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Original Research Article

Diversity of Native Hydrocarbon Degrading Bacterial Strains and Their Potential in Bioremediation of Soil Polluted by Crude Oil in Khuzestan Province (A response Surface Methodology Approach)

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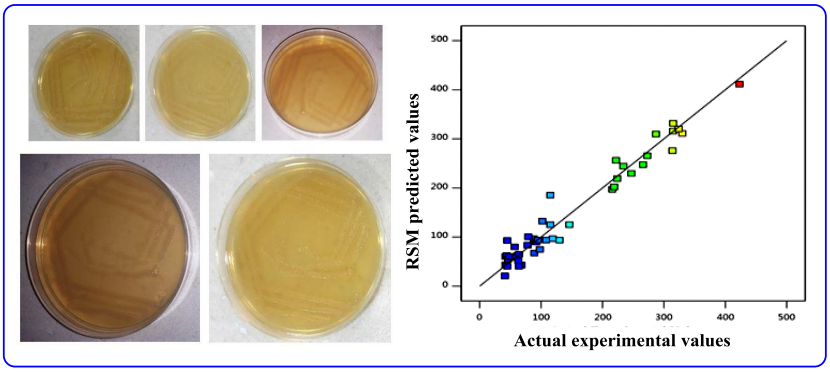
ABSTRACT

Many bioremediation strategies have been developed to help improve soil clearance from oil and its derivatives. The use of native bacteria to decompose crude oil-contaminated soils has been studied by many researchers. Along with this line of research, in the present study, a consortium of bacteria isolated from three different soil types in the Khuzestan region was used after identification. Finally, the optimal conditions for bioremediation of each soil type were determined. The results showed that the isolated strains were five unique strains, including Cupriavidus metal lidurans, Bacillus pacificus, Lysinibacillus fusiformis, Brevibacillus borstelensis, and Ralstonia metallidurans. These had good growth potential in contaminated soils and could effectively reduce TPH. Among the factors examined, the amount of moisture and nitrogen were two essential factors that reduce the amount of TPH. In optimal conditions, the moisture percentage was 135, 147, and 142, and the input nitrogen was 512 ppm, 513 ppm, and 617 ppm, for three types of soil, in Andimeshk, Ahvaz, and Abadan, respectively. NH_4NO_3, as the best source of nitrogen, had the best performance. In confirmatory experiments with 2.5% crude oil in optimal conditions, the rate of TPH reduction after 56 days was 48.4%, 53.4% and 56.4% for Abadan, Ahvaz, and Andimeshk soils, respectively. This study confirms the efficiency of native bacteria isolated from the soils of Khuzestan province for biodegradation of crude oil and introduces Bacillus pacificus bacteria as a new species that can bioremediation.

GRAPHICAL ABSTRACT



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Introduction

Crude oil is a complex mixture of hydrocarbons and other organic compounds that can cause serious environmental problems if it leaks [4]. Given the high absorption of crude oil, its accumulation in soil causes changes in the soil's physical, chemical, and microbial properties [5]. In this respect, one way to improve soil is the use of bioremediation by native microorganisms that reduce hydrocarbons [5-10]. Microorganisms can decompose a wide range of petroleum compounds, and they are abundant in these environments. Understanding the factors affecting the amount of biodegradation is very important to determine the primary strategies for designing biological reclamation systems [37, 40].

Experimental designs are measures taken by modeling and optimizing reaction variables using statistical methods to increase production efficiency without increasing its price [18, 26]. In traditional methods, only one factor is considered variable and the other factors are fixed, which is called one variable at a time technique. In this method, the interaction effects between the variables are not studied and the full effects of the process cannot be displayed. It also requires a significant amount of testing [9,32]. Response Surface methodology has been developed to study optimization to overcome this problem. It actually includes a set of mathematical and statistical techniques useful for modeling and analyzing issues in which a reaction is affected by several independent variables, and its goal is to optimize the response and is an ideal way to optimize a product or process [26,38].

