STRUCTURAL, PHYSICOCHEMICAL AND BIOLOGICAL CHARACTERIZATION OF BIOSURFACANT FROM SSC2
2.1 INTRODUCTION AND LITERATURE REVIEW

Demand for new specialty chemicals in the agriculture, cosmetic, food, pharmaceutical and environmental industries is steadily increasing. Because these chemicals must be both effective and environmentally compatible, it is natural to turn to the microbial world to try to meet this demand. One such microbially produced chemical of widespread use in all aspects of biotechnology and industry are the biosurfactants.

2.1.1 Biosurfactants defined

Biosurfactants are microbially produced amphipathic molecules with both hydrophilic and hydrophobic moieties that partition preferentially at the interface between fluid phases with different degrees of polarity and hydrogen bonding and reduce surface and interfacial tensions. These are structurally diverse and are produced by all forms of microorganisms including bacteria, yeasts and fungi. They are environmentally friendly, biocompatible, biodegradable and exhibit remarkable stability at extremes of temperature, pH and salinity. These, combined with the fact that they can be produced from a variety of substrates, make them the molecules of choice of the biotechnology industry. Apart from their structural diversity, biosurfactants offer a number of distinctive advantages as compared to their synthetic counterparts including:

Higher biodegradability and biocompatibility (environment friendly)
Ability to be synthesized from renewable/waste resources
Activity at extremes of temperatures, pH and salinity
Low toxicity and high specificity and selectivity in their action

(Georgion et al., 1990; Ishigami 1997; Makkar and Cameotra 2003)

2.1.1.1 Surface Tension and Critical Micelle Concentration

Because of their amphipathic nature, biosurfactants tend to accumulate at air water or oil water interfaces and reduce interfacial and surface tensions. Due to the
presence of a surfactant, less work is required to bring a molecule to the surface and the surface tension is reduced. In an assessment by Bodour et al., (2003), (i) Under identical growth conditions same amount and type of biosurfactant is produced and this is reflected in a similar reduction in surface tension (ii) a single isolate often produces chemical variants of the same surfactant, resulting in the production of a surfactant mixture with an associated characteristic surface tension.

As the interfacial tension is reduced and the aqueous surfactant concentration increased, the monomers aggregate to form micelles. The concentration at which micelles first appear is known as the critical micelle concentration (CMC) and corresponds to the lowest surface tension by the surfactant. The CMC for any surfactant is dependent on the surfactant structure as well as the pH, ionic strength, and temperature of the solution. CMCs of biosurfactants typically range from 5 to 200mg/l (Lang and Wagner 1987). Surface Tension and CMC are two very important criteria for assessing biosurfactant efficiency.

2.1.2 Diversity of biosurfactants

There are different types of biosurfactants known and documented in literature and hence biosurfactant diversity has been the subject of research of various authors (Vardar-Sukan and Kosaric 2000; Maier 2003). All biosurfactants are amphiphilic in nature, containing at least one hydrophilic and one hydrophobic moiety. The hydrophilic moiety can be an ester, hydroxyl, phosphate, carboxyl, or carbohydrate group and is either neutral or negatively charged. There have been no cationic biosurfactants reported.

Structurally, biosurfactants have been classified into the following classes, each class generally produced as a mixture of closely related compounds:

2.1.2.1 Glycolipids

These biosurfactants are usually carbohydrate in combination with long chain aliphatic acids or hydroxyl aliphatic acids and range in molecular weight from 500-1500. Among the glycolipids, the best known are rhamnolipids (Jarvis and Johnson
1949), trehaloselipids (Suzuki et al., 1969) and sophorolipids (Gorin et al., 1961). Among all these, rhamnolipids are the most studied and extensively used glycolipid. Rhamnolipids contain one or two rhamnose units and in general β-hydroxydecanoic acid residues and are produced extracellularly by almost all Pseudomonas aeruginosa strains under identical environmental conditions. In liquid cultures, P. aeruginosa primarily produces two forms of rhamnolipids: rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (monorhamnolipid) and rhamnosyl-rhamnosyl-β-hydroxydecanoyl-hydroxydecanoate (di-rhamnolipid) (Lang and Wagner 1987). However, a small proportion of up to 28 different congeners that have variable length hydrocarbon chains (C_{10}-C_{14}) are also produced (Deziel et al., 1999, 2000). In general, P. aeruginosa isolates, either clinical or environmental, produce a mixture of mono and dirhamnolipids under specific environmental conditions, but there are a few exceptions. For example, P. aeruginosa ATCC9027 produces only monorhamnolipid (Zhang and Miller 1992) and P. aeruginosa 158 was reported to produce rhamnolipid methyl esters (Hirayama and Kato 1982). Hence proper assessment of the rhamnolipid mixture is essential before applying the biosurfactant for any use especially in the biomedical field since different compounds exhibit different physicochemical properties.

2.1.2.2 Peptide and amino acid containing lipids

Lipopeptides and lipoproteins are a heterogenous class of biologically active peptides with a protein moiety attached to a fatty acid. The protein moiety can be neutral or anionic and the amino acids are often arranged in a cyclic structure. Best known among this group are those produced by Bacillus species including surfactin, iturin, fengycin and lichenysin.

2.1.2.3 Phospholipids, neutral lipids and acidic lipids

There are numerous lipid containing molecules integral to cell structure that are also surface active and have activities normally associated with biosurfactants. For example, phospholipid rich and lipopolysaccharide (LPS) rich vesicles, produced
by *Acinetobacter* sp. HOI-N upon growth on hexadecane, were known to function in enhancing the solubility of hexadecane (Kappeli and Finnerty 1979). *Candida tropicalis* and *Debaromyces polymorphus* produce a bioemulsifiers upon growth on hexadecane, which consists of at least 40% lipid moiety (Singh and Desai 1989). *Arthrobacter paraffineus* KY 4303 was found to produce a primary fatty alcohol when grown on paraffin (Suzuki and Ogawa 1972). A neutral lipid is produced by *Nocardia erythropolis* ATCC 4277 during growth on 4% hydrocarbon (MacDonald *et al*., 1981).

2.1.2.4 Polymeric biosurfactants

These are a group of highly heterogeneous polymers characterized by their high molecular weight, ranging from 50,000 to greater than 1,000,000 and with properties like high viscosity, high tensile strength and resistance to shear. These polymers can be carbohydrate or protein based and in addition usually contain lipids. In some cases the polymer may be a mixture of carbohydrate, protein and lipid and are noted for their emulsification abilities. The best characterized of the polymeric biosurfactants is emulsan produced by *Acinetobacter calcoaceticus* RAG1 (Rosenberg *et al*., 1979).

