Original Article

Optimization of Degradation of Petroleum Crude Oil by *Lysinibacillus* sp. SS1 in Seawater by Response Surface Methodology

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Abstract
Introduction: The prevalence of petroleum oil spills in oceans and seas is on the rise in India, resulting in widespread detrimental effects on the environment. Bioremediation by bacteria is an eco-friendly and safe technique for the removal of these pollutants from the seawater.
Materials and Methods: An indigenous bacteria, isolated from garage soil was grown on Bushnell Agar plate with Petroleum Crude Oil (PCO) as a carbon source. It was identified by biochemical characterization and 16S rRNA sequencing. The effect of factors such as concentrations of PCO, inoculum and glucose, agitation speed, pH, and degradation time on the growth of bacteria and PCO degradation in seawater was studied by one factor at a time approach. Screening and optimization were performed by Factorial Design and Central Composite Design respectively.
Results: According to findings, isolated bacteria degraded PCO within 48 h and could decolorize 6-dichlorophenol indophenol within 36 h. It was identified as a novel *Lysinibacillus* sp. SS1, which grew in the pH range of 4.0 to 10.0 and tolerated salinity of 6.0% w/v. Significant factors (concentrations of glucose, inoculum and pH) were optimized and optimum levels were 11.7% v/v inoculum, 11.36 g/L glucose, and pH 8.6. Maximum degradation of 84 ± 0.13% was achieved when grown in seawater supplemented with 4.0% v/v PCO, at 27 ± 2 °C at 80 RPM in 28 days at optimized conditions.
Conclusions: The present study is the first study reporting optimization of degradation of PCO by *Lysinibacillus* species. *Lysinibacillus* sp. SS1 could effectively degrade petroleum hydrocarbons in extreme conditions of seawater and can be applied for the treatment of oil spills.
Keywords: Biodegradation, Factorial Design, Central Composite Design, Seawater
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Introduction

Petroleum Crude Oil (PCO), a composite mixture of alkanes (paraffins), cycloalkanes (naphthenes) and various aromatic hydrocarbons is a chief source of energy and raw material for many industries such as plastics, fibres, detergents, pharmaceuticals and cosmetics.[1] The increase in global oil demand has led to the transportation of PCO via the sea route. Accidental spills and dumping of oily waste, transporting and refinery activities, oil pumping operations annually releases about 3 million tons of oil in the sea.[2,3] This harms the marine ecosystem and has adverse effect on marine life and human health as some Petroleum Hydrocarbons (PH) are toxic, mutagenic and carcinogenic. About 500 major oil spillage accidents exceeding 700 tons in the world from 1974 to 2016 have been reported, which have caused havoc on the ecology of the marine environment.[4] Cleaning contaminated sites is the priority at the moment and is frequently done by physico-chemical methods, which are expensive and require the restoration of pollutant sites.[5]

Micro-organisms, present at the site of pollution, acclimatize themselves and gain the ability to utilise the complex hazardous pollutants, as source of carbon and energy.[3] Bioremediation thus includes the use of these micro-organisms to transform the hazardous pollutants into non-toxic compounds.[6] Bioremediation is currently gaining significance as it is feasible, reliable and economic as compared to the conventional physico-chemical methods.[7]

Degradation of PH by micro-organisms in contaminated water is dependent on various factors like temperature, pH, oxygen, micro-organisms and inoculum, salinity, nutrients, bioavailability etc.[8] Studies have shown that the presence of easily assimilated carbon source such as glucose had a positive influence on the degradation of hydrocarbons.[9] Even though there are numerous studies on the effect of these factors on the degradation of PH in soil, marine and river water samples,[10–12] studies on the optimization of factors for maximum degradation in marine environment is scarce.[13] Additionally, the influence of these factors is organism-specific and has to be evaluated when novel PH degrading

strains are isolated and identified.

This study deals with the isolation and identification of a novel PCO degrading *Lysinibacillus* species from automotive service station soil contaminated with PH. The effect of factors such as PCO concentration, inoculum concentration, glucose concentration, agitation speed, pH and time of incubation on the growth of *Lysinibacillus* species and degradation of PCO in seawater was evaluated by the One Factor At a Time (OFAT) approach. Significant factors were identified by full factorial design and their levels were further optimized by a central composite design. To the best of our knowledge, this is the first report on PCO degradation in seawater by *Lysinibacillus* species.

Materials and Methods
Collection of Soil Samples
Soil sample was collected from an automotive service station established about 10 years ago situated in Moodabidri, Karnataka, India. The collected sample was kept in sterile falcon tubes and stored in the laboratory at 4 °C till use.

Substrates and Chemicals
The redox indicator 6-dichlorophenol indophenol (DCPIP) was procured from SRL (SRL chemicals, Bangalore, India). The PCO was received from a petroleum refinery industry situated in Mangalore, Karnataka, India. All other chemicals, solvents and reagents used in the study were of analytical grade, unless mentioned.

Isolation of Bacteria and Estimation of PH Degrading Ability
Soil sample (1 g) was vortexed in 10 ml of sterile saline (0.85% w/v) and was allowed to settle. The clear solution was serially diluted and spread plated onto nutrient agar medium. The plates were incubated at 37 ± 1 °C for 72 h. The bacterium under study was isolated based on the difference in morphological characteristics and stored as glycerol stock at -20 °C.

The isolate was then streaked on Bushnell Hass (BH) plate (Table 1) overlaid with 100 µl of PCO and incubated at 37 °C for six days and was observed for growth and zone of clearance around the colonies. Additionally, the ability of the isolate to reduce DCPIP (blue to colourless) on oxidation of PH in PCO in the medium was estimated. The isolate was grown in BH medium supplemented with 1% v/v PCO with a DCPIP indicator (0.0125% w/v) at 37 ± 1 °C for four days and a decrease was observed in DCPIP concentration at 600 nm.[14]

Identification of Bacterium by Biochemical Characterization and 16S rRNA Sequencing
The growth of the bacterial isolate was checked by growing the bacterium in a nutrient broth at 37 ± 1 °C maintained at different conditions of temperature, pH and NaCl concentration. Bacterial isolate was biochemically characterized by subjecting it to different tests such as oxidase, catalase, urease, nitrate reduction, hydrogen sulphide production, phenyl alanine deamination, esculin hydrolysis, methyl red (MR), voges proskauer (VP) tests, utilisation tests such as lysine, alanine, ornithine and malonate and sugar hydrolysis tests such as arabinose, xylose, glucose, lactose, raffinose, rhamnose, cellobiose, saccharose, melibiose, adonitol and trehalose.

