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Biodesalination and biodegradation of crude oil in aqueous solutions by *Halobacillus halophilus*

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ABSTRACT

Today, freshwater scarcity is one of the serious problems of human societies. Given the abundant reserves of saline water across the world, seawater desalination is one of the promising ways to supply freshwater. Another problem is the oil pollution of seawater caused by the extraction of petroleum from the seabed. Bacteria can be employed to desalinate seawater and to clean oil pollution. In this context, the effect of *Halobacillus halophilus* was assessed on the desalination and degradation of crude oil in aqueous solutions. The results indicated that maximum growth rate occurred within 6 d after culturing where the optimum temperature and pH for maximum growth of *Halobacillus halophilus* were 25°C and pH = 7.5. Under optimum temperature and pH, *Halobacillus halophilus* could desalinate seawater with 3%–15% of NaCl and degrade 1%–7.5% crude oil. A high absorption rate was observed in 5% NaCl (48% ± 2.3) and 5% crude oil (36 ± 1.2). In the final step, the simultaneous absorption of NaCl and oil degradation was measured. The total NaCl and crude oil removal rate were similar to the case where the bacterium performed each of these processes separately (NaCl: 42 ± 12.6, Oil degradation rate: 35.4 ± 3.5). Thus, the bacteria could desalinate water and remove oil pollution both separately and simultaneously.

Keywords: Desalination; Degradation of crude oil; Seawater; *Halobacillus halophilus*

1. Introduction

Freshwater is one of the most essential needs of humans [1]. Today, freshwater scarcity has become a major threat. Although a wide area of the Earth is covered with water, over 97% of this water is saline water and unpotable [2]. Out of the 3% drinkable water, only about 0.5% can be found as freshwater which is not evenly distributed across the world. Many countries are facing freshwater scarcity [1]. The demand for drinking water is one of the motivations for saline water desalination [3]. Another problem is seawater pollution with oil spills [4]. Oil extraction from resources located at seabed has polluted seawater and endangered the marine ecosystem [5]. Numerous physicochemical desalination or oil spill cleanup methods have been developed, but these methods demand a large amount of energy and time, and many of them fail to completely remove oil spills or desalinate seawater [6].

Numerous studies have been conducted to find an efficient way to simultaneously perform these processes to reduce energy and waste of time as well as to enhance efficiency [7,8]. Recent studies have shown that living microorganisms could offer smart desalination mechanisms with low energy demand to adapt to their surrounding environments. Many of them can well decompose crude oil by producing biosurfactants. They exclusively utilize hydrocarbons as carbon and energy sources [6]. Nowadays, researchers are more interested in biodesalination and biodegradation of oil spills to reduce the costs, energy, and environmental effects of desalination plus oil spill clean-up from seawater

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[9,10]. For this purpose, hydrocarbonoclastic bacteria which can tolerate high concentrations of NaCl are the best choice to assess these processes concurrently [11].

As known so far, halophilic and hydrocarbonoclastic bacteria require NaCl for their growth and metabolism; they can tolerate a wide range of near saturation marine concentrations with similar growth rates [12]. They have also the potential to adjust the metabolism to a wide range of salinities [13]. These bacteria require high NaCl concentrations to maintain their hemostasis. So they absorb high NaCl concentrations from their environment through complex mechanisms [14]. Unlike cyanobacteria, some of these halophilic bacteria supply their energy from carbon sources such as oil spills [15].

The literature review on the biotechnological approaches has indicated that *Halobacillus halophilus* [16] could be used as a salt-tolerant strain for oil degradation for simultaneous assessment of growth rate, biodesalination, and biodegradation of oil spills in brackish water [17–20] at various NaCl and oil spill concentrations through its biological membrane. The impact of physical factors such as temperature and PH on this process could be determined.

2. Materials and methods

2.1. Organism and growth experiments

Halobacillus halophilus (*Sporosarcina halophila*) was prepared as an active-growing culture from the Iranian Biological Resource Center (IBRC-M No.: 10217). It was kept in Bacto Marine Broth + 10% NaCl (Difco 2216). The cultures were incubated on a rotary shaker (150 rpm) at 30°C for 5 min to form bacterial colonies. To determine the optimum pH, 0.05 M Tris (pH 7–10.5) or 0.05 M succinate (pH 5–6.5) were added to the medium, with pH adjusted to 7.2 using HCl or NaOH.

2.2. Effect of incubation period on growth rate

The organisms were incubated at 30°C on a rotary shaker under aerobic conditions. The bacterial growth was measured every 24 h for 8 d using a spectrophotometer (UNICO 2150-UV) [21].