2.1.3 Factors affecting rhamnolipid biosynthesis and secretion

Rhamnolipids are the most extensively studied biosurfactants and are in part the subject of research of this work. Rhamnolipid biosynthesis and production is dependent on a number of cellular and environmental parameters including pH, temperature, salt concentration, agitation etc.

2.1.3.1 Nutritional factors: dependence on carbon, nitrogen and phosphate levels

Hydrocarbons are commonly used as a substrate for the production of biosurfactants. It has been postulated that the biological function of surface active compounds is related to hydrocarbon uptake, and therefore a spontaneous release
occurs with these substrates (Hisatsuka et al., 1971; Kappeli et al., 1980). Most biosurfactant studies have hence used hydrocarbons as substrates for growth. However, rhamnolipids are known to be produced from a variety of carbon sources including waste substrates (Robert et al., 1989; Benincasa et al., 2002). In the study by Robert et al., (1989), P. aeruginosa 44T1 produced rhamnolipids when grown on C12 n-alkanes or olive oil but not with other hydrocarbons tested. Best results were obtained with olive oil as carbon source. In a study by Arino et al., (1996), glycerol yielded higher productions than hydrophobic carbon sources. Cell hydrophobicity decreased during growth on glycerol and on n-hexadecane whereas glycolipid production was found to increase. In one of the earliest efforts for the production of rhamnolipids in continuous cultures, upto 1.5gml⁻¹ rhamnolipid was obtained from a formulated media containing a starting glucose concentration of 18.2 gml⁻¹ (Guerra-Santos et al., 1984). Presence of yeast extract was found to be inhibitory for rhamnolipid production while limiting concentrations of nitrogen favoured its production.

Rhamnolipid formation by P. aeruginosa occurs under limiting concentrations of nitrogen and iron during the late exponential and stationary phases of growth and nitrate was found to support maximum biosurfactant production (Guerra-Santos et al., 1986; Macelwee et al., 1990). Also of importance was the available phosphate concentration. Mulligan et al., (1989) established a phosphate limiting medium, proteose peptone/glucose/ammonium salts medium (PPGAS) (Cheng et al., 1970) as the best producer of rhamnolipids, yielding rhamnolipid with a surface tension of 29mNm⁻¹ and a concentration of approximately 30 times the Critical Micelle Concentration. Both nutrient broth and the minimal medium which contained substantially high levels of inorganic phosphate were very poor media for biosurfactant production.

2.1.3.2 Biosurfactant production in heterologous hosts

Not much success has been achieved towards high levels of production of rhamnolipids in heterologous hosts. In one such prominent study by Ochsner et al.,
(1994), only 10% of the activity as compared to the wild type was detected in an *E. coli* strain transformed with the gene coding for rhamnolipid (*rhlAB* genes). Levels of rhamnolipid production were only slightly increased when *P. putida* and *P. flourescens* were used as heterologous hosts (Ochsner *et al.*, 1995). Hence a better strategy would be to use *P. aeruginosa* wild strain itself for commercial purposes and enhance rhamnolipid production *in situ* by manipulating the culture and or environmental conditions.

### 2.1.4 Biosurfactant producers: scope for new molecules

A wide variety of microorganisms can produce biosurfactants ranging from *Pseudomonas*, *Bacillus* and *Acinetobacter* species among bacteria, *Arthrobacter* and *Rhodococcus* species among actinomycetes, *Candida* strains among yeasts and *Penicillium* and *Ustilago* among fungi being some of the commonest biosurfactant producers (Maier 2003). Several anaerobic bacterial strains are known to produce biosurfactants but the observed reduction in surface tensions (45-50 mN/m) was not as great as those produced by aerobic organisms (27-30 mN/m) (Cooper 1986).

Biosurfactant producing microorganisms have been isolated from a number of environments including soil, sea water, marine sediments, and oil fields (deep subsurface environments). In a study by Bodour *et al.*, (2003), 21 different sites, including uncontaminated, hydrocarbon contaminated, metal contaminated and hydrocarbon-metal contaminated soils were screened, and 20 of the 21 soils contained at least 1 biosurfactant producing isolate even when using a very limited screening methodology.

Hydrocarbono-clastic microorganisms often make and secrete one or more surface active agents. Any new or unexamined hydrocrabono-clastic environmental isolate hence offers a potential for the discovery of a new biosurfactant structure. Mercade *et. al.*, (1996) screened and selected biosurfactant producing microorganisms from hydrocarbon contaminated soil. Forty four isolates were able to use waste lubricating oil as a substrate with only 10% being able to produce biosurfactants. The strains were mainly *Bacillus* sp. producing lipopeptides and
Rhodococcus sp. producing trehalose lipids. This is an excellent example of the approach required to obtain new biosurfactants that can be applied for numerous commercial purposes.

2.1.5 Diverse uses of biosurfactants (Rhamnolipids)

The numerous advantages of biosurfactants over other synthetic surfactants makes them the molecules of choice for diverse industrial and commercial purposes. Hester (2001) of Technical insights, a division of Frost and Sullivan, non-ionic surfactants, estimated that biosurfactants could capture 10% of the surfactant market by the year 2010 with sales of $200 million in US. A number of reviews are available on the commercial and industrial uses of various biosurfactants specially rhamnolipids (Lang and Wullbrandt 1999; Maier and Soberon-Chavez 2000; Banat et al., 2000) including use in environment remediation, enhanced oil recovery, food and cosmetic industry, biomedical field etc.

<table>
<thead>
<tr>
<th>Area</th>
<th>Use</th>
<th>Effect</th>
</tr>
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<tbody>
<tr>
<td>Metals</td>
<td>Concentration of ores, cutting and</td>
<td>Wetting, foaming, emulsifying, lubrication, corrosion inhibition in rolling and cutting oils, in mold release additives</td>
</tr>
<tr>
<td></td>
<td>forming, casting, rust and scale removal, plating</td>
<td></td>
</tr>
<tr>
<td>Paper</td>
<td>Pulp treatment, paper machine</td>
<td>Deresinification, washing, defoaming, color leveling and dispersion, coating and coloring</td>
</tr>
<tr>
<td>Paint and protective coating</td>
<td>Pigment preparation, latex paints, waxes and polishes</td>
<td>Dispersion and wetting of pigment during grinding, emulsification, stabilize latex, retard sedimentation and pigment separation, stabilize emulsions</td>
</tr>
<tr>
<td>Petroleum products and production</td>
<td>Drilling fluids, work over of production wells, second recovery, refined products</td>
<td>Emulsify oil, disperse solids, modify rheological properties of drilling fluids for oil and gas wells, emulsify and disperse sludge and sediment in cleanout of wells, demulsifying crude petroleum, preferential wetting detergent,</td>
</tr>
</tbody>
</table>

