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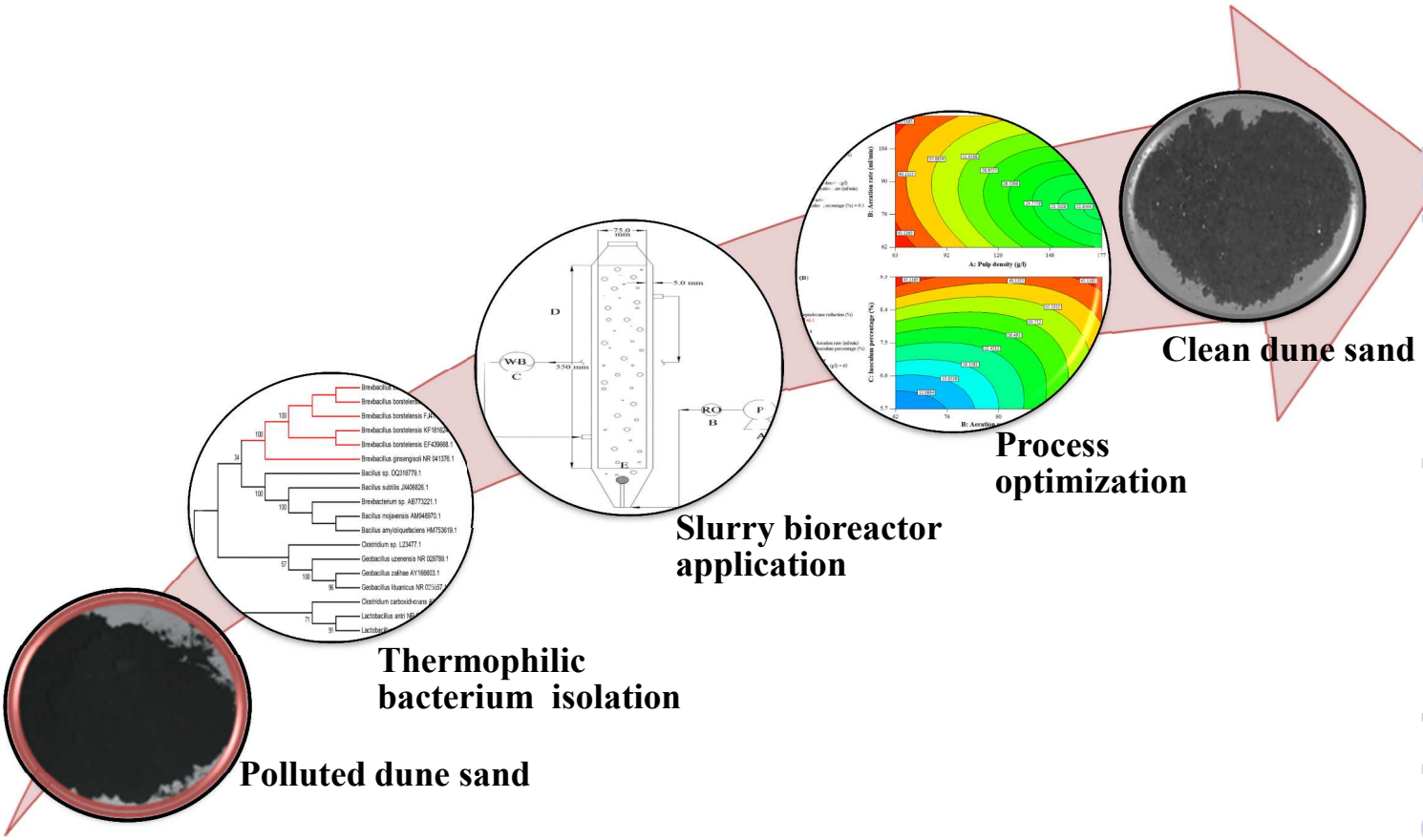

# Biodegradation of heptadecane in hydrocarbons polluted dune sands using a newly-isolated thermophilic bacterium, *Brevibacillus borstelensis* TMU30: Statistical evaluation and process optimization

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## Abstract

An enrichment culture was established to isolate a thermophilic hydrocarbon-degrading bacterium from contaminated soil samples from the Tehran Petroleum Refinery. The bacterium was characterized based on 16S rRNA and identified as *Brevibacillus borstelensis* TMU30. It is registered at NCBI under accession number KF181624.1. The capability of the bacterium for degradation of heptadecane as a representative contaminant in the polluted dune sand was evaluated in a slurry bubble column bioreactor. The aeration rate, inoculum content and pulp density were optimized to maximize the degradation of heptadecane using central composite design of response surface methodology. The results showed that maximum heptadecane reduction reached 48% at an aeration rate of 62 ml/min, inoculum content of 9.3% (v/v) and pulp density of 63 g/l only after 4 days. This study highlights an important potential use of thermophilic degradative bacterium to eliminate contamination in a slurry bioreactor while shortening dramatically the treatment time.

**Keywords:** Biodegradation; Hydrocarbons polluted dune sand; Thermophilic bacterium; Slurry bioreactor; Statistical optimization.

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## 1. Introduction

Petroleum contamination results from accidental spills, during oil loading and from past disposal practices. Petroleum hydrocarbons are major environmental pollutants because of their abundance, persistence and high toxicity to all biological systems. It is estimated that 0.08% to 0.4% of global petroleum production eventually reaches the sea.[1]

Aliphatic and polycyclic aromatic hydrocarbons (PAHs) are some of the earliest environmental carcinogens recognized. Although they are chief pollutants of air, the soil acts as the ultimate repository of these chemicals. PAHs are reported to be highly toxic and mutagenic chemicals that are ubiquitous in the environment. They have a detrimental effect on the flora and fauna of affected habitats and result in the uptake and accumulation of toxic chemicals in food chains and, in some instances, in serious health problems and/or genetic defects in humans. The US Environmental Protection Agency has identified 16 kinds of PAHs as priority pollutants.[2] The clean-up of hydrocarbon-contaminated soils remains a priority for the restoration of the natural environment.

