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DISCOVERY OF THE NEW OIL-DEGRADING BACTERIA WITH BIOSURFACTANT PRODUCTION ABILITY FROM OILY TAILINGS PONDS WASTE, OIL-POLLUTED SOIL, AND LIGHT AND HEAVY CRUDE OILS FOR REMEDIATION OF CRUDE OIL IN WATER

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Abstract: In this study, oil-degrading bacteria with biosurfactant production ability were discovered from oily tailings pond waste, refinery-contaminated soil, and light and heavy crude oils using the enrichment culture technique with Bushnell-Hass media. The ability of bacteria in crude oil biodegradation were also determined by conducting batch tests in flasks containing mineral salt medium (350 ml, pH 7, salinity of 30 ppt) and crude oil as the only carbon source (2 mL). Samples were incubated at a temperature of 26°C and 120 rpm for five weeks. The level of oil degradation at different periods of biodegradation was monitored weekly by analysis of the remaining of total petroleum hydrocarbons using a gas chromatograph (GC-FID). Moreover, the produced biosurfactants physicochemical properties, quality, and quantity were determined using surface tension (ST) and oil-displacement methods. A total of five oil-degrading bacteria were discovered from oily tailings ponds waste (1 species), refinery-contaminated soil (two species), light crude oil (one species) and heavy crude oil (one species). All species showed biosurfactant production ability. The lowest surface tensions of supernatants were between 55 mN/m to 40 mN/m at the end of the five-week biodegradation period. The minimum ST was belonged to the BS produced by species discovered from oily tailings pond waste (40 mN/m). Moreover, crude oil biodegradation of nearly 70% was obtained with the isolated bacteria from samples during the five-week biodegradation period. This study confirmed the fast and effective biodegradation of crude oil by the isolated bacteria with biosurfactant production as the main mechanism of oil uptake.

1. INTRODUCTION

Soil and groundwater contamination by petroleum hydrocarbons are the most common pollution resulting from the leakage of underground storage tanks (UST) (Paria, 2008), broken oil pipelines, oil refineries and storage facilities, oil spills in chemical plants and transport processes (Sherman and Stroo, 1989). Bioremediation is one of the most widely used biological methods, but the efficacy of microbial biodegradation is generally limited by the low bioavailability of hydrocarbons to microorganisms (Van Hamme et al., 2003). Biosurfactants are a class of natural chemicals that are produced by microorganisms including oil-degrading bacteria. Biosurfactants have an amphiphilic nature due to the presence of hydrophilic and hydrophobic parts, which allow biosurfactants to interact with air/water and hydrophobic compounds. This can reduce surface and/or interface tension (Mulligan and Gibbs, 2004; Van Hamme et al., 2003). Biosurfactants also have low toxicity with potentials in decontamination of environmental pollutants (Mulligan and Gibbs, 2004). To reduce costs and expand the use of biosurfactants, more productive strains with less expensive substrates are needed. The aim of this study was to discover

potential oil-degrading bacteria with biosurfactant production ability from oily tailings pond waste, refinery-contaminated soil, and light and heavy crude oils and determine their ability for crude oil biodegradation.

2. MATERIALS AND METHODS

2.1 Isolation of oil-degrading bacteria and biosurfactants characterization

Laboratory tests were conducted with samples including tailings pond waste (Calgary, Canada), light and heavy crude oils (Petro-Canada, Montreal) and refinery-contaminated soil (DaGang oil field, China). The potential oil-degrading bacteria with biosurfactant production ability were isolated using a minimal salt medium (MSM) enrichment culture technique (Chandankere et al., 2013). Samples were incubated on the rotary shaker at 200 rpm for 7 days at 28°C. After five consecutive enrichment cycles, 1 mL of cultivation from each sample was diluted (10^{-5}) and placed on solidified MSM containing crude oil as the sole carbon source. Bacterial that were grown on the plates were used for the biodegradation tests and further analysis tests.

2.1.1 Oil-displacement test

For this test, 10 µl of crude oil were added to the surface of 40 ml of distilled water in a petri dish (d, 2.5 cm) and allowed to form a thin oil layer. Then, 10 µl of culture supernatant were gently placed on the center of the oil layer. If biosurfactant was present in the supernatant, the oil was displaced, and a clearing zone was formed (Morikawa et al., 2000).

2.1.2 Emulsification capacity assay

Some biosurfactants show emulsification capacity (EC). Therefore, the ECA was used to determine the EC of biosurfactants produced by the isolated bacteria (Cooper and Goldenberg, 1987). Crude oil and supernatants were added to centrifuge tubes (15 ml) containing water. The mixture was vortexed at high speed for 2 minutes. After 24 hours, the height of the emulsion layer was measured. The emulsion index (E24) was calculated as the ratio of the height of the emulsion layer and the total height of liquid (Cooper and Goldenberg, 1987).

