A Study on Biosurfactant Production from Marine Bacteria

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Abstract- Biosurfactants are surface-active substances produced by microorganisms. They are amphiphiles and consist of a polar (hydrophilic) and non polar (hydrophobic) group. Due to their amphiphilic structure biosurfactants tend to increase the surface area of hydrophobic water-insoluble substances and this makes surfactant an excellent emulsifier, foaming and dispersing agents. They are environmental friendly, biodegradable, less toxic and non-hazardous. Because of their potential advantages, biosurfactants are widely used in many pharmaceutical and industrial applications. The present study was carried out on biosurfactant production by *Halobacterium salinarum* isolated from marine source. *Halobacteria* are microorganisms that require high salt concentrations to survive and reside naturally in habitats such as salt lakes and salt marshes. The water sample was collected from marina beach, Chennai and *Halobacterium salinarum* was isolated using a selective media (HS media) and identified by Gram staining technique and biochemical test .The biosurfactants were extracted and subjected to thin layer chromatography. Drop collapse assay and oil spreading assay to check their stability .Biosurfactant concentration was estimated by means of phenol sulphuric acid method and further characterized by FTIR analysis. The biosurfactant produced was then tested for its antimicrobial activity & its efficiency was tested by using in Avocado smoothie preparation, where the naturally available oil content was emulsified using the biosurfactant extracted from *Halobacterium.*

Index Terms- Biosurfactant; Drop collapse assay, Fourier Transform Infrared Spectroscopy, Halobacterium , Biosurfactant, Thin Layer chromatography.

I. INTRODUCTION

Halophilic bacteria produce wide range of products with different properties and applications. Biosurfactant are one such extracellular amphiphilic compounds produced by Halobacteria especially when grown on hydrophobic substrates. Biosurfactants are a structurally diverse group of surface-active substances produced by microorganisms(Desai and Banat 1997). All biosurfactants are amphiphiles and consist of a polar (hydrophilic) and a non polar (hydrophobic) group. They are used as emulsifiers, foaming and dispersing agents. In comparison to the chemically synthesized biosurfactants they are considered to be environmental friendly, biodegradable and non-hazardous. They are active at extreme temperatures, pH and salinity and can be produced from industrial wastes and by-products. Because of their potential advantages they are widely used in industrial and medicinal applications. Biosurfactants can also be produced from cheap raw materials like rapeseed oil, potato process effluents, oil refinery waste, cassava flour wastewater, curd whey and distillery waste, sunflower oil etc. (Muthusamy*et al.,* 2008).

In recent years, much attention has been directed towards biosurfactants due to their broad range of functional properties and diverse synthetic capabilities of microbes. The most significant advantage of a microbial surfactant over chemical surfactant is its ecological acceptance (Desai J.D and Banat I.M., 1997). Biosurfactants can be efficiently used in handling industrial emulsions, control of oil spills, biodegradation and detoxification of industrial effluents and in bioremediation of contaminated soil. Due to their unique properties and application, identification of new biosurfactant producing microbes are in great demand. Nine different methods have been reported for screening biosurfactant producing microbes such as hemolytic assay, bacterial adhesion to hydrocarbons (BATH) assay, drop collapse assay, oil spreading assay, emulsification assay, surface tension measurement, titled glass slide test, blue agar plate and hydrocarbon overlay agar assay. These methods can have both advantages and disadvantages.

This study was aimed at identifying a sensitive, reproducible and simple method for screening the biosurfactant producing bacteria. Screening method used in this study were Drop collapse assay, Oil spreading assay and Emulsification assay. Totally 6 different bacterial strains were screened and checked for their biosurfactant producing ability using the aforementioned three methods and it was observed that the drop collapse assay and oil spreading assay directly correlated to each other and were easy to perform without any specialized equipments. Apart from above mentioned screening methods, this study also analyzes the credibility of the use of hydrophobic substrates like crude oil for the isolation of biosurfactant producing bacteria from the environment. Thus the present study was carried out to determine the antimicrobial and emulsifying applications of biosurfactant extracted from *Halobacterium salinarum* isolated from sea water.

II. MATERIALS AND METHODS

2.1 Collection of Sample:

Sea water samples were collected from different locations in Marina beach, Chennai, Tamilnadu, India.The samples were collected in different sterile glass bottles, and immediately transported to laboratory for further analysis.

