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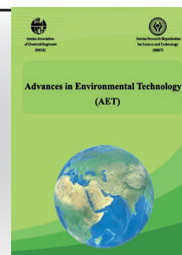

Indigenous production of biosurfactant and degradation of crude oil by *Bacillus sp.*

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ABSTRACT

The present study investigated the isolation and identification of biosurfactant producing bacteria from Iranian oil wells. The biosurfactant production of bacteria isolates was evaluated and confirmed using haemolysis and emulsification tests. The biodegradation of crude oil was studied using GC and HPLC analysis. A total of 45 strains have been isolated. These strains showed less than a 40 mN m^{-1} reduction in surface tension. The effects of different pH (4.2-9.2), salinity concentrations (1%-15%), and temperatures (25-50) in biosurfactant production of isolated strains were evaluated. One of the strains (*Bacillus sp.* NO.4) showed a high salt tolerance and a successful production of biosurfactant in a vast pH range. Its maximum biomass production (about 3.1 g L^{-1} dry weight) was achieved after 60 hours of growth. The surface tension of the culture broth dropped rapidly after inoculation and reached its lowest value (36 mN m^{-1}) during the exponential phase after about 36-48 hours of growth. The study of the GC graphs showed that higher aliphatic reduction occurred in fractions with C_{14} to C_{24} hydrocarbons. The depicted results of the HPLC graphs indicated a 100% degradation of chrysene and fluorine. In this study, we demonstrated the useful capacities of the isolates in removing oil pollutants and their application in MEOR in vitro.

1. Introduction

Surfactants are amphiphilic molecules that consist of a hydrophilic head and a hydrophobic tail. They are the active ingredients found in soaps and detergents with the ability to concentrate at the air–water interface and are commonly used to separate oily materials from a particular media. Surfactants are classified as ionic and non-ionic with varying chemical structures according to their hydrophilic group. For industrial applications, the ionic surfactants are classified based on the charge they carry when dissociated in water at their neutral pH. These classifications are namely anionic, cationic, zwitterionic, or amphoteric [1]. Among the surfactants, biosurfactant, or surface-active compounds are a heterogeneous group of surface active molecules produced by microorganisms, which either adhere to cell surface or are excreted extracellulary in the growth medium [2-5]. These molecules reduce surface tension and Critical Micelle Dilution (CMD) in both aqueous solutions and hydrocarbon mixtures. These properties create micro-emulsion in which micelle formations occur where hydrocarbons can solubilize in water or vice-versa [6]. Several types of biosurfactant have been isolated and characterized, including glycolipids, phospholipids, lipopeptides, natural lipids, fatty acids, and lipopolysacharides. The majority of known biosurfactants are synthesized by microorganisms grown on water immiscible hydrocarbons, but some have been produced on such water- soluble substrates such as glucose, glycerol, and ethanol [6,7]. Chemically-synthesized surfactants have been used in the oil industry to aid in the clean up of oil spills, as well as to enhance oil recovery from oil reservoirs. These compounds are not biodegradable and can be toxic to the environment. Biosurfactants have advantages over their commercially manufactured counterparts because of their lower toxicity, biodegradable nature, effectiveness at extreme temperature, pH, salinity, and ease of synthesis. They are potential candidates for many commercial applications in the pharmaceutical, food processing, and oil recovery industries [4,8,9]. With the growing interest in surfactant applications in environmental remediation, many researchers have studied the removal of single and double components of petroleum hydrocarbons [6-10]. The biosurfactant mechanism occurs at a concentration below the surfactant CMC (critical micelle concentrations).

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The phenomena associated with this mechanism include a reduction in: surface and interfacial tension, capillary force, wet ability, and contact angle. At concentrations below CMC, the surfactants reduce the surface and interfacial tension between air/water, oil/water, and soil/water systems. This paper describes the isolation of autochthonous biosurfactant producing bacteria for use in MEOR and biodegradation of crude oil [1,11-14].

2. Materials and methods

2.1. Microbial culture

The isolation of biosurfactant producing bacteria from Iranian oil wells was performed according to Tabatabaee, et al., 2012 [5]. The microorganisms were isolated by adding 1 ml of oil samples to 99 ml of SNB medium in 250 ml flasks. The mixture was placed on a reciprocal shaker at 200 rpm for 2 hours at 30 °C to produce a well-dispersed suspension in order to isolate the biosurfactant producing bacteria. The plates were incubated at 30 °C; subsequently, the numbers of colonies were stabilized and viable cell counts of all of the strains were determined. A streak culture was performed on the isolates to obtain pure isolates. Forty-five bacteria were isolated and the isolates were maintained on Nutrient Agar slants [1].

