Biosynthesis of novel Sophorolipids using *Candida bombicola* ATCC 22214: Characterization and applications

A THESIS SUBMITTED BY

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.....Dedicated to my beloved parents

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled: "**Biosynthesis of novel Sophorolipids using *Candida bombicola* ATCC 22214: Characterization and applications**", submitted by Reetika Gupta, for the Degree of ***Doctor of Philosophy***, was carried out by the candidate under my supervision at Division of Biochemical Sciences, National Chemical Laboratory, Pune 411008, India. Materials that have been obtained from other sources are duly acknowledged in the thesis.

Asmita Prabhune

(Research Guide)

DECLARATION BY RESEARCH SCHOLAR

I hereby declare that the thesis entitled "**Biosynthesis of novel Sophorolipids using *Candida bombicola* ATCC 22214: Characterization and applications**", submitted by me for the Degree of ***Doctor of Philosophy*** to the University of Pune, has been carried out by me at Division of Biochemical Sciences, National Chemical Laboratory, Pune, India, under the guidance of Dr. Asmita Prabhune. The work is original and has not formed the basis for the award of any other degree, diploma, associate ship, fellowship and titles, in this or any other University or other institution of higher learning.

I further declare that the materials obtained from other sources have been duly acknowledged in the thesis.

Reetika Gupta

(Research Scholar)

C O N T E N T S

A C K N O W L E D G E M E N T

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……..Reetika Gupta

LIST OF ABBREVIATIONS

°C	:	degree centigrade
Å	:	Angstrom
µg	:	microgram
µl/µL	:	microlitre
µm	:	micro meter
a.m.u.	:	Atomic mass unit
ALA	:	α-linolenic acid
ATCC	:	American Type Culture Collection
CID-MS	:	Collision induced dissociation mass spectrmetry
Conc.	:	Concentrations
Da	:	Dalton
ESI-MS	:	Electrospray ionization mass spectrometry
g	:	gram
h	:	hour
kV	:	kilo volts
kDa	:	kilo dalton
l/L	:	liter
LB	:	Luria-Bertani
LCMS	:	Liquid chromatography mass spectrometry
LNNSL	:	Linolenic acid derived sophorolipid
LNNSLME	:	Sophorolipid methyl ester of Linolenic acid
m	:	metre
m/z	:	mass to charged ratio
mg	:	milli gram
MGYP	:	Malt extract-glucose-yeast extract-peptone media
min	:	Minute
mm	:	milli meter
ml/mL	:	milli liter
mN	:	milli Newton
MS	:	Mass spectrometry
NCIM	:	National Collection of Industrial Microorganisms.

NMR	:	Nuclear magnetic resonance
nm	:	Nano metre
OD	:	Optical density/Absorbance
Pt	:	Platinum
rpm	:	revolution per minute
SL	:	Sophorolipid
TOF MS	:	Time of flight mass spectrometry
TLC	:	Thin layer chromatography
TMS	:	Tetramethyl silane

Abstract

Preparation of new sophorolipid (SL) analogues with different functionalities has widespread use in pharmaceutical and industrial applications. SL composition can be modified by using both *in vivo* and *in vitro* methods. Different lipophilic substrates have been used by researchers for SL production such as oleic acid, stearic acid, palmitic acid and different vegetable oils. To our knowledge, there is no such report on the analysis of individual SL molecule produced using pure α-linolenic acid (ALA) as a lipophilic substrate. SL production using α-linolenic acid as the lipophilic substrate may become a valuable product of interest. In order to biosynthesize novel SLs using *Candida bombicola*, Linolenic acid was used as the lipophilic and glucose as a hydrophilic source in the fermentation medium. The present study is expected to provide information on the production under optimized fermentation conditions, purification , characterization and application of the SL derived using α-linolenic acid (ALA) as the lipophilic source.

The thesis will be divided in to the following chapters:

Chapter 1: General Introduction

The first chapter is the general introduction of the thesis and it gives detailed literature survey, significance of the sophorolipids (SLs) and objectives of the study. It also describes the biosynthesis and various industrial applications of different sophorolipids derived using various lipophilic substrates. Chapter specific introduction will be discussed in respective chapters.

Chapter 2: Optimization of Fermentation parameters for production of linolenic acid derived Sophorolipids from *Candida bombicola* ATCC 22214

Sophorolipid production is regulated by various parameters of culture condition and media components. This chapter describes the fermentation parameters of *Candida bombicola* (ATCC 22214) for the production of linolenic acid derived Sophorolipids. These parameters included testing of different medium, medium pH, temperature, inoculum size and age, glucose, fatty acid and yeast extract concentration etc. as well as time kinetics of SL and biomass production.

Chapter 3: Production and Purification of Linolenic acid derived Sophorolipids

The sophorolipid was produced using optimum fermentation condition in shake flasks. The SL from *Candida bombicola* is secreted in the culture broth. After fermentation, culture broth was centrifuged and the supernatant was extracted twice with equal volumes of ethyl acetate, the organic layer was dried over anhydrous sodium sulphate and the solvent was removed by rotary-evaporation. The brownish semi-crystalline product (SL mixture) was washed twice with n-hexane to remove un-reacted fatty-acid and was stored at 4°C. Sophorolipid mixture (LNNSL) was analyzed by reverse phase high performance liquid chromatography (HPLC) with a Waters 2487 separation module (Waters Co. Milford, Massachusetts) using 250 × 4.6 mm^2 analytical symmetry C18, 5 µm column. The gradient solvent elution profile used was as follows: water/ acetonitrile (95:5, v/v) holding for 10 min; to a final composition of water/acetonitrile (5:95, v/v) with a linear gradient over 50 min and holding for 10

min. The flow rate was 0.5 ml min^{-1}. The peaks were detected at 220 nm wavelength by absorbance detector. Fractions from different peaks were collected and pooled separately in many runs. Further, for the ease of purification, chemical esterification reaction from the literature was followed in order to get single homogenous product (Bisht et al., 1999).

Chapter 4: Structural Determination and physical properties of Linolenic acid derived Sophorolipid and its methyl ester form

Positive and negative ESI and CID (collision induced dissociation) mass spectra were obtained with an API QSTAR PULSAR hybrid MS/MS quadrupole TOF system (Applied biosystems). Samples from different fractions (collected from HPLC) were injected separately into mass spectrometer for analysis. ESI and CID mass spectral analysis confirmed that the *Candida bombicola* when grown on glucose and α-linolenic acid produces a mixture of glycolipids consisting of free acid, lactone and diacetylated lactone forms of C18:3 (linolenic acid) SLs as well as diacetylated lactone form of C18:1 SL. The composition of the fermentation product (SL mixture) was 7.5 % free acid, 80 % lactone and 4.5 % diacetylated lactone of C18:3 molecules and 8 % of diacetylated lactone of C18:1 SL molecules. This composition was determined from the initial crude SL loaded on the column. Further, for the ease of purification, chemical esterification reaction from the literature was followed in order to get single homogenous product (Bisht et al. 1999). SL mixture was converted into the sophorolipid methyl ester and ESI-MS analysis confirmed the presence of C18:3 moieties in the fatty acid chain of this product and further the structure of this SL methyl ester (LNNSLME) was confirmed by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy which showed the presence of ester group in LNNSLME. Physical properties such as surface

tension and critical micelle concentration were determined for SL mixture containing 80 % Lactone and its purified sophorolipid methyl ester form and it was found that both the compounds are good surface active agents.

Chapter 5: Antibacterial properties of Linolenic acid derived Sophorolipid and its methyl ester form

Antibacterial properties of Sophorolipid mixture containing 80% Lactone of C18:3 fatty acid (linolenic acid) and its chemically derived Sophorolipid methyl ester form were checked against Gram-positive (*B. subtilis*) and Gram-negative (*E. coli* and *P. aeruginosa*) bacteria. Antibacterial tests of SL mixture (LNNSL) and sophorolipid methyl ester were performed using standard dilution micromethod. SL mixture and its methyl ester form were diluted to 5, 10, 20 μg ml^{-1} with sterile millipore water. Bacterial suspensions at a concentration of 10^6 CFU ml^{-1} were added into each of these dilutions of SL mixture and SL methyl ester separately and incubated for 6 h at their respective temperatures. 100 μl aliquots were taken out from the respective suspensions at 2 h intervals and plated on LB agar plates followed by incubation at their respective temperatures. Colonies were visualized after 24 h and digital images of the plates were captured. The effectiveness of compounds was analyzed by plotting percentage cell survival versus incubation time. It was observed that bacterial colonies of both the Gram-positive and Gram-negative bacteria decreased with increasing amount of compounds as well as increasing incubation time. SL mixture (LNNSL) containing 80 % Lactone of C18:3 molecules were found more effective as compared to Sophorolipid methyl ester against both Gram-positive and Gram-negative bacteria.

The antibacterial action of LNNSL containing 80 % Lactone of C18:3 fatty acid (linolenic acid) and its methyl ester derivative on Gram-positive and Gram-negative bacteria is investigated with the help of atomic force microscopy (AFM). Mode of action of SL mixture containing 80 % Lactone and Sophorolipid methyl ester was in agreement with other biosurfactants that act on the integrity of cell membrane (Baek et al. 2003) which involves bactericidal action as confirmed from AFM study. Further, The peptidoglycan cell wall of bacteria are mainly open networks of macromolecules and generally do not offer significant permeability barriers to compounds of molecular mass less than 50 kDa. So it was hypothesized that sophorolipids due to their amphiphilic nature and molecular mass in the range of 600-800 Da can make entry into the bacterial cell through both the lipid bilayer as well as through porin channels. At some concentrations, sophorolipid becomes toxic to the bacterial cell and show bactericidal action. Sophorolipid mixture (LNNSL) containing 80 % Lactone and Sophorolipid methyl ester (LNNSLME) both showed the similar mode of bactericidal action.

Summary and Conclusions

This study successfully demonstrated the analysis of chemically distinct forms in the SL mixture produced by *Candida bombicola* when grown on glucose and α-linolenic acid as well as its conversion into the single homogenous product by chemical esterification reaction. These novel Sophorolipids were good surface active and antibacterial agents. They have potential for various applications and offer the advantages of further modifications and can produce functionalized SLs according to their applications. Future aspects of these novel SLs will be discussed in this section of the thesis.

Chapter 1

General Introduction

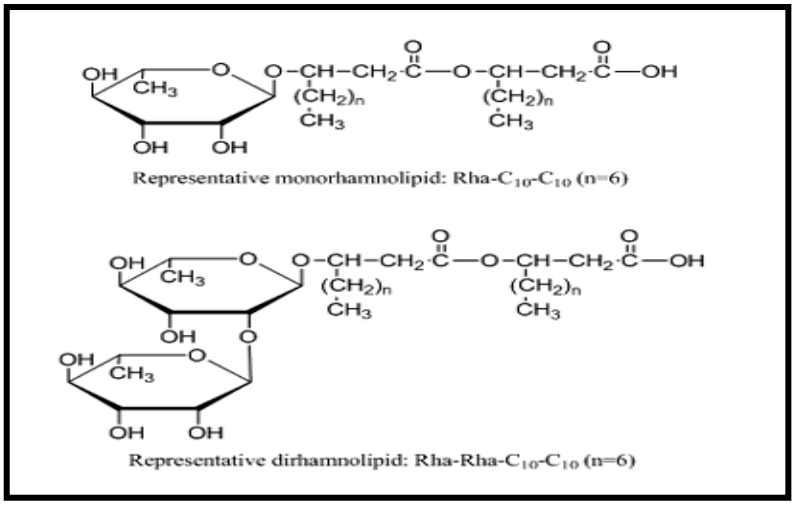
1.1 Introduction

Sophorolipids (SLs) are extracellular surface-active agents produced by various yeasts, such as *Torulopsis bombicola* (Cooper and Paddock, 1984; Gobbert et al., 1984), *Torulopsis apicola* (Tulloch et al., 1967), *Torulopsis petrophilum* (Cooper and Paddock, 1983) and *Candida bombicola* (Asmer at al., 1988; Davila et al., 1992 and Albrecht et al., 1996) etc. SLs are one of the types of glycolipid biosurfactants that are gaining importance for commercial purposes due to their biodegradability, low eco-toxicity and production based on renewable resources (Muller-Hurtig et al., 1993; Mulligan, 2005; Van Bogaert et al., 2007 and 2011). These glycolipids are low molecular weight biosurfactants and have been considered as secondary metabolites (Stodola et al., 1967; Bentley and Campbell, 1968 and Rosenberg and Ron, 1999) and are produced in the late exponential and stationary phases of the producer organism (Hommel et al., 1987). Growing environmental awareness has attracted the attention towards the production of glycolipid biosurfactants from renewable resources through fermentation processes (Makkar et al., 2011). The production of glycolipid biosurfactants through fermentation has replaced the first generation glycolipids (e.g., alkylpolyglucosides, APGs) as well as other synthetic surfactants produced by chemical means (Desai and Banat, 1997). The presence of a carbohydrate and a lipid moiety within the same molecule of glycolipid are responsible for its amphiphilic nature and is one of the important characteristic of a biosurfactant. The well studied and effective glycolipid biosurfactants from the point of view of surface-active properties are Rhamnolipid and sophorolipids (Fig. 1.1 A and B).

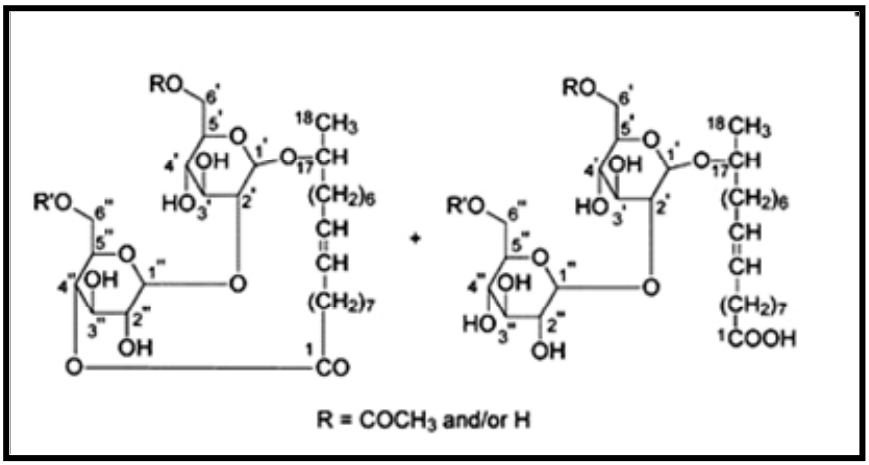
Production of rhamnose-containing glycolipids was first described in *Pseudomonas aeruginosa* by Jarvis and Johnson (1949) and are named as rhamnolipids. Rhamnolipids are composed of one or two molecules of rhamnose, linked to one or two molecules of β-hydroxy decanoic acid. L-rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate and L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate, referred to as rhamnolipid 1 and 2, respectively, are the principal glycolipids produced by *P. aeruginosa* (Fig. 1.1 A). The major drawback towards rhamnolipids production is that the productive strains are pathogenic bacteria such as *Pseudomonas aeruginosa*. In contrast to Rhamnolipids, sophorolipids' productive strains are non-pathogenic yeasts and also the high production yield of sophorolipids makes it favorable for commercial production and use. The focus of the present work is on sophorolipids so it will be discussed in detail.

1.2 Sophorolipids

SLs were first described in the early sixties by Gorin et al. (1961) as extracellular glycolipids synthesized by the yeast *Torulopsis magnoliae*. Later, Tulloch and Spencer in 1968 reported that the producing strain was actually *Torulopsis apicola*, currently known as *Candida apicola*. Another SL producing yeast discovered was *Candida bombicola* (Spencer et al., 1970). *Candida bombicola* is osmophilic yeast isolated from the honey of bumble-bees (Barnett et al., 1983 and Rosa and Lachance, 1998). *Candida bombicola* ATCC 22214 is known to produce high yields (400 g l^{-1}) of sophorolipids (Van Bogaert et al., 2011). In the present work, *Candida bombicola* ATCC 22214 is used for SL production because of its high production yields and non pathogenic nature.



(A) Rhamnolipids



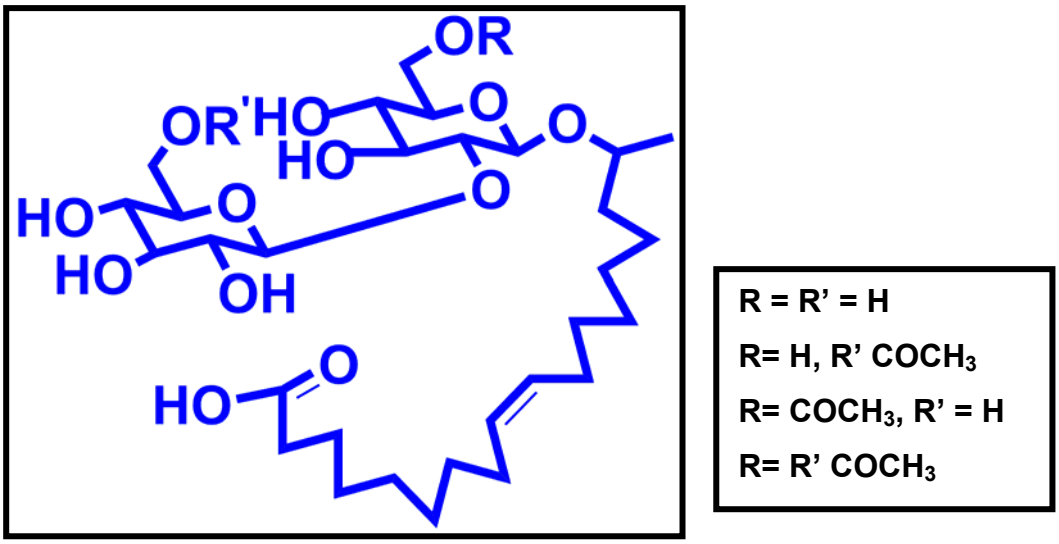
(B) Sophorolipids

Fig. 1.1: Structures of different glycolipids, A) Rhamnolipids and B) Sophorolipids (*Image courtesy from google; http://images.google.com)*

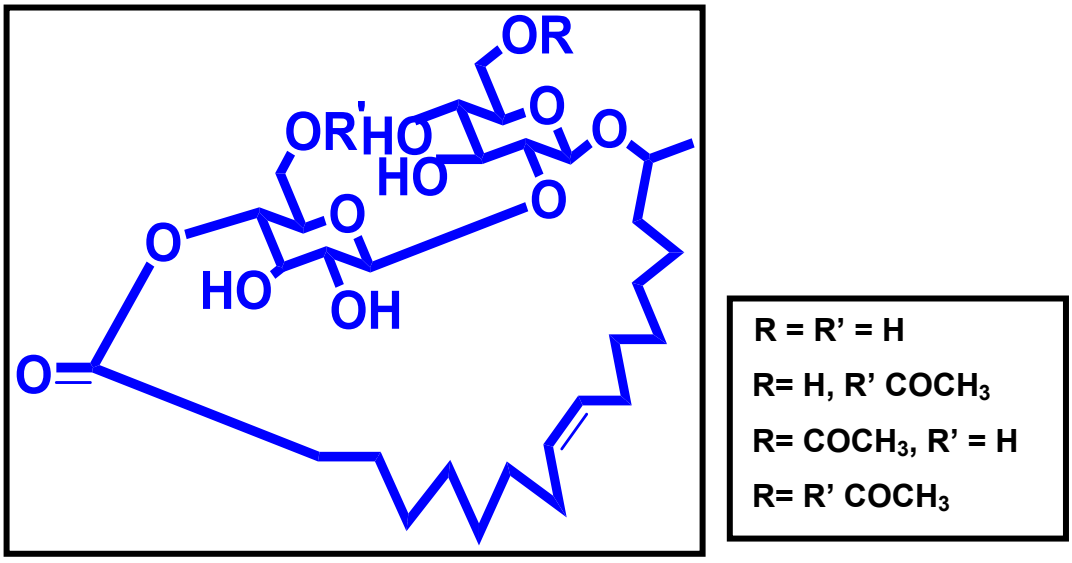
1.2.1 Sophorolipid structure

The sophorolipid structure is composed of a disaccharide and a hydroxy fatty acid. The disaccharide is a sophorose (2'-*O*-*β*-D-glucopyranosyl-*β*-D-glucopyranose) typically with the 6' and 6" acetylated hydroxy groups (Gorin et al., 1961 and Tulloch et al., 1962). The sophorose moiety is linked to the fatty acid through a glycosidic bond between carbon 1'

and the terminal (ω) or sub-terminal (ω-1) carbon of a long chain fatty acid (Gorin et al., 1961 and Weber et al., 1990). The sophorolipids produced by *Candida bombicola* are always a mixture of acidic and lactone forms (Ito et al., 1980; Hommel et al., 1987; Asmer at al., 1988; Davila et al., 1992, 1994 and 1997; Casas and Garcia-Ochoa, 1999; Hu and Ju, 2001 and Rau et al., 2001). The carboxylic acid end is free in the acidic or open ring form while it is internally esterified to the 4" position of the sophorose moiety in lactone or closed ring form (Fig. 1.2). These two acidic and lactone forms of SL further vary in their structures in terms of acetylation pattern at the 6' and 6" positions and differences in the fatty acid part (chain length, saturation and position of hydroxylation). Asmer et al. (1988) was the first who separated the SL mixture produced using oleic acid and glucose as substrates by *Candida bombicola* into six components based on the lactonization and acetylation pattern using medium pressure liquid chromatography and thin layer chromatography.



(A) Acidic form of SL



(B) Lactone form of SL

Fig. 1.2: Typical structures of sophorolipids, (A) Acidic form; (B) Lactone form (Asmer et al., 1988)

1.2.2 Properties of sophorolipids

Being a biosurfactant sophorolipids possess all the properties of a surface active molecule. The criteria for evaluating the biosurfactant activity are surface tension and critical micelle concentration (Rosen, 1978). SLs are reported to reduce the surface tension of water from 72.8 m N m^{-1} down to 40 to 30 m N m^{-1}, with a critical micelle concentration (CMC) of 11 to 250 mg/l (Develter and Lauryssen, 2010). The surface tension lowering and CMC values of SLs are comparable to those of commercially available surfactants such as surfactin (Arima et al., 1968). As stated above, that SLs are produced as a mixture of acidic and lactone forms. The properties and applications of SLs depend mainly on the abundance of certain structural form in the SL mixture such as lactone and acidic form (Inoue, 1988 and Klekner and Kosaric, 1993). Ratio of the acidic and lactone forms in the SL mixture varies with the growth conditions and differentiated supply of lipophilic substrates (Cavalero and Cooper, 2003). SL mixtures are typically viscous brown oils and are denser than water (Gorin et al., 1961; Tulloch et al. 1962 and Cavalero and Cooper, 2003). When the lactone form is present in the high concentration in SL mixture, it results in the formation of crystals instead of the more common viscous oil. For example, Cavalero and Cooper (2003) reported the formation of crystalline SLs, when alkanes such as hexadecane and heptadecane were used as substrates.

The French company Soliance (2004) reported that SLs are unstable at pH values higher than 7.0-7.5 for long term storage and beyond this point irreversible hydrolysis of the acetyl groups and ester bonds is observed. Soliance (2004) also reported the effect of pH on the solubility of SLs. They

reported that SLs are dispersible in water at pH 5.0 or lower values. SLs solubility improved beyond pH 5.6 to 5.8 and at pH 6.0, SLs are completely soluble in water even at high concentrations (Soliance, 2004). SLs were also checked for their solubility in organic solvents by some researchers. For example, Hu and Ju (2001) reported that SLs dissolve very well in ethanol (> 20% at room temperature). Furthermore, Bluth et al. (2006) reported its (SLs) solubility in polar solvents such as methanol, ethyl acetate and acetonitrile. SLs are also dispersible in mineral oil, vegetable oil, glycerol and propylene glycol due to its surfactant property (Soliance, 2004). Hirata et al. (2009) reported that the surface-active properties of SLs are not influenced by high salt concentrations. SLs are found to be active across a wide temperature range (Nguyen et al., 2010).

Different activities of SLs such as emulsifying ability, foaming, wetting and antimicrobial effects all are dependent on its surface-active properties. Surface-active properties also changes with the variations in the SL structure. For example, lactone forms have better surface tension lowering and antimicrobial activity as compared to acidic forms, while acidic forms shows better foam production and solubility (Van Bogaert et al., 2007). Shah et al. (2005) reported the spermicidal and virucidal activity of SLs. They demonstrated the membrane permeabilizing action of SLs to be responsible for these antimicrobial effects. They tested different analogs of SLs and observed that diacetate ethyl ester of SL to be most potent spermicidal and virucidal agent. Antiviral activity of SLs was also reported by Gross et al. (2004), Shah et al. (2005) and Gross and Shah (2007). Further, antibacterial effects of SLs were also reported by Gross and Shah (2003), Shah et al.

(2007) and Sleiman et al. (2009). SLs are also reported for their antifungal (Gross and Shah, 2004 and 2009) and anti-cancerous activities (Chen et al., 2006a and 2006b).

1.2.3 Biosynthesis of Sophorolipids

The building blocks for sophorolipid biosynthesis by *Candida bombicola* are hydrophilic and lipophilic carbon sources. The hydrophilic source generally used for SL production is glucose, while lipophilic sources may be alkanes, alcohols, aldehydes or fatty acids. In the first step, lipophilic substrates (alkanes, alcohols, aldehydes) are first oxidized into fatty acid. The second step is the hydroxylation of fatty acid. The hydroxylation of fatty acid may take place by any one of the two pathways. In the first pathway, fatty acid is broken down by β-oxidation pathway. The oxidized substrate is metabolized two carbons at a time and release acetyl CoA each time. The acetyl CoA may be used in cellular respiration or may be involved in the synthesis of larger biomolecules. The importance of this pathway is the generation of new long chain fatty acid. The fatty acid thus produced may be hydroxylated to produce hydroxy fatty acid. The synthesis of hydroxy fatty acid in this manner is said to be *de novo*. The hydroxy fatty acid will then get incorporated into the sophorolipid.

The second pathway for hydroxy fatty acid synthesis is that when the oxidized substrate is not broken down but is instead immediately hydroxylated. The chain length of the resulting hydroxy fatty acid will be the same as that of lipophilic substrate. The hydroxy fatty acid will then get incorporated into the sophorolipid. This pathway is referred to as direct incorporation.

There are a number of reports on direct incorporation and *de novo* synthesis of hydroxy fatty acid by *Candida* species (Tulloch et al., 1962 and 1967; Brakemeier et al., 1995 and 1998 and Linton, 1991). The extent of direct incorporation of hydroxy fatty acid is increased when an additional substrate such as glucose is provided, which may be used to maintain cellular respiration (Cooper and Paddock, 1984; Hommel et al., 1994 and Linton, 1991).

There is no evidence of direct incorporation of different sugars into the glycolipids synthesized by *Candida bombicola*. There are reports on the use of sugars such as sucrose, fructose, galactose and lactose etc. but regardless of the sugar type only sophorose was observed in the glycolipid product (Gobbert et al., 1984 and Zhou and Kosaric, 1993 and 1995). These studies suggested the possible *de novo* synthesis for sophorose sugar.

After the formation of hydroxy fatty acid, two UDP-activated glucose molecules are added in a serial way to complete the SL synthesis. The enzymes catalyzed SL biosynthesis is described below.

The enzyme catalyzed biochemical pathway for SL synthesis by *Candida* species is shown in Figure 1.3 (Van Bogaert et al., 2007). In yeasts, cytochrome P450 monooxygenase enzymes are found (Kappeli, 1986), which belongs to CYP52 family and are capable of hydroxylating fatty acids or alkanes at the terminal (ω) or sub-terminal (ω-1) positions (Van Bogaert et al., 2009 and 2010). Van Bogaert et al. (2007) isolated and characterized cytochrome P450 reductase gene (CPR) from *Candida bombicola.* The cytochrome P450 reductase is required to reduce the heme centre of cytochrome P450 monooxygenase, which in turn is required to activate

molecular oxygen. As shown in Figure 1.3, the fatty acids are converted to a terminal (ω) or sub- terminal (ω-1) hydroxy fatty acid through the action of a membrane bound nicotinamide adenine dinucleotide phosphate (reduced form; NADPH) dependent monooxygenase enzyme, cytochrome P450 (Jones, 1968).

Studies by Breithaupt and Light in 1982 on the cell-free extracts of sophorolipid producing yeast, *Rhodotorulla bogoriensis* demonstrated two different glucosyltransferases. Van Bogaert et al. (2011) reported the involvement of the same (glucosyltransferase) enzyme in the sophorolipid synthesis by *Candida bombicola*. Further, it was confirmed by Saerens et al. (2011a, 2011b) that there are two independent glucosyltransferases in *Candida bombicola* which are involved in SL synthesis. As shown in the pathway (Fig. 1.3) glucose is glycosidically coupled (position C 1') to the hydroxy group of the fatty acid through the action of a specific glucosyltransferase I. The transferase reaction requires nucleotide-activated glucose (uridine diphosphate glucose or UDP-glucose) as glucosyl donor (Breithaupt and Light, 1982). In a next step, a second glucose is glycosidically coupled to the C 2' position of the first glucose moiety by glucosyltransferase II (Van Bogaert et al., 2007 and 2011). The SLs obtained after the action of glycosyltransferase II are detected in the SL mixture as the acidic, non-acetylated molecules. Further, modifications in the SL structure may also occur by both internal esterification (lactonization) and by acetylation of the sophorose head. Lactonic SLs are formed by an esterification reaction of the carboxyl group of the hydroxy fatty acid with a hydroxy group of sophorose at the 4" position catalyzed by a specific lactone esterase (Asmer et al., 1988).

The acetylation at the 6'- and/ or 6"- position is carried out by an acetyl-coenzyme A (CoA) dependent acetyl transferase (Esders and Light, 1972 and Bucholtz and Light, 1976). The pathway shows that SL synthesized by the yeast (*C. bombicola*) is a mixture of acidic, lactonic and their acetylated forms. Figure 1.3 gives a schematic overview of the biochemical pathways involved in sophorolipid synthesis.

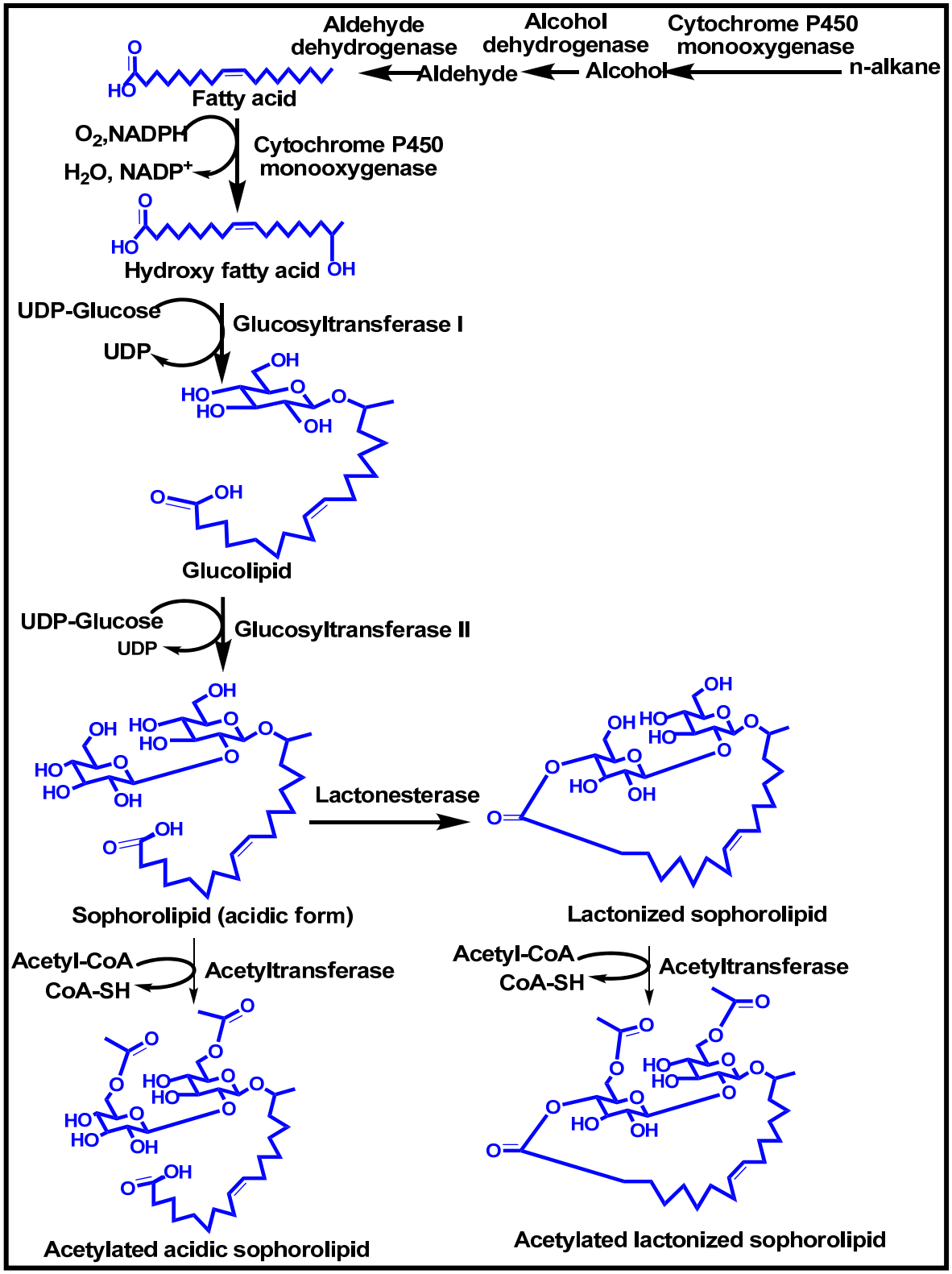


Fig. 1.3: Sophorolipid biosynthetic pathway (proposed by Asmer et al., 1988)

1.2.4 Effect of carbon sources on sophorolipid synthesis

Candida bombicola can synthesize sophorolipids even when no carbon source is provided but to a very lesser extent. It was reported that when no lipophilic substrate is provided in the medium, there will be *de novo* synthesis of fatty acid from the acetyl CoA derived from glycolysis (Van Bogaert et al., 2007). Studies carried out by many researchers have reported that SL synthesis increases when both the primary (hydrophilic substrate) and secondary (lipophilic substrate) carbon sources are provided in the medium (Cooper and Paddock, 1984; Asmer et al., 1988; Rau et al., 1996 and Davila et al., 1997). Hommel and Huse (1993) reported that the yield of SL is much larger, when both sugars (hydrophilic carbon source) and n-alkanes (hydrophobic carbon source) are used than when only sugar was used as the sole carbon source. According to Linton (1991) when sugar is supplied along with the lipophilic substrate, then the large amount of sugar will be available both for energy supply as well as a direct precursor of sophorose, which will be energetically favourable than using sugar alone for both the energy supply and as lipid precursor.

