Biosynthesis of novel glycolipids: Basic and Applied aspects

A Thesis submitted by

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.....Dedicated with love
to my parents
Because of whom
I am who I am!

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled: "**Biosynthesis of novel Glycolipids: Basic and Applied aspects"** submitted by Kasturi Joshi-Navare, 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 Glycolipids: Basic and applied aspects"**, 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.

Kasturi Joshi-Navare
(Research Scholar)

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Thank you.

Kasturi Joshi-Navare

List of abbreviations

SL	-Sophorolipid
µM	-Micromolar
CMC	-Critical micelle concnetration
SLJO	-Sophorolipid derived from Jatropha oil
SLPO	-Sophorolipid derived from pongamia oil
TLC	-Thin layer chromatography
HPLC	-High performance liquid chromatography
MALDI/MS	-Matrix assisted laser desorption ionization mass spectroscopy
NMR	-Nuclear magnetic resonance
SEM	-Scanning electron microscopy
MIC	-Minimum inhibitory concentration
SLOA	-Sophorolipid derived from oleic acid
SLLA	-Sophorolipid derived from linoleic acid
MTT	-3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
FTIR	-Fourier transform infra-red
HR-MS	-High resolution mass spectroscopy
XRD	-X-ray diffraction
Rpm	-Revolutions per minute
MGYP	-Malt glucose yeast peptone
MXYP	-Malt xylose yeast peptone
NA	-Nutrient agar

Abstract

Sophorolipids (SLs) belong to the category of glycolipid biosurfactants. They consist of a dimeric sugar- sophorose as a carbohydrate head and a long hydroxy fatty acid chain as tail. This amphiphilic nature allows them to form unique structures such as micelles and bilayers in heterogenous systems. SLs find applications in wide range of fields including Petroleum, food, pharmaceuticals, cosmetics, laundry and bioremediation. SLs are synthesized by employing non pathogenic yeast strains and they confer several physiological advantages to the producer. Biosurfactants are coming up as emerging class of biomedical compounds. They are a suitable alternative to synthetic medicines and antimicrobial agents, and could be used as safe and effective therapeutic agents or probiotics, especially at a time when drug resistance among causal organisms for many life-threatening diseases is on the rise. SLs offer the advantages of biodegradability, low ecotoxicity and the production based on renewable-resource substrates. The US FDA has also approved biosurfactants/sugar esters for the use in food and pharmaceuticals. Keeping this in mind; several basic and applied aspects of sophorolipids have been explored and the work has been reported in the thesis.

The thesis has been divided into 5 chapters

Chapter I. Introduction

The ***first chapter*** is an introduction to the thesis. It includes brief account on surfactants, current market share of Biosurfactants, types, their advantages over chemical ones and producing microorganisms. SL stands as a promising biosurfactant. The features which make it so are described along with its physiological role and the biosynthetic pathway. Owing to the unique nature; sophorolipids find applications in wide range of areas. The chapter gives brief literature review of the applications. With this background, the scope and objectives of the thesis have been defined.

Chapter II. Production of sophorolipids using non edible oils and their use as an alternative/ additive to laundry detergents

The ***second chapter*** is about the attempt to reduce the SL production cost by using non-edible oils namely Jatropha and Pongamia. Through optimization of

parameters and resting cell method, the yields 15.25g/l and 19.3g/l could be achieved for Jatropha oil derived SL (SLJO) and Pongamia oil derived sophorolipid (SLPO) respectively. Both SLs showed good surfactant property with the CMC values 9.5mg/l for SLJO and 62.5mg/l for SLPO. Keeping the prospective use of these SLs in mind, the physicochemical characterization, emulsion stability, antibacterial and stain removal studies in comparison with commercial detergent were done. Based on the results, it can be said that SLs have utility as fabric cleaner with advantageous properties such as skin friendly nature, antibacterial action and biodegradability. SLs enhance the detergent performance, so less quantity of detergent can be sufficient for desired cleaning effect. Thus the harm caused to environment through detergent usage can be reduced by replacing synthetic surfactants with green surfactant molecules.

Chapter III(a). Sophorolipid biosurfactants act in synergy with antibiotics to enhance their efficiency

In the ***third chapter (part a)*** the effect of co-administration of SL and antibiotics has been evaluated. SLs are amphiphilic in nature and therefore can span through the bacterial cell membrane. Thus the co-administration is expected to facilitate the entry of antibiotic molecules. During the experiments, effect of SL-antibiotic co-administration was evaluated against 3 different index bacteria namely, *Escherichia coli*, *Staphylococcus aureus, Pseudomonas aeruginosa.* Also the antibiotics differed in their mode as well as site of action. In accordance with the anticipation, SL-antibiotic combination achieved the bacterial inhibition within shorter period as compared to the antibiotic alone. The Scanning electron micrographs of the bacterial cells treated with combinations confirmed the damage to cell membrane with all 3 test bacteria at sublethal concentrations of both inhibitory agents.

Chapter III(b). Exploration of antiviral activity of SLs

The ***third chapter (part b)*** describes antiviral action of Oleic, Linoleic acid derived SL against viruses differing in their genetic makeup such as plus stranded RNA, segmented single negative stranded RNA and double stranded DNA viruses. The antiviral activity was tested in 3 different modes- direct treatment of virus, rapid

culture assay for Influenza virus and treatment of host cell line with SL prior to viral challenge in order to check if SL gives any antiviral immunity. Coxsackieviruses CV (B1-CVB6, CA7, CA9), murid herpesvirus, strain MHV-68, Influenza virus A/Mississippi/1/85 (H3N2) have been used for the studies.

It was observed that direct treatment of virus indicated 1 log_{10}- 4.5 log_{10} reduction in the virus titers. Pretreatment of cell cultures GMK and Hep-2 prior to Coxsackie virus infection showed a reduction in virus titer 1log_{10} - 2 $logs_{10}$. Similar results were obtained on the VERO, BHK and 3T3 cells with gamma-herpesvirus MHV-68. Visible reduction of Influenza A virus replication on MDCK cells was obtained at the concentration 100 µg/ml of SLLA (SL derived from Linoleic acid). Thus it can be concluded that direct treatment of viruses was more effective than indirect treatment and the inhibitory action can be attributed to the amphiphilic structure of SL which might be killing the viruses by disturbing the membrane lipid order.

Chapter IV. Differentiation inducing ability of SLs against glioma cells

The ***forth chapter*** deals with the differentiation inducing ability of SLs. The effects have been investigated on LN229 - a glioma cell line which has been reported for the first time. In response to different SL forms, marked difference in cell density was observed as compared to the control cells along with various morphological changes such as formation of long thread like extensions arising from ends of the cells, cell alignment, cell elongation and bundle formation in dose dependent manner. In this chapter the morphological evidence of the potential of SLs as differentiation inducers has been presented. The finding suggests the utility of SLs as a pharmaceutical agent for the treatment of glioblastomas. With the use of SL, the cancerous cells undergoing uncontrolled proliferation can be forced to differentiate thus tumor progression may be arrested.

Chapter V(a). Glycolipid production by a novel yeast- *Pichia caribbica* (HQ222812) with xylose as a head group and its advantageous properties

The ***fifth chapter (part a)*** deals with the use of a new xylose fermenting yeast- *Pichia caribbica* for biosurfactant production so as to achieve the less explored head group diversity in SL structure. The media and fermentation parameters

have been optimized to achieve maximum yield of 7.5g/l. The physicochemical properties of the xylolipid biosurfactant have been assessed. It reduced the surface tension of distilled water from 70mN/m to 35.9mN/m with the remarkably low CMC value 1.0 mg/l as compared to typical SLs (reported CMC range-40-100mg/l). Structural characterization was done using FTIR and HR-MS to identify the structure putatively. 17-L-[(β-D-xylopyranosyl)-oxy]-Δ9-heptadecanoic acid correlated to m/z 415 majorly constituted the product. Control experiment was performed in which glucose was provided as the hydrophilic carbon. This product was also subjected to HR-MS analysis to determine its chemical nature and found to be different from xylolipid. Presence of xylose as head group was anticipated to give altered physicochemical and biological activities. In accordance to the same, low CMC value and better inhibitory action was demonstrated against *Staphylococcus aureus*, a gram positive bacterium.

Chapter V(b). Crystalline xylitol production by a novel yeast- *Pichia caribbica* (HQ222812) and its application for quorum sensing inhibition in gram negative marker strain *Chromobacterium violecium* CV026

The ***fifth chapter (part b)*** is about quantitative production of xylitol from D-xylose with the yield of 0.852 gm/gm and volumetric productivity of 1.83 gm/l/h. A safe procedure for product extraction has been described. The ability of xylitol to act as a quorum sensing antagonist in gram negative marker strain *Chromobacterium violaceum* CV026 has been demonstrated for the first time.

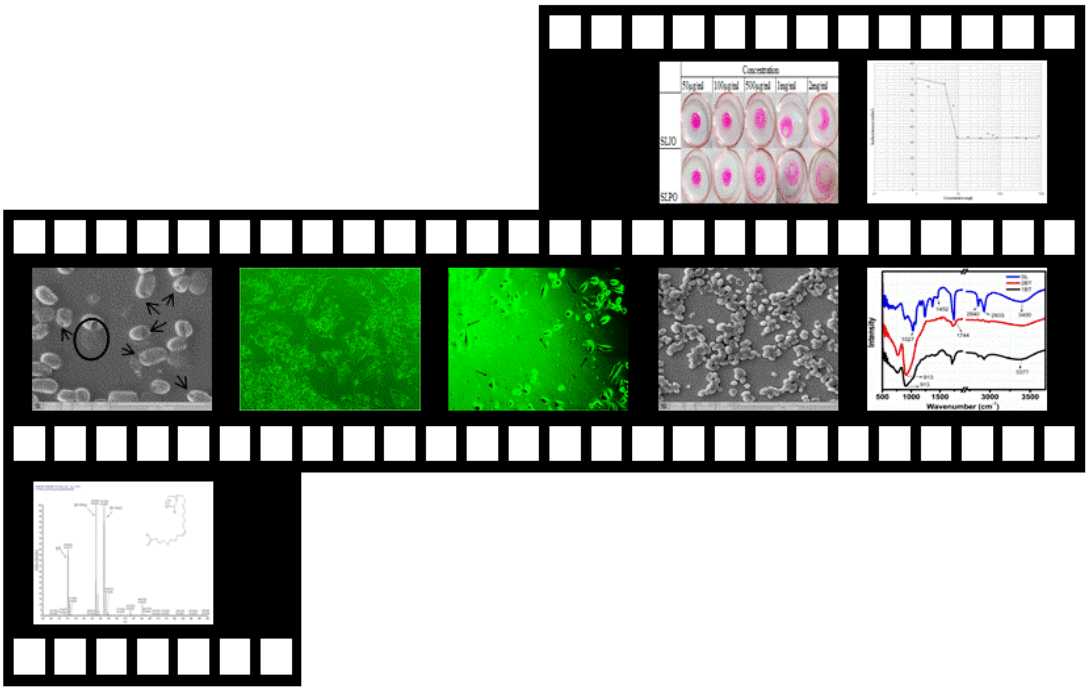
Conclusions

The last chapter summarizes the work presented in the thesis and emphasizes on possible further research in this area.

Chapter I

Introduction

Biosynthesis of novel glycolipids: Basic and Applied aspects



As the title suggests; the thesis deals with the production of different glycolipid molecules using the conventional sophorolipid producing yeast- *Candida bombicola* ATCC 22214 as well as novel strain *Pichia caribbica* MTCC 5703.

The molecules mentioned here belong to structural class of Sophorolipids (SLs) and related molecules which differ from classical SLs because of variation in either hydrophilic head group or hydrophobic tail group.

The work involves production of the glycolipids, optimization of media and fermentation parameters for maximum yield, characterization and their applications.

SLs are important biosurfactants and are known for their antimicrobial and anticancer potential. Owing to these facts; in this thesis we have explored the potential of economically produced SLs as fabric cleaner, advantage of conjugative administration of SL along with antibiotics for better handling of bacterial infections, antiviral potential of SLs and differentiation inducing ability of SLs against glioma cells which will have implications in treatment of glioblastoma- a kind of Central Nervous System tumor resistant to conventional chemotherapy.

1.1 About Surfactants

Definition of surfactants: The term is derived from 'Surface Active Agents'. Surfactants are amphiphilic compounds that reduce the free energy of the system by replacing the bulk molecules of higher energy at the interface. They contain a hydrophobic portion with little affinity for the bulk medium and a hydrophilic group that is attracted to the bulk medium.

Thus surfactants have the abilities to lower surface tension, increase solubility, detergency power, wetting ability and foaming capacity. Based on these abilities; surfactants find numerous applications. They are potentially useful in every industry dealing with multiphasic systems. Surfactants have been used industrially as adhesives, flocculating, wetting, foaming agents, de-emulsifiers and penetrants [1]. Therefore surfactants stand as one of the most important class of industrial bulk chemicals.

Current surfactant market: The current worldwide production of surfactants amount to 23 million metric tonnes with expected future growth rates of 3-4% per year globally. The individual applications of surfactants are classified as represented in following Figure 1.1 [2].

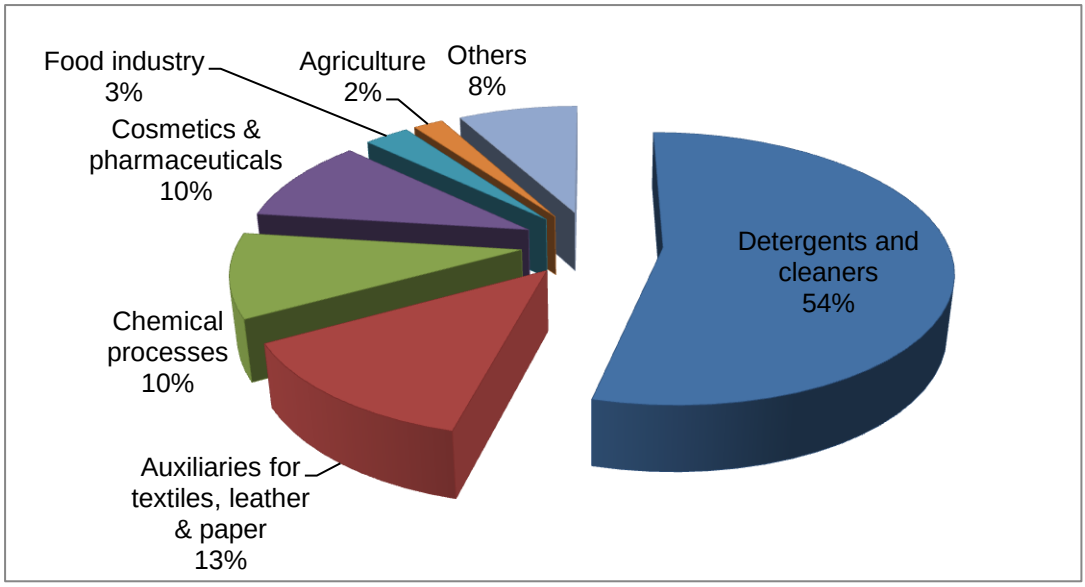


Figure 1.1 Usage of surfactants in various fields and their relative share

Large majority of the currently used surfactants are petroleum-based and produced by chemical means. Major types of synthetic surfactants include Linear

alkyl benzene sulphonates, alcohol sulphates, alcohol ether sulphates, alcohol glyceryl ether sulphonates, α-olefin sulphonates, alcohol ethoxylates and alkyl phenol ethoxylates [1]. These compounds are often toxic to the environment, and their use may lead to significant ecological problems, particularly in washing applications as these surfactants inevitably end up in the environment after use. The ecotoxicity, bioaccumulation, and biodegradability of surfactants are therefore issues of increasing concern which have lead to withdrawal of several surfactants [1, 3]. Still the low rate of biodegradation and high aquatic toxicity of synthetic surfactants remains the cause of major concern. This has dictated the development and market entry of the synthetic surfactants with better environmental profile. Such surfactants include alkyl polyglucosides, alkyl polyglucamides and fatty ester methyl ester ethoxylates. The shifted trend has been represented in the Figure 1.2.

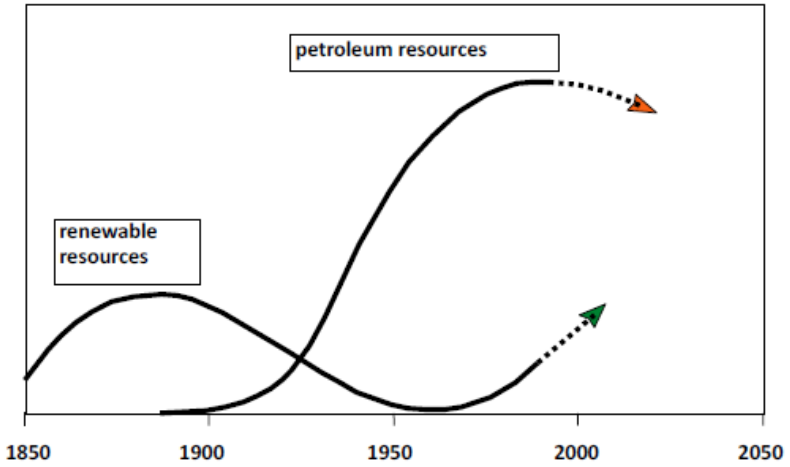


Figure 1.2 Feedstock utilization for production of surfactants

These molecules are not totally originated from renewable resources and still imply chemical synthesis steps. In contrast, microbial surfactants i.e. Biosurfactants can be produced from renewable feedstock or even waste streams through a natural fermentation process. Furthermore, they are readily degradable and display low eco-toxicity [4].

1.2 Types of Biosurfactants

Biosurfactants are biologically produced by yeasts or bacteria from various substrates including sugars, oils, alkanes and even organic waste material. They can be potentially as effective with some distinct advantages over the highly used

synthetic surfactants including high specificity, bio-degradability and biocompatibility. Biosurfactants are grouped as glycolipids, lipopeptides, phospholipids, fatty acids, neutral lipids [5]. The main types of biosurfactants have been listed in Table 1.1 along with their brief characteristics. Apart from the types mentioned in the Table, there is a category of polymeric surfactants comprising of Mannan-lipid protein, Carbohydrate-lipid protein and protein PA. Another category of biosurfactants i.e. particulate surfactants include vesicles, fimbriae and whole cells. Most of these compounds are either anionic or neutral. Only a few are cationic such as those containing amine groups. The hydrophobic part of the molecule is based on long chain fatty acid, hydroxyl fatty acids or α-alkyl- α-hydroxy fatty acids. The hydrophilic portion can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol. A wide variety of microorganisms can produce these compounds. The Critical Micelle Concentration i.e. CMC of the biosurfactants generally range from 1 to 200mg/l and their molecular mass from 500 to 1500 daltons. CMC is defined as the concentration of surfactant above which micelles form. After reaching CMC, surface tension remains relatively constant.

Biosurfactant	*Producers*	*Minimum Surface tension*	*Highest reported yield*	*Reference*
Glycolipids				
Rhamnolipids	*Pseudomonas aeruginosa, Pseudomonas sp., Burkholderia sp., Acinetobacter sp., Enterobacter sp.*	29mN/m	100g/L	C. Jiani *et. al.*, 1997, G. Sobero'n-Chavez 2011, J. Desai *et. al.*, 1997 [5, 6, 7]
Trehalolipids	*Rhodococcus erythropolis, Mycobacteria, Arthrobacter sp., Nocardia sp. etc*	32-36 mN/m	10.9g/L	A. Franzetti *et. al.*,2010, J. Desai et. al, 1997 [5,8]
Sophorolipids	*Candida bombicola, C. apicola, Candida sp.*	30-33 mN/m	422g/L	H. Daniel *et. al.*,1998 [9]
Cellobiolipids	*Ustilago zeae, U. maydis*	-	-	J. Desai et. al, 1997 [5]
Mannosyl erythritol lipids	*Pseudozyma sp., Ustilago sp.*	33.8 mN/m	Above 100g/L	J Arutchelvi *et. al.*,2008 [10]

Lipopeptides and Lipoproteins				
Licheniformin	*Bacillus licheniformis*	38 mN/m	-	D. Biria, *et. al.* 2010 [11]
Serrawettin	*Serratia marcescence*	28-33 mN/m	-	G. Soberon Chavez, 2011 [12]
Viscosin	*Pseudomonas fluorescence*	27 mN/m	170mg/l	M. Laycock et. al., 1991[13]
Surfactin	*Bacillus subtilis*	27 mN/m	4.3g/L	N.Shaligram *et. al.*,2010 [14]
This category of biosurfactants also include Subtilisin, Gramicidins, Polymixins, viscosinamide, amphisin, massetolides A-H which have not been explored in detail				
Fatty acids, Neutral lipids and Phospholipids				
Fatty acids	*Corynebacterium lepus*	20 mN/m	-	D. Cooper *et. al.*,1981 [15]
Neutral Lipids	*Nocardia erythropolis*	33 mN/m	-	C. Macdonald *et. al.*,1981 [16]
Phospholipids	*Thiobacillus thioxidans*	-	-	A. Prakash, 2012 [17]
Polymeric surfactants				
Emulsan	*Acinetobacter calcoaceticus*	-	-	Y. Shabtai *et. al.*, 1986[18]
Biodispersan	*Acinetobacter calcoaceticus*	-	-	E. Rosenberg *et. al.*,1988 [19]
Liposan	*Yarrowia lipolytica*	-	220mg/l	R. Pinchuk *et. al.*, 2000[20]

Table 1.1 Different types of Biosurfactants

Majority of biosurfactants are synthesized through the utilization of hydrocarbon substrates as well as from carbohydrates. They can either be extracellular or cell membrane associated. Production of biosurfactants can be growth associated but usually they are secondary metabolites, produced during the late logarithmic and/ or stationary growth phase as represented in Figure 1.3 [1]. Biosurfactants are categorized mainly by their chemical composition and their microbial origin [5].

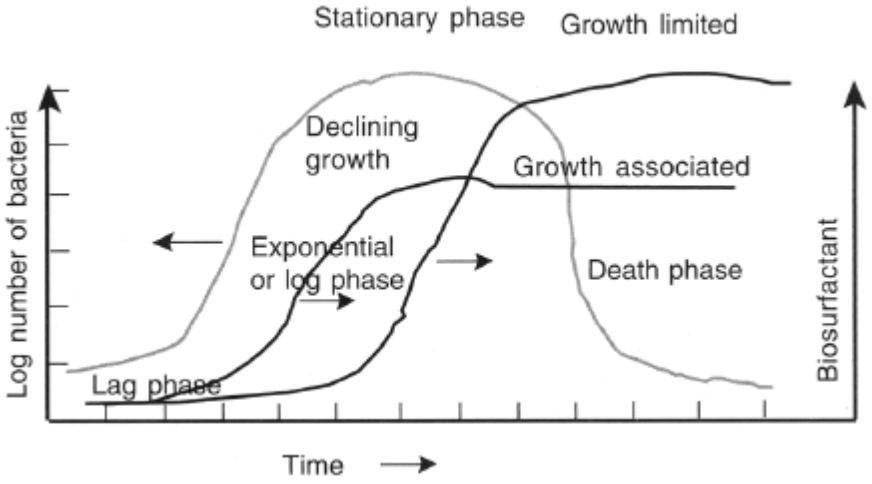


Figure 1.3 Typical biosurfactant production with reference to phases of growth-adapted from [1]

Glycolipidic biosurfactants: A great number of simple glycolipids are found in bacteria, yeasts, and lower marine invertebrates. These compounds are composed of a glycosyl moiety (one or several units) linked to a hydroxyl fatty acid or to one carboxyl group of a fatty acid (ester linkage). Glycolipids form the largest structural class of biosurfactants. The types of glycolipidic biosurfactants include Rhamnolipids, Sophorolipids, Trehalose lipids and Mannosyl erythritol lipids. Rhamnolipids i.e. glycolipids containing rhamnose and β-hydroxydecanoic acid were found for the first time by Bergstrom *et. al.,* 1946 in *Pseudomonas pyocyanea* after growth on glucose. Trehalose lipids are fatty acid glycosides containing a nonreducing disaccharide a-D-trehalose which occurs mainly in bacteria, fungi, algae, and insects, in which it seems to have a role as an energetic reserve compound. Mannosylerythritol lipids (MEL), yeast glycolipids, are produced from vegetable oils by *Pseudozyma* (previously *Candida*) *antarctica* T-34. MEL exhibits excellent surface-active and vesicle-forming properties [21]. Sophorolipids have been discussed in detail in coming sections of the thesis. Depending upon the variation in carbohydrate head group there are Glucose lipids, Fructose lipids, Cellobiose lipids and Saccharose lipids [1].

Other than glycolipidic biosurfactants, there are Lipopeptides, Lipoproteins, Fatty acids, Phospholipids and Neutral lipids. Some polymeric and particulate molecules are also known to behave as biosurfactants [5].

Economy is often the bottleneck of biotechnological processes, especially in the case of biosurfactant production. The success of biosurfactant production depends on the development of cheaper processes and the use of low cost raw materials, which account for 10–30% of the overall cost. Biosurfactants have to compete with surfactants of petrochemical origin in three aspects: cost, functionality and production capacity to meet the need of the intended application in common use sector. A high production cost can be tolerated for a biosurfactant used in low volume, high priced, products such as cosmetics, medicines, etc. But in applications such as enhanced oil recovery, which require high volumes of low priced surfactants, the high costs are incompatible with their use [22]. Biosurfactants have been identified as potentially useful sustainable ingredients for a number of commercial products. The next step is to determine whether the biosurfactant can be produced at a price which will make it competitive with existing chemical surfactants. Production costs for biosurfactants will depend on the cost of fermentation feedstock used, the yield in the fermentation broth and the cost of downstream processing and the interaction between each of these factors [23].

As can be seen from the maximum achievable yield values in Table 1.1; sophorolipids (SLs) are probably one of the most promising biosurfactants. Another advantage is that they are produced by non pathogenic yeast strains. In the contrary, rhamnolipids- another commercially available glycolipid surfactant that can be produced at relatively high amounts are produced by the opportunistic pathogen *Pseudomonas aeruginosa*. These properties confer advantage to SLs over rhamnolipids. Furthermore high production yields of SLs (over 400 g/L) and substrate conversion (70%) can be achieved [4].

1.3 About Sophorolipids (SLs)

SLs have been known for over 50 years, but because of growing environmental awareness, they recently regained attention as biosurfactants. Gorin *et. al.*, 1961 first described SLs as an extracellular glycolipid synthesized by the yeast *Torulopsis magnolia* [24]. Later in 1968, authors declared the strain to be actually *Torulopsis apicola*, currently known as *Candida apicola*. The structure of this biosurfactant was elucidated as a partially acetylated 2-O-β-D-glucopyranosyl- D-glucopyranose unit attached β-glycosidically to 17-L-hydroxyoctadecanoic or 17-L-

hydroxy-Δ9-octadecenoic acid by Tulloch *et.al*, 1962 and Tulloch and Spencer, 1968 [1, 25, 26].

1.3.1 SL structure: SLs are particularly produced by *C. bombicola* and *C. apicola*. Generally they occur in mixtures comprising usually 8 major and up to 15 minor components. Sophorose, a disaccharide of glucose with β1-2 linkage, forms the hydrophilic head of the molecule that may or may not be acetylated with one or two acetyl groups. The fatty acid chain typically has 16 or 18 carbon atoms with different degrees of saturation (none, one or two double bonds). SL molecules exist either in the acidic or lactonic form; in the latter, the carboxylic end of the fatty acid is esterified at the 4'', or less frequently at the 6' or 6'' position, of the sophorose unit as represented in Figure 1.4. The possible variants make the SL mixture produced by *Candida* sp.; very complex, although lactonic SL with 17-hydroxy-octadecanoic acid is reported to be the predominant congener [23].

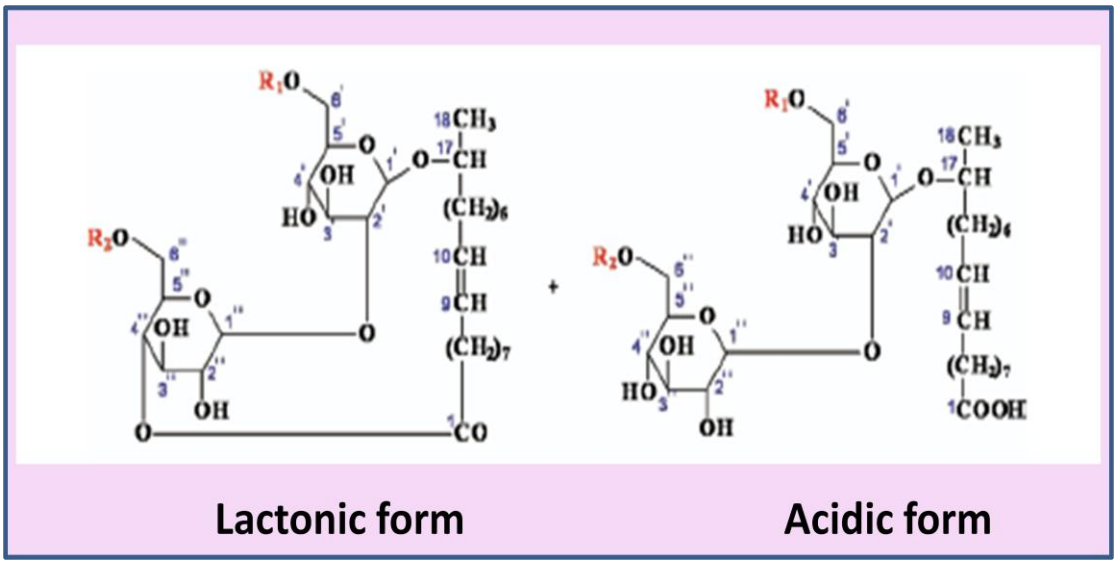


Figure 1.4 Structural frameworks for SL- lactonic conformation and acidic conformation. R_1 and R_2 are the positions of acetylation.

SL mixture comprises of the forms differing in position of hydroxylation, chain length, unsaturation, lactonization and acetylation pattern. Representative chemical composition of the SL mixture produced by *C. apicola* ATCC 96134 using oleic acid as the major carbon source has been provided in the Table 1.2.
The data is based on HPLC analysis.

No.	Possible structure	R.T. (min)	m/z	Abundance (%)
1	Acidic C18:1	6.93	621	6.5
2	Acidic C18:1, 1Ac	7.93	663	4.9
3	Acidic C18:2, 2Ac	9.84	703	2.8
4	Acidic C18:1, 2Ac	12.13	705	48.1
5	Acidic C18:0, 2Ac	16.54	707	2.8
6	Lactonic C18:1, 1Ac	24.69	645	3.06
7	Lactonic C18:2, 2Ac	36.45	685	2.7
8	Lactonic C18:2, 2Ac	39.25	685	2.2
9	Lactonic C16:0, 2Ac	44.21	661	1.1
10	Lactonic C18:1, 2Ac	47.73	687	4.6
11	Lactonic C18:1, 2Ac	49.68	687	10.0
12	Lactonic C18:0, 2Ac	67.92	689	4.1

Table 1.2 Chemical composition of SL synthesized by *C. apicola*. Table adapted from [23]

The physicochemical properties of SLs are governed by the structural features. Lactone conformation SLs have different biological and physicochemical properties compared to acidic forms. Generally, lactonic SLs have better surface tension lowering and antimicrobial activity, whereas the acidic ones display a better foam production and solubility. Presence of acetyl groups render the molecules less water soluble, but enhance their antiviral and cytokine stimulating effects. For instance, di- or mono-acetylated lactonic SLs display better antibacterial activity compared to non-acetylated lactonic ones and acidic forms and di-acetylated lactonic SLs posses a lower CMC and surface tension as compared to non-acetylated acidic molecules [4].

As mentioned in Table 1.2, SLs are produced as mixture of several structures differing in extent of saturation, position of hydroxylation i.e. ω or ω-1, acetylation pattern and lactonization. In spite of this it is possible to predict the SL structures based on secondary carbon feeding. Thus depending upon the desired properties we can choose the appropriate fatty acid substrates. Because of this reason; SLs are called as 'tailor made molecules'.

1.3.2 SL producers: As discussed in section 1.3, SLs are mainly synthesized by the members of the genus *Candida*, mainly *C. bombicola* and *C. apicola*. Few other related strains producing SLs have also been discovered. Chen *et.al.,* 2006 have discovered a new strain from oil contaminated waste water. The strain was identified to be *Wickerhamiella domercqiae* and it was able to produce large amounts of SL [27]. Thaniyavarn *et. al.,* 2008 isolated a thermo tolerant *Pichia anomala* strain from fermented food capable of synthesizing SLs [28]. Konishi *et. al.,* 2008 reported SL production using *C. batistae* CBS 8550. The product formed majorly consisted of acidic conformation and SLs were more hydrophilic [29]. Kurtzman *et. al.,* 2010 studied 19 yeasts of the *Starmerella* clade for their ability to synthesize significant amounts of SL. 5 such strains were identified namely, *S. bombicola*, *C. apicola*, *C. riodocensis*, *C. stellata* and a new species, *Candida sp.* NRRL Y-27208. Phylogenetic analysis of sequences for the D1/D2 domains of the nuclear large subunit rRNA gene placed all SL-producing species in the *S. bombicola* subclade of the Starmerella clade [30]. The new genus Starmerella with a single species- *Starmerella bombicola* is proposed to accommodate the teleomorph of *C. bombicola*. Yang *et. al.* 2012 reported SL production using *C. albicans*-0-13-1 using sugarcane molasses as the substrate [31]. Till date, *Rhodotorula bogoriensis* (formerly referred to as *C. bogoriensis*) is the only basidiomycete reported to synthesize SLs [4].

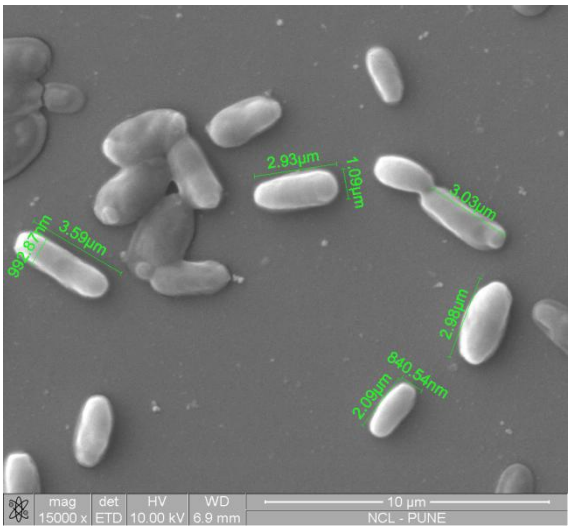


Figure 1.5 Typical SL producer yeast *C. bombicola* (ATCC22214)

1.3.3 Biosynthetic pathway for SL production: The pathway adapted from I. N. A, Van Bogaert *et. al.,* 2007 has been represented in Figure 1.6. In a first step, the

fatty acids are converted to a terminal (ω) or subterminal (ω-1) hydroxy fatty acid through the action of a membrane bound nicotinamide adenine dinucleotide phosphate (reduced form; NADPH) dependent monooxygenase enzyme, cytochrome P450 4A. For *C. bombicola* ATCC 22214, the same research group has identified five different cytochrome P450 monooxygenase genes belonging to the CYP52 family. In a second step, glucose is glycosidically coupled (position C1') to the hydroxyl group of the fatty acid through the action of a specific glycosyltransferase I. In a subsequent step, a second glucose is glycosidically coupled to the C2' position of the first glucose moiety by glycosyltranferase II. Lactonic sophorolipids are formed by an esterification reaction of the carboxyl group of the hydroxy fatty acid with a hydroxyl group of sophorose. The acetylation at the 6'- and/or 6''-position is carried out by an acetyl-coenzyme A (CoA) dependent acetyl transferase [3].

Reports on the biosynthetic pathway of SL synthesis by *C. apicola* by inhibition studies with cerulenin and by biotransformations of ^{13}C labeled hydrophobic substrates revealed that the ω and ω-1 fatty acid hydroxylation was associated with the stationary phase of cell growth. It has also been shown that the microsomal fatty alcohol oxidase and the fatty aldehyde dehydrogenase were induced in late logarithmic cells when either glucose or a mixture of glucose and n-hexadecane were used as carbon source. These enzymes mediate the conversion of alkane to corresponding fatty acid in stepwise manner [32].

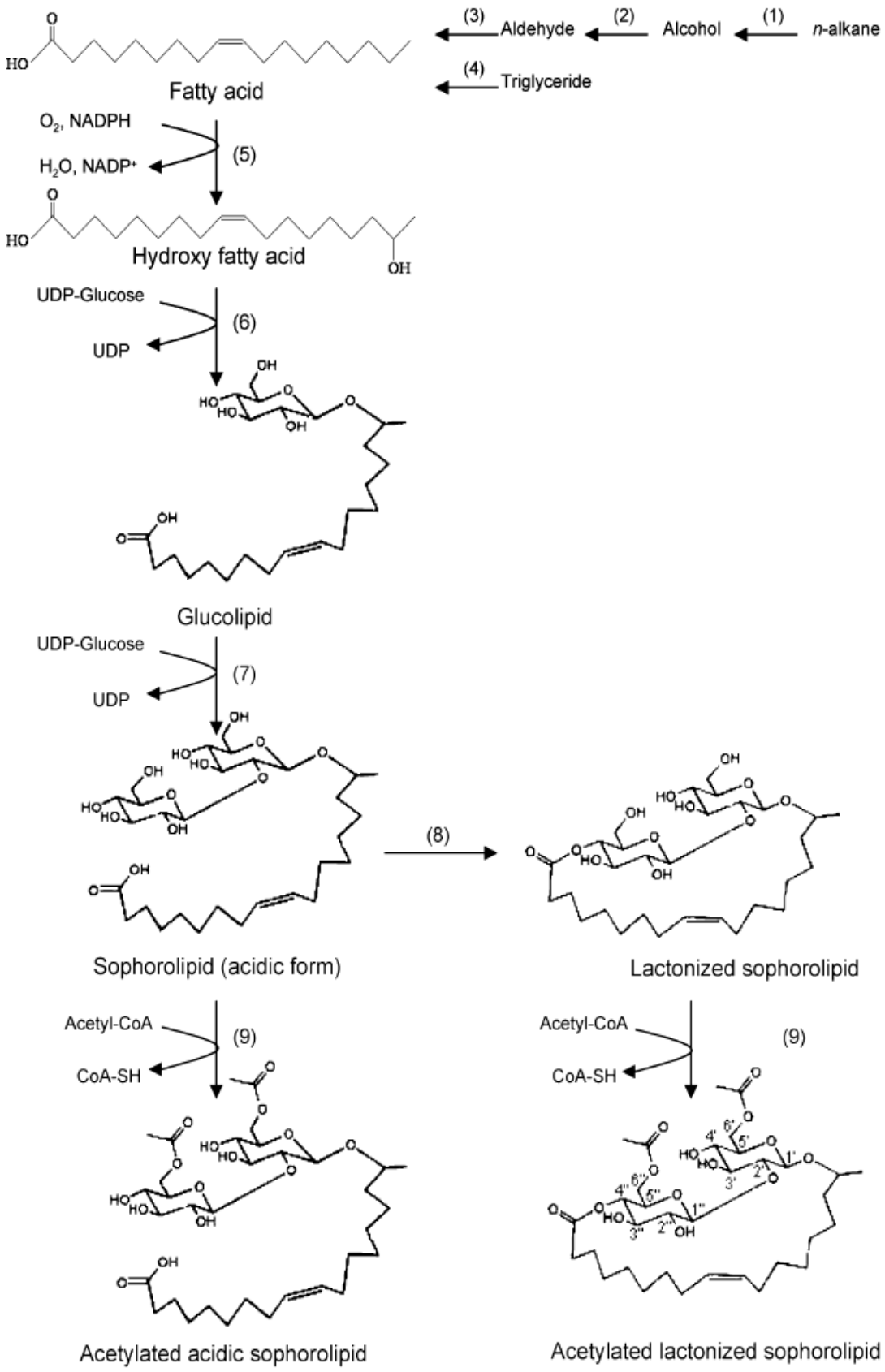


Figure 1.6 Proposed biosynthetic pathway for SL synthesis (1) Cytochrome P450 monooxygenase, (2) alcohol dehydrogenase, (3) aldehyde dehydrogenase, (4) lipase, (5) cytochrome P450 monooxygenase, (6) glucosyl transferase I, (7) glucosyl transferase II, (8) lactoneesterase and (9) acetyltransferase -adapted from [3]

1.3.4 Physiological role of SLs: SLs are physiologically important to the producer in 3 main aspects. One of the perceived benefits of SLs to the producing organisms is to help access and utilize lipophilic substrates [33]. As SL synthesis is associated with nitrogen starvation. It is suggested that formation of glycolipids is some sort of overflow metabolism, by means of extracellular storage material [34]. As the classical SL producers- *C. bombicola* and *C. apicola* are originally isolated from environments with high osmotic strength, SL production may be a way of dealing with the high sugar concentrations by sequestration. The SL producers can make the sugar less available for other organisms by modifying and storing it. SLs also possess antimicrobial properties against various genera [3]. Therefore SL production may also be regarded as a kind of defence mechanism against competing microorganisms. Ratio of the acidic and lactone forms in the SL mixture varies with the growth conditions and differentiated supply of lipophilic substrates [35]. Probably the nature of hydrophobic substrate dictates the need for raising defence. In presence of species competing for same substrates; yeast gains edge by producing the lactone form of SL which is biologically more active.

1.3.5 Variety of substrates used for production of SLs: Even when no hydrophobic carbon source is available, *C. bombicola* can still produce SLs. These SLs are formed de novo; the required fatty acids are derived by de novo synthesis by the fatty acid synthase (FAS) system, hydroxylated and subsequently incorporated into SLs. The de novo SLs also possess a C16 or C18 fatty acid tail and even in the presence of a suitable hydrophobic carbon source for SL production, de novo formation of SLs is still observed. Fatty acid synthesis can be blocked by the antifungal agent cerulenin, an inhibitor of the fatty acid synthase (FAS) complex. I.N.A. Van Bogaert *et. al.* (2008) used Cerulenin for inhibiting the de novo fatty acid synthesis and achieved reduced complexity of SL mixture [36]. When the yeast cells are supplied with only one type of carbon source, such as glucose or n-alkanes, SL formation is observed. The production is however considerably higher when two types of carbon sources i.e a hydrophilic (glycidic) and a hydrophobic (lipidic) carbon sources, are provided [3]. As represented by Figure 1.6; it may be understood that the SL producing yeasts do possess necessary enzymes required to convert different lipidic substrates to respective

fatty acid. This justifies the organism's ability to utilize n-alkanes, alcohols, aldehydes as well as triglycerides as SL precursors. SL producers prefer for direct incorporation of hydrocarbons having a chain length between 15 and 18 carbon atoms if these are in the medium [37]. Despite their overall heterogeneity—little variation in the lipid tail length of the *C. bombicola* SLs has been observed. The range is limited to C16–C18 fatty acids and is governed by the specificity of a cytochrome P450 monooxygenase. Two strategies to circumvent this C16–C18 preference are described by I.N.A. Van Bogaert *et. al.*, 2010. The first one skips the controlling action of the cytochrome P450 enzyme by supplying the yeast with already hydroxylated substrates, while the other method is based on the deception of the enzyme by presenting it substrates structurally resembling stearic acid [38].

1.3.5a Effect of hydrophilic carbon source: Hommel and Huse (1993) studied the effects of different carbohydrates namely glucose and galactose on SL production. It was found that the amounts of SL were much larger with glucose as source of carbon. In presence of alkane as hydrophobic carbon source in addition to carbohydrate; glucose served as energy supply as well as a direct precursor of sophorose. This was energetically favorable than using the sugar skeleton both for energy supply and as lipid precursor [39]. Zhou and Kosaric (1993) studied the applicability of cheese whey as a precursor for SL synthesis. Cheese whey is rich in lactose; a disaccharide of galactose and glucose. Therefore the sugars-galactose and lactose were also checked for SL production. In presence of lactose or cheese whey alone the organism did not grow, but when supplemented with hydrophobic substrate there was some SL production along with intracellular lipids. Galactose with olive oil also resulted in intracellular as well as extracellular lipids production. As compared to others, glucose showed maximum extracellular lipid i.e. SL production [40]. However it was found that *C. bombicola* did not consume lactose. Daniel *et. al.*, 1999 developed a two stage process in which oleaginous yeast *Cryptococcus curvatus* was grown on deproteinized whey to accumulate high amounts of single cell oil. Further this crude cell extract was used directly for growth and SL production by *C. bombicola* in order to make the process economical [41]. Solaiman *et. al.* (2004, 2007) reported the use of low cost soy-molasses, which is a co-product of soybean oil processing in place of

glucose [42, 43]. 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 [44]. The same group also directed synthetic dairy waste water supplemented with sugarcane molasses for SL synthesis and could achieve 93% COD removal efficiency [45].

1.3.5b Effect of hydrophobic carbon source: A lot of substrates can act as hydrophobic carbon source such as oils, fatty acids, and their corresponding esters, alkanes, etc. The level of SL formation during fermentations based on alkanes as hydrophobic feed-stock largely depends on the chain length of the used substrate. Hexadecane and octadecane give the best production yields. They appear to be directly converted into hydroxy fatty acids and incorporated into the SL molecules, in this way strongly influencing the fatty acid composition of the SL mixture [3]. The amount of direct incorporation increased with increasing alkane chain length to a maximum for pentadecane, hexadecane and heptadecane. As the length of the alkane substrate increased further, the amount of direct incorporation then decreased until there was no apparent incorporation for eicosane [35]. 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 [46]. Tulloch *et. al.*, (1968) utilized variety of alkanes namely, hexadecane, octadecane, eicosane, docosane, tetracosane and esters of different fatty acids i.e. palmitic, stearic, linoleic and eicosenoic as lipophilic or hydrophophobic substrates for SL synthesis by employing *Torulopsis magnolia* [26]. The corresponding fatty acids- palmitic, stearic, oleic, linoleic acid have also been used for SL synthesis [33,47,48,49] Because free fatty acids can disturb the electron balance of the cells, sometimes fatty acid methyl or ethyl esters, or triglycerides are used. In this case, esterases mediate the gradual release of fatty acids [3]. However *C. bombicola* is capable of utilizing the free fatty acids for SL synthesis. Prabhune *et. al.* 2002, 2012 have reported the production of SLs with non-typical fatty acids, Linolenic (18:3) and Arachidonic acid (20:4) with higher degree of unsaturation [50, 51]. The same

research group i.e. Prabhune *et.al*., 2012 have also reported the use of fatty alcohols with chain length C12-C14 such as lauryl alcohol as hydrophobic carbon source for SL synthesis. These precursors confer better skin compatibility to the resultant SL [52]. Most common vegetable oils such as rapeseed, sunflower, olive, safflower, soybean, meadowfoam, coconut and corn oil—which are rich in C16–18 fatty acids—are highly suitable and are readily incorporated into the SL molecule [53]. Wadekar *et. al.*, 2012 have tried out jatropha, karanj and neem oil for SL production but could end up with low yields [54]. As a part of the work included in this thesis, we have also tried out the non-edible oils Jatropha and Pongamia as an attempt to reduce SL production cost. Through the use of resting cell method and repeated use of cell mass, process could be made efficient [unpublished data]. Recently Li *et. al.*, 2013 reported synthesis of SLs with Docosahexaenoic acid and Eicosapentaenoic acid using *Wickerhamiella domercqiae*. In this study, fish oil being the rich source of long chain polyunsaturated fatty acids components was used [55].

1.3.5c Use of low cost substrates for improving economics of SL production: Efforts have been put further in order to utilize cheap substrates generated as waste from other processes/ activities. These can be regarded as the attempts to make the SL production process economical. Ashby *et.al.* (2005) synthesized 60g/l SL from Biodiesel co-product stream [56]. Deshpande *et.al.* (1995) reported 120g/l SL using animal fat from meat processing industry [57]. Fleurackers *et.al.* (2006) demonstrated SL yield 49g/l using waste frying oil [58]. Shah, V *et.al.*(2007) reported utilization of restaurant waste oil as a precursor for SL production (yield obtained was 34g/l) [59]. Also Cheese whey, Molasses, corn steep liquor, residues from vegetable oil refinery have been reported as raw material for SL production (Makkar *et. al.* 2002) [22].

1.3.6 Synthesis of novel structure SLs: Reports on studies of SLs of more structural diversity are rare [60]. Few research groups have attempted to modify the fundamental SL skeleton; driven by the fact that more structural derivatives of SLs are needed to better define structure-biological activity relationships and thereby enhance SL efficacy and its spectrum of action [61]. To a certain extent, structural variation (and hence physical properties) can be achieved by changing

the lipidic carbon source, which alters the SL fatty acid content [33]. Amino acid conjugated SLs at tail end were synthesized by chemical method and the derivatives were shown to have superior antibacterial as well as anti viral activity [61]. There have been attempts to change the SL head group to glucose by action of enzymes-glycosidases, naringinase, hesperidinase [49, 62, 63]. Shah V. *et. al.*, 2007 replaced the glucose with different sugars namely- Fructose, Xylose, Ribose, Lactose, Mannose, Arabinose and Galactose in order to get different glycolipid molecules bearing these sugars as head groups. The antibacterial activity of thus formed compounds was checked. The nature of the carbohydrate head was shown to have an influence on the antibacterial activity of the glycolipid [64]. We have checked a novel yeast isolate- *Pichia caribbica* (MTCC5703) for its ability to synthesize a glycolipid bearing xylose as head group [unpublished data].

1.3.7 Detection of SLs: SLs can be detected by anthrone reagent method at 620nm in which the carbohydrate part of the molecule is involved [65]. Alternatively, SLs can be analysed by thin layer chromatography on silica gel plates. Resolution can be achieved using the chloroform, Methanol based solvent system. For visualization, iodine vapor and molish reagent are typically used for detection of lipids and carbohydrates respectively [28].

1.4 Applications of Sophorolipids

On account of their surfactant property; SLs find application in various fields ranging from cleaning purposes, petroleum, cosmetics, foods, therapeutics, bioremediation etc. As seen earlier in section 1.1, the growing environmental awareness is driving the current market trend from petrochemical origin surfactants to biologically synthesized surfactants. The advantages associated with biosurfactants are that they can be produced from renewable feedstock or even waste streams through a natural fermentation process. Furthermore, they are readily degradable and display low eco-toxicity. In the forthcoming sections various applications of SLs have been discussed.

1.4.1 SLs for cleaning purposes: Applicability of SLs as an ingredient of laundry detergent was identified previously by Hall *et. al.* 1996 and Futura *et. al.,* 2002 [66, 67]. 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 SLs to additional surfactant used in detergent formulations is generally 4:1 to 3:2 [66]. SLs preserve their surface lowering properties despite high salt concentrations. Few multinational companies namely Belgium-based Ecover, France-based Groupe Soliance, Japan-based Saraya and South Korea-based MG Intobio are developing SL and other biosurfactants' based products [68, 69, 70]. Saraya has already commercialized its SL produced from fermented sugar and soybean oil in a low-foam dishwasher detergent called Sophoron.

1.4.2 SLs in petroleum industry: SLs also may be used as active surfactants in formulations for enhanced oil recovery [71]. Biosurfactant mixtures consisting of SL, Rhamnolipid and lecithin were used in vegetable oil extraction for biofuel application by Nguyen *et.al.*, 2011. Higher extraction efficiency was obtained with diesel-based reverse micellar microemulsions of biosurfactant mixtures than with either diesel itself or conventional hexane [72]. SLs are also useful in removing hydrocarbons from drill material, and in the regeneration of hydrocarbons from dregs and muds [73, 74]. SLs can also be applied for decontaminating porous media such as soils and groundwater tables polluted by hydrocarbons [3].

1.4.3 Bioremediation using SLs: Nickel contaminated stearic fatty acid residue was transformed to value added SL product while nickel was sequestered primarily in biomass for safe removal and recovery. SL product obtained from nickel contaminated lipid wastes had low nickel levels (<5 mg/l) that could allow its use in low-end consumer products and household applications [71]. SL washing with 4%concentration from *Torulopsis bombicola* removed 25% of the copper and 60% of the zinc from the heavy metal contaminated sediments. SLs could remove the carbonate and oxide-bound zinc. In this study Rhamnolipid was effective than SL but SLs were superior to surfactin [75].

