# Surface activity of salt-tolerant *Serratia* spp. and crude oil biodegradation in saline soil

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**ABSTRACT**

An ideal strain for crude oil degradation in saline soils would be one with high salt-tolerance. A novel bacterial strain, *Serratia* sp. BF40, was isolated from crude oil contaminated saline soils. Its salt-tolerance, surface activity and ability to degrade crude oil in saline soils were evaluated. It can grow in liquid culture with NaCl concentration less than 6.0%. Its surface activity characterized as an efficient surface tension reduction, was significantly affected by salinity above 2.0%. BF40 inoculation could decrease surface tension of soil solutions and facilitate crude oil removal in soils with 0.22–1.20% salinity, but the efficiency was both significantly lower than its biosurfactant addition. The BF40 strain has a high potential for biodegradation of crude oil contaminated saline soils in view of its high surface activity and salt-tolerance, which is the first report of biosurfactant producing by the genus *Serratia* for petroleum degrading. We suggest that biosurfactant addition is an efficient strategy. Simultaneously, the growing status of the strain and how to boost its surface activity in saline soils should deserve further studies in order to achieve a continuous biosurfactant supply.

**Keywords**: strain; biosurfactant; surface tension; degradation potential

Oil spills in terrestrial and aquatic environments are increasingly common and cause significant ecological damage and civil challenges (Das and Mukherjee 2007, Thavasi et al. 2011). Due to pipeline leakage, mismanagement, and offshore oil production, petroleum- and salt-contaminated soils are often found in oilfields and coastal zones (Pezeshki et al. 2000, Nicholson and Fathepure 2004). Salinity is a key factor which can inhibit microbial growth and subsequent degradation of petroleum contaminants (Hua et al. 2010), and high salt concentrations (400 mmol NaCl) can inhibit microbial growth in soils by more than 90% (Rousk et al. 2011). Rhykerd et al. (1995) found that salt concentrations of 200 dS/m can decrease oil mineralization in soils by 20–44%. Consequently, bioremediation of petroleum- and salt-contaminated soil requires microbe strains to be salt-tolerant, and the degradation process can be complicated and time-consuming. Saline soil pollution from crude oil presents substantial hazards to coastal ecosystems (Maki 1991, Pezeshki et al. 2000). Compared with decontamination of non-saline soils, relatively few studies investigated bioremediation for crude oil polluted saline soils.

For these reasons we propose a study to isolate microbe strains from crude oil polluted saline soils and evaluate each based on their ability to thrive on crude oil as a carbon (C) source. Suitable strain will then be selected and evaluated for salt-tolerance and ability to degrade crude oil contaminants in saline soils.

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## MATERIAL AND METHODS

**Screening and isolation of petroleum-degrading microbial strains**. Crude oil (48.4% saturated hydrocarbons, 27.5% aromatics, 6.1% asphaltenes, and 17.0% non-hydrocarbon compounds) used throughout the study was collected from the Binnan Oil Production Factory of the Shengli Oilfield. Crude oil-polluted saline soil samples were collected from the Shengli Oilfield Production Area, located in the coastal zone of Bohai Bay. Screening and isolation of oil degrading microbial strains were conducted using a mineral salts medium and serial dilution-agar plating technique on nutrient agar medium with crude oil as the sole C source (Kennedy et al. 1975).

39 microbial strains were isolated from crude oil polluted saline soils. Based on their growth on nutrient agar medium with crude oil as the sole C source, one strain was selected and nominated as BF40, which was rod-shape and able to utilize crude oil as C and energy sources. Sequencing of the 16S rDNA gene for an unknown pure microorganism appeared as the predominant strategy in literature for strain classification (Nie et al. 2010). For BF40 strain, 1475 base pairs were analyzed. A BLAST search was then conducted through the National Center for Biotechnology Information (NCBI) database (Altschul et al. 1990), which revealed high similarity of BF40 strain with the 16S rDNA gene from the genus *Serratia* (98% match). Therefore, the pure isolate of BF40 strain was nominated as *Serratia* sp. BF40.

**Salt tolerance experiment**. To evaluate salt tolerance, BF40 strain was grown in Luria-Bertani (LB) medium with NaCl additions, i.e., 0, 2.0, 4.0, 6.0, and 8.0% at 30°C on a rotary shaker at 150 rpm (Hua et al. 2010). Growth was monitored through spectrophotometry at optical density 630 ($OD_{630}$) at 2, 3, 4, 5, 6, 8, 10, 12, 14, 18, 22, 26, and 32 h.

