

# Production of Biosurfactant by *Bacillus Licheniformis* Strain Isolated from Contaminated Soil

Ahmed A. Burghal

*University of Basrah, College of Science, Biology Department, Iraq*

Wijdan H. Al-Tamimi

*University of Basrah, College of Science, Biology Department, Iraq*

Alyaa S. Jasim

*University of Basrah, College of Veterinary Medicine, Microbiology Department, Iraq*

**Abstract:** Biosurfactants are active surface components act as stimulants for biodegradation of the hydrophobic substrate available for microbial attachment. These molecules reduce interfacial tension in aqueous solutions, therefore the important application of biosurfactants is in oil industry. Biosurfactant producing *Bacillus* were isolated from hydrocarbon contaminated soil and identified by morphological, biochemical and genetic characterization. DNA was extract and the 16S rRNA was amplified by PCR using universal primer. The PCR product was sequenced and used BLAST program to assess the DNA similarities. The analysis of biosurfactant production by strain were performed by drop collapse technique, Oil Spreading, emulsification index method to determined the activity of biosurfactant. The results were showed the drop collapse was (++), Oil Spreading 14 mm, emulsification about 40%, also the oil biodegradation was 82%. According to the results from the present study the *Bacillus sp.* has potential ability are used especially for Microbial Enhanced Oil Recovery and bioremediation of hydrocarbons, so we can hope that looking for new strains can play an important role in their application.

**Keywords:** Biosurfactant, *Bacillus licheniformis*, oil contaminated soil

## I. INTRODUCTION

The microbial biosurfactant is a group of biological amphiphilic compounds produced by various microorganisms such as bacteria, fungi and molds to the media in which they grow to reduce the interfacial tension or on their cell surface [1-3]. The characterization of biosurfactants produced by various microorganisms have been extensively studied [4,5]. It appears in the form of the following structures: glycolipids, lipopolysaccharides, lipoprotein or lipopeptides, phospholipids, or the microbial cell surface itself [6,7]. Chemically synthetic surfactants are toxic to some degrading microorganisms and can be hardly for biodegraded, moreover, it is derived from petroleum so likely to be contaminated and cause disorder to the environment [2]. Biosurfactants are highly efficient and ecologically friendly. As a result, it can potentially replace virtually any synthetic surfactant [8].

Biosurfactants now have a wide range of applications in several industrial processes, such as oil, food, pharmaceutical, medicine industries and pollutant removal [9-12]. Addition to lubrication, fixing dyes, making emulsions, stabilizing dispersions, preventing foaming [13]. The main application is for enhancement of oil recovery techniques, especially the trapped residual oil [14], oil bioremediation due to their biodegradability and low critical micelle concentration (CMC) [13,15].

One of the most important bacteria producing biosurfactants are *Bacillus* spp. which are main use for biotechnological applications. Sheshtawy *et al.* [16] were production of biosurfactant from *Bacillus licheniformis* and their used for enhanced oil recovery and inhibition of the sulfate reducing bacteria. They produce a variety of products such as extracellular enzymes, biosurfactants, biopesticides, and so forth from renewable resources and are ecofriendly. Biosurfactants are produced from *Bacillus* spp. possess the property of functionality under extreme conditions of pH, temperature, and salinity addition to being from renewable resources and are ecofriendly [17-19].

Because of the of the lipopeptide biosurfactants produced by *Bacillus* spp. have highly ability to reduced the surface tension, so the one potential uses in the oil industry and bioremediation [20]. The objective of this study was to production of biosurfactants by *Bacillus* spp. isolated from contaminated soil and the identification of strain by amplified 16S rRNA gene.

## II. MATERIAL AND METHODS

**Soil samples:** The samples were taking from the contaminated region surrounding South Refineries Company located in the Basrah governorate, Iraq. Soil samples with high concentration of hydrocarbon from refineries processes were

collected from the layer 15-20 Cm below the surface in sterile plastic bags and transport to the laboratory, preserved at 4°C and bacteriological analysis were conducted within 24-48 h.