1.4.4 Cosmetics with SLs: SLs possess antibacterial, antioxidant, moisturizing, wetting foaming and emulsifying activity. Apart from these they do stimulate dermal fibroblasts. All these properties make SL a suitable ingredient of cosmetic formulations such as lotions, moisturizers, body washes, hair products, , lip colour, eye shadow, acne treatment creams, deodorant, skin smoothing and anti wrinkle products [76]. There are several patents on the use of SLs for cosmetic purposes

[77-80]. SLs are also effective for reducing the subcutaneous fat overload [81]. Soliance, a French company has commercialized skin and body cosmetics with SL as an active ingredient [69]. The high production costs can be tolerated for biosurfactants when used in low-volume specialty markets (e.g. cosmetics, healthcare, etc.) [71] as represented in Figure 1.7.

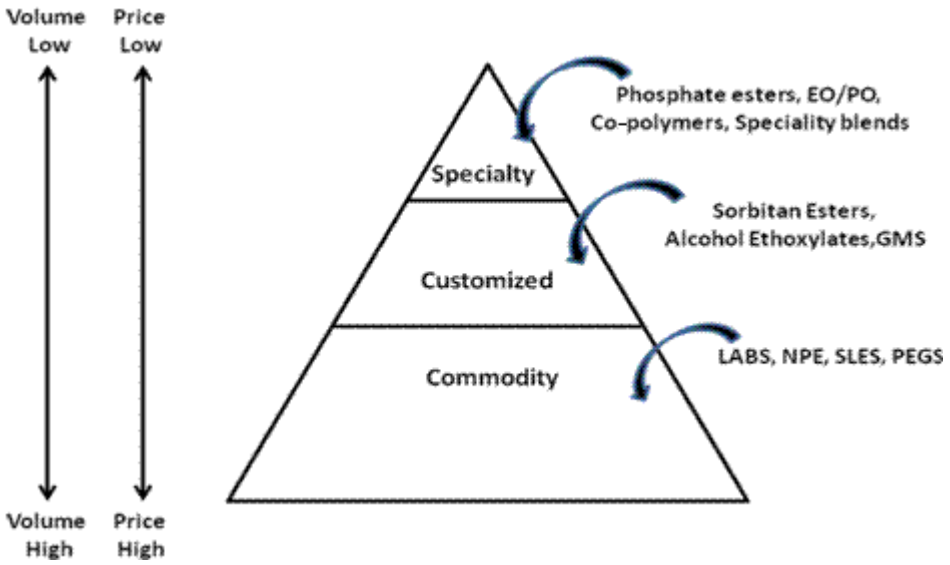


Figure 1.7 Pyramidal distribution of surfactants

1.4.5 SLs for Enzyme action enhancement: R. Gross *et. al.* (2005) reported the use of SL as protein inducer and protein repressor. They found that in presence of SL, *Bacillus subtilis* produced higher amount of amylase. Similarly *Pleurotus ostreatus* 473 strain when grown in presence of SL showed increased production of laccase while manganese peroxidase production decreased [82]. Menon *et.al.* (2010) reported that the rate of hydrolysis with thermostable xylanase was increased by 20% with the addition of SL at 1%w/v [83].

1.4.6 SLs in Agriculture and Foods: Biosurfactants can be used 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 [84]. This way SLs can improve the quality of wheat flour products [85]. In bakery and ice cream formulations biosurfactants play role in controlling consistency, retarding staling and solubilising flavour oils. They are also utilized as fat stabilizer and antispattering agent during cooking of oil and fats [84]. SLs can be used for cleaning fruits and vegetables because of their antimicrobial action [86]. They are

also useful against plant pathogenic fungi namely *Phytophthora sp.* and *Pythium sp.*[87]. Stanghellini *et. al.* (1996) have reported the use of Rhamnolipids for controlling plant pathogenic zoosporic fungi [88]. Similarly SLs can also be expected to show such utility though there are no reports. Moreover the surfactant property can allow their usage as adjuvant for pesticides etc. to facilitate the spreading and better penetration through the waxy surfaces of insects, fungi or plant leaves.

1.4.7 SLs as therapeutics: The US FDA has also approved biosurfactants/sugar esters for the use in food and pharmaceuticals. Tests with SLs pointed out that they are not irritating to the skin, do not trigger allergic reactions and have an oral safety level which is greater than or equal to 5ml/kg weight. Cytotoxicity was evaluated with human epidermal keratinocytes and was proven to be low [4]. These facts dictate the suitability of SLs for various therapeutic uses.

1.4.7a Antimicrobial property- against bacteria, algae, viruses: SLs are found to exhibit antimicrobial action against range of classes. The proposed primary mechanism of action of these surfactants is membrane lipid order perturbation, which compromises the viability of microorganisms [61]. Antibacterial property of SLs has been reported widely and generally they are more effective against gram positive organisms. Also the variation in structure i.e. fatty acid moiety or hydrophilic sugar moiety has some influence on the antibacterial action. Also the lactone form of SL is considered to be biologically more active [52, 64, 89, 90]. SL derived from lauryl alcohol was reported to exhibit potent antibacterial action against gram positive bacteria as compared to gram negative ones. It also inhibited the pathogenic yeast- *C. albicans* [91]. Sun, X. *et.al.*, 2004 have demonstrated the use of SL with loess for harmful algal bloom mitigation [92]. V Shah *et. al.*, 2005 have documented in vitro spermicidal and anti-HIV virucidal activities of SL analogs wherein they evaluated the antiviral activity of acidic, lactonic as well as different ester analogues of sophorolipids. The concentration range used was 9µg/ml to 330µg/ml. The diacetate ethyl ester was found most effective which brought down the virus titer by 5 log. Acidic form was more virucidal which was in contrast to the antiviral activity displayed by lactonic form [93]. The same research group; Gross *et. al.,* 2007 have explored the antiviral

activity of SLs. The world patent WO2007/130738 A1 by Gross R., 2007 has covered the use of SLs as antiviral agents for treating the infections caused by herpes and related viruses [94]. Chattopadhyay *et. al.* in 2002 have checked the action of different chemical surfactants on survival and sorption of viruses. They found that surfactants have a significant impact not only on the survival of viruses in soil and subsurface systems but also on their transport. Survival of viruses was found to decrease in the presence of synthetic surfactants. The surfactants reduce the sorption of virus particles by competing adsorption sites on different sorbents [95]. This can possibly be the mechanism of action for SLs in addition to membrane lipid order perturbation.

1.4.7b Induction of terminal differentiation: SLs are structurally similar to the eukaryotic cell membrane components-glycosphingolipids and gangliosides. Similar to these two; SLs can also mediate several cellular processes. One of those is cellular differentiation. Isoda *et. al.,* (1997) have shown that the microbial extracellular glycolipids induced differentiation and inhibition of the protein kinase C activity of human promyelocytic leukemia cell line HL60. Also the SL caused Myelogenous leukemia cell line K562 to differentiate into megakaryocytes and Basophilic leukemia cell line KU812 to differentiate into granulocytes [96].

1.4.7c Anticancer activity: Chen *et.al.* have reported a new strain for SL synthesis (*Wickerhamiella domercqiae*) and showed that the SL induced apoptosis in H7402 human liver cancer cells in dose and time dependent manner [27]. Fu *et.al.* (2008) showed that SLs induced human pancreatic adenocarcinoma cell line i.e. HPAC to undergo necrosis [97].

1.4.7d Cytokine stimulation and Immunoregulation: SLs have been reported to function as immunomodulators for the treatment of endotoxic (septic) shock by cytokine downregulation [98]. Administration of SLs after induction of intra-abdominal sepsis resulted in significantly decreased mortality in rat model. This may be mediated in part by decreased macrophage production and modulation of inflammatory responses [99]. SLs are new, promising modulators of immune response in animal models. These compounds act by suppressing the production of IgE and decreasing the lung inflammation in a mice model. They are capable of

reducing sepsis related to mortality after 36 h in mice with septic peritonitis by modulating nitric oxide, molecule adhesion, and production of cytokines and decrease the production of IgE in vitro of U266 cells, possibly by affecting the activity of plasmatic cells. SLs decreased the production of IgE in U266 cells by down-regulating important genes implicated in the biopathology in a synergic manner [100].

1.4.7e Antiadhesive action or biofilm inhibition: The bioconditioning of surfaces through the use of microbial surfactants have been suggested as a new strategy to reduce adhesion. The use of biosurfactants, which disrupts biofilms and reduce adhesion, in combination with antibiotics could represent a novel antimicrobial strategy, once antibiotics are in general less effective against biofilms than planktonic cells; the disruption of biofilm by biosurfactant can facilitate the antibiotic access to the cells [84].

1.4.7f SLs as intermediates for synthesis of 19-HETE and 20-HETE: SLs synthesized using Arachidonic acid as hydrophobic carbon source on acid hydrolysis; lead to production of the vasoactive compounds- 19-hydroxy-5Z, 8Z, 11Z, 14Z-eicosatetraenoic acid (19-HETE) and 20-hydroxy-5Z, 8Z, 11Z, 14Z-eicosatetraenoic acid (20-HETE). These compounds have pharmaceutically beneficial properties such as autoregulation of blood pressure, vascular tone and other physiological roles. The present biotransformation route allowed by-passing of expensive, multistep and hazardous chemical route [51].

1.4.8 Self assemblies of SLs: The amphiphilic nature of SLs confers the ability to arrange itself in the form of structures such as micelles, bilayes or vesicles. Zhau *et. al.* (2004) were the first to report self assemblies of SLs. Supramolecular structures of the self-assembled aggregates of SL molecules form at different pH values. In acidic conditions (pH < 5.5), giant twisted and helical ribbons of 5-11 μ width and several hundreds of micrometers length were observed for the first time. Increase in solution pH values slowed ribbon formation, decreased ribbon yield, and increased the helicity and entanglements of the giant ribbons [101]. Unusual, pH induced self assembies of SLs have been explained by Baccile *et. al.*. (2012) [102]. Zini *et. al.* 2008 reported ROMP polymerization of natural

diacetylated SLs gave a high molecular weight polymer with asymmetric bola amphiphilic repeating units. Upon annealing at 80°C the poly SL recrystallized and concomitantly the disaccharide units space out again at 2.44nm [103]. Stimuli-responsive surfactants are a class of compounds which have recently attracted major interest in polymer chemistry and material science with applications in wide range of fields namely- Stabilization of emulsions, suspensions or foams, drug encapsulation and delivery, hard-surface cleaning, personal care applications [102]. Recently P.K. Singh *et.al*., 2013, have reported the formation of highly spherical mesoscale molecular assembly of SLs created by a pulsed laser processing. These structures exhibited strong green fluorescence suggesting its utility for biomedical imaging in addition to hyperthermia induction [104]

1.4.9 SLs in nanotechnology: Kasture *et. al.* (2007) demonstrated the application of SLs obtained from oleic acid as a capping agent for Cobalt nanoparticles. Upon capping the nanoparticle surface, the sugar moiety of these SLs is exposed to the solvent environment, making the nanoparticles stable and water redispersible for biomedical applications. It is imperative that these nanoparticles are dispersed in the aqueous solutions. The synthesis and stabilization/phase transfer of nanoparticles to an aqueous medium in addition to magnetic properties pose major bottlenecks in this field [105]. Similarly, SL reduced/capped silver nanoparticles have been synthesized by Singh *et. al.,* 2009 Such SL capped Ag nanoparticles demonstrated highly potent antibacterial activity against both Gram-positive and Gram-negative bacteria. These SL capped silver nanoparticles can be obtained as a stable powder that can be re-dispersed in water as desired [106]. The same research group has also demonstrated the construction of Ag nanoparticle studded porous polyethylene scaffolds through the use of SLs. These scaffolds can be useful in tissue engineering as they support the growth of mammalian cells while inhibiting bacteria [107]. Baccile *et.al.* (2013) reported the synthesis of functional iron oxide nanoparticles (NP) in a one and a two-step method using a natural functional SLs. The final carbohydrate coated iron oxide nanoparticles represent interesting potentially biocompatible materials for biomedical applications [108].

1.5 Scope of the work

Economy is often the bottleneck of biotechnological processes. Biosurfactants have to compete with surfactants of petrochemical origin in three aspects: cost, functionality and production capacity. The success of biosurfactant production depends on the development of cheaper processes and the use of low cost raw materials, which account for 10–30% of the overall cost. So attempts are being made to explore cheap and renewable sources for SL production [22]. When SLs are solved in water, they lower the surface tension from 72.8 mN/m down to 40 to 30 mN/m, with a critical micelle concentration of 40 to 100 mg/l. The hydrophilic/lipophilic balance is 10 to 13, making SLs useful as detergents or as stabilizers for oil-in-water emulsions [3]. Thus this amphiphilic molecule- SL stands as potential candidate to replace chemical surfactants in detergents. Here we have explored the utility of non-edible oils Jatropha and Pongamia as cost saving raw materials and further checked the applicability of the synthesized SLs as fabric cleaner.

As mentioned before; SLs exhibit antimicrobial properties against range of microbial classes. We have tried to explore the antiviral potential of SLs against different classes of virus and checked if SL triggers any self defence mechanisms in host cells. Mannosyl Erythritol Lipid-A, a type of glycolipidic biosurfactant containing cationic liposomes promoted the gene transfection efficiency five to seven times with mammalian cultured cells [109]. Sun, X. *et.al.*, 2004 have demonstrated the synergistic effects of combination of SL and loess for harmful algal bloom mitigation to bring down the effective dose of both when used individually [92]. We have attempted to check the conjugative effect of antibiotic and SL in the expectation that the co-administration will facilitate the entry of antibiotic molecules across the cell membrane so there will be better handling of bacterial infections.

The ubiquitous membrane components glycosphingolipids and gangliosides are reported to modulate cell growth, adhesion and trans membrane signaling. Dramatic changes in glycosphingolipids composition and metabolism of glycosphingolipids were observed during oncogenesis, differentiation and oncogenic transformation. Certain gangliosides are reported to induce

differentiation of leukemia cells and regulate some protein kinases in cells. The glycolipids are similar in structure with gangliosides as both of them are amphiphilic in nature [96]. We have checked the differentiation inducing activity of SLs against glioma cell line which will have implications in treatment of glioblastoma- a kind of Central Nervous System tumor resistant to conventional chemotherapy.

The different structural classes of SL cause wide variation in physicochemical as well as biological properties. Few research groups have attempted to modify the fundamental SL skeleton; driven by the fact that more structural derivatives of SLs are needed to better define structure-biological activity relationships and thereby enhance SL efficacy and its spectrum of action [61]. So here, a xylose fermenting yeast which was identified to be *Pichia caribbica* (MTCC5703) has been employed for the biosurfactant production. The non- typical SL producing yeast strain was checked if it can produce a different glycolipid bearing xylose as head group i.e. xylolipid. The product was characterized for its physicochemical, antibacterial properties and structural information with various analytical tools.

1.6 Outline of the thesis

The thesis has been divided into 5 chapters

Chapter I. Introduction

The ***first chapter*** is an introduction to the thesis. The chapter gives brief literature review about different surfactants, SL- the biosurfactant which is the focus of the thesis and various applications of SLs. With this background, the scope and objectives of the thesis have been defined.

Chapter II. Production of sophorolipids using non edible oils and their use as an alternative/ additive to laundry detergents

The ***second chapter*** is about the attempt to reduce the production cost by using non-edible oils namely Jatropha and Pongamia. The work involves optimization experiments to maximize the yields of SLs. Further, both the SLs have been characterized in detail regarding their physicochemical properties. Also the emulsifying property has been studied with reference to changes in temperature,

pH and in hard water. To check the utility as fabric cleaner; detergency test has been performed.

Chapter IIIa. Sophorolipid biosurfactants act in synergy with antibiotics to enhance their efficiency

In the ***third chapter (part a)*** the effect of co-administration of SL and antibiotic has been evaluated. SLs are amphiphilic in nature and have been reported to form micellar, vesicular structures. Therefore experiments were carried out using different antibiotics against different index bacteria with the expectation that co-administration will improve the efficacy. Topographical and morphological changes were also studied with Scanning electron microscopy.

Chapter IIIb. Exploration of antiviral activity of SLs

The ***third chapter (part b)*** describes antiviral action of Oleic, Linoleic acid derived SLs against different families of viruses such as plus stranded RNA, segmented single negative stranded RNA and double stranded DNA.

Chapter IV. Differentiation inducing ability of SLs against glioma cells

The ***forth chapter*** deals with the differentiation inducing ability of SLs. The effects have been investigated on LN229 - a glioma cell line which has been reported for the first time. In response to different SL forms, various morphological changes were observed. The finding suggests the utility of SLs as a pharmaceutical agent for the treatment of glioblastomas [110].

Chapter Va. Glycolipid production by a novel yeast- *Pichia caribbica* (HQ222812) with xylose as a head group and its advantageous properties

The ***fifth chapter (part a)*** deals with the use of a new xylose fermenting yeast- *Pichia caribbica* for biosurfactant production so as to achieve the less explored head group diversity in SL structure. It includes optimization experiments, structural and physicochemical characterization of the product and also determination of its antibacterial activity. Presence of xylose as head group was anticipated to give altered physicochemical and biological activities. In

accordance to the same, low CMC value and better inhibitory action was demonstrated against *Staphylococcus aureus*, a gram positive bacterium.

Chapter Vb. Crystalline xylitol production by a novel yeast- *Pichia caribbica* (HQ222812) and its application for quorum sensing inhibition in gram negative marker strain *Chromobacterium violecium* CV026

The ***fifth chapter (part b)*** is about quantitative production of xylitol from D-xylose along with a safe procedure for product extraction. The ability of xylitol to act as a quorum sensing antagonist has also been demonstrated [111].

Conclusions

The last chapter summarizes the work presented in the thesis and emphasizes on possible further research in this area.

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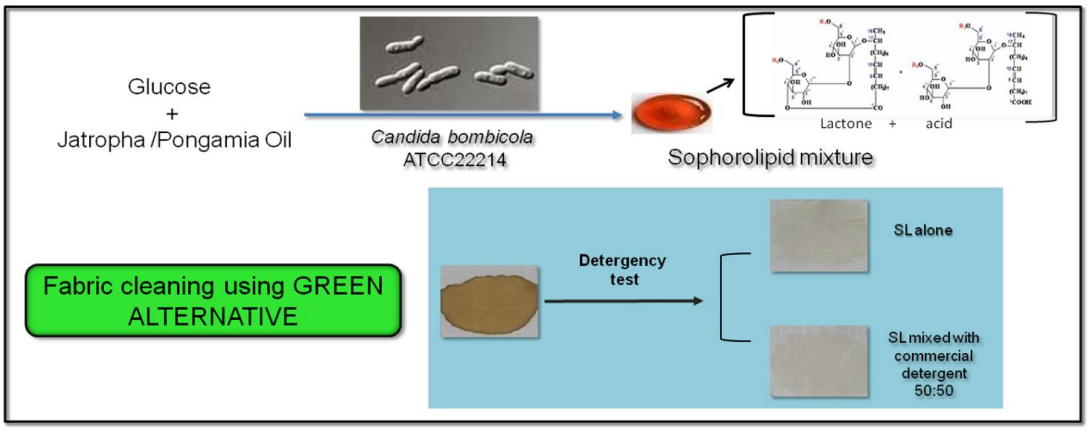
Chapter II

Production of sophorolipids using non edible oils and exploration of their potential as an alternative/ additive to laundry detergents



Sophorolipids (SLs) are glycolipidic biosurfactants suitable for various biological and physicochemical applications. In order to compete with the synthetic surfactants, production cost of SLs should be lowered. For the same purpose, the attempt has been made to reduce the raw material cost by using non-edible oils namely Jatropha and Pongamia. Fermentation parameters were optimized and also resting cell method was used so as to maximize the yields. Thus the yields 15.25g/l and 19.3g/l could be achieved for Jatropha oil derived SL (SLJO) and Pongamia oil derived SL (SLPO) respectively with 1% v/v oil feeding. SLs obtained from both oils displayed good surfactant property with the Critical Micelle Concnetration (CMC) values 9.5mg/l for SLJO and 62.5mg/l for SLPO. Keeping the prospective use of these SLs in mind, the physicochemical properties were checked along with emulsion stability under temperature, pH stress and in hard water. Also, antibacterial action and stain removal capabilities in comparison with commercial detergent were demonstrated. SLs obtained using both oils; Jatropha and Pongamia enhanced the detergent performance. Based on the results, it can be said that these SLs have utility as value fabric cleaner with advantageous properties such as skin friendly nature, antibacterial action and biodegradability. These properties of SLs make them potential green molecules to replace synthetic surfactants in detergents so as to reduce harm caused to environment through excessive detergent usage.

2.1 Introduction

In terms of production volume, surfactants belong to the most important classes of industrial chemicals with a current total world production exceeding 23 million tonnes per year [1]. About half that volume is used in household and laundry detergents and the other half in a wide variety of industrial sectors, particularly the chemical, textile, food, paper industry, cosmetics, personal, health care, and agriculture, etc. The majority of the currently used surfactants are petroleum-based and are produced by chemical routes. These surfactants in washing applications inevitably end up in the environment after use. They are often toxic and hazardous to the environment, and their use may lead to significant ecological problems [2]. The ecotoxicity, bioaccumulation, and biodegradability of surfactants are therefore issues of increasing concern. Most laundry detergents in India are phosphate based. Phosphates are a major source of water pollution and have become the direct cause of more than 40% of human and animal diseases [3]. These phosphates also contribute to eutrophication condition. With an increase in phosphates, especially in the absence of species feeding upon algae, algal blooms grow splendidly on the excess phosphorus and can produce toxins, killing fish, dolphins and plants. They can also indirectly cause oxygen depletion at greater depths, through microbial breakdown of dead algal cells [4].

Surfactant forms 15% portion of the detergent formulation [5]. Xenobiotic surfactants comprise a very important group of potentially toxic compounds that are believed to be harmful due to disruption of the function and structure of bacterial membranes. LABS i.e. Linear alkyl benzene sulphonates is a commonly used laundry surfactant. Brandt *et. al.* 2001 have studied the toxicity of LABS against *Nitrosomonas europaea* cells - Autotrophic ammonia-oxidizing bacteria (AOB) which have been considered ideal microbial indicators of perturbations caused by pollutants in natural environments. It was observed that cells pre-exposed to sublethal level of LABS (10 mg/l) showed severely affected cell functions evident by cessation of growth, loss of viability, and reduced NH_4 oxidation activity. Thus it was demonstrated that long-term incubation even at sublethal LABS levels was detrimental and the organism was unable to adapt to this compound [6]. Ginkel *et. al.*, 1989 reported construction of microbial consortium to achieve complete degradation of LABS [7]. Thus LABS is difficult to

be degraded by normal microbial flora of water bodies and causes harm to the environment.

Attempts should be made to reduce the detergent load in to the environment. There is an increasing demand for mild, non toxic, biodegradable surfactants made from renewable or 'natural' raw materials. Consumers are demanding products that function well at ambient physical conditions as well as multifunctional products that allow them to save money and reduce amounts of chemicals added to wastewater.

SLs (SLs) are a kind of microbial extracellular biosurfactants produced by non-pathogenic yeasts, such as *Candida bombicola, Yarrowia lipolytica, Candida apicola* and *Candida bogoriensis* [8]. These SLs are generally present in the form of disaccharide sophoroses (2-O-β-D-glucopyranosyl-D-glucopyranose) linked β glycosidically to the hydroxyl group at the penultimate carbon of fatty acids.When SLs are solved in water, they lower the surface tension from 72.8 mN/m down to 40 to 30 mN/m, with a critical micelle concentration of 40 to 100 mg/l. The hydrophilic/lipophilic balance is 10 to 13, making SLs useful as detergents or as stabilizers for oil-in-water emulsions [2]. Thus this amphiphilic molecule- SL satisfies above mentioned criteria and stand as an ideal candidate to replace chemical surfactants in detergents.

However like other biotechnological processes; economy is the bottleneck in case of biosurfactants as well. Biosurfactants have to compete with surfactants of petrochemical origin in three aspects: cost, functionality and production capacity (Makkar *et. al.* 2002). The success of biosurfactant production depends on the development of cheaper processes and the use of low cost raw materials, which account for 10–30% of the overall cost [9]. So attempts are being made to explore cheap and renewable sources for SL production.

Daverey *et.al.* (2009) produced 63.7g/l SLs using low cost media based on sugarcane molasses and soybean oil, sunflower oil or olive oil with oil feeding 100g/l [10]. Daniel *et.al.* (1998) used whey concentrate and rapeseed oil for SL production (yield obtained was 422g/l with 100g/l oil feeding) [11]. Ashby *et.al.* (2005) synthesized 60g/l SL from Biodiesel co-product stream (100g/l precursor feeding) [12]. Deshpande *et.al.* (1995) reported 120g/l SL using animal fat from

meat processing industry (100g/l precursor feeding) [13]. Fleurackers *et. al.* (2006) demonstrated SL yield 49g/l using waste frying oil (37.5g/l oil feeding) [14]. Shah, V *et.al.*(2007) reported utilization of restaurant waste oil as a precursor for SL production (yield obtained was 34g/l with 40g/l oil feeding)[15]. Also Cheese whey, Molasses, corn steep liquor, residues from vegetable oil refinery have been reported as raw material for SL production (Makkar *et. al.* 2002) [9]. However the non-edible oils namely Jatropha and Pongamia oil have not been studied in detail for purposes other than biofuel production. These oils are not suitable for human consumption due to their unfavourable odour, colour, composition and are, therefore, available at much cheaper rates. During SL synthesis; the vast majority of fatty acids are either elongated or metabolized to C16 or C18 fatty acids by the *Candida bombicola* ATCC 22214. The best yields are obtained using oleic acid (C18:1) as lipophilic precursor [2]. These above mentioned vegetable oils are majorly composed of saturated or unsaturated fatty acids with chain lengths of 16 or 18 carbon atoms, making them an ideal substrate for direct incorporation and the consequent high SL production and yield. Jatropha oil contains up to 80-95% of fatty acids ranging between C16 to C18 [16] while Pongamia oil contains 68-82% of C16-C18 chain length fatty acids [17]. The percentages of constituent fatty acids are displayed in the following Tables 2.1 and 2.2.

Fatty acid	Wt % in Jatropha oil
Palmitic acid (C16:0)	15.40
Stearic acid (C18:0)	6.27
Oleic acid (C18:1)	44.93
Linoleic acid (C18:2)	33.41
Arachidic acid (C20:0)	Trace
Docosanoic acid (C22:0)	Trace
Tetracosanoic acid (C24:0)	Trace

Table 2.1 Fatty acid composition of Jatropha oil

Fatty acid	Wt % in Pongamia oil
Palmitic acid (C16:0)	10.6
Stearic acid (C18:0)	6.8
Oleic acid (C18:1)	49.4
Linoleic acid (C18:2)	19.0
Arachidic acid (C20:0)	4.1
Docosanoic acid (C22:0)	5.3
Tetracosanoic acid (C24:0)	2.4

Table 2.2 Fatty acid composition of Pongamia oil

The typical SL producing yeast strain *Candida bombicola* (ATCC22214) is a robust organism and it can survive and produce SL in presence of the alkaloids and phenolic compounds in these non edible oils. There is a single recent report on the use of these non edible oils but the yields reported are 6.0g/l of SLJO and 7.6g/l of SLPO with 10% w/v oil feeding practising growth associated SL production [18].

Owing to the amphiphilic nature of SLs, their applicability as an ingredient of laundry detergent has been identified previously by Hall *et. al.* 1996 and Futura *et. al.* 2002 [19, 20] but still not in wide scale practice as a commodity product with the exception of few SL based cleaners by some multinational companies. In this chapter we are reporting production of SLs from non edible oils as renewable resources. The actual data evaluating washing performance of SLs or combination of SLs with some detergent formulations is being reported in this chapter for the first time. Thus applicability of SLs to be used in detergent formulations as an alternative to harmful chemical surfactants has been shown.

2.2 Materials and Methods

2.2.1 Microorganism and its maintenance

Candida bombicola (ATCC 22214) was used for the production of SLs. It was maintained on MGYP (Malt extract- 0.3g%, Glucose- 2g%, Yeast extract- 0.3g%,

Mycological peptone- 0.5g% and Agar- 2.0g%) slants. The microorganism was sub cultured in every 4 weeks and maintained at 4°C in a refrigerator [21].

2.2.2 Chemicals and reagents

All chemicals and solvents used in this study were of analytical grade and supplied by either Hi-media pvt. Ltd., India or Merck India ltd. The non-edible oils Jatropha and Pongamia were purchased from local market in Pune, India in a single batch. All buffers used in this study were essentially made in glass distilled water.

2.2.3 Optimization of media and fermentation parameters for maximum yield

2.2.3.1 Effect of medium constituents

In addition to primary i.e. hydrophilic and secondary lipophilic carbon sources; media contain nitrogen source, growth factors, buffer components and other minerals which show significant effect on SL yields. Different media previously reported for maximum production of SL were tried. The media compositions have been mentioned in Table 2.3.

Media components	Medium A [22]	Medium B [23]	Medium C [24]	Medium D [25]	Medium E [22]	Medium F [21]
Potassium di-hydrogen phosphate	0.1	-	0.1	0.6	0.1	-
Di-potassium hydrogen phosphate	-	-	0.016	-	-	-
Magnesium sulphate	0.5	0.03	0.07	0.03	0.5	-
Calcium chloride	0.01	-	0.027	-	0.01	-
Sodium chloride	0.01	-	0.05	-	0.01	-
Yeast extract	0.5	0.1	0.1	0.4		0.3
Ammonium nitrate	0.05 mole	-	-	-	0.05 mole	-
Glucose	10	10	10	15	10	5
Di-sodium hydrogen phosphate	-	0.2	-	0.2	-	-
Sodium di-hydrogen phosphate	-	0.7	-	-	-	-
Ammonium sulphate	-	0.1	-	-	-	-
Sodium citrate	-	-	0.5	-	-	-
Ammonium chloride	-	-	0.15	-	-	-
Urea	-	-	-	0.2	-	-
Peptone	-	-	-	-	0.5	0.5
Malt extract	-	-	-	-	-	0.3

Table 2.3 Media compositions- A to F values mentioned in g%

Seed culture was prepared by inoculating 5 ml of respective media with loopful growth from *Candida bombicola* (ATCC 22214) slant, followed by incubation at 28˚C, 180 rpm for 24 hours. This seed culture was transferred to 45 ml of fresh medium along with 0.5 ml of vegetable oil dispersed in 0.5ml of absolute ethanol. Incubation was continued further. 25ml of broth was withdrawn and processed after 96h and remaining 25ml was processed after 120h for SL estimation. This was done so as to check the optimum period of incubation for SL production.

The SLs were harvested by the procedure previously reported by Shah *et. al.,* 2007. Culture medium was centrifuged at 5,000rpm, at 10°C for 20 minutes The cell pellet was washed with ethyl acetate to recover the SLs precipitated and adhered to cells during centrifugation. The supernatant was extracted twice with equal volumes of ethyl acetate, the organic layer was dried over anhydrous Na_2SO_4, and the solvent was removed by rotary evaporation. The yellowish brown semi-crystalline product was washed twice with n-hexane and yields were determined [25].

2.2.3.2 Effect of primary, secondary carbon feed concentration

The medium and incubation period giving maximum yield were fixed and the glucose concentrations were varied as 5, 7 and 10%w/v keeping the oil feeding constant. In case of secondary carbon source, oil feeding was varied within the range 1 to 5%v/v. Glucose concentration was maintained at optimum value while performing secondary carbon feed optimization.

2.2.3.3 Effect of physical parameters

The medium which gave maximum SL yield was used for further experiments. According to the reports, optimal temperature for *Candida bombicola* ATCC 22214 is 28.8˚C (information from the National Collection of Yeast Cultures, UK) [2]. To check the effect of incubation temperature, the fermentations were carried out at different temperatures namely, 28, 31 and 33°C. To check the effect of initial pH on SL production; the pH values namely 4.0, 5.0, 6.0 and 7.0 were checked.

The fermentation, extraction procedures were essentially done as mentioned before in section 2.2.3.1. The experiments were carried out in triplicates in 250ml Erlenmeyer flasks containing 50 ml of the production media.

2.2.3.4 Production of SLJO and SLPO by Resting cell method

SL is known to be the stationary phase metabolite. It is desirable that majority of the carbon source provided be utilized for SL synthesis and not for biomass increase or cell maintenance purpose. In order to achieve so, the cells were pregrown in optimum medium and growth conditions *i.e.* medium F which also gave maximum biomass production and then these cells were subjected to the SL production medium containing the precursors for SL production i.e. glucose and non-edible oil as per the optimized conditions.

Seed culture of *Candida bombicola* ATCC 22214 was inoculated in 10 ml of freshly prepared Medium F (Malt extract 0.3g%, Yeast extract 0.3g%, Glucose 5.0g%, Mycological peptone 0.5g%) and incubated for 24h at 28°C under shaking condition (180 rpm). This pre-inoculum was added to 90 ml of MGYP nutrient medium in a 500 ml Erlenmeyer flask and incubated for 48 h at 28°C at 180 rpm. Cells were washed twice with glass distilled water under sterile conditions. The cell pellets (biomass ~ 6.0g wet weight or 1.5g dry weight for 100ml medium) were re-dispersed in simple production medium which is the sterile solution of 10% glucose with 1 ml of fatty acid (dispersed in 1 ml of ethanol) and the flask was kept at 180 rpm at 28°C for 96h. After 96h of incubation, a brown, viscous liquid (crude SL) could be seen settled at the bottom of the flask. The brown viscous phase was separated and subjected to extraction procedure as mentioned in section 2.2.3.1 [26-28]. The cells were separated from the broth by centrifugation at 5000 rpm, 10°C for 20 minutes. These separated cells can be again dispersed in production medium i.e. sterile solution of 10% glucose supplemented with non edible oils and sterile solution of 10% glucose to produce SLs. This way the biomass was reused up to 3 times with satisfactory yield of SL.

2.2.4 Characterization of SLJO and SLPO

After optimizing the media and fermentation parameters, the MALDI-MS (Matrix Assisted Laser Desorption/ Ionization – Mass spectrometry), NMR analysis was performed to know about structural composition of the SL samples. Further the surface active properties of SLJO and SLPO were evaluated.

2.2.4.1 Structural characterization of SLJO and SLPO

*2.2.4.1a MALDI-MS analysis of SLJO and SLPO:*SL samples 1mg were dissolved in 1 ml of methanol. Further 5 µl of SL sample was mixed with 20 µl of Dithranol matrix and MALDI-MS study was done on AB SCIEX TOF/TOF 5800.

2.2.4.1b SL structure confirmation with ^{1}H NMR: Two milligrams of samples namely SLJO and SLPO were dissolved in 0.5 ml of deuterated chloroform. ^{1}H NMR (200 MHz) spectra was recorded by Bruker AC200 at 25ºC. Chemical shift was expressed in ppm. Tetramethylsilane was used as an internal standard.

After structural characterization, the surface active properties namely, Surface tension reduction, CMC value, contact angle, wetting property, wetting performance with synthetic surfactants, emulsifying property, effect of pH, temperature, water hardness on SL functionality, antibacterial action of SLPO and SLJO were evaluated described in various sections below.

2.2.4.2 Minimum surface tension and critical micelle concentration (CMC)

Minimum surface tension and critical micelle concentration of SLs in the study were estimated using a KRUSS surface tensiometer K11 by Wilhelmy plate method.

Stocks of SLs were prepared in MilliQ water (pH 7.0) and diluted appropriately to get desired concentrations. The concentration range used was 0.95- 850mg/l for SLJO and 0.1-150mg/l for SLPO. The surface tension was measured at 26.7 °C by Wilhelmy plate method. A clean, dry 100 ml glass beaker was filled with the desired solution for surface tension measurement. The beaker was placed on the sample platform of the Kruss K11 tensiometer. The platinum surface tension probe was removed from the tensiometer hook and rinsed with deionized water and dried with the blue part of the flame from the propane torch. The probe was then air cooled and reinserted onto the tensiometer hook. The surface tensions of the desired solutions were measured as described in the Kruss K11 tensiometer operating manual. All surface tension measurements were the average value of 4 readings recorded at an interval of 30 seconds.

To determine Critical Micelle Concentration (CMC), the surface tension was measured as a function of surfactant concentration. Surface tension was then plotted vs. log surfactant concentration. The resulting curve had a nearly horizontal portion at concentrations higher than the CMC and had a negative steep slope at concentrations less than the CMC. The CMC was calculated as the concentration of the curve where the flat portion and the extrapolated steep slope intersected. The Surface Tension beyond CMC was the value in the flat portion of the curve.

2.2.4.3 Emulsification activity and stability

Emulsification activity and stability of the produced SLs was tested with oleic acid as an organic solvent using a modified method of Cirigilano and Carman [29,30]. 1ml sample containing the SL (0.5mg/ml) was mixed with 1ml of the oil substrate (oleic acid) to check emulsification activity. SL stock was essentially made in double distilled water pH 7.0. This solution was shaken vigorously in a vortex mixer for 2 minutes and allowed to settle for 10 minutes before measuring its absorbance at 600nm. The readings were noted in duplicates and the average values were calculated. Emulsification activity was expressed as the absorbance of the mixture at 600 nm (A_{600}). The stability of the resulting emulsion was expressed as the decay constant (k_d) estimated from the linear relationship between absorbance (A_{600}) and time (days) as represented in the following equation:

$$\log A_{600} = - k_d \times t$$

Effect of environmental parameters on emulsifying property

The effect of environmental parameters such as water hardness, pH and temperature on surface activity was determined by varying the levels of the individual parameters one-at-a-time by keeping the other parameters at a fixed level. The modified method of Daverey *et. al,.* 2010 has been used [30].

2.2.4.3a Effect of water hardness on emulsifying property

To study the effect of water hardness, simulated hard water has been used. The simulated hard water was prepared as follows- stock of 10mg/ml was prepared using equal amounts of calcium chloride ($CaCl_2.2H_2O$) and magnesium sulfate

($Mg(SO_4).7H_2O$). Then stock was diluted using distilled water to prepare moderately hard water (0.12mg/ml) and hard water (0.18mg/ml).

To test the effect of hardness on emulsification activity and stability of SLJO and SLPO, 1ml SL solutions (0.5mg/ml) were prepared in hard and moderately hard water. Further the solutions were incubated for 1 hour at 30˚C and then emulsification activity and stability was checked as mentioned in earlier part of the section.

2.2.4.3b Effect of pH on emulsifying property

For testing the effect of pH on emulsification activity and stability of SLJO and SLPO, 1ml SL (0.5mg/ml) solutions were prepared in acetate/ phosphate buffer solutions having various pH values in the range of 4.0 to 8.0 and incubated for 1 hour at 30˚C and emulsification activity and stability was checked.

Buffer preparation-

For pH 4.0 and 5.0; acetate buffer was used. Stocks of 0.2M ware prepared for buffer components namely, acetic acid (solution A) and sodium acetate (solution B). For pH 4.0 buffer, 20.5ml of solution A and 4.5ml of solution B were mixed. For pH 5.0 buffer, 7.4ml of solution A was mixed with 17.6ml of solution B. Further both the buffers were diluted with double distilled water to make up the volume up to 50ml.

For pH 6.0, 7.0 and 8.0; phosphate buffer was used. Stocks of 0.2M were prepared for buffer components namely, Sodium di-hydrogen phosphate (solution C) and Di-sodium hydrogen phosphate (solution D). For pH 6.0 buffer, 21.92 ml of solution C and 3.07 ml of solution D were mixed. For pH 7.0 buffer, 9.75 ml of solution C was mixed with 15.25 ml of solution D. 1.325 ml of solution C was mixed with 23.675 ml of solution D were mixed to make the buffer of pH 8.0. Further all 3 buffers were diluted with double distilled water to make up the volume up to 50ml.

2.2.4.3c Effect of temperature on emulsifying property

For checking the effect of temperature, 1ml stock solutions of each; SLJO and SLPO (stock strength-0.5mg/ml) were incubated for 30 minutes at various

temperatures in the range 20-80˚C and emulsification activity and stability was then evaluated using oleic acid as the substrate as described in section 2.2.4.3.

2.2.4.4 Evaluation of antibacterial property of SLs

Staphylococcus aureus (ATCC 29737) and *Escherichia coli* (ATCC 8739) were used as the test organisms- representatives of Gram positive and Gram negative genera.

Following protocol was followed to check the bacterial inhibition by SLs. Bacteria were grown in Nutrient Broth for 24 h at 37° C, 180 rpm and cell counts were quantified by measuring the A600 values. Appropriate dilution of bacterial cell suspension giving countable colonies (~5 X 10^3 CFUs/ml) was added to different SL concentrations (50-500µg/ml). The mixtures were incubated for 4h at 28˚C, 180 rpm. Then 50µl of mixture was spread plated on nutrient agar plates and incubated at room temperature for 24 hours and colonies were counted. Experiments were done in triplicates and the average values were noted.

2.2.5 Evaluation of surface active properties of SLPO and SLJO

In view of the intended use of SLs as detergent additive; wetting property and contact angle reduction was examined.

2.2.5.a Examining the wetting property

Wetting property or wettability was measured by canvas disc method (C. A. Wilham, *et. al.*; 1973). The test was carried out by measuring the sinking time of canvas disk [31]. The wetting property of SLPO, SLJO along with synthetic surfactants such as SDS (Sodium Dodecyl Sulphate) and Triton X-100 (Octyl phenol ethoxylate) was evaluated for comparison. 100 ml test solutions of above mentioned surfactants with concentration 0.01g%, 0.1g% and 1g% were prepared. Canvas disc of 1 inch diameter was placed in Gooche funnel and it was then inverted in a beaker containing test solution. Sinking time *i.e.* time required for the canvas disc to sink to the bottom of beaker was measured.

2.2.5.b Determination of contact angle

Contact angle measurements were performed using goniometer: G-10 contact angle meter. SLPO and SLJO stock solutions of 1µg/ml were prepared in double distilled water. 10µl volume was dropped onto the test surface. 3 typical surfaces namely, Glass, Teflon and Stainless steel have been tried out. θ_C values were measured as explained below (Refer to figure where θ_C has been marked).

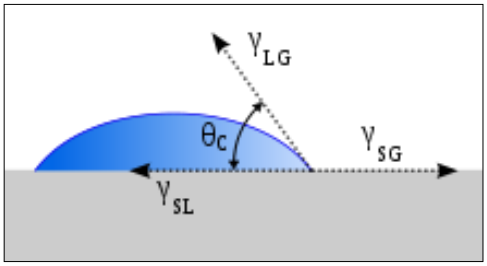


Figure 2.1 Contact angle determination

A liquid droplet rests on solid surface and is surrounded by gas. The contact angle, θ_C, was observed as the angle formed by a liquid at the three phase boundary where the liquid, gas and solid intersect.

2.2.6 Comparative performance assessment of SLs with synthetic surfactants, commercially available detergent

2.2.6.1 Wetting property of SLs in combination with synthetic surfactants

Wetting property of combinations of SLJO and SLPO with synthetic surfactants SDS and Triton X-100 was checked since we aim to use these SLs as an alternative to synthetic surfactants in detergents. Stock solutions of SLs (0.01g%) and chemical surfactants (0.01g%) were prepared. SLs and chemical surfactants were mixed in three ratios; SL: chemical surfactant-25:75 (1), 50:50 (2) and 75:25 (3) and their wetting property was assessed.

2.2.6.2 Detergency test

In detergency test, the comparative performance of SLJO, SLPO, a commercial detergent preparation were evaluated. Also the performances of SLPO, SLJO in combination with commercial detergent were checked. The performances of each candidate and the combinations thereof were checked against 4 different stains namely, coffee, turmeric, oil, poster color on 2 different types of fabrics namely- cotton and polyester.

Following method was practised -Pieces of 2" X 2" were cut of cotton and polyester cloth. Cloth pieces were placed on saran wrap and equal volumes of each type of stain solutions were put on them. Another piece of saran wrap and heavy weight was put on it for 5-10 minutes to assure stain absorption and then stains were allowed to dry overnight. Next day, stained pieces of cloth were soaked individually in 0.1g% solution of SLJO, SLPO and commercial detergent for 10 minutes. Soaked pieces of cloth were hand washed for approximately 1-2 minutes. Excess water from cloth was squeezed out and cloth pieces were allowed to dry normally and results were noted [32, 33]. Same procedure was carried out to test the stain removal capacity of SLJO in combination with commercial detergent (mixed in proportion 1:1 respectively) and SLPO in combination with commercial detergent (1:1) with appropriate controls.

2.3 Results and discussion

2.3.1 Process optimization for maximum production of SLs

2.3.1.1 Optimization of media and fermentation parameters for maximum yield

In order to maximize the product yield, 6 media differing in the proportion of sugar, nitrogen source, presence of buffer components etc. were chosen. Media compositions have been described in section 2.2.1. SLJO yields in different media have been presented in Figure 2.2 while those of SLPO have been represented in Figure 2.3.

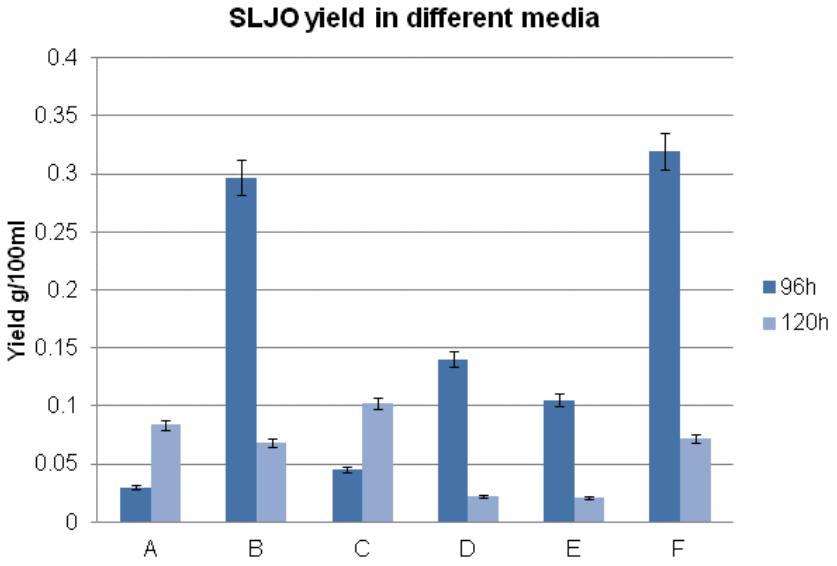


Figure 2.2. SLJO yield in different media A-E in g/100ml.

It is evident from Figure 2.2 that, the SLJO yield values decrease after 96h of incubation except in case of Medium A and C. Medium F was found to give highest SLJO yield with 96h of incubation. Similar results were obtained when Pongamia oil was used as Lipogenic precursor.

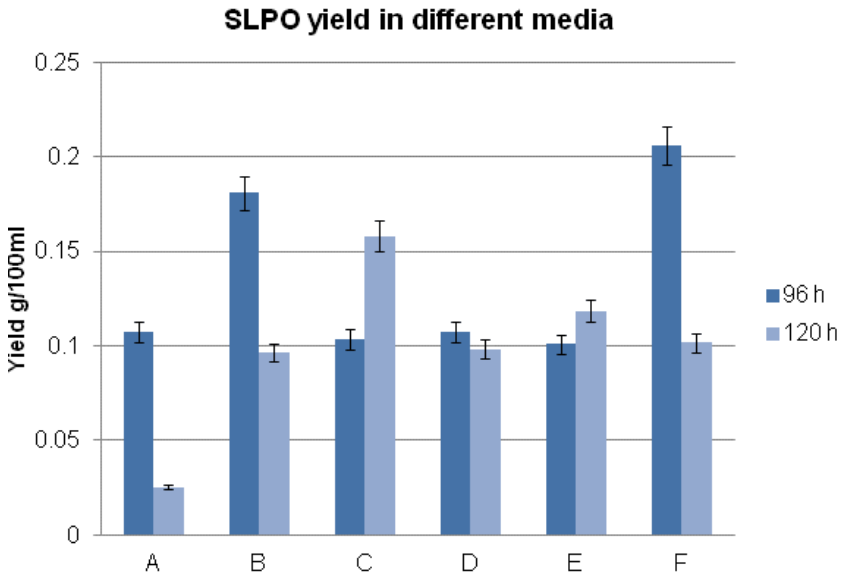


Figure 2.3. SLPO yield in different media A-E in g/100ml.

The Figure 2.3 shows that the SLPO yield values decrease after 96h of incubation except in case of Medium C and E. Out of the 6 media used, Medium F was found to give highest SLPO yield with 96h of incubation.

The data on optimization of fermentation parameters namely- incubation temperature, initial pH, Glucose concentration and precursor feeding volume has been displayed in Figure 2.4 and Tables 2.4-2.6.

Temperature of incubation	Yield (g/L)	
	SLJO	SLPO
28˚C	**1.433**	**1.388**
31˚C	0.375	0.78
33˚C	0.101	0.685

Table 2.4 Effect of incubation temperatures on SLJO and SLPO yield

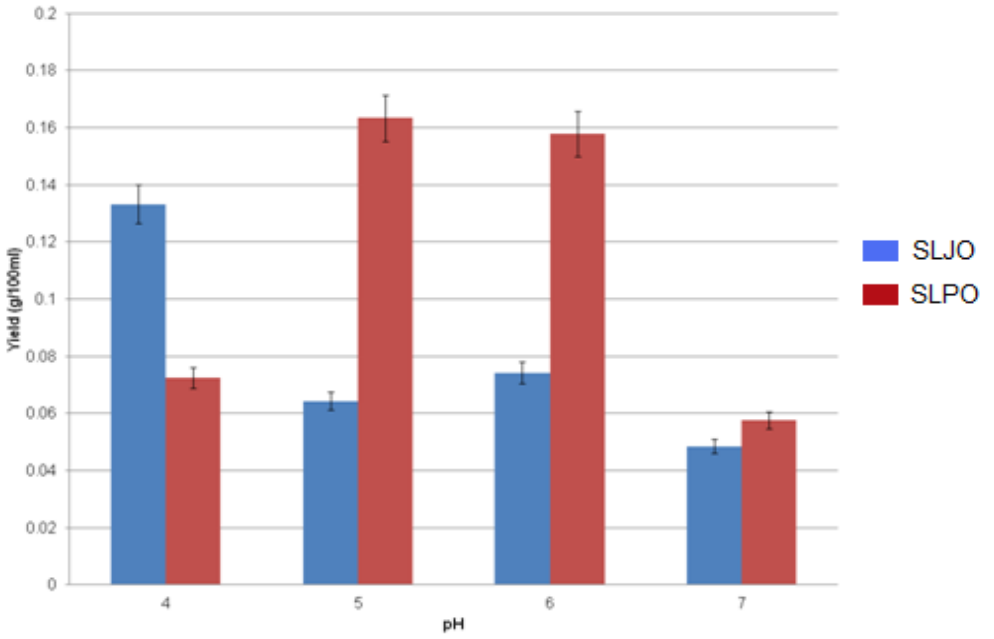


Figure 2.4 Effect of initial pH on SLJO and SLPO yield

Glucose concentration (grams/100ml)	Yield (g/L)	
	SLJO	SLPO
5	1.004	0.59
7	1.085	1.013
10	**1.537**	**1.562**

Table 2.5 Effect on Glucose concentration on SLJO and SLPO yield

Precursor volume (ml/100ml)	Yield (g/L)	
	SLJO	SLPO
1	**1.336**	**1.666**
3	0.868	0.812
5	0.484	0.156

Table 2.6 Effect of precursor volume on SLJO and SLPO yield

The set of optimized parameters for the production of SLJO are temperature 28°C, initial pH 4.0, glucose concentration 10g% and fatty acid precursor volume 1%v/v. The fermentation parameter values for maximum SLPO production were temperature 28°C, pH 5.0, glucose concentration 10g%, fatty acid precursor volume 1%v/v.

