4. RHAMNOLIPID-DETECTION AND ITS INFLUENCE ON GROWTH AND CELL SURFACE HYDROPHOBICITY

4.1. Introduction

4.1.1. Background

Biodegradation of organic compounds with its limited water solubility is slow because of the low availability of these compounds to microbial cells. The availability of slightly soluble organic compounds can be enhanced by microbial produced surfactants called biosurfactants, which can increase aqueous dispersion by many orders of magnitude. In many instances, biosurfactants also stimulate the biodegradation of organic compounds. For example, alkane degradation is stimulated by rhamnolipid (Zhang and Miller, 1994). Biosurfactants are biologically synthesized surface-active agents. They are produced on microbial cell surface or excreted extracellularly and are amphipathic molecules consisting of both a hydrophobic and a hydrophilic domain. When the surfactants are extracellular, they cause the emulsification of the hydrocarbon. When they are all cell wall associated, they facilitate the penetration of hydrocarbons to the periplasmic space. The structure allows biosurfactants, to decrease interfacial tensions, such as the interfacial tension between water and non aqueous phase liquids (eg. alkanes), thereby allowing the formation of hydrocarbon in water emulsions. The hydrophobic group in biosurfactants is the hydrocarbon chain of a fatty acid, whereas the hydrophilic group is derived from the functional group based on the type of biosurfactant. It is the ester or alcohol group in case of neutral lipids, the carboxylate group for fatty acid and
amino acids or carbohydrates in the case of glycolipids (Cameotra and Makkar, 2010). Kosaric (1992) classified biosurfactants based on their structure namely, hydroxylated and cross linked fatty acids, polysaccharide lipid complexes, glycolipids, lipoproteins and phospholipids. On the other hand Biermann et al. (1987) classified the biosurfactants based on the groups as glycolipids, lipopeptides, phospholipids, fatty acids, neutral lipids polymeric and particulate compounds.

4.1.2. Mechanism of action

Biodegradation suggests that there are two mechanisms by which biosurfactants enhance the biodegradation of slightly soluble organic compounds. Firstly biosurfactants can solubilize the hydrophobic compounds within micelle structures, effectively increasing the apparent aqueous solubility of the organic compound and its availability for uptake by a cell. Secondly it can cause the cell surface to become hydrophobic thereby increasing the association of the cell with the slightly soluble substrates. Since the second mechanism requires very low level of added biosurfactants, it is the more interesting of the two mechanisms from the perspective of enhancing the biodegradation process (Shreve et al., 1995). To successfully optimize the use of biosurfactants in the bioremediation process, their effect on cell surfaces must be understood. Biosurfactants causes the cell surface of organisms to become hydrophobic through release of lipopolysaccharide (LPS). The amount of LPS was found to be dependent on rhamnolipid concentration, but significant loss occurred even at concentration less than the critical micelle concentration (Al-Tahhan et al., 2000).
The outer leaflet of *P. aeruginosa*, composed of LPS, with three components. The first component is the lipid A tail, which is anchored into the hydrophobic region of the outer membrane. The second is the core oligosaccharide, which contains a unique 8-carbon sugar called 2-keto-3-deoxyoctonic acid (KDO). The third component is the O antigen which consists of 15 to 20 repeating monomers of a 3-5 sugar subunits (Rocchetta *et al.*, 1999). The presence of smooth LPS results in a relatively hydrophilic cell surface that is permeable to small hydrophilic molecules (molecular weight < 600), but excludes hydrophobic molecules. The O-antigen region contacts the surrounding environment and directly impacts nonspecific cell surface properties, such as hydrophobicity. *P. aeruginosa* coexpress two distinct LPS is composed of 2, 3 D-Rhamnose trisaccharide repeating units, while the longer B-band LPS contains numerous monosaccharide, arranged as di- to pentasaccharide units. While the B-band LPS often marks the underlying A-band molecules, variations in growth condition can alter LPS expression, resulting in cell surface properties (Norman *et al.*, 2002). LPS mutants which have lost the O-antigen component have increased affinity for hydrophobic probes. The loss of LPS from the outer leaflet of the outer membrane may cause a temporary exposure of the hydrophobic phospholipid fatty acid tails associated with the inner leaflet of the outer membrane. Alternatively loss of LPS may decrease the compaction of the outer leaflet of the outer membrane, allowing increased passage of large hydrophobic compounds (Al-Tahhan *et al.*, 2000).
4.1.3. Microorganisms

Biosurfactants are produced by certain bacteria and by a number of yeasts and filamentous fungi (Table 4.1). They include low-molecular weight glycolipids, lipopeptides, and high-molecular-weight lipid-containing polymers such as lipoproteins, LPS-protein complexes, and polysaccharides. Among the hydrocarbon utilizing bacteria, *P. aeruginosa* is one of the most frequently isolated organisms from hydrocarbon impacted environment and capable of producing metabolites (i.e. alginate, rhamnolipid, pyocyanin) that enhance its competitiveness and survival (Sarubbo *et al.*, 2006). The ability of *Pseudomonas* sp. to utilize hydrocarbons as a source of energy is well known. In most cases, however, the rate of utilization is slow when compared to readily soluble compounds like sugars. Studies have shown that biosurfactants, in particular rhamnolipid produced by *P. aeruginosa*, enhances hydrocarbon biodegradation rates. Rhamnolipid not only increased apparent hydrocarbon solubility, but also modified the cell surface, resulting in increased hydrophobicity. The addition of rhamnolipid at concentrations less than the critical micelle concentration (CMC) induced formation of multicellular aggregates, implying that the cells forming these aggregates are hydrophobic in nature (Al-

Tahhan *et al.*, 2000).
Table 4.1. Types of biosurfactants produced by various microorganisms

<table>
<thead>
<tr>
<th>Microbes</th>
<th>Types of surfactants produced</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Torulopsis bombicola</em></td>
<td>Glycolipid (sophorose lipid)</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Glycolipid (Rhamnose lipid)</td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em></td>
<td>Lipoprotein (surfactin)</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>Lipoprotein (surfactin)</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp. DSM 2874</td>
<td>Glycolipids (Rhamnose lipid)</td>
</tr>
<tr>
<td><em>Arthrobacter paraffineus</em></td>
<td>Sucrose and Fructose Glycolipids</td>
</tr>
<tr>
<td><em>Arthrobacter</em> sp.</td>
<td>Glycolipid</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td>Rhamnose lipid</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp. MUB</td>
<td>Rhamnose lipid</td>
</tr>
<tr>
<td><em>Torulopsis petrophilum</em></td>
<td>Glycolipid and/or protein</td>
</tr>
<tr>
<td><em>Candida tropicalis</em></td>
<td>Polysaccharide-fatty acid complex</td>
</tr>
</tbody>
</table>

**Source:** Kosaric (1992)

### 4.1.4. Rhamnolipids

Among various biosurfactants rhamnolipids are considered to be the most in degrading hydrocarbons. For example, the biodegradation of long chain alkanes was stimulated by addition of rhamnolipid. The facilitated biodegradation is probably due to the increase of cell surface hydrophobicity after extraction of lipopolysaccharides from cellular envelop by rhamnolipid, which subsequently stimulates uptake via direct contact between cells and hydrocarbons droplets. Thus, the interaction
between addition of rhamnolipid and biodegradation of hydrocarbon seems to be highly specific (Itoh and Suzuki, 1972).

