BIOTRANSFORMATION:

IN SYNTHESIS OF BIOACTIVE MOLECULES,

19-HETE AND 20-HETE,

FROM ARACHIDONIC ACID

A THESIS

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BY

SACHIN N. SHAH

DIVISION OF BIOCHEMICAL SCIENCES

NATIONAL CHEMICAL LABORATORY

PUNE -411 008 (INDIA)

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……Dedicated to my beloved parents

and my mentor

DECLARATION BY RESEARCH GUIDE

Certified that the work incorporated in the thesis entitled:

"**Biotransformation: In Synthesis of Bioactive molecules, 19-HETE and 20-HETE, from Arachidonic acid**", submitted by Mr. Sachin N. Shah, 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 411 008, India. Material that has been obtained from other sources is duly acknowledged in the thesis.

Dr. Asmita Prabhune
(Research Supervisor)

DECLARATION BY RESEARCH SCHOLAR

I hereby declare that the thesis entitled "**Biotransformation: In Synthesis of Bioactive molecules, 19-HETE and 20-HETE, from Arachidonic acid**", submitted 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 411 008, India, under the supervision of Dr. Asmita Prabhune. The work is original and has not been submitted in part or full by me for any other degree or diploma to any other University.

Sachin Shah
(Research Scholar)

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ABSTRACT

The major objectives of the thesis were to explore the biotransformation of arachidonic acid for production of 19-HETE and 20-HETE using *Candida bombicola*. A brief summary of the thesis is given below.

Chapter 1. Introduction

Chapter one presents a general introduction about eicosanoids and biotransformation of arachidonic acid metabolites. 20-hydroeicosatetraenoic acid (20-HETE) and 19-hydroxyeicosatetraenoic acid (19-HETE) are omega and omega–1 hydroxylated products of arachidonic acid which are important in autoregulation of blood pressure, vascular tone and other physiological roles. Different methods were discussed for the production of 20-HETE and 19-HETE. The intermediate form, sophorolipid was discussed in brief. Based on these reviews, the scope and objective of the present work have been outlined.

Chapter 2. Fermentation parameters and Characterization techniques

Chapter two describes the fermentation parameters of *Candida bombicola* (ATCC 22214) for the production of arachidonic acid derived sophorolipids. The derived sophorolipids on acid hydrolysis liberated 19-HETE and 20-HETE. The different experimental techniques and analytical tools were used during the course of the present work are discussed in detail.

Chapter 3. Dry column chromatography technique used for the purification of different forms of Sophorolipids produced by *Candida bombicola*

Chapter three deals with purification of the mono-, di- acetate forms of lactonic and acidic sophorolipids produced by *Candida bombicola* grown on glucose and long chain fatty acid i.e. arachidonic acid. The derived sophorolipids were isolated by silicagel

chromatography using dialysis tubing. Different analytical tools were used to characterize the purified sophorolipids and their derived products. This chapter also describes the detailed study of purified sophorolipids structure and presence of 19-hydroxyeicosatetraenoic acid and 20- hydroxyeicosatetraenoic acid in the sophorolipid form.

Chapter 4. Immobilization of whole cells on biocompatible materials: use as enzymes source for the biotransformation of arachidonic acid to 19-HETE and 20-HETE

Chapter four focuses on the continuous transformation of 19-HETE and 20-HETE from arachidonic acid by immobilized *Candida bombicola* on different biocompatible materials. The patterned thermally evaporated octadecylamine (ODA) lipid films and free-standing organic-gold nanoparticles embedded in a polymeric membrane provide a biocompatible surface for the immobilization of whole cells. The presence of gold nanoparticles in the membrane enables facile modification of the surface properties of the membrane and has been used as enzyme sources for the transformation of arachidonic acid to 19-HETE and 20-HETE. The attachment of the cells to the ODA film surface occurs possibly through nonspecific interactions such as hydrophobic interactions between the cell walls and the ODA molecules. The enzyme cytochrome P450 present in the immobilized yeast cell membrane was used to catalyze the biotransformation of the arachidonic acid to sophorolipids and thereafter, acid hydrolysis to yield 19-hydroxyecosatetraenoic acid (19-HETE) and 20-hydroxyecosatetraenoic acid (20-HETE). These biocomposite materials could be easily separated from the reaction mixture and exhibit excellent reusability.

Chapter 5. Summary and Conclusions

Chapter five presents the summary of the results obtained and the conclusions drawn are presented in this chapter.

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Abbreviations

μ-Cp	Microcontact printing
μL	Micro liter
17-ODYA	17-Octadecynoic acid
19-HETE	19-hydroxyeicosatetraenoic acid
20-HETE	20-hydroxyeicosatetraenoic acid
AA	Arachidonic acid
AAS	Atomic absorption spectroscopy
amu	Arbitrary mass units
ATCC	American Type Culture Collection
BSTFA	bis (trimethyl sylyl)-trifluroacetamide
DAEE	bis (2-(4-aminophenoxy)ethyl)ether
Di HETE	Dihydroxyeicosatetraenoic acid
EDAX	Energy dispersive analysis of X-ray
EET	Epoxyeicosatetraenoic acid
FID	Flame ionized detector
FTIR	Fourier transforms infrared spectroscopy
GC	Gas chromatography
h	Hour
HETE	Hydroxyeicosatetraenoic acid
HPETE	Hydroperoxyeicosatetraenoic acid
HPLC	High performance liquid chromatography
HySL	Hydrolyzed sophorolipids

KI	Potassium iodide
kV	Kilovolt
LC-ESI-MS	Electrospray soft ionization Liquid chromatography mass spectroscopy
LCMS	Liquid chromatography mass spectroscopy
m/z	mass to charged ratio
mA	milli ampere
MGYP	Malt extract- glucose- yeast extract -peptone media
min	Minute
MS	Mass spectroscopy
GCMS	Gas chromatography mass spectroscopy
MYP	Malt extract-yeast extract-peptone
NADPH	Nicotinamide adenine dinucleotidephosphate (reduced)
ng	Nanograms
ODA	Octadecylamine (CH_3-$(CH_2)_{17}$-NH_2)
PGI2	Prostacyclin
PGs	Prostaglandins
PUFA	Polyunsaturated fatty acid
QCM	Quartz crystal microgarvimetry
SAMs	Self assembled monolayers
SEM	Scanning electron microscopy
SL	Sophorolipids
TEM	Transmission electron microscopy

TLC	Thin layer chromatography
TXA2	Thromboxane A2
UV-Vis	Ultra violate-visible
XRD	X-ray diffraction

Chapter 1

Introduction

This chapter presents a general introduction about eicosanoids and biotransformation of arachidonic acid metabolites. 20-hydroxyeicosatetraenoic acid (20-HETE) and 19-hydroxyeicosatetraenoic acid (19-HETE) are omega and omega-1 hydroxylated products of arachidonic acid.

1.1. Introduction

Biotransformations are organic reactions utilizing biological catalysts. These biocatalysts can be either whole cells or enzymes. They are used under many different conditions like free enzymes, whole cells, immobilized enzyme / whole cells, aqueous and two phase systems. Biocatalysts can be used for regio- and stereoselective reactions, or to introduce chirality in ways that would be very difficult or impossible for classical synthetic processes. Biotransformations were observed by humans well before they were appreciated as having an underlying microbial cause. For example, food rot and the products of microbial fermentations have been enjoyed for thousands of years, respectively. In 1858, Louis Pasteur provided evidence for the role of specific microorganisms conducting favorable and unfavorable fermentations of grape juice[1]. The biotransformation of ethanol to acetic acid (vinegar) by *Acetobacter* was most likely developed concomitantly with ethanol production from fermentable sugars by our ancestors. Ethanol to vinegar was probably also the first true biotransformation process applied in an industrial manner. The properties of enzymes, the principle biocatalysts, became generally appreciated from kinetic studies conducted in the early 1900s[2]. An important industrial-scale fermentation to produce acetone to meet the war-time needs of Great Britain was developed in 1916[3]. **Table 1.1.** gives some randomly chosen milestones in the history of applied biotransformations. Microbial transformation offers many advantages over the conventional chemical methods. In general, yeast- mediated transformations in particular, have been extensively used since the early days of mankind for the production of bread, dairy products and alcoholic beverages.

Table 1.1.

Some selected milestones of industrially relevant biotransformation and biocatalytic processes[4].

Year	Process
5000 BC	Vinegar production
800 BC	Casein hydrolysis with chymosin for cheese production
1670	"Orlean" process for the industrial bio-oxidation of ethanol to acetic acid
1680	Antoni van Leeuwenhoek first to see microorganisms with his microscope.
1897	E. Buchner discovers yeast enzymes converting sugar into alcohol
1934	Regioselective biooxidation of sorbite to sorbose for the Reichstein Vitamin C synthesis
1940	Sucrose inversion using an invertase
1950	Bioconversion of steroids
1970	Hydrolysis of penicillin to 6-aminopenicillanic acid
1973	First successful genetic engineering experiments
1974	Glucose to fructose isomerisation with immobilized glucose isomerase
1985	Enzymatic process for the production of acrylamide
1990	Hydrolysis by protease (trypsin) of porcine insuline to human insuline
1995	3000 ton pa plant for the biotransformation of nicotinonitrile to nicotinamide

These are all early applications used in mixed cultures of microorganism and all of these biotechnology operations have primarily been directed in the area of agriculture and human nutrition. In 1862 Pasteur[5] laid a scientific foundation of one of these early applications, namely the oxidation of alcohol to acetic acid by using a pure culture of *Bacterium xylinum.*[6] Oxidation of glucose to gluconic acid by *Acetobactor aceti* and sorbitol to sorbose by *Acetobactor* sp. have been studied by Boutroux, L. in 1880 and Bertrand, G. in 1896.[7] The reducing action of fermenting yeast *Saccharomyces cerevisiae* was first observed by Dumas in 1874.[8] He showed that on addition of finely powdered sulfur to a suspension of fresh yeast in a sugar solution, hydrogen sulfide was liberated. The anaerobic condition of fermentation, the reduction of furfural to furfuryl alcohol by living yeast, was the first "phytochemical reduction".[9] Several other enzymatic or microbial biotransformations, bioconversions, biodegradations and fermentations followed, that invariably accompanies the birth of a new field.[10] Biotransformations were hailed as a solution that would ultimately displace traditional organic chemistry.[11] Biochemical methods represent a powerful synthetic tool to complement other methodology in modern synthetic organic chemistry. Biotransformations have number of advantages when compared to the corresponding chemical methods. Economically some biotransformations can be cheaper, more direct than their chemical analogues and the conversion normally proceeds under conditions that are regarded as environmentally acceptable.[12] In biotransformation, the enzymes or whole cells provide a remarkable enhancement in reaction rates as well as specificity to exhibit high stereoselectivity over the corresponding reactions.[13] Microbial transformation offers the advantages of highly selective operation at nonextreme pH, near room temperature and reduced levels of toxic

waste products.[14] Biotransformations with recombinant microbial enzymes have been widely used, including applications for the production of hormones, antibiotics, and speciality chemicals.[15] Enzymes or whole cells represent the most efficient catalytic systems known for conventional chemical reactions. Over the past few years, biotransformation processes have gained an importance as intermediate in chemical synthesis predominantly if reactions are not possible or only possible with high efforts. These reactions are very well documented in racemic resolutions and well directed implementation of individual functional groups of molecules. These reactions are feasible due to enzyme properties like reaction specificity, regio selectivity and stereo selectivity. Microorganisms have been used with considerable success in biotechnological applications and their impact on the chemicals industry.

1.2. Why biotransformation?

Bacteria or yeast produced large amount of biomass and a great varity of different enzymes in a short time with the chemo-, regio-, and enantioselectivity of enzymes. Because of their small size, bacteria have largest surface-to-volume ratio in the living world, which allows them to maximize their metabolic rates because of a high exchange of molecules and metabolites through their surface. With the right cultivation condition, microorganisms grow exponentially according to the equation

$$A_t = A_o\, e^{(\mu^* t)}$$

A_o is the biomass concentration at time zero or the start of cultivation. A_t is the biomass concentration at the time of harvest. μ is a strain specific growth rate. Some of the fastest growing bacteria weighing maybe 10^{-12} g are theoretically able to duplicate and grow so fast that their biomass would reach the mass of the earth (9×100^{54} tons) in less than a

week. This means, that if a bacterial strain produces an enzyme which can be used industrially, large amounts of enzymes can theoretically be produced economically.

Most microorganisms are also able to grow under varying conditions and on a great variety of substrates and renewable sources. This metabolic flexibility facilitates these microorganisms to produce hundreds of different enzymes for different reactions. However, these enzymes are not naturally overproduced but are regulated according to the physiological needs of the cells. Also, enzymes are produced and active working under given environmental conditions. Microorganisms might be useful in cases where there is no chemical solution or might allow extension of the arsenal of chemical transformations.

1.2.1. Advantages and disadvantages of biotransformation and bioconversion processes

Advantages:

Transformations are carried out with very efficient biocatalyst (enzyme/ whole cells) and are environmentally acceptable.

- Biocatalyst operates under mild conditions of ambient temperatures 20 to 40 ºC, atmospheric pressure and at or near neutral pH in the range 5.0 to 8.0.
- As biocatalyst is compatible, several biotransformation reactions can be preformed in one flask. It is applicable for the sequential reactions using multienzyme system as the isolation of unstable intermediates can be omitted.
- They showed specific selectivity, chemoselectivity, regioselectivity and enantioselectivity.

- Biocatalyst can carry out reactions which is not possible through conventional chemical synthesis or not economically feasible by traditional chemical synthesis.
- Biotransformations are producing a "nature-like", biodegradable compounds which is "Green Chemistry".

Disadvantages:

- In biotransformation, biocatalyst requires narrow operation parameters. If a reaction proceeds slowly under given parameters, there is only a narrow scope of alteration as it leads to deactivation of the enzyme/protein.
- Biocatalyst reaction shifting from an aqueous medium to an organic medium is most of the time not feasible and reduces its activity. Biocatalyst displays its highest catalytic activity in aqueous condition, which is the least desired solvent for most of organic compound.
- Many biocatalytic reactions are prone to substrate or product inhibition, which cause the enzymes / whole cells to cease to work at higher substrate or product concentrations.
- Enzymes as a biocatalyst are provided by nature in one enantiomeric form and sensitive to environmental extremes of temperature, pH etc.
- Some biocatalysts may cause allergies.

1.3. Classification of biochemical reactions of relevant biocatalyst

Enzymes are the tools of biocatalysis and are classified by the Enzyme Commission, International Union of Biochemistry and Molecular Biology, which is a subdivision of the Federation of Biochemistry.[16] Enzymes are known to catalyze a myriad of different chemical reactions and are divided into five classes accordingly, shown in Table 1.2.

1. Oxidoreductases: Oxidation-reduction, oxygenation of C-H, C-C, C=C bonds, or overall removal or addition of hydrogen atoms equivalents.
2. Transferases: Transfer of groups, aldehydic, ketonic, acyl, sugar, phosphoryl or methyl.
3. Hydrolases: Hydrolysis, formation of ester, amides, lactone, lactams, epoxides, nitriles, anhydride, glycosides, organohalides.
4. Lyases: Addition-elimination of small molecules on C=C, C=N, C=O bonds.
5. Isomerases: Isomerization such as racemization, epimerization, rearrangement.

The large majorities of enzymes used for biotransformation in organic chemistry are employed in a crude form and are relatively inexpensive. The preparations typically contain only about 1 to 30 % of actual enzyme. The remaining are inactive proteins, stabilizer, buffer salts or carbohydrates from the fermentation broth from which they have been isolated. Sometimes crude preparations are often more stable than purified enzymes.

Table 1.2. Function based classification of some enzyme groups

Enzyme group	Reaction types	Potential products
1. Oxidoreductase	Oxidation	Alcohol, Epoxide, Lactone, Amino Acid, Sulphoxide
	Reduction	Alcohol, Lactone
2. Transferase	Hydroxy methyl transfer	Hydroxyamino acid
	Amino group transfer	Amino acid, Amine
3. Hydrolase	Ester hydrolysis	Alcohol, Carboxylic acid,
	Trans-esterification	Carboxylic ester
	Nitrile/amide hydrolysis	Alcohol, Carboxylic acid,
	Hydantoin hydrolysis	Carboxylic ester
	Alkylhalide hydrolysis	Carboxylic acid
		Amino acid
		Haloalkanoic acid, Alcohol, Epoxide
4. Lyase	C-C bond formation	Amino acid, Acyloin, Cyanohydrin
	C-O bond formation	Alcohol, Amino acid
	C-N bond formation	Amino acid
5. Isomerase	Lactone formation	Lactone

1.3.1. Enzymes can catalyze a broad spectrum of reactions

There is an enzyme-catalyzed process equivalent to almost every type of organic reaction. For example: *Hydrolysis-synthesis* of esters,[17] amide,[18] lactones,[19] lactams,[20] ether,[21] acid anhydrides,[22] epoxide[23] and nitriles;[24] *Oxidation* of alkanes,[25] alkenes,[26] aromatics,[27] alcohol,[28] aldehyde,[29] ketones,[30] sulfide and sulfoxide;[31] *Addtion-elimination* of water,[32] ammonia,[33] hydrogen cyanide;[34] Halogenation and dehalogenation,[35] alkylation and dealkylation,[36] carboxylation[37]and decarboxylation,[38] isomerization,[39] acylation,[40] aldol reaction,[41] and Michael-addition.[42]

1.4. Comparison between enzyme and whole cell systems

The physical state of biocatalysts that are used for biotransformations can be very diverse in nature. Isolated enzyme systems or intact whole cells may be used for biotransformation depending on the factors **a)** The type of reaction **b)** The requirement of co-factors. **c)** The scale in which the biotransformation has to be performed.

Each approach has its own advantages and disadvantages. Many isolated enzyme systems are now commercially available or are relatively easy to isolate, they can be stable, easy to use and often give single product. However for some reactions where cofactors are used or the need to regenerate the cofactor can be an added complication. Whole cells do not have this disadvantage and although they do tend to give more than one product. They are often cheaper to use than isolated enzyme systems.[43] The advantages and disadvantages for both the methods are given in the Table 1.3.

Table 1.3.
Comparison of isolated enzyme and whole cell

Biocatalyst	Form	Advantages	Disadvantages
Isolated enzymes	Any	Simple apparatus, simple work-up, better yield due to higher concentration tolerance	Cofactor recycling necessary
	Dissolved in water	High enzyme activities	Side reactions possible, lipophilic substrates insoluble, work-up requires extraction
	Suspended in organic solvents	Easy to perform, easy work-up, lipophilic substrates soluble, enzyme recovery easy	Low activities
	Immobilized	Enzyme recovery easy	Loss of activity during immobilization
Whole cells	Any	No cofactor recycling necessary	Expensive equipment, tedious work-up due to large volumes, low productivity due to lower concentration tolerance, low tolerance of organic solvents, side reactions likely due to uncontrolled metabolism
	Growing culture	Higher activities	Large biomass, more byproducts, process control difficult
	Resting cells	Work-up easier, fewer byproducts	Lower activities
	Immobilized cells	Cell reuse possible	Lower activities

1.5. Hydroxylation of polyunsaturated fatty acids (arachidonic acid)

Hydroxylation, the conversion of a carbon–hydrogen to a carbon–hydroxyl bond, is one of the most widespread of enzyme activities, occurring in all forms of life from bacteria to humans. The reaction is a key part of the oxidative metabolism of many organic compounds.[44] Oxygenation of polyunsaturated fatty acids specifically of arachidonic acid has been extensively investigated due to its biological importance. Interest in this work has been stimulated by the discovery of different eicosanoids which act as potent vasoactive compounds. The transformation of arachidonic acid occur enzymetically into variety of oxygenated species called eicosanoids. These are bioactive lipids and involved in a number of signal transduction pathways in biological regulatory mechanisms.

1.5.1. General background on oxylipins (eicosanoids)

Oxygenated lipids are collectively known as oxylipins. One of the most biologically important groups of oxylipins in mammals is the eicosanoids. Eicosanoids (*Gr. Eicosa*, twenty) is the name given to unsaturated lipids derived from arachidonic acid ($C_{20:\,4}$), or similar 20-carbon polyunsaturated fatty acids by oxygenase reactions.[45] Eicosanoids give rise to a wide range of products with remarkable physiological activity. The pathways for the oxygenation of arachidonic acid are collectively known as arachidonic acid cascade.[46]

Lipids play important role in cells and organ biology in addition to their accepted structural importance as the building blocks of cellular membranes. An extensive study has demonstrated that fatty acids, glycerolipids, glycerophospholipids, ceramides, etc. participate as mediators in a variety of transmembrane signaling cascades. They are also

involved in cell differentiation, replication and apoptosis. The functional significance of these mediators was further emphasized by their proposed roles in the pathophysiology of diseases,[47] inflammation, asthma, cancer, diabetes, and hypertension.[48] These findings have stimulated an intensed research into the biochemistry, enzymology, and the regulation of lipid metabolism. All mammalian cells except erythrocytes convert arachidonic acid into bioactive eicosanoids using some or all of the following three-enzymatic pathways[49] shown in **Figure 1.1.** Oxygenated eicosanoids are produced through three different pathways which include **i.** the cyclooxygenase pathway that leads to the synthesis of numerous prostaglandins, prostacyclins and thromboxanes likes PGI2, PGE2, PGJ2, PGF2α, PGD2 and TXA2[50] **ii.** the lipooxygenase pathway that results in accumulation of hydroperoxides (HPETEs) and leukotrienes [51] **iii.** the arachidonic acid monooxygenase pathway creates EETs and HETEs and consists of cytochrome P450s possessing epoxygenase, lipooxygenase-like or ω/ω-1 hydroxylase activity.[52] Cytochrome P450 epoxygenases synthesize four regio-epoxy isomers; 5, 6-EET, 8, 9-EET, 11-EET, 12-EET and 14, 15-EET which can be further converted by epoxide hydrolases to corresponding dihydroxyeicosatrienoic acids.[53] Lipoxygenase-like cytochrome P450s create 5-HETE, 8-HETE, 9-HETE, 11-HETE, 12-HETE and 15-HETE.[52] ω/ω-1 hydroxylase converts arachidonic acid into 20-HETE, 19-HETE, 18-HETE, 17-HETE or 16-HETE.[52] All of the above eicosanoids can be further modified into additional eicosanoids.[54, 55] These are provided as a premier illustration of the role that lipid-derived mediators play in cell and organ function.

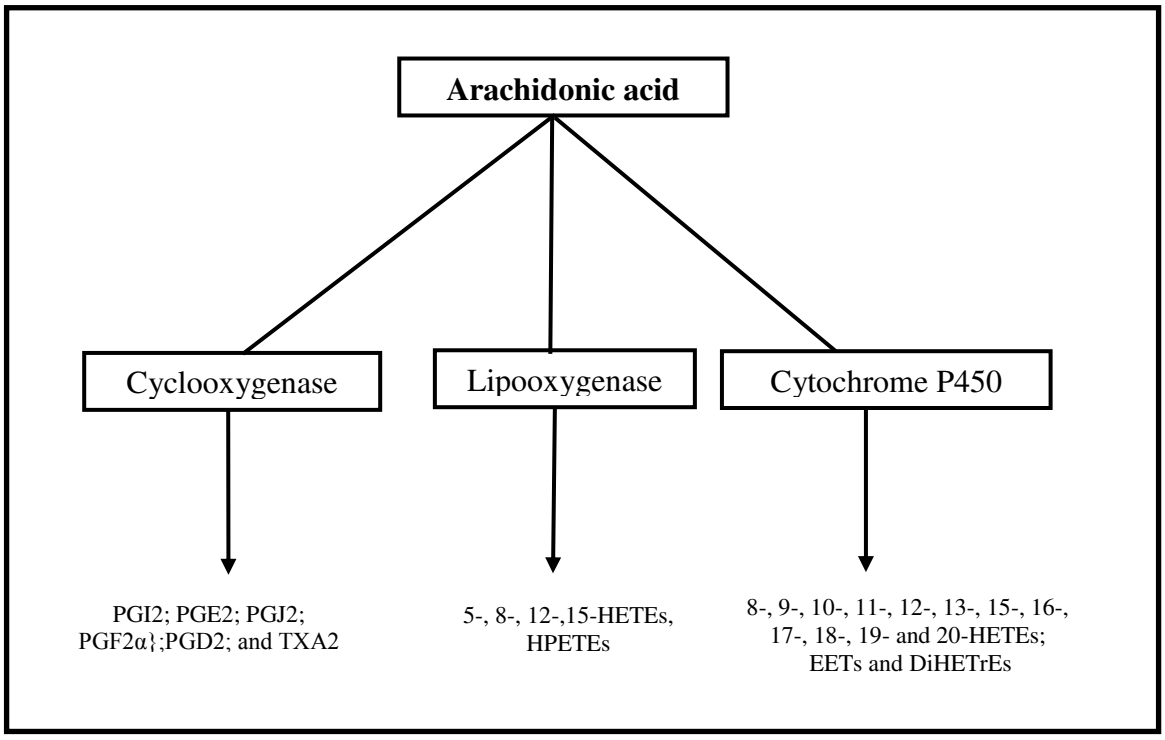

Figure 1. 1. Selective pathways for the metabolism of arachidonic acid

These eicosanoids occur in trace concentrations in many microorganisms and tissues including the mammalian brain and play important diverse roles as physiologic and pathophysiologic mediators.

1.5.2. Cytochrome P450 and eicosanoids metabolism

Cytochrome P450 designates a group of hemi-thiolate proteins, which catalyses oxygenation of xenobiotics and endogenous compounds such as fatty acids, lipid hydroperoxides and cytokines. Cytochrome P450s are intracellular hemeproteins that activate molecular oxygen for the oxidative metabolism of a great variety of lipophilic organic compounds. In eukaryotic cells the cytochrome P450s exist as membrane-bound hemeproteins, each containing about 500 amino acids with iron-protoporphoryrin IX as

the prosthetic group The amino-terminus of the protein is rich in hydrophobic amino acids and is believed to act as a domain for binding the protein to membranes. The role of the present thiol group as a ligand alters the electron density of the resonant porphoryrin ring of the heme, thereby providing an electronic center for the activation of molecular oxygen.

Many of the reactions catalyzed by cytochrome P450s have been characterized showing the great diversity of action of these remarkable catalysts. Coon *et al.* in 1996 have identified different types of reactions catalyzed by cytochrome P450s[56] (e.g., hydroxylation of aromatic and aliphatic chemicals, the N- and O-dealkylation of secondary and tertiary amines and O-methyl derivatives, the b-scission of hydroperoxides etc.). The number of chemicals that can serve as substrates metabolized by cytochrome P450s is enormous and certainly greater than 1000. Arachidonic acid, linoleic acid and other polyunsaturated fatty acids (PUFA) can be catalysed in several ways: **1.** hydroxylations of the ω-side chain (ω1, ω2, ω3, etc.); **2.** hydroxylations of bisallyic carbons; **3.** epoxidation; **4.** hydroxylations of allylic carbon; **5.** hydroxylations with double bond migration.

The cytochrome P450s catalyze the NADPH and oxygen dependent oxidative transformation of a large number of different chemical compounds. In general a specific cytochrome P450 will catalyze the metabolism of a limited number of chemical structures such as steroids and fatty acids while other cytochrome P450s have a broad substrate specificity suggesting a role for a unique "active site geometry" for a cytochrome P450.

1.5.2.1. NADPH-dependent metabolism of eicosanoids

The NADPH-dependent metabolism of several eicosanoids by cytochrome P450 is well established. The biological importance of these reactions resides in the fact that they: **(a)** increase eicosanoid structural diversity and, hence informational content, **(b)** may alter the pharmacological profile of the substrate, and **(c)** may participate in the regulation of steady state and / or stimulated levels of physiologically relevant molecules.[57] Cytochrome P450 catalyzed metabolism of prostanoids, leukotrienes, HETEs,[58] and epoxyeicosatrienoic acids (EETs)[59] results in the hydroxylation of these eicosanoids at the ultimate (C20 or ω carbon) or penultimate carbon atoms (C19 or ω -1 carbon).[60]

In arachidonic acid metabolism, the cytochrome P450 enzyme system is NADPH-dependent. The redox coupled activation of molecular oxygen is carried out by its delivery to the substrate ground state carbon skeleton. This feature, i.e., the NADPH-dependent redox coupled activation of molecular oxygen, as opposed to the free radical-mediated activation of carbon atoms, distinguishes the cytochrome P450 enzyme system from the other enzymes of the arachidonate cascade.

1.5.2.2. NADPH-independent metabolism of eicosanoids

Cytochrome P450 is an active peroxidase that catalyzes the metabolism of a wide variety of organic hydroperoxides including fatty acid hydroperoxides.[61, 62] This peroxidase activity was initially described by O'Brien and collaborators in 1974.[63] It is associated with the ferric, Fe^{3+} state of microsomal cytochrome P450, does not involve electron transfer from NADPH and exhibits high catalytic rates. The mechanism of peroxide O-O bond cleavage is homolytic or heterolytic scission.[62] The catalytic outcome

determines these reactions and is highly dependent on: **(a)** the nature of the cytochrome P450 isoform, and **(b)** the chemical properties of the organic hydroperoxide and the oxygen acceptor.[61] A hemolytic pathway was proposed to account for the formation from 15-hydroperoxyeicosatetraenoic acid (15-HPETE) of 11-hydroxy-, 13-hydroxy-, 14 & 15-epoxyeicosatrienoic acids by rat liver microsomes.[64]

The cytochrome P450s are members of the class of enzymes called oxygenases. Specifically, the cytochrome P450s are monooxygenases[65] or mixed function oxidases. [66] The cytochrome P450s are widely distributed in nature including mammals, plants, insects, yeast and some bacteria.[67]

1.6. Arachidonic acid ω and ω-1 hydroxylase reaction

The catalysis of fatty acid omega and omega-1 oxidation (ω and ω-1) is one of the best stablished cytochrome P450 reactions.[68] The cytochrome P450-catalyzed hydroxylation of arachidonic acid at its ω and ω-1 carbons was first documented by Morrison and Pascoe in 1981, when rabbit kidney cortex microsomes were shown to catalyze the NADPH-dependent formation of 19- hydroxyeicosatetraenoic acids and 20-hydroxyeicosatetraenoic acids (19-HETE and 20-HETE respectively).[69] Since then these hydroxylation reactions has been demonstrated in several tissues including human liver and kidney.[52] More recently the 16-, 17-, 18 and 19-hydroxyeicosatetraenoic acids have been added to the list of products generated by the cytochrome P450 which is arachidonic acid ω and ω-1 hydroxylase activity.[70, 71] Prabhune *et. al.,* in 2002 have shown that the yeasts *Candida bombicola* and *Candida apicola* can synthesize sophorolipids of arachidonic acid in which sophorose, as a diglucoside, is linked glycosidically to the terminal (ω) or (ω-1) hydroxy group of a hydroxy fatty acid.[72] These sophorolipids on

acid hydrolysis liberated 19-hydroeicosatetraenoic acid (19HETE) and 20-hydroeicosatetraenoic acid (20-HETE) shown in **Figure 1. 2.** These hydroxy fatty acids are produced by direct hydroxylation of exogenous chain fatty acids, which can be supplemented as such or as oils.[73] These fatty acids are usually either saturated or monounsaturated.[74]

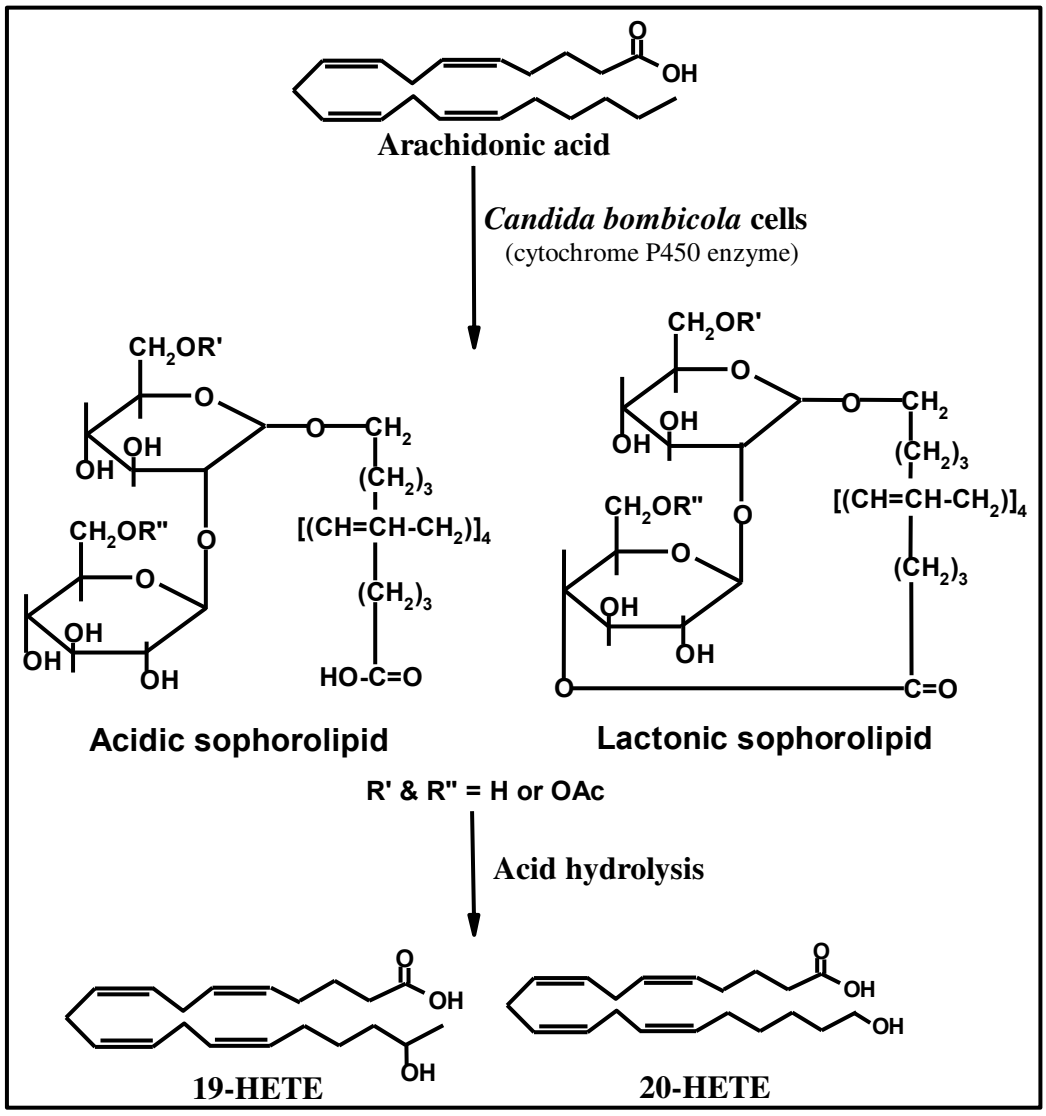

Figure 1.2. Arachidonic acid transformation to ω and ω-1 hydroxylase reaction mediated by cytochrome P450 enzyme present in *Candida bombicola* cells

The oxygen chemistry and reaction mechanisms responsible for the ω and ω-1 hydroxylation of arachidonic acid and other saturated fatty acids are similar. For arachidonic acid, these reactions impose additional steric requirements on the cytochrome P450 protein catalyst. Hydroxylation of arachidonic acid takes place at the thermodynamically less reactive C16 through C20 carbon. But it was not chemically comparable at the carbon C2 through C4 arachidonic acid. It suggested a highly rigid and structured binding site for arachidonic acid template. Thus the binding site must be capable of positioning the acceptor carbon atoms not only in optimal proximity to the hemebound active oxygen but also with complete separation of the reactive 5,6-, 8,9-, 11,12-, and 14,15-olefins and of the bis-allylic C7, C10 and C13 methylene carbons of arachidonic acid.