2.3.1.2 *Production of SLs using resting cell method*

SL production yields have been improved through the use of optimized parameters combined with resting cell method. With 1%v/v oil feeding, 15.25g/l of SL yield from Jatropha oil and 19.3g/l of SL yield from Pongamia oil could be obtained. Resting cell method allows the use of same biomass for up to 3 times making the process still more economic. After that SL production with same cell mass dropped considerably.

2.3.2 Characterization of SLJO and SLPO

2.3.2.1 Structural characterization of SLJO and SLPO

Typical structure of SLs consists of a sophorose (dimeric sugar) linked β-glycosidically to terminally or sub-terminally hydroxylated fatty acid with chain length 16-18 [2]. SL occurs as a mixture of compounds differing in their acetylation, lactonization and position of hydroxylation. This way even a pure precursor fatty acid leads to formation of different forms of SL in crude product. Starting material in present case is a mixture of fatty acids hence multiple forms of SL molecules are expected in the formed product. This was evident from the TLC analysis for SLJO and SLPO.

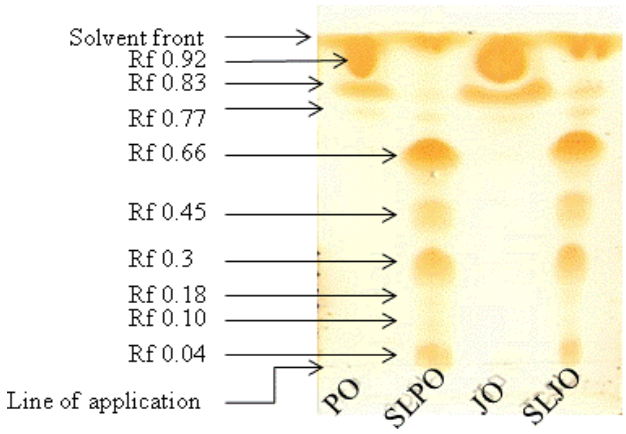


Figure 2.5 Thin layer chromatogram of crude SLs synthesized using Pongamia and Jatropha oil along with the precursor oils.

2.3.2.1a MALDI/MS analysis of SLJO and SLPO

Presence of multiple SL forms derived from different fatty acids was confirmed with the MALDI-MS analysis of the samples. Prominent peaks from the mass spectrum were correlated to sodium adducts [$M^+ + H^+ + Na^+$] of the expected forms of SLs. Different forms of SLs derived from Palmitic (C16:0), Stearic

(C18:0), Oleic (18:1), Linoleic (C18:2) and trace amounts of Arachidic (C20:0) acid were detected. In both the SLs, the Di-acetylated SL of C18:1 i.e. Oleic acid was detected as the most abundant structural form. The MALDI/MS spectra of SLJO and SLPO are presented in Figures 2.6 and 2.7 respectively.

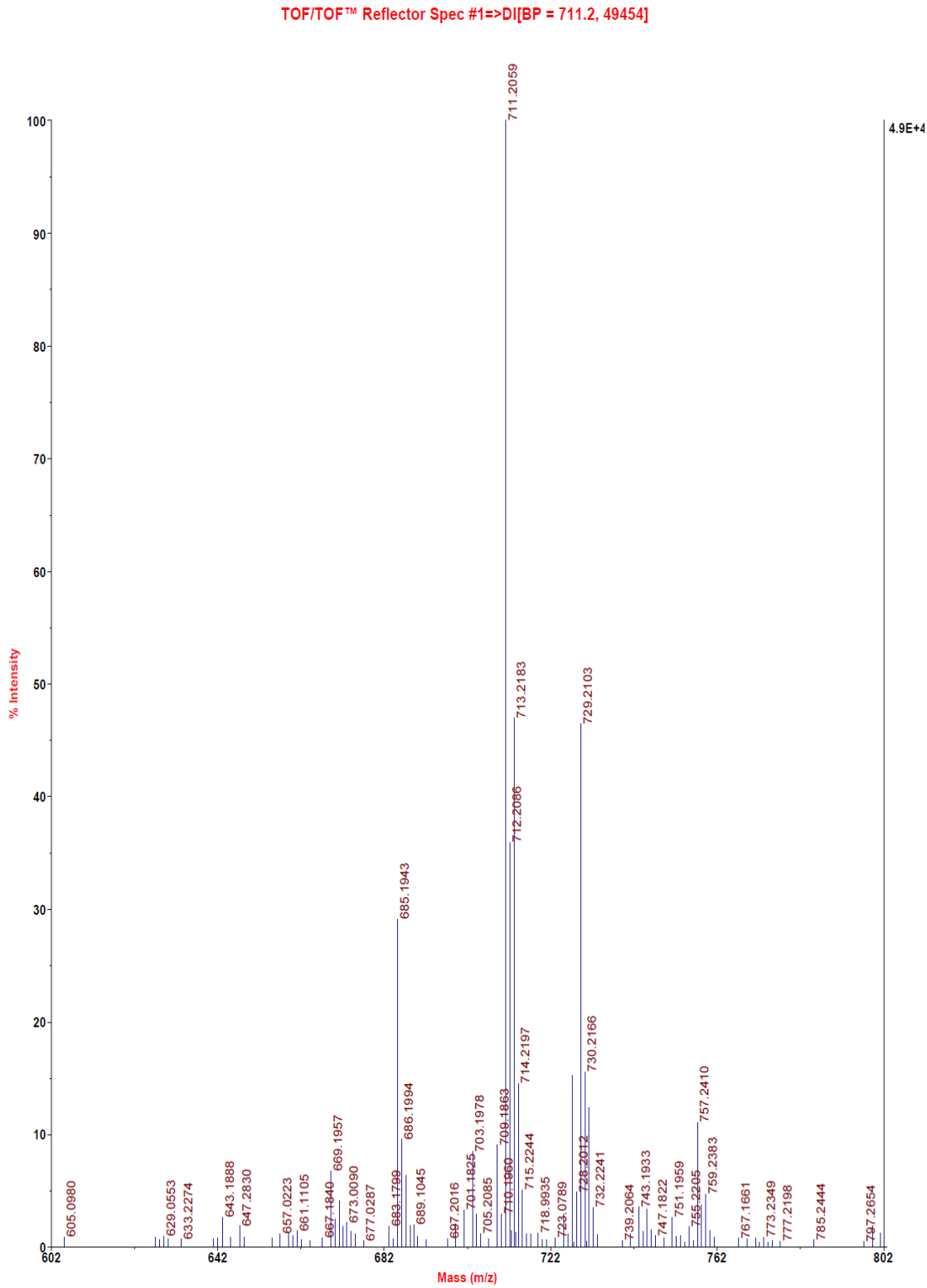


Figure 2.6 MALDI/MS spectrum of SLJO preparation

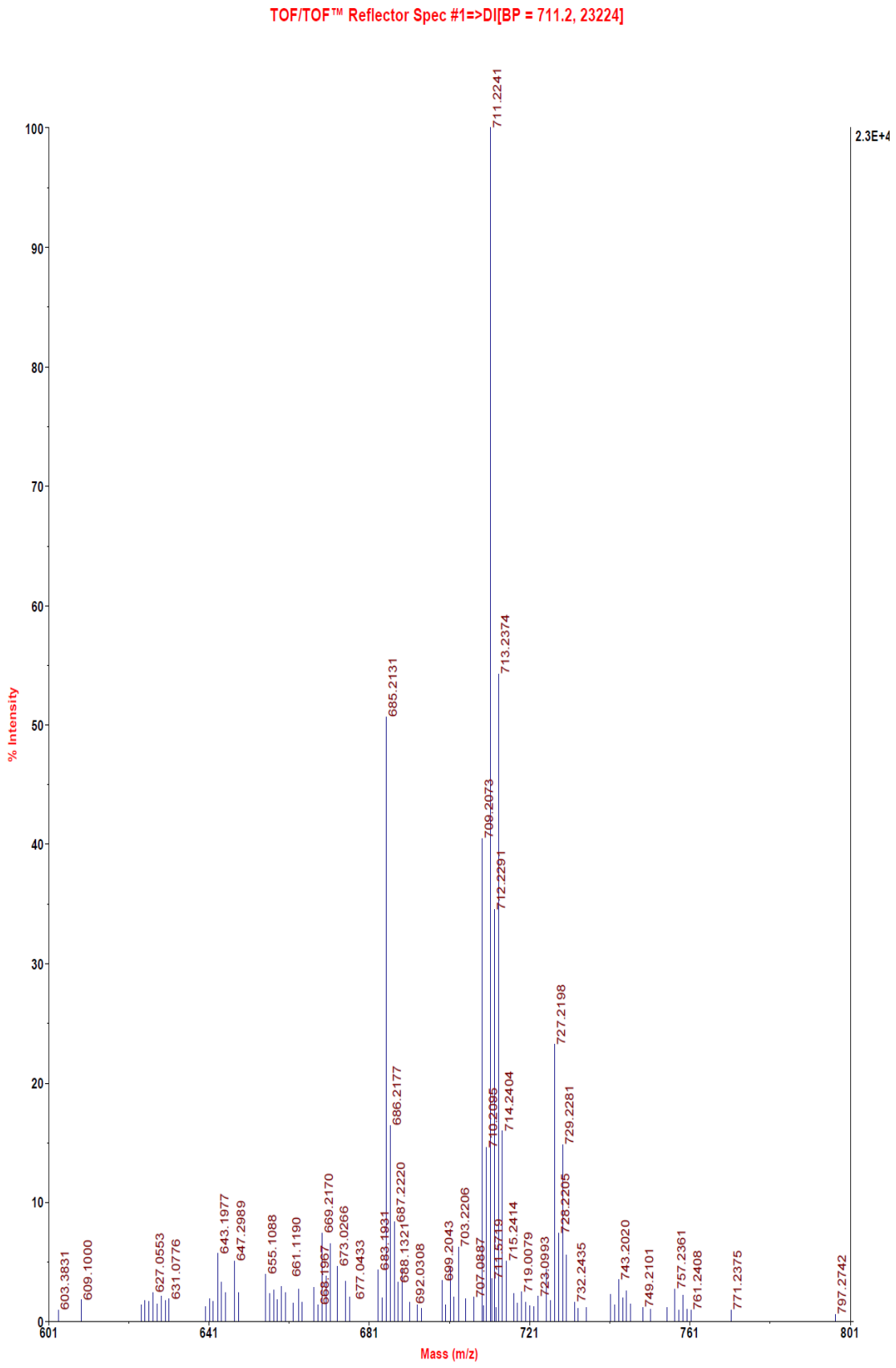


Figure 2.7 MALDI/MS spectrum of SLPO preparation

Structural composition of both SLs based on MALDI/MS data has been represented in Table 2.7a and 2.7b

SL structural forms	m/z	[M^++H^++Na^+]	SLPO		SLJO	
			Relative abundance	Approximate % composition	Relative abundance	Approximate % composition
Non-acetylated SL of C18:0, acidic form	623	647	5.08	1.62	1.86	0.67
Mono-acetylated SL of C18:1, lactonic form	645	669	7.4	2.35	6.75	2.45
Di-acetylated SL of C16:0, lactonic form	661	685	50.65	16.11	29.1	10.54
Mono-acetylated SL of C18:1, acidic form	663	687	8.32	2.65	6.41	2.32
Di-acetylated SL of C16:0, acidic form	679	703	6.25	1.99	8.47	3.07
Di-acetylated SL of C18:2, lactonic form	685	709	40.43	12.86	9.04	3.27
Di-acetylated SL of C18:1, lactonic form	687	711	100	31.81	100	36.22
Di-acetylated SL of C18:0, lactonic form	689	713	54.24	17.25	46.95	17.00
Mono-acetylated SL of C20:0, acidic form	692	716	1.77	0.56	1.19	0.43
Di-acetylated SL of C18:2, acidic form	703	727	23.2	7.38	15.21	5.51
Di-acetylated SL of C18:1, acidic form	705	729	14.82	4.71	46.41	16.81
Di-acetylated SL of 20:0, acidic form	735	759	2.21	0.70	4.73	1.71

Table2.7a Comparative data on structural composition of SLPO and SLJO

	SLPO	SLJO
C16:0	18.09%	13.61%
C18:0	18.87%	17.67%
C18:1	41.51%	57.79%
C18:2	20.24%	8.78%
C20:0	1.26%	2.14%
C16:C18:C20	18 : 80.6 : 1.3	13.6 : 84.2 : 2.14
Saturated: Unsaturated	38.22 : 61.75	33.42 : 66.57
Acidic: Lactonic	19.59 : 80.37	30.51 : 69.47

Table 2.7b Comparative data on structural composition of SLPO and SLJO-inference

2.3.2.1b *^{1}H NMR analysis of SLJO and SLPO*

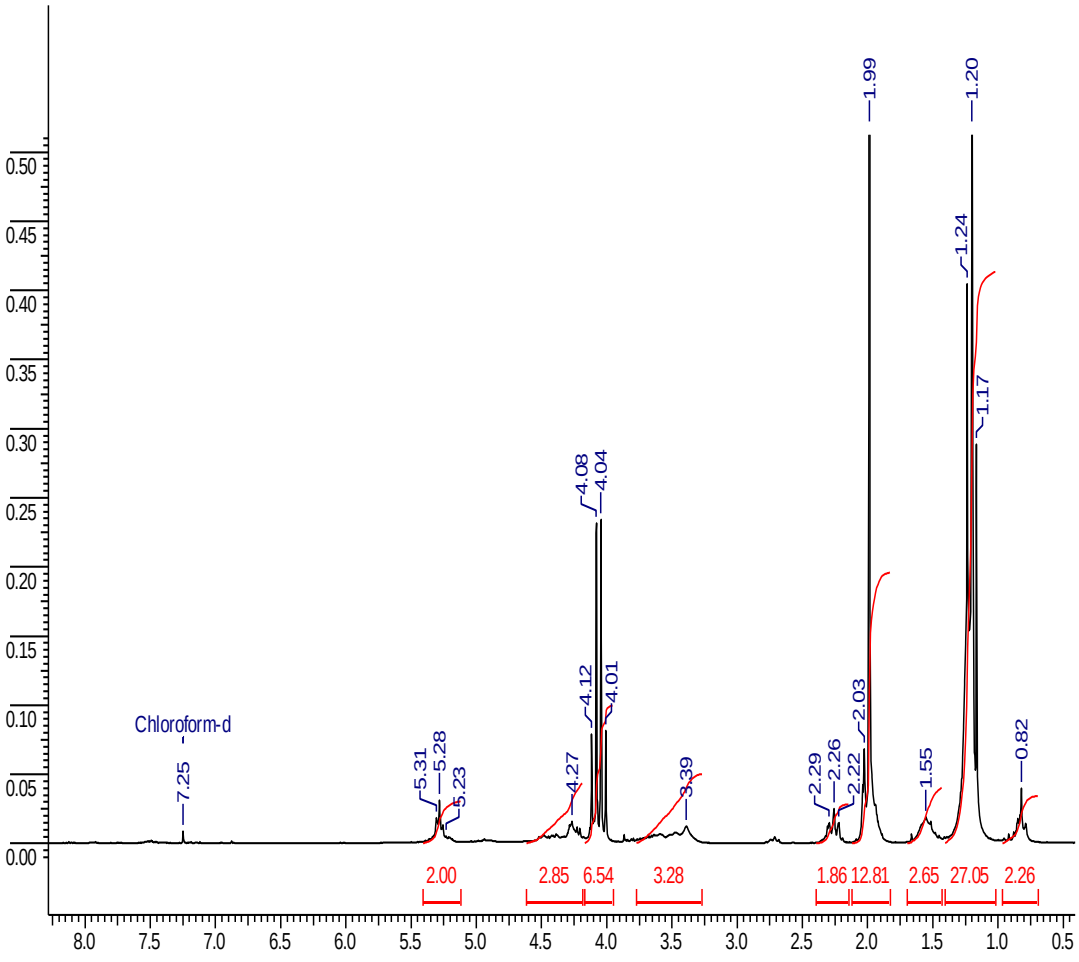


Figure 2.8 ^{1}H NMR pattern of SLJO

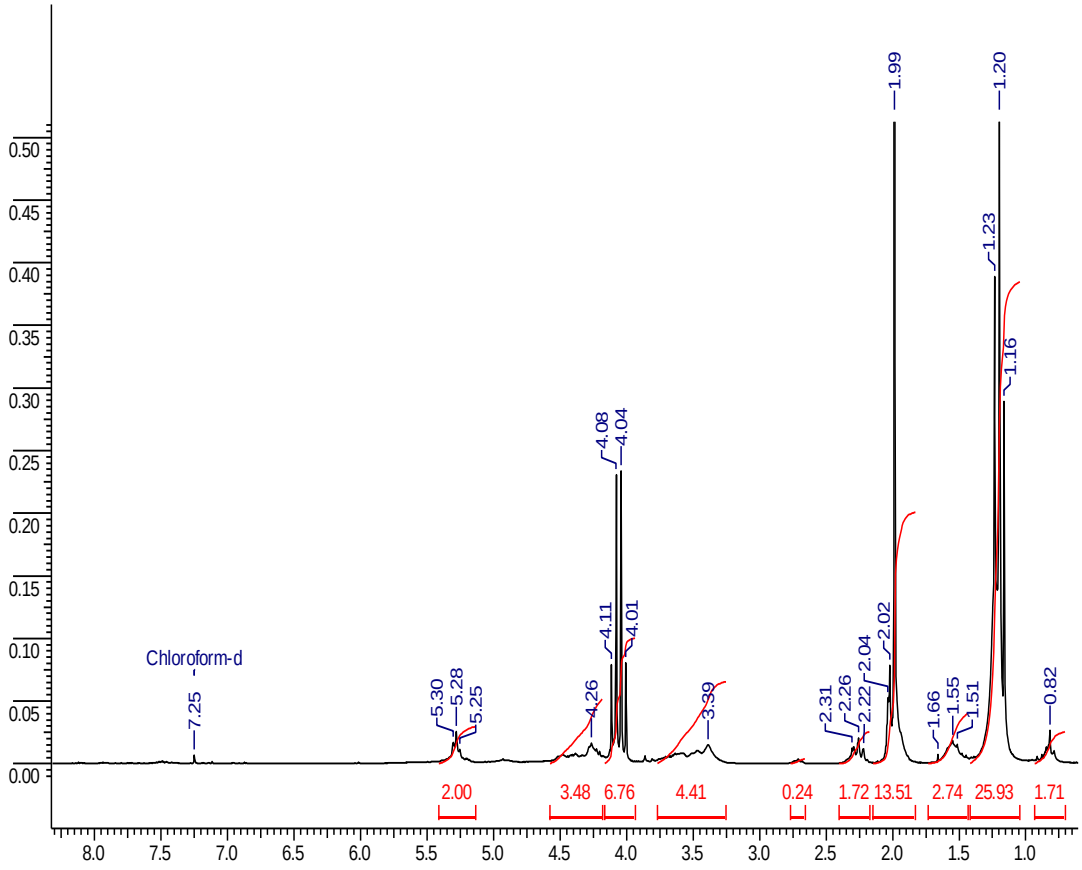


Figure 2.9 ^{1}H NMR pattern of SLPO

The ^{1}H NMR data as shown in Figures 2.8 and 2.9 indicated the presence of characteristic proton chemical shift peaks. Also the relative peak areas matched with previously reported SL-NMR data from relevant references [18, 28]

^{1}H chemical shift (ppm)	Multiplicity	Assignment
1.21	Doublet	-C**H**$_3$ (at ω position of fatty acids moiety)
1.31	Multiplet	-(C**H**$_2$)$_n$ (on fatty acid moiety)
2.03	Multiplet	-C**H**$_2$-C(=O)-O (on fatty acid moiety)
2.09	Multiplet	- C**H**$_3$ (on acetic acid moiety)
2.61	Doublet	-CH=CH-C**H**$_2$-CH=CH- (on fatty acid moiety, only for SL-1, C18:2)
3.25-3.70	Multiplet	-C**H**-OH (on sophorose moiety)
3.76	Multiplet	-(CH$_2$)-C**H**(-O-sophorose)-CH$_3$(on fatty acid moiety)
4.17 and 4.34	Multiplet	-CH$_3$-C(=O)-O-C**H**$_2$-CH(-O)-CH-OH (on sophorose moiety)
4.46 and 4.55	Doublet	-O-C**H**(-O-)-CH-OH (on sophorose moiety)
5.35-5.37	Multiplet	-CH$_2$-C**H**=C**H**-CH$_2$ (on fatty acid moiety)

Table 2.8 ^{1}H NMR chemical shift data for SL components-table adapted from [18]

2.3.2.2a Surface tension lowering ability of SLs

The surfactant property of both SLs namely, SLJO and SLPO was confirmed by 'oil displacement test' qualitatively and quantitatively. Dose dependent response was seen in the concentration range 50 µg/ml to 2mg/ml as shown in Figure 2.10. For lowest concentration, 2mm diameter halo was observed while for 2mg/ml; 15mm diameter halo of oil displacement was seen for SLJO. In case of SLPO; 2mm diameter halo of oil displacement was seen at lowest concentration while for highest concentration it was 20mm. Further the surface active properties were checked using different experimental and analytical methods.

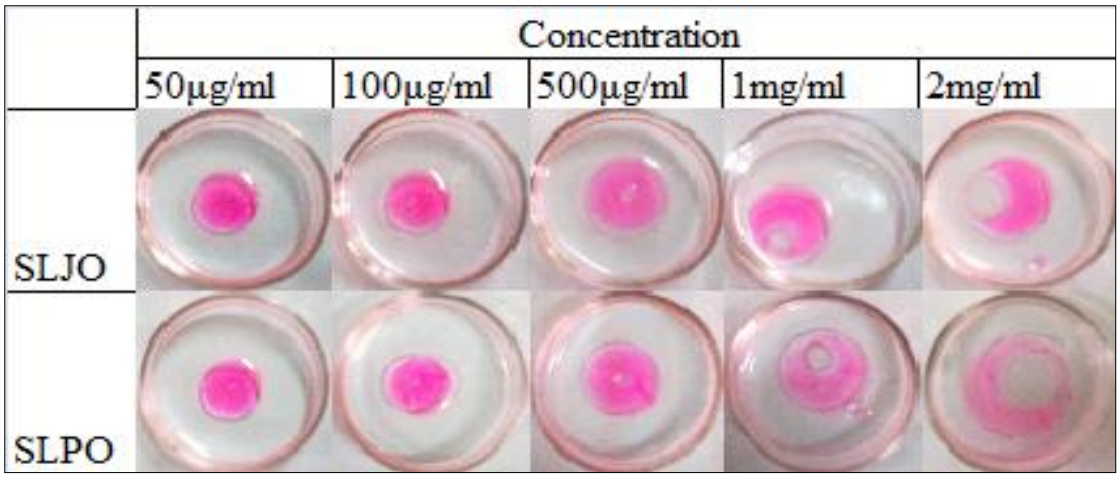


Figure 2.10 Concentration dependent responses of SLJO and SLPO in oil displacement test

2.3.2.2b Minimum surface tension and critical micelle concentration (CMC)

The surface tension reduction curve for SLJO has been depicted in Figure 2.11. It can be observed from the curve that SLJO reduced the surface tension of distilled water from 70.714mN/m to 33.512mN/m at the concentration of 9.5mg/l and the surface tension value stabilized beyond this concentration. Therefore CMC value of SLJO is 9.5mg/l.

Figure 2.12 represents the surface tension reduction curve for SLPO. According to the curve, SLPO reduced the surface tension of water from 70.714mN/m to 33.3mN/m. The CMC value of SLPO was determined to be 62mg/l.

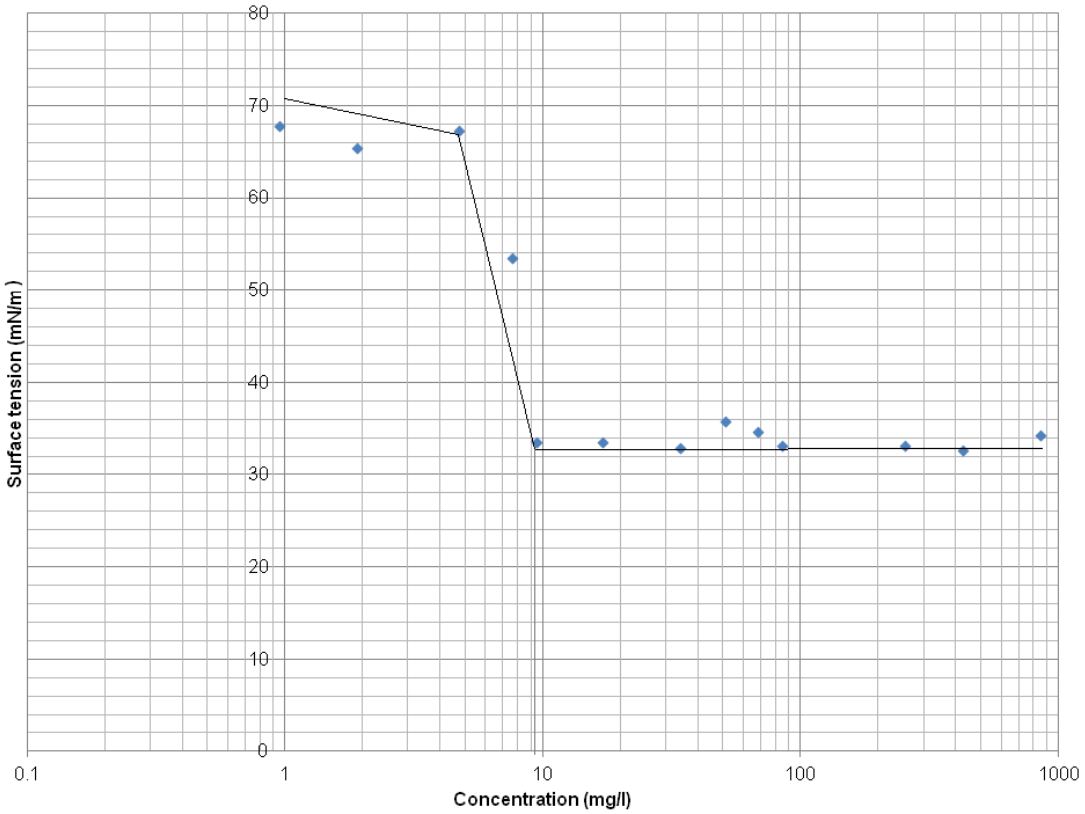


Figure 2.11 Surface tension reduction curve for SLJO

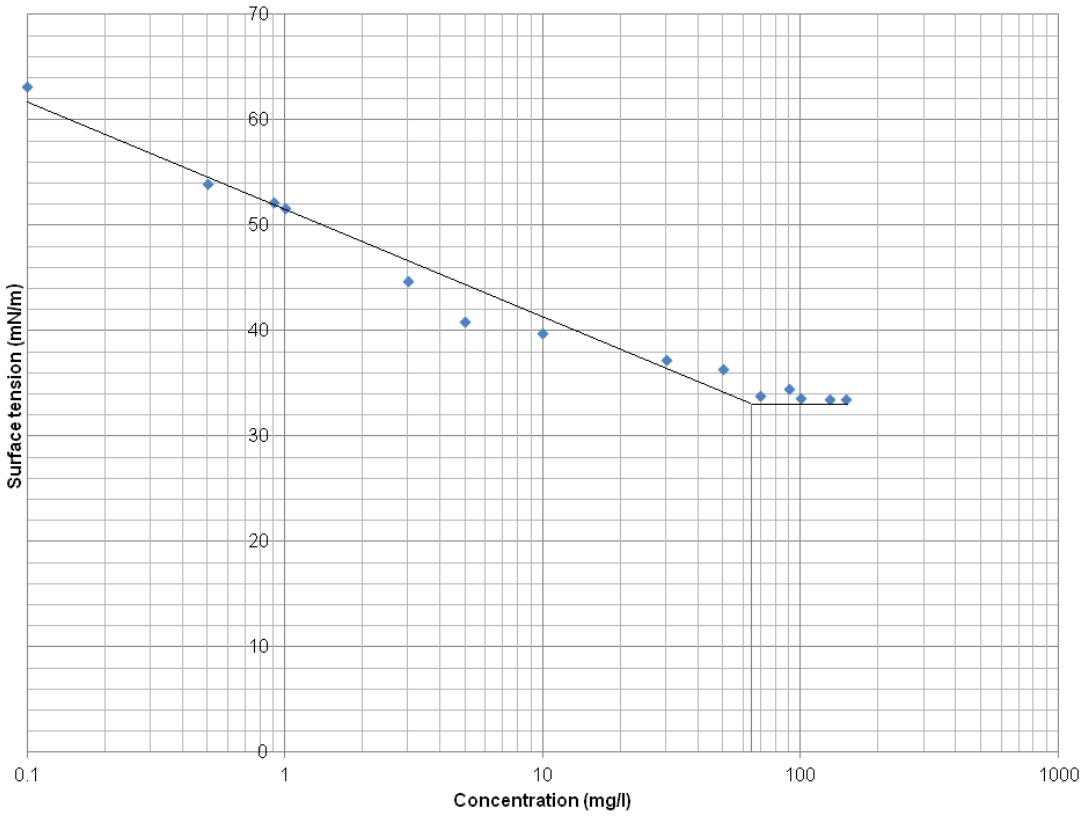


Figure 2.12 Surface tension reduction curve for SLPO

Typically the CMC values of SLs fall within the range 40-100mg/l [2]. Thus it is worth to mention that SLJO and SLPO are showing a low CMC value. Natural synergism between different forms of SLs creates a better balance for many interfacial properties [34]. On the other hand, SDS- a common synthetic surfactant lowers the surface tension of double distilled water up to 25mN/m with the CMC value 2240mg/l (0.008M). Another common surfactant Triton X-100 shows the CMC value 187.5 mg/l and lowers the surface tension of double distilled water up to 32mN/m.

2.3.2.2 Emulsification activity and stability

The emulsification activity and stability of SLs and synthetic surfactants namely, SDS and Triton X-100 have been noted in Table 2.9. Stock concentration used for each of the samples was 0.5mg/ml. Emulsification activity and stability of SLJO was observed to be better than that of standard chemical surfactant- Triton X-100; whereas, emulsification activity of SLJO was less than that of SDS but stability of emulsion was superior to SDS. Similar emulsification activity and stability was displayed by SLPO. It was better than Triton X-100 in terms of emulsification activity and stability while SDS showed superior emulsification activity but poor stability when compared to SLPO. The trend observed for stability of emulsion was: SLJO>SLPO>Triton X-100>SDS.

	Emulsification activity (A_{600})	Decay constant (kd)
SLJO	1.9725	-1.3824
SLPO	1.8695	-1.7568
Triton X-100	0.789	-1.8432
SDS	2.250	-5.7312

Table 2.9. Emulsification activity and k_d values of SLs and synthetic surfactants

Effect of environmental parameters on emulsifying property

As an ingredient of detergent, SLs should perform satisfactorily in extreme physical conditions and different water qualities. So the effect of different parameters on emulsifying property was explored.

2.3.2.2a Effect of water hardness on emulsifying property

Results mentioned in Table 2.10 imply that emulsification index i.e. A_{600} value was affected by the hardness of water. Also, water hardness adversely affected stability of emulsions. This was found to be true in case of all the 3 surfactants mentioned below i.e. SLJO, SLPO and Triton X-100. Stability of emulsions formed by SLJO and SLPO was better than that of Triton X-100. Stability of emulsion formed by SLPO was better than that of SLJO and Triton X-100 in moderately hard water. Stability of emulsion formed by SDS in distilled water was very low as compared to SLs and Triton X-100, but it was observed to behave erroneously in hard water.

Hardness	SLJO		SLPO		Triton X-100	
	Emulsification activity (A_{600})	Decay constant (K_d)	Emulsification activity (A_{600})	Decay constant (K_d)	Emulsification activity (A_{600})	Decay constant (K_d)
Distilled water	1.9725	-1.3824	1.8695	-1.7568	0.789	-1.8432
Moderately hard water	1.846	-1.8432	1.807	-1.6992	1.0665	-3.6288
Hard water	0.779	-3.0528	1.6335	-2.9088	1.531	-3.312

Table 2.10 Effect of water hardness on emulsifying property and stability of SLs

2.3.2.2b Effect of pH on emulsifying property

pH is known to be one of the most important environmental factors influencing the performance of any surfactant. pH alters the net charge on surfactant molecule and thus its orientation at the interface.

The experiment was carried out in triplicates and the emulsification indices were calculated from the average A_{600} values. It can be observed from Figure 2.13 that emulsification activity of SLJO and SLPO was comparable between pH values of 5.0-8.0. There was evident drop in SL emulsifying index value at pH 4.0 and the value was found to be 6 times low as compared to other pH values. Classically detergent formulations make the pH alkaline owing to the presence of builders such as sodium carbonate which releases hydroxyl ions on reacting with water. SLs are unstabe at pH higher than 7.0 to 7.5. Beyond this point, irreversible hydrolysis of the acetyl groups and ester bonds is observed as reported by I. N. A.

Van Bogaert *et. al.*, 2007 [2]. SLs were found to retain the surfactant activity at pH 8.0 thus making it suitable for combination with commercial detergents.

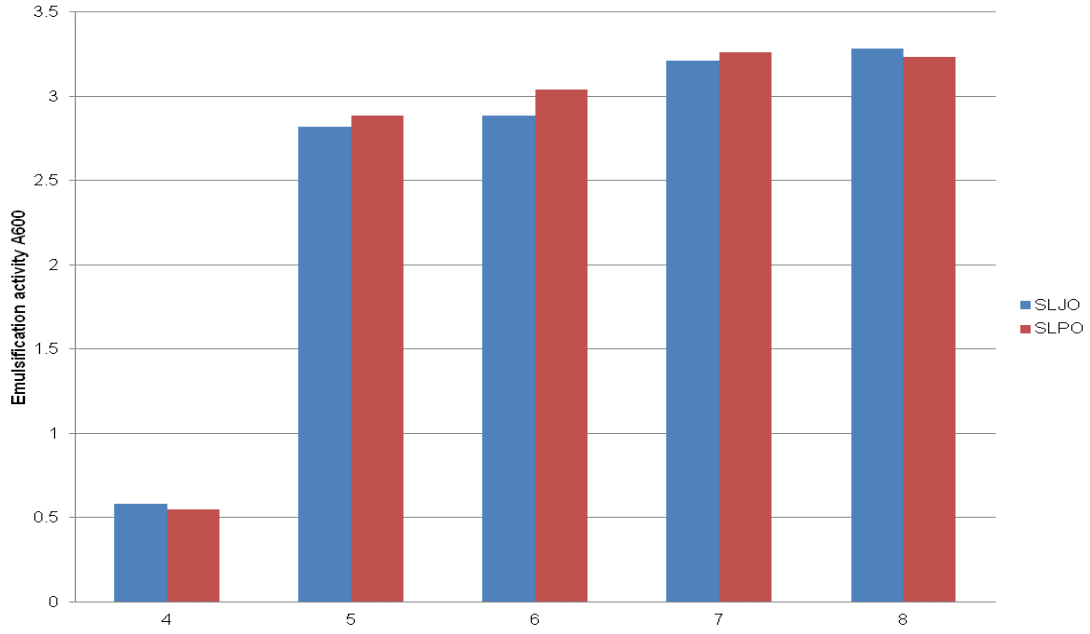


Figure 2.13 Emulsification activities of SLs at different pH values

It can be observed from Figure 2.14 that emulsions formed by SLJO and SLPO were stable within the pH range of 5.0-8.0 with maximum stability at pH 8.0 for both SLJO and SLPO. At pH 4.0, emulsion with SLPO is comparatively stable than that of SLJO. The decay constant of SLJO is -11.3184 at pH 4.0 while that of SLPO is -4.1184.

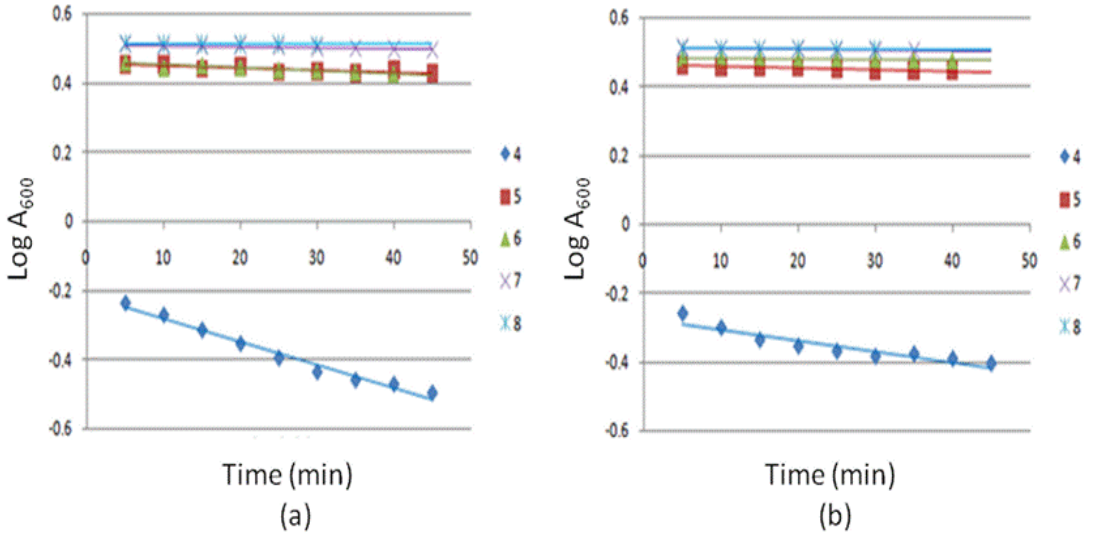


Figure 2.14 Stability of SLJO (a) and SLPO (b) emulsions at different pH values with respect to time

2.3.2.2c Effect of temperature on emulsifying property

It was observed from the Table 2.11 that for both SLs, emulsifying property is best at 60°C and then goes on decreasing. Probably temperature rise enhances micellarization and beyond 60°C destruction occurs. When the emulsion stability was assessed, it was found that the stability is best at 20°C and then it decreased with increasing temperature. Generally better emulsification by surfactants is often associated with higher temperatures. But both SLs behaved equally well and to some extent even better hence SLs do not dictate the requirement of hot water for improved performance. So the energy costs in washing processes can be lowered.

Temperature (˚C)	SLJO		SLPO	
	Emulsification activity (A_{600})	Decay constant (k_d)	Emulsification activity (A_{600})	Decay constant(k_d)
20	1.902	-0.9216	1.727	-1.1088
40	2.010	-3.2256	1.9885	-1.872
60	2.249	-4.1472	2.0765	-1.9728
80	1.807	-6.3072	1.9085	-2.1888

Table 2.11 Effect of temperature on Emulsification activity and stability of SLJO and SLPO

2.3.2.3 Evaluation of antibacterial property of SLs

SLs are known to posses antimicrobial properties [2]. On account of structural similarity SLs cause membrane lipid order perturbation and thus cause the intracellular contents to leak out.

The Minimum Inhibitory Concentration required to inhibit 90% of the organisms i.e. MIC_{90} values were determined. Against *Staphylococcus aureus,* MIC_{90} of SLPO was 100µg/ml and that of SLJO was 300µg/ml as depicted in Figure 2.15.

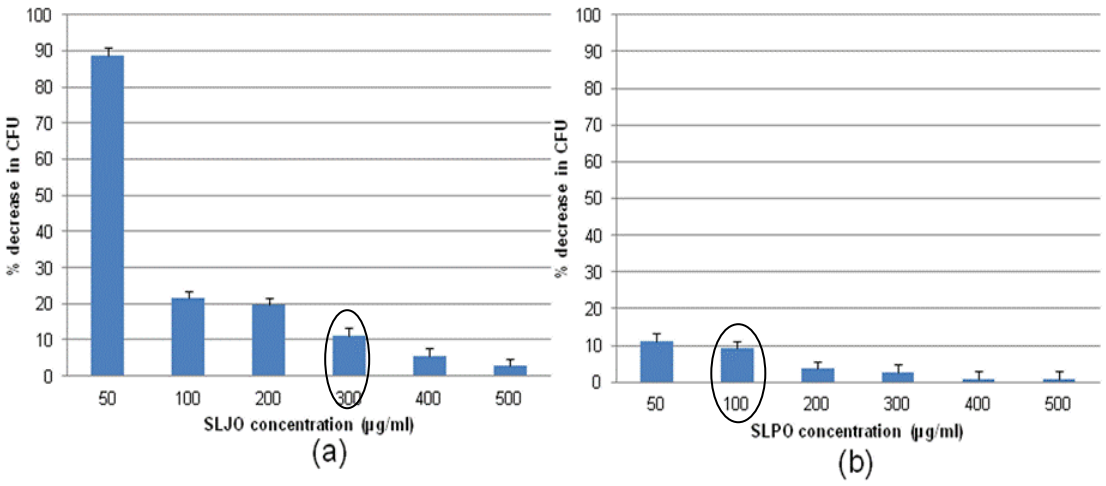


Figure 2.15 Antibacterial action of SLJO (a) and SLPO (b) against *Staphylococcus aureus*

Against *Escherichia coli*; MIC_{90} of SLPO was 400µg/ml while SLJO couldn't achieve 90% inhibition till 500µg/ml as shown in Figure 2.16.

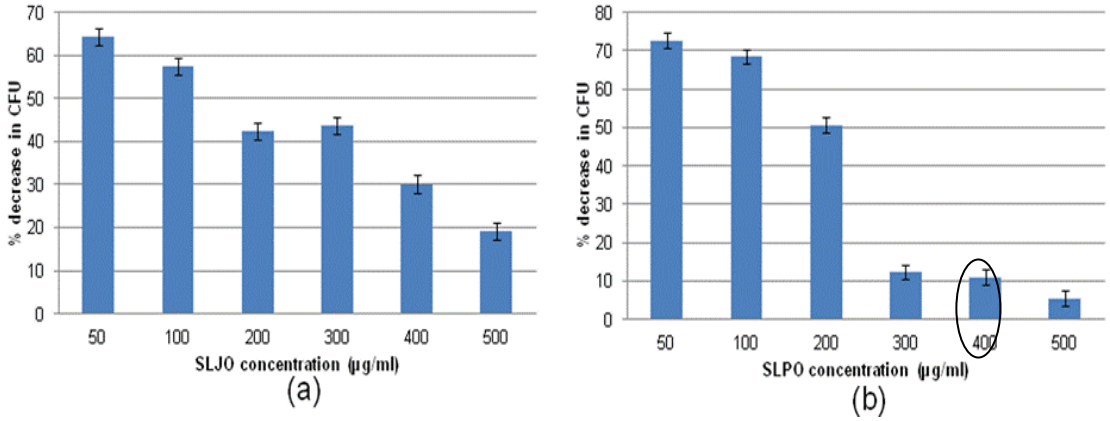


Figure 2.16 Antibacterial action of SLJO (a) and SLPO (b) against *Escherichia coli*

Thus SLPO exhibited better antibacterial action and both SLs were more effective against gram positive bacterium- *S. aureus* as compared to gram negative bacterium- *E. coli*. Gram negative bacteria have a complex cell envelope comprising of an outer membrane and an inner membrane that delimit the periplasm [35]. This can be the possible reason for less inhibitory potential of SLs against Gram negative bacteria. The antibacterial character of SLs is an additional advantage while using them in detergent formulations.

The observations on physiochemical and biological properties of SLJO and SLPO were assessed comparatively. It was observed that SLPO exhibited substantially higher antibacterial activity as compared to SLJO. SLPO was found to be largely composed of lactonic forms i.e. 80% while SLJO contained 69% of Lactonic forms. Lactonic form of SL is known to be biologically more active [2]. Therefore this finding can be justified on the basis of higher percentage of Lactonic forms. In terms of surfactant property, SLJO performed superior as compared to SLPO. This was evident from the data on CMC value, emulsification activity, stability and contact angle. The comparative structural composition data in Table2 revealed that SLJO contained higher percentage of long chain fatty acid derived SLs and greater extent of unsaturation as compared to that of SLPO. Both these characters can enhance the hydrophobicity of SL preparation which will in turn alter the hydrophilic-lipophilic balance and render the compound a better surfactant property.

2.3.3 Evaluation of surface active properties of SLPO and SLJO

2.3.3.1 Determination of contact angle

The contact angle value is dictated by the interaction between surfactant molecule and the solid surface. Acute contact angle correlate to free spreading of liquid over the given surface while the obtuse contact angles are observed generally in case of hydrophobic surfaces. Presence of surfactants in the given liquid reduces the solid-liquid interfacial tension thus better spreading is achieved in presence of surfactant. SLJO and SLPO were able to improve spreading and reduce the contact angle. Develter *et. al.* 2010 reported the contact angle reduction from 110° to 80° on Polyvinylchloride surface. This was achieved at the concentration 36mg/l of SL synthesized using glucose and rapeseed oil [36]. Thus SLs are suitable for use in dishwasher.

Sample	Contact angle value		
	Glass	Teflon	Stainless steel
Control (distilled water)	37°	95°	85°
SLPO	30°	63°	55°
SLJO	38°	56°	42°

Table 2.12 Change in contact angle of water on addition of SLJO, SLPO with different surfaces

2.3.3.2 Examining the wetting property

Dose dependent wetting performance of SLs was assessed using canvas disc method. At 0.01% concentration of SLs, 6-8.8 minutes were required for sinking. While at 1g% concentration sinking time decreased to 1.56-1.65 minutes. In the first phase of washing, textile fibres and soil must be wetted as thoroughly as possible by the wash liquor. Wetting is a complex process which is determined by the interaction of the different interfacial tensions between the solid surface, the liquid and the gas phase. A contact angle θ between the solid and a drop of a liquid applied to its surface is formed and this can be taken as a measure of wetting [37]. From the results of contact angle determination and wetting; it can be expected that SLs will reduce the soaking time required during washing process thus making the interface for stain removal available within shorter period (Refer to Figure 2.17).

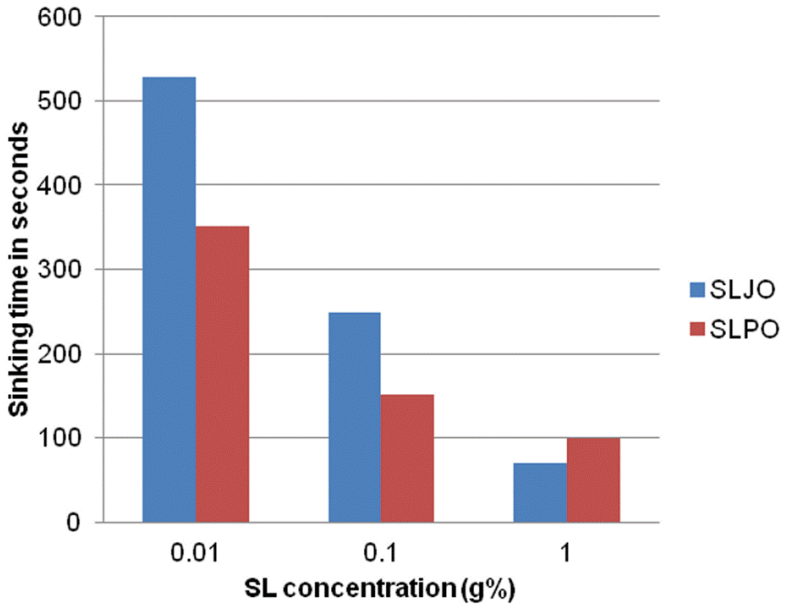


Figure 2.17 Wetting property of SLJO and SLPO

2.3.4 Comparative performance assessment of SLs with synthetic surfactants, detergent

2.3.4.1 Wetting property of SLs in combination with synthetic surfactants

Sinking time required in case of combinations of SL with SDS and Triton X-100 was less than the time required for individual SLJO, SLPO, SDS and Triton X-100 as can be seen from Figure 2.18. When used alone at concentration 0.01g% SDS showed the sinking time 751 seconds while Triton X-100 showed the sinking time as 387 seconds. After incorporation of SLs in combination with these synthetic

surfactants, sinking time decreased suggesting that SL enhances the wetting property of SDS and Triton X-100. Lowest sinking time was observed for the combinations of SLs: synthetic surfactants mixed in ratio 75:25. SLJO: SDS (75:25) showed the sinking time of 265 seconds while SLPO: SDS (75:25) sinking time was recorded as 88 seconds. SLJO: Triton X-100 (75:25) reduced the sinking time for canvas disc to 130 seconds while SLPO: Triton X-100 (75:25) caused the canvas disc to sink to bottom within 80 seconds.

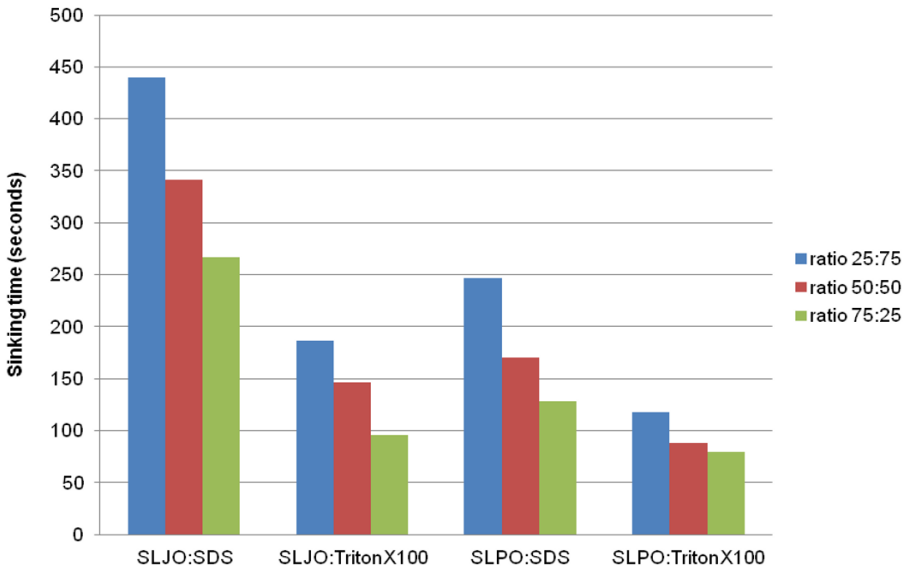


Figure 2.18 Effect of SL addition to improve wetting property of synthetic surfactants.

2.3.4.2 Detergency test

Figure 2.19 is the representative of detergency test results demonstrating comparative washing performances of SLs, commercial detergent and the SL-detergent combinations against coffee stain. It can be said that performance of SLs was nearly equal to that of detergent as with polyester fabric, total stain removal was achieved with all the combinations. With cotton, total coffee stain removal was not achieved with commercial detergent or SLs but SL-detergent combinations resulted in better stain removal and SLPO with commercial detergent performed best. Therefore potential of SLs to substitute for commercial synthetic detergent was demonstrated.

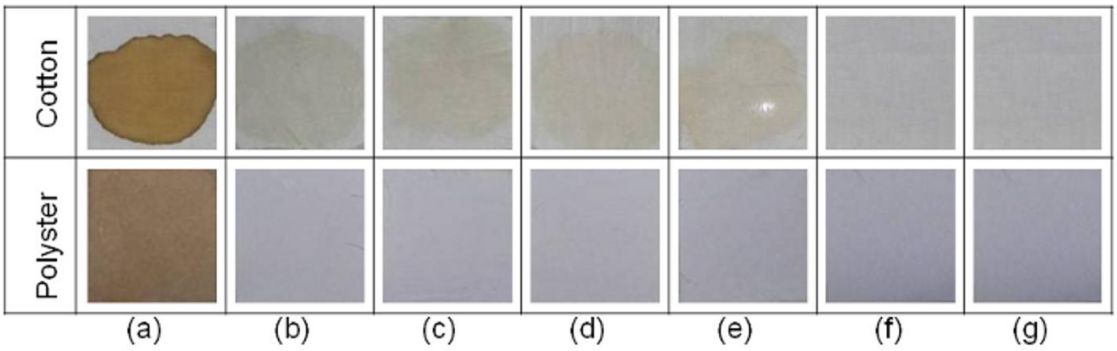


Figure 2.19 Detergency test results-Cleaning performances of SLs and commercial detergent and their combination against coffee stain. (a) coffee stained fabrics (b) washed with commercial detergent (c) washed with SLJO (d) washed with SLJO and commercial detergent 1:1 (e) washed with SLPO (f) washed with SLPO and commercial detergent 1:1 (g) unstained fabric

As per the results depicted in Figure 2.20, with oil stained cotton, any sole or combination of surface active agent couldn't achieve total cleaning with present protocol. Longer soaking and washing time might work for desired results. This can be attributed to the high absorption capacity of cotton towards oil. However with polyester when the absorption capacity was less, equally well cleaning was achieved with all the surface active agents.

