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Strain Improvement for the Production of Biosurfactants from *Pseudomonas Species* Isolated from Oil Contaminated Sites of Ajmer (Rajasthan) and Analysis by HPLC

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Abstract:*Biosurfactants are produced mainly by microorganisms such as bacteria, fungi and yeasts. They have a surface active property which is mediated by the amphiphilic nature of molecules. Biosurfactants have hydrophobic and hydrophilic regions which allowing them to act assurfactants. This action of surfactant is mediated at the interfaces between aqueous and non-aqueous components in a complex system and at the liquid gas interface. Recently biosurfactants gained importance in various fields because of their low toxicity, high biodegradability and powerful surfactant properties. The present study was aimed to improve production of biosurfactants from Pseudomonas species which mainly produce Rhamnolipid biosurfactant which provide significant opportunities to replace chemical surfactants with sustainable biologically produced surfactants. In continuation of these studies, the present work intended to purify characterize the biosurfactant produced by the bacterial species Pseudomonas.*

Keywords:*Biosurfactants, Amphiphilic, Hydrophobic and Hydrophilic regions, Rhamnolipid, Pseudomonas species.*

I. Introduction:

Biosurfactants are produced by different microorganisms such as bacteria, fungi and yeast. They are amphiphilic compounds produced on living surfaces, mostly microbial cell surfaces or excreted extracellular and contain hydrophobic and hydrophilic moieties that reduce surface tension between individual molecules at the surface and interface respectively. These microbially produced surface-active compounds possess the ability to reduce the surface and interfacial tension between two immiscible fluid phases. They are found in the nature in a wide variety of chemical structures, including Glycolipids, Lipopeptides and Lipoproteins, Fatty Acids, Neutral lipids, Phospholipids, Polymeric and Particulate Lipids (Desai and Banat, 1997).

In 1949, a crystalline glycolipid was isolated from *Pseudomonas aeruginosa* bacteria which showed antibiotic activity against tuberculosis in mice (Jarvis and Johnson, 1949). Biological compounds are being recognized increasingly as an alternative to synthetic ones because of characteristics such as low toxicity, biodegradability and mild process conditions. Biological surfactants, or biosurfactants, are an example of such compounds. They are produced by micro-organisms to fulfil various natural functions. They have a unique chemical structure, which consists of a hydrophobic and hydrophilic moiety, giving them detergency as well as emulsifying, foaming and dispersing traits. They are basically detergents. They dissolve water insoluble compounds by reducing their surface tension and form micro emulsions at the interfaces. Rhamnolipid is one of the types of glycolipids, in which one or two molecules of Rhamnose are linked to one or two molecules of hydroxyl decanoic acid while the OH group of one of the acids is involved in glycoside linkage with the reducing end of the Rhamnose disaccharide, the OH group of the second acid of involved in ester formation. Rhamnose is produced by *Pseudomonas aeruginosa*, a gram negative, motile, non-spore forming bacteria (Benincasa, Abalos & Oliveria, 2004).The present study focused on the biosurfactant production by *Pseudomonas aeruginosa* isolated from oil contaminated areas of Ajmer (Rajasthan)using different oils as substrate and the biosurfactant production was screened by Oil Spreading Technique and Emulsification Index test. Isolated biosurfactants were analyzed by using TLC and HPLC method.

II. Materials and Methods:

Isolation of *Pseudomonas aeruginosa*: In this study, oil contaminated soil samples were collected from various sites of Ajmer (Rajasthan). Two samples were selected for further experiments. These samples were from-Saras Dairy Ajmer and HMT factory Ajmer. Samples of oil contaminated soil were taken in sterile plastic bags. The temperature of the soil during collection was 38°C.Serial dilution and Pour PlateTechniquewere used to isolate bacterial colonies from the soil.

Selection of Oil Degrading Bacterial Strains: Six gram negative bacterial colonies were selected for screening of Biosurfactant production.The Bushnell Haas broth is used for examination of fuels for microbial contamination and hydrocarbon deterioration by bacteria. Medium contains all the nutrients except a carbon

source, which is necessary for the growth of bacteria. The bacteria can decompose a variety of hydrocarbons such as kerosene, mineral oil, paraffin wax and gasoline (Bushnell &Haas, 1941). For testing the liquid hydrocarbon, it is layered on the surface of the nutrient media. For testing the volatile hydrocarbon, petri plates containing medium are inverted and the hydrocarbon is poured into the lid of Petri Plate. Magnesium sulfate, Calcium chloride and Ferric chloride provide trace elements. Ammonium nitrate is a nitrogen source while Mono potassium phosphate and di-Potassium phosphate buffers the medium. Bacterial strains which produce biosurfactants can utilize hydrocarbon as a source of carbon (Abalos et al., 2004).