There are many reports of the cytochrome P450 4A gene family from rat, rabbit and human, which have been purified, cloned and expressed.[75] In humans, about 40 different cytochrome P450 are present and these play critical roles by catalyzing reactions in: (a) the metabolism of drugs, environmental pollutants and other xenobiotics; (b) the biosynthesis of steroid hormones; (c) the oxidation of unsaturated fatty acids to intracellular messengers; and (d) the stereo- and regio-specific metabolism of fat-soluble vitamins. The individual cytochrome P450 isoforms show regioselectivity for either the ω or the ω-1 hydroxylation. To date all the cytochrome P450 isoforms characterized, metabolize arachidonic acid to either 20-hydroxyeicosatetrenoic acid (20-HETE) or to mixtures of 20-hydroxyeicosatetrenoic acid (20-HETE) and 19-hydroxyeicosatetrenoic acid (19-HETE); i.e., none of these cytochrome P450 isoforms show exclusive regioselectivity for the fatty acid C19 carbon position of arachidonic acid. Falck *et al.,* in

1990 and Laethem *et al.,* in 1993 showed effect of inducers on microsomal cytochrome P450. The reconstitution of the arachidonic acid hydroxylase with purified enzymes showed that cytochrome P450s family (1A1, 1A2 and 2E1) might be responsible for the hydroxylation occurring at the C16 through C19 carbon atoms of arachidonic acid.[76, 71] Schwartzman *et al.,* in 1991 showed that 20-hydroxyeicosatetraenoic acid (20-HETE) excretion in human urine confirmed the participation of the cytochrome P450 in the ω - hydroxylation of endogenous arachidonic acid.[77] Prakash *et al.,* in 1992 studied that the majority of the 20- hydroxyeicosatetraenoic acid (20-HETE) in rat urine is found conjugated to glucuronic acid, an established route for the excretion of hydroxylated compounds.[78]

A wide range of biological effects of 19-hydroxyeicosatetraenoic acid 20-hydroxyeicosatetraenoic acid metabolites of arachidonic acid have been occurring in liver,[79] kidney,[53] lungs,[80] platelets,[81] vasculature,[82] heart,[83] central nervous system,[84] pituitary,[85] adrenals, pancreas and ovaries.[86]

1.6.1. The arachidonic acid epoxygenase reaction

There are many reports on the catalysis of epoxyeicosatetraenoic acid (EET) formation by purified cytochrome P450s, microsomal fractions, or isolated cell preparations. This has been confirmed in numerous tissues, including liver, kidney,[87] pituitary, brain, adrenal, endothelium, pancreas, and ovaries.[46, 52] Oliw *et al.,* in 1982 showed that the catalysis of arachidonic acid epoxidation by cytochrome P450 was secondary by the isolation of 11,12- and 14,15-dihydroxyeicosatrienoic acids (DHETs) from incubates containing kidney cortex microsomes, arachidonic acid and NADPH.[88] Chacos *et al.,* in 1982, demonstrated the rat liver microsomes to catalyze the NADPH-

dependent epoxidation of arachidonic acid to 5,6-, 8,9-, 11,12-, and 14,15-EET.[89] In mammals the epoxidation of polyunsaturated fatty acids to bis-allylic, cis-epoxides is unique to the cytochrome P450 enzyme system and at difference with the fatty acid ω and ω-1 oxygenase, which is more or less selective for arachidonic acid. Finally the fact that the cytochrome P450-dependent epoxidation of arachidonic acid generates only cis-epoxides, suggests that epoxidation proceeds by a determined pathway or that alternatively a rigid active site binding geometry restricts the freedom of C-C rotation for the transition state.[90]

1.6.2. Physiological role of 20-hydroxyeicosatetraenoic acid (20-HETE) and 19-hydroxyeicosatetraenoic acid (19-HETE)

The study of physiological roles for the metabolites of the cytochrome P450 arachidonic acid monooxygenase has developed into and intense research and the list of biological activities attributed to these metabolites has grown considerably during the last few years.

***Vascular properties*:** There are significant numbers of reports demonstrating that arachidonic acid metabolites play an important role in regulating vascular tone. Harder *et al.,* in 1994 has shown that 20-HETE inhibits the activity of Ca^{2+} activated K^+ channels (K_{Ca}) and depolarizes cerebral and renal arterial muscles.[91] Thus, 20-HETE appears to function as an endogenous modulator of $K_{Ca.}$[92] Such a modulator role of 20-HETE on K_{Ca} was revealed when 17-Octadecynoic acid (17-ODYA) was used as a specific mechanism based inhibitor of the metabolism of arachidonic acid. This was reverted by external addtion of 20-HETE, which showed increased K^+ channel activity in cerebral, and vascular smooth muscle cells.[93] 20-hydroxyeicosatetraenoic acid or products of its

oxidative metabolism are powerful vasoconstrictors while 19- hydroxyeicosatetraenoic acid is a stereospecific vasodilator.[46] On the other hand, the epoxyeicosatetraenoic acids (EETs) are in vitro systemic vasodilators with 5,6-EET as the most potent regeoisomer while 8(S), 9(R)-EET, the endogenous enantiomer in rat kidney, is a stereoselective renal vasoconstrictor.[52]

***Ion transport*:** Several reports identify the role for 20-HETE in the regulation of renal tubular ion transport. In the cells of the thick ascending limb of the rat kidney, 20-HETE decreases the open state probability of K^+ channel, thus regulating K^+ recycling across the membrane and Na^+ reception.[94] 19-hydroxyeicosatetraenoic acid and 20-hydroxyeicosatetraenoic acid were observed to play key role in stimulation and inhibition of renal Na^+, K^+ /ATPase. In rabbit mTALH cells, 20-HETE and 20-COOH arachidonic acid blocked the Na^+, K^+, 2Cl-cotransporter and Na^+, K^+ ATPase.[48, 53] Additionally the epoxyeicosatetraenoic acids (EETs) and 5,6-EET in particular, increased the cytosolic Ca^+ concentrations in several cell systems as well as in the single channel open probability of Ca^+ activated K^+ channels.[95, 96] 20- hydroxyeicosatetraenoic acid, identified as an endogenous inhibitor of Ca^+ activated K^+ channels is a powerful vasoconstrictor[97] (Escalante *et al.,* in1993; Ma *et al.,* in 1993) and behaves, therefore, as an EET functional antagonist.[98, 92] Falck J. R. *et al.,* in 2000 showed that of 20-HETE and 19(s)-HETE affect rabbit proximal straight tubule volume transport.[99] The proximal tubule is a major site of renal cytochrome P450.[100, 101] It has been estimated that the majority of 19 (S)-HETE and 20-HETE produced by the kidney comes from the proximal tubule.[102] 20-HETE inhibits Na-K-ATPase and is thought to play an important role in the effect of parathyroid hormone and dopamine to inhibit transport in this segment.[103, 104] 19(S)-

HETE which is the major ω-1 product of ω-hydroxylase showed to stimulate rat renal Na-K-ATPase activity,[105] which is exactly opposite function of 20-HETE.

Effect of NO: Recent studies have indicated that the tonic release of nitric oxide (NO) plays a central modulatory role in the regulation of renal tubular and vascular function and the long-term control of arterial pressure.[106, 107, 108] Nitric oxide-20-hydroxyeicosatetraenoic acid interactions play important role in the regulation of K^+ channel activity and vascular tone in renal arterioles. The inhibition of cytochrome P450 enzyme activity and the formation of 20-HETE contributed to the activation of K^+ channels and vasodilator effects of nitric oxide (NO) in renal arterioles. Roman R. J. *et. al.,* in 1998 have shown the addition of NO donor to the cytochrome P450 family enzyme (that produces 20-HETE) increased visible light absorbance at 440 nm indicating that NO binds to heme in this enzyme. NO donors are dose-dependently inhibited the formation of 20-HETE in microsomes preparation from renal arterioles.[109] In contrast, inhibition of the formation of 20-HETE with 17-Octadecynoic acids (17-ODYA) activated K^+ channel and masked the response to NO. Preventing the NO-induced reduction in intracellular 20-HETE levels also blocked the effects of NO on this channel. This indicates that the inhibition of the formation of 20-HETE contributes to the activation of K^+ channels and the vasodilator effects of NO in the renal microcirculation.

Cirrhosis: 20-hydroxyeicosatetraenoic acid (20-HETE) participates in key mechanisms that regulate the renal circulation and extracellular fluid volume. These metabolites are vasoactive and affect transport in kidney. McGiff J. C. *et. al.,* in 1997 demonstrated that excess production of 20-HETE, which constricts the renal vasculature and contributes to the renal functional disturbances in patients with hepatic cirrhosis, particularly the

depression of renal hemodynamics. In cirrhotic patients 20-HETE was excreted as glucuronide conjugate. Urinary excretion rate of 20-HETE was highest in patients in cirrhotic patients without ascites and in normal

1.7. Scope and objectives of the thesis

One of the important areas of biotechnology is the biotransformation process for the synthesis of bioactive molecules. The current research is directed towards the development of different experimental protocols for the synthesis of bioactive molecules using biological sources like yeast, fungi, free enzymes, whole cells, immobilized enzyme / whole cells. The uses of enzymes or whole cells for the transformation of organic compounds by immobilization technique have created in researcher to look at the biological system. Thus, there is a need to develop eco-friendly processes that do not employ toxic chemicals in the synthesis protocols. However, such biotransformation-based biocompatible materials synthesized strategies would have greater commercial viability. Recent studies have indicated that 20-HETE and 19-HETE play an important role in autoregulation of renal blood flow, tubuloglomerular feedback, renal sodium transport, and pulmonary function. They also affect mitogenic and vasoconstrictor responses to numerous vasoactive hormone and growth factors. Towards this objective, the Thesis will focus on the biotransformation of arachidonic acid to 20-hydroxyeicosatetraenoic acid (20-HETE) and 19- hydroxyeicosatetraenoic acid (19-HETE). The yeasts, *Candida bombicola* (ATCC 22214) and *Candida apicola* (ATCC 96134) are used for the first time to transform arachidonic acid into these monohydroxyeicosatetraenoic acids (19-HETE and 20-HETE) from arachidonic acid. Interest in this work has been stimulated by the discovery of different eicosanoids, which

acts as potent vasoactive compounds. The transformation of arachidonic acid occurs enzymatically into variety of oxygenated compounds. Our approach here is to address the problem, the difficulties associated in eicosanoids chemical synthesis, which are expensive as well as hazardous in nature. The chemical synthesis of 20-HETE and 19-HETE involves more than 10 steps starting from arachidonic acid. Out of these 10 steps, some steps in reactions require cryogenic temperatures and chirally active precursor. These chemical methods are not commercially feasible for the production of 19-HETE and 20-HETE. In this context the *Candida bombicola* as such and immobilized cells on the biocompatible materials not only help to permit simpler method but safer and economical way to produce these biologically active compounds.

We have demonstrated the immobilization of *Candida bombicola* yeast cells on the patterned thermally evaporated octadecylamine (ODA) films[110] and the hydrophobic nanogold membrane.[111] The immobilized yeast cells were biologically active and cytochrome P450 enzyme present in the C*andida bombicola* cells could be used to transform arachidonic acid to 19- hydroxyeicosatetraneoic acid (19-HETE) and 20-hydroxyeicosatetraneoic acid (20-HETE). The biocomposite assemblies are easily separated from the reaction medium for additional reuses. The binding of the cells to the biocompatible film as well as to the nanogold membrane is strong enough to prevent the leaching of the whole cells from the surface. Hence the whole cells immobilized membranes are reused for the biotransformation.

1.8. Outline of the thesis

The **THESIS** will be presented in **Five (5)** chapters, a brief summary of which is given below.

Chapter 1. Introduction

Chapter 1 presents a general introduction about eicosanoids and biotransformation of arachidonic acid metabolites. 20-hydroeicosatetraenoic acid (20-HETE) and 19-hydroxyeicosatetraenoic acid (19-HETE) are omega and omega-1 hydroxylated products of arachidonic acid, which are important in autoregulation of blood pressure and vascular tone. There are different methods discussed for the production of 19-HETE and 20-HETE. The intermediate product sophorolipid was discussed in brief. Different supports used for the immobilization of whole cell were discussed in brief. Based on these reviews, the scope and objective of the present work have been outlined.

Chapter 2. Fermentation parameters and characterization techniques

Chapter 2 presents the fermentation parameters of *Candida* Sp. for the production of sophorolipids and their derived products 19-HETE and 20-HETE. The different experimental techniques and analytical tools used during the course of the present work are discussed.

Chapter 3. Dry column chromatography technique used for the purification of different forms of sophorolipids produced by *Candida bombicola*

Chapter 3 deals with purification of the mono-, di- acetate forms of lactonic and acidic sophorolipids produced by *Candida bombicola* grown on glucose and long chain fatty acid arachidonic acid using the dry column chromatography technique.

Chapter 4. Immobilization of whole cells on biocompatible material: use as enzymes source for the biotransformation of arachidonic acid to 19-HETE and 20-HETE

Chapter 4 focuses on the continuous conversion of 19-HETE and 20-HETE from arachidonic acid by immobilization of *Candida bombicola* on different biocompatible materials. The patterned thermally evaporated octadecylamine (ODA) lipid films and Free-Standing Organic–Gold nanoparticles embedded in a polymeric membrane provide a biocompatible surface for the immobilization of whole cells. The presence of gold nanoparticles in the membrane enables facile modification of the surface properties of the membrane and this act as enzyme sources for the transformation of arachidonic acid to 19-HETE and 20-HETE. The attachment of the cells to the ODA film surface occurs possibly through nonspecific interactions such as hydrophobic interactions between the cell walls and the ODA molecules. The enzyme cytochrome P450 present in the immobilized yeast cell membrane was used to catalyze the biotransformation of the arachidonic acid to sophorolipids and thereafter, acid hydrolysis to yield 19-hydroxyecosatetraenoic acid (19-HETE) and 20-hydroxyecosatetraenoic acid (20-HETE). These biocomposite materials could be easily separated from the reaction mixture and exhibit excellent reusability.

Chapter 5. Summary and conclusions

The summary of the results obtained and the conclusions drawn are presented in this chapter.

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Chapter 2

Fermentation parameters and characterization techniques

This chapter describes the effect of different fermentation parameters for the production of sophorolipids and experimental techniques used during the course of the present work.

2.1. Introduction

The yeasts, *Candida bombicola* (ATCC 22214) and *Candida apicola* (ATCC 96134) can synthesize a mixture of sophorolipids. During stationary phase *Candida* sp. secretes sophorolipids when grown on glucose and supplemented with long chain fatty acid.[1] The sophorolipids consisted of sophorose, a diglucoside, linked glycosidically to the terminal (ω) and sub terminal (ω -1) hydroxy group of a hydroxy-fatty acid.[2] 19-hydroxyeicosatetraenoic acid (19-HETE) and 20- hydroxyeicosatetraenoic acid (20-HETE) were obtained from the yeast *Candida bombicola* and *Candida apicola* in glycosilated form when grown on glucose and arachidonic acid. Free hydroxylated fatty acids, 19-HETE and 20-HETE were liberated by hydrolysis of diglucoside linked glycosidically to the terminal (ω) and sub terminal (ω -1) of the fatty acid. Interest in this work was stimulated by the important biomedical role of hydroxy fatty acids (oxylipins), 19-HETE and 20-HETE. These oxylipins play an important role in modulating variety of cardiovascular events. As mentioned in the Chapter 1, there are evidences that 19-HETE and 20-HETE play important role in regulation of renal, cerebral and pulmonary blood flow via signal transduction pathways. For example, inhibition of 20-HETE formations in the kidneys and brain abolishes autoregulation of blood flow and arterial blood pressure. Though these oxylipins play such important role in human physiology there are no reports on production of these compounds other than chemical synthesis. Most of the reports were on chemical synthesis and some reports on mammalian cell lines were used for the production of these oxylipins.[3] Both methods are tedious and cost incurring (Sigma Chemicals USA and Cyman Chemicals Co. Ann. Arbor, MI, price of 20-HETE for 10 mg is $250).

In this chapter we are reporting for the first time, production of these vasoactive biomolecules by microbial transformation. 20-HETE and 19-HETE are produced as derivatives of arachidonic acid in the form of sophorolipids. As stated in the general introduction, standardization of fermentation parameters is prerequisite for any cost effective development of the process. In light of the above significances, variable parameters affecting fermentative production of sophorolipids by *Candida bombicola* and *Candida apicola* were optimized to obtain maximum yield. Conditions were optimized for maximum production of arachidonic acid derived sophorolipids. This part of the thesis describes the growth conditions for high arachidonic acid derived sophorolipids production by *Candida bombicola* and *Candida apicola*.

2.2. Materials and Methods

2.2.1. Chemicals

Arachidonic acid was obtained from Martek Corporation, MD 21045,USA, as a free acid of 80 % purity and the other fatty acids were gamma linolenic acid, linoleic acid and oleic acid. 1 g/ml working stock was freshly prepared by diluting with absolute ethanol. Glucose was obtained from Qualigens, India; yeast extract, malt extract, and peptone were from Hi-media, India. All other chemicals were of high purity or analytical grade and procured from commercial sources. Arachidonic acid of 100 % purity obtained from Cyman Chemicals Co. Ann. Arbor, MI, used as standard for all experiments.

2.2.2. Microorganism and maintenance

The yeast *Candida bombicola* ATCC 22214 and *Candida apicola* ATCC 96134 were procured from American Type Culture Collection, USA. Both the cultures were

maintained by periodic transfer onto medium containing 2 % agar with 5 % glucose MYP medium in petri plates or slants. The cultures were incubated at 30 °C for 48 h.

2.2.3. ***Effect of medium composition on production of arachidonic acid derived sophorolipids by Candida bombicola and Candida apicola***

Media A, B, C, D and E, varying in composition were screened for maximal production of arachidonic acid derived sophorolipid.

Yeast cultures were optimized using the following media:

(A) Medium A: (g l^{-1} of distilled water) Glucose, 50; Yeast extract, 3.0; Malt extract, 3.0; Peptone, 5.0; pH adjusted to 5.5.

(B) Medium B: reported by Prabhune *et al*. in 2002 for sophorolipids and sophorolipids derived products by *Candida* sp. having the following composition: (g l^{-1} of distilled water) Glucose, 50; Yeast extract, 1.0; $MgSO_4$, 0.3; Na_2HPO_4, 0.2; NaH_2PO_4, 7; $(NH_4)_2SO_4$, 1; pH adjusted to 5.5.[4]

(C) Medium C: (g l^{-1} of distilled water) Glucose, 150; Yeast extract, 4.0; $MgSO_4$, 0.3; KH_2PO_4, 6; Na_2HPO_4, 2; pH adjusted to 5.5.

(D) Medium D: (g l^{-1} of distilled water) Glucose, 150; Yeast extract, 4.0; $MgSO_4$, 0.3; KH_2PO_4, 6; Na_2HPO_4, 2; Urea, 2; pH adjusted to 5.5.

(E) Medium E: (g l^{-1} of distilled water) Glucose, 0.5 g/l; $MgSO_4$, 10 mg/l; KH_2PO_4, 125 mg/l; Na_2HPO_4, 60 mg/l; $CaCl_2$, 10 mg/l; $MnSO_4$, 0.5 mg/l

Fermentative procedure for production of arachidonic acid derived sophorolipids:

10 ml inoculum was developed by growing the yeast cells in respective medium for 24 h at 30 °C and 160 rpm orbital shaking. Starter culture was prepared by

transferring the inoculum in 50 ml medium followed by incubation at 30 °C for 24 h with 160 rpm orbital shaking.

The fermentative production was initiated by transferring the starter culture into 250 ml of the respective medium in 1000 ml conical flask followed by incubation at 30 °C with 160 rpm orbital shaking. The medium was supplemented with 1 g of 80 % arachidonic acid dissolved in 1 ml of ethanol to allow formation of arachidonic acid derived sophorolipid. The fermented broths were examined for the production of arachidonic acid derived sophorolipids at regular intervals of 24, 48, 72, 96 & 120 h. Highest activities were obtained on medium B on the basis of production of sophorolipids and respective oxylipins, hence was used further for variation in glucose concentration, pH, temperature and metal ion effect.

2.2.4. Effect of glucose concentration on production of arachidonic acid derived sophorolipids by *Candida bombicola* and *Candida apicola*.

The effect of glucose concentration on production of sophorolipids was tested by varying the concentration in the range 5 % to 10 % in medium B. Fermentative procedure employed for the experiment was same as described previously (2.2.3.)

2.2.5. Effect of pH on production of arachidonic acid derived sophorolipids by *Candida bombicola*

The effect of pH on production of sophorolipids was tested by adjusting pH of medium B in the range pH 4.0 to 7.0. Fermentative procedure employed for the experiment was same as described previously (2.2.3.). As glucose concentration showed marked effect on sophorolipid yield, pH variation was also studied in presence of 5 % and 10 % glucose in medium B.

2.2.5.1. Effect of pH on production of arachidonic acid derived sophorolipids by *Candida apicola*

The effect of pH on production of sophorolipids was tested by adjusting pH of medium B in the range pH 4.0 to 7.0. Fermentative procedure employed for the experiment was same as described previously (2.2.3.).

2.2.6. Effect of temperature on production of arachidonic acid derived sophorolipids by *Candida bombicola* and *Candida apicola*

The effect of temperature on production of sophorolipids and biomass was tested by incubating the cultures at different temperatures in the range 25 °C to 36 °C. Fermentative procedure employed for the experiment was same as described previously (2.2.3). As glucose concentration showed marked effect on sophorolipid yield, temperature variation was studied in presence of 5 % and 10 % glucose in medium B.

2.2.7. Effect of ferric ions on the production of arachidonic acid derived sophorolipids by *Candida bombicola*

Experiments were carried out to see the effect of Fe^{+3} (in FeCl3) and Fe^{+2} (in $FeSO_4$) on sophorolipid production in both cultures. In medium B, 10-30 mM concentration of Fe^{+3} and Fe^{+2} were added respectively and the fermentative procedure employed for the experiments was same as describe previously (2.2.3.)

2.3. Results and discussion

2.3.1. Effect of media composition on production of arachidonic acid derived sophorolipids by *Candida bombicola* and *Candida apicola*

Table 2.1. Composition of media used for screening maximal production of arachidonic acid derived sophorolipids by *Candida bombicola* and *Candida apicola*

	Medium (A)	**Medium (B)**	**Medium (C)**	**Medium (D)**	**Medium (E)**
Glucose	50 g/l	100 g/l	150 g/l	150 g/l	5 g/l
Yeast extract	3.0 g/l	1.0 g/l	4.0 g/l	4.0 g/l	----
Malt extract	3.0 g/l		----	----	----
Peptone	5.0 g/l		----	----	----
$MgSO_4$	----	0.3 g/l	0.3 g/l	0.3 g/l	10 mg/l
KH_2PO_4	----			6 g/l	125 mg/l
Na_2HPO_4	----	2 g/l	2 g/l	2 g/l	60 mg/l
NaH_2PO_4	----	7 g/l	----	----	----
Urea	----	----	----	2 g/l	----
$(NH_4)_2SO_4$	----	1 g/l	----	----	----
$CaCl_2$	----		----	----	10 mg/l
$MnSO_4$	----		----	----	0.5 mg/l

Table 2.2. Effect of media composition and time kinetics of sophorolipid production by *Candida bombicola*

	24 h	48 h	72 h	96 h	120 h
Medium (A)	**0.301**	**0.301**	**0.353**	**0.421**	**0.423**
Medium (B)*	**0.331**	**0.332**	**0.388**	**0.485**	**0.486**
Medium (C)	**0.502**	**0.541**	**0.692**	**0.798**	**0.802**
Medium (D)	**0.511**	**0.552**	**0.714**	**0.783**	**0.882**
Medium (E)	**0.203**	**0.232**	**0.253**	**0.271**	**0.273**

(Arachidonic acid derived sophorolipids obtained from 250 ml media, yield in grams)

Table 2.3. Effect of media composition and time kinetics of sophorolipid production by *Candida apicola.*

Medium	24 h	48 h	72 h	96 h	120 h
Medium (A)	**0.203**	**0.222**	**0.254**	**0.361**	**0.363**
Medium (B)*	**0.181**	**0.202**	**0.245**	**0.370**	**0.372**
Medium (C)	**0.412**	**0.448**	**0.562**	**0.628**	**0.632**
Medium (D)	**0.431**	**0.482**	**0.602**	**0.683**	**0.684**
Medium (E)	**0.134**	**0.162**	**0.213**	**0.231**	**0.233**

(Sophorolipids obtained from 250 ml media, sophorolipid yield in grams)

(* Prabhune *et al*. in 2002)

Highest yields of sophorolipid production by both *Candida bombicola* and *Candida apicola* were observed in media C and D. Medium A showed marginal increase in the sophorolipid production as compared to medium E. Medium E supported least production of sophorolipids as well as cell mass. In all media except medium D, the rate of production reached maxima at about 96 h of incubation. Media C and D, which supported highest sophorolipid formation, included the highest amount of glucose content. Glucose concentration showed marked effect on sophorolipid production as well as cell mass. Though both these media showed better yield of sophorolipids, concentration of hydroxylated arachidonic acid i.e. 19-HETE and 20-HETE were lower than that obtained in medium B. Sophorolipid produced in medium B showed the highest content of 19-HETE and 20-HETE, which was confirmed by TLC, GC and GC-MS. Consequently, medium B was chosen for all further experiments. Yield comparison revealed that *Candida bombicola* produced 1.3 times more arachidonic acid derived sophorolipids than *Candida apicola* under optimal conditions. The decrease in yield of sophorolipids proved more difficult to extract in case of *Candida apicola* as it formed stable emulsion when the cultures were extracted with ethyl acetate.

2.3.2. Effect of glucose concentration on production of arachidonic acid derived sophorolipids by *Candida bombicola* and *Candida apicola*

Table 2.4. Effect of Glucose concentration of medium (B) on growth of *Candida bombicola* for arachidonic acid derived sophorolipids production

Time period	Glucose (5 %)	Glucose (7 %)	Glucose (10 %)
24 h	**0.331**	**0.350**	**0.540**
48 h	**0.332**	**0.372**	**0.543**
72 h	**0.388**	**0.581**	**0.652**
96 h	**0.485**	**0.602**	**0.701**
120 h	**0.487**	**0.604**	**0.705**

(Sophorolipids obtained from 250 ml media, yield in grams)

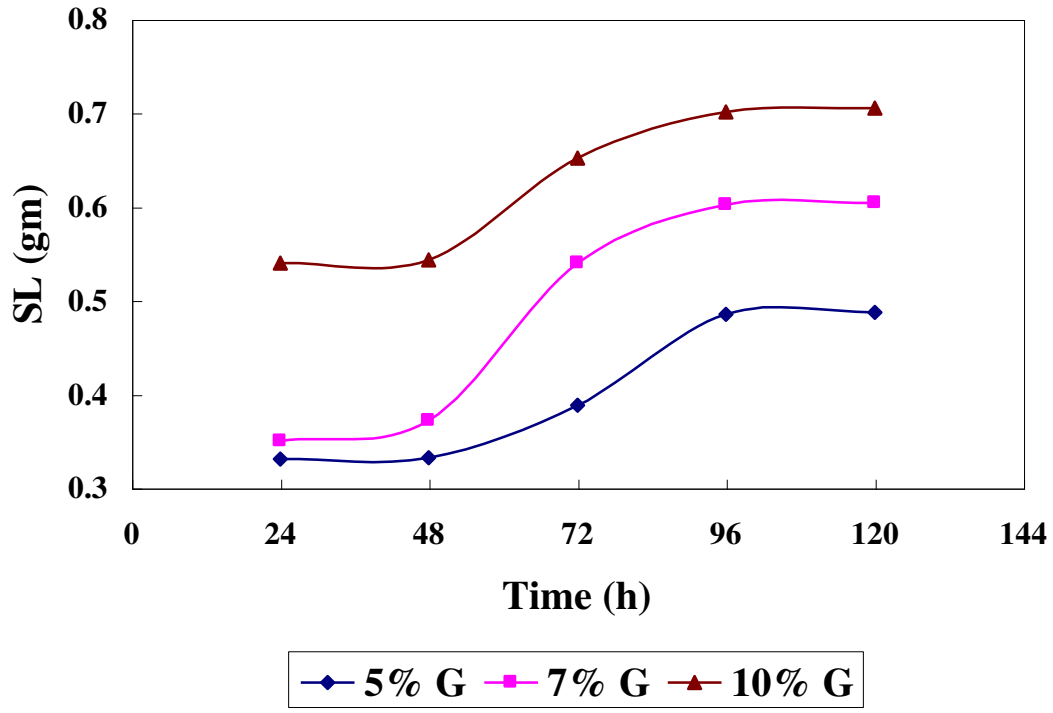


Figure 2.1. Effect of Glucose (G) concentration of medium (B) on growth of *Candida bombicola* for arachidonic acid derived sophorolipids (SL) production

Table 2.5. Effect of Glucose concentration of medium (B) on growth of *Candida apicola* for arachidonic acid derived sophorolipids production

Time period	Glucose (5 %)	Glucose (7 %)	Glucose (10 %)
24 h	0.181	0.26	0.413
48 h	0.202	0.335	0.421
72 h	0.245	0.384	0.5
96 h	0.37	0.483	0.624
120 h	0.371	0.487	0.627

(Sophorolipids obtained from 250 ml media, yield in grams)

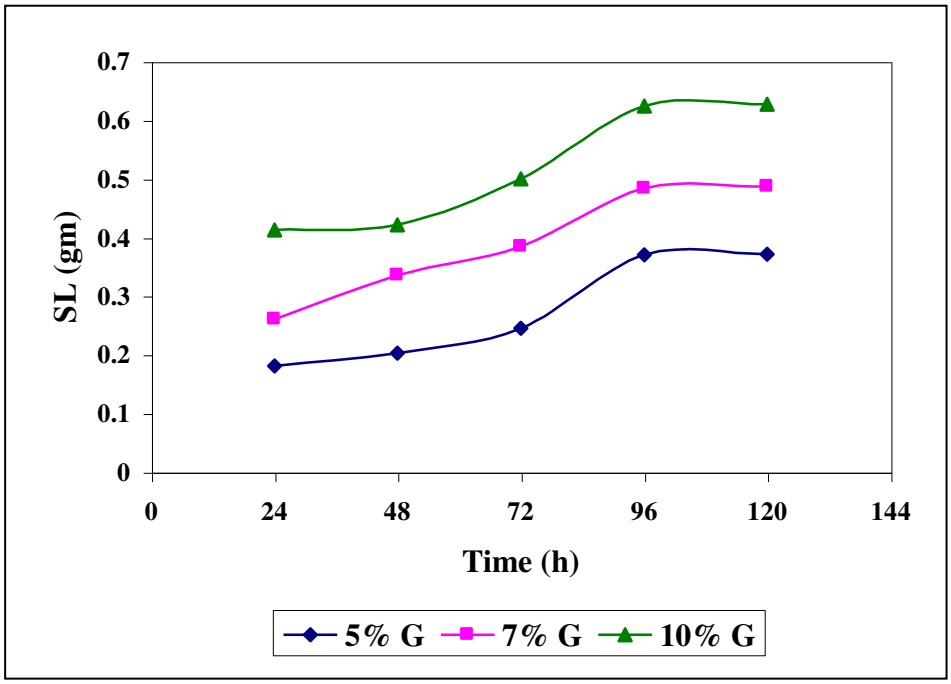


Figure 2.2. Effect of carbon source (Glucose, G) on growth of *Candida apicola* for arachidonic acid derived sophorolipids (SL) production in medium (B)

For production of sophorolipids two distinct enzymes are involved in formation of sophorose moiety, Glycosyl transferase I and Glycosyl transferase II which further activates hydroxylation of fatty acids.[1, 5] As per **Figure 2.1 & 2.2** it was observed that increase in glucose concentration increased the yield of sophorolipid production by *Candida bombicola* and *Candida apicola* by 1.5 times and 1.7 times respectively. However the final yields were higher with *Candida bombicola* (0.701 g) than that of *Candida apicola* (0.624 g) in medium B after 96 h incubation. Sophorolipid production showed a typical sigmoid behaviour involving lag phase upto 48 h, followed by a log phase extending to 96 h and thereafter stabilization into a stationary phase. In case of *Candida bombicola*, rate of log phase increase was higher at 7 % glucose concentration, however final yield did not raise upto the levels produced at 10 % glucose concentration.

