

## Effect of Phytogetic Biosurfactant on the Microbial Community and on the Bioremediation of Highly Oil-Polluted Desert Soil

Ali Diab\* and Maram Sandouka

Faculty of Biotechnology, October University for Modern Sciences and Arts (MSA), Egypt.

[Alidiab.maramsan@live.com](mailto:Alidiab.maramsan@live.com)

**Abstract:** The effect of a phytogetic surfactant on the microbial community and on the biodegradation of crude oil in a highly polluted desert soil (8%) were investigated. The addition of this biosurfactant increased total heterotrophic bacteria (THB) to reach the range of 4.3 – 20.3 CFUx10<sup>8</sup>/g dried soil, with increased factor of 7.18 – 10.38. Oil-degraders were in the range of 2.48-30.2 CFUx10<sup>7</sup> in presence of the biosurfactant, this in a range of 27.2-143.8 increased factor. Higher percentages of 5.7-17.6% of the oil degraders were recorded in presence of biosurfactant. In presence of biosurfactant the biodegradation rate of the oil increased to reach 23.8-30.0% after 90 days, this is in contrast to 3.8-10% in the absence of this biosurfactant. The maximum biodegradation of the saturates and the aromatic fraction were 92.8% and 41.8% respectively in presence of the biosurfactant. Based on these results it is advisable to use this cost-effective phytogetic surfactant for cleaning the highly oil-polluted sites especially in the absence of NP fertilizer.

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### 1. Introduction

Crude oil and some of its derivatives contain significant amounts of polycyclic aromatic hydrocarbons (PAHs). Soil contaminated with such compounds are of concern, since some of the petroleum hydrocarbon especially PAHs have a variety of mutagenic and carcinogenic effects on microorganisms, plants and animals, and are classified as compounds with hazardous effects on human health (Kalf *et al.*, 1997).

Bioremediation has been accepted as a cost-effective and important method for the treatment of oil-contaminated sites. Living organisms primarily bacteria are able to degrade the oil hydrocarbons converting them to non-toxic and harmless compounds.

Most hydrocarbons are strongly adsorbed to soil particles and their removal depends on their bioavailability. Zhang *et al* (2005) reported that one of the important factors in the biodegradation rates is its solubility. This related to the bioavailability of the contaminant to the microbial attack. Zhang *et al* (2010) reported that the efficiency of bioremediation is limited when the residual hydrocarbons especially in aged contaminated soil are strongly adsorbed by soil particles which led to lower biodegradation.

A promising approach for the increasing of the biodegradation rates of hydrocarbon compounds with low water solubility is the addition of biosurfactant (Xu and Obbard, 2004; Zhang *et al.*, 2005, 2010; Calvo *et al* 2008; Chang *et al.*, 2008; Yin *et al.*, 2009). Biosurfactants are important biotechnological

products with a wide range of applications in many industries (Kosaric, 2001). Biosurfactants are surface active compounds produced by microorganisms. These compounds have the property of enhancing oil recovery and bioremediation of hydrocarbon-polluted soils (Salihu, *et al.*, 2005).

Biosurfactants have other biological functions. They are important in commercial application in food, pharmaceuticals and biological industries such as biocontrol agents in agriculture applications, in health and beauty products for cosmetic industries (Banincasa, *et al.*, 2004; Tugrul and Consumar, 2005; Mukherjee, *et al.*, 2009; Nayak, *et al.*, 2009). Biosurfactants when compared to chemical surfactants have the following properties:

- Possibility of cost-effective production.
- Their biodegradability.
- They are active under extreme conditions of pH, temperature and salinity.
- They have low toxicity and low irritation.
- They have the ability to increase the bioavailability of poorly soluble hydrocarbons.

Oleszczuk *et al* (2007) reported that the root exudates of the plants they studied simultaneously affect the increase of the bioavailability and mineralization of the pollutants. Root exudates have the potential to selectively encourage the growth of pollutant-degraders in the rhizosphere. The positive influence of the root exudates could also be related to a phenomenon similar to that observed in the case of the influence of biosurfactants on pollutants (Bonat, 1995, Oleszczuk, 2003). Some plants are also

capable of producing this type of compounds (the so called phytogetic surfactant. **Fava and Gioia (2001)** studied the effect of the phytogetic surfactant soya lecithin (SL) on the aerobic biodegradation of polychlorinated biphenyls (PCBs), they found that in the presence of SL higher PCBs biodegradation and higher biphenyl and chlor-benzoic acid degrading bacteria were observed. Similar experiments have also been carried out by **Soeder et al (1996)**. The results presented by these authors suggested that some phytogetic surfactants might improve the bioavailability of PAHs in the rhizosphere.