The identification of the isolated bacterium was carried out by 16S RNA sequencing.[15] The obtained 16S sequence was subjected to homology analysis by Blast program (www.ncbi.nlm.nih.gov/blast) and a phylogenetic tree was constructed using the neighbour joining method considering 16S sequences of bacteria showing >98% homology using the Bioedit software.

PCO Degradation in Seawater by Lysinibacillus sp. SS1
Sea water (100 ml) was spiked with PCO (1.0% v/v) as a sole carbon source in Erlenmeyer flasks (250 ml) and sterilised at 121 °C at 15 lbs pressure for 30 minutes. Bacterium (16 h old) was inoculated into the sterilised sea water at a concentration of 5.0% v/v (1×10^8 CFU/ml) and was incubated at 25 °C for seven days. Seawater with PCO and without the inoculum was used as a control. Residual PCO from the culture broth and control were extracted with hexane in ratio 1:4.[16] The extracted residual PCO was subjected to gas chromatography analysis as described earlier.[6] The % degradation of PCO was determined as per the following equation:

$$\% \, Degradation = \frac{(A_o - A_S)}{A_o} * 100$$

Where A_o and A_s are the areas under the peaks for control and sample respectively.

Effect of Culture Conditions and Medium Components on Degradation of PCO in Seawater by Lysinibacillus sp. SS1
The culture conditions and medium components such as PCO concentration (1.0-6.0% v/v), inoculum concentration (2.5-15.0% v/v), glucose concentration (2.0-10.0 g/L), agitation speed (60-120 RPM), pH (7.5-8.5), time of incubation (7-28 days) were varied in order, as per levels given in Table 2 using the OFAT approach. The OFAT approach involves studying the effect of one factor on the output by varying the factor and fixing all the other factors. When the next factor is varied, the value of the previously fixed factor is maintained at the level at which the maximum output is achieved and thus the process is continued.[17] The degradation of pH in seawater is maximum at the temperature range of 20-30 °C.[8] Therefore, all the experiments were carried out at the temperature of 27 ± 2 °C.

The effect of these factors on the biodegradation of PCO by *Lysinibacillus* species in seawater was studied by measuring % degradation as per section 2.5. Additionally, the growth of the bacterium in these conditions was determined by checking the OD of the broth at 600 nm.

Optimization of PCO Degradation by Lysinibacillus sp. SS1 in Seawater

Factorial Design for Screening of Factors

Factors such as PCO concentration (X1, %v/v), inoculum concentration (X2, %v/v), glucose concentration (X3, g/L) and pH (X4) were screened for significant effects on degradation of PCO in seawater by *Lysinibacillus* sp. SS1 by a two-level (2^4) full factorial design. The factors were maintained at levels shown in Table 4 and 16 experiments were performed as per Table 5. *Lysinibacillus* sp. SS1 was inoculated into 100 ml of seawater supplemented with PCO and incubated at 27 ± 2 °C for 28 days at 80 RPM. The PCO, glucose, inoculum and pH were maintained as per levels mentioned in Table 5. The % degradation was calculated as described in section 2.5 and was designated as the main response (Y). Factors showing significant effect were optimized further by CCD.

Table 1. Growth of SS1 Isolated from Automotive Service Station Soil at Different Culture Conditions

Organisms/Tests	Growth at Different Temp				Growth at Different pH						Growth at Different NaCl Conc (%w/v)				
	4 °C	25 °C	37 °C	45 °C	2.0	4.0	6.0	8.0	10	12	4	6	8	10	12
SS1	-	+	+	+	-	+	++	++	++	-	++	+	-	-	-

Table 2. Biochemical Characterization of Ss1 Isolated from Automotive Service Station Soil

Organisms/Tests	Oxidase	Urease	Nitrate	H₂S	Phenylalanine deamination	Methyl Red (MR)	Voges Proskauer (VP)	Esculin Hydrolysis	Utilisation Tests			
									Lysine	Ornithine	Citrate	Malonate
SS1	+	+	-	+	-	-	-	+	-	-	+	+

Table 3. Sugar Hydrolysis Results of SS1 Isolated from Automotive Service Station Soil

Organisms/Tests	Sugar Hydrolysis Tests										
	Arabinose	Xylose	Glucose	Lactose	Raffinose	Rhamnose	Cellobiose	Sachharose	Melibiose	Adonitol	Trehalose
SS1	-	+	+	-	-	-	-	-	+	-	-

Table 4. Minimum and Maximum Levels of Factors Used in Factorial Design

Factors	Notations	Levels	
		-1	+1
PCO (%v/v)	X1	3	5
Inoculum (%v/v)	X2	5	10
Glucose (g/L)	X3	6	10
pH	X4	8.1	8.5

Table 5. Factorial Design for Screening of Significant Factors Along with % Degradation Values

Runs	X1	X2	X3	X4	Y
1	3	5	6	8.1	38.39
2	3	5	6	8.5	28.12
3	3	5	10	8.1	34.09
4	3	5	10	8.5	19.23
5	3	10	6	8.1	58.54
6	3	10	6	8.5	39.82
7	3	10	10	8.1	48.28
8	3	10	10	8.5	28.26
9	5	5	6	8.1	35.97
10	5	5	6	8.5	28.84
11	5	5	10	8.1	30.56
12	5	5	10	8.5	24.12
13	5	10	6	8.1	54.35
14	5	10	6	8.5	47.09
15	5	10	10	8.1	38.44
16	5	10	10	8.5	22.68

CCD for Optimization of Significant Factors

Factors such as inoculum concentration (X1, %v/v), glucose concentration (X2, g/L) and pH (X3) were optimized for maximum degradation of PCO in seawater by *Lysinibacillus* sp. SS1 by 2^3 CCD as per five levels in Table 6. The CCD consisted of 20 runs comprising of six star runs and six central runs (Table 7). The *Lysinibacillus* sp. SS1 was inoculated into 100 ml of seawater supplemented with 4.0%

v/v PCO and incubated at 27 ± 2 °C for 28 days at 80 RPM. Glucose, inoculum and pH were maintained as per levels mentioned in Table 7. The % degradation was calculated as described earlier and was selected as response (Y).

