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Growth and Optimization Processes of Mixed Microbial Population Degrading Chrysene

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Abstract

The response surface methodology (RSM) of chrysene removal was studied using mixed microbial culture. The effects of process factors such as initial chrysene concentration, agitation rate and, pH on chrysene degradation were also investigated. Maximum chrysene degradation rates of 60 and 50% were achieved at 300 rpm agitation rate and pH 8, respectively. Chrysene recoveries were within 51.22% to 72.12%. The numerical optimization process indicated that the optimum values for agitation rate, pH, and initial chrysene concentration were 334.3 rpm, 8.8 and 40.6 mg/L, respectively, with the desirability of 0.955. However, the second order quadratic model was statistically significant for the chrysene removal process with good agreement between the R^2 (0.9642), adjusted R^2 (0.9320), and predicted R^2 (0.8289). Finally, the results obtained from this study highlighted the optimal conditions for chrysene removal from an aqueous medium.

1. INTRODUCTION

Different forms of environmental contaminants are steadily being discharged or released into the environment through various means [1]. These contaminants are solely responsible for the deterioration of the environment through their toxic nature [2,3]. Chrysene, a poly-aromatic hydrocarbon (PAHs) with four benzene rings has existed in various forms in the environment and has contributed immensely to environmental deterioration [4].

Like most PAHs, chrysene is formed during the incomplete combustion of materials associated with the use of crude oil, natural gas, coal and wood for a domestic and industrial source of energy [5]. Existing conventional methods used to remove PAHs from aqueous and soil sediments such as soil vapor extraction, ultraviolet extraction treatment, groundwater pump and treat, and air sparing might be capital intensive and ecologically unacceptable [6,7]. On the other hand, biodegradation of PAHs using microorganisms has been proven to be an efficient way to degrade PAHs into less toxic forms [8,7]. This method is relatively cheap, easily managed and eco-friendly [9].

However, microorganisms isolated from contaminated environments are capable of degrading PAHs. This is due to their increased cell affinity to hydrophobic substances, which enable them to absorb and utilize the PAHs [10,11]. Also, microorganisms especially fungi are known to produce varieties of enzymes including lignin peroxidase and manganese peroxidase, which converts the PAHs into less harmful and simpler forms [7]. In this present study, mixed microbial culture isolated from coal mine contaminated soil was used to degrade chrysene in a liquid medium. However, mixed cultures were always preferable and can degrade a wide range of PAHs in comparison with single microbial strain [12,13]. The catabolic

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activities and synergetic interactions in mixed culture can lead to the complete mineralization of chrysene [14].

Previous works on the potentials of microbial culture and surfactants produced from microbial culture to degrade chrysene in a liquid medium have been reported. However, in the degradation of phenanthrene, chrysene, and benzo[a]pyrene in a liquid medium, [15] reported that laccase of *Polyporus sp. S133* was able to degrade most of the PAHs tested with phenanthrene (89%) degrading highest followed by chrysene (66%) and benzo[a]pyrene (55%) during a 72 hours incubation.

Also, [16] reported that *pseudoxanthomonas sp. PNK-04* was able to grow on chrysene as the sole carbon source and reduced chrysene concentration in the medium from 400 to 140 mg/L, i.e. degrading 60% of chrysene during 12 days incubation. Accordingly, this present study focused on chrysene degradation in a liquid medium using a novel mixed microbial culture isolated from coal mine contaminated soil. The effects of process factors such as pH, agitation rate and chrysene concentration on chrysene degradation were also investigated. Furthermore, response surface methodology (RSM) was employed in order to evaluate the optimum conditions of the studied process factors on chrysene degradation.

2. EXPERIMENTAL

2.1. Sample collection

The microbial strain used in this study was isolated from an abandoned coal mine contaminated soil (Onyeama Mine) located in Eke Udi Local Government Area, Enugu State Nigeria. Analytical grade chrysene (92% purity) used in this study was purchased from Bristol Scientific Company a Sigma-Aldrich authorized chemical distributor in Nigeria.

2.2. Preparation of culture media

Nutrient agar and broth purchased from Oxoid, Basingstoke UK were used for plating and culturing the microbial strain, respectively. The mixed culture was prepared by growing them on a malt extract agar plates at 25 °C for 30 days in the dark. In order to investigate the ability of the mixed microbial culture to degrade chrysene, the culture was grown on a 250 ml Erlenmeyer flask containing 50 ml of mineral salt medium (MSM). The MSM had the following compositions (g/L): 0.01 $(NH_4)_2SO_4$, 0.2 NH_4Cl, 0.25 K_2HPO_4, 0.02 $MnSO_4$, 0.5 $MgSO_4.7H_2O$, and 0.01 $CaCl_2$. The pH was maintained at 7.0±0.2. The MSM containing chrysene as a sole carbon source [17] was incubated for 30 days on a rotary shaker (VXR, Vibrax shaker, 220V, 50/60 Hz) set at 150 rpm and 37 °C. This was in order to obtain cultures with a stable chrysene degrading ability.

2.3. Batch Biodegradation of chrysene

The ability of the cultured microbial strain to degrade chrysene was investigated under sterile conditions in a 250 ml Erlenmeyer flask containing 50 ml of MSM. The MSM contained stock solutions of the initial chrysene concentration ranging from 10 to 50 mg/L. After 30 days of incubation, 1% v/v of the cultured strain was added to the biodegradation flask containing the MSM supplemented with chrysene. The effect of chrysene initial concentration on the MSM was investigated at pH 8 and agitation rate of 300 rpm for a period of 30 hours.

In order to study the effect of pH, a series of 5 batch experiments were carried out in 250 ml Erlenmeyer flasks containing 50 ml of the MSM. The pH of the medium was adjusted from 2 to 10 using 0.1 M HCl or NaOH, agitation rate of 300 rpm and 50 mg/L initial chrysene concentration. The effect of agitation rate on chrysene degradation was also investigated by incubating 250 ml Erlenmeyer flasks containing 50 ml of the MSM on a rotary shaker (VXR, Vibrax shaker, 220V, 50/60 Hz) at varying agitation rates of 100, 200, 300, 400, and 500 rpm. In order to monitor chrysene degradation, samples were taken from each flask at a specified time interval to follow up changes in the process conditions.