**Cohen et al (2004)** used the entire plant of the water fern *Azola* and seed meal of *Brassica nopus* as promising amendments for the bioremediation of contaminated soil. Seed meal of *Brassica* stimulated >100 fold increase in population of resident *Streptomyces* spp. and suppressed fungal infection of roots subsequently cultivated in the amended soil.

For increasing the production yield and expanding the commercial use of biosurfactant, it is essential to use low cost bioprocess and cost effective raw materials (**Wei et al, 2005; Pornsunthorntawe, et al, 2009**).

The objective of the present study was to obtain a phytogetic surfactant from cost effective raw materials such or sunflower seed meal, and to apply this biosurfactant in the bioremediation of a highly oil-polluted desert soil.

## 2. Materials and Methods

### 1. Isolation and Characterization of the Biosurfactant:

The biosurfactant found in twenty grams of sunflower seed meal were extracted two times, each with 100 ml chloroform using the shaking method described by **Chen et al (1996)**. The extracts were combined in a preweighed dish and the solvent was evaporated leaving a viscous yellow colored biosurfactant product. This crude biosurfactant was used in the biodegradation process.

### 2. Collection of Soil Samples:

Rhizosphere soil samples were collected from soil adjacent to the root of *Cynodon* plant growing in clean area in the garden of Modern Sciences and Arts (MSA) University, from a depth of 15 cm.

Non-rhizosphere soil samples were also collected from non-vegetated area in the same location from depth of 15 cm.

At least 10 samples of each of the rhizosphere soil (RH) and non-rhizosphere soil (S) were collected from different spots in the same area. Each group of soil were mixed thoroughly to form one composite sample. The soil samples were air-dried and sieved through 2mm diameter opening. The air-dried soil

samples were mixed thoroughly with crude petroleum oil and left in the laboratory for 60 days for wheathering, giving a concentration of 8% crude oil (w/w soil).

### Soil Treatments:

Soil microcosm test for each of rhizosphere soil (RH) and non-rhizosphere soil (S) was designed to include 4 treatments in duplicates. Each consisting of 500 ml beaker contains 100 g of the polluted soil and treated as in the following table:

Treatment	Amendments		
	biosurfacatant	NH <sub>4</sub> NO <sub>3</sub>	K <sub>2</sub> HPO <sub>4</sub>
1	+	+	+
2	-	+	+
3	+	-	-
4	-	-	-

The fertilizer used was NH<sub>4</sub>NO<sub>3</sub> (100mg/100g soil) and K<sub>2</sub>HPO<sub>4</sub> (50 mg/100 g soil) Biosurfactant was added in a concentration of 150 mg/100 g soil.

A small glass rod was introduced to each beaker for tilling the soil. The moisture content of each treatment was adjusted at 50% of the water holding capacity. All of the treatments were covered by thin aluminum foil to reduce the evaporation of water, and were incubated at 30°C. The loss of water due to evaporation in each treatment was determined at the beginning of the experiment and after 2-3 days, the amount of water lost was added.

From each of the above treatments samples were taken at the beginning of the experiment and after 90 days incubation period for microbiological analysis and for extraction and determination of the loss of oil as a result of biodegradation.

### Microbiological Analysis:

Total heterotrophic bacteria were counted using the usual dilution plate method. The counting medium was nutrient agar (Oxoid) supplemented with 0.4% (w/w) soluble starch. The agar plates were incubated at 30°C for 4-5 days after which the colonies appeared were counted and expressed as colony forming units (CFU/g air-dried soil).

For counting oil-degrading microorganisms, the three tube Most Probable Number (MPN) method was used as described by **Chaineau et al (1996)**.

### Extraction and Determination of the Residual Oil:

At the beginning of the experiment (O-time) and at the end of 90 days incubation period, 3g of the air-dried soil was mixed with 3g of anhydrous sodium sulfate to remove moisture, and then the residual oil in the soil sample was extracted by chloroform using the shaking method described by **Chen et al, (1996)**. The extract was pooled and evaporated in a

preweighed dish, and the amount of the residual oil was determined.