Statistical Analysis

All the experiments were performed in duplicates. Statistica (Version 10, Trial version) was used for design of screening

Table 6. Factors and Levels Implemented in CCD

Factors	Notations	Levels				
		-α	-1	0	+1	+α
Inoculum (%v/v)	X1	3.29	5	7.5	10	11.7
Glucose (g/L)	X2	4.63	6	8	10	11.36
pH	X3	7.96	8.1	8.3	8.5	8.63

Table 7. CCD for Optimization of Significant Factors Along with % Degradation Values

Runs	X1	X2	X3	Y
1	5.00	6.00	8.10	24.95
2	5.00	6.00	8.50	24.62
3	5.00	10.00	8.10	25.38
4	5.00	10.00	8.50	24.55
5	10.00	6.00	8.10	26.27
6	10.00	6.00	8.50	31.73
7	10.00	10.00	8.10	55.13
8	10.00	10.00	8.50	64.81
9	3.29	8.00	8.30	24.12
10	11.70	8.00	8.30	39.9
11	7.50	4.63	8.30	27.12
12	7.50	11.36	8.30	38.15
13	7.50	8.00	7.96	23.3
14	7.50	8.00	8.63	30.56
15 (C)	7.50	8.00	8.30	24.71
16 (C)	7.50	8.00	8.30	23.23
17 (C)	7.50	8.00	8.30	27.1
18 (C)	7.50	8.00	8.30	23.71
19 (C)	7.50	8.00	8.30	24.31
20 (C)	7.50	8.00	8.30	24.86

and optimization experiments. The experimental results were analysed by ANOVA to determine the effects of factors on PCO degradation. Response surface plots were designed to visualize the interactive effects of factors. Regression equations were generated to calculate the response at different levels of factors within the optimized range. Optimum conditions of factors were obtained from desirability profiles.

Validation
Degradation experiments were carried out at optimum levels and random levels of factors within the optimized range. The optimized second order model was validated by comparison of experimental response with the model predicted values.

Results and Discussion
Isolation of Bacterium and Determination of PH Degrading Ability
The bacterium designated as SS1 was isolated from the oil contaminated soil of an automotive service station in Moodabidri, Karnataka. It was a creamy white, flat colony with irregular edges (Figure 1A). On grams staining it was found to be gram positive rod (Figure 1B).

The isolate grew on BH plates spread with PCO as a sole carbon source within 72 h and showed a zone of clearance around its growth indicating its ability to utilise PCO as the sole carbon source (Figure 2A). When the isolate was grown in 1.0% v/v PCO with DCPIP as the redox indicator it was observed that the colour of DCPIP changed from blue to colourless within 36 h (Figure 2B). This implies that the bacterium oxidised pH in PCO during which the electrons are transferred to electron acceptors (sulphates, nitrates and oxygen), which in turns reduces DCPIP turning it colourless.[14] *Pseudomonas* sp. WDE11, isolated from refinery effluent decolourised DCPIP in 48 h when grown in BH broth supplemented with 1.0% v/v as a sole carbon source.[18]
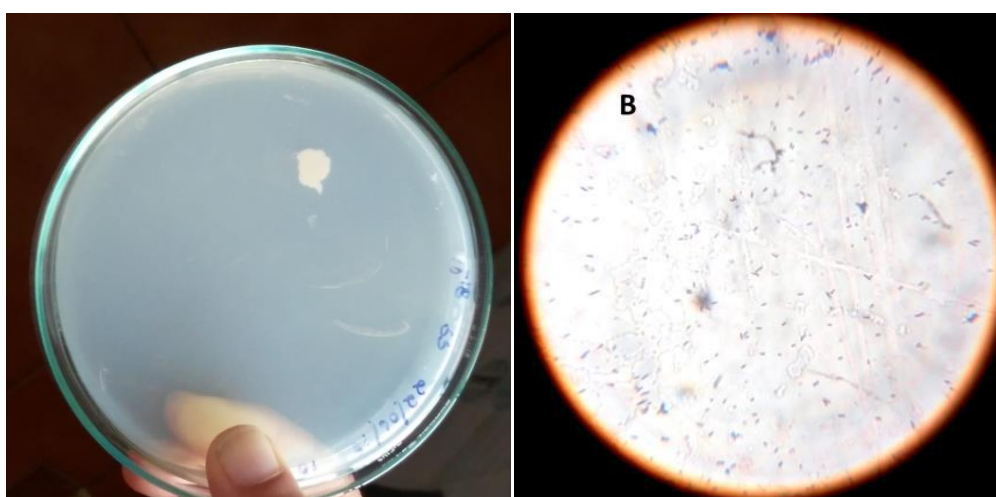

Figure 1. Bacterial Isolate Grown on Nutrient Agar **(A)**; and Microscopic Observation Post Gram's Strain **(B).**

Identification of Bacterium by 16S rRNA Sequencing and Biochemical Characterization
The identification of the isolate by molecular sequencing of *16S RNA* gene was performed and the gene was analysed by BLAST. It was revealed that bacterium exhibited maximum similarity (99.28% identity) to four strains of
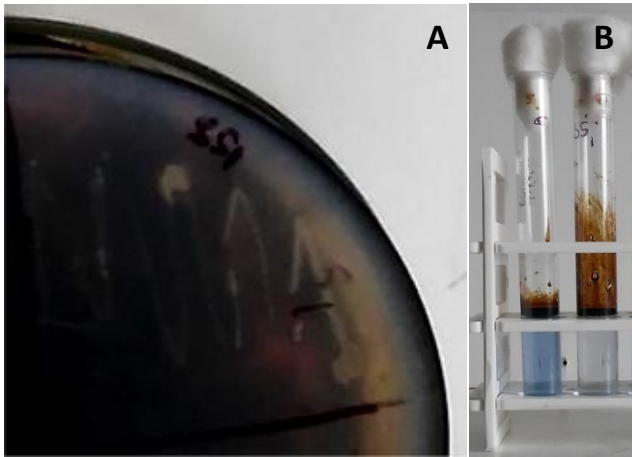
Figure 2. Petroleum Hydrocarbon Degradation Potential of Bacterial Isolate: Clearance of PCO around bacterial isolate within 48 h **(A)**; Decolourisation of DCPIP by bacterial isolate within 36 h (left: Control; right: SS1) **(B)**.