*Isolation of bacteria:* One gram of soil sample was dissolved in 10ml of normal saline to making tenfold serial dilution of sample for enumeration of Bacteria. The dilution factor expressed in colony forming unit (cfu) [21]. Suspension was applied for especial *Bacillus* isolation by heating sample at 70°C for 15 minute to reduced any vegetative bacterial cells. Serial dilutions were prepared and plated on nutrient agar medium supplied with 80µg/ml of cycloheximide and incubated at 30°C for 2-3 days for growth of the spores forming bacteria colonies. The bacterial colonies were purified and sub-cultured on the same medium for identified and biosurfactant production.

*Identification of bacteria:* The morphological characteristics of the isolate was identified under light microscope after staining by gram stain. The biochemical reactions include oxidase test and catalase production reaction, identified based on morphological and biochemical characteristics presented in Bergey's Manual of Determinative Bacteriology [22].

*DNA extraction:* The cell pellets from *Bacillus* sp. isolate was used to extracted genomic DNA using Wizard Genomic Isolation DNA kit (Promega, USA) by following the manufacturer's instructions and DNA extracted was used as the template for PCR. Specific bacterial primers and universal bacterial primers 1492R(5'-AGAGTTTGATCMTGGCTCAG-3') and 27F (5'-CGGCTACCTTGTTACTT-3') respectively were used for PCR amplification of 16S rRNA gene [23].

*Amplification of 16S-rRNA genes:* Polymerase chain reaction (PCR) was used to amplify of 16S rRNA genes fragment. The PCR master mix contain 10pmol of each primer ( 2 µl ) and 25 µl of 2x master mix (Promega, USA ) mixed with purified DNA as template ( 4 µl ). Sterile d. H<sub>2</sub>O was added to a final volume of 50 µl . Thermal cycler (Bioneer, Korea ) program was used to incubate reaction as following: 94 °C for 2 min., 94 °C for 1 min., primer annealing at 55 °C for 30 se., and primer extension at 72 °C for 1 min., the number of cycles was 35 cycle and finally PCR reaction time was 72 °C for 10 min [24].

*PCR products:* The yield of PCR products approximately 1500 bp, successful amplified of 16S rRNA gene was confirmed by agarose gel electrophoresis with 100 bp DNA ladder marker at 65V and 120mA for 40 min. using TBE buffer 1x, the gels were stained with ethidium bromide solution, visualized under the UV light.

*Sequence analysis:* The PCR products of isolate sequenced using the same primer as described for the amplification process. DNA Sequence were obtained using a ABI 3730XL Genetic Sequencer (NICEM, USA). The bacterial 16S rDNA sequences were tested with known 16S rDNA sequences using 3730 automatic sequencing equipment. Phylogenetic affiliation of the 16S rDNA sequence was initially estimated using the program BLAST "a basic search tool of the National Center for Biotechnology Information" (<http://www.ncbi.nlm.nih.gov/BLAST>) [25], to determined approximate phylogenetic position and percent homology scores were obtained to identify bacteria.

*Cultivation media:* Culture media of mineral salt medium (MSM, pH 7.2) composed (g/l) (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 2.00g, KH<sub>2</sub>PO<sub>4</sub> 2.4g, K<sub>2</sub>HPO<sub>4</sub> 4.8g, MgCl<sub>2</sub> 0.08g, (NH<sub>4</sub>)<sub>2</sub>MO<sub>7</sub>O<sub>24</sub>.4H<sub>2</sub>O 0.01g, CaCl<sub>2</sub>.2H<sub>2</sub>O 0.03g, Citric acid 0.4g, Carbon source (2%) crude oil in one liter, Trace element solution 2ml contained (g/l) FeSO<sub>4</sub>.7H<sub>2</sub>O 1g, NaMoO<sub>4</sub> 2g, MnCl<sub>2</sub> 1g, ZnSO<sub>4</sub>.7H<sub>2</sub>O 0.03g, CuSO<sub>4</sub>.5H<sub>2</sub>O 0.25g, CoCl<sub>2</sub>.6H<sub>2</sub>O 0.25g for crude oil biodegradation [26,27].

