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Optimization of *Enterobacter cloacae* (KU923381) for diesel oil degradation using Response Surface Methodology (RSM)

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Efficiency of *Enterobacter cloacae* KU923381 isolated from petroleum hydrocarbon contaminated soil was evaluated in batch culture and bioreactor mode. The isolate were screened for biofilm formation using qualitative and quantitative assays. Response surface methodology (RSM) was used to study the effect of pH, temperature, glucose concentration, and sodium chloride on diesel degradation. The predicted values for diesel oil degradation efficiency by the statistical designs are in a close agreement with experimental data (R^2 = 99.66%). Degradation efficiency is increased by 36.78% at pH = 7, temperature = 35°C, glucose = 5%, and sodium chloride concentration = 5%. Under the optimized conditions, the experiments were performed for diesel oil degradation by gas chromatographic mass spectrometric analysis (GC-MS). GC-MS analysis confirmed that *E. cloacae* had highly degrade hexadecane, heptadecane, tridecane, and docosane by 99.71%, 99.23%, 99.66%, and 98.34% respectively. This study shows that rapid bioremoval of hydrocarbons in diesel oil is acheived by *E. cloacae* with abet of biofilm formation. The potential use of the biofilms for preparing trickling filters (gravel particles) for the degradation of hydrocarbons from petroleum wastes before their disposal in the open environment is highly suggested. This is the first successful attempt for artificially establishing petroleum hydrocarbon degrading bacterial biofilm on solid substrates in bioreactor.

Keywords: hydrocarbon, biofilm, response surface methodology, biodegradation

Introduction

Diesel is a kind of engine fuel, fractional distilled compound obtained from petroleum oil. It contains rich in light weight hydrocarbons with the range of C_8-C_{26} and polyaromatic hydrocarbons (PAHs). Diesel spillage causes serious effects on ecosystem because of a few components which are potential carcinogenic and toxic nature against biotic factor in that environment (Price *et al*., 1993; Sadiq *et al*., 2002). Similarly ecological attributes of that area which can make the diesel seep into soil and water sources leads to series effects. A recent research has shown that the biological way of decontaminating the soil and water and combination with other methods is the best endowed practice for removing diesel oil spills. One of the biological ways to decontaminate the soil and water is the use of microorganisms, its metabolic processes remove pollutants and detoxify the hazardous nature of the compounds (Saadoun, 2002; Geetha *et al*., 2013). Response surface methology (RSM) is a combination of mathematical and statistical techniques that is useful for analyzing the effects of several independent variables on the system response without the need of a predetermined relationship between the objective function and the variables. To achieve the best results for growth and activity of bacterium, the composition of the media and growth conditions were optimized by using four different culture conditions with the help of RSM. A numerical approach was used to estimate the kinetic and film thickness parameters as a consequence of the validation of the process model with the aid of measured data. Several factors like pH, substratum, rate of agitation, hydrocarbon content, temperature, glucose concentration, and sodium chloride concentration, affect the hydrocarbon degradation and biofilm formation. Therefore, it is essential to quantify the optimal conditions to design the efficient diesel oil degradation system. RSM is used to determine the most feasible and optimum combination of these parameters (Wang *et al*., 2011; Bakkiyaraj *et al*., 2015; Kumar *et al*., 2015). Using RSM, various operating conditions can be considered simultaneously taking less number of experimental runs than conventional procedures (Sarve *et al*., 2015).

Wang *et al*. (2008) amended the process by RSM and a central composite rotatable design (CCRD) was applied in biodiesel production to understand the effects of dosage of the enzyme, ratios of both t-butanol to oil (v/v) and methanol to oil (mol/mol) on the FAME yield during methanolysis. Vidhyalakshmi and Vallinachiar (2012) isolated, characterized and optimized to enhance EPS formation of *Pseudomonas stutzeri* from sewage samples using central composite design (CCD) and RSM. They observed that EPS of *P. stutzeri*, binds heavy metals and also shows good emulsifying activity towards against hydrocarbons at optimized conditions and serve as better tool in bioremediation and waste management.

