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Characterization of Efficient Biosufactants Producing Bacteria and its Efficacy on Bioremediation of Crude Oil

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ABSTRACT

The present work was aimed to isolate efficient hydrocarbon degrading and biosurfactant producing bacteria strain from oil contaminated site. Biosurfactant are low molecular surface active compounds involved in the degradation of hydrocarbons. Biosurfactant activity of isolated strain were detected by using different screening method. The study showed that efficient biosurfactant producing bacteria were identified as *Arthrobacter globiformis.* More over, crude oil degradation as consortium degraded more effectively than individual strain.

Key words: *Bioremediation, Biosurfactants, Hydrocarbon degrading bacteria.*

Introduction

Pollution causes adverse change in natural environment it can be take place in the form of energy or chemical substances. One of the environmental pollution such as petroleum derived product (hydrocarbon) is one of the main cause of global contamination. Petroleum is used as a conventional energy source even though it cause global environmental pollution. Petroleum also known as crude oil is a multifarious mixture of many petroleum hydrocarbon of which Polycyclic Aromatic Hydrocarbons (PAH) is a major fraction which is toxic, mutagenic and carcinogenic in nature. Oil spillage may arise either accidentally (or) operationally during production, transportation, storage, processing (or) when used at sea or on land. Oil released into the environment production affects many plants and humans.

The recovery of spilled crude oil can be achieved by physical and chemical method but it can remove oil spillage of about 10 - 15%. Some common methods are used for soil remediation but it is expensive and lead to incomplete decomposition of contamination. To overcome these problems microbial bioremediation is the only way to preserve our nature. Bioremediation is a microbial processes to degrade and detoxify environmental contaminants is a cost effective, eco- friendly method in which highly hazardous oily materials are easily converted into harmless end product by using suitable microbial strain.

Biosurfactant are surface active compounds of heterogenous groups of surface active molecules produced by microorganism which either adhere to cell surface or are extracted extracellularly in the growth medium (Fietcher *et al.*, 1992; Cameotra *et al.*, 1998).

Biosurfactant are commonly employed in crude oil recovery (Banat *et al.*, 2010) hydrocarbon degradation in soil (Nitschke *et al.*, 2011). In some of the

studies reported that immobilized cells compared with free living bacteria more effective, have longer shelf life, lower cost price and higher crude oil degrading activity in various areas. Accordingly, the aim of this study is to isolate bacterial strain capable of producing biosurfactant and to obtain potential hydrocarbon degrader.

Materials and Methods

Sterilization

Glass ware and media were sterilized in autoclave at 121°C with 15 lbs pressure for 20 mins. Sample collection Sea water and soil sediment was collected from oil contaminated site from Ennore sea. Soil was collected in sterile ziplock bag and labelled. Sea water was collected in sterile bottle and both were brought to the laboratory and stored at 4 ºC till further analysis (Deepti Gulati *et al.,* 2017).

Isolation, identification and screening of biosurfactant producing bacteria

The oil degrading bacteria was isolated by enrichment technique. Bushnell Hass agar with 1.0% crude oil were prepared. 1g of soil sediment was weighed aseptically and 1ml of oil contaminated sea water was taken and both were added separately to the test tubes containing 9 ml of sterile saline. Serial dilutions of samples were prepared. 0.1 ml of each dilution was plated on duplicate BH agar plates using spread plate method. The petri plates were then incubated at 37 ºC for 72 hours. The colonies obtained on plates were marked and numbered. They were then streaked onto BH agar plates for purification and identification. Bacterial pure culture was maintained on BH agar slant and broth, stored at 4 ºC and sub cultured every month (Deepti Gulati *et al.,* 2017).

The isolated colonies were identified by following preliminary tests and biochemical tests. Preliminary tests such as Grams staining and motility test. . Biochemical test such as Indole, Methyl red-Voges Proskeur (MR-VP) test, Citrate utilization test, Catalase test, Oxidase test, Urease test, Hydrogen sulphide production test and starch hydrolysis test were carried out for the identification. Organisms were identified according to the Bergey's Manual of systematic bacteriology.

