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SCREENING OF MICROBES WITH ABILITY TO PRODUCE SURFACE-ACTIVE BIOMOLECULES FROM PETROLEUM CONTAMINATED SITES.

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ABSTRACT
Different types of surface activebiomolecules likeglycolipids, lipopeptides, phospholipids, neutral lipids, fatty acids or lipopolysaccharidesaresynthesizedby certain microorganisms e.g. bacteria, yeasts and moulds. In the present study isolation and screening of bacteria from petroleumcontaminated soil (PCS)from Wardha was carried out.The ability of producing these biomolecules was tested by drop collapse test,oil spreading test, measurement of surface tension, hemolytic assay, emulsification index, emulfication activity and methylene blue agar plate method. Five soil samples from oil contaminated soil of various automobile workshops, petrol pumps and saw mills were collected. Total 25 bacterial strains were isolated, out of which 8 potential bacterial strains with ability to produce surface activebiomoleculeswere selected for further study. On the basis of cultural, morphological and biochemical characteristics these 8 bacterial strains were identified as belongs to *Pseudomonas, Citrobacter* and *Klebsiella species(spp).*Amongall,*Pseudomona spp.* was found to be the potent producer of surface activebiomolecules.It is evident from the results that surface activebiomoleculescan be used in place of synthetic compound due to their efficiency and ecofriendly nature.

KEYWORDS:surface active biomolecules, surface tension, emulsification index.

INTRODUCTION
Chronic release of oil in the soil and water from numerous natural and anthropogenic sources poses a continuous serious threat for the environment. Oil spills have become a global problem particularly in industrialized and developing countries. Use of chemical surfactant for this purpose is one of the common methods[i]. Chemically-synthesized surfactants have been used in the oil industry to aid clean up of oil spills, as well as to enhance oil recovery from oil reservoirs. But, chemical surfactants are hazardous, toxic, non biodegradable and hence having many disadvantages in environmental as well as application point of view[ii].Bioremediation has proven to be an alternative to diminish the effects caused by hydrocarbon contamination of soil and water, using the metabolic capacities of microorganisms that can use hydrocarbons

as source of carbon and energy, or that can modify them by cometabolism. The efficiency of removal is directly related to the compounds' chemical structure, to its bioavailabity (concentration, toxicity, mobility and access) and to the physicochemical conditions present in the environment[iii].Naturally occurring surfaceactive compounds derived from microorganisms are called biosurfactants. They can be produced as part of the cell membrane by a variety of yeast, bacteria and filamentous fungi[iv].The ability to reduce surface tension is a major characteristic of surfactants.

Surface active biomolecules are group of secondary metabolites; amphiphilic compounds produced on living surface mostly by microbial cells and contain hydrophobic and hydrophilic moieties that decrease surface tension between individual molecules at the surface and interface respectively[v].Surfactants are key ingredients used in detergents, shampoos, toothpaste and a number of other industrial products. The surface active biomolecules are complexmolecules covering a wide range of chemical types including peptides, fatty acids, phospholipids, glycolipids, antibiotics, lipopeptides etc.[vi].Surface active biomolecules have several advantages over the chemical surfactants, such as lower toxicity, higher biodegradability better environmental compatibility,higher foaming), higher selectivity and specific activity at extreme temperatures, pH and salinity and the ability to be synthesized from renewable.

The surface active biomolecules could be used as an emulsifier to form emulsion between water and hydrocarbon such as palm oil, benzene, toluene with various stability.For this purpose, an effort has been made to replace chemical surfactant with the eco-friendly surface active biomolecules,which is produced from an isolated bacterial strains from petroleum contaminated soil of Wardha region.

MATERIALS AND METHODS

Sampling Sites and Sample Collection: The soil sample was collected from petroleum contaminated soil,e.g automobile workshops, Petrol Pumps and Saw mills. Under asceptic condition the petroleum contaminated site samples were transported to the laboratory and were stored at 4^0C till further use.

