applied sciences

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Simultaneous Application of Biosurfactant and Bioaugmentation with Rhamnolipid-Producing *Shewanella* for Enhanced Bioremediation of Oil-Polluted Soil

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Received: 6 August 2019; Accepted: 30 August 2019; Published: 9 September 2019

Abstract: In the present study, a combined treatment strategy involving the addition of rhamnolipid, rhamnolipid-producing bacteria (*Shewanella* sp. BS4) and a native soil microbial community for the remediation of hydrocarbon-contaminated soil under pilot-scale conditions was adopted. The isolate BS4 (rhl+), demonstrating the highest emulsification activity and surface tension reduction efficiency, was identified based on 16 S rDNA sequencing as *Shewanella* sp. strain. Growth conditions for rhamnolipid production were optimized based on Central Composite Design (CCD) as 2.9% crude oil, a 54×10^6 CFU g^{-1} inoculation load of soil, a temperature of 30.5 °C, and a pH of 6.7. In situ bioremediation experiments, conducted using hydrocarbon-contaminated soil treated with the combination of rhamnolipid and rhamnolipid-producing bacteria, showed that the inoculated *Shewanella* sp. BS4, along with the indigenous soil microbial community, supported the highest hydrocarbon-degrading bacterial population and soil respiration activity, and this treatment resulted in 75.8% hydrocarbon removal efficiency, which was higher compared to contaminated soil devoid of any treatment.

Keywords: bioremediation; biosurfactant; hydrocarbon-contaminated soil; rhamnolipids; *Shewanella* sp.

1. Introduction

Heavy dependence on petroleum products as a major energy source has led to the contamination of soils by oil-derived hydrocarbons to a great extent [1]. Soil contamination by hydrocarbons can be a result of an improper production process (i.e., oil spills from drilling), transport (i.e., oil spills from tankers or pipelines), storage (i.e., leaking storage tanks), or an improper disposal process. Soil hydrocarbon contamination can affect the functional equilibrium that exists between soil biota and their natural environment. Although numerous physicochemical remediation techniques can be applied to clean up oil-polluted soils, these techniques are expensive and disruptive [2]. The application of biological remediation techniques, such as biostimulation and bioaugmentation, as well

as plant-assisted bioremediation for hydrocarbon-contaminated soils, offers generally cheaper and more environmentally friendly solutions for clean-up of polluted sites [3,4].

Though the use of different hydrocarbon-degrading bacteria, including *Pseudomonas* spp., for the bioremediation of hydrocarbon-contaminated soils, has been successfully implemented, the main problem these bio-degraders encounter is the low solubility and high hydrophobicity of the oil-derived pollutants [5] due to the fact that hydrocarbons are strongly bonded to soil particles and are not available to the bacteria [6]. In this context, the use of biosurfactants for the enhancement of the bioremediation of soil hydrocarbon contamination has been recommended due to their non-toxic nature and biodegradability with no compromise in foaming properties [7]. Biosurfactant application can increase the bacterial cell surface hydrophobicity, allowing the hydrophobic substrates to interact with bacterial cells more effectively [5]

Rhamnolipid-type biosurfactants belong to a group of anionic surfactants comprising of l-(+)-rhamnose and β-hydroxyalkanoic acid units [8]. *Pseudomonas aeruginosa* is the most widely studied rhamnolipid-producing bacterium known for its ability to metabolize a variety of hydrophobic substrates, including n-alkanes, hexadecane, and oils [9]. Di-rhamnolipid synthesis in *P. aeruginosa* occurs in three consecutive enzymatic reactions: 3-(3-hydroxyalkanoyloxy) alkanoic acid (HAA) synthesis catalyzed by rhamnosyltransferase I (*Rhl*A), a glycosyltransfer reaction catalyzed by glycosyltransferase (*Rhl*B) to form mono-rhamnolipids, and the reaction catalyzed by rhamnosyltransferase II (*Rhl*C) that yields di-rhamnolipids [10]. The first two enzymes encoded by *Rhl*A and *Rhl*B genes are both located in the *Rhl*AB operon but are independently involved in rhamnolipid biosynthesis [11].

Studies on the combined use of rhamnolipids and rhamnolipid-producing bacterial strains for the bioremediation of hydrocarbon-contaminated soils are scarce; at the same time, their role in the remediation of crude oil-contaminated sites has been documented by many researchers [12–14]. Though much work has been conducted on rhamnolipid production focused on *P. aeruginosa* and other *Pseudomonas* strains, rhamnolipid production has also been reported for bacteria belonging to other genera, such as *Acinetobacter* [15,16] *Enterobacter* [16], *Pantoea* [16], and *Serratia* [17] Studies have shown that bioaugmentation is most efficient in cases when the augmented bacteria are inherent in the polluted soils [3,18].

On 28 January 2017, near the entire region of the Tamil Nadu shoreline of India, the collision of two merchant vessels caused the spill of hazardous oil and liquid petroleum gas (LPG). This oil spill, accounting for a quantity of about 1.1 metric tonnes, has caused severe damage to the ecosystem, marine life, and presents health hazards to the residents living in the vicinity of the seashore. Moreover, with the operation of more than 700 wells for the extraction of oil and gas in the state of Tamil Nadu and along with 31 additional contract areas awarded as a part of discovered small fields (DSF) by the Cabinet Committee of Economic Affairs (CCEA), the possible soil hydrocarbon contamination and bioremediation of such affected areas is receiving considerable attention in the state of Tamil Nadu in India today.

The aims of the present study were: (1) to isolate and characterize rhamnolipid-producing bacterial strains from various hydrocarbon-contaminated soils in Tamil Nadu, India; (2) to optimize the rhamnolipid production of the obtained bacterial strains; (3) to assess the efficiency of the application of rhamnolipid-producing strains and produced rhamnolipids singly and in different combinations in the bioremediation of oil-polluted soil.

2. Materials and Methods

2.1. Rhamnolipid-Producing Bacterial Strain Isolation and Screening

2.1.1. Soil Sampling and Strain Isolation

To obtain bacterial strains capable of rhamnolipid production, the soil was collected from hydrocarbon-contaminated areas of gasoline refueling stations and automobile service stations located

in Tamil Nadu, India. Bacteria from one gram of soil from contaminated site was enriched using an M9 minimal salt medium comprising sodium phosphate (dibasic) hepahydrate-25.6 gL−^{1}, monopotassium phosphate-6 gL^{-1}, sodium chloride-1 gL^{-1}, ammonium chloride-2 gL^{-1} supplemented with 2% crude oil. The contents were maintained in an orbital shaker at 200 rpm for 7 days. The cultures were isolated using the same M9 minimal salt medium supplemented with 1.5% agar. Plates were incubated at 30 °C for 120 h, and the isolates that showed positive growth were subjected to PCR-based screening for the presence of the *Rhl*AB operon.

