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Statistical Optimization of Crude Oil Biodegradation by *Marinobacter* sp. Isolated from Qeshm Island, Iran

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Background: Hydrocarbons degradation is principally achieved by microorganisms in natural environments. The extent of hydrocarbons biodegradation is mainly conditioned by environmental factors and its success depends on the optimal condition for the crude oil degrading isolates.

Objectives: The aims of the current study was to isolate and identify crude oil degrading bacterium from surface sediments of Qeshm Island, Iran and to evaluate the efficiency of a statistically-based experimental design for the optimization of crude oil degradation performed by the isolated strain.

Materials and Methods: Crude oil degrading bacteria were isolated by serial dilutions of bacterial consortium. In order to optimize crude oil biodegradation by isolated strains, Plackett-Burman experimental design was used to evaluate nine factors affecting crude oil biodegradation in twelve experimental trials. To observe the best yield in crude oil biodegradation, factors that had higher effects were considered for the next stage in the biodegradation optimization process using the Taguchi experimental design.

Results: A gram-negative bacterium strain named as the KK1- strain (with 98% homology with *Marinobacter* litoralis) was isolated from enrichment consortium. Among the various variables screened using the Plackett-Burman experimental design, pH, temperature, salinity and NH_4Cl were determined as the most significant factors and considered for the next stage of the biodegradation optimization process using the Taguchi experimental design. Theoretically, the optimum degradation conditions were determined to be: pH = 8, temperature = 35°C, salinity = 30 ppt and $NH_4Cl = 1\ g.L^{-1}$. The validity of the predicted optimized condition was tested by conducting experiments considering the predicted criteria. Biodegradation efficiency of 58.32±5.57% was achieved under the suggested conditions, which was significantly higher than that achieved by the primary conditions (35%).

Conclusions: Indigenous bacteria from surface sediments of Qeshm Island were found to be able to degrade crude oil. Our results showed that a combination of the Plackett-Burman and the Taguchi experimental designs may be successfully used to find the optimal amounts of factors required for crude oil biodegradation.

Keywords: Biodegradation Optimization; *Marinobacter litoralis*; Plackett-Burman; Taguchi

1. Background

Hydrocarbons-spillage in the environment, caused by accidents or human activities, is among the most common environmental pollutants (1). Several cleanup techniques have emerged to remove petroleum hydrocarbons from the soil in recent years of which bioremediation processes have received the most attention, because of their simplicity, high efficiency and inexpensiveness when compared to other technologies. Petroleum bioremediation is carried out by microorganisms capable of utilizing hydrocarbons as source of energy and carbon (2). *Pseudomonas*, *Achromobacter*, *Flavobacterium*, *Acinetobacter*, *Vibrio*, *Bacillus*, *Arthrobacter*, *Norcardia* and *Micrococcus* are considered as common oil degraders in marine environments (3). Although a variety of bacteria using crude oil as the sole carbon and energy source have been isolated from the ocean, successful bioremediation depends on environmental factors which could be optimized to achieve greater efficiency (4).

Conventional optimization procedures are performed by altering one parameter at a time and keeping all other parameters at fixed levels. Consequently, the impact of that particular parameter on the process can be assessed. However, these procedures are time consuming, require more experimental data sets, and do not provide information about the mutual interactions of the parameters. Therefore, statistical methods should be used for an ac-

Implication for health policy/practice/research/ medical education:
Application of bacterial biodegradation process would result in relatively fast and successful detoxification of public health threatening environmental contaminants. Petroleum contamination is a persistent and widespread problem ravaging almost all compartments of the environment and imposing serious health implications and ecological disturbances. The results of this study can be used in the bioremediation of petroleum-contaminated soil in the Iranian coasts of the Persian Gulf.

curate study of interactions among different factors. The use of multivariate experimental design techniques is becoming increasingly widespread in applied biotechnology. Multivariate designs, which allow the simultaneous study of several control variables, are faster to implement and more cost effective than traditional univariate approaches (5). Persian Gulf is one of the most important water pathways in the world. The Persian Gulf has been remarkably developed for crude oil production, transportation, and exportation. It is now well established that such activities result in contamination of the marine environment by petroleum and its products. Thus, it is expected that oil pollution has distributed on the Persian Gulf and has seriously endangered the marine ecosystem (6).