1.2.4.1 Effect of hydrophilic substrates on SL production

Influence of hydrophilic substrates such as different sugars was investigated by many researchers. For example, Klekner et al. (1991) used a disaccharide, sucrose in place of glucose as a hydrophilic substrate, but the obtained SL yield was lower and no effect on the structure of sugar part of SL was observed. Further, Zhou and Kosaric (1993 and 1995) investigated the effect of galactose and lactose on SL synthesis. They reported that there was no growth when only lactose was provided in the medium but when

hydrophobic substrates such as canola, olive or safflower oil were supplemented both the growth and SL formation were observed. For reducing the substrate costs, Daniel et al. (1998a) used deproteinized whey concentrate along with the rapeseed oil for SL production. They observed that the lactose (main sugar component of whey) was not consumed during the fermentation and the high yield of SL obtained was due to consumption of rapeseed oil only. In the same year, Daniel et al. (1998 b) reported a method to use deproteinized whey concentrate as a hydrophilic substrate by a two stage cultivation process. In the first stage, Daniel et al. (1998b) cultivated the oleaginous yeast *Cryptococcus curvatus* on the whey. The yeast cells accumulated a high level of single cell oil. These cells were then harvested and disrupted and served as a lipophilic substrate for the *Candida bombicola* cells. Solaiman et al. (2004 and 2007) reported the use of low cost soy-molasses, which is a co-product of soybean oil processing in place of glucose. Soy molasses contains about 30 % (w/v) carbohydrates. The main components of carbohydrates of soy molasses are raffinose, stachyose and disaccharide sucrose other minor amount of monosacharrides. Lower yields of SL were observed. Daverey and Pakshirajan (2009) reported sugarcane molasses as a substitute of glucose and soybean oil as lipophilic substrate for SL production. Sugarcane molasses contains about 62 % sugar content, of which 35 % (w/v) is sucrose and remaining content is of glucose and fructose. The yield of SL was less as compared to the experiment where only glucose was used as a substrate. Perkin et al. (2005) used honey as a hydrophilic substrate but at the end of fermentation process when the initially added glucose was consumed. Gobbert et al. (1984) used different mono-, di- and

trisaccharides such as glucose, fructose, mannose, maltose and raffinose, but the yield was lower as compared to SL yield obtained from the experiment using glucose as a substrate. Another observation was that there is no influence on the sugar part of SL. It means that all the sugars are metabolized into two glucose units in every case and incorporated as a sophorose moiety in the SL structure. In all the cases of use of different sugars and cheap low cost substrates in place of glucose, the SL yields were lower. It means that glucose is the preferred hydrophilic substrate of choice.

1.2.4.2 Effect of lipophilic substrates on SL production

There are several evidences on influence of lipophilic carbon sources on SL structure and yield. The lipophilic portion of the SL may vary in its structure in terms of chain length and presence of unsaturations. Different lipophilic carbon sources such as alkanes, fatty acids, fatty acid esters and vegetable oils have been used for SL production. Cavalero and Cooper (2003) reported that when alkanes were used as substrate, as the chain length is increased from C12 to C18 the percentage of hydroxy fatty acids having the same length as the substrate is increased and get directly incorporated into the SL structure. Hexadecane and octadecance gave the best yields of SLs. The same trend for incorporation of fatty acids or their esters into the SL molecule was found. Free fatty acids and their corresponding methyl or ethyl esters can be used as hydrophobic carbon sources (Davila et al., 1994). Among different fatty acids used till now, oleic acid was found to be best for production yield of SLs (Asmer et al., 1988). Different vegetable oils such as sunflower, corn, soybean, safflower and rapeseed oil and animal fat has been used as a lipophilic substrate for SL

production (Cooper and Paddock, 1984; Deshpande and Daniels, 1995; Kim et al., 1997; Casas, 1996; Casas and Garcia-Ochoa, 1999; Daniel et al., 1998a and 1998b; Rau et al., 1999, Rau et al., 2001 and Kim et al., 2005). Cheap lipophilic substrates such as restaurant waste oil and waste frying oil were also used for SL production (Shah et al., 2007 and Fleurackers, 2006) but the yield was low as compared to the fermentation where oleic acid was used as lipophilic substrate. Different lipophilic carbon sources used by various researchers for SL production are shown in the Table 1.1.

Table 1.1

List of different lipophilic carbon sources and organisms used for SL production

LIPOPHILIC SOURCES	PRODUCER ORGANISMS	REFERENCES
Alkanes		
Hexadecane, octadecane, eicosane, docosane, tetracosane	*Torulopsis magnoliae*	Tulloch et al., 1962; Tulloch and Spencer, 1968
Fatty acid esters		
Esters of Palmitic acid, stearic acid, linoleic acid, Eicosenoic acid	*Torulopsis magnoliae*	Tulloch et al., 1962
Fatty acids		
Palmitic acid, stearic acid, oleic acid, linoleic acid	*Candida bombicola*	Ashby et al., 2008
Oleic acid	*Candida bombicola*	Asmer et al., 1988; Rau et al., 1996 ; Rau et al., 2001
Arachidonic acid	*Candida bombicola*, *C. apicola*	Prabhune et al., 2002
Linolenic acid	*Candida bombicola*	Gupta and Prabhune, 2012
Vegetable oils		
Rapeseed oil	*Candida bombicola*	Daniel et al., 1998; Rau et al., 1999 ; Rau et al., 2001
Safflower oil	*Torulopsis bombicola*	Zhou et al., 1992
Sunflower, olive, corn and coconut oil	*Candida bombicola*	Casas and Garcia-Ochoa, 1999
Cheap substrates		
Biodiesel by-product streams	*Candida bombicola*	Ashby et al., 2005
Corn oil by-product	*Candida bombicola*	Kim et al., 2005
Waste frying oil, restaurant waste oil	*Candida bombicola*	Fleurackers, 2006; Shah et al., 2007

1.2.5 Physiological role of sophorolipids

It was reported by authors that microbial extracellular surface-active products or biosurfactants are produced for the assimilation of hydrophobic or water insoluble substrates by microorganisms. Biosurfactants emulsify those substrates in water phase and thus make them available for the microorganisms (Ito et al., 1980; Inoue and Itoh, 1982 and Hommel and Ratledge, 1993). It has been suggested that the sophorolipid synthesis by *Candida bombicola* is related to uptake of carbon sources (Ito et al., 1980 and Otto et al., 1999).

1.2.6 Applications of sophorolipids

Sophorolipids offer an environmental friendly alternative for the chemically derived surfactants because of their biodegradability, low eco-toxicity and the production based on renewable-resource substrates. SLs applications in various fields can be summarized in following points below.

1.2.6.1 Cosmetic industry

SLs exhibit moisturizing, antibacterial, antioxidant and other properties such wetting, foaming and emulsifying, which make them useful component for various cosmetic formulations (Klekner and Kosaric, 1993; Shete et al., 2006 and Lourith and Kanlayavattanakul, 2009). For example, SLs exhibited lower cytotoxicity than surfactin, which is a commercialized cosmetic ingredient (Yoshihiko et al., 2009). SL products are FDA (Food and drug administration) approved and are available on commercial level, for example the French company Soliance (http://www.groupesoliance.com) produces SL based cosmetics for body and skin. SL acts as an emulsifying agent as well as a bactericidal agent in the treatment of acne, dandruff and body odors

(Magar et al., 1987). Magar et al. (1987) also reported the use of SL lactone as a component of cosmetic formulations. SLs are also effective ingredient of pharmaco-dermatological products. They stimulate the dermal fibroblast metabolism and collagen neosynthesis, inhibit free radical and elastase activity, possess macrophage-activating and fibrinolytic properties, and act as desquamating and depigmenting agents (Hillion et al., 1998; Borzeix, 1999; Maingault, 1997 and1999 and Concaix, 2003). There are a number of patents on the use of SL esters in cosmetics (Inoue et al., 1980; Abe et al., 1981 and Kawano et al., 1981a, b).

1.2.6.2 Cleaning industry

Sophorolipids are fermentation products of yeasts and their production is based on renewable raw materials, hence they are biodegradable after use in various industrial applications. The biodegradability and surface-active properties make them favorable for household cleaning and detergent formulations. For example, The Japanese Company Saraya (http://www.saraya.com) has commercialized sophoron, a dish washer containing SLs as cleaning agent (Futura et al., 2002). SLs can also be applied in laundry detergents (Hall et al., 1996). Detergent compositions comprise of at least two surfactants of different characteristics and at least one of which must be a glycolipid biosurfactant (Hall et al., 1995 and 1996). The glycolipid biosurfactants such as sophorolipids, rhamnolipids, trehalose lipids etc. are active ingredient in the detergent compositions (Hall et al., 1996). Free acid and lactone form of SL or the mixtures of these two forms are utilized as components in detergent formulations. The weight ratio of sophorolipids to additional surfactant used in detergent formulations is

generally 4:1 to 3:2 (Hall et al., 1996). SLs preserve their surface lowering properties despite high salt concentrations.

1.2.6.3 Petroleum industry

Surface active and emulsifying properties of SLs can be exploited in the petroleum industry. The use of biosurfactants in petroleum industry is for enhanced oil recovery. The presence of biosurfactant lowers the surface and interfacial tensions of oil in the reservoir, which facilitates oil flow and penetration through pores in the reservoir during water, steam or fire flooding operations in enhanced oil recovery (Brown et al., 1986; Banat, 1995 and Marchal et al., 1999). Furthermore, SLs are useful in removing hydrocarbons from drill material and in the regeneration of hydrocarbons from dregs and muds (Baviere et al., 1994; Marchal et al., 1999 and Pesce, 2002). In the oil drilling process, the cuttings are pulled out by the tool are taken up to the surface by the up flow of drilling fluid injected through the channel of the drill string. Discharge of such hydrocarbon impregnated drill cuttings into sea is environmentally not safe. The patent filed by Baviere et al. 1994, reported the use of cleaning solution containing sophorolipids to clean these drill cuttings impregnated with polluted fluid comprising hydrocarbons before their discharge into sea. Baviere et al. 1994 reported that the cleaning solution for polluted cuttings comprises sophorolipids at concentrations ranging between 0.1 and 30 g l^{-1}. The properties of SLs which allows their use in such applications are biodegradability and surface-active nature.

1.2.6.4 Sophorolipids in bioremediation process

According to Head (1998), bioremediation is a process which involves the contaminant specific treatment in order to reduce the concentration of

individual or mixed environmental contaminants. There are several reports on the applications of biosurfactants in the treatment of hydrocarbon polluted soils (Bartha, 1986; Van Dyke et al., 1993; Zhang and Miller, 1994 and 1995; Banat, 1995; Deziel, 1996; Volkering et al., 1997; Bruheim et al., 1997 and Whang et al., 2008). The contamination of soils and groundwater tables by hydrocarbons may occur by leakage of petroleum tanks or pipes or may be by accidental spillage at ground level. The risks associated with contaminated soils are related to health and environment which may be caused by the vaporization of hydrocarbons in the environment as well as by the presence of aromatic hydrocarbons such as benzene, toluene, xylene etc. in the underground water.

SLs are reported for their solubilising action on poorly soluble aromatic compounds present in soil and water for enhanced degradation by microorganisms (Schippers et al., 2000). SLs are also reported for controlling the harmful algal blooms in water bodies by their antialgal action (Sun et al., 2004).

Ducreux et al. (1997) reported the use of SLs for decontaminating the hydrocarbon polluted soils and groundwater tables. They demonstrated that SLs can remove hydrocarbons from soil by forming hydrocarbon emulsions in water as well as by enhancing the biodegradation of hydrocarbons in soil by bacteria.

Miller (1995) reported that the addition of biosurfactant may promote desorption of heavy metals from soils in two ways. The first is through complexation of the free form of the metal residing in solution which decreases the solution-phase activity of the metal and therefore promotes

desorption. The second occurs under conditions of reduced interfacial tension; the biosurfactant accumulate at the solid- solution interface, which may allow direct contact between the biosurfactant and sorbed metal. SLs can also be used in the removal of heavy metals from sediments (Mulligan et al., 2001).

1.2.6.5 Food Industry

Sophorolipids are also used in food industry as a food formulation ingredient. In food formulations, apart from their obvious role as agents that decrease surface and interfacial tensions, thus promoting the formation and stabilization of emulsions, biosurfactants can have some other functions too. For example, to control the agglomeration of fat- globules, stabilize aerated systems, improve texture and shelf- life of starch- containing products, modify rheological properties of wheat dough and improve consistency and texture of fat based products (Kachholz and Schlingmann, 1987 and Nitschke and Coast, 2007). SLs are reported for their use in the food industry to improve the quality of wheat flour products (Akari and Akari, 1987) and in the cold storage transportation in air conditioning systems for the prevention of ice particle formation (Masaru et al., 2001). Furthermore, chemically modified derivatives of SLs such as sophorolipid alkyl esters (Allingham, 1971) are reported to enhance the characteristics of prepared food products (bakery and oily emulsions).

1.2.6.6 Sophorolipids as therapeutic agents

There are so many reports on the antimicrobial activities of biosurfactants such as lipopeptides and glycolipids (Scholz et al., 1998; Bernheimer et al., 1970; Itoh et al., 1971; Marahiel, 1993 and Tsuge et al.,

1996). The antimicrobial properties of biosurfactants have direct connection with their amphiphilic properties. Most of the biosurfactants, such as rhamnolipids produced by *Pseudomonas aeruginosa* (Itoh et al., 1971) and surfactin produced by *Bacillus subtilis* (Bernheimer and Avigad, 1970), function as antibiotics by solubilizing the major components of cell membranes. SLs are also reported for the same kind of solubilizing action on cell membranes (Gi, 2004 and Shah et al., 2005). SLs are reported to inhibit harmful algal blooms (Gi, 2004) and possess the antihuman immunodeficiency virus and spermicidal activities (Shah et al., 2005). SLs are used as a component in germicidal mixtures suitable for cleaning fruits and vegetables, skin and hair by lysing microbes attached to the object surface (Pierce and Heilman, 1998; Solaiman, 2005 and Yuan et al., 2011). They also act as antifungal agents against plant pathogenic fungi such as *Phytophthora* sp. and *Pythium* sp. (Yoo et al., 2005).

SLs are also finding importance in medicine because of beneficial effects such as their ability to induce cell differentiation instead of cell proliferation and the inhibition of protein kinase C activity of the human promyelocytic leukemia cell line HL 60 (Isoda et al., 1997). The anti-cancerous action of SL is attributed to a specific interaction with the plasma membrane (Isoda et al., 1997). Joshi-Navare et al. (2011) also reported the cell differentiation ability of SLs against the human tumorigenic glioma cell lines (LN-229). These cell lines were derived from grade IV glioblastoma, which is one of the frequent tumors of central nervous system and are resistant to conventional chemotherapy. Cell differentiation inducing ability of SLs in such brain tumors may become one of the preventive measures. Chen

et al. (2006a) reported the anti-cancerous activity of di-acetylated lactone form of SL against several human cancer cell lines. It was found by Chen et al. (2006b) that the cytotoxic effect of SL on the human liver cancer cells H7402 was due to its ability to induce apoptosis. SLs are also reported to decrease the mortality caused by septic shock in rat model (Bluth et al., 2006; Napolitano, 2006).

1.2.6.7 Sophorolipids as a source of specialty chemicals

SLs are a source of rare and expensive components ω and ω-1 hydroxy fatty acids (Rau et al., 2001). These fatty acids are non-toxic, biodegradable and can be produced in large amounts and are important for industrial use (Ashby et al., 2005). Inoue and Miyamoto (1980) reported the use of these fatty acids in polymerization reactions and their lactonization into macrocyclic esters, which find application in the perfume and fragrance industry. Prabhune et al. (2002) reported the synthesis of bioactive molecules, such as 19-Hydroxyeicosatetraenoic acid (19-HETE) and 20-Hydroxyeicosatetraenoic acid (20-HETE) by acidic hydrolysis of SLs produced using arachidonic acid. 19-HETE and 20-HETE are penultimate and terminal hydroxylated fatty acids of arachidonic acid and have pharmacological applications. The pharmacological applications involves the role of 19- and 20-HETE in stimulation of renal Na^+/K^+ - ATPase and role of 20-HETE as a secondary messenger in cellular processes such as autoregulation of renal blood flow, tubuloglomerular feedback and effects on Na^+ transport etc. (Oliw et al., 1981; Escalante et al., 1988; Alonso-Gaicia et al., 1999 and Lasker et al., 2000).

1.2.6.8 Sophorolipids role in Nanotechnology

SLs have been reported to have various applications in nanotechnology as well. Metal nanoparticles find applications in various fields such as mechano- and electrical applications, catalysis and biomedical use. These nanoparticles are stabilized with a capping agent to prevent aggregation and allow dispersion in organic solvents or water. For biomedical applications, the capped nanoparticles need to be dispersible in aqueous solutions. When acidic de-acetylated SLs are used to shield cobalt or silver particles, their fatty acid tail will interact with the metal particle either through the terminal –COOH or through the double bond. As the sugar moiety is exposed to the solvent, the nanoparticles acquire hydrophilic properties and are readily dispersible. SLs also act as a reducing agent, eliminating the necessity for an exogenous reducing agent (Kasture et al., 2007 and 2008). These SL capped nanoparticles can act as carriers for various bioactive molecules and have medicinal and diagnostic applications (Singh et al., 2010). For example, Singh et al. (2009) demonstrated the antibacterial activity of SL coated silver nanoparticles as such against both Gram positive and Gram negative bacteria. Britto et al. (2011) reported the synthesis of silver ions studded polymer scaffolds. In the preparation of polymer scaffold they incorporated sophorolipid on the polymer surface through covalent bonding. Then the prepared scaffold was studded with silver ions. The purpose of the use of SL on scaffold was to use it as a reducing and a capping agent for silver ions, and help in liberating the silver nanoparticles. These nanoparticle then act as a antibacterial agent for treating the medical implants such as catheters, surgical equipments, which are susceptible to bacterial infections.

1.2.6.9 Sophorolipids as an inducer in enzyme synthesis

In the literature, there are some reports about the role of SL as an inducer of several enzymes. For example, Lo and Ju (2009) reported that SLs act as inducer in cellulase production in *Hypocrea jecorina*. They demonstrated the degradation of SLs into the inducer sophorose by the fungus. They also reported that Cellulase synthesis could be further increased when *C. bombicola* was co-cultured with *H. jecorina* (Lo and Ju, 2009). SLs are also reported to induce amylase production in *Bacillus subtilis* and laccase and manganese peroxidase production in *Pleurotus ostreatus* (Gross and Shah, 2007).

1.2.6.10 Sophorolipids in self assembly and polymer formation

Zhou et al. (2004) reported the formation of supramolecular structures of self assembled aggregates of SL molecules under different pH conditions. They reported that acidic form of SL molecules represent a novel type of asymmetrical bolaamphiphiles because of the presence of disaccharide and carboxylic acid group as polar end groups, a kinked hydrophobic core (*cis*-9-octadecenoic chain), and a non-amide polar-nonpolar linkage. Bolaamphiphiles are the molecules which bears the polar groups at both the ends of a hydrophobic moiety. These bolaamphiphiles are the mimics of natural trans-membrane lipids and are important for the stability of membrane proteins in order to study their biochemical and structural features (Li et al., 2009). Lots of study has been carried out on the synthesis of symmetrical bolaamphiphiles bearing amino acids, monosaccharides, nucleotides as head groups and linear methylene chains or diacetylene chains as the hydrophobic core and supramolecular structures of monolayer vesicles, helical fibers,

helical ribbons, nanotubules, and rigid rods have been observed (Kogiso et al., 1998; Masuda et al., 2000 and Iwaura et al., 2002). Zhou et al. (2004) reported that asymmetrical bolas of SLs offers greater structural and chemical versatility over symmetrical bolas and bring unique structural and physicochemical properties as well as functionality to self assembled materials. By varying the solution pH values, Zhou et al. (2004) observed the formation of giant helical ribbons in acidic conditions (pH< 5.5). The giant ribbons were produced from interdigitated lamellar packing of SL-COOH molecules (Fig 1.4).

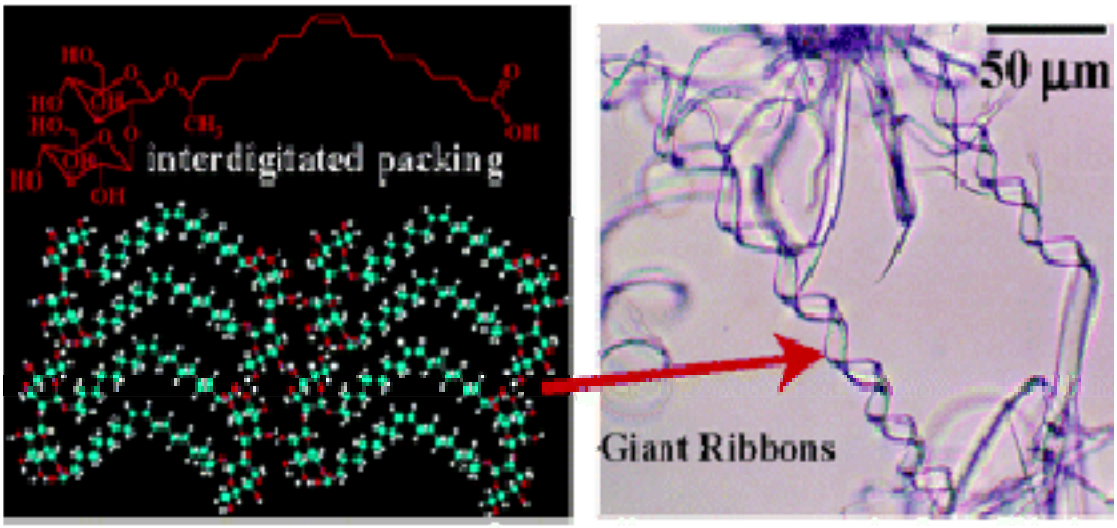


Fig. 1.4: Giant helical ribbon formation at acidic pH (< 5.5) by an interdigitated lamellar packing of acidic SL−COOH molecules shown in right panel, stabilized by both the strong hydrophobic association between the *cis*-9-octadecenoic chains and strong disaccharide−disaccharide hydrogen bonding shown in left panel (Zhou et al., 2004)

1.3 Objectives of the present work

The modification of SL structural composition can be done by using different lipophilic substrates, which in turn bring unique physicochemical properties and functionality to SL molecules (Davila et al., 1994; Nunez et al., 2001; Glenns and Cooper, 2006). Lipophilic substrates such as fatty acids and vegetable oils have been used for SL production as already discussed before. There are several reports on the analysis of sophorolipids produced using oleic acid and different vegetable oils with the help of analytical methods such as FAB-MS, APCI-MS and ESI-MS techniques (Asmer et al., 1988; Koster et al., 1995; Nunez et al., 2001 and Nunez et al., 2004). There is no such report on the analysis of individual SL molecule produced using pure α-linolenic acid (ALA) as a lipophilic substrate. SL production using α- linolenic acid as the lipophilic substrate may become a valuable product of interest with many enhanced beneficial properties. The objective of the present work was to synthesize novel SLs using *Candida bombicola*. In this study, effect of α-linolenic acid on the composition of SL mixture using different optimization parameters in fermentation, purification and characterization of individual SL molecule present in the SL mixture was done. Further, Physical and antibacterial properties were also checked in order to evaluate the potency of these SLs. *Candida bombicola* as a producer organism was used because of its non-pathogenic character and high production yields (Van Bogaert et al., 2007).

1.3.1 Alpha Linolenic acid (α-Linolenic acid)

α-Linolenic acid is a carboxylic acid with an 18-carbon chain and three *cis* double bonds (Fig. 1.5). In terms of its structure, it is named *all-cis*-9, 12,

15-octadecatrienoic acid. In physiological literature, it is given the name 18:3 (*n*−3).

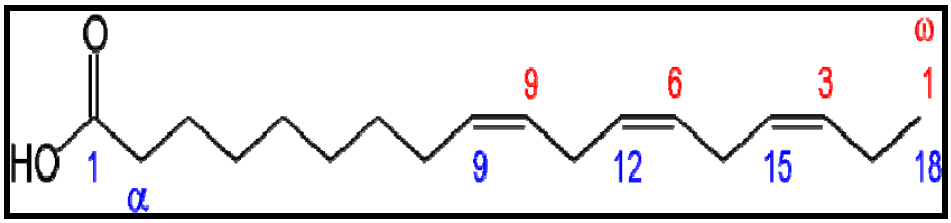


Fig. 1.5 Structure of α-Linolenic acid

The first double bond is located at the third carbon from the methyl end of the fatty acid chain, known as the *n* end. Thus, α-linolenic acid is a polyunsaturated *n*−3 (omega-3) fatty acid. It is an isomer of gamma-linolenic acid, a polyunsaturated *n*−6 (omega-6) fatty acid. Flax is a rich source of α-linolenic acid. α- Linolenic acid, an *n*−3 fatty acid, is a member of the group of essential fatty acids (EFAs), so called because they cannot be produced within the body and must be acquired through diet. Most seeds and seed oils are much richer in an *n*−6 fatty acid, linoleic acid. Linoleic acid is also an EFA, but it, and the other *n*−6 fatty acids, compete with *n*−3s for positions in cell membranes and have very different effects on human health. Studies have found evidence that α-linolenic acid is related to a lower risk of cardiovascular disease (Corner et al., 2000; Etherton et al., 2002). Further, α-linolenic acid as a ω- 3 fatty acid is also anticancerous (Bernard et al., 2002) and an important ingredient of skin care products (Brenner et al., 2004).

Beneficial properties and applications of SLs in different commercial fields are already discussed. The advantage of incorporating α-linolenic acid into the SL structure will give new functionality for different applications. α-linolenic acid in the form of SLs become more accessible and potent.

Chapter 2

Optimization of Fermentation parameters for production of linolenic acid derived Sophorolipids from Candida bombicola ATCC 22214

2.1 Summary

This chapter deals with the optimization of different process parameters to produce linolenic acid derived sophorolipids (designated as LNNSL). These process parameters include testing of different medium used by researchers for production of SLs using other lipophilic substrates, medium pH, effect of temperature, inoculum size and age as well as the optimum concentrations of glucose, fatty acid and yeast extract concentration in the medium. Results showed that out of six tested medium (A, B, C, D, E and F), medium A was chosen for LNNSL production and was used for optimization of different parameters (pH, temperature, inoculum size and age, optimum concentrations of glucose, fatty acid and yeast extract) which influenced SL production.

2.2 Introduction

Sophorolipid, a glycolipid biosurfactant is proving its importance in various industrial and pharmaceutical fields and are thus commercially beneficial (Baviere et al., 1994; Schippers et al., 2000; Masaru et al., 2001; Futura et al., 2002; Shah et al., 2005; Lourith and Kanlayavattanakul, 2009 and Britto et al., 2011). Some companies are using it on commercial scale because of its eco-friendly nature and surface-active properties in place of chemically synthesized surfactants as discussed in Introduction chapter (Soliance, 2004). Lot of research work is going on to increase the functionality of SLs in order to increase its usefulness in almost most of the industrial and pharmaceutical sectors according to need. For example, Shah et al. (2005) reported about the role of natural SLs obtained from fermentation in crude form and its chemical derivatives such as, methyl, ethyl and hexyl esters and

monoacetate and diacetate ethyl esters of SL as potent spermicidal and virucidal agents. In 2006, Bluth et al. reported some new chemo-enzymatically modified forms of SLs, which are effective septic shock antagonists. Further, some authors reported amino acid conjugated SLs (Azim et al., 2006), but these were having low microbicidal activity as compared to those reported by Shah et al. in 2005. Efforts were made not only in chemical and enzymatic modifications of SLs but also in modifications generated by introducing different hydrophilic and lipophilic carbon sources in the fermentation media in order to generate new structural analogues differing in their compositions (Tulloch et al., 1962; Gobbert et al., 1984; Cooper and Paddock, 1984; Klekner et al., 1991; Davila et al., 1992; Zhou and Kosaric, 1993 and 1995; Casas, 1996; Kim et al., 1997; Daniel et al., 1998a and 1998b; Casas and Garcia-Ochoa, 1999; Prabhune et al., 2002 and Daverey and Pakshirajan, 2009 and 2010). In the Introduction chapter it was described about the reports on the use of different hydrophilic substrates such as different mono-, di- and trisaccharides as well as waste cheap substrates of soy and sugarcane molasses and whey but the composition of the sugar part of SL was not influenced and it was always sophorose (Gobbert et al., 1984; Klekner et al., 1991; Davila et al., 1992, 1993; Daniel et al., 1998a and 1998b; Solaiman et al., 2004 and Daverey and Pakshirajan, 2009 and 2010). Then, the possible mechanism was put forward that sugars are metabolized into two glucose units, to form sophorose (Gobbert et al., 1984). It was also observed by the researchers that the yield of SL was lower as compared to yield obtained by using glucose as a substrate and thus glucose was the preferred substrate of choice (Gobbert et al., 1984).

Work has been done using several lipophilic substrates also. It was reported by the researchers that lipophilic substrates has profound effect on the composition of SL molecules (Asmer et al., 1988; Cavalero and Cooper, 2003 and Davila et al., 1994).

In the present work, the fermentative production of α-linolenic acid derived SL (designated as LNNSL) molecules is presented. In order to produce these novel SL molecules fermentation parameters must be standardized. In this chapter, the optimization of different parameters such as testing of different medium, medium pH, temperature, effect of concentrations of glucose, α-linolenic acid and yeast extract concentration etc. is presented.

The biosynthesis was carried out using *Candida bombicola* ATCC 22214 as a producer organism. In the fermentation media glucose was used as a hydrophilic, primary carbon source and α-linolenic acid was used as a lipophilic, secondary carbon source. This is the first report on the synthesis of α-linolenic acid derived SLs. *Candida bombicola* is used for SL production because of its non-pathogenicity (Asmer et al., 1988; Solaiman et al., 2004 and Van Bogaert et al., 2007).

2.3 Materials and methods

2.3.1 Materials

Malt extract, glucose, yeast extract and peptone used in this study were purchased from Hi-media, India; α-Linolenic acid was purchased from Sigma-Aldrich (USA). Sodium sulphate was purchased from Merck, India. Organic solvents such as ethyl acetate and n-hexane used were of analytical grade and were purchased from Rankem India.

2.3.2 Microorganism and maintenance

The yeast used for sophorolipid (SL) production was *Candida bombicola* (ATCC 22214). It was procured from American type culture collection, USA. *Candida bombicola* was grown for 48 h at 28 °C incubation on agar slants containing: malt extract, 0.3 %; glucose, 5.0 %; yeast extract, 0.3 %; peptone, 0.5 % and agar, 2.0 %. The microorganism was sub-cultured monthly and maintained at 4 °C in a refrigerator.

2.3.3 Media optimization

Six different media varying in composition were tested for optimal production of linolenic acid derived SLs (LNNSL). These six media tested were used by various researchers for SL production using different lipophilic substrates. These six media screened for LNNSL production are summarized in Table 2.1.

Table 2.1

Different media used for maximal production of Linolenic acid derived Sophorolipids by *Candida bombicola* prepared in distilled water (components are given in g l^{-1}). Initial medium pH was adjusted to 6.0

	Medium (A)[1]	Medium (B)[2]	Medium (C)[3]	Medium (D)[4]	Medium (E)[5]	Medium (F)[6]
Glucose	50	100	200	10	50	100
Yeast extract	3.0	10	12.5	1.0	1.0	5.0
Malt extract	3.0	---	---	---	---	---
Peptone	5.0	---	---	---	---	---
Urea	---	1.0	2.0	0.1	---	---
$(NH_4)_2SO_4$	---	---	---	---	1.0	3.3
$MgSO_4$	---	---	---	---	0.3	5.0
KH_2PO_4	---	---	---	---	---	0.2
Na_2HPO_4	---	---	---	---	0.2	7.0
NaH_2PO_4	---	---	---	---	7.0	---
$CaCl_2.2H_2O$	---	---	---	---	---	0.1
NaCl	---	---	---	---	---	0.1

References

[1]Singh et al., 2009. [2]Tulloch et al., 1962; Daverey and Pakshirajan, 2010. [3]Gorin et al., 1961. [4]Glenns et al., 2006; Cavalero and Cooper, 2003. [5]Prabhune et al., 2002. [6]Lee et al., 1993.