Primary Screening for Biosurfactants Production: Primary screening for biosurfactants production was done by Oil Spreading Test.

Oil Spreading Test: Oil spreading experiment was performed using Oil Spreading Test (Morikawa et al., 1993). 20 ml of distilled water was added to a Petri Plate followed by the addition of 20 µl of crude oil to the surface of the water. 10 µl of cell free culture broth was then added into the center of the oil layer. If biosurfactant present in the cell free culture broth, the oil will be displaced with an oil free clearing zone in oil layer and diameter of this clearing zone indicates the surfactant activity. A negative control was maintained with distilled water (without surfactant), in which no oil displacement or clear zone was observed and Triton X-100 was used as the positive control.

Characterization of Pseudomonas species by various biochemical tests: Catalase Test, IMViC Test (Methyl Red,Vogues Proskauer and Indole Test), Oxidase Test, Carbohydrate Fermentation Test, Casein Hydrolysis Test, Citrate Utilization Test, Lipid Hydrolysis Test, Starch Hydrolysis Test, Gelatin Hydrolysis test, Urease Test, and Hydrogen Sulfide Test were performed for identification of Pseudomonas sp.

Production of Biosurfactants: Bushnell Haas Medium was used for production of Biosurfactants.1000-1000 ml Bushnell Hass Broths was taken in four flasks and autoclaved these for 20 minutes at 121.6°C at 15 psi pressure. 400 ml engine oil was autoclaved for pouring on Bushnell Haas medium. When the temperature of Bushnell Haas medium reached 40°C, 4 different types of Gram Negative Bacillusstrainswere inoculated in each flask containing Bushnell Hass Broth. Then 100-100ml autoclaved engine oil was poured into each flask. These flasks incubated in shaking incubator at 120 RPM at 37°C for 20 days. Oil layer in each flask was measured with a scale. A control sample (blank) was maintained for identifyingfurther differences.

Optimization of Biosurfactant Production: Different types of culture medium, Bushnell Hass and Kay's (Mulligan & Gibbs, 1989) & (Zhang & Miller, 1992) were used for Optimization. Different concentrations of Ammonium nitrate in Bushnell Hass broth were also used. Chemical mutation by Ethidium bromide and Physical mutation by Ultraviolet light were applied. A dual mutation method was also used in this study. In this method a single strain was selected for chemical and physical both types of mutations. These all strains of Pseudomonas were used for extraction of biosurfactants.

Extraction of Biosurfactants: Acid Precipitation Method (Deziel et. al, 1996) was used for extraction of biosurfactants. After 1 month incubation, Bushnell Hass Broths inoculated with Pseudomonas sp.were centrifuged at 10000 RPM in a cooling centrifuge. Pellets were removed and supernatantscentrifuged at 12000 RPM. Again pellets were removed and supernatants were stored below 4°C for 24 hrs. Next day supernatants were again centrifuged at 8000 rpm. Pellets were removed and supernatants of all tubes were also stored at 4 degree centigrade for HPLC process. Remaining supernatants of all samples were taken in 1 liter beaker, pH was maintained 2.0 with 1M H_2SO_4 and then mixed equal volume of chloroform: methanol in 2:1 ratio. These supernatants were putted overnight for evaporation. After evaporation white color sediments were found in each beaker were biosurfactants. Biosurfactant was put in oven for 5 minutes for complete evaporation.

Quantitative Analysis of biosurfactants: Sterile petri plates were taken and the weight of the plates was measured. Now the sediments were poured into plates. They were placed into the hot air oven for drying at 100°C for 30 minutes. After drying plates were weighted. The dry weights of the biosurfactants were calculated by the following formula:

Dry weight of biosurfactant = (weight of plate after drying - weight of empty plate**)**

Characterization of Biosurfactants:

TLC (Thin Layer Chromatography): Primary characterization of biosurfactants was done by TLC (thin layer chromatography) TLC has been used for detection and composition of Rhamnolipids in culture broth extracts. Under normal phase TLC conditions mono Rhamnolipid and di Rhamnolipid are separated into two bands

HPLC (High Performance Liquid Chromatography): High performance liquid chromatography (HPLC) was used to measure Rhamnolipid concentrations. The Rhamnolipid molecules were hydrolyzed to release the Rhamnosemolecules, which were analyzed by HPLC. Two different hydrolysis methods were investigated. The first hydrolysis method was adapted from the hydrolysis step of the orcinol method (Koch et al. 1991). In this method 100µl of culture supernatant was transferred to a micro centrifuge tube and 900µl of a 53 % sulfuric acid solution was added. Then the tubes were inserted into a floating rack and were kept in boiling water on a hot plate for 30 min.