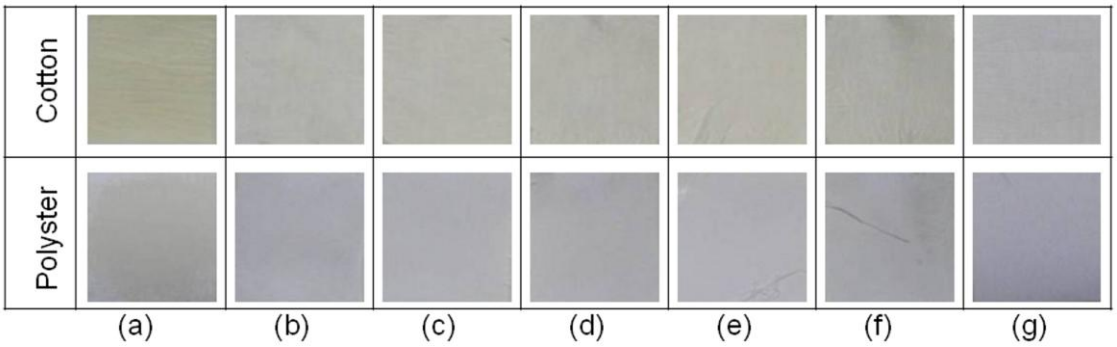


Figure 2.20 Detergency test results-Cleaning performances of SLs and commercial detergent and their combination against oil stain. (a) oil stained fabrics (b) washed with commercial detergent (c) washed with SLJO (d) washed with SLJO and commercial detergent 1:1 (e) washed with SLPO (f) washed with SLPO and commercial detergent 1:1 (g) unstained fabrics

As shown in Figure 2.21, poster color stained cotton fabric was cleaned with almost 80% efficiency with all the candidates. SLPO excelled in the performance as compared to others. In case of polyester fabric, reasonable stain removal was not achieved with any surface active agent. But it can be claimed that performances of SLs and commercial detergent were nearly same or comparable.

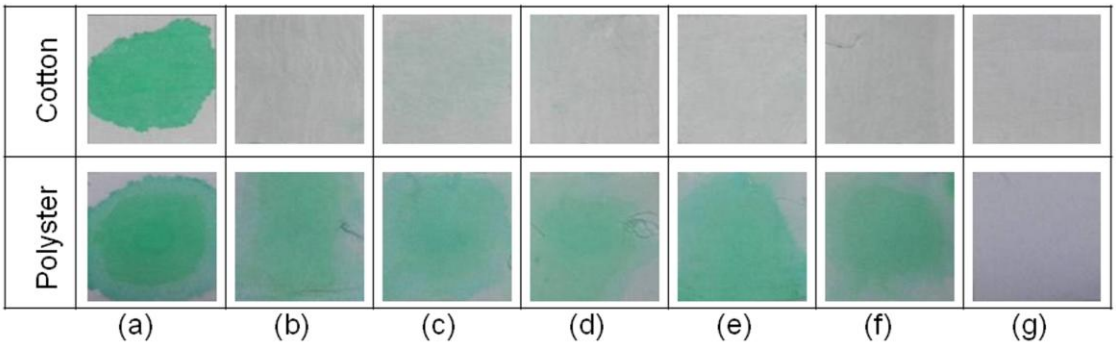


Figure 2.21 Detergency test results-Cleaning performances of SLs and commercial detergent and their combination against poster color stain. (a) poster color stained fabrics (b) washed with commercial detergent (c) washed with SLJO (d) washed with SLJO and commercial detergent 1:1 (e) washed with SLPO (f) washed with SLPO and commercial detergent 1:1 (g) unstained fabric

Turmeric stains are known to be notoriously difficult to treat. Conventionally bleach or acids are used for these stains which damage the fabric. As per the Figure 2.22, the turmeric stains with curcuminoids as major ingredients were found toughest to clean as compared to the other stains. During single washing, stains were not cleaned satisfactorily. In case of cotton fabric commercial detergent and its combinations performed better. While in case of polyester fabric, the performances were somewhat similar though there was no complete removal of turmeric stain.

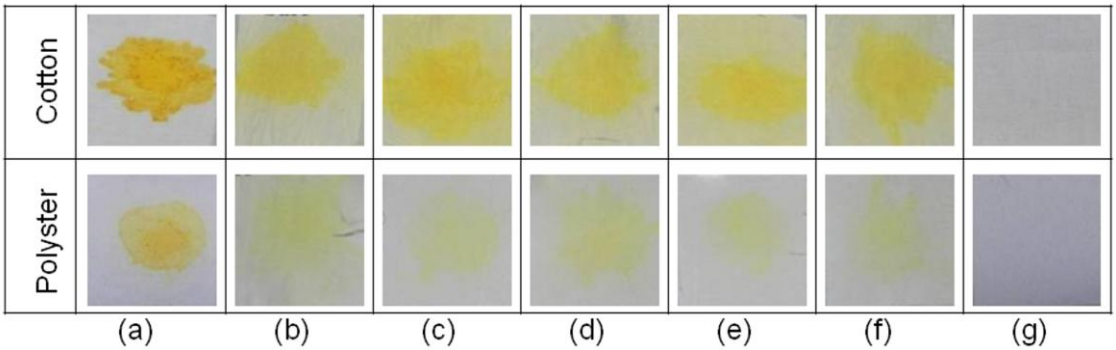


Figure 2.22 Detergency test results-Cleaning performances of SLs and commercial detergent and their combination against turmeric stain. (a) turmeric stained fabrics (b) washed with commercial detergent (c) washed with SLJO (d) washed with SLJO and commercial detergent 1:1 (e) washed with SLPO (f) washed with SLPO and commercial detergent 1:1 (g) unstained fabric

In the detergency test, stains differing in their chemical nature have been used which were considered to be notorious such as Cafeic acid- a yellow solid containing phenolic, acrylic group in coffee stain and curcuminoids in turmeric. Conventionally bleach or acids are used for these kinds of tough stains which damage the fabric on the other hand, SLs are skin friendly.

The results can be summed up as – there was an indication that for majority of stains, SLJO as well as SLPO could work as good as commercial detergents. Through standardization of the washing procedures, washing performance can be improved further.

It was clearly seen from the results obtained for different classes of stains that SLs when added along with commercial detergents (SL was added in 1:1 proportion) markedly enhanced the cleaning efficiency. This way, reduction in the detergent load to half is really attractive and will have big positive impact.

Thus, to conclude, in the present chapter SL, a type of biosurfactants has been produced using non edible oils derived from seeds of *Jatropha curcas* and *Pongamia pinnata*. The present report can be regarded as one of the first reports with reference to utilization of these oils for reducing SL production cost. The yield value obtained for SLJO was 15.25g/l and for SLPO, it was 19.3g/l with optimized conditions of 1%v/v oil feeding. These yields are remarkable when compared to the single previous report wherein the values were 6.0g/l and 7.6g/l for Jatropha and Pongamia oil respectively with 10% oil i.e. fatty acid precursor feeding. For production of SLs, Jatropha and Pongamia oil, containing alkaloids and phenolics have been used. Detoxification of these oils has been achieved by exploiting the robust organism- *Candida bombicola* and a valuable molecule has been produced from renewable stock. We have used resting cell method for the SL production in contrast to growth associated SL production. Resting cell method is a cost efficient method as cells could be used several times, reducing the cost of media as well as time.

The surface active properties have been checked and these SLs were found to work at relatively low CMC values- SLJO-9.5mg/l and SLPO-62mg/l. Other desired properties of SL to work as good detergent include wetting property, contact angle reduction, antibacterial action etc which were found satisfactory. Also the use of mixture of SLs is probably enhancing the surfactant property as against to the SLs produced using the typical fatty acid precursor- oleic acid. The emulsification property of SLs has been evaluated with special reference to changes in environmental parameters pertaining to different water qualities to show that these SLs can be used in variable conditions.

In this chapter we are reporting the use of SLs for cleaning the fabric stains in comparison with commercial detergent formulation. With coffee, oil, poster color stains and turmeric; SLs alone (SLJO or SLPO) could perform equally well as that of commercial detergent. In case of coffee, poster color and turmeric SLs performed even better. In case of turmeric stains though complete removal was not achieved; there was scope for improving the performance by modifying soaking, temperature and washing time. SLs when used with detergent, showed improved performance as compared to the performances of single cleaning agent. The present data suggests that SLs have the potential to partially replace synthetic petroleum based detergents thus reducing the load and harm caused to environment. Synthetic detergents exhibit half life values as long as 16 days which is detrimental to aquatic life and badly affects the ecological balance of water bodies. On the contrary, the biosurfactant- SLs are biodegradable, ecofriendly and non toxic.

In present chapter shake flask conditions have been standardized for SL production. It is possible to increase the yield further by using modern bioengineering methods such as scale up, continuous fermentation under controlled pH and oxygen conditions. Present washing performances were achieved using crude SL hence a cost incurring step of purification has been avoided. Moreover SLs are approved by US FDA as suitable for food, pharmaceutical and cosmetics applications therefore SL containing detergents will be skin friendly. With all these benefits, remarkable surfactant properties and economical production from renewable raw material SLs stand as promising alternatives to synthetic surfactant based detergents.

In preliminary experiments done, *C. bombicola* produced similar SLs when crushed seeds of Jatropha and Pongamia were used. Further detailed studies will be useful to reduce the production cost of SL.

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Chapter III

Chapter III is about evaluation of antimicrobial properties of SLs. In this chapter, the SLs derived from pure fatty acids i.e. Oleic acid (18:1) and Linoleic acid (18:2) have been used. Use of pure fatty acids ensured formation of limited number of SL structural variants rather than some vegetable oil containing various fatty acids.

This Chapter has been divided into 2 sub-chapters as follows-

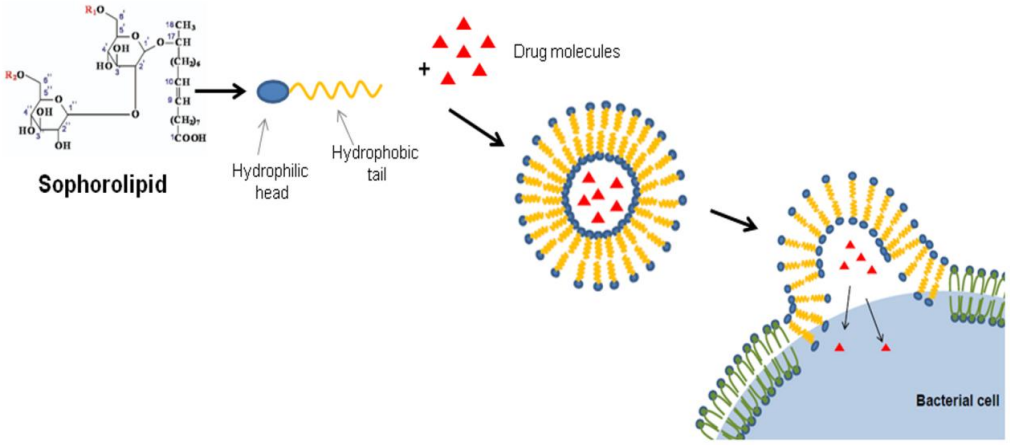
Chapter III(a)- Sophorolipid biosurfactants act in synergy with antibiotics to enhance their efficiency

and

Chapter III(b)-Exploration of antiviral activity of sophorolipids

Chapter III(a)

Sophorolipid biosurfactants act in synergy with antibiotics to enhance their efficiency



To cope up with the problem of antibiotic resistance development among major bacterial populations, various approaches are being tried out. Sophorolipids (SLs) are US FDA approved biosurfactants with antimicrobial properties. SLs have been used along with antibiotics as a different approach to improve antibiotic efficiency. Here the synergistic action of SL has been demonstrated with different antibiotics to get enhanced inhibitory action. The index bacteria used include *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The combination was observed to be more effective against the gram positive bacterium as compared to the gram negative bacteria owing to the ease of antibiotic entry and better inhibitory action of SLs against Gram positive bacteria. In case of *S. aureus*; antibiotic (tetracycline) alone couldn't achieve total inhibition till the end of 6h while the SL- antibiotic combination achieved total inhibition before 4h of exposure under same set of conditions. The inhibition caused by exposure of bacterium to SL-antibiotic combination was approximately 25% more as compared to SL alone. In spite of known robustness of Gram negative bacteria, SL- antibiotic (Cefaclor) combination proved efficient against *E. coli.* In case of *Pseudomonas aeruginosa*, only marginal improvement was seen. The combination showed 48% more inhibition within 2h of exposure as compared to antibiotic alone. The mechanism of action has been speculated about SL facilitating the entry of drug molecules through the structurally alike cell membrane. SLs being amphiphilic in nature, can form micellar self assemblies which can enclose the water soluble drugs and deliver it to cell interior. The data has been supplemented with Scanning electron micrographs showing bacterial cell membrane damage and formation of pores.

3a.1 Introduction

The advent of antibiotics for treating bacterial infections is considered one of the major advances in modern medicine. However, compared with other drugs, the lifetime of antibiotics for clinical use has been substantially limited by the phenomenon of antibiotic resistance [1]. Today, clinically important bacteria are characterized not only by single drug resistance but also by multiple antibiotics resistance caused by the use and misuse of antimicrobials during past decades [2]. Serious infections caused by bacteria that have become resistant to commonly used antibiotics have become a major global healthcare problem in the 21st century, involving all major microbial pathogens and antimicrobial drugs. Infections due to multidrug resistant pathogens are difficult to manage due to bacterial virulence factors and because of a relatively limited choice of antimicrobial agents [3]. Such infections are more severe and require longer and more complex treatments, but they are also significantly more expensive to diagnose and to treat [2, 4]. In this scenario, it is imperative to discover fresh antimicrobials or new practices that are effective for the treatment of infectious diseases caused by drug-resistant microorganisms [3]. In order to control multi-drug resistant pathogens such as tuberculosis and MRSA, clinicians have increasingly turned to multi-antibiotic therapies [5].

For the problem of antibiotic resistance, several approaches are being tried out ranging from nanotechnology, genomics to combined antibiotic therapy. Combining antibiotics with nanoparticles restores the ability of antibiotics to destroy bacteria that have acquired resistance to them. Furthermore, nanoparticles tagged with antibiotics increase the concentration of antibiotics at the site of bacterium–antibiotic interaction, and to facilitate binding of antibiotics to bacteria [6]. Comparative genomic analysis provides relevant information on the evolution of resistant strains and on resistance genes with cognate genetic elements. Moreover, bacterial genomics, including functional and structural genomics, is proving to be instrumental in the identification of new targets, which is a crucial step in new antibiotic discovery programs [1]. As mentioned above, combined antibiotic therapy is being practiced against Tuberculosis for over fifty years involving the drugs with different modes of action [7]. Based on this

approach, the drug synergism between antibiotics and bioactive plant extracts has been demonstrated.

As mentioned earlier the strategies based on genomic approach and nanotechnology require detailed knowledge of each drug and response by target organism. Additionally they are specific in nature. Moreover interactions of nanoparticles with biological systems are relatively unknown. The high surface to volume ratio, render nanoparticles highly reactive and catalytic. They are also able to pass through cell membranes in organisms [8]. These tiny particles are able to enter the body through the skin, lungs or intestinal tract, depositing in several organs and may cause adverse biological reactions by modifying the physiochemical properties of living matter at the nanolevel. In addition, the toxicity of nanoparticles will also depend on whether they are persistent or cleared from the different organs of entry and whether the host can raise an effective response to sequester or dispose off the particles. Recently, a number of investigators have found nanoparticles responsible for toxicity in different organs. All these facts limit the applicability of nanoparticles [9].

The strategies with wide applicability are being looked for. The drug delivery solutions are based on minimization of drug dosage, controlled localization and action of drugs. Liposomes can be considered as the promising candidates based on this approach. Liposomes are among the most widely used type of pharmaceutical nanocarriers for small and poorly water-soluble drug molecules. Conventional liposomes were predominantly used as long-circulating transport vehicles, followed by a second generation that improved the circulation time further by decorating the surface with PEG-chains (stealth liposomes). Third-generation liposomes are now being engineered to contain targeting ligands and to carry out stimuli-sensitive triggering of the drug release [10]. Alternatively, SLs; a kind of biosurfactant are amphiphilic in nature and tend to form self assemblies which upon modification can possibly be tuned into liposomes. Thus they have promising potential to be used as a drug delivery system. Moreover they possess range of beneficial properties which make them suitable for pharmaceutical applications.

Biosurfactants are coming up as emerging class of biomedical compounds. They are a suitable alternative to synthetic medicines and antimicrobial agents, and could be used as safe and effective therapeutic agents or probiotics [11]. SL is a promising candidate for such applications being produced by non pathogenic yeasts, such as *Candida bombicola, Candida apicola* and *Candida bogoriensis*. They are generally present in the form of disaccharide sophoroses (2-O-β-D-glucopyranosyl-D-glucopyranose) linked β glycosidically to the hydroxyl group at the penultimate carbon of fatty acids [12]. Antimicrobial property of SLs further makes them suitable for the present purpose. The antimicrobial action is not merely restricted toward bacteria; they also act as antifungal, antialgal, antimycoplasma and antiviral agents [13]. The proposed primary mechanism of action of these surfactants is membrane lipid order perturbation, which compromises the viability of microorganisms [14]. Moreover SLs offer the advantages of biodegradability, low ecotoxicity and the production based on renewable-resource substrates. The US FDA has also approved biosurfactants/sugar esters for the use in food and pharmaceuticals. Tests with SLs pointed out that they are not irritating to the skin, do not trigger allergic reactions and have an oral safety level which is greater than or equal to 5ml/kg weight. Cytotoxicity was evaluated with human epidermal keratinocytes and was proven to be low [15]. As SLs possess antimicrobial properties, their applications include incorporation in germicidal mixtures suitable for cleaning fruits and vegetables. Other medically beneficial effects include ability to trigger cell differentiation, apoptosis in cancerous cells. On account of its role as emulsifier and skin compatibility, they find application in various cosmetic formulations. The French company Soliance produces SL based cosmetics for body and skin [13].

Sun, X. *et. al*.., 2004 have demonstrated the synergistic effects of combination of SL and loess for harmful algal bloom mitigation to bring down the effective dose of both when used individually. In the field test, the effective concentration of loess and SL in the combination group was reduced to 10% and 25%, respectively [16]. Mannosyl Erythritol Lipid-A, a type of glycolipidic biosurfactant containing cationic liposomes promoted the gene transfection efficiency five to seven times with mammalian cultured cells [17].

Therefore the evidences of synergistic action of bioactive agents along with antibiotics lead us to speculate about the use of SLs in combination with antibiotics for efficient infection control. The amphiphilic nature of molecule is a key feature that is expected to mediate the facilitated entry of drug molecules across the cell membrane on account of its structural similarity with cell membrane lipid bilayer. In the present chapter the synergistic effect of SL with antibiotics differing in their modes of action has been demonstrated against representative index bacteria.

3a.2 Materials and methods

3a.2.1 Microorganisms and their maintenance

(a) *Candida bombicola* (ATCC 22214) was used for the production of SLs. It was maintained on MGYP (Malt extract- 0.3g%, Glucose- 2g%, Yeast extract- 0.3g%, Peptone- 0.5g% and Agar- 2.0g %) slants. The microorganism was sub cultured in every 4 weeks and maintained at 4°C in a refrigerator as described in earlier chapter, section 2.2.1.

(b) The test microorganisms- *Escherichia coli* (ATCC- 8739), *Pseudomonas aeruginosa* (NCIM-5031) and *Staphylococcus aureus* (ATCC- 29737) were procured from National Collection of Industrial Microorganisms, NCL. The cultures were maintained on Nutrient agar slants. The microorganisms were sub cultured in every 4 weeks and maintained at 4°C in a refrigerator till required further.

3a.2.2 Chemicals and reagents

All media, chemicals and solvents used in this study were of analytical grade and supplied by either Hi-media pvt. Ltd., India or Merck India ltd.

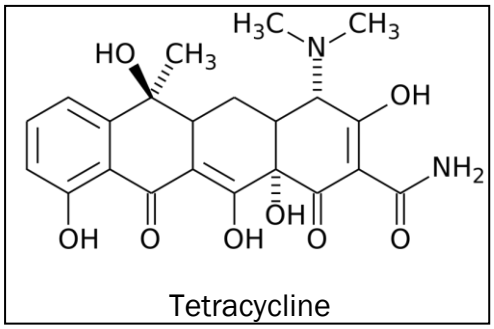
The fatty acid precursor- Oleic acid was purchased from Sigma Aldrich. The antibiotics- Cefaclor, Tetracycline-HCl and Ciprofloxacin were also purchased from Sigma and stored in refrigerator till required.

About the antibiotics used here-

Tetracyclines are a group of broad spectrum antibiotics whose general usefulness has been reduced with the onset of bacterial resistance [18]. Tetracycline is

soluble in water and the solubility decreases in non-polar solvents such as alcohol, acetone.

Structure



Tetracycline

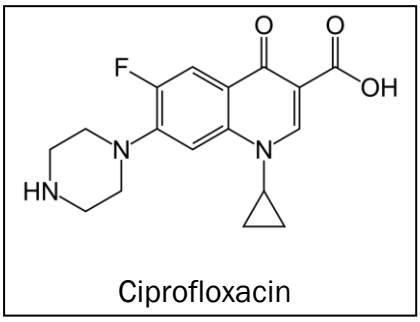
Mechanism of action: Tetracyclines bind to the 30S subunit of microbial ribosomes. They inhibit protein synthesis by blocking the attachment of charged aminoacyl-tRNA to the A site on the ribosome. Thus, they prevent introduction of new amino acids to the nascent peptide chain. The action is usually inhibitory and reversible upon withdrawal of the drug.

Resistance: Resistance to the tetracyclines results from changes in permeability of the microbial cell envelope. In susceptible cells, the drug is concentrated from the environment and does not readily leave the cells. In resistant cells, the drug is not actively transported into the cells or leaves it so rapidly, inhibitory concentrations are not maintained. This is often plasmid-controlled mechanism. Tetracyclines have a broad spectrum of antibiotic action. Originally, they possessed some level of bacteriostatic activity against almost all medically relevant aerobic and anaerobic bacterial genera. The susceptible genera included both Gram-positive and Gram-negative bacteria, barring a few exceptions, such as *Pseudomonas aeruginosa* and *Proteus spp.*, which displayed intrinsic resistance. However, many pathogenic organisms have acquired resistance by now. Thus the formerly vast versatility of this group of antibiotics has greatly eroded. Resistance amongst *Staphylococcus* spp., *Streptococcus* spp., *Neisseria gonorrhoeae*, members of the Enterobacteriaceae and several other previously sensitive organisms is now quite common.

Ciprofloxacin is a second generation fluoroquinolone antibiotic. Its spectrum of activity includes most strains of bacterial pathogens responsible for respiratory,

urinary tract, gastrointestinal, and abdominal infections, including both gram-negative and gram-positive bacterial pathogens [19].

Structure:



Ciprofloxacin

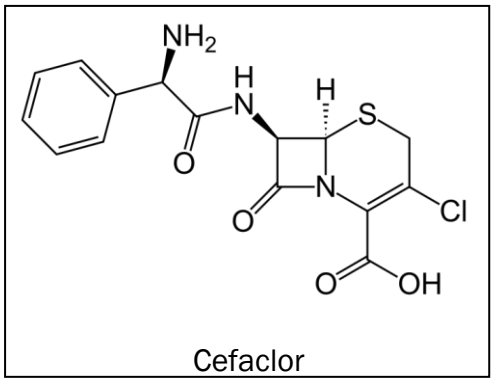
Mechanism of action: Ciprofloxacin is a broad spectrum antibiotic active against both Gram positive and Gram negative bacteria. It functions by inhibiting DNA gyrase, a type II topoisomerase, and topoisomerase IV, enzymes necessary to separate bacterial DNA, thereby inhibiting cell division.

Resistance: Ciprofloxacin was once considered a powerful antibiotic of last resort, used to treat especially tenacious infections. But there have been widespread use of the antibiotic to treat minor infections as well as non-approved uses. As a result in recent years many bacteria have developed resistance to this drug, leaving it significantly less effective than it would have been otherwise.

Resistance to ciprofloxacin and other related fluoroquinolones may evolve rapidly, even during a course of treatment. Numerous pathogens, including *Staphyloccus aureus*, enterococci, *Streptococcus pyogenes* and *Klebsiella pneumonia* now exhibit resistance worldwide.

Cefaclor, is a second-generation cephalosporin antibiotic used to treat certain infections caused by bacteria such as pneumonia and infections of ear, lung, skin, throat, and urinary tract. Cefaclor is slightly soluble in water but insoluble in alcohol and chloroform [20, 21].

Structure:



Cefaclor

Mechanism of action: Cephalosporins are bactericidal and have the same mode of action as other beta-lactam antibiotics (such as penicillins). Beta lactam antibiotics target the Penicillin Binding Proteins or PBPs- a group of enzymes found anchored in the cell membrane. These enzymes are involved in the cross linking of the cell wall. Cephalosporins are less susceptible to β-lactamases. Cephalosporins disrupt the synthesis of the peptidoglycan layer of bacterial cell walls.

Resistance: Resistance to cephalosporin antibiotics can involve either reduced affinity of existing penicillin-binding-protein components or the acquisition of a supplementary beta-lactam-insensitive penicillin-binding-protein. Currently, some *Citrobacter freundii, Enterobacter cloacae* and *Escherichia coli* strains are resistant to cephalosporin. Some *Morganella morganii, Proteus vulgaris, Providencia rettgeri,Pseudomonas aeruginosa* and *Serratia marcescens* strains have also developed resistance to cephalosporin to varying degrees. Beta-lactamases are the enzymes which render the producer bacteria resistant to beta-lactam antibiotics. These enzymes break open the beta-lactam ring thus deactivating the molecule's antibacterial properties.

3a.2.3 Synthesis, extraction and characterization of sophorolipid

SL was produced according to the procedure mentioned in Chapter 2, section 2.2.3.4 by resting cell method. For SL production, seed culture was prepared by inoculating 10 ml of fresh MGYP nutrient medium with *C. bombicola* ATCC 22214 followed by incubation at 30°C, 180 rpm for 24 h. This pre-inoculum was added to 90 ml MGYP nutrient medium in a 500-ml Erlenmeyer flask and incubated

further for 48 h. Cells were harvested, washed twice with sterile distilled water. The cell pellets (biomass ~1.5 g dry weight or 6.0 g wet weight in 100 ml medium) were re-dispersed in sterile 100 ml 10% glucose with 1 ml of Oleic acid (dispersed in 1 ml ethanol) and again incubation was continued for 96 h when a brown and viscous SL mass was seen settled at the bottom of the flask. The SLs were harvested by the procedure previously reported by Shah *et. al.* 2007. Culture medium was centrifuged at 5,000 rpm, at 10°C for 20 minutes. The supernatant was extracted twice with equal volumes of ethyl acetate, the organic layer was dried over anhydrous Na_2SO_4, and the solvent was removed by rotary vacuum evaporation. The yellowish brown semi-crystalline product was washed twice with n-hexane to remove unconverted fatty acid [22] as per the procedure mentioned in Chapter 2 section 2.2.3.1.

3a.2.4 Structural characterization of SL

3a.2.4.1 HPLC analysis- The SL sample was subjected to HPLC analysis to get an idea about relative percentages of lactonic and acidic component based on standard sample run. The chromeline-Hitachi HPLC system was used along with C18 column (5 µm, 150 x 4.6 mm). The solvent system used was MilliQ water-Acetonitrile(ACN) . Total run time was 65 minutes. For the first 15 minutes, ACN was maintained at 20% then it was gradually raised to 80% upto 40 minutes and was brought to 100% till 50 minutes and thereafter maintained for 15 minutes. The run was performed at flow rate 0.5ml/min and 25°C. The compounds were detected by L-2490 UV detector at 207nm.

3a.2.4.2 MALDI-MS (Matrix Assisted Laser Desorption/Ionization- Mass spectrometry) study- SL sample 1mg was dissolved in 1ml of methanol. Further 5µl of the sample was mixed with 20 µl of dithranol matrix and MALDI-MS study was done on AB SCIEX TOF/TOF 5800.

3a.2.5 Conjugative effect of SL and tetracycline against S. aureus

For the assay of conjugative action of SL and antibiotic, 3 different antibiotics were chosen differing in their site of action as described earlier.

(a) Stock preparation: Tetracycline stock was prepared by dissolving Tetracycline-HCl in sterile distilled water at the stock strength 1mg/ml. SL (Sophorolipid of Oleic acid mixture of lactonic and acidic form) stock was prepared in sterile

distilled water by dissolving the appropriate amount of SL in sterile distilled water supplemented with 3% v/v alcohol. SL stock strength used was 10mg/ml.

(b) Determination of Minimum Inhibitory Concentrations of individual SL and Tetracycline: In the first step, A_{600} values i.e. absorbances of bacterial suspension were correlated to number of Colony Forming Units i.e. CFUs by spread plate technique. Thus the A_{600} value giving isolated, countable colonies was fixed and the same A_{600} was maintained throughout the experiment. Based on the prior experimentation, the SL concentration range fixed for MIC determination was 100- 600µg/ml while the concentration range used for tetracycline was 5-100µg/ml. Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of compound that inhibits visible growth of microorganisms on the culture plate [23]. MIC of tetracycline against *S. aureus* (ATCC-29737) was found to be 150µg/ml while that of SL was found to be 400 µg/ml. Based on the results of MIC determination experiments, the concentrations of SL (300µg/ml) and Tetracycline (15µg/ml) were fixed. These are the sublethal concentrations of both bioactive compounds which are used so as to rightly evaluate the conjugative effect.

(c) Time dependent assay of synergistic action along with controls: Four test reactions were set up. The dilution scheme was designed as mentioned in Table 3a.1

Sr. no.	Test reaction description	Volume of SL stock (µl)	Volume of antibiotic stock (µl)	Volume of sterile distilled water (µl)	Volume of bacterial suspension (µl)	Total volume (µl)
1	Control	-	-	800	200	1000
2	SL alone	30	-	770	200	1000
3	SL + Tetracycline	30	15	755	200	1000
4	Tetracycline	-	15	785	200	1000

Table 3a.1. Dilution scheme used for the assay of conjugative action of SL and Tetracycline against *S. aureus*

During the sequential additions, SL and Tetracycline were mixed thoroughly followed by addition of sterile distilled water and suspension. The cells were exposed to Tetracycline alone, mixture of SL and tetracycline and SL alone.

Reaction mixtures as described in Table 3a.1 were incubated at 28°C, 180 rpm for 6h. The samples were removed at periodic intervals- 2, 4 and 6h and no. of CFUs were determined by spreading 50µl of mixture on Nutrient agar plates. The plates were incubated at 28°C and colonies were visualized after 24h. All antibacterial activity tests were performed in triplicates to certify the reproducibility. Colonies were counted and percentage cell survival was calculated using following formula [24].

% cell survival = no. of colonies on test plate X 100 /no. of colonies on control plate.

3a.2.6 Conjugative effect of Ciprofloxacin and SL against Pseudomonas aeruginosa

Similar protocol as mentioned in the above experiment, section 3a.2.4 was followed. The SL stock was prepared in sterile distilled water at 10mg/ml concentration while Ciprofloxacin stock was prepared at 1mg/ml concentration in sterile distilled water. The concentration range used for MIC determination experiments of Ciprofloxacin against *P. aeruginosa* was 5-40µg/ml while the concentration range used for SL was 200-1000µg/ml.

MIC of Ciprofloxacin against *P. aeruginosa* (NCIM 5031) was observed to be 25µg/ml. Based on these results; the sub lethal concentrations of SL and Ciprofloxacin were decided to be 1000µg/ml and 15µg/ml respectively. Four test reactions were set namely- control, SL alone, SL with Ciprofloxacin and Ciprofloxacin alone as described in Table 3a.2. The sampling intervals and the protocol and data evaluation method were same as mentioned earlier in previous section 3a.2.5.

Sr. no.	Test reaction description	Volume of SL stock (µl)	Volume of antibiotic stock (µl)	Volume of sterile distilled water (µl)	Volume of bacterial suspension (µl)	Total volume (µl)
1	Control	-	-	800	200	1000
2	SL alone	100	-	700	200	1000
3	SL + Ciprofloxacin	100	15	685	200	1000
4	Ciprofloxacin	-	15	785	200	1000

Table 3a.2. Dilution scheme used for the assay of conjugative action of SL and Ciprofloxacin against *P. aeruginosa*

3a.2.7 Conjugative effect of Cefaclor and SL against Escherichia coli

Similar protocol as mentioned in the above experiment section 3a.2.5 was followed. The SL stock was prepared in sterile distilled water at 10mg/ml concentration while cefaclor stock was prepared at 1mg/ml concentration. The concentration range used for MIC determination of Cefaclor against *E. coli* was 20-80µg/ml while the concentration range used for SL was 100-1000µg/ml.

MIC of Cefalor against *E. coli* (ATCC 8739) was found to be 200µg/ml while SL alone was not inhibitory to *E. coli*. Based on the results of MIC determination experiment, the sub lethal concentrations of SL and Cefaclor were decided to be 500µg/ml and 50µg/ml respectively. 4 test reactions were set namely- control, SL alone, SL with Cefaclor and Cefaclor alone as shown in Table 3a.3. The sampling intervals and the protocol and data evaluation method were same as mentioned earlier in previous section 3a.2.5.

Sr. no.	Test reaction description	Volume of SL stock (µl)	Volume of antibiotic stock (µl)	Volume of sterile distilled water (µl)	Volume of bacterial suspension (µl)	Total volume (µl)
1	Control	-	-	800	200	1000
2	SL alone	50	-	750	200	1000
3	SL + Cefaclor	50	50	700	200	1000
4	Cefaclor	-	50	750	200	1000

Table 3a.3. Dilution scheme used for the assay of conjugative action of SL and Cefaclor against *E.coli*

3a.2.8 Scanning Electron Microscopy of treated cells

The cells were subjected to the action of SL and antibiotic combinations in respective proportions. Tube assay procedure was followed for the same. After 4h incubation with SL-antibiotic combination, the bacterial cell suspension was centrifuged. Cell pellet was resuspended in 200µl of sterile distilled water and 10-15 µl of it was drop casted on to a silicon wafer for easier locating of bacterial cells and allowed to air dry. Samples were sputter coated till a fine layer of 10nm was formed. (Sputter coater; make- EMITECH, source- Au-Pd, Gas-Argon). The E-SEMs of the samples were then recorded at the resolution 3nm at 30kV under high vacuum. (SEM; make- FEI, model-Quanta 200 #D Dual beam ESEM with EDAX, source- Tungsten thermionic emission). The untreated healthy cells prepared as described earlier were also subjected to SEM for reference.

3a.3 Results and discussion:

Till date, no studies have evaluated the use of SLs in combination with antibiotics. Thus it is not known if these compounds might have antagonistic or synergistic effects when administered with antibiotics [25]. We have used the SL mixture during the study, knowing that natural synergism between SLs creates a better balance for many interfacial activities [26].

3a.3.1 Structural characterization data

(a) As per the HPLC analysis, it was found that the SL sample contains around 83% of lactone form and remaining 17% of acidic form. The acidic SL forms get eluted first while the lactonic SLs, especially the acetylated ones, show longer retention times because of higher hydrophobicity [27]. Therefore the acidic SLs got eluted within 20minutes while the peaks eluting after 35minutes were corresponding to lactonic forms.

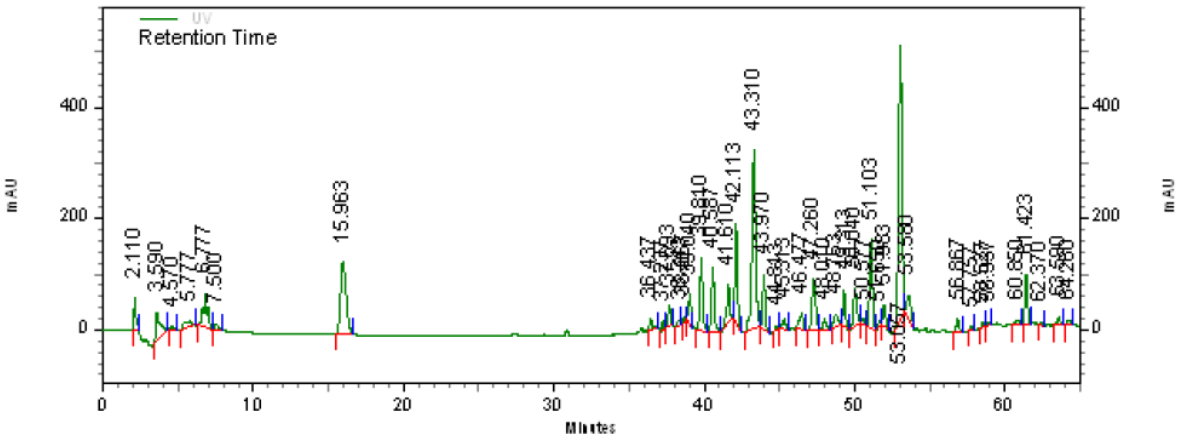


Figure 3a.1 HPLC elution pattern of SL preparation

(b) MALDI-MS study of SL- Prominent peaks from the mass spectrum were correlated to sodium adducts [$M^+ + H^+ + Na^+$] of the expected forms of SLs. Four different forms of Oleic acid derived SLs were detected. Di-acetylated lactonic SL of Oleic acid i.e. 17-L-(-oxy)-octadecanoic acid 1,4″-lactone 6′,6″-diacetate was detected with maximum % abundance. This was followed by di-acetylated acidic form. Mono-acetylated lactonic SL and mono-acetylated acidic SL were also detected in relatively small proportions. Apart from Oleic acid derived SLs, different SL structures having Linoleic (C18:2), Stearic (C18:0) and Palmitic (C16:0) acid as the hydrophobic part were also detected

as summarized in Table 3a.4. The finding was in accordance with previous reports [28]

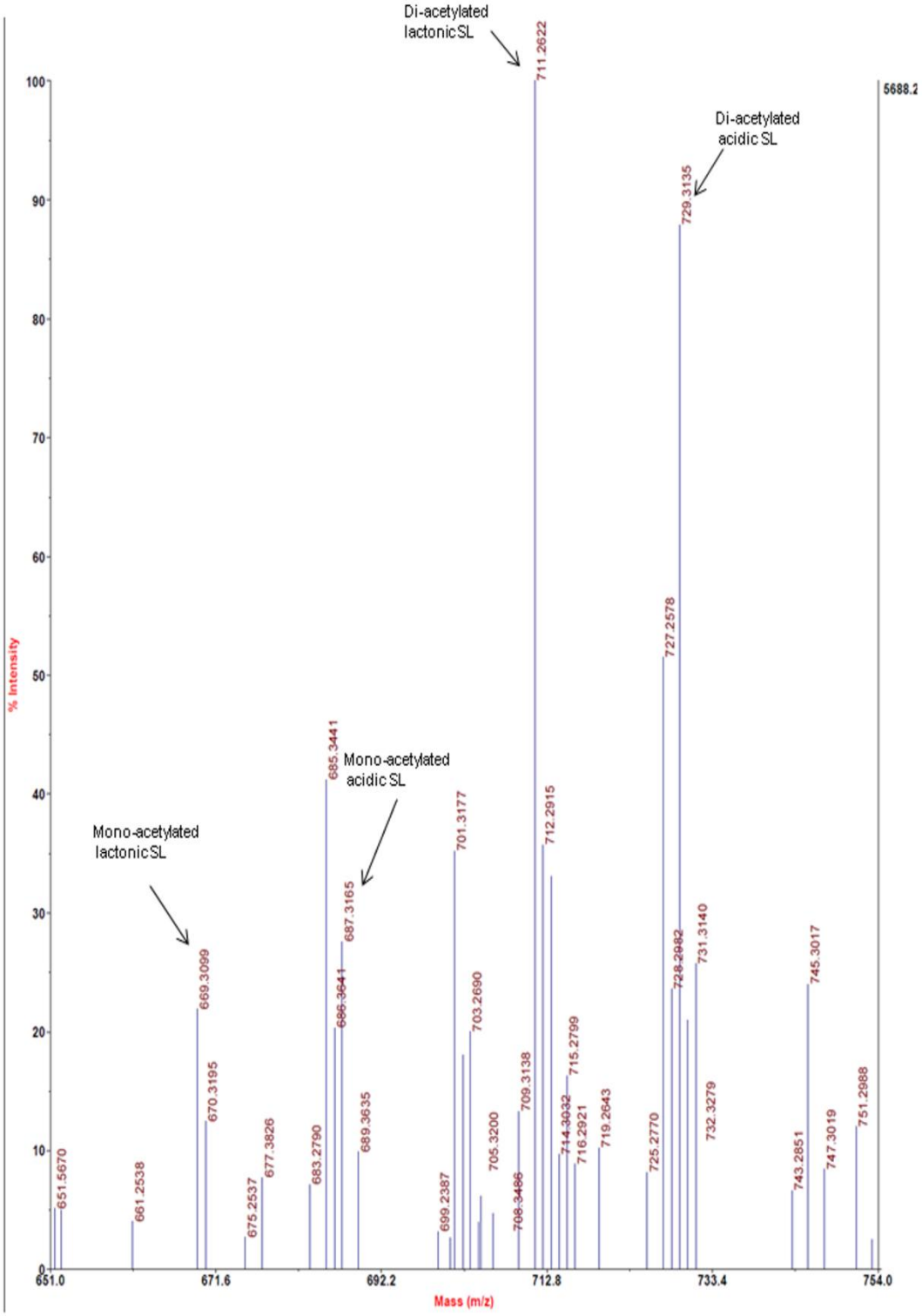


Figure 3a.2 MALDI-MS data of SL preparation. [M^++H^++Na^+] i.e. sodium adducts of the different structural forms of Oleic acid derived SLs have been indicated.

SL structure	m/z	M^++H^++Na^+
Di-acetylated SL of C18:2 Acidic form	703	727
Di-acetylated SL of C18:0 Acidic form	707	731
Di-acetylated SL of C18:2 Lactonic form	685	709
Di-acetylated SL of C16:0 Lactonic form	661	685

Table 3a.4 Peaks corresponding to SLs having fatty acids other than Oleic acid

3a.3.2 Conjugative effect of SL and tetracycline against S. aureus

In this study, three different index organisms were chosen to evaluate the synergistic action of SLs with different antibitics. These organisms are commonly occuring pathogens. *Staphylococcus aureus* is a gram-positive coccus, currently responsible for the majority of skin and soft tissue infections (SSTIs) in the United States. This bacterium is commonly found asymptomatically in healthy individuals, colonizing the anterior nares and other sites of the body, such as the skin and gastrointestinal tract. However, *S. aureus* can be extraordinarily pathogenic, causing a broad range of morbid states from serious skin infections, such as cellulitis and abscesses, to endocarditis and sepsis. *Staphylococcus aureus* is rapidly evolving resistance to contemporary topical as well as systemic antibiotics [29].

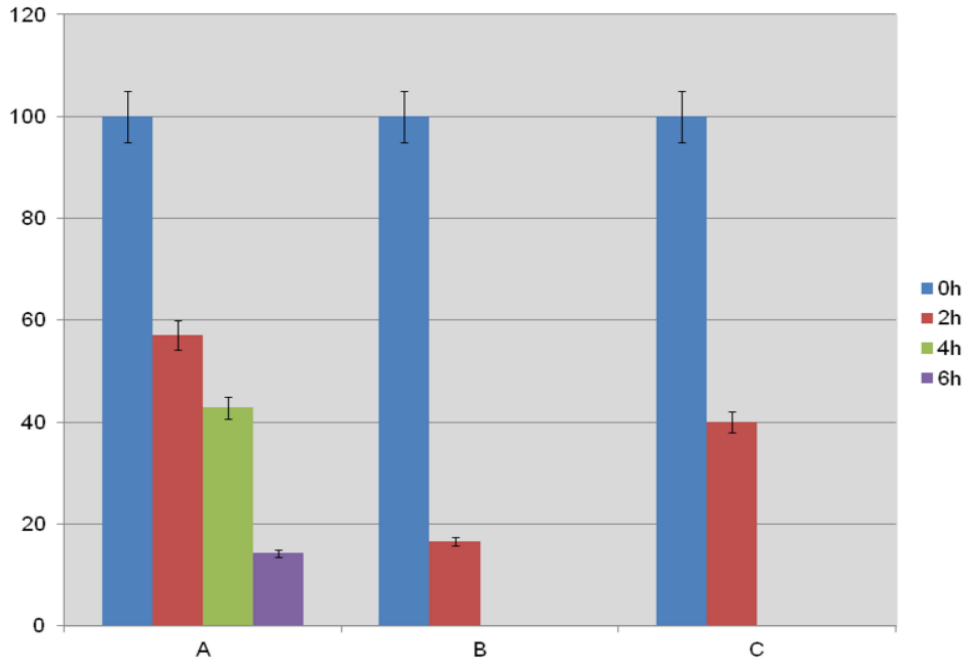


Figure 3a.3. Synergistic action of Tetracycline and SL against *S. aureus*: The graph represents % reduction in colony forming units on exposure to different bioactive agents A- Tetracycline(15µg/ml), B- Tetracycline(15µg/ml) with SL(200µg/ml), C- SL(200µg/ml).

It can be observed from Figure 3a.3 that Tetracycline, the protein synthesis affecting antibiotic alone cannot achieve total inhibition even after 6h of exposure. Whereas SL alone at 200µg/ml was efficient against *S. aureus* and showed total inhibition within 4h. However it was worth noting that when both agents were used in combination, at 2h exposure ~22% more inhibition was observed. i.e. when these 2 inhibitory agents were used together, improved efficiency was demonstrated.

3a.3.3 Conjugative effect of SL and Ciprofloxacin against P. aeruginosa

Pseudomonas aeruginosa, a gram negative rod shaped bacterium is an opportunistic pathogen of immune compromised individuals was chosen as another index bacterium. It typically infects the pulmonary tract, urinary tract, burns, wounds and also causes other blood infections. As a nosocomial pathogen it causes the highest mortality rate than any other bacterium. Bacteremia, pneumonia, osteomyelitis and endocarditis caused by bacterium have failure and mortality rates higher than those for other bacterial pathogens. High incidence, infection severity as well as resistance developed by the bacterium to conventional antimicrobial treatments are the problems while handling *P. aeruginosa* infections [30, 31].

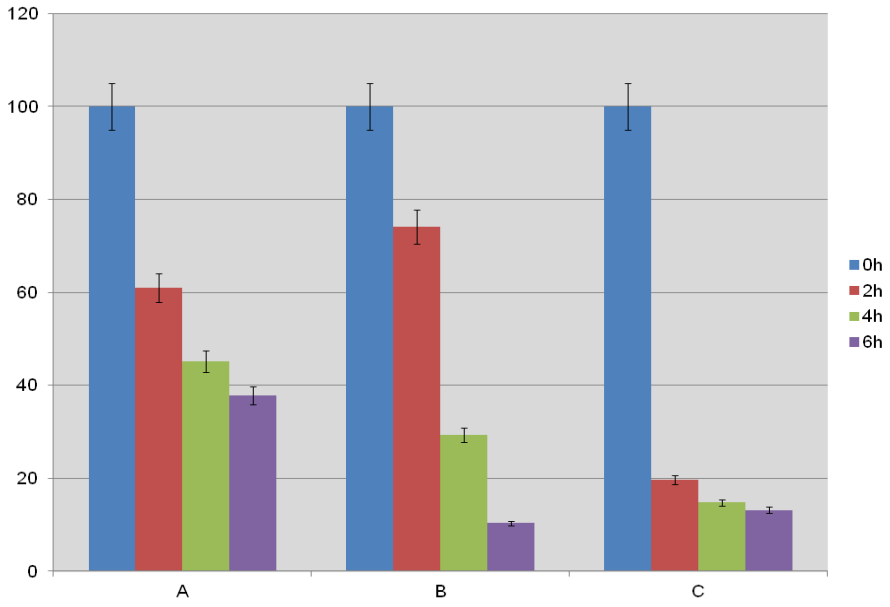


Figure 3a.4.Synergistic action of Ciprofloxacin and SL against *P. aeruginosa*: The graph represents % reduction in colony forming units on exposure to different bioactive agents A- Ciprofloaxacin (15µg/ml), B- Ciprofloaxacin (15µg/ml) with SL(100µg/ml), C- SL(100µg/ml).

As represented in Figure 3a.4, total inhibition has not been achieved in any of the test mixtures but maximum inhibition has been achieved in case of Ciprofloxacin-SL combination which was however just slightly better than that of ciprofloxacin alone which is the nucleic acid synthesis affecting antibiotic.

3a.3.4 Conjugative effect of SL and Cefaclor against E. coli

Another index bacterium used in present study, *E. coli* is found in the lower intestine of warm blooded animals. *Escherichia coli* is more than just a harmless intestinal inhabitant; it can also be a highly versatile pathogen who can be frequently deadly. Several different *E. coli* strains cause diverse intestinal and extraintestinal diseases by means of virulence factors that affect a wide range of cellular processes [32]. To include the bacterium here was important so as to observe the antimicrobial effect of cefaclor and cefaclor in combination with SL on the Gram negative bacteria that have a thin peptidoglycan layer adjacent to the inner cytoplasmic membrane, which makes them have little resistance against cefaclor.

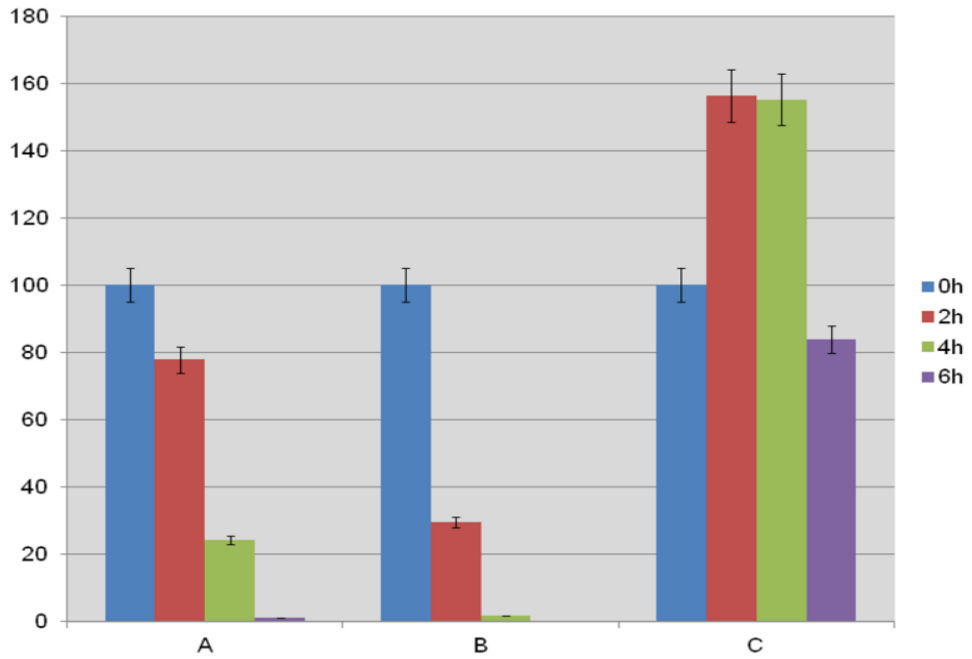


Figure 3a.5.Synergistic action of Cefaclor and SL against E. *coli*: The graph represents % reduction in colony forming units on exposure to different bioactive agents A- Cefaclor (50µg/ml), B- Cefaclor (50µg/ml) with SL (500µg/ml), C- SL(500µg/ml)

Figure3a.5 represents the comparative inhibitory action of cefaclor, SLs and their combinations against *E. coli*. Cefaclor, the cell wall synthesis affecting antibiotic has achieved almost total inhibition at the end of 6h exposure. SL alone was

unable to inhibit the bacterial growth totally but when administered along with the antibiotic; resulted in faster killing of the bacterium. It is noteworthy SL-cefaclor together could achieve ~98% killing within 4h while with Cefaclor alone it required 6h exposure to get equivalent effect.

