CHAPTER 4

BIOSYNTHESIS OF FUNGAL SURFACTANTS

This chapter deals with the biosynthesis of surfactants by a fungal strain *Fusarium proliferatum* and its characterization by various chromatographic and spectrometric techniques. The bio-surfactant was also analyzed for its MEOR potential and antioxidant activity.

4.1 Experimental

4.1.1 Materials

All the chemicals used were of reagent grade. The rice-bran and sludge were collected from a local rice-bran oil industry, A.P. Organics Private Limited, Dhuri, Sangrur (India).

4.1.2 Strain Isolation and preparation of seed culture

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 by translation elongation factor gene sequence data, at IMTECH, Chandigarh. TEF gene region was amplified using standard PCR reaction. The primer pair EF-1 (5’-ATGGGTAAGGA(A/ G)GACAAGAC-3’) and EF-2 (5’-GA(G/A)GTACCAGT(G/C)ATCATGTT-3’) was used in a PCR reaction using annealing temperature 53 °C [Geiser et. al., 2004]. The amplified product was sequenced using ABI PRISM ® Big Dye Terminator Cycle sequencing.

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.

4.1.3 Fermentation medium

The fermentation media for the production of bio-surfactants had composition (g/L): K$_2$HPO$_4$ (2.2), 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. temperatures (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 flasks containing 150 mL of the fermentation media. At the end of the fermentation, samples were taken from the media to determine surface tension.

4.1.4 Extraction of bio-surfactant

In the extraction process of Bio-surfactants, ultrasonication (Ultrasonic bath, LINCO, 40 MHz) was also performed in the procedure to check the yield difference with and without this step. After 120 h of incubation the flasks were withdrawn and kept in an ultrasonic bath for 30 min and then centrifuged at 5800 rpm for 20 min. the supernatant was filtered for the next step and the settled rice bran was discarded. The supernatant served the source of crude bio-surfactant. The pH of the supernatant was adjusted to 2.0 with 6N HCl and kept overnight for precipitation. The bio-surfactant was extracted thrice with various solvent systems viz. acetone, chloroform, ethyl acetate, methanol, varying ratios of ethyl acetate to methanol at room temperature and the organic layer was transferred to a rotary evaporator at 40 °C. The dried residues were washed with hexane to remove unutilized fatty acids and re-dissolved in methanol; sodium sulfate was added to remove the traces of water. 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 product obtained was purified using column chromatography.
4.1.5 Surface tension and 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 ori 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. The results of the surface tension and CMC measurements of the bio-surfactant produced by Fusarium proliferatum were 36.6 mN/m and 0.33 mg/mL, respectively.

4.1.6 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]. Table 4.1 gives the emulsification activity of various hydrocarbons by fungal strain *Fusarium proliferatum*.

**Table 4.1:** Emulsification activity of the fungal bio-surfactant on various hydrocarbons

<table>
<thead>
<tr>
<th>Hydrocarbons</th>
<th>Emulsification activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fusarium proliferatum</em></td>
<td></td>
</tr>
<tr>
<td>Olive oil</td>
<td>94</td>
</tr>
<tr>
<td>Rice-bran oil</td>
<td>95</td>
</tr>
<tr>
<td>Coconut oil</td>
<td>95</td>
</tr>
<tr>
<td>Kerosene</td>
<td>90</td>
</tr>
<tr>
<td><em>n</em>-dodecane</td>
<td>43</td>
</tr>
<tr>
<td>Hexane</td>
<td>37</td>
</tr>
</tbody>
</table>