2.3.3. Effect of pH on production of arachidonic acid derived sophorolipids by *Candida bombicola*

Table 2.6. Effect of pH on growth of *Candida bombicola* for arachidonic acid derived sophorolipids production in medium (B)

pH of the medium during fermentation	Yield of Sophorolipid In gm/250ml	Hydrolyzed Sophorolipid (mixture of oxylipins) (gm)
4	0.293	0.154
5	0.487	0.255
6	0.432	0.227
7	0.291	0.194

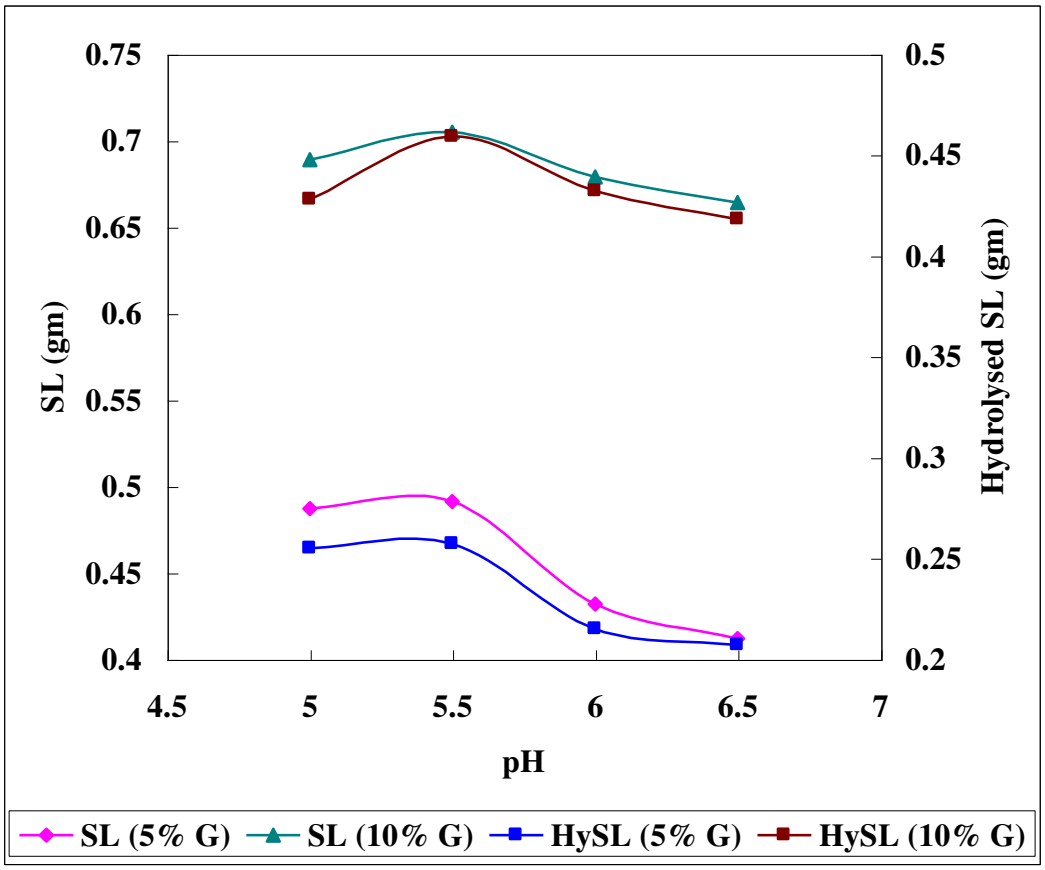
Effect of pH on sophorolipid production, 5 % Glucose

5	0.487	0.255
5.5	0.491	0.257
6	0.432	0.215
6.5	0.412	0.207

Effect of pH on sophorolipid production, 10 % Glucose

5	0.689	0.428
5.5	0.705	0.459
6	0.679	0.432
6.5	0.664	0.418

* In all the experiments cultures were incubated for 96 h. sophorolipid yields, in grams.



(G: Glucose, SL: Sophorolipids, HySL: Hydrolysed Sophorolipids)

Figure 2.3. Effect of pH on growth of *Candida bombicola* for arachidonic acid derived sophorolipids production in medium (B)

2.3.3.1. Effect of pH on growth of *Candida apicola* for arachidonic acid derived sophorolipid production

Table 2.7. Effect of pH on growth of *Candida apicola* for arachidonic acid derived sophorolipids production in medium B

pH of the medium during fermentation	Yield of Sophorolipid In mg /250 ml	Hydrolyzed Sophorolipid (mixture of oxylipins) gm
4	0.273	0.149
5	0.402	0.170
6	0.455	0.205
7	0.275	0.140

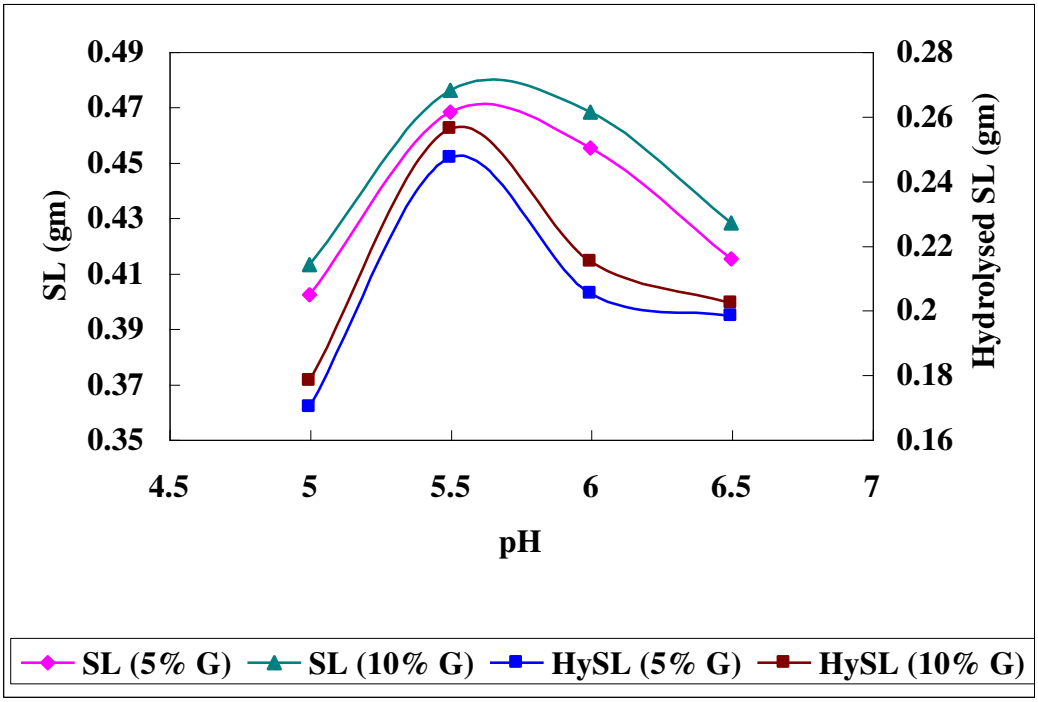
Effect of pH on sophorolipid production, 5% Glucose

5	0.402	0.170
5.5	0.468	0.247
6	0.455	0.205
6.5	0.415	0.198

Effect of pH on sophorolipid production, 10 % Glucose

5	0.583	0.348
5.5	0.626	0.396
6	0.568	0.345
6.5	0.538	0.332

* In all the experiments cultures were incubated for 96 h. sophorolipid yield in grams.



(G: Glucose, SL: Sophorolipids, HySL: Hydrolysed Sophorolipids)

Figure 2.4. Effect of pH on production of arachidonic acid derived sophorolipids by *Candida apicola* in medium (B)

Variation in fermentation pH revealed that pH 5.0 and 6.0 supported the highest production of sophorolipids in both *Candida bombicola* and *Candida apicola.* Same behavior was observed in yield of hydrolyzed sophorolipids. Deviation of pH from these optimal values reduced the yield by almost half the amount. The result obtained for the culture of *Candida bombicola* suggested maximum yield between pH 5.0 and 6.0 along with a shoulder peak at pH 4.0, which needed to be explained. This was true for the production of both sophorolipid as well as hydrolyzed sophorolipids.

In both the cultures, there was considerable difference between production of sophorolipid at pH 5.0 and pH 4.0. At pH 5.0 almost 1.7 times more sophorolipid was obtained as compared to pH 4.0 in *Candida bombicola* with 5 % glucose concentration. In case of *Candida apicola* the production of sophorolipids at pH 5.0 was almost 1.5 times more as compared to pH 4.0.

Interestingly the results obtained for *Candida bombicola* had distinct pH maxima with a shift towards pH 6.0 while maintaining the shoulder peak at pH 4.0 similar to *Candida apicola*. In case of both cultures at pH 5.0 the relative comparison at different glucose concentration 5 % and 10 % was 1.4 times more productions of sophorolipids in 10 % glucose. In *Candida bombicola* at pH 5.5 the relative comparison of different glucose concentration 5 % and 10 % was 1.5 times more productions of sophorolipids in 10 % glucose concentration. In case of *Candida apicola* it was 1.3 times more production of sophorolipids in 10 % glucose.

Both cultures showed maximum yield of sophorolipids and hydrolyzed sophorolipids at pH 5.5 in the presence of 10 % glucose. In case of *Candida apicola* the arachidonic acid derived sophorolipids provide more difficulty to extract and purify as it

formed stable emulsion when the cultures were extracted with ethyl acetate. Further work was therefore conducted with only *Candida bombicola*.

2.3.4. Effect of temperature on production of arachidonic acid derived sophorolipids by *Candida bombicola* and *Candida apicola*

Here we have examined the biomass and sophorolipid production at distinct temperatures ranging from 25 ºC to 36 ºC. This experiment was done in parallel in two conditions with 5 % and 10 % of glucose concentrations. Investigations with *Candida bombicola* have shown maximum biomass at temperature 30 ºC whereas the maximum yield of sophorolipid was obtained at 28 ºC. This point should be noted that almost similar biomass at temp 28 ºC was observed when 10 % of glucose was used in media. Although the biomass kept on increasing even after temp 28 ºC till 30 ºC, the sophorolipid production declined significantly. The double concentration of glucose helped in higher production of sophorolipids at all the levels.

Table 2.8. Effect of temperature on production of arachidonic acid derived sophorolipids by *Candida bombicola* in medium B

5 % Glucose *Candida bombicola*

Temperature (ºC)	Biomass (gm)	Sophorolipid (gm)
25	**1.10**	**0.101**
27	**2.45**	**0.325**
28	**2.90**	**0.460**
30	**3.85**	**0.310**
32	**3.50**	**0.281**
34	**3.15**	**0.101**
36	**2.90**	**0.090**

10 % Glucose *Candida bombicola*

Temperature (ºC)	Biomass (gm)	Sophorolipid (gm)
25	**1.9**	**0.310**
27	**3.18**	**0.632**
28	**3.38**	**0.708**
30	**4.84**	**0.625**
32	**4.16**	**0.478**
34	**3.82**	**0.221**
36	**3.15**	**0.202**

* In all the experiments cultures were incubated for 96 h.

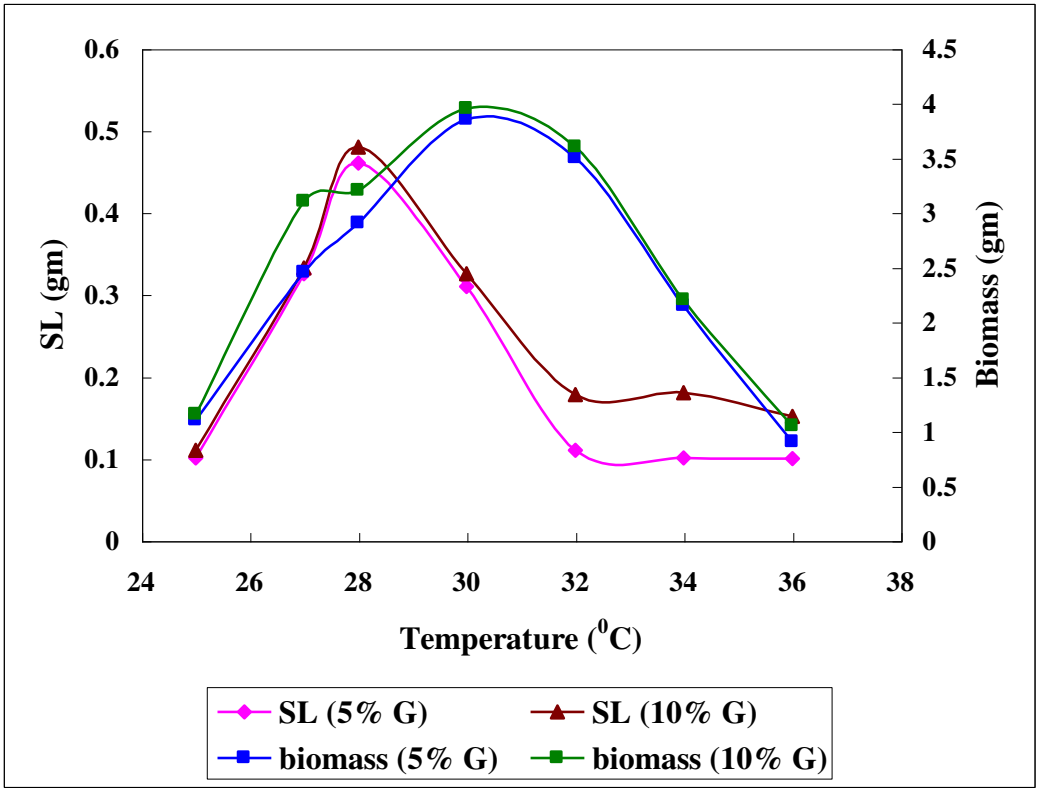


Figure 2.5. Effect of temperature on production of arachidonic acid derived sophorolipids (SL) by *Candida bombicola* in medium (B)

2.3.4.1. Effect of temperature on production of arachidonic acid derived sophorolipids by *Candida apicola*

Table 2.9. Effect of temperature on production of arachidonic acid derived sophorolipids by *Candida apicola* in medium B

5 % Glucose *Candida apicola*

Temperature (ºC)	Biomass (gm)	Sophorolipid (gm)
25	1.08	0.081
27	1.85	0.312
28	2.30	0.432
30	3.18	0.290
32	2.90	0.264
34	1.95	0.094
36	1.80	0.081

10 % Glucose *Candida apicola*

Temperature (ºC)	Biomass (gm)	Sophorolipid (gm)
25	1.12	0.204
27	3.18	0.487
28	3.31	0.626
30	4.67	0.56
32	3.92	0.368
34	2.68	0.211
36	2.15	0.198

* In all the experiments cultures were incubated for 96 h.

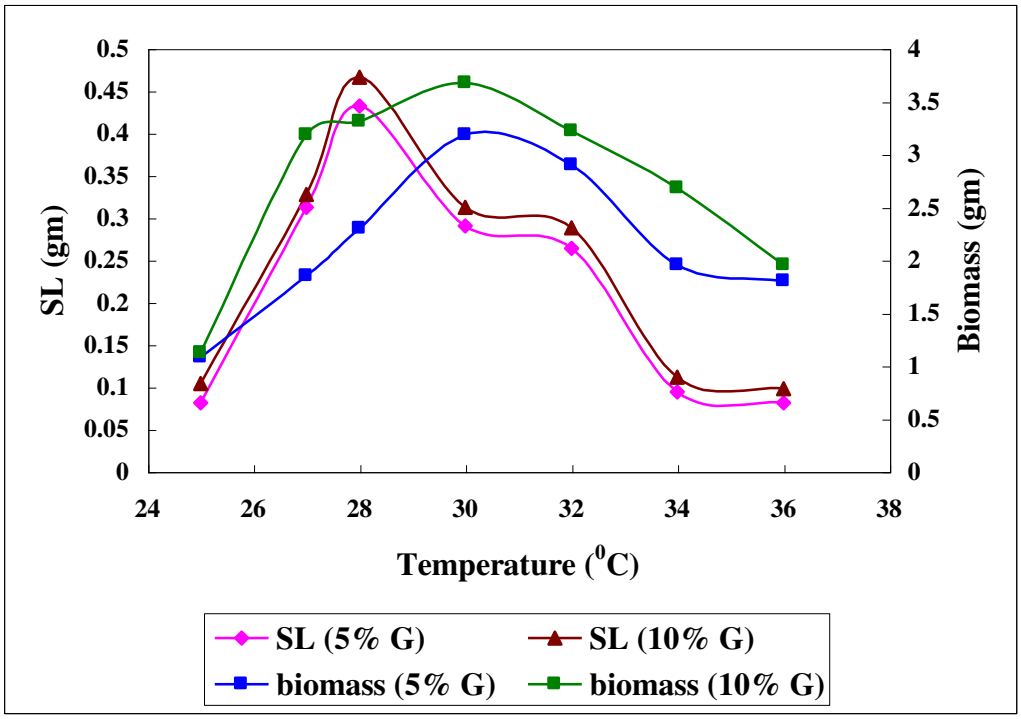


Figure 2.6. Effect of temperature on production of arachidonic acid derived sophorolipids by *Candida apicola* in medium (B)

Experiment was carried out as described in section 2.2.6 to study the dual effect of temperature and glucose in medium B. As per **Figure 2.6** similar patterns was observed for *Candida apicola*, though the biomass still persevered at 36 ºC, which declined significantly in *Candida bombicola*. In *Candida apicola* a sharp peak at 28 ºC was obtained for highest production of sophorolipid along with a shoulder peak at 32 ºC, which was slightly different from *Candida bombicola.* Though in both cases, 30 ºC showed maximum biomass, 28 ºC showed better sophorolipids production.

2.3.5. Effect of ferric ions on the production of arachidonic acid derived sophorolipids by *Candida bombicola*

Table: 2.10. Effect of Fe^{+3} on Sophorolipid production by *Candida bombicola* (5 % glucose, 250 ml of the medium B)

Fe^{+3} (mM)	Biomass (gm)	Yield of Sophorolipid (gm)
10	5.52	0.464
20	4.84	0.473
30	4.29	0.469

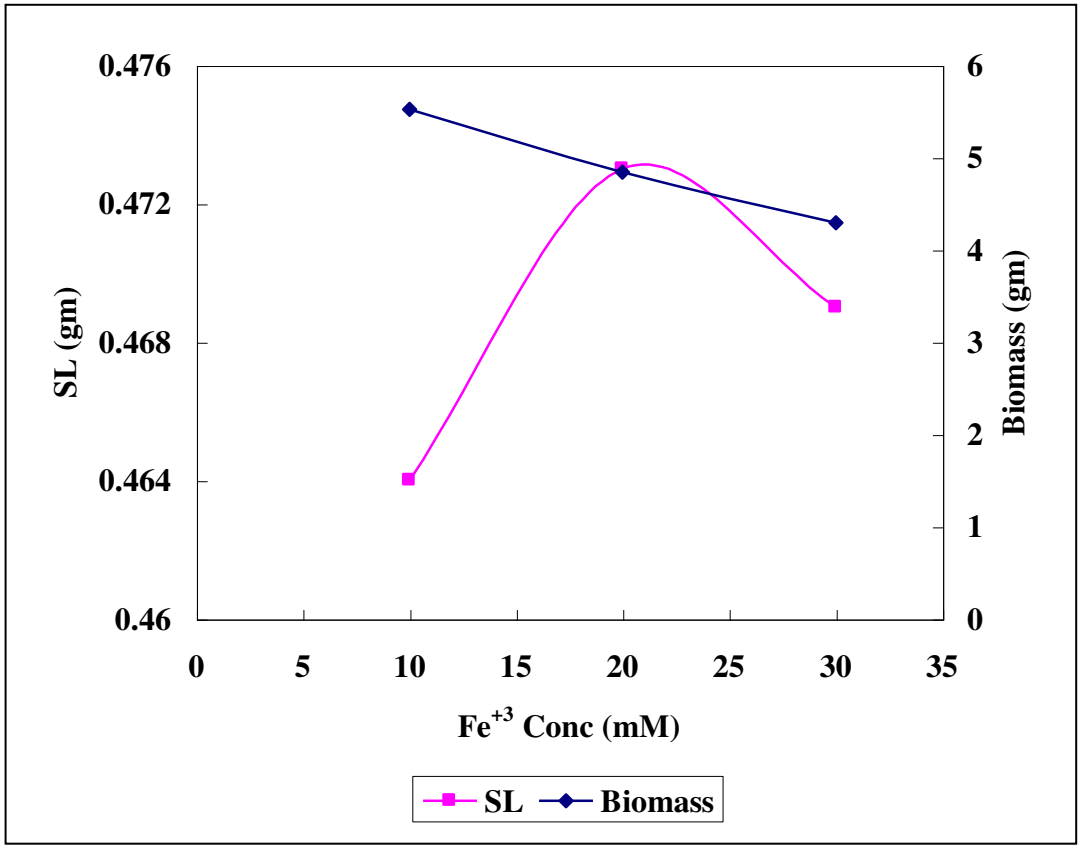


Figure 2.7. Effect of ferric ions on the production of arachidonic acid derived sophorolipids (SL) by *Candida bombicola*

2.3.5.1. Effect of ferric ions on the production of arachidonic acid derived sophorolipids by *Candida apicola*

Table: 2.11. Effect of Fe^{+3} on Sophorolipid production by *Candida apicola* (5 % glucose, 250 ml of the medium B).

Fe^{+3} (mM)	Biomass (gm)	Yield of Sophorolipid (gm)
10	5.21	0.438
20	4.42	0.462
30	4.20	0.449

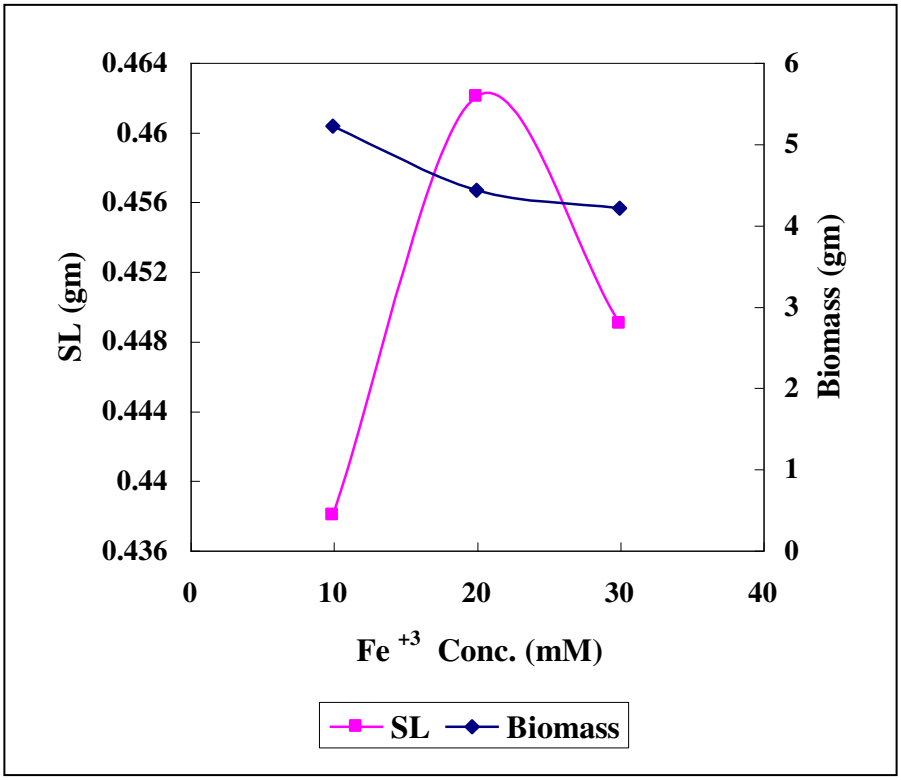


Figure 2.8. Effect of metal ions on growth of *Candida apicola* for arachidonic acid derived sophorolipids (SL) production

As described in Chapter 1, arachidonic acid derivatives are implicated in a number of physiological processes. Arachidonic acid is converted into its oxygenated form especially oxylipins, through cytochrome P450 pathway. Since cytochrome P450 are heme containing enzymes, effect of Fe was checked on efficiency of the reaction and overall conversion.

In case of *Candida bombicola*, 20 mM concentration of Fe^{+3} in the form of ferric chloride ($FeCl_3$) showed maximum yield of sophorolipids with increased biomass as compared to the cells where Fe was not supplemented. *Candida bombicola* showed better yield in sophorolipid production than *Candida apicola.* It was observed that in both the cultures, on addition of Fe^{+2} in the form of Ferrous sulphate (Fe_2SO_4), no significant increase was observed in sophorolipid as well as biomass. Though Fe increased the sophorolipid levels in both the cultures, hydrolyzed product showed no significant increase in oxylipins hence was not used for further work. Additionally, Fe ($FeCl_3$) showed formation of emulsions in *Candida bombicola*, which was not observed when grown without Fe.

2.4. Conclusion

The media used in the present studies included complex organic sources of carbon and nitrogen, such as yeast extract, beef extract in various combinations and levels in addition to glucose and arachidonic acid. Sophorolipid production on the five test media has been shown in Table 2.1. In five test media, medium B showed significant biomass and highest activity of oxylipins were almost similar in *Candida bombicola* and *Candida apicola.* Concentration of glucose affected the biomass as well as sophorolipid production in both yeast species. Highest activity was obtained in medium B with 10 %

glucose concentration at pH 5.5 for 96 h incubation at 28 ºC, on the basis of arachidonic acid derived sophorolipid, biomass and liberated 19-HETE and 20-HETE, which was in the ratio of 3: 1. In case of *Candida apicola* the arachidonic acid derived sophorolipids provided more difficulty to extract and purify as it formed stable emulsion when the culture broth was extracted with ethyl acetate. Further work was therefore conducted with only *Candida bombicola*.

Characterization techniques

The different experimental techniques used during the course of the present work are discussed in this chapter.

2.5. Characterization techniques

This part of the chapter presents the different techniques that have been used to characterize the biotransformation reaction of arachidonic acid to 19-HETE and 20-HETE. A number of characterizations have been done for different biocompatible materials and *Candida bombicola* cell immobilization on thermally evaporated fatty acid/amine lipid films and on the surface modified gold nanoparticle polymeric membrane.

Various characterization techniques such as Thin layer chromatography, Gas Chromatography (GC) and Gas Chromatography / Mass Spectrometry (GC-MS), UV-visible spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), Quartz crystal microgravimetry (QCM), Scanning Electron Microscopy (SEM), Atomic Force microscopy (AFM), Transmission Electron Microscopy (TEM), X-ray diffraction, Contact angle measurements and Biocatalytic activity measurements have been used to characterize these biocompatible material and biotransformation of arachidonic acid to 19-HETE and 20-HETE.

2.5.1. Thin layer chromatography (TLC)

This is probably the only separative analytical technique that has been most extensively used. It is also the quickest, simplest and cheapest of the various analytical techniques. However, the resolution is not as good in the case of HPLC and GC. Silica gel is the preferred adsorbent for the thin-layer chromatography for arachidonic acid, arachidonic acid derived product, sophorolipids and monohydroxy arachidonic acid compounds, 19-hydroxyeicosatetraenoic acid and 20-hydroxyeicosatetraenoic acid. Commercially available standard silica gel coated on aluminum plates (Merck,

Darmstadt, Germany; silica layer thickness 2 mm) is used for TLC. Glass micro-capillary is used for spot-application of samples. R_f values are frequently cited to confirm the presence of sophorolipids and their derived products, eicosanoids. The solvent mixture used to develop thin-layer chromatography plates requires constituents of different polar and nonpolar solvents for analysis of arachidonic acid derived products. Many solvent systems have been developed for the separation of the sophorolipids and HETE's on silica. Less polar system, such as diethyl ether / petroleum ether / acetic acid (50: 50: 1, by vol.), diethyl ether / hexane / formic acid (60: 40: 1, by vol.) and methanol / chloroform (10: 90, by vol.) are used for the compounds like arachidonic acid and the monohydroxy fatty acids compounds. Different methods are used for the detection of arachidonic acid and their derived products sophorolipids with various eicosanoids (HETE's) on TLC. Ultra visible absorbing compounds can be visualized under detection at 254 nm and 365 nm UV light. The plates are coated with silica impregnated with the fluorescent indicator F254 nm. Chemical spray reagent with the different charring solution like anisaldehyde solution, sulfuric acid / methanol (50: 50 by vol.) can be used. The simplest of these is staining with iodine vapors. This only works for the compounds with double bonds and less sensitive for compounds with only one double bond.

2.5.2 Gas Chromatography (GC) and Gas Chromatography / Mass Spectrometry (GC-MS)

Gas Chromatography (GC) with mass spectrometry (MS) detection is the most powerful technique for the analysis of eicosanoids. It has the advantages of very high sensitivity, excellent resolution and that it provides two parameters for characteristic fragment ions by mass spectrometry. Fragmentation of molecules into characteristic ions

can be used for the identification of compounds. This technique has been widely used for both, structure elucidation and quantification of very low concentrations of eicosanoids derived from transformation of arachidonic acid using *Candida* sp.

In 1919, Aston invented the first mass spectrometer although his apparatus is known as a mass spectrograph. Mass spectrometers are used to measure the masses of atoms and molecules with great accuracy.[6] They are also capable of detecting remarkably small amounts of an element or compound in the range $10^{-6} – 10^{-12}$g. Mass spectrometry is based on the formation of ions and their separation in a magnetic or electrostatic field. Charge is prerequisite for analysis. There are two principal types of mass spectrometers commercially available – magnetic sector and quadrupole (electrostatic) instruments. The main difference between these mass spectrometers lies in the principles governing the separation of ions. In quadrupole mass spectrometer, ions are generated in the source with low energy, which is ejected into the mass analyzer consisting of four symmetrically positioned rods. Since only ions are mass analyzed, the neutral molecule must be ionized. Electron impact, chemical, electron capture negative ion chemical and soft ionization are the techniques of ionization employed. Electron impact ionization is the most commonly used technique.

In this thesis, the biotransformation of the arachidonic acid to sophorolipids by using cytochrome P450 enzyme present in the yeast *Candida bombicola and Candida apicola* is described. Thereafter, the acid hydrolysis of these sophorolipids yields 19-hydroxyeicosatetraenoic acid (19-HETE) and 20-hydroxyeicosatetraenoic acid (20-HETE). The hydroxyl group present at the 19^{th} and 20^{th} carbon position are confirmed by derivatives of 19-HETE and 20-HETE to their respective methyl ester silyl ether of 19-

hydroxy-5Z, 8Z, 11Z, 14Z eicosatetraenoic acid and 20-hydroxy-5Z, 8Z, 11Z, 14Z eicosatetraenoic acid and thereafter analyzed by mass spectrometer.[7, 8] These measurements were done on Shimadzu GCMS QP 5050 automated quadrupole mass spectrometer operating in the electron impact mode. GC parameters: column used, BP-5 fused silica column (30 m x 0.25 mm, 0.25 mm, 0.25 mm coatings); He carrier at 14 kPa head pressure; injector at 250 °C; column initially at 150 °C for 1 min (rate 35 °C/min) and then increased to 220 °C for 5 min (rate 5 °C/min) and then at 280 °C and then held at these conditions for 10 min; injection volume, 1 μL. MS parameters: interface temperature 250 °C, ionization mode electron impact, scan range 70-800 amu (arbitrary mass units) s-1.