2.2 Enrichment of *Halobacterium* sp:

A selective media (HS media) was used as enrichment medium for isolation of Halobacterium. 20 ml of the each

collected sample were inoculated in 100mlof HS medium prepared in 500 ml Erlenmeyer flasks , kept in a rotator shaker at 35°C or 38°C for 1-2 weeks and the growth was observed. On obtaining a confluent growth 10 ml of the primarily enriched medium was withdrawn and transferred to a new flask and incubated under same conditions mentioned above.

2.3 Isolation of *Halobacterium* spp

The enriched culture was diluted and plated on HS media for viable count. The plates were incubated at 37ºC for 5-7 days. After incubation colonies with different morphology were selected and subcultured further to obtain pure organisms.

2.4 Characterization and identification of isolates

Morphological description of colonies, Gram stain,(Ekrakene T and Igeleke C.L,2007; Ramos J. L, 2004) mobility tests and identification keys.(Ainsworth G.C, et al., 1973 ; Barnett H.L, and Hunter B.B, 1998) were used for bacterial identification.

2.5 Biochemical Characterization :

The discrete colonies from subculture plates were taken and series of biochemical tests were done for identification of bacterial species. The bacterial isolates were characterized by different biochemical reactions i.e. Oxidase, Catalase, Indole, MR, VP, Citrate, TSI, Fermentation of sugars etc., for the identification of bacterial species, standard methods were followed in described by Holt *et al*., 1994 ; Sherman N., Cappuccino J.G, 2005 ; Oyeleke S.B, Manga S.B, 2008 ; Kumar A, 2011 ; Adebayo Tayo B.C , 2012.

2.6 Screening for Biosurfactants:

2.6.1 Extraction of Biosurfactant: Rhamnolipids were purified by first separating the cells from supernatant by centrifugation at 6,800 rpm for 20 minutes. The supernatant was then acidified using 0.1 N hydrochloric acid to pH 2.0.The precipitated rhamnolipid was then collected by centrifugation at 12,100 rpm for 20 minutes. Rhamnolipids were extracted three times with chloroform-methanol (2:1) mixture. The organic phase was separated and the extraction was repeated twice. This was then evaporated leaving behind relatively pure rhamnolipids. The white residues of rhamnolipids obtained after drying were further dissolved in an appropriate volume of methanol and transferred to a previously weighed container (K.Santhini*et al.*, 2014).

2.6.2 Thin layer chromatography: Silica gel plate was taken and the sample was spotted on to the plate at a distance of about 2.5cm from one end of the edge using a capillary tube and allowed to dry. The solvent system- chloroform:methanol (2:1) was poured into a tank and allowed to stand with a cover plate on the top. This ensures that the atmosphere within the tank becomes saturated with the solvent. After equilibration the silica plate was kept vertically immersed in the tank and is kept closed. The solvent rises up the plate due to capillary action. Once the solvent reaches the top of the plate it is removed from the tank and is kept for drying. Once dried the plates were sprayed with anthrone reagent (1.5ml Sulphuric acid,1.5ml Phenol & 7ml Ethanol)and the appearance of brown spot indicates the presence of biosurfactant and was compared with the standard (S.K.Arora *et al.*,2015).

2.6.3 Drop collapse assay:5µl of cell suspension or culture or supernatant was added to the oil coated solid surface. The shape of the drop on the oil surface was inspected after a minute. If the liquid contains surfactants, the drops spread or even collapse because the force or interfacial tension between the liquid drop and the hydrophobic surface is reduced. If the liquid does not contain surfactants then the polar water molecules are repelled from the hydrophobic surface and the drops remain stable (Jain *et al*,1991).

2.6.4 Oil spreading assay: 10 µl of the crude oil is added to the surface of 40ml distilled water in a Petri dish to form a thin oil layer.10 µl of culture or culture supernatant is then added, oil is displaced and a clearing zone is formed**.** If biosurfactant is present in the supernatant, the oil is displaced and a clearing zone is formed. (Morikawa M *et al.*, 2000).

