EVALUATION OF DIFFERENT SCREENING METHODS FOR BIOSURFACTANT PRODUCERS ISOLATED FROM EGYPTIAN FRESH WATER SAMPLES CONTAMINATED BY OIL SPILLS USING BACILLUS SUBTILIS AND BACILLUS LICHENIFORMIS

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ABSTRACT

In all countries, oil exploration and use threatens the health of the environment and living creatures including humans. An oil spill is the release of petroleum hydrocarbon into the environment. One of the most applicable and safe method is the bioremediation treatment, using microorganisms .This work aims at treating oil spills in fresh water and to compile information on types and properties of biosurfactant. It also describes factors affecting these biosufactants production. Different screening methods e.g. oil spreading assay, Emulsification index (E24), Drop collapse assay, Blood agar test, Hemolysis, MaCconkey agar test were tested. Two isolates gave highly Emulsification tests named O3, O11, which were identified as Bacillus subtilis and Bacillus licheniformis respectively, were isolated from petroleum hydrocarbon contaminated water. Optimization of two isolates were done and highest biosurfactant activity at temperature ranges from 10 oC to 50 oC and pH ranges from 3 to 10 and salinity ranges from 0%to 30%. Also, the biosurfactant was characterized by Fourier transform infrared spectroscopy (FTIR) and surface tension measurement. FTIR showed the production of biosurfactant from Bacillus subtilis similar to surfactin, while the biosurfactant from Bacillus licheniformis similar to lichenysins. The use of microbial biosurfactants significantly decreases the hydrophobicity and increases the rate of hydrocarbon biodegradation. Biosurfactants obtain

demonstrated good surface tension reduction capacity by Bacillus subtilis and Bacillus licheniformis up to 30 and 36Nm/m and have emulsifying activity 71% and 65% respectively.
Keywords: Bioremediation-Biosurfactant-Oil spill-Surface tension-FTIR.

INTRODUCTION

Crude oil is liquid petroleum containing thousands of hydrocarbon components. Each component has a unique chemical behavior that makes it either easily biodegradable, quite difficult to digest or not degradable at all .Petroleum hydrocarbon molecules can be grouped into four broad categories: saturates (branched, unbranched and cyclic alkanes), aromatics – ringed hydrocarbon molecules such as monocyclic aromatic hydrocarbons (MAHs) and polycyclic aromatic hydrocarbons(PAHs), resins and asphalthenes. In the structural arrangement of the four main hydrocarbon components of crude oil, saturates make up the outermost layer of the oil whilst asphalthenes constitute the innermost portion of the oil due to their greater molar masses(Macaulay and Rees, 2014).

The problem is that petroleum is a highly complex mixture of thousand of compounds, many of which cause toxicity to living organisms. Petroleum causes a variety of impacts on animals, including impaired reproduction, decreased resistance to disease, anemia, cancer, neurological damage, and birth defects in offspring. Petroleum impairs photosynthesis, and many physiological processes of Phytoplankton and plants cumulating in inhibition of growth or death. Birds and mammals become coated with oil and their feathers and fur lost property of insulation. Feathers and fur provide insulation by trapping a layer of air between the skin and the external

environment, and thus protects animals from cold (Ramesh and Somashaker, 2014).

To clean-up oil and grease, so many methods were used before which are economically very costly and will affect the environment (Ramesh and Somashaker, 2014). A primary way to contain and collect oil is by the use of containment boom and barrier; the oil can then be recovered by a skimmer (Walther and Henry, 2014). Dispersants were also used as well as Absorbents, while Burning was used successfully on beaches and on isolated slicks (Al-Majed *et al*., 2012), then came Bioremediation (Wang *et al*., 2011).

Bioremediation is a technology based on the activation of microbial degradation of pollutants in contaminated sites by optimizing environmental factors (Skladany and Metting, 1993).

