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Application of statistical experimental methodology to optimize bioremediation of n-alkanes in aquatic environment

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ABSTRACT

Response surface methodology (RSM) was employed to optimize nitrogen and phosphorus concentrations for removal of n-alkanes from crude oil contaminated seawater samples in batch reactors. Erlenmeyer flasks were used as bioreactors; each containing 250 mL dispersed crude oil contaminated seawater, indigenous acclimatized microorganism and different amounts of nitrogen and phosphorus based on central composite design (CCD). Samples were extracted and analyzed according to US-EPA protocols using a gas chromatograph. During 28 days of bioremediation, a maximum of 95% total aliphatic hydrocarbons removal was observed. The obtained Model *F*-value of 267.73 and probability $F < 0.0001$ implied the model was significant. Numerical condition optimization via a quadratic model, predicted 98% n-alkanes removal for a 20-day laboratory bioremediation trial using nitrogen and phosphorus concentrations of 13.62 and 1.39 mg/L, respectively. In actual experiments, 95% removal was observed under these conditions.

1. Introduction

In 1946, Zobell from Scripps Institution of Oceanography discovered that many microorganisms are capable of utilizing hydrocarbons as the sole source of energy for metabolism [1]. Historically, the most valuable data about hydrocarbon bioremediation were collected during bioremediation of the Exxon Valdez oil spill in 1989 [2].

Petroleum hydrocarbons can typically be divided into four classes: saturates, aromatics, asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins), and resins (pyridines, quinolines, carbazoles, sulfoxides, and amides). Biodegradation rates have been shown to be highest for the saturates, followed by the light aromatics, with high-molecular-weight aromatics and polar compounds exhibiting extremely low rates of degradation [3–5]. In contrast, higher naphthalene biodegradation than hexadecane from a freshwater lake were reported [6]. Rapid biodegradation of aromatics were also reported in a petroleum bioremediation process [7], and dissimilar degradation rates have been documented in literature [2,4,8,9]. Rontani et al. [10] investigated the trends of n-alkanes removal and showed that the disappearance of low molecular-weight alkanes is stimulated by fertilizer addition. It is generally thought that n-alkanes of shorter chain length are more easily used as an energy source than the longer chain ones [11].

Biostimulation involves the addition of nutrients (mainly nitrogen and phosphorus) to accelerate the biodegradation process [12]. In most marine ecosystems heavily contaminated with hydrocarbons, nitrogen and phosphorus are limiting factors in oil biodegradation. Bioremediation of oil spills has therefore focused on countering this limitation by adding fertilizers to petroleum-contaminated marine environments [13]. Several laboratory experiments have shown that the addition of nutrients might be effective for increasing the biodegradation of organic compounds because it stimulates bacterial growth [14,15]. The predominant mechanism of n-alkanes degradation involves terminal oxidation of the corresponding alcohol, aldehyde, or fatty acid functional groups. Branched alkanes are less readily degraded in comparison to n-alkanes [2].

Since several variables are involved in the biological degradation of hydrocarbons, investigation of bioremediation can be very time consuming if the parameters are optimized following the classical approach of changing one parameter at a time [16]. Additionally, synergistic and antagonistic effects of process parameters may not be reflected and may lead to biased results. Hence, the statistical tool of response surface methodology (RSM) was employed in this study. This method is based on polynomial approximation and its design is in accordance with statistical requirements [17].

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Table 1
Experimental matrix and results for n-alkanes removal (%).

