IMPACT OF ENVIRONMENTAL FACTORS ON CANDIDA ALBICANS BIOFILM

Ph.D THESIS

by

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of

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in

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"IMPACT OF ENVIRONMENTAL FACTORS ON CANDIDA ALBICANS BIOFILM"** in partial fulfilment of the requirements for the award 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 a period from December, 2010 to June, 2016 under the supervision of Dr. Vikas Pruthi, Professor and Dr. R. Prasad, Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

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.

(Suma Chaitanya Pemmaraju)

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

(Vikas Pruthi)
Supervisor

(R. Prasad)
Supervisor

Dated: _____

ABSTRACT

Biofilms are complex, interdependent community of surface associated microbial cells enclosed in an extracellular matrix. They can adhere to any surface ranging from industrial and aquatic water systems to medical devices and host tissues. The unique feature of biofilm structure is its increased resistance to conventional antimicrobial agents and immune system which made them difficult to treat from clinical point of view. Biofilms formed by *Candida* sp., *Staphylococcus* sp., *Streptococcus* sp., and *Escherichia coli*, *Pseudomonas aeruginosa* are responsible for majority of nosocomial infections. Among these, *Candida albicans* is the predominant fungal species associated with biofilm related infections.

C. albicans, dimorphic yeast of human microbiota colonizing oral, gastrointestinal and urogenital tracts of healthy individuals. Nevertheless, *C. albicans* is the most common fungal pathogen causing superficial and systemic infections. During colonisation, *C. albicans* is exposed to a variety of environmental factors at diverse host niches such as nutrient resources, oxidative and osmotic stresses, innate immune secretory factors and other co-infecting microorganisms. The environments encountered by *C. albicans* within a host depend on the niche it occupies. Adaptation to these different environments is crucial for *C. albicans* virulence as it increases the survival of this pathogen. In adaptation process, these environmental factors can influence the cell physiology, morphology, adherence and architecture of biofilms which results in coordinated changes in expression of virulence factors and cell wall composition. The altered virulence traits in the biofilm can influence the pathogenicity of *C. albicans*. Keeping the significance of above facts in view, the main objective of the work carried out in this thesis was to investigate the effect of environmental factors on *C. albicans* biofilm and how this in turn affects the virulence factors and cell wall composition of this fungal pathogen during biofilm growth.

C. albicans needs to assimilate locally available or alternative nutrients for their survival and multiplication in the dynamic environments. During infection, carbon sources play a central role in metabolism and critical for colonization in *C. albicans*. The role of different carbon sources such as glucose, lactate, sucrose, and arabinose on *C. albicans* biofilm development and virulence factors was investigated. Quantitative analysis of biofilm formation was analyzed by XTT (2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5- carboxanilide) reduction assay. Qualitative analysis of biofilm development was determined by confocal laser scanning, scanning electron and atomic force microscopy. Glucose grown cells exhibited the highest

metabolic activity during adhesion among all carbon sources tested. However, cells exposed to sucrose exhibited highest biofilm formation and matrix polysaccharides secretion. Exposure to lactate induced hyphal structures with the highest proteinase activity while arabinose grown cells formed pseudohyphal structures possessing the highest phospholipase activity. β-glucans are the major structural components of the cell wall of *C. albicans* as well as most important fungal pathogen associated molecular patterns (PAMPs). Therefore, structural changes in β-glucan was characterised by Fourier transform infrared (FT-IR) spectroscopy. Curve fitting analysis of the spectrum revealed a significant changes in β(1→6) to β(1→3) glucan ratio in the carbon sources tested. These results signify that carbon sources influence *C. albicans* biofilm development, modulate virulence factors and structural organization of cell wall component β-glucan.

During colonisation, *C. albicans* encounters environmental stresses namely osmotic and oxidative stresses. Therefore, the study was focused on the impact of these stress factors on *C. albicans* biofilm formation and virulence factors. *C. albicans* cells were incubated in the presence of 5 mM H_2O_2 and 2 M NaCl to induce oxidative and osmotic stresses. Oxidative stress enhanced extracellular DNA secretion into the biofilm matrix *via* reactive oxygen species mediated cell lysis, increased chitin levels, and reduced the extracellular phospholipase as well as the proteinase activity. While osmotic stress notably stimulated biofilm formation with increased proteinase and decreased phospholipase activity. FT-IR and nuclear magnetic resonance (NMR) spectroscopy analysis of the cell wall component mannan revealed a decrease in mannan content and reduced β-linked mannose moieties under stress conditions. The ability of *C. albicans* to survive in the presence of sub-lethal concentrations of oxidative and osmotic stress inducing agents can be attributed to the highly resistant biofilm mode of growth response by *C. albicans*.

