

## Enhancement Biodegradation of n-alkanes from Crude Oil Contaminated Seawater

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**ABSTRACT:** The aim of this research was to optimize bioremediation of seawater samples spiked with 1000 mg/L crude oil for removal of n-alkanes ( $C_{12}H_{26}$  to  $C_{34}H_{70}$ ). Bioaugmentation experiments were performed at laboratory scale: each bioreactor contained 250 ml dispersed crude-oil-contaminated seawater, indigenous acclimatized microorganism and nitrogen and phosphorus at concentrations based on central composite design (CCD) calculations. Three independent variables, time, nitrogen and phosphorus, were investigated and the experimental data obtained were fitted to a second-order polynomial mathematical model with multiple regressions. The obtained Model F-value of 97.12 and probability  $F < 0.0001$  implies the model is significant. Hydrocarbon analyses were carried out using a gas chromatograph equipped with flame ionization detector (GC/FID). During 28 days of experimentation, a maximum of 85.35% total n-alkane removal was observed. Numerical optimization was achieved based on desirability functions. Using 188.71 mg/L nitrogen and 18.99 mg/L phosphorus, design of experiment (DOE) software predicted 91.00% removal. A removal of 92.04% was observed experimentally, in close agreement with the predicted value.

**Key words:** Bioremediation, Bioaugmentation, Paraffines, Petroleum, Marine pollution

### INTRODUCTION

Crude oil is a complex mixture of many compounds including alkanes, aromatics, resins and asphaltenes which potentially could be eliminated from contaminated environments by microbial degradation. Different components of crude oil are degraded at different rates: n-alkanes, also known as n-paraffins, are oxidized more rapidly than either aromatics or naphthenes (Ijah and Antai, 2003; Fingas, 2001). Crude oil and hydrocarbon fuels (jet fuel, kerosene, gasoline, diesel fuel, ect.) contain large amounts of n-alkanes. Therefore, enhanced biodegradation of these compounds is extremely important in the case of hydrocarbon spills. Several factors may affect hydrocarbon degradation and, in particular, the oil concentration is an important consideration in determining whether bioremediation is a viable option. Very low concentrations of hydrocarbons may be ineffectually attacked by microorganisms (Foght and Westlake, 1987). In contrast, high concentrations of hydrocarbons can cause inhibition of biodegradation due to toxic effects, although the inhibitory concentration varies with oil composition. Hence, there is an optimum oil concentration range for bioremediation applications (Zhu, 2001).

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Different types of nutrients (primarily nitrogen and phosphorus) have been applied to improve petroleum hydrocarbon degradation, including classic (water soluble) nutrients and oleophilic and slow-release fertilizers. Application of nutrients for hydrocarbon biodegradation has been widely investigated (Delille *et al.*, 2009; Ruberto *et al.*, 2009; Kim *et al.*, 2008; Ramirez *et al.*, 2008; Bagherzadeh-Namazi *et al.*, 2008; Nikolopoulou and Kalogerakis, 2008; Salas *et al.*, 2006; Knezevich *et al.*, 2006), and each nutrient type exhibits various advantages and disadvantages (Jean *et al.*, 2008; Onwurah *et al.*, 2007; Das and Mukherjee, 2007). However, the literature is still inconclusive regarding what nutrient conditions are sufficient for different environments.

Low nutrient concentrations reduce the rate of biodegradation; whereas, high nutrient concentration may be toxic for marine biota and cause eutrophication and red tide. Hydrocarbon-utilizing microorganisms are ubiquitously distributed in marine ecosystems following oil spills (Atlas, 1995). For ex-situ bioremediation, addition of acclimatized naturally occurring microorganisms (bioaugmentation) enhances biodegradation of hydrocarbons. As dissolved hy-

drocarbon are more available for microbiological degradation, application of dispersants and surfactants to increase the bioavailability significantly enhance oil degradation as reported by (Zahed *et al.*, 2010). Other factors (e.g., climate, salinity, pH, ect.) have considerable effects on biodegradation of petroleum hydrocarbons in the marine environments as well.