57
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<thead>
<tr>
<th>Industry</th>
<th>Application</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Textiles</td>
<td>Preparation of fibers, dyeing and printing, finishing of textiles</td>
<td>Detergent and emulsifier in raw wool scouring, dispersant in viscous rayon spin bath, wetting penetration, solubilization emulsification, dye leveling, finishing formulations</td>
</tr>
<tr>
<td>Building and construction</td>
<td>Paving, concrete, ceramic</td>
<td>Improve bond of asphalt to gravel and sand, promote air entrainment</td>
</tr>
<tr>
<td>Agriculture</td>
<td>Phosphate fertilizers, spray application</td>
<td>Prevent caking during storage, wetting, dispersing, suspending of powdered pesticides and emulsification of pesticides solutions</td>
</tr>
<tr>
<td>Elastomers and plastics</td>
<td>Emulsion polymerization, latex adhesive, plastic articles, plastic coating and laminating</td>
<td>Wetting, solubilization, emulsification of monomers, introduction of air, control of cell size, improve bond strength,</td>
</tr>
<tr>
<td>Food and beverages</td>
<td>Food processing plants, fruits and vegetables, bakery and ice cream, crystallization of sugar, cooking fat and oils</td>
<td>For cleaning and sanitizing, improve removal of pesticides and wax coating, solubilization control consistency, improve washing, reduce processing time, prevent spattering due to heat and water</td>
</tr>
<tr>
<td>Industrial Cleaning</td>
<td>Janitorial supplies, descaling, soft goods</td>
<td>Detergents and sanitizers, wetting agents and corrosion inhibitors in acid cleaning of boiler tubes</td>
</tr>
<tr>
<td>Cosmetics and pharmaceuticals</td>
<td>Insect repellent, antacid, bath products, acne pads, antidandruff products, contact lens solution, hair color and care products, denture cleaner, adhesives, toothpastes, baby products, antisepsics, shampoo, conditioner, moisturizer, in toiletries, health and beauty products</td>
<td>Emulsifier, foaming agent, solubilizer, wetting agent, cleanser, antimicrobial agent, mediator of enzyme action</td>
</tr>
<tr>
<td>Pollution control</td>
<td>Soil bioremediation, oil storage tank cleanup, removal of oil spills</td>
<td>Emulsifiers, deemulsifiers</td>
</tr>
</tbody>
</table>

Table 2.1: Various industrial and environmental applications of biosurfactants
(Adopted from Vardar-Sukan and Kosaric 2000)
We describe below an overview of some of the most important application of biosurfactants with special emphasis on application potential of rhamnolipids.

2.1.5.1 Production of fine chemicals

Rhamnolipids and chemically modified rhamnolipids are being investigated as alternatives to high value synthetic chemicals whose use may have toxic environmental impacts. For example, Ishigami and Suzuki (1997), report the synthesis of pyrenacylester or rhamnolipid (R-PE) for use in monitoring the polarity and fluidity of solid surfaces and the attendant impact of coating on these surface properties. An exciting use of rhamnolipids is as a cheap source of rhamnose, used as a fine chemical in scientific and industrial settings, as a flavoring agent, as a component in chemical reactions, and as a starting material in the synthesis of organic compounds. (Linhardt et al., 1989). The cosmetic and health care industry uses rhamnolipids in a number of cosmetic products as a low irritant / anti-irritant additive.

2.1.5.2 Bioremediation

The most widespread use of rhamnolipids is in the field of environmental pollution remediation. These are the most effective surfactants when applied for the removal of hydrophobic compounds from contaminated soils. They possess low average minimum surface tension (30-32mN/m), low CMC (5-65mg/l) and high affinity for hydrophobic organic molecules (Arino et al., 1996). A number of studies have successfully established the role of rhamnolipids in cleaning up and as a flushing agent of sites polluted with either organics/metal/metal-organics. Addition of rhamnolipids to pure cultures or in soil systems has been shown to enhance biodegradation of a number of hydrocarbons including hexadecane (Oberbremer et al., 1990; Zhang and Miller 1992, 1994; Herman et al., 1997a, 1997b). Similar results were obtained at sites co-contaminated with organics and toxic metals in both pure cultures and in soil systems (Sandrin et al., 2000; Maslin and Maier 2000). Rhamnolipids have been shown to have a high affinity for a variety of toxic metals of concern and have the potential to be used for the removal of toxic metal from the
contaminated site by complexing (Herman et al., 1995; Betts 1997). They are also used for flushing of pesticide contaminated soils.

2.1.5.3 Biological activities of biosurfactants

Another major upcoming market opening up for biosurfactants is in the area of biomedical sciences. Increasing emergence of new diseases and increasing incidences of drug resistant pathogen related cases have instigated research towards finding novel molecules for use in the medical field. One such molecules are the structurally and functionally diverse biosurfactants. Lipopeptides are the most studied molecules for their role in the field of medicine. Important among them are iturins an surfactins. Iturin was found to be having strong antifungal activities and has been proposed to be an effective antifungal agent for profound mycosis. Surfactin apart from having antifungal and moderate antibacterial activity was also known to inhibit fibrin clot formation, exhibit antiviral and antitumor activities and also possess antimycoplasmal activity apart from other biomedical properties (Singh and Cameotra 2004). Apart from this, biosurfactants especially those produced by members of Lactobacilli group have been known to possess excellent antiadhesive properties, inhibiting adhesion of pathogenic organisms, for example uropathogenic bacteria, to susceptible surfaces or body parts (Boris and Barbes 2000; Reid 2001). Apart from these, the other biosurfactant group receiving recent attention for their biomedical applications are members of the glycolipid group, including rhamnolipids.

2.1.6 Glycolipids as biological agents

Among glycolipids, mannosylerythritol lipids (MEL) from Candida strains and rhamnolipid from Pseudomonas aeruginosa strains are the most commonly studied for their role in the biomedical field. MEL caused significant cellular differentiation in human promyelocytic leukemia cell line HL60 (Isoda et al., 1997) and also caused significant neurite outgrowth in PC-12 cells (Isoda et al., 1999). Also, exposure of B16 cells to MEL resulted in the events observed in apoptosis (Zhao et al., 1999). All this raises the possibility of their use for the treatment of
cancer cells. Apart from this MEL also raises the possibility of the use of glycolipids as agents for gene transfection, as an alternative ligand for immunoglobulins and as an affinity ligand material (Cameotra and Makkar 2004).