2.1.3 Surface tension measurement

The Du-Nouy-Ring method was used to determine the surface activity of the cell-free supernatants (Tadros, 2006). Tap water and/or MSM were used to calibrate the device. The surface tension of cell-free culture supernatants with no dilution was measured at least three times.

2.1.4 Blood agar assay

The hemolysis test developed by Mulligan et al. (1984). Isolated oil-degrading bacteria were inoculated on plates containing sheep blood agar and incubated at 25°C for 2 days. Positive strains were selected based on the bacterial ability to cause blood cell lysis and display a colorless, transparent ring around the colonies.

2.1.5 Total petroleum hydrocarbon analysis

Four mL of samples were taken from each of the biodegradation flasks. The samples were transferred to separatory funnels and 2 mL of n-hexane (95% Sigma-Aldrich) were used for extraction. The mixture was shaken vigorously for 1 min and allowed to settle for 3 minutes or until the two layers were separated. The water layer was then eluted into a 100 mL Erlenmeyer flask through the stopcock and the process repeated three times. The hexane collected was dried using 2 g of sodium to remove residual water and filtered via a Whatman No. 40 filter. The extracts were then transferred to an amber vial of 20 mL and stored at 4°C until analysis with gas chromatography (CP-3800 VARIAN, GC - FID). Helium was used as a carrier gas with a constant flow rate of 2 ml/min and a flow rate of 30 ml / min. The hydrogen gas and air flow rates were 30 mL/min and 300 mL / min. Injector and detector temperatures remained constant at 250°C. The

oven temperature was set at 50°C for 2 minutes, increased to 250°C at 8°C / min and maintained at 250°C for 6 minutes (total run time of 33.25 minutes). The total oil hydrocarbon under the GC-FID chromatogram has been determined.

3. RESULTS AND DISCUSSION

3.1 Isolation and identification of oil-degrading bacteria

Five different crude-oil-degrading strains (Table 1) that showed growth on crude oil were isolated from different samples including tailings ponds waste, light and heavy crude oils, and refinery-contaminated soil. In general, one strain was isolated from each of the B-H media inoculated with the tailing ponds waste, and light and heavy crude oils and two strains were isolated from the B-H media inoculated with the refinery-contaminated soil. Visual examinations of the strains showed that all strains were circular (cocci) in shape and their size ranged from 0.5 to 3 µm. The color of bacteria was different and ranged from white to shiny yellow to pink-reddish.

Table 1 Morphological characteristics of the isolated oil-degrading strains from different samples

	Strain isolated from				
Characteristics	Tailings pond waste	Light crude oil	Heavy crude oil	Refinery-contaminated soil; strain I	Refinery-contaminated soil; strain II
Shape (form)	Circular	circular	Circular	Circular	circular
Size	Moderate	moderate	Moderate	moderate	moderate
Appearance	Shiny	shiny	Shiny	dull	dull
Pigmentation	Yellow	white	Yellow	pink	pink

3.2 Biosurfactant characterization

Table 2 summarizes the characteristics of biosurfactants produced by the oil-degrading strains in this study. The results of oil-displacement test after two weeks of incubation period showed that the biosurfactants produced by the isolated strains from refinery-contaminated soil, red and white colonies, had the lowest oil displacement. A 4 mm and 5 mm of crude oil was displaced respectively, as the cell-free culture supernatant from these strains were dropped on the liquid medium. The biosurfactants produced by the strains isolated from the tailings pond waste, heavy and light crude oils had the oil displacement diameters of 10 mm, 17 mm, and 20 mm, respectively. Moreover, the emulsification tests showed that none of the produced biosurfactants by the isolated strains showed a strong emulsification property (<10%). Surface tension measurements also showed that all strains had surface tension properties and all five strains could lower the surface tension of the tap water/LB/MSM. The lowest surface tensions of supernatants obtained were between 55 mN/m to 40 mN/m after five weeks of incubation period. The minimum ST belonged to the biosurfactant produced by strain isolated from the tailings ponds waste (40 mN/m). Moreover, blood agar results showed that all the five isolated oil-degrading strains had strong Hemolytic activity.

Table 2 Characteristics of biosurfactants produced by the oil-degrading strains in this study

Isolated strains ID	Isolated Environments	* Oil spreading method, (diameter, mm)	Emulsification activity, (E24%)	** Minimum ST (mN/m)	Blood agar lysis
T – 1	Tailings pond waste	10	<10	40.0	++++

L – 1	Light crude oil	20	<10	43.1	++++
S - I (red colony)	Refinery-contaminated soil	5	<10	54.6	++++
S - II (white colony)	Refinery-contaminated soil	4	<10	53.0	++++
H – 1	Heavy crude oil	17	<10	46.0	++++
Control - 1	Bacillus Subtilis	ND	ND	51.4	ND

