STUDIES ON EXTRACELLULAR POLYMERIC SUBSTANCES FORMED BY BIOFILM FORMING CANDIDA SPECIES

A THESIS

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **STUDIES ON EXTRACELLULAR POLYMERIC SUBSTANCES FORMED BY BIOFILM FORMING CANDIDA SPECIES** in partial fulfilment of the requirements for the award of degree of Doctor of Philosophy, submitted in the Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from July 2004 to December 2008 under the supervision of Dr. Vikas Pruthi, Assistant Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

The matter embodied in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

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This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

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ABSTRACT

Candida albicans is an opportunistic pathogenic fungus capable of causing a wide variety of infections ranging from superficial to life-threatening systemic infections. Candidiasis is often associated with the formation of biofilms on the surface of inert or biological materials. These biofilms are spatially organized communities of cells encased in a matrix of Extracellular Polymeric Substances (EPS) on a substrate and are particularly characterized by increased resistance to antimicrobials. The present study reports for the isolation and biochemical characterization of EPS produced by a clinical strain of *C. albicans* isolated from infected clinical samples. About 53.4% of the clinical samples studied showed high incidence of *Candida* infection, among which *C. albicans* was found in maximum percentage (41%). Chromogenic agar medium was successfully utilized in the identification of these *Candida* species from infected clinical samples. On microscopic examination, *C. albicans* appeared as gram positive, ovoid, budding yeast having mould like hyphae and large refractile chlamydospores. Amongst 16 *C. albicans* isolates, maximum biofilm forming isolate, *C. albicans PLV12* was selected on the basis of XTT reduction assay. The assay was used to explore three developmental phases of *C. albicans PLV12* biofilm formation. In the first phase (early phase), adherence of candidal cells to the PVC surface takes place; in the second phase (intermediate phase), formation of a matrix with dimorphic switching from yeast to hyphal forms was observed while the last phase (maturation phase) showed an increase in the matrix material (EPS) giving three-dimensional architecture to the biofilm. Quantitative analysis of *C. albicans PLV12* EPS showed that biofilms contained significantly reduced content of total carbohydrate (40±8.5%), protein (5.0±1.2%), extracellular DNA (0.2±0.1%) and enhanced amount of glucose (16±3.4%), hexosamine (4.0±1.3%), uronic acid

(0.5±0.1%) and phosphorus (0.7±0.2%) in contrast to its planktonic counterparts. Microscopic examination using SEM, AFM and CLSM revealed that *C. albicans PLV12* biofilms have a highly heterogeneous architecture composed of cellular and non-cellular elements with EPS distributed in the cell-surface periphery or at cell-cell interface. The synthesis of EPS during the formation of *C. albicans PLV12* biofilm was found to be highly dependent on the conditions of incubation and types of substrates used. Under static conditions, EPS synthesis was minimal but was greatly enhanced under mild shaking (15 rpm) conditions. Parameter optimization indicated that maximum EPS yields were achieved at pH 6.3 and temperature 30°C using arabinose as a carbon substrate for growth.

Partial characterization of *C. albicans PLV12* EPS using chromatographic techniques showed presence of both negatively (D-glucuronic acid) and positively (N-acetylglucosamine) charged components in the exopolysaccharide chain. The major sugars units in the exopolysaccharide chain as revealed by HPLC and GC analysis were found to be glucose, mannose, galactose, rhamnose, N-acetyl glucosamine and D-glucuronic acid. Gel permeation chromatography determined that *C. albicans PLV12* produces a heteropolysaccharide having a molecular weight of ~300 KD. Structural analysis using FTIR showed presence of β-glucans (β (1→6) and β (1→3) and mannans in the exopolysaccharide chain. These were later assigned to α- and β-D-glucose, α-D-mannose, α-L-rhamnose and N-acetyl glucosamine (β-D-GlcNAc) by ^{1}H and ^{13}C NMR studies.

EMS (3%) treatment showed 19 *C. albicans PLV12* mutants having more than 50% reduction in their biofilm forming ability. Of which, the selected mutant strain, *pm4,* resulted in 75.2% and 71% reduction in biofilm formation and EPS production respectively, compared with the wild strain. This mutant strain showed bluish-green colonies with rough

morphology on HiChrom Candida agar medium. SEM analysis further explored morphological alterations in biofilms formed by this mutant strain. Comparative analysis of mutant (*pm4*) EPS with that of wild strain revealed reduction in total carbohydrate, protein and glucose content. The antifungal susceptibility testing indicated increased susceptibility of mutant strain towards Fluconazole, Itraconazole, Ketoconazole and Amphotericin B in contrast to its wild strain.

Various control strategies used in the study showed their potential towards *C. albicans PLV12* biofilm. Out of which Eucalyptus and Peppermint oils showing 80.87% and 74.16% reduction in *C. albicans PLV12* biofilm formation were found to be promising agents. Addition of metabolic inhibitors like DNP and bismuth dimercaprol to the culture medium also resulted in a significant decrease in viable cell counts and EPS yield. Studies with biosurfactant (rhamnolipid) showed 78.6% and 66.79% reduction in biofilm and EPS yield, acting directly on the biofilm matrix to disrupt and solubilize its components and incorporating the matrix into micelles. Thus, prove to be an attractive agent in overcoming *C. albicans* biofilm-induced resistance. Use of enzymes like Alginate Lyase, Cellulase, Chitinase, Proteinase K, and DNase I also revealed a significant decrease in *C. albicans PLV12* biofilms by partially degrading matrix material and causing biofilm detachment from the surfaces of the MTP. Interestingly, Lyticase hydrolyzing β-1, 3 glucan moiety of *C. albicans PLV12* EPS resulted in maximum (82%) reduction in biofilms. Further, *C. albicans PLV12* biofilms were found to be resistant to almost all the antifungal agents (Fluconazole, Itraconazole, Ketoconazole and Flucytosine) used even at concentrations greatly in excess of their MICs. Only Amphotericin B showed an inhibitory effect on the activity of biofilm cells (62% reduction) but no significant reduction in EPS production was noticed indicating that

EPS does not contribute a barrier to the penetration of antifungal agents of differing chemical structure.

The work presented in the thesis would be helpful in unveiling mystery behind *C. albicans* polymeric matrix and may provide means to design novel therapies against *C. albicans* biofilm-based infections.

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LIST OF ABBREVIATIONS

%	:	Percentage
µl	:	Microlitre
µm	:	Micrometer
°C	:	Degree centigrade
α-DMG	:	α-D-methyl glucoside
AFM	:	Atomic Force Microscopy
BSA	:	Bovine serum albumin
cfu	:	Colony forming unit
CLSM	:	Confocal Laser Scanning Microscopy
CMC	:	Carboxy methyl cellulose
ConA	:	Concavalin A
conc.	:	Concentration
CSF	:	Cerebrospinal fluid
D_2O	:	Deuterium Oxide
DEAE	:	Diethylaminoethyl
DNase 1	:	Deoxyribonuclease 1
DNP	:	2,4-Dinitrophenol
EMS	:	Ethyl Methanesulfonate
EPS	:	Extracellular polymeric substances
EtBr	:	Ethidium Bromide
FDA	:	Fluorescein Diacetate
FID	:	Flame ionization detector
FITC	:	Fluorescein Isothiocynate
FTIR	:	Fourier Transform Infrared spectroscopy
g	:	Gram
GAA	:	Glacial acetic acid
GC	:	Gas chromatograph
GOD/POD	:	Glucose oxidase/peroxidase
h	:	Hour
HEPES	:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	:	High performance liquid chromatography
IIT	:	Indian Institute of Technology
IMTECH	:	Institute of Microbial Technology
IUD	:	Intra uterine device
KD	:	Kilo Dalton
kHz	:	Kilohertz
l	:	Litre
M	:	Molar
mg	:	Milligram
MIC	:	Minimum inhibitory concentration
min	:	Minute
ml	:	Millilitre
mM	:	Millimolar
MTCC	:	Microbial Type Culture Collection

MTP	:	Microtitre Plate
N	:	Normal
N/m	:	Newton/meter
NCCLS	:	National Committee for Clinical Laboratory Standards
nm	:	Nanometer
NMR	:	Nuclear Magnetic Resonance
OD	:	Optical density
PBS	:	Phosphate buffer sulphate
PI	:	Propidium Iodide
ppm	:	Parts per million
PVC	:	Polyvinyl chloride
RI	:	Refractive Index
rpm	:	Rotations per minute
RT	:	Room temperature
SDA	:	Sabouraud Dextrose agar
SEM	:	Scanning Electron Microscopy
sp.	:	Species
TCA	:	Trichloroacetic acid
v/v	:	Volume/volume
vol	:	Volume
w/v	:	Weight/volume
XTT	:	2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide
YPD	:	Yeast Extract Peptone Dextrose
ZOI	:	Zone of inhibition

CHAPTER 1

INTRODUCTION

Biofilms have been studied extensively over the past two decades and have only recently been recognized for their complexity and ability to resist just about everything in our current antimicrobial armoury (Kathryn, 2004). These biofilms are defined as structured communities of microbial species embedded in a biopolymer matrix on either biotic or abiotic substrata (Jiang and Pace, 2006). In the process of biofilm formation, microorganisms irreversibly attach to and grow on a surface and produce extracellular polymers that facilitate attachment and matrix formation, resulting in an alteration in the phenotype of the organisms with respect to growth rate and gene transcription (Donlan, 2001). Biofilm formation is a clinically significant process in view of the fact that (1) they are resistant to antimicrobial agents; (2) they may be a persistent source of infection; (3) they may harbor pathogenic organisms, and (4) they may allow exchange of resistance plasmids (Donlan, 2001).

It is now estimated that 65% of microbial infections are caused by microorganisms growing on surfaces rather than in the free living planktonic state (Thomas, 2006). Although the majority of implant related infections are caused by bacteria like *Staphylococcus epidermidis, Staphylococcus aureus, Pseudomonas aeruginosa* and *E. coli*, fungal infections are becoming increasingly important. They are most often caused by pathogenic *Candida* genus, particularly *Candida albicans*. *C. albicans* is a pleiomorphic fungus that can exist either as a commensal or an opportunistic pathogen and is capable of causing superficial to life-threatening infections. Predisposing factors for *C. albicans* infections include immunosuppressive therapy, antibiotic therapy, human immunodeficiency virus (HIV) infection, diabetes and old age. In addition, structured microbial communities attached to surfaces, commonly referred to as biofilms, have increasingly been found to be the sources of *C. albicans* infections (Cao *et al.*, 2008). The increase in *Candida* infections in last decades has

almost paralleled the increased use of a broad range of medical implant devices in patients with impaired host defenses. Even with current antifungal therapy, mortality of patients with invasive candidiasis can be as high as 40% (Dominic *et al.*, 2007). Importantly, *C. albicans* is ranked as fourth in the cause of nosocomial infections, third in catheter-related infections, second highest in colonization to infection rate and highest overall in crude mortality (Subha and Gnanamani, 2008).

Biomaterials such as stents, shunts, prostheses (voice, heart valve and knee prostheses), implants (lens and breast implants and dentures), endotracheal tubes, pacemakers and various types of catheters have all been shown to facilitate *C. albicans* colonization and biofilm formation (Cao *et al.*, 2008). Candidiasis associated with these indwelling medical devices can result in serious complications and expensive care, which is noted as a frequent factor limiting the prolonged use of central venous catheters. When they are introduced in the host, indwelling medical devices are quickly overlaid with proteins, and microorganisms such as *C. albicans* (blastospores) can adhere onto this substrate to form a biofilm potentially responsible for fungal infection (Cateau *et al.*, 2008). Development of biofilm confers resistance to antimicrobials and host defense mechanisms. The subsequent realization of the role of candidal biofilms in candidiasis has prompted considerable interest in *C. albicans* biofilm structure, physiology, and regulation among researchers.

The EPS matrix is one of the most distinctive features of a microbial biofilm. It is a dynamic environment in which the component microbial cells appear to reach homeostasis and are optimally organized to make use of all available nutrients (Sutherland, 2001). EPS form a three-dimensional, gel-like, highly hydrated and often charged biofilm matrix, in which the microorganisms are embedded and are more or less immobilized (Wingender *et al*, 1999). It

creates a microenvironment for sessile cells which is conditioned by the nature of the EPS matrix. The matrix therefore shows great microheterogeneity, within which numerous microenvironments can exist. Matrix-enclosed microcolonies, sometimes described as 'stacks' or 'towers', are separated by water channels which provide a mechanism for nutrient circulation within the biofilm (Donlan and Costerton, 2002).

EPS are mainly responsible for the structural and functional integrity of biofilms and are considered as the key components that determine the physiochemical and biological properties of biofilms like gel formation, flocculation, emulsification, absorption, film formation, and protection. The EPS matrix helps the biofilm cells to resist multiple stress conditions, such as water or nutrient shortages, presence of biocides and antimicrobial agents (Kives *et al.*, 2006).

In general, the proportion of EPS in biofilms can vary between roughly 50 to 90% of the total organic matter (Wingender *et al*, 1999). The composition of the matrix varies according to the nature of the organisms present. The major matrix components are microbial cells, polysaccharides and water, together with excreted cellular products. Although exopolysaccharides provide the matrix framework, a wide range of enzyme activities can be found within the biofilm, some of which will greatly affect structural integrity and stability (Sutherland, 2001). Many of these exopolysaccharides are negatively charged due to the presence of carboxyl, sulphate or phosphate groups. Smaller amounts of proteins, nucleic acids and lipids can also be present (Al-Fattani and Douglas, 2006). Extracellular polysaccharides and proteins are shown to be the key components of the biofilm matrix. An earlier report indicates that extracellular DNA plays an important role in the establishment of biofilm structure (Whitchurch *et al.*, 2002). Also, dead cells have been observed in some biofilms,

suggesting that cell detritus can be considered part of the extracellular matrix. The role played by DNA and dead cells in biofilm represents exciting new directions for future studies of biofilm matrices (Branda *et al.*, 2005).

The EPS produced by *C. albicans* remains either attached to the cell surface or gets released into the extracellular medium in the form of slime. Studies by Baillie and Douglas, 1998; Critchley and Douglas, 1987; McCourtie and Douglas, 1981, suggested that *C. albicans* biofilm EPS plays a crucial role in candidal adhesion and antifungal resistance. Ballie and Douglas 2000, proposed that matrix of extracellular material, sometimes known as glycocalyx, may exclude or limit the access of a drug to organism deep in the biofilm. Possible drug exclusion by the matrix of biofilms seems to depend upon a number of factors, including the nature of the antibiotic and the binding capacity of the matrix towards it (Ballie and Douglas 2000). Analysis of EPS isolated from biofilm and planktonic cultures of *C. albicans* by our group revealed quantitative and quantitative differences providing contact-induced gene expression in *C. albicans* biofilm (Lal *et al.*, 2008). Recent study performed by Al-Fattani and Douglas, 2006, showed that matrix polymers significantly contribute towards drug resistance in both single-species and mixed-species biofilms containing *Candida*, especially under the flow conditions which prevail in many implant infections. However, biofilm resistance overall is likely to be multifactorial, involving, in addition, drug-resistant physiologies such as dormant 'quiescent' cells and expression of efflux pumps (Gilbert *et al.*, 2002).

Although a lot of literature exits for *C. albicans* biofilm formation, information regarding its polymeric substances is still limited. The present study is therefore aimed at the characterization of the biofilm EPS using a clinical isolate of *C. albicans* as a model system with a view to formulate a strategy for its regulation and control. Investigations whether EPS

plays an important role in biofilm resistance to antifungal agents would also be undertaken. Since little is currently known about the chemical composition and structure of the *C. albicans* EPS, it would be significant to study these polymeric matrix substances involved in the formation of complex architecture of biofilm. Quantitative measurements of biofilm and planktonic EPS were done for carbohydrate, protein, extracellular DNA and phosphorus content. The chemical structure of *C. albicans* exopolysaccharide was explored with the help of analytical techniques like GC, HPLC, FTIR and NMR. Control strategies targeting biofilm EPS were investigated using various antifungal agents (azoles, polyenes, and base analogs), herbal preparation like plant oils, biomolecules such as enzymes, biosurfactants and inhibitors like DNP, bismuth dimercaprol etc. Overall study will advance our knowledge towards *C. albicans* EPS which may assist in the development of novel therapeutics aimed at disrupting these biofilms and translate into improved clearance of related infections.

OBJECTIVES OF THE STUDY

1. Isolation of biofilm forming *Candida* species from clinical samples procured from hospitals and nursing homes.
2. To study various physio-chemical parameters contributing to EPS production in isolated *Candida* species.
3. Characterization of *Candida* species mutants defective in EPS production.
4. Purification and Quantification of EPS produced by *Candida* species.
5. To study the effect of various antifungal agents, herbal preparation, biomolecules such as enzymes, biosurfactants and inhibitors etc. on biofilm and EPS production by *Candida* species.

CHAPTER 2

LITERATURE REVIEW

2.1 Biofilms on indwelling medical devices

It is now widely recognized that in natural settings microbial cells are most often found in close association with surfaces and interfaces, in the form of multicellular aggregates commonly referred to as biofilms. This proclivity towards multicellularity makes microbial cells similar to many other types of living cells capable of unicellular existence and yet generally residing within multicellular communities (Branda *et al*, 2005). Biofilms serve as a nidus for disease and are often associated with high-level antimicrobial resistance of the organism (Kuhn *et al.*, 2002). Many of these are implant-related infections in which adherent microbial populations can be demonstrated on the surfaces of devices such as catheters, prosthetic heart valves, joint replacements and dental acrylic (Table 1). Recently, it has been documented some 65% of all human infections involve biofilms (Calderone, 2002). According to "National Institutes of Health" biofilms cause 80% of infections. Moreover, catheter-related blood stream infections are associated with increased mortality, prolonged hospitalization, and higher medical costs, in excess of $6000 per patient (Schachter, 2003). As a result, research in the biofilm field has vastly increased over the past 10 years in an attempt to study how biofilms form and how they can be controlled.

Microbial biofilms may pose a public health problem for persons requiring indwelling medical devices. The ability of microorganisms to adhere to biomaterials is a key factor in the initiation of disease processes. Biofilms on indwelling medical devices may be composed of gram-positive or gram-negative bacteria or yeasts. Fungi most commonly associated with such disease episodes are in the genus Candida, most notably *Candida albicans*, which causes both superficial and systemic disease (Chandra *et al.*, 2001).

2.2 Biofilm formation by *Candida albicans*

Most *Candida* biofilms are associated with an implanted device such as an intravascular or urinary catheter, an endotracheal tube, or a dental prosthesis on which they develop (Maki and Tambyah, 2001; Crump and Collignon, 2000; Adair *et al.*, 1999). One reason for this is that biofilms are notoriously difficult to eradicate due to the biofilm-specific properties such as their enhanced resistance to antimicrobials (Chandra *et al.*, 2001). The subsequent realization of the role of candidal biofilms in candidiasis has stimulated considerable interest among researchers.

In the initial stage (adhesion phase) of *C. albicans* biofilm formation, there are no complex structures but a few layers of adherent cells in the budding-yeast phase of growth. With biofilm development, the yeast community starts to differentiate. At first germ tubes appear, and distinct microcolonies come into being after 3 to 11 h as revealed by scanning electron microscopy (SEM) (Hawser and Douglas, 1994) and confocal laser scanning microscopy (CSLM) (Chandra *et al.*, 2001). After 18 to 24 h, the *C. albicans* biofilms are characterized by a mixture of yeasts, germ tubes and young hyphae, and an intracellular matrix of Extracellular polymeric substances (EPS). Mature biofilms can be seen after 48 h of incubation showing a dense network of yeasts, germ tubes, pseudohyphae and hyphae. Another feature of candidal biofilms is the presence of water channels which develop as a result of the detachment of individual microcolonies from the biofilm bodies (Stoodley *et al.*, 1999). Such structures permit waste disposal and nutrient influx into biofilms so that even the deeply embedded yeast cells have access to nutrients and oxygen (Ramage *et al.*, 2001).

IMPLANTS	ORGANISMS	SYMPTOMS
Prosthetic valves	*S. epidermidis*, *S. sanguis*	Prosthetic valve endocarditis
Intrauterine device	*C. albicans, CoNS, Enterococcus sp., S. aureus*	Device failure
Contact lenses	*P. aeruginosa*, *S. epidermidis*	Keratitis
Intravascular catheters	*S. epidermidis*, *S. aureus, C. albicans,*	Septicemia, endocarditis
Voice prostheses	*Streptococci, Staphylococci, C. albicans, CoNS*	Prosthesis failure
Urinary catheters	*E. coli*, *CoNS, P. aeruginosa*, *E. faecalis*, *P. mirabilis*	Bacteriuria
Joint replacements	*S. epidermidis*, *S. aureus*	Septicemia, device failure
Endotracheal tube	*P. aeruginosa*, *E. coli*, *S. epidermidis*, *S. aureus*	Pneumonia

Table 1. Biofilm associated implant infections.

2.3 Methods for studying *Candida* adhesion and biofilm formation

2.3.1 Cell counting

Cell number in the adhesion assays can be determined by counting the cells, usually under a light microscope. For this purpose, the adherent cells are stained with dyes such as crystal violet (Weibel, 1969). Multiple fields are then randomly counted, and the mean number of yeasts per field finally expressed as yeasts per unit square mm (Radford *et al.*, 1998; Ellepola and Samaranayake, 1998). Sometimes, microscopic cell counting yields inaccurate results due to yeast cell aggregation. One technique that overcomes this drawback is a fluorometric method in which the adherent yeasts are first stained with a fluorescent dye such as calcofluor white. As the fluorescence level is directly proportional to the cell number, the latter can be quantified using a fluorescence reader (Borg-von Zepelin *et al.*, 2002; Millan *et al.*, 2000).

2.3.2 Crystal violet assay

This assay which has been widely used to quantify cell adhesion (Weibel, 1969) can also be used for biofilm quantification. Djordjevic *et al.* 2002; Merritt *et al.*, 1998, have employed this method to measure biofilms formed by bacteria. In this method, the developed biofilms are stained with 1% crystal violet, then washed with distilled water to remove the extra dye, and finally destained with ethanol. The resultant destaining solution was collected and its optical density (OD_{492}) readings were measured. The latter reflects quantitatively the level of biofilm formation.

2.3.3 Tetrazolium salt assay

Another colorimetric method, the tetrazolium metabolic assay has been widely used in quantification of *Candida* biofilm formation. The most commonly used is tetrazolium salt 2,3- bis (2 - methoxy - 4 - nitro - 5 - sulfophenyl) - 5 - [(phenylamino) carbonyl] - 2H - tetrazolium hydroxide (XTT) reduction assay. XTT is a yellow salt and can be reduced by mitochondrial dehydrogenases of metabolically active yeast cells. The color change during the reaction reflects the number of viable cells, and can be assessed visually at 490-492 nm using either a microtiter plate reader or a spectrophotometer (Kuhn *et* al., 2002; Hawser, 1996; Tellier *et al.*, 1992). A linear relationship between incubation time and color development has been noted when the incubation duration is not longer than 5 h (Cory *et al.*, 1991; Buttke *et al.*, 1993). Based on this finding, workers usually incubate samples with XTT for 2 h to permit adequate depth of color change and also save experimental time. Since XTT salt is water soluble, the assay procedures can circumvent steps such as centrifugation, addition of lysis buffer, solubilization and sonication, and hence are carried out with relative ease. On the other hand, there are some pitfalls with the XTT reduction assay. XTT metabolic rate may vary from species to species since the XTT metabolism of *C. parapsilosis* has been found to be lower than that of *C. albicans* (Kuhn *et al.*, 2002). Thus, XTT reduction assay cannot be universally applied, especially for inter-species comparison of candidal biofilms.

2.3.4 CFU (Colony forming unit)

Notably, all the foregoing methods are based on cellular metabolism and estimate biofilm volume indirectly. Alternatively, biofilm cells can be enumerated directly by counting the CFUs of resuspended biofilm cells. Yet the latter technique is time-consuming and labor-

intensive and, therefore, not widely used. However, counting CFUs has an advantage in that it is not influenced by the metabolic status of the cells. Some workers have used this technique to measure the biofilm formation of *Pseudomonas aeruginosa* (Singh *et al.*, 2002).

2.4 The Biofilm Matrix

2.4.1 Composition of biofilm matrix

The biofilm matrix has some of the qualities of a mythological creature and its basic composition remains in debate (Stewart, 2006). Much of the biofilm matrix up to 97% is actually water, although, as with all aspects of biofilms, this will depend on the specific system under examination. The water can be bound within the capsules of microbial cells or can exist as a solvent whose physical properties such as viscosity are determined by the solutes dissolved in it (Zhang *et al.*, 1998). Mobility and water binding within the biofilm matrix are integral to the diffusion processes that occur within the biofilm (Schmitt and Flemming, 1999). The biofilm matrix, apart from water, is a complex of secreted polymers, absorbed nutrients and metabolites, products from cell lysis and even particulate material and detritus from the immediate surrounding environment (Sutherland, 2001). The major component, polysaccharides are widely acknowledged to be important matrix polymers. However, proteins and nucleic acids as well as lipids and phosphorus have also shown to appear in significant amounts (Table 2; Costerton *et al.,* 1981). Extracellular DNA has been also been found in the biofilm matrix and implicated in cohesion (Whitchurch *et al.*, 2002). Thus, all major classes of macromolecule – proteins, polysaccharides, DNA and RNA can be present in addition to peptidoglycan, lipids, phospholipids and other cell components (Sutherland, 2001).

COMPONENT	PERCENTAGE OF MATRIX
Water	upto 97%
Microbial cells	2-5%
Polysaccharides (homo and hetero)	1-2% (neutral and polyanionic)
DNA/RNA	<1-2%
Proteins	<1-2% (including enzymes)

Table 2. Composition range of biofilm matrix.

Various new methods have been developed for probing the composition of the biofilm matrix. For example, Johnsen *et al.*, 2000, used fluorescently labelled lectins to localize carbohydrate-containing polymers within biofilms. Microsensor probes can be used to determine dissolved oxygen and pH of the biofilm environment. Recently, CLSM studies by Stoodley *et al.*, 1999, has provided some new information on the structural complexity of biofilms and has confirmed their heterogeneity. The consensus from such studies is that biofilms comprise aggregates of microbial cells within a matrix of EPS and interstitial voids and channels separate the microcolonies. Studies conducted by Allison and Sutherland, 1987, indicate that EPS is not necessarily required for the initial attachment of microbial cells to surfaces, but its production is essential for the development of the architecture of any biofilm matrix. The EPS molecules provide the framework into which microbial cells are inserted. As EPS synthesis continues following cell attachment and as EPS might even provide a nutrient source for some of the cells, these macromolecules are dynamic components of the biofilm (Danese *et al.*, 2000). Recently, use of dual labelling with GFP followed by CLSM located the positions of bacterial cells within a biofilm matrix and confirmed the heterogeneity of their distribution (Moller *et al.*, 1998). The study indicated that the matrix was sufficiently fluid to permit redistribution of the two cell types within its structure during growth and development. This is probably a feature of matrices in which the polysaccharides are relatively fluid, whereas in others in which the polymers form more rigid gels, cells can remain effectively immobilized (Moller *et al.*, 1998).

2.4.1.1 Exopolysaccharides

Common to virtually all biofilm matrices are extracellular polysaccharides or exopolysaccharides, regarded as the major structural component of the matrix, providing a

framework for the biofilm complex (Allison, 2003). At an individual cell level, exopolysaccharides occur in two basic forms, *viz.*, capsular, whereby the exopolysaccharide is intimately associated with the cell surface, and as slime which is only loosely associated with the cell. Chemically, exopolysaccharides are highly heterogeneous polymers containing a number of distinct monosaccharides and non-carbohydrate substituents, many of which are strain specific (Whitfield, 1988; Sutherland, 1985). As with all polysaccharides, those produced by microorganisms can be divided into homopolysaccharides and heteropolysaccharides.

Most homopolysaccharides are neutral glucans, whilst the majority of heteropolysaccharides appear to be polyanionic (Sutherland, 1990). Homopolysaccharides can possess three different structures, namely linear molecules comprised of a single linkage type, linear repeat units possessing a one sugar side chain, and branched structures.

Majority of the microbial heteropolysaccharides are composed of repeating backbone units varying in size from disaccharide to octasaccharide. Structural diversity and, hence, rheological properties is increased by non-carbohydrate substituents (e.g. acetyl, pyruvate, sulphate groups) and linkage types. A wide range of microorganisms produce heteropolysaccharides, which are composed of repeating units (Fig. 1). The monosaccharide composition of exopolysaccharides in lactic acid bacteria are mostly galactose and glucose, along with small amounts of rhamnose, fructose, mannose, and galactosamine (Van den Berg *et al.* 1995). Generally, the heteropolysaccharides are synthesized intracellularly at the cytoplasmic membrane utilizing sugar nucleotides as precursors for the assembly of polysaccharide chains (Cerning, 1995). It is also worth noting that several species of bacteria are able to synthesize more than one chemically distinct exopolysaccharides, although

normally only one type is expressed under any set of specific growth conditions (Allison, 2003).

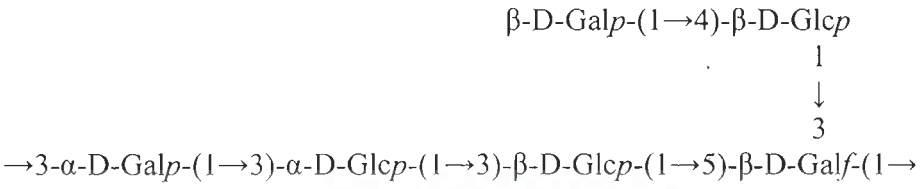
The polysaccharide chains vary in size from 10^3–10^8 KD and contain subunit configurations which may also be both functionally- and species-specific (Sutherland, 1985). Depending upon the components of the repeat units polysaccharides are usually negatively charged, sometimes neutral or rarely positively charged. Furthermore, polysaccharides may be hydrophilic but can also have hydrophobic properties (Neu and Poralla, 1990). Indeed, many polymers are heterogeneous with respect to lipophilicity and hydrophobicity, let alone a fully-formed, multi-component biofilm matrix (Sutherland, 1997). Polymer hydrophobicity can, therefore, play an important part in determining the behaviour of the polysaccharide at the cell surface or at an interface.

Exopolysaccharides are also subject to environmental modulation with respect to composition and molecular mass (Tait *et al.*, 1986) which in turn can affect their capacity to interact with other polymers and cations (Sutherland, 1990). Thus, in order to fully characterize any biofilm polymer, it is essential to be able to mimic in situ growth conditions that lead to exopolysaccharide production.

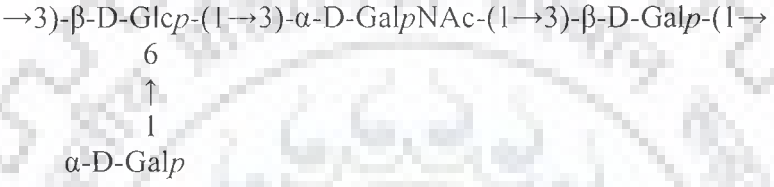
2.4.1.2 Proteins

Proteins are abundant in the biofilm matrix but their function is still unclear; how much these proteins are structural components and how much they serve functions independent of mechanical integrity (Stewart, 2006). The major function of extracellular proteins have been in biofilms is mostly seen in their role as enzymes performing the digestion of exogenous macromolecules and particulate material in the microenvironment of the immobilized cells

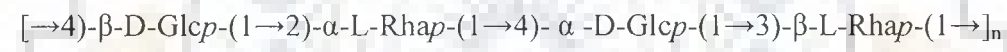
(A)



(B)



(C)



(D)

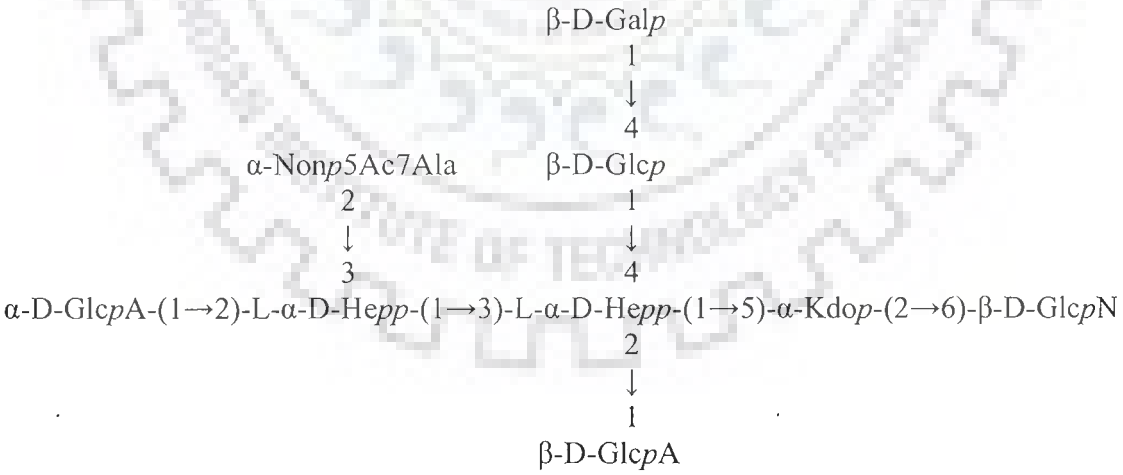


Figure 1. Structures of the exopolysaccharides produced by: (A) ***Lactobacillus helveticus*** **TN-4; (B)** ***Streptococcus thermophilus*** **Sfi6 (C)** ***Burkholderia tropica*** **Ppe8 (D)** *Vibrio parahaemolyticus* KX-V212.