The present study accesses the hydrocarbon degradation ability of *Enterobacter cloacae* (KU923381) isolated from petroleum hydrocarbon contaminated soil under *in vitro* conditions. The degradation of complex hydrocarbon diesel oil was investigated under a range of pH, temperature, and co-substrate concentrations. This is the first report on hydro-

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carbon degradation with aid of biofilm formation and the optimizations were done by CCD of RSM system.

Materials and Methods

Sampling and isolation of diesel oil degrading bacteria

Hydrocarbon contaminated water and soil samples from refinery outlet, petrol pump station, and generator Store room at Tanjore, Tamilnadu, India were collected. Then the samples were processed by 5 serial cycles of the modified enrichment method of Prakash and Irfan (2011), where Bushnell Haas medium (pH 7.0) was inoculated with isloates and incubated at 37°C in a shakin incubator at 120 rpm (Cooper *et al*., 1987; Bodour and Maier, 1998; Sugumar *et al*., 2014). At the end of the enrichment processes, the cultures were plated on to nutrient agar (Hi-media) as well as Bushnell Haas Agar medium (Hi-media, Constituents:1 g KH_2PO_4, 1 g K_2HPO_4, 1 g NH_4NO_3, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.05 g $FeCl_3$, 0.02 g $CaCl_2{\cdot}2H_2O$, 15 g/L of agar).

Screening of bacterial isolates

Bacterial isolates from the enrichment process were screened and the strains which shows highest emulsification index were used for further study (Youssef *et al*., 2004; Sriram *et al*., 2011). Biofilm formation assays were performed to identify biofilm forming potential strains by both qualitatively and quantitatively. Tube method and Modified Tissue culture methods were used for biofilm formations (Christensen *et al*., 1995; Magesh *et al*., 2013).

Identification of potential isolates

The bacterial isolates were identified based on Gram's staining, motility, and other biochemical tests according to Bergey's Manual of Systematic Bacteriology (Holt *et al*., 1994). Confirmation was done by 16S rRNA gene. The total genomic DNA was extracted from bacterial colonies from the nutrient agar slant, and then suspended in 0.5 ml of sterilized saline, after that it was centrifuged at 10,000 rpm for 10 min. The pellets (microbial cells settled from culture medium during centrifugation) were suspended in 0.5 ml of InstaGene Matrix (Bio-Rad); subsequently they were incubated at 56°C for 30 min and then heated 100°C for 10 min. The primers PC27F/1492R (27F-AGAGTTTGATCT TGGCTCAG) and (1492R-TACGGCTACCTTGTTACGA CTT) underwent 35 amplification cycles at 94°C for 45 sec, 55°C for 60 sec, and 72°C for 60 sec and amplified approximately 1,400 bp. Then the products were purified by using Montage PCR Clean up kit (Millipore). The purified PCR products were sequenced by using 2 primers (518F-CCAG CAGCCGCGGTAATACG and 800R-TACCAGGGTATCT AATCC) following instructions in Big Dye terminator cycle sequencing kit (Applied Biosystems). Sequencing products were analyzed on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied Biosystems) (Sugumar *et al*., 2014).

Experimental design and optimizations

The main advantage in using RSM is the speed and reliability of its outcome. All the statistical analyses were conducted using Minitab statistics software (Version 16.2.2, Minitab Inc.). Analysis of variance (ANOVA) was performed on the data to test the effects of the parameters and their interactions. Tukey's multiple tests were performed to determine the differences among the levels of each parameter. The α-level chosen was 0.05. To develop the model, four input parameters were considered as temperature, pH, sodium chloride concentration and glucose concentration. The potential bacterial isolate from overnight culture at the log phase of growth were transferred to 500 ml conical flasks, each containing 200 ml of sterile mineral salts medium with (2% v/v) diesel oil. A control sample of 200 ml of sterile mineral salts medium with only hydrocarbons where kept to analyse the photoxidation during diesel oil degradation studies.