2.5.3. Thermal evaporation of amine lipids

Thermally evaporated amine lipid films have been used for whole cells immobilization for the transformation of arachidonic to 19-HETE and 20-HETE. In this thesis, thermally evaporated amine lipid films have been deposited using Edwards E306 coating unit. The coating unit consists of a rotary pump used for backing and roughing the chamber where deposition is done and can produce a vacuum upto 10^{-3} Torr. Below this pressure, oil diffusion pump is employed to produce high vacuum of 10^{-7} Torr [9] and the deposition rate was 10 Å /min. Both these pumps are used in conjunction for backing and roughing of the deposition chamber. A liquid nitrogen trap was also used. Deposition of organic thin films is done under vacuum. The quality of deposition is better due to the increased mean free path of a molecule under vacuum as compared to atmosphere, resulting in a linear trajectory of the thermally evaporated molecule. The melting point of amine lipids is reduced under vacuum, enabling low current requirements for thermal

evaporation. The amine lipids (amphiphilic molecules) used for deposition, were taken in a molybdenum boat and subjected to low tension DC of about 20 amps under 10^{-7} Torr vacuum and heated. The substrates such as Si (111), glass and quartz were kept at a suitable distance above the molybdenum boat. The molecules when heated evaporated and condensed onto these substrates giving nearly same thickness of the uniform films on each substrate. The rate of deposition and the thickness of the lipid films was monitored *in-situ* using Edwards FTM5 quartz crystal microbalance. The films were tested for stability by using infrared spectroscopy, and it was found that the films did not decompose on deposition in vacuum. An amphiphilic molecule has a hydrophobic and a hydrophilic part. The hydrophobic end is normally a long chain of hydrocarbons called the tail and the hydrophilic moiety is called the polar head group as shown in **Figure 2.9.**

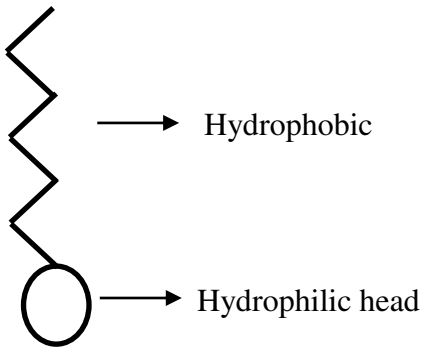


Figure 2.9. Schematic representation of an amphiphilic molecule showing the hydrophobic long chain hydrocarbons and hydrophilic head group. The amphiphilic molecules we have used in this work is octadecylamine [ODA; CH3-(CH2)17-NH2].

2.5.4. Quartz crystal microgravimetry (QCM)

In this thesis, QCM have been used for calculating the amount of entrapped whole cells within the stacks of lipid bilayers. The knowledge of amount of the immobilized biocatalyst is important to compare the activity of the immobilized whole cells with the similar amount of the free cells in solution. In 1880, Jacques and Pieree Curie discovered that mechanical stress applied to the surfaces of various crystals such as quartz, rochelle and tourmaline afforded a corresponding electrical potential across the crystal whose magnitude was proportional to the applied stress.[10] This behavior is referred to as the *piezoelectric effect*. This property exists only in materials that are acentric that is those crystallize in noncentrosymmetric space groups. A single crystal of an acentric material will possess a polar axis due to dipoles associated with the orientation of atoms in the crystalline lattice. When stress is applied across an appropriate direction, there is a shift of dipoles resulting from the displacement of atoms. This atomic displacement leads to a corresponding change in the net dipole moment producing a net change in electrical charge on the faces of the crystal. The validity of the converse of this effect was also established wherein application of a voltage across these crystals afforded a corresponding mechanical strain. This inverse piezoelectric effect is the basis of the quartz crystal microgravimetry (QCM) technique. AT-cut quartz resonator, in which thin quartz wafer is prepared by slicing a quartz rod at an angle of 35 degree with respect to the X-axis of the crystal, resonates in the *thickness shear mode*.

The work described in this thesis, we have used gold-coated AT-cut 6 MHz quartz crystal. The frequency counter used was an Edwards FTM5 instrument operating at a frequency stability and resolution of ± 1 Hz. At this resolution and with the type of quartz

crystal used, the mass resolution would be 12 ng/cm^2. The different thicknesses of lipid films were thermally evaporated on the QCM crystals. These crystals were immersed into *C. bombicola* cells suspension for different time intervals and the frequency changes were measured *ex-situ* after thorough washing with deionized water and drying in flowing nitrogen. The frequency changes were converted to a mass uptake by using the standard Saurbrey formula.[11] The "leaching out" of the cells were also studied by QCM by immersing the enzyme incorporated lipid films in appropriate buffer solution and measuring the frequency change at different time intervals The work discussed in this thesis, QCM plays an important role in determining the amount of *Candida bombicola* cells entrapped within the stacks of lipid bilayers. The thick ODA films of thickness 250 Å on gold-coated AT-cut quartz crystals were used in this study.

2.5.6. UV-visible spectroscopy (UV-vis)

It deals with the study of electronic transitions between orbital or bands of atoms, ions or molecules in gaseous, liquid and solid state.[12] The work described in this thesis, UV-Vis spectroscopy was used to monitor the binding of gold nanoparticles embedded in the polymeric membrane. UV-vis spectra of gold nanoparticle polymeric membrane transferred on quartz substrate were recorded in transmission mode. These measurements were done on a JASCO V570 UV/VIS/NIR operated at a resolution of 1 nm.

2.5.7. X-ray diffraction measurements (XRD)

We have used XRD for the determination of gold nanoparticles embedded in the polymeric membrane was recorded. XRD measurements of as synthesized nanoparticles and before and after functionalized with octadecylamine were done. These measurements

were done on a Philips PW 1830 instrument operating at 40 kV and a current of 30 mA with Cu Ká.

2.5.8. Fourier transforms infrared spectroscopy (FT-IR)

Infrared (IR) spectroscopy is a chemical analytical technique, which measures the infrared intensity versus wavelength (wavenumber) of light. Based upon the wavenumber, infrared light can be categorized as far infrared (4 ~ 400cm^{-1}), mid infrared (400 ~ 4,000cm^{-1}) and near infrared (4,000 ~ 14,000cm^{-1}). Infrared spectroscopy detects the vibration characteristics of chemical functional groups in a sample. When an infrared light interacts with the matter, chemical bonds will stretch, contract and bend. As a result, a chemical functional group tends to adsorb infrared radiation in a specific wave number range regardless of the structure of the rest of the molecule. A Fourier Transform Infrared (FTIR) spectrometer obtains infrared spectra by first collecting an interferogram of a sample signal with an interferometer, which measures all of infrared frequencies simultaneously. An FTIR spectrometer acquires and digitizes the interferogram, performs the FT function, and outputs the spectrum. The energy required exciting the bonds in a compound and making them to vibrate more energetically occurs in the infrared region of the spectrum (ca. 400-4000 cm-1). If we pass a beam of infrared radiations of varying frequency through a sample then the energy of the beam is absorbed. This happens when the energy matches the difference between vibrational energy levels belonging to the bonds.

Peak assignments for fatty lipids used in this thesis are octadecylamine (ODA). The two bands at 2920 and 2850 cm-1 have been assigned to the unsymmetric and symmetric methylene (CH2) stretching vibrations respectively and two weak bands at

about 2960 and 2875 cm^{-1} to the asymmetric/degenerate and symmetric methyl (CH3) stretching vibrations respectively. The position of the peaks and the increase in intensity of the methylene stretching vibrations relative to methyl stretching vibration with chain length indicates structural integrity of the molecule. More interestingly, actual peak values of the symmetric and unsymmetric CH2 stretching vibrations can be used as a sensitive indicator of the ordering of the alkyl chains. Typical peaks for the free amine are seen at 3333 cm-1 of octadecylamine.[13]

The work described in this thesis, 250 Å thick thermally evaporated amine lipid films were deposited on a Si (111) wafer. The silicon substrates were used in this study since they are chemically stable and generally are not reactive. It is excellent for optical studies of deposited films in the visible region using reflection techniques. It does not have strong lattice absorption bands in the useful regions of the infrared and thus can be used for transmission studies in this region. To correct for the lattice absorption bands in silicon, a reference silicon sample is used as a reference. FTIR measurements of 250 Å thick amine biocomposite lipid films on Si (111) substrates were carried out *ex-situ* after immersion of the films in the aqueous suspension of *Candida bombicola* cells dispersed in deionized water. Through washing and drying of the films was done prior to FTIR measurements. For comparison FTIR measurements of as-deposited amine lipids films were also recorded. FTIR measurements of gold nanoparticle polymeric membrane before and after binding with octadecylamine were also recorded by transferring the membranes on Si (111) substrate. These measurements were done in the diffuse reflectance mode on a Perkin-Elmer Spectrum One FTIR spectrometer operated at the resolution of 4 cm^{-1}.

2.5.9. Scanning electron microscopy (SEM)

Scanning Electron Microscopy is extremely useful for the direct observations of surfaces because they offer better resolution and depth of field than optical microscope. The study of SEM in our case is important in order to rule out any surface attachment of whole cells *Candida bombicola* on the lipid surface. SEM and EDAX measurements were also used to confirm the attachment of *Candida bombicola* on biomaterial surfaces. Samples for SEM and EDAX measurements were prepared by drop-coating a film on a Si (111) substrate. SEM and EDAX measurements were also used to confirm the binding of whole cells to the patterned thermally evaporated fatty lipid films and octadecylamine bound to gold nanoparticle polymeric membrane. SEM images of whole cells bound to as-prepared gold nanoparticle polymeric membrane. The Binding of Fe nanoparticles to *Candida bombicola* cells and similarly, the attachment of *C. bombicola* on nanoporous alumina membrane were also recorded. These measurements were performed on a Leica Stereoscan-440 scanning electron microscopy (SEM) equipped with a Phoenix EDAX attachment.

2.5.10. Transmission electron microscopy (TEM)

The work described in this thesis, TEM is used to determine the particle size of gold nanoparticles embedded in polymeric membrane. Samples for TEM analysis were prepared by transferring the gold nanoparticle polymeric membrane from the liquid-liquid interface onto carbon-coated copper TEM grids. TEM micrographs of the gold nanoparticles leached from the polymeric membrane and only the polymeric membrane were also recorded. These measurements were performed on a JEOL Model 1200EX instrument operated at an accelerating voltage of 120 kV. TEM is a method of producing

images of a sample by illuminating the sample with electronic radiation (under vacuum) and detecting the electrons that are transmitted through the sample.

2.6. References

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Chapter 3

Dry column chromatography technique used for the purification of different forms of sophorolipids produced by *Candida bombicola*

This chapter presents the purification of arachidonic acid derived sophorolipids produced from the yeast *Candida bombicola* (ATCC 22214) when grown on glucose and secondary carbon source, arachidonic acid. The crude product was a heterogeneous mixture of sophorolipids, which are glycolipids of sophorose, linked to the fatty acid through glycosidic bond between ω and ω-1 carbon of arachidonic acid. The derived sophorolipids were isolated by silicagel chromatography using dialysis tubing. Acid hydrolysis of the resolved sophorolipids was analyzed and it showed the presence of 19-hydroxyeicosatetraenoic (19-HETE) and 20-hydroxyeicosatetraenoic (20-HETE). These are the compounds of pronounced pharmaceutical importance.

Part of the work presented in this chapter has been accepted in: *Biotech. Lett.* **2006. (In press)**

Sachin Shah and Asmita Prabhune.

3.1.Introduction

Sophorolipids are extracellular surface-active glycolipids that are produced by yeast genus *Candida* (formally *Torulopsis*) such as *Candida bombicola*, *Candida apicola* and *Candida gropengiesseri.*[1] The yeasts *Candida bombicola* ATCC 22214 and *Candida apicola* ATCC 96134 when grown on primary carbon sources, usually glucose and long chain fatty acid as a secondary carbon source produce sophorolipids. These hydroxy fatty acids are produced by direct hydroxylation of long chain fatty acids (C_{16} to C_{18}) which are supplied exogenously[2] and are usually either saturated or monounsaturated[3, 4] or can be derived by oxidation of n-alkanes.[5, 6] The sophorolipids are consisting of a sophorose moiety (2-*O*-β-D-glucopyranosyl-β-D-glucopyranose) linked glycosidically to a hydroxyl fatty acid residue. These are categorized in two main types as acidic and lactonic forms. It has been found that nine different structural classes of oleic acid derived sophorolipids are observed.[7] The mixture of sophorolipids is typically in the form of brown oil or semisolid and denser than water in nature. The sophorolipid production occurs in the presence of a simple carbohydrate substrate.[8] It has been established that the highest sophorolipid yields are obtained when an additional, hydrophobic substrate is provided.[9] The hydroxy fatty acid moiety of the acidic sophorolipids remains a free acid and the moiety of the lactonic sophorolipids forms a macrocyclic lactones ring with the 4”-OH group of the sophorose. The water-soluble substrate is used primarily for cellular metabolism and for synthesis of the hydrophilic sophorolipid moiety, while the lipophilic substrate is used exclusively for production of the hydroxy carboxylic acid moiety. This is related to the observation that *Candida* sp. is capable of directly incorporating fatty acids into the sophorolipid product.[10] The fatty acid moiety of the sophorolipid can be

synthesized by direct incorporation and it is more efficient to produce sophorolipid.[9] In this process, the lipophilic substrate is not broken down via the b-oxidation pathway, but instead it is immediately hydroxylated and subsequently incorporated into the sophorolipid.[11, 12]

The properties as well as specific applications of these sophorolipids are dependent on their respective forms.[13] Acetylated lactonic sophorolipids have been reported as commercial and scientific importance as they show biocide activity,[14] anticancer activity[15] and have been used in cosmetics as antidandruff, bacteriostatic agents, deodorant,[16] shampoo and moisturizing agent.[17] They are also utilized as agents for stimulating skin fibroblast metabolism.[18, 19]

Acidic sophorolipids have found applications in the therapeutics active for skin diseases,[20] such as in fibrinology, healing, desquamation, depigmenting, and also for macrophage activation.[21]

As the heterogeneous mixture of sophorolipids has varied activity and amphiphilic properties, their exploitation requires purification of the chemically distinct forms. Presently available methods for purification of lactonic and acidic sophorolipids include crystallization with aqueous buffer by Hu & Ju in 2001[4] and organic solvents like ethanol by Tulloch et al. in 1968,[22] respectively. Ito et al. in 1980, Stuwer et al. in 1987 have described conventional silica gel based column chromatography for purification of sophorolipids.[23, 24] These methods require large amount of organic solvents and are cumbersome

This chapter demonstrates purification and characterization of sophorolipids produced by the yeast *Candida bombicola* (ATCC 22214) grown on primary carbon

source, glucose and secondary carbon, arachidonic acid (shown in **Figure 3.1.**). The crude sophorolipids were a heterogeneous mixture, which are glycolipids of sophorose linked to the fatty acid through glycosidic bond between ω and ω-1 carbon of arachidonic acid. The derived sophorolipids were isolated by dialysis tubing silica gel column chromatography with UV-visible GF 254_{nm} silica gel of 100-200 mesh size. This chromatography method was simple and quick for resolution of arachidonic acid derived sophorolipids. This method allows easy and rapid resolution of lactonic and acidic sophorolipids derived from arachidonic acid. Being stable in the form of sophorolipids (either lactonic or acidic) at ambient temperature, this method permitted to generate commercially useful amount of 19-hydroxyeicosatetraenoic acid (19-HETE) and 20-hydroxyeicosatetraenoic acid (20-HETE) in sophorolipid form. Sophorolipids of 19-HETE and 20-HETE can be stored at 10 ˚C without significant loss of activity and yielded respective fatty acid on acid hydrolysis. A mixture of these sophorolipids eventually can be purified and are relatively stable at 4 ˚C for more than one month. Purified arachidonic acid derived sophorolipids on the acid hydrolysis yielded 19-hydroxyeicosatetraenoic acid (19-HETE) and 20-hydroxyeicosatetraenoic acid (20-HETE). These oxylipins have pronounced pharmaceutical importance.

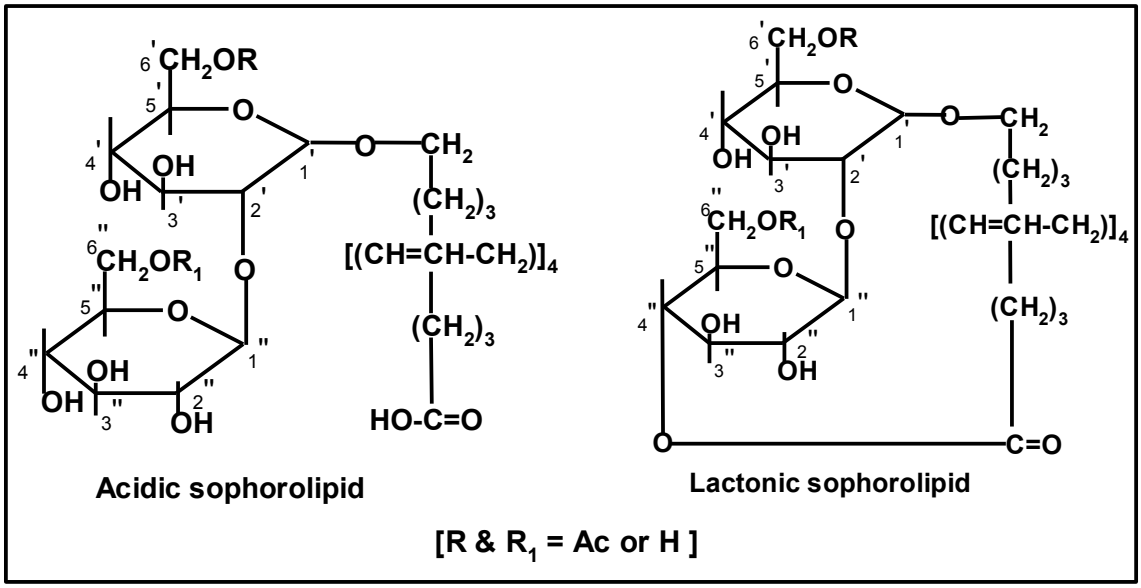


Figure 3.1. Structures of the sophorolipids synthesized by *Candida bombicola* when grown on glucose and arachidonic acid ($C_{20:4}$)

3.2. Experimental

3.2.1. Material and methods

Arachidonic acid (90 % purity) was obtained from Sigma. Methyl alcohol, chloroform, ethyl acetate were obtained from E-Merck, Laboratory grade. Dialysis tubing was obtained from Arthur H. Thomas Co. Philadelphia. Pa., U.S.A. Silica gel UV-GF 254_{nm} of 100-200 mesh size and standard Kiesel-gel 60 F 254 TLC plates were obtained from Spectrochem, India and E-Merck, respectively.

The yeasts, *Candida bombicola* ATCC 22214 and *Candida apicola* ATCC 96134 were obtained from American Type Culture Collection and maintained at 4 ºC on MGYP containing 5 % glucose agar slants. Stock cultures were maintained by subculturing at monthly intervals.

3.2.2. Production of arachidonic acid ($C_{20:4}$) derived sophorolipids

Candida bombicola (ATCC 22214) was pre-cultivated in 50 ml medium at 30 ºC consisting of (g l^{-1}): glucose, 100; yeast extract, 1; $(NH_4)_2SO_4$, 1; $MgSO_4 \cdot 7H_2O$, 0.3; Na_2HPO_4, 2; NaH_2PO_4, 7; pH 5.5 and shaking at 160 rpm. The media were sterilized before cultivation by autoclaving at 121 °C for 15 min. The process was initiated by inoculating an overnight grown culture into 1 l conical flask containing 250 ml media, composition as mentioned above. To optimize the production of sophorolipid, different media and effect of glucose concentration were checked to increase the sophorolipid production. Glucose plays important role in sophorolipid production as described in Fermentation Chapter 2. (2.3.2.). Each 1 l flask was supplemented with 0.5 g of 90 % arachidonic acid (10 % other fatty acid), obtained from Sigma and diluted in 1 ml ethanol. *Candida apicola* (ATCC 96134) was grown under identical conditions as

described for *Candida bombicola.* The process was initiated by inoculating an overnight grown culture into 1 l conical flask containing 250 ml media of composition as mentioned above. The secondary carbon source arachidonic acid was supplemented in each of 1 l flask as 0.5 g of 90 % arachidonic acid (10 % other fatty acid), diluted in 1 ml ethanol. The flasks were kept at 28 °C for 96 h at 160 rpm. It was observed and reported that the maximum growth of *Candida bombicola* and *Candida apicola* were at 30 °C but maximum sophorolipids production occurred at 28 °C. For the qualitative analysis of the sophorolipids, experiment was performed by comparing with control, where no secondary carbon source arachidonic acid has been added in 250 ml media of same composition as mention above. The control flask was inoculated with respective culture and same conditions as mentioned above.

3.2.3. Isolation of arachidonic acid ($C_{20:4}$) derived sophorolipids

Fermented broth was centrifuged at 6,000 × g, at 10 °C for 20 min after 96 h incubation. The cell-free broth was extracted twice with equal volumes of ethyl acetate. The anhydrous sodium sulfate was added to the ethyl acetate layer to remove residual water, filtered and ethyl acetate was subjected to rotary evaporation to remove the solvent. The semi crystalline product was washed twice with 5 ml of cold n-hexane to remove unmetabolized arachidonic acid. In a typical batch, 2.0 g of arachidonic acid, supplemented to 1 l batch for 96 h at 28 °C and with 160 rpm produced 1.44 g of arachidonic acid derived sophorolipid by *Candida bombicola.* A similar yield of 1.25 g of arachidonic acid derived sophorolipid was obtained using *Candida apicola.* Sophorolipids obtained from *Candida apicola* were difficult to extract with ethyl acetate due to formation of stable emulation.

3.2.4. Identification of arachidonic acid ($C_{20:4}$) derived sophorolipids by TLC

Sophorolipids were detected by thin layer chromatography (TLC) on pre-coated silica gel of standard Kiesel-gel 60 F_{254} Merck plates. Plate was developed with chloroform/methanol/acetic acid (95:5:0.5, v/v) solvent system. The spots were visualized with the UV-visible illumination at 254 $_{nm}$ and with detecting reagent: anisaldehyde: acetic acid: fuming sulphuric acid: ethanol (6.9: 2.5: 8.32: 250, v/v). The comparison assignment was made according to the method described by *Asmer et al.,* in 1988.[7] Nine bands were observed, out of which five bands were different than the standard arachidonic acid and control without secondary carbon source. It was observed that arachidonic acid derived sophorolipids showed four prominent bands of R_f values of 0.16, 0.54, 0.82 and 0.90 which served as reference for the identification of sophorolipids.

Plate was developed with chloroform/methanol/acetic acid (95: 5: 0.5, v/v.) solvent system. Five bands with R_f value of 0.16, 0.54, 0.82, 0.90 and 0.93 were identified against the standard arachidonic acid and control where no secondary carbon source arachidonic acid was added. Standard arachidonic acid showed single spot on TLC and control with two spot as shown in **Figure 3.2.**

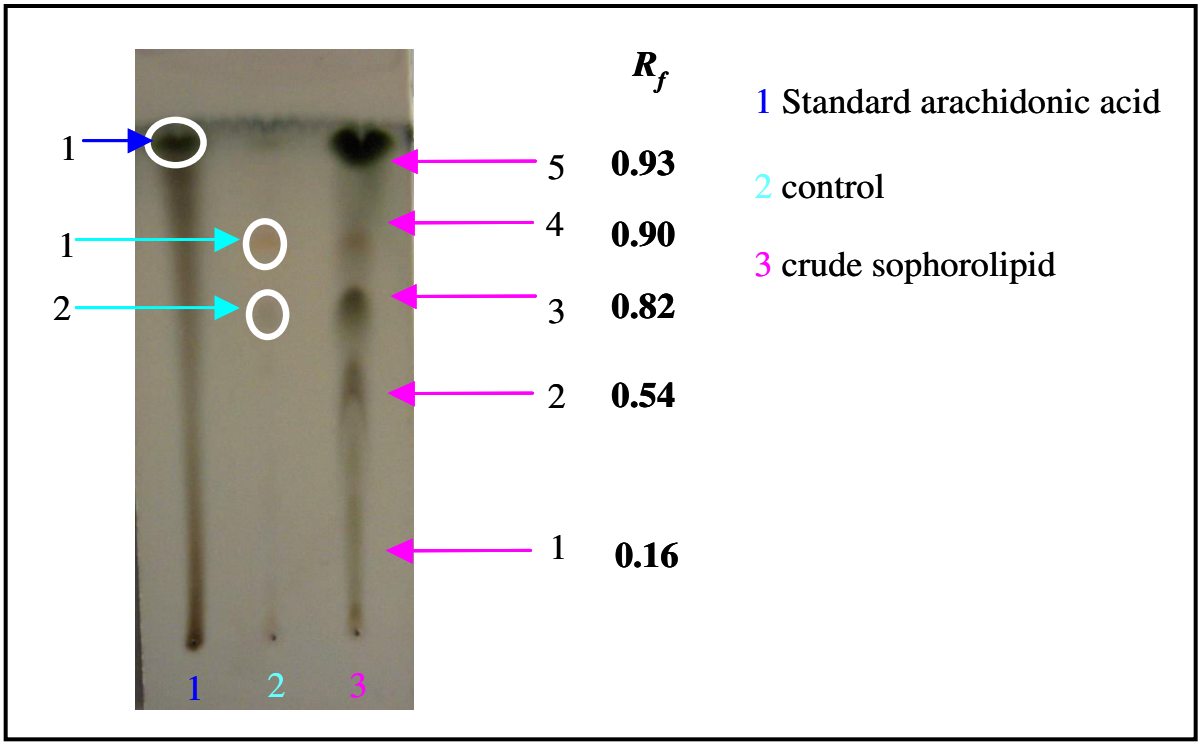


Figure 3.2. Thin Layer Chromatography of arachidonic acid ($C_{20:\ 4}$) derived sophorolipids. TLC Plate was developed with chloroform/methanol/acetic acid (95: 5: 0.5, v/v.) solvent system.

3.3. Purification of arachidonic acid ($C_{20:\ 4}$) sophorolipids by Dry silicagel chromatography using dialysis tubing

Conventionally, silica gel glass column chromatography has been used for purification of sophorolipids but for large-scale purification of sophorolipids it is impractical and challenging.[25] Glass column chromatography method required large amount of organic solvent for eluting the sophorolipid components through the column. Y. Hu and L. Ju in 2001 described sophorolipids purification via crystallization using aqueous buffers.[4] It was observed that there were various aqueous buffers having pH range from 4 to 8 for dissolving and crystallization of different type of sophorolipids. In this method sophorolipids were dissolved in different pH buffer and kept overnight for the formation of crystals. The other method was crystallization with organic solvents, which showed significant loss of sophorolipids. For large-scale purification such methods were time consuming with relatively low yield of desired sophorolipids.

A new method for purification of arachidonic acid derived sophorolipids was developed with the dry silicagel chromatography using dialysis tubing. There is a simple modification in conventional silica glass chromatography. Instead of glass column, dialysis bag was used for packing UV-visible GF $254_{\ nm}$ with 100-200 mesh size silica gel. Before packing, the silica gel was activated at 110 ˚C for 4 h. 100 g of such activated silica gel was mixed thoroughly with 10 ml distilled water and packed in 4x 40 cm. dialysis tubing which was sealed at one end. 10 ml distilled water was mixed to maintain the same moisture level and reproducibility of the R_f value. 1 g of crude sophorolipid was dissolved in ethyl acetate and mixed with an equal amount of dry activated silica gel and the resultant slurry was packed on the top of the column. 100 ml of chloroform/methanol

(95: 5, by vol.) solvent system was allowed to migrate till it reached the end of the column. Time taken for migration was 2 h for the size of the column mentioned earlier. The column was examined under UV-visible illumination at 254 $_{nm}$. Nine distinct bands were identified and were cut with surgical knife to separate the bands, as shown in **Figure 3.3**.

The silica gel slices were carefully separated after removing dialysis tubing. Each silica gel fraction was extracted with 10 to 15 ml ethyl acetate to get purified forms of arachidonic acid derived sophorolipids which were dehydrated with 2.0 gm of sodium sulfate. Supernatant was decanted and filtered. For the concentration of sophorolipid, N_2 gas was plunged for evaporation of excess ethyl acetate. Total 840 mg sophorolipid compound was recovered from nine distinct silica gel slices. After repeating the experiments it was observed that 80 to 85 % of sophorolipids can be recovered and the band position showed shift of ± 1.5 cm due to manual error in packing the column

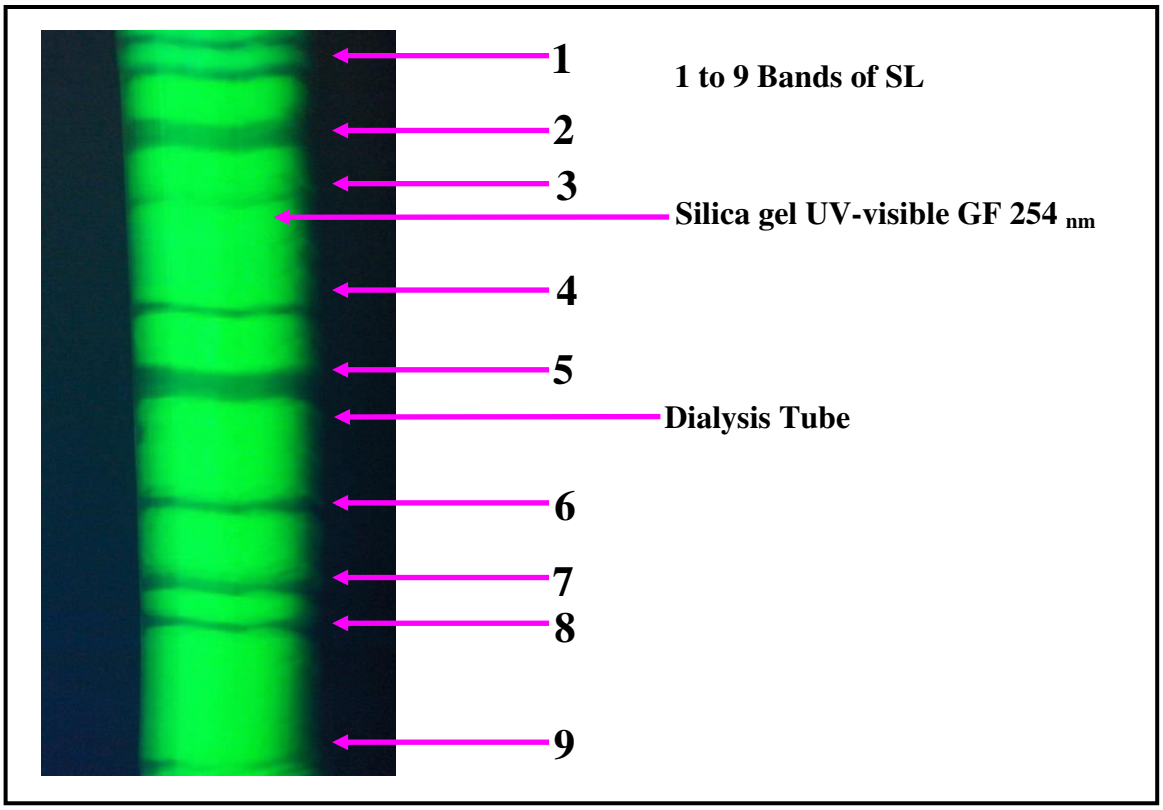


Figure 3.3. Picture of silicagel chromatography using dialysis tubing packed with silica gel UV-visible GF 254 $_{nm}$ for purification of arachidonic acid derived sophorolipids. Nine different bands were visualized under UV-visible illumination at 254 $_{nm}$

3.3.1. Hydrolysis of purified sophorolipids and isolation of hydroxylated fatty acids

Purified sophorolipids were hydrolyzed with 1M HCL in N_2 atmosphere for 12 h at 25 °C and liberated hydroxy fatty acids were extracted with an equal volume of chloroform. Hydroxylated fatty acids were purified on 500 mg Aminopropyl Sep-Pak Cartridges (Waters). Samples in 0.5 mL chloroform were applied to cartridge pre-equilibrated with 5 mL n-hexane. Neutral lipids were eluted with 25 mL chloroform/2-propanol (2:1, v/v), mono-hydroxylated fatty acids with 25 mL 2% (v/v) acetic acid in diethyl ether and phospholipids with 25 mL methanol. The hydroxy fatty acid fraction was rotary evaporated and the residue taken up in a small volume of chloroform. Hydroxy fatty acids were purified by TLC on standard Kiesel-gel 60 plates and developed with petroleum ether (b.p. 60-80 ^{0}C) diethyl ether/acetic acid (50:50:1, v/v). Iodine vapour was used to visualize fatty acids and the corresponding bands were immediately eluted with methanol/chloroform (2:1 v/v) and derivatized to their methyl ester silyl ethers for GC and GC-MS analysis.[3, 26]

3.4. Characterization

3.4.1. LC-ESI-MS of purified arachidonic acid derived sophorolipids

The purified sophorolipids were identified by LC-MS for the structure determination of sophorolipid forms. LC-MS Thermo Finnigan associated with electrospray soft ionization mass spectroscopy (LC-ESI-MS) system was used to identify molecular weight of sophorolipids produced with glucose and arachidonic acid. The molecular ions were collected in an ion trap and the mass/charge (m/z) values were detected. 10 mg of purified sophorolipids obtained from silica gel using dialysis tubing chromatography of R_f values 0.16 (220 mg), 0.54 (80 mg) and 0.82 (350 mg) was

dissolved in 500 µl of ethyl acetate. 1 µl sample was subjected to LC-MS**.** The significant ions occurred at m/z 710 and m/z 728 of fractions of R_f valve 0.16 and 0.82 respectively. The structures were determined as the lactonic and the acidic forms of the diacetate sophorolipids of arachidonic acid, respectively (**Figure 3.4.A. and 3.4.B.**). At m/z 668, significant ions of fraction R_f value 0.54 showed monoacetate lactonic form of sophorolipid of arachidonic acid. (**Figure 3.4.C.)**.