#### Determination of the Different Fractions of the Residual Oil:

A known weighted of the recovered residual oil was suspended in n-hexane and filtered through a tarred filter paper to remove the non-soluble hexane fraction i.e. the asphaltene fraction. The hexane soluble part was fractionated into saturates and aromatics by liquid-solid chromatography on 100-200 mesh activated silica gel column (15x1.5cm) by successive elution with 60 ml aliquotes of n-hexane and benzene (Chaineau *et al* 1995).

The solvents were evaporated, and each fraction was estimated gravimetrically.

#### 4. Results and Discussion

Results obtained in the presence and in the absence of the biosurfactant and/or NP fertilizer are found in Tables (1-4) and illustrated in Figs. (1 and 2). The results after 90 days incubation period can be summarized as follows:

- Addition of the phytogetic biosurfactant to the different treatments dramatically increased the counts of total heterotrophic bacteria and oil-degrading microorganisms (Table 1-2).
- Total bacterial counts (CFUx10<sup>7</sup>/g soil) in presence of this biosurfactant (Table 1) were in the range of 43.2 to 203.2. This is in contrast to 5.43-23.9 in the absence of the biosurfactant, i.e.

with increased factor of 7.18-10.38. From these results it can be suggested that in addition to the surface active property of the biosurfactant, bacteria in the present work may be able to utilize the biosurfactant and to protect themselves from the toxicity of high oil concentration (8% w/w).

- Oil-degraders (CFUx10<sup>7</sup>/g soil) after the addition of the biosurfactant (Table 2) also increased and were in the range of 2.48-30.20, this is in contrast to 0.09-0.30 in the absence of this biosurfactant, i.e. with increased factor of 27.2-143.8. As for the percentages of the oil degraders (relative to total heterotrophic bacteria), it can be seen (Table 2, Fig. 1) that higher percentages of 5.17-17.6% were recorded in presence of the biosurfactant as compared to its absence (0.09-2.0%). Dibble and Bartha (1979) reported increase in CO<sub>2</sub> evolution over the range of 1.25-5% (w/w) oil and no increase was observed at a level of 10%. Bartha (1986) found that the maximum petroleum hydrocarbons biodegradation were maintained if the concentration of hydrocarbons are 5% (w/w) or slightly higher, at 10% concentration, biodegradation activity was inhibited. Diab and Sandouka (*unpublished data*) found that increasing oil pollutant from 4.3% to 7.8% (w/w) resulted in the decrease of the counts of CFU/g soil of total heterotrophic bacteria and oil-degrading microorganisms, and biodegradation activity was reduced.

**Table 1:** Counts of total heterotrophic bacteria (CFUx10<sup>7</sup>/g soil) in the different treatments of the rhizosphere and non-rhizosphere soil in presence and in absence of the biosurfactant. Increased factor (F) i.e. data in presence of the biosurfactant relative to that in the absence of the biosurfactant is also given. S: polluted soil only, S(+)= polluted soil amended with NP fertilizer, RH=polluted rhizosphere soil only, and RH(+)= polluted rhizosphere soil amended with NP fertilizer. ±= Standard deviation (n=3). Values within the same column followed by the same letter are non significantly differ (P=0.05).

Treatments	CFUx10 <sup>7</sup> /g air-dried soil			"F"
	O-time	90 days		
		Biosurfactant added	No biosurfactant	
S	0.57±0.03 <sup>a</sup>	43.2±1.4	5.43±0.20	7.96
S(+)	0.55±0.05 <sup>a</sup>	87.6±4.7	12.2±0.24	7.18
RH	2.94±0.04 <sup>b</sup>	155.7±18.0	15.0±1.40	10.38
RH(+)	2.91±0.05 <sup>b</sup>	203.2±4.8	23.0±0.92	8.50

**Table 2:** Counts of oil-degrading bacteria (CFU/g air-dried soil) in the different treatment of rhizosphere and non-rhizosphere soil, in presence and in the absence of the biosurfactant. Increased factor (F) is also given. ±= Standard deviation (n=3). Values within the same column followed by the same letter are non significantly differ (P=0.05).

