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Isolation and Identification of Biosurfactant-Producing Bacteria in the Waters of Bandar Abbas and Determination of the Surface Tension Activity

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Biosurfactants are in the category of surface active molecules (or surfactants), but have a different structure and are produced by microorganisms. These molecules reduce the surface and interfacial tension in the environments of liquid and mixed with hydrocarbons that have a potential properties in biological therapy. They caused the increased solubility and removal of hydrophobic contaminants and this leads to many applications in biodegradation of these materials as well as oil recovery and the process of de-emulsification. The produced surfactants by microorganisms are preferred compared to the chemical surfactants because of biodegradation, low toxicity, very high efficacy of heat, acidity and salinity. Biosurfactants have antimicrobial, antifungal and antiviral effects as well. So, Biosurfactants can be used to combat diseases caused by pathogens (including bacteria and fungi). General use and popularization of the use of these materials depend on the better understanding and abundant production of these materials on an industrial scale. Therefore, the present study was conducted aimed to isolate, identify and evaluate the activities of the bacteria producing biosurfactants from the waters of Persian Gulf coasts. First, water and sediment sampling was done from three stations in Bandar Abbas coasts. Then, the biosurfactants-producing bacteria were isolated through biochemical and phenotypic tests. Biosurfactants were isolated and surface tension and the effect of environmental conditions was tested on their activities. The results of study showed that the isolated bacterium is Pseudomonas aeruginosa. This isolate reduces the surface tension from 71.1 mN/m to 21.38 mN/m. it was found that the biosurfactants are produced by this bacteria, thus this bacteria can be used to produce biosurfactants aims to help the scientific and practical improvement of industries in the Islamic Republic of Iran.

Keywords: Biosurfactant, Surface tension, Bandar Abbas.

Biosurfactants are a group of surfactants that are under the study and are a little applied in industries, agriculture, medicine and a little in environmental biomedicine. Biosurfactants are in the group of surface active molecules (or surfactants), but have a different structure and are produced by microorganisms. Due to today's concerns of the environment, research on biosurfactants is very important. These molecules reduce the surface and interfacial tension in the environments of liquid and mixed with hydrocarbons that have a potential properties in biological therapy. They cause an increase in solubility and removal of hydrophobic contaminants and this leads to many applications in biodegradation of these materials as well as oil recovery and the process of de-emulsification. Surfactants produced by microorganisms are

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preferred compared to the chemical surfactants because of biodegradation, low toxicity, very high efficacy of heat, acidity and salinity[9].

Another advantage of microbial biosurfactants is in their production ability on a bed of cheap raw materials including the waste of industrial, agricultural and food technologies and etc which leads to a lower cost of production. Although the interest in biosurfactants is increasing, they are not industrially cost-effective and economic yet, compared to the synthetic surfactants[4]. The success in their production depends on cheap processes and the use of cheap raw materials for 10-30% of the total cost of the technologic process of their production[5].

Previous studies have shown that a wide range of carbon resources such as renewable agricultural sources to produce biosurfactants are usable without harmful effects on the ecology and also with the best characteristics of surfactants with respect to the status of their application. The best production is obtained when carbohydrates and vegetable oils are used to produce biosurfactants[10]. Anderson (2013) from Utah University studied the characteristics of surfactants produced by the isolated bacteria from manure. He studied on Pseudomonas lurida. The objective of his study was to survey biosurfactant production in an effort to produce the surface active materials as a green way in the fertilizer manufacturing industry instead of using synthetic surfactants[3]. From the 16 isolated bacteria in the research environment, one bacterium had the activity of surfactant production. When it was cultured on the liquid and solid mediums and identified by reducing the surface tension of the liquid. Molecular identification by PCR showed that this bacterium is Pseudomonas lurida. Surfactant materials were isolated from the surface of grown cells on the solid medium. This product was not toxic to soil and bacteria in the soil. However, it caused an increase in cumulative activity of root colony bacteria such as Pseudomonas chlororaphis 06. So, it may contribute to the death of germs in the soil and in deeper levels. Surfactants produced by Pseudomonas lurida caused an increase in water infiltration in the agricultural soil, but did not have any effect on very hydrophobic soils produced by the fire in the nature.

So trying to reach alternative solutions to fight the oil and non-oil pollution in the waters of Persian Gulf is necessary. The present study takes steps in the direction to identify the bacteria producing biosurfactants and amount of their surfactant activities in the waters of Bandar Abbas coast.

The present study seeks to achieve the overall objective of biosurfactant producing bacteria in the waters of Bandar Abbas and the determination of their biosurfactant activities.