3a.3.5 Scanning Electron Microscopy images of the bacterial cells treated with SL and antibiotic mixture

Figure 3a.6 represents the Scanning Electron Micrographs (SEM) of *S. aureus* cells. These include control cells which were not exposed to any inhibitory agent and appeared as intact, healthy cells. The cells exposed to the mixture of SL and Tetracycline were also subjected to Scanning Electron Microscopy. Treated cells exhibited disturbed cell integrity which is evident from damaged cell membranes. Also leakage and accumulation of cytoplasmic contents was observed.

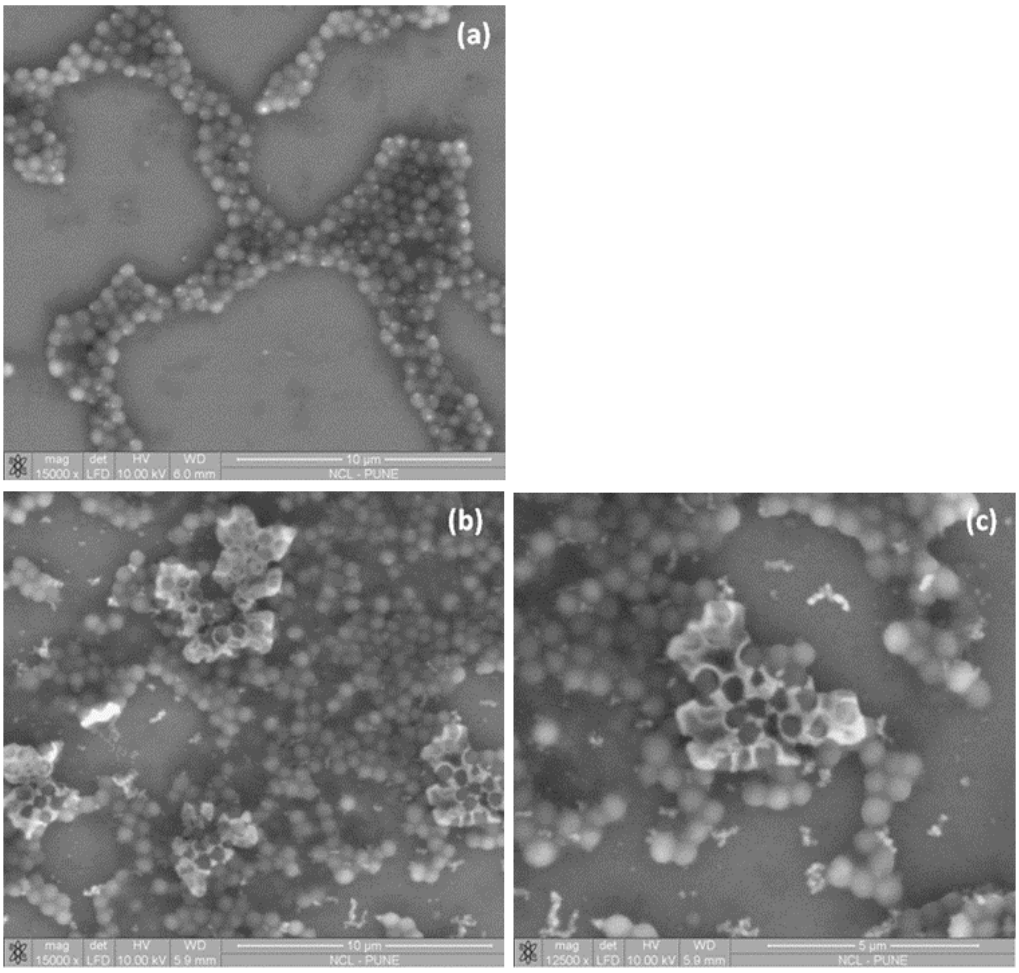


Figure 3a.6 Scanning Electron Micrograph of the *S. aureus* cells- (a) control- untreated cells (b) and (c) cells exposed to the Mixture of SL and Tetracycline. Damge to cell membranes is evident from the images. Also leakage of cellular contents can be observed

Figure 3a.7 contains the SEMs of *P. aeruginosa* cells. Cells were found shrunk and rounded suggesting divergence from original shape. Probably this has happened due to loss of cell membrane integrity and coalescence with neighboring cells. Formation of membrane pores was also observed.

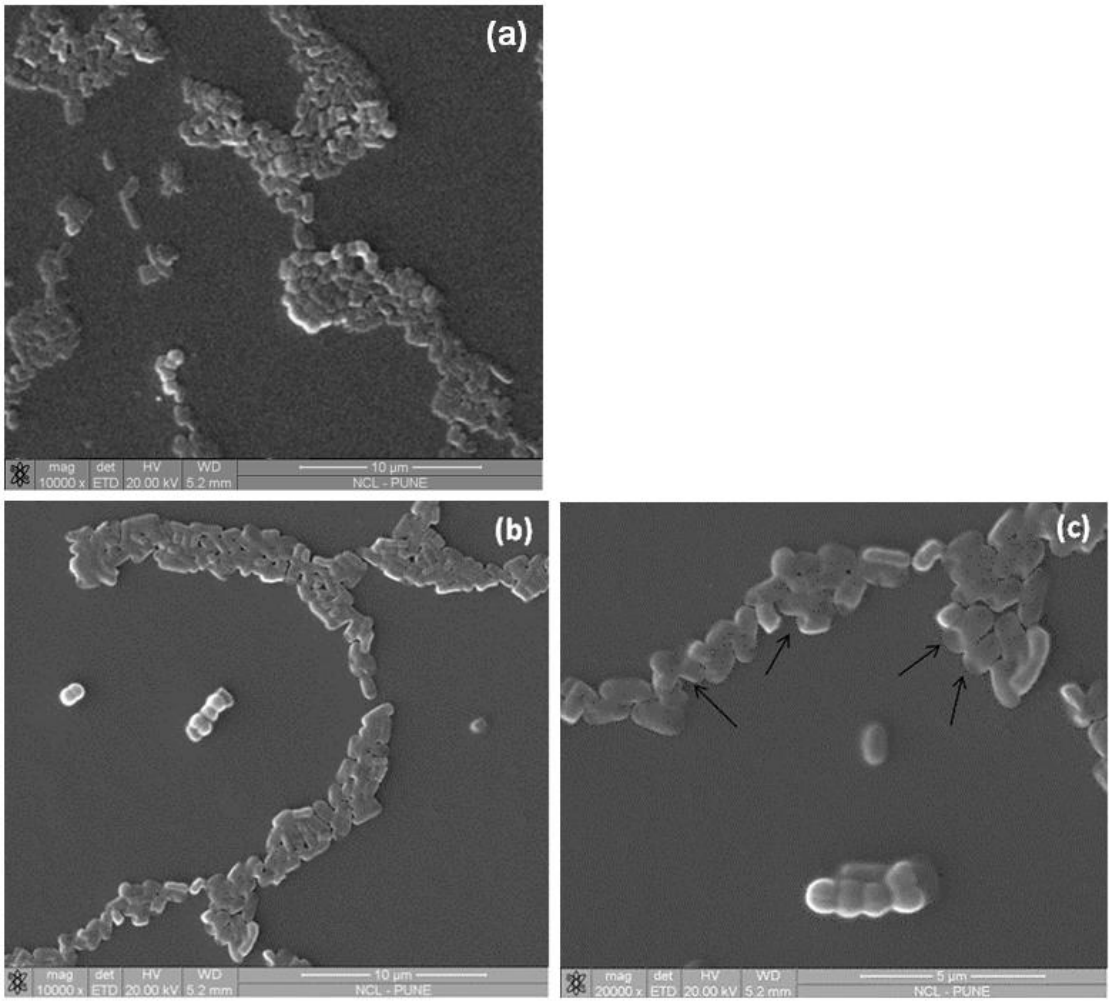


Figure 3a.7. Scanning Electron Micrograph of the *P. aeruginosa* cells- (a) control- untreated cells (b) and (c) cells exposed to the Mixture of SL and Ciprofloxacin. Overall cell shrinkage could be observed suggestive of damge to cell membranes. Also formation of cell membrane pores can be observed.

Figure 3a.8 shows the SEMs of *E. coli* cells without the treatment of any inhibitory agent and treated with SL- Cefaclor mixture. The cells exposed to the action of this mixture show damaged cell membranes and formation of pores through it. These pores are marked by the arrows while leakage of cytoplasmic contents is marked by circles.

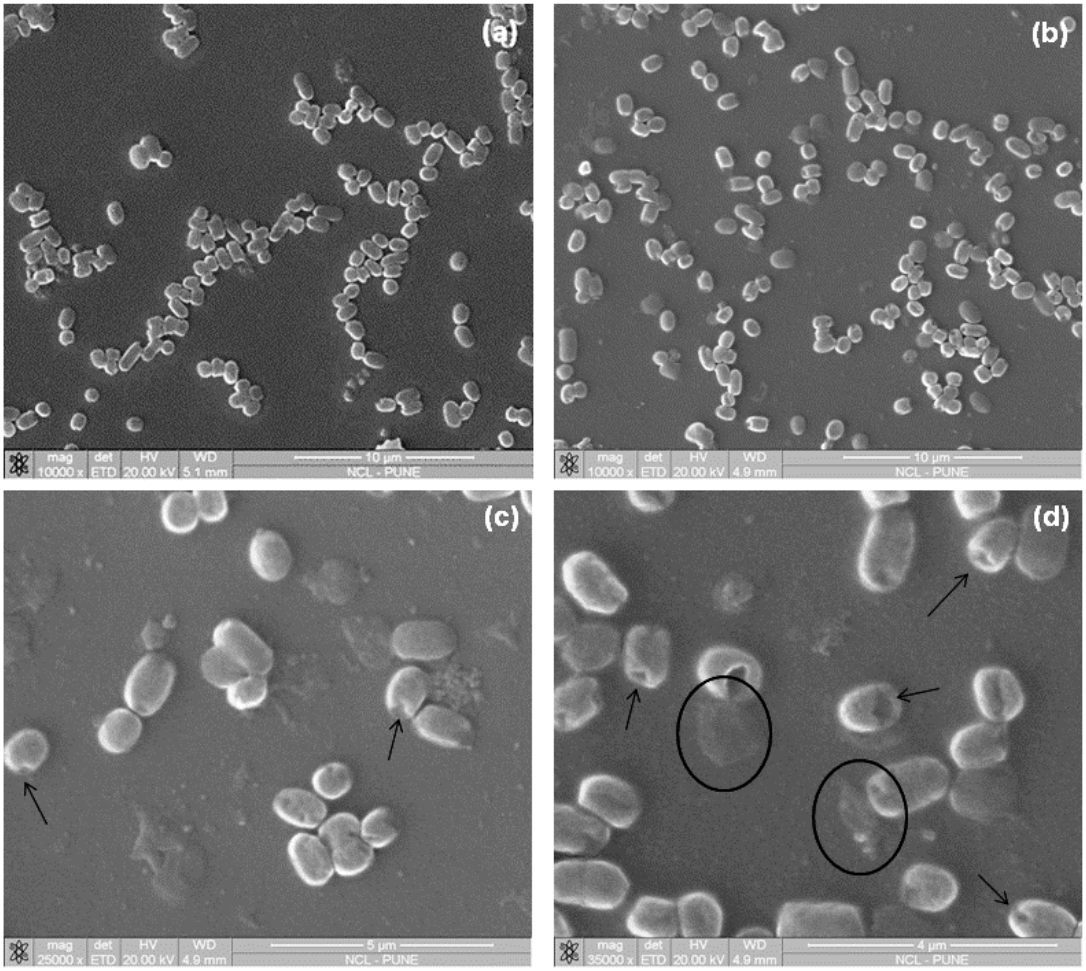


Figure 3a.8. Scanning Electron Micrograph of the *E. coli* cells- (a) control- untreated cells (b) (c) and (d) cells exposed to the Mixture of SL and Cefaclor at different magnification. Damge to cell membranes is evident causing membrane pores and leakge of cellular contents through them.

Figures 3a.6 to 3a.8 are the scanning electron micrographs of the different bacterial cells treated with the respective antibiotic- SL mixture. Range of morphological changes were observed suggestive of damage to cell membrane and the consequences of disturbed cell mebrane integrity such as formation of membrane pores leading to leakage of cytoplasmic contents, overall shrinkage of cells, accumulation of cell debris were noted. It should be noted that though the antibiotics differ in their action; similar features of cellular damage were observed in all 3 cases. So it can be said that the inhibitory action involved cell membrane lipid order perturbation in addition to the action of antibiotic. The inhibitory effect of antibiotics was observed at lower concentrations when co-administered with SLs. Probably SLs have enhanced the drug action by facilitating the entry across cell membrane thus achieving requisite intracellular antibiotic concentration at low dosage.

3a.3.6 Hypothesis about mechanism of drug entry facilitation by sophorolipids

A hypothesis has been proposed about the entry of antibiotic molecules in presence of SL as schematically represented in Figure 3a.9.

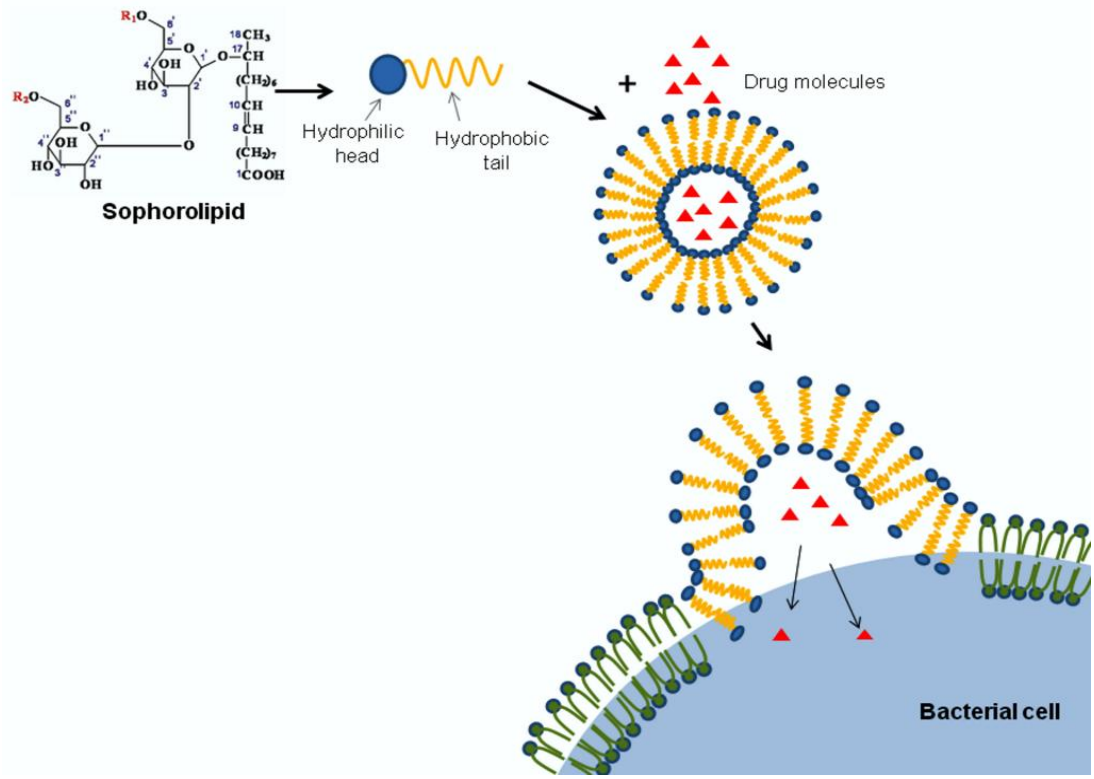


Figure 3a.9. Schematic diagram displaying the proposed mechanism of SL mediated drug entry facilitation across cell membrane.

Naturally evolution does not provide any active transport for antibiotics, and a passive diffusion process facilitated by channels must be invoked [33]. Antibiotic agents are thought to diffuse freely through the cell wall of gram-positive bacteria. However, in gram-negative bacteria the diffusion of a given antibiotic agent depends on the permeability of the outer membrane. This permeability is determined by the particular structure of the membrane, which is composed of proteins and an asymmetric lipid bilayer [34]. The outer membrane of bacteria contains various protein channels, called porins, which are involved in the influx of various compounds, including several classes of antibiotics. Bacterial adaptation to reduce influx through porins is an increasing problem worldwide that contributes, together with efflux systems, to the emergence and dissemination of antibiotic resistance. Gram-negative bacteria are responsible for a large proportion of antibiotic-resistant bacterial diseases. These bacteria have a complex cell envelope that comprises an outer membrane and an inner membrane that delimit the periplasm [35]. Thus while addressing the issue of

antibiotic resistance, enhancing the permeability of drugs is of fundamental importance.

The SLs, on account of their amphiphilic nature are capable of forming micelles, bilayer structures and self assemblies which can enclose the water soluble drugs. When administered together, SLs can span through the structurally alike cell membrane lipid bilayer and deliver the drug molecules to the cell interior. SLs are known to have better antibacterial action against Gram positive bacteria while it requires large doses of the SLs to demonstrate any antibacterial activity, especially with Gram-negative bacteria [31]. In agreement with the fact, SL alone couldn't inhibit the growth of *E. coli*, but supported it. But in case of cells treated with the combination of SL and Cefaclor, total inhibition was achieved much faster as compared to the sample treated with Cefaclor alone. Therefore it can be concluded that the enhanced inhibitory effect is not due to additive action of two antimicrobial agents. Hence the enhanced efficiency of Cefaclor- SL combination against *E. coli* can be considered as a proof for the argument- better performance is due to facilitation of entry of drug molecules by SLs.

Combined antibiotic therapy has been shown to delay the emergence of bacterial resistance and also produce desirable synergistic effects in the treatment of bacterial infections [7]. Also in case of nanoparticles, when they are used together with antibiotics; advantage is conferred that if bacteria have resistance against one of the components, a further component could kill them in a different manner [23]. Similarly SLs being antimicrobial in nature, when co-administered with antibiotics, the bacteria have to combat against two agents hence reducing the likelihood of bacterial survival as well as development of resistance. Therefore it can be expected that the co-administration of SL and antibiotic will handle the infection efficiently. Additionally according to toxicity studies, SLs can be safely administered till considerably high dosage i.e. 5ml/kg of body weight. Also because of enhanced entry of antibiotic molecules, the desired inhibitory effect may be achieved at low concentration of antibiotic.

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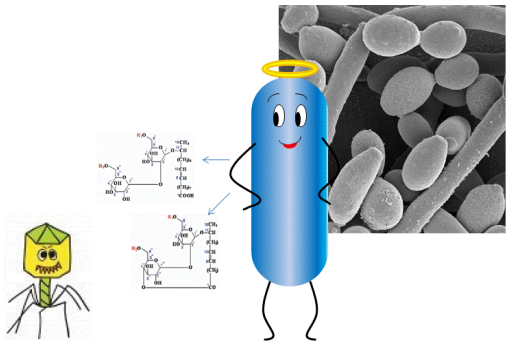
Chapter III(b)

Exploration of antiviral activity of Sophorolipids



In the present chapter, the antiviral action of Oleic and Linoleic acid derived sophorolipids (SLs) against different families of viruses such as plus stranded RNA, segmented single negative stranded RNA and double stranded DNA has been reported. The antiviral activity was tested in 3 different modes- direct treatment of virus, rapid culture assay for Influenza virus and treatment of host cell line with SL prior to viral challenge in order to check if SL gives any antiviral immunity. Coxsackieviruses CV (B1-CVB6, CA7, CA9), murid herpesvirus, strain MHV-68, Influenza virus A/Mississippi/1/85 (H3N2) have been used for the studies.

It was observed that direct treatment of virus indicated 1 $\log_{10}$- 4.5 $\log_{10}$ reduction in the virus titers. Pretreatment of cell cultures GMK and Hep-2 prior to CV infection showed a reduction in virus titer 1$\log_{10}$ - 2 $\log s_{10}$. Similar results were obtained on the VERO, BHK and 3T3 cells with gamma-herpesvirus MHV-68. Visible reduction of IAV replication on MDCK cells was obtained at the concentration 100 µg/ml of SLLA (SL derived from Linoleic acid). Thus it can be concluded that direct treatment of viruses was more effective than indirect treatment and the inhibitory action can be attributed to the amphiphilic structure of SL which might be killing the viruses by disturbing the membrane lipid order.

- *This part of work was done in collaboration with- Dr. S. Bopegamage, Head, Enterovirus Laboratory, Virology Dept., Slovak medical university, Limbova 12, 8330 Bratislava,Slovak republic*
- *The part of work reported in the chapter has been presented as a poster during Budapest RECOOP meeting (March 30th –April 1st, 2012)- Antiviral effect of sophorolipid linoleic acid Borsanyiova M., Kollarcikova V.,* ***Joshi-Navare K.****, Stipalova D., Storcelova M., Mistrikova J. Zavodska E., Vareckova E., Prabhune A. Bopegamage S.*
  (http://www.szu.sk/userfiles/file/Aktuality/Zborn%C3%ADkBudapest_RECOOP_2012.pdf)

3b.1 Introduction

As mentioned earlier in Chapter-3a; biosurfactants are coming up as emerging class of biomedically important compounds. They can serve as suitable alternative to synthetic medicines, antimicrobial agents, probiotics and therapeutic agents. [1]. SL is a promising candidate for such applications being produced by non pathogenic yeasts, such as *Candida bombicola, Candida apicola* and *Candida bogoriensis*. They are generally present in the form of disaccharide sophoroses (2-O-β-D-glucopyranosyl-D-glucopyranose) linked β glycosidically to the hydroxyl group at the penultimate carbon of fatty acids [2]. Antimicrobial action of SLs is not merely restricted toward bacteria but they also act as antifungal, antimycoplasma and antialgal agents [3]. The proposed primary mechanism of action of these surfactants is membrane lipid order perturbation, which compromises the viability of microorganisms [4]. Like other biosurfactants, SLs possess the advantages of biodegradability, low ecotoxicity and the production based on renewable substrates. SLs also form a part of cosmetic formulations owing to its antimicrobial property and beneficial effects to skin. Biosurfactants/sugar esters have been approved for the use in food and pharmaceuticals by US FDA. Safety of SLs has been confirmed through the cytotoxicity tests with human epidermal keratinocytes [5].

As mentioned in above paragraph, SLs exhibit antimicrobial action against various life forms. In a similar way, surfactant like molecules are expected to bring about membrane lipid order perturbation in viruses.

Chattopadhyay *et. al.,* 2002 have checked the action of different chemical surfactants on survival and sorption of viruses. They found that surfactants have a significant impact not only on the survival of viruses in soil and subsurface systems but also on their transport. Survival of viruses decreases in the presence of surfactants. Among them the ionic surfactants were more effective than the nonionic surfactant (Triton X-100). It is known that ionic surfactants molecules can bind to biomolecules, such as proteins, leading to denaturation and loss of biological activity. In contrast, nonionic surfactant molecules do not bind to biomolecules to a significant extent and also do not denature proteins. The surfactants reduce the sorption of virus particles by competing adsorption sites on

different sorbents. Sorption is the first step that initiates viral infection. It is mediated by the binding of host cell receptors and viral envelope. On the basis of the hydrophobicity data, it is likely that sorption of hydrophobic viruses (such as T-2) will be favored by hydrophobic sorbents (such as hectorite, hydrophobic iron oxides) while the sorption of hydrophilic viruses will be favored by hydrophilic sorbents [6].

SLs have been reported to function as immunomodulators for the treatment of endotoxic (septic) shock by cytokine downregulation [7], and display anticancer activity [8]. Maingault, 1997 [9] has proposed that SLs can be used to treat skin diseases. V Shah *et. al.* have documented in vitro spermicidal and anti-HIV virucidal activities of SL analogs wherein they evaluated the antiviral activity of acidic, lactonic as well as different ester analogues of SLs. The concentration range used was 9µg/ml to 330µg/ml. The diacetate ethyl ester was found most effective which brought down the virus titer by 5 log. Acidic form was more virucidal as compared to lactonic form [10]. The same research group; Gross *et. al.* 2007 have explored the antiviral activity of SLs. There is a world patent WO2007/130738 A1 describing the use of SLs as antiviral agents for treating herpes-related viruses and virus infections [11].

Thus in the light of literature review; we decided to further check the antiviral property of SLs against different classes of viruses namely, Coxsackie viruses (plus stranded RNA virus), Influenza virus (segmented single negative stranded RNA virus) and Murid Herpes virus 68 (double stranded DNA virus)

We intended to check-

a) if the SLs show direct virucidal action

b) whether SLs are affecting sorption of viruses and

c) whether SLs are capable of triggering the host cell defenses such as cytokines against viral pathogen.

3b.1.a Coxsackie viruses:

Coxsackievirus is a virus that belongs to a family of non enveloped, linear, positive sense ssRNA viruses, *Picornaviridae* and the genus *Enterovirus*. Coxsackieviruses

are divided into group A and group B viruses based on early observations of their pathogenicity in mice. Group A coxsackieviruses were noted to cause a flaccid paralysis (which was caused by generalized myositis) while group B coxsackieviruses were noted to cause a spastic paralysis (due to focal muscle injury and degeneration of neuronal tissue). At least 23 serotypes (1-22, 24) of group A and six serotypes (1-6) of group B are recognized.

In general, group A coxsackieviruses tend to infect the skin and mucous membranes, while Group B coxsackieviruses tend to infect the heart, pleura, pancreas, and liver. Few other disease conditions are suspected to be associated with coxsackie virus infections such as development of insulin-dependent diabetes (IDDM) and Sjogren's syndrome.

There is no FDA-approved specific therapy for Coxsackie virus infection [12]. However a recent study has demonstrated that Fluoxetine appears to inhibit replication of viral RNA in vitro [13].

3b.1.b Influenza A virus:

Influenza A virus causes influenza in birds and some mammals, and is the only species of influenzavirus A. The genus Influenzavirus A belongs to the *Orthomyxoviridae* family of viruses.

Influenza A viruses are negative-sense, single-stranded, segmented RNA viruses. The several subtypes are labeled according to an H number (for the type of hemagglutinin) and an N number (for the type of neuraminidase). There are 17 different H antigens (H1 to H17) and nine different N antigens (N1 to N9).

Each virus subtype has mutated into a variety of strains with differing pathogenic profiles; some are pathogenic to one species but not others, some are pathogenic to multiple species [14].

Treatments for influenza include a range of medications and therapies that are used in response to disease influenza. Treatments may either directly target the influenza virus itself; or instead they may just offer relief to symptoms of the disease, while the body's own immune system works to recover from infection. The two main classes of antiviral drugs used against influenza are neuraminidase inhibitors, or inhibitors of the viral M2 protein. These drugs can reduce the

severity of symptoms if taken soon after infection and can also be taken to decrease the risk of infection. However, viral strains showing drug resistance to both classes of drug have emerged [15].

3b.1.c Murid Herpes virus 68:

Murid herpesvirus 68 (MHV-68) possesses double stranded DNA as its genetic material. It is an isolate of Murid herpes virus 4 which belongs to the genus *Rhadinovirus*. It is a member of the subfamily *Gammaherpesviridae* in the family of *Herpesviridae*. MHV-68 serves as a model for study of human gammaherpesviruses which cause significant human disease including B-cell lymphoma and Kaposi's sarcoma [16].

Gamma herpes viruses are latent in replication competent lymphocytes and do not require TK (thymidine kinase) or RNR (Ribonucleotide reductase) for reactivation. Therefore Acyclovir treatment which proves effective against other herpes virus has little effect against Gamma herpesvirus. There are no established effective therapies to control the Gamma herpes virus infections [17].

3b.2 Materials and methods

3b.2.1 Materials used

(a) Host cell lines: GMK (Green Monkey Kidney cell line), Hep2 (Human epidermoid cancer cells), MDCK (Madin Darby Canine Kidney cells), VERO (African Green Monkey Kidney cells), BHK-21 (Syrian Hamster's Kidney cells) and NIH 3T3 (derived from the embryos of BALB/c 3T3 mice)

(b) Viruses: Cox-sackie viruses B1 to B6, A7, A9, INFLUENZA A/MISS/1/85/M1/E4 clones 2X HT 2048, Murid Herpes virus 68

(c) Media: MEM-E (Minimum Essential Medium-Earles), DMEM (Dulbecco's Modified Eagle's Medium) were used for cultivation of host cell lines and wherever required, the media were supplemented with 5 or 10% fetal bovine serum and HEPES 50mM.

(d) Reagents: MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Crystal violet

During Rapid culture assay for Influenza virus titrations, Monoclonal antibodies against specific viral haemagglunin antigen coupled with Horse radish peroxidase conjugated anti-antibodies were used.

(e) Sophorolipid (SL): SLs were synthesized using *Candida bombicola* ATCC 22214 and Oleic or Linoleic acid as the fatty acid precursor through Resting cell method mentioned in Chapter 3a, section 3a.2.3 [18]. Thus referred to as SLOA (Sophorolipid derived from Oleic acid) or SLLA (Sophorolipid derived from Linoleic acid). The crude mixtures were further analysed by HPLC and the SLOA was found to contain 83%lactone and 17% acidic form while SLLA was found to be composed of 56% lactone and 44% acidic form.

3b.2.2 Evaluation of antiviral action of SLLA against Cox-sackie viruses

GMK (Green Monkey Kidney cell line), Hep2 (Human epidermoid cancer cells) and L_{929} were chosen as the host cell lines for the experimentation with Cox-sackie viruses. Cox-sackie viruses B1 to B6, A7 and A9 were used. Growth medium used for cultivation is MEM-E (Minimum Essential Medium-Earles) supplemented with 2% fetal bovine serum and HEPES 50mM.

MTT assay was performed to determine the cytotoxicity of SL on the host cell lines. The concentrations showing no morphological change were used for testing the antiviral activity of SLs.

(a) For evaluation of the **effect of SL on virus adsorption**; the cell suspension and the pre-grown cell monolayer were treated with SL and then challenged with virus infection.

GMK and Hep2 cell suspensions were treated for 30 minutes with 100µg/ml SLLA. To 50µl of these pretreated cells, 50µl of different virus dilution were added. Incubation was continued till 5 days. Then the virus titres were determined by plaque assays.

And in case of pregrown cell monolayer; To the 24h grown cell monolayers, 100 µg/ml of SLLA was added. After 30 minutes exposure the medium containing SLLA was discarded and virus dilutions were added. Virus titres were determined by plaque assays.

(b) Cell monolayer was grown in presence of SL and then challenged with viral infection. This was expected to give some clue about **ability of SL to induce cytokine production or to trigger any other kind of cellular defences**.
The cell monolayers were grown up in the presence of the 100 µg/ml SLLA. After 24 hours, the old medium was removed and virus dilutions were added. Virus titres were determined by plaque assays.

(c) For evaluation of **direct virucidal action**; the virus suspensions were incubated with SL and then it was used to induce infection.
For this experiment, the viral suspension was maintained with 250µg/ml SLLA for 50 minutes. And then the plaque forming ability of treated viral suspension was checked.

3b.2.3 Evaluation of antiviral action of SLOA against Influenza A virus

The strain used for experiments was INFLUENZA A/MISS/1/85/M1/E4 clones 2X HT 2048. MDCK (Madin Darby Canine Kidney cells) were used for virus cultivation. Culture medium used was DMEM i.e. Dulbecco's Modified Eagle's Medium supplemented with 5% fetal bovine serum.
To determine the tolerable dose of SLOA against MDCK cells, SLOA was dissolved in sterile distilled water (stock strength 1mg/ml). The concentrations within the range- 5 to 100µg/ml were used.
The 24h grown MDCK cells were washed with physiological saline (pH 7.2) and SLOA- requisite volume was added to the cells followed by virus dilutions (The virus dilutions used were- 1:2000, 1:4000, 1:8000). Rapid Culture Assay was used for testing the effect of SLs on influenza virus replication based on the use of monoclonal antibodies (mouse origin, concentration 1.5µg/ml) against specific viral haemagglunin antigen coupled with Horse radish peroxidase conjugated anti-antibodies (Goat anti mouse- HRP conjugate procured from Biorad, dilution 1:5000) .

3b.2.4 Evaluation of antiviral action of SLLA against Murid Herpes virus68

VERO (African Green Monkey Kidney cells), BHK-21 (Syrian Hamster's Kidney cells) and NIH 3T3 (derived from the embryos of BALB/c 3T3 mice) were used as host cell line for testing the antiviral effect of SLs against MHV-68. MTT assays

were performed to determine the safe concentration of SLLA against all the 3 cell lines for the concentration range 125-500µg/ml.

(a) To check if SLLA affects the virus adsorption with the cells in suspension form; the host cells, virus dilution and requisite concentration of SLLA were mixed together and subjected to plaque assay.

(b) To check the effect of SLLA on virus adsorption with the adherent host cells; cells were allowed to grow and form monolayer and then SLLA and virus dilution were added to the cells and plaque assays were observed

(c) To check the inhibitory effect of SLLA on virus i.e. Direct virucidal effect; the virus dilution and SLLA in requisite concentration were maintained together at 4°C for 24h. And then plaque forming ability of treated virus dilution was checked against pregrown cell monolayer

(d) To check if the SLLA triggers any host defence mechanism such as interferon production; the cell were grown in presence of SLLA for 24 h and then challenged with the virus infection.

Plaque assays were performed with crystal violet staining for visualization.

3b.3 Results and discussion

3b.3.1 Evaluation of antiviral action of SLLA against Cox-sackie viruses

(a) Effect of SL on virus adsorption

Cox-sackie viruses	GMK		Hep2	
	SLLA	Without SLLA	SLLA	Without SLLA
B1	$10^{7.25}$	$10^{7.25}$	$10^{10.5}$	$10^{10.5}$
B2	$10^{7.5}$	$10^{7.5}$	**10^{8}**	$10^{8.75}$
B3	$10^{7.25}$	$10^{7.25}$	**$10^{10.5}$**	$10^{11.75}$
B4	10^{7}	10^{7}	**$10^{7.75}$**	10^{9}
B5	$10^{8.25}$	$10^{8.25}$	$10^{10.75}$	$10^{10.75}$
B6	$10^{8.25}$	$10^{8.25}$	**$10^{9.75}$**	10^{11}
A7	$10^{8.25}$	$10^{8.25}$	$10^{9.5}$	$10^{9.75}$
A9	$10^{8.25}$	$10^{8.25}$	$10^{9.25}$	$10^{9.25}$

Table 3b.1 Effect of SLLA on adsorption of Cox-sackie viruses- GMK and Hep2 cell suspensions were exposed to SLLA prior to viral challenge. Highlighted boxes indicate reduction in virua titre due to action of SLLA.

Thus we can observe from the above mentioned results (Table 3b.1) that antiviral action of SL was not evident with GMK cell line. But in case of Hep2 cell line;

remarkable antiviral activity was observed showing up to 1.25 log reduction in virus titre.

So we can say that 100 µg/ml SLLA triggered cell defenses against the Cox-sackie viruses B2, B3, B4, B6 and marginally against A7 in Hep2 cells but not in GMK cell line.

Cox-sackie viruses	GMK		Hep2	
	SLLA	Without SLLA	SLLA	Without SLLA
B1	$10^{9.75}$	$10^{10.25}$	$10^{11.5}$	$10^{11.5}$
B2	$10^{10.25}$	$10^{10.5}$	$10^{11.25}$	$10^{11.25}$
B3	$10^{8.5}$	$10^{8.5}$	$10^{12.75}$	10^{13}
B4	$10^{8.25}$	$10^{9.25}$	10^{7}	10^{7}
B5	$10^{7.25}$	$10^{7.25}$	$10^{11.25}$	$10^{11.25}$
B6	$10^{7.5}$	$10^{7.5}$	10^{8}	10^{8}
A7	$10^{9.25}$	$10^{9.5}$	10^{12}	10^{12}
A9	$10^{9.75}$	$10^{10.5}$	$10^{10.25}$	$10^{10.5}$

Table 3b.2 Effect of SLLA on adsorption of Cox-sackie viruses- GMK and Hep2 pre-grown cell monolayers were exposed to SLLA prior to viral challenge. Highlighted boxes indicate reduction in virus titre due to action of SLLA

The effect of SLLA on adsorption of Cox-sackie viruses when the cell monolayer was exposed to SL, prior to viral chellenge has been represented in Table 3b.2. The GMK cell line in monolayer form was found receptive towards SLLA rather than in suspension form and as a result of 30 minutes exposure to 100µg/ml SLLA prior to viral challenge; adsorption of viral particles got affected. But the reduction in virus titer was marginal 0.25 to 1 log reduction was observed. Maximum effect was observed against Cox-sackie B4 virus. Increasing the contact period with SLLA may further impair the viral adsorption.

Hep 2 cells were more receptive to SLLA in suspension form. The adhered cells showed only marginal defence to the cox-sackie viruses which resulted in 0.25 log reduction in virus titer that too just with B3 and A9 virus.

(b) Evaluation of the ability of SL to trigger cellular defences

The results have been represented in Table 3b.3. They were interpreted as-the longer period of exposure of adhered cells to SLLA triggered the defence mechanisms probably cytokine production in GMK cell line to some extent in addition to the effect on virus particle adsorption This resulted in up to 1.5 log

reduction in Cox-sackie virus titer. The effect was evident against the B2, B3, B5 and B6.

Against Hep2 cells; as a result of longer exposure to SLLA, 1.25 log reduction was observed which could be attributed to improved cell defences against B5 virus.

Cox-sackie viruses	GMK		Hep2	
	SLLA	Without SLLA	SLLA	Without SLLA
B1	$10^{9.5}$	$10^{9.75}$	10^{12}	10^{12}
B2	10^{8}	$10^{9.5}$	$10^{10.5}$	$10^{10.5}$
B3	$10^{9.25}$	$10^{10.25}$	$10^{10.5}$	$10^{10.5}$
B4	$10^{9.5}$	$10^{9.5}$	$10^{7.25}$	$10^{7.25}$
B5	$10^{7.5}$	$10^{8.25}$	$10^{10.75}$	10^{12}
B6	$10^{8.25}$	$10^{9.5}$	$10^{9.5}$	$10^{9.5}$
A7	10^{10}	$10^{10.5}$	10^{11}	10^{11}
A9	10^{9}	10^{10}	$10^{8.25}$	$10^{8.25}$

Table 3b.3 Effect of SLLA treatment on GMK and Hep2 cell line's response to Cox-sackie virus infection. Highlighted boxes indicate reduction in virus titer

(c) Evaluation of direct virucidal action of SLLA

Cox-sackie viruses	Without SLLA Diluted virus 1:1000	With SLLA 250µg/ml
B1	$10^{6.75}$	$10^{6.25}$
B2	$10^{7.75}$	10^{6}
B3	10^{9}	$10^{5.25}$
B4	$10^{7.25}$	$10^{5.5}$
B5	10^{7}	$10^{5.25}$
B6	$10^{9.5}$	$10^{5.25}$
A7	10^{9}	$10^{6.25}$
A9	$10^{7.5}$	$10^{6.25}$

Table 3b.4 Reduction in virus titer as result of exposure to SLLA. Values of reduced titers are highlighted

As per the data represented in Table 3b.4, the direct treatment of virus with SLLA 250 µg/ml showed marked reduction in virus titer wherein 1.25 to 4.25 log reduction was observed. Maximum reduction i.e. 4.25 log reduction was observed in case of B6 virus.

3b.3.2 Evaluation of antiviral action of SLOA against Influenza A virus

As the outcome of experiment to determine the tolerable dose of SLOA; no toxic effects were observed against MDCK cells till 200 µg/ml. Beyond 200 µg/ml; it

turned toxic to the MDCK cells. At 5, 10, 20 and 50 µg/ml; no inhibitory effect on virus replication was observed. At 100µg/ml; slight inhibitory effect on virus replication was observed wherein ~ 87% inhibition was observed as compared to the untreated control. Whereas exposure to 200µg/ml SLOA was found to be detrimental to the host cells as well. The images are represented in Figure 3b.1.

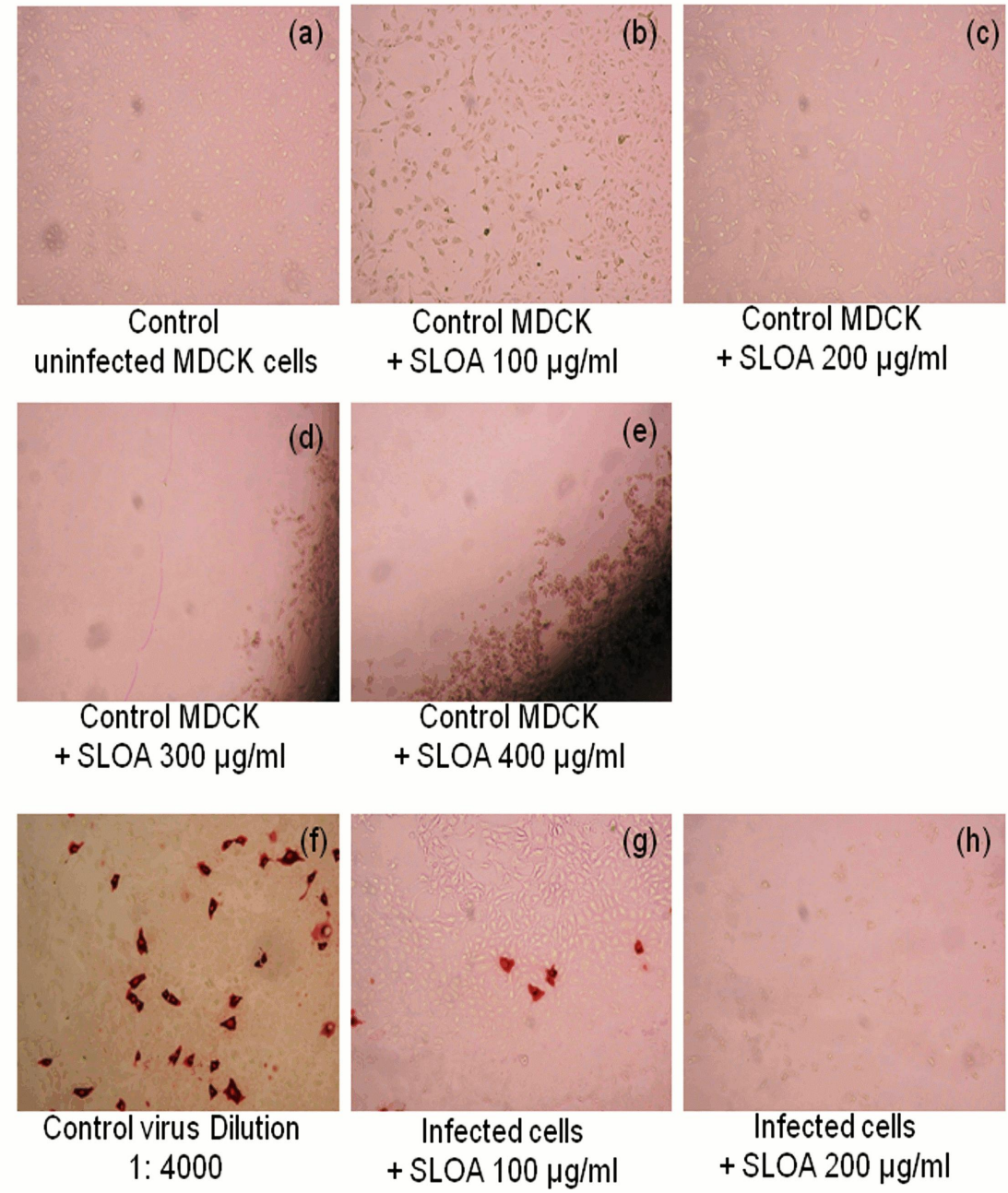


Figure 3b.1 (a) to (e) SLOA toxicity testing against MDCK cells; (f) to (g) MDCK infected with Influenza virus- effect of SLOA along with control

3b.3.3 Evaluation of antiviral action of SLLA against Murid Herpes Virus 68

SLLA concentration (µg/ml)	24h	48h	120h	144h
VERO				
500	+ + +	+ + + +	+ + + +	+ + + +
250	-	-	-	-
125	-	-	-	-
BHK-21				
500	+	+ +	+ +	+ + + *
250	-	-	-	-*
125	-	-	-	-*
NIH3T3				
500	+ + + +	+ + + +	+ + + +	+ + + +
250	-	-	-	+
125	-	-	-	-

Table 3b.5 SLLA toxicity testing against the host cell lines. (+) indicates cytotoxic effects while (-) indicates absence of toxic effects

The results depicted in the table 3b.5 indicate that SLLA at concentration as high as 500µg/ml affects all the host cells adversely while 250 µg/ml is safe for the host cells till 120h of exposure.

(a) Effect of SLLA on virus adsorption with cells in suspension form

VERO			BHK-21			NIH 3T3		
Without SLLA	SLLA 250 µg/ml	SLLA 125 µg/ml	Without SLLA	SLLA 250 µg/ml	SLLA 125 µg/ml	Without SLLA	SLLA 250 µg/ml	SLLA 125 µg/ml
10^6	10^5	$10^{5.5}$	10^7	10^6	$10^{6.5}$	10^5	10^4	$10^{4.5}$

Table3b.6 Effect of SLLA on virus adsorption with cells in suspension form

Thus it can be observed from the data shown in Table 3b.6 that – SLLA marginally affects the adsorption of virus. 1 log reduction was achieved with 250µg/ml of SLLA while 125µg/ml caused 0.5 log reduction in $TCID_{50}$.

(b) Effect of SLLA on virus adsorption with cells in adherent form

VERO			BHK-21			NIH 3T3		
Without SLLA	SLLA 250 µg/ml	SLLA 125 µg/ml	Without SLLA	SLLA 250 µg/ml	SLLA 125 µg/ml	Without SLLA	SLLA 250 µg/ml	SLLA 125 µg/ml
10^5	$10^{4.5}$	10^5	10^6	10^5	10^6	10^5	10^4	10^5

Table3b.7 Effect of SLLA on virus adsorption with cells in adherent form

Thus it can be observed from the data shown in Table 3b.7 that – SLLA marginally affects the adsorption of virus. 0.5 log reduction was achieved with 250µg/ml of SLLA while 125µg/ml did not cause any virus inhibition.

(c) Effect of direct virucidal treatment

For the experiment, virus dilution was maintained with SLLA for 24h at 4°C. The experiment was performed only with VERO cells. The exposure resulted in 1 log reduction for both concentrations of SLLA namely, 250µg/ml and 125µg/ml. This reduction can be regarded as only the marginal inhibition.

(d) Ability of SLLA to induce self defence mechanism in host cells

The experiment was performed with Vero cell line. The cells were grown in presence of SLLA for 24h and then virus dilution was added. The results indicated that SLLA did not trigger the production of any self defense molecules and the virus titre remained the same in case control and SLLA exposed cells.

In this study, 3 modes of action of SLs namely ability to impede virus sorption, direct inhibitory action on viruses and ability to trigger defence mechanisms in host cell lines were checked. Overall results suggested that direct virucidal action proved to be most effective against Cox-sachie as well as influenza virus. Virus sorption was also affected to some extent in case of Cox-sachie as well as Influenza virus. Triggered host cell defences were observed in Green Monkey Kidney cell line against Cox-sachie viruses. Against Murid herpes virus -68 only a marginal inhibition was observed with all 3 modes of SL action.

Studies on the effect of SLs on virus receptors on host cells which are involved in the attachment and sorption of viruses will be taken up in future.

3b.4 References

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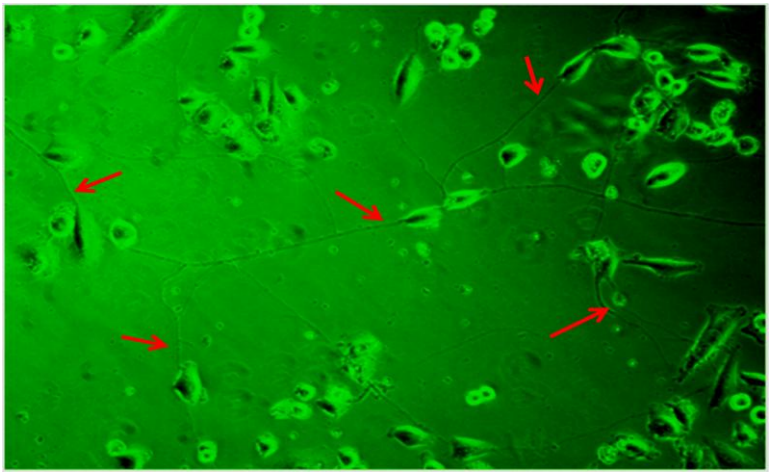
Chapter IV

Exploration of differentiation inducing ability of SLs against glioma cells



Sophorolipids (SLs) are biosurfactants produced by non pathogenic yeasts. They show structural similarity with the membrane components of mammalian cells-glycosphingolipids and gangliosides which are involved in the processes such as signaling, oncogenesis, differentiation. SLs have been reported to induce differentiation in number of leukemic cell lines and cell death via apoptosis in human liver cancer cell line and necrosis in pancreatic adenocarcinoma cell line.
The effects of precursor fatty acids and SLs of oleic, linoleic acids -pure acidic and crude forms, were investigated on LN229 - a glioma cell line which we are reporting for the first time. In response to different SL forms, various morphological changes were observed such as formation of long thread like extensions arising from ends of the cells, cell alignment, cell elongation and bundle formation in dose dependent manner.
In this chapter we are presenting the morphological evidence of the potential of SLs as differentiation inducers.

Kasturi Joshi-Navare, Anjali Shiras, and Asmita Prabhune, ***Differentiation-inducing ability of sophorolipids of oleic and linoleic acids using a glioma cell line****. Biotechnol. J. 2011, 6, 509–512.*

4.1 Introduction

Biosurfactants could be used as safe and effective therapeutic agents or probiotics [1]. SL is a promising candidate for such applications being produced by non pathogenic yeasts, such as *Candida bombicola, C. apicola* and *C. bogoriensis*. Antimicrobial property of SLs further makes them suitable for the therapeutic purpose. The antimicrobial action is not merely restricted toward bacteria; they also act as antifungal, antialgal, antimycoplasma and antiviral agents [2]. Moreover SLs offer the advantages of biodegradability, low ecotoxicity and the production based on renewable-resource substrates. The US FDA has also approved biosurfactants/sugar esters for the use in food and pharmaceuticals. Tests with SLs pointed out that they are not irritating to the skin, do not trigger allergic reactions and have an oral safety level which is greater than or equal to 5ml/kg weight. Cytotoxicity was evaluated with human epidermal keratinocytes and was proven to be low [3]. On account of its role as antimicrobial agent and emulsifier in addition to skin compatibility SLs find application in various cosmetic formulations. Other medically beneficial effects include ability to trigger cell differentiation, apoptosis in cancerous cells [2]. In this chapter the cell differentiation triggering ability of SLs has been explored.

SLs are generally present in the form of disaccharide sophoroses (2-O-β-D-glucopyranosyl-D-glucopyranose) linked β glycosidically to the hydroxyl group at the penultimate carbon of fatty acids. Being a surfactant molecule, SLs posses carbohydrate head and a lipid tail. Thus these amphiphilic molecules interact with the phase boundary in heterogenous systems. SLs mainly occur as mixtures of close ring (macrolactone form) and open ring (acidic form) structures, which undergo acetylation to various extents at the primary hydroxyl group of the sophorose ring [4,5].

