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Original Article

Bioremediation of Crude Oil Using Bacterium from the Coastal Sediments of Kish Island, Iran

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Abstract

Background: Much of the environment is affected by petroleum contamination. It imposes serious health problems for humans as well as serious environmental impact. Bioremediation is an important consideration for removing environmental pollutants because, compared with other technologies, it incurrs lower costs and is environmentally compatible.

Methods: Crude oil degrading bacteria were isolated using serial dilutions of a bacterial consortium. The Taguchi experimental design L_{16} (4^5) was used to optimize the biodegradation process of crude oil by the isolated strain. This investigation applied the parameters of temperature, salinity, pH, NH_4Cl and $FeSO_4.7H_2O$. Modeling the kinetics of crude oil biodegradation included five batch cultivation experiments (2.5 ml/L to 40 ml/L) using crude oil as a single limiting substrate.

Results: *Halomonas* sp. MS1 was identified using identification tests. Maximum biodegradation efficiency was predicted to occur at pH=9, temperature=30 ˚C, salinity=2%, NH_4Cl concentration=0.4 g/L and $FeSO_4.7H_2O$=0.04 g/L. After optimization, biodagradation was significantly (P<0.05) higher (i.e. 90.65%) than it results under the original conditions. Furthermore, growth kinetics modelling of bacteria in various concentrations of crude oil showed a positive correlation between increased concentration, up to 10 ml/L and bacterial growth, but this was not evident at higher concentrations (20-40 mL/L)

Conclusion: Overall, bacteria in surface sediment samples from Kish Island have been determined as having good potential for application in oil biodegradation. Optimum amounts of the studied factors were determined successfully by applying the Taguchi experimental design and the models of Teissier and Haldane are suggested as kinetic models to describe the batch crude oil degradation behavior of MS1.

Keywords: Bioremediation, Halomonas sp, Taguchi, Growth kinetics, Iran

Introduction

Environmental pollution from hydrocarbons is a problem threatens human health as well as terrestrial and marine ecosystems. Observation clearly shows that sites near oil-related industries such as production, transportation and storage both at sea and on land are particularly susceptible to the effects of petroleum pollution (1, 2). Accordingly, several technologies have been developed and proposed to control and reduce hydrocarbon contamination in such environments (3). Among these approaches, bioremediation has received much research attention because it is environmentally friendly and economically viable; it is also

very effective in detoxification of contaminants (4-6).

Bacterial strains in marine environments are diverse and widely distributed in the ocean, and mainly involved in the process of hydrocarbon biodegradation. Among reported hydrocarbon-degrading bacteria, they mostly belong to the genera of *Pseudomonas, Acinetobacter, Nocardia, Vibrio, Achromobacter, Alcanivorax, Marinobacter, Sphingomonas and Micrococcus* (7, 8). Efficacy of the biodegradation procedure can be affected by several important variables such as accessibility of nutrients, O_2 concentration, pH value and importantly, bioavailability of the contaminants (9-11). Likewise, successful performance of the bioremediation process relies on presence of microorganisms with good ability for hydrocarbon degradation as well as appropriate environmental conditions (4, 12).

Single factor optimization presents some limitation on the optimization process. Statistical experimental designs such as the Taguchi approach are commonly applied to optimize parameters affecting the process. The Taguchi method uses orthogonal array to study a large number of variables. In this method, fewer trials are required, thereby saving time and cost (10, 13, 14). The method has been successfully adapted for process optimization of various environmental parameters (14, 15).

In addition to the optimization process, investigation of the kinetics of the biodegradation process is considered necessary to determine an appropriate model by making correlations of the specific growth rate (μ) and crude oil concentration (16). This information is particularly important for characterizing the concentration of a chemical that remains at any specific time interval and allows predictions of levels that are likely to be present at future intervals. The Persian Gulf is an important maritime traffic route and has undergone considerable exploitation by crude oil-related industries such as production, transportation and exportation. Unsurprisingly, it is now common knowledge that such activities have resulted in an adverse effect on the surrounding marine environment (17).

