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STATISTICAL EVALUATION AND PROCESS OPTIMIZATION OF BIOTREATMENT OF POLYCYCLIC AROMATIC HYDROCARBONS IN A BIOREACTOR

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Abstract

Application of a biological treatment system using white rot fungi is an interesting alternative for treatment of water and wastewater contaminated with persistent organic pollutants, such as polycyclic aromatic hydrocarbons (PAHs). A packed bed bioreactor using the white rot fungi, *Phanerochaete chrysosporium* (*P. chrysosporium)* was evaluated for degradation of pyrene and phenanthrene in polluted wastewater. White rot fungi have significant potential to metabolize organic pollutants such as PAHs by means of their ligninolytic enzymes. This study examined the effect of feed flow rate (0.14 to 0.55 mL min^{-1}) and initial PAHs concentrations in feed (50 to 100 mg L^{-1}). Response surface methodology (RSM) was applied to predict the degradation of PAHs available in influent and enzyme activity. The RSM results showed that the best model for PAHs removal efficiency and enzyme activity is the reduced quadratic model. The optimum region, identified based on four critical responses, was an influent flow of 0.35 mL min^{-1} and initial pyrene and phenanthrene concentrations of 60 mg L^{-1}. This resulted in 90% removal efficiency for pyrene and 87% for phenenthrene and enzyme activity of 57 U L^{-1} for MnP and 426 U L^{-1} for LiP.

Keywords: ligninolytic enzyme, packed bed bioreactor, *Phanerochaete chrysosporium*, polycyclic aromatic hydrocarbons, response surface methodology

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are heterogeneous chemicals consisting of two or more fused benzene rings, generated during incomplete combustion of solid (such as coal and wood) and liquid (such as oil and petrol) fuels or derived from industrial activity (Abdel-Shafy and Mansour, 2016). Many PAHs are considered to be mutagenic and potentially carcinogenic, and should be removed from environment (Guieysse et al., 2000; Savic et al., 2016).

Their limited dispersion by vaporization or solubilization makes them poorly available to microorganisms as nutrients. The lack of bioavailability in addition to the lack of suitable microbial enzymes are the main reasons for the recalcitrance of PAHs biodegradation (Guieysse et al., 2000).

An environmentally friendly approach for PAHs degradation, implements white rot fungi, which are known to degrade a variety of lignin model compounds by their complex enzyme system (Eibes et al., 2007; Santi et al., 2015). The bioremediation of wastewater using aquatic species has been performed recently as well (Malschi et al., 2018).

Fungi are widely used to treat water, wastewaters and soil. *Phanerochaete chrysosporium* (*P. chrysosporium*) promises degradation of a diverse

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range of persistent and toxic pollutants, including petroleum hydrocarbons, phenols, chlorinated phenolic compounds, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, dyes, chlorinated pesticides and insecticides (Vidali, 2001).

P. chrysosporium produces several extracellular peroxidases, including lignin peroxidase (LiP) and manganese peroxidase (MnP) and is involved in lignin biodegradation (Zouari et al., 2002). Expression of ligninolytic enzymes by *P. chrysosporium*, is an idiopathic event triggered by nitrogen or carbon limitation and is highly dependent on culture and medium compositions to allow biodegradation (Shim and Kawamoto, 2002).

Immobilization of microorganisms in the form of biofilm has been used for biotechnological processes in environmental studies (Tekere et al., 2007; Zilouei et al., 2006). Lewandowski et al. (1990) obtained kinetic rates and studied different reactor configurations for chlorophenol removal, using *P. chrysosporium*. Fixed bed biofilm reactors are suitable for treatment of large amounts of diluted effluent contaminated with PAHs. Fixed bed bioreactors have been used in the past, for biological treatment of wastewater such as detoxification of industrial phenolic wastewater (Shim and Kawamoto, 2002), removal of chlorinated phenols using mixed bacterial consortium (Zilouei et al., 2006), detoxification of PAHs contaminated wastewater using white rot fungal isolate *DSPM95* (Tekere et al., 2007), decolorization of distillery and brewery waste using *Coriolus* (FitzGibbon et al., 1998) and bioremediation of dye industry effluents using *Pseudomonas aeruginosa* and *Fusarium oxysporum* (Arumugam and Sivakami, 2016). Feijoo et al. (1995) reported that fungal packed bed bioreactors operating in continuous mode are better solutions to scaling-up enzyme production than batch cultures. Moreira et al. (2003) operated a packed bed bioreactor inoculated with *P. chrysosporium* to produce lignin and manganese peroxidase for extended periods of time to detoxify colored effluents. They used oxygen pulses in the input stream to shear away excess mycelium growth and achieved a decolorization efficiency of 80-90%. One approach used by Chander and Arora (2007) is to extract enzymes to overcome high adsorption by fungi.