Central composite design experiments: Central composite design (CCD) was applied for optimization of diesel oil degradation using *Enterobacter cloaceae.* From the previous an-

Table 1. GCMS analysis of diesel oil

S. No	Compound	Mass	(µg/g)
1	Naphthalene	128	353
2	Methylnaphthalenes	142	1560
3	Decahydro-trans- Naphthalene	138	2975
4	Fluorene	166	59
5	Anthracene	178	54
6	Phenanthrene	178	57
7	Methylphenanthrenes	192	159
8	Methylanthracene	192	6.0
9	Pyrene	202	64
10	tert-butyl-Benzene	134	1.2
11	Heptacosane	380	62
12	Hexadecane	226.5	34
13	Tridecane	184.37	2.5
14	C1-201 PAHs	216	290
15	C3-178 PAHs	220	120
16	Three PAH species	228	6.0

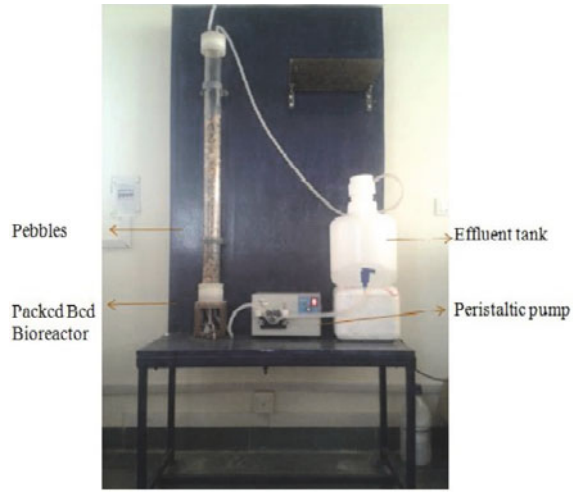

Fig. 1. Fabricated continuous flow packed bed biofilm reactor.

alysis four experimental factors were chosen for the present studies, namely, pH, temperature, glucose, and NaCl concentration. The variables optimized were pH in the range of 7–10, temperature 20–35°C, glucose concentration 5–10% (w/v) and NaCl concentration 5–10% (w/v) respectively. 2^3 factorial central composite experimental design leading to a set of 31 experimental runs was used to optimize diesel degradation.

RSM is a scientific approach to Taylor first order and second order series with experimental data for optimization (Moghadam *et al.*, 2013; Natalia *et al.*, 2014). The surface of Taylor expansion curve was determined using RSM and this describes the response. It is of the form (Eqs.1):

$$\text{Respone} = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x^2 + \Sigma\beta_{ij} x_i x_j \quad (1)$$

Where,
β_0 = Regression constant
β_i, β_{ii}, β_{ij} = Regression coefficients
x= Process variable

Experimental setup for Continuous flow packed bed Biofilm bioreactor

A Pilot scale Continuous flow packed bed Biofilm reactor was fabricated (Fig. 1) and experiments were conducted at optimized conditions. The bioreactor was 100 cm height with 6 cm internal diameter of cylindrical Borosil make glass column. Pebbles with range of 2.5 cm to 3.5 cm were used as inert support media for biofilm growth and inbuilt support at the base of bioreactor to hold pebbles. The culture grown in Minimal salt medium prepared from Merck chemicals [1.4 g $(NH_4)_2SO_4$, 1.0 g $MgSO_4{\cdot}7H_2O_2$, 0.015 g $CaCl_2{\cdot}2H_2O_2$, 1.0 ml trace element solution, 1.0 ml stock A solution, and 35.2 ml 1.0 M phosphate buffer]. The potential isolate culture of about 1% (v/v) was inoculated into artificial wastewater containing MSM with diesel oil of about 2% (v/v) at optimized conditions. After two weeks of incubation, the degradation studies were conducted and compared with batch scale studies.