Enrichment culture technique for isolation of Bacteria :1 gram soil was inoculated in 250 ml Erlenmeyer flask containing 100 ml Minimal Salt Medium having composition (g/l):$NaNO_3$ 2.5g; KCl 0.1g; $KH2PO_4$ 3.0g; $K2HPO_4$ 7.0g; $CaCl_2$ 0.01g; $MgSO_4.7H_2O$ 0.5g and 5ml of trace element solution contains: $FeSO_4.7H_2O$ 0.116g/l; $H3BO_3$ 0.232g/l; $CoCl_2.6H_2O$ 0.41g/l; $CuSO_4.5H_2O$ 0.008g/l; $MnSO_4.H_2O$ 0.008g/l; $[NH_4]6Mo7O_{24}$ 0.022g/l; $ZnSO_4$ 0.174g/l with 2% kerosene oil as a sole source of carbon and were incubated at 30^0C at 200rpm on rotary shaker for 7days and same procedure was successively repeated for 3 time for enrichment to isolate desirebacteria[vii].

Isolation and Identification of bacteria: After enrichment the soil samples were inoculated on solid nutrient agar plate. After incubation at 37^0C and 24 hrs, well grown, isolated and morphologically distinct microbial colonies were selected and transferred on agar slants. The stock culture was preserved at 4^0C for further study. All these isolates were further identified on the basis of cultural, morphological and biochemical characterization as per Bergey's manual of systematic bacteriology (9^{th} edition).

Preliminary screening for surface active biomolecules production:

Surface tension measurement: Reduction in surface tension of cell-free culture wasmeasured according to the drop counting method using stalagmometer. For this purpose, five mL of inoculums of the bacterial culture were added to 250 mL Erlenmeyer flask containing 100 mL Nutrient broth with 2% kerosene oil as a carbon source. The experimental flasks were incubated at 30^0C on rotary shaker at 200 rpm. After 5 days of incubation, broth was centrifuged at 8000 rpm for 20 min for cell removal and cell free supernatant was collected in sterile flask. The reduction in surface tension of cell free broth was determined by using stalagmometer by drop counting method. The stalagmometer was calibrated before each measurement using distilled water[viii].

Oil displacement assay: The assay was carried out by addition of 20 µl of kerosene oil to the surface of 50 ml of distilled water in a beaker to form a thin oil layer. Then10 µl of culture supernatant was gently placed on the centre of the oil layer. If surface active biomolecule is present inthe supernatant, the oil is displaced and a clearing zone is formed. The diameter of this clearing zone on the oil surface correlates to surfactant activity, also called oil displacement activity[ix].

Blue agar plate method: Minimal 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 surface active biomolecules. 30 µl of cell free supernatant was loaded into the each well prepared in methylene blue agar plate using cork borer (4 mm) and these plates were incubated at 30^0C for 3 days.If anionic surfactants are secreted by the microbes growing on the plate, they form a dark blue, insoluble ion pair with cetyl-trimethylammonium bromide and methylene blue. Thus, productive colonies are surrounded by dark blue halos. A dark blue halo zone around the culture was considered positive for anionic surface activebiomolecules production[viii].

Blood agar hemolysis: Blood agar hemolysis method is used to screen surface active biomoleculeproducing strain. This method is based on the fact that biosufactants are able to haemolyse the red blood cell present in blood. Pure culture of bacterial isolates were streaked on the freshly prepared blood agar and incubated at 30°C for 48-72 hour. 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. This zone of hemolysis indicates production of surface active biomolecules [x].

Foaming activity: Isolated strains were grown separately in 250 mL Erlenmeyer flasks, each containing 100 mL of nutrient broth medium. The flasks were incubated at 30°C on a shaker incubator (200 rpm) for 72 hour. Foam activity was detected as duration of foam stability, foam height and foam shape in the graduated cylinder.

Emulsificationassay: Emulsification activity of culture supernatant was calculated by emulsification index known as E24. Emulsification assay was carried by adding 2ml kerosene oil in 1ml cell free supernatant which was obtained after the centrifugation and then it was vortexed for 5 minutesconfirming regular mixing of both the liquids. The emulsification activity was observed after 24 hours.

It was calculated by using the formula:

E24 = Total height of the emulsion layer/height of the aqueous layer * 100

Confirmatorymethod

Phenol: sulfuric acid method: Surface active biomolecules producing strains selected from above screening methods were inoculated in Minimal salt medium broth and incubated at 30^0C on rotary shaker for 4-5 days. After incubation, broth was centrifuged at 10,000 rpm for 15 min and supernatant was collected while pellet was discarded. 1ml collected supernatant was

mixed with 1ml of 5% phenol then 5ml of concentrated H_2SO_4 was added in drop wise manner. Presence of surface active biomolecules in supernatant produces orange color from yellow color.

