MOLECULAR CHARACTERIZATION OF BIOFILM FORMING CANDIDA SPECIES ON BIOMATERIALS

A THESIS

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"MOLECULAR CHARACTERIZATION OF BIOFILM FORMING CANDIDA SPECIES ON BIOMATERIALS"** in fulfillment of the requirements for the awards of the **Degree of Doctor of Philosophy** and submitted in the **Department of Biotechnology** of the **Indian Institute of Technology Roorkee, Roorkee** is an authentic record of my own work carried out during the period July, 2004 to January, 2008 under the supervision of Dr. Vikas Pruthi.

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

Dated: 15th Jan 2008

(Vishnu Agarwal)

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

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ABSTRACT

Biofilms are composed of microbial communities that are attached to a biotic or abiotic surface in which microorganisms usually encase themselves in an extracellular polysaccharide or slime matrix. These biofilms are of serious concern especially in implant associated infections. These infections are believed to be caused by the introduction of pathogenic microorganisms during the implantation or through blood stream infections onto the surface of the newly inserted device. Among them *Candida* species most notably *Candida albicans* are the major fungal pathogen of humans and are responsible for significant morbidity and mortality. This dimorphic fungus ranked fourth among biofilm forming agents behind *Enterobacteriaceae*, *Staphylococcus aureus*, and *Pseudomonas species*. *C albicans* in its yeast form exists as commensal in many healthy human beings, living mainly in the gastrointestinal and vaginal tract, and in the oral cavity. However, it becomes a major pathogen in immunocompromised hosts infected with HIV, or those in intensive care receiving organ transplants. *C. albicans* biofilm formation on biomaterials is a major problem in medical transplants which leads to health risks, and economic loss. Keeping the significance of above facts in view we initiated our research work with the objectives dealing with the molecular analysis of biofilm formed by *C. albicans* on biomaterials used in medically implanted devices. Percentage distribution of fungal pathogen in clinical samples was obtained by plate dilution method. Data showed high incidences of *Candida* infections (60%). On microscopic examinations these clinically isolated *Candida spp.* appeared as ovoid, budding yeast, sometimes having mould like hyphae, occasionally large, refractile spores, chlamydospores were also noticed. Species identification of these strains were performed by both biochemical and microbiological procedures. On selective medium CHROMagar clinical isolates of *Candida* spp. adhering to medically implanted

biocompatible devices appeared as green, dark violet, blue-gray, lavender and pink colony color for *C. albicans, C. glabrata, C. tropicalis, C. parapsilosis* and *C. krusei* respectively out of which *C. albicans* out breaks were found to be maximum. Scanning electron microscopy (SEM) and light microscopic data showed that *C. albicans* biofilm has sticky dense network of extracellular polysaccharide (EPS). Biochemical and colorimetric analysis data depicted that hexosamine, glucose, mannose, fructose, xylose, arabinose and proteins are major constituents of *C. albicans* biofilm. XTT (2, 3-bis [2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) reduction assay data showed maximum adherence with 48hr grown culture. Data also suggested that biofilm were highly metabolically active in its first 24h. However, as the *Candida* biofilm matures and become more complex (48 to 72 h), the metabolic activity reached its plateau reflecting high number of cells that constitute the mature biofilm. Maximum colonization strength of *C. albicans* biofilm was observed at a pH 6.5 and temperature optimum of 37°C after 48h. The *in vitro* activity of clinically used antifungal agents fluconazole, ketoconazole, clotrimazole, itraconazole, nystatin, caspofungin, variconazole and amphotericin B against pre-formed *C. albicans* biofilms was assessed using the XTT-reduction assay. Experiments revealed that activity of nystatin, caspofungin and other azole derivative like ketoconazole, clotrimazole, itraconazole, variconazole against biofilms was reduced about 27, 11, 100, 3, 2, >250 times respectively compared with their activities against planktonic cultures. Importantly, complete killing of cells within the biofilms was never achieved, as reflected by residual metabolic activity of biofilms. Studies were undertaken to analyze *C. albicans* biofilm on commonly used biocompatible biomaterial such as polypropylene, polystyrene, polyvinylchloride, silicone rubber, polyacrylamide and steel. These biofilm were analyzed by Confocal laser scanning microscopy (CLSM) using fluorescent staining with Propidium iodide (PI; 8 μM) for biofilm

residing *Candida* cells and fluorescein isothiocynate-concanavalin A (FITC-ConA; 50 µg/ml) to visualize EPS. Biofilm thickness analysis was done by z-sectioning for each biomaterial revealed that number of microorganisms and EPS produced on different layers varies with biomaterials. Green (FITC-Con A) and Red (PI) fluorescent intensity graph and XTT tetrazolium reduction assay showed differential expression pattern in term of metabolic activity and thickness of biofilm. Quantitative CLSM studies revealed that polymer surface properties and chemical interactions affects adherence and hence biofilm development. Biomaterial surface hydrophobicity and roughness was studied by Goniometric analysis and Atomic force microscopy (AFM) respectively. Data revealed that among all the biomaterials selected PVC was found to possess highest hydrophobicity (97^0), roughness (134nm), and colonization (117.5 µm biofilm thickness). Information retrieve from Candida Genome Database (CGD) shows that multiple genes are responsible for *C. albicans* biofilm regulation. During the biofilm formation induction of drug efflux pumps get induced and this make the biofilm more resistant towards antifungal therapy, but role of these pumps during biofilm formation remain unexplored, keeping the fact in view role of *Candida* ABC transporter (cdr1) on biofilm formation was also analyzed. Cloning and characterization of CDR1 gene (*Candida* ABC transporter) of biofilm forming *C. albicans* was done in to vector pSKPDR5PPUS at SpeI site and transformed to *Sachcaromyces cereviceae* host AD1-8u$^-$ *(MATa pdr1-3 his1 ura3 Δyor1::hisG Δsnq2::hisG Δpdr5::hisG Δpdr10::hisG Δpdr11::hisG Δycf1::hisG Δpdr3:hisG Δpdr15::hisG)* by lithium acetate transformation method. The cloned product was designated as Apcdr1. Cloning was confirmed through drug susceptibility tests, PCR, and SDS-PAGE which gave overexpressed protein (cdr1p) band of 170kd. Role of CDR1 gene in biofilm formation was tested by studying effect of different metals like Copper, Zinc, Magnesium, Phosphorus, Manganese, and Cadmium on biofilm

formed by Apcdr1 with its control along with drug susceptibility tests. Apcdr1 was shown to be hypersensitive towards azole drugs and have demonstrated its inability to form complete biofilms under metal stress condition thereby depicting the involvement of drug efflux pump towards biofilm formation. Prevention and control of *C. albicans* biofilm achieved by using rhamnolipids, plant oils, enzymes and silver coating. Rhamnolipid (8 µg or 4% v/v concentration) isolated from *Pseudomonas aeruginosa* by acid precipitation technique was able to reduce 91%, 86%, 85%, and 68% of biofilm formed on silicone rubber, polystyrene, polyvinylchloride, and polypropylene respectively. The inhibitory effect of 30 plant oils (almond, alsi, babchi, babuna, cade, castor, chaulmoogra, clove, coconut, eucalyptus, ginger grass, ginger, jasmine, jojoba, juniper, jyotishmati, khus, lavender, mahua, malkangani, musturd, neem, ocimum, peppermint, rose, tea tree, til, tulsi, walnut and wheatgerm) was evaluated against *C. albicans* by standard disc diffusion assay, eighteen (babchi, castor, clove, coconut, eucalyptus, ginger grass, ginger, jasmine, juniper, lavender, mahua, malkangani, musturd, ocimum, peppermint, rose, tea tree, and tulsi) among the thirty plant oils selected were found to be effective. The Minimum Inhibitory Concentrations (MICs) values were calculated by agar dilution and broth macro dilution assay respectively. Effect of these oils was further evaluated against *C. albicans* biofilm results depicted that eucalyptus, peppermint, ginger grass, and clove oils can reduce 80.87, 74.16%, 40.46% and 28.57% biofilm respectively. Enzymatic activities of polygalactouronse, pectin lyase, cellulase, arabinase, alginate lyase, proteinase and chitinase were used against *C. albicans* biofilm taking fluconazole as positive control. Data showed maximum 70.7% reduction in *C. albicans* biofilm with alginate lyase while 66.6%, 49.4%, 33.2%, 29.6%, 19%, 14.8%, and 13.3%% reduction was achieved by pectin lyase, cellulase, chitinase, polygalactouronse, arabinase, proteinase glucose oxidase, and proteinase respectively. *C. albicans* biofilm

inhibition on PVC surface was studied by silver-coating for different time intervals of 5,10,15,20 and 25 sec. followed by FE-SEM, AFM and goniometric analysis. Data showed that 15 sec of silver coating was sufficient to complete prevention of *C. albicans* biofilm on PVC surface. Work presented in this thesis may prove very useful to combat with *Candida albicans* biofilm related infections.

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CHAPTER1 INTRODUCTION

Transplantation procedures, immunosuppression, the use of indwelling devices (e.g. dental implants, catheters, heart valves, vascular bypass grafts, ocular lenses, artificial joints and central nervous system shunts) and prolonged intensive care unit stays have increased the prevalence of fungal diseases. The reason involves that biomaterial surfaces are not well protected by host defenses and hence provide not only a focal point for infecting pathogens but also share a common property to be colonized by different microbial flora under wide range of conditions (Costerton 1999; Mukherjee et al., 2004; Hawser et al., 1998). Frequently, failure of such devices stems from fungal biofilm build up which is extremely resistant to host defense mechanism and antimicrobial treatment.

Candida species, notably *Candida albicans* is the major fungal pathogen in humans, is a dimorphic fungus capable to cause superficial mucosal infections, as well as systemic infections in immunocompromised individuals. It causes infection in its biofilm mode of growth and has taken centre stage with the increasing recognition of its role in human infections due to the development of resistant or phenotypic adaptation within the biofilm (Jain et al., 2007).

The factors responsible for its pathogenesis are still not well understood. The candidal proliferation is involves sequence of different steps and the first is the adhesion to the biomaterial surface. Adhesion follows the growth and the biofilm development which is able to protect the microbial cells from host defense mechanism and external agents (Gristina et al., 1993; Hendricks et al., 1999; Gristina 1987. Since years, azole drugs and derivatives continue to dominate as antifungal agents of choice against *Candida* related infections, as topical applications or as oral drugs. Even though very widely acclaimed for their efficacy,

these drugs are known to have side effects (Jain et al., 2007; Meyer et al., 2003; Sheehan et al., 1999; Bruzual et al., 2007; Sanglard et al., 2003). Besides this, the action of antifungal may be limited by their penetration and chemical reaction into biofilm matrix, the extracellular polymeric material (Jain et al., 2007; Meyer et al., 2003; Sanglard et al., 2003).

Biofilm formed by *C. albicans* are 10–1000 times resistant to host defense system, phagocytosis and antimicrobial therapy than their planktonic counterparts. The increasing resistance of *C. albicans* towards these antifungal compounds and the reduced number of available drugs led to the search of new therapeutic alternatives among plants and their essential oils, empirically used by antifungal proprieties (Cavaleiro et al., 2006). Among all the strategies which have been identified to overcome drug resistance, the exploration of new and effective natural products showing antifungal activity against *C. albicans* biofilm cells with low cytotoxicity, is likely to significantly impact the treatment, as well as the management, of biofilm-associated fungal infections (Deans et al., 1991). Recently, the use of biosurfactants (as antimicrobial and/or anti-adhesive agents) have shown a promising candidature for preventing the biofilm associated infections (Rodrigues et al., 1994; Busscher et al., 1997).

It is also known that the microbial susceptibility to pharmacological treatments is strongly decreased in presence of polymeric devices (Bridgett et al., 1993; Higashi et al., 1998; Klueh et al., 2000). In such cases to get rid off infections requires the removal of the polymeric implant which has deep consequences on the patient discomfort and on the cost of the medical cures (Di Tizio et al., 1998). The combinations of medical advances associated with invasive procedures and the widespread use of broad spectrum antibiotics are likely to be responsible for escalating the occurrence of infectious complications related to medical

devices. The medical consequence of these device related infections can be life threatening that may lead to device removal and thus making its management a difficult and costly affair (Kojic and Darouiche, 2004). An increasing proportion of these device related infections are being caused by *Candida albicans* (Bridgett et al., 1993; Tebbs et al., 1994; Ramage et al., 2006). This opportunistic dimorphic fungus can populate and penetrate implanted surfaces to form biofilm, a structured community composed of a mixture of cell types (yeast, pseudohyphal, hyphal cells) in a extracellular matrix comprising of polysaccharides and proteins (Richard et al., 2005; Douglas, 2003). These implanted devices provide the necessary platform for the biofilm architecture to build up starting from surface colonization, growth and proliferation to produce extracellular matrix material by its biofilm. Mature *Candida* biofilm exhibits a complex three dimensional structures and extensive heterogeneity with typical micro-colony/ water channels encased in exopolymer material (Chandra et al., 2001; Kuhn et al., 2002; Chang and Meritt, 1992). From the clinical perspective, the sessile cells in biofilm display phenotypic trends that are dramatically different from their planktonic counter parts (Ramage et al., 2006; Howser et al., 1998; Christensen et al., 1994; Higashi et al., 1998; Klueh et al., 2000; Barton et al., 1996). Though, much work has been done on morphological and physiological stages of *Candida* biofilm formation however, the molecular and physical interactions that govern adhesion to biomaterials, their relation to polymer surfaces, chemical and physical forces, molecular and physical interactions have not been understood in detail. There are still lack of effective treatment strategies, understanding of molecular mechanism, drug resistance and its correlation with gene involved.

Keeping this in view the following objectives were performed for investigating in to molecular characterization of Candida biofilm:

1) Isolation of *Candida albicans* from clinical samples

2) Morphological analysis, physico-chemical characterization and optimization of growth conditions for *C. albicans* biofilms

3) Studies of molecular interaction of *C. albicans* biofilms formed on different biocompatible material surfaces

4) Cloning and characterization of gene involve in biofilm development

5) Effect of Biosurfactants, Plant oils, Enzymes, Silver coatings and Antifungal agents on *C. albicans* biofilm

CHAPTER 2 REVIEW OF LITERATURE

Microorganisms in natural environments occur commonly as a complex community attached to a surface in the form of a structure called biofilm. Within biofilm, microbial cells are enclosed within a self-produced polymeric matrix and adherent to an inert or living surface rather than living in a free swimming, planktonic state (Costerton et al., 1978). The fully developed biofilm has a three-dimensional structure made up of a polysaccharide matrix that contains water channels for the transfer of nutrients and for the removal of wastes (Marshall et al., 1992; Davey et al., 2000). It has been observed that microbial surface thermodynamics, surface structures, different physio-chemical interactions affect biofilm formation (Fowkes, 1963; Donlan & Costerton, 2002).

These microbial biofilms impose enormous cost on human health. According to "National Institutes of Health" report biofilms cause 80% of infections. Another report released in 2000 by "The Biomedical Market Newsletters" states that hospital-acquired infections in the United States affect around 2 million people annually. 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).

2.1 BIOFILM: DEVELOPMENTAL MECHANISM

Microbial adhesion to surfaces consists of the initial attachment of the cells by surface conditioning as a result of nutrients adsorption (Katsikogianni et al., 2004; Rijnaarts et al., 1995). The physical forces which drag microbes towards material surface are Brownian motion, van der Waals and gravitational forces (Gottenbos et al., 2002). In addition, effect of surface characteristics like electrostatic charge, roughness, hydrophobic interactions,

chemoattractants (chemotaxis and haptotaxis) also contributes to this process (Kirov et al., 2003).

Microbial adhesion also depends upon physical interactions which may be long range (>50 nm) or short range (<5 nm). The long-range interactions, generally considered as non specific distances, are a function of the distance and free energy. Short-range interactions become effective when the cell and the surface come into close proximity. These interactions can be separated into chemical bonds (such as hydrogen bonding), ionic and dipole interactions and hydrophobic interactions (Mayer et al., 1999). Table 1 shows different physical interactions involved during developmental stages of biofilm formation. Microbes are initially pulled to approach the material surface by long-range interactions and after a threshold distance (<5 nm), short-range interactions come in to play. This initial attachment of bacteria to surfaces is the initial part of adhesion leading to biofilm development. In later stages of biofilm development, other cell-molecule interactions dominate. This interaction implies a stronger microbial adhesion to surface by induction of selective bonding of microbial cell surface extracellular polymeric substances (EPS), facilitating cell surface to bind with material surfaces (Katsikogianni et al., 2004).

Table 1. Role of different physical interactions in biofilm developmental stages

Biofilm stages	Physical forces	Types	Role
Surface conditioning	Vander Waals interactions, Hydrogen bonding, Surface hydrophobicity	Weak	Decide surface wettability
Reversible adhesion	Vander Waals interactions, Electrostatic forces	Moderate	Brings microbes together
Irreversible adhesion	Covalent bonding, Electrostatic forces	Very strong	Make microbes intact to each other with surfaces
Biofilm development	Covalent bonding, hydrogen bonding, Electrostatic interactions between sessile cells	Very strong	Make microbes intact to each other even under shear conditions

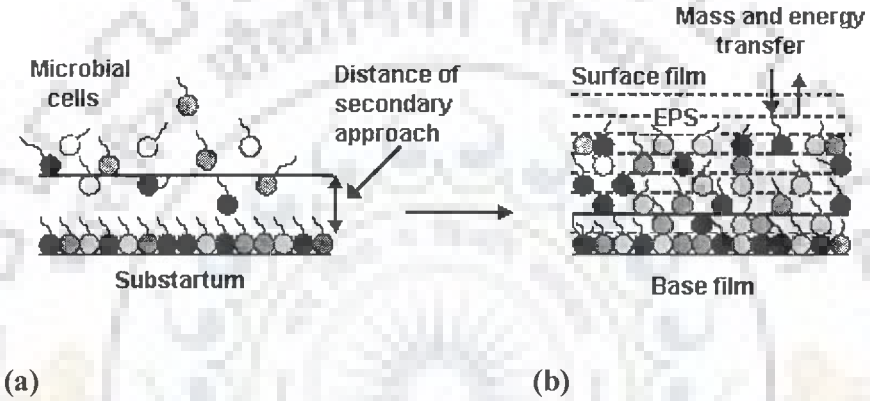

Fig. 1: Diagrammatic representation of the process of biofilm formation showing: (a) microbes concentrated at the distance of secondary approach forced to get adhered to the surfaces; (b) Fully developed biofilm showing mass and energy transfer with EPS production.

Studies by van Loosdrecht *et al.*, 1989 on physiochemical interactions during bacterial adhesion suggested that biofilm develops when deposited bacterial cells interact with suspended bacterial cells in the solution. When suspended bacterial cells get close to deposited bacterial cells within the range of secondary approach, attractive van der Waals interactions develops and begins to contribute for hydrodynamic dispersion or molecular diffusion (Fig. 1). At this distance, van der Waals interactions dominate over Lewis acid–base and electrostatic interactions. As Lewis acid–base interactions are a short range force and electrostatic interactions are weak, under these conditions of secondary approach where reversible adsorption occurs microorganisms have a more favorable adsorption tendency to deposited cells than porous media and thus biofilm is developed.

Biofilm formation is an important aspect of many microbial diseases, including native valve endocarditis, osteomyelitis, dental caries, middle ear infections, medical device-related infections, ocular implant infections, and chronic lung infections in cystic fibrosis patients (Table 2; Donlan et al., 2002). Biofilms are 10–1000-times resistant for antimicrobial agents than planktonic microorganisms, and also extraordinarily resistant to phagocytosis, which makes biofilms extremely difficult to eradicate from living hosts (Lewis et al., 2001).

Consequently, therapy for biofilm-related infections that appear to respond to a therapeutic course of antibiotics may delay for weeks or even months. Darwin's theory of evolution says that the only true driving force behind the course of action of any organism is reproductive fitness that means any action that increases proliferation will endure within a species (Caldwell et al., 1999). Therefore, when we talk about the force work behind biofilm formation we find that outside of the laboratory microorganisms rarely find themselves in an environment as nutrient rich as culture media and under these conditions they find them

selves better in biofilm mode of growth. Microorganisms have many advantages while residing under the biofilm associated matrix which is described below.

Table 2. Biofilm associated implant infections

Implants	Organisms	Symptoms
Prosthetic valves	*S. epidermidis*, *S. sanguis*	Prosthetic valve endocarditis
Contact lenses	*P. aeruginosa*, *S. epidermidis*	Keratitis
Intravascular catheters	*S. epidermidis*, *S. aureus*	Septicemia, endocarditis
Urinary catheters	*E. coli*, *P. aeruginosa*, *E. faecalis*, *Proteus mirabilis*	Bacteriuria
Joint replacements	*S. epidermidis*, *S. aureus*	Septicemia, device failure
Endotracheal tube	*P. aeruginosa*, *E. coli*, *S. epidermidis*, *S. aureus*	Pneumonia
Voice prostheses	*Streptococci, Staphylococci*	Prosthesis failure

Donlan et al., 2002

2.1.1 Degree of Shelter

This is a mode of defense to organisms within biofilms as they can withstand nutrient deprivation, pH changes, oxygen radicals, disinfectants, and antibiotics better than planktonic organisms. Biofilms are also resistant to phagocytosis, biofilm-specific characteristics including slow growth and physiologic heterogeneity of the inhabitants. Another important trait that fortifies biofilm resistance is the sticky matrix predominantly composed of EPS. The important role of EPS in both the early and late stages of biofilm formation is exemplified by the conspicuous presence of genes involved in polysaccharide synthesis for example algC, the gene required for alginate synthesis plays a role in *Pseudomonas aeruginosa* biofilms (Davies et al., 1993). Glucan binding protein GbpA is a glucosyltransferase that has been implicated in sucrose-dependent polysaccharide production and biofilm formation in *Staphylococcus mutans* (Loo, 2003). In addition, the intercellular adhesin locus (icaADBC) in *Staphylococcus aureus* and *Staphylococcus epidermidis* encodes the gene products responsible for the synthesis of a β-1-6-linked poly-N-acetylglucosamine polymer called PNAG or PIA (polysaccharide intercellular adhesin) (Heilmann, 2003). Certain bacterial species may have evolved to switch on transcription of genes required for EPS synthesis in response to certain environmental stimuli that are encountered upon host entry, before the immune system mounts a specific attack (Davey et al., 2000; Van Loosdrecht et al. 1989; Davies et al., 1993). For example, certain stimuli human body, such as iron deprivation and osmotic stress, induce the expression of genes encoding proteins that

synthesize EPS in Staphylococci and Enterococci (Deighton et al., 1993; Baldassarri et al., 2001).

2.1.2 Requisition for Nutrition

Microorganisms have a number of strategies to reside inside the host body, or attachment. Microbial surface proteins that bind to host extracellular matrix proteins such as fibronectin, fibrinogen, vitronectin, and elastin are referred to as MSCRAMMs (microbial surface component recognizing adhesive matrix molecules) and often play a key role in initial adherence of bacteria to solid surfaces within the host (Patti et al., 1994). *S. aureus* is notable for the abundance of MSCRAMMs that can produce with clumping factors A and B (ClfA/B), fibronectin binding factors A and B (FnBA/ B), and a collagen binding protein (Cna). *S. epidermidis* produces at least two autolysin–adhesins that bind to fibronectin and the fibrinogen binding protein Fbe (Heilmann, 2003). *Streptococcus pyogenes* contains genes for fibronectin (prtF) and fibrinogen (emm) binding proteins (Patti et al., 1994). Findings suggests that once the biofilm is established expression of the adhesins and motility factors is suppressed which shows that the main role of adhesins, pili, and flagella only in initial attachment. Exopolysaccharide expression and biofilm formation are markedly enhanced in certain microorganisms such as Pseudomonads, *V. cholerae*, and *E. coli*, the Staphylococci and the Streptococci, with abundant amount of glucose or another readily utilizable carbon source (Davies et al., 1993; Deighton and Borland, 1993). When nutrient sources are depleted, the microorganisms detach and become planktonic these findings suggest that nutrient deprivation is a trigger to move on, in search of a better habitat. Glucose-induced exopolysaccharide production may be multi-functional as glucose may simply serve as a

substrate in the EPS synthesis pathway as well as in EPS elaboration at the level of transcriptional regulation rather than at the level of EPS synthesis (Jefferson, 2004).

2.1.3 Evolution of Communal Behavior

Biofilms are the adaptation to enjoy the benefits of multicellularity and major steps in the way compatible with the mainstream theory of evolution, as suggested by mathematical modelling (Kreft, 2003). There are indeed similarities between biofilm microorganisms and multicellular organisms. For instance, microorganism can sense their surroundings; adjust their metabolic processes for efficient use of available substrates and protection from detrimental conditions. When microbes grow within a biofilm there are changes in gene expression result in phenotypic heterogeneity within the biofilm which can be interpreted as specialization or division of labor similar to cellular differentiation seen in multicellular organisms with the secretions referred to as autoinducing signals, which influence gene expression and may be a means by which cells communicate with one another. Sessile microorganisms also exhibit altruistic behavior and can undergo a process similar to programmed cell death, suggests adaptation towards multicellularity (Rice et al., 2003).

2.1.4 Division of Labor

Whereas early colonizers of the oral cavity are aerobic or facultatively anaerobic, limited oxygen diffusion through the biofilm provides an environmental niche allowing for later colonization by obligate anaerobes (Loo, 2003). Division of labor is coordinately regulated within biofilms through intercellular communication by autoinducing signals which are small molecules, generally homoserine lactones in gram-negatives and peptides in gram-positives. These are constitutively released by microbes and at a critical concentration, induce the expression of certain genes and so frequently referred to as quorum-sensing

signals which relay information to another microbial cell by local diffusion rates (Redfield, 2002). In biofilm development role of autoinducers is suspicious. One study found a role for the LuxS system in *S. mutans* but other indicated that neither LuxS required for biofilm formation, nor lasR/lasI quorum sensing system in *P. aeruginosa* is necessary (Wen et al., 2002; Pesci et al., 1997). Furthermore, there is evidence that the accessory gene regulator (Agr) which is involved in quorum-sensing in the Staphylococci actually affects biofilm formation depends on the flow strength over the biofilm. Under static conditions, Agr reduces biofilm formation, does not affect biofilm under low to moderate flow and under very strong flow it increases biofilm formation (Vuong et al., 2003).

2.1.5 Selfless Approach

Experimental evidence from mathematical modeling supports the concept that microorganisms enjoy a number of benefits due to their community mode of growth capable of exhibiting unselfish or even altruistic behavior (Kreft, 2003). While this may initially appear to defy the rules of survival of the fittest, the models indicate that unselfish behavior in biofilm inhabitants can increase the overall growth yield. Therefore, altruistic bacteria, despite a sacrifice in growth rate are, under certain conditions, actually more benefited. Process similar to apoptosis may actually occur in microorganisms due to the presence of homologues of pro-apoptotic genes such as caspases among bacteria (Bayles, 2003). Presence of several toxin–antitoxin cassettes, similar to those that ensure plasmid maintenance, have been found within the chromosomes of gram negative and gram-positive bacteria, and it has been suggested that programmed cell death may occur within the biofilm community (Hayes, 2003). Microorganism within biofilm matrix reduces the metabolic load

and increases nutrient availability to the survivors presents a good example for altruistic behavior (Bayles, 2003).

2.2 ARCHITECTURE OF *C. albicans* BIOFILMS

Although research on bacterial biofilms has been fairly advanced (Donlan and Costerton, 2002; Costerton et al., 1987, 1995, 1999) studies on fungal biofilms have only recently been initiated. In vitro and in vivo models of *C. albicans* biofilms formed on bioprosthetic surfaces were recently developed and used to gain insight into fungal biofilm biology (Chandra et al., 2001; Kuhn et al., 2002). These studies demonstrate the heterogeneous and highly complex interactions influencing the formation of *Candida* biofilms, a theme also observed in bacterial biofilms. *C. albicans* biofilm formation proceeds in an organized fashion through distinct early, intermediate and maturation developmental phases. Fluorescence and confocal scanning laser microscopy (CSLM) utilizing carbohydrate-specific dyes (e.g., calcofluor and concanavalin A, Con-A) indicated that the *C. albicans* biofilms are encased within a polysaccharide-rich extracellular matrix (ECM) (Chandra et al., 2001). Similar stages of development and the presence of extracellular polysaccharide matrix have also been reported for bacterial biofilms (Davey and O'Toole, 2000; O'Toole et al., 2000; Sauer et al., 2002). Recent in vivo studies using a rabbit model of biofilms formed by *C. albicans* on intravascular catheter demonstrated the formation of biofilms in vivo (Schinabeck et al., 2004). Structural heterogeneity of architecture appears to be a common property of biofilms formed by almost all microbes (Wimpenny et al., 2000), and those formed by *C. albicans* also share this characteristic (Chandra et al., 2001b; Baillie and Douglas, 1999). Different studies have shown that architecture of *C. albicans* biofilms is affected by the substrate surface. For example, biofilms formed on flat hydrophobic surfaces

of silicone elastomer or polyvinyl chloride (PVC) discs have a distinct biphasic structure composed of an adherent blastospore layer covered by hyphal elements embedded within layer of ECM (Chandra et al., 2001; Kuhn et al., 2002). In contrast, *C. albicans* biofilm formed on roughedged and irregular surfaces of polymethylmethacrylate denture strips appear as dense tracks of cells growing along the raised rough edges of the surfaces at early phase of development, with an overgrowth of cells and ECM in mature biofilms (Chandra et al., 2001). Recently, Mukherjee et al. (2004) reported the EDTAbased isolation of ECM from *C. albicans* biofilm grown to different developmental stages, and showed that the isolated ECM consists of protein, carbohydrates, and DNA. Furthermore, the total protein (TP) and total carbohydrate (TC) composition of the ECM varies with different developmental stages of *C. albicans* biofilm formation. TC was significantly lower in ECM of biofilm grown to early phase, when compared with ECM of biofilm grown to mature phase (96.4 mg/g versus 153.0 mg/g, respectively, $P < 0.05$). This was consistent with earlier studies which revealed that biofilm maturation is accompanied with abundant synthesis of carbohydrate-rich ECM (Chandra et al., 2001). Sizebased analyses revealed that ECM contains two groups (high and low molecular weight) each of TP and TC (Mukherjee et al., 2004). However, the role of the individual protein and carbohydrate components of ECM in *C. albicans* biofilm formation and drug resistance has not been investigated. Environmental factors (including fluid shear, pH, oxygen availability and redox-gradients) have been proposed to account for heterogeneous architecture in bacterial biofilms (Wimpenny et al., 2000; Rasmussen and Lewandowski, 1998; Stoodley et al., 1997; Stoodley et al., 2001; Xu et al., 1998) Although the role of these factors in *C. albicans* biofilm formation has not been investigated in detail. Garcia-Sanchez et al. (2004) showed that biofilm populations which are formed in different

environments display very similar and specific transcript profiles. These investigators also identified two clusters of genes encoding for amino acid biosynthesis, and showed that Gcn4p (a regulator of amino acid metabolism) was required for normal biofilm growth. The architecture of fungal biofilms can also be speciesdependent. Kuhn et al. 2002 showed that biofilm formed by *C. parapsilosis* do not exhibit biphasic arrangement of discrete layers as seen with *C. albicans*, but rather consist of patches of mushroom-shaped biofilm communities. Additionally, *C. parapsilosis* biofilms consisting of ECM was significantly less than those formed by *C. albicans*.

2.3 FACTORS INFLUENCING MICROBIAL ADHESION

Many physical, biological and chemical factors like hydrodynamic shear, mass transfer, detachment, substratum texture, physiology of cells, microbial population, EPS particulate matter substrate concentration, physico-chemical environment, type of substrate and nutrients affect the biofilm structure to various extents (Rosenberg et al., 1986). Taylor et al., 1990 and Picioreanu et al., 2000 made Conceptual and mathematical modeling to describe the effect of different factors on biofilm structure. Below are some factors which affect biofilm formation.

2.3.1 Surface Thermodynamics

Surface thermodynamic theory is the fundamental theory in interpreting microbial hydrophilicity or hydrophobicity, microbial attachment to porous media, and microbial biofilm development (Rosenberg et al., 1986; Corpe, 1980). Surface thermodynamics depends on surface tension which is determined by various independent forces like dispersion, dipolar induction, hydrogen-bonding and metallic interactions (Fowkes, 1963). Effect of nutrient conditions, exopolymer production, growth rates and physiological states

on microbial surface thermodynamics have also been extensively investigated (Azeredo et al., 1999; Chen et al., 2002).