Mineral salt medium (MSM, pH 7.2) composed (g/l) KH<sub>2</sub>PO<sub>4</sub> 0.5g, K<sub>2</sub>HPO<sub>4</sub> 1g, NaNO<sub>3</sub> 4g, MgSO<sub>4</sub>.7H<sub>2</sub>O 0.5g, KCl 0.1, FeSO<sub>4</sub>.7H<sub>2</sub>O 0.01, Yeast extract 0.01, Carbon source (2%) crude oil , Trace element 0.05 ml solution 2ml contained (g/l) NaMoO<sub>4</sub> 0.1g, MnCl<sub>2</sub> 1.75g, ZnSO<sub>4</sub>.7H<sub>2</sub>O 3.1g, CuSO<sub>4</sub>.5H<sub>2</sub>O 2g, H<sub>3</sub>BO<sub>3</sub> 1.5g for biosurfactant production [28-30].

*Screening of the biosurfactant producer:* The bacterial strain maintained on nutrient agar at 4°C was inoculated in 50 ml of seed medium (nutrient broth ) in a 100 ml Erlenmeyer flask and incubated in a rotary shaker at 120 rpm at 30 °C. after growth for 18 h standard inoculum 5% were used in the fermentation medium which is equivalent to 1x10<sup>8</sup> CFU/ml. For crude oil biodegradation and biosurfactant production, MSM supplied with 2% crude oil as the sole carbon and energy source. The media inoculated with 5% (v/v) inoculum of isolate and incubated at 30°C on a rotary shaker (120 rpm) for 7 days [31]. Culture were centrifugated (Eppendorf cooling centrifuge, Hamburg , Germany) at 6000 rpm for 15 min. in 4 °C and supernatant was collected for extracellular biosurfactant assay by different methods and biodegradation percentage.

*Biodegradation efficiency:* After the end of incubation period the culture media taken from the flasks were placed in the centrifuge at 6000 rpm for 15 min. to cells remove, supernatant was mixed with equal volumes of chloroform in separating funnel, and shaken to extract residual crude oil [32]. Residual hydrocarbon was measured using the method described by [33].

*1-Drop collapse method:* The drop collapse was determined by adding 100 µl mineral oil to the 96 well microtiter plates and incubate for an one hour at room temperature for equilibration then added 10 µl of cell free culture was added to the surface of the oil and leaved 1 min, the shape of the drop on the surface of the mineral oil was observed and interpreted as partial spreading on the oil surface (+) to complete spreading on the oil surface. (+++) [34].

*2-Oil spreading method:* the Oil spreading technique was determined by addition 100 µl of crude oil to the to the Petri plate contain 50 ml of distilled water. About 10 µl of cell free culture broth was carefully dropped on the crude oil surface in the plate, while the control sample used 10 µl of distilled water. The Oil spreading was determined as the diameter of clear zone ( mm ) on the oil surface and compared to control [34].

*3-Oil dispersion test:* Bacillus strain were grown for 48 h in nutrient broth at 30°C then 5ml of growth culture was added to 250 ml Erlenmeyer flasks containing 50 ml of MSM supplied with 1% crude oil, The flasks were incubated at 30°C on a shaker incubator (120 rpm) for 7 days, after incubation 5ml of suspension was added to the same MSM, mixing with a shaker for 15 min, and left standing for 15 min. The oil dispersion observed and recorded the results as forming high small dispersed flock and dense liquid partial to complete.