2.1.2. Screening for the Presence of the *Rhl*AB operon

The 842-bp fragment of the *Rhl*AB operon covering regions of both RhlA and RhlB genes was amplified using the primers *Rhl*ABf (50–CAGGCCGATGAAGGGAAATA–30 and *Rhl*ABr (50–AGGACGA CGAGGTGGAAATC–30), as described by Ochsner et al. [10]. The reactions were carried out in a 25-µL volume of a reaction mixture (25 mM $MgCl_2$, 10 × PCR buffer, 10 mM dNTP, 5 U/µL Taq, 0.2 µM each primer, and 0.1 µg of template genomic DNA). The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of 30 s of denaturation at 95 °C, then 1 min of primer annealing at 50 °C and 2 min of extension at 72 °C, and a final extension step at 72 °C for 10 min. Separation of PCR products was carried out using agarose gel electrophoresis, and the product testing was performed at 305 nm using a Gel Documentation System (Alpha Imager HP, Protein Simple, San Jose, CA, USA).

2.1.3. Screening of the Bacterial Strains for Biosurfactants Production Ability

A qualitative drop-collapse test was applied for the screening of selected strains for biosurfactant production ability using the method proposed by Bodour and Miller-Maier [19]. The result was considered positive for biosurfactant production when the drop was flat and negative when cultures produced rounded drops [20].

An oil-spreading assay was carried out, as described by Morikawa et al. [21]. In brief, 10 µL of oil was added to the surface of 40 mL distilled water to form a thin oil layer. A total of 10 µL of culture supernatant was gently placed on the center of the oil layer. Clearing zone due to the displacement of oil by the supernatant indicated the positive presence of biosurfactant.

An emulsification index, which measures the emulsification ability of the biosurfactant in the culture broth, was measured according to the method described by Cooper and Goldenberg [22]. Briefly, the assay was carried out in a test tube by adding kerosene to the cell-free culture broth at a ratio of 1:1, followed by which the tubes were vortexed vigorously for two minutes. The mixtures were allowed to settle for 24 h before the percentage of the volume occupied by the emulsion was determined. The equation used to determine the emulsion index (EI_{24}) was as follows:

$$EI_{24}(\%) = \frac{\text{The height of the emulsion layer}}{\text{The height of the total solution}} \times 100 \quad (1)$$

The surface tension of the liquid was measured with a Traube Stalagmometer, as described by Plaza et al. [23]. The surface tension (mN m^{-1}) was calculated using the following formula:

$$\sigma_L = \frac{\sigma w * Nw * \rho L}{NL * \rho w} \quad (2)$$

where σ_L is the surface tension of the liquid under investigation, σ_W is the surface tension of water, N_L is the number of drops of the liquid, N_W *is* the number of drops of water, ρ_L is the density of the liquid, ρ_W is the density of water.

2.1.4. Selected Bacterial Strain Identification

For identification of the biosurfactant-producing bacteria, the 16 S rRNA gene was amplified using the universal primers 8f (5′–AGAGTTTGATCCTGGCTCAG–3′) and 1489r (5′–TACCTTGTTACGACTTCA–3′), as described by Eden et al. [24], with the conditions described below. The reaction was carried out in 25-µL volume containing 25 mM $MgCl_2$, 10 × PCR buffer, 10 mM dNTP, 5U/µL Taq, 0.2 µM of each primer, and 0.1µg of template genomic DNA. The following PCR conditions were applied: initial denaturation at 94 °C for 3 min, followed by 25 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at 70 °C for 2 min, with a final extension at 72 °C for 7 min. The reaction was conducted using Palm cycler^{TM} (Corbet Life Science, Sydney, Australia). The PCR products were purified using a HipureA^{TM} gel purification kit (Himedia, Mumbai, India) and were loaded on 2% agarose gel. The obtained 16S rDNA gene sequence was identified based on the Ribosomal Database Project (RDP) classifier [25] and phylogenetic tree construction was performed using the software MEGA 5.2, using the neighbor-joining (NJ) method with 1000 bootstrap replications, as described by Tamura et al. [26]. The 16S rRNA gene nucleotide sequence was deposited in NCBI under accession number KT763389.

2.2. Rhamnolipid Production of the Isolate Under Laboratory Conditions

2.2.1. Optimization of Rhamnolipid Production

A central composite design (CCD) was applied to optimize the rhamnolipid production of the selected bacterial strain (Box and Wilson, 1951). The experiment was carried out in 500-mL Erlenmeyer flasks containing 200 mL of mineral salt medium (MSM), amended with crude oil as a sole carbon source. Thirty treatments factors (runs) with variable substrate concentrations, pH, temperature, and inoculation loads were the factors used in the experiment (Table S1). The coded values were–α,-1,0,1,+,α, with the corresponding values of parameters: for A (crude oil concentration) 0, 1, 2, 3, and 4%; B (inoculation load) 35×10^2, 10×10^4, 29×10^5, 16×10^7, and 33×10^8 CFU mL^{-1}; C (pH) 3.5, 5.0, 6.5, 8.0, and 9.5; D (temperature) 12.5, 20.0, 27.5, 35, and 42.5 °C. The agitation was maintained at 150 rpm, 120 h, during the experiment. Rhamnolipid production was quantified as rhamnose equivalents (RE) using the phenol-sulfuric acid method with a standard curve prepared using rhamnose [27]. The critical micelle concentration (CMC) of the biosurfactant was determined based on a plot of surface tension vs. surfactant concentration (expressed mg mL^{-1}) [28].

The results of the experiments were analyzed using Design-Expert 9.0 software (Stat-Ease, Inc., Minneapolis, MN, USA), and the following second-order polynomial regression model equation was obtained:

$$Y = \beta_o + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A + \beta_{22} B^2 + \beta_{33} C^{2+} \beta_{44} D^{2+} \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD \quad (3)$$

where Y represents the predicted response (rhamnolipid production in RE), b0 is an intercept, A is the crude oil concentration (%), B is the inoculation load (CFU mL^{-1}), C is pH, and D is the temperature (°C). β1, β2, β3, and β4 are the linear coefficients; β11, β22, β33, and β 44 are the squared coefficients; β12, β13, β14, β23, β24, β34 are the interaction coefficients; A^2, B^2, C^2, D^2, and AB, AC, AD, BC, BD, CD represent the interactions between factors A, B, C, and D.