2. Objectives

Among Iranian islands in the northern Persian Gulf, Qeshm Island is the largest and is especially known for its geographical location and economical importance. The aims of the current study were as follows; 1) to isolate and identify crude oil degrading bacteria from surface sediments of Qeshm Island, and 2) to evaluate the efficiency of the Plackett-Burman and Taguchi experimental designs for optimizing crude oil biodegradation performed by the isolated strain.

3. Materials and Methods

3.1. Sampling and Bacterial Enrichment

To isolate crude oil degrading bacteria, coastal surface sediments were collected from different sites of Qeshm Island, Iran. To enrich bacteria capable of utilizing crude oil, 5 mL of soil samples were added to 250 mL conical flasks containing 50 mL mineral salt medium (MSM) (0.5 $g.L^{-1}$ K_2HPO_4, 1 $g.L^{-1}$ NH_4Cl, 0.01 $g.L^{-1}$ $FeSO_4.7H_2O$, 1000 mL filtered 40 ppt local seawater) with 1% Iranian light crude oil as the sole source of carbon. The flask was incubated at 30 °C on a rotary shaker at 140 rpm and adjusted to pH=7. Oil utilization in the enriched cultures was monitored by a decrease in the amount of crude oil concentration and an increase in bacterial biomass; next, 5 mL of the enriched culture was transferred to fresh medium and incubated under the same conditions. This process was repeated four times to obtain the enriched crude oil degrading consortium.

3.2. Isolation and Identification of Bacterial Strains

At the end of the enrichment procedure, serial dilutions of bacterial consortium were prepared and 100 µl of each dilution was spread on nutrient agar plates incubated at 30 °C for 48 hours. Then, bacterial colonies were collected and purified by repetitive streaking onto nutrient agar plates (Quelab Laboratories Inc. Montreal, Canada). Purified strains were then identified by morphological and biochemical tests (7). Further molecular identification of the strains was also performed by 16S rDNA gene sequence analysis. For this purpose, DNAs of the isolated bacteria were extracted using the bacterial DNA extraction kit (Roche®- Germany). The isolated strains were then identified by 16S rDNA gene sequence analysis after amplification of the gene by PCR using a set of universal primers 27F (5- AGA GTT TGA TCC TGG CTC AG-3) and 1510R (5-GGT TAC CTT ACG ACT T-3). The reactions were cycled in a Primus 25 advanced® thermocycler with an initial denaturation step at 95°C for 5 minutes followed by 35 cycles of denaturation at 94 °C for 1 minutes, annealing at 52°C for 1.30 minutes and extension at 72°C for 1 minute, and a final extension step at 72°C for 15 minute. DNA sequences of the cloned 16S rDNA fragments were compared using BLAST from the http://blast.ncbi.nlm.nih.gov/Blast.cgi website maintained by the National Center of Biotechnology Information (NCBI).

3.3. Biodegradation of Crude Oil by Isolated Strains

In order to assess the utilization rates of crude oil using the isolated strains, microbial inoculums with final optical density (OD600 nm) of 0.15 were passed to the MSM containing 1% crude oil. The same MSM without any microbial inoculum was also retained. The experimental cultures were performed at 30 °C at 140 rpm for 7 days. The entire medium for each trial was used for analysis of the rest of the crude oil concentrations.

3.4. The Plackett-Burman Experimental Design

The Plackett-Burman experimental design was used to search for the relative importance of various factors on crude oil degradation in MSM. The design included eleven variables (nine independent variables with two blank variables), which were examined in 12 runs in a Plackett-Burman design. Temperature (20 and 30 °C); salinity (20 and 40 ppt); pH (7 and 9); rpm (100 and 140); K_2HPO_4 (0.1 and 0.5 $g.L^{-1}$); NH_4Cl (0.2 and 1 $g.L^{-1}$); Fe_2SO_4 (0.01 and 0.04); inoculum size (0.05 and 0.2 OD600); medium volume (50-100 mL) and two blanks. The assays were performed in duplicates. The strength of the variables is shown in Table 1. Statistical analysis was carried out with the Statistica 8.0 software (SoftStat, Inc.).

