International Journal of Scientific Engineering and Research (IJSER)
ISSN (Online): 2347-3878
Index Copernicus Value (2015): 56.67 | Impact Factor (2017): 5.156

Burkholderia cepacia MSA47, A New Strain Isolated from the Rhizosphere of an Egyptian Salt Marsh Plant, Produced Potent Biosurfactant Applied for the Bioremediation of Oil Sludge-Polluted Soil

Diab A.A[1], Kord M[2], Badawy M[3], Shereen Sami[4]

[1, 4]Faculty of Biotechnology, October University for Modern Science and Arts (MSA), Egypt

[2, 3]Faculty of Science, Cairo University, Egypt

Abstract: ***During the screening of bacteria isolated from the rhizosphere of an Egyptian salt marsh plant for the production of biosurfactants, it was found that a bacterial strain MSA47 was able to produce active biosurfactant (produced 176.6 cm^2 ODA) in presence of waste frying oil (a cheap substrate). This strain was gram negative rods and identified as Burkholderia cepacia MSA47. This bacterial strain was able to utilize 5 different vegetable oils with the production of active biosurfactants (116.9 ±5.4 – 176.6 ±0.0 cm^2 ODA). Results of the effect of different soluble sugars showed that these substrates were not promising for the production of biosurfactant as compared to the hydrophobic substrates; this indicates that this B.cepecia strain prefers the hydrophobic substrates for the production of active biosurfactant. It was found that NH_4NO_3 and $NaNo_3$ were best nitrogen sources, this was followed by the organic nitrogen source ''peptone''. The results also show that E_{24} values were higher against crude oil+Kerosene (74.4%) and crude oil (66.3%). This B.cepecia strain was able to produce biosurfactant stable at wide range of temperature (up to 121 °C), wide range of pH values (2-12), and different NaCl concentrations (0-25% w/v). These properties give the biosurfactant produced by B.cepacia MSA47 a potential use in petroleum industries. Accordingly, the sterilized supernatant was used for enhancing the biodegradation of total hydrocarbons and PAHs during the bioremediation process to 69.0 ±6.9% and the biodegradation of the total of the 6 carcinogenic PAHs to 94.0%. In presence of NP alone, TPH degradation was 48.9%, and total carcinogenic PAH degradation was 86.6%. While in presence of a combination of BRNP, TPHs were degraded by 60.7%, and the total carcinogenic PAHs were 97.4%. These results indicate that the promising factor for enhancing the biodegradation of TPHs and PAHs compounds is the presence of BR. The results show that BR alone or in combination with NP fertilizers highly stimulated the native microorganisms in the oily sludge polluted soil to highly or completely degrade the carcinogenic PAH individuals found in the oily sludge. The use of BR will reduce the amount of fertilizers needed for the biodegradation process. On the other hand the use of BR alone enhanced the biodegradation of PAHs without fertilizers. This will reduce the cost of the bioremediation process.***

Keywords: Burkholderia cepecia, Biosurfactants, Salt marsh plant, Bioremediation, TPH, PAHs

1. Introduction

In natural ecosystems and in managed ecosystems, bacteria are common inhabitants of plants. The most diverse and environmental adapted plant associated bacteria were found to be members of the genus *Burkholderia* **(Compant *et al*, 2008).** Strains of *Burkholderia* are able to live in the rhizosphere of plants as epiphytic or endophytic symbiosis **(Coeny and Vandamme, 2003; Janssen, 2006).**

Burkholderia strains are widely distributed in many habitats, being found in water, soils and plant surfaces. These bacteria are exploited for bio-control, bioremediation and plant growth promotion **(Coeny and Vandamm, 2003).** *Burkholderia* is a gram negative genus; it includes more than 80 species, some of which may be used in biotechnology such as bioremediation of polluted sites and bio-besticidal agents. The genus *Burkholderia* represents a rapidly expanded group of gram negative, non-fermentative bacteria which are widely distributed in many environments. This genus includes closely related species known as *Burkholderia cepacia complex* (Bcc).

The natural habitats of *B.cepacia* strains are soil, water and vegetation. These species are used for biological control and bioremediation of polluted soils. *B.cepacia* is able to produce antimicrobial agents that inhibit plant pathogens **(Tabachioni *et al*, 2002).** Several *B.cepacia* strains are able to degrade diesel fuel **(Rodreques *et al*, 2012)** and PAHs **(Palleroni *et al*, 2005).** *B.cepacia* strains are able to control seedlings and root diseases and can be the alternative of using chemical substances. These strains can colonize the rhizosphere of many crop plants such as corn, maize, rice, pea, sunflower, radish and many others, leading to an increase in the crop yield.

From the literature it can be seen that no *Burkholderia* sp. were recorded from the rhizosphere of wild plants (except for tropical legumes) such as the salt marsh plants and desert plants. Accordingly in our work, it is the first time to isolate *B.cepacia* from the rhizosphere of salt marsh plant; moreover it is the first time to isolate this bacterium from Egyptian habitats. The aim of the present work was to study the production, characterization and application of the crude biosurfactant for the bioremediation of the oily sludge contaminated desert soil.

2. Materials and Methods

1) Soil Samples

Samples from the rhizosphere soil of salt marsh plant namely *Urospermum pecroides* were collected. By using the

usual dilution plate method, six bacterial isolates were isolated as dominant colonies appeared on nutrient agar plates. The six isolates were purified by the streak method.

2) Seed Culture

Screening the purified bacterial strains for the production of biosurfactants

Inorganic salt medium (ISM) supplemented with waste frying oil (2% w/v), was used. For each bacterial strain, two 250 ml conical flasks each containing 50 ml of the ISM supplemented with 2% waste frying oil were inoculated with 5 ml of the seed culture of the bacterium.

The inoculated flasks were incubated at 32 °C on a shaker operated at 140 rpm, for a period of 48-96h. At the end of the incubation period, the bacterial cultures were sterilized and centrifuged at 6000 rpm for 30 min for removing the bacterial cells. The culture free cells (supernatant) were tested for the production of biosurfactant by using the oil displacement area (ODA), (**Techaoei *et al*, 2011)**.

The most active biosurfactant-producer was selected and further studied using the blood hemolysis test **(Rahman *et al*, 2010)**, CTAB test **(Satpute *et al*, 2010)** and parafilm test **(Morita *et al*, 2007).** The emulsification index of the produced biosurfactant was also carried out against hydrocarbon oils and vegetable oil **(Techaoei *et al*, 2011; Diab *et al*, 2017).** The composition of ISM is as follows (g/L distal water):

NH_4NO_3	3.0	$MgSO_4$	0.5
K_2HPO_4	2.0	NaCL	0.1
KH_2PO_4	1.0	$CaCL_2$	0.1

Trace Salt Solution 1 ml
pH 7-7.2

3) Effect of different carbon sources on the production of biosurfactant

a) Effect of vegetable oils

Three 250 ml conical flasks, each containing 50 ml of ISM were prepared for each vegetable oil (2% w/w) and inoculated with 3 ml of the seed culture of the selected bacterial strain. The inoculated flasks were incubated at 32 °C on a rotary shaker operated at 140 rpm, for a period of 48h, after which the production and activity of the biosurfactant were measured by the ODA method. The vegetable oils used were: corn oil, olive oil, soybean oil, sunflower oil and waste frying oil.

b) Effect of different soluble substrates

Three 250 conical flasks, each containing 50 ml of ISM were prepared for each soluble substrate, and inoculated with 3 ml of the seed culture of the organism, the production and activity of the produced biosurfactant were tested as mentioned above. The soluble substrates used were: glucose, glycerin, fructose, sucrose, and soluble starch.

c) Effect of mixtures of the vegetable oils with the soluble substrates

50 ml of the ISM were prepared for each mixture (1:1), inoculated with the organism, incubated on a shaker at 140 rpm at 30 °C for 48h, and then tested for the production and activity of the biosurfactant as described before. Waste frying oil was used for the preparation of the mixtures.

4) Effect of different nitrogen sources

Three flasks 250 ml each containing 50 ml of the ISM were prepared for each nitrogenous source (2 g/L) and incubated with 3 ml of the seed culture of the organism. The flasks were incubated at 32 °C on a shaker at 140 rpm for 48h. The production and activity of the produced biosurfactant were tested as mentioned before. The nitrogen sources used were (2 g/L): Ammonium chloride, ammonium nitrate, ammonium phosphate, ammonium sulphate, sodium nitrate and peptone.

5) Stability Test

a) Thermo-stability

This test was carried out according to **Techaoei *et al* (2011)** and **Diab and El-Din (2013).**

b) Effect of different pH values

The supernatant of the culture free broth was adjusted at different pH values (2-12 pH). The activity of the biosurfactant in each pH was measured **(Diab and El-Din, 2013).**

c) Effect of salinity

The effect of different concentrations of NaCl on the activity of the biosurfactant was carried out according to **Diab and El-Din (2013).**

6) Extraction of the biosurfactant produced by bacterial strain MSA47

The bacterial strains MSA47 was grown on NB medium supplemented with 2% waste frying oil, the culture was incubated at 32 °C for 48h on rotary shaker operated at 140 rpm. At the end of incubation period the culture broth was centrifuged at 6000 rpm for 20 min to remove bacterial cells. The biosurfactant in the cell free supernatant was extracted 2 times with methylene chloride-methanol (2:1) at room temperature.