Process optimization can be conducted using response surface methodology (RSM) (Bas and Boyaci, 2007), which is a collection of mathematical and statistical techniques to design experiments, develop models, evaluate factors, and optimize conditions. It also can reflect the role of each component of a process (Amani et al., 2011). The use of RSM for design optimization decreases the cost of expensive analysis methods. RSM also makes it possible to design different projections to provide graphic illustrations and allow visual interpretation of the functional relations between the response and experimental variables.

Central composite design (CCD) has been used extensively to build second order RSMs (Anderson and Whitcomb, 2000; Myers and Montgomery, 2002). Some studies have examined RSM for a packed bed fungal reactor to decolorize wastewater. Li and Jia (2008) investigated statistical modelling and optimized the removal rate in a packed bed fungal bioreactor. They investigated the role of hydraulic retention time and pollutant concentration.

No information was found in the literature on the optimization of PAHs removal from wastewater in packed bed fungal bioreactors. The objective of the present study is to investigate more thoroughly the biodegradation of PAHs contaminated water in a packed bed fungal bioreactor. Pyrene and phenanthrene were chosen as model contaminants. The effects of initial flow rate and influent pollutant concentrations were studied using RSM to enhance the enzyme activity of the system and consequently, degradation of PAHs.

2. Experimental

2.1. Cultivation of microorganism and preparation of inoculum

P. chrysosporium was purchased from the biotechnology laboratory of Amirkabir University of Technology. Fungi were maintained at low temperature at 4°C, on 2.5% malt extract agar slants. Subcultures were made every 2 months. The inoculum consisted of conidial suspensions, and was diluted with distilled water so that the absorbance at 650 nm in a 1 cm path-length cuvette was 0.5. The number of spores was enumerated using a Thoma counting-cell under an optical microscope at ×1000 magnification (2.4×10^6 cells mL^{-1}).

The nitrogen limited synthetic growth medium with the following composition (per litter of distilled water) was used in the present study: 10 g glucose; 2g KH_2PO_4; 0.5 g $MgSO_4.7H_2O$; 6 mg $CaCl_2$; 0.03 g $MnSO_4$; 0.06 g NaCl; 6 mg $FeSO_4.7H_2O$; 6 mg $CoCl_2$; 6 mg $ZnSO_4.7H_2O$: 6 mg $CuSO_4$; 0.6 mg $AlK(SO_4)_2.12H_2O$; 0.6 mg H_3BO_3; 0.6 mg $Na_2MoO_4.2H_2O$; 12 mg yeast extract; 0.2 g di-ammonium tartarate ($C_4H_{12}N_2O_6$); 1 mg thiamine; 0.07 g veratryl alcohol and 0.5 g Tween 80. The pH of the medium was adjusted to 4.5 using 20 mM sodium acetate. This growth medium was based on the culture composition suggested by Tien and Kirk (1988).

2.2. Packed bed bioreactor set up and operation

Fig. 1 shows the laboratory-scale packed bed bioreactor used. A jacketed column with a working volume of 300 mL was filled with crushed perlite carriers. The packed bed had a porosity of 67%. Influent was fed to the reactor through the bottom and an up-flow was created in the reactor. The reactor was equipped with recycled flow with a peristaltic pump. The reactor was aerated from the bottom directly to the reactor using air passing through filter-sterile at about 60 mL min^{-1}. The assembled reactor was autoclaved for 20 min at 121°C before runs. The spore solution was added at 10% v/v to the feed medium. The reactor

operated as a closed system for the first 4 days. After this initial period, medium was contaminated with different concentrations of pyrene and phenanthrene fed continuously into the reactor. The peristaltic pump recycled 50% of initial flow at each run. The recirculation maintained a high glucose concentration and yielded a lower protease concentration. Recirculation increases the mass transfer of oxygen and nutrients to the biofilm, on the other hand. Sampling from the reactor effluent was done after 24 h and 48 h of continuous performance of the reactor. The primary analysis revealed that 24 h was sufficient time to reach a steady state of pollutant concentration in the outlet flow. A similar reactor without inoculation was used as a control throughout the study.

A comprehensive set of experiments was designed to study the effect of changing factors. All mathematical and statistical calculations were done using experiment design software. After each run the reactor was cleaned, autoclaved and cultivated again for the next run. The packing and its fungal biofilm were dried in an oven to measure the dry weight of biomass at the end of each run and to assure that the fungal mass grown on packing was more or less the same for all runs.