2.2. Hemolysis test

The bacteria strains were incubated at 30 °C for 72 h for hemolytic activity by plating the cell on Blood Agar [10].

2.3. Emulsion formation

The biosurfactant solutions were prepared at varying concentrations between 0.05% and 5% of biomass to determine their emulsification stability in crude oil. The solutions were put into test tubes with crude oil at different volume ratios of 3:1, 1:1, and 1:3 (biosurfactant solution: crude oil). The test tube containing the mixture of oil and water was shaken with a vibrating shaker at about 800 rpm for two minutes at 20 ˚C and then allowed to settle for 24 hours for phase separations to observe the stability of the emulsions formed. Thereafter, the volumes of the different phases were measured. The type of emulsion formed was investigated by diluting 2 g of the emulsions in 10 cm^3 of either water or crude oil. The systems were agitated for about 30 seconds and allowed to settle for about five minutes. The dilution effect of the emulsions in the different phases was observed. Also, the conductivity of the emulsions was determined at 15 °C.

2.4. Measurement of surface tension

The surface tensions of the surfactant solutions were measured using a Du Nouy tensiometer by a platinum ring method. The concentration of the solutions used was between 0.00001 and biomass-10%. The surfactant preculture of the bacteria strains were prepared in NB in OD600=1.1 ml of Inocula and were added to 100 ml MSS and 1% filtered oil as the hydrocarbon source. The mixtures with control samples were incubated at 30 °C on a shaker at 150 rpm for 3 days. The surface tension was measured using a KRUSS F6 tensiometer [7].

2.5. Investigation of the effect of different salt concentrations on surface tension

The effect of salinity on surface tension was determined by adding different concentrations (1%-15%) of NaCl to the Minimal Cooper's medium. The mixtures were incubated at 30 °C on a shaker at 150 rpm for three days [1].

2.6. Investigation of pH effect on surface tension

The effect of pH on surface tension was carried out by changing the pH (4.2-9.2) of Minimal Cooper's medium (pH=7.2). In these experiments, timely variations in biomass surface tension (ST), emulsification, and Critical Micelle Dilution (CMD-1, CMD-2) were recorded over a period of 72 h. The biomass was measured by the dry weight method. The surface tension and CMD were measured by the Ring method using a du Nouy tensiometer.

2.7. Crude oil analysis

The dispersion of crude oil was observed in the medium which suggested the ability of bacterium on oil degradation. The bacteria were cultured for 72 hours in a reciprocal shaker at 150 rpm with a temperature of 30 °C. The culture medium was separated for aliphatic and PAH analysis. The medium was mixed with an equal volume of chloroform and transferred to a separating funnel. The suspension was shaken firmly to dissolve the crude oil fraction of the culture medium in the solvent phase. The solvent phase was separated and evaporated in a vacuumed chamber for 24 hours. The residue was weighed to determine the amount of the total crude oil. The total aliphatic was measured with a GC (UNICAM model with FID detector and 30 m x 0.25 mm capillary tube). The aromatic fractions of the oil was extracted and analyzed according to [5] using the Waters HPLC system. The residue was dissolved in n-hexane and filtered. Two ml of the filtered solution was loaded to a 1 x 25 cm glass column filled with Silica gel (20 cm) and sodium sulfate (5 cm as a moist capturing material). The column was prewashed with n-hexane, and 10 ml of n-hexane was used as mobile phase to release aliphatic fractions. The fraction was collected and the solvent was evaporated. The residue was weighed to determine the amount of the total aliphatic fractions and GC analysis. To release aromatic fractions from the column, 10 ml of n-hexane/dichloromethane (1:1, v/v) was used as the mobile phase; the aromatic fractions were collected and the solvent was evaporated. The residue was weighed to determine the amount of the total aromatic fraction; it also used for HPLC analysis.

3. Results and discussions

By sampling crude oil from the south of Iran, the screening resulted in 45 aerobic *Cocci* and *Bacilli* (Gram positive and negative) bacterial isolates. The isolated strains were tested for haemolytic activity. Among the isolates, 35 strains showed haemolytic activity. The selected strains were used for further screening as shown in Table 1. Our results were similar to that of et al Barkay et al. [9]. The isolated cultures were designated as strain 4 (*Bacillus* sp.), strain 5 (*Bacillus* sp.) and strain 30 (Gram negative bacilli, rod shape).