Rhamnolipid too have been known to exhibit antibacterial and antiphytoviral effects. In a study by Itoh et al., (1971), mono- and di-rhamnolipids produced by *P. aeruginosa* strains growing on a mixture of hydrocarbons possessed antibacterial, antimycoplasmal and antiviral activities with the monorhamnolipid component being more active especially against some gram positive bacteria. Abalos et al., (2001) identified seven rhamnolipids in cultures of *P. aeruginosa* AT10 from soybean oil refinery waste and found them to exhibit excellent antifungal properties against various fungi. Most recently, Benincasa et al., (2004) and Haba et al., (2003), isolated rhamnolipids from *P. aeruginosa* grown on soapstock and waste frying oil respectively, and found excellent antibacterial properties. Rhamnolipids have been shown to be effective against a number of zoosporic plant pathogens also including *Pythium aphanidermatum, Phytophthora capsici* and *Plasmopara lactucae-radicis* causing cessation of motility and lysis of the entire population in less than 1 min. (Stanghellini and Miller 1997). Algicidal activity has also been observed from *Pseudomonas aeruginosa* rhamnolipids (Wang et al., 2005).

All this confirms the possibility of the application of rhamnolipids in the control of bacterial diseases. Keeping in mind the structural diversity of rhamnolipid, it is essential to evaluate each molecule according to its activity and possible biomedical use.

### 2.1.7 Dual activity of biosurfactants

Most of the biosurfactants known possess dual activity: surface active behavior and biological activity. Neu and Poralla (1990) were the first to report compounds like surfactin, iturin and viscosin as emulsifying agents involved in bacterial growth on hydrophobic surfaces. These compounds were discovered initially as biological agents: surfactin as haemolysin (Kakinuma and Arima 1969) and iturin and viscosin as antibiotics (Besson et al., 1978; Leisinger and Margraff 1979). This
functional versatility makes them the most sought after compounds of the recent times, finding extensive use in both environment biotechnology and biomedical science. Similar is the case with rhamnolipids. Initially discovered as surface active agent, these compounds are now known to possess excellent biological activity. However, their potential in the field of biomedical science is still in the infancy as compared to the work done on other biosurfactants like surfactin. Also of importance is the fact that the structural diversity of rhamnolipid holds great promise for the discovery of new biological agents. Moreover, microorganisms, having diverse metabolic synthetic capabilities, offer potential in expanding range of surfactants with novel structures, thereby providing new possible applications.

This chapter of the thesis deals with the identification/ characterization of rhamnolipid(s) produced by the oil field isolate *P. aeruginosa*, used for crude oil sludge remediation in soil under natural environmental conditions. The antimicrobial property of the isolated product was also the area of study for assessing the potential application of rhamnolipid in biomedical field.
2.2 METHODOLOGY

2.2.1 Assessing rhamnolipid production in different media

Rhamnolipid production by *Pseudomonas aeruginosa* SSC2 was assessed in the presence of different carbon sources:

Proteose peptone glucose acid salt (PPGAS) media
Minimal media with different hydrocarbons as sole carbon source: n-hexadecane, n-dodecane, n-pentadecane and a mixture of n-dodecane and pristane
Minimal media with a carbohydrate (glucose) as sole carbon source

Reduction in surface tension was taken as the criteria for assessing biosurfactant production. Surface tension of cell and hydrocarbon free broth after different time intervals was measured by DuNouy Tensiometer. The process involved has been described in section 1.2.1.2. of chapter 1.

Biomass buildup upon growth in the two chosen substrates were also measured.

2.2.2 Isolation (extraction of rhamnolipids)

*Pseudomonas aeruginosa* SSC2 was grown in two different media: Minimal media with n-hexadecane as the sole carbon source and a proteose peptone glucose acid salt (PPGAS) media. After growth of SSC2 for 48-60hr in PPGAS and 3-4 days in minimal medium (the time at which lowest surface tension was recorded), cells were removed by centrifugation, surface tension of cell free broth taken and pH lowered to 2.0-3.0 using 3N HCl. After overnight incubation at 4°C, the residue obtained was recovered by centrifugation, dissolved in 50mM Sodium-bi-carbonate buffer (pH 8.6), reacidified to pH 2.0-3.0 and incubated at 4°C again for 5-6 hr. Final residue obtained was recovered by centrifugation and rhamnolipid extracted repeatedly using a mixture of Chloroform: Methanol (2:1). Approximately 100-150ml of the solvent was used for the extraction. All the extracts were pooled together and solvent evaporated in rotavapour. The viscous residue obtained was re-dissolved in methanol and filtered through 0.22μm filter (Millipore). Methanol was again evaporated and
the final residue (partially purified rhamnolipid) obtained was weighed, and dissolved in a small volume of methanol.

2.2.3 Physicochemical characterization

2.2.3.1 Critical Micelle Concentration (CMC)
Different concentrations of rhamnolipid mixture (obtained upon growth in PPGAS media and on n-hexadecane as carbon source) were made ranging from 0.05g/ml to 5g/ml. CMC was taken as the point in the range of concentrations at which surface tension displayed an abrupt change in value. For both the biosurfactant solutions, experiment was done twice and in triplicate for each concentration.

2.2.3.2 Stability at different pH and temperatures
Stability of the rhamnolipid mixture was analyzed at different temperatures (4°C to 65°C) and at different pH values (2.0 to 12.0) at different time intervals for 1 week. Change in surface tension was the criteria for monitoring change in rhamnolipid stability. Different sets were made at 2CMC value in water. For each temperature and pH value, samples were made in triplicate and the whole experiment performed twice.

2.2.4 Separation and purification of rhamnolipid mixture
Surface active rhamnolipids were separated by a method modified from a previous work of Sim et al., (1997). Different components of the rhamnolipid mixture were separated and analyzed by two methods: Thin Layer Chromatography for analyzing small amount of mixture and column chromatography using silica gel for separation of compounds in large volumes of rhamnolipid mixture.
2.2.4.1 TLC for rhamnolipid mixture separation

Different components in the mixture of rhamnolipid were resolved by TLC on precoated silica gel plates 60 F-254 (Merck). Rhamnolipid mixture was loaded by capillary onto TLC plates and resolved in a solvent system consisting of Chloroform, methanol and acetic acid. Two detection techniques were used: exposure to iodine vapours and vapourization with the Molish reagent (Arino et al., 1996). Plates were incubated for 15-20 minutes at 50°C to 60°C after spraying the plates with Molish reagent for the coloured spots to appear. A total of 10 different solvent systems were tried including:

1. Chloroform: Methanol: Acetic acid (75:5:2)
2. Ethyl acetate: n-Butanol: Methanol: Water (16:3:3:2)
3. Ethyl acetate: Acetic acid: Methanol: Water (6:1.5:1.5:1)
5. Acetone: Water (9:1)
8. Chloroform: Methanol: Acetic acid (70:10:2)
9. Chloroform: Methanol: Acetic acid (72:8:2)
10. Chloroform: Methanol: Acetic acid (65:15:2)

Of these, solvent system no. 8 Chloroform: Methanol: Acetic acid (70:10:2) gave best resolution and was the solvent of choice for later experiments. Rhamnolipid standard (1mgml⁻¹) procured earlier in laboratory and rhamnose solution (10mgml⁻¹) were used as positive controls and surfactin solution (1mgml⁻¹), hexadecane and hexadecanoic acid was used as a negative control for confirming the effectiveness of detection of sugar containing compounds by the Molish reagent.