7) Application of the biosurfactant produced by the selected bacterial strain for the bioremediation of oil sludge-polluted soil

a) Soil Treatment

Oily sludge samples from sludge generated during the periodical cleaning of oil storage tanks at Abu-Dhabi were kindly provided. Analysis of this sludge in the laboratories of the Faculty of Biotechnology-MSA University revealed the presence of 18-20% crude oil. For successful bioremediation process the concentration of the oil must be reduced to 4-5%.

Accordingly, non-polluted desert soil sample was collected and thoroughly mixed with the oily sludge so as to give 4-5% (w/w). This polluted soil was treated as follows: **(Diab *et al*, 2017),** soil microcosm test was designed to include 4 treatments in duplicates. Each consisting of 500 ml glass beaker containing 100 g of the polluted soil, and treated as found in Table (1).

Table 1: Different treatments of the polluted soil

Treatments	Biosurfactant (BR)	NP	BRNP	Oil
1	-	-	-	+
2	+	-	-	+
3	-	+	-	+
4	-	-	+	+

The NP fertilizer was $NaNO_3$ (100 mg/100g soil) and K_2HPO_4 (60 mg/100 g soil). Biosurfactant produced by the studied bacterial strain was added (5 ml/100 g soil) in the form of sterilized supernatant. Small glass rod was introduced to each beaker for tilling the soil. The moisture content was adjust at 5% by adding tap water for treatment ''1'' & ''3'' and by adding the supernatant for treatment ''2'' and ''4''.

All the treatments were incubated at temperature 30 °C. The loss of moisture due to evaporation was determined at the beginning of experiment and every 2-3 days. All of the treatments were covered by thin aluminum foil to reduce evaporation of moisture.

b) Extraction and determination of the residual oil

At the beginning of the experiment at the end of 90 days incubation period, 4 g of the air-dried soil was mixed with 4 g of anhydrous sodium sulphate, and the residual oil was extracted by n-hexane using the shaking method described by **Chen *et al* (1996).** The extract was collected and evaporated in a pre-weighted dish, and then the amount of residual oil was calculated.

c) HPLC analysis for the resolution of the different PAH individuals found in the residual oil

The listed US-EPA 16 polycyclic aromatic hydrocarbons (PAHs) in the n-hexane extract was analyzed and quantified by the help of the Egyptian Petroleum Research Institute (EPRI) using high performance liquid chromatography (HPLC), Model agilent-1200 series equipped with DAD detector (model 1260 infinity) according to standard test method (EPA 530 and EPA 610). Calibration curve was done by EPA PAH mix standards (Supleco, USA, 99%).

8) Identification

Identification of the selected bacterial strain (MSA47) was carried out by using: BBL Crystal Identification System [Gram Negative ID Kit-Non-Fermentative ID System].

9) Statistical analysis

All values were averages of 3 or 2 readings and expressed as mean ±SD, for determining significance of differences among the means, data were analyzed for significant differences ($P<0.05$) between treatments.

3. Results and Discussion

During the screening of bacterial isolates for the production of biosurfactant using the ODA cm^2 method, a bacterial strain MSA47 showed very good surface activities (Table 2). This strain was able to produce higher ODA cm^2 value (135.6) in the presence of waste frying oil (Fig 1A), was positive for parafilm test (Fig 1B), positive for blood hemolysis test (Fig 1C) and positive for CTAB test (Fig 1D). This strain was selected and studied, it was gram negative rods, produces glistering yellow colonies with entire edge when grown on nutrient agar plates. It was identified as *Burkholderia cepecia* with the aid of the BBL Crystal Identification System – Gram negative ID Kit (Non-fermentative ID System).

It was found that most of the *Burkholderia* strains were inhabitant of the rhizosphere of crop plants, and no reports were available on the isolation of *B.cepacia* from the rhizosphere of desert and salt marsh plants. It is of interest to find out that this *B.cepacia* strain was isolated for the first time from rhizosphere of salt marsh plant; furthermore, it is the first time to isolate this *B.cepecia* strain from Egyptian habitats. **Almatawah (2017)** isolated strain of B.cepecia KISRQC from oil contaminated soil from Kuwait.

Compant *et al* (2008) reported that in nature, bacteria are common inhabitants of plants, and the most environmentally adapted plant associated bacteria are members of the genus *Burkholderia*. Species of this genus may be found in plant rhizosphere as epiphytic or endophytic symbiotic associations **(Coenye and Vandamme, 2003; Janssen, 2006).**

Results of the effect of different vegetable oils on the production of biosurfactant by this *B.cepecia* strain are found in Table (2) and illustrated in Fig (2). The results show that this bacterial strain was able to utilize all of the 5 vegetable oils tested (corn oil, olive oil, soybean oil, sunflower oil and waste frying oil) for the production of active biosurfactants (116.9 ±5.4 – 176.7 ±0.0 cm^2 ODA). Waste frying oil was promising substrate for the production of biosurfactant with the highest surface activity (176.7 ±0.0 cm^2 ODA) when grown on ISM, this was followed by soybean oil (130.7 ±2.7 cm^2 ODA). **Almatawah (2017)** found that B.cepecia was able to produce biosurfactant from olive oil.

Results of the effect of different soluble substrates (e.g. glycerin, glucose, fructose, sucrose and soluble starch) on the production of biosurfactants (Table 3, Fig 3) show that glycerin was able to produce biosurfactant of higher surface activity (176.7 ±0.0 cm^2 ODA). Other substrates were able to produce less surface active biosurfactant (0.0 – 105.0 ±7.1 cm^2 ODA). When each of the soluble substrates was mixed with waste frying oil, the results (Table 3, Fig 3) show that no effects of the sugars on the surface activity of the produced biosurfactant was observed. These results indicate that the new *B.cepecia* strain prefers the hydrophobic substrates (vegetable oil) over the hydrophilic substrates.

Wattanaphon *et al* (2008) found that *Burkholderia cenocepacia* BSP3 was able to grow in the presence of a mixture of glucose and oil (2%), but it failed to grow on vegetable oils alone. **Anyanmu (2010)** found that *P.aeruginosa* LS-1 was able to utilize both water soluble and water-immiscible substrates for the production of biosurfactant and they found that the water soluble sources were promising for the production of active biosurfactants than the water immiscible substrates. **Techaoei *et al,* (2011)** reported that *P.aeruginosa* was able to produce maximum biosurfactant activity (38.5 cm^2 ODA) when grown on glucose, this is in comparison to sucrose (0.22 cm^2 ODA),

galactose (2.75 cm^2 ODA), and sorbose (0.68 cm^2 ODA). On the other hand, when vegetable oils were used, ODA values were 2.93 cm^2 in corn oil, 1.94 cm^2 in soybean oil and 2.75 cm^2 in coconut oil. **Jain *et al* (2013)** found that a strain of *Klebseilla* sp was able to utilize carbohydrates for the production of biosurfactants (e.g. glucose, sucrose, fructose, galactose, starch and xylose), but at the same time this strain failed to use vegetable oils for the production of biosurfactants. **Pansiripat *et al* (2010)** found that high biosurfactant yield was obtained when non water-soluble carbon sources were used by *P.aeruginosa* UG2, while **Wu *et al* (2008)** showed that water-soluble substrates such as glucose and glycerol were promising for the production of higher yield of biosurfactant by *P.aeruginosa* EMI. On the other hand, **Pansiripat *et al* (2010)** reported that both hydrophilic and hydrophobic substrates were used together to increase the production of biosurfactant. It appears from the above results that bacterial strains may differ for the chosen of the carbon sources according to bacterial species and strains. Inside one species, different strains may differ in their utilization of the different carbon sources.

A part from studies on the production of biosurfactants using single or mixture of sugars and oils, the research on the selection of more economic process in which wastes of oils may be of economic approach **(Makkar *et al,* 2011).** In the present work waste frying oil was promising substrates for the production of biosurfactant of the highest surface activity (176.6 cm^2 ODA). Waste frying oils are cheaper substrates and nutrient rich. Large volumes of waste frying oil are generated in restaurants, and may be available as cost-effective substrates for the production of different biosurfactants.

Results of the effect of different nitrogen sources on the production of biosurfactant by *B.cepecia* MSA47 (Table 4, Fig 4) show that NH_4 NO_3 and $NaNO_3$ were best nitrogen sources for the production of biosurfactant of the highest ODA values (176.6 ±0.0). This was followed by an organic nitrogen source (153.0 ±7.0 cm^2 ODA), $(NH_4)_2SO_4$ was not promising substrate (0.8 cm^2 ODA). **Adamczak and Bendarsici (2000)** found that *Arthrobacter paraffineus* preferred ammonium salt and urea for the production of biosurfactant. **Techaoei *et al* (2011)** found that *P.aeruginosa* SCMU 106 was able to use ammonium phosphate for the production of biosurfactant of 143.67 cm^2 ODA. On the other hand $(NH_4)_2SO_4$, NH_4NO_3 and KNO_3 were less promising, their ODA values were: 10.83 ±0.29 cm^2, 9.83 ±0.76 cm^2 and 11.17 ±0.2 cm^2 respectively. Results of the effect of different concentrations of $NaNO_3$ (Table 4, Fig 5) show that the produced biosurfactant was active in the presence of wide range (0.5-5% w/v) of NaNO3 (90.4 ±4.1 – 176.7 ±0.0 cm^2 ODA). At concentration of 2-4% w/v $NaNO_3$, maximum ODA values of 176.6 ±0.0 cm^2 were recorded.