Lysinibacillus genus such as *Lysinibacillus boronitolerans, Lysinibacillus pakistanensis, Lysinibacillus xylanilyticus* and *Lysinibacillus macroides*. Hence it was identified as *Lysinibacillus* species and deposited in GenBank as *Lysinibacillus* sp. SS1 (Accession: MW897754.1) (Figure 3).

The identified *Lysinibacillus* species could grow at a temperature range of 25 °C - 45 °C and in pH from 4.0 to 10.0 (Table 1). Previous reports have shown that isolates of *Lysinibacillus* genus could grow between pH 6.0-10.0.[19] It could tolerate NaCl up to 6% w/v and gave a positive result for oxidase, catalase test, urease, citrate utilisation and esculin hydrolysis (Table 2). The bacterium gave contrasting results for hydrogen sulphide production and nitrate reduction (positive), compared to other isolates of *Lysinibacillus* genus.[20] Among the 11 sugars tested for hydrolysis, the bacterium could only hydrolyse glucose, xylose and cellobiose (Table 3).
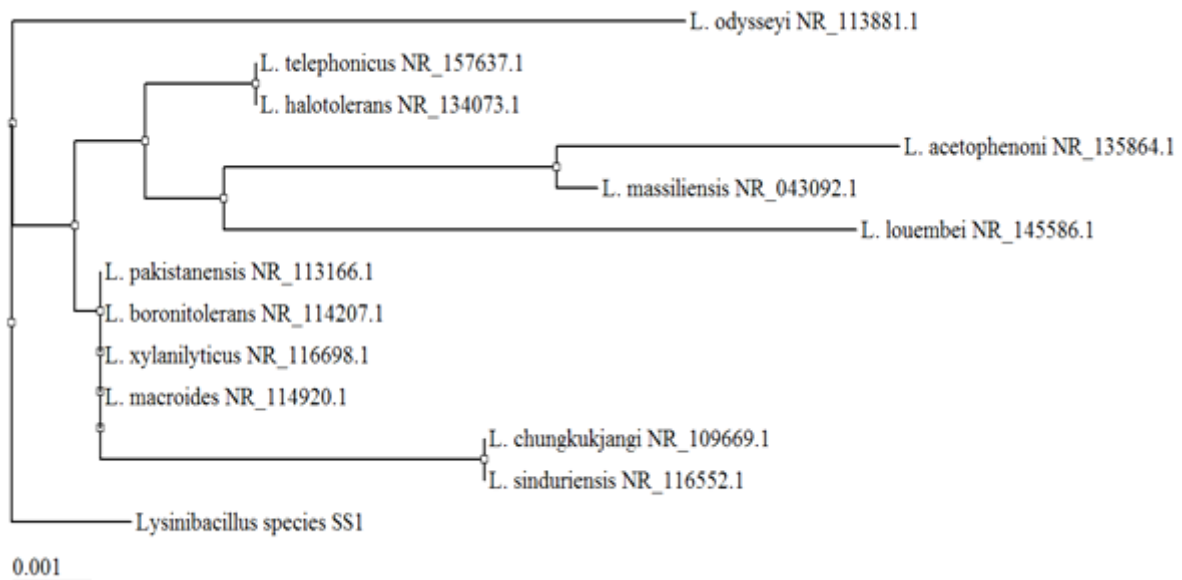
**Figure 3.** Phylogenetic Tree Showing the Relationship of Isolated Lysinibacillus sp. SS1 with Similar Organisms.

PCO Degradation in Seawater by Lysinibacillus sp. SS1

Lysinibacillus sp. SS1 (5.0 %v/v) was grown in seawater supplemented with 1% v/v PCO for seven days. Residual PCO in the treated sample and the un-inoculated control was extracted with hexane. It was observed that *Lysinibacillus* sp. SS1 could degrade 14.16 ± 0.08% of 1.0% v/v PCO. *Pseudomonas aeruginosa, Bacillus subtilis* and *Bacillus cereus*, isolated from oil contaminated sites in Saudi Arabia degraded 12%, 18% and 17% of pH in 1.0% v/v PCO in liquid medium.[21]

Effect of Culture Conditions and Medium Components on Degradation of PCO in Seawater by Lysinibacillus sp SS1

The PCO concentration varied from 1.0 to 6.0% v/v at 5.0% v/v inoculum, 2.0 g/L glucose at 100 RPM for seven days.