*4-Emulsification activity:* The emulsification index of culture samples was determined by adding 5 ml of hydrocarbon (Crude oil) to the same amount of cell free culture (supernatant) in a graduated test tube. The tube was mixed with a vortex at high speed for 2min and left to stand. After 24 h [35,36]. the emulsification activity (E24) was calculated using following formula  $E24 (\%) = \text{total height of the emulsified layer (cm)} / \text{total height of the liquid layer (cm)}$ .

### III. RESULTS AND DISCUSSION

The soil samples showed a high levels of contamination with hydrocarbon originating from the refinery (2500 mg.Kg<sup>-1</sup> dry soil). However, it seems that soil bacteria are adapted to this level because they have shown high numbers. The rate of total heterotrophic bacteria was  $1.6 \times 10^6$  cfu.g<sup>-1</sup> of indigenous soil bacteria when plated on nutrient after two days at 30°C The number of bacteria in soil samples was agreed with Jazeh *et al.* [37] who observed the presence high number of biosurfactant producing bacteria in oil contaminated habitat.

*Identification of bacterial strain:* The morphological characteristics of the isolate was identified under light microscope after staining by gram stain. The biochemical reactions include oxidase test and catalase production reaction, identified based on morphological and biochemical characteristics presented in Bergey's Manual of

Determinative Bacteriology [22]. The image taken by an optical microscope under 100× oil immersion lens showed that bacteria cells are gram positive, rod-shaped spores forming, biochemical test showed that the strain catalase and oxidase positive, isolate was identified as *Bacillus spp.* These morphology characters are in accordance with the previous report about *Bacillus spp.*, by Maliji *et al.* [31].

*Genetic identification:* The partial 16S-rRNA gene sequences that were determined depending on the deposited data in the GenBank, by using BLAST search in the NCBI data bank, sequences homologous to 16S rRNA gene of the isolate 40B was collected. Comparison of 16S rRNA nucleotide gene sequence of the strain with corresponding *Bacillus* sequences clearly showed that the isolate was closely related to the type strain of *Bacillus licheniformis* WSE-KSU302 (HM753626.1). sharing a 16S rRNA gene sequence similarity of 99%.

*Hydrocarbon biodegradation and biosurfactant production:* When strain 40B was cultivated with 2% crude oil as the sole carbon source in the MSM to determine their growth rate, biosurfactant yield, and biodegradation activity. After 7days of incubation, the Biodegradation activity of bacterial isolates revealed a varying hydrocarbon biodegradation to 82% as show in figure 1. Different species of *Bacillus* such as *Bacillus cereus*, *B. subtilis* and *B. licheniformis* were ability for crude petroleum oil biodegradation isolated from oil contaminated site [38,39,31]. Biosurfactant are produced by many bacterial strains that showed a strong ability to grow on a hydrocarbons as sole carbon source and degrade or transform of these compounds [40].

Many microorganisms produce biosurfactant such as *Bacillus spp.* which produce highly activity biosurfactant [41]. In this study a biosurfactant producing bacteria belong to *Bacillus licheniformis* WSE-KSU302 strain was obtained from hydrocarbons contaminated soil. The results showed that this strain could utilize 80% of crude oil in MSM during 7 days as the carbon source. Study by Ramos *et al.* [42] have shown that the survival of microorganisms in petroleum hydrocarbons medium after their inoculation is an important factor in the biodegradation of hydrocarbons in liquid medium.

**Drop collapsing:** The result of drop collapsing test by used mineral oil in microtiter was showed positive reaction (++) between drop of surfactant producing culture and mineral oil surface causing flattened on oil surface (Figure 2). Full spreading due to the reduction in force tension between the liquid drop and the hydrophobic surface. The surface tension an essential factor in the stability of the drops, reduce this factor dependent on surfactant concentration and correlates with surface. To screen bacterial colonies for potential biosurfactant production a rapid drop collapsing test was employed [40,43]. The drop of cell free suspension if contained biosurfactants when placed on an oil-coated surface it was observed to collapse compared with non biosurfactant-containing drops which remained stable [17].