2.3.4 Fermentative procedures

2.3.4.1 Inoculum development

Inoculum was developed by transferring a loopful of *C. bombicola* cells from slants in MGYP (malt extract, 0.3%; glucose, 5.0%; yeast extract, 0.3%; peptone, 0.5%) medium and incubated for 24 h at 28 °C with 180 rpm orbital shaking.

2.3.4.2 Fermentative production

All the optimization experiments were performed in 500 ml Erlenmeyer flasks containing 100 ml media by varying the medium composition (Table 2.1). The fermentative production of SLs was initiated by transferring the inoculum (10%, v/v) into 100 ml of respective medium (A, B, C, D, E and F) followed by incubation at 28 °C with 180 rpm orbital shaking. Each medium (A, B, C, D, E and F) was supplemented with 1 ml of α-linolenic acid dispersed in 1 ml of ethanol.

The fermented broths were examined for the estimation of SLs at regular intervals (24, 48, 72, 96, and 120….. up to192 h). For this, 100 mL culture broths of each tested media (A, B, C, D, E and F) were examined at regular intervals (24, 48, 72, 96, and 120….. up to192 h).

2.3.4.3 Sophorolipid estimation

100 ml culture broths of each tested media (A, B, C, D, E and F) at regular time intervals (24, 48, 72, 96, and 120….. up to192 h) were centrifuged at 5000 rpm for 20 min at 25 °C and supernatant was separated. The supernatant (aqueous phase) collected were extracted twice with ethyl acetate in separating funnel. The ethyl acetate layer (organic phase layer) was collected and rotary-evaporated at 40 °C to remove solvent. The residue

obtained was washed with n-hexane two times to remove the un-reacted fatty acid. After the washing with n-hexane the partially purified SL residue was dried under vacuum and weighed to estimate the SL content.

2.3.4.4 Study of media pH optimization on LNNSL production by C. bombicola

To study the effect of media pH on SL formation, 500 ml Erlenmeyer flasks were taken and each containing 100 ml of medium A were autoclaved at 121 °C for 15 min. The composition of medium A as already mentioned above was: glucose, 5.0 %; malt extract, 0.3 %; yeast extract, 0.3 %; peptone, 0.5 % and this composition was kept constant in all the 500 ml flasks containing 100 ml media while varying the pH. The pH tested was 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 respectively. The experimental media flasks were supplied with 1 ml of α-linolenic acid dispersed in 1 ml of ethanol. All the flasks were inoculated with 10 % (v/v) inoculum and kept for SL formation in incubator shaker at 180 rpm for 168 h at 28 °C.

After incubation period, all the flasks were taken out and 100 ml culture broth of each flask were centrifuged separately to remove cells and supernatant was separated. The supernatants collected from each of the flasks were extracted twice with ethyl acetate separately. The ethyl acetate layer (organic layer) of each media flask was dried over anhydrous sodium sulphate and the solvent was removed by rotary-evaporation separately at 40 °C. The residues obtained were washed separately with n-hexane to remove unused fatty acid. The partially purified SL residues thus obtained from each flask were measured gravimetrically to estimate SL production. The experiment was performed in triplicates.

2.3.4.5 Temperature optimization study on LNNSL production by C. bombicola

After the optimization of medium pH, the experiment for temperature optimization was performed by varying the temperature of incubator shaker for the estimation of maximum SL production. Composition of media A (glucose, 5.0 %; malt extract, 0.3 %; yeast extract, 0.3 %; peptone, 0.5 %), media pH at 6.0, supply of fatty acid (1 ml of α-linolenic acid dispersed in 1 ml of ethanol) and inoculum size and age (10 %, v/v; 24 h grown inoculum) to each of the experimental flasks (500 ml flasks containing 100 ml of media A) were kept constant. Separate runs of the experiment at different incubator temperatures were performed. The temperatures used for the study were 26, 28, 30, 32, 34 and 36 °C. The experiment was carried out at 180 rpm for 168 h.

After the incubation period of 168 h, all the experimental flasks were taken out of the incubator shaker. 100 ml culture broths of each experimental flask were centrifuged to remove cells from supernatant. The supernatants of each experimental flask were extracted twice with ethyl acetate separately and dried over anhydrous sodium sulphate and the solvent was rotary-evaporated at 40 °C. The SL residue obtained was washed twice with n-hexane to remove unused fatty acid. The SL was dried under vacuum and weighed gravimetrically. The experiment was performed in triplicates.

2.3.4.6 Optimization of inoculum size for LNNSL production by C. bombicola

Effect of inoculum size was performed by varying the volume of 24 h grown inoculum while keeping rest of the parameters such as composition of

media A (glucose, 5.0 %; malt extract, 0.3 %; yeast extract, 0.3 %; peptone, 0.5 %), media pH (6.0), supply of fatty acid substrate (1 ml of α-linolenic acid dispersed in 1 ml of ethanol) and incubation period (168 h) constant. Experimental flasks (500 ml Erlenmeyer flasks) containing 100 ml of medium A was inoculated separately with different inoculum sizes such as 5.0 %, 10 % and 20 % (v/v). All the experimental flasks were kept in incubator shaker at 180 rpm for 168 h at 28 °C.

After 168 hours of incubation period, flasks were taken out. Cells were removed by centrifugation and the supernatants were separated. Supernatants (100 ml) obtained from each experimental flask were extracted twice with equal volumes of ethyl acetate and solvents were removed by rotary-evaporation at 40 °C. Residues were washed twice with n-hexane and dried under vacuum and weighed gravimetrically. Three sets of parallel experiments were performed.

2.3.4.7 Optimization study of inoculum age for LNNSL production by C. bombicola

The age of inoculum was also checked for its influence on SL production, while keeping other parameters such as composition of media A (glucose, 5.0 %; malt extract, 0.3 %; yeast extract, 0.3 %; peptone, 0.5 %), media pH (6.0), supply of fatty acid substrate (1 ml of α-linolenic acid dispersed in 1 ml of ethanol), inoculum size (10 %, v/v), incubation period (168 h) and temperature of incubator shaker (28 °C) constant. Experimental flasks (500 ml Erlenmeyer flasks) containing 100 ml media A were inoculated separately with 10 % (v/v) inoculum of 24, 48, 72 and 96 h grown cultures of

C. bombicola. All the flasks were kept for incubation at 180 rpm for 168 h at 28 °C.

The steps involving separation and isolation of SL from each experimental flask were same as described above in each optimization parameters. The SL was measured gravimetrically. Experiment was performed in triplicates.

2.3.4.8 Optimization study of glucose concentration for LNNSL production by C. bombicola

After the optimization for medium pH, temperature, inoculum size and age, the effect of glucose concentration on SL production was checked by using different concentrations of glucose in medium A such as, 5.0 %, 7.5 %, 10 %, 12.5 % and 15 % (w/v) while rest of the media components such as malt extract, 0.3 %; yeast extract, 0.3 %; peptone, 0.5 % were kept constant. The other parameters such as media pH (6.0), supply of fatty acid substrate (1 ml of α-linolenic acid dispersed in 1 ml of ethanol), inoculum size and age (10 %, v/v; 72 h grown culture), incubation period (168 h) and temperature of incubator shaker (28 °C) were kept constant. Experimental flasks (500 ml Erlenmeyer flasks) were supplied with 100 ml of media A containing varying concentrations of glucose (5.0 %, 7.5 %, 10 %, 12.5 % and 15 %, w/v) separately and were autoclaved at 121 °C for 15 min. All the experimental flasks were supplied with 1 ml of α-linolenic acid dispersed in 1 ml of ethanol and inoculated with 10 % (v/v), 72 h grown inoculum separately. The flasks were incubated at 28 °C for 168 h at 180 rpm.

The flasks were taken out after the incubation period of 168 h. 100 ml culture broths of each flask were centrifuged separately to remove cells from

supernatants. The supernatants were collected separately and extracted twice with equal volumes of ethyl acetate. The ethyl acetate layer containing SLs and unused fatty acids were dried over anhydrous sodium sulphate and were rotary-evaporated to remove solvent. The obtained SL residues of each experimental flask were washed twice with n-hexane to remove unused fatty acid and dried under vacuum. The SL residues obtained were measured gravimetrically. Experiment was performed in triplicates.

2.3.4.9 Optimization study of fatty acid concentration for LNNSL production by C. bombicola

The fatty acid concentration (secondary carbon source) was also checked for the maximal SL production. For this purpose, different concentrations of fatty acid were used such as 0.1 %, 0.2 %, 0.3 %, 0.4 %, 0.5 % and 0.6 % (v/v) while keeping the other parameters such as composition of media A (malt extract, 0.3 %; yeast extract, 0.3 %; peptone, 0.5 % and optimal glucose conc. 10 %, w/v), media pH (6.0), incubation temperature (28 °C), inoculum size and age (10 %, v/v; 72 h grown culture) constant. 500 ml Erlenmeyer flasks containing 100 ml of media A were supplied with varying amounts of α-linolenic acid such as 0.1 %, 0.2 %, 0.3 %, 0.4 %, 0.5 % and 0.6 % (v/v) dispersed in equal volumes of ethanol and inoculated separately with 10 % (v/v), 72 h grown inoculum of *C. bombicola*. The flasks were kept for incubation at 180 rpm for 168 h at 28 °C.

The experimental flasks were taken out after the incubation period (168 h) and 100 ml culture broths of each flask were centrifuged to remove cells from supernatants. The supernatants were collected separately and extracted twice with equal volumes of ethyl acetate. The ethyl acetate layers collected

were dried over anhydrous sodium sulphate and were rotary-evaporated separately to remove solvent. The SL residues obtained were washed twice with n-hexane to remove un-used fatty acid. After washing the SL residues were dried under vacuum and weighed gravimetrically. Experiment was performed in triplicates.

2.3.4.10 Optimization study of yeast extract concentration for LNNSL production by C. bombicola

Yeast extract concentration was also optimized for maximal SL production. Different yeast extract concentrations used for optimization study were 0.1 %, 0.2 %, 0.3 %, 0.4 % and 0.5 % (w/v) and all the other parameters such as composition of media A except yeast extract concentration (malt extract, 0.3 %; peptone, 0.5 % and optimal glucose conc. 10 %, w/v), media pH at 6.0, incubator temperature (28 °C), inoculum size and age (10 %, v/v, 72 h grown culture), α-linolenic acid (0.4 %, v/v) supply and incubation time (168 h) were kept constant. 500 ml Erlenmeyer flasks containing 100 ml of media A were supplied with 0.4 % (v/v) α-linolenic acid dispersed in equal volume of ethanol and inoculated with 10 % (v/v), 72 h grown culture of *C. bombicola* separately. All the flasks were kept for incubation at 28 °C, 180 rpm for 168 h for SL production.

After completion of incubation period of 168 h, all the flasks were taken out and the culture broths of each flask were centrifuged to remove cells from supernatant. The supernatants were collected separately and extracted twice with equal volumes of ethyl acetate. The ethyl acetate layers from each experiment were dried over anhydrous sodium sulphate and rotary-evaporated to remove solvent. The residues were washed with n-hexane to

remove un-used fatty acid. After washing SL residues were dried under vacuum and weighed gravimetrically. Experiment was done in triplicates.

2.4 Results and discussion

Results of the effect of six different media and time kinetics on sophorolipid production is summarized in Table 2.2.

Table 2.2

Effect of media composition and time kinetics on sophorolipid production by *Candida bombicola*

	24 h	**48 h**	**72 h**	**96 h**	**120 h**	**144 h**	**168 h**	**192 h**
Medium (A)	1.20	1.46	1.51	1.54	1.67	1.74	1.88	1.84
Medium (B)	1.96	2.59	2.68	2.72	2.86	2.96	3.07	2.92
Medium (C)	2.01	2.65	2.76	2.82	2.90	3.00	3.96	3.84
Medium (D)	0.70	0.12	0.17	0.22	0.27	0.31	0.38	0.28
Medium (E)	1.01	1.15	1.22	1.29	1.35	1.40	1.59	1.48
Medium (F)	1.42	1.54	1.68	1.74	1.83	1.90	2.00	1.86

*Sophorolipid obtained from fermentation in 100 ml media and the values are shown in g l^{-1}.

As shown in the Table 2.2, medium B, C and F gave good yields of SL while medium D showed lowest production of SL. In all media, the production of SL was found maximum at about 168 h of incubation. Medium B, C and F, which supported highest SL formation, included the highest amount of glucose content. The SL obtained from all media were analyzed by mass

spectrometry studies and it was found out that peaks of α-linolenic acid derived SLs were maximum in medium A (the details are discussed in chapter 4). Therefore, medium A was used for further optimization of different parameters such as pH, temperature, glucose concentration, time kinetics etc. Maximum SL production was observed up to 168 h, so for further optimization studies, batches were carried out up to 168 h.

2.4.1 Effect of media pH on LNNSL production by C. bombicola

At pH value 6.0 the optimal production of SL was observed. SLs obtained at other pH values were also quantified and the graph was plotted. As shown in Figure 2.1, it can be seen that as the medium pH is increased from 4.5 to 5.5, SL production increased, and the optimum production of SL was obtained at pH 6.0. Beyond pH 6.0, decrease in the yield was observed. The optimal SL production at pH 6.0 is in agreement with the earlier studies on SL production using different lipophilic substrates. For example, Daverey and Pakshirajan (2009 and 2010) obtained maximum SL production at media pH 6.0 using glucose and rapeseed oil as well as sugarcane molasses and rapeseed oil in their study. Further, Ashby et al. (2005 and 2008) also obtained maximum SL production at starting media pH at 6.0 using fatty acids such as palmitic, stearic and oleic acid as substrates. The possible reason may be that the enzymes involved in cellular metabolism of SL formation become more active at media pH 6.0.

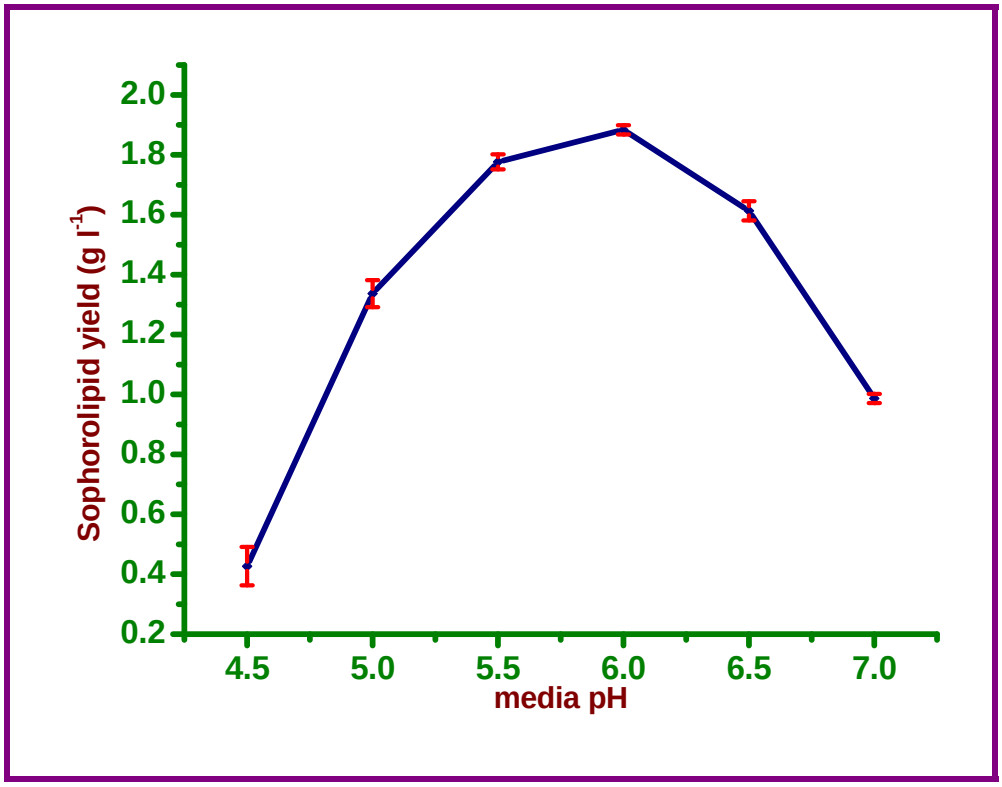


Fig. 2.1: Effect of media pH on LNNSL production by *C. bombicola* (rest of the parameters were kept constant). The data points are the average of the triplicate experiments and the error bars (red in color) are standard deviations.

2.4.2 Effect of temperature on LNNSL production by C. bombicola

The effect of temperature variation on SL production was checked at temperatures 26, 28, 30, 32, 34 and 36 °C. The optimal temperature range for SL production was observed to be 28-30 °C (Fig. 2.2). Figure 2.2 showed that after 30 °C the SL production decreased. The optimal temperature range of 28-30 °C obtained was in agreement with the studies done by various researchers on SL production. In their studies they used 28 or 30 °C temperature for maximal SL production (Asmer et al., 1988; Davila et al., 1992, 1994 and 1997; Singh et al., 2009; Daverey and Pakshirajan, 2009 and

2010 etc.) The possible reason is same as that for pH optimization that the enzymes responsible for cellular metabolism of SL formation are more active at the temperature range of 28-30 °C. As shown in Figure 2.2, after 32 °C sudden drop in SL production was observed at 34 and 36 °C, which indicated the possibility of low/non functionality of the enzymes responsible for SL formation.

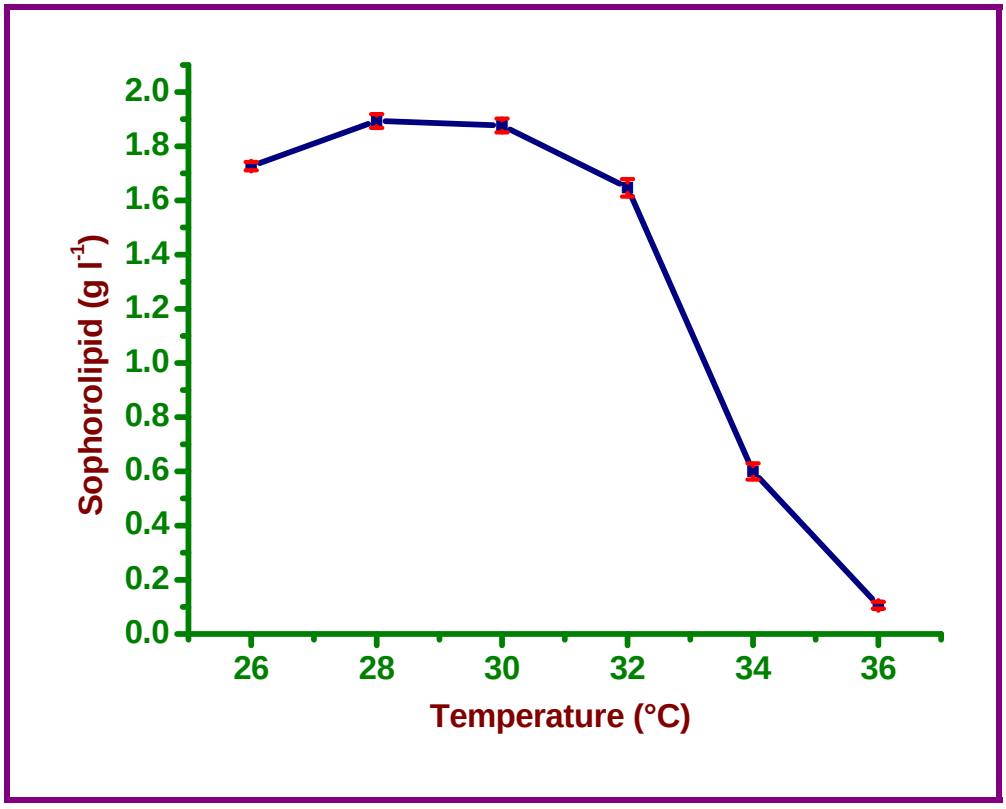


Fig. 2.2: Effect of temperature on LNNSL production by *C. bombicola* (rest of the parameters were kept constant). The data points are the average of the triplicate experiments and the error bars (shown in red) are standard deviations.

2.4.3 Effect of inoculum size on LNNSL production by C. bombicola

Graph of inoculum size versus SL production was plotted. As shown in Figure 2.3, the optimal inoculum size for maximum SL production was observed to be 10 %. At 20 %, decrease in production was observed. Significant increase in SL production was observed when the inoculum size was increased from 5.0 % (v/v) to 10 % (v/v) meaning that inoculum size also affects the SL production. 10% inoculum size was found to be optimum for SL production by other authors too (Cavalero and Cooper, 2003).

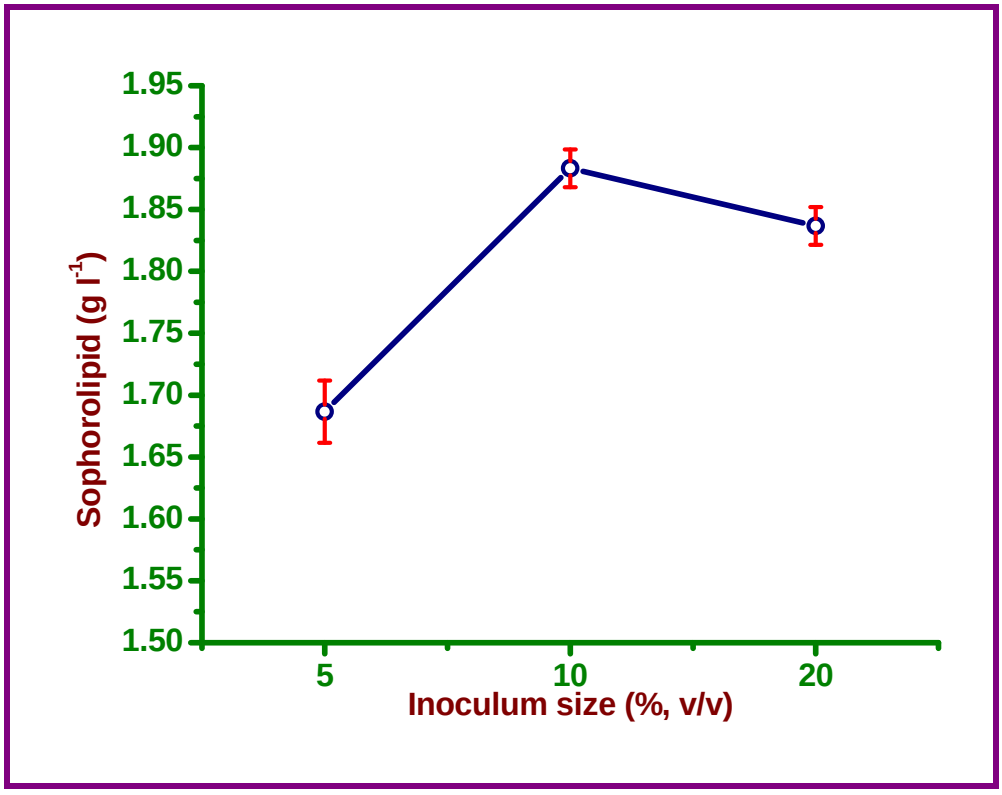


Fig. 2.3: Effect of inoculum size on LNNSL production by *C. bombicola* (rest of the parameters were kept constant). The data points are the average of the triplicate experiments and the error bars (shown in red) are standard deviations.

2.4.4 Effect of inoculum age on LNNSL production by C. bombicola

As shown in figure 2.4, SL production is not much influenced by varying the inoculum age and this is in agreement to earlier studies where some authors used 48 h (Asmer et al., 1988; Daverey and Pakshirajan, 2009, 2010 and Nunez et al., 2001) grown inoculum while some used 72 h (Koster et al., 1995) grown inoculum for SL production As there is little increase in production at 72 h (Fig. 2.4). Therefore, 72 h grown culture was used for further experiments.

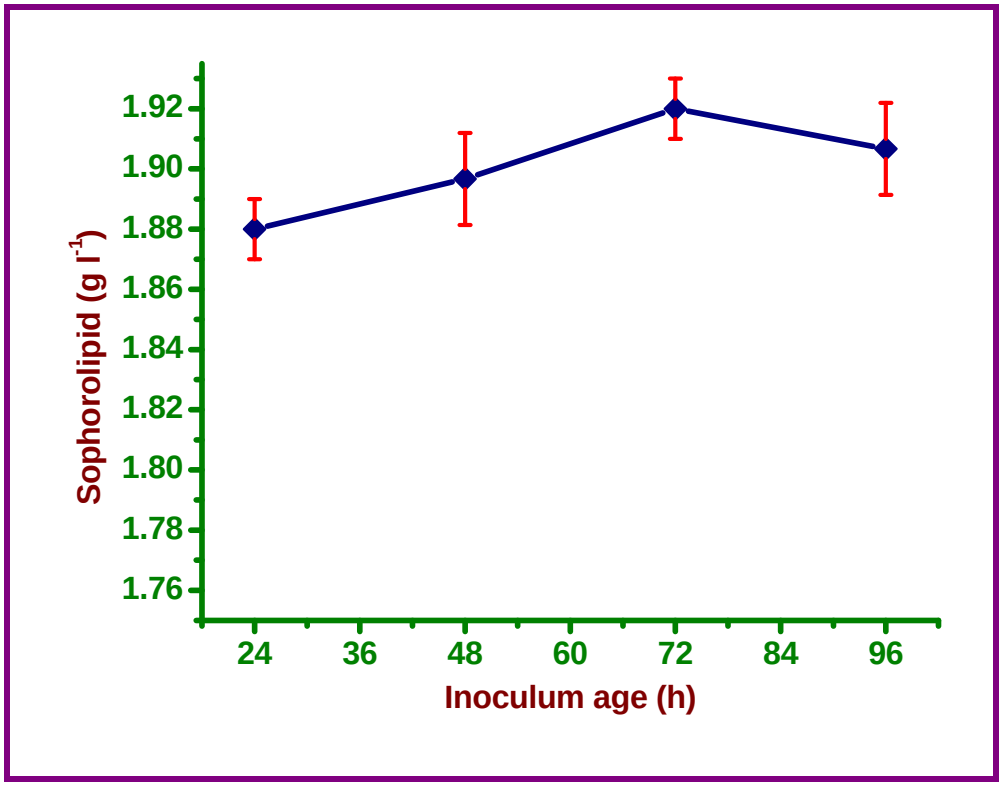


Fig. 2.4: Effect of inoculum age on LNNSL production by *C. bombicola* (rest of the parameters were kept constant). The data points are the average of the triplicate experiments and the error bars (shown in red) are standard deviations.

2.4.5 Effect of glucose concentration for LNNSL production by C. bombicola

As shown in Figure 2.5, 10 % of glucose concentration in medium A was optimal for maximum SL production. About two times increase in SL production was observed when the glucose concentration was changed from 5.0 % to 10 %. It showed that glucose has a profound effect on SL production because it is required for both the cellular metabolism and the formation of sophorose sugar of SL molecules (Linton, 1991). The optimum concentration of glucose (10 %) obtained in this case of LNNSL production is in agreement to the previous studies by Tulloch et al. (1962), Daverey et al. (2010) and Lee et al. (1993) who used 10 % of glucose concentration as the optimum concentration required for SL production using other lipophilic substrates.

2.4.6 Effect of fatty acid concentration on LNNSL production by C. bombicola

As shown in figure 2.6, 0.4 % (v/v) concentration of fatty acid was found to be optimum for maximal SL production. Studies on SL production by researchers using various lipophilic (secondary carbon sources) sources indicated the involvement of these secondary carbon sources in maximal production of SLs but after the certain limit of feeding lipophilic substrates the production decreased which may be due to inhibition of proper aeration from un-metabolized oils or fatty acids. It means that there is a certain limit (optimal concentration) of these substrates that the organism can intake. As shown in Figure 2.6 with the increase in fatty acid concentration 0.1 to 0.3 % (v/v) the SL production increased and at 0.4 % concentration become optimum for SL production. When the feeding of fatty acid content increased to 0.5 % (v/v), SL

production started decreasing and at further additions (0.6 %, v/v) SL production decreased.

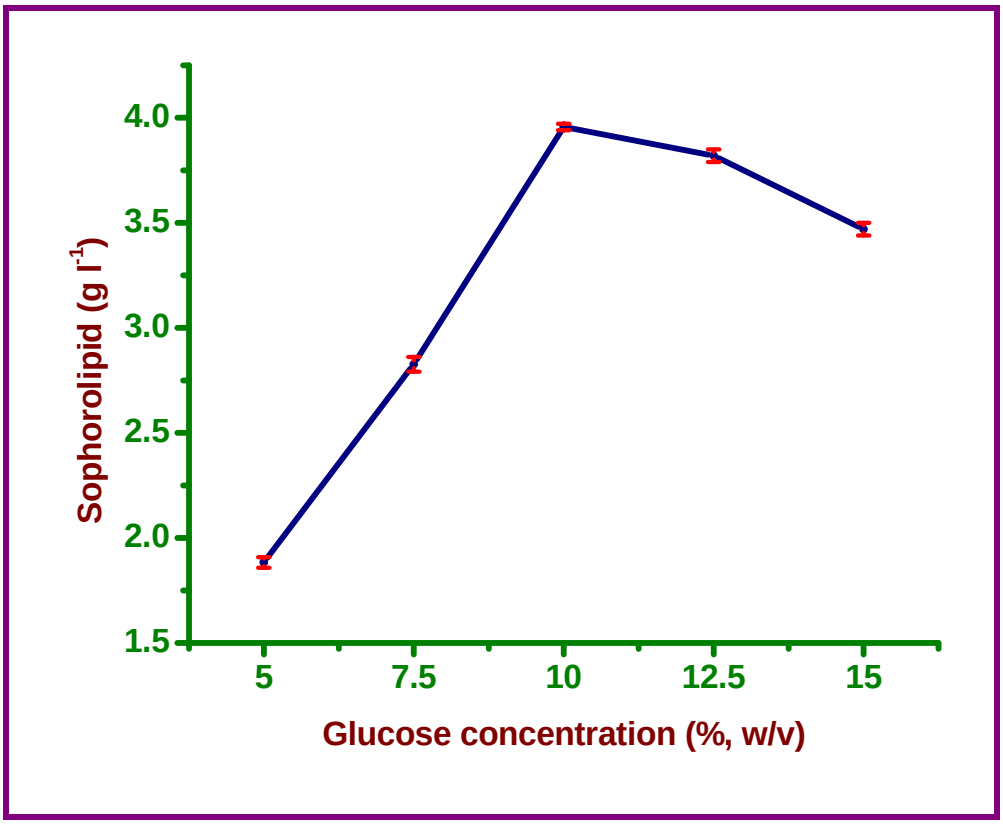


Fig. 2.5: Effect of Glucose concentration on LNNSL production by *Candida bombicola* (rest of the parameters kept constant). The data points are the average of the triplicate experiments and the error bars (shown in red) are standard deviations.

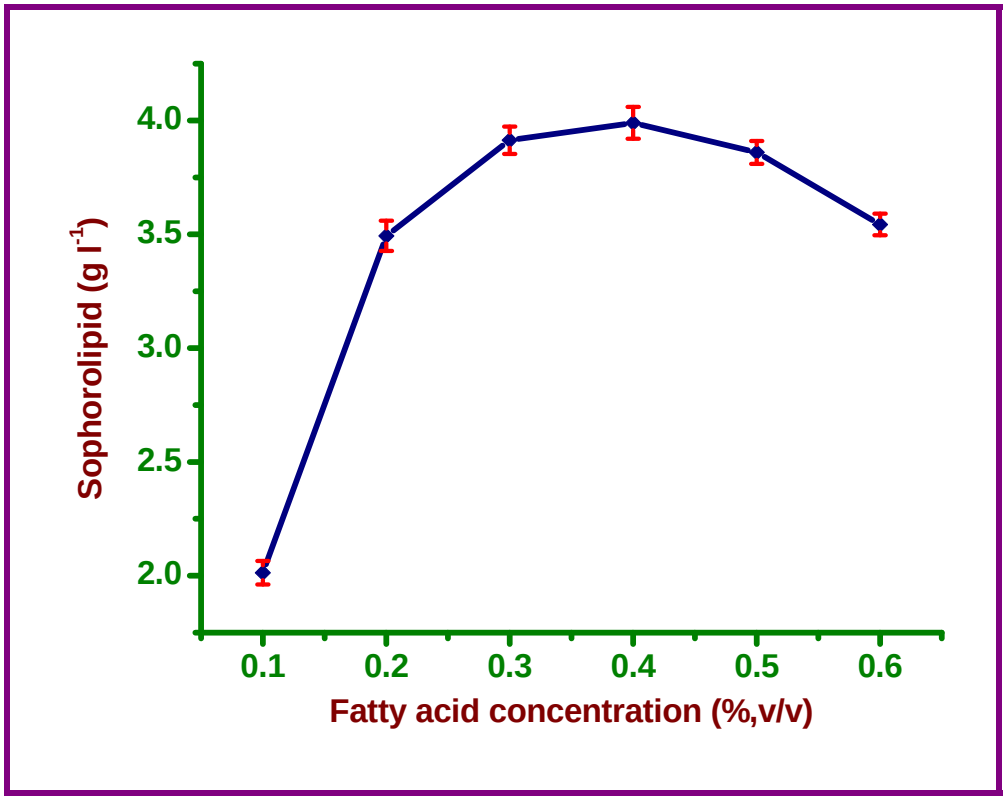


Fig. 2.6: Effect of fatty acid concentration on LNNSL production by *Candida bombicola* (rest of the parameters kept constant). The data points are the average of the triplicate experiments and the error bars (shown in red) are standard deviations.