Biosurfactants have gained increased attention because of their biodegradability, low toxicity, ability to be produced from cheap raw materials and effectiveness at extreme conditions of temperature, pH and salinity (Badour *et al*., 2002).

Screening and selection of bio-surfactant candidate bacterial strains from diverse habitats is an interesting area of research that enhances both our collective knowledge and cache of microorganisms that can serve industrially significant processes. A number of approaches are employed in the isolation and screening of bacterial isolates that are capable of degrading petroleum hydrocarbons via bio surfactant production (Hassan *et al*., 2018).

This work aims at treating oil spills in fresh water and to compile information on types and properties of biosurfactant, microbial screening methods as well as biosynthesis, extraction of biosurfactant. It also describes

factors affecting these biosufactants production. Two bacterial strains from eight, Bacillus subtilis and Bacillus lichenformis isolated from petroleum hydrocarbon contaminated water.

MATERIALS AND METHODS

Sampling: For the isolation of biosurfactant producing bacteria, two oil polluted samples were collected one from Ismailia canal near mustard and the other one a little bit far by few metres. Eight microorganisms were obtained from the polluted samples with oil spills.

Materials:

LB (Luria-Bertani) MEDIUM

Tryptone 10.0 g

Yeast extract 5.0 g

NaCl 10.0 g in case of sea water

Agar 20.0 g

Distilled water 1000.0 ml

A pH to 7.0.with modification yeast extract10g in both cases and NaCl 5g in case of fresh water (Biniarz and Lukaszewicz., 2017) supplemented with glucose 5 mg as a sole Carbon source.

Methods:

Isolation of Crude Oil Bacteria Producing Biosurfactant: The sterilized media was spread on sterile petri-plates under aseptic conditions, after that the samples were added by direct method. Only 1ml from each water polluted with oil. The plates were incubated at 30 ^{o} C until the microbial colonies developed (April *et al*., 2000).

Purification of the Isolates: The purification procedure of these crude oil utilizing microbial isolates were carried out by agar streak method according to (Bertani, 2004).

Morphology of Isolate and Gram Staining: Morphology and Gram staining were done according to (Ismail, 2008).

Growth on MacConkey Agar Medium: All isolates were streaked on MacConky agar medium which is a selective and differential medium designed to isolate and differentiate enterics based on their ability to ferment lactose. Bile salts and crystal violet inhibit the growth of Gram positive organisms. Lactose provides a source of fermentable carbohydrate, allowing for differentiation. Incubation at 30° C for 24hr; the growth of the tested isolates was investigated after that incubation time according to (Athar *et al*., 2014).

Oil Spreading Assay: This method was employed to check the efficacy of the culture medium in displacing the oil layers, 1 ml of crude oil was added to the surface of 30 ml of distilled water in a Petri-dish to form a thin oil layer, 20 µl of culture supernatant was gently dropped on the centre of the oil layer, after one minute if the sample was +ve (containing biosurfactant), the oil is displaced and a clearing zone was measured (Morikawa *et al*., 2000).

Drop Collapse Assay: It is a rapid and crude method to assess thesurfactant activity In brief, about 10 µl of cell free broth was added inthe center of an oil drop (20 µl of any oil) taken in a clean glass slide. The collapse of oil drop hasbeen visualized and the less time taken indicates the higher activity of surfactant (Jain *et al*., 1991).

Emulsification Index: The emulsion index was calculated after 24 hours; hence it is also called E24. Emulsion index was measured in percentage by diving the height of the emulsion to the total height of the mixture. The procedure is described as follows:

- 6 ml Distilled water+2 ml immersion oil
- The sample was vortexed for 10 min
- The mixture was allowed to settle down
- 2 ml crude biosurfactant sample was added
- Again the sample was vortexed for 10 min and settled for 24 hrs.

