Research Article

Application of Bioand Chemical Surfactants in Bioremediation of Crude Oil Fractions: Part II

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ABSTRACT

The synthesized of chemical nonionic surfactant and biosurfactant produced by *Bacillus licheniformis* showed a potential to enhance the biodegradation of crude oil. The presence of surfactants in the growth medium (mineral salt medium) increases the solubility of different fractions of crude oil and hence increases their bioavailability. Moreover, the biodegradation percentage was demonstrated at 50% of microcosm containing bio and chemical surfactant with saturates fraction separately. On the other hand, the biodegradation percentage of aromatics and resins fractions was presented at 60% with biosurfactant after 7 days incubation period. These results indicated that the bacterial strain has a potential to be applied in the bioremediation of petroleum contaminated sites.

Keywords: Biosurfactants, chemical surfactant, biodegradation, crude oil, *Bacillus licheniformis*

INTRODUCTION

Petroleum components have traditionally been divided into four fractions: saturated hydrocarbons, aromatic hydrocarbons, compounds containing nitrogen, sulphur and oxygen (NSO) and asphalthenes. The relative proportions of these fractions depend on the crude type, and the susceptibility of specific crude to microbial degradation can be predicted from its composition[1]. Bioremediation of contaminated aquatic and soil environment has arisen as an effective technology, with a range of advantages compared to traditional methods. Bioremediation of waste materials, which contain hydrocarbons and their derivatives, is based on the ability of microorganisms, when present in a high biomass concentration, to degrade the waste materials to non-toxic products, such as H_2O and CO_2[2]. One of the main factors affecting the biodegradation efficiency of complex oily compounds is the low availability of contaminants for microbial attack.

An alternative method to expand bioavailability and contaminant metabolism is increasing substrate solubilization by using bio and chemical surfactants. The complex compounds such as oily sludge, these compounds are difficult to degrade. It shows that the economically and environmentally interest as use of microorganisms that showing excellent degradation capability together with the production of biosurfactant. This alternative method has a great advantage over the use of bacteria which only present the degradative capacity[3].

The surfactant can be classified into nonionic and ionic surfactants; ionic surfactants could be further categorized into cationic, anionic and zwitterionic surfactants according to the ionizability of the polar headgroup. The toxicity of surfactants is depending on its molecular structure. The negatively charged surface of the bacterial cells makes the cells more sensitive to introduce the charged surfactants, especially positively charged cationic surfactants. Generally, nonionic surfactants are less toxic to microorganisms than ionic surfactants[4].

The surfactant molecules at a concentration (near or above critical micelle concentration, CMC) form mixed micelles with membrane lipids may solubilize cell membranes and lead to lysis of cells. At concentrations below the CMC, the surfactant molecules cannot form mixed micelles with lipid molecules, then the incorporation of surfactant into the cell membrane is enough to impair the barrier function of cell membrane[5].

Degradation of wide range of hydrocarbons and crude oil are crucial importance for the bioremediation of oil contamination sites and microbial enhanced oil recovery (EOR)[6]. Indeed, searching for microbial strains capable of degrading a wide range of hydrocarbons is scarce.

Moreover, the native microbial populations in contaminated sites may have insufficient catabolic capabilities to achieve extensive, rapid, and sustainable biodegradation; especially in soils where no previous exposure to contaminants has occurred and/or contaminant concentrations are low[7]. For these reasons, the *Bacillus licheniformis* ATCC 10716 is investigated as a novel biodegrading strain in this work.

The aim of the present study is to enhance the biodegradation process of different fractions of crude oil (saturates, aromatics and resins) (SAR) in the presences of bio and chemical surfactants.

The bacterial count and some surface properties of surfactant in different microcosms were measured. The bioremediation of crude oil as a whole in the presences of bio and chemical surfactants was determined in part I[8].

