International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2015): 78.96 | Impact Factor (2015): 6.391

Serratia marcescens P25, A New Strain Isolated From The Phycoplane of the Red Marine Alga *Punctaria* sp Produced Potent Biosurfactant Used for Enhancing the Bioremediation of Spent Motor Oil-Polluted Soil

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Abstract: ***Samples of marine algae were collected from a coastal area at Abokir, Alexandria, and identified as: Punactraia sp (brown alga), Colpomenia sp (brown alga), Jania sp (red alga) and Ulva sp (green alga). 25 bacterial strains were isolated and purified from the phycoplane of Punctaria sp and were screened for the production of biosurfactants using cheap substrate (waste frying oil). The production of biosurfactants was tested by the plug agar and the ODA method. 60% and 80% of the tested strains were biosurfactant producers when the plug agar and the ODA method respectively used. Five red pigmented strains were very active biosurfactant producers (158-167.6 Cm^2 ODA) were selected and studied for their emulsification activity and their stability at wide range of temperature (0-121°C), pH values (2-12) and salinity (5-25% NaCl-W/V). The selected five bacterial strains were identified as members of Serratia marcescens. Strain P25 was characterized by producing a biosurfactant of more stability at wide range of temperature, pH and salinity, this is in addition to its ability to produce high emulsion activity against spent motor oil. The produced emulsion was stable at 7-30 days. The above characters give this Serratia marcescens P25 strain a potential application in petroleum industry such as cleaning oil storage tanks, recovery of oil from oily sludge, microbial enhanced oil recovery (MEOR),washing oil- contaminated soil and enhancing the bioremediation of hydrocarbon- contaminated sites. The cell free culture broth (supernatant) containing the biosurfactant that was produced by strain P25 was sterilized and applied for the bioremediation of spent motor oil- contaminated soil. The result show that the addition of the sterilized supernatant alone increased the biodegradation of the oil to 65.0±5.2%. Addition of NP fertilizer alone failed to increase the biodegradation more than 46.0±2.0%, while in the presence of a mixture of biosurfactant and NP (BRNP) the biodegradation increased to 60.0±5.0%. Statistically, no significant difference between the result in presence of BR in the presence of BRNP (P>0.05). It can be concluded that the promising factor in the biodegradation of spent motor oil is the addition of BR alone or in combination with NP.***

Keywords: Serratia marcescens, Marine algae, Biosurfactant, Bioremediation, Spent motor oil

1. Introduction

The marine environment supports good sources for discovering bioactive compounds such as biosurfactants, drugs, antibiotics and others. Most of the biosurfactants produced by marine microorganisms are characterized by antibacterial, anti-adhesive and anti-biofilms activities against different pathogens **(Gudina *et al*, 2016).**

Marine microorganisms have characteristic activities that stimulate the production of unique new bioproducts such as biosurfactant and other bioactive material. However, marine biosurfactants are not widely explored mainly due to difficulties associated with the isolation and growth of their producing microorganisms **(Gudina *et al*, 2016).**

Marine invertebrates such as sponge, coral, mollusks and tunicales are important sourcesof new bioactive compounds. Most of these compounds are synthesized by the symbiotic microorganisms, and not by the host itself **(Silvin *et al*, 2009; Dusane *et al*, 2012; Mabrouk *et al*, 2014).** Several publications on the production of biosurfactants from sponge associated microorganisms **(Gundhimathi*et al*, 2009)** and from coral associated marine bacteria **(Mabrouk *et al*, 2014)** were reported.

To the best of our knowledge no pervious publications on the isolation and characterization of biosurfactants produced by the marine algae- associated bacteria available, especially from the Mediterranian Sea habitat at Alexandria. Accordingly, the aim of the present work is the study of the marine algae associated bacteria for the production of potent biosurfactant, and to apply the crude biosurfactant for enhancing the bioremediation of spent- motor oil contaminated soil.

Used (spent) motors oil are common environmental contaminants. Unused motor oil consisting of hydrocarbons (80%-90%) and additives (10%-20%). During use in the motor of vehicles, they are altered due to the breakdown of the constituents **(Ugoh and Moneke, 2011).** Spent motor oil includes heavy metals, aliphatic and aromatic hydrocarbons such as polycyclic aromatic hydrocarbons (PAHs) **(Jain *et al*, 2009).** These compounds are highly toxic to plants, animals and humans when released to the environment **(Mandri and Lin, 2007, Wu *et al*, 2008).**

In most developing countries the spent motor oils are illegally disposed by dumbing in landfill, drainage systems, open vacant plots and farmland **(Objegba and Sadiq, 2002; Gracia-Hernandez *et al*, 2007; Akoachere *et al*, 2008).**

Spent motor oil causes harmful conditions for living organisms in the soil due to poor aeration of the soil, immobilization of nutrients and lower the pH **(Atuanya, 1987).** PAHs, and heavy metals found in the spent motor oil are able to alter soil biochemistry, soil microbial properties, pH, O_2 and nutrient availability **(Objegba and Sadiq, 2002, Ugoh and Moneke, 2011).** Exposure to oil contaminated environment causes headache, skin irritation and itchy eyes. Exposure to spent motor oil for a long time increases the risk of liver, kidney, bone marrow damage and caner development **(Vazquez- Duhalt, 1989; Mishra *et al*, 2001; Lloyed and Cakette, 2001).**

For the protection of the environment, it is of important to remove or detoxify the pollutants by using non-expensive bioremediation process which involves the capacity of the natural microorganisms to degrade the hydrocarbons of the waste oils. This bioremediation technology is environmental- friendly, and cost effective. On the other land this technique in nature is slow and needs long periods of time, thus it may be time consuming. For enhancing the bioremediation process, biosurfactants, maybe added to the contaminated sites in presence of nutrients.

In the present work, the biosurfactant produced by *Serratia marcescens* P25 was used for enhancing the bioremediation of spent motor oil-polluted soil, this is in the presence and in absence of nitrogen and phosphorus fertilizers.

2. Materials and Methods

1) Collection of the marine algae

Samples of marine algae were collected from a sea shore location at Abo-Kir, Alexandria. The collected algae were introduced into sterile 2L conical flasks containing sea water from the area of collection, transferred to the laboratory as soon as possible and kept at 4°C for further studies.

2) Screening the collected marine algae for biosurfactnat production

The collected algae were washed several times with sterilized sea water collected from the same area, for removing marine bacteria, leaving behind the attached bacteria of the phycoplanes (surface of algae). Portions of each of the washed algae (5 grams) were introduced into 250 ml conical flask containing 100 ml sterilized inorganic salt medium (ISM) containing soybean oil (2% W/V) and incubated at 25°C on a shaker operated at 140 rpm for a period of 7 days, after which the production of the biosurfactant was tested using the oil displacement area (ODA) method. The composition of the ISM was as follows (gm/L water):

$NaNO_3$, 2.0; K_2HPO_4, 2.0; $KHPO_4$, 1.0; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $CaCl_2$, 0.1; yeast extract, 0.1; sea water, 200 ml, trace salt solution, 1ml. The pH was 7. The trace salt solution was as follows (g/L dist water):
$FeSO_4.7H_2O$, 0.1; H_3BO_3, 0.2; $CoCl.6H_2O$,0.3; $CuSO_4.5H_2O$, 0.1; $MnSO_4.2H_2O$, 0.01; $ZnSO_4$, 0.10; $(NH_4)_6MO_7O_{24,}$ 0.10.