*Pseudomonas* sp. are well known for their ability to produce rhamnolipid biosurfactants with potential surface active properties when grown on different carbon substrates and therefore is a promising candidate for large scale production of biosurfactants. Among *Pseudomonas* sp., *P. aeruginosa* is widely studied for the production of glycolipid type biosurfactants. However, glycolipid type biosurfactants are also reported from some other species like *P. putida* and *P. chlororaphis* (Das and Chandran, 2011). Four different rhamnolipid homologous (Fig.4.1), produced by *P. aeruginosa*, have been identified and characterized. The rhamnolipid consists of one or two L-rhamnose units and one or two units of β-hydroxy decanoic acid. RL-1 and RL-3 are the principal rhamnolipid produced. RL-2 and RL-4 are biosynthesized under certain cultivation conditions only (Tahzibi *et al.*, 2004).

Jarvis and Johnson (1949) were first described the rhamnolipid from *P. aeruginosa*, however the biosynthesis of rhamnolipid was carried out *invitro* by Hauser and Karnofsky (1954), who showed that these glycolipids were secreted into the medium during the stationary phase of growth. Hauser and Karnofsky (1958) also defined the optimal conditions for rhamnolipid production by *P. aeruginosa* from various radioactive precursors, such as acetate, glycerol, glucose and fructose. Burger *et al.* (1963) described the complete enzymatic synthesis of a rhamnolipid by extracts of *P. aeruginosa*. Since *P. aeruginosa* is known to produce two kinds of hemolytic activities i.e. the heat-labile hemolysin, which is a potent phospholipase
and the heat-stable hemolysins, which are rhamnolipids. The structure and mechanism of synthesis of this product have been elucidated by biochemical studies, whereas its hemolytic activity and other toxic manifestations have been the focus of interest of several investigations by microbiologists.

**Fig. 4.1. Structure of Rhamnolipid**

![Structure of Rhamnolipid](image)


Biosurfactants can act as emulsifying agents by decreasing the surface tension and forming micelles. The micro droplets encapsulated in the hydrophobic microbial cell surface are taken inside and degraded. Fig.4.2. demonstrates the involvement of biosurfactant (rhamnolipids) produced by *Pseudomonas* sp. and the mechanism of formation of micelles in the uptake of hydrocarbons. Although
biosurfactants have many interesting properties, their industrial importance is dependent upon ease of production. Low yields of biosurfactant are major factor jeopardizing its popularity. Recently, efforts have been made to increase yield of biosurfactant by modifying nutritional and environmental factors (Tahzibi et al., 2004).

**Fig. 4.2. Involvement of rhamnolipid produced by *Pseudomonas* sp in the uptake of hydrocarbons**

![Diagram showing the involvement of rhamnolipid in the uptake of hydrocarbons](image)

**Source**: Das and Chandran (2011)

The present chapter (chapter 4) deals with the screening of the isolate *P. aeruginosa* JQ062961 for its production of rhamnolipid. *P. aeruginosa* JQ062961 was selected, since it was optimised to exhibit highest crude oil degrading efficiency of 65% among the three isolates tested in Chapter 3. The production of rhamnolipid
was detected both by qualitative and quantitative methods. Qualitative detection was
demonstrated by hemolytic activity. Quantitative analysis was carried out by orcinol
assay and by a series of chromatographic analysis such as Thin Layer
Chromatography (TLC), High Performance Thin Layer Chromatography (HPTLC)
and Gas Chromatography-Mass Spectrometry (GCMS). Also growth experiments to
investigate the rhamnolipid-induced chemical and structural changes of the cell
surface were performed simultaneously by measuring the cell growth, cell surface
hydrophobicity, and LPS release.

4.2. Materials and Methods

4.2.1. Screening for rhamnolipid production

The isolate P. aeruginosa JQ06296, which was already optimized for crude oil
degrading ability was screened for rhamnolipid production by qualitative and
quantitative methods. Qualitative analysis was performed by hemolysis and
quantitative analysis was carried out by orcinol assay and chromatographic methods.
P. aeruginosa MTCC 1034 was used as a standard reference in the detection of
rhamnolipid production by hemolytic activity (qualitative), orcinol assay
(quantitative) and TLC (chromatographic).

The P. aeruginosa JQ062961 isolate and P. aeruginosa MTCC 1034 were
inoculated individually into two 100ml Erlen Meyer flasks containing 50ml of Kay’s
minimal medium and incubated for 24hr at 30°C with orbital shaking at 250 rpm.
1ml each of the inoculum was withdrawn from Kay’s minimal medium from both
groups and inoculated into 100ml of sterile Proteose Peptone-Glucose-Ammonium Salts (PPGAS) medium individually and incubated for 72hr at 30°C with orbital shaking at 250 rpm. The culture was then centrifuged at 10000 rpm for 30 min. The supernatant obtained from each group was stored in sterile screw cap containers separately and stored at 4°C for further analysis.

4.2.2. Qualitative analysis (Haemolytic activity)

10ml each of culture supernatants (pH 6.5) of *P. aeruginosa* JQ062961 and *P. aeruginosa* MTCC 1034 were concentrated individually by the addition of 75mM ZnCl₂. The precipitate obtained were dissolved in 10ml of 0.1M sodium phosphate buffer (pH 6.5) and extracted twice with equal volumes of diethyl ether. The pooled organic phases were evaporated to dryness, and the pellets were dissolved individually in 100μl of ethanol. 10μl of concentrated culture supernatants of both the strains were applied aseptically to the sterile Himedia filter paper discs (6.0mm Whatman AA) individually and dried. The discs were then placed on sterile Blood agar plates prepared by adding 5% v/v, sterile defibrinated sheep blood to sterile blood agar base, incubated at room temperature for 2 days and observed for zone (mm) of hemolysis. Uninoculated sterile filter paper disc was used as a control (Tuleva *et al.*, 2002).

4.2.3. Quantitative analysis (Orcinol assay)

Rhamnolipid concentration of *P. aeruginosa* JQ062961 and *P. aeruginosa* MTCC 1034 were estimated individually in triplicates. 333μl of the culture supernatants were extracted twice with 1ml of diethyl ether. The ether fractions were
evaporated to dryness and 0.5ml of distilled water was added. To 100µl of each sample 900µl of solution containing 0.19% orcinol in 53% H₂SO₄ was added; after heating for 30min at 80°C, the samples were cooled for 15min at room temperature and the absorbance was measured by UV spectrophotometer at 421nm. The rhamnolipid concentrations were quantified from the standard L-rhamnose calibration curve between 0 and 0.5g·l⁻¹ and the results were expressed as RE (rhamnose equivalents; g·l⁻¹) by multiplying rhamnose values by a coefficient of 3.4, obtained from the correlation of pure rhamnolipids/rhamnose. The uninoculated broth processed by the same procedure was used as a blank (Chandrasekaran and Bemiller, 1980).