Results of the emulsification activity as measured by the emulsification index (E_{24}) (Table 5, Fig 5) show that the higher E_{24} values were recorded against crude oil+kerosene (71.4%) and crude petroleum oil (66.3%). **Lima *et al* (2011)** proposed that the emulsification activity of a biosurfactant is considered stable if its E_{24} is equal to 50% or more. **Techaoei *et al* (2011)** reported that it is desired to obtain a biosurfactant with high and stable E_{24}, which may represent a potential product for enhanced oil recovery and biodegradation of hydrocarbons. **Anyanwu *et al* (2011)** reported that the ability of biosurfactant to emulsify hydrocarbon-water mixture has been demonstrated to increase hydrocarbon biodegradation and is potentially useful in enhanced oil recovery. The above authors suggested that the ability of biosurfactant to form stable emulsion with different hydrocarbons and vegetable oils, gives this product many advantages in petroleum and pharmaceutical industries.

Based on the above discussions, *B.cepacia* MSA47 strain may be promising candidate for oil recovery, cleaning of oil storage tanks, recovery of oil from sludge and help in the bioremediation of oil polluted sites. At the same time, this bacterial strain was also able to form E_{24} values of more than 50% (53% - 58%) against vegetable oils, suggesting that this bacterial strain may be also valuable in food and pharmaceutical industries.

Results of the stability of the biosurfactant produced by *B.cepacia* MSA47 (Table 5, Fig 7-8) show that this biosurfactant was stable at wide range of pH values (Fig 7), but with different retained activity (RA). Optimum pH values were pH7 and pH8 (100% RA) followed by pH9 and pH10 (87.1% RA). Results of the stability of the produced biosurfactant in presence of different concentration of NaCl (Table 5, Fig 8) show that this biosurfactant was stable at different NaCl concentration (0-25% w/v). The retained activity was 100% in presence of 2-5% NaCl, and 87.1%, 81.0% and 75.1% in presence of 10%, 15%, 20% and 25% NaCl concentration respectively. It retained 75.1% of its activity at 20-25% NaCl and it was found also that this biosurfactant retained 100% of its activity at 121°C. **Diab and El-Din (2013)** found that *Bacillus sp* SH26 showed 95.3%, 88.4%, 83.9% and 44.4% retained activity (RA) at 5%, 10%, 15% and 20% NaCl, while *P.aeruginosa* SH30 showed 76.7% and 47.1% retained activity at 5 and 10% NaCl respectively, but at higher concentrations the stability dropped to 25.1% RA. **Techaoei *et al* (2011)** found that *P.aeruginosa* SCMU106 was able to produce a biosurfactant showing stability at 8% NaCl. **Rufino *et al* (2008)** found that *Candida lipolytica* produced biosurfactant of stability at 2-10% NaCl concentrations and at a wide range of temperature (0-121 °C).

The present results showed that biosurfactant produced by *B.cepacia* MSA47 was stable at wide range of pH values (2-12 pH), wide range of NaCl (2-25% w/v) and wide range of temperature (0-121 °C). These properties beside the good surface activity give the biosurfactant produced by *B.cepacia* MSA47, a potential use in petroleum and other industries. Accordingly, the sterilized supernatant containing the active biosurfactant was used for enhancing the biodegradation of total hydrocarbons and PAHs during the bioremediation of oil sludge polluted soil. **Al-Bahry *et al* (2012)** reported that oil reservoirs are one of the harsh environments, where temperature can range from 20 °C to 90 °C, from normal salinity to hyper saline and wide range of pH values. It can be seen from the present results that the biosurfactant produced by *B.cepacia* MSA47 strain meets these harsh conditions, because it produces a biosurfactant

of surface activity and stability at wide ranges of environmental factors. Accordingly, this biological product is useful for cleaning oil storage tanks; recovery of oil from oily sludge, washing oil-contaminated soils, microbial enhanced oil recovery (MEOR) and enhanced the bioremediation of oil-contaminated sites. The biosurfactant produced by *B.cepacia* MSA47organic phase was recovered from the supernatant by using methylene chloride-methanol (2:1). The extract was collected and evaporated to dryness leaving behind oily yellowish brown product. The production yield of this product was 12 gm/L, this bi-product was selected and kept for further purifications and studies.

Results of the biodegradation of the oily sludge polluting this type of soil are found in Table (6) and illustrated in Fig (9). The results show that addition of biosurfactant alone (BR) increased the biodegradation of the oil to reach 69.0 ±6.9% at the end of 90 days experimental period. Addition of NP alone failed to increase the biodegradation above 48.9 ±6.3%, while in presence of a combination of BR and NP, biodegradation of the oily sludge increased but did not exceed 60.7 ±6.5%. It is observed that statistically no significant difference between the results with BR alone and those with BRNP was observed (P=0.05). From these results it can be seen that the promising factor is the presence of BR.

Thavasi *et al* (2011) found that the addition of biosurfactant alone had significant oil degradation with little difference compared to the presence of BRNP, i.e BR alone was able to enhance the biodegradation process without using fertilizers. This will reduce the cost of the bioremediation process, and minimize the wash away problem when soluble fertilizers are used for the bioremediation of the aquatic environment. **Thavasi *et al* (2010)** found that a maximum of 82% of crude oil was degraded in presence of biosurfactant alone, while in presence of BRNP the biodegradation was 75%. ***Cameotra and Singh (2008)*** studied the effect of the crude biosurfactant and nutrient amendments on the biodegradation of oil sludge of different origins by applying a consortium made of two *Pseudomonas aeruginosa* strains and *Rhodococcus* sp during 8 weeks period. They found that the higher biodegradation (98%) was in presence of BRNP, while in presence of BR alone 73% only of the oil was degraded. On the other hand 63% of the oil was degraded in presence of NP alone. In the control experiment (no additives) 52% of the oil was degraded. **Whang *et al* (2008)** found that during 88 days experimental period, maximum biodegradation of TPH was 97% in presence of rhamnolipid, while in presence of surfactin, 76.3% of the oil was degraded. In the control experiment 47% of the oil was degraded.

Eruke and Udoh (2015) reported that the biodegradability of hydrophobic organic compounds by microorganisms is a limiting factor during bioremediation. Application of biosurfactants to the contaminated soil can potentially reduce the interfacial tension, increase the stability and bioavailability of the hydrocarbons and then facilitate the biodegradation of such compounds. The application of biosurfactants in the bioremediation process may be more acceptable from a social point of view due to their naturally occurring properties, the unusual structure diversity that may lead to unique properties, the possibility of cost-effective production and their biodegradability properties. Accordingly, biosurfactant are of promising choice for the application in enhancing hydrocarbon biodegradation. **Sorbinho *et al* (2013)** reported that the major market of biosurfactants is the petroleum industries, in which these biological compounds can be used for cleaning-up of oil spills, the removal of oil residues from storage tanks, in microbial-enhanced oil recovery (MEOR) and the bioremediation of hydrocarbon-polluted sites.

Helmy *et al* (2010) reported that hydrocarbon pollutants are removed from the environment primarily as a result of their biodegradability by the native microbial populations. Such process is a time consuming, and another technique have been developed such as the addition of biosurfactants which help to stimulate the indigenous microbial populations to degrade the hydrocarbons at rates higher than those achieved in presence of nutrients alone, this is through increasing the bioavailability of the pollutants to microorganisms. The above authors used crude biosurfactant in the form of cell free broth culture (supernatant) which was applied directly without purification of the biosurfactant. They found that in presence of the biosurfactant the removal efficiency of the pollutants was 20% compared to those without addition of biosurfactant.

In the present work polycyclic aromatic hydrocarbons (PAHs) compounds at the beginning and at the end of 90 days bioremediation period was identified and quantified by using HPLC analysis technique. The results (Table 7, Fig 10) demonstrated the resolution of 14 US EPA priority PAHs with a total of 1291.0 mg/kg oil sludge polluted at beginning of the experiment of which naphthalene was of higher content as compared to the other PAHs. This compound was represented by 312 mg/kg soil (i.e 24.2%). This was followed by phenanthrene (240.0 mg/kg soil, 16.6%) and flouranthene (205.8 mg/kg soil, 15.9%). Other PAHs are in range of 0.6-8.4%. **Diab and Sandouka (2010)** found that the highest PAH content in a Kuwaiti oil-polluted sample were pyrene (14.6%) and flouranthene (11.8%) relative to the 16 US EPA priority PAHs. **Cofield *et al* (2007)** found that at the initial of the experiment, the highest PAH content of the oil polluting a soil sample was benzo(a)pyrene (21.2%) of the total of the 16 PAHs, followed by flouranthene. **Al-Gounaim and Diab (2004)** found that flouranthene and pyrene were more frequent than the other PAHs of the 16 US EPA (15.3% and 12.4%) respectively. The results show that the sum of the 4-ringed PAHs and the sum of the 3-ringed PAHs were more frequent 386.4 mg/kg soil and 376.6 mg/kg soil respectively.

For simplicity the results in this paper is focused on the biodegradation of the carcinogenic PAHs found in this type of oily sludge. The results (Table 8, Fig 11) show that 6 carcinogenic PAH individuals were resolved from the oily sludge with a total (at the beginning of the experiment) of 461.8 mg/kg soil i.e with 35.8% (w/w). The most frequent carcinogenic PAH was flouranthene (205.0 mg/kg soil), it is represented by 44.6% of the total carcinogenic, this was followed by chrysene (21.7%) and benzo(a)flouranthene (14.7%). From this results it can be seen that the potent

carcinogenic PAH benzo(a)pyrene was absent from this type of oil sludge.