Biodegradation test

For optimization studies, the degradation percentage of the experiments was analyzed by gravimetric method and Merck chemical solvents were used in the experiment (Chang, 1998; Ganesh, 2009). The PAH in the compounds were detected by GCMS analysis (Sanyaolu *et al.*, 2012). The culture activities were stopped by adding 1% 1*N*-HCL. 50 ml of culture broth was mixed with 50 ml petroleum ether: acetone (1:1) in a separating funnel and was shaken vigorously to get a single emulsified layer. The top layer in separating funnel was taken in a clean preweighed beaker. The petroleum ether and acetone was evaporated on a water bath at 65°C. The mass of residual oil was measured and the percentage of degradation (DE%) was calculated by

$$\text{DE\%} = \frac{\text{Amount of hydrocarbon degraded}}{\text{Amount of hydrocarbon added in media}} \times 100$$

The sample which shows hydrocarbon degradation was further confirmed by GCMS analysis. Five millilitre of sample was transferred into a 50 ml separating funnel, and 5 ml of diethyl ether was added to it (Sanyaolu *et al.*, 2012). The sample was shaken vigorously for about 2 min with periodic venting to release vapour pressure. The organic layer was allowed to separate for 10 min and was recovered into the 50 ml beaker. The aqueous layer was re-extracted twice with 2 ml of the extract. The combined extract was dried by passing through the funnel containing the anhydrous sodium sulfate. The dried extract was concentrated with a stream of nitrogen gas. PerkinElmer Mass Spectrometer with a HP-5 MS column was used for GCMS analysis. Biodegradation efficiency (BE%) of individual compounds were calculated by formulae:

$$\text{BE\%} = 100 - \frac{\text{As} \times 100}{\text{Aac}}$$

where As = total area of peaks in each sample
Aac = total area of peaks in the appropriate abiotic control.

Statistical analysis

All experiments were conducted in duplicate. The values reported in this work are an average of three samples for every replication maintained. The data were statistically analyzed for differences in means at the 5% probability level by general linear models procedure in SAS institute (SAS, 1996) and by following Steel and Torrie (1980).

Results and Discussion

Samples from three hydrocarbon contaminated sites were enriched and seven morphologically distinct microbial isolates were obtained. Using emulsification test, three isolates showed highest diesel degrading abilities than the rest (Riser-Roberts, 1992; Satpute *et al.*, 2008; Perfumo *et al.*, 2010; Donio *et al.*, 2013). These three potential isolates were identified by morphologically, biochemically and confirmed by 16S rRNA sequencing as *Bacillus niacini* (KU925847), *Enterobacter cloacae* (KU923381), and *Stenotrophomonas maltophilia* (KU925846). The three isolates were further screened for biofilm formation (Table 2). In TCP method, *Stenotrophomonas maltophilia* (KU925846) and *Enterobacter cloacae* (KU923381) OD values were above 0.2 values (Christensen *et al.*, 1995; Magesh *et al.*, 2013). Among the three potential degrading isolates, *Enterobacter cloacae* (KU923381) (OD=0.27) showed highest biofilm formation in screening method were taken for further degradation studies.

In the present study, the optimum conditions for hydrocarbon degradation were determined by RSM. Here, 4-level-4-factor design was implemented, totally 31 experiments were done in duplicates (Goyal *et al.*, 2012). The prophase research results showed that the four independent variable parameters such as reaction temperature (T), hydrogen ion concentration (pH), glucose concentration (GLU), and sodium chloride concentration (NaCl) have important effects on hydrocarbon degradation. The feedback of hydrocarbon degradation in percentage was shown in Table 3.

Table 2. Screening of biofilm formation by the three isolates

S. No	Sample	TM	TCP OD @ 595 nm	Biofilm formation
1	*Enterobacter cloacae* (KU923381)	++	0.268 + 0.012	High
2	*Bacillus niacini* (KU925847)	+	0.131 + 0.006	Moderate
3	*Stenotrophomonas maltophilia* (KU925846)	++	0.211 + 0.003	High
4	Blank (Negative Control)	-	0	-