Run	Point type	Factors			n-Alkanes removal (%)		
		A:N (mg/L)	B:P (mg/L)	C:time (day)	Observed	Predicted	Residual
1	Fact	0.0	0.0	7	10.89	11.38	−0.49
2	Fact	20.0	0.0	7	21.92	20.13	1.79
3	Fact	0.0	2.0	7	18.12	17.34	0.78
4	Fact	20.0	2.0	7	36.66	38.02	−1.36
5	Fact	0.0	0.0	28	33.45	32.07	1.38
6	Fact	20.0	0.0	28	59.22	59.98	−0.76
7	Fact	0.0	2.0	28	52.73	54.50	−1.77
8	Fact	20.0	2.0	28	94.86	94.35	0.51
9	Axial	5.0	1.0	18	79.43	81.60	−2.17
10	Axial	15.0	1.0	18	91.03	93.75	−2.72
11	Axial	10.0	0.5	18	74.49	78.38	−3.89
12	Axial	10.0	1.5	18	92.07	88.46	3.61
13	Axial	10.0	1.0	12	69.13	70.63	−1.50
14	Axial	10.0	1.0	23	91.10	89.88	1.22
15	Center	10.0	1.0	18	88.58	87.67	0.91
16	Center	10.0	1.0	18	85.69	87.67	−1.98
17	Center	10.0	1.0	18	90.68	87.67	3.01
18	Center	10.0	1.0	18	89.04	87.67	1.37
19	Center	10.0	1.0	18	90.35	87.67	2.68
20	Center	10.0	1.0	18	87.07	87.67	−0.60
21	–	0.0	0.0	18	26.05	–	–

The main goal of the present study is to model and optimize bioremediation of n-alkanes in dispersed crude oil by performing a series of controlled laboratory experiments designed and analyzed by central composite design (CCD) and response surface methodology (RSM). The effects of both time and nutrients on biodegradation are investigated.

2. Materials and methods

2.1. Sampling and microorganism acclimitization

Seawater samples were collected from Butterworth Beach, Penang, Malaysia. A media containing 1 g/L NH_4NO_3, 1 g/L KH_2PO_4, 1 g/L K_2HPO_4, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.05 g/L $FeCl_3$, and 0.02 g/L $CaCl_2$ was used to culture bacteria [18,19]. The sample mixture of seawater, nutrients, and hydrocarbon was stirred, aerated and maintained at room temperature under natural light conditions and pH 7.0–7.8. Details of bacterial consortium acclimatization have been reported earlier [20].

2.2. Bioremediation experiments

Seawater samples were mixed with light crude oil (Shell Refining Company Berhad, Port Dickson, Malaysia) and the dispersant Corexit 9500 in a ratio of 20:1 (w/w). Erlenmeyer flasks were used as bioreactors, each containing 250 mL oil-contaminated seawater with initial oil concentration of 100 mg/L and different amounts of nitrogen and phosphorus as listed in Table 1. Furthermore, one extra test (Run 21) was carried out for determination of removal on day 18 due to natural attenuation. Ammonium nitrate and dipotassium hydrogen phosphate were used as nitrogen and phosphorus sources, respectively. Each bioremediation test reactor received 1 mL bacterial inoculum containing 1.2×10^7 cells/mL. The reactors were shaken and samples were collected at 7, 12, 18, 23 and 28 days for analysis.

2.3. Chemical analysis

Analytical grade chemicals were used and all analyses were done according to Standard Methods for the Examination of Water and Wastewater [21]. Samples were extracted three times by dichloromethane (DCM) following US-EPA test methods [22] and hydrocarbons quantification was performed using a GC 2000 series gas chromatograph equipped with a FID flame ionization detector (Fisons Instruments, Milan, Italy). A DB-5 capillary column (J&W Scientific, Folsom, CA, USA) (60 m × 0.25 mm I.D., film thickness 0.25 μm) was employed. Splitless mode injections were carried out with the purge valve opened at 1 min; injector and detector temperatures were set at 300 °C; helium (He) was used as carrier gas; make-up gas, N_2, flow rate was 30 mL/s; the oven tempera-

Table 2
Analysis of variance for response surface quadratic model terms.

Source	Sum of squares	DF[a]	Mean square	*F*-value	Prob > *F*	Remarks
Model	15375.10	8	1921.89	267.73	<0.0001	Significant
A	1254.67	1	1254.67	174.78	<0.0001	Significant
B	863.65	1	863.65	120.31	<0.0001	Significant
C	3150.94	1	3150.94	438.95	<0.0001	Significant
B^2	71.42	1	71.42	9.95	0.0092	Significant
C^2	217.18	1	217.18	30.25	0.0002	Significant
AB	71.22	1	71.22	9.92	0.0092	Significant
AC	183.65	1	183.65	25.58	0.0004	Significant
BC	135.71	1	135.71	18.91	0.0012	Significant
Residual	78.96	11	7.18			
Lack of fit	60.58	6	10.10	2.75	0.1437	Not significant
Pure error	18.39	5	3.68			
Cor total	15454.06	19				

[a] DF = degree of freedom.