Emulsion was observed (Rikalovic *et al*., 2012 and Mukherjee *et al*., 2006). Modification happens (1ml broth+5ml water + a drop of crude oil). The E24 index is calculated by using the following equation (Sidkey *et al*., 2016)

$$E24 = (\text{Height of emulsion formed x } 100)/(\text{Total height of the solution})$$

Hemolytic activity: A pure culture of each bacterial isolate was streaked on the freshly prepared blood agar and incubated at 37 o C for 48–72 Hrs. Results were recorded based on the type of clear zone observed (Plaza *et al*., 2006 and Youssef *et. al.*, 2004).

Oil Spreading Method: Oil spreading technique was carried out. Briefly, 5 mL of distilled water was added to the Petri plate followed by addition of 1 mL of crude oil to the surface of the water. Then, 2 mL of cell-free culture broth was dropped on the crude oil surface. The diameter of the clear zone on the oil surface was measured and compared to 10 mL of distilled water as a negative control (Sharma *et al*., 2014).

Fourier Transform Infrared Spectroscopy: The biosurfactant extract recovered from the supernatant of the bacterial isolate was characterized by Fourier transform infrared spectroscopy (FTIR). The FTIR spectra using Perkin Elmer – spectrum one, controlled by Spectrum Software Version 3.2. In the range of 650-4000 cm-1 (Egyptian Petroleum Research Institute) through HATR technique (Chandankere *et al*., 2013).

Surface Tension: The surface tension of the culture supernatants was measured using Theta Lite –surface tensiometer - Biolin Scientific ,
according to ASTM D1331 - 14 " Standard Test Methods for Surface and Interfacial Tension of Solutions of Paints, Solvents, Solutions of Surface-Active Agents, and Related Materials".

Biosurfactant Stability Tests: Stability studies (optimization) were carried as described by (Obayori *et al*., 2009). The stability of the biosurfactants against pH, temperature, salinity was determined as following with some modifications:

Optimization of the Isolate According to Temperature: Optimization is made by broth containing LB (Luria-Bertani) Broth. Adjust temperature as follows to 10oC, 20oC, 30oC, 40oC, 50oC .The broth is sterilized and put in different incubators for 7 days then 10 days.

Optimization of the Isolate According to pH: Optimization is made by broth containing LB (Luria-Bertani) Broth. Adjust pH as follows to 3, 4, 5, 6, 7, 8, 9, and 10. The broth is sterilized and put in incubators according to optimum temperature of each microorganism for 7 days then 10 days.

Optimization of the Isolate According to Salinity: Optimization is made by broth containing LB (Luria-Bertani) Broth. Adjust salinity as follows to 0 %,

3%, 5%, 10%,15%,20%,25%.30%. The broth is sterilized and put in incubators according to optimum temperature and pH of each microorganism for 7 days.

Bacteria identification was Done by Biolog (Bochner, 1989): Two strains of the isolated microorganisms were identified by Biolog GEN III MicroPlateTM.

RESULTS AND DISCUSSION

Biosurfactants have better surface activity, lower toxicity, they can bind heavy metals, have higher biodegradability, selectivity and biological activity, they are produced from renewable resources, can be produced through fermentation and can be reused by regeneration. The other advantages of microbial surfactants are eco-friendly, high foaming ability and efficiency at extreme temperatures, pH and salt concentrations (Sidkey *et al*., 2016) .From two contaminant fresh water samples by oil spill, eight isolates were selected for studying cell morphology as in table(1).

Table (1): Morphology of isolates, Forms of isolates, Gram staining, MaCconkey test

Fresh Isolates	Form	GRAM Staining	MaCconkey Test
6	Creamy	POSITIVE	POSITIVE
7	Creamy	NEGATIVE	NEGATIVE
8	Creamy	NEGATIVE	NEGATIVE
9	Transparent	POSITIVE	POSITIVE
O1	Creamy	POSITIVE	POSITIVE
O11	Yellowish	POSITIVE	POSITIVE
O2	Creamy	POSITIVE	POSITIVE
O3	Creamy	POSITIVE	POSITIVE

Several tests were done as oil displacement test, emulsifying activity, hemolytic activity, gram staining, oil collapse test, Optimization of the microorganisms according to pH, temperature, salinity to know the best conditions for these microorganisms to get the best yield of their biosurfactants for the eight isolated strains as shown in table 1, 2.

