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Isolation of Biosurfactant Producing Organisms from the Petroleum Contaminated Soil in Gujarat

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ABSTRACT

During the study of isolation of bio surfactant producing microorganism from the petroleum contaminated soil, 58 polycyclic aromatic hydrocarbons (PAH) degrading bacteria were isolated.14 isolates were screened for the biosurfactant production To check the potential of the utilization of different Hydrocarbons, the selected 5 isolates were identified and further assessed. The isolates were screened for ability to grow on petroleum fractions like petrol, diesel, kerosene, lubricant oil, paraffin oil, crude oil as sole source of carbon,on the basis of qualitative Study, 2 isolates (Ps5, Pc, two of them which were found high active to utilize crude oil and biosurfactant production. Two isolates were characterized using morphological and biochemical tests to pseudomonas spp. Two isolates showed the growths on mineral salts medium as the carbon source and energy as demonstrated by the increase in cells forming unit (CFUml) during the incubation period.The two isolates were tested to producing biosurfactant by four assays (E24 assay, Drop collapse test,Cell surface hydrophobicity, Bacterial Adhesion to Hydrocarbons (BATH test and CTAB Agar Test) these assays suggested that two isolates producing biosartactant. This study conclude the ability of pseudomonas spp. isolated from oil contaminated soil to grow on the enriched media with the hydrocarbon as a sole source of energy with high potential capacity of oil degradation and Biosurfactant production, it can be used for removal of oil from sand could enhance in situ bioremediation of soils contaminated with polycyclic aromatic hydrocarbons (PAHs)

Key word: *pseudomonas spp, Petrolium, Biodegradation, Biosurfactant, isolation of PHB degrading bacteria.*

INTRODUCTION

Crude oil, commonly known as petroleum, is a liquid found within the Earth comprised of hydrocarbons, organic compounds and small amounts of metal. While hydrocarbons are usually the primary component of crude oil, their composition can vary from 50%-97% depending on the type of crude oil and how it is extracted. Organic compounds like nitrogen, oxygen, and sulfur typically make-up between 6%-10% of crude oil while metals such as copper, nickel, vanadium and iron account for less than 1% of the total composition.

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Crude oil is created through the heating and compression of organic materials over a long period of time. Most of the oil we extract today comes from the remains of prehistoric algae and zooplankton whose remains settled on the bottom of an Ocean or Lake. Over time this organic material combined with mud and was then heated to high temperatures from the pressure created by heavy layers of sediment. This process, known as diagenesis, changes the chemical composition first into a waxy compound called kerogen and then, with increased heat, into a liquid through a process called cat agenesis.The most common method of crude oil extraction is drilling. Geologists will first identify a section of land they believe has oil flowing beneath it. There are a number of ways this can be accomplished, the most frequently used methods are satellite imagery, gravity meters, and magnetometers. Once a steady stream of oil is found, underground the drilling can begin. Oil can also be extracted from oil sands, often called tar sands. Oils sands are typically sand or clay mixed with water and a very viscous form of crude oil known as bitumen. The extraction process for oil sands is quite different from drilling due to the high viscosity of this extra-heavy oil. Petroleum utilization is a much more complex process than coal utilization. This is illustrated in Figure 8-5. In particular, the preparation of petroleum before it is sold to the consumers is very extensive. The reason for this is that, despite their similar elemental composition, the chemical structure of different crude oils may be very different, as discussed above. Most of them are used as fuels.The composition of particular petroleum product ranges from the very low molecular weight hydrocarbons to the very high. A hydrocarbons' chemical structure affects its biodegradation in two ways. First, the molecule may contain groups or substituents that cannot react with available or inducible enzymes. Second, the structure may determine the compound to be in a physical state where microbial degradation does not easily occur. Usually, the larger and more complex the structure of a hydrocarbon, the more slowly it is oxidized. Also the degree of substitution affects the degradation. Compounds that contain amine, methoxy and sulfonate groups, ether linkages, halogens and branched carbon chains are generally persistent. Adding aliphatic side -chains increases the susceptibility of cyclic hydrocarbons to microbial attack[48]**.** Also the concentration of contaminants will affect the number of organisms present. It has been shown that the higher concentrations of gasoline in contaminated water were related to higher counts of microorganisms.

Microorganisms: bacteria play the central role in hydrocarbon degradation. The driving force for petroleum biodegradation is the ability of microorganisms to utilize hydrocarbons to satisfy their cell growth and energy needs. A large number of studies report that low molecular weight alkanes are degraded most rapidly. Mixed cultures carry out more extensive biodegradation of petroleum than pure cultures[12,14,45,57] **.**In many ecosystems there is already an adequate indigenous microbial community capable of extensive oil biodegradation, provided that environmental conditions are favorable for oildegrading metabolic activity[29,47]. Accepted values for a mixed microbial population in the soil are C: N, 10:1; and C: P, 100:1. Nitrogen and phosphorus can be supplied with common inorganic fertilizers with the N: P ratio at 16:1 when the optimum nitrogen fertilization for a sandy matrix is lower than 100 mg N kg dry soilimates[29].

