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Re-Accredited 'A' Grade with CGPA 3.61 by NAAC)

Minor Research Project
On
Microbial production of Biosurfactants for enhanced oil recovery

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1. Introduction:

Marine ecosystems are among the largest of earth's aquatic ecosystem. They include oceans; salt marshes, intertidal zones, estuaries, lagoons, mangroves, coral reefs, deep sea & sea floor have high natural variability and are subject to ever-changing environmental phenomena such as storms, climatic anomalies as well as anthropogenic pressures. Major pollutant in marine water is an oil spill caused mainly by petroleum industry where oil is released into the ocean or coastal waters, but spills may also occur on land. Oil spills may be due to releases of crude oil from tankers, offshore platforms, drilling rigs and wells, as well as spills of refined petroleum products (such as gasoline, diesel) and their by-products, heavier fuels used by large ships such as bunker fuel, or the spill of any oily refuse or waste oil.

Within this environment massive animal & plants have varying degree of natural resilience to changes within their habitats. The natural adaptation of organisms to environmental stress, combined with their breeding strategies provides important mechanism for coping with daily & seasonal fluctuation from ambient condition. This is built resilience means that some plants & animals are able to withstand a certain amount of contamination by oil. Nevertheless spills are not the only anthropogenic on marine habitats. Widespread exploitation of natural resources & industrial pollution also contribute significantly to the degree of variability within marine ecosystem. Against a background of high natural variability, more subtle damage inflicted by oil such as or downturn in breeding success, productivity or biodiversity can be difficult to detect.

The fact remains that consistent increase in the level of pollutant has degraded marine ecosystem to levels of irreparable damage.oil spills at sea are extremely dangerous & life threatening to marine ecosystem & human.

Harmful effects of oil pollution

Oil penetrates into the structure of the plumage of birds and the fur of mammals, reducing its insulating ability, and making them more vulnerable to temperature fluctuations and much less buoyant in the water.

Animals that rely on scent to find their babies or mothers cannot due to the strong scent of the oil. This causes a baby to be rejected and abandoned, leaving the babies to starve and eventually die. Oil can impair a bird's ability to fly, preventing it from foraging or escaping from predators. As they preen, birds may ingest the oil coating their feathers, irritating the digestive tract, altering liver function, and causing kidney damage. Together with their diminished foraging capacity, this can rapidly result in dehydration and metabolic imbalance. Some birds exposed to petroleum also experience changes in their hormonal balance, including changes in their luteinizing protein(C. Michael Hogan ,2008). The majority of birds affected by oil spills die from complications without human intervention(Dunnet, G et al ,1982). Some studies have suggested that less than one percent of oil-soaked birds survive, even after cleaning, although the survival rate can also exceed ninety percent, as in the case of the Treasure oil spill(AC Wolfaardt , et al ,2009).

Heavily furred marine mammals exposed to oil spills are affected in similar ways. Oil coats the fur of sea otters and seals, reducing its insulating effect, and leading to fluctuations in body temperature and hypothermia. Oil can also blind an animal, leaving it defenseless. The ingestion of oil causes dehydration and impairs the digestive process. Animals can be poisoned, and may die from oil entering the lungs or liver.

Methods for cleaning up include

- Controlled burning can effectively reduce the amount of oil in water, if done properly.[19] But it can only be done in low wind, and can cause air pollution.[20]It is 95-98% efficient but causes heavy smoke

- Dispersants can be used to dissipate oil spills.[21] A dispersant is either a non-surface active polymer or a surface-active substance added to a suspension, usually a colloid, to improve the separation of particles and to prevent settling or clumping. They may rapidly disperse large amounts of certain oil types from the sea surface by transferring it into the

water column. They will cause the oil slick to break up and form water-soluble micelles that are rapidly diluted. The oil is then effectively spread throughout a larger volume of water than the surface from where the oil was dispersed. They can also delay the formation of persistent oil-in-water emulsions. However, laboratory experiments showed that dispersants increased toxic hydrocarbon levels in fish by a factor of up to 100 and may kill fish eggs.[22] Dispersed oil droplets infiltrate into deeper water and can lethally contaminate coral.

- Dredging: for oils dispersed with detergents and other oils denser than water.
- Skimming: Requires calm waters at all times during the process.
- Solidifying: Solidifiers are composed of dry hydrophobic polymers that both adsorb and absorb. They clean up oil spills by changing the physical state of spilled oil from liquid to a semi-solid or a rubber-like material that floats on water. Solidifiers are insoluble in water, therefore the removal of the solidified oil is easy and the oil will not leach out. Solidifiers have been proven to be relatively non-toxic to aquatic and wild life and have been proven to suppress harmful vapors commonly associated with hydrocarbons such as Benzene, Xylene, Methyl Ethyl, Acetone and Naphtha. The reaction time for solidification of oil is controlled by the surf area or size of the polymer as well as the viscosity of the oil. Some solidifier product manufactures claim the solidified oil can be disposed of in landfills, recycled as an additive in asphalt or rubber products, or burned as a low ash fuel
- Vacuum and centrifuge: oil can be sucked up along with the water, and then a centrifuge can be used to separate the oil from the water - allowing a tanker to be filled with near pure oil. Usually, the water is returned to the sea, making the process more efficient, but allowing small amounts of oil to go back as well. This issue has hampered the use of centrifuges due to a United States regulation limiting the amount of oil in water returned to the sea.[53]
- Use of skimmers to separate oil from water
- Centripetal force –as water is heavier than oil hence spun out so that oil can be pumped out
- Wrangling out the oil that clings to oleophilic surface. This technique is most widely used as it is least destruction ,but it is oily 10-15% efficient under even best circumstances

- Sorbents- Remove oil with absorbent sponges made from diaper like substances. some sorbents are made from natural materials straw, grass, coconut husk/wood chips
- Bioremediation: use of micro organisms[23] or biological agents[24] to break down or remove oil; such as the oil eating bacteria[25] or providing needful facilities .

