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ORIGINAL ARTICLE

Studies on Biosurfcatants Produced by Microorganisms Isolated from Different Locations

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ABSTRACT

Background: *Biosurfactants are amphiphilic compounds which have the significant advantages over synthetic counterparts. The advantages of biosurfactants over their synthetic derivatives and a wide range of applications have attracted a strong interest of scientific community. These have wide range of potential applications in areas of environmental applications and management, crude oil recovery, antimicrobial agents in health care and food processing industries.* ***Objectives:*** *the present study describes the Collection of different samples and isolation of microorganisms utilizing different hydrocarbons, purification and identification of the microbial isolates, screening of the ability of the used isolates for the effeciency in producing biosurfactants and studies for achieving optimum production of the biosurfactants and their chemical identification.* ***Methodology and Results:*** *In this study, we collected 15 samples from different locations from crude oil reservoires, waste industrial water, waste water, agricultural soil and automobile workshop. totally 1834colonies were obtained from all 15 samples from which 149 morphologically different colonies were tested for biosurfactants production and 50 strains showed a promising biosurfactants production. The two strains W1-S55 and SA3-S107 that presented the highest values of oil spreading test (65 and67mm), emulsification index (80 and85%), parafilm M test(8and10mm) and dropcollapse test(5and6mm) were identified as Pseudomonas aeruginosa and Bacillus subtilis, respectively by 16s rRNA sequence analysis and by Biochemical tests with Biolog system. 3 % Glyceroland %0.15 Glycine as carbon and nitrogen source, respectively was the optimal for biosurfactants producing condition by the isolate W1-S55, 16% molass and 0.5%sodium nitrate as carbon and nitrogen source, respectively was the optimal for biosurfactants producing condition by the isolate SA3-S107. The effect of pH; salinity and temperature on the produced biosurfactant were also evaluated.*

INTRODUCTION

Surfactants are surface active agents with wide ranging properties including the lowering of surface and interfacial tensions of liquids. Surface tension is defined as the free surface enthalpy per unit area [1] and is the force acting on the surface of a liquid leading to minimization of the area of that surface.

Surfactants or surface active agents(SACs) can be derived from both chemically based (chemical surfactants or synthetic surfactants) produced by organic chemical reactions and biologically based (biosurfactants) produced from natural source from plants animals and by biological processes being excreted extracellularly by microorganisms such as bacteria, fungi and yeasts. [2,3]

Biosurfactants are a group of secondary metabolite with surface active properties and are synthesized by a great variety of micro-organisms[4-6]. These metabolites are complex amphiphilic compounds which are produced mainly by hydrocarbon degrading microorganisms. The molecular structures of these comprise a hydrophilic portion, which may consist of mono-, oligo- or polysaccharides, amino acids or peptides or carboxylate or phosphate groups, and a hydrophobic portion, which is composed of saturated or unsaturated (hydroxy) fatty acids or fatty alcohols.[7,8]

The main physiological role of biosurfactants is to permit microorganisms to grow on water-immiscible substrates by reducing the surface tension at the phase boundary, thus making the substrate more readily available for uptake and metabolism, though the molecular mechanism related to the uptake of their substrates are still not clear and not fully understood[4].

Biosurfactants are categorized mainly by their chemical composition and their microbial origin.[9] In

general, their structure includes ahydrophilic moiety consisting of amino acids or peptides anions or cations; mono-, di-,or polysaccharides; and a hydrophobic moiety consisting of unsaturated, saturated, or fatty acid.[10-12] Desai and Banta[4], have been classified the biosurfactant into lipopeptides and lipoproteins, glycolipids, polymeric, fatty acids, phospholipid and neutral lipids and particulate biosurfactant.

METHODOLOGY

Chemicals:

Media and chemicals for isolation and biosurfactants assay like folin-ciocalteau and vanillin reagents were of high grades and purchased from Sigma and Hi-Media Laboratories, India, (all chemicals were analytical grade).

Isolation and purification of the microorganisms:

According to the procedures obtained by Bodour *et al.*,[13] A 5 gm in case of the soil sample and 5 ml in case of the liquid sample placed in to a 250ml flask containing 50 ml of sterile tape water and incubated at 30 ºC on a shaker at 200 rpm for 21 days. on days 3, 7 , 14 , 21 a sample of each soil slurry was serially diluted, plated on R_2A agar media, and incubated for one week at 30 ºC, after incubation, plates were Enumerated.

The bacterial colonies were purified by cross streaking on R2A agar plates at 30 ºC. after incubation, plates were enumerated, and morphologically different bacteria were selected for qualitative biosurfactant screening. The selected bacterial isolate were stored in R2A slants for further identification. these culture were kept in refrigerated condition (4 ºC) for further experimentation.