MATERIALS AND METHODS

Microorganism

Bacillus licheniformis ATCC 10716 was supplied by the microbial resources center (MIRCEN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

Crude Oil

The crude oil produced from Esh El-Malaha, Alexandria, Egypt was used in the present work.

Synthesis of Chemical Surfactant

Type the name of the surfactant in this study was synthesized as described in part I[8].

Culture Medium and the Production of Biosurfactant

The bacterial strain was streaked on a nutrient agar slant and incubated for 24h at 30°C. Two loops of culture were inoculated in 40ml of nutrient broth in a 100ml Erlenmeyer flask. This flask incubated in a rotary shaker 150rpm at 30°C for 8–12h until cell numbers reached 10^8CFU/ml. For biosurfactant synthesis, a mineral salt medium with the following composition was used (g/l): $NaNO_3$2.5, KCl 0.1, $KH_2PO_4$3.0, K_2HPO_4 7.0, $CaCl_2$0.01, $MgSO_4 \cdot 7H_2O$ 0.5, and 5 ml of a trace element solution. Trace element solution contained 0.116g/l of $FeSO_4 \cdot 7H_2O$, 0.232g/l of H_3BO_3, 0.41g/l of $CoCl_2 \cdot 6H_2O$, 0.008g/l of $CuSO_4 \cdot 5H_2O$, 0.008g/l of $MnSO_4 \cdot H_2O$, 0.022g/l of $[NH_4]_6Mo_7O_{24}$ and 0.174g/l of $ZnSO_4$[9,10]. The carbohydrate (glucose) was added to adjust the final concentration 2%. The concentration of yeast extract at 3% was used to increase the production of biosurfactant[11]. Cultivation study has been done in 500ml flasks containing 150ml medium at 30°C for 72h.

Biodegradation of Different Crude Oil Fractions

Different crude oil fractions (saturates, aromatics and resins, SAR) (1g) and/or 0.1 g of chemical surfactant were added separately to the mineral salt medium MSM (100 ml) in 500 ml Erlenmeyerflasks. The flasks were autoclaved at 120°C for 20min. The bacterial strain (1× 10^7CFU/ml) an aliquot of 2 ml inoculum was inoculated into the mineral medium. The cultures were incubated at a temperature controlled shaking incubator at 150   rpm at 30°C for 7days. The biosurfactant was produced using MSM medium with (1g) glucose as a sole carbon source for 72h. Adding sterile (1g) of (SAR) separately until the end of the incubation period. A sample without inoculum was used as a negative control[12]. At the end incubation period, the bacterial count and surface properties were determined. The crude oil samples were then extracted from the different microcosms. Consequently, a gravimetric analysis was then determined.

Extraction of Different Fractions of Crude Oil after Treatment by Bacterial Strain and Gravimetric Estimation

At the end of incubation period (7 days), the bacterial broth (100 ml) was thoroughly shacked with n-hexane (50 ml 3 times) in a separating funnel. The three samples were then collected in case of saturates fraction samples.

While, the samples, containing aromatics fraction was extracted using benzene. Methylene chloride was used in the case of the resin fraction. The collected organic layer was dried over anhydrous sodium sulphate. The solvent was removed on a rotary evaporator until a constant weight[13].

The oil sample was accurately weighed. Percentage of the biodegraded oil was calculated and alterations in its chemical composition were studied by chromatographic analysis (GC and HPLC)[14].

Gas Chromatographic Analysis

The biodegradation of the crude oil was monitored using an Agilent 6890 gas chromatographic instrument equipped with flame ionization detector according to the testing method of[15].

The type of column, the condition of injector and the oven temperature was mentioned in part I[8].

High Performance Liquid Chromatographic (HPLC) Analysis

The crude oil remained after the biodegradation and the corresponding control sample was analyzed using a HPLC. The type of column and the condition of separation by this column was described in part I[8].