Need for Biodegradation: Dissolved aromatic components of petroleum even at a low ppb concentration, disrupts the chemoreception of some marine organisms. As feeding and mating responses in marine life forms largely depend on chemoreception, such disruption can lead to elimination of many species from the polluted area, even when the pollutant concentration is far below the lethal level as defined in the conventional sense. Another disturbing possibility is that some condensed polynuclear components of petroleum that are carcinogenic and relatively resistant to biodegradation may move up marine food chains and taint fishes or shellfishes, which further can be used as food or feed.

Direct or indirect exposure of living organisms to hydrocarbon compounds can have varying ecological and/or economic impacts, Economic impact, The National Wildlife Federation reported that already more than 150 threatened or endangered sea turtles are dead. And 316 sea birds, mostly brown pelicans and northern gannets, have been found dead along the gulf coast as a result of the spreading oil (http://www.care2.com/causes/10-mosthorrifying-facts-about-the-gulf-oill-spill.html; last accessed on 14.2.2013).

REVIEW OF LITRATURE:

Approaches to Biodegradation of HCs

There have been several approaches to treat soil and water contaminated with HCs:Phytoremediation: This strategy makes use of plants for bioremediation, but is outside the scope of this review.Use of microbial consortium: This involves use of multiple microbial species together rather than relying on catabolic capacity of any single species. Consortium can be in form of microbial biofilms, where production of certain biosurfactants can enhance oil degradation by increasing its bioavailability. Biofilms may be single-species or multi-species. Some organisms apply behavioral strategies such as adhesion and biofilm formation to acquire carbon and energy from hydrophobic organic compounds (HOCs) contained in marine aggregates. HOCs are weakly soluble in water providing for low bioavailability. *Marinobacter hydrocarbanoclasticus SP17* form biofilms at HOC-water interface enhancing bioavailability of HOCs[16]**.** Physiological and proteomic studies revealed that biofilm formation is an efficient strategy to colonize hydrophobic interfaces[58,59] .Stimulation of anaerobic degradation using alternative electron acceptor: Anaerobic degradation can be used as an alternative where aerobic conditions cannot be maintained. Several alternative electron acceptors have been proposed for use in anaerobic degradation, including nitrate, sulphate, iron(Fe3+), and carbon dioxide[34] **.** Nutrient augmentation (Biostimulation): Large amount of contaminants enter the soil most commonly from leaking underground storage tanks, landfills, waste disposal ponds etc. Assimilation of HCs may be done by microorganisms already present in soil, albeit at a slow pace. However relying solely on this small population of naturally present organisms is not sufficient for effective bioremediation in most cases. Natural environments are often deficient in nutrients. Microbial growth rate can be accelerated to enhance soil remediation by providing exogenous supply of nutrient, especially nitrogen and phosphorous to the contaminated soil. Other nutrients present in limiting concentrations can also be added. This approach is known as nutrient augmentation. HC degradation in contaminated soil can be enhanced by applying surfactants (along with other nutrients) to soil, which makes HCs more easily available to microorganisms. After applying nutrients, they are mixed with soil by convenient means such as tilling, disking, rototilling, etc . Bioremediation of contaminated soil can be carried out either *in situ* or *ex situ*. In latter case contaminated soil is excavated and treated at a separate treatment facility, whereas in the former organisms are added directly to the contaminated site. One of the *In situ* methods for treating soil contaminated dominantly with volatile pollutants includes soil venting. Bioventing, which is applicable in case of semi-volatile and non-volatile contaminants, involves addition of oxygen directly to a site of contamination in the unsaturated zone[34].*Ex situ* methods include composting, slurry-phase bioreactors, land treatment (a form of land farming), etc. Both *ex-situ* and *in-situ* approaches may include a combination of biological and non-biological processes. Bioavailability of a particular compound depends on its solubility in water. A water soluble contaminant can be rapidly degraded, however degradation can be limited if contaminant is sparingly soluble in water resulting in reduced bioavailability. Microorganisms growing on organic compounds with limited water solubility face a problem as they will obtain only a fraction of the substrate from surrounding aqueous phase.

With a low solubility compound the opportunity for contact between degrading organism and organic compound becomes limited. Liquid HCs form separate phase above or below water surface being less or more dense than water. For example, polychlorinated biphenyls (PCBs) are denser than water and form a separate phase below the water surface, whereas benzene and petroleum being less dense form a separate phase above the water surface[34].

Microorganisms Known to Degrade HCs: A wide variety of organisms are known to degrade HC compounds under different environmental conditions. These organisms belong to different groups like thermophiles, alkaliphiles, halophiles, etc. Many of these organisms are more effective in form of consortium. *Pseudomonas putida* is well known for HC degradation. *P. putida* can efficiently degrade benzene and toluene[15]. *P. oleovorans* is reported to degrade tetrahydrofuran (THF). *P. oleovorans* DT4 can also utilize and biotransform THF and BTEX compounds[72].

Many of these HC degraders employ their plasmid encoded machinery for degradation. The TOL plasmid pWWO *xyIN* gene product from *Pseudomonas putida* was reported to be involved in *m*-xylene uptake[28] .Alkylbenzoate degradation genes in *P. putida* are located on its TOL plasmid[26].. *P. putida* is considered to be more suitable for bioremediation application, as it is not known to be human pathogen, unlike *P. aeruginosa*. We are currently investigating growth of *Viribacillus salarius* isolated by us from saline soil of Khambhat, India[53].