Testing method

Sampling method

In this study, water and sediment sampling was done from 3 stations of Bandar Abbas coast in the south of Iran. 3 samples were taken from each station and totally 9 samples were collected. In two stages during the April 2015 to July 2015, samples were collected. A total of 18 samples were collected and transferred to the Microbiology Laboratory of Islamic Azad University, Science and Research branch in Fars. The distance from the sampling site to the laboratory was less than 4 hours and samples were transported to the laboratory in a portable

Table 1. Total viable cells

Dilution	Number of bacterial colonies	Dilution factor	THB (CFU/ml)
1	280	10^{-2}	2.8×10^5
2	200	10^{-3}	2.0×10^5
3	160	10^{-4}	1.6×10^5

Fig. 1. Sample of a pour plate of the studied isolate

refrigerator. Then, to analyze and identify the biosurfactant producing bacteria were cultured on a mineral salt medium.

Bacteria isolation

Mineral-base mediums of Di potassium hydrogen phosphate, ammonium chloride, manganese sulfate and 1 ml of micronutrients with 1000 ml of distilled water were used to isolate the bacteria. After incubation of the primary culture at 30ºC for 3 days on a shaking incubator, colonies with distinguishable morphological properties and form were isolated and cultured on ENDO and Maconkey agar mediums. The colonies obtained from the secondary culture were used as a pour plate to test biosurfactant production and oil analysis. The isolated strains through strain culture on the nutrient agar medium were kept at 4ºC.

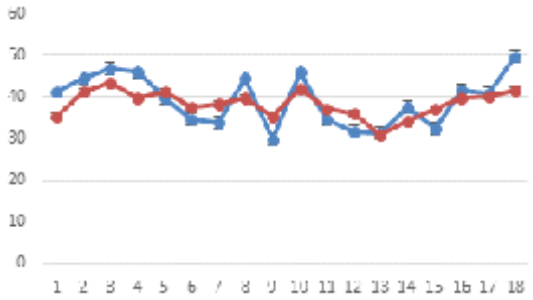

Chart 1. The frequency distribution of surface tension rate of the studied isolate culture plates

Table 2. phenotypic identification of the studied isolate

Test	Result
Gram staining	negative
Spore staining	negative
motility	positive
Catalase	Positive
Oxidase	Positive
Urease	Positive
Oxidative/fermentative	Negative
Melting gelatin	Positive
Glucose fermentation	Positive
Lactose fermentation	Negative
Sucrose fermentation	Negative
Methyl red	Negative
Voges-Proskauer	Positive
The use of citrate	Positive
Indole production	Positive
H2S production	Positive
Hydrolysis of starch	Positive

Viable cell counts

Colonies derived from the primary culture were serially diluted with sterile distilled water to 10^{-6} and 100 µl of each dilution was inoculated on to the culture plates. These plates were incubated for one night (12-18 hours) at 37ºC. Then, the result in terms of the number of viable colonies counts were counted using the total viable colonies count method (7).

Prescott and Harley formula to count the viable cell colonies is in the following:

The primary sample (CFU/ml) = Number of plates/ number of colonies × (1/ml of inoculum size) × dilution factor

Determining the ability of biosurfactant production by the isolates

The isolated bacteria were cultured on mineral salt agar medium (containing 1% oil as the carbon source). To do this, one ml of the overnight culture (18 hours) of the bacteria on a BHI broth was removed by the sampler and released on

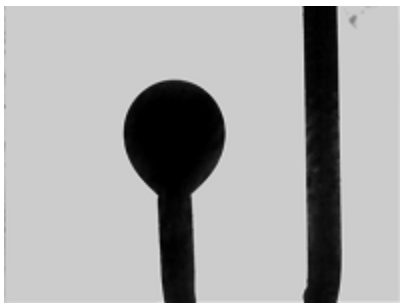

Fig. 2. An image of the hook of the device measuring the Neuer surface tension

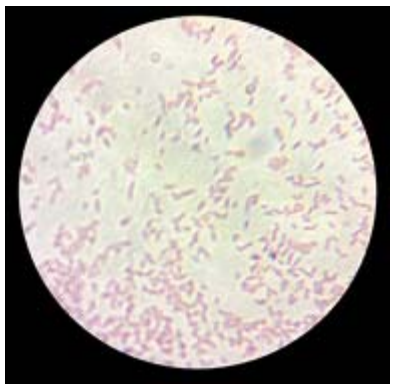

Fig. 3. Light microscope image of the isolate

mineral agar medium. Then, it was incubated at 30ºC for 7-10 days (10).