2.2.2. Extraction, Partial Purification, and Characterization of Rhamnolipids

Crude biosurfactant extraction was carried out, as described by Yakimov et al. [29] Briefly, centrifugation (15 min at 10,000× *g*) was applied to obtain a cell-free supernatant from bacterial cultures grown for 24 h. Cultures with more than 40×10^6 CFU mL^{-1} were used. HCl was added to the supernatant to reduce the pH to 2, and then the suspension was kept at 4 °C for 24 h. The biosurfactant

was extracted with chloroform-methanol (3:1, v:v). Finally, the precipitate was decanted, and the solvent was evaporated.

Thin-layer chromatography (TLC) was used for the identification of the biosurfactants, according to the protocols of Smyth et al. [30]. Silica gel plates were prepared by adding 30 g of silica-G (200–245 mesh) to 60 mL distilled water followed by mixing. Microscopic slides were coated by the slurry and air-dried. Ten microliters of the purified biosurfactant fraction were applied at the point of origin (near the bottom) of each preparative silica gel plate. The silica gel plates containing biosurfactant were eluted using a solution mix of chloroform-methanol-acetic acid (65:15:2, v/v/v) and visualized using iodine vapors.

The retention factor (R_f) value of BS was calculated according to the following formula:

$$R_f = \frac{\text{Distance travelled by the substance}}{\text{Distance travelled by the solvent}} \quad (4)$$

For partial rhamnolipid purification, rhamnolipids specifying TLC spots were dissolved in 1 mL methanol and centrifuged at 15,000× g for 3 min at 4 °C to remove the silica gel. The supernatant was collected, air-dried for 24 h, followed by dissolving the dried fraction in 25 µL methanol. Finally, the rhamnolipids were separated by centrifugation at 1000× g for 10 min and air-drying. The dried fraction was further purified on a silica gel (60–120 mesh) column eluted with a gradient of chloroform and methanol, as described by Sharma et al. [31].

Fourier transform infrared spectroscopy (FT-IR) was used for the characterization of extracted rhamnolipids. The IR spectra of TLC-purified rhamnolipids were recorded using an FT-IR spectrometer (AVATAR 330 FFT-IR system, Thermo Electron Corporation, Madison, WI, USA) in the 4000–400 cm^{-1} spectral region using potassium bromide solid cells. The spectra were recorded for the different pellets and analyzed using the standard methods described previously by Yin et al. [32].

2.3. Soil Bioremediation Study

2.3.1. Experimental Design

A 120-day experiment evaluating rhamnolipid suitability for enhanced oil recovery was carried out with artificially contaminated soil (supplemented with 10% of crude oil), as described by Silva et al. [33]. The soil (normal soil) used in the bioremediation experiment was sandy clay loam soil with a bulk density of 129.6 g cm^3, a pH of 7.4, and an electric conductivity of 0.21 dsm^{-1}, and contained 74.2 mg kg^{-1} of available N, 149.6 mg kg^{-1} of available K, 6.4 mg kg^{-1} of available P, and 0.32% of organic matter. The microbial biomass-C was 321 µg g^{-1}, total bacterial abundance was 24 × 10^5 CFU g^{-1}, fungal abundance was 40 × 10^3 CFU $g^{—1}$, and autochthonous hydrocarbon-degrading bacterial abundance was 30 × 10^2 CFU g^{-1} in the normal soil. The following treatments were included in the experiment: (1) contaminated soil (CS); (2) contaminated soil + normal soil (1:1) (CS+NS); (3) rhamnolipid-treated contaminated soil + normal soil (TS+NS) (1:1 ratio); (4) contaminated soil + normal soil + rhamnolipid-producing bacteria (*Shewanella* sp. BS4) (CS + NS + RPB); (5) contaminated soil + rhamnolipid-producing bacteria (CS+RPB); (6) rhamnolipid-treated contaminated soil + rhamnolipid-producing bacteria (TS + RPB); (7) rhamnolipid-treated contaminated soil + normal soil + rhamnolipid-producing bacteria (TS + NS + RPB). The rhamnolipid-treated soil (TS) was obtained by mixing CS and biosurfactant at a concentration of 2% (v/w). The bacterial treatment was established by adding *Shewanella* sp., 24 × 10^8 CFU g^{-1} at 1% v/w (volume/weight). In all treatments, the soil moisture content was maintained at 50% during the whole experiment. The soil used for the CS and TS treatments was sterilized before the bioremediation experiment.

2.3.2. Estimation of Bacterial Abundance and Activity in Treated Soils

The abundance of hydrocarbon-degrading bacteria was estimated in MSM using 0.1% diesel as a carbon source based on the protocol of Benincasa [34]. The plates were maintained at 28 ± 2 °C for 21

days. Soil basal respiration (SR) was measured according to the method proposed by Bekku et al. [35]. Briefly, in a 250 mL conical flask, 20 g of moist soil was adjusted to a 60% water-holding capacity and amended with 0.2 N NaOH in a 10 mL tube placed inside the flask to trap the CO_2 resulting from mineralization. Ten milliliters of $BaCl_2$ was added to the NaOH trap and incubated in the dark for seven days. The amount of CO_2 production was then measured using titration with 0.1 N HCl, and the respiration rate was calculated (expressed as mg of CO_2 g^{-1} of soil h^{-1}).

Dehydrogenase (DHA) activity in the differently treated soils was determined using 2,3,5-triphenyltetrazoliumchloride (TTC) as a substrate, according to Pepper et al. [36]. The samples were incubated for 24 h at 37 °C, the formation of 1,3,5-triphenyl formazan (TPF) was determined photometrically at 485 nm, and the results are expressed as mg TPF g^{-1} of soil h^{-1}.

2.3.3. Estimation of the Total Petroleum Hydrocarbon Content in Soil

The total petroleum hydrocarbon (TPH) content in contaminated soils was estimated gravimetrically, as described by Peng et al. [37]. Briefly, 25 mL of dichloromethane was added to five grams of petroleum-contaminated soil in a centrifuge tube, and the contents were centrifuged at 10,000× *g* for 5 min. The supernatant was then transferred to an Erlenmeyer flask for the evaporation of dichloromethane at 65 °C, and the amount of residual TPH was determined gravimetrically.

2.3.4. Germination Assay

To test the toxic effect of differently treated soils on plants, a germination assay was carried out according to the methods of Marecik and Biegańska-Marecik [38]. Briefly, 40 g of soil from each treatment was placed into a Petri dish (150 mm) containing sterile soil with a 65% water holding capacity (adjusted with distilled water). Twenty red pepper seeds were gently pressed into the soil and incubated in covered Petri dishes at 25 °C under dark conditions for 72 h. The number of germinated seeds was recorded at periodic intervals of 0, 3, 6, 9, 12, 24 days, and the germination percentage was calculated.