3) Isolation of bacteria associated with the phycoplane of *Punctaria* sp

Ten grams of the washed algae with sterilized sea water were introduced into 250 ml conical flask containing 100 ml of sterilized sea water. The flasks were shaken at 150 rpm on a shaker for one hour, after which serial dilutions were made using sterilized sea water. One ml of each of the suitable dilutions was used to inoculated a plate of ISM agar medium. From each dilution three plates were inoculated. The plates were incubated at 25°C for a period of 7 days, after which different colonies were isolated, purified by the streaking method and subcultured on slants of nutrient agar medium supplemented with 200 ml sea water/L.

4) Screening the purified bacterial strains isolated from the marine alga *Punctaria* sp for the production of biosurfactants

A loop of each of the purified bacterial strains found on a slant, was inoculated into 50 ml ISM in 250 ml conical flask supplemented with soybean oil (2% W/V). The inoculated flasks were incubated at 25°C on a shaker operated at 140 rpm for a period of 7 days. At the end of the incubation period, each culture was sterilized and then was centrifuged at 6000 rpm for a period of 30 min for the removal of the bacterial cells. The cell- free broth culture, (supernatant) was tested for production of biosurfactants by the following methods:

a) Oil displacement area (ODA)

This method was carried out according to **Techaoei *et al*, (2011)** as follows:

- 40 ml dist water introduced into a petridish (15 Cm diameter).
- 20 µl of light crude oil or used motor oil was spread over the water surface.
- 10 µl of the sterilized supernatant was placed on the center of the oil film.
- The diameter of the developed clear circle was measured.
- The area of the circle was calculated as oil displacement area (ODA) as follows:

ODA=3.14x r^2 where "r" is half of diameter.

b) The plug agar method

This method was developed by **Diab and El-Din (2013)** and used as follows:

- The bacterial strain was grown on ISM agar medium supplemented with glucose (2%W/V). From the bacterial growth, agar discs were cut by a sterilized cork-boarer (10 mm).
- Agar disc were transferred and located on the surface of agar plates coated with thin oil layer.
- After 10-15 min the clear zones developed were observed and measured.

c) Emulsification index

This method was carried out according to**Techaoei *et al*, (2011)** as follows:

- In a screw caped tube, 3ml of the supernatant was added to 3ml of each of the following oils: crude light oil, kerosene, a mixture of crude oil and kerosene (1:1), used motor oil, olive oil and soybean oil.

- The tubes were vortexed at high speed for two minutes. The mixture was left for 24h and E24 was measured. The tubes also were left for 7-30 days and the emulsification activity (E7-E30d) was measured.

d) Stability of the biosurfactants produced by the tested strains

Thermostability

This method was carried out according to Techaoei et al, 2011 Haddad et al (2009) as follows:

- Ten ml portions of each biosurfactant were exposed to various temperatures (50-121°C) for 30 min, allowed to cool at room temperature. The activity of each product was measures by the ODA method.

Effect of different pH values

- Portions of each biosurfactant were adjusted at different pH values (2-12 pH). The activity of each one was measured by the ODA method.

e) Effect of salinity

The effect of NaCl concentrations was carried out by adding different concentrations of NaCl (0-25% W/V) to different portions of each biosurfactant, allowed to stand for 20 min, after which the activity of each biosurfactant in each NaCl concentration was measured by the ODA method.

f) Applications of the biosurfactant produced by the S.marcescens P25 strains for the bioremediation of spent motor oil polluted soil.

Soil treatment

Polluted soil sample was collected from a mechanic shop and treated as follows: Soil microcosm test was designed to include 5 treatment in duplicates. Each consisting of 500 ml glass beaker containing 100 gm of the polluted soil, and treated as found in **Table (1)**.

Table 1: Different treatment of the polluted sample

Treatment	Amendments			
	Biosurfactant (BR)	NP	BRNP	Sterilized culture medium (5 ml)
1	+	-	-	-
2	-	+	-	-
3	-	-	+	-
4	-	-	-	+
5	-	-	-	-

The NP fertilizer was NH_4NO_3 (100 mg/ 100 g soil) and K_2HPO_4 (50 mg/ 100g soil). Biosurfactant was added (5 ml/ 100 g soil) in the form of sterilized supernatant containing the biosurfactants. Control 1 included 5 ml of the sterilized culture medium (without inoculation). 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 treatment were covered by thin aluminum foil to reduce evaporation of water. All of the treatment were incubated at room temperature (25-28°C). The loss of water due to evaporation was determined at the beginning of the experiment and every 2-3 days.

From each of the above treatment, samples were taken at the beginning of the experiment (0-time) and after 40 days incubation period for the determination of the loss of oil due to biodegradation.

Extraction and determination of the residual oil

At the beginning of the experiment (0-time) and at the end of 40 days incubation period. Four grams of the air dried soil was mixed by the same amount of anhydrous sodium sulphate. The residual oil in the soil was extracted by n-hexane using the shaking method described by **Chen *et al*, (1996)**. The extract was collected and evaporated in a preweighed dish, and the amount of residual oil was determined.

1) **Identification of the bacterial strains**
   Bacterial strains were identified according to Bergey's Manual of Determinal Bacteriology **(Holt et al, 1994)** and **Grimont and Grimont (2006).**
2) **Statistical analysis**
   All values were averages of three 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

Samples of marine algae were collected from a coastal area at Abu-Kir, Alexandria. The collected algae were identified as: *Punctaria* sp (brown algae), *Colpomenia* sp (brown algae), *Ulva* sp (green algae) and *Jania* sp (red algae).

The collected algae were washed several times with sterilized sea water collected from the same area, for removing bacterial cellls of the sea water adhering to the algae, and leaving behind the bacterial strains that are associated with the surfaces of the algae (phycoplanes). Portions of each of the washed algae (nearly of the same weight) were inoculate to sterilized ISM mediums supplemented with 200 ml sea water per L and waste frying oil (2%). The cultures were incubated at 27°C on a shaker operated at 140 rpm for 7 days. The results **(Table 2, Figure 1)** show that the brown algae *Punctaria* sp was able to produce highly active biosurfactantanl of 171.2 Cm^2 ODA followed by *Colpomenia* (79.0 Cm^2 ODA). The other two algae *Ulva* sp and *Jania* sp produced less active biosurfactants (14.7 and 3.3 ODA Cm^2 respectively).

Ramanan *et al* (2016) reported that the term phycorsphere was used for the first time in **1972** by **Bell and Mitchell** to indicate the zone which existing outwards from the algal organisms, in which the bacterial growth is stimulated as a result of the extracellular products of the algae. **Diab and Metwalli (1982)** were the first to use the term "phycoplane" to indicate the active surface of the algae supporting higher bacterial populations as compared to the sea water surrounding the algae.

The above results indicate that the marine algae may represent rich sources for the production of highly active biosurfactant. To the best of our knowledge no previous reports on the isolation and characterization of biosurfactant from the phycoplanes of marine algae were available. Because the phycoplane of the brown algae *Punctaria* sp

was characterized by producing biosurfactant of higher activity as compared to the other three marine algae, it was selected and further studied for the isolation of biosurfactant- producers form its phycoplane.

Twenty five bacterial strains were isolated and purified from the phycoplane of *Punctaria* sp, and were screened for the production of biosurfactant by using the plug agar assay method **(Diab and El.Din, 2013)** and by the oil displacement area (ODA) method. Many workers used for the screening process, measurement of the surface tension reduction by a tensiometer. However, tensiometer is not feasible to apply for large numbers of isolates at preliminary screening level **(Satpute *el al*, 2010).**

When the plug agar method was used 15 out of the 25 isolates tested (60%) were biosurfactant producers. Some of the methods currently used for the detection of biosuractants are based on the reduction of surface tension between two immiscible liquids. The agar plug method is based on lowering the interfacial tension between oil and the agar layer, i.e. between liquid phase and solid phase. This method is sensitive, required small amount of the biosurfactant, and the clear zone developed are clearly observed and recorded **(Figure 2)**.