Dune sand is used to absorb hydrocarbons to prevent contamination of groundwater sources. Polluted dune sands have a detrimental effect on human and the environment; therefore, it is necessary to find an appropriate method for their disposal. Of the techniques employed to remediate polluted sites, the environmentally-friendly technologies of bioremediation with their obvious advantages are gaining in prominence.[3]

The main advantages of bioremediation are its safety and cost-efficiency when compared to conventional techniques including physico-chemical remediation methods such as soil washing, soil vapor extraction, land farming, soil flushing, solidification or stabilization, thermal desorption and also phytoremediation which may lead to secondary contaminations and need

expensive substances and equipments.[4] Bioremediation can lead to complete mineralization of a pollutant into inorganic minerals, water and carbon dioxide by aerobic bacteria and lower concentrations of contaminants where physical and chemical methods are not feasible.[5,6] If petroleum contains compounds with complex structures, it cannot be completely mineralized to water and carbon dioxide and complex residues will remain.[7]

Diverse bioremediation approaches have been developed to accelerate biological degradation. The success of bioremediation generally relies upon addressing the types of contaminant, soil characteristics, appropriate microorganisms and their degradation properties and multiple environmental factors for degradation. Temperature is the most important factor controlling bioavailability of low-solubility hydrocarbons and the nature and extent of microbial metabolism.[3,8,9] Increasing the temperature increases biodegradation in response to decreased viscosity, increased mass transfer of hydrophobic contaminants to the cell and increased solubility.[10,11]

Finding a thermophilic bacterium that can metabolize hydrocarbons would make the process more efficient. Five thermophilic genera have been found: *Thermoleophilum sp*., *Thermomicrobium sp*., *Bacillus sp*., *Geobacillus sp*. and *Thermus sp*. Most studies have focused on the use of these thermophilic bacteria to degrade a petroleum fraction or light crude oil. Isolating crude oil-degrading bacteria to degrade high concentration crude oil and bioremediation of oil-polluted soil have been rarely reported.[9,12] Studies have focused on slurry phase bioremediation because of its advantages of simplicity and efficiency.[13-15] In slurry bioreactors, an increase in the water-to-soil ratio increases the solubility of contaminant and bioavailability, which can shorten treatment time.[4,16,17]

Process modeling and optimization studies can be conducted using response surface methodology (RSM). RSM helps evaluate important factors in building models to study the interaction between variables and to select optimum values for variables and desirable responses. This approach decreases the number of experiments required, increases possible statistical interpretations and indicates the interaction between multiple variables. Central composite design (CCD) is used to build second-order response surface models.[18-20]

There is little focus on the remediation of hydrocarbons polluted dune sand because of its unique properties such as density and difficulties like sedimentation during its remediation in slurry systems. The novelty of present study is isolation and characterization of a potent thermophilic bacterium (TMU30) and evaluation of its ability to degrade heptadecane as a representative component in polluted dune sand in a slurry bubble column bioreactor. The simultaneous use of thermophilic bacterium and a slurry bubble column bioreactor can shorten biodegradation time as a critical point. The effects of the operating parameters of aeration rate, pulp density and inoculum content and their interactions on heptadecane degradation in a bioreactor were also evaluated using RSM.

## 2. Materials and Methods

### 2.1. Microorganism enrichment and isolation

Samples used for bacterial enrichment were collected from Tehran petroleum refinery. Nutrient broth medium for enrichment of the bacteria in the samples and nutrient agar medium were used to purify and maintain the bacteria. A specific culture medium was prepared for the bacteria that used hydrocarbons as the sole carbon and energy sources for growth. The composition of the medium is listed in Table 1. The enrichment medium was 100 ml nutrient broth and was

sterilized in each of 8 Erlenmeyer flasks. Water samples (3 ml) and soil and sludge samples (3 g each) were added to the flasks as inoculum. The flasks were incubated at 55°C and 200 rpm for 1week. After that time, 3 ml of each flask was added to100 ml fresh medium as inoculum.

To isolate the microorganisms, 100ml of medium was added to each of 8 Erlenmeyer flasks and then 2g of hydrocarbon-polluted soil that was sterilized using ultraviolet light was added as a carbon source to the flasks. Next, 3 ml of each sample prepared during the enrichment step was added to the flasks as an inoculum. Serial dilutions of 0.1 to $10^{-7}$ ml were performed on the samples. Each diluted sample was spread onto plates of nutrient agar medium. The plates were kept at 55°C for 24 h. Four types of bacteria were grown; the one with the highest growth rate was purified and selected as a target for further investigation.

**2.2. Microorganism identification and phylogenetic analysis**

DNA extraction was performed using DNeasy Blood and Tissue Kits (Qiagen, The Netherlands). The 16S rDNA was amplified by PCR using pAF 5′-AGAGTTTGATCCTGGCTCAG-3′ forward primer and pAR 5′-AAGGAGGTGATCCAGCCGCA-3′ reverse primer. PCR amplification consisted first of a denaturation step of 5 min at 94 °C followed by 35 cycles of 1 min at 94 °C for denaturation, then at 58 °C for 1 min for annealing and finally at 72 °C for 2 min for extension. Final extension was done at 72 °C for 10 min.

The PCR product (1500 bp) was purified with high-purity PCR Product Purification Kit (Fermentas, Canada) according to manufacturer instructions and used for DNA sequencing. The closest matches to the sequence were determined using the BLASTN sequence similarity search

tool in the NCBI GenBank.[21] The phylogenetic tree was constructed with MEGA 4.0,[22] using the neighbor-joining method.[23]

Statistical support for the branching nodes was estimated using bootstrap values based on 1000 replicates. The 16S rDNA gene and 12 related sequences obtained from GenBank were selected for use as references for phylogenetic tree construction.[23] The neighbor-joining tree represents the nucleotide sequence of 16S rDNA gene and shows the relationships between the TMU30 (KF181624.1) strain and closely-related taxa with accession numbers. The percentages at the nodes indicate the levels of bootstrap support based on the neighbor-joining analyses of the 1000 resampling datasets.