2.4.7 Effect of yeast extract concentration on LNNSL production by C. bombicola

Yeast extract as a source of nitrogen is useful for yeast growth and SL production. Therefore, yeast extract concentrations were varied in medium A to know the optimal yeast concentration. As shown in Figure 2.7, SL production was low at 0.1 % yeast extract concentration and as the concentration increased to 0.2 %, SL production also increased and at 0.3 % yeast extract concentration optimal production of SL was observed. Beyond 0.3 % yeast extract concentration, the yield of SL decreased slightly, it may be

due to the fact that SLs have been reported to accumulate under nitrogen limited conditions (Inoue, 1988 and Asmer et al., 1988). It was reported by Ratledge and Evans in 1989 that vitamins such as pantothenic acid, thiamin and pyridoxin present in yeast extract decrease the amount of SLs produced by yeast. Not only in the case of SLs but also other glycolipids such as Rhamnolipids are produced in maximum quantity under limited nitrogen supply (Ochsner et al., 1995). Therefore, the limited nitrogen supply directs the cellular metabolism towards maximum SL formation and the present study is in agreement to the previous studies (Inoue, 1988 and Asmer et al., 1988).

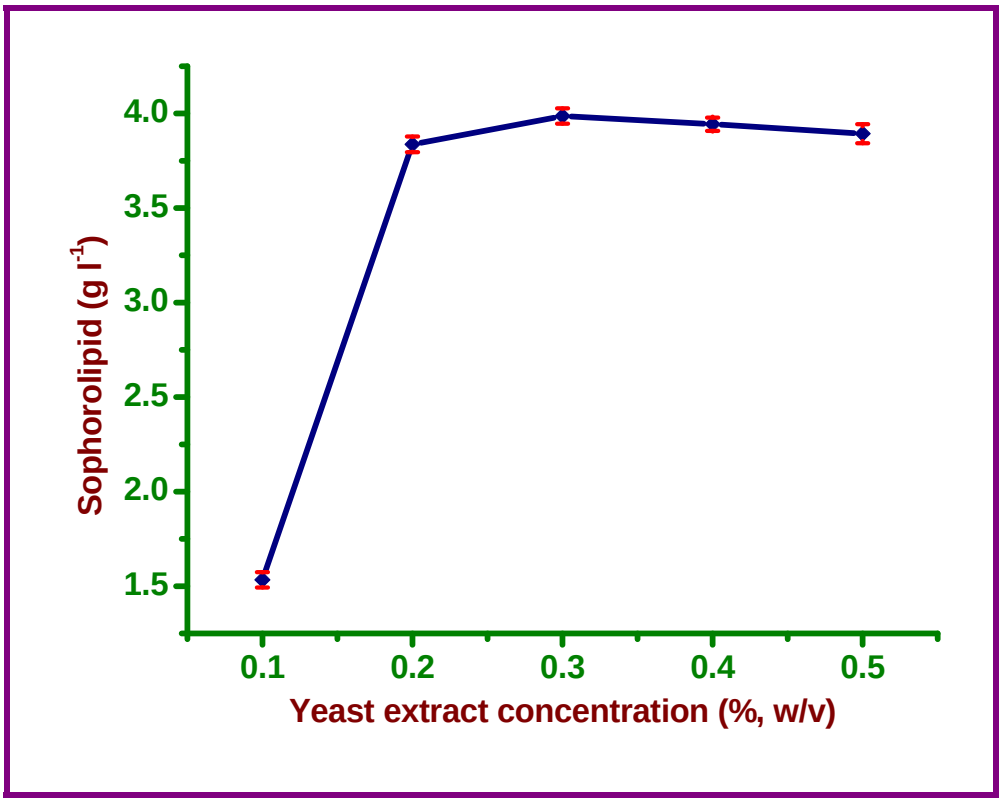


Fig. 2.7: Effect of Yeast extract concentration on LNNSL production by *Candida bombicola* (rest of the parameters kept constant). The data points are the average of the triplicate experiments and the error bars (shown in red) are standard deviations.

The optimum levels of process parameters were decided and summarized in the Table 2.3.

Table 2.3

Optimum levels of the process parameters chosen for LNNSL production

Process parameter	Optimum level
Medium pH	6.0
Temperature	28 °C
Inoculum size	10 %
Inoculum age	72 h
Glucose concentration	10 % (w/v)
Fatty acid concentration	0.4 % (v/v)
Yeast extract concentration	0.3 % (w/v)
Fermentation period	168 h

2.4.8 Time kinetics on LNNSL/Biomass production

Under the optimized fermentation parameters, time-kinetics on LNNSL and biomass production was performed at regular time intervals (24, 48, 72, 96, 120, 144, 168 and 192 h). The production of LNNSL and biomass in g l^{-1} is shown in Table 2.4 and a graphical representation between time (h) and LNNSL/biomass production (g l^{-1}) is shown in Figure 2.8.

Table 2.4

Time kinetics on Sophorolipid and biomass production under optimized conditions. The values are the average of the triplicate experiments with their standard deviation

Time (hours)	SL (g l^{-1})	Biomass* (g l^{-1})
24	1.89 ± 0.037	2.01 ± 0.061
48	1.91 ± 0.047	2.17 ± 0.035
72	2.20 ± 0.021	2.55 ± 0.035
96	2.53 ± 0.071	2.84 ± 0.050
120	2.98 ± 0.078	3.11 ± 0.040
144	3.43 ± 0.060	3.11 ± 0.031
168	3.98 ± 0.045	3.11 ± 0.035
192	3.84 ± 0.061	2.83 ± 0.045

***** After the separation of supernatant, cells were washed two times with ethyl acetate to remove the glycolipids and oil. Then the cell biomass was washed with distilled water. The cells were centrifuged twice and collected and then dried in an oven at 70 °C for 48 h and weighed to measure the biomass.

Time-kinetics performed on LNNSL/Biomass production showed that LNNSL production started after 48 hours but increase in SL production was observed from late exponential phase (after 96 h). Maximum accumulation of LNNSL was observed in stationary and late stationary phases and reached its maximum level at 168 h after which SL production decreased. Biomass growth started after 24 hours and continued till 120 hours. No increase in growth was observed after 120 hours and the biomass remained constant. Biomass decreased after 168 h.

The yield of LNNSL was estimated to be 3.98 g l^{-1}. Though the yield is lower as compared to other mono unsaturated fatty acid derived sophorolipid such as oleic acid and saturated alkanes of hexa-, hepta- and octa-decanes (Asmer et al., 1988; Cavalero and Cooper, 2003; Davila et al., 1993). There are reports on poly unsaturated fatty acid derived sophorolipids using *Candida bombicola* by Prabhune et al. (2002) which also showed the lower yield. But the yield can be compromised when required in low volumes in medicinal field. There is possibility that higher yields can be obtained by screening other sources as well as mutant strains for the production of LNNSL.

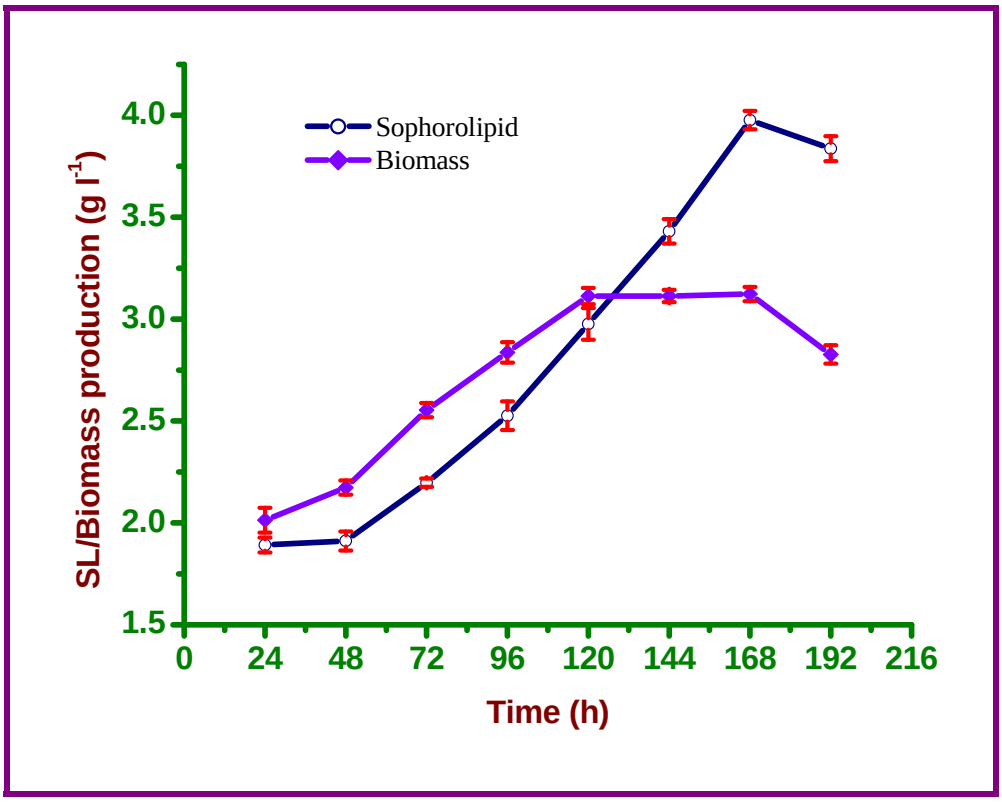


Fig. 2.8: Time course of Sophorolipid (LNNSL) and Biomass production under optimized conditions. The data points are the average of the triplicate experiments and the error bars (shown in red) are standard deviations.

2.5 Conclusions

The study of different process parameters showed that about 7 days (168 h) are required for maximum production of LNNSL. The optimum physiological pH and temperature for production was found to be 6.0 and 28 °C respectively. Inoclulum size of 10 % (v/v) was found optimum, but inoculum age did not have much influence on LNNSL production. Glucose at 10 % (w/v) gave maximum yield and fatty acid supply of 0.4 % (v/v) was found to be optimum. Yeast extract at 0.3 % (w/v) was required for optimum production. The maximum accumulation of SLs in stationary and late stationary phases is in agreement with the previous reports on SL production using fatty acids (Hommel et al., 1987) other than the linolenic acid and also in agreement with the other glycolipid biosurfactants (Stodola et al., 1967; Bentley and Campbell, 1968 and Rosenberg and Ron, 1999).

Chapter 3

Production and Purification of Linolenic acid derived Sophorolipids

3.1 Summary

In chapter 2, the optimization of different process parameters such as pH, temperature, effect of glucose, fatty acid and yeast extract concentrations on SL production using glucose and α-linolenic acid as carbon substrates by *Candida bombicola* was demonstrated and the optimum medium for SL production was decided. This chapter deals with the study on production of LNNSL using 1 liter batches under optimized conditions to see the effect on yield of SL. Further, in this chapter purification of LNNSL is described using high performance liquid chromatography and by chemical modification of SL mixture following esterification reaction. Results showed that there is no change in the yield of SL when the production is carried out for 1 liter batch. The results from HPLC showed the presence of three prominent peaks which must be characterized to identify different SL molecules present in LNNSL mixture. The characterization of these peaks is described in the chapter 4. The esterification reaction of LNNSL mixture produce single product as confirmed from thin layer chromatography. The complete characterization of this product is also described in the chapter 4.

3.2 Introduction

It was reported earlier by Tulloch et al. (1962) that the composition of fatty acid portion of SL varies according to the substrate added into the medium. They worked on fatty acid esters of palmitic, stearic, oleic and linoleic acid and found that the maximum incorporation was observed for stearic and oleic acid into the lipophilic portion of the SL molecule by means of acid methanolysis and gas liquid chromatography. Asmer et al. (1988) reported the production (fermentor level) and structural studies of SLs

produced by *Candida bombicola* using glucose and oleic acid and oleic acid alone in the medium. They reported that the yield of SL in the medium where only oleic acid was used as a carbon source was maximum (77 g l^{-1}) as compared to the medium where both the glucose and oleic acid were used as carbon sources (38 g l^{-1}) and the structure elucidation was done with the help of medium pressure liquid chromatography and thin layer chromatography. This study is in contrast to other studies which showed that both the hydrophilic (sugars) and lipophilic (fatty acids) carbon sources are required for maximum production of SLs (Cavalero and Cooper, 2003). Davila et al. (1992) reported that the production of SL using rapeseed oil fatty acid (main component of oil is oleic acid) esters and glucose generated the major product to be the diacetylated lactone form of SL having oleic acid in the lipophilic portion and they showed that both the carbon sources glucose and fatty acid are required for maximum yield of SLs. Studies done by several researchers showed that both the carbon sources are necessary for maximum yield of SLs (Gobbert et al.,1984; Linton, 1991 ; Zhou et al., 1992; Davila et al., 1994; and Hommel et al., 1994). These authors suggested that the hydrophilic carbon source such as glucose is used primarily for cellular metabolism and the formation of sophorose moiety of SL, while the lipophilic carbon source is used for the synthesis of hydroxy-carboxylic acid moiety. Different analytical techniques were used for purification of SL. For example, Asmer et al. (1988) used thin layer chromatography and medium pressure liquid chromatography. Nunez et al. (2001, 2004) and Davila et al. (1994) used high performance liquid chromatography and purification by silica gel chromatography using dialysis tubing method was used by Shah and

Prabhune (2007) in place of tedious and time taking silica gel chromatography.

The structural analysis and applications of the SLs produced using glucose and α-linolenic acid could be done only when the sufficient amount of the product is available. Therefore, the production was done using one liter batches under optimized fermentation conditions. Further, the purification of LNNSL was done by two methods (1) High performance liquid chromatography of LNNSL mixture. (2) Chemical esterification of LNNSL mixture in order to produce single homogenous product following chemical esterification reaction given in literature (Bisht et al., 1999).

3.3 Materials and methods

3.3.1 Materials

The yeast *Candida bombicola* ATCC 22214 was used for the production of α-linolenic acid derived sophorolipids (designated as LNNSL). This yeast was procured from American type culture collection. Glucose, yeast extract, malt extract and peptone were purchased from Hi-media, India and sodium sulphate and acetonitrile was procured from Merck, India. α-linolenic acid (≥99%, purity, C18:3) was purchased from Sigma-Aldrich. Solvents such as chloroform and methanol were purchased from Rankem, India.

3.3.2 Growth conditions and microorganism maintenance

Candida bombicola ATCC 22214 was grown for 48 h at 28 °C on agar slants containing (g l^{-1}): malt extract, 3.0; glucose, 50; yeast extract, 3.0; peptone, 5.0 and agar, 20. The microorganism was sub-cultured every month and maintained at 4 °C.

3.3.3 Inoculum development

Inoculum was developed by growing *Candida bombicola* (ATCC 22214) cells at 28 °C in 100 ml MGYP medium containing (g l^{-1}): malt extract, 3.0; glucose, 100; yeast extract, 3.0; peptone, 5.0; pH 6.0 and shaken at 180 rpm for 72 h.

3.3.4 Production of sophorolipids

LNNSL production was carried out in 1 liter Erlenmeyer flasks containing 250 ml of optimized media of composition (g l^{-1}): malt extract, 3.0; glucose, 100; yeast extract, 3.0; peptone, 5.0. Media pH was adjusted to 6.0. All the flasks containing 250 ml of media were supplied with 1 ml of α-linolenic acid dispersed in 1 ml of ethanol separately. Total 4 ml of fatty acid was used for 1 liter batch. The flasks were then inoculated separately with 10 % (v/v, 25 ml inoculum to each 250 ml media) 72 h grown inoculum and kept for SL production in incubator shaker at 180 rpm for 168 h at 28 °C.

3.3.5 Isolation of sophorolipids

After incubation of 168 h it was observed that brownish oily viscous layer of SL settled down at the bottom of the culture broth. Culture medium was centrifuged at 5000 rpm for 20 min. The supernatant was separated and extracted twice with equal volumes of ethyl acetate. To ensure the complete isolation of SLs, the cells were also washed with ethyl acetate so as to remove the glycolipids and fatty acid adhered to cells. Then ethyl acetate layer obtained from both the supernatant extraction and washing of cells were pooled and was dried over anhydrous sodium sulphate. The solvent was removed by rotary-evaporation at 40 °C. The brownish viscous product was obtained, which was washed twice with n-hexane to remove the un-reacted

fatty acid and was dried under vacuum. SL yield was calculated gravimetrically. The product was stored at 4 °C till further use.

3.3.6 Microscopic analysis of SL mixture

A small amount of SL mixture obtained above was dissolved in water and drop casted on a glass slide and covered with a cover slip. It was observed under different optical zooms and digital pictures were taken.

3.3.7 Qualitative analysis of SL mixture by thin layer chromatography (TLC)

Thin layer chromatography (TLC) is the simplest, quickest and cheapest of the various analytical techniques that have been used extensively for the qualitative analysis of compounds. Preferred adsorbent used for TLC is silica gel. Commercially available standard silica gel coated on aluminium plates (Kiesel-gel $60_{F254,}$ Merck, Darmstadt, Germany, silica layer thickness 2 mm) are used for the analysis of SL mixture. Glass micro-capillary is used for the spot application of samples. Many solvent systems have been used for the separation of compounds. Ultra-visible absorbing compounds can be visualized under detection at 254 nm of UV light. The plates are coated with silica impregnated with the fluorescent indicator F254 nm. The other methods used for detection are charring with anisaldehyde solution and staining with iodine vapours. The simplest, staining with iodine vapours works best for compounds with double bonds. The ethyl acetate extract of SL mixture was used for the determination of number of components present in the mixture by means of thin layer chromatography. Developing system used was $CHCl_3$:CH_3OH:H_2O in the ratio of 95:4:1, v/v/v. Spots were visualized by either of the two detection techniques: exposure to iodine vapour and the second

one is charring with anisaldehyde solution (anisaldehyde:sulphuric acid:acetic acid = 0.5:1:50, v/v/v (Asmer et al., 1988).

3.3.8 Purification of sophorolipids

The brownish viscous product obtained in step 3.3.5 was used for the purification of sophorolipids by two methods:

- ❖ High performance liquid chromatography
- ❖ Purification by chemical esterification of SL mixture

3.3.8.1 High performance liquid chromatography (HPLC)

Sophorolipid mixture obtained from fermentative production was dissolved in acetonitrile and analyzed by reversed-phase high performance liquid chromatography (HPLC) with a Waters 2487 separation module (Waters Co. Milford, Massachusetts) using analytical symmetry C18, 5 µm column (250 × 4.6 mm^2). The gradient solvent elution profile used was as follows: water**:**acetonitrile (95:5, v/v) holding for 10 min; to a final composition of water**:**acetonitrile (5:95, v/v) with a linear gradient over 50 min and holding for 10 min. The flow rate was 0.5 ml min^{-1}. The peaks were detected at 220 nm wavelength by absorbance detector. Fractions from different peaks were collected and pooled separately in many runs.

3.3.8.2 Purification by chemical esterification of SL mixture

Synthesis of Sophorolipid methyl ester

Synthesis of SL methyl ester was done by following the methodology for chemical esterification reaction given by Bisht et al. in 1999 (for conversion of oleic acid derived SLs into single homogenous esterified product). Steps involved in the reaction are shown in the flow sheet form in Figure 3.1. Chemical esterfication reaction is shown in Figure 3.2.

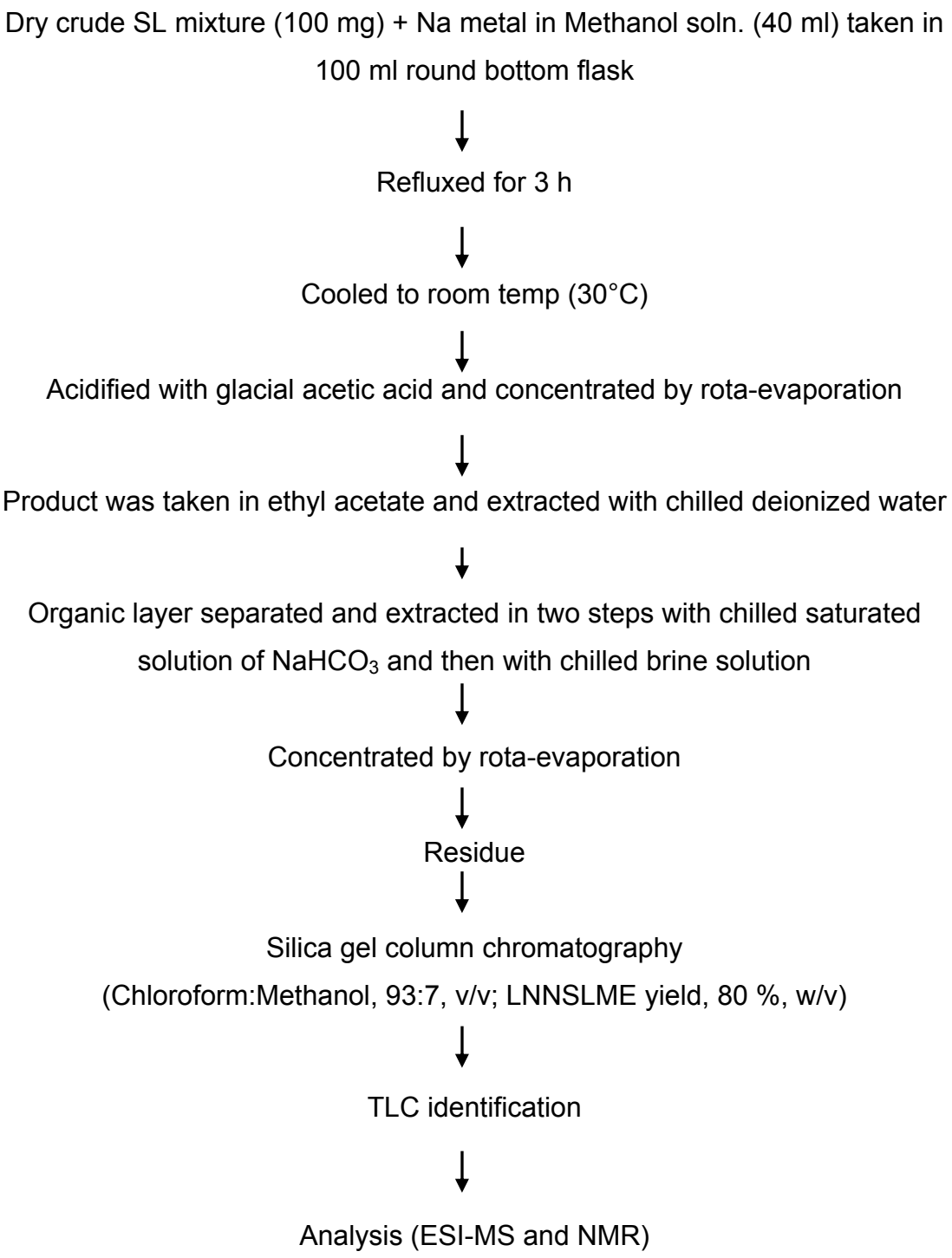


Fig. 3.1: Flow sheet diagram of reaction steps involved in chemical esterification of LNNSL mixture

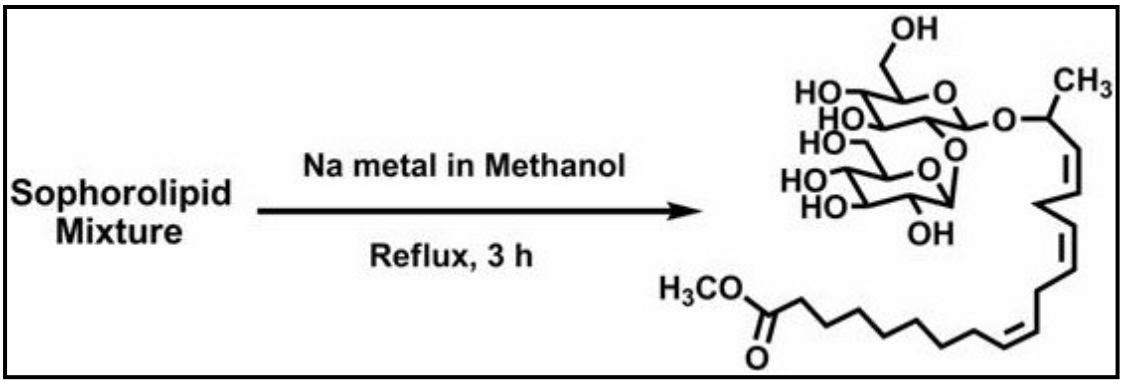


Fig. 3.2: Chemical esterfication reaction for conversion of sophorolipid mixture into its methyl ester form (LNNSLME)

3.4 Results and discussion

3.4.1 SL yield

The SL obtained after fermentative production using 10 % glucose and 4 ml of α-linolenic acid was 3.98 g $l^{-1}$of culture medium. The semi crystalline viscous nature of obtained SL is shown in Figure 3.3.

3.4.2 Microscopic analysis of SL

The SL mixture was analyzed microscopically at 5X, 10X and 20X optical zoom (Fig. 3.4). It was found that the mixture was semi-crystalline in nature.

Fig. 3.3: Semi crystalline viscous LNNSL layer in Petri-plate containing culture broth

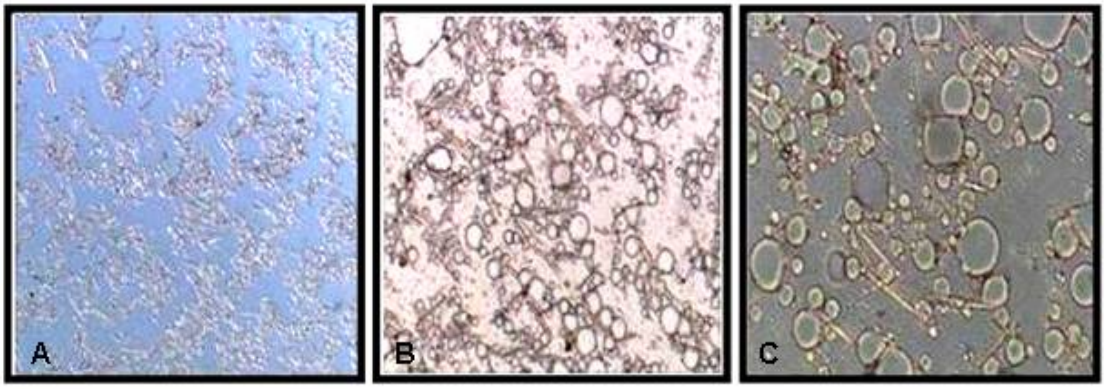


Fig. 3.4: Microscopic analysis at (A) 5X (B) 10X and (C) 20X, optical zoom showing presence of semi crystalline viscous nature of LNNSL.

3.4.3 Analysis of SL mixture by thin layer chromatography

This brownish semi-crystalline SL was analyzed by thin layer chromatography using TLC plates. The development of TLC plate after spotting the small amount of ethyl acetate extract of LNNSL mixture was done using $CHCl_3$:CH_3OH:H_2O in 65:15:2 ratio as mentioned in the materials and methods section 3.3.7 and the detection of these spots was done using iodine

vapours. The mixture gets resolved into four different components at different positions and having different R_f values. As shown in Figure 3.5, the four components were named as SL-1, SL-2, SL-3 and SL-4 and their R_f values are shown in Table 3.1.

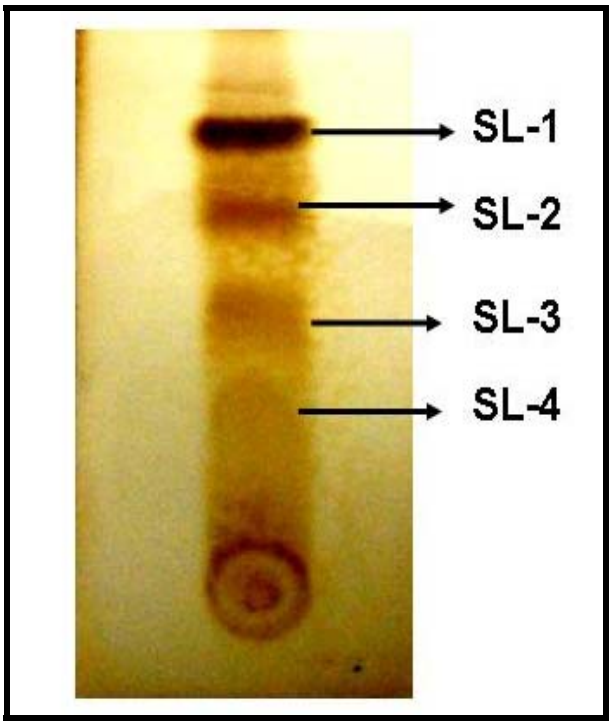


Fig. 3.5: Thin layer chromatography of SL mixture (LNNSL) using silica gel GF254 plates

Table 3.1

R_f values of components of linolenic acid derived SL

LNNSL components	*R_f values
SL-1	0.77
SL-2	0.63
SL-3	0.48
SL-4	0.37

***R_f** (retardation factor) is the ratio between the distance moved by analyte from the origin and the distance moved by solvent front from the origin

3.4.4 High performance liquid chromatography

The LNNSL mixture was analyzed by HPLC. Chromatogram showed three major peaks as illustrated in Figure 3.6. The three major peaks were eluted at 6.8 min, 14.8 min and 27.6 min respectively.

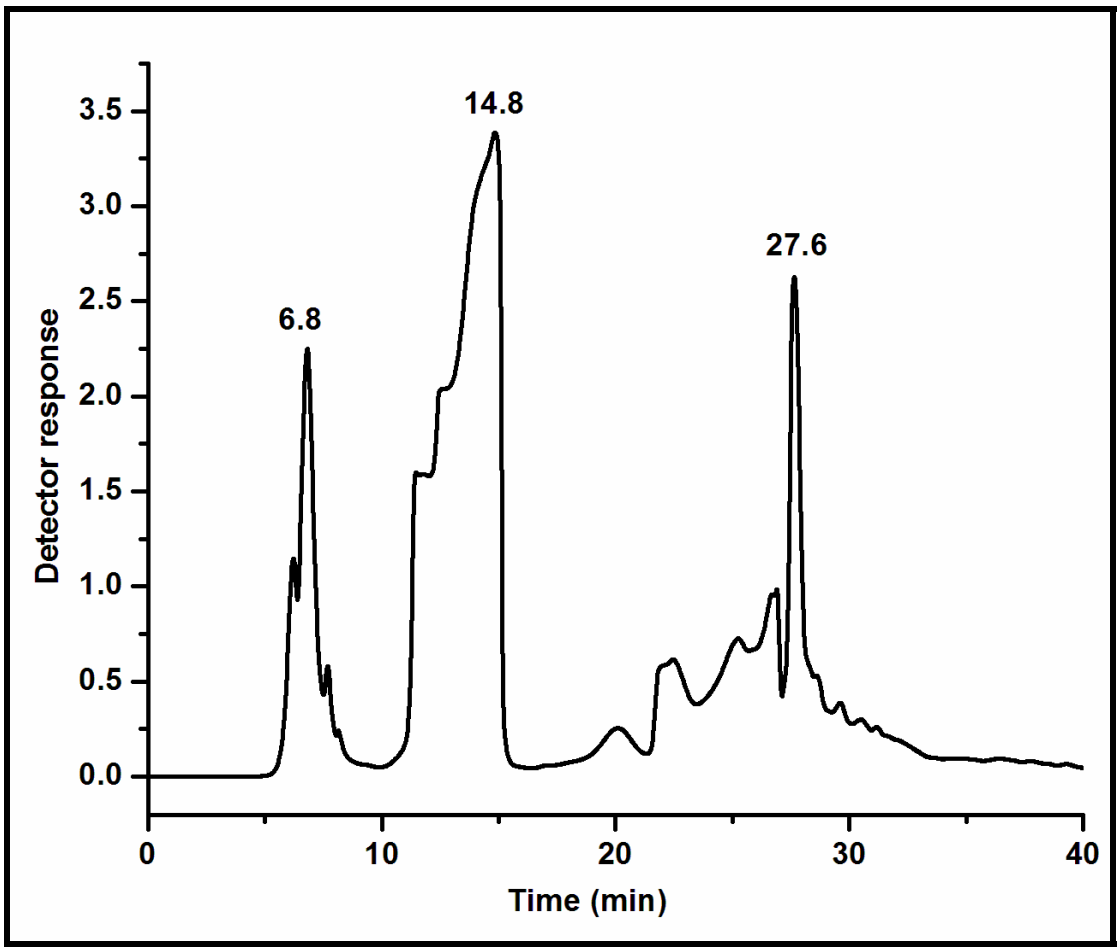


Fig. 3.6: Chromatographic profile of LNNSL mixture showing three major peaks at 6.8 min, 14.8 min and 27.6 min respectively

As shown in Figure 3.6 the most prominent peak was observed at 14.8 min. The fractions from these peaks were collected separately in number of runs in order to collect substantial amount of sample for further characterization.