On the other hand when the oil displacement area (ODA) method was used **(Table 3 and Figures(3-4)**, the results show that out of the 25 bacterial strains screened 20 (80%) were biosurfactant producers. The 20 positive strains were divided according to the different biosurfactant activities (ODA cm2) into 4 groups:

Group A, very active group, it includes 7 bacterial strains (28%) of 158-176.6 Cm^2 ODA **(Figure 4 a)**.
Group B, this group contains 3 strains (12%) of good biosurfactant activities, producing 90-130.7 Cm^2 ODA **(Figure 4b)**.
Group C, it includes 7 bacterial strains (28%) of moderately active biosurfactants (60-89.4 Cm^2 ODA) **(Figure 4c).**
Group D, it represents a weakly active group, it includes 3 bacterial strains (12%) of 12-40.8 Cm^2 ODA **(figure 4d)**.

The oil displacement area (ODA Cm^2) is also more sensitive for detection of lower concentration of biosufactants found in the supernatant of the broth culture as compared to the other methods currently used **(Youssef *et al*, 2004; Hamza *et al*, 2013).** The oil displacement area (ODA) is better predictor of biosurfactant production than the drop collapse method and the blood agar method, because it is very sensitive, required small sample value, rapid, easy to be carried out and does not require any specialized equipment **(Youssef *et al*, 2004; Plaza *et al*, 2006).** The ODA method also depends on decreasing the water-oil interfacial tension caused by the biosurfactant regardless of its structure **(Morikawa *et al*, 2000).** By using the ODA method, very active biosurfactant producer group (producing 158-176.6 ODA Cm^2) was selected. Their values of ODA cm2 represent the higher values as compared to those obtained by other investigators. **Techoaei *et al* (2011)** recorded the highest value of ODA (143.2 Cm^2 and 110.6 Cm^2) for *Pseudomonas aeurginosa* SCMC/06.

Hamza *et al* (2013) screened 20 bacterial strains for biosurfactant production using the oil spread method, micro plate method and drop collapse method. They found that 45% of the strains were positive for the oil spread method. They recorded the results as follows:
+: clear zone diameter of 5-9 mm (i.e. 0.2-0.6 Cm^2 ODA)
++: clear zone diameter of 10-15 mm (i.e. 0.8-1.8 Cm^2 ODA)
+++: clear zone diameter of >21-<30 mm (i.e.> 3.4- < 7.1 Cm^2 ODA)
Tambkar *et al* (2013) screened 14 bacterial strains for the production of biosurfactant using the oil spread method, the drop collapse method and β hemolysis test. They considered 5 mm (i.e. 0.2 Cm^2 ODA) and 10 mm (i.e. 0.8 Cm^2ODA) as positive biosurfactant production by the oil spread method. They recorded 92.9% of the strains were positive for this method.

Techoaei *et al* (2011) isolated 25 bacterial strains from garage site, all of these isolates were found to be biosurfactant producers when tested by the oil displacement area (ODA) method. They recorded their results as follows:
+: oil displacement area of 0.1 – 3.14 Cm^2.
++: oil displacement area of 3.14 – 12.57 Cm^2.
+++: oil displacement area of 12.57 – 28.28 Cm^2.
++++: oil displacement area of > 28.28 Cm^2.

It must be mentioned that in the present work the value 3.14 Cm^2 ODA i.e. 20 mm clear diameter zone was considered negative for the ODA method. All of the positive biosurfactant producers recorded in the present work were able to produce biosurfactants of 12 – 176.6 Cm^2**(Table 3). Sarmentia and Gazol (2012**) reported that algae and bacteria are dominant communities in the sea. It was found that bacteria play important role in algal growth and survival **(Kim *et al*. 2014; Amin *et al*, 2015).**

The present works show that for the first time to isolate biosurfactant producing bacteria from the phycoplane of the brown alga *Punctaria* sp. This brown alga may represent a rich source of the production of bioactive materials such as biosurfactants and antibiotics. Many reports were available on the production of antimicrobial substances from the marine algae associated bacteria, but at the same time no reports were available on the production of biosurfactants and their applications from the phycoplanes of the marine algae.

Out of the 20 biosurfactant producing bacteria isolated from the phycoplane of the brown alga, 5 red pigmented bacterial strains produced very active biosurfactant of 158 – 176.6 Cm^2ODA, on using waste frying oil as substrate, were selected and studied for their emulsification activity, and their stability at wide range of temperature, pH and salinity. The selected bacterial strains were identified as belonging to *Serratia marcescens*.

Several publications reported the isolation of *S. marcescens* from different habitats e.g. from row milk **(Abdu, 2003),** semiarid soils **(Helvia *et al.*, 2010),** petroleum hydrocarbon-polluted soil **(Anyanwu *et al.*, 2011; Elemba *et al*, 2015; Munachimso *et al.*, 2015),** hard coral **(Dusane *et al.*, 2011),** the gut of American cookroach **(Ahmed and Hassan, 2013),**

and from the gut and reproductive apparatus of the red palm weevil **(Scrascia *et al.*, 2016).** *S. marcescens*A4B was repeatedly isolated from the diseased honeybee **(El Sanoussi *et al.*, 1987).** The red pigmented *Serratia* was reported to be associated with the fig was **(Grimont *et al.*, 1981) Iverson *et al*(1984)** showed that *S.marcescens*strains were found in sugar-beet-maggot development stage, suggesting an insect microbe symbiosis, as well as a nutritional inter dependence. On the other hand **Griment *et al* (2006)** indicated that pigmented *S.marcescems*biotypes were rarely isolated from plants.

To the best of our knowledge, no reports are available on the isolation of the red pigmented *S. marcescens*from the phycoplanes of the brown algae. Accordingly, 5 strains of *S. marcescens* were selected and used for the production of active biosurfactant by using waste frying oil as cheap substrate.

Results of the emulsification activity of the 5 identified strains as measured by the emulsification index E24 **(Table 4 Figure 5-9)** show that all of the 5 *Serratia* strains were able to emulsify the hydrocarbon oils and vegetable oils but with different emulsification E24 values. All of the *S. marcescens* were able to emulsify crude oil, producing 60.2% - 100% E24 values, and mixture of crude oil and kerosene (1:1) with values of 59.0% - 88.5% E24. The highest E24 values were 88.3% - 100% recorded with used motor oil. For vegetable oils E24 values were in the range of 20.0% - 60%.

This result shows that E24 values were higher with hydrocarbon oil (88.3 – 100% E24) than with vegetable oils (20% - 60% E24). Thus it appear that the biosurfactant produced by the five *S. marcescens* strains are of potential application in the bioremediation of soil polluted with used (spent) motor oil. The ability of biosurfactant to emulsify oils and hydrocarbons increase the biodegradation of the hydrocarbons. This makes the biosurfactant of potential use in oil spill management and enhanced oil recovery **(Neto *et al*, 2008).**

Willumsen and Karison (1997) and **Lima *et al* (2011)** proposed that an emulsification character is considered stable if its E24 corresponds to 50% or more. **Techaoei *et al* (2011)** reported that one of the desirable characteristics of a biosurfactant is its emulsification properties. **Anyanwu *et al* (2011)** reported that the ability of a biosurfacant to emulsify hydrocarbon makes this biosurfactant of potential use in the biodegradation of hydrocarbons and in microbial enhanced of recovery (MORE).