2.4. Statistical Analysis

A minimum of six replications was maintained for each experiment unless mentioned specifically. Data were subjected to one-way ANOVA (analysis of variance) and tested for significance at a *p*-value of 0.05. For the bioremediation experiment, the experimental design adopted was RBD (randomized block design).

3. Results and Discussion

3.1. The Characteristics of Isolated Rhamnolipid-Producing Bacterial Strain

3.1.1. Genetic Characterization of the Strains

In the present study, we isolated rhamnolipid-producing bacterial strains from soils from different hydrocarbon-contaminated sites. Among a total of 37 morphologically and biochemically distinct isolates that were able to grow on a mineral salt medium supplemented with 2% crude oil, the presence of the *Rhl*AB operon was confirmed for four isolates by PCR analysis (Figure S1). Among these four strains, one isolate-labeled BS4 was selected for further study based on its higher biosurfactant production ability. This isolate exhibited a positive reaction to the drop collapse test and had maximum oil displacement—7 mm in diameter. This strain showed a surface tension value of 20.8 mN m^{-1} in MSM and yielded EI_{24}(%) values of 62.4 % and 11.4 % with respect to cell and cell-free extracts, respectively. In comparison, *Pseudomonas aeruginosa* strain MTCC 2453 provided a surface tension value of 26.7 mN m^{-1} and EI of 62.7%. The strain BS4 was identified as *Shewanella* sp. based on 16S rRNA gene analysis. A comparative phylogenetic analysis of this strain with other members of the *Shewanella* genus is provided in Figure 1.

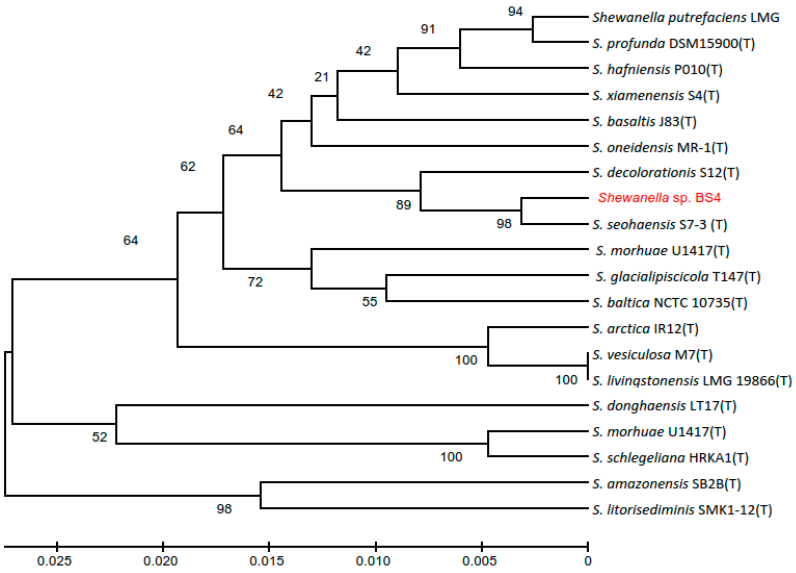


Figure 1. Phylogenetic tree showing the position of *Shewanella* sp. BS4 and its closest neighbors with a bootstrap value of 1000.

The frequent occurrence of *Shewanella* spp. in hydrocarbon-contaminated sites and their role in bioremediation has been reported in the literature [39–41]. For example, *Shewanella putrefaciens* was isolated from the Prestige oil spill in Spain [42], and *Shewanella alga* and *Shewanella upenei*, among predominant biosurfactants-producing bacteria, were isolated from the crude oil-contaminated sediments and seawater samples collected from five stations in the Persian Gulf [43]. Much has been studied with respect to the *Rhl*AB operon that harbors both *Rhl*A and *Rhl*B enzymes that are responsible for mono-rhamnolipid biosynthesis in *P. aeruginosa* [10,11,44,45]. The presence of the *Rhl*AB operon in *Serratia rubidaea* strain SNAU02, responsible for rhamnolipid synthesis, was confirmed by Nalini and Parthasarthy [17].

3.1.2. Production of Biosurfactant by the Isolate under Optimized Conditions

In the present study, four parameters, namely, oil concentration, inoculation load, temperature, and pH, were evaluated for their influence on rhamnolipid production by the isolated bacterial strain. Second-order polynomial equation fitted using multiple regression analysis was applied for the optimization of rhamnolipid production. A second-order response model obtained after regression analysis for rhamnolipid production was described by the following equation:

$$R1 = +2.02 + 0.52 \times A + 0.24 * B + 0.054 * C + 0.021 * D - 0.17 * AB + 0.019 * AC + 0.019 * AD - 0.031 * BC - 6.25 * BD + 0.031 * CD - 0.18 * A^2 - 0.028 * B^2 - 0.12 * C^2 - 0.12 * D^2 \quad (5)$$

where A is the oil concentration, B the inoculation load, C the temperature, and D the soil pH. The parameters of the experimental Central Composite Design (CCD) runs, and the corresponding results are provided in Supplementary Table S1.

The predicted R^2 value (0.827) was in reasonable agreement with the adjusted R^2 value (0.935) since the difference was less than 0.2 (Table 1). Based on the results obtained from the experiment designed according to the CCD model, the optimum concentration of crude oil was 2.9%, the inoculation load was 54×10^6 CFU mL^{-1}, the temperature was 30.5 °C, and the pH value was 6.7. The results of the experiment showed that an increase in crude oil concentration and inoculation load increased rhamnolipid production at a fixed pH and temperature (6.5 and 27.5 °C, Figure 2a). The predicted and experimental values for rhamnolipid production were close to a straight line (Figure 2b).

Table 1. Results of ANOVA for the response surface quadratic model.

Source	Sum of Squares	Degrees of Freedom	Mean Square	*f* Value	*p*-Value
Model	9.73	14	0.69	30.73	<0.0001
A-Crude oil	6.51	1	6.51	287.93	<0.0001
B-Inoculation load	1.35	1	1.35	59.87	<0.0001
C-Temperature	0.07	1	0.07	3.11	0.0980
D-pH	0.01	1	0.01	0.46	0.5076
AB	0.46	1	0.46	20.15	0.0004
AC	5.62	1	5.62	0.25	0.6252
AD	5.62	1	5.62	0.25	0.6252
BC	0.01	1	0.01	0.69	0.4189
BD	6.25	1	6.25	0.02	0.8702
CD	0.01	1	0.01	0.69	0.4189
A^2	0.87	1	0.87	38.49	<0.0001
B^2	0.02	1	0.02	0.96	0.3428
C^2	0.37	1	0.37	16.22	0.0011
D^2	0.37	1	0.37	16.22	0.0011
Residual	0.34	15	0.02		
Lack of Fit	0.29	10	0.02	3.01	0.1179
Pure Error	0.04	5	9.66		
Cor Total	10.07	29			

When bacterial growth and rhamnolipid production were studied in CCD-optimized growth medium, the highest bacterial abundance, 58×10^6 CFU mL^{-1}, was attained after 96 h of incubation, and the rhamnolipid production was observed to be 2.5 mg cell dry weight (CDW) (Figure 2c). The CMC value of the biosurfactant was 27.4 (Figure 2d).

