OCCURRENCE OF RHAMNOLIPID SYNTHESISING GENES IN POLYCYCLIC AROMATIC HYDROCARBON (PAH) UTILIZING *Pseudomonas*

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CERTIFICATE

This is to certify that the project report titled **"Occurrence of Rhamnolipid synthesizing genes in Polycyclic Aromatic Hydrocarbon (PAH) utilizing *Pseudomonas*"** submitted by **Ms. Renu Sharma** to the Department of Life Sciences, National Institute of Technology, Rourkela in partial fulfillment of the requirements for the degree of Masters of Science in LIFE SCIENCE is a bonafide record of work carried out by her under my supervision. The contents of this report in full or parts have not been submitted to any other Institute or University for the award of any degree or diploma.

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DECLARATION

I hereby declare that the major thesis entitled "OCCURRENCE OF RHAMNOLIPID SYNTHESISING GENES IN POLYCYCLIC AROMATIC HYDROCARBON (PAH) UTILIZING *Pseudomonas*" submitted to Department of Life Science, National institute of technology, Rourkela in the partial fulfillment of the requirement for the degree of Master of Science (Life Science) is an authentic record of the work carried out by me and no part of this thesis has been presented for the award of any degree, diploma, fellowship or any other similar title.

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III. ABSTRACT

As promising biotechnological products, rhamnolipids are the most studied biosurfactants. The present study focused on the distribution of *rhlI* among polycyclic aromatic hydrocarbon (PAHs) utilizing bacterial isolates as a tool for the identification of rhamnolipid synthesizing *Pseudomonas* species. Physical and biochemical characterization were done through Gram staining, Macconkey agar test, oxidase test, cell surface hydrophobicity, sulphur reduction indole production mannitol fermentation test, mannitol motility nitrate test. All the isolates show different morphological aspects in terms of Gram staining, color of colonies, motility, sulphur reduction, indole production and mannitol fermentation. Polymerase chain reaction (PCR) amplification with *rhlI* specific primers revealed the presence of *rhlI* coding gene in *Pseudomonas aeruginosa* ATCC 15442, *Pseudomonas aeruginosa* PAO1, N6, NBH1, NBH4, NPD6 and the size of amplified product is 155 bp.

Keywords – Biosurfactant, amplification, *rhlI* gene, *Pseudomonas aeruginosa*

Chapter 1: Introduction

1.1 RHAMNOLIPID - A glycolipid biosurfactant

Biosurfactant are the peculiar group of compounds which shows notable distinction from chemical surfactant in terms of chemical structure and composition (Ron and Rosenberg 2001). Biosurfactants are microbial secondary metabolites that appear to play a role whenever a microbe encounters an interface. They play important role in motility, biofilm formation, solubilization of aromatic hydrocarbon. Biosurfactants have several advantages over the chemical surfactants like lower toxicity, higher biodegradability, better environmental compatibility, higher foaming, high selectivity and specific activity at extreme temperatures, pH and salinity. They are functional amphipatheic molecules which comprise a variety of chemical structures such as glycolipid, lippopeptide, polysaccharide protein complexes, phospholipid and neutral lipid.

Based on the structural features, biosurfactants are classified into five types: (1) glycolipids, (2) phospholipids, neutral lipids and fatty acids, (3) lipopeptides and lipoproteins, (4) flavolipids and (5) polymeric biosurfactants (Ron and Rosenberg 2001; Bodour, Guerrero-Barajas et al. 2004). Glycolipids are generally composed of a carbohydrate group and one or more aliphatic acids or hydroxyl aliphatic acids. The mostly and widely studied glycolipids are sophorolipids and rhamnolipids.

Phospholipids, neutral lipids and some fatty acids are the components of cell structures and generally they have surface activity that is usually associated with biosurfactants. Lipoproteins are comprised of two moieties that are a protein and a fatty acid, for example surfactin. Flavolipids are the biosurfactant of acid tail groups (Bodour, Guerrero-Barajas et al. 2004). These all four groups are low molecular weight biosurfactants and the molecular weight of these biosurfactant ranges from 500 to 3000 and they all are capable of efficiently reducing surface and interfacial tension. Polymeric biosurfactants are polysaccharide-protein complexes and they all are identified by their high molecular weight which ranges from 50,000 to greater than 1,000,000. They are generally more effective in stabilizing emulsions of oil-in water but do not have the capacity to lower the surface tension as much. Biosurfactants can be divided into two classes: low-molecular-mass molecules, which have the capability to lower surface and interfacial tension and high-molecular- mass polymers, which are well known as emulsion

stabilizing agents. The classes of low-mass surfactants include glycolipids, lipopeptides and phospholipids whereas high-mass ones include polymeric and particulate surfactants. Biosurfactants have remarkably low CMCs (Cell micelle concentration) when they are compared to structurally similar synthetic surfactants and they could be used at much lower concentrations. For example, at pH 4, monorhamnolipid mixtures (nonionic) with the heptyl chain as the major congeners have CMC values ranging from < 1 μM to ~ 10 μM depending on ionic strength of solution. Inspite of that, the nonionic alkyl glucosides and glucamides of comparable alkyl chain length that are structurally similar to the rhamnolipids at pH 4 have CMC values in the order of 10^{-3}.

Commercially available biosurfactants are rhamnolipids and surfactin but these surfactants have limited availability. The limitation may be because of following reasons: (1) the lack of control over distribution of congener for each class of biosurfactants, which generally makes it difficult to obtain pure and simple materials as well as understand the properties of each congener for facilitating the use of these molecules; (2) high cost of production and downstream processing. The production costs of biosurfactants are reported as approximately 3 to 10 times higher than that of the synthetic surfactant. Biosurfactant shows highly friendly nature because of their ability to solubilize hydrophobic compounds and they are excellent agents for bioremediation of contaminants present in the environment (Kosaric 2001). Hence, they are widely used in several branches of industries like agriculture, cosmetics, pharmaceutics and food additives (Muthusamy et al., 2008; Banat et al., 2010). First and mostly important fact of biosurfactant is that they are able to interact with poorly soluble contaminants and improve their transfer into the aqueous phase. This is required for the mobilization of recalcitrant pollutants which have been embedded in the soil matrix and their subsequent removal. A mostly and widely studied biosurfactant is rhamnolipid (Soberon Chavez et al., 2005; Abdel Mowgoud et al., 2009) which is generally serve as a model biosurfactant for scientific experiment. All biosurfactants share a common property that is amphiphilic nature. Generally, biosurfactants exist in anionic or non-ionic form.

Rhamnolipids (RLs), the glycolipid biosurfactants were first isolated from *Pseudomonas aeruginosa* and well described by Jarvis and Johnson in 1949 (Jarvis and Johsan 1949). They display relatively high surface activities and are produced in relatively high yields in relatively short incubation periods. *P. aeruginosa* is known to have a relatively large genome at

approximately 6.29 Mbp containing 5,570 predicted open reading frames including a large number of genes which are involved in the catabolism, transport and interchange of organic compounds as well as four potential chemotaxis systems. This reported *P. aeruginosa* with versatile metabolic capabilities making it important to utilize a wide variety of substrates and some of which are not easily degraded by other organisms. The oil wastes, soap stock and other wastes from vegetable oil refineries and food industries are known to the low cost raw materials for rhamnolipid production. They are one of the virulence factors contributing to the pathogenesis of *P. aeruginosa* infections. *P. aeruginosa* is an opportunistic human pathogen having capability of producing several destructive toxins and causing a range of human diseases mainly effecting immune system of individuals producing cystic fibrosis in individual, burn victims, and leukemic patients. *P. aeruginosa* is capable of growing and promoting rhamnolipid production under certain range of different carbon source and the highest levels of rhamnolipid production result from using vegetable-based oils such as carbon sources, including soybean oil, corn oil, and olive oil. Inspite of that, rhamnolipids exhibit several usefull industrial application like emulsification, detergency, wetting, foaming, dispersing, solubilization, antimicrobial and antiadhesive activities in different areas from bioremediation to food additives. Rhamnolipids produced from *Pseudomonas aeruginosa* are known to possess antifungal activity against some plant, *Magnaporthegrisea and Phytophthoracapsici*. The rhamnolipids inhibit spore germination and also prevent hyphal growth in *P. capsici* at concentrations of 50 μg ml^{-1}.The ability of rhamnolipids in disrupting *Bacillus pumilus* biofilms by removing exopolymeric substances. Rhamnolipids have capability to disrupt *Y. lipolytica* biofilms as compared to chemical surfactants, SDS and CTAB. They are powerful natural emulsifiers capable of reducing the surface tension of water about upto 25 mN/m. They help in removing oil spills. Rhamnolipid produced by *P. aeruginosa* have long been known as the heat-stable extracellular hemolysin (Sierra 1960; Kurioka and Liu 1967; Johnson and Boese-Marrazzo 1980; Fujita et al. 1988) and recently, a rhamnolipid congener produced by *Burkholderia pseudomallei* was identified to display hemolytic and cytotoxic activities. Rhamnolipid have been used for the synthesis and stabilization of nanoparticles (Xie et al. 2006; Palanisamy and Raichur 2009), the preparation of microemulsion (Xie et al. 2007; Nguyen and Sabatini 2009), as an antiagglomeration agent as dispersing agent, in cleaning soap mixtures and also as a source of rhamnose. The principal rhamnolipids are mono-rhamno-di-lipidic congener and di-rhamno-di-lipidic congener consisting

of one or two L-rhamnose units and two units of β-hydroxydecanoic acid while mono-rhamno-mono-lipidic congener and di-rhamno-mono-lipidic congener consisting of one or two L-rhamnose and one unit of β-hydroxydecanoic acid (Lang and Wullbrandt, 1999). The vegetable oils such as soybean oil, corn oil, canola oil and olive oil are the major sources for the the highest productivity of rhamnolipid. Increased concentration of C/N and C/P ratios promote the production of rhamnolipids while high concentrations of iron are inhibitory for rhamnolipid production. The production of rhamnolipids are inhibited by the presence of NH_4^+, glutamine, asparagine, and arginine as nitrogen source and are promoted by NO_3^-, glutamate and aspartate. Apart from having tensioactive properties, rhamnolipids are glycolipids which play a vital role in regulating the cell population density-dependent control of genes expression which is termed as quorum sensing (QS) or cell-to-cell communication.

Polycyclic aromatic hydrocarbons are hydrophobic compounds and their presence in the environment is mainly due to their low water solubility. It is necessary to enhance the solubility of PAHs for successful remediation processes. The solubilities of both polycyclic aromatic hydrocarbons (PAHs) like pyrene and phenanthrene were increased linearly with the rhamnolipid concentration at above critical micelle concentration. Rhamnolipid is one such biosurfactant suitable for the biodegradation of pyrene and phenanthrene. The solubility of phenanthrene in a mixed system is lower than that in a single PAH system whereas the solubility of pyrene in a mixed system is enhanced. This is because the hydrophobicity of pyrene is more as compare to than that of phenanthrene. So, pyrene is dominant in the competitive solubilization. The effectiveness and capability of rhamnolipid biosurfactants to enhance the removal of absorbed contaminants from soil was determined using column studies. *Pseudomonas aeruginosa* is known to produce rhamnolipid that is involved in its swarming motility behaviour such as rhamnolipids and their precursors-3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs). In *P. aeruginosa* PAO1, swarming motility is generally inhibited by some fatty acids including branched-chain fatty acids and unsaturated fatty acids. This type of motility is characterized by a multicellular movement in viscous environments like semisolid agar medium and is totally different from flagella-dependent swimming motility which is characterized by individual cell movement in aqueous environments. The general characteristics of swarmer cells are elongation, hyperflagellation and the increased production of extracellular wetting agents which promote reduction of surface tension and promote surface movement. Hence, it has been widely utilized

in industries like agriculture, enhanced oil recovery and bioremediation of oil-contaminated sites. Rhamnolipids also have antibacterial and antifungal activities having possible roles in the medical and agricultural fields. Also, recently a strain of *Pseudomonas putida* was found to be naturally capable of producing rhamnolipid. Rhamnolipids are "secondary metabolites" and their production starts with the onset of the stationary phase. There are different techniques for the characterization of isolated rhamnolipid. The isolated rhamnolipids were characterized by techniques like infrared (IR) spectroscopy and electrospray ionization mass spectrometry (ESIMS). The infrared spectra confirmed that the isolated compound corresponds to the rhamnolipid structure whereas mass spectroscopy indicated that the isolated preparation are a mixture of mono-rhamno-mono-lipidic, mono-rhamno-di-lipidic and di-rhamno-di-lipidic congeners. Iron is an essential and important element for life but also serves as an environmental signal for biofilm development in the opportunistic human pathogen *Pseudomonas aeruginosa*. Under iron-limiting conditions, *P. aeruginosa* promotes enhanced twitching motility and forms flat unstructured biofilms. Under iron limitation, the timing of rhamnolipid expression is shifted to the initial stages of biofilm formation (versus later in biofilm development under iron conditions) and it results in increased bacterial surface motility. The extraction of rhamnolipid include several processes like chloroform–methanol (Kim et al., 1997; Nitschke and Pastore, 2006) and chloroform–ethanol mixtures (Zhang and Miller, 1992; Costa et al., 2006), dichloromethane (Cooper et al., 1981; Joshi et al., 2008), ethyl acetate (Déziel et al., 1999; Chayabutra and Ju 2001), and methanol (Ohno et al., 1995; Ghojavand et al., 2008). Hence, it is necessary to develop a more economical and environmental friendly technique to recover the biosurfactant products (Sen and Swaminathan 2005; Chen et al., 2007 2008).The foam fractionation technique basically provides both a high biosurfactant recovery efficiency and a high enrichment ratio and most of the research has only focused on the recovery of the surfactin, a lipopeptidebiosurfactant produced by *Bacillus subtilis* strains The foam fractionation in batch mode was used for the recovery of rhamnolipid that is well known a glycolipid biosurfactant produced by *Pseudomonas aeruginosa SP4* (Pornsunthorntawee et al., 2008a).

