Surfactant-enhanced Bioremediation of *n*-Hexadecane-contaminated Soil Using Halo-tolerant Bacteria *Paenibacillus glucanolyticus* sp. Strain T7-AHV Isolated from Marine Environment

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A halo-tolerant bacterial strain *Paenibacillus glucanolyticus* sp. strain T7-AHV isolated from marine environment was used for bioremediation of *n*-hexadecane-contaminated soil. Soil/water ratio, initial inoculums volume, surfactant addition, *n*-hexadecane concentration, and salinity were investigated. The possibility of biosurfactant production by isolated strain was also studied, and the results demonstrated that it was not a biosurfactant producer, based on measurement of the surface tension of culture broth. Both tween 80 and rhamnolipid enhanced the biodegradation of *n*-hexadecane significantly up to 44 and 46 %, respectively. A biodegradation rate of 39.7 % was observed at salinity level of up to 2 %, and the biodegradation efficiency decreased significantly at higher salinity concentrations. A natural hydrocarbon-contaminated soil sample with total petroleum hydrocarbon (TPH) concentration of 1437 mg kg^{-1} was subjected to bioremediation using the selected conditions of operational parameters, and a biodegradation rate of 22.1 % was obtained.

Keywords:
bioremediation, soil contamination, *n*-hexadecane, *Paenibacillus glucanolyticus* sp. strain T7-AHV, surfactant

Introduction

Different hydrocarbons (HCs) are discharged to the soil, shorelines, and seas through industrial petroleum-related activities, such as wastes originated from refineries, petroleum drilling in shoreline areas, production, marine transportation, and accidental spills. Alkanes, which are saturated HCs, are the major parts of mineral oil in which *n*-hexadecane would be the major substance. *n*-Hexadecane has low solubility of $5.21 \cdot 10^{-5}$ mg L^{-1} at 15 degree of siliceous and high partitioning coefficient of 9.1 log K_{ow} in water. In addition, regarding its high inflammability, *n*-hexadecane has the lowest reactivity among the organic compounds[1].

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Remediation of hydrocarbon-contaminated sites has been practiced using different processes, which have showed either economic or operational restrictions. Of the applied methods, bioremediation has been known as a reliable technique for decontamination of polluted environments. An efficient bioremediation technique requires overcoming restrictions like slow response in cold climatic conditions, high salinity of the contaminated site, hydrophobic nature of HC which limits the bioavailability, and inefficiency in the case of refractory contaminants[2,3]. Furthermore, biodegradation of HCs in contaminated resources using microorganisms is considered a time-consuming process. Isolation and acclimation of bacteria capable of HC degradation is an acceptable and efficient way to overcome this limitation[4,5]. Microbial strains are reported as microorganisms capable of decomposing different HCs[6]. Bacterial strains belonging to different genera, including *Alcanivorax, Trichoderma, Bacillus, Acinetobacter, Burkholderia, Cellulomonas, Klebsiella, Marinobacter, Microbacterium, Micrococcus, Achromobacter,* and *Pseudomonas* have been reported in biodegradation of HCs[7–14]. In addition to isolation of HC-degrading microorganisms, the efficiency of bioremediation highly correlates with the inherent biodegradability of the organic contaminant, solubility, and consequently, the bioavailability of HCs to bacteria and providing a desired environment[15]. Generally, two factors are effective in *n*-alkanes biodegradation: i) activation of metabolic enzymes, even in extreme environments such as high saline medium, and (ii) transportation of alkanes into the bacterial cell[16].

Bioavailability of HCs would be increased through either dissolution and emulsification or adherence and direct HC uptake from the oil–water interface. Surfactants have a significant effect on increasing the cell uptake through pseudo-solubilization of HC and increasing the interfacial area by lowering the surface tension of the solution[17]. In other words, a mass transfer occurs where HC complexes with surfactant and then complex becomes dissociated within a hydrophobic compartment of the cell wall. High salinity content of medium can negatively impact the bacterial metabolism and cause plasmolyzation of bacterial cells[18,19]. The negative influence of salinity on the efficiency of biological approaches like bioremediation systems in soil and marine environments have been reported by researchers[20–22].

Since HC-contaminated soils and sediments are usually highly saline environments, in the present work, the role of newly isolated halo-tolerant bacteria from contaminated marine areas for *n*-hexadecane degradation through involvement of the external addition of rhamnolipid biosurfactant and chemical surfactant was investigated. Based on literature review, isolation and characterization of *Paenibacillus glucanolyticus* sp. strain T7-AHV as a halo-tolerant strain capable of HC degradation has not been reported, and this is the first study demonstrating its oxidative degradation capabilities for bioremediation of HC in saline contaminated soils.

Materials and methods

Materials

Rhamnolipid biosurfactant was externally created by *Pseudomonas aeruginosa* SP4, according to the method described in our previous study[23]. GC-grade *n*-hexadecane, *n*-hexane, methanol, trichloromethane and tween 80, sodium dodecyl sulphate (SDS), sulphuric acid (95–97 %), sodium hydroxide and constituents of culture media were all of analytical grade and obtained from Merck, Germany. Deionized water was used for the preparation of working solutions.

Isolation of *n*-hexadecane-degrading bacteria

For isolation of bacterial strain, 50 mL of seawater from contaminated areas of the Persian Gulf, Iran, was added to a 500-mL flask containing 200 mL phosphate mineral salt (PMS) medium. The medium was stirred for 30 min and 10 mL of the settled solution was transferred to a 500-mL flask containing 200 mL pre-sterilized PMS. The PMS medium constituents were as follows: (g L^{-1}): K_2HPO_4: 6.3, $CaCl_2{\cdot}H_2O$: 0.1, $MgSO_4{\cdot}7H_2O$: 0.1, $MnSO_4{\cdot}H_2O$: 0.1, $FeSO_4{\cdot}7H_2O$: 0.1, and 1 mL L^{-1} of trace elements solution. The ingredients of solution containing trace elements were as follows (g L^{-1}) H_3BO_3: 0.03, $CoCl_2{\cdot}6H_2O$: 0.02, $ZnSO_4{\cdot}7H_2O$: 0.01, $CuSO_4{\cdot}2H_2O$: 0.001, Na_2MoO_4: 0.006. *n*-Hexadecane was applied for providing energy and carbon to enrich *n*-hexadecane-degrading inoculums, and poured into the medium at a concentration of 2 % (V/V)[23]. Salinity was set to 0.5 % using NaCl. All of the culture mediums were sterilized before experiments. The incubation of flask was carried out at 37 °C and 150 rpm for 7 days. Growth was monitored by measurement of absorbance at 600 nm. After the first run of incubation (7 days), 10 mL of culture medium was transferred into a new 500-mL flask containing 95 mL fresh *n*-hexadecane + PMS medium. Enrichment and refreshing of the growth medium was repeated eight times. For isolation of pure *n*-hexadecane-degrading bacterial strains, 1 mL of culture medium was diluted to 10^{-4} times, spread onto *n*-hexadecane-coated PMS agar plates, and incubated at 37 °C for 72 h. The salinity was adjusted to the desired values. Some colonies appeared, and