2.4. Extraction and recovery of residual chrysene

Soxhlet extraction process was used to extract 5 ml of the liquid culture using acetone/dichloromethane (1:1v/v) as the solvent. After extraction, the solvent was left to evaporate and the remaining residue was dissolved in 5 ml of dichloromethane. The chrysene concentration was estimated using a gas chromatography coupled with a mass spectrometer (GC-MS Shimadzu QP-500). The carrier gas was helium (purity > 96.9%) at a constant flow rate of 1.5 ml min^{-1} with column pressure at 100 kpa. The injection volume and temperature were 1 µl and 80 °C, respectively. The concentrations of chrysene were determined after calibrations of the method with standard chrysene samples at different concentrations. Chrysene degradation was calculated using Eqn. (1). In order to calculate chrysene recoveries, blank samples were spiked with 20 µl aliquots of chrysene standard solutions. Calibration solutions containing the same chrysene concentration were prepared in triplicates [27]. The % recovery of chrysene was calculated using the expression in Eqn. (2)

$$\%\text{Chrysene degradation} = \frac{\text{initial chrysene concentration} - \text{final chrysene concentration}}{\text{initial chrysene concentration}} \text{ x } 100 \quad (1)$$

$$\%\text{Chrysene recovery} = \frac{C_{PS} \times C_{SC}}{C_{SS} \times C_{PC}} \times 100 \quad (2)$$

Where; C_{PS} is the chrysene standard peak area in the sample, C_{SC} is the syringe standard peak area in the calibration solution, C_{SS} is the syringe standard peak in the sample, and C_{PC} is the chrysene standard peak area in the calibration solution.

2.5. Determination of microbial biomass

The microbial cell density in the sample was evaluated using a UV-Visible spectrophotometer (Agilent Cary 8454) by measuring the absorbance (OD) at 600nm wavelength. The measured OD_{600} was converted to dry weight using a calibration curve (plot of dry weight of microbial biomass versus OD_{600}). In order to determine the microbial biomass as dry weight, samples were centrifuged for 20 min in a 5 ml microcentrifuge tube. The biomass pellets formed were dried at 60 °C for 24 hours, allowed to cool and weighed (W_1). The empty weight of the centrifuge tube was also recorded (W_2). The microbial biomass dry weight (mg/L) was determined using the difference between the first and second weight [17].

2.6. Experimental design and statistical analysis of data

Response surface methodology (RSM) of design expert (version 7.0.0, Stat-ease, Inc., Minneapolis U.S.A software) was used in exploring the second order quadratic model, which was used in relating the independent factors and the response [18]. in order to obtain the response, experimental data was fitted to the second order quadratic model of Eqn. (3)

$$Y = \beta o + \sum_{i=1}^{n} \beta_j X_j + \sum_{i=1}^{n} \beta_{jj} X_j^2 + \sum_{i=1}^{n-1} \sum_{j=2}^{n} \beta_{ij} X_i X_j \quad (3)$$

According to Eqn. (3), Y is the response factor, βo is the intercept term β_j, β_{jj}, and β_{ij} are the linear, quadratic and interactive terms, respectively. However, in this study, the central composite design (CCD) of RSM was used to optimize the process factors for the chrysene removal. The coded levels of the process factors were determined using Eqn. (4).

$$Zj = \frac{Xi - Xj}{\Delta x} \quad (4)$$

where Zj is the coded value of the independent process factor, xi is the uncoded value of the independent process factor, xj is the uncoded value of the independent process factor at the center point and Δx is the step change of the independent process factor. In design expert, the four different models, which can be used for parameter estimation, include linear, two-factor interaction (2FI), quadratic, and cubic. The

independent process factors studied in order to optimize the removal of chrysene were initial chrysene concentration (mg/L) (A), agitation rate (rpm) (B), and pH (C).

The experimental levels of the process factors were coded with the notations ─1, 0, and +1 as shown in Table 1. The axial level denoted as α, (- 1.6817, +1.6817) was determined according to the number of experimental runs displayed by design expert 7.0 software. Table 2 shows the experimental design matrix with process factor combinations and responses for chrysene removal. However, the CCD of RSM was used to generate 20 experimental runs, which were carried out in a randomized manner to represent the chrysene removal process.

Table 1. *Experimental factors and their levels*

		Range and levels		
Independent process factors	unit	-1 (low)	0 (center)	+1 (high)
Chrysene concentration (A)	mg/L	10	30	50
Agitation rate (B)	rpm	100	300	500
pH (C)		2	6	10

Table 2. *Experimental design matrix*

	Factor A (mg/L)	Factor B (rpm)	Factor C	Responses	
Std order	chrysene conc	Agitation rate	pH	Actual	predicted
1	10 (-1)	100 (-1)	2 (-1)	58.6	69.5
2	50 (1)	100 (-1)	2 (-1)	55.9	60.3
3	10 (-1)	500 (1)	2 (-1)	57.2	57.7
4	50 (1)	500 (1)	2 (-1)	59.4	59.1
5	10 (-1)	100 (-1)	10 (1)	60.4	59.9
6	50 (1)	100 (-1)	10 (1)	70.1	69.6
7	10 (-1)	500 (1)	10 (1)	55.6	58.1
8	50 (1)	500 (1)	10 (1)	78.9	81.3
9	10 (-α)	300 (0)	6 (0)	54.3	52.8
10	50 (α)	300 (0)	6 (0)	70.7	71.3
11	30 (0)	100 (-α)	6 (0)	50.6	51.2
12	30 (0)	500 (α)	6 (0)	53.2	53.1
13	30 (0)	300 (0)	6 (-α)	70.9	71.3
14	30 (α)	300 (0)	10 (α)	73.6	75.4
15	30 (0)	300 (0)	6 (0)	83.9	86.1
16	30 (0)	300 (0)	6 (0)	81.4	81.5
17	30 (0)	300 (0)	6 (0)	84.9	88.2
18	30 (0)	300 (0)	6 (0)	85.5	87.4
19	30 (0)	300 (0)	6 (0)	88.9	89.1
20	30 (0)	300 (0)	6 (0)	82.2	82.3