The model predicted was correlated to coefficients of interactions, linear and quadratic effects. The correlation coefficients for each model and variable significance were measured by the probability values. All the factors and their square interactions ($P < 0.05$) were significant at the 95% statistic confidence level. The effects of suitable variable level interactions on hydrocarbon degradation as a function of two variables were observed by making a three dimensional plot on the response surface curves (while retaining the other variable constant). The regression equation obtained after analysis of variance gives the level of diesel removal as a function of the different degradation variables: temperature (T), hydrogen ion concentration (pH), glucose concentration (GLU), and sodium chloride concentration (NaCl). All terms regardless of their significance are included in the following Equation (2):

$$\text{Degradation percentage} = 198.678 + 4.13552 \times T - 30.2282 \times pH - 7.51521 \times NaCl - 23.8037 \times GLU - 0.0269192 \times T^2 + 1.87691 \times pH^2 + 0.310467 \times NaCl^2 + 0.755987 \times GLU^2 - 0.202317 \times T \times pH + 0.07149 \times T \times NaCl - 0.03643 \times T \times GLU - 0.398083 \times pH \times NaCl + 1.13052 \times pH \times GLU + 0.44467 \times NaCl \times GLU \quad (2)$$

CCD showed that along with experimental and model predicted values of hydrocarbon degradation on the 14th day for all 31 combinations (Table 3). As a result of optimizing media components of MSM by CCD using RSM, a regression model equation was developed which predicted maximum hydrocarbon degradation (55%) that closely matched the experimentally measured value (54.20%) as obtained on the 14th day. Normally, a regression model considered as high correlated, goodness of the fit and experiments are reproducible when R^2 value of the model is higher than 0.9 (Laxmi *et al.*, 2011; Bakkiyaraj *et al.*, 2015; Dave *et al.*, 2015). To examine the accuracy of predicted model by computer software, the graph representing experimental values for hydrocarbon removal efficiency against the predicted model was prepared. The high correlation, close to unity, is more than desired and verifies the second order model satisfactory conformance to the experimental data. Analysis of variance is showed in table 4 (F-test) for this experiment and value of R^2=99.66% indicates that of the variability in the response could be explained accuracy of the model was good. This analysis of the response trends using the model was considered to be reasonable and the *P*-values observed in the model is significantly coefficient.

The results revealed the statistically significant ($P < 0.05$) effects of pH, temperature, Glucose concentration, NaCl concentration on hydrocarbon biodegradation. Maximum biodegradation efficiency of 55% was obtained at pH = 7, temperature = 35°C, Glucose = 5%, and NaCl = 5%. In all experiments, the practically achieved biodegradation rates were consistent with predicted values. Leahy and Colwell (1990) reported that the range commonly fitted for most of petroleum degrading bacterial species have degrading property at pH = 6–8, but the optimum degradation abilities is observed at pH 7. Likewise increasing temperature favours the hydrocarbon degradation commonly in the range of 30–40°C. At 5% glucose concentration in the medium showed highest degradation and high biofilm formation. Samuel and Oladipupo (2012) observed that nutrient (NPK fertilizer), surfactant (Tween 80) and mixed culture (biomass) variations plays vital role in crude oil degradation using RSM gives significant results in batch culture.