Treatments	Biosurfactant added		No biosurfactant		"F"
	CFUx10 <sup>7</sup> /g soil	(%) oil-degraders	CFUx10 <sup>7</sup> /g soil	(%) oil-degraders	
S	2.48±0.40	5.7	0.09±0.017	1.7	27.6
S(+)	7.30±0.70	8.3	0.15±0.014	1.2	48.6
RH	27.4±1.30 <sup>(a)</sup>	17.6	0.30±0.04	2.0	91.6
RH(+)	30.20±3.30 <sup>(a)</sup>	14.9	0.21±0.04	0.9	143.8

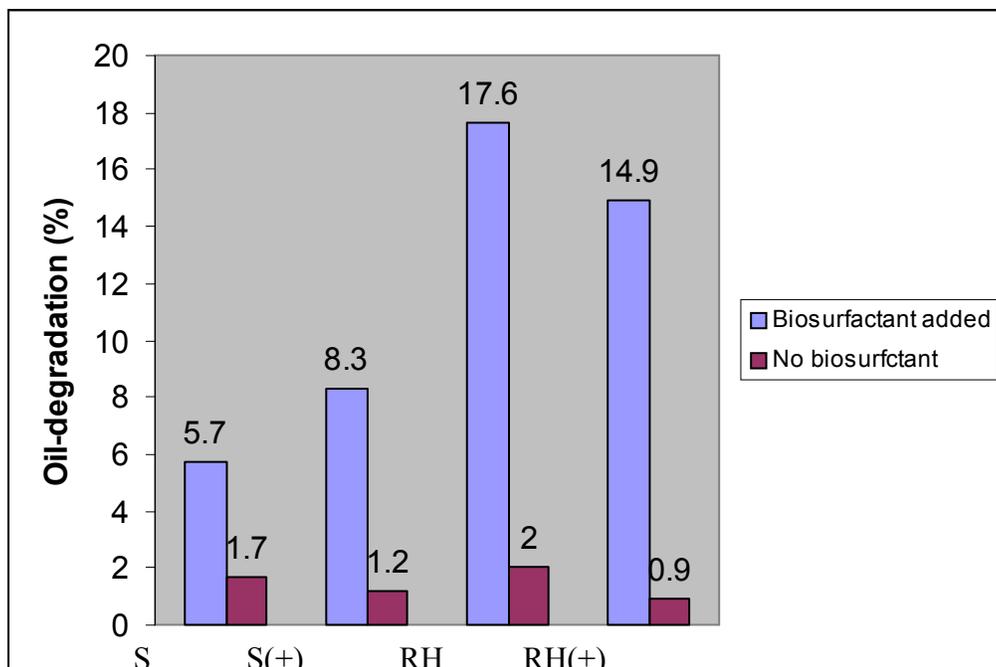


Fig. 1. Percentage of oil-degraders in the different treatments in presence and in absence of biosurfactant.

To overcome the adverse effects of the high oil concentrations, it can be suggested (based on our present work) that toxic effects of high concentrations of oil may be neutralized by the application of a suitable phyto-genic biosurfactant.

The present results show that in the presence of the phyto-genic biosurfactant the rhizosphere soil of *Cynodon sp* plant stimulated higher counts of total bacteria and oil-degraders as compared to the non-rhizosphere soil. It appears from these results that a beneficial combined effects of the biosurfactant and the rhizosphere nutrients were able to neutralize the toxic effect of the oil pollutant. The positive rhizosphere effect could also be related to a phenomenon similar to that observed in the case of the influence of biosurfactant on pollutant (Banat *et al*, 1995; Oleszczuk, 2003). Some plants are capable of producing this type of compounds, the so called

phyto-genic surfactant (Soeder, *et al* 1996; Fava and Gioia, 2001).

Kosaric (2001) reported that biosurfactants are important biotechnological products which are characterized by lowering surface and interfacial tensions, penetrating action, spreading and enhancing microbial growth. Priya and Usharani (2009) indicated that biosurfactants cause emulsification of the hydrocarbons and facilitate the utilization of such compounds by microorganisms.

Results of the capacity of the natural microbial population of the studied treatments to degrade total petroleum hydrocarbons (TPHCs) in presence and in the absence of the phyto-genic surfactant and/or NP fertilizer are found in Table (3) and illustrated in Fig. (2). In the absence of the biosurfactant the biodegradation (% loss) of TPHs were 3.8-10%.

**Table 3:** Biodegradation (loss %) of total petroleum hydrocarbons (TPHCs) as affected by the presence or absence of biosurfactant. Increased factor (F) is given.  $\pm$  Standard deviation (n=3). Values within the same column followed by the same letter are non significantly differ (P=0.05).