Several studies have utilized CCD for the successful optimization of rhamnolipid production in the case of *P. aeruginosa* strains, such as AT10 [46], ATCC9027 [46], and *P. aeruginosa* SG [47], but no study on rhamnolipid production using *Shewanella* species has been carried out previously. We adopted the response surface methodology for maximization of rhamnolipid production using the optimization of operational factors [48]. Sharon [49] reported that *Shewanella putrefaciens* strain LH4:18 could grow on crude oil under anaerobic conditions without the addition of any other carbon source. However, *Shewanella* species are shown to be facultative aerobes using either oxygen under aerobic conditions or oxidized metals under anaerobic conditions as the terminal electron acceptor [50].

3.1.3. The Characteristics of Rhamnolipids Produced by the Isolate

TLC analysis revealed the presence of mono- and di-rhamnolipids based on the spots at *Rf* values of 0.31 and 0.81 in the biosurfactant mixture produced by the isolated *Shewanella* strain (Figure 3a). These results are per the findings of Nalini and Parthasarathi [17], who observed characteristic bands of the di- and mono- rhamnolipid-type biosurfactants at *Rf* values of 0.38 and 0.85, respectively. FTIR analysis also confirmed the presence of a rhamnolipid-type biosurfactant based on the peaks located at 2929, 1636, 1405, 1222, and 1061 cm^{-1}, which corresponded to the presence of CH stretching vibration, C = O stretching vibration, CH/OH deformation, CH deformation, and C-O stretching, respectively (Figure 3b). Leitermann et al. [51] showed that double bands at 2921 and 2855 cm^{-1} corresponded to C-H vibrations, which represent the hydroxydecanoic acid chain tails of rhamnolipid, while the C=O stretching band at 1730 cm^{-1} is due to the presence of esters and carboxylic acid groups. The area between 1200 and 1460 cm^{-1} represents C-H and O-H deformation vibrations, which are characteristic features of carbohydrate molecules. The other peaks might have occurred due to the protein contamination that occurred during the extraction process.

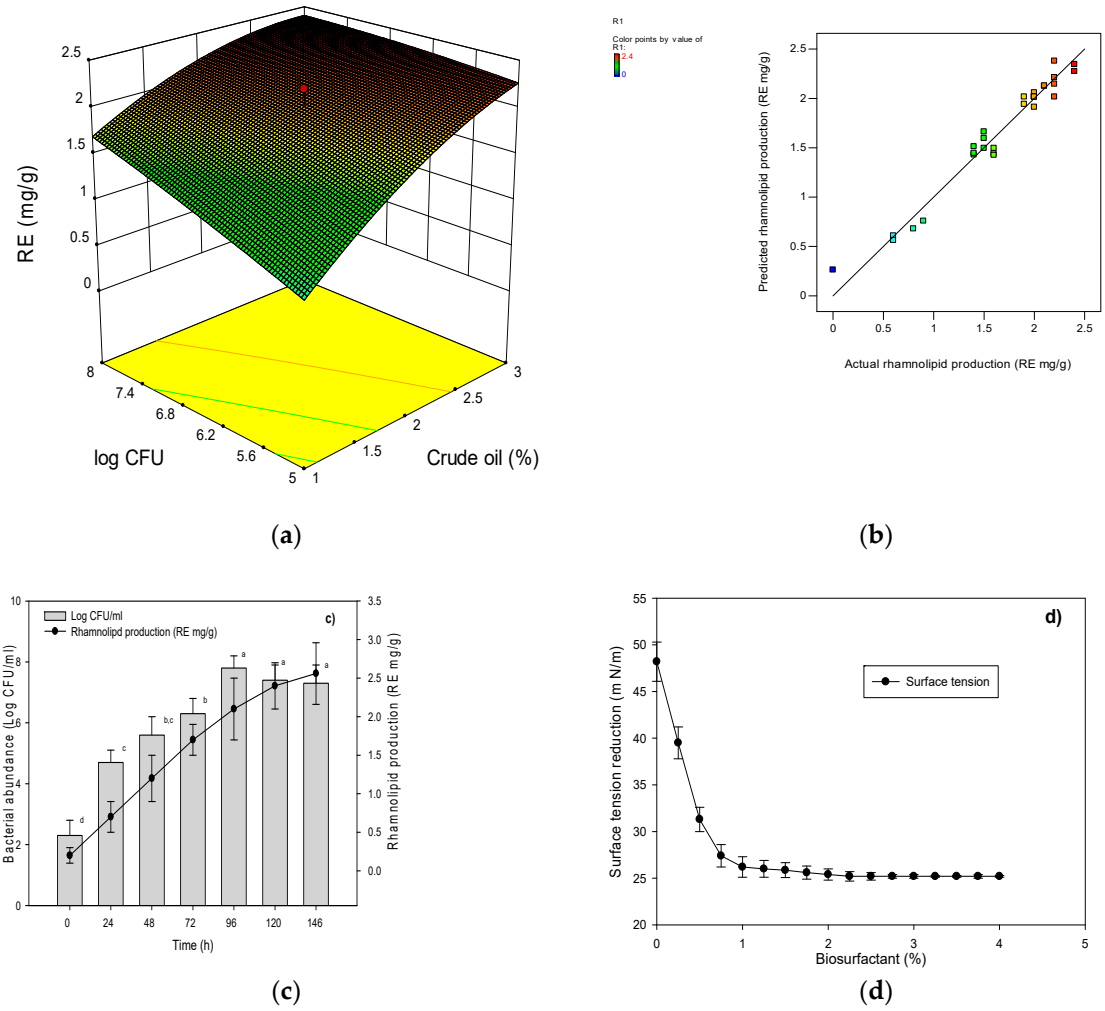


Figure 2. (**a**) Three-dimensional contour plot for maximum rhamnolipid production expressed as RE (rhamnose equivalents) mg g^{-1} cell dry weight as a function of inoculation load and crude oil (%), (**b**) Predicted vs. actual values for rhamnolipid production based on response surface methology (**c**) Growth and rhamnolipid production in RSM optimized media, (**d**) Graph of surface tension vs. concentration of the rhamnolipid. Different lower case letters after values indicate a significant difference at a *p*-value of 0.05 as determined by Duncan's multiple range test.