### 2.3. Contaminated dune sand sampling and analysis

Large particles of dune sand (>5 μm) were sifted out using a soil sieve. The hydrocarbons were extracted from the dune sand using hexane as a solvent and the extract was analyzed by gas chromatography/mass spectrometry (GC/MS), used in many similar investigations to accurate contamination detection and measurement.[24,25] Table 2 shows the results of GC/MS analysis of the contaminated dune sand. The polluted sands contained linear hydrocarbons (alkanes); no polycyclic aromatic hydrocarbon was detected.

### 2.4. Experiments in the bioreactor

The experiments were carried out in a bubble column bioreactor made of glass. The column height was 55 cm and its internal diameter was 7.5 cm. To maintain bioreactor operation at 55 °C, water was heated in a bath and pumped into the jacket around the bioreactor. The air required for bacterial growth and to prevent sedimentation of the sands due to their high density was

provided by a compressor and entered to the medium through a sparger at the bottom of the bioreactor. It was adjusted using a rotameter according to the experimental design.

The bioreactor was supplied with a condenser to prevent evaporative loss. Control experiment (without inoculation) was performed to determine the decrease in heptadecane from probable evaporation under optimal conditions. The isolated bacterium (5% to 10% v/v) was inoculated into the bioreactor, which was filled with a mixture of dune sand and medium (40 to 200 g/l). The aeration rate was set at 50-133 ml/min. Fig. 1 shows the schematic of the bioreactor set-up.

### 2.5. Heptadecane measurement

The heptadecane concentration was measured every day from a sufficient volume of liquid containing hydrocarbons from the bioreactor. To extract the heptadecane from each sample, 5 ml dichloromethane was added as a solvent to 1 ml of sample in a vessel. The vessel was shaken for 24 h; then 1 ml of supernatant was analyzed by GC/MS to ascertain the heptadecane concentration. After extraction of the heptadecane from the dune sand using dichloromethane (1 g sand mixed with 5 ml solvent), the extract was filtered through Whatman paper No. 42 and the heptadecane concentration was measured by GC.

### 2.6. Experimental design

The effectiveness of bioremediation depends on identification of the rate-limiting factors and their optimization in feasibility studies, since the effect of different factors are considered and discussed in many investigations.[25] Heptadecane was used as a model hydrocarbon to monitor biodegradation behavior accurately. Aeration rate, pulp density and inoculum content and their

interactions were selected to determine process efficiency and optimal conditions to evaluate the ability of the isolated bacterium to degrade aliphatic hydrocarbons.

The experimental designs were based on RSM with three factors in five levels (Table 3). RSM is a collection of mathematical and statistical technique for empirical model building. The objective of careful design of experiments is to optimize a response (output variable) that is influenced by several independent variables (input variables). The CCD used a total of $2^k+n_\alpha+n_0$ trials where $k$ is the number of independent variables, $2^k$ is the number of factorial points, $n_\alpha=2k$ is the number of axial points and $n_0$ is the number of center points.[26,27] Fractional CCD was performed in 13 trials. Design-Expert software (version 7.1.4) was used to develop the experimental design. The behavior of the system is explained by the quadratic polynomial empirical model:

$$y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i<1}^{3} \sum_{j=1}^{3} \beta_{ij} X_i X_j + \varepsilon \quad (1)$$

where $y$ is the expected value of the response variable; $\beta_0, \beta_i, \beta_{ii}, \beta_{ij}$ are the model parameters; and $X_i$, $X_j$ are the coded factors.[28] Here, $y$ represents the heptadecane degradation content of the empirical models.

### 2.7. Validation of statistical model

An experiment was performed under the predicted optimal conditions and the experimental heptadecane degradation was compared with the predicted results to verify the validity of the statistical model. After validation, RSM was used to assist in the modeling and enhancement of heptadecane removal efficiency.

## 3. Results and Discussion

### 3.1. Bacterial strain identification

Fig. 2 shows that the 16S rDNA sequence similarities and BLAST analysis confirm that the TMU30 strain belonged to *Brevibacillus borstelensis* with 100% similarity. The partial sequences (1400 bp) obtained were deposited in the GenBank under accession number KF181624.1. An unrooted phylogenetic tree for 16S rDNA was constructed using the sequence *B.borstelensis* (KF181624.1) and 17 reference sequences from the NCBI GenBank. Phylogenetic analysis and bootstrap resampling (100%) confirmed that *B.borstelensis* (KF181624.1) is in the group with *B.borstelensis* strain BP8 (KC879112.1), *B.borstelensis* strain 1CK49 (JQ229800.1), *B.borstelensis* strain P35 (FJ417406.1) and *B.borstelensis* strain S3 (EF439668.1).

### 3.2. Statistical analysis

Thirteen experiments were designed according to fractional factorial CCD with 3 center points. Table 4 shows the experimental conditions and results obtained in each trial in the bioreactor. In each run, the amount of heptadecane in the sand sample (before bioremediation) was compared with the amount of its concentration in the treated sample to determine the percentage of heptadecane degradation after 4 days. Table 5 shows the results of ANOVA used to investigate the effect of aeration rate, pulp density and inoculum content on the response of the system. This statistical tool is required to test the significance and adequacy of the model. The mean squares (MS) are calculated as MS = SS/DF, where SS is sum of squares of each variation source and DF is the respective degrees of freedom.

The Fischer variation ratio (F-value) is a statistically valid measure of how well the factors describe the variation in the data about its mean. It can be calculated from ANOVA as F-value =

MS (model variation)/MS (error variance). Normally, the data varies around its mean value; the greater the F-value from unity, the more acceptable is this variation.[29] In general, the calculated F-value should be several times greater than the tabulated value. Table 5 shows that the calculated F-value for the heptadecane removal response model was 50.75, which exceeds the tabulated F-value at 95% confidence level and indicates that the treatment differences are highly significant. The P-value shows that the heptadecane degradation model is statistically significant. ANOVA indicates that all factors (aeration rate, inoculum content and pulp density) are significant at $p< 0.05$.