2.3.2 C/N Ratio

Microbial growth depends upon a stoichiometric ratio of carbon and nitrogen to synthesize new cellular materials (Chen et al., 2002). Different carbon and nitrogen ratio yield different cell growth rates and hence surface thermodynamic properties. It has been found that for the same carbon source, ammonia and nitrate yields different components of surface tension and when carbon is limited, surface tension also get affected while zeta potential change is not reported with growth conditions (Corpe, 1980).

2.3.3 Surface Attributes

Microbial surface characteristics and physiological states contribute to surface thermodynamics and are governed by physico-chemistry of bacterial surfaces and depend on macromolecular components like lipo-polysaccharide, protein and exopolymers, varying in quantity with growth conditions and from strains to strains (Chen et al., 2001; Grasso et al., 1996).

2.3.4 Secondary Approach

Microbial transport depends on deposition coefficient and retardation factor related to interaction of free energies between bacteria and media at different distances. Total interaction free energies between microorganisms and media at the closest and secondary approach are usually found to be negative, i.e., attractive; while in between are positive, i.e., repulsive, if both microorganisms and media are negatively charged. Microbial deposition on a clean medium particle during transport is achieved by van der Waals interactions, balance

hydrodynamic dispersion and molecular diffusion, surface shear forces, electrostatic interactions and Lewis acid-base interactions (Fig. 2; Mayer et al., 1999).

2.3.5 Microbial Cell Surface Structure

Many bacterial cell surface structures like Flagella, pili, and fimbriae have also been implicated in adherence of *Vibrio cholerae, E. coli, P. aeruginosa*, and *Salmonella enterica* (Desvaux et al., 2004).

2.3.5.1 Flagella

The role of flagellar filaments, motility and chemotaxis in biofilm formation has been investigated by inducing well-defined mutations, eg., insertional mutation in *fliC*, which encodes flagellin, or in *flhDC*, encoding the master regulator required for the expression of all other genes of the flagellar regulon (Pratt et al., 1998; Soutourina et al., 2003). Deletion of the chemotaxis genes *cheA* through *cheZ*, resulted in motile but non-chemotactic cells. The influence of these mutations was investigated under stagnant culture conditions and it was concluded that non-chemotactic cells formed biofilms indistinguishable from wild-type cells but motility was critical for the initiation of *E. coli* biofilm formation. It has also been suggested that motility, but not chemotaxis, promotes initial cell-to-surface contact (Pratt et al., 1998).

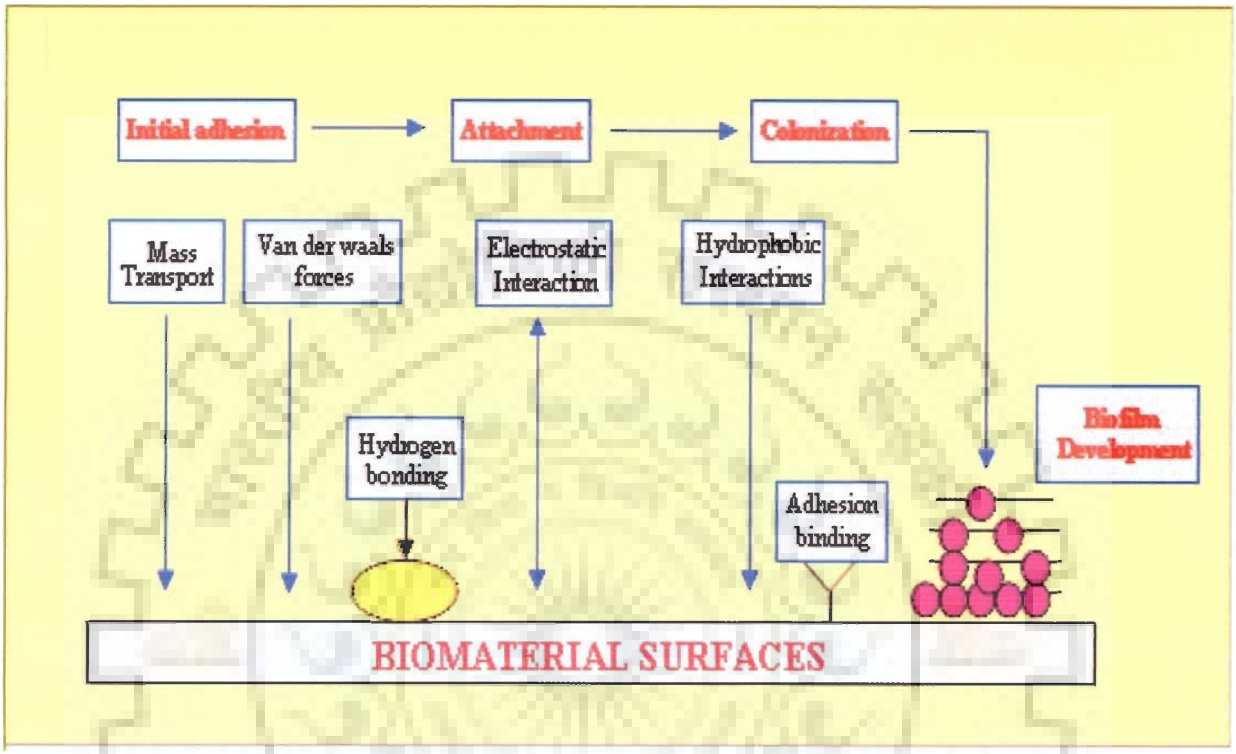

Fig. 2: Different forces involved during biofilm formation on biomaterial surfaces

2.3.5.2 Fimbriae

Fimbriae are also associated with host tissue adhesion among which Type 1 fimbriae are the most common found in *Enterobacteriaceae* and appeared to be necessary for early biofilm formation in rich culture medium (Klemm et al., 1994). Their influence on biofilm formation has been well studied and it was found that *fim* gene cluster encodes the structural components of the fimbrial organelle as well as the fimbrial biosynthesis machinery. It also has been seen that *fim* mutants are defective in initial attachment to abiotic surfaces such as polyvinylchloride (PVC) under stagnant culture conditions in rich culture medium (Pratt et al., 1998).

2.3.5.3 Secretory Proteins

Autotransporter proteins are secretory proteins which contain all the requirements for secretion across the cytoplasmic and the outer membrane to the bacterial cell surface in their primary structure. All proteins that are secreted by this autotransporter (type V) secretion mechanism possess an overall unifying structure, comprising (i) an N-terminal leader peptide (for secretion across the inner membrane), (ii) the secreted mature protein (or passenger domain), (iii) a linker region necessary for translocation of the passenger domain through the outer membrane, and (iv) a dedicated C-terminal domain involved in the formation of a transmembrane pore (Desvaux et al., 2004). Adhesive phenotypes have been attributed to a subfamily of *E. coli* autotransporters like Ag43, AIDA and TibA. Antigen 43 (Ag43) is a prominent surface protein of *E. coli* and is the product of the *agn43* gene (also called *flu*). This autotransporter protein is a self recognizing adhesin which contains both receptor recognition and receptor target and protrudes approximately 10 nm beyond the outer membrane and establishes autoaggregation of cells through Ag43–Ag43 interactions by an

intercellular handshake mechanism (Klemm et al., 2004). Antigen 43 promotes bacterial biofilm formation by its ability to induce microcolony formation (Danese et al., 2000).

2.3.5.4 Curli

Curli are heteropolymeric proteinaceous filamentous appendages which are composed of a major (CsgA) and minor (CsgB) subunit and influence the adherence properties of several biofilm-forming *E. coli* strains (Olsen et al., 1989). The genes for curli production are organized in the operons *csgBAC* and *csgDEFG*. The involvement of curli in biofilm formation followed from the observation that overexpression of the curlin encoding gene *csgA* resulted in a biofilm-forming phenotype, whereas a knockout mutation caused loss of adherence (Vidal et al., 1998). Furthermore, its overexpression resulted in the development of a differentiated, mature biofilm with channels and pump compared to an undifferentiated biofilm formed by the isogenic wild-type strain (Vidal et al., 1998).

2.3.5.5 Production of EPS

Biofilms are primarily composed of microbial cells and EPS which may account for 50% to 90% of the total organic carbon of biofilms and primarily composed of polysaccharides. It has been investigated that EPS are the key components responsible for resistance, protection, virulence, and altered growth profile of biofilm residing microorganism. Some of these polysaccharides are neutral or polyanionic, as is the case of gram-negative bacteria due to the presence of uronic acids (such as D-glucuronic, D-galacturonic, and mannuronic acids) or ketal-linked pryruvates (Hussain et al., 1993). This property is important because it allows association of divalent cations such as calcium and magnesium, which have been shown to cross-link with the polymer strands and provide greater binding force in a developed biofilm (Sutherland, 2001). In the case of some gram-

positive bacteria, such as the staphylococci, the chemical composition of EPS may be quite different and may be primarily cationic. The composition and structure of the polysaccharides determine biofilm primary conformation for example many bacterial EPS possess backbone structures that contain 1, 3- or 1,4-β-linked hexose residues and tend to be more rigid, less deformable, and in certain cases poorly soluble or insoluble (Hussain et al., 1993).

2.3.5.6 Conjugative pili

Bacterial conjugation is a process of horizontal gene transfer that involves intimate cell-to-cell contact, in which a conjugative plasmid is transmitted from a donor to a recipient cell through a specialized conjugative pilus encoded on the plasmid. Some bacteria carrying conjugative plasmids from different incompatibility groups induced the formation of a thick biofilm (Ghigo, 2001).

2.3.6 Physiological States and Nutrition of Bacteria

Development of biofilms is of great interest with respect to substrate conversion and the degradation of substances. Very little is known about the dependence of the biofilm density (gram dry mass per biofilm volume) on the growth conditions of the biofilm system. Biofilm nutrition affect due to different surface structures, there is still a lack of knowledge on mass transfer with in biofilm matrix. It has also been found that biofilm density is affected by substrate load and hydrodynamic conditions (Kwok et al., 1998).

2.3.7 Environmental Factors

Temperature, time of exposure, microbial concentration, and presence of toxins affect microbial adhesion and their reproductive potential. Generally, the number of bonds that can form between microbial cell surfaces to a material surface (substratum) will be a function of

densities of functional groups as well as interacting forces. Concentrations of nutrients, electrolytes and pH value in the culture environment also influence adhesion (Bunt et al., 1995; McWhirter et al., 2002). Bunt *et al.* (1993) showed that the pH and the ionic strength of the suspending buffer influence the cell surface hydrophilicity. It was found to be significantly higher at higher pH and low ionic strength and lower at lower pH and ionic strength. Greatest adhesion to hydrophobic surfaces was found at pH between 2.2 and 4, in the range of the isoelectric point when bacteria are uncharged, and ionic strength 1 M (Katsikogianni et al., 2004).

2.3.8 Substratum Characteristics

The factors influencing bacteria adherence to a surface include chemical composition of the material, surface charge, hydrophobicity, surface roughness and contact angle measurements. Surface chemistry influences microbial adhesion and proliferation. Presence of different functional groups on materials surfaces affects microbial adhesion by influencing material hydrophilicity and charge (Gottenbos et al., 2000). Scheuerman *et al.*, 1998, reported that irregularities of material surfaces promote microbial adhesion and biofilm formation whereas the ultra smooth surface does not. This may be due to presence of greater surface area and depressions on the roughened surfaces which provide more favorable sites for colonization. The implant site infection rates are different both in case of porous and dense materials and increase with porosity. However, microbes prefer grooved and porous materials to adhere due to increased surface area (Medilanski et al., 2002).

The angle of contact made by a droplet of liquid at a solid surface is a reflection of the surface free energy of the substratum (Lawrence et al., 1990). The liquid may spread on the surface or remain as a discrete drop, making a finite angle of contact with the surface.

Instruments are available for the measurement of contact angles. Substratum wettability may be expressed in terms of water contact angles, the work of adhesion between water and the substratum, bubble contact angles, or the results of applying the equation of state approach (Neumann et al., 1974; Pringle et al., 1983). Although it is preferable to measure bubble contact angles on wetted surfaces, problems may arise from changes to the liquid surface tension upon the addition of the macromolecules (Lawrence et al., 1990).

2.4 THE GENETIC FRAMEWORK OF BIOFILMS

Genetic adaptation is the basis of fitness and survival which can occur from mutations well as recombination within genes, acquisition of new genetic material, or from the regulated expression of existing genetic material. Flexibility in microbial gene expression permits survival in environments with rapidly changing conditions, and microorganisms, being particularly adaptable, have flourished in nearly every environmental niche on our planet body. Scientific interest in the process of microbial biofilm formation has erupted in recent years and studies of the molecular genetics of biofilm formation have begun to shed light on the driving forces behind the transition to the biofilm mode of existence. A number of target gene-directed as well as global proteomics- and genomics-based studies have lead to the identification of a plethora of genes associated with biofilm development. Tables 3, presents a compilation of genes from various clinically relevant microorganisms that have been implicated in the biofilm mode of growth. It lists the genes that appear to be required for biofilm formation (Jefferson, 2004).

In case of Candida biofilm, information retrieve from Candida Genome Database (CGD) shows that multiple genes are responsible for *C. albicans* biofilm regulation. During the biofilm formation induction of drug efflux pumps get induced and this make the biofilm

more resistant towards antifungal therapy, but role of these pumps during biofilm formation remain unexplored.

Ramage et al. (2002) evaluated resistance in biofilms formed by *C. albicans* mutants deficient for Cdr1p, Cdr2p or Mdr1p, at intermediate as well as mature phases (24 and 48 h) of growth and demonstrated that the *CDR1* and *CDR2* genes were up-regulated in intermediate and mature *C. albicans* biofilms. In case of prokaryotes, reports are there about involvement of ABC transporters of *Pseudomonas* species in adherence and biofilm formation but, the question that why drug resistance genes (including CDR1) be up regulated and what correlation they have with biofilms are still remain unanswered.

Information from Candida genome data base showed that drug resistance and biofilm formation may link together. Many of genes are associated both with biofilm formation and drug resistance, for example ALS2 gene which is known for biofilm formation and germ tube induction, is induced by ketoconazole whereas KRR1 gene which generally shows lower transcription in azole resistance strains that over expresses MDR1 get induced upon biofilm formation. ERG11, GPM1 gene get induced both by azole or biofilm formation while PDR16 gene show over-expression with CDR1, CDR2 and azole resistance is fernasol down regulated during biofilm formation. Table 4 shows the genes and their associated functions that are involved in Candida drug resistance and biofilm formation.

In Candida CDR1 is a 4506 nuleotide long major ABC transporter of *C. albicans* which involve in multiple drug resistance. The gene involve in many important metabolic functions and is a multidrug transporter of ATP-binding cassette (ABC) superfamily which transports phospholipids in an in-to-out direction; transcription induced by beta-estradiol, progesterone, corticosteroid, or cholesterol.

The *CDR1* gene encodes an integral plasma membrane (PM) protein of 1,501 amino acids, with a predicted molecular mass of 169.9 kDa (fig. 3). On the basis of its amino acid sequence, Cdr1p is predicted to consist of two homologous halves, each comprising one N-terminal hydrophilic domain followed by a C-terminal hydrophobic domain. The hydrophilic domain comprised a conserved ABC region, including the ATP-binding motifs known as the Walker A and Walker B motifs and another highly conserved motif, ABC signature, preceding the Walker B motif. Cdr1p has a similar topology to its close homologues Pdr5p and Snq2p of *Saccharomyces cerevisiae*.

Table 3: Genes involved in biofilm formation

Species	Gene	Role
E. coli	agn43	Antigen protein involved aggregation
S. epidermidis	atlE	Autolysin/adhesin
C. albicans	ALS1-ALS7, ALS9	Adhesion to host surface
S. aureus	hla	Hemolytic toxin
S. gordonii	sspA/B	Human salivary protein and collagen binding
S. aureus, S. epidermidis	icaADBC	Intercellular adhesin synthesis
S. aureus	clfA	Clumping factor A, fibrinogen binding protein
S. mutans	gbpA	Polysaccharide formation
E. faecalis	bopABCD	Biofilm on plastic surfaces operon
S. mutans	tarC	Regulator of glucosyltransferase S and glucan binding protein
S. epidermidis	aap	Accumulation associated protein
P. aeruginosa	lasI	Synthesis of 3OC12-HSL quorum-sensing signal
E. faecalis	esp	Enterococcal surface protein
S. gordonii	comX	Competence
S. mutans	luxS	Quorum sensing
S. gordonii	PBP2B, PBP5, glmM, bacA	Peptidoglycan synthesis
S. aureus, S. epidermidis	σ^B	Alternate sigma factor-stress response
S. mutans	brpA	Possible regulator of autolysis
S. mutans	ccpA	Carbon catabolite control protein
P. aeruginosa	crc	Global carbon metabolism regulator
E. coli	tra	Conjugative pilus of F plasmid
E. coli	rpoS	Regulator involved in slow growth
S. mutans	dgk	Stress response regulator, antibiotic regulator
S. gordonii	mutT	DNA mismatch repair
S. epidermidis	purR	Regulator of purine synthesis, metabolism

Table 4: Genes involved in biofilm and associated drug resistant functions in *Candida*[#]

Description(s)	Associated Gene(s)
ALS family protein; role in adhesion, biofilm formation, germ tube induction; expressed at infection of human buccal epithelial cells; putative GPI-anchor; induced by ketoconazole, low iron and at cell wall regeneration; regulated by Sfu1p	ALS2
Decreased transcription is observed upon benomyl treatment or in an azole-resistant strain that overexpresses MDR1; induced upon biofilm formation	KRR1
Lanosterol 14-alpha-demethylase, member of cytochrome P450 family that functions in ergosterol biosynthesis; target of azole antifungals; may contribute to drug resistance; azole- or biofilm-induced; subject to hypoxic regulation	ERG11
Phosphatidylinositol transfer protein; increased transcription correlates with CDR1 and CDR2 overexpression and azole resistance; induced by fluphenazine, 17-beta-estradiol, ethynyl estradiol, nitric oxide; farnesol-downregulated in biofilm	PDR16
Protein described as a sulfate transporter; transcription is negatively regulated by Sfu1p; induced upon biofilm formation; amphotericin B induced	SUL2
Protein described as phosphoglycerate mutase; enzyme of glycolysis; antigenic during murine, human infection; biofilm-, fluconazole-, or amino acid starvation (3-aminotriazole treatment) induced; regulated by Efg1p, Gcn4p	GPM1
Protein similar to S. cerevisiae Vps16p, which is involved in protein-vacuolar targeting; likely to be essential for growth, based on insertional mutagenesis; downregulated in biofilm or in azole-resistant strain that overexpresses MDR1	VPS16
Putative ATP sulfurlyase of sulfate assimilation; repressed by Met or Cys, Sfu1p, or in fluconazole-resistant isolate; strongly induced on biofilm formation, even in presence of Met and Cys; Hog1p-, caspofungin-, possibly adherence-induced	MET3
Putative C-4 methyl sterol oxidase with role in C4-demethylation of ergosterol biosynthesis intermediates, based on similarity to S. cerevisiae Erg25p; fluconazole-induced; upregulated in biofilm and in azole-resistant strain	ERG25
Putative chaperone of Hsp70 family; role in sensitivity to beta-defensin peptides; heat-shock, amphotericin B, Cd, ketoconazole-induced; farnesol-downregulated in biofilm; surface localized in yeast-form and hyphal cells; antigenic in host	HSP70
Putative role in phosphate transport; biofilm-regulated expression; amphotericin B repressed	PHO88
Putative secreted acid sphingomyelin phosphodiesterase; induced upon biofilm formation; possible Kex2p substrate; increased transcription is observed in an azole-resistant strain that overexpresses MDR1	ASM3

Source Candida Genome Database: www.candidagenome.org/

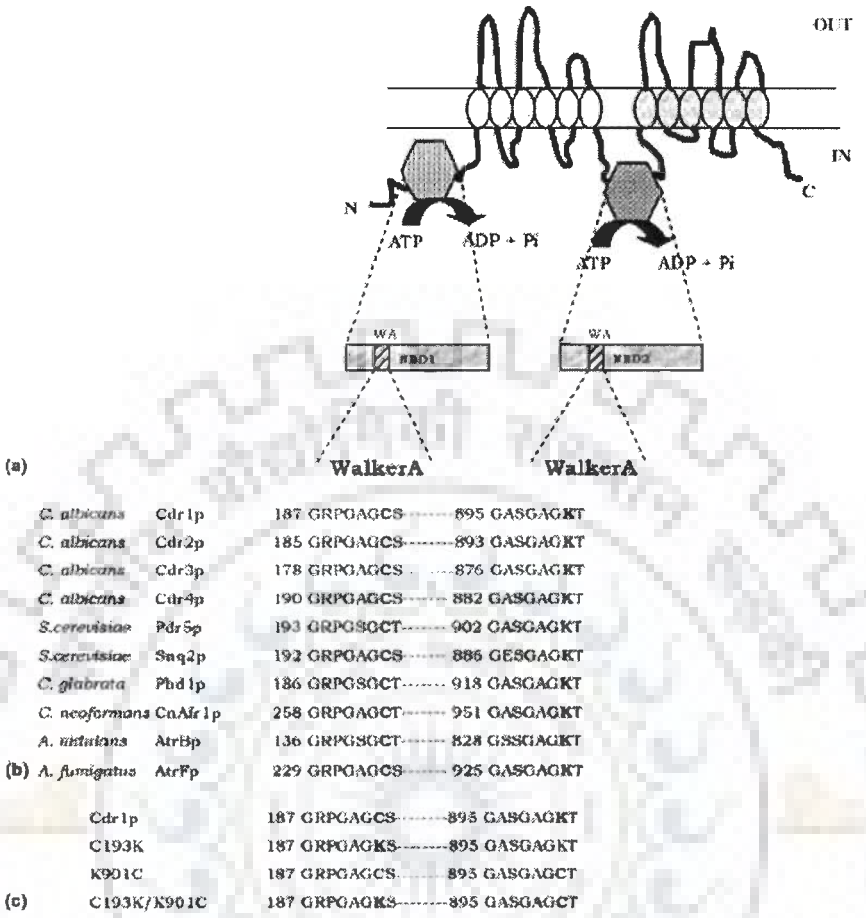

Fig. 3: (a) Topological model of Cdr1p showing two putative transmembrane domains (TMD) and two nucleotide-binding domains (NBD). Each TMD comprises six a-helices panning the lipid membrane. The cytoplasmic domains of Cdr1p, i.e. NBD1 and NBD2, are located at the N- and Cterminus, respectively. (b) Amino acid sequence alignment of NBD1 and NBD2 of Cdr1p with other fungal ABC type transporters, highlighting (in bold) the conservation of the cysteine and lysine residues within Walker A.

(Jha et al., FEMS Yeast Research 5 (2004) 63–72)

Cdr1p protein sequence[#]

MSDSKMSSQDESKLEKAISQDSSSENHSINEYHGFDAHTSENIQNLARTFTHDSFKDDSSAGLLKYLTH

MSEVPGVNPYEHEEINNDQLNPDSENFNAKFWVKNLRKLFESDPEYYKPSKLGIGYRNLRAYGVAND

SDYQPTVTNALWKLATEGFRHFQKDDDSRYFDILKSMDAIMRPGELTVVLGRPGAGCSTLLKTIAVNT

YGFHIGKESQITYDGLSPHDIERHYRGDVIYSAETDVHFPHLSVGDTLEFAARLRTPQNRGEGIDRETYA

KHMASVYMATYGLSHTRNTNVGNDFVRGVSGGERKRVSIAEASLSGANIQCWDNATRGLDSATALE

FIRALKTSAVILDTTPLIAIYQCSQDAYDLFDKVVVLYEGYQIFFGKATKAKEYFEKMGWKCPQRQTTA

DFLTSLTNPAEREPLPGYEDKVPRTAQEFETYWKNSPEYAELTKEIDEYFVECERSNTRETYRESHVAK

QSNNTRPASPYTVSFFMQVRYGVARNFLRMKGDPSIPIFSVFGQLVMGLILSSVFYNLSQTTGSFYYRG

AAMFFAVLFNAFSSLLEIMSLFEARPIVEKHKKYALYRPSADALASIISELPVKLAMSMSFNFVFYFMV

NFRRNPGRFFFYWLMCIWCTFVMSHLFRSIGAVSTSISGAMTPATVLLLAMVIYTGFVIPTPSMLGWSR

WINYINPVGYVFESLMVNEFHGREFQCAQYVPSGPGYENISRSNQVCTAVGSVPGNEMVSGTNYLAG

AYQYYNSHKWRNLGITIGFAVFFLAIYIALTEFNKGAMQKGEIVLFLKGSLKKHKRKTAASNKGDIEA

GPVAGKLDYQDEAEAVNNEKFTEKGSTGSVDFPENREIFFWRDLTYQVKIKKEDRVILDHVDGWVKP

GQITALMGASGAGKTTLLNCLSERVTTGIITDGERLVNGHALDSSFQRSIGYVQQQDVHLETTTVREAL

QFSAYLRQSNKISKKEKDDYVDYVIDLLEMTDYADALVGVAGEGLNVEQRKRLTIGVELVAKPKLLL

FLDEPASGLDSQTAWSICKLMRKLADHGQAILCTIHQPSALIMAEFDRLLFLQKGGRTAYFGELGENCQ

TMINYFEKYGADPCPKEANPAEWMLQVVGAAPGSHAKQDYFEVWRNSSEYQAVREEINRMEAELSK

LPRDNDPEALLKYAAPLWKQYLLVSWRTIVQDWRSPGYIYSKIFLVVSAALFNGFSFFKAKNNMQGL

QNQMFSVFMFFIPFNTLVQQMLPYFVKQRDVYEVREAPSRTFSWFAFIAGQITSEIPYQVAVGTIAFFC

WYYPLGLYNNATPTDSVNPRGVLMWMLVTAFYVYTATMGQLCMSFSELADNAANLATLLFTMCLN

FCGVLAGPDVLPGFWIFMYRCNPFTYLVQAMLSTGLANTFVKCAEREYVSVKPPNGESCSTYLDPYIK

FAGGYFETRNDGSCAFCQMSSTNTFLKSVNSLYSERWRNFGIFIAFIAINIILTVIFYWLARVPKGNREK

KNKK*

[#]Source: Candida Genome Database

CDR1 gene sequence[#]

4506 nucleotides

ATGTCAGATTCTAAGATGTCGTCGCAAGATGAATCTAAATTAGAAAAGGCAATTAGTCAAGACTC
TTCTTCAGAAAACCATTCCATTAATGAATACCACGGGTTTGATGCCCATACAAGTGAAAACATTCA
GAATTTAGCCAGAACTTTCACTCATGATTCTTTCAAAGATGACTCGTCAGCAGGTTTATTGAAATA
CTTAACCCATATGTCAGAAGTGCCCGGGGTCAATCCATATGAACATGAAGAAATAAATAATGACC
AATTGAATCCCGATTCAGAAAATTTCAATGCCAAATTTTGGGTTAAAAATTTAAGAAAATTGTTTG
AATCAGATCCTGAATATTATAAACCTTCGAAATTGGAATTGGATATAGAAATTTAAGAGCATATG
GTGTTGCTAATGATTCTGATTATCAACCAACTGTTACTAACGCACTTTGGAAATTAGCCACAGAAG
GTTTCAGACATTTCCAAAAAGATGATGACTCAAGATATTTTGATATTTTGAAATCAATGGATGCCA
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TATCCCCACATGATATTGAACGTCATTACCGTGGTGATGTTATTTATTCTGCTGAAACCGATGTTCA
TTTCCCTCATTTGAGTGTTGGTGATACTTTGGAATTTGCCGCTAGATTAAGAACTCCACAAAACAG
AGGTGAAGGTATCGACAGAGAAACTTATGCTAAACACATGGCTAGTGTTTATATGGCAACCTATG
GGTTATCACACACAAGAAATACTAATGTTGGTAACGATTTCGTCCGTGGTGTTTCCGGTGGTGAAA
GGAAAAGAGTGTCAATTGCTGAAGCTTCTTTGAGTGGTGCTAATATCCAATGTTGGGATAATGCCA
CTAGAGGGTTAGATTCTGCTACTGCTTTGGAATTCATTAGAGCTTTGAAAACTTCAGCAGTCATTTT
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GTTGTCTTGTATGAAGGTTATCAAATTTTCTTTGGTAAAGCCACCAAGGCTAAAGAATATTTTGAA
AAGATGGGTTGGAAATGTCCTCAAAGACAAACTACTGCTGATTTCTTGACTTCCTTAACCAATCCA
GCTGAAAGAGAACCATTACCAGGTTATGAAGATAAAGTGCCAAGAACTGCTCAAGAATTTGAAAC
ATATTGGAAAAATTCTCCCGAATATGCTGAATTAACTAAGGAAATTGATGAATATTTTGTTGAATG
TGAACGATCCAACACCAGGGAAACTTATCGTGAATCTCATGTTGCCAAACAATCCAACAATACAA
GACCAGCATCTCCATATACTGTATCATTCTTCATGCAAGTGAGGTATGGTGTTGCGAGAAATTTCC
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ATTGTCGTCGGTGTTCTATAATCTTAGTCAAACCACTGGATCGTTTTATTATAGAGGTGCTGCCATG
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CCACCAATACTTTCTTGAAATCAGTTAATTCATTATAC
AGTGAAAGATGGAGAAATTTCGGTATTTTCATTGCTTTCATTGCTATTAATATCATTCTTACGGTGA
TCTTTTATTGGTTAGCTAGAGTTCCAAAGGGTAACAGAGAGAAAAAAAATAAGAAATAA

#Source: Candida Genome Database

2.5 BIOFILM RESISTANCE

The development of antimicrobial resistance is not well understood till date. Different studies revealed that drug resistance is a complex phenomenon in which different mechanism work in same direction and produce more complicated upshots. These include physical or chemical diffusion barriers to antimicrobial penetration into the biofilms, activation of the general stress response, emergence of a biofilm-specific phenotype and more interestingly sacrifice of growth rate for mutualistic benefits to gain resistance tolerance and adaptation towards reduced struggle for survival. Presence of matrix, associated components and their reactions with antimicrobials prevents the access of antimicrobials to the microbial cells embedded in the community. In fact, the penetration profile was suggestive of a substrate being consumed within the matrix. Chlorine, a commonly used disinfectant, did not reach >20% of the bulk media's concentration within a mixed *Klebsiella pneumoniae* and *P. aeruginosa* biofilm, as measured by a chlorine-detecting microelectrode. Suci *et al.* showed that the rate of transport of the antibiotic ciprofloxacin to the surface of a colonized surface was reduced compared with transport to a sterile surface using infrared spectroscopy suggesting that the ciprofloxacin was binding to the biofilm components (Suci, 1994).

To resist antimicrobial substances generally microbes slows their growth (Brown, 1988; Wentland, 1996). Since cells growing in biofilms are expected to experience some form of nutrient limitation, it has been suggested that this physiological change can account for the resistance of biofilms to antimicrobial agents. Gilbert and colleagues in their studies with *P. aeruginosa*, *Escherichia coli* and *S. epidermidis* showed growth-rate-related effects under controlled growth conditions for planktonic cultures and biofilms (Evans, 1991; Duguid, 1992; Duguid 1992). They made the general observation that the sensitivities of both

the planktonic and biofilm cells to either tobramycin or ciprofloxacin increased with increasing growth rate, thus supporting the notion that slow growth rate of biofilm cells protects the cells from antimicrobial action.

Nutrient gradient, waste products and signaling factors are responsible for different growth rate, heterogeneity and resistance within the biofilm. Staining methods utilizing propidium iodide (PI) and Fluorescein isothiocynate-concavalin A (FITC-ConA) were employed to identify regions of biofilms that contain rapidly or slowly growing cells as well as matrix polymer production based on their relative RNA–DNA content or sugar moieties (Fig. 4; Agarwal et al, 2006).

This heterogeneity within biofilms is also responsible for protein synthesis and respiratory activity, whereas DNA content remained relatively constant but necessary (Whitchurch et al., 2002) throughout the biofilm. Sometimes microbes produce unpredicted phenotypes or physiological state to tolerate '*toxoids*'. For example when biofilm cells were treated with the antibiotic fleroxocin, cell elongation was observed and was most extreme in cells located close to the exposed side of the biofilm (Korber, 1994). To resist the detrimental effects of heat shock, cold shock, changes in pH and many chemical agents in *E. coli* there is expression of σ factor and RpoS which expresses only in stationary phase (Adams et al., 1999).