Data analysis

The gathered data was analyzed using descriptive and inferential statistics and IBM SPSS software version 22 and reported in section 4 as the tables and charts.

Test results

Collecting samples and isolation of the bacteria

After the initial culture of the samples, the colonies with morphologically identified and distinguished appearance were isolated and cultured on ENDO and Maconkey agar mediums as the pour plate. To determine the viability and proliferation of the isolated bacteria, viable cell count was carried out and the results is reported in table 1.

Viable cell count

Total count of viable cells was performed on the plates with 40 to 300 colonies (table 1-4). Total viable cells was calculated using the following formula:

(THB) = number of colonies × colony size based on CFU/ml

Results of the initial culture led to isolate of two isolates with distinguishable morphological appearance that only one colony had viability and the ability to be cultured and showed the most reduction in surface tension. This isolate reduced the surface tension from 71.1 mN/m to 31.4 mN/m. This colony was removed from the primary culture plate and cultured on the secondary culture plates as the pour plate. The isolated colony was used for all subsequent tests. In the following figure, a sample of a pour plate of the studied isolate is displayed:

Determining the biosurfactants producing ability of isolates

To determine the biosurfactant-producing ability of the studied isolate, the tests of measuring surface tension, emulsification index, drop collapse test, hemolytic activity and expanding oil test were conducted. The results of these tests are as follows:

Measuring surface tension

Surface tension is used to confirm the secretion of surfactants. First, surface tension of the sample is controlled and then surface tensions of the test sample are measured. Reducing surface tension by strain gauge K6 was measured. In chart 1, the results of measuring surface tension are shown.

As chart 1 shows, the most reduction in the surface tension in the first section is related to the isolate 9. Also in the second section of sampling, the most reduction in the surface tension was observed in the isolate 13. Both isolated had the same lowest surface tension and were considered as the basic isolate. In this chart, isolate 13 shows the reduction of surface tension in 31.4 mN/m. To determine a variable in a group, one-sample t-test was used. One-sample t-test showed that the studied isolate has the ability to produce biosurfactant with the probability of $p<0.05$. In this study, the studied isolate with an average of 31.39 and a standard deviation of 25.6 showed that has the ability to reduce the surface tension ($df=17$, $t=24.67$, $p=0.000$).

Identification of bacteria

The suitable isolate was selected according to the highest production of biosurfactants and the ability to be cultured through the microbial and biochemical tests was identified. The results of physical and biochemical (phenotypic) tests using for identification of the studied isolate was as follows:

According to the results of physical, morphologic and biochemical tests, it was found that the studied isolate in this study is Pseudomonas aeruginosa.

DISCUSSION

In the present study, biosurfactant-producing bacteria were isolated from the Persian Gulf coastal sediments where hydrocarbons contaminants and heavy metal pollution caused by maritime transport can be seen. The isolated bacteria by expanding oil test (Violetta, 2011), collapse of the drop (Rosenberg et al, 2011) and emulsification index (Anandaraj, 2010) showed that this bacterium has a high ability of emulsification. Biosurfactant produced by this bacterium reduced the surface tension significantly which indicates their potential application in different industries. The extracted biosurfactants from the culture of this bacterium had a dry biomass about 7.3 g/ml which corresponded to the results of Arji et al (2013). The results of this study showed that the studied isolate can use the environmental salts as

the nitrogen source and glucose, phenol and glycerol as the carbon source. Most biosurfactant production in the presence of glycerol as a carbon source. These results are consistent with the findings of Anderson's study (2013). The study results showed that the best temperature for biosurfactant production by Pseudomonas aeruginosa is 37ºC and this bacterium has the most production of biosurfactants in the acidity of 5.6-5.7 which is consistent with Hamze et al study (2013) and Zank et al study (2012). To identify, the common physical and biochemical tests were used. Phenotypic actions led to the identification Pseudomonas aeruginosa in this study which are similar to the methods using in Violetta study (2011). Therefore, identification of bacteria has had the correct path. Results of the present study showed that Pseudomonas aeruginosa has viability and culturability properties which is corresponded to Zank et al study (2012).

CONCLUSION

Today, surfactants are widely used around the world in different industries from oil and gas big industries to the small industries such as production of cosmetics, from pharmaceuticals to food additives. Synthetic surfactants are associated with major problems such as lack of biological decay, health problems caused by preservatives and chemical compounds and etc. so, the world is looking for conversion ways to replace surfactants. One of these conversion ways is the use of produced biosurfactants by microorganisms. In the present study, Pseudomonas aeruginosa was isolated by phenotypic methods (physical and biochemical) from the coastal waters of Persian Gulf.

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