The second hydrolysis method is a general method for the rupture of oligosaccharides (Sluiter et al. 2005). In which 115 µl of a 53 % sulfuric acid was added to 2 ml of supernatant in a bottle. Then the solution was autoclaved at 121°C for 30 min and allowed to cool to room temperature.

Removal of proteins from biosurfactant samples: The hydrolyzed samples were treated to remove proteins, since proteins present in the supernatant can damage the HPLC column. For this 1 ml of hydrolyzed sample was centrifuged at 14 000 rpm (Eppendorf Minispin Plus) for 5 minutes. After that31. 5 µl of a 60 % Perchloric acid solution was added to 900 µl of the supernatant. Then the sample was incubated on ice for 10 minutes. After that 49.5 µl of a 7M KOH solution was added to the sample. The sample was incubated on ice for 10 minutes. The sample was centrifuged at 14 000 rpm for 5 minutes. Each sample was filtered using a 0.22 µm syringe filter (Millipore).

Samples of biosurfactants and L-rhamnose standards were filtered through a 0.22 µm syringe filter (Millipore). Settings for the column of HPLC are listed in Table-1. Standards containing L-rhamnose and glucose with concentrations between 100 and 5000 ppm were injected to construct a standard curve.

Table-1: HPLC Settings.

Detection	244 nm.
Column:	C-18
Column dimensions:	300 mm x 7.8 mm
Mobile phase:	Acetonitrile: water
Flow rate of mobile phase:	01 ml/min

III. Results and Discussion:

Isolation of Pseudomonas species: Six Gram Negative Bacillus strains were isolated from soil samples.

Selection of Oil Degrading Bacterial strains: Primary selection of oil degrading and biosurfactant producing bacterial strains was done by Bushnell Hass broth. Four strains show growth in Bushnell Hass broth. These strains were selected for further studies.

Primary Screening for Biosurfactants Production by Oil Spreading Test: Oil Spreading Test for screening of biosurfactant activity show positive results. All four strains show oil clearance zones in oil layer. Oil clearance zones were measured by a scale in cm. Results were shown in Figure-1.

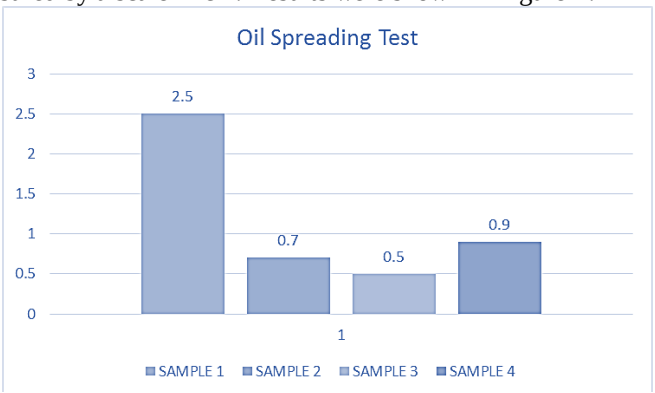


Figure-1: Primary Screening of Biosurfactant Production.

Characterization of Pseudomonas species by various Biochemical Tests: Various Biochemical Tests were applied for the Characterization of Pseudomonas species. Results of these biochemical tests were shown in Table-2.

Table-2: Results of various biochemical tests.