An emerging idea in the field is that a biofilm-specific phenotype induced with in sessile community result in the expression of active mechanisms to combat the detrimental effects of antimicrobial agents (Cochran, 2000; Gilbert, 1997; Maira-Litran, 2000a; Maira-Litran, 2000b). Resistant phenotypes might be induced by nutrient limitation, certain types of stress, high cell density or a combination of these phenomena.

2.5.1 Possible Drug Resistance Mechanism of Candida Biofilm

The mechanisms of biofilm resistance to antimicrobial agents have not been fully understood in details. Possible mechanisms include: (1) restricted penetration of drugs through the biofilm matrix; (2) phenotypic changes resulting from a decreased growth rate or nutrient limitation; and (3) expression of resistance genes induced by contact with a surface (Sheehan et al., 1999; Schrtliff et al., 2002). Multiple mechanisms of drug resistance operates in bacteria and vary with bacterial species, strains and nature of the antimicrobial agent being administered (Chandra et al., 2001). Extracellular biofilm matrix polymeric material plays a vital role to exclude or limit the access of drugs to biofilm residing organisms. Most studies with biofilms indicate that the matrix does not form a major barrier for drug diffusion but penetration of certain antimicrobials can be delayed (Sheehan et al., 1999). Studies with biofilms produced under flow conditions showed that resuspended cells without matrix were less resistant to amphotericin B than intact biofilms, suggesting that the matrix might play a minor role in drug resistance (Douglas, 2003). Biofilm residing cells generally sacrifice their growth rate and grow slowly because of the limited availability of nutrients, and develop a decreasing nutrient gradient from upper to basal layer of biofilm. A slow growth rate is generally resulting in changes in cell surface composition that could, in turn, affect the susceptibility of the icroorganisms to antimicrobial agents. To investigate effect of growth rate on C. albicans biofilm, a perfused biofilm fermenter was used to generate biofilms at different growth rates, and the susceptibility of the biofilm cells to amphotericin B was compared with that of planktonic organisms grown at the same growth rate in a chemostat. The results showed that biofilms were resistant to the drug at all growth rates tested whereas planktonic cells were resistant only at low growth rates. The study gives

important outcome that biofilm resistance is not only the result of a low growth rate but depends on some other feature of the biofilm mode of growth. When microorganisms attach to a surface and form a biofilm they express an altered phenotype and surface-induced expression of resistance genes also play important role in biofilm resistance. The genes that are activated or repressed in *Candida* biofilms compared with planktonic cells, and there is particular interest in genes that might contribute to drug resistance. For example, upregulation of genes coding for multidrug efflux pumps would result in a multidrug-resistant phenotype (Ramage et al., 2002). *C. albicans* possesses two different types of efflux pump: ATP-binding cassette (ABC) transporters and major facilitators, which are encoded by CDR and MDR genes, respectively. A recent study has demonstrated that genes encoding both types of efflux pump are upregulated during biofilm formation and development.

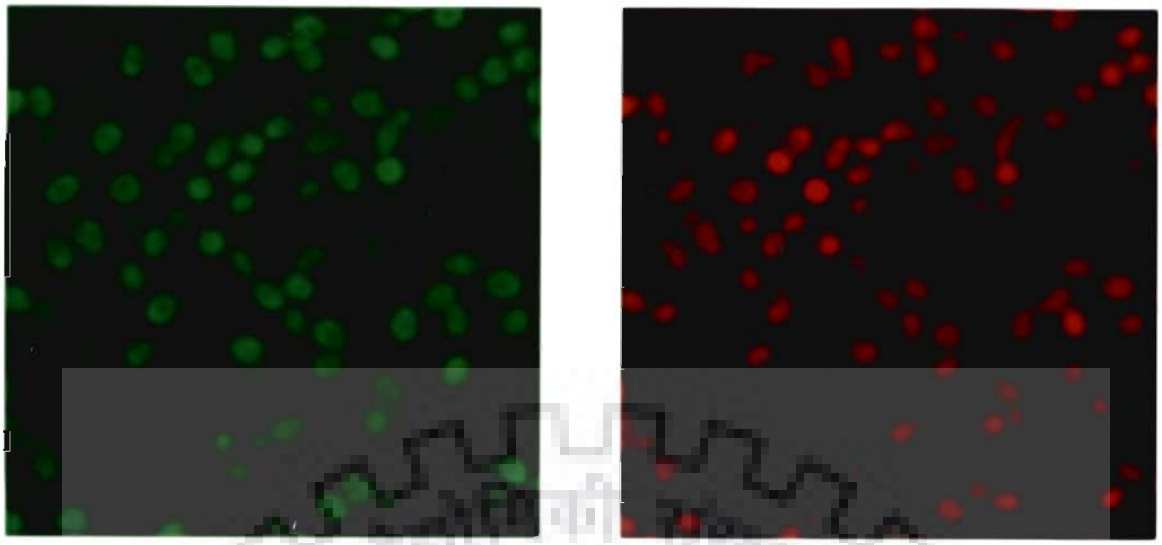
However, mutants carrying single or double deletion mutations of these genes were highly susceptible to fluconazole when growing planktonically but still retained the resistant phenotype during biofilm growth. These results strongly suggest that drug resistance in *C. albicans* biofilms is a complex process that cannot be explained by a single molecular mechanism and may be a result of combined mechanisms working in same direction.

2.6 NOVEL APPROACHES TO COMBAT BIOFILMS

Since years, many drugs and derivatives continue to dominate as antimicrobial agents of choice against biofilms. Even though very widely acclaimed for their efficacy, these drugs are known to have side effects (Jain et al., 2007; Meyer, 2003; Sheehan et al., 1999; Bruzual et al., 2007; Sanglard et al., 2003). Besides this, the action of these drugs may be limited by their penetration and chemical reaction into biofilm matrix, the extracellular polymeric material (Jain et al., 2007; Meyer, 2003; Sanglard et al., 2003). The increasing resistance of

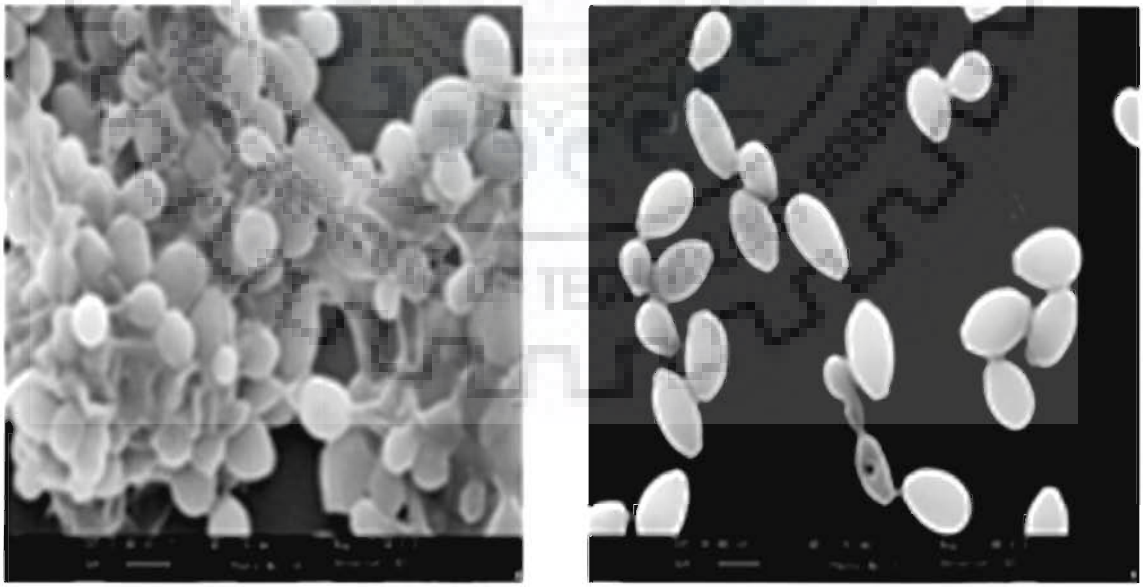
biofilm residing microbes towards these compounds and the reduced number of available drugs led to the search of novel therapeutic alternatives.

Our recent preventive trials against *C. albicans* biofilm demonstrated that peppermint, eucalyptus, ginger grass and clove oils act as potent antifungal agents against *C. albicans* biofilm and eucalyptus oil was found to be a potentially superior antifungal agent in compared to fluconazole (fig. 5). In an another study, we found that 2,4 dinitrophenol (DNP), a blocking agent of EPS production, when added to the culture medium resulted in a reduced EPS yield, marked compositional changes along with altered morphology of sessile cells suggesting that DNP may be a useful agent in controlling *C. albicans* biofilm associated infections in future studies.



(a) (b)

Fig. 4: CLSM images of *C. albicans* biofilm on PVC surface. Image showing metabolically active cells in red color (a) while green (b) indicates presence of polysaccharides in biofilm



(a) Control (b) With Eucalyptus oil

Fig. 5: Effect of Eucalyptus oil on *C. albicans* biofilm

CHAPTER 3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Clinical Isolates of *Candida* species

Among forty eight Candida spp. isolates obtained *C. albicans* was use for further investigations. To compare the activity of plant oils against biofilm formed by screened isolate with fluconazole, a reference *C. albicans* strain MTCC 227 (Microbial Type fCulture Collection) was obtained from Institute of Microbial Technology (IMTECH),

3.1.2 Rhamnolipid Producing Strain

Rhamnolipid was isolated and characterized from *Pseudomonas aeruginosa* MTCC 2642 was obtained from Institute of Microbial Technology.

3.1.3 Strains and Growth Media for CDR1 Gene Studies

Plasmids were maintained in *Escherichia coli* DH5α. The *CDR1* gene was obtained from clinical isolate of *C. albicans*. The *S. cerevisiae* strains used in the study was AD1-8u⁻ *(MATa pdr1-3 his1 ura3 Δyor1::hisG Δsnq2::hisG Δpdr5::hisG Δpdr10::hisG Δpdr11::hisG Δycf1::hisG Δpdr3:hisG Δpdr15::hisG),* kindly provided by Richard D. Cannon, University of Otago, Dunedin, New Zealand. *E. coli* was cultured in Luria-Bertani medium (35). *C. albicans* was maintained on YEPD (yeast extract, 10 g/liter; Bacto Peptone, 20 g/liter; glucose, 20 g/liter), and *S. cerevisiae* was maintained on YNB, complete synthetic medium (CSM; Himedia, India.), or CSM without uracil as required.

3.1.4 Biomaterials

Commercially available biopolymer namely silicone rubber, polyvinyl chloride (PVC), polypropylene (PP) and polystyrene (PS), and polymethylmethacrylamide (PMMA) were used to study *C. albicans* biofilm. Squares of 1.0cm x 1.0cm were cut from these

materials for use in experiments. Before use, the biomaterials were decontaminated by 2% Decon (SD Fine chemicals, India) with mild agitation (30rpm) and finally soaked in Milli-Q water. These samples were then dried in a laminar airflow hood, and sterilized using 100% ethylene oxide (Speranza et al., 2003).

3.1.5 Culture Media

Sabouraud Dextrose medium, Yeast Extract Peptone Dextrose medium, Luria-Bertani medium, CHROMagar medium, Yeast Nitrogen Base medium, RPMI-1640 medium was used from Himedia, India. Distilled water was added to make up to required amount and pH.

3.1.6 Chemical Reagents and Diagnostic Kits

The entire chemicals used were obtained from commercial sources and were of analytical grades. Sources of the fine chemicals used in this study have been listed below.

- Castor, eucalyptus, peppermint, oils were purchased from Himedia chemicals (Himedia, India) while twenty seven plant oils were derived from steam distillation— almond, alsi, babchi, babuna, cade, chaulmoogra, clove, coconut, ginger grass, ginger, jasmine, jojoba, juniper, jyotishmati, khus, lavender, mahua, malkangani, musturd, neem, ocimum, rose, tea tree, til, tulsi, walnut and wheatgerm (Boutekedjiret et al., 2003).
- DNA-modifying enzymes were purchased from Genei.
- Protease inhibitors, Amphotericin B, Miconazole, Nystatin, Cycloheximide, Anisomycin, Rhodamine 6G, Sigma Chemical Co. (St. Louis, Mo.).
- Fluconazole was kindly provided by Pfizer pharmaceutical group, USA.

- XTT (2,3-Bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carbox-anilide), Menadione, Tetramethylene diamine (TEMED), Sodium Dodycel Sulfate (SDS), Ammonium Persulfate, Mercaptoethanol. Sigma Chemical Co. (St. Louis, Mo.).
- All other culture media were from Himedia, India and solvents were from Ranbaxy, India.

3.2 METHODS

3.2.1 Isolation of *C. albicans* from Clinical Samples

A total of forty eight yeast isolates from sputum, urine, oropharyngeal swabs, Inter uterine devices (IUDs), blood, pus, CSF from patients at IIT Roorkee hospital, were tested by the conventional methods. These included isolates of *C. albicans* (n = 15), *C. tropicalis* (n = 11), *C. glabrata* (n =6), *C. krusei* (n = 4), *C. lusitaniae* (n = 3), *C. parapsilosis* (n = 3), *C. guilliermondii* (n = 2), *C. kefyr* (n = 2), *C. firmetaria* (n = 1), and *C. rugosa* (n = 1). Each specimen was randomly assigned a number from 1 to 48 and sub cultured twice onto Sabouraud dextrose agar (SDA; Himedia, India) to ensure isolation of pure colonies. After 48 h of incubation, a sample of each specimen was suspended in 5 ml of sterile normal saline and adjusted by transmittance at 530 nm (spectro name) to a concentration approximately $1x10^6$ to $5x10^6$ yeast cells/ml (NCCLS, 1997). A 1:100 dilution of each yeast suspension was then performed to produce a final concentration of $1x10^4$ to $5x10^4$ yeast cells/ml. A volume of 0.1 ml of each isolate was introduced into set of 10ml YPD broth to make a final inoculum of approximately $1x10^3$ to $5x10^3$ yeast cells. Concentrations were verified by plating onto SDA and performing counts of serial dilutions and placed at 35°C with continuous agitation at 120rpm. A 0.1-ml aliquot was withdrawn from each positive bottle and plated (four quadrant streaking) onto CHROMagar Candida (CHROMagar, Himedia, India) medium along with a control. The specimens were then incubated at 30°C for 1-4 days. The incubation temperature of 30°C was selected, as this temperature has been used successfully in prior studies with CHROMagar Candida (Baurngartner et al., 1996; Freydiere et al., 1997; Hospenthal et al., 2002; Huang et al., 2001; Merlino et al., 1998; Odds et al., 1994; Pfaller et

al., 1996). Each plate was read on days 1 to 4 with emphasis placed on recording the colony color, size, texture, and presence of color diffusion into the surrounding agar.

3.2.2 Biofilm Formation and Quantification

Biofilm was formed either directly on the wall of capped polypropylene tubes, on micro titer plate (MTP) or on the biomaterial surfaces depending up on experimental requirement, quantification was performed using modified XTT reduction assay (Baillie et al., 1999). In case of biomaterials, 1cm2 pieces of different biomaterials were dipped in 1 ml of culture with 5 x 10^8 cfu/ml of 48h grown *C. albicans* and placed for 90 min of adhesion phase at 37^0C. The biomaterial pieces were then washed with sterilized PBS (0.1 M; pH 7.2) to remove loosely adherent cells. To the washed pieces 1 ml of sterilized YEPD broth was added and incubated at 37^0C for 48 h. Biofilm thus formed was then quantified using XTT reduction assay as described by Kuhn et al., 2002.

3.2.2.1 *XTT Reduction Assay*

XTT (Sigma, St. Louis, Mo.) solutions (1 mg/ml in PBS) was prepared, filter sterilized through a 0.22 μm poresize filter, and stored at -70°C. Menadione (Sigma) solution (0.4 mM) was prepared and filter sterilized 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 were first washed five times with 1 ml of PBS, and then 1 ml of PBS and 60 μl of the XTT-menadione solution were added to each of the pre-washed and control tubes having different biomaterial samples. The tubes were then incubated in the dark for 2 h at 37°C. Following incubation the color change in the solution was measured spectrophotometrically at 492 nm (Varian, USA).

3.2.3 Effect of Preconditioning with Serum and Saliva on *C. albicans* Biofilm

The horse serum (Himedia, India) was aliquoted and stored at –70 °C. Saliva was collected by chewing parafilm to stimulate salivary glands for 1 h and collected in 50 ml Falcon tubes on ice, then centrifuged at 3,000 g. The saliva was pooled, aliquoted, and stored at –70 °C. Serum and saliva were prepared at 50% (v/v) in sterile PBS, and were individually dispensed (100 µl) into six wells of a microtiter plate. The serum and saliva solution were then incubated in the wells overnight at 4 °C. Excess serum and saliva were then aspirated, and the adsorbed conditioning film washed once in sterile PBS. *C. albicans* was washed in PBS and re-suspended at a concentration of 2×10^6 cells per milliliter in YPD, and dispensed (100 µl per well) into the 96-well microtiter plates and incubated for 30 min, 4 h and 24 h at 37 °C. For each time point the wells were aspirated and washed three times in sterile PBS, and adhesion measured by the XTT reduction assay and by light microscopy (Ramage et al., 2001). Experiments were performed twice with six replicates for each condition, with similar results. The effect of serum and saliva conditioning films on *C. albicans* adherence and biofilm formation as compared to control wells was assessed by using the Student's t test. $P < 0.05$ was considered statistically significant.

3.2.4 Effect of Shaking Conditions and Biofilm Growth Rate

Effect of different shaking conditions to biofilm formation was studied by incubating the biofilm for different time interval of 0.5, 2, 4, 6, 8, 12, 24, 48, and 56 h under different shaking conditions of 5, 10, 20, 30, 40, and 50 rpm followed by biofilm quantification (Ramage et al., 2001).

3.2.5 Effect of Temperature and pH

To analyze effect of temperature and pH on biofilm formation C. albicans biofilm was formed by incubating at different incubating temperatures of 4, 15, 25, 35, 37, 45, and 55^0C and pH 3.5, 4.5, 5.5, 6.5, 7.5 and 8.5 and keeping other factors unaffected.

3.2.6 Planktonic MIC Determination

Planktonic MIC values were determined using the Clinical and Laboratory Standards Institute (CLSI) broth microdilution methods (NCCLS 1997; CLSI, 2002). Each isolate was plated on Sabouraud dextrose agar and incubated at 37^0 C for 24 h. Amphotericin B deoxycholate, caspofungin, fluconazole, ketoconazole, clotrimazole, itraconazole, nystatin and voriconazole diluted to concentration ranging from 25-0.01 µg/ml in sterile RPMI 1640 medium with l-glutamine and without sodium bicarbonate (Sigma), buffered with 3-[N-morpholino)-propanesulfonic acid. The lowest concentration inhibiting any visible growth at 48 h was used as the MIC.

3.2.7 Sessile MIC (SMIC) Determination

SMIC values were determined using an XTT assay, which has demonstrated good correlation between the reduction of metabolic activity by 50% and the MIC50 determined by CLSI M27-A methodology (Hawser et al., 1998). Mature *C. albicans* biofilms were formed on microtiter plates as previously described. Wells containing 48 h grown *C. albicans* biofilm were then filled with 100 µL of serial dilutions of drugs in RPMI. Untreated biofilm wells and negative control wells were filled with 100 µL of RPMI without antifungal agents. Microtiter plates were incubated for an additional 48 h at 37^0 C and then metabolic activity was determined using XTT reduction assay. The reduction of absorption by 50% and 80% was reported as the SMIC50 and SMIC80, respectively.

3.2.8 Isolation of EPS

Batches of medium (50ml in 250ml flasks) were inoculated with 24h grown *Candida* cultures incubated at 35^0C for 24 hrs in an orbital shaker operating at 120 rpm and harvested by centrifugation, pellets were suspended in fresh media (cell number was determined by using a hemocytometer and adjusted to 2 x10^4 cfu ml^{-1}) and used for biofilm formation.

For EPS analysis culture supernatants were added to 1.5 volume of chilled acetone and after 2h at 4^0C the resultant precipitate was collected by centrifugation at 17000g for 30 min. The pellets were redissolved in small volume of distilled water and any insoluble material was removed by centrifugation. Water soluble polymers was recovered by acetone precipitation (3 vols, 2 h at 4^0C), washed with ether and evaporated to dryness *in vacuo*. The dried material was weight and the yield was calculated as a percentage of combine dry weight of yeast cells and EPS. Crude EPS was dissolved in a minimum volume of distilled water and dialysed at 4^0C for 24h against three changes distilled water. EPS in the retentate was precipitated with 3 vol. of acetone, dried and weighed. Sample were stored at -20^0C and used for EPS analysis (McCourtie and Douglas 1985).

3.2.9 Quantification of Biofilm Bound EPS

To quantify the amount of exopolysaccharides (EPS) produced by *C. albicans* biofilm on the surface of different biomaterials, the sample pieces were sonicated and centrifuged (5000g, 5 min). The supernatant so obtained was treated with 1.5 volumes of chilled acetone. After 2h incubation at 4^0C the resultant precipitate was collected by centrifugation (17000g, 30 min). The pellet was redissolved in minimum volume (500µl) of distilled water and recentrifuged twice. The water soluble fraction was then recovered and washed with ether and evaporated to dryness *in vacuo*. Crude EPS thus obtained was redissolved in a minimum

volume of distilled water. The sample was dialyzed at 4^0C for 24h against three changes of distilled water. EPS in the retentate was precipitated with 3 volumes of acetone, dried and weighed (McCourtie et al., 1985).

3.2.10 EPS Analysis

EPS preparation were analysed quantitatively for total carbohydrate by Phenol Sulphuric acid method (Dubois et al., 1956), Pentoses by ferric orcinol method , Phosphorus by ascorbic acid ammonium molybdate method (Ames et al., 1968), Protein by Lowry method, Glucose by GOD/POD method, hexosamine by Elson-Morgon assay and reducing sugars by Dinitrosalicylic acid method.

3.2.11 CLSM Studies

C. albicans biofilm on the different biomaterials was fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.2) for 1.5h. After washing with PBS, biofilm were visualized using fluorescent staining with Propidium iodide (PI; 8 µM) solution. Extracellular polysaccharide in *C. albicans* biofilm was visualized with fluorescein isothiocynate-concanavalin A (FITC-ConA; 50 µg/ml) in 10 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES)/ 0.1 M NaCl, containing 0.1 mM Ca^2 and 0.01 mM Mn^2 (Takenaka et al., 2001; Watanabe, 1995). CLSM (Radiance 2100, BioRad) was performed under a Nikon microscope (objective Plan Apo 60X/1.4 oil, Japan). The excitation wavelength for PI fluorescence was 543nm (Green He Ne laser) and fluorescence was detected through emission filter HQ 590/70 (High quality band pass), centered at 590nm with 70nm bandwidth. Image processing and graph analysis was carried out with Lasersharp2000 (BioRad) and photoshop 5.5 (Adobe Systems, San Jose, CA) was used for the final image

assembly. The digitized images were threshold manually at the same level to define biofilm boundaries for analysis.

3.2.12 Scanning Electron Microscopy (SEM)

C. albicans biofilm were fixed with 2.5% (v/v) glutaraldehyde in PBS 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 with ethanol series (30%, 50%, 70%, 90% and 100%). All samples were dried to critical point by Polaron critical point drier, coated with gold and viewed under SEM (Leo 435, England).

3.2.13 Biomaterials Surface Properties

Surface characteristics were analyzed using atomic force microscopy (AFM) and contact angle measurements (Bavejaa et al., 2004).

a) Atomic Force Microscopy

Dimension 3000 Scanning Probe Microscope with tapping mode AFM was used to analyze roughness of biomaterial samples (5x5 μm). The average roughness was then calculated by Nanoscope software and represented as the arithmetic average of the deviation from center plane. Three points were analyzed on each square and results were expressed as mean± standard deviation.

B) Contact Angle Measurement

Hydrophobicity of biomaterial surface was determined by contact angle measurement using Rame-Hart contact angle goniometer (Mountain Lakes, NJ, USA; Model 100-00). Distilled water was used as contact angle liquid. A drop of distilled water was placed on the surface of biomaterial and image was captured by a camera equipped with the Rame-Hart

2001 imaging software. The angle made by drop with respect to biomaterials was calculated and results were expressed as mean ± standard deviation for contact angle of 5 drops.

3.2.14 Plasmid Construction and Yeast Transformation

PCR amplification kit (Bangalore Genei, India) was used to amplify by PCR the *CDR1* open reading frame (ORF) and transcriptional termination region (4.8 kb) from *C. albicans* genomic DNA with primers containing *Spe*I restriction sites, primers 5'-CTTTAAAAGGTCAACTAGTAAAAAATTATG-3' and 5'-CAATAATACACTAGTTTGCAACGGAAG-3'.

The PCR product was digested with *Spe*I and was cloned into plasmid pSKPDR5PPUS that had previously been cut with *Spe*I and dephosphorylated with alkaline phosphatase (Sigma, USA). The orientation of the *CDR1* ORF was confirmed by sequencing to be the same as that of *PDR5p*, and the plasmid was designated as ApCDR1. Plasmid ApCDR1 was linearized with *Xba*I and was used to transform *S. cerevisiae* AD1-8u$^-$ for uracil prototrophy (Ura$^+$) by the lithium acetate transformation protocol. The entire CDR1 ORF DNA in ApCDR1 was sequenced, and the CDR1 ORFs from *C. albicans* and *S. cerevisiae* AD1-8u$^-$/ ApCDR1 transformant were amplified from genomic DNA by PCR with *Pfu* DNA polymerase (Biolabs, india) and sequenced.

3.2.15 Genomic DNA Extraction and Southern Analysis of *CDR1* Gene Integrated into *S. cerevisiae* Genome

Genomic DNA was prepared from *S. cerevisiae* cells as described previously (Scherer and Stevens 1987). Genomic DNA (5 µg) was digested with a restriction endonuclease (*Eco*RV, *Spe*I, *Bam*HI, *Pst*I, or *Eco*RI; New England Biolabs), separated in a 0.75% agarose gel, and transferred to a Hybond_ nylon membrane (Amersham). Membranes were

hybridized with a [α^{-32}P]dCTP-labeled, *C. albicans CDR1*-specific probe under high-stringency conditions (Cannon et al., 1994).

3.2.16 MIC Determination

The MICs of antifungal agents for *S. cerevisiae* cells were determined by a microdilution test based on the macrodilution reference method of the National Committee for Clinical Laboratory Standards (NCCLS 1997). Cells (10µl cell suspension, 2x 10^5 cells/ml) were inoculated into 90 µl of CSM-URA buffered with 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) and 18 mM *N*-2-hydroxyethylpiperazine-*N*_-2-ethanesulfonic acid (HEPES, pH 7.0) and containing 0.67% (wt/vol) yeast nitrogen base (YNB) in microtiter plate wells. For uracil-requiring strain AD1-8u$^-$, the medium was supplemented with 0.02% (wt/vol) uridine. The wells contained doubling dilutions of antifungal agents (final concentrations were as follows: fluconazole, 30 to 0.058 µg/ml; itraconazole and ketoconazole, 8 to 0.016 µg/ml). The microtiter plates were incubated at 30°C for 48 h with shaking, and then the growth of cells in individual wells (the optical density at 590 nm (OD590)) was measured with a microplate reader (Oasis, 310) The MIC at which 80% of cells were inhibited (MIC80) was the lowest concentration of drug that inhibited the growth yield by at least 80% compared to the growth found for a no-drug control.

3.2.17 NTPase Assays

Yeasts were grown in YEPD (pH 5.5) at 30°C until they reached the late exponential phase of growth (OD600, 7), washed twice in ice cold distilled water, and incubated on ice for 2 h to minimize glucose-stimulated Pma1p activity. The cells were resuspended in homogenizing medium (50 mM Tris [pH 7.5], 2 mM EDTA, 1 mM phenylmethylsulfonyl

fluoride) and disrupted with a Braun homogenizer. Cell debris and unbroken cells were removed by centrifugation at 3,000 x *g* at 4°C for 10 min. A crude membrane fraction was isolated from the cell-free supernatant by centrifugation at 30,000 x *g* at 4°C for 45 min. Plasma membranes were prepared by centrifugation of the supernatant obtained after selective precipitation of mitochondria at pH 5.2, as described by Goffeau and Dufour (Goffeau and Dufour 1988). The plasma membranes were resuspended in 10 mM Tris (pH 7.0)–0.5 mM EDTA–20% (vol/vol) glycerol and were stored at -80°C. The protein concentration was determined by a micro-Bradford (Bio-Rad Laboratories, Hercules, Calif. (Bradford et al., 1976]) assay with bovine gamma globulin as the standard. Nucleoside triphosphatase (NTPase) activity was measured by incubating the plasma membrane fractions (10 µg) at 30°C in a final volume of 120 µl containing 6 mM nucleoside triphosphate (NTP) and 7 mM MgSO4 in 59 mM MES-Tris buffer (pH 6.0 to 8.0). To eliminate possible contributions from nonspecific phosphatases and vacuolar or mitochondrial ATPases, 0.2 mM ammonium molybdate, 50 mM KNO3, and 10 mM NaN3 respectively, were included in the assay mixtures (Monk et al., 1991). Other ATPase inhibitors (20 µM oligomycin, 20 µM aurovertin B, or 100 µM vanadate) were added to the reaction mixture where indicated. After 30 min the reaction was stopped by the addition of 130 µl of a solution containing 1% (wt/vol) SDS, 0.6 M H2SO4, 1.2% (wt/vol) ammonium molybdate, and 1.6% (wt/vol) ascorbic acid. The amount of inorganic phosphate released from NTPs was measured at 750 nm after 10 min of incubation at room temperature.

3.2.18 Rhodamine 6G efflux by *S. cerevisiae* Cells

The efflux of rhodamine 6G (Sigma) from intact *S. cerevisiae* cells was determined by adapting the method described by Kolaczkowski et al., 1996. Yeast cells from YEPD

cultures in the exponential growth phase (OD600, 0.5) were collected by centrifugation (3,000 x *g*, 5 min, 20°C) and washed three times with water. The cells were resuspended at a concentration of 0.5x 10^6 to 1.0x 10^7 cells per ml in HEPES-NaOH (50 mM; pH 7.0) containing 5 mM 2-deoxyglucose and 10 M rhodamine 6G. In some experiments fluconazole (10 μM) was also added. Cell suspensions were incubated at 30°C with shaking (200 rpm) for 90 min to allow rhodamine accumulation under glucose starvation conditions. The starved cells were washed twice in HEPES-NaOH, and portions (400 μl) were incubated at 30°C for 5 min before the addition of glucose (final concentration, 2 mM) to initiate rhodamine efflux. At specified intervals after the addition of glucose, the cells were removed by centrifugation, and triplicate 100μl volumes of the cell supernatants were transferred to the wells of 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark). The rhodamine fluorescence of the samples was measured with a Cary Eclipse spectrofluorimeter (Varian Inc., Mulgrave, Victoria, Australia). The excitation wavelength was 529 nm and the emission wavelength was 553 nm.

3.2.19 Use of Stock Metal Solutions Against Biofilm

Silver nitrate ($AgNO_3$), aluminum sulfate ($Al_2(SO_4)_3 \cdot 18H_2O$), zinc sulfate ($ZnSO_4 \cdot 7H_2O$), stannous chloride ($SnCl_2 \cdot 2H_2O$) and copper sulfate ($CuSO_4 \cdot 5H_2O$) were obtained from Fisher Scientific Company of Fairlawn, NJ. Nickel sulfate ($NiSO_4 \cdot 6H_2O$), mercuric chloride ($HgCl_2$), cobalt chloride ($CoCl_2 \cdot 6H2O$), lead nitrate ($Pb(NO_3)_3$), potassium tellurite (K_2TeO_3) and sodium selenite (Na_2SeO_3) were obtained from Sigma Chemical Company of St Louis, MO. Cadmium chloride ($CdCl_2 \cdot 5/2H_2O$) was purchased from Terochem Laboratories of Edmonton, AB, and manganous sulfate ($MnSO_4H_2O$) from BDH of Toronto, ON. Reagent grade metal and metalloid compounds were purchased for the

purposes of this study to minimize the potential influence of contaminating, residual metals. All stock metal solutions, with the exception of Sn^{2+}, were made up in double-distilled water at 5 times the highest concentration desired in the challenge plates. These stock solutions were passed through a 0.22 μm syringe filter into sterile glass vials and stored at room temperature. Sn^{2+} was dissolved in 50% ethanol and stored in a sterile polypropylene tube. Flat-bottom 96-well microtiter plates (Nunc) with a final volume of 200 μl. Serial two-fold dilutions of metals in YNB, complete synthetic medium (CSM; Himedia, India. with 0.02% (wt/vol) uridiidine), or CSM without uracil along the length of these microtiter plates, allowing the first well of each row to serve as a sterility control, and the last well to serve as a growth control. The plates were incubated for 24h at 35°C. The MBEC was determined by reading the optical density of the plate at 650 nm (OD650).