Thus, the aim of this study was to investigate potential hydrocarbon degradation capacity and to determine the crude oil biodegradation kinetics of bacteria associated with the surface sediment of the Persian Gulf near the Kish Island.

Material and Methods

Chemicals

All chemicals and solvents were purchased from Merck chemical company. The light crude oil was obtained from an oil company in Iran.

Sediment sampling and screening

Samples of Kish coastal surface sediment were collected from different sites to isolate crude oil degrading bacteria. Collected sediment samples were transferred to pre-sterilized bottles and kept at 4 ˚C during transportation to the laboratory. A first series of tests was done to screen and enrich bacteria that were capable of utilizing crude oil; a 5 mL amount of sediment sample was added to a 250 mL conical flask containing 50 mL mineral salt medium (MSM; 0.5 g/L K_2HPO_4, 1 g/L NH_4Cl, 0.01 g/L $FeSO_4.7H_2O$, 1000 ml filtered 40 ppt local seawater, adjusted to pH=8) with 1% crude oil as the sole source of carbon. The flask was then incubated at 30 ˚C on a rotary shaker at 140 rpm for 10 d. Oil utilization in the enriched samples was investigated by monitoring amounts of crude oil concentration and culture biomass using the appropriate method. Then 5 ml of the enriched sample was transferred to the new medium and incubated. This process was repeated 4 times in order to obtain enriched crude oil degrading cultures.

Isolation and identification of the candidate isolate

At the end of the enrichment process, using serial dilution and nutrient agar, bacterial strains were purified at 30 ˚C for 48 h. Then, bacterial colonies were picked off, and purified by successive cultures onto nutrient agar plates. Isolated strains were first cultivated in nutrient broth and incubated for 2 d at 30 ˚C and 140 rpm and pelleted by centrifugation at 8000 rpm for 15 min followed by washing in sterilized seawater. These isolated

strains of MSM containing 1% crude oil were used to determine utilization rates of crude oil. Mediums were incubated at 30 ˚C and 140 rpm. The same MSM, without any microbial inoculum was considered as the control treatment. At the end of the experiment, the entire medium for each candidate culture was used for analysis of the remaining crude oil concentrations. The most efficient crude oil degrading strain was selected from among the isolated strains and then identification was made based on biochemical, morphological and 16S rDNA gene sequencing.

DNA of the strains was extracted using the bacterial DNA extraction kit (Roche-Germany). Amplification of the 16S rDNA gene was done by PCR using the 27F (5-AGA GTT TGA TCC TGG CTC AG-3) and 1510R (5-GGT TAC CTT ACG ACT T-3) primers. Each PCR reaction contained 2 µL DNA template, 5 µL Buffer, 0.8µL dNTP, 2 µL of each primer, 0.8 µL DNA polymerase, made up to 36.4 µL with deionized sterile distilled water. DNA amplification was done under the following conditions: 94 ˚C for 5 min, 33 cycles at 94 ˚C for 30 sec, 56 ˚C for 40 sec, 72 ˚C for 45 sec and a final extension at 72 ˚C for 10 min. PCR amplification product was checked by agarose gel electrophoresis (70V, 35 min) and then sent to a commercial provider for sequencing. With BLAST (http://blast.ncbi.nl-m.nih.gov/Blast.cgi) and comparisons were made among gene sequences of the strains.

Optimization of biodegradation

Optimization of crude oil degradation was performed using the Taguchi experimental design, L_{16} (4^5). The Taguchi experimental trials and levels of experimental factors show in Table 1 and 2.