3.4.5 Chemical esterification of LNNSL mixture

Bisht et al. in 1999 reported that SL mixture can be converted into single homogenous product by using some chemical modifications. In their report they demonstrated the conversion of SL mixture derived using oleic acid and glucose as carbon sources into single homogenous esterified product by chemical esterification with sodium methoxide. For the ease of purification and also to derive new SL product containing ester of Linolenic acid in the fatty acid portion, this chemical esterification reaction was followed. The details of the reaction were described in Materials and methods section. The residue obtained after rota-evaporation was measured gravimetrically and about 80 % (w/w) yield was obtained that of starting material (100 mg).

Thin layer chromatography performed on this product revealed single spot on charring with anisaldehyde solution (Fig. 3.7). This product was used for further characterization using mass spectrometry technique, which is described in chapter 4.

Fig. 3.7: Thin layer chromatography of esterified product (LNNSLME) using silica gel GF254 plates showing single spot

3.5 Conclusions

The conclusions drawn from the above study indicated that the yield of LNNSL was not affected when the production was carried out using one liter batches. The yield of LNNSL from one liter batch was 3.98 g l^{-1}. The LNNSL mixture obtained from fermentation was subjected to microscopic analysis which showed that it is semi crystalline viscous in nature. The semi crystalline viscous nature of LNNSL mixture is indicative of the presence of both the lactones and acidic forms in the mixture as lactones are known to exhibit crystalline property while acidic forms exhibit viscous nature (Cavalero and Cooper, 2003). Further, analysis of LNNSL mixture by thin layer chromatography showed that it consists of four different SLs (SL-1, SL-2, SL-3, SL-4) having R_f values 0.77, 0.63, 0.48, 0.37 respectively. The component adjacent to solvent front or migrating first (SL-1) was most non-polar or hydrophobic while that migrated in last (SL-4) was most polar or hydrophilic in nature. This analysis indicated that both lactones (hydrophobic) and acidic (hydrophilic) SLs were present in LNNSL mixture. Analysis by HPLC had shown three major peaks at 6.8, 14.8 and 27.6 min. Peak detected at 14.8 min was more prominent. Fractions from these three different peaks were collected and pooled separately in number of runs for further characterization studies which are described in chapter 4.

For the ease of purification LNNSL mixture was subjected to chemical esterification reaction. The yield of methyl ester of linolenic acid SL (designated as LNNSLME) was 80 % (w/w). Thin layer chromatography revealed a single spot. The characterization of this product is described in chapter 4.

Chapter 4

Structural Determination and physical properties of Linolenic acid derived Sophorolipid and its methyl ester form

4.1 Summary

This chapter deals with the analysis of chemically distinct forms present in the heterogeneous mixture of LNNSL as well as the analysis of its methyl ester form (LNNSLME). The structural characterization of individual form present in the LNNSL mixture was done with the help of mass spectrometry technique. The mass spectrometry technique used for the structural determination was Electro-spray ionization (ESI) and further the structures were confirmed using fragmentation patterns obtained from tandem mass spectra involving collision induced dissociation mass spectrometry. Analysis of LNNSLME was done using ESI-MS and further structural confirmation was done by NMR studies.

Physical properties such as surface tension and critical micelle concentration of LNNSL and its methyl ester, LNNSLME were also determined using Wilhelmy plate method.

4.2 Introduction

In the literature, there are several reports on analysis and structural determination of individual components in SL mixture produced using different lipophilic substrates. Different analytical techniques have been used by researchers to identify chemically distinct forms of SLs. For example, Asmer et al. (1988), Koster et al. (1995) and Nunez et al. (2001 and 2004) identified SL structures derived using oleic acid as lipophilic substrate with the help of fast atom bombardment mass spectrometry (FAB-MS), atmospheric pressure chemical ionization mass spectrometry (APCI-MS) and electro-spray ionization mass spectrometry (ESI-MS) and NMR respectively. Cavalero and cooper (2003) used gas chromatography mass spectrometry for the

identification of SLs generated using different alkanes, palmitoleic and oleic fatty acids and sunflower oil. They observed the maximum direct incorporation (95 %) for C18:1 fatty acid moiety into the SL structure. Mass spectrometry techniques have vast application in the field of structural determination of biomolecules (Tomer et al., 1983 and Adams, 1990).

SL offers great structural diversity and hence different physicochemical and biological properties of the distinct functional forms (Glenns and Cooper, 2006). For the exploitation of individual functional form in the industrial, pharmaceutical and other sectors, these forms must be fully characterized. Therefore, in this chapter structural characterization of the three major fractions collected from three prominent peaks observed in HPLC and the esterified product obtained by following chemical esterification reaction (Bisht et al., 1999) was also characterized with the help of mass spectrometry is presented. Further, the surface tension and critical micelle concentration, which are the most widely used criteria for evaluating biosurfactant activity were determined for LNNSL and its methyl ester, LNNSLME with the help of Wilhelmy plate method. This is the first report on the characterization of linolenic acid derived SLs.

4.3 Materials and methods

4.3.1 Chemicals

Acetonitrile used was of HPLC grade and was procured from Merck, India. CD_3OD (Deuterated methanol) was purchased from Sigma.

4.3.2 ESI and CID-MS analysis of LNNSL fractions

Positive and negative ESI and CID mass spectra were obtained with an API QSTAR PULSAR hybrid MS/MS quadrupole TOF system (Applied

biosystems). ESI mass spectra were obtained with time-of-flight mass spectrometry (TOF MS). Ion source was set at 3.8 kV. Tandem mass spectra (MS/MS) were acquired by selecting the desired precursor ion with Q1, which fragments ions in a collision cell (Q2). N_2 gas was used in the collision cell as collisionally activated dissociation gas. These fragment ions were detected in the time of flight section. Preliminary identifications of different SL forms present in LNNSL mixture was done using ESI-MS analysis and further structural confirmation of these forms were done from fragmentation patterns obtained from CID-MS analysis.1 µl samples from different fractions of LNNSL (collected from HPLC) were injected separately into mass spectrometer for analysis using both ESI and CID-MS technique.

4.3.3 ESI and NMR (nuclear magnetic resonance) analysis of LNNSLME

LNNSLME was analyzed by ESI-MS analysis and for this 1 µl of LNNSLME (stock solution of 2-3 mg dissolved in acetonitrile) was injected into the mass spectrometer for analysis.

Further structural confirmation of LNNSLME was also done using NMR technique. ^{1}H and ^{13}C NMR spectra were recorded using Avance Bruker-200 MHz spectrometer. Chemical shifts are given in parts per million downfield from 0.00 ppm using tetramethylsilane (TMS) as the internal reference. The ^{1}H NMR spectra of LNNSLME was taken in CD_3OD (deuterated methanol). The following abbreviations are used to present the spectral data: **s** = singlet, **m** = multiplet, **d** = doublet.

4.3.4 Surface tension and critical micelle concentration determination

SL solutions (100 ml) of different concentrations were prepared using distilled water and kept for two hours for equilibration. The surface tension

(S.T.) measurements of aqueous solutions of sophorolipids against air were done using the Wilhelmy plate method on Kruss K-11 tensiometer (automated tensiometer) at room temperature (25 °C). Different concentrations ranging from 0 to 200 mg l^{-1} of LNNSL and its methyl ester (LNNSLME) were used for the measurement of surface tension. Before measuring surface tension of SL solutions, the surface tension of water was determined.

4.3.4.1 Wilhelmy plate method

In this method, thin platinum (Pt) plate was used to measure equilibrium surface tension at the air-liquid interface. The plate is cleaned thoroughly and attached to a balance via a thin metal wire. During the experiment, the plate is lowered to the surface of sophorolipid solutions until a meniscus is formed, and then raised so that the bottom edge of the plate lies on the plane of the undisturbed surface. This method determines surface tension by measuring the force bringing the plate downward via a counter balance (Fig. 4.1) and used to calculate the surface-tension (σ) using the Wilhelmy equation:

$$\sigma = F / (\cos\theta * L)$$

where,

σ = surface tension

F = force

θ = contact angle of the sample against the Pt plate

L = wetted perimeter (2w+2d) of the Wilhelmy plate

For almost all liquids against Pt, θ=0 and so the equation simplifies to:

$$\sigma = F / L$$

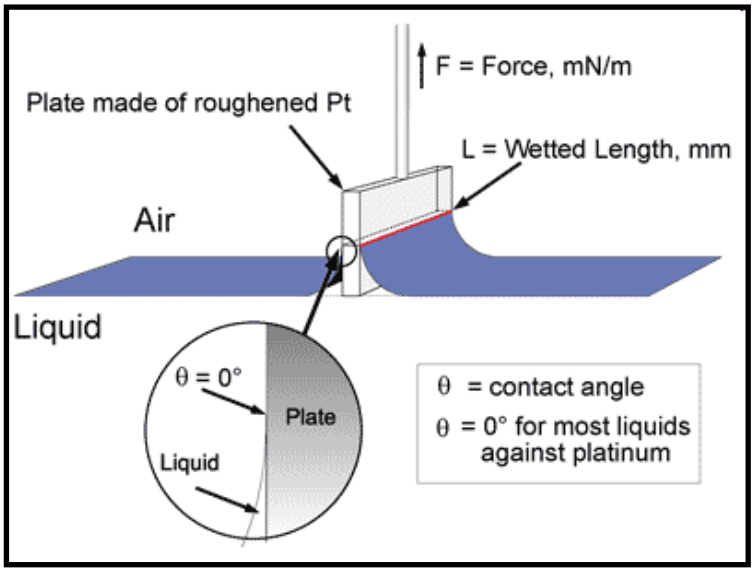
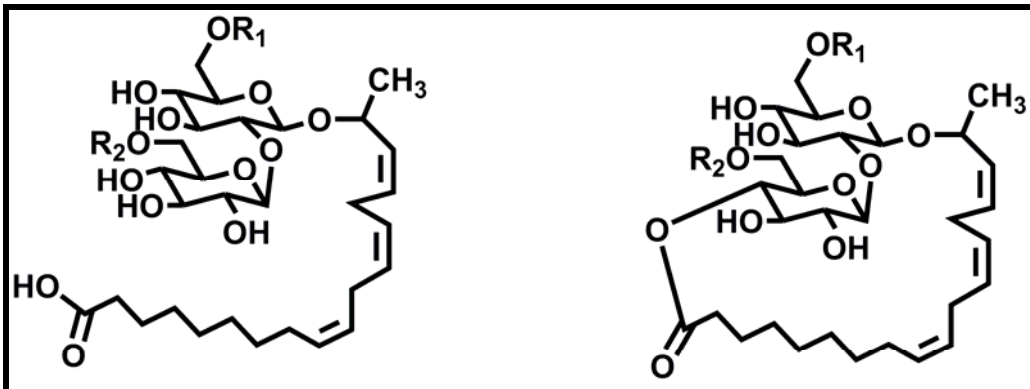


Fig. 4.1 Schematic diagram of measurement of surface tension using Wilhelmy plate method (*Image courtesy: http:// images.google.com*)

The plots of SLs concentration versus S.T. were plotted and the break point in S.T. with varying SLs conc. was taken as critical micelle concentration (CMC) and reported.

4.4 Results and discussion

Different possible forms of α-linolenic acid derived SLs are shown in Figure 4.2.



Compound	R_1	R_2	Form	Notation	M_r
1	H	H	Lactone	Glc-Glc-1',4"-C18:3	600
2	H	H	Free acid	Glc-Glc-1'-C18:3	618
3	H	Acetyl	Lactone	GlcOAc-Glc-1',4"-C18:3	642
4	Acetyl	H	Lactone	Glc-GlcOAc-1',4"-C18:3	642
5	H	Acetyl	Free acid	GlcOAc-Glc-1'-C18:3	660
6	Acetyl	H	Free acid	Glc-GlcOAc-1'-C18:3	660
7	Acetyl	Acetyl	Lactone	GlcOAc-GlcOAc-1',4"-C18:3	684
8	Acetyl	Acetyl	Free acid	GlcOAc-GlcOAc-1'-C18:3	702

Fig. 4.2: Possible structures of SLs with α-linolenic acid (ALA). Encircled areas are showing the presence of lactone, free acid and diacetylated lactone forms of C18:3 SL molecules in the mixture

4.4.1 Mass spectrometric analysis of LNNSL fractions

4.4.1.1 Mass spectra of first fraction obtained at retention time 6.8 min

The positive mode ESI mass spectrum of the first fraction (eluting at 6.8 min) is shown in Fig. 4.3. Ions corresponding to protonated and sodiated SL molecules were observed at m/z 619 $[M+H]^+$ and m/z 664 $[M+2Na]^+$ respectively. Some pseudo-molecular ions of higher masses due to the formation of solvent adducts were also detected at m/z 655 $[M+H+2H_2O]^+$, m/z 679 $[M+2H+H_2O+CH_3CN]^+$ and m/z 700 $[M+2CH_3CN]^+$ (Fig. 4.3). The protonated and sodiated molecular ions as well as pseudo-molecular ions

confirmed the presence of free acid form of C18:3 SL molecules in the SL mixture (Fig. 4.2, compound 2).

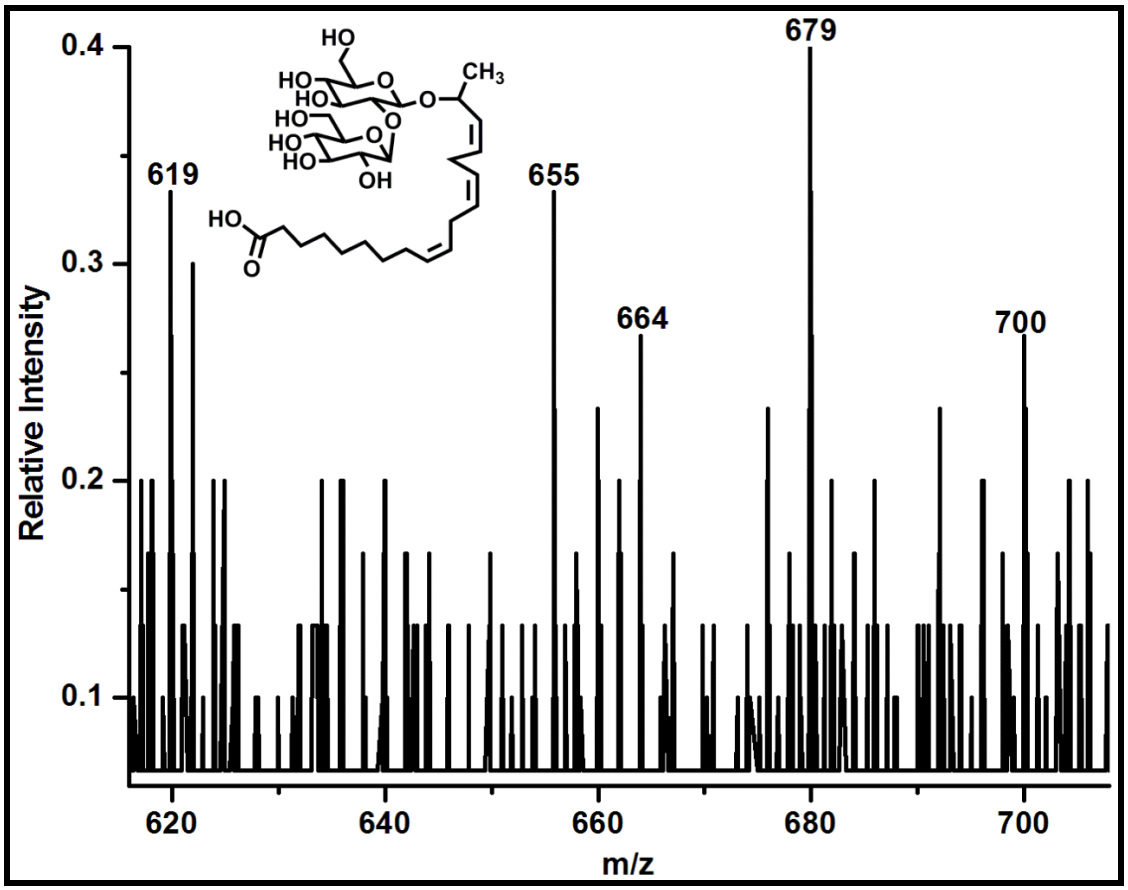


Fig. 4.3: Positive mode ESI spectra of first fraction from 6.8 min showing presence of free acid form of C18:3 SL molecules (Fig. 4.2, compound 2)

The negative mode ESI mass spectrum was also recorded for this fraction. The spectrum consists of $[M-H]^-$ ion at m/z 617 with no apparent fragmentation (Fig. 4.4). CID-MS analysis of m/z 617 $[M-H]^-$ ion gave sufficient information on the molecular structure of free acid form (Fig. 4.5). CID mass spectrum of $[M-H]^-$ ion showed a rich diversity of fragment ions and consists of a uniform pattern of 14 a.m.u. equidistant peaks (Fig. 4.5). This phenomenon is known as charge remote fragmentation because bond cleavage takes place at a site in the ion remote from the charge site and is

well recognized in the CID spectra of fatty acid carboxylate anions (Adams, 1990). Characteristic charge-remote fragmentation of ions containing a saturated hydrocarbon chain involve losses of the elements of C_nH_{2n+2} from the alkyl terminus resulting in a regular series of peaks with a spacing of 14 mass units. The presence of a double bond and its position are established by the absence of specific C_nH_{2n+2} losses, namely those which would arise as a result of vinylic hydrogen transfer or double bond cleavages (Tomer et al., 1983). As shown in Fig. 4.5 the charge remote fragment ion series results from collisionally induced losses of $C_nH_{2n+1}COO^-$ from m/z 617, yielding peaks at m/z 558, 544, 530, and so on. The series is interrupted between m/z; 488 and 434, 434 and 394, 394 and 355, indicative of the presence of un-saturation between C9 and C10, C12 and C13, C15 and C16 of the fatty acid alkyl chain. The low abundances of peaks at interruption sites are most likely caused by the relatively high energy requirement for vinylic hydrogen transfers and double bond cleavages.

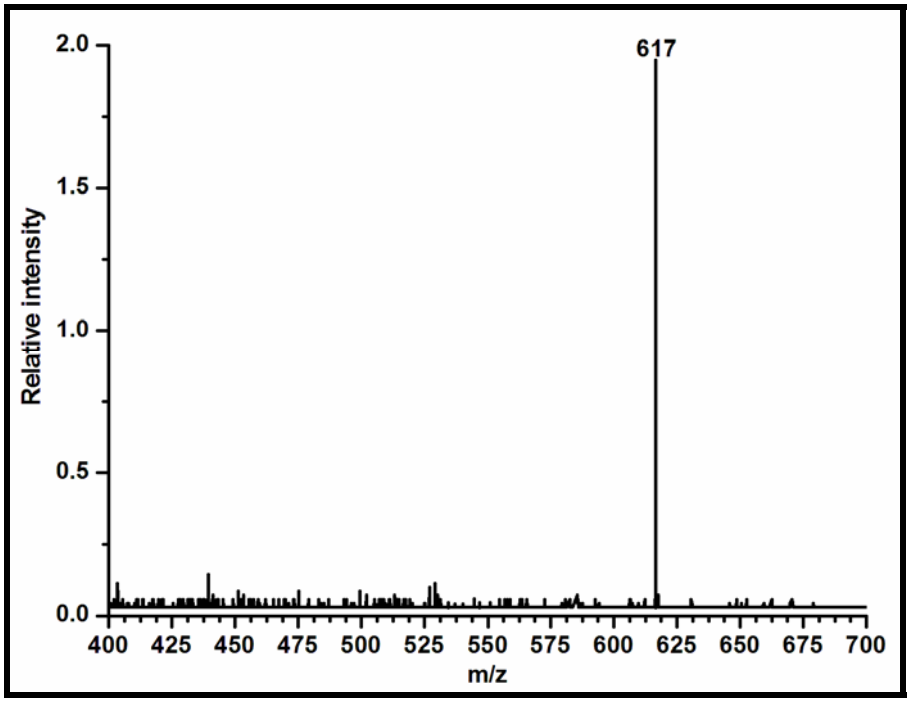


Fig. 4.4: Negative mode ESI spectra of first fraction from 6.8 min showing presence of free acid form of C18:3 SL molecules

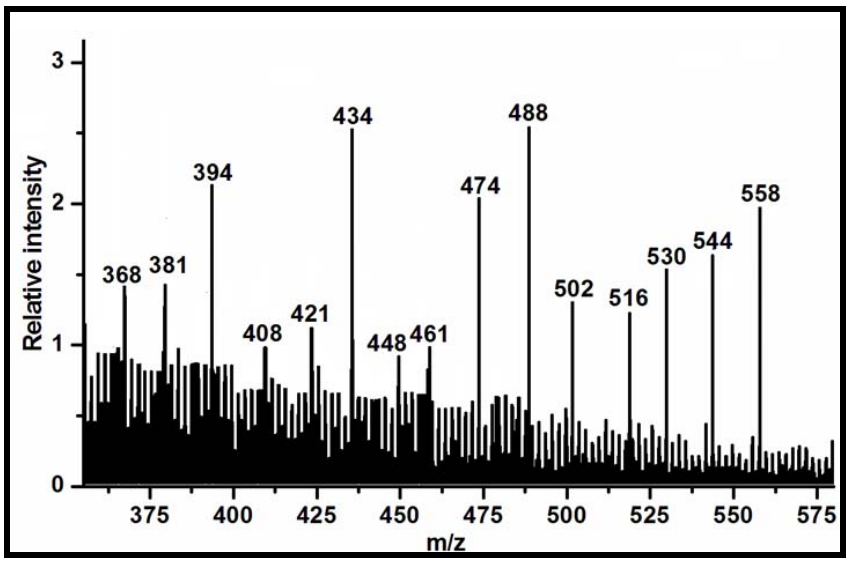


Fig. 4.5: CID spectrum of the m/z 617 $[M-H]^-$ ion

4.4.1.2 *Mass spectra of second fraction obtained at retention time 14.8 min*

The positive mode ESI mass spectrum of second fraction (eluting at 14.8 min) is shown in Fig. 4.6. The protonated molecular ion was observed at m/z 602 $[M+2H]^+$ that corresponds to the lactone form of SL (Fig. 4.2, compound 1). The loss of $2H_2O$ from the protonated $[M+H]^+$ molecular ion generated the peak at m/z 565 in the same spectra (Fig. 4.6) that gave the evidence for the presence of lactone form of C18:3 SL molecule in the SL mixture (Fig. 4.2, compound 1). Further structural evidence on this form was obtained by the CID-MS analysis of the protonated molecular ion $[M+H]^+$. There is a report on the CID-MS analysis of isolated fractions of SLs produced by *C. bombicola* using glucose and oleic acid as substrates by Koster et al. (1995). In positive CID-MS analysis, they reported the characteristic consecutive loss of 3 H_2O molecules from protonated molecular ion and an important ion generated by the loss of $C_6H_{10}O_5$ fragment. They proposed that the loss of $C_6H_{10}O_5$ fragment occurs from the reducing end of the disaccharide that leads to the formation of oxonium ion.

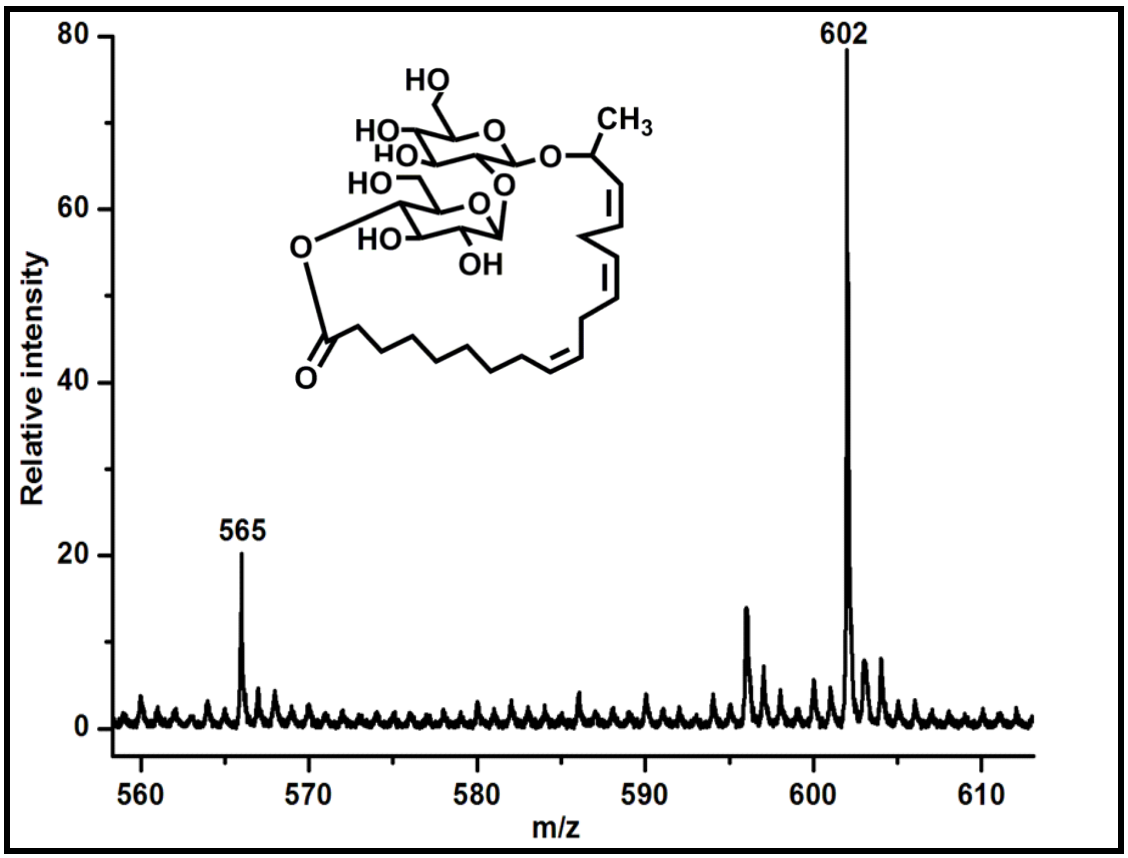


Fig. 4.6: Positive mode ESI spectra of second fraction from 14.8 min showing presence of lactone form of C 18:3 SL molecules (Fig. 4.2, compound 1)

The similar kind of ions was observed in the CID-MS spectra of protonated molecular ion (m/z 601) using our conditions (Fig. 4.7). The base peak in the spectrum at m/z 439 results from the fragmentation of $[M+H]^+$ ion by the loss of a $C_6H_{10}O_5$ molecule which follows the scheme given by Koster et al. (1995). Fragment ion peaks at m/z, 421, 403, 385 and 367 were obtained as the result of consecutive losses of H_2O molecules from m/z 439. The other less intense peak at m/z 277 ($C_{18}H_{29}O_2$) indicates the loss of H_2O molecule from the protonated hydroxy fatty acid ($C_{18}H_{31}O_3$, m/z 295). The protonated hydroxy fatty acid peak at m/z 295, which is the characteristic peak of acidic form is absent in the spectra of this lactonic form. Spectrum

also contains fragment ion peaks at m/z, 565 and 547 which were the results of losses of two and three H_2O molecules from m/z 601 respectively.

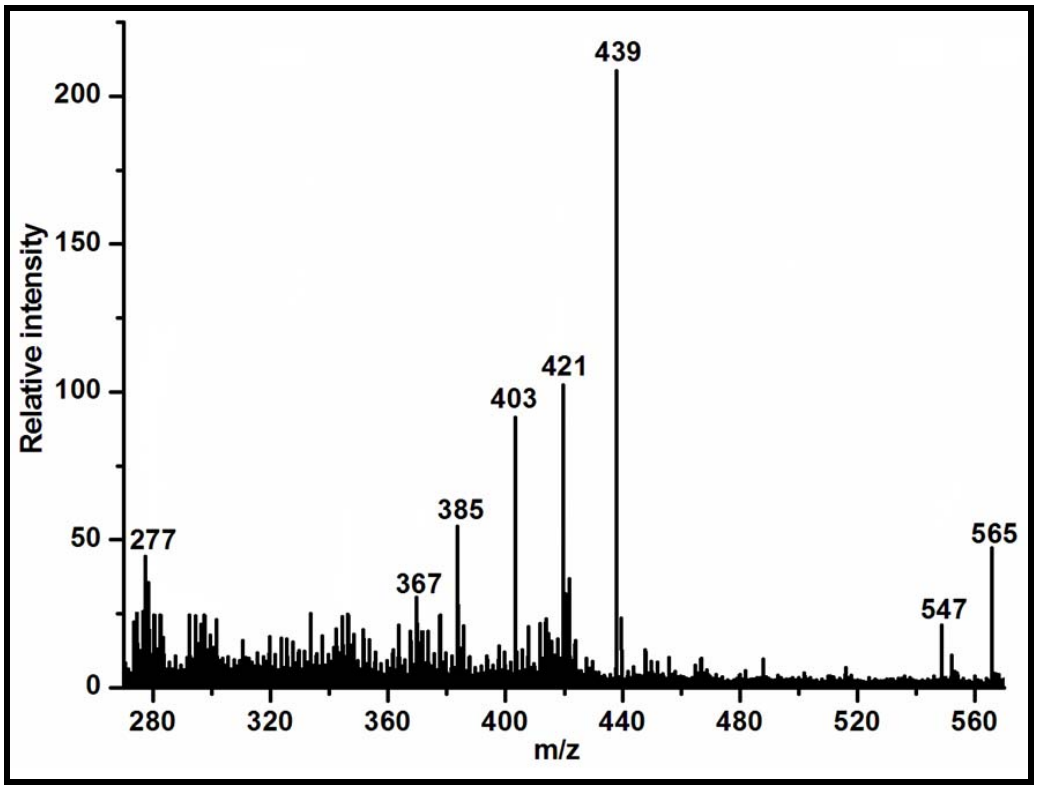


Fig. 4.7: CID spectrum of the protonated molecular ion, m/z 601

4.4.1.3 Mass spectra of third fraction obtained at retention time 27.6 min

The positive mode ESI mass spectrum of third fraction (eluting at 27.6 min) is shown in Fig. 4.8. The protonated molecular ion peak was observed at m/z 685 $[M+H]^+$ that corresponds to the diacetylated SL lactone having C18:3 in its fatty acid chain (Fig. 4.2, compound 7). In the same spectra, one more peak was observed at m/z 711 $[M+Na]^+$ (Fig. 4.8) that corresponds to the diacetylated SL lactone having C18:1 in its fatty acid chain. The CID-MS spectrum of m/z 685 molecular ion gave more evidence for its presence (Fig. 4.9). Fragment ion peaks at m/z 667, 649 and 631 were generated by

consecutive losses of H_2O molecules from m/z 685. The fragment ion peak at m/z 409 was obtained by the loss of hydroxy fatty acid moiety from m/z 685. Less intense peak observed at m/z 205 is the characteristic of the compound with an acetate group at C-6" atom of the nonreducing component. Fragment ion peak at m/z 277 ($C_{18}H_{29}O_2$) indicates the loss of H_2O molecule from the protonated hydroxy fatty acid ($C_{18}H_{31}O_3$, m/z 295).