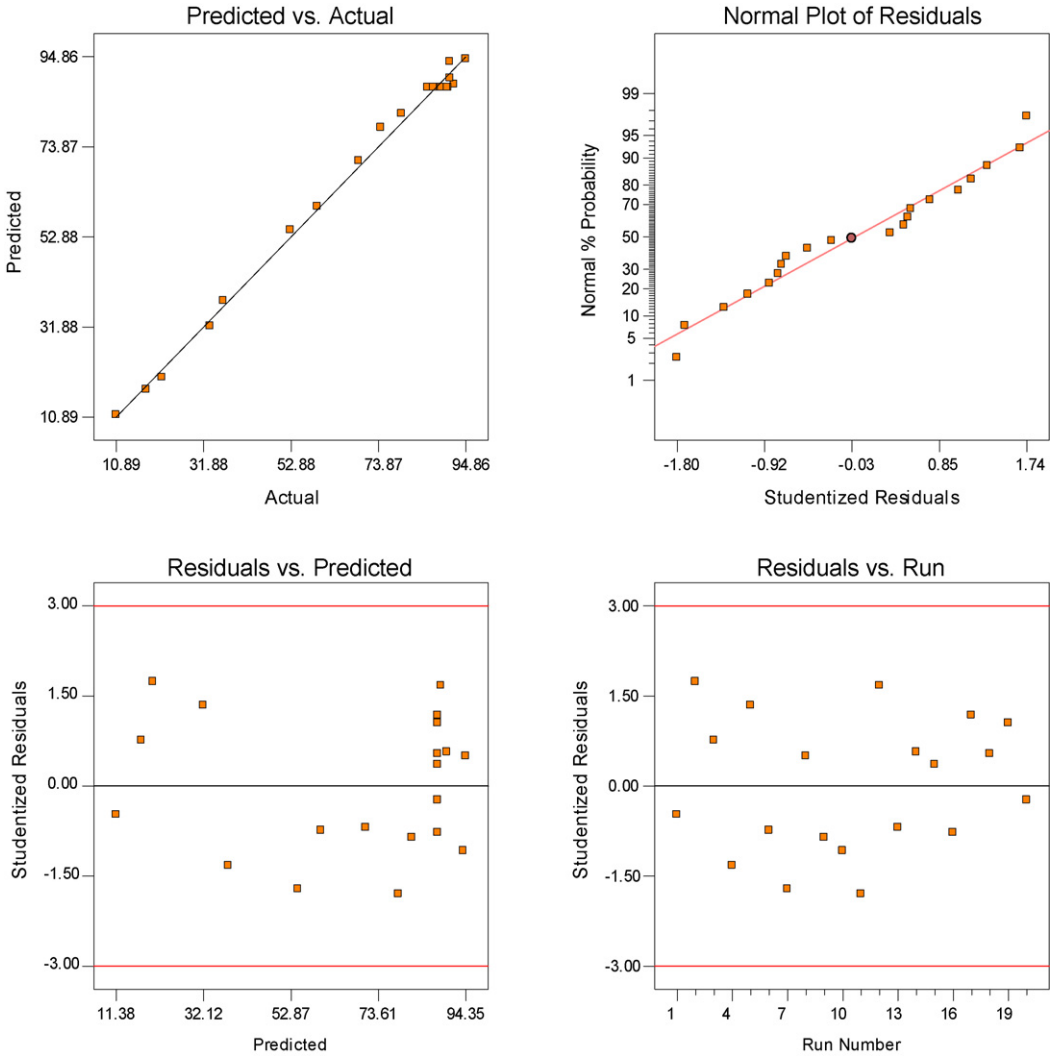

Fig. 1. Diagnostics plots for n-alkanes degradation (a) predicted versus actual, (b) normal plot of residual, (c) residual versus predicted, and (d) residual versus run.

ture program was 1 min at 60 °C, increasing by 10 °C/min up to 160 °C then 10 min at this temperature followed by 4 °C/min up to 300 °C, and finally 10 min at 300 °C. Chrom-Card version 2.1 software (Thermo Electron, Rodano, Italy) was used for data analysis.

