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Evaluation of biosurfactant production by *Sporosarcina halophila* and its application in crude oil remediation

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Abstract

Background: Biosurfactants are valuable microbial products that have significant applications in various industries. The advantages of these compounds are biodegradability, low toxicity, activity in difficult environmental conditions, and the ability to produce oil residues and compounds from the surface of seawater and soils contaminated with oil compounds. The aim of this study was to evaluate the ability of biosurfactants production by *Sporosarcina halophila*.

Methods: For this purpose, to detect the production of biosurfactant by *Sporosarcina halophila*, quantitative and qualitative screening methods such as hemolysis, oil spreading test and emulsification index test were used. Finally, different concentrations of crude oil in the bacterial growth medium were used to see that this strain can decompose crude oil using biosurfactant production to continue its growth or not.

Results: The results showed that this strain is able to produce biosurfactants in oil hemolysis and spreading test with emulsifying activity of more than 30%, indicating that this strain is a suitable strain for biosurfactant production. Also, this strain could grow in the presence of crude oil in its medium as only carbon resource by biosurfactant production.

Conclusion: This study showed that the metabolites derived from *Sporosarcina halophila* strain have emulsifying properties and can be considered as a potent strain in the production of biosurfactants. Also, it was concluded that these biosurfactants are applicable for many different industrial or environmental fields such as bioremediation of crude oil from soil or water by *Sporosarcina halophila* strain.

Keywords: Biosurfactant, Sporosarcina, Surface-active agents, Petroleum

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Introduction

Biosurfactants or bioemulsifiers are compounds that reduce surface tension produced by many bacteria and fungi (1,2). The importance of these compounds in comparison with synthetic surfactants is in non-toxicity, environmental compatibility, activity at temperature, pH, and high salinity. Biosurfactants are a group of chemicals produced by bacteria that reduce the surface tension of oil between the surface of oil and water or air by having polar and non-polar regions (3). Bacteria can break down oils by producing these substances. The biosurfactants produced by the oil-degrading bacteria actually reduce the surface tension of the oil, breaking and emulsifying the oil layer into small pieces of oil in the water, and then, the bacterial enzymes can affect and break down the oil spot (4,5). The literature review on the biotechnological approaches indicated that *Sporosarcina halophila* could be used as a strain for oil degradation to in brackish water (6-10) at various oil spill concentrations through its biological membrane. In our pervious study, it was indicated that this strain could remove oil pollution from seawater (11). For this purpose, the aim of this study was to assess the production of biosurfactant by *S. halophila* and prove that this strain has the ability to produce biosurfactants and can be used as a novel strain in the biodegradation of oil spot.

Material and Methods

Isolation and identification of bacteria

Halobacillus Sporosarcina halophila was purchased as an active culture from the National Center for Genetic and Biological Resources of Iran (IBRC-M No: 10217). Bacteria were cultured in bacto-marine broth with 10% NaCl. The inoculated bacteria were then kept in a shaker incubator (150 rpm) at 30°C.

Oil spreading test

The oil spreading method is an efficient and fast test for the

initial screening of biosurfactant-producing strains. For this purpose, 30 mL of distilled water was poured into a petri dish, then, 20 µL of crude oil was placed in the center of the plate, so that a layer of crude oil was formed on the surface of the water inside the plate. 10 µL of the bacteria supernatant was poured into the center of the oil layer. The removal of oil and the formation of a halo by adding bacteria supernatant were considered as an indicator of the production of biosurfactant. In this method, the diameter of the created halo is an index for concentration of biosurfactant production by bacteria. The removal of oil and the observation of a clear area on the water surface confirmed the presence of biosurfactants (12).

Hemolysis test

The ability of bacteria to produce surfactant was also assessed by hemolytic activity test. In this method, after 16 hours of bacterial culture, 100 µL of culture medium was spread on blood agar plate containing 5% blood and incubated at 28°C for 96 hours. After incubation, the presence of a clear halo on the plate was investigated as an indicator of biosurfactant production. Hemolysis halo formation was monitored every 24 hours (13).

Emulsification index test (E24)

Two milliliters of bacterial culture fluid and 1 ml of liquid oil were poured into a test tube and mixed vigorously for 2 minutes using vortex, then, placed at 30°C for 24 hours. Finally, using a millimeter ruler, the emulsification index was calculated by dividing the height of the emulsified layer to the total height of the mixture based on the following formula. The higher the height of the emulsified layer compared to the total height of the mixture, the higher the emulsion index (14).

$$E24 = \frac{Height\ of the\ emulsified\ layer}{Height\ of the\ total\ mixture} \times 100 \qquad \text{Eq. (1)}$$

Assessment of petroleum hydrocarbon on the growth rate by S. halophila at different crude oil concentrations

For degradation studies, total petroleum hydrocarbon was measured spectrophotometrically according to the method used by Rahman et al (15). For this purpose, organisms were inoculated in 3 replicates with 20 mL Bacto Marine Broth and various crude oil concentrations (1%, 2.5%, 5%, and 7.5%) (Assaluyeh Petrochemical Co.) as the only carbon resource for bacteria, and finally, the samples were placed on a shaker at 150 rpm and incubated for 8 days at the optimum temperature and pH was measured in pervious sections. To measure the bacterial growth at different crude oil concentrations, optical density was read at 600 nm every 24 hours.