Table 1: Combinations of the five factors and four levels on crude oil biodegradation by MS1

Variables	Levels		
Salinity (%)	2	3	4
Temperature (˚C)	25	30	35
pH	7	8	9
$FeSO_4.7H_2O$ (g/L)	0.01	0.02	0.04
NH_4Cl (g/L)	0.8	1.6	2

Table 2: Experimental conditions for crude oil biodegradation based on the orthogonal design form L_{16} (4^5)

Experimental number	Salinity (%)	pH	Temperature (˚C)	$FeSO_4 7H_2O$ (g/L)	NH_4Cl (g/L)	Crude oil biodegradation %
1	1	1	1	1	1	8.61 ± 0.54
2	1	2	2	2	2	36.42 ± 18.51
3	1	3	3	3	3	54.18 ± 9.82
4	1	4	4	4	4	60.83 ± 9.46
5	2	1	2	3	4	37.81 ± 14.75
6	2	2	1	4	3	37.85 ± 7.17
7	2	3	4	1	2	54.38 ± 8.88
8	2	4	3	2	1	73.40 ± 6.96
9	3	1	3	4	2	46.51 ± 5.12
10	3	2	4	3	1	56.62 ± 9.83
11	3	3	1	2	4	41.38 ± 11.00
12	3	4	2	1	3	56.13 ± 4.37
13	4	1	4	2	3	43.16 ± 8.50
14	4	2	3	1	4	51.31 ± 7.20
15	4	3	2	4	1	56.28 ± 9.75
16	4	4	1	3	2	36.09 ± 5.34

During the optimization process (n=3), 250 mL conical flasks containing 50 mL MSM and 10 mL/L amounts of crude oil were used. Incubations were done at 140 rpm. In order to reach primary inoculum sizes, the candidate isolate was first cultured in MSM supplemented with nutrient broth, shaken at 140 rpm at 30 ˚C for 3 d. Bacteria were then centrifuged at 8000 rpm for 15 min, washed twice with sterilized sea water and then used in the designed trials. The same experimental trials without bacteria were performed as controls for checking possible abiotic loss of crude oil during the investigated intervals. Following incubation, flasks were analyzed to measure amounts of oil removed. Statistical analysis was done using Statistica software (version 8). Accordingly, calculations were made for impact of the individual variables and determinations were made for optimum conditions for operation of bacteria in oil degradation. After determination of optimal conditions, the isolated strain was inoculated into a medium containing 10 mL/L crude oil and residual oil was measured after 3 day.

Modeling the kinetics of crude oil biodegradation

Five batch cultivation experiments were done using the selected isolates with different concentrations of crude oil as a single limiting substrate for the isolated strain. To compare results, specific growth rate (μ) (h^{-1}) was calculated for each experiment and data were fitted in deterministic models as shown in Table 3. Kinetic parameters of the models (μ_{max}= maximum specific growth rate (h^{-1}), K_s= half-saturation constant (mg/L) and K_i= inhibition constant (mg/L) were estimated using non-linear regression in MATLAB (version 7.11).

Extraction of residual oil

Extraction of residual oil was performed using the method of Mishra (18) with some modification. Culture media were acidified to pH<2 and extraction was repeated two times in a seperatory funnels using 25 mL n-hexane per each extraction. These two extracts were then combined and dried at room temperature by evaporation under nitrogen gas. Then, the amount of residual crude oil was determined gravimetrically.

Table 3: Example of kinetic models for substrate inhibition

Source	Equations
Haldane (Andrews, 1968)	$\mu = \frac{\mu_{max}}{(1+\frac{K_S}{S})(1+\frac{S}{K_{i,S}})}$
Aiba et al. (1968)	$\mu = \mu_{max}\frac{S}{S+K_S}e^{\frac{-S}{K_{i,S}}}$
Tessier (Edwards, 1970)	$\mu = \mu_{max}(e^{\frac{-S}{K_{i,S}}} - e^{\frac{-S}{K_S}})$

μ = specific growth rate (h^{-1}), μ_{max} = maximum specific growth rate (h^{-1}), K_s = half-saturation constant (mg/L), S= substrate concentration (mg/L), K_i= inhibition constant (mg/L)

Estimation of biomass concentration

Bacterial biomass was determined using the dry weight method, as described (19).