2.3. Effect of temperature and pH on growth rate

To assess the effect of pH and temperature on bacterial growth, different pH and temperatures were used. For this purpose, 5 mL sterile Bacto Marine Broth was inoculated with the test organisms in test tubes with three replicates and incubated at different temperatures (15°C, 25°C, 35°C, and 45°C) for 8 d. Every 24 h, the growth rate was measured using a UNICO 2150-UV spectrophotometer at 600 nm. To assess the effect of pH, the organisms were inoculated in three replicates and incubated at different pHs (5, 7.5, 8, 9.5) using NaOH or HCl at 30°C for 8 d. Finally, the growth rate was measured at 600 nm every 24 h.

2.4. Effect of salt removal by Halobacillus halophilus at different NaCl concentrations on growth rate and absorption rate

NaCl absorption by bacteria was assessed at various NaCl concentrations. In this section, 1 mL bacteria was inoculated in three replicates with 20 mL Bacto Marine Broth containing 3%, 5%, 7%, 10%, and 15% NaCl. Then, the flasks were incubated on a shaker at 150 rpm at optimum pH and temperature. To measure the bacterial growth rate at different NaCl concentrations, the optical density was read at 600 nm every 24 h. In addition, to assess desalination, the samples were centrifuged at 14,000 rpm to separate bacteria from the medium. Finally, NaCl concentration in each medium was daily determined by atomic absorption spectrophotometer (AAS) (Aurora Biomed) for 8 d [22]. NaCl absorption was estimated as the difference between the initial and final concentrations whereby the absorption rate was calculated. Each sample was read three times.

2.5. Assessment of the effect of petroleum hydrocarbon on growth rate and removal rate by Halobacillus halophilus in different crude oil concentrations

For degradation studies, total petroleum hydrocarbon measurement was spectrophotometrically used based on Rahman et al. method [23]. For this purpose, the organisms were inoculated in three replicates with 20 mL Bacto Marine Broth with various crude oil concentrations (1%, 2.5%, 5%, and 7.5%) (Assaluyeh Petrochemical Company) used as the only carbon resource for bacteria. Finally, the samples were placed on a shaker at 150 rpm and incubated for 8 d at optimum temperature and pH. To measure bacterial growth at different crude oil concentrations, optical density was read at 600 nm every 24 h. In addition, every 24 h, the samples with different crude oil concentrations were mixed with an equal volume of toluene to extract hydrocarbons. After centrifugation (4,000 rpm for 5 min), the extracted hydrocarbons were spectrophotometrically detected at 420 nm. A standard curve was prepared using known concentrations of crude oil to estimate the hydrocarbon contents of the samples. The degradation was estimated as the difference between the initial and final concentrations of total hydrocarbons measured with optical density (Relation 1).

$$\text{Initial concentration of hydrocarbons} - \frac{\text{Final concentration of hydrocarbons}}{\text{Initial concentration of hydrocarbons}} \times 100 \quad (1)$$

2.6. Simultaneous effect of salt removal and crude oil degradation on growth and absorption rates by Halobacillus halophilus

To obtain the growth rate and to compare NaCl removal and crude oil degradation rates simultaneously, the bacteria were inoculated with 20 mL Bacto Marine Broth, 12% NaCl, and 5% crude oil. The samples were incubated in three replicates for 8 d on a shaker at 150 rpm at optimum temperature and pH. Within 8 d, the bacterial growth was measured by a spectrophotometer at 600 nm. Crude oil degradation and NaCl absorption were also measured every 24 h. Finally, the total NaCl absorption and oil degradation were calculated and compared with the total values of the previous sections.

2.7. Data analysis

To compare NaCl and crude oil absorption data, statistical analysis was used. Initially, the normality of data distribution was determined by the Kruskal–Wallis test. Statistical analysis was carried out using one-way analysis of variance (ANOVA) as implemented in SPSS 11.5. Mean values of various treatments were tested. To determine the significance, Tukey's test was also used, where the significance level was considered 0.05. The diagrams were drawn in Excel.

3. Results

3.1. Bacterial growth rate at different incubation times

The effect of different incubation times on bacterial growth was measured every 24 h for 8 d. According to the results, *Halobacillus halophilus* indicated the highest growth rate of 1.629 ± 0.15 at pH = 7.2 and 30°C. The results revealed that this strain reached its maximum growth after 6 d of incubation. Then, its growth and following that, the bacterial population declined (Fig. 1).