3.5. Optimization of Crude Oil Biodegradation Using Taguchi Experimental Design

To observe the best yield in crude oil biodegradation, parameter optimization was performed using the Taguchi experimental design, L_9 (3^4) selected from the design module of Statistica 8.0 (SoftStat, Inc.). A total of nine experiments, were used to investigate the effect of four factors, including pH, temperature, NH_4Cl concentration and salinity (Table 2). During the optimization

process, crude oil was used as the sole carbon source and spiked in to the 250 mL conical flask at 10 mL.L^{-1}. Predetermined salinity levels were achieved by adding distilled water or NaCl to the sea water. Inoculation was done as mentioned in Table 2 with a final optical density (OD600nm) of 0.20. All inoculated flasks were shaken at 140 rpm for one week. For each experiment a flask containing similar ingredients without bacterial inoculation was prepared as the control to determine abiotic degradation. All experiments were carried out in triplicates. The entire medium for each trial (50 mL) was used for analysis of the rest of the crude oil concentrations. In order to evaluate the accuracy of the Taguchi experimental design, the optimized culture conditions, were then applied to biodegradation of crude oil and residual oil was estimated after 7 days.

3.6. Extraction of Residual Oil

Analytical procedures were performed according to the process suggested by Mishra et al. with minor modifications (8). Briefly, culture media were acidified to pH < 2 and extracted two times in a separatory funnel using 25 mL n-hexane for each step. Extracts were mixed together and dried at room temperature by evaporation of solvents under a gentle nitrogen stream under a fume hood. Then after, the amount of residual crude oil was determined gravimetrically.

Table 1. Plackett-Burman Experimental Design for the Evaluation of Nine Independent Variables with two Blank Variables for Crude Oil Biodegradation

Trial Number	Variables											Percentage of Crude Oil Biodegradation (mean±SD)
	Temperature (°C)	Salinity (ppt)	pH	rpm	K_2HPO_4 (g.L^{-1})	NH_4Cl (g.L^{-1})	Fe_2SO_4 (g.L^{-1})	Inoculum Size (OD600)	Medium Volume (mL)	Blank	Blank	
1	30	20	9	100	0.1	0.2	0.04	0.05	100	1	1	13.54±3.66
2	20	20	9	140	0.5	1	0.01	0.05	100	1	-1	26.85±3.46
3	20	40	9	100	0.1	1	0.01	0.20	50	1	1	32.19±5.75
4	20	40	7	140	0.1	0.2	0.04	0.20	100	1	-1	27.41±3.25
5	30	40	7	140	0.5	0.2	0.01	0.05	50	1	1	34.13±2.56
6	20	20	9	140	0.5	0.2	0.04	0.20	50	-1	1	14.94±1.68
7	30	40	9	100	0.5	0.2	0.01	0.20	100	-1	-1	34.19±5.74
8	20	40	7	100	0.5	0.1	0.04	0.05	100	-1	1	35.02±2.68
9	30	20	7	140	0.1	1	0.01	0.20	100	-1	1	38.18±2.93
10	30	20	7	100	0.5	1	0.04	0.20	50	1	-1	38.68±7.88
11	30	40	9	140	0.1	1	0.04	0.05	50	-1	-1	32.31±2.32
12	20	20	7	100	0.1	0.2	0.01	0.05	50	-1	-1	13.61±4.04

Table 2. Experimental Conditions for Crude Oil Biodegradation Based on the Taguchi Design L_9 (3^4) and Results of Crude Oil Biodegradation at the End of the Experiments