Production of surface active biomolecules: Surface active biomolecules was produced by using minimal salt medium.As formulated by Makkar and Cameotra (Makkar, 1998), surface active biomolecules production was carried out in 250mL conical flasks containing 50mL of a minimal salt medium containing 2% kerosene oil as the sole source of carbon. The temperature of the medium was maintained at 30°C with shaking at 150rpm. Culture medium samples were drawn for estimation of surface activebiomolecules production once every 24 hours for five days. Surface active biomolecules concentration in the culture broth was estimated after its crude extraction and concentration. A conical flask without the kerosene oil was maintained as control. All the experiments were performed in triplicate.

Extraction of surface active biomolecules :The culture broth was centrifuged at 5000rpm, 4^0 C for 20 minutesto obtain a cell-free supernatant. The pH of the supernatant was adjusted to 2 using 6N HCl and was subjected to acid precipitation by placing it at 4°C overnight.The off-white precipitate was separated by centrifugation at 10000 rpm for 30 min at 4°C. The precipitate was extracted thrice with a 2: 1 chloroform-ethanol mixture. The organic phase was removed and the surface activebiomolecule was concentrated using a rotary evaporator at 40°C. The solvents were evaporated leaving behind relatively pure surface activebiomolecules as a viscous light brown matter.

Measurement ofsurface activebiomolecules concentration: The surface active biomolecules concentration was measured indirectly by measuring the absorbance of surface active biomolecules methylene blue complex at wavelength of 500nm. Methylene blue with a concentration of 0.015mg/ml was prepared and its optical density was measured using spectrophotometer. The solution was used as standard. 2 ml of the broth culture was pipetted into a test tube containing 2 ml of the standard and 1 ml of chloroform was added to the mixture. The mixture was vortexed for two minutes and allowed to stand. The surface active biomolecules -methylene blue complex was siphoned using pasteur pipette into a cuvette and the absorbance was measured using spectrophotometer [ix].

Fourier Transform Infrared Spectroscopy: To understand the overall chemical nature of the extracted surface active biomolecules,Fourier transform infrared spectroscopy (FTIR) was employed. The technique helps to explore the functional groups and the chemical bonds present in the crude extract. The analysis was done using [Bruker (Alpha)] Samples were prepared by homogeneous dispersal of 1mg of the surface activebiomolecules sample in pellets of potassium bromide. IR absorption spectra were obtained using a built-in plotter. IR spectra were collected over the range of 450–4500cm^{-1} with a resolution of 4cm^{-1}. The spectral data were the average of 50 scans over the entire range covered by the instrument. The spectrum was studied to interpret the chemical nature of the surface activebiomolecules fraction.

RESULTS AND DISCUSSION

Total 25 bacterial strains were isolatedfromfive samples collected from soil of various automobile workshops, petrol pumps and saw mills. Out of 25 isolates,8 potential bacterial strains (UN1, UN2,UN3,UN4, UN5, UN6, UN7, UN8) with ability to produce surface active biomolecules were selectedfor further study.The study comprises of biomass yield, synthesis of surface activebiomolecules and reduction in surface tension of the culture media. The isolates were screened by oil displacement method, blue agar plate method, blood agar heamolysis, foaming activity etc. On the basis of cultural, morphological and biochemical characterization, isolates UN1, UN2,UN3,UN4, UN5, UN6, UN7, UN8 were tentatively identified as *Pseudomonas sp., Citrobacter sp., Klebsiella sp.* The isolates were further

characterized for their ability to produce surface active biomolecules by using 2% kerosene oil as a sole source of carbon.All the eight isolates showed oildisplacement and drop collapse test positive. *Pseudomonas, Citrobacter* and *Klebsiella sp.* showed oil displacement zone of 1.0cm, 0.5cm, 1.4cm, 2.0cm, 0.7cm, 1.5cm, 1.0cm and 1.5cmrespectively. Further emulsification index test showed that *Pseudomonas, Citrobacter* and *Klebsiella spp.*hasemulsification index of 64.70%, 64.51%, 63.33%, 63.33%, 60.00%, 63.63%, 63.63% and 62.50%respectively. Kerosene oil as a carbon source favored extracellular production of surface active product by *Pseudomonas, Citrobacter, Klebsiella sp.* which was indicated by the reduction in surface tension of the broth. *Pseudomonas* sp. was found to reduce the surface tension of broth from 65N/m to 39N/m. while *Citrobacter and Klebsiella sp.* were found to reduce the surface tension upto 41.5 and 42 N/m respectively. Whereas the surface tension of control sample was found to be 48.89 N/m.FTIR spectra of surface activebiomolecule was performed to identify the types of functional groups present in the compound. Characteristic absorption bands corresponding to functional groups typically forming parts of rhamnolipids could be observed (Fig 5). The characteristic peak at 3429 cm^{-1} represents the O-H stretching (free hydroxyl of phenolic groups).Presence of N-H stretch was observed at 3450 $cm^{-1.}$ Presence of CH2 and CH3 at 1476.56 cm^{-1} was observed. Absorption around 1775.78 cm^{-1}represents ester (C=O) and carbonyl (COO^{-}) groups.