1.2 PSEUDOMNAS SPECIES

The opportunistic human pathogen *Pseudomonas aeruginosa*is responsible for producing a variety of virulence factors including exotoxin A, elastase, alkaline protease, alginate, phospholipases, and extracellular rhamnolipids. The previously characterized *rhlABR* gene is

responsible for encoding a regulatory protein (*RhIR*) and a rhamnosyltransferase (*RhLAB*) and both of which are required for rhamnolipid synthesis. Another gene whose name is *rhlI*, has now been identified downstream of the *rhlABR* gene cluster. The putative *RhlI* protein have common significant sequence similarity with bacterial autoinducer synthetases of the *Luxl* type. A *P. aeruginosa rhilI* mutant strain carrying a disrupted *rhlI* gene and it is unable to produce rhamnolipids and lacked rhamnosyl transferase activity. In *Pseudomonas aeruginosa*, the *LasR-LasI* system regulates the expression of the *lasB* gene, encoding elastase, and it is the medium to serve as a means for cell-to-cell communication to allow the coordinated expression of virulence- associated genes (Gambello and Iglewski1991). *P. aeruginosa*produced two types of rhamnolipid which are the monorhamnolipid,rhamnosyl-b-hydroxydecanoyl-b-hydroxorhydecanoate (Rha-C10-C10) and the dirhamnolipid, rhamnosyl-rhamnosyl-b-hydroxydecanoyl-b-hydroxydecanoate (Rha-Rha- C10-C10).The biosynthesis of rhamnolipid occurs through the three sequential reactions. *RhlA* promotes the synthesis of the fatty acid dimer moiety of rhamnolipids and (HAA) while rhamnosyl transferases *RhlB* and *RhlC* promotes the transfer of dTDP-L-rhamnose to either HAA or previously generated mono-rhamnolipid respectively. Restriction in the availability of a number of nutrients, except the carbon source, is known to promote the production of rhamnolipids (Guerra-Santos et al. 1986). The transcription of *rhlAB* and the production of rhamnolipid are inversely proportional to the concentration of iron (Fe) available to the bacterial cells. *P. aeruginosa* produces two classes of signal molecules that are the acyl homoserinelactones (AHLs) and the 4-hydroxy-2-alkylquinolines (HAQs). The most abundant AHLs are N-(3-oxododecanoyl)-L-HSL and N-butanoyl-L-HSL while the HAQs include 3,4-dihydroxy-2-heptylquinoline [Pseudomonas Quinolone Signal (PQS)]and its precursor 4-hydroxy-2-heptylquinoline (HHQ)). *RhlA* is responsible for the synthesis of the HAAs [the fatty acid dimers, from two 3-hydroxyfatty acid precursors (Zhu and Rock 2008)]. The membrane-bound *RhlB* rhamnosyl transferase utilizesd TDP-L-rhamnose and an HAA molecule as precursors synthesizing mono-RL and these mono-rhamnolipid are in turn the substrates and together with dTDP-L-rhamnose of the *RhlC* rhamnosyl transferase to produce di-Rhamnolipids. Recently, the genes *rhlA*, *rhlB*, and *rhlC* were found in the rhamnolipid producing species such as *Burkholderia thailandensis* and *B. pseudomallei*. *RhlA* and *RhlB* are encoded by genes arranged in a cluster in an operon, which is controlled by the regulatory genes *rhlR* and *rhlI*. The expression of *rhlAB* is positively controlled in a cell-density manner by a cell-

to-cell communication system called quorum sensing (Ochsner and Reiser 1995).The gene regulation by quorum sensing reflects that bacteria produce and release chemical signal molecules for which increases in their external concentrations reflects the cell-population density.

1.2.1 QUORAM SENSING IN PSEUDOMONAS SPECIES

P. aeruginosa regulates the transcription of the cluster of genes in an operon by quorum sensing. Multiple systems of quorum sensing (QS) regulates the control of rhamnolipid synthesis genes (*rhl*A, *rhl*B and *rhl*C). Two quoram sensing systems such as *LasR/I* and *RhlR/z* depend on acyl homoserine lactones (AHL) ligands, N-3-oxododecanoyl-HSL and N-butanoyl-HSL, respectively which bind to their cognate transcriptional regulators that are *LasR* and *RhlR*, respectively, for regulation of expression of several genes, among which are rhamnolipid biosynthesis genes *LasR/I* and *RhlR/I* activate the expression of their own autoinducer synthase genes, *lasI* and *rhlI* respectively as a positive feedback. A large proportion of these are directing the production of virulence factor including proteases, lectins, HCN, phenazines and rhamnolipid (Williams and Camara 2009). In case of rhamnolipid biosynthesis, the product of *rhlI* is the signal butanoyl-homoserine lactone, C4-HSL which acts as the activating ligand for the transcriptional regulator *RhlR*. The *RhlR/C4-HSL* complex then binds to the specific sequence in the *rhlAB* regulatory region for the purpose of activating the transcription. *P. aeruginosa* quoram sensing is not only dependent on cell density but also on a wide variety of environmental signals like hyper osmotic stress, soils, marshes and marine coasts. Thus osmoadaptation is critical to *P. aeruginosa* survival in the environment and might play a role in its pathogenicity. Bacteria generally survive under hyperosmotic conditions by accumulating low molecular mass molecules that are compatible with cellular processes at high internal concentrations (Sleator et al., 2002). *P. aeruginosa* PAO1 was known to synthesize and accumulate glutamate, trehalose and N-acetylglutaminyl glutamine amide (NAGGN) in the absence of exogenous osmoprotectant, but preferentially accumulated glycine betaine (GB) when the latter was added to the culture medium. The osmotic stress studies are generally performed in minimal media since components of rich media can bring osmoprotectants in an uncontrolled way. Hyperosmotic condition (0.5 M NaCl) moderately affects growth of *P. aeruginosa* and it lead to intra-cellular accumulation of compatible solutes. The production of signal molecules was delayed and their highest concentrations were 2.5 to 5 fold lower than in NaCl-free PPGAS, except for HHQ and the

highest concentration of which was increased. The presence of NaCl prevents rhamnolipid synthesis. It was found that, when the osmoprotectant glycine betaine was added to PPGAS/NaCl medium and it was imported by the cells without being metabolized. This did not improve growth. Inspite of that, it reestablished the time-courses of HSL and HHQ accumulation and partially restored the HSL and PQS levels. It also partially or fully restored rhamnolipid production. The quantification of mRNAs encoding enzymes involved in HSL, PQS, and rhamnolipid biosyntheses confirmed the effect of hyperosmotic stress and glycine betaine at the level of gene expression.There are different techniques for the extraction of rhamnolipid. By 2010, one company, Rhamnolipid Inc. in St. Petersburg (USA) was known to produce rhamnolipids commercially and according to some scientists, the actual price for rhamnolipids ranges between 200 (20% solution) to 6000 USD/kg (98% pure). Rhamnolipid have also been found as extracellular product in *Burkholderia* species, a genus closely related to pseudomonas (Toribio et al., 2010). *Burkholderia* species produced rhamnolipid which is characterized (Haubler et al., 1998) and it is a causative agent of systemic infectious disease of animals and plants. Rhamnolipids have also been detected and characterized in *Burkholderia plantarii*. Recently, mono and di- rhamnolipid homolouges identified from *Burkholderia thailandensis*. *Burkholderia kururiensis* is one of the degrading bacterium and it is isolated from the polluted site of Japan (Zhang et al., 2010). *Burkholderia* species is associated to the wide range of geographic distribution having the ability to colonize diverse host plants at distantly related environment (Perrin et al., 2006; Elliott et al., 2007). The principal objective of present work is occurrence of rhamnolipid synthesizing genes in polycyclic aromatic hydrocarbon utilizing *pseudomonas*.

Chapter 2: Review of Literature

Rhamnolipid are amphiphilic biological compounds produced extracellular or as part of the cell membrane by a variety of *Pseudomonas* bacteria which connects the hydrophilic microorganism living in the environment with water insoluble hydrophobic hydrocarbon. Surfactants are compounds that lower the surface tension of a liquid, the interfacial tension between two liquids or also between a liquid and a solid. But they are not environment friendly as they are highly toxic. This problem does not seen in biosurfactant due to their diversity, environmentally friendly nature, possibility of large-scale production, selectivity, performance under extreme conditions and potential applications in environmental protection. Rhamnolipid is one such kind of glycolipid biosurfactant.

It reduces surface tension, critical micelle concentration (CMC) and industrial tension in both aqueous solutions and hydrocarbon mixtures. It is the most intensively studied biosurfactant as it displays relatively high surface activities and are produced in relatively high yields after relatively short incubation periods. They are one of the virulence factors contributing to the pathogenesis of *P. aeruginosa* infections. Consequently, many aspects of rhamnolipid biosynthesis have been investigated to control their production and effects.

2.1. ***RHAMNOLIPID STRUCTURE****-*

In 1946, an oily glycolipid was produced by *Pseudomonas pyocyanea* which is now well known as *Pseudomonas aeruginosa* grown on glucose. This oily substance was named pyolipic acid and its structural units were found as L-rhamnose and b-hydroxydecanoic acid (Bergstrom et al. 1946; Jarvis and Johnson 1949; Hauser and Karnovsky 1954). Then, the exact chemical nature of this biomolecule was studied and identified by Jarvis and Johnson (1949) followed by Edwards and Hayashi (1965). The glycon part of rhamnolipid is composed of one (for mono-RLs) or two (for di-RLs) rhamnose units linked to each other through a-1,2-glycosidic linkage (Edwards and Hayashi 1965) explained in fig.1. The aglycon part is composed of mainly one or two but in few cases, three (Andra et al., 2006) b-hydroxyfatty acid chains. Their chain lengths vary from C8 to C16 (De´ziel et al., 2000; Abalos et al., 2001). The first identified rhamnolipid was Rha-Rha-C10-C10. Recently, about 60 different rhamnolipid congeners and homologs have been reported by (Abdel-Mawgoud et al., 2010).

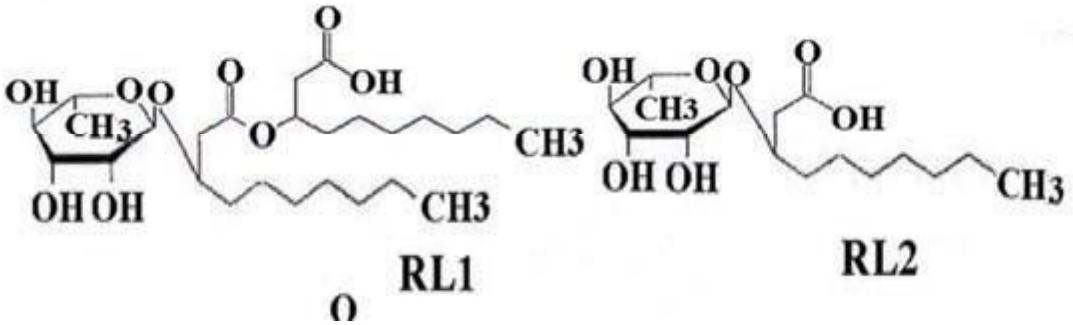


Fig.1. **Structure of rhamnolipid: RL1 (mono-rhamno-di-lipidic congener), RL2 (mono-rhamno-mono-lipidic congener)**

2.2 ***RHAMNOLIPID METHOD OF DETECTION AND ANALYSIS*** *–*

There are several methods with variable precision and purposes available for the detection and analysis of RLs. Some are qualitative and some are quantitative methods. The most widely used qualitative method for the screening of rhamnolipid producing strains is cetyltrimethyl ammonium bromide (CTAB) agar test (Siegmund and Wagner 1991; Pinzon and Ju 2009). Another indirect way to detect rhamnolipid is based on their hemolytic properties. It is performed in solution using an erythrocyte suspension to which the rhamnolipid solution is added done by (Johnson and Boese-Marrazzo 1980). Rhamnolipid production can also be tested using blood agar plates on which the bacteria are directly inoculated (Carrillo et al., 1996) form halo around the colony. The drop collapsing test (Jain et al., 1991) is also one of the sensitive method for the rapid screening of RL production by various isolates. A similar method is used in the oil spreading test in which a drop of bacterial supernatant is added on top of an oil/ water interface (Morikawa et al., 2000). The presence of a surface-active molecule will cause the oil to be repelled and form a clear zone.

One of the most widely used quantification methods for rhamnolipid is the orcinol test. The rhamnose groups of rhamnolipid are hydrolyzed and transformed into methyl furfural, which then reacts with the orcinol to produce a blue-green color that can be measured in spectrophotometre at 421 nm (Chandrasekaran and BeMiller 1980; Koch et al., 1991). Sometimes anthrone is used (9, 10-dihydro-9-oxoanthracene) instead of orcinol to create a dye that can be quantified at 625 nm (Helbert and Brown 1957; Hodge and Hofreiter 1962). Several

chromatographic methods are also adopted to measure rhamnolipid like thin layer chromatography, gas chromatography, liquid chromatography etc. Infrared (IR) has been used mostly to quantify complex mixtures of congeners (Gartshore et al., 2000). It is based on the relatively broad IR absorption bands corresponding to various hydroxyl, ester, and carboxylic groups present in rhamnolipid. Nuclear Magnetic Resonance (NMR) measures structural analysis of purified congeners (Haba et al., 2003a; Monteiro et al., 2007).

2.3.***RHAMNOLIPID BIOSYNTHESIS (at genetic level)*** –

Pseudomonas are generally Gram-negative bacteria whose rhamnose is a component of the cell wall lipopolysaccharide (Burger et al., 1963; Rahim et al., 2000). The carbon of rhamnose is derived from glycerol and not from acetate by the condensation of two three-carbon units formed from glycerol without cleavage or rearrangement of its carbon–carbon bonds (Hauser and Karnovsky 1957). The biosynthetic conversion of glucose to rhamnose in *P. aeruginosa* was fully clarified with the in vivo and in vitro studies of (Southard et al., 1959 Glaser and Kornfeld 1961). Other carbon sources are more efficient for rhamnolipid production, such as mannitol (De´ziel et al., 1999b), vegetable oils (Trummler et al., 2003), glycerol, or ethanol (Chen et al., 2007a). Three enzymatic reactions are required for the final rhamnolipid biosynthesis in *P. aeruginosa* (Sobero´n-Cha´vez et al., 2005): (1) *rhlA* is involved in the synthesis of the HAAs, the fatty acid dimers from two 3-hydroxyfatty acid precursors (De´ziel et al., 2003; Zhu and Rock 2008); (2) the membrane-bound *rhlB* rhamnosyltransferase uses dTDP-L-rhamnose and an HAA molecule as precursors produce mono-RL; (3) these mono-RLs are in turn the substrates together with dTDP-L-rhamnose of the rhlC rhamnosyltransferase to produce di-RLs. Recently, the *rhlA*, *rhlB*, and *rhlC* genes were identified in the rhamnolipid producing species *Burkholderia thailandensis* and *B. pseudomallei* (Dubeau et al., 2009). *RhlA* and *RhlB* are encoded by genes organized in an operon which is regulated by the regulatory genes *rhlR* and *rhlI*. The expression of *rhlAB* is positively controlled in a cell-density manner by a cell-to-cell communication system called quorum sensing (Ochsner and Reiser 1995; Pearson et al., 1997). The production of secondary metabolites and virulence factors such as antibiotics and proteases are generally controlled by quorum sensing (Miller and Bassler 2001).

2.4 ***MEDIUM COMPONENTS AFFECTING ON GROWTH AND RHAMNOLIPID PRODUCTION BY P.aeruginosa***

2.4.1 ***CARBON SOURCE***

The carbon source used in bacterial culture plays significant role in rhamnolipid production. The carbon source used in rhamnolipid synthesis is categorized into three groups carbohydrates, hydrocarbon and vegetable oils. Water soluble carbon sources like glycerol, glucose, ethanol and manintol were all used for rhamnolipid production by *Pseudomonas* species. However, rhamnolipid production was interior to that obtained with water- immiscible substrate such as n- alkane and olive oil (Robertson et al 1994) showed that large amount of rhamnolipid production bound to *Bacillus licheniformis* cells when grown on mineral salts medium containing glucose and 0.1 % yeast extract. The surface tension of medium was reduced from 70 to 74 mN/m to as low as 28 mN/m due to the production of rhamnolipid.