3.2.20 Rhamnolipid Isolation and Coating

Rhamnolipid was isolated and characterized from *Pseudomonas aeruginosa* (MTCC 2642) as described previously (Busscher et al., 1997; Santa et al., 2002; Siegmund 1999]. Polymer materials were coated with rhamnolipid using physical absorption. Briefly, a stock solution of the rhamnolipid (100mg/ml) was prepared in ethanol and 100μl amount was absorbed to both side of 1.0cm^2 test sample. The ethanol was allowed to evaporate resulting in biosurfactant absorbed. Control samples were coated on both sides with ethanol without biosurfactant added.

3.2.21 Screening of Plant Oils

The YPD agar plates were prepared and 50 μl, $5x10^8$ cfu/ml of the two strains of *C. albicans* (CA I- fluconazole resistance and CA II-clinical isolate) 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 30^0C. After 48 h of cultivation, the diameter of the zone was measured and considered as zone of inhibition (ZOI).

3.2.22 Determination of Minimum Inhibitory Concentration (MIC) of Plant Oils

MIC of plant oils was determined by agar dilution assay (NCCLS, 1997). The agar plates were prepared by in triplicates by adding YEP agar containing different concentrations of plant oils (i.e., 0.03–3% v/v). Tween-20 (0.5% v/v) was added to enhance the oil solubility. The plates were inoculated with 10^3 cfu, using the inocula of *C. albicans* prepared as above and incubated for 48 h at 30^0C. Plates with Tween-20 but without any plant oil were used as control. Number of colonies was counted after 48 h of incubation. The lowest concentration of plant oil required to completely inhibit the growth of *C. albicans* was designated as the MIC.

3.2.23 Silver Coating

The combination of magnetron sputtering with a neutral atom beam source to deposit silver coatings on thermally sensitive polymer substrates. The advantage of this combination is that the metal can be deposited at a substrate temperature of 70^0 C rather than 200–300^0 C as obtained by combining magnetron and r.f. plasma (substrate holder bias) sources. The atom beam (argon plasma current 120 mA at 0.1 Pa) was used both to activate the polymer surface for 60 s prior to coating deposition and also during the silver deposition process. The polymer samples were mounted approximately 35 cm from this source. The silver coatings were deposited using a 6.5 cm diameter cylindrical target and the deposition current used was 0.4 A. Coatings were deposited on pieces Polyvinylchloride (PVC).The coating deposition rate was obtained using glass slide samples mounted on the substrate holder beside the polymer sheets. The coating thickness was measured using glancing angle X-ray diffraction.

3.2.24 Statistical Analysis

The effect of each concentration of metal on *C. albicans* biofilm formation compared to biofilms formed in the absence of plant oils was analyzed using Student's t-test. Data with a P-value <0.05 were considered statistically significant.

CHAPTER 4 RESULTS

4.1 ISOLATION OF BIOFILM FORMING *C. albicans* FROM CLINICAL SAMPLES

Identification to the species level of yeast isolated from clinical specimens is often problematic for diagnostic laboratories. Reference procedures that use biochemical, morphological and temperature studies are often not practicable for the clinical laboratory because they are labor intensive and run over several weeks. Chromogenic agar medium is a simple, cost effective, selective and differential medium in which chromogenic substrate can be incorporated directly into the growth medium. This facilitates rapid detection of different *Candida* species from mixed culture on the basis of coloration and colony morphology.

4.1.1 Isolation of *Candida* species from Clinical Samples

Among thirty-six isolates of the four *Candida* spp. known to generate a distinct, identifiable color on CHROMagar Candida, including *C. albicans* (n = 15), *C. tropicalis* (n = 11), *C. glabrata* (n = 6), and *C. krusei* (n = 4) (Table 5, fig. 7). All the *C. albicans* and *C. tropicalis* isolates were readily identifiable by 48 h. All four *C. krusei* isolates produced the distinctive large, dry, pink colonies by 24 h. *C. glabrata* isolates form small colonies that produced a pink to dark purple coloration, sometimes with diffusion of pigment into the surrounding agar after 48 h of incubation. Other eleven isolates of *Candida* including *C. lusitaniae* (n = 3), *C. parapsilosis* (n = 3), *C. guilliermondii* (n = 2), *C. kefyr* (n = 2), and *C. firmetaria* (n = 1) typically produce small, creamy colonies that are white, pale pink, or light lavender on CHROMagar- Candida and are not distinguishable to the species level (Table 5, fig. 7). A distinct appearance produced by *C. rugosa* on CHROMagar Candida. *Candida rugosa* grew in small, dry colonies that possessed a pale or white border.

4.1.2 Screening of *C. albicans* for Biofilm Formation

All fifteen isolates of *C. albicans* were screened for biofilm formation by incubating them for 48h at 37^0C. Data showed that isolate no. 12 form maximum biofilm (Fig. 6). This strain of *C. albicans* was used for further investigations.

Table 5: Colony characteristics of *Candida* isolates grown on Chromogenic Candida agar

Species	No. of isolates	Colony characteristic(s)
Candida albicans	15	Green
Candida krusei	4	Pink, flat, rough, pale border
Candida tropicalis	11	Light green
Candida glabrata	6	Dark violet
Candida parapsilosis	3	small, creamy colonies, pale pink, or light lavender in color.
Candida lusitaniae	3	
Candida guilliermondii	2	
Candida kefyr	2	
Candida firmetaria	1	
Candida rugosa	1	

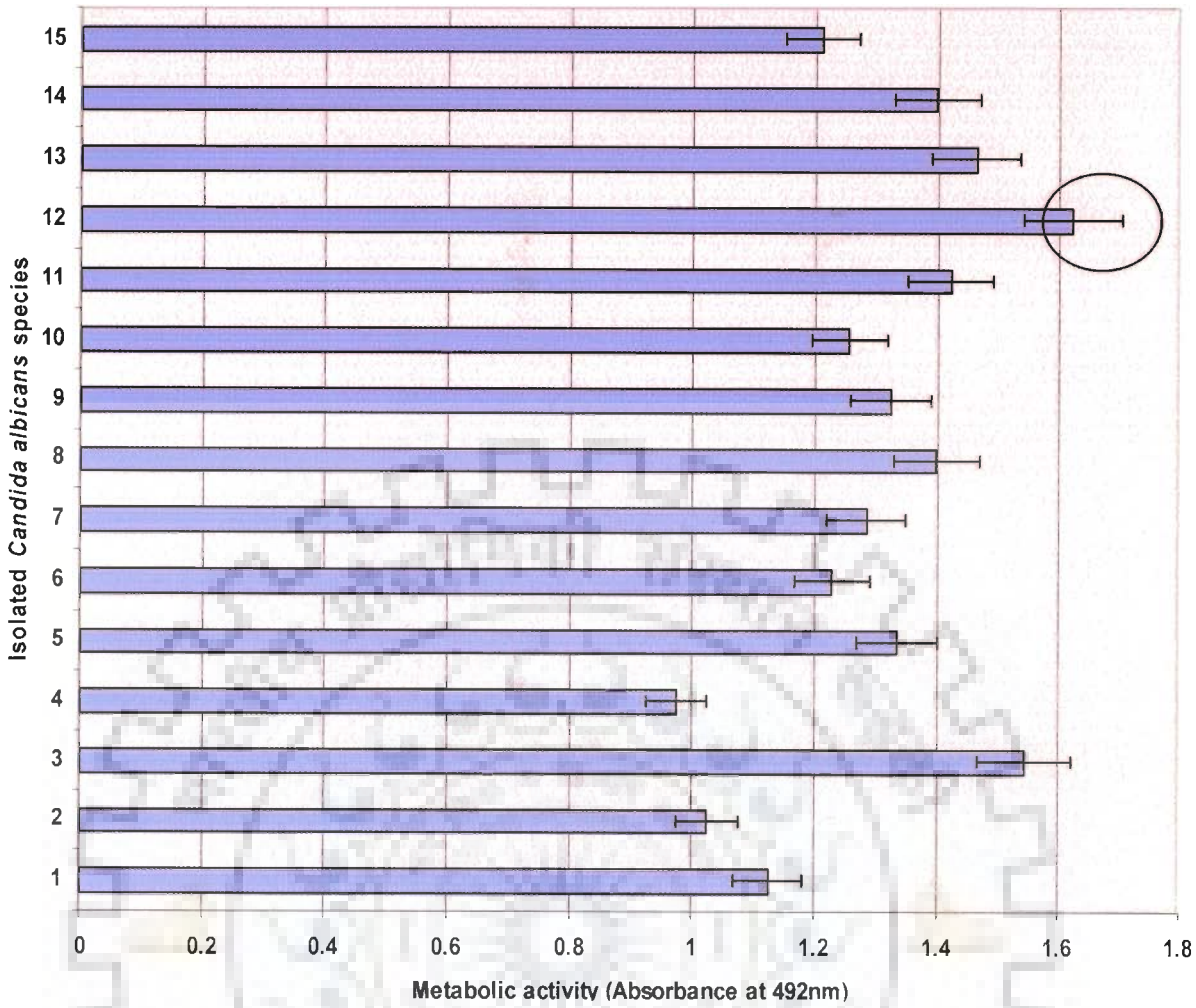
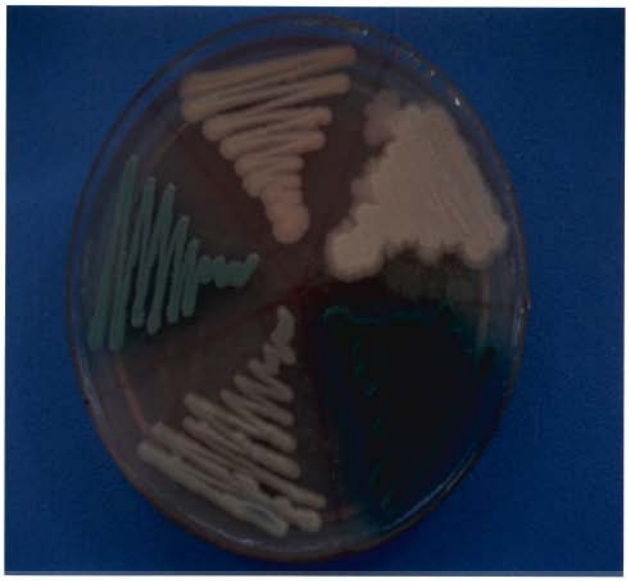


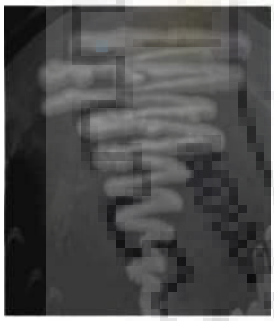
Fig. 6: Biofilm formation by different *C. albicans* isolates after 48h



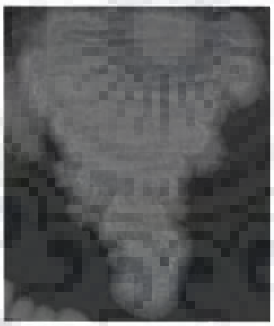
Chromogenic agar plate

Candida albicans

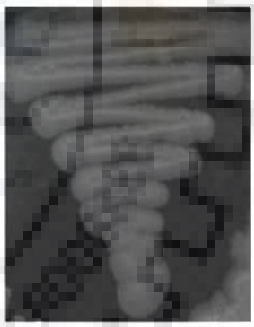
Candida tropicalis



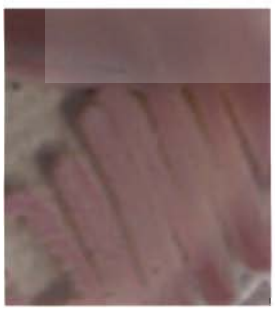
Candida lusitaniae



Candida kefyr



Candida guilliermondii



Candida krusei

Candida glabrata

Candida parapsilosis

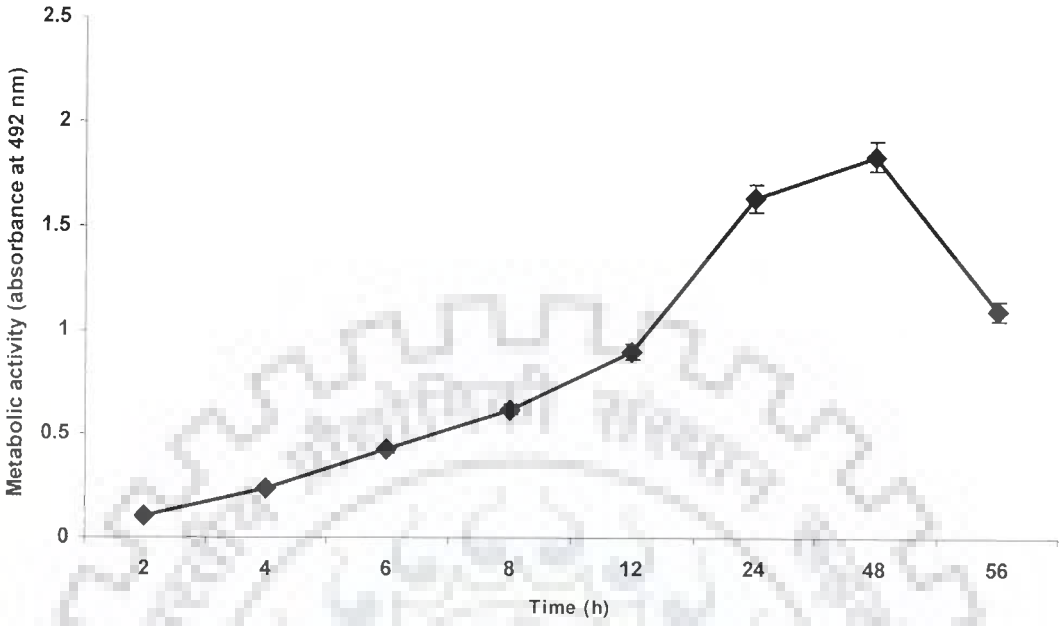
Fig. 7: Growth of different *Candida* species on Chromogenic-candida agar

4.2 PHYSICO-CHEMICAL CHARACTERIZATION, OPTIMIZATION OF GROWTH CONDITIONS AND MORPHOLOGICAL ANALYSIS OF *C. albicans* BIOFILMS

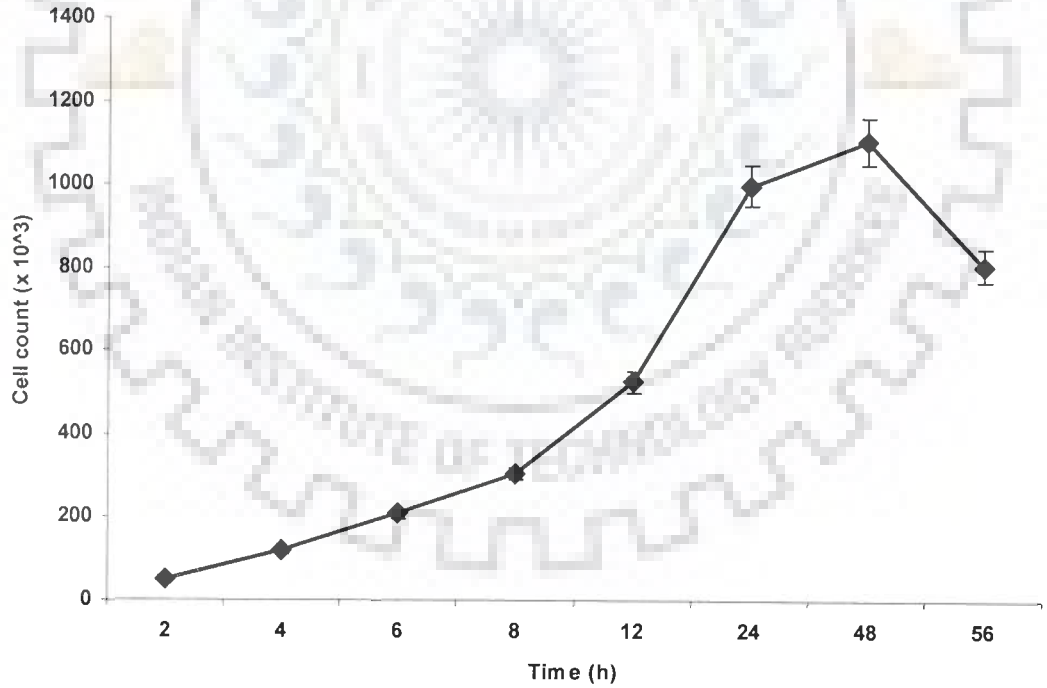
C. albicans is capable of invading virtually every site on the body, including deep tissues and organs, upper sites such as skin, nails and mucosa. Superficial infections, such as acute pseudomembranous infections of the oral cavity or vagina, are some of the most frequently encountered. To look deep inside the biofilm, study of biofilm characteristics, morphology and different growth condition was carried out.

4.2.1 Characteristics of Biofilm Formed by *C. albicans*

Adherence and subsequent biofilm formation by *C. albicans* on the surface of polystyrene wells over 56 h, as determined by the colorimetric XTT-reduction assay, and cfu counts are illustrated in fig. 8A and B. The production of the soluble colored formazan salt from sessile cells, a direct reflection of cellular metabolic activity, increased over time with the increased sessile cellular density. The biofilms were highly metabolically active in the first 8 h, but as the biofilm matured and the complexity increased (24 to 48 h) the metabolic activity reached a plateau, but remained high probably reflecting the increased number of cells that constituted the mature biofilm. Experiments were performed in sets of eight replicates, with similar results obtained in all experiments. As biofilm maturation occurred (24 and 48 h growth), the complexity of the biofilm increased into a multilayered biofilm matrix.



(A) XTT reduction assay



(B) Biofilm cell count

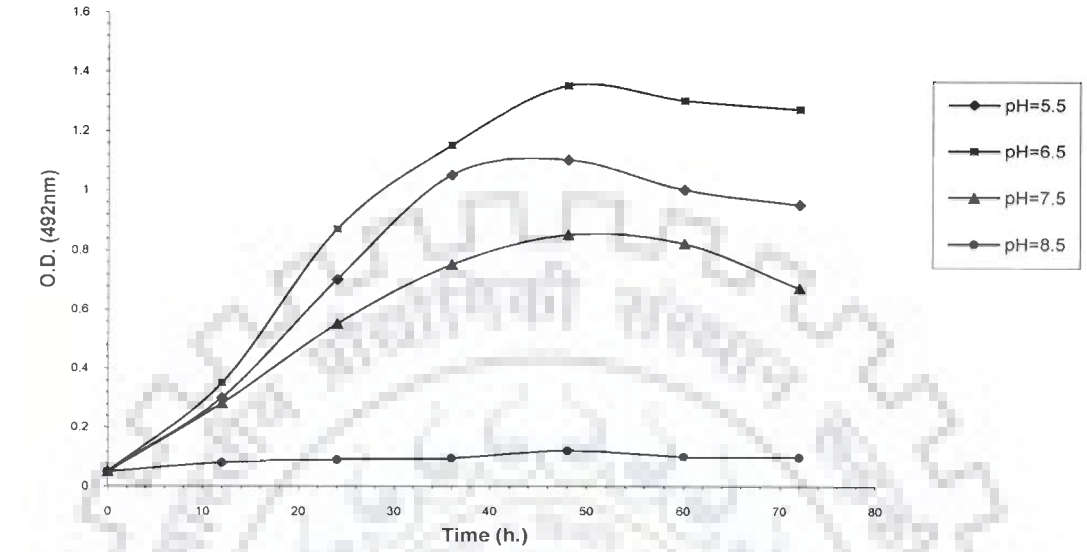
Fig. 8: *C. albicans* biofilm formation at different time intervals. (A) Biofilm quantification by XTT reduction assay, (B) Biofilm cell count

4.2.2 Temperature and pH Optimization for *C. albicans* Biofilm

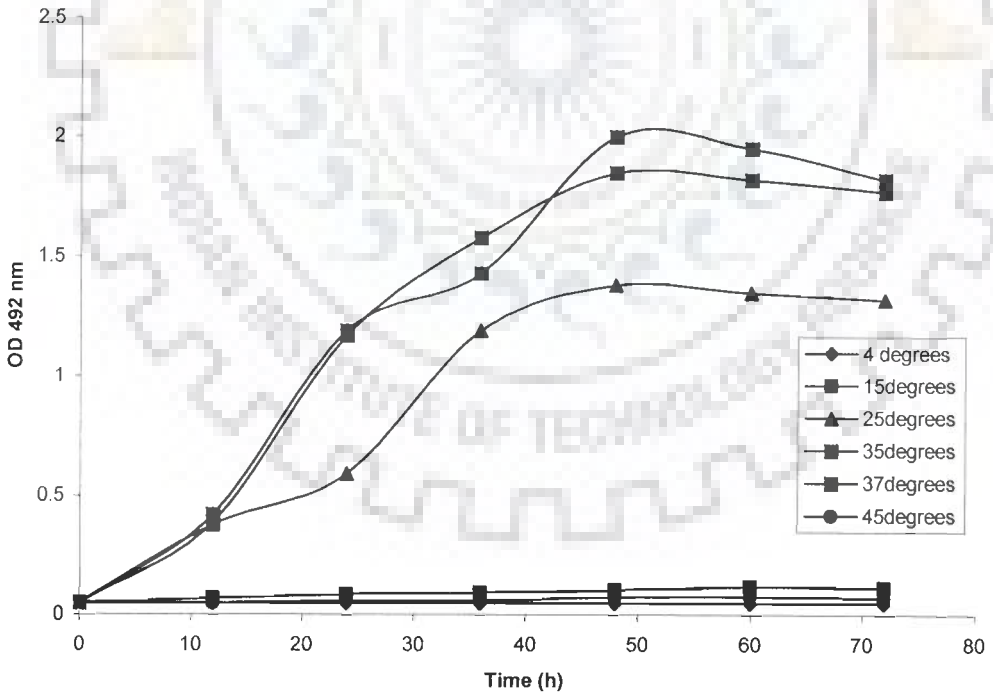
Our studies showed that maximum biofilm formation takes place at 35^0 C and 6.5 pH. Also, results didn't show much difference in biofilm formed at 35 or 37^0C which revealed a better adaptation of isolated *C. albicans* towards biofil formation near to range of body temperature (fig. 9A, B).

4.2.3 Quantitative Measurement of 48 h Grown *C. albicans* Biofilm with Shaking or Static Incubation

Data revealed that maximum biofilm formation takes place under shaking condition at 20 rpm. Biofilm formation under shaking condition shows better conditioning of media component to biofilm surfaces, better homogenization and hence better availability of nutrients and oxygen (Table 6).



(A)



(B)

Fig. 9: Effect of pH (A) and Temperature (in ^{0}C) (B) on *C. albicans* biofilm

Table 6: Effect of different shaking conditions on 48 h grown *C. albicans* biofilm

Shaking speed (rpm)	Dry weight (mg)	XTT reduction assay (absorbance at 492 nm)
0	1.9	1.84
5	1.92	1.90
10	1.95	2.00
20	2.21	2.12
30	2.05	2.01
40	1.63	1.61
50	1.21	1.10

4.2.4 Effect of Different Carbon Sources on Biofilm Formation and EPS Yield

To study the effect of different carbon sources for the EPS production and biofilm formation, *C. albicans* biofilm was grown in media containing different carbon sources at a concentration of 2% (w/v) for 48 h. Among six carbon sources examined, arabinose was found to increase biofilm formation and EPS production while nearly same amount of EPS was produced with galactose, mannose and sucrose.

Data revealed that with increase in EPS production biofilm formation also increases. Maximum biofilm formation was observed with arabinose which was found to be 42% more than that of control (dextrose) and minimum in case of sucrose which showed 60% reduction in biofilm (Table 7).

4.2.5 Effect on EPS Composition

Major changes in EPS composition with response to different carbon sources were observed. Fructose, sucrose, arabinose and xylose showed an increase in total sugar content which is a major component of matrix polymer, while glucose content was reduced in all cases. A significant increase in pentose and phosphorus content was observed with arabinose, fructose and xylose. Hexosamine content was reduced to nearly half when Galactose and mannose were used as sole carbon source (Table 8).

Table 7: *C. albicans* biofilm formation and EPS production with different carbon sources

Carbon sources	EPS (g/l)	Biofilm formation (A_{492})
Control	0.852	2.0040
Arabinose	0.974	2.8252
Fructose	0.816	1.8532
Galactose	0.670	1.0100
Mannose	0.694	1.0900
Sucrose	0.642	0.9180
Xylose	0.746	1.6001

*Control means the medium containing 2% dextrose

Table 8: EPS composition of 48 h grown *C. albicans* biofilm in response to different carbon sources

Carbon source	Total Sugars	Glucose	Hexosamine	Pentose	Protein	Phosphorus
Control*	84.2	4.20	0.14	0.99	6.86	0.26
Arabinose	87.2	1.69	0.12	5.01	5.76	2.59
Fructose	88.9	3.26	0.13	6.27	7.69	0.29
Galactose	82.6	0.99	0.06	0.43	6.92	1.28
Mannose	82.2	2.59	0.06	5.85	7.93	0.49
Sucrose	91.8	0.88	0.18	0.68	5.48	0.86
Xylose	92.5	0.87	0.21	4.24	4.44	2.09

* Control means the medium containing 2% dextrose.

4.2.6 Effect of Serum and Saliva Conditioning Films on *C. albicans* Adhesion and Biofilm Formation

The effect of serum and saliva conditioning films on *C. albicans* adherence and biofilm formation is shown in fig. 10. When serum was provided as a conditioning film the level of *C. albicans* adherence was significantly elevated in comparison to that observed in untreated wells. The effect was most clearly demonstrated during early adherence at 30 min there was a 314% compared to control values adherence when compared to the control ($p < 0.0001$). After 2 h, 4 h, 6 h, 8 h, 12 h and 24 h there was a smaller difference observed between biofilm formation in the presence or absence of the serum pellicle (representing 152%, 151%, 124%, 152%, 139%, 113% and 123% of the corresponding values for binding to polystyrene respectively), which did not represent statistically significant differences and no significant differences were observed after 48 h and 56 h (fig. 10). In contrast to serum, the effect of saliva preconditioning films was minimal. Initial adhesion at 30 min was marginally increased with 79% increase when compared to the control, while after consecutive time intervals there was only a minimal effect in biofilm metabolic activity, with differences not being statistically significant ($p=0.7843$).

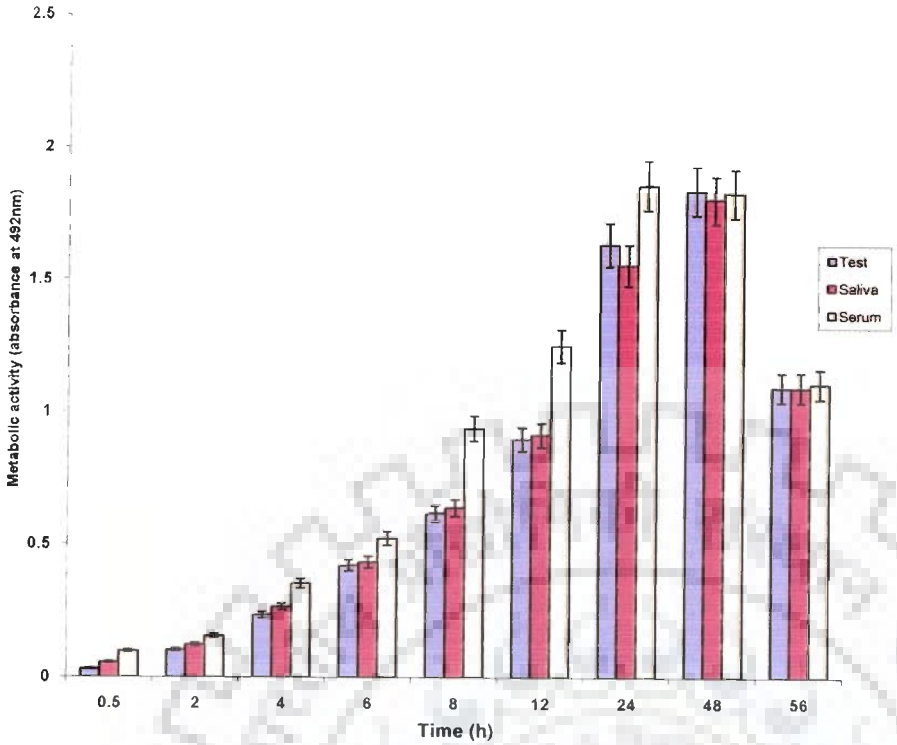


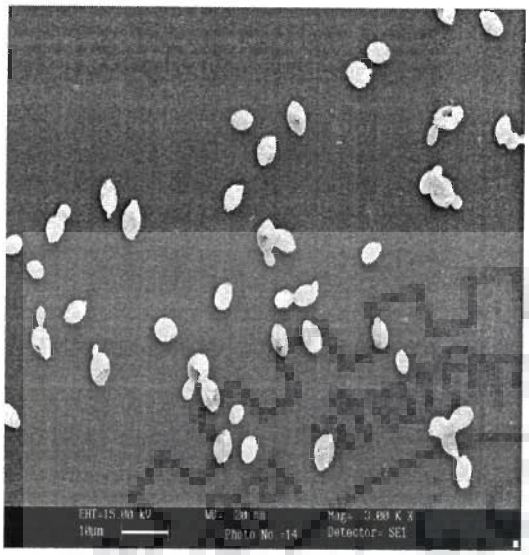
Fig.10: Effect of serum and saliva conditioning on *C. albicans* adhesion and biofilm formation

4.2.7 SEM and CSLM Visualization of *C. albicans* Biofilms

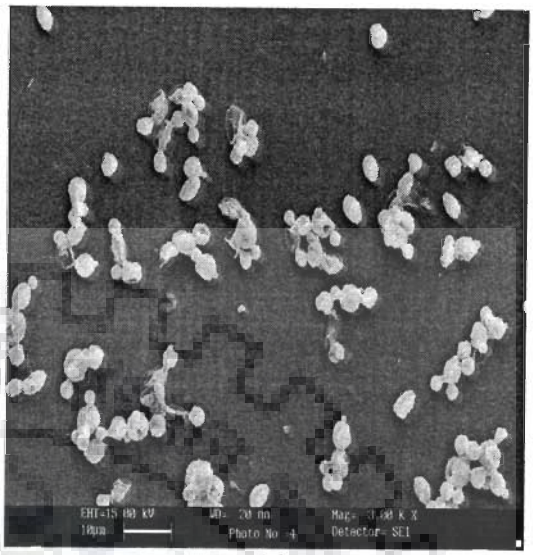
Biofilm formation by *C. albicans* on PP discs was monitored by SEM (Fig. 11). Despite its destructive nature, SEM observations provided useful information on the different cellular morphologies present in the biofilm structure. Initial adherence of yeast cells was followed by germ tube formation and subsequent development of hyphae (12 h and 24 h). Mature biofilms consisted of a dense network of cells of all morphologies, deeply embedded in matrix consisting of expolymeric material (24 h and 48 h). Biofilm with less cells density was observed after 56 h depicting biofilm detachment and reduction due to accumulation of metabolites and waste products.

The non-invasive CSLM technique enabled imaging of intact biofilms and visualization of the three dimensional distribution of labelled *C. albicans* cells in the context of the complex biofilm community. Significant channeling and porosity were observed. Overall, results indicated that mature *C. albicans* biofilms displayed typical microcolony/water channel architecture with extensive spatial heterogeneity. A three dimensional reconstruction of a 24 h old, 26.6 μm thick *C. albicans* biofilm after staining with FITC-ConA (green, fig. 12a) and PI (red, fig. 12b) resulting from the compilation of a series of individual *xy* sections taken across the *z* axis.