the morphologically distinct colonies were determined using repetitive streaking onto the *n*-hexadecane + Agar PMS medium, and selected based on growth ability.

Identification of bacterial isolate using 16S rRNA

Genetic identification of the isolated bacterium was done by 16S ribosomal DNA (rDNA) sequencing using universal 27F and 1525R primers of the genomic DNA[24]. The genomic DNA was extracted using boiling method[25]. Polymerase chain reaction (PCR) was carried out using Biometra thermal cycler (Whatman Biometra, Göttingen, Germany). A 50-μL solution containing Lyophilized I-Taq Maxime PCR Premix (iNtRON Biotechnology, Korea), 41 μL double distilled H_2O, 5 μL extracted DNA and 2 μL of each primer (10 pmol) was applied for performing the reaction of amplification.

The PCR conditions were as follows: initial denaturation step (95 °C, 5 min) followed by 35 cycles of denaturation (94 °C, 20 s), annealing (56 °C, 30 s), polymerization (72 °C, 90 s), and a final extension step at 72 °C for 15 min. The purification of PCR products was carried out by means of QIAquick Gel Extraction kit (Qiagen, Germany), and direct sequencing in both strands via an ABI PRISM 3730xl DNA Analyzer and the BigDye Terminator v3.1 cycle sequencing kit chemistry (Applied Biosystems, USA) under contract by Bioneer Inc. (South Korea). The edition and assembling of Sanger sequence reads were carried out by means of DNA Sequence Assembler v4 (2013). The analysis of sequence data was performed using BLASTn from NCBI (http://www.ncbi.nlm.nih.gov), and classified using the EzTaxon server http://ezbiocloud.net/eztaxon. The phylogenetic analysis was performed in MEGA 6.0 by neighbour-joining algorithm using Kimura-2-parameter model and 1000-bootstrap replication.

Biosurfactant production assay

The possibility for presence of biosurfactant was examined with oil displacement method. Briefly, 50 μL crude oil was added to a 15-cm diameter plate containing 40 mL distilled water. The supernatant of culture broth (15 μL) was then added to the oil surface. The clear zone diameter was investigated and compared with sodium dodecyl sulfate (SDS) as standard. Development of a hole in the surface of the oil layer is an indication of biosurfactant production[26]. In addition, measurement of culture broth surface tension was performed as a valid indication for presence of biosurfactant during incubation period. A tensiometer (Khushboo Sci. Co., Mumbai) was used to determine the surface tension of the solution according to principles of Du-Noüy's ring method. Du-Noüy ring method is used for measuring the surface tension of a liquid and the interfacial tension between two liquids. The force referred to the wetted length acting on a ring as a result of the tension of the withdrawn liquid lamella when moving the ring from one phase to another is measured in this method.

The average value of three data was reported as a final value. All measurements were made on cell-free supernatant obtained after centrifuging the culture medium at 10000 g for 10 min[27,28].

Table 1 – *Characteristics of soil sample*

Characteristic	Value (%)	Characteristic	Value (%)
Sand	35.18	Na_2O	5.58
Clay	25.64	MgO	2.42
Silt	39.18	Al_2O_3	6.31
Moisture content	8.22	SiO_2	55.86
Fe_3O_4	4.98	P_2O_5	0.65
Sr	0.023	K_2O	1.34
Zr	0.007	CaO	9.39
L.O.I	9.58	Cl	3.86

Soil preparation

All soil samples derived from the contaminated soils near the marine oil fields in the southwest of Iran. Soil samples were prepared using soil cores from surface layers (0–40 cm), which were air-dried and sieved to reach 2 mm particle size distribution. Homogenization of soil samples was obtained by simultaneous shaking and rinsing with acetone to remove any possible organic matter, followed by sterilization and storing in plastic cold boxes. Samples were synthetically contaminated with *n*-hexadecane at desired pollution levels of 500, 1000, and 5000 mg kg^{-1} (dry weight). The pure *n*-hexadecane was first solubilized at *n*-hexane and then added to the samples. It was mixed vigorously and left under the hood for 24 h for complete evaporation of *n*-hexane. X-ray fluorescence (XRF) analysis was performed to determine the soil chemical characteristics. The characteristics of soil samples are presented in Table 1. The studied soil was characterized as a clay-loam type based on texture assay analysis with specific surface area of 11.42 $m^2\ g^{-1}$. In addition, a naturally contaminated soil sample of the same origin and without processing was used as a naturally contaminated sample.

Biodegradation experiments

Batch experiments were performed in 500-mL flasks. In brief, for each experimental sample, 20 g

(dry weight) pre-prepared soil was transferred to considered flasks. Effect of operational parameters, including varying volumes of inoculums with $OD_{600\ nm}$ = 1 (5, 10, 15 and 20 mL), water content (50 %, 100 % and slurry), surfactant kind (rhamnolipid biosurfactant, Tween 80 and SDS), salinity levels (0.5, 1, 1.5 and 2 %), and different initial *n*-hexadecane concentrations (500, 1000 and 5000 mg kg^{-1}) were investigated based on one factor at a time experimental design[15,23]. The flasks were shaken vigorously at 180 rpm and 37 °C. Sampling for determination of residual *n*-hexadecane concentrations and microbial growth was carried out on a weekly basis with total duration of 35 days.