The constant flow of saliva in the oral milieu containing innate immune secretory factors acts as a chemical barrier to restrict pathogens. The response of *C. albicans* to the innate immune defense factors like mucin, lactoferrin, and lysozyme was tested. In addition, the effect of extracellular adenosine tri-phosphate (eATP) released by damaged cells to signal 'danger' to immune system were also studied. Experiments were conducted by incubating *C. albicans* in the presence of different test compounds mentioned above and biofilm formation was evaluated using XTT assay. Biofilm formation was suppressed by lysozyme and lactoferrin in a dose dependent manner. However, lactoferrin and lysozyme were more effective at higher concentrations (≥2 mg mL^{-1}) in disrupting preformed *C. albicans* biofilm. Mucin is another host derived secretory compound that suppressed biofilm formation and virulence traits in *C.*

albicans by inhibiting cell attachment to polystyrene surface. On the other hand, danger signalling molecule extracellular dATP/ATP (500 µM) stimulated biofilm formation and the release of eDNA into *C. albicans* biofilm matrix. Treatment of *C. albicans* with dATP activated ROS mediated cell lysis thereby releasing DNA extracellularly into matrix. Overall, results indicated that modulation of *C. albicans* biofilm formation by these host innate immune factors can alter the dynamic interplay between host immune system and the clearance of the pathogen.

Biofilm communities are far more complex than recognised. Microorganisms present in the surrounding environment can have a major impact largely on the growth of other microbes. The influence of pathogenic bacteria namely *Pseudomonas aeruginosa Staphylococcus aureus* and *Escherichia coli* on *C. albicans* biofilm formation was studied. Different responses were observed when different pathogenic bacteria were co-incubated with *C. albicans*. Both *P. aeruginosa* and *E. coli* suppressed *C. albicans* biofilm formation, conversely *S. aureus* did not exert any inhibitory effect on *C. albicans* biofilm growth. The specific activity of phospholipase and proteinase was also reduced in mixed species biofilms as compared to *C. albicans* biofilm.

Along with the above studies, attempts were made to identify the agents that interfere with the adhesion and biofilm formation by *C. albicans*. Several phytocompounds were selected from the chemical library available in the literature and screened for their antifungal activity. Antifungal activity of certain plant extracts and phytochemicals is due to the presence of biologically active compounds like terpenes and polyphenolic substances. Hence, in this investigation the role of terpenes namely eugenol, menthol and thymol and fluconazole (FLA) on *C. albicans* biofilm inhibition were studied. The minimum inhibitory concentration evaluated by broth micro-dilution method showed antifungal activity against *C. albicans* at a concentration of 0.12 % (v/v) for both thymol and eugenol as compared to 0.25 % (v/v) for menthol. Thymol and eugenol were more effective in inhibition of preformed biofilm than menthol. Synergistic studies using checkerboard micro-dilution assay showed fractional inhibitory concentration index between thymol/FLA effectively against pre-formed *C. albicans* biofilms. Thymol with fluconazole showed highest synergy in reduction of biofilm formation than eugenol and menthol which was not observed when their activities were observed independently. Adherence studies and microscopic analysis showed reduction in cell number and alteration in morphology of *C. albicans.*

The distinct ability of *C. albicans* to grow at diverse host niches is attributed to the adaptive response generated with respect to the environmental conditions it encounters in a specific

niche. Significant findings from these studies indicated that host derived environmental factors like nutrient resources and stresses promote biofilm formation. It also affects virulence factors and cell wall composition in *C. albicans* biofilm. On the other hand, microbe derived environments can inhibit biofilm formation and virulence factors in *C. albicans*. Thus, biofilm formation in response to different host environmental factors is integral part of *C. albicans* pathogenicity. The cell phenotypes of *C. albicans* within biofilms are highly resistant to antifungal agents and innate immune factors. To counteract this, several natural phytochemicals in form of combinatorial therapy either by using conventional antifungal agents or by innate immune secretory factors like mucin, lactoferrin or lysozyme could be a promising medication in the treatment of biofilm infections.

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1. **Suma C Pemmaraju**, Parul A Pruthi, R. Prasad and Vikas Pruthi. **2015**. Extracellular dATP/ATP induces eDNA release and biofilm formation in *Candida albicans*. *Oral and poster* presentation at Fourth European Congress on Microbial Biofilms (EUROBIOFILMS), held at Brno, Czech Republic from June 23-26.