Table (2): Screening tests to the isolates Blood agar test, oil spreading assay, oil collapse assay, E24 test

Fresh Isolates	Blood Agar Test	Lactose Fermentation	Oil Spreading Assay Diameter	Oil Collapse Assay	E24%
6	Beta	None	2cm	Positive	Negative
7	Gamma	Lactose Fermentor	7cm	Positive	Negative
8	Beta	Lactose Fermentor	2cm	Positive	Negative
9	Beta	None	7cm	Positive	Negative
O1	Beta	None	6cm	Positive	57%
O11	Gamma	None	6cm	Positive	65%
O2	Beta	None	5cm	Positive	Negative
O3	Beta	None	6.5cm	Positive	71%

Then the most potent microorganisms O3, O11 were identified by Biolog as Bacillus subtilis, Bacillus licheniformis respectively.

Hemolytic activity: The culture supernatants of Bacillus subtilis and Bacillus lichenformis strains exhibited hemolytic activity, the first beta hemolytic while the second is gamma. They produced B-hemolysis pattern, i.e. complete lysis of red blood cells. These hemolytic zones were related to the ability of these bacterial strains to produce biosurfactants which was in agreement with Plaza *et al.*2006; Youssef *et al.*2004 who reported that the

existence of a relationship between the hemolytic activity and biosurfactant production. They suggested the use of blood agar test, hemolysis as a primary method for screening as in Table(3).

Drop Collapse Assay: Results of wetting activity investigated by using the drop collapse test revealed that Bacillus subtilis and Bacillus licheniformis strains were capable of producing biosurfactant as in Table(3). The drop collapsing assay relies on the capacity of surfactants to destabilize the liquid droplets on an oily surface which is in agreement with Jain *et al*., 1991.

Growth on MacConkey Agar Medium: The strains B. subtilis and B. licheniforms showed no growth on MacConkey and showed no lactose fermentation as in Table (3), Such trend was in agreement with Athar *et al*., 2014.

Oil Spreading Assay: The oil displacement method measures the surface activity of a surfactant solution tested against crude oil; the larger the diameter of displaced circle the higher surface activity of the surfactant . Biosurfactant produced by Bacillus subtilis culture showed higher surface activity, the diameter of displaced circle was 6.5cm while in case of Bacillus licheniformis strains were 6 cm as in Table(3). This was in agreement with Kiran *et al*., 2010.

Emulsification Index (E24 %): E24 of the eight isolates ranges from 57% to 71%. The cell-free culture broth of both strains was successfully emulsified crude oil. The highest E24 value recorded by Bacillus subtilis biosurfactant was 71% compared to 65% for Bacillus licheniformis ,so it could be stated that the bacterial strains under study can produce biosurfactants and

bioemulsifier as in Table(3),This is in agreement with study of (Sidkey *et al.* , 2016).

Table (3): Comparison between B. subtilis and B. licheniformis in growth criteria and activity

Test	Bacillus subtilis	Bacillus lichenformis
Gram stain	gram +ve	gram +ve
Blood agar (hemolysis)	Beta	Gamma
Growth on MaCconky	Negative	Negative
Lactose fermentation	None	None
Oil collapse	Positive	Positive
Oil spreading assay	6.5cm	6cm
Optimum temperature for growth	40	20
Optimum pH for growth	6	7
Optimum Salinity for growth	5g/l	0g/l
Emulsification	71%	65%

In comparison between two isolates Bacillus subtilis and Bacillus licheniformis, was found in Blood hemolysis and temperature range and Emulsification index.