Several options for treating oil-contaminated sites, including physical, chemical, and biological methods. Physical and chemical methods are relatively expensive because the contaminant extracted or the soil burned still has to be treated or disposedf. Over the past decade, bioremediation of oil-contaminated soils has been the subject of many environmental studies. Many bioremediation strategies have been developed to help improve soil clearance from oil and its derivatives [22]. The use of native bacteria to decompose crude oil-contaminated soils has been studied by many researchers [2, 32,36,, 41]. However, creating flooding conditions, using various sources of input nitrogen in different concentrations, and using the RSM method are some of the innovations of this work. This study used a consortium of bacteria isolated from three different soil types in the Khuzestan region after identification. Finally, the optimal conditions for bioremediation of each soil type were determined.

Materials and Methods

Study area and Soil Sampling

The composite sampling method was used in the present study. To this aim, 250 grams of soil were collected from depths up to 10 cm from each contaminated site. Then, all samples were mixed and used for further work [8,12]. Sampling was performed in oil transfer centers in three cities of Khuzestan province, namely Abadan, Ahvaz, and Andimeshk, as shown in Figure 1.

Figure 1: Map of Khuzestan province and sampling locations

Preparation of Culture Medium

All chemicals used in this study were high purity and obtained from reputable commercial sources (Sigma-Aldrich). To stimulate the growth of petrophilic bacteria, the Bushnell House culture medium was constructed by combining 1 gr KH_2PO_4, 1 gr K_2HPO_4, 1 gr NH_4NO_3, 0.2 gr $MgSO_4.7H_2O$, 0.02 gr $CaCl_2$, 0.05 gr $FeCl_3$ and 1% by volume of crude oil as the only source of carbon and energy, in one liter of distilled water. Then pH was set on seven. After that, 100 ml of

the culture medium was added to a 250 ml Erlenmeyer flask and autoclaved at 121 °C for 15 minutes at 20 PSI [28].

Isolation of Oil Degrading Bacteria

In the primary inoculation, 10 g of each contaminated soil was added to the culture medium and incubated in an incubator shaker for one week at 37 °C and rotation rate of 100 rpm. After one week, three Erlenmeyer flasks were reconstituted in completely sterile conditions, and each flask was diluted with 5 CC of the supernatant from old flasks. This was continued for 8 weeks to ensure that the oil degrading bacteria have grown up [2]**.** After the growth of petrophilic bacteria on petroleum specific medium, 4 colonies from each sample (12 colonies from all three samples) were selected. These were transferred to the Trypticase Soy Agar medium in completely sterile conditions for single-colonization and other identification steps. Purified colonies grown on TSA medium were transferred to nutrient broth medium for DNA extraction and stocking [3,4].

Then, all colonies were classified based on morphological characteristics such as gram-negative and gram-positive, size and shape according to Bergey's Manual of Systematic Bacteriology [6]. Then biochemical tests including Catalase, Oxidase, Indole, and Citrate were performed on the colonies [31].

Identification of Oil Degrading Bacteria

Polymerase Chain Reaction (PCR) by Universal 27F and 1492R primers of the genomic DNA was used to identify bacteria by sequencing of the 16s rRNA gene that was present in all bacteria and had a constant common region among all bacterial species [21,27].

DNA extracted by the boiling method was used according to the following instruction. (1) Microbial stocks were cultured on TSA medium and incubated for 24 h at 37 °C. (2) Pure mono-isolates were dissolved in a tube containing neutrino broth medium and incubated for 24 h. (3) In a completely sterile condition, one ml of the resulting suspension was distributed in a 1.5 mL micro tube. (4) Centrifuged at 6000 rpm for 15 min. (5) Boiled at boiling water at 100 °C for 10 min. (6) Kept at -20 °C for 10 min. (7) Put in boiling water for ten minutes again, then centrifuged at 12,000 rpm for 5 min. (8) Supernatant was discarded. (9) 1 mL ethyl alcohol 95% was added to the resulting precipitate and centrifuged again at 12,000 rpm for 10 min. (10) The alcohol was discarded, and the microtube was stored at laboratory temperature until complete drying. (11) 100 µL of sterile distilled water was added and centrifuged for 10 min at 12,000 rpm. (12) After centrifugation, the supernatant containing pure DNA was transferred to a 0.2 mL microtube and stored at -20 °C [11,3]. After preliminary identification of bacteria based on morphological characteristics, identification was performed by sequencing the gene encoding 16s rRNA. For this purpose, the bacterial genome was extracted, and then the 16s rRNA region was reproduced using Polymerase Chain Reaction (PCR) [13]. Sequences were analyzed by MEGA 6 software, and the final identification was done using the Blast program at NCBI. Then the sequences were selected and recorded based on maximum sequence alignment with reference bacteria. To isolate the sequences obtained in the corresponding branch, the phylogenetic tree was drawn using the (http://www.ebi.ac.uk) database.