3. RESULTS AND DISCUSSION

3.1. Effect of initial chrysene concentration on microbial growth

The growth profile of the mixed microbial culture at different initial chrysene concentrations are shown in Figure 1. It was found that the mixed microbial culture utilizing chrysene showed a lag phase before the exponential growth. The lag phase was observed to vary at different initial chrysene concentration indicating that the mixed microbial culture was not well adapted in the MSM containing chrysene. However, Figure 1 indicated that the growth of the mixed microbial culture was low at 10 mg/L indicating that the chrysene in the medium might not be sufficient for microbial growth.
Similarly, [4] reported that of all the carbon sources tested during chrysene degradation, the lowest rate of chrysene degradation (35%) was observed at the lowest glucose concentration of 2%. The exponential phase at initial chrysene concentrations of 10 mg/L, 20 mg/L, and 30 mg/L commenced after 5 hours of microbial adaptation to chrysene in the MSM (lag phase). On the other hand, at initial chrysene concentrations of 40 mg/L and 50 mg/L, the exponential growth phase commenced after 10 hours. These observations suggested that the time for exponential growth phase increased with an increase in initial chrysene concentration [19].

The high demand for energy needed to overcome the effects of chrysene inhibition at higher concentrations [19] could be contributing to the increased time for the exponential growth phase. However, maximum microbial growth and chrysene degradation (64%) was obtained at 50 mg/L initial chrysene concentration and it was chosen for further studies. Consequently, it was assumed that the majority of the chrysene degradation took place during the exponential growth phase when the microbial cells are actively diving and microbial cellular respiration is at its peak [10]. However, external nutrients sources were not added during the experiment. This was in order to investigate the growth of the mixed microbial culture in the MSM containing chrysene as a sole carbon and energy source [20, 21].

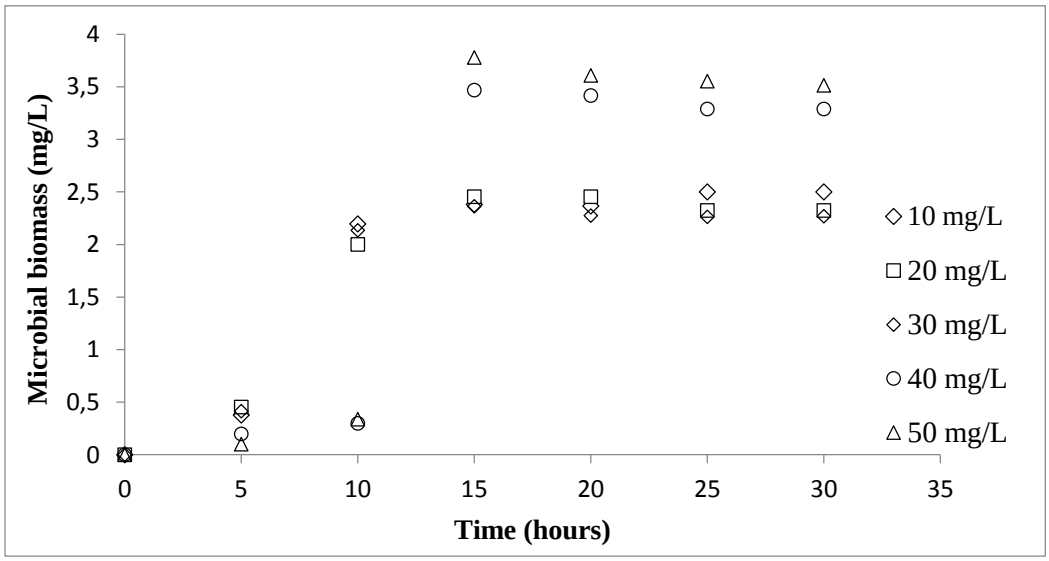


Figure 1. *Mixed microbial growth at different initial chrysene concentration*

3.2. Effect of agitation rate on chrysene degradation

The effects of agitation rate on chrysene degradation in the MSM was carried out at 50 mg/L initial chrysene concentration, pH 8 and agitation rates of 100, 200, 300, 400, and 500 rpm (Figure 2). From Figure 2, chrysene degradation increased with an increase in agitation rate and was highest (60%) at 300 rpm. The maximum mixing of the dissolved oxygen at 300 rpm could be contributing to the increased chrysene degradation. Another reason was attributed to the even distribution of the initial chrysene

concentration in the MSM at 300 rpm, thereby increasing the growing number of the microbial population [22].

However, during the agitation tests, the flasks were not perfectly sealed; this was to ensure adequate mass transfer of oxygen. On the other hand, chrysene degradation at agitation rates of 100 and 200 rpm were 20% and 25%, respectively. The decrease in chrysene degradation below 300 rpm could be due to insufficient mixing of the initial chrysene concentration. However, the restriction of oxygen to the microbial cells at lower agitation rates (100 and 200 rpm) in the MSM could inhibit the oxidative microbial enzymes and/or slow down chrysene degradation [4]. [23] reported that dissolved oxygen concentration was notably lower at 100 and 200 rpm, which resulted in oxygen limitation and lower microbial density in the maximum production of L-glutaminase by *Bacillus cereus (MTCC 1305).*

A decrease in chrysene degradation was also observed at 400 and 500 rpm as shown in Figure 2. The higher agitation rates (400 and 500 rpm) could have aggravated microbial cell damage, which in turn led to the inactivation of microbial enzyme and subsequently decreased chrysene degradation [24]. Also, [25] found that the lowest cell stability of *β-manganese* was at the agitation rate of 750 rpm, which corresponded to the lowest enzyme stability in the medium.