The pH of seawater was maintained at 8.1 (measured pH of seawater). It was observed that both the growth and % degradation was maximum at 4.0% v/v and decreased with an increase in concentration (Figure 4A). Maximum growth and degradation activity was observed at 2.0% v/v of PCO in BH broth for *Pseudomonas aeruginosa* strain isolated from seawater.[11] The effect of inoculum concentration on the growth and % degradation between 2.5% v/v to 15.0% v/v was studied at 4.0% v/v, 2.0 g/L glucose, pH at 8.1 at 100 RPM for seven days in seawater. The growth of *Lysinibacillus* species increased with an increase in inoculum concentration whereas % degradation increased up to 7.5% v/v inoculum and then decreased there after (Figure 4B). The degradation of PCO by *Yarrowia lipolytica* increased with an increase in biomass concentration in liquid medium supplemented with
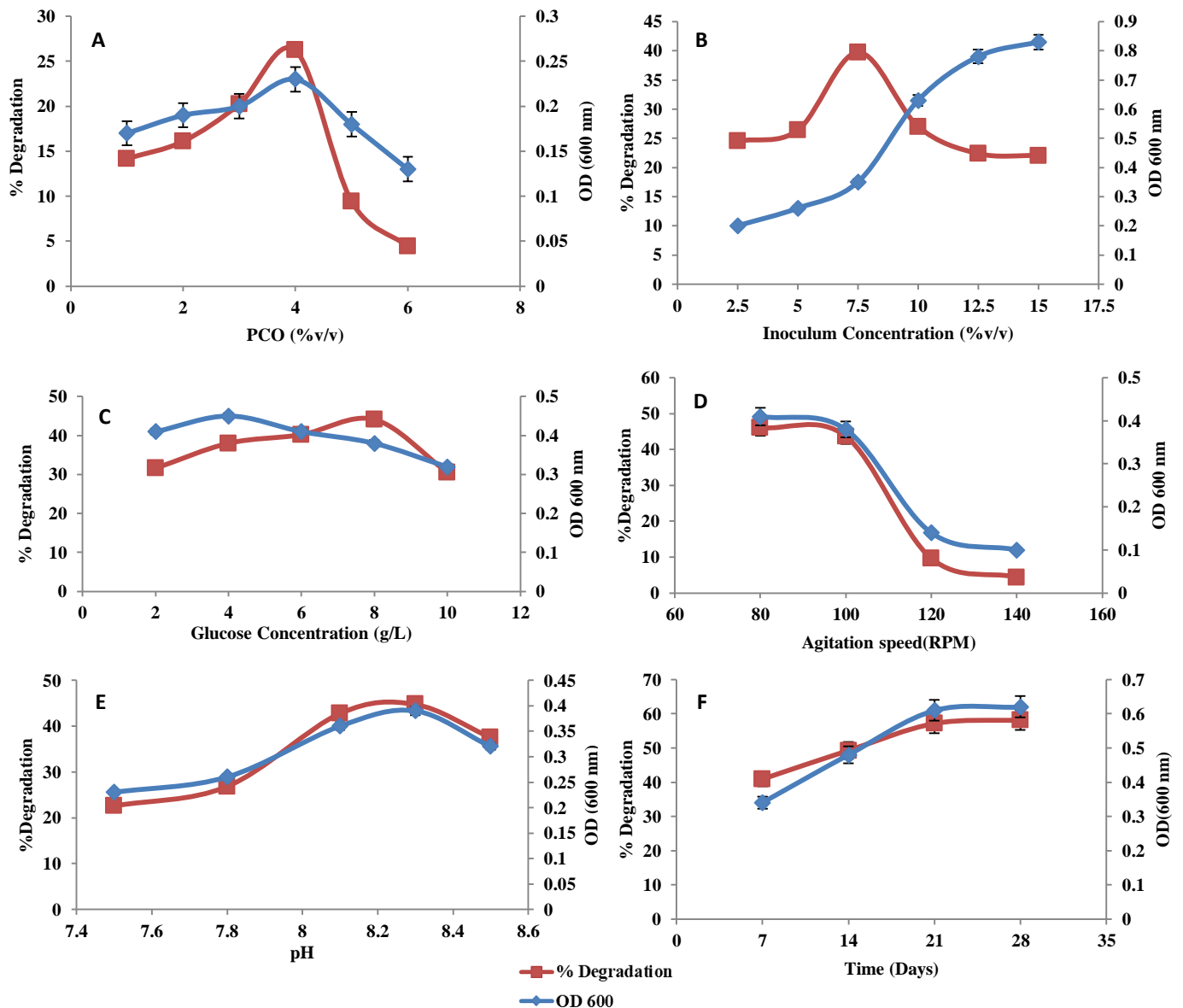
Figure 4. Effect of PCO (A); Inoculum (B); Glucose (C); Agitation Speed (D); pH (E) and Incubation Time (F) on the Degradation of PCO by *Lysinibacillus* sp.SS1 in Sea Water.

3.0% v/v PCO.[22] The degradation of PCO (4.0% v/v) in seawater maintained at pH with 7.5% v/v inoculum, kept at 100 RPM for seven days was performed in the presence of different glucose concentrations (2.0-10.0 g/L). The growth of *Lysinibacillus* species and % degradation was found to be maximum at 6.0 g/L and 8.0 g/L respectively (Figure 4C). When the glucose concentration varied from 5.0 g/L to 15.0 g/L in the presence of 1.5% v/v in seawater, the oil removal by *Pseudomonas* sp. sp48 increased with an increase in the glucose concentration.[13]

The effect on agitation speed (60-120 RPM) on the degradation of PCO (4.0% v/v) in seawater maintained at pH 8.1 with 7.5% v/v inoculum with 8 g/L glucose for seven days was studied. The growth and TPH reduction reached maximum at 80 RPM, beyond which a decrease was noted

(Figure 4D). Above the speed of 80 RPM, the PCO agglomerated on the surface of seawater, hence, the contact between the bacterial cells and PCO was minimal. Also, at higher agitation speeds there is a chance of shearing bacterial cells. Hence, a drastic decrease in the growth and TPH reduction was observed beyond agitation speed of 100 RPM. The pH of the seawater varied between 7.5 and 8.5 since the pH of seawater fluctuates between this range. The % degradation and growth of *Lysinibacillus* species (inoculated at 7.5% v/v) was measured in seawater supplemented with 4.0% v/v PCO and 8.0 g/L glucose maintained at 80 RPM for seven days. The growth and % degradation showed a maximum of 0.39 and 44.4% at pH 8.3 (Figure 4E). The growth and degradation of crude oil was favoured in the presence of more alkaline conditions.

Similar results were reported when *Pseudomonas* sp. sp48 was grown in the presence of PCO in liquid medium.[13] The growth and % degradation increased with an increase in the number of days and reached maximum at 21 days (Figure 4F).

Among six factors, concentrations of PCO, inoculum and glucose along with pH were considered for screening experiments as a bell-shaped pattern in % degradation was observed on their variation indicating scope for further optimization. Agitation speed and time of incubation were fixed at 80 RPM and 28 days as maximum % degradation was obtained at that level.

Table 8. ANOVA Table for Degradation of PCO as Affected by the Independent Factors in Factorial Design

	SS	df	MS	F	*p**
(1)X1	10.04174	1	10.04174	0.438705	0.521384
(2)X2	601.8467	1	601.8467	26.29355	0.000329
(3)X3	456.7047	1	456.7047	19.95257	0.000952
(4)X4	630.872	1	630.872	27.56161	0.000273
Error	251.7847	11	22.88952		
Total SS	1951.25	15			

*Indicates Significance at 95% Confidence Interval

Table 9. ANOVA Table for the Degradation of PCO as Affected by the Independent Factors in CCD

|	SS	df	MS	F	*p**
(1)X1 (L)	806.8198	1	806.8198	37.96898	0.000107
X1 (Q)	166.6741	1	166.6741	7.843689	0.018773
(2)X2 (L)	478.322	1	478.322	22.50986	0.000787
X2 (Q)	189.22	1	189.22	8.904701	0.013712
(3)X3 (L)	50.15568	1	50.15568	2.360328	0.15547
X3 (Q)	37.19012	1	37.19012	1.750168	0.215308
1L by 2L	473.908	1	473.908	22.30213	0.000813
1L by 3L	33.2847	1	33.2847	1.566379	0.239218
2L by 3L	1.721035	1	1.721035	0.080992	0.781764
Error	212.4945	10	21.24945		
Total SS	2394.388	19			