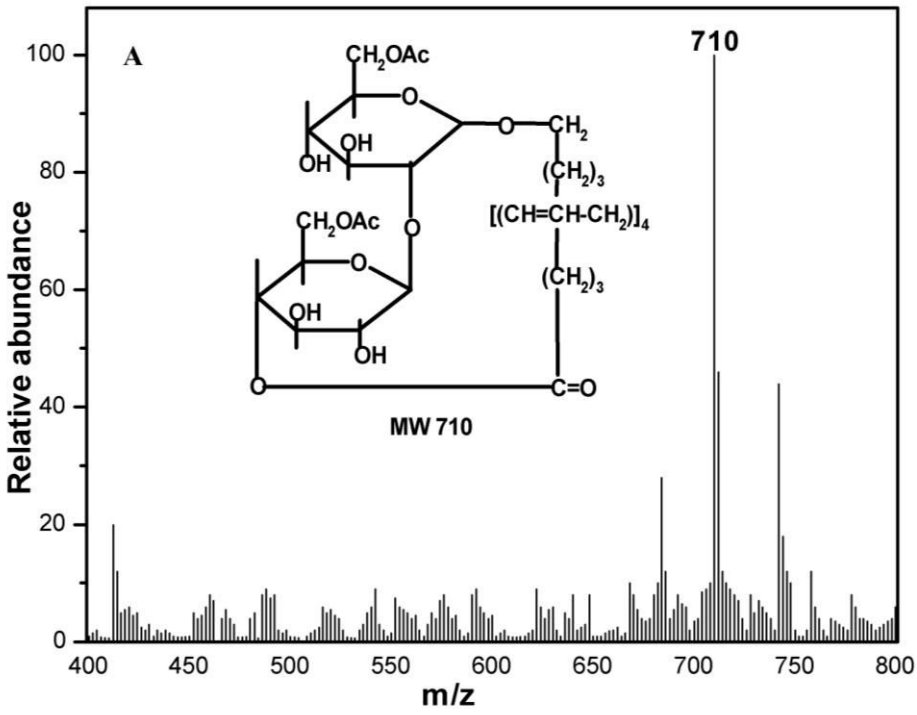
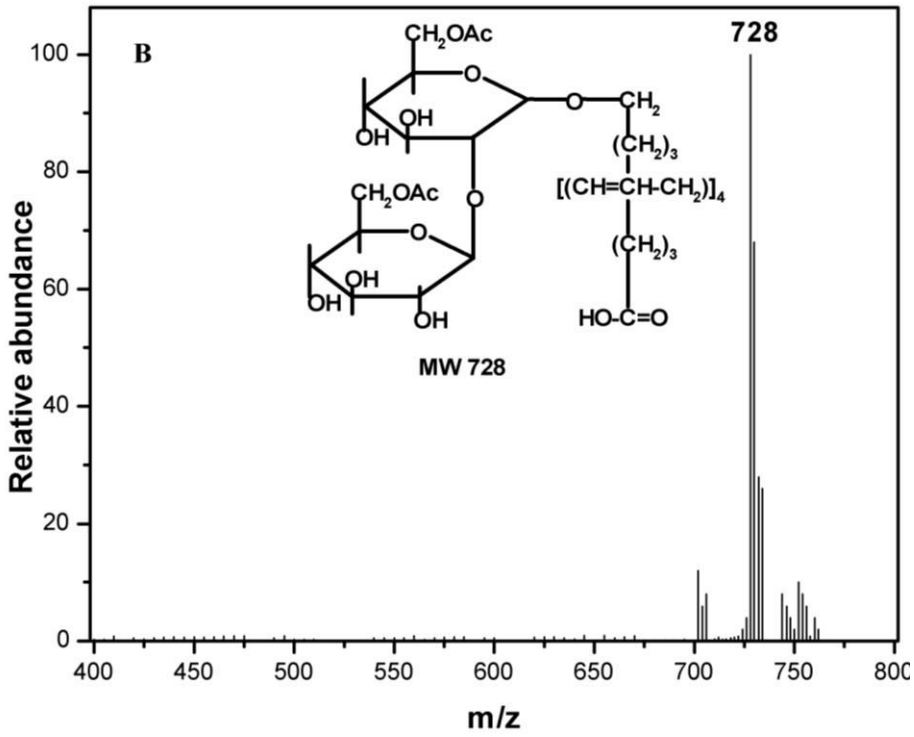
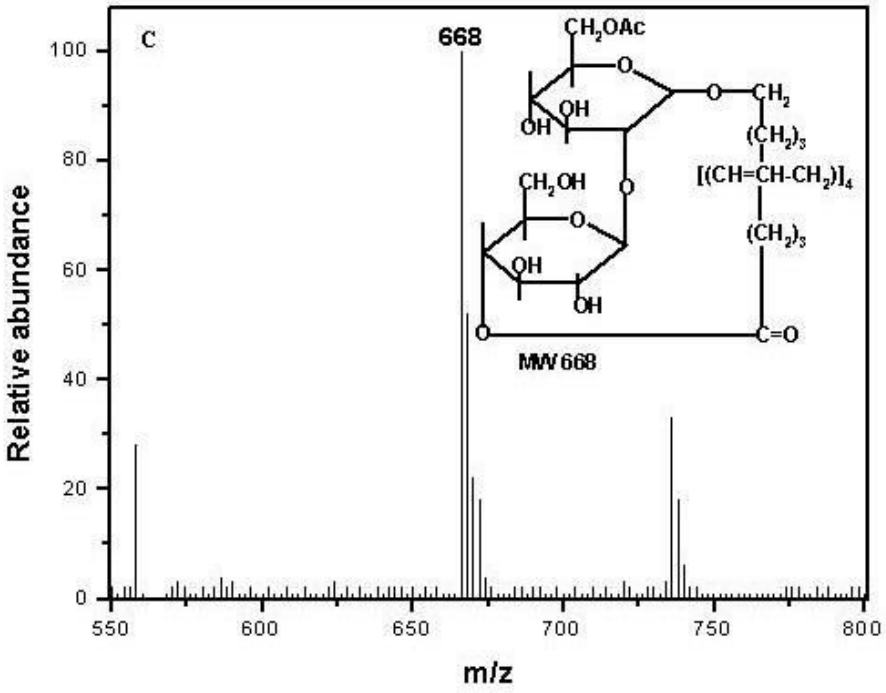


Figure 3.4. The mass spectrum of purified arachidonic acid derived sophorolipids produced from glucose and arachidonic acid by dry silicagel chromatography using dialysis bag.

A. Mass spectrum of diacetate lactonic arachidonic acid derived sophorolipid (Mol.Wt.710)



B. Mass spectrum of diacetate acidic arachidonic acid derived sophorolipid (Mol.Wt.728)



C. Mass spectrum of monoacetate lactonic arachidonic acid derived sophorolipid (Mol.Wt.668)

3.4.2. FT-IR spectroscopy of arachidonic acid derived sophorolipids

FTIR is a powerful tool to study the different forms of sophorolipids. FT-IR measurements of the purified sophorolipids formed on Si (111) substrate were done on a Perkin-Elmer spectrum-1 FTIR spectrometer operated in the diffuse reflectance mode at a resolution of 4 cm^{-1}. **Figure 3.5.** show FTIR spectrum of the purified arachidonic acid derived sophorolipid by dry silica chromatography method (curve 1 and 2) and as synthesized sophorolipids from *Candida bombicola* (curve 3). In the entire spectrum, similar absorption arising from the O-H stretching vibrations occurs in the region of 3435 cm^{-1}. The carbonyl functional group (C=O) had a strong peak in the region of 1744 cm^{-1} may include contributions from that of lactones, esters, or acids (curve1, 2, and 3). The asymmetrical stretching ($\nu_{as}CH2$) and symmetrical stretching ($\nu_{s}CH2$) of methylene occurs at 2926 and 2850 cm^{-1}, respectively (curve1, 2, and 3). The 1624 cm^{-1} band is from stretching of the unsaturated C-C bonds (curve 3). Lactones and esters have two strong absorption bands arising from C-O and C-O stretching. The stretch of C-O band of C (-O)-O-C in lactones appears at 1157 cm^{-1} (curve 1), while that from the acetyl esters at 1247 cm^{-1} (curve 1, 2 and 3). Sugar C-O stretch of C-O-H groups is at 1048 cm^{-1} (curve 1, 2 and 3) (Mantsch and Chapman, 1996).[27] The primary difference between the spectra of the as synthesized sophorolipids (curve 3) and purified sophorolipids (curve 1 and 2) was the band at 1445 cm^{-1} that corresponds to the C-O-H in plane bending of carboxylic acid (–COOH) (Silverstaein and Webster, 1998).[28]

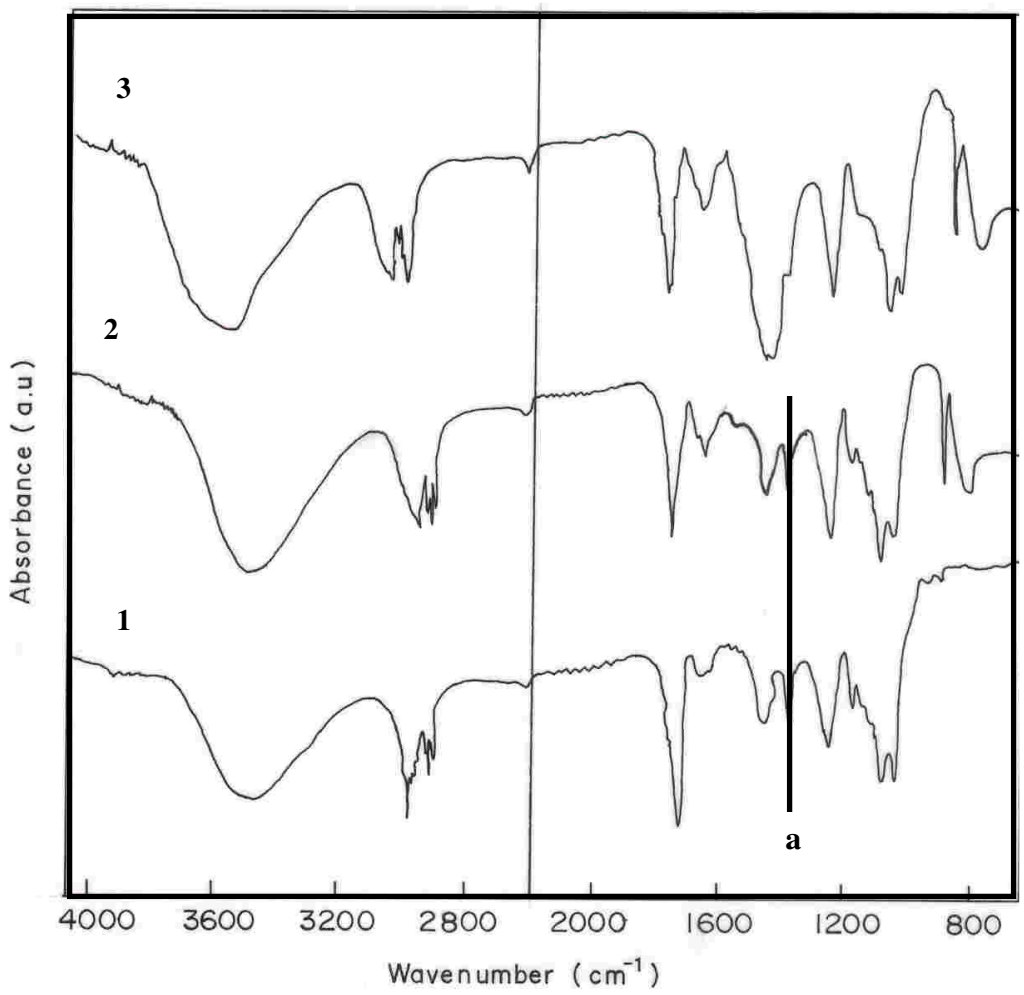


Figure 3.5. FTIR spectra demonstrate, curve (1 and 2) of purified sophorolipids by dry silicagel chromatography using dialysis tubing and curve (3) as synthesized arachidonic acid derived sophorolipids produced from *Candida bombicola.* The band at 1445 cm^{-1} (a) that corresponds to the C-O-H in plane bending of carboxylic acid is shown in curve (1 and 2)

3.4.3.Identification of hydroxylated fatty acids by Gas Chromatography

A Shimadzu 17A FID detector was used to analyze free hydroxylated fatty acids obtained from purified sophorolipids. For the GC analysis 10 µl of hydroxylated fatty acid sample was converted to methyl ester by the addition of 20 µl diazomethane reagent. Vials were kept at room temperature for 15 min. After evaporation to dryness under nitrogen, trimethylsylyl ether of free hydroxyl groups were prepared by reaction with 20 µl of bis (trimethylsylyl)-trifluoracetamide (BSTFA) and kept at 45 °C for 30 min. Samples were dissolved in 20 µl chloroform, and 1 µl aliquots were analyzed by GC and GC/MS. Gas chromatography was performed on a BP-5 fused silica column (30 m; 0.25 mm inner diameter; 0.25 µm film coatings). Helium as a carrier gas at 14 kPa head pressure. GC temperature program ranging from initially at 150 °C for 1 min (rate 35 °C/min) then increased to 220 °C for 5 min (rate 5 °C /min) and then at 280 °C and then held at these conditions for 10 min; injector at 250 °C; detector at 300°C column; sample injection volume 1 µl. The samples were identified by comparison of gas chromatographic retention times with authentic standard of 20-HETE from Cayman Chemicals Company. Standard 20-HETE was derivatized to their methyl ester silyl ether with BSTFA as mention above. As shown in **Figure 3.6.** the typical gas chromatogram spectra of experimental sample (b) and standard authentic 20-HETE (a). Standard authentic derivatized 20-HETE was illustrated by peak at gas chromatographic retention time 18.6 min. on comparison with experimental sample was elutes at same retention time 18.6 min. and at 19.3 min. of 19-HETE. The free hydroxyl groups were quantitated by calculating the ratio of relative abundance with standered authentic 20-HETE.

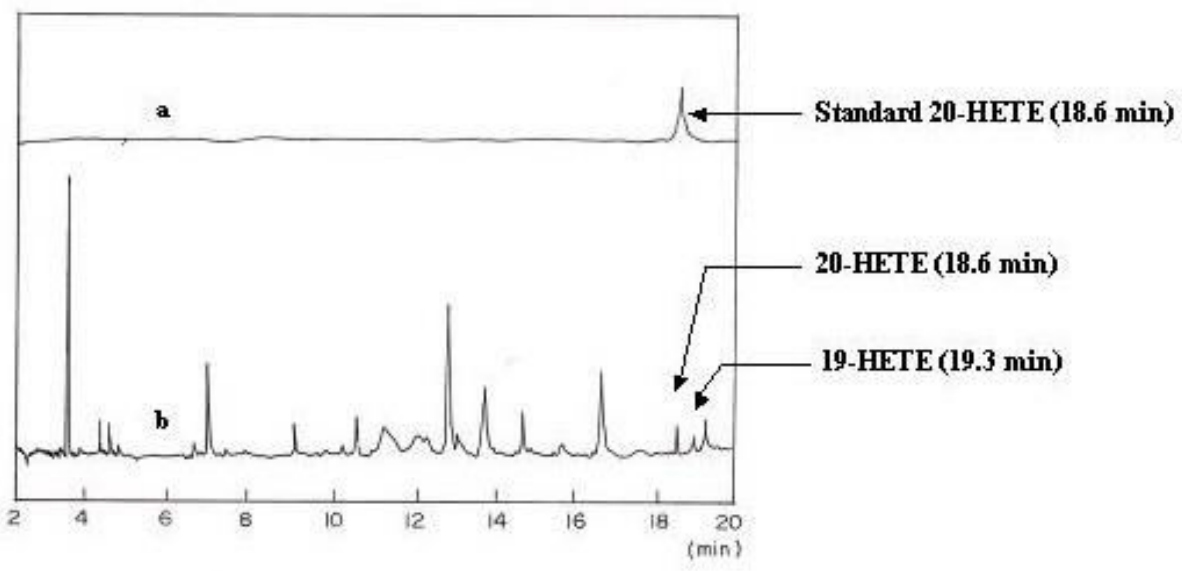


Figure 3.6. Representative Gas chromatography spectra of the methyl ester silyl ether of 20-hydroxy 5Z, 8Z, 11Z, 14Z -eicosatetraenoic acid (a) and methyl ester silyl ether of free hydroxylated fatty acid obtained from lactonic diacetate sophorolipids (b) illustrating the peak at gas chromatographic retention time 18.6 min. and 19.3 min.

3.4.4. Identification of hydroxylated fatty acids by Gas Chromatography-Mass Spectroscopy (GC-MS)

A Shimadzu GCMS QP 5050 automated quadrupole mass spectrometer operating in the electron impact mode. GC parameter**:** column used, BP-5 fused silica column (30 m x 0.25 mm, 0.25 mm, 0.25 mm coatings). Helium as a carrier gas at 14 kPa head pressure; injector at 250 °C; column initially at 150 °C for 1 min (rate 35 °C/min) then increased to 220 °C for 5 min (rate 5 °C /min) and then at 280 °C and then held at these conditions for 10 min; injection volume 1 µl. MS parameters**:** Interface temperature 250 °C, ionization mode electron impact, scan range 70 to 450 amu (arbitrary mass units) s^{-1}.

Acid hydrolysis of the sophorolipid yielded hydroxy fatty acids. The 20-hydroxy-5Z, 8Z, 11Z, 14Z-eicosatetraenoic acid (20-HETE) and 19-hydroxy-5Z, 8Z, 11Z, 14Z-eicosatetraenoic acid (19-HETE) were formed and detected by gas chromatography with mass spectrophotometer. The partial mass spectrum of the hydroxylated fatty acids released from the sophorolipids was compared with standard 20-HETE mass spectrums. **Figure 3.7.** has shown the partial mass spectrum of 20-HETE as detected by GC-MS. Significant ions of methyl ester silyl ether of 20-HETE occurred at *m/z* [406 M^+], 391 [M^+ - 15], 316 [M^+ - 90], 304 [(M^+ +1) – 103]. Selective ion monitoring also showed a prominent signal at m/z 103 [$(CH_3)_3$-Si-O^+-CH_2]. The structures of the significant ions occurred of methyl ester silyl ether of 20-HETE shown in **Table 3.1**. The mass spectrum thus clearly indicated the hydroxyl group at the C20 position and the compound was 20-hydroxy 5Z, 8Z, 11Z, 14Z -eicosatetraenoic acid (20-HETE). Confirmation of the identity of 20-HETE was obtained by the observed co-elution of standard 20-HETE by GC and GC-MS with the isolated material.

The mass spectrum indicated a hydroxyl group at position C19. The mass spectrum ions occurred at m/z 117 due to cleavage from C19 to C20; [$(CH_3)_3$ -Si- O^+-CH-CH_3] and 73 [base ion; $(CH_3)_3$-Si] and loss of 220 [M^+ - 186] due to rearrangement and loss of – CH=CH$(CH_2)_3$-CH[O^+-Si-$(CH_3)_3$]-CH_3, 201 {M^+ - [131 + 74 (silyl group + H)]}. The structure of the significant ions occurred of methyl ester silyl ether of 19-HETE shown in **Table 3.2.** The compound was identified as 19-hydroxy-5Z, 8Z, 11Z, 14Z-eicosatetraenoic acid.

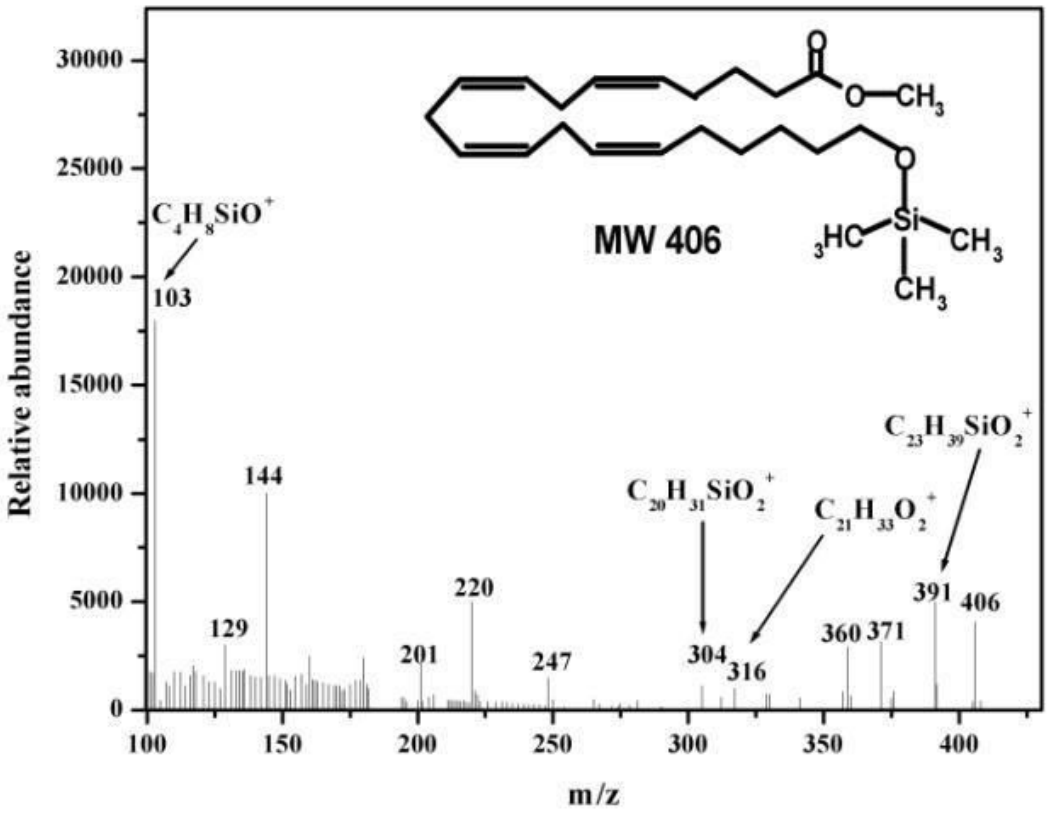


Figure 3.7. Partial electron impact mass spectrum of monohydroxylated derivative of arachidonic acid as detected by GC-MS. The compound was extracted from diacetate lactonic arachidonic derived sophorolipid and identified as the methyl ester silyl ether of 20-hydroxy 5Z, 8Z, 11Z, 14Z -eicosatetraenoic acid.

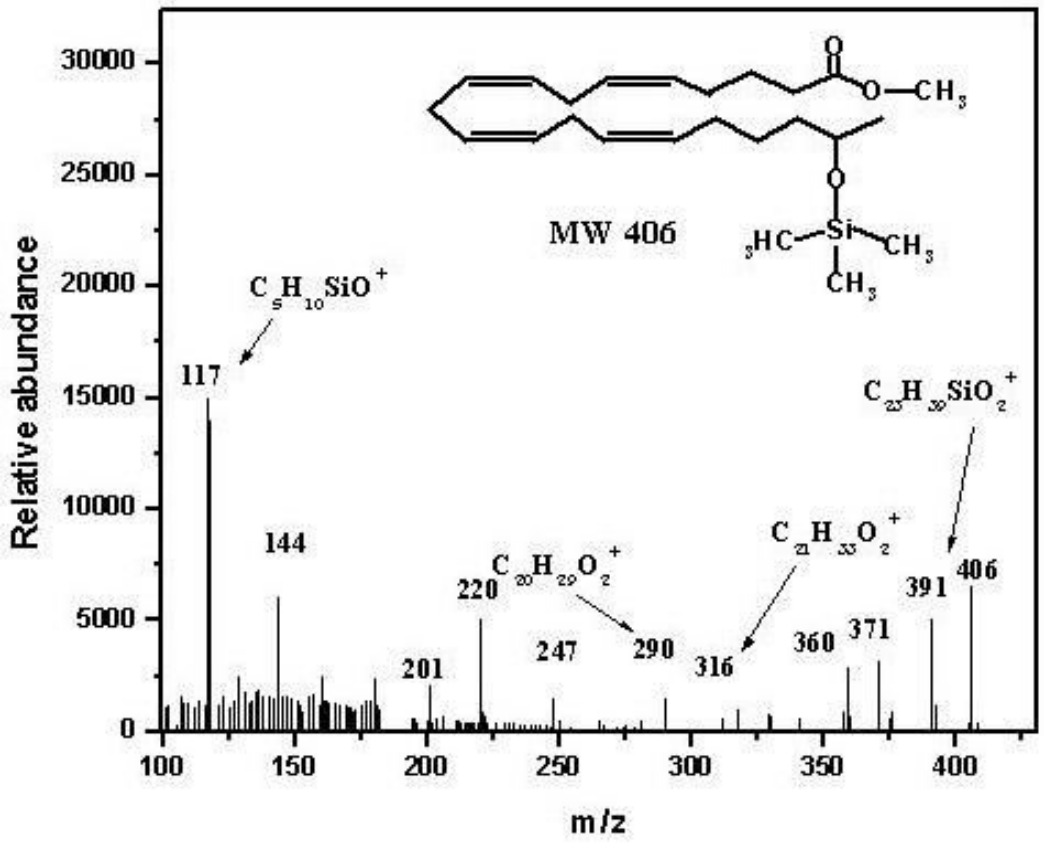
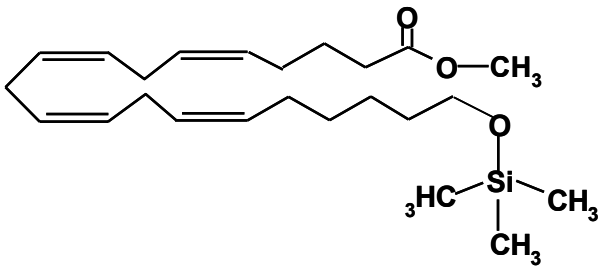
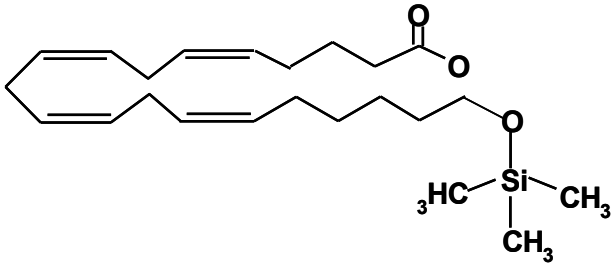
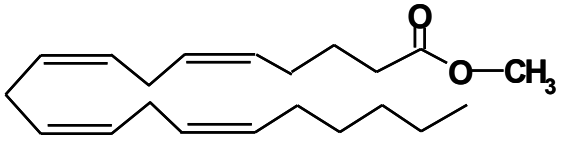
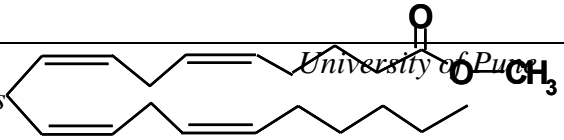

Relative abundance
m/z
MW 406
C5H10SiO+
C23H39SiO2+
C21H33O2+
C20H29O2+
117
144
201
220
247
290
316
360
371
391
406

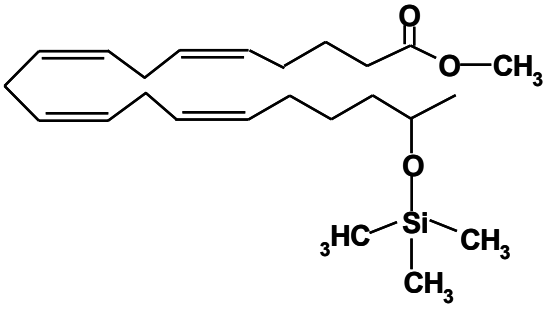
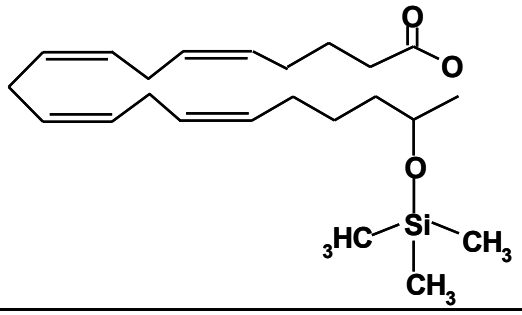
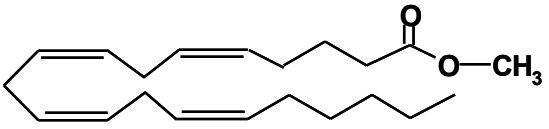
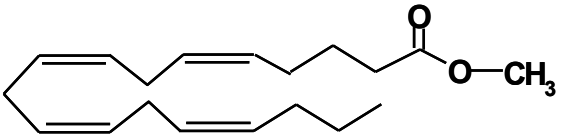
Figure 3.8. Partial electron impact mass spectrum of monohydroxylated derivative of arachidonic acid as detected by GC-MS. The compound was extracted from diacetate lactonic arachidonic derived sophorolipid and identified as the methyl ester silyl ether of 19-hydroxy 5Z, 8Z, 11Z, 14Z -eicosatetraenoic acid.

Table 3.1. The structures of the significant ions occurred of methyl ester silyl ether of 20-HETE.

Structure	m/z
	**406**
	**391**
	**316**
	**304**

Selective ion monitoring also showed a prominent signal at m/z 103, [$(CH_3)_3$-Si-O^+-CH_2]. It clearly indicates that bond cleavage from C 19 to C 20 and it confirm that the hydroxyl group is at the C 20 position and that the compound was 20-HETE.

Table 3.2. The structures of the significant ions occurred of methyl ester silyl ether of 19-HETE.

Structure	m/z
	**406**
	**391**
	**316**
	**290**

Selective ion monitoring also showed a prominent signal at m/z 117, [$(CH_3)_3$ -Si-O^+-CH-CH_3]. It clearly indicates that bond cleavage from C 18 to C 19 and it confirm that the hydroxyl group is at the C 19 position and that the compound was 19-HETE.

3.5. Conclusion

In this chapter, it has been shown for the first time the dry silica column chromatography using dialysis tube. This gives simple method of separation and purification of different forms of arachidonic acid derived sophorolipids. The ESI-MS and FT-IR spectrum of sophorolipids provided a straightforward identification of mono- and di-acetate of the lactonic form and diacetate of the acidic form of arachidonic acid ($C_{20:\ 4}$) sophorolipids. It has been observed that dry column chromatography using dialysis tubing was cost effective and a quick process for purification of different forms of sophorolipids. High yields of purified lactonic and acidic sophorolipids with commercial importance can be obtained by this method. Silica gel used for the column can be reused after washing with appropriate solvents and calcination. Purified lactonic diacetate sophorolipids yielded hydroxy fatty acids 19-HETE and 20-HETE which are pharmacologically important. Mixture of 19-hydroxy and 20-hydroxy lactonic sophorolipids could be further purified to get respective sophorolipid and respective fatty acid on hydrolysis. This will be an alternative for storing these hydroxy fatty acids in sophorolipids form as these sophorolipids showed better stability at higher temperature. This method can be used for purification of other fatty acids derived sophorolipids for commercial exploitation.

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Chapter 4

Immobilization of whole cells on biocompatible materials: Use as Enzyme Source for the Biotransformation of Arachidonic acid to 19-HETE and 20-HETE

4.1. Summary

A variety of materials can be utilized for immobilization of *Candida bombicola* based on different immobilization protocols. These biomaterials can be used as enzyme sources for the transformation of arachidonic acid to vasoactive compounds 19-hydroxyeicosatetraenoic acid (19-HETE) and 20-hydroxyeicosatetraenoic acid (20-HETE). In general few points need to be considered before choosing the materials for the whole cell immobilization.

1. The materials should be stable, robust and inert. It must be biocompatible and should not interfere with bioreaction.
2. The materials should protect the whole cell containing reactive enzyme against microbial deterioration and render the enzyme accessible to cofactor, metal ions etc.
3. The materials must permit substrate accessibility to immobilized whole cells and thus avoid mass transfer problem.
4. The materials should have high immobilization whole cell loading factor for the efficient transformation reaction.
5. The immobilization process must be simple, quick, inexpensive and ecofriendly.

The studies in this chapter are focused on immobilization of *Candida bombicola* on novel biocompatible supports as an enzyme sources for the transformation of arachidonic acid to bioactive molecules, 19-hydroxyeicosatetraenoic acid (19-HETE) and 20- hydroxyeicosatetraenoic acid (20-HETE). This chapter is divided into two subchapters for three different biocompatible materials used for immobilization of *Candida bombicola*.

4.2. Introduction

Impressive advances are being made in the synthesis of chemically functionalized and patterned biocompatible surfaces for the immobilization of biomolecules such as whole cells and enzymes of microorganisms such as bacteria and yeast.[1, 2] Development of methodologies for the entrapment and immobilization of whole cells has important implications in a range of applications, examples of which include basic cell biology,[3, 4] biosensing,[5] tissue engineering[6] and treatment of diseases by controlled delivery of biological products.[7] One of the challenges in this area is to develop protocols wherein the immobilization of the cells is to be spatially controlled, preferably on a submicron to micron scale by designing surfaces of varying "adsorptivity" of the biological system. This gives an insight into the effect of cell shape and cell function[3] which enhances our ability to control the cellular environment and helps in understanding fundamental cell biology. Such patterned surfaces for immobilization of cells have been obtained using microcontact printing (μ-CP) on reactive[3] and mixed self-assembled monolayers (SAMs).[8] SAMs of alkanethiolates[9] and alkylsilanes[10] were obtained by the sol-gel technique[11] and using elastomeric membranes.[12] Groves *et. al.,* in 2001 have demonstrated that phospholipid bilayers act as biomimetic surfaces and modulate the assembly and growth of cells.[13] Recently μ-CP of organic monolayers and subsequent polymer functionalization has been used to develop patterns in the seeding of bacterial cells.[14]

An important application of bacterial and fungal cells (genetically engineered and otherwise) is use as "factories" for the production of industrially and medically important enzymes and metabolites.[15] Here we have been interested in assembly of specific cells on

surfaces from the point of view of using the cells as sources of enzymes for biotransformations and synthesis of new materials. The enzyme cytochrome P450 present in the yeast cells was used to catalyze in situ ω and ω -1 hydroxylation of arachidonic acid. Cytochrome P450, the enzyme of interest is unstable outside the cellular environment and in such cases, immobilization of the whole cells was important to catalyze reactions that are dependent on the unstable enzymes. As part of our search for newer and more versatile materials, tailorable surfaces for cell immobilization has been used. This chapter presents the synthesis of different biocompatible materials whose surface may readily be modified to render it compatible for a variety of biocatalytic applications.