For extraction of HC, 3-g air-dried soil samples were subjected to shaking at 180 rpm for 30 min, and sonication for 10 min using a 30-mL solvent mixture of trichloromethane and methanol (2:1, v/v). The extracted solutions were evaporated. The residual was solved in 4 mL *n*-hexane for quantitative analysis.

The TPH values of the samples were determined with gas chromatography equipped with a flame ionization detector (GC-FID) (Model: Chrompack CP 9001) using (HP-5) capillary column (30 m length, 0.32 mm inner diameter, and 0.2 mm film thickness). Helium was used as carrier gas at a constant flow rate of 1 mL min^{-1}. The temperature program was as follows: the column temperature was held at 50 °C for 1 min and then ramped at 15 °C min^{-1} to 280 °C and held for 5 min. The injector and detector temperatures were set at 250 and 320 °C, respectively. The injection volume was 1 μL. Density of bacteria was evaluated based on most probable number (MPN)[29]. Briefly, 1 mL of microorganism suspension diluted tenfold to 10^{-10} in ringer solution (8.5 g NaCl L^{-1} DW) was added to 9 mL of sterile nutrient broth in five replicates in 10 series.

Removal of *n*-hexadecane due to biodegradation was calculated using Eq. (1):

$$\text{Removal}\ \% = \frac{C_0 - C_t}{C_0} \cdot 100 \qquad (1)$$

where C_0 (mg kg^{-1}) and C_t (mg kg^{-1}) represent the initial and final *n*-hexadecane concentrations, respectively. *n*-Hexadecane recovery rate was about 87 % immediately after spiking. Considering the value of 13 % as a non-extracted portion of *n*-hexadecane from soil, all raw data were multiplied by a factor 1.13.

Finally, a set of bioremediation experiments was conducted on the unwashed soil sample in selected conditions. The various HCs were detected via gas chromatography mass spectrometry (GC-MS) analysis (Model: Agilent 7890, USA) with HP-5MS capillary column (30 m × 0.25 mm × 0.25 mm film thickness, 5 % phenyl- 95 % methyl siloxane phase). The carrier gas was fed at 1 mL min^{-1} steady state flow rate. The oven temperature was firstly set to 40 °C min^{-1}, which was increased to 300 °C at 5 °C min^{-1} for around 3 min. Ultimately, 10:1 splitting ration was observed for injecting sample in the instrument.

Results and discussion

n-Hexadecane-degrading isolate

According to 16S rRNA gene sequences and Neighbor-joining phylogenetic analysis (Fig. 1), the isolate was identified as *Paenibacillus glucanolyticus* sp. strain T7-AHV. This strain was a gram-positive, strictly aerobic, motile, endospore-forming, halo-tolerant bacterium. Its sequence was registered in Genbank under accession number MH248272.

Neighbor-joining method with MEGA 6.0 was applied for constructing tree. Bootstrap values over 70 % (1000 replications) were shown at each node. *Escherichia coli* (LN831047) was used as the outgroup.

Biodegradation assay

Effect of initial seed volume

The results indicated that the *n*-hexadecane biodegradation was affected by the initial inoculum volume. A slow startup can be attributed to low initial bacterial density and vice versa. The trend of *n*-hexadecane biodegradation against time showed an enhancing effect along with seed volume until 15 mL, and further increase in the seed volume caused no significant effect in promoting *n*-hexadecane biodegradation (Fig. 2). High bacterial density requires more nutrition, and insufficient substrate limits the growth and metabolism of bacterial strains[30]. Bioaugmentation is a successful strategy that plays an important role in bioremediation of hydrocarbon-contaminated soil, especially in slurry phase bioreactors[1]. In this regard, the pure strains of *Paenibacillus glucanolyticus* sp. strain T7-AHV demonstrated the best performance in biodegradation of *n*-hexadecane with initial inoculum volume of 15 mL (OD_{600} = 1), corresponding to log MPN value of 6.55. *n*-Hexadecane removal rates (1000 mg kg^{-1}, dry weight) at the end of incubation period were 15.7, 19, 21.4, and 22.5 %, for the seed volumes of 5, 10, 15, and 20 mL, respectively. Since an insignificant difference was observed in the removal efficiencies between 15 and 20 mL of seed volumes (p value > 0.05), the value of 15 mL (log MPN 6.73) was selected for the subsequent tests. Findings are in accordance with literature[31]. Qin *et*