4.2.4. Chromatographic analysis

To gravimetrically detect rhamnolipids in a liquid medium, it requires a preliminary separation step prior to the analysis inorder to eliminate other interfering compounds that may contain in the supernatant (Ahmad et al., 2011). The remaining supernatants of *P. aeruginosa* JQ062961 and *P. aeruginosa* MTCC 1034 were individually adjusted to pH 2.0 with concentrated HCl and allowed to stand overnight at 4°C. Then they were centrifuged separately at 10000 rpm for 20min. Following centrifugation, the precipitates were dissolved individually in 0.4M HCl and extracted twice with chloroform – ethanol (2:1v/v) mixture, for 4hr. The mixture was shaken vigorously and left static for phase separation. The organic phase was evaporated at 40°C and the crude rhamnolipid was left over as a residue. The crude rhamnolipid obtained from both the strains were individually dissolved in 0.05M
sodium bicarbonate (pH 8.6) and stored at 4°C until further analysis (Monteiro et al., 2007).

4.2.4.1. Analysis by Thin layer chromatography (TLC)

TLC plates were prepared using silica gel as the absorbent, and the prepared plates were activated at 110°C for 30min, cooled in a desiccator. 50µl each of the samples of *P. aeruginosa* JQ062961 and *P. aeruginosa* MTCC 1034 were spotted separately. The plates were developed in petroleum ether: diethyl ether: glacial acetic acid (80:20:1) solvent system till they travel up to 1cm from the opposite side of the plate. The plates were dried and solution containing 50% Orcinol H₂SO₄ was sprayed over the plates and heated in hot air oven at 110°C for 10min and observed for brown color band formation (Monteiro et al., 2007).

4.2.4.2. High performance thin layer chromatography (HPTLC) analysis

HPTLC was carried out using a CAMAG TLC system, USA composed of an automatic TLC sampler (CAMAG LINOMAT IV), automatic development trough (20x20cm), detector (CAMAG TLC Scanner II), and an electronic integrator (CATS 3.20 version software). An aliquot of 15µl of the crude rhamnolipid sample obtained from isolate *P. aeruginosa* JQ062961 was applied on to a 20 x 20 cm aluminum sheet, precoated with silica gel 60 F₂₅₄ Merck plate of 0.2mm thickness. The sample was loaded at a dosage speed of 50 nl/s under nitrogen stream and developed using 10 ml of the mobile phase of CHCl₃/CH₃OH/H₂O (65:25:4) in plates preconditioned for 3min, to a migration distance of 85mm. The plate was dried and sprayed with orcinol reagent (0.19% orcinol in 53% H₂SO₄), and then put in a
hot-air oven at 120°C for 15min. The developed chromatogram was scanned at 550nm. From the signals recovered from the scanner the Rf values were automatically calculated using the CATS software.

4.2.4.3. Gas Chromatography and Mass Spectrophotometric (GC/MS) analysis

GC/MS analysis was carried out using The JEOL GCMate II, Japan, is a high resolution double focusing magnetic GC mass spectrometer equipped with ion sources for electron impact (EI), chemical ionization (CI) and fast atom bombardment (FAB) by direct insertion probe and is capable of exact mass measurements for elemental composition determinations. The spectrometer is equipped with high energy Collision Induced Dissociation (CID) linked scan unit allowing MS-MS fragmentation experiments (product ion scans, precursor scans, constant neutral loss scans) for use in structural analysis. The mass spectrometer's data processing software automatically bridges to the NIST Mass Spectral Database Library for searching unknown compounds. Because of the high molecular weight, rhamnolipids cannot be directly analysed by GCMS. The sample need to be acid hydrolyzed inorder to break the cleavage between the carbohydrate and lipid portions and subsequent derivatization of the resulting fatty acid chains to fatty acid methyl esters (FAME) and further conversion to trimethylsilyl (TMS) ether derivatives (Yakimov et al., 1995). The positive spots of the isolate P. aeruginosa JQ062961 from the TLC plate were scraped and to this 1ml of chloroform and 0.85 ml of methanol were added. The sample was centrifuged at 6000 rpm for 10min to remove the silica gel particles. 0.15 ml of sulfuric acid was added and heated at 100°C for
140 min. Mixed with 1 ml of distilled water and vortexed vigorously for 1 min. Left to stand for phase separation. The bottom layer (chloroform layer) containing the FAME was removed. FAME was then recovered with hexane and then concentrated to 1 ml under nitrogen. Rhamnolipid derivatives were analyzed after trimethylsilylation at 70°C for 1 hr with 50% BSTFA (bistrimethylsilyl trifluoroacetamide). 1 μl of the sample was injected into JEOL GC MATE II, instrument with the injection temperature 225°C, column HP5, carrier gas helium, column oven temperature range 70-250°C and the rate of temperature at 20°C.

4.2.5. Growth experiments

Growth experiments were performed simultaneously to relate the influence of rhamnolipid addition to the medium on cell growth, cell surface hydrophobicity, and Lipopolysaccharide (LPS) release. Cell growth was determined by optical density (OD) and protein analysis was performed to understand the viability of the cells throughout the experimental period. Cell surface hydrophobicity was measured by bacterial adherence to hydrocarbon (BATH) assay as more release of LPS into medium will make the bacterial cell surface hydrophobic, thereby increasing the passage of large hydrophobic (hydrocarbons). LPS release to the supernatant was assessed by 2-keto-3-deoxyoctonic acid (KDO) analysis (Al-Tahhan et al., 2000; Norman et al., 2002).

The effect of rhamnolipid on the above parameters was performed for the test strain *P. aeruginosa* JQ062961 and *P. aeruginosa* MTCC 1034 was used as a
standard reference. Both the strains were grown on a soluble (1% [wt/vol] glucose) and a slightly soluble (1% [vol/vol] hexadecane) substrates. All growth experiments were performed in Mineral salt medium (MSM) in the presence and absence of rhamnolipid. The medium was adjusted to pH 7.2 and supplied with 1% glucose or hexadecane. The test isolate and the standard MTCC culture were prepared individually in MSM and grown to late exponential phase for 24hr at 30°C. In each experiment 1% inoculum was supplied and was performed in triplicates in 1 liter flasks containing a total volume of 200ml of culture.

**4.2.5.1. Growth in Mineral Salt Medium (MSM) containing glucose**

The test isolate and the standard MTCC culture were inoculated individually in two 1000ml Erlen Meyer flasks containing 20 ml of Mineral salt medium (MSM), at 7.2 pH with 1% glucose. The inoculated flasks were incubated at 30°C with shaking at 200 rpm. One uninoculated 1000ml flask containing 200ml of sterile MSM with 1% glucose was kept as control. From each flask, the culture was aseptically sampled for analytical procedures once in every 4hr, until the growth reached to the stationary phase.