Bioremediation is a multi-variable process and optimization through classical methods is inflexible, unreliable and time-consuming. To overcome these disadvantages, response surface methodology (RSM) was used. This widely used technique is a practical mathematical and statistical tool for analyzing the effects of several independent variables on a process (Draper and John, 1988, Myers and Montgomery, 2002). Rotatable central composite design (CCD), the most commonly used RSM, has been used recently for hydrocarbon biodegradation optimization research, including naphthalene biodegradation using *Pseudomonas* sp (Pathak *et al.*, 2009), biodegradation of weathered crude oil in coastal sediments (Mohajeri *et al.*, 2010), nutrient and inoculums optimization for petroleum hydrocarbons biodegradation (Vieira *et al.*, 2009), optimization of nutrient components for diesel oil degradation (Huang *et al.*, 2008) and biodegradation phenan-

threne by mixed culture consortia (Nasrollahzadeh *et al.*, 2007).

The objective of this research was enhancement of n-alkane biodegradation of dispersed crude oil in laboratory-scale experiments by optimizing nitrogen and phosphorus concentration employing full factorial CCD and RSM.

**MATERIAL & METHODS**

Indigenous bacteria were collected from Butterworth Beach, Penang, Malaysia. Bacteria were cultured in 1g/L NH<sub>4</sub>NO<sub>3</sub>, 1g/L KH<sub>2</sub>PO<sub>4</sub>, 1g/L K<sub>2</sub>HPO<sub>4</sub>, 0.2g/L MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.05g/L FeCl<sub>3</sub>, and 0.02g/L CaCl<sub>2</sub> (Ghazali *et al.*, 2004; Dutta and Arayama, 2000) at room temperature under natural light conditions and pH 7.0-7.8 with stirring and aeration. Bacterial inoculums characteristics were reported before (Mohajeri *et al.*, 2009; Zahed *et al.*, 2010). Erlenmeyer flasks (bioreactors) contained 250 ml seawater with an initial concentration of 1000 mg/l light crude oil (Shell, Port Dickson, Malaysia) and the dispersant Corexit 9500 in a ratio of 20:1 (w/w) as well as the amounts of nitrogen and phosphorus listed in Table 1. Run 21 (an extra test) was carried out to determine the amount of removal from natural attenuation. NH<sub>4</sub>NO<sub>3</sub> and K<sub>2</sub>HPO<sub>4</sub> were used as nitrogen and phosphorus sources, respectively.

**Table 1. Experimental matrix for central composite design (CCD) for overall optimization**

Run No.	Point type	Factors		
		N(mg/L)	P(mg/L)	Time(day)
1	Fact	0.0	0.0	7
2	Fact	200.0	0.0	7
3	Fact	0.0	20.0	7
4	Fact	200.0	20.0	7
5	Fact	0.0	0.0	28
6	Fact	200.0	0.0	28
7	Fact	0.0	20.0	28
8	Fact	200.0	20.0	28
9	Axial	50.0	10.0	18
10	Axial	150.0	10.0	18
11	Axial	100.0	5.0	18
12	Axial	100.0	15.0	18
13	Axial	100.0	10.0	12
14	Axial	100.0	10.0	23
15	Center	100.0	10.0	18
16	Center	100.0	10.0	18
17	Center	100.0	10.0	18
18	Center	100.0	10.0	18
19	Center	100.0	10.0	18
20	Center	100.0	10.0	18

Bioactors were shaken and samples were taken at 7, 12, 18, 23 and 28 days.

Nutrients were determined using Standard Methods (APHA, 2005) and hydrocarbon analysis was performed using EPA procedures (US-EPA, 1991). Samples were extracted three times with dichloromethane (DCM) using analytical grade chemicals. Selected n-alkane quantification was carried out using a GC 2000 Series gas chromatograph equipped with a FID flame ionization detector (Fisons Instruments, Milan, Italy) using a DB-5 capillary column (J&W Scientific, Folsom, CA, USA) (60m×0.25mm I.D., film thickness 0.25 μm). Injector and detector temperatures were set to 300°C; carrier gas, He, flow rate was 30 cm/s; make-up gas, N<sub>2</sub>, flow rate was 43 cm/s; the oven temperature was programmed for 2 min at 70°C, increasing by 5°C/min up to 180°C and by 10°C/min up to 270°C, and finally 3 min at 270°C.