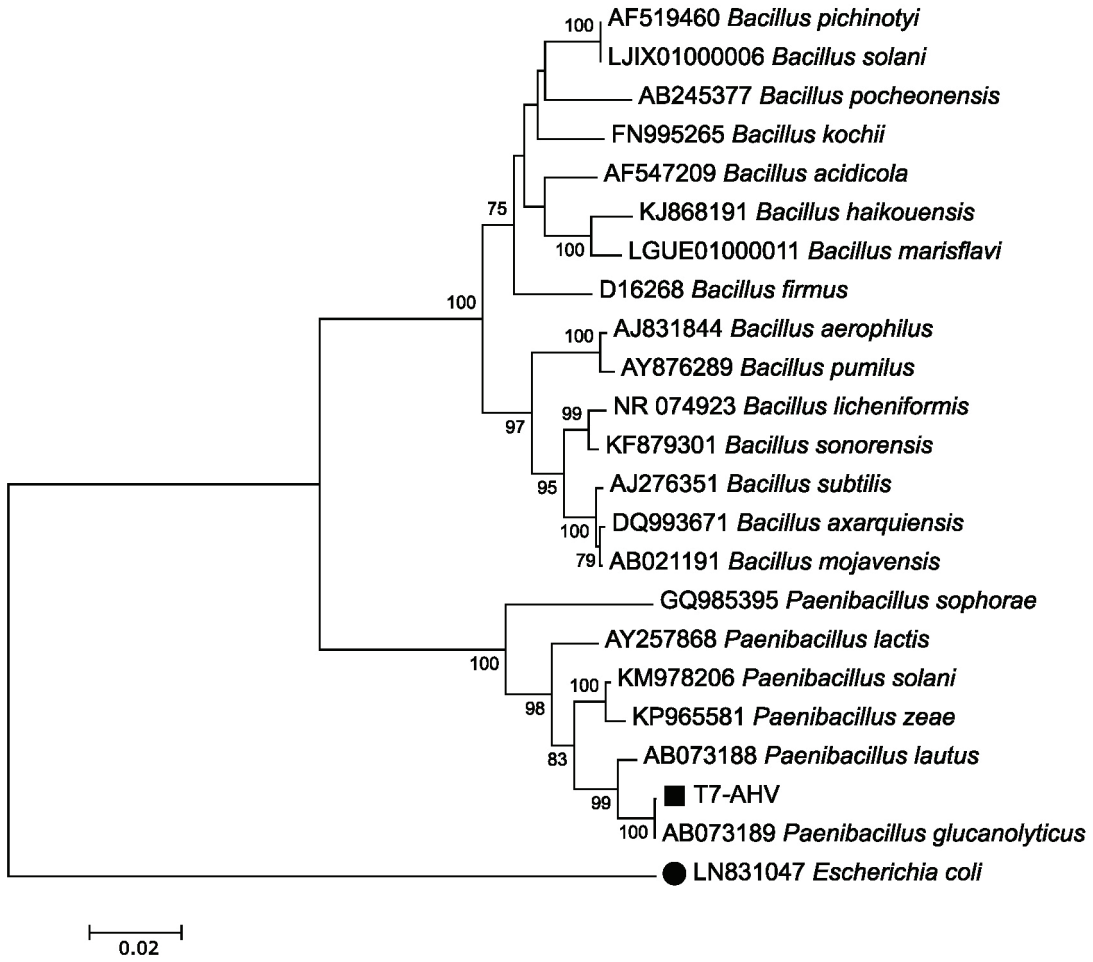

Fig. 1 – *16s rRNA sequences phylogenetic tree. Only the bootstrap values above 70 % are shown. The 16S rRNA gene fragment of Escherichia coli was used as outgroup.*

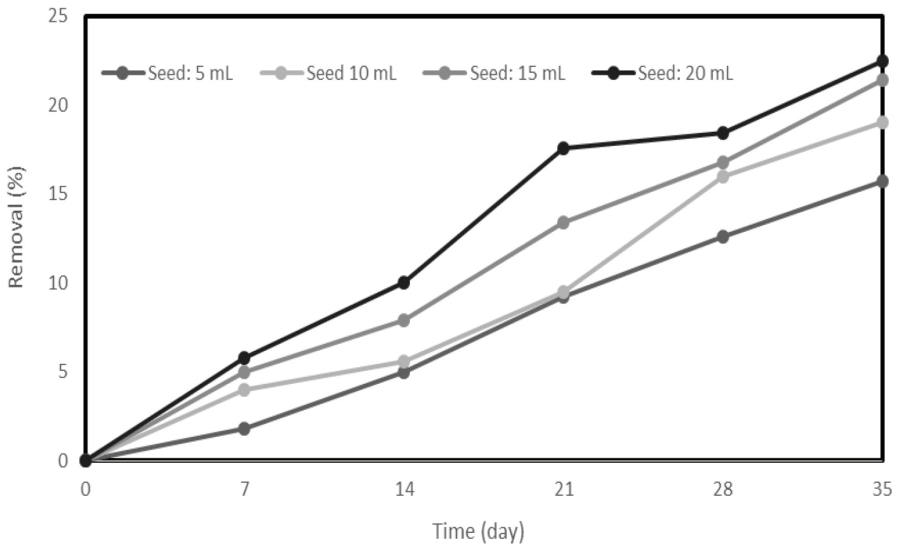

Fig. 2 – *Effect of initial inoculums on n-hexadecane biodegradation in synthetically contaminated soil, moisture: 100 %, and salinity: 0.5 %*

al. showed that the petroleum degradation rate with microbial inoculation was higher than that of treatment without microbial inoculation[32].

Effect of moisture content

Soil moisture is an index showing the amount of water present in the soil, which depends on the organic matter in the soil and soil particle size[33]. Moisture content and solubility of contaminant are major factors in biodegradation of toxic compounds by microorganisms in soil which provide a uniform environment, in terms of nutrients, oxygen, biomass, and pollution in a high gradient medium such as soil[34]. Water has an important effect on providing the essential physiological conditions for microorganism growth and nutrients, and metabolic by-products transport in/out of cell. Thus, there is a strong relationship between the biological activity of soil and the sufficient amount of water in soil. In addition, the bacterial community of soil has high tendency of living in natural soil moisture conditions[35]. In slurry phase, the removal efficiency of pollutant depends on the soil/water ratio, which is influenced by the mass transfer rate of pollutant[36]. Effect of soil moisture content on *n*-hexadecane biodegradation was investigated. The *n*-hexadecane biodegradation efficiencies at different water contents of 50, 100, and 300 % (slurry) were 9, 22 and 27.5 %, respectively (Fig. 3). Accordingly, biodegradation of *n*-hexadecane increased in slurry phase, which facilitated the metabolism of *Paenibacillus glucanolyticus* sp. strain T7-AHV through enhancing the bioavailability and solubilizing the *n*-hexadecane in soil. The obtained findings are in line with literature[37,38]. Previous research on soil-slurry conditions showed that the higher moisture contents of soil led to an increase in biodegradation of PAH compounds, and slurry conditions enhanced the bioremediation of contaminated soil[39,40].

Effect of external biosurfactant addition

A major obstacle in biodegradation of HCs is their inherent hydrophobicity, which limits the contaminant's bioavailability and consequently decreases biodegradation rate[41]. Surfactants can enhance the HC biodegradation rate through increasing the bioavailability either by promoting the solubilization or adherence and uptake of HC from the HC–water interface[42]. Results of mass *n*-hexadecane degradation compared to samples with addition of external surfactant are shown in Fig. 4a. At the beginning of cultivation, the *n*-hexadecane concentration in the cultures containing rhamnolipid biosurfactant, SDS, tween 80, and the control sample decreased gradually over 7 days. The decrease did not stop for samples with or without surfactant until the end, but was observed to be significantly faster for rhamnolipid- and tween 80-containing cultures, with removal efficiencies of 46 and 44.4 %, respectively.

Rhamnolipid facilitates *n*-hexadecane uptake and degradation through enhancing the cell surface hydrophobicity, and thus the adhesion of cells to mass *n*-hexadecane[43]. Low removal rate between 28–35 days indicates the necessity for periodical addition of external surfactant to obtain more acceptable results. Presumed mechanism of rhamnolipid function in enhancing the biodegradation efficiency is presented in Fig. 5.