Optimization of Crude Oil Biodegradation Using RSM

To obtain the optimization conditions for oil degradation, the response surface methodology by optimal design, a flexible design structure to accommodate custom models, categorical factors, and irregular (constrained) regions that runs are determined by a selection criterion Chosen during the build and was used to study influential factors in decomposing oil, including Bacterial consortium type, Moisture, Nitrogen input, Nitrogen source, and soil salinity. The TPH degradation was considered the response [36,32]. The levels of these factors are revealed in Table 1. Design experiments were carried out using Design-Expert program 11. After entering the factors and their levels, 46 runs had to be tested.

Table 1: Levels of Factors for the Experimental Design

Factor	Symbols	Type	Levels		
Moisture %	A	Numeric	50	100	200
Nitrogen (ppm)	B	Numeric	0	500	1000
Soil type	C	Categorical	Salty (EC>16)	Half salty (EC: 4-16)	Not salty (EC: 0-4)
Bacteria source	D	Categorical	AB*	AH*	AN*
Nitrogen source	E	Categorical	NH_4NO_3	$(NH_4)_2 SO_4$	NH_4Cl

AB: A strain isolated from the soil of Abadan
AH: A strait isolated from the soil of Ahvaz
AN: A strait isolated from the soil of Andimeshk

Result and Discussion

Identified Bacteria from Soil Contaminated by Crude Oil

In this study, 11 bacterial strains were isolated from three different soil types. Table 2 presents the colony characteristics of isolated strains from three different soils. Based on the 16S rRNA gene sequences and Neighbor-joining phylogenetic analysis (Figure 2), the isolated strains were five unique strains, including Cupriavidus metallidurans, Lysinibacillus fusiformis, Brevibacillus borstelensis, Ralstonia metallidurans, and Bacillus pacificus. The pure culture of bacteria is demonstrated in Figure 3.