2.4.2 ***NITROGEN SOURCES***

Medium constituents other than carbon sources also effect the production of biosurfactant. The structure of surfactin was influenced by the L- glutamic acid concentration in the medium to produce either val- 7 or Leu- 7 surfactin. It isobserved that nitrate is the best source of the nitrogen for rhamnolipid production by *Pseudomonas strain 44T1*and *Rhodocuccus strain ST- 5* growing on olive oil and paraffin. The production has started around after 30 h of growth when the culture reached nitrogen limitation and continued to increase upto 60 h of fermentation. In *Pseudomonas aeruginosa*, a simultaneous increase in rhamnolipid production and glutamine synthatase activity was observed when growth slowed as the culture become nitrogen limiting. (Guerra-Santos et al., 1984) showed maximum rhamnolipid production at nitrogen limitation of C : N 16 : 1 to 18 : 1 and no rhamnolipid production below C : N at 11 : 1 (Kim et al., 1997) showed that the best yield of rhamnolipid production was obtained using NH_4HCO_3 as the main nitrogen source. The final culture ph measured after 3 days culture indicated that the high yield of rhamnolipid resulted from the prevention of the decrease in the ph. The maximum rhamnolipid concentration was attained when 13.5 g/l of NH_4HCO_3 was used.

Iron concentration has a dramatic effect on rhamnolipid production by *P. aeruginosa* resulting in a threefold increase in production when cells were shifted from medium containing 36 µM to iron to medium contain 18 µM .

2.4.3 ***ENVIRONMENTAL FACTORS***

Environmental factors and growth conditions like pH, temperature, agitation and oxygen availability also effect rhamnolipid production through their effects on cellular growth and activity. Rhamnolipid production in *Pseudomonas species* is maximum at the range of pH from 6.0 to 6.5. and decreased sharply above pH 7.0 (Gurerra- santos et al., 1984). (Sheppard and cooper 1990) concluded that oxygen transfer was one of the key parameters for the process optimization and scale up of surfactin production in *B. subtilis*. Increasing the aeration rate to 169.9 h^{-1} caused an increase in rhamnolipid production by *P.aeruginosa* (Benincasa et al., 2002)

2.5 ***REGULATION OF RHAMNOLIPID PRODUCTION BY ENVIRONMENTAL FACTORS (Link Between Quorum Sensing and the Environment)***

The transcription of *rhlAB* and the production of rhamnolipid are inversely proportional to the concentration of iron (Fe) available to the bacterial cells (De´ziel et al., 2003; Glick et al., 2010). The expression of *lasIR* (Bollinger et al., 2001; Kim et al., 2005., Duan and Surette 2007; Duan and Surette 2007) and *rhlIR* (Bredenbruch et al., 2006; Jensen et al., 2006) is enhanced by iron limitation. The production of rhamnolipid is drastically inhibited by the presence of $NH4^{+}$, glutamine, asparagine and arginine as nitrogen source and promoted by NO3-, glutamate, and aspartate (Mulligan and Gibbs 1989; Venkata Ramana and Karanth 1989; Kohler et al., 2000; Van Alst et al., 2007;). (De´ziel et al., 2003) observed that nutritional conditions can dominant over cell-to-cell communication in rhamnolipid production.

2.6 ***EXTRACTION OF RHAMNOLIPID***

Most methods of recovery of rhamnolipid have been very well refreshed by (Heyd et al., 2008). One of the easiest method of recovery is by acid (Zhang and Miller 1992; Van Dyke et al., 1993; De´ziel et al., 1999b) or aluminum sulfate precipitation (Schenk et al., 1995). Aluminum sulfate precipitates rhamnolipid by salting out process. The rhamnolipid are recovered by centrifugation. The most adopted method was solvent extraction (Schenk et al,. 1995; Mata-Sandoval et al., 1999; Le´pine et al., 2002). Other methods are downstream processing in continuous fermentative production processes include adsorption (Dubey et al. 2005), ion exchange chromatography (Reiling et al., 1986; Schenk et al., 1995; Abadi et al., 2009), ultrafiltration (Mulligan and Gibbs 1990; Haussler et al., 1998), and foam fractionation

(Gruber 1991; Sarachat et al., 2010). The chromatographic methods are usually the best methods for separation of specific rhamnolipid congeners in a pure form. Thin layer chromatography (TLC) is also a good method for purification (Sim et al., 1997; Monteiro et al., 2007). For large scale downstream processing, preparative column chromatography using silica gel is a better method for extraction (Burger et al., 1966; Monteiro et al,. 2007).

2.7. ***POTENTIAL APPLICATION OF RHAMNOLIPID***

2.7.1 ***AS ANTIMICROBIAL AGENTS*** –

Rhamnolipid are known to display antibacterial activities against plant and human pathogenic bacteria. Rhamnolipids are known to be active against the Gram-negative bacteria *P. aeruginosa*, *Enterobacter aerogenes*, *Serratia marcescens* and *Klebsiella pneumonia*, as well as against Gram-positive *Micrococcus* sp., *Streptococcus* sp., *Staphylococcus* sp. and *Bacillus* species (Benincasa et al., 2004). RLs have direct influence on bacterial cell surface structures. (Al-Tahhan et al., 2000) observed a loss in lipopolysaccharides (LPS) in *P. aeruginosa* strains when treated with RLs at low concentrations and this resulted in increased cell surface hydrophobicity. Recently, (Sotirova et al., 2009) showed that RLs from *Pseudomonas sp. PS-17* on interacting with *P. aeruginosa* causing a reduction in LPS content and changes in the outer membrane proteins of the bacteria observed. (Sotirova et al., 2009) concluded that RLs from *Pseudomonas sp*. PS-17 have a potential application in the field of biomedicine against pathogenic bacteria. Several studies described antifungal activity of RLs mainly against phytopathogens including *Botrytis sp., Rhizoctonia sp., Pythium sp., Phytophtora sp. and Plasmopara sp.* Additionally, RLs were also shown to be active against *Mucor miehei* and *Neurospora crassa*. The main mode of action of RLs against zoospore-producing plant pathogens is the direct lysis of zoospores via the intercalation of rhamnolipid within plasma membranes of the zoospore which are not protected by a cell wall. Recent studies also demonstrated an effect of rhamnolipid in the reduction of mycelia growth of *Pythium myriotylum* and *Botrytis cinerea*. These data suggest that rhamnolipid may also have an adverse effect on cell structures that are protected by a cell wall. Properties of RLs against the algae *Heterosigma akashiwo*, viruses, amoeba like *Dictyostelium discoideum* and mycoplasma have also been reported. However, rhamnolipid applications have no significant effects on yeasts. In addition to

their in vitro antimicrobial activity, RLs have proven to be also efficient in in vivo plant systems. Treatments with RLs have been shown to protect pepper plants from Phytophthora blight disease and also prevent the development of Colletotrichum orbiculare infection on leaves of cucumber plants. However, rhamnolipid applications have no significant effects on yeasts. In addition to their in vitro antimicrobial activity, RLs have proven to be also efficient in in vivo plant systems. Treatments with RLs have been shown to protect pepper plants from Phytophthora blight disease and also prevent the development of Colletotrichum orbiculare infection on leaves of cucumber plants. Yoo et al. 2001 investigated RLs as alternative antifungal agents against typical plant pathogenic oomycetes, including *Phytophthora sp*. and *Pythium sp*. They showed that RLs significantly decrease the incidence of water-borne damping-off disease. Sharma et al., 2000 obtained similar results in field trials on chili pepper and tomato. Using bacterial mutants, clearly showed that phenazine and RLs interact in the biological control of soil-borne diseases caused by *Pythium spp*. Recent studies also demonstrated that a combination mixture of SRE (Syringomycin E) and rhamnolipid is efficient against pathogenic and opportunistic fungi recovered from diseased grape.

2.7.2. ***RHAMNOLIPID IN PLANT AND ANIMAL IMMUNITY***

During the last decade, pattern recognition emerged as a fundamental process in the immune response of plants and animals. Perception by pattern recognition receptors (PRRs) of molecular signatures that identify whole classes of microbes but are absent from the host allows this nonself recognition. Once recognized, these molecular signatures, conventionally named microbe-associated molecular patterns (MAMPs), trigger complex signaling pathways leading to transcriptional activation of defense-related genes. In mammals, MAMP perception leads to the inflammatory response with the production of cytokines including interleukins and the tumor necrosis factor (TNF). Years ago, lipopeptides were shown to stimulate human innate immune responses through the PRR Toll-like receptor TLR2 perception, by activating the transcriptional activator of multiple host defense genes NFkB, the production of interleukin (IL)-12 and the respiratory burst. Lipopeptides are also involved in the stimulation of innate immunity in plants. It is quite recent that RLs have been shown to be involved in triggering plant and animal defense responses and can be described as a new class.

2.7.3 ***RHAMNOLIPID AS STIMULATOR OF HUMAN AND ANIMAL IMMUNITY***

RLs have been long known as exotoxins produced by the human pathogen *P. aeruginosa* and several recent papers have highlighted their role in the stimulation of innate immunity in animal cells. The heat-stable Rha-Rha-C14-C14 produced by *Burkholderia plantarii* and some synthetic derivatives have been particularly studied. Rha-Rha-C14-C14 is structurally quite similar to the RL exotoxin from *P. aeruginosa* and identical to the RL of *Burkholderia pseudomallei*, the causative agent of melioidosis, an infectious disease of humans and animals leading to skin infection, lung nodules and pneumonia. This RL exhibits strong stimulatory activity on human mononuclear cells to produce TNF, a pleiotropic inflammatory cytokine. Such a property has not been noted so far for RL exotoxins but only for the lipopolysaccharide (LPS) bacterial endotoxins. Like LPS, the cell stimulating activity of this RL could be inhibited by incubation with polymyxin B. Interestingly, immune cell activation by Rha-Rha-C14-C14 does not occur via receptors that are involved in LPS (TLR4) or lipopeptide signaling (TLR2). Synthetic RLs derived from B. plantarii Rha-Rha-C14-C14 were also analyzed for their immune cell activation. These synthetic RLs differ by variations in the length, stereochemistry, number of lipid chains, number of rhamnoses and the occurrence of charged or neutral groups. The authors also compared these synthetic RLs to the well-characterized LPS MAMP from *Salmonella minnesota*. Immunostimulatory properties of RLs were monitored by assaying the secretion of TNF and the induction of chemi luminescence in monocytes. Howe et al. 2000 found that biological test systems showed large variations, depending on particular chemical structures and physicochemical parameters. LPS were, however, more efficient to induce luminescence and TNF production than the RLs tested. Furthermore, they found that biologically inactive RLs with lamellar aggregate structures antagonize the induced activity in a way similar to lipid A-derived antagonists of LPS. An extended study on structure-activity relationships of synthetic RLs derivatives also indicated a specific, recognition-based mode of action, with small structural variations in the RLs resulting in strong effects on the immune stimulatory activities. RLs also stimulated the release of interleukin IL-8, granulocyte-macrophage colony-stimulating factor, and IL-6 from nasal epithelial cells at non-cytotoxic levels. Interestingly, it was recently demonstrated that RLs could also potentiate the recognition of other MAMPs by the human innate immune system. Several MAMPs of *P. aeruginosa* are known to activate the innate immune system in epithelial cells, particularly the production of antimicrobial peptides such as

the human beta-defensin-2 (hBD-2) and proinflammatory cytokines such as interleukin (IL)-8. In this study, RLs were found to interact with the well-known MAMP flagellin. The authors provide evidence that RLs are responsible for the release of flagellin from the flagella. Their findings indicate that upon adhesion to surfaces, *P. aeruginosa* may alter the outer membrane composition in an RL-dependent manner, thereby shedding flagellin from the flagella. In turn, epithelial cells recognize flagellin leading to synthesis of anti-microbial peptides as well as recruitment of inflammatory cells by induction of proinflammatory cytokines.

2.7.4 ***RHAMNOLIPID AS STIMULATORS OF PLANT IMMUNITY***

Rhamnolipids have very recently been characterized as new MAMPs involved in non-specific immunity in plants. They have been also shown to induce resistance in plants, which is effective against a broad range of pathogens. It is demonstrated that Rha-C10-C10 and Rha-Rha-C10-C10 from *P. aeruginosa* and Rha-Rha-C14-C14 from *B. plantarii* trigger strong defense responses in grapevine including early events of cell signaling like Ca^{2+} influx, reactive oxygen species (ROS) production and MAP kinase activation. These RLs also induce a large battery of defense genes including some pathogenesis-related protein genes and genes involved in oxylipins and phytoalexins biosynthesis pathways. Interestingly, depending on the concentrations tested, RLs were able to activate a programmed cell death reminiscent of animal apoptosis. It was also demonstrated that RLs potentiate defense responses induced by other elicitors (i.e., chitosan and a culture filtrate of the fungus B. cinerea). Another novel role of RLs consists in protecting grapevine against the necrotropic pathogen *B. cinerea*. RLs are also active in other plant species. They are able to stimulate defense genes in tobacco, wheat and Arabidopsis thaliana. RLs are also potent protectors in monocotyledonous plants against biotrophic fungi. To date, it is not known whether the perception of RLs requires specific receptors in the plant plasma membrane. Interestingly, lipopeptide biosurfactants, which are lipid derivatives with similar properties to RLs, have also been described as potent MAMP elicitors. Surfactin, the most studied cyclic lipopeptide from Bacillus subtilis, has been shown to trigger early signaling events and late defense responses in tobacco cell suspensions. As for RLs, it is yet unclear whether the induction of defense responses by lipopeptides requires specific receptors in the plant plasma membrane. An alternative hypothesis is that lipopeptides could induce defense responses by membrane disturbance and this could also be the case for RLs.

2.7.5 ***POTENTIAL OF RHAMNOLIPIDS IN AGRICULTURE AND BIOMEDICAL FIELD***

Major breakthroughs allowing production, separation and purification of RLs in industrial quantities and laboratory purities have allowed the application of these molecules in different fields from cosmetic to industrial and more recently from agriculture to medicine. As previously stated, the major advantage of using rhamnolipid biosurfactants, which have diverse roles in plant and animal systems, is that they are natural and organic biodegradable compounds, originating from a large number of bacteria. RLs have also been proposed to be used in food industry applications. RLs have a direct biocide action on bacteria and fungi. They also increase the susceptibility of certain Gram-positive bacteria to specific antibiotics. RLs have been demonstrated to control zoosporic pathogens through lysis of their zoospores. Clinical trials using RLs for the treatment of psoriasis, lichen planus, neurodermatitis and human burn wound healing have confirmed excellent ameliorative effects of RLs when compared to conventional therapy using corticosteroids. RLs also display differential effects on human keratinocyte and fibroblast cultures. The advantages of these biosurfactants are low irritancy and even anti-irritating effects, as well as compatibility with human skin. Moreover, RLs have permeabilizing effects on Gram-positive and Gram-negative human bacterial strains, reinforcing their potential in biomedicine. An important issue to be taken into account is the study of side effects of biosurfactants on plants and animals. Attention should be paid while using surfactants on plants as the latter could be affected in many different ways. Parameters like negative impact on crop yield or other important agronomical traits should not be neglected and should be studied in parallel to avoid any impact on plant growth or metabolism, while boosting plant immunity. For instance, it is known that high concentrations of RLs cause necrosis in plants. Dose/response experiments in the field are a necessity in order to ensure use of non-toxic concentrations of RLs. In addition, in animal systems, RLs are known as virulence factors especially for immune compromised patients and individuals suffering from cystic fibrosis (CF). At some concentrations, RLs also have hemolytic activity. Thus, care should be taken in the use of RLs, albeit some applications such as fungicide and bactericide are already considered especially for skin treatments.