Munachimso and Josiab (2015) reported that the E24 values differed according to differences in the type of bacteria and the type of hydrocarbons. For *Serratia marcescens* UEO 15, E24 values were 78.9 ± 3% and 66.79 ± 2.3% against diesel and engine oil respectively. For *Bacillus firrnus* UEO 9, 56.63 ± 1.39% and 52.22 ± 0.5% E24 against diesel and engine oil respectively were recorded.

Anyanwu *et al* (2011) found that the emulsification activity of the biosurfactant produced by *S. marcescens* was higher with vegetable oil (88 ± 2.2 – 98.0 ± 0.8%) than with hydrocarbons (50.0 - 90.0 %)**Ahmed and Hassan (2013)**found that *S. marcescens* 510 isolated from the gut of American cockroach was able to produce bio emulsifier of 87% E24 against kerosene. Results of the stability of the biosurfactant produced by the *S. marcescens* strains after time intervals (E24h – E30d) are found in **Table (4)** and illustrated in **Figures (5-9)**. It can be seen from the results that emulsions formed with used motor oil were more stable (55.0 ± 3.1% - 83.3 ± 2.0% emulsification) after 30 days as compared to the other oils. Higher E30d of 83.3 ± 2.0% , 82.0 ± 3.9% and 81.0 ± 2.9% were recorded against used motor oil with the biosurfactant produced by *S. marcescens* P25, *S. marcescens* PC2 and *S. marcescens* P24 respectively.

Peter *et al* (2014) showed that the biosurfactant produced by *S. marcescens* was able to give E24 of 40.9% with motor oil, which decreased to 29.16% after 96h. This organism showed less than 50% E24 with kerosene (9.1%), petrol (0%) and diesel (22.22%) which decreased after 48-96h. With vegetable oils such as coconut oil and jasmine oil this organism gave 54.16% and 52.0% E24 respectively, while with soybean oil and mustard oil E24 values were 43.75% and 30.76% respectively.

The application of biosurfactant in industries depends on their stability at wide range of temperatures, pH values and salinity **(Khopade *et al*, 2012)**. Results of thermostability of the biosurfactant produced by the five *S. marcescens* strains showed that these products were able to maintain its surface activity (as measured by the ODA Cm^2 method) unaffected in a wide range of temperature even after heating at the autoclave temperature of 121ºC for 30 minutes. This character gives this new biological compound a potential use in food, cosmetics and pharmaceutical industries, where heating to achieve sterility is required **(Abouseoud *et al*, 2008).**

The thermostability of thesebiosurfactantswill increase their potential application in conditions where high temperature prevails such as microbial enhanced oil recovers **(Khopade *et al*, 2012).**

Results of the stability of the biosurfactant produced by the five *S. marcescens* strains at different pH values **(Table 5, Figure 10)** show that all of them were stable at wide range of pH (2-12) but with different retained activities. It can be seen that strain P20 and strain PC2 showed their optimum activities at pH 7, (153.9 ± 7.2) and (143.1 ± 2.3 Cm^2 ODA) respectively. Strain P20 showed higher retained activities (93. % - 86.2% at pH 8-12) i.e. at the alkaline side more than the acidic side (62.9%-93.0% at pH 2-6). On the other hand strain PC2 showed higher retained activities (85.7 - 96.9% at pH 2-6) while at pH 8 – 12 the retained activities are 92.8% - 72.5% indicating more activities at the acidic side than at the alkaline side. As for the other 3 strains (P21, P24 and P25) the results show that their optimum activities were at pH 8 (153.9 ± 5.5, 165.0 ± 8.2 and 165.0 ± 8.1 ODA Cm^2 respectively). Strain P21 retained more of its activity at pH 9-12 (93.0 – 73.4% respectively) than at the acidic side pH 2-6 and the neutral side pH 7 (12.7 – 61.7%).On the other hand strain P24 retained more of its activities at pH 2-7 (57.6% - 74.4%) while at pH 9-12 the retained activities

were lower (68.5% - 17.1%). As for strain P25, more retained activities were observed at the alkaline pH value. (76.7% - 74.4% at pH 9-12).

As for the stability of the biosurfactant produced by the five *S. marcescens* strains in the presence of different concentration of NaCl theresults **(Table 6 and Figure 11)** show that the activities of the biosurfactants differ with differences in NaCl concentrations(5 – 25% w/v).All of the five strains retained 79.7% (for P24) – 100% (for P20) in presence of 5% NaCl.Strain P20 retained 93.2% - 68.5% in presence of 10-25% NaCl, strain P25 and PC2 retained 96.2% - 68.5% and 98.2% - 72.2% respectively of their activities at 10 – 25% NaCl. The other 2 strains retained less activities as compared to the other strains. It is if important to observe that 4 strains (P20, P21, P25 and PC2) were found to retain more than 66% of their activities in presence of 25% NaCl. Three strains (P20, P25, PC2) retained more than 74% of their activities in presence 20% NaCl. The above results confirmed the stability of the biosurfactant produced by the five *S. marcescens* strains at wide range of salinity (5 – 25% NaCl w/v). Oil reservoirs are one of the harsh environments, where temperature can range from 20 to 90ºc, normal salinity to hyper saline and wide range of pH values **(Al-Bahry *et al*, 2012)**.

The biosurfactant produced by the five *S. marcescens* strains meet these harsh conditions, because they showed surface activity and stability over wide range of environmental factors. These biological products are useful for cleaning oil-storage tanks, recovery of oil from oily sludge, washing oil-contaminated soils, microbial enhanced oil recovery and enhancing the bioremediation of oil-contaminated sites.

One of the five *S. marcescens* strains, strain P25 was characterized by producing a biosurfactant of more stability at wide range of temperature, pH and salinity, this is in addition to its ability to produce higher emulsification activity against used motor oils as compared to the other 4 strains. The above characters give this P25 strain a potential use in the bioremediation of polluted sites. Accordingly, this strain was selected and further studied for the production of biosurfactant using cheap carbon source such as waste frying oil. The sterilized supernatant containing the biosurfactant was applied for enhancing the bioremediation of spent motor oil-contaminated soil.

Dhial and Jasuja (2012) reported that the cell free culture broth (supernatant) containing the biosurfactant may be applied directly to the contaminated site without necessarily characterization of its chemical structure. **Plociniczak *et al* (2011)** indicated that the biosurfactant is very stable and effective while it is in the culture medium that was used for their synthesis.

Results of the biodegradation of the spent motor oil polluting this type of soil are found in **Table (7)** and illustrated in **Figure (12)**. It can be seen from these results that after 40 days incubation period the addition of biosurfactant (BR) alone (synthesized by *Serratia marcescens* P25) increased the biodegradation of the oil to 65.5% ± 5.2%. Addition NP alone failed to increase the biodegradation more than 46.0 ± 2.0%, while in presence of a mixture of biosurfactant and NB (BRNP) the biodegradation increased, but did not exceed 60.5 ± 5.0%. Statistically no significant difference between the results in presence of biosurfactant and that in the presence of BRNP ($P > 0.05$). In the presence of broth cell free culture medium used for the production of the biosurfactants (control 1), 38.0 ± 2.0% of the spent motor oil was degraded, while in control (2) (without any treatment), 24.3 ± 2.1% of this pollutant was degraded.

It can be concluded from the above results that the promising factor in removing the spent motor oil, is the addition of the biosurfactant alone or in combination with NP nutrients. The present results are in agreement with the results of **Thavasi *et al* (2011)** who found that biosurfactant alone are capable of enhancing biodegradation without added fertilizers, this cause reduction in the cost of the bioremediation process and overcome problems encountered when water soluble fertilizers are used for the bioremediation of the aquatic environments. On the other hand **Thavasi *et al* (2010)** found that the maximum crude oil biodegradation was 82% when biosurfactant alone was applied, while in presence of a combination of biosurfactant and fertilizers the biodegradation decreased to reach 75%. **Comeotra and Singh (2008)** studied the effect of crude biosurfactant and nutrient amendment on the biodegradation of oil sludge during 8 weeks period using a consortium made of two *P. aeruginosa* strains and *Rhodococcus* sp. They found that 98% of the oil sludge was degraded in presence of BRNP, while in presence of BR alone 73% of the oil was degraded. On the other hand 63% of the oil was degraded in presence of NP alone. In the control experiment (received no treatments) only 52% of the oil was degraded.

