CHAPTER 2

MATERIALS AND METHODS

2.1 METAL FATTY ACID SALTS AS CARBON SOURCE FOR RHAMNOLIPID PRODUCTION

2.1.1 Organism

*Pseudomonas aeruginosa* was obtained from MTCC2297 (IMTECH, India). The culture was maintained using standard laboratory procedure.

2.1.2 Preparation of different metal fatty acid salts

Metal fatty acid salts were prepared using standard Laboratory procedure.

2.1.2.1 Preparation of calcium stearate

Materials used:

- Stearic acid
- Potassium hydroxide
- Calcium chloride
Protocol

Equimolar concentration of stearic acid (MW: 282.47) and potassium hydroxide (MW: 56) was mixed and stirred to produce potassium stearate (MW: 32.47). Calcium chloride (MW: 147) was mixed to it in the ratio of 1:2 to produce calcium stearate and potassium chloride. Potassium chloride was washed out with distilled water. The derived calcium stearate was dried and then used.

\[
\text{CH}_3\text{(CH}_2\text{)}_{16}\text{COOH} + n\text{KOH} \rightarrow \text{CH}_3\text{(CH}_2\text{)}_{16}\text{COOK} + \text{H}_2\text{O} + (n-1)\text{KOH}
\]
\[
\text{Potassium Stearate}
\]

\[
\text{CH}_3\text{(CH}_2\text{)}_{16}\text{COOK} + \text{CaCl}_2 \rightarrow \text{CH}_3\text{(CH}_2\text{)}_{16}\text{COOCa} + 2\text{KCl}
\]
\[
\text{Calcium Stearate}
\]

2.1.2.3 Preparation of zinc stearate:

Materials used:

- Stearic acid
- Potassium hydroxide
- Zinc chloride

Protocol

Equimolar concentration of stearic acid (MW: 282.47) and potassium hydroxide (MW: 56) was mixed and stirred to produce potassium stearate (MW: 32.47). Zinc chloride (MW: 136.29) was mixed to it in the ratio of 1:2 to produce zinc stearate and potassium chloride. Potassium chloride was washed out with distilled water. The derived zinc stearate was dried and then used.
2.1.2.4 Preparation of cadmium stearate

Materials used:

- Stearic acid
- Potassium hydroxide
- Cadmium chloride

Protocol

Equimolar concentration of stearic acid (MW: 282.47) and potassium hydroxide (MW: 56) was mixed and stirred to produce potassium stearate (MW: 32.47). Cadmium chloride (MW: 136.29) was mixed to it in the ratio of 1:2 to produce cadmium stearate and potassium chloride. Potassium chloride was washed out with distilled water. The derived cadmium stearate was dried and then used.

2.1.2.5 Preparation of iron stearate

Materials used

- Stearic acid
- Potassium hydroxide
- Ferric chloride

Protocol

Equimolar concentration of stearic acid (MW: 282.47) and potassium hydroxide (MW: 56) was mixed and stirred to produce potassium stearate (MW: 32.47). Ferric chloride (MW: 136.29) was mixed to it in the ratio of 1:2 to produce ferric stearate and potassium chloride. Potassium
chloride was washed out with distilled water. The derived ferric stearate was dried and then used. Similarly other fatty acid salts were also prepared.

2.1.3 MEDIA USED

1 Modified PPGAs (phosphate-limited protease peptone-glucose-ammonium salts medium) (Zhang Y et al 1992)
2 Nutrient broth
3 M9+ cadmium Stearate
4 M9+ iron stearate
5 M9+ calcium stearate
6 M9+ zinc stearate

2.1.3.1 Preparation of Modified PPGAS medium

(Composition for 1000 ml)

\[
\begin{align*}
\text{NH}_4\text{Cl} & \quad - 1.09 \text{ g} \\
\text{KCl} & \quad - 2.18 \text{ g} \\
\text{Tris HCl} & \quad - 18.9 \text{ g} \\
\text{MgSO}_4 & \quad - 3.96 \text{ g} \\
\text{Glycerol} & \quad - 30 \text{ ml} \\
\text{Peptone} & \quad - 10 \text{ g} \\
\end{align*}
\]

All components except glycerol were added to distilled water and made up to 1000 ml. The pH of the medium was adjusted to 7.2 by using 0.1N sodium hydroxide solution. Glycerol was added to this medium. Medium was autoclaved for 15 min at 15-psi pressure.
2.1.3.2 Preparation of 5X M9 mineral salt preparation

(Composition per 1000 ml)

KH$_2$PO$_4$ - 15 g
NaHPO$_4$.7H$_2$O - 64 g
NaCl - 2.5 g
NH$_4$Cl - 5 g

All components were added to double distilled water and made up to 1000 ml. The solution was mixed thoroughly and pH was adjusted to 7.0. The solution was autoclaved for 15 min at 15 psi pressure.