2.8 ***ALTRERNATIVES TO P.aeruginosa FOR RHAMNOLIPID PRODUCTION***

As *p.aeruginosa* is opportunistic pathogen, so to adopt heterologous production of rhamnolipid brings two major advantages as compared to the production with *P. aeruginosa*. The first is the increment in safety during handling large amounts of culture broths. The second is the possibility of constitutive rhamnolipid production inspite of the very tightly regulated production in *P. aeruginosa*. (Ochsner et al., 1995) cloned the *rhlAB* rhamnosyltransferase gene into various hosts like *Pseudomonas fluorescens, Pseudomonas oleovorans, Pseudomonas putida*, and *E. coli*. The best rhamnolipid production was 60 mg/L that was achieved with *P. putida*. Rhamnolipid producing species belong to the closely related genera *Pseudomonas* and *Burkholderia* in the phylum proteobacteria (Walter et al., 2010). The genus *Burkholderia* arose from the genus *Pseudomonas* and in 1992 it was classified as a new genus based on 16S rRNA sequence analysis (Yabuuchi et al. 1992). The bacterial isolates such as *Acinetobacter calcoaceticus, Enterobactersp* (Rooney et al. 2009), *Pseudoxanthomonas sp*. (Nayak et al., 2009), *Pantoea sp*. (Vasileva-Tonkova and Gesheva 2007; Rooney et al., 2009), *Renibacterium salmoninarum* (Christova et al., 2004), *Cellulomonascellulans* (Arino et al., 1998b), *Nocardioides sp.* (Vasileva-Tonkova and Gesheva 2005), and *Tetragenococcus koreensis* (Lee et al., 2005) have been reported to produce rhamnolipid. The major non-pathogenic rhamnolipid producers from the genus *Pseudomonas* are *P. chlororaphis* (Gunther et al., 2005), *P. alcaligenes* (Oliveira et al., 2009) and *P. putida* (Tuleva et al., 2002; Martinez Toledo et al., 2006) and from the genus *Burkholderia* are *B. glumae* (Pajarron et al., 1993), *B. plantarii* (Andra et al., 2006), and *B. thailandensis* (Dubeau et al., 2009).

Chapter 3: Objectives

3.1 Isolation and screening for PAHs utilizing *Pseudomonas*

3.2 Preliminary screening for biosurfactant

3.3 Characterization of Isolates.

3.4 Amplification of rhamnolipid synthesizing gene in *Pseudomonas*

3.5 Extraction and qualitative analysis of biosurfactant

Chapter 4: Material and Method

4.1 Isolation and screening for PAHs utilizing bacteria

4.1.1. Study site and sample collection

Water was collected from Bhitarkanika, Paradeep port and Rushikulya estuary of Orissa. Samples were collected in sterile phycol tubes, stored on ice and transferred to the laboratory immediately. Prior to enrichment and screening.

4.1.2. Culture media, chemical and Strains:

- **Bushnell Haas Media (BHM):** (composition per liter): $MgSO_4$ - 0.20g, $CaCl_2$-0.02g, K_3PO_4 - 1.00g , K_2HPO_4 – 1g, NH_4NO_3 – 1g, $FeCl_3$ -0.05g, pH- 7.2.
- **Seawater Nutrient agar medium (SWNA):** (composition per liter): NaCl- 15g, Peptone- 5g, Beef extract- 3g and 1.5% Agar, pH- 7.2.
- **Luria–Bertani (LB) medium**: (composition per liter): NaCl- 10g, Peptone- 10g, Yeast extract- 5g, pH 7.
- **Stock solution of PAHs:** 20 mg/ml stock solution of PAHs (Napthalene, Phenanthrene and Pyrene, AR, Sigma Aldrich) was prepared in acetone.
- **BHM- PAHs agar plates:** To solidified BHM agar plates (25ml), 0.5 ml of PAH solution was poured forming a uniform layer of PAH over agar surface after complete evaporation of acetone.
- ***Pseudomonas Agar*:** (per lit.)Enzymatic digest of gelatin – 20mg, $MgCl_2$ - 1.4g, K_2SO_4 - 10g, Agar - 13.6g.
- ***Pseudomonas Agar base :*** (per lit.)Casein enzymichydrolysate - 10g, Pancreatic digest of gelatin - 16g,K_2SO_4 - 10g, $MgCl_2$ -1.4g, Agar - 11g, pH 7.2.
- ***Macconkey's Agar :*** Bile salts- 1.5 g, Crystal violet dye - 0.001g, Lactose- 10.0g, Neutral pH red indicator- 0.03g and Peptone- 3.0g.
- ***Strains:****Pseudomonas aeruginosa* ATCC *15442 and Pseudomonas aeruginosa* PAO1

4.1.3. Isolation and screening

1 ml of water sample was transferred to 100 ml of BHM (Bushnell haas medium) supplemented with 100 mg /L of Pyrene, Phenanthrene and Naphthalene.For further enrichment of PAHs utilizing consortia, after 15 days, 1ml inoculum was transferred to 100 ml BSM supplemented with 200 mg/l of PAHs. After 15 days it was serially diluted (10^{-1}, 10^{-2} and 10^{-3}) and 100 µl aliquot was spread out on SWNA late supplemented with 50 mg/l of PAHs (to keep the isolates under PAHs stress). Isolated colonies with distinct morphology were streaked on BSM-PAHs agar plates of Pyrene, Phenanthrene and Naphthalene. Plates were incubated at 37^{o}C and growth was monitored for 3-7 days. Isolates were further screened for biosurfactant..

4.2. Preliminary screening for biosurfactant

4.2.1. Drop collapse assay

The drop collapsing method is based on the principle that a drop of a culture containing a rhamnolipid biosurfactant will collapse and spread completely over the surface of immersion oil. The polystyrene coated glass plate was taken and immersion oil was spreaded over it. Then bacterial culture grown in MMMF broth was dropped (2 µl) over immersion oil coated polystyrene glass plate one by one. After 1 min, the shape of the drop on the surface of the immersion oil was observed. The bacterial cultures that gives flat drops after 1 minute with scoring system ranging from '+' to '+ + + +' indicating partial to complete spreading on immersion oil surface indicates the ability of rhamnolipidbiosurfactant production of the cultures and those cultures that give '-' indicates the non-capability of biosurfactant synthesis by the bacterial culture (Jain et al ., 1991).

4.2.2. Emulsification assay

The emulsifying activity of biosurfactant was determined according to (Cooper and Goldenberg, 1987). About 2 ml of Luria bertani broth (LB) containing 10mM of $MgCl_2$ grown bacterial culture was added into the test tubes. After that 1 ml of n-octane was added into each test tube. Then test tube containing bacterial culture was vortex for 2 min. After that heights in the test tubes were measured with scale just after vortex. And again after 24 h, height was measured to test emulsifying activity. Emulsifying activity was expressed as the percentage of

the total height occupied by the emulsion in the test tube. The mathematical equation used to measure emulsification index is as follows. Emulsification index is denoted as E_{24}%

Emulsification index E_{24} % = $\frac{\text{HEIGHT OF THE EMULSION LAYER}}{\text{TOTAL HEIGHT}} X\ 100$

4.2.3. Oil spread assay

The overnight grown bacterial culture in LB broth (Luria bertani broth, himedia) was taken as a source for performing the oil spread technique. About 20 ml water was taken in petridish and about 200 µl immersion oil was put over the water. Then 20 µl bacterial culture was put over the immersion oil. It was found that rhamnolipid producing bacterial culture spread over the oil and produced clear zone.

4.2.4. Growth kinetics

The bacterial strains were inoculated in Luria bertani broth and kept in the incubator shaker for 32 h, in order to monitor the growth at 595 nm absorbance in spectrophotometer.

4.3. Cell surface hydrophobicity(math assay)

Cell-surface hydrophobicity and cell surface charge of bacteria is recognized as measurable physicochemical variable for evaluating bacterial adhesion to surfaces. About 10 ml culture was taken and pellet was collected and then resuspended with 5 ml distilled water. Then, absorbance was measured at 595 nm. After that, 300 µl n-octane was mixed with 3 ml culture and left for 15 min. Again after 15 min, absorbance was measured.

Cell surface hydrophobicity = $1- A_F/A_O$

4.4. TENTATIVE SELECTION FOR PSEUDOMONAS SPECIES

4.4.1 Growth on pseudomonas agar and pseudomonas agar base–

According to the protocol of Bergy's manual of microbiology, about 3.8 g of *pseudomonas* agar and 1ml glycerol was added in 100ml distilled water. Similarly, about 4.8 *pseudomonas* agar base and 1 ml glycerol was added in 100 ml distilled water. The bacterial isolates of similar morphology and colony forming units were sub cultured on *Pseudomonas* agar and *pseudomonas* agar base separately. The species of *Pseudomonas* are mainly identified by their capability of green fluorescent pigment formation and *Pyocyanin* when grown on *Pseudomonas* agar, *pseudomonas* agar base and blood heamolysis. *Pseudomonas aeruginosa* ATCC (15442) was taken as positive control

4.5. Amplification of rhlI gene –

4.5.1. Inoculation of strains

Strains were inoculated in the Luria bertani media and left overnight for growth.

4.5.2. Preparation of lysate

- About 400 µl culture of each bacterial strain was taken in the eppendroff or centrifuge tube.

- Then eppendroff tube was placed under centrifugation at 6000 rpm for 10 min at 10°c.

- After that supernatant was discarded from each eppendroff tube and pellets was resuspended in 300 µl of miliQwater (autoclaved).

- Then eppendroff tube was placed in waterbath (100°c) for 10 min.

- Then, transferred into the icebath for 5 min.

- Then, again eppendroff tube was centrifuged for 6000 rpm at 4 °c for 10 min.

- Supernatant was used as lysate for PCR.

4.5.3. Preparation of reaction mixture

Master mix contains water, dNTPs, buffer, primers and Taq DNA Polymerase in a single tube, which can then be aliquoted into each individual tubes. After that $MgCl_2$ and template DNA solutions was added which is well described in Table 1. PCR was performed in a 25 µl reaction mixture containing 1x PCR buffer, 25 Mm $Mgcl_2$, 100pmol of each primer, 0.050 U/µL of DNA Taq polymerase, 2.5 % of DMSO.

TABLE1. Preparation of reaction mixture

REACTION MIX	MASTER MIX (6)	
WATER	10.5 µl	64.8 µl
BUFFER 10 X	2.5 µl	15 µl
MgCl2	1.5 µl	9 µl
dNTPS	0.6 µl	3.6 µl
Primer F'(rhlI)	1.5 µl	9 µl
Primer R'(rhlI)	1.5 µl	9 µl
DMSO	0.6 µl	3.6 µl
Taq polymerase	1µl	6µl

Primer *rhlI f*= 5'ttcatcctccttagtcttccc 3'

Primer *rhlI r*= 5'ttccagcgattcagagagc 3'

4.5.4. Methodology

- Genomic DNA was amplified in sterile PCR tubes with reaction volume of 25µl.

- Then, these tubes were placed in the thermal cycler and amplification was carried out.

- The PCR products were then stored at 4ºC for further use in future.

TABLE 2.**PCR CONDITIONS**

		TEMPERATURE	TIME
	Initial denaturation	95°C	5 min
30 CYCLES	Denaturation	94°C	3 min
	Annealing	57°C	1 min
	Extension	72°C	1 min
	Final extension	72°C	5min

4.5.5. Gel preparation

- In 40 ml TAE buffer, 0.4 g Agarose was taken.

- Heated for 1 min in oven for complete mixing

- 2 µl of EtBr (Ethidium bromide) was added

- Pour
- Left untill gel get dryed.
- 10 µl sample and 2 µl loading dye was added in each well of the gel.

4.6 .Extraction of biosurfactant

- Isolates were grown in 10ml Luria bertani broth and incubated at 37°C for 12 h.
- Bacterial cell mass was harvested by centrifugation at 6000 rpm/RT/10 min.

- Bacterial supernatant was discarded and remaining pellet was transferred to 50 ml of Bushnell has media containing 100 mg/L of phenanthrene as carbon source.
- It was incubated at 37 °C/dark for 7 days.
- Growth was monitored at regular interval in terms of absorbance at 595 nm.
- After 7 days incubation, the whole culture was centrifuged at 6000 rpm/RT/10 min.
- The culture supernatant was collected and pH was adjusted at 2 by using 2M HCl. Using chloroform : methanol (2: 1), biosurfactant was precipitated.
- It was kept at 4°C undisturbed for 12 to 18 hours.
- Biosurfactant was collected as a precipitating layer between aqueous and organic phase.
- It was dried at 40°C and used for FTIR analysis.
- 2 mg of dried extracted crude biosurfactant was mixed with 200 mg of KBr and it was palletized. IR spectra was collected in between 600 nm and 4000 nm.

4.7 Biochemical assay

4.7.1 Gram staining

Gram staining technique was performed to get the preliminary idea about the rhamnolipid producing bacteria. First the slide was heat fixed, than the bacterial smear was flodded with crystal violet solution and left for 1 min. Then, crystal violet was washed with distilled water. After that, iodine solution was added and left for 1 min. Then again slide was rinsed off with distilled water. After that, decolorizer was added and it was allowed to remin for 1 to 5 seconds. Then, it was rinsed off with distilled water. After that, safranin was added and was allowed to remain in the slide for 30 seconds. Again safranin was washed out with distilled water and lastly slide containing bacterial smear was observed under microscope.

4.7.2. Macconkey agar test

It is used for the isolation and differentiation of Gram-negative enteric bacilli from the positive ones. Enzymatic Digest of Gelatin, Enzymatic Digest of Casein, and

Enzymatic Digest of Animal Tissue are the nitrogen as well as vitamin sources in MacConkey Agar. Lactose is the fermentable carbohydrate in it. During Lactose fermentation, a local pH drop around the colony causes a color change in the pH indicator, Neutral Red, and bile precipitation. Bile Salts Mixture and Crystal Violet are the selective agents inhibiting Gram-positive cocci and allowing Gram-negative organisms to grow. Sodium Chloride maintains the osmotic environment and agar is the solidifying agent present in macconkey agar.

4.7.3. Oxidase test

This oxidase test is a key test to differentiate between the families of *Pseudomonadaceae* (ox+) and *Enterobacteriaceae* (ox-) and is useful for differentiation and identification of several bacteria, those that have to use oxygen as the final electron acceptor in aerobic respiration. The enzyme cytochrome oxidase is involved with the utility of reduction of oxygen at the end of the electron transport chain. It was performed by adopting following methodology:-

- A good-sized amount of inoculum (already incubated and grown) from a plate culture or slant culture was taken and placed it on a piece of filter paper.
- One drop of the oxidase reagent was dropped.
  .A positive reaction will usually occur within 10-15 seconds, and will become blusish- purple.