Trial Number	pH	Temperature (°C)	NH_4Cl (g.L^{-1})	Salinity (ppt)	Percentage of Crude Oil Biodegradation (mean±SD)
1	6.00	25.00	0. 5	30.00	10.04±1.76
2	6.00	30.00	1	40.00	14.00±1.15
3	6.00	35.00	2	50.00	15.15±3.13
4	7.00	25.00	1	50.00	28.20±4.19
5	7.00	30.00	2	30.00	36.29±3.32
6	7.00	35.00	0.5	40.00	38.30±2.70
7	8.00	25.00	2	40.00	37.26±7.63
8	8.00	30.00	0. 5	50.00	34.09±3.74
9	8.00	35.00	1	30.00	61.27±5.49

4. Results

4.1. Identification of Microbial Strain

A gram negative, rod shaped, aerobic, catalase and oxidase positive bacteria, forming raised colorless colonies were isolated and signed as the KK1- strain. Differential phenotypic characteristics of the isolated strain are shown in Table 3. The 16S rDNA sequence obtained from the isolated strain was deposited in the GenBank database (accession number: KF484912) and showed 98% similarity to *Marinobacter litoralis*.

4.2. Screening of Important Factors for Crude Oil Biodegradation by KK1

The biodegradation percentage of crude oil by KK1 was found to vary from 13.54% (trial number 1) to 38.68% (trial number 9) according to the Plackett-Burman experimental design (Table 1). Our results showed that temperature (˚C), salinity (ppt), rpm, K_2HPO_4 (g.L^{-1}), NH4Cl (g.L^{-1}), inoculum size (OD600 nm) and medium volume (mL) had positive effects, whereas pH and Fe_2SO_4 (g.L^{-1}) had negative effects within the tested ranges (Table 4). The Pareto chart illustrates the order of significance of the variables affecting crude oil biodegradation (Figure 1). The order of significance as indicated by the Pareto chart was NH_4Cl (g.L^{-1}), salinity (ppt), temperature (˚C), pH, inoculum size (OD600 nm) and K_2HPO_4 (g.L^{-1}). Among the significant factors, NH_4Cl, salinity, temperature and pH, which had greater effects, were considered for the next stage in the biodegradation optimization process using the Taguchi experimental design.

4.3. Factors Affecting Biodegradation of Crude Oil and Optimal Condition

During the Taguchi experiment the highest crude oil biodegradation percentage was achieved in trial 9 and the changes in crude oil concentrations in control trials were negligible (Table 2). The ANOVA results of Signal/Noise ratio comparisons at the end of the experiment are shown in Table 5. All examined factors showed significant effects ($P < 0.05$) on crude oil biodegradation. Theoretically, the optimum degradation conditions were determined as: pH= 8, temperature = 35 ˚C, salinity = 30 ppt and NH_4Cl = 1 g.L^{-1}. The validity of the predicted optimized condition was tested by conducting experiments considering the predicted criteria. Biodegradation efficiency of 58.32±5.57% was achieved under the suggested conditions, which was significantly higher than the primary conditions (35%).

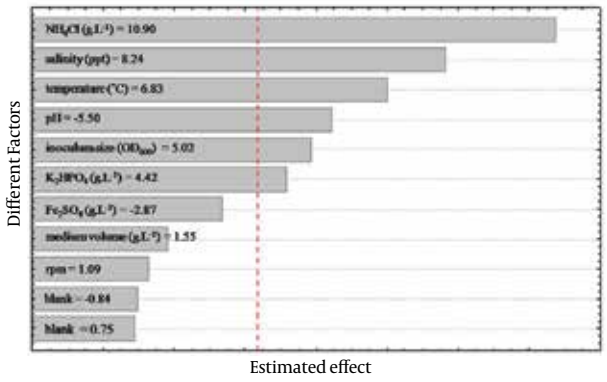

Figure 1. The Pareto Chart for Determination of Significant Factors which Influenced Crude Oil Biodegradation. Alpha is 0.05

Table 3. Differential Phenotypic Characteristics of Strain KK1, *M. litoralis* SW-45, *M. hydrocarbonoclasticus* and *M. aquaeoleiaquaeolei*