**4.1.7 TLC-Column Chromatography**

The crude bio-surfactant was analyzed by thin layer chromatography (TLC) using silica gel plates (Silica gel 60, F	extsubscript{254}, Merck, Germany). The chromatograms were developed by using chloroform, methanol (9:1) solvent system and visualized with the reagents (i) iodine vapors and (ii) 5% sulfuric acid. Further the crude bio-surfactant was purified using column chromatography. For this purpose, column (60×2.5 cm) was packed with 500 g silica gel (60-120) with wet packing using chloroform. 500 mg of the recovered bio-surfactant was dissolved in the minimum amount of chloroform and loaded into the column, dry silica and cotton was placed after pouring the sample into the column, in order to avoid the disturbance of silica while running the solvent. The compounds were eluted in a stepwise fashion with chloroform: methanol in varying ratios (100, 99:1, 98:2, 97:3…..90:1, 80:20, 70:30, 60:40, and 50:50). In order to obtain the purified fractions, TLC of each fraction was performed by developing in the solvent system.
chloroform: methanol (9:1) and visualizing with iodine vapors and 5% sulfuric acid. All the fractions with the same pattern of spots were pooled and dried by rotary evaporation to obtain the purified fractions. The purified compound showed a single spot in TLC analysis at $R_f$ 0.66 (chloroform: methanol, 9:1). **Figure 4.1** explains a systematic approach for the purification of bio-surfactant from *Fusarium proliferatum*.

![Schematic procedure of bio-surfactant purification from *Fusarium proliferatum*](image)

**Figure 4.1**: Schematic procedure of bio-surfactant purification from *Fusarium proliferatum*

### 4.1.8 FTIR

The purified bio-surfactant was analyzed by FTIR spectroscopy and the spectrum was recorded in an FTIR system (Nicolet, USA) using KBr disc.
FTIR Spectral data: Wave number (cm\(^{-1}\)); 3409, 3077, 2923, 2852, 1718, 1641, 1508, 1463, 1377, 992, 909 and 720.

4.1.9 NMR

The bio-surfactant was subjected to further analysis with the help of NMR spectroscopy. All the \(^1\)H, \(^{13}\)C NMR, HSQC, COSY, HMBC spectra was recorded on Ultrashield 400 NMR spectrometer (Bruker, Germany) equipped with a 5 mm multinuclear inverse probe head. Preliminary data processing was carried out with Bruker software, TOPSPIN 3.2. The NMR samples were prepared immediately prior to NMR analysis by dissolving the purified bio-surfactant (15 mg/0.6 mL) in deuterated chloroform. Table 4.2 gives the \(^1\)H and \(^{13}\)C NMR spectral data of the purified bio-surfactant produced by *Fusarium proliferatum*.

4.1.10 LC-ESI-HRMS Analysis

The purified bio-surfactant was analyzed using a Thermo-Finnigan LCQ ion-trap MS (Arcade, New York, USA) using the syringe-infusion pump. The collected samples were dissolved in acetonitrile and infused in the LC-ESI source. An ESI spectrum (positive mode) was recorded from \(m/z\), 50-1500. The MS/MS profiles were obtained by collision-induced dissociation (CID) using argon as collision gas. The positive ions of compounds were obtained with capillary at 4200 V, cone 45 V, spray voltage 4.03 kV, spray current 5.2 μA, dry heater temperature 250 °C, nebulizer pressure 1.2 Bar and sheath gas flow rate 7.0 mL/min. The fragmentation was achieved with the collision
energy at 18 and 20 V. the mass spectrum data of the purified bio-surfactant is as below:

**Mass Spectral data:** m/z 871.1974, 701.4782, 634.4374, 605.2707, 475.3173, 453.3367.

**Table 4.2:** $^1$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 (t, $J= 6.6$ Hz)</td>
<td>14.1</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>22.7</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>28.6</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>29.1</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>29.3</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>29.5</td>
</tr>
<tr>
<td>7</td>
<td>1.2-1.4 (merged broad signal)</td>
<td>29.6</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>29.6</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>29.7</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>30.2</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>31.6</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>31.9</td>
</tr>
<tr>
<td>13</td>
<td>2.0 (dd, $J=7.48$)</td>
<td>33.8</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>34.2</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>34.7</td>
</tr>
<tr>
<td>16</td>
<td>4.9 (5H, qdd, $J= 7.9, 1.6, 10.4$)</td>
<td>114.0</td>
</tr>
<tr>
<td>17</td>
<td>6.6 (1H, d, $J= 8.2$ )</td>
<td>115.9</td>
</tr>
<tr>
<td>18</td>
<td>7.1 (1H, dd, $J=8.2$)</td>
<td>123.5</td>
</tr>
<tr>
<td>19</td>
<td>7.3 (1H, d, $J= 2.4$)</td>
<td>124.0</td>
</tr>
<tr>
<td>20</td>
<td>-</td>
<td>135.1</td>
</tr>
<tr>
<td>21</td>
<td>5.9 (3H, td,dd, $J= 6.6, 16.9; 3.2, 16.9)</td>
<td>139.2</td>
</tr>
<tr>
<td>22</td>
<td>-</td>
<td>151.8</td>
</tr>
</tbody>
</table>
4.1.11 Applications