Further analysis of typical micro-colony inside the 24 h grown biofilm showed that the structures are covered with dense EPS matrix (FITC-ConA, green color) produced by cells inside them(fig. 12c). Staining of the micro-colony with PI showed the cells as red color and EPS containing spaces or cavities in black color spot (fig. 12d) suggesting secretion of EPS as cell over-layers.

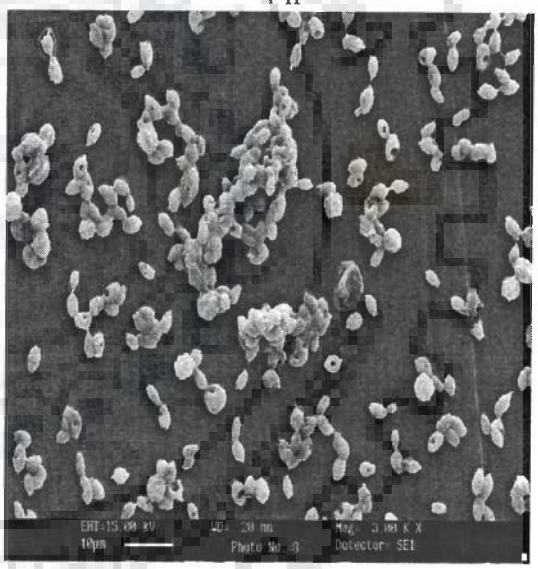


2 h



4 h

6 h



8 h

(Continued….)

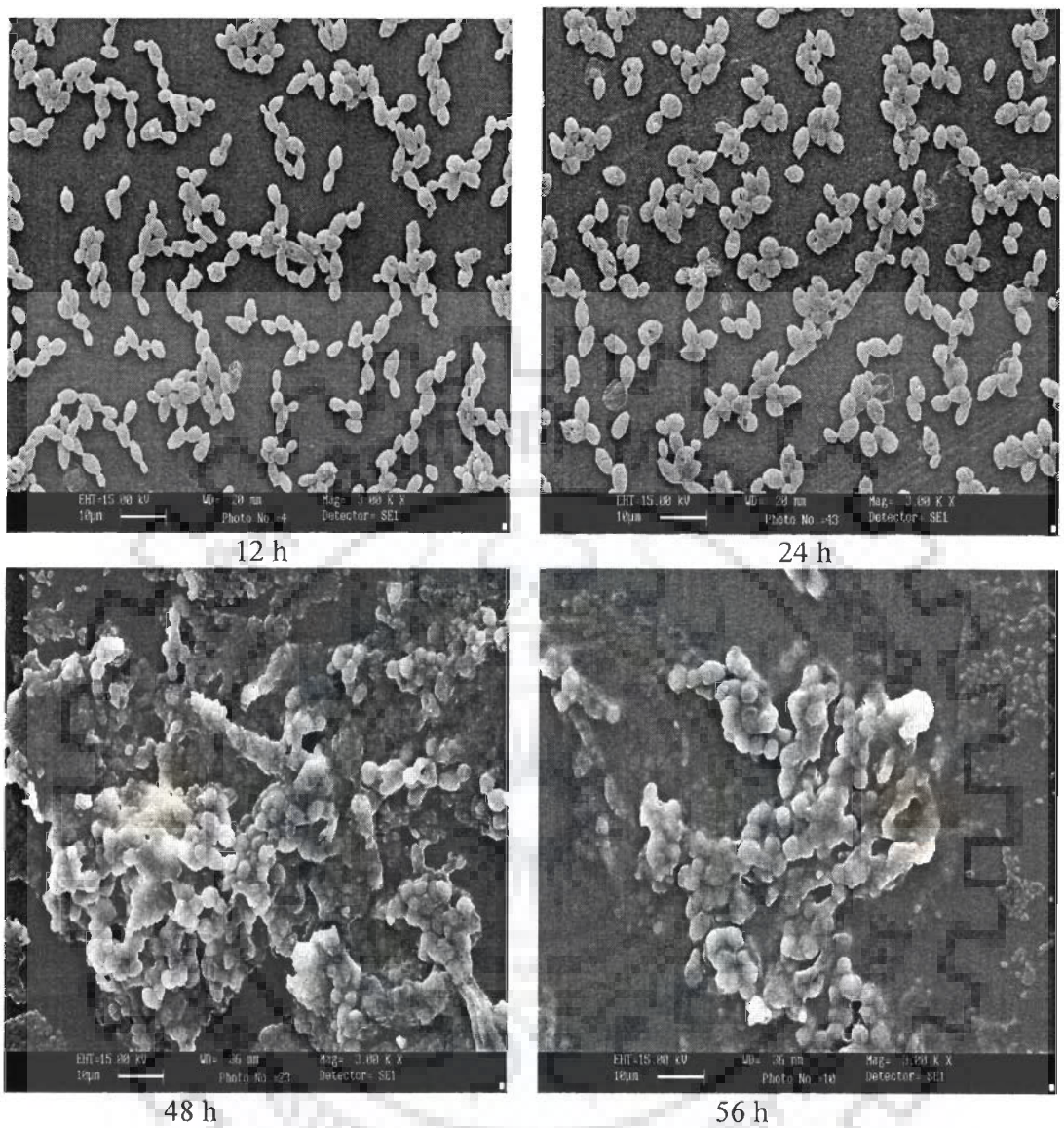


Fig. 11: Scanning electron microscopy of *C. albicans* biofilm at different time intervals

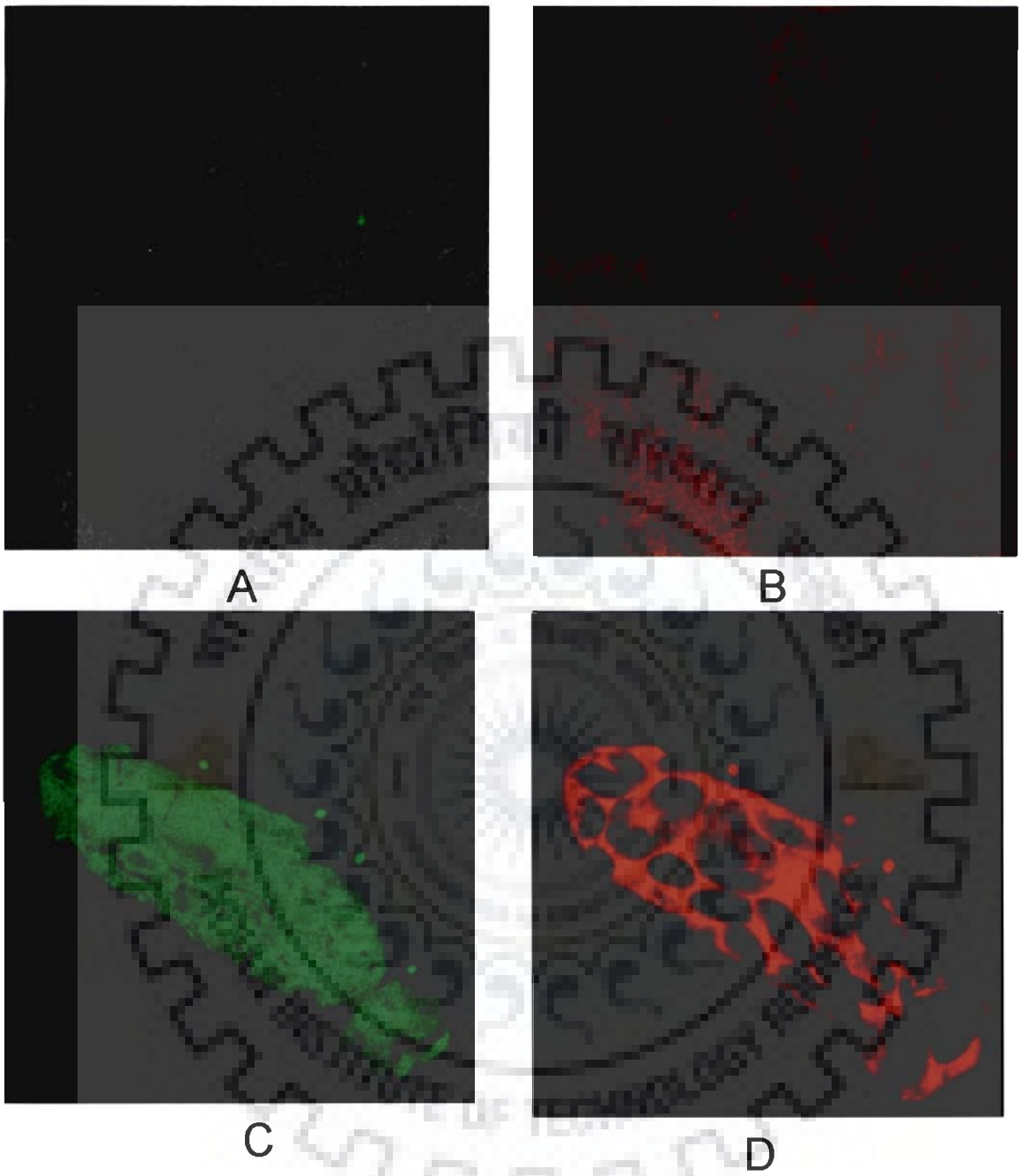


Fig. 12: CLSM photographs of 48 h *C. albicans* biofilm after staining with (A) FITC-ConA, (B) PI and (C & D) germ tube containing germinating candidal cells

4.2.8 Susceptibility Testing of *C. albicans* Biofilms Against Clinically Used Antifungal Agents

The *in vitro* activity of clinically used fluconazole, ketoconazole, clotrimazole, itraconazole, nystatin, caspofungin, variconazole and amphotericin B against pre-formed *C. albicans* biofilms was assessed using the XTT-reduction assay. Experiments revealed the increased resistance of sessile *C. albicans* cells compared to their planktonic counterparts (Table 9).

The antifungal agents tested showed less activity against 48 h biofilms compared to planktonic MIC's, as the SMIC50s and SMIC80s were generally much greater than the concentration of antifungal required to inhibit planktonic cells. Data revealed that *C. albicans* biofilms were intrinsically resistant to fluconazole (MICs >25 μg/ml), and the activity of nystatin, caspofungin and other azole derivative like ketoconazole, clotrimazole, itraconazole, variconazole against biofilms was reduced about 27, 11, 100, 3, 2, >250 times respectively compared with their activities against planktonic cultures. Amphotericin B demonstrated certain activity against *C. albicans* biofilms, as indicated by SMIC80s (1.9 μg/ml), but this concentration is generally regarded as resistance, due to the high toxicity of this drug. Importantly, complete killing of cells within the biofilms was never achieved, as reflected by residual metabolic activity of biofilms at concentrations up to 25 μg/ml.

Table 9: Drug susceptibility of *C. albicans* planktonic and sessile cells

Drug	Planktonic		Sessile	
	MIC_{50}	MIC_{80}	$SMIC_{50}$	$SMIC_{80}$
Fluconazole	3.5	4.5	>25	>25
Ketoconazole	0.05	0.06	1.5	6.0
Clotrimazole	1.0	1.2	2.0	3.2
Itraconazole	0.5	1.2	0.8	2.0
Nystatin	0.2	0.3	2.5	8.0
Caspofungin	0.5	0.7	0.3	7.5
Variconazole	0.06	0.1	5.0	>25
Amphotericin B	0.8	1.0	0.9	1.9

4.3 STUDIES OF MOLECULAR INTERACTION OF *C. albicans* BIOFILMS FORMED ON DIFFERENT BIOCOMPATIBLE MATERIAL SURFACES

Though, much work has been done on morphological and physiological stages of *Candida* biofilm formation on different biomaterial surfaces however, the molecular and physical interactions that govern adhesion to biomaterials has yet to be deciphered. In the present investigation we have focused on the impact of *C. albicans* biofilm on different biomaterial surfaces, and tried to establish its correlation with its surface properties.

4.3.1 Surface Properties of Biomaterials

The roughness and hydrophobicity of biomaterial samples were measured in order to determine surface characteristics which may account for adhesion of *Candida* cells. Among different biomaterial samples tested the order of roughness recorded was PS<SR<PP<PMMA<PVC (Table 10). AFM data of different biomaterials depicted maximum number of grooves and crusts in case of PVC while no such deformities were reported in case of PS (Fig. 13). PVC with maximum contact angle (97^0) indicated higher hydrophobic nature in comparison to other biomaterials tested (Table 10).

4.3.2 Biofilm Quantification

Biofilm quantification on different biomaterial samples were performed using XTT reduction assay to measure variation in metabolic activity of biofilm residing *C. albicans* cells. Maximum biofilm formation was measured in case of PVC followed by PS and PP, while very less candidal adherence was observed in case of SR and PMMA when compared to PVC (Fig. 14).

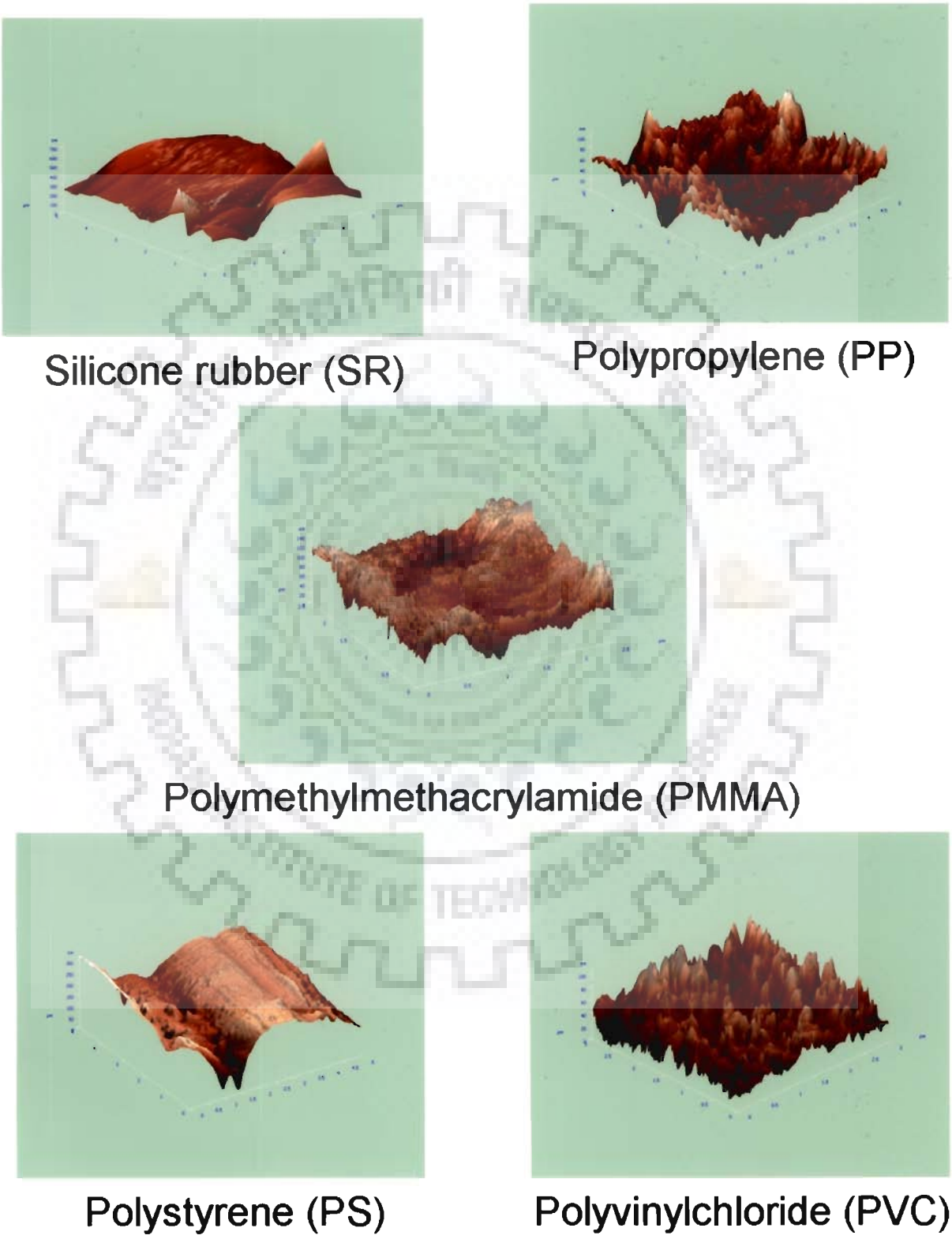
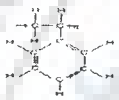
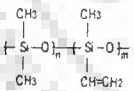
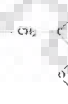


Fig. 13 : Atomic force microscopy of biomaterial surfaces showing roughness in 5x5 µm area

Table 10: Average contact angle, roughness, amount of exopolysaccharides produced and thickness of *C. albicans* biofilm on different biomaterial surfaces. Data represents result of three individual experiments plotted in triplicates and shown as ± standard deviation

Biomaterials	Structures	Contact angle±SD (°)	Roughness (nm)	EPS (μg /cm^2 area of polymer)	Biofilm thickness (μm)
Polyvinylchloride		97±1.20	134±0.8	11.45±1.2	117.5±3.2
Polystyrene		91±0.07	24±2.2	9.41±0.9	66±1.7
Polypropylene		84±1.2	43±1.6	8.65±0.6	26.6±2.3
Silicone rubber		77±1.3	27±0.6	6.95±0.7	10.5±1.8
PMMA		0±0	48±1.3	3.92±0.5	9.6±1.1

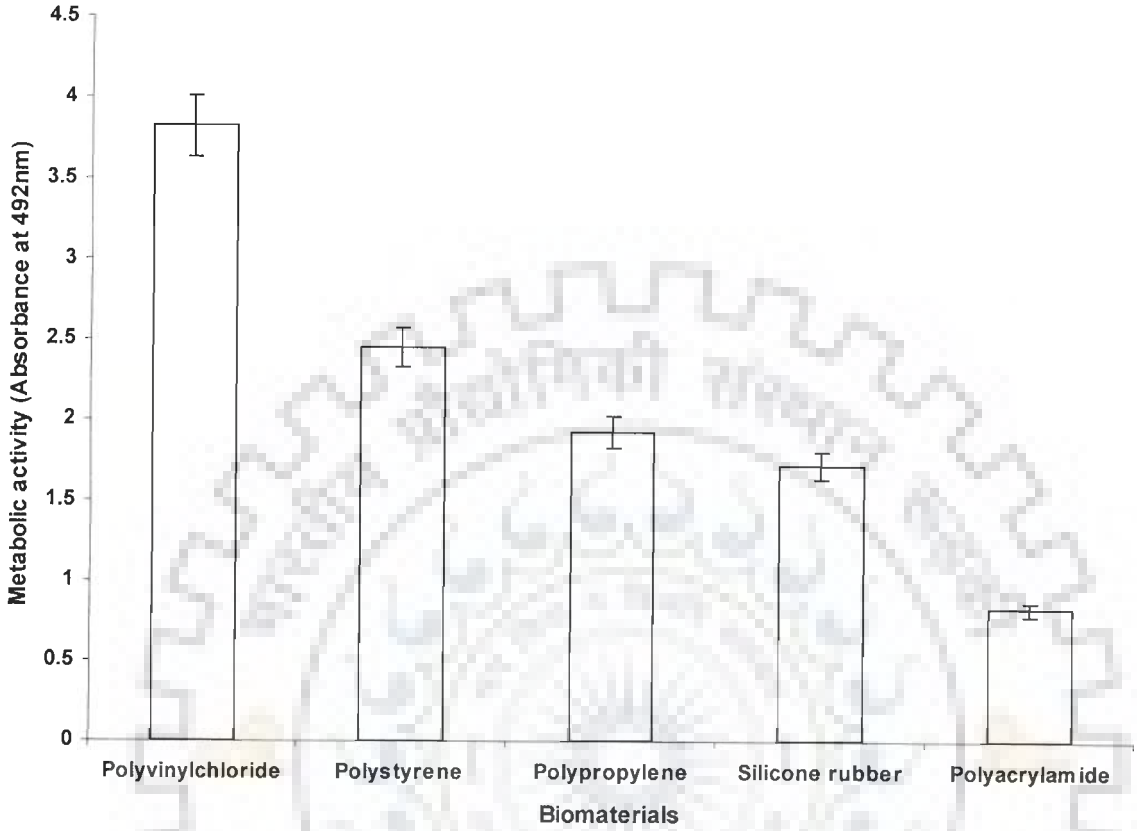


Fig. 14: Quantification of *Candida* biofilm by XTT reduction assay $P < 0.05$ (n=3) on different biomaterial surfaces

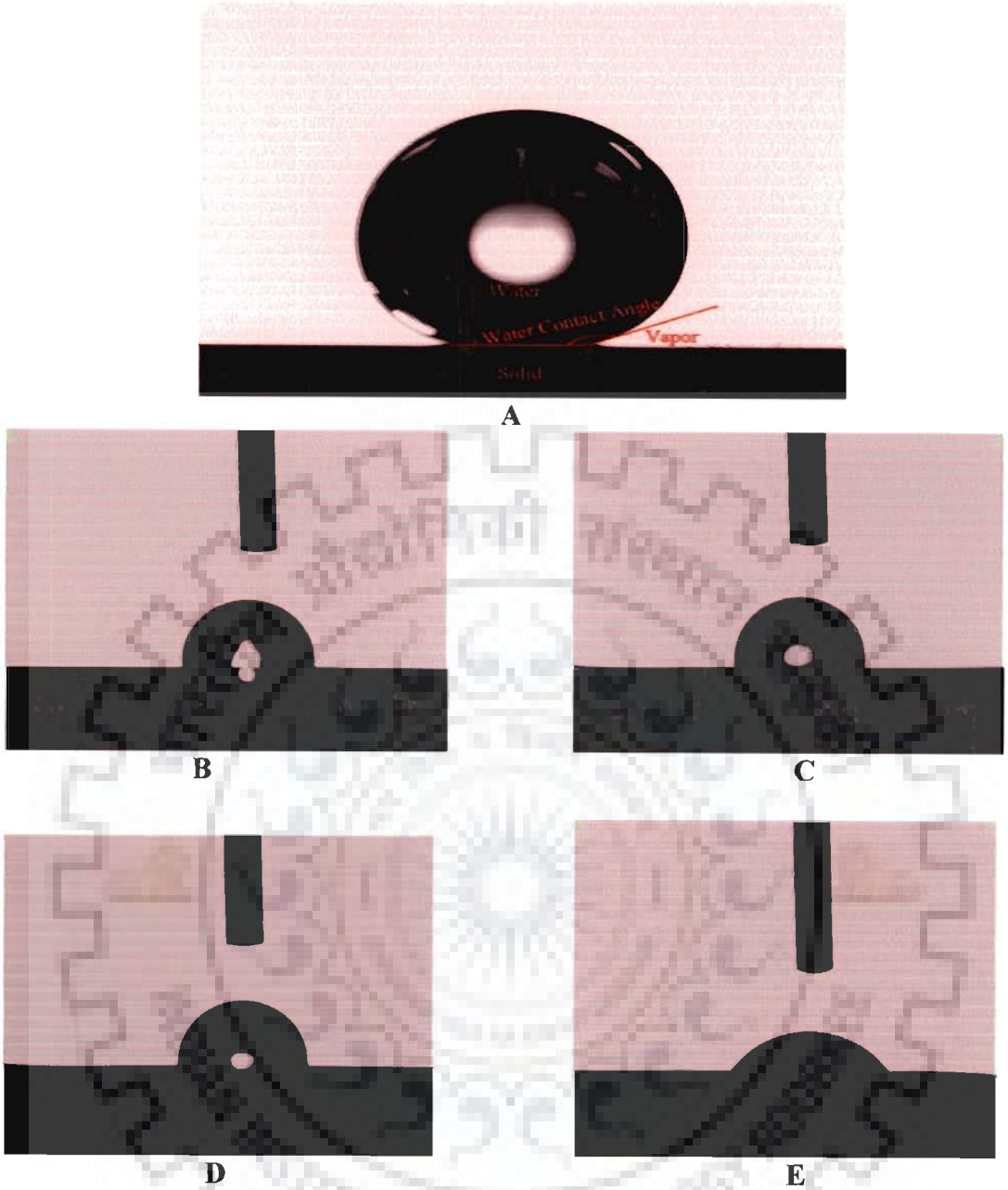


Fig. 15: Goniometric analysis of surface-water contact angle measurement. (A)Reference figure (B) PVC (C) PS (D) PP (E) Silicone rubber.

4.3.3 CLSM Studies of Biofilm Growth Patterns on Different Biomaterials

z sectioning of *C. albicans* biofilm on different biomaterial surfaces as well as red (PI) and green (FITC-ConA) intensity gave pattern of change of intensities along with biofilm thickness. The intensity-thickness graph data depicted differential growth pattern of both the cells and EPS in term of surface dependant growth of biofilm (fig 16).

PVC and PS showed similar pattern of biofilm growth. In both the cases green fluorescent intensity found to increase towards upper layers with slight increase in red intensity at mid-thickness of biofilm. The data clearly demonstrates that a higher EPS production takes place in case of upper layers of *C. albicans* biofilm when grow on PVC or PS surfaces.

Again similar growth pattern was observed both in case of PP and silicone rubber in which both the red and green intensities were found to increase proportionally with enormous increase in green intensity near upper layers. PMMA showed a different biofilm growth pattern with decreasing green intensity gradient from basal to top layers. Data shows that cells at the basal layers secrete more EPS than cells of upper layers.

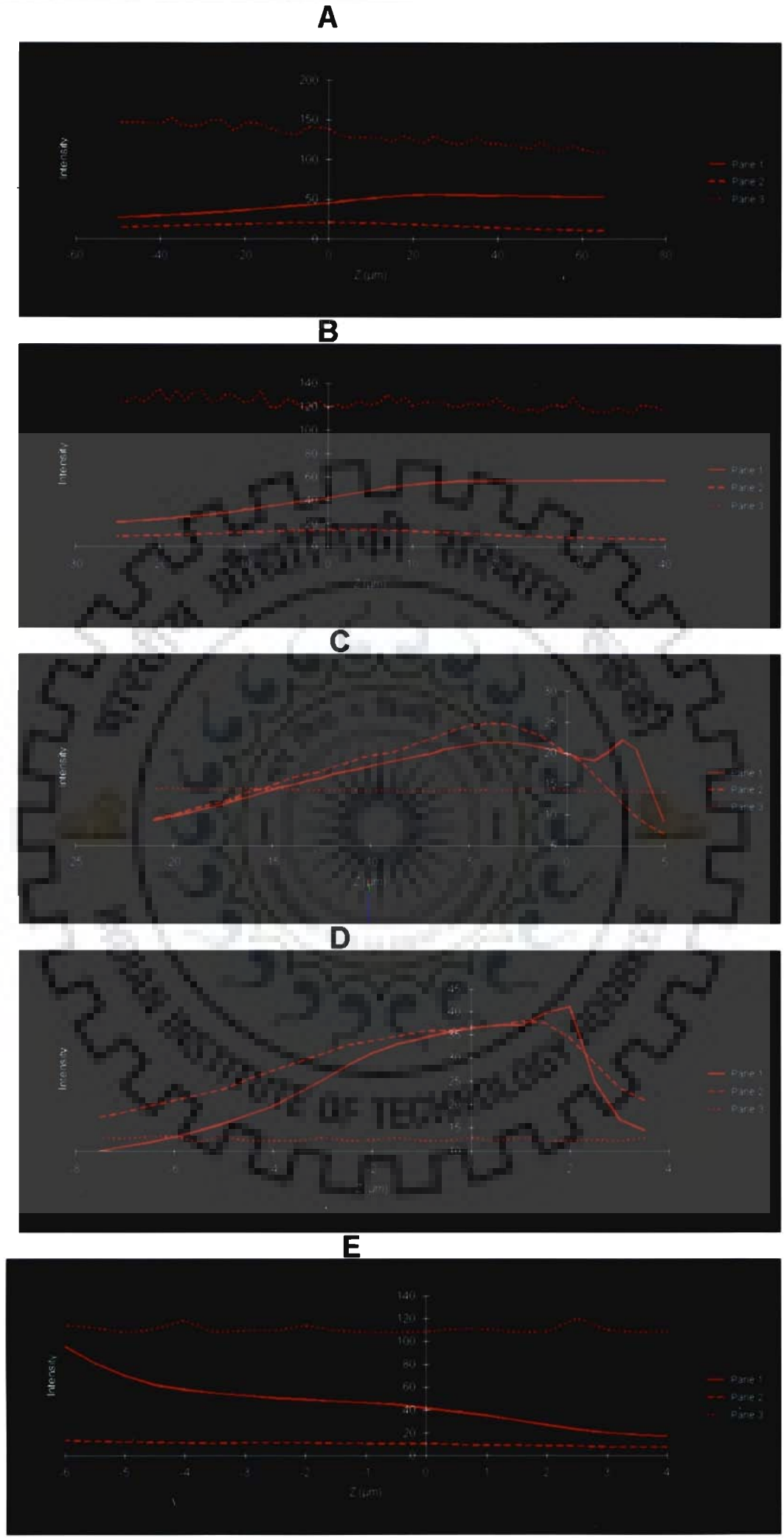


Fig.16: Thickness-intensity graph of *C. albicans* biofilm on different biomaterial surfaces (A) PVC, (B) PS, (C) PP, (D) Silicone rubber, and (E) PMMA

4.3.4 EPS Quantification

Amount of EPS produced on 1.0 cm^2 pieces of biomaterial samples were quantified and it was found that maximum 11.45 µg EPS was produced over the PVC surface while the lowest amount of EPS was produced in case of PMMA (Table 10).

4.3.5 CLSM Studies of Surface Biofilm Growth

C. albicans biofilm on biomaterial samples were observed using CLSM. The z-sectioning for biofilm thickness, intensity measurements and exopolysaccharides production after staining with PI, and FITC-ConA illustrated that PVC had maximum fluorescent intensities followed by PS, PP, SR and PMMA (Fig. 17). When CLSM images with red and green fluorescent intensities were superimposed, yellow color (green + red) revealed that the extracellular FITC-ConA – reactive polysaccharide (green) was produced in the intracellular spaces (red) indicating thereby that extracellular polysaccharides were produced as a capsular component in biofilm. The z-sectioning of biofilm formed on biomaterials was done and thickness was calculated by 0.5µm^2 sectional analysis. Results showed maximum biofilm thickness in case of PVC was 117.5 µm followed by 66.0, 26.6, 10.5, and 9.6µm with PST, PP, SR, and PMMA respectively (Table 10). Interestingly green intensity was recorded to be more in case of PMMA than to SR surface while red intensity was found to be more in case of SR surface.

4.3.6 SEM Analysis

The XTT reduction data was further confirmed by SEM analysis. The data showed different biofilm structures on different biomaterials surfaces with maximum colonization in case of PVC (fig. 18). Least adherence was observed in case of PP and SEM of PMMA could not performed due altered surface structure and shrunken of PMMA surface (PMMA) during SEM analysis.