Characteristic	Strain KK1	*M. litoralis*	*M. hydrocarbonoclasticus*	*M. aquaeolei*
Motility	+	+	+	+
Urease	+	+	-	+
Nitrite reduction to N_2	-	-	+	-
Hydrolysis of gelatin	-	-	-	+
Hydrolysis of starch	-	-	-	ND
Hydrolysis of Tween-80	+	+	ND	+
Growth at/in				
pH 5.0	-	+	-	+
0 % NaCl	-	-	-	+
20% NaCl	+	-	+	+
growth at 4 ˚C	-	+	-	-
Maximum temperature for growth (˚C)	50	46	45	50
Optimum temperature for growth (˚C)	35	30-37	32	30

Table 4. The ANOVA Results and Effect Estimates of Crude Oil Biodegradation from the Results of the Plackett-Burman Design

Factor	Effect	$F_{1,23}$	Sum of Squares
Temperature (°C)	6.83	15.95	280.37
Salinity (ppt)	8.24	23.18	407.55
pH	-5.50	10.33	181.61
rpm	1.09	0.41	7.24
K2HPO4 ($g.L^{-1}$)	4.42	6.69	117.66
NH4Cl ($g.L^{-1}$)	10.90	40.57	713.18
Fe2SO4 ($g.L^{-1}$)	-2.87	2.82	49.68
Inoculum size (OD600nm)	5.02	8.60	151.20
Medium volume (mL)	1.55	0.82	14.52
Blank	0.75	0.19	3.42
Blank	-0.84	0.24	4.26
Error			210.93

Table 5. The ANOVA Results of Signal/Noise Ratio at the End of the Experiment

Factor	$F_{2,18}$	Sum of Squares
Temperature (°C)	24.97	846.180
pH	134.45	4555.274
Salinity (ppt)	13.59	460.534
NH4Cl ($g.L^{-1}$)	6.88	233.424
Error		304.923

5. Discussion

It has now been well established that the overall efficiency of oil spill bioremediation processes relies on the development of optimal conditions for enhanced oil biodegradation rates in contaminated media. A large array of factors may influence the oil biodegradation rates of which presence of microorganisms with the appropriate metabolic capabilities plays a key role during the process. By far, optimized hydrocarbon biodegradation using appropriate microorganisms is achieved by providing adequate physiochemical conditions for the microorganisms (9).

In the current study, a bacterium strain KK1 (with 98% similarity to *Marinobacter litoralis*) responsible for crude oil degradation was isolated and characterized using classical and molecular methods from surface sediments of Qeshm Island. In general, species belonging to the genus *Marinobacter* are omnipresent in the marine environment, of which *M. aquaeolei* VT8 (10), *M. algicola* DG893 (11), *M. maritimus* (12) and *M. hydrocarbonoclasticus* (13) have been demonstrated to be important hydrocarbon degraders in various marine habitats.

The appropriate use of statistical experimental design can allow the experimentalist to reduce the complexity of a problem by identifying factors which significantly affect the outcome and focusing future experiments on these factors (14). Due to the large number factors in this study, running experiments would cost time and money, so a Plackett–Burman design was chosen to screen important factors for crude oil biodegradation. The effect of nine medium components and operating conditions were studied and among them NH_4Cl ($g.L^{-1}$), salinity (ppt), temperature (°C), pH, inoculum size (OD600 nm) and K_2HPO_4 ($g.L^{-1}$) were found to be significant for crude oil biodegradation by KK1. Among the significant factors, NH_4Cl, salinity, temperature and pH, which had higher effects, were considered for the next stage of the biodegradation optimization process using the Taguchi experimental design.

In the present study, according to the Taguchi experimental design pH was found as the most effective factor in crude oil degradation (F=13.69, Figure 2), which may be because of its effects on microbial activity (possibly through altering the enzymatic activity), transport processes and the nutrient solubility (15). In the current study, degradation was maximal at pH=8, though the biodegradation process was active from pH 6 to 8. Similarly, Hambrick et al. (16) found increased rates of microbial mineralization of octadecane when pH was increased from 6.5 to 8.0. However, Leahy and Colwell (17) have argued that the most petroleum degrading bacterial species can perform degrading action at pH= 6-8 in general.

