CHAPTER 3

BIOSYNTHESIS OF BACTERIAL SURFACHTANTS

This chapter deals with the biosynthesis of surfactants from three bacterial strains Lysinibacillus chungkukjangi, Pseudomonas indica MTCC 3714, and Pseudomonas cepacia MTCC 7099. The synthesized bio-surfactants were characterized with the help of various chromatographic and spectroscopic techniques and their MEOR and anti-oxidant potential was checked.

3.1 Materials and methods

3.1.1 Materials

All the solvents used for extraction were of laboratory grade. All chromatographic purifications were performed with silica gel #60-120, #230-400 and silica gel G (CDH India Ltd.) TLC analyses were performed on silica gel coated Kieselgel 60 F<sub>254</sub>, 0.2 mm thick plates (Merck). The hydrocarbon n-Dodecane was purchased from HIMEDIA and other oils were purchased from the local market. The rice-bran residues were collected from a local rice-bran industry, A.P. Organics Private Limited, Dhuri, Sangrur (India).

3.1.2 Isolation of bio-surfactant producing microorganism

The strain was isolated from the rice bran oil industry sludge. 1mL of sludge sample was taken and grown in the minimal salt medium (MSM) containing rice-bran oil for microbial isolation. Serial dilutions with 1mL of MSM were made in the sterilized
distilled water (10 mL) up to $10^{-6}$ dilutions. From each dilution, agar plates were streaked. The dilution numbers from $10^{-2}$ to $10^{-6}$ single colonies were picked and grown in nutrient broth (composition (g/50 mL); peptone, 0.5; beef extract, 0.25; NaCl, 0.25) at 30 °C in a New Brunswick Scientific (Innova 42- Eppendorf) incubator shaker at a shaking speed of 150 rpm for 24-48 h of incubation. The strains were again streaked on nutrient agar slants to check the purity. Isolated pure strains were grown in the fermentation medium for the bio-surfactant production and surface tension was monitored to get the bio-surfactant producing strains. Finally, the strain which reduced the surface tension to maximum was characterized and found to be *Lysinibacillus chungkukjangi*, which was deposited in the Genbank under the accession number KP877500 in Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India.

### 3.1.3 Procurement of bacterial strains and their screening for bio-surfactant production

The bacterial strains *Pseudomonas indica*, MTCC 3714 and *Pseudomonas cepacia*, MTCC 7099 were procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The strains were grown in the media as described by MTCC; composition (g/L): Tryptone (10.0); Yeast Extract (5.0) and NaCl (10.0) in 1.0 L distilled water. The stains were grown in the fermentation media for the bio-surfactant production and surface tension was monitored, luckily, both the strains were found to produce Bio-surfactants.
3.1.4 Storage of bacterial strains and preparation of seed culture

In the laboratory, the cultures were stored in 20% glycerol at 4 °C as well as maintained on nutrient agar plates and was transferred to 50 mL of nutrient broth to prepare the seed culture. The cultivation conditions for the seed culture were 30 °C, 150 rpm, and 24-48 h of incubation.

3.1.5 Optimization of bio-surfactant synthesis

The optimization of the biosynthesis of surfactants for each bacterial strain is as follows:

3.1.5.1 *Lysinibacillus chungkukjangi*

The fermentation media for the production of bio-surfactant was as follows (g/L): K$_2$HPO$_4$ (2.2), NaCl (0.01), MgSO$_4$ (0.6), CaCl$_2$ (0.04), FeSO$_4$ (0.02). In order to perform optimization, the media were supplemented with different carbon sources (de-oiled rice bran, rice-bran, fatty acids, waxes and glucose) and nitrogen sources (urea, yeast extract, NaNO$_3$ and NH$_4$NO$_3$) in the concentration of 2-10% (w/v, carbon source) and 0.2-1.0% (w/v, nitrogen source) respectively. The other physico-chemical factors were also optimized viz. temperature (20-40 °C), pH (5.0 to 8.0 with 6N HCl), inoculation volume (1-10%, w/v), shaking speed (0-250 rpm) and fermentation time (24-192 h). The media were sterilized by autoclaving at 15 psi for 20 min. Before inoculation of the microbial culture, 1% of the kerosene was added for induction. The reactions were carried out in 1 L Erlenmeyer flasks containing 350 mL of the fermentation media.
3.1.5.2 *Pseudomonas indica*

The fermentation media for the production of bio-surfactants was as follows (g/L): K$_2$HPO$_4$ (2.2), KH$_2$PO$_4$ (0.14), NaCl (0.01), and 0.5 mL of trace element solution containing (g/L): ZnSO$_4$.7H$_2$O (2.32), MnSO$_4$.4H$_2$O (1.78), H$_3$BO$_3$ (0.56), CuSO$_4$.5H$_2$O (1.0), Na$_2$MoO$_4$.2H$_2$O (0.39), CoCl$_2$.6H$_2$O (0.42), EDTA (1.0), NiCl$_2$.6H$_2$O (0.004), KI (0.66). In order to perform optimization, the media were supplemented with different carbon sources (de-oiled rice bran, rice-bran, fatty acids, waxes and glucose) and nitrogen sources (urea, yeast extract, NaNO$_3$ and NH$_4$NO$_3$) in the concentration of 2-10% (w/v, carbon source) and 0.2-1.0% (w/v, nitrogen source) respectively. The other physico-chemical factors were also optimized viz. temperature (20-40 °C), pH (5.0 to 8.0 with 6N HCl), inoculation volume (1-10%, w/v), shaking speed (0-250 rpm) and fermentation time (24-192 h). The media were sterilized by autoclaving at 15 psi for 20 min. Before inoculation of the microbial culture, 1% of the kerosene was added for induction. The reactions were carried out in 1 L Erlenmeyer flasks containing 350 mL of the fermentation media.

3.1.5.3 *Pseudomonas cepacia*

The fermentation media for the production of bio-surfactants were as follows (g/L): K$_2$HPO$_4$ (2.2), KH$_2$PO$_4$ (0.14) NaCl (0.01), MgSO$_4$, CaCl$_2$, FeSO$_4$. In order to perform optimization, the media were supplemented with different carbon sources (de-oiled rice bran, rice-bran, fatty acids, waxes and glucose) and nitrogen sources (urea, yeast extract, NaNO$_3$ and NH$_4$NO$_3$) in the concentration of 2-10% (w/v, carbon source) and 0.2-1.0% (w/v, nitrogen source) respectively. The other physico-chemical factors were
also optimized viz. temperature (20-40 °C), pH (5.0 to 8.0 with 6N HCl), inoculation volume (1-10%, w/v), shaking speed (0-250 rpm) and fermentation time (24-192 h). The media were sterilized by autoclaving at 15 psi for 20 min. Before inoculation of the microbial culture, 1% of the kerosene was added for induction. The reactions were carried out in 500 mL Erlenmeyer flask containing 150 mL of the fermentation media.

3.1.6 Extraction of bio-surfactant

The extraction of the bio-surfactant was performed according to the previous reports by slight modifications [Anandraj and Thivakaran, 2010; Pansiripat et. al., 2010; Thavasi et. al., 2009]. The procedure for all the bacterial strain is as follows:

3.1.6.1 *Lysinibacillus chungkukjangi*

The bio-surfactant was extracted from the culture media after cell removal by centrifuging (Eppendorf, Centrifuge, 5810 R) at 5800 rpm for 20 min. The supernatant pH was maintained to 2.0 with HCl (6N) with a Thermo Scientific, ORION 2 STAR, pH Benchtop. The bio-surfactant was extracted till organic layer had no color with various solvent systems (viz. chloroform, methanol, ethyl acetate, petroleum ether, and their varying ratios) and after that solvent layer was transferred to an RBF and concentrated in vacuum under reduced pressure at 40 °C (IKA RV 10, Germany). The dried viscous product was washed with hexane, again dissolved in chloroform and anhydrous Na₂SO₄ was added to get rid of residual water. After two hours, the chloroform layer was filtered using grade 1 filter paper (G1), and evaporated in a rota evaporator. The product obtained (crude bio-surfactant) was characterized with different
chromatographic and spectroscopic techniques and was characterized as the lipopeptide mixture.