The normal probability plot of the residual revealed that

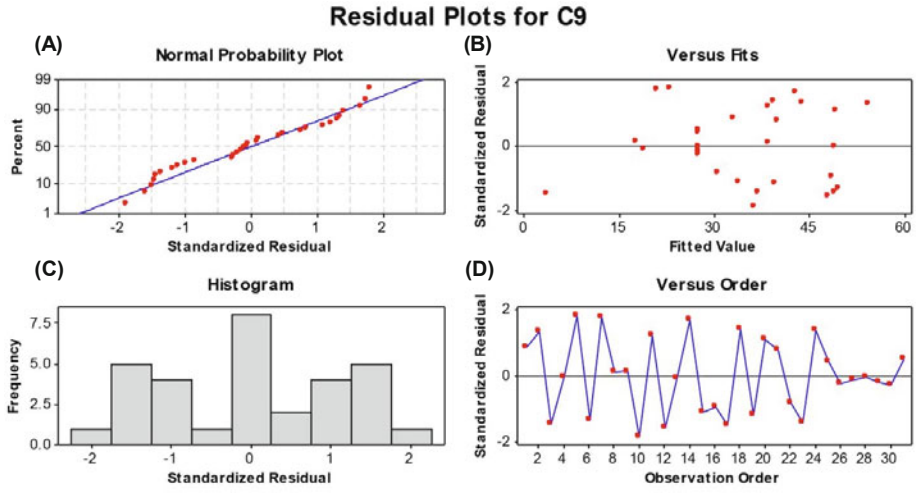

Fig. 2. Residual plots of RSM-CCD experiment. (A) Normal probability plot of the difference between the observed and the predicted value. (B) Difference between the observed and the predicted value versus fitted value when the variance is constant. (C) Histogram of the difference between the observed and the predicted value. (D) Difference between the observed and the predicted value versus the order.

Table 3. Experimental results based on central composite design

Std Order	Run Order	Pt Type	Blocks	Temp	pH	NaCl	GLU	DE% (Expt.)	DE% (Pred.)
1	1	1	1	20.0	7.0	5.0	5.0	33.499	32.99238
2	2	1	1	35.0	7.0	5.0	5.0	55.000	54.20305
3	3	1	1	20.0	10.0	5.0	5.0	36.000	36.87772
4	4	1	1	35.0	10.0	5.0	5.0	48.963	48.98413
5	5	1	1	20.0	7.0	10.0	5.0	24.118	23.0342
6	6	1	1	35.0	7.0	10.0	5.0	48.793	49.60662
7	7	1	1	20.0	10.0	10.0	5.0	22.000	20.9483
8	8	1	1	35.0	10.0	10.0	5.0	38.470	38.41646
9	9	1	1	20.0	7.0	5.0	10.0	17.790	17.71485
10	10	1	1	35.0	7.0	5.0	10.0	35.060	36.19328
11	11	1	1	20.0	10.0	5.0	10.0	39.290	38.558
12	12	1	1	35.0	10.0	5.0	10.0	46.977	47.93216
13	13	1	1	20.0	7.0	10.0	10.0	18.813	18.87342
14	14	1	1	35.0	7.0	10.0	10.0	43.720	42.7136
15	15	1	1	20.0	10.0	10.0	10.0	33.077	33.74532
16	16	1	1	35.0	10.0	10.0	10.0	47.893	48.48123
17	17	-1	1	12.5	8.5	7.5	7.5	2.524	3.425375
18	18	-1	1	42.5	8.5	7.5	7.5	40.219	39.37196
19	19	-1	1	27.5	5.5	7.5	7.5	38.811	39.52119
20	20	-1	1	27.5	11.5	7.5	7.5	49.830	49.17417
21	21	-1	1	27.5	8.5	2.5	7.5	40.380	39.92172
22	22	-1	1	27.5	8.5	12.5	7.5	30.000	30.51261
23	23	-1	1	27.5	8.5	7.5	2.5	48.092	48.96154
24	24	-1	1	27.5	8.5	7.5	12.5	44.564	43.74879
25	25	0	1	27.5	8.5	7.5	7.5	27.830	27.45549
26	26	0	1	27.5	8.5	7.5	7.5	27.240	27.45549
27	27	0	1	27.5	8.5	7.5	7.5	27.330	27.45549
28	28	0	1	27.5	8.5	7.5	7.5	27.410	27.45549
29	29	0	1	27.5	8.5	7.5	7.5	27.280	27.45549
30	30	0	1	27.5	8.5	7.5	7.5	27.210	27.45549
31	31	0	1	27.5	8.5	7.5	7.5	27.880	27.45549

all the points approximate a straight line proving no severe indication of non normality and confirms that the experiments is a good fit of the model (Fig. 2A). A residual plot allowed visual assessment of each observation separated from the fitted line. The residuals randomly scattered in a constant width band about the zero line (Fig. 2B). Histogram of the residuals showed visual assessment of the assumption. The measurement errors in the response variable were normally distributed (Fig. 2C). This ensured model was suitable to navigate the design space and a satisfactory model to the experimental data.