(Dignac *et al.*, 1998). Thus, they provide low-molecular weight nutrients which can readily be taken up and metabolized by the cells. On the basis of their relatively high negatively charged amino acid content, proteins are supposed to be more involved than sugars in electrostatic bonds with multivalent cations, revealing their role in the floc structure (Dignac *et al.*, 1998).

2.4.1.3 Lipids

The role of EPS components remained to be established. However, it is expected that nucleic acids and lipids significantly influence the rheological properties and thus the stability of biofilms. Extracellular lipids from *Serratia marcescens* with surface-active properties have been proposed to help bacteria in surface environments to overcome the strong surface tension of surrounding water, thus facilitating growth on solid surfaces (Matsuyama and Nakagawa, 1996), thus involved in the interaction between bacteria and interfaces (Neu, 1996).

2.4.1.4 Phosphorus

Sathasivan *et al.*, 1997, studied the effect of phosphorus on the formation of biofilms in a pilot scale experiment. In phosphorus limited drinking water, supplementation with low levels (1 µg/l) of phosphate led to an increase in the concentration of microbes present in the biofilms. Additions of more than 1 µg/l had minor effect on the viable counts of heterotrophic bacteria and total bacterial numbers, but content of ATP increased with increasing content of phosphorus (up to 5 µg/l), showing the phosphorus limitation in biofilms. The addition of phosphorus increased the proportion of gram-negative bacteria in

biofilms and also changed the community structure of gram-negative bacteria. Also, in phosphorus limiting water the formation of biofilms was affected by the content of phosphorus (Sathasivan *et al.*, 1997).

2.4.1.5 Extracellular DNA

Extracellular DNA has been found in the biofilm matrix and implicated in cohesion (Whitchurch *et al.*, 2002 and Sutherland, 2001). This extracellular DNA has been presumed to be derived from lysed cells and has not been thought to represent an important component of biofilm structure (Whitchurch *et al.*, 2002). However, it has been known for many years that some bacteria, including *P. aeruginosa*, produce substantial quantities of extracellular DNA through a mechanism that is thought to be independent of cellular lysis and that appears to involve the release of small vesicles from the outer membrane. These studies suggested that extracellular DNA is required for the initial establishment of *P. aeruginosa* biofilms and perhaps biofilms formed by other bacteria that specifically release DNA.

2.4.2 Functions of EPS

The importance of EPS has long been recognized and a variety of functions (Table 3) have been attributed to them as far as the benefits they provide to cells, either as single organisms, in binary associations, or in heterogenous communities. Some important functions are discussed below:

2.4.2.1 Role in Cellular associations

Consortial activities are required for many microbial processes which are not possible with

single species populations (Geesey and Costerton, 1986). Microorganisms maximize their metabolic capabilities through co-operative interactions between two or more populations sharing a mutual habitat (Wolfaardt *et al.*, 1999). EPS play an important role in these interactions by facilitating "communication" between cells through participating in cell-cell recognition, by serving as an adhesion and the establishment of favorable micro-environment (Kolen-Brander, 1989).

EPS are viewed as important mediators in the adhesion of bacteria and other microorganisms to surfaces (Sutherland, 1984; Marshall, 1985). In addition to their potential role in the initial attachment of cells during biofilm formation, they are also involved in maintaining the structural integrity of biofilms (Vandevivere and Kirchman, 1993; Marshall, 1984; Costerton *et al.*, 1981). Stewart *et al.*, 1995, using a combination of probes to differentially stain cellular nucleic acids and EPS, showed that EPS distributions in biofilms shows much variety and they did not always overlap. Considering the importance of EPS in the attachment of cells to surfaces and the fact that attached cells produce more EPS than their planktonic counterparts (Vandevivere and Kirchman, 1993), it could be argued that EPS also play a role, at atleast indirectly, in the exchange of genetic material between cells. Lebaron *et al.*, 1997, studied plasmid mobilization between *E. coli* strains in biofilms as well as other environments. They concluded that bacterial adhesion is among the factors that can be related to plasmid transfer and further the hydrodynamic conditions within biofilms could affect transfer potential.

2.4.2.2 Role in Nutrition

It has been suggested that microorganisms survive in environments where nutrients are

EFFECT OF EPS COMPONENT	COMPONENT	ROLE IN BIOFILM
Surface-active	Amphiphilic Membrane vesicles	Interface interactions Export from cell, Sorption
Sorptive	Charged/hydrophobic Polysaccharides	Ion exchange, sorption
Nutritive	Various polymers	Source of C, N, P
Informative	Lectins Nucleic acids	Specificity, recognition Genetic information, structure
Redox active	Bacterial refractory polymers	Electron donor or acceptor
Active	Extracellular enzymes	Polymer degradation

Table 3. Functions of EPS in Biofilm.

available at levels below the threshold concentrations required to remain viable, or under feast-and-famine conditions characterized by fluctuations in available nutrient sources (Wolfaardt *et al.*, 1999). Patel and Gerson, 1974, demonstrated the capacity of a *Rhizobium* strain to re-utilize its own EPS and concluded that the enzymes involved were extracellular enzymes and that a little number of organisms appear to possess the enzymes required to depolymerize their own EPS. Pirog *et al.*, 1997, proposed that EPS may function as a food reservoir at least in ways: (1) production of EPS as nutrient reserve and (2) accumulation of nutrients by EPS.

As suggested by Patel and Gerson, 1974, EPS may also be created for long-term storage of carbon and energy in *Rhizobium sp.*, Roberson and Firestone, 1992, noted that some polysaccharide carbon was used by the bacteria for protein production- in other words; carbon was shuttled between proteins and polysaccharides as the water status changed. In addition to the role in the accumulation of carbon reservoirs, EPS may also be important mediator for concentration of growth factors. Studies by De Philippis *et al.*, 1991, demonstrated enhancement of EPS production induced by Mg^{2+} deficiency indicating the role of anionic polysaccharides as chelating agents for cations essential to cellular metabolism.

2.4.2.3 Role in the Interaction of microorganisms with their biological, physical and chemical environment

Pirog, 1997, found that EPS formed under favorable growth conditions protected *Acinetobacter* from extreme pH values, elevated temperature, drying, freezing, biocides, heavy metal ions (Cu^{2+}, Pb^{2+} and Cr^{3+}), and detergents. Studies by Lawrence *et al*, 1995;

Decho, 1990, suggested that EPS facilitate interactions, not only among microbial populations but also between microorganisms and the environment in which they live.

The production of EPS is another possible survival strategy employed by bacteria to cope with fluctuations in water content. Roberson and Firestone, 1992, suggested that the hygroscopic properties of EPS and the resulting maintenance of higher water content in the microenvironment around microbial communities may also be responsible for an increase in nutrient availability to the cells and by providing high water content, may maintain transport of nutrients to cells at diffusion rates close to that in water. In addition, EPS matrix may indirectly contribute to biocide resistance when other factors associated with biofilms, such as the establishment of nutrient and oxygen gradients, lead to reduced growth rates (Gilbert and Brown, 1995).

The concentration of extracellular enzymes by EPS to facilitate extracellular cleavage of high molecular weight carbon sources before uptake into cells is well documented in literature (Decho, 1990; Costerton, 1984; Lamed *et al.*, 1983). The accumulated compound may serve as a carbon source during periods of nutrient limitation. Frolund *et al.*, 1995, utilized cation exchange to extract EPS from activated sludge and proposed that a large proportion of the exoenzymes were immobilized in it by absorption in the EPS matrix. Thus exoenzymes should be considered to be an integrated part of the EPS matrix.

The ability of the bacteria to establish themselves in the micro-niche where they were protected from the antibiotics and host defenses is well known. Delighton *et al.*, 1996, studied the contribution of EPS to infections by *Staphylococcus epidermidis* in the presence and absence of medical implants. They found that strains capable of producing matrix of extracellular polysaccharide in which the cells are encased resulted in significantly more

abscesses, which persisted longer than abscesses caused by EPS-negative strains. Finally they concluded that slime or its components appeared to delay the clearance of *Staphylococcus epidermidis* from host tissues.

2.4.2.4 EPS and the Macroenvironment

Bacteria assist in the establishment of growth conditions in soil through the EPS production which are favorable to microbial and higher life forms (Roberson *et al.*, 1995). In the same manner, microbial EPS stabilizes aquatic sediments. Yallop *et al.*, 1994, demonstrated that the stability of sediments was greatly increased by the presence of a microbial mat. Welch and Vandevivere, 1994, found increased mineral dissolution rates when fresh microbial EPS were tested at water-rock interfaces. They attributed the increased rates to two mechanisms: (1) an increase in the solubility of the mineral by soluble organic ligands; and (2) the formation of metal-organic complexes at the mineral surface, thereby weakening metel-oxygen bonds to the bulk material. Chakrabarti and Banerjee, 1991; Southam and Beveridge, 1993, suggested charged and hydrophobic regions of EPS play a role in bacterial-mineral interactions, and is of similar importance as EPS-mediated attachment of cells to metals during corrosion.

Microorganisms generally contribute to mineralization of contaminants to CO_2 and H_2O by utilizing contaminants as sources of carbon and energy (Ascon-Cabrera and Lebeault, 1993; Lapppin *et al.*, 1985). These organic contaminants are accumulated in EPS by the same mechanisms proposed for the accumulation of naturally-occuring nutrients. Mittleman and Geesey, 1985, demonstrated that EPS which accumulated copper could serve as a route of entry for this metal into environmental food chain and transfer to higher

organisms by biomagnification.

2.4.3 Industrial and clinical importance of EPS

Bacterial extracellular polysaccharides are of commercial interest in biotechnology for various industrial and biomedical applications or are clinical relevance as virulence factors participating in infection processes of plants, animals and man (Wingender *et al.*, 1999). Polysaccharides like xanthan, gellan, cellulose, hyaluronic acid and several β-D-glucans have various commercial importance whereas newly discovered polysaccharides and some chemically modified polysaccharides offer the potential of novel applications in future (Becker *et al.*, 1998; Jonas and Farah, 1998; Sutherland, 1998; De Phillipis and Vincenzini, 1998). Exoenzymes and toxin proteins are involved as virulence factors in the colonization, invasion and destruction of host tissues and in the interference with host immune response mechanisms (Wingender *et al.*, 1999). On Industrial scale, among extracellular proteins such as starch-hydrolyzing enzymes, proteases, cellulases, pectinases and lipases find use in the hydrolysis of macromolecules and offer great promise for biosynthetic purpose (Jager and Reetz, 1998; Priest, 1992).

2.4.4 Control and Regulation of EPS synthesis

The types of EPS, their amount and rate of synthesis may all have a bearing on the adhesion and biofilm forming properties of an invading organism, yet the underlying molecular mechanisms are unknown. The complexity surrounding the genetics of EPS synthesis may in part be related to the relatively large monosaccharide repeat units produced by some bacteria and the ability of others to produce more than one EPS. Recombinant DNA technology is

helping to resolve this gap in our understanding. The control and regulation of alginate synthesis in *P. aeruginosa* has been explored by May *et al.*, 1991. Essentially, alginate synthesis in *P. aeruginosa* occurs when non-mucoid strains are environmentally stressed. Transcription of a critical alginate biosynthetic gene, *algD*, is triggered by environmental signals such as high osmolarity, dehydration and nutrient deprivation and is known to be controlled by regulatory proteins AlgR1, AlgR2 and AlgR3 (May *et al*, 1994). AlgR1 is a member of the family of response regulators of the phosphorylation-dependent two-component bacterial signal transduction system which control the cellular responses to changes in the surrounding growth environment. In response to the presence of a solid surface, the *algD* gene of *P. aeruginosa* is transcriptionally activated when grown as a biofilm (Hoyle *et al.*, 1993). Indeed, using a *LacZ algC* of *Pseudomonas aeruginosa*, Davies and Geesey, 1995, demonstrated alginate synthesis to be upregulated within minutes of the cell attaching to a surface.

Recent analyses have shown that the conversion of mucoidy is frequently caused by mutations in the *mucA* gene which is located within the *algU mucABCD* cluster (Boucher *et al.*, 1997, 1996). In *P. aeruginosa* AlgU controls extreme stress responses and is required for transcription of *algD* and *algR*. MucA, MucB, MucC and MucD are negative regulators of AlgU activity (Boucher *et al.*, 1997, 1996; Schurr *et al.*, 1996).

The influence of the growth environment on EPS production through the use of continuous culture techniques has been studied in other systems (Tait *et al*, 1986; Ombaka *et al*, 1983). A common observation is the ability of bacteria to produce greater quantities of EPS when grown on solid media compared to those cultivated in a liquid broth (Allison, 1994; Abu *et al.*, 1991). Other factors such as growth rate and nutrient deprivation may also

contribute to this response, whereby biofilm cells cultivated at slow rates of growth produce substantially more EPS than their planktonic counterparts (Allison, 1994; Evans *et al*, 1994). Again, this may be a survival strategy.

Recently, it has also been suggested that in some Gram-negative organisms, the production of EPS may be under the control of signal substances in the form of *N*-acyl substituted homoserine lactones (HSL) (Beck von Bodman and Farrand, 1995). These are global regulators of transcriptional activation in bacteria (Salmond *et al.,* 1995; Passador *et al.*, 1993; Williams *et al.*, 1992) responsible for cell-cell signaling and implicated in cell-density mediated events. Production of such small, diffusible signals enables the bacterial population to monitor its own density and to trigger a coordinate and unified response at the population level (Swift *et al.*, 1992). In *P. aeruginosa* the protease genes *lasA* and *lasB* are coordinately regulated by a quorum or density sensing system (Passador *et al.*, 1993). For the *algD* promoter the opposite trend was observed. However, Storey *et al.*, 1997, reported a positive correlation between *algD* expression and expression of both *lasB* and *lasA*. Possible explanations might be that these genes either respond to the same environmental or physiological stimuli, such as growth as a biofilm (Costerton *et al.*, 1987), or that they have a regulatory element in common. Under certain conditions these genes may be controlled by a stress-induced sigma factor (Boucher *et al.*, 1997, 1996). HSLs may also act as global regulators of biofilm-specific physiology. In biofilms signal substances such as HSL might be concentrated within the slow-growing core of a thick microcolony, in so doing stimulating increased EPS production. In this respect the strength of bacterial attachment to surfaces during the early stages of biofilm formation has been shown to be significantly enhanced by the presence of specific HSLs (Heys *et al.*, 1997).

2.5 Characterization of Microbial Exopolysaccharides

2.5.1 Morphological analysis:

2.5.1.1 Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy has been important for high resolution visualization of biofilms. In this technique, biofilm specimens are prepared by fixation, staining, drying and conductively coating prior to imaging under high vacuum. The image shows strands of polymeric material in between the microorganisms and the surface to which they are attached to (Costerton *et al.*, 1987; Richards and Turner, 1984). While any pretreatment can alter specimen morphology, drying appears to significantly alter biofilms due to EPS polymers collapsing (Kachlany *et al.*, 2001; Fassel and Edmiston, 1999; Little *et al.*, 1991). Hawser and Douglas, 1994, with the help SEM demonstrated that on catheter surface mature *C. albicans* biofilms consists of a dense network of yeasts, germ tubes, pseudohyphae, hyphae and extracellular polymeric material was visible on the surfaces of some of these morphological forms.

2.5.1.2 Atomic Force Microscopy (AFM)

Atomic Force Microscopy has garnered much interest in recent years for its ability to probe the structure, function and cellular nanomechanics inherent to specific biological cells (Cross *et al.*, 2006). AFM force measurements of cell-solid and cell-cell interactions using functionalized probes have shown to be a promising approach to study the initial bacteria attachment (Dufrene, 2002). Bowen *et al.*, in 2001, developed an AFM "colloid probe" technique involving immobilization of a particle at the tip of the cantilever to measure the incident forces as a function of separation distance at the surface. They used this technique to

study protein-protein interactions and adhesion of *Saccharomyces cerevisiae* vegetative cells and *Aspergillus niger* spores to surfaces such as mica. Razatos *et al.*, 1998 determined interactions between *E. coli* and PMMA (polymethylmethacrylate) by forming confluent layer directly onto the cantilever tip.

2.5.1.3 Confocal Laser Scanning Microscopy (CLSM)

The technique was developed in the 1980s and allows examination of biofilms without the limitations imposed by SEM or TEM. Fully hydrated biofilms are analyzed by progressive laser scans at different focal planes within the sample. Computer analysis of the scanned images permits a recreation of the three-dimensional structure of the biofilm. The application of CLSM combined with a number of staining fluorescent techniques provides an important and effective tool to analyze the composition and structure of hydrated biofilms *in situ*, nondestructively and in real time (Lawrence and Neu, 1999). Viability and distribution of cells within the biofilm may be analyzed with CLSM. When using CLSM, the choice of suitable fluorescent stains is critical in order to increase the contrast between the organisms and the exopolymers in the biofilms. Zang *et al.*, 2004, used CLSM for quantification of EPS by measuring the total cell volume from a 3-D image of a seawater-grown biofilm after staining cells with fluorescent dye acridine orange specific for nucleic acids. In a recent study, Chen *et al.*, 2007, performed multiple fluorochrome experiments to probe total cells (Syto 63), dead cells (Sytox blue), proteins (FITC), lipids (Nile red), α-polysaccharides (ConA) and β-polysaccharides (calcofluor white) for exploring detailed structure of bioaggregates.

2.5.2 Structural analysis

The primary structure of an exopolysaccharide is defined by its monomer composition (both absolute and anomeric configurations), the sequence and ring size of the constituting monosaccharides, the location of the glycosidic linkages, and the type and location of non-carbohydrate substituents. A combination of several techniques is necessary to determine the structure of an exopolysaccharide (Sletmoen *et al.*, 2003). Some of them are described below:

2.5.2.1 High Performance Liquid Chromatography (HPLC)

It is used for the identification and quantification of mono- and oligosaccharides resulting from the partial hydrolysis of exopolysaccharide. The retention mechanism of HPLC anion exchange columns is based on the formation of weak complexes between the monosaccharides and ions (higher affinity to the immobilized ions in the matrix means longer retention times). The main disadvantages of this method are the low resolving power and the use of a nonselective and low sensitivity RI detection system. In the High performance anion exchange chromatography (HPAEC) system, the columns have polymer-base matrices characterized by high chemical stability and selectivity (determined by the mobile phase used). Separation is made at high pH with strong alkaline solutions and detection is achieved by monitoring the change in the electric current due to the oxidation of the saccharides in the surface of the gold working electrodes located in the pulse amperometric detector. This procedure is very sensitive and selective for the analysis of sugar compounds in their native state.

2.5.2.2 Gas Chromatography/Mass Spectrometry (GC/MS)

The most extensively used technology for the analysis of the monomer composition of exopolysaccharide is GC-MS. After acid hydrolysis of exopolysaccharide molecules, generally with trifluoroacetic acid, the resulting monosaccharides are derivatized to alditol acetates (Blakeney *et al.*, 1983) or trimethylsilylated glycosides (Gerwig *et al.*, 1979), which are then separated by gas chromatography using different gas carriers (He, N_2, H_2), columns, and temperature programs. The identification and quantification of monosaccharides can be carried out with a flame ionization detector or a mass spectrometer, using inositol as an internal standard. The monomeric composition of several exopolysaccharides produced by different lactic acid bacteria strains was determined using the GC-MS method (Ruas-Madiedo and Reyes-Gavilan, 2005). The sugar linkage analysis is achieved by methylation of exopolysaccharide, hydrolysis followed by reduction with sodium borodeuteride, and further acetylation. The partially methylated deuterated alditol acetates obtained can be identified by GC-MS (Harding *et al.*, 2003; Faber *et al.*, 2001). Finally, to obtain the 3-D structure of an exopolysaccharide molecule, both the ring size (pyranose/furanose) of the monosaccharide residues and the relative orientations of the adjacent monosaccharides have to be determined.

2.5.2.3 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR is a non destructive technique that allow rapid characterization of structural features of complex, polymeric material vibrational and rotational motions of atoms in molecules (Nichols, 1985). The carbohydrates show high absorbencies in the so-called fingerprint region 1200–950 cm^{-1}, where the position and intensity of the bands are specific for every polysaccharide, allowing its possible identification. Due to absorbance overlapping in this

region, it has been very difficult to assign the absorbencies at specific wave numbers to specific bonds or functional groups. Kacurova and Wilson, 2001, described FTIR spectroscopy as a powerful research tool for elucidating the structure, physical properties and interactions of carbohydrates. This technique provided a new interpretive and experimental framework for the study of complicated systems of natural polymers. FTIR have been used to study antibiotic penetration into biofilms and to determine the composition of the exopolymeric matrix. Schmitt and Flemming, 1998, discussed use of FTIR technique as a means to investigate microorganisms in biofilm which presented a new addition to taxonomic and genetic methods. Furthermore, FTIR-attenuated total reflection (ATR) technique allowed observation of biofilms forming directly on the interface of an ATR crystals such as germanium.

2.5.2.4 Nuclear Magnetic Resonance (NMR)

NMR is the technique most often used to study the conformation of molecules in solution and allows elucidation of the type of glycosidic linkages and the structure of the repeating units that build the exopolysaccharide molecules. To date, the structure of several exopolysaccharides synthesized by *Pseudoalteromonas ruthenica* (Saravanan and Jayachandran, 2008), *Vibrio harveyi* (Bramhachari and Dubey, 2006), *Sargassum fusiforme* (Mao *et al.*, 2004), *Bacillus sp.* (Kumar *et al.*, 2004), *Staphylococcus arueus* (Joyce *et al.*, 2003), *S. thermophilus* (Lemoine *et al.*, 1997), several *Lactobacillus* species (Harding *et al.*, 2003) etc., has been determined by NMR spectroscopy.

NMR spectroscopy relies on the interaction of radio-frequency electromagnetic radiation with magnetically active nuclei in a strong magnetic field. The radio frequencies

used range from 200 to 800 MHz, corresponding to magnetic fields from 4.7 to 18.8 Tesla. ^{1}H and ^{13}C are the spin-active nuclei most frequently encountered in carbohydrates. ^{1}H and ^{13}C NMR spectroscopy, including one- (1D) and two-dimensional (2D), is a powerful tool for structural studies of carbohydrates (Widmalm, 1998). Leeflang *et al.*, 2000, discussed the applicability of ^{1}H and 2D NMR spectroscopy for the determination of the primary structure and tertiary structure of carbohydrate-containing molecules. 1D ^{1}H NMR spectroscopy can be used for rapid identification or to check the purity of a polysaccharide sample. Signals in the anomeric region (about 4.3-5.5 ppm) of the spectrum and the coupling of the anomeric protons (*J*H1, H2) may provide useful information about the number of residues in a repeating unit, and the anomeric configuration, respectively. Since the natural abundance of ^{13}C is very low (1.1% relative to ^{12}C), the peak intensity of ^{13}C has to be enhanced in 1D ^{13}C NMR spectroscopy by using a large number of pulses, by taking advantage of the Nuclear Overhauser Effect (NOE), or by using distortionless enhancement by polarization transfer (DEPT) experiments. The values of ^{13}C chemical shift and ^{13}C-^{1}H coupling (^{1}JC, H) provide structural information of the polysaccharides (Widmalm 1998).

The 1D NMR techniques are often used for the assignment of signals in the anomeric region. For detailed assignment for the spin system of sugar residues, 2D techniques are needed. These techniques include ^{1}H-^{1}H-Correlated Spectroscopy (COSY), Total Correlation Spectroscopy (TOCSY), Homonuclear Hartmann-Hahn Spectroscopy (HOHAHA), gradient selected Heteronuclear Single Quantum Coherence (gHSQC), gradient selected Heteronuclear Multiple-Bond Correlation (gHMBC) experiment, and Nuclear Overhauser Effect Spectroscopy (NOESY). The sequence of the monosaccharide residues in a repeating unit can be established by 2D NOESY and HMBC experiments. The former experiment

gives information about the inter residue linkage from observation of the NOE between anomeric protons and the protons at the substituted positions of neighboring sugar residues. The latter experiment gives rise to cross peak between proton and carbon atoms that are long-range scalar coupled (Widmalm 1998).

2.5.3 Biosynthesis of Exopolysaccharides

The biosynthetic pathway for exopolysaccharide production has been extensively studied in many microorganisms. For instance, exopolysaccharide production by *Streptococcus thermophillus* has been studied at the genetic level and specific genes (*eps*) are found to be involved in the exopolysaccharide production (De Vuyst and Degeest, 1999; Stingele *et al*, 1996; Poolman *et al*, 1990). These polysaccharides are made by polymerizing repeat unit precursors formed in the cytoplasm (Escalante, 2002). Several enzymes which are not unique to exopolysaccharide production are involved in the biosynthesis and secretion process (Fig. 2).

Sugar nucleotides UDP-glucose and UDP-galactose play an essential role in EPS biosynthesis due to their role in sugar activation, which is necessary for monosaccharide polymerization, as well as sugar modification (epimerisation, decarboxylation, dehydrogenation, etc; Escalante, 2002). Sutherland, 1990 found that the loss of the capacity to synthesize the sugar nucleotide usually leads to loss of EPS production. The enzymes that participate in sugar activation and modification play an important role in both the formation of building blocks and the final EPS composition (De Vuyst and Degeest, 1999). It has been determined that UDP-glucose pyrophosphorylase is associated with EPS production in some *S. thermophilus* strains in cultures grown in glucose or lactose as sole carbon source.

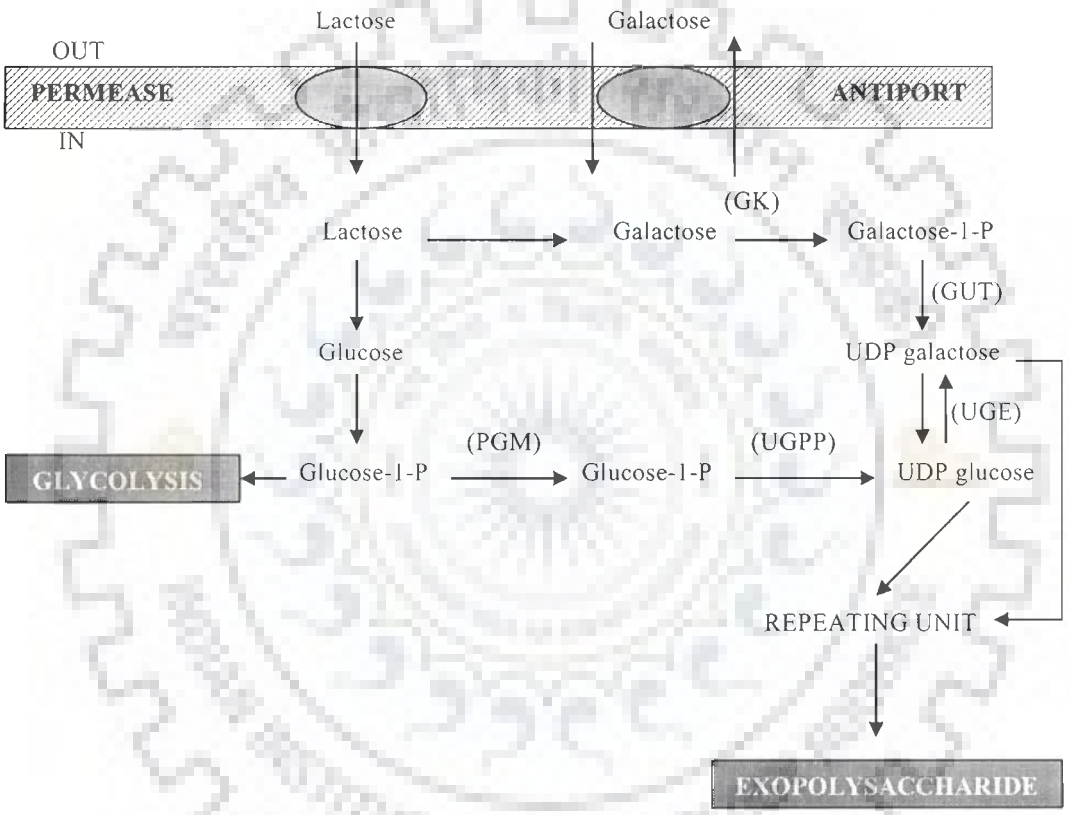


Figure 2. Pathway involved in the biosynthesis of EPS precursors in *S. thermophilus*. Abbreviations refer to: GK, galactokinase; GUT, galactose 1-P uridyl transferase; PGM, phosphoglucomutase; UGPP, UDP-glucose pyrophosphorylase; UGE, UDP-galactose 4-epimerase.

De Vuyst and Degeest, 1999; Escalante *et al.*, 1998, studied that the most likely role of both Leloir enzymes UDP-galactose 4-epimerase and UDP-glucose pyrophosphorylase as the precursors for biosynthesis of EPS. They observed that loss of production and overproduction of EPS in *S. thermophilus* is related to a variation in the activity of the enzymes involved in the synthesis of nucleotide-sugar precursors. Additionally, a mutant strain with increased activity levels of the enzymes phosphoglucomutase, UDP-galactose 4-epimerase, and UDP-glucose pyrophosphorylase was obtained by mutagenesis, leading to an EPS overproducing ropy strain.

Recently, Wang *et al.*, 2006, reported the identification and characterization of the *ste* (*Streptomyces eps*) gene cluster of *Streptomyces sp.* 139 required for exopolysaccharide biosynthesis. They designed degenerate primers to amplify an internal fragment of the priming glycosyltransferase gene that catalyzes the first step in exopolysaccharide biosynthesis. Screening of a genomic library of *Streptomyces sp.* 139 with the polymerase chain reaction product as probe allowed the isolation of a *ste* gene cluster containing 22 open reading frames similar to polysaccharide biosynthesis genes of other bacterial species. Further, they confirmed involvement of the *ste* gene cluster in exopolysaccharide biosynthesis by disrupting the priming glycosyltransferase gene in *Streptomyces sp.* 139 to generate non-exopolysaccharide-producing mutants.

2.5.4 Genetics of Exopolysaccharide production

De Vuyst, 2001 and Van Kranenburg, 1999, suggested that the genes encoding exopolysaccharide synthesis might be located in the plasmid, such as in *Lactobacillus lactis* and *Lactobacillus casei*, or located on the chromosome, as in all the thermophilic Lactic acid bacteria. In *S. thermophilus* Sfi6, Stingele *et al.*, 1996, identified the *eps* genetic locus revealing

a 15.25 kb region encoding 16 open reading frames (ORFs), within which a 14.52 kb region encodes 13 genes (*eps*A to *eps*M) capable of directing exopolysaccharide synthesis. Homology searches of the predicted proteins showed a high level of homology (40–68% identity) for *eps* A, B, C, D and E with the genes encoding capsular polysaccharide (CPS) in *Streptococcus pneumoniae* and *Streptococcus agalactiae*.

Van Kranenburg *et al.*, 1997, determined that all the essential information needed for the biosynthesis of exopolysaccharide in *L. lactis* NIZO B40 was encoded in a single 12 kb gene cluster located on a 40 kb plasmid (*eps*RXABCDEFGHIJKL), driven by a promoter upstream of *eps* R (Fig. 3). The gene products of 11 of the 14 genes were homologous in sequence to gene products involved in exopolysaccharides, CPS, lipopolysaccharide (LPS) or teichoic acid biosynthesis of other bacteria and putative functions were assigned to these genes. Further study of the *eps* gene cluster of *Lactobacillus delbrueckii* subsp. *bulgaricus* by Lamothe *et al.*, 2002, revealed a 18 KB DNA region consisting of 14 genes (*epsA* to *epsN*) with a similar genetic organization to other Lactic acid bacteria. The genes in the clusters were oriented in one direction and transcribed as a single mRNA (Jolly and Stingele, 2001; Van Kranenburg *et al.*, 1997; Stingele *et al.*, 1996). The functional sequences of these clusters in Gram-positive bacteria, which synthesize polysaccharide at the cell surface, appear to follow a similar trend of regulation, chain-length determination, biosynthesis of the repeating unit, polymerization and export (Jolly and Stingele, 2001).