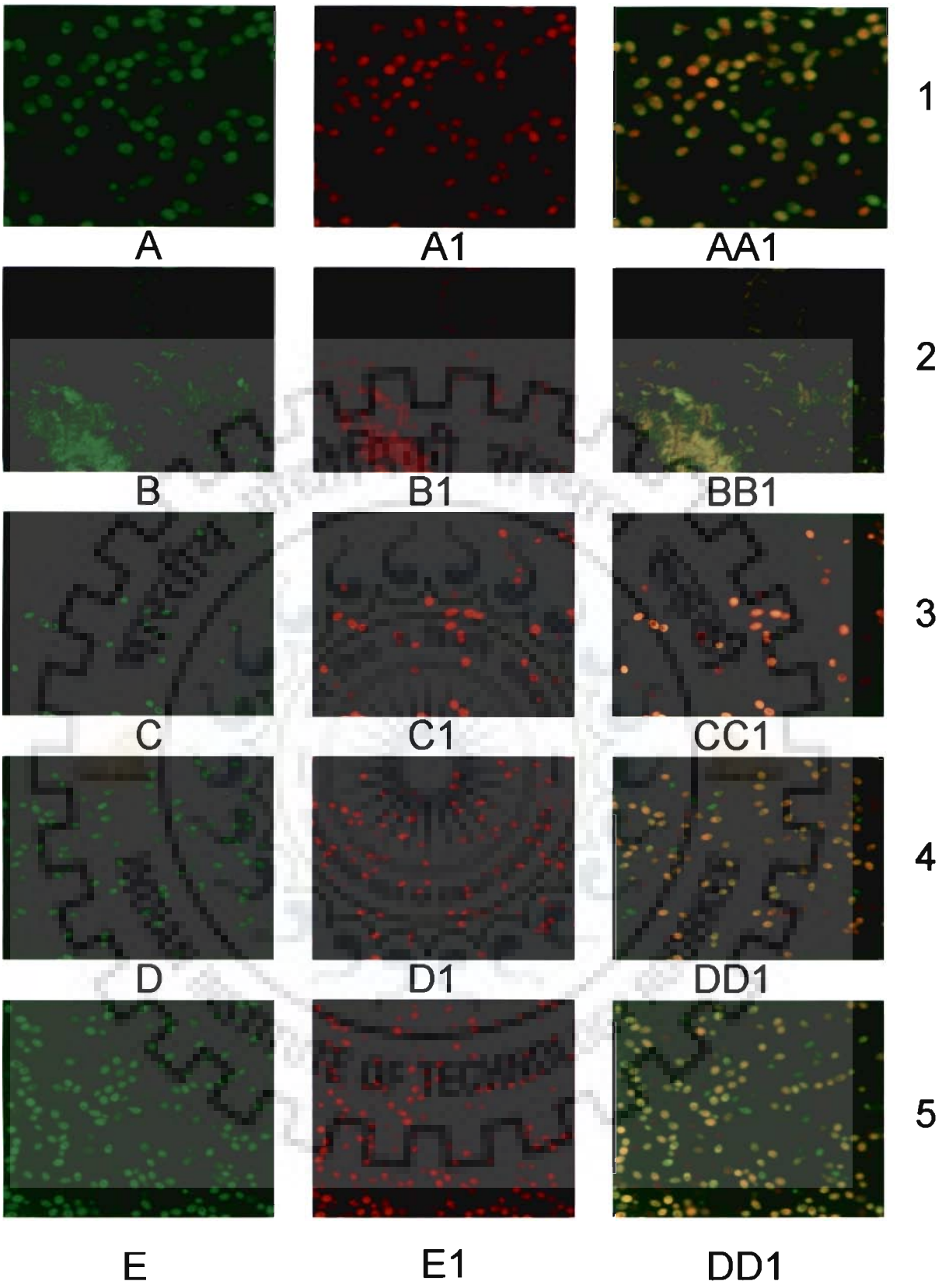


Fig. 17: CLSM images of Candida biofilm formed on different biomaterial surfaces, row 1, 2, 3, 4, and 5 shows PP, SR, PMMA, PS, and PVC respectevely. Green images shows exopolysaccharides by FITC-ConA staining (A-E), Biofilm forming PI stained sessile cells in red (A1-E1), resulting image (AA1-EE1) produced by overlap of green and red images showing production of exopolysaccharide as capsular component in yellow

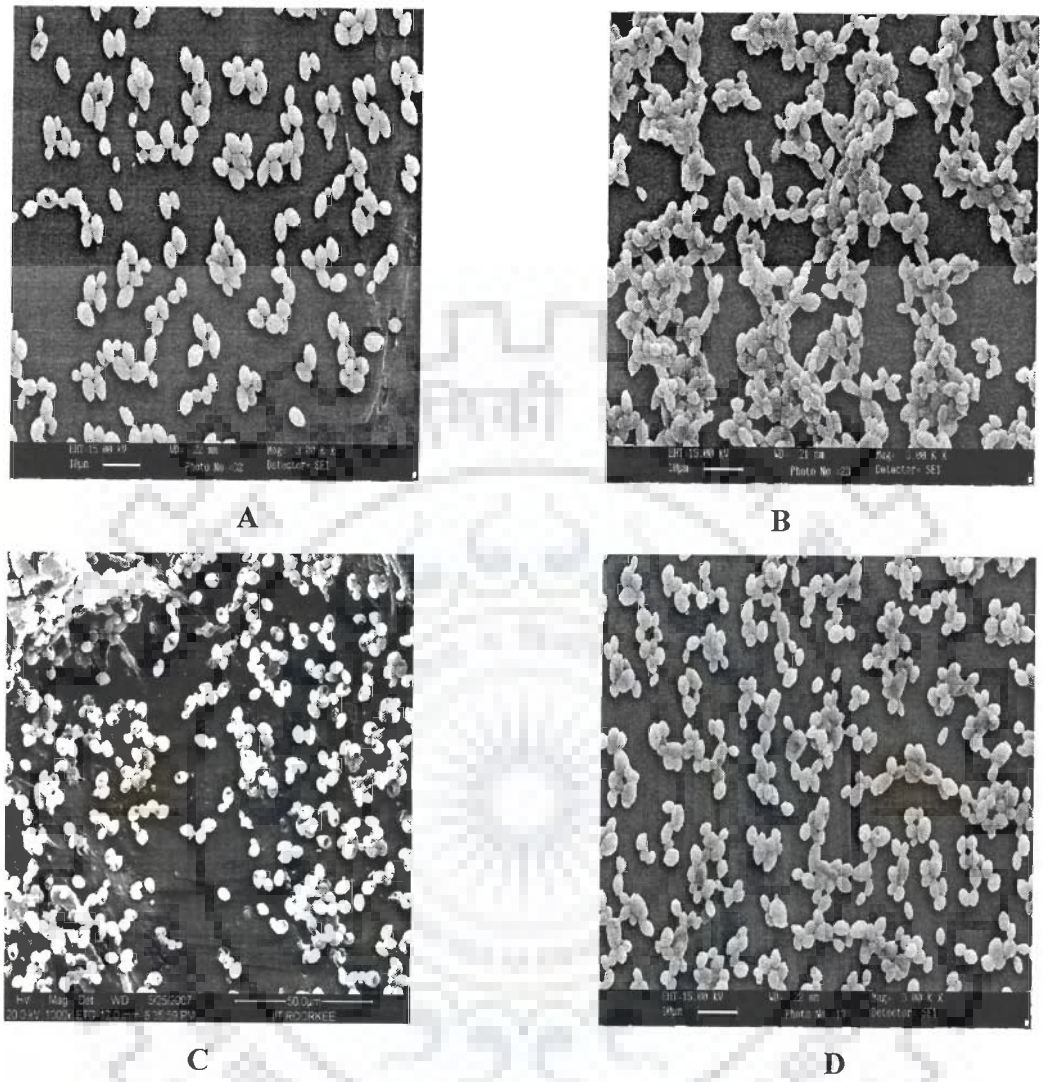


Fig. 18: SEM of *C. albicans* biofilm formed on different biomaterial surfaces (A) PP (B) PVC (C) silicone rubber (D) PS

4.4 CLONING AND CHARACTERIZATION OF GENE INVOLVE IN BIOFILM DEVELOPMENT

Information retrieve from Candida Genome Database (CGD) shows that about thirty genes are reported to be responsible for *C. albicans* biofilm formation but roles of many of the genes remain still unknown. Also, during the biofilm formation induction of drug efflux pumps become more active and this make the biofilm more resistant towards antifungal therapy, but role of ABC transporters in biofilm formation remain unexplored.

To study the role of ABC transporters in biofilm development, CDR1 (candida drug resistance gene) was cloned in to *S. cerevisiae* AD1-8u$^-$ host and biofilm studies were carried out for its role in biofilm development or resistance.

4.4.1 Integration of *C. albicans CDR1* Gene at the *PDR5* Locus in *S. cerevisiae* AD1-8u$^-$.

The function of *C. albicans* Cdr1p was studied with ABC transporter by expressing *CDR1* in the *S. cerevisiae pdr1-3* mutant AD1-8u$^-$*(MATa pdr1-3 his1 ura3 Δyor1::hisG Δsnq2::hisG Δpdr5::hisG Δpdr10::hisG Δpdr11::hisG Δycf1::hisG Δpdr3:hisG Δpdr15::hisG)*, from which seven major ABC transporters have been deleted. protein over expression system (Decottignies et al., 1997) that uses the multidrug resistance regulatory mutation *pdr1-3* to up-regulate the *PDR5* promoter and that results in overexpression of the Pdr5p protein primarily located in plasma membranes (Balzi et al., 1994; Decottignies et al., 1997). Hyperinduction of Cdr1p was achieved by integrating the *CDR1* ORF at the *S. cerevisiae* AD1-8u$^-$ *PDR5* locus downstream from the *PDR5* promoter. First, the *CDR1* ORF and its transcription terminator region was amplified from *C. albicans* genomic DNA by PCR and cloned into the *Spe*I site in plasmid pSK-PDR5PPUS, which is located between the

PDR5 promoter and the *PDR5* stop codon (Fig. 19). The resulting plasmid was linearized with *Xba*I and transformed into *S. cerevisiae* AD1-8u$^-$ (Δ*PDR5*; from which nt -360 to 1163 were deleted) with selection for Ura$^-$ transformants. This selection protocol was made possible by the presence of the *S. cerevisiae URA3* gene in the *PDR5* terminator region of ligated vector. The Ura$^+$ *S. cerevisiae* transformants (ApCDR1) demonstrated lower levels of sensitivity to azoles than the parental strain and was selected for further analysis. The doubling time of ApCDR1 in YEPD and CSM-based media was the same as that for the parental strain. To confirm the integration of *CDR1* at the *PDR5* locus in ApCDR1, uncut total DNA and restricted genomic DNAs were hybridized with a *C. albicans CDR1*-specific probe. The probe hybridized with uncut genomic DNA, and there was no evidence of episomal plasmid. The hybridization of the probe with single bands of genomic DNA of the expected sizes after digestion with five separate restriction endonucleases (*Eco*RV, 5,467 bp; *Spe*I, 4,776 bp; *Bam*HI, 4,272 bp; *Pst*I, 2,236 bp; *Eco*RI, 1,042 bp) indicated that a single integration event had occurred at the *PDR5* locus.

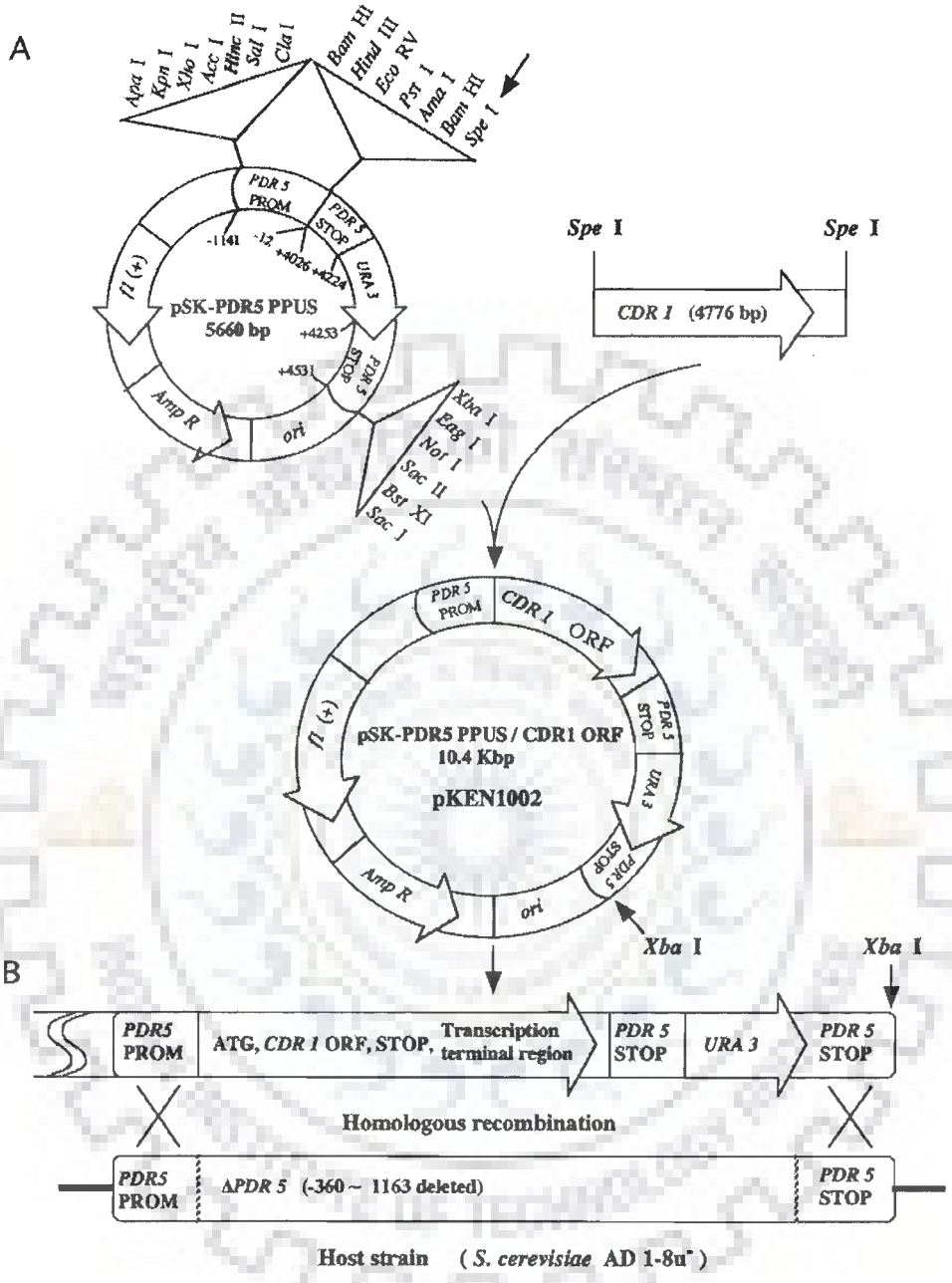


Fig. 19: Cloning strategy of CDR1 gene to S. cerevisiae AD1-8u⁻ strain

(Source: Nakamura et al., Antimicrobial agenta and chemotherapy, 2001;45(12): 3366-3374)

4.4.2 Expression of *C. albicans CDR1* in *S. cerevisiae*

Expression of Cdr1p (fig. 20) was examined by SDS-polyacrylamide gel electrophoresis (PAGE) analysis of plasma membrane proteins (Decottignies et al., 1994). No major plasma membrane protein bands of the size expected for ABC transporters (170 kDa (Decottinies et al., 1997; Krishnamurthy et al., 1998; Oulette et al., 1994)) were detected by Coomassie blue staining in samples from parental strain AD1-8u$^-$. This confirmed the depletion of endogenous pumps in this strain with multiple deletions. In contrast, samples from both ApCDR1 and AD124567 contained a major protein band at 170 kDa which accounted for 10 to 20% of the Coomassie blue-stained plasma membrane protein. Only the 170-kDa protein from APCDR1 reacted with anti-Cdr1p antibodies (Fig. 20).

Fig. 20: (I) Cloning of *C. albicans* CDR1 in vector pSK-PDR5 PPUS. Lane (1) molecular weight marker (2) CDR1 gene (3) Ligated vector (4) linearized vector, (II) Plasma membrane proteins seperated through 8% polyacrylamide gel and stained with Coomassie blue Lane (M) molecular weight marker (P) purified cdr1p(A), western blot for cdr1P (B)

4.4.3 Antifungal Sensitivities of *S. cerevisiae* cells Expressing Cdr1p

The phenotypic effects of Cdr1p expression in *S. cerevisiae* strains with a depleted ABC transporter background on antifungal sensitivity were measured. Parental strain AD1-8u⁻ was exquisitely sensitive to fluconazole, ketoconazole, and itraconazole (Table 11). Transformant ApCDR1 was markedly less sensitive to fluconazole, ketoconazole, and itraconazole, for which there were 46, 35, and 230-fold increases in the MICs, respectively. Thus, expression of Cdr1p in this transformant conferred cross-resistance to different azole antifungal drugs, as has been shown in other *S. cerevisiae* strains and in *C. albicans* (Albertson et al., 1996; Oulette et al., 1994). These results, together with those of SDS-PAGE, Western, indicated that the *C. albicans* drug resistance gene, *CDR1*, is functionally overexpressed in *S. cerevisiae* ApCDR1.

4.4.4 NTPase Activity of ApCDR1

Plasma membrane fractions from *S. cerevisiae* ApCDR1 possessed at least an order of magnitude higher oligomycin-sensitive ATPase activity than parental strain AD1-8u⁻ over the pH range 6.0 to 8.0 (Fig. 21A). This activity had a pH optimum of about 7.5, and thus, the ATPase activity of APCDR1 was readily distinguished from the Pma1p ATPase activity of *S. cerevisiae*, which has a pH optimum of 6.0 (Decottinies et al., 1994). Furthermore, the activity of Pma1p is insensitive to oligomycin (Marchetti et al., 2000) and is specific for ATP (Decottinies et al., 1994). *C. albicans* Cdr1p expressed in *S. cerevisiae* ApCDR1 also showed oligomycinsensitive UTPase, CTPase, and GTPase activities similar to the ATPase activity, and all these NTPase activities had alkaline pH optima. The activity of each NTPase of ApCDR1 was sensitive to 100 μM vanadate but was insensitive to 20 μM aurovertin B. Mitochondrial ATPase activity therefore made a negligible contribution to the ATPase

activities of these membrane preparations. The ATPase activities of ApCDR1 were unaffected by the addition of fluconazole (up to 80 μM) to the assay mixture, indicating that this substrate does not stimulate ATP hydrolysis.

4.4.5 Cdr1p-Mediated Rhodamine Efflux

The glucose-dependent efflux of rhodamine 6G from *S. cerevisiae* ApCDR1 was demonstrated (Fig. 21B). Efflux from deenergized (by incubation with 2-deoxyglucose), rhodamine 6G-preloaded cells of ApCDR1 required the presence of glucose; by 10 min following glucose addition the extracellular rhodamine 6G concentration had increased more than sixfold. In contrast, efflux of rhodamine 6G from parental AD1-8u$^-$ cells was undetectable in the presence of glucose or the absence of glucose. Both strains showed similar survival rates following rhodamine pretreatment and accumulated equivalent amounts of rhodamine during pretreatment in the presence of 2-deoxyglucose, as demonstrated by measurement of the amount of rhodamine 6G released following cell lysis. Fluconazole inhibited rhodamine efflux from ApCDR1 cells. The addition of fluconazole (10 μM) during preincubation of ApCDR1 cells with rhodamine in the presence of 2-deoxyglucose resulted in a 35% reduction in the concentration of released fluorescence 10 min following the addition of glucose.

Table 11: Antifungal sensitivities of *S. cerevisiae* cells expressing Cdr1p

S. cerevisiae strain	MIC_{80} (μg/ml)		
	Fluconazole	Ketoconazole	Itraconazole
AD1-8u^{-*}	0.650	0.024	0.02
ApCDR1$^{\#}$	29.9	0.84	4.6

*Parental strain.

#Strain expressing Cdr1p (the MICs were unaffected by supplementing the medium with uridine [0.02%; wt/vol]).

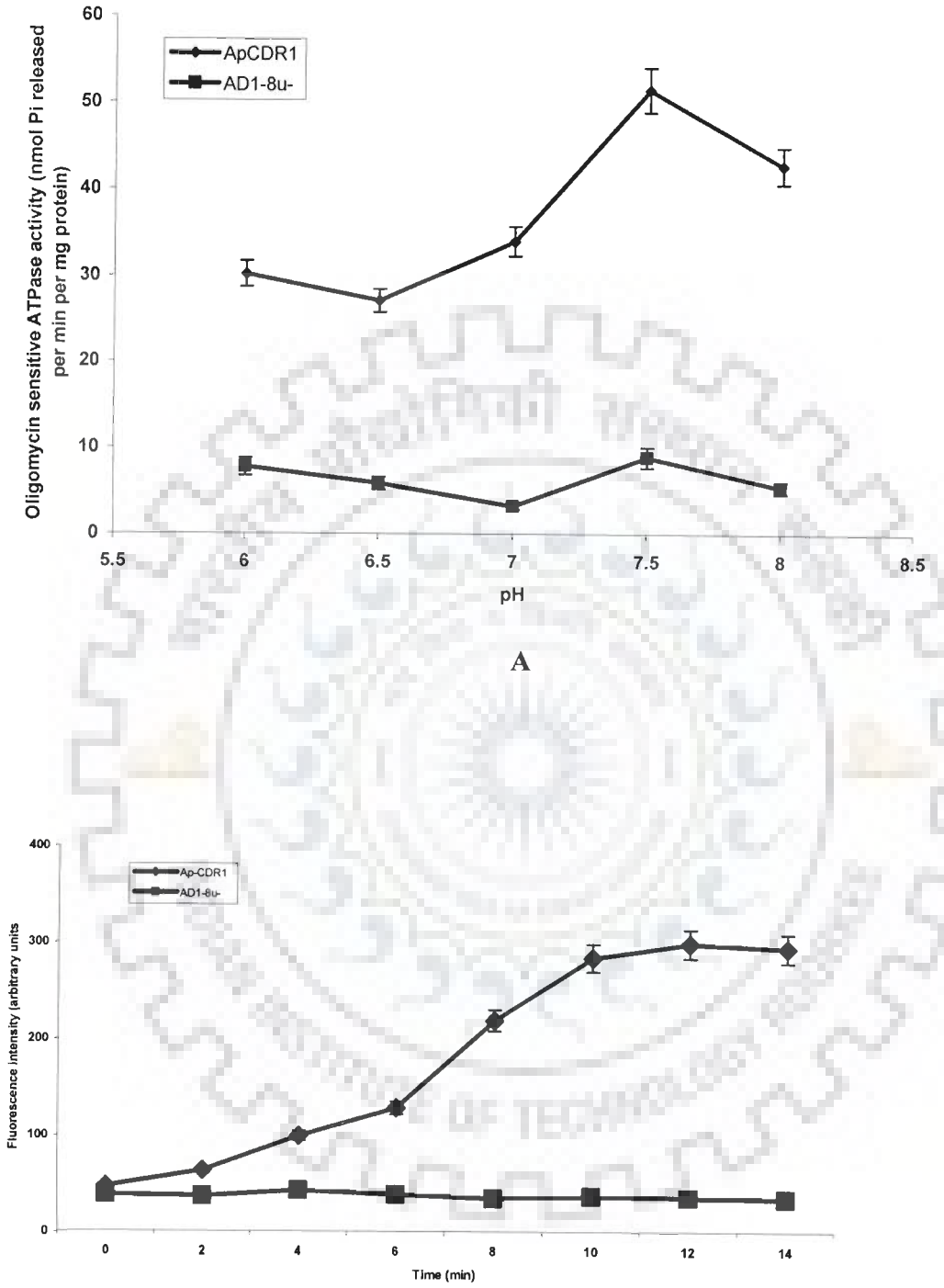


Fig. 21: NTPase activity (A), and Rhodamine efflux (B) pattern by ApCDR1 and its parental strain (AD1-8u$^-$).

4.4.6 Effect of CDR1 Gene on Metal Resistance of Biofilm

Biofilms of ApCDR1 and Ad-8u$^-$ strains were prepared with concentration $5x10^6$ cells/ml. Mean and standard deviation calculations for cell densities were based on pooled data from all growth controls performed (i.e. from 5 replicates each). Mean viable cell counts and standard deviation (SD) for the cell counts for each row were pooled and compared using one-way analysis of variance (ANOVA). The cell density of biofilms grown on the different rows was found to be equivalent to $p = 0.991$ for ApCDR1; $p = 0.692$ for Ad-8u$^-$.

The mM concentration of metal ions tested which found to inhibit 90% biofilm growth ($MBEC_{90}$) was different for both ApCDR1 and AD1-8u$^-$. Maximum difference in metal susceptibility was observed in case of Hg^{2+} for which ApCDR1 was about 11 times more than AD1-8u$^-$ followed by Mn^{2+}, Zn^{2+}, Cu^{2+}, Ag^{2+}, Co^{2+}, Ni^{2+}. Al^{+3} got precipitated and its effect could not determine.

In general and MBEC values were greater in rich medium than in minimal medium. This was probably due to the chelation of metal ions by phosphates and organic matter present in the growth medium. Collectively, these data suggest that CDR1 gene provides biofilm tolerance to metal cations and CDR1 cloned ApCDR1 become up to 11 times (for Hg^{2+}) more resistance to metal than its respective host AD1-8u$^-$ (Table 12).

Table 12: Effect of metal ions on Planktonic cells and biofilm formed by ApCDR1 and AD1-8u$^-$

Metal Cations	Biofilm Inhibitory Concentrations Value (mM)			
	ApCDR1		AD1-8u$^-$	
	Biofilm	Planktonic	Biofilm	Planktonic
Co^{2+}	142 ± 10.1	74±2.6	52 ± 4.2	48±4.3
Ni^{2+}	108 ± 11.2	48±3.2	41 ±2.3	38±3.1
Mn^{2+}	96±9.4	21±1.4	12 ±1.0	10±2.2
Cu^{2+}	58 ± 4.8	14±1.1	09 ± 1.3	10±1.9
Ag^{+}	43 ± 5.2	09±0.6	07 ±1.5	07±1.2
Hg^{2+},	34 ± 3.1	11±2.1	03 ±0.4	04±0.9
Zn^{2+}	41 ± 5.1	08±1	06 ±1.0	05±0.6
Al^{3+}	ppt	ppt	ppt	ppt

4.5 EFFECT OF BIOSURFACTANTS, PLANT OILS, ENZYMES, SILVER COATINGS AND ANTIFUNGAL AGENTS ON *C. albicans* BIOFILM

4.5.1 Effect of Biosurfactant

4.5.1.1 Effect of Different Concentrations of Rhamnolipids

To analyze the effect of rhamnolipids on *C. albicans* biofilm formation different concentration (1 µg to 10 µg) of rhamnolipids was added to preformed biofilm on MTP plate. XTT reduction data showed that 8 µg (4% v/v) concentrations of rhamnolipid was sufficient to inhibit 90% biofilm growth (fig. 22). Complete inhibition was never achieved even up to 20 µg concentration.

4.5.1.2 *Effect of Rhamnolipids on C. albicans Biofilm Formed on Biomaterial Surfaces*

After 48 h of biofilm formation all biomaterial pieces were found to have tightly bound *C. albicans* biofilm along with extensive exopolysachcharide production. These biomaterials pieces were carried out for XTT reduction assay to measure variation in metabolic activity among biofilm encased *Candida* cells attached to biomaterial surfaces. Maximum biofilm formation was measured in case of Polyvinylchloride followed by polystyrene and polypropylene, while very less candidal adherence was observed in case of silicone rubber when compared to polyvinylchloride. It was found that rhamnolipid coating lead to a decrease in *C. albicans* biofilm on biomaterial surfaces. Results showed that maximum 91% biofilm reduction was measured in case of silicone rubber followed by 86%, 85%, 68%, reduction with polystyrene, polyvinylchloride, and polypropylene respectively (fig. 23).

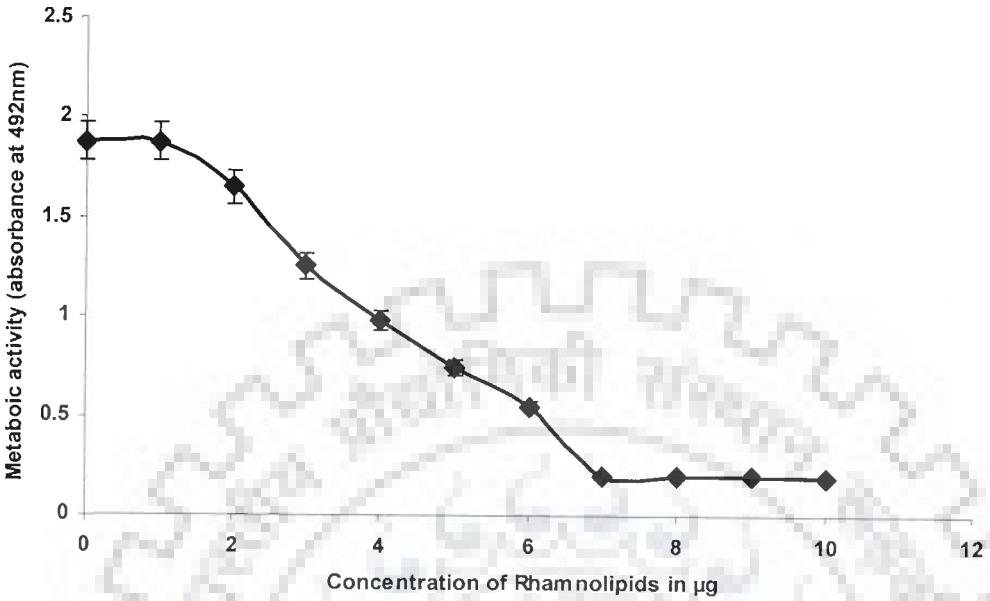


Fig. 22: Effect of different concentrations of Rhamnolipids on *C. albicans* biofilm growth ($p<0.05$; n=5)

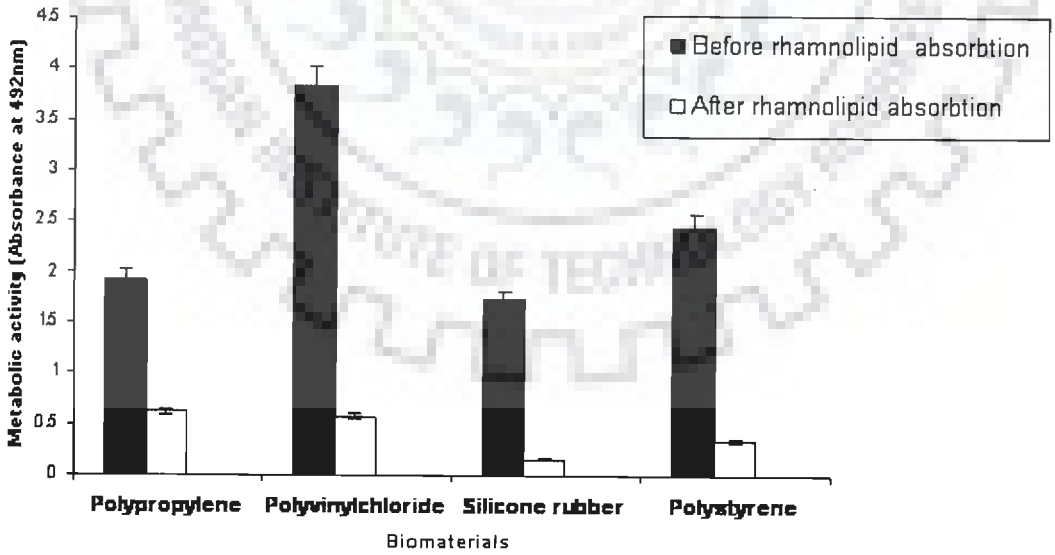


Fig. 23: Effect of Rhamnolipid absorbed on biomaterial surfaces on *C. albicans* biofilm *($p=0.05$; $n=3$)*

4.5.1.3 Roughness and Hydrophobicity/Hydrophilicity of the Biomaterials

The roughness and contact angle measurement for hydrophobicity or hydrophilicity of biomaterials was done in order to determine surface characteristics which may account for adhesion of *Candida* cells in present studies.

The roughness and contact angle measurements for the biomaterials are shown in Table 13. PS was the smoothest material while PVC was the roughest one with an average roughness measurement of 23nm 134nm respectively.

Contact angle measures the hydrophobicity or hydrophilicity of materials. A higher surface/water contact angle is an indication of a more hydrophobic material. The highest contact angle was 97^0 for PVC and the lowest was for silicone rubber, showing that PVC was the most hydrophobic biomaterial. There were substantial differences appeared both in roughness and hydrophobicity or hydrophilicity among the biomaterials after rhamnolipid coatings, in all the cases they reduced without any order or definite value (Table 13).

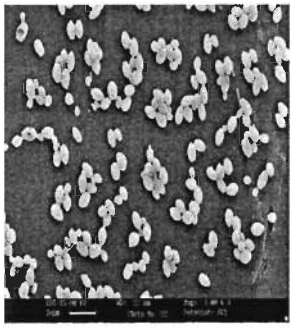
4.5.1.4 Scanning Electron Microscopy

SEM analysis studies on biofilm growth on biosurfactant absorbed and control biomaterial surfaces (polypropylene, polystyrene, polyacrylamide and silicone rubber) revealed intense biofilm formation with high cell number in control samples while rhamnolipid absorbed polymer pieces showed significantly few adhering cells (fig 24).