**Oil-spreading technique:** The result of the oil-spreading technique showed that the supernatant of the *Bacillus* strain when added to oil in plates containing distilled water the diameter of the clearing zone of displacement of 14 mm after 30 s, due to displaced the oil surface (Figure 3). Method of the oil spreading is rapid and simple way can be carried out easily without the need for special equipment and require a small amount of the sample and the activity and quantity of biosurfactant is low [44]. Youssef *et al.* [36] demonstrated that the oil spreading technique is a reliable method for testing biosurfactant production by various microorganisms. reported by Morikawa *et al.* [45] who showed that the concentration of the biosurfactant in the solution evidenced by area of oil displacement in oil spreading assay. While the oil dispersion result was reaction about (++) as showed in figure 4.

**Emulsification assay:** This strain showed obvious effect in the emulsification of crude oil was done by mixing 5 ml of crude oil to 5 ml of culture supernatant and kept overnight. Around 40% emulsification was observed after 24 h as shown in figure 5. Studies by Okerentugba and Ezerony [46] showed that strains isolated from areas with permanent contamination had better emulsification activity. The *Bacillus licheniformis* strain have high emulsification activity and biosurfactant production. Bento *et al.* [47] observed that *Bacillus* spp. which have these abilities isolated from contaminated soil. Also Joshi *et al.* [19] and El Sheshtawy *et al.* [16] have showed that the bacteria species such as *Bacillus licheniformis* have ability to produce efficient biosurfactant. Thavasi *et al.* [48] explained emulsification test is an indirect method used to screen biosurfactant production and detected if the cell free culture broth used in this assay contains biosurfactant then it will emulsify the hydrocarbons present in the test solution.

However, there are many studies of microorganisms producing surface-active compounds when growing on crude petroleum-oil hydrocarbons [49,37]. The production of biosurfactant by the producing microbes is depended on the utilization of available hydrophobic substrates as a result of decreasing the surface tension of substrates and increasing their apparent solubility [50-52].

Table 1: *Bacillus* strain with an evaluation of biosurfactant production

Index	<i>Bacillus licheniformis</i>	Control
Drop collapse	++	-
Oil separation mm	14	0
Oil dispersion	++	-
Emulsification E24%	40	0



Figure 1: Biodegradation of oil

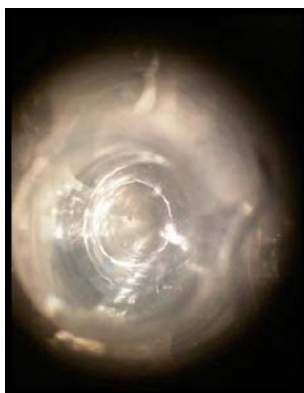


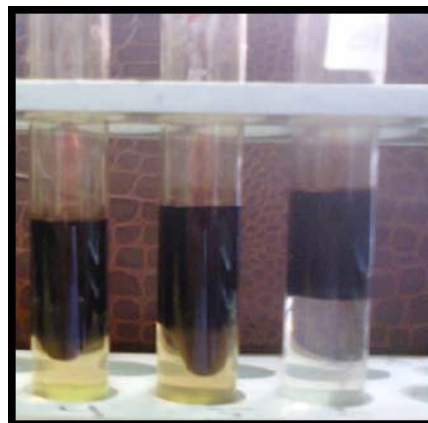
Figure 2: Drop collapse



Figure 3: Oil separation



Figure4: Oil dispersion

Figure 5: Emulsify of oil (E<sub>24</sub>)

#### IV. CONCLUSION

In the present study the *Bacillus* strain was isolated from contaminated soil showed a wide distributed in this habitats which has ability for biosurfactant production in MSM supplied with crude oil 2%. This strain identified as *Bacillus licheniformis* genetically depending on 16S rRNA gene. Biosurfactant properties were measured by different methods such as emulsification index, drop collapse and oil spreading.

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