For preparative purposes, a small portion of the developed plate was stained with Molish reagent, to give an idea of the position of different compounds on the plate and from the rest of the unstained plates, different spots were scraped off. Particular rhamnolipid compound was extracted twice using 10ml of methanol and the extracts pooled together. Extraction included overnight incubation in the solvent, spinning
down silica gel by centrifugation and pipetting out the solvent. The extracts after concentration were analyzed on TLC for purity of spots.

2.2.4.2 Column chromatography for separation of rhamnolipid mixture

For separation of large volumes of rhamnolipids, liquid chromatography was utilized. A glass column (3cm × 60cm) was prepared with 60gm of activated silica gel (230-300 mesh) chloroform slurry. Rhamnolipid extract in methanol (from growth on PPGAS medium) was evaporated to dryness and dissolved in chloroform before loading. Column was washed with 100ml of chloroform to remove all neutral lipids. Chloroform: methanol mobile phases were then applied in sequence: 50:3 (1200ml), 50:5 (400ml), 50:50 (400ml) and a final wash with pure methanol was given to bring out all the rhamnolipids. 20ml fractions were collected. Detection of particular component in each fraction was done using TLC upon staining with Molish reagent. Similar fractions were pooled together and dried under vacuum in a rotavevaporator at 35-40°C. Purity of the final fractions was again confirmed by TLC.

2.2.5 Analyzing the presence of different rhamnolipid congeners in the biosurfactant mixture using HPLC-ES-MS

For identification of different rhamnolipid congeners in the mixture of rhamnolipid obtained from both the medium, HPLC-ES-MS was carried out according to the method described by Haba et al., 2003. Samples (20µl) dissolved in methanol were analyzed by HPLC (Finnigan MAT, UK) using a Shimpack C8 column (Shimadzu; 4.6x 250mm; 100A), SpectraSYSTEM (AS3000) autosampler and a SpectraSYSTEM (P4000) pump. An acetonitrile-water gradient (both containing 0.1% acetic acid) was used: 30% acetonitrile for 2 min, followed by a ramp of 30-100% acetonitrile for 30 min, standby for 5 min, and then return to initial conditions. HPLC flow rate was 1ml per minute. MS was performed by Finnigan MAT system. Full scan data was obtained by scanning from m/z 100-1000 in centroid mode with a maximum injection time of 50mSec and 5 total microscans. The working conditions for the ES source was as follows: drying nitrogen was introduced into the source body.
at a flow rate of 400NL/h, nebulizing nitrogen was set at 20NL/h, and capillary temperature was 270°C. Cone voltage used was -30V. Electron spray ion source (ES) and a negative ion mode was used for analyses.

### 2.2.6 Identification of the isolated compounds

Different techniques including IR, NMR (C¹⁴ and H¹) and MS were done to conclusively elucidate the structures of different mixture components obtained. For NMR analyses, Bruker AVANCE DPX 300 model was used with H¹ NMR at 300Mhz and C¹³ at 75Mhz. MS in direct injection mode and HPLC-ES-MS of the purified compounds was done as described above to conclusively ascertain the structure.

### 2.2.7 Biological activity of rhamnolipids

Antimicrobial activity of the isolated rhamnolipid mixture and the separated components were assayed by the Kirby Bauer agar diffusion assay (Bauer et al., 1966) and determined according to MIC values, defined as the lowest concentration of antimicrobial agent that inhibits the development of visible growth after 24 hours of incubation (time varied for some organisms with late growth). The following organisms, procured from MTCC, were used for the assay, on solid media plates specified for their growth in MTCC catalogue with incubation at their optimum temperatures. These were:

<table>
<thead>
<tr>
<th>BACTERIA</th>
<th>MTCC no.</th>
<th>Media</th>
<th>Incubation temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter calcoaceticus</td>
<td>127</td>
<td>Nutrient Agar</td>
<td>30°C</td>
</tr>
<tr>
<td>Alcaligenes faecalis</td>
<td>126</td>
<td>Nutrient Agar</td>
<td>30°C</td>
</tr>
<tr>
<td>Bacillus mycoides</td>
<td>2910</td>
<td>Nutrient Agar</td>
<td>25°C</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>121</td>
<td>Nutrient Agar</td>
<td>30°C</td>
</tr>
<tr>
<td>E. coli</td>
<td>1610</td>
<td>Nutrient Agar</td>
<td>37°C</td>
</tr>
<tr>
<td>Yeast/Actinomycete</td>
<td>Code</td>
<td>Media</td>
<td>Temperature</td>
</tr>
<tr>
<td>------------------------</td>
<td>-------</td>
<td>---------------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Lactococcus lactis</td>
<td>1484</td>
<td>Tomato Juice Agar</td>
<td>25°C</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>1934</td>
<td>Nutrient Agar</td>
<td>37°C</td>
</tr>
<tr>
<td>Serratia marcescens</td>
<td>97</td>
<td>Nutrient Agar</td>
<td>30°C</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>1430</td>
<td>Nutrient Agar</td>
<td>37°C</td>
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<tr>
<td>Streptococcus lactis</td>
<td>440</td>
<td>Tomato Juice Agar</td>
<td>37°C</td>
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<tr>
<td><strong>YEASTS</strong></td>
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<td></td>
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</tr>
<tr>
<td>Candida albicans</td>
<td>1637</td>
<td>Yeast extract Peptone Dextrose Media (YEPD)</td>
<td>25°C</td>
</tr>
<tr>
<td>Kluyveromyces marxianus</td>
<td>242</td>
<td>Malt Yeast Agar</td>
<td>25°C</td>
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<td>Rhodotorula glutinis</td>
<td>1151</td>
<td>Malt Yeast Agar</td>
<td>30°C</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>172</td>
<td>Yeast extract Peptone Dextrose Media (YEPD)</td>
<td>30°C</td>
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<td><strong>ACTINOMYCETES</strong></td>
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<td>Arthrobacter protophormiae</td>
<td>2682</td>
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<td>30°C</td>
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<td>Micrococcus luteus</td>
<td>106</td>
<td>Nutrient Agar</td>
<td>30°C</td>
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<tr>
<td>Rhodococcus rhodochrous</td>
<td>265</td>
<td>Nutrient Agar</td>
<td>25°C</td>
</tr>
<tr>
<td>Streptomyces aureofaciens</td>
<td>325</td>
<td>CM media</td>
<td>30°C</td>
</tr>
</tbody>
</table>