The R2 A agar media consist of (g/L): Yeast extract,0.5; preteose peptone,0.5; Casein hydrolysate,0.5; glucose,0.5; soluble starch,0.5; sodium pyruvate,0.3; K2HPO4,0.3; MgSO4.7H2O,0.05 and Agar15.0.

Identification of the most potent microorganism isolates producing biosurfactant:

The most potent biosurfactants producing bacteria were identified by biochemical tests with Biolog system and 16S rRNA gene sequence analysis.

16S rRNA gene sequence analysis

Crude bacterial lysates were prepared directly from culture plates by suspending bacteria from a clonal culture in 100 of PCR grade water (approximately McFarland Standard 2.0) and placing in a hot block at 100 ºC. for 10 min. A-1400 bp fragment of the 16S rRNA gene of the bacterial stains was amplified using the universal primer pair 27F 5/AGAGTTTGATCCTGGCTCAG3/ and1389R 5/ACGGGCGGTGTGTACAAG 3/. Resulting of PCR amplicons were sequenced in- house with forward and reverse primers 5GTTGCGCTCGTTGCGGGACT3/ and 5/ CTCCTACGGGAGGCAGCAG 3/ using an 310 DNA Analyzer and standard sequencing methods. The consensus sequences were then used to compare with online data bases.[14]

Biochemical tests with Biolog INC.

In the majority of cases, a single colony was selected and emulsified into 'inoculating fluid A' (Biolog) for subsequent inoculation on to the Microplate test plate (Biolog). More fastidious organisms, including capnophilic strains, were cultured on alternative media, according to the manufacturer's instructions and inocula prepared to a specified transmittance using a trubidimeter, as specified in the user guide. For each isolate 100 of the cell suspension was inoculated into each well of the MicroPlate, using a multichannel pipette and incubated at 37 ºC for 20h, aerobically. microPlates were read in the MicroStation semi-automated reader after 20h and results interpreted by the identification system's software (GEN III database).

Characterization and identification of the biosurfactant:

1- Biochemical composition of biosurfactant

Chemical composition of the biosurfactant was analyzed following standard methods. Carbohydrate content of the biosurfactant was determined by the phenol sulfuric acid method using D-glucose as standard.[15] Protein content was determined by Lowry method using bovine serum albumin as a standard.[16] Lipid content was estimated adopting the procedure of Folch[17].

Lowry method for protein estimation:[16]

- Different concentrations (0 -100) µg /ml of Bovine serum albumin (BSA) were prepared; the final volume was 1 ml.
- Four ml of solution 2 (Analytical reagents) was added to each tube and left to stand for 10 min.
- 0.4 ml from diluted Folin-Ciocalteus phenol reagent was added with rapid agitation and left to stand for 30 min.
- The absorbency (O.D) was measured at 600 nm.
- The relation between the absorption (O.D) and the protein concentration was drawn to obtain the standard curve for the BSA,
- The unknown protein samples were treated in the same manner and the protein concentration was determined according to the standard curve.

Phenol sulfuric acid method for carbohydrate estimation[15]:

- Different concentrations ranged from (0-100) µg/ml was prepared from the stock solution of glucose; the final volume was 1 ml.
- One ml of phenol solution was added to each tube with shaking.
- Five ml of concentrated sulphuric acid was added to the mixture, mixed well and left to cool at room temperature.
- The absorbency (O.D) was measured for each tube at 490 nm.

- The relation between absorbency (O.D) and glucose concentration was drawing to obtain the standard curve of glucose.
- The unknown carbohydrate samples were treated in the same manner and the carbohydrate concentration was determined according to the standard curve of glucose.

Note: The unknown samples used for protein and carbohydrate estimation were prepared by dissolving 1mg of emulsifier powder in 1ml of Tris – Mg or Tris – HCl with mixing by magnetic stirrer.

Colorimetric (Kits) method for lipid estimation[17]:

- 15 µL of samples or standards were placed into microcentrifuge tubes or a 96-well plate.
- For samples in DMSO, steps 2 and 3 were skipped and proceed to step 4.
- Samples and standards were incubated uncovered at 90°C for 30 minutes to completely evaporate organic solvents.
- This step is optional for aqueous, non-organic based samples, which will not evaporate during heating.
- Samples were transferred to 4°C for 5 minutes and 150 µL of 180 M sulfuric acid were added
- Samples were incubated at 90°C for 10 minutes then transferred to 4ºC for five minutes.
- 100 µL of each standard and unknown sample were placed into a clean 96-well plate and read at OD 540 nm to determine background.
- 100 µL of Vanillin Reagent were added, then incubated at 37ºC for 15 minutes mixed carefully.
- Samples were read then subtracted background from signal at OD 540 nm to determine signal.

Spectroscopic analysis

The IR and UV viz.- spectra-, Mass-spectrum and HNMR were determine at the Microanalysis Center (Cairo university).

