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Enterobacter cloacae MSA4, A New Strain Isolated from the Rhizosphere of a Desert Plant, Produced Potent Biosurfactant Used for Enhancing the Biodegradation of Polycyclic Aromatic Hydrocarbons (PAHs) During the Bioremediation of Spent Motor Oil-Polluted Sandy Soil

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Abstract: ***Ten bacterial strains were isolated from the rhizosphere of a desert plant and screened for the production of biosurfactant using three screening tests. One of the isolated strains (MSA4) was able to produce a potent biosurfactant; it was selected and identified by the 16S rRNA sequence determination and phylogenic analysis as Enterobacter cloacae MSA4. The crude biosurfactant produced by strain MSA4 was characterized by higher E_{24} values against crude oil (82.0 ±2.8%) and spent motor oil (96.0 ±2.1%). Accordingly, it was used for enhancing the biodegradation of PAHs during the bioremediation of spent motor oil-polluted sandy soil. Results of the HPLC analysis show that the potent carcinogenic PAH benzo(a)pyrene was of higher concentrations in the spent motor oil (112.1 ±1.4 mg/kg soil), followed by the two carcinogenic PAHs, benzo(b)flouranthene (110.2 ±2.5 mg/kg soil) and flouranthene (82.4 ±1.7 mg/kg soil). When the crude biosurfactant was applied to the spent motor oil-polluted soil, 98.5% of the US EPA 14 priority PAHs was degraded. On the addition of NP fertilizer alone no increase of the biodegradation above 89.5% was recorded, while the addition of BRNP, the biodegradation increased from 89.5% to reach 98.2%. This result indicates that the promising factor for enhancing the biodegradation of PAHs is the presence of BR. The present results show that the application of BR alone or in combination with NP fertilizer highly stimulated the native microorganisms in the polluted soil to highly or completely degrade different PAH individuals of the spent motor oil. The use of BR in combination with NP will reduce the actual amount of fertilizers needed for the biodegradation process. On the other hand the use of BR alone promoted the biodegradation of PAHs without using fertilizers; this will reduce the cost of the bioremediation process.***

Keywords: *Enterobacter cloacae* (MSA4), rhizosphere, biosurfactant, spent motor oil, biodegradation, PAHs

1. Introduction

Spent motor oils are common environmental pollutants, especially in many of the developing countries, in which these oils are illegally disposed by dumping in landfill, open vacant plots, drainage systems and farmland **(Gracia-Hernandez *et al*, 2007; Akoachere *et al*, 2008).** Un-used motor oil contains hydrocarbons (80-90%) and additives (10-20%). During use inside the motors, they are altered due to the breakdown of their constituents **(Ugoh and Moneke, 2011).** The used (spent) motor oils contain heavy metals, aliphatic and aromatic hydrocarbons such as PAHs **(Jain *et al*, 2009).** The spent motor oils when polluted the soil cause poor aeration, immobilization of nutrients and decrease the pH of the polluted sites **(Atuanya, 1987).** Heavy metals and PAHs found in the spent motor oil can alter soil properties and microbial community **(Objegba and Sadiq, 2002; Ugoh and Moneke, 2011).** Many of the PAHs found in these oils are highly toxic to plants, animals and humans when released to the environment **(Mandri and Lin, 2007; Wu *et al*, 2008).** Exposure to spent motor oils for a long time increases the risk of liver, kidney, bone marrow damage and cancer development **(Vazquez-Duhalt, 1989; Mishra *et al*, 2001; Lloyed and Cakette, 2011).**

It is well known that hydrocarbons may be adsorbed to soil particles, and their removal depends on their bioavailability to microorganisms. The root exudates of certain plants may increase the bioavailability of the pollutants and may selectively encourage the growth of the hydrocarbon-degraders in the rhizosphere of such plants. Microorganisms and plants have complementary role in the phytoremediation of oil polluted soil **(Joner *et al*, 2004).** The main factor for phytoremediation of hydrocarbon contaminated soil is the rhizosphere microflora **(Muratova *et al*, 2003).**

For the protection of the environment, oil waste pollutants must be removed or detoxified using safe and cost effective bioremediation process through which the native natural microorganisms can be adapted to degrade the hydrocarbons. The bioremediation technology in nature is slow and needs long period of time and may be time consuming, this is because these pollutants may be strongly adsorbed to soil particles, and are not available to microorganisms. For enhancing the bioremediation process, biosurfactant may be added to the polluted site in presence or in absence of fertilizers.

In the present work the biosurfactant produced by *Enterobacter* sp which was isolated from the rhizosphere of

a desert plant was used for enhancing the bioremediation of spent motor polluted desert soil.

2. Materials and Methods

1) Collection of rhizosphere soil sample.
Soil samples were collected from the rhizosphere (soil in direct contact with roots) of a desert salt marsh plant located at Borg Al Arab 30 km of Alexandria. Five samples were collected from different rhizosphere of the same plants. The samples were kept in ice box and transferred to the laboratory as soon as possible. In the laboratory the 5 samples were mixed to form one composite sample.

2) Isolation of the bacterial strains from the rhizosphere soil.
Five grams of the composite soil sample were introduced into 250 ml glass bottle containing 100 ml sterilized saline solution, and shaked on a shaker operated at 130 rpm for 15 min, then, serial dilutions 10^{-3}- 10^{-6} were prepared. One ml of each suitable dilution (10^{-3}- 10^{-6}) was used to inoculate a plate of inorganic salt agar medium. Three plates were inoculated from each dilution. After incubation of the plates at 30 °C for 7 days, ten colonies were isolated, purified and subcultured on slants of NA agar medium.