Biosurfactant

Biosurfactants are biological surface-active agents capable of reducing interfacial tension between liquids, solids and gases, thereby allowing them to mix and disperse readily in water or other liquids. (Bio) surfactants are amphiphilic molecules consisting of a hydrophilic and a hydrophobic moiety that interacts with the phase boundary in heterogeneous systems. The non-polar "tail" is typically a hydrocarbon chain whereas the polar "head" appears in many different varieties such as carbohydrates, amino acids or phosphates. Surface active compounds (SACs) are one of the most commonly used chemicals in everyday life.

In addition to their use as a formulation aid, certain surfactants can also be used as an active compound with antimicrobial, antitumor, antiviral or immunological properties or as inducers of cell differentiation. This has resulted in a number of potential applications and related developments in biomedical sciences. Biosurfactants such as rhamnolipids (source produced)are known to have very high and specific antimicrobial activity against the zoospores of Phytophtora, one of the most important phytopathogenic fungi.

Types of Biosurfactants

The microbial surfactants (MS) or biosurfactans are complex molecules covering a wide range of chemical types including peptides, fatty acids, phospholipids, glycolipids, antibiotics,

lipopeptides, etc. Microorganisms also produce surfactants that are in some cases combination of many chemical types: referred to as the polymeric microbial surfactants (PMS).

Table 1:Examples of Biosurfactants produced by different micro organism

Biosurfactant	**Producing organisam**	**Carbon source**	**localization**
Cellobios lipid	*Ustilago sp.*	Vegetable oil	(Cell free(extracellular) production of biosurfactant F.sineriz,R.K hommel,HP clebber.)
Corynomycolate	*Arthrobacter sp.*	Different sugar	Cell bound (production of biosurfactant F.sineriz,R.K hommel,HP clebber.)
Mannosyl erythroyiol lipid	*Candida sp.*	Glucose,soyaben oil	Cell free (production of biosurfactant

			F.sineriz,R.K hommel,HP clebber.)
Rhamnoel lipid	*Pseudomonas sp.*	Glycerol	Cell free (production of biosurfactant F.sineriz,R.K hommel,HP clebber.)
Sopherose lipid	*Torulopsis sp.*	Glucose,vegetable oil.	Cell free (production of biosurfactant F.sineriz,R.K hommel,HP clebber.)
Trehalose corynomycolate	*Rhodococcus erythropolis*	Carbohydrate	Cell free (production of biosurfactant F.sineriz,R.K hommel,HP clebber.)
Lipopeptide	*Bacillus licheniformis*	Glucose	Cellfree (production of

			biosurfactant F.sineriz,R.K hommel,HP clebber.)
Surfactin	*Bacillus subtilis*	Glucose	Cell free (production of biosurfactant F.sineriz,R.K hommel,HP clebber.)

Properties of Biosurfactants

- **Biosurfactant Toxicity**

Toxicity tests are rather a part of wider research over applicational functions.In spite of these biosurfactants are commonly considered as slow or non toxic. Biosurfactants in comparison with synthetic surfactants pose haemolytic activity to human erythrocytes lower than cationic surfactants(Esterquats,Mono alkyl quaternary systems)and anionic SDS. They do not pose detrimental effects to heart,lung,liver and kidney and interfere in blood coagulation in normal clotting time.

- **Biosurfactnts Biodegradability**

Biosurfactants seems to be more easily biodegradable than their synthetic equivalents.The biodegadibility test of sophorolipids biosurfactants produced by non pathogenic yeast *Candida bombicola*.Moerover biodegradability expressed in a form of BOD/TOD(biological oxygen demand to total oxygen demand,for sophorolipids after 8 days of cultivation has reached the level of 61%.Two other biosurfactants (surfactin and anthrofactin)examined were also has easy biodegradable as sophorolipids ,while synthetic surfactants showed no biodegradability after 8 days.

- **Physico- Chemical Property**

Some investigation showed that surface activity of biosurfactants is comparable with surface activity of synthetic surfactants.For example biosurfactants are able to reduce surface tension of water to 29.0 Mn/m.Moreover water in oil emulsion of palm ,crude, coconut and olive oil with biosurfactants were comparably or even more stable than that with synthetic surfactansts.

Applications of Biosurfactants

Biomedical and Therapeutic Applications of Biosurfactants

Some of the biosurfactants were described for their potential as biological active compounds and applicability in the medical field. Therefore, they are a suitable alternative to synthetic medicines and antimicrobial agents and may be used as safe and effective therapeutic agents. Recently,

there has been an increasing interest in the effect of biosurfactants on human and animal cells and cell lines. Lipopeptides produced by *Bacillus subtilis* and *Bacillus licheniformis*, mannosylerythritol lipids produced by *Candida antartica* and rhamnolipids produced by *Pseudomonas aeruginosa,* have been shown to have antimicrobial activities.

- **Environmental application**

Organic aqueous wastes (e.g., pesticides), organic liquids, oils (e.g., petroleum-based) and organic sludges or solids (e.g., paint-derived) are common environmental organic chemical hazards and are source of soil and aquatic contaminations that are normally difficult to be removed.

