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ISOLATION, CHARACTERIZATION AND MEOR ABILITY OF THE BIOSURFACTANT PRODUCED FROM SERRATIA MARCESCENS UEO15

ELEMBA, ODENAKACHI MUNACHIMSO
Department of Biology/Microbiology/Biotechnology,
Federal University Ndufu Alike Ikwo, P.M.B. 1010
Ebonyi Nigeria

IJAH, UDEME JOSHUA JOSIAH
Department of Microbiology, Federal University of Technology, Minna. P.M.B. 65, Minna,
Nigeria.

CHIBUNNA, MIRACLE
Imo state university, owerri,
Nigeria

ABSTRACT

Serial dilution of soil sample and subsequent plating on nutrient agar resulted in isolation of 15 different bacteria isolates. Haemolytic activity on blood agar plate, three isolates UEO1, UEO9, UEO15 produced a clear zone around the colonies causing lysis of the blood. In the drop-collapsing test all the three strains collapsed the oil drop thus producing a flat drop and also produced a clear zone around the oil indicating ability to displace oil at the range between 5.2-5.7cm when tested using oil displacement method. All these results confirmed the ability of the three strains to produce surface active molecules. But when these isolates where subjected to emulsifications assay there activity varied having an emulsification index of 78.90%, 58.80% and 56.63% for UEO15, UEO1 and UEO9 respectively. The isolates were identified as *Serratia marcescens* UEO15, *Bacillus firmus* UEO9 and *Bacillus licheniformis* UEO1; these isolates (*Serratia marcescens* UEO15, *Bacillus firmus* UEO9 and *Bacillus licheniformis* UEO1) were subjected to biosurfactant production and they produced different amount 12.5(1.34g/l), 5.78 (0.27g/l), and 9.16(0.6g/l) respectively for both crude and purified. Since the aim of the research was on *Serratia marcescens*, this isolate was concentrated upon and further characterized. The TLC has an Rf value of 0.75 and gave red colour spot when sprayed with ninhydrin classifying it as a lipopeptide; the GCMS revealed the presence of palmitic acid, oleic acid, 1-[[[(2-aminoethoxyhydroxyphosphonyl]oxy] which are characteristic features of phospholipids; the FTIR revealed important functional groups (phosphine,P-H_3; amine, N-H and carboxylic acid, C=O) that defined the surfactant to be a phospholipid. The effectiveness of the phospholipid of SMUEO15 in oil recovery was also tested and the result revealed that 78% and 59% of crude oil and kerosene was recovered as compared to 10% and 25% obtained by distilled water respectively. This result confirms that biosurfactant are active biomolecules that can be used in oil recovery and bioremediation of hydrocarbon polluted environment.

Keywords: **Biosurfactant, TLC, GCMS, FTIR, Isolates,**

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1. INTRODUCTION

Biosurfactant are surface active molecules produced by microorganisms majorly bacteria, yeast and few fungi. They have two active ends that are referred to be amphipathic, with one end being the hydrophilic (water-loving) moiety comprising of carbohydrates, alcohol and their derivatives while the other end called the hydrophobic (water-hating) moiety is composed of fatty acids, ester and their derivatives [1].

The range of industrial applications of biosurfactants include enhanced oil recovery, crude oil drilling, lubricants, bioremediation of pollutants, health care and food processing [2; 3].

Different microorganisms produced different biosurfactants which are influenced by the type of carbon substrates used, the environment in which organism was isolated and the nitrogen content of the medium to which the organism was cultured, these leads to their classification and characterization based mainly on their chemical structure and microbial origin [3; 4,5].

Environmental pollution caused by oil spill and hydrocarbon waste are subject of concern and this has led to search for effective and environmentally friendly way by which this pollutants can be removed and this study focussed on the use of biosurfactant which are biodegradable, biocompatible and non-toxic in the recovery of soil polluted with oil to test the efficacy of biosurfactant.

2. MATERIALS AND METHODS

2.1 Sample collection

crude oil contaminated soil was collected (using soil auger) at the depth of 10-15cm from NNPC depot in Chanchaga Local Government Area, Minna, Niger state, Nigeria and was aseptically transported to the laboratory in sterile sample container and stored at ambient temperature in the laboratory before it was used for bacteria isolation. The isolation of the bacterial isolates was carried out in Microbiology Laboratory Federal University of Technology Minna.