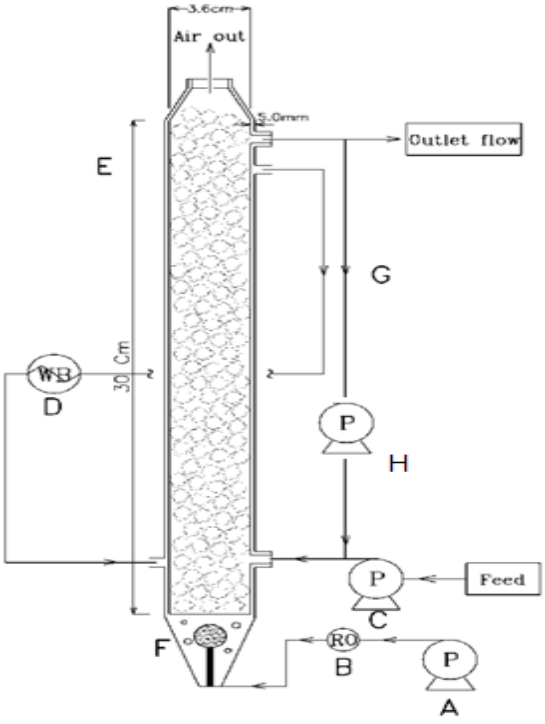


Fig. 1. Schematic diagram of packed bed bioreactor: A) Aeration pump, B) Flow meter, C) Peristaltic pump, D) Water bath, E) Bioreactor column, F) Sparger, G) Recycling stream, H) Peristaltic pump

2.3. Analyses

Residual PAHs in the samples obtained from the reactor effluent were extracted with dichloromethane at 1:10 v/v (dichloromethane to sample). After being agitated for 10 h in a tube shaker, dichloromethane was separated and analyzed by high performance liquid chromatography (HPLC). A C18 PAHs column (*Waters*, USA) was used. The samples were eluted at 1 mL min^{-1} with a mobile phase composed of acetonitrile and water (80:20) for 10 min. UV detection was performed at 254 nm (Kanchanamayoon and Tatrahun, 2008).

2.4. Enzyme assay

LiP activity was determined based on the oxidation of veratryl alcohol to veratraldehyde. The standard reaction mixture consisted of 1 mL of 125 mM sodium tartrate buffer (pH 3.0), 500 µL of 10 mM veratryl alcohol, 500 µL of 2 mM hydrogen peroxide solution and 500 µL of sample enzyme extract. The reaction was initiated by the addition of hydrogen peroxide and the change in absorbance at 310 nm was monitored for 1 min. One unit of enzyme activity was defined as 1 µmole of veratraldehyde produced per minute per ml of culture filtrate (Arora and Gill, 2000). The MnP assay was based on the oxidation of phenol red. The reaction mixture contained 1.0 mL of sodium succinate buffer (50 mM, pH=4.5), 1.0 mL of sodium lactate buffer (50 mM, pH=5.0), 0.4 mL of manganese sulfate (0.1 mM), 0.7 mL phenol red (0.1 mM), 0.4 mL of H_2O_2 (50 µM), 1 mg mL^{-1} of gelatin and 0.5 mL of sample (enzyme extract). The reaction was initiated by adding H_2O_2. Absorbance at 610 nm was monitored for 4 min. One unit of MnP enzyme activity was defined as 1 µmol of phenol red oxidized per min per mL of culture filtrate (Arora et al., 2002).

2.5. Experimental design

In this work, 2^3=8 factorial points and 6 star points with 6 central points were designed with Design-Expert 7.0. The behavior of the system is described by Eq. (1) as a second-order polynomial empirical model:

$$y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i<1}^{3} \sum_{j=1}^{3} \beta_{ij} X_i X_j + \varepsilon \quad (1)$$

where y is the response, β_0 is the constant coefficient, X_i $(i = 1-n)$ are non-coded variables, β_i are the linear interaction coefficients, β_{ii} are the quadratic interaction coefficients, β_{ij} (i and $j = 1-n$) are second-order interaction coefficients, and ε is the residual for each experiment (Trinh and Kang, 2010).

The data was processed for Eq. (1) using Design-Expert 7.0; ANOVA was used to evaluate the interaction between the process variables and the response. The fit of the polynomial model was expressed as the coefficient of determination (R^2), and its statistical significance was checked using a F-test in the same program.

3. Results and discussion

3.1. Start up

The decrease in PAHs in the packed bed bioreactor system over time was monitored by sampling every hour to determine the time necessary to reach a steady state for pollutant removal. In all

these runs, the system reached steady state within 9-12 h. Fig. 2 shows the results over time for 90 mg L^{-1} of pyrene and phenanthrene at an initial flow rate of 0.35 mL min^{-1} to approximate wastewater with a high pollution load. The sharp decrease in pollutant at the beginning may be a response to the dilution of feed influent to the reactor or lack of uniform distribution of pollutant in the medium. A low rate of PAHs removal was observed during operation of the control reactor probably as a result of adsorption onto the carriers. The results for the control reactor shown in Fig. 2 indicate that 8% of removal rate was the result of adsorption of pyrene and 11% for phenanthrene. This means that 82% of removal was due to the fungal bioremediation of pyrene and 87% of phenenthrene. After 7 days steady state operation of the reactor, the run was stopped because excessive growth of the biomass had clogged the system. These preliminary results indicate that 24 h is sufficient time for sampling and analysis of polluted wastewater.

3.2. Process optimization and statistical analysis

The flow rate of influent to the reactor and initial pollutants concentrations (pyrene and phenanthren) were chosen as experiment design variables. A three-factor-five-level CCD was used to determine the optimal values of the variables. Table 1 lists the ranges and levels of the variables.

