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Production of lipopeptide biosurfactant by *Bacillus* Sp. and its application in crude oil biodegradation

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Lipopeptide biosurfactant was produced by hydrocarbon degrading bacterial isolates. These biosurfactant producing isolates isolated from the Egyptian soils. Biosurfactants are valuable microbial amphiphilic molecules (consisting of molecules having a polar water-soluble group attached to a water-insoluble hydrocarbon chain) with effective surface-active and biological properties applicable to several industries and processes. In recent years, natural biosurfactants had attracted attention because of their low toxicity, biodegradability, and ecological acceptability. *Bacilli* species were tested for their abilities to produce biosurfactants by measuring their emulsification activity, emulsification index, oil displacement test, drop collapse and spreading over the blood agar plates. Results revealed that both *Bacilli* isolates were able to produce biosurfactant by using Soy bean and diesel oils as a carbon sources for energy. Bacterial isolates *were* identified by16srRNA as *Bacillus amyloliquefaciens* for MG 8 isolate, and *Bacillus subtilis* for MG13 isolate. The use of crude oil degradation assay, carried out by using individual microbe and\or consortium, the produced biosurfactant enhanced the degradation of the used polycyclic aromatic hydrocarbons (PAHs) oil, the results confirmed the ability of both *Bacilli* strains to degrade oil. Microbial oil recovery experiment was carried out by using artificially contaminated sand with Kerosene, and the results provided the ability of supernatant of both strains to recover kerosene by different ratios but *B. amyloliquefaciens* was more than *B. subtilis*.

Keywords: Lipopeptide biosurfactant, *Bacilli* isolates identification, crude oil biodegradation.

INTRODUCTION

The expansion in environmental carefulness has led to serious consideration of biological surfactants as the most promising alternative to existing product (Henkel et al., 2012) Biosurfactants are considered as one of the high values of microbial products, which have gained considerable interest in recent years that have become an important product of biotechnology for industrial and medical applications (Makkae et al., 2011). Polycyclic aromatic hydrocarbons biosurfactants are extracellular macromolecules produced by bacteria, yeast, and fungi and, in particular, by natural and recombinant bacteria when grown on different carbon sources. Specifically, *Bacillus* species are their well known for its ability to produce lipopeptides type biosurfactants with potential surface-active properties when grown on different carbon substrates (Ghojavand et al., 2008). polycyclic aromatic hydrocarbons that would contribute to chronic hazards including mutagenicity and carcinogenicity (Mandri et al., 2007). Compared to physico-chemical methods, biological methods

have gained increased acceptance in cleaning up the hydrocarbon contaminated sites because these are environmentally friendly, cost-effective, and efficient (Chandankere et al., 2014). However, the general low bioavailability of hydrocarbons, which are highly recalcitrant molecules that can persist in the environment due to their hydrophobicity and low water solubility, is a hindrance to microbial degradation (Xia et al., 2014). Biosurfactants are classified as glycolipids, lipopeptides, phospholipids, fatty acids, neutral lipids, and polymeric or particulate compounds(Khopade et al., 2012). Lipopeptides are among the most commonly isolated and characterized biosurfactants. Among the many classes of biosurfactants, lipopeptides represent a class of microbial surfactants with remarkable surface properties and biological activities, such as surplus crude oil recovery, efficient removal of petroleum hydrocarbons and heavy metals from contaminated soils (Ayde et al., 2014). Release of biosurfactants is one of the strategies used by microorganisms to influence the uptake of PAHs and hydrophobic compounds in general (Johnsen et al., 2005). The lipopeptides produced by numerous *Bacillus* spp. are classified into both families depending on their amino acids sequence: surfactins, iturins and fengycins (Ayde et al., 2014). There are many reports describing the effect of exogenously added microbial biosurfactants in enhancing the bioremediation of crude oil-polluted soils by indigenous microbes (Noparat et al., 2014). PAHs represent the group of compounds in oil that has received the greatest attention due to their carcinogenic and mutagenic properties (Pampanin et al., 2013).

There for the aims of this study are isolate potent hydrocarbon-degrading and biosurfactant-producing bacterial strains and to assess their abilities to produce that may assist biosurfactant produced in assisting the biodegradation of representative PAHs in crude oil.

MATERIALS AND METHODS

Bacilli isolates and source

Two *Bacilli* isolates were isolated from Egyptian soils, cultured and laboratory maintained on nutrient agar medium (Difico, 1984). The isolates were exposed to biosurfactant production tests.

Biosurfactants production

Inoculum preparation: A loop of each tested isolate was inoculated into 50 mL of nutrient broth in a 250-mL conical flask and incubated in a rotary shaker at 28°C, 200 rpm for 16-18 hours until the O.D. became 1.00 at 600nm. The biosurfactant production was performed in 250 mL conical flasks containing 100 mL of the modified Mckeen medium (Phitnaree et al., 2008) inoculated with 10% (v/v) from each isolate. The flasks were incubated in a rotary shaker at 28 °C, 150 rpm for 96 hours.

Biosurfactant activity tests

The emulsification activity of the supernatant of bacterial isolates as well as the pure aqueous solution of biosurfactant was tested by using two methods:

1) Emulsification activity test

Two ml of cell free supernatant were added into a screw-capped tubes containing 2ml of distilled water. The solution was mixed with 1-ml of a substrate (soybean or diesel oils). After a vigorous vortex for 2 min, the tubes were allowed to stand for one hour to separate aqueous and oil phases, before measuring the absorbance at 540 nm (Satpute et al., 2008). Aqueous phase was withdrew carefully and O.D. at 540 nm was measured and compared with those of un-inoculated broth used as negative control. Emulsification activity was defined as the measured optical density at 540nm. Assays were carried out in triplicates.