4.7.4. SIM (Sulphur Reduction Indole Production Motility Test)

This test is used to identify the bacteria capable of reducing sulphur, capable of producing indole through the utility of tryptophanase and to detect the presence of flagella. The strains were inoculated into the SIM media and placed in incubator at 37°C for 48 hours. After that, 4 drops of Kovac's reagent was added. The development of black ppt indicates the sulphur reduction and formation of red colour indicates the indole production. The positive motility is observed when the growth is observed away from the line of inoculation.

4.7.5. Mannitol motility nitrate test

This test is used to identify that whether they are capable of mannitol fermentation and nitrate reduction. The positive result indicates the development of yellow colour which shows their capability of fermenting mannitol and after the addition of 4-5 drops of each sulphanilic acid and alpha naphthalene into the culture tubes, red colour develop due to nitrate reduction which shows positive result.

Chapter 5: Results

5.1. ISOLATION AND SCREENING

1 ml of water sample was transferred to 100 ml of BHM (Bushnell Haas medium) supplemented with 100 mg /L of Pyrene, Phenanthrene and Naphthalene. For further enrichment of PAHs utilizing consortia, after 15 days, 1ml inoculum was transferred to 100 ml BSM supplemented with 200 mg/l of PAHs. After 15 days it was serially diluted (10^{-1}, 10^{-2} and 10^{-3}) and 100 µl aliquot was spread out on SWNA lates supplemented with 50 mg/l of PAHs (to keep the isolates under PAHs stress). Isolated colonies with distinct morphology were streaked on BSM-PAHs agar plates of Pyrene, Phenanthrene and Naphthalene. Plates were incubated at 37°C. and then results were obtained of isolation and screening as mentioned in Table 3.

TABLE3. ISOLATION AND SCREENING OF ISOLATES

ISOLATES	**NAPHTHALENE**	**PHENANTHRENE**	**PYRENE**
NBH1	+	±	±
NBH2	+	+	+
NBH3	-		
NBH4	+	+	+
NBH5	-		
NBH6	+	+	+
NBH7	+	±	+
NBH8	+	-	±
N6P	+	+	+
NPD1	+	+	+
NPD2	+	+	+
NPD3	±	±	±
NPD4	±	±	-
NPD5	+	+	+
NPD6	+	+	+
NPD7	+	±	+
NPD8	±	-	±
NE3B02	+	+	+
*Pseudomonas sp.*NP202	+	+	+
Pseudomonas pseudoalcaligenis NP103	+	+	+
Pseudomonas mendocina NR802	+	+	+

+ : Satisfactory, ± : Variable, - : Negative

5.2. Preliminary screening for biosurfactant

5.2.1. Drops collapse assay

If the culture does not contain biosurfactant, the polar water molecules are repelled from the hydrophobic surface and the drops remain stable. If the culture contains surfactant, the drops spread or even collapse because the force or interfacial tension between liquid droplet and the hydrophobic surface is reduced as shown in fig.2. Drop collapse assay was performed with different isolates and it was found that almost all the strains have positive response as mentioned in Table 4.

TABLE4. The results of preliminary screening assay i.e, drop collapse assay of isolates for biosurfactant is mentioned in Table4.

STRAINS	**RESULT**
Pseudomonas aeruginosa ATCC (15442)	+
NR802	+
NE3B02	+++
NP202	+
NP103	-
NE3B01	-
Pseudomonas sp. N6P	+++
NBH4	+++
NBH1	+++
Pseudomonas aeruginosa PAO1	+++

STRAINS	RESULT
NBH2	+
NBH3	+
NBH4	+
NBH5	+
NBH6	-
NBH7	-
NBH8	+
NPD1	+
NPD2	+
NPD3	+
NPD4	+
NPD5	+
NPD6	+
NPD7	-
NPD8	-

+ : PARTIAL SPREAD

+++ : COMPLETE SPREAD

- : NO SPREAD

1. The cultures that gives flat drops were scored POSITIVE **– ABLE TO PRODUCE RHAMNOLIPID**
2. The intact drops were scored NEGATIVE- **UNABLE TO PRODUCE RHAMNOLIPID**

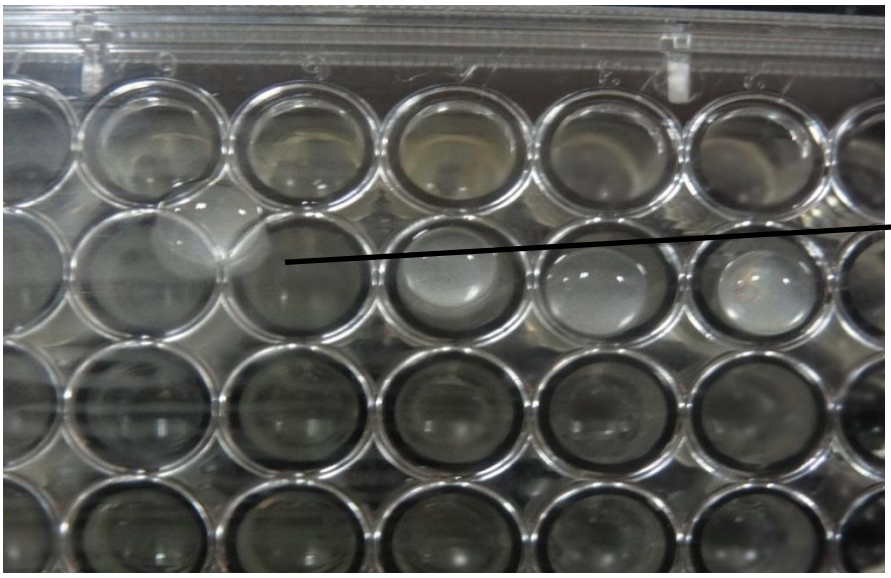


*Fig.**2. Drop collapse assay of Pseudomonas aeruginosa*** **ATCC 15442.** As flat droplet is appeared on immersion oil that means it is able to synthesize rhamnolipid.

5.2.2 . Emulsification assay

An emulsion is a mixture of two or more liquids that are normally immiscible. After putting the value of emulsification height and total height in the below mentioned formula i.e, the following results were obtained mentioned in Table5. It is found that emulsification ability of *Pseudomonas aeruginosa* ATCC 15442 and NE3B02 is better as compared to other isolates which indicates the presence of rhamnolipid as shown in fig.3.

EMULSIFICATION INDEX E_{24} = HEIGHT OF THE EMULSION LAYER X 100

TOTAL HEIGHT

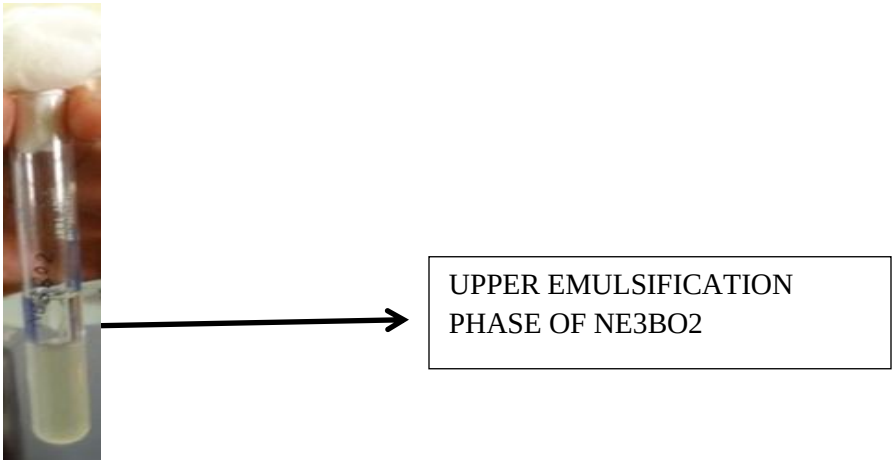


Fig.3. Emulsification assay of NE3B02

TABLE 5. The following results indicates that different isolates has different extent of emulsification activity. Emulsification results show that biosurfactant from *Pseudomonas aeruginosa* ATCC 15442 has good capability of emulsifying hydrocarbon source.

STRAINS	TOTAL HEIGHT	EMULSIFICATION HEIGHT	EMULSIFICATION INDEX
NE3B01	4.2 cm	1.6 cm	38.095 %
NP202	4.2 cm	0.9 cm	21.42 %
Pseudomonas pseudoalcaligenis NP103	3.9 cm	0.7	17.94 %
Pseudomonas aeruginosa ATCC 15442	4.0 cm	1.2 cm	30 %
Pseudomonas mendocina NR802	4.3 cm	1.5 cm	34.88 %
NE3B02			
Pseudomonas aeruginosa PAO1	4.0 cm	1.2 cm	30 %
N6P	4.2 cm	1.6 cm	38.095 %
NPD1	3.9 cm	0.7 cm	17.94 %
NPD2	3.9 cm	1.2 cm	30.76 %
NPD3	3.7 cm	0.5 cm	13.51 %
NPD4	3.8 cm	0.6 cm	17.94%
NPD5	3.7 cm	1.3 cm	35.13 %
NPD6	3.7 cm	1.2 cm	32.43 %
NPD7	3.5 cm	1.3 cm	37.14 %
NPD8	3.7 cm	0.5 cm	13.51 %
NBH1	3.7 cm	0.5 cm	13.51 %
NBH2	3.5 cm	0.5 cm	14.28 %
NBH3	3.5 cm	0.5 cm	14.28 %
NBH4	3.7 cm	0.9 cm	24.32 %
NBH5	3.5 cm	0.5 cm	14.28 %
NBH6	3.4 cm	1.1 cm	11.42 %
NBH7	3.5 cm	0.5 cm	14.28 %
NBH8	3.7 cm	1.3 cm	32.13%

5.2.3. Oil spread assay

The oil spread technique was performed on overnight grown bacterial culture in Luria bertani broth. 20 ml water was taken in petridish and about 200 µl immersion oil was put over the water. Then 20 µl bacterial culture was put over the immersion oil. It was found thatrhamnolipid producing isolate spread over the oil and produced clear zone. Almost all the isolates produced clear zone in maximum level as results are mentioned in Table6.**TABLE**6. RESULTS OF OIL SPREAD ASSAY

STRAINS	**RESULT**
Pseudomonas aeruginosa ATCC (15442)	+
Pseudomonas mendocina NR802	-
NE3B02	+
NE3B01	+
Pseudomonas sp.NP202	+
Pseudomonas pseudoalcaligenis NP103	+
N6P	+
NBH4	+
NBH1	+
Pseudomonas aeruginosa PAO1	+

+ :clear zone on the oil surface

-: Bacterial culture remain intact on the oil surface

5.2.4. Growth kinetics

The isolates were inoculated in Luria bertani broth and kept in the incubator shaker for 32 hours, in order to monitor the growth at 595nm absorbance in spectrophotometer. The growth kinetics of isolates named as *Pseudomonas aeruginosa* ATCC 15442, NP103, NR802, NE3B02, NP202, JV502, JB022 were observed for 32 hours and the results are mentioned in Table.7. Pseudomonas aeruginosa ATCC 15442 has log phase for longer time as shown in fig.4.

TABLE7. GROWTH KINETICS OF ISOLATES

STRAINS	1 hr	4 hr	10 hr	24 hr	26 hr	30 hr	32 hr
Pseudomonas aeruginosa ATCC 15442	0.0345A	0.141A	0.6155A	1.0945A	1.167A	1.2415A	1.2855A
NP103	0.0325A	0.2995A	0.7455A	1.077A	1.17A	1.1615A	1.153A
NR802	0.0325A	0.242A	0.785A	1.194A	1.294A	1.328A	1.3385A
NE3B02	0.033A	0.242A	0.695A	1.125A	1.3085A	1.23A	1.312A
NP202	0.041A	0.296A	0.576A	0.852A	0.9585A	1.027A	0.9725A
JV502	0.0305A	0.0495A	0.249A	0.9905A	1.241A	1.1045A	1.1085A
JB022	0.0295A	0.1005A	0.581A	1.3215A	1.511A	1.398A	1.437A

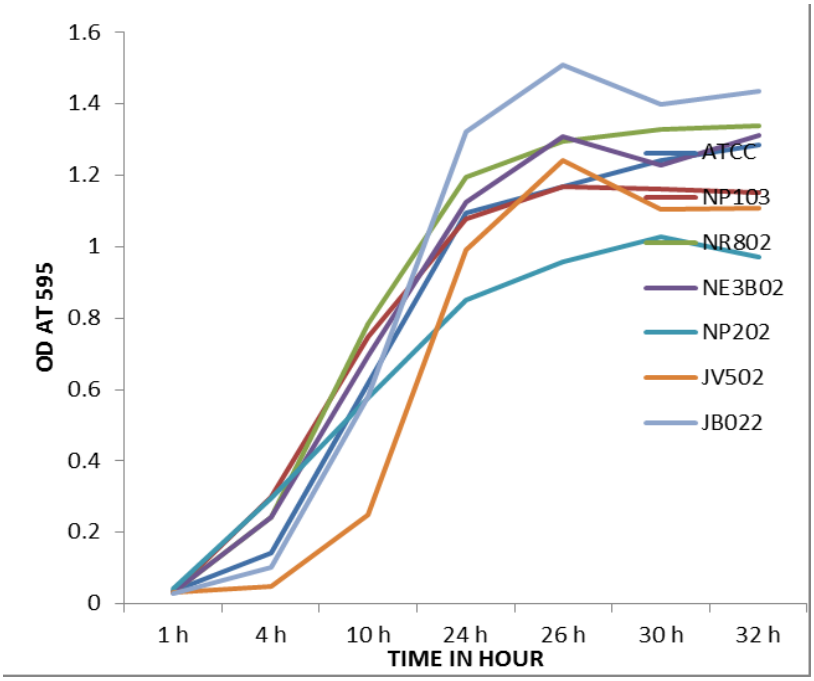


Fig.4. Growth of 7 isolates monitored at 595 nm. In comparison to all bacterial strains, the exponential phase or log phase of JV502, JB022 and *Pseudomonas aeruginosa* ATCC 15442 remain for longer time. *Pseudomonas sp.*NP202 is showing the slowest growth.