*Indicates Significance at 95% Confidence Interval

Screening of Significant Factors for Degradation of PCO in Seawater by *Lysinibacillus* sp. SS1

Factorial design was used as a screening design to identify the most significant factors on the degradation of PCO by *Lysinibacillus* sp. SS1 for further optimization.[23] The observed values of % degradation along with 16 experiments involving the factorial design is depicted in Table 5. The significance of factors on PCO degradation was tested by Student's t-test for ANOVA (Table 8).[24] Inoculum level (X2), glucose concentration (X3) and pH (X4) were selected as factors having statistically significant positive effect on degradation of PCO ($p<0.05$) and were chosen for further optimization by CCD. pH and glucose depicted negative effect during optimization of degradation of diesel by *Enterobacter cloacae*.[25] The PCO concentration was found to be insignificant and was maintained at 4.0% v/v (center point) for the rest of the experiments.

Optimization of Significant Factors for Maximum Degradation of PCO

Inoculum concentration (X1), glucose concentration (X2) and pH (X3) were optimized for maximum degradation of PCO in seawater by *Lysinibacillus* sp. SS1 using CCD. The observed values of % degradation along with 20 experiments of CCD is depicted in Table 7. Accuracy of results were depicted based on the observation that consistently same values of % degradation was obtained for central runs (Runs 13-20). From the ANOVA table (Table 9) it was observed that both linear and quadratic effects of inoculum and glucose along with their interaction effect had a significant effect on % degradation ($p<0.001$). Linear, quadratic and interaction effects of pH was insignificant ($p>0.05$).
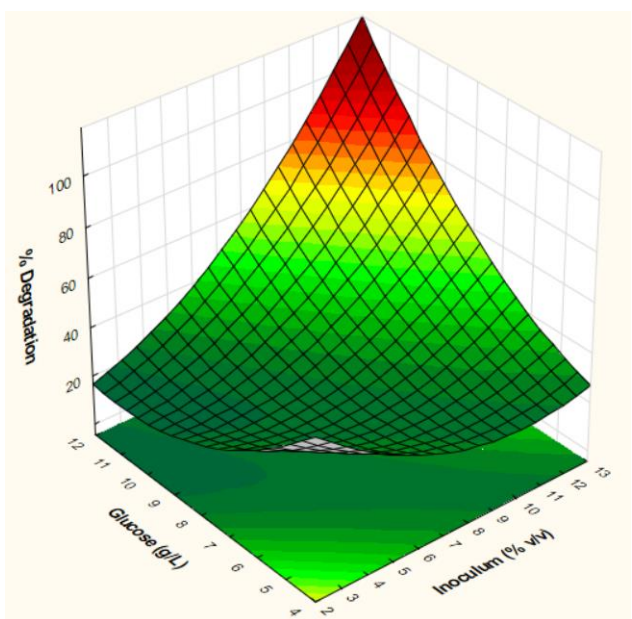
Figure 5. Response Surface Plot Showing the Effects of Interaction of Glucose and Inoculum on % Degradation of PCO in Seawater by *Lysinibacillus* sp. SS1.

Response Surface Graph (RSG) is a three-dimensional plot that displays the effect of the interaction of two independent factors on the dependent response visually.[26] RSG was obtained by plotting inoculum and glucose on *x* and *y* axis respectively while plotting % degradation on the *z* axis (Figure 5). The % degradation was observed to maximum at high concentrations of inoculum and glucose. However, at lower levels of significant independent factors, insignificant variations were observed in % degradation values.

The second order regression equation modelling the degradation of PCO by *Lysinibacillus* species SS1 in seawater as a function of independent factors is given as follows:

$$Y = 3176.8 - 51.26*X1 - 32.70*X2 - 696.96*X3 + 0.544*X1^2 + 0.906*X2^2 + 40.1*X3^2 - 1.539*X1*X2 + 4.08*X1*X3 + 1.16*X2*X3.$$

Determination coefficient (R^2) is a measure of variation between the experimental and predicted values.[27] High R^2 values indicate low variations and high accuracy of the model. An R^2 value of 0.91 was obtained for model describing the degradation of PCO by *Lysinibacillus* sp. SS1

indicating its suitability in predicting % degradation at different levels of factors under an optimized range.

The optimum levels of glucose, inoculum and pH were determined from the desirability profiles. The level of factors achieving desirability of 1.0 was chosen as the optimum level (Figure 6). The optimized conditions were selected as 11.7% v/v inoculum, 11.36 g/L glucose and pH 8.6 at which maximum % degradation of PCO can be accomplished.