2.2 Isolation of bacteria

Adopting the method used by [6] and [7], ten grams of crude oil polluted soil sample was inoculated into 40ml of distilled water, these was then diluted serially from 10^{-1} to 10^{-6} in distilled water. Then 1ml of the diluent from 10^{-6} was inoculated onto the surface of sterile nutrient agar containing filter niacin to inhibit growth of fungi [8] and a sterile glass rod was used to spread the liquid over the agar. The nutrient agar plates were then incubated at 37^{o}C for 24 hours. Morphologically different colonies that appeared on the surface of the agar plates was sub-cultured repeated on sterile NA to obtain pure cultures of the isolates

2.3 Biosurfactant assay

Biosurfactant activity of the isolates was evaluated by the following methods

2.3.1 Haemolytic activity

This was carried out using blood agar plate in accordance to the method used by [6,8]. Blood agar was prepared with 5% (v/v) human blood and blood agar base. The nutrient agar base was sterilized by autoclaving at 121ºC at 15lbs pressure for 15 minutes, allowed to cool before blood was added and allowed to solidify. The isolates were streaked on the blood agar and the plates were incubated at 28^{o}C for 48 hours. Isolates which were haemolytic by showing clear zone was further subjected to the following test for confirmation.

2.3.2 Drop collapsing test

Adopting the method used by [9] 2µl of crude oil was added to 96-well microtitre plate and equilibrated for 1 hour at 37ºC. Then 5µl of the culture supernatant was added to the surface of the crude oil in the well. The shape of drop on the oil surface was noted after 1 minute. The culture supernatant that collapsed the oil drop was indicated as positive and the culture supernatant which failed to collapse the oil drop was indicated as negative. Distilled water was used as negative control.

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2.3.3 Oil displacement method

In this test, 17 ml distilled water was added to a petri dish which is 90 mm in diameter. 100 µl of engine oil was added to the water surface, followed by the addition of 20 µl of cell culture supernatant on to the oil surface. The diameter and the clear halo visualized under visible light were measured after 30 seconds [10].

2.3.4 Emulsification capacity

Emulsification capacity of the biosurfactants towards two hydrocarbons (i.e. diesel and engine oil) was done using the [11] method. A mixture of 5ml of hydrocarbon and 2 ml of cell free extract obtained after the centrifugation of culture were taken into a test tube and homogenized by vortexing for 2 minute. This was allowed to stand undisturbed for 24hours after which the emulsion activity was investigated and the emulsification index (E24) was calculated by the total height of the emulsion layer divided by the total height of the aqueous layer and then multiplying by 100. The results were compared with SDS as positive control.

2.4 Characterization and identification of biosurfactant producers

Isolates were characterized based on Gram's reaction, and biochemical tests. The biochemical tests performed included reduction of nitrate, spore formation, utilization of citrate, production of indole, and methyl red-voges proskauer test (MR-VP). The ability of the isolates to utilize the following carbohydrates was tested: glucose, arabinose, inositol, xylose, fructose, mannitol and sucrose. The probable identities of the isolates were determined using the schemes of Krieg *et al.*2000 and Holt *et al*., 2004.

2.5 Production and Extraction of biosurfactants

One hundred millilitres of mineral salt medium of [14] was dispensed in conical flasks containing 1 ml of diesel was sterilized by autoclaving at 121°C for 15 minutes, the medium was allowed to cool before being inoculated with 2mI of nutrient broth culture of the bacterial isolates. Two of the flasks were left uninoculated and served as a control. The flasks were incubated shaking at 250rpm at room temperature (30^{o}C) and pH 7.1. For the period of 8 days. After which the cultures were centrifuged at 500rpm for 30minutes, then it was filtered using whatman No.1 filter paper. The supernatants was collected and subjected to solvent extraction three times using Chloroform and Methanol in the ratio of 2:1.two phase separation occurred (the upper solvent and the whitish bottom phase which is the crude biosurfactant), the bottom sediment was collected using separating funnel and washed with distilled water, after which the solvent was allowed to evaporate to dryness over a water bath at 45^{o}C for 24hours. Quantity of the dried biosurfactant was determined by measuring the dry weight using the formula; dry weight – initial weight of the plate.

Quantity of biosurfactant= Weight of the plate after drying -weight of the empty plate

2.6 Characterization of the biosurfactant produced

The biosurfactants produced was purified and characterized using TLC, FTIR and GCMS respectively.