Table l. Hemolytic activity of maintained *Bacillus Sp*. at different conditions.

Maintained condition	Hemolytic activity (mm)
At the time of isolation	5.2
-80 °C	5.1
Lyophilized	5.3
Room Temperature	5.3

The best isolate i.e. *Bacillus sp*., were tested for hemolytic activity (Table 1), which is regarded by some authors as indicative of biosurfactant production and used as a rapid method for bacterial screening [1,6,8,14].

The identification of biosurfactant producing bacteria can be further confirmed by the measurement of the surface tension. The reduction of surface tension measurements by isolated bacteria from Iranian crude oil reservoirs indicates the production of surface-active compounds at different conditions (Table 2) after the lyophilization, kept at -80 °C and room temperature.

Table 2. Stability of surface tension, emulsification activity, CMC, of *Bacillus sp*. Over 7 years of maintenance conditions (- 80 °C , Room temperature, Lyophilization).

Maintained Conditions	Surface tension (mN m^{-1})	Emulsification activity (%)	CMC (mg L^{-1})
At the time of isolation	36.0± 0.2	70	40
-80 °C	36.0± 0.2	70.03	42
Lyophilized	36.08± 0.2	70.09	41
Room temperature	36.0± 0.2	71.0	40

Similar results were obtained by Banat and his co-worker (1991). The isolated strain, showed the ability to reduce culture-broth surface tension to values below 40 mN m^{-1}(Table 2).

3.1. Environmental factors and growth conditions affecting biosurfactant production

The salt concentration on the optimization of biosurfactant production conditions and its impact on microbial activity of microorganisms was evaluated as a parameter. The results showed that the biosurfactant production was