In the present study flouranthene is more frequent (44.6%) as compared to other carcinogenic PAHs. This PAH compounds represents the most abundant PAH in environmental sample, it was reported to be cytotoxic, mutagenic and potentially carcinogenic **(McElory *et al*, 1989; Irvin and Martin, 1987).** This compound is considered more of a potential health hazard by virtue of its abundance than the widely studied but less abundant carcinogenic benzo(a)pyrene. The IRAC (International Agency for Research on Cancer) has identified 6 of the 16 US APA PAHs as potential carcinogenic compounds. The following PAHs: benzo(a)anthracine, chrysene, benzo(b)flouranthene, benzo(k) flouranthene, benzo(a)pyrene and dibenzo(ah)anthracene, were classified by the US EPA (2002) as probable human carcinogens (Class B). The IRAC classified benzo(a)anthracene as probable human carcinogen (Class A). The following PAHs, benzo(b)flouranthene, benzo(k)flouranthene and benzo(a)pyrene are classified as possible human carcinogen Class B **(Chauhan, *et al* 2008).**

Results of the biodegradation of the carcinogenic PAH compounds as affected by the addition of biosurfactant alone (BR), NP fertilizer and a combination of BRNP are found in Table (8) and illustrated in Fig (11). After 90 days bioremediation period, the biodegradation of the total 6 carcinogenic PAHs was 94.0%, 86.6% and 97.4% in presence of biosurfactant alone (BR), NP and BRNP respectively. While the sum of the 5-ringed PAHs were less degraded (81.6%, 73.0% and 97.8% respectively). **Blyth *et al* (2015)** found that the addition of BR alone to polluted soil significantly increased the biodegradation of the total US EPA 16 priority PAHs (78.7% compared to 62% in the control). The same authors found that the biodegradation of 3-, 4- and 5- ringed PAH was 75%, 76% and 85% respectively in presence of BR, while in the control the biodegradation was 42%, 60% and 82% respectively.

It was found in the present results that one PAH compound namely benzo(b)flouranthene was completely degraded in presence of BRNP (100%). The two PAHs flouranthene and chrysene were highly degraded in presence of BR (99.5% and 96.6% respectively) and BRNP (99.3% and 95.0% respectively). It was observed that dibenzo(ah)anthracene was highly degradable in presence of BRNP only (93.6%), while in presence of BR alone and NP alone, this compound was weakly degradable (70% and 65.3% respectively).

It is of interest to observe that flouranthene, the most abundant cytotoxic, nutagenic and carcinogenic PAH was highly degraded in presence of BR (99.5%) and BRNP (99.3%). The present results show that the application of biosurfactant alone or in-combination with NP fertilizer stimulated the native microorganism in this oily sludge-polluted soil to highly degrade the carcinogenic PAH individual found in the oily sludge pollutants. **Diab *et al* (2017)** found similar results with regard to the biodegradation of PAH individual found in spent motor oil polluting soil sample.

The use of biosurfactant in combination with fertilizers reduces the actual amount of fertilizers needed for the bioremediation process. On the other hand, biosurfactants alone are able to promote the biodegradation process without using fertilizers. This will reduce the cost of the biodegradation process **(Thovasi *et al*, 2011, a, b).** Accordingly, the presence of BR alone or in combination with NP fertilizer is the main promising factor in the biodegradation of carcinogenic PAH individuals.

Eruke and Udoh (2015) reported that the use of biosurfactant for the bioremediation of hydrocarbon polluted sited is more acceptable, because these biological compounds are natural products, biodegradable and of no or low toxicity, cost effective and other useful characters. These characters make the biosurfactant the alternative for the chemically synthesized surfactants.

4. Acknowledgement

This study has been conducted in the laboratories of the Faculty of Biotechnology at October University for Modern Sciences and Arts (MSA). The Authors acknowledge the top management of MSA, Professor *Nawal El-Degwi*, and Professor *Khayri Abdelhamid* for their help and encouragement and Professor *Ali Diab* for his full support. We would like to thank the faculty members and the lab technicians in the Faculty of Biotechnology at MSA.

References

[1] **Adamczak, M., Bednarski, W. 2000.** Influence of medium composition and aeration on the synthesis of biosurfactant by *Candida antratica*. Biotechnol. Letters. 22:313-316.

[2] **Al-Gounaim, M.Y., Diab, A. 2004.** Simple bioremediation treatment for the removal of polycyclic aromatic hydrocarbons (PAHs) from the polluted dessert soil of Kuwait. Arab.Gulf.J.Sci.Res.22:66-73.

[3] **Almatawah, Q. 2017.** An indigenous biosurfactant producing Burkholderia cepacia with high emulsification potential towards crude oil. J. Environ. Anal. Toxicol. an open access journal. ISSN: 2161-0525. 7(6).

[4] **Anyanwu, C.U., Obi, S.K.C., Okolo, B.N. 2011.** Lipopeptide biosurfactant by *Serratia marcescens* NSK-1 strain isolated from petroleum contaminated soil. J. Appl. Sc. Res. 7: 79-87.

[5] **Anyanwu, C.U. 2010.** Surface activity of extracellular products of *Pseudomonas aeruginosa* isolated from petroleum contaminated soil. Int. J. Environ. Sci. 1(2): 225-235.

[6] **Anyakora, C., Ogeche, A., Coker, H., Ukpo, G., Ogah, C., 2004.** A screen for benzo(a)pyrene, a carcinogen in the water samples from the Niger Delta using GC-MS. Niger J.Hosp.Med.14:288-293.

[7] **Blyth, W., Shahsavari, E., Morrison, P.D., Ball, A.S. 2015.** Biosurfactant from red ash trees enhances the bioremediation of PAH contaminated soil at a former gas work site. J.Environ.Management. 162:30-36.

[8] **Cameotra, S.S., Singh, P. 2008.** Bioremediation of oil sludge using crude biosurfactants. In Biodeter. Biodegrad. 62:274-280.

[9] **Chauhan, A., Fazlurrahman, Oakesholt, J.G., Jain, R.K. 2008.** Bacterial metabolism of polycyclic aromatic hydrocarbons: Strategies for bioremediation. Int.J.Microbiol. 48:95-113.

[10] **Chen, C.S., Rao, P.S.C., Lee, L.S. 1996**. Evaluation of extraction methods for determining polynuclear aromatic hydrocarbons from contaminated soil. Chemosphere. 32:1123-1132.

[11] **Coenye, T., Vandamme, P. 2003**. Diversity and significance of *Burkholderia* spp occupying diverse ecological niches. Environmental Microbiology. 5(9):719-729.

[12] **Cofield, N., Schwab, A.b., Banks, M.K. 2007**. Phytoremediation of polycyclic aromatic hydrocarbons in soil. Part I. Dissipation of target contaminants. Int.J.Phytorem.9:366-370.

[13] **Compant, S., Nowak, J., Coenye, T., Clement, C., Barka, E.A. 2008**. Diversity and occurrence of *Burkholderia* spp in the natural environment. FEMS Microbiology Review. 32(4):607-626.

[14] **Diab, A., Ageez, A., Gardoh, I. 2017.** *Serratia marcescens* P25, A new strain isolated from the phycoplane of the brown algae *Punctaria* sp, produced potaent biosurfactant used for enhancing the bioremediation of spent motor oil-polluted soil. International Journal of Science and Research (IJSR) 6(1):1034-1047.

[15] **Diab, A., Sandouka, M. 2010.** Biodegradation of polycyclic aromatic hydrocarbons (PAHs) in the rhizosphere soil of *Cyerus congumeratus*, an Egyptian wiled desert plant. Nature and Science, 8(12):144-153.

[16] **Diab, A., El-Din, S.G. 2013**. Production and characterization of biosurfactants produced by *Bacillus* spp and *Pseudomonas* spp isolated from the rhizosphere soil of an Egyptian salt marsh plant. Nature and Science 11(5):103-112.

[17] **Eruke, O.S, Udoh, A.J. 2015.** Potential for biosurfactant enhanced bioremediation of hydrocarbon contaminated soil and water. A Review. Advances in Research. 4(1):1-4.

[18] **Helmy,Q., Kardena,E., Nuracman,Z., Wisjnuprapto, 2010**. Application of biosurfactant produced by *Azotobacter vinelandii AVOI* for enhancing oil recovery and biodegradation of oil sludge. International Journal of Civil and Environmental Engineering IJCEE 10(1):7-14.

[19] **Irvin, T.R., Martin, J.E. 1987.** *In vitro* and *in vivo* embryo toxicity of flouranthene, a major prenatel toxic compound of diesel soot. Teratology, 35:65 A.

[20] **Jain, R.M., Mody, K., Joshi, N., Mishra, A., Jha, B. 2013**. Production and structural characterization of biosurfactant produced by alkaliphilic bacterium, *Klebsiella* sp. Evaluation of different carbon sources. Colloids and surfaces B: Biointerfaces, 108: 199-204.

[21] **Jenssen, P.H. 2006**. Identifying the dominant soil bacterial taxa in libraries of 16s rRNA and 16s rRNA genes. Appl. Environ. Microbiol. 72(3):1719-1728.

[22] **Lima,T.M.S., Procopio, L.C., Brandao, F.D., Carvelho, A.M.X., Totola, M.R., Borges, A.C. 2011.** Biodegradability of bacterial surfactants. Biodegradation. 22:585-592.

[23] **Lima ,T.M.S., Fonseco,A.F., Leao,B.A., Hounteer,A.H., Totola,M.R., Borges,A.C. 2011.** Oil recovery from fuel oil storage tank sludges using biosurfactants. J. Bioremed. Biodegrad. 2:125-137.