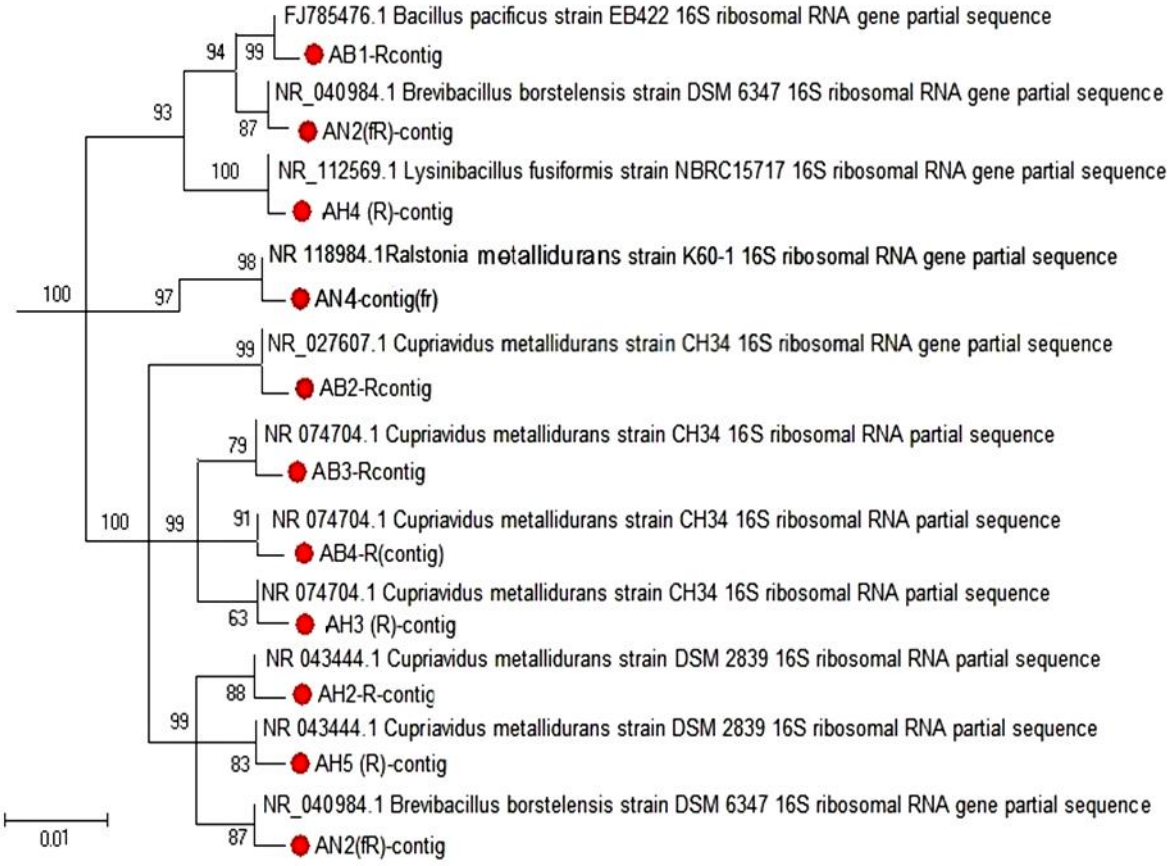

Figure 2: Phylogenetic tree of 16s rRNA sequences

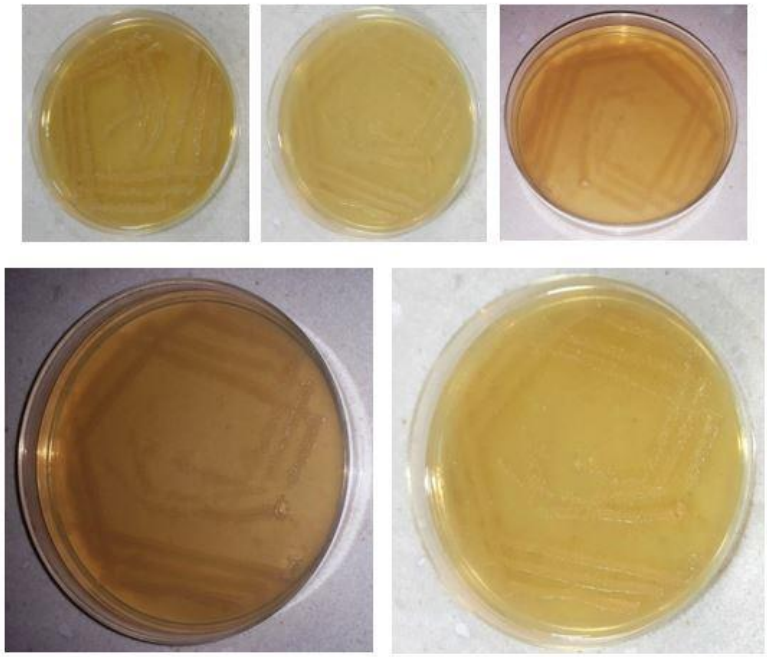

Figure 3: Pure culture of bacteria isolated from soils of Khuzestan province

Table 2: Colony Characteristics of Isolated strains from three different soils

Soil Type	Colony Name	Strain Name	Size	Shape	Gram Test	Motility	Catalase	Oxidase	Indole	Citrate
AB	AB1	Bacillus pacificus	coarse	rod	+	-	+	+	-	-
	AB2	Cupriavidus metallidurans	tiny	rod	-	+	-	+	-	+
	AB3	Cupriavidus metallidurans	tiny	rod	-	+	-	+	-	-
	AB4	Cupriavidus metallidurans	tiny	rod	-	+	-	+	-	-
AH	AH2	Cupriavidus metallidurans	tiny	rod	-	+	-	+	-	-
	AH3	Cupriavidus metallidurans	tiny	rod	-	+	-	+	-	-
	AH4	Lysinibacillus fusiformis	coarse	rod	+	+	+	+	-	-
	AH5	Cupriavidus metallidurans	tiny	rod	-	+	-	+	-	-
AN	AN2	Brevibacillus borstelensis	coarse	rod	+	-	+	+	-	-
	AN3	Cupriavidus metallidurans	tiny	rod	-	+	-	+	-	-
	AN4	Ralstonia metallidurans	tiny	rod	-	+	-	+	-	-