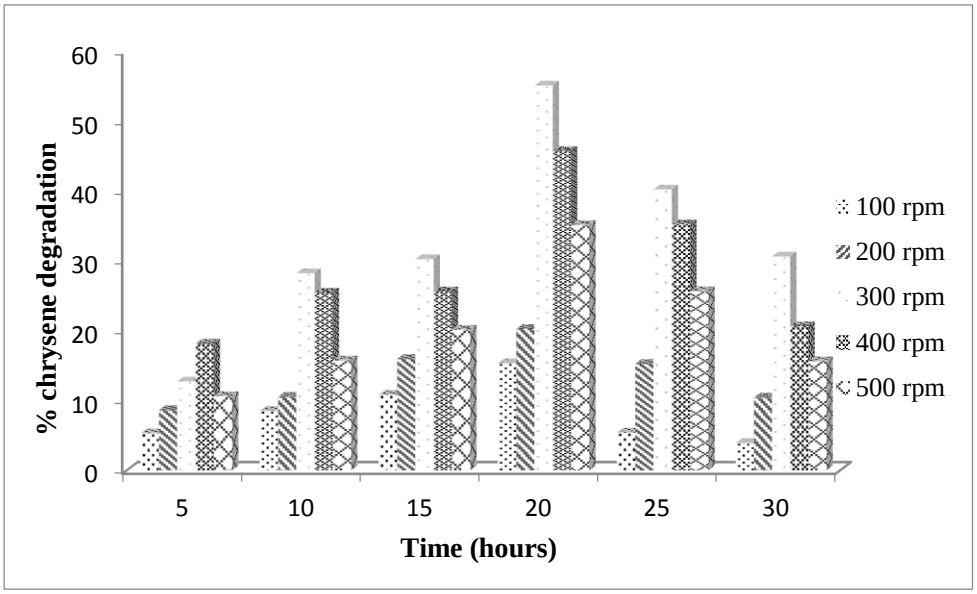


Figure 2. *Effect of agitation rates on chrysene degradation by mixed microbial culture*

3.3. Effect of pH on chrysene degradation

Chrysene degradation by mixed microbial culture was investigated at pH range varying from 2 to 10 at 50 mg/L initial chrysene concentration and the agitation rate of 300 rpm (Figure 3). Maximum chrysene degradation of 50% was achieved at pH 8 and optimum contact time of 20 hours suggesting that the mixed microbial culture exists predominantly as alkaline species. Consequently, under alkaline conditions, chrysene has a high oxidation potential that can lead to a rapid redox reaction leading to the production of oxygen [26].

Chrysene degradation rates of 20%, 30%, and 40% were observed at pH 2, 4, and 6, respectively. These observations indicated poor chrysene degradation in acidic medium. The disturbance of the MSM through the addition of HCl could be responsible for the observed low chrysene degradation. Another reason for the observed low chrysene degradation at pH values less than 8 could be due to the different reaction pathway, which led to the formation of chrysene intermediate [26]. Chrysene degradation was also observed to decrease at pH 10, which was attributed to the increase in ionic interaction with OH^- present in the alkaline pH 10.

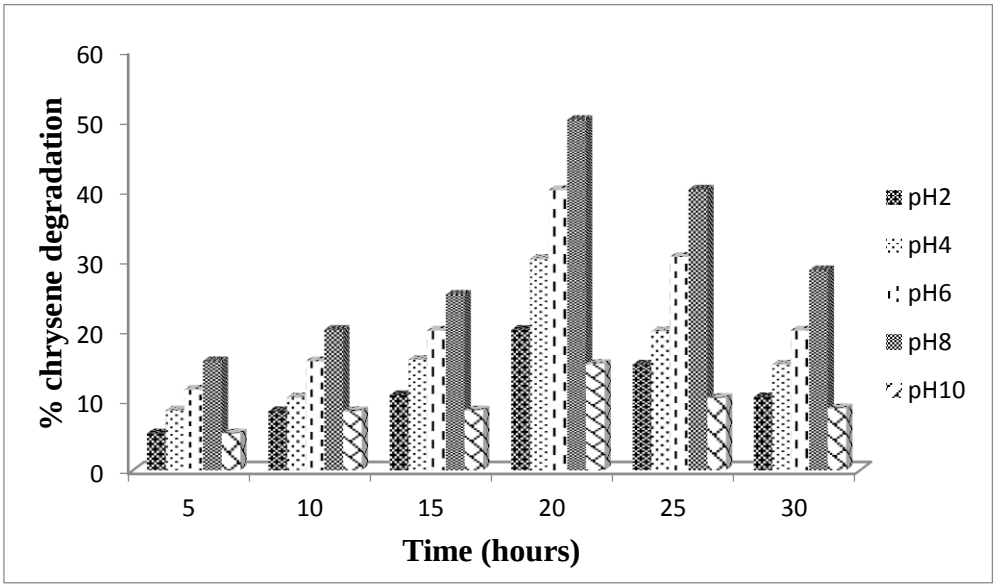


Figure 3. *Effect of pH on chrysene degradation by mixed microbial culture*

3.4. Residual chrysene recovery

Chrysene recoveries and relative standard deviations (RSDs) were calculated for each initial chrysene concentration [27] as shown in Table 3. However, it is recommended that the average recoveries and RSDs should be in accordance with European Union (EU) regulation 333/2007 [28, 29]. The results for the chrysene recoveries from GC-MS extraction procedure indicated that the recovery values varied for each chrysene concentration. The varied recovery was attributed to the volatility and solubility of chrysene in the acetone/dichloromethane solution used as solvent [30].

Chrysene recoveries from Table 3 ranged from 53.42% to 72.12% and were within the recommended values (50 to 120%) by EU regulation [27]. The values of RSDs varied from 2.19% to 9.30% indicating a good precision of the analytical method. Also, in the recovery of PAHs from cocoa and chocolate sample, [31] reported an RSD lower than 20%, and authors concluded that the data were in accordance with the EU requirements. Table 4 shows the comparisons of chrysene degradation with previous works from literature. It can be observed that different strains of microbial mixed culture and surfactant were able to degrade chrysene. Also, the mixed cultures were able to grow on different culture media containing chrysene (Table 4). However, it can be said that the results of chrysene degradation using mixed culture isolated from coal mine contaminated soil was comparable with previous works with culture conditions of pH, agitation, and temperature.