Chrom-Card version 2.0 software (Thermo Electron, Rodano, Italy) was used for data analysis. The results were confirmed by gas chromatography/mass spectrometry using 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). The chromatographic conditions were as follows: carrier gas (He) flow rate was 50 cm/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. Chromatographic peaks of samples were identified by mass spectra and compared to the standards. Supelco (Sigma-Aldrich, Bellefonte, PA, USA) standard mixture of aliphatic hydrocarbons was used. To check the accuracy and precision of the analytical procedure, triplicate analysis of certified reference material (CRM) was occasionally performed. Other quality assurance and quality control were performed according to US-EPA procedures (US-EPA, 1991).

The statistical software DESIGN EXPERT® 6.0.7 (Stat-Ease Inc., Minneapolis, USA) was used for RSM and rotatable CCD and a quadratic design model was suggested by the software. Coded and actual values of variables of the design of experiments for overall n-alkanes degradation optimization are shown in Table 2.

## RESULTS & DISCUSSION

Total n-alkane bioremediation results, predicted values and diagnostics case statistics including Residual, Cook's Distance and Outlier-T are listed in Table

3. Detailed analysis of variance (ANOVA) for the model and terms are listed in Table 4.

The selected independent variables were coded according to equation (1):

$$x_i = \frac{X_i - X_0}{\Delta X} \quad (1)$$

$$i = 1, 2, \dots, k$$

where  $x_i$  refers to coded value of the  $i^{\text{th}}$  independent variable,  $X_0$  is the value of  $X_i$  at the center point and  $\Delta X$  is the step change value (Montgomery, 2008). The empirical equation was obtained from multiple regression analysis through the least squares method. The second order polynomial multiple regression model was fitted to the response (n-alkanes removal), giving equation (2):

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

where Y is the response (n-alkanes removal),  $\beta_0$  is the value of the fixed response at the center point of the design;  $\beta_i$ ,  $\beta_{ii}$ , and  $\beta_{ij}$  are the linear, quadratic and interaction effect (cross product coefficients) regression terms, respectively;  $X_i$  and  $X_j$  are the coded values of independent variables; and k denotes the number of independent variables (in this research 3) and  $\varepsilon$  is the random error.

Final equation in terms of coded factors is equation (3):

$$Y = 67.24 + 10.88A + 7.65B + 19.43C - 31.37C^2 + 6.83AC + 4.65BC \quad (3)$$

Values of Probability F less than 0.05 indicate model terms are significant. The obtained Model F value of 97.123 implies the model is significant. There is only a 0.01% chance that a Model F value this large could occur due to noise. The Lack of Fit F-value of 1.78 implies the lack of fit is not significant relative to the pure error. There is a 27.29% chance that a lack of fit F-value this large could occur due to noise.

The quality of the fit of the polynomial model was expressed by the coefficient of determination R<sup>2</sup>, adjusted coefficient (R<sup>2</sup><sub>Adj</sub>) and predicted coefficient (R<sup>2</sup><sub>Pred</sub>). The predicted R-squared value of 0.9475 is in logical agreement with the adjusted R-squared value of 0.9681. Adequate Precision measures the signal to noise ratio: a ratio greater than 4 is desirable. The ratio

**Table 2. Coded and actual values of variables of the design of experiments for overall n-alkanes degradation optimization**

Symbol	Factor	Coded levels of variables				
		-1.00	-0.5	0	0.5	1.00
A	Nitrogen (mg/L)	0	50.0	100.0	150.0	200.0
B	Phosphorus (mg/L)	0	5.0	10.0	15.0	20.0
C	Time (day)	7	12	18	23	28

**Table 3. Results and diagnostics case statistics for n-alkane degradation**

n-alkane removal (%)					
Run No.	Observed	Predicted	Residual	Cook's Distance	Outlier T
1	8.85	9.69	-0.84	0.058	-0.379
2	18.52	17.77	0.75	0.046	0.337
3	17.21	15.69	1.52	0.192	0.697
4	22.15	23.78	-1.63	0.221	-0.750
5	27.11	25.59	1.52	0.193	0.700
6	58.18	61.00	-2.82	0.662	-1.365
7	48.15	50.20	-2.05	0.349	-0.957
8	86.35	85.62	0.73	0.045	0.331
9	60.14	62.10	-1.96	0.005	-0.497
10	77.28	72.98	4.30	0.024	1.136
11	65.50	63.71	1.79	0.004	0.452
12	73.23	71.37	1.86	0.005	0.472
13	53.20	49.98	3.22	0.009	0.815
14	77.46	69.41	8.05	0.057	2.416
15	63.54	67.54	-4.00	0.015	-1.030
16	69.57	67.54	2.03	0.004	0.506
17	64.01	67.54	-3.53	0.012	-0.900
18	65.51	67.54	-2.03	0.004	-0.506
19	67.99	67.54	0.45	0.000	0.111
20	60.18	67.54	-7.36	0.051	-2.134