Data analysis

To compare the growth rate of bacteria at various crude oil concentrations, statistical analysis was used. First, data normalization was determined by the Kruskal-Wallis test. Statistical analysis was carried out using one-way analysis of variance (ANOVA) by SPSS version 11.5. The mean values of various treatments were tested. To determine the significance, Tukey's test was also used. The significance level was considered at $P < 0.05$. The diagrams were drawn in Excel.

Results

In the oil spreading test, studies showed that this strain was able to remove the oil layer on the water surface and create a clear area, while phosphate buffered saline (PBS) as a negative control on the oil surface did not change anything (Figure 1). This confirms that these strains are capable of producing biosurfactants.

Hemolysis method was also used as a primary method to investigate the production of biosurfactant by this strain. During the hemolysis test, the strain produced a clear halo, indicating the production of surfactant by this strain (Figure 2A).

Also, microscopic examination of the plate containing blood agar showed that emulsifying drops were observed under a microscope with 40X magnification (Figure 2B). The results of emulsification activity also showed that the strain has more than 30% of emulsifying activity, indicating that it is a suitable strain for biosurfactant production. Studies have shown that this bacterium emulsifies diesel oil up to about 32%, crude oil up to about 37%, and sunflower oil and olive oil up to about 35% (Figure 3). A higher index will indicate that the emulsifying agent is more powerful and can combine the two phases of the emulsion with better efficiency.

Bacterial growth rate was measured at different crude oil concentrations by calculating optical density in all groups. In addition, crude oil degradation by bacteria, and then, total hydrocarbons were measured. The results showed that the maximum growth occurred in the 6th day 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 (Figure 4). These data showed that *S. halophila* can degrade crude oil by production of biosurfactant.

Discussion

In the present study, the production of biosurfactants by *Halobacillus halophilus* (*Sporosarcina halophila* strain) and its growth in the presence of crude oil were investigated. The method of spreading oil and hemolysis on agar medium and the emulsifying activity were investigated and the supernatant of this culture medium showed significant results in the production of biosurfactant by this strain in all three tests. The growth rate analysis at different concentrations of crude oil showed that *S. halophilus* can degrade crude oil by production of biosurfactant. In fact, *S. halophilus* secretes biosurfactant, which reduces the surface tension of oil

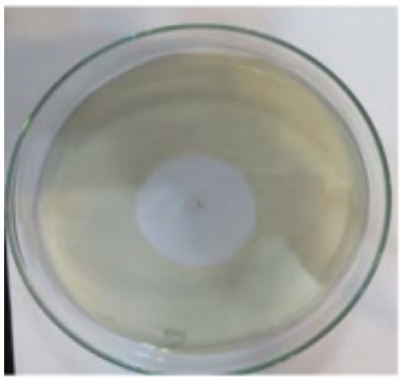

Figure 1. The clear halo resulting from the removal of oil from the water surface by *S. halophila*

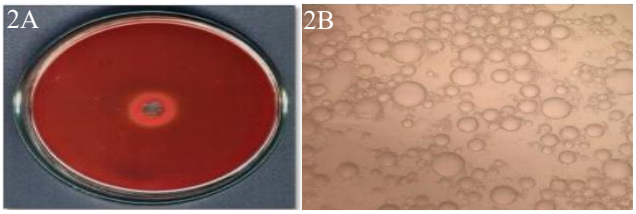

Figure 2. (A) The formation of a halo in the center of the plate, indicating the activity of bacterial hemolysis. (B) The formation of emulsion droplets under a microscope with 40X magnification

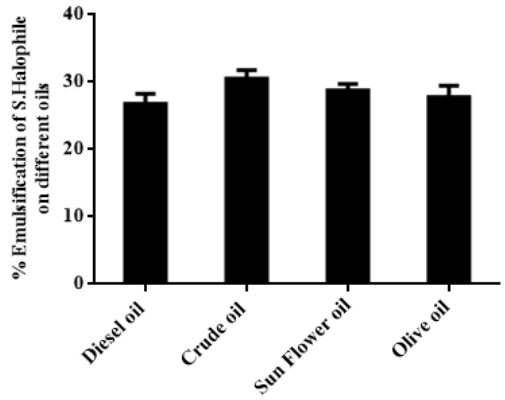

Figure 3. Percentage of emulsification of *S. halophila* based on E24 index on different oils. Bacterial emulsification was measured above 30%