Results

Isolation and identification of the microbial strain

Sediment from Kish Island in the Persian Gulf was screened to assess the ability of its associated micro biota to utilize crude oil. Of all the tested cultures, four of the isolates exhibited crude oil degrading behavior. Based on these results, MS1 showed the highest efficiency of crude oil degradation and was therefore selected for further experiments. Morphological and biochemical characteristics of MS1 are shown in Table 4. Analysis of nearly full-length sequencing of 16S rDNA gene revealed the higher similarity of the isolate with *Halomonas* sp. DNA sequence was deposited in Gen Bank under accession numbers KF452283.

Table 4: Morphological characteristics and biochemical tests of MS1 strain

Characteristic	*Halomonas* sp.
Morphology	Short rod
pigmentation	Cream
Gram	-
Anaerobic	+
Oxidase	+
Catalase	+
NO_3 reduction	+
Salt range(% w/v)	0.5-25
pH range	5-12
Temperature range (˚C)	4-45
Growth on	
Glucose	+
Citrate	+
Glycerol	+
Acetat	+
O/F	O
Hdrolysis of	
Starch	-
Gelatine	-

Biodegradation optimization process

To determine the optimal biodegradation condition of *Halomonas* sp. MS1 the Taguchi experimental design L_{16} (4^5) was applied for 16 experiments (Table 2). As can be seen in Table 2, maximum (73±6.96%) and minimum (9±0.54%) percentages of crude oil removal were observed on trials 8 and 1, respectively. Statistical analysis (ANOVA) of S/N ratio (Fig. 1) indicated that factors of temperature, pH and salinity each had a significant effect (P<0.05) on biodegradation percentage (Table 5). Theoretically optimized levels for the considered factors were predicted as follows: salinity=2%, pH=9, temperature=30 ˚C, $FeSO_4.7H_2O$= 0.04 g/L and NH_4Cl=0.4 g/L. interestingly, adjusting the crude oil degrading experiment with the above obtained optimized parameters, resulted in increased oil removal efficiency to 90.65% in the adjusted experiment, in 3 days.

Kinetic models and parameters estimation

Results of the biodegradation and cell biomass curve at different initial crude oil concentrations are shown in Fig. 2 & 3.