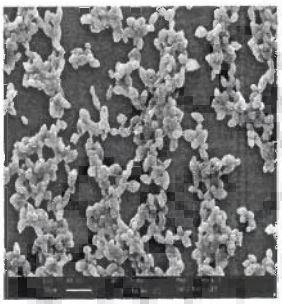
Table 13: Average contact angle and roughness of polymer surfaces before (control) and after (test) Rhamnolipid coatings. Data are result of three individual experiments plotted in triplicates and shown as ± standard deviation

Biomaterials	Contact angle±SD (^{o})		Roughness ±SD ((nm)	
	Control	Test	Control	Test
Polypropylene	84±1.2	68±0.3	43±1.6	39±0.8
Polyvinylchloride	97±1.2	66±0.8	134±0.8	95±1.7
Silicone rubber	77±1.3	52±1.6	27±.0.6	20±1.2
Polystyrene	91±0.07	74±1.1	24±2.2	19±0.9

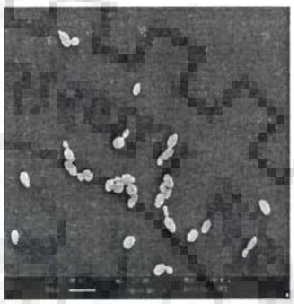
PP (A)



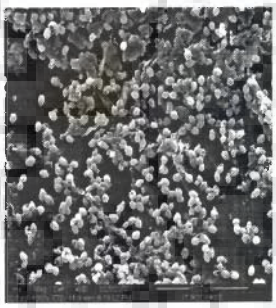
PP (B)



PVC (A)

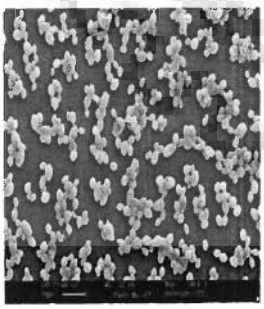


PVC (B)

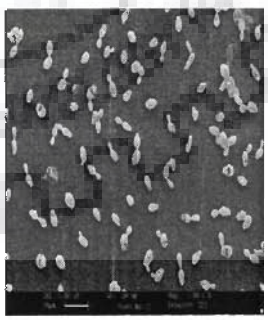


Silicone rubber (A)

Silicone rubber (B)



PS (A)



PS (B)

Fig. 24: SEM of *Candida* biofilm formed on different biomaterial surfaces PVC, PS, PP and silicone rubber (A) represents control and (B) reduction by absorption of biosurfactant respectively

4.5.2 Effect of Plant Oils on *C. albicans* Biofilm

Azole drugs and derivatives continue to dominate as antifungal agents of choice against *C. albicans* biofilm related infections, as topical applications or as oral drugs. Even though very widely acclaimed for their efficacy, these drugs are known to have side effects. Besides this, the action of antifungal may be limited by their penetration and chemical reaction into biofilm matrix, the extracellular polymeric material. The increasing resistance of *C. albicans* towards these antifungal compounds and the reduced number of available drugs led to the search of new therapeutic alternatives among plants and their essential oils, empirically used by antifungal proprieties. Of all the strategies which have been identified to overcome drug resistance, the exploration of new and effective natural products showing antifungal activity against *C. albicans* biofilm cells with low cytotoxicity, is likely to significantly impact the treatment, as well as the management, of biofilm-associated fungal infections. In this work we report the anti-Candida activity of thirty plant oils (almond, alsi, babchi, babuna, cade, castor, chaulmoogra, clove, coconut, eucalyptus, ginger grass, ginger, jasmine, jojoba, juniper, jyotishmati, khus, lavender, mahua, malkangani, musturd, neem, ocimum, peppermint, rose, tea tree, til, tulsi, walnut and wheatgerm). Chosen oils were further assessed using XTT (2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) reduction assay followed by Scanning electron microscopy (SEM). Since fluconazole is widely given against various *C. albicans* infections, is chosen as a control. The outlined work investigates the potential of plant oils and their effect on biofilm formation of *C. albicans* strain (CA I) previously isolated from clinical samples.

4.5.2.1 Screening of Plant Oils for Anti-Candida Activity

A clinical isolate and a fluconazole resistant strain demonstrated differential sensitivity to tested oils. Majority of oils were found to be effective and showed anti-Candida activity even at very low concentrations. Eighteen among 30 oils tested gave 1-30 mm zone of inhibition (ZOI). Table 14 shows ZOI for plant oils in response to CA I and CA II. For CA I, 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 14).

4.5.2.2 Effect of Plant Oils on Biofilm Growth

Screened plant oils were further checked against CA I biofilm up to 5% (v/v) concentration. XTT reduction assay showed that 72 µg/mL concentration of fluconazole (used as standard drug) was able to reduce 78% biofilm (Fig. 25a) whereas 0.84% concentration of eucalyptus oil and 1.16% concentration of peppermint oil gave 80.87% and 74.16% biofilm reduction (Fig. 25b). About 40.46%, 28.57%, reduction in CA I biofilm was achieved by ginger grass and clove oils with 1.68% and 4.8% concentration respectively. Other oils did not show any activity against biofilm 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 CA I biofilm than fluconazole (Table 15).

4.5.2.3 Determination of MIC of Plant Oils

MICs of four effective oils and fluconazole were determined for CA I and CA II. The oils exhibited concentration dependent inhibition of growth. Fluconazole at 4 µg/mL concentration was able to inhibit total growth of CA I. Strain CA II was highly resistant to fluconazole 3000 µg/mL, i.e., 750-fold the MIC of CA I required for a partial growth inhibition. Eucalyptus oil was found to be most effective, 0.05% concentration was enough to completely inhibit growth of both the strains tested. Peppermint oil was found to have same MIC values both for CA I and CA II (Table 15).

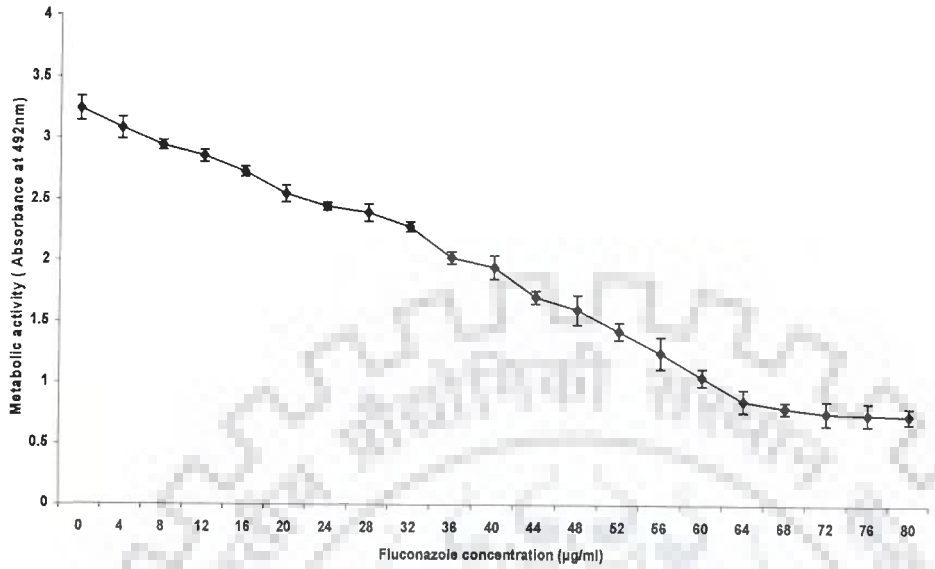
4.5.2.4 Scanning Electron Microscopy of CA I Biofilm

To evaluate the relevance of XTT reduction assay, SEM was employed. Scanning electron microscopic analysis of control biofilms and those treated with the plant oils are shown in Fig. 26. Visualization of the ultrastructure in general revealed that most reduction to biofilm constituents was caused by eucalyptus oil followed by peppermint oil (Fig. 26 c, d) as compared to the untreated biofilm with fluconazole control (Fig. 26 a, b). Eucalyptus and peppermint oil treated cells demonstrated reduction in adhering cells and biofilm development. This suggests that despite the relative minimal diffusion, eucalyptus and peppermint oils compared with other plant oils may be exerting a metabolic interference in biofilm. Clove and ginger grass oil treated biofilm residing cells also demonstrated some degree of reduction.

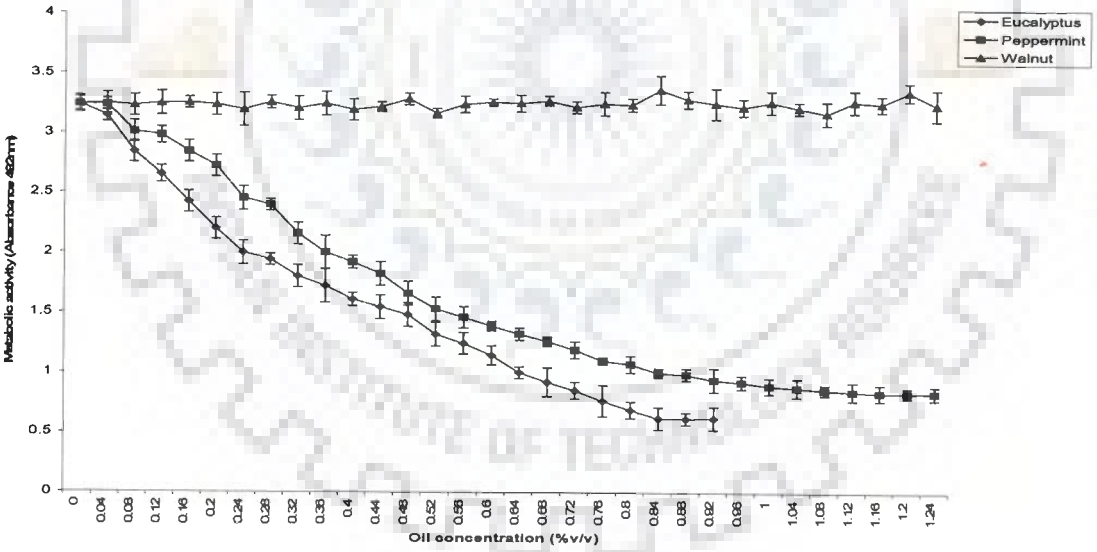
Table 14: Effect of plant oils on *C. albicans* taking fluconazole as standard

Botanical name	Plant oils	#ZOI (mm)	
		CA I	CA II
Eucalyptus globulus	Eucalyptus	26.7± 2.2	27.2±0.7
Mentha piperita	Peppermint	22.2±1.6	21.8± 1.2
Cymbopogan martini	Ginger grass	16.0±0	15.2± 0.7
Eugenia caryophyllus	Clove	13.8±1.3	13.0±0.9
Ocimum sanctum	Tulsi	11.3±1.2	11.6±1.3
Melaleuca alternifolia	Tea tree	11.0± 0.7	9.5± 0.8
Ocimum basiliscum	Ocimum	9.8± 0.8	10.2± 1.1
Ricinus communis	Castor	7.8± 0.9	5.2±0.3
Juniperus chinensis	Juniper	5.6±0.5	5.8± 0
C. anthelminticum	Malkangni	5.3± 0.6	5.5±0.5
Cocos nucifera	Coconut	4.0±0.7	4.2± 0.3
Psoralea corylifolia	Babchi	3.4± 0.4	3.9± 0.2
Madhuca indica	Mahua	3.2± 0	3.0± 0.2
Z. officinalis	Ginger	2.6± 0	2.1± 0
Brassica juncea	Mustard	2.3± 0	2.0± 0
R. officinalis	Rose	2.1± 0	2.2±0
Jasminum nudiflorum	Jasmine	1.4± 0	1.0± 0
Lavandula angustifolia	Lavender	1.2± 0	1.3± 0
Linum usitatissimum	Alsi	0± 0	0± 0
Azadirachta indica	Neem	0± 0	0± 0
Matricaria chamomilla	Babuna	0± 0	0± 0
Sesamum indicum	Til	0± 0	0± 0
Celastrus paniculata	Jyotishmati	0± 0	0± 0
Simmondsia chinensis	Jojoba	0± 0	0± 0
Juglans regia	Walnut	0± 0	0± 0
Prunus glandulosa	Almond	0± 0	0± 0
Triticum vulgare	Wheatgerm	0± 0	0± 0
Vetiveria zizanoides	Khus	0± 0	0± 0
Juniperus oxycedrus	Cade	0± 0	0± 0
Taraktogenos kurzli	Chaulmoogra	0± 0	0± 0
Control (disc without oil)		0± 0	0± 0
Fluconazole (positive control)		24.3± 2.4	0± 0

#Mean of six replications, diameter of disc = 5 mm, significant at ±1% ZOI



(a)



(b)

Fig. 25: Biofilm formation by *C. albicans* (CA I) with different concentrations of (a) Fluconazole (b) Eucalyptus and Peppermint oil along with Walnut as ineffective oil control. Experiments are the results of three individual experiments performed in triplicate. ($n=3$, $p<0.05$)

Table 15. *C. albicans* (CA I) biofilm reduction achieved in response to most effective plant oils (MIC value to 5% v/v)

Plant oils	Biofilm reduction (%)	Concentration (% v/v)	MIC value (% v/v)	
			CA I	CA II
Eucalyptus	80.87±4.2	0.84	0.05	0.05
Peppermint	74.16±3.1	1.16	0.08	0.08
Ginger grass	40.46±2.2	1.68	0.08	0.09
Clove	28.57±1.8	4.80	0.33	0.35
*Fluconazole	78.0 ±3.3	72.0	4.0	>3000

*Values in µg/mL

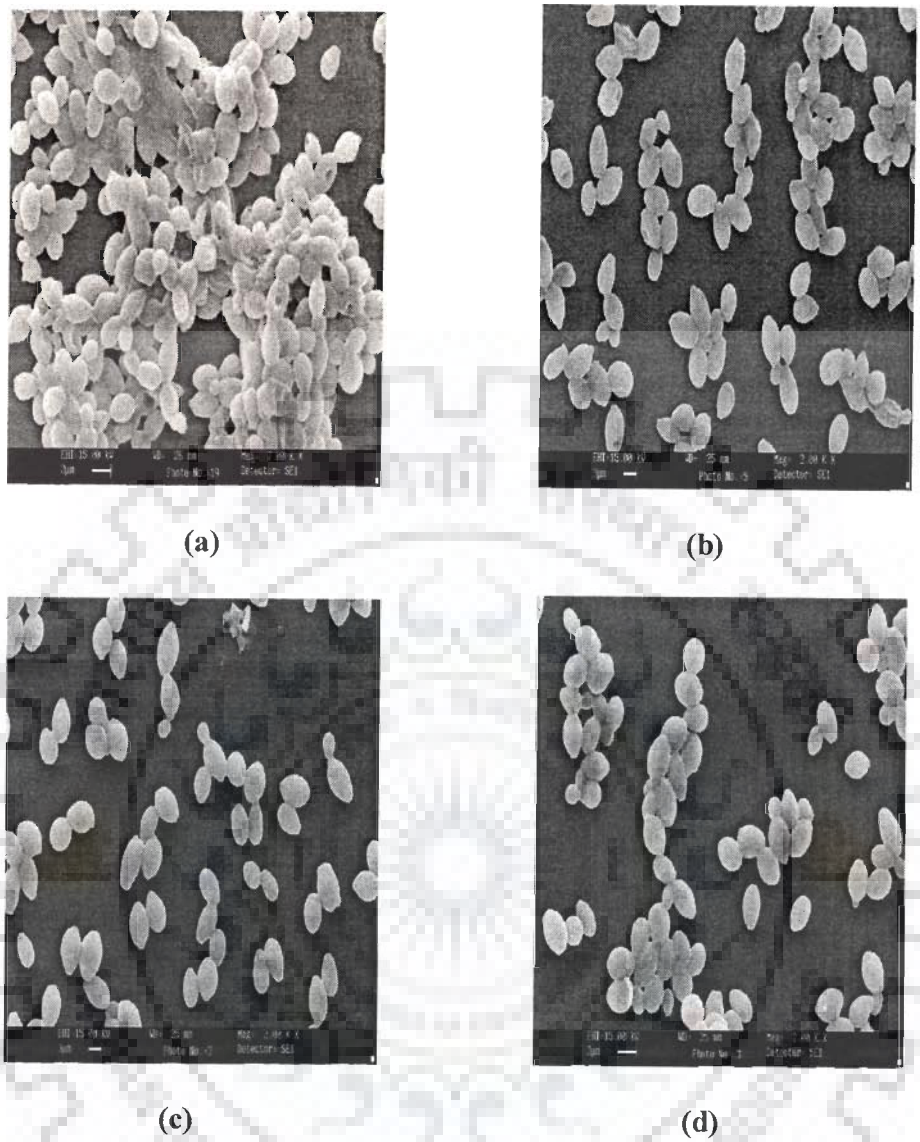


Fig. 26: Scanning electron micrographs showing reduction in *C. albicans* (CA I) biofilm with plant oils and antifungal agent. (a) Control, without oil (b) Fluconazole (c) Eucalyptus oil and (d) Peppermint oil, with 2.0 KX magnification

4.5.3 Effect of Enzymes

Enzymes can be used for degradation of biofilm, but due to the heterogeneity of the extracellular polysaccharides in the biofilm, a mixture of enzyme activities may be necessary for a sufficient degradation of bacterial biofilm. The purpose of this study was to assess the applicability of commercially available enzymes for removal of biofilm as well as for their antimicrobial activity against cells in biofilm.

In our studies, alginate lyase was found to be most effective enzyme to inhibit biofilm formation with maximum 70.7% reduction. Pectin lyase, cellulase, chitinase, polygalactouranase, arabinase, proteinase and glucose oxidase showed 66.6%, 49.4%, 33.2%, 29.6%, 19%, 14.8%, and 13.3% reduction after 24h respectively (fig. 27). Alginate lyase, pectin lyase, polygalactouranase, and glucose oxidase showed their maximum inhibition between 25-35 units of enzymes while other (cellulase, chitinase, and arabinase) showed inhibition at about 35-45 units and become constant at 50 unit enzyme concentration.

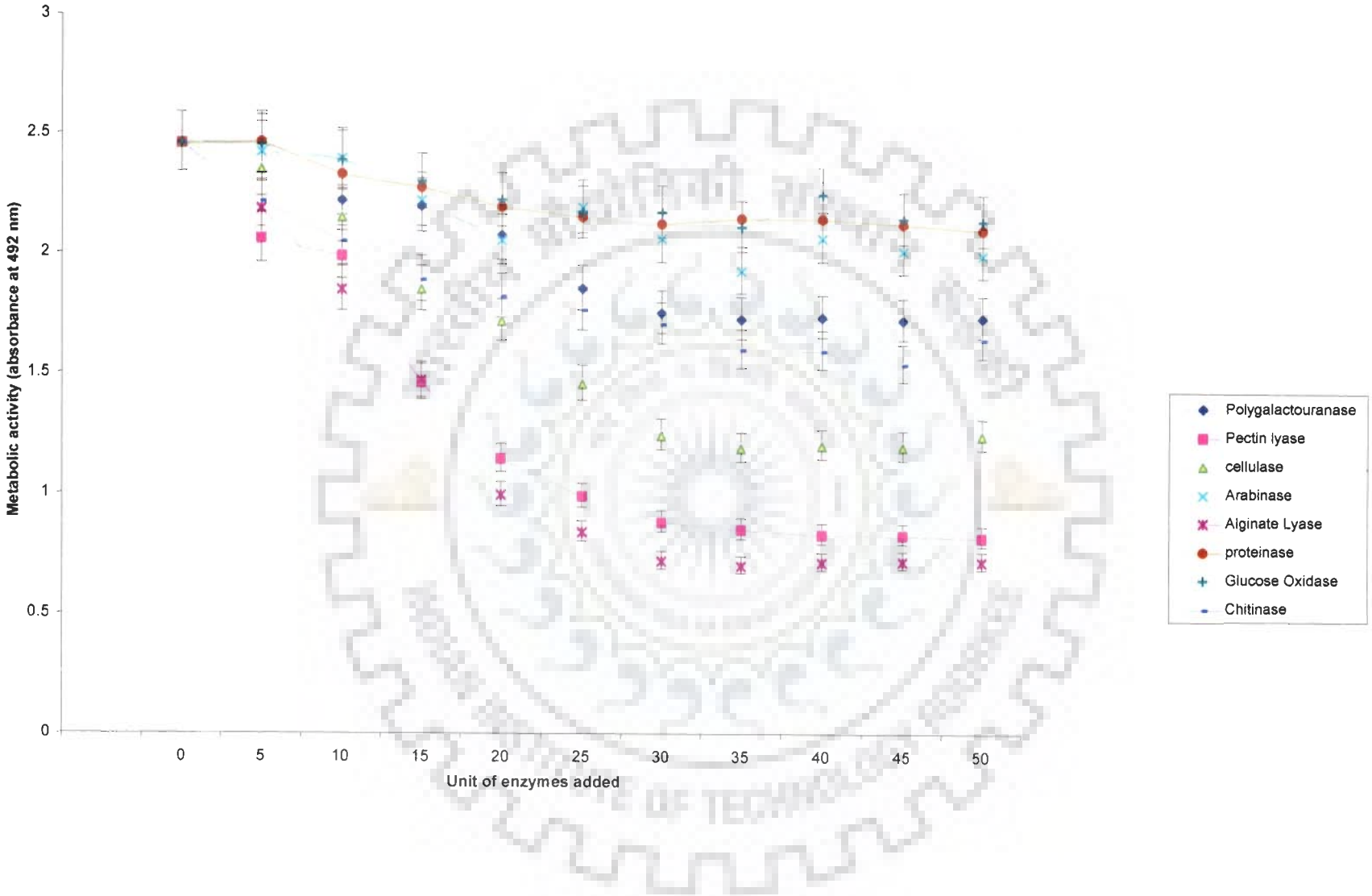


Fig. 27: Effect of different enzymes on *C. albicans* biofilm

4.5.4 Effect of Silver Coatings on *C. albicans* Biofilm Biomaterials

Silver has long been known to be a potent antibacterial agent with a very broad spectrum of activity and has been used safely in medicine for many years. Silver surface coating results thin metal layer strongly bounded to underlying substratum which may limit toxicity to host and control infections. A lot of work has been done with bacteria and silver coatings but from the best of our knowledge no study has been carried out with fungal infections notably *Candida albicans* biofilm. In the present work, we have studied effect of silver coating on *C. albicans* biofilm and surface properties.

4.5.4.1 Roughness and Hydrobhobicity Measurement

To check the effect of silver coating over the PVC pieces on surface roughness and hydrophobicity was measured with AFM and goniometric analysis respectively. Initially uncoated PVC sample (control) had roughness 134nm while it decrease significantly and reach up to 34nm with 15 seconds of coating (Table 16). AFM microscopy photograph showed variation in surface topography with silver coatings (Fig. 28).

Goniometric data showed that contact angle does not affect significantly with silver nanoparticles deposition ie surface hydrophobicity/hydrophilicity remain nearly same during the coating process (Table 16).

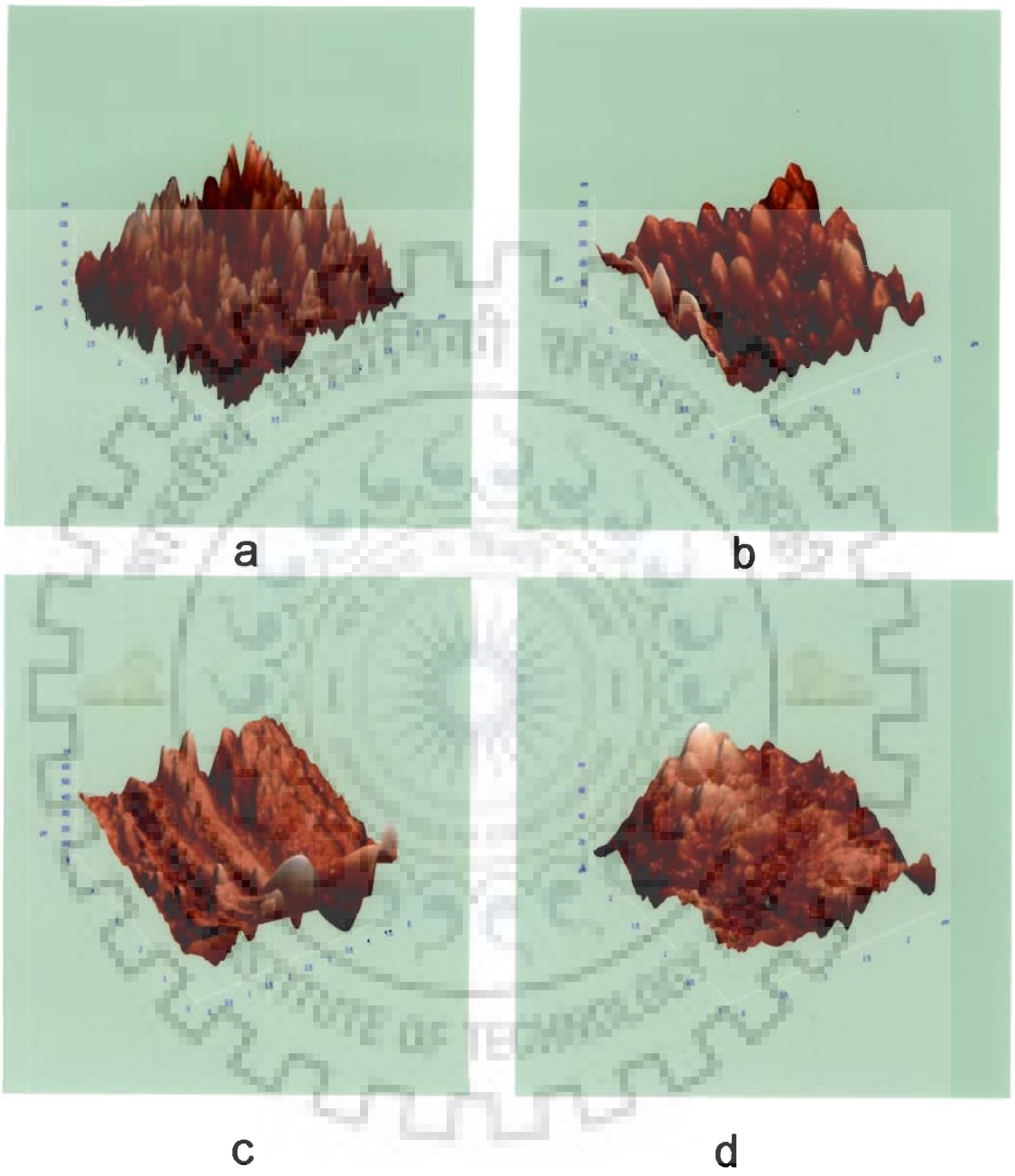


Fig. 28: AFM of PVC surfaces after silver coating: (a) Control, (b) 5 sec, (c) 10 sec, (d) 15 sec

Table 16: Average thickness, contact angle and roughness of silver coated PVC surfaces. Data are result of three individual experiments plotted in triplicates and shown as ± standard deviation

Time (Sec)	Coating thickness (nm)	Roughness	Contact angle
0	0	134±5.8	91±2.2
5	4±0.8	88±6.9	82±1.8
10	8±1.2	52±5.2	79±1.3
15	12±1.3	34±4.7	90±1.6

4.5.4.2 Coating Thickness Measurement

The coating thickness was measured with surface profilometer and was found to be 4, 8, 12nm after 5sec, 10 sec and 15 sec with constant deposition rate of sprutter.

4.5.4.3 Scanning Electron Microscopy

Scanning electron microscopy of silver coated PVC samples before (at 14kx magnification) and after biofilm formation (at 3kx magnification) was performed with silver uncoated control sample. SEM results revealed that deposited silver nanoparticles are compactly, equally and randomly distributed throughout the surface. The density of silver nanoparticles seems to increase with the thickness or the time of coating. (fig. 29 A-D).

C. albicans biofilm formed on 4 and 8nm thick silver coated samples showed few scattered cells with altered morphology. PVC piece with 12nm silver coat showed no any adherent *Candida* cells. (fig. 29 E-H).

4.5.4.4 Biofilm Quantification:

XTT reduction assay was used to measure metabolic activity of biofilm residing candidal cells. Data showed 85%, 93% and complete reduction in *C. albicans* biofilm formed on samples with 4, 8, and 12nm silver coatings respectively (fig. 30).

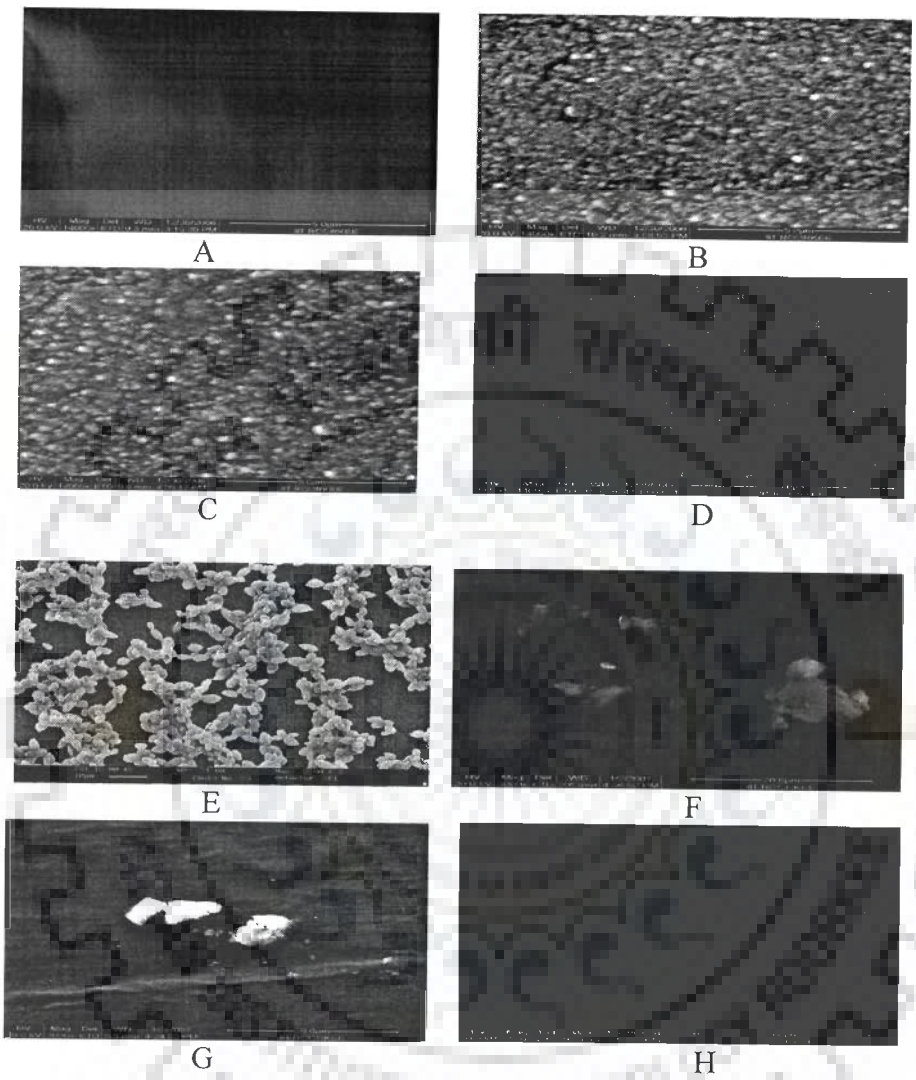


Fig. 29: SEM of PVC surfaces silver coated for different time intervals of 0 sec, 5 sec, 10 sec, and 15 sec (A-D). Biofilm formation by *C. albicans* on these silver coated surfaces E-H respectively.

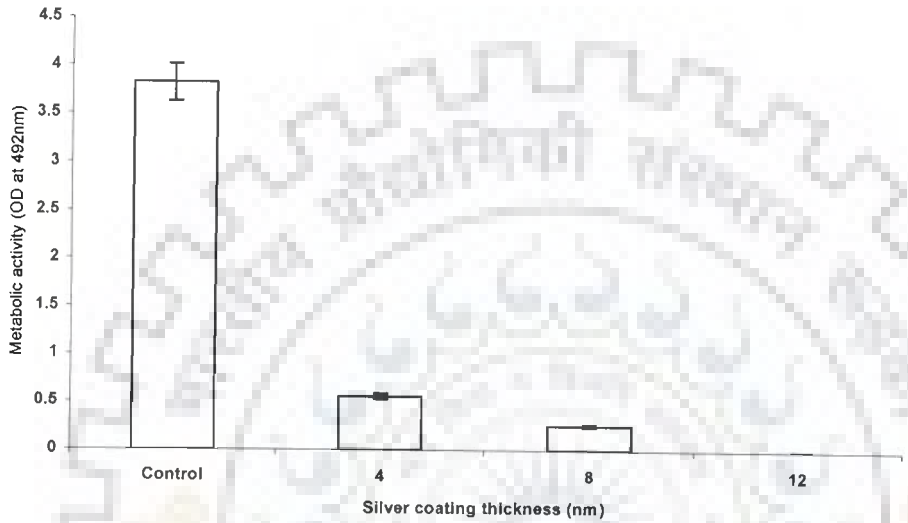


Fig. 30: XTT reduction assay of *C. albicans* biofilm formed on silver coated PVC pieces.