5.3. Cell surface hydrophobicity (math assay)

Cell-surface hydrophobicity and cell surface charge of bacteria is recognized as measurable physicochemical variable for evaluating bacterial adhesion to surfaces. The presence of rhamnolipid increases cell surface hydrophobicity. About 10 ml culture was taken and pellet was collected and then resuspended with 5 ml distilled water. Then, absorbance was measured at 595 nm. After that, 300 µl n-octane was mixed with 3 ml culture and left for 15 minutes. Again after 15 min, absorbance was measured. Then by using mathematical formula, cell surface hydrophobicity was measured and following observations were made as shown in fig.5.(A)

Cell surface hydrophobicity = $1- A_F/ A_i$

Where A_F= Final absorbance

A_i = Initial absorbance

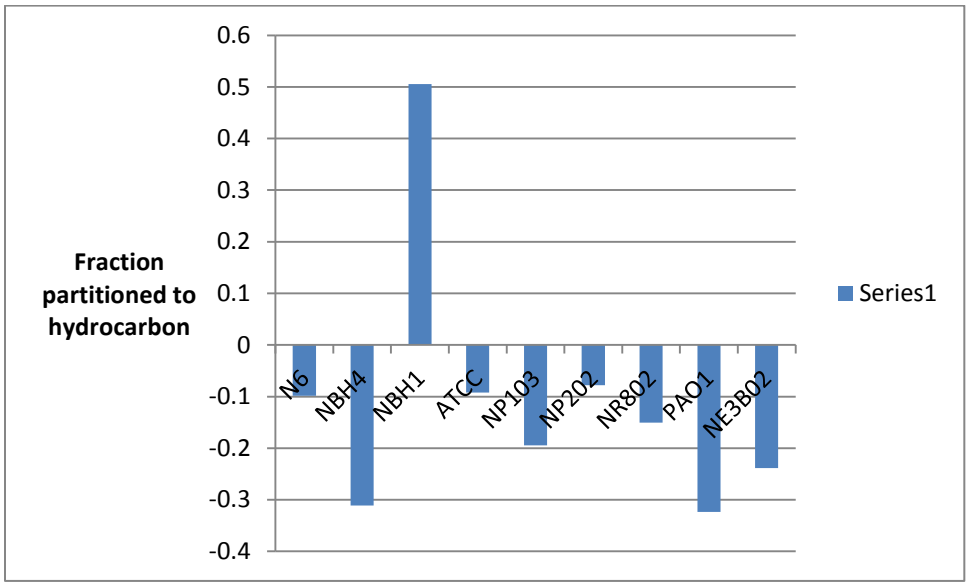


Fig.5.(A) Cell surface hydrophobicity of N6, NBH4, NBH1, *Pseudomonas aeruginosa* ATCC 15442, NP103, NP202, NR802, *Pseudomonas aeruginosa* PAO1, NE3B02

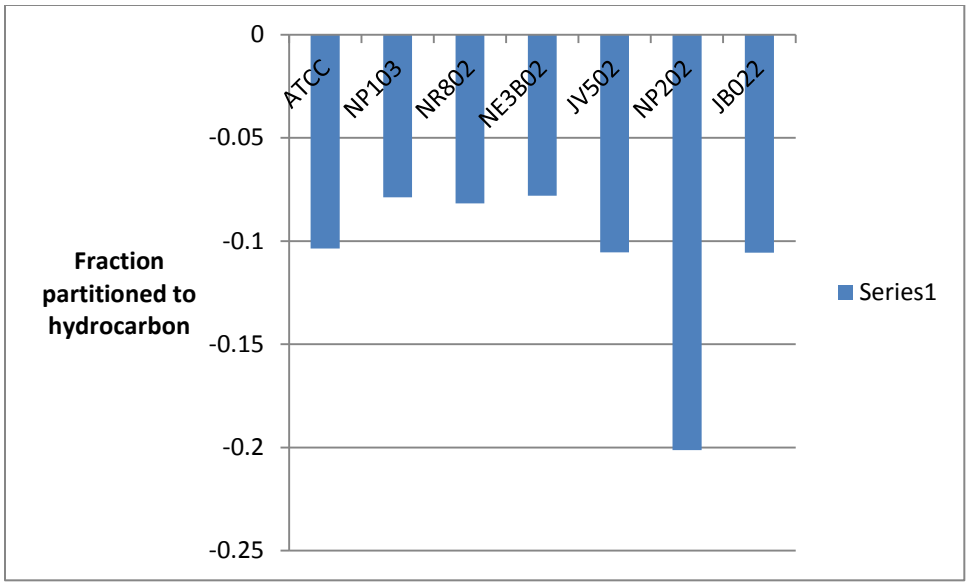
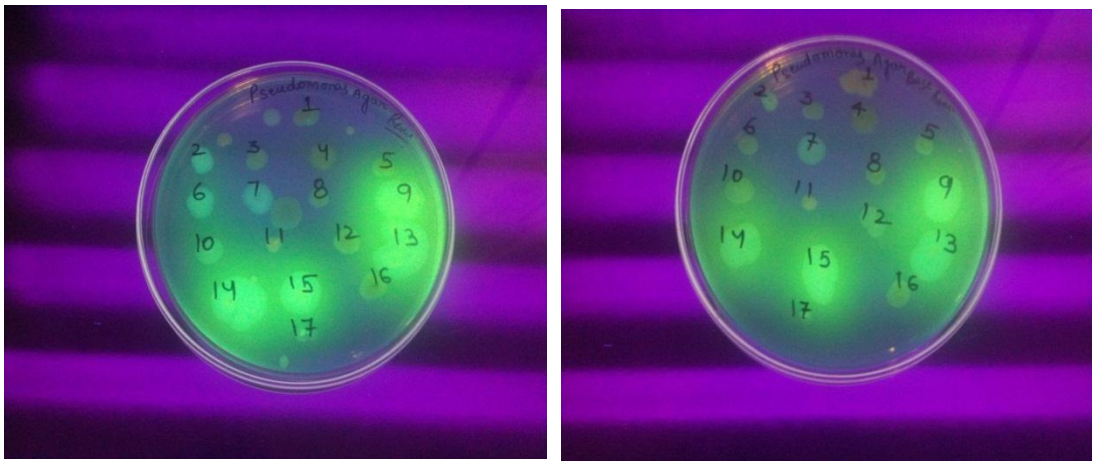


Fig.5.(B). Cell surface hydrophobicity in *Pseudomonas aeruginosa* ATCC 15442 is moderate as compare to other isolates.

5.4.1 Growth on pseudomonas agar and pseudomonas agar base

The bacterial isolates of similar morphology and colony forming units were sub cultured on *Pseudomonas* agar and pseudomonas agar base separately. The species of *Pseudomonas* are mainly identified by their capability of green fluorescent pigment formation as mentioned in Table8. when grown on *Pseudomonas* agar and *Pseudomonas* agar base as shown in fig.6. and fig.7.



A **B**

Fig.6 Growth on *Pseudomonas* agar (A) and *Pseudomonas* agar base (B). Isolates streaked on *Pseudomonas* agar and *Pseudomonas* agar base

1 – 8= Isoates are NBH1,NBH2,NBH3,NBH4,NBH5, NBH6, NBH7, NBH8

9-16 =Isolates are NPD1,NPD2, NPD3, NPD4, NPD5, NPD6, NPD7, NPD8

17= PAO1

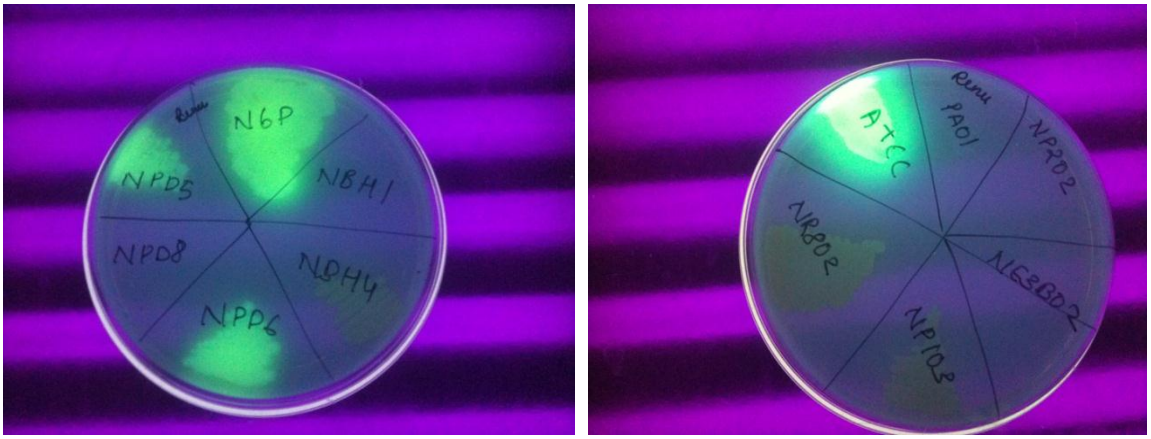


Fig.7. Pseudomonas aeruginosa* ATCC 15442 *taken as positive control

On the basis of the growth of isolates on *pseudomonas* agar and *pseudomonas* agar base(green fluorescent colour is observed), isolates named as N6 P, NPD5, NPD6are selected tentatively and then amplified with *rhlI* for the purpose of confirmation of their ability of synthesizing

rhamnolipid. On the basis of the presence of fluorescein pigment, the results of the tentative selection of isoltes are mentioned in Table8.

TABLE8. Growth on *Pseudomonas* agar and *Pseudomonas* agar base

STRAINS	**RESULT**
NBH1	-
NBH2	-
NBH3	-
NBH4	-
NBH5	-
NBH6	-
NBH7	-
NBH8	-
NPD1	+
NPD2	-
NPD3	-
NPD4	-
NPD5	+
NPD6	+
NPD7	+
NPD8	-
*Pseudomonas aeruginosa*ATCC 15442	+
*Pseudomonas aeruginosa*PAO1	-
*Pseudomonas sp.*NP202	-
Pseudomonas pseudoalcaligenis NP103	-
Pseudomonas mendonica NR802	-
NE3B02	-
N6P	+

Where + indicates Isolate give green fluorescence colour

_ indicates Isolate do not give green fluorescence colour

5.5. Amplification of rhlI gene

After the drying of gel, 10 µl sample and 2 µl loading dye was added in each well of the gel as shown in fig.8. and fig.9

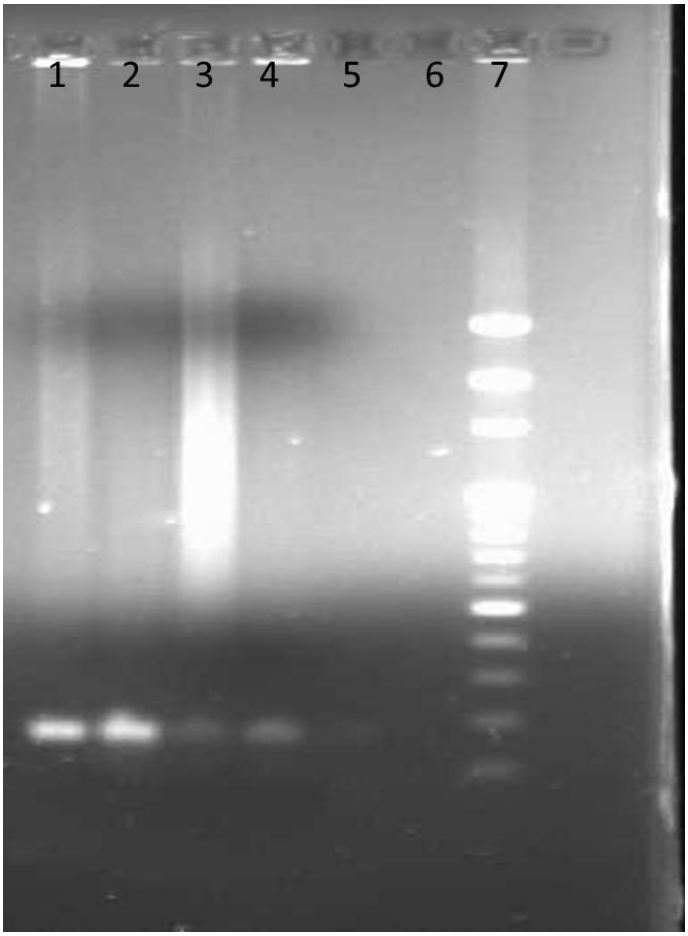


Fig.8. Gel photograph showing amplification of *rhlI*. Isolates in 7 lanes are

1- *Pseudomonas aeruginosa* ATCC 15442, 2- *Pseudomonas aeruginosa* PAO1, 3-NBH4, 4-N6, 5-NBH1, 6-NPD6 and 7- Ladder. The size of the amplified product is 155 bp.

Primer *rhlI-f* = 5’ ttcatcctcctttagtcttccc 3’

Primer *rhlI- r* = 5’ ttccagcgattcagagagc 3’

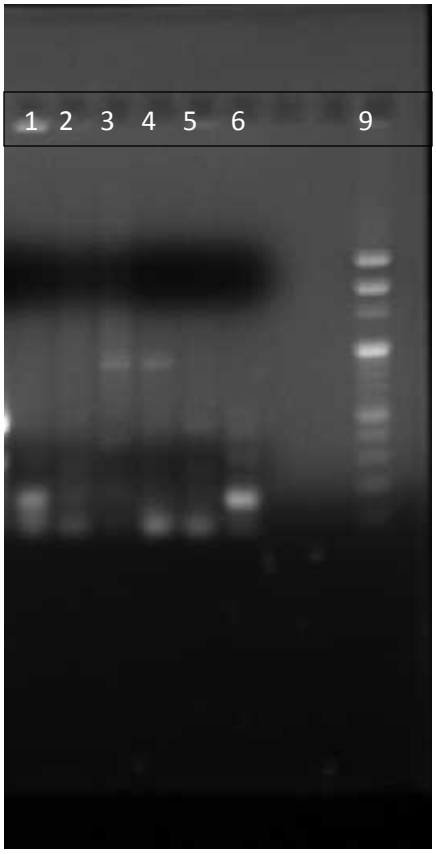


Fig.9. Gel photograph showing amplification of *rhlI*. Isolates in lanes are NBH1(1), NPD7(2), NPD5(3), NPD6(4), NPD1(5), PAO1(6) and ladder (9)

5.6 Extraction of biosurfactant

Pellet was formed from the extracted biosurfactant and then analysed under FTIR (Fourier Transform Infra-Red Rradiation) in the range of 600nm to 4000nm. Different functional groups were observed within the range of IR spectrum like Primary amine, secondary amine, Carboxylic group, ester etc.