4.1.11.1 DPPH (1,1-diphenyl-2-picryl hydrazyl) 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\(_{50}\) (the half maximal inhibitory concentration) was also determined. IC\(_{50}\) denotes to the concentration of sample required to scavenge 50% of the DPPH free radicals. IC\(_{50}\) was calculated to be 18.81 mg/mL. Table 4.3 represents the percentage inhibition of the bio-surfactant produced by fungal strain Fusarium proliferatum. The results were not promising for their future anti-oxidant applications.

4.1.11.2 Microbial Enhanced Oil Recovery (MEOR)

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. The MEOR potential of the Fusarium proliferatum is given in Table 4.4.

Table 4.3: Percentage inhibition of the DPPH by fungal 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>0.84</td>
</tr>
<tr>
<td>4.</td>
<td>4.00</td>
<td>5.30</td>
</tr>
<tr>
<td>5.</td>
<td>5.00</td>
<td>7.00</td>
</tr>
</tbody>
</table>

Table 4.4: MEOR potential of the distilled water and the bio-surfactant

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Recovery with distilled water (%)</th>
<th>Recovery with bio-surfactant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Less than 50</td>
<td>95</td>
</tr>
</tbody>
</table>

4.2 Results and Discussion

4.2.1 Characterization of the strain

The fungal strain was characterized by translation elongation factor (TEF) and was found to be *Fusarium proliferatum*. The strain was found to reduce the surface tension
to 36.6 mN/m, respectively. The TEF gene sequence data of the *Fusarium proliferatum* strain was sequenced using ABI PRISM ® Big Dye Terminator Cycle sequencing. The TEF gene sequence data are as follows:

GTCGACTCTGGCAAGTCTGATCTGTGAGTACTACCCTGGACGATGAGCTTATCTGCCATC
GTGATCTGACCAAGATCTGCGGGGTACATCTTGGAAGACAATATGCTGACATCGCTTCACA
GACCGGTCACTTGATCTACCAGTGCGGTGATCGAACAAGCGAACATCGAGAAGTTCGAGA
AGTTAGTCACTCTTCCCTCGATCGCGGTCTCTGCCCCACCGATTTTACTTTCGATTGAAAC
GTGCCTGCTACCCCCTGCTCGAGACCCAAAAATTTTTGCGATATGACCGGAATTTTTTTGTTGGGC
ATTATACCGGCACCTGCAGCGATGAGCGCGTTTTTGCCCTTTCCTGTCCACAAACCTCAATGAGC
GCATTGTCAGTGTAAGCGAGCGACTAACCATCAGAATAGAAGGCTGAGCTCGGTAA
GGTTTCTAAGTACGCTGGTTCTTGACAAGGCTAAGCGGAGCGTGAGCGTGGTATCA
CCATCGATATTGCTCTGGAAGTTCGAGACTCCTCGCTACTATGGTCACCGTATGATGTGTT
GTCGCTCATACCTCATCTCTTCTCATACTACTTAAACATCATACGATCGGCTCCCCTACAC

4.2.2 Optimization of bio-surfactant production

The optimized factors for the bio-surfactant production by *Fusarium proliferatum* were as follows:

4.2.2.1 Carbon source and its percentage (% \( w/v \))

The fungal strain *Fusarium proliferatum* was able to produce optimum yields (90-95%) of bio-surfactants when the carbon source was rice bran (3%) in combination with fatty acids (2.5%) and glucose (2%) in a concentration of 7.5% (w/v). There are so many reports in the literature which suggests the production of bio-surfactants on
mixed substrates [Cooper and Paddock, 1983]. The yield of the bio-surfactant recovered after solvent extraction was 17.6 g/L and at this concentration the surface tension was 36.6 mN/m. The yield was found to be better as compared to the previously studied fungi *Candida lipolytica* where the optimum yield was 8.0 g/L and 4.5 g/L [Sarubbo et. al., 2007; Rufino et. al., 2007]. Figure 4.2 represents the optimization of nutritional parameters with respect to the optimal yields and detailed discussion of the environmental factors is as below:

4.2.2.2 Nitrogen source and its percentage (%, w/v)

The optimum yields were obtained when the nitrogen source was yeast extract (1.5%) in combination with ammonium nitrate (0.5%) in the concentration of 2% (w/v). The emulsions with the respective carbon and nitrogen sources were stable up to 15 days. Yeast extract was found to be a good choice of nitrogen source in the previous studies on *Candida* spp. done by Cavalero and Cooper, [2003], Kim et. al., [1997]; Cooper and Paddock, [1983].
Figure 4.2: Optimization of bio-surfactant production for *Fusarium proliferatum*

### 4.2.2.3 C/N ratio

A balanced carbon to nitrogen ratio in the nutrient media plays a significant role in the optimum bio-surfactant production. *Fusarium proliferatum* grown on the rice-bran industry’s waste (rice-bran and fatty acids) as the carbon source and yeast extract plus ammonium nitrate as the nitrogen source gave optimum bio-surfactant production with a C/N ratio 3.75. The yield of the bio-surfactant at this C/N ratio was found to be 17.6 g/L.

### 4.2.2.4 Incubation time, inoculation volume, and shaking speed

All these environmental factors also give equal contribution to the optimum yields of Bio-surfactants. The inoculation volume, incubation time, and shaking speed were 10%, 96 h, and 150 rpm respectively. The results were comparable to the previous studies of when *Candida lipolytica* produced bio-surfactant with shaking at 150 rpm [Sarubbo et. al., 2007] and *Candida bombicola* with 10% inoculum size [Deshpande and Daniels, 1995] and *Pseudomonas aeruginosa* in 96 h of incubation time [Xia et. al., 2012].

### 4.2.2.5 pH and temperature

The optimized pH and temperature for the fungal strain *Fusarium proliferatum* when grown on rice-bran industry’s waste were 6.5±0.2 and 30 °C respectively. These results
were similar to the previous reports by Benincasa et. al., [2002], Kim et. al., [1997], Fox and Bala, [2000] and Deshpande and Daniels, [1995].

4.2.2.6 Ultrasonication

The yield of bio-surfactants increased up to 30% on ultrasonication of the media for 30 min before solvent extraction. This significant yield enhancement can be understood by considering the fact that bio-surfactants are extracellular and intracellular in nature and liberated into the fermentation medium during the growth of microorganisms. The extracellular bio-surfactants are easily recovered from the media after cell removal but the intracellular bio-surfactants can be liberated into the medium only after the cell rupture [Baker and Chen, 2010]. So, when the media was ultrasonicated prior to centrifugation, cell rupture may occur and the yield comes out to be more as compared to when it is done without ultrasonication [Suslick, 1990].

4.2.3 Bio-surfactant characteristics

The bio-surfactant produced by *Fusarium proliferatum* was dark brown honey like in color and the best solvent system for the optimal extraction were ethyl acetate and methanol. The crude bio-surfactant was further purified by using column chromatography technique.