BIOSURFACTANTS: Surfactants are surface active compound that reduce the interfacial tension between two liquids, or that between a liquid and a solid. Surfactants are organic compound that contain both hydrophobic (head part of the surfactant) and hydrophilic (tail part of the surfactant) moieties. Thus surfactant contains both water insoluble i.e. water repellent group as well as water soluble i.e. water loving group. Biosurfactants are also surface active compound like chemical surfactants but unlike the chemical surfactant, biosurfactant are synthesized by microbes like bacteria, fungi and yeast. Biosurfactants comprise the properties of dropping surface tension, stabilizing emulsions, promoting foaming and are usually non-toxic and biodegradable. Recently interest in biosurfactant has increased because of its diversity, flexibility in operation, and more ecofriendly then chemical surfactant. Furthermore possibility of their production on large scale, selectivity, performance under intense conditions and their future applications in environmental fortification also these have been increasingly attracting the attention of the scientific and industrial community. These molecules have a potential to be used in a variety of industries like cosmetics, pharmaceuticals, humectants, food preservatives and detergents. But the production of biosurfactant on industry level is still challenge because of using high costly synthetic media for microbial growth. Biosurfactants are classified on the basis of diversity in their structure and their microbial origin.

Classification of Biosurfactant:

Biosurfactants are classified in to two major group one is low molecular weight surface active agent call biosurfactant and high molecular weight substance called bio-emulsifier that is especially used as enhancement of emulsification of hydrocarbon. Further these two major group is divided in to six major group known as glycolipids, lipopolysaccharides, lipoproteins-lipopeptides, phospholipids, hydroxylated and cross linked fatty acids.

Properties of Biosurfactant: Biosurfactants are surface active compound that accumulate at the boundary between two immiscible fluids or between a fluid and a solid. By reducing surface (liquid-air) and interfacial (liquid-liquid) tension they reduce the repulsive forces between two different phases and allow them to mix and thus enhance the solubility properties like chemical surfactant. The most effective biosurfactants can reduce the surface tension of water from 72 to 30 mN·m−1 and the interfacial tension between water and *n*-hexadecane from 40 to 1 mN·m−1 Bio surfactant produces from *B.subtilis* is able to lower the surface tension of water to 25 mN/m and interfacial tension of water/hexadecane to <1 mN/m. Furthermore, biosurfactants are more effective and efficient and CMC of biosurfactant is about 10–40 times lower than that of chemical surfactants, so less amount surfactant is required to get a maximum decrease in surface tension as compare to the chemical surfactant.

In many cases it was found that biosurfactant activities are not influenced by environmental condition such as temperature and pH. In 1990 McInerney suggested that lichenysin produced by *B. licheniformis* was not affected by temperature (up to 50°C), pH (4.5–9.0) and by NaCl and Ca concentrations up to 50 and 25 g/l respectively. Apart from theses above properties biosurfactant can be easily degraded unlike the chemical surfactant and thus and they are chiefly suited for the environmental applications such as bioremediation, and dispersion of oil spills. The toxicity of biosurfactants is much lower and some of the researcher consider as these are nontoxic compounds. Very few literature are available that describes the toxicity of biosurfactant and their direct bad impact on environment. Therefore they are appropriate for pharmaceutical, food and cosmetic uses. A study suggested that a synthetic anionic surfactant (Corexit) showed an LC50 (concentration lethal to 50% of test species) against Photobacterium phosphoreum ten times lesser than rhamnolipids, suggesting the larger toxicity of the chemically derived surfactant. In a particular study where toxicity of six biosurfactants was comparing with the toxicity of, four of the synthetic surfactants and two commercial dispersants, it was observed that mostly biosurfactants degraded quicker, except for a synthetic sucrosestearate that showed structure homology to glycolipids and was degraded more rapidly than the biogenic glycolipids. A biosurfactant from *P. aeruginosa* was compared to a synthetic surfactant that is widely used in the industry, regarding toxicity and mutagenic properties. Both assays indicated a higher level of toxicity and mutagenic effect of the chemically derived surfactant, whereas the biosurfactant was considered to be slightly non-toxic and no mutagenic.

Microorganisms producing biosurfactants: Biosurfactants produced by a variety of microorganisms mainly bacteria, fungi and yeasts are diverse in chemical composition and their nature and the amount depend on the type of microorganism producing a particular biosurfactant. Many microorganisms for industrial utilization for waste products have been isolated from contaminated soils, effluents and waste water sources. Thus, these have an ability to grow on substrates considered potentially noxious for other non-producing microorganisms.

Economic factors of Biosurfactant production:To overcome the expensive cost constraints associated with biosurfactant production, two basic strategies are generally adopted worldwide to make it costeffective: (i) the use of inexpensive and waste substrates for the formulation of fermentation media which lower the initial raw material costs involved in the process; (ii) development of efficient and successfully optimized bioprocesses, including optimization of the culture conditions and cost-effective recovery processes for maximum biosurfactant production and recovery. As millions of tons of hazardous and non-hazardous wastes are generated each year throughout the world, a great need exists for their proper management and utilization. The residues from tropical agronomic crops such as cassava (peels), soybeen (hull), sugar beet, sweet potato (peel and stalks), potato (peel and stalks), sweet sorghum , rice and wheat bran and straw); hull soy, corn and rice; bagasse of sugarcane and cassava; residues from the coffee processing industry such as coffee pulp, coffee husks, spent coffee grounds; residues of the fruit processing industries such as pomace and grape, waste from pineapple and carrot processing, banana waste; waste from oil processing mills such as coconut cake, soybean cake, peanut cake, canola meal and palm oil mill waste; saw dust, corn cobs, carob pods, tea waste, chicory roots etc. have been reported as substrates for biosurfactant production . Additional substrates used for biosurfactant production include water-miscible wastes, molasses, whey milk or distillery wastes. .The various substrates previously reported for biosurfactants production are listed with their advantages.