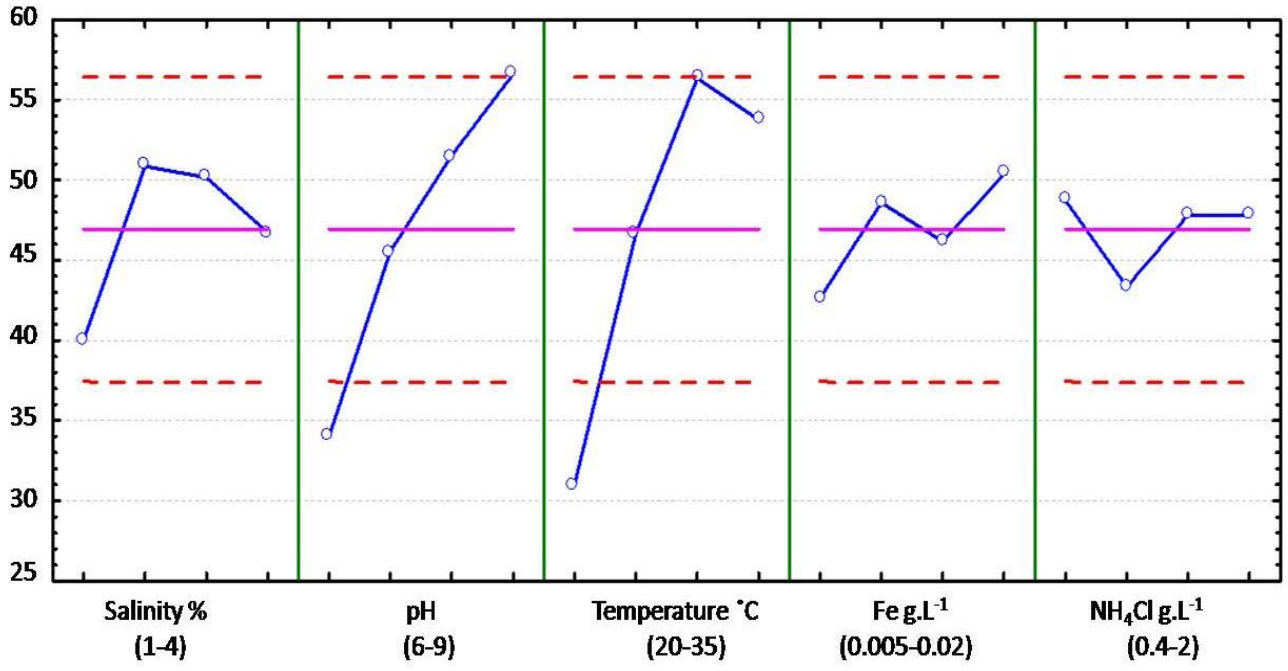

Fig. 1: Responding diagrams for each controlling factor. The S/N ratios of five factor demonstrated optimal combination of Salinity 2%, pH 9, Temperature 30 ˚C, **$FeSO_4$ $7H_2O$** 0.04g/L, **NH_4Cl** 0.4g/L

Table 5: ANOVA for oil degradation % in the L_{16} (4^5) orthogonal array experiment

Factor	SS	DOF	F	*P*-value
Salinity	883.076	3	3.28	0.03
pH	3399.379	3	12.62	0.00
Temperature	4669.017	3	17.33	0.00
$FeSO_4$ $7H_2O$	403.961	3	1.50	0.23
NH4Cl	212.793	3	0.79	0.51

SS=sum of squares of deviation, DOF = degree of freedom, F= a ratio of the mean of the squared deviation to the mean of squared error