4.2.4 Surface tension and CMC measurements

The surface tension and CMC measurements of the bio-surfactant were determined. The de-ionized water used for calibration purposes had the surface tension, 71.2 mN/
m. The surface tension and CMC of the purified bio-surfactant were calculated to be 36.6 mN/m and 0.33 mg/mL respectively. The CMC value was determined by the plot of surface tension with varying concentration of purified bio-surfactant as given in Figure 4.3. The bio-surfactant produced by *Candida lipolytica* grown on industrial residue lowered the surface tension to 32 mN/m, while the bio-surfactant produced by Nocardiopsis sp. and *Candida lipolytica* grown on olive oil and canola oil reduced the surface tension to 29 and 30 mN/m respectively [Rufino et. al., 2007; Khopade et. al., 2012; Sarubbo et. al., 2007].

![Figure 4.3: Surface tension reduction trend in pure bio-surfactant solution](image)

**4.2.5 Emulsification Activity**

The bio-surfactant produced was able to emulsify various hydrocarbons *viz.* coconut oil, refined oil, kerosene, n-dodecane. Among all the hydrocarbons, coconut and refined oil were the best substrates and n-dodecane was the poorest (Figure 4.4). The ability of bio-surfactants to emulsify coconut and refined oil suggests its potential
applications as cleaning and emulsifying agents in the food industry. The emulsification order of hydrocarbons by the culture broth of *Fusarium proliferatum* was in the order coconut oil= refined oil (95%) > olive oil (94%)> kerosene (90%) > n-dodecane (43%) > hexane (37%). The results show the bio-surfactant could emulsify different hydrocarbons which confirmed their applicability against different hydrocarbon pollution, such that it enhances the availability of the recalcitrant hydrocarbons [Aparna et. al., 2012].

![Emulsification activity of bio-surfactant produced by Fusarium proliferatum on various hydrocarbons](image)

**Figure 4.4:** Emulsification activity of bio-surfactant produced by *Fusarium proliferatum* on various hydrocarbons

### 4.2.6 TLC- Column Chromatography Analysis

The TLC analysis performed for the crude extract showed the presence of many compounds after charring with 5% sulfuric acid and iodine vapors. The product obtained was purified using column chromatography. Light brown colored viscous compound was isolated in 15 mg amount. The pure bio-surfactant thus isolated
showed surface active properties. The purity of the compound was checked by performing TLC analysis, which showed a single spot at Rf value 0.66 (chloroform: methanol, 9:1).

4.2.7 FTIR analysis

The functional groups present in the purified bio-surfactant were identified on the basis of FTIR spectra recorded in the spectral region of 4000-400 cm\(^{-1}\). In Figure 4.5, a strong band at 3409 cm\(^{-1}\) occurred due to stretching vibration of the N-H group. The peak at 3077 cm\(^{-1}\) corresponded to the presence of vinyl =C-H- group. The symmetric stretching vibrations of -CH, -CH\(_2\) and -CH\(_3\) occurred in the region 2923-2852 cm\(^{-1}\) in line with the previous results reported by Biria et. al., [2009]. The band at 1718 and 1508 cm\(^{-1}\) indicated the stretching and bending vibrations of secondary amide group. The band at 1641 cm\(^{-1}\) could be assigned to the stretching vibrations of the imine group (-C=N-). The presence of bending modes in the region 1463- 1377 cm\(^{-1}\) indicates the presence of methyl and methylene groups which is similar to the results reported by Hazra et. al., [2015]. The strong bands at 992 and 909 cm\(^{-1}\) show the bending vibrations of monosubstituted double bonds (vinyl) of alkenes. The absorption band at 720 cm\(^{-1}\) indicates the presence of cis substitution of the double bonds. The above FTIR spectral data demonstrate the presence of functional groups amide, imine, alkane and alkene (wave numbers 3409, 1641, 2923-2852 and 992-909 cm\(^{-1}\) which is similar to the results reported by Maneerat et. al., [2006].