### 3.1.6.2 Pseudomonas indica

The samples from fermentation broth were withdrawn, kept in ultrasonication bath for 30 min and then centrifuged at 5000 rpm for 30 min. The supernatant served as the source of crude bio-surfactant. The pH of the supernatant was adjusted to 3.0 with 6N HCl and kept overnight for precipitation. For the complete extraction of bio-surfactants the supernatant was extracted thrice with different solvent systems viz. chloroform, methanol, ethyl acetate and varying ratios of these solvents, at room temperature. The organic layer was concentrated in a rotary evaporator at 40 °C, transferred in a flask and sodium sulfate was added to remove the traces of water present. After one hour the organic layer was filtered with Whatman filter paper no. 1, again concentrated in the rotary evaporator, the yield was noted and the crude bio-surfactant obtained was used for further analysis. The bio-surfactant was characterized as a mixture of di-rhamnolipids.

### 3.1.6.3 Pseudomonas cepacia

The bio-surfactant was extracted from the culture medium after ultrasonication (Ultrasonic bath, LINCO, 40 MHz) prior to centrifugation to remove cells for 20 min at 5800 rpm. The pH of the supernatant was adjusted to 2.0 with 6N HCl and mixture was extracted vigorously with various solvents till color disappears and the organic phase was concentrated in a rotary evaporator at 40 °C. The yellowish brown colored dried residue was washed with hexane and dissolved in methanol and again concentrated in
a rotary evaporator, the yield was noted and purification was done by column chromatography.

3.1.7 Surface tension and critical micelle concentration (CMC) measurements

The surface tension of the purified bio-surfactant was measured in an Easy Dyne tensiometer (KRUSS, Germany) using the du Nöuy ring method at room temperature. Critical micelle concentration (CMC) was calculated by plotting surface tension of serially diluted bio-surfactant solutions as a function of surfactant concentration. Tensiometer determines surface tension readings with the help of an optimally wettable platinum orii suspended from a measuring probe. In the ring method, the liquid is raised until contact with the surface is recorded. The sample is then brought down again so that the film produced beneath the liquid is stretched for the maximum force which is used to estimate the surface tension of the liquid. The instrument was calibrated against de-ionized water and after each reading, washed with de-ionized water, acetone and heated till red hot in a Bunsen burner. The readings were taken in triplicates and average was considered. Table 3.1 provides the results of surface tension and CMC values of all the three bacterial Bio-surfactants.

Table 3.1: Surface tension and CMC values of the bacterial Bio-surfactants

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Surface tension (mN/m)</th>
<th>Critical Micelle Concentration (CMC) (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysinibacillus chungkukjangi</td>
<td>27.9</td>
<td>50</td>
</tr>
<tr>
<td>Pseudomonas indica</td>
<td>26.4</td>
<td>20</td>
</tr>
<tr>
<td>Pseudomonas cepacia</td>
<td>33.2</td>
<td>250</td>
</tr>
</tbody>
</table>
3.1.8 Emulsification activity

The emulsification activity was checked with the cell free supernatant obtained after the completion of fermentation reaction. To check the emulsification activity, 2 mL of the hydrocarbons (olive oil, rice-bran oil, coconut oil, kerosene, n-dodecane and hexane) were added to 2 mL of the cell free supernatant in a test tube and vortexed for 2 min at high speed. The emulsification activity was measured by the height of the emulsion layer divided by the height of the total solution [Aparna et. al., 2012; Pruthi and Cameotra, 1995]. The results of emulsification activity on various hydrocarbons are given in Table 3.2.

Table 3.2: Emulsification activity of the bacterial bio-surfactants on various hydrocarbons

<table>
<thead>
<tr>
<th>Hydrocarbons</th>
<th>Lysinibacillus chungkukjangi</th>
<th>Pseudomonas indica</th>
<th>Pseudomonas cepacia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olive oil</td>
<td>100.0</td>
<td>62.0</td>
<td>89.0</td>
</tr>
<tr>
<td>Rice-bran oil</td>
<td>85.71</td>
<td>65.0</td>
<td>88.0</td>
</tr>
<tr>
<td>Coconut oil</td>
<td>84.32</td>
<td>65.0</td>
<td>89.0</td>
</tr>
<tr>
<td>Kerosene</td>
<td>34.48</td>
<td>7.0</td>
<td>43.0</td>
</tr>
<tr>
<td>n-dodecane</td>
<td>14.28</td>
<td>56.0</td>
<td>36.0</td>
</tr>
<tr>
<td>Hexane</td>
<td>8.33</td>
<td>5.0</td>
<td>25.0</td>
</tr>
</tbody>
</table>

3.1.9 Purification of bio-surfactant produced by *Pseudomonas cepacia*

The extracted bio-surfactant was purified by column chromatography and analyzed by thin layer chromatography (TLC). The mixture obtained was analyzed by thin layer chromatography (TLC) using silica gel plates (Silica gel 60, F254, Merck, Germany) and chloroform, methanol (9:1) as the solvent system. Visualization was done with iodine
vapors and 5% sulfuric acid and the mixture of bio-surfactants was purified on silica gel (60-120) column (60×2.5 cm) eluting various compounds with a chloroform/ methanol gradient ranging from 100 to 50:50 ratios. Each fraction was monitored by TLC to get the pure compound and visualization was done with iodine vapors and 5% sulfuric acid when chloroform: methanol (9:1) was the solvent system. The pure fractions having same TLC profile were pooled and dried in a rotary evaporator (Buchi, Switzerland). The schematic procedure of isolation and purification of bio-surfactants produced by *Pseudomonas cepacia* is given in Figure 3.1.

### 3.1.10 Characterization of bio-surfactant

The chemical structures of the bio-surfactants were determined by following techniques:

#### 3.1.10.1 FTIR Spectroscopy

The structural analysis of the purified bio-surfactant was performed by infrared spectroscopy in the spectral region 4000-400 cm\(^{-1}\) and the spectrum was recorded on a Nicolet (USA) FTIR system using KBr disc and a Bruker Vertex 70 FTIR spectrometer equipped with a diamond attenuated total reflectance (ATR) crystal accessory was used to provide a chemical analysis of the Bio-surfactants. All the bacterial bio-surfactants from *L. chungkukjangi*, *P. indica* and *P. cepacia* were characterized by FTIR spectroscopy and the spectral data is given below:

**FTIR spectral data of *L. chungkukjangi* bio-surfactant:** Wave numbers (cm\(^{-1}\)); 3414, 2925, 2854, 2363, 2343, 1618, 1458, 1377, and 1107.
Figure 3.1: Isolation and purification scheme of the purified bio-surfactant from *Pseudomonas cepacia*

**FTIR spectral data of *P. indica* bio-surfactant:** Wave numbers (cm\(^{-1}\)); 3446, 3100, 2900, 1717, 1650, and 1056.

**FTIR spectral data of *P. cepacia* bio-surfactant:** Wave numbers (cm\(^{-1}\)); 3367, 2924, 2853, 1724, 1459, 1408, 1377, 1266, 1219, 771, and 731.

### 3.1.10.2 NMR Spectroscopy

The known bio-surfactants (*lipopetides and rhamnolipids*) were characterized for \(^1\)H and \(^{13}\)C spectra using a JEOL ACX spectrometer set at 300 MHz with deuterated chloroform as solvent while the unknown bio-surfactant (*pentanedioate*) was
characterized for nuclear magnetic resonance (NMR) analysis $^1$H NMR, $^{13}$C NMR, heteronuclear single quantum correlation (HSQC), $^1$H-$^1$H correlation spectroscopy (COSY), heteronuclear multiple bond correlation (HMBC) with a Bruker, Ultrashield 400 NMR (Germany) spectrometer using CDCl$_3$. The bacterial bio-surfactants from *L.chungkukjangi* and *P. indica* were analyzed on JEOL 300 MHz spectrometer and *P. cepacia* on Bruker 400 MHz spectrometer. The spectral data is as given below:

**NMR spectral data of *L.chungkukjangi* bio-surfactant:** $^{13}$C NMR (CDCl$_3$, 100MHz): $\delta$ 210.55, 181.59, 181.52, 177.80, 81.43, 77.60, 73.08, 71.10, 43.12, 40.81, and 31.95-11.55. $^1$H NMR (CDCl$_3$, 300MHz): $\delta$ 4.17-4.22, 1.98-2.53, 0.79-1.34.