2.6.1 Purification was done using thin layer chromatography: Thin layer chromatography was carried out by spotting the crude biosurfactant on TLC Plates already pre-coated (G60, Merck, Darmstadt, Germany) the plate which was commercially prepared was developed and activated by placing it in an hot air oven at a temperature of 100^{o}C for 30minutes before the surfactant were spotted on the plate using capillary tube. Chloroform-methanol-acetic acid and water (85:10:5:1) was used to separate the spots [15].

The TLC plates were placed inside the tank containing the solvents the tank was cover with lid and was observed for movement. After one hour the movement stopped and was assumed separation has ended. The plates were removed from the tank allowed to air dried and then was viewed under a UV light to identify the separated fractions. After was the plates were sprayed with anthrone reagent and ninhydrin solution.

The spots were scraped from the plate and extracted with chloroform-methanol mixture.

The quantity produced after this was also weighed and measured.

The Rf value was determined by using the formula, distance travelled by the test sample divided by the distance travelled by the solvent

$$Rf = \frac{\text{Distance travelled by the test sample}}{\text{Distance travelled by the solvent}}$$

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2.6.2 Infrared Spectroscopy

The Infrared (IR) spectroscopy of the biosurfactant was carried out using 8400S Fourier transform infrared spectrophotometer by Shimadzu, Japan and the IR spectra were scanned between 500 and 4500cm^{-1} wave numbers (per cm) with a resolution of two measures per wave number using potassium bromide as background reference.

2.6.3 Gas chromatography mass spectroscopy

The GCMS analysis was carried out using GCMS QP2010 Plus Shimudza, Japan equipped with capillary column and selective detector (AOC-20i) which was set to scan from m/z 20 to m/z 310 at a scan rate of 1.5 scans per seconds with an initial oven temperature of 80^{o}C for three minutes with a carrier gas (Helium) at a flow rate of 1.58ml/min and a split ratio of 50:1.0.

2.7 OIL RECOVERY ABILITY OF THE BIOSURFACTANT

Oil recovery ability of the biosurfactant was carried out using soil column study method by [16]. Two Glass columns were packed with 100g of sandy loam soil each and they were saturated with 20ml of crude oil and kerosene each this was allowed to stand for five days. The efficiency of the biosurfactant solution in releasing the oil from the soil was tested by adding 100 ml aqueous solution of 1.0% of the biosurfactant solution to the column. Distilled water only was used as control.

2.8 Statistical Analysis

All values are averages of three readings and have been shown as mean ± SD. Analysis of variance (ANOVA) was used in determining the significance of differences among the means.

3. RESULTS

3.1 Identification and characterization of the bacterial isolates

Serial dilution of crude oil contaminated soil sample and subsequent plating on Nutrient agar resulted in isolation of 15 colonies of bacteria, which were all identified using the scheme of Holt *et al.,* (2004) and (Table 1)

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GLOBAL JOURNAL OF ADVANCED RESEARCH
(Scholarly Peer Review Publishing System)

Table 1: Identification and characterization of the bacterial isolates

Isolate code	Gram reaction	Catalase	Lactase	Glucose	Sucrose	Citrate	Mobility	Indole	Urease	HS	Gas	Mr	Vp	Spore	Oxidase	Confirmed isolates
UEO1	+ rod	+	–	+	+	–	+	–	+	+	–	–	+	+	–	*Bacillus licheniformis* UEO1
UEO2	+ rod	+	+	+	+	–	+	–	–	–	–	+	–	+	–	*Bacillus circulans* UEO2
UEO3	+ rod	+	+	+	+	_	+	–	–	–	–	+	–	+	–	*Bacillus circulans* UEO3
UEO4	+ rod	+	–	+	-	-	+	–	+	–	–	+	–	+	–	*Bacillus lentus* UEO4
UEO5	- rod	+	+	+	+	+	+	–	–	–	–	–	–	–	–	*Pseudomonas nautica* UEO5
UEO6	+cocci	+	–	+	+	–	+	–	–	+	–	–	+	–	–	*Micrococcus* sp UEO6
UEO7	+rod	+	–	+	+	–	+	–	_	_	_	+	_	+	–	*Bacillus* Sp UEO7
UEO8	- rod	+	–	+	–	–	+	–						–	+	*Pseudomonas* sp UEO8
UEO9	+rod	+	–	+	+	–	+	–	–	–	–	+	–	+	–	*Bacillus firmus* UEO9
UEO10	-rod	+	–	+	–	+	+	–	–	–	–	–	+	–	–	*Achromobacter oryzihabitan* UEO10
UEO11	-rod	+	–	+	+	ND	ND							–	–	*Proteus mirabilis* UEO11
UEO12	-rod	+	–	+	+	ND	ND							–	–	*Proteus vulgaris*
UEO13	+rod	+	–	+	–	–	+	–	+	–	–	+	–	+	–	*Bacillus lentus*
UEO14	-rod	+	–	+	_	+	+	–	–	–	–	–	+	–	–	*Serratia marcescens*UEO14
UEO15	-rod	+	–	+	+	+	+	–	+	–	–	+	+	–	–	*Serratia marcescens*UEO15