Biochemical Tests	**Sample 1 Results**	**Sample 2 Results**	**Sample 3 Results**	**Sample 4 Results**
Catalase test	Positive	Positive	Positive	Positive
Oxidase test	Negative	Negative	Positive	Positive
MR test	Positive	Negative	Negative	Negative
VP test	Negative	Negative	Negative	Negative
Indole test	Negative	Negative	Negative	Positive
Sugar fermentation	Negative	Negative	Positive (in glucose and sucrose)	Negative
Casein hydrolysis	Positive	Positive	Positive	Positive
Citrate utilization	Positive	Negative	Negative	Negative
Lipid hydrolysis	Positive	Positive	Positive	Positive
Starch hydrolysis	Positive	Positive	Negative	Positive
Gelatin hydrolysis	Negative	Negative	Positive	Negative
Urease test	Positive	Positive	Positive	Positive
H_2S TEST	Negative	Negative	Negative	Negative

Production of biosurfactants:

Production of biosurfactants was carried out in flasks. Each flask containing 1000ml Bushnell Haas Broth in the addition of 100 ml Engine Oil. These flasks were incubated at 37°C for 20 days. Oil Spreading Test and Emulsification Index Test were applied to measure surfactant activity in the medium. Results of Oil Spreading Test and Emulsification Index were shown in Figure-2 and Figure-3.

Oil Spreading Test: Oil Spreading Test results are shown in Pie chart in which each color shows sample no. and oil clearance zone shown in cm. and percentage.

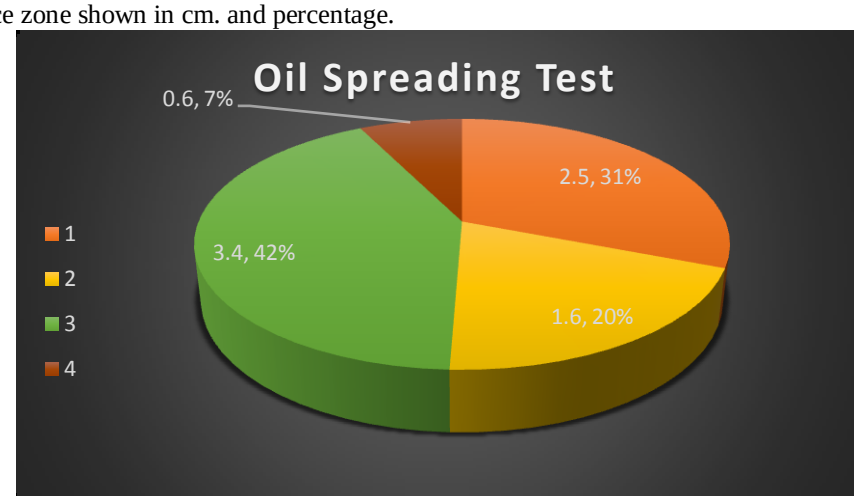


Figure-2: Oil Spreading Test

Emulsification Index Test: Each Column shows Sample no.

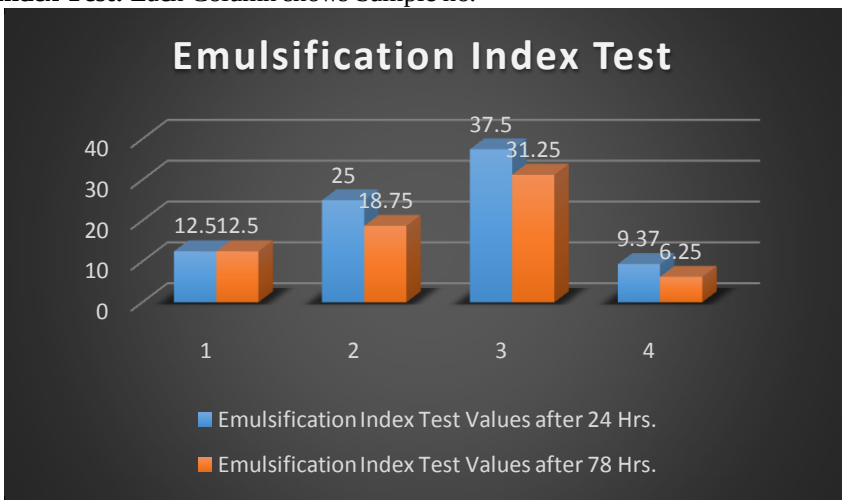


Figure-3: Emulsification Index Test

Optimization of Biosurfactant Production: Optimization of biosurfactant production achieved by various methods. Results are shown in Table-3, Table-4, Table-5, Table-6, and Table -7.

Table-3: Effects of Media on Growth:

Nutrient Media	Sample 1	Sample 2	Sample 3	Sample 4
Bushnell Haas Media	Poor growth	Poor growth	Maximum Growth	Good Growth
Kay's Media	Good Growth	Good Growth	Maximum Growth	No Growth

Table-4: Effect of Various Concentration of Ammonium Nitrate:

Concentration Of Ammonium Nitrate gm./Liter	Growth of Sample 3
10 gm./liter	Maximum growth.
15 gm./liter	Good growth.
20 gm./liter	Poor growth.
25 gm./liter	No growth.