**4.2.5.2. Growth in MSM containing glucose and rhamnolipid**

The test isolate and the standard MTCC culture were inoculated individually into two 1000ml Erlen Meyer flasks containing 200ml of MSM (pH 7.2) with 5mM rhamnolipid and 1% glucose. The inoculated flasks were incubated at 30°C with shaking at 200 rpm. One uninoculated 1000ml flask containing 200ml of sterile MSM with 5mM rhamnolipid and 1% glucose was kept as control. From each flask
the culture was aseptically sampled for analytical procedures once in every 4hr, until the growth reached to the stationary phase.

**4.2.5.3. Growth in MSM containing hexadecane**

The test isolate and the standard MTCC culture were inoculated individually in to two 1000ml Erlen Meyer flasks containing 200ml of MSM (pH 7.2) with 1% hexadecane. The inoculated flasks were incubated at 30°C with shaking at 200rpm. One uninoculated 1000 ml flask containing 200ml of sterile MSM with 1% hexadecane was kept as control. From each flask the culture was aseptically sampled for analytical procedures on day 0 and day 1, followed by once in every 4 days, until the growth reached to the stationary phase.

**4.2.5.4. Growth in MSM containing hexadecane and rhamnolipid**

The test isolate and the standard MTCC culture were inoculated individually in to two 1000ml Erlen Meyer flasks containing 200ml of MSM (pH 7.2) with 5mM rhamnolipid and 1% hexadecane. The inoculated flasks were incubated at 30°C with shaking at 200 rpm. One uninoculated 1000ml flask containing 200ml of sterile MSM with 5mM rhamnolipid and 1% hexadecane was kept as control. From each flask the culture was aseptically sampled for analytical procedures on day 0 and day 1, followed by once in every 4 days, until growth reached to the stationary phase.

**4.2.5.5. Measurement of growth (Optical density - OD)**

1ml of the sample was withdrawn from each of the flask of the above experiments (4.2.5.1 to 4.2.5.4) and OD was measured at 540nm at periodic intervals as mentioned above for the respective substrates.
4.2.5.6. Protein analysis

1ml of cell suspension from each flask was withdrawn individually, washed twice and then resuspended in sterile water. 0.1N NaOH was added, and the cell suspension was heated at 100°C to lyse cells. The hydrolysate was cooled to room temperature and 1ml of freshly mixed complex forming reagent (2% Na₂CO₃, 1% CuSO₄, 2% sodium potassium tartarate) was added. The solution was allowed to stand at room temperature for 10min. 1ml of folin ciocalteu reagent was added and vortexed and the mixture was allowed to stand at room temperature for 30-60min. The absorbance was read at 750nm. Standard curve of absorbance was plotted as a function of initial protein concentration and used to determine the unknown protein concentration (Lowry et al., 1951).

4.2.5.7. BATH assay

A sample volume of 4.0ml was withdrawn from each flask individually and washed with Mineral salt medium (MSM) five times to remove the interference of the added rhamnolipid from the cell pellet. Cells were resuspended in MSM and adjusted to an OD of 1.0 ±0.01 at 400 nm. 1ml of hexadecane was added to 4 ml of the adjusted cell suspension in a 16 x 100mm test tube and vortexed for 1min. The mixture was then allowed to settle and separate for 30min and the turbidity of the aqueous phase was measured at 600nm in a spectrophotometer. Hydrophobicity was expressed as the percentage of cell adherence to crude oil calculated as follows:

\[
\frac{1-\text{OD of the aqueous phase}}{\text{OD of the initial cell suspension}} \times 100
\]
For a given sample, three independent determinations were made and the mean value was accounted (Zhang and Miller, 1992).

4.2.5.8. LPS analysis by thiobarbituric acid method (KDO assay)

Sample volume of 1.0ml was withdrawn from each flask individually and centrifuged at 6000 rpm for 10min. 0.1ml of the supernatant from each sample was withdrawn and placed in a screw-cap tube, and 0.1ml of 0.036N H₂SO₄ was added. The mixture was hydrolyzed at 100°C for 20min to liberate 2-keto-3-deoxyoctonic acid (KDO) and then cooled. The mixture was further acidified by adding 0.25ml of 0.025N HIO₄ in 0.125N H₂SO₄ and allowed to stand for 20min at room temperature. Sodium arsenate (2%, 0.5ml in 0.5N HCl) was added with shaking, and the tubes were allowed to stand for 2min, followed by addition of 2.0ml of 0.3% thiobarbituric acid (pH 2) with stirring. The tubes were then heated at 100°C for 10min, allowed to cool, centrifuged, and the absorbance of each sample was measured at 548nm (Osborn et al., 1972).

4.2.5.9. Statistical Analysis

The data obtained in the present study were expressed as Mean ± SD and were analysed using student ‘t’ test at 5% level of significance using computer software STATISTICA 6.0 (Statsoft, Bedford, UK).
4.3. Results
4.3.1. Haemolytic activity

The result showed that the rhamnolipid produced by both the test isolate
*P. aeruginosa* JQ062961 and the reference strain *P. aeruginosa* MTCC 1034 was
found to exhibit the zone of clearance in red blood cells in sheep blood agar.

*P. aeruginosa* JQ062961 recorded a maximum haemolytic zone of 22 ±1.0mm and
*P. aeruginosa* MTCC 1034, exhibited only 17 ±1.0mm (Plate 4.1).

Plate 4.1. Haemolytic activity on sheep blood agar exhibited by *P. aeruginosa*
JQ062961 and *P. aeruginosa* MTCC 1034

4.3.2. Orcinol assay (Rhamnolipid production)

The quantification of rhamnolipid by orcinol assay method revealed that
*P. aeruginosa* JQ062961 utilized glucose as a substrate and produced rhamnolipid
(rhamnose equivalent). *P. aeruginosa* JQ062961 produced 0.277 ±0.020g/l\(^1\) rhamnolipid, which is relatively higher than that of rhamnolipid produced (0.268
±0.010g/l\(^1\)) by the reference *P. aeruginosa* MTCC 1034 (Table 4.2).
Table 4.2. Orcinol assay -Production of rhamnolipid (g l\(^{-1}\)) by experimental and reference \textit{P. aeruginosa} strains

<table>
<thead>
<tr>
<th>S.no.</th>
<th>Strains</th>
<th>Rhamnolipid production (g l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>\textit{P. aeruginosa} JQ062961</td>
<td>0.277 ± 0.020</td>
</tr>
<tr>
<td>2</td>
<td>\textit{P. aeruginosa} MTCC 1034</td>
<td>0.268 ± 0.010</td>
</tr>
</tbody>
</table>

4.3.3. Analysis by Thin layer chromatography (TLC)

The rhamnolipids produced by \textit{P. aeruginosa} JQ062961 and \textit{P. aeruginosa} MTCC 1034 were confirmed through TLC. The result showed that \textit{P. aeruginosa} JQ062961 had produced two orcinol-positive spots, the higher one (low molecular weight) representing mono-rhamnolipid and the lower spot (high molecular weight) representing di- rhamnolipid at \textit{Rf} 0.51 and \textit{Rf} 0.32, respectively. Similarly \textit{P. aeruginosa} MTCC 1034 had also produced two orcinol positive spots, at \textit{Rf} 0.33 and 0.51 corresponding to di and mono rhamnolipids, respectively (Table 4.3 and Plate 4.2).