3) Preparation of the seed cultures.
Conical flasks (150 ml), each containing 40 ml of nutrient broth medium were inoculated with a loop of the purified bacterial strains found growing on a slant. The inoculated flasks were incubated at 30 °C for 24-48 h, after which one ml of each culture was used as an inoculum.

4) Screening for the production of the biosurfactant by the purified bacterial strains.
Conical flask (250 ml) each containing 100 ml of inorganic salt medium (ISM) supplemented with waste frying oil (2% v/v), each was inoculated by one ml of the seed culture (24-48 h old). The inoculated flasks were incubated at 30 °C on a shaker operated at 130 rpm for a period of 7 days. At the end of the incubation period, the cultures were sterilized and then were centrifuged at 6000 rpm for a period of 20 min for the removal bacterial cells. The cell-free broth culture (supernatant) was tested for the production of biosurfactant by the oil displacement area (ODA) as described by **Techaoei *et al* (2011),** blood hemolysis test **(Rahman *et al*, 2010)** and CTAB test **(Satpute *et al*, 2010).** The emulsification index (E_{24}) of each broth culture (supernatant) was carried out against crude oil, spent motor oil, olive oil and corn oil according to **Techaoei *et al* (2011)** and **Diab *et al*, (2017)**. The bacterial strain showing higher biosurfactant activities was selected and identified. The supernatant of the identified strain (containing the biosurfactant) was used for enhancing the biodegradation of PAHs during the bioremediation of spent motor oil polluted soil. The composition of the ISM medium was as follows (g/L water): $NaNO_3$, 2.0; K_2HPO_4, 2.0; $KHPO_4$, 1.0; KCl, 0.5; $CaCl_2$, 0.1; yeast extract, 0.1; sea water, 200 ml, trace salt solution, 0.1 ml. The pH was 7.0. The trace salt solution was as follows (g/L dist. Waster): $FeSO_4.7H_2O$, 0.1; H_3BO_3, 0.2; $CaCl_2.6H_2O$, 0.3; $CuSO_4.5H_2O$, 0.1; $MnSO_4.2H_2O$, 0.1; $ZnSO_4$, 0.1; $(NH_4)6Mo7O_{24}$, 0.1.

5) Treatment of spent motor oil polluted-soil.
A sandy soil sample was polluted in the laboratory by spent motor oil (3.5% w/w). For comparison a soil sample was polluted by 3.5% (w/w) non-used motor oil. The polluted soil was treated as follows: Soil microcosm test was designed to include 4 treatments in duplicates. Each consisting of 500 ml glass beaker containing 100 gm of the spent motor oil-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/100 g soil) and K_2HPO_4 (50 mg/100 g soil). Biosurfactant produced by *E.cloacae* was added (5 ml/100 g soil) in the form of sterilized supernatant. A small glass rod was introduced to each beaker for tilling the soil. The moisture content was adjusted at 5% by adding tap water. All of the treatments were covered with thin aluminum foil to reduce evaporation of water. All the treatments were incubated at temperature (30 °C). The loss of water due to evaporation was determined at the beginning of the experiment and every 2-3 days.

6) Extraction and determination of the residual oil
At the beginning of the experiment and at the end of 40 days incubation period, 5 grams of the air dried soil was mixed by the same amount of anhydrous sodium sulfate. The residual oil in the soil was extracted by n-hexane by using the shaking method **(Chen *et al*, 1996).** The extract was collected and evaporated in a pre-weighted dish, and then the amount of residual oil can be determined.

7) HPLC analysis of the n-hexane extract for the resolution of different PAH individuals found in the residual spent motor oil.
The listed US-EPA 16 polycyclic aromatic hydrocarbons (PAHs) in n-hexane extract was analyzed and quantified by using High Performance Liquid Chromatography (HPLC), Model agilent-1200 series equipped with DAD detector (model 1260 infinity) according to standard test method (EPA 550 and EPA 610). Calibration Curve was done by EPA PAH mix standards (Supelco, USA, 99%) of known concentrations.

8) Identification of the bacterial strains.
The bacterial strain MSA4 was identified according to Bergy's Manual of Determinative Bacteriology **(Holt *et al*, 1994),** and was confirmed by Amplification of 16S rRNA gene. Genomic DNA of the bacterial cells was isolated using Wizard® Genomic DNA Purification Kit (Promega® Corporation, USA). The Primers [5'-AGA GTT TGA TCC TGG CTC AG-3'] and [5'-AAG GAG GTG ATC CAG CC-3'] were used to amplify nearly full-length 16S rRNA gene (1500 bp). PCR was performed using the standard reaction mixture (50 µl) containing: 1 X PCR buffer, 1.5 Mm $MgCl_2$, 5% dimethyl sulfoxide, 200 mM of each dNTPs, 15 pmol of each primer, 1 U of Taq polymerase enzyme (Promega®)

and 50 ng of DNA template. PCR reaction conditions were: an initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 54 sec, and extension at 72 °C for 2 min. Reactions were performed in a PCR Thermo cycler machine (Biorad®, USA). PCR products were separated on 1% agarose gel. DNA fragments were further purified and cleaned up by Q1Aquick Gel extraction kit, Qiagen® according to the kit manual instructions.

9) 16S rRNA sequencing and phylogenetic analysis

16S rRNA fragment was sequenced using the specific primers. The sequence reads were edited and assembled using the DNASTAR software (Lasergene, Madison, WI). Sequence similarity searches were performed at the National Center for Biotechnology Information (NCBI) server using BLASTIN (http://www.ncbi.nlm.nih.gov/blast). The sequences were aligned using Clustal W version 1.8 and subjected to phylogenetic tree in the context of 16S rRNA sequences was conducted using the Neighbor-Joining (NJ) method and 1000 bootshap replication to assess branching confidence.