The results were confirmed by gas chromatography/mass spectrometry. For this purpose, a 5890 Hewlett Packard GC Series II with a 5972 Mass Selective Detector (Palo Alto, CA, USA), equipped with DB-5 MS column (30 m × 0.32 mm, 0.25 μm film thickness) was employed. The chromatographic conditions were as follows: carrier gas (He) flow rate was 50 mL/s; the initial column temperature was 65 °C (held for 2 min) and was raised to 220 °C at a rate of 9 °C/min and then held for 20 min; the injector and transfer-line temperature was 300 °C; the injection volume was 1 μL and the split ratio was 1:10. MS detected at voltage 1.05 kV, EI 70 eV, scan field 35–350 *m*/*z*, and ion source temperature 200 °C. Data acquisition and processing was controlled by HP Chemstation software. Chromatographic peaks of samples were identified by mass spectra and by comparison with the standards. Supelco (Sigma–Aldrich, Bellefonte, PA, USA) standard mixture of aliphatic hydrocarbons was used. Selected n-alkanes from $C_{12}H_{26}$ to $C_{34}H_{70}$ were target analytes. To check the accuracy and precision of the analytical procedure, triplicate analysis of certified reference material (CRM) was carried out periodically; an average error of 4.2 ± 2.1% was observed during this study. Other quality assurance and quality control were performed according to US-EPA procedures [22].

2.4. Statistical methods and data analysis

For experimental design and data analysis, Design-Expert® 6.0.7 (Stat-Ease Inc., Minneapolis, USA) using CCD and RSM was employed. The design model was quadratic. Coded representations of variables of the design of experiments for overall n-alkanes degradation were A: for nitrogen (mg/L), B: for phosphorus (mg/L), and C: for time (day). The selected independent variables were coded according to Eq. (1):

$$x_i = \frac{X_i - X_0}{\Delta X}, \quad i = 1, 2 \ldots, k \tag{1}$$

where x_i refers to coded value of the ith independent variable, X_0 is the value of X_i at the center point and ΔX is the step change value. The objective of using this software was to understand the influence of factors on the bioremediation process and to establish the optimum conditions for n-alkanes bioremediation.