### 3.3. Heptadecane degradation

#### 3.3.1. Statistical model

Eq. (2) was obtained from the 13 batch run using Design-Expert software (version 7.1.4). Multiple regression analysis of the experimental data fit the experimental results of the CCD to a reduced quadratic equation. The empirical relationship between heptadecane reduction and the three test variables in coded units obtained using RSM is:

$$\text{Heptadecane reduction} = 9.88 - 9.02A + 10.95B + 11.95C + 3.45AB - 8.17BC + 5.48A^2 + 6.71B^2 + 5.91C^2 \quad (2)$$

where A is pulp density, B is aeration rate and C is inoculum content.

The statistical significance of the reduced quadratic model was evaluated using ANOVA and the results are shown in Table 5. The relatively high $R^2$ (0.99) value indicates that the reduced quadratic equation for heptadecane reduction is capable of representing the system under the given experimental conditions. Fig. 3 shows good agreement between the data predicted by the

model and the experimental values. As seen, the model fit the experimental data well. The results indicate that this regression was statistically significant at $p < 0.05$ (95% confidence interval).

### 3.3.2. Contour plots

Interaction terms were selected at $p < 0.05$ using the largest absolute coefficients in the fitted model (BC= aeration rate× inoculum content; AB = pulp density×aeration rate) for the axes of the contour plots to account for the curvature of the surfaces. Fig. 4 shows interaction plots, which indicate that the effect produced by changing one variable depends on the level of other variables. Fig. 4(a) shows that, at a lower inoculum content, an increase in the aeration rate strongly affected the efficiency of heptadecane removal; however, at higher inoculum content, heptadecane removal remained nearly constant with an increase in aeration rate. A low inoculum content could result in an aeration rate is less than that required for the biological system and that increasing the inoculum content increases biodegradation of heptadecane. Higher inoculum content provides sufficient agent for heptadecane removal so the increase in aeration has less effect.

Fig. 4(b) shows that raising the pulp density can decrease the heptadecane removal at both low and high aeration rates, but that increasing pulp density at a low aeration rate is more effective. Also, heptadecane removal decreased in response to the sedimentation of sand at the bottom of the bioreactor.

Fig. 5 shows the relationship between different parameters at different values for heptadecane reduction. Fig. 5(a) shows that a maximum heptadecane reduction of >45% was observed at a pulp density of 63 g/l, aeration rates of 62 to 118 ml/min when the inoculation content was held constant at 9.3%. This indicates that the effect of low pulp density on the aeration rate (62 - 118

ml/min) was not significant. As shown in the figure, the amount of heptadecane reduction increased as the pulp density decreased from 177 to 63 g/l at a constant aeration rate.

Fig. 5(b) shows the interaction between inoculum content and aeration rate affected heptadecane reduction at a constant pulp density 63 g/l. A maximum reduction of heptadecane of >45% was observed at an aeration rate of 118 ml/min, inoculation content of 9.3% and a constant pulp density of 63 g/l. This figure indicates that the increase in inoculation content (5.7 to 9.3%) and aeration rate (62 to 118 ml/min) increased the percentage of heptadecane reduction.

### 3.4. Process optimization using desirability function

In numerical optimization, minimum and maximum levels must be provided for each parameter. These goals are combined into an overall desirability function. Desirability is an objective function that ranges from zero outside the limits to one at the goal. The model seeks to maximize this function. Chances improve for finding the best local maximum by starting at several points in the design space.[29] The multiple response method was applied to optimize any combination of the three goals (aeration rate, pulp density, inoculum content). Numerical optimization finds a point at which the desirability function is maximized. The levels of all parameters within the range of investigation were set for maximum desirability. Fig. 6 shows a ramp desirability generated from optimum point by numerical optimization. The best local maximum was a pulp density of 63 g/l, inoculum content of 9.3% and aeration rate of 62 lit/min. This generated a desirability of 1.00 that resulted in 49.65% heptadecane removal. The desirability value shows that the estimated function may present the experimental model and desired condition.

### 3.5. Model validation

To test the validity of the optimized conditions given by the model, an experiment was carried out with the parameters suggested by the model. Table 6 presents the results of the experiment conducted under optimal conditions and verifies that the experimental and predicted values from fitted correlations were in close agreement at a 95% confidence interval. The model for heptadecane reduction predicted the optimal values of inoculum content at9.3%, aeration rate at 62 ml/min and pulp density at 63 g/l resulted in maximum heptadecane reduction. Under these conditions, the experimental value for the heptadecane reduction was 48% on the fourth day of the experiments. These results confirmed the validity of the model; the experimental values were determined to be quite similar the predicted values.

An important limitation of bioremediation methods is that they are time-consuming. A significant factor for the choice of thermophilic bacterium in this study was to address this limitation. As seen, a biodegradation efficiency of approximately 50% after only 4 d can be considered a success when compared with biodegradation with much longer treatment times using Dietzia strain DMYR9.[18] This short biodegradation time is also in close agreement with those reported by Zhang *et al.*[31] using an aliphatic hydrocarbon-degrading bacterium SH-1.

## 4. Conclusion and future prospects

Slurry phase bioremediation of hydrocarbon-contaminated dune sand using a newly-isolated bacterium, *Brevibacillus borstelensis* TMU30, was studied. Statistical design of experiments was applied to determine the effective factors and their interactions on the heptadecane bioremediation. Aeration rate, pulp density and inoculum content were identified as significant factors in maximizing heptadecane reduction. The predicted results indicate that the optimal

values were aeration rate at 62 ml/min, inoculum content at 9.3% and pulp density at 63 g/l. The optimal heptadecane removal predicted by this model was confirmed experimentally. The results illustrate the success of this novel isolated bacterium for aliphatic hydrocarbons degradation while dramatically decreasing bioremediation time.