**NMR spectral data of *P. indica* bio-surfactant:** $^{13}$C NMR (CDCl$_3$, 100MHz): $\delta$ 210.19, 171.25, 139.24, 128.72, 128.80, 114.13, 73.08, 60.47, and 34.50-10.95. $^1$H NMR (CDCl$_3$, 300MHz): $\delta$ 5.23, 4.26, 4.22, 4.20, 4.19, 4.06, 3.92, 3.76, 3.68, 3.68, 3.67, 3.61, 3.58, 3.45, 2.33, 2.31, 1.94, 1.23-1.44, 1.14-1.42, 1.14-1.42, and 0.89.

**NMR spectral data of *P. cepacia* bio-surfactant:** $^1$H NMR (CDCl$_3$, 400MHz); $^{13}$C NMR (CDCl$_3$, 100 MHz): Table 3.3 gives the chemical shift values of $^1$H and $^{13}$C NMR of the purified bio-surfactant.

### 3.1.10.3 LC-MS analysis

Dual-AJS-ESI in positive mode was performed using an Agilent G-6550A MS Q-TOF mass spectrometer. The collected samples were dissolved in methanol and infused in the Dual-AJS-ESI source with a flow rate of 0.2 mL/min. Dual-AJS-ESI spectra (positive mode) was recorded from m/z 200-2000 for 25 min. Operating conditions were as
follows: gas temperature, 150 °C, gas flow 11 L/min, nebulizer, 40 psi; sheath gas temperature 250 °C. *Lysinibacillus chungkukjangi* and *Pseudomonas indica* biosurfactants were subjected for the LC-MS analysis and the results are as follows:

Table 3.3: $^1$H and $^{13}$C NMR data of the purified bio-surfactant

<table>
<thead>
<tr>
<th>C No.</th>
<th>$^1$H (δ ppm)</th>
<th>$^{13}$C (δ ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.8 (J=6.56)</td>
<td>14.1</td>
</tr>
<tr>
<td>2</td>
<td>1.2 (merged)</td>
<td>22.7</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>28.9</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>29.1</td>
</tr>
<tr>
<td>5</td>
<td>1.2-1.3 (merged)</td>
<td>29.3</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>29.5</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>29.6</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>29.7</td>
</tr>
<tr>
<td>9</td>
<td>2.0 (9H, dd, J= 10.84; 7.56);</td>
<td>30.2</td>
</tr>
<tr>
<td>10</td>
<td>2.3 (2H, s)</td>
<td>31.9</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>33.8</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>77.0</td>
</tr>
<tr>
<td>13</td>
<td>4.9 (7H, qdd, J= 7.96, 1.6, 10.4)</td>
<td>114.0</td>
</tr>
<tr>
<td>14</td>
<td>6.6 (1H, d, J=8.2)</td>
<td>115.9</td>
</tr>
<tr>
<td>15</td>
<td>7.3 (2H, dd, J= 2.4, 8.2)</td>
<td>123.5</td>
</tr>
<tr>
<td>16</td>
<td>7.1 (1H, d, J=2.4)</td>
<td>124.0</td>
</tr>
<tr>
<td>17</td>
<td>5.8 (3H, td,dd, J=6.68, 16.9)</td>
<td>139.2</td>
</tr>
</tbody>
</table>
LC-MS Spectral data of *L. chungkukjangi* bio-surfactant: m/z 1397, 939, 872, 871, 653, 639, 481, 393, 202.

**LC-MS Spectral data of *P. indica* bio-surfactant:** Retention time (RT), 1.067 (m/z 694, 650, 606, 414, 400, 398); RT, 5.369 (m/z 808, 807, 806); RT, 8.17 (m/z 783, 782, 760, 759, 758); RT, 18.191, (m/z 1343, 1342, 680, 664, 663).

### 3.1.10.4 LC-ESI-HRMS Analysis

A Thermo-Finnigan LCQ ion-trap MS (Arcade, New York, USA) using a syringe-infusion pump was used for the molecular weight analysis of purified bio-surfactant. The purified sample was suspended in acetonitrile and introduced in LC-ESI source. The spectrum was observed in a positive mode and scanning was done from 50-1500 m/z range.

**Mass spectrum data of *P. cepacia* bio-surfactant:** m/z 905.6730, 724.5662, 681.5162, 491.3010, 475.3269.

### 3.1.11 Applications

#### 3.1.11.1 DPPH scavenging activity

The bio-surfactant was analyzed for anti-oxidant potential using the DPPH (1,1-diphenyl-2-picryl hydrazyl) method [Yalcin and Cavusoglu, 2010]. In the present experiment, the standard and reagent used were ascorbic acid and stable DPPH radical respectively. The aliquots of 100 µL from 1-5 mg/mL concentration of bio-surfactant
and standard ascorbic acid were added to 3.0 mL of the 0.004% (w/v) methanolic solution of DPPH. The samples were incubated for 30 min in dark at the room temperature and absorbance at 517 nm was determined against a blank in a UV-Visible Spectrophotometer (SHIMADZU, UV 1800). The percentage inhibition (I %) of free radical DPPH was evaluated using the following formula.

\[ I \% \text{ [DPPH radical]} = \left( \frac{A_c - A_s}{A_c} \right) \]

Where, \( A_s \) = absorbance of the sample and \( A_c \) = absorbance of the control. IC\textsubscript{50} (the half maximal inhibitory concentration) was also determined. IC\textsubscript{50} denotes to the concentration of sample required to scavenge 50% of the DPPH free radicals. Table 3.4, Table 3.5 and Table 3.6 gives the results of percentage inhibition of DPPH by bacterial bio-surfactants produced by Lysinibacillus chungkukjangi, Pseudomonas indica, and Pseudomonas cepacia respectively. Among all the bacterial strains Lysinibacillus chungkukjangi showed the best DPPH scavenging activity.

**Table 3.4:** Percentage inhibition of DPPH by *Lysinibacillus chungkukjangi* bio-surfactant

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Concentration (mg/mL)</th>
<th>Percentage inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.2</td>
<td>5.83</td>
</tr>
<tr>
<td>2.</td>
<td>0.4</td>
<td>10.93</td>
</tr>
<tr>
<td>3.</td>
<td>0.6</td>
<td>26.96</td>
</tr>
<tr>
<td>4.</td>
<td>0.8</td>
<td>27.7</td>
</tr>
<tr>
<td>5.</td>
<td>1.0</td>
<td>37.7</td>
</tr>
</tbody>
</table>

**Table 3.5:** Percentage inhibition of DPPH by *Pseudomonas indica* bio-surfactant

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Concentration (mg/mL)</th>
<th>Percentage inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1.00</td>
<td>No inhibition</td>
</tr>
<tr>
<td>2.</td>
<td>2.00</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>3.00</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>4.00</td>
<td>-</td>
</tr>
<tr>
<td>5.</td>
<td>5.00</td>
<td>0.74</td>
</tr>
</tbody>
</table>
Table 3.6: Percentage inhibition of DPPH by *Pseudomonas cepacia* bio-surfactant

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Concentration (mg/mL)</th>
<th>Percentage inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1.00</td>
<td>0.95</td>
</tr>
<tr>
<td>2.</td>
<td>2.00</td>
<td>2.12</td>
</tr>
<tr>
<td>3.</td>
<td>3.00</td>
<td>9.55</td>
</tr>
<tr>
<td>4.</td>
<td>4.00</td>
<td>12.10</td>
</tr>
<tr>
<td>5.</td>
<td>5.00</td>
<td>19.21</td>
</tr>
</tbody>
</table>

3.1.11.2 Microbial Enhanced Oil Recovery (MEOR)

The sand pack test was performed to check the MEOR as proposed by Pruthi and Cameotra [1997] with slight modifications. A column (60×2.5 cm) was used to demonstrate the sandpack test. 135 g of washed sand was poured into the column with slight tapping to maintain the homogeneity in packing and saturated with 100 mL kerosene. A vacuum pump was connected to the column to maintain the constant flow. First time only distilled water was flooded into the column and second time 200 mg of bio-surfactant was dissolved in 10 mL of distilled water and flooded into the column to recover entrapped kerosene. The results were compared when only distilled water was used for the recovery of kerosene and when bio-surfactant was dissolved into distilled water. **Table 3.7** provides the result of sandpack test performed for kerosene recovery.