Table-1: Surface tension, Biomass and Biosurfactant yield for the isolates.

Isolates	Oil displacement zone	Emulsification index (E24%)	Biomass at 600nm (gL-1)	Biosurfactant yield at 500nm (gL-1)
UN1	1.0 cm	64.70	0.601	2.256
UN2	0.5 cm	64.51	0.554	-1.091
UN3	1.4 cm	63.33	0.438	0.970
UN4	2.0 cm	63.33	0.397	0.397
UN5	0.7 cm	60.00	0.540	3.603
UN6	1.5 cm	63.63	0.369	0.364
UN7	1.0 cm	63.63	0.615	2.721
UN8	1.5 cm	62.5	0.000	-0.620

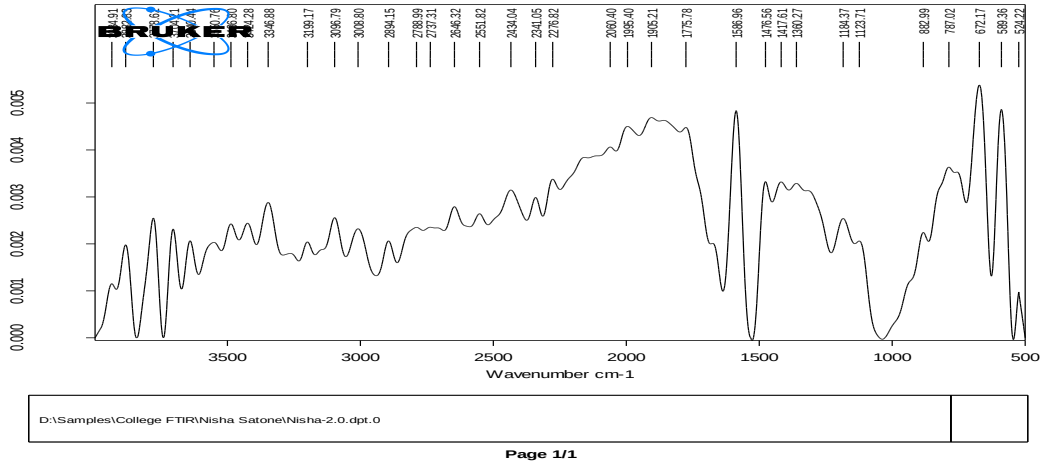

Fig 1: FTIR analysis of surface active biomolecule

Fig2:CTAB methylene blue agar test

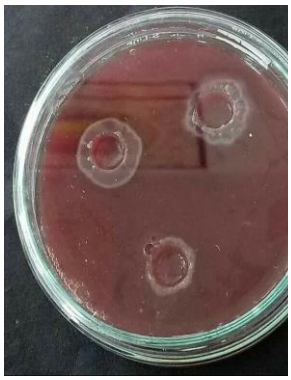

Fig3:Blood haemolysis test

Fig4:Foaming activity

Fig5: Oil spreading test

Conclusion

The present study is an attempt to isolate,characterizeandscreenoutsurface active biomoleculeproducing bacteria from petroleum contaminated sites.*Pseudomonas*strain screened from the environment gave maximum surface active biomolecule production (0.20 g/L-^{1}) with kerosene oil as sole source of carbon and could reduce the surface tension of brothfrom 65mN/m to 39 mN/m. It is evident from the results that surface active biomolecule can be used in place of synthetic surfactants due to their efficiency and ecofriendly nature.Petroleumcontaminated sites can be considered as enrichment environments for selection of microbes with ability to produce surface-active biomolecules.

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