Optimization of the Isolate According to pH, Temperature, Salinity: Optimization of two isolates Bacillus subtilis and Bacillus licheniformis were done.The results of optimization agreed with Desai and Banat (1997) who elucidated that, environmental factors and growth conditions such as pH, temperature affect biosurfactant production through their effects on cellular growth or activity.

Several studies have aimed to optimize the biosurfactant production process by changing the variables that influence the type and amount of biosurfactant produced by a microorganism. Important variables are carbon and nitrogen sources (Santos *et al*., 2002), also the use of glucose as a sole

carbon source enhances the production of high yield of Biosurfactants (Fooladi *et al* .2013). Potential nutrient limitations and other physical and chemical parameters such as oxygen (Kronemberger *et al*., 2008), temperature and pH (Mukherjee *et al*., 2006) were considered.

Fig.1 shows that optimum temperature for Bacillus subtilis is 40 ^{o}C and for Bacillus licheniformis is 20 ^{o}C, also shows that optimum temperature for Bacillus subtilis is 30 ^{o}C, 40 ^{o}C, 50 ^{o}C and Bacillus licheniformis is 20 ^{o}C after incubation for 10 days.

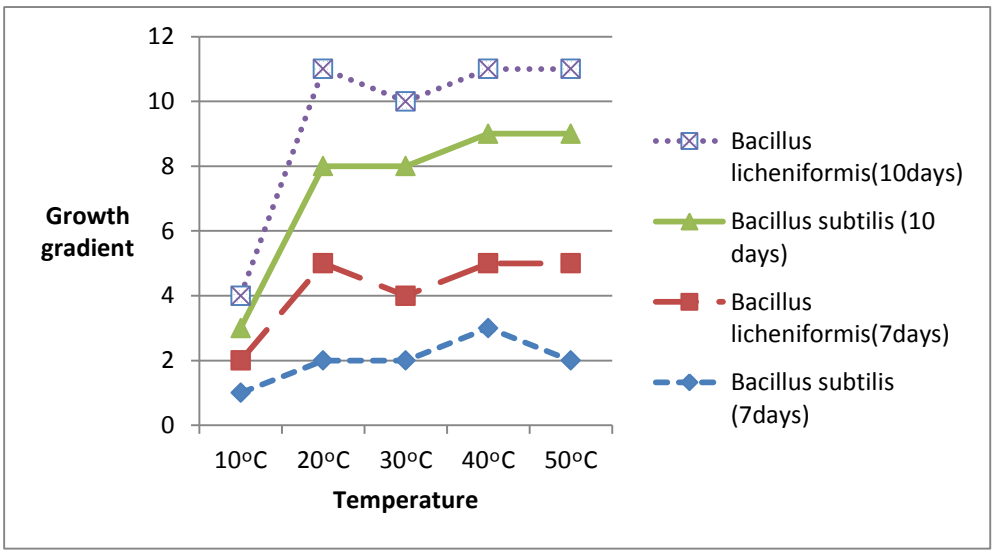


Fig (1): Growth Gradient by temperature after incubation period of 7 & 10 days

Fig (2): shows that optimum pH for Bacillus subtilis is 6 and for Bacillus licheniformis is 7, also shows that optimum pH for Bacillus subtilis and Bacillus licheniformis is 7 after incubation for 10 days.