Fourier Transform Infrared (FTIR) Analysis

FTIR spectra of the resins samples (a film of each sample on KBr pellet) were obtained using a Nicolet IS-10FTIR spectrometer. FTIR spectra were collected between 4,000 and 500cm^{-1} with a resolution of 1cm-1[16].

Deasphalting of Crude Petroleum Oil

Deasphalting of the extracted from crude oil sample was carried out using the standard test method of[17].

Separation of Maltene from its Hydrocarbon Fractions

Separation of the maltene into its components; saturate, aromatics and resins was carried out using liquid column chromatography[18].

Apparatus: Double jacket glass column of 100cm length and 2cm internal diameter with a 1liter reservoir at the top and tapered at the bottom and thermostat bath.

Chemicals: aluminum oxide neutral (70-230 mesh) activated by heating to 300°C for 24h, n-heptane, benzene, methylene chloride, n-hexane were produced from (Honeywell specialty chemicals seelze GmbH).

The process of separation was carried out according to the method used by Zewen.[18]

Identification of the different hydrocarbon components is achieved according to refractive index, Saturates <1.48, monoaromatic 1.48-1.53, diaromatic 1.53-1.59, polyaromatic >1.59 and resins dark.

Surface Properties

Surface properties including surface tension, Emulsification index (E_{24}) and Foaming, as indicator for the production of biosurfactant were determined.

RESULTS AND DISCUSSION

Chemical Structure Confirmation of the Synthesized Nonionic Surfactant

FTIR spectra of 2-(dodecylcarbamoyl) benzoic acid, Mass spectra of 2 (dodecylcarbamoyl) benzoic acid and FTIR spectra of 41-hydroxy-3,6,9,12,15,18,21,24,27,30,33,36,39 tridecaoxahentetracontyl 2-(dodecylcarbamoyl) benzoate were described in part I[8].

Liquid Column Chromatography for Fractionation of Crude Oil

Liquid column chromatography is a useful tool for the separation of petroleum components, namely saturates, aromatics, resins and asphaltene. It is used as a marker for the characterization and identification of Esh El-Malaha sample through its hydrocarbon types distribution. Table (1) showed that saturates content at 55.653%. On the other hand, the aromatic hydrocarbons content was detected at 19.093%. In addition, resin components which considered as a polar fraction, the value of resins contents was calculated at 17.840%. Indeed, asphaltene was observed at 7.414%. Asphaltene is a significant fraction of higher molecular weight consisting of hydrocarbons, oxygen, sulphur and nitrogen compounds[19].

Table 1: Hydrocarbons type analysis of crude oil of Esh El-Malaha

Fractions	Weight (g/l)	(%)
Maltene fraction	10.3798	92.586
Saturates	6.2353	55.653
Aromatics	2.1405	19.093
Resins	2.00	17.840
Asphaltene fraction	0.8312	7.414
Total	11.211	100

Evaluation the Growth of Bacterial Strain and Some Surface Properties of Bio and Chemical Surfactants after Biodegradation of Different Crude Oil Fractions

Bacillus licheniformis was used for the biodegradation of different crude oil fractions, using bio and chemical surfactants separately. The bacterial count and measurement of surface tension and emulsification power was represented in Table (2) in different microcosms. The results in Table (2) showed that, at the end of incubation period, the same bacterial counts were observed in microcosms containing saturates fraction and bio and chemical surfactants separately. In addition, the best reduction in the surface tension and increasing in the emulsification power have given with the microcosm

containing saturate fraction and chemical surfactant which reached a value of 37mN/m and 56%, respectively. While in the microcosms containing aromatics, resins fraction separately and biosurfactant shows better bacterial growth, reduction of surface tension and increasing of emulsification power than other microcosms. It has been concluded that, during the biodegradation of saturates fraction; the chemical surfactant has the ability to decrease the surface tension and increase the emulsification power. On the other hand, during the biodegradation of aromatics and resins fraction, the biosurfactant decrease the surface tension and increase the emulsification power. However, the chemical and biosurfactant leads to increase the bioavailability and subsequent biodegradation of the hydrocarbons[20]. The solubilization ratio of the biosurfactant was better than several synthetic surfactants reported in the literature[21]. Additionally, the present study showed direct relationships between the emulsification activity (E_{24}) and the decrease in the surface tension with increasing the growth rate on hydrocarbons[21].