Surface tension and emulsification activity were employed to evaluate the surface-active properties using culture supernatant. Based on biosurfactant production, a high biosurfactant production liquid medium (BPLM) supplied with glucose was selected in our pilot study (Nie et al. 2010). After 0, 12, 24, 36, 48, 60, and 72 h, culture supernatant of BF40 strain was prepared to determine surface tension and emulsification activity. BF40 strain was also grown in BPLM at NaCl concentrations 0.5, 1.0, 2.0, 3.0, and 4.0% for 36 h to investigate the influence of salinity on the surface-active properties. Surface tension was determined with a surface tensiometer (JK99B, Pingxuan, Shanghai). Emulsification activity was assessed by the protocol of Cooper and Goldenberg (1987). A 15 mL graduated clear tube was filled with 5 mL supernatant and 5 mL liquid paraffin. After thorough vortex mixing at maximum speed for 2 min, the tube was left standing undisturbed at room temperature for 24 h. The height of the liquid paraffin layer was measured and divided by the total height of liquid paraffin and aqueous phases. The ratio was multiplied by 100 to obtain the emulsification index (EI24). All measurements were taken in triplicate.

**Isolation of biosurfactant**. Isolation of biosurfactant was conducted according to Nie et al. (2010). 1000 mL of the BF40 supernatant were harvested at 36 h by centrifugation at 4000 rpm and 4°C for 15 min to remove cells. Supernatant was then acidified to pH 2.0 with concentrated HCl and kept at 4°C overnight. The precipitate was recovered by centrifugation at 4°C and 12 000 rpm for 30 min and then washed twice with aqueous HCl (pH 2.0). Precipitates were dissolved in 1 mol/L NaOH and adjusted to pH 7.0. Crude biosurfactant thus obtained was dried in vacuum and stored at –20°C until use.

**Biodegradation of crude oil in soils**. Soil used was collected from topsoil (0–20 cm) near the oil well of the Shengli Oilfield. Crude oil concentration in soil was less than 100 mg/kg. Soil salinity and pH were 0.22% and 7.90. Soil organic matter and total N and P contents were 7.78, 0.63 and 0.65 g/kg, respectively. Available N, P and K contents were 27.3, 24.1, and 227.4 mg/kg, respectively.

Soil samples were adjusted to 0.60% and 1.20% through the addition of NaCl. Soils were then contaminated with 1.2% (w/w) crude oil. For each contaminated sample, 100 g (dry weight) was weighed and placed in a 200 mL Erlenmeyer flask. At each saline level (0.22, 0.60, and 1.20%), three treatments were arranged: soil samples inoculated with 10 mL ($10^9$ cells/mL) medium of BF40 strain; soil samples supplied with 20 mg/kg biosurfactant from BF40 strain; and soil samples supplied with 10 mL medium without BF40 cells as the control. Soil moisture was adjusted to 60% through the addition of distilled water. After 70 day incubation, surface tension of soil solution (1:2.5) was determined using a surface tensiometer. Total petroleum hydrocarbons (TPH) in the soil were determined gravimetrically as described below. Each treatment was conducted in triplicate.

**TPH analysis**. Soil TPH was extracted using a Speed Extractor (E-916, Büshi, Flawil, Switzerland). 10 g of dry soil was placed in an extraction cell mixed with 2 g anhydrous $Na_2SO_4$ extracted with

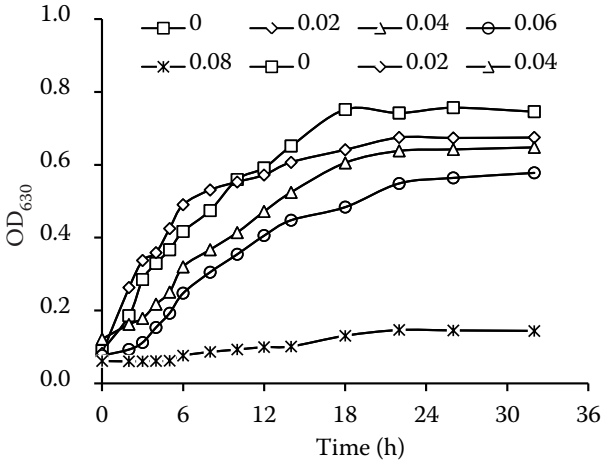


Figure 1. Growth curves of *Serratia* sp. BF40 in medium with different NaCl concentration

100 mL dichloromethane. The temperature and pressure were set at 100°C and 120 bars. After 2 cycles, the extract was condensed to 2 mL in a rotary evaporator (N-1001V, EYELA, Tokyo, Japan) and dried at room temperature under a gentle N stream in a fume hood. The amount of residual TPH was determined gravimetrically.