Chapter 4. A

Candida bombicola cells immobilized on patterned lipid films as enzyme sources for the transformation of arachidonic acid to 19-HETE and 20-HETE

Chapter 4. B

Nanogold membrane as scaffolds for whole cell immobilization as enzyme source for biotransformations of arachidonic acid to 19-HETE and 20-HETE

Chapter 4. A: Candida bombicola cells immobilized on patterned lipid films as enzyme sources for the transformation of arachidonic acid to 19-HETE and 20-HETE

4.A.1. Introduction

Chapter 4.A. part presents the assembly of *Candida bombicola* yeast cells onto patterned thermally evaporated fatty amine thin films (octadecylamine, ODA) and the use of the enzyme cytochrome P450 present in the yeast cells to catalyze *in-situ* the ω-hydroxylation of arachidonic acid (AA) to 19- hydroxyeicosatetraenoic acid (19-HETE) and 20-hydroxyeicosatetraenoic acid (20-HETE)[16, 17] (see **Figure 4.A.1.**). Cytochrome P450 is a membrane bound protein and is known to be highly unstable outside the cells.[17] Thus, rather than immobilizing the unstable purified enzyme within a lipid film, we demonstrate here the immobilization of *Candida bombicola* yeast cells carrying the enzyme cytochrome P450.[16, 17] This immobilization affords a cheaper, renewable and more versatile alternative for carrying out the transformation of arachidonic acid to 19-HETE and 20-HETE. In view of the vasoactive and renal pharmacological activity of 19-HETE and 20-HETE,[18] the large-scale production of this molecule is of commercial interest and the methodology presented herein assumes added importance.

Part of the work presented in this chapter 4.A. has been published in: *Biotechnol. Prog.* **2003**, *19*, 1659-1663.

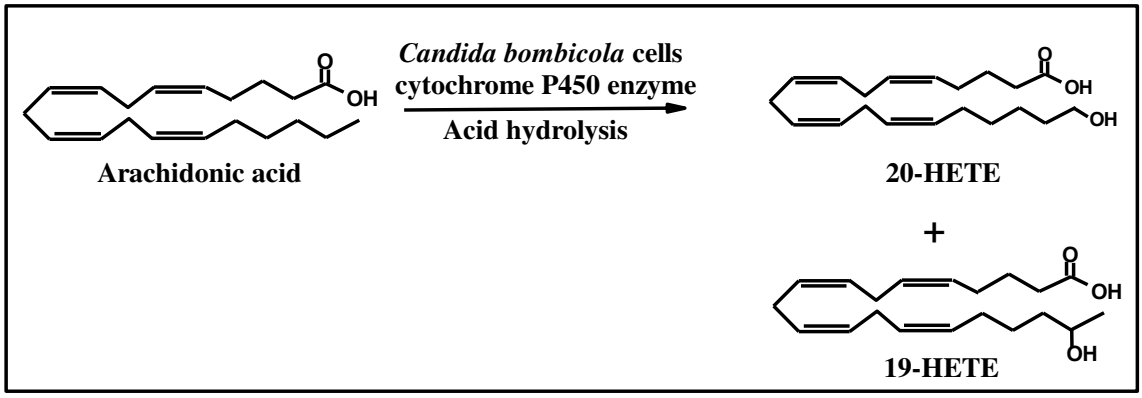
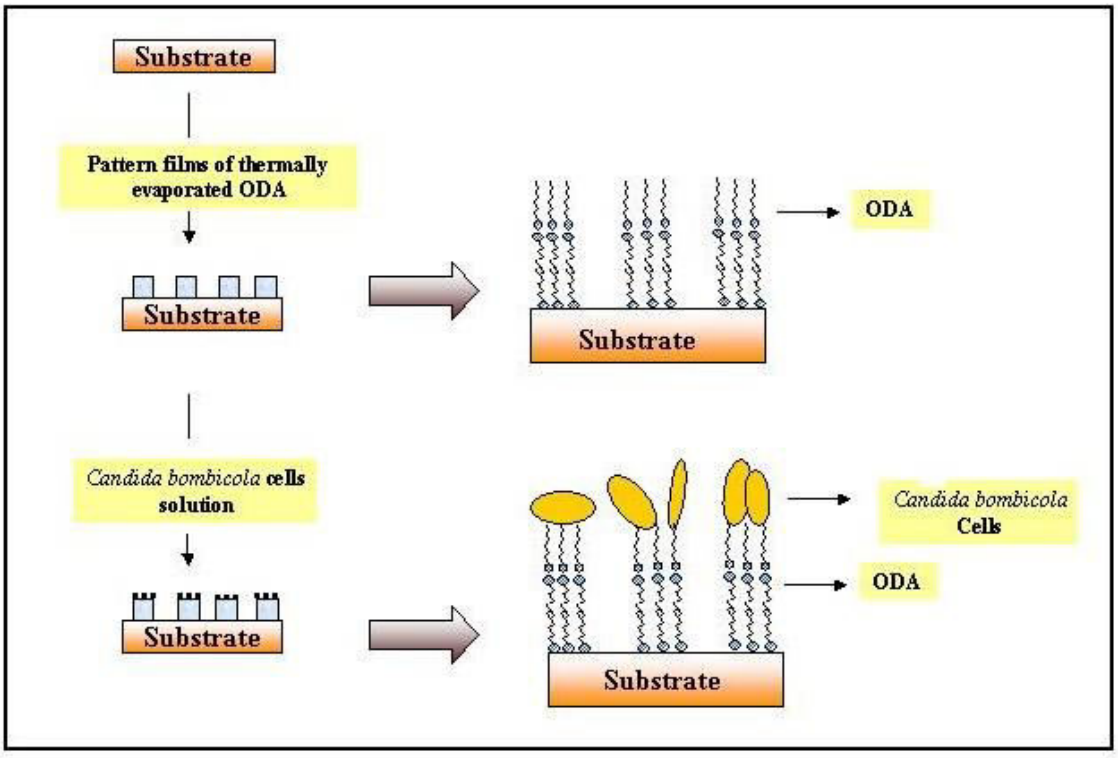


Figure 4.A.1. Transformation of arachidonic acid to 20-hydroxyeicosatetraenoic acid (20-HETE) and 19-hydroxyeicosatetraenoic acid (19-HETE) mediated by cytochrome P450 enzyme present in *Candida bombicola* cells

The immobilization of the *Candida bombicola* cells was accomplished by simple immersion of the patterned ODA film into an aqueous dispersion of the cells as illustrated in **Scheme 4.A.1.** The assembly of the cells on the ODA surface occurs possibly through hydrophobic interactions between the cell wall and the ODA molecules **(Scheme 4.A.1.)** and thus provides a rational strategy for assembly of the cells. The adhesion of the cells to the ODA surface is sufficiently strong and permits reuse of the immobilized cells in the biochemical transformation of arachidonic acid to 20-HETE.

Scheme 4.A.1. Illustration of immobilization of *Candida bombicola* yeast cells on thermally evaporated ODA film surface



4.A.2. Materials and Methods

4.A.2.1. Chemicals

Arachidonic acid of 99 % purity and octadecylamine (ODA) of 97 % purity were obtained from Sigma and Aldrich Chemicals respectively and used as received. All reagents were from standard commercial sources and of highest quality available.

4.A.2.2. Growth of Candida bombicola cells

Candida bombicola cells ATCC 22214 were pre-cultivated in 50 mL medium at 30 ^{0}C consisting of (g l^{-1}): glucose, 100; yeast extract, 1; $(NH_4)_2SO_4$, 1; $MgSO_4 \cdot 7H_2O$, 0.3; Na_2HPO_4, 2; NaH_2PO_4, 7; pH 5.5 and shaken at 160 rpm. Cells from the late growth phase (24 h) were inoculated into 1 lit. flasks containing 400 mL medium. Fermentative procedure employed for the experiment was same as described previously in Chapter 2 section (2.2.3.).

4.A.2.3. Deposition of octadecylamine films

250 Å thick films of ODA (CH_3-$(CH_2)_{17}$–NH_2) were thermally evaporated onto 6 MHz AT-cut quartz crystals for quartz crystal microgravimetry (QCM) measurements, Si (111) wafers (for Fourier transform infrared (FTIR) spectroscopy and scanning electron microscopy (SEM) measurements) in an Edwards E308 vacuum coating unit. A 40 μm x 40 μm mesh size transmission electron microscope (TEM) grid was used as a mask in the deposition of patterned ODA films on the Si (111) substrate. The deposition was done at a pressure of 1 x 10^{-7} Torr and the film deposition rate and thickness were monitored in-situ using an Edwards quartz crystal thickness monitor.

4.A.2.4. Immobilization of Candida bombicola cells onto the thermally evaporated ODA films

The immobilization of the yeast cells on the ODA surface was followed by QCM by immersion of the 250 Å thick ODA film on gold-coated AT cut quartz crystals for different time intervals in an aqueous dispersion of the cells (~ 10^8 cells/mL) and measuring the frequency change of the crystals *ex-situ* after thorough washing and drying of the crystals. The frequency counter used was an Edwards FTM5 instrument operating at a frequency stability and resolution of $\pm$ 1 Hz. For a 6 MHz crystal used in the investigation, this translates into a mass resolution of 12 ng/cm^2. The frequency change was converted to mass loading using the Sauerbrey formula.[19] The 250 Å thick ODA films on Si (111) substrates were immersed in the *Candida bombicola* cells dispersed in deionized water for 4 h. This optimum time of immersion was estimated from QCM measurements. For the reusability of the cells immobilized on 250 Å thick ODA films surface, were washed 3 times by deionized water prior to reuse.

4.A.2.5. FTIR measurements

FTIR measurements of the 250 Å thick ODA films deposited on Si (111) substrates before and after immobilization of the *Candida bombicola* cells were made on a Perkin Elmer Spectrum-1 FTIR spectrometer operated in the diffuse reflectance mode at a resolution of 4 cm^{-1}.

4.A.2.6. Scanning electron microscopy (SEM)

The immobilization of the *Candida bombicola* cells on the patterned ODA films was also studied by scanning electron microscopy (SEM) on a Leica Stereoscan-440 electron microscope. Presence of cells on the patterned ODA film surface after incubation of the cells immobilized on ODA surface after 96 h in the reaction medium was confirmed from SEM images.

4.A.2.7. Synthesis of sophorolipids

The yeast cells immobilized on the 250 Å thick ODA films were immersed in the reaction medium containing 5 mL 10% sterile glucose and 30 mg arachidonic acid and was incubated for 96 h at 30 ^{0}C under slow shaking. After the reaction, the supernatant was decanted and used for extracting the sophorolipid.[17]

4.A.2.8. Hydrolysis of glycolipid and isolation of 19-HETE and 20-HETE

Acid hydrolysis of the sophorolipids under N_2 with 1M HCl for 12 h at 25 ^{0}C liberated the fatty acids, which were extracted with an equal volume of chloroform. Hydroxylated fatty acids were purified on 500 mg Aminopropyl Sep-Pak Cartridges (Waters). Samples in 0.5 mL chloroform were applied to cartridge pre-equilibrated with 5 mL n-hexane. Neutral lipids were eluted with 25 mL chloroform /2-propanol (2:1, v/v), mono-hydroxylated fatty acids with 25 mL 2% (v/v) acetic acid in diethyl ether and phospholipids with 25 mL methanol. The hydroxy fatty acid fraction was rotary evaporated and the residue taken up in a small volume of chloroform. Hydroxy fatty acids were purified by TLC on standard Kiesel-gel 60 plates and developed with petroleum ether (b.p. 60-80 ^{0}C) diethyl ether/ acetic acid (50:50:1, by volume). Iodine

vapors were used to visualize fatty acids and the corresponding bands were immediately eluted with methanol/chloroform (2:1 v/v) and derivatized to their methyl ester silyl ethers.[17, 20]

4.A.2.9. Gas chromatography mass spectroscopy (GC-MS)

A Shimadzu GCMS QP 5050 automated quadrupole mass spectrometer operating in the electron impact mode. GC parameters: column used, BP-5 fused silica column (30m x 0.25 mm, 0.25mm, 0.25mm coatings). He gas as a carrier at 14 kPa head pressure; injector at 250 ^{0}C; column initially at 150 ^{0}C for 1 min (rate 35 ^{0}C/min) then increased to 220 ^{0}C for 5 min (rate 5 ^{0}C/min) and then at 280 ^{0}C and then held at these conditions for 10 min; injection volume 1 µL. *MS parameters*: Interface temperature 250 ^{0}C, ionization mode electron impact, scan range 70 to 450 amu (arbitrary mass units) s^{-1}.

4.A.3. Results and discussions

4.A.3.1. QCM studies

The kinetics of cell immobilization onto the thermally evaporated ODA films was monitored by immersion of a 250 Å thick ODA covered QCM crystal for different time intervals in the cells dispersed in deionized water and monitoring the change in resonance frequency of the crystal *ex-situ* after thorough washing and drying of the crystals. Since the mass of the individual cells is not known, we have used the frequency change alone as an indicator of the attachment of the cells on the hydrophobic ODA surface. **Figure 4.A.2. (A)** has shown the QCM mass uptake data recorded during immobilization of *Candida bombicola* cells to the ODA film surface. The error bars are based on an analysis of three separate QCM measurements. It is observed from the **Figure 4.A.2. (A)** that there is a rapid attachment of the cells initially with 90 % of the cells being immobilized within the first 100 minutes of immersion. The cell density on the ODA film surface eventually reaches saturation after 4 h of immersion in the yeast cell suspension. In all further experiments, this optimum time of immersion (4 h) in the *Candida bombicola* cells solution was used to obtain films of the immobilized cells.

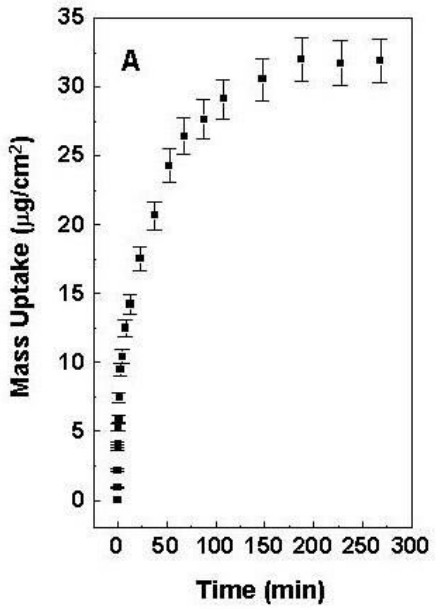


Figure 4.A.2. (A) QCM mass uptake data recorded during immobilization of *Candida bombicola* cells onto a 250 Å thick ODA film deposited on an AT-cut 6 MHz quartz crystal as a function of time of immersion in the cell suspension. The error bars indicate 10 % deviation to the data from their mean values as determined from the three separate measurements.

4.A.3.2. FTIR Studies

FTIR spectroscopy provides a convenient means of monitoring the attachment of the *Candida bombicola* cells via fingerprint signatures of cellular components. **Figure 4.A.2. (B)** has shown the FTIR spectra recorded from a 250 Å thick as-deposited ODA film (curve **1**) and the ODA film after immersion in *Candida bombicola* cells solution for 4 h (curve **2**). Two prominent features labeled *a* (917 cm^{-1}) and *b* (1110 cm^{-1}) in the figure can be seen for the cell-immobilized ODA film (curve **2**) which are clearly absent in the as-deposited ODA film (curve 1). These two absorption bands *a* and *b* are characteristic of excitation of deoxyribose-phosphate vibration modes and vibrations in the deoxyribose groups in the DNA molecules of the yeast cells respectively.[21] The FTIR results thus present additional evidence for the presence of the *Candida bombicola* cells on the ODA film surface.

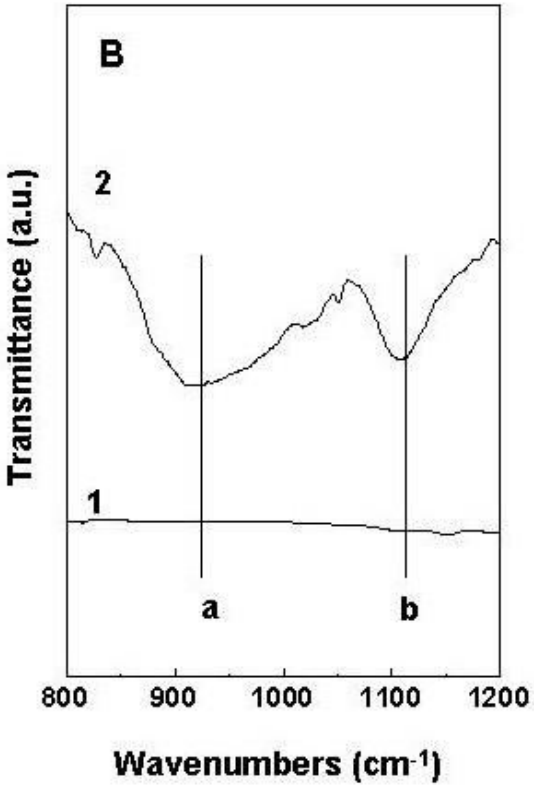


Figure 4.A.2. (B) FTIR spectra recorded from an as-deposited 250 Å thick ODA film (curve 1) and the ODA film after complete immobilization of the *Candida bombicola* cells (curve 2) on Si (111) substrates

4.A.3.3. SEM measurements

Figure 4.A.3. (A) has shown the SEM image of the as-deposited ODA film on a Si (111) substrate using a TEM grid as a mask. It is seen that well-defined individual hexagonal elements of the ODA film have been deposited on the substrate. **Figure 4.A.3. (B)** and **(C)** have shown energy dispersive analysis of x-rays (EDAX) spot profile analysis on masked (marked as **x** in Figure 4.A.3. (A)) and exposed substrate (marked as **+** in Figure 4.A.3. (A)) of the patterned surfaces of ODA lipid films. **Figure 4.A.3. (C)** has shown nitrogen signal from the exposed surface, hence confirms the deposition of the ODA, however nitrogen signals were absent from the masked region, this confirms that ODA is not deposited in this region. Thereafter, this film was immersed in an aqueous suspension of *Candida bombicola* cells for 4 h and washed thoroughly prior to imaging by SEM.

Figures 4.A.4. (A) and (B) have shown the low and high magnification SEM image recorded after immobilization of the *Candida bombicola* cells onto the hexagonal ODA patterns. It is clearly seen from SEM image that the yeast cells are immobilized extremely faithfully on the ODA elements with negligible binding of the cells to the exposed silicon surface.

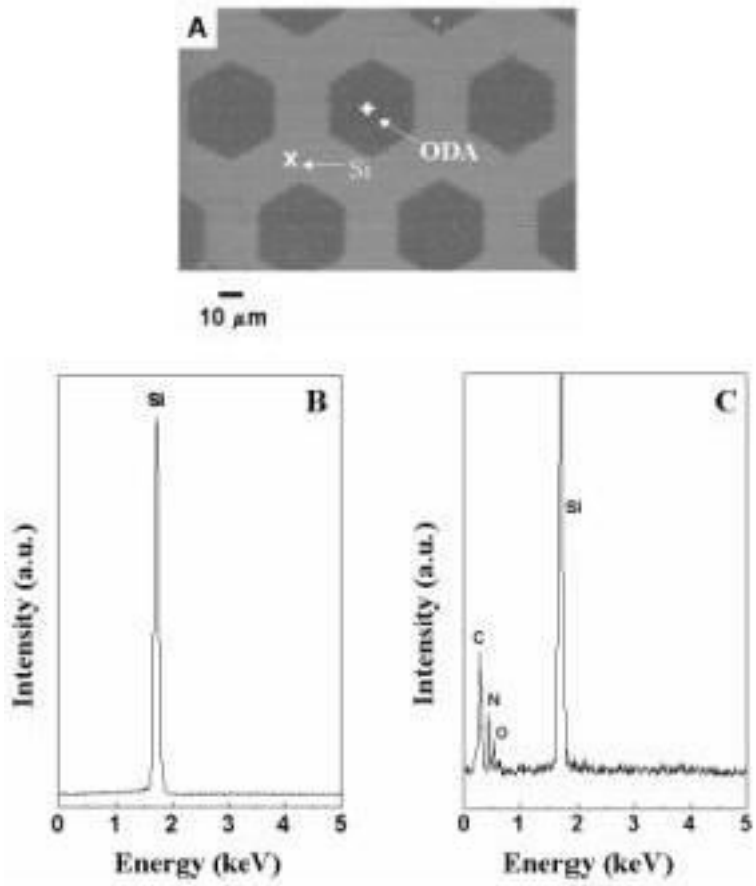
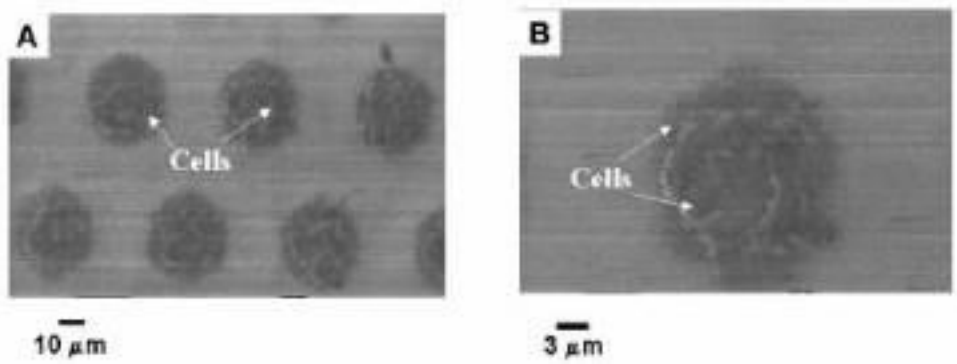


Figure 4.A.3. (A) SEM images recorded from patterned thermally evaporated ODA thin film. **(B)** and **(C)** shows EDAX spot profile analysis on masked. [In **Figure 4.A.3. (A)** marked as **x** and exposed surface of patterned ODA lipid films (marked as +)]



Figures 4.A.4 (A) and (B). low and high magnification of SEM images after immobilization of *Candida bombicola* cells onto the ODA film surface.

4.A.3.4. Synthesis of sophorolipids

As mentioned briefly in the introduction, our interest in *Candida bombicola* cells centres on the ability of the cells to catalyse the transformation of arachidonic acid to 20-HETE (Figure 1) and therefore, it is of paramount importance to establish the viability of the immobilized cells in performing this biochemical function. Films of the immobilized cells were reacted with arachidonic acid and the sophorolipids were isolated from the reaction medium and subjected to acid hydrolysis as described in detail in the experimental section. The immobilized yeast cells transformed 75 % of the arachidonic acid to sophorolipid, which was then subjected to acid hydrolysis to yield 20-HETE [17]. The overall reaction leading to the formation of 20-HETE from arachidonic acid was shown in **Figure 4.A.1.**

4.A.3.5. Acid hydrolysis of sophorolipids and isolation of 19-HETE and 20-HETE

As mentioned briefly in the introduction, our interest in *Candida bombicola* cells centers on the ability of the cells to catalyze the transformation of arachidonic acid to 19-HETE and 20-HETE. The sophorolipids formed during the biotransformations of arachidonic acid were subjected to acid hydrolysis to yield 19-HETE and 20-HETE compounds. The hydroxyecosatetraenoic acids were reacted with diazomethane solution and thereafter with the bis silyl trimethyl fluroacetamide (BSTFA) to give methyl ester silyl ether of hydroxyecosatetraenoic acid.

Figure 4.A.5. has shown the partial mass spectrum of 20-HETE as detected by GC-MS. Significant ions occurred at *m/z* [406 M^+], 391 [M^+ - 15], 316 [M^+ - 90], 304 [(M^+ +1) – 103].[17] Selective ion monitoring also showed a prominent signal at m/z 103 [$(CH_3)_3$-Si-O^+-CH_2].[17] The mass spectrum thus clearly indicates that the hydroxyl group

is at the C20 position and that the compound was 20-hydroxy 5Z, 8Z, 11Z, 14Z-eicosatetraenoic acid (20-HETE). Confirmation of the identity of 20-HETE was obtained by the observed co-elution of 20-HETE standard by GC-MS with the isolated material.

The mass spectrum indicated a hydroxyl group at position C19. The mass spectrum of ions occurred at m/z 117 due to cleavage from C19 to C20; [$(CH_3)_3$ -Si- O^+-CH-CH_3] and 73 [base ion; $(CH_3)_3$-Si] and loss of 220 [M^+ - 186] due to rearrangement and loss of –CH=CH $(CH_2)_3$-CH[O^+-Si-$(CH_3)_3$]-CH_3, 201 {M^+ - [131 + 74 (silyl group + H)]}. **Figure 4.A.6.** has shown the structures of the significant ions occurred of methyl ester silyl ether of 19-HETE. The compound was identified as 19-hydroxyeicosatetraenoic acid.[17] Also yeast cells such as *Candida apicola* have been shown to synthesize sophorolipids in which sophorose, as a diglucoside is linked glycosidically to the terminal (n-) or sub terminal (n-1) hydroxyl group of a hydroxyl fatty acid.[17] We would like to add that the film of the immobilized cells could be reused after reaction and thorough washing with only a marginal loss in activity (5 % cells over 5 reuse cycles) indicating that the cells were strongly bound to the underlying ODA film surface and that there is little leaching out of the cells during reaction. This is confirmed by imaging the ODA patterned surface after incubating the cells in the reaction medium for 96 h. the presence of the cells on the ODA film surface after reaction.

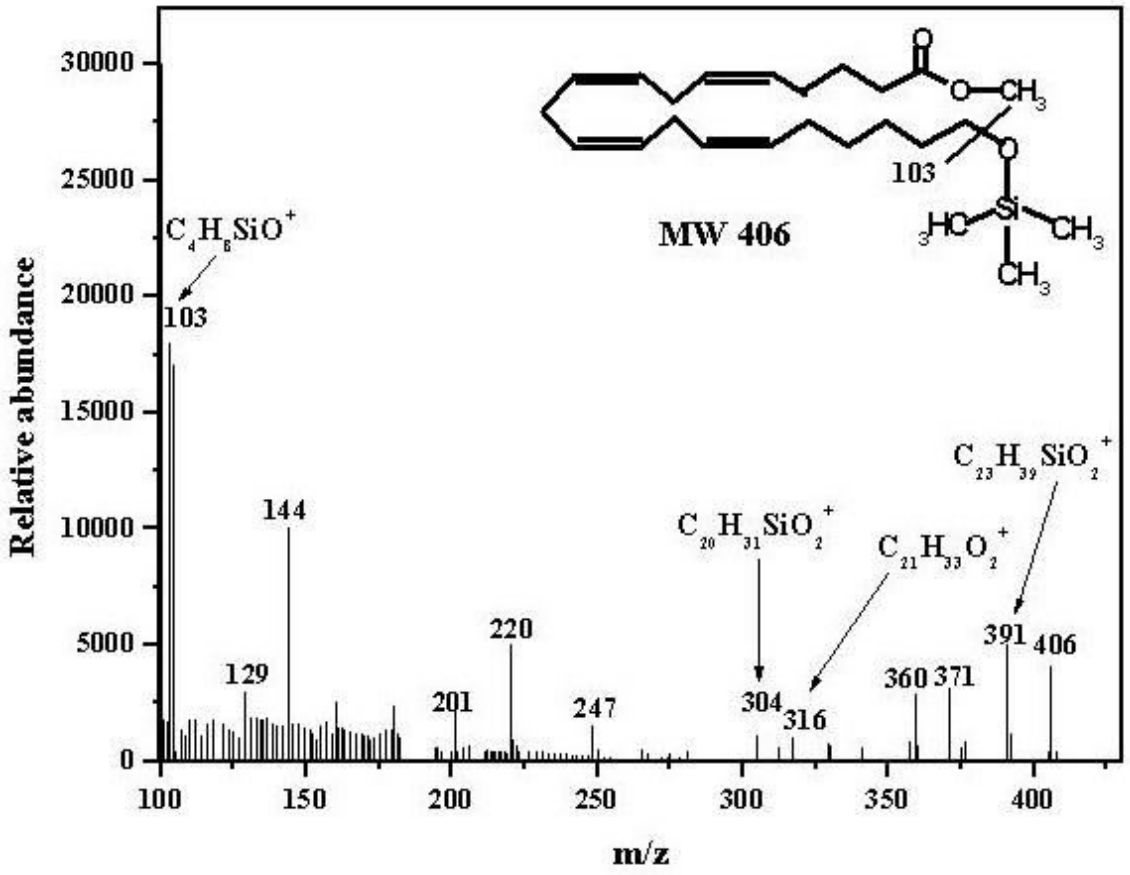


Figure 4.A.5. Partial electron impact mass spectrum of 20-HETE as detected by GC-MS. The compounds were extracted from sophorolipids, synthesized by *Candida bombicola* cells and was identified as the methyl ethyl silyl ether of 20-hydroxy-5Z, 8Z, 11Z, 14Z-eicosatetraenoic acid (20-HETE)

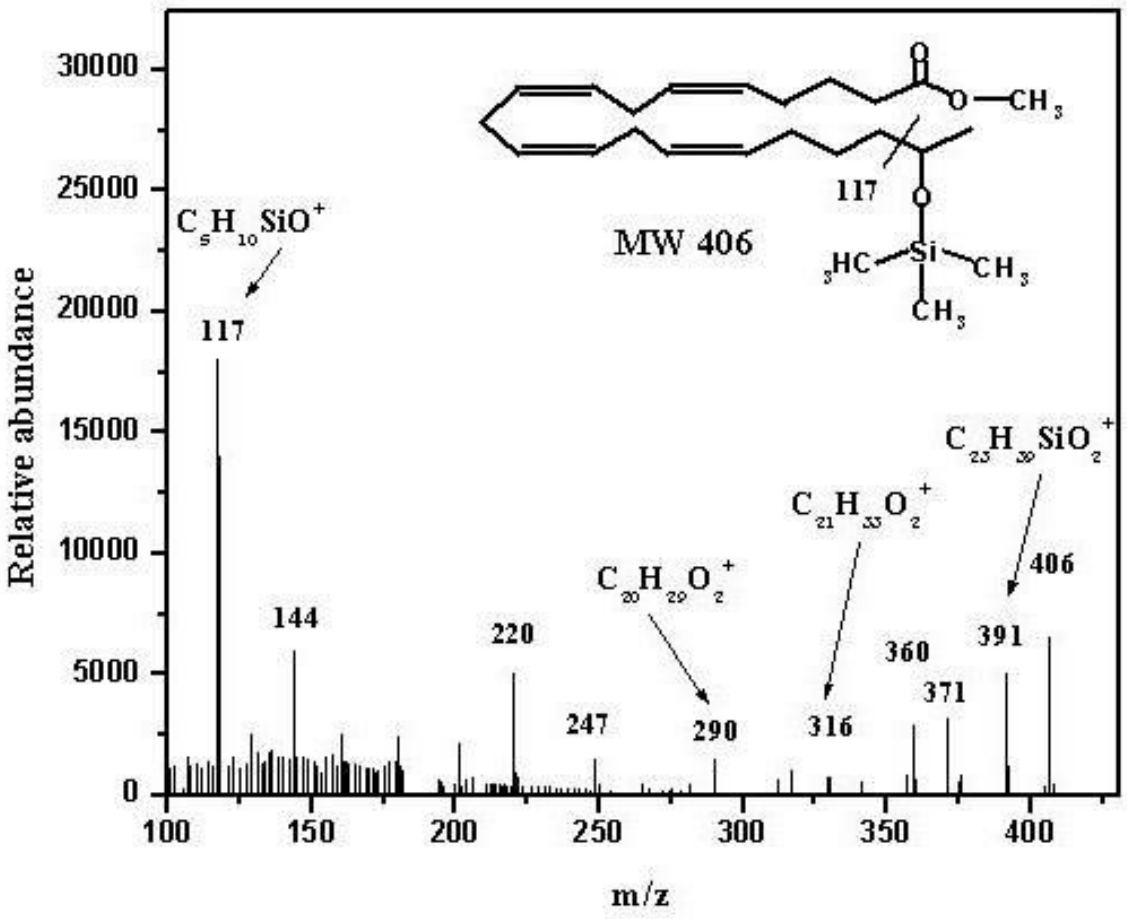


Figure 4.A.6. Partial electron impact mass spectrum of 19-HETE as detected by GC-MS. The compounds were extracted from sophorolipids, synthesized by *Candida bombicola* cells and was identified as the methyl ethyl silyl ether of 19-hydroxy-5Z, 8Z, 11Z, 14Z-eicosatetraenoic acid (19-HETE)

Figure 4.A.7. (A) and **(B)** have shown SEM images of different regions of the *Candida bombicola* cells bound to ODA lipid films after one cycles of reaction (incubating in the reaction medium for 30 ^{o}C for 96 h). Thus, the yeast cells were strongly bound to the hydrophobic ODA lipid film permitting excellent reuse. The films of the immobilized cells could be reused after reaction and through washing with only a marginal loss in biocatalytic activity ca. 10 % after 5 cycles, indicating that the cells are strongly bound to the ODA lipid films.