Table 4.3. TLC result on \textit{Rf} values of rhamnolipid produced by experimental and reference \textit{P. aeruginosa} strains

<table>
<thead>
<tr>
<th>S.no.</th>
<th>Strains</th>
<th>\textit{Rf} value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Spot 1 (Di-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rhamnolipid)</td>
</tr>
<tr>
<td>1</td>
<td>\textit{P. aeruginosa} JQ062961</td>
<td>0.32</td>
</tr>
<tr>
<td>2</td>
<td>\textit{P. aeruginosa} MTCC 1034</td>
<td>0.33</td>
</tr>
</tbody>
</table>
Plate 4.2. Rhamnolipids produced by *P. aeruginosa* JQ062961 and *P. aeruginosa* MTCC 1034 on TLC plate

4.3.4. High performance thin layer chromatography (HPTLC) analysis

The rhamnolipid produced by *P. aeruginosa* JQ062961 was analysed by HPTLC using CAMAG TLC system. The result showed the presence of both the homologues mono and di-rhamnolipids having the respective *Rf* values of 0.14 and 0.05 with the area of percentage of 69.62 and 30.38%, respectively (Figure 4.3).

4.3.5. Gas chromatography –mass spectrometry (GCMS) analysis

The fatty acid methyl ester (FAME) mass spectroscopic analysis of the rhamnolipid produced by *P. aeruginosa* JQ062961 showed the presence of five different types of silyl and ester derivatives of decanoic acid as illustrated in Table 4.4 and Fig. 4.4a to 4.4f, which confirmed that the biosurfactant is of rhamnolipid type.
Fig. 4.3. HPTLC spectrum of mono and di-rhamnolipids produced by *P. aeruginosa* JQ062961

Spectrum: M: FES_RHAM
Method: Scan
Integration: Calibration Spectrum Data END
Analysis b: RHAMNOLIPID

Wavelength: 254 nm
Track: 2, noise level: 0.063mV, raw data file: FES_RHAM
Track 2, Analysis b: RHAMNOLIPID
The compound with the major abundance was found to be silyl and ester derivatives of Octadecatrienoic acid (60.72%), followed by ester derivative of Decadienoic acid (12.14%), ester derivative of Hexadecanoic acid (12.15%), silyl and ester derivative of Eicosanoic acid (8.09%) and finally ester derivative of Tetradecanoic acid (8.09%).

**Table 4.4. GCMS analysis of silyl and ester derivatives of rhamnolipid produced by *P. aeruginosa* JQ062961**

<table>
<thead>
<tr>
<th>S.no</th>
<th>Fatty acid</th>
<th>Molecular weight</th>
<th>Molecular formula</th>
<th>Retention time</th>
<th>Area</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9,12,15-Octadecatrienoic acid, 2-[(trimethylsilyl)oxy]-1-[(trimethylsilyl)oxy]methyl] ethyl ester</td>
<td>496.87</td>
<td>C_{27}H_{52}O_4S_{i2}</td>
<td>11.58</td>
<td>992017</td>
<td>60.72</td>
</tr>
<tr>
<td>2</td>
<td>2,6-Decadienoic acid, 8-methyl-4-propyl, ethyl ester</td>
<td>252.39</td>
<td>C_{16}H_{28}O_2</td>
<td>15.38</td>
<td>198403</td>
<td>12.14</td>
</tr>
<tr>
<td>3</td>
<td>Hexadecanoic acid, methyl ester</td>
<td>270.45</td>
<td>C_{17}H_{34}O_2</td>
<td>17.37</td>
<td>198433</td>
<td>12.15</td>
</tr>
<tr>
<td>4</td>
<td>Eicosanoic acid, 2,3-bis[(Trimethyl silyl)oxy] propyl ester</td>
<td>530.97</td>
<td>C_{29}H_{62}O_4S_{i2}</td>
<td>18.77</td>
<td>132269</td>
<td>8.09</td>
</tr>
<tr>
<td>5</td>
<td>Tetradecanoic acid, 12-methyl-1- methyl ester</td>
<td>256.42</td>
<td>C_{16}H_{32}O_2</td>
<td>20.13</td>
<td>132269</td>
<td>8.09</td>
</tr>
</tbody>
</table>
Fig. 4.4a. GC-MS spectra of rhamnolipid and its principle fragmentations produced by *P. aeruginosa* JQ062961
Fig. 4.4b. Mass spectrum of 9,12,15-Octadecatrienoic acid, 2-[(trimethylsilyl)oxy]-1-[(trimethylsilyl)oxy]methyl]ethyl ester fragment produced by *P. aeruginosa* JQ062961

9,12,15-Octadecatrienoic acid, 2-[(trimethylsilyl)oxy]-1-[(trimethylsilyl)oxy]methyl]ethyl ester

![Mass spectrum graph](image)

RT 11.58

C_{27}H_{52}O_4Si_2

Mass-to-charge ratio (m/z)
Fig. 4.4c. Mass spectrum of 2,6-Decadienoic acid, 8-methyl-4-propyl, ethyl ester fragment produced by *P. aeruginosa* JQ062961
Fig. 4.4d. Mass spectrum of Hexadecanoic acid, methyl ester fragment produced by *P. aeruginosa* JQ062961
Eicosanoic acid, 2,3-bis[(trimethylsilyl) oxy] propyl ester fragment produced by P. aeruginosa

**Mass spectrum of Eicosanoic acid, 2,3-bis((trimethylsilyl) oxy) propyl ester**

- **RT**: 18.77
- **Mass-to-charge ratio (m/z)**

The mass spectrum shows the fragmentation pattern of the Eicosanoic acid, 2,3-bis((trimethylsilyl) oxy) propyl ester fragment produced by P. aeruginosa, with the mass-to-charge ratio (m/z) values indicated along the y-axis and the percentage values on the x-axis.
Fig. 4.4f. Mass spectrum of Tetradecanoic acid, 12-methyl, methyl ester fragment produced by P. aeruginosa JQ062961
4.3.6. Growth experiments with glucose as substrate

4.3.6.1. Growth analysis by turbidometry (Optical density)

In the present study growth of the strains *P. aeruginosa* JQ062961 and *P. aeruginosa* MTCC 1034 in 1% glucose was closely related both with and without the addition of rhamnolipid. Also there was no difference observed in the phases of growth. The lag phase was observed till 4\(^{th}\) hr, exponential phase lasted till 20\(^{th}\) hr, followed by the stationary phase till 28\(^{th}\) hr and the decline phase commenced at 32\(^{nd}\) hr (Fig.4.5).