2) Emulsification index (E_{24} %) test

Two ml of cell free supernatant and 2 ml of diesel oils were added to a screw cap tubes and vortexed at high speed for 2 min. The mixtures were incubated at room temperature for 24-hours. The emulsion index (E_{24} %) was then calculated from the ratio of the height of the emulsion zone to the total height of the oil, emulsion, and aqueous zones (Yeh et al., 2005)**.**

Screening methods for potential biosurfactant-producing bacteria

Biosurfactant-producing isolates were screened using the oil spreading technique developed by (Morikawa et al., 2000), 30 mL of distilled water was taken in a Petri dish to which 1 mL of oil was added to the center. Twenty μL of the culture supernatant from the broth were added on top of the oil layer. The Petri dishes were closely observed for a zone of displacement in the oil, and the diameter of displacement was measured. Drop collapse method, 25 µl of extracted biosurfactant was pipetted as a droplet onto parafilm; the flattening of the droplet and the spreading of the droplet on the parafilm surface was followed over seconds or minutes.

Subsequently, methylene blue (which had no influence on the shape of the droplets) was added to stain the water and allowed to dry and the diameter of the dried droplet was recorded (Tugrul and Cansunar 2005). For the hemolytic activity analysis: the isolates were screened for their ability to decompose blood on blood agar plates (Merck) containing 5% (v/v) sheep blood and incubated at 37°C for 48 h. Hemolytic activity was detected by the presence of a clear zone around bacterial colony (Plaza et al., 2006).

Molecular identification of bacterial isolates

Molecular characterization of the bacterial isolates was done by using 16S rRNA gene sequencing with the help of Solgent Company, Daejeon South Korea. Bacteria were first cultivated on nutrient agar medium at 28°C for 3 days. A small amount of each isolate was individually scraped and suspended in 100µl autoclaved distilled water in 2ml sterile eppendorf tubes and boiled at 100° C for 15 minutes. These non-living bacteria were sent to Sol Gent Company for DNA extraction using SolGent purification bead. Prior to sequencing, the ribosomal rRNA gene was amplified using the polymerase chain reaction (PCR) technique in which two universal bacterial primers 27F (forward) and 1492R (reverse) which were incorporated in the reaction mixture. Primers used for gene amplification have the following composition: 27F (5' AGAGTTTGATCMTGGCTCAG3'), and 1492R (5' TACGG YTACCT GTTA CG ACTT 3') as recommended by (Jiang et al., 2006). The purified PCR products (amplicons) were reconfirmed using a size nucleotide marker (100 base pairs) by electrophoreses on 1% agarose gel. Then these bands were eluted and sequenced with the incorporation of dideoxynucleotides in the reaction mixture. Each sample was sequenced in the sense and antisense directions using 27F and 1492R primers (White et al., 1990). Sequences were further analyzed using Basic Local Alignment Search Tool from the National Center of Biotechnology Information website. Phylogenetic analysis of sequences was done with the help of MegAlign (DNA Star) software version 5.05.

Characterization of the biosurfactant

Extraction of lipopeptide biosurfactant

This extraction technique is a combination of acid precipitation and solvent extraction (Vater et al., 2002). First cells were removed by centrifugation at 13,000 rpm for 15 min at 4°C. The supernatant was acidified by addition of concentrated HCl to pH 2.0 and allow to precipitate at 4°C overnight, then, centrifuge at 13,000 rpm for 15 min at 4°C to obtain pellet. Supernatant was removed to extract the pellet with methanol for 2 h while stirring continuously. Finally, filter methanol to remove remaining material and evaporate to dryness using rotary evaporation.

Thin layer chromatography (TLC)

TLC is a simple method, which can be used to detect the presence of lipopeptides, while preparative TLC can be used to purify small quantities (Symmank et al., 2002). Dissolve a small quantity of crude extract in chloroform and apply 10 µl onto a TLC plate (silica gel 60) and apply at point of origin near the bottom of the plate Once dried, develop plate in solvent system of chloroform: methanol: water (65:25:4). When plate developed completed and removed, it allowed to air-dry in a fume cupboard. Spray the plate evenly with water or a solution of 5% H_2SO_4 and place in an oven at 110°C for 20 min to visualize spots.

High performance liquid chromatography (HPLC-MS)

LC-MS/MS was performed using a Waters Acquity UPLC system coupled to a Waters Synapt G2 mass spectrometer. Prior to analysis the MS was calibrated in both positive and negative ion resolution mode over the mass range of 100–2000 Da typically using sodium formate clusters. In this method, 5 µL of extract was injected onto a Waters C18 BEH 1.7 m (2.1 × 100 mm) column. The mobile phase consisted of A: Water (0.1% formic acid), B: Acetonitrile (0.1% formic acid) run with a 20-min gradient of (30%, v/v Acetonitrile for 5 min; 30–100%, v/v Acetonitrile for 8 min and 100%, v/v Acetonitrile for 2 min; 100–30%, v/v Acetonitrile for 1 min; 30%, v/v Acetonitrile for 4 min) at a flow rate of 0.2 L/min. The eluent flowed directly into the ESI source. The MS instrument was operated in MSE mode collecting both low energy (4V) precursor and high energy (ramp: 20 - 40 V) product spectra. A 2 mg/L solution of leucineen kephalin was used as the lock mass with a constant flow rate of 5 L/min. Mass spectra were attained in both positive and negative ion mode using the following parameters: scan time of 0.5 s, cone voltage of 30 V, capillary voltage of 2.8 kV, source temperature 100 °C, dissolving temperature 300 °C, cone gas 100 L/h, dissolving gas 500 L/h.