**Table 4 . Analysis of variance for Response Surface Reduced Quadratic Model Terms**

Source	Sum of squares	DF <sup>1</sup>	Mean square	F-Value	Prob > F	Remarks
Model	9702.01	6	1617.00	97.12	< 0.0001	significant
A	1005.53	1	1005.53	60.39	< 0.0001	significant
B	498.05	1	498.05	29.91	0.0001	significant
C	3210.32	1	3210.32	192.81	< 0.0001	significant
C <sup>2</sup>	4441.47	1	4441.47	266.75	< 0.0001	significant
AC	373.46	1	373.46	22.43	0.0004	significant
BC	173.17	1	173.17	10.40	0.0066	significant
Residual	216.45	13	16.65			
Lack of Fit	160.13	8	20.02	1.78	0.2729	not significant
Pure Error	56.32	5	11.26			
Cor Total	9918.46	19				

<sup>1</sup> DF= Degree of freedom

of 31.45 indicates an adequate signal. This model can be used to navigate the design space. Summary statistics for the model including coefficient of variation (CV), Predicted Residual Sum of Squares (PRESS) and standard deviation are listed in Table 5.

Table 5. Summary statistics for the model

Standard Deviation	4.08
Mean	54.21
Coefficient of variation (CV)	7.53
R-Squared	0.9782
Adjusted R-Squared	0.9681
Predicted R-Squared	0.9475
PRESS	520.82
Adequate Precision	31.45

The model adequacy can be judged by applying the diagnostic plots. The model diagnostics plots for n-alkanes degradation are illustrated in Figs.1 (a) - (b). Predicted versus actual plot is presented in Fig.1 (a). Predicted values were calculated in accordance to the model and actual values were determined empirically in bioremediation experiments. Observed and predicted values are similar.

The normal plot of residual (Fig.2 (b)) was obtained by plotting studentized residuals versus normal probability percent. Residual points fall in a nearly straight line, confirming the normality assumption. Three-dimensional response surface plots were generated to visu-

alize possible interactions between variables for higher yield of n-alkane removal. Fig. 2(a) illustrates the effect of nitrogen and phosphorus concentration at day 18: positive effect for both nutrients is clearly demonstrated. This suggests that increasing phosphorus and nitrogen concentration can increase n-alkane removal. As see in Fig 2(b). the optimized predicted removal was obtained at a phosphorus concentration of 20 mg/L at approximately 24 days. Due to dominating interaction effects of time and phosphorus, higher, levels of these variables increase biodegradation up to 23 days. Optimum levels of nutrient are both economically and ecologically important: high nutrient concentrations may cause eutrophication and harmful algal blooms (HABs) in the aquatic ecosystems (Tam *et al.*, 2009; Atlas, 1995).

In a 7 day experiment, the highest nutrient concentration (run 4) exhibited the highest removal. Up to 25.35% light paraffin compounds from n-Dodecane to n-Octadecane were removed in run 1 (natural attenuation); the highest elimination was observed for n-Octadecane (12.34%). At day 18, runs 14 and 16 exhibited detectable degradation for all medium chain n-alkanes from n-Dodecane to n-Docosane. The highest removal was observed for run 8, which was supplemented with 200 mg/L nitrogen and 20 mg/L phosphorus. The removal of most n-alkanes was extremely high, up to 98%; average removal of total n-alkanes was 85.35% while Natural attenuation removed only 27.11% of n-alkanes. The highest degradation in center point (N=100, P=10 in 18 days) was observed for run 16:

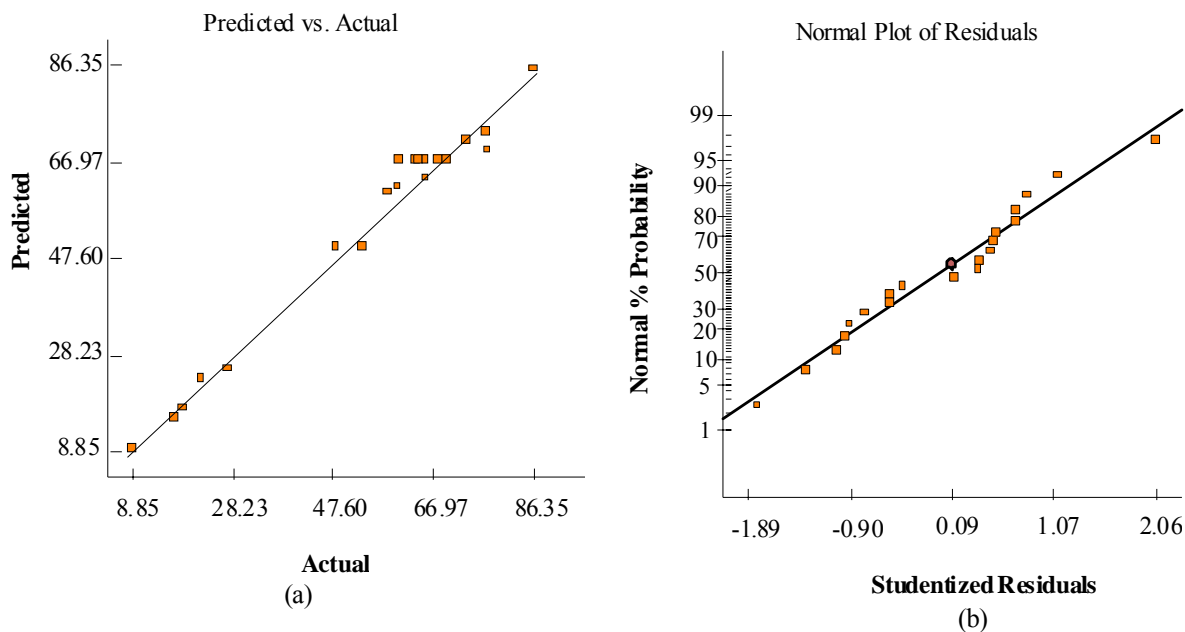
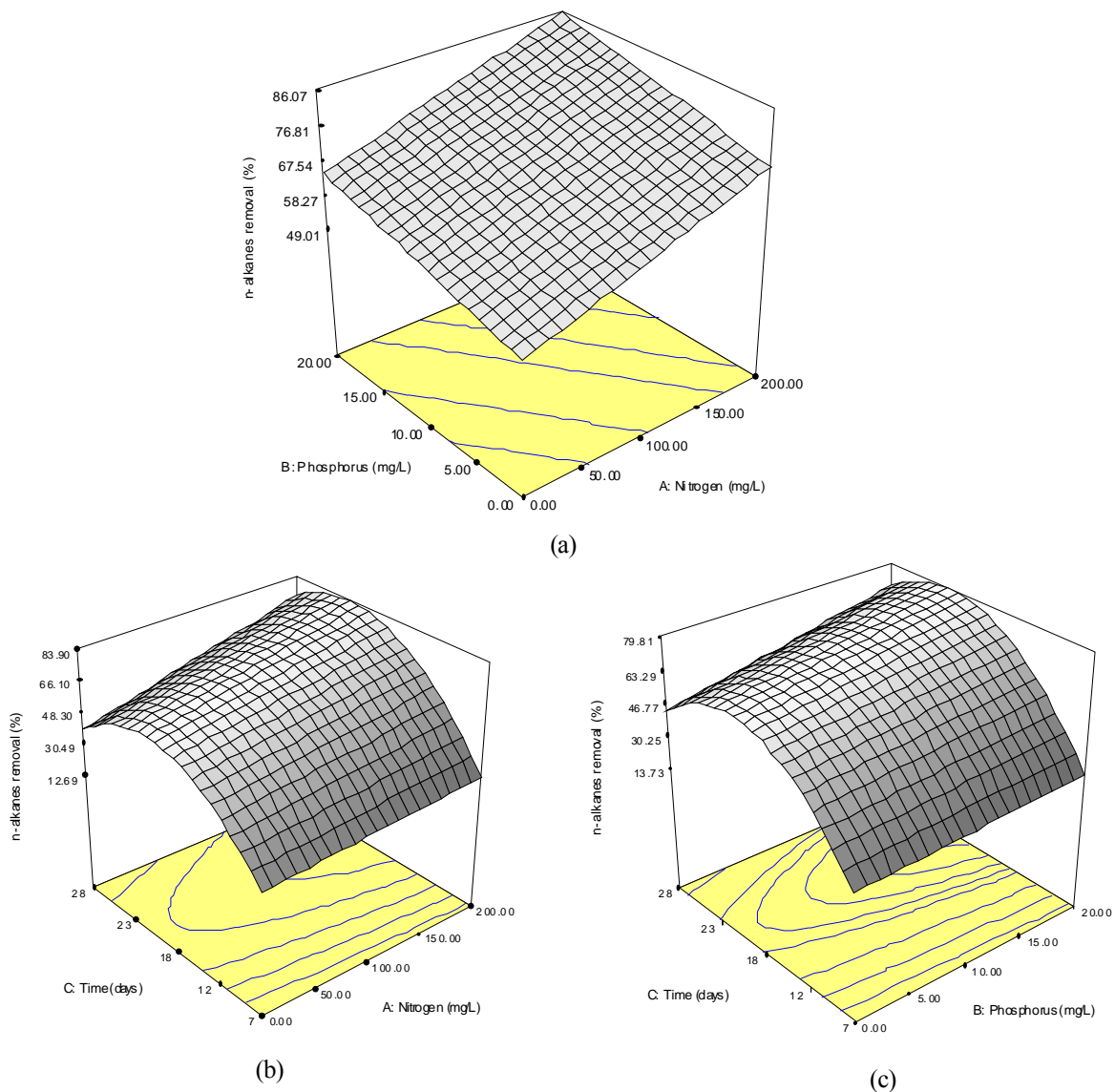