Validation of Optimized Model

The experimental values of % degradation was close to model predicted values indicating the validity of the model. At optimized conditions, 84.3 ± 0.13% degradation of 4.0% v/v PCO was achieved in 28 days which was close to the model predicted value (91%). Maximum degradation of 81.63% by *Enterobacter* sp. S-1 was attained at optimum conditions of 30 °C, pH 7.14 and TPH 4.83 g/L.[28] Optimization improved the degradation of PCO in seawater drastically i.e. from 14.16 ± 0.08% (1.0% v/v PCO) to 84.3 ± 0.13% (4.0% v/v PCO). *Pseudomonas* strain, a marine bacteria showed a 2.4 fold increase in degradation of PCO on optimization by a Box Behnken design in seawater.[13]
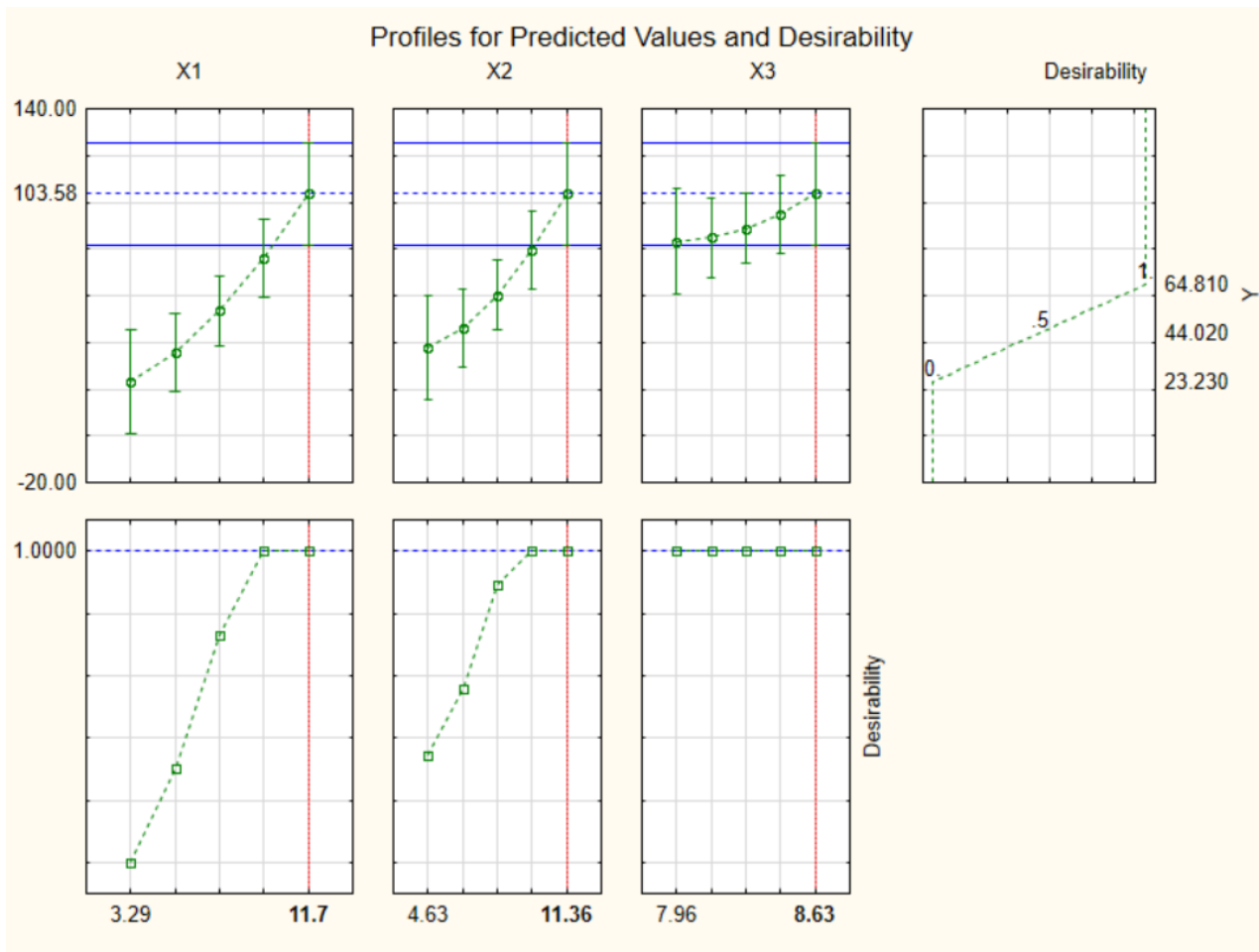
Figure 6. Desirability Profiles Showing the Optimum Levels of Inoculum (X1), Glucose (X2) and pH (X3) for Maximum % Degradation (Y) of PCO by *Lysinibacillus* sp. SS1 in Seawater.

Conclusion

Lysinibacillus sp. SS1, a novel bacterium isolated from automotive motor garage soil sample, contaminated with petroleum hydrocarbons showed the potential to degrade petroleum crude oil in complex conditions of seawater. The effect of factors influencing the process of degradation was studied by using the OFAT approach. The conditions at which maximum degradation of petroleum crude oil occurs in seawater was determined by optimisation using RSM. This study revealed that indigenous micro-organisms present at the pollutant site could be exploited for bioremediation of petroleum crude oil by application of optimisation.

Authors' Contributions

Louella Concepta Goveas: Conceptualization, Investigation, Methodology, Validation, Writing; Melita Alva: Investigation; Jenishia Menezes: Investigation; Amrutha Krishna: Investigation; Ananya Salian: Investigation; Shyama Prasad Sajankila: Validation.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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References

1. Varjani SJ, Upasani VN. Carbon spectrum utilization by an indigenous strain of *Pseudomonas aeruginosa* NCIM 5514: Production, characterization and surface active properties of biosurfactant. Bioresour Technol. 2016;221:510-6. doi:10.1016/j.biortech.2016.09.080

2. Brooijmans RJ, Pastink MI, Siezen RJ. Hydrocarbon-degrading bacteria: the oil-spill clean-up crew. Microb Biotechnol. 2009;2(6):587-94. doi:10.1111/j.1751-7915.2009.00151.x

3. Varjani SJ, Joshi RR, Senthil Kumar P, Srivastava VK, Kumar V, Banerjee C, et al. Polycyclic aromatic hydrocarbons from petroleum oil industry activities: effect on human health and their biodegradation. In Waste bioremediation, 2018 (pp. 185-99). Springer, Singapore. doi:10.1007/978-981-10-7413-4_9

4. Chandra S, Sharma R, Singh K, Sharma A. Application of bioremediation technology in the environment contaminated with petroleum hydrocarbon. Ann Microbiol. 2013;63(2):417-31. doi:10.1007/s13213-012-0543-3

5. Varjani S, Upasani VN. Influence of abiotic factors, natural attenuation, bioaugmentation and nutrient supplementation on bioremediation of petroleum crude contaminated agricultural soil. J Environ Manage. 2019;245:358-66. doi:10.1016/j.jenvman.2019.05.070

6. Goveas LC, Sajankila SP. Effect of yeast extract supplementation on halotolerant biosurfactant production kinetics coupled with degradation of petroleum crude oil by *Acinetobacter baumannii* OCB1 in marine environment. Bioresour Technol Reports. 2020;11:100447. doi:10.1016/j.biteb.2020.100447

7. Al-Hawash AB, Alkooranee JT, Abbood HA, Zhang J, Sun J, Zhang X, et al. Isolation and characterization of two crude oil-degrading fungi strains from Rumaila oil field, Iraq. Biotechnol Reports. 2018;17:104-9. doi:10.1016/j.btre.2017.12.006