Antimicrobial activity of the biosurfactant:

96 well microtitre plates were used for determining the minimum inhibitory concentrations (MICs) of the surface active metabolites (Biosurfactants).prepare stock solution of the surface active metabolite (Biosurfactant) by dissolving 500µg of it in one ml of sterilized distilled water then make several dilutions (500,250,125,62.5,31.25,15.63,7.81,3.9) by serial dilution method . A 50 µl of each dilution of the solution was dispensed in to each well of the microtiter plates. Add 50 µl of the tested microorganism contain 10^6 (0.5 McFarland) to each well of the microtiter plates. Add 100 µl of the Mueller Hinton broth media in case of bacteria,Sabaroud broth medium in case of yeasts and Dox broth medium in case of fungi to each well of the microtiter plates incubate the microtiter plates at 37^{o}C for 24 hours in case of bacteria, 30 ^{o}C for 24 hours in case of yeasts and 25 ^{o}C for 72 hours in case of fungi. Read the microtiter plates after the end of the incubation period by using microtiter plates reader (ELISA Reader)[18].

RESULTS AND DISCUSSIONS

Isolation and purification of the microorganisms:

This study began by the isolation and purification of 149 bacterial colonies from different localities in Egypt e.g- Arma oil company, Cairooil soap Company , waste water, waste industeaial water and Kafr El-sheikh, Giza, El-Gharbia, Elmenofia governorate.

Twenty four bacterial isolates were obtained from oils samples (15 isolate from Arma company, 9 isolates from Cairooil&soap company).

Twinty three isolates obtained from waste industrial water (6 isolates obtained from arma company, 17 isolates from Cairo oil &soap company).

Thirty four isolates obtained from waste water stations (10 isolates from El-Badrashein station isolates from, 15 isolates from Elwasta station, 9 isolates from El-ameria station).

Fifty-seven isolates obtained from Agricultural soil (12 isolates from KafrEl-sheikh, 21 isolates from Giza governorate, 10 isolates from El-Gharbia governorate, 14 isolates from El- menofia governrate).

Eleven isolates obtained from the automobil workshop (Sakara- Giza).

The collected bacterial isolates were subjected to a process of purification using the R2 a agar medium.

Table 1: Isolation, purification and enumeration of microbial count

Sample	Days of incubation	CFU/ml	Total No of colonies	Colonies selected for screening
1	3	$7X10^3$	7	2
	7	$3\ X10^2$	3	2
	14	$1X10^1$	1	1
	21	$1X10^1$	1	1
2	3	$31X10^3$	31	3
	7	$19X10^2$	19	2
	14	$10\ X10^1$	10	2
	21	$3X10^1$	3	2
3	3	$55X10^3$	55	3
	7	$31X10^2$	31	2
	14	$17X10^1$	17	2
	21	$5X10^1$	5	2
4	3	$33X10^2$	33	2
	7	$19X10^2$	19	2
	14	$7x10^1$	7	1
	21	$3X10^1$	3	1
5	3	$45X10^2$	45	4
	7	$23X10^2$	23	2
	14	$1x10^1$	1	1
	21	$2X10^1$	2	1
6	3	$23X10^2$	23	4
	7	$17X10^2$	17	3
	14	$9x10^1$	9	1
	21	$2X10^1$	2	1
7	3	$122X10^4$	122	6
	7	$18X10^3$	18	2
	14	$20X10^2$	20	1
	21	$4X10^1$	4	1
8	3	$143X10^4$	143	6
	7	$55X10^3$	55	4
	14	$38X10^2$	38	3
	21	$15X10^1$	15	2
9	3	$97X10^4$	97	4
	7	$15X10^3$	15	2
	14	$6X10^2$	6	2
	21	$9X10^1$	9	1
10	3	$88X10^3$	88	5
	7	$39X10^2$	39	3
	14	$10X10^2$	10	2
	21	$3X10^1$	3	2
11	3	$50X10^3$	50	3
	7	$19X10^2$	19	2
	14	$12X10^2$	12	2
	21	$7X10^1$	7	2
12	3	$110X10^3$	110	6
	7	$57X10^2$	57	3
	14	$31X10^2$	31	1
	21	$9X10^1$	9	2
13	3	$70X10^3$	70	4
	7	$31X10^2$	31	3
	14	$27X10^1$	27	2
	21	$5X10^1$	5	1
14	3	$109X10^3$	109	5
	7	$78X10^2$	78	3
	14	$40X10^2$	40	4
	21	$8X10^1$	8	2
15	3	$70X10^4$	70	5
	7	$42X10^3$	42	3
	14	$9X10^2$	9	2
	21	$1X10^1$	1	1
Total number of colonies / plate			**1834**	**149**

Identification of the most potent microorganism isolates producing biosurfactant

The two most potent strains were identified by biochemical tests with Biolog INC and by 16S rRNA, the first strain W1-S55 identified as *Pseudomonas aeruginosa*, the second strain SA3-S107 identified as *Bacillus subtilis*.