Substrates for commercial microbial production: Despite possessing many industrially attractive properties and advantages compared withsynthetic ones, the production of biosurfactants on industrial scale has not been undertaken due to high investment costs.

This necessitates their profitable production and recovery on a large scale. Various aspects of biosurfactants, such as their biomedical and therapeutic properties their natural roles[36], their production on inexpensive alternative substrates and their industrial potential, have been reviewed. However their cost of production continues to remain very high. Using low-cost raw materials is a possible solution for this obstacle. Another approach is to use renewable low cost starting materials from various sources including industrial wastes from frying oils, oil refinery wastes, molasses, starch rich wastes, cassava waste water and distilled grape marc

Biosurfactants and Degradation of Petroleum Hydrocarbons

The first biosurfactant to be chemically characterized was a rhamnolipid (branched glycolipid) that is produced by *Pseudomonas aeruginosa*. At the time, this lipid was studied only with respect to its toxicity[24]**.** It was not until later that it was shown to dramatically improve the growth of *P. aeruginosa* in *n*hexadecane media and its surface active properties came to light[20] and the ability of biosurfactants to lower surface tension and increase emulsification was conclusively demonstrated. Biosurfactant research in the 80s focused on growth promotion increases responding to enhanced bioavailability of *n*alkanes[13,46]. Production of many biosurfactants was also shown to be stimulated by the presence of aliphatic hydrocarbons, indicating that the compounds were being actively induced as a means to increase access to hydrophobic molecules[55]. This was established mainly by comparing the relative amounts of biosurfactants that are produced when bacteria were grown on *n*-alkanes as compared to water-soluble substrates such as glucose[41]. By the 1990s, it was widely accepted that biosurfactants can play important roles in petroleum hydrocarbon degradation.Surfactant molecules have an amphiphilic structure in which a hydrophilic portion on one end of the molecule allows biosurfactants to be soluble in water and a hydrophobic part of the molecule allows intraction with hydrophobic substances. This property also leads to the concentration of surfactant molecules at interfaces, including the air-water interface[61].

PAHs can be transported to the surface of bacterial cells only if they are mobilized into the aqueous phase by association with a surfactant[64,65]. When micelles form, solid PAHs may partition into the interior of the micelle, which increases the hydrocarbon's apparent solubility[42]. This is considered to be one of the major mechanisms in which biosurfactants enhance the bioavailability of PAHs[51]**.** Once PAH molecules are associated with the surfactant, they may remain entrapped in the micellar core (also known as the micellar pseudophase), or directly released to the bacterial cell if the cell wall is sufficiently hydrophobic to interact with the surfactant (Fig. 1.2.4). Non-micellar transport is possible through cell surface modifications by surfactant molecules[3] and interactions between pollutants and micelle-like aggregates or single surfactant molecules. These modes of action are expounded upon in biosurfactant-based bioremediation studies. For instance, most solubilization studies suggest that the percentage of PAH solubilized is dependent on biosurfactant concentration. Solubilization of petroleum hydrocarbons by biosurfactants generally leads to an increase in biodegradation in liquid media[23,70] and soil[62]. However, there are also studies thatshow biosurfactants can inhibit PAH degradation[17,18,52]. This may occur when bacteria are unable to access PAHs that are contained in the center of the micelles and the cell envelope is not compatible to interact with the surfactant in a manner that allows the release and diffusion into the cell membrane for uptake by the bacteria.

When microorganisms are able to interact with the surfactant, the presence of the biosurfactant will frequently lead to enhanced growth of the microorganism, which accesses the carbon contained in the PAH, or possibly by use of the surfactant as a substrate for growth[62] **.** Synthetic surfactants are considered to have greater potential for toxicity[56], possibly by causing delipidation of the cell membrane of bacteria that are exposed to certain surface active agents. Thus, biosurfactants can also have antimicrobial activities aside from serving as a carrier for hydrophobic substance mobilization[6,19] During micellar-dependent mobilization, growth reduction may be concentrationdependent especially at supra-CMC levels where the PAH is sequestered in the micellar pseudophase[17,18].

Some synthetic surfactants share the degradation-enhancing properties that occur with biosurfactants and due to their low production costs dominate the $23 billion global surfactant industry. Commercially, synthetic surfactants have proved useful for inclusion in cleaning products, for delivery of pharmaceuticals, and for applications in biological sciences, personal hygiene, oil drilling, and many other markets. However, with respect to environmental applications, biosurfactants are increasing in popularity for several reasons. Chemical surfactants are created from petroleum feedstocks; whereas, biosurfactants can be generated from microorganisms, provided that inexpensive feedstocks for microbial culture can be used [38]**.** Other advantages include higher biodegradability, lower toxicity, higher selectivity and specific activity at extreme temperatures, pH, and salinity. The diversity of biosurfactants that may reside in nature thus offers molecules having many interesting and potentially useful chemical properties for a wide range of applications (Table 1.2.4). Current research is developing biotechnologies that can increase yields and lower costs associated with biosurfactant production to compete with the synthetic market.