2.6 *C. albicans* biofilm control strategies:

2.6.1 Antifungal agents

Pretreatment of *C. albicans* with various antifungals is known to suppress candidal adhesion

to denture acrylic (Ellepola and Samaranayake, 1998). One possible reason for this is the "post-antifungal effect", which refers to the persisting inhibition of cell growth following limited exposure to sub-inhibitory concentration (sub-MICs) of antimicrobials even though the agents have been removed. Ellepola and Samaranayake, 1998, demonstrated that the inhibition of metabolic activity in the drug-exposed yeasts during the post-antifungal period may result in decreased yeast adhesion. Alternatively, antifungals such as polyenes and azoles may inhibit the adhesion of yeasts by reducing the hydrophobicity of yeast cells although contradictory results have been shown by others (Anil *et al.*, 2002). In addition, antifungal drugs (particularly azoles) can suppress the yeast-to-hypha transition in *C. albicans* (Ha and White, 1999) which in turn affects their adhesion. Interestingly, the adhesion inhibitory effect of antifungals seems to vary between drug-resistant and susceptible *Candida* cells. For example, the foregoing inhibition of yeast filamentation by antifungals has only been observed in azole susceptible isolates of *C. albicans*, and no such effect on resistant isolates has been documented (Ha and White, 1999). More recent evidence also indicate that pretreatment with the new antifungal caspofungin results in decreased adhesion in all fluconazole-susceptible *C. albicans* strains tested but in only 60% strains that were resistant to fluconazole (Soustre *et al.*, 2004). Moreover, even the pretreatment of substrata (Egusa *et al.*, 2000; McCourtie *et al.*, 1986) with antifungals such as chlorhexidine can lead to a reduction in candidal adhesion, indicating that antifungals act on both substrata and yeast cells so as to synergistically inhibit candidal adhesion. Not surprisingly, antifungals can also affect biofilm formation of *Candida*. It has been demonstrated that preincubation of *C. albicans* cells with sub-MICs of various antifungal agents (azoles, nystatin, and chlorhexidine) significantly decreases the degree of biofilm formation, as reflected by dry

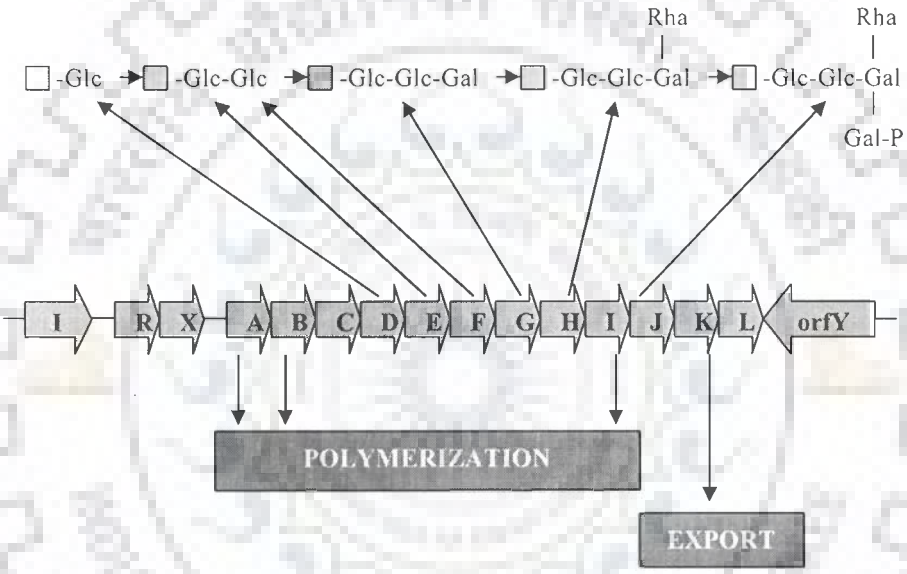


Figure 3. Organization of the *eps* gene cluster in *Lactococcus lactis*.

weight measurement (Lal *et al.*, 2008; Kuhn *et al.*, 2002). Efforts have also been made to investigate the effect of antifungal pretreatment of substratum on *Candida* biofilm formation. It has been found that coating the substratum with caspofungin results in a lower degree of *C. albicans* biofilm formation (Bachmann *et al.*, 2002). In addition to the pretreatment of yeasts or substrata with antifungals, the effect of antifungals on developing *Candida* biofilms has also been investigated. A SEM study has shown that the yeast cells of caspofungin-treated biofilms develop less hyphal forms than those of the untreated ones (Bachmann *et al.*, 2002), thus providing one clue to the inhibitory mechanism of this antifungal agent.

2.6.2 Plant oils

Plant oils traditionally used for domestic and therapeutic purpose are increasingly claimed to have broad spectrum antimicrobial properties due to their phenolic, alcoholic and terpenoid constituents (Hammer, 1998; Hili, 1997; Deans, 1991). However, azole antifungal agents and derivatives continue to dominate as drugs of choice against *Candida* infections, as topical applications or as oral drugs, repeated use of which on biofilms can select drug-resistant microbes (Chandra *et al.*, 2001; Sheehan, 1999). Studies by Williams *et al.*, 1993, and Williams and Home, 1995 demonstrated potency of tea tree oil with higher levels of terpinen-4-ol against *C. albicans*. Devkatte *et al.*, 2005, examined efficacy of 38 plant oils for the in vitro anti-Candida activity against four isolated of *C. albicans*. Out of which six plant oils; cinnamon, lemongrass, clove, Japanese mint, Geranium, Motiarosha and gingergrass were found to be most effective against all *C. albicans* isolates (Devkatte *et al.*, 2005). For future, more experiments need to be conducted on plant oils showing potential anti-Candida activity to identify their active components.

2.6.3 Enzymes

As such a large proportion of the structure of biofilms is composed of polysaccharides secreted by the constituent microorganisms, the presence of enzymes (polysaccharases) acting on these polymers will inevitably have a marked effect on the structure and on the integrity of the biofilm (Sutherland, 1999). Microbial EPS are substrates for a wide range of enzymes most of which are highly specific. They may be degradable by polysaccharide hydrolases or by polysaccharide lyases. Polysaccharide lyases cleave the linkage between a neutral monosaccharide and the C_4 of uronic acid with simultaneous introduction of a double bond at the C_4 and C_5 of the uronic acid. Both types of enzyme are commonly found to degrade microbial EPS as well as eukaryotic polymers (Sutherland, 1995). Although the enzymes degrading microbial exopolysaccharides may be endo- or exo-acting leading to rapid or slow breakdown of the polymer chain respectively (Sutherland, 1999). These polysaccharases may be derived from three major sources:

(1) endogenously from the polysaccharide-synthesizing microorganisms;

(2) exogenously from a wide range of other eukaryotic or prokaryotic microorganisms;

(3) from bacteriophage particles or phage-induced bacterial lysates.

Most of the enzymes acting on microbial EPS are obtained de novo, while few are commercially available. Zhang and Bishop, 1998, performed a study to examine the biodegradability of biofilm exopolysaccharide by the microorganisms from the original biofilm (its own producers) and from activated sludge (other microorganisms). They observed four distinctive phases during EPS biodegradation. In the first phase, instantaneous concentration increase of carbohydrate and protein in the test solutions were observed when EPS was added; in the second phase, easily biodegradable EPS from the added EPS was

quickly utilized; in the third phase, microorganisms began to produce soluble EPS, using the minimally biodegradable EPS left from the previously added EPS; in the fourth phase, cells consumed the newly produced EPS and microbial activity gradually stopped, suggesting that EPS can be used as a substrate, and that the EPS carbohydrate can be utilized faster than the EPS protein. They suggested that EPS can be used as a substrate, and that the EPS carbohydrate can be utilized faster than the EPS protein.

2.6.4 Biosurfactant

These are amphiphilic compounds produced on living surfaces, mostly microbial cell surfaces, or excreted extracellularly and contain hydrophobic and hydrophilic moieties that reduce surface tension and interfacial tensions between individual molecules at the surface and interface, respectively (Karanth *et al.*, 1999). Bussher *et al.*, 1997, studied inhibition of *Candida* spp. adhesion on silicone rubber by biosurfactant produced by *Streptococcus thermophilus*. They found that biosurfactant released by Streptococci reduces yeast adhesion not only to silicone rubber without a salivary conditioning film but also to silicone rubber with a salivary conditioning film, despite the fact that adsorbed salivary components themselves had already decreased adhesion of *C. albicans* and stimulated detachment of *C. tropicalis* after the passage of an air bubble. Chen and Stewart, 2000, suggested that surfactants such as SDS, Triton X-100, and Tween 20 might disrupt hydrophobic interactions involved in cross linking of the biofilm matrix. Rodrigues *et al.*, 2004, found that biosurfactants from probiotic bacteria *Lactococcus lactis* 53 and *Streptococcus thermophilus* A were able to reduce microbial numbers on voice prostheses and also induces a decrease in the airflow resistance that occurs on voice prostheses after biofilm formation. They suggested

that it is more effective to decrease the viability of biofilms by acting directly on the EPS than to reduce the number of organisms on the esophageal surface of voice prostheses. Their study presents a promising strategy for prolonging the lifespan of voice prostheses. Studies by Boles *et al.*, 2005, found that both exogenous addition of rhamnolipids (biosurfactant) and induced expression of rhamnolipid genes can produce detachment in wild-type *P. aeruginosa* biofilms. Interestingly, the rhamnolipid-mediated detachment mechanism involves the formation of cavities within the centre of biofilm structures (Boles *et al.*, 2005). Further they suggested that these rhamnolipids could also disrupt cell surface structures that act as adhesions.

2.6.5 Polysaccharide degrading agents

Biofilm exopolysaccharides are the major cause of infection which acts as a potential barrier, hindering or preventing biocides from reaching target organisms (Sutherland, 1990; 2001). Inhibition of the production of these biofilm exopolysaccharides can be used as a principal strategy to control biofilm formation. Among several compounds available, Bismuth compounds are potential agents which are well known to reduce exopolysaccharide production and biofilm formation in several gram-negative bacteria like *Pseudomonas aeruginosa*, *Klebsiella pneumoniae, Staphylococci, E. coli* etc (Huang and Stewart, 1999). Huang and Stewart, 1999, found that in biofilm cultures of *P. aeruginosa* ERC1, 1×MIC of BisBAL (bismuth dimercaprol) was able to kill attached cells along with reduction in polysaccharide production. Their results indicated that efforts to develop agents that block polysaccharide production may be valuable in the battle against biofilm infection. Studies by Zhang *et al.*, 2005, also explored the role of bismuth dimercaprol in preventing bacterial

adherence and biofilm formation on the surface of biliary stents. Cammarota and Sant Anna Jr, 1998, studied metabolic blocking of exopolysaccharide synthesis by 2, 4-dinitrophenol (DNP) and its effect on microbial adhesion and biofilm formation. They found that addition of chemical reagent that uncouples phosphorylation of carbohydrate prevented both synthesis of polysaccharide and adhesion. Further they indicated that there is direct relationship between the amount of exopolysaccharide produced and adhesion in the multi-species biofilm. Recent studies by Jain *et al.*, 2007, indicated that in addition to EPS production, DNP treatment affects the marine bacterial cell surface hydrophobicity, thereby reducing bacterial adhesion to glass and polystyrene surfaces.

CHAPTER 3

MATERIALS & METHODS

3.1 MATERIALS

3.1.1 Microorganisms

Among 39 *Candida sp.* isolates obtained from infected clinical samples, *Candida albicans PLV12* strain was used in the investigation while *Pseudomonas aeruginosa* strain (MTCC 2642) was obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India.

3.1.2 Biopolymers for biofilm formation

Commercially available biopolymer, polyvinylchloride (PVC) and glass slides were cut into 1 cm^2 and sterilized before use.

3.1.3 Culture media

Yeast Extract Peptone Dextrose (YPD) medium (Appendix-I), Yeast Extract Phosphate (YEP) medium (Appendix-II), Sabouraud Dextrose agar (SDA) medium (Appendix-III) and HiChrom Candida agar medium (Appendix-IV) were purchased from Himedia Chemicals, India.

3.1.4 Chemical Reagents and Diagnostic Kits

Some of the fine chemicals and kits used in the study have been listed below:

- 2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide(XTT); Menadione; Ethidium Bromide (EtBr); Amphotericn B; Diethylaminoethyl (DEAE); Carboxy methyl cellulose (CMC); Sephacryl S200 HR; Alcohol dehydrogenase, carbonic anhydrase, Deuterium Oxide (D_2O); Ethyl Methanesulfonate (EMS), Proteinase K, Lyticase, Cellulase, Deoxyribonuclease I (DNase 1), Chitinase, Lipase and Alginate Lyase

from Sigma Chemicals, USA.

- Peppermint, Castor, Eucalyptus oils were purchased from Himedia Chemicals, India while 27 oils derived from steam distillation are: Alsi, Almond, Babchi, Babuna, Cade, Chaulmogra, Clove, Coconut, Ginger grass, Ginger, Jasmine, Jojoba, Juniper, Jyotishmati, Khus, Lavender, Mahua, Malkangani, Musturd, Neem, Ocimum, Rose, Tea Tree, Til, Tulsi, Walnut and Wheatgerm.
- Fluorescein Diacetate (FDA); Propidium Iodide (PI); Fluorescein Isothiocynate (FITC); Bismuth dimercaprol; Fluconazole; Itraconazole; Ketoconazole; 2,4-Dinitrophenol (DNP), Blue dextran and Dextran from Himedia Chemicals, India.
- Concavalin A CL Agarose and Bovine serum albumin (BSA) from Bangalore Genei, India.
- GOD/POD kit from Excel diagnostics, India.
- All culture media and solvents were obtained from Himedia Chemicals and Ranbaxy, India.

3.2 METHODS

3.2.1 Isolation and identification of biofilm forming *Candida* isolates procured from clinical samples

Isolation and identification of *Candida* species from 73 samples of infected IUD (n=23), urine (n=12), oropharyngeal swab (n=9), sputum (n=7), blood (n=13) and CSF (n=9) from patients at I.I.T (Indian Institute of Technology) Roorkee hospital and local nursing homes was done by conventional methods (Freydiere *et al.*, 2001). The isolated 39 *Candida sp.* include *C. tropicalis* (n=8), *C. albicans* (n=16), *C. krusei* (n=5), *C. gulier* (n=7) and *C. glabrata* (n=3). Isolates were numbered and sub cultured on SDA medium to obtain pure colonies. 48 h grown isolates were suspended into 5 ml of sterile normal saline and adjusted to concentration approximately 1×10^6 to 5×10^6 yeast cells/ml (NCCLS standards, 1997) at 540 nm. A 1:100 dilution of each isolate suspension was then performed to produce a final concentration of 1×10^4 to 5×10^4 yeast cells/ml. 0.1 ml of each isolate suspension was plated onto HiChrom Candida agar plates and incubated at 35°C for 48 h. Each plate was observed after 48 h for colony morphology, color, size and texture. These isolates were further identified upto species level by standard biochemical tests and germ tube formation studies.

3.2.2 Biofilm formation and Quantification

For biofilm formation, biopolymer pieces (1 cm^2) were dipped in 1.0 ml of culture with 5×10^8 cfu/ml and placed for 90 min of adhesion phase at 35°C. The pieces were then washed with sterilized PBS (pH 7.2) to remove loosely adherent cells. 1.0 ml of sterilized YPD broth was added to the washed pieces and incubated at 35°C for 24 h. Quantification of the biofilms thus formed was performed using XTT reduction assay as described below.

3.2.3 XTT reduction assay

XTT solution (1 mg/ml in PBS) was filter sterilized through a 0.22 μm pore size filter and stored at -70°C. Menadione solution (0.4 mM) was filter sterilized and prepared immediately before each assay. Prior to each assay, XTT solution was thawed and mixed with the menadione solution at a ratio of 5 to 1 by volume. The biofilms on biopolymer pieces were first washed five times with 1.0 ml of PBS and then 60 μl of the XTT-menadione solution were added to each of the pre-washed and control tubes. The tubes were then incubated in the dark for 2 h at 35°C. The XTT salt is reduced by *C. albicans* mitochondrial dehydrogenase to brown color water-soluble tetrazolium formazan product which was measured spectrophotometrically (Varian, USA) at 492 nm. The biopolymer pieces were analyzed microscopically for biofilm formation.

3.2.4 *C. albicans PLV12* Extracellular polymeric substances (EPS) isolation

3.2.4.1 Planktonic EPS

Planktonic EPS was isolated from culture supernatants of *C. albicans PLV12* by acetone precipitation method using modified protocol of McCourtie and Douglas, 1985. The 48 h grown culture supernatant was treated with 1.5 volume of chilled acetone and centrifuged at 17000 g for 15 min, the pellet so obtained was redissolved in a small volume of distilled water and centrifuged to remove any insoluble material. Water soluble polymer was reprecipitated with 2 volume of chilled acetone for 2 h at 4°C. The precipitate was finally washed with ether and evaporated to dryness in warm air current before being weighed. The percentage yield was calculated as a dry weight of EPS divided by the combined dry weight of cells and EPS.

3.2.4.2 Biofilm EPS

Biofilm EPS was isolated following a slight modification of protocol given by Baillie and Douglas, 2000. Biofilms grown on biopolymer surface were scraped off by sterile tooth picks and transferred to sterile capped PVC bottles containing 2 ml of distilled water. The tubes were sonicated on ice for 20 sec at 3.5 Hz frequency (Hielscher Ultrasound Technology, USA) and vortexed for 60 s to disrupt the biofilms. The sonication procedure did not promote cell lysis. Cell suspensions were then pooled and centrifuged. The supernatants were concentrated to one-tenth of the original volume using an Amicon DC2 hollow-fibre system (Millipore Ltd, Watford, UK) and dialyzed at 4°C for 3 days against five changes of distilled water. The retentates were freeze-dried.

3.2.5 Analysis of *C. albicans PLV12* EPS

Quantitative analysis of *C. albicans PLV12* planktonic and biofilm EPS was done for:

- Total carbohydrate by phenol sulphuric acid method (Dubois *et al.*, 1956; Appendix V),
- Hexosamine by Blumerkrantz and Asboe-Hansen method (Chaplin and Kennedy, 1986; Appendix-VI),
- Uronic acid by Carbazole assay (Appendix VII),
- Glucose by GOD/POD kit (Appendix VIII),
- Phosphorus by Ames *et al.*, 1968 method (Appendix IX),
- Protein was determined by Lowry's method (Appendix X) and
- Extracellular DNA by Allesen-Holm *et al.*, 2006 method (Appendix XI).

3.2.6 Scanning electron microscopy (SEM)

SEM was performed in accordance with the procedure given by Hawser and Douglas, 1994. *C. albicans PLV12* biofilms formed on biopolymer pieces (1.0 cm^2) were fixed with 2.5% (v/v) glutaraldehyde in PBS (pH 7.2) for 2 h at room temperature. They were then treated with 1% (w/v) uranyl acetate for 1 h, and washed with distilled water. The samples were dehydrated in ethanol series (50%, 80%, 90% and 100%). All samples were dried to critical point by Polaron critical point drier, gold coated and viewed under Scanning Electron Microscope (Leo 435, England).

3.2.7 Confocal scanning laser microscopy (CLSM)

Biofilms formed on biopolymer surface were fixed with 2.5% glutaraldehyde in PBS for 1.5 h and visualized with fluorescent stains with 8 µM PI and 50 µg/ml FITC-ConA in 10 mM HEPES/ 0.1 M NaCl, containing 0.1 mM Ca^2 and 0.01 mM Mn^2. Confocal laser scanning (Radiance 2100, BioRad) was performed using Nikon microscope (objective Plan Apo 60X/1.4 oil, Japan). The excitation wavelength for PI fluorescence was 543 nm (Green He Ne laser) and fluorescence was detected through emission filter HQ 590/70 (High quality band pass), centered at 590 nm with 70 nm band width. Image processing and graph analysis was carried out using Lasersharp2000 (BioRad) and photoshop 5.5 (Adobe Systems, San Jose, CA).

3.2.8 Atomic force microscopy (AFM)

Images of biofilms formed on biopolymer surfaces were obtained with a commercial atomic force microscope (NTEGRA NT-MDT, Russia) in semi-contact mode using sharpened silicon nitride cantilevers NSG10S with spring constant about 10 N/m. The cantilevers had

an amplitude range 5-15 nm, tip radius 10 nm and cone angle of 22 degree. Height and deflection images were simultaneously acquired at a scan rate of 250 kHz. Data analysis was done using NOVA software.

3.2.9 Fluorescent microscopy

Biopolymer pieces containing biofilms were dipped in suspension containing 1:1 mixture of Fluorescein Diacetate (FDA; 1 mg/ml in PBS) and Ethidium Bromide (EtBr; 0.5 mg/ml in PBS) solution. Samples were incubated at 25°C for 30 min. After incubation, samples were examined under fluorescence microscope (Zeiss, Axiovert 25, Japan).

3.2.10 Effect of shaking conditions on EPS production

Effect of shaking conditions on EPS production was studied by incubating *C. albicans PLV12* cultures for 48 h under different shaking conditions (5, 10, 20, 20, 40, 50 and 60 rpm) respectively.

3.2.11 Effect of pH and temperature on EPS yield

To study effect of pH and temperature on EPS production, *C. albicans PLV12* culture was incubated for different pH (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) and temperatures (5, 10, 15, 20, 25, 30, 35, 40, 45 and 50°C) respectively.

3.2.12 Effect of carbon sources on EPS yield

To check effect of carbon sources on EPS production, *C. albicans PLV12* was grown in minimal salt medium containing different seven carbon sources namely arabinose, galactose,

glucose, fructose, sucrose, xylose and mannose, at a concentration of 2% (w/v) for 48 h to study their effect on EPS yield. Glucose was used as a control in the study.

3.2.13 Ion exchange chromatography of EPS:

Before passing through the columns dried EPS was hydrolyzed in 2 M HCl at 100°C for 3 h to its monomeric subunits.

3.2.13.1 Diethylaminoethyl (DEAE)

Anion-exchange chromatography was carried out on a DEAE sephadex A_{50} column (1.5 × 11 cm) equilibrated with 20 mM Tris buffer (pH 7.6). Elution of charged fractions was performed at a flow rate of 1.4 ml/min with a linear gradient of 0.05 to 0.4 M NaCl, 2 ml fractions were collected and assayed for D-glucuronic acid using carbazole assay.

3.2.13.2 Carboxymethylcellulose (CMC)

Cation-exchange chromatography was performed on a CMC sephadex A_{50} column (1.5 × 5 cm) equilibrated with 20 mM Tris buffer (pH 7.6). Charged fractions were eluted with a linear gradient of 0.05 to 0.4 M NaCl, 2 ml fractions were collected and assayed for N-acetylglucosamine using Morgan-Elson assay.

3.2.14 Affinity chromatography of EPS

Affinity chromatography was done with Concavalin A CL Agarose column (1.5 × 5 cm) equilibrated with 5 mM sodium acetate buffer (pH 5.2). Fractions were eluted at a flow rate of 0.6 ml/min with a linear gradient of 0.1 to 0.5 M α-D-methyl glucoside (α-DMG) in sodium acetate buffer and assayed for Glucose activity using GOD/POD kit.

3.2.15 Gel permeation chromatography of EPS

The homogeneity and molecular weight of the EPS were determined by Gel permeation chromatography using Sephacryl S 300 HR column (60 x 2.5 cm) at a flow rate of 1.2 ml/min and eluted with 50 mM Tris buffer (pH 7.5). Calibration curve was prepared using (V_e, elution volume) molecular weight standards like Dextrans, Alcohol dehydrogenase (14.4 KD), Carbonic anhydrase (30 KD), Bovine serum albumin (66.2 KD). The void volume was determined by using Blue dextran (2000 KD) solution. From the plot of log M_w vs. V_e/V_o, the approximate molecular weight of the fraction was computed.

3.2.16 Gas chromatography of EPS

Analysis of hydrolyzed EPS as alditol acetate (Chaplin and Kennedy, 1986) derivatives was performed on HP 5890 Gas chromatograph fitted with an automatic injector, a flame ionization detector and an OV 225 packed column (0.32 mm × 60 m). Hydrogen was used as the carrier gas. The GC oven was temperature programmed as follows: 50°C for 1 min then an increase of 20°C/min until 120°C, followed by a gradient of 2°C/min until 240°C. Sample peaks were identified by matching their retention time with that of standard sugars.

3.2.17 High performance liquid chromatography of EPS

Monosaccharides were identified by Waters HPLC equipment fitted with Waters high performance carbohydrate column (4.6 mm x 250 mm) and Waters 2414 Refractive Index detector. The experiment was performed at 30°C with a flow rate of 1.4 ml/min using mobile phase of acetonitrile: water (70:30). EPS samples were hydrolyzed to their monomeric subunits with 4 M trifluoroacetic acid at 120°C for 2 h.

3.2.18 Fourier Transform Infrared spectroscopy of EPS

For FTIR analysis, dried EPS was ground with KBr powder and pressed into pellets for FTIR spectra measurement in the frequency range of 4000-500 cm^{-1}. The EPS sample was characterized using FTIR spectrophotometer (Thermo-Nicolet, USA) equipped with OMNIC software for data analysis.

3.2.19 Nuclear Magnetic Resonance spectroscopy of EPS

The ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Avance 500 MHZ FT NMR spectrometer. The freeze-dialyzed EPS sample was dissolved in 600 µl of D_2O. The observed chemical shifts (^{1}H and ^{13}C) were reported relative to internal reference tetramethylsilane (TMS). ^{1}H NMR measurements were obtained at 298 K with spectral width 10330.578 Hz, digital resolution 0.157 Hz, acquisition time of 3.17 s and number of scans 128. ^{13}C NMR spectra were also obtained at 298 K with spectral width 26455.02 Hz, digital resolution 1.61 Hz, acquisition time of 0.30 s and number of scans 1024.

3.2.20 Mutational studies using random mutagenesis approach

In order to find out EPS deficient mutants, a random mutagenesis approach was undertaken using Ethyl methanesulfonate (EMS). Mutagenesis was carried out using EMS in the concentration range 1-5% using protocol given by Sleep *et al.*, 1991. 100 µl of treated culture was plated evenly onto the YEP agar plates and incubated at 35°C for 24 h. Colonies thus formed were picked and grown in YPD broth medium and screened for EPS production.

The antifungal susceptibility of *C. albicans* wild and mutant strains was checked on HiChrom Candida agar medium using commercially available antifungal discs. The paper

discs containing 10 µg of Fluconazole, Itraconazole and Ketoconazole and 100 Unit/disc of Amphotericin B were placed onto HiChrom Candida agar medium plates seeded with the *C. albicans PLV12* wild and mutant suspension. The agar plates were incubated at 37°C for 48 h.

3.2.21 Control Strategies for *C. albicans* biofilm and EPS

3.2.21.1 Effect of plant oils:

3.2.21.1.1 Screening of plant oils

Thirty plant oils namely peppermint, castor, eucalyptus, alsi, almond, babchi, babuna, cade, chaulmogra, clove, coconut, ginger grass, ginger, jasmine, jojoba, juniper, jyotishmati, khus, lavender, mahua, malkangani, mustard, neem, ocimum, rose, tea tree, til, tulsi, walnut and wheatgerm were tested against *C. albicans PLV12* biofilm and its EPS. The YPD agar plates were prepared and 50 µl, $5x10^8$ cfu/ml of *C. albicans PLV12* were spread aseptically over agar surface, 5 µl of plant oils were spotted onto 5 mm discs, transferred to the seeded plate and incubated at 35°C. After 48 h of cultivation, the zone of inhibition was measured.

3.2.21.1.2 Biofilm formation and quantification

For biofilms formation, 200 µl of 5 x 10^8 cfu/ml of *C. albicans PLV12* was suspended in YPD and added to 96-well MTP for 90 min of adhesion phase. The wells were washed with sterilized PBS to remove loosely adhered cells. To the washed wells, 5% (v/v) concentration of plant oils and sterilized YPD broth was added to 200 µl final volumes and incubated at 37°C for 24 h. Quantification of biofilm formed was done using XTT reduction assay using MTP reader (Oasys UVM 340) at 492 nm. Testing was performed in triplicate. Fluconazole was used as a positive control in the study. Plant oils found to reduce biofilm at 5% (v/v) conc.

were considered as biofilm preventive and subjected to MIC determination.

3.2.21.1.3 Determination of minimum inhibitory concentration (MIC) of plant oils

MIC of plant oils was determined by agar dilution assay. The agar plates were prepared in triplicate by adding YPD agar containing different concentrations of plant oils (0.03-5% v/v). Tween-20 (0.5% v/v) was added to enhance oil solubility. The plates were inoculated with 10^3 cfu, using inocula of both the strains prepared as above and incubated for 48 h at 35°C. Plates with Tween-20 but without any plant oil were used as control. Number of colonies were counted after 48 h of incubation. The lowest concentration of plant oil required to completely inhibit the growth of *C. albicans PLV12* was designated as the MIC.

3.2.21.2 Effect of 2,4-Dinitrophenol (DNP)

C. albicans PLV12 was grown in chemically defined medium (Appendix-XII) containing MIC of DNP used as a sole carbon source. The culture medium was buffered to pH 7.0 with 10 mM phosphate buffer. To examine microbial accumulation, PVC pieces (1.0 cm^2) were placed in the culture flasks. Samples were withdrawn at different time intervals (8, 16, 24, 32, 40, 48, 56 and 64 h).

3.2.21.2.1 MIC of 2,4-Dinitrophenol

The MIC of DNP was determined by tube dilution method. About 10^7 cells were inoculated into tubes containing chemically defined medium with varying DNP concentrations (175 to 675 µM) and shaken at 120 rpm at 35°C for 24 h.

3.2.21.3 Effect of Biosurfactant

Biosurfactant (Rhamnolipid) was isolated and characterized from *Pseudomonas aeruginosa* strain (MTCC 2642) by method described previously by Makkar and Cameotra, 1997. To study its effect on biofilm formation and EPS production, different concentrations (5 to 30 mg/ml) of biosurfactant were added to preformed biofilms on 96-well MTP surfaces and biofilm formation and EPS yield were calculated.

3.2.21.4 Effect of Enzymes

Seven enzymes: Proteinase K, Lyticase, Cellulase, Deoxyribonuclease 1, Chitinase, Lipase and Alginate lyase were used to assess their effect on biofilm formation and EPS production in *C. albicans PLV12*. Enzyme solutions were prepared immediately before use. All enzyme treatments were carried out for 2 h at 35°C with a final enzyme concentration of 50 µg/ml. The assays were performed using protocol given by Kaplan *et al.*, 2004.

3.2.21.5 Effect of Bismuth dimercaprol

C. albicans PLV12 was grown in chemically defined medium containing 20 mg/l glucose as sole carbon source and MIC of Bismuth dimercaprol. To examine microbial accumulation, PVC pieces (1.0 cm^2) were placed in the culture flasks. Samples were withdrawn at different time intervals (8, 16, 24, 32, 40, 48, 56, 64, 72 and 80 h) to investigate effect on biofilm formation and EPS production.

3.2.21.5.1 MIC of Bismuth dimercaprol

Bismuth dimercaprol was prepared by dissolving bismuth nitrate in dimercaprol containing

solution at a molar ratio of 1:1.6. The MIC of bismuth dimercaprol was determined by tube dilution method. About 10^7 cells were inoculated into tubes containing chemically defined medium with varying bismuth dimercaprol concentrations (5-50 μM) and shaken at 120 rpm at 35°C for 24 h.

3.2.21.6 Susceptibility of *C. albicans PLV12* biofilms and EPS to antifungal agents

C. albicans PLV12 biofilms were tested for susceptibility to Fluconazole, Amphotericin B, Itraconazole, Ketoconazole, Flucytosine using method described earlier by Baillie and Douglas, 1998. Itraconazole, Ketoconazole, Fluconazole were used at ≤ 95 μg/ml, Amphotericin B was used at ≤ 50 μg/ml and Flucytosine was used at ≤ 250 μg/ml. The MICs of antifungal agents for *C. albicans PLV12* were determined by NCCLS M-27A broth microdilution method (National Committee for Clinical Laboratory Standards, 1997). To check susceptibility of biofilms and the role of EPS matrix in the resistance of *C. albicans PLV12* to antifungal agents, 48 h grown preformed biofilms on PVC pieces (1.0 cm^2) were incubated in 96-well MTP containing different concentrations of various antifungal drugs for 5 h at 37°C. After 48 h of incubation, the metabolic activity of the biofilms was measured using XTT reduction assay and compared with that of control biofilms incubated in the absence of drugs.

