Discovery of indigenous biosurfactant-producing oil-degrading species from oily tailings ponds waste and its biodegradation ability

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ABSTRACT
Numerous oil-degrading bacteria with biosurfactant production ability have been isolated from oil-related environments. However, less attention has been paid to the application of these compounds in oil-related activities. The objective of this study was to discover oil-degrading bacteria with biosurfactant production ability and to determine the contribution of the discovered oil-degrading bacteria in crude oil biodegradation. An oil-degrading strain was discovered from the oily tailings pond waste with biosurfactant production as the main mechanism of oil uptake. The culture supernatant lowered the surface tension of minimal salt medium (MSM) from 56.7 mN/m to 42.3 mN/m. Moreover, maximum crude oil biodegradation of 70% was obtained with the strain during the five weeks of the biodegradation period. This study suggests the management of the oily tailing ponds waste by indigenous oil-degrading bacteria capable of biosurfactant production as an effective and low-cost remediation method.

RÉSUMÉ
De nombreuses bactéries dégradant le pétrole et capables de produire des biosurfactants ont été isolées d'environnements liés au pétrole. Cependant, on a accordé moins d'attention à l'utilisation de ces composés dans les activités liées au pétrole. L'objectif de cette étude était de découvrir des bactéries dégradant les huiles ayant une capacité de production de biosurfactant et de déterminer la contribution des bactéries dégradant les huiles découvertes dans la biodégradation du pétrole brut. Une souche dégradant les hydrocarbures a été découverte dans les déchets de bassin de résidus d'hydrocarbures avec la production de biosurfactant comme principal mécanisme d'absorption d'huile. Le surnageant de culture a abaissé la tension superficielle du MSM de 56,7 mN/m à 42,3 mN/m. De plus, une biodégradation maximale du pétrole brut de 70% a été obtenue avec la souche pendant les cinq semaines de la période de biodégradation. Cette étude suggère que la gestion des déchets de bassins de résidus d'hydrocarbures par des bactéries indigènes dégradant les huiles, capables de produire des biosurfactants, constitue une méthode de restaurationefficace et peu coûteuse.

1 INTRODUCTION

Petroleum contamination of the environment occurs frequently (Paria 2008). Bioremediation of petroleum-contaminated environments can be considered as an effective remediation method when hydrocarbons are available to microorganisms (Van Hamme et al. 2003). Biosurfactants are a class of natural surface-active chemicals produced by several oil-degrading microorganisms (Mulligan and Gibbs, 2004; Van Hamme et al. 2003). Biosurfactants have amphiphilic nature (Mulligan and Gibbs, 2004); that is there are both hydrophilic and hydrophobic parts in their molecular structure (Mulligan and Gibbs, 2004). This allows interaction between biosurfactants with air/water and hydrophobic compounds (Mulligan and Gibbs, 2004). As a result, the surface and/or interfacial tension is reduced (Mulligan and Gibbs, 2004). Moreover, biosurfactants' low toxicity provides great opportunities to apply them for decontamination of environmental pollutants (Mulligan and Gibbs, 2004). However, one limitation to their environmental applications is their high cost. Strategies including production from cheap materials and search for microorganisms with biosurfactant production ability can reduce the costs associated with their production. The aim of this study was to discover potential hydrocarbon-degrading bacteria with biosurfactant production ability from oily tailings pond waste. Moreover, the crude oil biodegradation with the discovered oil-degrading bacteria was also assessed.

2 MATERIALS AND METHODS

An environmental sample including oily tailings pond waste (Calgary, Canada) was used for isolation of oil-degrading bacteria without any pretreatment or modification.

2.1 Microorganism

The microbial strain used in this study was isolated from oily tailings pond waste by the culture-dependent technique using sterilized crude oil as the sole carbon source.

2.2 Media and Chemicals

Bushnell Haas (BH) and minimal salt medium (MSM, Fisher Scientific, Canada) were used in this study (Chandankere 2013).

2.3 Experimental Design

The experimental phase was comprised of three steps including: (1) isolation of crude oil-degrading bacteria, (2) verification and characterization of biosurfactant-producing bacteria and (3) determining the contribution of isolated oil-degrading bacteria in crude oil biodegradation.