Table 3.7: MEOR potential of various bacterial bio-surfactants

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Kerosene recovery without bio-surfactant (%)</th>
<th>Kerosene recovery with bio-surfactant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. chungkukjangi</em></td>
<td>Less than 50</td>
<td>90</td>
</tr>
<tr>
<td><em>P. indica</em></td>
<td>-</td>
<td>70</td>
</tr>
<tr>
<td><em>P. cepacia</em></td>
<td>-</td>
<td>90</td>
</tr>
</tbody>
</table>
3.2 Results and Discussion

3.2.1 Optimization of the bio-surfactant production

The nutrition given to the microorganisms plays important role in their growth as well as better bio-surfactant yield. Various parameters were screened and best were chosen for the best yields because even a little change in the media composition and growth conditions, had a significant impact on bio-surfactant production. The main purpose of the optimization studies was to isolate bio-surfactant in greater yields which reduces the surface tension of the media and was able to emulsify various hydrocarbons. The details are discussed as follows:

3.2.1.1 Carbon source and its percentage (w/v)

3.2.1.1.1 Lysinibacillus chungkukjangi

In case of Lysinibacillus chungkukjangi, among all the carbon sources (de-oiled rice bran, rice-bran, fatty acids, waxes, and glucose), optimum yields (90-95%) were obtained when rice-bran (3%) was used in combination with glucose (1%) in the concentrations of 4% (w/v). The yield of the bio-surfactant recovered after solvent extraction was 6.1 g/L and at this concentration, the reduction in surface tension was 27.9 mN/m. The yield of bio-surfactant obtained in the present study was found to be better as compared to the previous reports where low cost carbon sources were used in the production of bio-surfactants by Pseudomonas sp., and the yield obtained was 4.97 g/L while it was less than the yield (8.0 g/L) obtained when Candida lipolytica was grown on the inexpensive carbon source [Aparna et. al., 2012; Rufino et. al., 2014].
Figure 3.2 represents the optimization of nutritional parameters with respect to the optimal yields.

3.2.1.1.2 Pseudomonas indica

In case of *Pseudomonas indica*, the bio-surfactant yield was optimum (90-95%) when media were supplemented with 4% (w/v) carbon sources which were rice-bran (3%) and de-oiled rice bran (3%) in combination with glucose (1%); the only difference was in the reduction of surface tension. When the media were supplemented with glucose as the sole carbon source the reduction in surface tension values was optimum (25.4 mN/m) but the yields were 9.6 g/L and the reduction in surface tension of the culture broth was 26.4 and 31.6 mN/m respectively. Hence, in the present work, rice-bran was found to be the best carbon source for the production of bio-surfactants by *Pseudomonas indica*. Figure 3.3 represents the optimization of nutritional parameters with respect to the optimal yields of bio-surfactant. Various researchers have reported optimum yields when the conventional (n-dodecane) and unconventional (soapstock) carbon sources were used for bio-surfactant production. The bio-surfactant production by the bacteria *Pseudomonas putida*, *P. diminuta* and *P. aeruginosa*, grown on 1% n-dodecane was observed to be 6.2, 6.0, 6.1 g/L respectively. Also, when *Pseudomonas aeruginosa* was grown on soapstock as a sole carbon source the rhamnolipid produced were 15.9 g/L respectively [Pruthi and Cameotra, 1995; Benincasa et. al., 2002; Makkar and Cameotra, 1997]. The increase in the yield of the bio-surfactant on addition of rice-bran as carbon source probably is due to its high content of fatty acids/esters which may help in the biosynthesis of the rhamnolipids.
Figure 3.2: Optimization of bio-surfactant production for *Lysinibacillus chungkukjangi*

Figure 3.3: Optimization of bio-surfactant production for *Pseudomonas indica*
3.2.1.3.3 *Pseudomonas cepacia*

*Pseudomonas cepacia* was able of producing optimum yields (90-95%) of bio-surfactant when the carbon source was de-oiled rice-bran (3%) with glucose (1%) in the concentration of 4% (w/v). The yield obtained after solvent extraction was found to be 5.3 g/L and lowering in surface tension on this concentration was 33.2 mN/m. The yield obtained was compared with the previous report where low cost carbon source was used in the production of bio-surfactants by *Pseudomonas* sp., and the yield obtained was 4.97 g/L [Aparna et. al., 2012] and also with our results with the bacterial strain *Lysinibacillus chungkukjangi* where the yield was 6.1 g/L. Figure 3.4 demonstrates the optimization of nutritional parameters with respect to the optimal yields of bio-surfactants produced by *Pseudomonas cepacia*.

![Figure 3.4](image_url)

**Figure 3.4**: Optimization of bio-surfactant production for *Pseudomonas cepacia*
3.2.1.2 Nitrogen source and its percentage (w/v)

All the three bacterial strains (*Lysinibacillus chunkukjangi*, *Pseudomonas indica*, and *Pseudomonas cepacia*) produced optimum yields of bio-surfactant when yeast extract in varying concentrations (0.2, 0.4, and 0.4%, w/v) was used among all nitrogen sources *viz.* urea, yeast extract, NaNO$_3$, NH$_4$NO$_3$. Also, the stable emulsions were obtained with yeast extract. The results were in accordance with the bio-surfactant production by *Bacillus* sp., when the yeast extract was used as a nitrogen source [Gnanamani et. al., 2010; Kim et. al., 1997].

3.2.1.3 C/N ratio

The carbon to nitrogen ratio is a very important factor as it enhances the bio-surfactants yield. The optimum C/N ratio for *Lysinibacillus chunkukjangi*, *Pseudomonas indica* and *Pseudomonas cepacia*, were 20, 10 and 10 respectively. The results were in accordance with the previous reports. When *Pseudomonas fluorescence* was observed for the bio-surfactant production, C/N ratio 20 was observed, which was similar to our study where we too got the same in case of *Lysinibacillus chungkukjangi* [Govindammal, 2014]. The results of Abouseoud et. al., [2007] were in accordance with the present study in case of *Pseudomonas indica* and *Pseudomonas cepacia* where they too found the optimum C/N ratio 10 in case of *Pseudomonas fluorescence*, when grown in the media containing olive oil and NH$_4$NO$_3$ as carbon and nitrogen sources respectively [Abouseoud et. al., 2007].
3.2.1.4 Incubation time, inoculation volume and shaking speed

Besides of all the environmental factors, these physico-chemical factors also gave an equal contribution in the optimum yields of bio-surfactant production. The time period of fermentation is called as incubation time, which is a key factor in the bio-surfactant production as there are two phases, one in which the microbial cells grow and another where the bio-surfactant production increases, achieves its maximum and becomes stationary. While the inoculum volume means the concentration of the microbial cells taken for the fermentation process and in the present study, we observed that the amount of inoculation volume did not affect the reduction in surface tension values to a greater extent, but the yields of bio-surfactant were optimum at a particular concentration. Shaking speed is the revolutions per minute given to the fermentation media so that there remains equalization in nutrient distribution which again is an important factor to consider. Our isolated strain, *Lysinibacillus chungkukjangi* produced optimum yields of bio-surfactant when, the incubation time, inoculation volume and shaking speed were 168 h, 2%, and 150 rpm respectively. The results were in accordance with the previous report of when *Bacillus subtilis* produced lipopeptide bio-surfactant at temperature 30 °C and 150 rpm [Kim et al., 1997]. There is a previous report where, *Bacillus subtilis* bacterium was found to produce lipopeptide bio-surfactant after 168 h of incubation, 200 rpm shaking speed and temperature 35 °C [Yalcin and Cavusoglu, 2010]. The bacterial strain *Pseudomonas indica* produced optimum bio-surfactants with the inoculation volume of 5% (v/v) after 7 days of incubation at a shaking speed of 200 rpm, which is same as observed for the production of bio-surfactants by *Lactobacillus delbrueckii* when grown on peanut oil
cake [Thavasi et. al., 2011]. The another bacterial strain *Pseudomonas cepacia* produced optimum bio-surfactants with the inoculation volume of 2% (v/v) after 4 days of incubation at a shaking speed of 150 rpm. When the fermentation was carried out for more than desired time the values of surface tension was observed to be increased instead of decreasing. The amount of inoculation volume did not affect the reduction in surface tension values to a greater extent, but does affect the yields of Bio-surfactants.