4.3.6.2. Protein estimation

Similarly in protein estimation, the presence of rhamnolipid didn’t found to have any prominent effect for both the strains with glucose as substrate. Maximum protein concentration by *P. aeruginosa* JQ062961 and *P. aeruginosa* MTCC 1034 in the presence of rhamnolipid was 51.0 ±0.82 and 44.0 ±1.63µgml\(^{-1}\), respectively and in the absence of rhamnolipid it was 50.0 ±2.48 and 42.0 ±1.63µgml\(^{-1}\), respectively (Fig.4.5). The student ‘t’ test revealed that the difference on protein production by the test organisms grown in glucose as well as glucose plus rhamnolipid substituted media were statistically more significant (*t* = 36.30 to 94.00; *P* <0.001 to *P*< 0.0001) (Table 4.5).

4.3.6.3. Cell surface hydrophobicity (BATH assay)

In terms of cell surface hydrophobicity as measured by BATH assay, both the organisms, grown on glucose in the absence of rhamnolipid exhibited a relatively low cell surface hydrophobicity at all stages of growth, recorded a maximum of
29 ±1.68 and 28 ±1.63% at the late stationary phase by *P. aeruginosa* JQ062961 and *P. aeruginosa* MTCC 1034, respectively. In contrast, rhamnolipid addition caused cell surface hydrophobicity with slow increase throughout the exponential phase and reached 32.0 ±1.63% adherence in the late exponential phase, then rapidly increased to 71.0 ±2.81% adherence at the end of the stationary phase, followed by 69.0 ±1.27% in the decline phase for *P. aeruginosa* JQ062961. The cell surface hydrophobicity of *P. aeruginosa* MTCC 1034 also increased throughout the exponential phase to reach 28.0 ±1.63 % adherence in the late exponential phase and then rapidly increased to 58.0 ±1.45% adherence at the end of the stationary phase, followed by 56.0 ±0.82% in the decline phase (Fig.4.5). The statistical student ‘t’ test revealed that the difference on cell surface hydrophobicity (BATH) by the test organisms grown in glucose as well as glucose plus rhamnolipid substituted media were highly significant (*t* = 17.320 to 53.766; *P* <0.01 to *P*< 0.0001) (Table 4.5).
Fig. 4.5. Growth, protein estimation, cell surface hydrophobicity (BATH), LPS release (KDO) of *P. aeruginosa* JQ062961 and *P. aeruginosa* MTCC 1034 grown on 1% glucose in the presence and absence of rhamnolipid

Table 4.5. Summary table comparison of growth experiment on
different parameters with glucose and hexadecane as substrates between experimental *P. aeruginosa* JQ062961 and *P. aeruginosa* MTCC 1034

<table>
<thead>
<tr>
<th>Growth experiment with substrates</th>
<th>Parameters</th>
<th>t - value</th>
<th>P - value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>Protein</td>
<td>94.00</td>
<td>P&lt; 0.0001*</td>
</tr>
<tr>
<td></td>
<td>BATH</td>
<td>17.320</td>
<td>P&lt; 0.001*</td>
</tr>
<tr>
<td></td>
<td>LPS release</td>
<td>2.1650</td>
<td>P&gt; 0.05**</td>
</tr>
<tr>
<td>Glucose + Rhamnolipid</td>
<td>Protein</td>
<td>36.30</td>
<td>P&lt; 0.001*</td>
</tr>
<tr>
<td></td>
<td>BATH</td>
<td>53.766</td>
<td>P&lt; 0.0001*</td>
</tr>
<tr>
<td></td>
<td>LPS release</td>
<td>6.298</td>
<td>P&lt; 0.01*</td>
</tr>
<tr>
<td>Hexadecane</td>
<td>Protein</td>
<td>62.353</td>
<td>P&lt; 0.0001*</td>
</tr>
<tr>
<td></td>
<td>BATH</td>
<td>7.784</td>
<td>P&lt; 0.01*</td>
</tr>
<tr>
<td></td>
<td>LPS release</td>
<td>25.980</td>
<td>P&lt; 0.001*</td>
</tr>
<tr>
<td>Hexadecane + Rhamnolipid</td>
<td>Protein</td>
<td>44.538</td>
<td>P&lt; 0.001*</td>
</tr>
<tr>
<td></td>
<td>BATH</td>
<td>21.065</td>
<td>P&lt; 0.001*</td>
</tr>
<tr>
<td></td>
<td>LPS release</td>
<td>34.641</td>
<td>P&lt; 0.001*</td>
</tr>
</tbody>
</table>

* Statistically significant ; ** Statistically non significant

### 4.3.6.4. LPS release (KDO assay)

KDO analysis of LPS release to the supernatant by *P. aeruginosa* JQ062961 was found to be a maximum of 1.0 ±0.08μg in glucose alone supplied medium during decline phase. At the same time, the values for *P. aeruginosa* MTCC 1034 strain was also found to be similar (0.9 ±0.06μg) on the same medium. However,
LPS release was found to be comparatively higher for both the strains when exposed to glucose and rhamnolipid supplied medium i.e., 2.1 ± 0.021µg for *P. aeruginosa* JQ062961 and 1.7 ± 0.016µg for *P. aeruginosa* MTCC 1034 (Fig.4.5). The student ‘t’ test revealed that the difference on LPS released by the test organisms grown in glucose supplemented medium was statistically non-significant (t = 2.165; P> 0.005), whereas the LPS released by the test organisms when grown in glucose plus rhamnolipid supplemented medium was statistically significant (t = 6.298; P< 0.001) (Table 4.5).

4.3.7. Growth experiments with hexadecane as substrate

A similar set of experiments were performed with 1% hexadecane as the substrate.

4.3.7.1. Growth analysis by turbidometry (Optical density)

The growth of both the strains *P. aeruginosa* JQ062961 and *P. aeruginosa* MTCC 1034 in 1% hexadecane was better on addition of rhamnolipid and there was a difference in the phases of growth. The lag phase was observed till 24\textsuperscript{th} hr, exponential phase lasted till 288\textsuperscript{th} hr, followed by the stationary phase till 480\textsuperscript{th} hr and the decline phase commenced at the 576\textsuperscript{th} hr in the absence of rhamnolipid. Whereas in the presence of rhamnolipid, the growth seems to be accelerated for both the isolates that the exponential phase started at 24\textsuperscript{th} hr and lasted till 192\textsuperscript{nd} hr, followed by stationary phase till 384\textsuperscript{th} hr and the decline phase commenced at 480\textsuperscript{th} hr (Fig.4.6).
Fig.4.6. Growth, protein estimation, cell surface hydrophobicity (BATH), LPS release (KDO) of *P. aeruginosa* JQ062961 and *P. aeruginosa* MTCC 1034 grown on 1% hexadecane in the presence and absence of rhamnolipid.