3.2. Temperature and pH optimization for Halobacillus halophilus growth

To assess the effect of temperature and pH on the growth rate, different temperatures (15°C, 25°C, 35°C, and 45°C) and pH values (5, 7.5, 8, 9.5) were considered. The results indicated a maximum growth rate at 25°C after 6 d (Fig. 2). At lower (15°C) and higher (35°C and 45°C) temperatures, however, the growth rate declined in comparison with the temperature of 25°C in all days. In addition, the highest bacterial growth rate was observed at pH = 7.5. In more acidic or more alkaline media, the growth rate diminished as expected (Fig. 3).

3.3. Effects of different NaCl concentrations on growth and removal rate by Halobacillus halophilus

As displayed in Fig. 4, the bacterial growth rate increased by elevating the NaCl concentration. The maximum growth rate was detected at 5% NaCl. The growth of many bacteria dropped at high NaCl concentrations, but *Halobacillus halophilus* was resistant even to NaCl values greater than 5%. The NaCl concentration in seawater was about 3.5%. These data showed that *Halobacillus halophilus* could tolerate brackish water whose growth rate increased until 5% NaCl concentration. Further, the results indicated the maximum NaCl absorption at 5% NaCl (Fig. 5). Although the optimum growth and absorption were determined at NaCl content of 5% in this study as shown in Figs. 4 and 5, there was no significant difference between 3% and 5% of NaCl as depicted in Fig. 6.

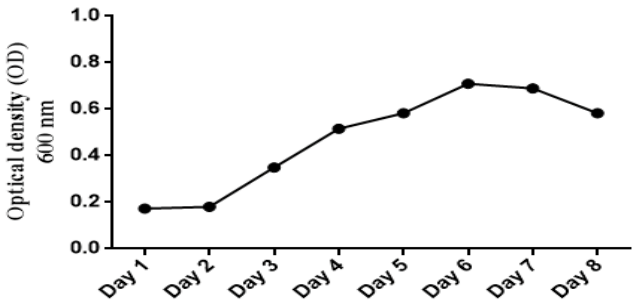

Fig. 1. Effect of incubation time on bacterial growth rate; Data showed that bacterial population increased after 6 d at pH = 7.2 and 30°C on a rotary shaker (150 rpm). After this time, a decline was observed in bacterial growth. Bacterial growth was measured in OD_{600}.

3.4. Effect of petroleum-hydrocarbon concentration on growth and removal rate by Halobacillus halophilus

The bacterial growth rate was measured at different crude oil concentrations by calculating the optical density

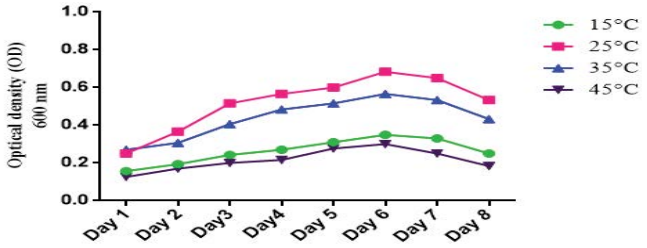

Fig. 2. Effect of different temperatures on bacterial growth; According to the results, the optimum temperature for *Halobacillus halophilus* growth was 25°C. After 6 d the maximum growth occurred in all groups, but at 25°C the values were higher than other groups (pH = 7.2; temperature = variable; incubation time = 8 d; OD_{600}).

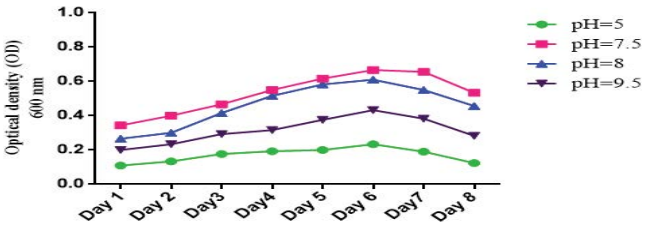

Fig. 3. Effect of pH on bacterial growth; Data showed that the optimum pH for *Halobacillus halophilus* growth was pH = 7.5. This indicated that *Halobacillus halophilus* grew in environments with neutral pH. Changes in the medium pH strongly affected bacterial growth (temperature = 25°C; pH = variable; incubation time = 6 d; OD at 600).