Zafar *et al.* (2010) and Kuntiya *et al.* (2005) stated that indicate that slightly increased concentrations of sodium chloride than the optimized conditions favours the cell growth and degradation of naphthalene and phenol by the microbial strain. Annuar *et al.* (2006) stated that optimized medium ensures moderate concentration into maximum biodegradation of naphthalene. Degradation of atrazine was carried out in batch reactors using microorganisms with aid of RSM

Table 4. Analysis of variance for CCD model fitting to the biodegradation data of diesel oil

Source	Sum of squares	D.F*	Mean square	*F* value	*P* value
Model					
Residual	13.99	16	0.87		
Lack of fit	13.51	10	1.35	17.04	0.001
Pure error	0.48	6	0.08		
Corr. Total		30			
R^2 = 0.9966					
Adj. R^2 = 0.9936					

*D.F, degrees of freedom

Table 5. Diesel degradation efficiency of *Enterobacter cloacae* (KU923381) under different parameters

S. No	Sample	Parameters				Degradation%
		pH	Temp	NaCl	Gluco. Conc.	
1	Initial	7.5	37	1%	5%	40.21 + 0.05
2	Optimized in batch study	7	37	5%	5%	55 + 0.01
3	Optimized in bioreactor	7	37	5%	5%	57.94 + 0.88

Table 6. Biodegradation efficiency percentage BE (%) of 9 detectable peaks from GCMS analysis

S. No	Name of peaks	Retention time	*Enterobacter cloacae*
1	*Name: Tridecane Formula: C13H28 MW: 184*	12.76	99.66
2	*Name: Hexadecane Formula: C16H34 MW: 226*	20.40	99.71
3	*Name: Heptadecane Formula: C17H36 MW: 240*	23.23	99.23
4	*Name: Nonadecane Formula: C19H40 MW: 268*	28.44	98.25
5	*Name: Heneicosane Formula: C21H44 MW: 296*	33.27	98.22
6	*Name: Eicosane Formula: C20H42 MW: 282*	30.93	98.01
7	*Name: Docosane Formula: C22H46 MW: 310*	35.52	98.34
8	*Name: Hexacosane Formula: C26H54 MW: 366*	43.96	96.54
9	*Name: Heptacosane Formula: C27H56 MW: 380*	46.11	97.56

observed that pH, temperature, inoculum concentration, and agitation speed are the parameters interacts in degradation process (Debasmita, 2013). El-Bestawy *et al.* (2014) stated that availability of essential nutrients for microbial growth may also limit the rate at which microorganisms degrade pesticides and concentration of the pesticide must not be so high as to be toxic nor so low such that biodegradation does not proceed due to a lack of induction of appropriate degradative enzymes.

About 57.94% of degradation efficiency was observed for hydrocarbon degradation in bioreactor at 1 h HRT using gravimetric method (Table 5). In bioreactor, HRT is one of important factor in biochemical processes. Optimization of HRT may leads to increase the removal efficiencies of hydrocarbon and decrease effluent concentrations. *Enterobacter cloaecea* were previously shown degradation potential against pentaerythritol tetranitrate, alkanes, and diesel oil (Saadoun, 2002; Mariano *et al*., 2008). The biofilm reactor provides best substratum for biofilm formation of bacteria or other microorganisms, microbes over the surface where continuously exposed to wastewater containing the contaminants favours bacterial growth and converts the contaminants into harmless products (Rao *et al*., 2010).