Crude oil biodegradation experiment

To study the petroleum oil degradability by the bacteria isolates, shake flask biodegradation experiments were carried out in 100 mL Erlenmeyer flasks containing 50 ml of sterilized MSM medium. An experiment was setup in 100 ml conical flasks, containing 50 ml of minimal salt medium (Mcinerney et al., 1990) medium and 0.5 ml of pre-sterilized petroleum oil to give a final concentration of 2%.Sterilized MSM was inoculated with 10% (v/v) microbial cell suspension and the culture flasks were incubated on an orbital shaker at 150 rpm and 35◦C for 3 weeks. The flasks without inoculation were kept as control.

Gas chromatographic (GC-MS) analysis of residual oil

The contents of the whole flasks were extracted with hexane, dried over anhydrous sodium sulfate, re-dissolved in hexane, and analyzed using an Aglient 6890 gas chromatograph equipped with an Algient mass spectrometric detector, with a direct capillary interface and fused silica capillary column PAS-5 ms (30m x 0.32mm x 0.25µm film thickness).Samples were injected under the following conditions:

Helium was used as carrier gas at approximately 1.0 ml/min, pulsed splitless mode. The solvent delay was 3min and the injection size was 1.0µl. The mass spectrometric detector was reported in electron impact ionization mode with an ionizing energy of 70e.v. scanning from m/z 50 to 500. The ion source temperature was 23°C. The electron multiplier voltage was maintained 1650v above auto tune. The instrument was manually tuned using perfluorotributyl amine. The GC temperature program was started at 60°C (2min) then elevated to 300°C at a rate of 5°C/min, the injector temperature was set at 280°C, respectively. Wiley and Wiley Nist spectral data base was used in the identification of the separates peaks.

Application of the biosurfactants in recovery of spiked used keroseen from sand

The application of the product in microbiologically enhanced oil recovery was evaluated using sand pack technique described by (Abu Ruwaida et al.,1991). A glass column (40×2.5cm) was packed with 100 g of acid-washed sand. The column was then saturated with 100 ml of kerosene. The activity of the isolated surfactant in oil recovery was estimated by pouring 100 ml of aqueous solution of biosurfactant onto the column. The per cent of oil released was evaluated.

RESULTS

Emulsification activity and emulsification activity

Two bacterial isolates obtained, two were found to produce biosurfactant producer when exposed to emulsification activity and emulsification index ($E_{24\%}$) tests. Results indicated the emulsification activity and emulsification index for the bacterial isolates under the effect of different carbon sources. Results revealed that the both tested isolates were able to produce biosurfactants due to their emulsification activity and emulsification index. The highest emulsification activity of 1.980 was due to the isolate MG 8 followed by the isolate MG 13 (1.850) when they grow on soybean oil as carbon source. While, they recorded low emulsification activity when it cultured on a medium containing diesel oil as sole carbon source, however emulsification index ($E_{24\%}$) of isolate MG 13 was (76%) higher than isolate MG8 (74 %) in using diesel oil as carbon source.

Screening methods for potential biosurfactant-producing bacteria

Biosurfactant producer isolates when tested by drop-collapse, oil spread and hemolytic activity to improve their ability for biosurfactant production. Bacterial isolate MG8 and MG13 were found to possess maximum biosurfactant production ability. To determine the concentration of the biosurfactant produced by the bacterial isolates, the Petri dishes were closely observed for a zone of displacement in the oil, and the diameter of displacement was measured in cm. Both samples had significantly displaced the oil layer and started to spread in the water. Figuer (1) showed a zone of displacement for *Bacillus* MG13 was displacement diameter 7.5 cm against displacement diameter 6 cm for *Bacillus* MG8.

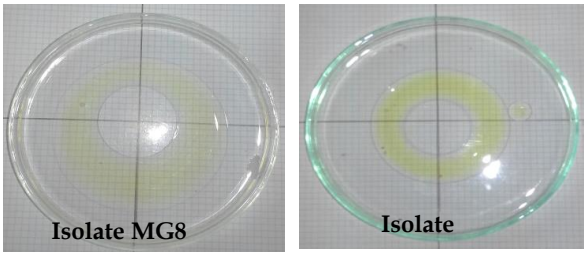

Figure.(1): Oil displacement test of the biosurfactant produced by bcterial isolates.

The drop collapse method depends on the principle that a drop of liquid containing a biosurfactant collapses and spreads over the oily surface. There is a direct relationship between the diameter of the sample and concentration of the biosurfactant and in contrast, the drop lacking biosurfactant remains beaded due to the hydrophobicity of the oil surface that cause aggregation of droplets (Tugrul and Cansunar, 2005). In drop collapse assay, no activity was detected for distilled water as predicted. The biosurfactant droplets do result in a collapsed droplet (Fig.2), indicating their effects on reduction of surface tension, in this study the isolate MG13 showed the biggest drop collapse than the other tested isolate.

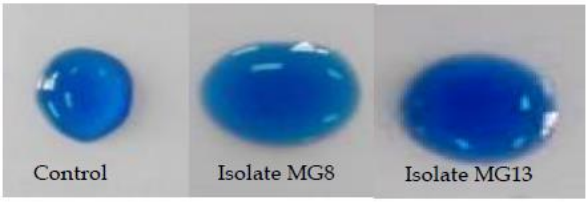

Figure. (2). Drop collapse assay. Collapsed droplets.