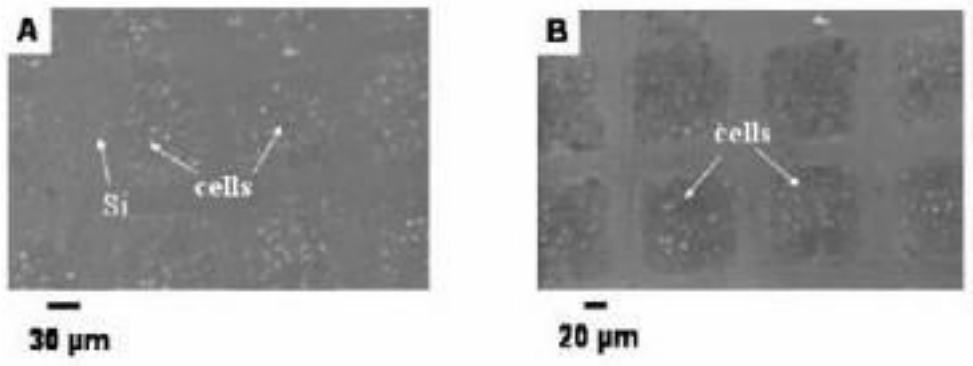


Figure 4.A.7. Low **(A)** and high **(B)** magnification SEM images of *Candida bombicola* whole cells immobilized on thermally evaporated octadecylamime lipid films after one cycle of reaction.

4.A.4. Conclusion

In this study, we have demonstrated the immobilization of *Candida bombicola* yeast cells on patterned thermally evaporated ODA films, the assembly of cells to ODA films possibly driven by hydrophobic interactions between cell walls and ODA molecules. The immobilized yeast cells were biologically active and cytochrome P450 enzyme present in the *Candida bombicola* cells could be used to transform arachidonic acid to 19-hydroxyeicosatetraenoic acid (19-HETE) and 20-hydroxyeicosatetraenoic acid (20-HETE). The biocomposite films are easily separated from the reaction medium for additional reuse.

Chapter 4. B: **Nanogold membrane as scaffolds for whole cell immobilization as enzyme source for biotransformations of arachidonic acid to 19-HETE and 20-HETE**

4.B.1. Introduction

Chapter 4.B. focuses on the preparation of chemically functionalized biocompatible polymeric membrane embedded by the gold nanoparticles and hydrophobized by using octadecylamine (ODA). The free standing hydrophobic nanogold membrane provides a biocompatible surface for the immobilization of whole cells. The attachment of the cells to the ODA bound to the nanogold membrane occurs possibly through the nonspecific interactions such as hydrophobic interactions between the cell walls and the ODA molecules. The enzyme, cytochrome P450 present in the immobilized yeast cells on the ODA film surface was used for the transformation of the arachidonic acid (AA) to sophorolipids and thereafter sophorolipids were acid hydrolyzed to liberate 19- hydroxyeicosatetraneoic acid (19-HETE) and 20-hydroxyeicosatetraneoic acid (20-HETE). The advantage of using whole cells is that it limits us to use cofactors such as NADPH for the synthesis of sophorolipids.

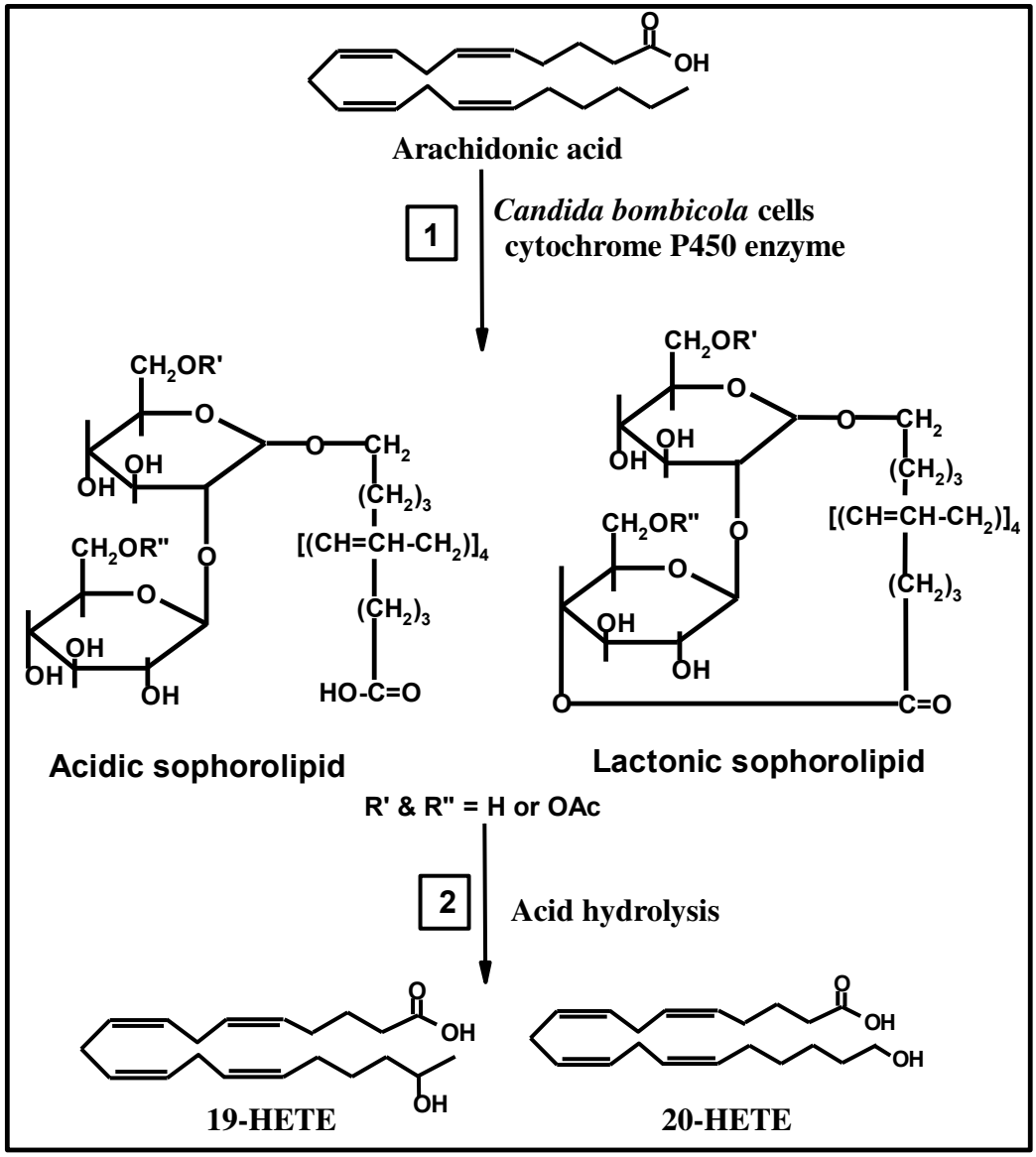
The synthesis of a free-standing gold nanoparticle membrane at the interface between chloroform containing bis (2-(4-aminophenoxy)ethyl)ether (DAEE) and aqueous chloroauric acid solution.

Part of the work presented in this chapter 4.B. has been published in: *Biotechnol. Prog.* **2004**, *20*, 1817-1824.

Thereafter the nanogold membrane is hydrophobized by binding the octadecylamine (ODA) molecules to the gold nanoparticles embedded on the polymeric membrane and were used for the immobilization of whole cells.

The membrane is formed spontaneously by the reduction of $AuCl_4^-$ ions by DAEE at the liquid-liquid interface, this process leading to the formation of gold nanoparticles.[22] The concomitant process of oxidation of DAEE leads to the creation of a polymeric matrix in which the gold nanoparticles were embedded **(Figure 4.B.1, step 1)**. The gold nanoparticle membrane was extremely stable, robust, easily handled, malleable and can be grown over large areas and thickness by suitably varying the experimental conditions.[22] Hydroxylation was done by simple immersion of the nanogold membrane in octadecylamine (ODA) in ethanol solution for 12 h resulted in binding of ODA molecules to the gold nanoparticles through the amine groups. The immobilization of the *Candida bombicola* cells was accomplished by simple immersion of the hydrophobic nanogold membrane into an aqueous dispersion of the cells **(Figure 4.B.1, step 2).** The assembly of the cells on the hydrophobic nanogold membrane surface occurred possibly through hydrophobic interactions between the cell wall and the ODA molecules **(Figure 4.B.1, step 2)** and thus provides a rational strategy for assembly of the cells. The adhesion of the cells to the ODA surface is sufficiently strong and permits reuse of the immobilized cells in the biochemical transformation of arachidonic acid to 20-HETE **(Scheme 4.B.1.).** Moreover, the free standing nanogold membrane could be easily separated from the reaction medium and were reused.

Scheme 4.B.1. Transformation of Arachidonic acid to 20-hydroxyeicosatetraneoic acid (20-HETE) mediated by cytochrome P450 enzyme present in *Candida bombicola* yeast cells



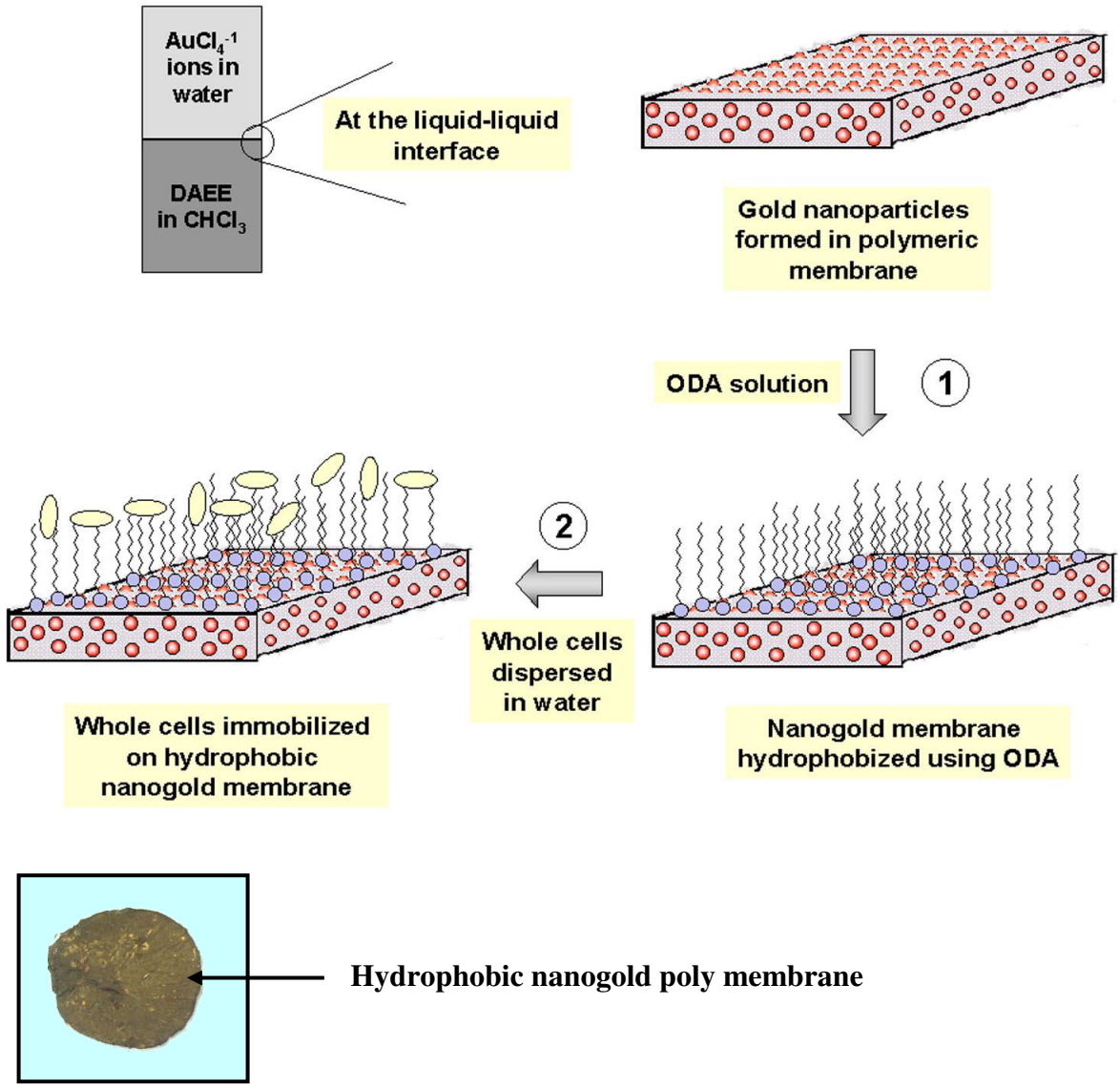


Figure 4.B.1. Illustration of hydrophobization of nanogold membrane using octadecylamine and thereafter, immobilization of the *Candida bombicola* whole cells on the hydrophobic nanogold membrane

4.B.2. Materials and Methods

4.B.2.1. Chemicals

Arachidonic acid (99 % purity), ODA (CH_3-$(CH_2)_{17}$-NH_2) were obtained from Sigma and Aldrich Chemicals respectively and used as-received. Ethyl alcohol (99.5%) was obtained from E-Merck, Germany. Bis (2-(4-aminophenoxy)ethyl)ether (DAEE) was prepared as: A mixture of potassium 4-nitrophenoxide (3.0 g, 16.9 mmol), 2-chloroethyl ether (1.21 g, 8.4 mmol) and ethylene glycol (15 mL) was stirred at 130 ^oC for 3 h. The cooled mixture was poured into ice-cold water. The pale yellow solid separated was filtered and recrystallized from ethanol. Yield 60 %, m. p. 155-156 ^oC. (Lit.1 154-157 ^oC)

Bis (2-(4-nitrophenoxy) ethyl) ether (0.8 g, 2.28 mmol) 10 mL of ethanol and 1 mg of 5 % Pd /C catalyst were placed in a flask fitted with a reflux condenser. Hydrazine hydrate (64 %, 2 mL) was added to the reaction mixture over a period of 10 min at room temperature and then the reaction mixture was refluxed for 16 h. The hot mixture was filtered and cooled. The solvent was stripped off to yield a waxy solid. Yield 92 % m. p. 59-61 ^oC. (Lit.2 59-60 ^oC)

4.B.2.2. Growth of Candida bombicola cells

Fermentative procedure employed for the experiment was same as described previously in Chapter 2 section (2.2.3.)

4.B.2.3. Nanogold membrane synthesis

In a typical experiment, 100 mL of 10^{-2} M concentrated aqueous solution of chloroauric acid ($HAuCl_4$) was mixed with 100 mL of 10^{-2} M DAEE in chloroform for 30 min. The membrane formed at the interface was separated and repeatedly washed with deionized water and were used for enzyme immobilization. The amount of gold

nanoparticles in the membrane was determined by atomic absorption spectroscopy (AAS). 10 mg of nanogold membrane was dissolved in 20 mL freshly prepared saturated I_2 solution in KI and volume was made up to 100 mL using deionized water. The solution was analyzed by a Varian Spectra AA 220 atomic absorption spectrometer (AAS) and was compared with the standard of gold solution to estimate the weight percent of gold nanoparticles in the membrane. The gold nanoparticles leached from the polymeric membrane were also used for the immobilization of whole cells.

4.B.2.4. Hydrophobization of nanogold membrane

10 mg of the nanogold membrane was dispersed in 10^{-2} M ODA solution prepared in ethyl alcohol for 12 h. The nanogold membranes were then washed with copious amount of alcohol and chloroform and dried in the air for further use.

4.B.2.5. UV-Vis spectroscopy studies

UV-visible spectra of gold nanoparticles embedded in the polymeric membrane were recorded on a quartz substrate using a Jasco V570 UV/VIS/NIR spectrophotometer operated at a resolution of 1 nm. The probable structure of the nanogold membrane is illustrated in **Figure 4.B.1.**

4.B.2.6. Transmission Electron Microscopy (TEM) measurements

TEM measurements were performed on a JEOL Model 1200EX instrument operated at an accelerating voltage of 120 kV. Samples for TEM analysis were prepared by transferring a nanogold membrane from the liquid-liquid interface on carbon-coated TEM copper grids. The mixtures were allowed to dry for 1 min following which the extra solution was removed using a blotting paper. TEM measurements of gold nanoparticles leached from the nanogold membranes were also recorded.

4.B.2.7. FTIR measurements

FTIR measurements of the nanogold membrane formed on Si (111) substrates before and after binding of octadecylamine (ODA) were made on a Perkin Elmer Spectrum-1 FTIR spectrometer operated in the diffuse reflectance mode at a resolution of 4 cm^{-1}.

4.B.2.8. Scanning electron microscopy (SEM) and energy dispersive analysis of X-rays (EDAX) measurements

The immobilization of the *Candida bombicola* cells on the as prepared and hydrophobic nanogold membrane were studied by scanning electron microscopy (SEM) on a Leica Stereoscan-440 electron microscope. Spot-profile energy dispersive analysis of X-rays (EDAX) measurements were performed to test the faithfulness of cell immobilization onto the surface of the nanogold membranes using a Phoenix EDAX attachment connected to the scanning electron microscope. Presence of cells on the hydrophobic nanogold membrane surface after incubation of the cells after 96 h in the reaction medium was confirmed by SEM images. SEM images of the gold nanoparticles leached from the polymeric membrane before and after immobilization of cells were also recorded.

4.B.2.9. X-ray diffraction measurement (XRD)

XRD measurements of gold nanoparticles bound to the polymeric membrane were done on a Philips PW 1830 instrument operating at 40 kV and a current of 30 mA with Cu K_α radiation.

4.B.2.10. Immobilization of Candida bombicola cells onto the hydrophobic nanogold membrane

20 mg of hydrophobic nanogold membranes were then immersed in an aqueous dispersion of the cells (~ 10^8 cells/mL) for 4 h (the optimum time of immersion was estimated from our earlier experiments).[23] The amount of cells immobilized on the hydrophobic nanogold membrane was estimated from the initial and the final cell counts after immobilization. To determine the confidence limit, separate measurements were made for 3 different hydrophobic nanogold membranes. For the reusability of the cells immobilized on surface of hydrophobic nanogold membrane, it was washed 3 times by deionized water prior to reuse. Nanogold membrane and gold nanoparticles leached polymeric membrane were also used for the immobilization of the whole cells.

4.B.2.11. Synthesis of sophorolipids

The yeast cells immobilized on the 20 mg of hydrophobic nanogold membrane were immersed in the reaction medium containing 5 mL 10% of sterile glucose and 30 mg arachidonic acid in 200 µl alcohol and was incubated for 96 h at 30 ^{0}C under slow shaking. After the reaction, the supernatant was decanted and used for extracting sophorolipid.[17, 23]

4.B.2.12. Hydrolysis of glycolipid and isolation of 20-HETE and 19-HETE

Acid hydrolysis of the sophorolipids under N_2 with 1M HCl for 12 h at 25 ^{0}C liberated the fatty acids, which were extracted with an equal volume of in chloroform.[17,23] Hydroxylated fatty acids were purified as described in section 4.A.2.8. of chapter 4.

4.B.2.13. Gas chromatography mass spectroscopy (*GC-MS*)

A Shimadzu GC-MS QP 5050 automated quadrupole mass spectrometer operating in the electron impact mode was used as described in section 4.A.2.9. of chapter 4.

4.B.3. Results and discussions

4.B.3.1. Preparation of the nanogold membrane material

Figure 4.B.2. (A) has shown the UV-Vis spectrum recorded in the transmission mode from the thin film of nanogold membrane transferred onto a quartz substrate. A strong absorption band centered at 540 nm is observed. This absorption was due to excitation of surface plasmons in gold nanoparticles and is responsible for their vivid pink-purple colour.[24] The amine groups of DAEE molecules at the interface were protonated (pH of $HAuCl_4$ solution ~ 3.2) leading to electrostatic complexation with $AuCl_4^-$ ions. That the electrostatic complexation with gold ions was a crucial step in the formation of the gold nanoparticle membrane was indicated by the control experiment where a similar interfacial reaction was carried out with the aqueous $HAuCl_4$ solution maintained at pH 9. At this pH, the amine groups in DAEE would not be protonated and no membrane formation was observed even after 12 h of reaction. Reduction of chloroaurate ions takes place at the interface and the oxidized DAEE molecules cap the spontaneously formed gold nanoparticles preventing their further aggregation. The nanogold membrane could be formed either by simple cross-linking of the gold nanoparticles through the terminal groups of oxidized DAEE or through formation of a polymeric network of the oxidized DAEE molecules. The estimation of gold nanoparticles in polymeric membrane was done by leaching the gold nanoparticles using

a saturated I_2 solution in KI and solutions were analyzed by atomic absorption spectrometry (AAS) as described in the experimental section. The nanogold membrane was kept in the iodine solution for more than 5 hours. The mass loading of the gold nanoparticles in the polymeric membrane was estimated as 30 weight %.

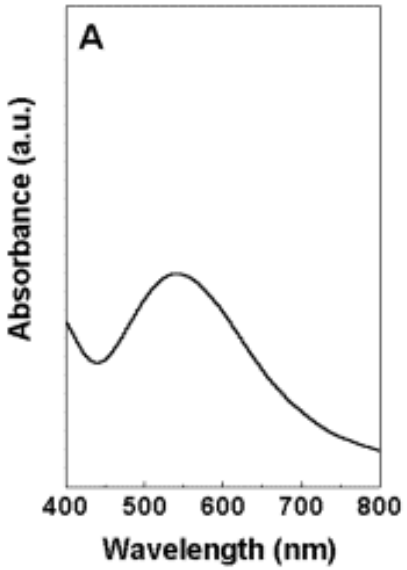


Figure 4.B.2. (A) UV-vis spectra recorded from the as-prepared nanogold membrane on quartz substrate

4.B.3.2. FTIR studies

Figure 4.B.2. (B) has shown the FTIR spectra recorded from a nanogold membrane before (curve **1**) and after (curve **2**) hydrophobization with the ODA molecules by immersion in 10^{-2} M ODA solution formed in absolute alcohol for 12 h. Two prominent features labeled *a* (2850 cm^{-1}) and *b* (2920 cm^{-1}) were due to the methylene antisymmetric and symmetric vibrations from the hydrocarbon chains of octadecylamine molecules bound to the nanogold membranes which were clearly absent in the as prepared nanogold membranes (curve **1**). The frequency of these resonance indicated the ODA molecules on the gold particle surface were in closed-packed state. The hydrophobization of gold nanoparticles using octadecylamine molecules and the binding of the ODA molecules to the nanogold membranes occured through amine groups.[24, 25]

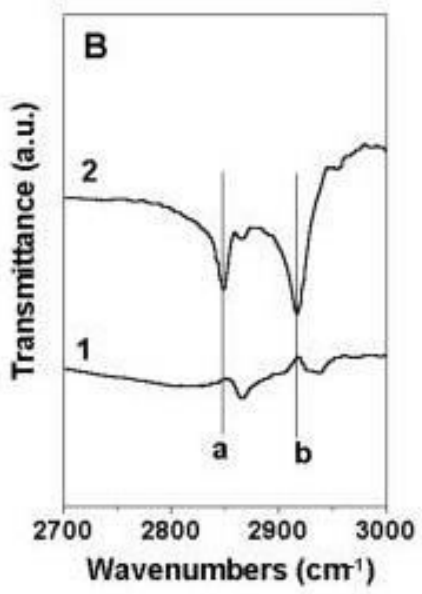


Figure 4.B.2. (B) FTIR spectra recorded from the nanogold membrane before **(curve 1)** and after **(curve 2)** hydrophobization with the octadecylamine (ODA).

4.B.3.3. XRD and EDAX measurements

Figure 4.B.3. has shown the powder XRD pattern of the nanogold membrane. The Bragg reflections in the nanogold membrane clearly correspond to presence of gold.[22] The presence of intense (311) reflection in the XRD pattern suggested oriented growth of the gold nanoparticles in the polymeric membrane along these crystallographic planes. This confirmed the reduction of chloroaurate ions at the liquid-liquid interface for the formation of gold nanoparticles. Spot profile EDAX measurements were done on the nanogold polymeric membrane. The prominent Au signal confirms the fidelity of gold nanoparticles in the polymeric membrane. However, the chlorine signals were also seen which were attributed to the unreduced gold ions ($AuCl_4^-$) were present in the membrane, presumably bound to the surface of the gold nanoparticles.

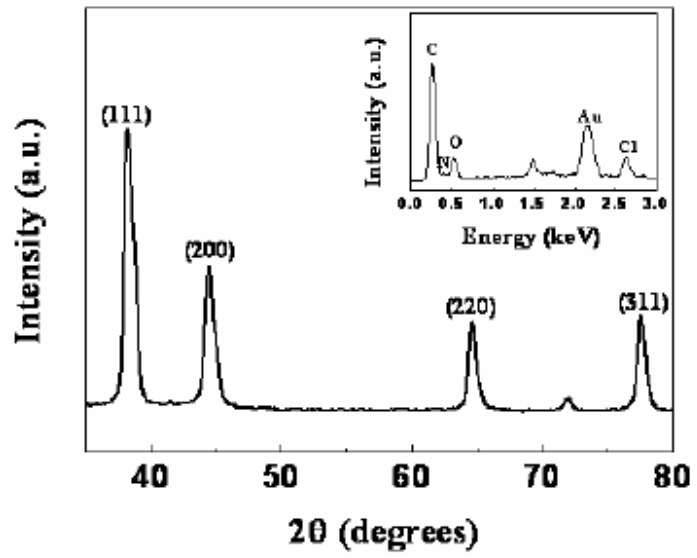


Figure 4.B.3. XRD patterns recorded from the gold nanoparticle membrane. Inset shows the spot profile EDAX recorded form the gold nanoparticle polymeric membrane

4.B.3.4. TEM measurements

Figure 4.B.4 (A) and (B) have shown the low and high magnification of TEM micrographs respectively. It was seen that the gold nanoparticles were fairly dispersed in the polymeric membrane with little extent of aggregation in the membrane. As mentioned previously the chloroaurate ions formed electrostatic complex with the protonated amine groups of DAEE molecules and then are reduced. After reducing the chloroaurate ions the oxidized DAEE molecules formed a polymer and oxidized DAEE molecules cap the spontaneously formed gold nanoparticles preventing their further aggregation. The gold ions were electrostatically bound to protonated DAEE molecules and were highly localized at the liquid-liquid interface. Reduction of the gold ions by DAEE must clearly lead to oxidation of DAEE molecules in a highly localized manner. DAEE molecules possessed two terminal aniline segments, which were known to be good reducing agents. Oxidation of DAEE most probably proceeded through formation of a polymeric network derived from DAEE at the liquid-liquid interface. The gold atoms formed by the reduction of $AuCl_4^-$ ions diffused along the polymeric network, aggregate into larger gold nanoparticles as seen in the **Figure 4.B.4 (A) and (B)**, thereby yielding a polymeric network with inclusions of gold nanoparticles at the liquid-liquid interface. As shown in **Figures 4.B.4. (C) and (D)** the gold nanoparticles leached from the nanogold membranes by iodine treatment as described in the 4.B.2.6 section. The dark spots seen in **Figures 4.B.4. (A) and (B)** corresponding to the gold nanoparticles were not seen in the gold nanoparticles leached membrane. However, the dark regions in **Figures 4.B.4. (C) and (D)** corresponded to the entrapped iodine molecules during the leaching the gold nanoparticles. This was confirmed from the diffraction measurements.

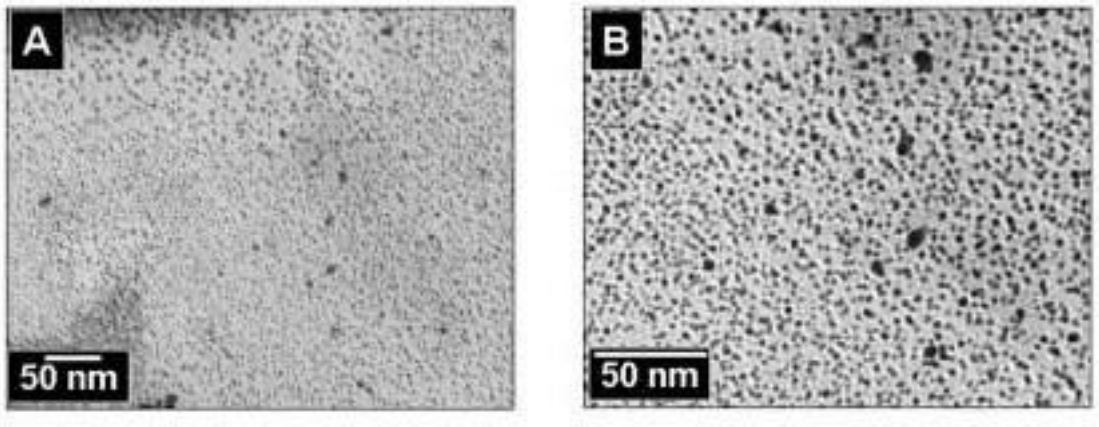
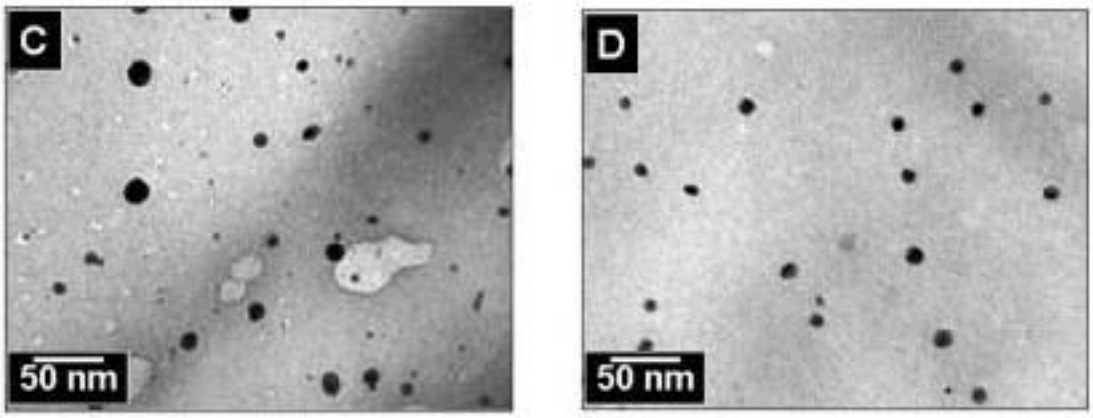


Figure 4.B.4. (A) and (B) Low and high magnification of TEM micrographs of the free standing nanogold membrane.



Figures 4.B.4. (C) and (D) The TEM micrographs of the gold nanoparticles leached from the nanogold membrane

4.B.3.5. SEM

Figure 4.B.5. (A) and (B) have shown the low and high magnification of SEM images of the nanogold membrane transferred from the liquid-liquid interface on the Si (111) substrates. It was seen that surface of the nanogold membranes was not smooth. Moreover, a mesh like structures were seen to form which indicated the simple cross-linking of the gold nanoparticles through the terminal groups of oxidized DAEE or through formation of a polymeric network of the oxidized DAEE molecules. As mentioned briefly earlier, the first step in the formation of the membrane was electrostatic complexation of $AuCl_4^-$ ions with protonated amine groups of DAEE molecules at the liquid-liquid interface and incomplete reduction of the gold ions would explain their presence in the membrane.

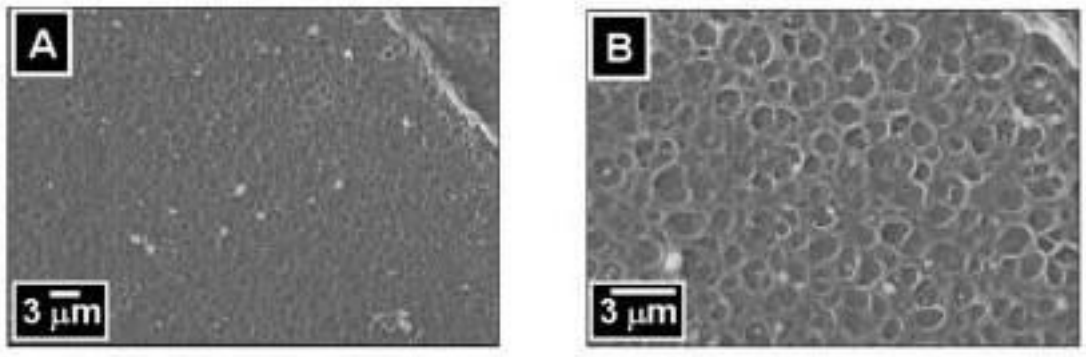


Figure 4.B.5. (A) and (B) Low and high magnification of SEM images of nanogold membrane synthesized at the liquid-liquid interface and transferred on Si(111) substrate.

Thereafter, this nanogold membrane was immersed in an aqueous suspension of *Candida bombicola* cells for 4 h and washed thoroughly prior to imaging by SEM. **Figure 4.B.6. (A) and (B)** have shown the SEM images recorded after immobilization of

the *Candida bombicola* cells on to the hydrophobic nanogold membrane. It was clearly seen from the SEM images that yeast cells were immobilized extremely faithfully on the surface of the hydrophobic nanogold membranes. It was well known that these *Candida bombicola* cells bound to hydrophobic regions.[23] The process of attachment of the cells to the hydrophobic nanogold membranes has been illustrated in **Figure 4.B.1.** The low and high magnification of SEM images recorded after immobilization of the *Candida bombicola* cells on to the as prepared nanogold membrane as shown in **Figure 4.B.6. (C) and (D)**. The density of the cells was less as compared to the cells immobilized on the hydrophobic nanogold membranes. It was well known that the cells bind to hydrophobic regions of the surface[23] and thus the use of octadecylamine bound to the nanogold membranes provided the hydrophobic surface for the binding of the *Candida bombicola* yeast cells. The process of the attachment of the *Candida bombicola* yeast cells to the hydrophobic nanogold membrane has been illustrated in **Figure 4.B.1.** In order to understand the interactions between the polymer membrane and the *Candida bombicola* yeast cells, the gold nanoparticles leached polymeric membrane was used for the immobilization of whole cells. Care was taken to wash the polymeric membranes with copious amount of chloroform and after with deionized water prior to use.