2. **Suma C Pemmaraju**, Parul A Pruthi, R. Prasad and Vikas Pruthi. **2014.** Antibiofilm activity of polyphenolic nutraceutical compounds from Citrus fruits on *Candida albicans*. Poster presented at International Conference on Pharmaceutical Sciences, held at SGRRITS, Dehradun, India from February 14-15.

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6. **Suma C. Pemmaraju**, Nivedita Singh, Priya Vashisth and Vikas Pruthi. **2011**. *Candida albicans* biofilm formation on contact lenses and its susceptibility to Eugenol. Poster presented at 7th International Conference on Yeast Biology held in Indian Institute of Technology Bombay, Mumbai, India, from December10-13, pp. 80.

7. Deepak Sharma, Nivedita Singh, **Suma C. Pemmaraju**, Richa Panwar, Swaranjit S Cameotra and Vikas Pruthi. **2011**. Production of microbial surfactants from oily sludge contaminated soil by *Bacillus subtilis* DSVP23. Poster presented at International Conference on New horizons in biotechnology and 8th Annual convention of The biotech research society, India organized by National institute for interdisciplinary science and technology (NIIST), CSIR, Trivandrum, India, from November 21-24, pp. 166.

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ABBREVIATIONS

ABC	ATP-binding cassette
AFM	Atomic force microscopy
AIDS	Acquired immunodeficiency syndrome
ALS	Agglutinin-like sequences
ANOVA	Analysis of variance
AP-1	Activator protein
ATCC	American type culture collection
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
CFU	Colony forming unit
CLR	C-type lectin receptors
CLSI	Clinical and Laboratory Standards Institute
CLSM	Confocal laser scanning microscopy
Con A	Concanavalin A
conc.	Concentrated
CVC	Central venous catheters
CWP	Cell wall protein
DAMP	Damage-associated molecular pattern
dATP	2′-deoxyadenosine 5′- triphosphate
DCFDA	2′,7′- dichlorodihydrofluorescein diacetate
dCTP	2′-deoxycytodine 5′- triphosphate
dGTP	2′-deoxycytosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleic triphosphate
dTTP	2′-deoxythymidine 5′-triphosphate
eDNA	extracellular deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substances
FIC	Fractional inhibitory concentration
FITC-ConA	Fluorescein isothiocyanate-concanavalin A

FLA	Fluconazole
FTIR	Fourier transform infrared spectroscopy
GCN	General amino acid control
GCS	Glutathione synthetase
GlcNAc	*N*-acetyl-D-glucosamine
GLR	Glutathione reductase
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPI	Glycosylphosphatidylinositol
h	Hour(s)
HBEC	Human buccal epithelial cells
HIV	Human immunodeficiency virus
HSD	Honestly significant different
HWP	Hyphal wall protein
IL	Interleukin
Log	Logarithm
LB	Luria-Bertani
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
MBL	Mannose-binding lectin
MHz	Megahertz
MIC	Minimum inhibitory concentration
Min	Minute
mM	Millimolar
MOPS	3-(N-morpholino)propanesulfonic acid
MR	Mannose receptor
MTCC	Microbial type culture collection
MTP	Microtiter plate
NADH	Nicotinamide adenine dinucleotide
NLR	NOD-like receptor
NMR	Nuclear magnetic resonance
OD	Optical density
Ost	Osmotic stress

Oxt	Oxidative stress
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PI	Propidium iodide
Pir	Protein with internal repeat
PKA	Protein kinase A
PKC	Protein kinase C
PL	Phospholipase
PLGA	Poly($_{D,L}$-lactide-*co*-glycolide)
PMN	Polymorphonuclear leukocyte
PVE	Prosthetic valve endocarditis
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediate
RNS	Reactive nitrogen species
ROI	Reactive oxygen intermediate
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPMI	Rosewell park memorial institute
SAP	Secreted aspartyl proteinase
SAPK	Stress-activated protein kinase
SD	Standard deviation
SDA	Sabouraud dextrose agar
SDS	Sodium dodecyl sulphate
SDS-Mer	Sodium dodecyl sulphate-mercaptoethanol
SEM	Scanning electron microscopy
SMIC	Sessile minimum inhibitory concentration
TCA	Tricarboxylic acid
TE	Tris-EDTA
TLR	Toll-like receptors
TSP	3-(trimethylsilyl) propionic-2, 2, 3, 3-d_4 acid sodium salt
XTT	2, 3-bis (2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide
YNB	Yeast nitrogen base
YPD	Yeast peptone dextrose