Table 3. *Recoveries and relative standard deviation of residual chrysene*

Analyte	Concentration (mg/L)	%Recoveries	%RSDs
Chrysene	10	53.42	2.41
	20	51.22	2.19
	30	62.77	7.32
	40	68.31	6.06
	50	72.12	9.3

Table 4. *Comparison of chrysene degradation in various microbial cultures*

Microbial species/surfactant	**PAHs**	**Culture Media**	**Culture conditions**	**Time**	**% Degradation**	**Ref.**
Fungus (*Polyporus sp. S133*)	chrysene	MSB	pH (5.7) Agitation (120 rpm) Temperature (25°C)	30 days	65%	[4]
Surfactant (Tween 80)	chrysene	MSB	pH (5.7) Agitation (120 rpm) Temperature (25°C)	30 days	49%	[4]
Pseudoxanthomonas sp. PNK-04	chrysene	PMS	pH (7) Agitation (180 rpm) Temperature (37°C)	12 days	60%	[16]
Mixed cultures: *A. xylosoxidans* *Pseduomonas sp.* *Sphingomonas sp.*	chrysene	BHM	pH (7) Agitation (150 rpm) Temperature (4°C)	15 days	 54% 33% 17%	[14]
Mixed culture (bacteria and fungus)	chrysene	MSM	pH (7.5) Agitation (150 rpm) Temperature (30°C)	30 days	82%	[20]
Mixed cultures: *P. Chrysosporium* *P. Ostreatus* *Fusarium sp F092*	chrysene	MELM	Temperature (25°C)	30 days	 52% 45% 48%	[21]
Mixed culture	chrysene	MSM	pH (7) Agitation (150 rpm) Temperature (37°C)	30 days	64%	present study

MSB (Mineral salt broth), PMS (Phosphate-buffered salt medium), BHM (Bushnell-Haas medium), MSM (Mineral salt medium), MELM (Malt extract liquid medium)

3.5. Diagnostics plots for statistical validation

The normal probability plot of residuals was retrieved from design Expert 7.0 software. The normal probability plot, which was used to check the adequacy of the statistical model, determines if the residual follows a normal distribution [32]. Accordingly, the normal probability plot of residuals follows a straight-line, indicating the normal distribution of experimental data (Figure 4a). However, it was observed that the residuals fluctuated randomly around the center line confirming the validity of the statistical model (Figure 4b). The plot of residuals versus predicted values in Figure 4c indicated a good agreement between the predicted and actual response values as the predicted response were scattered within the range of residuals. [33] found that if the model is adequate, the residuals should contain no obvious pattern within the range of predicted response values.

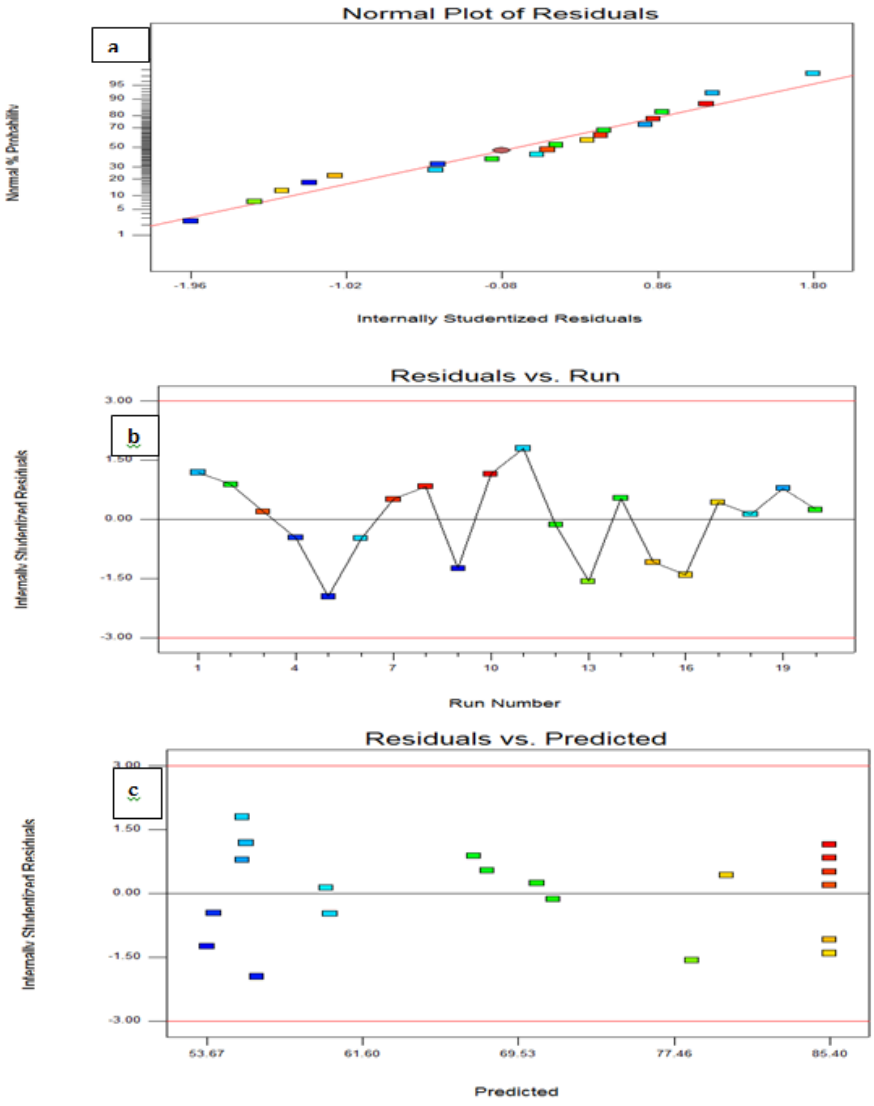


Figure 4. *Residual diagnostics plots for chrysene removal (a) normal probability plot (b) residuals vs. run number plot (c) residuals vs. predicted plot*

3.6. ANOVA for the Response Surface Quadratic Model

The ANOVA of the quadratic model showed an F-value of 29.92, which indicated that the model is significant with $P< 0.05$ (Table 5). The F-value and P- value of the model lack of fit were 1.19 and 0.4263, respectively, showing that the lack of fit is non-significant and relative to pure error [34]. There is a 43.63% possibility that the lack of fit can occur due to noise, indicating that the model is good. [35] reported that for the desired model to fit, a non-significant lack of fit is good. According to Table 6, the quadratic model with a non-significant lack of fit was suggested, while the cubic model was aliased. The quadratic model equations, relating the response (%chrysene removal) with the independent process factors for coded and actual factors, are shown in Eqn. (5) and Eqn. (6), respectively.