3. Result and Discussion

Ten bacterial strains were isolated from the rhizosphere of a desert plant and were screened for the production of biosurfactant. The results (Table 2, Figure 1) show that 80% of the screened strains were positive for the ODA method, while 40% of these isolates were positive for the production of biosurfactant by using both CTAB and blood hemolysis test. One strain (MSA4) produced the highest ODA value (158.4 ±6.3 cm^2, Figure 1, a), followed by strain (MSA7) which produced 116.9 ±5.4 cm^2. The two strains showed positive results for CTAB (31 ±1.2mm) and for blood hemolysis (34 ±1.6mm) (Figure 1, b-c).

Using the 16S rDNA specific primers, nearly full-length 16S rDNA gene region was successfully amplified and sequenced. The partial length 16SrRNA was submitted to GenBank under accession number KY744226. According to the sequences similarity, MSA4 isolate was identified as members of *Enterobacter cloacae.* A phylogenetic tree representing different *Enterobacter* species with the local isolate was constructed (Figure 2) by Neighbor-Joining (NJ). It showed that MSA4 isolate showed high degree of genetic resemblance to *Enterobacter cloacae.*

This strain was then studied for its E_{24} and stability tests at wide range of pH values and salinity (Table 3). The results show that *E.cloacae* MSA4 was able to produce higher E_{24} values against crude oil and spent motor oil (82.0 ±2.8 and 96.0 ±2.1% respectively). At the same time, the biosurfactant produced by this strain was stable at high temperature (0-121 °C), wide range of pH values (2-12) and wide range of salinity (5-20% w/v NaCl). The sterilized supernatant containing the biosurfactant was used for enhancing the biodegradation of polycyclic aromatic hydrocarbons (PAHs) during the bioremediation of spent motor oil polluted sandy soil.

Polycyclic aromatic hydrocarbons (PAHs) content of the used motor oil at the beginning and at the end of 40 days bioremediation period were identified and quantified using HPLC analysis technique. As a comparison PAH contents of the unused motor oil was also identified and quantified by the same method. The results (Table 4) demonstrate the resolution of 14 different US EPA priority PAHs, both from spent and un-used oils with total 696.1 mg/kg soil and 458.4 mg/kg soil respectively. It is important to find out that in the used oil the potent carcinogenic PAH namely benzo(a)pyrene is of higher concentration (112.1 ±1.4 mg/kg soil, with 16.1% of the total) this is in comparison to 60.0 ±0.7 mg/kg soil (13.1 %) found in non-used oil. Followed benzo(a)pyrene in the spent oil, benzo(b)flouranthene (110.2 ±2.5 mg/kg soil, with 15.8% of the total) and flouranthene (82.4 ±1.7 mg/kg soil, with 11.8%). It was observed from Table (4) that the unused motor oil is characterized by the presence of 0.1% anthracene and 23.6% benzo(a)anthracene, while in used motor oil these two PAHs are absent. On the other hand, benzo(ghi)perylene and acenaphthene are absent in the unused oil, and present in the used oil (with 1.9% and 1.6% respectively). All of the other individual PAHs are more frequent in the spent motor oil than in the un-used oil. **Cofield *et al* (2007)** found that at the beginning of the experiment, benzo(a)pyrene was of higher content in the oil polluted soil sample (21.2%) followed by pyrene and flouranthene. **Diab and Sandouka (2010)** found that the highest PAH content in an oil-polluted soil sample were pyrene (14.6%) and flouranthene (11.8%) relative to the 16 US EPA priority PAHs. Al-**Gounaim and Diab (2004)** found that flouranthene and pyrene were more frequent in oil polluted soil sample (15.3% and 12.4% respectively) than the other PAHs of the 16 US EPA.

In the present work, flouranthene was represented by 11.8% and 2.6% (relative to the 14 US EPA PAHs) in used oil and un-used oil respectively. Flouranthene has been reported to be cytotoxic, mutagenic and potentially carcinogenic (**McElroy *et al*, 1989' Irvin and Martin, 1987**). While there are hundreds of PAHs, the US EPA has priority list of 16 PAHs that pose the most concern of the environment **(Ball and Truskewycz, 2013).** The following PAH: benzo(a)pyrene, 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 International Agency for Research on Cancer (IRAC) classified benzo(a)anthracene as probable human carcinogens (Class A) and the following PAHs: benzo(b)flouranthene, benzo(k)flouranthene and benzo(a)pyrene as possible human carcinogens (Class B) **(Chauhan *et al*, 2008). Anyakora *et al* (2004)** reported that the exposure to PAHs has been linked to obesity, cancer, lower IQ, inhibition growth, endocrine disruption and diabetes. **Irrigaray *et al* (2006)** indicated that benzo(a)pyrene impaired adipose tissue lipolysis which let to weight gain and increased appetite in mice. They found also that after the withdrawer of benzo(a)pyrene, the mice retained their evaluated weight.