3.2. Treatment Efficiency of the Contaminated Soil Under Different Bioremediation Conditions

The bioremediation experiment revealed significant differences in the removal efficiencies of hydrocarbons between all treatments. The highest hydrocarbon removal efficiency of 75.8% was observed in the TS + NS + RPB treatment (Figure 4a). Soil respiration (0.6 mg CO_2 g^{-1} h^{-1}) (Figure 4b), DHA activity (5.2 µg TPF g^{-1} soil 24 h^{-1}) (Figure 4c), and the highest hydrocarbon-degrading bacterial abundance (42 × 10^6 CFU g^{-1}) (Figure 4d) were obtained in the TS + NS + RPB treatment by the 90th day of the experiment, and this activity was 44.4%, 80.6%, and 39.5% higher, respectively, than that in the CS alone treatment. At the end of the experiment on day 135, there were no significant increases in hydrocarbon-degrading bacterial population and DHA activity, and a decrease of 17.5% in soil respiration activity was observed.

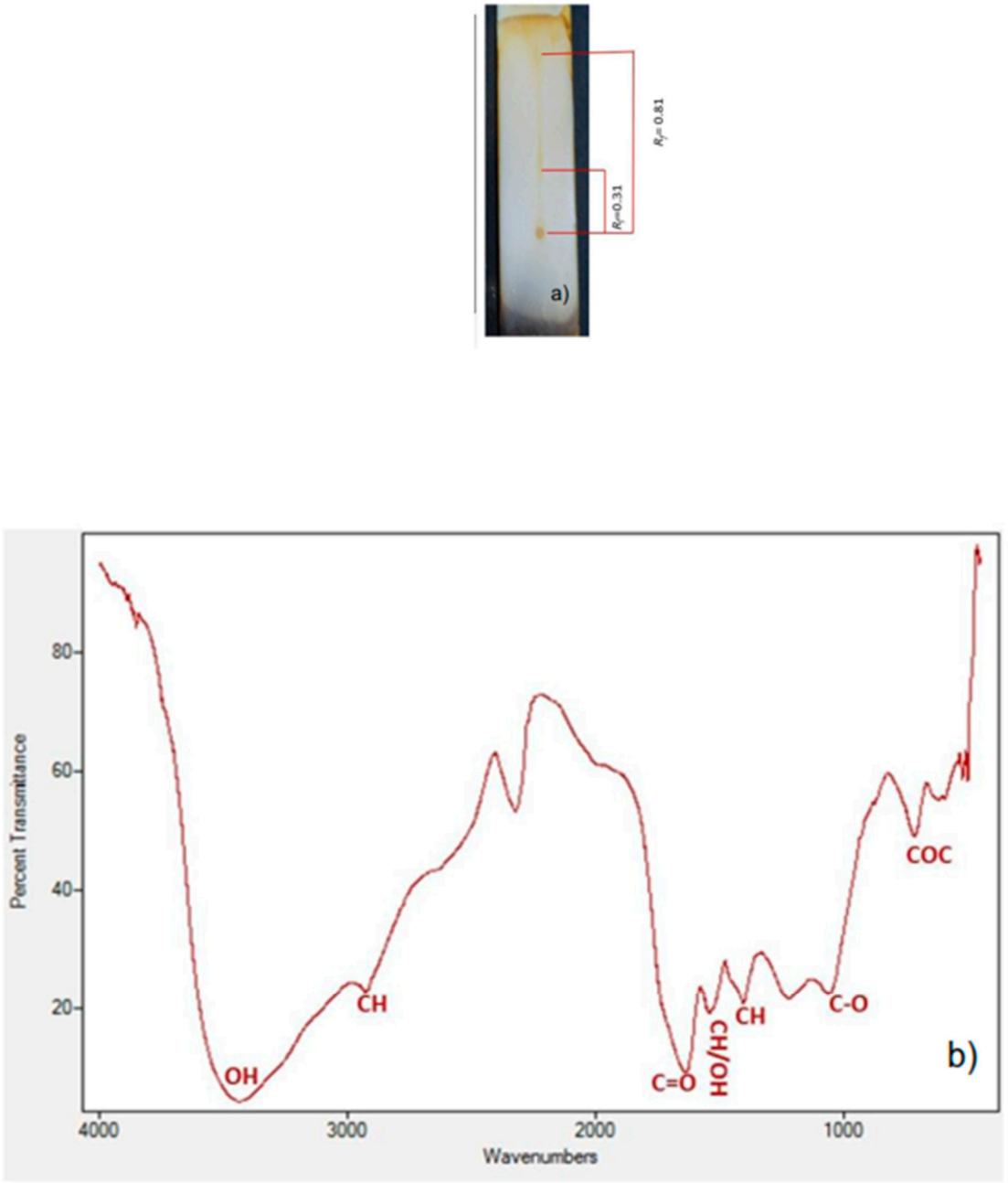
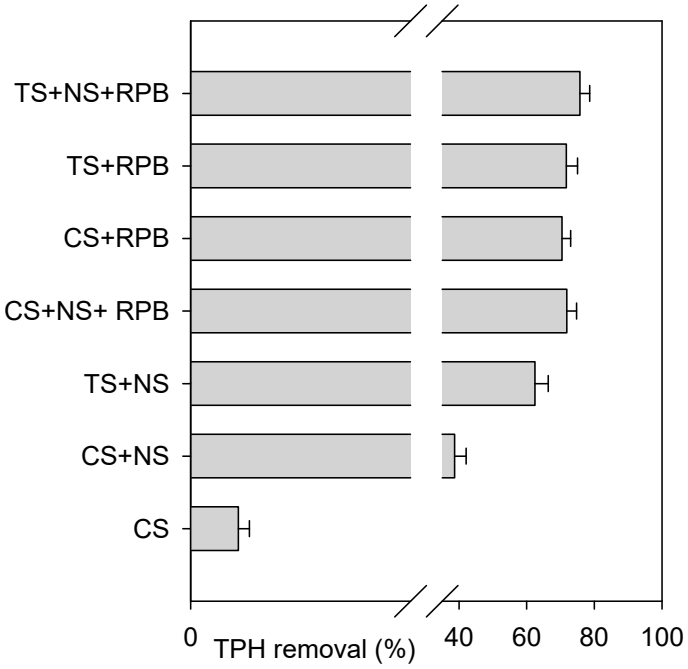
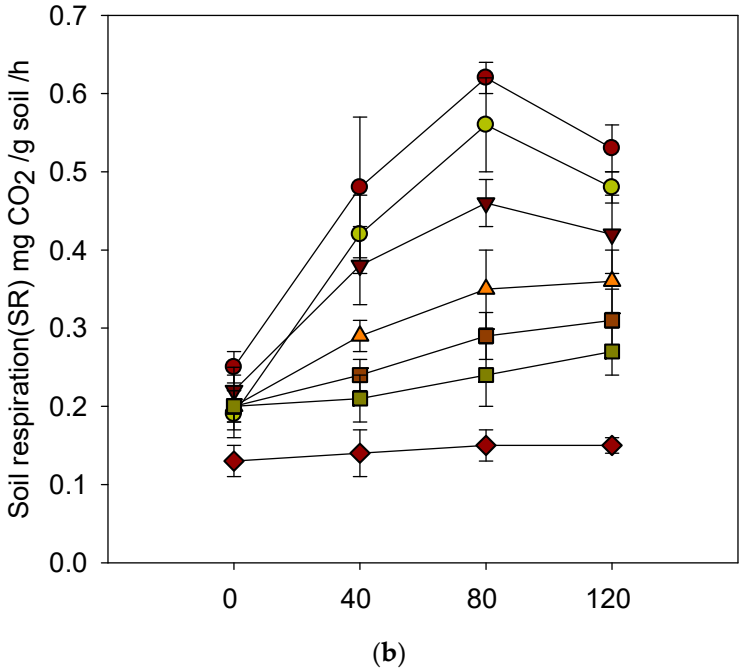


