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Characterization of novel biosurfactant producing strains of *Bacillus* spp. isolated from petroleum contaminated soil

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ABSTRACT

Background and objectives: Biosurfactants are surface active agents with broad range commercial applications in various industries and have considerable advantages over their chemical counterparts.

Materials and Methods: In this study, bacteria were isolated from contaminated and uncontaminated soil and selected during preliminary screening using hemolytic activity, oil spreading and oil collapsed techniques. Isolates with at least more than one positive response to these three methods were subjected to complementary screening by measuring surface tension reduction as well as emulsification capacity. The criteria for selection of potent isolates were surface tension reduction below 40 mN/m and emulsification capacity of more than fifty percent.

Results: Using these stepwise screening procedures, two biosurfactant/bioemulsifier producing isolates have been successfully selected that were able to reduce surface tension effectively and one of which formed a stable emulsion.

Conclusion: Phylogenic relationships of the two potential candidates were determined comparing the 16Sr DNA gene sequences, revealing them as two isolates of *Bacillus subtilis* and *Bacillus cereus* that can be used in pilot scale for industrial production of new biosurfactant/bioemulsifier.

Keywords: *Bacillus*, biosurfactant, emulsification, petroleum, soil.

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INTRODUCTION

Surfactants of both biological and chemical origin are amphipathic molecules that accumulate at the interfaces and reduce surface and interfacial tension at the interfaces between liquids, solids, and gases thereby allowing them to mix or disperse readily as emulsions in water or other liquids (1-3). Surface active compounds are divided into two main types: those that reduce surface tension at the air-water interface (Biosurfactant) and those that reduce the interfacial tension between immiscible liquids or at solid- liquids interface (Bioemulsifier). Biosurfactants usually exhibit emulsifying capacity but bioemulsifiers do not necessarily reduce surface tension (4). Many microorganisms produce extracellular or membrane-associated surface active compounds that play essential roles in the survival of the producing microorganisms either through facilitating nutrient transport or providing microbe-host interaction or playing role as biocides (5, 6).

Biosurfactants are categorized mainly by their chemical composition and microbial origin. Generally, their structures include a hydrophilic moiety consisting of amino acids or peptides, mono-, di- or polysaccharides and hydrophobic moiety comprising unsaturated or saturated fatty acids. Accordingly, the major classes of biosurfactants include glycolipids, lipopeptides, lipoprotein,

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phospholipids, fatty acids, polymeric biosurfactant and particulate biosurfactants (7, 8). This wide range of structural diversity results in broad spectrum of potential industrial applications including production of food, cosmetics, and pharmaceuticals, agriculture, mining, enhanced oil recovery, transportation of crude oil, cleaning oil storage tanks and pipelines and soil remediation (9-11). Biosurfactant producing microorganisms belong to different genera including: *Arthrobacter* spp., *Bacillus* spp., *Candida* spp., *Clostridium* spp., *Corynebacterium* spp., *Nocardia* spp., *Pseudomonas* spp., *Rhodococcus* spp. and more other genera have been reviewed (2-12).

The enormous market demands for surfactants are currently met by numerous synthetic mainly petroleum-based, chemical surfactants. These compounds are usually toxic to the environment and non-degradable. Tightening environmental regulations and increasing awareness for the need to protect the ecosystem have effectively resulted in an increasing interest in biosurfactants as possible alternative to chemical surfactants (2) . They have advantages over their chemical rivals in bioavailability, biodegradability, activity under extreme condition, lower toxicity, ecological acceptability, structural diversity, productivity on cheap and renewable substrates, capacity for modification and mass production through biotechnology and genetic engineering (2, 5, 12).

At present few biosurfactants have been used on an industrial scale due to the lack of cost effective production processes. Therefore the search for biosurfactant producing microorganisms that can be grown economically on industrial scale continues (4). Thus, the main goal of this study was to introduce novel microorganisms with potential for biosurfactant/ bioemulsifier production.

MATERIALS AND METHODS

Soilsamples. Soil samples included oil contaminated soil and an active farm soil. The physical and chemical characteristics including temperature, pH, moisture and electrical conductivity were determined using a portable analytical instrument (HACH Company) (13). Direct isolation of the microorganisms was carried out using serial dilution on (up to 10^{-7}) of soil samples in 0.85 % sterile saline. Using the spreading method on nutrient agar plates, total bacterial count was measured after incubation at 30°C for 24 hours. Morphologically distinct colonies were isolated and purified by replicating on the same solid medium to obtain pure cultures.

Preliminary screening of biosurfactant/ bioemulsifier producing bacteria. Pure isolates were cultured in E-medium at 30°C and 200 rpm for 9 days (14).The broth cultures were centrifuged at 22000×*g* for 45 min (15). The supernatant was subsequently subjected to the preliminary screening methods using oil spreading and oil collapse methods as below.