µ	Micron
µL	Microliter
µm	Micrometer
µM	Micromolar

CHAPTER-1

1. Review of literature

1.1 General Introduction

In healthy individuals, *Candida* species are commensal fungi that form a part of normal microbiota of skin, oral, gastrointestinal as well as vagina surfaces. However, *Candida* species are at present recognized as major agents of nosocomial infections (Sarvikivi et al. 2008). The genus *Candida* consists of nearly 200 species, of which *C. albicans, C. krusei, C. glabrata, C. parapsilosis* and *C. tropicalis* are considered medically significant (Pfaller & Diekema 2010). Among these, *C. albicans* is the largely identified (50-80%) opportunistic pathogen (Segal 2005; Pfaller & Diekema 2004). Reports state that 30% to 75% of humans are asymptomatic carriers of *C. albicans* (Pappas et al. 2009).

In a commensal state, the stable interaction with host immune system creates an inhospitable environment that requires a homeostasis between *Candida* growth rate and its clearance by the host defence mechanisms. Slight instability in host immune system or mammalian microflora alters *C. albicans* from commensal to a pathogen causing systemic and life threatening infections. *Candida* infections are prevalent in immunocompromised people, such as patients with HIV infection, cancer, transplant recipients, and endocrine disorders such as diabetes mellitus (Samaranayake et al. 2006). Prolonged antibiotic therapy may also alter the normal endogenous microflora and enable development of *Candida* infections. *C. albicans* nosocomial infections are increasing alarmingly due to their ability to grow as biofilm on implanted medical devices. Device associated *C. albicans* infections have high mortality rates, as well developed biofilms are resistant to conventional antifungal agents (Fox & Nobile 2013). Along with biofilm formation, *C. albicans* possess armoury of virulence factors such as morphological transitions, phenotypic switching, adhesins and secreted hydrolases that aid in hematogenously disseminated infections in immunocompromised hosts (Mayer et al. 2013).

During infection, *C. albicans* encounters stresses in different ways from host defences, host environment and intrusion of antifungal agents. *C. albicans* has a unique ability to grow as a commensal in several anatomically distinct host niches, each with its own specific set of environment. The structure of biofilm in *C. albicans* is highly dependent on the environmental conditions. For instance, *C. albicans* encounters different environmental forces such as physical factors (carbon and nitrogen sources, pH, temperature, and serum), stresses (osmotic,

nitrosative and oxidative stress), innate immune defences (epithelial cells, antimicrobial peptides, mucin, macrophages and leucocytes) and co-infecting microorganisms during colonisation and dissemination (Fig.1.1). These environmental factors can influence the cell physiology, morphology, adherence and architecture of biofilm which results in coordinated changes in expression of virulence factors and cell wall composition. Thus, pathogenicity of *C. albicans* is complex and depends on the host physical and physiological status and virulence traits. Several studies have reported independently on the role of environmental factors on *C. albicans* growth but how the above mentioned environmental factors drive the *C. albicans* biofilm formation is still in its infancy. Hence, the present study focused on the impact of environmental factors on *C. albicans* biofilm. Special emphasis was given to *C. albicans* cell wall, a dynamic structure that protects the cell from environmental stresses. Understanding the pathogenicity of *C. albicans* biofilms might help in development of new antibiofilm agents and preventive procedures.