Kang *et al* (2010) studied the effect of the biosurfactant sphorolipid in the biodegradation of aliphatic, aromatic hydrocarbons and Iranian crude oil. They found that addition of the biosurfactant increased the biodegradation of TPH to reach 85.96%. Because bioaugmentation of the polluted sites with microorganisms follows restrict regulations, and the overall low feasibility associated with on – site production of biosurfactant, the introduction of the externally produced biosurfactant to the polluted sites is considered a more solid approach (**Lawniczak *et al*.,2013)**.

Eruke and Odoh (2015) reported that biodegradability of hydrophobic organic compounds by microorganisms could be a limiting factor during bioremediation. Application of biosurfactants to contaminated soil can potentially reduce the interfacial tension, increase the solubility and bioavailability of the hydrocarbons and then facilitate their biodegradation. The application of biosurfactant 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. These properties make biosurfactant apromising choice for application in enhancing hydrocarbon biodegradation.

Helmy *et al*, (2010) indicated that hydrocarbon pollutants are removed from the environment primarily as a result of their biodegradation by the native microbial population.

Such process is a time consuming and new technologies have been developed. For example the addition of biosurfactant which help to stimulate the indigenous microbial populations to degrade hydrocarbons at rate higher than those achieve in presence of nutrients alone. This is through increasing the bioavailability of the pollutant to microorganisms.

It is well known that petroleum hydrocarbons polluting the soil are hydrophobic compounds and are strongly adsorbed to soil particles. The biodegrading of these compounds is limited by their poor solubility and bioavailability. It may be possible to enhance the biodegradation of these pollutant by introducing biosurfactant. **Mulligan and Gibbs (2004)** explained that biosurfactant are able to enhance the biodegradation of hydrocarbons by increasing their bioavailability for microorganisms and by interaction of the biosurfactent with the bacterial cell surface to increase the hydrophobicity of the surface, thus allowing the hydrocarbons to easily associate to the bacterial cells.

Table 2: The production of biosurfactant from the marine algae where pieces of each were inoculated into ISMW medium. ± = Standard deviation, n =3

	Algae		*ODA cm²*
1.	*Punctaria* sp	(brown algae)	171.2±5.8
2.	*Colpomenia* sp	(brown algae)	97.0±1.7
3.	*Ulva* sp	(green algae)	14.7±2.1
4.	*Jania* sp	(red algae)	3.3±0.4
Sea water			……

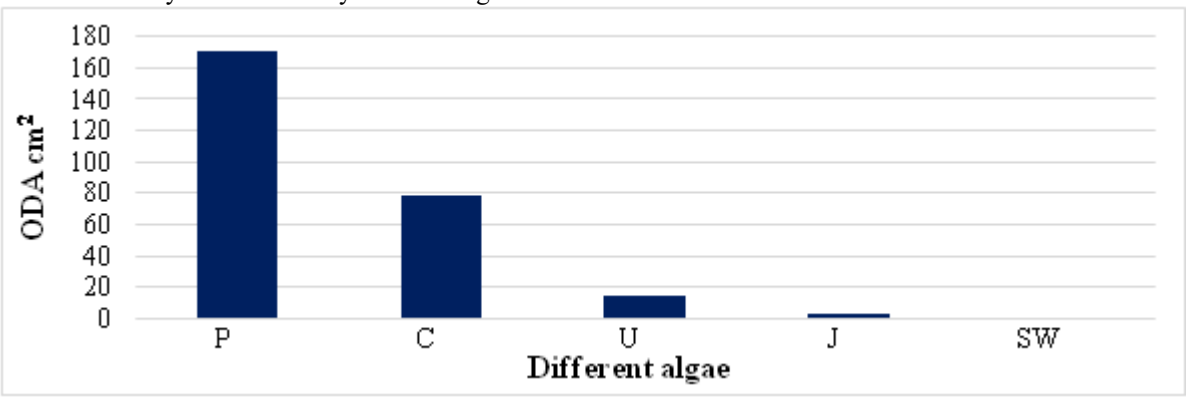

Figure 1: Production of biosurfactant from the phycoplanes of marine algae.
P= *Puctaria* sp, C= *Colpomenia* sp, U= *Ulva* sp, J= *Jania* sp, SW= Sea water

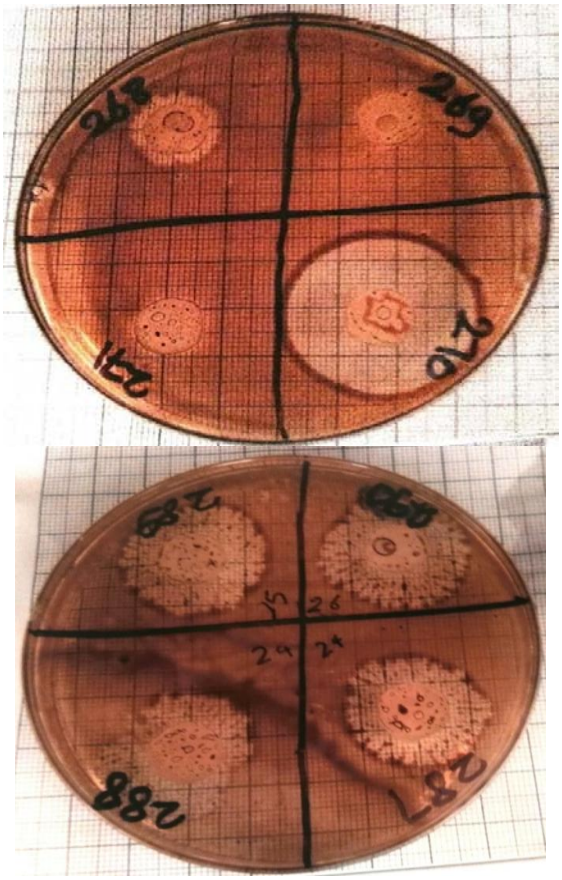

Figure 2: Photographs showing the production of biosurfactants by using the plug agar method

Table 3: Dividing the biosurfactant producing bacterial strain detected by the ODA cm^2 method into different groups according to their biosurfactant activity (ODA cm^2)

Activity range (ODA cm^2)	*Total No. of strains*	*(%)*	*Comment*
A- 158-176.6 cm^2	7	28	Very good activity
B- 90-130.7 cm^2	3	12	Good activity
C- 60-89.4 cm^2	7	28	Moderate activity
D- 12-40.8 cm^2	3	12	Weak activity
E- 0.0 cm^2	5	20	No activity

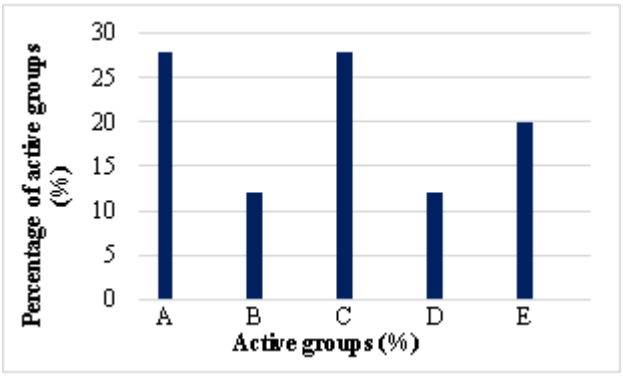

Figure 3: Percentages of the different active biosurfactant producing groups (A-D) comparing to the non-biosurfactant producer (E)