2.1.3.3 Preparation of M9+ metal fatty acid salt medium

(Composition for 100 ml)

M9 salt solution - 20 ml
*MgSO$_4$ - 0.2 ml
*CaCl$_2$ - 10 µl

(*added after autoclaving and prior to inoculation)

The M9 salts were mixed, made up to 100ml using distilled water. 1% of different metal fatty acid salts like calcium stearate, cadmium stearate, zinc stearate, iron stearate were added to this composition in separate flasks for preparation of respective metal fatty acid salts medium. The medium was autoclaved for 15 min at 15 PSI pressure.

2.1.4 GROWTH CURVE

_Pseudomonas aeruginosa_ was inoculated in 3 different media such as nutrient broth, PPGAs and M9 minimal media for growth curve analysis.
Freshly prepared media were inoculated with 1% of overnight grown *Pseudomonas aeruginosa* culture. The turbidity of the culture was analyzed using model U3200 UV-Visible spectrophotometer (HITACHI) by taking samples from the culture at regular intervals till it reached a stationary phase in its growth.

2.1.5. **RHAMNOLIPID EXTRACTION**

Rhamnolipids were purified by first pelleting the cells by centrifugation at 6000 rpm for 10 min. Supernatant was separated from the pellet and acidified to pH 2.0 using concentrated hydrochloric acid or sulphuric acid. Acidified or protonated samples were incubated at 4°C for one hr to precipitate rhamnolipids. It was centrifuged at 15,000 rpm for 15 min to pellet crude rhamnolipids. Both mono and di rhamnolipids were extracted from the pellet using chloroform:methanol (2:1) solvent system. After extraction, solvents were evaporated to get rhamnolipids.

2.1.6 **ORCINOL ASSAY**

The rhamnolipid was isolated from the supernatant by acid precipitation followed by solvent extraction. The rhamnolipid produced was quantified by standard orcinol assay (Chandrasekaran and Bemiller, 1980).

2.1.6.1 **Materials used**

- Orcinol - 0.19% in 53% H$_2$SO$_4$
- HCl
- Diethyl ether
- Rhamnose (10mg/ml)
2.1.6.2 Protocol

500µl of *Pseudomonas aeruginosa* culture was taken from medium in separate 1.5ml tube. The culture was centrifuged at 6000 rpm for 10 min to remove the cells. The pellet was removed. The pH value of the supernatant was adjusted to 2 with concentrated hydrochloric acid or sulphuric acid and incubated for 1 hr in the refrigerator to precipitate the rhamnolipid. It was centrifuged at 12,000 rpm and 4°C for 20 min. The resulting pellet was extracted with diethyl ether twice. The organic solvent was evaporated, leaving behind yellow colored rhamnolipid. 1 ml of distilled water was added and vortexed. 1ml of 0.19% orcinol in 53% sulphuric acid was added and heated at 80°C for 20 mins. It was cooled at room temperature for 10 min. Absorbance was read at 421 nm using HITACHI UV-Visible spectrophotometer.

2.1.7 Thin layer chromatography

The obtained rhamnolipids from different media were separated by thin layer chromatography (TLC 60 F 254 Merck, India) using a solvent system of chloroform:methanol:acetic acid in the ratio of (65:15:2) (Wilhelm et al 2007). Presence of monosaccharide and di-saccharide polar head groups as mono-and di-rhamnolipids were observed.

2.1.8 Derivatization of rhamnolipid

Equimolar concentrations of extracted and partially purified rhamnolipids from different media were reacted with sodium bicarbonate (NaHCO₃) to produce sodium salt of rhamnolipid. Equal molar concentration of 4-Bromophenacyl bromide dissolved in 70% acetonitrile, was added to the sodium salt of rhamnolipid and heated at 70 °C for 1 hr to derivatize rhamnolipid to form rhamnolipid bromo phenacyl ester, which was used as such for detection at 265 nm in HPLC analysis.
2.1.9 High performance liquid chromatography:

Derivatized rhamnolipids were analyzed using HPLC from Agilent technologies 1200 model. The sample was passed through zorbax reverse phase C18 (21.2 x 250mm) column with mobile solvent gradient system of acetonitrile and water from 70% to 100%. Eluted rhamnolipids were detected at 265 nm using diode array detector (DAD) and fractions were collected respectively.

2.1.10 Mass spectrometry

Rhamnolipid fractions collected from HPLC were analyzed by mass spectrometry 6100 series single quadrupole LC/MS. Samples were dissolved in methanol and subjected to analysis. Samples were ionized in the positive mode. Fragments were separated according to their charge to mass ratio. Since partially purified rhamnolipids were a mixture of both mono and di-rhamnolipids, m/z values were calculated accordingly.

2.2 SYNTHESIS OF RHAMNOLIPID CAPPED NANOPARTICLES

2.2.1 Synthesis of rhamnolipid capped CdS nanoparticles

The ratio of cadmium to rhamnolipid taken was 0.45mg Cd\(^{2+}\) / 10mg rhamnolipid and used for the formation of stable complex (Francisco et al 2001). The reaction was carried out by dissolving 10mM Cd(NO\(_3\))\(_2\) with 470mg of rhamnolipid to form rhamnolipid-Cd complex. It was found that agglomeration occured at neutral pH, to prevent this, the pH of the reaction mixture was adjusted to 10 using 1M NaOH. Rhamnolipid-Cd\(^{2+}\) complex was formed after 15 min of continuous stirring at room temperature. The sodium sulphide solution equal to the concentration of Cd(NO\(_3\))\(_2\) was added drop by drop to the rhamnolipid-Cd\(^{2+}\) mixture. The mixture was continuously stirred
for 12 h at 80°C. These particles were then harnessed for further characterization studies.