MATERIALS AND MATHOD

COLLECTION OF SOIL SAMPLES

Hydrocarbon contaminated Soil samples were collected from the vicinity of various petroleum hydrocarbon contaminated sites like Oil well, Petrol pump and Garages.

PETROLEUM HYDROCARBONS AS CARBON SOURCE

Following petroleum hydrocarbon sources were used as source of carbon in various experiments described below (table :1).

ISOLATION OF HYDROCARBON DEGREDING BACTERIA:

To isolate the existing micro flora in the samples, enrichment technique was employed The Soil samples were suspended in sterile distilled water and the supernatant was used as inoculums. 2% (1 ml) supernatant was inoculated in 50 ml Bushnell and Hass medium (HIMEDIA laboratories M350) and mineral salt medium (Appendix - 1) in 250 ml flasks. These flasks are supplemented with 2% (1 ml) carbon source, *viz.* Crude oil and lubricant oil. Incubation was done at room temperature for 24-48 hrs. After one incubation, the medium was centrifuged to obtain the cell mass and two such Subsequent transfers were given in both the media for various carbon sources, to enrich the growth of hydrocarbon degrading organisms in the samples.

ENRICHMENT OF PETROLEUM HYDROCARBON DEGRADING BACTERIA:

After the enrichment step, the media was centrifuged at 10000 rpm for 10 min to obtain the cell mass Further this cell mass was suspended in Nutrient broth, and incubated for three hrs. Nutrient agar plates were streaked from it. isolated colonies of the organisms obtained (table 3)

IDENTIFICATION: After obtaining them in pure, the organisms were identified morphologically culturally and biochemically. Morphological Characteristics: Colony characteristics of the organisms were identified by shape, size, edge, Pigmentation, color, opacity, elevation, surface, texture of colony. Gram staining, Capsule staining, Flagella staining and motility test was performed to study morphology of the organisms and Grams reaction. Biochemical identification of bacteria: The Biochemical tests performed for identification.

ASSESSMENT OF HYDROCARBON UTILIZATION EFFICIENCY:

Five hydrocarbon fractions were used for this purpose, viz. Petrol, diesel, kerosene, paraffin oil and lubricant oil. 250 ml flasks were filled with 50 ml BHM Broth and were supplemented with the respective petroleum fraction. These flasks were incubated in the shaking condition to facilitate proper aeration and agitation. The O.D at 600nm was read periodically. From the data obtained by this study, the efficiency of the organisms to utilize petroleum hydrocarbons was assess.

PRODUCTION AND EXTRACTION OF BIO-SURFACTANT:

The selected 5 organisms were inoculated in N broth flasks and incubated, centrifuged to obtain cell mass at A600=1. Now they were inoculated in fresh BHM medium supplemented with 3% of carbon sources like glucose, glycerol, mannitol and kerosene and incubated for five days, after centrifuged at 350g for 20 min. The supernatant was collected and the cell pellet thus obtained was preserved for the tests. The pH of supernatant was adjusted at 2 by addition of 5 moles/l of H_2SO_4 for the precipitation of bio-surfactant. The precipitates were extracted with two volumes of diethyl ether/methanol (1:1 v/v) mixture. The solvent was evaporated. The extract thus obtained is called the crude extract and it is used in some of the tests given below.

DEGRADATION OF B.T.E.X:

Assessment of capacity to degrade various hydrocarbons and crude oil fractions by selected bacteria from 30 bacterial isolates, ps5 and PC having better efficiency to utilize crude oil (As interpreted from whole cell protein) were further assessed for their capability to degrade 5 crude oil fractions (like petrol, Diesel, Lubricant oil, kerosene and paraffin oil), as well as monocyclic Aromatic volatile Hydrocarbons (like phenol, Toluene, Benzene, Ethylbenzene and xylene), and Polycyclic Aromatic Hydrocarbons (PAHs) like Naphthalene (as model, example) (fig 1) (table 5 and 6).

ASSAY METHODS FOR DETECTION OF BIOSURFACTANTS:

The tests for the analysis of bio surfactant production qualitative and quantitative measures.

Drop collapse test: Two ml of hydrocarbon sources like paraffin oil, diesel, crude oil and kerosene were added to each well of a 96-well microliter plate lid. The lid was equilibrated for 1 hour at room temperature and then 5 µl of culture supernatant from all four sample sets was added to the surface of oil. The shape of the drop on the oil surface was inspected after 1 min. Biosurfactant producing cultures giving flat drops were scored as positive. Those cultures that gave rounded drops were scored as negative (fig.2).

Emulsification activity test (E24 test): Emulsification activity was measured according to the method of Cooper and Goldenberg with a slight modification. 4ml of culture supernatant from all 4 sample sets including crude extract was taken. 4ml of n-hexadecane or crude oil were added and vortexed at high speed for 2 min. The mixture was allowed to stand for 24 hrs. The emulsification activity is defined as the height of the emulsion layer divided by the total height and expressed as percentage (fig 3).