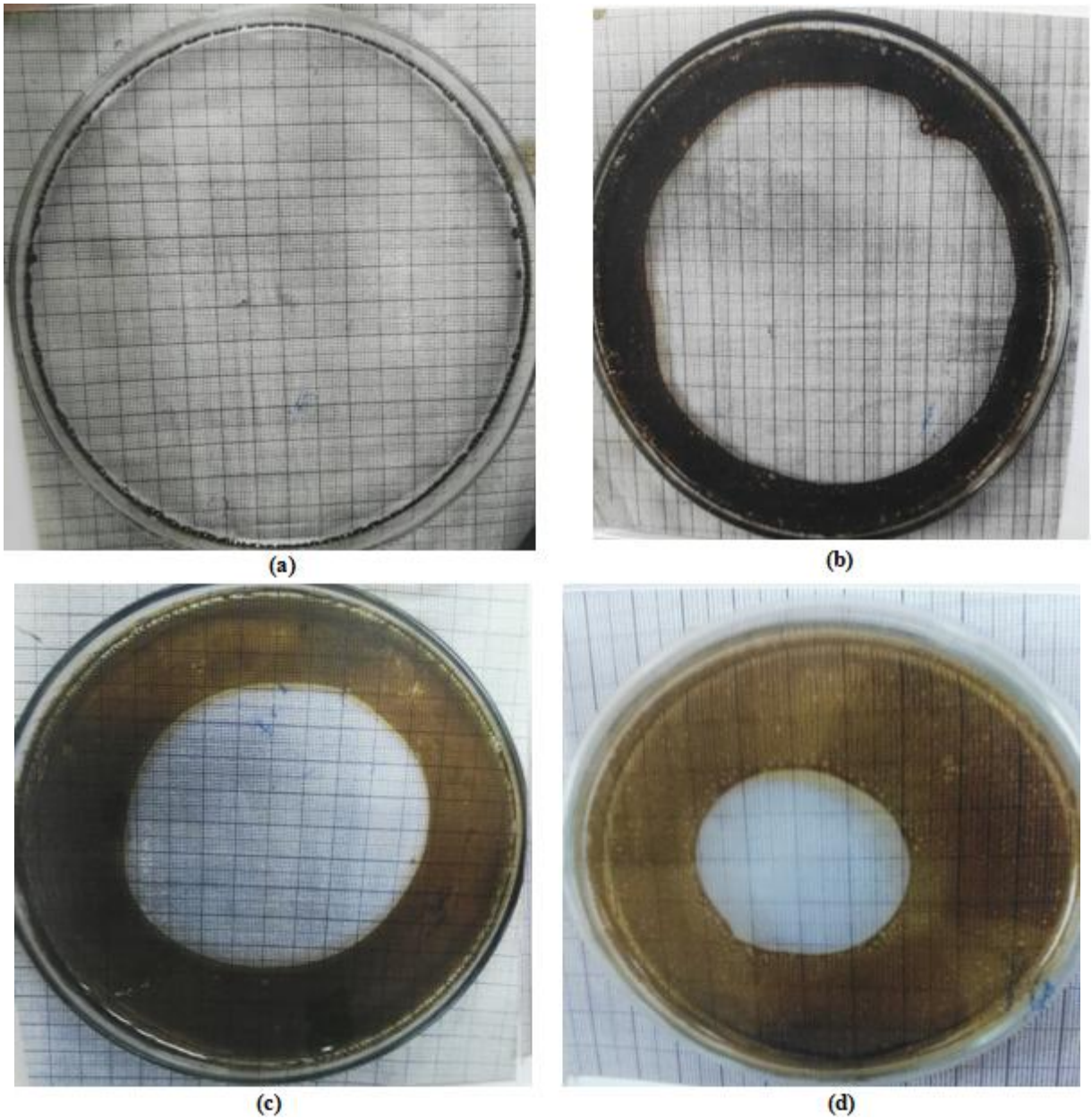

Figure 4. Photographs showing the production of biosurfactant by using the oil displacement area (ODA):
a- A representative of group "a" active biosurfactant (165.8 cm^2)
b- A representative of group "b" active biosurfactant (113.0 cm^2)
c- A representative of group "c" active biosurfactant (63.6 cm^2)
d- A representative of group "d" active biosurfactant (12.6 cm^2)

Table 4: Emulsification activities (%) of the different *Serratia* strains on different hydrocarbons oils and vegetable oils. Stability of the emulsification during 7-30 days are also given. ± = Standard deviation. n=2.

Oils	*Serratia* strain P20					*Serratia* strain P21				
	E24h	E7d	E15d	E21d	E30d	E24h	E7d	E15d	E21d	E30d
Crude oil	100.0±0.0	74.1±4.1	74.0±3.1	60.2±1.6	40.0±2.1	68.7±4.2	61.8±1.7	56.6±2.1	57.1±1.9	40.0±1.1
Kerosene	8.0±0.1	12.0±0.5	20.0±1.2	20.0±1.0	20.0±1.0	50.0±3.2	10.0±0.1	20.0±1.0	20.0±0.4	20..0±0.9
Crude oil+ Kerosene	88.5±4.1	52.0±2.5	56.0±4.2	40.1±2.1	25.0±1.4	66.7±3.3	58.0±1.8	57.0±1.9	25.0±0.7	25.0±1.1
Used motor oil	100.0±0.0	100.0±0.0	68.2±1.8	60.4±4.1	60.1±3.1	100.0±0.0	100.0±0.0	60.0±2.1	55.5±1.5	55.0±3.1
Olive oil	60.0±3.1	60.0±2.2	40.0±4.1	36.2±3.0	36.0±1.2	55.6±3.0	55.2±2.1	50.4±1.8	40.6±1.6	40.0±1.6
Soybean oil	52.0±3.0	37.0±2.1	30.0±2.2	21.0±1.2	20.0±1.2	51.0±2.1	50.3±2.2	45.3±2.1	40.2±1.9	38.0±1.2

Oils	*Serratia* strain P24					*Serratia* strain P25				
	E24h	E7d	E15d	E21d	E30d	E24h	E7d	E15d	E21d	E30d
Crude oil	60.2±1.7	60.0±1.3	60.0±3.0	50.0±3.1	37.0±1.0	78.6±2.3	60.0±1.8	60.0±2.0	60.0±1.8	60.0±2.0
Kerosene	14.2±0.7	11.4±0.9	11.0±0.8	12.2±0.8	20.0±1.0	6.7±0.5	7.7±0.4	8.0±0.4	8.0±0.4	8.0±0.5
Crude oil+ Kerosene	60.0±1.4	40.0±1.4	40±1.8	40.0±2.1	32.1±1.2	59.5±1.8	40.0±0.8	40.0±1.9	40.0±1.0	20.0±0.9
Used motor oil	88.3±2.2	88.3±2.8	88±3.6	88.0±3.2	81.0±2.9	96.0±3.1	86.0±2.0	86.0±2.0	84.0±1.9	83.3±2.0
Olive oil	21.7±1.0	20.0±1.1	20.0±0.9	15.0±0.9	15.0±0.7	48.0±1.4	47.0±1.1	41.8±1.1	40.0±1.4	15.0±0.6
Soybean oil	20.0±1.1	20.0±1.0	20.0±1.1	15.0±0.4	20.0±1.4	56.0±1.6	52.7±1.6	41.7±1.6	40.0±0.9	20.0±0.8