### 3.2.1.5 pH and temperature

The pH and temperature play a very important role in bio-surfactant synthesis as the microorganisms become inactive after a particular range of pH and temperature. The optimized pH and temperature observed for *Lysinibacillus chungkukjangi*, *Pseudomonas indica* and *Pseudomonas cepacia* grown on rice-bran industry waste were 6.5±0.2 and 30±1 °C respectively. The results resembled with the previous reports where the bacterial strain *Bacillus subtilis* also showed maximum bio-surfactant production at temperature 30°C [Kim et. al., 1997; Fox and Bala, 2000] and pH 6.5 was comparable to the results when *Pseudomonas indica* was grown for the bio-surfactant production [Bhardwaj et. al., 2015b]. The results were also comparable with the studies of Benincasa et al. [Benincasa et. al., 2002] when bio-surfactant was produced by *Pseudomonas aeruginosa* grown on soapstock as the sole carbon source.

### 3.2.1.6 Ultrasonication

Ultrasonic irradiation leads to enhancement in the yields of bio-surfactant. When experiments were performed using ultrasonication the improvement in the production
of bio-surfactants were observed up to 20% in the case of all the bacterial strains [Suslick, 1990]. The reason may be the efficient recovery of bio-surfactant attached to the microbial cell wall. Hence, ultrasonication was always performed prior to centrifugation in all experiments.

3.2.2 Bio-surfactant Characteristics

3.2.2.1 Lysinibacillus chungkukjangi

The bacterium *Lysinibacillus chungkukjangi* could produce bio-surfactant when grown on the fermentation media supplemented with rice-bran (carbon source), and yeast extract (nitrogen source). The best solvent system for the extraction of bio-surfactant was chloroform: methanol (2:1) and the product obtained was viscous, oily, and light brown in color. Kim et. al., [1997] have also reported the same ratio of the solvent system for the extraction of lipopeptide bio-surfactant produced by *Bacillus subtilis*. This extracted product was partially characterized and studied for its MEOR potential and anti-oxidant property.

3.2.2.2 Pseudomonas indica

The best solvent system for the extraction of bio-surfactants from *Pseudomonas indica* was found to be chloroform: methanol in the ratio 3:2. The bio-surfactant appeared as viscous and dark brown in color. This crude bio-surfactant was used for the further analysis for the chemical structure elucidation.
3.2.2.3 *Pseudomonas cepacia*

The physical appearance of the bio-surfactant produced by *Pseudomonas cepacia* when grown on de-oiled rice-bran was dark yellowish brown in color. The solvent systems used for the maximum extraction of the bio-surfactant from the fermentation media were chloroform and varying ratios of chloroform and methanol. Further, this honey colored bio-surfactant mixture was purified using column chromatography.

3.2.3 Surface tension and CMC measurements

3.2.3.1 *Lysinibacillus chungkukjangi*

The bio-surfactant produced by *Lysinibacillus chungkukjangi* reduced the surface tension of the media to 27.9 from 72 mN/m and CMC was calculated to be 50 mg/L (Figure 3.5). The present results were comparable with the previous studies, when *Pseudomonas cepacia*, *Bacillus licheniformis*, and *Bacillus subtilis* was examined for the bio-surfactant production and reduction in surface tension was observed to be 27.5 mN/m, 28 mN/m and 28.2 dyne/cm [Silva et. al., 2014; Yakimov et. al., 1995; Kim et. al., 1997].

3.2.3.2 *Pseudomonas indica*

The bio-surfactant produced by *Pseudomonas indica* exhibited good values of reduction in surface tension. The surface tension and CMC (Figure 3.6) of the bio-surfactant was measured and were found to be 26.2 from 71.2 to mN/m and 20.0 mg/L, respectively. The surface tension values obtained were comparable with the
results obtained by Shavandi et. al., [2011], Khopade et. al., [2012], Pruthi and Cameotra, [1997], in case of bacterial strains *Rhodococcus* sp., *Nocardiopsis* sp., and *Arthrobacter protophormiae*, respectively.

![Figure 3.5: Surface tension versus concentration of bio-surfactant](image1)

**Figure 3.5:** Surface tension versus concentration of bio-surfactant

![Figure 3.6: Reduction in surface tension with respect to concentration](image2)

**Figure 3.6:** Reduction in surface tension with respect to concentration
3.2.3.3 *Pseudomonas cepacia*

The surface tension and CMC (Figure 3.7) of the bio-surfactant produced by *Pseudomonas cepacia* was observed to be 33.2 from 71.2 mN/m and 25 mg/mL. The surface tension values were comparable with the previous studies when *Lactococcus lactis* and *Pseudomonas aeruginosa* was examined for the bio-surfactants production and the reduction in surface tension was 33.3 mN/m [Zhao et. al., 2015; Rodrigues et. al., 2006].

![Figure 3.7: Surface tension versus concentration of bio-surfactant](image)

**Figure 3.7**: Surface tension versus concentration of bio-surfactant

3.2.4 Emulsification activity

3.2.4.1 *Lysinibacillus chungkukjangi*

The *Lysinibacillus chungkukjangi* bio-surfactant was capable of emulsifying various hydrocarbons viz. olive oil (100%), rice-bran oil (85%), coconut oil (84%), kerosene
(34%), n-dodecane (14%), and n-hexane (8%) (Figure 3.8). In view of the results, it is anticipated that the bio-surfactant obtained could be used for solubilizing high-fat and oil prevalent in dairy industry wastewater and in cosmetic industries [Hazra et. al., 2015; Williams 2009].

Figure 3.8: Emulsification activity of bio-surfactant produced by Lysinibacillus chungkukjangi on various hydrocarbons

3.2.4.2 Pseudomonas indica

The Pseudomonas indica bio-surfactant was able to emulsify various hydrocarbons viz. olive oil (62%), rice-bran oil (65%), coconut oil (65%), kerosene (7%), n-dodecane (56%), and n-hexane (5%). Pseudomonas indica showed highest emulsification activity for n-dodecane, this special feature of Pseudomonas indica is because it can utilize butane (hydrocarbon) for its growth. Due to this reason it may be possible that it showed highest emulsification activity for n-Dodecane rather than other strains. The
maximum emulsification activity was observed with the olive oil, coconut oil and rice-bran oil and the emulsions were stable up to 15 days at room temperature. The details are shown in Figure 3.9. The results were comparable with previous reports which show the emulsification of 58.5 and 60% when bio-surfactant production was observed from *Pseudomonas aeruginosa* and *Arthobacter protophormiae* [Desai and Banat, 1997; Pruthi and Cameotra, 1997].