Identification of the isolate W1-S55:

a- by 16S rRNA

Sequencing of 16s rRNA for the isolate W1-S55

GTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCC
TGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGA
TCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAG
GCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTAC
GGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGA
AGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTCCAACAG
AATAAGCACCGGCTAACTTCGTGCCAGA

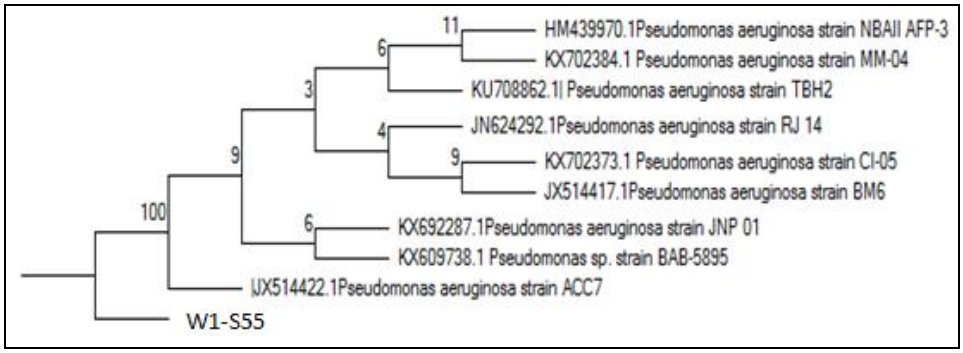

Fig. (1): Phylogenetic tree of the isolate**W1-S55.**

b- by Biochemical tests using Biolog INC method.

Table 2: Biochemical tests for the isolate W1-S55.

Test	Result	Test	Result	Test	Result
Netive control	-Ve	D-Raffinose	-Ve	α-D-Glucose	I
Dextrin	-Ve	D-Lactose	-Ve	D-Manose	-Ve
D-maltose	-Ve	D-Melibiose	-Ve	D-Fructose	I
D-Trehalose	-Ve	β-Methyl-D-Glucoside	-Ve	D-Galactose	-Ve
D-Cellobiose	-Ve	D-salicine	-Ve	3-Methyl Glucose	-Ve
Gentiobiose	-Ve	N-acetyl-D-Glucoseamine	I	D-Fucose	-Ve
Sucrose	-Ve	N-acetyl-D-Mannosamine	-Ve	L-Fucose	-Ve
D-Turanose	-Ve	N-acetyl-D-Galactosamine	-Ve	L-Rhamnose	-Ve
Stachyose	-Ve	N-acetylNeuraminic Acid	-Ve	Inosine	-Ve
possitive control	+Ve	1%NaCl	+Ve	10%Sodium Lactate	+Ve
pH6	+Ve	4%NaCl	-Ve	Fucidic Acid	+Ve
PH5	+Ve	8%NaCl	-Ve	D-Serine	-Ve
D-Sorbitol	-Ve	Gelatin	-Ve	Pectin	-Ve
D-Mannitol	I	Glycerol-L-Proline	I	Galactourronic Acid	I
D-Arabitol	-Ve	L-Alanine	I	GalactonicAcidLacton	-Ve
Myo-Insitol	-Ve	L-Arginine	I	D-Gluconic Acid	I
Glycerol	I	L-Aspartic Acid	I	D-Glucouronic Acid	-Ve
D-Glucose-6-PO_4	-Ve	L-Glutamic Acid	I	Glucouronamide	I
D- Fructose -6-PO_4	-Ve	L-Histidine	I	Mucic Acid	-Ve
D-Aspartic Acid	-Ve	L-PyroglutamicAcid	I	Quinic Acid	I
D-Serine	-Ve	L-Serine	-Ve	D-Saccharic Acid	-Ve
Troieandomycine	+Ve	Lincomycine	+Ve	Vancomycine	+Ve
Rifamycine	+Ve	Guanidine HCl	+Ve	Tetrazolium Violet	+Ve
Minocyclinc	+Ve	Niaproof 4	+Ve	Tetrazolium Blue	+Ve
P-Hydroxy-phenylacetic Acid	I	Methylpyruvate	-Ve	D-LacticAcidMethylEster	-Ve
L-Lactic Acid	I	Citric Acid	I	α-Keto-Glutaric Acid	+Ve
D-Mallic Acid	-Ve	L- Mallic Acid	+Ve	BromoSuccinicAcid	-Ve
Nalidexic Acid	+Ve	Lithium Chlorid	-Ve	Potassium Tellurite	+Ve
Tween 40	I	Amin-Butyric Acid	I	Hydroxy-ButyricAcid	+Ve
Hydroxy-D,L Butyric Acid	I	Keto-Butyric Acid	-Ve	Acetoacetic Acid	-Ve
Propionic Acid	I	Acetic Acid	+Ve	Formic Acid	I
Aztreonam	-Ve	SodiumButyrate	-Ve	Sodium Bromat	-Ve