Recommendations for future researches are to isolate other species which may have a great potential to degrade several PAHs components simultaneously. Although biodegradation time in this study is short in comparison with biodegradation times reported by other researchers, absolutely there are many potent species to isolate which are able to shorten biodegradation time and may result in lower amount of contamination due to their metabolite activity and pathway. In addition, regarding to laboratory conditions, considering as many factors as possible to be optimized can have a huge effect on responses such as biodegradation time and efficiency. Optimizing these critical factors would help this process to be industrialized.

By a well designed consortium of symbiotic bacteria, biodegradation can be improved substantially due to complementary catabolic pathways and enhancement of bioavailability caused by biosurfactant production. As an example Rahman et al.[30] isolated five species including *Micrococcus sp*., *Corynebacterium sp*., *Flavobacterium sp*., *Bacillus sp*., and *Pseudomonas sp*. for bioremediation of contaminated soils. The mixed culture degraded 78% of the crude oil in the sample, compared with 43% for single species *Micrococcus sp*. Utilizing symbiotic culture including the isolated bacteria can significantly increase the mineralization rate of various components of petroleum.

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**Tables and Figures Captions**

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**Table 2.** GC/MS analysis of polluted dune sands

**Table 3.** Factors and their levels in designed experiments

**Table 4.** The CCD used in RSM and the values of heptadecane reduction as a response

**Table 5.** ANOVA results for heptadecane reduction.

**Table 6.** Optimum condition and the model verification

**Fig. 1.** Schematic of the laboratory scale slurry bubble column bioreactor used in this study. A: Aeration pump; B: Rotameter; C: Water bath; D: Jacket; E: Sparger

**Fig. 2.** A neighbor-joining tree representing the nucleotide sequence of 16S rDNA gene showing relationships between strain TMU30 (KF181624.1) and closely related taxa with accession numbers. Percentages at the nodes indicate levels of bootstrap support based on neighbor joining analyses of 1000 resampling datasets

**Fig. 3.** Predicted versus actual data for heptadecane degradation

**Fig. 4.** Interaction plots of heptadecane reduction for factors: (a) B and C, and (b) A and B, A: pulp density, B: aeration rate, C: inoculum.

**Fig. 5.** Contour plots of heptadecane reduction as functions of: (a) A and B, and (b) B and C. A: pulp density, B: aeration, C: percentage of inoculums.

**Fig. 6.** Desirability ramps for numerical optimization of the condition for maximum heptadecane reduction

**Table 1**

| Chemical compound | Concentration (g/l) |
|---|---|
| $KH_2PO_4$ | 0.1 |
| $K_2HPO_4$ | 0.2 |
| $Na_2HPO_4.2H_2O$ | 0.3 |
| $NH_4Cl$ | 0.005 |
| $MgSO_4.7H_2O$ | 0.025 |
| $CaCl_2$ | 0.03 |
| $FeCl_3.6H_2O$ | 0.025 |

**Table 2**

| Compound | Concentration (mg/kg contaminated soil) |
|---|---|
| Decane | 412 |
| Dodecane | 350 |
| Tetradecane | 580 |
| Heptadecane | 710 |
| Octadecane | 700 |
| Eicosane | 577 |
| Docosane | 352 |
| Tetracosane | 166 |
| Hexacosane | 45 |

**Table 3**

| Design Factors | Levels | | | | |
|---|---|---|---|---|---|
| | **+α= +1.414** | **+1** | **0** | **-1** | **-α= -1.414** |
| **A: Pulp density (g/l)** | 200 | 180 | 120 | 60 | 40 |
| **B: Aeration rate (ml/min)** | 130 | 118 | 90 | 62 | 50 |
| **C: Inoculum content (%v/v)** | 10.0 | 9.3 | 7.5 | 5.7 | 5.0 |

**Table 4**

| Trial | Factors | | | Response |
|---|---|---|---|---|
| | Pulp density (g/l) | Aeration rate (ml/min) | Inoculum content (%) | Heptadecane reduction (%) |
| 1 | 63 | 118 | 9.3 | 46.3 |
| 2 | 63 | 62 | 5.7 | 9.1 |
| 3 | 177 | 62 | 9.3 | 24.4 |
| 4 | 120 | 90 | 7.5 | 13.1 |
| 5 | 177 | 118 | 5.7 | 27.6 |
| 6 | 120 | 90 | 5.0 | 5.9 |
| 7 | 120 | 90 | 7.5 | 10.5 |
| 8 | 120 | 50 | 7.5 | 7.7 |
| 9 | 120 | 130 | 7.5 | 41.1 |
| 10 | 120 | 90 | 7.5 | 10.1 |
| 11 | 200 | 90 | 7.5 | 9.2 |
| 12 | 120 | 90 | 10.0 | 39.7 |
| 13 | 40 | 90 | 7.5 | 34.7 |

**Table 5**

| Response | Model | ANOVA | | | | | |
|---|---|---|---|---|---|---|---|
| | | Source | SS | DF | MS | F-value | P-value |
| **Heptadecane reduction (%)** | Reduced quadratic model<br>$R^2$=0.99<br>Adjusted $R^2$=0.97 | Model | 2881.62 | 8 | 360.20 | 50.75 | 0.0002 |
| | | A | 325.13 | 1 | 325.13 | 45.81 | 0.0011 |
| | | B | 959.98 | 1 | 959.98 | 135.27 | 0.0001 |
| | | C | 571.22 | 1 | 571.22 | 8.49 | 0.0003 |
| | | AB | 23.81 | 1 | 23.81 | 3.35 | 0.1265 |
| | | BC | 133.35 | 1 | 133.35 | 18.79 | 0.0075 |
| | | $A^2$ | 225.23 | 1 | 225.23 | 31.74 | 0.0024 |
| | | $B^2$ | 337.18 | 1 | 337.18 | 47.51 | 0.0010 |
| | | $C^2$ | 261.52 | 1 | 261.52 | 36.85 | 0.0018 |
| | | Residual | 35.48 | 5 | 7.1 | | |

