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IMPROVED OIL RECOVERY BY USING BIOSURFACTANTS PRODUCED FROM BACILLI BACTERIA ISOLATED FROM OIL RESERVOIRS IN IRAQ

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ABSTRACT

Isolation of bacterial species from produced water of oil field and exhibited ability for biosurfactants production and then evaluate the efficiency of biosurfactants in improving oil recovery. Three bacterial species identified as *B. cereus* (M1), *B. cereus* (M2) and *B. licheniformis* (M3) were recovered from produced water of oil fiel. There was a significant reduction in surface tension by *B. cereus* (M1) 53 mN/m to 38 mN/m, *B. cereus* (M2) 35 mN/m and 32.9 mN/m for *B. licheniformis* (M3). The highest oil displacement of *B. cereus* (M2) was 40mm while *B. cereus* (M1) was the lowest rate. The strongest emulsification activity (100%) was for both M1 and M2, while the highest oil recovery in test tubes (25%) and beakers (52%) was by M1. The highest degradation rate was 66% *B. licheniformis* (M3) followed by 63% and 57% for *B. cereus* (M1) and *B. cereus* (M2) respectively. The GC analysis of crude oil n-alkanes showed that all species have biodegradation efficiency comparing with the control. The study showed a significant ability of these strains for producing biosurfactants that can apply for Microbial Enhanced Oil Recovery MEOR.

KEY WORDS: Bacilli bacteria, Biosurfactants, Microbial Enhanced Oil Recovery (MEOR).

INTRODUCTION

Growing attention has been given for microbial manipulation in field of biotechnology such as Microbial Enhanced Oil Recovery (MEOR) which is an alternate recovery method that conducts microorganisms and their metabolic products resulting in shifting use of common chemical and thermal ways to cost effective and eco-friendly methods (Bachmann *et al.,* 2014; Bezza and Chirwa, 2017). The microorganisms produced surface-active organic compounds when grown on water immiscible substrates that help to reduce surface and interfacial tension are called Biosurfactants (Batista *et al.,* 2006). Predominantly they are secreted either extracellular or attached to parts of cells during growth on water immiscible substrates, the substrate will be more readily available for uptake and metabolism because of the biosurfactants reduce the surface tension at the phase boundary (Desai and Banat, 1997). The biosurfactants have unique chemical structure that vary from glycolipids, phospholipids, polymeric and lipopeptides (Yin *et al.,* 2009; Makkar and Rockne, 2003). Specific microorganisms such *Acinetobacter sp.*, *Candida antartica* and *Bacillus sp.*, have to ability for biosurfactants production (Fakruddin, 2012). *Bacillus subtilis* has a certain importance in field of biotechnology and MEOR due to their ability in producing a wide range of metabolites including enzymes, antibiotics, amino acids, insecticides, biopolymers, bacteriocins and biosurfactants (Perez

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et al., 2017). A certain attention has given to surfactin which is a lipopeptide biosurfactant (Sanjana *et al.,* 2017). Lipopeptides are known as promising microbial surfactants and have been successfully used in enhancing oil recovery in extreme environmental conditions (Zhang *et al.,* 2016). The impact of these products associated with their functionality under extreme conditions of temperature, salinity and pH (Banat *et al.,* 1995). MEOR processes used the injected microorganisms to increase the reservoir pressure and reduce the oil viscosity by produce CO_2, CH_4, H_2, acids, solvents and biosurfactants that have significant effects on oil quality (Youssef *et al.,* 2009).

MATERIALS AND METHODS

Sample collection

Oil samples were collected from Nahran Omer reservoirs which is located in northern part of Basra, Iraq. Samples were collected in sterile glass bottles and transferred directly to laboratory for bacteriological analysis experiments.