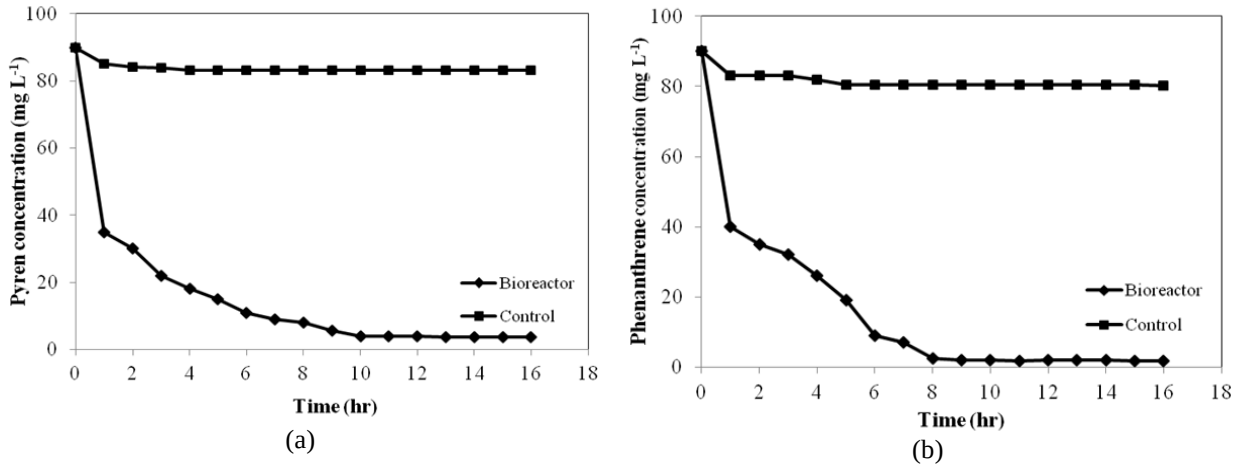


Fig. 2. Changes in concentrations of (a) pyrene and (b) phenanthrene over time in inoculated and control reactors

Table 1. Experimental range and levels of independent test variables

Variable	*Low axis (-α)*	*Low factorial (-1)*	*Centre point (0)*	*High factorial (-1)*	*High axis (+α)*
Influent flow rate (mL min^{-1}): A	0.14	0.22	0.35	0.46	0.55
Pyrene initial concentration (mg L^{-1}): B	50	60	75	90	100
Phenanthrene initial concentration (mg L^{-1}): C	50	60	75	90	100

Table 2. Experimental plan and results

Run	*Influent flow rate (mL min^{-1})*	*Pyrene initial concentration (mg L^{-1})*	*Phenanthrene initial concentration (mg L^{-1})*	*Pyrene effluent concentration (mg L^{-1})*	*Phenanthrene effluent concentration (mg L^{-1})*	*MnP activity (U L^{-1})*	*LiP activity (U L^{-1})*
1	0.47(+1)	90(+1)	60(-1)	5.48	0.95	15	392
2	0.35(0)	75(0)	75(0)	4.11	0.61	29	351
3	0.35(0)	75(0)	75(0)	0.62	0.77	48	323
4	0.22(-1)	90(+1)	60(-1)	0.00	0.00	13	429
5	0.22(-1)	60(+1)	90	0.03	0.00	17	310
6	0.35(0)	75(0)	75(0)	3.48	0.95	28	200
7	0.47(+1)	90(+1)	90(+1)	3.91	0.77	12	100
8	0.55(+α)	75(0)	75(0)	4.24	0.91	18	256
9	0.22(-1)	90(+1)	90(+1)	0.14	0.07	15	80
10	0.35(0)	75(0)	75(0)	3.06	0.40	20	170
11	0.47(+1)	60(+1)	60(-1)	1.22	0.47	76	454
12	0.35(0)	75(0)	100(+α)	0.05	0.29	12	150
13	0.47(+1)	60(+1)	60(-1)	0.00	0.02	36	100
14	0.47(+1)	60(+1)	90(+1)	4.002	0.73	17	320
15	0.35(0)	75(0)	75(0)	3.52	0.26	31	398
16	0.35(0)	50(-α)	75(0)	1.01	0.32	41	461
17	0.35(0)	75(0)	50(-α)	0.19	0.52	51	600
18	0.35(0)	75(0)	75(0)	2.87	0.45	35	351
19	0.35(0)	100(+α)	75(0)	4.91	0.60	10	100
20	0.14(-α)	75(0)	75(0)	0.00	0.00	28	370