Key: + =positive; – =negative: HS=hydrogen sulphide; Mr= methyl red; Vp= voges proskauer; ND= not determined

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3.2 Haemolytic activity of isolates

On blood agar plate, three isolates UEO1, UEO9, UEO15 produced a transparent clear zone (β-haemolysis) around the colonies causing lysis of the blood (Table 2); this is a clear indication that those isolates are potent producers of biosurfactant

Table 2: Haemolytic ability of isolates

S/No	Isolates	Beta(β)-Haemolysis	Alpha (α)-Haemolysis
1	*Bacillus licheniformis* UEO1	+	
2	*Bacillus circulans* UEO2		+
3	*Bacillus circulans* UEO3		+
4	*Bacillus lentus* UEO4		+
5	*Pseudomonas nautica* UEO5		
6	*Micrococcus* sp UEO6		+
7	*Bacillus* Sp UEO7		+
8	*Pseudomonas* sp UEO8		+
9	*Bacillus firmus* UEO9	+	
10	*Achromobacter oryzihabitan* UEO10		+
11	*Proteus mirabilis* UEO11		+
12	*Proteus vulgaris* UEO12		+
13	*Bacillus lentus* UEO13		+
14	*Serratia marcescens*UEO14		+
15	*Serratia marcescens*UEO15	+	

3.3 Displacement and Emulsification capacity of the isolates

The diameter and zone of displacement by these isolates *(Bacillus licheniformis* UEO1, *Serratia marcescens*UEO15, and *Bacillus firmus* UEO9) were 5.7, 5.6, and 5.2cm respectively and the emulsification index rages from 58.90 to 78.90% (Table 3)

Table 3: Diameter of displacement and Emulsification capacity of the isolates

Isolates	Diameter of displacement(cm)	Emulsification capacity ($\%E_{24}$) (for diesel)	Emulsification capacity ($\%E_{24}$) for engine oil	R_f
*Serratia marcescens*UEO15	5.6 ± 0.01^{ab}	78.90 ± 3.0^{c}	66.79 ± 2.2^{c}	0.75
Bacillus firmus UEO9	5.2 ± 0.01^{a}	56.63 ± 1.39^{a}	52.22 ± 0.5^{a}	0.46
Bacillus licheniformis UEO1	5.7 ± 0.11^{ab}	58.80 ± 0.64^{ab}	58.10 ± 0.02^{b}	0.53

++ wide degree of spread, + the spread is not wide

Comparing the diameter of displacement among the three isolates the statistical analysis showed that there were no significant difference ($p\geq0.05$) between the isolates, but there were significant difference between their emulsification index within the row ($p\leq0.05$)

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(Scholarly Peer Review Publishing System)

3.4 Quantity of biosurfactant produced

The quantity/amount of crude and purified biosurfactant produced by these isolates differ from one another, with *Serratia marcescens* having the largest amount (12.5 and 1.34g/l) of biosurfactant as compared to Bacillus licheniformis and B. firmus with 9.16; 0.27g/l and 5.78; 0.6g/l respectively (Figure 1)