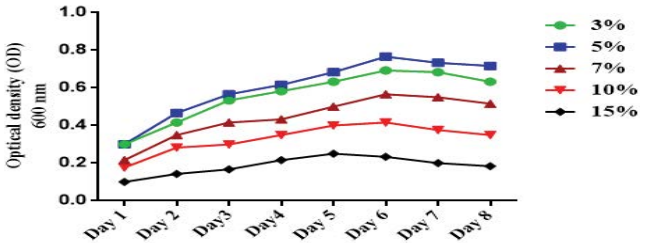

Fig. 4. Effect of NaCl concentration on bacterial growth rate; Data showed that at 5% NaCl concentration, the growth rate was much better in comparison of other NaCl concentrations. The maximum growth rate occurred in day 6 of bacterial growth with NaCl concentration of 5% (temperature = 25°C; pH = 7.5; incubation time = 8 d; OD at 600 nm).

in all groups. In addition, crude oil degradation by bacteria and then total hydrocarbons were also evaluated. The results showed that maximum growth occurred on day 6 of bacterial growth with 5% crude oil. These findings proved that crude oil (as the only source of carbon in media) could alter the bacterial growth diagram (Fig. 7). Furthermore, the data indicated that hydrocarbon degradation was maximum in the group containing 5% crude oil. It is therefore confirmed that maximum growth in 5% crude oil could affect hydrocarbon degradation and as a result, degradation was higher at 5% concentration (Fig. 8). There was a significant difference between the groups regarding the total crude oil degradation. There was maximum degradation in 5% crude oil (36% ± 1.2) (Fig. 9).

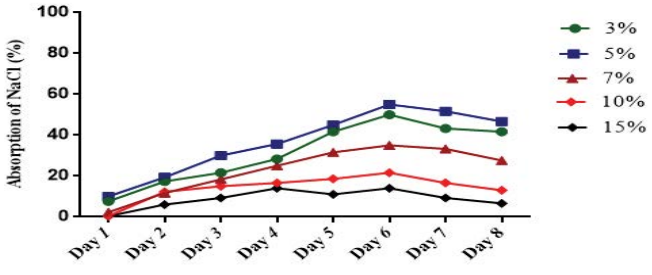

Fig. 5. NaCl absorption by bacteria at different concentrations; This figure shows that the NaCl absorption rate was maximum in the group with 5% NaCl concentration (temperature = 25°C; pH = 7.5; incubation time = 8 d; OD at 600 nm).

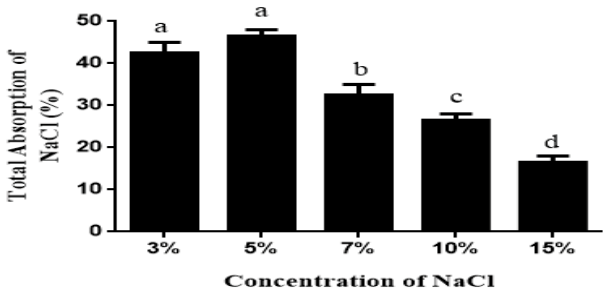

Fig. 6. Total NaCl absorption. Data indicated the maximum NaCl consumption in 5% NaCl (48% ± 2.3, 44% ± 3.5). There was no significant difference between 3% and 5% NaCl in terms of NaCl absorption. Asymmetric letters indicate a significant difference between groups. One-way ANOVA was used with Tukey's post hoc test. Data are represented as mean ± scanning electron microscopy.

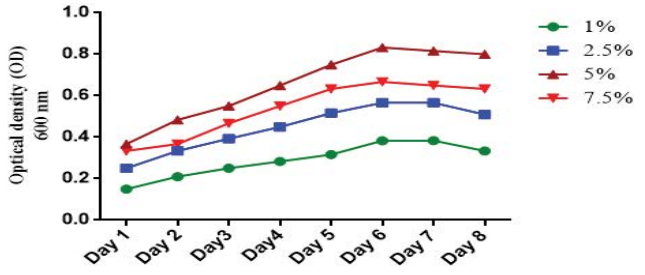

Fig. 7. Effect of different crude oil concentrations on bacterial growth rate; Data showed that in 5% crude oil, the bacterial growth was much better than the other groups. Maximum growth rate occurred on day 5 of bacterial growth with 5% crude oil (temperature = 25°C; pH = 7.5; incubation time = 8 d; OD at 600 nm).

