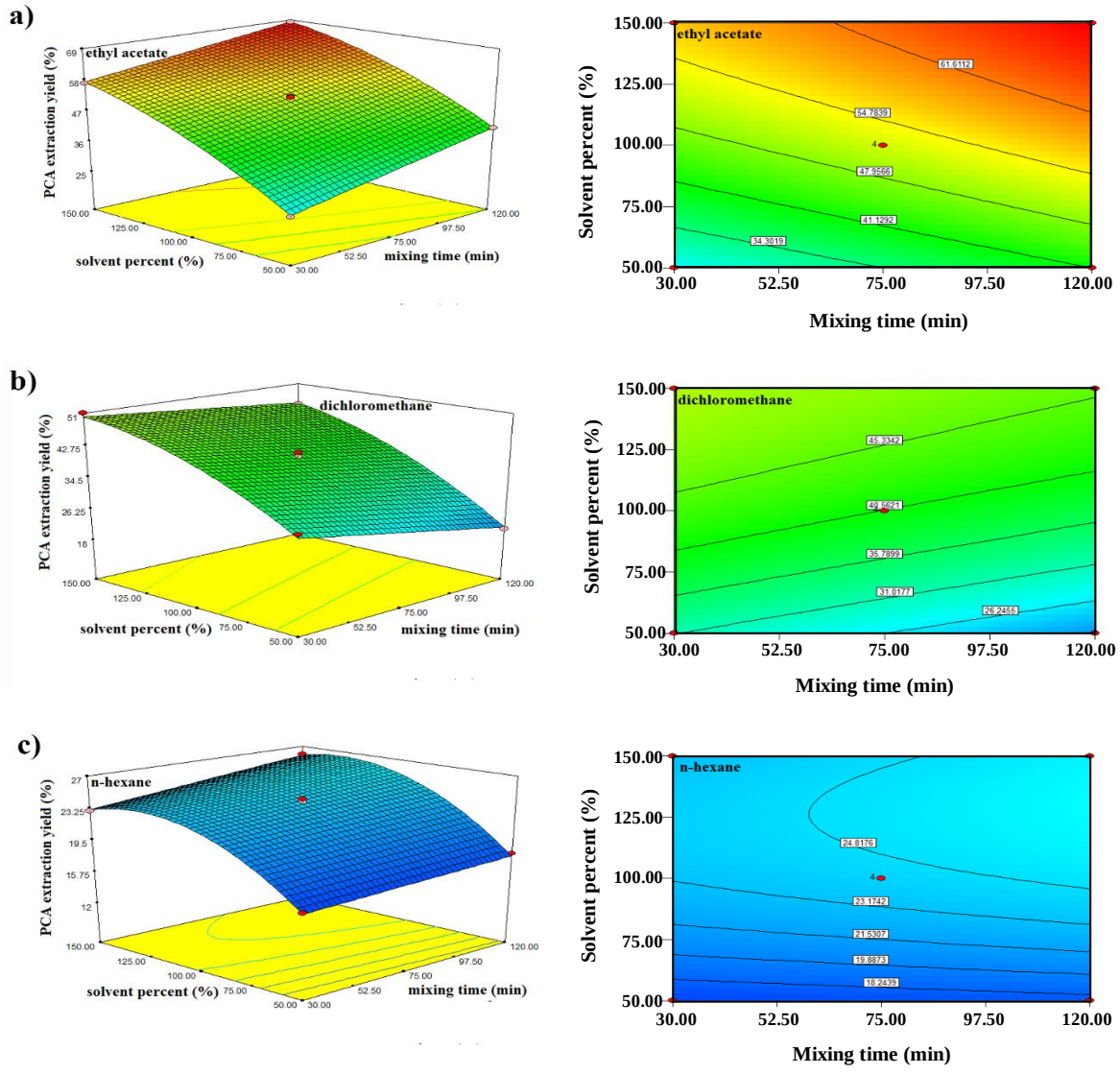

Fig. 6: The effect of investigated parameters on the PCA extraction yield. a) ethyl acetate, b) dichloromethane and c) n- hexane.

The interaction of both mixing time and solvent percent for each solvent is presented in Fig. 6. Ethyl acetate showed the most appropriate extraction yield and there was a direct relationship between the considered

Table 4: The maximum analytical and theoretical extraction yield comparison for each solvent.

Solvent type	Solvent percent (%)	Mixing time (min)	Theoretical extraction yield (%)	Analytical extraction yield (%)	Inhibition zone (mm)
ethyl acetate	150	120	68.36	68	9.2
dichloromethane	150	30	50.10	48.75	8
n- Hexane	130	120	26.47	25.2	7.3

parameters and the experimental responses. Accordingly, increasing the mixing time and the solvent percent to the maximum level raised the PCA extraction yield to about 68%. The results were consistent with *Thoo et al.* [43].

In addition, the solvent percent showed the same effect on the PCA extraction yield as ethyl acetate when dichloromethane was applied. Meanwhile, increasing in the mixing time showed a negative impact on the extraction process. As it is shown in Fig. 6.c, n-hexane showed a poor performance in the antibiotic extraction which reached 27% at the optimum condition.

To verify the obtained results, the maximum theoretical and analytical antibiotic extraction yield was compared at the optimized factors (Table 4). According to the data presented in Table 4, the negligible difference between analytical and theoretical PCA extraction obtained from two selected samples revealed the accuracy of the proposed model. Additionally, R-squared and Adj R-squared were 99.90% and 99.84%, respectively for the model. Therefore, the accuracy of the presented model was proved according to the obtained R-squared and Adj R-squared [63-65]. Moreover, to study the antibacterial efficiency of the extracted PCA, a disk diffusion test was applied. The results of the disk diffusion are presented in Table 4.

CONCLUSIONS

In this study, the effect of solvent type, solvent percent and mixing time on the solvent-solvent extraction efficiency of phenazine 1–carboxylic acid (PCA) from *P. aeruginosa* MUT3 broth have been investigated by central composite rotatable design. The obtained results showed better performance for ethyl acetate in PCA extraction. In addition, the extraction yields for ethyl acetate, dichloromethane and n-hexane were measured around 68, 48.75 and 25.2%, respectively. An acceptable agreement was obtained between analytical and theoretical data (R-squared and adj R-squared were 99.90% and 99.84%, respectively). Moreover, the disk diffusion assay proved the validity of the HPLC results on the extracted PCA at the optimized condition. Accordingly, ethyl acetate was the solvent with higher efficiency for PCA extraction (around 68% yield) and dichloromethane showed better performance (48.75%) than n-hexane (25.2%). Consequently, the present study optimized the PCA extraction process and the optimum conditions were obtained at 150% of solvent and 120 min mixing time for ethyl acetate. The results indicated that the extraction process plays a key role in the production of the biological components and the extraction efficiency is able to be maximized by optimization of the process.

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