16SrRNA Sequence Analysis

From the isolate genomic DNA was isolated and obtained genomic DNA was estimated using Nano Drop 2000 spectrophotometer. Polymerase chain reaction was performed using the taq DNA polymerase with isolated genomic DNA as the template. Universal 16S rRNA primers were used for amplification. PCR amplicons were electrophorsed on 1.2% agarose gel and visualized with ethidium bromide and sequenced with both forward and reverse primers. Sequence aligned using CLUSTRAL Omega software. Coding sequence was searched in database with BLAST.

Screening Isolates for Biosurfactant Production

Mineral salt medium (MSM)broth was prepared and autoclaved for 121°C at 15 lbs for 15 mins. Inoculum was inoculated into 100 ml MSM broth with 10 ml of crude oil as carbon source and incubated at 37 °C for 5 days. After 5 days of incubation culture was centrifuged at 8000 rpm for 15mins at 4 °C. Supernatant was used for screening of biosurfactant production by different methods (Asia Fadhile Almansoory *et al.,* 2014)

Drop Collapse Test

2 µl of crude oil was added to 96 well micro plate and left for 1 hour to equilibrate at 37 °C. 5 µl of culture supernatant was added to the well. Shape of drop was observed after 1 minute. Collapsed drop indicates positive result for the presence of biosurfactant. Distilled water was used as control (Deepti Gulati *et al.,* 2017)

Oil Spreading Method

50 ml of distilled water was filled in sterile petriplate. On to the water surface, 20 µl of crude oil and 10 µl of culture supernatant were added. Apperance of Clear zone indicates positive result. Distilled water was used as control. (Deepti Gulati *et al.,* 2017).

Emulsification Index (E 24)

E 24 of culture was determined by adding 4ml of crude oil and 4ml of supernatant were added into test tube. Test tube was vortexed for 2 minutes and emulsion activity was observed after 24 hours. Percentage of E 24 were calculated as Height of emulsion layer (in cm) divided by total height (in cm) , multiplied by 100. Results were compared with 1% SDS as control (Deepti Gulati *et al.,* 2017).

Blood Hemolysis Test

Nutrient agar was prepared and sterilized for 121 °C at 15 lbs for 15 minutes. Then allow the media to cool at 50 °C. Nutrient agar was supplemented with 5% blood and poured into sterile petriplate. After solidification, 50 µl of supernatant were added and incubated at 37 °C for 48- 72 hr. Clear zone surrounding culture supernatant indicated the presence of biosurfactant producing organisms (Asia Fadhile Almansoory *et al.,* 2014).

Extraction of Biosurfactant

After screening for biosurfactant producing bacteria, selected isolates were used for the extraction of Biosurfactants. Culture was inoculated into 100 ml of MSM broth with 10% crude oil and incubated for days at 37 °C. After incubation, centrifugation was done at 8000 rpm for 15 min at 4 °C. Supernatant was taken and pH was adjusted to 2, using 1N HCL. Then equal volume of Chloroform and methanol were added. This mixture were shaken well for mixing and left overnight for evaporation. White colored sediment was obtained as Biosurfactant.

Dry weight of biosurfactant were calculated by using formula Dry weight of biosurfactants = weight of the plate after drying with biosurfactant – weight of the empty plate (Asia Fadhile Almansoory *et al.,* 2014).

Characterization of Biosurfactants

Chemical nature of produced biosurfactant by isolates were identified by performing Thin layer chromatography. A drop of biosurfactant extract was spotted in TLC plate. TLC tank were filled with Chloroform: methanol: water (65: 15:2) it were used as solvent. Anthrone reagent were sprayed to detect lipopeptide biosurfactant as red spots. Ninhydrin reagent was sprayed to detect glycolipid as yellow spot. (Ali Ebadi *et al.,* 2017).