Accordingly, in the present study, *Bacilli* strains displayed excellent hemolytic activity (Fig. 3), (Rodrigues et al., 2006) scored the hemolytic activity. The hemolytic strains to lower the surface tension. Hemolytic activity appears to be a good screening criterion in the search for biosurfactants produced bacteria (Carrillo et al.,1996). Lysis of blood agar has been recommended as a method to screen the biosurfactant activity. This method is useful in predicting the promising strains regarding biosurfactant production. Since, in most cases, the degree of lysis of red blood cells is directly proportional to the concentration of biosurfactant production (Youssef et al., 2004). Hemolysis was included in this study, since it is widely used in screening the biosurfactant production and in some cases, it is the sole method used (Yonebayashi et al., 2000).

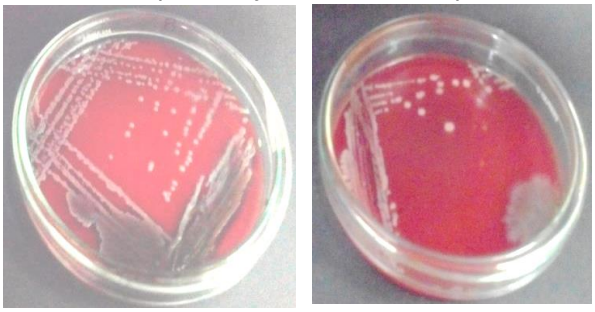

Figure. (3). Hemolytic activity of bacterial isolates.

Identification of bacterial isolates

The most powerful new method for screening of microbial diversity for complex environmental samples is based on the cloning and sequencing of 16S rRNA gene (Balcázar et al., 2007). The molecular approach has been used for bacterial phylogeny and is of great importance for definition and identification of species (Mignard and Flandrois, 2006)). The positive result obtained from the confirmatory assays confirmed the biosurfactant production by both isolates. The partial 16S rDNA gene sequences retrieved from the both isolates were grouped using a sequence match program to show their corresponding phylogenetic positions. Although the 2 clones could be affiliated to the genus *Bacillus*. They exhibited, unexpectedly, similarity to several *Bacillus* species. That based on the 16S rRNA gene sequences and using the Gene Bank BLAST tool, (http://www.ncbi.nlm.nih.gov/BLAST) (Tamura et al., 2013). Results in this study demonstrated that the 16S rRNA sequence of the isolate MG8 was identified as *Bacillus amyloliquefaciens,* it was 100% identical to the type strain KR149334.1 *Bacillus amyloliquefaciens* of South China (Fig. 4). While, the isolate MG13 identified as *Bacillus subtilis,* it was 100% identical to the type strain KP196795 *Bacillus subtilis* of South Africa (Fig. 5).

Identification of biosurfactant

The most common technique used for the extraction of lipopeptides is acid precipitation with the formation of the precipitate overnight (Alvarez et al., 2012). The direct extraction from cell-free broth was beneficial and higher lipopeptide concentrations which were in general reached when a successive extraction was performed, this was also confirmed by (Yuan et al., 2012). Thin-layer chromatography characterization revealed a pink spot with R_f value of 0.62 when sprayed with ninhydrin reagent, indicating the presence of amino acids. No spot was observed when sprayed with p-anisaldehyde confirming the absence of sugar moiety. The above result confirmed the lipopeptidal nature of the biosurfactant. Similar results were reported by (Sriram et al., 2011). Results of LC_MS analysis indicated that both *Bacilli* strains can produce lipopeptide compounds. In methanolic extract of the liquid cultures supernatant supplemented with oil.

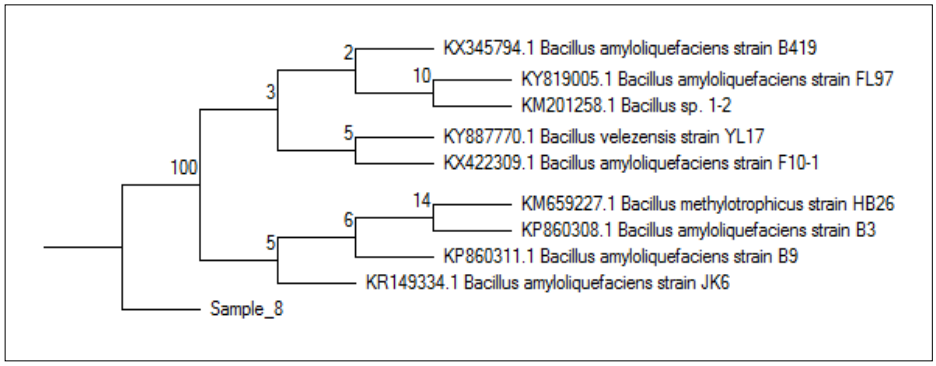

Fig. (4): Phylogenetic dendrogram based up on 16S rRNA sequence (1500bp) of *Bacillus amylolicoficiens* MG8 strain compared with the sequence of standard strains obtained from the Gene Bank database .