The remediation of contaminated sites is usually performed via soil washing or in situ flushing, in case of soil contamination, and bioremediation or use of dispersants, in case of aquatic areas. Soil washing/flushing is heavily dependent on the solubility of the contaminants, which can be very challenging when dealing with poorly soluble hazards. Hydrophobic contaminants usually require use of detergents or dispersants, both in soil or aquatic environment, and the process is often followed by their biodegradation. The surfactant degrade the hydrophobic pollutants.

Eg:Rhamnolipids

Surfactants in removal of hydrophobic pollutants

Highly hydrophobic contaminants can bind very tightly to soil, therefore inaccessible to biodegradation. Surfactants have the potential to promote desorption of the contaminants from soil. Usually, 1-2% (w/w) of surfactant is used for washing contaminant soil, whereas in aqueous solution the concentration of surfactant can be as low as 10 times less than in soil. Rhamnolipids were effective in removing polycyclic aromatic hydrocarbons (PAHs) and pentachlorophenol

from soil with 60-80% removal efficiency, which varied with contact time and biosurfactant concentration.

- **Surfactants in petroleum industry**

Indigenous or injected biosurfactant-producing microorganisms are exploited in oil recovery in oil-producing wells. Microbial enhanced oil recovery (MEOR) is often implemented by direct injection of nutrients with microbes that are able of producing desired products for mobilization of oil, by injection of a consortium or specific microorganisms or by injection of the purified microbial products (e.g., biosurfactants). These processes are followed by reservoir repressurization, interfacial reduction of tension/oil viscosity and selective plugging of the most permeable zones to move the additional oil to the producing wells. Oil recovery was showed to be increased by 30-200 % with injection of biosurfactants, bacteria (e.g., *P. aeruginosa*, *X. campestri*, B. *licheniformis*) and nutrients .However, application of MEOR requires a thoroughly research on a case-by-case basis taking in account the physical-chemical conditions and soil and rock formation characteristics. The characteristics of the oil that has been already recovered from the well will also impact the MEOR application. MEOR is a powerful technique to recover oil, especially from reservoirs with low permeability or crude oil with high viscosity, but the uncertainties on the results and costs are a major barrier to its widespread.

Oilfield emulsions are formed at various stages of petroleum exploration, production and oil recovery and processing, and represent one of the major problems for the petroleum industry, which requires a de-emulsification process in order to recover oil from theses emulsions.

- **Other applications**

Rhamnolipids have potential microbial activity. It has been shown that these biosurfactants are very efficient bacteriostatic agent against *Listeria monocytogenes*, an important food related pathogen, and showed synergistic effect when combined with nisin, a broad-spectrum bacteriocin . Both biosurfactants and surfactin were shown to be able to reduce bacterial adhesion to polystyrene surfaces more efficient than the chemical surfactant sodium dodecyl sulfate. Moreover, purified rhamnolipid inhibited virtually 100% of the growth of strongly adherent L. monocytogenes strain . Moreover, rhamnolipids were shown to significantly reduce the rate of deposition and adhesion, in rinsed chamber with these biosurfactants, of several bacterial and yeast strains isolated from explanted voice prostheses . Rhamnolipids are also known to remove heavy metals. It was shown that these biosurfactant was able to remove nickel and cadmium from soils with efficiencies of 80-100%, under controlled environment, and 20-80% removal was reported in field samples.

2. Aim and objectives

With the above views, the objectives of the present studies were:

- To isolate biosurfactant producing microorganism from natural environment.
- Screening of biosurfactant producing microorganism through enrichment culture.
- To analyze reduced interfacial tension created biosurfactant producing micro organism on oil surface.

3. Review of literature

Human activities pollute the oceans with oil through land run off, vessel accidents, periodic tanker discharges, and bilge discharges. Oil spills are environmental disasters that impact humans, plants,and wildlife including birds,fishes,mammals. According to a review by D Dave&A E Ghaley,2011, the international guidelines for preventing oil spills & impact of disasters caused were analyzed through characteristics of oil spills. A comparative analysis were performed on the currently available remediation technologies using ten evaluation criteria that includes cost, efficiency, time, impact on wildlife, reliability, level of difficulty, oil recovery weather effect of physical or chemical characteristics of oil and needed for further treatment. The primary objectives of response to oil spills are to prevent the spills from moving on to shore, reduce the impact on marine life, speed the degradation of any unrecovered oil. There are several physical, chemical, mechanical remediation technologies for oil spills including blooms, skimmers, sorbents, dispersants, in situ burning and bioremediation. Based on the comparative analysis oil recovery with mechanical method and application of dispersants followed by bioremediation is the most effective response for marine oil spills(D Dave,2011)

Mechanical recovery using weir skimmer is one of the most important techniques in combating oil spills. The enhancement of weir skimmer capacity on oil spill recovery by introducing a tangential water jet along the inside bottom of a weir chamber. The jet creates a strong ,stable vortex inside the weir.(A H Hammoud,2005).The weir skimmer oil recovery rate increased as the water jet flow rate increases, oil viscosity decreases, film thickness increases. The maximum oil recovery height was attained when the weir crest was at the same level as the oil water separation surface.

surfactants are usually organic compounds containing both hydrophilic and hydrophobic group. Surfactants can be derived from both chemically based(chemical or synthetic)or biologically based(biosurfactants) sources. It also includes various classes of surfactants based on their origin and introduce a few of the most widely used biosurfactants. Typical examples of biosurfactants

derived from animal source include lecithin, gelatin, casein, wool fat, and wax. Many surface active compounds are derived from renewable plant resources (Qinggyi Xu,Zengshe Liu,2011).The European surfactant market in 2004 estimated at 25 M metric tons,25% of which are plant derived like saponins,lecithin etc.

surfactants used in synthetic detergents are chemically engineered cleaning products not containing soap but showing very low biodegrable ability of low toxicity considering to be a slow process and therefore causes environmental problems. Day to day increase in use of surfactants in almost all routine and household purposes has led to deposition of surfactants on land oceans, rivers etc. These deposited surfactants were found to be toxic and harmful to various living creature. Thus there arose a need of biosurfactants which can be called environmental friendly surfactants(Saraswathi et al,2014).Biosurfactants are surface active compounds are produced by micro organisms. These molecules reduce surface tension both aqueous and hydrocarbon mixtures.