Model equation in terms of coded factors:

$$\%\text{chrysene removal} = 85.40 + 4.24A + 0.100B + 2.10C + 3.00\,AB + 5.00\,AC + 3.00BC - 7.80A^2 - 11.16B^2 - 3.73C^2 \quad (5)$$

Model equation in terms of actual factors:

$$\%\text{chrysene removal} = 49.41759 + 0.78214*\text{chrysene conc.} + 0.12288*\text{Agitation rate} + 0.32627*\text{pH} + 7.50000E\text{-}004*\text{chrysene conc.}*\text{Agitation rate} + 0.062500*\text{chrysene conc.}*\text{pH} + 3.75000E\text{-}003*\text{Agitation rate}*\text{pH} - 0.019499*\text{chrysene conc}^2 - 2.78962E\text{-}004*\text{Agitation rate}^2 - 0.23337*\text{pH}^2 \quad (6)$$

However, the ANOVA Table 5 indicated that the agitation rate (B) was non-significant ($P > 0.05$). Therefore, eliminating B from the coded Eqn. (5) and agitation rate from the actual Eqn. (6), the rearranged model Eqns. (7) and (8), were obtained. Eqns. (7) and (8) represent the final significant model equations for the studied chrysene removal process.

Final significant model equation in terms coded factors:

$$\%\text{chrysene removal} = 85.40 + 4.24A + 2.10C + 3.00\,AB + 5.00\,AC + 3.00BC - 7.80A^2 - 11.16B^2 - 3.73C^2 \quad (7)$$

Final significant model equation in terms actual factors:

$$\%\text{chrysene removal} = 49.41759 + 0.78214*\text{chrysene conc.} + 0.32627*\text{pH} + 7.50000E\text{-}004*\text{chrysene conc.}*\text{Agitation rate} + 0.062500*\text{chrysene conc.}*\text{pH} + 3.75000E\text{-}003*\text{Agitation rate}*\text{pH} - 0.019499*\text{chrysene conc}^2 - 2.78962E\text{-}004*\text{Agitation rate}^2 - 0.23337*\text{pH}^2 \quad (8)$$

Eqns. (7) and (8) could explain the significant effects and interactions of the process factors on chrysene removal. From the ANOVA Table 5, the values of the Prob > F for all coefficient terms in the significant model Eqns. (7) and (8) were less than 0.05, indicating the significance of the model terms at 95% confidence limit. However, the R^2 (0.9642) and adjusted R^2 (0.9320) are close to each other, indicating the maximum adequacy of the quadratic model and good relation between the calculated and observed data [36]. The adequate precision (13.06) in Table 5 was greater than 4, which showed an adequate signal of the model. Therefore, this model can be used to navigate the design space for chrysene removal efficiency using mixed microbial culture. Consequently, the model summary statistics in Table 7 showed that design expert suggested the quadratic model with good agreement between the R^2 (0.9642), adjusted R^2 (0.9320) and predicted R^2 (0.8289). The low PRESS (560.08) further confirmed the high significance and statistical validity of the quadratic model (Tables 5 and 7).

Table 5. *ANOVA for Response Surface Quadratic Model*

Source	Sum of Squares	DF	Mean Square	F Value	p-value Prob > F	
Model	3156.59	9	350.73	29.92	0.0001	significant
A-chrysene conc	245.93	1	245.93	20.98	0.001	
B-Agitation rate	0.14	1	0.14	0.012	0.9163	
C-pH	60.43	1	60.43	5.16	0.0465	
AB	72	1	72	6.14	0.0326	
AC	200	1	200	17.06	0.002	
BC	72	1	72	6.14	0.0326	
A^2	876.72	1	876.72	74.8	0.0001	
B^2	1794.37	1	1794.37	153.09	0.0001	
C^2	200.92	1	200.92	17.14	0.002	
Residual	117.21	10	11.72			
Lack of Fit	63.71	5	12.74	1.19	0.4263	not significant
Pure Error	53.5	5	10.7			
Cor Total	3273.8	19				

Std. Dev	3.42	R^2	0.9642
Mean	69.9	Adjusted R^2	0.932
CV%	4.9	Predicted R^2	0.8289
PRESS	560.08	Adeq precision	13.106

Std. Dev (Standard deviation), CV% (coefficient of variation)

Table 6. *Lack of fit test*

Sum of Source	Mean Squares	F (DF)	P-value square	F-value	Prob >F	
Linear	2913.8	11	264.89	24.76	0.0012	
2FI	2569.8	8	321.23	30.02	0.0008	
Quadratic	63.71	5	12.74	1.19	0.4263	Suggested
Cubic	33.71	1	33.71	3.15	0.1361	Aliased
Pure Error	53.5	5	10.71			

Table 7. *Models Summary Statistics*

Source	Std Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	13.62	0.9360	-0.0763	-0.3291	4351.06	
2FI	14.2	0.1987	0.1711	0.6698	5466.57	
Quadratic	3.42	0.9642	0.932	0.8289	560.08	Suggested
Cubic	3.81	0.9734	0.9156	-1.2931	7507.3	Aliased

3.7. Optimization process for the quadratic model

Numerical optimization process was employed to optimize the independent process factors (pH, agitation rate, and chrysene concentration) for chrysene removal. The algorithm involves the setting of the desired goal and response with weight and importance for each goal (Table 8). However, during the optimization process, the set goal was to find the optimum values of the independent process factors in order to maximize chrysene removal. The optimum values obtained for the process factors were chrysene concentration (40.6 mg/L), agitation rate (334.3 rpm), and pH (8.8). Maximum chrysene removal at these optimum values was 87.3% with the desirability of 0.955 (Table 8). Furthermore, the ramps for the chrysene removal process in Figures. (5a), (5b), and (5c) showed the points where the optimum values were obtained from the starting and final values of the process factors during the studied chrysene removal process.