3.2.22 Statistical analysis

All experiments were performed in triplicate and results were expressed as means ± standard deviations. Statistical analysis of the differences between mean values obtained for experimental groups was performed using student's t-test. P-values of 0.05 or less were considered significant.

CHAPTER 4

RESULTS

4.1 Isolation and identification of *Candida* species from clinical samples

In the present investigations, 73 different clinical samples of Intrauterine Devices (n=23), Urine (n=12), Oropharyngeal swab (n=9), sputum (n=7), Blood (n=13) and CSF (n=9) were studied. Out of which, thirty nine *Candida* species namely *C. tropicalis* (n=8), *C. albicans* (n=16), *C. krusei* (n=5), *C. gulier* (n=7), *C. glabrata* (n=3) were isolated and identified by selection on Chromogenic selection medium (HiChrom Candida agar), standard biochemical tests and germ tube formation studies. The use of differential media, HiChrom Candida agar, allows rapid and selective identification of different *Candida* species based on color reaction and colony morphology from mixed cultures with high degree of accuracy compared with standard identification procedure (Lal *et al.*, 2008).

When grown on differential medium, HiChrom Candida agar, the clinical isolates: *C. tropicalis*, *C. gulier*, *C. glabrata*, *C. krusei* and *C. albicans*, appeared as light green, yellowish, cottony white, pink and green colour colonies respectively (Fig. 4). Identification of the *Candida* species performed by morphological and biochemical analysis indicated highest incidence of *C. albicans* infection in the clinical samples examined (Table 4). On microscopic examination, at 100X (Fig. 5A) and 10X (Fig. 5B) magnification, *C. albicans* appeared as gram-positive, thin-walled, ovoid, budding yeasts sometimes having mould like hyphae and occasionally with large refractile chlamydospores.

4.2 Screening of *C. albicans* isolates for biofilm formation

Among sixteen *C. albicans* isolates screened, maximum biofilm formation was achieved using XTT reduction assay by isolate *PLV12* at 48 h of incubation, revealing its maximum ability to form biofilm compared to all other isolates (Fig. 6). This strain (*PLV12*) was

selected for further investigation.

4.3 *C. albicans PLV12* biofilm formation

The adherence and subsequent biofilm formation by *C. albicans PLV12* on the surface of MTP wells over 72 h were determined by XTT reduction assay (Fig. 7). The production of the soluble colored formazan salt from sessile cells during biofilm formation is a direct reflection of cellular metabolic activity which increased with time with increased cell counts. In the initial phase (8 h), *C. albicans PLV12* biofilms were highly metabolically active but the complexity increases as the biofilm matures (24 to 48 h) and the metabolic activity reached a plateau, reflecting the increased number of cells. These mature biofilms as demonstrated by SEM were characterized by a mixture of yeasts, germ tubes and young hyphae, and an intracellular matrix of EPS (Fig. 8C). Biofilms with less cell density were observed after 56 h depicting biofilm detachment and reduction due to accumulation of metabolites and waste products.

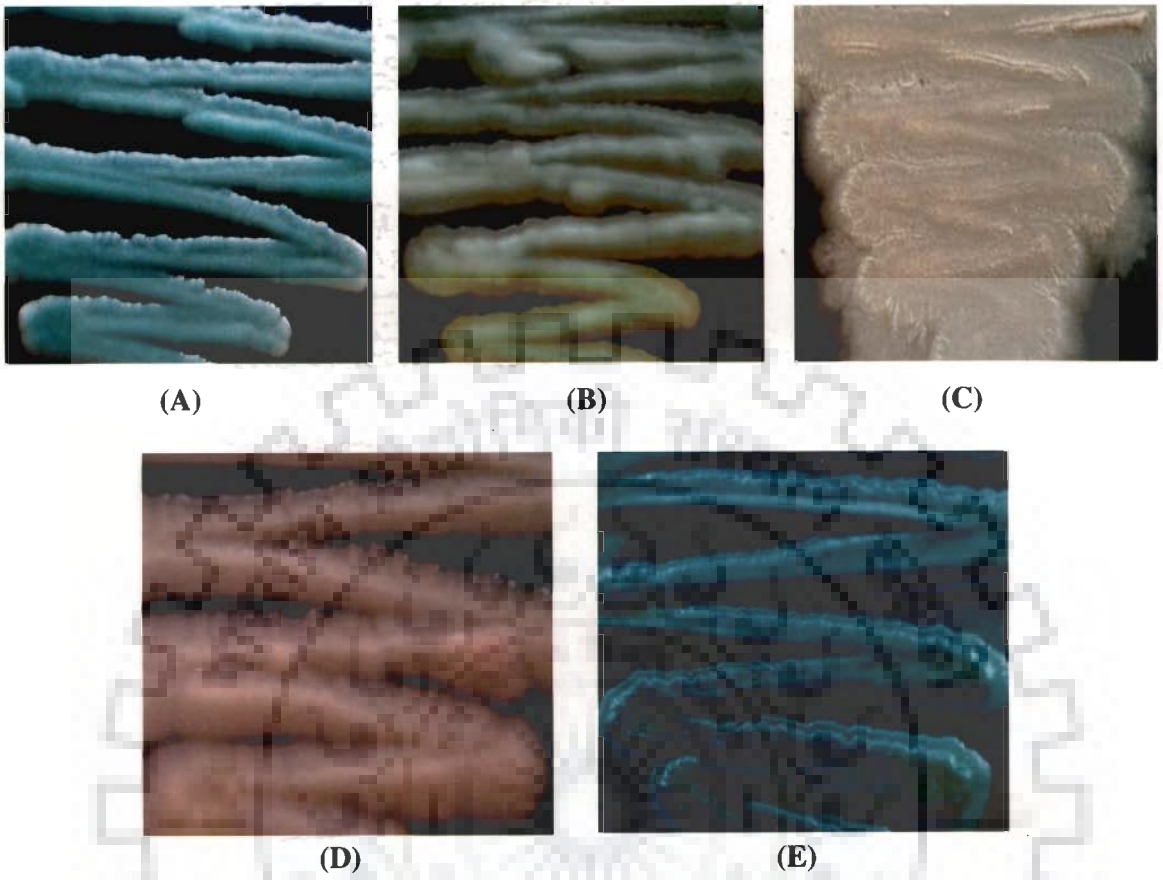


Figure 4. Image showing *Candida* species isolates from clinical samples on Hichrom Candida agar: (A) *C. tropicalis* (B) *C. gulier* (C) *C. glabrata* (D) *C. krusei* (E) *C. albicans.*

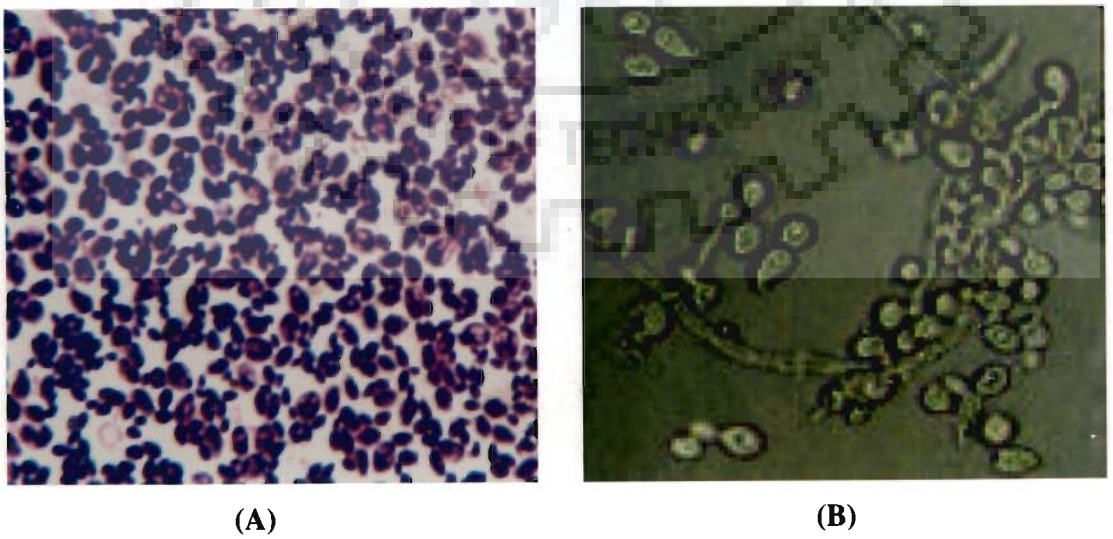


Figure 5. Gram staining (A) and Light microscopy (B) of *Candida albicans.*

Candida sp.	No. of isolates	Colony characteristics	Germ tube formation	Chlamydospore formation	Pseudohyphae formation
Candida albicans	16	Creamy to yellow, bright, soft, smooth colonies; Cocci to oval shaped cells	+	+	+
Candida gulier	7	White, creamy, shiny, smooth or wrinkled colonies; elongate, oval to cylinder cells	–	–	+
Candida krusei	5	Off-white, dull, smooth to lobed margin colonies; oval elongate cells	–	–	+
Candida tropicalis	8	Creamy, border colonies; oval cells	–	+/–	+
Candida glabrata	3	White, smooth, glistening colonies; round to oval cells	–	–	+/–

Table 4. Identification of *Candida* species.

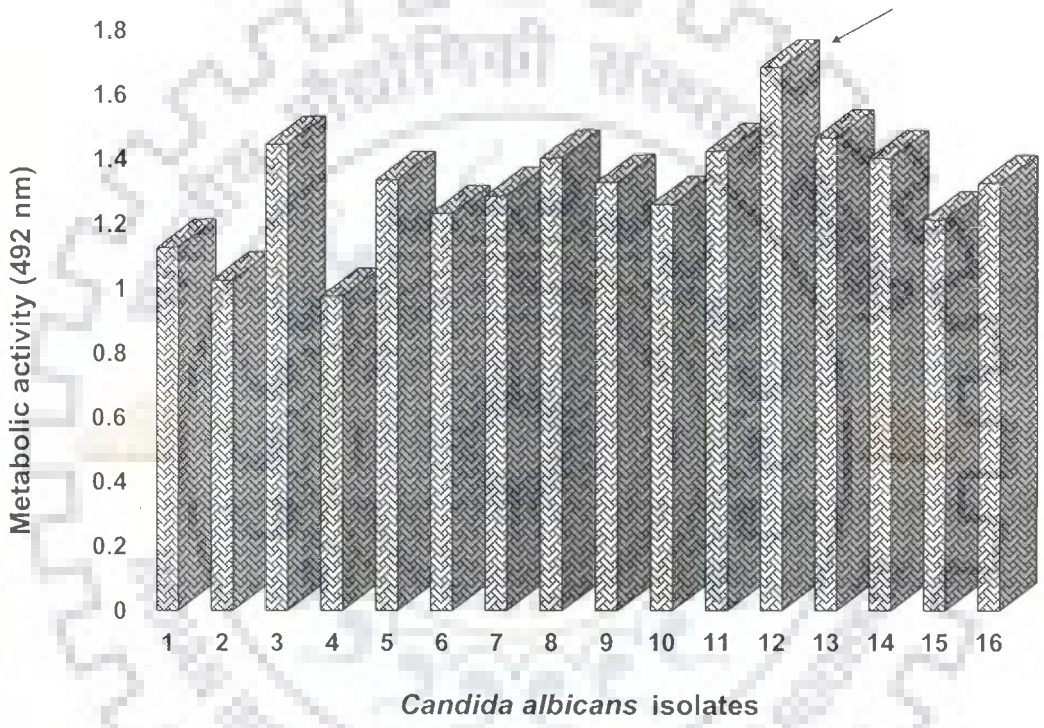


Figure 6. Biofilm formation by *C. albicans* isolates at 48 h of incubation using XTT reduction assay. Arrow indicating maximum biofilm forming isolate.

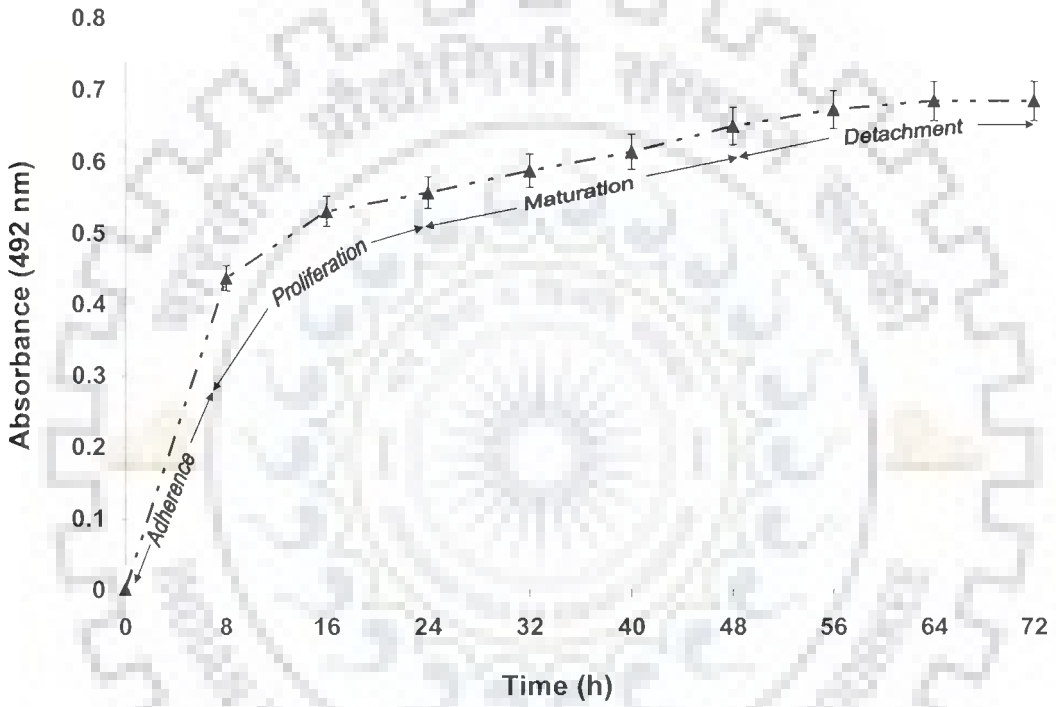


Figure 7. ***C. albicans PLV12*** **biofilm formation as determined by XTT reduction assay.**

4.4 EPS production by *C. albicans PLV12*

EPS surrounding *C. albicans PLV12* cells in biofilms was isolated and its composition was compared with that of EPS obtained from culture supernatants of planktonically grown *C. albicans PLV12*. Data revealed that planktonic EPS consisted largely of total carbohydrate (86±6.5%), protein (9.0±1.5%), phosphorus (0.4±0.1%), hexosamine (0.6±0.2%), uronic acid (0.2±0%) and extracellular DNA (0.5±0.1%). Biofilm EPS on the other hand, contained significantly less total carbohydrate (40±8.5%), protein (5±1.2%) and extracellular DNA (0.2±0.1%) with an increase in glucose (16±3.4%), phosphorus (0.7±0.2%), hexosamine (4.0±1.3%) and uronic acid (0.5±0.1%) content (Table 5).

4.5 Microscopic analysis of *C. albicans PLV12* biofilm

Microscopic analysis of *C. albicans PLV12* biofilm formation on PVC surface was performed using SEM (Fig. 8). Data showed that initial adherence of yeast cells was followed by germ tube formation and subsequent development of hyphae at 12 (Fig. 8A) and 24 h (Fig. 8B) respectively. Mature biofilm of *C. albicans PLV12* formed after 48h (Fig. 8C) consists of dense network of matrix consisting of exopolymeric material.

C. albicans PLV12 biofilm formation was further explored by CLSM. Fig. 9 shows intense green fluorescence resulting from ConA (Concavalin A) binding to polysaccharides of *C. albicans PLV12* while PI (Propidium iodide) penetrates only cells with damaged membranes and stains the cells red. Thus, areas of red fluorescence represent dead cells and green fluorescence indicates dense network of polysaccharides. The yellow color formed as a result of overlapping of red and green images depicts production of exopolysaccharides as capsular components. These analyses revealed a highly heterogeneous architecture of mature

COMPONENT (%)	PLANKTONIC EPS	BIOFILM EPS
Total carbohydrate	86±6.5	40±8.5
Protein	9.0±1.5	5.0±1.2
Glucose	5.0±1.6	16±3.4
Extracellular DNA	0.5±0.1	0.2±0.1
Hexosamine	0.6±0.2	4.0±1.3
Phosphorus	0.4±0.1	0.7±0.2
Uronic acid	0.2±0	0.5±0.1

Table 5. Percentage composition of planktonic and biofilm EPS obtained from *C. albicans PLV12*.

C. albicans PLV12 biofilms in terms of the distribution of fungal cells (indicated by red color) and polysaccharides (green coloration). AFM analysis of *C. albicans PLV12* biofilm on glass surface in semi-contact mode is shown in Fig. 10. The 3D image depicted that candidal cells were embedded within sticky thin colorless layer of EPS distributed around the cell surface, particularly at the cell-substrate periphery. The image significantly gives a better image resolution sensing height and texture variations of biofilm on the glass surface in contrast to the optical microscopy. In a 500 nm^2 area, the biofilm cells were seen as ridges being typically 2-10 nm above the lowest point in the image. These ridges may be formed due to variable production of EPS, such that EPS is sometimes produced in such a great quantity that it protrudes around the cells and become more pronounced after drying of biofilm.

4.6 Quantitative measurement of *C. albicans PLV12* biofilm and EPS under shaking and static conditions

Quantitative measurements of *C. albicans PLV12* biofilm with shaking incubation showed that slow shaking (5 rpm) increased the synthesis of EPS which seemed to form a slimy matrix encasing candidal cell colonies (Fig. 11). At slightly higher speeds (15 rpm), EPS was very extensive (1.12 g/l) and completely covered the cells while statically grown biofilms contained less EPS (Fig. 11 and Fig. 12). At 60 rpm, biofilm formation and EPS production was completely inhibited most probably due to intense shear forces generated at high speed (Fig. 12). In contrast to static conditions, under shaking conditions, there was better homogenization and conditioning of media components to biofilm surfaces which resulted in better availability of nutrients and oxygen to the residing microorganisms.

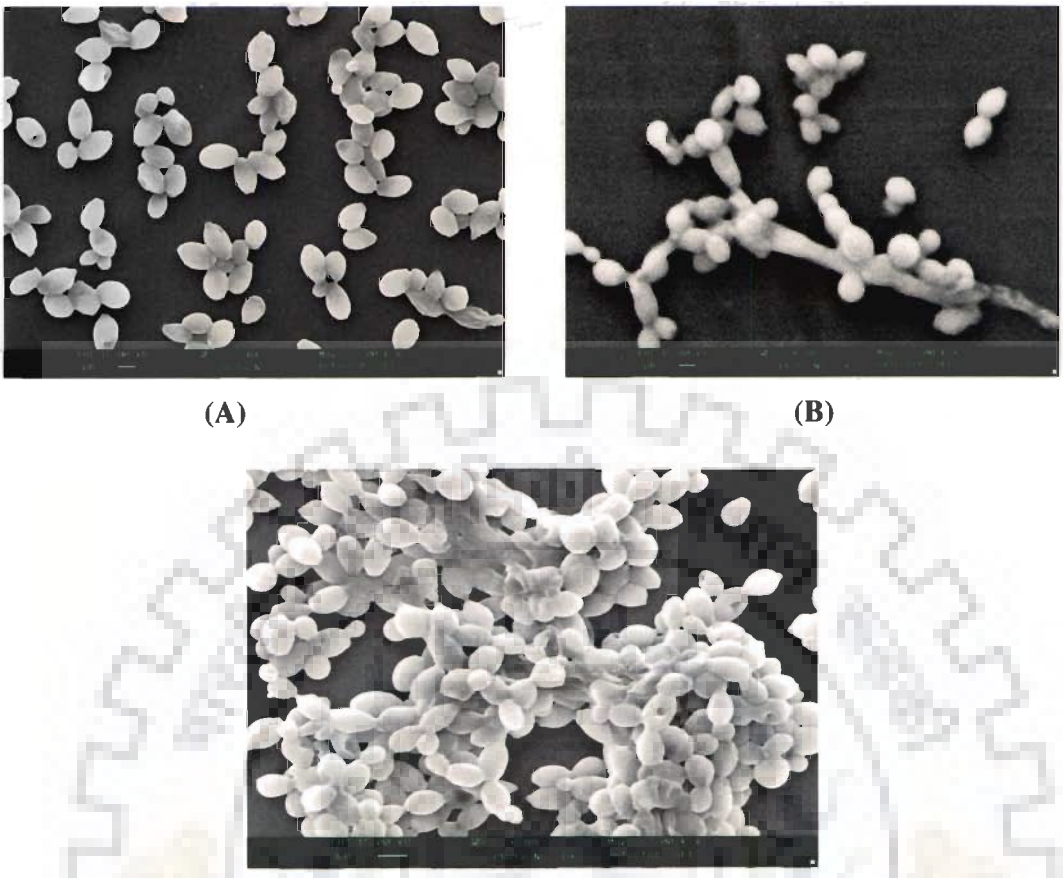


Figure 8. Scanning electron micrographs of *C. albicans PLV12* biofilm on PVC surface at: (A) 12 h, (B) 24 h, (C) 48 h of incubation.

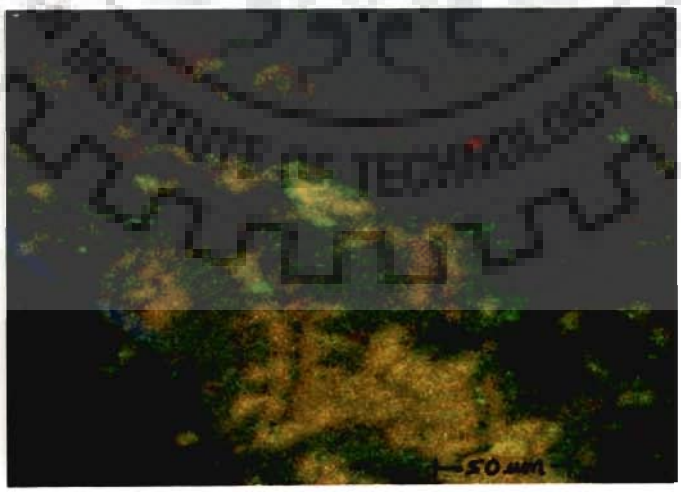
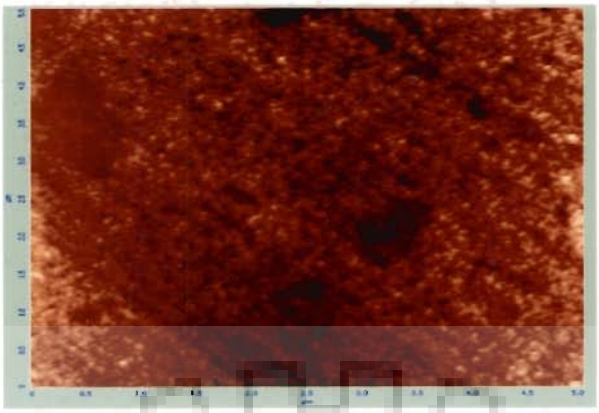
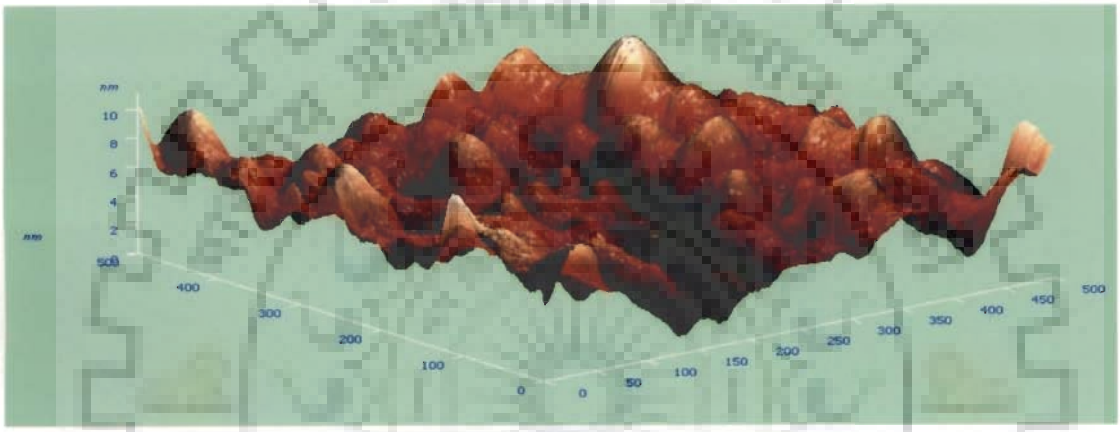


Figure 9. CLSM image of 48 h grown *C. albicans PLV12* biofilm showing PI stained dead cells in red color and FITC-Con A stained polysaccharides in green color while yellow color reflects exopolysaccharide production as capsular components.

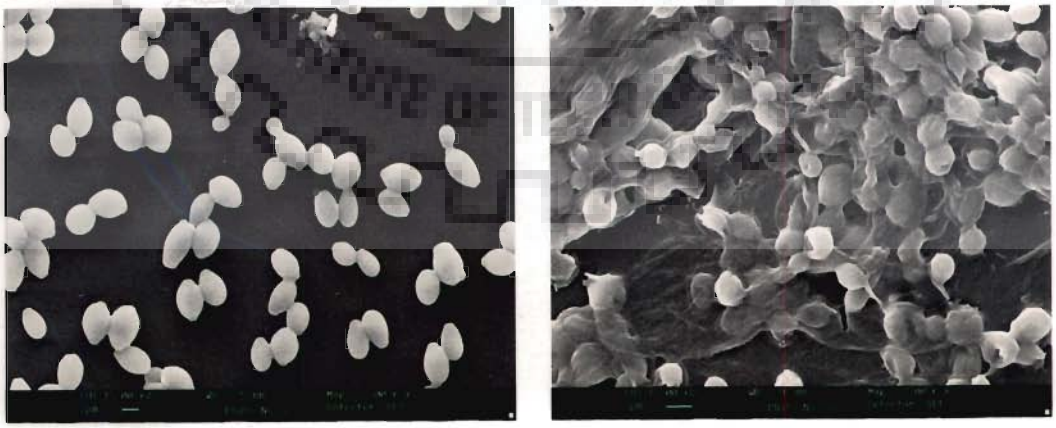


(A)



(B)

Figure 10. (A) AFM image of 48 h grown *C. albicans PLV12* biofilm; (B) 3-D image of *C. albicans PLV12* biofilm depicting the ridges formation having Z (height) scale 30 nm and X-Y scales 2 µm.



(A) (B)

Figure 11. SEM images of 48 h grown *C. albicans PLV12* showing biofilm formation and EPS production on PVC surfaces under (A) static (B) shaking conditions.

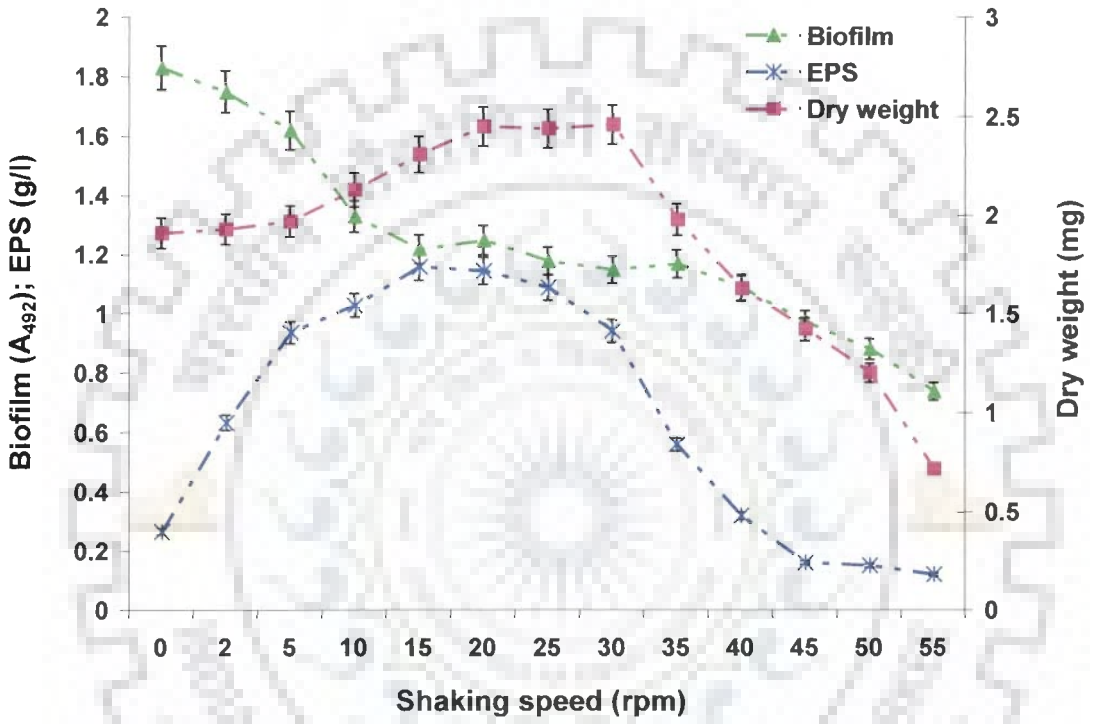


Figure 12. Quantitative measurement of *C. albicans FLV12* biofilm, EPS and dry weight under different shaking speed.

4.7 Effect of pH and temperature

To examine the effect of pH and temperature on EPS production, *C. albicans PLV12* was cultivated in minimal salt medium at different temperature (5.0-50°C) and pH (3.0-8.0) conditions. It was observed that maximum EPS production was observed at 30°C (Fig. 13A), below the optimum growth temperature (35°C) for *C. albicans PLV12*. On the other hand, pH values close to the neutral pH (7.0) were favorable for EPS production in *C. albicans PLV12*. At acidic pH, EPS production was low but at pH 6.3, EPS production reaches its highest point (Fig. 13B).

4.8 Effect of carbon sources

To study the effect of carbon sources on biofilm formation and EPS production, *C. albicans PLV12* was cultivated in the minimal salt medium containing 2% (w/v) glucose, fructose, sucrose, galactose, arabinose, mannose and xylose as carbon sources. Glucose was taken as control in the experiment. Among carbon sources tested, biofilm formation and EPS production were found to be maximum in medium containing arabinose as a carbon source followed by glucose, fructose and xylose (Table 6; Fig. 14). It was also noticed that nearly similar amounts of EPS was produced with galactose, mannose and sucrose i.e., 0.675 g/l, 0.694 g/l and 0.621 g/l, out of which sucrose was found to be the least efficient carbon source showing 32% reduction in EPS yield compared with the control (Table 6).