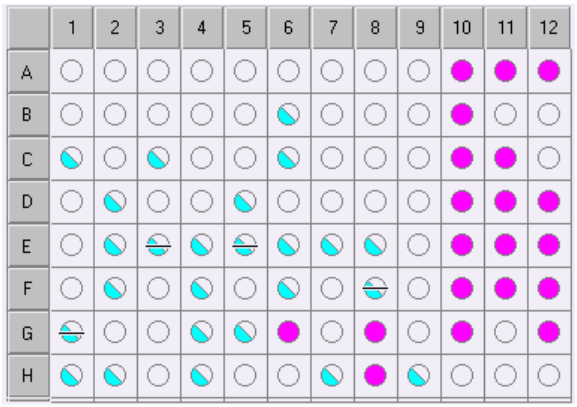

Fig. 2: Identification of the isolate **W1-S55** by biochemical tests with biolog.

Characterization and identification of the biosurfactant:

1- Biochemical composition of biosurfactant

Preliminary biochemical characterization of the biosurfactant produced by the isolate W1-S55 pseudomonas aeruginosa growing on Glycerol mineral salts medium. Showed that the product contains 51.72% (w/w) of lipids and 12.20% (w/w) of proteins and 36.10 (w/w) of carbohydrates this result suggests that the surfactant has aglycolipid nature, probably related to the rhamnolipid family of surfaces active compounds which are characteristics of some pseudomonas aeruginosa.These results similar to the results obtained by Chandran *et al* [19]which showed that the biosurfactant produced by the yeast *Trichosporon asahii* contain 68.20% of lipid, 0.0% of protein and 31.85% of carbohydrates.

Table 3: Bovine serum albumin standard curve

Bovine serum albumin(µg/ml)	Absorbance
10	0.113
20	0.136
30	0.193
40	0.290
50	0.339
60	0.455
70	0.500
80	0.511
90	0.592
100	0.746

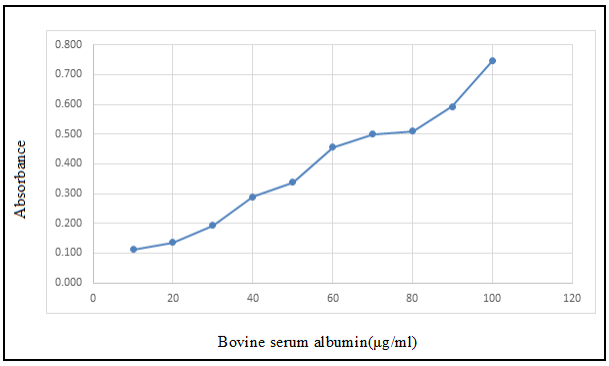

Fig. 3: Bovine serum albumin standard curve.

Table 4: Sugar standard curve.

Sugar concentration(µg/ml)	Absorbance
10	0.134
20	0.231
30	0.387
40	0448
50	0.499
60	0.538
70	0.666
80	0.775
90	0.821
100	0.942

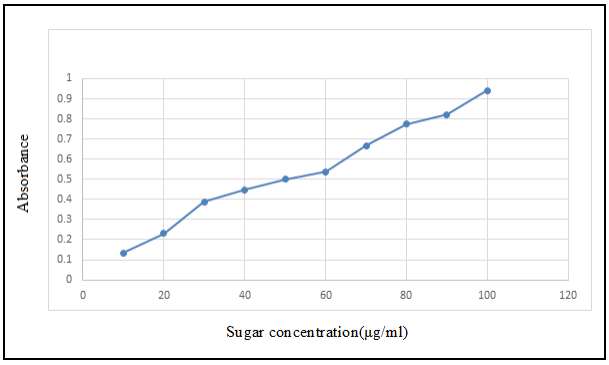

Fig. 4: sugar standard curve.

Table 5: Purified lipid standard curve.

Purified lipid(mg/dL)	Absorbance
2500	0.651
1250	0.486
625	0.404
313	0.378
156	0.248
78	0.199
39	0.103
0	

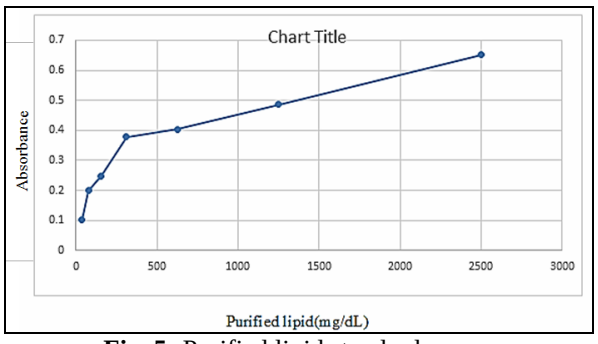

Fig. 5: Purified lipid standard curve.