Oil spreading method. Oil spreading technique was carried out according to the method described previously by Youssef *et al.* (14) and Plaza *et al.* (6). Briefly, fifty milliliter of distilled water was added to the petri dishes followed by addition of 100 µl of crude oil to the surface of water. Then 10 µl of cell free culture broth was dropped on to the crude oil surface. The diameter of clear zone on the oil surface was measured and compared to 10 µl of distilled water as negative control.

Oil collapse method. A modified oil collapse method was carried out using 96 well microtiter-plates containing 100 µl mineral oil which was equilibrated for an hour at room temperature. Ten microliter of supernatant of culture broth was added to the surface of a well and the picture captured after 1 minute using 10 × objective lens of microscope. Biosurfactant production was considered positive when the drop diameter was at least 0.5 mm larger than those produced by distilled water and also by culture medium as negative controls (6- 14).

Hemolytic activity. Isolates were screened on blood agar plates containing 5% (v/v) sheep blood and incubated at 37°C for 48 h. Hemolytic activity was detected as the presence of a clear zone around bacterial colonies (6- 14).

Complementary screening. Isolates which were positive for at least more than one of preliminary methods were subjected to the complementary screening to verify their ability to produce biosurfactant / bioemulsifier.

Surface tension measurements. Surface tension reduction was measured using Krüss Hamburg Nr2215 Tensiometer and by submerging the platinum ring in the cell free culture broth and recording the force required to pull it through the air–liquid interface (16). The results were compared to distilled water

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Table 1. Microbial and physiochemical characteristics of soil samples.

Soil samples	Total count (CFU g^{-1})	pH	Moisture (%)	Electrical conductivity (mS)	Temperature (°C)
Oil contaminated	10^4×3.1	7.35	9.25	0.60	28
Newly harvested farmland	10^5×1.1	7.67	16.27	0.29	29

and medium composition (as negative control) and Tween 20 (as a positive control). The criterion used for selecting biosurfactant-producing agents was the ability of the isolates to reduce surface tension below 40 mN/m (4- 17).

Emulsification capacity (E24). Emulsification activity test was performed according to Krepsky *et al.* (18). The results were compared with water and pure E-medium as negatives controls and 1% solution of SDS (a common chemical bioemulsifier) as positive control. A criterion cited for emulsion stabilizing capacity is the ability to maintain at least 50% of original emulsion volume 24 hours after formation (4). Two-way ANOVA analysis ($p < 0.05$) was performed on emulsion activity data to determine significant differences in emulsion activity in the presence of different hydrocarbon sources while the difference between isolates were a blocked factor in this analysis. Statistical analysis was performed using Minitab (Version 15) software.

Biochemical and genetic analysis of superior isolates. Partial sequencing of 16S rRNA of two superior isolates was carried out in Pasteur Institute Laboratories, France. Morphological and biochemical identification tests were also performed following directions in Bergey's Manual of Systematic Bacteriology (19).

RESULTS

Two soil samples were screened for biosurfactant-producing microorganisms. To depict the environment from which the bacteria were isolated, physical and chemical characteristics of soil samples were determined (Table 1).

The grown population of the oil-contaminated and farmland soil were 3.1 ×10^4 CFU/g and 1.1×10^5 CFU/g, respectively. The initial isolation yielded a total of 102 pure isolates which were grown on nutrient agar. Among them, 16 isolates gave positive response to hemolytic activity; 32 positive results were obtained for oil spreading and 16 positive responses were obtained when oil collapse method was used; and in total 14 isolates or 13.72% putative biosurfactant producer were obtained with positive responses to more than one of the above preliminary screening methods.

The putative biosurfactant producing isolates were screened in complementary stage using two methods. Results from these experiments indicate that the surface tension varies from 23.3 mN/m to 57.6 mN/m and the emulsion activity ranging from 0 to 100% for different hydrocarbon sources (Table 2).

It is interesting that a given isolate showed different and distinct emulsifying response when exposed to each of hydrocarbon sources. Following complementary screening, five potential biosurfactant producing strains were isolated, of which 2 were gram positive and were further characterized (Table 3). Sequencing of 16S rDNA produced by polymerase chain reaction of bacterial DNA using universal primers revealed that superior gram positive biosurfactant-producing isolates were closely related to two *Bacillus* spp., including *Bacillus subtilis*, and *Bacillus cereus* (16) . Determination of the morphological and biochemical traits of these isolates confirmed the results of phylogenetic studies.

DISCUSSION

Biosurfactants are amphiphilic molecules with great diversity, environmental acceptability and broad spectrum of functions and industrial applications which make them interesting bio-products. Soil is a known habitat and source of versatile microorganisms and since the microorganisms capable of emulsifying and solubilizing hydrophobic agents have an apparent advantage over their competitors, sampling of this nature provides a source rich in microorganisms with desired characteristics.