Table 3. ANOVA for response surface models applied to the pollutant removal

Response	***Model***	***ANOVA***					
		Source	***Sum of square***	***DF***	***Mean square***	***F value***	***prob>F***
Pyrene concentration in effluent (mg L^{-1})	Reduced quadratic model	***Model***	***62.86***	***9***	***6.98***	***10.11***	***0.0006***
		A	31.07	1	31.07	44.95	<0.0001
		B	10.24	1	10.24	14.82	0.0032
		C	0.34	1	0.34	0.49	0.4980
		AB	1.15	1	1.15	1.67	0.2254
		BC	1.33	1	1.33	1.92	0.1955
		A^2	2.23	1	2.23	3.23	0.1024
		C^2	17.53	1	17.53	25.36	0.0005
		Residual	6.91	10	0.69		
		(R^2= 0.91, R^2_{adj}=0.81)					
Phenanthrene concentration in effluent (mg L^{-1})	Reduced quadratic model	***Model***	***1.65***	***9***	***0.18***	***4.39***	***0.0152***
		A	1.39	1	1.39	33.32	0.0002
		B	0.079	1	0.079	1.88	0.1998
		C	5.219E-003	1	5.219E-003	0.13	0.7309
		AB	0.028	1	0.028	0.67	0.4336
		BC	0015	1	0.015	0.36	0.5616
		A^2	0.042	1	0.042	1.02	0.3373
		B^2	0.038	1	0.038	0.91	0.3635
		C^2	0.074	1	0.074	1.78	0.2120
		Residual	0.42	10	0.042		
		(R^2= 0.85, R^2_{adj}=0.80)					

Table 4. ANOVA for response surface models applied to enzyme activity

Response	***Model***	***ANOVA***					
		Source	***Sum of square***	***DF***	***Mean square***	***F value***	***prob>F***
MnP activity (U L^{-1})	Reduced quadratic model	***Model***	***4859.11***	***13***	***373.78***	***4.90***	***0.0307***
		A	5.082	1	51.82	0.68	0.4415
		B	465.13	1	465.13	6.09	0.0486
		C	758.55	1	758.55	9.94	0.0198
		AB	218.20	1	218.20	2.86	0.1418
		AC	255.38	1	255.38	3.335	0.1171
		BC	741.13	1	741.13	9.71	0.0207
		A^2	173.66	1	173.66	2.28	0.1822
		B^2	100.75	1	100.75	1.32	0.2943
		Residual	457.98	6	76.33		
		(R^2= 0.91, R^2_{adj}=0.72)					
LiP activity (U L^{-1})	Reduced quadratic model	***Model***	***3.543E+005***	***13***	***27256.24***	***3.23***	***0.0790***
		A	6447.94	1	6447.94	0.77	0.4154
		B	65254.39	1	65254.39	7.74	0.0319
		C	1.012E+005	1	1.012E+005	12.01	0.0134
		AB	18184.71	1	18184.71	2.16	0.1922
		AC	10307.28	1	10307.28	1.22	0.3111
		BC	64202.24	1	64202.24	7.62	0.0329
		B^2	4259.53	1	4259.53	0.51	0.5038
		C^2	3766.08	1	3766.08	0.45	0.5287
		Residual	50568.6	6	8428.10		
		(R^2= 0.87, R^2_{adj}=0.76)					

Each factor was varied at five levels while the other parameters were kept constant. Once the desired ranges had been defined, they were coded to lie at ±1 for factorial points, 0 for center points, and ±α for axial points. A CCD for 3 independent variables, each at 5 levels, was fit to the model. A total of 20 experiments were carried out. Table 2 lists the experimental conditions and their responses. The experiments were performed in a random order to preserve the unbiased nature of the results. The software designs experiments to obtain reliable response and also checks the repeatability of the results. Table 3 lists the ANOVA results for the reduced quadratic model for PAHs removal at $p<0.1$ for a 90% confidence interval. Table 4 lists statistical factors and calculation for the models, as well as the ANOVA results for the reduced quadratic model for enzyme activity. The low p-value of the model verifies accuracy of the model and the significance of the results.

3.2. PAHs removal

Eqs. (2) and (3) are the model equations for coded values in a quadratic model fitting the experimental results for PAHs removal.

Pyrene concentration =

$+3.44 + 1.51A + 0.87B - 0.16C + 0.38AB - 0.41BC - 0.39A^2 - 1.10C^2$ (2)

Phenanthrene concentration =

$0.58 + 0.32A + 0.076B - 0.020C + 0.059AB - 0.043BC - 0.054A^2 - 0.051B^2 - 0.072C^2$ (3)

where A is the influent flow rate (mL min^{-1}), B is the initial pyrene concentration (mg L^{-1}) and C is the initial phenanthrene concentration (mg L^{-1}).

Figs. 3(a) and 3(b) demonstrate suitable agreement between the experimental and predicted values for pyrene and phenanthrene concentration in effluent, respectively. The legend is based on the color so that red signifies the highest; blue signifies the lowest and green signifies the moderate concentration. The R^2 for pyrene was 0.90 and adjusted R^2 (R^2_{adj}) was 0.81. The R^2 for phenenthere was 0.85 and R^2_{adj} was 0.80, indicating that the actual and predicted values were in agreement.