The isolation and identification of biosurfactant producing bacteria were assessed(A Tabatabee,M Mnzaheri Assadi in 2002).The potential application of these bacteria in petroleum industry was investigated. Samples were collected from oil wells and 45 strains were isolated. To confirm the ability of isolates in biosurfactant production screening method employed include haemolysis test, emulsification test and measurement of surface tension .Further the effect of various physiological factor including PH ,salinity concentration, temperature on biosurfactant production also evaluated .Among the isolated strains NO.4 *Bacilli sp* showed high salt tolerance and their successful production of biosurfactant in a vast PH & temperature domain and reduced surface tension to value between 40Mn/M.

Twenty hydrocarbon degrading bacteria were screened for biosurfactant production. All the bacterial isolates grown in mineral salt medium with addition of 1% tapis crude oil as carbon source. The presence of biosurfactant was determined by drop collapse test, micro plate analysis, oil spreading and emulsification assay. Only one isolates *pseudomonas aeruginosa* UKMP14T was found to be positive .It showed that only *pseudomonas aeruginosa* UKMP14T have an ability biodegrade hydrocarbon concurrently and produce biosurfactant.(Ainon Hamzah&Shahidan Radiman,2013)

Seema Dhail,2012,isolated and identified biosurfactant producing bacteria from oil spilled sea water collected from harbors of Mumbai. The potential application of this bacteria in microbial enhanced oil recovery was investigated. To confirm biosurfactant production various assay test and measurement of surface tension were conducted. Effect of different PH, salinity, temperature on biosurfactant production were also studied. Among the entire isolated strains *Pseudomonas sp* showed high salt tolerance and successful biosurfactant production.

To isolates biosurfactant producing bacteria and optimize the condition like temperature and PH for maximum biosurfactant production. Samples were collected from 8 selected points of oil contaminated region from Iran and Sri lanka. Tests like haemolytic activity ,drop collapse and oil spread method were performed and species with best results were picked for complimentary screening test like emulsification method, foaming and surface tension measurement. Totally 160 sp were isolated. During primary and complimentary screening test 59 sp showed haemolytic activity 46 had drop collapse activity and 18 species had positive in emulsification. Finally 2 bacilli sp were found to be able to release surface tension more than 30 Mn/m.(Ghayyomi Jazeh,2012)

In the study of (Manoj Kumar&Olaf A Lizins,2006)the biodegradation of oil by hydrocarbon degrading ability shown by *Pseudomonas putida* in the presence of biosurfctant producing bacterium was investigated.The co-culture of test organism exhibited improved degradation capacities ,in a reproducible fashion in aqueous and soil matrix in comparison to the individual bacterium culture.Result indicate that in situ biosurfactant production not only resulted in increased emulsification of oil but also change the adhesion of hydrocarbon of cells of other bacterium.

Different screening methods such as emulsification method,oil spreading method,hydrocarb on overlay method, modified drop collapse methods were used to detect biosurfactant production by hydrocarbon degrading *Arthrobacter sp N3* strain.To investigate biosurfactant production,batch cultivation of *Arthrobacter sp N3* was carried out in a fermenter with a complex nutrient medium supplemented by sunflower oil as a carbon source.The highest oil displacement activity was achieved when *Arthrobacter sp N3* was cultivated in 2 stages.Then two forms of biosutfactant was achieved from the culture fluid.It was indicated that oil spreading was the most reliable method to detect biosurfactant production.(Vilma Cipnyte,Saulius Grigiskis,2011).

Effect of biosurfactant produced by bacterium *Pseudomonas sp* strain GU104,a quinoline degrading marine bacterium tested on the Acetylcholine esterase ,lactate dehydrogenase (LDH) phenol oxidase & alpha amylase activity of green muscle perna viridis showed no significant effect on the physiology of perna viridis. Jounita Coelho,C.U Rivonkar ,2003).

4. Materials and methods

4.1 Collection of soil samples and Isolation

Oil contaminated soil samples from oil mill and petrol bunk were collected at 5 different locations in and out Ujire. The sample was taken in sterile polythene bag and was taken to the laboratory and analyzed. Along with isolated samples pure cultures of *Bacillus sp* & Lactic Acid Bacteria were also taken for study. Organism was enriched by inoculating into sterile mineral salt medium (MSM). one gram of each soil sample was inoculated into 50 mL of minimal salt medium (Tahzibi *et al.*, 2004) containing (g/L); 15 g NaNO3, 1.1 g KCl, 1.1 g NaCl, 0.00028 g FeSO4.7H2O, 3.4 g KH2PO4, 4.4 g K2HPO4, 0.5 g MgSO4.7H2O, 0.5 g yeast extract at 37°C in shaker incubator (100 rpm). After 48 h of incubation, the samples were serially diluted using sterile saline (0.85% NaCl) and different bacterial isolates were selected based on the colony morphology on enrichment media. The selected isolates were screened for the production of biosurfactants using different screening methods.

4.2 Enrichment & Screening of biosurfactant producing organisms

The collected sample was serially diluted and were grown aerobically in 500 ml Erlenmeyer flask with different mineral salt medium containing (g l-1)Nazina media, Mc Inerney medium, Coopers medium,Mukherjees medium(Table 1) etc this containing.