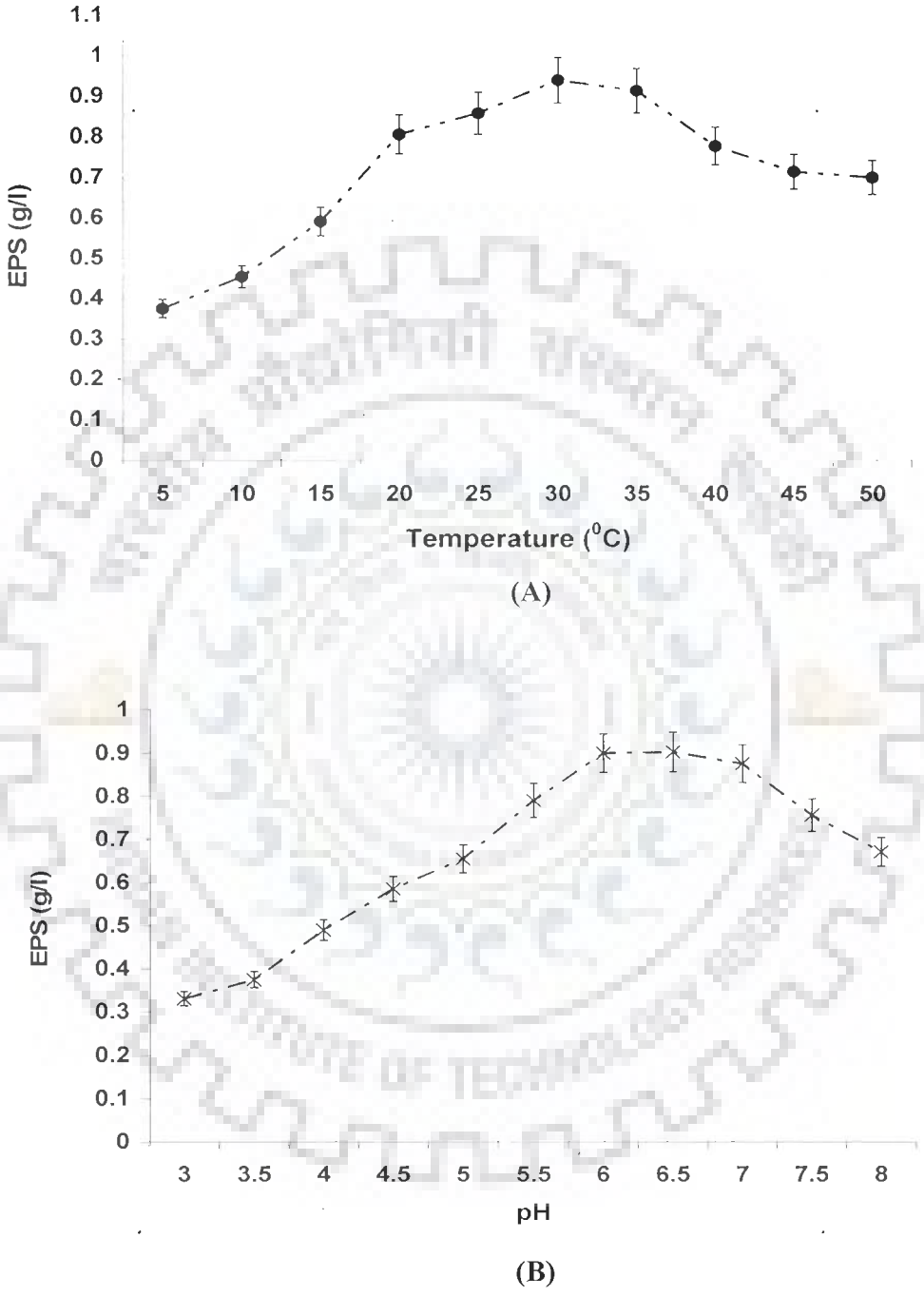


Figure 13. ***C. albicans PLV12*** **EPS yield at different temperatures (A) and pH values (B).**

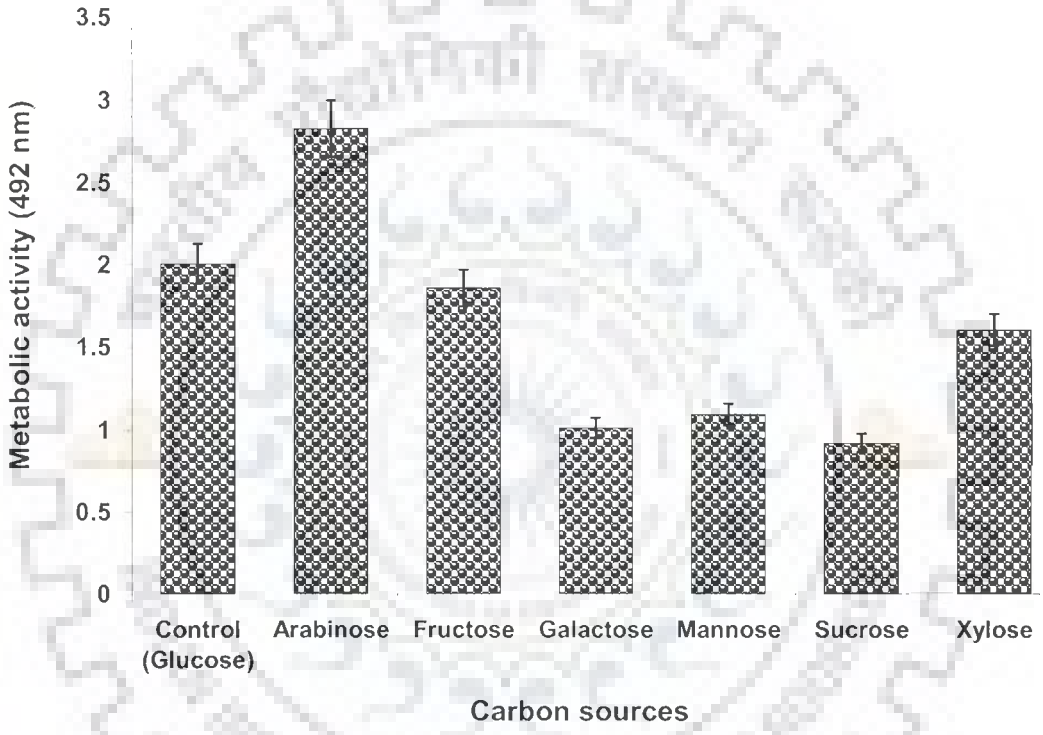


Figure 14. Effect of carbon sources on *C. albicans PLV12* biofilm formation.

CARBON SOURCE	EPS YIELD (g/l)
Control (Glucose)	0.912
Fructose	0.815
Arabinose	0.984
Sucrose	0.621
Xylose	0.793
Galactose	0.675
Mannose	0.694

Table 6. *C. albicans PLV12* EPS yield in response to carbon sources.

Purification of *C. albicans PLV12* EPS components:

4.9 Ion exchange chromatography

Purification of EPS was done by ion exchange chromatography using DEAE Sephadex A_{50} and CMC Sephadex A_{50} ion exchange columns. Since isocratic elution was not able to elute the charged fractions, gradient elution was performed using different concentrations of NaCl in the range from 0.05 M to 0.4 M. In order to detect the anionic component, hydrolyzed *C. albicans PLV12* EPS was passed through DEAE Sephadex A_{50} (1.5 × 11 cm) column. The elution profile of the hydrolyzed EPS is shown in Fig. 15A. Fractions were collected and assayed for D-glucuronic acid using carbazole assay. A major peak obtained at fraction 34 with 0.3 M NaCl gave highest D-glucuronic acid activity. The fraction was further passed on Waters High Performance Carbohydrate column (4.6 mm x 250 mm) with RI detector to confirm D-glucuronic acid activity (Fig. 15B).

In order to detect cationic component, the above fraction collected was concentrated and further purified by passing through CMC Sephadex A50 (1.5 × 5 cm) column with NaCl gradient. The fractions thus collected were assayed for N-acetylglucosamine content. Data revealed a major peak (Fig. 16A) at fraction 26 with 0.2 M NaCl gradient. Again, the fraction was passed through the HPLC column to confirm N-acetylglucosamine activity. HPLC graph shows a major peak at 3.397 min equivalent to N-acetylglucosamine (Fig. 16B).

The elution of both anionic and cationic fractions through ion exchange and HPLC columns showed presence of both positively (N-acetylglucosamine) and negatively (D-glucuronic acid) charged sugar moieties in the exopolysaccharide chain of *C. albicans PLV12*.

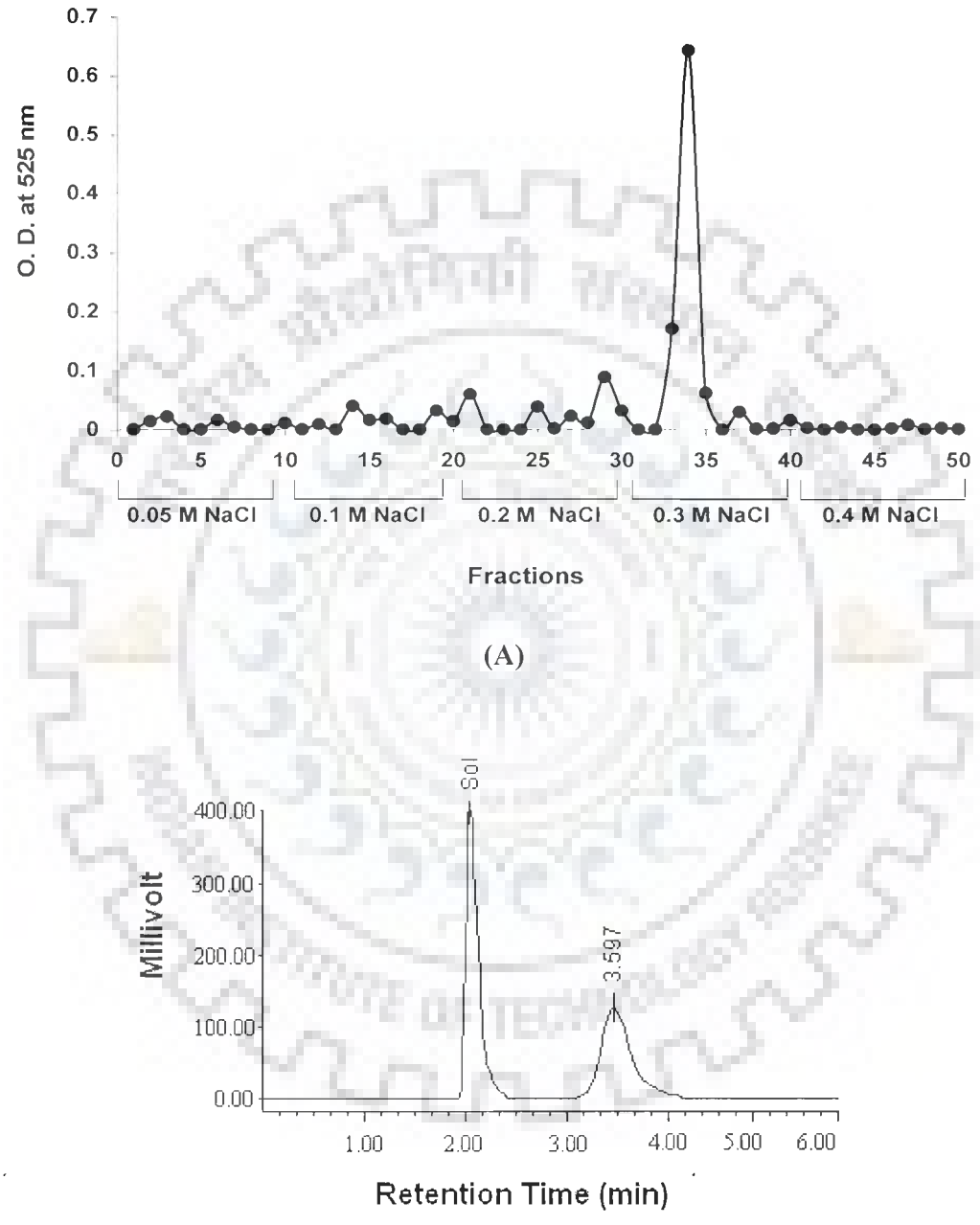


Figure 15. (A) DEAE elution profile of hydrolyzed EPS and (B) HPLC elution profile of D-glucuronic acid on Waters High Performance Carbohydrate column.

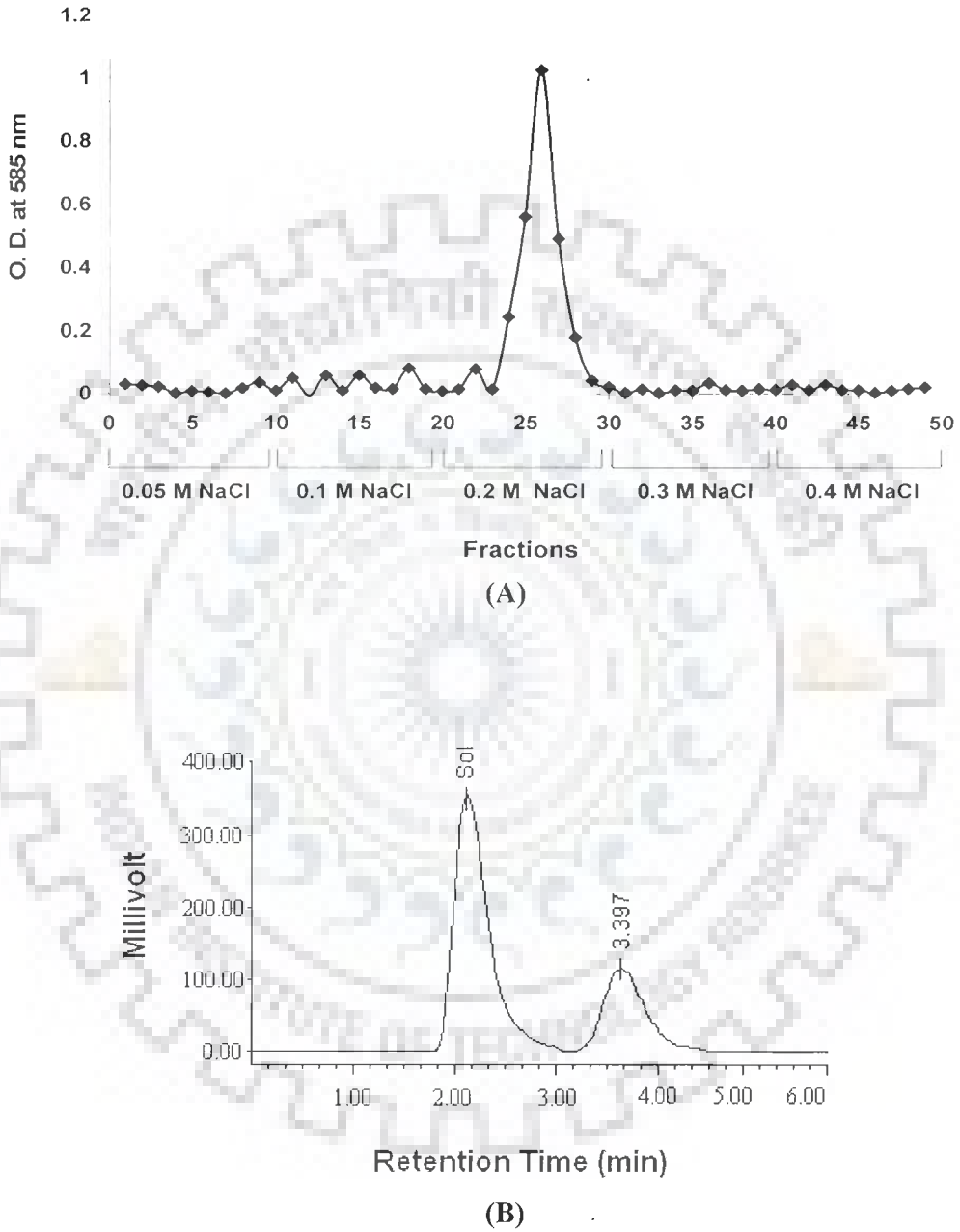


Figure 16. (A) CM Cellulose elution profile of hydrolyzed EPS; (B) HPLC elution profile of N-acetylglucosamine on Waters High Performance Carbohydrate column.

4.10 Affinity chromatography

ConA CL agarose is known to interact specifically with branched polysaccharides and glycoproteins, the non-reducing end chains of which comprise α-D-glucose and α-D-mannose residues. ConA CL agarose column was used to check its affinity towards glucose moiety present in the exopolysaccharide chain. Fractions were eluted with a linear gradient of 0.1 to 0.5 M α-D-methyl glucoside (α-DMG) in sodium acetate buffer. The fractions collected were assayed for Glucose activity with the help of GOD/POD kit. Fig. 17 shows elution profile of hydrolyzed EPS on ConA CL agarose column. Results revealed a major peak with 0.4 M α-DMG at fraction no. 32 showing highest glucose activity.

4.11 Gel permeation chromatography

The elution profile of *C. albicans PLV12* EPS on gel permeation column (2.5 by 60 cm) using sepharcryl S200 HR is shown in Fig. 18. Results showed one major peak eluted with absorption maximum at OD_{485} as 0.272 in fraction number 44 with 50 mM Tris buffer (pH 7.5). This peak corresponds to a high molecular-weight fraction. The relative molecular weight value of the fraction was calculated with the help of calibration curve made by using molecular weight standards. Using calibration curve, the relative molecular weight of *C. albicans PLV12* EPS was found to be approx. 300 KD.

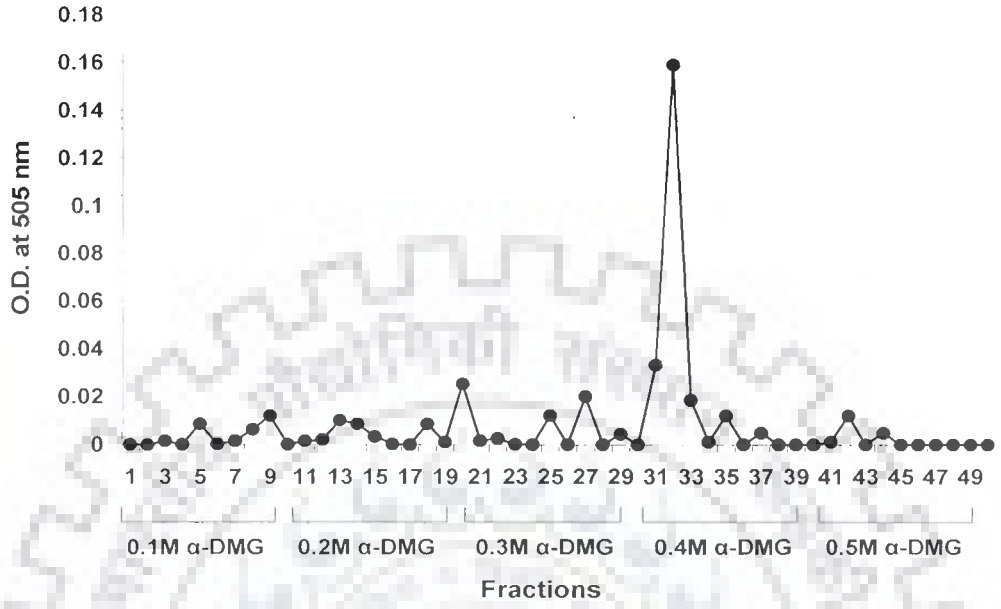


Figure 17. ConA CL agarose elution profile of *C. albicans PLV12* hydrolyzed EPS.

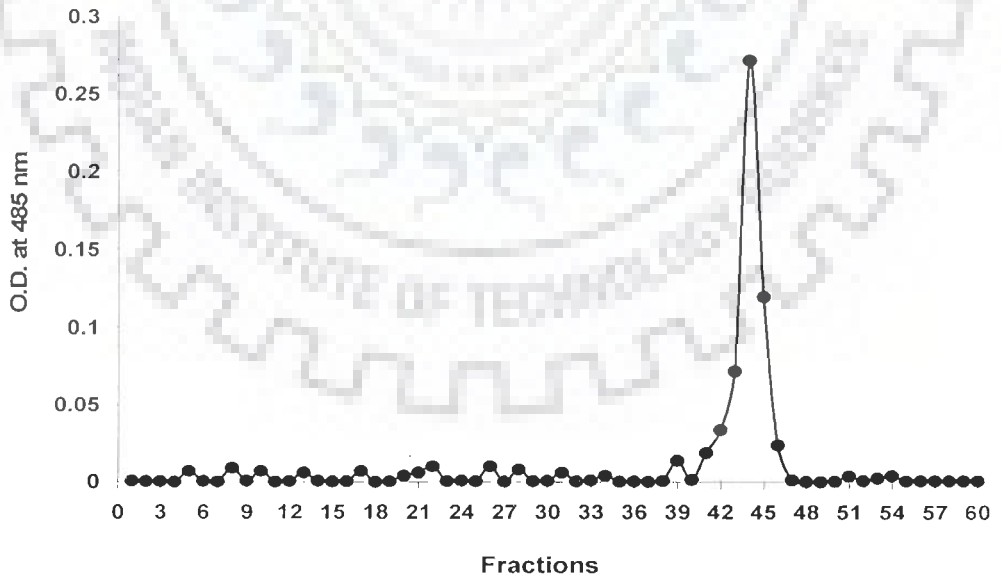


Figure 18. Elution profile of *C. albicans PLV12* EPS in Sephacryl S200 HR column.

4.12 Gas chromatography

The sugar composition of dialyzed *C. albicans PLV12* EPS was analyzed by HP 5890 Gas Chromatogram fitted with OV 225 packed column equipped with FID. GC profile of monosaccharide units as their alditol acetate derivatives are shown in Fig. 19. The GC chromatogram showed presence of five individual sugars namely Glucose, Mannose, Rhamnose, D-glucuronic acid and N-acetylglucosamine detected at 1.768, 3.524, 3.947, 5.057 and 6.144 retention time respectively. Results revealed that glucose, mannose and rhamnose were the most abundant neutral sugars in EPS produced by *C. albicans PLV12* (Fig. 19).

4.13 High performance liquid chromatography

HPLC analysis of acid hydrolyzed EPS on Waters high performance carbohydrate column equipped with RI detector revealed presence of six monosaccharide units in *C. albicans PLV12* EPS (Fig. 20). Results showed presence of N-acetyl glucosamine (at 3.363 min), glucose (at 3.825 min), D-glucuronic acid (at 4.330 min), mannose (at 4.553 min), galactose (at 5.346 min) and rhamnose (at 5.776 min) in the polysaccharide chain. Two unidentified sugars were also observed at retention time 6.879 min and 8.156 min. Fig. 20 clearly indicates that glucose which eluted at 3.825 min constitutes the major portion of the *C. albicans PLV12* EPS.

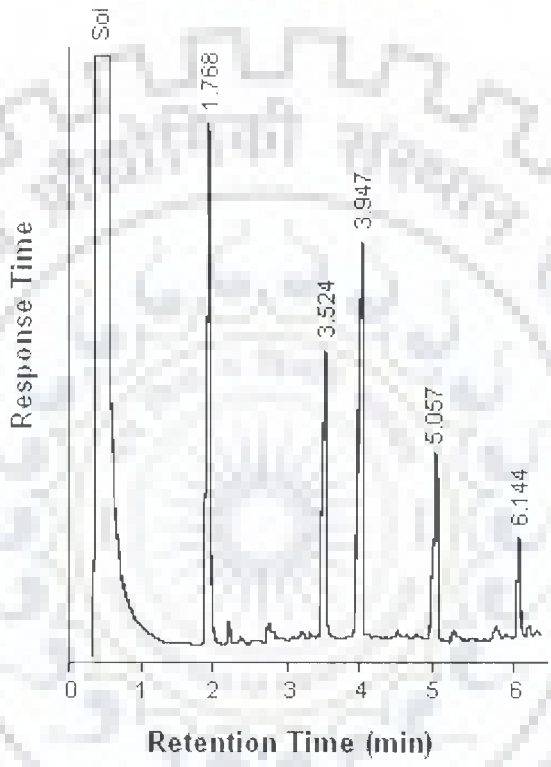


Figure 19. Gas chromatogram of alditol acetate derivatives of hydrolyzed *C. albicans PLV12* EPS on OV 225 column.

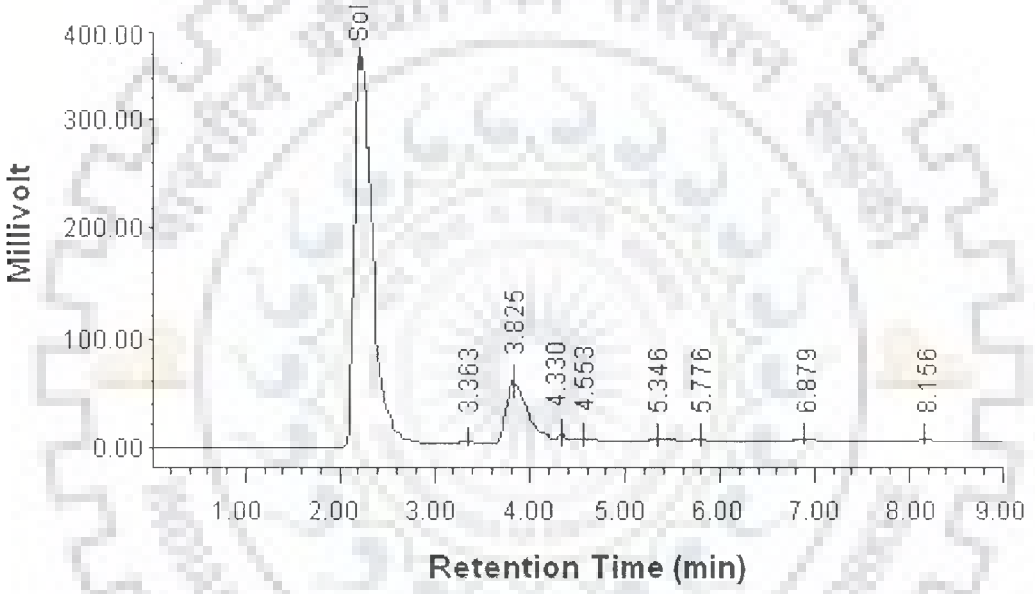


Figure 20. HPLC profile of *C. albicans PLV12* hydrolyzed EPS on Waters High Performance Carbohydrate column.

4.14 Fourier Transform Infrared spectroscopy

Distinctive absorption band maxima in the mid-infrared region at 1200-800 cm^{-1} were found to be useful for the identification of *C. albicans PLV12* exopolysaccharide. The FTIR spectra shown in Fig. 21 exhibited specific absorbance of O–H stretching at 3426 cm^{-1}, weak C–H stretching at 2921 and 2847 cm^{-1}, O=C=O stretching at 2365 and 2343 cm^{-1}, C=O stretching of carboxylate at 1630 cm^{-1}, C-C ring stretching at 1460 cm^{-1} and C–N stretching of primary aromatic amine at 1269 cm^{-1}. The absorption peaks for sugars in mid infra red region were detected at 818, 863, 890, 954, 992, 1017, 1067 and 1128 cm^{-1}. These peaks clearly indicated presence of β-glucans (β (1→6) and β (1→3)) and mannans in the *C. albicans PLV12* exopolysaccharide which was further confirmed by NMR data.

4.15 Nuclear Magnetic Resonance spectroscopy

The structural analysis of *C. albicans PLV12* EPS was performed by means of ^{1}H and ^{13}C NMR spectroscopy. One of the difficulties associated with the modeling of carbohydrate ^{1}H spectra is that most signals lie in a narrow region. This was clearly evident from Fig. 22A; the region between 3.4 and 4.1 ppm features all signals except H1 (most deshielded). The proton spectrum of *C. albicans PLV12* in D_2O consists of three different regions as shown in Fig. 22A. The anomeric region, from approx. 4.3 to 6.0 ppm contains the anomeric proton signals (H1) from all the sugar rings, since these protons are shifted downfield from the other ring protons due to the close proximity of two adjacent oxygen atoms. The 5 anomeric signals, at shifts of 4.944, 4.995, 5.017, 5.041 and 5.196 ppm, were assigned easily by inspection. All of the other ring protons were found overlapped in a narrow region (non-anomeric protons) to form a broad hump of signals from approximately 3.5 to 4.2 ppm. The

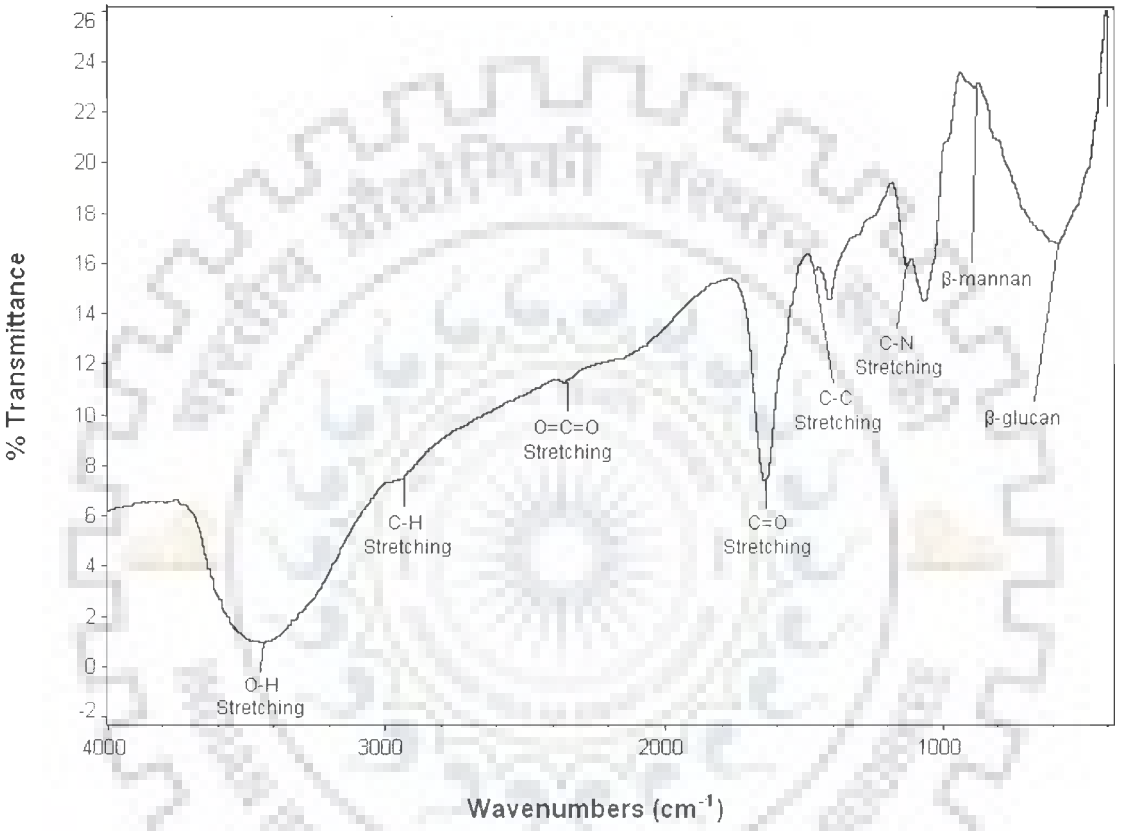


Figure 21. FTIR spectrum of *C. albicans PLV12* EPS.

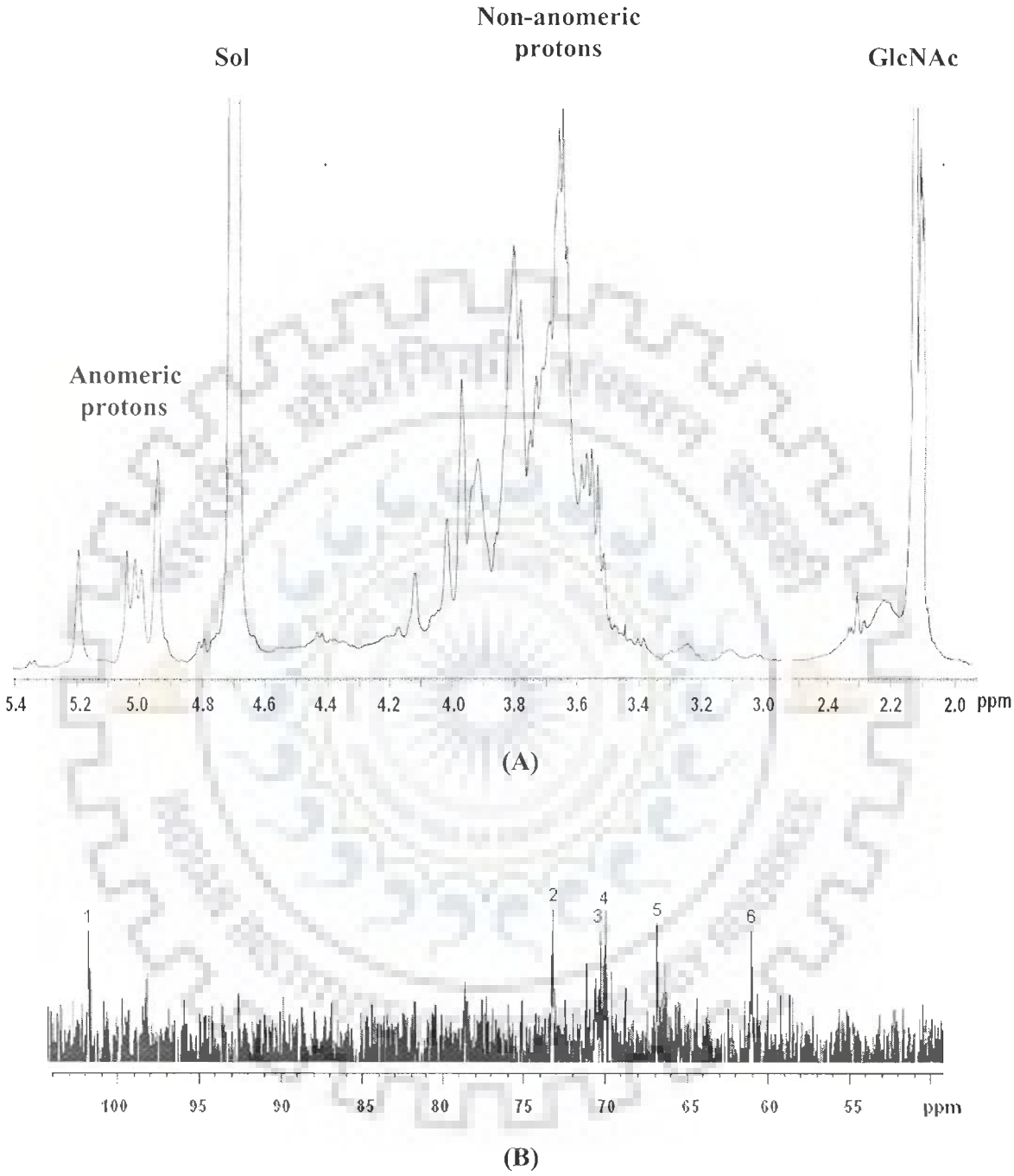


Figure 22. (A) ^{1}H NMR and (B) ^{13}C NMR spectrum of *C. albicans PLV12* EPS.

signal for residual D_2O in the sample occurred at the edge of the anomeric region in the spectrum at 4.699 ppm. The signal for N-acetyl-glucosamine (GlcNAc) was observed at 2.119 ppm. Since the 'hump region' in the spectrum was too complex to interpret, initial analysis was focused on the anomeric region. Analysis of anomeric region showed presence of pyranose ring form in α- anomeric configuration. Glucose exhibited singlet for α anomer at chemical shift 5.196 ppm, assigned at H1. Another singlet was observed at 4.944 ppm due to H1 proton of α- anomer of mannose. A triplet of α-rhamnose was detected at chemical shift 4.995, 5.017 and 5.041 ppm in the spectrum.