2.7 Estimation of Biosurfactant:

Phenol - Sulphuric acid method: Biosurfactant producing test samples were selected and inoculated in HS broth & incubated at 37º C on rotary shaker for 4-5 days. After incubation, broth was centrifuged at 10,000 rpm for 15 mins, the supernatant was collected, while pellet was discarded.1ml of collected supernatant was mixed with 1ml of 5% Phenol then 5ml of concentrated Sulphuric acid was added in drop wise manner. Presence of biosurfactant in supernatant produces orange color from yellow color.

2.8 Characterization by Fourier Transform Infrared Spectroscopy (FTIR):

Fourier Transform Infrared Spectroscopic analysis (FTIR) was carried out using crude biosurfactant extract obtained from the acid precipitation of the cell free culture supernatant. IR Prestige- 21Fourier Transform Infrared spectrophotometer (Samadzku, Japan) was used to determine the chemical nature of the biosurfactant by the KBr pellet method (Das *et al.*, 2009a, b; Mukherjee *et al.*, 2009).

2.9 Antimicrobial activity: The antibacterial and antifungal activity of the extracted biosurfactant was determined against 5 bacterial organisms namely *E. coli, Bacillus* spp, *Staphylococcus aureus, Streptococcus* spp and *Pseudomonas* spp and 2 fungal organisms namely *Candida albicans* and *Aspergillus niger* respectively. About 20ml of Muller Hinton agar (MHA) medium was prepared and sterilized for antibacterial activity while 20ml of Sabouraud Dextrose Agar (SDA) medium was prepared and sterilized for antifungal activity and poured onto the petriplates and was allowed to solidify. Once solidified the bacterial cultures and the fungal cultures were swabbed onto the agar medium using a sterile cotton swab. The wells were punctured using a sterile cork borer. The biosurfactant extracted from all the strains were dispensed in to the wells marked as H1, H2, H3, H4, H5, H6 and standard Rhamnose served as the control. The petriplates were then incubated at 37°C for 24 hours for bacteria and 37°C for 3-4 days for fungi and observed for the zone of inhibition. The diameter of the zone of inhibition was measured in mm.

2.10 Application: The biosurfactant produced was tested for its efficiency by using in Avocado smoothie preparation,

where the naturally available oil content was emulsified using the biosurfactant from *Halobacterium* spp. *A*vocado pulp was collected and the oil is separated and kept aside in an eppendorf tube . 10µl of extracted biosurfactant was added to the eppendroff and kept under observation for the emulsifying property of the added biosurfactant.

III. Results and Discussion

3.1 Isolation of *Halobacterium* spp from Marine water sample: Numerous pigmented colonies such as yellow, red, white spongy, white radial and dirty white colonies were isolated from the marine water sample and were numbered serially from H1-H6 for further identification **(Fig 1).**The shape, size, elevation, margin and color of the colony were observed in the culture plates with *Halobacterium salinarum* (HS) medium. Extremely halophilic species have been isolated from distinct saline regions of Marina beach.

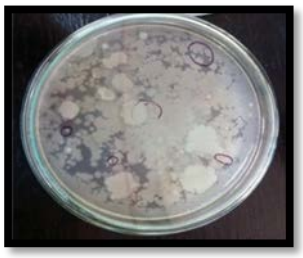

Figure 1: HS agar plate showing growth of *Halobacterium*

3.2 Characterization of Isolated bacteria:

A) Colony Morphology: The colony morphology of isolated *Halobacterium* strains were observed to be circular,smooth, rhizoid, filamentous, lobate, dry,irregular and entire. They were also motile and aerobic in nature.

B) Gram staining: The isolated strains were further characterized by means of Gram staining technique wherein all the six strains(H1, H2, H3, H4, H5 and H6) were identified to be Gram negative rod and cocci **(Fig 2).**

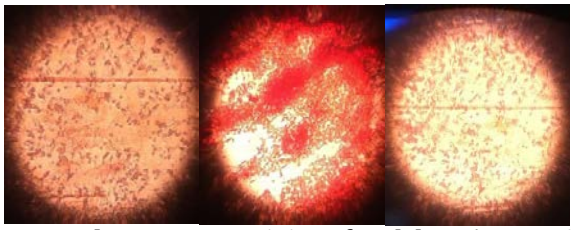

Figure 2:Shows Gram staining of *Halobacteium* strains

C) Biochemical test: The isolated pure cultures were subjected to various biochemical characterization test and the results were tabulated **(Table 1).**In the present study, the bacterial colonies isolated from marine water samples showed positive results for almost all the biochemical tests except a few. The results of the morphological and biochemical characteristic studies have confirmed the presence of *Halobacterium sp.*