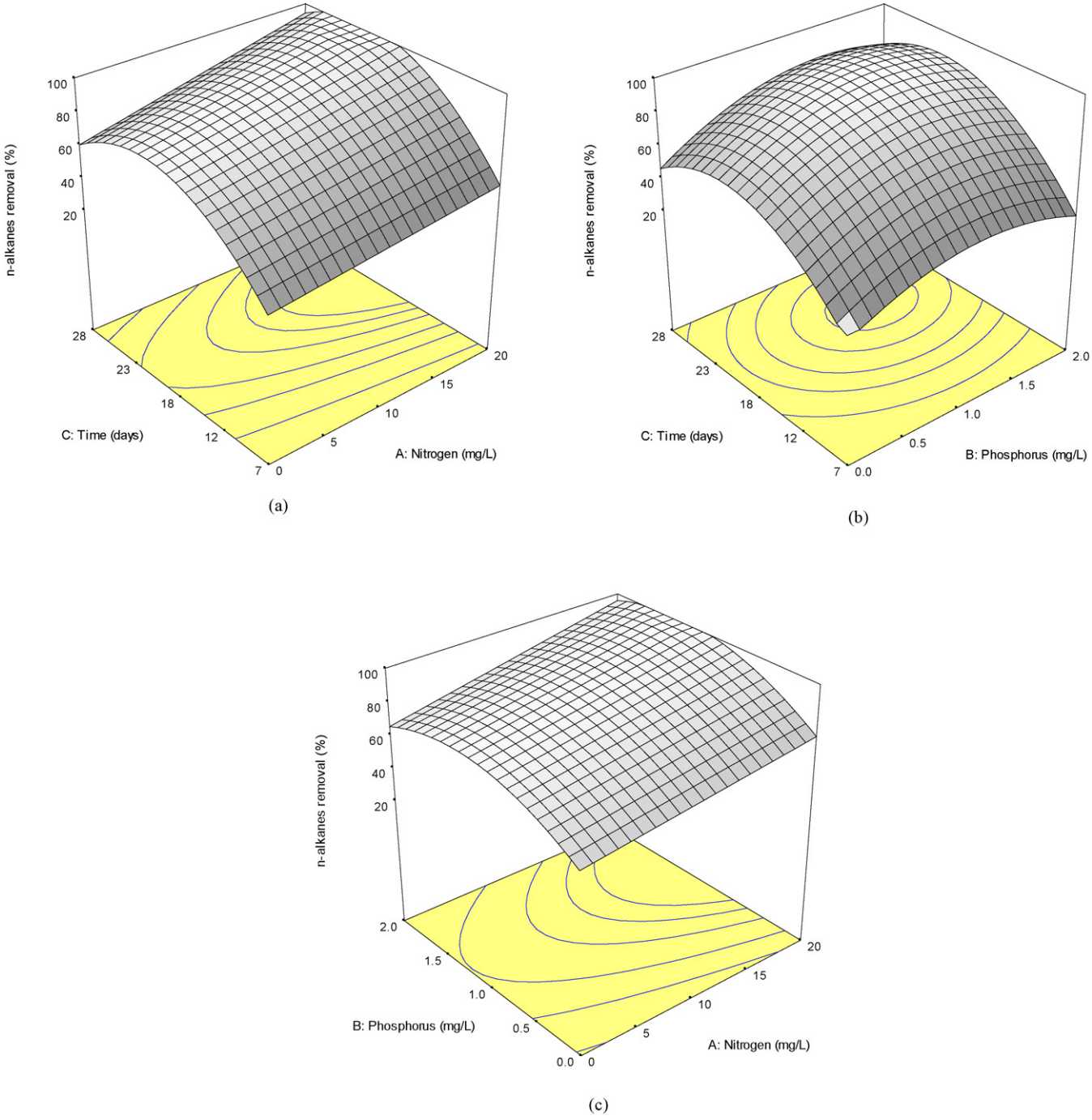

Fig. 2. Three-dimensional surface graph of n-alkanes degradation (a) effect of nitrogen concentration and time with phosphorus concentration 1 mg/L, (b) effect of phosphorus concentration and time with nitrogen concentration 1 mg/L, and (c) effect of nitrogen and phosphorus concentration at time 18 days.

3. Results and discussion

3.1. Statistical analysis and modeling

The response function (Y) was measured as the percentage of n-alkanes removal in accordance with the following equation [17]:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n} \beta_{ii} x_i^2 + \sum_{i \neq j=1}^{n} \beta_{ij} x_i x_j + \varepsilon \quad (2)$$

where β_0 is the value of the fixed response at the center point of the design; β_i, β_{ii} and β_{ij} are the linear, quadratic and interaction effect regression terms, respectively; x_i denotes the level of the independent variable; n is the number of independent variables; and ε is the random error. In the present study, the following second order polynomial equation was used:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \varepsilon \quad (3)$$

Eq. (4) represents n-alkanes degradation in terms of coded factors:

$$Y = 87.67 + 12.15A + 10.08B + 19.25C - 17.02B^2 - 29.68C^2 + 2.98AB + 4.79AC + 4.17BC \quad (4)$$

Results for n-alkanes degradation are summarized in Table 1 and analysis of variance (ANOVA) for the Model and terms are shown in Table 2. The obtained Model *F*-value of 267.73 implies the model is significant. There is only a 0.01% chance that a Model *F*-value this large could occur due to noise. Values of probability $F < 0.05$ indicate model terms are significant. The lack of fit *F*-value of 2.75 implies the lack of fit is not significant relative to the pure error. There is a 14.37% chance that a lack of fit *F*-value this large could occur due to noise. Standard deviation was 2.68, predicted residual sum of squares (PRESS) was 659.00 and mean and coefficient of variation were 67.83 and 3.95 respectively. *R*-squared was 0.9949. The predicted *R*-squared value of 0.9574 is in reasonable agreement with the adjusted *R*-squared value of 0.9912. Adeq precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 46.161 indicates an adequate signal; hence this model is relaiable and can be used to navigate the design space.