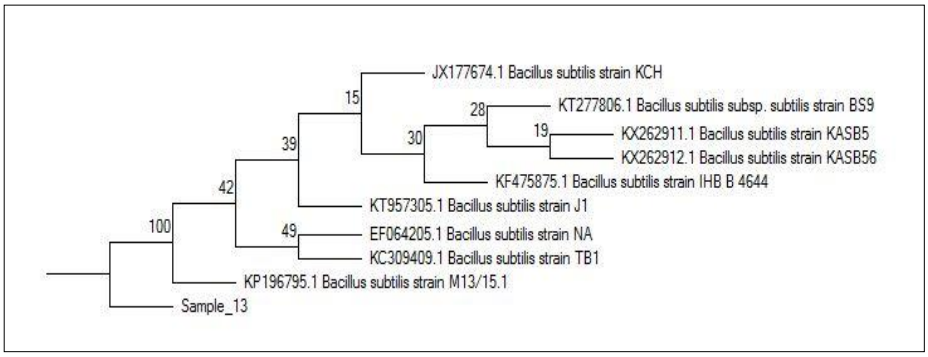

Fig. (5): Phylogenetic dendrogram based up on 16S rRNA sequence (1500bp) of *Bacillus subtilis* MG13 strain compared with the sequence of standard strains obtained from the Gene Bank database.

Bacillus amyloliquefaciens strain LC–MS–MS spectral analysis of the purified fraction, a series of protonated ions were observed for the fraction of the purified sample eluting at 4.89 min, 5.41 min and 5.56 min , compounds fell into masses of 1022, 1031 and 1045 m/z that were identified as surfactin lipopeptide (Chen et al., 2008) also at 6.01 min and 6.27 min at 1059 and 1079 m/z were identified as iturin lipopeptide (Pyoung et al., 2010), Fig. (6).

While compounds were found in the methanolic extract of *Bacillus subtilis* strain, the purified fraction, a series of protonated ions were observed for the fraction of the purified sample eluted at 5.41 min 5.57min and 6.03 min respectively, the masses of 1069 -1079 m/z were identified as iturin lipopeptides (Stein 2008), Figure (7). Some examples of lipopeptide biosurfactants that are produced by members of the *Bacillus subtilis* group consistes of: lipopeptides of surfactin (Youssef et al., 2007) bacillomycin and plipastatin (Roongsawang et al., 2002), fengycin, iturin from strains of *B. subtilis* subsp. *subtilis*; lipopeptides of BL86, lichenysin A and lichenysin C from strains of *B. licheniformis* and lipopeptide of bamylocin A from the *B. amyloliquefaciens* strain (Lee et al., 2007). The co-production of two lipopeptides or more by the same bacterial strain could be advantageous, since a synergistic effect may occur. The simultaneous production of surfactin and iturin A substances has been only reported for *B. subtilis* (Ahimou et al., 2000), and possibly for *B. amyloliquefaciens* (Souto et al., 2004).

Crude oil biodegradation experiment

One of the major environmental problems today is hydrocarbon contamination resulting from the activities related to the petrochemical industry. Bioremediation functions basically on biodegradation, which may refer to complete mineralization of organic contaminants into carbon dioxide, water, inorganic compounds, and cell protein or transformation of complex organic contaminants to other simpler organic compounds by biological agents like microorganisms.