**Statistical analysis**. All statistical analysis was conducted using SPSS 11.0 (SPSS Inc., USA). To detect significant differences among treatments, ANOVA and least significant difference (*LSD*) were calculated. All significant differences reported are $P \leq 0.05$ unless otherwise noted.

## RESULTS AND DISCUSSION

**Effect of salinity on microbial growth**. Based on spectrophotometry at $OD_{630}$, BF40 strain grew well in medium with NaCl concentration less than 4.0%, showing slight change within 0–4.0% NaCl (Figure 1). When NaCl concentration increased to 6.0%, the reproduction time significantly increased and growth plateaus ($OD_{630}$) decreased by around 22.5%, which indicated that growth was inhibited to a certain extent by salt stress. At 8.0% NaCl, cultivation for 32 h resulted in only a slight increase in $OD_{630}$, which suggested that growth almost completely halted. Therefore, the BF40 strain can grow in liquid culture with NaCl concentration less than 6.0%. Salinity may affect growth processes, and salt-tolerant strain should be further tested for their ability to facilitate bioremediation under saline conditions.

**Effect of salinity on surface activity**. Growth of BF40 strain produced the lowest surface tension after 24 h (around 30.0 $mN/m^2$), which was less than half the BPLM without microbial seeding (70.2 $mN/m^2$, Figure 2A). Surface tension was not significantly different when NaCl concentrations were less than 2.0%, but above 2.0% it was positively correlated with NaCl increase (Figure 2B). To our knowledge, *Serratia* has never been mentioned in scientific literature as a biosurfactant producing microorganisms for crude oil degrading, thus, this finding expands scientific knowledge of the diversity of biosurfactant producers. Another approach for screening potential biosurfactant producing microbes is the estimation of emulsifying activity. In this study, EI24 of BF40 strain was less than 45.0% in all treatments (Figure 2), which was much lower than the efficient emulsification activity of 65.0% reported by Bento et al. (2005). Salt concentrations had a significant effect on EI24, which decreased sharply with NaCl concentrations greater than 1.0%.

Calvo et al. (2009) point out that two different types of biosurfactants are produced by microbes. One type consists of low molecular weight molecules that efficiently lower surface tension and interfacial tension, and the other type consists of high molecular weight polymers that have high emulsification activity at low concentrations. Both

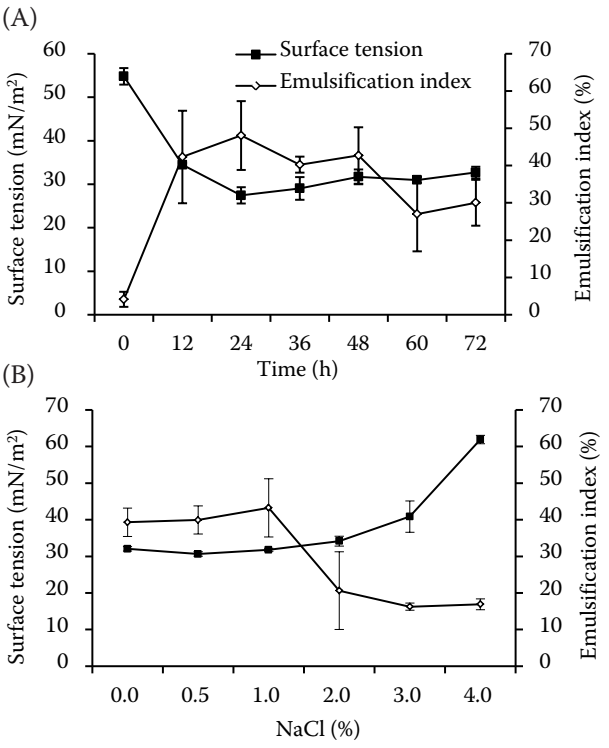


Figure 2. Surface activity of culture supernatant of *Serratia* sp. BF40 at different times (A) and NaCl concentrations (B). Bars in each curve represent standard deviation ($n$ = 3)