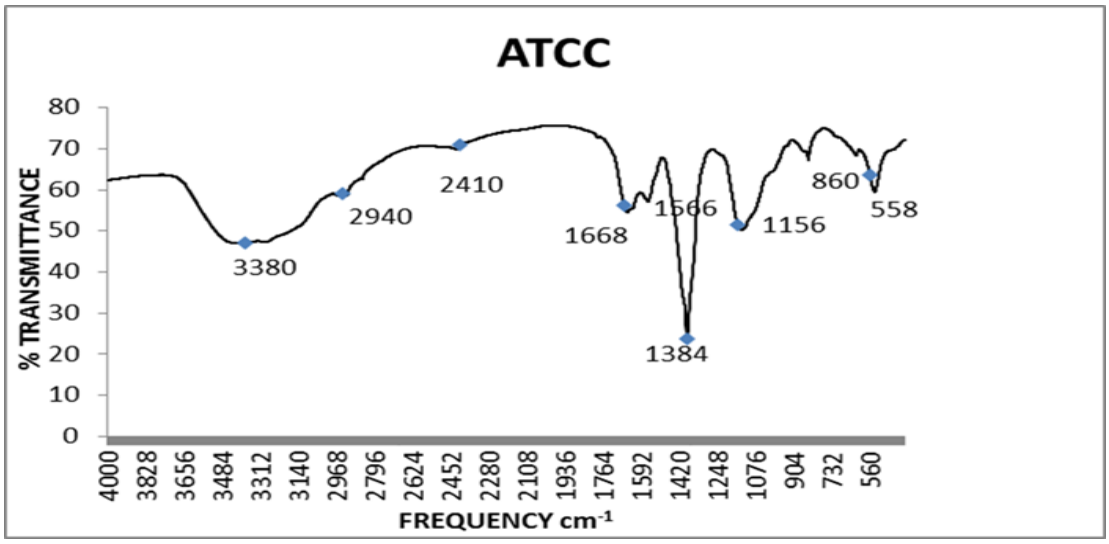


Fig.10. Fourier transform IR spectrum of *Pseudomonas aeruginosa* ATCC 15442

Functional group	ABSORPTION cm-1	Type of vibration
Phenol and alcohol	3380	Hydrogen bonded OH stretch
Alkanes	2940	H-C-H asymmetric stretch
Carboxylic acids	2410	OH stretch
Ethers	1156	C-O stretch
Nitro groups	1566	N=O stretch

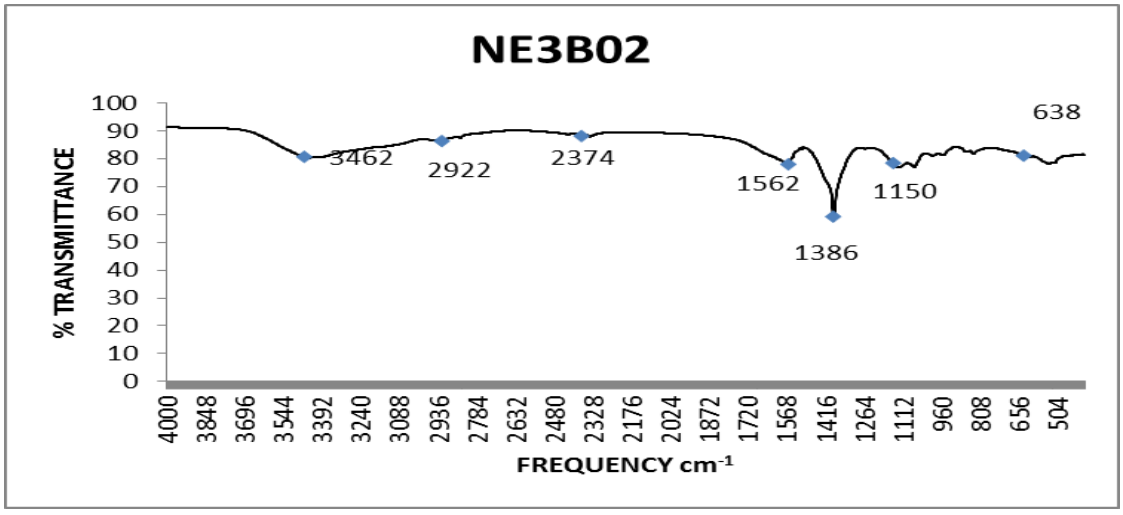


Fig.11. Fourier transform IR spectrum of NE3B02

Functional group	ABSORPTION cm-1	Type of vibration
Amines-primary	3462	N-H stretch
Alkanes	2922	H-C-H stretch
Alkanes	2374	H-C-H stretch
Amines-primary	1562	N-H bend
Nitro groups	1386	N=O group
Esters	1150	C-O stretch

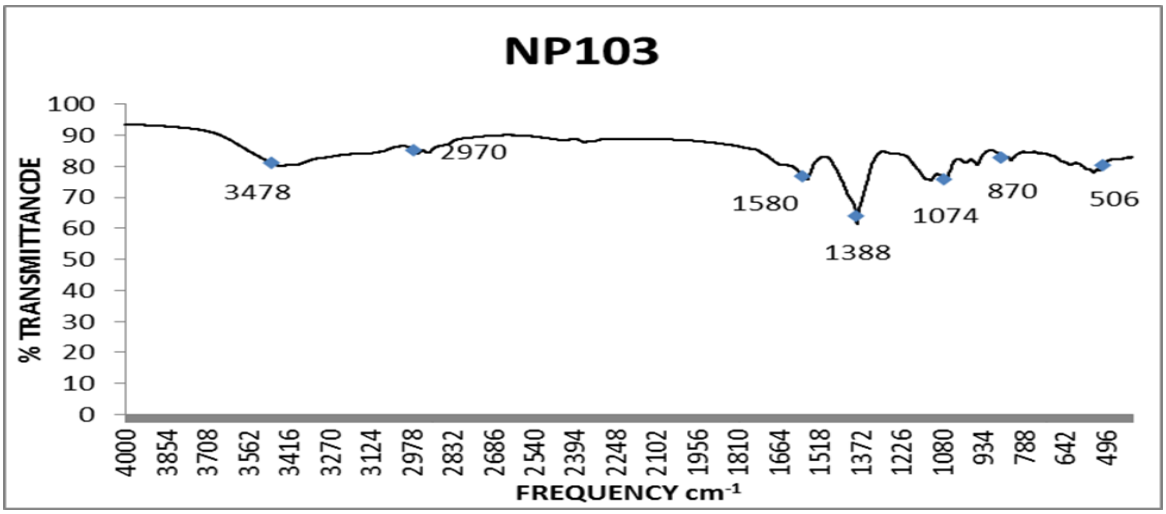


Fig.12. Fourier transform IR spectrum of *Pseudomonas pseudoalcaigenis* NP103

Functional group	ABSORPTION cm-1	Type of vibration
Phenol & alcohol	3478	Hydrogen O-H stretch
Alkanes	2970	H-C-H asymmetric stretch
Aromatic ring	1580	C-C=C symmetric
Nitro group	1388	N=O bend
Ester	1074	C=O stretch

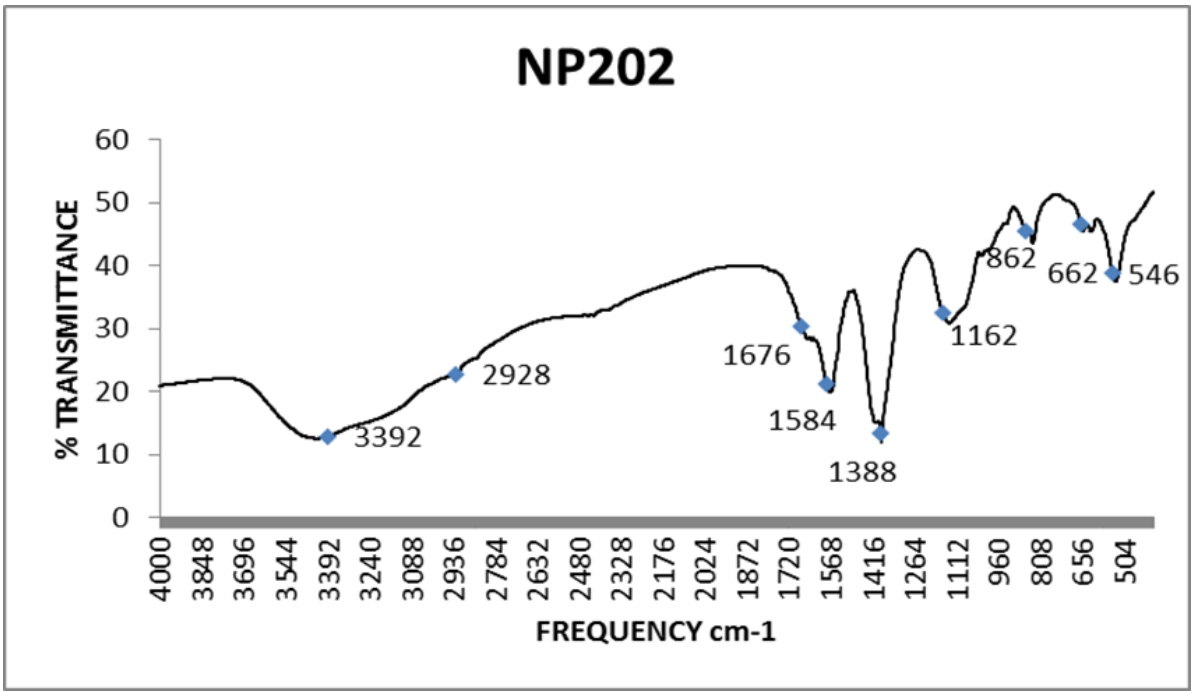


Fig.13. Fourier transform IR spectrum of *Pseudomonas sp.* NP202

Functional group	**ABSORPTION cm-1**	**Type of vibration**
Carboxylic acid	**3392**	**O-H stretch**
Alkanes	**2928**	**H-C-H asymmetric**
Alkenes	**1676**	**C-C=C symmetric**
Aromatic ring	**1584**	**C-C=C symmetric**
Nitro group	**1388**	**N=O bend**
Ethers	**1162**	**C-O stretch**

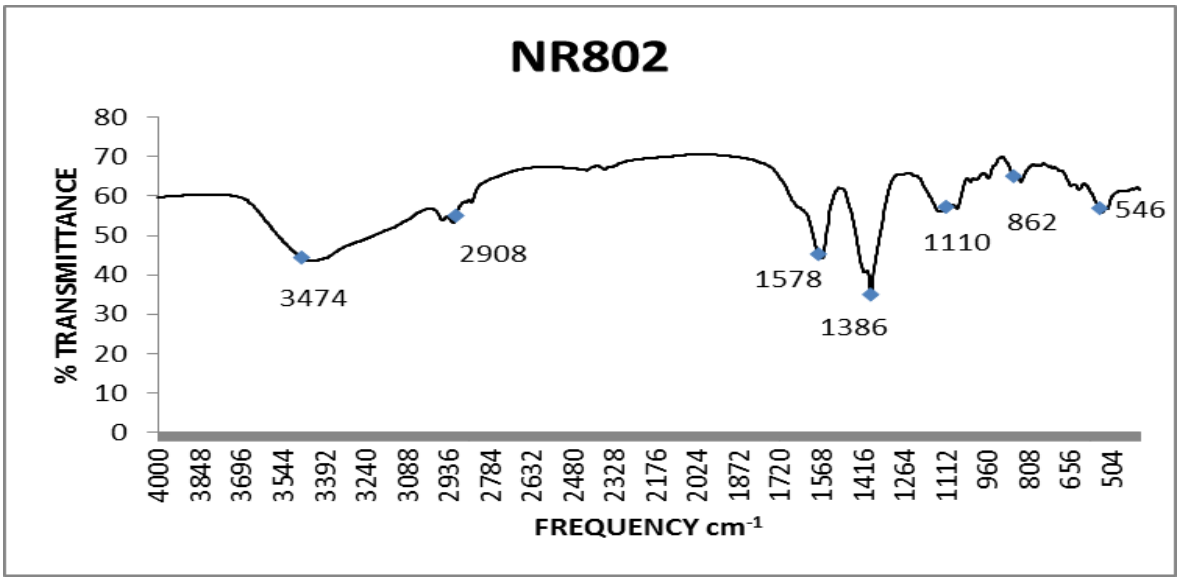


Fig.14. Fourier transform IR spectrum of *Pseudomonas mendonica* NR802

Functional group	**Absorption cm-1**	**Type of vibration**
Amines-secondary	**3474**	**N-H stretch**
Alkanes	**2908**	**H-C-H asymmetric & symmetric stretch**
Aromatic ring	**1578**	**C-C=C symmetric**
Nitro groups	**1386**	**N=O bend**
Ethers	**1110**	**C-O stretch**

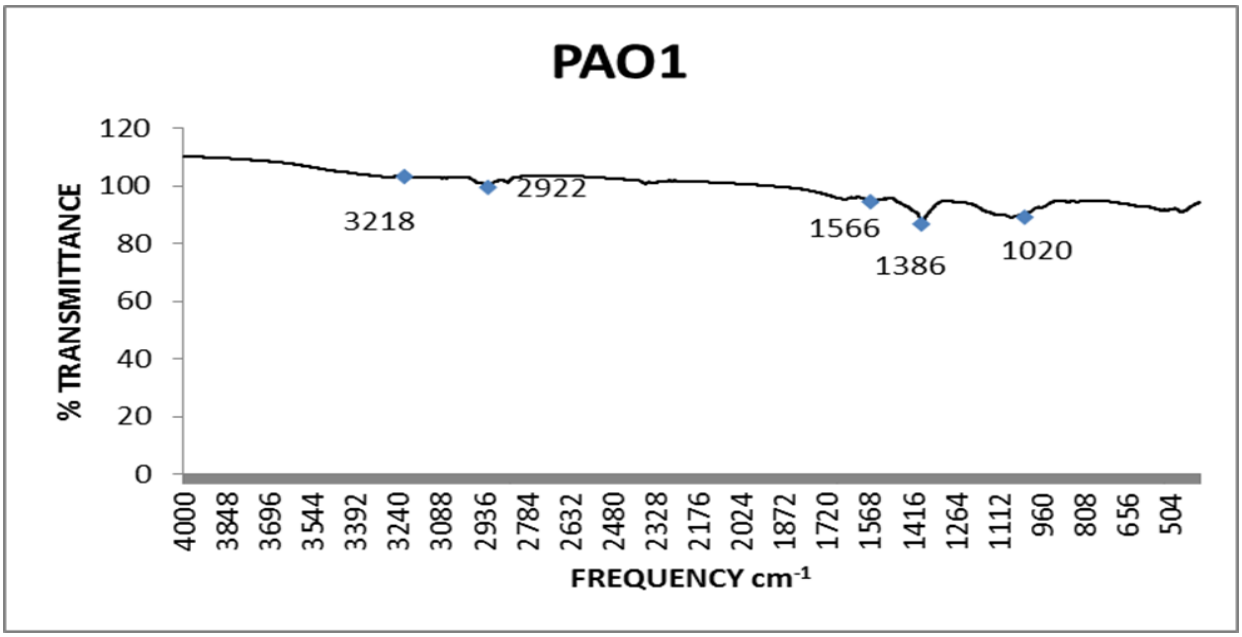


Fig.15.Fourier transformIR spectrum of *Pseudomonas aeruginosa* PAO1

Functional group	**Absorption cm-1**	**Type of vibration**
Alkynes	**3218**	**ΞC-H stretch**
Alkanes	**2922**	**H-C-H asymmetric & symmetric**
Nitro groups	**1566**	**N=O stretch**
Nitro groups	**1386**	**N=O bend**
Ethers	**1020**	**C-O stretch**

5.7. Biochemical assay

The physical characterization of isolates were done by different biochemical assay like gram staining, oxidase test, Macconkey agar test, oxidase test, SIM (Sulphur Reduction Indole Production Motility Test), Mannitol motility nitrate test . All the isolates show different morphological aspects in terms of Gram staining, color of colonies, motility, sulphur reduction, indole production and mannitol fermentation.

5.7.1 Gram Staining

All were Gram negative having rod shape as shown in fig.16. The results of gram staining is mentioned in Table9.