[24] **Makkar, R.S., Cameotra, S.S., Banat, I.M. 2011.** Advances in utilization of renewable substances for biosurfactant production. AMB Express 2011, 1:5.

[25] **McElory, A.E., Farringoton, J.W. Teal, J.M. 1989.** Bioavailability of PAH in the aquatic environment. In metabolism of polycyclic aromatic hydrocarbons in the aquatic environment (Varanasi, U. Ed Vol2, PP:1-39 CRC Press, Boca Raton Fla.

[26] **Morita, T., Konishi, M., Fukuoka, T., Imura, T., Kitamoto, D. 2007**. Physiological differences in the formation of the glycolipid biosurfactant, mannosyle eythrital lipids, between *Pseudomonas antaritica* and *P.aphidis*. Appl. Microbiol. Biotechnol. 74:307-315.

[27] **Palleroni, N. 2005.** Genus I. *Burkholderia cepecia*. In: Brenmer, D.J., Krieg, N.R., Staley, J.T., editors Bergy Manual of Systematic Bacteriology. New York Springer; P 575-600.

[28] **Pansiripat, S., Pornsunthorntawee, O., Rujiravnit, R., Kitiyanan, B., Somboonthanate, P., Chavadej, S. 2010**. Biosurfactant production by *Pseudomonas aeruginosa* SP4 using sequencing batch reactor: Effect of oil-to-glucose ratio. Biochem. Eng. J. 49:185-191.

[29] **Rahman, P.R.S.M., Pasirayi, G., Auger, V., Ali, Z. 2010.** Production of rhamnolipid biosurfactant by *Pseudomonas aeruginosa* DS10-129 in a microfluidic bioreactor. Biotech. Appl. Biochem. 55(1):45-52.

[30] **Rodrigues-Rodrigues, C.E., Zunigo-Chancon, C., Barbaza-Solano, C. 2012.** Evaluation of growth in diesel fuel and surfactants production ability by bacteria isolated from fuels in Costa Rica. Revesta de la SoCIATED Venezolana de Microbiologia, 32:116-120.

[31] **Rufino, R.D., Sarubbo, L.A., Neto, B.B., Campos-Takaki, G.M. 2008**. Experimental design for the production of tension-active agent by *Candida Lipolytica*. J. Ind. Microbiol. Biotechnol. 35:907-914.

[32] **Satpute, S.K., Banpurkar, A.G., Dhakephalkar, P.K.M Banat, I.M., Chopade, B.A. 2010.** Methods for investigating biosurfactants and bioemulsifiers: a review. Critical Reviews in biotechnology. 30(2)127-144.

[33] **Sorbinho, H.B., Luna, J.M., Rufino, R.D., Porto, A.L.F., Sarubbo, L.A. 2013.** Biosurfactant: classification, properties and environmental application. In Recent Development in Biotechnology. 1st ed; Studium Press LLC: Houston, T.x., U.S.A. Vol 11, pp:1-29.

[34] **Tabachioni, S., A., Bevivino, C., Dalmastri and L., Chiarini. 2002.** Burkholderia cepecia complex in the rhizosphere: a mini review. Abstract. Volume (52). Issue (2). http://www.annmicro.uniml.it/abstr52.htm. Accessed on January 4, 2003.

[35] **Techaoei, S., Lumyong, S., Prathumpai, W., Santiarwarn, D., Leelapornoisid, P. 2011**. Screening, characterization and stability of biosurfactant produced by *Pseudomonas aeruginosa SCMU*106 isolated from soil in Northern Thailand Asian J.Biological Sciences 4(4):340-351.

[36] **Thavasi, R., Jayalakshmi, S., Banat, I.M., 2011**. Effect of biosurfactant and fertilizer on biodegradation of crude oil by marine isolate of *Bacillus megaterium*,

Corynbacterium kutscheri and *Pseudomonas aeruginosa*. Biores. Technol. 102:772-778.

[37] **Thavasi, R., Subramanyam, Nambaru, V.R.M., Jayalakshmi, S., Blasubramanian, T., Banat, I.M., 2010.** Biosurfactant production by *Pseudomonas aeruginosa* from renewable sources. Indian J. Microbial. 5(7):200.

[38] **Thavasi, R., Jayalakshmi, S., Banat, I.M. 2011(a).** Effect of biosurfactant and fertilizers on biodegradation of crude oil by marine isolates by *Bacillus megaterium*, *Corynbacterium kutscheric* and *Pseudomonas aeruginosa*. Bioresour.Technol.102:772-778.

[39] **Thavasi, R., Jayalakshmi, S., Banat, I.M. 2011(b).** Application of biosurfactants produced from peanut oil cake by *Lactobacillus delbrueckii* in biodegradation of crude oil. Bioresour.Technol. 102(3):3366-3372.

[40] **Wattanaphon, H.T., Kerdsin, A., Tammacharoen, C., Sangvanich, P., Vangnai, A.S. 2008.** A biosurfactant from *Burkholderia cenocepecia* and its enhancement of pesticide solubilization. J. Appl.Microbiol. 105:416-423.

[41] **Whang, L.M., Liu, P-WG, Ma, C.C, Cheng, S.S. 2008.** Application of biosurfactants, rhamnolipid and surfactin, for enhanced biodegradation of diesel contaminated water and soil. J. Hazard. Mater. 151:155-163.

[42] **Wu, Y., Luo, Y., Zou, D., Ni, J., Liu, W., Teng, Y. 2008**. Bioremediation of polycyclic aromatic hydrocarbons contaminated soil with *Monilinia* sp. Degradation and microbial community analysis. Biodegradation. 19:2547-257.

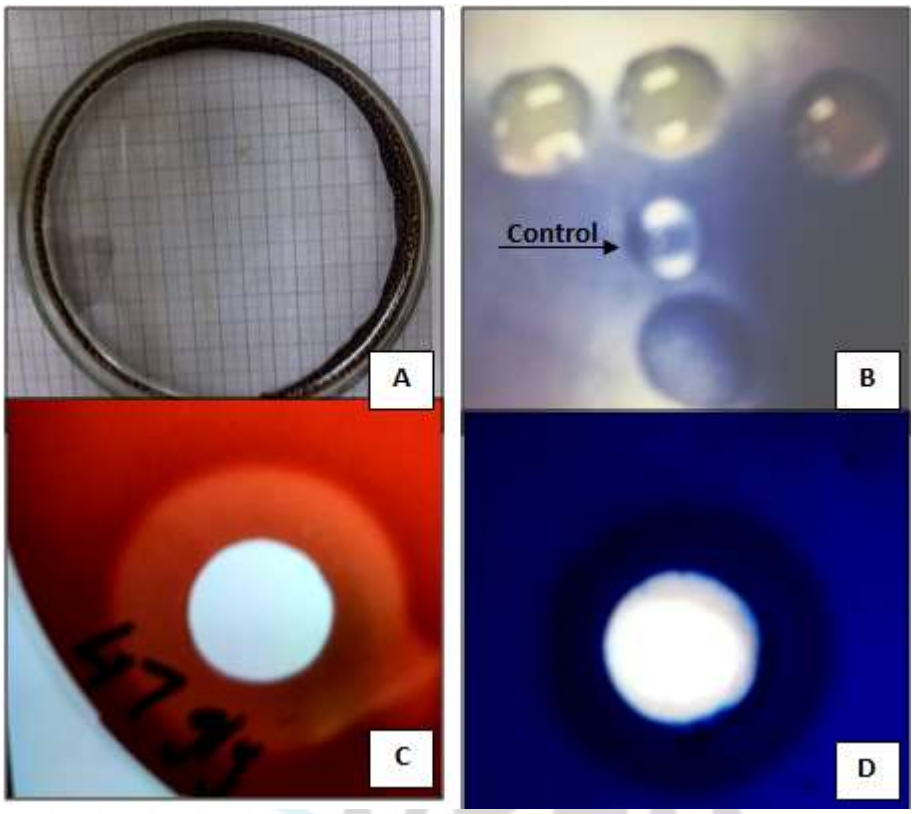

Figure 1: Photographs showing
[A] Oil Displacement Area test is of 135 cm^2
[B] Parafilm Positive test
[C] Blood Hemolysis Positive test (27.0 mm)
[D] CTAB positive test (33.0 mm)

Table 2: Effect of different vegetable oils (2% w/v) on the production of biosurfactant by *Burkholderia cepecia* MSA47 as measured by the ODA method cm^2 when grown on NB and ISM media.
± = standard deviation, n = 2, NB = Nutrient Broth, ISM = Inorganic Salt Medium

Media	ODA cm^2				
	Corn Oil	Olive Oil	Soybean Oil	Sunflower Oil	Waste Frying Oil
NB	81.9 ±4.5	176.0 ±0.0	159.5 ±7.8	166.2 ±1.6	135.8 ±4.4
ISM	117.9 ±7.6	116.9 ±5.4	130.7 ±2.9	123.9 ±4.5	176.0 ±0.0

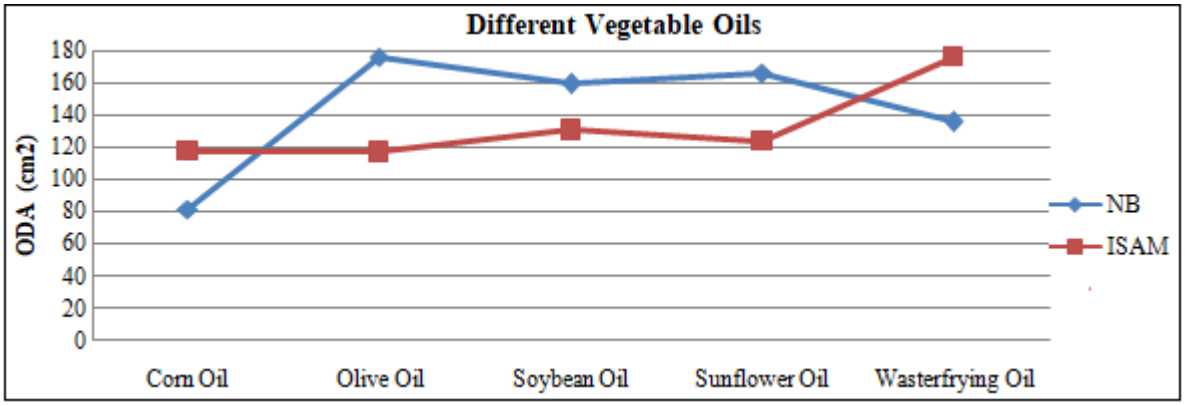

Figure 2: Effect of different vegetable oils on the production of biosurfactant by *Burkholderia cepecia* MSA47.