Biochemical Test	**H1**	**H2**	**H3**	**H4**	**H5**	**H6**
Indole	Positive	Positive	Positive	Positive	Positive	Positive
Methyl red	Positive	Positive	Positive	Positive	Positive	Positive
Voges Proskauer	Negative	Negative	Negative	Negative	Negative	Negative
Citrate	Positive	Positive	Positive	Positive	Positive	Positive
Triple Sugar Iron	Positive	Positive	Positive	Positive	Positive	Positive
Catalase	Positive	Negative	Negative	Positive	Positive	Negative
Carbohydrate	Negative	Negative	Negative	Negative	Negative	Negative

Table 1:Biochemical test results of Isolated Halobacterium species (H1 –H6)

.3 Screening of Biosurfactant:

3.3.1 Extraction of Biosurfactant: The bacterial cultures were inoculated in Mineral Salt medium and incubated for 48 hrs. After incubation period, cultures were centrifuged and the supernatant was collected and screened for biosurfactant activity (K.Santhini *et al.*, 2014).The collected supernatant was mixed with Chloroform: Methanol in the ratio of 2:1. **(Fig 3).**The lower organic phase was found to contain biosurfactant exhibiting a oily nature .(S.K.Aroraet *al.*, 2015).

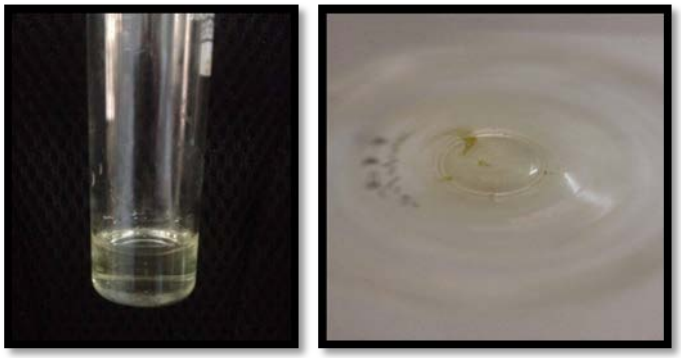

Figure 3: Extraction of Biosurfactant using Chloroform: Methanol (2:1)

3.3.2 Thin layer chromatography: The biosurfactant produced by all the six strains (H1 - H6) were subjected to TLC and compared with the standard biosurfactant Rhamnolipid. The Sediments obtained on extraction were spotted onto the TLC plate and the plates when sprayed with Phenol sulphuric acid reagent showed brown spots and the R_f value was found to be 0.65 the spot was concluded as a lipid moiety containing the compound of lipopeptide. (Anyanwu *et al*., 2011) cand this indicates the presence of biosurfactant in the sample **(Fig 4)**(S.K.Arora *et al.*,2015).

Figure 4: Shows the development of Brown spots

3.3.3 Drop collapse assay: The H1- H6 samples showed dispersion and collapse of oil in water. Among the samples H2 & H6 showed immediate dispersion collapsing the oil sample. This assay relies on the destabilization of liquid droplets by surfactants. Therefore when drops of cell suspension or culture supernatant are placed on oil coated solid surface, Absence of biosurfactants in the liquid, the polar water molecules get repelled from the hydrophobic surface and the drops remain stable. If the liquid contains surfactants, the drops spread or even collapse because the force or interfacial tension between the liquid drop and the hydrophobic surface is reduced **(Fig 5).** The stability of drop is dependent on surfactant concentration and correlates with surface and interfacial tension (Jain *et al.*, 2014).