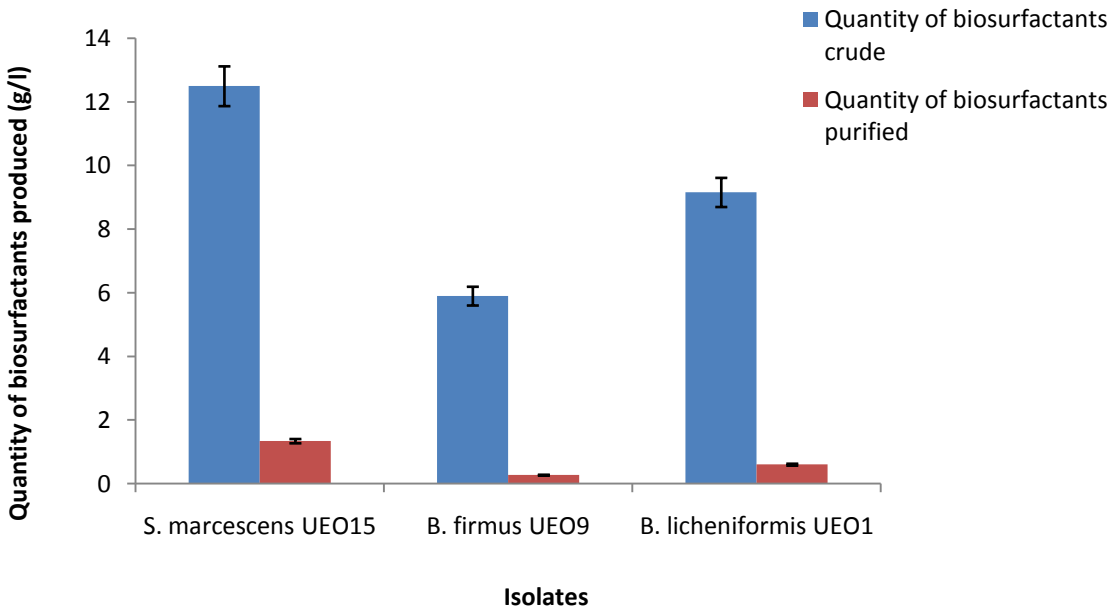

Figure 1: quantities of biosurfactant produced

3.5 Characterization of biosurfactant from *S.marcescens* UEO15

S.marcescens UEO15 was further characterized using GCMS and FTIR this isolate was chosen because of the reasonable amount of surfactant recovered after purification; so that it can effectively be subjected and used in further analysis.

3.5.1 The Infra-Red (FTIR) Analysis

Infrared analysis of the biosurfactants produced by *Serratia marcescens* is shown in Table 4 (Figure 1). Revealed strong band of CH_2 stretching in the region with wave number 2908.65cm^{-1} which is typical of alkanes (C-H), weak bands that stretched at 2400.85 cm^{-1} confirms the presence of phosphines (P-H_3). Conjugated weak bands stretching at 1662.69 cm^{-1} revealed characteristic for amines (N-H). Carboxylic acids and their derivatives (C=O) had strong bands that stretched at 1105.25 cm^{-1}. The alcohols and phenols (OH) were also proved from the broad bands stretching at 978.74 cm^{-1}. Weak bands that stretched at 500.34 cm^{-1} indicated disulphides. Which are characteristic component of Sewarratin (biosurfactant from *S. marcescens*)

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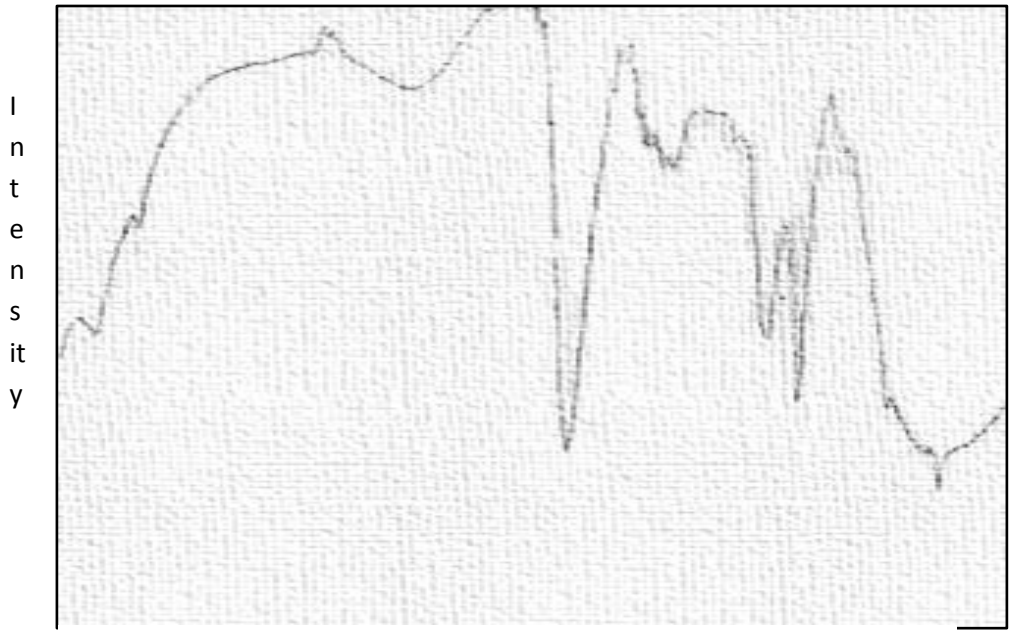