Figure 3. The thin-layer chromatographic slide proving mono- and di-rhamnolipid presence in the extracted mixture (**a**) and Fourier transform infrared spectroscopic (FT-IR) spectra confirming the presence of a rhamnolipid-type biosurfactant (**b**). *Rf*–retention factor (0.31 for di-rhamnolipid and 0.81 for mono-rhamnolipid).

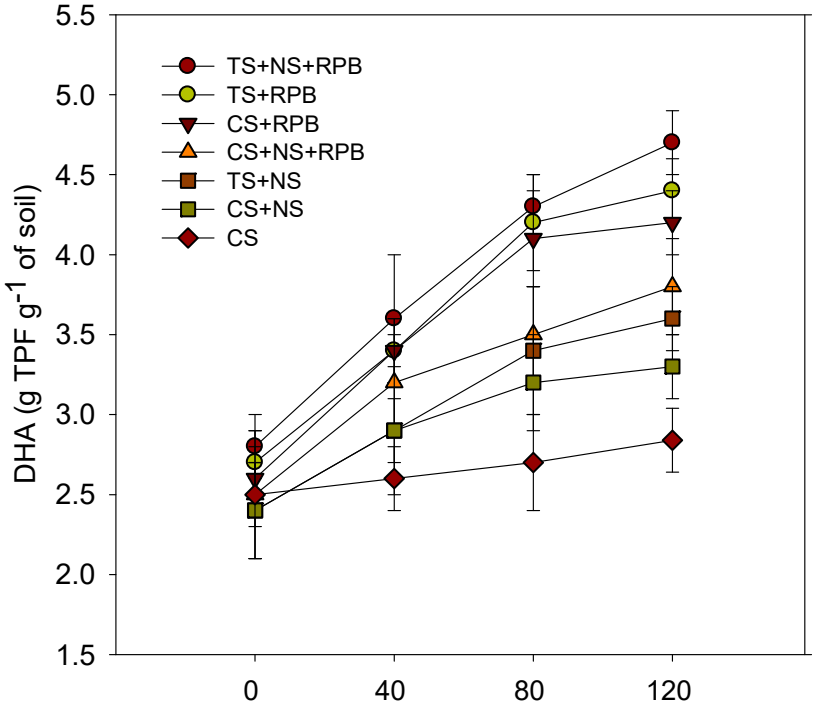


(**a**)

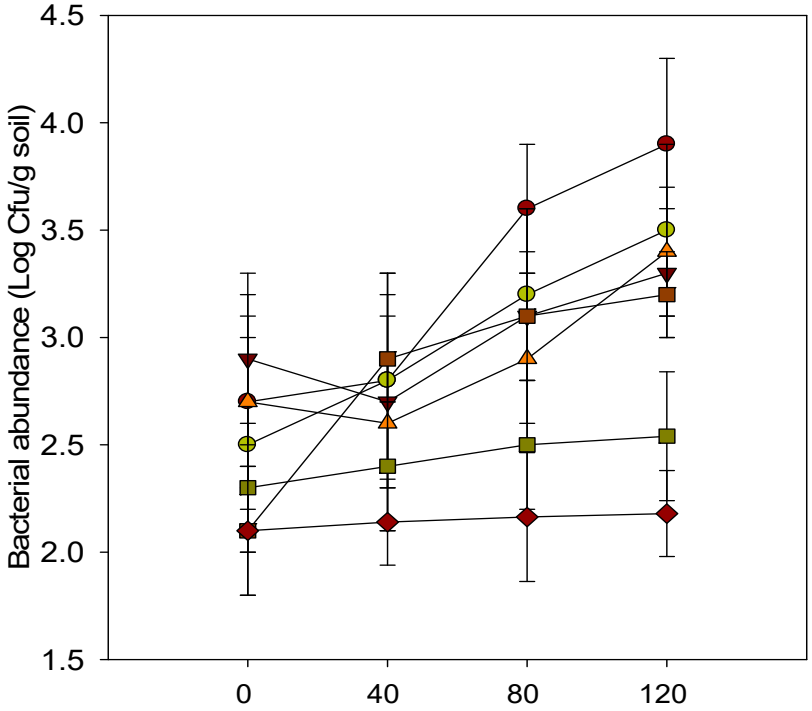


(**b**)