The ^{13}C NMR spectrum of *C. albicans PLV12* EPS as shown in Fig. 22B demonstrated presence of six carbon signals at 102, 73.25, 70.30, 70.02, 66.87 and 61.06 ppm, corresponding to the six ring carbons in the polysaccharide chain. The chemical shift for α-L-rhamnose was observed at 102 ppm in the spectrum. The carbon signals at 73.25 and 70.02 ppm were assigned to α-D-glucose while carbon signal for β-D-glucose was detected at 66.87 ppm. Two carbon signals for β-pyranose were also seen in the ^{13}C spectrum at chemical shift 70.30 and 61.06 respectively. Overall results indicated presence of glucose, mannose, rhamnose and N-acetyl glucosamine moieties in *C. albicans PLV12* exopolysaccharide.

Random mutagenesis approach:

4.16 Mutational studies for *C. albicans PLV12*

Maximum number of colonies with varied morphology were obtained with 3% EMS treatment of *C. albicans PLV12* strain (wild). Out of 183 mutant colonies obtained, 19 colonies (*pm1-pm19*) were selected on the basis of their difference in colony morphology compared with the wild strain. The selected mutant colonies were screened for their biofilm forming ability using XTT reduction assay. Data showed that out of 19 colonies screened, only 9 colonies namely *pm1, pm4, pm6, pm7, pm11, pm12, pm13, pm16* and *pm18* gave more than 50% reduction in metabolic activity compared with the wild strain (Table 7). Since mutant *pm4* gave maximum reduction (75.2%) in biofilm formation, it was chosen for comparative study with parental strain. Fig. 23 shows comparative morphology of wild (*PLV12*) and mutant (*pm4*) strains on HiChrom Candida agar medium, *pm4* showed bluish-green colonies having rough morphology compared to green and smooth colonies of wild strain. Parameter optimization for mutant strain (*pm4*) revealed that maximum EPS production takes place at 48 h of incubation as shown in Fig. 24. At this time period, EPS yield by mutant strain (0.23 g/l) was found to be 71% less than that of the wild strain (0.923 g/l).

SEM analysis of 48 h grown *C. albicans PLV12* (wild) and *pm4* (mutant) strains was done to detect morphological alteration in the biofilm formation. Images showed that mutant strain generated scattered and thinner biofilm than compact and thicker biofilm generated by the wild strain (Fig. 25).

Compositional analysis of *pm4* EPS showed 38% reduction in total carbohydrate content while protein and glucose contents were reduced by 5% and 3% (Fig. 26). No observable changes in uronic acid, hexosamine and phosphorus content of mutant (*pm4*) EPS

were observed.

The antifungal susceptibility of *C. albicans* wild and mutant strains against Fluconazole, Itraconazole, Ketoconazole and Amphotericin B was checked on HiChrom Candida agar plates. Data revealed that after 48 h incubation, the zone of inhibition for *C. albicans* mutant (*pm4*) strain were found to be (26.2±2.2) mm, (17.3±1.2) mm, (9.7±0.3) mm and (5.6±0) mm for Amphotericin B, Fluconazole, Itraconazole and Ketoconazole in comparison to (24.0±1.9) mm, (16.2±1.1) mm, (7.3±0.5) mm and (5.1±0.2) mm for wild strain (Fig. 27). Results revealed increased susceptibility of *C. albicans* mutant strain towards antifungal agents in contrast to its wild strain.

MUTANT COLONY	% BIOFILM FORMATION
Control	100
pm1	30.97
pm2	49.90
pm3	70.50
pm4	24.80
pm5	55.91
pm6	47.64
pm7	48.10
pm8	53.40
pm9	52.37
pm10	50.94
pm11	47.05
pm12	40.36
pm13	48.63
pm14	50.81
pm15	52.96
pm16	37.80
pm17	59.06
pm18	41.15
pm19	58.67

Table 7. Percentage biofilm formation by *C. albicans PLV12* mutants.

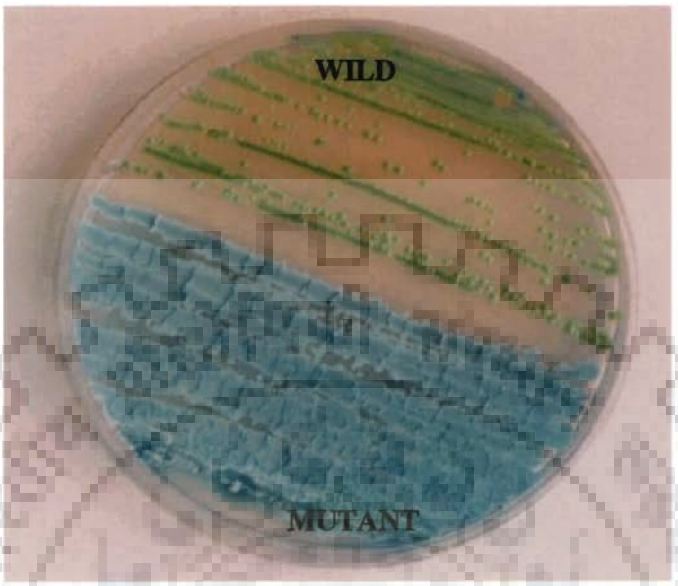
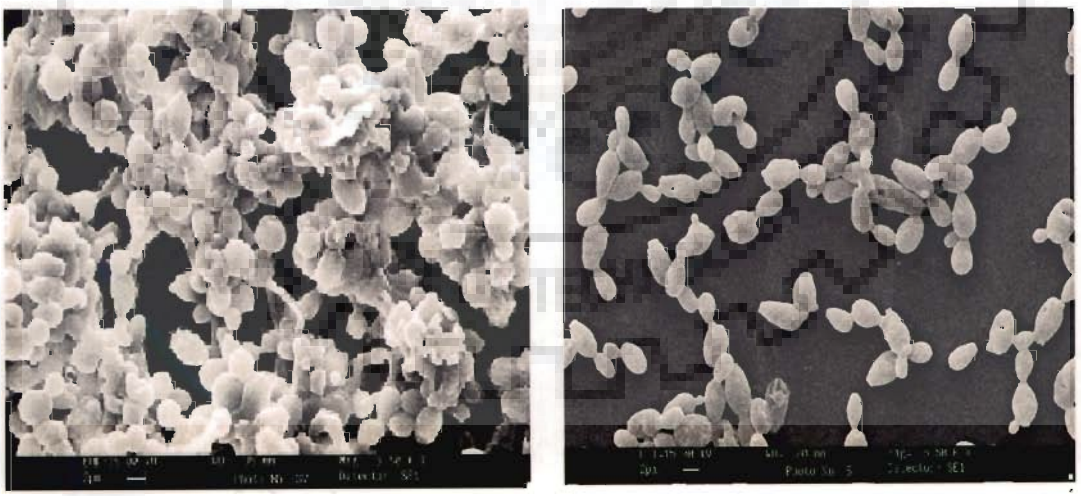


Figure 13. Comparative colony morphology between *C. albicans PLV12* (wild) and Mutant (*pm4*) strains on HiChrom Candida agar.



(Wild) **(Mutant)**

Figure 15. Scanning electron micrograph of *C. albicans PLV12* (wild) and Mutant (*pm4*) strains at 5.5 KX magnification.

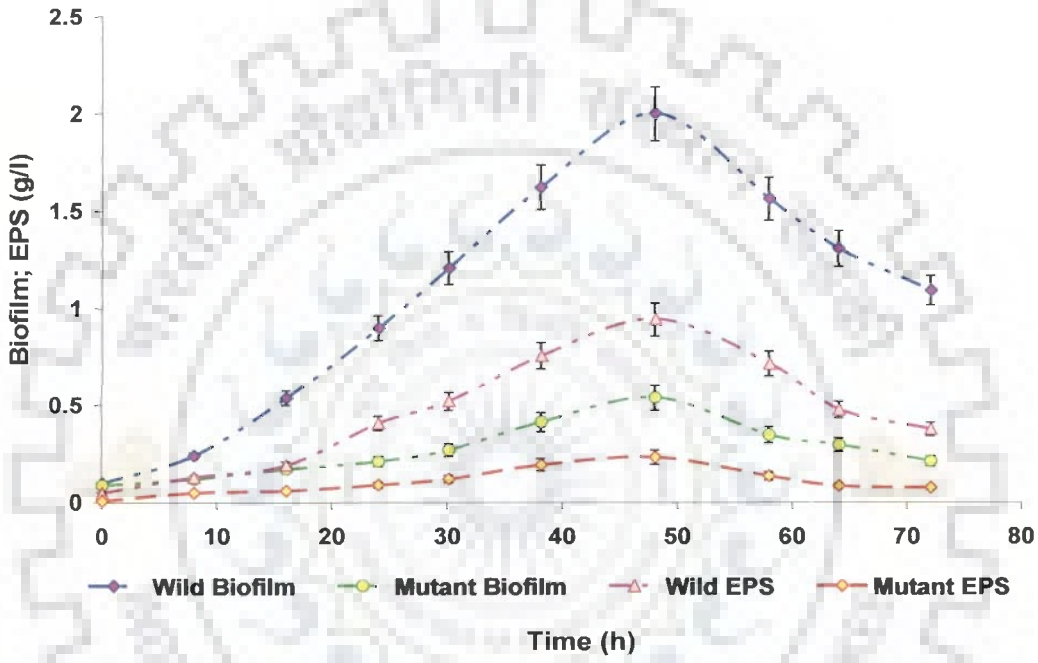


Figure 24. Biofilm formation and EPS production by *C. albicans PLV12* (wild) and Mutant (*pm4*) strains with time.

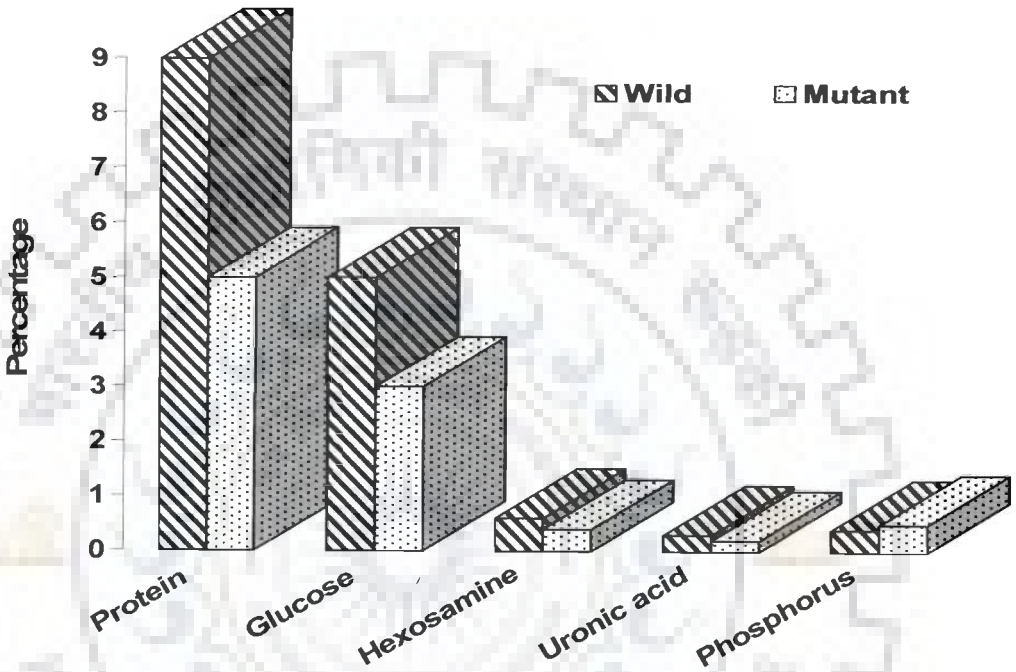


Figure 26. Comparative analysis of EPS composition of Wild and Mutant (*pm4*) strains.

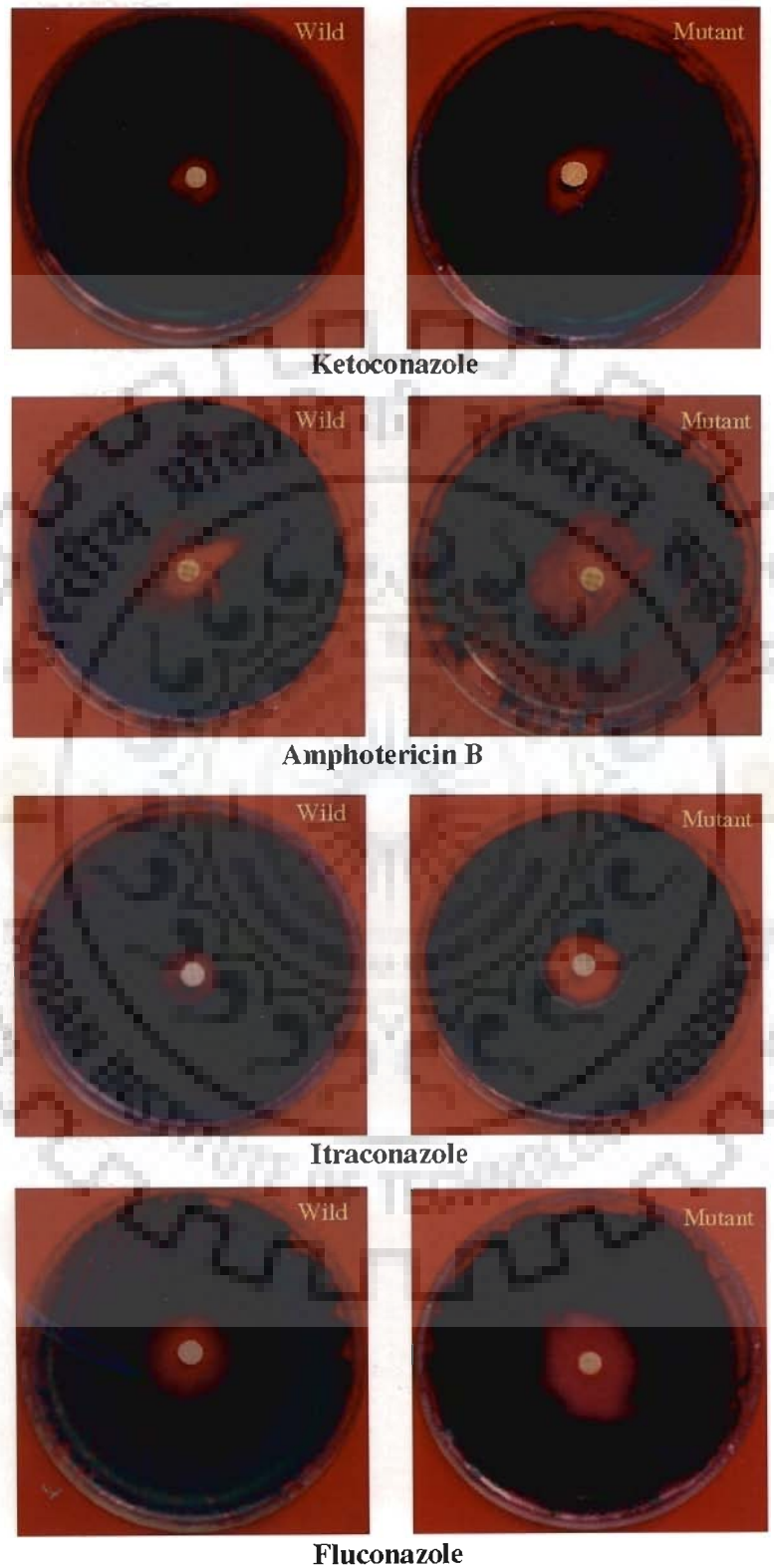


Figure 27. Antifungal susceptibility of *C. albicans* wild (*PLV12*) and mutant (*pm4*) strains on HiChrom Candida agar medium.

Control strategies against *C. albicans PLV12* biofilm and EPS:

4.17 Effect of plant oils on Biofilm formation and EPS yield

Screening of plant oils for anti-Candida activity

Majority of the plant oils were found to be effective and showed anti-candida activity even at very low concentrations. Zone of inhibition (ZOI) for plant oils in response to *C. albicans PLV12* is shown in Table 8. Eucalyptus and peppermint oil resulted in 26.7 and 22.2 mm ZOI, four oils (clove, ginger grass, tea tree, tulsi) showed 10-20 mm ZOI, twelve oils (babchi, castor, coconut, ginger, jasmine, juniper, lavender, mahua, malkangani, musturd, ocimum, rose) showed 1-10 mm ZOI while twelve oils (almond, alsi, babuna, cade, chaulmoogra, jojoba, jyotishmati, khus, neem, til, walnut, wheatgerm oil) were found to be non-effective (Table 8).

Determination of MIC of plant oils

MICs of four effective oils (eucalyptus, peppermint, clove and ginger grass) and fluconazole were determined for *C. albicans PLV12*. The oils exhibited concentration dependent inhibition of growth. Fluconazole at 4.0 µg/ml concentration was able to inhibit total growth of *C. albicans PLV12*. Eucalyptus oil was found to be most effective, 0.05% concentration was enough to completely inhibit growth of *C. albicans PLV12* (Table 9).

Effect of plant oils on biofilm growth and EPS yield

Screened plant oils were further checked against *C. albicans PLV12* biofilm at 5% (v/v) concentration. XTT reduction assay showed that 72 µg/ml concentration of fluconazole (used as control) was able to reduce 78% biofilm whereas 0.84% concentration of Eucalyptus oil

PLANT OILS	BOTANICAL NAME	*ZOI (mm)
Eucalyptus	*Eucalyptus globulus*	26.7± 2.2
Peppermint	*Mentha piperita*	22.2±1.6
Ginger grass	*Cymbopogan martini*	16.0±0
Clove	*Eugenia caryophyllus*	13.8±1.3
Tulsi	*Ocimum sanctum*	11.3±1.2
Tea tree	*Melaleuca alternifolia*	11.0± 0.7
Ocimum	*Ocimum basiliscum*	9.8± 0.8
Castor	*Ricinus communis*	7.8± 0.9
Juniper	*Juniperus chinensis*	5.6±0.5
Malkangni	*C. anthelminticum*	5.3± 0.6
Coconut	*Cocos nucifera*	4.0±0.7
Babchi	*Psoralea corylifolia*	3.4± 0.4
Mahua	*Madhuca indica*	3.2± 0
Ginger	*Z. officinalis*	2.6± 0
Mustard	*Brassica juncea*	2.3± 0
Rose	*R. officinalis*	2.1± 0
Jasmine	*Jasminum nudiflorum*	1.4± 0
Lavender	*Lavandula angustifolia*	1.2± 0
Alsi	*Linum usitatissimum*	0
Neem	*Azadirachta indica*	0
Babuna	*Matricaria chamomilla*	0
Til	*Sesamum indicum*	0
Jyotishmati	*Celastrus paniculata*	0
Jojoba	*Simmondsia chinensis*	0
Walnut	*Juglans regia*	0
Almond	*Prunus glandulosa*	0
Wheatgerm	*Triticum vulgare*	0
Khus	*Vetiveria zizanoides*	0
Cade	*Juniperus oxycedrus*	0
Chaulmoogra	*Taraktogenos kurzli*	0
Control (disc without oil)	-	0
Fluconazole (positive control)	-	24.3± 2.4

*Mean of six replications, diameter of disc = 5 mm, significant at 1% level

Table 8. Screening of plant oils for anti-Candida activity.

PLANT OILS	BIOFILM REDUCTION (%)	CONCENTRATION (% v/v)	MIC VALUE (% v/v)
Eucalyptus	80.87±4.2	0.84	0.05
Peppermint	74.16±3.1	1.16	0.08
Ginger grass	40.46±2.2	1.68	0.08
Clove	28.57±1.8	4.80	0.33
*Fluconazole	78.0 ±3.3	72.0	4.0

*Values in µg/ml

Table 9. MIC values of effective plant oils against *C. albicans PLV12*.

and 1.16% concentration of Peppermint oil gave 80.87% and 74.16% biofilm reduction (Table 9). About 40.46% and 28.57% reduction in biofilm was achieved by ginger grass and clove oils with 1.68% and 4.80% concentrations. Fig. 28 shows that peppermint, eucalyptus, ginger grass and clove oils reduces EPS yield by 93.2%, 94.47%, 92.7% and 91.9% respectively. Other oils did not show any activity against biofilm and EPS even up to 5% (v/v) concentration. It was found that concentration of fluconazole and eucalyptus oil responsible for maximum reduction in biofilm was 18 times and 16.4 times the MIC values respectively i.e. eucalyptus oil was found to be more effective against *C. albicans PLV12* biofilm than fluconazole (Table 9).

Further, visualization of *C. albicans PLV12* biofilm ultrastructure by SEM revealed that major damage to the biofilm constituents was caused by eucalyptus and peppermint oil (Fig. 29B, 29C) compared with the untreated control biofilm (Fig. 29A). This suggests that despite the relative minimal diffusion, these oils compared with other oils may be exerting a metabolic interference in *Candida* biofilm. Results revealed that active components of eucalyptus and peppermint oils have strong potential to affect *C. albicans PLV12* cell growth, function, biofilm formation and development by interfering with any of steps involved in biofilm development.

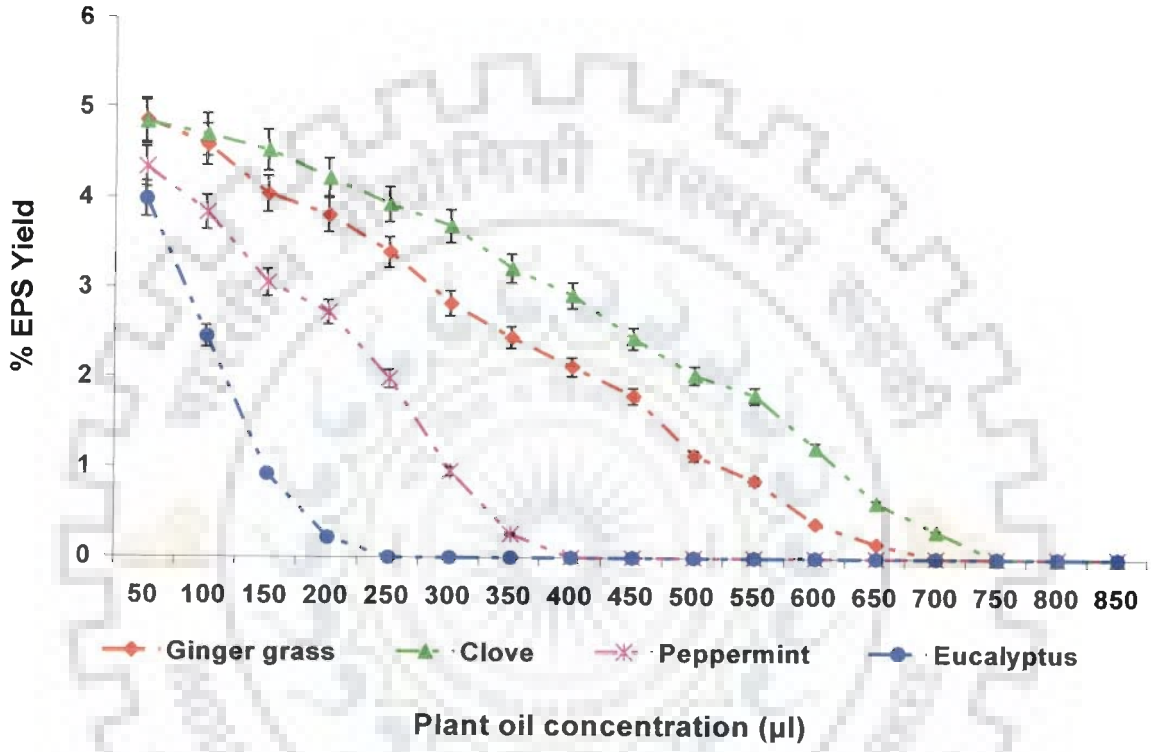
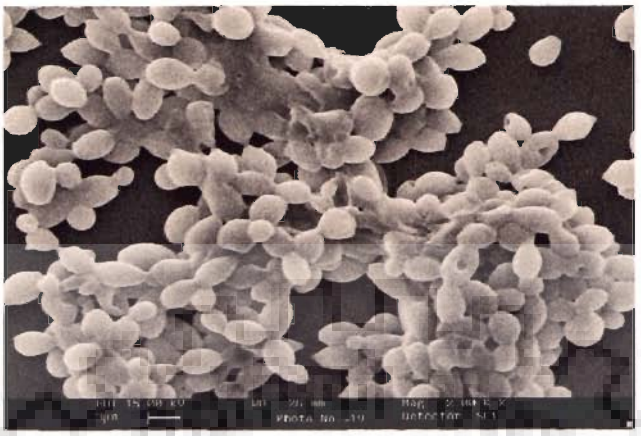
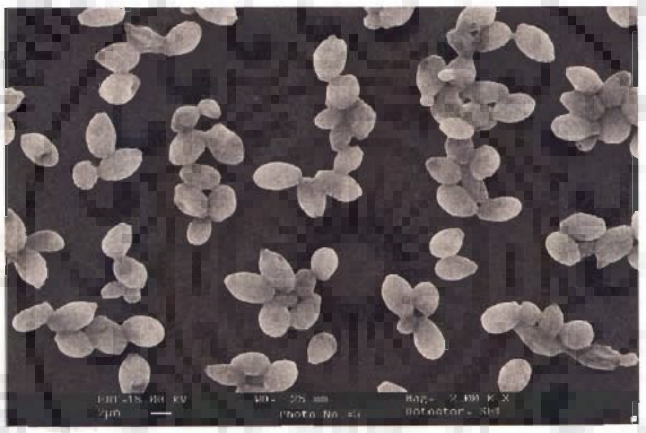


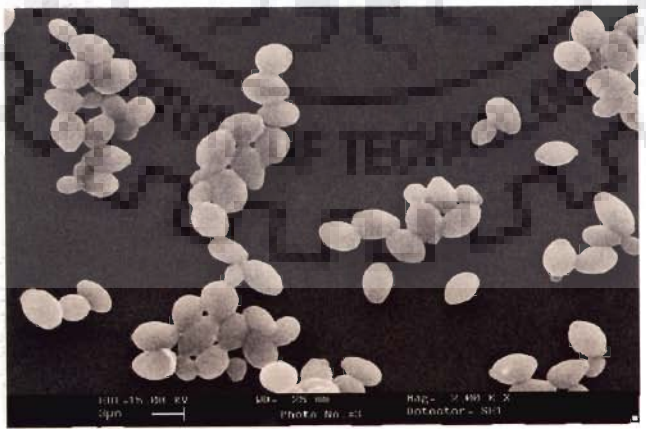
Figure 28. ***C. albicans PLV12*** **EPS yield in response to plant oils.**



(A)



(B)



(C)

Figure 29. Scanning electron micrographs of *C. albicans PLV12* biofilm (A) Control (without oil) (B) Eucalyptus oil treated and (C) Peppermint oil treated at 2.0 KX magnification.

4.18 Effect of 2,4-Dinitrophenol (DNP) on biofilm formation and EPS production

DNP is a metabolic blocking agent that uncouples oxidative phosphorylation of carbohydrate by carrying protons across the mitochondrial membrane, leading to a rapid consumption of energy without generation of ATP. To monitor effect of DNP on biofilm formation and EPS production, *C. albicans PLV12* was grown in chemically defined medium containing $5x10^{-4}$ M (MIC) of DNP used as a sole carbon source. Analysis of PVC pieces showed that in control experiment, maximum biofilm formation and EPS production (5.44 mg/cm^2) were observed after 48 h. On the other hand, 35% reduction in biofilm formation and 66.3% reduction in EPS yield were achieved in response to DNP (Fig. 30).

Effect of DNP on ultrastructure of *C. albicans PLV12* biofilm after 48 h was observed using SEM analysis. Images revealed that DNP not only caused considerable decrease in cell aggregation but also demonstrated a degree of changes in the cell morphology (Fig. 31B) in comparison to the control biofilm (Fig. 31A).

4.19 Effect of Biosurfactant on Biofilm and EPS production

To evaluate the effect of biosurfactant (rhamnolipid) produced by *Pseudomonas aeruginosa* on *C. albicans PLV12* biofilm, different concentrations of biosurfactant from 5 to 30 mg/ml were added to preformed biofilm on MTP. It was observed that biosurfactant showed a high antimicrobial activity against *C. albicans PLV12* at 15 mg/ml concentration which was sufficient to inhibit 78.6% of biofilm growth (Fig. 32). About 66.79% reduction in EPS yield showed that biosurfactant not only interferes with the adhesion of *C. albicans* onto MTP surface but also has the ability to reduce EPS production. Results suggest that biosurfactant could act directly on the *C. albicans PLV12* biofilm matrix to disrupt and solubilize its components, perhaps even incorporating the matrix into micelles.