Effects of chemical mutation: Chemical mutation was done by Etbr (Ethidium bromide). It is a carcinogen chemical which causes mutation.

Table-5: Effects of Chemical Mutation

Concentration of Etbr (µl/ 10 ml.)	Growth After Mutation
10 µl	No growth
20 µl	Growth occurs
30 µl	No growth
40 µl	No growth
50 µl	No growth

Growth occurs in only 20µl /10ml concentration of Etbr in chemical mutation, hence selected for extraction of biosurfactants.

Table-6: Effects of physical mutation: Physical mutation was done by ultraviolet light.

UV Treatment Time	Growth of Pseudomonas Spp.
5 Minutes.	Growth Occur
10 Minutes.	No Growth
15 Minutes.	No Growth
20 Minutes.	No Growth

Effects of Dual mutation: This method was applied during the study for optimization of biosurfactant production. The chemical Mutant strain was selected for U.V mutation for 2 minutes. Growth was occurred in dual mutant strain.

Final Screening for Biosurfactant Production: Five strains of Pseudomonas spp. were selected for final screening by oil spreading test these strains are shown in Table-7.

Table-7 Strains selected for Biosurfactant Production.

Bacterial Samples	Strain Type
Sample No. 1	10 gm. /Liter of Ammonium Nitrate.
Sample No. 2	15 gm. /Liter of Ammonium Nitrate.
Sample No. 3	20µl/ 10ml of Etbr (Chem. Mut.)
Sample No. 4	5 Min. U.V Treatment (Physical Mut.)
Sample No. 5	Dual Mutation (20µl/10 ml. Etbr+ 2 Min UV)

Table-8: Results of Oil Spreading Test.

Sample No.	Quantity of Water in Petri plate.	Quantity of Engine Oil Layered on Water.	Total Oil Layer In Centimeter.	Oil Displacement Area (Zone)
1.	20 ml.	20µl.	6 cm.	1.6 cm.
2.	20 ml.	20µl.	5.7 cm.	2.0 cm.
3.	20 ml.	20µl.	5.3 cm.	0.7 cm.
4.	20 ml.	20µl.	5 cm.	1.8 cm
5.	20 ml.	20µl.	6 cm.	**5.1 cm**

The dual mutant strainshows highest zone of oil displacement. It shows that strain of Pseudomonas spp. was improved. It almost completely displaced the oil layer when added in small quantity only. It displaced **5.1 cm**. area of oil layer.

Extraction of biosurfactant: After extraction of biosurfactants dry weight of biosurfactants was taken. Dry weight of biosurfactants is shown in Table-9.

Table-9: Dry Weight OF biosurfactants.

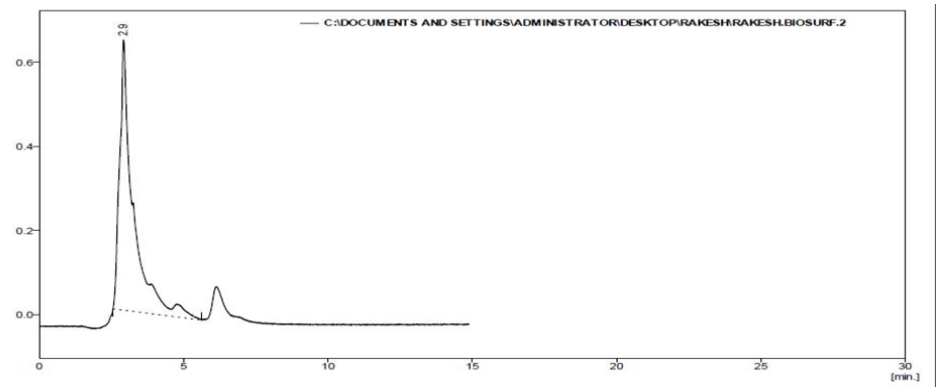
Samples	Weight of Plate	Weight of Plate After Drying of Biosurfactant	Total Dry Weight of Biosurfactant In 30 ml. Supernatant
10 gm./liter ammonium nitrate in BH broth	47.121 gm.	47.023 gm.	0.098 gm.
15 gm./liter ammonium nitrate concentration in BH broth	46.00 gm.	46.533 gm.	0.533 gm.
Chemical mutation 20µl /10 ml Etbr	45.970 gm.	46.012 gm.	0.042 gm.
Physical mutation 5 minutes UV treatment	48.130 gm.	48.566 gm.	0.0436 gm.
Dual mutation (20µl Etbr +2 minutes UV	45.170 gm.	46.195 gm.	**1.025 gm.**

Dual mutant strain of Pseudomonas spp. produce 1.025 gm. /30 ml supernatant biosurfactants it shows that strain was improved for production of biosurfactants.