Cell surface hydrophobicity, Bacterial Adhesion to Hydrocarbons (BATH test):The bacterial adhesion of hydrocarbon assay was used to determine for 4 carbon sources. Bacteria were harvested from growth cultures by centrifugation at 8000 rpm for 10 min at 4°C, washed twice and suspended in PUM buffer to an initial absorbance at 400nm to 1.0 Kerosene and cell suspensions were vortexed in a test tube for 2 min and equilibrated for 15 min. The bottom aqueous phase was carefully removed with a Pasteur pipette and A400 was measured. The adherence was expressed as the percentage decrease in optical absorbance of the lower aqueous phase following mixing procedure compared with that of a cell suspension prior mixing.

$$H = \left[\frac{1-A}{A_0}\right].100\%$$

Whereas;

A_0: is the absorbance of the bacterial suspension without hydrophobic phase. A: the absorbance after mixing with hydrophobic phase.

CTAB Agar Test: The CTAB agar plates were streaked with the cell pellet obtained previously. After the incubation, the plates were observed for the dark blue halo surrounding the growth of the organism.

Hemolysis Test: The blood agar plates were inoculated with the cultures in two different patterns. Sterile paper discs were immersed in the cultures and placed on the surface of the blood agar plate. Alternatively, the plates were inoculated in a cross pattern.The plates were observed for the zone of hemolysis around the growth of organism (fig .4)

RESULT AND DISCCUTION

Isolation of Biosurfactant producing bacteria

Table 2 shows the details of various sites, types of samples collected and the no of biosurfactant producing bacteria isolated. The sites selected have been under the constant pressure of hydrocarbon contamination. These samples where enriched in two different media supplemented with crude oil as sole source of carbon and energy. Isolation of the grown bacterial strains in the selective medium was continued 3-4 times in the fresh medium. From the broth, isolation of bacterial Species was made on N- agar plates. Repeated transfers of the single colony on the agar plates were made to obtain pure culture. Screening of the hydrocarbon Contaminating environment like petrol pump, oil well vicinity, garage, etc.

Enrichment and screening of hydrocarbon utilizers

Out of 58 isolates 14 bacterial isolates were screened for utilization of various hydrocarbons. To check the potential of the utilization of different Hydrocarbons, the selected isolates were identified and further assessed. The isolates were screened for ability to grow on petroleum fractions like petrol, diesel, kerosene, lubricant oil, paraffin oil, crude oil as sole source of carbontable . Growth was compared visibly and graded ++++, +++, ++ and + for luxurious, good fair and moderate growth .on the basis of qualitative Study, 2 isolates (Ps5, Pc,) were subjected for further assays.The 5 isolates showed considerable biodegradation \ utilization of various petroleum hydrocarbon fractions, as listed in **Table 4**.

Identification

Morphological Identification:The initial Identification was done morphologically, cell morphology and Gram's reaction were studied.

Motility testThe motility of both the isolates studied by stab agar as well as hanging drop method Ps5 and Pc motile organism

Capsule and flagella staining:Capsule staining is performed by Hiss method and flagella staining is performed by manval method Capsules are not found to be present and flagella staining is positive.

Biochemical identification further identification is done on the basis of the diverse biochemical reactions and the response of the isolates towards it

Microbial degradation of BTEX: Liquid monocyclic aromatic hydrocarbons such as benzene, toluene, and phenol are toxic to bacteria when present in liquid phase. However, if they are introduced in vapor phase good growth can be obtained[15]. Measurment of increases in cell mass in terms of turbidity and whole cell protien directly indicates the utilization of various volatile monocyclic aromatic hydrocarbons as they are added as the sole source of carbon in individual BHM flask (fig 1) (table 5 and 6)

Many organisms have been reported to use various volatile monocyclic petroleum hydrocarbons, (including BTEX) as their sole carbon and energy substrate, despite their extreme insolubility in the aqueous phase. Numerous genera of bacteria are known as good hydrocarbon degraders. They tolerate high concentration of the hydrocarbons and have a high capability for their degradation. Most of them belong to *Pseudomonas, Spinghomonas, Aeromonas, and Alkalizes. Acinetobactor, Arthobactor, Brevibecterium, Xantomonas, Mycobacterium, Rhodococcus* and *bacillus species*[2].

Assay methods for detection of biosurfactant: Drop collapse test Jain *et al.*[21] developed the drop collapse assay. This assay relies on the destabilization of liquid droplets by surfactants. Therefore, drops of a cell suspension or of culture supernatant are placed on an oil coated, solid surface. If the liquid does not contain surfactants, the polar water molecules are repelled from the hydrophobic surface and the drops remain stable. If the liquid contains surfactants, the drops spread or even collapse because the force or interfacial tension between the liquid drops and the hydrophobic surface is reduced. The stability of drops is dependent on surfactant concentration and correlates with surface and I terfacial tension fig.2.