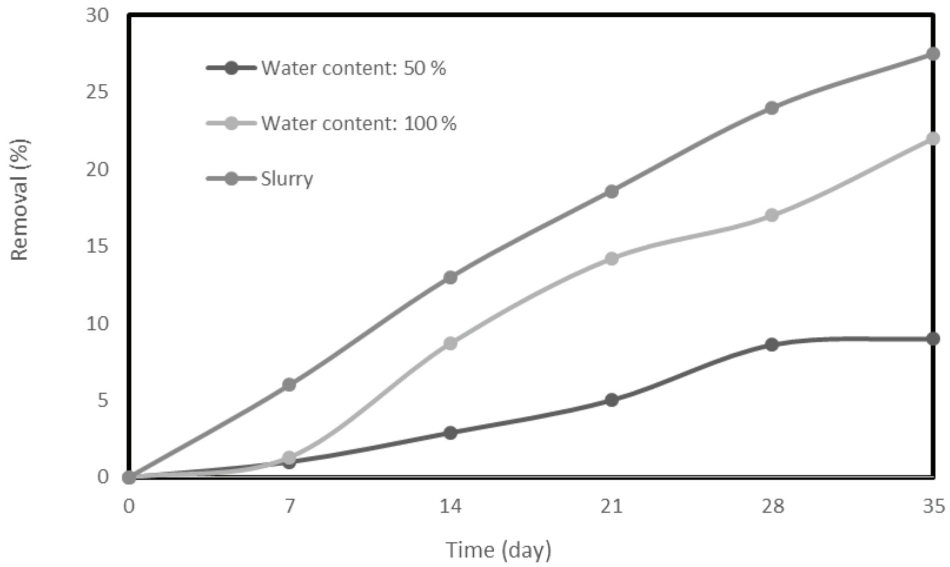

Fig. 3 – *Effect of water content on n-hexadecane biodegradation in synthetically contaminated soil, initial seed volume: 15 mL, and salinity: 0.5 %*

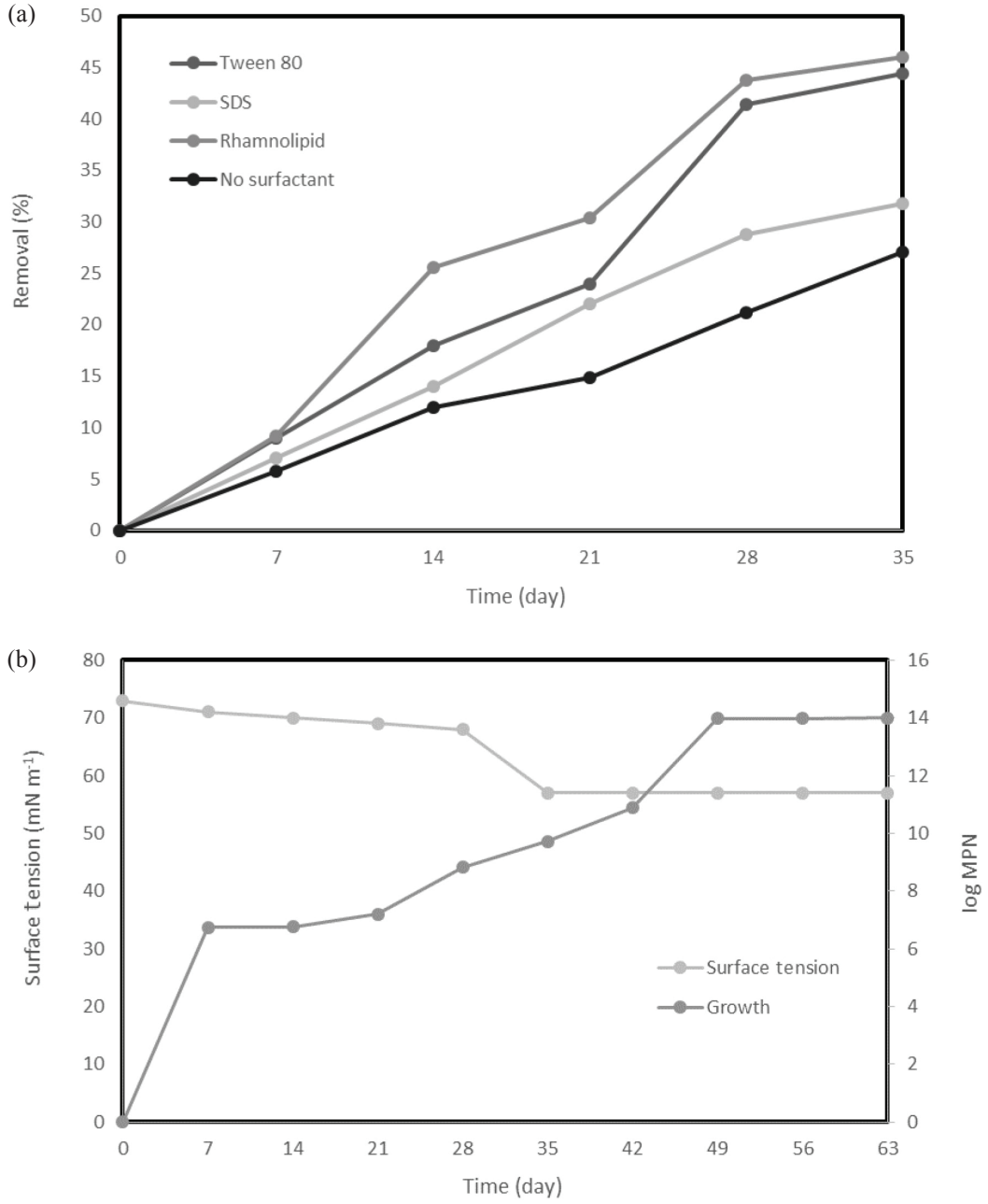

Fig. 4 – *a) Effect of surfactant on n-hexadecane biodegradation in synthetically contaminated soil, and b) variations of surface tension in culture broth versus bacterial growth in the sample free of external surfactant addition, seed volume: 15 mL, moisture: slurry and salinity: 0.5 %*

The observed removal of 27 % for *Paenibacillus glucanolyticus* sp. strain T7-AHV with no surfactant addition indicates the special enzymatic capabilities of this halo-tolerant for biodegradation of HCs as well as cell membrane characteristics to pass the *n*-hexadecane into the cell[44]. Furthermore, decrease in surface tension of culture broth of the control sample (Fig. 4b) demonstrates a low potential for production of exo-polymeric substances into solution.