Fig. 1. Diagnostics plots for n-alkanes degradation (a) Predicted versus actual (b) Normal plot of residuals



**Fig. 2. Three dimensional surface graph of n-alkanes biodegradation (a) the effect of Nitrogen and phosphorus concentration in day 18 (b) the effect of Nitrogen concentration and time in phosphorus concentration 10 mg/L (c) effect of phosphorus concentration and time in Nitrogen concentration 100 mg/L**

total n-alkanes were removed at 69.57%. Most n-alkanes were degraded efficiently; removal of n-Octadecane, n-Eicosane and n-Docosane was also observed in this run.

Comparison average biodegradation of medium chain n-alkanes ( $C_{12}H_{26}$  to  $C_{22}H_{46}$ ) and long chain n-alkanes ( $C_{24}H_{50}$  to  $C_{34}H_{70}$ ) at different days are presented in Figs.3 (a) to (c). In all experiments, the greatest removal was observed for medium chain n-alkanes. Although n-tetracosane, n-hexacosane and n-octacosane are classified as long chain n-alkanes, they showed reasonable removal both in 23 and 28 days.

Removal was lowest for n-Triacontane, n-Dotriacontane and n-Tetratriacontane, due to their high molecular weight and lower biodegradability.

Biodegradation of n-alkanes in crude oil contamination depends on several parameters, including nature of oil, type of matrix (seawater, sediments, ect.), bioavailability and bioremediation strategy. Delille et al. (1998) reported that the aliphatic fractions decreased through bioremediation of oil contaminated coastal seawater. Degradation of n-octadecane from crude oil at 64–98% was demonstrated by (Radwan *et al.*, 2002). Nikolopoulou and Kalogerakis (2008) enhanced bio-

degradation of crude oil by combining fertilizers, biosurfactants and molasses. They found that the use of biosurfactants resulted in an increased removal of petroleum hydrocarbons (96% removal of C19–C34 n-alkanes) within a period of 18 days as well as in a reduction of the lag phase. The biodegradation of hydrocarbon pollutants was observed by Knezevich et al. (2006). Removal of selected n-alkanes (C10, C12, and C14) was more than 95%; whereas, removal of longer chain n-alkanes (C16 to C36) was 63% to 87%. Da Silva et al. 2009 investigated C15 C30 n-alkane removal in soil at 30 and 60 days. They reported that up to 50% n-alkanes can be removed in 30 days. Moreover, average biodegradation is more than 90% in 60 days. Morris and co authors (2009) observed over 82% removal of diese-range organics (C8 to C25 n-alkane)

over 21 days while natural attenuation was about 31%. Rahman *et al.*, (2003) reported that n-alkanes in the range of C8–C11 were degraded completely after 56 days of treatment. (Riser-Roberts, 1992) confirmed observations that shorter chain length n-alkanes are more easily used as an energy source than the longer chains. Numerical optimization was carried out for maximizing n-alkane removal based on desirability functions. Variables were set to “in range” and response (n-alkanes removal) with the goal to maximize removal. Table 6 presents the optimum conditions suggested by the Design Expert software for n-alkanes bioremediation. Highest degradation, 94.90%, was observed at 20 days with 13.62 mg/L and 1.39 mg/L nitrogen and phosphorus, respectively. The highest removal predicted by the software was 97.76%.