Cell surface hydrophobicity: Bacterial Adhesion to Hydrocarbons Assay (BATH): Rosenberg *et al*[5],developed the bacterial Adhesion to hydrocarbons method, a simple photometrical assay for measuring the hydrophobicity of bacteria. The method is based on the degree of adherence of cell to various liquid hydrocarbons. For measuring this trait, a turbid, aqueous suspension of washed microbial cells is mixed with a distinct volume of a hydrocarbon, e.g., hexadecane or ocatane. After mixing for 2 minutes, the two phases are allowed to separate. Hydrophobic cells become bound to hydrocarbon droplets and riae with the hydrocarbon. They are removed from the aqueous phase. The turbidity of the aqueous phase is measured. The decrease in the turbidity of the aqueous phase correlates to the hydrophobicity of the cell (table 8)

Emulsification activity assay [E_{24}test]: This test was developed by Cooper and Goldenberg[39]. For measuring this trait, kerosene is added to an aqueous sample. The mixture is vortexes at high speed for 2 minutes. After 24 hours, the height of the stable emulsion layer is measured. The emulsion index E24 is calculated as the ratio of the height of the emulsion layer and the total height of liquid. E24 correlates to the surfactant concentration .Evaluating the emulsification capacity is a simple screening method suitable for a first screening of biosurfactant producing microbes (table 9). It is applied in many screening,[21,10,35,36] whereas the kerosene can be replaced with other hydrophobic compounds, e.g., hexadecimal. But surface activity and emulsification capacity do not always correlate. Consequently, this method gives just an indication on the presence of biosurfactants fig 3.

CTAB Agar plate: The CTAB agar plate method is a semiquantitative assay for the detection of extracellular glycolipids or other anionic surfactants. It was developed by Siegmund and Wagner[2]**.** The microbes of interest are cultivated on a light blue mineral salts agar plate containing the cationic surfactant Cetyl-trimethyl ammonium bromide and the basic dye methylene blue. If anionic surfactants are secreted by the microbes growing on the plate, they from a dark blue, insoluble ion pair with Cetyl-trimethyl ammonium bromide and methylene blue. Thus, productive colonies are surrounded by dark halos.The microbial culture grown on BHM with added 2% (v/v) crude oil was streaked on the surface of a Cetyl-trimethyl ammonium bromide (CTAB) Methaylene blue agar plate. After 24 hours of incubation, the plate was inspected for the formation of a dark blue halo around the culture spot which indicated the occurrence of a specific reaction between the cationic CTAB methylene blue complex and the anionic biosurfactants. No blue halo was observed on CTAB agar plate .Hemolysis test:Biosurfactants can cause lysis of erythrocytes. This principle is used for the hemolysis assay which was developed by mulligan et al.58 Culture are inoculated on sheep blood agar plates and incubated fir 2 days at 25 ^{0} C. positive strains will cause lysis of the blood cells and exhibit a colorless, transparent ring around the colonies. Hemolysis can also be shown with purified biosurfactant fig.5.The blood agar method is often used for a preliminary screening of microorganism for the ability to produce biosurfactant on hydrophilic media[68].

Table 1: Hydrocarbons used and its source	
Hydrocarbon	**Source**
Petrol	Ankur Petroleum, Dhansura
Diesel	Ankur Petroleum, Dhansura
Kerosene	Market yard, Dhansura
Paraffin oil	College Laboratory
Lubricant oil	2T oil, Racer 2 HP
Hydrocarbon contaminated soil sample	Ambika Garage, Dhansura

Table 2: Types of samples collected and No. of isolates obtained			
NO.	**LOCATION**	**TYPES OF SAMPLE**	**NO. OF ISOLATES**
1	Sangh Petrol pump Dhansura	Soil sample	01
2	Lakshmi garage Dhansura	Soil sample	01
3	Road transport petrol pump, Modasa	Soil sample	01
4	Soil from Ambika garage Dhansura	Soil sample	01
5	Soil from Krishna garage Modasa	Soil sample	01
6	Maruti garage Dhansura	Soil sample	01
7	Soil sample from Jay mataji garage Modasa	Soil sample	01
8	Soil sample from Manva garage Modasa	Soil sample	01
9	Ankur Petroleum Dhansura	Soil sample	01
10	Thakkar petrol pump Modasa	Soil sample	01
11	Sahiyog petrol pump Modasa	Soil sample	01
12	Chamunda garage Dhansura	Soil sample	01
13	Uma garage Dhansura	Soil sample	01
14	Jalaram garage Dhansura	Soil sample	01
	TOTAL		**14**

TABLE 3 Description of the samples of petroleum fractions		
NO.	**LOCATION**	**TYPE OF SAMPLE**
1	Oil Well, Mehsana	Crude oil
2	Racer 2 HP	Lubricant oil
3	Ankur Petroleum, Dhansura	Petrol
4	Ankur Petroleum, Dhansura	Diesel
5	Racer 2 HP	Paraffin oil
6	Market yard, Dhansura	Kerosene

TABLE 4: Assessment of hydrocarbon utilizing organisms (In terms of OD at 600nm)

Fraction Organism	Petrol	Diesel	Kerosene	Paraffin oil	Lubricant oil	Crude oll
Ps5	-	++	+	++	++	+
Pc	+	+	-	++	+	-

++++ Luxurious, +++ good, ++ fair and + moderate growth
Where Ps5 = *Pseudomonas spp*
Pc = *Pseudomonas spp*

Table 5: Details of Flasks

Flask no	Name of the hydrocarbons
1	Crude oil
2	Diesel
3	Paraffin oil
4	Lubricant oil
5	Kerosene
6	Petrol

Ps5=*Pseudomonas spp.*
Pc=*Pseudomonas spp.*

Table 6: Degradation of various Monocyclic Aromatic Hydrocarbons by two selected bacteria isolates Ps5 and Pc in terms of Turbidity

S. No.	Volatile hydrocarbon	Turbidity(A_{600}) Ps5	Turbidity(A_{600}) Pc
1	Benzene	++	++
2	Toluene	+	-
3	Ethyl-benzene	+	+
4	Xylene	+++	++
5	Phenol	++	++

Where: - indicates no growth, + indicates poor growth, ++ indicates moderate growth and +++ and ++++ indicates luxuriant growth in terms of turbidityAs shown in the **Table** the cell mass (in terms of turbidity) has been found to be better in case of Ps 5 as compared to Pc. This indicates that even volatile monocyclic aromatic hydrocarbons are utilized by both bacterial isolates Ps 5 and Pc in significant amount.