Effect of salinity

Effect of soil salinity on the *n*-hexadecane biodegradation rate was investigated. One of the parameters that has an adverse effect on pollutants biodegradation by microorganisms is the level of salinity. Literature indicates that both microbial growth and biodegradation can be affected by NaCl, which is in line with literature[45,45]. The removal efficiencies of *n*-hexadecane (initial concentration of

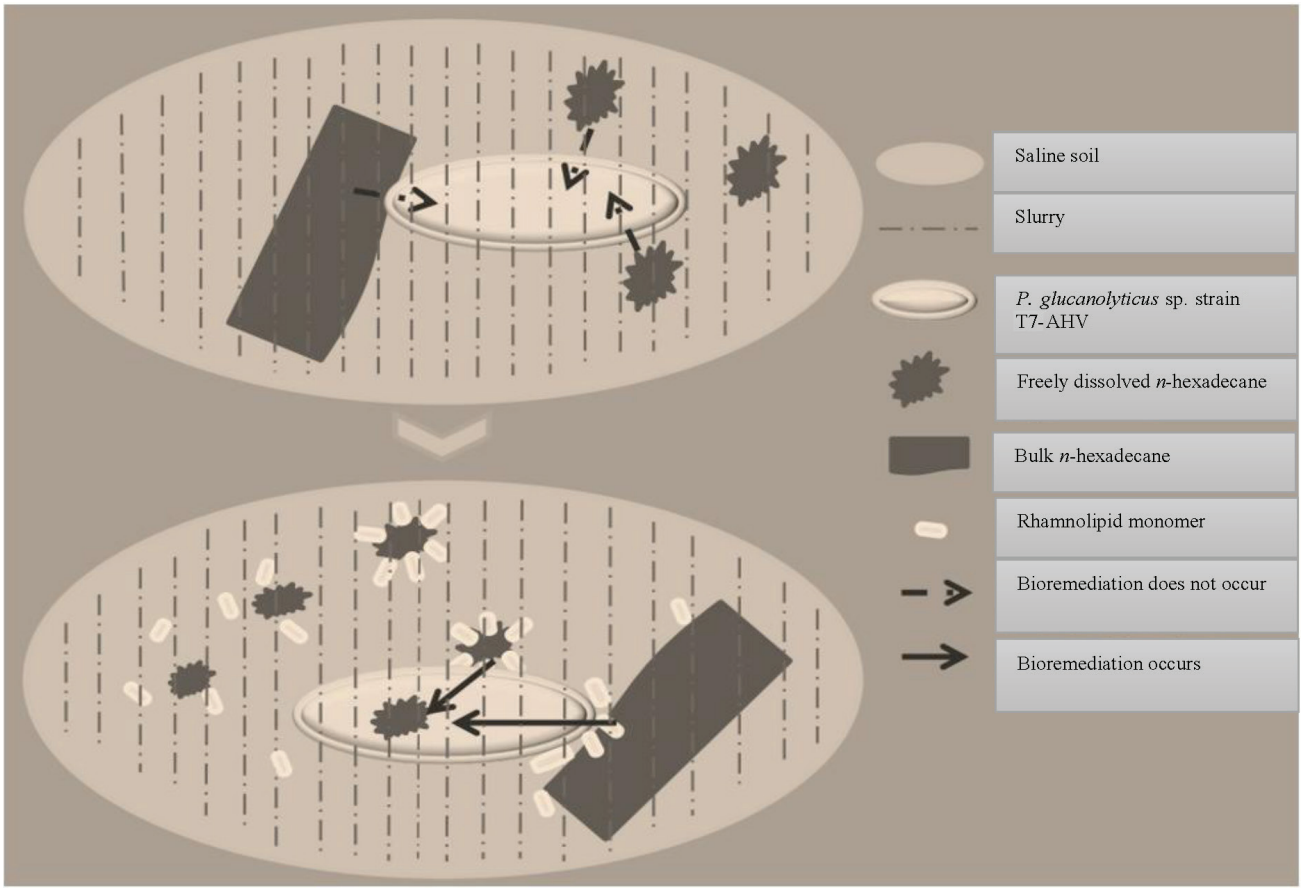

Fig. 5 – *Mechanisms of rhamnolipid for enhancing the bioavailability of n-hexadecane by Paenibacillus glucanolyticus sp. strain T7-AHV*

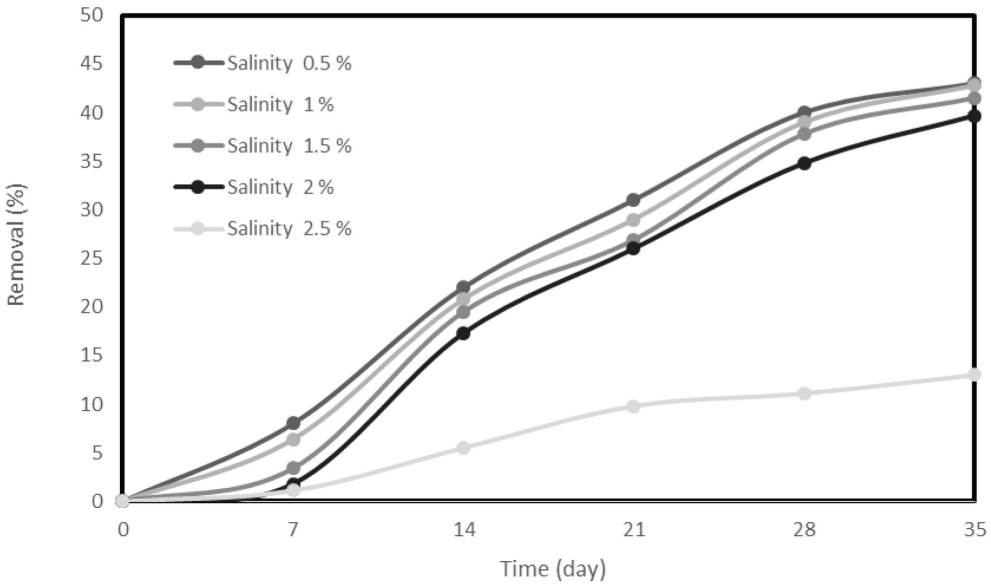

Fig. 6 – *Effect of salinity on biodegradation rate of n-hexadecane in synthetically contaminated soil, seed volume: 15 mL, moisture: slurry, and surfactant: rhamnolipid*

1000 mg kg^{-1}, dry weight) at the end of the fifth week for salinity levels of 0.5, 1, 1.5, 2, and 2.5 % were 43, 42.8, 41.5, 39.7, and 13 %, respectively (Fig. 6).