Fig. 1(a) presents the predicted versus actual plot for n-alkanes biodegradation. Actual values were obtained from experiments while the predicted values were calculated according to the model (Eq. (4)). The plot indicates that the actual and predicted values are close to each other. Generally, residual analysis guarantees that the statistical assumptions fit the analytical data. As can be seen in Fig. 1(b), the normal plot of residual for n-alkanes biodegradation is reasonably good. This figure was obtained by plotting studentized residuals versus normal percentage of probability of the experiments. Fig. 1(c) shows residual versus predicted values for n-alkanes biodegradation. In this research, points of observed runs were scattered randomly within the constant range of residuals across the graph. Thus, it revealed no obvious pattern and unusual structure. In other words, the model is adequate and there is no reason to suspect any violation of the independence or constant variance assumption in all runs. The studentized residuals versus run plot represented in Fig. 1(d) shows points scattered randomly between −1.800 and +1.736; the errors were normally distributed and insignificant.

3.2. Interaction among variables

Three-dimensional response surface plots were used to graphically represent the regression equation. The 3D design expert plot, nitrogen versus time, in Fig. 2(a), shows significant mutual interaction between nitrogen concentration and time for n-alkanes removal ($F = 25.58$, $P = 0.0004$). The interactive effect of phosphorus concentration and time for n-alkanes degradation is shown in Fig. 2(b); very strong relations ($F = 18.91$, $P = 0.0012$) were observed. It is comprehensible from the figure that n-alkanes degradation increased substantially at phosphorus concentration and time up to 1.5 mg/L and 18 days respectively. The optimum duration appears to be 18 days as any longer period increases n-alkanes degradation only marginally. Significant synergistic interaction ($F = 9.92$, $P = 0.0092$) was exhibited between nitrogen and phosphorus concentrations. The interaction graph of nitrogen and phosphorus concentrations (Fig. 2(c)), shows n-alkanes removal to be affected by both nutrients; more strongly by nitrogen than phosphorus. Presence of excess amount of nutrients decreased n-alkanes removal; furthermore it will increase the cost of bioremediation and possibly cause eutrophication in aquatic ecosystems.

Table 3
Numerical optimization criteria for maximum n-alkanes removal.

Criteria	Ultimate goal	Lower limit	Upper limit
n-Alkanes removal (%)	Maximize	15	100
Nitrogen (mg/L)	In range	0	20.0
Phosphorus (mg/L)	In range	0	2.0
Time (day)	In range	7	28

3.3. Optimization and method verification

Numerical conditions optimization was carried out as suggested by the Design-Expert® software for n-alkanes bioremediation. The goal was set to maximize removal for response as presented in Table 3. The highest degradation of 94.90% was obtained at 20 days. Standard deviation and percent error were calculated to ensure the validity of model at the optimum point. Optimum conditions found by design expert and observed results for n-alkanes bioremediation are tabulated in Table 4. An error of less than 5% shows CCD reliably optimized n-alkanes degradation in crude oil contaminated seawater.

3.4. Improvement of n-alkanes degradation

Run numbers 1, 21 and 5 correspond to the natural attenuation study on 7, 18 and 28 days respectively. The amount of n-alkanes removed in 7 days was mainly due to evaporation of light (low molecular weight) compounds.

Run numbers 2–4 were carried out for 7 days only, providing insufficient time to microorganism for both medium and long chain n-alkanes utilization. Thus, low n-alkanes removals were observed in these runs. Although Runs 6 and 7 were performed for sufficient time, the lack of nutrient (nitrogen for run 6 and phosphorus for run 7) hindered efficient n-alkanes removal. Runs 8–20 exhibited good n-alkanes bioremediation for the experimental conditions.