Table 7: Result of Drop Collapse Test

Isolate no.	Dextrose	Glycerol	Mannitol	Kerosene
Ps5	+	+	+	+
Pc	+	+	+	+

+ = Liquid contains surfactants,
- = Liquid does not contains surfactants
Where Ps5= *Pseudomonas spp*.
Pc= *Pseudomonas spp*

TABLE 8: Results of BATH Test				
Isolate No.	**Glucose**	**Glycerol**	**Mannitol**	**Kerosene**
Ps5	69.3%	81%	68.2%	62%
Pc	65%	76.4%	67.4%	58%

Adherence capacity of cells to various hydrocarbons in percentage
Ps5= *Pseudomonas spp.*
Pc= *Pseudomonas spp.*

Table 9: Results of Emulsification Assay				
Isolation No	Glucose	Glycerol	Mannitol	Kerosene
Ps 5	6%	8%	-	-
Pc	8%	7%	5%	7%

E_{24}obtained in percentage
Where Ps 5 = Pseudomonas spp.
Pc = Pseudomonas spp

Fig. 1: Control for the various hydrocarbons in BHM medium
Growth of Ps 5 in different hydrocarbons in the BHM medium

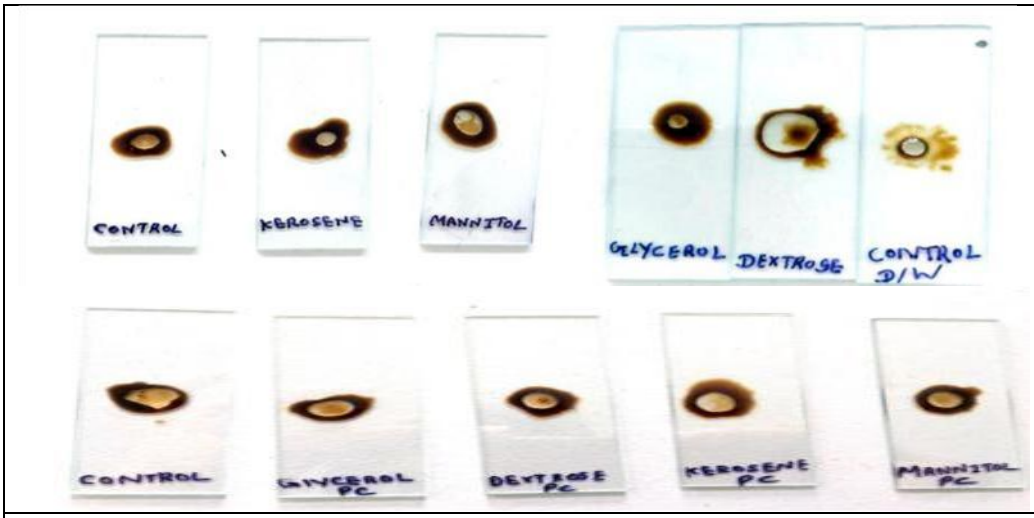
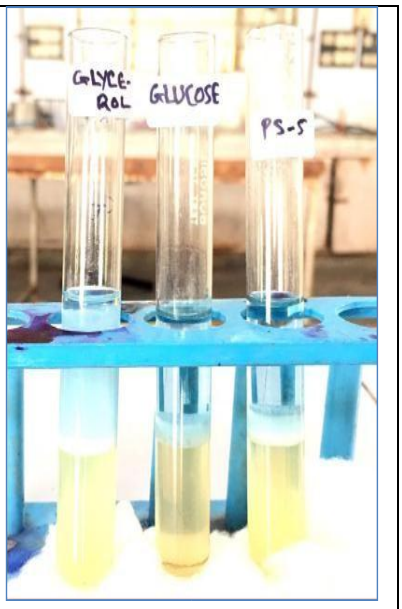


Fig. 2: Drop collapse test of Ps5 and Pc organism

Fig. 3: E_{24}test for Pc organism



For Ps5 organism

Fig. 4: Blood agar plates showing beta hemolysis due to bio-surfactant production by the Ps 5 isolate

From the all above result bio surfactant producing pseudomonas app, is present in oil contaminated soil they use PHB as their nutrient source , Application of biosurfactants in removal of oil from sand.The performance of biosurfactants and chemical surfactants in oil recovery was studied using crude oil contaminated sand. As can be seen from the results obtained, the different biosurfactants at a concentration of 1 g/l recovered between % and % of oil. Ps 5 having 40% while Pc having 35%oil recovery. This study can conclude the ability of pseudomonas spp. isolated from oil contaminated soil to grow on the enriched media with the hydrocarbon as a sole source of energy with high potential capacity of oil degradation and bio surfactant production.

Conflict of interest: nil

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