Since the removal efficiency at the salinity content of 2.5 % was low, and there was no significant difference for *n*-hexadecane removal between 0.5, 1, 1.5, and 2 % salinity levels (p value > 0.05)

on the one hand, and the objective of the current work to remove *n*-hexadecane from saline environments on the other hand, the salinity level was considered as 2 %. This study showed that the bioremediation of *n*-hexadecane was positively correlated with salinity until the value of 2 %, while the metabolic function of *Paenibacillus glucanolyticus* sp. strain T7-AHV decreased with higher salinity concentrations.

Few studies have examined the biodegradation of *n*-hexadecane[18] by halo-tolerant microorganisms. Findings reveal the significant influence of salinity on bioremediation, and the effectiveness of adding inoculated consortium on enhancement of biodegradation of HCs in the soil. Findings of other researchers have proven adverse effects of high salinity biodegradation and bacterial density[46].

Effect of n-hexadecane concentration

The biodegradation was affected by initial *n*-hexadecane concentrations. Lower removal efficiencies were observed for higher *n*-hexadecane concentrations. In the current work, the inhibitory effect of initial *n*-hexadecane was not observed for the studied range of 500–5000 mg kg^{-1}, dry weight (Fig. 7a), which demonstrates the superior characteristics of isolated strain *Paenibacillus glucanolyticus* sp. strain T7-AHV. It was observed in the literature[6] that the biodegradation efficiency decreased along with increasing the initial concentration of *n*-hexadecane. Generally, researchers have proved the relationship between pollutant concentration and biological activity. The removal efficiency of *n*-hexadecane for initial concentrations of 500, 1000, and 5000 mg kg^{-1} (dry weight) were 73.6, 62, and 24 %, respectively at the end of week 10 (Fig. 7). Dibble and Bertha reported that higher petroleum concentration inhibits the microbial activity[47]. There are some claims for decreasing biodegradation with increasing initial concentration of pollutant, when it would be adsorbed on the soil particles surface, which hinders the microbial growth and causes toxic effects that decrease the microbial activity, slow the metabolic rate, and thus exhausts the survival ability of microbial populations[48]. Even for *n*-hexadecane concentration of 5000 mg kg^{-1}, a removal of 24 % (mass: 1200 mg kg^{-1}) has been obtained, which indicates the high ability of *Paenibacillus glucanolyticus* sp. strain T7-AHV for biodegradation of *n*-alkanes. A possible reason for the high performance of the system would be the appearance of a higher bacterial density when a high concentration of carbon source is present. As illustrated in Fig. 7b, bacterial density for initial *n*-hexadecane concentration of 5000 mg kg^{-1} was significantly higher, compared to the two other studied *n*-hexadecane concentrations.

n-Hexadecane mineralization

A qualitative GC-MS analysis was carried out for investigating the mineralization rate and metabolites of *n*-hexadecane biodegradation using *Paenibacillus glucanolyticus* sp. strain T7-AHV. The main metabolites of *n*-hexadecane biodegradation are hexadecanol, hexadecanoic acid, methyl octanoate, methyl decanoate, methyl dodecanoate, methyl hexadecanoate, hexadecane[49–51] which were also observed in our analysis with some differences. The results indicated that the peak area decreased gradually, which demonstrates the efficiency of the isolated halo-tolerant strain in removal of alkanes. The short height of the peaks related to metabolites, as well as low number of metabolites, again proves the high mineralization rate obtained by biological function of *Paenibacillus glucanolyticus* sp. strain T7-AHV. According to the literature, detection of mentioned metabolites indicated the metabolization of *n*-hexadecane through monoterminal oxidation pathway[52]. The results are in accordance with findings of Piccolo *et al.* Monooxygenase belonging to cytochrome P450 families in cells of bacterial strains was observed for *n*-hexadecane metobolizing using both monoterminal and subterminal oxidation[53]. Versatile microbial oxidases are stereoselective and regioselective, indeed leading to individual degradation pathways[54].

According to observed metabolites including alkanes, methyl-related compounds, and fatty acids, it seems that *n*-hexadecane was biologically attacked on the terminal methyl by the monooxygenase. Alvarez declared that the putative fate of *n*-hexadecanoic acid, which is channeled to bioxidation, causes production of carboxylic acid derivatives shortened by a two-carbon atom fragment as acetyl-CoA, that acts as a precursor of the tricarboxylic acid cycle, lipids biosynthesis, and triacylglycerol accumulation, which provides carbon and energy sources[55]. Alvarez stated that bioxidation is the main reason for biosynthesis of lipids that provides good conditions for microbial cells metabolism, although at low amounts of nutrients[55].