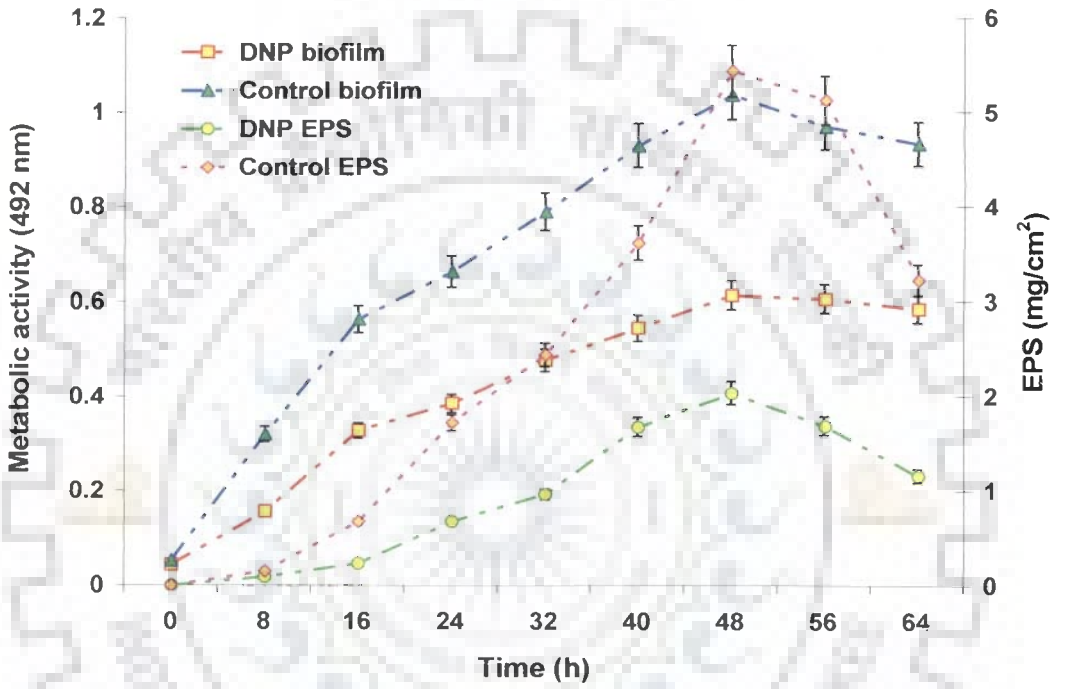
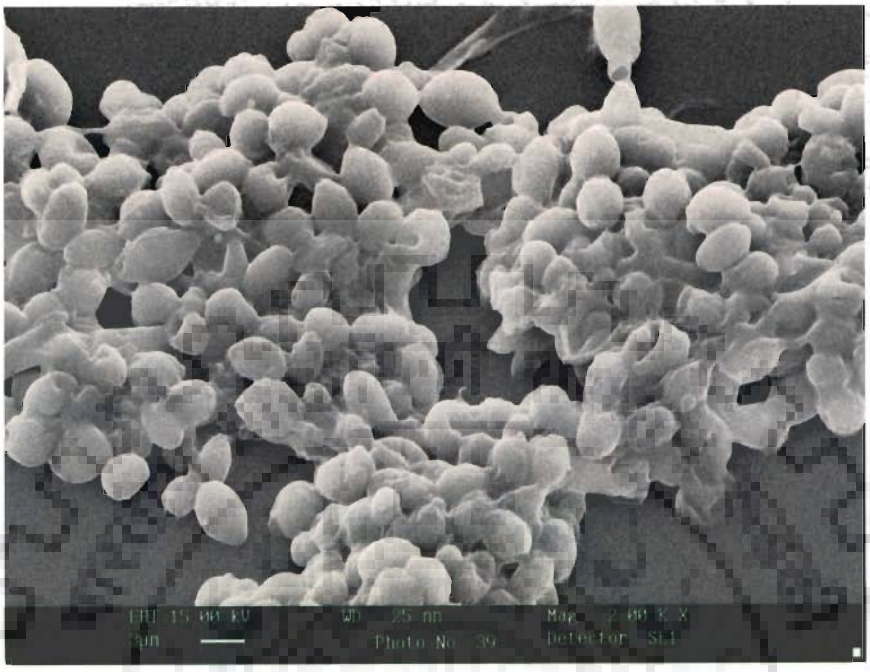
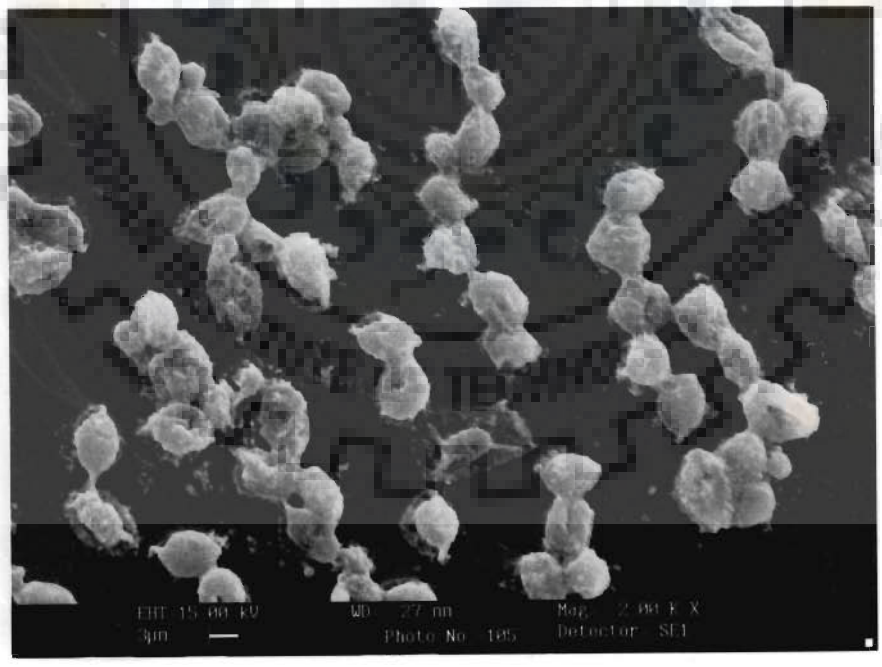


Figure 30. ***C. albicans PLV12*** **biofilm formation with and without DNP (control) at different incubation period.**



(A)



(B)

Figure 31. SEM images of *C. albicans PLV12* biofilm: (A) Control biofilm (B) DNP treated biofilm.

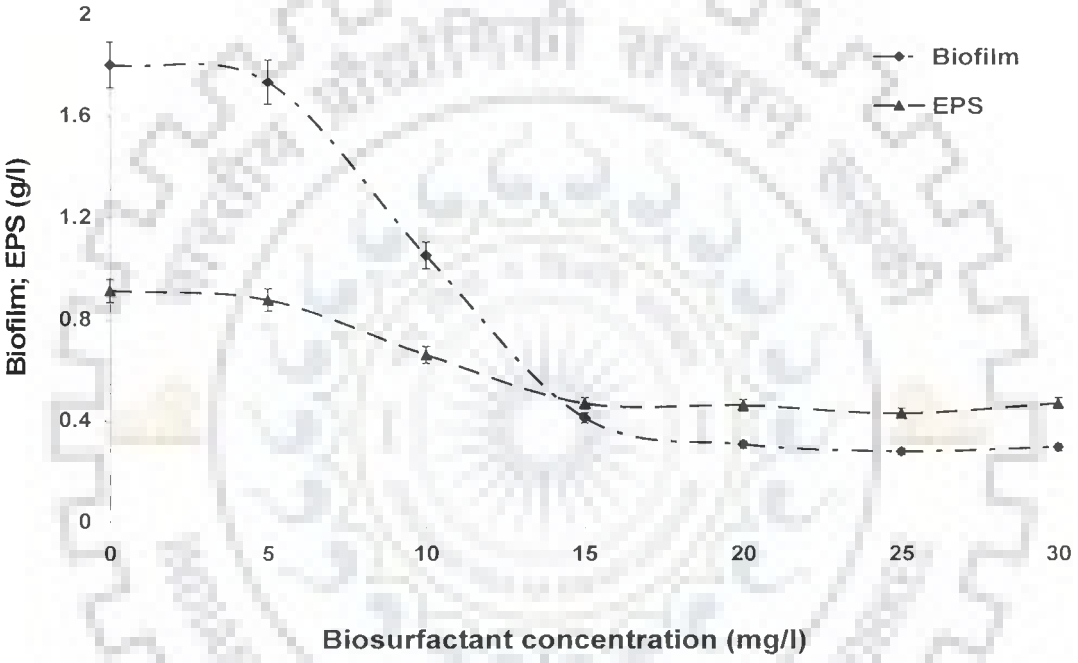


Figure 32. Effect of different concentrations of biosurfactant on *C. albicans PLV12* biofilm and EPS.

4.20 Effect of enzymes on Biofilm and EPS production

As a large proportion of the biofilm matrix is composed of polysaccharides, proteins, lipids and sugar derivatives secreted by the constituted microorganisms, the presence of enzymes acting on these polymers will inevitably have a marked effect on the structure and on the integrity of the biofilm. Seven enzymes namely Chitinase, Lyticase, Alginate lyase, Cellulase, Proteinase K, Deoxyribonuclease 1 (DNase 1) and Lipase were used to assess their effect on biofilm formation and EPS production in *C. albicans PLV12*. It was observed that *C. albicans PLV12* biofilms were unaffected by Lipase enzyme (Table 10). Treatment with Lyticase, Alginate lyase, Cellulase, Chitinase, Proteinase K, and DNase 1 resulted in a significant decrease in metabolic activity, suggesting that these enzymes partially degraded matrix material and caused some biofilm detachment from the surfaces of the MTP. Interestingly, Lyticase had by far the greatest effect, causing about 82% reduction in metabolic activity measured at OD_{492}. By contrast, Chitinase showed 27.8% reduction in biofilm formation thereby suggesting that most of the hexosamine present in *C. albicans PLV12* matrix unlikely to be in the form of chitin. DNase 1 also caused some degree of detachment in *C. albicans PLV12* biofilm.

ENZYME	BIOFILM REDUCTION
Lyticase	82.0%
Alginate Lyase	71.1%
Cellulase	65.3%
DNase I	35.5%
Proteinase K	32.4%
Chitinase	27.8%
Lipase	8.9%

Table 10. Detachment of *C. albicans PLV12* biofilms after exposure to different enzymes.

4.21 Effect of Bismuth dimercaprol on EPS production and biofilm formation

Bismuth dimercaprol, prepared by dissolving bismuth nitrate in dimercaprol, 1:1.6 ratio was used to check the effect on *C. albicans PLV12* EPS production and biofilm formation. Results showed that for concentrations <13.5 μM, the viable cell counts increased at least 1-log while cell counts for those treated with >13.5 μM did not changed significantly. Therefore, 13.5 μM was determined as the MIC of bismuth dimercaprol for *C. albicans PLV12*. The decrease in *C. albicans PLV12* metabolic activity in response to MIC of bismuth dimercaprol is shown in Fig. 33. In control experiment, biofilm density increased significantly during the first 48 h then maintained a steady state thereafter. For the bismuth dimercaprol treated biofilm, biofilm density increased initially with time but started to decrease after 40 h of treatment. A 3-log reduction was observed in comparison with the untreated control at 80 h.

Reduction in viable cell count was also checked using fluorescent viability test using two fluorescent dyes, FDA and EtBr. In control biofilms, only green coloration appeared due to absence of dead cells (Fig. 34A), viable cells showed well defined intense green fluorescence in areas such as the cell wall and vacuolar region that may be due to acetylesterase activity (Medzon and Brady, 1969). Consequently it was assumed that structures containing these enzymes showed intense green fluorescence. It was also noticed that fluorescence was concentrated at one point inside the viable cells and that the surrounding intracellular components showed a weak fluorescent staining. In case of Bismuth dimercaprol treated biofilms (Fig. 34B), reduction in viable cell count (green colored cell) was clearly observed with dead cells depicting a bright light red fluorescence.

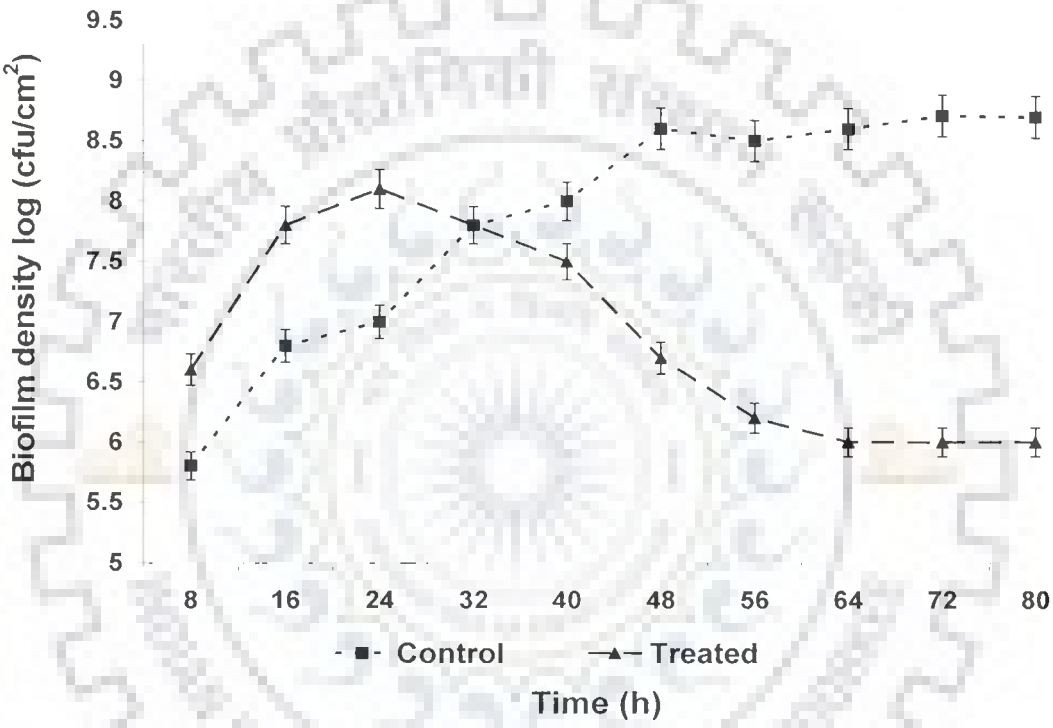
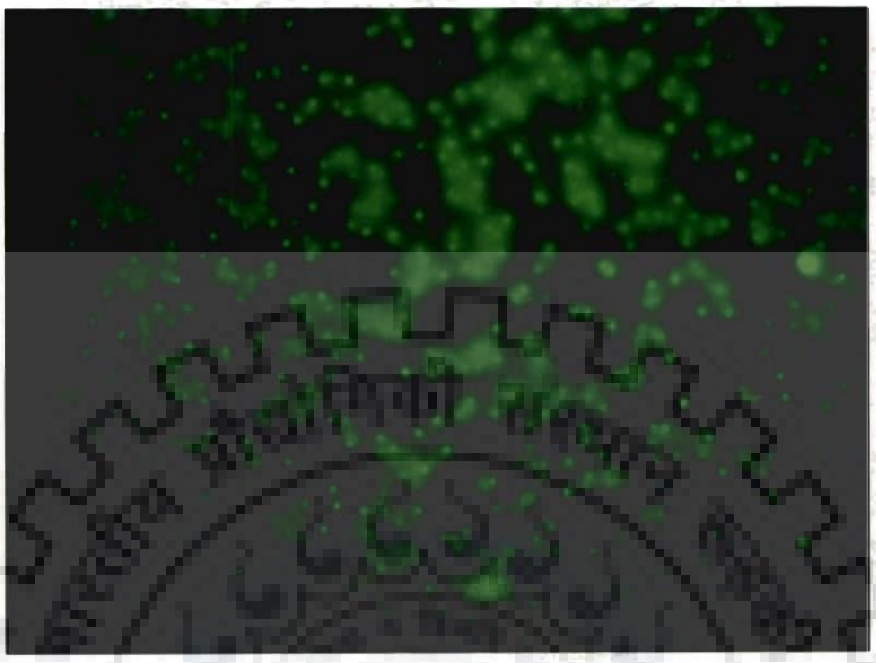


Figure 33. ***C. albicans PLV12*** **biofilm density in response to 13.5 µM Bismuth dimercaprol treatment at different time period.**

Fig. 35 shows EPS production by *C. albicans PLV12* with and without Bismuth dimercaprol treatment. Analysis of PVC pieces in control experiment showed that EPS yield increases with time and reached 24 mg/cm^2 after 80 h of incubation. With exposure to 13.5 μM bismuth dimercaprol, EPS production increased for the first 24 h, then continued to decrease and after 80 h, 1.5 mg/cm^2 EPS was obtained.



(A)

(B)

Figure 34. Fluorescent micrographs of *C. albicans PLV12* biofilm showing viable (green) and dead (red) cells in (B) Control (B) and Bismuth dimercaprol treated biofilms.

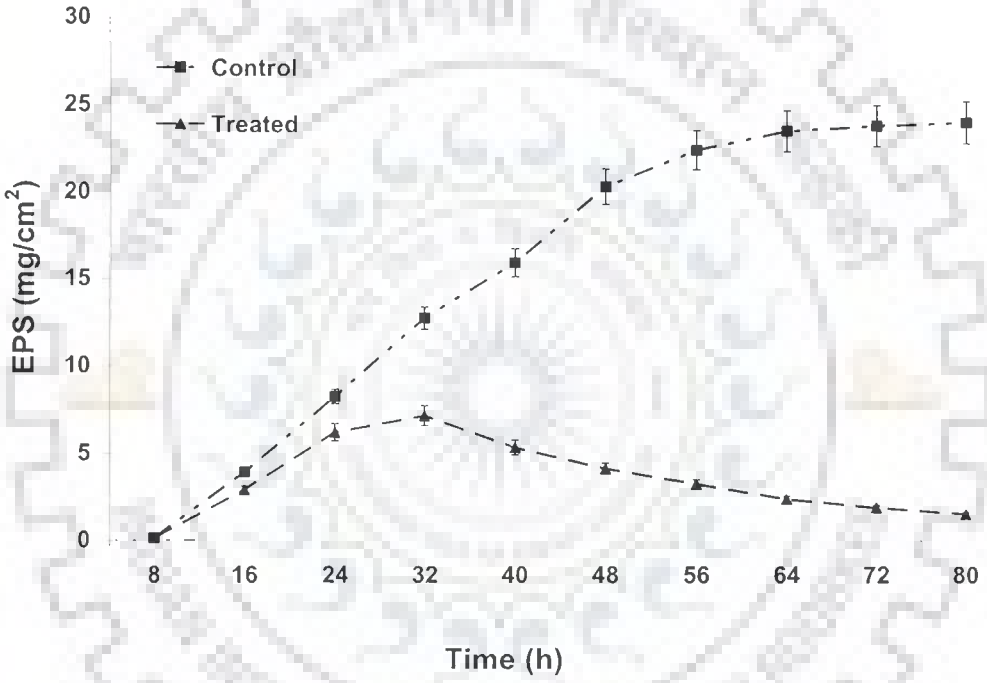


Figure 35. Effect of MIC of Bismuth dimercaprol on *C. albicans PLV12* EPS production at different time period.

4.22 Susceptibility testing of *C. albicans PLV12* against antifungal agents

The susceptibility of *C. albicans PLV12* against antifungal agents and the role of EPS matrix in the resistance of *C. albicans PLV12* to antifungal agents was performed using antifungal agents: Fluconazole, Amphotericin B, Itraconazole, Ketoconazole and Flucytosine. The MICs of antifungals against *C. albicans PLV12* biofilms using NCCLS M-27A broth microdilution method is shown in Table 11. Our data revealed that Ketoconazole, Itraconazole, Flucytosine and Fluconazole were not effective even in excess of their MICs. Only Amphotericin B was able to inhibit 62% of biofilms at concentration around 1.2 µg/ml (MIC; Fig. 36). In the similar manner, EPS yield did not changed significantly with different concentrations of antifungals (Fig. 37). Results suggest that matrix formation did not affect the susceptibility of biofilms to antifungal agents and drug resistance is unrelated to the extent of matrix formation in *C. albicans PLV12*.

DRUG	MIC (μg/ml)
Fluconazole	0.4
Itraconazole	≤0.025
Amphotericin B	1.2
Flucytosine	0.3
Ketoconazole	≤0.025

Table 11. Antifungal susceptibility of *C. albicans PLV12* biofilms by NCCLS broth microdilution method after 48 h of incubation on 96-well MTP.

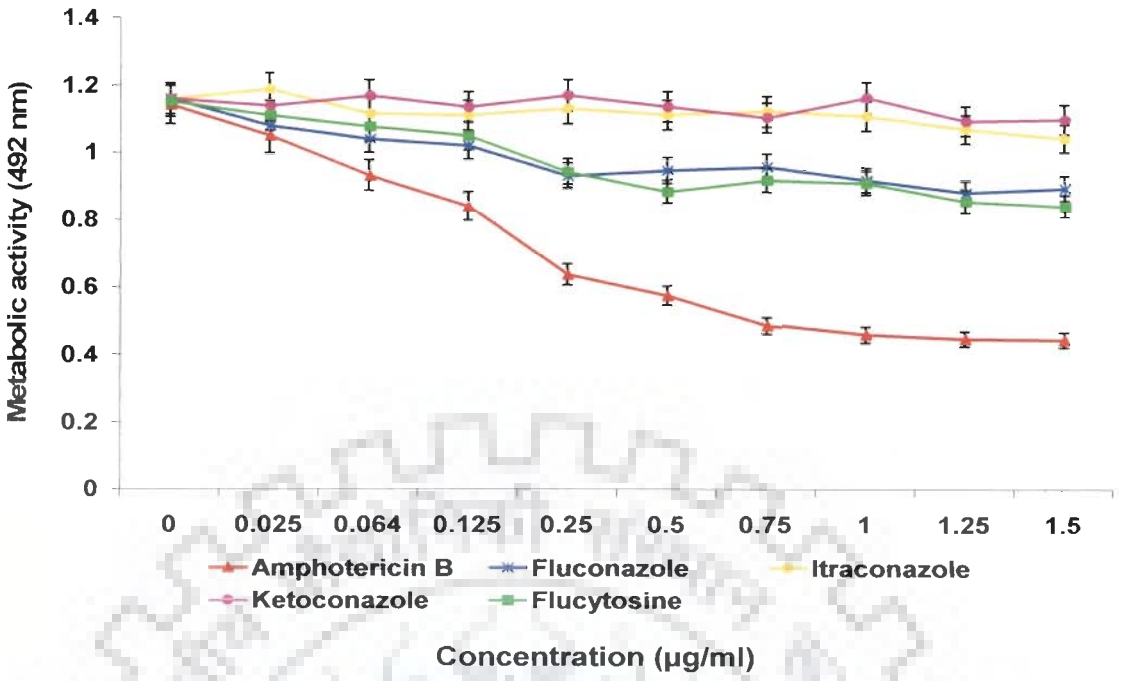


Figure 36. Effect of different concentrations of antifungal drugs on *C. albicans PLV12* biofilm formation.

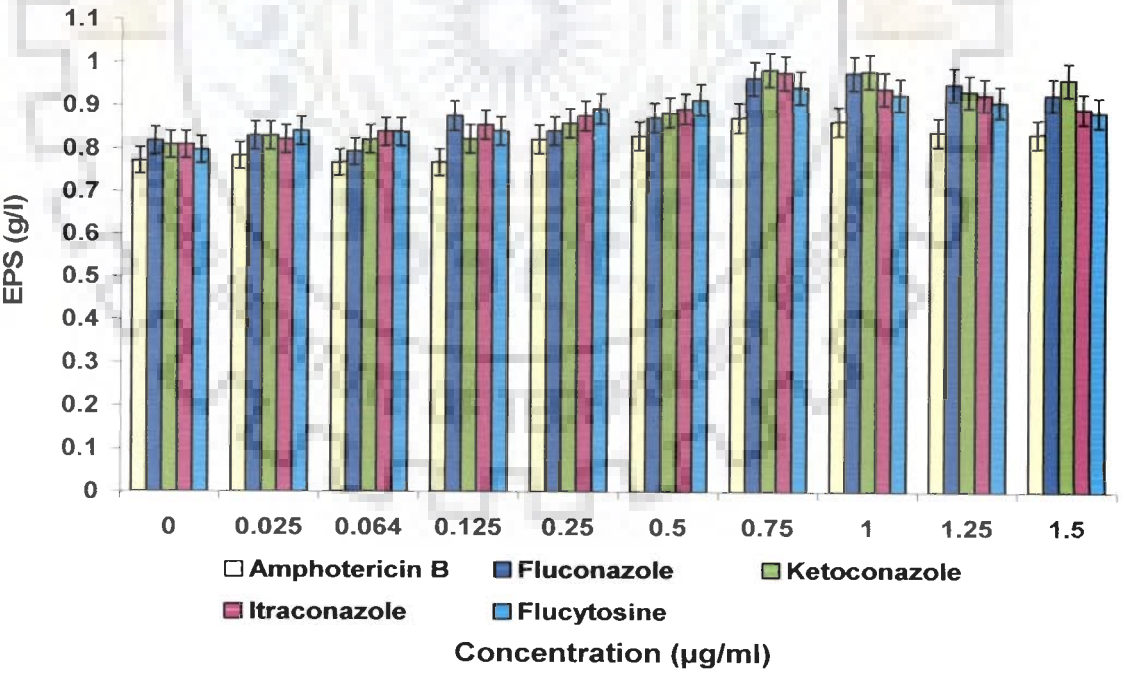


Figure 37. Effect of different concentrations of antifungal drugs on *C. albicans PLV12* EPS yield.

CHAPTER 5

DISCUSSION

Amongst the 150 known human fungal pathogens, *Candida* species are the only yeasts that form part of the normal microbiota of humans which facilitates their encounter with most implanted biomaterials and host surfaces (Ahidjo *et al.*, 2008; Dominic *et al.*, 2007). Despite reports that a wider range of species are now being implicated as causes of medical implant infection, most cases of infections are still due to *Candida* species (Inabo, 2006; Dominic *et al.*, 2007; Jabra-Rizk, 2004; Kojic and Darouiche, 2004). Therefore, accurate identification of *Candida* species is crucial for successful clinical management and effective therapeutic strategies. Several methods are available for the presumptive identification and differentiation of *Candida* species. Out of which, use of differential medium like Hichrom Candida agar allows selective identification of *Candida* species based on color reaction and colony morphology with high degree of accuracy in mixed cultures from clinical samples (Odds, 1979). The selective action relies on the specific hydrolysis of a chromogenic substrate by the enzyme N-acetyl-β-D-glucosaminidase secreted by microorganisms producing colonies with various pigmentations (Bauters *et al.*, 1999). This hydrolysis results in the different coloration of *Candida* species. Hichrom Candida agar media not only facilitates the detection and identification of *C. albicans* from mixed cultures but also provides results 24 to 48 h sooner than standard isolation and identification procedure. In the present studies, thirty nine isolates of *Candida* species procured from clinical samples were analyzed using Hichrom selection medium. On selective medium, *C. albicans*, *C. tropicalis*, *C. gulier, C. glabrata* and *C. krusei* appeared as green, light green, yellowish, cottony white and pink colored colonies respectively. In a similar study, Cooke *et al.*, 2002, found Chromogenic agar medium to be useful in presumptive identification of *Candida* sp. from clinical specimens, although there was variation in the range of species differentiated and in

the sensitivity and specificity for target groups.

Colorimetric assays to discriminate between living and dead cells based on the metabolic activity of viable cells are important tools in the study of eukaryotic cell activity. Various viability stains involve the use of Tetrazolium salts, including 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) and 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) (Peeters *et al.*, 2008; McCluskey *et al.*, 2005; Gabrielson *et al.*, 2002). Out of these, the XTT assay has been used extensively for the quantification of viable cells in planktonic cultures (Gabrielson *et al.*, 2002) and yeast biofilms (Honraet *et al.*, 2005). Use of this assay constitutes a new, simple, rapid and inexpensive method of assaying viability of *C. albicans* in biofilm systems. The XTT assay is based on the mitochondrial dehydrogenase activity of live, metabolically active cells that cleaved the tetrazolium ring of the yellow colored XTT salt to produce its brown formazan water-soluble derivative (Kuhn *et al.*, 2003). The color change during the reaction reflects the number of viable cells, and can be assessed visually at 492 nm using either a microtiter plate reader or a spectrophotometer (Kuhn *et al.*, 2002; Hawser, 1996; Tellier *et al.*, 1992). Despite its popularity, problems regarding intra- and interspecies variability have been reported by some workers (Honraet *et al.*, 2005; Kuhn *et al.*, 2003).

The XTT reduction assay was successfully utilized in the study for selecting biofilm forming *C. albicans* strains among clinical isolates. Our studies revealed that biofilm formation by the isolate, *C. albicans PLV12*, has three developmental phases: adherence of candidal cells to the PVC surface (early phase), formation of a matrix with dimorphic switching from yeast to hyphal forms (intermediate phase) and increase in the matrix

material taking on a three-dimensional architecture (maturation phase). The fully mature *C. albicans PLV12* biofilms had a mixture of morphological forms and consist of a dense network of yeasts, hyphae, and pseudohyphae in a matrix of polysaccharides, protein, and unknown components. Similar three phases were observed by Chandra *et al.*, 2001, for two clinically relevant *C. albicans* biofilm models formed on bioprosthetic materials. Similar distinct phases have also been reported for biofilm formation by other such groups (Davey and O'Toole, 2000; Watnick and Kolter, 2000). Thus, microorganisms appear to share common basic steps during biofilm formation. These growth phases later transformed adherent blastospores to well-defined cellular communities encased in a polysaccharide matrix. Ramage *et al.*, 2001, described a linear relationship between cellular density of the biofilm and metabolic activity for *C. albicans* biofilm using XTT assay.

Biofilm matrix is one of the most distinctive features of a microbial biofilm. It forms a three-dimensional, gel-like, highly hydrated and locally charged environment in which the micro-organisms are largely immobilized (Flemming *et al.*, 2007; Al-Fattani and Douglas, 2006). The composition of EPS varies according to the nature of the organisms present. Quantitative analysis of *C. albicans PLV12* EPS showed that major part of it consists of carbohydrate along with protein, phosphorus and extracellular DNA during both planktonic as well as biofilm mode of growth (Lal *et al.*, 2008). The carbohydrate part, commonly referred as 'exopolysaccharide' is regarded as the major structural component of the matrix and provides a framework to the biofilm complex. The content of which is found to reduce in biofilm mode while an increase in glucose, phosphorus and hexosamine was noticed in the study. These values largely confirm those reported by Al-Fattani and Douglas, 2006, who also reported higher percentage of glucose (32.2%), phosphorus (0.5%) and hexosamine

(3.3%) in biofilm matrix along with reduction in total carbohydrate (39.6%) content. Our studies in accordance with Al-Fattani and Douglas, 2006; Ballie and Douglas, 2000, demonstrated that glucose is the major sugar present component of *C. albicans* matrix material. Jabra-Rizk *et al.*, 2004, suggested that *C. albicans* might produce biofilm-specific EPS by differentially regulating genes encoding enzymes involved in carbohydrate synthesis (Chandra *et al.*, 2001; Baillie and Douglas, 2000). The presence of extracellular DNA in the *C. albicans PLV12* matrix can presumed to be derived from lysed candidal cells and possibly be required for the initial establishment of biofilm as proposed by Whitchurch *et al.*, 2002, for *P. aeruginosa* biofilms. Proteins on the other hand, are the structural components, which play major role as enzymes performing digestion of exogenous macromolecules and particulate material in the microenvironment of the immobilized cells (Dignac *et al.*, 1998). They are supposed to be more involved than sugars in electrostatic bonds with multivalent cations, revealing their role in the floc structure within the EPS matrix as suggested by the studies of Dignac *et al.*, 1998.

In the present studies, the increased magnification and resolution power associated with the SEM technique permitted a more detailed examination of *C. albicans PLV12* biofilm. The mature biofilms consist of a mixture of yeast and filamentous forms embedded within exopolymeric material (Lal *et al.*, 2008). Similar observations were noted by Ramage *et al.*, 2001, for *C. albicans* 3153A biofilm. Previous studies by Hawser *et al.*, 1998, also described the production of extracellular matrix polymers by *C. albicans* biofilm under static and shaking incubation.

The application of confocal laser scanning microscopy combined with fluorescent

stains provided an important and effective tool to analyze the composition and structure chemistry of hydrated *C. albicans PLV12* biofilms *in situ*, non-destructively and in real time. The major advantage of using CLSM is the possibility of non-destructive, three-dimensional, optical sectioning of fully hydrated living biofilm systems (Lawrence and Neu, 1999). The *in situ* analysis of EPS in the studies relied on probes (FITC-ConA and PI) which are more or less specific for representative EPS molecules or characteristic chemical groups of EPS. FITC-ConA is known to selectively bind to the mannose and glucose residues of biofilm polysaccharides. PI is a fluorescent nucleic-acid stain that enters dead/nonviable by binding to double-stranded nucleic acids through intercalation between base pairs. The combined effects of FITC-ConA and PI stained images of *C. albicans PLV12* biofilm showed that EPS is produced both in cell bound as well as secreted form (Lal *et al.*, 2008). CLSM suggested that *C. albicans PLV12* extracellular material is predominantly composed of cell wall-like polysaccharides containing mannose and glucose residues, based on staining with dyes that specifically bind these carbohydrates. Analysis also demonstrated that *C. albicans PLV12* possess structural heterogeneity and display a typical micro-colony/water channel architecture similar to that of bacterial biofilms (Watnick and Kolter, 2000). Our results inferred that CLSM coupled with fluorescent stains could be successfully used to probe viability and extracellular matrix within *C. albicans PLV12* biofilms.

In the past few years, Atomic Force Microscopy (AFM) has been used to image film morphologies and probe surface properties like ligand and receptor interactions and viscoelasticity (Ahimou *et al.*, 2007). It can provide real-time *in situ* quantitative morphological information of biological samples as well as measure the interaction forces between a sharp tip and the surface of interest (Mendez-Vilas *et al.*, 2007). Furthermore, as

no stains or coatings are needed in this method, biofilms may be observed *in situ*. AFM is also a useful tool to predict the potential for microorganism to attach and form biofilms on surfaces, without the time-consuming sample preparations and tedious counting required for SEM (Arnold and Bailey, 2000). In addition, proteins and other cell-surface features, which may be involved in biofilm development, can be studied. Fang *et al.*, 2000, studied the adhesion forces and cell elasticities of biofilm-forming sulphur-reducing bacteria on mica surfaces using atomic force microscopy while Nunez *et al.*, 2005, successfully explored the interactions and predation by *Bdellovibrio bacteriovorus* on *E. coli* biofilms. Within the medical context, atomic force microscopy has been used to observe the effect of modified catheter surfaces on bacterial biofilm development (Lindsay and Holy, 2006). Emerson and Camesano, 2004, investigated pathogenic microbial adhesion to biomaterials by measuring the local interaction forces between an immobilized cell and both biomaterial and biofilm surfaces. All of these studies and measurements provide important information on single-cell properties; nevertheless, they do not provide information on the properties of whole biofilms. The AFM-based methodology was shown to be sensitive enough to dissect the effect of subtle changes in overall *C. albicans PLV12* cell surface composition on the initial interaction with biomaterials. Although, AFM did not revealed the detailed *C. albicans PLV12* biofilm structure but it potentially depicted the structural surface information regarding EPS "blanket" surrounding *Candida* cells and its biofilm (Lal *et al.*, 2008). In future, AFM might also be used to determine the importance of surface morphology and chemistry on the diversity of pathogenic microbes associated with surface biofilms.