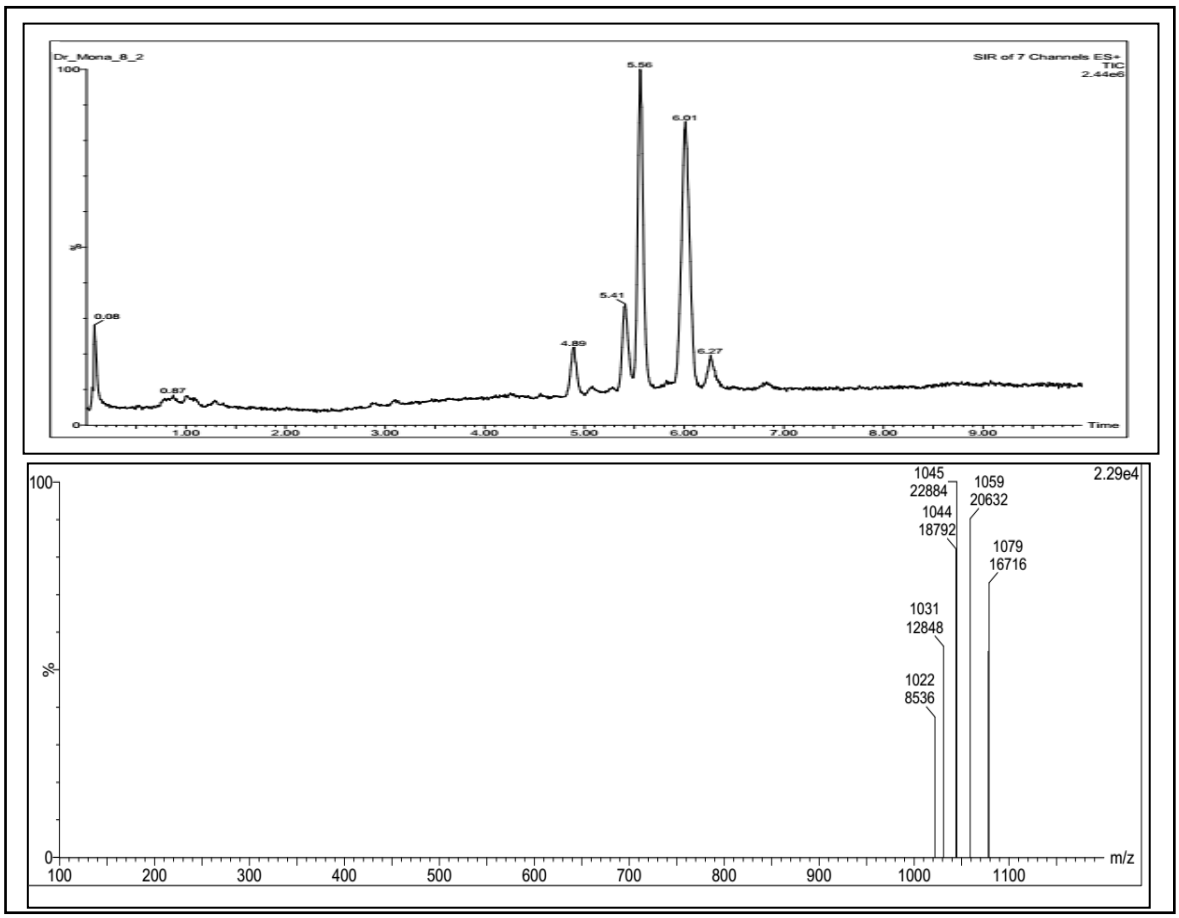

Fig. (6): LC-ESI/MS analysis of the methanolic extract from a *Bacillus amyloliquefaciens strain*. a) Chromatograms of lipopeptides, b) ESI-MS analysis of lipopeptides produced

Many indigenous microorganisms in water and soil are capable of degrading hydrocarbon contaminants (Das and Chandran 2011). The results of crude oil biodegradation experiment showed that the ability of both *Bacilli* strains to degrade crude oil either each in individually or in mixed culture using MSM medium and crude oil as a carbon source. Low solubility of many hydrocarbons reduces the bioavailability to microorganisms and limits the biodegradation process. There is an assumption that biosurfactants can be used to enhance the biological accessibility to such substances. In fact, biosurfactants increase the solubility of the hydrocarbon materials and provide an environment for bacteria growth (Helmy et al., 2010). The reason for that some microorganisms produce biosurfactant has not been found clearly and is the debating topic of many scientists. However, biosurfactants like other natural compounds such as antibiotics were considered as means of survival (Abouseoud et al., 2008). In this study the results of crude oil biodegradation experiment showed the ability of both *Bacilli* strains to degrade crude oil either individual or mixed culture using MSM medium and crude oil as a carbon source. To find out the degradation of the hydrocarbons GC/MS analysis was carried out. GC/MS study revealed the presence of certain hydrocarbon compounds, which might be produced due to degraded by bacterial degradation of crude oil. Results indicated the disappearance ratio of aliphatic compounds. Compound heptadecane (C_{17}) is completely disappeared in all treatments. Compounds C_{11}, C_{12}, C_{13}, C_{14}, C_{15}, C_{16} compounds are completely disappeared in *B. amyloliquefaciens* treatment, also compounds C_{29} and C_{30} are disappeared completely in *B. subtilis* individual treatment, while these compounds also disappeared by different ratios in other individual and mixed treatments.