Isolation of biosurfactants producing bacteria

Bacterial isolation was done according to procedure of Tabatabaee and his workers (2005). Briefly, one mL of oil sample was added to 99 mL of SNB medium in sterile conical flasks 250 mL in volume. The suspension was mixed by shaking flasks at 120 rpm for 24 h at 35 °C on a flat-bed shaker. After that, 0.1 mL of the mixed suspension was spread on nutrient agar plates then were incubated at 35 °C for 24 h. *Molecular identification of isolated bacteria.* Recovered bacterial isolates were pelleted and conducted for genomic DNA extraction using Promega (Cat. No. A1120, USA) following manufacture instructions. Isolated bacteria were identified by amplification and sequencing of the 16S rRNA gene using the universal bacterial specific primer 1492R and 27F (Lane, 1991). The PCR cycling parameters were: initial denaturation for 2 min at 94 °C, 35 cycles of 40 sec at denaturation at 94 °C, 30 sec. of annealing at 55 °C, and 1 min of elongation at 72 °C. Cycling was completed by a final elongation step at 72 °C for 10 min. The PCR cycling parameters were: an initial denaturation step for 2 min at 94 °C; 35 cycles of 40 sec at 94 °C (denaturation), 30 second at the 55 °C (annealing), 1 min at 72 °C (extension), followed by a final 10 min extension step at 72 °C. DNA bands in size of approximately 1500 kb of 16S rRNA were purified and sequenced at Macrogen laboratories, Korea. Species identity was predicted based on the 16S rRNA gene of recovered bacterial species to species recorded in Gen Bank of National Centre for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST).

Screening of biosurfactant producing bacteria

The bacterial isolates were streaked on nutrient agar plates and incubated for 24 h at 35 °C. For liquid culture, bacterial cells at a concentration of 1×10^8 cell per mL or a loop-full of bacterial growth were inoculated into 100 mL conical flask containing 50 mL of nutrient broth. Flasks were then incubated in a rotary shaker at 120 rpm at 35 °C (El-Sheshtawy *et al.,* 2015). For biosurfactant production, 5% (v/v) of cultivated bacterial growth was seeded into an inorganic salt medium (ISM) containing (Diaband Gamal, 2013). The medium was modified by adding 0.03% glucose, pH was adjusted at 7.0, and then supplemented with crude oil (1% v/v). Following flasks incubation for 7 days at 30 °C on shaker at 120 rpm.

Evolution of biosurfactant production by bacterial isolates

To evaluate bacterial isolates ability in producing biosurfactant, surface tension, oil spreading, and emulsifying activity was studied.

Measuring surface tension

Ring method was used to measure the Surface tension on 125 mL of each bacterial supernatant using Tensiometer (703D, Sigma) at room temperature (Batista *et al.,* 2006 and Cha *et al.,* 2008). The results were compared to sterile medium negative control.

Emulsification test E24

2mL of crude oil and 2mL of free cell supernatant were inoculated to test tube and homogenized by vortexing at high speed for 2 min. The emulsification activity was calculated after 24 hr. using following formula (Lai *et al.,* 2009): E24=the height of the emulsified layer (mm) /the total height of the liquid layer (mm)*100.

Oil spreading test

Oil spreading technique was carried out according to (Youssef *et al.,* 2004). Surfactant activity can be determined by measuring the diameter of clear zone on the oil surface comparing to distilled water as a negative control.

Experimental design of crude oil recovery

Oil reservoir system was mimicked by developing an experiment microcosm design through using large tubes 3x20 cm in dimension (oil pipelines) and beakers 100 mL (tanks). The developed microcosms were flashed using sterile distilled water and left to dry. Then were filled with crude oil and left to settle for 24 h. After crude oil was gently discarded leaving oil residual contamination in inner-side of microcosms, the system was weighed before and after gently adding 3 mL of free cell supernatant into inner surfaces of the developed system. The same volume of water was used as control (Diaband Gamal, 2013).

Biodegradation of petroleum hydrocarbon by bacterial isolates

Crude oil degradation was determined by inoculated fresh growth of bacterial isolates into 100 mLconical flasks, each containing 50 mL of basic mineral medium (Rojas-Avelizapa *et al.,* 1999). The medium was modified by adding 1 mL of sterile trace element stock solution (Linhardt *et al.,* 1989). Flasks were incubated at a rotary shaker for 10 days at 120 rpm and 35 °C. The results were determined by gravimetric method (Saxena, 1990).