Cupriavidus metallidurans

It is a gram-negative and rod-shaped bacterium that can break down resistant organic matter. In one study, a strain of Cupriavidus was used to decompose toxins such as chlorpyrifos and TCP (chlorpyrifos and 3, 5, 6-trichloro-2-pyridinol). Their results showed that the bacterium could degrade chlorpyrifos and TCP 94% and 100% in soil, respectively, compared to soil without the bacterium, which had only degradation rates of 28.2% and 19%, respectively [24]. In another study, Cupriavidus was investigated as a bacterium capable of cadmium tolerance, and results indicated that it can convert soluble cadmium to insoluble form ($CdCl_2$) [35].

These and other studies, revealed that Cupriavidus, as a strain isolated in this study, can be involved in the decomposition of crude oil, which is of paramount importance [33,7].

Lysinibacillus fusiformis

It is a gram-positive, motile, rod-shaped bacterium that can tolerate soil boron. It can also grow in crude oil with 5% by volume of salt. It was first discovered by Ahmed in 2007 [1]. It is a potential organism for producing many commercially viable products through

biotransformation. And that is used in the production of critical industrial materials such as wax, nylon, plastic resin, lubricants, and cosmetics, as additives in painting and coating, and as a precursor to lactones [20]. The study of the biodegradability of crude oil by this bacterium was tested in a study in 2012. The results showed that this bacterium grows well in the environment containing crude oil and uses alkanes and aromatic hydrocarbons as a carbon source [29].

Brevibacillus Borstelensi

Another bacteria identified in this study is Brevibacillus borstelensis. This bacterium is a gram-positive, aerobic, rod-shaped bacterium that forms the endospore of the genus Brevibacillus. It is a thermophilic strain that can degrade and use polyethylene as the primary source of carbon. This strain can reduce the amount of polyethylene by 30% at a temperature of 50 °C for 30 days and can even destroy non-degradable plastics such as polyethylene under suitable conditions [14]. A study has also used the bacterium as a native bacterium isolated from soil contaminated with crude oil for soil bioremediation. That study showed that the combined use of native crude oil-degrading bacteria with nutrients can effectively regenerate crude oil-contaminated soil on a large scale [30]. This study and other research in this field show that Brevibacillus borstelensis have the potential to be used as bacteria that can biodegrade soil and decompose petroleum products, and show that native bacteria isolated in Our study also has this capability and confirms the results obtained in this study [15,19].

Ralstonia Metallidurans

This bacterium is a gram-negative, aerobic, chemolytotrophic bacterium that does not form spores. However, it is well adapted to environments contaminated with high levels of heavy metals. The compatibility of this bacterium is mainly due to the presence of two large plasmids in it, which are highly resistant to the micromolar amounts of heavy metal ions such as cadmium, cobalt, zinc, thallium copper, lead, nickel, mercury, and chromate. It can also use different substrates as a carbon source, and its optimum growth temperature is 30 °C [25]. Due to its ability to resist toxic metals, this bacterium is used for biodegradation in contaminated areas. In addition, new findings show that the lead-binding protein isolated from this bacterium plays a crucial role in preventing lead poisoning [4]. This bacterium has been used in various biodegradation studies [34,1].

Bacillus Pacificus

It is a gram-positive, optional, anaerobic, rod-shaped bacterium that is 1.2-1.6 μm wide and 3-4 μm long. Its colony is white, circular, opaque, and 2-3 mm in diameter. This bacterium is catalase and oxidase positive. It grows at a temperature of 15-45 °C, which is optimally 30 °C, and also grows in soil with 9% by volume of salt. This bacterium is one of the 9 new bacteria known in Bacillus cereus group is isolated independently in soil or seabed sediments [23].

Due to the novelty of this strain, no studies have been done on the degradability of petroleum products by this species, but considering that this strain is a member of the Bacillus family and because this bacterium has a high ability to survive in harsh and salty conditions. Potentially, according to the results of this study, it can be considered a new species in the decomposition of crude oil. However, more specific research is still needed.