Table 4(continue)

Oils	*Serratia* strain PC2				
	E24h	E7d	E15d	E21d	E30d
Crude oil	74.4±1.4	60.4±2.0	60.0±2.1	56.1±2.2	56.0±1.6
Kerosene	0.0	0.0	3.7±0.6	3.0±0.1	4.0±0.2
Crude oil+ Kerosene	59.0±2.4	60.0±1.9	55.6±3.2	50.4±3.6	40.0±2.1
Used motor oil	96.4±3.2	87.0±3.1	84.0±4.2	84.0±3.1	82.0±3.9
Olive oil	56.0±1.6	20.0±0.9	4.0±0.7	4.0±0.4	8.0±0.6
Soybean oil	60.0±2.1	52.0±1.4	51.9±2.6	50.2±2.1	12.0±0.8

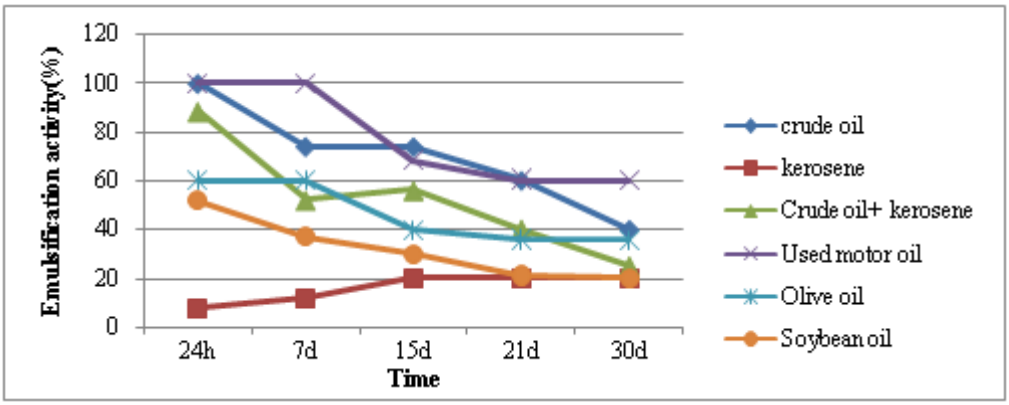

Figure 5: Emulsification activity (%) of *Serratia* strain P20 against different oils at different times intervals

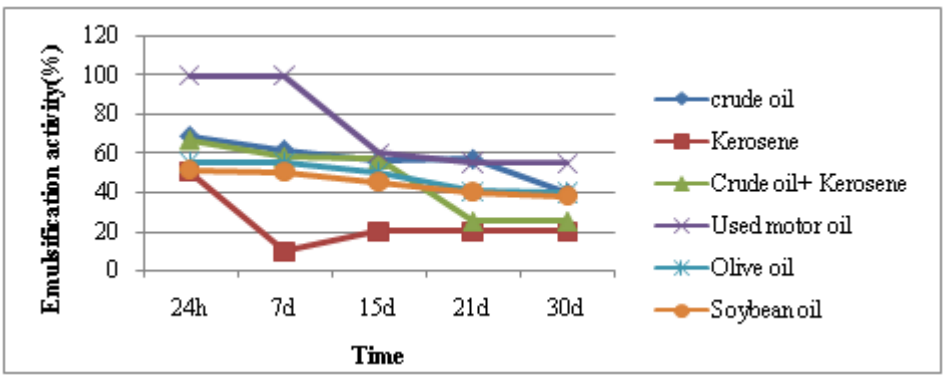

Figure 6: Emulsification activity (%) of *Serratia* strain P21 against different oils at different times intervals

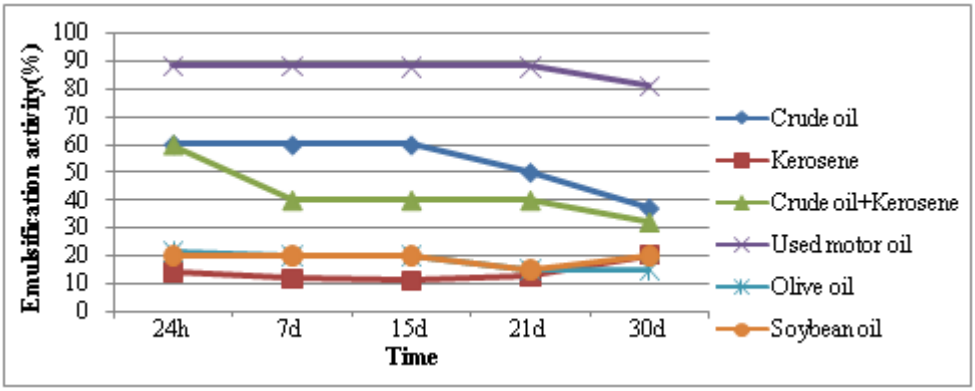

Figure 7: Emulsification activity (%) of *Serratia* strain P24 against different oils at different times intervals

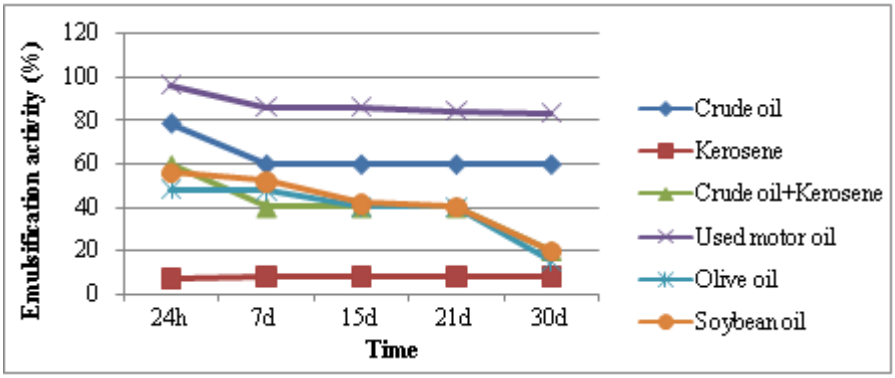

Figure 8: Emulsification activity (%) of *Serratia* strain P25 against different oils at different times intervals

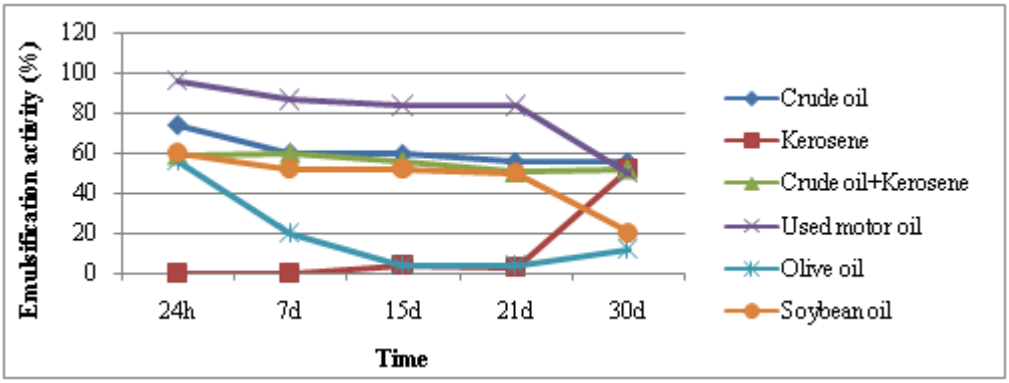

Figure 9: Emulsification activity (%) of *Serratia* strain PC2 against different oils at different times

Table 5: Effect of the different pH values on the activities of the biosurfactants produced by the different *Serratia* strains, as measured by the ODA cm^2. Retention activities (RA%) are also given relative to each optimum pH value. ±= standard deviation, n=2.