Table 2: Mineral salt medium

CHEMICAL	NAZIMA MEDIA	Mc INERGNEY MEDIA	COOPER SMESH MEDIA	MUKERJES MEDIA
NaCl	7g/l	50g/l	-	0.01g/l
K_2HPO_4	0.9g/l	13.9g/l	-	2.2g/l
KH_2PO_4	0.21g/l	2.7g/l	4.083g/l	0.14g/l
NH_4Cl	0.8g/l	-	-	-

$MgCl_2.5H_2O$	0.2g/l	-	-	-
$CaCl_2.2H_2O$	0.5g/l	-	-	0.04g/l
$NaHCO_3$	2.2g/l	-	-	-
$(NH_4)_2SO_4$	-	1g/l	-	-
$MgSO_4$	-	0.25g/l	0.197g/l	0.6g/l
Glucose	-	-	20g/l	20g/l
Sucrose	20g/l	20g/l	-	-
NH_4NO_3	-	-	4.002g/l	3.3g/l
$NaHPO_4$	-	-	7.119g/l	-
$FeSO_4$	-	-	-	0.2g/l
Trace element	1ml/l[a]	10ml/l[b]	1ml/l[c]	0.5ml/l[d]

Table 3: Trace element

CHEMICAL	l[a]	l[b]	l[c]	l[d]
H_3BO_3	2.86g/L	0.01g/l	-	0.56g/l
$MnCl_2$	1.68g/l	-	-	-
$ZnSO_4$	0.022g/l	-	-	-
$CuSO_4.5H_2O$	0.08g/l	-	-	1.0g/l
$COCL_2.6H_2O$	0.06g/l	-	-	-
$NaMO_4.2H_2O$	25g/l	-	-	-
Na_2EDTA	-	1.0g/l	0.00148g/l	1.0g/l
$MnSO_4$	-	3g/l	-	1.78g/l
$FesO_4.7H_2O$	-	0.1g/l	0.0011g/l	-
$Cacl_2$	-	0.1g/l	0.00077g/l	-
$CoCL_2.6H_20$	-	0.1g/l	-	0.42g/l
$ZnSO_4.7H_2O$	-	0.1g/l	-	2.32g/l
$ALK(SO_4)_2$	-	0.01g/l	-	-
Na_2MO_4	-	0.01g/l	-	0.34g/l
$MnSO_{4.4}H_2O$	-	-	0.00067g/l	-
$NiCL_2.6H_2O$	-	-	-	0.002g/l
KI	-	-	-	o.66g/l

4.3 Identification of microorganisms

The isolated microorganisms were identified by Gram staining and Biochemical Tests. The isolated colonies were tested for their biosurfactant production by following methods

4.3.1 Gram staining

To cross check the isolated microorganism for gram positive or gram negative.

- Made thin smear of the simple culture on a clean glass slide and heat fixed the smear.
- Flooded the smear with crystal violet for 30 seconds to 1 minute.
- Washed the slide with distilled water for a few seconds, using wash bottle.
- Flooded the smear with gram's iodine solution for 30 seconds to 1 minute.
- Wash off the iodine solution with isoprapanol. Added the isopropanol drop by drop, until no more colour flows from the smear.
- Wash the slides with distilled water and drained.
- Flooded the smear with safranin for 30 seconds to 1 minute (counter staining)
- Wash with distilled water and blot dry with blotting paper, and kept for air drying observed microscopically.

4.3.2 Biochemcal test

Methyl red :

Materials:

- MR-VP broth
- Methyl red pH indicator
- Nutrient broth.
- Test tube
- Inoculation loop
- Cotton etc

Methodology:

- Prepare MR-VP broth in test tube and sterilize at appropriate temperature.
- Inoculate the test organisms using sterile technique.
- Incubate tubes at 37°C for 24-48 hours .
- At the end of incubation ,add 5 drops of methy red indicator directly to the medium.
- Gently shake the medium.
- Observe for colour change.

Voges-proskauer tests:

Materials:

- MR-VP broth
- Nutrient broth.
- Potassium hydroxide
- Alpha napthol
- Test tube
- Inoculation loop
- Cotton etc

Methodology:

- Prepare MR-VP broth in tests tube and sterilize at 121°C for 10 minutes.
- Inoculate the test organisams using sterile techniques.

- Incubate tubes at 37°C for 24-28 hours.
- At the end of incubation ,add 0.5 ml of alpha napthol ,followed by 0.2ml of KOH.
- Shake the tube gently to expose the medium to atmospheric oxygen and allow the medium to stand for 10-15 minutes.
- Observe for colour change and record the result.

Indole test

Materials:

- SIM agar or peptone water
- Kovacs Indole reagent
- Test tube
- Cotton
- Conical flask
- pH paper/pH meter etc

Methodology:

- Prepare peptone water medium and sterilize at 121 ^{0}C for 15 minutes.
- Inoculate the medium with test organism by using suitable techniques.
- Incubate the medium at 37^{0}C for 24 hours.
- Look for growth after 24 hours and add 1ml of kovacs indole reagent.
- Observe colour change and record the result.

Citrate utilization test:

Materials:

- nutrient broth
- simmon's citrate agar medium
- Sterile inoculation loop
- Micro pippetes
- Spirit lamp

Methodology:

- Inoculated and incubated test bacterial colony in nutrient broth, at 37°C overnight on orbital shaker.
- Inoculated & incubated 50µl overnight grown bacterial culture in simmon's citrate agar medium at 35^0C, for 4 days.