4.2.8 NMR analysis
The structure of the purified bio-surfactant was elucidated using $^1$H, $^{13}$C NMR, HSQC, $^1$H-$^1$H COSY, and HMBC experiments. The $^1$H NMR (Figure 4.6) indicated the presence of six methyl signals at $\delta$ 0.8 (t, $J$=6.64) which appeared as triplets suggesting their attachment with methylene protons. Another four methyl proton signals was overlapped with the methylene signals at $\delta$ 1.2. Four $sp^3$ –CH- (methine) signal also overlapped at $\delta$ 1.2. Forty three methylene signals appeared in the region $\delta$ 1.2-1.4 suggesting the long chain hydrocarbons. Ten methylene signals appeared in the region $\delta$ 1.5 and 2.0 suggesting their allylic nature. 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]. A doublet at $\delta$ 7.3 ($J$=2.4) suggested the attachment of $sp^2$ methine protons with an electron withdrawing functional group. At $\delta$ 7.1 ($J$=8.2) and 6.6 ($J$= 8.2) doublet of doublet and a doublet showed the presence of -HC=CH- and its attachment somewhere in the proximity of the electron withdrawing group. Two signals at $\delta$ 5.8 and 4.9 suggested the presence of isolated olefinic protons in the long hydrocarbon chain.

The $^{13}$C NMR (Figure 4.7) showed 22 peaks, in which seven signals appeared in the downfield region of the spectrum, which indicated olefinic carbons ($\delta_c$ 114.0, 115.9, 123.5, 124.0, 135.1, 139.2, 151.8) and fifteen signals in the upfield region of the spectrum, which indicated the methyl and methylene carbons ($\delta_c$ 14.1, 22.7, 28.6, 29.1, 29.3, 29.5, 29.6, 29.6, 29.7, 30.2, 31.6, 31.9, 33.8, 34.2, 34.7). Combined analysis of $^1$H, $^{13}$C NMR, and HSQC indicated the presence of seven signals in the olefinic
region, one was quaternary of imine moiety, and remaining six signals indicated the presence of three double bonds.

**Figure 4.5:** FTIR spectrum of the purified bio-surfactant

**Figure 4.6:** $^1$H NMR spectrum
**Figure 4.7:** $^{13}$C NMR spectrum

The protons and the carbons present in the $^1$H, $^{13}$C NMR spectrum were correlated with the help of HSQC experiment. **Figure 4.8**, represents the HSQC spectrum and the lines connecting proton and carbon represents the respective proton to carbon ($^1$H$\rightarrow^{13}$C) attachments.

The correlations between the protons were observed from the $^1$H-$^1$H COSY experiments (**Figure 4.9**). The $^1$H-$^1$H COSY spectrum presented correlations between proton signals $\delta$ 7.3/ 7.1 (19-H/18-H) and at $\delta$ 7.1/ 6.6 (18-H/17-H) and at $\delta$ 5.8/4.9 (21-H/ 16-H) and $\delta$ 2.0/5.8 (13-H/21-H) which suggested the presence of 19-18, 18-17, 16-21, and 13-21 proton connectivities.
The HMBC spectrum showed three types of proton to carbon ($^1\text{H} \leftrightarrow ^{13}\text{C}$) 19-H/18-H, 13-H/16-H, 20-H/17-H correlations. Figure 4.10, represents the HMBC spectrum and lines drawn shows the connections.

Figure 4.8: HSQC spectrum
Figure 4.9: $^1$H-$^1$H COSY spectrum
Combined analysis of the FTIR, $^1$H, $^{13}$C NMR, HSQC, $^1$H-$^1$H COSY, and HMBC experiments have led to the tentative structure represented in Figure 4.11. The $^1$H-$^1$H COSY, and HMBC correlations are also given in Figure 4.12 and 4.13. The IUPAC name of this compound generated by ChemBioDraw Ultra 12.0 was (Z)-5,9,18-trimethyl-N-((Z)-N’-((Z)-5-methyltetradec-2-en-1-yl)-N-((2Z,4Z,9Z,11Z,17Z)-5,11,18-trimethyltriaconta-2,4,9,11,17-pentaenoyl)carbamimidoyl) dotriacont-7-enamide. The proposed structure correlates to the composition of fatty acid chains of rice-bran which gets utilized in the biosynthesis of bio-surfactants by the fungus *Fusarium proliferatum*. The rice-bran contained protein, fat and crude fiber contents [Rosniyana et. al., 2007]. The major fatty acids present in rice-bran oil were palmitic, oleic, and linoleic acids, which were in the ranges of 13.9–22.1, 35.9–49.2, and 27.3–41.0%, respectively [Goffman et. al., 2003].
Further, support to the proposed structure was obtained through MS studies. The ESI/HRMS of the compound (Figure 4.14) gave peaks at m/z 871.1974, 701.4782, 634.4374, 605.2707, 475.3173, 453.3367, for the possible fragments as shown in
Figure 4.15. The exact mass of the tentative structure as calculated in ChemBioDraw Ultra 12.0 was 1236.1963. The fragmentation of the proposed structure was also done in ChemBioDraw Ultra 12.0 and respective peaks were matched with the molecular ion peaks in the ESI-HRMS spectrum (Figure 4.14). The parent ion peak was not observed in the ESI-HRMS spectrum due to longer fatty acid chains and higher molecular weight. The fragment ion m/z 870.7816 appeared when m/z 365.4147 gets removed from the molecular weight 1236.1963, which gave M+1 peak at m/z 871.1974 (Figure 4.14).