Table 3: Effect of different soluble substrates in presence and absence of waste-frying oil on the production of biosurfactant by *Burkholderia cepecia* MSA47 as measured by the ODA method cm^2

± = Standard deviation, n=2

Substrates / ODA cm^2										
Oil	Gly	Gly Oil	Gl	Gl Oil	Fr	Fr Oil	Su	Su Oil	St	St Oil
133.4±3.3	176.6±0.0	176.6±0.0	105.0±7.1	176.6±0.0	52.3±2.4	176.6±0.0	15.1±0.6	85.1±3.4	-	150.0±0.0

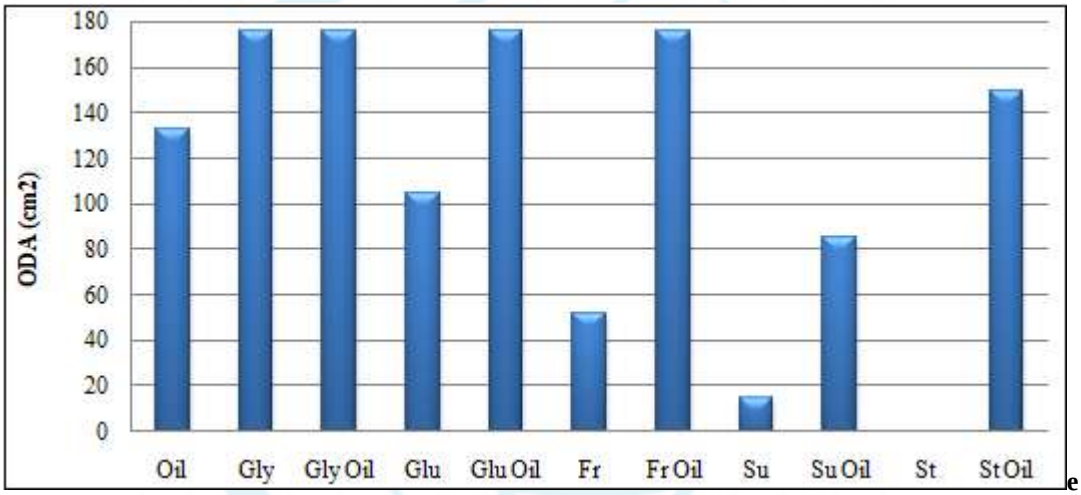

Figure 3: Effect of different soluble substrates in presence and absence of waste-frying oil on the production of biosurfactant by *Burkholderia cepecia* MSA47

Table 4. Effect of different nitrogen source on the production of biosurfactant by *Burkholderia cepecia* MSA47 as measured by the ODA method cm^2. Effect of different concentrations of sodium nitrate are also given

± = Standard deviation, n=2

Nitrogen Source	ODA cm^2	Concentration of Sodium Nitrate ($NaNO_3$) %	ODA cm^2
Ammonium Chloride	132.7 ±4.2	0.5	90.4 ±4.1
Ammonium Nitrate	176.6 ±0.0	1	153.8 ±8.2
Ammonium Phosphate	0.8 ±0.0	2	176.6 ±0.0
Ammonium Sulfate	113.0 ±0.0	3	176.6 ±0.0
Sodium Nitrate	153.0 ±6.4	4	176.6 ±0.0
Peptone	153.0 ±7.0	5	155.6 ±7.4

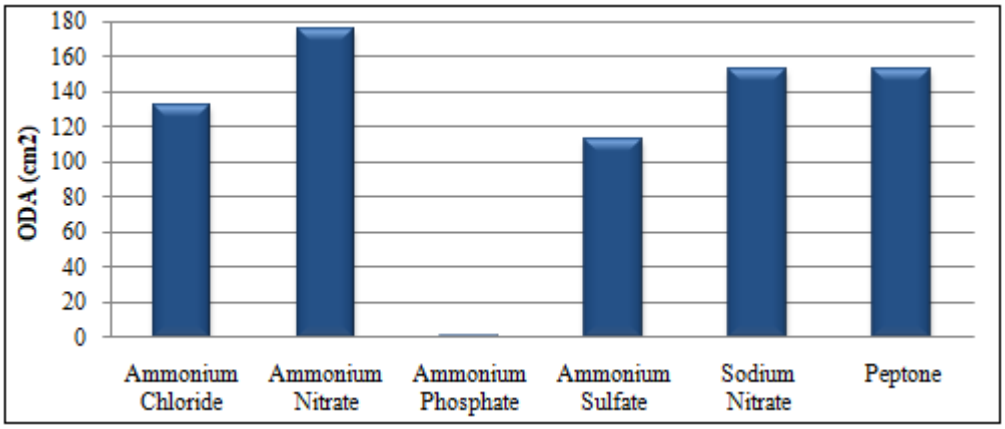

Figure 4: Effect of different nitrogen source on the production of biosurfactant by *Burkholderia cepecia* MSA47.

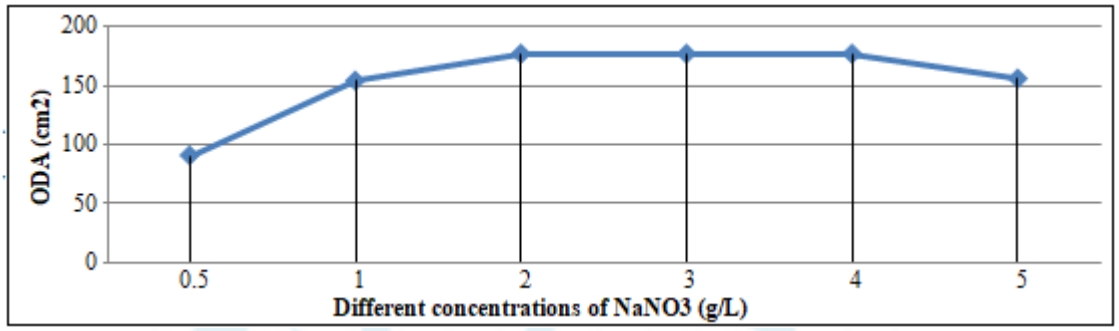

Figure 5: Effect of different concentrations of sodium nitrate on the production of biosurfactant by *Burkholderia cepecia* MSA47 as measured by the ODA method cm^2.

Table 5: Emulsification activity (E_{24}) and stability of the biosurfactant produced by *Burkholderia cepecia* MSA47 at wide ranges of salanity and pH values. (±)= Standard deviation, ODA= Oil displacement area, (n)= 2, (RA)= retained activity

Emulsification Activity		Stability Tests					
Oils	E_{24} (%)	pH	ODA cm2	RA (%)	NaCl (%)	ODA cm2	RA (%)
1. Crude Oil	66.3 ±1.1	2	113.0 ±3.2	64.0	0.0	176.7 ±0.0	100.0
2. Kerosene	57.8 ±3.1	5	132.7 ±2.1	75.1	2	176.7 ±0.0	100.0
3. Kerosene+Oil (1:1)	71.4 ±4.5	6	153.9 ±5.8	87.1	5	176.7 ±0.0	100.0
4. Paraffin Oil	54.8 ±1.7	7	176.7±0.0	100.0	10	153.9 ±4.6	87.1
5. Sunflower Oil	52.0 ±2.8	8	176.7 ±0.0	100.0	15	143.1 ±4.1	81.0
6. Olive Oil	58.5 ±1.8	9	153.9 ±5.2	87.1	20	132.7 ±3.6	75.1
7. Corn Oil	53.0±1.2	10	153.9 ±6.1	87.1	25	132.7 ±3.8	75.1
		11	122.7 ±4.1	69.4			
		12	122.7 ±3.8	69.4			

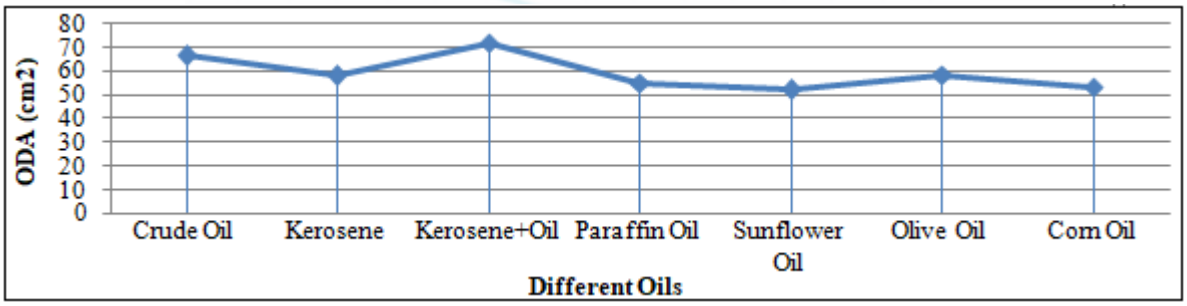

Figure 6: Emulsification Index (E_{24}%) of the biosurfactant produced by MSA47 strain against hydrocarbon oils and vegetable oils