Table 4: Interpretation of FT-IR for *S. marcescens* UEO15

e No (cm^{-1})	**nsity**	**tified functional Group**
.65	51	(s) stretch of Alkane
.85	10	phine P-H_3(w) stretch
.69	00	ne (N-H)
.25	23	oxylic acids (C=O) and their derivatives
74	42	hols and phenols (OH)
34	31	lphide (w)

Key: s=strong; w=weak

3.5.2 The gas chromatography and mass spectroscopy analysis

Eight major peaks (labelled 1-8) were revealed in the GCSM analysis(Figure 3) showing the important fatty acid content (Table 5) of the biosurfactant proving hydrophobic nature of the surfactant.

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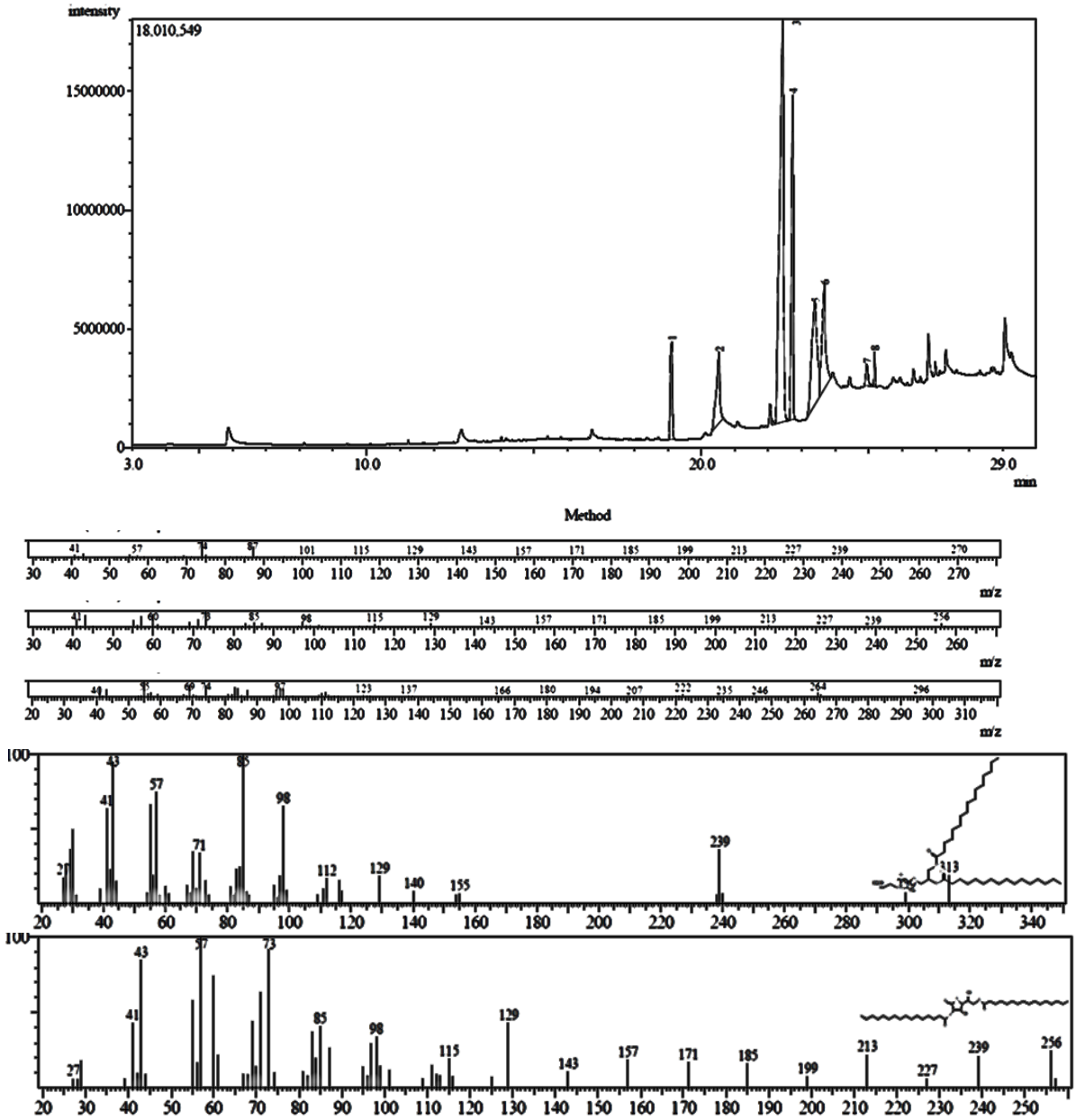