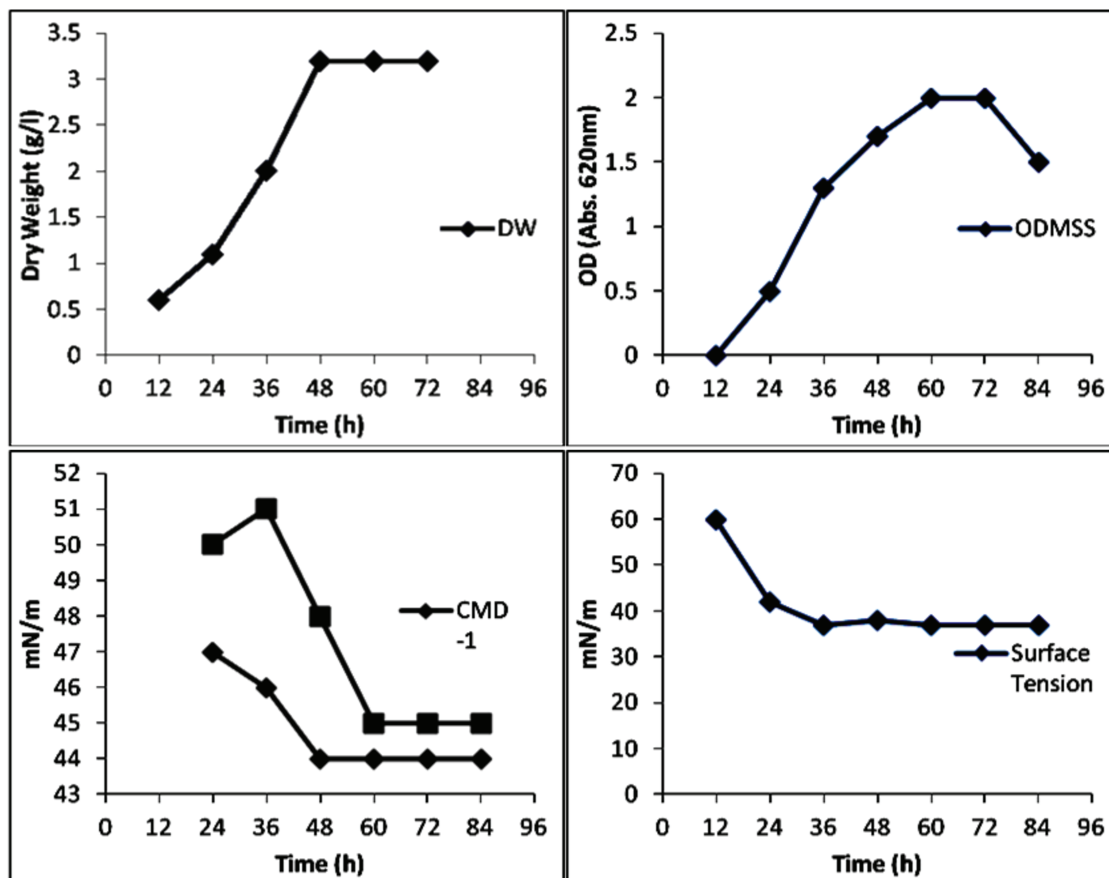

Fig. 1. Typical batch fermentation of oil by strain 4 bacterial culture which was isolated from an Iranian crude oil reservoir at 30 °C and a pH of 7.2. ST (surface tension); CMD-1 and CMD-2 (Critical Micelle Dilution); DW (Dried Wet).

optimal at 5% NaCl, which is close to the results obtained by Yakimov et al. [15]. He isolated *Bacillus licheniformis* BAS50 which grew and produced a lipopeptide surfactant when cultured on a variety of substrate at salinities of 13% NaCl.

The depicted results in showed that (Fig.1), the production of the biosurfactant and biomass during the growth of strain 4 on a mineral salt solution containing 1% oil at different time intervals, surface tension, and Critical Micelle Dilution (CMD-1 and CMD-2). Maximum biomass production (about 3.1 g L^{-1} dry weight) was achieved after 60 hours of growth. The surface tension of the culture broth dropped rapidly after inoculation and reached its lowest value (36 mN m^{-1}) during the exponential phase after about 36-48 hours of growth. Mnif et al. reported a decreased surface tension (ST) from 68 to 35.1 mN m^{-1} for an isolated *Pseudomonas aeruginosa* [16].

The CMD plot, a measure of biosurfactant concentration, showed that insufficient surfactant was initially present to form micelles. At 14-36 hours of growth, the surfactant concentration started to increase, reaching its maximum after about 36 hours.

The pH, temperature, agitation, and oxygen availability also effect biosurfactant production through their effects of cellular growth or activity. In our investigation using *Bacillus sp.* (Fig. 2), showed that the different pH has effect on surface tension. Comparable results were obtained by other scientists [17,18]. They found that the surface tension reducing activity of *Bacillus subtillis* C_9 was stable over the range of a pH of 5.0-9.5.

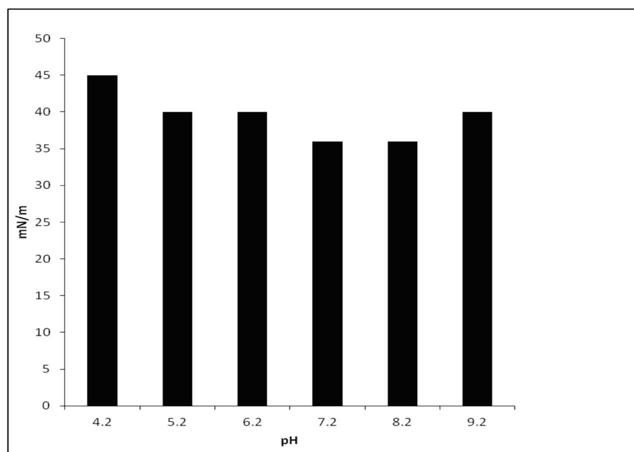

Fig. 2. Effect of pH on surface tension using isolated *Bacillus sp.*

Also, Abu-Ruwida et a1. (1991) observed the biosurfactant production of *Rhodococcus* at pH 6.5-7.2 that was determined by the differences in surface tension; they found the surface tension reducing activity of *Rhodococcus*. In this study, *Bacillus sp*. reduced surface tension in different temperatures but the optimum temperature for the selected strains was between 30- 40 °C. Also, Abu-Ruwaida et al. 1991 found the optimum biosurfactant production of *Rhodococcus sp*. at 37 °C. The kinetics investigation of biosurfactant production from oil indicates that it predominantly occurs during the exponential growth phase, suggesting that biosurfactant is produced as a primary metabolite accompanying biomass formation. Similar results were obtained by Abu-Ruwaida et al. in 1991 for *Rhodococcus*, strain ST-5.