Appropriateness of Process Models and Statistical Analysis

Response surface methodology (RSM) is a powerful optimization tool used to estimate the relationship between the experimental and predicted results. In this work, optimization was done using Design-Expert Version 11 software. Five important variables were optimized by optimal design. Overall, 46 experiments were done based on the variables and levels stated in Table 1. The experiments are shown in Table 3. Results of RSM depicted that, there is a relationship between response and variables. The final equation for coded factors is as follows (Equation 1). Positive and negative values respectively indicate synergistic effect and antagonistic effect on the response [17].

$$Y=+48.42 -105.3A -23.72B -9.5C -3.59D-12.09E+21.36AB+13.31AE +3.55 CE -11.83DE+ 114.94A^2 \quad (1)$$

Where Y is TPH concentration, A is Moisture, B is Nitrogen concentration, C is soil type, D is bacteria type and E is Nitrogen source type. As a result, the Quadratic model was suggested.

Table 3: Different run conditions and the percentage of TPH removal

Run	Moisture %	Nitrogen amount (ppm)	Soil type	Bacterial source	Nitrogen Source	TPH removal %
1	100	500	Salty	AB*	$(NH_4)_2 SO_4$	79
2	200	0	Half salty	AB	NH_4NO_3	85
3	50	500	Salty	AB	NH_4Cl	66
4	50	0	Not salty	AB	$(NH_4)_2 SO_4$	51
5	100	500	Half salty	AH*	$(NH_4)_2 SO_4$	68
6	200	1000	Not salty	AN*	NH_4NO_3	82
7	200	0	Half salty	AN	$(NH_4)_2 SO_4$	82
8	50	1000	Salty	AH	$(NH_4)_2 SO_4$	41
9	50	0	Half salty	AN	NH_4NO_3	37
10	100	1000	Half salty	AH	NH_4Cl	71
11	100	0	Not salty	AH	$(NH_4)_2 SO_4$	78
12	200	1000	Salty	AH	$(NH_4)_2 SO_4$	88
13	200	0	Salty	AB	$(NH_4)_2 SO_4$	91
14	50	1000	Salty	AB	NH_4NO_3	67
15	200	0	Not salty	AH	NH_4NO_3	80
16	100	500	Not salty	AB	NH_4NO_3	81
17	100	500	Not salty	AB	NH_4NO_3	80
18	50	0	Salty	AB	NH_4NO_3	23
19	100	1000	Not salty	AN	$(NH_4)_2 SO_4$	82
20	100	500	Salty	AB	$(NH_4)_2 SO_4$	76
21	100	500	Salty	AH	NH_4NO_3	74
22	200	1000	Half salty	AB	$(NH_4)_2 SO_4$	85
23	50	500	Half salty	AB	NH4NO3	26
24	100	500	Half salty	AH	$(NH_4)_2 SO_4$	79
25	50	1000	Half salty	AN	NH4NO3	18
26	200	500	Salty	AB	NH_4Cl	88
27	50	0	Salty	AH	$(NH_4)_2 SO_4$	52
28	200	0	Half salty	AH	NH_4Cl	90
29	100	0	Not salty	AB	NH_4Cl	83
30	50	0	Half salty	AH	NH_4NO_3	31
31	200	1000	Not salty	AH	NH_4NO_3	86
32	100	500	Salty	AH	NH_4NO_3	81
33	200	0	Salty	AN	NH_4NO_3	91
34	100	1000	Salty	AN	NH_4Cl	76
35	50	0	Half salty	AN	NH_4Cl	40
36	50	500	Not salty	AH	NH_4Cl	39
37	200	1000	Salty	AB	NH_4NO_3	88
38	200	500	Not salty	AB	$(NH_4)_2 SO_4$	87
39	50	1000	Not salty	AB	NH_4Cl	48
40	50	500	Salty	AN	$(NH_4)_2 SO_4$	38
41	200	1000	Not salty	AN	NH_4Cl	85
42	50	1000	Half salty	AB	$(NH_4)_2 SO_4$	24
43	50	0	Not salty	AN	NH_4Cl	42
44	200	1000	Half salty	AN	NH_4NO_3	86
45	200	1000	Not salty	AN	NH_4NO_3	86
46	100	0	Salty	AH	NH_4Cl	87