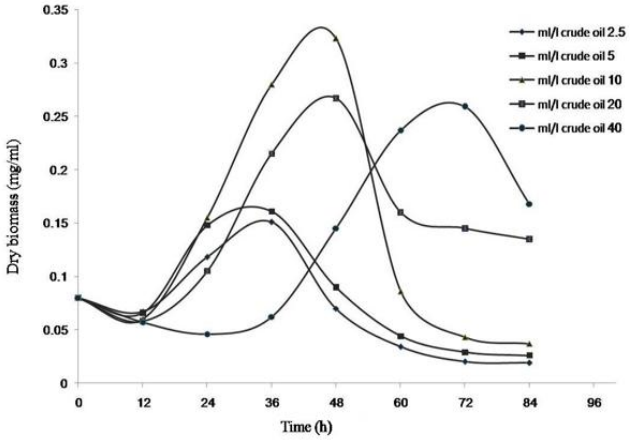

Fig. 2: The cell biomass curve at different initial crude oil concentrations

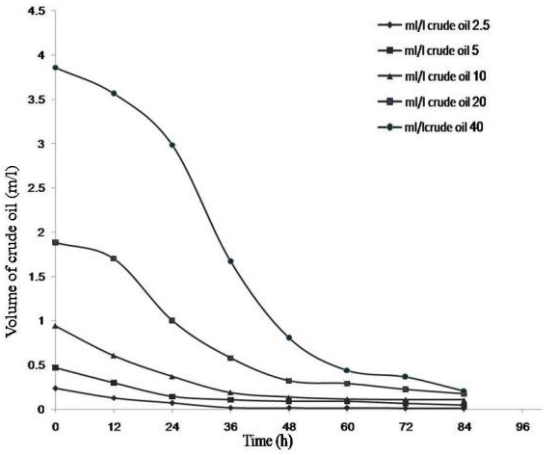

Fig. 3: Biodegradation curve at different initial crude oil concentrations

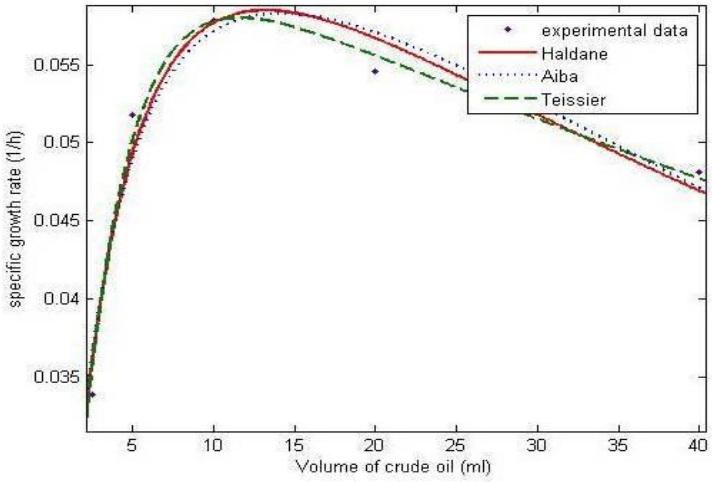

Fig. 4: Specific growth rates of MS1 strain on different initial crude oil concentrations with fitting of various kinetic models

To measure the various parameters of the model, the effect of different concentrations of crude oil on the biomass concentration was studied for various test periods. The value of specific growth rates (μ) of biomass at the exponential growth phase were calculated for different initial crude oil concentrations based on the slopes of time-series plots of lnX. The experimental values of μ were plotted against initial concentrations of crude oil in Fig. 4.

This Figure shows a typical trend that value of μ tended to increase as the crude oil concentration was increased and reached a peak and then declined. In this figure, the value of μ_{max} was equal to 0.0578 h^{-1} and achieved at the initial crude oil concentration of 10 ml/L. The decline trend of the plot beyond 10 ml/L, indicates that crude oil is an inhibitory type substrate and its inhibition effect is predominant above the level of 10 ml/L. Three inhibition kinetic models of Haldane, Aiba and Tessier were fitted to the experimental data by plotting the specific growth rate as shown in Fig. 3. Based on the highest correlation coefficient (r^2) and the least root mean square error (RMSE) the model with best fit was selected. The model parameters of (μ_{max}, K_S and K_i) were estimated by the non-linear regression in MATLAM version 7.11. These results, together with those of r^2 and RMSE are given in Table 6. These results revealed that among the three models Tessier and Haldane had r^2 greater than 0.90 and the least RMSE, indicating very good fit to the batch experimental data.

Table 6: Parameter estimation and regression statistics for various substrate-inhibition models

Model	*μ_{max} (h^{-1})*	*K_S (mg/L)*	*K_i (mg/L)*	*r^2*	*RMSE*
Haldane	*0.091*	*3.78*	*50*	*0.95*	*0.0028*
Aiba	*0.086*	*3.282*	*100*	*0.87*	*0.0032*
Tessier	*0.064*	*0.331*	*142.85*	*0.96*	*0.0017*

Discussion

It is important to isolate bacteria with a high capacity to degrade hydrocarbon contamination in order to perform bioremediation in contaminated areas (20). In this current study, MS1 with high capacity for crude oil degradation was isolated from the surface sediment of Kish Island and identified as *Halomonas* sp.

The genus *Halomonas*, initially suggested by Vreeland et al. (21) and includes more than 20 species, are among the larger moderate halophilic bacterial groups with biodegradation potential of hydrocarbon pollutants (22, 23). In this study, the optimum culture condition for crude oil biodegradation by MS1 was determined by the Taguchi method and indicated that using statistical experimental design produced appropriate information for optimization of the parameters using a minimum number of experiments (13, 24). Optimum degradation conditions were determined as follows; salinity=2%, pH=9, temperature=30 ˚C, $FeSO_4.7H_2O$=0.04 g/L and NH_4Cl=0.4 g/L. Under these conditions, the MS1 strain reached a maximum crude oil degradation of approximately 90% within 3 days. According to the Taguchi experimental design, temperature was determined as an effective factor in crude oil biodegradation.