- **Growth (OD)**
- **Protein (µg/ml)**
- **BATH assay (% adhered)**
- **LPS (µg)**

**Time (hrs)**
4.3.7.2. Protein estimation

Protein estimation data showed the addition of rhamnolipid found to have a notable effect for both the strain with hexadecane as substrate. Maximum protein content observed in the presence of rhamnolipid was 83.0±0.49μgml⁻¹ and 74.0 ±0.41μgml⁻¹, respectively by *P. aeruginosa* JQ062961 and *P. aeruginosa* MTCC 1034 at 288th hr. But in the absence of rhamnolipid, the protein content observed was 69.0 ±2.45μgml⁻¹ and 60.0 ±1.63μgml⁻¹ respectively by *P. aeruginosa* JQ062961 and *P. aeruginosa* MTCC 1034 at 384th hr. Thus the addition of rhamnolipid had documented a faster protein production with hexadecane as a substrate (Fig.4.6). The statistical student ‘t’ test revealed that the difference on protein production by the test organisms grown in hexadecane as well as hexadecane plus rhamnolipid substituted media were more significant (t = 44.538 to 62.353; P <0.001 to P< 0.0001) (Table 4.5).

4.3.7.3. Cell surface hydrophobicity (BATH assay)

The cell surface hydrophobicity of cells grown on hexadecane alone showed a slow and steady increase from 5.0 ± 0.05 to 37.0 ±1.45%, at 384th hr. for *P. aeruginosa* JQ062961, after which the percentage of adherence declined to 31.0 ±0.82% at the end of 576th hr. Likewise for *P. aeruginosa* MTCC 1034, it increased from 3.0 ± 0.016 to 33.0 ±1.63%, at 384th hr, followed by a decline to 28.0 ±1.27% at the end of sampling at 576th hr. The addition of rhamnolipid to the growth medium caused a rapid increase in cell surface hydrophobicity from 15.0 ±0.41% to 83.0 ±2.45% for *P. aeruginosa* JQ062961 and from 12.0 ±0.63% to 74.0 ±1.23% for *P.aeruginosa* MTCC 1034 at 288th hr. Decline in the hydrophobicity from 83.0 to
63.0 ±1.45% and from 74.0 to 59.0 ±1.63% was observed for *P. aeruginosa* JQ062961 and *P. aeruginosa* MTCC 1034, respectively during 576\textsuperscript{th} hr of incubation (Fig.4.6). The statistical student ‘t’ test revealed that the difference on cell surface hydrophobicity (BATH) by the test organisms grown in hexadecane as well as hexadecane plus rhamnolipid substituted media were more significant (t = 7.784 to 21.065; P <0.01 to P< 0.001) (Table 4.5).

4.3.7.4. LPS release –KDO assay

Results on LPS release in the presence of hexadecane without rhamnolipid was found to be 3.0 ±0.022\(\mu g\) for *P. aeruginosa* JQ062961, which was slightly higher than that of *P. aeruginosa* MTCC 1034 (2.70 ±0.08\(\mu g\)) at 576\textsuperscript{th} hr of incubation. At the same time, in the presence of rhamnolipid, there was an increase in LPS release of 4.90 ±0.013\(\mu g\) and 4.70 ±0.016\(\mu g\) for *P. aeruginosa* JQ062961 and *P. aeruginosa* MTCC 1034, respectively, during the same incubation period. It was observed that there was no reduction in the LPS concentration till the end of the incubation period of 576\textsuperscript{th} hr for both the organisms (Fig.4.6). The student ‘t’ test revealed that the difference on LPS released by the test organisms grown in hexadecane as well as hexadecane plus rhamnolipid substituted media were statistically significant (t = 25.980 to 34.641; P <0.001) (Table 4.5).

In general the data showed that the addition of rhamnolipid enhanced growth, protein concentration, percentage of adherence and LPS release. Of the two strains tested in the present study, the test isolate *P. aeruginosa* JQ062961 was found to exhibit better cell surface hydrophobicity and LPS release to the supernatant than the reference strain *P. aeruginosa* MTCC 1034.
4.4. Discussion

Hemolysis of sheep’s erythrocytes by biosurfactant was used as screening experiment to identify the biosurfactant producing *Bacillus subtilis* (Bernheimer and Avigad, 1970). They demonstrated the presence of biosurfactants caused the lysis of red blood cells in agar. In the present study the presence of rhamnolipid was qualitatively detected by hemolysis of erythrocytes in 5% sheep blood agar. The result showed that the rhamnolipid produced by *P. aeruginosa* JQ062961 contained fairly large amount of factors (hemolysins) with hemolytic activity as shown by large diameter and maximum areas of zone of clearance (22 mm) than the reference strain *P. aeruginosa* MTCC 1034 (17 mm). Similarly, Thavasi *et al.* (2011) have reported the hemolytic activity of 105 bacterial strains test, their results inferred that among the tested 105 strains, 101 (96.1%) strains were positive for hemolysis and within those positive strains, *P. aeruginosa* displayed the maximum hemolytic activity of 30.50mm. Tuelva *et al.* (2002) also evidenced a hemolytic zone of 11 mm by *P. putida* 21BN isolated from the industrial waste water sample. The diameter of the zone of hemolysis could be attributed to the amount of hemolysins released by the bacteria in the supernatant (Rahman *et al.*, 2010). Anandaraj and Thivakaran (2010) have reported a clear zone of hemolysis in *P. aeruginosa* isolated from automobile workshop. Likewise, Tahzibi *et al.* (2004) had demonstrated the hemolytic zone formation by mutant *P. aeruginosa* PTCC 1637. Twenty three isolates from 270 samples of oil contaminated soil collected from the Daqing oil field in 90 different areas showed an obvious hemolytic zone on the blood agar plate was reported by Liu *et al.* (2011).
Orcinol assay is used as a direct assessment of the amount of glycolipid in the culture sample (Asshifa et al., 2012). In the present study it showed that there was no much significant difference in the concentration of rhamnolipid produced by *P. aeruginosa* JQ062961 and *P. aeruginosa* MTCC 1034 and it was nearly identical with 0.277 and 0.268gl⁻¹, respectively. Various studies have reported on different concentration of rhamnolipid produced by *Pseudomonas* sp. For instance Zhang et al. (2005) stated the rhamnolipid production of 0.45gl⁻¹ by *P. aeruginosa* after 3 days of incubation with 3% glucose as carbon source. Similarly Tuelva et al. (2002) reported 1.2gl⁻¹ of rhamnolipid produced by *P. putida* in the stationary phase with 2% glucose as carbon source.