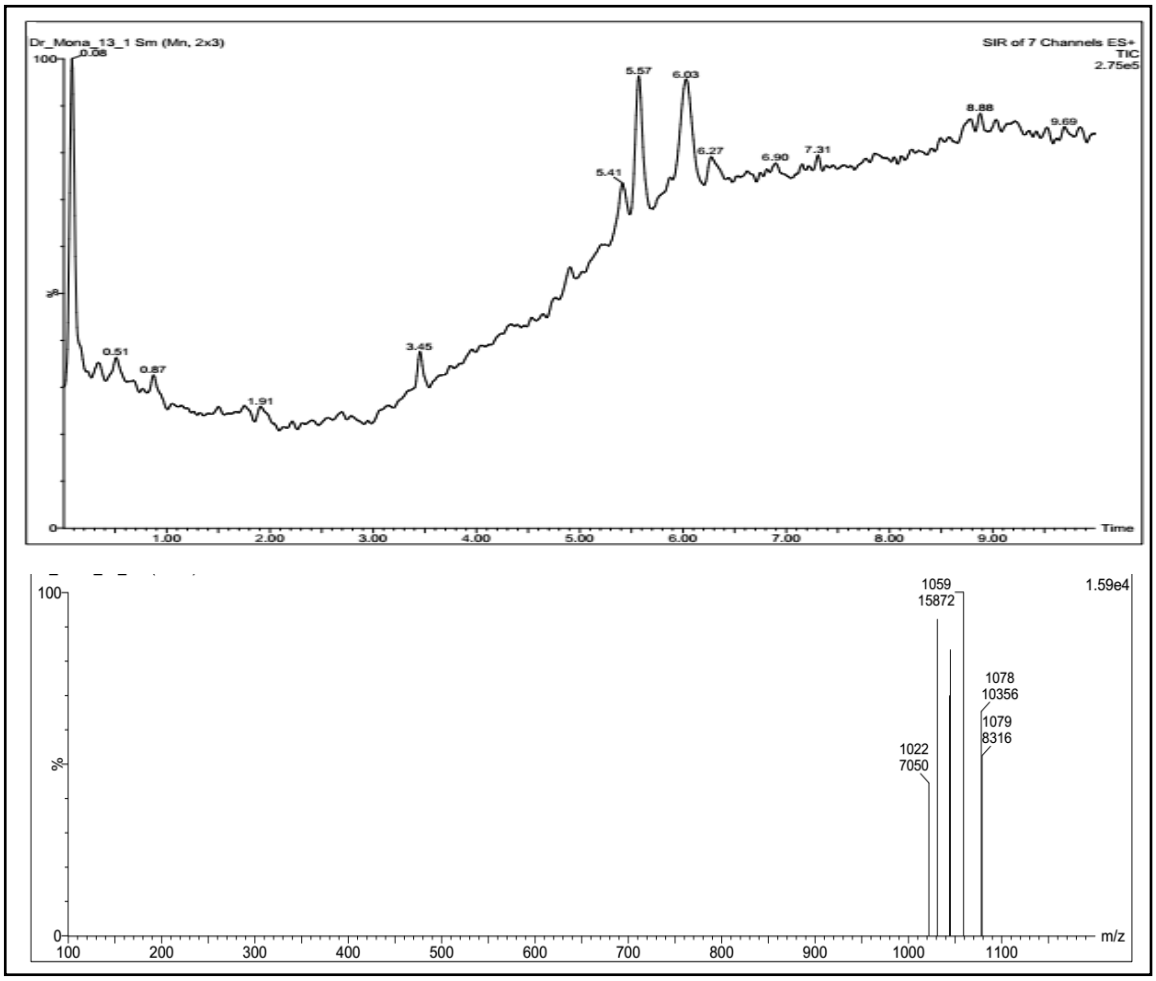

Fig. (7): LC-ESI/MS analysis of the methanolic extract from *Bacills subtilis* strain. a) Chromatograms of lipopeptides, b) ESI-MS analysis of lipopeptides produced iturin group.

Other compounds from C_{18} to C_{30} are disappeared by different ratios in individual and mixed treatments. The strain showed a much more enhanced biodegradation of different aliphatic compounds was *B. amyloliquefaciens*, then mixed treatments and *B. subtilis.*

Microorganisms use long-chain n-alkanes as carbon and energy source and higher rate of hydrocarbons utilization is also reported by bacterial consortium from oil exposed environments (Geetha et al., 2013). *n*-Alkanes are the main constituents of mineral oil contaminations (Hinchee et al.,1994). Long-chain *n*-alkanes (C_{10}- C_{24}) are degraded most rapidly Short-chain alkanes (less than C_9) are toxic to many microorganisms, but they evaporate rapidly from petroleum contaminated sites. Oxidation of alkanes is classified as being terminal or ditermina Aliphatic hydrocarbons become less water soluble with increasing chain length. Hydrocarbons with a chain length of C_{12} and above are virtually water insoluble. Two mechanisms are involved in the uptake of these lipophilic substrates: the attachment of microbial cells at oil droplets and the production of biosurfactants (Hommel 1990). The uptake mechanism linked to attachment of the cells is still unknown, whereas the effect of biosurfactants might have an important role.

The results in Fig (9) indicated that the difference between aromatic compounds in control sample and treated sample, some of them had hidden such as: 2,6 di-methylnaphthalene, 1,3dimethylnaphthalene,1, and 1,4,6 Tri-methylnaphthalene and other degraded by different ratios. Crude oil is the major source of energy in the world. The technologies, which improve the recovery efficiency and reduce environmental impacts, will be promising alternative to traditional oil recovery technologies.

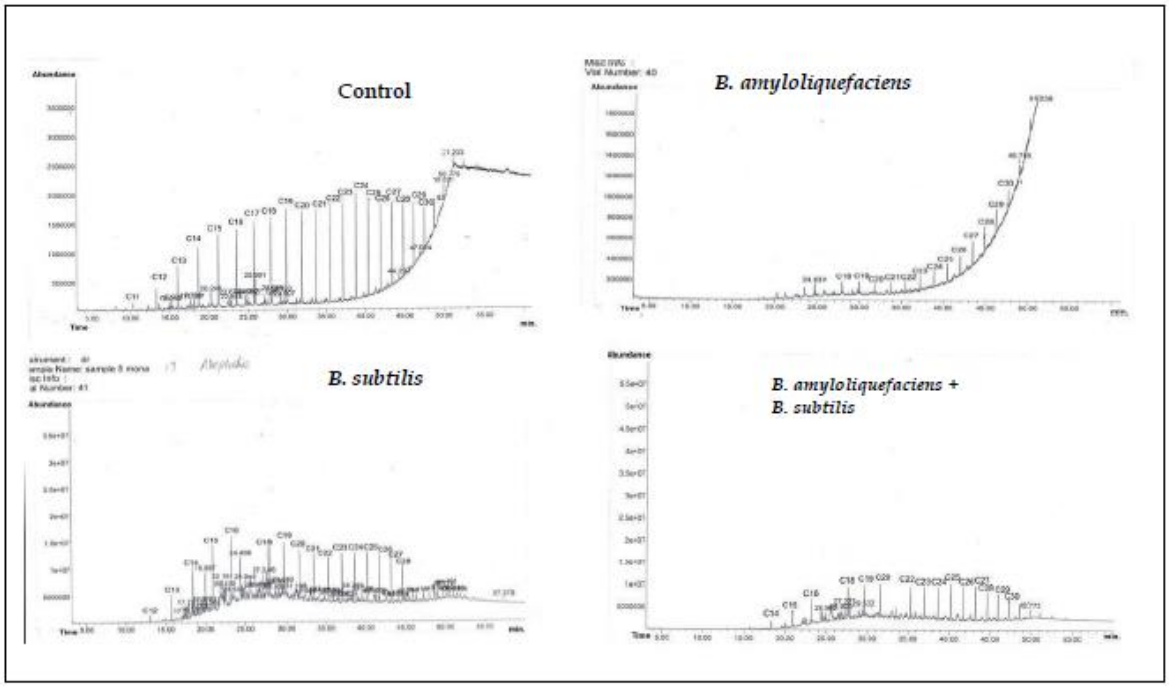

Fig. (8): GC chromatograms of aliphatic compounds residues of petroleum oil in liquid MSM medium