Bioremediation of unwashed soil

Reliability of isolated halo-tolerant strain *Paenibacillus glucanolyticus* sp. T7-AHV in biodegradation of various HCs of a real unwashed contaminated soil was also evaluated in selected conditions, including slurry phase and addition of rhamnolipid. HCs present in the soil sample were

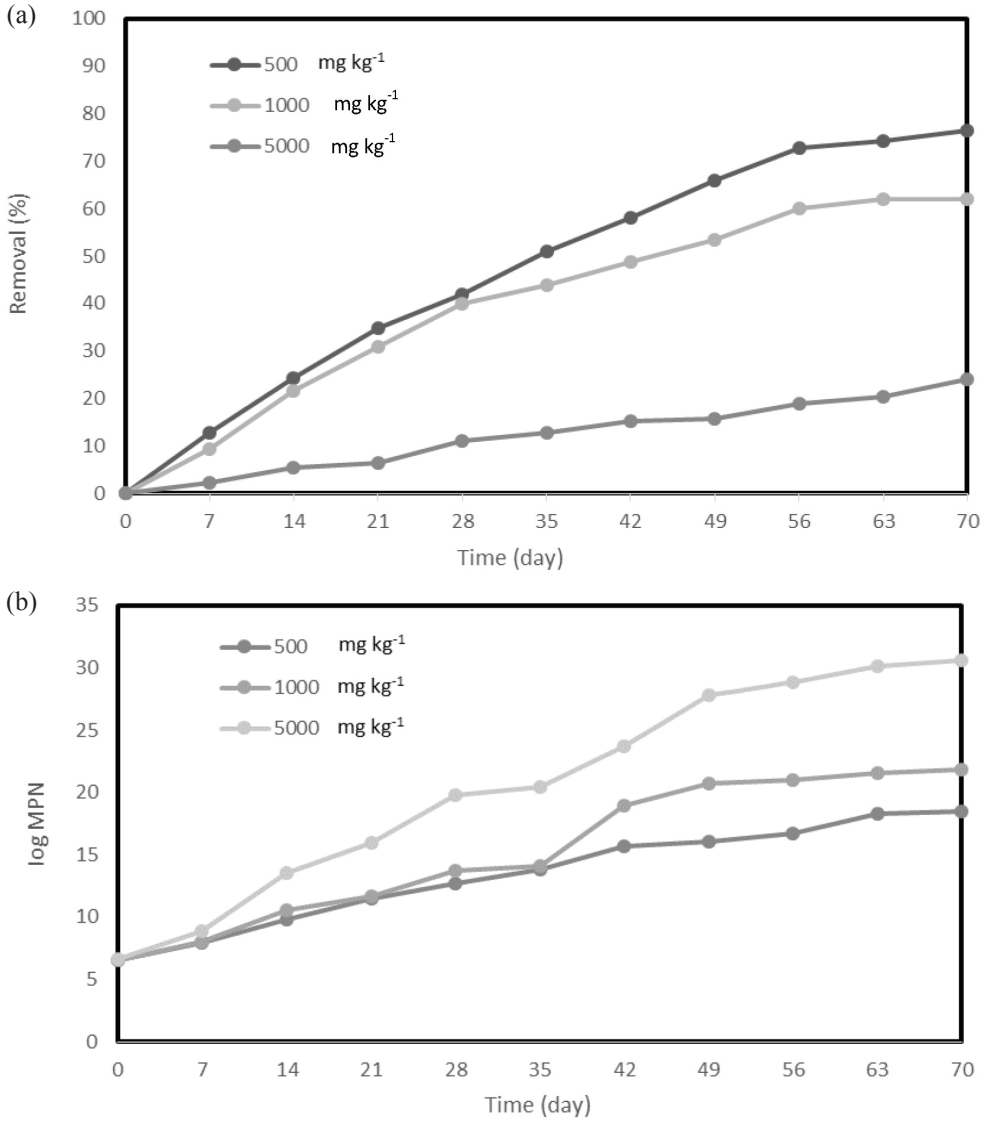

Fig. 7 – *a) Effect of n-hexadecane concentration on biodegradation efficiency in synthetically contaminated soil (seed volume: 15 mL, slurry phase, salinity: 2 % and surfactant: rhamnolipid), b) comparison of bacterial density in different initial n-hexadecane concentrations*

determined via GC-MS analysis. Initial HC concentration of 1437 mg kg^{-1} decreased to 1119 mg kg^{-1} (dry weight) (22.1 % removal) during 60 days and in the selected conditions. The main identified HCs in unwashed soil were *n*-hexadecane, hexadecane, pentadecane, octadecane, heptadecane, nonadecane, and docosane. As may be seen in Table 2, removal efficiencies between 18.6 % – 25 % were observed for various HCs using *Paenibacillus glucanolyticus* sp. T7-AHV. The variety of contamination and high cumulative concentrations of different HCs in unwashed soil are the main reasons for lower removal efficiency of isolated strain compared to synthetically contaminated samples. The obtained findings are in line with our previous study on soil remediation with the modified Fenton oxidation[56] and with the literature on purification of hydrocarbon-polluted soil[57,58].

Table 2 – *Removal efficiency of different HCs in unwashed soil through bioremediation using Paenibacillus glucanolyticus sp. strain T7-AHV with external addition of rhamnolipid in slurry phase*

Hydrocarbon	Initial concentration (mg kg^{-1})	Final concentration (mg kg^{-1})	Removal (%)
n-Hexadecane	234 ± 13	174	25.64
Heptadecane	110 ± 9	86	21.8
Pentadecane	136 ± 18	102	25
Octadecane	208 ± 14	158	24.03
Hexadecane	350 ± 32	280	20
Nonadecane	215 ± 22	175	18.6
Docosane	184 ± 7	144	21.72
Total HC	1437	1119	22.13

Conclusions

This study was the first research into introducing *Paenibacillus glucanolyticus* sp. T7-AHV as a halo-tolerant strain capable of *n*-alkane biodegradation. High saline environment and contamination with HCs are two important issues limiting natural decontamination of soils close to the oil and gas industries. Our results suggested that *Paenibacillus glucanolyticus* sp. T7-AHV is an efficient and reliable strain for bioaugmentation of saline hydrocarbon-contaminated sites, such as soil, shoreline, and sea, alone or as part of a mixed consortium. In summary, the addition of rhamnolipid, operation in slurry phase, and a larger initial seed volume enhanced the biodegradation of *n*-hexadecane by *Paenibacillus glucanolyticus* sp. T7-AHV significantly. The isolated strain was not a biosurfactant producer, and since mass *n*-hexadecane was applied for providing carbon and energy, the bioavailability of *n*-hexadecane occurred through direct contact of cells with biosurfactant as a stimulating factor, which facilitated interfacial uptake of HCs or enhancement cell surface permeability for HC degradation. Removal efficiency of about 22 % for unwashed soil polluted with different *n*-alkanes proves the high potential of introduced bioremediation process for full-scale applications.

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