*AB: A strain isolated from the soil of Abadan

*AH: A strait isolated from the soil of Ahvaz

*AN: A strait isolated from the soil of Andimeshk

Analysis of variance (ANOVA) was used to check whether the model parameters were significant or not, and the details are given in Table 4. For a model to be used, it must be significant, and the lack of fit must be insignificant. In this study, the F-value was 34.13, implying that the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise, and P-values less than 0.05 indicate that model terms are significant. In this case A, B, C, E, AB, DE, A^2 are significant model terms. Values greater than 0.05 indicate the model terms are not significant. The lack of fit F-value was 3.28, which means the lack of fit was not significant relative to the pure error [16,32].

Table 4: ANOVA Analysis of the Quadratic Model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	4.800E+05	20	24000.70	34.13	< 0.0001	Significant
A-Moisture	2.288E+05	1	2.288E+05	325.41	< 0.0001	Significant
B-Nitrogen	16823.34	1	16823.34	23.92	< 0.0001	Significant
C-Soil type	11997.91	2	5998.95	8.53	0.0015	Significant
D-Bacteri type	861.63	2	430.81	0.6127	0.5498	Not significant
E-Nitrogen Sorce	4777.48	2	2388.74	3.40	0.0495	Significant
AB	9850.11	1	9850.11	14.01	0.0010	Significant
AE	3488.36	2	1744.18	2.48	0.1041	Significant
CE	6396.79	4	1599.20	2.27	0.0896	Not significant
DE	11234.92	4	2808.73	3.99	0.0122	Significant
A^2	79418.94	1	79418.94	112.94	< 0.0001	Significant
Lack of Fit	16335.51	20	816.78	3.28	0.0957	Not significant
Residual	17579.51	25	703.18			
Lack of Fit	16335.51	20	816.78	3.28	0.0957	Not significant
Pure Error	1244.00	5	248.80			
Core Total	4.976E+05	45				
R2 = 0.9647	Adjusted R^2=0.9364	Predicted R^2= 0.8784		Adeq Precision=21.7408		

Figure 4 shows the ratio of predicted data to actual data to reduce TPH. As can be seen, the actual values obtained were constantly consistent with the values predicted by the response surface method, and the value of R^2 increased. The correlation coefficients also show that the selected model is well coordinated with the data. The model was analyzed in terms of R^2 and adjusted R^2, where both values should be close to 1 for an appropriate correlation between the experimental and predicted values [18, 36].

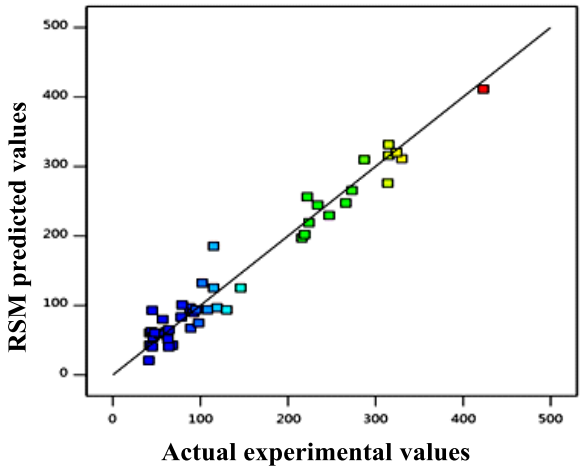

Figure 4: Actual Values Confronting to RSM Predicted Values for TPH Reduction

The Effect of Independent Variables on Response

To make the effect of the independent variables on the answer evident, it is beneficial to provide three-dimensional and contour diagrams. By

examining these graphical forms, we can examine the effect of the independent variables on the response at different points. Thus by finding the intersection point between the variables, we can record the exact value of the measured feature called the response. To illustrate these diagrams, two independent variables and one dependent variable were depicted while the other independent variables were kept constant at their central value [32,28]. As shown in Figure 5, the amount of TPH decreases with an increase in moisture content. This increase remains almost constant after 140% moisture and shows that after that amount of moisture, an increase in moisture is energy loss. On the other hand, by increasing the amount of input nitrogen, the amount of TPH decreases. The optimal amount of it is about 600 (mg/kg), considering the moisture percentage. Using software, the optimal values for each soil type were also suggested, as shown in Table 5.