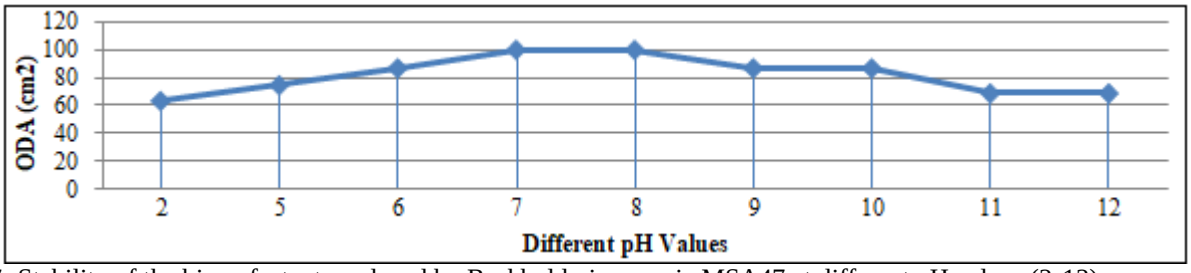

Figure 7: Stability of the biosurfactant produced by Burkholderia cepecia MSA47 at different pH values (2-12).

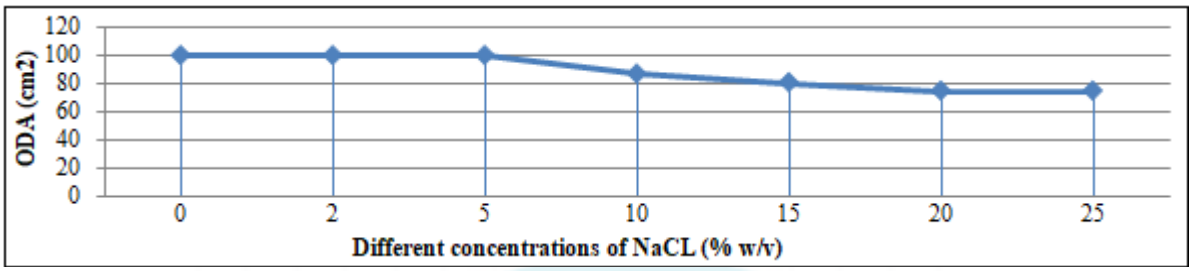

Figure 8: Stability of the biosurfactant produced by *Burkholderia cepecia* MSA47 at wide range of salinity (0-25% w/v).

Table 6: Biodegradation of total petroleum hydrocarbons (TBH) present in the oily sludge polluted the soil, after 90 days incubation period as affected by the addition of biosurfactants (BR), NP and BRNP.
(±) = Standard deviation, n= 3.

Treatments	Loss (%)
1. Biosurfactant alone (BR)	69.0 ±3.9
2. Nitrogen-Phosphorous (NP)	48.9 ±6.3
3. BR+NP	60.7 ±6.5
4. Control – 1 (culture medium free)	40.5 ±7.3
5. Control – 2 (no additives)	16.1 ±2.6

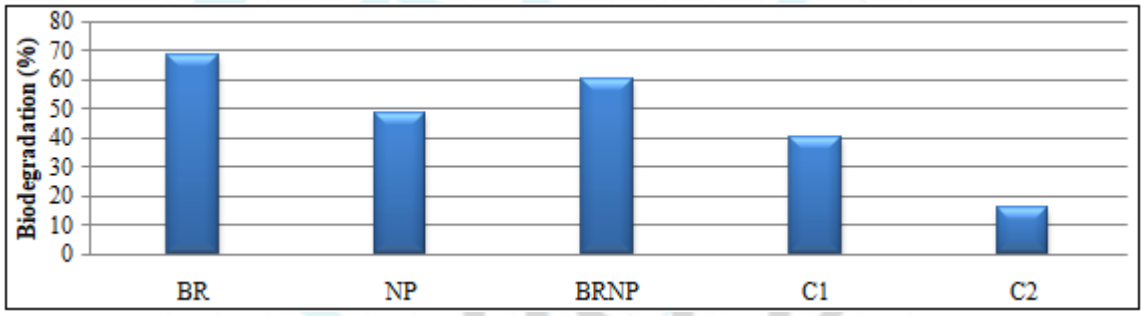

Figure 9: Biodegradation of TPH present in the oily sludge (Loss %) as affected by different treatments, after 90 days incubation.

Table 7: Biodegradation of PAH compounds present in the oily sludge as affected by the presence of biosurfactants (Br), NP and BRNP after 90 days incubation period. * A newly developed PAHs during treatments (not included in the recovered PAHs at 0-time).

PAHs	No. of Rings	Zero-time mg/kg soil	BR		NP		BRNP	
			Residual PAHs mg/kg soil	Loss %	Residual PAHs mg/kg soil	Loss %	Residual PAHs mg/kg soil	Loss %
1. Naphthalene	2	321.0	1.3	99.6	5.2	98.3	2.3	99.3
2. Acenaphylene	3	*	3.9*	*	-	-	-	-
3. Acenaphthene	3	100.0	-	100	-	100	5.2	94.8
4. Flourene	3	36.6	3.2	91.3	8.3	77.3	-	100.0
5. Phenanthrene	3	240.0	6.1	97.9	4.8	98.0	2.7	98.9
6. Anthracene	3	*	6.1*	-	3.4*	-	-	-
Total		**376.6**	**19.3**	**97.5**	**16.5**	**96.5**	**7.9**	**97.9**
7. Flouranthene	4	205.8	1.1	99.5	15.9	92.2	1.5	99.2
8. Pyrene	4	32.6	4.3	86.8	1.0	96.9	6.3	80.7
9. Benzo (a) anthracene	4	48.0	3.1	93.5	7.8	83.8	2.2	95.4
10. Chrysene	4	100.0	3.4	96.6	8.8	91.2	5.0	95.0
Total		**386.4**	**11.9**	**96.9**	**33.5**	**91.3**	**15.0**	**96.1**
11. Benzo (b) flouranthene	5	68.0	7.0	89.7	11.4	83.2	-	100
12. Benzo (k) flouranthene	5	8.0	3.3	58.6	6.7	16.3	1.1	86.3

13. Benzo (a) pyrene	5	*	2.3*	-	1.4*	-	2.7*	-
14. Dibenzo (a,h) anthracene	5	32.0	9.6	70.0	11.1	65.3	2.0	98.1
Total		**108.0**	**22.2**	**81.6**	**30.6**	**73.0**	**5.8**	**97.1**
15. Benzo (ghi) perylene	6	108.0	-	100	19.4	82.0	-	100.0
16. Indeno (1,2,3.c,d) pyrene	6	-	*	-	5.2*	-	-	-
Total		**108.0**	**-**	**100**	**24.6**	**82.0**	**-**	**100**
Overall Total		**1291.0**	**54.7**	**96.7**	**110**	**92.4**	**31**	**97.8**