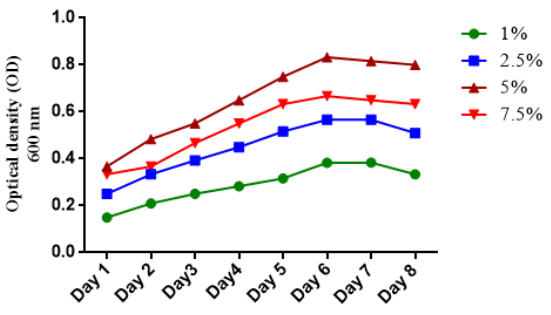

Figure 4. Effect of different crude oil concentrations on bacterial growth rate. Data showed that in 5% crude oil, bacterial growth was much better than other groups. The maximum growth rate occurred in the 6th day of bacterial growth with 5% crude oil (temperature = 25°C, pH = 7.5, incubation time = 8 days, OD = 600 nm)

and emulsifies it. The emulsified oil is broken down by bacteria, and turning into carbon dioxide and simpler substances that are absorbed by microbes in nature (16). Due to the nature of biosurfactants, these compounds are able to break down red blood cells by interfering with cell membranes. *S. halophilus* is one of the relatively most well-known salt-desire bacteria (9,16). Different strains of this bacterium have also been considered for their ability to remove polluting petroleum hydrocarbons (6). One of the methods of bioremediation is to add microorganisms to the environment that consume petroleum pollutants as a source of energy and carbon and convert it to carbon dioxide and water (17-19). Studies showed that oil pollution in nature is gradually degraded by microorganisms in the environment (20,21). Various microorganisms have been found in the areas contaminated with petroleum products, many of which could decompose oil and use it as a source of carbon and energy (22). Numerous studies on oil degradation by microorganisms have been conducted to find the best bacterial strains capable of decomposing pollutants in water and soil (22,23). *S. halophilus* is a heterotrophic bacterium (24). So, it requires carbon resources to obtain energy from the environment. Bioremediation has been used for many years in the world. Biological methods are compatible with the environment, and also, have a significant economic advantage over other methods of treatment (physical and chemical) (25). Bioremediation with the lowest cost is the ability to permanently remove contaminants by converting them into safe materials (25, 26). Numerous studies have been performed on various bacilli to clean up oil pollution. In a study of 15 species of crude oil-decomposing *Bacillus* isolated from oil-contaminated areas and their optimal growth conditions were evaluated (27). The surface tension and total carbon content, nitrogen and hydrogen in the crude oil were measured before and after exposure to the bacteria. The results show the effect of biodegradation of bacilli on petroleum hydrocarbons and their ability to use oil as the only source of hydrocarbons and energy by their surfactant production (28,29). In another study, *Halobacillus* licheniformis has been shown to have a significant effect on the removal of oil spots in seawater (30). Bacterial adhesion to hydrocarbons increases the solubility of hydrocarbons and stimulates their degradation (27). Also, in our pervious study, it was revealed that *S. halophilus* could degrade crude oil with high efficacy and can be classified as an *Alcanivorax* (alkane-degrading marine bacterium) bacterium (11). This study approved that *S. halophilus* is capable of producing biosurfactant and can be widely used in many different environmental and industrial fields.

Conclusion

According to the results of this study, the metabolites

obtained from *S. halophila* strain have emulsifying properties and can be considered as a potent strain in the production of biosurfactants, and the surfactant produced by this strain is a suitable option for use in industry or in environmental bioremediation.

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Ethical issues

The present study was approved by the Ethics Committee of Babol Noshirvani University of Technology, Babol, Iran (Ethical code: IR.Noshirvani.REC.1398.394).

Competing interests

The authors declare that they have no conflict of interests.

Authors' contributions

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Formal Analysis: Majid Zamani Beidokhti, Daryoush Yousefi Kebria.
Funding acquisition: Majid Zamani Beidokhti, Daryoush Yousefi Kebria.
Investigation: Majid Zamani Beidokhti.
Methodology: Majid Zamani Beidokhti, Daryoush Yousefi Kebria.
Project administration: Daryoush Yousefi Kebria.
Resources: Majid Zamani Beidokhti, Daryoush Yousefi Kebria.
Software: Majid Zamani Beidokhti, Daryoush Yousefi Kebria.
Supervision: Majid Zamani Beidokhti, Daryoush Yousefi Kebria.
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Visualization: Majid Zamani Beidokhti, Daryoush Yousefi Kebria.
Writing – original draft: Majid Zamani Beidokhti.
Writing – review & editing: Daryoush Yousefi Kebria.

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