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Table 2. Detection of biosurfactant producing isolates by preliminary and complementary screening methods.

Preliminary methods				Complementary methods			
Isolate	Hemolytic activity	Oil collapse	Oil spreading	Surface tension (mN/m)	E24 In presence of Kerosene	E24 In presence of n-Hexadecane	E24 In presence of Crud oil
SN1	-	+	++	23.3±0.05	53.59±0.56	63.18±2.06	100±0
SN12	+	-	+	26.2±0.05	2.17±0.23	2.22±0.25	67.53±3.98
Cw20	-	+	+	44.6±0.82	52.59±3.42	57.93±3.93	100±0
CY7	-	+	+	51.1±1.23	0±0	0±0	72.85±1.01
FY1	-	+	+	51.6±1.69	2.35±0.30	0.81±0.58	94.04±4.86
Fy4	+	+	+	43.1±0.80	2.90±0.46	2.61±0.37	82.9±6.48
Fw1	++	+	-	57.6±2.29	2.27±1.23	3.32±0.91	100±0
FY13	+	+	+	37.8±0	0±0	1.47±1.18	72.62±5.24
Fw13	+	+	+	52.0±2.35	0±0	0±0	67.33±6.22
Cw29	-	+	+	48.4±3.48	0±0	0±0	61.73±0.54
NP1	-	+	+++	57.5±1.01	3.12±1.62	2.13±0.21	80.6±8.11
FY11	+	-	++	31.9±0.04	0±0	0±0	69.98±9.06
Cw26	+	+	-	53.3±2.43	58.73±1.62	65.93±0.89	61.51±3.20
To1	+	-	+	35.08±0.91	0±0	0±0	51.54±0.83
Controls							
Culture medium	-	-	-	65.8±1.32	0±0	0±0	77.23±0
water	-	-	-	70.1±0.07	3.91±1.90	2.21±0.27	100±0
Tween20	-	-	-	35.2±0	-	-	-
1% SDS	-	-	-	-	68.50±1.78	65.87±3.24	66.50±0.62
Sea water 4% NaCl	-	-	-	-	0±0	0±0	100±0

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Table 3. Biochemical characterization of superior biosurfactant producing isolates

Characteristics	SN1	SN12	Characteristics	SN1	SN12
Cell morphology	Bacilli	Bacilli	Utilization of		
Gram reaction	+	+	Citrate	+	-
Spore forming	+	+	Egg-yolk lecithinase	-	+
Catalase	+	+	Formation of Indole	-	-
Oxidase	-	-	Growth at pH 6.8, nutrient broth	+	+
Motility	+	-	Growth at pH 5.7, nutrient broth	+	+
Voges-Proskaur test	+	-	Growth in NaCl		
Acid from			2%	+	+
D-Glucose	+	+	5%	+	+
L-Arabinose	+	-	7%	+	+
D-Xylose	+	-	10%	+	-
D- Manitol	+	-	Growth at		
Gas from glucose	-	-	5°C	-	-
Hydrolysis of			10°C	-	-
Casein	+	+	30°C	+	+
Gelatin	+	-	40°C	+	-
Starch	+	+	50°C	+	-
Esculin	+	+	55°C	-	-
Urea	-	-	65°C	-	-
Tween80	-	+	Nitrate reduction to Nitrite	+	+

- : negative results , +: positive results

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Pure isolates were cultured in production medium and following centrifugation, supernatants were used for preliminary screening since excretion type bacteria that release biosurfactants to the culture medium are more interesting from the industrial point of view than bacteria with adherent biosurfactants due to the simplicity and economical aspects of the recovery process (4- 20).

The primary screening of biosurfactant producing bacteria was carried out using hemolytic activity, oil collapse and oil spreading techniques. Selection of these methods was due to their strong advantages including simplicity, low cost, quick implementation and use of relatively common equipment that is accessible in almost every microbiological laboratory; however, as expected, these methods are not perfect or flawless. As previously mentioned by Youssef *et al.* (6) and Plaza *et al.* (14) in the hemolytic method, there are many bio-products that can cause red blood cell lysis which do not necessarily have to be surface actives molecules.

The drop collapse method depends on the principle that a drop of liquid containing a biosurfactant collapses and spreads over the oily surface. There is a direct relationship between the diameter of the sample and concentration of the biosurfactant and in contrast, the drop lacking biosurfactant remains beaded due to the hydrophobicity of the oil surface that cause aggregation of droplets (9- 21) but this method is not sensitive in detecting low levels of biosurfactant production. The results of our experiments indicate, 15% of total isolates were positive for hemolytic activity or oil collapse and 31% were considered positive based on oil spreading and since these methods have shown differences, the isolates with more than one positive response were exposed to complementary screening. The latter screening stage included surface tension and emulsion activity measurements.