Table 8. *Numerical optimization process for chrysene removal*

Name	Goal	Lower limit	Upper limit	Weight	Weight	Importance
Chrysene conc (mg/L)	is in range	10	50	1	1	3
Agitation rate (rpm)	is in range	100	500	1	1	3
pH	is in range	2	10	1	1	3
%chrysene removal	Maximize	51	89	1	1	3

Number of solution	Chrysene conc (mg/L)	Agitation rate (rpm)	pH	%chrysene removal	Desirability	
1	40.6	334.3	8.8	87.3	0.95	selected

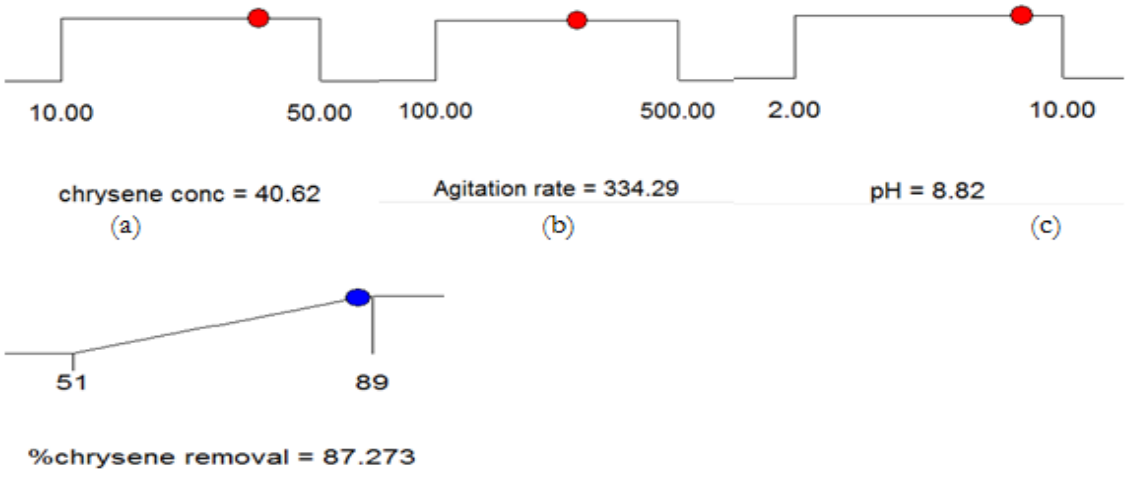
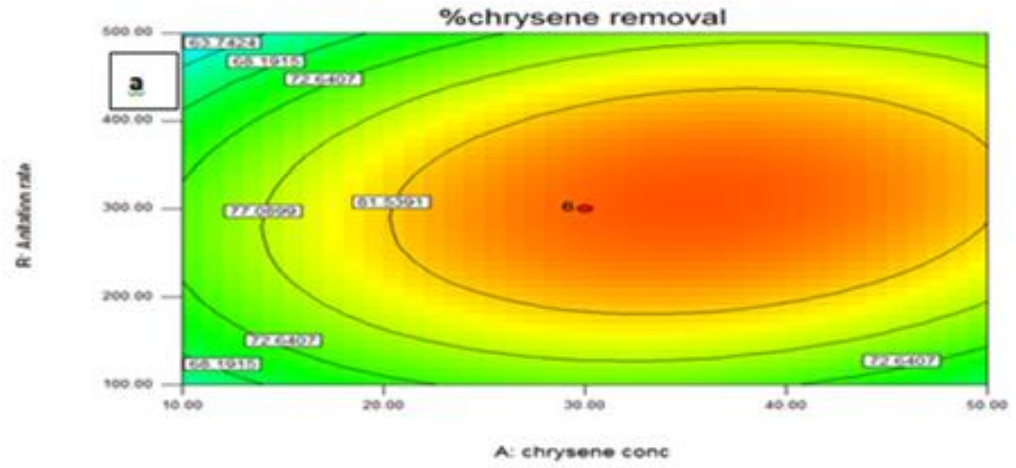


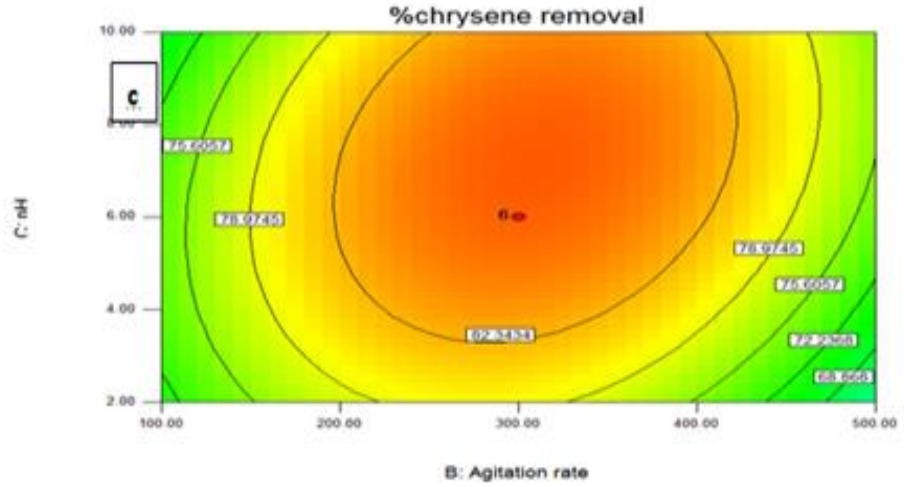
Figure 5*. Ramps of the chrysene removal optimization process at (a) chrysene concentration (mg/L), (b) agitation rate (rpm), and (c) pH*