4.4 Screening for biosurfactant production

Blood hemolytic test:

Pure culture of bacterial isolates were streaked on the freshly prepared blood agar and incubated at 37°C for 48-72 h. Results were recorded based on the type of clear zone observed i.e. α-hemolysis when the colony was surrounded by greenish zone, β-hemolysis when the colony was surrounded by a clear white zone and γ-hemolysis when there was no change in the medium surrounding the colony .

Drop collapsing test:

Screening of biosurfactant production was performed using the qualitative drop-collapse test described by Crude oil was used in this test. Two microlitres of oil was smeared to all over the surface of glass slide left to equilibrate for 24 h. Five micro liters of the 48 h culture, before and after centrifugation at 12,000 g for 5 min to remove cells, was transferred to the oil-coated well regions and drop size was observed after 1 min with the aid of a magnifying glass. The result was considered positive for biosurfactant production when the drop was flat and those cultures that gave rounded drops were scored as negative, indicative of the lack of biosurfactant .

Emulsification test (E24):

Several colonies of pure culture were suspended in test tubes containing 2 mL of mineral salt medium after 48 h of incubation, 2 mL hydrocarbon (oil) was added to each tube. Then, the mixture was vortexed at high speed for 1 min and allowed to stand for 24 h. The emulsion index (E24) is the height of the emulsion layer (cm) divided by total height (cm), multiplied by 100

$$\text{Emulsification index (E24)} = \frac{\text{Height of the emulsion layer}}{\text{Total height}} \times 100$$

Oil spreading method

In this method,20 ml of distilled water was added to a plastic Petri dish followed by addition of 20 µl of crude oil to the surface of the water. 10 µl of cell free culture broth was then added to the oil surface. If biosurfactant is present in the cell free culture broth, the oil will be displaced with an oil free clearing zone and diameter of this clearing zone indicates the surfactant activity, also called oil displacement 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.

Hydro carbon overlay method :

Hydrocarbon overlay agar method was performed with some modifications. Mineral agar plates were coated individually with 100 µl of crude oil. Plates were inoculated with isolates and incubated at 30 ºC for 48–72 h. Colony surrounded by an emulsified halo was considered being positive for biosurfactant production**.**

Blue agar plate method:

Mineral salt agar medium supplemented with glucose as carbon source (2%) and cetyltrimethylammonium bromide (CTAB: 0.5 mg/mL) and methylene blue (MB: 0.2 mg/mL) were used for the detection of anionic biosurfactant (Satpute et al., 2008). Thirty microlitre of cell free supernatant was loaded into the each well prepared in methylene blue agar plate using cork borer (4 mm). The plate was then incubated at 37°C for 48-72 h. A dark blue halo zone around the culture was considered positive for anionic biosurfactant production

5. Result

The basis of isolating biosurfactant micro organism is to collect sample from oil spilled area & identify its ability to grow on mineral salt media, where most of the hydrocarbon degrading

micro organisms grow when crude oil is provided as a sole source of carbon. Further screening for biosurfactant ability can be analyzed by hemolytic test, blue agar test, drop collapsing and emulsification index. more than one screening methods e included in the primary screening helps to identify potential biosurfactant producers.Amongst screening technique results on blood agar media hemolytic assays are not reliable and sensitive, because this method will categorize microbes in two groups as hemolytic and non-hemolytic. Strains that are hemolytic are believed to be biosurfactant producers, but there are other products such as virulence factors that can lyse the blood cells and also biosurfactants with poor diffusion in agar may not be able to lyse the blood cells.

5.2 Growth of biosurfactant producing microorganisam:

The isolates were grown on different mineral salt solution like Nazina medium, Mc Inergney medium, Coopers medium, Mukherjee medium with varying trace element. All these isolates have shown high growth in McInergney medium compare to all other medium. Among these isolates soil sample 3 shown high growth in Mc Inergney medium. All these isolates were named as S1,S2,S3,(Siol sample1,Soil sample2,Soil sample3) and also pure cultures of Bacillus and Lactobacillus were added.

Table 4 : Growth of isolates on Mineral salt solution(Trace elements)

Sl.No	Samples	Nazina	Mc Inergney	Coopers	Mukherjee
01	Soil 1	0.06	0.23	0.06	0.05
02	Soil 2	0.05	0.22	0.05	0.03
03	Soil 3	0.06	0.26	0.03	0.01
04	Bacillus sp	0.01	0.29	0.03	0.03
05	LAB	0.01	0.29	0.02	0.01

Table 5: Growth of isolates on Mc Inergney medium with & with out oil

Sl.No	Without oil	With oil
01	0.23	0.8
02	0.22	0.74
03	0.26	0.86
04	0.29	0.79
05	0.29	0.76

Then the Mc Inergney medium was selected to grow the different isolates.The growth was carried out by adding with and with out oil for checking the hydro carbon degrading ability.The O.D result was measured for checking the growth of the micro organisms.The Bacillus and Lactobacillus have shown high growth in Mc Inergney medium without oil. They have shown the growth up to 0.29. The mico organisam S3(soil sample 3)has shown high growth in in Mc Inergney medium with oil. The growth has recorded up to 0.86.The lest growth has shown by soil sample 1(S1) ie,o.8.

5.3.2 Identification of micro organism

Table 6 Biochemical Characterization of micro organism

Sl.No	Samples	Gram	Biochemical test

		staining	MR	VP	I	CUT
01	**Soil 1**	**G+C**	**+++**	**+++**	**++**	**++**
02	**Soil 2**	**G+R**	**+++**	**+++**	**++**	**++**
03	**Soil 3**	**G+R**	**+++**	**++**	**+++**	**++**
04	**Bacillus sp**	**G+R**	**+++**	**+++**	**+++**	**++**

+++ =Good result

++ =Moderate

- =Nil

Identification of microorganism was done through different methods like gram staining and different biochemical tests.Through gram staining all the samples like sample 2,3, *Bacilli* and *lactobacilli* were found to be gram positive rod except sample 1,which was gram positive cocci.