When the total concentrations of the carcinogenic PAH found in the used (spent) motor oil are compared with the un-used oil, the results show that high concentration of 417.3 mg/kg soil (with about 60% of the total 14 PAHs) was recorded from the spent motor oil, while 236.4 ml/kg soil (51.6%) was resolved from the un-used oil. The used oil included 7 carcinogenic PAHs (Table 7) of which the most

frequent PAH was benzo(a)pyrene (112.1 ±1.4 mg/kg soil and of 26.9% of the total of the 7 PAHs). On the other hand, 8 carcinogenic PAHs were recorded from the un-used motor oil of which benzo(a)anthracene was most frequent (108.0 mg/100 kg soil and of 45% of the total 8 PAHs). It is of interest to observe that benzo(a)anthracene was recorded only from the un-used oil, and disappeared while the motor of the vehicle is working. On the other hand the used motor oil contained higher level of carcinogenic PAHs as compared to the unused oil. Accordingly, spent motor oil contaminations need more attention due to its hazardous effects. Hence for the sake of human health and environment these harmful compounds must be removed or detoxified by using a safe and cost effective strategy such as bioremediation.

Results of the application of crude biosurfactant (BR), NP and a combination of BRNP on the biodegradation of the US EPA 14 priority PAHs are found in Table (5) and illustrated in Figure (3). From these results the following can be summarized:

When biosurfactant (BR) was applied to the spent motor oil-polluted soil, 98.5% of the total US EPA 14 priority PAHs was degraded. On the addition NP fertilizer alone, no increase of the biodegradation above 89.5% was recorded, while addition of a combination of BRNP, the biodegradation increased from 89.5% to reach 98.2% which is equal to that obtained in the presence of BR alone. These results indicate that the promising factor for enhancing the biodegradation process is the presence of BR.

As for the biodegradability of each individual PAH, the result (Table 5, Figure 3) show that the biodegradation of each PAH was affected by the different types of treatments and the number of aromatic rings in each individual. For simplicity results are summarized in Table (3) and illustrated in Figure (4), according to the biodegradability of each individual as follows:

a) Completely Degradable Group (99.9-100% degradation)

In presence of BR alone, 6 PAHs (of which 3 are carcinogenic) of this group were degraded, while in presence of BRNP 7 PAHs (of which 2 PAHs are known to be carcinogenic) were degraded. On the other hand in presence of NP alone, only one PAH of this group was degraded.

b) Highly Degradable Group (95-98% degradation)

This group includes 5 PAHs highly degraded in presence of BR alone, of which 3 individuals are carcinogens. In the presence of BRNP, 5 individuals are highly degraded, of which 4 individuals are carcinogens. On the other hand no individuals of this group are recorded in the presence of NP alone.

c) Moderately Degradable Group (80-94% degradation)

In presence of BR alone, three individual PAHs were moderately degraded, of which one individual only is a carcinogen. In presence of BRNP only one individual was recorded, while in presence of NP alone 8 individuals were moderately degraded of which 5 individuals are carcinogenic.

d) Weakly Degradable Group (Less than 80% degradation)

This group was absent in presence of BR alone, and one individual only was recorded in presence of BRNP, while 5 individuals (of which two are carcinogenic) were weakly degraded in presence of NP alone.

The above results leads to the conclusion that the presence of NP fertilizer alone failed to stimulate the completely degradable and the highly degradable PAH individuals as compared to the presence of BR and BRNP, while NP alone was only able to stimulate the moderately and weakly biodegradable PAHs.

As for the biodegradation of the carcinogenic PAH individuals found in the spent motor oil polluting this type of soil, the results (Table 7, Figure 5) show that seven carcinogenic PAHs were resolved. As a total, they are represented by 417.3 mg/kg soil (59.9% of the total 14 priority PAHs). After 40 days bioremediation period the biodegradation of the total was 98.6% in presence of BR alone. In presence of NP fertilizer alone the biodegradation failed to increase above 87.6%. On the other hand the addition of a combination of BRNP, the biodegradation increased to reach the same level of biodegradation that was achieved in presence of BR alone. **Blyth *et al* (2015)** found that the addition of biosurfactant alone to a polluted soil significantly increased the biodegradation of the total US EPA 16 priority PAH to 78.7%, compared to62% in the absence of biosurfactant. The same authors found that the biodegradation of the 3-4 and 5 ringed PAHs was 75%, 76% and 85% respectively in presence of biosurfactant, while in the control the biodegradation was 42%, 60%, and 82% respectively.

The results (Table 7, Figure 5) show that the six ringed PAH indeno(1,2,3-c,d)pyrene and the 5-ringed PAH benzo(a)pyrene were completely degraded (100% and 99.6%) in presence of BR alone and BRNP. The PAH dibenzo(ah)anthracene was less degraded in presence of NP alone (52.0%) and BRNP (60%) while other PAHs were highly degraded (95.3%-99.1%) in presence of BR alone and BRNP respectively. **Patowary *et al* (2015)** found that a biosurfactant-producing strain of *Pseudomonas aeruginosa* was able to degrade 79.16% TPH in four weeks, and completely degraded 8 PAH individuals of which two carcinogenic PAHs were dibenzo(ah)anthracene and indeno(1,2,3-c,d)pyrene.

The present results show that the application of biosurfactant alone or in combination with NP fertilizer highly stimulated the native organisms in the spent motor oil-polluted soil to highly or completely degrade the different PAH individuals of the spent motor oil. The use of biosurfactant in combination with fertilizers reduces the actual amount of fertilizer needed for the bioremediation of the polluted site. On the other hand, biosurfactant alone are able to promote the biodegradation process without using fertilizers. This will reduce the cost of the bioremediation process (**Thavasi *et al*, 2011, a, b). Eruke and Udoh (2015**) reported that the application of biosurfactant for the bioremediation of hydrocarbon-polluted sites is more acceptable due to the

natural properties of these compounds such as their unusual structure, biodegradability, low or no toxicity, cost effective and other useful characters. This makes the biosurfactant as alternative to the chemically synthesized surfactants for enhancing the biodegradation of hydrocarbons in polluted soils and water.