Activity against methanol was also tested for each organism to rule out inhibition due to methanol (used as a solvent) rather than the compound itself. Mother culture of all the organisms to be tested were first prepared in Tryptic Soy Broth to an OD$_{600}$ of 0.5-0.8 and used as inoculum to prepare culture plates by spread plate method. Inoculum size was first standardized for each culture and the amount showing best growth used for all subsequent experiments. Wells were bored in each plate under sterile conditions. Different dilutions of individual compounds and mixture were prepared and different amounts added in bored wells to give different concentrations of the compound to be tested on the plate. This ranged from 2.5μg to 800μg for each compound.
2.3 RESULTS AND DISCUSSION

Biosurfactants are the compounds of the century owing to their great structural and functional versatility. They are produced by a wide range of microorganisms from a wide variety of carbon sources including waste substrates. From among all classes of biosurfactants rhamnolipids produced by members of *Pseudomonas* group are the most widely studied (Lang and Wullbrandt 1999). They are the most widely applied biosurfactants for use in environmental biotechnology for pollution remediation. However, despite the fact that rhamnolipid production from *Pseudomonas aeruginosa* is well documented, enormous possibility is there for discovery of new rhamnolipid congeners with altered number of rhamnose moiety or different fatty acids. Keeping this in mind, work was initiated for structural, biochemical and biological characterization of the biosurfactant isolated from *Pseudomonas aeruginosa* SSC2.

**Isolation and physicochemical characterization of biosurfactant from two media**

*Pseudomonas aeruginosa* has been known to produce rhamnolipids on a variety of substrates ranging from the hydrophobic hydrocarbons to the water soluble carbohydrates like glucose (Robert *et al.*, 1989; Guerra-Santos *et al.*, 1984). We evaluated production of rhamnolipids by SSC2 in a variety of media on the basis of the reduction in surface tension. Both the hydrophobic substrates tested and hydrophilic compounds used, exhibited biosurfactant production as indicated by the reduction in surface tension (*Figure 2.1*). This is in agreement with the observations of Haferburg *et al.*, (1986), where biosurfactant was also reported upon growth on water soluble substrates. Reduction in surface tension is an indication of biosurfactant production with maximum biosurfactant production indicated by lowest surface tension (Pruthi and Cameotra 1995). Maximum and fastest reduction in surface tension was observed upon growth of SSC2 on PPGAS (29dynescm⁻¹), a media reported to yield maximum rhamnolipid production (Mulligen *et al.*, 1989). Media containing two different hydrocarbons (n-dodecane and pristane), as expected, produced maximum rhamnolipid from hydrophobic substrate (surface tension 28.5dynescm⁻¹). However, two different substrates, PPGAS media and n-hexadecane
Figure 2.1: Change in Surface Tension by SSC2 on different growth media
in minimal medium, were chosen for analyzing the functional and structural diversity of rhamnolipids produced by *Pseudomonas aeruginosa*.

Although PPGAS media is reported to yield high amounts of rhamnolipids, yield was more in case of growth on n-hexadecane (1.4gml\(^{-1}\) as compared to 0.8gml\(^{-1}\) for PPGAS media). Biosurfactants play a very vital role in the uptake of hydrophobic compounds and hence growth on hexadecane was accompanied by a large amount of biosurfactant production indicated by the reduction in surface tension.

An evaluation of the efficiency (surface tension reduction) and effectiveness (Critical Micelle Concentration) of rhamnolipid produced from the chosen media revealed the following pictures:

<table>
<thead>
<tr>
<th>Biosurfactant producing media</th>
<th>Lowest Surface Tension (dynescm(^{-1}))</th>
<th>Maximum biomass (gL(^{-1}))</th>
<th>Maximum biosurfactant yield (gL(^{-1}))</th>
<th>CMC (mgL(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPGAS</td>
<td>29</td>
<td>1.69</td>
<td>0.8</td>
<td>1-2</td>
</tr>
<tr>
<td>Minimal media with n-hexadecane as carbon source</td>
<td>30.4</td>
<td>0.812</td>
<td>1.4</td>
<td>5-6</td>
</tr>
</tbody>
</table>

Each value is a mean of two independent sets each comprising of 3 samples.

Reduction of air water surface tension indicates the surfactant’s ability to reduce the interfacial force that holds oil and soil together. CMC is the aqueous concentration of the surfactant at which the surface tension of the solutions first shows the smallest tensional force. From an environmental standpoint, low values of CMC and highest reduction in surface tension would be the optimal characteristics a surfactant should possess in order to promote remediation of contaminated ecological niches.

Differential behaviour of rhamnolipid solution obtained from both the media was
observed with a strong effect on surface tension at low concentrations and negligible effect at high concentrations. Rhamnolipid obtained from growth on peptone (PPGAS media) rapidly lowered the surface tension from 74 dynes cm\(^{-1}\) to 32.4 dynes cm\(^{-1}\) till 2mg\(\text{L}^{-1}\). Further increase in rhamnolipid concentration only slowly decreased the surface tension from 32.4 to 29.6 dynes cm\(^{-1}\) (Figure 2.2). Similar results were obtained for rhamnolipid from hexadecane with 6mg\(\text{L}^{-1}\) being the concentration after which no significant decrease in surface tension was observed. CMC of rhamnolipid obtained from PPGAS media and growth on hexadecane was thus deduced to be 2mg\(\text{L}^{-1}\) and 6mg\(\text{L}^{-1}\) respectively. The rhamnolipid isolated thus holds potential for use in environmental biotechnology. Positive effects of surfactant has been observed at concentrations both higher and lower than CMC (Herman \textit{et al.}, 1997b; Deshpande \textit{et al.}, 1999). However, this effect is dependent on the bacterial isolates involved and their interaction with the hydrocarbon in soil/sand system. Nevertheless CMC is one of the primary criteria for evaluating biosurfactant effectiveness.

Temperature and pH stability of rhamnolipid mixture from PPGAS and hexadecane was studied at 8mg\(\text{L}^{-1}\) and 10 mg\(\text{L}^{-1}\) concentration (2CMC) respectively. Both the products were found to be stable from pH 2.0 to 8.0 (Figure 2.3) and 4\(^0\)C to 50\(^0\)C temperatures (Figure 2.4). At pH 12.0 rhamnolipids were found to lose their stability as indicated by an increase in surface tension with product from hexadecane exhibiting more instability than the product from PPGAS. On the contrary, rhamnolipid from hexadecane was stable even at 65\(^0\)C, while the product from PPGAS exhibited marked instability. Nevertheless considering the excellent surface tension reducing ability and low CMC value, rhamnolipid isolated from \textit{P. aeruginosa} SSC2 holds great potential for commercial use.