Biodegradation of Crude Oil

100 ml sea water was added in conical flask and kept for sterilization for 121 °C at 15 lbs for 15 minutes. After sterilization 1ml of crude oil and 1 ml of culture were added. 0.1g of Hexachloroethane was added as internal standard and incubated for 7 days in shaker condition. 100 ml sea water was taken in conical flask and sterilize for 121 °C at 15 lbs for 15 min after sterilization, 1 ml crude oil and 0.1g of hexachloroethane were added and used as control.

Gravimetric Analysis

Gravimetric analysis is used to study the efficiency of oil degradation. After 7 days, the flask was taken out and the bacterial activities are stopped by adding 1% 1N Hcl. For extraction of crude oil, 50 ml of culture broth was mixed with 20 ml of petroleum ether: acetone(1:1) in a separating funnel and was shaken vigorously to get a single emulsified layer. Acetone was then added to it and shaken gently to break the emulsification, which is resulted in three layers. Top layer was mixed with petroleum ether, oil and acetone; clumping cells makes the middle layer and bottom aqueous layer contains acetone, water and biosurfactant insoluble form. The petroleum ether and acetone were evaporated on a water bath. The gravimetric estimation of residual oil left after biodegradation was made by weighing in a tarred beaker.

The percentage of degradation was calculated as

Weight of residual oil = weight of beaker containing extracted oil – weight of empty beaker. Amount of crude oil degraded = weight of oil added – weight of residual oil.

%Degradation = Amount of oil degraded media x 100.

Gas Chromatography - Mass Spectroscopy Analysis

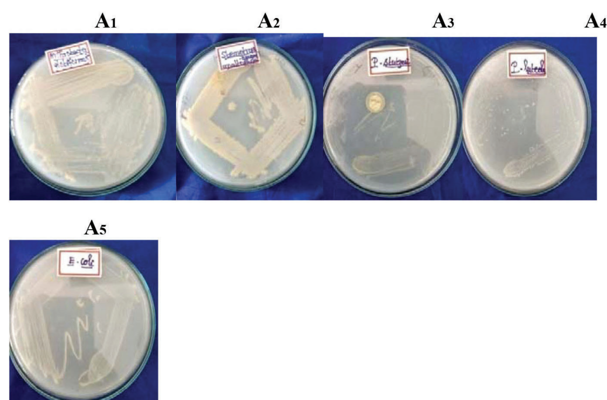
It is an analytical method that combines the feature of Gas chromatography and mass spectrometry to identify the different compounds present in test sample. Degraded compound can be identified by GC-MS analysis of sample. GC-MS equipped with RTX -5 capillary column (30 long, 0.25 mm) internal diameter and highly pure nitrogen constituted the carrier gas. Sample was then analysed and compared with standard graphs.

Results

Table 1. Identification of Biosurfactant producer

Isolate	Name of the organism
A1	*Arthrobacter gloformis*
A2	*Stenotrophomonas maltophilia*
A3	*Pseudomonas stutzeri*
A4	*Pseudomonas luteola*
A5	*Escherichia coli*

PURE CULTURE



16S rRNA SEQUENCE ANALYSIS

Pseudomonas luteola strain NBRC 103146 16S ribosomal RNA, partial sequence

Sequence ID: NR_114215.1 Length: 1462 Number of Matches: 1

Range 1: 55 to 596 GenBank Graphics ▼ Next Match ▲

Score	Expect	Identities	Gaps	Strand
1002 bits(542)	0.0	542/542(100%)	0/542(0%)	Plus/Plus

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Query  1    TGCTCTCTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  55   TGCTCTCTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGG  114

Query  61   GACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGTGGGGGATC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  115  GACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGTGGGGGATC  174

Query  121  TTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAACGGC  180
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Sbjct  175  TTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAACGGC  234

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Query  241  CACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCT  300
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Sbjct  295  CACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCT  354

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Query  361  AGGAAGGGTTGTAACCTAATACGTTGCAGCTTTGACGTTACCAGCAGAATAAGCACCGGC  420
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Query  541  GA  542
            ||
Sbjct  595  GA  596
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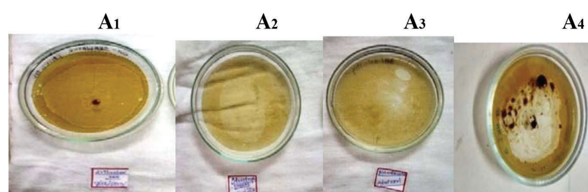
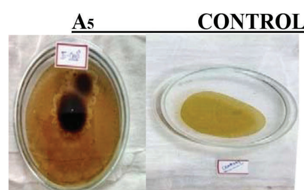
Results