![Figure 3.9](image.png)

**Figure 3.9:** Emulsification activity of bio-surfactant produced by *Pseudomonas indica* on various hydrocarbons

### 3.2.4.3 *Pseudomonas cepacia*

The *Pseudomonas cepacia* bio-surfactant was capable of emulsifying various hydrocarbons viz. olive oil (89%), refined oil (88%), coconut oil (89%), kerosene (46%), n-dodecane (36%) and hexane (25%). The best results were obtained with oils and least with hydrocarbons and details are given in Figure 3.10. The results corresponded
to good emulsification ability of the bio-surfactant, hence, their potential applications in the hydrocarbon pollutions [Aparna et. al., 2012; Bhardwaj et. al., 2015a].

3.2.5 Purification of bio-surfactant produced by *Pseudomonas cepacia*

The TLC analysis of the mixture demonstrated many spots after charring with 5% sulfuric acid and iodine vapors. The major spot was purified by column chromatography as described in materials and methods. The yield of the pure compound obtained by column chromatography was approximately 25 mg.

![Emulsification activity](image)

**Figure 3.10:** Emulsification activity of bio-surfactant produced by *Pseudomonas cepacia* on various hydrocarbons

The $R_f$ value of the pure compound was observed to be 0.62 (chloroform: methanol, 9:1). The similar $R_f$ was also measured when the same industrial waste was used for the production of bio-surfactant by *Fusarium proliferatum* which suggest its structural relations with the previously reported compound [Bhardwaj et. al., 2015a].
3.2.6 Characterization of the bio-surfactant

3.2.6.1 Fourier Transform Infra Red (FTIR) Spectroscopy

3.2.6.1.1 Lysinibacillus chungkukjangi

The functional groups present in the bio-surfactant were identified on the basis of the FTIR spectrum recorded in the spectral region of 4000-400 cm\(^{-1}\). In Figure 3.11, a strong band at 3414 cm\(^{-1}\) occurred due to stretching vibration of the N-H group present in the peptide bond. The peak at 2343 and 2363 cm\(^{-1}\) occurred due to the presence of carbon dioxide in the atmosphere. The symmetric stretching vibrations of -CH, -CH\(_2\) and -CH\(_3\) occurred in the region 2925-2854 cm\(^{-1}\). The band at 1618 cm\(^{-1}\) occurred due to the stretching vibrations of the carbonyl group. The presence of aliphatic chains was indicated by the -CH stretching band at 1458-1377 cm\(^{-1}\). The peak at 1107 cm\(^{-1}\) corresponded to the –C-N bond. The above FTIR spectral data clearly demonstrate the lipopeptide (wave numbers 3414, 1618 and 1107 cm\(^{-1}\) for NH, CO and CN respectively) nature of the bio-surfactant as reported by Biria et. al., [2009] and Anyanwu et. al., [2011].

3.2.6.1.2 Pseudomonas indica

There are several reports on the production and characterization of bio-surfactants by Pseudomonas sp. which reveals the presence of rhamnolipids as their chemical structure [Abouseoud et. al., 2007]. The functional groups present in di-rhamnolipids produced by Pseudomonas indica were identified on the basis FTIR spectra recorded in the spectral region of 4000-400 cm\(^{-1}\). A strong absorption band at 3446 cm\(^{-1}\) occurred
due to stretching vibration of –OH group [Aparna et. al., 2012]. C-H symmetric bands of –CH₂- and –CH₃ groups of aliphatic chain were observed in the region 3100-2900 cm⁻¹. Carbonyl stretching band observed at 1717 cm⁻¹ is characteristic for ester compounds [Pornsunthorntawee et. al., 2008]. The absorption at 1650 and 1056 cm⁻¹ was because of stretching vibration of COO- and –C-O-C groups respectively [Saikia et. al., 2012] (Figure 3.12).

![Figure 3.11: FTIR spectrum of the lipopeptide bio-surfactant by Lysinibacillus chungkukjangi](image)

**Figure 3.11:** FTIR spectrum of the lipopeptide bio-surfactant by *Lysinibacillus chungkukjangi*

![Figure 3.12: FTIR spectrum of the rhamnolipids produced by Pseudomonas indica](image)

**Figure 3.12:** FTIR spectrum of the rhamnolipids produced by *Pseudomonas indica*
3.2.6.1.3 Pseudomonas cepacia

The molecular composition of the purified bio-surfactant was identified on the basis of Fourier transform infrared spectroscopy. In the spectrum (Figure 3.13), a medium band at 3367 cm\(^{-1}\) was due to the presence of -OH group. The band at 2924 and 2853 cm\(^{-1}\) were due to the symmetric stretch of sp\(^3\) hybridized –CH, –CH\(_2\) and –CH\(_3\) [Bhardwaj et. al., 2015a]. The band at 1724 cm\(^{-1}\) was due to the presence of the ester group [Chander et. al., 2012]. The bands at 1459, 1408, and 1377 cm\(^{-1}\) was due to the presence of –CH\(_3\) bending vibrations [Hazra et. al., 2015]. The bands at 1266, 1219 and 771 cm\(^{-1}\) suggests the C-O vibrations. A long chain band was appeared at 731 cm\(^{-1}\) which suggests the bending motion associated with four or more –CH\(_2\) groups in an open chain which suggests it to be a long chain hydrocarbon.

![FTIR spectrum of the purified bio-surfactant produced by Pseudomonas cepacia](image)

Figure 3.13: FTIR spectrum of the purified bio-surfactant produced by Pseudomonas cepacia
3.2.6.2 Nuclear Magnetic Resonance (NMR) Spectroscopy

3.2.6.2.1 *Lysinibacillus chungkukjangi*

Figure 3.14, represents $^1$H NMR the spectra of the bio-surfactant produced by *Lysinibacillus chungkukjangi* grown on the rice-bran as a carbon source. In the $^1$H NMR spectra, a singlet at δ 7.25 corresponded to the peak of CDCl$_3$. Other peaks in the region δ 6.90-7.25 revealed the presence of aromatic rings in the structure of biosurfactant. The peak at δ 5.14 revealed the presence of –CH=CH- moieties attached with the electronegative groups. The peaks in the region δ 4.17- 4.22 revealed the presence of -NH, –CH$_2$O-, -OCH$_2$- and –OCH$_3$ groups. The upfield signals, in the region δ 1.98-2.53 shows the presence of -CH$_2$-C=O group and δ 0.79-1.34 corresponded to sp$^3$, CH, CH$_2$ and CH$_3$ aliphatic protons [Morikawa et. al., 1993].

![Figure 3.14: $^1$H NMR spectrum](image)

When $^{13}$C NMR spectra (Figure 3.15) of the bio-surfactant produced by *Lysinibacillus chungkukjangi* analyzed, the three solvent peaks of CDCl$_3$ appeared in
the region $\delta_c$ 77.38, 77.18, and 76.76. Adjacent to this, the peaks at $\delta_c$ 71.10, 73.08, 77.60 and 81.43 corresponded to the presence of oxygen (–O) and nitrogen (–N) atoms attached to the carbon atom through a single bond. In the upfield region, the peaks at $\delta_c$ 11.55-31.95 showed the presence of $sp^3$, -CH$_3$, -CH$_2$ and -CH$_3$ groups. In the downfield region, the peaks $\delta_c$ 177.80, 181.52, 181.89 revealed the presence of carboxylic acid derivatives. Far left in the spectrum, the peak at $\delta_c$ 210.55 showed the presence of a C=O group of aldehydes or ketones.

![Figure 3.15 13C NMR spectrum](image)

3.2.6.2.2 *Pseudomonas indica*

The NMR is an efficient technique for the structural elucidation of bio-surfactants. The $^1$H NMR spectra obtained for the bio-surfactants by *Pseudomonas indica* was well correlated with that of the di-rhamo-di-lipidic congeners by *Pseudomonas aeruginosa* [Raza et. al., 2009]. Figure 3.16, shows the $^1$H NMR of rhamnolipids which shows
various peaks of rhamnose rings and fatty acid chain in the spectra. The details of the peaks are given in the Table 3.8.