**Structural characterization of rhamnolipid mixture components**

Analyses of biosurfactant from SSC2 confirmed the product to be glycolipid as was evident by the specificity of stain used and comparison with the standard. Rhamnolipid isolated by acid precipitation from both the above selected media were found to be mixtures of at least 5 compounds as was evident by TLC analysis using Molish reagent as the staining agent (Figure 2.5). In Molish test, Conc. H\(_2\)SO\(_4\) breaks
Figure 2.2: Critical Micelle Concentration of the isolated biosurfactants
Figure 2.3: Stability of the biosurfactant produced upon growth on PPGAS medium and n-hexadecane at different pH. Change in surface tension was monitored at 2CMC for 7 days. No change in surface tension indicates stability of the biosurfactants. Only at pH 12.0, after 5 days biosurfactants were found to lose their stability indicated by an increase in surface tension, with biosurfactant from growth on n-hexadecane being slightly more unstable than that produced upon growth of SSC2 on PPGAS medium.
Figure 2.4: Stability of the biosurfactants produced at different temperatures. Surface Tension was monitored at 2CMC. At 65°C, biosurfactant produced from PPGAS showed instability.
the glycoside bond, dehydrating the formed monosaccharide to furfural derivatives after which reaction with α-naphthol results in purple condensation compound. Molish reagent failed to stain other compounds apart from rhamnose or rhamnolipid (surfactin, hexadecane, hexadecanoic acid and fructose solutions were the other compounds tested). Out of the five compounds detected on TLC, only three could be separated and purified for structural analyses by NMR and ES-MS. HPLC-ES-MS of the separated rhamnolipid congeners was performed in negative ion mode for identification of homologs. In negative electron spray ionization, only negatively charged molecules are observed. Under these conditions, molecules having an organic acid fraction, such as rhamnolipid, show intense (M-H)⁻ ions. Out of the 3 compounds characterized, mono and dirhamnolipid were the prominent species. Apart from these, we report the presence of a new rhamnolipid species (P2) containing C₁₄ (Rha-Rha-C₁₀-C₁₄) as was revealed by the mass spectra (m/z 705) and fragments obtained (479, 226). The mass of this compound was detected to be that formed upon addition of a water molecule to the compound (m/z 723; 705+H₂O). Further, presence of fragments confirmed the true identity of the compound. HPLC-ES-MS of the compounds finally revealed the following structures with P2 being the newly reported species:

P1: Rha-Rha-C₅₀-C₁₀ (m/z 649)
P2: Rha-Rha-C₁₀-C₁₄ (m/z 705)
P4: Rha-C₁₀-C₁₀ (m/z 503)  
(Figure 2.6)

Several structural homologs of rhamnolipids produced by different strains of *Pseudomonas aeruginosa* have been identified so far with the mono- and the dirhamnolipid molecules being prominent. While an extra ring confers more hydrophilicity to the rhamnolipid, additional carbon in the fatty acid chains can increase their hydrophobicity. These properties can affect the stability of rhamnolipid in the aqueous phase (as monomers or micellar conglomerates), their capability to solubilize hydrophobic organic compounds, and the bioavailability of such compounds. Hence proper elucidation of the structure assumes importance for proper usage of the surfactant in any field. Recently HPLC has emerged as an important tool
Figure 2.5: Analyses and separation of different compounds of the rhamnolipid mixture. TLC analyses revealed similar rhamnolipid congeners produced upon growth of SSC2 on PPGAS medium and on minimal medium with n-hexadecane as carbon source. Molish reagent was used as staining agent. Silica gel (230-300 mesh) was used for column chromatographic separation of mixture components.
Figure 2.6. ESI Mass Spectra of different compounds isolated from rhamnolipid mixture produced by SSC2. Apart from the parent compound, a number of smaller fragments also observed.
for the analyses of different rhamnolipid homologs with a maximum of 28 congeners reported so far (Deziel et al., 1999; Mata-Sandoval et al., 1999; Deziel et al., 2000). However, keeping in mind the great metabolic diversity of bacterial systems, more structural homologs can be expected.

Rhamnolipid mixtures obtained from both the substrates, contained a number of compounds (a total of around 13 probable congeners) as indicated in the HPLC profiles in Figure 2.7 and Figure 2.8. The corresponding m/z values were also obtained. Analyses of their masses revealed some difference in the compounds present in the each mixture according to the carbon source used (Table 2.2). With both substrates, the fatty acids found varied from C₈ to C₁₄, C₁₀ being the most abundant. The observation is in league with other works reporting several rhamnolipid homologs.

Hence rhamnolipids should always be regarded as a mixture of structurally diverse congeners and every product has to be properly structurally characterized.

**Biological activity of rhamnolipid**

The antibacterial activity of the isolated rhamnolipid mixture and individual compounds is listed in Table 2.3. Different antibacterial profile was obtained for each member of the mixture as compared to the mixture as a whole. Rhamnolipid mixture showed good antibacterial activity against gram positive bacteria and actinomycetes including *Bacillus mycoides* (6.5µg), *B. subtilis* (13µg), *S. aureus* (35µg) and *Streptomyces aureofaciens* (2.5µg) as compared to gram negative organisms. Lang and Wagner (1993) reported activity of rhamnolipid mixture against gram positive organism *Bacillus* to be 35µgml⁻¹ and Abalos et al., (2001) to be 64µgml⁻¹. Lowest MIC values reported so far for the RL mixture against gram positives (*Bacillus* strains) is 4-8µgml⁻¹ (Benincasa et al., 2004).