Biodegradation of Different Crude Oil Fractions by Bacterial Strain

Bacillus licheniformis was grown on 1% of different crude oil fractions separately. After 7 days of cultivation period, the percentage biodegradation of different fractions crude oil were estimated by gravimetric analysis. As reported in Table(2), the bacterial strain could degrade up to 50% for saturates fraction in medium containing bio and chemical surfactants separately. While, the highest percentage degradation of aromatics and resins fractions were reached up to 60 and 50% with bioand chemical surfactants. Verma[22] reported that, the oily sludge degradation capacity of *Pseudomonas* sp. SV 17, which degraded approximately 60% of saturates and aromatics fractions after 5days, was achieved biosurfactant production.

The Gas Chromatographic analysis (GC) of the residual saturates fraction in a medium containing bio and chemical surfactants separately were determined. The negative control (sample crude oil without treatment by microorganism) and positive control (sample crude oil treatment by microorganism without any type of surfactant) were also illustrated in Table (3). Table 4 showed that, the microcosm containing chemical surfactant gives better degradation in the shorter chain length (C_{14}–C_{20}) of n- and iso-paraffins than other microcosms. However the degradation percent of the medium chain length (C_{21}–C_{30}) n-paraffins decreased in the different microcosms. On the other hand, the long chain length of (C_{31}–C_{42}) iso-paraffins decreased in all different microcosms. Alkanes are major the components of crude oil. Alkane hydroxylase is a key enzyme involved in alkane degradation. The enzyme which introduces oxygen atom derived from molecular oxygen into the alkane substrate plays an important role in crude oil

bioremediation[23]. Moreover, the total n-paraffins concentration decreased more than iso-paraffins in all different microcosms. This action is normal for the susceptibility of microbial attack to different hydrocarbons[24]. Toledo[25] demonstrated that the fraction containing n-alkanes is usually more susceptible to biodegradation process. This action illustrated that saturates fraction containing branched alkanes is less vulnerable to microbial attack. Paudyn[26], Barthakar[1] reported that isoprenoids are relatively resistant to biodegradation and are more slowly degrade when compared to linear ones due to their branched nature.

The biodegradation potential of the bacterial strain on polyaromatics was detected using HPLC analysis after 7 days (see Table 4). The polyaromatics extents of the control crude oil sample were Naphthalene, Acenaphthylene, Acenaphthene and Fluorine (2-member rings polyaromatics), Phenanthrene and Anthracene (3-member rings polyaromatics), Fluoranthene, Pyrene and Benzo (a) anthracene (4-member rings polyaromatics), Benzo (b) fluoranthene and Benzo (k) fluoranthene (5-member rings polyaromatics).

These compounds also mean a potential risk to human health, as many of them are carcinogens[27]. It has been found that the microcosm containing positive control, Acenaphthylene and Benzo (k) fluoranthene were fully utilized by bacterial strain. The polyaromatics were fully utilized in microcosm containing biosurfactant including Naphthalene, Acenaphthylene and pyrene which represents at 72.03 % of the untreated sample. Additionally, the 5-member rings polyaromatics were completely utilized. Providenti[28] reported that biosurfactants have been also reported as enhancers for

polyaromatics hydrocarbon biodegradation in the liquid media. On the other hand, in microcosm containing chemical surfactant, Fluorine and Benzo (k) fluoranthene were fully utilized by the bacterial strain.