Fig. 4 shows the pyrene concentration of effluent in contour plots. It is clear from Fig. 4 that there is a combined effect of influent flow rate and initial pollutant concentration on the effluent pyrene concentration. When the influent flow rate increased (decreasing retention time), at a constant initial pollutant concentrations, the effluent pyrene concentration increased, which indicates lower removal efficiency. It is evident that the increase in initial pollutant concentration deteriorates the biodegradation process since it may have an inhibitory effect on fungus function and removal rate. Fig. 5 show the phenanthrene concentration of effluent in contour plots. The effect of initial variables on phenantrene concentration is the same as pyrene.

3.3. Enzyme activity

The model equation for coded values in the quadratic model that fits the experimental results for MnP activity is shown in Eq. (4) and for LiP in Eq. (5):

MnP activity =
$-31.91 - 3.03A - 9.07B - 11.58C - 5.22AB - 5.65AC + 9.63BC - 3.47A^2 - 2.64B^2$ (4)

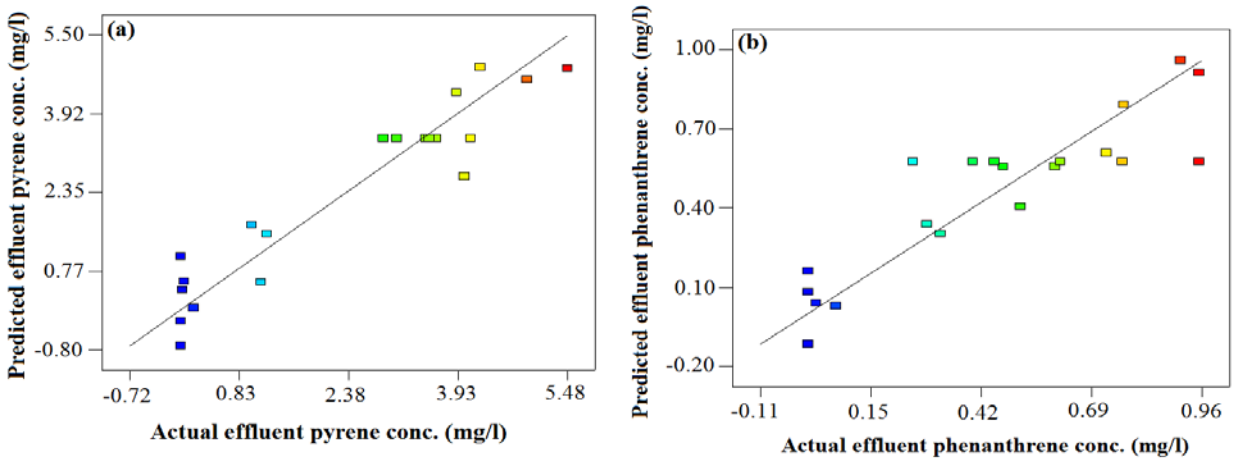


Fig. 3. Predicted versus actual diagram (a) effluent pyrene concentration, b) effluent phenanthrene concentration

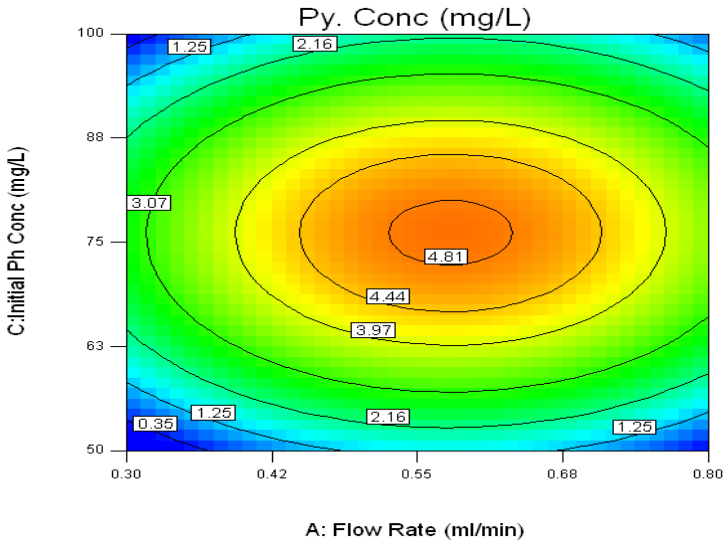


Fig. 4. Effect of flow rate and initial pollutant concentration on pyrene concentration in effluent (considering initial pyrene concentration of 60 mg L^{-1})