The chromatogram of biodegradation process at: (a) day 7, (b) day 18, (c) day 28 is presented in Fig. 4 as a sample. As can be seen, at the final stage of bioremediation experiment, most of the n-alkanes were eliminated from the samples while other heavier compounds were still present.

Maximum removal by natural attenuation was 33.45%. The highest n-alkanes removal in un-optimized condition was 94.86% in 28 days (Run 8) while process optimization showed 94.90% removal in 20 days. Namkoong et al. [23] evaluated the bioremediation of diesel-contaminated soil with composting; they report 98.1% degradation of hydrocarbons within 30 days. Furthermore, Morris et al. [24] reported over 82% biodegradation of diesel-range organics (C8–C25 n-alkanes) and 31% natural attenuation over 21 days at 30 °C. Over 90% removal of normal aliphatic hydrocarbons via bioremediation in intertidal sediments has also been reported [25].

A comparison of average degradation of medium chain n-alkanes ($C_{12}H_{26}$ to $C_{22}H_{46}$) and long chain n-alkanes ($C_{24}H_{50}$ to $C_{34}H_{70}$) is illustrated in Fig. 3. It clearly shows that long chain com-

Table 4
Optimum conditions found by Design-Expert® for n-alkanes bioremediation.

Factors			n-Alkanes removal (%)			StD[a]	De[b]
N (mg/L)	P (mg/L)	Time (day)	Observed	Predicted	Error (%)		
13.62	1.39	20	94.90	97.76	−3.01	±2.02	1.00

[a] Standard deviation.
[b] Desirability.

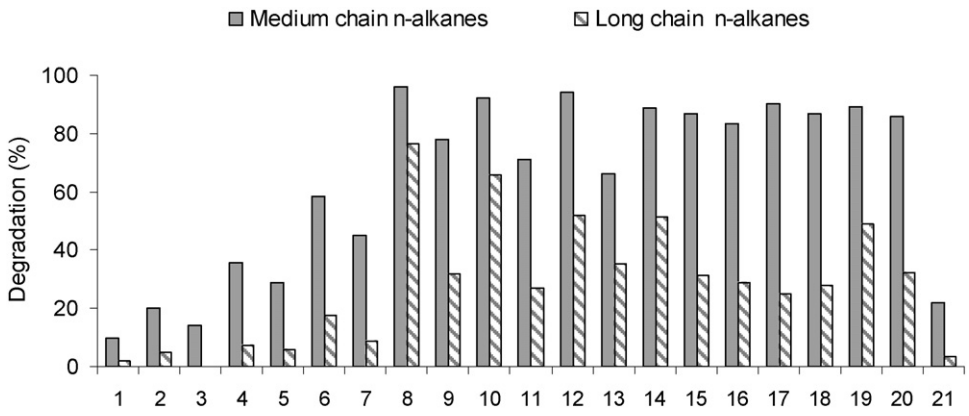

Fig. 3. Comparison degradation of medium chain and long chain n-alkanes in different runs.

pounds degraded slower than medium chain ones. Similar results were reported by other researchers [26,27].

The extent of the degradation of n-alkanes longer than C36 was not always clear because of their extremely low concentration in crude oil [28]. Rahman et al. [29] reported that n-alkanes in the range of C8–C11 were degraded completely after 56 days of treatment. Higher n-alkanes C12–C21, C22–C31 and C32–C40 were removed at the levels of 83–98%, 80–85% and 57–73%, respectively. Obuekwe and Al-Zarban [30] removed C22, C24 and C26 in soil up to 99.3%, 98.7% and 91.7% respectively.