In the recent study, the best biodegradation efficiency of crude oil was achieved at the highest temperatures (35°C). Temperature is known to play a major role in controlling the nature and extent of hydrocarbons being degraded by bacteria (18). At low temperatures, the viscosity of the oil is increased, the volatilization of toxic short-chain alkanes is reduced, and its water solubility is decreased, delaying the onset of biodegradation. In contrast, higher temperatures lead to increased hydrocarbon metabolism rates, typically between 30 and 40°C. At higher temperatures, the membrane toxicity of hydrocarbons may be increased (19). In addition, salinity was also found to have a significant effect on crude oil biodegradation (F=5.53, Figure 2). Our results propose 30 ppt salinity as the optimum level for crude oil

biodegradation. It has been emphasized that, environmental salinity affects the metabolism and growth of microorganisms. Shiaris (20) found a positive correlation between salinity and rates of biodegradation of phenanthrene and naphthalene in estuarine sediments. However, some researchers reported decreased rates of hydrocarbon metabolism when salinity was increased and concluded that this may be because of the negative effects of ions on metabolic rates of bacterial cells (15). Finally, the NH_4Cl concentration had a significant effect on biodegradation percentage according to the Taguchi experimental design (F=3.97, Figure 2). It is now well established that the availability of nitrogen and phosphorus limits the microbial degradation of hydrocarbons in marine environments (9). Treating petroleum-contaminated soil with nitrogen (N) load can increase growth rate of the cell, decrease the microbial lag phase, help maintain microbial populations at high activity levels, and increase the rate of hydrocarbon degradation (21, 22). However, as found in our experiment, it has been argued that N concentration has ambiguous effects and excessive levels of N can result in deleterious/no effects (23); for example, Leys et al. (24) revealed that the high concentrations of nutrients may result in inhibited biodegradation of polycyclic aromatic hydrocarbons by Sphingomonas and Mycobacterium strains. In essence, the nutrient requirements for bacterial growth are not constant, and depend on bacterial type, carbon source metabolized and the environmental conditions.

In conclusion, the findings of this study showed that indigenous bacteria from Qeshm Island have the potential to degrade crude oil. Complete biodegradation could not be achieved within our experimental period (7 days). However, it can be speculated that increased biodegradation percentages could have been achieved if the treatment time had been prolonged. The statistical methodology, combination of the Plackett-Burman and Taguchi designs, was demonstrated to be effective in selecting statistically significant factors and finding the optimal concentration of factors for crude oil biodegradation.

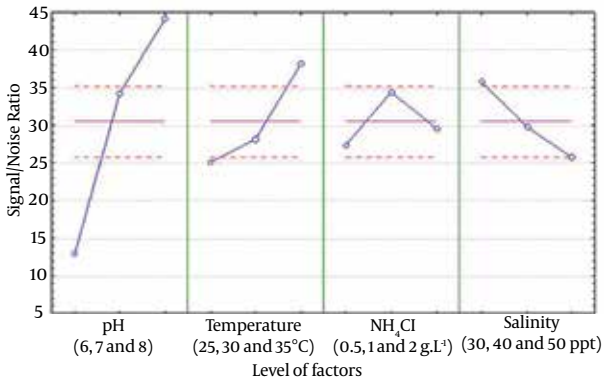

Figure 2. Level Effects of Each Factor on the Biodegradation of Crude Oil

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Authors' Contribution

Mohsen Shahriari Moghadam contributed to the statistical analysis and interpretation of the data, wrote the manuscript, and was the guarantor. Gholamhossein Ebrahimipour and Behrooz Abtahi contributed to the original idea and development of the protocol and critical revision of the manuscript. Nafsa Khazaei and Negin Karbasi collected samples, cultured and laboratory analysis.

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