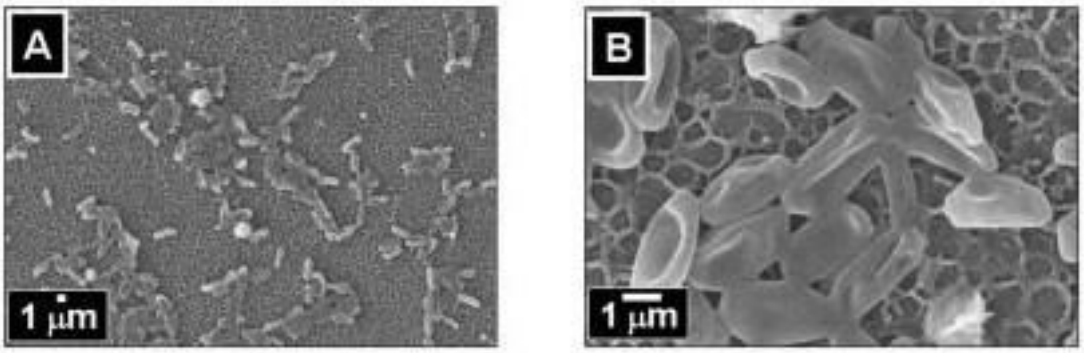


Figure 4.B.6. (A) and (B) The SEM images of the *Candida bombicola* cell immobilized on the hydrophobic nanogold membranes.

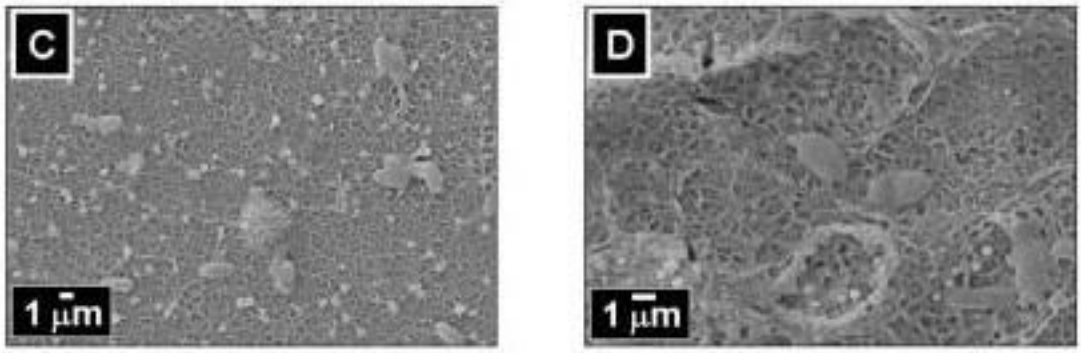


Figure 4.B.6. (C) and (D). The *Candida bombicola* cells immobilized on the as prepared nanogold membrane.

Figure 4.B.7. (A) and (B) have shown the SEM images after immersion of the gold nanoparticles leached polymeric membrane in aqueous dispersion of *Candida bombicola* cells in water for 4 h. It was seen that hardly any number of the cells attached to the polymeric surface. This confirmed the role of hydrophobic gold nanoparticles embedded in the polymeric membrane were responsible for the attachment of the cells to the surface of the nanogold membrane.

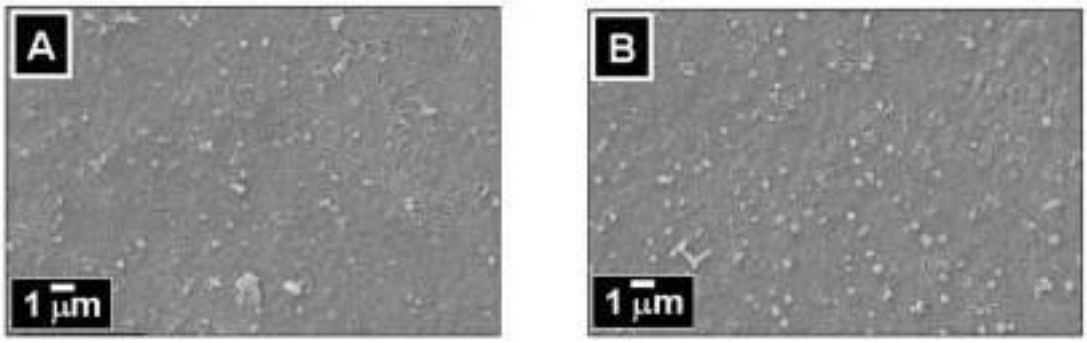


Figure 4.B.7. (A) and (B) The SEM images after the immobilization of the *Candida bombicola* cells on gold nanoparticles leached polymeric membrane.

4.B.3.6. Synthesis of sophorolipids

Enzymes of the cytochrome P450 monooxygenase family are known to metabolize hydroxylation of arachidonic acid.[26] Expressions of cytochrome P450 have been documented in liver, kidney and cerebral microvasculars. The earlier biochemical studies of the cytochrome P450 dependent arachidonic acid monooxygenase reaction showed that catalytic activity turnover was NADPH dependent.[27] This was an advantage of using whole cells, which limited us to use cofactors such as NADPH for the synthesis of sophorolipids. Moreover, the advantage in the use of immobilized whole cell system with cell bound activity was that this obviated the need for enzyme extraction and removal of the unwanted macromolecules released during the extraction process which were laborious and expensive.

Candida bombicola yeast cells are known to produce extracellular biosurfactants known as sophorolipids. Sophorolipids are produced as a mixture of acidic and lactonic forms. Sophorolipids obtained from the *Candida bombicola* yeast cells; the lactonic form represented the largest fraction of the products.[28] **Scheme 4.B.1.** has shown the transformation of arachidonic acid using *Candida bombicola* yeast cells to the acidic and lactonic form of the sophorolipids **(Scheme 4.B.1. step 1)**. Thereafter, acid hydrolysis of these sophorolipids yields 20-HETE **(Scheme 4.B.1. step 2)**. Nanogold membranes of the immobilized *Candida bombicola* yeast cells were reacted with arachidonic acid and the sophorolipids were isolated from the reaction medium and subjected to acid hydrolysis as described in details in the experimental section. The amount of cells immobilized on the 20 mg of hydrophobic nanogold membrane was estimated from the initial (~ 10^8 cells/mL) and the final cell counts after immobilization as described in the experimental

section, and was estimated to be 0.3 mg. The immobilized yeast cells transformed 75 w/v % of the arachidonic acid to sophorolipid compared with the same amount of free cells in solution. The sophorolipids were separated by thin-layer chromatography (TLC) on standard Kiesel-gel 60 plates and were detected by mass spectroscopy. **Figure 4.B.8. (A) and (B)** have shown the mass spectra of the sophorolipids formed from arachidonic acid. Significant ions occurred at *m/z* [710] and *m/z* [728], and the structures were determined to be lactonic and the acidic forms of the diacetate respectively.

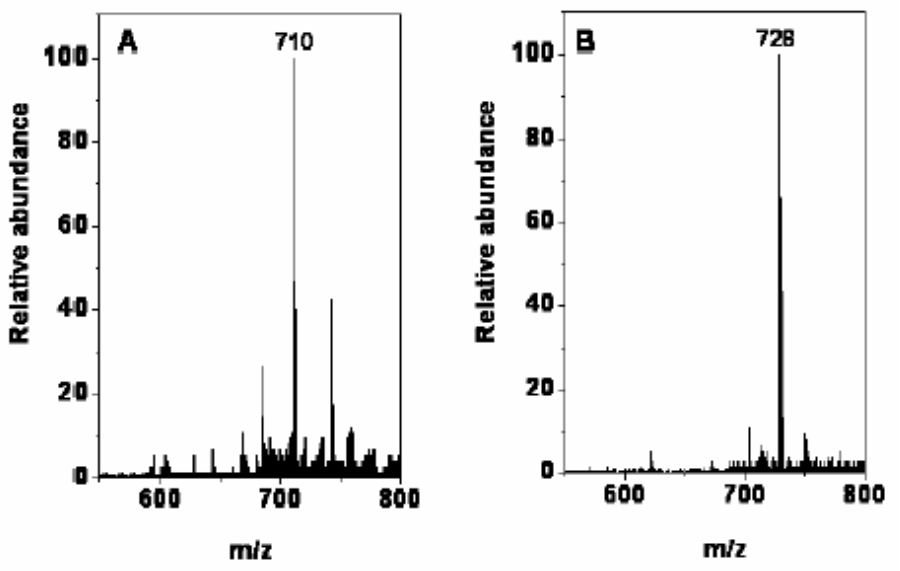


Figure 4.B.8. (A) and (B) Mass spectrum of the sophorolipids produced from the arachidonic acid. The sophorolipids are Lactonic (A) and Acidic (B) forms of diacetate as detected by mass spectroscopy.

The binding of the cells to the nanogold membranes was strong enough with marginal leaching of cells during the sophorolipid production. Hence, the immobilized cells on the hydrophobic nanogold membrane were reused for the five successive reaction cycles. The yeast cells bound to the hydrophobic nanogold membrane transformed 70 w/v % of the arachidonic acid to sophorolipid for the third cycle of reaction, while transformed 60 w/v % for the fifth cycle of reaction.

Figure 4.B.9. (A) and (B) have shown the low and high magnification of SEM images of different regions of *Candida bombicola* cells immobilized on hydrophobic nanogold membrane after one cycle of reaction (incubating in the reaction medium for 30 ^{0}C for 96 h). SEM images have shown the registry of the cells attached to the surface of the hydrophobic nanogold membranes. SEM confirms the fidelity of the yeast cells on the surface of the hydrophobic nanogold membrane after one cycle of reaction.

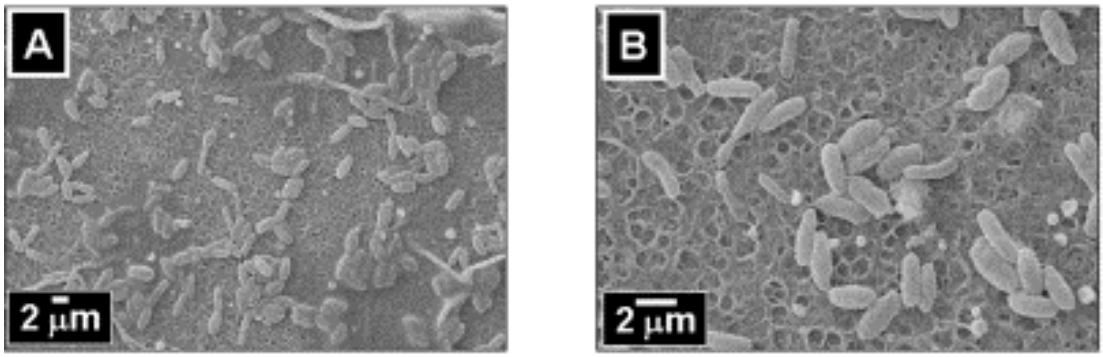


Figure 4.B.9. (A) and (B) Low and High magnification of SEM images of the immobilized *Candida bombicola* cells on the hydrophobic nanogold membrane after one reaction cycle

Acid hydrolysis of the sophorolipid yielded 19-HETE and 20-HETE. The 20-HETE formed was analysed by gas chromatography. As the hydroxylated fatty acids released from the sophorolipids. **Figure 4.B.10.** has shown the partial mass spectrum of 20-HETE as detected by GC-MS. Significant ions occurred at *m/z* [406 M^+], 391 [M^+ - 15], 316 [M^+ - 90], 304 [(M^+ +1) – 103].[17, 23] Selective ion monitoring also showed a prominent signal at m/z 103 [$(CH_3)_3$-Si-O^+-CH_2].[17, 23] The mass spectrum thus clearly indicated that the hydroxyl group was at the C20 position and that the compound was 20-hydroxy 5Z, 8Z, 11Z, 14Z-eicosatetraenoic acid (20-HETE). Confirmation of the identity of 20-HETE was obtained by the observed co-elution of 20-HETE standard by GC-MS with the isolated material.

The mass spectrum indicated a hydroxyl group at position C19. The mass spectrum of ions occurred at m/z 117 due to cleavage from C19 to C20; [$(CH_3)_3$ -Si- O^+-CH-CH_3] and 73 [base ion; $(CH_3)_3$-Si] and loss of 220 [M^+ - 186] due to rearrangement and loss of –CH=CH $(CH_2)_3$-CH[O^+-Si-$(CH_3)_3$]-CH_3, 201 {M^+ - [131 + 74 (silyl group + H)]}. **Figure 4.B.11.** has shown the structures of the significant ions occurred of methyl ester silyl ether of 19-HETE. The compound was identified as 19-hydroxyeicosatetraenoic acid.[17]

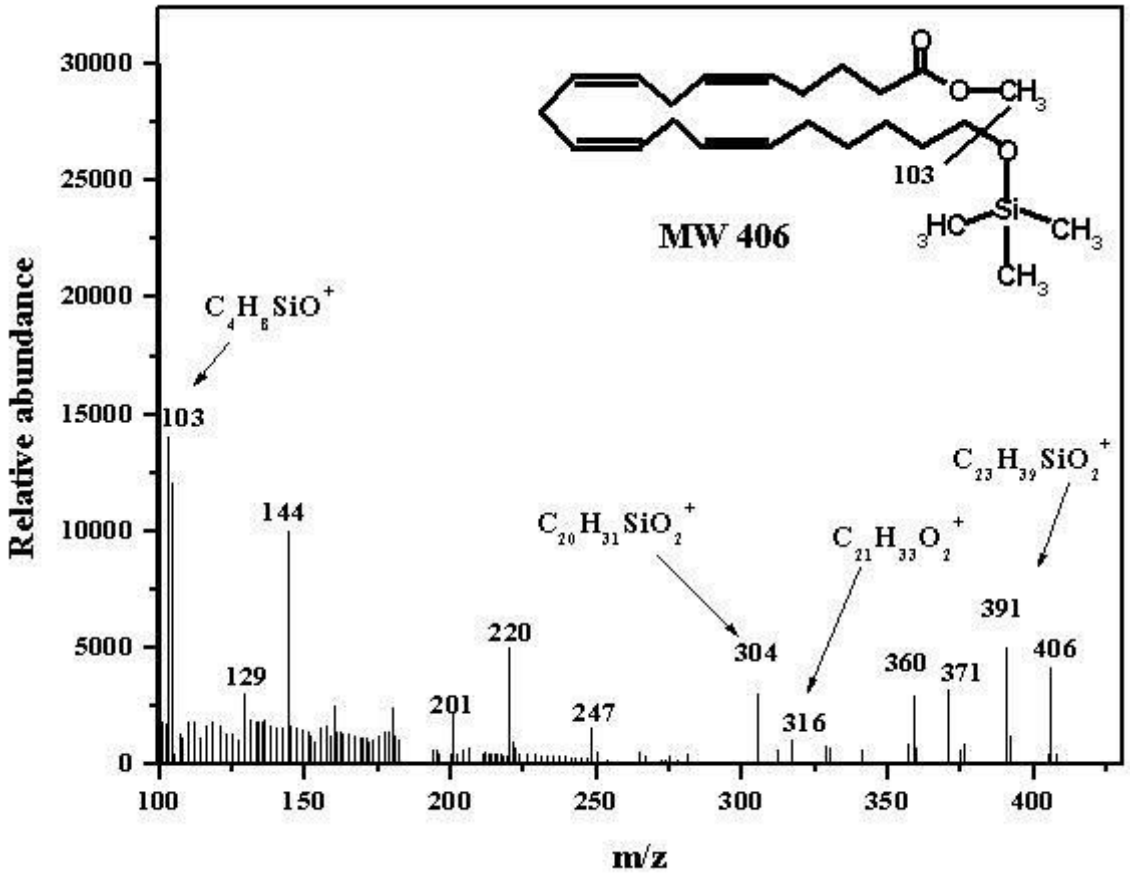


Figure 4.B.10. Partial electron impact mass spectrum of 20-HETE as detected by GC-MS. The compounds were extracted by from sophorolipids, synthesized by *Candida bombicola* cells and was identified as the methyl ester silyl ether of 20-hydroxy-eicosatetraenoic acid (20-HETE). The inset shows the structure of the methyl ester silyl ether of 20-hydroxy ecosatetraenoic acid (20-HETE)

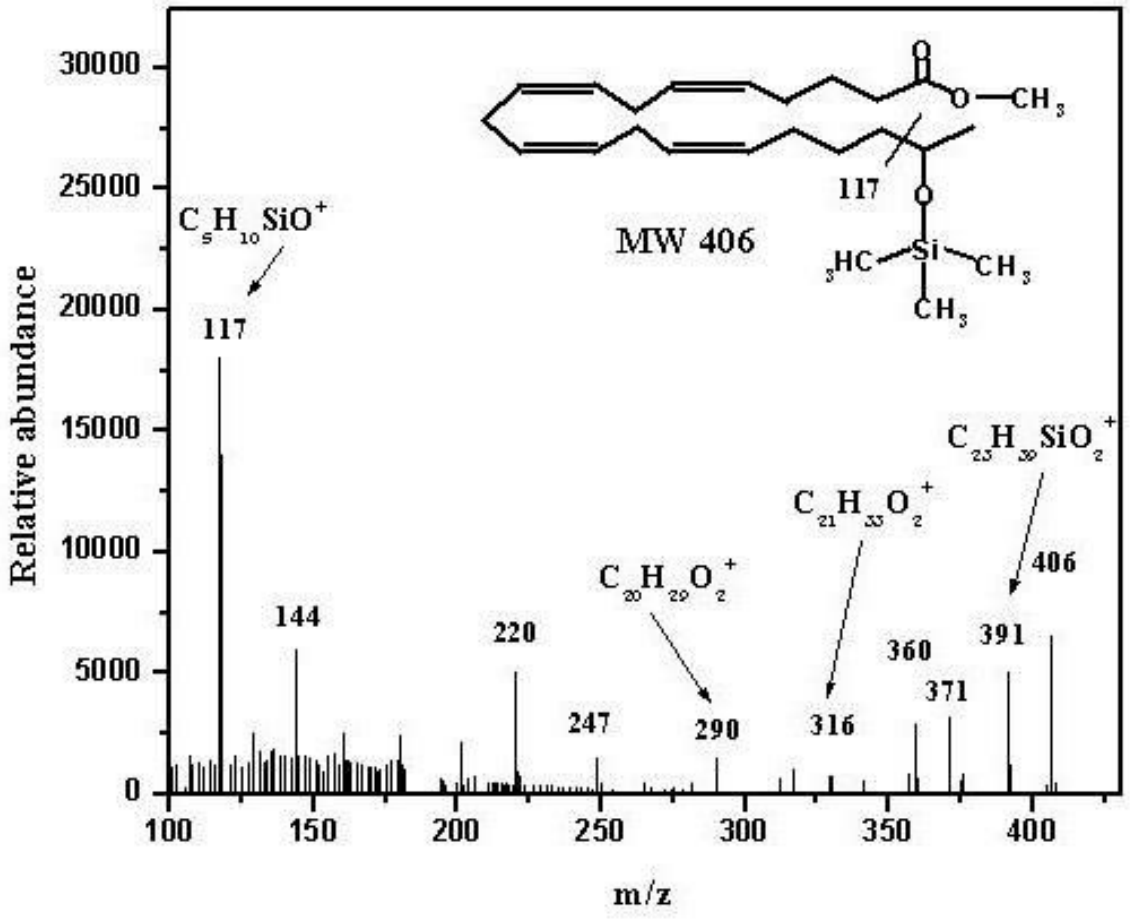


Figure 4.B.11. Partial electron impact mass spectrum of 19-HETE as detected by GC-MS. The compounds were extracted by from sophorolipids, synthesized by *Candida bombicola* cells and was identified as the methyl ester silyl ether of 19-hydroxy-eicosatetraenoic acid (19-HETE). The inset shows the structure of the methyl ester silyl ether of 19-hydroxy ecosatetraenoic acid (19-HETE)

4.B.4. Conclusion

In this study, we have demonstrated the synthesis of hydrophobic nanogold membrane and thereafter used for the immobilization of *Candida bombicola* yeast cells driven by hydrophobic interactions between the cell walls and the ODA molecules. Enzymes of the cytochrome P450 monooxygenase family present in the yeast cells were used for the hydroxylation of arachidonic acid for the production of sophorolipids. The binding of the cells to the nanogold membrane was strong enough to prevent the leaching of the whole cells from the surface. Hence the whole cells immobilized membranes were reused for the biotransformation.

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Chapter 5

Summary and Conclusions

The summary of the results obtained and the conclusions drawn are presented in this chapter with future potential developments in the area.

5.1 Summary of the work

The major objectives of the thesis were to explore the biotransformation of arachidonic acid for production of 19-HETE and 20-HETE using *Candida bombicola*. A brief summary of the thesis is given below.

Chapter one presents a general introduction about eicosanoids and biotransformation of arachidonic acid metabolites. 20-hydroeicosatetraenoic acid (20-HETE) and 19-hydroxyeicosatetraenoic acid (19-HETE) are omega and omega–1 hydroxylated products of arachidonic acid which are important in autoregulation of blood pressure, vascular tone and other physiological roles. Different methods were discussed for the production of 19-HETE and 20-HETE. The intermediate sophorolipid was discussed in brief. Based on these review the scope and objective of the present work have been outlined.

Chapter two describes the fermentation parameters of *Candida bombicola* (ATCC 22214) for the production of arachidonic acid derived sophorolipids. The derived sophorolipids on acid hydrolysis liberated 19-HETE and 20-HETE. The different experimental techniques and analytical tools were used during the course of the present work are discussed in detail.

Chapter three deals with purification of the mono-, di- acetate forms of lactonic and acidic sophorolipids produced by *Candida bombicola* grown on glucose and long chain fatty acid i.e. arachidonic acid. The derived sophorolipids were isolated by silicagel chromatography using dialysis tubing. Different analytical tools were used to characterized the purified sophorolipids and their derived products This chapter also describes the detailed study of purified sophorolipids structure and presence of 19-

hydroxyeicosatetraenoic acid and 20- hydroxyeicosatetraenoic acid in the sophorolipid form.

Chapter four focuses on the continuous synthesis of 19-HETE and 20-HETE by immobilization of *Candida bombicola* on different biocompatible materials. The patterned thermally evaporated octadecylamine (ODA) lipid films and free-standing organic–gold nanoparticles embedded in a polymeric membrane provide a biocompatible surface for the immobilization of whole cells. The presence of gold nanoparticles in the membrane enables facile modification of the surface properties of the membrane and has been used as enzyme sources for the transformation of arachidonic acid to 19-HETE and 20-HETE. The attachment of the cells to the ODA film surface occurs possibly through nonspecific interactions such as hydrophobic interactions between the cell walls and the ODA molecules. The enzyme cytochrome P450 present in the immobilized yeast cell membrane was used to catalyze the biotransformation of the arachidonic acid to sophorolipids and thereafter, acid hydrolysis to yield 19-hydroxyecosatetraenoic acid (19-HETE) and 20-hydroxyecosatetraenoic acid (20-HETE). These biocomposite materials could be easily separated from the reaction mixture and exhibit excellent reusability.

5.2. Conclusions of the work

Our approach here is to address the problem, the difficulties associated in eicosanoids (oxylipins) chemical synthesis, which expensive as well as hazardous in nature. The chemical synthesis of 19-HETE and 20-HETE involve more than 10 steps starting from arachidonic acid. These chemical methods are not commercially feasible. Presented work is aiming to reduce the price for the production of theses HETE's through biotransformation method.

Biotransformation of arachidonic acid by yeasts *Candida bombicola* (ATCC 22214) and *Candida apicola* (ATCC 96134) produced vasoactive molecules 19-hydroxyeicosatetraenoic acid (19-HETE) and 20-hydroxyeicosatetraenoic acid (20-HETE). These are omega and omega–1 hydroxylated products of arachidonic acid. 20-HETE and 19-HETE which play an important role in autoregulation of renal blood flow, tubuloglomerular feedback, renal sodium transport, pulmonary function and vasoconstrictor responses to numerous vasoactive hormones. This thesis describes the innovated methods for the continuous production of 19-HETE and 20-HETE. Different biocompatible materials were synthesized like patterned lipid films, and free-standing organic-gold nanoparticle poly membranes for the immobilization of *Candida bombicol*a whole cells.

Biomolecules may easily get denatured and loose their biocatalytic activity after adsorbing on solid surfaces during immobilization. This was primary reason to design completely new class of material for immobilization. These provide a biocompatible environment and can readily conserve the native structure of biomolecules. This thesis describes the use of patterned lipid films, gold nanoparticles embedded polymeric membrane for the immobilization of *Candida bombicola* whole cells. The immobilized cells show enhanced temporal and indicating protective nature offered to whole cells on biocompatible materials. The enzyme cytochrome P450 present in the yeast cells was used to catalyze in situ ω and ω -1 hydroxylation of arachidonic acid. Cytochrome P450, the enzymes of interest is unstable outside the cellular environment and in such cases, immobilization of the whole cells would be important to catalyze reactions that are dependent on the cofactors. These biocompatible materials were easily separated from

the reaction mixture and reused by simple distilled water washing. They showed excellent reuse characteristics for the biotransformation of arachidonic acid.

The yeasts *Candida bombicola* (ATCC 22214) and *Candida apicola* (ATCC 96134) when grown on primary carbon source glucose and secondary carbon source arachidonic acid produced mixture of sophorolipids of 19-HETE and 20-HETE. The derived sophorolipids are stable than liberated hydroxylated fatty acids. These sophorolipids on acid hydrolysis yielded 19-HETE and 20-HETE. We have shown separation of sophorolipids to give increase stability to these heat and light labile biomolecules.

Patterned thermally evaporated octadecylamine (ODA) films are used for the immobilization of *Candida bombicola* cells. The attachment of the cells to the ODA film surface occurs possibly through the nonspecific interactions such as hydrophobic interactions between the cell walls and the ODA molecules. The presence of gold nanoparticles in the polymeric membrane enables facile modification of the properties of the membrane. Cytochrome P450 is a membrane bound protein and is known to be highly unstable outside the cells environment. Since the yeast cells are used, the cofactors such as nicotinamide adenine dinucleotide phosphate (NADPH) is not required and is readily supplied by the cells along with the primary enzyme. This is a major advantage in using the whole cells rather than using enzyme. Hence it was of paramount interest to used biocompatible materials for yeast cells. Biocompatible environment for the immobilization of *Candida bombicola* have done the hydroxylation of arachidonic acid to form sophorolipids and thereafter, acid hydrolysis gives 19-hydroxyeicosatetraenoic acid (19-HETE) and 20-hydroxyecosatetraenoic acid (20-HETE).

This thesis also discuses about the intermediate arachidonic acid derived sophorolipids. These are extracellular surface active glycolipids produced by the yeasts *Candida bombicola.* The crude product was a heterogeneous mixture of sophorolipids, which are glycolipids of sophorose linked to the fatty acid through glycosidic bond between ω and ω-1 carbon of arachidonic acid. The derived arachidonic acid sophorolipids were isolated by silicagel chromatography using dialysis tubing. Acid hydrolysis of the resolved sophorolipids yielded 20-hydroxyeicosatetraenoic acid (20-HETE) and 19-hydroxyeicosatetraenoic acid (19-HETE), the compounds of pronounced pharmaceutical importance.

5.3. Scope for future work

The present study examined the importance of oxylipins 19-hydroxyeicosatetraenoic acid and 20-hydroxyeicosatetraenoic acid derivatives of arachidonic acid. Arachidonic acid is primarily metabolized by a cytochrome P450 to these HETE's. The importance of HETE's in the regulation of renal function, vascular tone, airway resistance and many other physiological significance. The chemical synthesis of 19-HETE and 20-HETE methods are commercially cumbersome. It is known that terminal carbon center hydroxylation (omega) by chemical method is difficult due to lack of reactivity at this carbon. In this context the most useful characteristic of microbial transformation is that offers unusual activation at normally unreactive carbon centers where no conventional chemistry is applicable. These microbes not only help to permit simpler methods but also safer and economical ways of producing these biologically active compounds.

Immobilization of whole cells on biocompatible materials enhances the production of HETE's. Thermally evaporated lipid films and gold nanoparticle polymeric membranes can act as scaffolds for growth of different cells in tissue engineering. These materials can be used for the growth of different microorganisms and thus suggests potential biomedical applications as biocompatible implants, grafting in bone surgery, drug delivery, etc. The facility of patterning biomaterials can be extended towards the patterned immobilization of DNA, and can be used for screening genomic libraries. Immobilizing various proteins on a single chip can easily perform multi-step biocatalytic reactions requiring many enzymes, wherein each enzyme would specifically react with its substrate giving desired final product. The replacing the chemical approaches for eicosanoids with microbial route will reduce the cost coupled with the possibility of obtaining other novel compounds. From these studies it is expected to obtain hitherto unknown but structurally relevant novel products which may posses biological activation far superior to the known product.

List of publications

1. *Candida bombicola* Cells Immobilized on Patterned Lipid Films as Enzyme Sources for the Transformation of Arachidonic Acid to 20-HETE

Sumant Phadtare, Parag Parekh, **Sachin Shah**, Amruta Tambe, Rohini Joshi, S. R. Sainkar, Asmita Prabhune and Murali Sastry

Biotechnol. Prog. **2003,** *19,* 1659-1663.

2. Immobilization of *Candida bombicola* Cells on Free-Standing Organic-Gold Nanoparticle Membranes and Their Use as Enzyme Sources in Biotransformations

Sumant Phadtare, **Sachin Shah**, Asmita Prabhune, Prakash P. Wadgaonkar and Murali Sastry

Biotechnol. Prog. **2004,** *20*, 1817-1824.

3. Purification by silicagel chromatography using dialysis tubing and characterization of sophorolipids produced from *Candida bombicola* grown on glucose and arachidonic acid

Sachin Shah and Asmita Prabhune

Biotechnol. Lett. **2006** (In Press)

Awards and honors

1. Best research paper selected for Oral presentation at 73rd Annual meeting of the Society of Biological Chemists (INDIA), G.B. Pant University of Agriculture & Technology, Pantnagar, **India.** (November 21-24, **2004**).

2. Best research paper (poster) award in Industrial Biotechnology "BIOTECH 2005", 3rd National Conference of Biotechnology Society "Concept to Commercialization", organized by the Center For Biotechnology, Jawaharlal Nehru University, New Delhi, **India.** (December 22-24, **2005**)

3. Research abstract selected for Oral presentation at 3rd International Congress of Nanotechnology (ICNT) 2006, San Francisco, **USA.** (October 30th, **2006**)

Patents Filed

1. A new process for production of succinic acid from glucose

Indian Patent no. (757/DEL/2005)

US Patent file reference (PCT 572, 2005)

Papers Presented in Conferences / Symposia

1. Nanogold membrane as scaffolds for whole cell immobilization as enzyme source for biotransformation of arachidonic acid to 20-HETE

Sachin Shah, Sumant Phadtare, Murali Sastry, Asmita Prabhune

73rd Annual meeting of the Society of Biological Chemists (INDIA),

G.B. Pant University of Agriculture & Technology, Pantnagar, India.

(November 21-24, **2004**) **(Best research paper selected for Oral presentation)**

2. Effect of culture conditions on growth and production of n-3 and n-6 fatty acids families by Mucor sp. Vs 1846

Gayatri Govitrikar, Sayali Sathe, **Sachin Shah**, Archana Pundle and Asmita Prabhune

73rd Annual meeting of the Society of Biological Chemists (INDIA).

University of Agriculture & Technology, Pantnagar, India.

(November 21-24, **2004**)

3. A Microbial production of Sophorolipids (Biosurfactant) and its novel method for purification and its industrial applications

Sachin Shah and Asmita Prabhune

74th Annual meeting of the Society of Biological Chemists (INDIA).

Central Drug Research Institute, Lucknow, India.

(November 7-10, **2005**)

4. Whole cell immobilization as enzyme source for biotransformation of arachidonic acid to 20-hydroxyeicosatetraneoic acid (20-HETE)

Sachin Shah and Asmita Prabhune

"BIOTECH 2005", 3rd National Conference of Biotechnology Society "Concept to Commercialization", organized by the Center For Biotechnology, Jawaharlal Nehru University, New Delhi, India.

(December 22-24, **2005**) **(Best research paper award in Industrial Biotechnology)**

5. *Candida bombicola* cells immobilization on biocompatible materials for biotransformation of arachidonic acid to 20-hydroxyeicosatetraneoic acid and 19-hydroxyeicosatetraneoic acid

Sachin Shah and Asmita Prabhune

"Life: Molecular Integration & Biological Diversity"

20th IUBMB International Congress of Biochemistry and Molecular Biology and 11th FAOBMB Congress, Japan.

(June 18-23, **2006, Kyoto Japan**)

6. Immobilization of *Candida bombicola* cells on porous alumina membrane and their use in transformation of arachidonic acid to 19-HETE and 20-HETE.

Sachin Shah, Sulabha Kulkarni, Asmita Prabhune.

3rd International Congress of Nanotechnology (ICNT) 2006, San Francisco, **USA.** (October 30th, **2006**, **San Francisco, USA.**) **(Research abstract selected for Oral presentation)**