It is necessary to explain that the increase in any compound may be attributed either to a real increase due to the formation of additional amounts of this polyaromatic hydrocarbon. This is only accepted in the cases of lower polyaromatics and the other probability to enhance consumption of other compounds. This leads to a relative accumulation of such ployaromatic hydrocarbon (Table4)[29,30]. Low percentage degradation toward the 2- and 4-member rings polyaromatics was obtained in microcosm containing a positive control. Nevertheless, the highest degradation percentage was observed in 5-member rings polyaromatics and accumulation in 3-member rings polyaromatics resulted from the breakdown of higher polyaromatics. It is clear that the highest degradation of 4-member rings polyaromatics was detected in the medium containing biosurfactant. While in microcosm containing chemical surfactant was observed better degradation of 2- and 3-member rings polyaromatics. Several studies on the degradation of polyaromatic hydrocarbons by bacteria demonstrated a tendency towards inverse relationship between the biodegradation rates and the number of aromatic rings and molecular mass[31]. According to the Vitte[32], under permanent toxic condition, the lighter PAHs (i.e., with 2 or 3 aromatic rings) present in oily sludge were almost completely removed after 10 days of incubation. In contrast, the concentration of fluoranthene, pyrene and chrysene (heavier PAHs) started to decrease only after 10 days incubation.

Table 2: Growth count and surface properties of bio and chemical surfactants using different fractions of crude oil as carbon sources separately

Sample	Log count	Surface tension (S.T.) mN/m	Emulsification power (E_{24}) (%)	Percentage biodegradation*
0 (inoculation time)	7.2	55	0	0
Saturate fraction				
Saturate fraction + *Bacilluslichenifomis(B.L.)*	7.6	44	0	20
Saturate + *B.L.* + Biosurfactant	8.0	39	50	50
Saturate + *B.L.* + Chemical surfactant	7.9	37	56	50
Aromatic fraction				
Aromatic fraction + *B.L.*	7.8	43	35	20
Aromatic + *B.L.* + Biosurfactant	8.2	37	72	60
Aromatic + *B.L.* + Chemical surfactant	7.8	44	36	50
Resin fraction				
Resin fraction + *B.L.*	8.0	47	0	40
Resin + *B.L.* + Biosurfactant	8.5	40	55	60
Resin + *B.L.* + Chemical surfactant	7.8	44	50	50

percentage biodegradation = Weight of original oil –wt. of residual/wt. of original oil 100

Table 3: Distribution of carbon number in the chromatogram of residual saturates fraction

Carbon number of paraffins	*Negative control		**Positive control		Sat. + microorganism + biosurfactant		Sat. + microorganism + chemical surfactant	
	% Paraffins							
	normal	iso	Normal	iso	normal	iso	normal	iso
C_{14}- C_{20}	22.44	21.86	22.57	15.91	18.92	13.15	16.92	12.64
C_{21}- C_{30}	36.47	6.14	34.18	14.62	32.49	22.96	33.27	22.91
C_{31}- C_{42}	11.93	1.16	12.04	0.68	11.81	0.67	13.29	0.97
Total paraffins	70.84	29.16	68.79	31.21	63.22	36.78	63.48	36.52

*Negative control: (sample crude oil without treatment by microorganism); **Positive control: (sample crude oil treatment by microorganism without any type of surfactant).

Table 4: Polyaromatics distribution in aromatics fraction after treatment by *Bacillus licheniformis* using HPLC