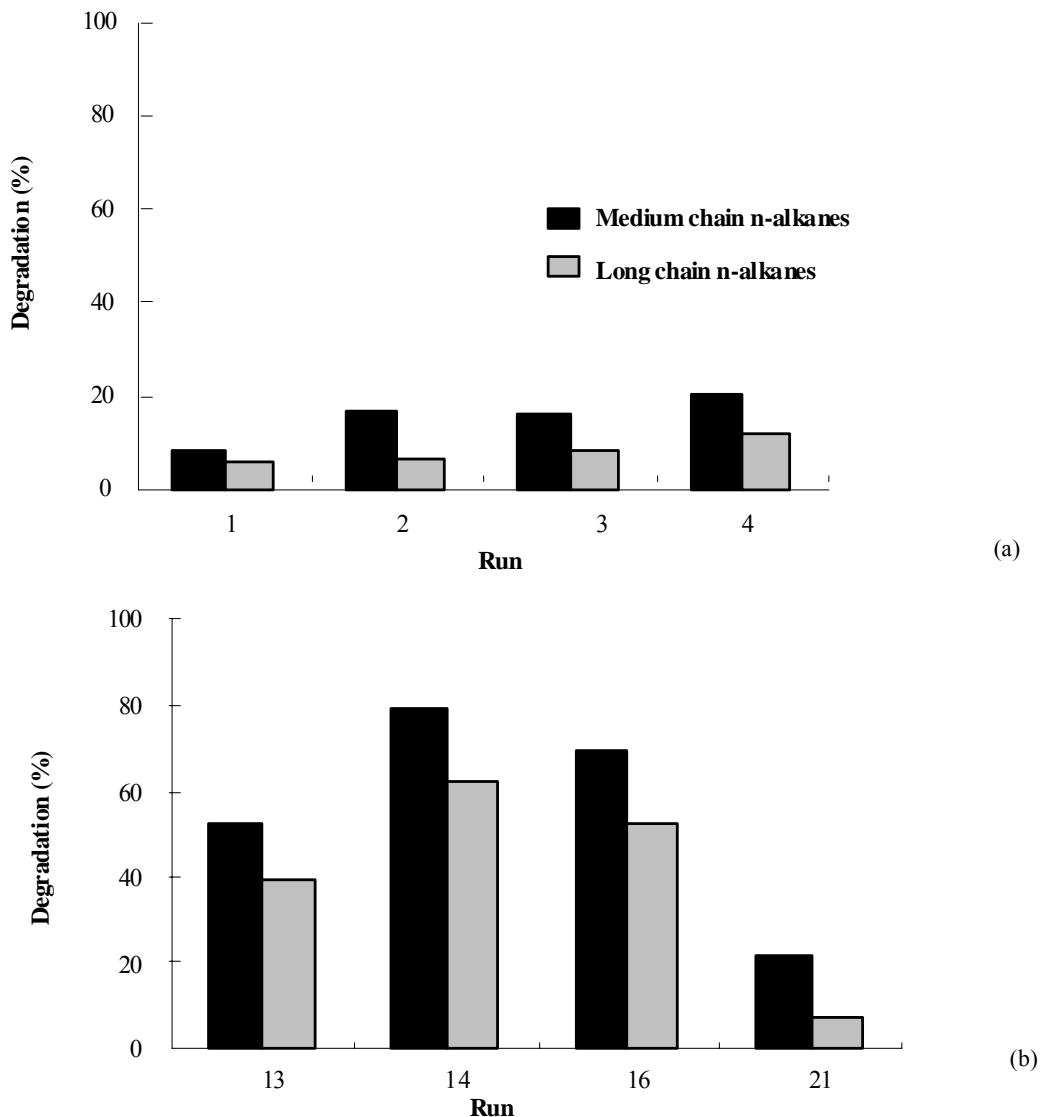


Fig. 3. Comparison biodegradation of medium chain and long chain n-alkanes in different days (a) day 7 (b) day 18 (c) day 28 (Continues)