**Figure 3.16:** $^1$H NMR spectrum of the rhamnolipid bio-surfactants produced by *Pseudomonas indica* grown on rice-bran as the carbon source

In $^{13}$C NMR spectra, the solvent peaks of CDCl$_3$ appeared at $\delta_c$ 77.10 to 77.30 (Figure 3.17). To the very right of the solvent peak, the peaks in the region $\delta_c$ 10.9522-34.5096 showed the presence of $sp^3$, $-\text{CH}_3$, $-\text{CH}_2$ and $-\text{CH}_3$ groups. Next to this region, the peaks at $\delta_c$ 60.4763 and 73.0868 corresponded to the presence of $-\text{CH}_2-\text{O}$ groups. On the left of the solvent peak, the peaks at $\delta_c$ 114.13, 128.7202, 128.8062 and 139.24 revealed the presence of $\text{C}=\text{C}$ groups. Far left in the spectra, the peaks at $\delta_c$ 171.2554 and $\delta_c$ 210.1959 showed the presence of the $\text{C}=\text{O}$ group of esters and carboxylic acids [Silva et al., 2014].
**Table 3.8**: $^1$H NMR chemical shifts (in ppm) of major di-rhamnolipids produced by *Pseudomonas indica*

<table>
<thead>
<tr>
<th>Moiety</th>
<th>Proton location</th>
<th>Chemical shift (δ ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhamnose (s)</td>
<td>C-1’</td>
<td>4.22</td>
</tr>
<tr>
<td></td>
<td>C-2’</td>
<td>3.92</td>
</tr>
<tr>
<td></td>
<td>C-3’</td>
<td>3.76</td>
</tr>
<tr>
<td></td>
<td>C-4’</td>
<td>3.68</td>
</tr>
<tr>
<td></td>
<td>C-5’</td>
<td>3.68</td>
</tr>
<tr>
<td></td>
<td>-CH₃ (ring)</td>
<td>1.23-1.44</td>
</tr>
<tr>
<td></td>
<td>C-1”</td>
<td>4.19</td>
</tr>
<tr>
<td></td>
<td>C-2”</td>
<td>3.67</td>
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<td>C-3”</td>
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<tr>
<td></td>
<td>C-5”</td>
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</tr>
<tr>
<td></td>
<td>-CH₃ (ring)</td>
<td>1.14-1.42</td>
</tr>
<tr>
<td>Hydroxy fatty acid</td>
<td>C-1</td>
<td>4.06</td>
</tr>
<tr>
<td></td>
<td>C-2</td>
<td>2.33</td>
</tr>
<tr>
<td></td>
<td>C-3</td>
<td>1.94</td>
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<td>-(CH₂)₅-</td>
<td>1.14-1.42</td>
</tr>
<tr>
<td></td>
<td>-CH₃</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>-CH₂-COO-</td>
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</tr>
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<td></td>
<td>-COO-CH₂-</td>
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</tr>
<tr>
<td></td>
<td>-O-C-H</td>
<td>4.20</td>
</tr>
</tbody>
</table>

**Figure 3.17** $^{13}$C NMR spectrum
3.2.6.2.3 *Pseudomonas cepacia*

The structural characterization of the purified bio-surfactant produced by *Pseudomonas cepacia* was made by performing 1D and 2D NMR techniques like $^1$H, $^{13}$C NMR, HSQC, $^1$H-$^1$H COSY, and HMBC experiments.

In $^1$H NMR (Figure 3.18) spectrum, six methyl signals at $\delta$ 0.8 (t, $J$=6.64) which appeared as triplets suggested their attachment with methylene protons. Three protons of one methyl group found to be overlapped with the protons of methylene which appeared at $\delta$ 1.2. The proton signal of the of long hydrocarbon chains was seen in the region $\delta$ 1.2-1.3, which were for seventy methylene. The upfield region was not clear due to overcrowding of long fatty acid chains protons in the structure of purified bio-surfactant, therefore, the number of protons was considered approximately as per mass of the molecule [Maneerat et. al., 2006]. Eight methylene protons signal at $\delta$ 1.4 and 2.0 suggested their allylic nature. In which, four protons of two methylene groups appeared at $\delta$ 2.0 and two protons of sp$^3$–CH- appeared at $\delta$ 2.3 which suggest its insertion between double bonds. A doublet of two protons at $\delta$ 7.3 (2H, $J$=2.4) revealed the attachment of –CH=CH- moiety’s protons with an electron withdrawing functional group. At $\delta$ 7.1 (1H, $J$=8.2) and $\delta$ 6.6 (1H, $J$= 8.2) doublet of doublet and a doublet also showed the presence of -HC=CH- and its attachment somewhere in the proximity of the electron withdrawing group. Signals of three and seven protons appeared at $\delta$ 5.8 and $\delta$ 4.9, in which two protons at $\delta$ 4.9 were for methylene protons of –CH$_2$OH and rest protons were for isolated olefinic protons in the long hydrocarbon chain. The –OH proton appeared as a broad singlet at $\delta$ 3.6.
Figure 3.18: $^1$H NMR Spectrum

In $^{13}$C NMR (Figure 3.19), 18 peaks appeared, out of which five peaks were in the downfield region ($\delta_c$ 114.07, 115.91, 123.51, 124.07, 139.29) of the spectrum, which indicated olefinic carbons and twelve signals ($\delta_c$ 14.12, 22.70, 28.96, 29.17, 29.37, 29.52, 29.63, 29.70, 30.29, 31.62, 31.93, 33.8) in the upfield region of the spectrum, which indicated the methyl and methylene carbons. One carbon signal got merged at $\delta_c$ 77.02 with the solvent peaks of CDCl$_3$. 
HSQC experiment ($^1\text{H} \rightarrow ^{13}\text{C}$) helped to correlate the protons ($^1\text{H}$ NMR) and the carbons ($^{13}\text{C}$ NMR) by joining lines in the spectrum (Figure 3.20). Also, the proton-proton correlation were observed from $^1\text{H}-^1\text{H}$ COSY experiments (Figure 3.21) and thus, the proton signals at δ 7.1/ 6.6 (17-H/15-H) and δ 5.8/ 4.9 (18-H/14-H) were connected which suggested the 18H-14H, 17H-15H proton connectivities. Lastly, HMBC experiment (Figure 3.22) was performed to see neighborhoods proton and revealed three types of proton to carbon correlations ($^1\text{H} \rightarrow ^{13}\text{C}$) 17-H/13-H, 14-H/3,8-H, 18-H/3,8-H.
Figure 3.20: HSQC Spectrum
Figure 3.21: $^1$H-$^1$H COSY Spectrum

Figure 3.22: HMBC Spectrum
Collective analysis of all the spectral data (FTIR, $^1$H, $^{13}$C NMR, HSQC, $^1$H-$^1$H COSY, and HMBC) have enlightened the tentative structure represented in Figure 3.23. The key $^1$H-$^1$H COSY and HMBC correlations are also shown in Figure 3.24 and Figure 3.25. The IUPAC name of the compound was generated in ChemBioDraw Ultra 12.0 which was as follows:

![Chemical structure]

**Figure 3.23:** Tentative structure of the purified bio-surfactant

(Z)-1-((1Z,4Z,16Z)-3,17-dimethyloctatetraconta-1,4,16-trien-1-yl) 5-((1Z,4Z,8Z,14Z)-3,8,15-trimethyloctatetraconta-1,4,8,14-tetraen-1-yl)-3-(2-hydroxyethylidene) pentanedioate. The proposed structure suggests the utilization of fatty acid chains of de-oiled rice-bran in the bio-surfactant production by *Pseudomonas cepacia*. A long chain enamide has already been reported by a research group Bhardwaj et. al., [2015a], which was isolated from the utilization of rice-bran by *Fusarium proliferatum*. The carbon source (de-oiled rice-bran) used in the present study was taken from the same industry, which has given a new pentanedioate compound during the biosynthesis of surfactants by *Pseudomonas cepacia* (Figure 3.23).
3.2.6.3 Liquid Chromatography-Mass Spectrometric (LC-MS) analysis

3.2.6.3.1 Lysinibacillus chungkukjangi

The mass spectrum (Figure 3.26) of the Lysinibacillus chungkukjangi bio-surfactant showed the presence of a mixture of lipopeptides with molecular weights between
m/z 202 and 1397. The parent ion peak at 1397 corresponded to the lipopeptide bio-
surfactant (putisolvin I and II) produced by *Pseudomonas putida* [Kuiper et. al., 2004].
The other fragment ions at m/z 939, 872, 871, 653, 639, 481, 393, 202 were also
typical of lipopeptide bio-surfactants [Yakimov et. al., 1995; Morikawa et. al., 2000;
Pecci et. al., 2010; Hathout et. al., 2000]. In conclusion, the whole spectroscopic and
chromatographic studies inferred to the presence of lipopeptide bio-surfactants.