The given bacterial sample was identified as a strain of *pseudomonas luteola.*

Drop Collapse Test

OIL SPREADING METHOD





EMULSIFICATION INDEX (E_{24})

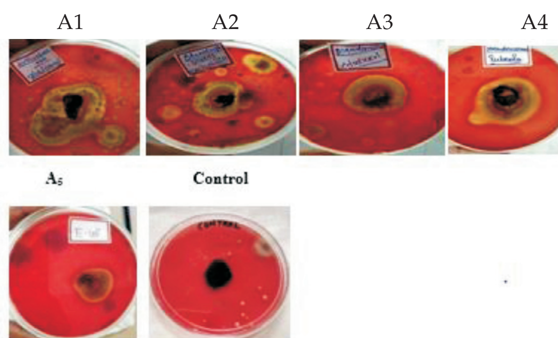
Table 1.2. Screening of Biosurfactant producer

Isolate	Drop collapse test	Oil spreading method (cm) (Extent of displacement)	Emulsification index (E24)	Blood hemolysis	Starch hydrolysis
A1	Positive	7.5 cm (+++)	93.4%	3.3cm	Presence of zone
A2	Positive	5.0 cm (++)	89.6%	3.1cm	Presence of zone
A3	Positive	4.3 cm (+)	53.5%	2.1 cm	Presence of zone
A4	Positive	6.0 cm (++)	66.6%	2.0cm	Presence of zone
A5	Positive	5.0 cm (+)	52.9%	2.8cm	Presence of zone

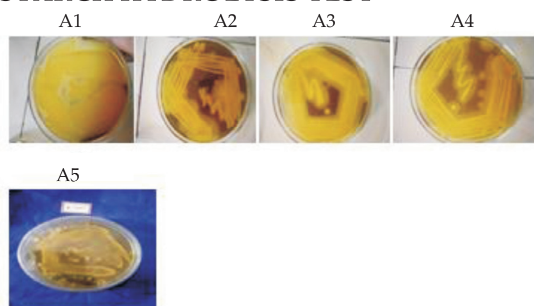
+++ (Bigger displacement), ++ (medium displacement), +(smaller displacement)

BLOOD HEMOLYSIS TEST

A1 A2 A3 A4



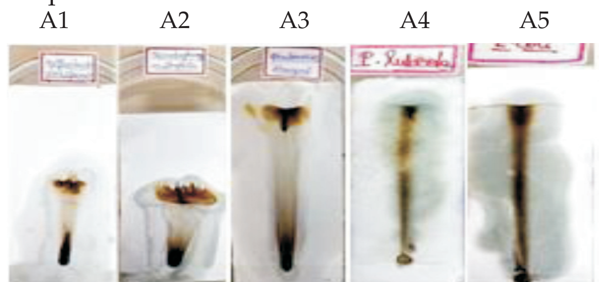
A5 Control



STARCH HYDROLYSIS TEST

A1 A2 A3 A4

A5



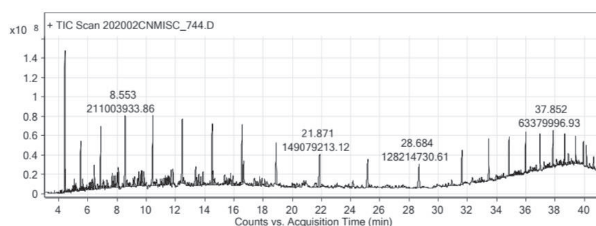
Thin Layer Chromatography

Thin layer chromatography was performed all the five isolates shows yellow colour spot after spraying with ninhydrin reagent. It indicates presence of glycolipid.