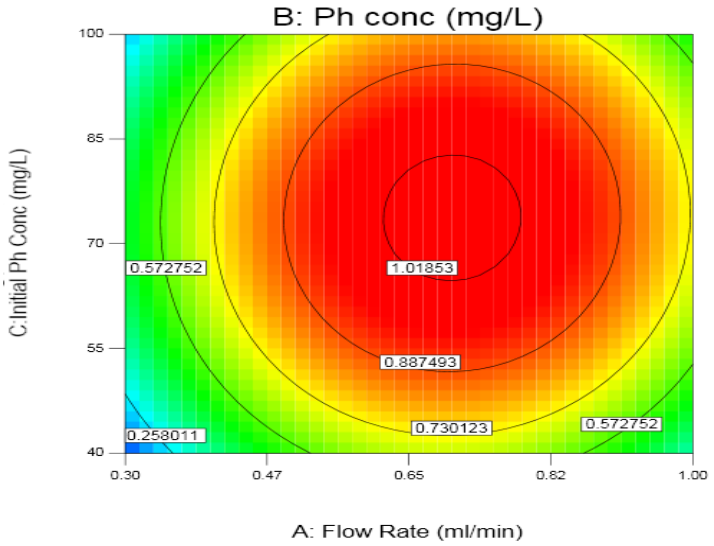
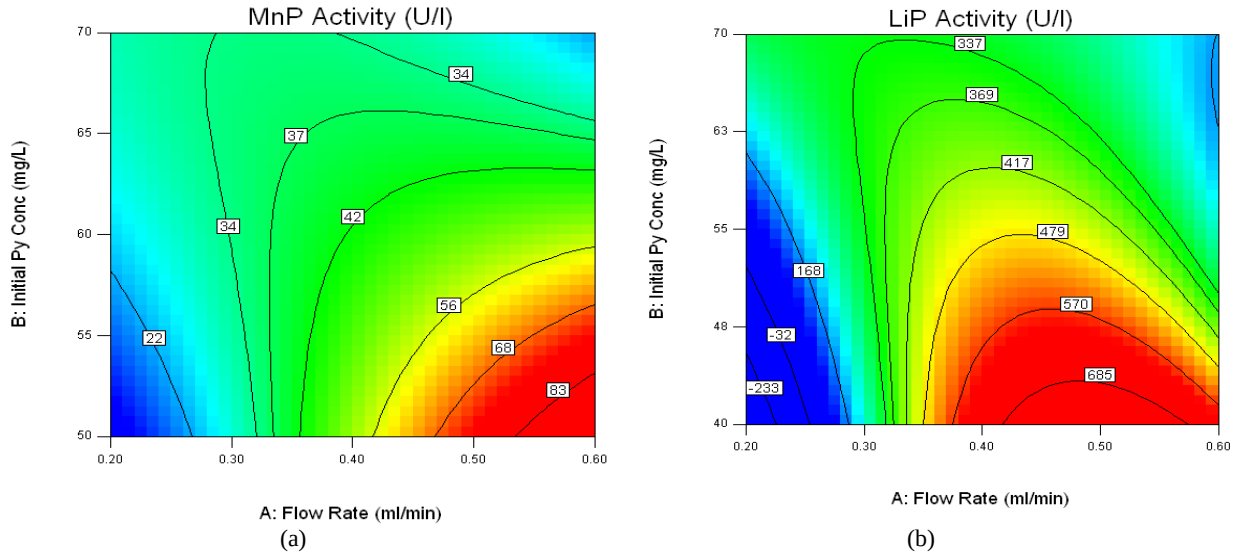


Fig. 5. Effect of flow rate and initial pollutant concentration on phenanthrene concentration in effluent (considering initial phenanthrene concentration of 60 mg L^{-1})



(a) (b)

Fig. 6. Effect of flow rate and initial pollutant concentrations on enzyme activity (a) MnP (b) LiP

LiP activity =
$300.31 - 33.76A - 107.40B - 133.77C - 47.68AB - 35.89AC - 89.58BC - 17.19B^2 + 19.17C^2$ (5)

where A is the influent flow rate in mL min^{-1}, B is the initial pyrene concentration in mg L^{-1} and C is the initial phenenthrene concentration in mg L^{-1}.

R^2 and R^2_{adj} for MnP were 0.91 and 0.72, respectively, for LiP, these values were 0.87 and 0.76, respectively. This confirms that the actual and predicted enzyme results were in agreement. The influence of different variables on MnP and LiP activities are depicted as contour plots in Figs. 6(a) and 6(b), respectively. Fig. 6(a) clearly shows the combined effect of influent flow rate and initial pyrene concentration on MnP activity. Increasing the initial pollutant concentration at constant influent flow, rate decreased the enzyme activity. This may be in response to the inhibitory effect of pollutant on the fungi and its enzyme activity. This decrease in activity may eventually lower the removal rate. Fig. 6(b) shows similar results for LiP activity.

3.4. Process optimization

When the process produces multiple responses, regions where the requirements simultaneously meet critical properties must be determined. Table 5 lists the regions with responses in the desirable range. The best compromise can be found visually by overlaying the critical response contours on a contour plot. Fig. 7 shows the graphical optimization of the overlay plot of the contour graphs that displays the areas of feasible response values in factor space.

The optimum conditions were identified by determining the minimum concentration of pollutants in the effluent and the maximum enzyme activity. It is evident in Table 6 that the optimal conditions are a flow rate 0.35 mL min^{-1} and initial pyrene and phenanthrene concentrations of 60 mg L^{-1} each. The

models predicted 1.65 mg L^{-1} of pyrene concentrations and 0.36 mg L^{-1} of phenanthrene concentration in effluent as response to the above optimal conditions. The enzymes activities were predicted to be 57 U L^{-1} for MnP and 426 U L^{-1} for LiP.