In biochemical tests almost all the samples showed high and good results.

5.4 Screening of microorganisms

Blood haemolysis test

Fig: 1Isolates showing growth on blood Agar media

Diffusion of culture supernatant on blood agar

Oil spreading method

Figure 2: Oil displacement activity shown by isolates

Table 7 Oil displacement activity of culture & supernatant

	Bacterial Culture					Culture Supernatant				
	S1	**S2**	**S3**	**Bac**	**LAB**	**S1**	**S2**	**S3**	**Bac**	**LAB**
Initial Measurement of oil surface in (mm)	**7**	**7**	**7**	**7**	**7**	**7**	**6.5**	**7**	**7**	**6**
Measurement of oil surface after addition of samples(mm)	**4**	**5**	**5**	**5**	**5**	**3**	**4**	**5**	**4**	**6**

In oil spreading method,for both bacterial culture and culture supernatant the initial measurement of oil surface was high compared to measurement of oil surface after addition of samples.

Drop collapse method

Figure 3 : Stability of drop(culture & supernatant) on oil coated surface

Table 8 Drop collapse measurement on oil coated surface

	Bacterial Culture					Culture Supernatant				
	S1	**S2**	**S3**	**Bac**	**LAB**	**S1**	**S2**	**S3**	**Bac**	**LAB**
Measurement of water drop size on oil coated surface in (mm)	**0.4**	**0.4**	**0.5**	**0.4**	**0.4**	**0.8**	**0.8**	**1**	**0.9**	**1**
Measurement of sample drop size on oil coated surface (mm)	**0.5**	**0.4**	**0.5**	**0.4**	**0.4**	**1**	**1**	**1.1**	**1**	**1**

In drop collapse method measurement of water drop size was taken for both bacterial culture and culture supernatant and sample drop size was also taken in mm.

Hydro carbon overlay method

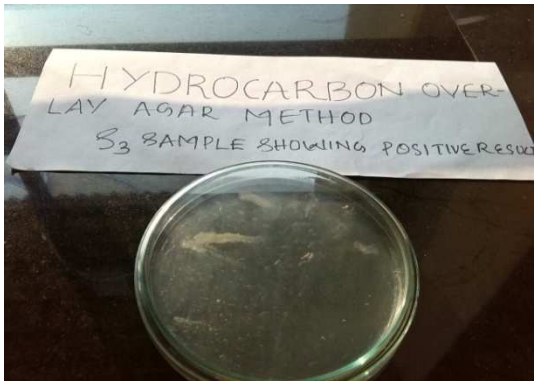

Figure 4 : Bacterial growth on oil coated media

In hydro carbon overlay method S3 sample showed high results.

Emulsification method

Table 9 Emulsification index

	Emulsification Index(%)				
	S1	S2	S3	Bac	LAB
Bacterial Culture	47.6	48.5	48.0	45	45
Culture Supernatant	52.38	43.56	56	53.33	53

In emulsification method,bacterial culture and culture supernatant were taken for all the samples like sample 1,2,3,bacilli and lactobacilli.

Emulsification index was calculated by the formula Height of emulsified layer/total height*100

Blue agar Method

Figure 5: A clear zone of halo around culture as well as supernatant on Blue agar medium

From Left: Culture, supernatant, control

Sample 1,Gram positive cocci showing growth on Blue agar media

- **Summary**

Oil spills are the major threat to marine system, they occur mainly due to anthropological activities during accidental discharge from oil tankers, offshore platforms, drilling rigs and wells, as well as spills of refined petroleum products (such as gasoline, diesel) and their by-products, heavier fuels used by large ships such as bunker fuel, or the spill of any oily refuse or waste oil. But marine forms show natural resilience by withstanding a certain amount of contamination by oil. The fact remains that consistent increase in the level of pollutant has degraded marine ecosystem to levels of irreparable damage.oil spills at sea are extremely dangerous & life threatening to marine ecosystem & human. There are several physical, chemical, mechanical remediation technologies for oil spills including blooms, skimmers, sorbents, dispersants, in situ burning and bioremediation. These methods vary from one another in terms of cost, efficiency, time, impact on wildlife, reliability, level of difficulty, oil recovery weather effect of physical or chemical characteristics of oil and needed for further treatment. The primary objectives of response to oil spills are to prevent the spills from moving on to shore, reduce the impact on marine life, speed the degradation of any unrecovered oil. Dispersant or Surfactant are compounds that lower the surface tension or interfacial tension between two liquid or between a liquid and a solid. Surfactant may act as detergent, wetting agent ,forming agents, emulsifier etc. The surfactants are usually organic compounds. They are amphiphilic, meaning they containing both hydrophobic group and hydrophilic group. Therefore, a surfactant contain both water soluble and water in soluble (oil soluble)components. Surfactant will diffuse in water and absorb at inter face between air and water or at the interface between oil and water, in the case where water is mixed with oil. The water-insoluble hydrophobic group may extend out of the bulk water phase, into the air or into the oil phase, while the water-soluble head group remains in the water phase.