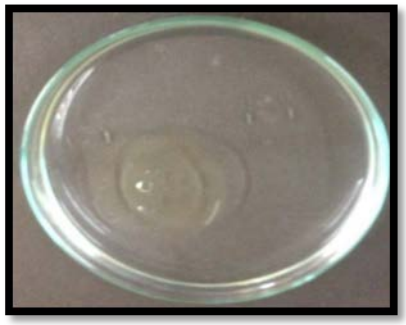

Figure 5: Shows the Drop collapse assay

3.3.4 Phenol-Sulphuric acid assay: Rhamnolipid undergoes dehydration in the presence of sulphuric acid to form derivatives of furfural that condenses with phenol to form a reddish orange coloured complex with an absorption maximum 490 nm (Abirami sivasubramni *et al.*,2015) .The Presence of rhamnolipid biosurfactant was confirmed in all the samples, whereas H2 & H5 showed a colour change to greenish yellow indicating a mere presence of biosurfactant **(Fig 6).**

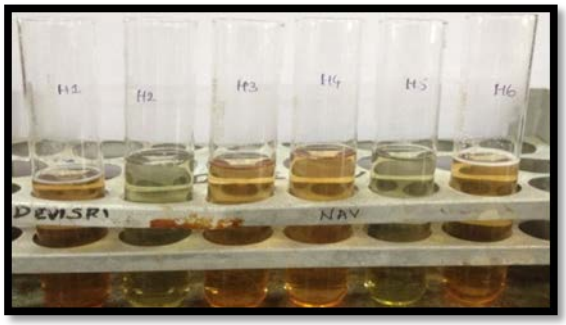

Figure 6: Shows the colour change of biosurfactants in Phenol sulphuric acid assay

3.4 Antimicrobial activity: The antibacterial activity was determined against five bacterial organisms such as *E. coli*, *Bacillus sps*, *Staphylococcus aureus*, *Streptococcus sps* and *Pseudomonas sps* and antifungal activity was determined against two fungal organisms namely *Candida albicans* and *Aspergillus niger*for the test samples H1- H6 **(Table 2).** Antimicrobial activity of purified biosurfactant showed broad spectrum of activity against the test organisms. The result showed that the highest activity of biosurfactant from H4 & H6 strain .H5 strain produced biosurfactant was active against *Bacillus* sp (0.85mm) followed by H6 strain biosurfactant against *S.aureus* and *Escherichia coli* (0.75mm). The least activity observed against *A.niger* (0.25mm).

S.No	Organisms	H1	H2	H3	H4	H5	H6	Control
1	*E.coli*	0.5	0.75	0.4	0.35	0.65	0.65	0
2	*Bacillus sps*	0.35	0.6	0	0.35	0.85	0.5	0
3	*Staphylococcus aureus*	0	0	0	0.75	0	0.75	0

4	*Streptococcus sps*	0.25	0.5	0	0.3	0.6	0.5	0
5	*Pseudomonas sps*	0	0.5	0	0.4	0.5	0.75	0
6	*Candida albicans*	0.6	0	0	0.4	0.45	0.5	0
7	*Aspergillus niger*	0.35	0.35	0.35	0.25	0.3	0.4	0

Table 2: Antimicrobial activity of the isolated strains

3.5 Characterization of Biosurfactant by FTIR: The biosurfactant produced by *Halobacterium* spp (H4 & H6) was classified as a lipopeptide .The most important peaks of FTIR analysis of the biosurfactant revealed that, the peak at 491 cm1 is due to C–I (Carbon–Iodine) bond , 3429 and 2360 cm1 denoted as the N–H group. The transmittance around at 1400 cm1 referred to the aliphatic chain of the C–H group. An intense stretching peak 1159, 1537 and 1626 cm1 indicates the presence of RNO2 groups. The availability of all these functional groups firmly substantiated that the biosurfactant is a peptide nature (Donio *et al.,* 2013a). The above information from the respective wave numbers confirmed the lipopeptide nature of the biosurfactant .(Fig 7 & 8).

Figure 7: Shows FTIR results of the biosurfactant produced by *Halobacteriumsp* H4 strain