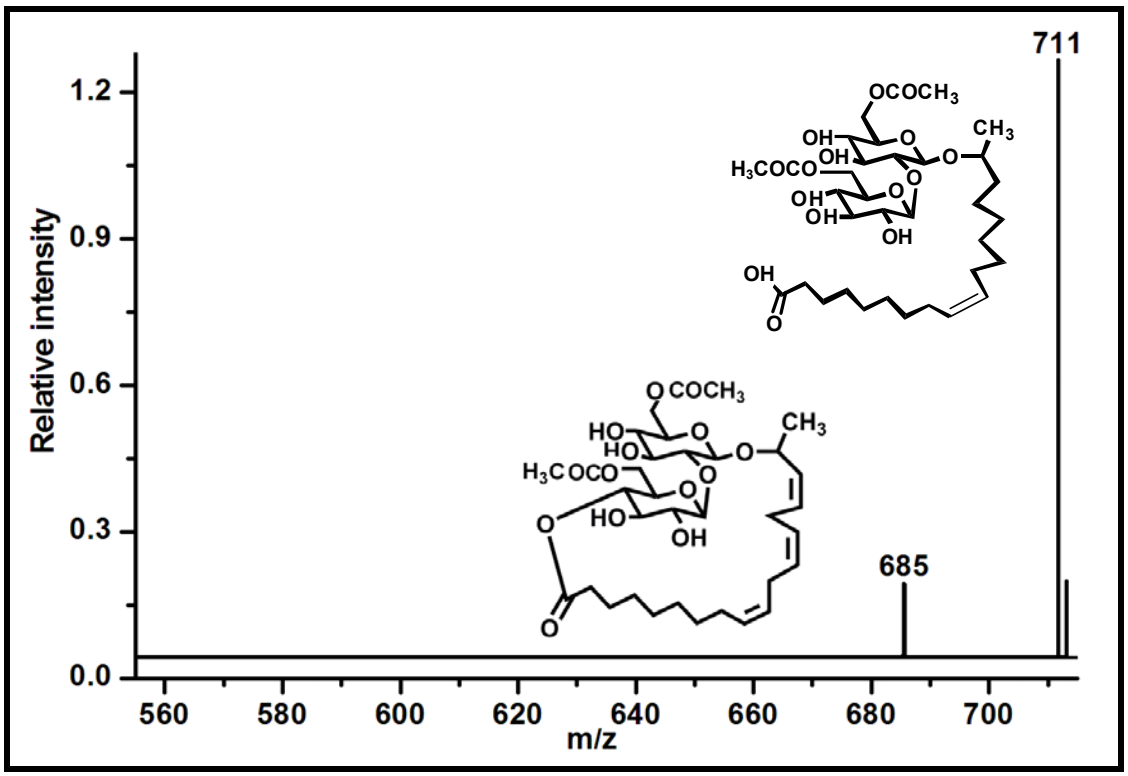


Fig. 4.8: Positive mode ESI spectra of third fraction from 27.6 min showing presence of diacetylated lactone of C18:3 SL molecules (Fig. 4.2, compound 7) and diacetylated lactone of C18:1 SL molecules

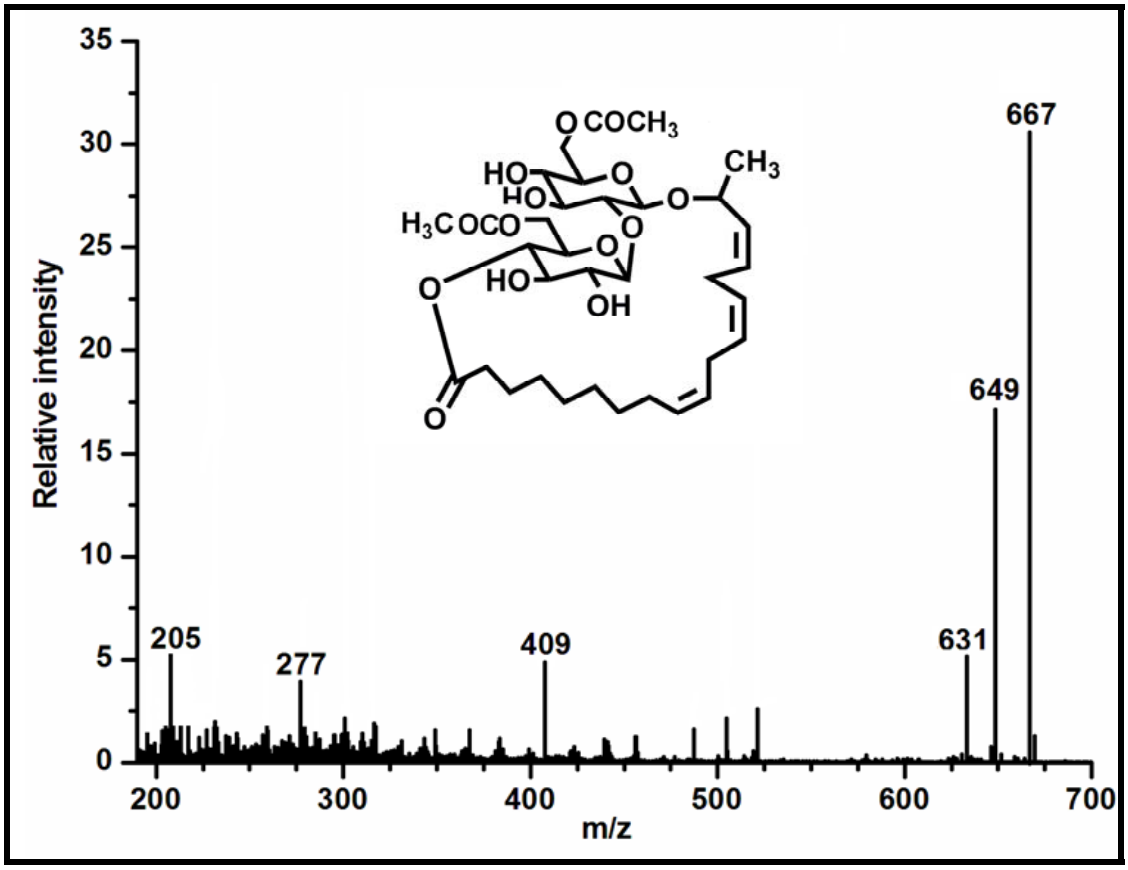


Fig. 4.9: CID spectrum of the protonated molecular ion, m/z 685

4.4.2 Mass spectrometric (Electro-spray ionization) analysis of SL methyl ester

Chemical esterification of LNNSL as described in Chapter 3 showed the yield of 80 % (w/w) LNNSLME. This esterified product was characterized by Electro-spray ionization mass spectrometry. The positive mode ESI mass spectrum of sophorolipid methyl ester is shown in Fig. 4.10. Two peaks were observed at m/z 633 $[M+H]^+$ and m/z 655 $[M+Na]^+$ respectively. These two peaks gave the evidence for the presence of SL methyl ester (m/z 632). The fatty acid chain of this compound contains C18:3 moieties.

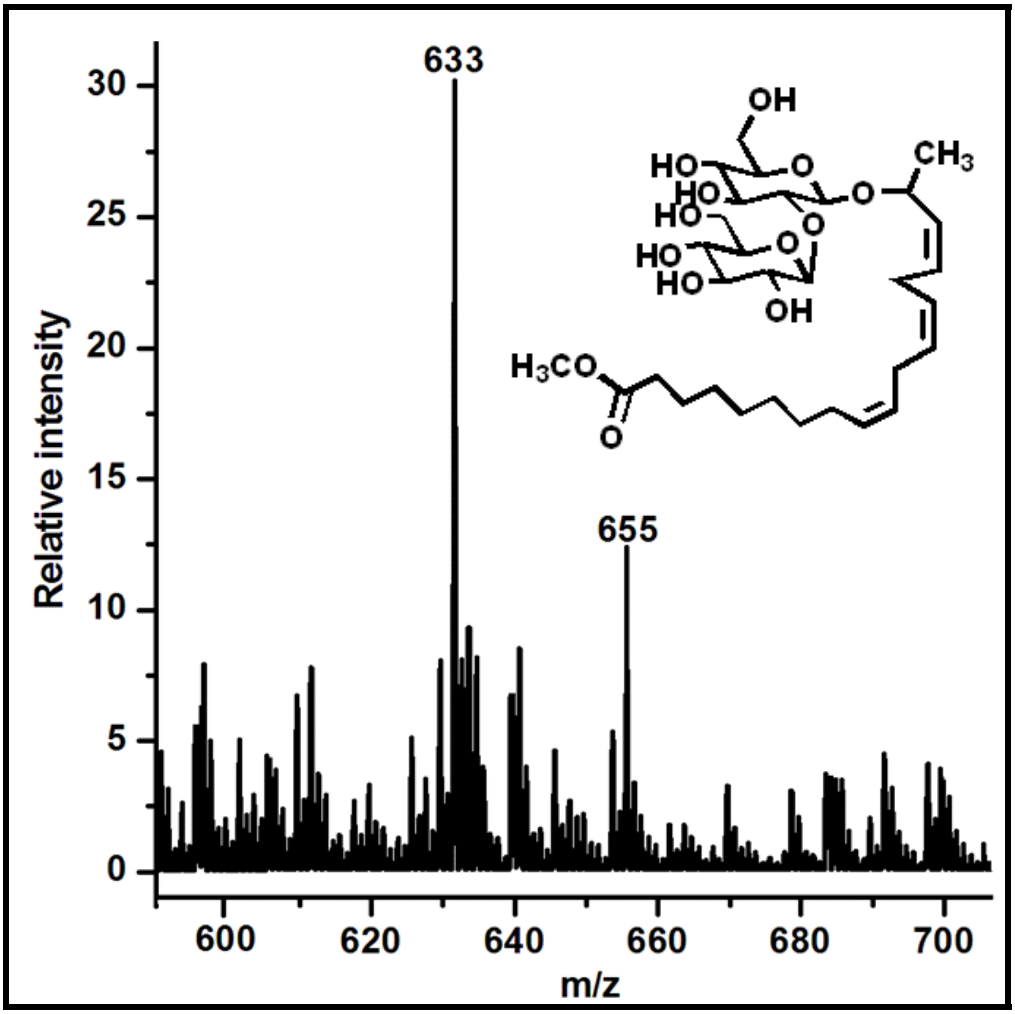


Fig. 4.10: Positive mode ESI spectra of SL methyl ester having C18:3 moieties in its fatty acid chain

4.4.3 NMR analysis of LNNSLME

^{1}H NMR spectra (Fig. 4.11) showed the characteristic ester peak at 3.65 ppm (3H, s, OC**H**$_3$). The protons of glucose- H-1' and glucose- H 1'' were identified at 5.03 (d, 1H) and 5.27(d, 1H) ppm respectively. The signals of protons from -CH=CH- groups were found at 5.37-5.69 (6H, m) ppm.

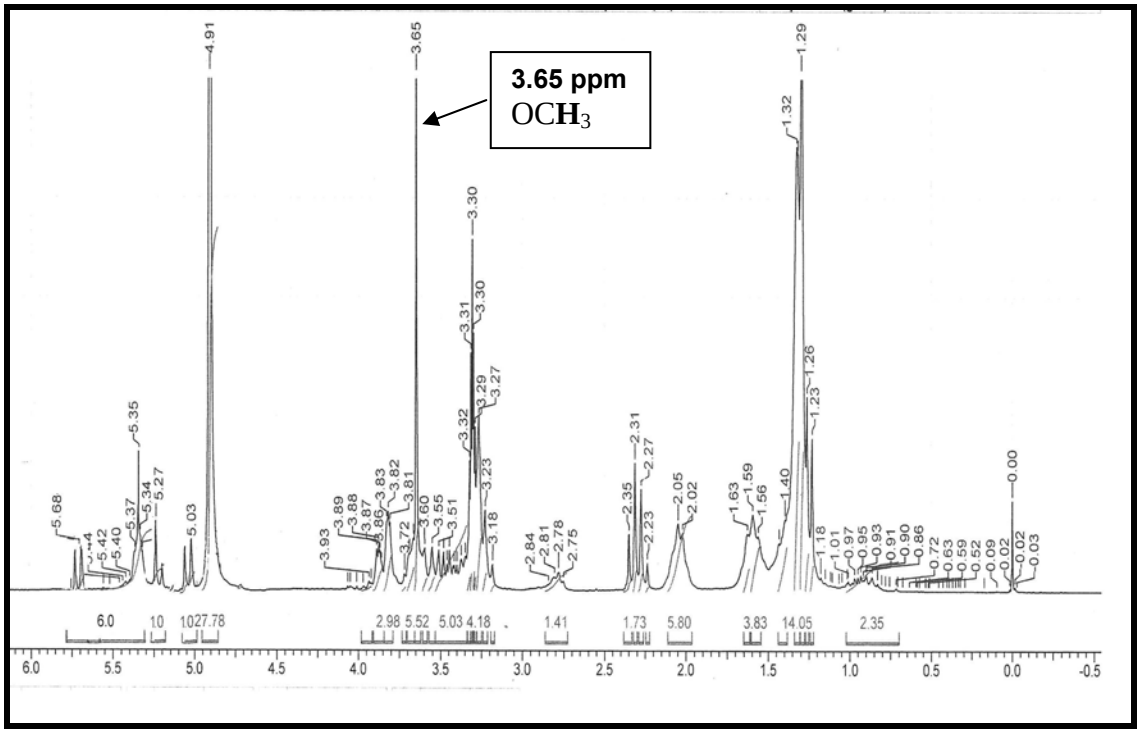


Fig. 4.11: ^{1}H NMR spectra of LNNSLME showing characteristic ester peak at 3.65 ppm

For further confirmation of molecular structure of LNNSLME ^{13}C NMR spectra measurement was performed, which showed that the resonance signals of CH_2 - groups present in fatty acid moiety were found at 26.07-37.86 ppm, carbons from --CH=CH-- group in fatty acid chain moiety at 129.07-130.97 ppm, glucose- C 1' and glucose- C-1" at 102.7 and 104.9 ppm and glucose-C-6' and glucose C- 6" at 62.75 and 63.11 ppm respectively. The signal at 176.09 ppm proves the presence of acid group in LNNSLME. The characteristic peak for ester carbon was found at 52.05 ppm.

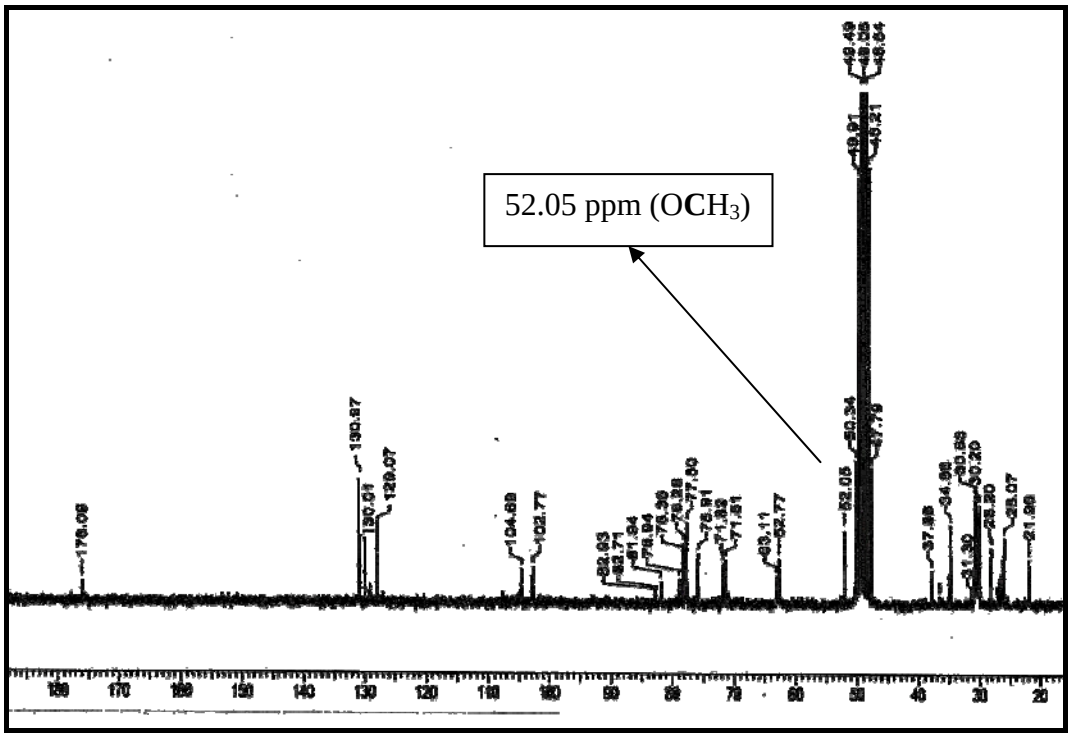


Fig. 4.12: ^{13}C NMR spectra of LNNSLME showing characteristic ester peak at 52.05 ppm

In the present work, ESI and CID mass spectral analysis confirmed that the *C. bombicola* when grown on glucose and ALA produces a mixture of glycolipids consisting of free acid, lactone and diacetylated lactone forms of C18:3 SLs as well as diacetylated lactone form of C18:1 SL. The presence of different lipophilic carbon sources (secondary carbon sources) influences the composition of SLs or in other words the fatty acids incorporated into the SL structure reflects the secondary carbon source on which the microorganism is grown (Tulloch et al., 1962; Nunez et al., 2001). Presence of the C18:3 SLs in the mixture indicates the direct incorporation of this fatty acid into the SL structure. *Candida* (*Torulopsis*) *bombicola* produces C18:1 SLs during growth on glucose and nitrogen source (Gorin et al., 1961). So, it may be justified that the C18:1 SL is produced from the yeast itself by utilizing glucose and

nitrogen sources from the medium. The approximate composition of the fermentation product (SL mixture) was 7.5 % free acid, 80 % lactone and 4.5 % diacetylated lactone of C18:3 molecules and 8 % of diacetylated lactone of C18:1 SL molecules. This composition was determined from the initial crude SL loaded on the column. Further, for the ease of purification, we followed the chemical esterification reaction from the literature in order to get a single homogenous product (Bisht et al., 1999). We observed the conversion of SL mixture into the SL methyl ester and ESI-MS analysis confirmed the presence of C18:3 moieties in the fatty acid chain of this product and ^{1}H and ^{13}C NMR study of LNNSLME also confirmed the formation of esterified product.

4.4.4 Surface tension and critical micelle concentration determination

The criteria for evaluating biosurfactants activity are surface tension and critical micelle concentration (CMC). The surface tension correlates with the concentration of the surface-active compound until the critical micelle concentration (CMC) is reached. The CMC is defined as the minimum surfactant concentration required for reaching the lowest surface tension values. Efficient surfactants have a low critical micelle concentration (i.e. less surfactant is necessary to decrease the surface tension or less surfactant is required to saturate interfaces between air/liquid or liquid/liquid interface). At concentrations above the CMC, amphiphilic molecules associate readily to form supra-molecular structures such as micelles, bilayers and vesicles. The surface tension and critical micelle concentration of linolenic acid derived SL (LNNSL) and its methyl ester form designated as LNNSLME were determined using Wilhelmy plate method. It was observed that with the increase in concentration of LNNSL, there is a decrease in the surface tension value and

as the conc. reached to 140 mg l^{-1}, the minimum surface tension (32.384, mN m^{-1}) was achieved and beyond this point the constant value of surface tension was obtained (Table 4.1, Fig. 4.13). The inflexion point between the minimum surface tension value (32.384, mN m^{-1}) and slope of surface tension is reported as the critical micelle concentration which is 140 mg l^{-1} (Fig. 4.13)

As shown in Table 4.2, the same phenomena of decrease in surface tension values were observed with the increase in the concentration of LNNSLME and a point is reached where the minimum value of surface tension (34.37, mN m^{-1}) was obtained after which the constant values of surface tension was observed and the inflexion point between the minimum surface tension and value (34.37, mN m^{-1}) and slope of surface tension was reported as the critical micelle concentration which is 100 mg l^{-1} (Fig. 4.14).

Table 4.1

Influence of the LNNSL on the surface tensions (against air) of water at 25 °C

[1]Concentration of LNNSL (mg l^{-1})	[2]Surface tension (mN m^{-1})
0	72.00
1	52.692
3	47.082
5	45.984
7	43.636
9	43.392
10	41.837
20	41.745
40	39.794
70	39.184
80	35.769
90	34.153
110	32.75
CMC 140	32.384
200	33.421

[1]The concentrations of LNNSL used between 140 to 200 mg l^{-1} and the corresponding [2]surface tension values are omitted from the table but are shown graphically.

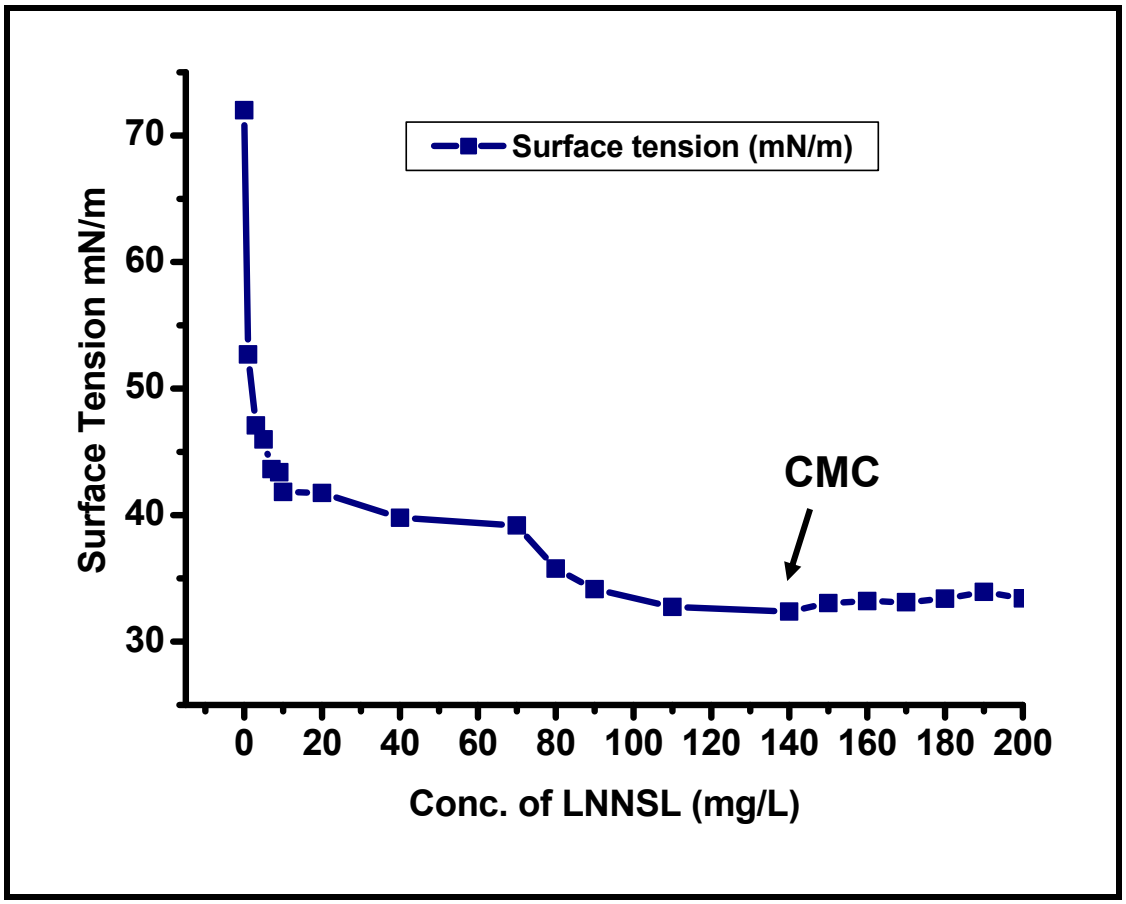


Fig. 4.13: Graphical representation of surface tension versus concentration of LNNSL

Table 4.2

Influence of the sophorolipid methyl ester of linolenic acid (LNNSLME) on the surface (against air) tension of water at 25 °C

[1]Concentration of LNNSLME (mg l^{-1})	[2]Surface tension (mN m^{-1})
0	72.00
1	61.42
3	56.57
5	54.81
9	50.37
10	50.32
30	41.98
50	37.86
70	36.37
90	36.64
CMC 100	34.37
200	35.05

[1]The concentrations of LNNSLME used between 100 to 200 mg l^{-1} and the corresponding [2]surface tension values are omitted from the table but are shown graphically.

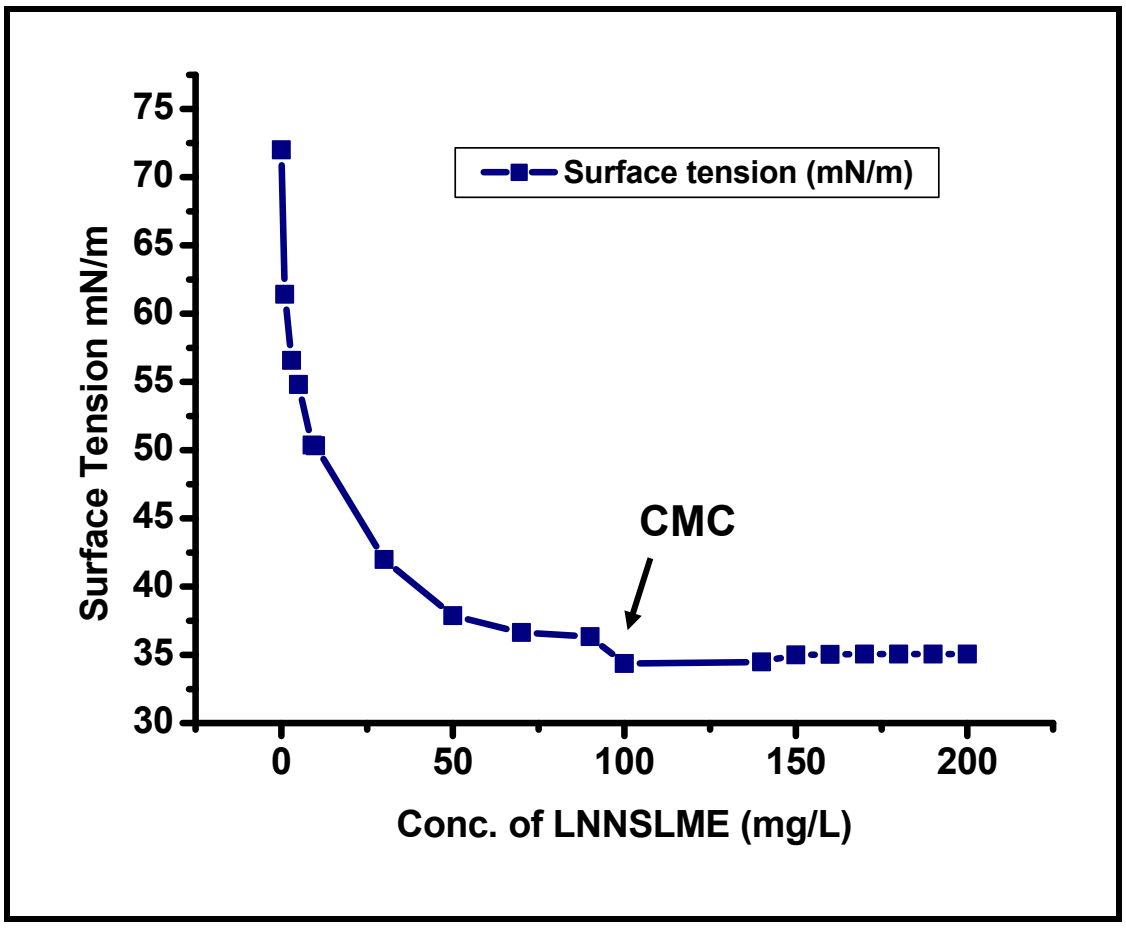


Fig. 4.14: Graphical representation of surface tension versus concentration of sophorolipid methyl ester (LNNSLME)

The surface tension and critical micelle concentrations of LNNSL and LNNSLME were compared to the other sophorolipids (Table 4.3) and it was found that these values were comparable to the SLs derived using oleic acid and surfactin (Zhang et al., 2004 and Arima et al., 1968). Oleic acid derived SLs and surfactin are used on commercial level. In this way SLs produced using linolenic acid can be used commercially.

Table 4.3
Surface- active properties of some biosurfactants

Biosurfactants	Microorganim	C - source	Surface tension (mN m^{-1})	CMC (mg l^{-1})	References
GLYCOLIPIDS					
Sophorolipids	*Torulopsis bombicola*	Glucose, oleic acid	33	na	Cooper and Paddock, 1984
	C. bombicola	Glucose, soybean dark oil	48	150	Kim et al., 1997
	C. bombicola	Deproteinized whey, rapeseed oil	39	130	Otto et al., 1999
Rhamnolipid	*P. aeruginosa*	Waste free fatty acids from soybean oil refinery	26	120	Abalos et al., 2001
LIPOPEPTIDES					
Surfactin	*Bacillus subtilis*	Glucose	27-32	23-160	Arima et al., 1968
Viscosin	*Pseudomonas fluorescens*	Glycerol	26.5	150	Neu et al.,1996
FATTY ACIDS					
Fatty acids	*Corynebacterium lepus*	Kerosene/alkanes	< 30	150	Cooper et al., 1978

4.5 Conclusions

The largest portion (about 80 %) of the LNNSL mixture was observed in the lactone form of C18:3 (linolenic acid) SL molecules. This is in agreement with the studies done by Tulloch et al. (1967), Asmer et al. (1988) who showed in their work using different lipophilic substrates that the lactones represent the largest fraction of the SL mixture. Weber et al. (1992) reported that yeasts belonging to *Candida* species are capable of directly incorporating fatty acids into the SL product and also Linton (1991) showed that direct incorporation of fatty acids supplied as a carbon source is more efficient over de novo synthesis of fatty acids. The present work showed the direct incorporation of C18:3 fatty acid moieties into the SL structure which is in agreement to the studies done by Linton (1991) and Weber et al. (1992) using other fatty acids. Chemical esterification reaction reported for the conversion of SL mixture into single homogenous product by Bisht et al. (1999) also proved efficient in conversion of LNNSL mixture into esterified product of linolenic acid SL (LNNSLME) and also good yield (80 %) of SL methyl ester (LNNSLME) was obtained. The ESI-MS analysis confirmed the presence of linolenic acid methyl ester in the fatty acid portion of SL molecules.

SLs when solved in water lower the surface tension from 72 mN m^{-1} to 40 to 30 mN m^{-1} (Van Bogaert et al., 2007) with a critical micelle concentration (CMC) of 11 to 250 mg l^{-1} (Develter and Lauryssen, 2010). Results showed that LNNSL and LNNSLME reduced the surface tension (ST) of water from72 mN m^{-1} to 32 and 34 mN m^{-1} respectively. The CMC values of LNNSL and LNNSLME required is 140 and 100 mg l^{-1} respectively. Thus the obtained values of surface tension and critical micelle concentration are comparable to

the reported values for different types of SLs discussed above in the Table 4.3 of results and discussion section. Thus, the linolenic acid derived SL as well as its methyl ester form both has the potential for industrial applications (foaming for food processing, emulsifying and phase dispersion activity for cosmetics, solubilization of agrochemicals, emulsification processes etc.) and can replace chemically synthesized surfactants because of their biodegradability and environment friendly nature.

Chapter 5

Antibacterial properties of Linolenic acid derived Sophorolipid and its methyl ester form

5.1 Summary

The objective of this study is to introduce new antimicrobial agents with high efficiency. This chapter is divided into two parts 5A and 5B. First part deals with the antibacterial activity of LNNSL and LNNSLME including the determination of minimum inhibitory concentrations of both the compounds against Gram negative and Gram positive bacteria and second part deals with the investigation of antibacterial action of these two compounds on Gram negative and Gram positive bacteria with the help of Atomic force microscopy.

5A Antibacterial activity

5B Insight into the antibacterial action using Atomic force microscopy

5A Antibacterial activity

5A.1 Summary

Sophorolipids (SLs), due to their surface-active properties, biocompatibility and various biological applications, have become a vital topic of research. SLs have been used extensively, especially for antimicrobial activity as most of the pathogenic microbes are gaining resistance to present day antibiotics. In this chapter, the antibacterial activity of α-linolenic acid derived sophorolipid, LNNSL containing 80 % lactone and its methyl ester (LNNSLME) against both Gram negative (*E. coli* and *P. aeruginosa*) and Gram positive (*B. subtilis*) bacteria is presented.

5A.2 Introduction

Sophorolipids (SLs) possess unique biological properties. Their antibacterial, antifungal, antiviral and anti-cancerous activities make them relevant molecules for applications in combating many diseases and as therapeutic agents (Van Bogaert et al., 2007). There are many reports on the use of SLs as antimicrobial agents in the literature. Shah et al. (2005) first reported the spermicidal, anti-HIV and cytotoxic activities of SL mixture and its chemical derivatives which are similar to commercially available nonoxynol-9 (nonionic spermicide). Further, Bluth et al. (2006) showed that SL mixture is effective septic shock antagonists. There are a number of patents on antibacterial, antiviral and antifungal activities of SL mixture and its alkyl ester forms (Gross and Shah, 2003, 2004, 2007 and 2009 and Gross et al., 2004). SLs have also been demonstrated to be effective anticancer agents against cancerous cell lines (Chen et al., 2006a, 2006b and Joshi-Navare et al.,

2011). Biosurfactant's use as an antimicrobial agent is still limited on commercial scale due to low cost of production of chemically synthesized antibiotics (Bonomo, 2000; Shah et al., 2005; Azim et al., 2006; Napolitano, 2006; Shah et al., 2007 and Singh et al., 2009). However, the occurrence of antibiotic-resistant pathogens increased the attention on other types of antimicrobial agents (Bonomo, 2000 and Rodrigues et al., 2006). SL products are FDA (Food and drug administration) approved and are available on commercial level, for example, the French company Soliance produces SL based cosmetics for body and skin (Van Bogaert et al., 2007 and Kaelynn, 2009). SLs act as emulsifier as well as a bactericidal agent in the treatment of acne, dandruff, and body odours (Magar et al., 1987). SLs are also known for their role as anti-adhesive agents against several pathogens which indicate their utility as suitable anti-adhesive coating agents for medical insertional materials leading to a reduction in a large number of hospital infections without the use of synthetic drugs and chemicals (Rodrigues et al., 2006). In this part of the chapter, antibacterial activity of LNNSL and LNNSLME against Gram positive and Gram negative bacteria is presented.

5A.3 Materials and methods

5A.3.1 Bacterial cultures and growth conditions

Bacterial cultures used were obtained from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India. *Bacillus subtilis* (NCIM 2063) was used as a representative of Gram positive bacteria and *Escherichia coli* (NCIM 2931) and *Pseudomonas aeruginosa* (NCIM 5029) were used as representatives of Gram negative bacteria. Luria

bertini (LB) medium was used for the growth of both Gram positive and Gram negative bacteria. The composition of LB medium used was in g l^{-1}: tryptone, 10.0; yeast extract, 5.0; NaCl, 10.0; agar, 20.0; pH, 7.0. *E. coli* was grown at 37 °C while *B. subtilis* and *P. aeruginosa* were grown at 30 °C for overnight. After overnight growth, at their respective temperatures, bacterial cultures were centrifuged at 5000 rpm for 4 min and the wet pellets were re-suspended in normal saline and were re-centrifuged at 5000 rpm for 4 min to remove the growth medium. The final pellets were again re-suspended in saline.