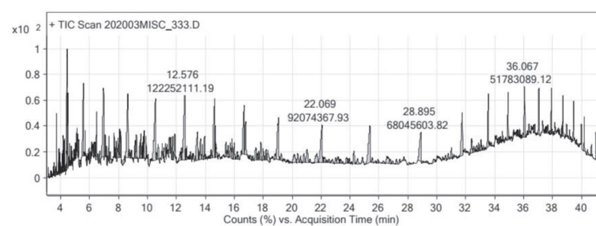
A1 A2 A3 A4 A5

Graphical Representation of GC – MS

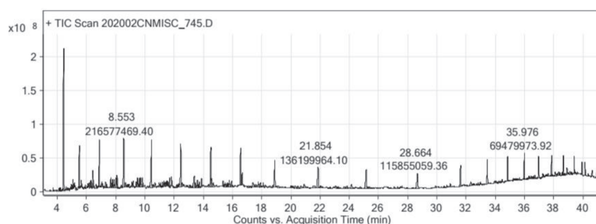
GC-MS Analysis of Crude Oil Degradation Using *Arthrobacter globiformis.*



GC-MS ANALYSIS OF CRUDE OIL DEGRADATION USING BACTERIAL CONSORTIUM



GC-MS RESULT FOR CONTROL



Discussion

This study was designed to isolate biosurfactant producing bacteria from oil contaminated soil sediment and sea water. Soil sediment and sea water were collected from Ennore sea. Sample were serially diluted and spread plated in Bushnell Hass medium with 1% crude oil as carbon source. 5 isolates were obtained and they were identified by MALTI –TOF. 5 isolates are *Pseudomonas stutzeri, Pseudomonas luteola, Arthrobacter globiformis, Stenotrophomonas maltophilia*, *Escherichia coli* were identified as Gram negative bacilli. After isolation the starch hydrolysis test was performed in order to

Table 1.3. Gravimetric Analysis
Degradation efficiency after 7 days was checked

S. No.	Organisms	Dry weight of breaker	Breaker containing extracted oil	Final weight	Weight of residaual oil (1 ml)	Percentage
1	*Arthrobacterglobiformis*	61.43	62.22	0.79	1	79%
2	Consortium	67.4	68.25	0.85	1	85%
3	Control	66.22	66.70	0.45	1	48%

determine the ability of organisms to breakdown complex polysaccharide such as starch. On characterization, 5 isolates produced biosurfactants after screening. Most of the researchers have used 2 to 3 screening methods before selecting biosurfactants producers. According to Yousef *et al.* (2004) single method is not suitable to identify all type of biosurfactants. Various methods are required for effective screening of biosurfactants producers. Drop collapse method were used and all the five isolate isolate showed positive result (Table 1) . Drop collapse method for screening of biosurfactant producers were used by many workers. Husain *et al.* (2008) found that drop collapse test was not able to reliably detect low concentration of biosurfactants. Oil spreading method– all the five isolates showed positive result. Among 5, *Arthrobacter globiformis* showed maximum extent of displacement (Table 1.1).

Morikawa *et al.* reported that the area of oil displaced circle was measured as activity of biosurfactant. Varjani *et al.* reported score ranging "+" to "+++" partial to complete displacement of crude oil. Emulsification index test, 5 isolates showed emulsification index ranging from 50% to 90%. *Arthrobacter globiformis* showing emulsification index more than 90%. (Table 1). In Blood haemolysis test all the five isolates are haemolytic and presence of zone with varying diameter were observed. Among 5, *Arthrobacter globiformis* showed maximum diameter (3.3 cm) (Table 1). Mullaigain et al recommended the blood agar method as a preliminary screening method. After extraction of biosurfactant, dryweight of biosurfactants were calculated. Analysis of component of biosurfactant by thin layer chromatography were done. Thin layer chromatography result suggest that the biosurfactant contain protein, carbohydrate as well as lipid. All the isolate shows yellow colour spots after spraying with Ninhydrin reagent. So the biosurfactant is qualitatively identified as Glycolipid in nature. After screening potent biosurfactants producing bacteria were identified as *Arthrobacter globiformis. Arthrobacter globiformis* is considered as a efficient biosurfactant producer after biodegradation As all the 5 isolates produce biosurfactant after biodegradation as Consortium for 7 days. For both the above organisms percentage of biodegradation of crude oil by gravimetric analysis were determined after 7 days of incubation. It was found that Arthrobacter globiformis degraded 79 % and in consortium 85% was degraded. Similar to my result Muthusamy sathishkumar *et al.* (2008) illustrated that microbial consortium degraded maximum at 1% crude oil concentration than indi-