The ubiquitous membrane components in mammalian cells, glycosphingolipids and gangliosides are known to have role in cell growth modulation, adhesion and trans membrane signaling. They undergo dynamic changes in composition and metabolism during oncogenesis, differentiation, oncogenic transformation. Certain gangliosides are involved in leukemic cell differentiation and regulation of some protein kinases (Refer to Figure 4.1).

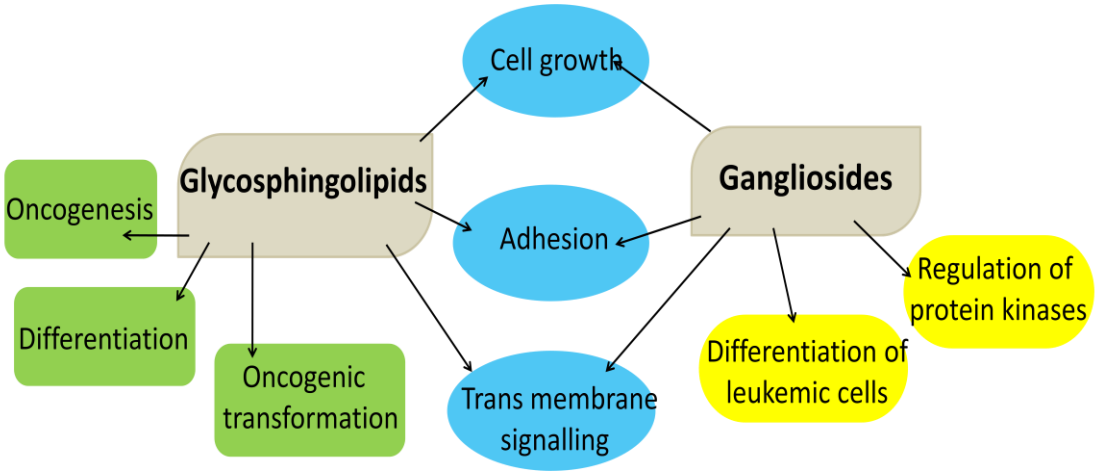


Figure 4.1 Roles of glycolipid-like cell membrane components summarized

The amphiphilic microbial glycolipids are structurally similar to above mentioned membrane components as they both possess sugar as well as lipid moiety. Thus glycolipids including SLs stand as potential candidates to be tested for their ability to control cell fate. Isoda *et. al.* (1997) have shown that the microbial extracellular glycolipids induced differentiation and inhibition of the protein kinase C activity of human promyelocytic leukemia cell line HL60. Also the SL caused Myelogenous leukemia cell line K562 to differentiate into megakaryocytes and Basophilic leukemia cell line KU812 to differentiate into granulocytes [6]. In this case, the SLs were produced using safflower oil and the product was crude hence the key molecule and mode of action are yet to be identified. Chen *et. al.* have reported a new strain for SL synthesis (*Wickerhamiella domercqiae*) and showed that the SL induced apoptosis in H7402 human liver cancer cells in dose and time dependent manner [7]. Fu *et. al.* (2008) showed that SLs induced human pancreatic adenocarcinoma cell line i.e. HPAC to undergo necrosis [8]. Another glycolipid produced by *C. antarctica*; Mannosyl Erythrito Lipid (MEL) has been shown to induce neuronal differentiation in Rat pheochromocytoma PC12 cells [9]

Glioblastoma is one of the frequent tumors of the Central Nervous System (CNS) and is highly resistant to conventional chemotherapy. LN-229 cells are derived from grade IV Glioblastoma. These cells are highly proliferative and tumorigenic and contain a mutated p53 [10, 11]. Use of novel compounds that can inhibit growth of glioma cells, induce apoptosis or promote their differentiation may offer new avenues for management of brain tumors. As mentioned above glycolipids

could induce differentiation in variety of cells. Thus in case of tumors with low promise from conventional cures, differentiation may provide the alternative way through which tumor progression can be arrested.

Thus after summarizing the therapeutic potential of SLs to modulate cell fate, previous reports about differentiation through use of different glycolipids and nature of glioblastomas as invasive CNS tumor; abilities of SLs to induce differentiation in a cell line derived from neuronal tissue were checked. The SLs synthesized by *C. bombicola* are in fact a mixture of over 20 related molecules with differences in the fatty acid part (chain length, saturation, and position of hydroxylation) and the lactonization and acetylation pattern.

The different structural classes cause wide variation in physicochemical properties. Lactonized SLs have different biological and physicochemical properties as compared to acidic forms [2]. In order to study the effect of SL structural variation on its differentiation inducing ability, 3 different SL preparations were used during the study namely- SL of oleic acid pure acidic form (SLOAA), SL of oleic acid crude form (SLOAC) and SL of linoleic acid crude form (SLLAC).

4.2 Materials and methods

4.2.1 Microorganisms, cell line and their maintenance

(c) *Candida bombicola* (ATCC 22214) was used for the production of SLs. It was maintained on MGYP (Malt extract- 0.3g%, Glucose- 2g%, Yeast extract- 0.3g%, Peptone- 0.5g% and Agar- 2.0g %) slants. The microorganism was sub cultured in every 4 weeks and maintained at 4°C in a refrigerator as mentioned in earlier Chapter 2, section 2.2.1[12].

(d) Human glioma cell line LN-229, ATCC number: CRL-2611 was procured from National Centre for Cell Sciences. The cell line was revived from p120 liquid nitrogen storage and subcultured regularly during use. Dulbacco's Modified Eagle's Medium supplemented with 5% FBS was used.

4.2.2 Chemicals and reagents

All microbial media, chemicals and solvents used in this study were of analytical grade and supplied by either Hi-media pvt. Ltd., India or Merck India ltd.
Fatty acid precursors and MTT reagent were purchased from Sigma Aldrich.
Cell culture media and Foetal bovine serum were purchased from Gibco.
The antibodies were purchased from Chemicon / Invitrogen.

4.2.3 SL production

SL synthesis was carried out using pure Oleic and Linoleic acid as fatty acid precursors according to the procedure mentioned in Chapter 3a, section 3a.2.3. [13, 14, 15]. The unconverted fatty acid was removed by several times washing with n-hexane.

4.2.4 Chemical purification of SL by alkaline hydrolysis method

This crude SL (a mixture of lactonic and acidic form) was refluxed in 5M NaOH at 100°C for 10minutesThe solution was quenched with 2N HCl (up to pH 6.0) to convert all SL in to acidic form. Further, it was extracted with n-pentanol to get the desalted product, evaporated till dryness and finally washed with diethyl ether to remove the impurities such as hydroxyl ions and dried under vacuum [16]. The so obtained product contains acidic form of deacylated SL

4.2.5 ^{1}HNMR analysis of the chemically purified SL

Two milligrams of sample was dissolved in 0.5 ml of deuterated Methanol. 1H NMR (200 MHz) spectra was recorded by Bruker AC200 at 25ºC. Chemical shift was expressed in ppm. Tetra Methyl Silane (TMS) was used as an internal standard.

4.2.6 HPLC analysis of the crude SL preparations

The SL samples were subjected to HPLC analysis to get the idea about relative percentages of lactonic and acidic component. The chromeline-Hitachi HPLC system was used along with Thermo C18 column (5 µm, 150 x 4.6 mm). The solvent system used was MilliQ water-Acetonitrile (ACN). Total runtime was 65 minutes. For the first 15 minutes, ACN was maintained at 20% then it was gradually raised to 80% upto 40 minutes and was brought to 100% till 50 minutes

and thereafter maintained for 15 minutes. The run was performed at flow rate 0.5ml/min and 25°C. The compounds were detected by L-2490 UV detector at 207nm.

4.2.7 MALDI/MS (Matrix Assisted Laser Desorption/Ionization- Mass spectrometry) analysis of the SL preparations

SL sample 1mg was dissolved in 1ml of methanol. Further 5µl of the sample was mixed with 20 µl of dithranol matrix and MALDI-MS study was done on AB SCIEX TOF/TOF 5800

4.2.8 Effect of precursor fatty acids on LN-229 cells

A 24 well plate was seeded with LN229 cells with cell density of approximately 7000 cells per well in 500 µl DMEM supplemented with 10% FBS, 100U/ml penicillin and 100µg/ml streptomycin sulfate. For checking the effect of the fatty acid precursors on cells, the pure fatty acids namely oleic and linoleic acid were added to the 24h pre-grown LN-229 cells in a 24 well plate which served as a control. The concentration range used for both fatty acids was 0.25µl, 0.5µl, 1µl, 2µl, 4µl, 8µl per 500µl DMEM with 10% FBS per well.

4.2.9 MTT assays for determination of sub lethal concentration of SLs against LN-229 cells

96 well plates were seeded with 3000cells/200µl/well followed by incubation for 24h. Then the culture medium was replaced with fresh one in case of control wells while in case of test wells, fresh medium along with requisite concentration of SL was added. The concentration range of SLs used was20µg/ml- 200µg/ml (stock 1mg/ml prepared in 3%ethanol). The readings were recorded at the intervals 0h, 24h, 48h and 72h. The Mitochondrial activity of viable cells was estimated in terms of reduction of MTT. To all wells 20µl MTT reagent (stock 5mg/ml) was added and incubated for 4h (5%CO_2, 37°C). Then the insoluble blue colored formazan complex was solubilized with 200µl of DMSO reagent and estimated colorimetrically using Bio-rad microplate reader (Model 680) at 570nm after 10 minutes incubation. The readings were done in triplicates.

4.2.10 Evaluation of differentiation inducing ability of different forms of SLs

A 24 well plate was seeded with LN229 cells with cell density of approximately 7000 cells per well in 500 µl DMEM supplemented with 10% FBS, 100U/ml penicillin and 100µg/ml streptomycin sulfate. The plate was incubated under 5% CO_2 atmosphere at 37°C for 48 hours till the requisite cell density was achieved and cells were properly adhered to the surface and healthy. The sublethal concentrations of SLs determined through MTT test were used during the plate assays.

Three different SL preparations namely SLOAA, SLOAC and SLLAC have been used for the experiment. The concentrations used for SLOAA were 5µg/ml, 10µg/ml, 20µg/ml, 40µg/ml, 80µg/ml, 160µg/ml, for SLOAC, the concentrations 5µg/ml, 10µg/ml, 20µg/ml, 40µg/ml, 80µg/ml, 160µg/ml were used while for SLLAC the concentrations- 200ng/ml, 400ng/ml, 1µg/ml, 5µg/ml, 10µg/ml, 20µg/ml were used. The stock solutions 1mg/ml were prepared in sterile distilled water and then filtered through sterile 0.25µ membrane filter. Various dilutions of SLs were prepared in DMEM + 2% FBS. Serum concentration was maintained low so as to avoid over-crowding of wells and individual cells could be observed. The plate was observed at regular interval of 24h and the morphological changes were noted. The supernatants (culture medium with requisite concentration of SLs) were replaced after every 48h. The experiment was continued till 96h and images were recorded.

4.2.11 Immunocytochemistry analysis for checking the expression of marker proteins

The assays were performed in 24well plates as described above with coverslips placed at the bottom of each well before seeding the plate. Then the cells were fixed and stained with antibodies for visualization of expression of protein markers as per the following protocol.

The supernatant medium was removed and wells were washed with 1X ice cold phosphate buffered saline (PBS).

↓

The cells were fixed onto the coverslips using 4% paraformaldehyde pH8.0 for 10 minutes at room temperature.

↓

Cells were exposed to 0.1% Triton X-100 for 3 minutes followed by 5% Bovine Serum Albumin (BSA) wash for 15 minutes. (Triton X-100 treatment allows us to observe cytoplasmic bodies while BSA blocks non specific binding)

↓

25µl of primary antibody (1:100 diluted with 0.5%BSA in 1X PBS) was allowed to bind for 1h. 1X PBS moistened tissue paper was placed inside the lid to prevent drying of antibody solution.

↓

Unbound antibodies were washed with 1X PBS and BSA each for 5minutes.

↓

Then 25µl of secondary antibody (1:50 diluted with 1X PBS) was allowed to bind for 45 minutes.

↓

Unbound antibodies were washed away with 1X PBS.

↓

Cells were further stained with 25µl of nuclear dye -4′, 6-diamidino-2-phenylindole (DAPI) for visualization of nuclear material. The plate was incubated in dark for 10minutes.

↓

Cells were then washed with 1X PBS twice.

↓

Coverslips were then mounted in 1, 4-diazabicyclo [2.2.2] octane (DABCO) (which prevents quenching of fluorescent dye labelled secondary antibody).

↓

Slides were then maintained in dark for 10minutes and then stored at 4°C till visualization with confocal microscope- Zeiss LSM 510.

4.3 Results and Discussion

4.3.1 ^{1}HNMR analysis of the chemically purified SL

As mentioned in materials and methods section, ^{1}H-NMR analysis was performed after SL synthesis, extraction and purification through alkaline hydrolysis. The ^{1}H-NMR pattern has been presented in figure 4.2 which confirmed the presence of pure acidic form of SL of oleic acid.

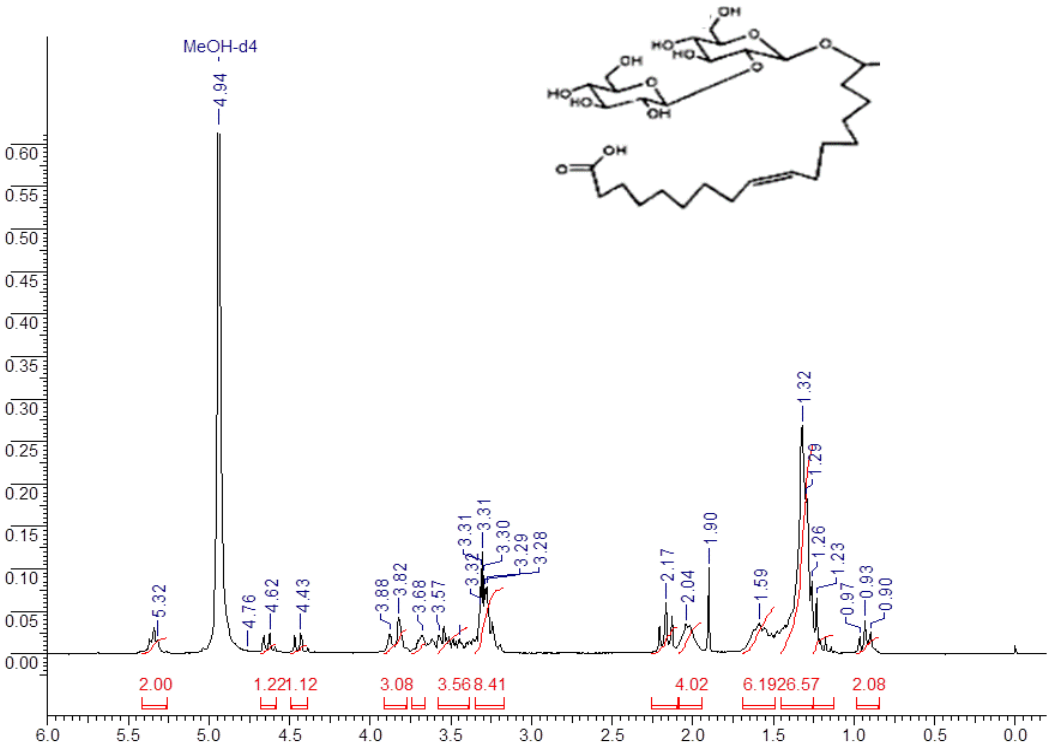


Figure 4.2 NMR pattern of Acidic form of deacetylated SL of oleic acid

4.3.2 HPLC analysis of the crude SL preparations

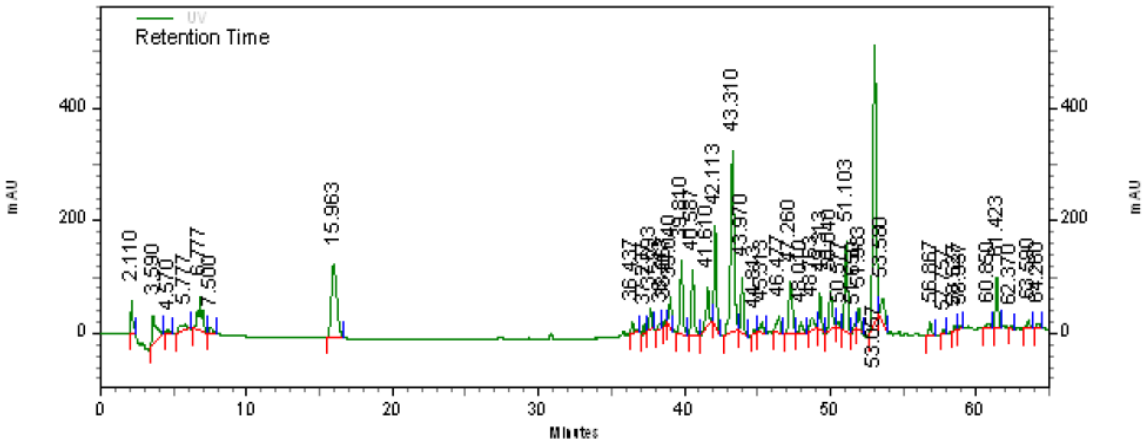


Figure 4.3 HPLC elution pattern of SLOAC- crude preparation

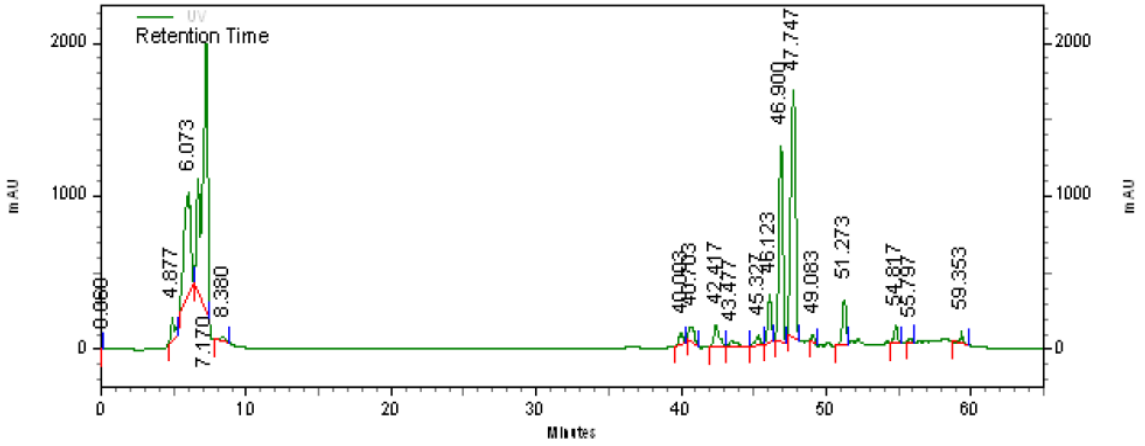


Figure 4.4 HPLC elution pattern of SLLAC- crude preparation

Figure 4.3 depicts the HPLC elution pattern of the SLOAC preparation. As per the HPLC analysis, it was found that the SLOAC sample contains around 83% of lactone form and remaining 17% of acidic form. While in case of SLLAC, the lactone form accounts for 56% and acid form accounts for 44% (Refer to figure 4.4). The acidic SL forms get eluted first while the lactonic SLs, especially the acetylated ones, show longer retention times because of higher hydrophobicity [15]. Thus the peaks lying in the later half region were considered to be of different lactonic forms.

The SL crude mixtures were used during the study, knowing that natural synergism between lactone and acid form of SLs creates a better balance for many interfacial activities [17].

4.3.3MALDI/MS analysis of the SL preparations

(a) MALDI/MS analysis of SLOA preparation

Please refer to the section 3a.3.1(b) from earlier chapter for the detailed data.

(b) MALDI/MS analysis of SLLA preparation

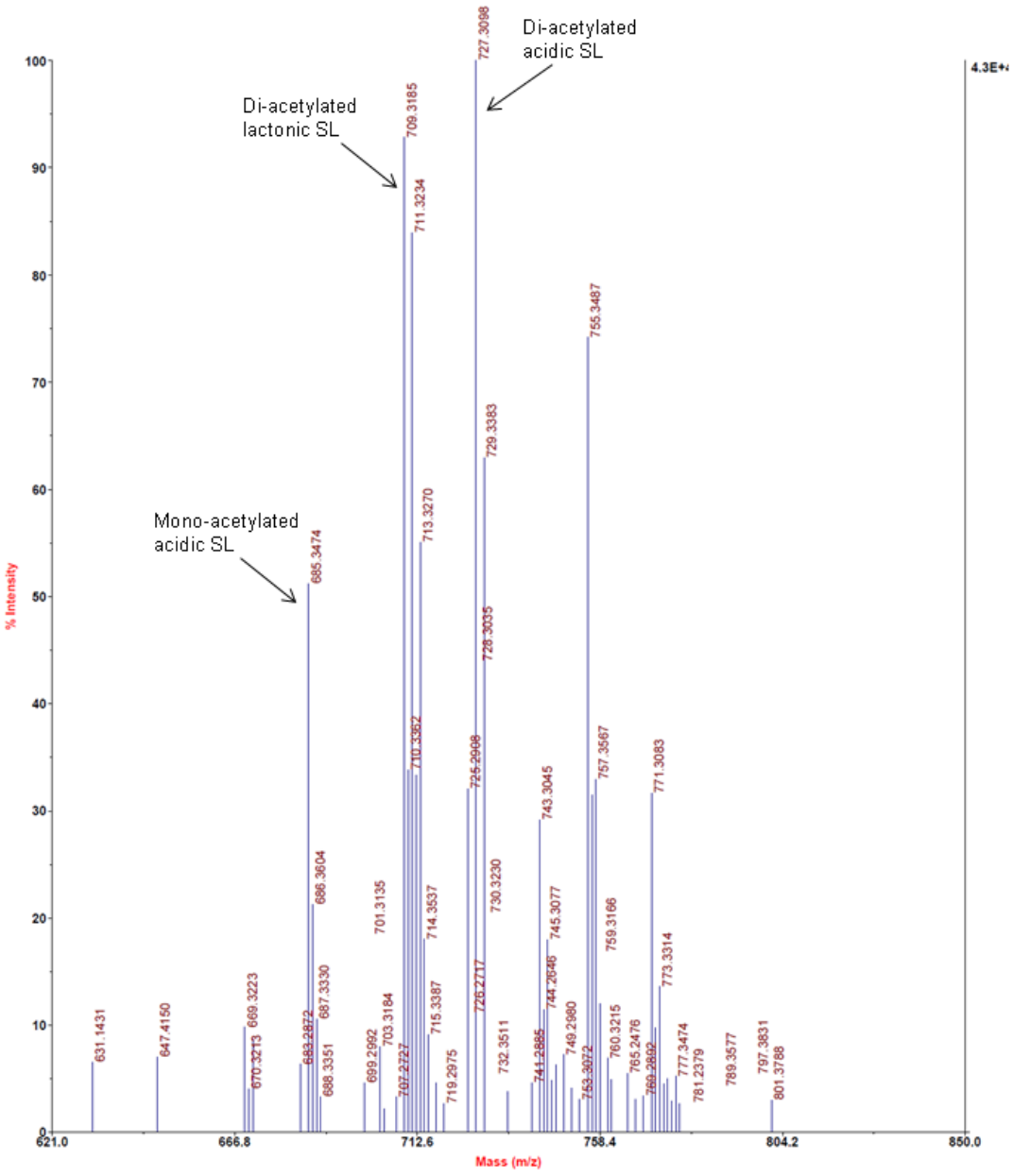


Figure 4.5 MALDI-MS data of SL preparation. [M^++H^++Na^+] i.e. sodium adducts of the different structural forms of SLLAC (Linoleic acid derived SL) have been indicated.

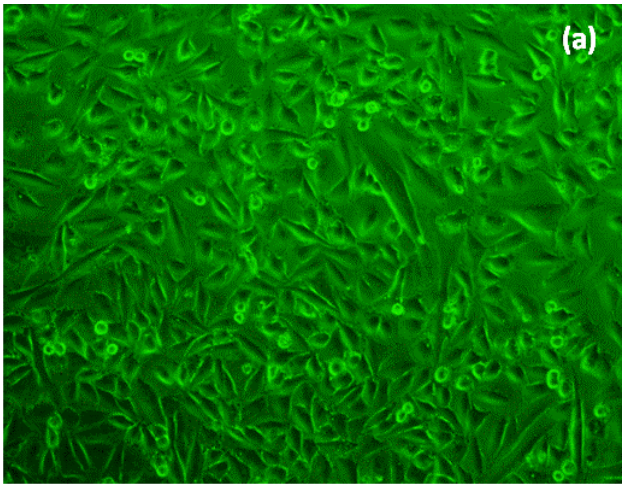
MALDI-MS study of SL- Prominent peaks from the mass spectrum were correlated to sodium adducts [M^+ + H^+ + Na^+] of the expected forms of SLs. Three different

forms of Linoleic acid derived SLs were detected. Di-acetylated acidic SL of Linoleic acid was detected with maximum % abundance. This was followed by di-acetylated lactonic form. Mono-acetylated acidic SL was also detected in relatively small proportions. Apart from Linoleic acid derived SLs, different SL structures having Oleic (C18:1), Stearic (C18:0) and Palmitic (C16:0) acid as the hydrophobic part were also detected as summarized in Table 4.1. The finding was in accordance with previous reports

SL structure	m/z	M^++H^++Na^+
Di-acetylated SL of C18:1 Acidic form	705	729
Di-acetylated SL of C18:1 lactonic form	687	711
Mono-acetylated SL of C18:1 acidic form	663	687
Di-acetylated SL of C18:0 Lactonic form	689	713
Di-acetylated SL of C16:0 Lactonic form	661	685

Table 4.1 Peaks corresponding to SLs having fatty acids other than Linoleic acid

4.3.4 Effect of precursor fatty acids on LN-229 cells



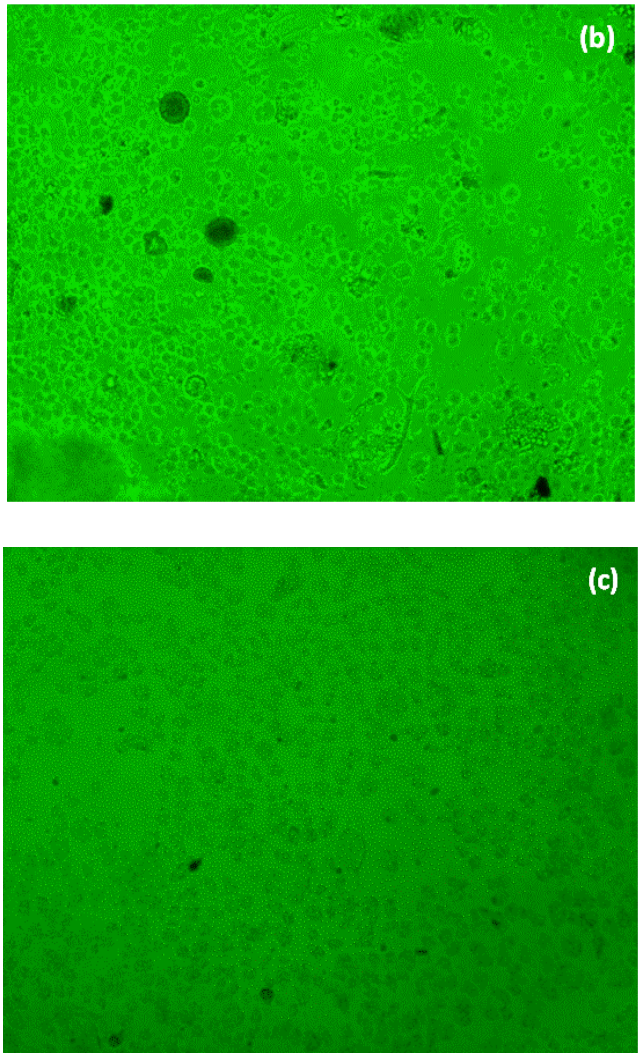


Figure 4.6 Effect of fatty acid precursors on LN229 cells (a) control cells (b) cells exposed to 2µl of oleic acid (c) cells exposed to 2µl of linoleic acid

Within 2h of exposure, even at lowest concentration i.e 2µl of oleic as well as linoleic acid, the cells lost their base adherence, native shape and turned round suggesting cell toxicity as represented in Figure 4.6.

4.3.5 Evaluation of differentiation inducing ability of different forms of SLs

At lower concentrations, no change in morphology was seen. The cells appeared same as those in control well. It was observed that the density of cells in the control wells was considerably high as compared to the other wells. Thus the SLs are having anti-proliferative action. In the wells SLOAA 20µg/ml, 40µg/ml (refer to Figures 4.7 and 4.8), SLOAC 10µg/ml (Figure 4.9) and SLLAC 400ng/ml, 1µg/ml,

5µg/ml, 10 µg/ml (Figures 4.10 to 4.13) morphological changes were seen after 48h.

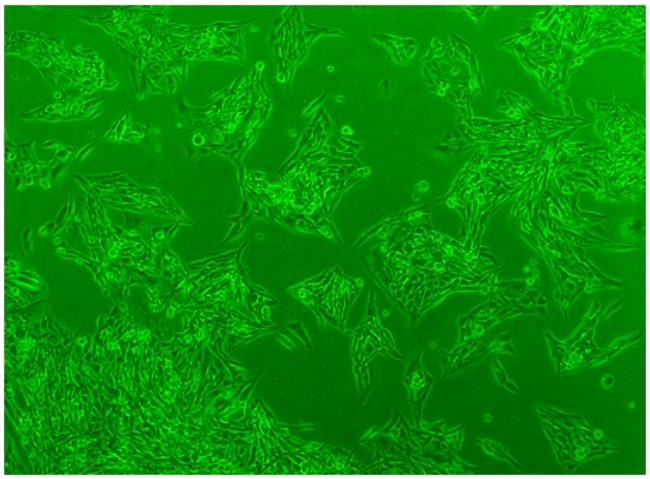


Figure 4.7 LN229 cells treated with pure oleic acid Sophorolipid i.e. SLOAA 40µg/ml- elongated cells can be seen (40X magnification)

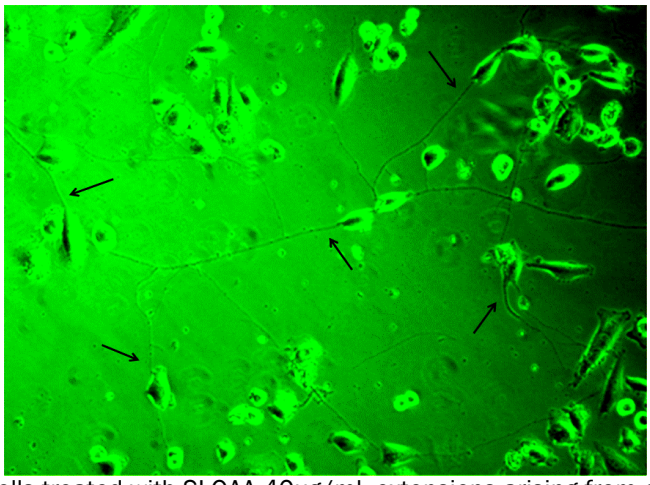


Figure 4.8 LN229 cells treated with SLOAA 40µg/ml- extensions arising from ends of cells can be seen (200X magnification)

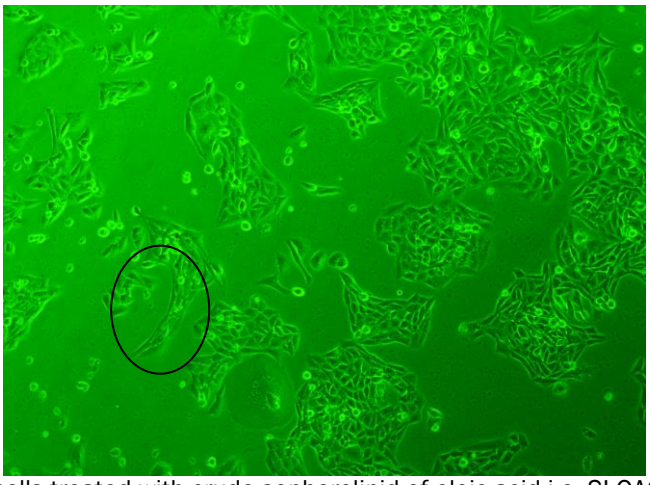


Figure 4.9 LN229 cells treated with crude sophorolipid of oleic acid i.e. SLOAC 10µg/ml. Bundle formation and presence of elongated cells was observed (40X magnification)

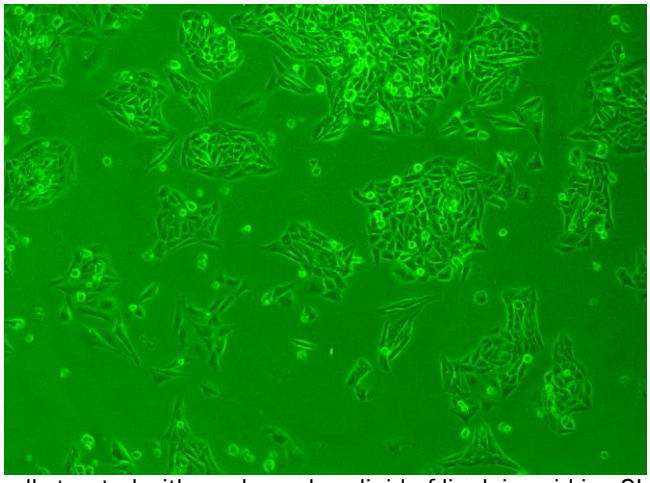


Figure 4.10 LN229 cells treated with crude sophorolipid of linoleic acid i.e. SLLAC 400ng/ml. Cell elongation and initiation of threadlike extensions was observed (40X magnification)

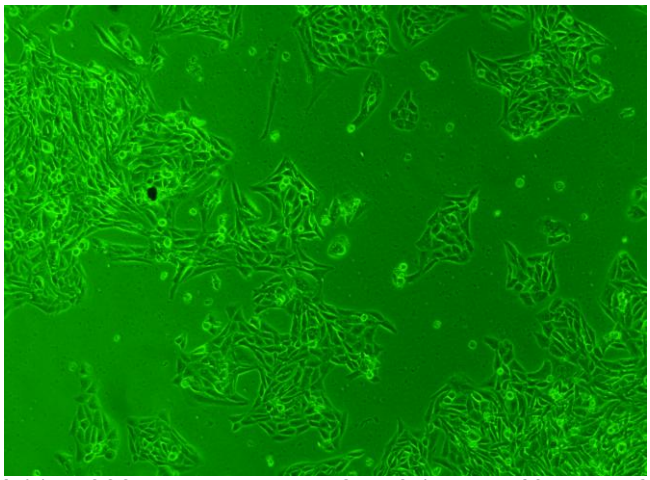


Figure 4.11 LN229 cells treated with SLLAC 1µg/ml (40X magnification)

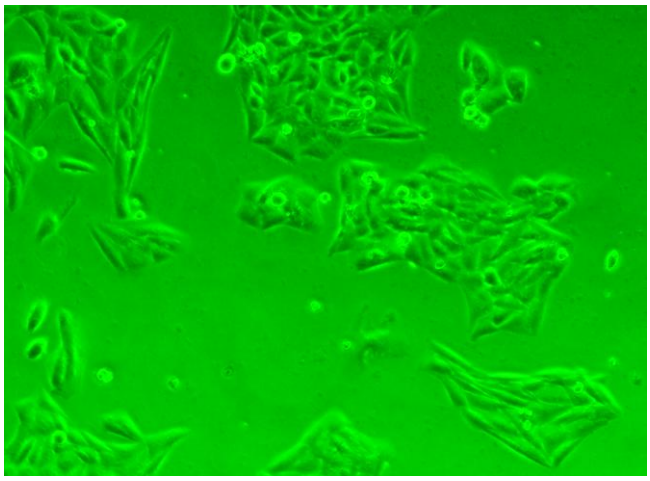


Figure 4.12 LN229 cells treated with SLLAC 5µg/ml. Cell elongation and initiation of bundle formation was seen (200X magnification)

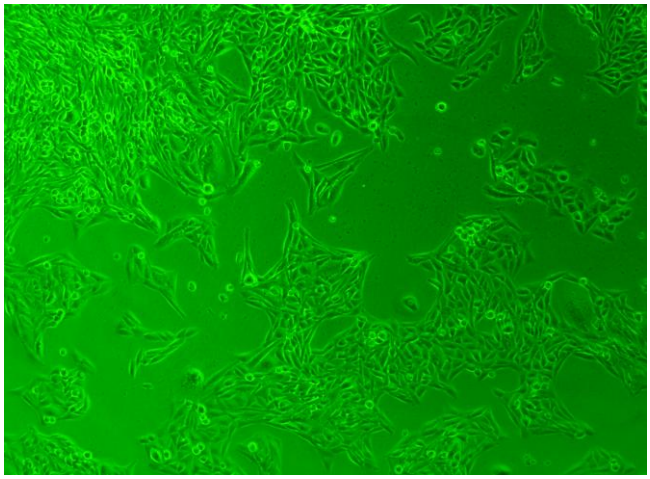


Figure 4.13 LN229 cells treated with SLLAC 10µg/ml (40X magnification)

The morphological features such as elongation of cells, formation of thread- like extensions arising from cell ends, bundle formation and aligning of cells were counted as indications of differentiation.

With SLOAA at 80µg/ml and beyond cell death was seen. With SLOAC, beyond 10µg/ml cell death was seen. With SLLAC, 20µg/ml turned toxic to cells.

4.3.6 Immunocytochemistry analysis for checking the expression of markers

In order to confirm the differentiation; Expression of below mentioned markers was checked in the LN229 cells exposed to 40µg/ml of SLOAA. Morphological changes indicative of differentiation were observed at this concentration of SL.

(1) GFAP- Glial Fibrillary Acidic Protein, a 50 kDa type III intermediate filament protein is a reliable marker of normal astrocytes. Mature astrocytes show increased expression of GFAP.

(2) Ki-67- This antigen is present during all active phases of the cell cycle but is absent from resting cells exclusively in the nuclei of cycling cells, the defined period of nuclear expression makes it a reliable marker of malignant proliferating cells. As the cell usually makes a choice between proliferation and differentiation; we expect the decreased expression of Ki-67 in differentiated cells which serves as the sort of negative confirmation.

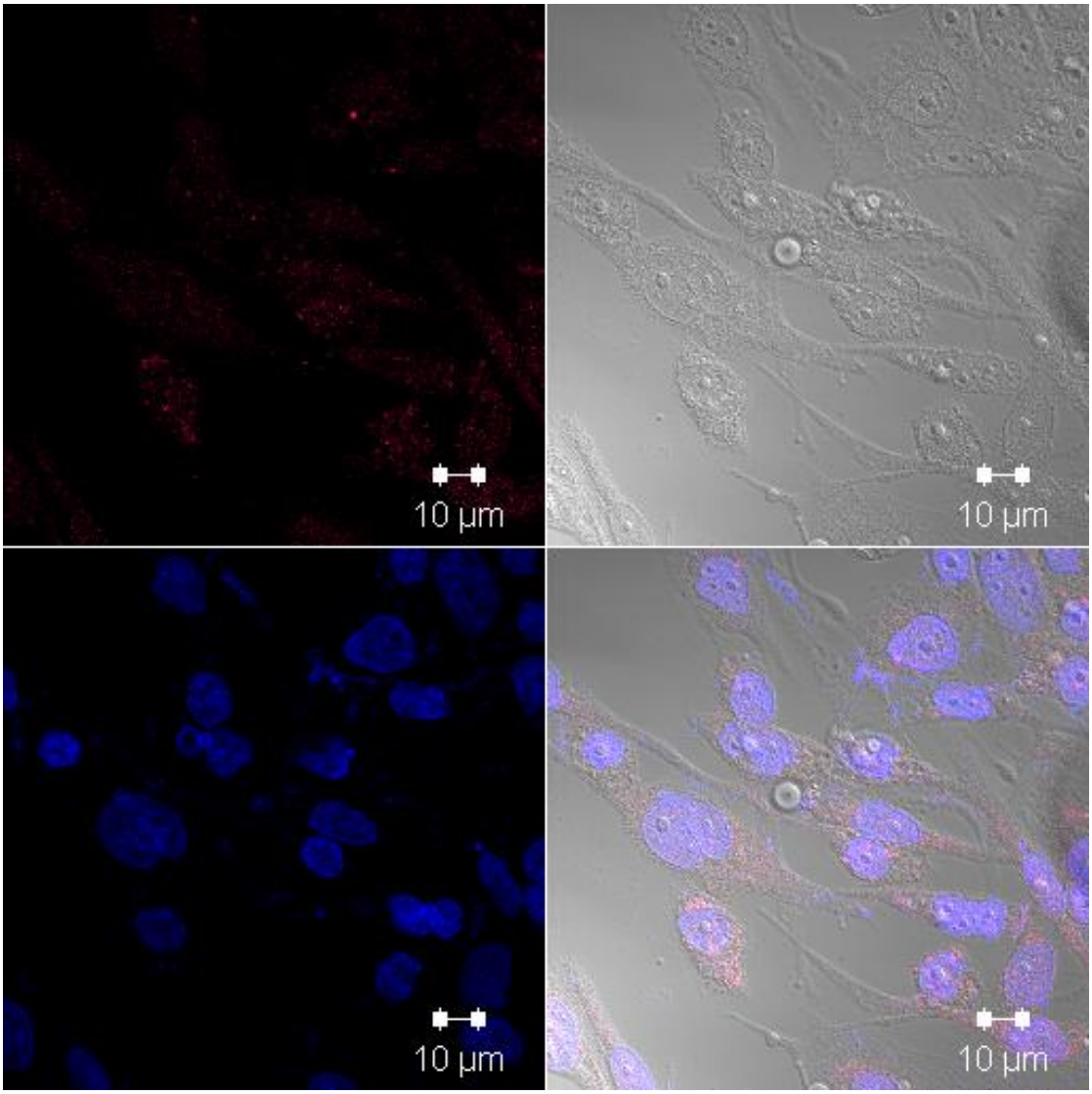


Figure 4.14 LN229 cells exposed to SLOAA 40µg/ml stained with the antibodies specific for GFAP

The confocal microscopy images represented in Figure 4.14 indicate that GFAP presence was detected in majority of the cells indicating cellular differentiation to astrocytes.

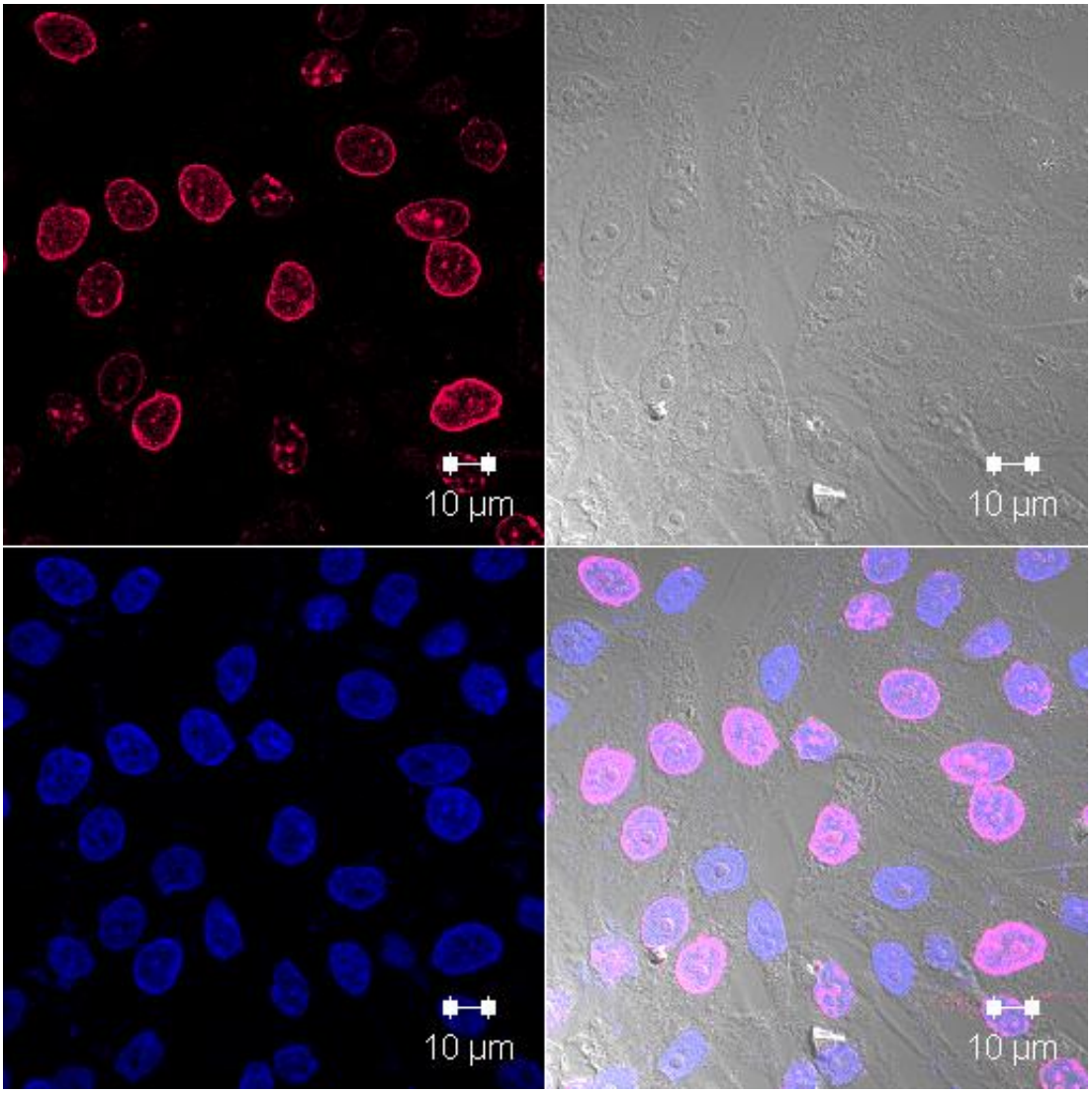


Figure 4.15 LN229 cells exposed to SLOAA 40µg/ml stained with antibodies specific for Ki-67

It was observed from Figure 4.15 that large population of cells showed presence of Ki-67 thus indicating the proliferation. Approximately 1/3rd of the cells did not show the binding of KI-67 specific antibodies indicating that they are on the path of differentiation. Thus we could not get confirmation for differentiation. Therefore further confirmation studies such as RT-PCR are necessary.

Thus to conclude- we found that the crude SLs which contain both acidic as well as lactonic form induce morphological changes at comparatively lower concentrations as compared to the pure acidic form. The known fact is Lactonized SLs have different biological and physicochemical properties as compared to acidic forms. In general, lactonic SLs have better surface tension lowering and

antimicrobial activity, whereas the acidic ones display better foam production and solubility [2]. Thus our findings can be justified.

Also with SLLAC; the features of differentiation-elongation, alignment of cells and bundle formation were seen at lowest concentrations of 400ng/ml, 1µg/ml, 5µg/ml and 10µg/ml. Probably this can be attributed to the higher extent of unsaturation as linoleic acid is 18:2.

Present study has provided directions for further investigations. Experiments need to be performed with purified SL forms to establish the effects of individual components. These studies will help us unravel the mechanisms underlying cellular differentiation as well as to develop potentially useful therapeutic agents. The differentiation inducing ability of SLs can prove its usefulness in regenerative medicine. The studies with neural cell line can provide clues for treatment of neurodegenerative disorders. Induction of terminal differentiation and growth arrest of cancer cells can be a possible approach for treatment of cancer.

Among published papers on SLs, only few are related to the pharmacological roles of SLs. Hence it is necessary to explore new applications of SLs as anticancer drugs. Bluth M. *et. al.* (2008) have verified the selective action of SLs against malignant cells without harming normal cells. This would be advantageous in minimizing the side effects which are commonly associated with the current therapeutic regimens [8]. Effect of these molecules has to be checked with normal cell lines to know if the compound is selectively affecting cancerous cells. As the compound is inducing cell death, it should be checked whether it is through apoptosis since we have shown that the SLs can induce differentiation and cell death using glioma cell line. Studies will be followed by flow cytometry.

4.4 References

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Chapter V

Chapter V is about the use of a novel yeast- *Pichia caribbica* (MTCC5703) for production of SL like molecule. The above mentioned yeast has been known for its ability to metabolize xylose. Therefore during production procedure; xylose was fed as hydrophilic carbon and as a result, glycolipidic molecule bearing xylose was anticipated.

During the experiment; unusual pigment was produced alongside by the same yeast. On exploring it to depth; xylitol production was detected with interesting property which makes the second half of this chapter.

The Chapter has been divided into 2 sub-chapters as follows-

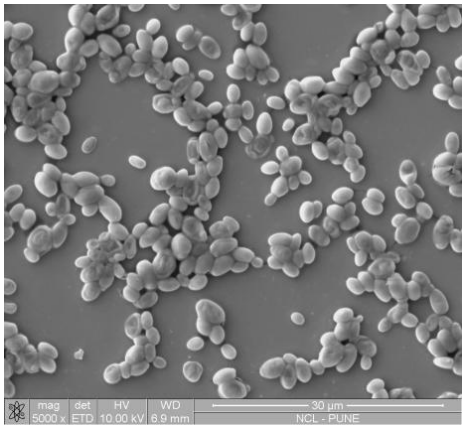
Chapter V(a)-Glycolipid production by a novel yeast- *Pichia caribbica* (HQ222812) with xylose as a head group and its advantageous properties

and

Chapter V(b)-Crystalline xylitol production by a novel yeast- *Pichia caribbica* (HQ222812) and its application for quorum sensing inhibition

Chapter V(a)

Glycolipid production by a novel yeast- *Pichia caribbica* (HQ222812) with xylose as a head group and its advantageous properties



Sophorolipids (SLs) belong to a class of glycolipidic biosurfactants exhibiting wide range of applications in various fields. The structural diversity in SLs gives rise to variation in physicochemical and biological properties. In order to achieve the less explored head group diversity in SL structure, a new xylose fermenting yeast- *Pichia caribbbica* has been employed for biosurfactant production. The media and fermentation parameters have been optimized to achieve maximum yield of 7.5g/l. The physicochemical properties of the xylolipid biosurfactant have been assessed. It reduced the surface tension of distilled water from 70mN/m to 35.9mN/m with the remarkably low CMC value 1.0 mg/l as compared to typical SLs (reported CMC range-40-100mg/l). Structural characterization was done using FTIR and HR-MS to identify the structure putatively. 17-L-[(β-D-xylopyranosyl)-oxy]-Δ9-heptadecanoic acid correlated to m/z 415 majorly constituted the product. Control experiment was performed in which glucose was provided as the hydrophilic carbon. This product was also subjected to HR-MS analysis to determine its chemical nature and found to be different from xylolipid. Presence of xylose as head group was anticipated to give altered physicochemical and biological activities. In accordance to the same, low CMC value and better inhibitory action was demonstrated against *Staphylococcus aureus*, a gram positive bacterium.

5a.1 Introduction

Biosurfactants are amphiphilic molecules produced from biological sources extracellularly and tend to reduce the interfacial and/or surface tension of a system. Because of their inherent biodegradability and broad array of functional properties (including emulsification, phase partitioning, wetting, foaming and surface activity), many of these materials are advantageous over currently used petrochemical-based products [1].

SLs are a kind of glycolipidic biosurfactant synthesized in high concentrations by non-pathogenic yeasts. SLs are amphiphilic molecules interacting with the phase boundary in heterogeneous systems. Typical structure of SLs consists of a sophorose (dimeric sugar) linked β-glycosidically to terminally or sub-terminally hydroxylated fatty acid with chain length 16-18. SL occurs as a mixture of compounds differing in their acetylation, lactonization and position of hydroxylation [2]. SLs like other biosurfactants exhibit a variety of useful properties and applications in various fields. These include the areas such as food and food-related industries (as emulsifiers, foaming, wetting, solubilizers, antiadhesive agents), biomedicine and therapeutics (as antimicrobial agents, immunoregulators and immunomodulators, owing to their possible role in signalling and cytotoxic activity) [3].