* Oil spreading tests were conducted after 14 days of incubation on Bushnell-Hass media.
** Surface tensions measurements were conducted after five weeks of incubation on Bushnell-Hass media.
(-) no hemolysis, (+) incomplete, (++) complete hemolysis with a diameter of lysis between 1 to 2 cm, (+++) hemolysis with a diameter of lysis between 2 to 3 cm, and (++++) complete hemolysis with a diameter of lysis between 3 to 5 cm.
ND: not determined

3.3 Biodegradation experiment

The results of biodegradation experiment showed that maximum crude oil biodegradation of 63 ± 4% (the average ± the standard deviation of the biodegradation of crude oil by all five strains) was obtained with the strains after five weeks of the incubation at 26°C. The maximum biodegradation percentage was obtained with the strains isolated from the light crude oil and tailings pond waste with the biodegradation percentages of 12.7% and 18.5%, respectively in the first week of the biodegradation. While the biodegradation percentage obtained by the strains isolated from the heavy crude oil was < 4.5%. The biodegradation percentage obtained by the two strains of refinery-contaminated soil (both red and white strains) and *Bacillus subtilis* (as the control) were <0.5%, respectively. However, the biodegradation of crude oil increased further to 48 ± 8%, 56 ± 5%, and 63 ± 4% in the following weeks (Figure 1). As shown in Fig. 1 the crude oil degradation has reached a plateau after five weeks of incubation possibly due to complete mineralization of available hydrocarbons, limited bioavailability of remaining hydrocarbons and/or nutrients (Zhang and Miller, 1992).

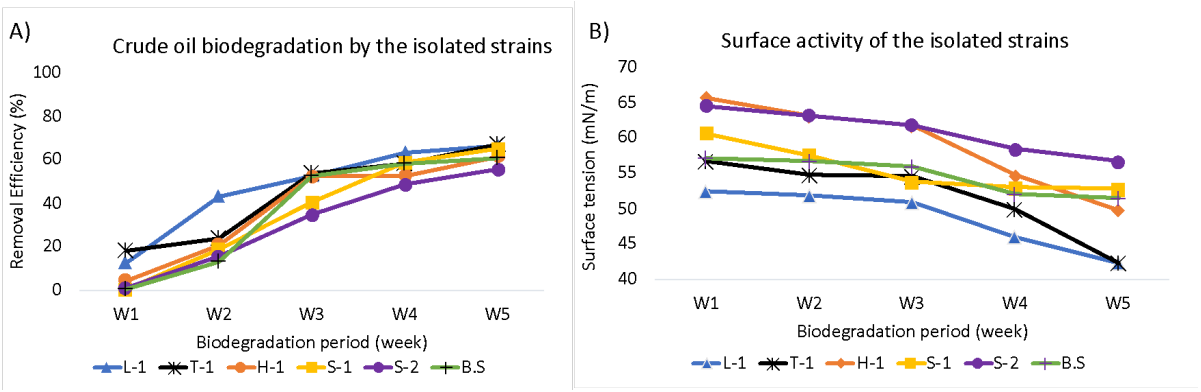

Figure 1 A) Biodegradation of crude oil in MSM (pH 7, salinity of 30 ppt) and B) surface tension values of cell-free culture supernatants over time by five different strains of oil-degrading strains isolated from the light crude oil (L-1), tailings pond waste (T-1), heavy crude oil (H-1), refinery-contaminated soil (S-1 and S-2) and *Bacillus subtilis* (B.S) as the control.

4. CONCLUSIONS

Worldwide, demand for surfactants is significantly increasing, but most of the surfactants available on the market are chemically based, primarily due to their easy availability, low price, and expanded areas of application. Now the market for biosurfactants is in its early stages of development, the use of biosurfactants has been restricted to a few specialized applications due to technical constraints and material costs.

Because knowledge on the physiology, genetics, and biochemistry of biosurfactant-producing strains needs to be expanded, screening of biosurfactant producing species and process technology development will help to reduce production costs. The present study reported the presence of biosurfactant-producing strains in oil-contaminated environments and their ability to biodegrade crude oil under the tested conditions (salinity of 30 ppt and temperature of 26°C). Five oil-degrading bacteria with biosurfactant production capacity were discovered using Bushnell-Hass media enrichment techniques from oily tailings pond waste, refinery-contaminated soil and light and heavy crude oils. All strains have high surface activity which could lower the surface tension. The lowest surface tensions obtained by cell-free supernatants ranged from 55 mN / m to 40 mN / m. The minimum surface tension was belonged to the strain isolated from oily tailings pond waste (40 mN /m). In addition, nearly 70% biodegradation of crude oil was obtained from samples with the isolated bacteria during the five-week biodegradation period. This study confirmed the isolated bacteria's rapid and effective biodegradation of crude oil with the production of biosurfactants as the main mechanism for oil absorption.

5. REFERENCES

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