3.2. Aliphatics and aromatics reduction

The GC analysis of aliphatic fractions is shown in Table 3. The depicted results show the aliphatic fraction at the start time. In the control sample (no bacteria), the aliphatic decreased after 3 days of incubation in comparison with the start time, whereas a reduction was observed in the first few hours in the treated sample (with bacteria). Further incubation revealed that after 3 days, 50%-75% reduction occurred between C_{14} and C_{15}, whereas only a 30%-50% reduction was detected on C_{18}, C_{26}, and C_{27}; the rest of the aliphatic was 30%.

Our results indicated that the isolated bacteria had a high efficiency for crude oil degradation. The isolates have used the crude oil as a sole source of carbon and energy. Since crude oil consists of aliphatics, aromatics, resins, and asphaltene, the aliphatic and aromatic fractions decreased more than the asphaltene occurred [1,17]. The study of the GC graphs showed that the decrease of the higher aliphatic reduction occurred in fractions with C_{14} to C_{24} hydrocarbons. The reduction of aliphatic fractions in the control sample suggests that volatilization played a major role in aliphatic fractions in the control sample. The results of the HPLC (Fig.3) indicated the 100% degradation of chrysene and fluorine. The biodegradation of the oily sludge components of the chain length C_{12}-C_{30} and the aromatic hydrocarbons was reported by *B. subtilis* DSVP23 after five days of incubation [19,20,15,3].

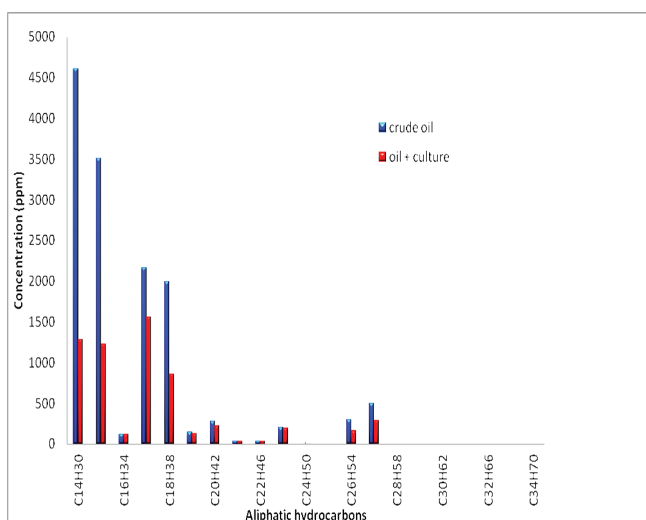

Fig. 3. Effect of biosurfactant on aliphatic fraction of crude oil chain length C_{14}-C_{27}. Blue & red columns stand for oil & oil + culture, respectively.

4. Conclusion

The isolation and identification of biosurfactant producing bacteria has been investigated. Samples (crude oil) were collected from Iranian oil wells and 45 strains were isolated using Cooper's mineral salt solution. To confirm the ability of isolates in biosurfactant production, a haemolysis test was performed. An emulsification test was monitored. Biosurfactant production carried out by the isolated strains was determined via the measurement of the surface tension (Ring method). The measurement of the surface tension was investigated using all the 45 bacterial isolates. Among these

Table 3. GC analysis of aliphatic fractions.

Component	Result (ppm)																				
	$C_{34}H_{70}$	$C_{33}H_{68}$	$C_{32}H_{66}$	$C_{31}H_{64}$	$C_{30}H_{62}$	$C_{29}H_{60}$	$C_{28}H_{58}$	$C_{27}H_{56}$	$C_{26}H_{54}$	$C_{25}H_{52}$	$C_{24}H_{50}$	$C_{23}H_{48}$	$C_{22}H_{46}$	$C_{21}H_{44}$	$C_{20}H_{42}$	$C_{19}H_{40}$	$C_{18}H_{38}$	$C_{17}H_{36}$	$C_{16}H_{34}$	$C_{15}H_{32}$	$C_{14}H_{30}$
Oil sample	nd	nd	nd	nd	nd	nd	nd	498	295	nd	9	205	35	35	273	141	1991	2165	119	3506	4601
Oil with microorganism	nd	nd	nd	nd	nd	nd	nd	286	160	nd	7	191	32	35	218	122	859	1555	115	1223	1282

*Non detected

strains, eight strains showed a reduction of less than 40 mN m^{-1} in surface tension. The surface tension results showed that ST is reduced. The emulsification characteristics of strain 4 and its stability over a wide range of pH and temperatures and high salt concentrations suggest that strain 4 is suitable for use in oil fields such as MEOR and the removal of oil pollutions. The aliphatic fractions have been biodegraded more efficiently than aromatics.

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