The different structural classes of SL cause wide variation in physicochemical properties. Lactonized SLs have different biological and physicochemical properties as compared to acidic forms. The biosurfactants' hydrophilic/lipophilic balance, foam formation capacity, and antimicrobial effects are all strongly influenced by the degree of lactone formation [2]. Modification of the lipophilic portion of the SL molecule can alter their physicochemical properties [4]. Also the composition of lipophilic tail affects the biological activity e.g. extent of unsaturation in the tail region of SL modulates the differentiation inducing activity against glioma cell line [5]. Thus the head and tail group diversity govern the properties of SLs. One type of SL may be more suitable for specific applications than the other type because of the structural difference [6].

Reports on studies of SLs of more structural diversity are rare [7]. Few research groups have attempted to modify the fundamental SL skeleton; driven by the fact that more structural derivatives of SLs are needed to better define structure-biological activity relationships and thereby enhance SL efficacy and its spectrum of action [8]. To a certain extent, structural variation (and hence physical properties) can be achieved by changing the lipidic carbon source, which alters the SL fatty acid content [1]. Amino acid conjugated SLs at tail end were synthesized by chemical method and the derivatives were shown to have superior antibacterial as well as anti viral activity [8].

There have been attempts to change the SL head group to glucose by action of enzymes-glycosidases, naringinase, hesperidinase. [9, 10, 11]. Shah V. *et. al.*. replaced the glucose with different sugars namely- Fructose, Xylose, Ribose, Lactose, Mannose, Arabinose and Galactose in order to get different glycolipid molecules bearing these sugars as head groups. The antibacterial activity of thus formed compounds was checked. The nature of the carbohydrate head was shown to have an influence on the antibacterial activity of the glycolipid [12]. However the report is without purification and characterization studies of individual glycolipids to understand the precise chemical structure in terms of glycosylation, acetylation and lactonization. Thus there is scope for further exploration of structural variants.

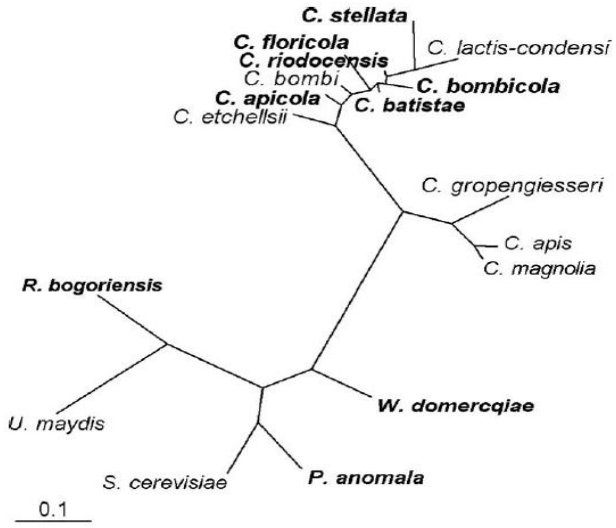


Figure 5a.1 Phylogenetic tree of sophorolipid producing (bold) and non-producing species based on the D1/D2 26S ribosomal region. The tree was constructed by running the DNA Maximum Likelihood program after alignment of the sequences. The marker bar below denotes the integer branch length. (Adapted from [16])

SL production is to some extent associated with yeasts closely related to *Candida bombicola* and *C. apicola.* Recently several new SL producing strains - *Wickerhamiella domercqiae, Pichia anomala* and few members of the genus *Candida* namely C*. batistae, C. riodocensis, C. stellata* and *Candida* sp. Y-27208 have been discovered. *Rhodotorula bogoriensis*, a basidiomycete is also reported as SL producer [13, 14, 15, 16].

Hande *et. al.*, (2012) from our research group have mentioned isolation of yeast from over-ripe banana by enrichment in xylose containing medium. The phylogenetic analysis of ITS1-5.8S-ITS2 region sequences of ribosomal RNA of isolate revealed that it shows affiliation to genus *Pichia* and clades with *Pichia caribbica*. Further ethanol production by this isolate on a xylose containing synthetic medium and sugarcane bagasse hemicellulosic hydrolysate has been evaluated. The isolate was maintained and regularly sub cultured on a medium containing xylose as the primary carbon source [17]. We have also demonstrated the conversion of xylose in to xylitol using this isolate [18].

In the present chapter, the same isolate has been employed for the biosurfactant production. It was speculated that being a member of the genus *Pichia*; the strain might be capable of producing SL like the available report [14]. Also the previous work suggested that –*P. caribbica* possesses enzymes necessary for metabolism of xylose. So the experiments were carried out in order to check if this isolate can synthesize glycolipidic molecules and incorporate xylose in place of glucose. Such surfactant molecule was expected to exhibit different physicochemical as well as biological reactivity. The media and fermentation conditions have been optimized for maximum yield of such modified biosurfactant. Further the product was characterized for its physicochemical, antibacterial properties and structural information with various analytical tools. The present SL-analogue was observed to possess advantageous physicochemical and antibacterial properties as compared to the typical SL.

5a.2 Materials and methods

5a.2.1 Microorganisms and their maintenance

(a) Xylose fermenting yeast, *Pichia caribbica* was used for the production of xylolipid. It was maintained on MXYP (Malt extract- 0.3g%, Xylose- 2g%,

Yeast extract- 0.3g%, Peptone- 0.5g% and Agar- 2.0g %) slants. The microorganism was sub cultured in every 4 weeks and maintained at 4°C in a refrigerator

(b) The test microorganisms- *Pseudomonas aeruginosa* (NCIM-5031) and *Staphylococcus aureus* (ATCC- 29737) were procured from National Collection of Industrial Microorganisms, NCL, Pune. The cultures were maintained on Nutrient agar slants. The microorganisms were sub cultured in every 4 weeks and maintained at 4°C in a refrigerator.

5a.2.2 Chemicals and reagents

All media, chemicals and solvents used in this study were of analytical grade and supplied by either Hi-media pvt. Ltd., India or Merck India ltd.

The fatty acid precursor- Oleic acid (technical grade 90% pure) was purchased from Sigma Aldrich.

5a.2.3 Optimization of media and fermentation parameters for production of biosurfactant by Pichia caribbica

(a) Media optimization

In addition to primary and secondary carbon sources; media contain nitrogen source, growth factors, buffer components and other minerals which are known to show significant effect on SL yields [2, 14]. Different media previously reported for maximum production of SL were tried. Glucose was replaced with xylose in order to get it as the head group in the biosurfactant molecule.

The seed culture was prepared by inoculating 10 ml of fresh MXYP nutrient medium (Malt extract- 0.3g%, Xylose- 2g%, Yeast extract- 0.3g%, Peptone- 0.5g%) with *Pichia caribbica* followed by incubation at 30°C, 180 rpm for 24 h. This pre-inoculum was added to 100 ml of following different media supplemented with 1ml of oleic acid dispersed in 1ml absolute ethanol in a 500-ml Erlenmeyer flask and incubated further for 7d i.e. 168h to ensure complete utilization of oil.

Media components	Media compositions (values in g%)		
	Medium A [19]	Medium B [20]	Medium C [14]
Potassium di-hydrogen phosphate	0.1	-	0.02
Magnesium sulphate	0.5	0.03	0.02
Calcium chloride	0.01	-	-
Sodium chloride	0.01	-	-
Yeast extract	0.5	0.1	0.1
Ammonium nitrate	0.05M	-	-
Xylose	10	10	4
Di-sodium hydrogen phosphate	-	0.2	-
Sodium di-hydrogen phosphate	-	0.7	-
Ammonium sulphate	-	0.1	-
Sodium nitrate	-	-	0.4

Table 5a.1 Media compositions used during optimization experiments. The media reported as per the references were modified by adding xylose in place of glucose

The product was harvested by the procedure previously reported by Shah S. *et. al.*. 2007. Culture medium was centrifuged at 5,000 rpm, at 10°C for 20 minutes The supernatant was extracted twice with equal volumes of ethyl acetate, the organic layer was dried over anhydrous Na_2SO_4, and the solvent was removed by rotary vacuum evaporation. The yellowish brown product was washed twice with n-hexane to remove unconverted fatty acid [21]. For qualitative confirmation of surfactant property, the collected product was subjected to Oil Displacement test which was primary test to confirm the product formation.

(b) Effect of primary, secondary carbon feed concentration on the biosurfactant yield by *Pichia caribbica*

The medium giving maximum yield was continued and xylose, the primary carbon concentrations were varied within the range 5 to 10%w/v. In case of secondary carbon source, oleic acid feeding was varied within the range 2 to 6%v/v (1ml ~ 0.895g of oleic acid).

(c) Effect of physical parameters on biosurfactant production by *Pichia caribbica*

To check the effect of incubation temperature, the fermentations were carried out at different temperatures namely, 25, 30 and 33°C. Typically the SL fermentations are run within the temperature range 25-30°C [2] so we have checked the yield at above mentioned temperatures. To check the effect of initial pH on SL production; the initial pH values were adjusted approximately (error $\pm$ 0.5) to 5.0, 6.0, 7.0 and 8.0 using 0.1 M HCl/NaOH.

(d) Effect of various nitrogen sources on biosurfactant production by *Pichia caribbica*

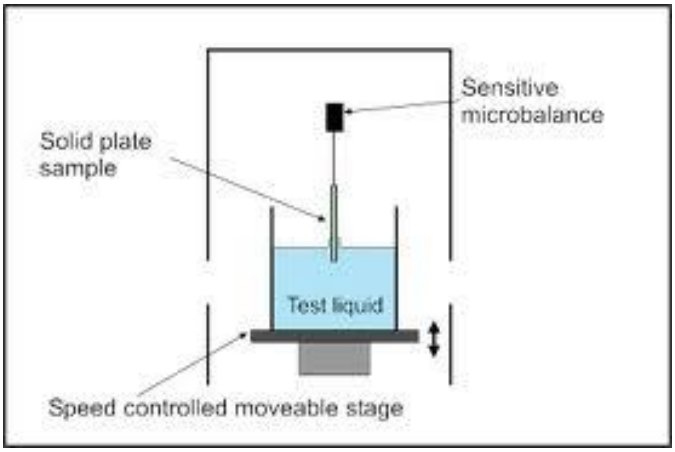
To identify the nitrogen source giving best yield in the chosen medium, different organic and inorganic nitrogen sources namely Ammonium sulphate, Ammonium chloride, Urea, Yeast extract, Sodium nitrate and Ammonium nitrate were used at the concentration 0.1g%.

The fermentation, extraction procedures were essentially done as mentioned before for the optimization experiments. The experiments were carried out in triplicates in 250ml Erlenmeyer flasks containing 50 ml of the production media. Control experiment was also performed without addition of fatty acid.

5a.2.4 Physicochemical characterization of the biosurfactant produced by Pichia caribbica

(a) Minimum surface tension and critical micelle concentration determination

'Oil displacement test' was performed as a qualitative confirmation of surfactant property of the *P. caribbica* synthesized xylolipid product. Method reported by Morikawa *et. al.* (1993) was followed [22]. Minimum surface tension and critical micelle concentration of biosurfactant was estimated using a KRUSS surface tensiometer K11 by Wilhelmy plate method.



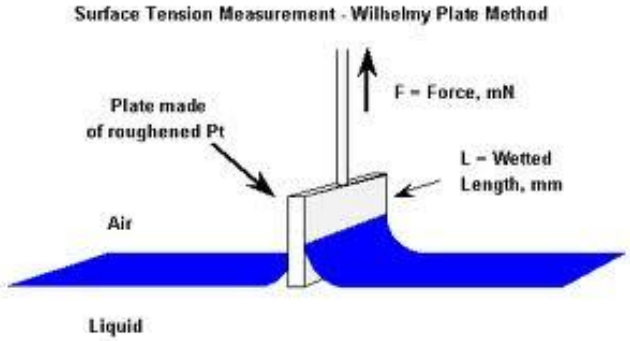


Figure 5a.2 Diagrammatic representation of surface tension measurement by Wilhelmy plate method

The Wilhelmy plate used for measuring surface tension is often made from glass or platinum which may be roughned to ensure complete wetting. The thoroughly cleaned plate is attached toa scale via a thin metal wire. The force on the plate due to wetting is measured via a tensiometer and used to calculate surface tension using the Wilhelmy equation:

$$\gamma = F/\ l * \cos\theta$$

Where *l* is the wetted perimeter of the Wilhelmy plate and *θ* is the contact angle between the liquid phase and the plate [23].

Stock of *Pichia caribbica* synthesized product (20mg/l) was prepared in MilliQ water and diluted appropriately to get desired concentrations. The concentration range used was 1ng/l to 20mg/l. The surface tension was measured at 26.7 °C by Wilhelmy plate method. A clean, dry 100 ml glass beaker was filled with the

desired solution for surface tension measurement. The beaker was placed on the sample platform of the Kruss K11 tensiometer. The platinum surface tension probe was removed from the tensiometer hook and rinsed with deionized water and dried with the blue part of the flame from the propane torch. The probe was then air cooled and reinserted onto the tensiometer hook. The surface tensions of the desired solutions were measured as described in the Kruss K11 tensiometer operating manual. All surface tension measurements were the average value of 4 readings recorded at an interval of 30 seconds.

To determine Critical Micelle Concentration (CMC), the surface tension was measured as a function of surfactant concentration. Surface tension was then plotted vs. log surfactant concentration. The resulting curve had a nearly horizontal portion at concentrations higher than the CMC and had a negative steep slope at concentrations less than the CMC. The CMC was calculated as that concentration of the curve where the flat portion and the extrapolated steep slope intersected. The Surface Tension value remains almost constant beyond CMC.

(b) *Determination of contact angle*

Contact angle measurement which signifies the wettability was studied for the *Pichia caribbica* synthesized product using Goniometer (G-10 contact angle meter). Measurements were carried out on three different surfaces namely; glass, Teflon and stainless steel. Biosurfactant stock solution of 1µg/ml was prepared in MilliQ water. 10µl volume was used for each measurement. The sessile drop method was used to estimate wetting properties of a localized region on a solid surface. The angle between the baseline of the drop and the tangent at the drop boundary was measured.

5a.2.5 Structural characterization of the biosurfactant produced by Pichia caribbica

For structural characterization, the biosurfactant was synthesized using resting cell method in which the pre-grown cell mass was subjected to biosurfactant production in presence of hydrophilic and hydrophobic carbon source according to the procedure mentioned in [5]. In one set of experiment; 10% xylose solution

was fed to the pregrown cells along with oleic acid while in the control experiment, 10% glucose solution was fed. Rest of the procedure was same as mentioned earlier.

(a) FTIR analysis: For characterization of *Pichia caribbica* synthesized biosurfactant with the Fourier-transformed infrared (FTIR) spectroscopy, the spectrum was recorded on a Perkin-Elmer Spectrum One in the frequency range of 4000 to 500cm^{-1}. To enhance the resolution, the samples were prepared as potassium bromide (KBr) pellets with about 0.32% (in weight) of sample.

(b) HR-MS analysis: HR-MS (High Resolution Mass Spectrometry): HR-MS mass spectra were recorded on a Thermo Scientific Q- Exactive, Accela 1250 pump. The samples were prepared by dissolving the products in methanol to a final concentration of 1mg/ml and 10µl volume was injected into the HR-MS system. Glycolipid mixtures were separated using Hypersil Gold (Thermo Scientific) 150 X 4.6mm column. During the elution program, isocratic solvent phase MilliQ water/Methanol (80:20 v/v) was maintained for 40 minutes. Capillary temperature was 30°C. The mixtures were scanned by Thermo Scientific Accela PDA detector from 200nm to 900nm. The effluent was connected to (HESI) Heated electrospray ionization. The full MS scan was performed using positive polarity. The mass spectra associated with chromatographic peaks were analyzed. The peaks with highest relative abundance values were correlated with [M]/ [$M+NH_3^+$]/ [$M+Na^+$] i.e. native molecular mass, ammonium adduct and sodium adduct. Thus based on the m/z (mass to charge ratio); the structures were putatively identified.

5a.2.6 Evaluation of antibacterial activity of the biosurfactant produced by Pichia caribbica

Glycolipids are known to have antibacterial activity. In order to check if the new to nature xylolipid also has similar property, *Pseudomonas aeruginosa* (NCIM 5031) and *Staphylococcus aureus* (ATCC 29737) were used as test organisms and agar plate method was used to determine the extent of antibacterial activity.

Antibacterial tests were carried out using the broth micro dilution and spread plate method.

Following protocol was followed to check the bacterial inhibition by synthesized xylolipid. Bacteria were grown in Nutrient Broth media for 24 h at 37° C, 180 rpm and cell counts were quantified by measuring the A600 values. Appropriate dilution of bacterial cell suspension giving countable colonies (~5 X 10^3 CFUs/ml) was added to different concentrations of *Pichia caribbica* synthesized biosurfactant (10µg-5mg/ml). The mixtures were incubated for 4h at 28˚C at 180 rpm. Then 50µl of mixture was spread plated on nutrient agar plates and incubated at room temperature for 24 hours and colonies were counted. Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of compound that inhibits visible growth of microorganisms on the culture plate. The concentration of biosurfactant at which no bacterial colony was observed on the plate was considered as minimum inhibitory concentration [24].

5a.3 Results and Discussion

5a.3.1 Optimization of media and fermentation parameters

In order to maximize the product yield, 3 media differing in the sugar percentage, nitrogen source and buffer components etc. were chosen as mentioned in materials and methods section. Out of the 3 media used, medium B performed best in terms of xylolipid yield (300mg/100ml). Along with these experiments, control experiment was also performed in which fatty acid was not added where no recoverable product was obtained.

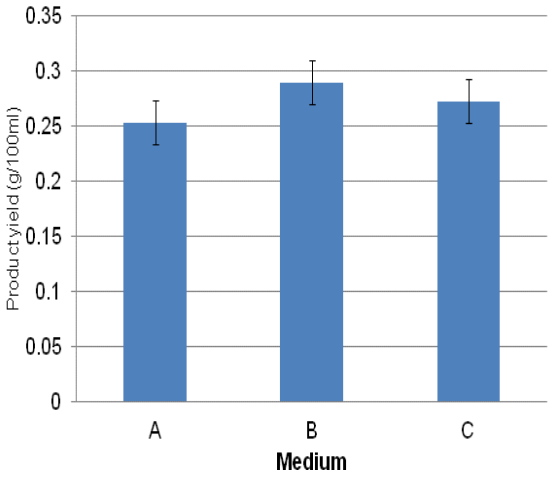


Figure 5a.3 Comparative xylolipid yield in different media

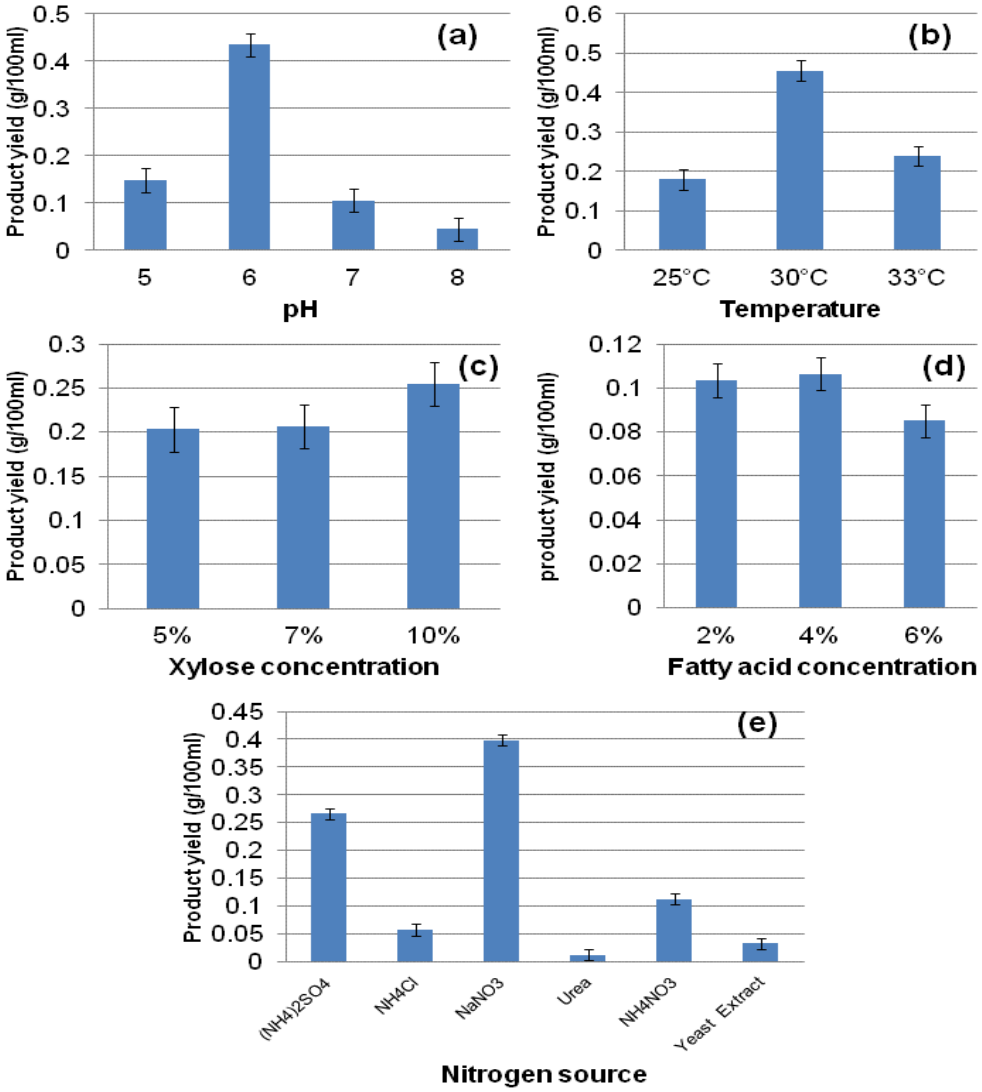


Figure 5a.4 Optimization of fermentation parameters for maximum xylolipid yield-(a) Effect of initial pH, (b) Effect of incubaton temperature, (c) Effect of xylose concentration, (d) Effect of Oleic acid concentration and (e) Effect of different nitrogen sources

Effect of fermentation parameters on xylolipid yield is represented in Figure 5.4. The set of fermentation parameters giving maximum yield are incubation temperature 30°C, pH 6.0, xylose concentration 10g%, Oleic acid concentration 4%v/v and sodium nitrate as nitrogen source. Through optimization of media and fermentation parameters, 750mg/100ml i.e. 7.50g/l yield could be achieved.

5a.3.2 Physicochemical characterization of the biosurfactant produced by Pichia caribbica

(a) Minimum surface tension and critical micelle concentration determination

The *Pichia caribbica* synthesized biosurfactant reduced the surface tension of distilled water from 70mN/m to 35.9mN/m at the CMC value 1 mg/l as represented in Figure 5a.5

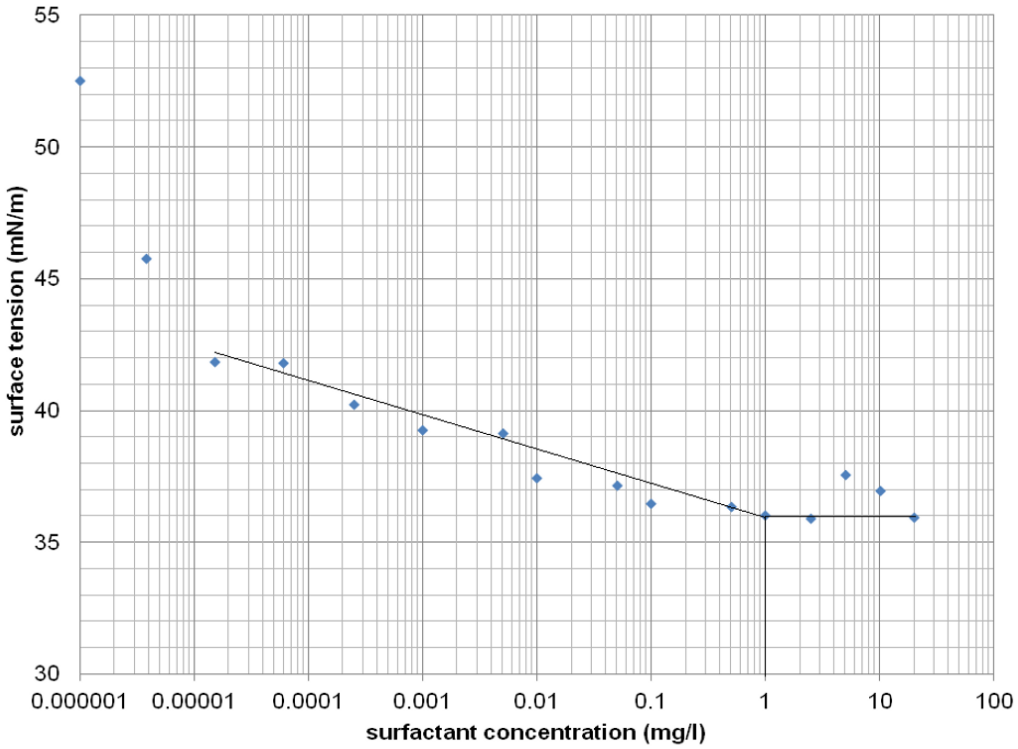


Figure 5a.5 Surface tension reduction and CMC determination of *Pichia caribbica* synthesized biosurfactant

Typically the CMC values of SLs fall within the range 40-100mg/l [2]. SL derived from Oleic acid crude mixture synthesized by *Candida bombicola* (ATCC22214) lowers the surface tension of water to 34mN/m with the CMC value of 120mg/l [25]. Thus it is worth to mention that the *Pichia caribbica* synthesized biosurfactant is showing a low CMC value as compared to the values mentioned in earlier reports with SL of Oleic acid where head group is sophorose i.e. two glucose moieties. On the other hand, SDS- a common synthetic surfactant lowers the surface tension of double distilled water up to 25mN/m with the CMC value 2240mg/l (0.008M) [26]. Another common surfactant Triton X-100 shows the CMC value ~150 mg/l (0.22-0.24mM) and lowers the surface tension of double distilled water up to 32mN/m [27].

Therefore the novel biosurfactant synthesized by *Pichia caribbica*, exhibits CMC value superior than the synthetic surfactants as well as the classical Oleic acid-SL.

(b) Determination of contact angle reduction

The contact angle value is dictated by the interaction between surfactant molecule and the solid surface. The *Pichia caribbica* synthesized biosurfactant

was able to improve spreading and reduce the contact angle on Teflon and Stainless steel but not on Glass as represented by the contact angle values in Table 5a.2. Thus the biosurfactant property would be suitable for hard surface cleaning applications.

	Glass	Teflon	Stainless steel
Control (DW)	37°	95°	85°
Pichia caribbica synthesized biosurfactant	43°	76°	75°

Table 5a.2 Contact angle reduction on different surfaces by the biosurfactant

5a.3.3 Structural characterization of the biosurfactant produced by Pichia caribbica

(a) FTIR analysis of the *Pichia caribbica* synthesized biosurfactant

The FTIR spectrum of the formed xylolipidic biosurfactant (represented by 1BT) is shown in Figure 5a.6 along with that of typical *C. bombicola* synthesized SL and *P. caribbica* synthesized glucolipidic biosurfactant (represented by 2BT).

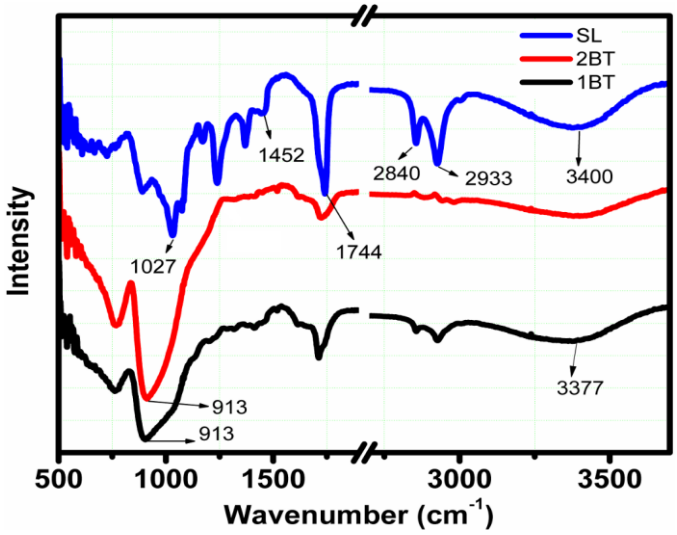


Figure 5a.6 FTIR pattern of the typical *C. bombicola* synthesized SL, *Pichia caribbica* synthesized glucolipidic biosurfactant and *Pichia caribbica* synthesized xylolipidic biosurfactant

The broad band at 3,377 cm^{-1} corresponded to the O-H stretching resulting from the OH groups of sugar. The peak resulting from Sophorose has been reported to occur within 3400-3430 cm^{-1}. The asymmetrical stretching ($masCH_2$) and symmetrical stretching ($msCH_2$) of methylene occurred at 2,933 and 2,860 cm^{-1} respectively [28]. These bands were detected with all the 3 compounds. The C=O

absorption band at 1744 cm^{-1} was observed in spectra of all 3 biosurfactants which may include contributions from that of lactones, esters, or acids [29]. The peak at 1452 cm^{-1} in SL spectrum corresponded to C-O-H in-plane bending of carboxylic acid –COOH, analogous tiny peaks were observed with other 2 compounds, thus suggesting the presence of acidic form of glycolipid compound. [30]. Band at 1147 cm^{-1} which specifies the lactones, was absent in case of xylolipid and glucolipid therefore the presence of acidic glycolipid was further confirmed [28, 30]. The peak observed at 1027 cm^{-1} in SL spectrum was found deviated towards lower wavelength in case of xylolipid and glucolipid, which appeared at 913 cm^{-1}. These peaks can be associated with the sugar C-O stretch of C-O-H groups. The variation in head group moiety in case of xylolipid and glucolipid possibly caused this deviation. The IR spectra of all 3 compounds revealed absorption band around 750 cm^{-1} which were correlated to the presence of C=C unsaturation in lipophilic tail of the synthesized compounds [31]. Thus the comparative FTIR spectra suggested that the newly synthesized compounds xylolipid and glucolipid are grossly similar in structure to typical SL with few deviations.

(b) HR-MS analysis:

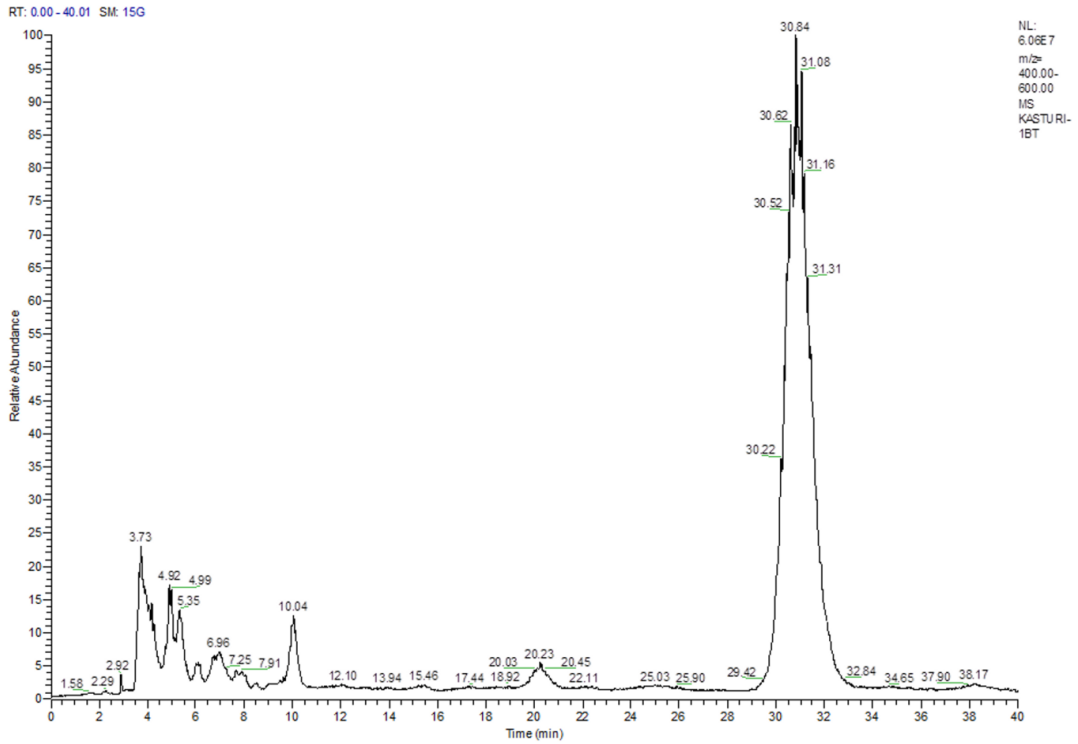


Figure 5a.7 HR-MS chromatogram for *P. caribbica* synthesized xylolipid biosurfactant. The chromatogram depicts a major peak at retention time 30.84 minutes

Figure 5a.7 illustrates the chromatogram obtained from the Photo diode array detector for the *P. caribbica* synthesized xylolipid. A major peak spanned over 4 minutes got eluted at 30.84minutes. Figure 5a.8 is the mass spectrum associated with this peak revealed the presence of 3 prominent peaks namely, m/z 415, 432 and 437. These were correlated to [M], [M+NH_3^+] and [M+ Na^+]. The observed m/z suggested the presence of 17-L-[(β-D-xylopyranosyl)-oxy]-Δ9-heptadecanoic acid.

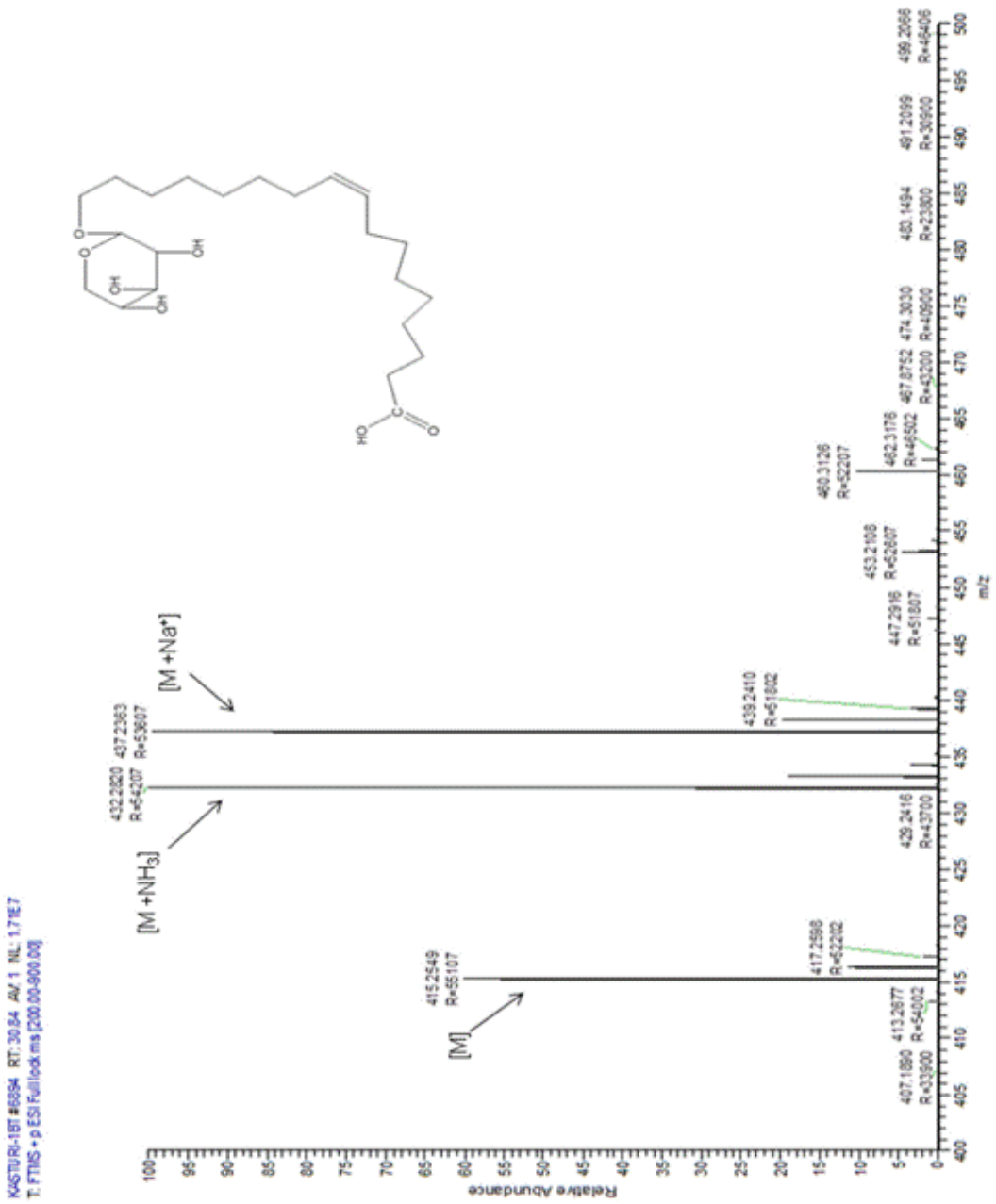


Figure 5a.8 Mass spectrum associated with the peak eluted at 30.84 minutes. The m/z values-415, 432 and 437 correlated with [M], [M+NH_3^+] and [M+ Na^+]. The observed m/z suggested the presence of 17-L-[(β-D-xylopyranosyl)-oxy]-Δ9-heptadecanoic acid

On the contrary, in case of glucolipid; 2 major peaks were observed. First one got eluted at 6.21 minutes spanned over 6 minutes. Another peak got eluted at 32.86 minutes which was spanned over 8 minutes (Refer to Figure 5a.9).

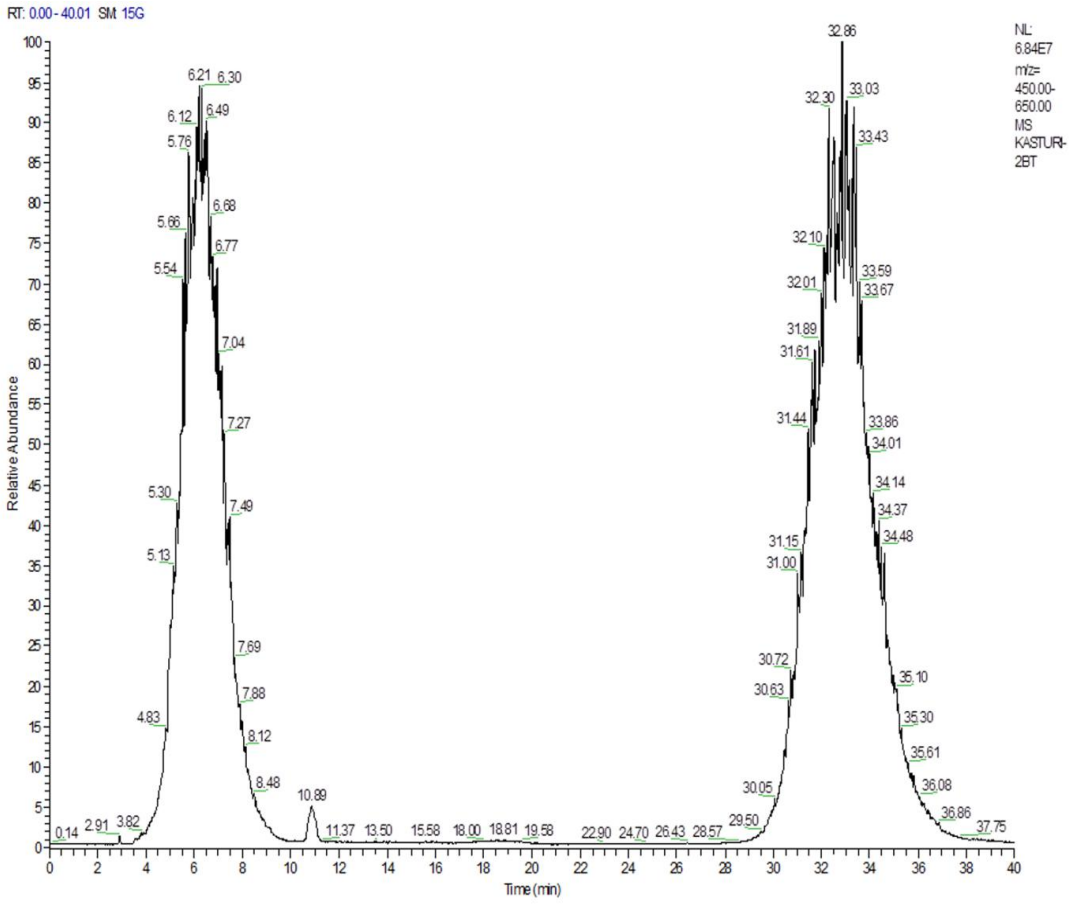


Figure 5a.9 HR-MS chromatogram for *P. caribbica* synthesized glucolipid biosurfactant. The chromatogram depicts 2 major peaks at retention time 6.21and 32.86 minutes

The mass spectrum associated with first peak represented by Figure 5a.10 revealed the presence of prominent peaks m/z= 459, 476 and 481. These were correlated to [M], [M+NH_3^+] and [M+ Na^+]. The observed m/z suggested the presence of 17-L-[(β-D-glucopyranosyl)-oxy]-Δ9-octadecanoic acid. The mass spectrum associated with second peak represented by Figure 5a.11 revealed the presence of prominent peaks m/z= 503, 520 and 525 which could be correlated to [M], [M+NH_3^+] and [M+ Na^+]. The observed m/z suggested the presence of 17-L-[(6'-acetyl, β-D-glucopyranosyl)-oxy]-Δ9-octadecanoic acid.

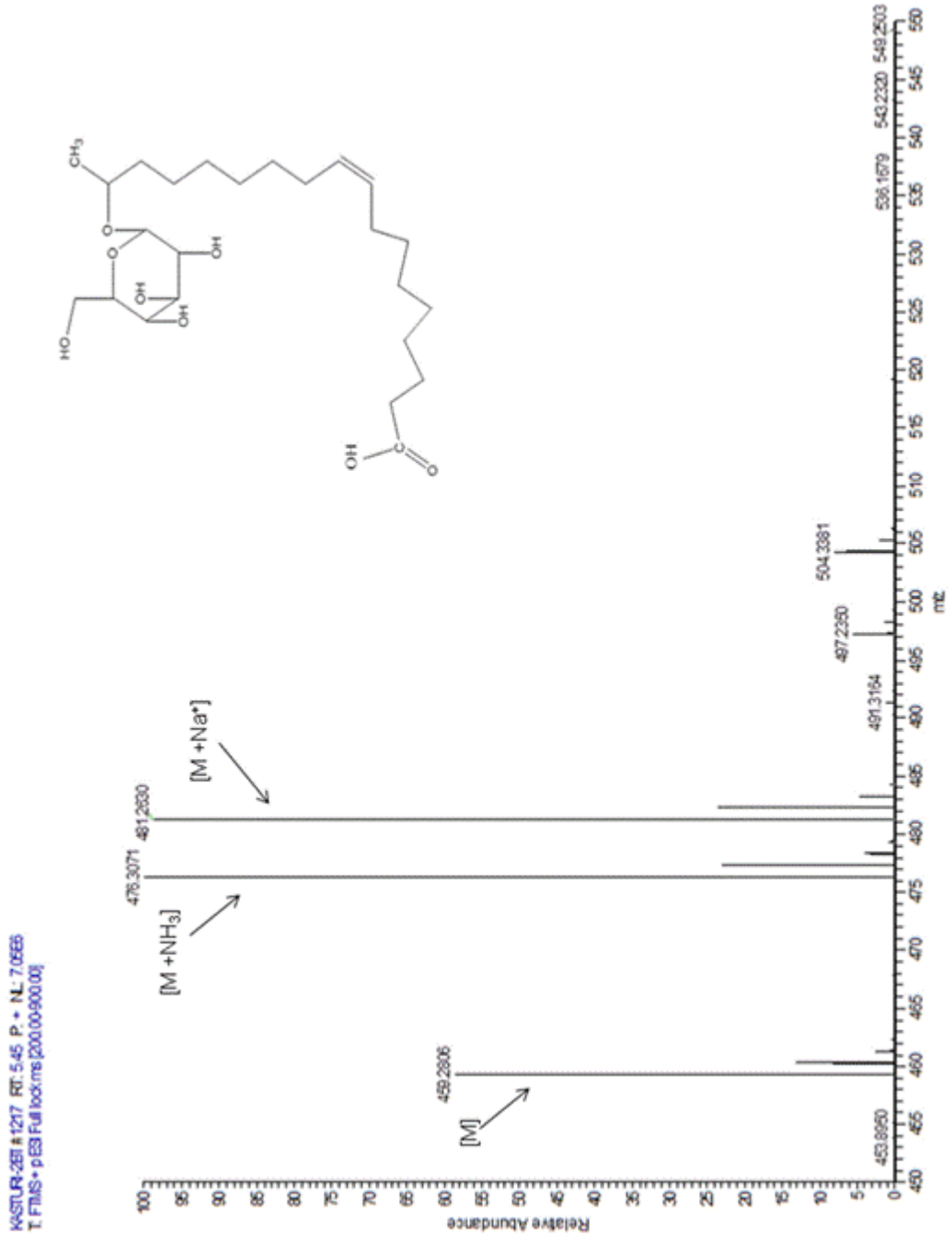


Figure 5a.10 Mass spectrum associated with the peak eluted at 6.21 minutes. The m/z values- 459, 476 and 481 correlated with [M], [M+NH_3^+] and [M+ Na^+]. The observed m/z suggested the presence of 17-L-[(β-D-glucopyranosyl)-oxy]-Δ9-octadecanoic acid

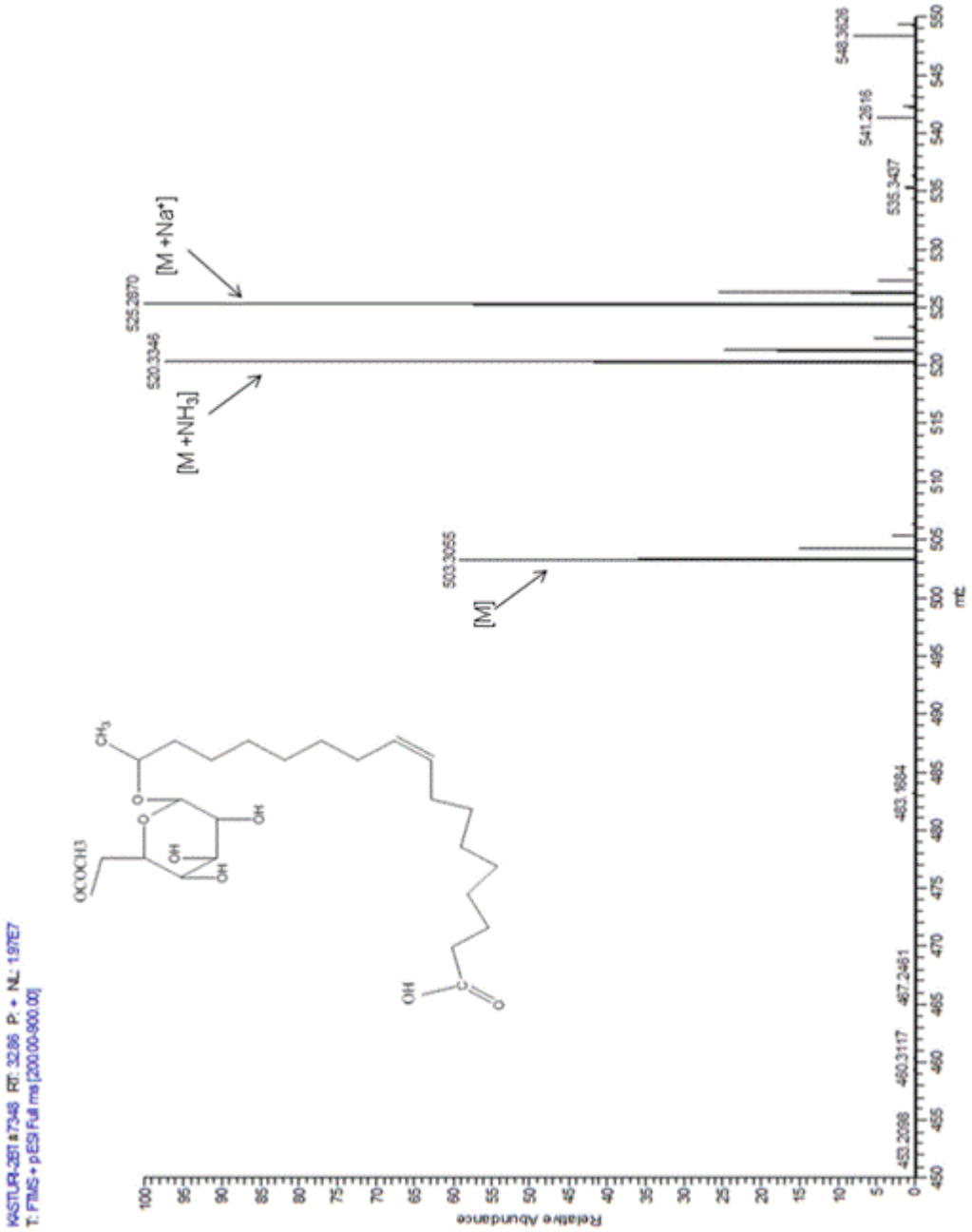


Figure 5a.11 Mass spectrum associated with the peak eluted at 32.86 minutes. The m/z values-503, 520 and 525 correlated with [M], [M+NH_3^+] and [M+Na^+]. The observed m/z suggested the presence of 17-L-[(6'-acetyl, β-D- -glucopyranosyl)-oxy]-Δ9-octadecanoic acid

Therefore in the chromatograms and mass spectra; clear distinction was observed for the products synthesized in presence of xylose versus glucose. When glucose was fed as the hydrophilic carbon source, organism synthesized 2 kinds of biosurfactants possessing single glucose. In the present case, the use of resting cell method by-passsed the possibility of de novo synthesis of glycolipidic product. It is worth mentioning that significant difference in yield values was observed. Resting cell method yielded 0.44g/l of xylolipid while the yield was 0.13g/l in case

of glucolipid. This finding was in accordance with the initial speculation for favored xylose utilization by *P. caribbica*.

5a.3.4 Evaluation of antibacterial activity of the biosurfactant produced by Pichia caribbica

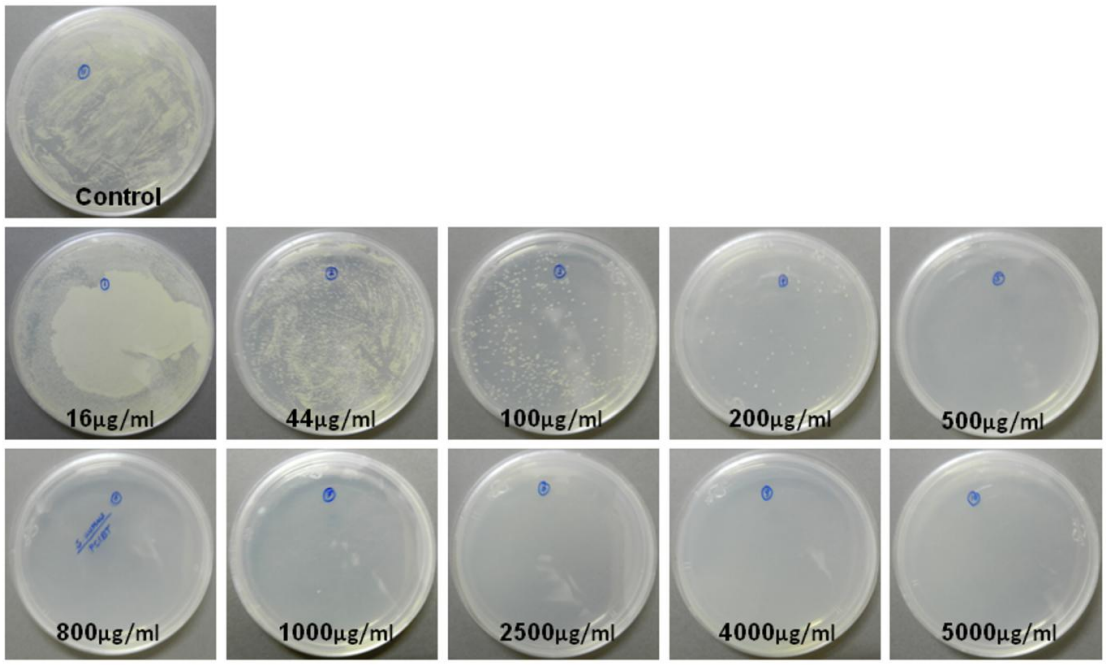


Figure 5a.12 Effect of xylolipid exposure on the growth of *Staphylococcus aureus*

Figure 5a.12 depicts the antibacterial action of *Pichia caribbica* synthesized xylolipid against *S. aureus* while Figure 5a.13 shows the effect of the compound against *P. aeruginosa*. The antibacterial property of the crude xylolipid was checked against *Staphylococcus aureus* and *Pseudomonas aeruginosa* as the representatives of Gram positive and Gram negative index bacteria as per the method described. These index bacteria were chosen because *S. aureus* is associated with serious skin infections, such as cellulitis and abscesses [32] and *Pseudomonas aeruginosa*, is also known to cause secondary skin infections [33].