![Figure 3.26 ESIMS spectrum of the lipopeptide bio-surfactants](image)

**Figure 3.26** ESIMS spectrum of the lipopeptide bio-surfactants

### 3.2.6.3.2 *Pseudomonas indica*

The crude bio-surfactant was analyzed by LC-MS and the MS spectra were acquired in
the [M+H]^+ positive ion mode. The LC-MS chromatogram of the bio-surfactants
showed various peaks out of which the peaks having the retention time 1.067 (m/z
694, 650, 606, 414, 400, 398), 5.369 (m/z 808, 807, 806), 8.17 (m/z 783, 782, 760, 759,
758) and 18.191 (m/z 1343, 1342, 680, 664, 663) were the major ones. Mass
spectrometry analysis revealed the presence of four major di-rhamnolipid bio-
surfactants having m/z values 650, 806, 758 and 663 respectively (**Figure 3.27**). These
corresponded to the di-rhamno-di-lipidic congeners, Rha-Rha-C_{10}-C_{10} (molecular
weight (MW), 650), Rha-Rha-C_{16}-C_{14}-CH_3 (MW, 806; Rha-Rha-C_{16}-C_{14} = 791, 791+15 (one -CH_3) = 806), Rha-Rha-C_{14:1}-C_{14:1} or Rha-Rha-C_{14:2}-C_{14} (Rha-Rha-C_{14}-C_{14} = 763 [Abdel-Mawgoud et. al., 2010]; one double bond in both the chains or two in a single chain will reduce four hydrogens and the MW will be equal to 758 or two double bonds in one chain= 758) and Rha-Rha-C_{10:1}-C_{10}-CH_3 or Rha-Rha-C_{10}-C_{10:1}-CH_3 (MW, 663) [Pantazaki et. al., 2011] respectively. The ions other than the major fragment ions may be their fragments or because of the impurities precipitated at the time of biosurfactant extraction.

(a) Retention time (1.067)

(b) Retention time (5.369)
(c) Retention time (8.17)

(d) Retention time (18.191)

Figure 3.27: MS of the major peaks of LC chromatogram having retention times (a) 1.067 (b) 5.369 (c) 8.17 (d) 18.191

3.2.6.4 LC-ESI-HRMS of Pseudomonas cepacia

Additional support for the proposed structure was attained through MS studies. The ESI/HRMS of the compound (Figure 3.28) gave important major peaks at m/z 905.6730, 724.5662, 681.5162, 491.3010, 475.3269, for the possible fragments as shown in Figure 3.29. ChemBioDraw Ultra 12.0 was used for the calculation of molecular masses of the fragment ions and individual peaks were matched with
molecular ion peaks in the ESI-HRMS spectrum of the pure compound. The slight difference in the calculated and experimental masses is because of the theoretical and experimental determinations. In the software the molecule is supposed to be gaseous in nature while in actual conditions it is viscous, so the results vary in real and theoretical studies/measured and calculated mass values. The molecular mass of the compound was calculated to be 1575.5239 but the parent ion peak was not observed in the ESI-HRMS spectrum due to longer fatty acid chains and higher molecular weight [Bhardwaj et. al., 2015a]. The calculated fragment ion m/z 905.7962 (appeared due to the removal of m/z 669.7277 from the molecular mass m/z 1575.5239) gave highest M+ peak in the mass spectrum at m/z 905.6730. Further, the fragment ion at m/z 723.7383 (appeared due to removal of m/z 182.0579 from m/z 905.7962) gave another M+1 peak at m/z 724.5662. The fragment ion m/z 681.7277 (appeared due to the removal of m/z 42.0106 from m/z 723.7383) gave a M+ peak at m/z 681.5126. The molecular ion fragment m/z 490.5478 (191.1800 gets removed from the m/z 681.7277) gave a M+1 peak at m/z 491.3010. The peak at m/z 475.3269 was supposed due to the removal of some portion from one chain in the structure of the purified bio-surfactant as shown in Figure 3.29.

**Figure 3.28:** ESI/HRMS of the purified bio-surfactant
The possible biochemical mechanism for the proposed structure may be as follows given in Figure 3.29b.

### 3.2.7 Applications

#### 3.2.7.1 DPPH assay results

The anti-oxidants are capable of seizing the free-radical chain reactions. If the absorbance of DPPH solution decreases as compared to the control upon the treatment with bio-surfactants, it reveals the anti-oxidant potential of bio-surfactant. The DPPH activity was checked using ascorbic acid as standard and IC₅₀ (the half maximal inhibitory concentration) calculated. The IC₅₀ of the standard was 0.056 mg/mL and that of the bio-surfactant produced by *Lysinibacillus chungkukjangi,*
Figure 3.29b: Suggested Biochemical Mechanism for the biosynthesis of bio-surfactants from *Pseudomonas cepacia*  

*Pseudomonas indica,* and *Pseudomonas cepacia* were 1.3, 50.97, and 11.23 mg/mL. As the concentration of bio-surfactant increased in the solution, the DPPH scavenging activity also increased simultaneously in case of all the bacterial strains. Among all the strains *Lysinibacillus chungkukjangi* showed the highest anti-oxidant potential while *Pseudomonas indica* possessed the least. The result for the bio-surfactant produced by *Lysinibacillus chungkukjangi* showed it to be a good scavenging agent, while, for *Pseudomonas indica* the applicability was found to be very less. Although, the result of the DPPH assay for *Pseudomonas cepacia* was not good enough, but was far better.
than the bio-surfactant produced by *Fusarium proliferatum* grown on the rice-bran of the same industry. When compared to the literature, all the bacterial strains possessed least anti-oxidant potential as compared to the surfactin (lipopeptide) bio-surfactant produced by *Bacillus subtilis*. IC$_{50}$ of surfactin was 0.25 mg/mL when butylated hydroxyl toluene (BHT) was standard in the DPPH assay [Bhardwaj et. al., 2015a; Yalcin and Cavusoglu, 2010].

### 3.2.7.2 Microbial Enhanced Oil Recovery (MEOR) potential

When the bio-surfactants produced by *Pseudomonas indica* were analyzed for the sandpack test, kerosene recovered was 70% higher as compared to distilled water alone. Hence, rhamnolipids can be used in various microbial enhanced oil recovery (MEOR) processes where the recovery is not efficient with other methods. The results obtained were comparable with the bio-surfactant produced by *Bacillus subtilis* which was able to recover 62% of oil [Makkar and Cameotra, 1999]. In other experiments, when *Lysinibacillus chungkukjangi* and *Pseudomonas cepacia* were analyzed for the sandpack test, the results were better when bio-surfactant was added to the distilled water as compared to the distilled water alone. As the crude bio-surfactant exhibited MEOR potential, it can be utilized directly in the mentioned process simply after extraction from the fermentation media. The both Bio-surfactants, recovered up to 90% of entrapped kerosene, which showed their excellent MEOR activity and makes their use possible in petrochemical industries, cleaning of oil-tanks, etc., where superior recovery is needed in order to enhance the process. Pruthi and Cameotra, [1997] reported the similar results when the bio-surfactant produced by *Arthrobacter protophormiae* was used to demonstrate the sandpack test. Nevertheless, the results
were comparatively better when the bio-surfactant produced by *Aspergillus ustus*, and *Serratia marcescens* was subjected for MEOR study, and recovery was approximately 75% and 51% of hydrocarbons and kerosene [Kiran et. al., 2009].