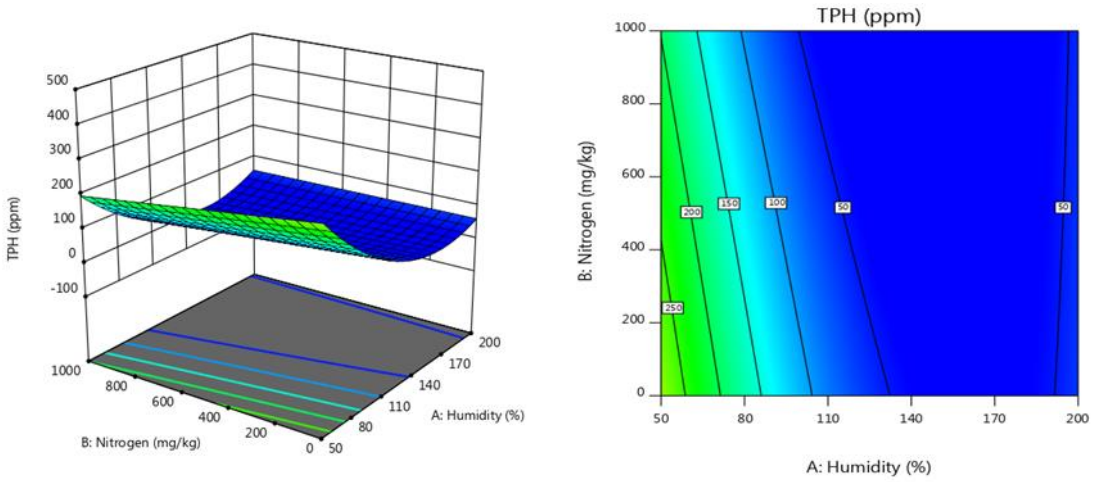

Figure 5: Interactive Effect of Moisture and Nitrogen Amount on TPH Degradation Rate

Table 5: Optimal conditions for each type of soil

	Soil type	Moisture %	Nitrogen (ppm)	Salinity	Bacterial Type	Nitrogen Source
Optimal Conditions for Each Type of Soil	*Andimeshk* soil	135	512	No salinity	AB	NH_4NO_3
	Ahvaz soil	147	513	Half salinity	AB	NH_4NO_3
	Abadan soil	142	617	Salinity	AB	NH_4NO_3

To confirm the optimal conditions, confirmatory tests with a concentration of 2.5% of crude oil and the conditions mentioned in Table 5 were performed. After 56 days, the TPH removal rate was 48.4%, 53.4%, and 56.4% for soils taken from Abadan, Ahvaz, and Andimeshk, respectively. These results confirm the optimal conditions described in Table 5.

Conclusion

Strains isolated from the three different types of soil in Khuzestan province can decompose crude oil and be considered bioremediation bacteria. On the other hand, the RSM model used is well coordinated with the obtained data and can be used to predict the results. This model identifies the optimal conditions for all three types of soil and can be used for further research in bioremediation. Among the variables used to determine the optimal conditions, moisture and nitrogen as two factors that reduce the amount of TPH by increasing their amount to the optimal level, have the greatest effect on reducing the amount of TPH, and can be effective as two independent variables. In general, the biological decomposition of TPH using local bacteria can be used as an expandable method to remove oil contamination from soil. Also introduces Bacillus pacificus bacteria as a new species that can bioremediation. However, more specific research is still needed.

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Authors' contributions
AN and AT supervision designed research, analyzed the data, and assisted to wrote the manuscript; SJ designed research, analyzed the data, and assisted to wrote the manuscript; GhG assisted to experiment, MRA assisted in carrying out the microbial experiments; PM designed research, carry out the experiments, analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Conflict of Interest
The authors declare that they have no conflict of interest.

Ethical approval:
This study does not contain any studies with human participants or animals performed by any of the authors.

Agreement for publication: All authors agree on the publication of this work.

Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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