5A.3.2 Bacteria

Escherichia coli is a Gram negative, rod shaped bacterium about 2.0 µm long and 0.5 µm in diameter with peritrichous flagella and are found in the lower intestine of warm blooded animals (Eckburg et al., 2005). Most of the *E. coli* strains are harmless but some strains may cause serious food poisoning in humans (Vogt and Dippold, 2005).

Pseudomonas aeruginosa is a Gram negative and rod shaped opportunistic pathogen. As a nosocomial pathogen it causes the highest mortality rate of any other bacterium. Bacteremia, pneumonia, osteomyelitis, and endocarditis due to *P. aeruginosa* have therapeutic failure and mortality rates are notably higher than those for other bacterial pathogens.

Bacillus subtilis is a Gram positive bacterium. They are commonly found in soil. They are non pathogenic to humans but sometimes may cause food contamination. Its spores can survive the extreme heating used to cook

food, and it is responsible for causing ropiness, caused by the bacterial production of long chain polysaccharides.

5A.3.3 Sample preparation

Antibacterial tests of LNNSL, containing 80 % lactone and LNNSLME were performed using standard dilution method and spread plate method. Bacterial suspensions at a concentration of 10^6 CFU/ml were taken in a test tube and LNNSL and LNNSLME to the final concentrations of 5, 10 and 20 µg ml^{-1} diluted in sterile Millipore water were added separately. Test tubes containing only respective cells without SL served as controls. All the test tubes were incubated for 6 h at their respective temperatures. 100 µl aliquots were taken out from the respective suspensions at every 2 h intervals and plated on LB agar plates followed by incubation at their respective temperatures (37 °C for *E. coli* and 30 °C for *B. subtilis* and *P. aeruginosa*). Colonies were visualized after 24 h and digital images of the plates were captured. Colonies were counted and percentage cell survival was calculated using the following formula:

$$Percentage\ (\%)\ cell\ survival = \frac{Number\ of\ colonies\ on\ test\ plate}{Number\ of\ colonies\ on\ control\ plate} \times 100$$

5A.3.4 Minimun inhibitory concentration

Minimum inhibitory concentration (MIC) is considered as that concentration of compound which inhibits more than 90 % of growth of bacterial colony. The concentration of sophorolipid at which no bacterial colony was observed on the plate was considered as minimum inhibitory concentration (Rai et al., 2010).

5A.4 Results and discussion

The effectiveness of sophorolipids was analysed by plotting percentage cell survival versus incubation time. It was observed that bacterial colonies of both the Gram negative and Gram positive bacteria showed the decrease in count with increasing amount of sophorolipids as well as increasing incubation time.

In the case of *E. coli* (Fig. 5.1), the cell survival dropped to 0.3 % within 6 h of exposure for lowest concentration (5 µg ml^{-1}) of LNNSL, while for the LNNSLME the highest concentration (20 µg ml^{-1}) is required for the same cell survival (0.3 %) in 6 h of exposure (Table 5.1, Fig. 5.1 and 5.2). The MIC values for LNNSL and LNNSLME were found to be 10 and 20 µg ml^{-1} respectively and incubation time of 6 h.

Table 5.1

Percentage cell survival of *E. coli* after treatment with LNNSL and its methyl ester form (LNNSLME)

Incubation time	Cell survival at different concentrations of LNNSL			Cell survival at different concentrations of LNNSLME		
	5 µg ml^{-1}	**10 µg ml^{-1}**	**20 µg ml^{-1}**	**5 µg ml^{-1}**	**10 µg ml^{-1}**	**20 µg ml^{-1}**
2 h	36.4 %	11.9 %	3.6 %	66.2 %	25.8 %	4.96 %
	(110 CFU)	(36 CFU)	(11CFU)	(200 CFU)	(78 CFU)	(15 CFU)
4 h	3.9 %	2.3 %	1.32 %	49.6 %	22.5 %	2.3 %
	(12 CFU)	(7 CFU)	(4 CFU)	(150 CFU)	(68CFU)	(7 CFU)
6 h	0.33 %	0	0	33.1 %	11.9 %	0.33 %
	(1 CFU)	(0 CFU)	(0 CFU)	(100 CFU)	(36CFU)	(1 CFU)

Control colonies: 302 CFU

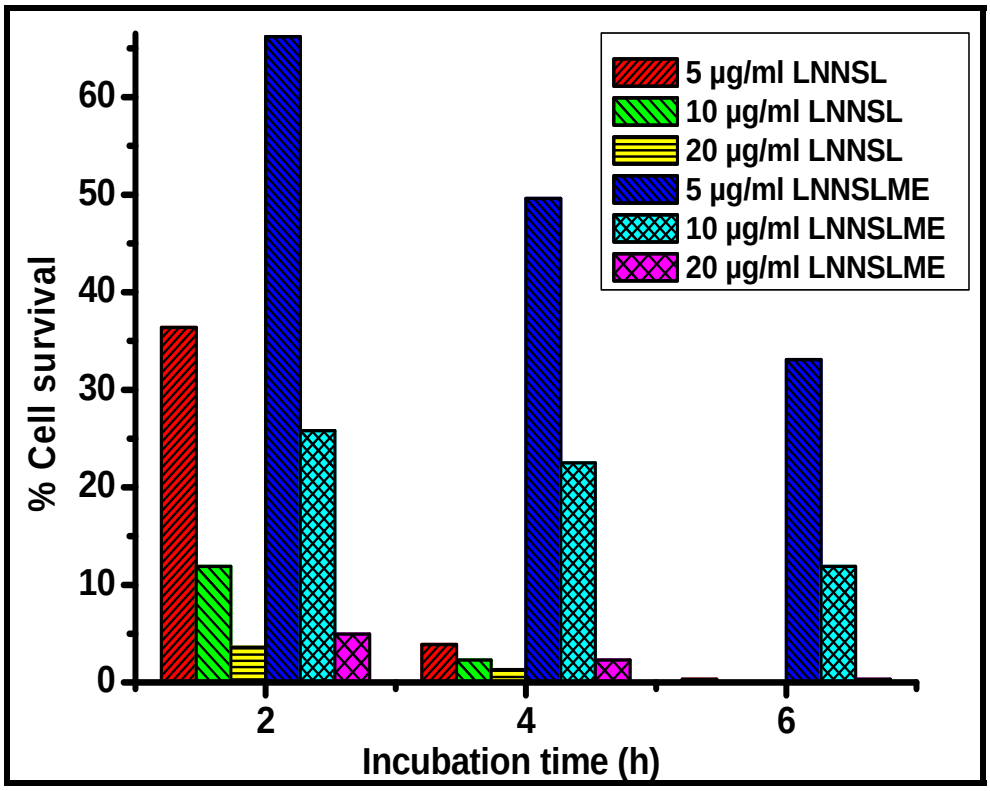


Fig. 5.1: Graphical representation of antibacterial activity of LNNSL and LNNSLME form against *E. coli* after different times of incubation at 37 °C

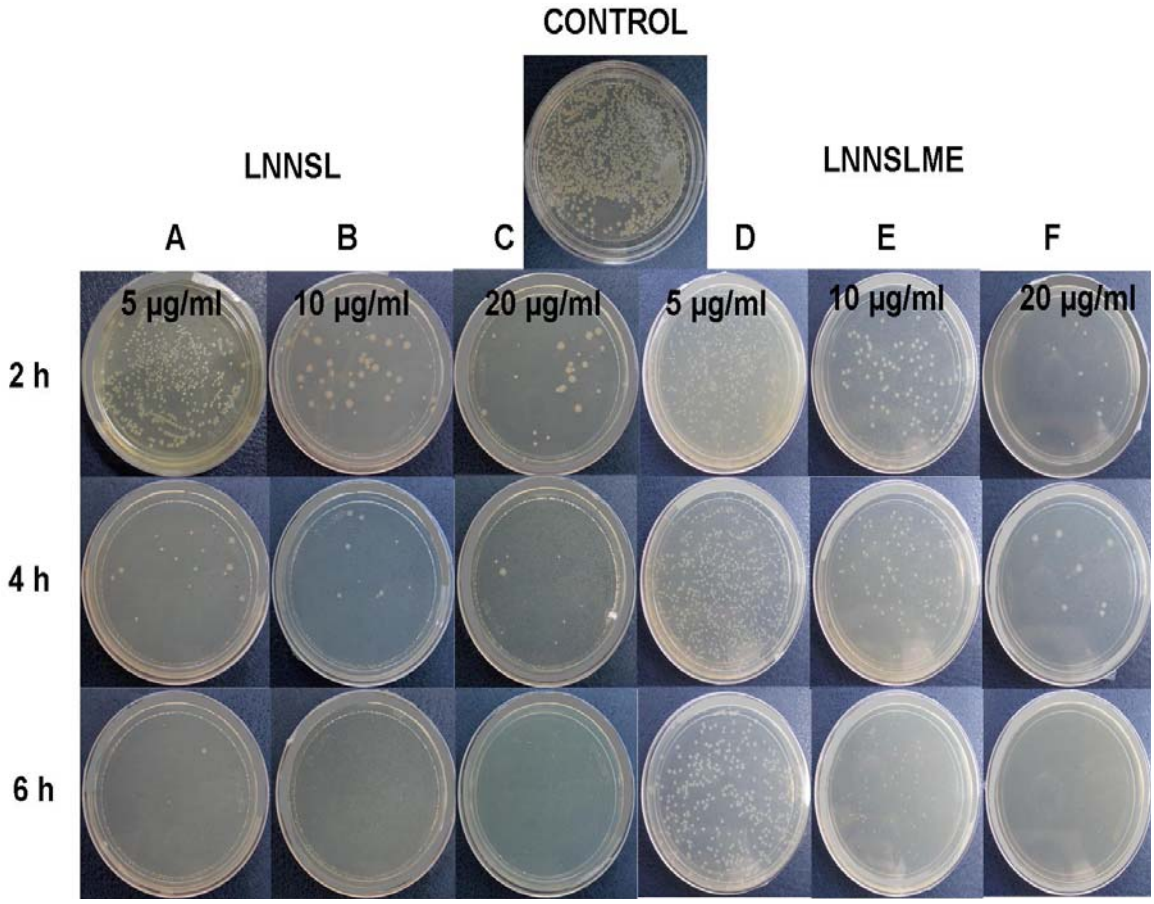


Fig. 5.2: Digital images of the grown bacterial plates (recorded after 24 h of incubation) of *E. coli* treated with LNNSL and its methyl ester (LNNSLME) showing time and concentration dependent decrease in number of colonies

For *P. aeruginosa* (Fig. 5.3) the cell survival dropped to 0.3 % within 6 h of exposure for lowest conc. (5 µg ml^{-1}) of LNNSL, while for its methyl ester (LNNSLME) the cell survival dropped to 3.9 % for the higher concentration (10 µg ml^{-1}) in 6 h of exposure (Table 5.2, Fig. 5.3 and 5.4). The MIC values for LNNSL and LNNSLME were found to be 10 and 20 µg ml^{-1} respectively and incubation time of 6 h.

Table 5.2

Percentage cell survival of *P. aeruginosa* after treatment with LNNSL and its methyl ester form (LNNSLME)

Incubation time	Cell survival at different concentrations of LNNSL			Cell survival at different concentrations of LNNSLME		
	5 µg ml^{-1}	10 µg ml^{-1}	20 µg ml^{-1}	5 µg ml^{-1}	10 µg ml^{-1}	20 µg ml^{-1}
2 h	62.9 %	26.4 %	9.9 %	79.4 %	69.5 %	62.9 %
	(190 CFU)	(80 CFU)	(30 CFU)	(240 CFU)	(210 CFU)	(190 CFU)
4 h	27.8 %	8.6 %	0.66 %	71.2 %	59.6 %	26.4 %
	(84 CFU)	(26CFU)	(2 CFU)	(215 CFU)	(180 CFU)	(80 CFU)
6 h	0.33 %	0	0	14.9 %	3.97 %	0
	(1 CFU)	(0 CFU)	(0 CFU)	(45 CFU)	(12 CFU)	(0 CFU)

Control colonies: 302 CFU

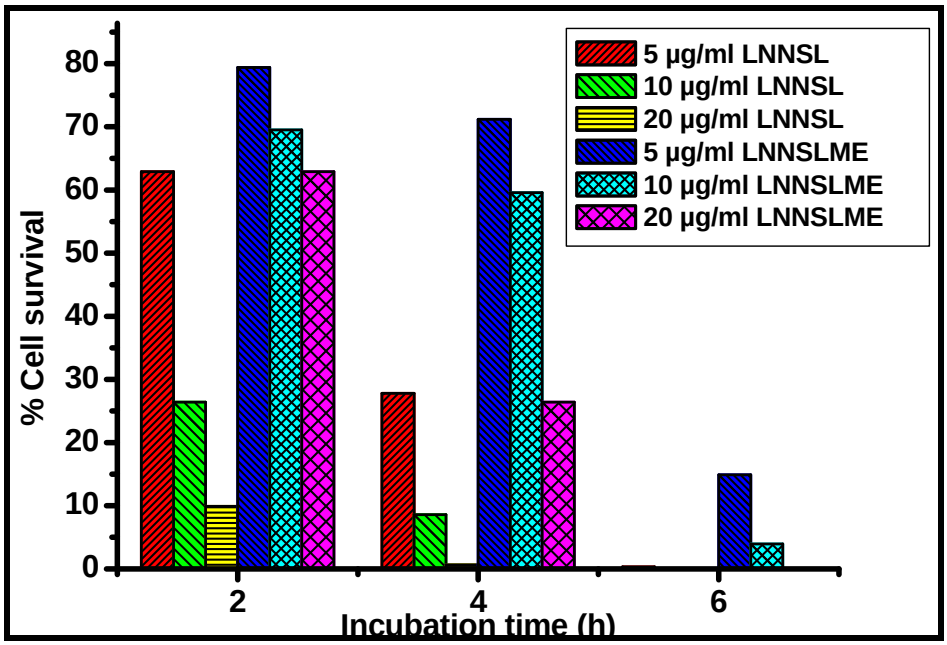


Fig. 5.3: Graphical representation of antibacterial activity of LNNSL and LNNSLME form against *P. aeruginosa* at different times of incubation at 30 °C

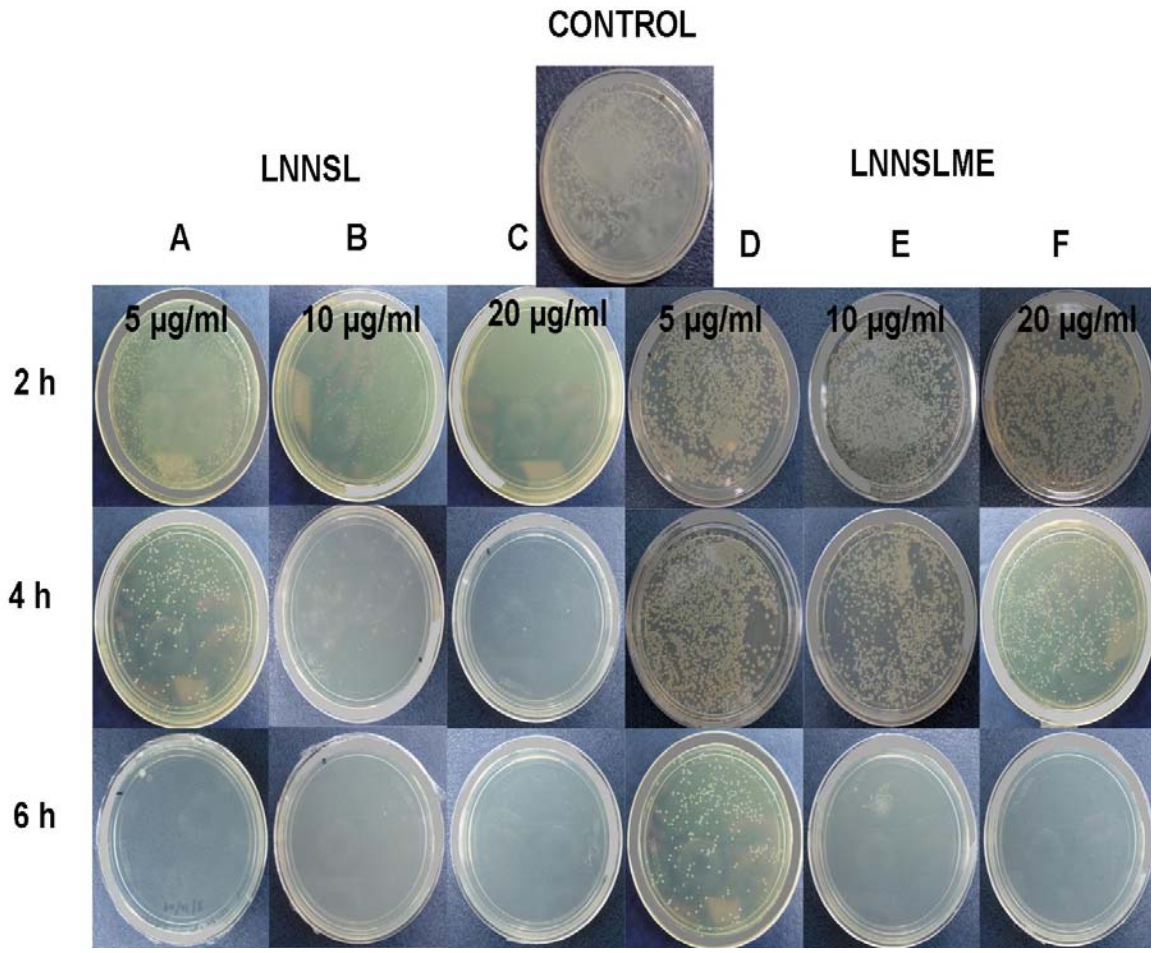


Fig. 5.4: Digital images of the grown bacterial plates (recorded after 24 h of incubation) of *P. aeruginosa* treated with LNNSL and its methyl ester (LNNSLME) showing time and concentration dependent decrease in number of colonies

Figure 5.5 showed that the percentage cell survival for *B. subtilis* dropped to 0.3 % within 6 h of exposure of LNNSL (20 µg ml^{-1}) while for LNNSLME (20 µg ml^{-1}) percentage survival dropped to 3.4 % within 6 h of incubation. It indicates that LNNSL (80 % lactone) is more effective as compared to its methyl ester form, LNNSLME (Table 5.3, Fig. 5.5 and 5.6). The MIC values for LNNSL and LNNSLME were found to be 20 and >20 µg ml^{-1} respectively and incubation time of 6 h.

Table 5.3

Percentage cell survival of *B. subtilis* after treatment with LNNSL and its methyl ester form (LNNSLME)

Incubation time	Cell survival at different concentrations of LNNSL			Cell survival at different concentrations of LNNSLME		
	5 µg ml^{-1}	10 µg ml^{-1}	20 µg ml^{-1}	5 µg ml^{-1}	10 µg ml^{-1}	20 µg ml^{-1}
2 h	25.5 %	12.4 %	2.06 %	28.96 %	15.17 %	8.27 %
	(74 CFU)	(36 CFU)	(6 CFU)	(84 CFU)	(44 CFU)	(24 CFU)
4 h	11.03 %	4.13 %	1.37 %	15.5 %	8.27 %	4.82 %
	(32 CFU)	(12 CFU)	(4 CFU)	(45 CFU)	(24 CFU)	(14 CFU)
6 h	1.7 %	0.68 %	0.3 %	10.3 %	6.8 %	3.4 %
	(5 CFU)	(2 CFU)	(1 CFU)	(30 CFU)	(20 CFU)	(10 CFU)

Control colonies: 290 CFU

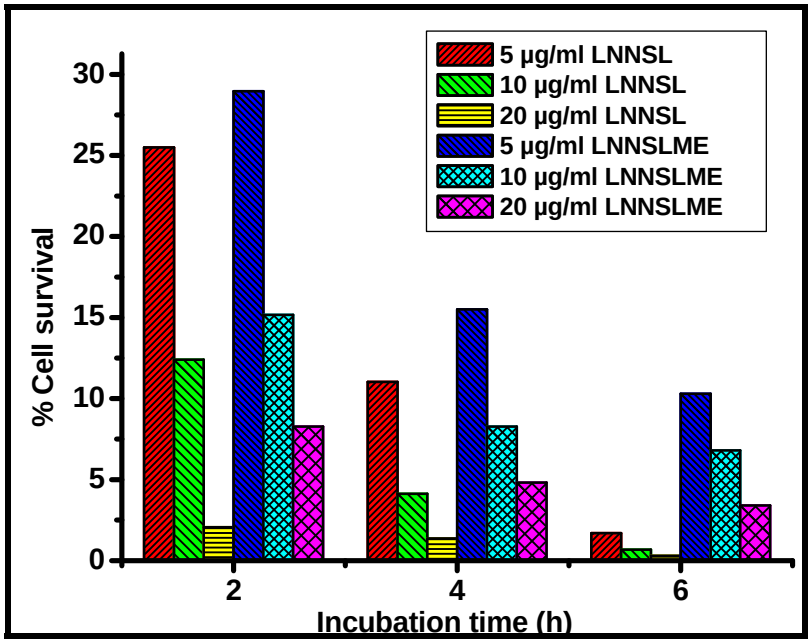


Fig. 5.5 Graphical representation of antibacterial activity of LNNSL and LNNSLME against *B. subtilis* after different times of incubation at 30 °C

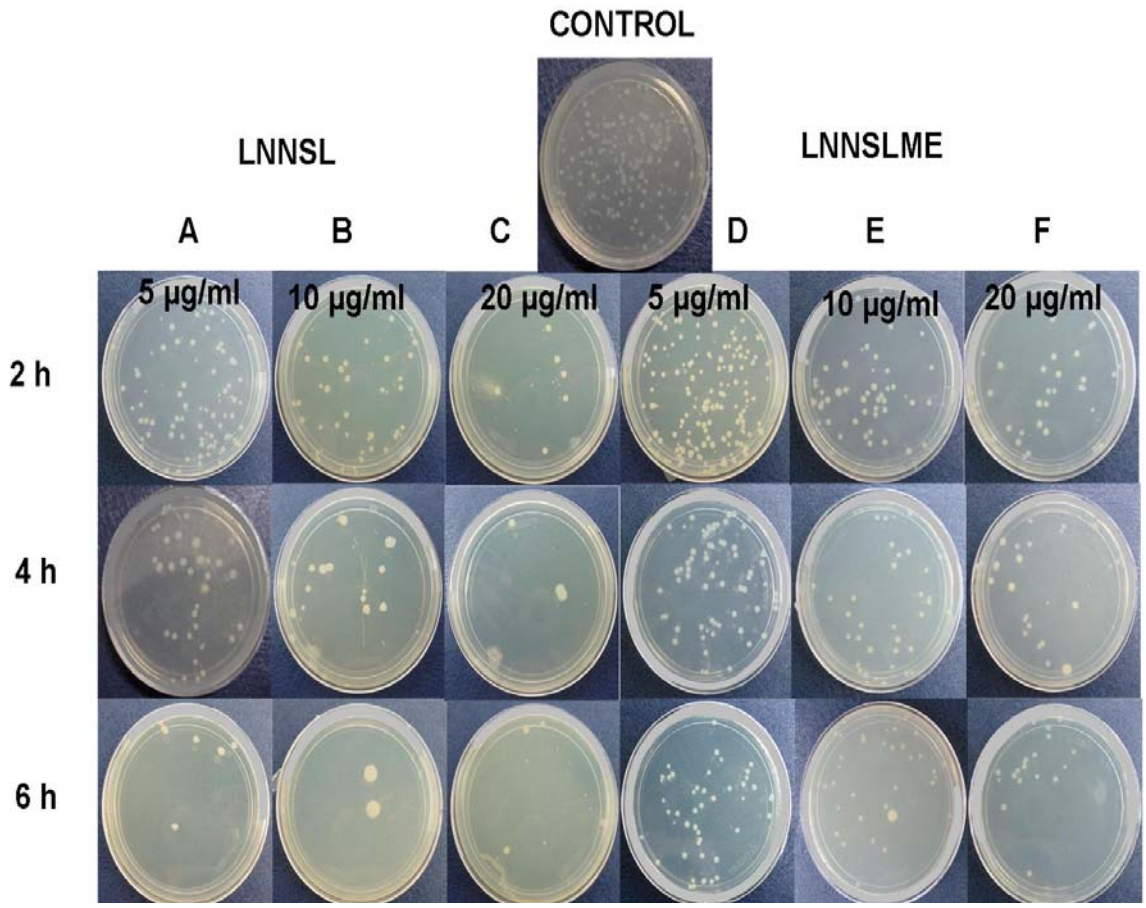


Fig. 5.6: Digital images of the grown bacterial plates (recorded after 24 h of incubation) of *B. subtilis* treated with LNNSL and LNNSLME showing time and concentration dependent decrease in number of colonies

It was clearly observed that in all the cases, LNNSL is more effective antibacterial agent as compared to its methyl ester form (LNNSLME). In chapter 3 and chapter 4, purification and mass spectrometry analysis of LNNSL had showed that it contains 80 % of lactone form of linolenic acid SL (Gupta and Prabhune, 2012). SL lactone forms are reported for their increased antimicrobial activity as compared to acidic forms (Cavalero and Cooper, 2003 and Van Bogaert et al., 2007). This study is in good agreement with the previous antibacterial studies of different lipophilic substrates derived

SL by various researchers (Cavalero and Cooper, 2003 and Van Bogaert et al., 2007). As methyl ester form had also showed its antibacterial activity against both Gram negative and Gram positive bacteria, it can also be used as antibacterial agent. The other observation made was that LNNSL and LNNSLME both inhibited Gram negative bacteria at lower concentrations as compared to Gram positive bacteria. So, these SLs of linolenic acid were found to be more potent against Gram negative bacteria as compared to other SLs reported in the literature which was more effective against Gram positive bacteria (Gross and Shah 2003). The more antibacterial effect towards Gram negative bacteria may be attributed to the less resistance offered by thin peptidoglycan layer adjacent to the inner cytoplasmic membrane as compared to the thick peptidoglycan layer of Gram positive bacteria which offer more resistance (Singh et al. 2009).

5A.5 Conclusions

The minimum inhibitory concentrations (MIC) of LNNSL, containing 80 % lactone against *B. subtilis*, *E. coli* and *P. aeruginosa* were found to be 20, 10 and 10 μg ml^{-1} respectively. The MIC values of methyl ester form (LNNSLME) against *B. subtilis*, *E. coli* and *P. aeruginosa* were determined to be >20, 20 and 20 μg ml^{-1} respectively. The results suggests that Linolenic SL mixture (containing 80% lactone) as compared to its methyl ester derivative showed good antibacterial activity towards both the Gram positive and Gram negative bacteria and was found to be more potent against Gram negative bacteria.

5B Insight into the antibacterial action using Atomic force microscopy

5B.1 Summary

This part of the chapter deals with the investigation of antibacterial action of LNNSL and LNNSLME with the help of atomic force microscopy (AFM). Nanometer scale images of both the control (without SL treatment) and treated bacterial cells showed the membranolytic effects of LNNSL and LNNSLME on the cell membrane of both the Gram negative and Gram positive bacteria. This is the first report on the investigation of antibacterial action of novel linolenic acid derived SL and its methyl ester (LNNSLME) using AFM study.

5B.2 Introduction

There are some reports on the antibacterial activity of sophorolipids as well as other glycolipids [Gross and Shah, 2003, 2004, 2007 and 2009 and Gross et al., 2004]. It is already reported that biosurfactants act on the integrity of cell membranes, which leads to cell lysis, (Cameotra and Makkar 2004). However, the ways in which the biosurfactants affect the membrane integrity differ. Rhamnolipids are thought to act on the lipid part of cell membranes or outer proteins causing structural fluctuations in the membrane (Rodrigues et al., 2006 and Sanchez et al., 2010). There are many evidences on the actions of antibiotics by means of the direct observation of morphological alterations by optical microscopy, scanning probe microscopy and atomic force microscopy (Klainer and Perkins, 1971; Nishino and Nakazawa, 1973; Nakao et al., 1981; Goi et al., 1985 and Carlo and Ricci, 1998). The great advantage of AFM compared to TEM or SEM is that it is

simple to operate in almost any environment such as aqueous solutions, but also other solvents, in air, vacuum, or other gases. It also provides the details of surface morphology with very high resolution without the need for complicated sample preparation (Binnig et al., 1986). AFM is an advanced non-destructive imaging technique which can be used to understand the nanoscale interactions between bacteria and different molecules. Since its introduction in 1986 (Binnig et al., 1986), AFM has been used extensively in the field of life sciences (Braga and Ricci, 1998 and Bonomo, 2000). AFM images can reveal the surface ultra structure of living microbial cells with unprecedented resolution (Braga and Ricci, 1998 and Dufrene et al., 1999).

AFM is a kind of scanning probe microscope and is based on the concept of near field microscopy which overcomes the problem of the limited diffraction related resolution inherent in conventional microscopes because in AFM the probe is located in the immediate vicinity of the sample itself (usually within a few nanometers), records the intensity and not the interference signal, and this greatly improves the final resolution. The principle of AFM is to scan the sample surface with the sharp probing tip (radius of few nanometers) attached to a cantilever. The three dimensional imaging is performed by measuring the displacement in x, y and z axis with the help of piezoelectric ceramic. When the probe tip is brought into the proximity of the sample surface, forces between the tip and the sample lead to a deflection of the cantilever according to the Hook's law. The forces between the probe tip and samples are detected through the deflection of the cantilever. The deflection of the cantilever is sensed using a laser beam reflected on the rear side of the cantilever and for this a position sensitive detector monitors the position of the

reflected beam. Forces that are measured in AFM include mechanical contact force, vander Waals forces, capillary forces, chemical bonding, electrostatic forces, magnetic forces and salvation forces etc. AFM is an advanced technique of scanning probe microscopes that can be used for observation of nonconductive surfaces and has thus opened the possibility for the surface analysis of various samples.

The aim of this study was to investigate this new tool (AFM) for analyzing surface and morphological alterations of Gram negative and Gram positive bacteria by examining the samples of bacteria exposed to linolenic acid derived sophorolipid (LNNSL) and its methyl ester form (LNNSLME). In the first part of this chapter (5A), the minimum inhibitory concentrations of LNNSL and LNNSLME were decided for both the Gram negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and Gram positive (*Bacillus subtilis*) bacteria. In this part, the effect of these concentrations (MICs) of LNNSL and LNNSLME on the morphology of both the Gram negative (*E. coli* and *P. aeruginosa*) and Gram positive (*B. subtilis*) bacteria were investigated with the help of AFM and also this is the first study on the visualization of morphological changes in bacteria treated with SLs using atomic force microscopy.

5B.3 Materials and methods

5B.3.1 AFM analysis

The atomic force microscopy measurements were performed using a multimode scanning probe microscope equipped with a Nanoscope IV controller (Veeco Instrument Inc., Santa Barbara, CA). Soft AFM cantilevers,

with resonant frequency of 30 kHz and a spring constant of 0.27 N/m, and a nominal tip radius of 15 nm were used to image both the controls and sophorolipid treated bacteria. The samples were imaged in air using the contact mode with settings of 512 pixels/line and 1 Hz scan rate. Larger and smaller scans were made in order to assess the exact position and nature of the bacterium, with further smaller scans being used to zoom in on any interesting features. In contact mode, topography is measured by sliding the probe's tip across the sample surface. Deflection and 3D constructions based on height images were acquired with the imaging software from Veeco. Schematic diagram of AFM is shown in Figure 5.7.

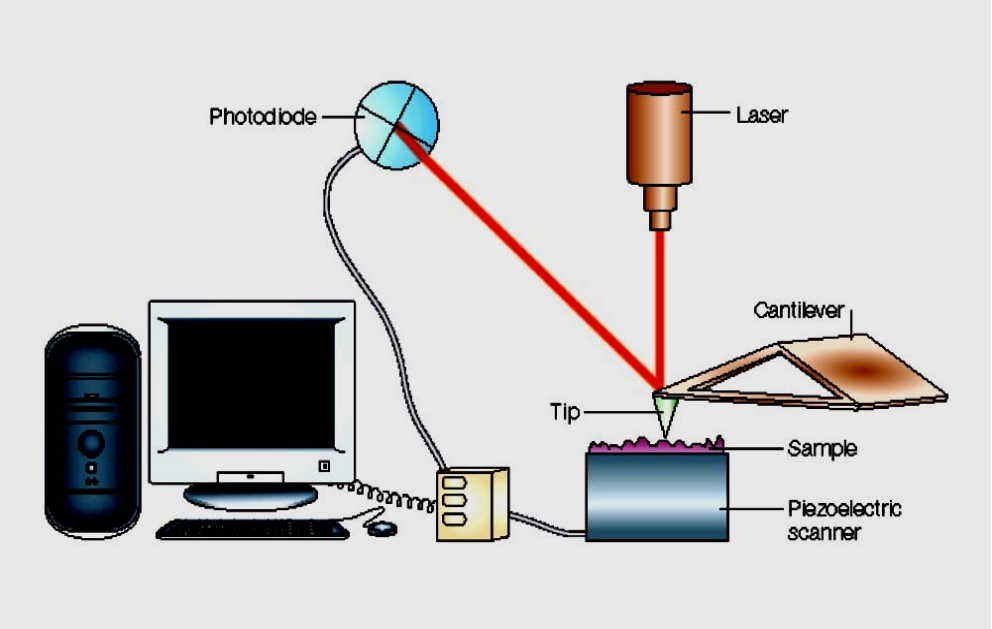


Fig. 5.7: Schematic representation of the components of an atomic force microscope (AFM) (*Image courtesy: http://images.google.com*)

5B.3.2 Sample preparation for AFM study

The bacterial concentration used was kept constant at 10^6 CFU/ml. The bacteria were treated with minimum inhibitory concentrations of LNNSL and LNNSLME (Table 5.4). Equal volumes of test compounds (LNNSL and LNNSLME) and bacteria (*E. coli*, *P. aeruginosa* and *B. subtilis*) were incubated in sterilized borosilicate tubes for 2 h. After incubation, 100 μl of treated and untreated bacterial suspensions were applied on a freshly cleaved mica surface and allowed to air dry for 5 min before imaging.