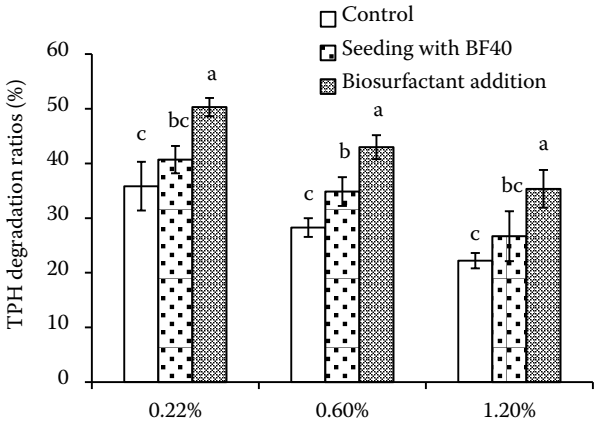


Figure 3. Crude oil degradation in different treatments. Columns represent means ($n$ = 3) and bars represent standard deviation; means with the same letter within groups are not different at $P < 0.05$. Control – un-inoculated; inoculation with BF40; biosurfactant addition – addition of biosurfactant from BF40; TPH – total petroleum hydrocarbons

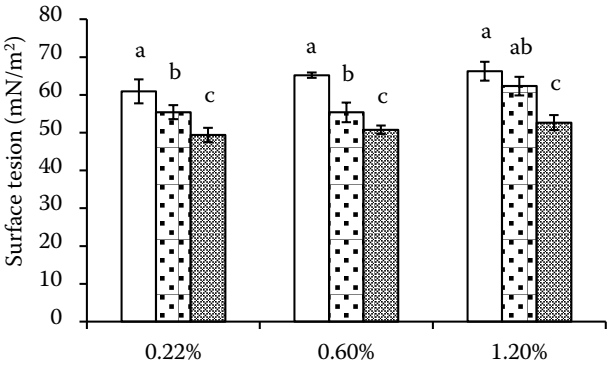


Figure 4. Surface tension of soil solutions in different treatments. Columns represent means ($n$ = 3) and bars represent standard deviation; means with the same letter within groups are not different at $P < 0.05$. Control – un-inoculated; inoculation with BF40; biosurfactant addition – addition of biosurfactant from BF40

low surface tension and high emulsification activity can enhance bioavailability of hydrophobic pollutants. The results indicated that BF40 strain was efficient biosurfactant producer, and belonged to the first type. Although BF40 strain exhibited salt-tolerance, surface activity was significantly affected by salt stress, which indicated that microbial growth and metabolic activity were more susceptible than their viability (Rhykerd et al. 1995).

**Biodegradation of crude oil in soils**. With soil salinity increased from 0.22% to 1.20%, TPH degradation ratios in the treatments significantly decreased by 29.8–38.0% (Figure 3). The greatest reduction was in the control, and the lowest in the biosurfactant addition treatment. Compared with the control, TPH degradation rates in soils with 0.22, 0.60, and 1.20% salinity increased by 13.6, 23.3, and 20.0% by BF40 inoculation, and increased by 40.4, 52.0 and 59.1% by biosurfactant addition, respectively. The increment of crude oil removal reached the significant level in the biosurfactant addition treatments. The results indicated that biosurfactant from BF40 can significantly facilitate biodegradation of crude oil in soil, especially under saline conditions.

The surface tension of soil solution from different treatments was also determined, and results showed that surface tension in the biosurfactant addition treatments was 50.0 mN/m$^2$ or so, showing little change with salinity increase, and was significant lower than that in the control (Figure 4). BF40 seeding also significantly lowered surface tension, but the decrease was insignificant in soil with 1.20% salinity. Thus, biosurfactant from BF40 strain was efficient in saline soils, and little affected by salt stress. Biosurfactant addition had advantage over its producer inoculation in removing crude oil in this study.

The addition of biosurfactant secreted by microbe can enhance the biodegradation by increasing the bioavailability of the hydrophobic organic pollutants involved crude oil (Mukherjee and Das 2005). Inoculation with biosurfactant producers likely offers the advantage of a continuous supply of surfactant to enhance crude oil degradation in soil. In this study, BF40 inoculation had the positive effect on the removal of crude oil and the reduction of surface tension of soil solution. However, the biodegradation efficiency of BF40 inoculation was significant lower than that of surfactant addition in soils with different salinity. The possible explanation was that microbial growing conditions, such as soil constitution (Syafruddin et al. 2010, Roldán-Carrillo et al. 2011) and salinity (Figure 2B, Figure 4), could significantly influence the microbial surface activity, although BF40 strain possessed salt-tolerant ability.

In conclusion, the BF40 strain has a high utilized potential for biodegradation of crude oil contaminated saline soils in view of its high surface activity and salt-tolerance. For crude oil contaminated saline soil bioremediation, using biosurfactant is an efficient strategy. Simultaneously, the growing status of BF40 strain and how to boost its surface activity in saline soils should deserve further studies in order to achieve a continuous biosurfactant supply.

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