Figure 4. *Cont.*

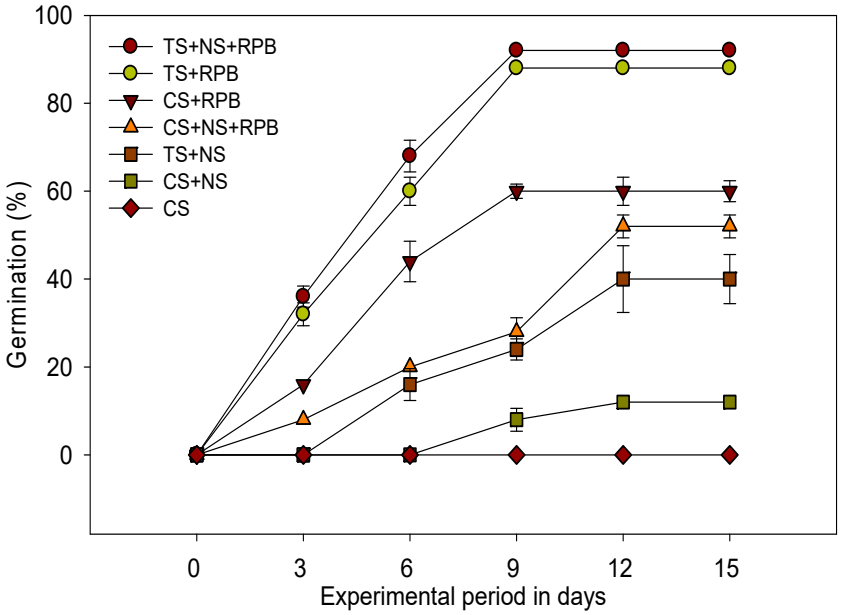


(**c**)



(**d**)

Figure 4. *Cont.*



(**e**)

Figure 4. Reduction in the total petroleum hydrocarbon (TPH, %) (**a**) as influenced by different treatments, soil respiration activity (mg CO_2 g^{-1}, soil h^{-1} (**b**) and soil dehydrogenase activity (mg TPF g^{-1} of soil h^{-1}) (**c**). Hydrocarbon-degrading bacterial abundance under different treatments (**d**) Germination rate (%) of red pepper seedlings (**e**) Different lower case letters after values indicate a significant difference at a *p*-value of 0.05 as determined by DMRT.

Phytotoxicity evaluation of treated soils based on germination studies revealed the highest germination (92%) in the TS + NS + RPB treatment, followed by the TSB + RPB treatment (Figure 4e). These treatments also supported better plant growth in these soils, which was evident by an 86.9% increase in germination percentage compared to contaminated soil (CS) (Figure S2). Soil respiration and DHA activity are the measures of total microbial activity in soil [52–54], and any change in organic matter quantity or quality can be observed as a change in soil respiration [54,55] and/or in DHA activity [56]. The results of the present study clearly showed that the addition of rhamnolipids improved the activity of *Shewanella* sp. BS4 and the native soil microbial community, which in turn improved the hydrocarbon bioremediation process. Although the native soil microbes did not play a major role in the bioremediation process, the oil-polluted soil bioremediation was more efficient in the case of the simultaneous application of rhamnolipid and *Shewanella* sp. BS4.

The results from this study showed that the addition of rhamnolipids might enhance oil biodegradation efficiency by acting as mediators, increasing the mass transfer rate, and thus making oil compounds more bioavailable for soil-indigenous microorganisms. Also, rhamnolipids may induce changes in the properties of cellular membranes of bacteria by enhancing hydrophobicity, which increases microbial adherence [57]. The single-use of rhamnolipids in oil bioremediation may require the addition of nutrients, such as nitrogen or phosphorus, to the soil. For example, the augmentation of rhamnolipids and nutrients enhanced crude oil reduction and increased the abundance and expression of the *alkB* gene in soil [58].

Our results indicated that the simultaneous application of rhamnolipids and rhamnolipid-producing bacteria enhanced the oil bioremediation process in soil more than the individual use of these treatments. However, the bioaugmentation of oil-polluted soil with the rhamnolipid-producing *Shewanella* BS4 strain without rhamnolipid amendment provided nearly as effective oil removal efficiency.

This approach seems especially promising in the case of in situ soil bioremediation, as biosurfactants are produced on-site, and external addition of biosurfactants can be avoided. However, adding bacterial consortia to contaminated soil at field scale always evolves competition with indigenous bacteria and putative loss of inoculum and wanted degradation efficiency, but that will depend on the soil conditions and quality of inoculum in each case. Szulc and co-workers [59] observed that bioaugmentation with a selected consortium of bacterial strains contributed to the highest diesel oil biodegradation efficiency, whereas the addition of rhamnolipids did not notably influence the treatment process in a field-scale study.

Biosurfactants, especially rhamnolipids, can be combined with phytoremediation for effective remediation of polluted soils [60]. Almansoory et al. [61] reported that the application of biosurfactants enhanced the phytoremediation efficiency of gasoline-contaminated soils by up to 93.5% under pilot-scale conditions.

4. Conclusions

In the present study, we adopted a new approach of using rhamnolipids along with rhamnolipid-producing bacteria for the bioremediation of hydrocarbon-contaminated soils. For the first time, a rhamnolipid-producing bacterial stain identified as *Shewanella* sp. was isolated from polluted soils of Tamil Nadu. Rhamnolipid production by this strain was dependent on the concentration of crude oil, the inoculation load, the temperature, and the pH of the growth media. Our results confirmed that simultaneous addition of rhamnolipids and a rhamnolipid-producing bacterial stain enhanced soil microbial activity and improved remediation of crude oil-contaminated soil. Though the results of the present study are encouraging, possible success under field conditions always depends upon the ability of the microbial strain to survive under harsh environmental conditions, ability to adapt to grow with the nutrients present under field conditions, and ability to successfully colonize in hydrocarbon-contaminated sites. Future studies at large scale are needed to assess how combined use of rhamnolipids along with rhamnolipid-producing bacteria could be linked to biostimulation and phytoremediation approaches for the treatment of oil-polluted soils. Also, such studies could provide data for performing cost-benefit analysis and life cycle assessment of such combined bioremediation approach.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/9/18/3773/s1, Figure S1: Screening of the *Rhl*AB gene with lane 3 representing the strain BS4. Amplification of 842 bp fragment was observed using the primers *Rhl*ABf and *Rhl*ABr, Figure S2: Photograph of 15-day-old red pepper seedlings under different treatments (no germination was observed in crude oil-contaminated soil with no amendments, and the seeds had a normal 97% germination rate under normal conditions). Table S1: Experimental central composite design (CCD) runs in design Design-Expert 7.1 and corresponding response (results).

Author Contributions: Conceptualization, M.M.J. and S.T.; methodology, M.M.J., A.B., and S.T.; writing—original draft preparation, M.M.J., S.T., R.G., A.B., D.S., P.R., A.J.H., J.T., and M.T.; writing—review and editing, M.M.J., J.T., and M.T.; funding acquisition, S.T.

Funding: This study was supported by the Estonian Research Council grant PRG548 and EU Horizon 2020 grant No. 679266.

Acknowledgments: The authors are grateful to VELS University for support. The authors also thank the Department of SAIF IIT Guindy, Chennai, India, for FTIR analysis.

Conflicts of Interest: The authors declare no conflict of interest.

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