2- Spectroscopic analysis

The spectrscopic analysis including (UV, IR, HNMR , Mass spectrum) of the both purified active substances produced by pseudomonas aeruginosa W1-S55 and *Bacillus subtlis* SA3-S107 were done .the substances has a molecular weight of about 650.00 and 1085.7483 gm/mol, respectively.

The product obtained by the isolate Pseudomonas aeruginosa W1-S55.

The ultraviolet(UV) absorption spectrum of the purified substance in ethyl alcohol using UV/Vis spectrophotometer, exhibit maximum absorption peaks at 400 nm, Fig(6).

The infrared (IR) absorbtion spectrum of the purified substance in ethyl alcohol showed maximum bands, Fig. (7). The IR analysis of the biosurfactants revelated that, the most important bands were located at 3342Cm^{-1} corresponds to the O-H stretch, 2926 cm^{-1} corresponds to the CH_2 strech, 1926Cm^{-1} corresponds to the unsaturated C=C bonds. Lactones and esters have the two strong absorbtion bands arising from C=O and C-O Streching the C=O absorption band at 1700 cm^{-1} includes contributions from that of lactones esters or acids.The stretch of C-O bond of C=O-O-C in lactones appears at1157 cm^{-1}. The sugar C-O stretch of C-O-H group is at1050 cm^{-1}. The above informations from the respective wave numbers confirmed the glycolipid nature of the biosurfactant.

The mass spectrometric analysis of the biosurfactant also complements the UV, IR the significant peaks observed at *m/z*=325 indicated the presence of lipids and at *m/z*=650 indicated the presence of carbohydrates moieties.

The proton nuclear magnetic resonance spectrum (H-NMR) of the active substance was shown in Fig.(9). the presence of long hydrocarbon chains and rhamnose(carbohydrates) rings is indicated by the appearance of the characteristic chemical shifts in the region of 0.8-1.4 and 3.3-5.5 ppm respectively.

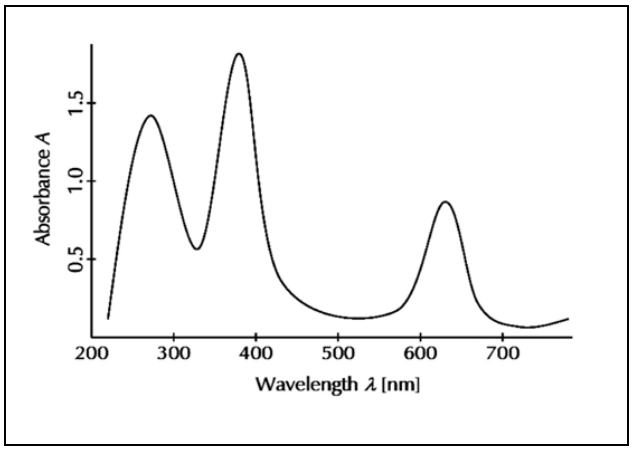

Fig. 6: TheUV-spectrm peaks of the purified active substance produced by Pseudomonas aeruginosa W1-S55.

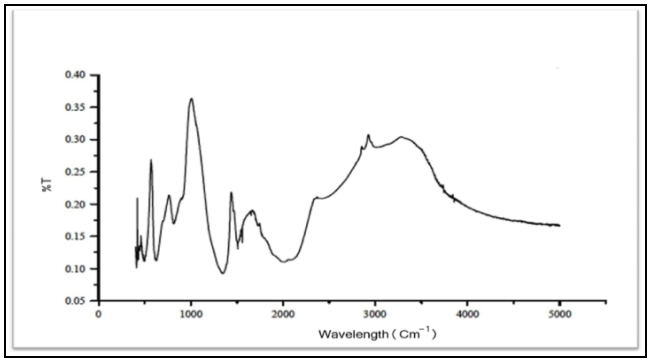

Fig. 7: The IR- spectrm bands of the purified active substance produced by *Pseudomonas aeruginosa* **W1-S55.**