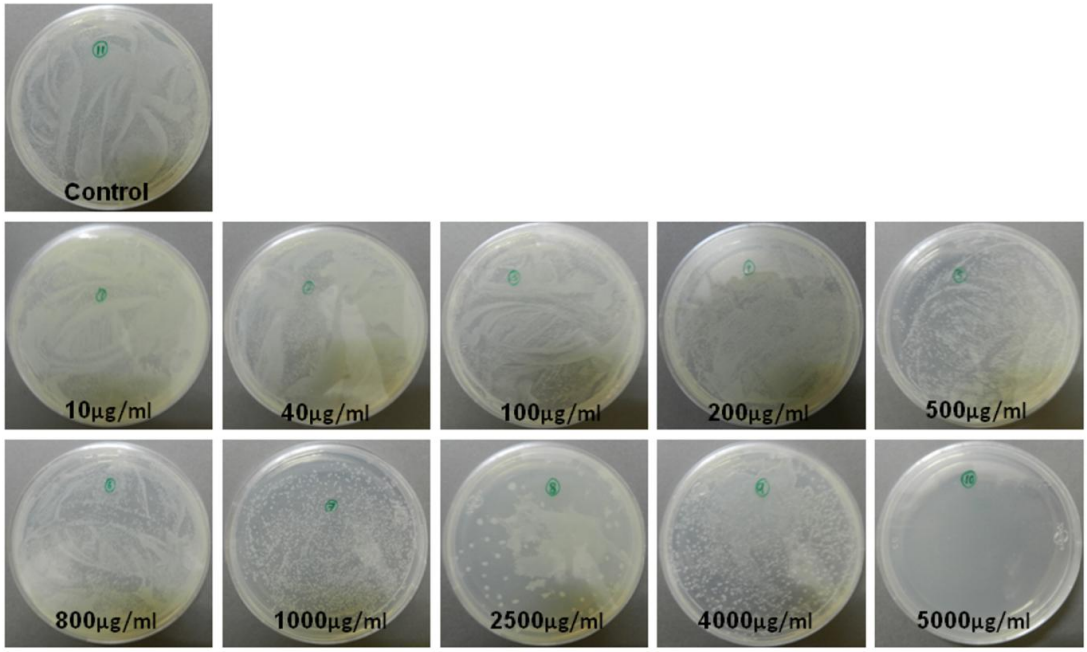


Figure 5a.13 Effect of xylolipid exposure on the growth of *Pseudomonas aeruginosa*

With *S. aureus*, Minimum Inhibitory concentration of xylolipid was found out to be 0.025 mg/ml i.e. 25µg/ml while in case of *P. aeruginosa*, it was 5.0mg/ml.

SLs are known to have antimicrobial property against range of microbial classes. The proposed primary mechanism of action of SLs is membrane lipid order perturbation, which compromises the viability of microorganisms [8]. SLs have been used in cosmetic formulations for its bacteriocidal action [2]. The Minimum Inhibitory concentration (MIC) values of *P. caribbica* synthesized xylolipid and its analogue- *C. bombicola* synthesized typical SL have been represented in the Table 5a.3. A comparison has been made between the MIC values against *S. aureus* and *Pseudomonas sp.* [12, 34].

Test bacterium	Minimum inhibitory concentration (MIC) value of biosurfactant	
	P. caribbica synthesized xylolipid biosurfactant	*Candida bombicola* synthesized SL derived from oleic acid
Staphylococcus aureus	0.025mg/ml	>0.512mg/ml [34]
Pseudomonas sp.	5mg/ml#	>6.25mg/ml* [12]

Table 5a.3 Comparative MIC values of *P. carbbica* synthesized biosurfactant and typical *C. bombicola* synthesized SL. #- Experiment was performed against *P. aeruginosa* *- Experiment was performed against *P. putida* and MLD_{50} value has been mentioned

It is worth to be noted that the newly synthesized xylolipid has markedly better inhibitory action against Gram positive bacterium. So its incorporation into skin formulations will be advantageous.

Thus to conclude in the present Chapter, production of a novel biosurfactant-xylolipid has been described by employing a new yeast *Pichia caribbica* for the first time. The media and fermentation parameters have been optimized. Thus the maximum yield of 7.5g/l was achieved using Medium B and optimized fermentation conditions (incubation temperature 30°C, pH 6.0, Xylose concentration 10g%, Oleic acid concentration 4%v/v and sodium nitrate as nitrogen source). The physicochemical properties of the xylolipid have been assessed. It reduced the surface tension of distilled water from 70mN/m to 35.9mN/m with the remarkably low CMC value of 1 mg/l. It also reduced the contact angle on surfaces, stainless steel and Teflon. Structural characterization was done using FTIR and HR-MS which revealed that the compound formed is majorly composed of 17-L-[(β-D-xylopyranosyl)-oxy]-Δ9-heptadecanoic acid with m/z =415. Head group diversity was anticipated to give altered physicochemical and biological activities. In accordance to the anticipation low CMC value and better inhibitory action (MIC value 25µg/ml) was demonstrated against *S. aureus*, a gram positive bacterium. The bacteriocidal action against *P. aeruginosa* (MIC value 5.0mg/ml) was marginally better as compared to typical SL.

Owing to the presence of xylose as the head group, the molecule was expected to exhibit different Hydrophilic –Lipophilic Balance value. Change in HLB value suggests different surfactant properties suitable for different applications. In case of m/z 415, the molecule with single xylose unit; greater variation in surfactant property can be expected. The molecule will also behave differently in response to different stimuli. Stimuli-responsive surfactants are a class of compounds which have recently attracted major interest in polymer chemistry and material science with applications in wide range of fields namely- Stabilization of emulsions, suspensions or foams, drug encapsulation and delivery, hard-surface cleaning, personal care applications [35]. Thus the new xylolipid molecule has to be studied in detail so as to explore new possibilities.

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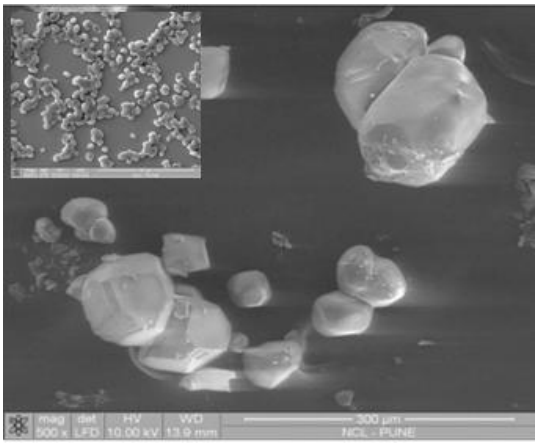
Chapter V(b)

Crystalline xylitol production by a novel yeast- *Pichia caribbica* (HQ222812) and its application for quorum sensing inhibition



Xylitol, a sugar alcohol is fast gaining ground over other artificial sugar substitutes owing to its advantageous properties. Xylitol is a safer alternative for diabetics because of insulin independent metabolism. It has beneficial properties suitable to form an important part of odontological formulations. Conventional commercial production of xylitol involves harsh chemical method operating at high temperature and pressure. Thus microbial production of xylitol is preferred over chemical method. Different yeast species have been extensively exploited for this purpose. In the present chapter quantitative production of xylitol from D-xylose with the yield of 0.852 gm/gm and volumetric productivity of 1.83 gm / l / h in crystalline form, using a novel yeast *Pichia caribbica* is reported. Also, a mild, safe procedure for product extraction is described. The ability of xylitol to act as a quorum sensing antagonist in gram negative marker strain *Chromobacterium violaceum* CV026 has been demonstrated for the first time.

- *Ruchira Mukherji, Kasturi Joshi-Navare, Asmita Prabhune* ***Crystalline xylitol production by a novel yeast- Pichia caribbica (HQ222812) and its application for quorum sensing inhibition in gram negative marker strain Chromobacterium violaceum CV026*** *Applied Biochemistry and Biotechnology 169 (2013)1753-1763*
- *International patent has been filed based on the present work (Provisional no.2190DEL2012).*
- *The culture has been deposited as per Budapest Treaty IDA guidelines (Culture accession number-MTCC-5703)*

As mentioned in Chapter 5a; a yeast- Pichia caribbica has been used for the production of novel biosurfactant. During the synthesis of biosurfactant, 'resting cell method' was tried out. The supernatant; at an intermediate step was observed as a bright yellow coloured clear fluid. The yeast pigment caught our attention. Interestingly it exhibited the 'quorum quenching activity' thus compelling us to further study and characterize it in detail. Later it was found that the quorum quenching activity was not associated with the pigment but another substance present along with. Characterization studies revealed it to be pure xylitol.

5b.1. Introduction

Xylitol is a five carbon sugar alcohol belonging to the polyol group which has been widely used as an ideal sweetener for diabetic patients because of its insulin independent metabolism and low glycemic index. The value of xylitol market reached $340 million with the global consumption 43,000 tons in 2005 [1]. According to the NovaGreen, a xylitol producing company; the current global xylitol market is $400-500 million [2]. The global market demand for polyols is expecting annual 2.7% increase. The advantageous physical and chemical properties of xylitol make it a highly sought after compound for pharmaceutical, odontological and food industries. A variety of interesting applications have been reported for xylitol especially in the field of odontology as an important component of toothpastes, mouthwashes and sugar free chewing gums [3]. Due to the anti-biofilm activity and negative heat of solution associated with it, xylitol forms an important part of odontologial formulations. It has been shown to prevent tooth decay, cause tooth re-hardening and remineralisation [4].There are several pharmaceutical applications of xylitol. It finds use in post-traumatic or post-operative states when efficient glucose utilization is inhibited, due to the induced resistance to insulin by excessively secreted stress hormones. Catabolic disorders can also be corrected due to the anabolic effects produced by xylitol. Xylitol, when used regularly in diet, limits obesity. It is used for parenteral nutrition in infusion therapy as it is inert to amino acids. It is also used in treatments for lipid metabolism disorders [5].

In nature xylitol is abundantly found in fruits and vegetables such as berries, corn husk, oat, lettuce, cauliflower, and mushrooms but its extraction from them is easier said than done owing to the fact that the resultant yield is very low [6]. Xylitol is industrially produced by chemical hydrogenation of pure xylose from the xylan fraction obtained after hemicellulose degradation at high temperature and under high pressure of about 50 atm using raney nickel as catalyst [7]. However the limited product yield of about 50-60% was associated with this process and the expensive downstream processing steps involved in product extraction and purification makes it an unsuitable method of choice [8]. Hence alternative methods involving fermentation by microorganisms and also enzymatic conversion of D-xylose to xylitol operating at ambient conditions are currently being explored. Conversion of lignocellulosic biomass into xylitol fermentatively has gained immense popularity despite it being a time consuming process involving optimization of several variables [9]. Yeasts have been touted to be the best xylitol producers from hemicellulosic or other xylan rich biomass among all the microorganisms investigated. Yeast of genera *Candida, Debaryomyces* and *Pichia* have been reported to give good xylitol yield from their fermentation medium [10]. Microbially, Xylitol is produced in a one step process by reduction of xylose using the enzyme xylose reductase in the presence of NADPH. Enzymatic conversion of xylose to Xylitol using this enzyme xylose reductase has been proposed to be a far superior process in terms of productivity for large scale production of Xylitol [11]. Nevertheless microbial conversion of renewable lignocellulosic biomass or xylose containing synthetic media into xylitol is the most preferred method of choice because the fermentation process can be easily controlled. In this chapter the production of Xylitol with 99.9% purity from a simple D xylose containing medium by a previously unreported organism *Pichia caribbica* with simple cost friendly recovery process has been described. The *Pichia* synthesized xylitol was characterized in detail with different analytical techniques namely XRD, NMR, FTIR, HPLC and SEM in comparison with standard xylitol.

Apart from the odontological activity reported so far, Xylitol is also known to possess antimicrobial activity. It averts ear and upper respiratory infections caused by

Streptococcus pneumoniae, which is responsible for 30% or more of such attacks, suppresses *Haemophilus influenzae*, another important pathogen implicated in these kinds of infections. A notable anti-biofilm effect of Xylitol has been previously recognized against *Streptococcus mutans*, the organism associated with dental decay. The polysaccharide capsule of *S. mutans* plays major role in tooth enamel adherence and subsequent formation of biofilm in the form of dental plaque. The capsule synthesis occurs through the utilization of dietary sugars like sucrose. D-xylitol cannot be utilized by *S. mutans* and thus capsule synthesis and further consequences are avoided [6]. Most reports published till date talk about anti- pathogenic effect of xylitol against gram positive organisms, however none of them mention about xylitol acting as a quorum sensing inhibitor in gram negative systems. In this article anti-AHL (Anti-acyl homoserine lactone) activity of ylitol has been reported using *Chromobacterium violaceum* CV026 as the test organism. This test organism was chosen since it serves as a representative gram negative system capable of responding to many natural and synthetic AHL molecules. Xylitol produced by *Pichia caribbica* reported here could inhibit Quorum sensing by three distinct molecules namely C6-HSL, C8-HSL and 3-oxo-C6-HSL all of which serve as important signalling molecules in virulence gene regulation of various pathogenic gram negative bacteria.

5b.2. Materials and Methods

5b.2.1 Reagents and media

All the media ingredients used were of analytical grade supplied by either Hi-media

pvt. Ltd., India or Merck India ltd. Acyl homoserine lactones were purchased from Cayman chemicals, USA. Standard Xylitol was purchased from Sigma with purity $\geq$99%.

5b.2.2 Culture condition /Maintenance of microbe

The culture *Pichia caribbica* (accession number HQ222812) [11] was maintained on MXYP agar slants (Malt extract- 0.3g%, Xylose- 2g%, Yeast extract-0.3g%, Peptone- 0.5g% and Agar- 2.0g%) slants. The microorganism was sub cultured in every 4 weeks and maintained at 4°C in a refrigerator. *Chromobacterium Violaceum CV026* was grown in Luria Bertani Medium supplemented with

100µg/ml Ampicillin and 30µg/ml Kanamycin. Culture was preserved in the form of glycerol stock and was revived whenever required.

5b.2.3 Inoculum development for Pichia caribbica

10 ml of MXYP broth (0.3g% malt extract, 0.3g% yeast extract, 0.6g% mycological peptone, 2g% D-xylose) was inoculated with *Pichia caribbica*. After 24h incubation at 28°C, 180 rpm it was added to 90 ml MXYP broth and incubation was continued further for 48h.

5b.2.4 Fermentation procedure for conversion of D-xylose to xylitol using resting cells

The cells were harvested from MXYP broth after 48 h by centrifugation at 5000rpm, 20 minutes, 10°C. These cells (wet weight 4.0 g dry weight approximately 1.0g) were then re-dispersed in100ml of 10% xylose solution. And the incubation was continued further till 120h with periodic monitoring of xylitol and residual xylose from fermentation broth using high performance liquid chromatography (HPLC) system of Chromeline-Hitachi. The column used was Waters sugar pak 6.5 X 300 mm. The mobile phase used was milli-Q water with 100 µM EDTA and 200 µM $CaCl_2$. The flow rate was maintained at 0.4 ml per minute and column temperature was maintained at 70°C. The sugar and sugar alcohol were detected with the help of Chromeline L-2490 refractive index detector [1].

5b.2.5 *Product harvesting*

After 72h of incubation the cells were separated by centrifugation at 5000rpm for 20 mins at 10°C and the yellow coloured supernatant was processed further to separate the xylitol. The cells obtained after fermentation were reused for up to 3 times without substantial loss of efficiency.

5b.2.5.1 *Concentration of product:* The Supernatant broth was subjected to rotary vacuum evaporation at 85°C to get a viscous fluid which was further concentrated by centrivac (LABCONCO centrivap concentrator) system. Further crystallization was induced by storing the product overnight in refrigerator at -20ºC.

5b.2.5.2 *Decolourization of product:* Decolourization was done using absolute ethanol. 5ml of absolute ethanol was added to 2g of semi-crystalline yellow coloured material. The material was washed with absolute ethanol which solubilised the pigment. Washing was repeated thrice over the period of 24 hours. After overnight contact; powder was again washed with the solvent. The intense yellow colour got eluted from the crystals into the solvent (ethanol) layer. The solvent was discarded and residual solvent was allowed to evaporate at room temperature to get complete dry white crystalline powder.

5b.2.6 *Characterization of Xylitol*

Complete characterization of the synthesized product was done with the help of XRD, H^1 NMR, FT-IR, HPLC and SEM. Purity of the compound was specifically determined by HPLC and ^1H NMR. Standard xylitol as a control was also subjected to the same procedures for comparison.

5b.2.6.1 X-Ray Diffraction analysis: To further confirm the identity of crystal X-ray diffraction (XRD, Philips X'Pert PRO) was done. For XRD analysis the crystalline sample was crushed in to a very fine powder in a mortar and pestle. It was then filled in a 1cm by 1cm size and 1mm deep square etched on a glass slide. The glass slide was carefully placed in an empty petri dish, taken to the x-ray diffractometer and its spectra were recorded. The diffraction pattern obtained was then matched against the standard JCPDS-PDF (database made available by The International Centre for Diffraction Data, an organization dedicated to collecting, editing, publishing and distributing Powder Diffraction Data) to confirm that the crystals were of xylitol.

5b.2.6.2 ^1H NMR: Two milligrams of samples namely *Pichia* xylitol and Sigma xylitol were dissolved in 0.5 ml of deuterated Methanol. 1H NMR (200 MHz) spectra was recorded by Bruker AC200 at 25ºC. Chemical shift was expressed in ppm. Tetramethylsilane was used as an internal standard.

5b.2.6.3 FTIR analysis: After separating the white crystalline compound from the spent broth it was subjected to preliminary identification by FTIR. The crystals were crushed with KBr, pelleted and the Fourier transform infra-red (FTIR) spectra

were recorded on a Perkin-Elmer Spectrum One in the frequency range of 4000 to 500cm^{-1}. Additionally FTIR spectra of pure xylitol from sigma and pure xylose were also recorded using similar procedure as described earlier.

5b.2.6.4 HPLC analysis: The HPLC method as described earlier in section 5b.2.4 was followed. The xylitol separated from fermentation broth and commercial xylitol were subjected to HPLC analysis to know about the purity of produced xylitol.

5b.2.6.5 Scanning electron microscopy (SEM): (FEI Quanta 200 3D) was used to visualize the crystal morphology. The powdered crystalline sample was sprinkled onto carbon tape. Excess particles were blown off. Sample was sputter coated till a fine layer of 10nm was formed. (Sputter coater; make- EMITECH, source- Au-Pd, Gas-Argon). The samples were then visualised.

5b.2.7 Violacein inhibition assay

5b.2.7.1 Violacein inhibition assay (qualitative) using Pichia synthesized Xylitol

This assay has been designed in such a way that a zone of inhibition of Violacein synthesis can be seen around the agar well containing the probable QS Inhibitor. *Chromobacterium violaceum* mutant CV026 is used as the test organism which has the ability to respond to a variety of signal molecules [13]. The QS signal molecule (namely C6-HSL, C8-HSL or 3-oxo-C6-HSL) at a concentration of 12.5 µM was added to 10 ml of Luria bertani soft agar containing 100µl of overnight culture of CV026. The soft agar was then overlaid onto basal LA plate, and a 4mm diameter well is dug in the centre of the plate using a sterile cork borer after the overlay was set. To the agar well 75 µl of appropriate concentration of the compound under investigation was added and the plates were incubated in upright position at 30^{o}C for 24 h. The diameter of zone of Violacein inhibition was then measured. Standard xylitol was used for comparative analysis.

5b.2.7.2 Violacein inhibition assay (quantitative):

This assay is based on a similar principle to the one described above, i.e. the presence of any quorum sensing inhibitor will quantitatively decrease the production of the purple pigment violacein which can then be estimated

colourimetrically [14]. In this tube assay 100µl of overnight grown culture of CV026 was inoculated in 10ml LB broth containing the QS signal molecule (namely C6-HSL) at appropriate concentrations. The test compound, *Pichia caribbica* synthesized xylitol in this case, was then added at increasing concentrations in a series of test tubes. The tubes were incubated at 30°C for 18h. The violacein produced was extracted from the culture broth by dissolution of the pigment in DMSO and separating it from the cell mass by centrifugation. The amount of xylitol required to inhibit or substantially decrease the purple pigment production could be quantitatively estimated by measuring the optical density at 570nm. Appropriate positive and negative controls were used. The tube without addition of xylitol served as positive control while the tube without C6-HSL or xylitol served as negative control. All experiments were done in triplicates for confirming reproducibility.

5b.3. Results and Discussion

5b.3.1 Product Harvesting, Concentration, Decolourization and yield calculation

The *Pichia caribbica* xylitol production was monitored at periodic intervals with HPLC. The trend observed is depicted in Figure 5b.1.

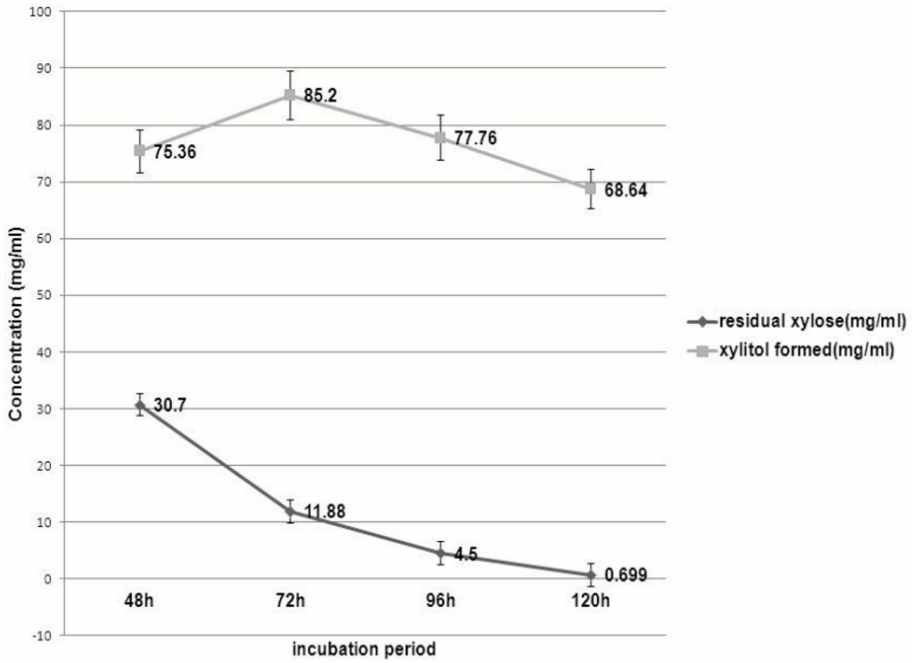


Figure 5b.1 HPLC monitoring of fermentation reaction

Maximum xylitol content was observed after 72h of incubation then there was a decrease which can be attributed to its utilization for cell maintenance purpose.

The optimum period of incubation to get maximum xylitol yield was found to be 72h. The harvested product was concentrated as per the procedure mentioned in previous section 5b.2.5.1. It appeared as bright yellow coloured crystalline substance.

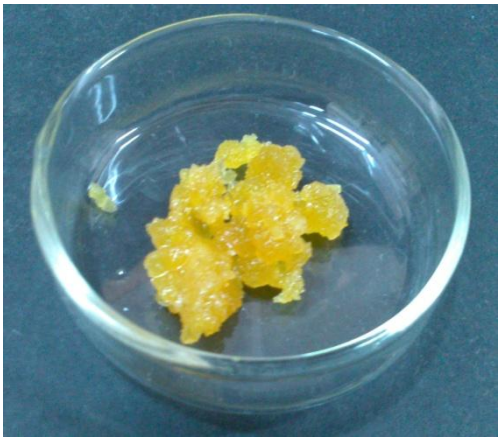


Figure 5b.2 Appearance of crude xylitol after concentration procedure

Further it was decolorized by the mentioned procedure in section 5b.2.5.2 leading to a product safe for food and pharmaceutical applications. After the decolorization procedure, white, dry crystalline powder of xylitol was obtained.

Figure 5b.3 Xylitol obtained after decolorization treatment

The maximum xylitol produced by free cells was 85.2g per 100 g/L of xylose at ambient pH and temperature with the yield of 0.852 gm/gm and volumetric productivity of 1.83 gm / l / h. The yield value reported in the present study; 85.2g/100g corresponded to 93.61% of theoretical yield value [15].

Microbial sources have been exploited extensively for xylitol production; among them yeasts have been the most promising. Yeasts are especially favoured

because they are very robust organisms and can easily grow on both chemically defined media as well as inexpensive renewable resources like hemicellulose. In the present work, xylitol synthesis has been achieved through fermentative conversion of D-xylose. The popular *Candida* species employed for D-xylose to xylitol conversion are *C. Tropicalis C. Gullerimondi, C. Parapsilosis* [10], *C. Peltata* [16]. Use of chemically defined medium could improve the yield to 0.87g/g in case of *Candida tropicalis* [17]. Xylitol yield was associated with longer incubation period i.e. 120 h which effectively reduced the volumetric productivity value. *Debaryomyces hansenii* has also been another organism of choice for xylitol production from D- xylose with the yield values falling in the range 0.76- 0.81 g/g. However this was achieved at 35-37ºC of incubation [18, 19, 1]. *Pichia stipitis* has been reported for xylitol production [10]. In this chapter, a new species of *Pichia* namely *P. caribbica* has been reported for the first time which is capable of quantitative conversion of pure xylose into xylitol fermentatively.

The theoretical yield value of 93.61% in present study is specifically remarkable as the xylitol yield values reported so far from D-xylose are in a range of 65–85% of the theoretical value [8]. Other contemporary reports on high xylitol yield involve extensive optimization of fermentation and genetic manipulation. On the other hand, in present report the high yield has been achieved without any genetic engineering and optimization of pH, temperature conditions [20, 21]. Reported here is a novel technique of separation of xylitol from its fermentation broth in almost pure crystalline form which reduces the cost of recovery associated with the downstream processing. The process involved simple rota-vaporization and speed vacuum concentration of the spent broth to achieve almost quantitative yield of synthesized xylitol, followed by low temperature storage for 48 h to induce crystal growth. The crystals in this case were associated with yellow pigment which was completely removed by treatment with only pure ethanol making it palatable and safer product. Use of low cost hemicellulosic raw material is preferred over pure D-Xylose for Xylitol production on basis of process economics. However the cost and time associated with present production, extraction and decolourization method could compensate for the higher cost of D-xylose as starting material. The decolourization procedures for xylitol obtained using hydrolysed hemicellulosic intermediates involve the use of activated charcoal or other adsorbents [22]. In

previous report by Sampaio *et. al.* 2006, it has been mentioned that the use of adsorbents generally causes around 20% loss of xylitol and make the crystallization difficult and time consuming [23] thus complicating the downstream processing and eventual product purification [24].

5b.3.2 *Characterization of Xylitol*

XRD spectrum of *Pichia* synthesized crystalline xylitol was recorded and the data was analyzed. On comparing the experimental spectra against the standard xylitol in the database, it was evident from Figure 5b.4 that the white crystalline compound was indeed xylitol as more than 98% of spectral peaks were overlapping with each other (JCPDS PDF no.34-1802).

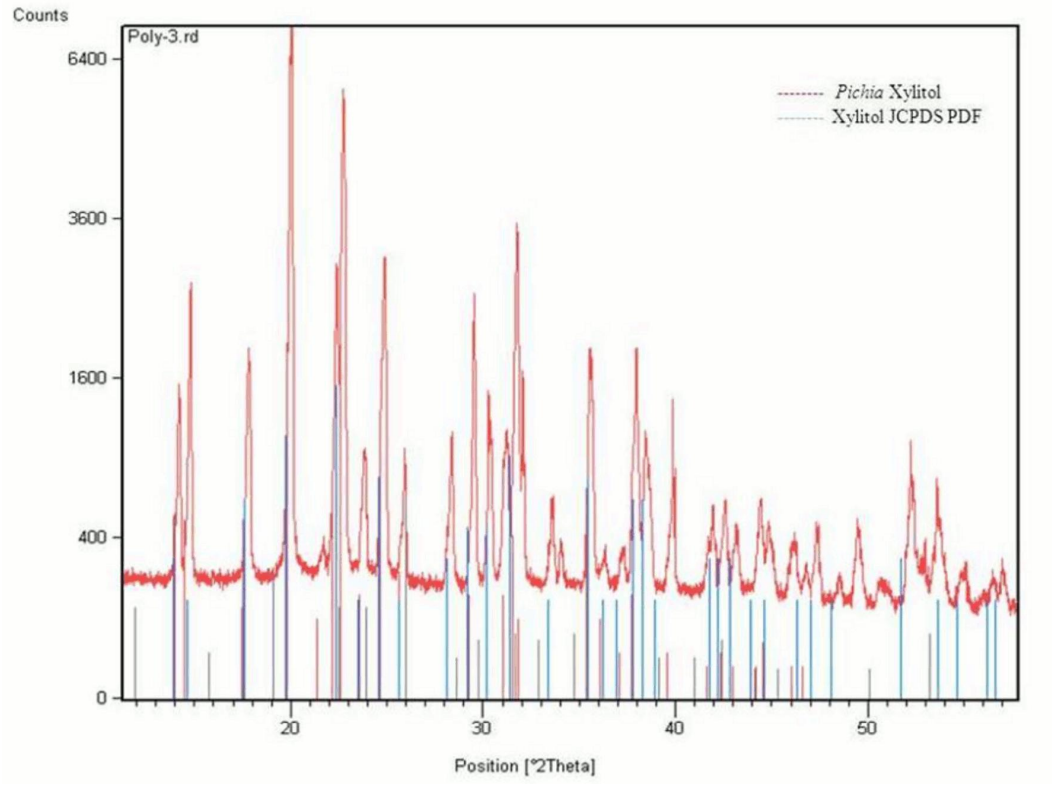


Figure 5b.4 Powder XRD spectra of Pichia Xylitol Compared with spectra of Standard Xylitol from JCPDS-PDF database

^{1}H NMR spectra recorded using Tetramethylsilane as an internal standard and deuterated methanol as solvent is depicted below in Figure 5b.5. Both Sigma xylitol and *Pichia* synthesized xylitol spectra showed similar peaks hence suggested that both compounds were essentially the same.

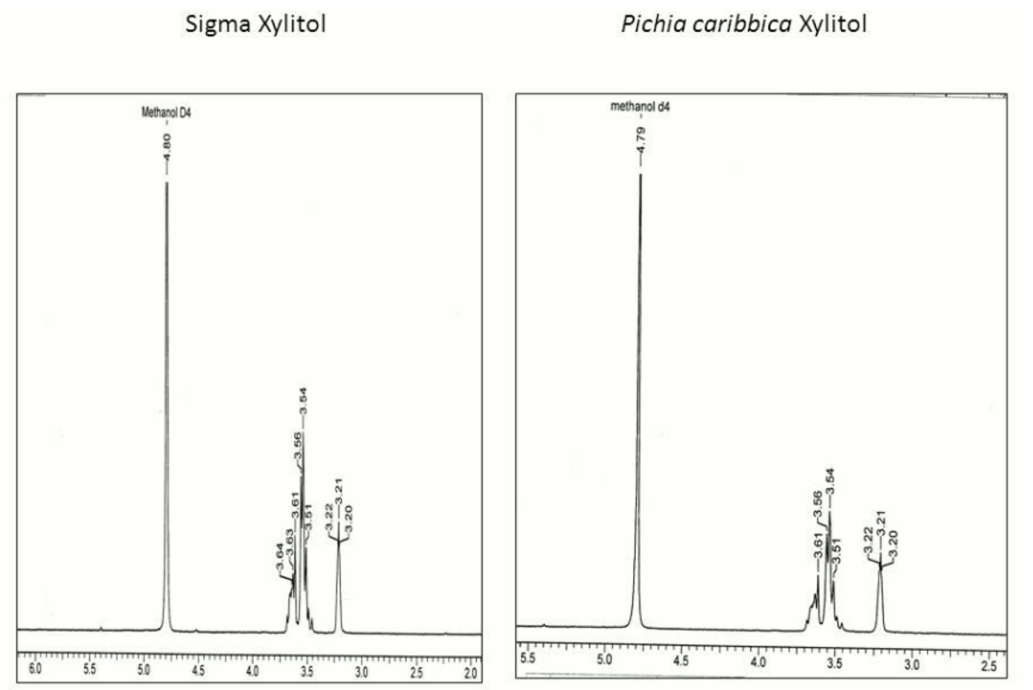


Figure 5b.5 ^{1} HNMR Spectra of both Standard Sigma Xylitol, and *Pichia caribbica* synthesized xylitol

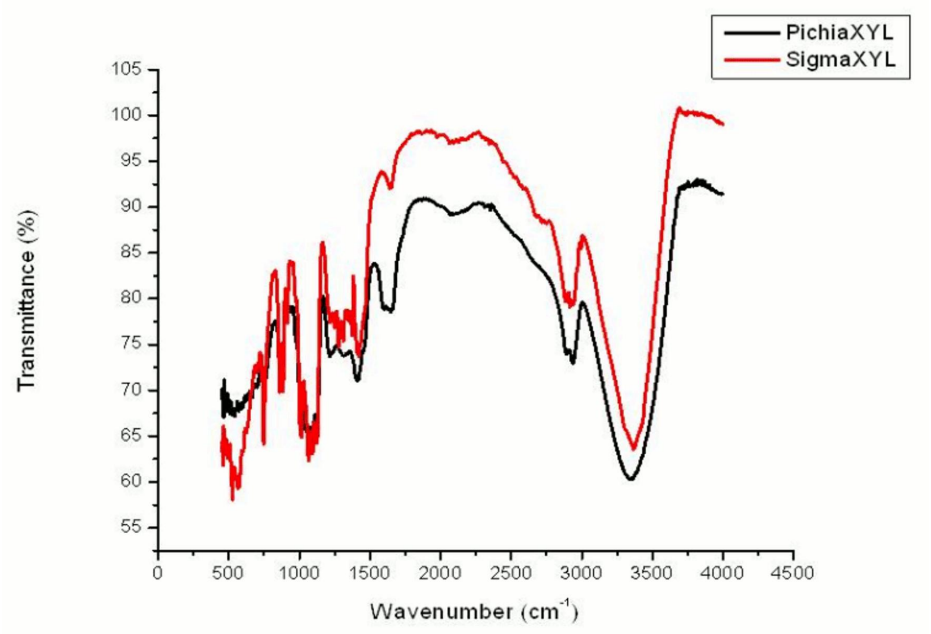


Figure 5b.6 FT-IR Spectra in Transmission mode was recorded for both Sigma xylitol and *Pichia caribbica* synthesized xylitol.

The Fourier transform-IR spectra of *Pichia* xylitol and pure Sigma xylitol were recorded as shown in Figure 5b.6. Upon analyzing the FT-IR data it was observed that the IR spectra represented a broad stretching around 3200–3400 cm^{-1} which is characteristic of hydroxyl group present in this sugar alcohol and a weak C–H stretching band at around 2932 cm^{-1} for both experimental and sigma xylitol.

The absorption band from 1300 cm^{-1} to 800 cm^{-1}, is called “finger print” region, and is related to conformation and surface structure of molecule. These bands have always been very hard to explain however in the spectra it can be seen that both *Pichia* and Sigma xylitol showed essentially similar peaks in the fingerprint region also. There was a strong characteristic peak around 1410 cm^{-1} and 2931 cm^{-1} which is typical of methylene groups present in the molecule. Thus after all physical and chemical analysis the compound was confirmed to be xylitol.

HPLC analysis revealed that *Pichia* synthesized xylitol showed similar retention time as that of standard Sigma xylitol under identical conditions. After analyzing the spent culture supernatant for residual xylose content it was observed that at 72 h, 90% of the xylose got utilized i.e. it was fermentatively converted into xylitol. This was indicative of quantitative xylitol production by this organism.

The crystal morphology seen under the scanning electron microscope (Refer to Figure 5b.7) was indicative of the purity of the compound and similar to that reported previously [25]. The shape of the crystals appeared hexagonal to round. The crystalline white compound obtained from the spent broth was determined to be xylitol using various modern characterization techniques. The purity of the *Pichia* synthesized xylitol was determined qualitatively.

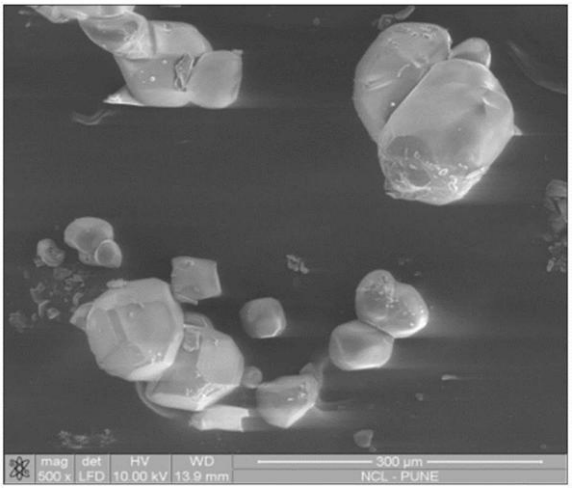


Figure 5b.7 Scanning electron micrograph of *Pichia caribbica* synthesized Xylitol revealed typical crystal morphology. (magnification 500X)

5b.3.3 Violacein inhibition assay (Qualitative and Quantitative)

In qualitative assay represented by Figure 5b.8, it was observed that xylitol could cause receptor antagonism using three different signal molecules and hence

inhibition of quorum sensing associated phenotype in *Chromobacterium violaceum* CV026. From the Figure 5b.8 we can see that *Pichia* synthesized xylitol could quantitatively inhibit Violacein production induced by the signal molecule C6-HSL in CV026. It was seen that increasing the concentration of xylitol was not inhibitory to the organism but it severely impaired quorum sensing signal reception. Similar results were obtained with standard xylitol.

Figure 5b.8 Qualitative Violacein synthesis inhibition by agar well diffusion method shows ability of *Pichia* xylitol to antagonize C6-HSL, quorum sensing signal reception in *Chromobacterium violaceum CV026*

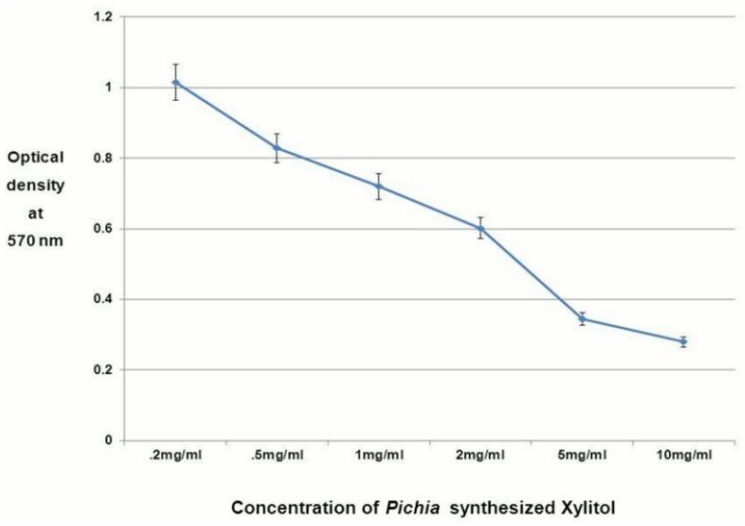


Figure 5b.9 Quantitative inihbition of violacein synthesis in the presence of increasing concentration of *Pichia* synthesized xylitol.

Figure 5b.9 showed that *Pichia* synthesized xylitol inhibited the violacein synthesis in quantitative manner too.

Due to a plethora of interesting characteristics xylitol has become a pharmaceutically important molecule. Xylitol has been shown to be an inhibitor of biofilm formation in gram positive dental pathogen *Streptococcus mutans* making

it an indispensible addition to dental hygiene product. It has also been reported to avert middle ear infection caused by *Streptococcus pneumoniae* and *Haemophilus influenzae* both of which are important gram positive pathogenic bacteria. But ability of xylitol to stop quorum sensing in gram negative organisms has not been explored before. The present work investigated the ability of *Pichia* synthesized xylitol to inhibit gram negative quorum sensing signal (acyl homoserine lactone) reception. Acyl homoserine lactones are the largest class QS signals which play a crucial role in gram negative cell to cell signalling and virulence gene expression. *Pichia* synthesized xylitol was able to antagonize not one but three different AHL signal and receptor interaction as determined using CV026 based AHL antagonism assay system.

Violacein synthesis in *Chromobacterium violaceum* is a quorum sensing associated phenotype, the signal molecule in this case being C6-HSL. Inhibition of quorum sensing or quorum sensing signal antagonism will not result in any Violacein synthesis which is a powerful indicator as it helps to screen for QSIs with efficiency. In this assay CV026 a violacein-negative, mini-Tn5 mutant of *C. violaceum* has been used which does not produce the purple pigment unless provided with an external signal molecule and is sensitive of all AHLs ranging C4-C8 in chain length. This is interesting since all of these molecules C6-HSL,C8-HSL and 3-oxo-C6-HSL are important signalling components for virulence genes activation in major gram negative pathogens. This finding is very crucial since broadens the scope of application of xylitol in various bio therapeutics.

Thus to conclude- in this Chapter, we have investigated the ability of newly isolated strain *Pichia caribbica* (MTCC 5703) to synthesize xylitol from D-xylose and it was found to give high yield of 0.852g/g of xylitol per gram of xylose using resting cell method. Also the synthesized xylitol could be extracted using a fast, simple procedure that is cost effective and acceptable for human consumption. The extracted xylitol was more than 98% pure and had the typical crystal morphology. Further it was seen that xylitol had the ability to inhibit quorum sensing mediated phenotype of violacein pigment production in gram negative marker strain *Chromobacterium violaceum* CV026.

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Conclusions

This chapter summarizes the work done. The highlights of the research work have also been noted along with their impact on the field. Also the scope for future potential developments and the prospective path of further investigation have been discussed.

The main aim of the thesis was to synthesize different glycolipidic compounds belonging to the structural class of SLs. Different media and precursors have been fed to the SL synthesizing yeasts to impart structural variation. Structural variation and its correlation with the properties of the molecule is in a way a less explored area. We have tried to contribute to the knowledge in this particular aspect. Further various physicochemical and biological applications of synthesized SLs have been studied.

The work mentioned in Chapter 2 comprised of SL synthesis using the non-edible oils namely, Jatropha and Pongamia as cost friendly precursors. Optimization of media, fermentation parameters have been reported along with resting cell method for maximizing the SL yield. This way SL yields could be improved. Such crude preparation can be considered as potential candidate for large scale commodity usage. In accordance with this fact, properties and performance of the SLs were assessed as a component of laundry detergent. It was found that in comparison to synthetic surfactants, SLs were effective at low dose, they retain functionality over a range of temperatures, pH and in hard water. Their antimicrobial property is advantageous as a part of detergent formulation. The washing performances of SLs and SLs in combination with commercial detergent were assessed against different kinds of stains, which were found satisfactory.

As a continuation of the work, production economics has to be worked out using the crushed seeds of Jatropha and Pongamia as a source of secondary carbon precursor. Scale-up experiments and use of fermenter will also be important in improving the process economics. The washing performances by SLs can be improved through the optimization of soaking and washing time durations.

Chapter 3 and 4 were concerning the biological activity of SLs derived from pure fatty acids which are typical precursors for SL synthesis- Oleic (18:1) and Linoleic acid (18:2).

To address the issue of global concern i.e. antibiotic resistance development in bacteria, a novel approach was tested as explained in Chapter 3a. The antibiotics were administered along with SLs against the pathogenic bacteria maintaining appropriate controls. As a result of this co-administration, bacterial inhibition was achieved faster and at lower concentration of antibiotic. This was attributed to the

amphiphilic nature of SLs which is responsible for facilitating the entry of antibiotic particles across the bacterial cell membrane lipid bilayer. The data was supplemented with scanning electron microscopy and damaged cell membrane integrity was confirmed. Hence the bacterial infections can be controlled in a better way by co-administration of SLs and antibiotics. This will possibly suppress the emergence of resistant forms as the pathogens will not be able to survive the simultaneous attack, develop the resistance and spread the resistance.

SLs are US FDA approved food and pharmaceutical additives. Cytotoxicity and skin irritation studies have established safety of SLs. Oral safety level has been determined as greater than or equal to 5ml/kg weight. All these facts support the usage of SLs with antibiotics. So far in vitro studies have been done which need to be supplemented with actual in vivo data. In order to achieve so, animal experiments have to be performed. Also the experiments pertaining to different classes of antibiotics against different pathogens have to be undertaken.

The sub-section of Chapter 3 i.e. 3b described the antiviral activity of SLs against different classes of viruses- Cox-sackie (+ssRNA), Influenza (segmented -ssRNA) and Murid Herpes virus (dsDNA). The study was done in collaboration with- Dr. S. Bopegamage, Head, Enterovirus Laboratory, Virology Dept., Slovak medical university, Bratislava,Slovak republic. The mode of antiviral action was also investigated to check if it occurs through direct contact, adsorption inhibition or cytokine induction. Out of the three, maximum efficiency of virus destruction was observed with direct contact. Action of SLs on viral/ host cell receptors involved in the attachment and sorption of viruses needs to be evaluated.

The ability of SLs derived from Oleic and Linoleic acid to induce terminal cell differentiation was checked in the Chapter 4. Being structurally similar to the eukaryotic cell membrane components- Gangliosides and Glycosphingolipids, SLs were hypothesized to have some effect on cell fate determination, oncogenic transformation and trans membrane signalling. LN229 cell line, derived from grade IV glioblastoma was chosen as the target. On exposure to SLs, these cells showed distinctive morphological features suggestive of differentiation. This potential can be exploited for the treatment of the glioblastomas which are invasive tumors of central nervous system and are known to be resistant to

conventional chemotherapy. Induction of terminal differentiation in tumor cells and thus arresting tumor growth can serve as a promising treatment approach.

Chapter 5a illustrated the use of novel yeast species- *Pichia caribbica* MTCC5703 for production of SL like molecule. The particular strain had been used for fermentation of xylose to ethanol. Therefore in the present case, in place of glucose; xylose was fed as the hydrophilic precursor for SL production, considering the possession of different enzyme array by this yeast; variation in SL structure was expected. Accordingly, the product formed was characterised through FTIR and HR/MS analysis and the molecular mass correlated with structure of 17-L-[(β-D-xylopyranosyl)-oxy]-Δ9-heptadecanoic acid. This altered molecule with single xylose unit as a head group was shown to exhibit enhanced physicochemical and biological activity as compared to typical *C. bombicola* synthesized SL derived from oleic acid. It reduced the surface tension of water at CMC value 1mg/ml and resulted in complete bacterial inhibition at 25µg/ml against *Staphylococcus aureus* while in case of *P. aeruginosa*, at 5.0mg/ml.

This altered glycolipid structure with single xylose as head group conferred different HLB i.e. Hydrophic-Lipophilic balance value. Therefore the structure can result in formation of different kind of self assemblies in response to the stimuli. These characteristics are potentially attractive for the application regarding stabilization of emulsions, suspensions or foams, drug encapsulation and delivery, hard-surface cleaning, personal care applications.

In Chapter 5b of the thesis, it was demonstrated that the yeast isolate *P. caribbica* can quantitatively synthesize xylitol from d-xylose. The present yield 0.85g/g matched with 93% of theoretical yield value. The product obtained was in crystalline form and analytical techniques revealed the 99% purity. A simple, safe method has been developed for its decolorization. Moreover a novel application of xylitol to act as quorum sensing inhibitor in a gram negative model organism has been demonstrated. Xylitol inhibited the 3 types of Acyl Homoserine Lactones which are the typical signalling molecules of major gram negative pathogens. Therefore the present work suggested the bio therapeutic utility of xylitol.

Publications:

- ***Kasturi Joshi-Navare**, Anjali Shiras, and Asmita Prabhune, Differentiation-inducing ability of sophorolipids of oleic and linoleic acids using a glioma cell line. Biotechnol. J. 2011, 6, 509–512*

- *Ruchira Mukherji, **Kasturi Joshi-Navare**, Asmita Prabhune, Crystalline xylitol production by a novel yeast- Pichia caribbica (HQ222812) and its application for quorum sensing inhibition in gram negative marker strain Chromobacterium violaceum CV026 Applied Biochemistry and Biotechnology 169 (2013)1753-1763*

- *Pradeep Kumar Singh, Ruchira Mukherji, **Kasturi Joshi-Navare**, Abhik banerjee, Rohan Gokhale, Satyawan Nagane, Asmita Prabhune, Satishchandra Ogale, Fluorescent sophorolipid molecular assembly and its magnetic nanoparticle loading: a pulsed laser process Green chemistry 15 (2013)943-953*

- ***Kasturi Joshi-Navare**, Asmita Prabhune, Sophorolipid biosurfactants act in synergy with antibiotics to enhance their entry (communicated)*

- ***Kasturi Joshi-Navare**, Poonam Khanvilkar, Asmita Prabhune, Jatropha and Pongamia oil derived SLs: Characterization and application in laundry detergents (ready to be communicated)*

- ***Kasturi Joshi-Navare**, Pradeep Kumar Singh, Asmita Prabhune, Production of sophorolipid-like biosurfactant with xylose as a head group by a new yeast isolate- Pichia caribbica (HQ222812) and demonstration of its enhanced physicochemical and biological activity (ready to be communicated)*

- *Debanjan Guin, Pooja Singh, **Kasturi Joshi-Navare**, Asmita Prabhune, Chemically conjugated sophorolipids on CdTe QDs: A biocompatible photoluminescent nanocomposite for theranostic applications (communicated)*

Patents

- *Ruchira Mukherji, **Kasturi Joshi-Navare**, Asmita Prabhune, Process for production of Crytalline xylitol using Pichia caribbica (HQ222812) and its application for quorum sensing inhibition in model gram negative bacterium*
  International patent has been filed based on the the work.
  Provisional no.2190DEL2012, Filing date: 16.7.2012

- ***Kasturi Joshi-Navare**, Asmita Prabhune, sphorolipid biosurfactant acts in synergy with antibiotics to enhance their entry*
  Patent filing is in process

Poster presentations

- ***Kasturi Joshi-Navare**, Anjali Shiras, Asmita Prabhune, Exploration of differentiation inducing ability of sophorolipids of oleic, linoleic acid using a cancerous cell line of neural origin at 'International conference on cellular and molecular bioengineering, Nanyang technological university, Singapore, 2nd-4th August, 2010*
  - *Recieved **best poster award** for the same during Science day poster presentation session, National chemical Laboratory*

- ***Kasturi Joshi-Navare**, Poonam Khanwilkar, Asmita Prabhune, Jatropha and Pongamia oil derived sophorolipids: Characterization and application in laundry detergents at '104th AOCS Annual meeting and expo', Montreal, Canada, 28th April-1st May, 2013*
  - *Abstract accepted*