Synthesis of extracellular matrix material (EPS) during the formation of *C. albicans PLV12* biofilms is highly dependent on the conditions of incubation. SEM analysis showed

that biofilms formed by *C. albicans PLV12* incubated statically on PVC surfaces consisted of a dense network of yeasts, germ tubes, hyphae and pseudohyphae. As reported previously by Hawser *et al.*, 1998, relatively little matrix material was visible in these biofilms, even when samples were prepared using a freeze-drying technique that gives improved preservation of the matrix. Our findings of statically grown *C. albicans PLV12* biofilms as revealed by SEM were in accordance with the observations of Hawser *et al.*, 1998. Both studies showed a dense network of matrix material around candidal cells after 48 h of incubation. By contrast, biofilms grown under gentle shaking showed marked increase in the synthesis of extracellular matrix material due to wettability and hydrophobicity of the surface while shear forces generated at higher speeds tend to inhibit matrix formation completely. These observations confirmed earlier finding which demonstrated that biofilms subjected to a liquid flow produces substantially more matrix material than those incubated statically (Hawser *et al.*, 1998). At high magnification, matrix material was clearly visible on the surface of the cells.

A range of environmental factors can influence adhesion properties, either through their effect on the physiology of the organism or on the physiochemistry of the adhesion interaction (Fridkin and Jarvis, 1996). The pH and temperature of culture broth is one of the most critical environmental parameters affecting growth and biosynthesis of EPS. However, the effect of pH and temperature on the biosynthesis of EPS and cell growth varies with different microorganisms, operational conditions and medium composition (McCourtie and Douglas, 1981). Our studies revealed that pH values (6.3) close to neutral pH are best suited for *C. albicans PLV12* EPS production. Dumitriu, 1998, reported that the control of pH values close to 6.0 can result in increased EPS production. Also, it was observed that an

incubation temperature (30°C) below the optimum growth temperature (35°C) resulted in greater *C. albicans PLV12* EPS yield. Since temperature values below 30°C resulted in lower growth rate and cell mass than at a higher temperature, long logarithmic phases of growth were promoted at low temperatures. Moreover, low cell mass might be associated with greater EPS production, because when cells grow slowly, the synthesis of cell wall polymer is slower and more isoprenoid phosphate is available for EPS synthesis (Dumitriu, 1998). Dumitriu, 1998, suggested that the difference between optimum growth temperature and optimal temperature for EPS production could be a result of increased activities of enzymes involved in the synthesis of EPS precursors.

The structure and physical properties of microbial EPS also depend on the culture medium composition and the growth conditions. Different biosynthetic pathways work in a coordinated manner and determine differential EPS composition and hence structural and functional variability of biofilm. These biosynthetic pathways get affected by carbon sources which either work as a substrate and/or inducer for transcriptional activation of genes responsible for EPS synthesis which result in variable composition of EPS. The carbon source used for growth determines both the quality and quantity of polysaccharide formation (Royan *et al.*, 1999). Sugar nucleotides play an important role in the EPS synthesis as activated precursors (Fett *et al.* 1995). Studies concerning effect of growth-limiting substrates on EPS synthesis clearly demonstrated that the growth medium composition can dramatically affect the specific rate of matrix polymer synthesis (Lacroix *et al.*, 1995; Heald and Kristiansen, 1985). The amount of carbon substrate converted to polymer by the microbial cells depends on the growth medium composition. Generally, media containing a high carbon-to-limiting-nutrient ratio are favoured for polysaccharide production

(Sutherland, 1998). During our investigations to find a suitable carbon source for *C. albicans PLV12* growth, we found that best biofilm and EPS recoveries were obtained with arabinose as a carbon source. Previous findings by Bahat-Samet *et al.*, 2004 and Burdman *et al.*, 2000, on a free living nitrogen fixing bacterium, *Azospirillum brasilense,* suggested that arabinose plays an important role in cell aggregation, attachment and colonization processes.

The understanding of microbial biofilms has advanced enormously in recent years. A substantial contribution to this understanding has come from the identification and analysis of biofilm-defective mutants. Such studies have defined roles for adherence, motility, and extracellular matrix materials in biofilm development (Davey and O'Toole, 2000; O'Toole *et al.*, 2000). The genetic characterization of random mutants is a challenge with asexual diploids like *C. albicans;* however, the *C. albicans* genomic sequence has provided a platform for molecular gene disruption technologies that cause partial or complete gene function defects (Richard *et al.*, 2005). In the present investigations, we attempt to isolate *C. albicans PLV12* mutants defective in EPS production by random mutagenesis approach. After 3% EMS treatment, we obtained 19 *C. albicans PLV12* mutants having altered colony morphology with more than 50% reduction in biofilm forming ability. Out of which, a selected mutant strain (*pm4*) showed distinctly lower levels of EPS production (0.23 g/l) with major reduction in total carbohydrate (38%), protein (5%) and glucose (3%) content. On SEM analysis the *pm4* showed lesser ability to adhere onto PVC surfaces in contrast to wild strain which exhibited more robust biofilm formation covering the entire surface of the PVC with a dense mass of Candidal colonies. Further, the mutant strain was found to be more susceptibility towards antifungal agents compared with the wild strain.

Till date, very little is known about the chemical nature of EPS produced by *Candida albicans*. So in order to gain a refined knowledge, characterization was done using chromatographic techniques. Chemical analysis of *C. albicans PLV12* EPS showed that major part of it comprises of carbohydrates containing both negatively (D-glucuronic acid) and positively (N-acetylglucosamine) charged components as revealed by ion exchange chromatography. Gel permeation chromatography determined that *C. albicans PLV12* produces only one class of exopolysaccharide with a molecular weight around 300 KD. Based on the results of the GC and HPLC analysis, it was concluded that the major monosaccharides for this EPS were glucose, mannose, rhamnose, galactose, N-acetylglucosamine and D-glucuronic acid. The remainder of the peaks could probably be fragments of partially hydrolyzed EPS or by-product of hydrolysis reaction (Dlamini *et al.*, 2007). A limited number of microorganisms have been reported to produce polysaccharides that yield rhamnose upon hydrolysis. For example, *P. elodea* produces gellan which has a repeat unit containing rhamnose and β-(1-3)-D-glucose (O'Neil *et al.*, 1983). *Acinetobacter calcoaceticus* produces a polysaccharide containing rhamnose, glucose and mannose (Morin *et al.*, 1987; Kaplan and Rosenberg, 1982). *Klebsiella sp.* produces a polysaccharide that contains rhamnose, galactose and glucuronic acid (Morin and Monsan, 1990). The tentative analysis of the *C. albicans PLV12* EPS has indicated that it possesses a unique composition. However, further work is required to confirm the primary structure and the significance of the sugars that constitute the EPS produced by *C. albicans PLV12.*

FTIR analysis of *C. albicans PLV12* EPS exhibited characteristic peaks for proteins, nucleic acid, and carbohydrates. The spectral profile in 800-1200 cm^{-1} region mainly reflected the absorption of sugars present in the *C. albicans PLV12* exopolysaccharide. The

component bands that are disclosed concern mainly β-glucans at 992, 890 cm^{-1} (β (1→6)) and 1067, 1017 cm^{-1} (β (1→3)); and mannans at 954, 863 and 818 cm^{-1}. Studies showed that FTIR spectroscopy affords a rapid and easy means of obtaining an indication of the nature of the major components of *C. albicans PLV12* EPS.

The 1D ^{1}H-NMR spectra of *C. albicans PLV12* EPS in D_2O showed two distinguishable groups of signals. The bulk signal contained mainly the non-anomeric protons, present in the narrow range from 3.5 to 4.2 ppm. The proton signal assignment in this region was hampered by signal overlap. Outside the bulk area several well-resolved signals were found in the anomeric region (4.3 to 6.0 ppm). Investigation of anomeric proton (H1) signals in this region indicated presence of pyranose ring in α- or β- configuration. Based on NMR spectra of carbohydrates, these peaks (4.944, 4.995, 5.017, 5.041 and 5.196 ppm) were assigned to α- and β-D-glucose, α-D-mannose and α-L-rhamnose sugars. The presence of N-acetyl glucosamine (β-D-GlcNAc) was also detected at 2.119 ppm in the ^{1}H spectrum. The ^{13}C-NMR data of *C.albicans PLV12* EPS showed six carbon signals. The chemical shifts indicated presence of α-L-rhamnose, α- and β-D-glucose in the exopolysaccharide chain. Importantly, results concerning ^{1}H and ^{13}C-NMR data were found to be consistent with FTIR data. Our studies predicted that *C. albicans PLV12* produces a heteropolysaccharide made up of four different monosaccharide units namely glucose, mannose, rhamnose and N-acetyl glucosamine. The exopolysaccharide structure of *C. albicans PLV12* was found to be somewhat similar to the exopolysaccharide formed by *Staphylococcus aureus*. Both the organisms are important biofilm colonizers on medically implanted devices and their exopolysaccharides contains glucose and hexosamine as major sugar units (Joyce *et al.*, 2003). Faillie *et al.*, 1992, utilized ^{1}H and ^{13}C NMR spectroscopy

for determining complete structure of *C. albicans* cell wall phosphopeptidomannans and found that it was composed of repeating units of β-mannose. The prediction of β-D-glucans moieties in the cell wall of *C. albicans* was also done by studies of Brown *et al.*, 1992. Additionally, studies by Lowman *et al.*, 2003, suggested that blastospores and hyphae of *C. albicans* are composed of repeating units of (1→3)-β-D-glucans using NMR spectroscopy.

In an attempt to find suitable control strategies for *C. albicans PLV12* biofilm formation, various herbal preparations like Plant oils, biomolecules (Enzymes and Biosurfactant), metabolic inhibitors (DNP and Bismuth dimercaprol) and antifungal agents (Fluconazole, Amphotericin B, Itraconazole, Ketoconazole and Flucytosine) commonly used against *C. albicans* were investigated. As *C. albicans* biofilms are much more resistant to antimicrobial agents than its planktonic counterpart (Ramage *et al.*, 2005; Chandra *et al.*, 2001) there is greater demand for new antifungal agents that would enhance the number of effective therapeutic alternatives (Alviano *et al.*, 2005). Plant oils are well known for their *in vitro* and/or *in vivo* antifungal properties, but their anti-biofilm activity has not been studied extensively (Dalleau *et al.*, 2008; Williams and Holme, 1995). The screening of plant oils for antimicrobial activity has shown that a great number of these plants contain active compounds. Many of the major components of plant oils including 1, 8-cineole, limonene and linalool, geranial, germacrene-D, and menthol have been reported to have antifungal activity (Grigoleit and Grigoleit, 2005; Ramage *et al.*, 2002; Mazzanti *et al.*, 1998).

In the present investigations, antifungal properties of 30 plant oils were assessed against *C. albicans PLV12* biofilm and its EPS. Our findings showed that treatment of eucalyptus, peppermint, clove and ginger grass oils exerted a metabolic interference in *C. albicans PLV12* biofilms (Agarwal *et al.*, 2008). As biofilm formation and development

involves a series of mechanisms, cell responses and interactions, change in any step may cause inhibition of biofilm formation. This suggests that active components of eucalyptus and peppermint oils have strong potential to affect *C. albicans PLV12* cell growth, function, biofilm formation and development by interfering with any of the steps involved in biofilm growth. The ineffectiveness of twelve oils in the study might reflect lack in activity of antimicrobial compounds against *C. albicans PLV12* strain.

Our findings clearly demonstrated that peppermint, eucalyptus, ginger grass, and clove oils not only act as a potent antifungal agent against *C. albicans PLV12* and its biofilm, especially eucalyptus oil is a potentially superior antifungal agent compared to fluconazole. Accordingly, reduction in *C. albicans PLV12* biofilm and EPS in response to plant oils used in our study can be explained on the basis of presence of these active components restricting biofilm development. It was also encouraging to note that the majority of the oils used in the study were fungicidal at low concentrations. Since the concentrations of plant oils used were within the toxicity range for mammalian cells (Araujo *et al.*, 2003), results suggested that these compounds may have a clinical application in the treatment of *Candida* related infections. Our results not only encourage examination of the efficacy of plant oils in other forms of systemic and superficial fungal infections, but also to explore its broad spectrum effect against other pathogenic manifestations including malignancies.

2,4-dinitrophenol (DNP) was used in the study as a control agent against *C. albicans PLV12* biofilm. It is a metabolic blocking agent that uncouples oxidative phosphorylation of carbohydrate by carrying protons across the mitochondrial membrane, leading to a rapid consumption of energy without generation of ATP. DNP is known to inhibit the bacterial adhesion to surfaces (Paul, 1984; Fletcher, 1980). However, it is well documented that initial

bacterial attachment to surfaces is influenced by several other factors such as cell surface hydrophobicity and cell surface charge (Sorongon *et al.*, 1991; Handley *et al.*, 1987; Van loosdrecht *et al.*, 1987; Fletcher, 1985). Recently, Jain *et al.*, 2007, reported that treatment of DNP influence bacterial cell surface hydrophobicity, which in turn, reduces bacterial adhesion to surfaces. Our studies with DNP revealed similar results as reported by Masak *et al.*, 2002; Cammarota and Sant'Anna, 1998, who showed that addition of blocking agent significantly affected biofilm accumulation by blocking EPS synthesis. Addition of the DNP to the culture medium resulted in 35% reduction in biofilm formation and 66.3% reduction in EPS yield on PVC surfaces in *C. albicans PLV12*. The values could confirm the assumption that the adhesion of candidal cells coincides with or follow the synthesis of polysaccharides as blocking synthetic pathway prevented the adhesion of cells and the subsequent biofilm formation.

Many bacteria like *P. aeruginosa* used in the present studies are well known to secrete biosurfactant (rhamnolipid) which can facilitate surface motility (Deziel *et al.*, 2003; Kohler *et al.*, 2000), mediate the assimilation of hydrocarbons as nutrient sources (Beal and Betts, 2000), affect biofilm architecture (Davey *et al.*, 2003) and alter cell surface polarity (Al-Tahhan *et al.*, 2000; Zhang and Miller, 1994). It is assumed that production of biosurfactants enables the microbial cells within biofilms to solubilize and utilize substrates that would otherwise be inaccessible. The excreted biosurfactants could also lead to loss of matrix material from a surface through alteration of the conditioning film by which it is attached. Rodrigues *et al.*, 2004, evaluated the extent of biofilm formation by a mixture of bacterial and fungal strains isolated from explanted voice prostheses and cultured on silicone rubber voice prostheses with an adsorbed biosurfactant layer. They observed that

biosurfactants greatly reduced microbial numbers on prostheses and also induced a decrease in the airflow resistance that occurs on voice prostheses after biofilm formation. It has been suggested that it is not the thickness of biofilms that is the most important factor in valve failure but rather the combined presence of EPS-producing bacterial strains and fungal species (Elving *et al.,* 2000). Apparently, it was more effective to decrease the viability of biofilms by acting directly on the EPS than to reduce the number of organisms on the esophageal surface of voice prostheses (Elving *et a*l. 2000). Our studies too revealed an observable antimicrobial activity of *P. aeruginosa* biosurfactant towards *C. albicans PLV12* biofilm and its EPS. About 78.6% reduction in biofilm growth and 66.79% reduction in EPS yield showed that biosurfactant not only interferes with the adhesion of *C. albicans PLV12* onto MTP surface but also has the ability to reduce EPS production. Results indicated that biosurfactant could act directly on the *C. albicans PLV12* biofilm matrix to disrupt and solubilize its components, perhaps even incorporating the matrix into micelles.

Microbial EPS are the substrates for a wide range of enzymes most of which are highly specific. Although the enzymes degrading microbial exopolysaccharides may be endo- or exo-acting leading to rapid or slow breakdown of the polymer chain respectively, almost all those studied in detail have proved to be endoglycanases or endo-acting polysaccharide lyases (Sutherland, 1999). In our studies, we used Lyticase, Alginate lyase, Cellulase, Chitinase, Proteinase K, Lipase and DNase 1 enzymes to investigate whether *C. albicans PLV12* biofilms could be enzymatically detached from MTP surfaces by degradation of the matrix polymers. Almost all enzymes except Lipase resulted in a significant decrease in *C. albicans PLV12* biofilms, suggesting that these enzymes partially degraded matrix material and caused some biofilm detachment from the surfaces of the MTP.

Interestingly, lyticase hydrolyzing β-1, 3 glucan moiety of matrix material resulted in 82% reduction in *C. albicans PLV12* biofilms. In a similar study Al-Fattani and Douglas, 2006, found that biofilms of *C. albicans PLV12* were partially detached by treatment with proteinase K, chitinase, DNase I, or β-N-acetylglucosaminidase, whereas *C. tropicalis* biofilms were only affected by lipase type VII or chitinase. They investigated possible lysis of *C. albicans* biofilm cells during their exposure to lyticase by resuspending the candidal cells after enzyme treatment in 1 M sorbitol buffer, and compared the optical density with that of suspensions of control (untreated) biofilm cells. They found that exposure to Lyticase reduced the optical density readings of the suspensions, suggesting that there could have been some cell lysis during the enzyme treatment or simply due to dissolution of some of the *C. albicans* matrix material. In our studies, DNase I, known to be a major matrix component in some microbial biofilms (Starkey *et al.*, 2004), caused only some degree of detachment in *C. albicans PLV12* biofilms. The presence of DNA in the *C. albicans PLV12* matrix would be consistent with the higher phosphorus content of the matrix of this organism.

Bismuth-containing remedies have been in regular use since centuries in numerous medical applications. Some workers discovered that bismuth: pyrithione complexes at dosages lower than generally required to be lethal to bacteria are effective against biofilm, able to penetrate bacterial slime and reduce slime formation even in non-biofilm bacterial colonies (www.wipo.org/pctdb/en/wo). Bismuth dimercaprol is one of several bismuth thiol agents with antibacterial activity upto 1000-fold greater than inorganic bismuth compounds (Domenico *et al.*, 1999). *Klebsiella pneumoniae* EPS expression was inhibited >90% by bismuth dimercaprol at 5 yM $Bi3^{+}$, *Pseudomonas* EPS expression was inhibited >90% by bismuth dimercaprol at 2.5 UM Bi3T and by 80W by bismuth: ethanedithiol at 4yM $Bi3^{+}$.

Our results concerning bismuth dimercaprol revealed a 3-log reduction in viable cell counts with 88.4% reduction in *C. albicans PLV12* EPS production as supported by fluorescent viability testing data. A possible reason for reduction in viable cell count and EPS may be the inactivation of redox enzymes by bismuth salt that resulted in both reduced energy levels and EPS synthesis (Domenico *et al.*, 1999). The role of bismuth dimercaprol in preventing bacterial adherence and bacterial biofilm formation on the surface of biliary stents explored has also been explored by Zhang *et al.*, 2005. Recently, Abdi-Ali *et al.*, 2008, studied anti-biofilm activity of Bismuth dimercaprol (BisBAL) against biofilm forming *Pseudomonas aeruginosa* strain. They observed complete eradication of planktonic cells with 64 fold MIC of BisBAL while complete eradication of biofilm was obtained with 1024–2048 fold MIC of BisBAL. Although the details of biofilm and EPS inhibition by bismuth dimercaprol were not fully understood, the compound may offer a promising approach towards *C. albicans* biofilm control.

Biofilm-mediated antifungal resistance is a well documented phenomenon among *Candida* species. The reason for such unrelenting resistance could be (1) physicochemical nature of biofilms and (2) perfusion ability of antifungal drugs. There are many predictions, hypothesis and theories with regard to the nature of biofilms and their resistance to antimicrobials: (1) production of antibiotic- degrading enzymes; (2) slow growth rate of biofilm cells because of limited availability of key nutrients, particularly at the base of biofilm and (3) extracellular polymeric material, that acts as an adsorbent or a reactant with antimicrobial (Kojic and Darouchie, 2004; Baillie and Douglas, 2000; Chandra *et al.*, 2001; Kuhn *et al.*, 2000). In the present studies, five antifungal agents belonging to all three major classes: azoles, polyenes and nucleoside analogs were used to investigate whether the biofilm

matrix acts as a barrier to drug penetration. The polyene antifungal agents which include amphotericin B, are fungicidal and have the broadest spectrum of antifungal activity of the available agents (Sheehan *et al.*, 1999; Andriole, 1999 and Wynn *et al.*, 1999). These causes fungal cell to die by intercalating into ergosterol-containing membranes, the major sterol in fungal membrane, to form channels and destroy the proton gradient in the cell with leakage of cytoplasmic content (Andriole, 1999 and Wynn *et al.*, 1999). The azoles comprising itraconazole, ketoconazole and fluconazole inhibits ergosterol biosynthesis through their interactions with the enzyme lanosterol demethylase, which is responsible for the conversion of lanosterol to ergosterol in the fungal cell membrane, leading to the depletion of ergosterol in the membrane. Lastly, flucytosine is a nucleoside analog and after its uptake into the fungal cell, it ultimately leads to the disruption of DNA and protein synthesis of the fungal cell (Andriole, 1999 and Wynn *et al.*, 1999).

Our findings showed that *C. albicans PLV12* biofilms were resistant to almost all the azoles and flucytosine even at concentrations greatly in excess of the MICs. Similar observations with *C. albicans* grown statically and under shaking conditions were found by Ballie and Douglas, 2000. In both the studies only amphotericin B showed an inhibitory effect on the activity of biofilm cells but no significant reduction in EPS production was noticed. Despite the improved activity of some newer antifungal agents, complete eradication of sessile organisms within mature biofilms has not been demonstrated (Cocuaud *et al.*, 2005; Bachmann *et al.*, 2002; Kuhn *et al.*, 2002; Ramage *et al.*, 2002). Our results supported this view, as wells containing high concentrations of antifungal agents continued to exhibit XTT activity.

Previous studies by Chandra *et al.*, 2001, showed that the progression of drug resistance in *C. albicans* was associated with increase in metabolic activity of the developing biofilm and was not a reflection of slower growth rate, which indicates that drug resistance develops over time, coincident with biofilm maturation. Our studies indicated that EPS does not contribute a barrier to the penetration of antifungal agents of differing chemical structure. Donlon, 2002 and Chandra *et al.*, 2001, suggested that the phenomena of drug resistance in *C. albicans* biofilms cannot be attributed solely to matrix exclusion or slow growth rate rather contact-induced gene expression for acquiring characteristic properties is probably an additional mechanism by which drug resistance is acquired. In addition, synthesis of new proteins occurs after *C. albicans* attaches to surfaces, which suggests that drug resistance might also arise as a consequence of specific surface-induced gene expression (Jabra-Rizk *et al.*, 2004). Recent studies by Subha and Gnanamani, 2008, raised the possibility that reduced diffusion rate and reactivity of nature of antifungal agents with Candidal strains might be the major reason for inability of the drugs to perfuse biofilm barrier. However, biofilm resistance overall is likely to be multifactorial involving in addition, drug-resistant physiologies such as dormant 'quiescent' cells and expression of efflux pumps (Gilbert *et al.*, 2002).

CHAPTER 6

CONCLUSIONS

Our present investigation proved that *Candida albicans* occupies the first place with regard to frequency (41%) of isolation in clinical samples compared to non-albicans *Candida* species. Chromogenic agar medium was found to be sensitive enough for detection and presumptive identification of *C. albicans* isolates from mixed cultures in the study. Biofilm forming capability of 16 *C. albicans* isolates was evaluated using XTT reduction assay. The assay established a linear relationship between metabolic activity and cellular density of biofilm in maximum biofilm producing strain, *C. albicans PLV12*. The microscopic examination of mature *C. albicans PLV12* biofilm employing SEM, CLSM and AFM revealed a highly heterogeneous architecture in terms of fungal cells distribution and extracellular material (EPS). Quantitative analysis of *C. albicans PLV12* EPS showed high content of glucose (16±3.4%), hexosamine (4.0±1.3%), uronic acid (0.5±0.1%) and phosphorus (0.7±0.2%) in planktonic EPS compared with the biofilm EPS. The difference in EPS composition pointed towards production of biofilm-specific EPS via differential regulation of genes encoding enzymes involved in carbohydrate synthesis in *C. albicans PLV12*.

Optimization of growth parameters for *C. albicans PLV12* indicated that synthesis of EPS during biofilm formation was highly dependent on the conditions of incubation, with pH values close to neutral (6.3) and incubation temperature below optimum growth temperature (30°C) resulting in greater EPS yield. Arabinose was found to be the most efficient carbon source for maximum biofilm formation and EPS production (0.984 g/l) in *C. albicans PLV12*. Furthermore, biofilms subjected to a liquid flow (shaking condition) produced substantially more EPS matrix (1.12 g/l) than those incubated statically (0.91 g/l).

Biochemical characterization of *C. albicans PLV12* EPS using chromatography, FTIR and NMR spectroscopy indicated production of a water soluble heteropolysaccharide made up of α- and β-D-glucose, α-D-mannose, α-L-rhamnose and N-acetyl glucosamine (β-D-GlcNAc) subunits. Gel permeation chromatography further revealed its molecular weight to be ~300 KD.

The random mutagenesis approach with 3% EMS was successfully utilized in the study to isolate a *C. albicans PLV12* mutant (*pm4*) showing 75.2% reduction in biofilm formation and 71% EPS production with enhanced susceptibility to antifungal agents.

Finally, studies concerning *C. albicans PLV12* biofilm control showed that Plant oils (particularly Eucalyptus and Peppermint oils), Metabolic inhibitors (DNP and Bismuth Dimercaprol), Biosurfactant (Rhamnolipid) coating, Enzymes (Lyticase, Alginate Lyase and Cellulase) hydrolyzing glucan and mannan moieties of EPS and Amphotericin B are potential agents against *C. albicans* biofilms. Future prospect of the present study could assist in the development of novel therapeutics aimed at disrupting *C. albicans* biofilms, which will translate into improved clearance of *Candida* related infections.

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APPENDIX

APPENDIX-I

Yeast Extract Peptone Dextrose medium:

Composition:-

Peptic digest of animal tissue	20.0 g/l
Yeast Extract	10.0 g/l
Dextrose	20.0 g/l
pH	7.0

Distilled water added to make up to 1 litre volume. Sterilize by autoclaving at 15 lbs pressure (121°C) for 30 min.

APPENDIX-II

Yeast Extract Phosphate medium:

Yeast extract	1.0 g/l
Disodium phosphate	0.2 g/l
Monopotassium phosphate	0.3 g/l
Phenol red	0.001 g/l
Agar	20.0 g/l
pH	7.0

Distilled water added to make up to 1 litre volume. Sterilize by autoclaving at 15 lbs pressure (121°C) for 30 min.

APPENDIX-III

Sabouraud Dextrose agar medium:

Composition:-

Peptone	10.0 g/l
Dextrose	40.0 g/l
Agar	15.0 g/l
pH	7.0

Distilled water added to make up to 1 litre volume. Sterilize by autoclaving at 15 lbs pressure (121°C) for 30 min.

APPENDIX-IV

HiChrom Candida agar medium:

Composition:-

Peptone	15.0 g/l
Yeast extract	4.0 g/l
Dipotassium hydrogen phosphate	1.0 g/l
Chromogenic mixture	7.22 g/l
Chloramphenicol	0.5 g/l
Agar	15.0 g/l
pH	7.0

Distilled water added to make up to 1 litre volume. Heat to boiling to dissolve the medium completely. Do not autoclave.

APPENDIX-V

Phenol sulphuric acid assay:

Reagents:-

A) Phenol (5% w/v)

B) Conc. H_2SO_4.

Procedure:-

Mix 200 µl of reagent A to 200 µl of sample solution

⇓

Add 1ml of reagent B rapidly and leave solutions undisturbed

for 10 min before shaking vigorously

⇓

After 30 min determine absorbance at 485 nm

APPENDIX-VI

Morgan-Elson assay:

Reagents:-

A) Dissolve 6.1 g of di-Potassium tetraborate tetrahydrate in 80 ml of water and make upto100 ml with d/w.

B) Add 1.5 ml of water to 11 ml of conc. HCl. Add a further 87.5 ml of GAA and dissolve 10 g of 4-(N,N-dimethylamino)-benzaldehyde in this mixture. Dilute 10 ml to 100 ml with GAA immediately prior to use.

Procedure:-

Add 50 µl of reagent A to 250 µl sample solution

⇓

Heat each mixture at 100°C for 30 min

⇓

After cooling rapidly to room temp, add 1.5 ml of reagent B,

washing down any condensate formed

⇓

Incubate the samples at 37°C for 20 min

⇓

After cooling to RT, determine absorbance at 585 nm

APPENDIX-VII

Carbazole assay:

Reagents:-

A) Dissolve 0.95 g of $Na_2B_4O_7 \cdot 10H_2O$ in 2 ml of hot water and add 98 ml of ice-cold conc. H_2SO_4 carefully with stirring.

B) Dissolve 125 mg of Carbazole (recrystallized from ethanol) in 100 ml of absolute ethanol to give a stable reagent.

Procedure:-

Cool 250 µl sample solution in an ice bath

⇓

Add ice-cold 1.5 ml reagent A with mixing and cooling in ice bath

⇓

Heat the mixtures at 100°C for 10 min

⇓

Cool rapidly in the ice-bath

⇓

Add 50 µl of reagent B and mix well

⇓

Reheat at 100°C for 15 min

⇓

Cool rapidly to RT and determine absorbance at 525 nm

APPENDIX-VIII

GOD/POD method:

Reagents:-

A) Phosphate buffer, 100 mM, pH 7.0, containing 0.05% (w/v) thimersal as preservative.

B) Color reagent (100 ml): Weigh and dissolve the following in the order, in 80 ml of phosphate buffer (100 mM, pH7.0), 4–Aminoantipyrine (16 mg), glucose oxidase (1.2 mg), peroxidase (0.5 mg), phenol (105 mg) and 0.01% (v/v) Tween-20. Mix and make up the volume with phosphate buffer to 100ml in a volumetric flask.

Procedure:-

Add 1 ml of reagent B to 10 µl of sample solution

⇓

Mix well and incubate at 37°C for 10 min

⇓

Determine absorbance at 505 nm

APPENDIX-IX

Ames *et al* method:

Reagents:-

A) Ascorbic acid (10% w/v).

B) Ammonium molybdate (0.42% w/v) in 1 N H_2SO_4.

C) Mix reagents A and B in 1:6 (v/v) ratio.

Procedure:-

Add 4.2 ml reagent C to 1.8 ml sample solution

⇓

Incubate at 45°C for 20 min in water bath

⇓

After cooling, determine absorbance at 820 nm

APPENDIX-X

Lowry's assay:

Reagents:-

A) 2% Na_2CO_3in 0.1N NaOH.

B) 1% Sodium Potassium Tartarate in d/w.

C) 0.5% $CuSO_4.5H_2O$ in d/w.

D) Mix reagents A, B and C in 48:1:1 (v/v) ratio.

E) Folin-Ciocalteau (1:1 v/v) in d/w.

Procedure:-

Add 2 ml of reagent D to 1 ml sample solution

↓

Incubate for 10 min at RT

↓

Add 0.2 ml Folin reagent and incubate for 30 min

↓

Determine absorbance at 600 nm

APPENDIX-XI

Allesen-Holm method:

Procedure:-

Culture samples were centrifuged at 10,000 rpm for 3 min

⇓

Supernatant was transferred to a new eppendorf tube

⇓

NaCl (0.25 M) was than added to the supernatant

⇓

Extracellular DNA was precipitated by adding 2:1 volume of ethanol

⇓

Precipitated extracellular DNA was dissolved in TE buffer

⇓

DNA concentration was determined spectrophotometrically (OD_{260}/OD_{280})

APPENDIX-XII

Chemically defined medium:

Composition:-

$CaCO_3$	1.0 mg/l
KNO_3	13.6 mg/l
$MgSO_4.7H_2O$	1.0 mg/l
$FeSO_4.7H_2O$	0.159
$(HOCOCH_2)_3N$	0.2 mg/l
$ZnSO_4.7H_2O$	0.142 mg/l
KH_2PO_4	205.0 mg/l
Na_2HPO_4	426.0 mg/l
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.0014 mg/l
$CuSO_4.5H_2O$	0.0028 mg/l
$Co(NO_3)_2.H_2O$	0.0023 mg/l
$Na_2B_40_7.10H_2O$	0.0014 mg/l
$MnSO_4.H_2O$	0.0114 mg/l
pH	7.0

Distilled water added to make up to 1 litre volume.