Table 1.4.1

Compound Name	RT	Molecular Formula	Molecular Weight	Peak Area (%)
Nonane 4, 5 dimethyl	4.099	C11H24	156.31 g/mol	57.1
Octane 3,3 -dimethyl	4.131	C10H22	142.28 g/mol	71.1
Nonane, 5 2 methylpropyl	4.219	C13H28	184.36 g/mol	57.1
1, 3, 8 p - Menthatriene	4.367	C10H14	134.22 g/mol	119.1
Ethane, hexachloro	4.423	C2Cl6	236.74 g/mol	201.1

Table 1.4.2. List of compounds identified in sterile crude oil (using bacterial consortium)

Compound name	RT	Molecular formula	Molecular weight	Peak area (%)
Dodecane, 1-fluoro	3.368	$C_{12}H_{25}F$	188.32 g/mol	57.1
Nonane, 4,5 dimethyl	3.475	$C_{11}H_{24}$	156.31 g/mol	57.1
Tridecane, 6-methyl	3.495	$C_{14}H_{30}$	198.39 g/mol	71.1
Dodecyl propylcarbonate	3.644	$C_{16}H_{32}O_2$	272.423 Da	105
Nonane,2,2,4,4,6,8,8- hetamethyl	3.718	$C_{16}H_{34}$	226.44 g/mol	57.1

Table 1.4.3. List of compounds identified in sterile crude oil (control) by GC-MS

Compound name	RT	Molecular formula	Molecular weight	Peak area (%)
Nonane 4,5 dimethyl	4.101	$C_{11}H_{24}$	156.31 g/mol	57.1
Benzene 1,4 diethyl	4.173	$C_{10}H_{14}$	134.22	57.1
Nonane, 5 propyl	4.98	$C_{12}H_{26}$	170.33 g/ml	57.1
Benzene 1,2,3,5 - tetramethyl	4. 751	$C_{10}H_{14}$	134.22 g/mol	119.1
Phosphorochloric acid, dipentyl ester	4.412	$C_{18}H_{24}O_4^{-2}$	304.4 g/mol	117.1

vidual strain. Raed S. Al – Wasify *et al.* (2014) demonstrated that individual bacterial strain showed lesser degradation than mixed bacterial consortium. The ability of biodegradation is better when microbial consortium is used. Al–Saleh *et al*. (2009) reported that individual strain degraded only limited range of hydrocarbon substrates and crude oil is made up of mixture of compounds, so for biodegradation as consortium degraded wide range of hydrocarbons than individual strain. Final degradation of crude oil after 7 days were checked by GC-MS analysis.

Arthrobacter globiformis degraded 414 compounds, consortium shows 251 compounds has degraded and in control 413 compounds were identified. As a result biosurfactant producing bacteria as a consortium degraded more complex compounds into simple compounds.

Conclusion

In the present study, 5 bacterial cultures were isolated from crude oil contaminated soil. Biosurfactant producing bacteria in environmental cleaning is a major role as revealed from present study. All the 5 isolate produced biosurfactant exhibited emulsification activity.

Among 5 isolates *Arthrobacter gloformis* showed high emulsification activity more than 90%. The potent biosurfactant producer *Arthrobacter gloformis* was selected for crude oil degradation for 7 days. Crude oil degradation as consortium was checked for 7 days. Final degradation of crude oil were checked for both by GC-MS analysis. Thus, consortium degraded crude oil more effectively than individual strain. Bacterial culture as consortium has potential application in environment biotechnologies.