**Table 6**

| Response | Pulp density (g/l) | Aeration rate (ml/min) | Inoculum content (%) | Predicted value (%) | Experimental value (%) | 95% CI low | 95% CI high |
|---|---|---|---|---|---|---|---|
| Heptadecane reduction (%) | 63.0 | 62.0 | 9.3 | 49.65 | 48.0 | 38.3 | 61.0 |

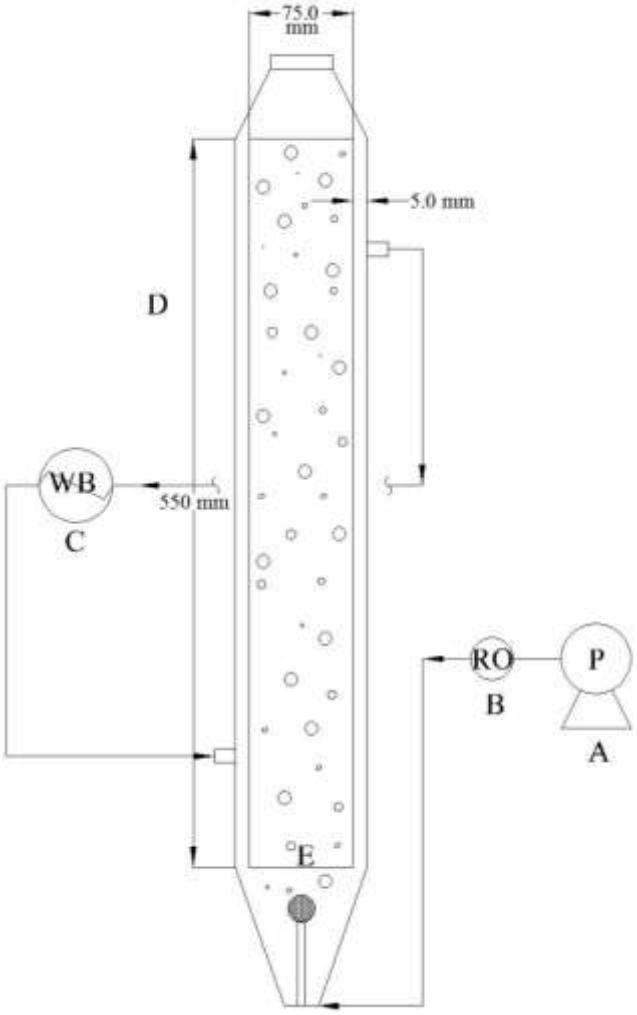


**Fig. 1**

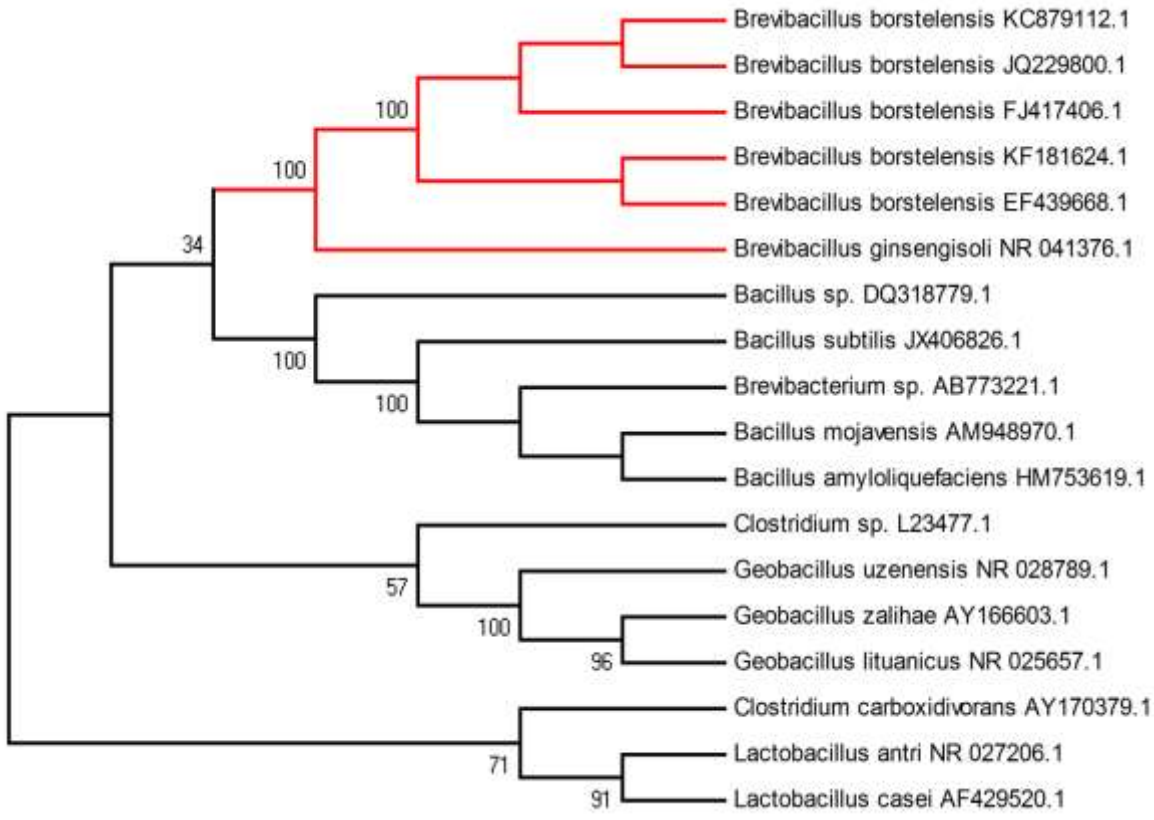