Treatments	Biodegradation (loss %)		“F”
	Biosurfactant added	No biosurfactant	
S	23.8 $\pm$ 2.5 <sup>(a)</sup>	3.8 $\pm$ 0.50	6.2
S(+)	26.5 $\pm$ 1.7 <sup>(a)</sup>	8.4 $\pm$ 0.84	3.1
RH	30.0 $\pm$ 2.0	10.1 $\pm$ 1.00	3.0
RH(+)	24.7 $\pm$ 3.1 <sup>(a)</sup>	7.4 $\pm$ 0.92	3.3

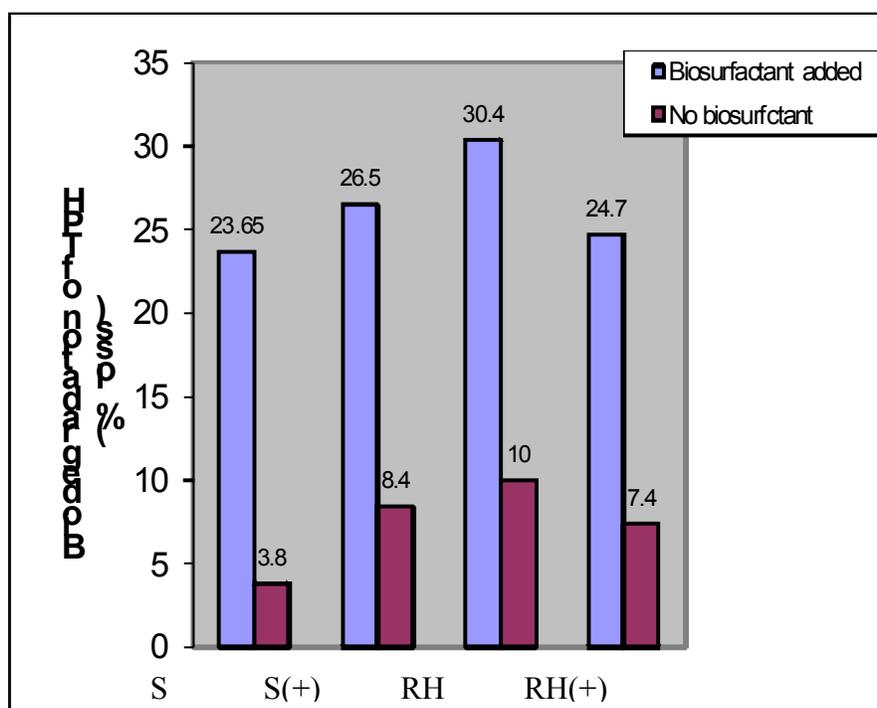


Fig. 2. Biodegradation of TPHs (% loss) in the different treatments as affected by the presence or absence of biosurfactant.

Addition of the biosurfactant increased the biodegradation rates to reach 23.6-30.0%, i.e. with increased factor of 3.0-6.2. Addition of NP fertilizer had no significant effects in the presence of the biosurfactant. A significant biodegradation rate (30.0%) was observed in the rhizosphere soil (RH) when NP fertilizer was absent. On the other hand no significant variation between the results obtained from S, S(+) and RH (+). This indicates that in presence of the biosurfactant NP fertilizer failed to enhance the biodegradation of the PHCs. These results indicate that this phyto-genic biosurfactant was more easily utilized than the inorganic NP fertilizer.

The effect of the phyto-genic surfactant soy lecithin (SL) on the aerobic biodegradation of polychlorinated biphenyls (PCBs) were studied by **Fava and Gioia (2001)** and **Soeder et al (1996)**. They found that in the presence of SL, a higher availability of biphenyls and chlorobenzoic acid degrading bacteria, and high PCB biodegradation and dechlorination yields were observed. It was found that some phyto-genic surfactant improved the bioavailability of polycyclic aromatic hydrocarbons (PAHs) in the rhizosphere soil of some plants (**Oleszczuk et al, 2007**). The intensity of the influence of phyto-genic surfactants on the degree of degradation of PAHs varied widely and depended on

the strain of bacteria and the conditions during the experiments (**Soeder et al, 1996**).

**Kosaric (2001)** reported that biosurfactants have been shown to promote biodegradation of hydrocarbons. In the presence of selected biosurfactants, a preferential and significant removal of PAHs was observed after only 22 days of bioremediation. Keeping in mind that bioremediation is a slow process, this results showed a significant reduction of the time required to bioremediation of contaminated sites. **Mulligan (2005)** showed that biosurfactants are widely used in bioremediation and waste treatments to remove hazardous materials. **Whang et al (2008)** found that the glycolipid and glycoprotein types of biosurfactants were able to increase the solubility and bioavailability of the petrochemical mixture, and also stimulated the indigenous microorganisms for enhanced biodegradation.