Analysis of n-alkane fraction by gas chromatography

Capillary Gas Chromatography-Flam Ionization Detector GC-FID in Nahran Omer laboratories (South oil company, Iraq) was used in order to analyzed the standard aliphatic compounds (C7-C40) and alkane fraction. The model of column was Agilent 19091J-101HP-5 for aliphatic separation 5% phenyl methyl siloxane was used with dimension (5 m x 0.2 mm x 0.33 mm). Helium gas used as carrier in liquid gas chromatography with a linear velocity of 1 mL min^{-1}. The Biodegradation Efficiency (BE) was then determined for each treatment (Michaud *et al.,* 2004).

RESULTS AND DISCUSSION

Isolation of biosurfactants producing bacteria

For this reason, the aim of this study focused on *Bacillus sp.* isolation from oil fields and test their ability in biosurfactants production that could enhance oil recovering. Our study showed that *Bacillus sp.* was the most dominant bacterial species recovered from collected and tested oil samples. Growing under aerobic condition, spore forming, Gram positive bacilli form were the main primary characteristic features of isolates species. Molecular identification of 16S rRNA gene showed that bacterial isolates M1, M2 and M3 belonging to two *B. cereus* and *B. licheniformis*. These species were identical to the *B. cereus* strain OF, *B. cereus* strain KNUC256 and *B. licheniformis* VMS13 at NCBI platform. Studies reported isolation of *Bacillus* species from petroleum reservoirs as the most predominant bacteria with ability for producing biosurfactants that is significantly used especially for MEOR and bioremediation of hydrocarbons (Burghal *et al.,* 2015; El-Sheshtawy *et al.,* 2015).

Measurement of surface tension

Measurement of surface tension assay is used to investigate the ability of biosurfactant compounds in reducing surface tension liquids. Our findings illustrated that the best surface tension reduction was related to *B. licheniformis* (M3) 32.9 mN/m, *B. cereus* (M2) 35 mN/m followed by *B. cereus* (M1) 38 mN/m comparing with the control 53 mN/m as presented in Table 1. Studies showed that biosurfactants produced by bacterial cultures effectively reduced surface tension of water from 72 to 27 mN/m (Banat *et al.,* 1991; Banat, 1995). Recently, these observations have been confirmed through reduction in surface tension from 72 to 28 mN/m due to the activity of *B. licheniformis* (Joshi *et al.,* 2013).

Emulsification test E24

These isolates showed obvious effect in the emulsification of crude oil the results were observed after 24 h. The emulsification activity as measured by the E24 index showed that the highest value of petroleum oil emulsification 100% by *B. cereus* (M1) and *B. cereus* (M2) followed by *B. licheniformis*(M3) 58% (Table 1). Emulsification test is an indirect method used to screen biosurfactant production in cell free culture broth through emulsifying hydrocarbons (Thavasi *et al.,* 2011). The best biosurfactant emulsifier can be achieved at E24 value equal or more than 50% (Lima *et al.,* 2011).

Oil spreading test

The biosurfactant produced by *B. cereus* (M2) exhibited the highest performance of oil displacement zone 40 mm followed by *B. cereus* (M1) and *B. licheniformis* (M3) 15 mm and 20 mm respectively in comparing with the control (Table 1).

Displacement occurs when hydrocarbon layers displacing as a result of the reduction in interfacial tension due to the biosurfactant concentration (Almansoory *et al.*, 2014).