**Fig. 2**

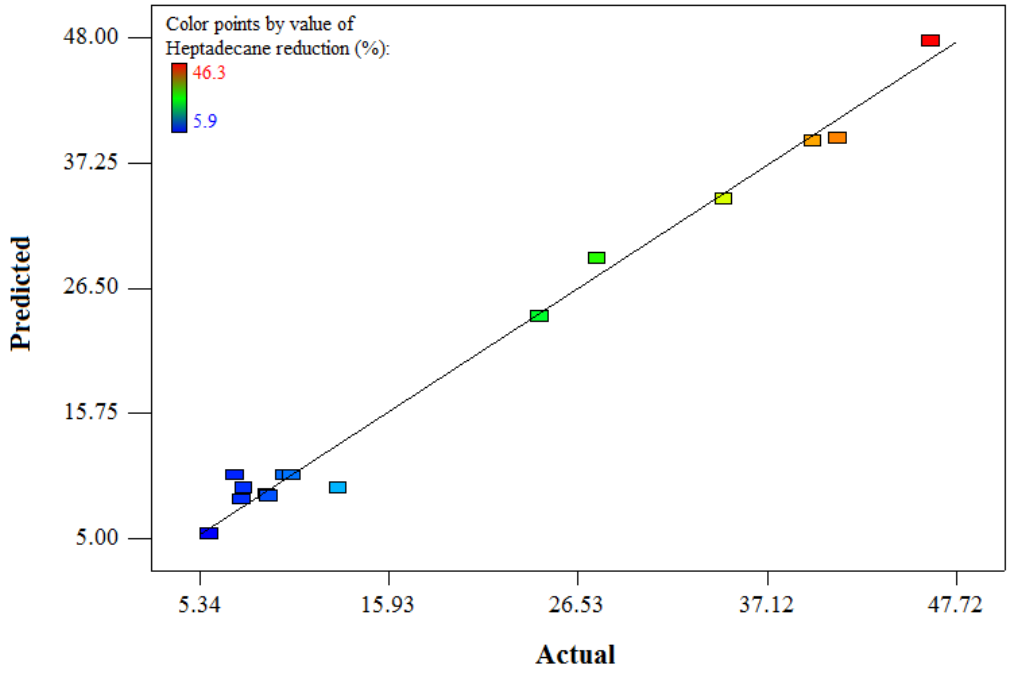


**Fig. 3**

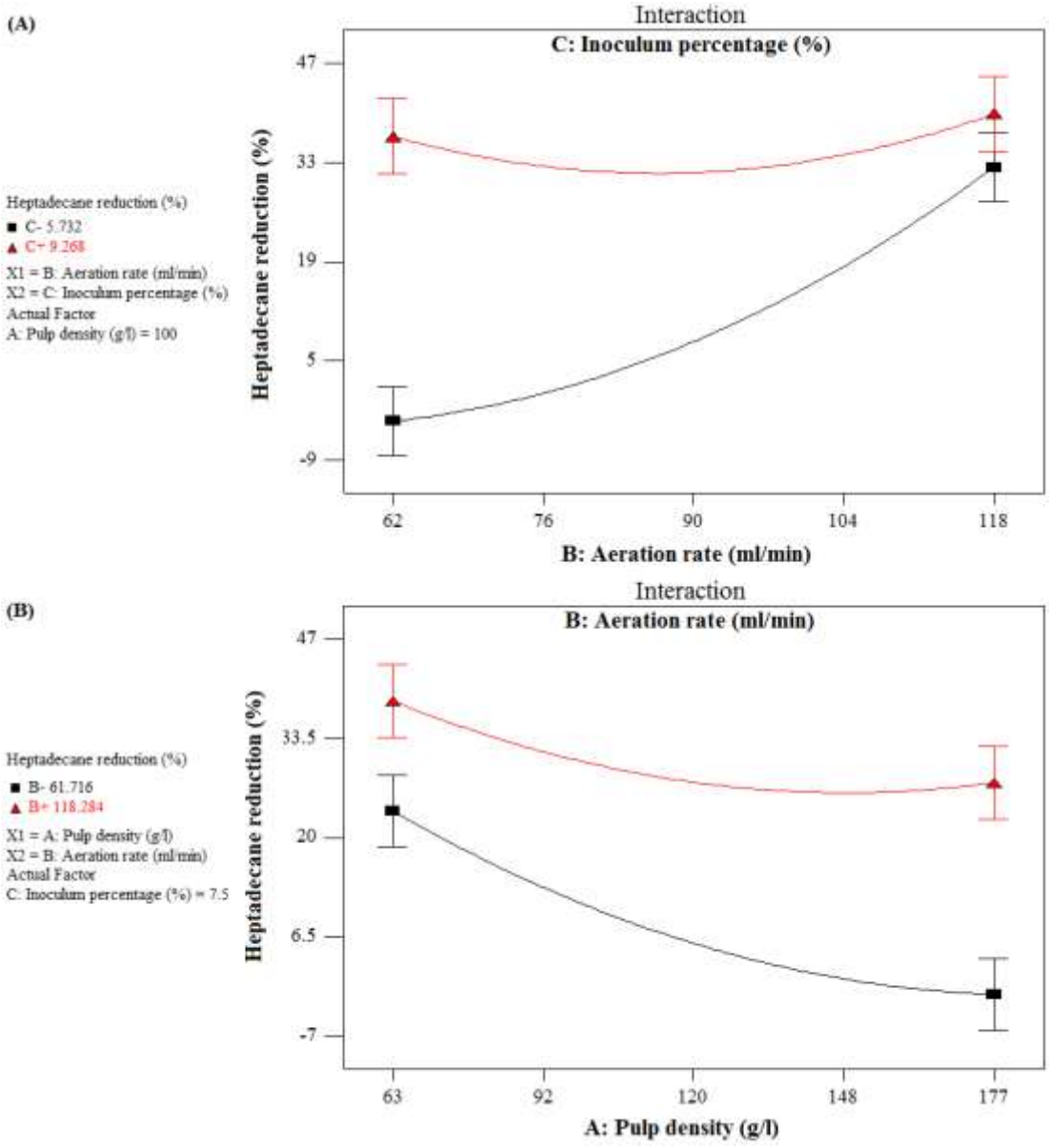


**Fig. 4**

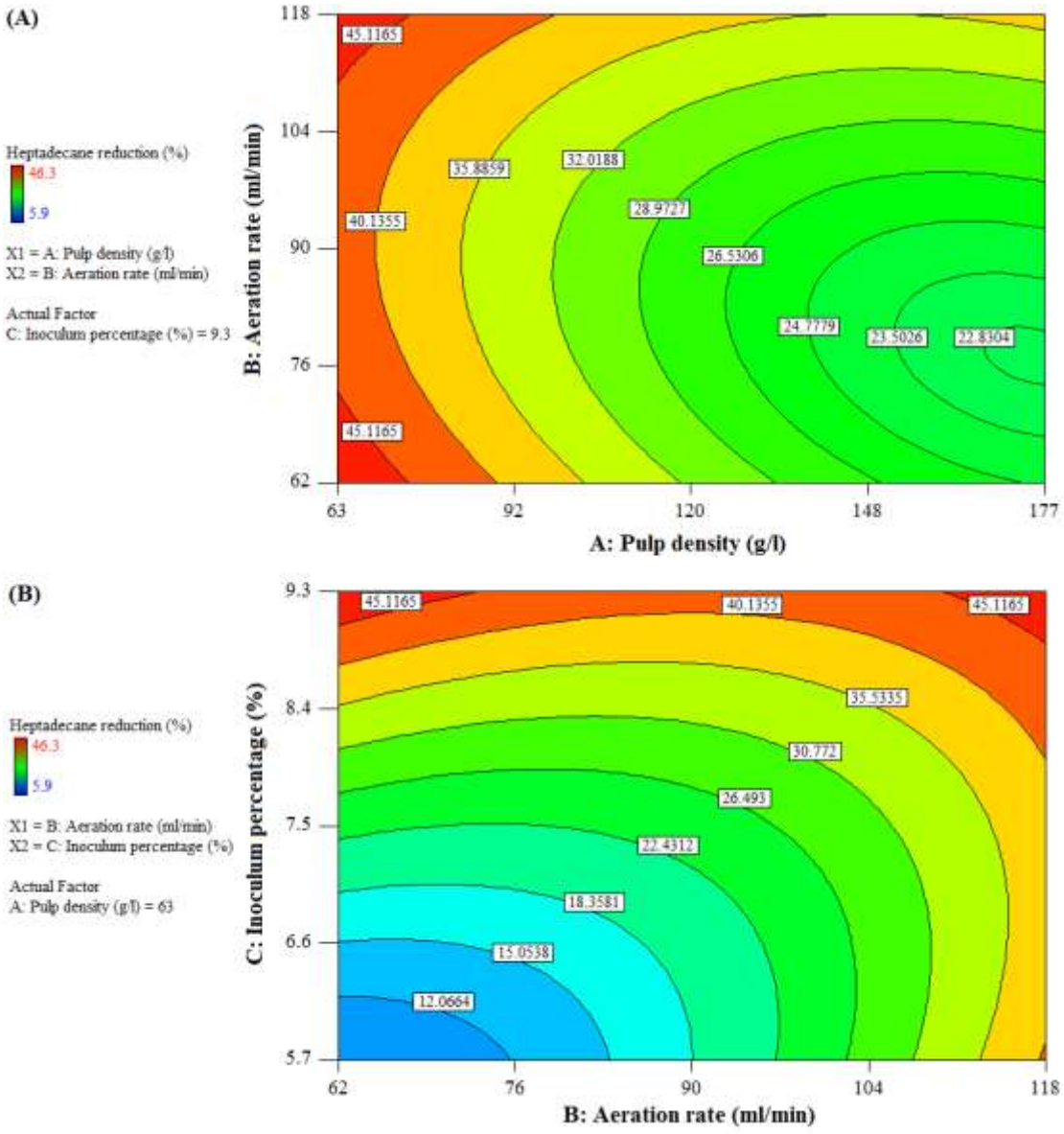


**Fig. 5**

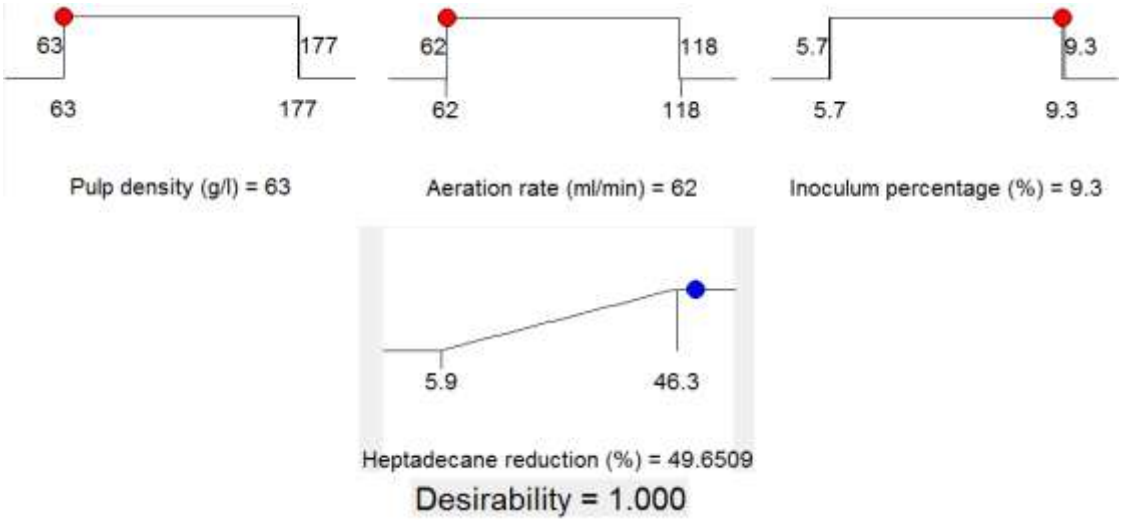


**Fig. 6**