pH values	Different *Serratia* strians									
	P20		P21		P24		P25		PC2	
	ODA cm^2	RA	ODA cm^2	RA	ODA cm^2	RA	ODA cm^2	RA	ODA cm^2	RA
2	103.8±4.1	62.9	19.6±0.9	12.7	95.0±4.1	57.6	113.0±6.2	68.4	122.7±2.6	85.7
4	122.7±5.6	79.9	19.6±0.8	12.7	103.8±6.4	62.9	122.7±3.3	74.9	132.7±1.9	92.2
6	143.1±3.7	93.0	26.4±1.0	17.2	113.0±7.1	68.5	122.7±4.2	74.9	143.1±1.8	100.0
7	153.9±7.2	100.0	95.0±2.1	61.7	122.7±4.2	74.4	132.7±6.1	80.4	143.1±2.3	100.0
8	143.1±6.6	93.0	153.9±5.5	100.0	165.0±8.2	100.0	165.0±8.1	100.0	132.7±3.1	92.8
9	143.1±6.6	93.0	143.1±6.4	93.0	113.0±5.6	68.5	143.1±4.4	86.7	103.8±2.1	72.5
10	132.7±5.6	86.2	132.7±4.2	86.0	86.5±4.1	52.4	132.7±2.1	80.4	103.8±4.5	72.5
12	132.7±5.8	86.2	113.0±5.6	73.4	28.2±1.4	17.1	122.7±1.9	74.4	103.8±2.2	72.5

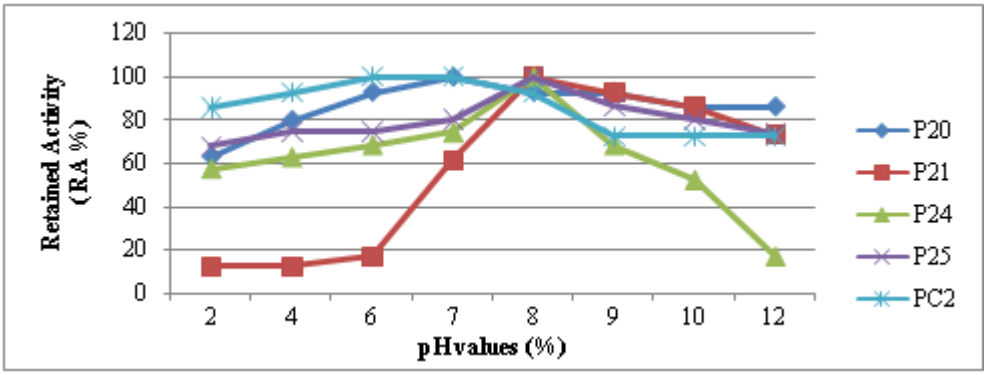

Figure 10: Retained activity (%) of biosurfactant produced by the different *Serratia* strains as affected by different pH value

Table 6: Effect of different concentrations of NaCl on the activity of biosurfactants produced by different *Serratia* strains as measured by the ODA cm^2 method. Retained activities (RA) (%) of each relative to 0% NaCl are also given ±= standard deviation, n=2

NaCl concentration (%W/V)	Different *Serratia* strains									
	P20		P21		P24		P25		PC2	
	ODA cm^2	RA	ODA cm^2	RA	ODA cm^2	RA	ODA cm^2	RA	ODA cm^2	RA
5	165.0±6.2	100.0	153.9±4.1	90.7	122.6±4.2	79.7	158.8±4.8	96.2	168.0±6.8	99.4
10	153.9±4.7	93.2	132.7±5.2	78.2	103.8±3.6	67.5	158.8±5.6	96.2	166.0±7.7	98.2
15	143.1±5.1	86.7	132.7±4.6	78.2	103.8±4.5	67.5	148.1±5.1	89.8	163.1±8.6	96.5
20	122.7±3.4	74.4	113.0±3.9	66.7	78.5±3.7	50.7	143.1±3.2	86.7	132.7±4.9	78.1
25	113.0±2.8	68.5	113.0±3.1	66.7	78.5±3.5	50.7	113.0±2.9	68.5	122.7±5.1	72.2
0.0	165.0±5.2	100.0	169.6±6.1	100.0	153.8±4.8	100	165.0±4.9	100.0	169.4±6.7	1000

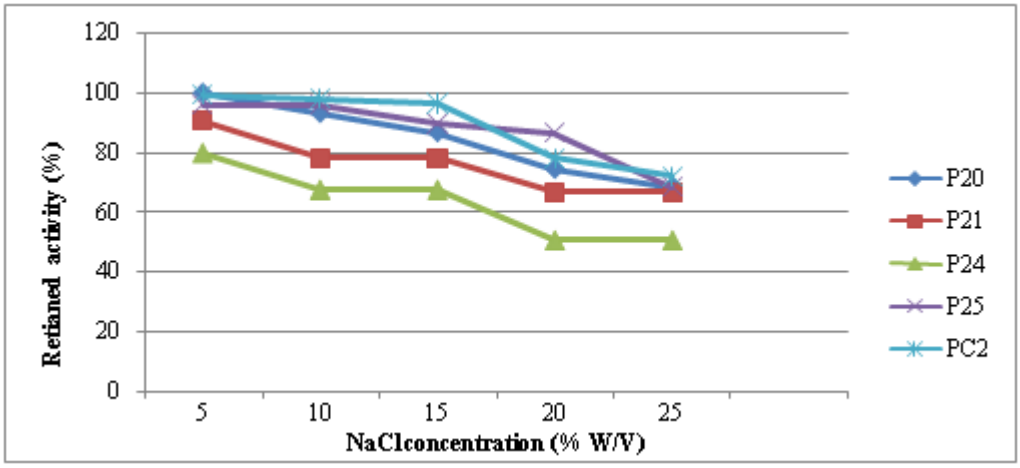

Figure 11: Retained activity of the biosurfactant produced by *Serratia* strains in the presence of different concentrations of NaCl (%W/V)

Table 7: Bioremediation of the spent motor oil polluted soil, as effected by the addition of biourfactant alone (BR), (NP) alone and a combination of (BR+NR) after 40 days inoculation period. The effect of the uninoculated medium (control 1) is also given.
±= Standard deviation, n=3. Data with same letter are of non-significant difference at p>0.05. At O-time the soil was contaminated with 2.1% spent motor oil.

Treatment	*Biodegradation loss (%)*
BR	65.1±6.2 (a)
NP	46.0±2.0
BRNP	60.0±5.0 (a)
Control (1)	38.0±2.0
Control (2)	24.3±2.1

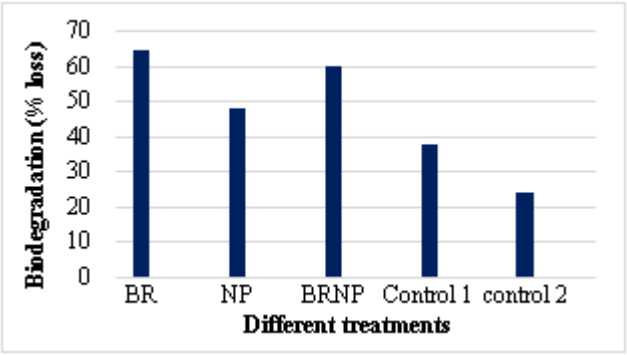

Figure 12: Biodegradation of the spent motor oil in presence of biosurfactant alone (BR), (NP) alone and a combination of BR and NP (BRNP) after 40 days incubation period

4. Acknowledgment

This study has been conducted in the laboratory 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 assistance and encouragement.

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