The best biodegradation efficiency was achieved at the temperature of 30 ˚C. Temperature affects the physical and chemical properties of oil and thus plays an important role in microbial biodegradation rate and composition of the microbial community (10). Aghamiri et al. (10) and Lin et al. (25) report optimum temperature for biodegradation of crude oil and naphthalene as 30 ˚C. Petroleum generally has higher degradation at temperatures in the range 30-40 ˚C in a soil environment; 20-30 ˚C in some freshwater environments and 15-20 ˚C in marine environments (26). Although biodegradation of hydrocarbons can happen in a wide range of temperatures, biodegradation rate generally decreases with decreasing temperature (26). So, in this study, the lowest rate of biodegradation was observed at the lowest temperature.

This is because crude oil has more viscosity at lower temperature while there is increased solubility of toxic short-chain alkanesare due to a decreasing rate of evaporation (26, 27). pH of the medium influencing diversity of microbial medium, enzyme activity and nutrient solubility (25, 27, 28). The highest oil biodegradation in the current study, was obtain at pH 9 but biodegradation was active at pH 6-9. Lin et al. (25) reported in pH 6 to 7.5 more than 90% of naphthalene was consumed by *Bacillus fusiformis*. Generally, in slightly alkaline conditions bacteria are better able to degrade hydrocarbons (29). Salinity was also observed as a parameter that exhibited a significant effect under the Taguchi design.

The result proposed 2% salinity as the optimum level for crude oil biodegradation. While some researchers reported a negative correlation between the rate of hydrocarbon metabolism with salinity (e.g. that an increasing amount of ions has a negative effect on the metabolism rate of bacteria) (25, 30), others such as Shiaris (31) reported a direct correlation between the rate of biodegradation by increasing salinity. Among the examined factors, $FeSO_4$ and NH_4Cl concentration had no considerable effect on optimization, indicating that biodegradation with small $FeSO_4$ and NH_4Cl concentration may be applicable. Substrate consumption by organisms with special enzymes is the main hypothesis of biodegradation kinetics. Therefore, the rate of substrate degradation depends on concentration of the substrate and on the substrate-degrading organisms (32). The batch experiments at various initial crude oil ranging from 2.5 to 40 ml/L were carried out using a MS1 strain microbial culture. Fig. 3 shows that, until 10 ml/L crude oil concentrations, specific growth rate of the culture increased. Specific growth rate showed a decrease at concentrations higher than 10 ml/L. The maximum specific growth rate was 0.0578 h^{-1} at an initial crude oil concentration of 10 ml/L. This result means that there was a small lag phase. As the initial crude oil concentration increased to 20 and 40 ml/L the lag phase was raised. In fact, tests showed that the lag phase was increased when the initial crude oil concentration was higher due to the slower cell adaptation. This shows that toxicity of crude oil inhibited MS1 strain at higher concentrations (19, 33).

Various types of kinetic substrate consumption and inhibition models have been used to explain the dynamics of microbial growth on different compounds, for example Phenol, Toluene, Benzene and p-cresol (16, 32, 34). To predict kinetics of microbial activity at substrate adaptation conditions, an effort was made to fit the kinetic rate data to proper kinetic models. In these models, value of K_s the affinity of microbes to substrate and K_i values showed culture medium sensitivity to inhibition of substrate. A larger K_i value indicates less inhibitory effect of the substrate on the medium (16, 35). Table 6 shows that best fit were achieved using Tessier and Haldane respectively, which explains the degradation of crude oil by MS1.

Conclusion

We isolated and characterized from surface sediments of the Kish Island a *Halomonas* sp. Strain MS1 after enrichment on crude oil. MS1 has significant ability to utilize crude oil as its sole carbon and energy sources. Optimum amounts of the studied factors were determined successfully by applying the Taguchi experimental design and the models of Teissier and Haldane are suggested as kinetic models to describe the batch crude oil degradation behavior of MS1.Obviously, the isolated strain may be seen as an important tool on bioremediation of hydrocarbon-contaminated sites.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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