In the present study, Thin layer chromatography (TLC) analysis reprapacented two orcinol positive spots, both for *P. aeruginosa* JQ062961 and *P. aeruginosa* MTCC 1034 with the *Rf* values of 0.51 & 0.32 and with the *Rf* values of 0.51 & 0.33 representing mono and di-rhamnolipids, respectively. The result was in agreement with Olesja et al. (2009), who confirmed the presence of rhamnolipids in the *P. aeruginosa* DS10-129 culture broth by TLC, by detecting two anisealdehyde positive spots at *Rf* 0.32 and *Rf* 0.52, corresponding to di- and mono-rhamnolipids, respectively. The data in the present study was further coincided by the study carried out by Zhang et al. (2005), where they obtained two clear orcinol-positive spots at *Rf* 0.32 and 0.52 on TLC from crude oil degrading *P. aeruginosa*. Separation of rhamnolipid by TLC produced by *P. aeruginosa* (DAUPE 614) isolated from a petrochemical plant from Brazil showed two orcinol positive spots,
with Rf 0.55 and 0.36, representing mono and di-rhamnolipids respectively also found to be in-line with the present data (Monteiro et al., 2007).

In the present investigation, High performance thin layer chromatography (HPTLC) result of rhamnolipids produced by P. aeruginosa JQ062961 showed the presence of both the homologues, mono and di-rhamnolipids accounting to 69.62 and 30.38%, respectively. Similarly HPTLC analysis of rhamnolipid extracted from P. aeruginosa Bs20 by Abel-Mawgoud et al. (2009), revealed that it contained two homologues mono and di rhamnolipid quantified as 56% and 44%, respectively. HPTLC analysis of rhamnolipid produced by P. desmolyticum NCIM 2112 (Pd 2112) showed the presence of only one homologue, mono rhamnolipid (Jadhav et al., 2011). The nature, quantity and ratio of the rhamnolipid homologues produced are strain dependent rather the components of the medium in which the organism grown (Abel-Mawgoud et al., 2009).

In the present study, Fatty acid methyl ester (FAME) GC-MS analysis of the rhamnolipid produced by P. aeruginosa JQ062961 showed the presence of silyl and/or ester derivative of decanoic acid as the major component in all the 5 fragmentation units. Similar result of decanoic acid as the major component of the 5 acid fragments isolated from rhamnolipid produced by Acinetobacter calcoaceticus, Enterobacter asburiae and P. aeruginosa was reported by Hoskova et al. (2013). Likewise Chitra et al. (2012) had extracted 17 different types of fatty acid esters by GC-MS from the biosurfactant produced by five different bacterial strains, isolated from oil contaminated sites of Naval harbor, Mumbai. Their data showed the
presence of methyl and ester derivatives of tetradecanoic acid, hexadecanoic acid, eicosane and octadecanoic acid, which supported the present GC-MS data. Biosurfactant analysis by GC-MS, isolated from oil degrading \textit{R. erythropolis} 3C-9 was characterized as esterified derivative of hexadecanoic acid and three kinds of octadecanoic acids by Peng \textit{et al.} (2007). They also stated that various decanoic acids isolated from \textit{Rhodococcus} sp., which included decanoic acid, undecanoic acid, tetradecanoic acid, octadecadienoic acid, 10-methyl-octadecanoic acid, pentadecanoic acid, heptadecanoic acid and eicosenoic acid. Arino \textit{et al.} (1996) also characterized the rhamnolipid produced by \textit{P. aeruginosa} GL1 as trimethyl silyl and ester derivatives of decanoic acid.

Growth experiments were carried out inorder to study the effect of rhamnolipid on the cell surface of the test isolate \textit{P. aeruginosa} JQ062961 and the standard reference strain \textit{P. aeruginosa} MTCC 1034 in the presence of soluble substrate glucose and slightly soluble hexadecane. Maximum growth, protein concentration, cell surface hydrophobicity (\% of adherence) and LPS release were exhibited by both the organisms only in rhamnolipid containing medium. Hexadecane being hydrophobic in nature, its utilization by \textit{P. aeruginosa} will be influenced by the presence of hydrophilic zone formation by the polysaccharide chains of LPS layer present on the surface of the cell wall (Nikaido and Nakae, 1979). The results of the present study showed that addition of rhamnolipid caused the release of LPS from the cellular envelope of the organisms, to the growth medium, thereby increasing the cell surface hydrophobicity. This mechanism
facilitated the uptake of hexadecane, indicated by increased growth and protein concentration in both the isolates. Noordman and Janssen (2002) reported similar results of increased LPS release from P. aeruginosa in the presence of rhamnolipid-hexadecane containing growth medium. In contrast experiments conducted with glucose (hydrophilic) as substrate showed that there was no much influence on growth of either strain on addition of rhamnolipid even though the cell surface hydrophobicity and LPS release was increased. These results in glucose were supported by similar growth experiments conducted by Al-Tahhan et al. (2000), who demonstrated the effect of rhamnolipid-glucose had no effect on growth of P. aeruginosa ATCC 9027.

The present study data showed that cell surface hydrophobicity of P. aeruginosa JQ062961 grown on hexadecane with rhamnolipid was higher (83%) than when grown on glucose (71%) and for P. aeruginosa MTCC 1034, it was 74% in hexadecane and 58% in glucose. The higher percentage of adherence was observed in the stationary phase for both the strains. These results were supported by Tulevaa et al. (2002), who reported that at the beginning of stationary phase, cell surface hydrophobicity of P. putida 21BN grown on hexadecane was slightly higher (72%) than when grown on glucose as the carbon source (60%). Similarly, the study conducted by Beal and Betts (2000) recorded 50.50% adherence in hexadecane and 78% in water soluble growth substrates by P.aeruginosa PG201 with the addition of biosurfactant. Zhang and Miller (1994) reported that the hydrophobicity of P. aeruginosa ATCC 9027 increased to 79% on hexadecane in the presence of the
dR-Me (methyl ester form of rhamnolipid). Al-Tahhan et al. (2000) stated that the addition of rhamnolipid with hexadecane as a substrate recorded a cell surface hydrophobicity of 75% at stationary phase.

LPS forms the major component of the outer membranes of gram-negative bacteria and are responsible for the cell permeability characteristics (Denyer and Maillard, 2002). In the present study, LPS release by KDO analysis showed that P. aeruginosa JQ062961 released 4.9μg of LPS in hexadecane with rhamnolipid which was slightly higher than 4.7μg of LPS released by P. aeruginosa MTCC 1034. In glucose medium with rhamnolipid, the release was 2.1μg for P. aeruginosa JQ062961 and 1.7μg for P. aeruginosa MTCC 1034. Similar study conducted by Sotirova et al. (2009) revealed that addition of rhamnolipid increased the release of extra cellular LPS and caused reduction of total cellular LPS content to 22% in P. aeruginosa NBIMCC 1390, grown in hexadecane containing medium. The LPS released by KDO analysis can be correlated with the percentage of adherence by BATH assay (Beal and Betts, 2000). The study data showed that the percentage of adherence declined from 32nd hr in glucose medium and 384th hr in medium containing hexadecane though there was an increase in LPS release was observed throughout the experiment. This phenomenon can be attributed to that these bacterial cells would have regenerated LPS to become more hydrophilic (hence there was decreased hydrophobicity) rather than taking up the released LPS from the growth medium (Al-Tahhan et al., 2000).