Characterization of Biosurfactants: Primary characterization of biosurfactants was done by TLC. The yellow color spotshows presence of Rhamnolipid biosurfactant.

HPLC (High Performance Liquid Chromatography): HPLC results of all five strains shown here-

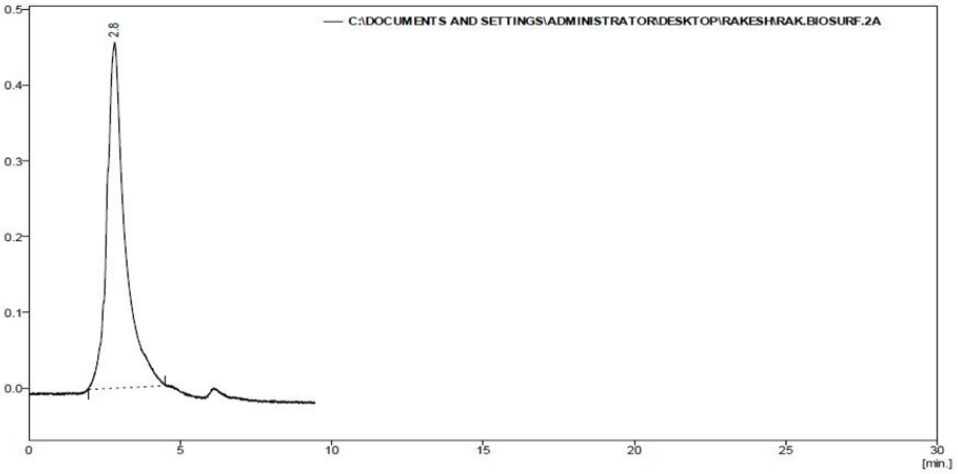
1. HPLC result of biosurfactants produced from sample 1st (10 GM. /Liter concentration of ammonium nitrate) in BH broth.



Result Table (Uncal - C:\DOCUMENTS AND SETTINGS\ADMINISTRATOR\DESKTOP\RAKESH\RAKESH.BIOSURF.2)

	Reten. Time [min]	Area [mV.s]	Area [%]
1	2.920	21.028	100.0
	Total	21.028	100.0

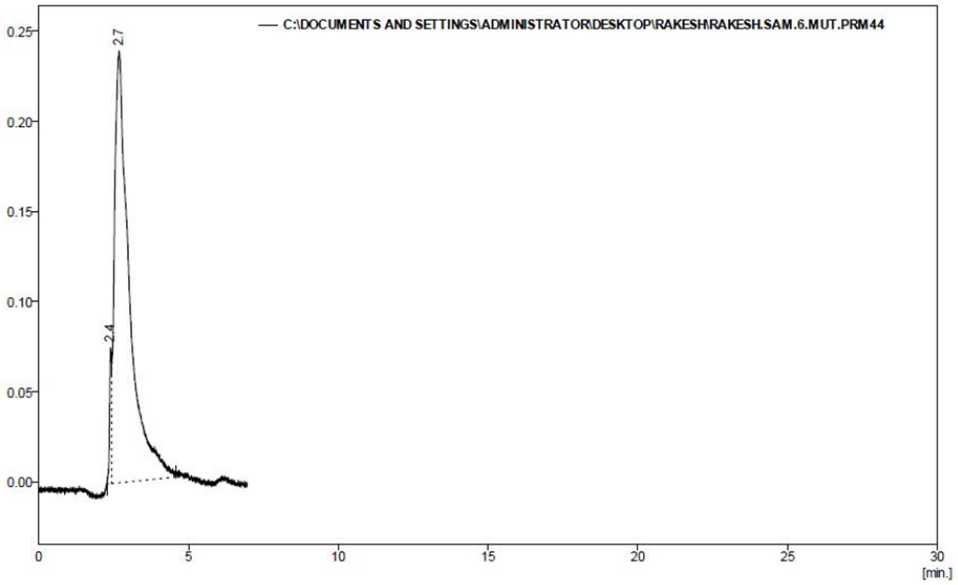
2. HPLC result of biosurfactants produced from sample 2nd 15 GM. / Liter ammonium nitrate concentration in BH broth.