8. Al-Hawash AB, Dragh MA, Li S, Alhujaily A, Abbood HA, Zhang X, et al. Principles of microbial degradation of petroleum hydrocarbons in the environment. Egypt J Aquat Res. 2018;44(2):71-6. doi:10.1016/j.ejar.2018.06.001

9. Kachieng'a L, Momba MN. Kinetics of petroleum oil biodegradation by a consortium of three protozoan isolates (*Aspidisca* sp., *Trachelophyllum* sp. and *Peranema* sp.). Biotechnol Reports. 2017;15:125-31. doi:10.1016/j.btre.2017.07.001

10. Goveas LC, Menezes J, Salian A, Krishna A, Alva M, Basavapattan B, et al. Petroleum hydrocarbon degradation in soil augmented with used engine oil by novel *Pantoea wallisii* SS2: Optimisation by response surface methodology. Biocatal Agric Biotechnol. 2020;25:101614. doi:10.1016/j.bcab.2020.101614

11. Thavasi TR, Jayalakshmi S, Thangavel B, Banat IM. Effect of salinity, temperature, pH and crude oil concentration on biodegradation of crude oil by *Pseudomonas aeruginosa*. J Biol Environ Sci. 2007;1(2):51–7

12. Bravo-Linares C, Ovando-Fuentealba L, Mudge SM, Loyola-Sepulveda R. Application of response surface methodology to oil spill remediation. Fuel. 2013;103:876-83. doi:10.1016/j.fuel.2012.09.034

13. Farag S, Soliman NA, Abdel-Fattah YR. Statistical optimization of crude oil bio-degradation by a local marine bacterium isolate *Pseudomonas* sp. sp48. J Genet Eng Biotechnol. 2018;16(2):409-20. doi:10.1016/j.jgeb.2018.01.001

14. Hanson KG, Desai JD, Desai AJ. A rapid and simple screening technique for potential crude oil degrading microorganisms. Biotechnol Tech. 1993;7(10):745-8. doi:10.1007/BF00152624

15. Yan Z, Zhang Y, Wu H, Yang M, Zhang H, Hao Z, et al. Isolation and characterization of a bacterial strain *Hydrogenophaga* sp. PYR1 for anaerobic pyrene and benzo[a]pyrene biodegradation. RSC ADV. 2017;7(74):46690-8. doi:10.1039/C7RA09274A

16. Hamouda RA, Sorour NM, Yeheia DS. Biodegradation of crude oil by *Anabaena oryzae*, *Chlorella kessleri* and its consortium under mixotrophic conditions. Int Biodeterior Biodegrad. 2016;112:128-34. doi:10.1016/j.ibiod.2016.05.001

17. Nayak S, Sajankila SP, Goveas LC, Rao VC, Mutalik S, Shreya BA. Two fold increase in synthesis of gold nanoparticles assisted by proteins and phenolic compounds in *Pongamia* seed cake extract: response surface methodology approach. SN Appl Sci. 2020;2(4):634. doi:10.1007/s42452-020-2348-5

18. Goveas LC, Krishna A, Salian A, Menezes J, Alva M, Basavapattan B, et al. Isolation and characterization of bacteria from refinery effluent for degradation of petroleum crude oil in seawater. J Pure Appl Microbiol. 2020;14(1):473-84. doi:10.22207/JPAM.14.1.49

19. Ahmed I, Yokota A, Yamazoe A, Fujiwara T. Proposal of *Lysinibacillus boronitolerans* gen. nov. sp. nov., and transfer of *Bacillus fusiformis* to *Lysinibacillus fusiformis* comb. nov. and *Bacillus sphaericus* to *Lysinibacillus sphaericus* comb. nov. and Bacillus sphaericus to Lysinibacillus sphaericus comb. nov. Int J Syst Evol Microbiol. 2007;57(5):1117-25. doi:10.1099/ijs.0.63867-0

20. Lee CS, Jung YT, Park S, Oh TK, Yoon JH. *Lysinibacillus xylanilyticus* sp. nov., a xylan-degrading bacterium isolated from forest humus. Int J Syst Evol Microbiol. 2010;60(2):281-6. doi:10.1099/ijs.0.013367-0

21. Al-Dhabaan FA. Morphological, biochemical and molecular identification of petroleum hydrocarbons biodegradation bacteria isolated from oil polluted soil in Dhahran, Saud Arabia. Saudi J Biol Sci. 2019;26(6):1247-52. doi:10.1016/j.sjbs.2018.05.029

22. Ferreira TF, Coelho MA, Rocha-Lero MH. Factors influencing crude oil biodegradation by Yarrowia lipolytica. Brazilian Arch Biol Technol. 2012;55(5):785-91.

23. Uniyal S, Sharma RK, Kondakal V. New insights into the biodegradation of chlorpyrifos by a novel bacterial consortium: process optimization using general factorial experimental design. Ecotoxicol Environ Saf. 2021;209: 111799. doi:10.1016/j.ecoenv.2020.111799

24. Mishra S, Maiti A. Optimization of process parameters to enhance the bio-decolorization of Reactive Red 21 by *Pseudomonas aeruginosa* 23N1. Int J Environ Sci Technol. 2019;16(11):6685-98. doi:10.1007/s13762-018 -2023-1

25. Ramasamy S, Arumugam A, Chandran P. Optimization of *Enterobacter cloacae* (KU923381) for diesel oil degradation using response surface methodology (RSM). J Microbiol. 2017;55(2):104-11. doi:10.1007/s12275-017-6265-2

26. Oberoi DP, Sogi DS. Utilization of watermelon pulp for lycopene extraction by response surface methodology. Food Chem. 2017;232:316-21. doi:10.1016/j.foodchem. 2017.04.038

27. Ramakrishnan V, Goveas LC, Halami PM, Narayan B. Kinetic modeling, production and characterization of an acidic lipase produced by *Enterococcus durans* NCIM5427 from fish waste. J Food Sci Technol. 2015;52 (3):1328-38. doi:10.1007/s13197-013-1141-5

28. Sun S, Liu Q, Chen S, Yu W, Zhao C, Chen H. Optimization for microbial degradation of petroleum hydrocarbon (TPH) by *Enterobacter* sp. S-1 using response surface methodology. Pet Sci Technol. 2019;37(7):821-8. doi:10.1080/10916466.2019.1566256