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Table 2: Screening the 10 bacterial strains for the production of biosurfactant by using the ODA, CTAB and blood hemolysis tests. ± = Standard deviation, n = 2

Bacterial Strains	ODA (cm^2)	CTAB (mm)	Blood Hemolysis (mm)
MSA 1	29.2±1.3	20 ±1.0	22 ±1.1
MSA 2	39.6±1.6	-	-
MSA 3	34.8±2.2	-	-
MSA 4	158.4±6.3	31 ±1.2	34 ±1.6
MSA 5	-	-	-
MSA 6	-	-	-
MSA 7	116.9±5.4	31 ±1.4	22 ±1.4
MSA 8	13.3±0.7	19 ±0.8	20 ±0.7
MSA 9	16.6±1.0	-	-
MSA 10	6.9±0.4	-	-

Table 3: Emulsification activity (E_{24}) and stability of the biosurfactant produced by *E.cloacae* MSA4 at wide range of salinity and pH values. (±) = Standard deviation, n = 2.

Emulsification Activity		Stability Test			
Oils	E_{24} (%)	NaCl (%)	Activity (ODA CM^2)	pH	Activity (ODA CM^2)
1. Crude Oil	82.0±2.8	5	144.8±2.8	2	20.4±1.1
				5	34.8±2.3
2. Spent Motor Oil	96.0±2.1	10	118.9±7.8	6	81.7±4.3
				7	152.8±1.6
3. Olive Oil	61.0±1.4	15	94.3±1.0	8	133.7±1.0
				9	113.6±0.8
4. Corn Oil	60.5±2.1	20	52.8±3.7	10	62.0±2.3
				12	27.4±1.3

Table 4: HPLC analysis of used and un-used motor oil for the resolution of the different PAH individuals of the 16 US EPA priority PAHs, at the beginning of the experiment.
±= Standard deviation, n= 2. Percentages relative to the overall total are given.

PAHs	No. of Rings	Used Motor Oil		Un used Motor Oil	
		Mg/kg Soil	(%)	Mg/kg Soil	(%)
1. Naphthalene	2	**104.8±2.8**	**15.1**	**144.0±2.8**	**31.4**
2. Acenaphthylene	3	61.4±1.8	8.8	32.9±0.6	7.2
3. Acenaphthene	3	10.8±0.1	1.6	-	-
4. Flourene	3	23.7±0.6	3.4	5.5±0.1	1.2
5. Phenanthrene	3	54.0±1.4	7.8	27.0±0.6	5.9
6. Anthracene	3	-	-	0.3±0.01	0.1
Total		**149.9**	**21.6**	**65.7**	**14.3**
7. Flouranthene	4	82.4±1.7	11.8	12.7±0.3	2.6
8. Pyrene	4	11.2±0.3	1.6	8.0±0.3	1.7
9. Banzo(a)anthracene	4	-	-	108.0±1.4	23.6
10. **Chrysene**	4	56.2±1.3	8.1	14.1±0.4	3.1
Total		**149.8**	**21.5**	**141.2**	**30.8**
11. Benzo(b)flouranthene	5	110.2±2.5	15.8	24.0±0.8	5.2
12. Benzo(k)flouranthene	5	23.3±0.4	3.3	13.0±0.1	2.8
13. Benzo(a)pyrene	5	112.1±1.4	16.1	60.0±0.7	13.1
14. **Dibenzo(ah)anthracene**	5	2.5±0.1	0.4	5.2±0.3	1.1
Total		**248.1**	**35,6**	**102.2**	**22.3**
15. Benzo(ghi)perylene	6	12.9±0.4	1.9	-	-

16. **Indeno(1,2,3-c,d)pyrene**	6	30.6±0.6	4.4	4.4±0.1	1.0
Total		**43.5**	**6.2**	**4.4**	**1.0**
Overall Total		**696.1**		**458.4**	

Table 5: Biodegradation (Loss %) of the individual PAHs present in the spent motor oil as affected by the presence of biosurfactant (BR), NP and BRNP at the end of 40 days incubation period

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	**104.8**	**-**	**100.0**	**0.0**	**100.0**	**0.08**	**99.9**
2. Acenaphthylene	3	61.4	-	100.0	6.0	90.7	-	100.0
3. Acenaphthene	3	10.8	1.2	88.9	3.0	72.2	2.1	88.6
4. Flourene	3	23.7	0.2	99.2	8.0	66.2	0.2	99.2
5. Phenanthrene	3	54.0	1.2	97.8	8.0	85.2	2.6	95.2
Total		**149.9**	**2.6**	**98.3**	**25.0**	**83.3**	**4.9**	**96.7**
6. Flouranthene	4	82.4	1.4	98.3	17.0	79.4	1.5	98.2
7. Pyrene	4	11.2	1.1	90.1	2.6	76.8	-	100.0
8. Chrysene	4	56.2	1.1	88.0	4.6	91.8	2.6	95.3
Total		**149.8**	**3.6**	**97.6**	**24.2**	**83.8**	**4.1**	**97.3**
9. Benzo(b)flouranthene	5	110.2	1.6	99.1	12.1	89.0	2.0	98.8
10. Benzo(k)flouranthene	5	23.3	1.0	95.7	2.1	91.0	0.4	98.3
11. Benzo(a)pyrene	5	112.1	0.5	99.6	11.6	89.7	-	100.0
12. Dibenzo(ah)anthracene	5	2.5	0.4	84.0	1.2	52.0	1.2	60.0
Total		**248.1**	**3.5**	**98.6**	**27.0**	**89.1**	**3.6**	**98.5**
13. Benzo(ghi)perylene	6	12.9	0.6	95.3	1.8	86.0	-	100.0
14. Indeno(1,2,3-c,d)pyrene	6	30.6	-	100.0	3.0	90.1	-	100.0
Total		**43.5**	**0.6**	**98.6**	**4.8**	**89.0**	**-**	**100.0**
Overall Total		**696.1**	**10.3**	**98.5**	**73.0**	**89.5**	**12.7**	**98.2**