Confirmation test was carried out to validate the optimization results. Under the optimized conditions, the experimental pyrene and phenanthrene concentrations in effluent were 1.32 and 0.36 mg L^{-1}, respectively, MnP activity was 55 U L^{-1} and LiP activity was 356 U L^{-1}. The results of analysis shown in Table 7 indicate that the experimental values are in good agreement with the predicted values. For statistical model validation, each response must be a value between CI high and CI low. Table 7 shows that the experimental result of confirmation test is in agreement with predicted optimal results.

Juan et al. (2008) found that the removal rate for phenanthrene was of 32.3% and for pyrene was 57.7% in an agitated batch culture. Tekere et al. (2007) examined flow rates and achieved 44.0% PAHs removal for phenanthrene and 77.0% for pyrene in a packed bed fungal bioreactor using fungal isolate *DSPM95* at a flow rate of 0.9 mL min^{-1}, initial PAHs concentration of 1 mg L^{-1} over 27 days. The result of current study confirmed that white rot fungi can be successfully used to treat polluted wastewater and the process could be modeled and optimized.

Table 5. Desirable values for four responses

Response	***Desirable***
Pyrene concentration in effluent (mg L^{-1})	1.0-3.0
Phenanthrene concentration in effluent (mg L^{-1})	0.3-0.7
MnP (U L^{-1})	20-50
LiP (U L^{-1})	150-450

4. Conclusions

This research shows that production of ligninolytic enzymes in a packed bed bioreactor is a suitable approach for treatment of wastewater contaminated with PAHs, This process can be used in large-scale industrial plants. It was also found that RSM was a suitable tool for modelling, optimization and prediction of PAHs removal behavior in such systems.

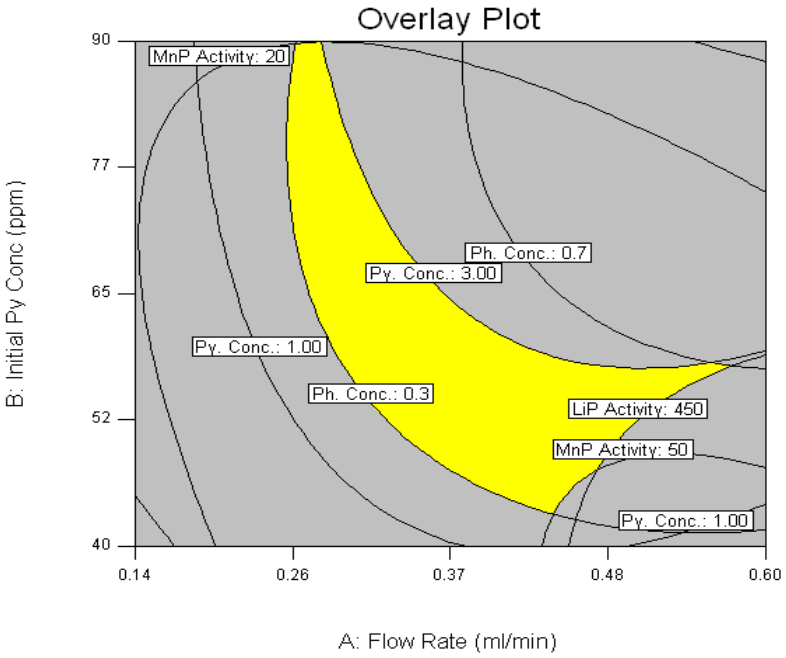


Fig. 7. Overlay plot for the desirable region

Table 6. Optimum conditions and predicted values for responses

Influent flow rate (mL min^{-1})	***Pyrene initial concentration (mg L^{-1})***	***Phenanthrene concentration (mg L^{-1})***	***Pyrene concentration in effluent (mg L^{-1})***	***Phenanthrene concentration in effluent (mg L^{-1})***	***MnP activity (U L^{-1})***	***LiP activity (UL^{-1})***	***Desirability***
0.35	60	60	0.36	0.65	57	426	0.89

Table 7. Confirmation test results

Response	***Target***	***Predicted result***	***CF result***	***CI low***	***CI high***
Pyren concentration in effluent (mg L^{-1})	Minimize	0.65	1.32	0.29	2.76
Phenanthrene concentration in effluent (mg L^{-1})	Minimize	0.36	0.63	0.25	0.86
MnP activity (U L^{-1})	Maximize	57	73	54.75	81.88
LiP activity (U L^{-1})	Maximize	426	526	286.4	621.9

Results from chemical analysis clearly indicated that the PAHs were largely removed in the inoculated reactor. However, pollutant loss was observed in the control reactor. This was believed to be the result of pollutant adsorption into the reactor.

The excessive growth of mycelia creates practical and technical considerations for this approach. It is necessary to study methods for the control and regulation of biomass extension.

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