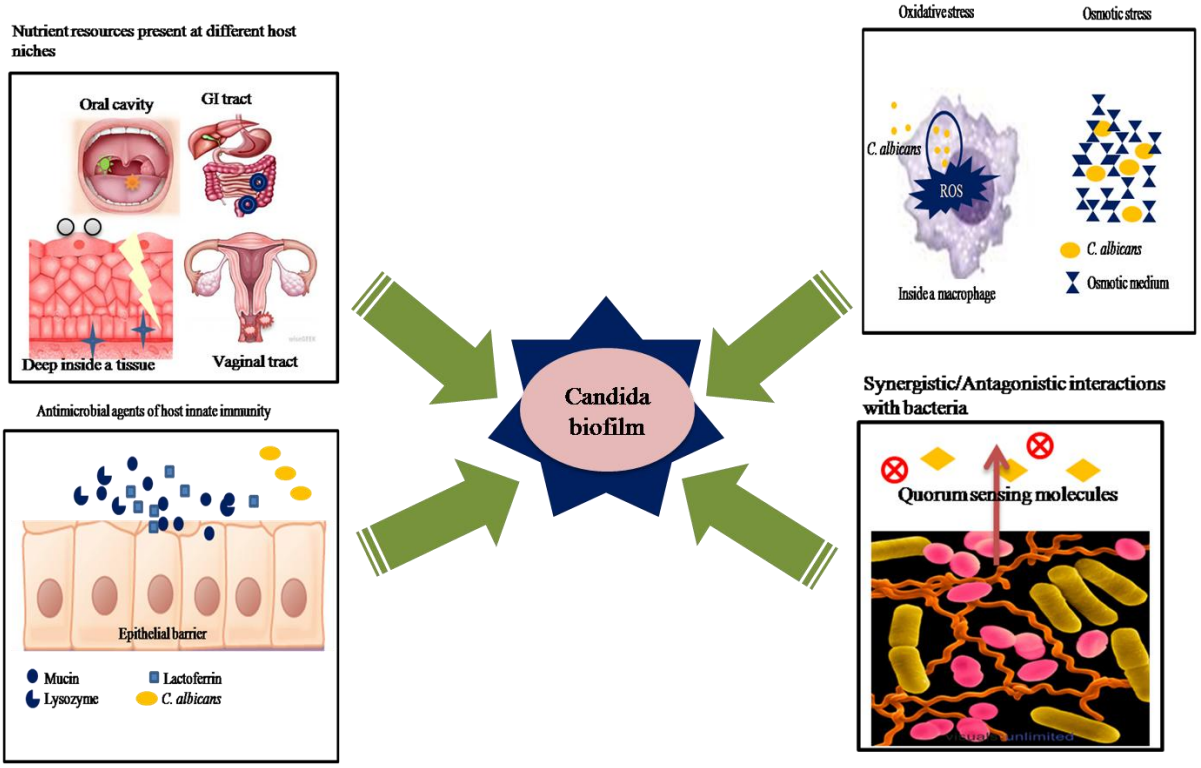


Figure 1.1 Environmental factors affecting *Candida* biofilm. *C. albicans* encounters different host environments such as nutrient resources, stresses, antimicrobial agents of innate immunity and other colonizing microorganisms which affect its biofilm.

1.2 *Candida* biofilm

1.2.1 Candida infections

The complex interaction of microorganisms with implanted devices and host factors are responsible for large proportion of nosocomial infections. Recent reports by the National Institutes of Health demonstrated that 80% of all microbial infections are associated to pathogenic biofilms (Nobile & Johnson 2015; Fox & Nobile 2013). A biofilm is a complex, interdependent community of surface associated microbial cells enclosed in an extracellular matrix. They can adhere to any surface (or air-liquid interfaces) ranging from industrial and aquatic water systems to medical devices and host tissues (Nobile & Johnson 2015). *C. albicans* remains the predominant fungal species associated with biofilm related infections isolated from medical devices including urinary and central venous catheters, joint prostheses, pacemakers, mechanical heart valves, dentures and contact lenses (Kojic & Darouiche 2004).

C. albicans biofilm acts as a reservoir for infections to seed candidemia that can lead to invasive systemic infections of tissues and organs (Fig. 1.2). These biofilms are highly resistant to conventional antifungal drugs and the host immune system. *C. albicans* is the third leading cause of infections related to catheter, second highest in colonization to infection rate and the overall highest crude mortality (Crump & Collignon 2000). Candidiasis related to devices is problematic as device removal is a costly affair and has serious implications (Kuhn et al. 2002).

Most of the device related fungal infections are associated with *Candida* biofilm (Kojic & Darouiche 2004). Central venous catheters (CVC) represent the highest risk related to device associated infections (Richards et al. 2000). *Candida* commonly colonizes the central venous catheter employed to administer nutrients and cytotoxic drugs in cancer patients (Kojic & Darouiche 2004; Richards et al. 2000). Biofilm formation on CVC is rapid as it is coated with plasma and various proteins. *Candida* biofilms on prosthetic heart valves (90%) cause prosthetic valve endocarditis (PVE) or infection of the valve surrounding the heart tissue (Kojic & Darouiche 2004).