Table 6: Grouping the biodegraded PAH individuals according to their biodegradability in presence of biosurfactants (BR), NP and a combination of BRNP. T=Total Number, C= Number of Carcinogenic PAHs.

Groups (Biodegradation %)		Numbers of Individual PAHs		
		BR	NP	BRNP
A. Completely Degradable (99.9%-100%)	T C (C%)	6 3 50%	1 - -	7 2 28.6%
B. Highly Degradable (95%-98%)	T C (C%)	5 3 60%	- - -	5 4 80%
C. Moderately Degradable (80%-94%)	T C (C%)	3 1 33.3%	8 5 62.5%	1 - -
D. Weakly Degradable (Less than 80%)	T C (C%)	- - -	5 2 40%	1 1 100%
Overall Total	**T C (C%)**	**14 7 50%**	**14 7 50%**	**14 7 50%**

Table 7: Biodegradation (Loss %) of the individual carcinogenic PAHs present in the spent motor oil as affected by the presence of biosurfactant (BR), NP and BRNP at the end of 40 days incubation period

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. Flouranthene	4	82.4	1.4	98.3	17.0	79.4	1.5	98.2
2. **Chrysene**	4	56.2	1.1	88.0	4.6	91.8	2.6	95.3
Total		**138.6**	**2.5**	**98.2**	**21.6**	**84.4**	**4.1**	**97.0**
3. Benzo(b)flouranthene	5	110.2	1.6	99.1	12.1	89.0	2.0	98.8
4. Benzo(k)flouranthene	5	23.3	1.0	95.7	2.1	91.0	0.4	98.3
5. Benzo(a)pyrene	5	112.1	0.5	99.6	11.6	89.7	-	100.0
6. **Dibenzo(ah)anthracene**	5	2.5	0.4	84.0	1.2	52.0	1.2	60.0
Total		**248.1**	**3.5**	**98.6**	**27.0**	**89.1**	**3.6**	**98.5**
7. Indeno(1,2,3-c,d)pyrene	6	30.6	-	100.0	3.0	90.1	-	100.0

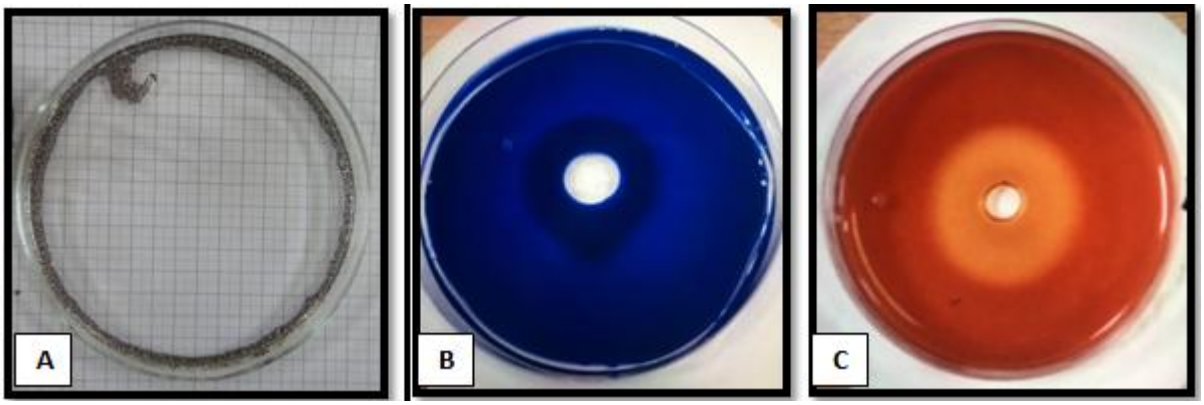

Figure 1: Photograph showing:

A. Activity of the biosurfactant produced by *E.Cloacae* as measured by ODA cm^2 method
B. Activity of the biosurfactant produced by *E.Cloacae* as measured by CTAB method
C. Activity of the biosurfactant produced by *E.Cloacae* as measured by Blood Hemolysis test