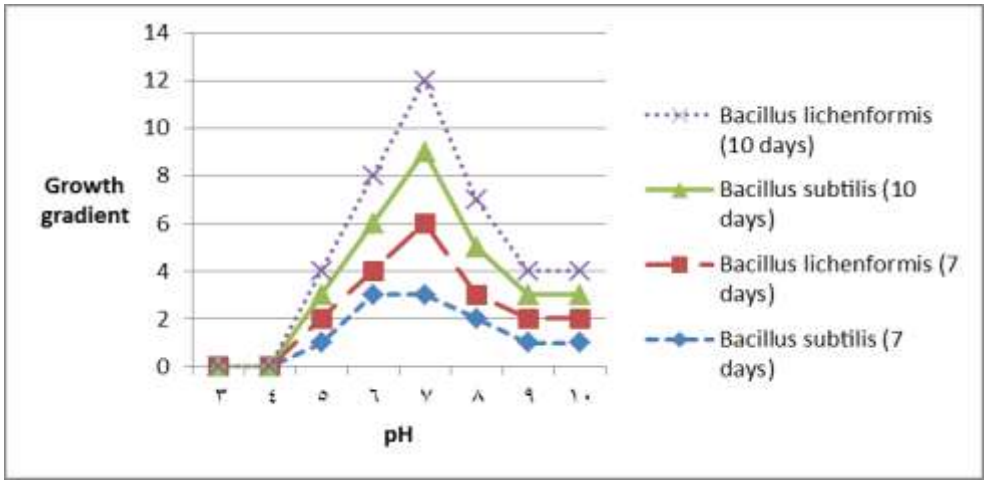


Fig. (2): Growth gradient according to pH value after incubation period of 7 & 10 days.

Fig. 3 shows the optimum salinity for Bacillus subtilis is 5g/land for Bacillus licheniformis is 0.

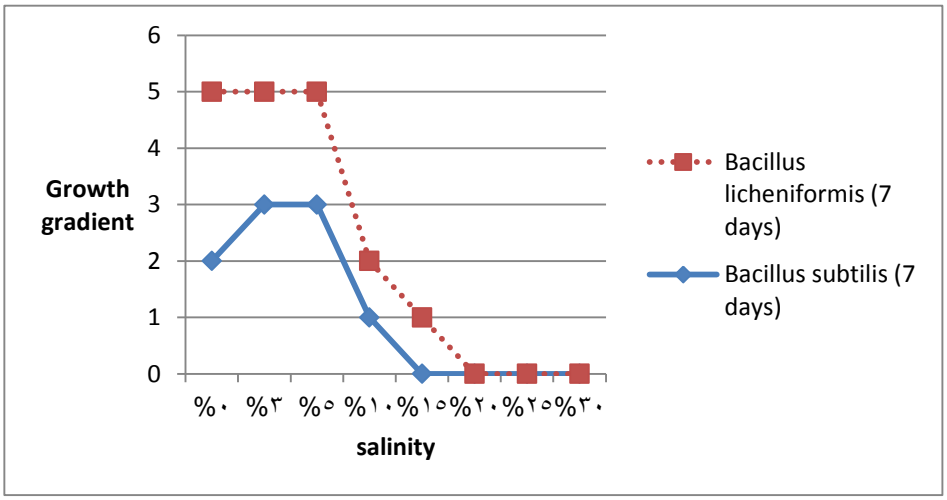


Fig. (3): Growth gradient according to salinity concentration g/l after incubation period of 7 days

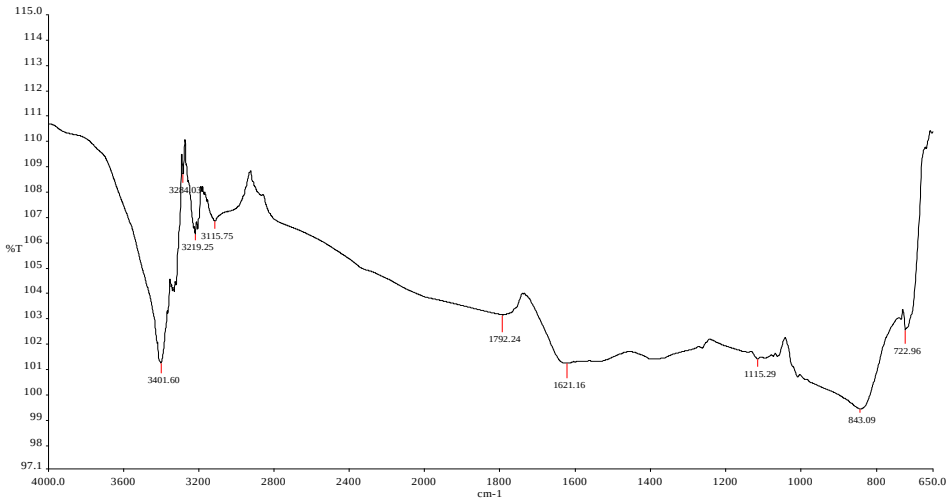
Surface Tension Measurement:

Name of Isolate	Results of Surface Tension by Nm/m
Control	49
Bacillus subtilis	30
Bacillus licheniformis	36

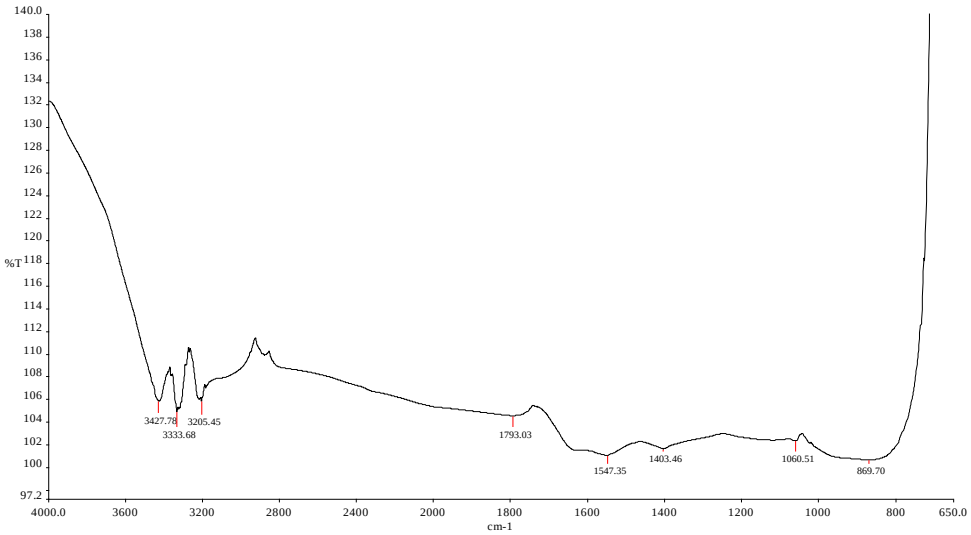
Based on surface tension measurement, the cell-free supernatants of strains showed a remarkable reduction in surface tension, Bacillus subtilis biosurfactant was observed to have higher surface tension reduction capacity up to (30 mN/m) than Bacillus lichenformis that was up to (36 mN/m). While the distilled water and control gave 72 and 49 mN/m, respectively compared with study of (Jenny *et al*., 1991).

FTIR Curves:

FTIR curves to the two strains are done as the following: The first FTIR curve(1) for Bacillus lichenformis containing amide-alcohol (-OH)-alcohol-amide-ketones (-C=O)-nitro-aldehyde (-NH)-terminal di substituted alkene-cis di substituted alkene groups (similar to lichenysins biosurfactant) compared by lipopeptide in study of (Suthar and Nerurkar, 2016).The second FTIR curve(2) for Bacillus subtilis containing amide –amide – alcohol – ketones - asymmetric nitro-symmetric nitro-mono substituted alkene-terminal di substituted alkene groups, similar to surfactin biosurfactant compared to lipopeptide in study of (Joshi *et al*., 2008).