3.5. Mutual effect of NaCl and oil absorption and their impact on Halobacillus halophilus growth

The effect of NaCl and oil absorption alone was evaluated in the previous sections. In the final step, the simultaneous effect of NaCl and oil absorption was assessed on the bacterial growth rate (Fig. 10). Fig. 10 indicates that bacteria can perform both processes without any noticeable effect on bacterial growth under ideal conditions. It is hoped that bacteria could perform these processes simultaneously without imposing any side effects on the growth curve. In this curve, similar to normal conditions, the maximum growth rate occurred on day 6 of culturing. Based on Fig. 11, the total NaCl and crude oil removal rates were similar to the case in which the bacterium performed each of these processes separately. It implies that these processes can operate independently without adversely affecting or disrupting each other (Table 1).

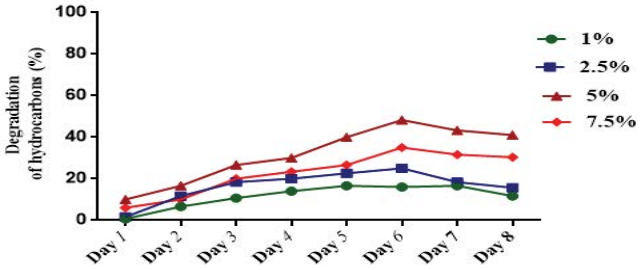

Fig. 8. Crude oil biodegradation at different concentrations; This figure shows that the crude oil degradation rate was maximum in the group containing 5% crude oil. In other groups, degradation increased with a rise in the bacterial population. However, the maximum growth in 5% crude oil could affect hydrocarbon degradation and as a result, the degradation rate was higher at 5% concentration (temperature = 25°C; pH = 7.5; incubation time = 8 d; OD at 420 nm).

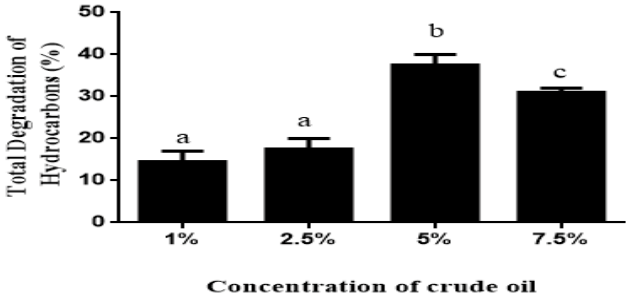

Fig. 9. Total hydrocarbon degradation. Data based on the total hydrocarbon degradation rate indicated a maximum hydrocarbon consumption in 5% crude oil (36% ± 1.2). Asymmetric letters show a significant difference between the groups. One-way ANOVA was used with Tukey's post hoc test. Data are represented as mean ± scanning electron microscopy.

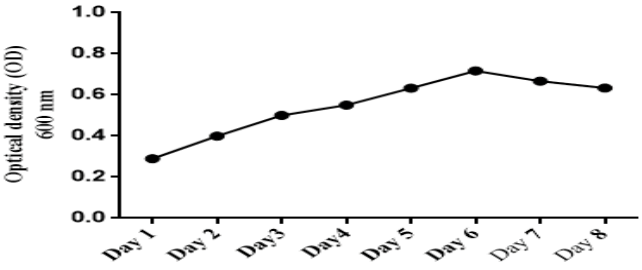

Fig. 10. Effect of NaCl and oil absorption on bacterial growth; 5% crude oil + 5% NaCl were added to the culture. The bacteria could perform both processes without any noticeable effect on their growth under ideal conditions. They could also undertake these two processes simultaneously without imposing any side effects on the growth curve (temperature = 25°C; pH = 7.5; incubation time = 8 d; OD at 420 nm).

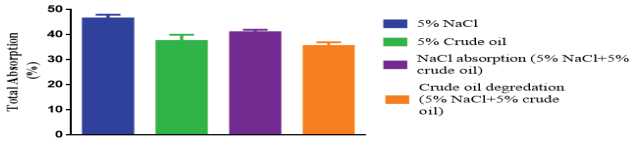

Fig. 11. Effect of NaCl and oil absorption on each other; Regarding the total absorption rate, there is no difference between the NaCl and crude oil absorptions alone or simultaneously. One-way ANOVA was used combined with Tukey's post hoc test. Data are represented as mean ± scanning electron microscopy.