2.3.1 Isolation of Oil-Degrading Bacteria

The isolation of oil-degrading bacteria was conducted by the culture-dependent method (Rahman et al. 2002). Two grams of oily tailings pond waste was inoculated in 200 mL of BH broth medium amended with 2 g of crude oil (sterilized by autoclaving at 120 °C for 20 min) as the sole carbon source. Samples were placed on an orbital shaker (200 rpm) and incubated at 26 ± 1 °C for 7 days. The sub-culturing was repeated two more times by inoculating fresh BH broth with 2 mL of cultures from the previous step and amended with sterilized crude oil. This step was followed by three consecutive strain purifications at which different dilutions (10^0 to 10^{-5}) of the cultures were made and 0.1 mL of dilutions were spread on the BH agar plates spiked with the sterilized crude oil. Plates were incubated at 26 ± 1 °C until distinct colonies were observed. Distinct colonies were then transferred to fresh BH plates for further purification and stored at 4 °C for further use.

2.3.2 Isolation and Characterizations of Biosurfactant-Producing Bacteria

The biosurfactant production ability of the isolated strain was assessed according to Chandankere (2013). Fresh MSM (200 mL) medium was inoculated with the isolated oil-degrading strain (1-5 distinct colonies) and amended with sterilized crude oil (2 g). Samples were incubated on the rotary shaker (200 rpm) for 7 days at 26 ± 1 °C. Several tests were conducted including oil displacement test, surface tension measurement, emulsification activity test, and blood agar test to characterize the produced biosurfactant by the isolated oil-degrading strain.

2.3.2.1 Oil Displacement Test

The oil displacement test was conducted according to Morikawa et al. (2000) with slight modifications. Briefly, 20 mL of distilled water was added to clean Petri dishes (d: 25 mm) followed by the addition of 20 µl of crude oil to the surface of the water. Then 10 µl of cell-free culture supernatant was gently placed on the crude oil surface. The diameter of the clear zone on the oil surface was visualized and measured (Morikawa et al. 2000).

2.3.2.2 Surface Tension Measurements

The surface activity of the biosurfactant produced by the isolated strain was assessed during biosurfactant production by a Du- Nouy Tensiometer (Fisher Scientific, Model 21) at room temperature (Tadros 2006).

2.3.2.3 Emulsification Activity of Biosurfactants

The emulsification index (E_{24}) of biosurfactant was determined according to Cooper and Goldenberg (1987). 5 mL of crude oil and 5 mL of cell-free culture supernatant were added to clean centrifuge tubes (15 mL). The mixture was vigorously vortexed for 2 min and kept undisturbed for 24 h at room temperature. Then the heights of the crude oil layer, the aqueous layer, and the emulsified layer were recorded. The E_{24} (in percent) was determined from the height of the emulsified layer divided by the height of the aqueous layer times 100.

2.3.2.4 Blood Agar Assay and CTAB Agar Plate Test

Hemolytic activity of strain is considered as an indication of biosurfactant production (Mulligan 1984) and the type of produced biosurfactants by the isolated strains was assessed (Siegmund and Wagner 1991). Isolated strains were grown on blood agar plates, containing 5% (v/v) human blood, and incubated at 30 °C for 48 h. After the incubation period, the hemolytic activity of strain was visually determined. The isolated strain was cultivated on the light blue agar plates containing cetyltrimethylammonium bromide (CTAB) (0.2 mg/mL) and methylene blue (5 mg/mL). The formation of dark blue halos around the colonies considered as a sign of anionic biosurfactant production.

2.3.3 Biodegradation of Crude Oil

The ability of isolated oil-degrading strain in crude oil biodegradation in water was studied. The biodegradation experiment was conducted at 26 ± 1 °C for five weeks (Saborimanesh and Mulligan 2015). Biodegradation experiment contained two treatments including (1) control (contained only MSM medium (350 mL, pH 7; salinity of 30 ppt) and sterilized crude oil) and (2) biotreatment (contained MSM medium (350 mL, pH 7; salinity of 30 ppt), sterilized crude oil and each of the isolated strains). Samples were placed on an orbital shaker (180 rpm) and incubated at 26 ± 1 °C for five weeks. Samples with *Bacillus subtilis* were used as the control. Samples (3 mL) covering days 0, 7, 14, 21, 28 and 35 were taken to analyze the total petroleum hydrocarbon (TPHs) and the surface activity of strains. TPH was measured using gas chromatography (GC-FID, CP-3800 VARIAN). 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).