Forty percent of positive isolates in preliminary screening have shown reduction of surface tension below the standard index (40 mN/m). The criterion used for selecting biosurfactant production is the ability to reduce surface tension below 40 mN/m (4 - 17) while some authors have suggested 30 mN/m (22, 23). As observed in Table 2, some of these results are quite impressive compared to the most potent biosurfactant producing microorganism among *Bacillus* spp. known to reduce the surface tension of water to 27 mN/m (12, 24). The second factor that has been studied in complementary stage was emulsion activity using three different hydrocarbons including crude oil, kerosene and n-hexadecane. About 13% positive isolates in preliminary stage showed emulsion activity. It is interesting that a given isolate showed different and distinct responses when it was exposed to each of hydrocarbon sources suggesting appropriate selection of hydrocarbon source is necessary for determination of emulsification capacity. Amiriyan *et al.* 2004 suggested that emulsifier activity depends on the affinity of bioemulsifier for hydrocarbon substrates which involves a direct interaction with hydrocarbon itself rather than an affect on surface tension of the medium (25). We used water and artificial sea water (4%NaCl) as negative controls which suggest that crude oil may not be an appropriate hydrocarbon source for the study of emulsion activity and statistical analysis has verified a significant difference between the response of a given isolate to the hydrocarbon source (Table 4).

In the present study, two superior isolates SN1 (*Bacillus subtilis*), SN12 (*Bacillus cereus*) with biosurfactant-producing ability and the former with emulsion capacity were isolated from petroleum-contaminated soil. Their ability to reduce surface tension and emulsion capacity makes them new potential candidates for biosurfactant and bioemulsion production. Further studies have been initiated to identify their properties and consequently determine the potential of their different industrial applications in particular enhanced oil recovery application.

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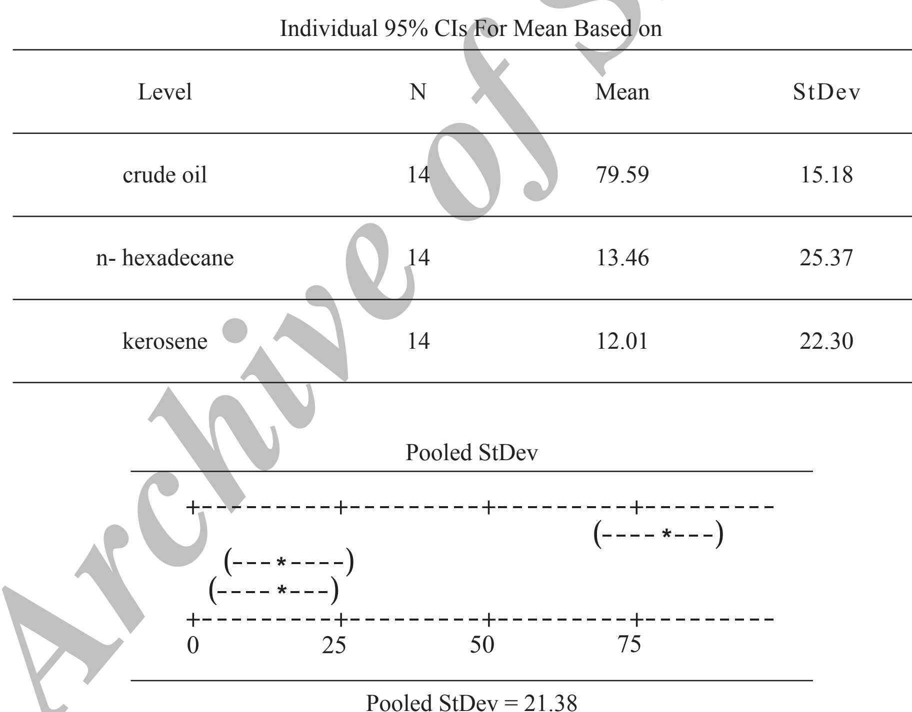
Table 4. Two-way ANOVA analysis for determining significant difference of emulsion activity in presence of different hydrocarbon sources using Minitab software.

Source	DF	SS	MS	F	P
hydrocarbon	2	44717.1	22358.6	108.01	0.000
block	14	13406.3	957.6	4.63	0.000
Error	28	5796.2	207.0		
Total	44	63919.6			
S = 14.39		R-Sq = 90.93%		R-Sq(adj) = 85.75%	



Individual 95% CIs For Mean Based on

Level	N	Mean	StDev
crude oil	14	79.59	15.18
n- hexadecane	14	13.46	25.37
kerosene	14	12.01	22.30

Pooled StDev

Pooled StDev = 21.38

DF:Degree of freedom, SS:Sum of square, MS:Mean of square, F:Fisher test.

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