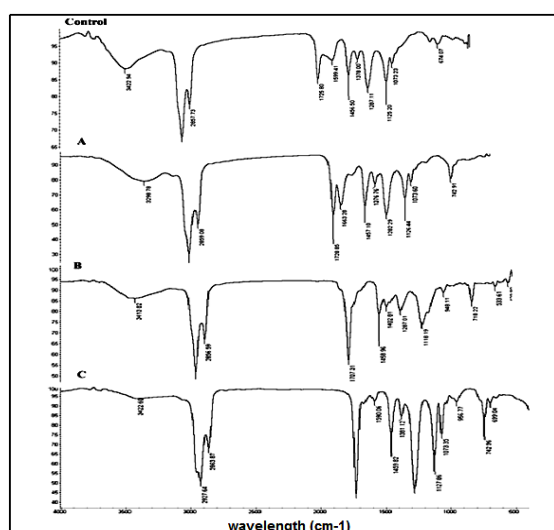| Number of rings	Compound	Negative control	Positive control	Aromatics + microorganism + biosurfactant	Aromatics + microorganism + chemical surfactant
2	Naphthalene	8.10	15.54	0	2.85
	Acenaphthylene	9.83	0	0	8.38
	Acenaphthene	1.00	1.51	41.45	1.2
	Fluorine	0.07	1.74	14.63	0
	Total concentration (%)	19.00	18.79	56.08	12.43
3	Phenanthrene	1.05	0.87	2.85	0.05
	Anthracene	0.61	7.31	20.34	0.11
	Total concentration (%)	1.66	8.18	23.19	0.16
4	Fluoranthene	0.18	68.09	6.10	0.29
	Pyrene	72.03	2.03	0	85.87
	Benzo (a) anthracene	1.03	1.28	14.63	0.85
	Chrysene	0	0.88	0	0.04
	Total concentration (%)	73.24	72.28	20.73	87.05
5	Benzo (b) fluoranthene	4.00	0.75	0	0.36
	Benzo (k) fluoranthene	2.10	0	0	0
	Total concentration (%)	6.10	0.75	0	0.36

FTIR Spectral Analysis

Figure 1: FT-IR absorption spectra for resins extracted from crude of Ash El-malaha. Control sample (sample resins without treatment by microorganism). A. Positive control (sample crude oil treatment by microorganism without any type of surfactant) B. Sample with chemical surfactant C. Sample with biosurfactant.

The compounds containing nitrogen, sulfur or oxygen (NSO) compounds and asphaltic fraction are most resistant to the biodegradation and are most persistent in ecosystems[33].

FTIR analysis for resin fraction extracted from Esh El-Malaha crude oil sample was shown in Fig. (1), the characteristic band for OH- stretching and NH-stretching was appeared at 3422.92cm^{-1} for aliphatic hydrocarbons. The highest decrease of this band appeared in sample containing biosurfactant. Strong bands at 2924 and 2857cm^{-1} appeared due to stretching vibration of methylene group[34]. Strong band was detected at 1728cm^{-1} due to carboxylic esters, the highest increase of this band was shown in the sample containing biosurfactant due to the strong oxidation process[35]. Sharp peak indicating the presence of C=C aromatic at 1599(cm^{-1})[36], sharply decrease of this band in sample containing

chemical surfactant. Strong bands were observed at 1456cm^{-1} due to bending vibration of CH_3 group and medium band at 1378(cm^{-1})[34]. The band was detected at 1456 cm^{-1} decrease in the sample containing chemical surfactant. Strong band was appeared at 1287 cm^{-1} denotes of carboxylic acid, this band sharply increase in the sample containing biosurfactant due to the oxidation process. Indeed, mono-substituted benzene and polynuclear aromatics at 718, 740cm^{-1}, the band at 718cm^{-1} appeared only in the sample containing chemical surfactant and the band at 740 cm^{-1} was only observed in the sample containing the bacterial strain without surfactant and the sample with the biosurfactant due to the oxidation process of the aromatic band.

CONCLUSION

This study has demonstrated that the biodegradation of different crude oil fractions with *Bacilluslicheniformis* using bio and chemical surfactants enhance under laboratory condition.

The total n-paraffins concentration decreased more than iso-paraffins in saturates fraction using bio and chemical surfactants. This bacterial strain could be completely utilizing and high percentage degradation of 5-member rings of polyaromatics compound in crude oil samples with bio and chemical surfactants, respectively.

These compounds considered as more toxic and carcinogenic for soil and aquatic environment. This bacterial strain could potentially be useful for bioremediation efforts of petroleum contaminated environment.

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