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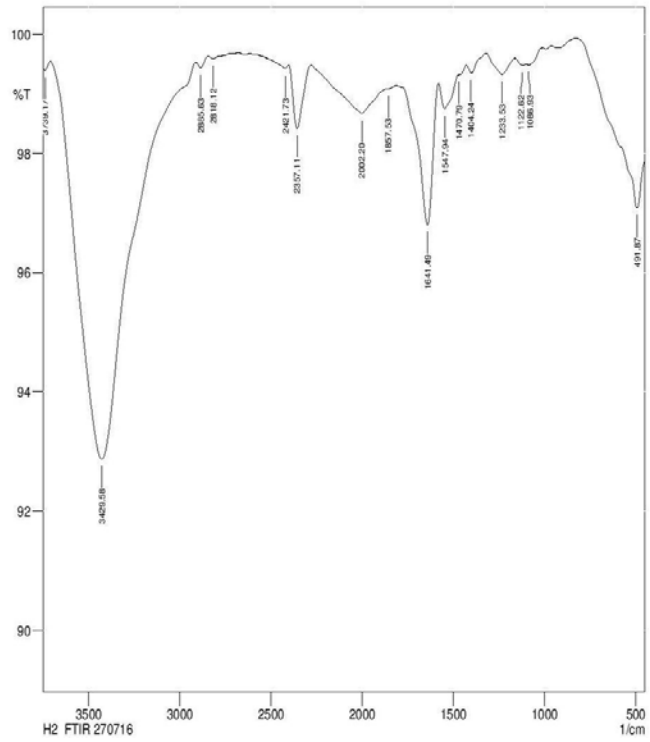
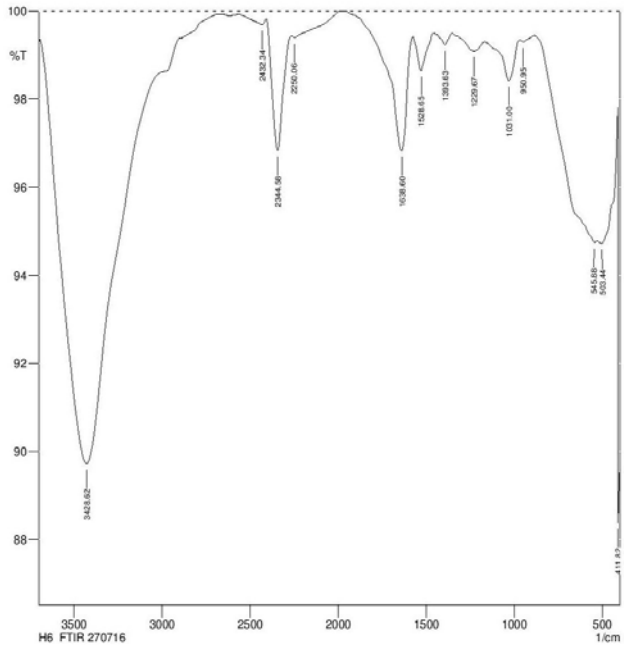


SHIMADZU

	Peak	Intensity	Corr. Inte	Base (H)	Base (L)	Area	Corr. Are
1	405.07	89.496	7.979	406.03	399.28	0.32	0.124
2	428.22	97.637	0.657	452.33	407	0.425	0.082
3	491.87	97.088	0.845	581.56	453.29	1.349	0.214
4	1086.93	99.486	0.048	1099.47	1018.46	0.146	0.016
5	1122.62	99.476	0.054	1163.13	1100.44	0.132	0.008
6	1233.53	99.329	0.308	1321.3	1164.09	0.358	0.113
7	1404.24	99.357	0.17	1430.28	1322.26	0.228	0.031
8	1470.79	99.318	0.004	1471.75	1431.24	0.11	0.004
9	1547.94	98.75	0.473	1581.7	1472.71	0.485	0.129
10	1641.49	96.804	2.356	1782.3	1582.66	1.622	0.879
11	1857.53	99.086	0.007	1863.32	1812.2	0.198	0.001
12	2002.2	98.673	0.544	2280.92	1864.28	1.792	0.49
13	2357.11	98.417	1.065	2404.37	2281.89	0.544	0.267
14	2421.73	99.424	0.076	2587.62	2405.34	0.352	0.017
15	2818.12	99.587	0.057	2842.23	2711.07	0.206	0.011
16	2885.63	99.431	0.139	2911.67	2843.2	0.143	0.019
17	3429.58	92.875	6.669	3709.27	2912.64	11.151	9.56
18	3739.17	99.396	0.228	3781.61	3710.24	0.149	0.037
19	3852.98	90.053	0.011	3857.8	3814.4	1.968	0.002
20	3895.41	90.008	0.006	3900.23	3858.76	1.892	0.001
21	3955.21	89.984	0.034	3986.07	3924.34	2.824	0.005

Figure 8: Shows FTIR results of the biosurfactant produced by *Halobacteriumsp* H6 strain.