Table 1
Absorption rate in different experiments

Total absorptions	Absorption rate
5% NaCl	48% ± 2.3
5% crude oil	36% ± 1.2
5% NaCl + 5% crude oil	NaCl = 42% ± 12.6 Crude oil = 35.4% ± 3.5

The total NaCl and crude oil absorption alone or simultaneously during 8 d of culturing showed that these processes can operate independently without imposing any adverse effect on one another

4. Discussion

This study explored the effect of environmental changes as well as NaCl and oil concentrations on *Halobacillus halophilus* growth. It was shown that *Halobacillus halophilus* could degrade oil and absorb NaCl from its medium. *Halobacillus halophilus* (*Sporosarcina halophila*) is an aerobic, rod-shaped, motile, endospore-forming, and gram-positive bacterium. It is a moderate halophile that grows optimally in 0.5 and 2.0 M NaCl though it can tolerate NaCl concentrations as high as 3.5 M, suggesting that these mechanisms effectively respond to a broad range of salinities [24,25]. The results indicated the significance of physiochemical factors in bacterial growth and survival. In this study, the effect of incubation time, pH, and the temperature were inspected on the bacterial growth rate. According to the results, this bacterium showed its maximum growth rate in 6 d of incubation at 25°C and pH = 7.5. Further, these bacteria had a maximum growth rate in neutral environments and room temperature. Carbon resources and specific concentrations of NaCl play a pivotal role in bacterial survival. In the present study, the bacterial tolerance was first tested at different NaCl concentrations; then bacterial growth and NaCl absorption rate by bacteria were investigated at different NaCl concentrations. The results showed that this bacterium is halotolerant and can tolerate salinity levels of 3%–15% NaCl. However, the maximum bacterial growth and NaCl absorption occurred at 5% NaCl concentration. NaCl concentrations beyond 5% could negatively affect the microorganism activity by increasing the osmotic pressure of the environment [26,27]. The results of this study revealed that the prolongation of the delayed stage in the bacterial growth process at high NaCl concentrations could be assigned to the inhibitory effects of NaCl on microorganism growth [28]. By elevating the salt concentration beyond the optimal level, the adaptation time of bacteria increased, so bacterial growth was reduced, decelerating the biomass production. Since the bacterium is halotolerant, it can pump excess NaCl ions out through the cell membrane and properly balance the concentration inside and outside the cell [29].

Studies have reported that oil pollution in nature is gradually degraded by microorganisms in the environment [30,31]. Various microorganisms have been found in areas contaminated with petroleum products, many of which could decompose oil and use it as a source of carbon and energy [32]. Specifically, these microorganisms grow under difficult conditions, so their efficiency is low under natural conditions [33]. However, under proper environmental conditions, they decompose oil pollution with considerable speed and use it as a source of carbon [34]. Numerous studies on oil degradation by microorganisms have been conducted to find the best bacterial strains capable of decomposing pollutants in water and soil [22,32]. *Halobacillus halophilus* is a heterotrophic bacterium [35]. Hence, it requires carbon resources to obtain energy from the environment. According to this study, the best oil degradation rate was obtained at 5% crude oil. High hydrocarbon concentrations caused the oil to disperse in water, inhibiting its biodegradation due to limited levels of oxygen or nitrogen [34,36]. In addition, it was confirmed that oil desalination and degradation mechanisms occur without interfering with one another. These two mechanisms can take place simultaneously (12% NaCl + 5% crude oil) by bacteria without reducing the efficiency of the other process. However, some studies have revealed that further increase in the salt concentration reduced bacterial activity and thus declined oil consumption [37]. Thus, the use of optimal oil and salt concentrations plays an important role for bacteria to simultaneously perform these processes without compromising the efficiency of the other. Alcanivorax secretes a substance called biosurfactant, which reduces the surface tension of oil and emulsifies it [32]. The emulsified oil is broken down by bacteria, turning it into carbon dioxide and simpler substances which are absorbed by microbes in nature [38]. Studies have reported that mixed microbial populations with a large total enzyme capacity could decompose complex hydrocarbons [39,40]. The mixed microbial population includes several microorganisms which can synthesize decomposing enzymes for

different sections of decomposition pathways of aromatic compounds and increase the chance of more comprehensive decomposition through metabolic pathways [39,41].

5. Conclusion

The results of the current research indicated that *Halobacillus halophilus*, as a halotolerant bacterium, succeeded in desalination of a wide range of NaCl concentrations in aqueous solutions. This bacterium could degrade crude oil with high efficacy and can be classified as an Alcanivorax bacterium. It can be considered as a new strain for future biodesalination and oil biodegradation applications. Future studies are recommended to employ a mixture of microorganisms to perform these (desalination and oil degradation) processes to boost the efficiency and provide a proper alternative for inefficient conventional methods.

Acknowledgments

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Conflicts of interest

The authors declare no conflicts of interest.

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