TABLE9. GRAM STAINING

STRAINS	RESULTS
NPD1	Gram negative
NPD2	Gram negative
NPD3	Gram negative
NPD4	Gram negative
NPD5	Gram negative
NPD6	Gram negative
NPD7	Gram negative
NPD8	Gram negative
Pseudomonas aeruginosa ATCC 15442	Gram negative
Pseudomonas aeruginosa PAO1	Gram negative
NP202	Gram negative
*Pseudomonas pseudoalcaligenis*NP103	Gram negative
NE3B02	Gram negative
Pseudomonas mendocina NR802	Gram negative
NBH1	Gram negative
NBH2	Gram negative
NBH3	Gram negative
NBH4	Gram negative
NBH5	Gram negative
NBH6	Gram negative
NBH7	Gram negative
NBH8	Gram negative

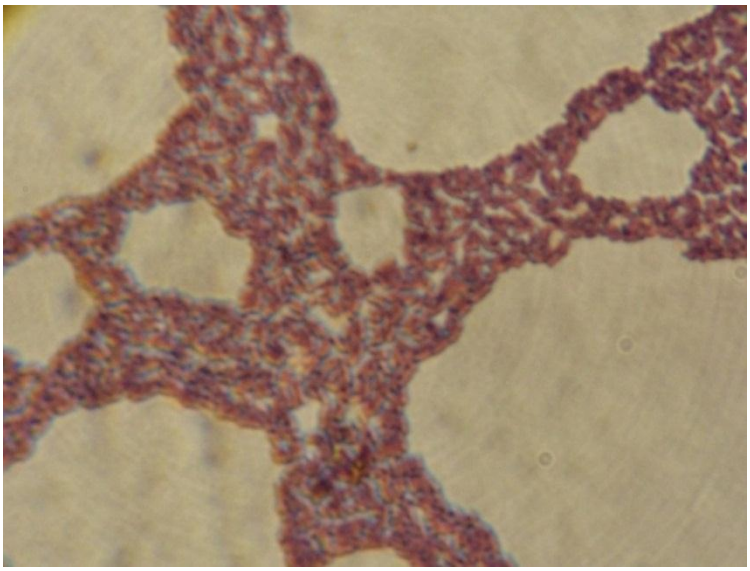


Fig.16. Gram Negative rod NPD1

5.7.2. Macconkey agar test

Macconkey agar test was performed for the isolation and differentiation of Gram-negative enteric bacilli from the positive ones. By utilizing the present component lactose in the medium, Lactose positive bacteria such as *Escherichia coli, Enterobacter and Klebsiella* produce acid in the medium, which lowers the pH of the agar below 6.8 and results in the appearance of red or pink colonies. The bile salts in the medium precipitate and promote the colony to become hazy. Non lactose fermenting bacteria such as *Pseudomonas aeruginosa* and *Shigella c*annot utilize lactose in the medium, and will use peptone instead. This results in the formation of ammonia, which raises the pH of the agar, and leads to the formation of white or colorless colonies in the plate as shown in fig.17.

Fig.17. Macconkey agar test. Appearance of white or colourless colonies.

5.7.3. Oxidase test

All the isolates show positive result (development of blue colour) as shown in fig.18. It means they use oxygen as the final electron acceptor in aerobic respiration and the enzyme cytochrome oxidase is involved with the utility of reduction of oxygen at the end of the electron transport chain and the results of remaining isolates were mentioned in Table10.

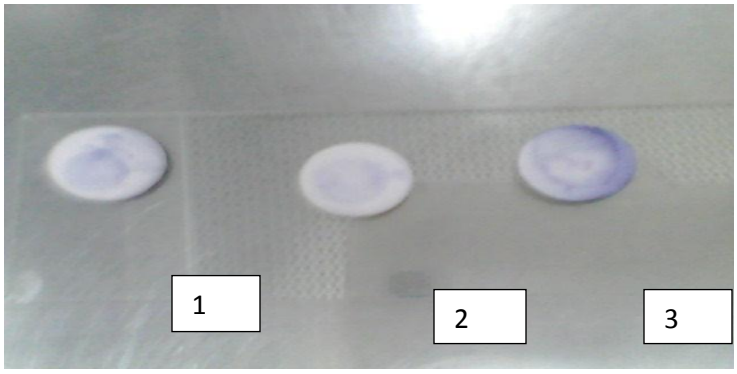


Fig.18.1- N6p, 2- *Pseudomonas aeruginosa* PAO1, 3- *Pseudomonas aeruginosa ATCC 15442*. Inoculum on a piece of filter paper

TABLE. 10 OXIDASE TEST

STRAINS	RESULT
Pseudomonas aeruginosa ATCC 15442	+ Blue
Pseudomonas aeruginosa PAO1	+ Blue
N6	+ Blue
NE3B02	+ Blue
Pseudomonas pseudoalcaligenis NP103	+ Blue
Pseudomonas species NP202	+ Blue
Pseudomonas mendocina NR802	+ Blue
NPD1	+ Blue
NPD2	+ Blue
NPD3	+ Blue
NPD4	+ Blue
NPD5	+ Blue
NPD6	+ Blue
NPD7	+ Blue
NPD8	+ Blue
NBH1	+ Blue
NBH2	+ Blue
NBH3	+ Blue
NBH4	+ Blue
NBH5	+ Blue
NBH6	+ Blue
NBH7	+ Blue
NBH8	+ Blue

5.7.4. SIM (Sulphur Reduction Indole Production Motility Test)

SIM (Sulphur reduction indole production and motility test) was performed to identify the bacteria capable of reducing sulphur, capable of producing indole through the utility of tryptophanase and to detect the presence of flagella. All the isolates show different results in Table11. *Pseudomonas* species have the capability of reducing sulphur as shown in fig.19.

TABLE11. SIM TEST

STRAINS	SULPHUR REDUCTION	INDOLE PRODUCTION	MOTILITY
*Pseudomonas aeruginosa*ATCC 15442	+ BLACK ppt.	+ RED COLOUR	+
*Pseudomonas aeruginosa*PAO1	+	+	+
NPD5	-	-	+
NPD6	-	+	+
NE3B01	+	+	+ (BEST)
NE3B02	-	-	+
Pseudomonas mendonica NR802	-	-	+
*Pseudomonas sp.*NP202	-	-	+
NPD4	-	-	+
NPD3	-	-	+
NPD2	-	-	+
NPD1	-	-	+
NBH1	-	-	+
NBH2	-	-	+
NBH3	-	-	+
NBH4	-	-	+
NBH5	-	-	+
NBH6	-	-	+
NBH7	-	-	+
NBH8	-	-	+

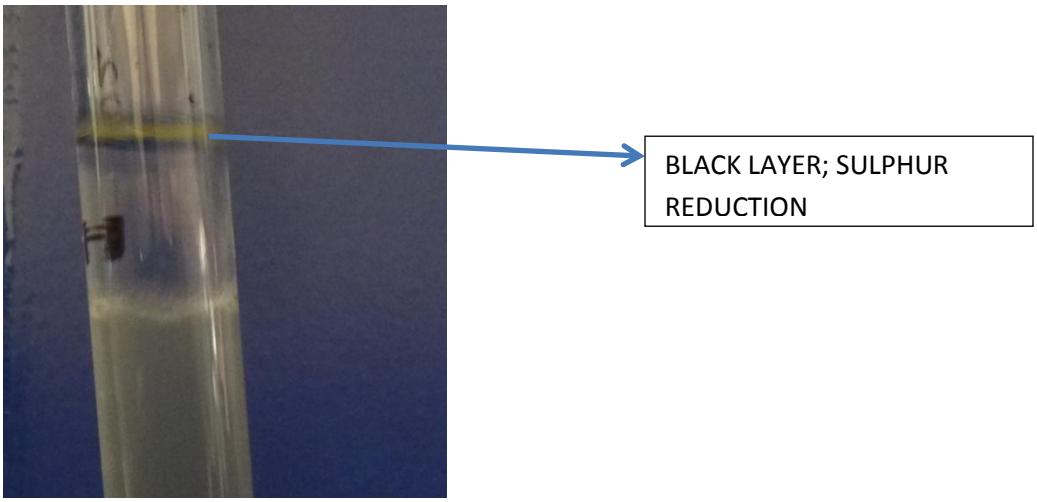


Fig.19. SIM TEST (Formation of black colour)

5.7.5 Mannitol motility nitrate test

This test is used to identify that whether they are capable of mannitol fermentation and nitrate reduction. The positive result in mannitol motility nitrate test indicates the development of yellow colour as shown in fig.20(A) which shows their capability of fermenting mannitol ; ther capability of spreading outward from their original position and after the addition of 4-5 drops of each sulphanilic acid and alpha naphthalene into the culture tubes, red colour develop due to nitrate reduction. The isolates show different result with this biochemical assay and the results are mentioned in Table12.

Fig.20.(A) Mannitol fermentation **Fig.20.(B) Motility of NE3B01**

Pseudomonas aeruginosa ATCC 15442 do not have the capability of mannitol fermentation but they have better capability of nitrate reduction. NE3B01 is highly motile as compare to other isolates as shown in fig.20.(B).

TABLE .12 MANNITOL MOTILITY NITRATE TEST

STRAINS	**MANNITOL FERMENTATION**	**NITRATE REDUCTION**
Pseudomonas aeruginosa ATCC 15442	-	+
Pseudomonas aeruginosa PAO1	+ Yellow	-
Pseudomonas sp.NP202	-	+
Pseudomonas mendonica NR802	-	+-
NE3B02	-	-
Pseudomonas pseudoalcaligenis NP103	-	-
NE3B01	+ Yellow	+
NPD1	-	-
NPD2	-	-
NPD3	-	-
NPD4	-	-
NPD5	-	-
NPD6	-	-
NPD7	-	+-
NPD8	-	-
NBH1	+ Yellow	-
NBH2	-	-
NBH3	-	-
NBH4	-	-
NBH5	-	-
NBH6	-	-
NBH7	-	-
NBH8	-	-

IN NITRATE REDUCTION :-

+ indicates red colour

-indicates yellow colour

+ indicates partial red and partial yellow colour

6. Discussion

The present study focused on the occurrence of *rhlI* among polycyclic aromatic hydrocarbon (PAHS) utilizing bacterial isolates as a tool for the identification of rhamnolipid synthesizing *Pseudomonas species*. After selective enrichment of PAHs degrading bacteria, isolates named as *Pseudomonas aeruginosa* ATCC 15442, *Pseudomonas aeruginosa* PAO1, NE3B02, NP202, NP103, NR802, NBH1, NBH2, NBH3, NBH4, NBH5, NBH6, NBH7, NBH8, NPD1, NPD2, NPD3, NPD4, NPD5, NPD6, NPD7, NPD8 were studied. The preliminary screening for biosurfactant was done by several assays like drop collapse assay, emulsification assay, oil spread assay and it was found that among all these strains *Pseudomonas aeruginosa* ATCC show positive result in all the above mentioned tests. *Pseudomonas aeruginosa* ATCC (15442) was taken as positive control and all the isolates were grown on *Pseudomonas* agar and *Pseudomonas* agar base. Among all the strains, N6P, NPD1, NPD5, NPD6, NPD7 show green fluorescent colour. Physical and biochemical characterization were done through Gram staining, macconkey agar test, oxidase test, cell surface hydrophobicity, sulphur reduction indole production mannitol fermentation test, mannitol motility nitrate test. All the isolates show different morphological aspects in terms of Gram staining, color of colonies, motility, sulphur reduction, indole production and mannitol fermentation. All were Gram negative having rod shape. Macconkey agar test was performed for the isolation and differentiation of Gram-negative enteric bacilli from the positive ones. Oxidase test was done in order to differentiate the families of *Pseudomonadaceae* and *enterobacteriaceae.* All the above isolates show positive result that means they use oxygen as the final electron acceptor in aerobic respiration and the enzyme cytochrome oxidase is involved with the utility of reduction of oxygen at the end of the electron transport chain. SIM (Sulphur reduction indole production and motility test was performed to identify the bacteria capable of reducing sulphur, capable of producing indole through the utility of tryptophanase and to detect the presence of flagella. All the above isolates show different results. The positive result in mannitol motility nitrate test indicates the development of yellow colour which shows their capability of fermenting mannitol and after the addition of 4-5 drops of each sulphanilic acid and alpha naphthalene into the culture tubes, red colour develop due to nitrate reduction which shows positive result. Polymerase chain reaction (PCR) amplification with *rhlI* specific primers revealed the presence of *rhlI* coding gene in *Pseudomonas aeruginosa*

ATCC 15442, *Pseudomonas aeruginosa* PAO1, N6, NBH1, NBH4, NPD6. The size of the product was found to be 155 bp . The extraction of biosurfactant was done with chloroform : methanol. After extraction, each sample was analysed in FTIR in between 600nm to 4000nm.

7. Summary and Conclusion

The aim of present study is the distribution of *rhlI* among polycyclic aromatic hydrocarbon (PAHs) utilizing bacterial isolates as a tool for the identification of rhamnolipid synthesizing *Pseudomonas* species. 1 ml of water sample was transferred to 100 ml of BHM (Bushnell haas medium) supplemented with 100 mg /L of Pyrene, Phenanthrene and Naphthalene. For further enrichment of PAHs utilizing consortia, after 15 days, 1ml inoculum was transferred to 100 ml BSM supplemented with 200 mg/l of PAHs. After 15 days, it was serially diluted (10^{-1}, 10^{-2} and 10^{-3}) and 100 µl aliquot was spread out on SWNA lates supplemented with 50 mg/l of PAHs (to keep the isolates under PAHs stress). Isolated colonies with distinct morphology were streaked on BSM-PAHs agar plates of Pyrene, Phenanthrene and Naphthalene. Plates were incubated at 37°C. Isolates named as *Pseudomonas aeruginosa* ATCC 15442, *Pseudomonas aeruginosa* PAO1, *Pseudomonas mendocina* NR802, NE3B02, *Pseudomonas sp*.NP202, *Pseudomonas pseudoalcaligenis* NP103, NBH1, NBH2, NBH3, NBH4, NBH5, NBH6, NBH7, NBH8, NPD1, NPD2, NPD3, NPD4, NPD5, NPD6, NPD7, NPD8, N6P were studied after selective enrichment. Physical and biochemical characterization were performed through Gram staining, Macconkey agar test, oxidase test, cell surface hydrophobicity, sulphur reduction indole production mannitol fermentation test, mannitol motility nitrate test. All were Gram negative having rod shape. Non-lactose fermenting bacteria such as *Pseudomonas aeruginosa* cannot utilize lactose in the medium, and will use peptone instead. This results in the formation of ammonia, which raises the pH of the agar, and leads to the formation of white or colorless colonies in the Macconkey agar plate. All the isolates show positive result i.e, development of blue colour that means they use oxygen as the final electron acceptor in aerobic respiration and the enzyme cytochrome oxidase is involved with the utility of reduction of oxygen at the end of the electron transport chain. Similarly in SIM test and MNN test, different pseudomonas species show different responses. Growth of strains was monitored at 595 nm and it was concluded that *Pseudomonas aeruginosa* ATCC 15442 has log phase for a longer time. The strains were grown in *Pseudomonas* agar and

Pseudomonas agar base for the tentative selection of the isolates. Polymerase chain reaction (PCR) amplification with *rhlI* specific primers (forward primer - 5'ttcatcctccttagtcttccc 3' and reverse primer - 5'ttccagcgattcagagagc 3') revealed the presence of *rhlI* coding gene in *Pseudomonas aeruginosa* ATCC 15442, *Pseudomonas aeruginosa* PAO1, N6, NBH1, NBH4, NPD6. The size of the amplified product is 155 bp. Than extraction of rhamnolipid was done with chloroform : methanol and in FTIR analysis, it was found that rhamnolipid has different functional groups like primary amine, secondary amine, alkane, carboxylic acid, ester.

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