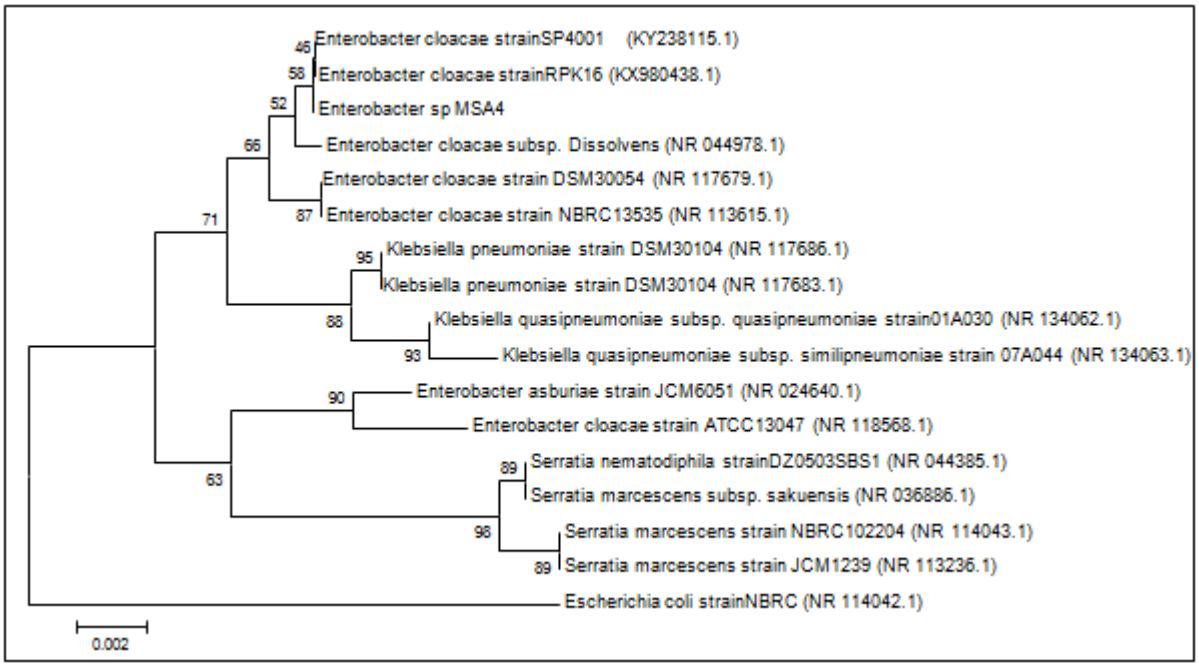

Figure 2: Phylogenic tree based upon the 16S rRNA sequences obtained by neighbor-joining method. The tree shows the phylogenetic positions of clone MSA4 to reference type strains of different species.

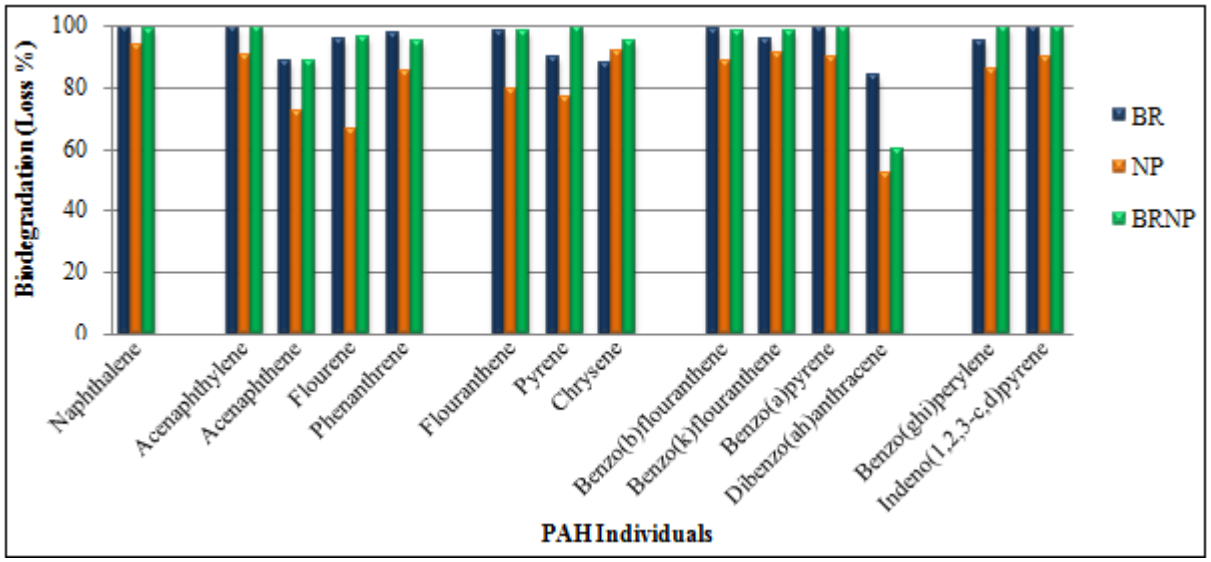

Figure 3: Biodegradation (Loss %) of the individual PAHs present in the spent motor oil as affected by the addition of biosurfactant (BR), NP and BRNP after 40 days of incubation period.