3 RESULTS AND DISCUSSION

3.1 Isolation and Identification of Oil-Degrading Bacteria

Table 1 summarizes the specifications of the crude-oil-degrading strain isolated from tailings ponds waste. Visual examinations of the strain showed that all strains were circular (cocci) in shape. The color of the strain was shiny yellow.

Table 1 Specifications of the isolated oil-degrading strain from tailings pond waste.

Parameters	Specifications
Isolated environment	Tailings pond waste
No. of isolated strains	One
Shape (form)	Circular
Size	< 3 mm
Appearance	Shiny
Pigmentation	Yellow

3.2 Biosurfactant Characterization

The results of the oil-displacement test after two weeks of incubation period implied the biosurfactant production by the isolated strain. The crude oil displacement of 10 mm was obtained with the biosurfactant produced by the isolated strain. Moreover, the produced biosurfactant by the isolated strain did not show a strong emulsification property (<10%). The strain had a strong surface activity and lowered the surface tension of tap water and/or MSM to less than 42 mN/m. Despite the low crude oil displacement of the biosurfactant isolated from the tailings pond after two weeks of incubation, the strong surface activity by the biosurfactant (42 mN/m) after five weeks of incubation implied that the biosurfactant was produced at the late phase of growth of strain on crude oil. Moreover, blood agar results showed that the isolated strain had strong hemolytic activity.

3.3 Biodegradation of Crude Oil

The results of biodegradation experiment showed the crude oil biodegradation by the strain during five weeks of the incubation at 26 °C. The maximum biodegradation of 67% was obtained with the strain isolated from tailings pond waste (Figure 1). However, the crude oil degradation reached a plateau after five weeks of incubation possibly due to the possible complete mineralization of available hydrocarbons and/or limited bioavailability of remaining hydrocarbons and/or nutrients (Zhang and Miller 1992).

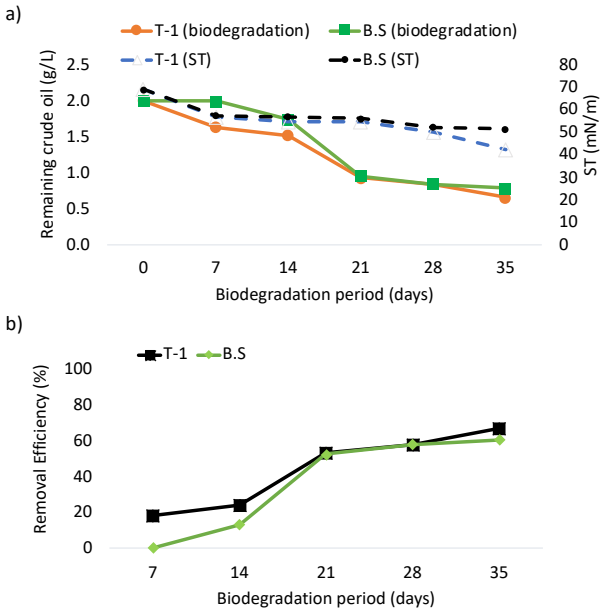

Figure 1. Remaining crude oil in g/L and crude oil removal efficiency after five weeks of biodegradation and corresponding surface activity (ST) of the strain isolated from oily tailings pond waste (T-1), and *Bacillus subtilis* which is a known biosurfactant producer (B.S, control).

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. The use of biosurfactants has been restricted due to technical constraints and material costs. The present study showed the presence of biosurfactant-producing strain in oily tailings pond waste and the strong surface activity of the produced biosurfactant as compared with biosurfactant produced by *Bacillus subtilis*, one of the known biosurfactant producers. Moreover, high crude oil biodegradation (67%) of this strain under the tested conditions (salinity of 30 ppt and temperature of 26 ± 1 °C suggests that this strain can be used for biodegradation of crude oil-contaminated aquatic environment.

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