Result Table (Uncal - C:\DOCUMENTS AND SETTINGS\ADMINISTRATOR\DESKTOP\RAKESH\RAK.BIOSURF.2A)

	Reten. Time [min]	Area [mV.s]	Area [%]
1	2.830	18.910	100.0
	Total	18.910	100.0

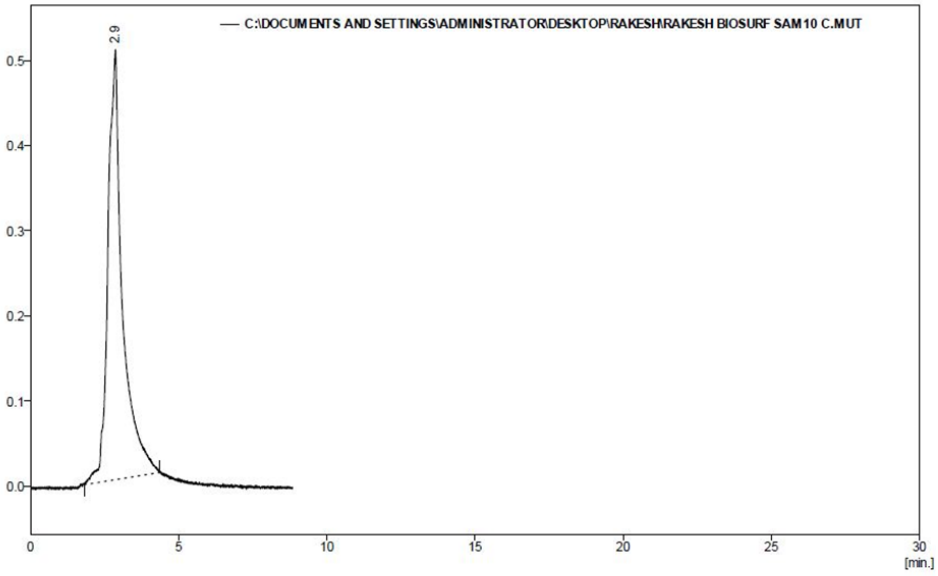
3. HPLC result of biosurfactants produced from chemical mutant strain (20µl. /10 ml. Etbr.)



Result Table (Uncal - C:\DOCUMENTS AND SETTINGS\ADMINISTRATOR\DESKTOP\RAKESH\RAKESH.SAM.6.MUT.PRM44)

	Reten. Time [min]	Area [mV.s]	Area [%]
1	2.397	0.335	3.9
2	2.680	8.298	96.1
	Total	8.633	100.0

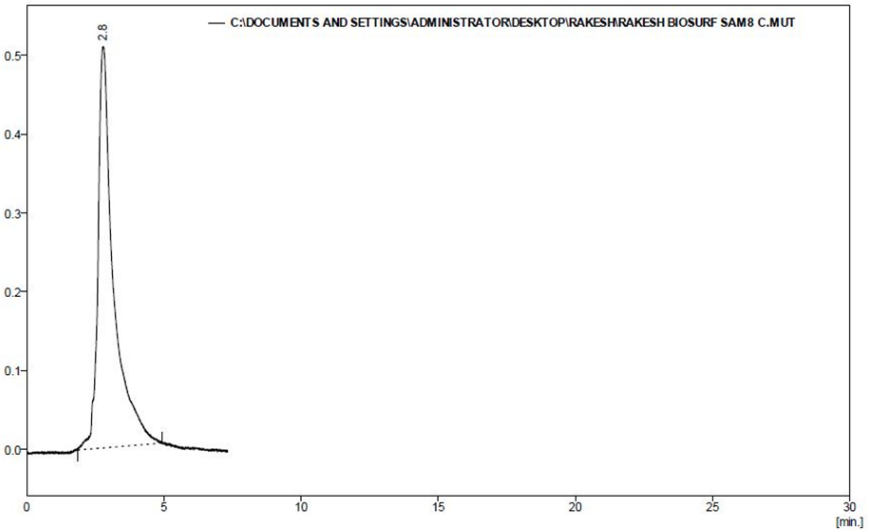
4. HPLC results of biosurfactant produced from UV mutant strain.