	Peak	Intensity	Corr. Inte	Base (H)	Base (L)	Area	Corr. Are
1	411.82	88.381	8.862	412.78	399.28	0.673	0.274
2	503.44	94.716	0.659	526.59	413.75	2.349	0.466
3	545.88	94.742	0.279	886.33	527.55	5.238	0.693
4	950.95	99.293	0.069	968.31	887.29	0.226	0.012
5	1031	98.418	0.907	1166.98	969.27	0.885	0.297
6	1229.67	99.092	0.258	1328.05	1167.95	0.534	0.091
7	1393.63	99.23	0.2	1420.63	1352.16	0.194	0.028
8	1528.65	98.652	0.797	1575.91	1462.11	0.459	0.19
9	1638.6	96.824	2.689	1908.65	1576.87	1.763	1.282
10	2250.06	99.392	0.068	2264.53	1987.73	0.418	0.078
11	2344.58	96.833	2.817	2409.19	2265.49	1.057	0.825
12	2432.34	99.689	0.154	2565.44	2410.16	0.131	0.048
13	3428.62	89.725	9.77	3692.88	2674.42	17.445	15.833
14	3731.45	98.876	0.486	3763.28	3693.84	0.272	0.079
15	3806.68	89.56	0.01	3807.65	3783.53	1.139	0
16	3823.08	89.441	0.012	3824.04	3809.57	0.696	0
17	3839.47	89.433	0.026	3847.19	3832.72	0.701	0.001
18	3909.88	89.733	0.032	3917.59	3905.06	0.589	0.001
19	3941.7	89.88	0.01	3950.38	3938.81	0.536	0
20	3971.6	89.98	0.005	3981.25	3969.67	0.53	0

3.6 Application: Avocado smoothie ingredients are prepared and its squeezed oil is added in avocado smoothie and also extracted biosurfactant is added in it, to confirm that it's an emulsifier and can be used in food. In control separate upper layer is formed in eppendorf tube while adding squeezed oil in avocado smoothie **(Fig 9).**

Figure 9: Shows Squeezed oil taken from avocado& Control for avocado smoothie

Test samples (H4& H6): In two separate fresh eppendorfs, Avocado squeezed oil and extracted biosurfactant from H4& H6 strains were taken and mixed well. After the addition of biosurfactant to the eppendorf the oil was emulsified and got mixed well in the preparation resembling as one single layer **(Fig 10).** The biosurfactant used in food industries helps in proper emulsification of the oil and increases the absorption of the essential oils in the system.

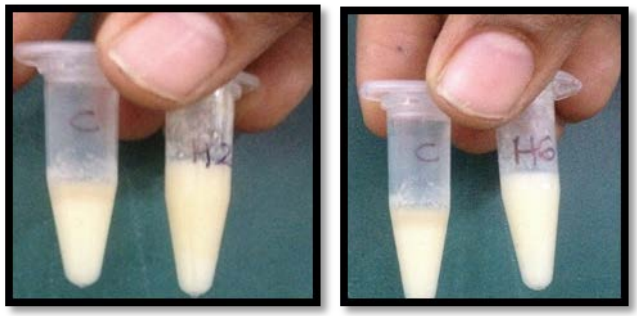


Figure 10: Shows biosurfactants from H4 and H6 added to the preparation.

IV. Conclusion

Biosurfactants have wide applications in various industries such as agriculture, food production, chemistry, cosmetics and pharmaceutics. Many properties of microbial surface active compounds such as emulsification/de-emulsification, dispersion, foaming, wetting and coating make them useful in physico-chemical and biological remediation technologies of both organic and metal contaminants. Biosurfactants were found to increase the bioavailability of hydrocarbon resulting in enhanced growth and degradation of contaminants by hydrocarbon-degrading bacteria present in polluted soil. The production of biosurfactants using the microbial sources have received much attention these days when compared to synthetic sources because of low toxic and non-hazardous nature but only limited works were carried out so mass production of biosurfactants from *Halobacterium* spp have to be taken from pilot study level to industrial level. Not much work has been reported on the biosurfactant production from *Halobacterium* spp due to the difficulty in isolation procedure. Yet the present study was one such attempt to explore the potential utility of *Halobacterium* for the goodness of mankind.

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