The gas chromatogram of diesel substrate retrieved from the inoculated medium after two week incubation is shown in Fig. 3A and B. It was found that *Enterobacter cloacae* (KU-923381) almost degraded hydrocarbons such as Tridecane, Hexadecane, Heptadecane, Heptacosane, decahydro-trans-

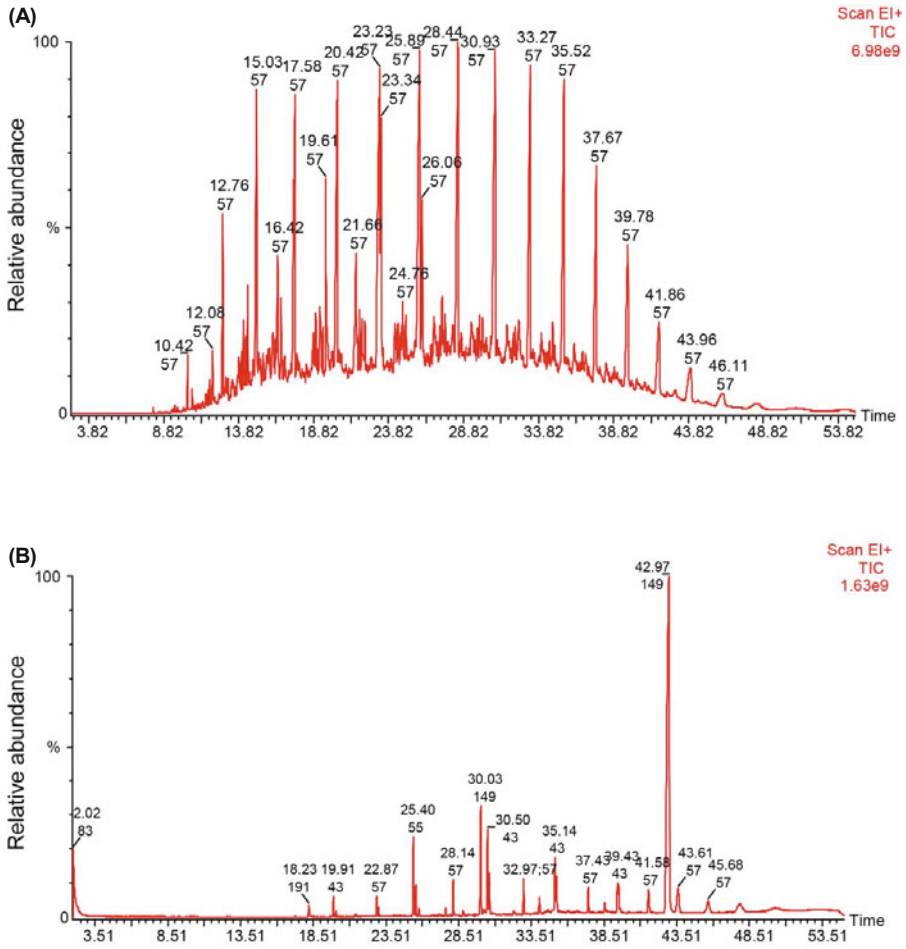

Fig. 3. GCMS analysis of hydrocarbon degradation. (A) GCMS analysis of controlled sample containing hydrocarbon. (B) GCMS analysis of treated sample by *Enterobacter cloacae* (KU923381).

Naphthalene, and tert-butyl-Benzene present in diesel fuel. Treated diesel substrate showed a decrease in the area of major peaks indicating breakdown of the main compounds; while new peaks leading to formation of short chain compounds represented breakdown products or presumed metabolites (Table 6) (Zafar *et al*., 2010; Ameen *et al*., 2016).

Conclusion

This study reveals the diesel oil degrading bacteria isolated from petroleum contaminated site could degrade most components of hydrocarbons. The bacteria could also form the biofilm on various substrates. So the bacteria will have most application in removal of complex hydrocarbons from effluent as well as contaminated soil. Diesel oil hydrocarbon degradation with aid of biofilm formation in continuous flow packed bed bioreactor and the optimizations were done by CCD of RSM system, is the innovative and pertinent approach in pollution treatment. The similarity of the predicted and the observed results has confirmed the validity and applicability of RSM (CCD) in optimization processes. Our results suggest that statistical optimum strategy is an effective tool to predict the biodegrading activity of diesel hydrocarbon and conditions fitted for bioremediation treatment process in industrial applications.

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