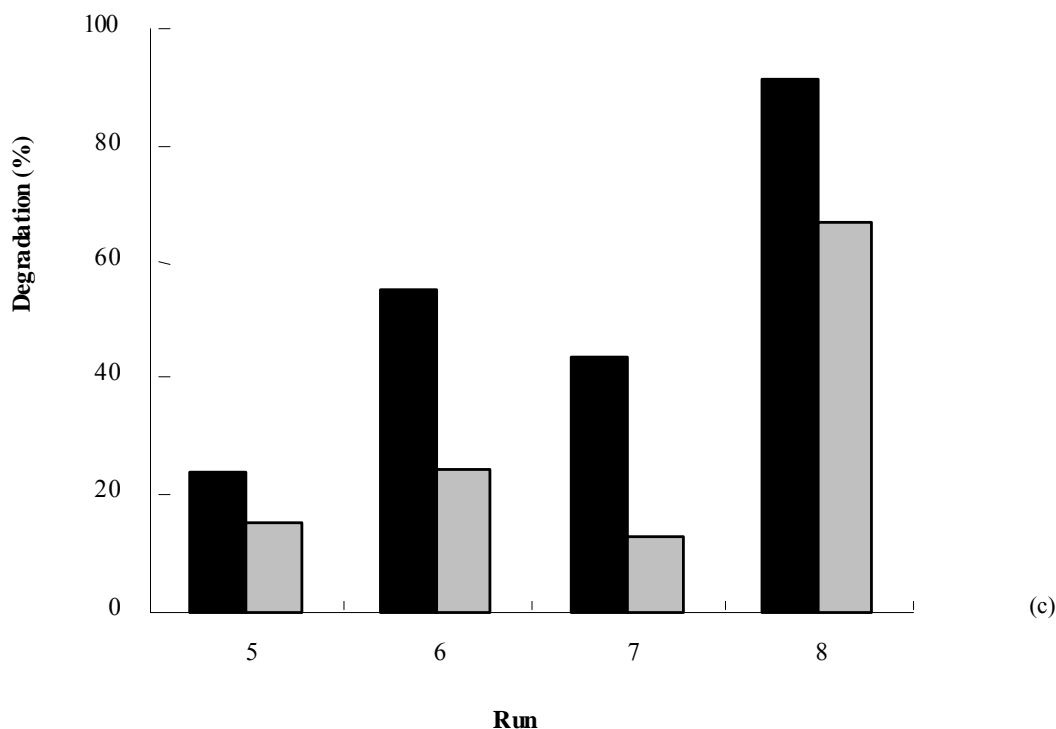


Fig. 3. Comparison biodegradation of medium chain and long chain n-alkanes in different days (a) day 7 (b) day 18 (c) day 28

Table 6. Optimum conditions found by design expert for n-alkane bioremediation

Matrix	N (mg/L)	P (mg/L)	Time (day)	Removal (%)			StD*
				Observed	Predicted	Error (%)	
n-alkane	188.71	18.99	25	92.04	91.00	1.13	±0.74

\*Standard deviation

Although model validation was confirmed by statistical tests, model verification was carried out both for the optimum condition and for a random test. Error was below 5% for all tests, indicated that process optimization by CCD were reliable for optimizing n-alkanes bioremediation in oil-contaminated seawater.

**CONCLUSION**

The need for efficient treatment technologies for major marine oil spills has been clearly demonstrated. Bioremediation, the process by which organisms degrade organic compounds to non toxic or less toxic substances, has been used with some success. The results of this study indicate that n-alkanes can be removed from contaminated seawater under laboratory conditions over a period of four weeks using indigenous microorganisms and suggest that efficacy of removal in natural marine environments may depend

on nutrient availability. Up to 92% n-alkane removal was observed after numerical optimization using a second-order polynomial mathematical model (generated with multiple regression analysis) for removal. The adequacy of the model was checked by ANOVA and diagnostic tests and also verified through optimization. This study indicates that CCD can be used effectively for modeling and optimizing biodegradation of petroleum contaminants in the aquatic environment.

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