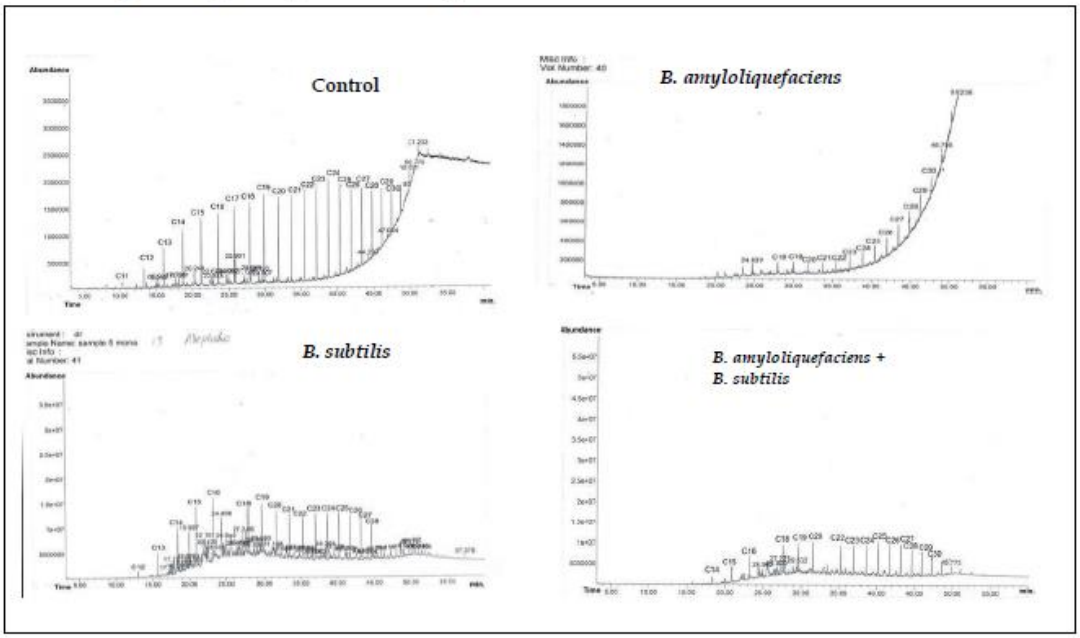

Fig. (9): GC chromatograms of aromatic compounds residues of petroleum oil in liquid MSM medium.

Biotechnology is considered as a new field recently introduced to the oil industry for various applications (Vazquez-Duhalt et al., 2008) Petroleum is a complex mixture of hydrocarbons and other organic compounds (Hamme et al.,2003). Ghazali et al., (2004) reported that different *Bacillus* spp. isolates played critical role as major component in microbial consortium to biodegrade environmental contaminants like crude oil, benzene, toluene, ethylbenzene, o-xylene and octanol. Therefore, hydrocarbon degrading bacteria with the ability to produce biosurfactant is widely recommended for fast degradation of crude oil (Kumar et al., 2006). A Reduction in the toxicity level of crude oil by using biosurfactant producing bacteria may also support the growth of other hydrocarbon degrading bacterial strains to augment the process of oil degradation a part from biosurfactants (Iwabuchi et al., 2002).

Microbial enhanced oil recovery (MEOR)

The highest percentage for MEOR was achieved by followed by the *B*. *subtlis* (58%) but for *Bacillus amyloliquefaciens* it was 50%. Microbial Enhanced Oil Recovery (MEOR) is a tertiary recovery technique, which involves the use of microbes and their metabolic products including biosurfactants, biopolymers, acids, solvents, gases and enzymes to increase the recovery of crude oil from depleted and marginal reservoirs (Sen, 2008). The alternative is a biologically based EOR process, also known as MEOR. Biosurfactants MEOR represent a promising method to recover a substantial proportion of the residual oil from marginal oil fields. Biosurfactants can be implemented in two ways: they can be produced either *ex situ* to be injected into the reservoir or *in situ* by indigenous or injected (Banat et al., 2010). The first approach involves the production of biosurfactants above ground by fermentation and therefore requires expensive equipment, including bioreactor and purification (Pereira et al., 2013)

CONCLUSION

Recently, increasing global environmental awareness has led to much more interest in biosurfactants compared to their chemical counterparts. It is due to unique properties of biosurfactants including biodegradability, low toxicity, mild production conditions, and environmental acceptability, lower critical micelle concentration, higher selectivity as well as better activity at extreme temperature, pH and salinity. *Bacillus* spp. can used in producing biosurfactant by using inexpensive carbon and nitrogen sources, to be used in bioremediation of contaminated sites by oil or heavy metals. Generally, biosurfactants are a very diverse group of biomolecules ranging from the low molecular weight to high molecular weight compounds such as extracellular polymeric substance or lipopolysaccharides. Not surprisingly, that the physicochemical properties of these molecules also vary greatly, which account for the wide range of results achieved due to using different combinations of biosurfactants and metal ions. One of the major advantages that regularly bandied about, due to the use of biosurfactants is their green credentials that lack of toxicity, biodegradability, and relative stability under a wide range of physicochemical environments. However, it must be considered that biosurfactants have significant biocidal and biostatic effects on certain groups of microorganisms. Therefore, the unrestricted addition of biosurfactants to natural environments may have unforeseen consequences.

CONFLICT OF INTEREST

The present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors contributed equally in all parts of this study

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