No activity of the rhamnolipid mixture was seen against yeasts. However, in spite of no activity shown by the rhamnolipid mixture against *Kluveromyces marxianus*, two of the purified compounds (P2 and P4) exhibited good to moderate activity. This could be attributed to the fact that the concentration of these compounds would be very low in the mixture, less than their MIC for these compounds to show their
Figure 2.7: HPLC profile of biosurfactant mixture from PPGAS medium. Figures in (red) signify the MS value of compound(s) detected at the particular Retention Time (RT).
Figure 2.8: HPLC profile of biosurfactant mixture from growth on hexadecane. Figures in red signify the MS value of compound(s) detected at the particular Retention Time (RT).
<table>
<thead>
<tr>
<th>RT</th>
<th>n- Hexadecane</th>
<th>PPGAS</th>
<th>m/z</th>
<th>Rhamnolipid Structure</th>
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<tbody>
<tr>
<td>11.04-11.22</td>
<td>-</td>
<td>+</td>
<td>621</td>
<td>Rha-Rha-C₈-C₁₀</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>Rha-Rha-C₁₀-C₈</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>703</td>
<td>Rha-Rha-C₁₂₁-C₁₂</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rha-Rha-C₁₀-C₁₄:₈:₁</td>
</tr>
<tr>
<td>12.8-12.95</td>
<td>-</td>
<td>+</td>
<td>695</td>
<td></td>
</tr>
<tr>
<td>13.39-13.92</td>
<td>+</td>
<td>+</td>
<td>649</td>
<td>Rha-Rha-C₁₀:C₁₀</td>
</tr>
<tr>
<td>15.7-15.8</td>
<td>+</td>
<td>+</td>
<td>675</td>
<td>Rha-Rha-C₁₀-C₁₂:₁</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rha-Rha-C₁₂₁:₁-C₁₀</td>
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<tr>
<td>15.88-16.20</td>
<td>+</td>
<td>+</td>
<td>503</td>
<td>Rha-C₁₀:C₁₀</td>
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<td>16.23-16.55</td>
<td>-</td>
<td>+</td>
<td>723</td>
<td>Rha-Rha-C₁₀:₁-C₁₄ (+H₂O)</td>
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<tr>
<td>17.76-17.9</td>
<td>+</td>
<td>+</td>
<td>677</td>
<td>Rha-Rha-C₁₀:C₁₂</td>
</tr>
<tr>
<td>18.5-18.6</td>
<td>+</td>
<td>-</td>
<td>529</td>
<td>Rha-C₁₀:C₁₂:₁</td>
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<td></td>
<td></td>
<td>+</td>
<td>Rha-C₈:₁-C₈</td>
</tr>
<tr>
<td>18.6-18.76</td>
<td>-</td>
<td>+</td>
<td>749</td>
<td></td>
</tr>
<tr>
<td>20.64-20.77</td>
<td>-</td>
<td>+</td>
<td>751</td>
<td>Rha-Rha-C₁₂:₁-C₁₄</td>
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<tr>
<td>20.69-20.78</td>
<td>+</td>
<td>-</td>
<td>531</td>
<td>Rha-C₁₀:C₁₂</td>
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<tr>
<td>25.05-25.18</td>
<td>+</td>
<td>-</td>
<td>679</td>
<td>677+2H (Rha-Rha-C₁₀:C₁₁:₁)</td>
</tr>
</tbody>
</table>

479: Rha-Rha-C₁₀(-OH); 333: Rha- C₁₀(-H); 163: Rhamnose (-OH);
169: C₁₀ (-H₂); 451: Rha-Rha-C₈; 507: Rha-Rha-C₁₂; 195: C₁₂₁:₁; 367: C₁₀-C₁₂/C₁₂-C₁₀

Table 2.2: Different separated rhamnolipid congeners identified according to their mass by HPLC-ES-MS
<table>
<thead>
<tr>
<th>Sl. no.</th>
<th>Name of the organism</th>
<th>MTCC no.</th>
<th>Mixture</th>
<th>P1</th>
<th>P2</th>
<th>P4</th>
<th>P5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BACTERIA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Acinetobacter calcoaceticus</td>
<td>127</td>
<td>15</td>
<td>30</td>
<td>20</td>
<td>6</td>
<td>20</td>
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<tr>
<td>2.</td>
<td>Alcaligenes faecalis</td>
<td>126</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>3.</td>
<td>Bacillus mycoides</td>
<td>2910</td>
<td>6.5</td>
<td>200</td>
<td>17</td>
<td>17</td>
<td>7</td>
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<tr>
<td>4.</td>
<td>Bacillus subtilis</td>
<td>121</td>
<td>13</td>
<td>&gt;600</td>
<td>500</td>
<td>20</td>
<td>15</td>
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<tr>
<td>5.</td>
<td>E. coli</td>
<td>1610</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>6.</td>
<td>Lactococcus lactis</td>
<td>1484</td>
<td>150</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
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<td>7.</td>
<td>Pseudomonas aeruginosa</td>
<td>1934</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
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<td>8.</td>
<td>Serratia marsescens</td>
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<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
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<td>9.</td>
<td>Staphylococcus aureus</td>
<td>1430</td>
<td>35</td>
<td>R</td>
<td>700</td>
<td>55</td>
<td>3</td>
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<td>10.</td>
<td>Streptococcus lactis</td>
<td>440</td>
<td>150</td>
<td>R</td>
<td>20</td>
<td>120</td>
<td>40</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>11.</td>
<td>Candida albicans</td>
<td>1637</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>12.</td>
<td>Kluyveromyces marxianus</td>
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<td>R</td>
<td>R</td>
<td>40</td>
<td>120</td>
<td>R</td>
</tr>
<tr>
<td>13.</td>
<td>Rhodotorula glutinis</td>
<td>1151</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>14.</td>
<td>Saccharomyces cerevisae</td>
<td>172</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>ACTINOMYCESETES</td>
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</tr>
<tr>
<td>15.</td>
<td>Arthrobacter protophormiae</td>
<td>2682</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>20</td>
</tr>
<tr>
<td>16.</td>
<td>Micrococcus luteus</td>
<td>106</td>
<td>25</td>
<td>R</td>
<td>R</td>
<td>70</td>
<td>15</td>
</tr>
<tr>
<td>17.</td>
<td>Rhodococcus rhodochrous</td>
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<td>6</td>
<td>150</td>
<td>8</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>18.</td>
<td>Streptomyces Aureofaciens</td>
<td>325</td>
<td>2.5</td>
<td>20</td>
<td>4</td>
<td>6.25</td>
<td>5</td>
</tr>
</tbody>
</table>

* Evaluated on the size of the halo formed around the well after 18-24 hours of incubation (48 hours in case of Streptococcus lactis)

Table 2.3: Antimicrobial activity of biosurfactant isolated from  
Pseudomonas aeruginosa
activity. This is the first report of a satisfactory activity of any rhamnolipid against any member of the yeast family.

Very few reports are there on the antibacterial properties of rhamnolipid and earlier works have focused on the use of rhamnolipid mixture as a whole (Haba et al., 2003; Benincasa et al., 2004). In lieu of increasing drug resistant cases, it is essential to develop new antibacterial agents with good activity at low concentrations. Biosurfactants with their biological origin and immense structural diversity offer a safe usage as a potential biomedical molecule. Different antimicrobial activity profile was obtained for the individual separated compounds. The overall general observation indicated the activity (in terms of lowest MIC) in the following order: P5 > P4 > P2 > P1, with the monorhamnolipid component (P4) being more biologically active than the dirhamnolipids (P1). Despite their ubiquitous presence and biological importance, biosurfactants have been the subject of systematic research for less than half a century, on the back of the works aimed at developing new antibiotics, when it was observed that many of the cultures had developed a surface active behavior at the end of the growth process. Considering the enormous structural diversity and biological origin of rhamnolipids and the fact that they are environmentally safe compounds, makes them an attractive candidate as new and unexplored antimicrobial agent for use in pharmaceutical industry and biomedical science. The rhamnolipid mixture isolated from SSC2 also holds great promise for both environmental applications (pollution remediation) and biomedical science (antimicrobial agent).
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