Chemical surfactants have disadvantage due to their toxicity and difficulty in being degraded in the environment . So biological surface-active agents capable of reducing interfacial tension between liquids, solids and gases, thereby allowing them to mix and disperse readily in water or other liquids. (Bio)surfactants are amphiphilic molecules consisting of a hydrophilic and a hydrophobic moiety that interacts with the phase boundary in heterogeneous systems. The microbial surfactants (MS)or biosurfactans are complex molecules covering a wide range of chemical types including peptides, fatty acids, phospholipids, glycolipids, antibiotics, lipopeptides, etc. Microorganisms also produce surfactants that are in some cases combination of many chemical types: referred to as the polymeric microbial surfactants (PMS).They have the advantage of easily biodegradable with reduced toxicity. Presently wide range of micro organism including fungi are know to produce biosurfactant among them some are pathogenic in nature .Hence forth in the present study an effort is made to screen biosurfactant production from non pathogenic microorganism. In the present study three different soil samples were collected from oil spilled areas s1:soil from petrol bunk,s2 :soil from oil mill,s3 petrol bunk of in & Ujire place and along with pure cultures of *Bacilli and lactobacilli* were also taken. The collected sample was serially diluted and were grown aerobically in 500 ml Erlenmeyer flask with different mineral salt medium containing (g l-1)Nazina media, Mc Inerney medium, Coopers medium, Mukherjees medium etc with varying elements like Na2EDTA,Mnso4,Feso4.7H2o,Cacl2,CoCl2.6H2o,ZnSo4.7H2o,CuSo4.5H2o,Alk(So4),H3Bo3,Na2Mo4,KI. All these isolates have shown high growth in McInergney medium compare to all other medium. Among these isolates soil sample 3 shown high growth in Mc Inergney medium. . Initially grown without oil & then same media with 1% crude oil. In

case of with out oil bacilli and lacto bacilli showing high results and in case of with oil sample 1 showing high result that is 0.8. The isolated microorganisms were identified by Gram staining and Biochemical tests. Through gram staining all the samples like sample 2,3, *Bacilli* and *Lactobacilli* were found to be gram positive rod except sample 1,which is gram positive cocci. In biochemical tests almost all the samples showed high and good results. The isolated colonies were tested for their biosurfactant production by following methods; like blood haemolysis, oil spreading, drop collapse method, hydro carbon overlay method, emulsification method etc.

In oil spreading assay, the measurement clear zone on the oil surface was measured. And for both bacterial culture and culture supernatant the initial measurement of oil surface was measured and it was found to be high compared to measurement of oil surface after addition of samples.

In drop collapse assay, increase in the surface area of the broth containing biosurfactant over the oil coated surface was measured. And measurement of water drop size was taken for both bacterial culture and culture supernatant and sample drop size was also taken in mm. It was found to be in case of water drop size and sample drop size S3 sample showing high results. .

In hydro carbon overlay assay, bacterial growth on the oil coated surface of mineral salt solution agar indicating a zone of halo around the streaked surface was recorded as positive. In this method also S3 sample was showing high results.

In emulsification method,bacterial culture and culture supernatant were taken for all the samples

like sample 1,2,3,bacilli and lactobacilli. Emulsification index was calculated by the formula

Height of emulsified layer/total height*100.

In blood haemolysis test,the bacterial colonies were observed for the presence of clear zone around the colonies. This clear zone indicates the presence of biosurfactant producing organisms. Among these methods like hemolytic assays are not reliable and sensitive, because this method will categorize microbes in two groups as hemolytic and non-hemolytic. Strains that are hemolytic are believed to be biosurfactant producers, but there are other products such as virulence factors that can lyse the blood cells and also biosurfactants with poor diffusion in agar may not be able to lyse the blood cells. Thus, the results from hemolytic assay on blood agar plate are not so reliable and sensitive. Remaining methods adapted for screening of biosurfactant

producing micro-organism were considerably good with reproducible results.

Out of all isolates inoculated onto Blue agar medium , only sample 1 ,Gram positive cocci both culture & supernantant showed anionic biosurfactant production by producing a zone of halo around the colonies.

From the study of soil samples taken from petrol bunks of in & around ujire , we successfully isolated three bacterial strains showing good biosurfactant from that soil sample 3 showed high biosurfactant production both from the culture as well as from the supernatant,followed by Bacillus species.It was also found that biosurfactant produced by sample 1 ,that is gram positive cocci is an anionic biosurfactant. So the present study was successful in isolating gram positive bacteria showing enhanced biosurfactant production, which could be used for oil recovery from spills.

- **Discussion**

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6. Conclusion

Biosurfactants are the surface active molecule which has a capability to reduce surface or interfacial tension between liquid, solid materials. It have a lot application in the present scenario, they reduce surface and interfacial tension by accumulating at the interface of immiscible fluids and thus increase the solubility. The use of biosurfactant is a promising alternative over the chemical surfactant as they are better biodegradable and do not pollute the environment . In the present study, analyzed the biosurfactant producing ability of different microorganism which were collected from different places. Among that Soil Sample 3 has shown high biosurfactant ability & when tested for type by growing on blue agar medium .Only sample 3 showed growth indicating anionic biosurfactant production. The screening analysis carried out yielded positive result by isolating non pathogenic (Gram positive micro organism) showing good ability to bring about emulsification of immiscible liquids, also such micro organisms can be used for enhanced oil recovery in oil spilled areas.

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10. Publication details

The following work was presented as a poster by **Ms Prarthana J*** entitled "Microbial production of Biosurfactant" in a National conference on "Advances in Environmental Biotechnology for sustainability"Organized by Department of Biotechnology Alvas College Modbidri,In assaociation with Karnataka State Pollution control Board Mangalore.Feb 6-7, 2015**(& received Best poster awarded)**

Findings of the work is published in *International Journal of Emerging Engineering Applications and Bioscience*

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