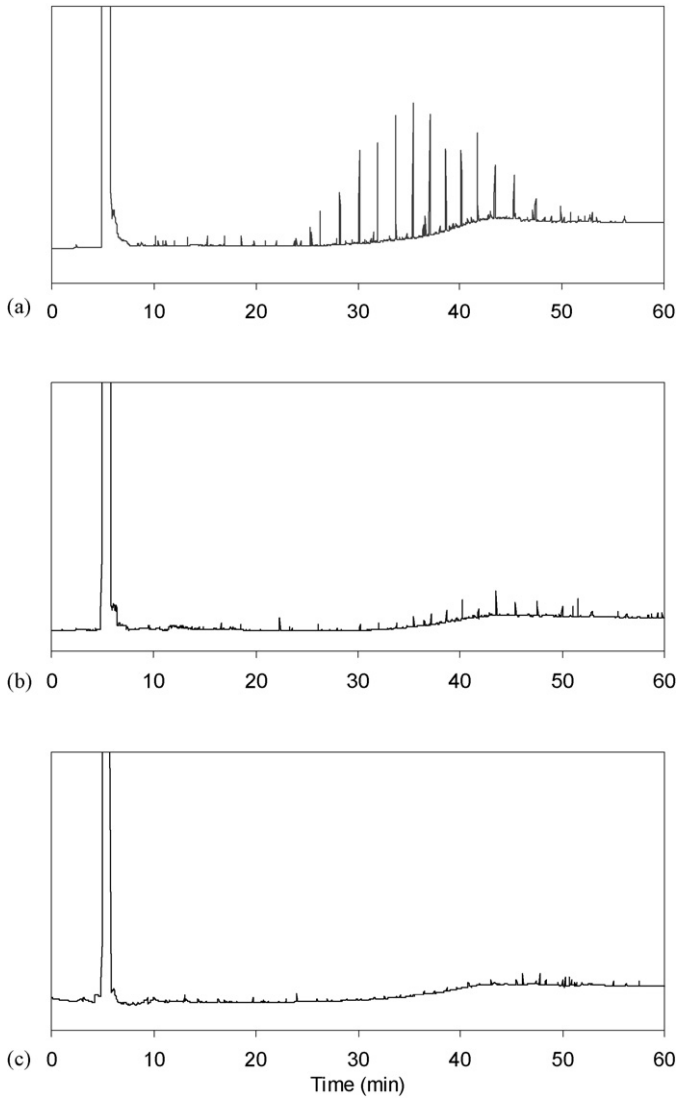

Fig. 4. Chromatogram of biodegradation process at: (a) day 7, (b) day 18, and (c) day 28.

Huang et al. [31] used a 5-level, 3-factor CCD to study the enhancement of diesel oil (n-alkanes) degradation in artificial seawater by nutrients (N and P) and yeast extract supplementation. The results showed increase in degradation from 12.6% to 75% within 7 days when 2.53 g/L $(NH_4)_2SO_4$, 2.75 g/L Na_2HPO_4, and 0.01 g/L yeast extract were supplied. The carbon, nitrogen and phosphorus (C:N:P) ratio of 200:10:1 was applied for selected n-alkanes ($C_{12}H_{26}$ to $C_{20}H_{42}$) in a diesel fuel contaminated soil bioremediation study [32]. The optimum nitrogen and phosphorus ratio of 100:20:2.7 for removal of petroleum hydrocarbons was reported [33] using RSM. The supplementation with fertilizer at 100:25:25 C:N:P ratio was documented for n-alkanes bioremediation in marine sediments [34].

In this study, optimization through RSM showed capability to remove n-alkanes in shorter time and with less nutrient consumption (compared to results obtained without using optimization through RSM) which is both economically and environmentally beneficial.

4. Conclusions

Response surface methodology was employed for evaluation and modeling of n-alkanes bioremediation in this study. Bioremediation exhibited a maximum n-alkanes removal of 36.7%, 92.1%, 94.9% at 7, 18 and 28 days respectively; compared to 10.9%, 26.0%, and 33.4% for natural attenuation. As well as incurring extra cost for bioremediation, the application of excess nutrients may cause eutrophication in the marine environment. Hence, numerical optimization based on desirability function was performed. 95% n-alkanes removal was observed in a 20-day experiment using 13.62 mg/L nitrogen and 1.39 mg/L phosphorus. The response surface methodology was found useful in relating the operating variables and their interactions with n-alkanes biodegradation in a statistically significant manner.

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