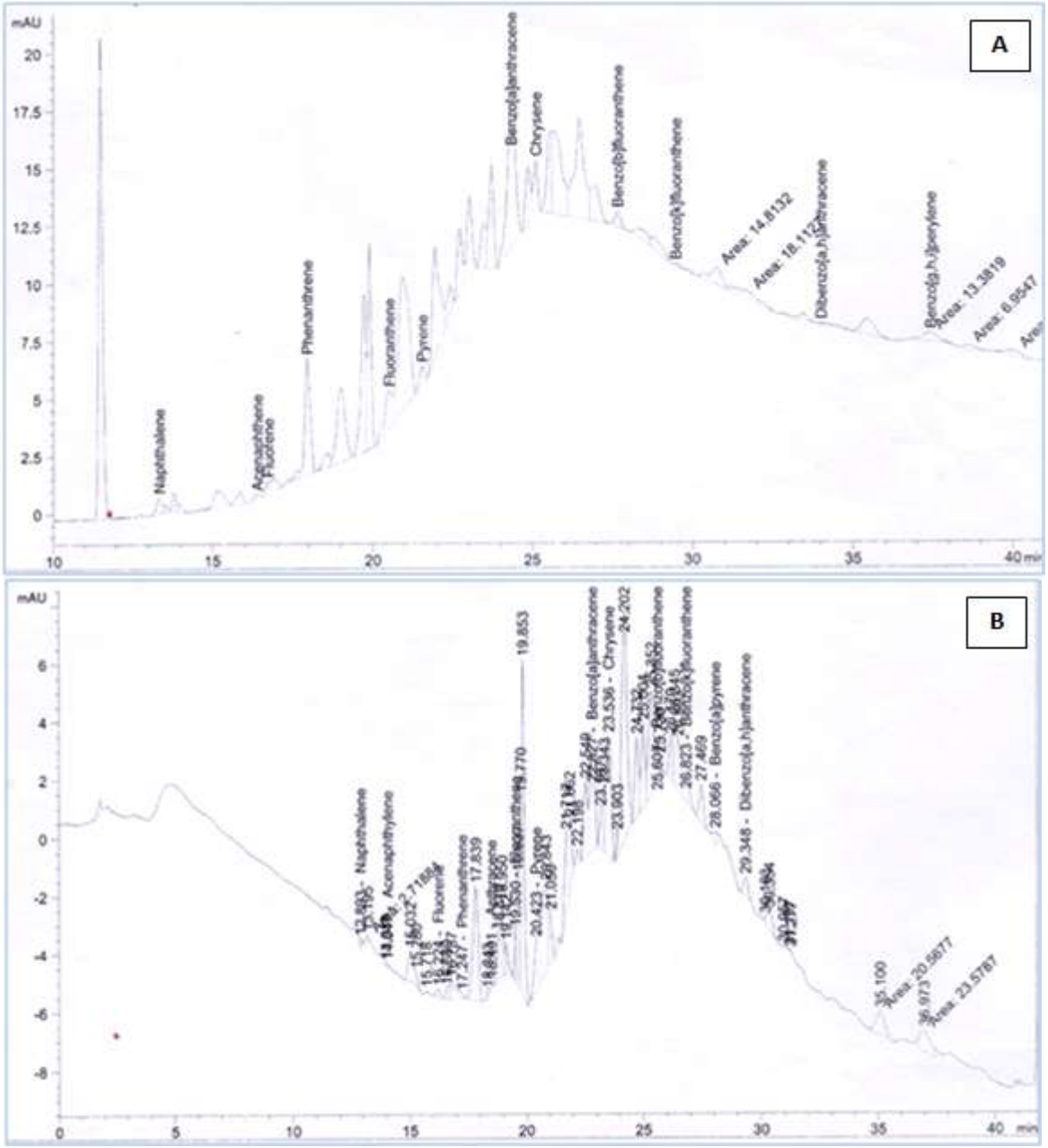

Figure 10: HPLC analysis of the residual PAH individuals present in the control (oil sludge only-A), oil sludge in presence of BR (B), oil sludge in presence of NP (C) and oil sludge in presence of a combination of BR and NP (BRNP)-D.

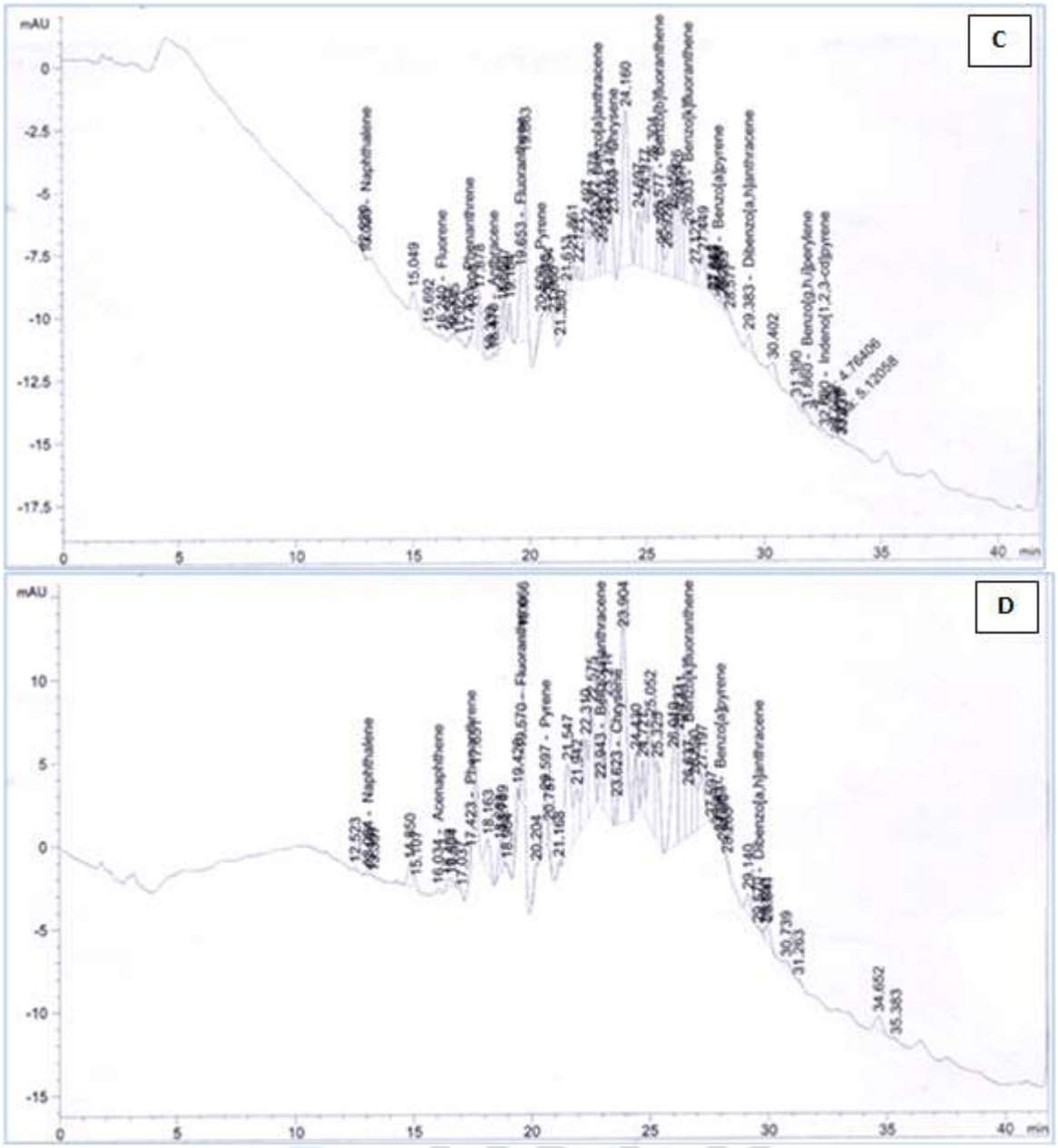

Figure 10: (Cont.)

Table 8: Biodegradation of the carcinogenic PAH individuals present in the oily sludge as affected by the presence of BR, NP and BRNP after 90 days incubation period. * = new developed PAHs during treatments (not included in the recovered PAHs at zero time).

PAHs	No. of Rings	Zero Time mg/kg	BR		NP		BRNP	
			Residual PAHs mg/kg	Loss %	Residual PAHs mg/kg	Loss %	Residual PAHs mg/kg	Loss %
Flouranthene	4	205.8	1.1	99.5	15.9	92.2	1.5	99.3
Benzo(a)anthracene	4	48.0	3.1	93.5	7.8	83.8	2.2	95.4
Chrysene	4	100.0	3.4	96.6	8.8	91.2	5.0	95.0
Total		353.8	7.6	97.9	32.5	90.8	8.7	97.5
Benzo(b)flouranthene	5	68.0	7.0	98.7	11.4	83.2	-	100.0
Benzo(k)flouranthene	5	8.0	3.3	58.6	6.7	16.3	1.1	86.3
Dibenzo(ah)anthracene	5	32.0	9.6	70.0	11.1	65.3	2.0	93.8
Total		108.0	19.9	81.6	29.2	73.0	3.1	97.1
Overall Total		461.8	25.2	94.0	55.1	86.6	11.8	97.4

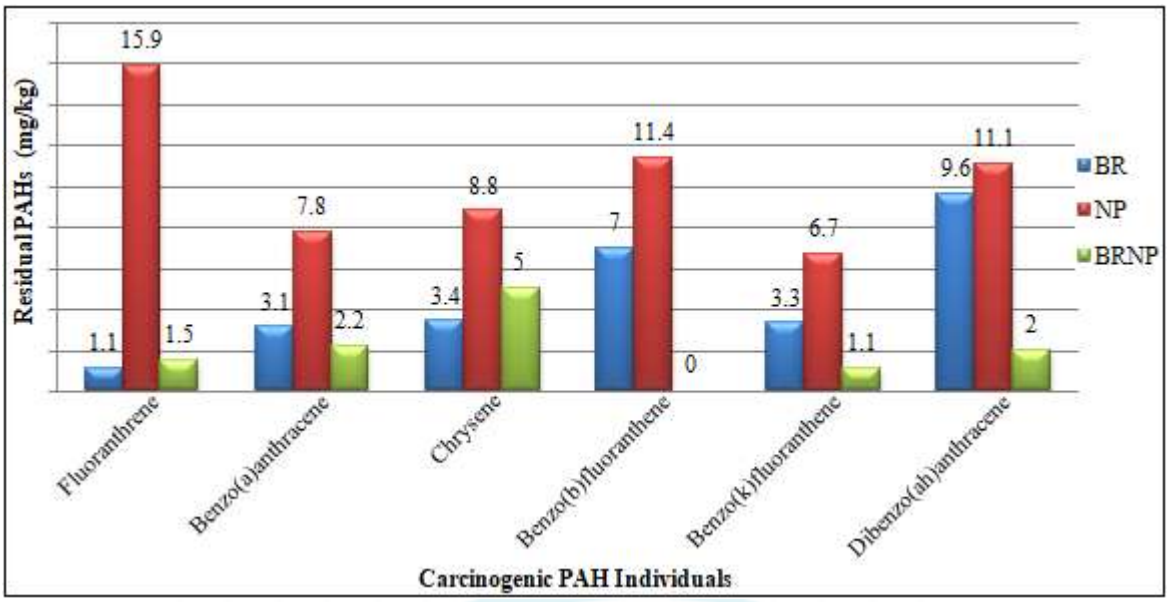

Figure 11: Residual carcinogenic PAH after treating the polluted soil with BR, NP and BRNP.