3.8. Response surface and contour plots

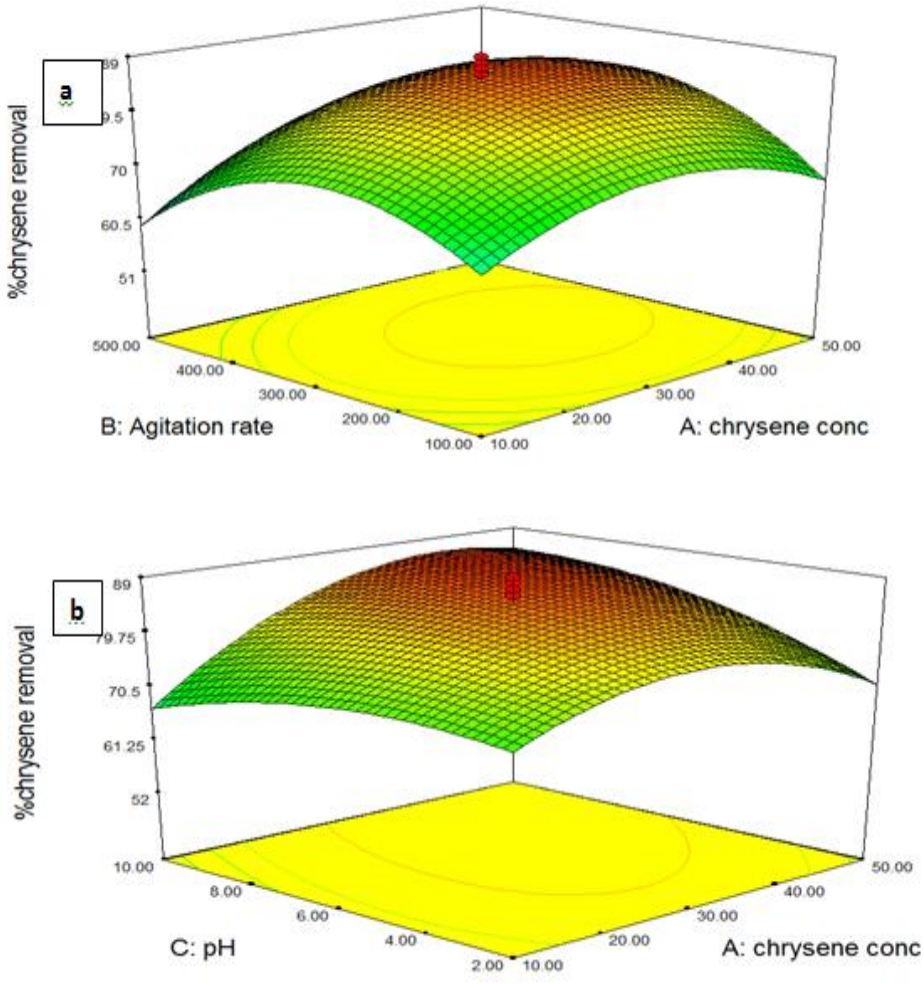
Three-dimensional response surface plots (3D) and two-dimensional contour plots (2D) were generated in terms of the actual factors using the model Eqn. (8). This was in order to investigate the interaction between the independent process factors on chrysene removal. The 2D and 3D contour and response surface plots for chrysene removal are shown in Figures 6 and 7, respectively. The 2D contour plots in Figures. (6a), (6b) and (6c) corresponds to a particular height of the response, which was defined for the levels of the combinations of the independent process factors [36]. Accordingly, the 2D contour plots depict that the highest height of the contour was at the agitation rate of 300 rpm, while the pH and chrysene concentration had little effect on chrysene removal.

The 3D surface plot in Figure 7a shows that the increase in pH and chrysene concentration to 8 and 40 mg/L, respectively increased chrysene removal indicating the alkaline pH tolerance of the mixed microbial culture [37]. Also, the increase in agitation rate and chrysene concentration to 300 rpm and 40 mg/L, respectively, increased chrysene removal (Figure 7b). It was obvious that the agitation rate of 300 rpm permits the maximum contact between the dissolved oxygen, microbial population and chrysene in the MSM. The increase in pH and agitation rate to 8 and 300 rpm, respectively, increased chrysene removal (Figure 7c). However, the optimization results on chrysene removal were experimentally validated at the optimum values of agitation rate (334 rpm), pH (8.8) and chrysene concentration (40.6 mg/L). The chrysene removal from the validated experimental result was 80%, which was close to the model predicted results of 87.3%. The closeness of the validated and model predicted results confirmed the validity of the quadratic model.





Figures 6. *2D Contour plots for (a) chrysene concentration and agitation rate, (b) chrysene concentration and pH, and (c) agitation rate and pH*



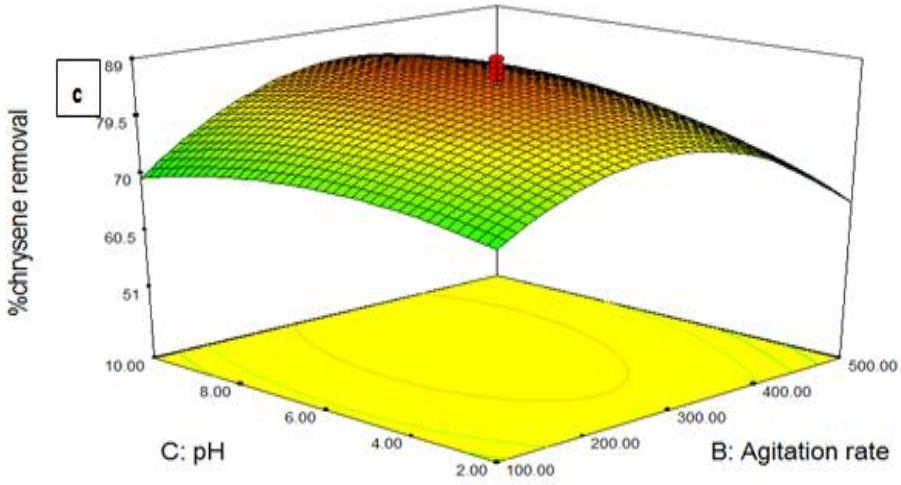


Figure 7*. 3D response surface plots for (a) chrysene concentration and agitation rate, (b) chrysene concentration and pH, and (c) agitation rate and pH*

4. CONCLUSION

The results obtained from this study showed that agitation rate, pH, and chrysene concentration had a significant effect on chrysene removal by mixed microbial culture. Maximum microbial growth was obtained at 50 mg/L chrysene concentration. The optimum values for chrysene removal, which were obtained from the numerical optimization process, were chrysene concentration (40.6 mg/L), agitation rate (334 rpm) and pH (8.8). It may be concluded the second quadratic model was sufficient to obtain and predict the optimum values of the independent process factors on chrysene removal.

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CONFLICTS OF INTEREST

No conflict of interest was declared by the authors.

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