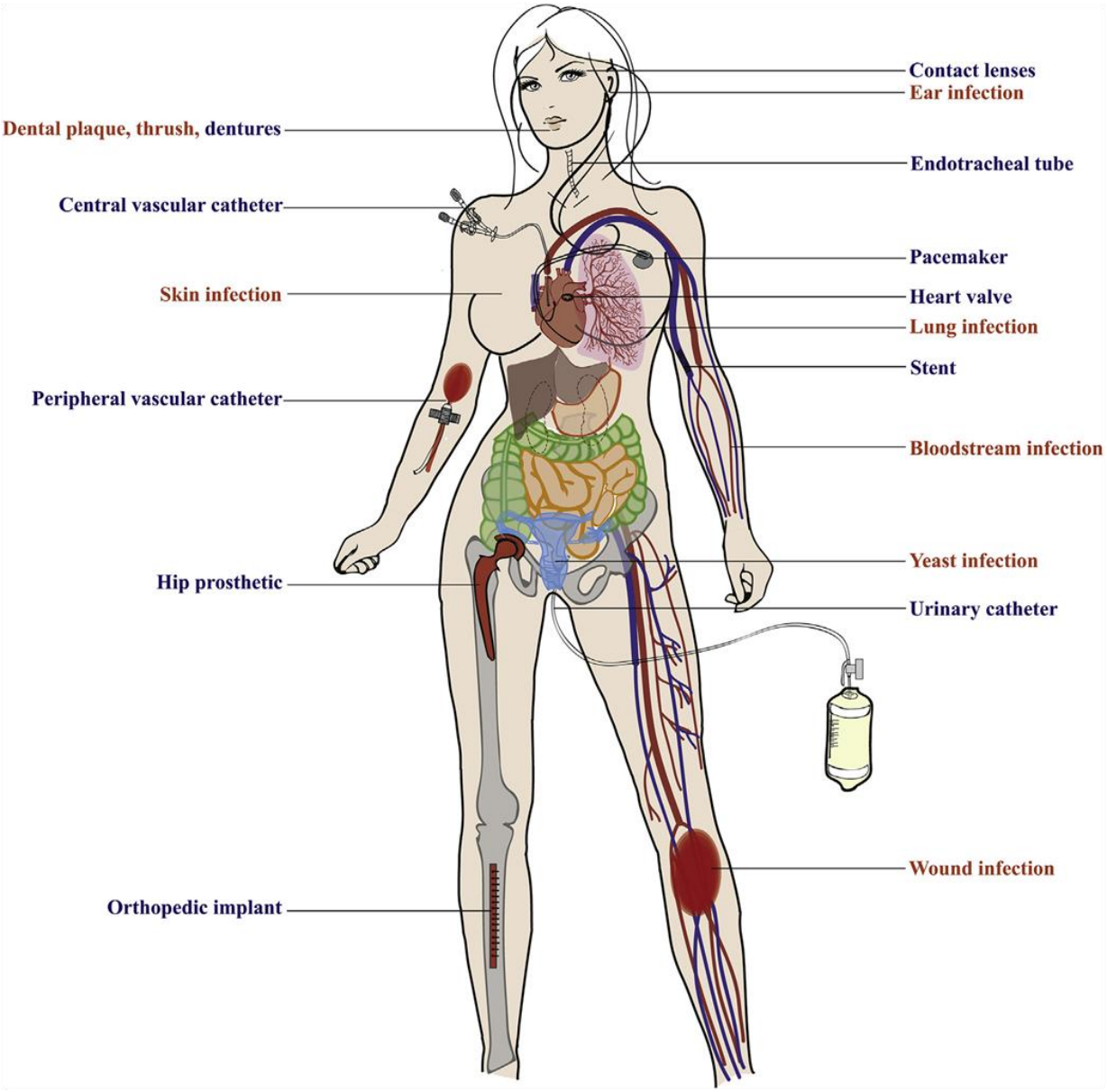


Figure 1.2 Biofilm-associated infections by *C. albicans*. Schematic representation of female body organs or regions susceptible to *C. albicans* infections either by direct biofilm formation on a implanted devices (text in blue) or from a localized and disseminated infection originating from a biofilm (text in red). (Figure taken from Gulati & Nobile 2016)

The exogenous risk factors for *Candida* infection could be due to contamination of catheter tip during insertion or by medical staff and patient's own skin (Sheretz 2000). *Candida* colonizing as commensal in gastrointestinal tract are able to penetrate intestinal mucosa and disseminate into blood, thus can seed catheter tip endogenously (Kojic & Darouiche 2004; Goldmann & Pier 1993). Other risk factors include immunocompromised state, arthritis, diabetes mellitus, prolonged antibiotic treatment, and prior surgery at site of device insertion (Kathju et al. 2014) . The use of intrauterine device has been linked to pelvic inflammatory disease. *Candida* co-exists with bacteria and other *Candida* sp., to form biofilms on epithelial surfaces causing superficial infections (Budtz-Jörgensen 1990). Some of the non-device associated infections that involve biofilms are *Candida* endocarditis, oral thrush, vulvovaginal candidiasis and denture stomatitis (Douglas 2003; Ferrer 2000). *C. albicans* accounts for 54% of systemic infections causing deep seated candidiasis in immunocompromised people (Wingard 1995).