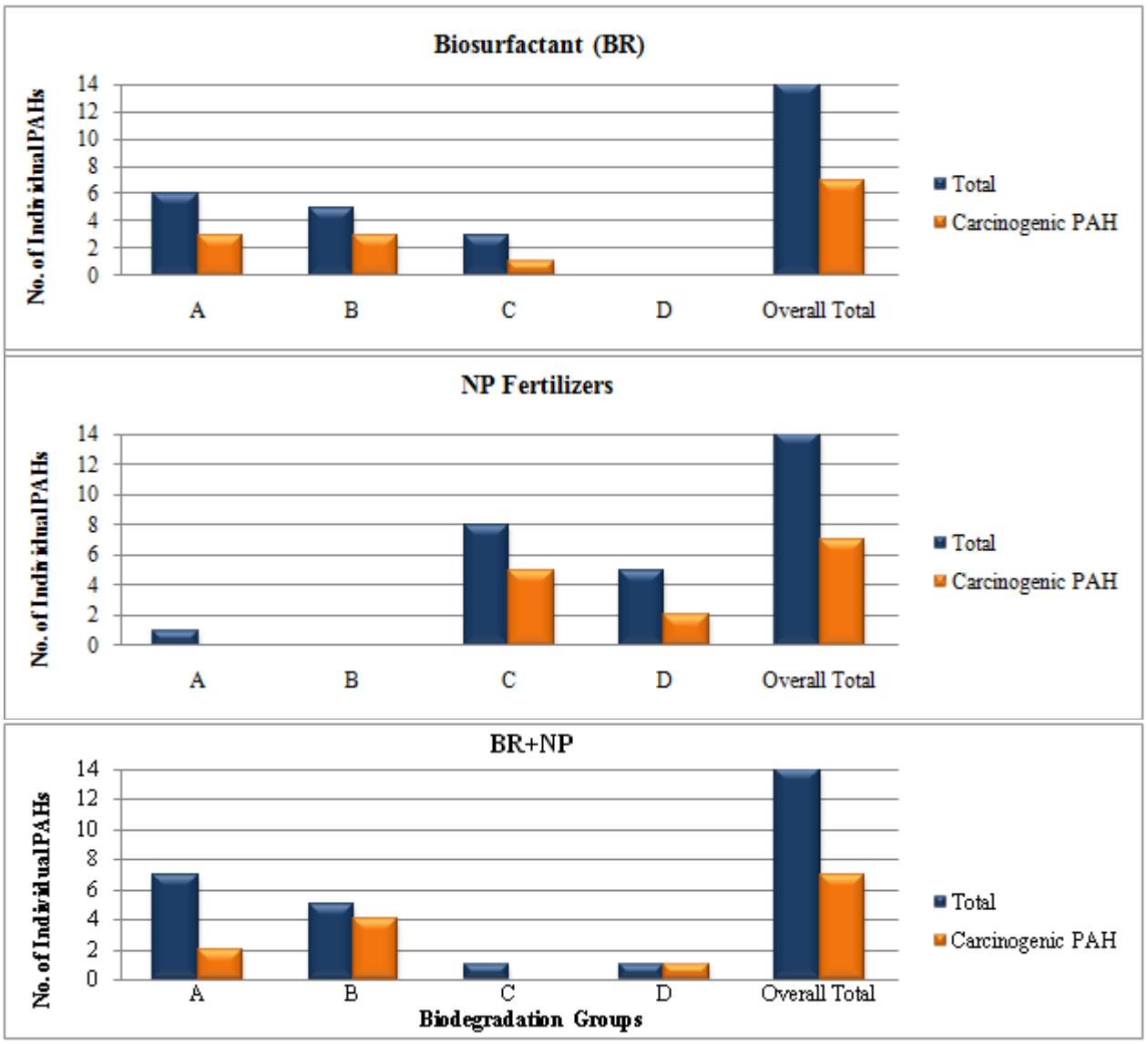

Figure 4: Numbers of PAH individuals in each biodegradation group. Numbers of the carcinogenic PAH (C) in each group are also found

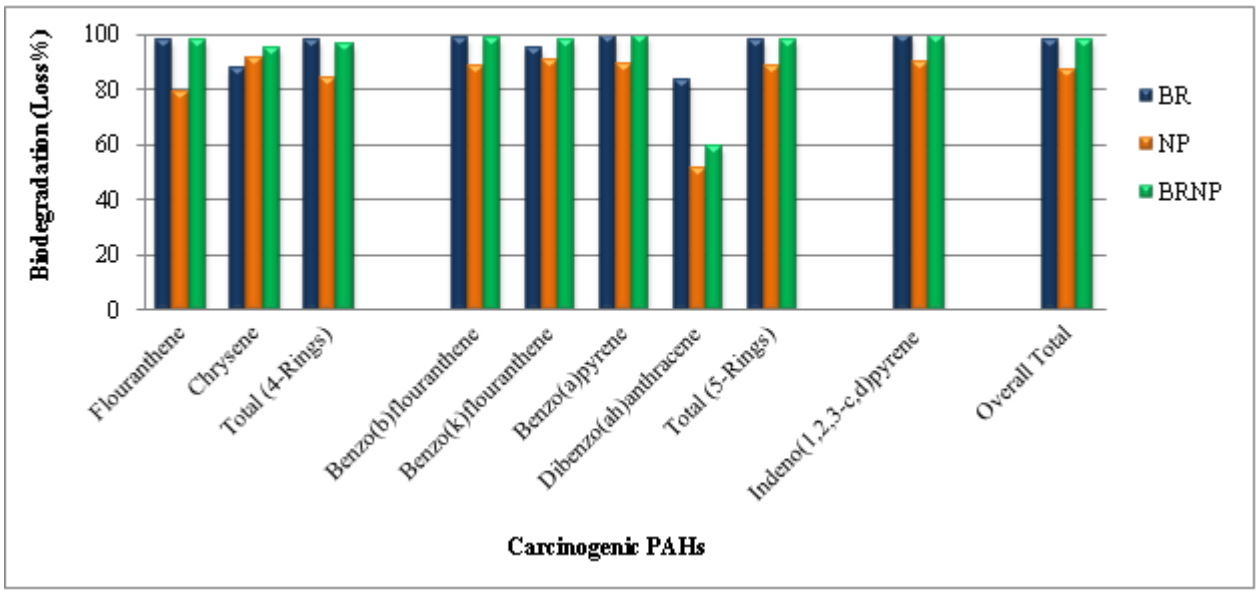

Figure 5: Biodegradation (Loss %) of the carcinogenic PAH individuals present in the spent motor oil as affected by the presence of biosurfactant (BR), (NP) and (BRNP) after 40 days of incubation period.