Crude oil recovery

Table 1 showed that the maximum of recovered oil from contaminated beakers and tubes (developed system) by supernatant method was approximately 52% and 25% respectively of the biosurfactant produced by *B. cereus* (M1) followed by *B. cereus* (M2) 15 % and 16% while, *B. licheniformis* (M3) exhibited the lower efficiency in oil recovery ~ 9% and 4%. Biosurfactants produced by bacterial culture were used for cleaning oil tanks, oil-contaminated tankers, barges, storage tanks, tank cars and trucks, pipelines and other containers used to transport or store crude oil or petroleum fractions (Gutnick *et al.*, 1989; Banat *et al.*, 1995). The oil was floated and remains as distinct phase, therefore, the biosurfactant monomers may accumulate at the solid-oil interface and reduce the capillary force holding oil and solid due to reduction in the interfacial tension. The oil will undergo to displace if the interfacial tension between oil-solid is highly reduced to overcome the capillary force (Urum, 2004; Diaband Gamal, 2013).

Biodegradation of petroleum hydrocarbon by bacterial strains

The percentage of hydrocarbon biodegradation rate was 66%, 63% and 57% of *B. licheniformis* (M3), *B. cereus* (M1) then *B. cereus* (M2) respectively (Table 1). The result illustrated that all isolates expressed their ability for crude oil degradation in liquid media. This outcome was similar (Ijah andUkpe, 2017) who showed that *Bacillus* strain 61B was able to cause a 50.4% decrease in the weight of crude oil after 20 days of incubation. Meanwhile, it is compatible with Nazinaand his group(2005) through obtaining hydrocarbon oxidizing *Geobacillus* strains isolated from formation water of oil fields.

Gas chromatography analysis

The percentage degradation of *n*-alkanes for tested samples was performed using GC (gas chromatography) assay comparing with control as shown in Figure 1. All isolated bacteria expressed their ability in *n*-alkanes (C15-C40) degradation. *B. licheniformis* (M3) has the highest degradation efficiencies for n-C13 to n-C24, while *B. cereus* (M1) was more active on n-C25 to n-C39. *B. cereus* (M2) exhibited identical activity with *B. licheniformis* (M3) in degrading n-C40 and less degradation than M1 and M3 isolates from n-C15 to n-C39. The results revealed that after biodegradation there was

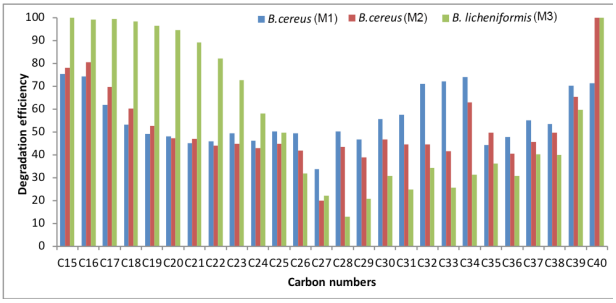

Fig. 1. Gas chromatography analysis of n-alkanes extracted from oil degraded in bacterial cultures.

complete degradation of some C40 because this fraction found in low percentage in crude oil and this makes them easy to use as food by bacteria (Al-Wasify and Hamed, 2016).

CONCLUSION

The Bacilli bacteria of *B. cereus* (M1), *B. cereus* (M2) and *B. licheniformis*(M3) were the common bacteria which can be isolated from petroleum reservoirs. These isolates which have produce a potent biosurfactants were able to reduce surface tension and have high displacement capacity, highly efficient emulsification activity with potency of oil recovery further the better degradation rate of *n*-alkanes. The heavy components of hydrocarbons

Table 1. Detection of biosurfactant producing isolates by screening methods.

Type of tests	*B. cereus* (M1)	*B. cereus* (M2)	*B. licheniformis* (M3)	Control
Surface tension (mN/m)	38	35	32	53
Oil emulsification (%)	100	75	58	0
Oil displacement (mm)	15	40	20	0
Oil recovery in tubes (%)	25	16	4	1
Oil recovery in beakers (%)	52	15	9	3
Oil degradation (%)	63	57	66	14

(high *n*-alkanes) can be used for nutrition the several kinds of bacteria in order to produce the biosurfactants which can be used in the bioremediation for most oil industries and Microbial enhanced oil recovery, MEOR processes.

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