1.2.2 Formation and characteristics of C. albicans biofilms

The ability of *C. albicans* to form densely aggregated biofilm communities is considered as a major virulence attribute. *C. albicans* forms highly structured biofilms consisting of oval budding yeast forms, pseudohyphal and long tubular hyphal cells (Fanning & Mitchell 2012). Extracellular matrix of biofilm is composed of polysaccharides, proteins, lipids, extracellular DNA (eDNA) and phosphorous. Experiments using i*n vitro* and *in vivo* systems have characterized *C. albicans* biofilm development as a series of four distinct sequential steps as shown in Figure 1.3 (Chandra et al. 2001; Finkel & Mitchell 2011; Desai & Mitchell 2015). Biofilm formation begins with the adherence of yeast cells to a solid substrate (adherence step). The initial adhesion is influenced by non specific factors such as electrostatic forces, chemical properties, surface hydrophobicity and roughness of the biomaterials used (Donlan & Costerton 2002; Agarwal et al. 2008a; Lal et al. 2008). Specific factors include fungal surface adhesins recognizing ligands such as salivary factors and serum proteins (fibronectin and fibrinogen). In addition, *C. albicans* can co-aggregate and/or bind to other colonizing microorganisms (Kumamoto 2002). The adhered yeast cells undergo proliferation, produce filaments (hyphae and pseudohyphae) and initiates biofilm development (initiation step). This stage is followed by biofilm maturation resulting in a complex three dimensional microcolony architecture of polymorphic cells encased in an extracellular matrix with extensive spatial heterogeneity (maturation step). This thick structure provides protection from physical and chemical injuries, influx of nutrients and efflux of waste products. Non-adherent yeast cells slowly detach or

disperse from the biofilm into surrounding medium to colonize new sites or disseminate in blood (dispersal step).