Figure 3: GC-MS of *S. marcescens* UEO15, showing the scans (mz) and probably structure present in the biosurfactant

Table 5: The hydrophobic component of the biosurfactant (SMUEO15) from *S. marcescens*

ι Number	ıpound name	ecular Formula (mol. weight)
	ıitic acid,	$[_{34}O_2$ (270)
	ıdecanoic acid	$[_{28}O_2$ (228)
	:tadecenoic acid,	$[_{36}O_2$ (296)

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decanoic acid	$[_{38}O_2$ (298)
: Acid	$[_{34}O_2$ (282)
decanoic acid, 2-(2-hydroxyethoxy)ethyl ester	$[_{44}O_4$ (372)
(2-aminoethoxy)hydroxyphosphinyl]oxy]methyl]-1,2-ethanediyl ester	$[_{74}NO_8P$ (691)
ıdecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$[_{38}O_4$ (330)

The presence of a Phosphine P-H_3(w) stretch as seen in IR spectrum and hydroxyphosphinyl group as revealed in peak 7 confirmed the biosurfactant to be a phospholipid**.**

3.6 Oil recovery ability of the biosurfactant

Soil column study, which tested the effectiveness of the biosurfactant in possible microbial enhanced oil recovery, revealed that the biosurfactant SMUEO15 recovered 78% and 58% of crude oil and kerosene fron soil as compared to that of distilled water (10% and 25%) respectively (Figure 4).