References

Al – saleh, E., Drobiova, H. and Obuekwe, C. 2009. Predominant culturable Crude oil- Degrading bacteria in the Coast of Kuwait. *Inter. Biodeter. Biodegrad.* 63: 400-406.

Ali Ebadi, Mohsen Olamaee, Nayer Azam Khoshkholgh Sima, Reza Ghorbani Nasrabadi and Maryam Hashemi, 2017. Isolation and characterization of biosurfactant producing and crude oil degrading bacteria from oil contaminated soils. *Iranian Journal of Science and Technology Transaction Science*. 42(12): 1149-1156.

Asia Fadhile Almansoory, Mushrifah Idris, Siti Rozaimah Sheikh Abdullah and Nurina Anuar, 2014. Screening for Potential Biosurfactant producing Bacteria from hydrocarbon degrading isolates. *Advances in Environmental Biology.* 8(3): 639-647.

Banat, I.M., Franzetti, A., Gandolfi, I., Betetti, G., Martinotti, M.G., Fracchia, L. and Marchant, R. 2010. Microbial biosurfactant production, applications and future potential. *Applied Microbiology and Biotechnology.* 87(2): 427-444.

Cameotra, S.S. and Makkar, R.S. 1998. Synthesis of biosurfactants in extreme condition. *Applied Microbiology and Biotechnology.* 50 : 520-529.

Deepti Gulati and Srishty Mehta, 2017. Isolation and identification of petrol degrading microorganisms from contaminated soil and comparison of their bioremediation potential. *International Research Journal of Pharmacy.* 8(2) : 34-38.

Fitcher, A. 1992. Biosurfactant moving towards industrial application. *Trends in Biotechnology.* 10 : 208-217.

Husain, D. R., Goutx, M., Acquaviva, M., Gilewicz, M. and Bertrand, J.C. 1997. *World J. of Microbiol. and Biotechnol.* 13: 587-590.

Morikawa, M., Hirata, Y. and Imanaka, T.2000. A study on the structure relationship of lipopeptide biosurfactants. *Biochimicaet Biophysica Acta.* 1488 : 211-218.

Mulligan, C., Cooper, D. and Neufield, R. 1994. Selection of microbes producing biosurfactants in media without hydrocarbon. *Fermentation Technology.* 62 : 311 – 314.

Muthuswamy Sathiskumar, Arthur Raj Binupriya, Sang-Ho Baik and Sei –Eok Yun, 2008. Biodegradation of crude oil by individual bacterial strains and mixed bacterial consortium isolated from hydrocarbon contaminated areas. *Wiley Online Library.* 36(1): 92-94.

Nitschke, M., Siddhartha, G. and Costa, J. Jonas 2011. Rhamnolipids and PHas: recent reports on pseudomonas derived molecules of increasing industrial interest. *Process Biochemistry.* 46 : 621-630.

Raed S. Al-wasify and Shimaa R. Hamed 2014. Bacterial biodegradation of crude oil using local isolates. *International Journal of Bacteriology.* 2014: 8.

Rahman, K., Rahman, T., Korkoutas, Y., Petsas, I., Marchant, R. and Banat, I. 2003. Enhanced bioremediation of alkane in petroleum sludge using bacterial consortium amended with rhamnolipid and macronutrients. *Bioresources Technology.* 90 (2): 159-168.

Saharan, B.S., Sahu, R.K. and Sharma, D. 2011. A review on biosurfactant fermentation, current developments and persperctives. *Genectic Engineering Biotechnology Journal.* 29: 1-39.

Varjani, S.J., Rana, D.P., Bateja, S., Sharma, M.C. and Upasani, V.N. 2014. Screening and identification of biosurfactant (bioemulsifier) producing bacteria from crude oil contaminated sites of Gujarat, India. *Int. J Innov Res. Science Eng Technol.* 3(2) : 9205-9213.

Youssef, O.H., Duncan, K.E., Nagel, D.P., Sava, K.N., Knapp, R.M. and Mclnerney, M.J. 2004. *J. of Microbiol. Method.* 56 : 339-347.