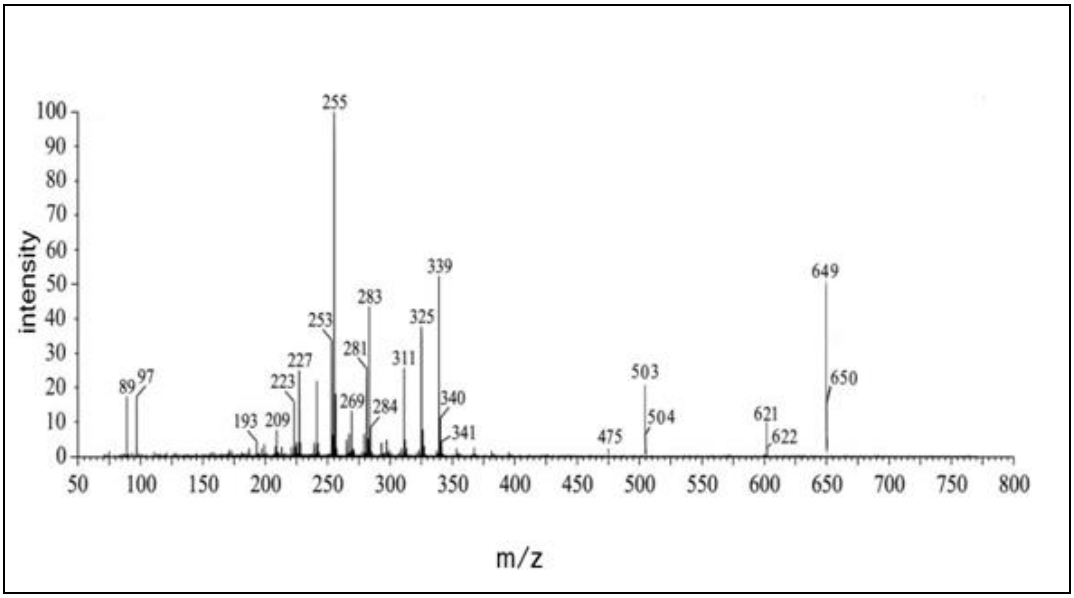

Fig. 8: The Mass- spectrm of the purified active substance produced by *Pseudomonas aeruginosa* **W1-S55.**

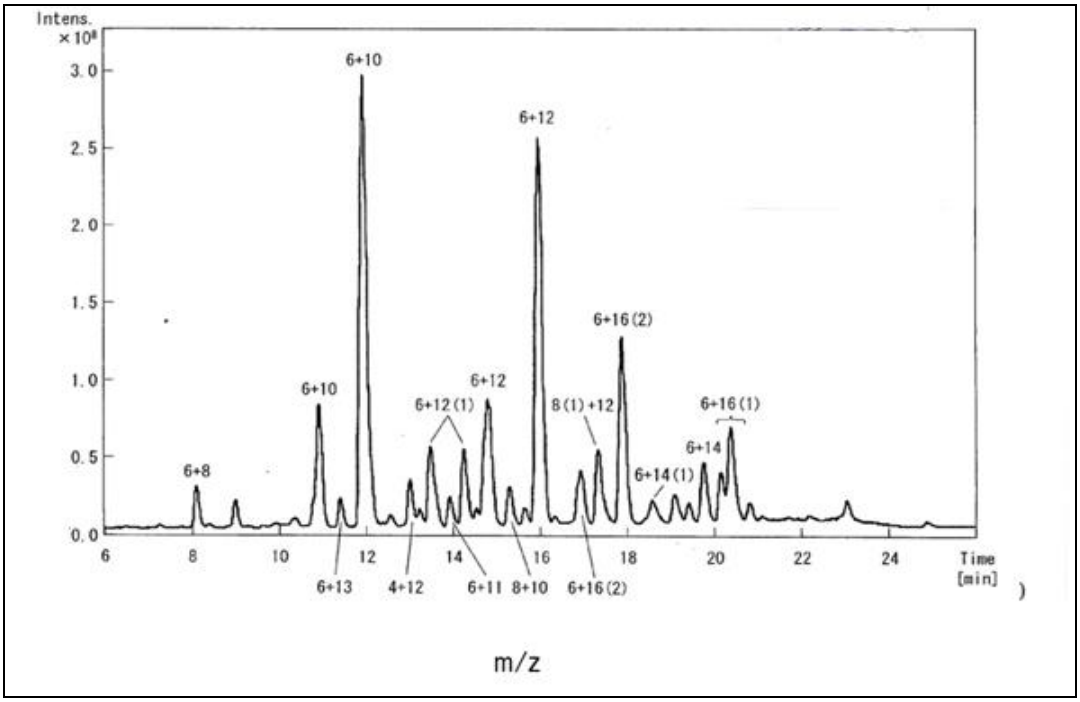

Fig. 9: The HNMR of the purified active substance produced by *Pseudomonas aeruginosa* W1-S55.