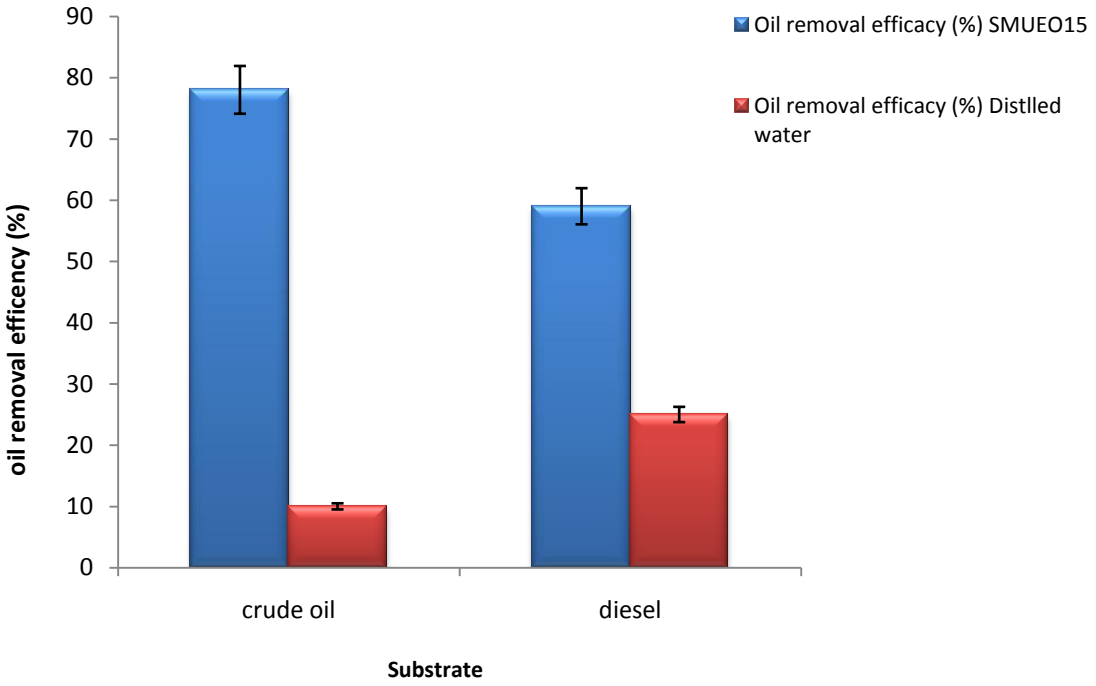

Figure 4: Oil recovery efficacy of biosurfactant SMUEO15

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4. DISCUSSION

Serial dilution of soil sample and subsequent plating on nutrient agar resulted in isolation of fifteen isolates. Strains were tested for haemolytic activity, which is regarded by some authors as indicative for biosurfactant production and used as a preliminary method for bacterial screening [17; 18; 8]. Haemolytic activity observed in all the fifteen isolated strains (Table 2); UEO1, 9 and 15 gave beta(β) (complete) haemolytic activity while the other strains had alpha (incomplete) haemolytic activity. Based on this only the three isolates were selected and subjected to other analysis.

In the drop-collapsing test all the three strains collapsed the oil drop thus producing a flat drop indicating that they are potent producers of biosurfactant [19]. Three of the isolates successfully displace the oil hence confirmation to production of biosurfactant. Displacement of oil clearly is a sign of extracellular surfactants present in the supernatant of cultures [8].
The emulsification index (78.90%) of the *S.marcescens* UEO15 in this study compares favourably with that of Yu-Hong Wei *et al.* (2008) who had similar result (77%) in their studies with *Serratia marcescens* as good emulsifiers, and (79.92%) *Serratia marcescens* UCP 1549 by Alves *et al.* [20]

The compounds present as revealed from the IR and GCMS confirm the presence of the bonds formed between carbon atoms and hydroxyl groups in the chemical structures of serrawettin as reported by Guo and Zang,[21] Therefore the biosurfactant (SMUEO15) produced and used in this study is a Serrawettin with phosphine esterified to hydroxyl-amine group (Table 5) which confirms it to be a phospholipid in contrast to lipopeptides reported by Anyanwu, *et al*.,[22] and Alves, *et al*.[20]; both produced from *Serratia marcescens* NSK-1and *Serratia marcescens* UCP 1549 respectively. These differences may be attributed to the different sources from where the bacteria were isolated, the strain used, the substrates used as carbon source during production and the environmental conditions of the different area were the research were carried out [23]; [24]. This corresponds with the statement that it is possible to obtain different types of biosurfactants from one species of microorganism [25; 26; 27].
Soil column study (Table 6) which tested the effectiveness of the biosurfactant in possible microbial enhanced oil recovery or remediation of contaminated soil revealed that 78% and 59% of crude oil and kerosene respectively were recovered by the addition of the biosurfactant solution while only 10% and 25% of crude oil and kerosene respectively were recovered with distilled water. Visual observation of the oil droplets in the column effluent showed that the oil removed existed as free product.
The removal efficacy obtained in this present study was higher than that reported by Anyanwu *et al*.[22] who recorded 60% and 51% removal of engine oil and kerosene respectively and also that reported by Scheibenbogen *et al*. [28] which ranged between 23% and 59%. However, Pruthi and Cameotra[16] reported much more efficiency recovery of 85% - 90% of the oil from a sand pack column using biosurfactant solution from *Arthrobacter protophormie*. These difference in efficacy maybe attributed to the different strains ability.
The results indicated that the biosurfactants were able to remove significant amount of crude oil from the contaminated soil. Most of the oil removed was due to mobilization, caused by the reduction of surface and interfacial tensions rather than the solubilization and emulsification effects [29]. Therefore *Serratia marcescens* UEO15 biosurfactant, can be concluded to have potential application in microbial enhanced oil recovery and bioremediation of oil polluted environment.

5. CONCLUSION

The results obtained in this present study showed that the isolate *S. marcescens* UEO15 was an effective biosurfactant producer which produced a phospholipid serrewattin type of biosurfactant named biosurfactant SMUO15. The study also led to the suggestion that the strain can be an effective agent for environmental clean-up in area of oil spill remediation and recovery.

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