Result Table (Uncal - C:\DOCUMENTS AND SETTINGS\ADMINISTRATOR\DESKTOP\RAKESH\RAKESH BIOSURF SAM10 C.MUT)

	Reten. Time [min]	Area [mV.s]	Area [%]
1	2.860	17.301	100.0
	Total	17.301	100.0

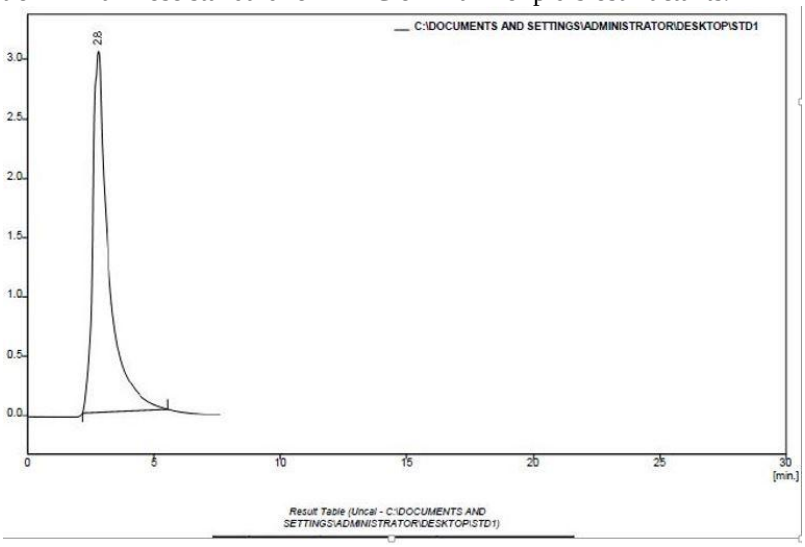
5. HPLC results of biosurfactant produced from the dual mutant strain of Pseudomonas spp.



Result Table (Uncal - C:\DOCUMENTS AND SETTINGS\ADMINISTRATOR\DESKTOP\RAKESH\RAKESH BIOSURF SAM8 C.MUT)

	Reten. Time [min]	Area [mV.s]	Area [%]
1	2.790	20.055	100.0
	Total	20.055	100.0

6. The HPLC result of L-Rhamnose standard for HPLC of Rhamnolipid biosurfactants.



Result Table (Uncal - C:\DOCUMENTS AND SETTINGS\ADMINISTRATOR\DESKTOP\STD1)

	Reten. Time [min]	Area [mV.s]	Area [%]
1	2.793	131.778	100.0
	Total	131.778	100.0

HPLC results showed that dual mutant strain produced Rhamnolipid biosurfactants. It was confirmed by matching with L-Rhamnose standard sample in HPLC.

IV. Conclusion:

The present study had been carried out by extensively to study the isolation of Pseudomonas spp. from oil contaminated sites of Ajmer (Rajasthan) for production of biosurfactants and improvement in Pseudomonas strain by various optimization processes and mutations experiments. Oil spreading test was used for identification of biosurfactants concentration. It was also used in primary screening for biosurfactants presence. We can say that this test gives root to this study because oil spreading test show that my bacterial strains produced biosurfactants. In oil spreading test large zone of the oil displacing areashowshigh concentrations of biosurfactant as well as good quality of biosurfactants also. Emulsification index test show quality of

biosurfactant. A good quality biosurfactant show high emulsification index after more than 48 hours also while low quality biosurfactants show less emulsification index. In many literatures reviews TLC used as a primary analysis tool for presence of Rhamnolipid biosurfactants. It gives a basic proof of the presence of biosurfactants. Literature research on Rhamnolipid studies did not reveal evidence of HPLC methodology for Rhamnose measurements. However, in this study, evidence for the accuracy of this method was demonstrated. Bushnell Hass Broth is the best suitable medium for production of biosurfactants. It also used for selection of oil degrading bacteria because this is a selective media in which no carbon source was present. Carbon source added separately as used engine oil to make an oil layer on broth. Decrease in oil layer show that bacteria use oil as carbon source and produced biosurfactants. Dual mutant strain of Pseudomonas sp. produce biosurfactants in high concentration. In Bushnell Hass Broth high concentration of ammonium nitrate decreases the production of biosurfactants. It also inhibits the growth of the organism when added in high concentration.

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