Antimicrobial activity of the biosurfactant:

Also the antimicrobial activity studies were done and the minimum inhibitory concentration determined by the microdilution method, the studies showed that the two biosurfactants having antimicrobial activity against different microbial test organisms such as G+Ve bacteria(*staphylococcus aureus* and *Bacillus subtilis*), G-Ve bacteria(*Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi*), filamentous fungi (*Aspergillus flavus* and *Aspergillus niger*) and Unicellular fungi (*Candid albicans*), and with MIC ranging from (31.25-250µg/ml). These results are similar to the results obtained by Benincasa et al[20] who represented that the surface active agent produced by *Pseudomonas aeruginosa* LBI having antimicrobial activity against G+Ve bacteria, G-Ve bacteria, filamentous fungi and unicellularv fungi with MIC (4-250 µg/ml) **.**

Table 6: The minimum Inhibitory concentration of the surface active compound produced by the isolate **W1-S55** by microdilution method.

Test organism	(µg/ml)MIC
staphylococcus aureus	31.25
Esherichia coli	125
Bacillus Subtilis	62.5
Pseudomonas aeruginosa	250
Salmonella typhi	31.25
Candid albicans	31.25
Aspergillus flavus	125
Aspergillus niger	62.5

REFERENCES

1. OECD. (1995): Surface tension of aqueous solutions OECD guideline 115. Paris: Organization for Economic Cooperation and Development.
2. Urum, K.; Pekdemir, T.(2004): Evaluation of biosurfactants for crude oil contaminated soil washing. Chemosphere. 57: 1139–1150.
3. Holmberg, Krister. (2001): "Natural surfactants." Current Opinion in Colloid & Interface Science 6.2: 148-159.
4. [4] Desai J. and BanatI. (1997): Microbial production of surfactants and their commercial potential. Microbiol. Mol. Biol. Rev. 61: 47–64.
5. Sim L.,Ward O. and Li Z.Y. (1997): Production and characterization of a biosurfactant isolated from *Pseudomonas aeruginosa* UW-1J. Indus. Microbiol. Biotechnol. 19: 232–238.
6. Lang S. and Wullbrandt D. (1999): Rhamnose lipids biosynthesis, microbial production and application potential. Appl. Microbiol. Biotechnol. 51: 22–32.
7. Panesar, R., Panesar, P. S., Hasija, D., Bera, M. B., & Kumar, H. (2009). Fermentative potential of *Pseudomonas aeruginosa* strain for biosurfactant production. In Biol. Forum-Inter. J .1:109.
8. Bodour, A.A. and Miller-Maier, R.M. (2002): Biosurfactants: Encyclopedia of Environmental Microbiology. Wiley, NY, pp: 750-770.
9. Banat IM, Makkar RS & Cameotra SS. (2000) : Potential commercial applications of microbial surfactants. Appl Microbiol Biotechnol. 53:495–508.
10. Falatko, David M., and John T. Novak.(1992):"Effects of biologically produced surfactants on the mobility and biodegradation of petroleum hydrocarbons." Water environment research 64.2: 163-169.
11. Karanth, N.G.K. et al., (1999): Microbial production of Biosurfactants and their Importance. Current Science, 77: 116-126.
12. Costa SG, Nitschke M, Haddad R, Eberlin MN, Contiero J.(2006): Production of *Pseudomonas aeruginosa* LBI rhamnolipids following growth on Brazilian native oils. Pro Biochem 41: 483-488.
13. Bodour A. Adria, Kevin P. Dress and Raina M. Maier.(2003): Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid southwestern soils. J. Appl. Env. Microbiol., 69(6):3280-3287.
14. (NCBI BLAST http://blast.ncbi.nlm.nih.gov/Blast.cgi)
15. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. T., & Smith, F. (1956): Colorimetric method for determination of sugars and related substances. Analytical chemistry, 28(3): 350-356.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951): Protein measurement with the Folin phenol reagent. J biol Chem, 193(1) : 265-275.
17. Folch, J., Lees, M., & Sloane-Stanley, G. H. (1957): A simple method for the isolation and purification of total lipids from animal tissues. J biol Chem, 226(1) : 497-509.
18. Mayrhofer, S., Domig, K. J., Mair, C., Zitz, U., Huys, G., & Kneifel, W. (2008) : Comparison of broth microdilution, Etest, and agar disk diffusion methods for antimicrobial susceptibility testing of *Lactobacillus acidophilus* group members. Applied and Environmental Microbiology, 74(12): 3745-3748.
19. CHANDRAN, PREETHY; DAS, NILANJANA.(2010): Biosurfactant production and diesel oil degradation by yeast species *Trichosporon asahii* isolated from petroleum hydrocarbon contaminated soil. Int J Eng Sci Technol, 2.12: 6942-6953.
20. Benincasa M, Abalos A, Oliveira I et al.(2004): Chemical structure, surface properties and biological activities of the biosurfactant produced by *Pseudomonas aeruginosa* LB1 from soapstock. Antonie Van Leeuwenhoek. 85: 1–8.