Further, the molecular ion fragment m/z 169.1956 gets removed from m/z 870.7816 and gave another fragment of m/z 701.5859, which showed M+ peak at m/z 701.4782 (Figure 4.14). This fragment ion m/z 701.5859 further dissociated into m/z 633.5233, by the removal of the m/z 68.0626 and gave M+1 peak at m/z 634.4374 (Figure 4.14).

The fragment ion m/z 633.5233, again dissociated into m/z 605.4920 by the elimination of m/z 28.0313, which gave M+ peak at 605.2707 (Figure 4.14).

Consequently, the fragment ions m/z 26.0157 and 127.1487 gets removed from the m/z 605.4920 to produce another fragment m/z 452.3277 which gave M+1 peak at m/z 453.3367 (Figure 4.14). The base peak at m/z 475.3173 (Figure 4.14) is supposed to be due to the removal of one chain in the structure of the purified bio-surfactant as shown in Figure 4.15.

Figure 4.14: ESI/HRMS of the purified bio-surfactant
Figure 4.15a: Possible fragment ions of the purified bio-surfactant

The possible biochemical mechanism for the proposed structure may be as follows:
Figure 4.15b: Suggested Biochemical Mechanism for the biosynthesis of bio-surfactants from *Fusarium proliferatum*

### 4.2.10 Applications

#### 4.2.10.1 DPPH assay results

The anti-oxidants are capable of seizing the free radical chain reactions. If the bio-surfactant possesses the anti-oxidant activity the absorbance of the solution decreases and thus IC$_{50}$ (the half maximal inhibitory concentration) was determined. The DPPH activity of the crude bio-surfactant was checked using ascorbic acid as standard. The
IC\textsubscript{50} of the standard was 0.056 mg/mL and that of the bio-surfactant produced was 18.81 mg/mL. The results suggest that the bio-surfactant produced by \textit{Fusarium proliferatum} grown on rice-bran possessed anti-oxidant activity to a lesser extent as compared to the surfactin bio-surfactant produced by \textit{Bacillus subtilis}. The IC\textsubscript{50} of the surfactin bio-surfactant reported was 0.25 mg/mL when BHT (butylated hydroxyl toluene) was used as the standard [Yalcin and Cavusoglu, 2010].

\textbf{4.2.10.2 Microbial enhanced oil recovery (MEOR) potential}

The bio-surfactant produced by \textit{Fusarium proliferatum} was found to have very promising MEOR potential. As compared to the distilled water alone the bio-surfactant was able to recover 95\% of the entrapped kerosene. The results obtained in the present study were comparable to the previous studies carried out by Pruthi and Cameotra, [1997], and Kiran et. al., [2009] with \textit{Arthrobacter protophormiae} and \textit{Aspergillus ustus} Bio-surfactants. Hence the bio-surfactant produced by \textit{Fusarium proliferatum} grown on rice-bran have promising MEOR potential and therefore, can be used at industrial level where enhanced recovery is needed.