CHAPTER 5 DISCUSSION

5.1 ISOLATION OF *C. albicans* FROM CLINICAL SAMPLES

The value of Hi-Chrom candida agar medium for the isolation of *Candida* spp. from samples containing low numbers of cells has been systematically addressed and improves the ability of the mycology laboratory to rapidly identify mixed yeast infections. The various identification systems have been introduced for yeast identification is based on color changes because of utilization of the several kinds of substrates by the metabolizing yeast. Several brands of chromogenic media have been developed to produce rapid yeast identification (Hospenthal et al., 2002). These media contain chromogenic substrates that react with enzymes secreted by microorganisms producing colonies with various pigmentations. These enzymes are species specific, allowing organisms to be identified to the species level by their color and colony characteristics (Odds et al., 1998; Aamlid et al., 1999; Ainscough et al., 1998).

After 24 to 48 h of incubation, contrasting colored colonies result from the cleavage of chromogenic substrates by species-characteristic enzymes. In our studies, the color and morphology characteristics of all forty eight isolates on CHROMagar Candida were observed and it was found that yeast growing directly over the first quadrant of streaking demonstrated a slightly darker hue compared to that seen in the other three quadrants. This color variation was slight and most apparent after 48h of incubation, waning over the following 7 days. This was a consistent finding with all 48 isolates. Inoculation by streaking or swabbing of a small sample volume is a sensitivity-limiting factor and may account for a certain false-negative results. In addition, the incubation time required for Chromogenic Candida agar is long in connection with the diagnosis of life-threatening candidemia. Routine use of chromogenic

media carries the potential for cost savings in the clinical microbiology laboratory. Odds and Bernaerts, in 1994 in their studies on Chromogenic candida agar reported that of 726 yeast strains isolated from clinical material, 285 C. albicans isolates gave distinctive green colonies that were not seen with any of 441 other yeast isolates representing 21 different species. From their data, the green colony color allowed the recognition of *C. albicans* with 100% sensitivity and specificity. In our studies *Candida rugosa* is uncommonly isolated from clinical specimens, and a study including more isolates is required to confirm this finding. Further evaluation of the technique with larger number of isolates representing additional species of yeasts needs to be done. Also, the cost effectiveness of routine use of Chromogenic Candida agar should be assessed depending upon the type of patients being catered to by a particular laboratory. Use of this medium could allow diagnosis laboratories to more rapidly identify clinically important *Candida* spp. at potentially decreasing laboratory costs (Ainscough et al.,1998; Koehler et al., 1999). More importantly, this capability will also enable clinicians to more rapidly make appropriate antifungal choices, decreasing patient morbidity and mortality.

5.2 CHARACTERISTICS OF BIOFILM FORMATION BY *C. albicans*

We used 96-well microtiter plate system that allows the growth of multiple independent biofilms, which showed no statistically significant differences to one another when assessed by an XTT metabolic reduction assay. This model, therefore, allows us to more easily observe and assess multiple isolates and multiple experimental parameters in a reproducible manner. The XTT reduction assay described herein, developed on the basis of the study by Tellier et al. in 1992, was used to assess the metabolic activity of biofilms grown over a range of time intervals. It was clearly shown that an increasing cellular density (biofilm formation) was directly related to increased colorimetric measurements with the XTT-reduction assay. Hawser et al in 1996 indicated this relationship by comparing total viable cell counts with XTT readings.

Initially, there was a period of adherence (0-2 h) and subsequent microcolony formation (2-4 h). Dimorphic switching occurred thereafter with a transition from budding-yeast forms to filamentous pseudo- and true-hyphal forms (4-6 h). Micro-colonies then became interlinked by the hyphal extensions, forming a confluent monolayer (6-8 h). The complexity of the biofilm increased with time, taking on a three-dimensional architecture with spatial heterogeneity as it matured (8–48 h). The biofilm after 24 h and 48 h consisted of a mixture of yeast cells, pseudohypahe and true hyphae. Production of EPS and micro-colony formation were the most important factor in the three-dimensional architecture, with yeast cells located in the basal layer as also reported by Baille and Douglas, 1999.

The different composition of EPS and biofilm formation in response to different sugars was investigated. It was revealed that the nutritional requirement for EPS production in *C. albicans* was not always consistent with that of mycelial growth. Findings also suggest

that EPS production and biofilm formation are correlated (Baillie and Douglas 2000). It was found that phosphorus content changed negatively with glucose amount, this may be due to the fact that phosphorus inhibits growth of microbial cells while glucose is utilized as a nutrient for biofilm residing microbes (Sutherland 2001; Baldwin 1998).

In addition to the cell-specific factors that are integral for adhesion and biofilm formation, other features can also play important defined roles. For example, biomaterial surface physico-chemistry and surface roughness has been reported to play a role in bacterial adherence (Gristina et al., 1993; Gorman et al., 1993). The effect of pre-conditioning films has also been reported to have a positive effect on initial adhesion and biofilm formation (Sen et al., 1997; Nikawa et al., 1997; Gristina et al., 1987). In this study we have shown that the presence of serum or salivary pellicles, which are normally found in the oral environment, increased the initial adherence of *C. albicans* cells to polystyrene microtiter plates. Our results indicated that biological conditioning films, particularly serum, may help provide receptor binding sites for planktonic *C. albicans*. Other investigators have previously shown that presence of serum and salivary pellicles can potentiate *C. albicans* colonization of acrylic strips and denture lining materials. *C. albicans* biofilm formation is a complex process dependant upon multiple variables, including proteinaceous preconditioning films during early adhesion events. Following initial adhesion, the structural integrity, and developmental characteristics of *C. albicans* biofilms was monitored using scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) techniques. The increased magnification and resolution power associated with the SEM technique permitted a more detailed examination of biofilms. *C. albicans* biofilms mature biofilms consist of a mixture of yeast and filamentous forms embedded within exopolymeric material.

Ramage et al. 2001, in their studies showed that biofilm generally emanated from small micro-colonies comprised predominantly of budding yeast cells (2-4 h). After 4 h the budding yeast cells began to filament, forming pseudo-hyphae and eventually true-hyphae. After 8 h, hyphae from neighboring micro-colonies, comprised from principally budding yeast cells, merged into an intricate network of spatially dispersed filamentous forms that intertwined to form a coherent monolayer of woven-like structures.

Hawser et al. 1998 also described the production of extracellular matrix polymers by *C. albicans* biofilms. The non-destructive CSLM technique permitted *in situ* visualization of hydrated biofilms and demonstrated that *C. albicans* biofilms possess structural heterogeneity and display a typical micro-colony/water channel architecture similar to what has been described for bacterial biofilms (Watnick et al., 2000). This structural complexity that was observed may represent an optimal spatial arrangement for influx of nutrients, disposal of waste products and establishment of micro-niches throughout the biofilm. CSLM provides the added advantage over all other analytical techniques in that it can used to accurately measure the depths of biofilms. In this study, a mean biofilm depth of 26.6 μm was measured for 24 h biofilms. Detailed information of this nature can also be used for comparative studies of biofilms formed under different environmental conditions.

Currently, there is a well described method by the NCCLS (M-27A) to determine antifungal susceptibilities for yeast planktonic cultures. Nevertheless, the susceptibility data generated from this approach does not account for the intrinsic resistance exhibited by sessile cells. For example, Hawser and Douglas in 1995 reported that a range of antifungals were between 30 and 2000 times less active against *C. albicans* biofilms than that of the planktonic MIC's. In agreement, here we have demonstrated the intrinsic resistance of *C.*

albicans biofilms to fluconazole, the most commonly used antifungal agent and their increased resistance to clinically used amphotericin B. Although amphotericin B exhibited a certain degree of activity against biofilms as indicated by SMIC50s, the SMIC80 values already fell into the resistant range according to interpretative break points (Rex et al., 1997; NCCLS 1997). Moreover, even at higher concentrations (up to 512 μg/ml) sterility was never achieved. The disparity between planktonic and sessile minimum inhibitory concentrations from an identical isolate may therefore explain why antifungal treatment may be ineffective in some instances and partially explain the lack of absolute correlation between clinical (*in vivo*) and mycological (*in vitro*) resistance. We have shown that *C. albicans* can form biofilms with relative ease and simplicity. However, macroscopic and microscopic evaluation of these biofilms by different techniques has revealed a level of complexity that could not have been mediated by random cell division. Importantly, *C. albicans* biofilms formed on different types of biomaterials showed similar architectural features and properties. We have shown that these communities of cells are metabolically active and encased in an extra-cellular polymeric substance. Moreover, they resist the actions of antifungal agents at concentrations that would kill them when free-floating. The spatial heterogeneity and complexity observed in the intact biofilms would lead us to consider that cell-cell communication and highly regulated gene-expression systems are present. Our observations corroborate those by Baille and Douglas in year 1999 and provide an excellent platform from which to initiate research in more focused avenues, such as the use of genetic approaches to study biofilm formation and the biofilm of *C. albicans*.

5.3 STUDIES OF MOLECULAR INTERACTION OF *C. albicans* BIOFILMS FORMED ON DIFFERENT BIOCOMPATIBLE MATERIAL SURFACES

Most important in the pathogenesis of foreign body associated infections is the ability of microbes to colonize the polymer surface by the formation of a thick multilayered biofilm (Decho and Kawaguchi, 1999; Christensen et al., 1994; Tacconelli et al., 1997; Raad, 1998). Depending on the kind of device, its insertion side, duration of insertion, and the nature of infecting microorganism biofilm constitute a major reason for infections to occur and persist at various sites in the human body (Reid G., 1999). It is evident that biofilm forming organisms attribute their properties towards pathogenesis more successfully than other organisms with more toxins and tissue damaging exoenzymes (Scierholz, 2001). During our investigation biofilm quantification was resoluted using the tetrazolium salt, 2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]2H-tetrazolium-carboxanilide (XTT) to monitor biofilm formation by *C. albicans*. The salt reduced by mitochondrial dehydrogenase of *C. albicans* to brown color water-soluble tetrazolium formazan product which depicted a linear relation ship with metabolic activity of *C. albicans* biofilm as reported earlier (Pruthi et al., 2003). The heterogenic complexity of biofilm which initiate with the adhesion phase provides a protective shield to the colonization of various surfaces, in fact it is the extracellular polymeric substances (EPS) composed of a wide variety of organic materials, including polysaccharides, proteins, nucleic acids, phospholipids, uronic acid, humic substances which provides protection against phagocytosis, interference with the cellular immune response and resistance against antibiotic effects (Scierholz, 2001). The EPS composition may be the results of active secretion, shedding of cell surface material, cell lysis, and adsorption from the environment. Researchers have only recently begun to acknowledge the important role of

EPS in microbial communities, which may include cell adhesion and aggregation, granulation, organic chemicals degradation, biofouling, cell-to-cell communication, biofilm structure, resistance to heavy metal toxicity, etc. However, little information is presently available on the chemical constituents, characteristics and functions of EPS content of the *C. albicans* (Douglas, 2003; Hoffman and Decho 1999). Our studies showed that maximum EPS produced by *C. albicans* biofilm was recorded on PVC surface followed by PS, PP, SR and PMMA. This suggests more pathogenic and resistant nature of *Candida* biofilm formed on PVC in contrast to other biomaterial surfaces.

Earlier researchers have shown that when the microorganisms reach the proximity of a surface, attachment is determined by physical and chemical interactions, which may be attractive or repulsive, depending upon the complex interplay of the chemistries of the microorganisms and substratum surfaces, and the aqueous phase (Rupp and Archer, 1994). The differential susceptibility of biomaterials towards *Candida* biofilm may involve many material surface properties. The biofilm formation was found to be the maximum with PVC followed by PS, PP, SR and PMMA i.e. decrease in hydrophobicity along with reduction in contact angles. Hogt et al., 1985 in their experiment showed that absorption of protein on hydrophobic surface was found to increase surface hydrophilicity, which resulted in the reduction of adhesion. Since the cell surface hydrophobicity of *Candida* cells is known to be an important factor in its adherence to acrylic surface this factor may also play a key role in the biofilm development on these biomaterials (Klapper et al., 2002). Our observations showed that *C. albicans* biofilm formation decreases with contact angles of biomaterials samples used in this study. PVC with contact angle 97^0 formed maximum biofilm while least biofilm developed was recorded on PMMA surface. These results are in agreement with

previous studies showing that the correlation between contact angle and biofilm formation is dependent on surface properties (Balazs et al., 2004; Briandet et al., 2001; Jansen et al., 1995; Jin et al., 2003; Jin et al. 2004). AFM analysis used to quantify the biomaterial surface roughness showed high surface area in case of PVC which may account for greater surface area and the depressions as roughened surfaces provide more favorable sites for *C. albicans* colonization (Webb et al., 1999). PMMA showed higher roughness with least colonization than SR this probably may be due to the negatively charged surface. A similar result has also been reported by Scheuerman et al., 1998. In their studies they have showed that *C. albicans* adhesion was reduced on the negatively charged PMMA/AA (acrylic acid), while it was increased on the positively charged PMMA/DMAEMA (dimethylamino ethyl methacrylate) in a manner depending on the co-monomer content. It has also been reported that surface materials with different porosity, groove and braid are with higher infection rates than flat ones, probably due to increased surface area (Wenn et al., 1999; Kiremitci et al., 1996). M. Katsikogianni et al., 2004 also reported that microbes preferentially adhere to irregularities that confirm to their size since this maximizes microbial surface area. Grooves or scratches that are on order of microbial size increase the contact area and hence the binding potential (Bos et al., 2000). The application of CLSM has provided a powerful non destructive method for biofilm analysis. CLSM made it possible to observe biofilm without destruction of the structures, and revealed that biofilm developed in both horizontal and vertical direction. CLSM has been adopted for biofilm analysis using fluorescent dye specific for nucleic acids (PI) and glycoconjugate fraction (FITC-ConA) of biofilm. PI binds to DNA by intercalating between the bases with little or no sequence preference and with a stochiometry of one dye per 4-5 base pairs of DNA. This dye is commonly used for identifying dead cells in a

population and as a counterstain in multicolor fluorescent techniques. CLSM coupled with fluorescence methods and image analysis provides images with better resolution thus giving more information pertaining to biofilm. *C. albicans* biofilm on biomaterial samples were observed using CLSM. Hence from the current work it can be inferred that these staining techniques could be use simultaneously to probe viable and non viable *Candida* cells. The combined effects of individual stained images showed EPS produced in cell bound as well as secreted form. This present scheme is expected to applicable to other disaggregate systems such as biofilm in waste water sludge floccules (Chen et al., 2007).

The different growth pattern of *C. albicans* biofilm on biomaterial surfaces showed involvement of material surface properties on biofilm growth. Our data showed that both the cell numbers (red intensities) and EPS (green intensities) vary with biomaterials as well as different layers. Since, biofilm structure depends on EPS our data also showed that biomaterial surface properties not only affect biofilm structure and morphology but also their metabolic activity and most probably gene expression level.

5.4 CLONING AND CHARACTERIZATION OF CDR1 GENE

Drug efflux pumps are major mechanism of drug resistance and *C. albicans*, like *S. cerevisiae*, contains many ORFs with homology to either ABC or MFS drug pumps. However, that they all are not involved in antifungal drug export; they may either be insufficiently expressed or have other physiological functions.

Integration of the *CDR1* ORF into genomic DNA resulted in stable inheritance of a single copy of the gene at the locus for the *S. cerevisiae* homologue *PDR5*. Fusion of the *CDR1* ORF to the *PDR5* promoter in a strain that contains the transcriptional regulator mutation *pdr1-3* ensured overexpression of Cdr1p. This overexpression was demonstrated in terms of increased levels of appearance of a new 170-kDa protein band that accounted for 10 to 20% of the plasma membrane protein which reacted with anti-*C. albicans* Cdr1p antibodies. This protein was functional. Its expression conferred on *S. cerevisiae* drug resistance, increased levels of plasma membrane NTPase activity, and energy-dependent rhodamine 6G efflux. The drug resistance phenotype was due to the overexpression of Cdr1p and not simply the *pdr1-3* mutation, as this mutation was also present in the hypersensitive parental strain, AD1-8u$^-$. Plasma membranes from the Cdr1p-overexpressing strain displayed oligomycin-sensitive NTPase activity with biochemical properties, including pH activity profiles, similar to those of Pdr5p, the *S. cerevisiae* multidrug efflux pump homologous to *C. albicans* Cdr1p (Decottignies et al., 1994). The pH optimum for UTPase activity (pH 7.0 to 8.0) was significantly higher than that (pH 6.5) reported by Krishnamurthy et al. with a plasmid-based expression system (Krishnamurthy et al., 1998).

Expression of Cdr1p in ApCDR1 conferred the ability to efflux rhodamine 6G. Rhodamine 6G efflux is associated with *S. cerevisiae* Pdr5p and Yor1p (Decottignies et al.,

1998; Kolaczkowski et al., 1996), but both genes that encode these proteins are deleted from ApCDR1. Rhodamine efflux has also been associated with azole resistance in *Candida* (Clark et al., 1996) and, in particular, with Cdr1p expression (Maesaki et al., 1999).

Our results showed that the Cdr1p in ApCDR1 functions normally and that rhodamine 6G is Cdr1p substrate. Expression of Cdr1p reduced the sensitivity of AD1-8u$^-$ to a variety of structurally unrelated compounds which could be pump substrates. The spectrum of compounds to which Cdr1p conferred resistance was similar to that to which Pdr5p confers resistance (Kolaczkowski et al., 1996). Recent experimental evidence suggests that Cdr1p may be involved in the distribution of phosphatidylethanolamine across the plasma membrane lipid bilayer (Dogra et al., 1999), analogous to the "flippase" activity ascribed to human Cdr1p homologues Mdr2p and Mdr3p (Higgins, 1994; Ruetz et al., 1994; van Helvoort et al., 1996). Other studies suggest that Pdr5p and Cdr1p might also transport membrane sterols (Kolaczkowski et al., 1996; Kontoyiannis, 2000; Krishnamurthy et al., 1998). These observations seem to indicate a broad Cdr1p substrate specificity that includes amphipathic molecules that contain both hydrophobic and hydrophilic domains. Plasma membrane ATP-binding cassette (ABC) transporter, short-lived multidrug transporter actively regulated by Pdr1p (a homologue of cdr1p) also involved in cation resistance during exponential growth (Miyahara et al., 1996). Recently, the role of these pumps has been explored in biofilm-associated resistance (Ramage et al., 2002; Mukherjee et al., 2003). Ramage et al., (2002) evaluated resistance in biofilms formed by *C. albicans* mutants deficient for Cdr1p, Cdr2p or Mdr1p, at intermediate and mature phases (24 and 48 h) of growth. These investigators demonstrated that the *CDR1* and *CDR2* genes were up-regulated in intermediate and mature *C. albicans* biofilms, but role of these pumps in case of biofilm

was not established till date. Reports are also there about involvement of ABC transporters of *Pseudomonas* species in adherence and biofilm formation but role major ABC transporter of *C. albicans* CDR1 was remained unexplored (Chandra ref).

Our data showed metal resistance incase of biofilm formed by CDR1 expressing ApCDR1 than its parental AD1-8u⁻ strain. This function of metal resistance is conferring by CDR1 gene. In the present studies we find the novel function of CDR1 to provide metal tolerance to biofilm. The study give two new important aspect of CDR1 gene first, it involved in cation transport and second, to provide metal resistance to biofilms. The study is important aspect of biofilm study since previously there was no report of involvement of CDR1 gene to biofilm and it was known to provide only drug resistance. More research needs to be carried out to uncover concealed relations between drug efflux pumps and biofilm formation.

5.5 EFFECT OF BIOSURFACTANTS, PLANT OILS, ENZYMES, SILVER COATINGS AND ANTIFUNGAL AGENTS ON *C. albicans* BIOFILM

5.5.1 Effect of Biosurfactant on *C. albicans* Biofilm

Most important in the pathogenesis of foreign body associated infections is the ability of microbes to colonize the polymer surface by the formation of a thick multilayered biofilm (Christensen et al., 1994; Scierholz et al., 2001). Depending on the kind of device, its insertion side duration of insertion and the nature of infecting microorganism, different syndrome generates several clinical presentations. It is evident that biofilm forming organisms attribute their properties towards pathogenesis more successfully than other organisms with more toxins and tissue damaging exoenzymes (Rupp et al., 1994). When the microorganisms reach the proximity of a surface, attachment is determined by physical and chemical interactions, which may be attractive or repulsive, depending upon the complex interplay of the chemistries of the microbes, substratum surfaces and the aqueous phase. To understand the forces that determine adhesion many concepts including DLVO model, the thermodynamic approach (Morra et al., 1997) and the extended DLVO theory (Jucker et al., 1998) have been given but still adhesion and surface interplay have not been fully understood. The reason behind that is involvement of environmental conditions (Kiapper et al., 2002), expression of genes (Shirtliff et al., 2002) altered growth rate (Chen et al., 2002) and surface properties (Pratt et al., 1998) of biofilm residing organism.

Hogt et al (1985) in their experiment showed that absorption of protein on hydrophobic surface was found to increase surface hydrophilicity, which resulted in the reduction of adhesion. In our studies goniometric analysis of rhamnolipid coated pieces showed reduction in contact angle (increased hydrophilicity) which may be a factor

responsible for biofilm reduction. Microbes with higher hydrophobic properties prefer hydrophobic material surface (Vacheethasanee et al., 2000), these findings explain that why these biofilm reduces with contact angle.

Our results are in agreement with previous studies showing that the correlation between contact angle and biofilm formation is dependent on surface (Samaranayake et al., 2004; Yip et al., 2003), and contrary to the finding of reports of no correlation (Jones et al., 1999) or reduction with hydrophobic surface (Balazs et al., 2004). Our results show *C. albicans* biofilms on biomaterials of different hydrophobic and roughness, results indicate that amount of biofilm produced depends on roughness and hydrophobicity/hydrophilicity but have no direct relationship with these properties.

Beside these, rhamnolipid coated biomaterials showed decreased surface roughness and biofilm formation. The probable reason of this may be due to a rough surface has a greater surface area and the depressions in the roughened surfaces provide more favorable sites for microbial colonization (Scheuerman et al., 1998). It has also been reported that surface materials with different porosity, groove and braid are with higher infection rates than flat ones, probably due to increased surface area (Scheuerman et al., 1998; Bos et al., 2000).

Harshey et al.(Mireles et al., 2001) during their studies on surfactin along with chemical surfactants (Tween 20 & Tween 80) showed the potential of biosurfactants in inhibiting the formation of biofilm by *Salmonella enterica* on PVC and urethral catheter. Similarly, Rodrigues et al. (Gristina et al., 1993) also reported efficacy of biosurfactant obtained from probiotic bacteria *Lactococcus lactis* and *Streptococcus thermophilus* against biofilm formed by *C. albicans,* and *C. tropicalis* on voice prostheses.

Earlier Busscher et al. (Busscher et al., 1997) have reported the inhibition of biofilm formed by *Candida* species on silicone rubber by biosurfactant produced by *Streptococcus thermophilus*. They showed interference of biosurfactant on adhesion phage of biofilm development. In fact, to prevent/remove biofilm residing organisms, the chemical compositions of EPS plays a vital role as it varies significantly among biofilms formed under different conditions (Mukherjee et al., 2004; Hawser et al., 1998). Similar findings are also shown by Arciola et al. (Arciola et al., 1995) during the studies on bacterial adhesion between seven different silicone-based materials. Ludwika et al. 1984, also noted a varying degree of bacterial attachment to different synthetic polymers including silicone rubber, polypropylene, polyetherurethane, and cellulose acetate.

5.5.2 Effect of Plant Oils on *C. albicans* Biofilm

Plant oils traditionally used for domestic and therapeutic purpose are increasingly claimed to have broad spectrum antimicrobial properties. Selected oils have been suggested to have potent antimicrobial activity, including anthelmintic, skin infections and insect bites, chicken pox, colds, flu and measles sinus congestion, asthma, bronchitis, pneumonia, tuberculosis and cholera properties, due to their phenolic, alcoholic and terpenoid constituents (Deans et al., 1991; Hammer et al., 1998; Hili et al., 1997). 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 (jain et al., 1997; Sheehan et al., 1999; Sanglard et al., 2003; Chandra et al., 2001). The biofilm formed by *C. albicans* are much more resistant to antimicrobial agents than planktonic cells (Ramage et al 2005, Chandra et al., 2001). In this context, new agents that can inhibit the growth of biofilm-associated microorganisms are

greatly needed and would enhance the number of effective therapeutic alternatives (Alviano et al., 2005). Taking into account this the present study was carried out to assess the antifungal properties of plant oils against C. *albicans* and its biofilm.

The tetrazolium salt, XTT was used to monitor effect of plant oils on *C. albicans* biofilm formation by colorimetric determination. The salt reduced by mitochondrial dehydrogenase to brown color water-soluble tetrazolium formazan product determined spectrophotometrically (absorbance at 492nm). Eucalyptus and peppermint oils gave 80.87 and 74.46% reduction in *C. albicans* biofilm formation using this assay. Further visualization of the biofilm ultrastructure by SEM revealed that damage to the biofilm constituents was caused by eucalyptus oil followed by peppermint oil as compared with the untreated biofilm with fluconazole control suggesting that despite the relative minimal diffusion, these oils compared with other oils may be exerting a metabolic interference in *Candida* biofilm. Clove, ginger grass oil treated cells also revealed some degree of destruction. Since biofilm formation and development involves a series of mechanisms, cell responses and interactions, and any change in any step may cause biofilm inhibition. This suggests that active components of eucalyptus and peppermint oils have strong potential to affect *C. albicans* cell growth, function, biofilm formation and development by interfering with any of steps involved in biofilm development.

Although, previous reports suggest that filamentation in *C. albicans* play a critical role in biofilm formation and virulence (Lopez 2005; Ramage et al., 2002), contrary to this, the biofilm forming clinical isolate of *C. albicans* used in the present study does not show hyphal development in liquid culture. The finding is important because it shows that hyphal formation is not an important factor for biofilm formation and virulence. The observation is

in support with the findings of Nobile and Mitchell 2005, who demonstrated for the first time that *C. albicans* mutant strain unable to form biofilm can form filaments normally in liquid culture, thus separating the processes of filamentation and biofilm formation.

The difference in the microbial susceptibility is attributable to the chemical composition of plant oils. The ineffectiveness of twelve oils in the present study might reflect lack in activity of antimicrobial compounds against the treated *C. albicans* strains. The findings may be a result of either absence or insufficient concentrations of anti-Candida components which needs to be effective. Data showed that eucalyptus and peppermint oil are not only able to kill *C. albicans* cells efficiently but also inhibits biofilm formation. Interference of peppermint oil with iron uptake is also reported, which shows its involvement with normal metabolic stage of cell (Gregoleit and Gregoleit, 2005).

Our results clearly demonstrate that peppermint, eucalyptus, ginger grass, and clove oils not only act as a potent antifungal agent against *C. albicans* and its biofilm, especially eucalyptus oil is a potentially superior antifungal agent compared to fluconazole. Accordingly, reduction in *C. albicans* biofilm in response to plant oils used in our study can be explained on the basis of presence of these active components restricting biofilm development. Many of the major components of plant oils including 1, 8-cineole, limonene and linalool (Mazzanti et al., 1998) geranial (Araujo et al., 2003), germacrene-D (Ngasspa et al., 2003), and menthol (Iscan et al., 2002) have been reported to have antifungal activity. Studies have also been done for toxicity level of these components which shows that effective concentrations of plant oils in our study are within the toxicity range for mammalian cells (Rosa et al., 2003). The 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. The study is an important contribution to the characterization of the anti-*Candida* activity of plant oils from the Indian flora. Subsequently, experiments need to be conducted on plant oils showing potential anti-Candida activity to identify the active components.

5.5.3 Effect of Enzymes on *C. albicans* Biofilm

The enzymatic reduction in biofilm may be a result of either damage in biofilm constituents or interference with metabolic activity of sessile cells. Biofilms are mainly composed of different types of sugars known as polysaccharides and these together with other elements form extracellular polymeric matrix responsible for biofilm growth, physiology and structure. The enzymes in the study were chosen on the basis of their predictable action on biofilm residing cells and their matrices. Alginate lyase showed maximum reduction in biofilm as this acts as mannuronate β-eliminase which breaks sugar polymers and hence change in both the physiology and structure followed by metabolic reduction of biofilm residing cells.

5.5.4 Effect of Silver Coating on *C. albicans* Biofilm

Use of sputter as silver coating agent results in distribution of nanoparticles of metallic silver throughout the PVC surface. Results showed that much of the antimicrobial activity is due to silver particles. Although during coating process surface roughness also reduced to its one forth and this definitely adds towards biofilm reduction but no significant variation in surface hydrophobicity was observed and most of the biofilm reduction is because of silver particles. Silver exhibits good anti-microbial properties and in recent years has been used on medical devices ranging from wound dressing to urinary catheters (Ahearn

et al., 1999; Christensen et al., 1995; Gabriel et al., 1995; Johnson et al., 1999)The antimicrobial activity of silver is dependent on the silver cation (Ag^+), which binds strongly to electron donor groups on biological molecules containing sulfur, oxygen or nitrogen and silver ions generally act by displacing other essential metal ions such as calcium or Zinc(Maki et al., 1998). Although relatively higher temperature causes considerable difficulties when coating medical devices, many of which contains thermally sensitive polymer parts. In this study problem of silver coating adhesion on thermally sensitive polymers was addressed by combining a magnetron sprutter source with a saddle field plasma source.

C. albicans is the most common human fungal pathogen associated with device related infections mainly in its biofilm mode of growth. Most of *C. albicans* infections are associated with biofilm which has taken central stage with the increasing recognition of their role in human infections. In our investigation, thirty-six isolates of *Candida* spp. known to generate a distinct, identifiable color on a simple, cost effective, selective and differential medium on chromogenic agar medium was used. This facilitates rapid detection of different *Candida* species from mixed culture on the basis of coloration and colony morphology. Among different isolated *Candida* species, *C. albicans* (isolate no. 15) was screened on the basis of its maximum biofilm forming capabilities. The parameter optimization associated with *C. albicans* biofilm showed maximum adherence at 37^0C which is near to human body temperature and hence depicted its adaptation to be human pathogen. EPS quantification and compositional studies of *C. albicans* biofilm studies in this investigation showed it to be mainly composed of glucose, hexosamine, pentose, phosphorus and protein and their relative concentration varies with carbon sources. CLSM studies, AFM data and goniometric analysis of biofilm on different biomaterial surfaces showed that polymer surface properties and chemical interactions affects adherence and hence biofilm development. PVC with highest hydrophobocity (97^0) and roughness (134 nm) was found to support maximum colonization of *C. albicans* which depicted that higher roughness and hydrophobocity enhance biofilm formation.

Studies based on cloning of CDR1 gene of *C. albicans* in to *S. cereviceae* host AD-8u$^-$ showed its involvement in metal resistance of biofilm and cation transport. The finding is important for better understanding of biofilm resistance and development.

Studies carried out for biofilm prevention and control showed that rhamnolipid, a biosurfactant, inhibits 91% biofilm on catheter (silicone rubber) at its 8 µg concentration. Among plant oils tested against *C. albicans* biofilm, eucalyptus oil was found to give 80.87% reduction and proved better than its chemical counterpart Fluconazole used as positive control. Data obtained from enzymatic reduction studies showed alginate lyase causes 70.7% drop in metabolic activity of biofilm residing *C. albicans* cells. Interestingly, 15 sec silver coating was sufficient to inhibit complete growth of *C. albicans* biofilm on PVC surfaces.

Future prospects of this investigation can lead to develop strategies to control *C. albicans* biofilm and analysis of novel cell receptors which may provides specific binding sites. Studies on CDR1 disruptant *C. albicans* may give more detailed view of relation between biofilm formation and drug efflux pumps. Further, approaches leading to alteration of biomaterial surfaces may inhibit *C. albicans* biofilm formation and colonization which would be helpful to eradicate biofilm associated device infection.

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