Various studies also have been carried out on the effects of biosurfactants on enhancing the recovery and biodegradation of petroleum hydrocarbons (**Nayak, et al, 2009; Calvo et al, 2008, Chang et al, 2008; Salihu et al, 2009; Helmy et al, 2010**). **Zhang et al (2010)** reported that it may be possible to facilitate phytoremediation efficiency by introducing biosurfactants to improve desorption and bioavailability of the hydrocarbons resulting in

enhanced biodegradation of aged hydrocarbons in soil.

Results of the biodegradation of petroleum soil fraction (saturates and aromatics) are found in Table (4). The results show that in the presence of the biosurfactant biodegradation of the saturates was in the ranges of 78.1±6.3% (in soil without nutrients) to 92.8±2.8% (in the rhizosphere soil without addition of nutrients).

On the other hand in the absence of the biosurfactant the biodegradation rate of the saturates

decreased and was in the range of 20.6±1.0% to 44.7±1.6 (in the rhizosphere soil in the absence of NP fertilizer).

As for the biodegradation of the aromatic fraction, the same trend of results were observed. In presence of the biosurfactant the biodegradation was in the range of 26.4±4.4% to 41.8±2.0% (in the rhizosphere soil without the addition of NP fertilizer). On the other hand in absence of biosurfactant, the biodegradation rate decreased to reach 9.6±1.0 to 23.5±2.2%.

Table 4: Biodegradation (mg/g soil and (%) loss) of the saturated and aromatic fractions of the oil pollutant as affected by different treatments and presence or absence of the phyto-genic biosurfactant. Original concentration (at O-time) of saturates and aromatic were 32mg/g and 28mg/g respectively. ± = standard deviation (n=3).

Treatments	Saturates				Aromatics			
	No biosurfactant		Biosurfactant present		No biosurfactant		Biosurfactant present	
	Biod. Mg/g	Loss (%)	Biod. Mg/g	Loss (%)	Biod. Mg/g	Loss (%)	Biod. Mg/g	Loss (%)
S	6.6±0.3	20.6±1.0	25.0±2.0 <sup>(a)</sup>	78.1±6.3 <sup>(a)</sup>	2.7±0.3	9.6±1.0	7.4±0.9	26.4±4.4 <sup>(a)</sup>
S(+)	11.6±1.4	36.3±4.4	26.2±1.1 <sup>(a)</sup>	81.9±3.6 <sup>(a)</sup>	4.1±0.5	14.6±1.6 <sup>(a)</sup>	8.4±0.6 <sup>(a)</sup>	30.0±2.2
RH	14.3±0.5	44.7±1.6	29.7±0.6	92.8±2.8	6.6±0.6	23.5±2.2	11.7±0.6	41.8±2.0
RH(+)	10.5±1.3	32.8±4.0	25.6±2.4 <sup>(a)</sup>	80.0±7.8 <sup>(a)</sup>	3.8±0.3	13.6±0.8 <sup>(a)</sup>	8.1±0.7 <sup>(a)</sup>	28.9±2.7 <sup>(a)</sup>

From the above results the following points could be summarized:

- The biodegradation rates of both the saturates and the aromatics clearly increased in presence of the biosurfactant.
- The saturates fraction was easily degradable than the aromatic fraction.
- Maximum biodegradation of the saturates (92.8±2.8%) and maximum biodegradation of the aromatics (41.8±2.0%) were recorded from the rhizosphere soil when NP fertilizer was absent. This may be due to the specific root exudates which were superior over NP fertilizer.
- Addition of NP fertilizer to the rhizosphere (RH+) decreased the biodegradation of both saturates and aromatics by (12%) and (12.9%) respectively.
- The above results lead to the conclusion that this phyto-genic biosurfactant represent a promising tool for the detoxification of the polluted soil from the toxic petroleum hydrocarbons.

The present work demonstrate that the application of this cost-effective phyto-genic surfactant to the highly polluted soil clearly stimulated the development of high counts of total heterotrophic bacteria and oil-degrading microorganisms which resulted in the enhancement of the biodegradation rate of the oil pollutant and its fractions especially in absence of NP fertilizer. Addition of this biosurfactant to the highly oil-polluted sites may change the balance of nutrients and provide the oil degraders with the essential

nutrients required for their growth and biodegradation activity.

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#### Corresponding author

Ali Diab

Faculty of Biotechnology, October University for Modern Sciences and Arts (MSA), Egypt

[Alidiab.maramsan@live.com](mailto:Alidiab.maramsan@live.com)

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