Table 5.4

Minimum inhibitory concentrations of LNNSL and LNNSLME against Gram negative (*E.coli* and *P. aeruginosa*) and Gram positive (*B. subtilis*) bacteria

Minimum inhibitory concentrations (MICs)		
	LNNSL (μg ml^{-1})	**LNNSLME (μg ml^{-1})**
E. coli	10	20
P. aeruginosa	10	20
B. subtilis	20	25 (>20)

5B.4 Results and discussion

5B.4.1 AFM images of untreated E. coli

AFM images of freshly prepared untreated (control) *E. coli* (5.9 × 5.9 µm scan size) is shown in Figure 5.8. Image of untreated *E. coli* bacteria showed characteristic rod shape with the distinctive peritrichous flagella. The length and width of untreated *E. coli* bacteria was determined to be 3.65 and 0.8 µm respectively.

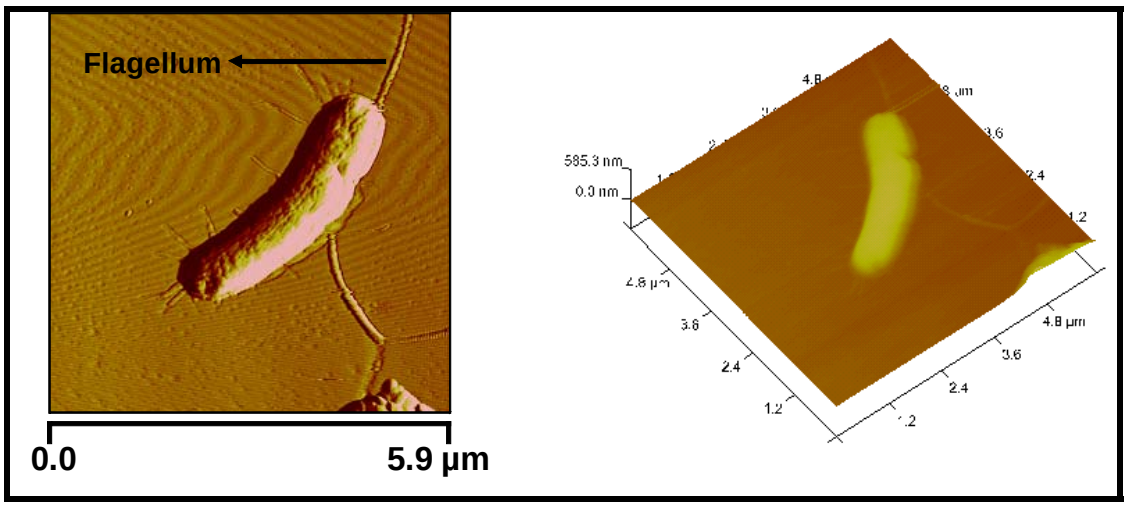


Fig. 5.8: Images (5.9 × 5.9 µm scan size) of untreated (control) *E. coli* (the left panels show deflection image and the right panel is 3D reconstruction based on height data) showing relatively smooth surface

5B.4.2 AFM images of E. coli bacteria treated with LNNSL

AFM images of *E. coli* bacteria treated with minimum inhibitory concentration (10 µg ml^{-1}) of LNNSL are shown in Figure 5.9 A, B and C. Figure 5.9 A is the large scan size (50 × 50 µm) image and captured in deflection and height mode. The images showed bacterial cells with copious amount of exuded fluid. Images were further zoomed in a particular area and smaller scan size images of (13.3 × 13.3 µm) and (4.0 × 4.0 µm) were taken in deflection and height modes (Fig. 5.9 A, 5.9 B and 5.9 C). Bacterial cells were severely damaged and the presence of copious amount of cytoplasmic fluid around the bacteria indicates the permeabilization of inner membrane due to the membranolytic action of LNNSL.

5 B.4.3 AFM images of E. coli bacteria treated with LNNSLME

AFM images of *E. coli* treated with minimum inhibitory concentration of 20 µg ml^{-1} of sophorolipid methyl ester, LNNSLME are shown in Figure 5.10 A and B. Both larger (26.9 × 26.9 µm) and smaller (8.3 ×8.3 µm) scan size images in deflection and height modes were taken. Figure 5.10 A and B showed damaged or lysed bacterial cells with the exuded cytoplasmic fluid from both the ends of the bacterial cell. It means that SL methyl ester exerted the similar mode of action of LNNSL on bacterial cells.

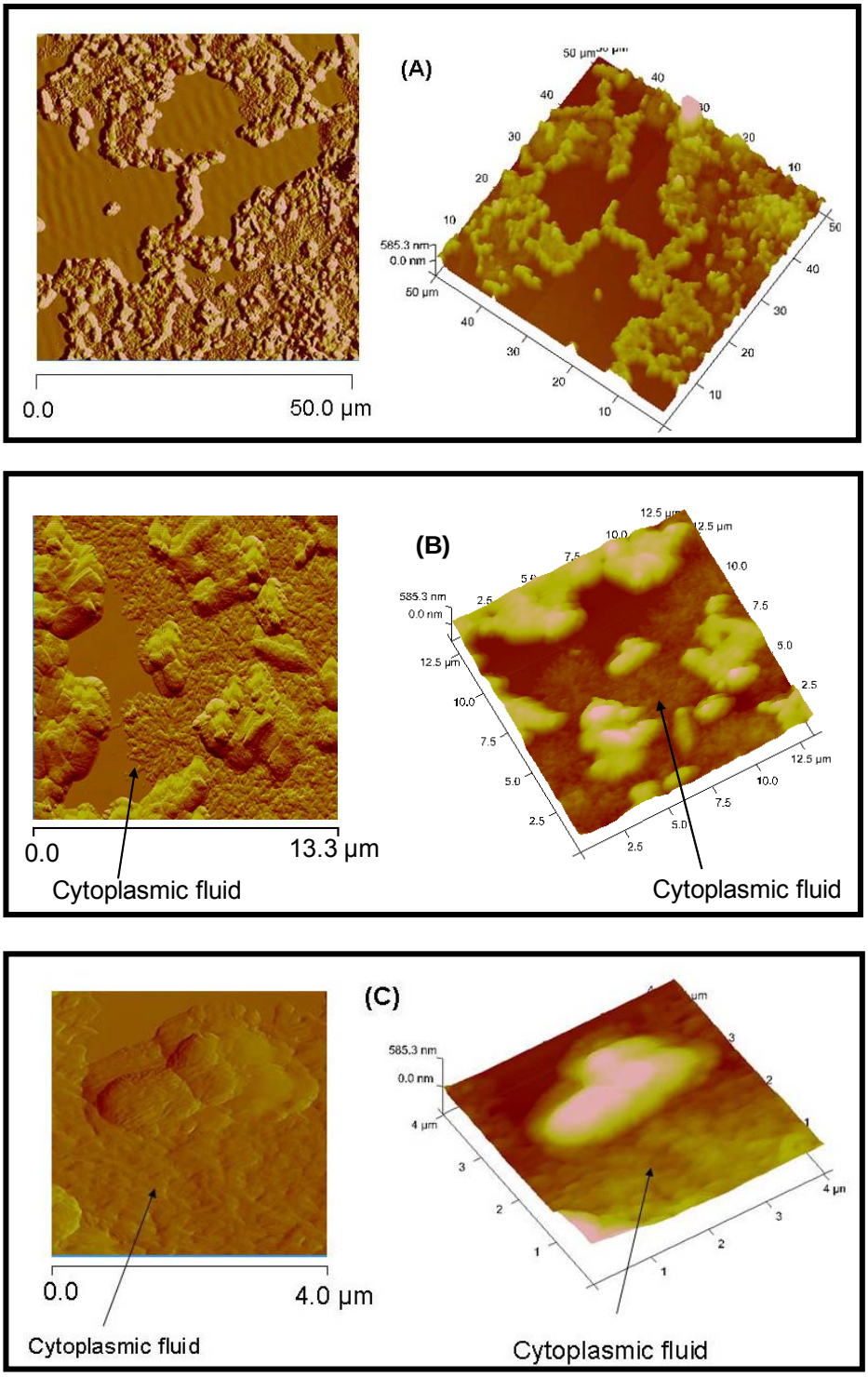


Fig. 5.9: Larger (A) 50.0×50.0 µm and smaller (B and C) 13.3×13.3 µm and 4.0×4.0 µm scan size images of LNNSL treated *E. coli* (the left panels show deflection images and the right panels are 3D reconstructions based on height data)

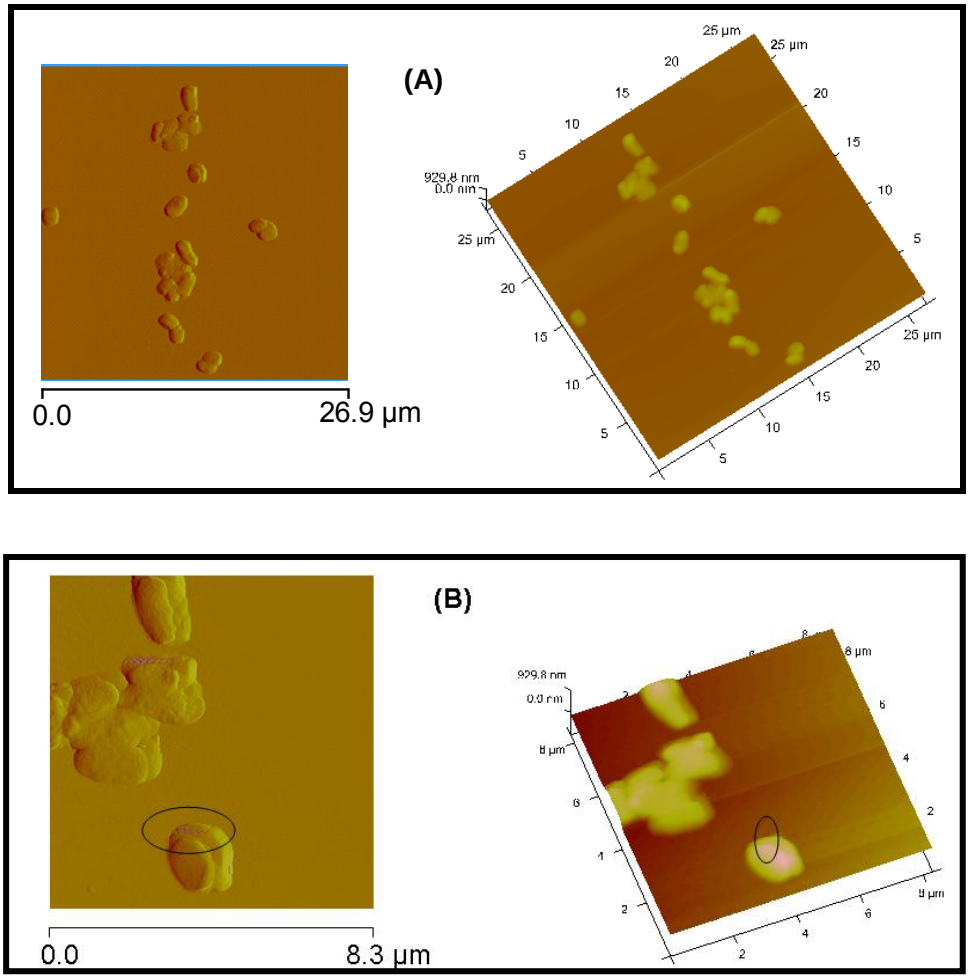


Fig. 5.10: Larger (A) 26.9×26.9 µm and smaller (B) 8.3×8.3 µm scan size images of LNNSLME treated *E. coli* bacteria. Encircled areas showing exuded cytoplasmic fluid from both the ends of damaged cells (left panel showing deflection images and right panel 3D reconstructions based on height data)

5B.4.4 AFM images of untreated P. aeruginosa

AFM images of freshly prepared untreated (control) *P. aeruginosa* are shown in Figure 5.11. Images showed the rod shaped structure with flagella. The length and width of bacteria was determined to be 1.92 µm and 0.8 µm respectively.

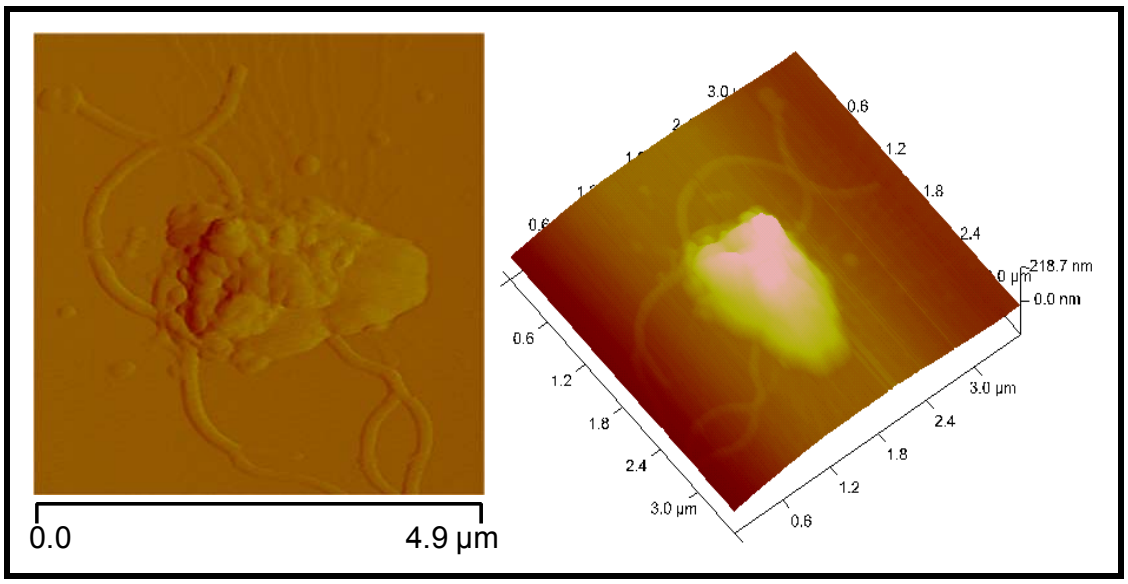


Fig. 5.11: Image (4.9×4.9 µm scan size) of untreated *P. aeruginosa* single cell (left panel showing deflection images and right panel 3D reconstructions based on height data)

5B.4.5 AFM images of P. aeruginosa bacteria treated with LNNSL

AFM images of *P. aeruginosa* treated with minimum inhibitory concentration of 10 µg ml^{-1} of LNNSL are shown in Figure 5.12. AFM images showed the swollen cells with the release of cytoplasmic fluid from bacteria. Cytoplasmic fluid can be seen around the bacteria (Fig. 5.12).

5B.4.6 AFM images of P. aeruginosa bacteria treated with LNNSLME

AFM images of *P. aeruginosa* treated with 20 µg ml^{-1} (MIC) of LNNSLME are shown in Figure 5.13. The bacterial surface showed ridges and grooves as shown in Figure 5.13 (Deflection and height images). It is also clearly visible from the deflection and height images that the bacterial cell is damaged and cellular contents have pulled away from the outer surface on the one side of the bacteria (Fig. 5.13 A and B).

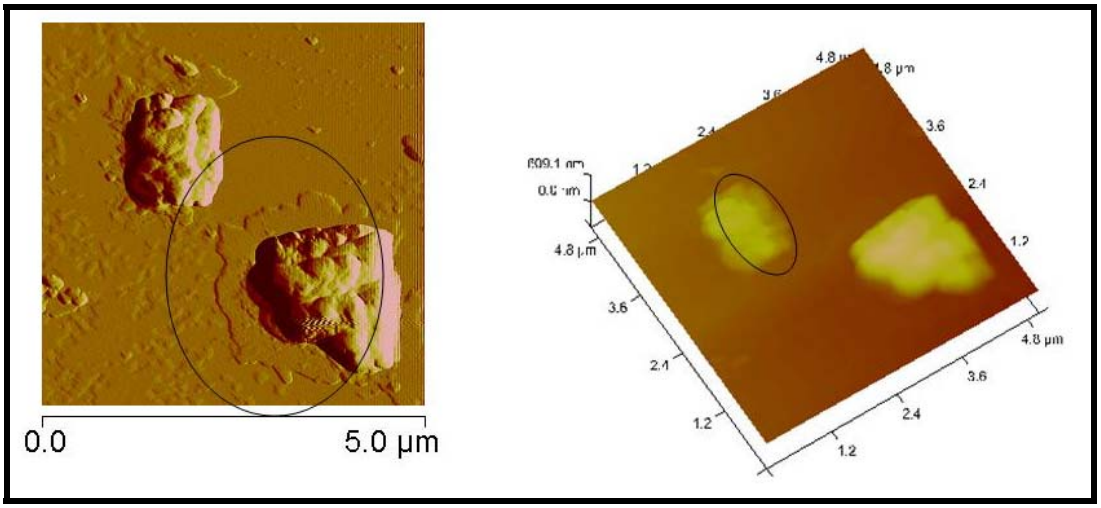


Fig. 5.12: Images (5.0×5.0 µm scan size) of LNNSL treated *P. aeruginosa* cells. Encircled areas show membranolytic action and release of cytoplasmic fluid (left panel showing deflection images and right panel 3D reconstructions based on height data)

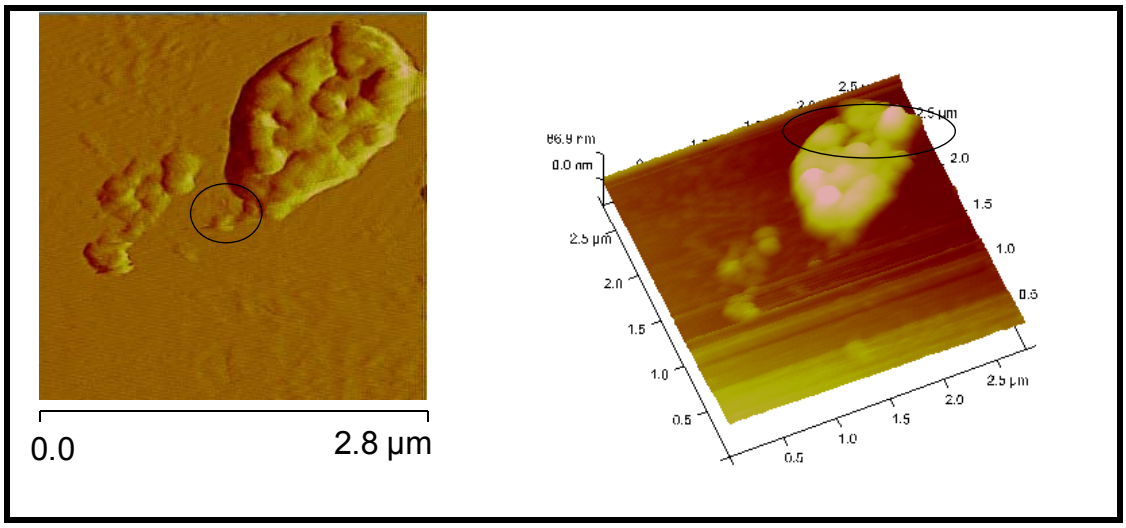


Fig. 5.13 Images (2.8×2.8 µm scan size) of LNNSLME treated *P. aeruginosa* cell (left panel showing deflection images and right panel 3D reconstructions based on height data)

5B.4.7 AFM images of untreated B. subtilis

AFM images taken in deflection and height modes of control (untreated) *B. subtilis* are shown in Figure 5.14. Images showed the rod shaped structure of length 2.05 µm and width of 0.9 µm of *B. subtilis* cells.

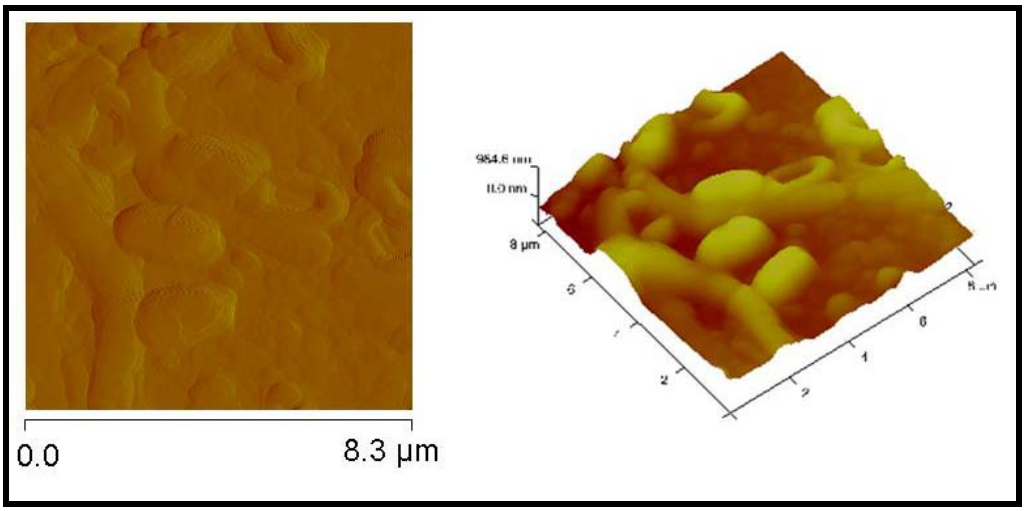


Fig. 5.14: Images (8.3×8.3 µm scan size) of untreated *B. subtilis* cells (left panel showing deflection images and right panel 3D reconstructions based on height data)

5B.4.8 AFM images of B. subtilis bacteria treated with LNNSL

AFM images of B. *subtilis* treated with 20 µg ml^{-1} (MIC) of LNNSL are shown in Figure 5.15. Treatment with LNNSL caused swelling of bacterial cells along with their lysis as shown in Figure 5.15.

5B.4.9 AFM images of B. subtilis bacteria treated with LNNSLME

AFM images of *B. subtilis* treated with 25 µg ml^{-1} (MIC) of sophorolipid methyl ester, LNNSLME are shown in Figure 5.16. As shown in Figure 5.16, the membrane of bacteria is collapsed and large amount of cytoplasmic fluid

is coming out of the bacterial cell which indicates the permeabilization of membrane by the action of sophorolipid methyl ester.

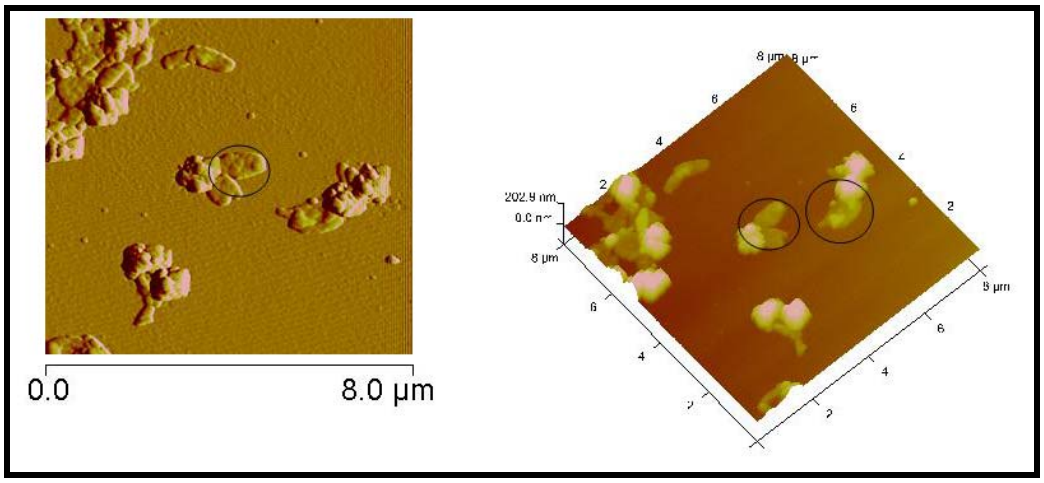


Fig. 5.15: Images (8.0×8.0 µm scan size) of LNNSL treated *B. subtilis* cells. Encircled areas show lysed cells. (Left panel showing deflection images and right panel 3D reconstructions based on height data).

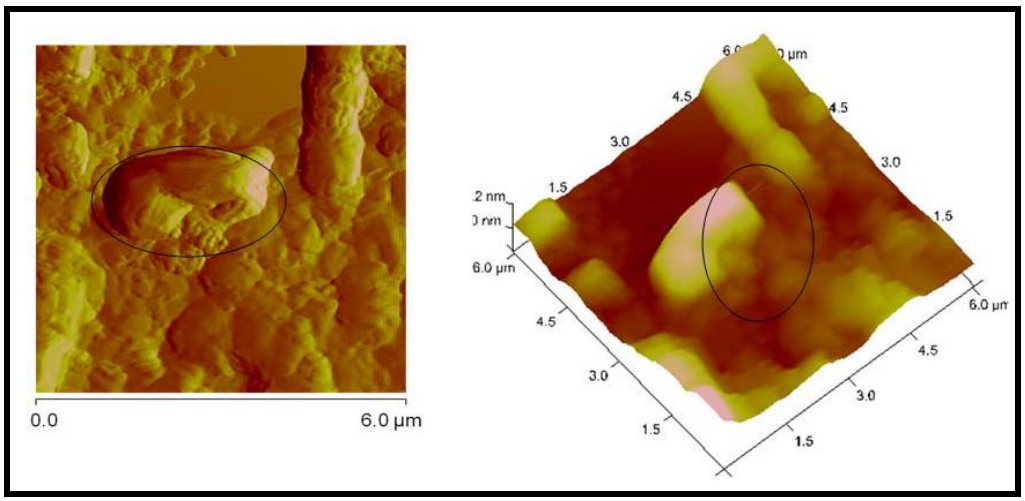


Fig. 5.16: Images (6.0×6.0 µm scan size) of LNNSLME treated *B. subtilis* cells. Encircled areas show release of cytoplasmic fluid from cells. (Left panel showing deflection images and right panel 3D reconstructions based on height data).

Lang et al. (1989) and Lang and Wagner (1993) reported that biosurfactants primarily affects the cell membranes of microorganisms. According to Hotchkiss (1946) if the membrane of particular cell is damaged there would be loss of intracellular constituents such as enzymes, essential ions, coenzymes and intermediates into the suspending medium. Disorganization of cell membranes by surface active agents has long been suggested as cause of their bactericidal action (Hotchkiss, 1946). Further, Kim et al. (2002) reported that upon treatment of *B. subtilis* with sophorolipid causes the release of an intracellular enzyme malate dehydrogenase, indicating the interaction of sophorolipid with cellular membrane and increased permeability. AFM results of *B. subtilis* treated with both the LNNSL and LNNSLME showed the similar mode of action of both the compounds leading to lysis and release of cytoplasmic fluid from the bacterial cell which indicated the possibility of increased permeability of cellular membrane upon the treatment with compounds. AFM results of *E. coli* and *P. aeruginosa* treated with LNNSL and LNNSLME also showed the damaged cells with the release of cellular contents indicating the similar mode of interaction of compounds with cellular membranes. It is clear from AFM study that LNNSL as well as LNNSLME possess bactericidal activity. Mode of action of LNNSL and LNNSLME was in agreement with other biosurfactants that act on the integrity of cell membrane (Lang et al., 1989 and Cameotra and Makkar, 2004) and exhibited bactericidal action. Further, the peptidoglycan layer of cell wall of bacteria are mainly open networks of macromolecules and generally do not offer significant permeability barriers to compounds of molecular mass less than 50 kDa (Lang and Wagner, 1993). Therefore, it was hypothesized

that sophorolipids due to their amphiphilic nature and molecular mass in the range of 600-800 Da can make entry into the bacterial cell through both the lipid bilayer as well as porin channels and at the particular concentration become bactericidal in nature. In our case minimum inhibitory concentrations of LNNSL and LNNSLME were found to be bactericidal against both Gram negative and Gram positive bacteria.

5B.4 Conclusions

There is no clear report on the AFM study of antimicrobial action of other glycolipids. This is the first report on the AFM study of action of Linolenic acid derived sophorolipid, LNNSL and its methyl ester form, LNNSLME. These data confirmed the membranolytic action on cell membranes of Gram negative and Gram positive bacteria. These compounds showed their potential as a bactericidal agent and thus can be used as a potential candidate against bacterial infections.

Summary and Conclusions

The work presented in this thesis mainly focuses on the biological synthesis of novel sophorolipids using *Candida bombicola* as a producer organism. Preparation of new sophorolipid (SL) analogues with different functionalities has widespread use in pharmaceutical and industrial applications. SL composition can be modified by using both *in vivo* and *in vitro* methods. Different lipophilic substrates have been used by researchers for SL production such as oleic acid, stearic acid, palmitic acid and different vegetable oils. SL production using α-linolenic acid as the lipophilic substrate may become a valuable product of interest of enhanced functionality. This is the first report on the linolenic acid derived sophorolipids (LNNSL).

The production of LNNSL was carried out under shake flask conditions. Physiological parameters like effect of pH, temperature, glucose, fatty acid and yeast extract concentrations were studied and optimized. As these parameters play an important role in the cellular metabolism and in turn affect the biosynthesis of sophorolipid. The production of LNNSL is dependent on optimum supply of hydrophilic and lipophilic substrate (glucose and α-linolenic acid). It was found out that *Candida bombicola* is capable of directly incorporating the Linolenic acid into the lipophilic portion of sophorolipid structure. The yield of LNNSL from optimized conditions was found to be 3.98 g l^{-1} and is comparable to the yields reported for polyunsaturated fatty acid derived sophorolipid. High production cost can be tolerated for biosurfactants used in low volumes specialty markets such cosmetics and health care. Therefore the use of pure α-linolenic acid for production of LNNSL can be beneficial for such application fields.

This study successfully demonstrated the analysis of chemically distinct forms in the SL mixture produced by *Candida bombicola* when grown on glucose

and α-linolenic acid as well as its conversion into the single homogenous product by chemical esterification reaction. These novel Sophorolipids were good surface active and antibacterial agents. They have potential for various applications and offer the advantages of further modifications and can produce functionalized SLs according to their applications.

LNNSL and its methyl ester form, LNNSLME showed potential against both Gram positive and Gram negative bacteria and are more effective against Gram negative bacteria which is an advantageous feature as compared to other SLs reported till now which are more effective against Gram positive bacteria. AFM has proved efficient for investigating the mode of action of LNNSL and LNNSLME. Their surface tension and critical micelle concentrations are comparable to the values reported for other commercially available surfactants and biosurfactants.

Future aspects

This study opened the opportunity for investigating the potential of these novel SLs as an antifungal and antiviral agent. LNNSL, containing three double bonds (polyunsaturated) can served as good candidate for self assembly and polymer formation. The other important feature of LNNSL and its methyl ester form is that it can serve as a mediator for providing linolenic acid in accessible form to applications such as cosmetics and food applications etc.

LNNSL and LNNSLME are also the sources of speciality chemicals such as ω and ω-1 fatty acids which possess beneficial properties and are important for polymer, perfumes and fragrance industries etc. Linolenic acid derived SLs may also checked for cell differentiation ability in cancerous cells.

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- Insight into the antibacterial action of Linolenic acid derived sophorolipid and its methyl ester form with the help of Atomic Force Microscopy. **Reetika Gupta** and Asmita A. Prabhune (Communicated)

- Optimiztion of fermentation parameters for production of Linolenic acid derived sophorolipids using *Candida bombicola* ATCC 22214. **Reetika Gupta** and Asmita A. Prabhune (Manuscript under preparation)

Posters presented

- **Synthesis of Linolenic sophorolipid using *Candida bombicola*: chemical esterification, characterization.** Oral presentation in Young research Conference, Institute of Chemical Technology, Mumbai, 14-16 Jan. 2010.

- **Antimicrobial property of newly synthesized sophorolipid methyl ester (17-[2' –O-β- D-glucopyranosyl- β- D- glucopyranosyl]- oxy)-cis,cis,cis-9,12,15-octadecatrienoate).** Poster presentation in Young

research Conference, Institute of Chemical Technology, Mumbai, 14-16 Jan. 2010.

- **Synthesis of Linolenic sophorolipid using *Candida bombicola*: chemical esterification, characterization and antimicrobial activity**. Poster presentation in "The Society of Biological Chemists (India), Bangalore". December 13-15, 2010.

- **Antimicrobial property of newly synthesized sophorolipid methyl ester (17-([2'-O-β-D-glucopyranosyl-β-D-glucopyranosyl]-oxy)-cis,cis,cis-9,12,15-octadecatrienoate).** Poster presentation in Science Day at National Chemical Laboratory, India (March, 2010)