FTIR curve (1): for Bacillus licheniformis biosurfactant similar to lichenysins



FTIR curve (2): for Bacillus subtilis biosurfactant similar to surfactin

CONCLUSION

The Bacillus subtilis and Bacillus lichenformis strains, isolated from fresh water contaminated with oil spill, are capable of producing high yield of biosurfactants and bioemulsfiers. The preliminary chemical characterization revealed that they belong to the lipopeptides. Bacillus subtilis strain are capable of producing a biosurfactant similar to surfactin and Bacillus lichenformis strain are capable of producing a biosurfactant similar to lichenysins. In terms of the surface activities, the crude biosurfactants showed comparable physicochemical properties. .Indeed, they are capable of forming stable emulsions with a variety of hydrophobic compounds, and their activities are not affected by exposure to exaggerated environmental conditions. The features of these biosurfactants and bioemulsfiers make them an interesting biotechnological product for many environmental and industrial applications such as bioremediation processes and Microbial enhanced oil recovery.

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تقييم طرق الفرز المختلفة لمنتجي خافضات التوتر السطحى الحيوية المعزولة من عينات المياه العذبة المصرية الملوثة بالانسكابات النفطية باستخدام البكتريا العصوية الرقيقة والبكتريا العصوية حزازية الشكل

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المستخلص

في جميع البلدان، يهدد التنقيب عن النفط واستخدامه صحة البيئة والكائنات الحية بما في ذلك البشر. ويُعرف الانسكاب النفطي على أنه تسرب للهيدروكربونات النفطية إلى البيئة. وتُعد المعالجة الحيوية واحدة من أكثر الأساليب المتبعة والآمنة باستخدام الكائنات المجهرية الدقيقة . ويهدف هذا البحث إلى معالجة الانسكابات النفطية في المياه العذبة وتجميع معلومات عن أنواع وخصائص خافضات التوتر السطحي الحيوية (المشتتات). ويصف البحث العوامل التي تؤثر على إنتاج خافضات التوتر السطحي الحيوية.لقد تم اجراء طرق الفرز المختلفة مثل مقايسة انتشار النفط ونشاط الاستحلاب واختبار انهيار الزيت والنشاط الانحلالى واختبار ماكونكي . قد أعطت اثنتين من العزلات أعلى نشاط استحلابى وهما العصوية الرقيقة والحزازية الشكل التى تم عزلهم من المياه الملوثة بالهيدروكربونات النفطية. وقد تم استمثال الكائنات الحية المجهرية بحسب الرقم الهيدروجيني من ٣ إلى ١٠، ودرجة الحرارة من ١٠ oC الى ٥٠ oC والملوحة من %صفر الى ٣٠%.كما تم تمييز خافضات التوتر السطحي الحيوية عن طريق التحليل الطيفي بمحول فورييه للأشعة تحت الحمراء (FTIR) وقياس التوتر السطحى الطيفي. وقد أثبت التحليل بمحول فورييه للأشعة تحت الحمراء أن خافضات التوتر السطحي الحيوية الناتجة عن العصوية الرقيقة تماثل السيرفكتين، في حين أن تلك الناتجة من العصوية حزازية الشكل تماثل ليشينيسيين، إن استخدام خافضات التوتر السطحي الناتجة

عن الميكروبات من شأنها أن تقلل بشكل كبير من عدم القابلية للذوبان في المياه وتزيد من معدل التحلل الحيوي للهيدروكربونات. وقد أثبتت خافضات التوتر السطحي الحيوية قدرتها الجيدة على خفض التوتر السطحي ونشاط الاستحلاب في النفط الخام حيث سجل انخفاض التوتر السطحي من العصوية الرقيقة والعضوية حزازية الشكل حتى ٣٠ و٣٦متر نيوتن/ متر بينما سجل نشاط الاستحلاب ٧١٪ و ٦٥٪ على التوالي.

الكلمات الدالة: المعالجة الحيوية – خافضات التوتر السطحي الحيوية – الانسكاب النفطي – التوتر السطحي – التحليل الطيفي بمحول فوريه للأشعة تحت الحمراء.