2.2.1.1 Atomic force microscopy analysis of rhamnolipid capped CdS nanoparticles

Size controlled rhamnolipid capped CdS nanoparticles were synthesized. The particle’s shape and size were observed through Atomic Force Microscopy (WSXM Nanoscope III A, Veeco Meterology).

2.2.2 Synthesis of rhamnolipid capped ZnS nanoparticles

Ratio of Zinc to rhamnolipid approximately 0.35mg Zn2+ / 10mg rhamnolipid was taken for the formation of stable complex (Francisco et al 2001). The reaction was carried out by dissolving 4.7mM ZnCl$_2$ with 470mg of rhamnolipid to form rhamnolipid-Zn complex. It was found that agglomeration occured at neutral pH to prevent this pH of the reaction mixture was adjusted to ten using 1M NaOH. Rhamnolipid-Zn$^{2+}$- complex was formed after 15 min with continuous stirring at room temperature. The sodium sulphide solution equal to ZnCl$_2$ was then added drop by drop to the Zn$^{2+}$ - rhamnolipid mixture while maintaining continuous stirring for 12h at 80°C. These particles were then harnessed for further characterization studies.

2.2.2.1 Fourier transform infra red spectroscopy (FTIR) analysis of rhamnolipid capped ZnS nanoparticles

Perkin elmer spectrum one: FT-IR spectrometer was used in the spectral region from 4000 to 400 cm$^{-1}$. was used to analyze capping of rhamnolipid with metal ion during synthesis of ZnS nanoparticles. The samples were prepared in potassium bromide (KBr) pellet in equal ratio. The spectra were recorded in Transmittance mode.
2.2.2.2 Size and shape analysis of capped ZnS nanoparticles by high resolution transmission electron microscopy (HRTEM)

Rhamnolipid capped ZnS nanoparticles were placed on a three-micron size carbon coated copper grid. The morphology and size distribution of rhamnolipid-capped ZnS particles were observed through high resolution TEM model (JEOL 3010).

2.2.2.3 Small angle X-ray scattering analysis (SAXS)

SAXS was employed to analyze the size and distribution of rhamnolipid capped ZnS particles using an Anton Paar slit collimation compact Kratky type SAXS camera fitted onto a rotating anode X-ray generator running at 50kV, 100mA. Copper Kα radiation was used for this study. The samples were prepared both as powder and aqueous solution.

2.2.2.4 UV-Vis / Fluorescence spectroscopy

Absorption peak for rhamnolipid capped ZnS nanoparticle was observed using Hitachi model U3200 UV-Visible spectrophotometer. Fluorescence emission peak was measured using Hitachi fluorescence model F-3010 spectrophotometer.

2.3 RHAMNOLIPID CAPPED ZnS NANOPARTICLE-BIOMOLECULES INTERACTIONS

Capped ZnS nanoparticles interactions with plasmid DNA, bovine serum albumin (BSA) and Candida rugosa lipase (CRL) were studied. 100 µg of each protein was taken for study. Individual proteins interacted with various concentrations of 5mM ZnS nanoparticles. Interactions with DNA were carried out with 2.9kb pRSET 41B+. 1µg of plasmid DNA was used for its interaction with capped ZnS nanoparticles.
2.3.1 Fluorescence spectroscopy analysis

Hitachi fluorescence model F-3010 spectrophotometer was used for the analysis of interaction between biomolecules and capped ZnS nanoparticles. Protein molecules were excited at 280 nm and showed emission at 340 nm in the control and experiment. Various concentrations of capped ZnS nanoparticles were conjugated with 100 µg of protein molecules during the analysis. For interaction with DNA, various concentrations of capped ZnS nanoparticles with 1 µg plasmid was taken.

2.3.2 Electrophoretic analysis

1% agarose gel electrophoresis was run for both bovine serum albumin and CRL along with capped ZnS nanoparticles. 5 µgs of protein was mixed with 10 µl of 5mM ZnS nanoparticles and loaded on to the gel. Tris glycine buffer pH 8.3 was used for running the gel at 75 V for 30 minutes. After electrophoresis, the protein gel was illuminated under ultra violet radiation and bands appeared as blue bands.

2.3.3 Zymography analysis

After electrophoresis, the gel containing protein-ZnS nanoparticle conjugates was equilibrated with Tris buffer pH 7.0 for 30 minutes. Agar solution containing 25mM Tris buffer pH 7.2, 125 µl tributyrin substrate, and a few drops of triton X 100 heated for emulsification. The agarose gel containing protein-ZnS conjugates was sandwiched between tributyrin agar solutions in a petridish. The gel was incubated at 37°C for the appearance of clear zone.