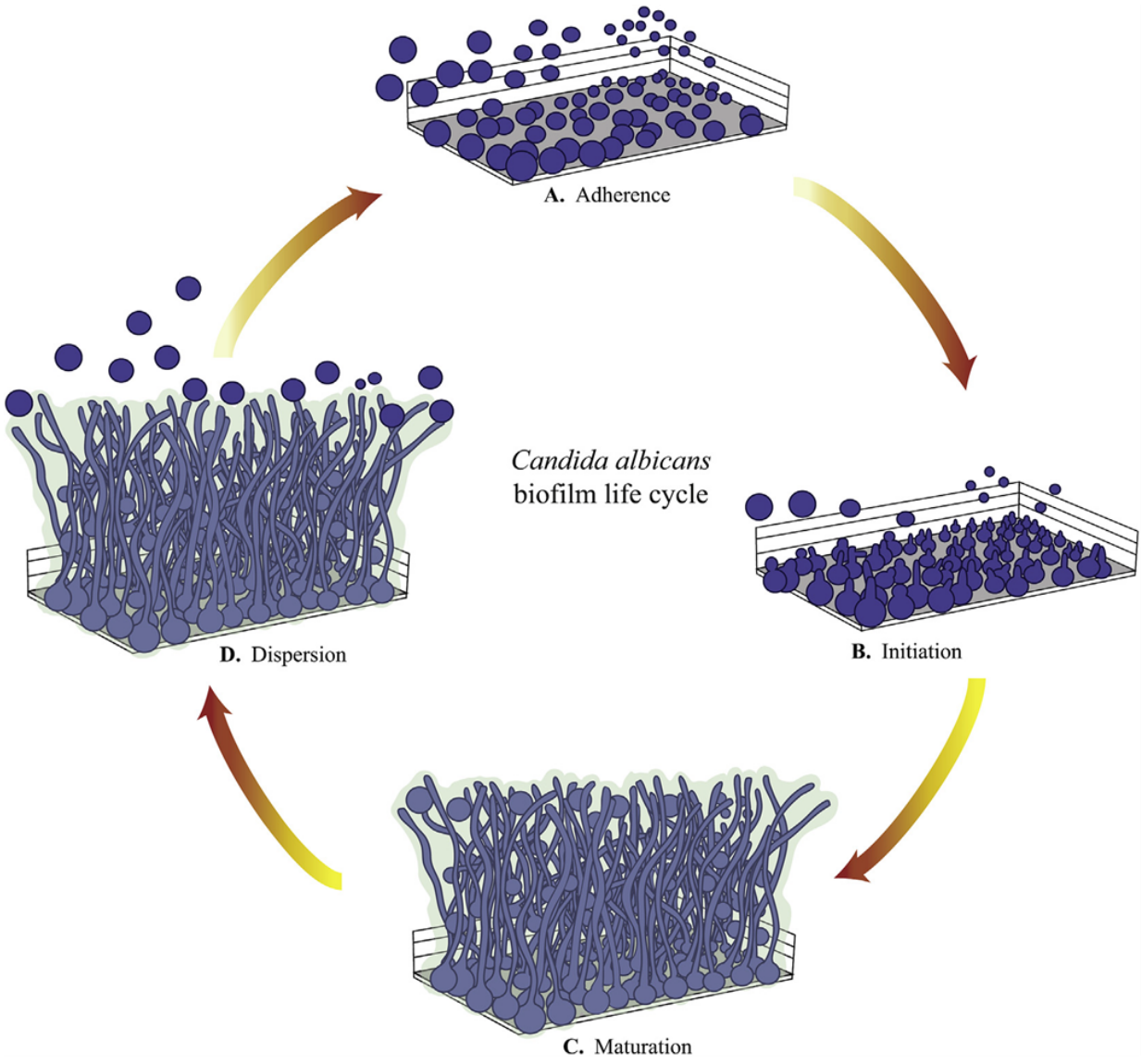


Figure 1.3 *C. albicans* biofilm life cycle. A. Adhesion of yeast cells to a surface. B. Initiation of biofilm formation, where cells proliferate to form a basal layer of adhered cells. C. Maturation of the biofilm, where complex filamentous and yeast cells develop and become encased in an extracellular matrix. D. Dispersion of yeast cells from mature biofilm to colonize new surface (Source: Gulati & Nobile 2016).

The ultra structure of biofilm formed on a solid substratum appears as thin basal yeast layer and upper hyphal layer with fibres of matrix surrounding the cells under scanning electron microscope (Baillie & Douglas 1999). The development of confocal microscopy allowed the examination of fully hydrated three dimensional architecture of live biofilm with typical microcolonies (Ramage et al. 2001a; Chandra et al. 2001). Studies of *C. albicans* biofilm using *in vitro* biofilm models has correlated well with the *in vivo* and *ex vivo* models in the time course of biofilm developmental phases and also had similar architectural properties isolated from patients with biofilm infections (Gulati & Nobile 2016).

1.2.3 Biofilm matrix and composition

The extracellular matrix is a distinctive feature of biofilm and its dynamic environment provides a homeostasis and nutrients to the microbial cells. It forms a three dimensional, hydrated, gel like structure in which microbes are enclosed more or less in an immobilized state (Fig. 1.4). The matrix is highly heterogeneous and the microcolonies often described as 'mushroom shaped stacks or towers' separated by water channels which provide nutrient cycling (Donlan & Costerton 2002). The thickness of mature biofilm ranges between 50 µm to 350 µm and matrix is mainly comprised of glycoprotein (55%), polysaccharides (25%), lipids (15%) and nucleic acids (5%) as has been reported earlier (Nobile & Johnson 2015; Zarnowski et al. 2014). Confocal and fluorescence microscopic studies using lectin binding dyes (Concavalin A and Calcofluor) displayed that *C. albicans* biofilms are enveloped in a thick extracellular matrix affluent in cell wall like polysaccharides containing glucose and mannose residues (Baillie & Douglas 2000). Due to the presence of carboxyl, phosphate and sulphate groups, these polysaccharides are negatively charged. The largest fraction of matrix polysaccharides consists of glucan-mannan complexes with β-1,3 glucan and α-1,2 and 1,6 linked mannan attached to β-1,6 glucan (Zarnowski et al. 2014). Environmental modulation can affect the composition and molecular mass of matrix polysaccharides and their capacity to interact with other polymers (Moryl et al. 2014).

The major function of matrix proteins is known to be involved in form of enzymes that can breakdown complex macromolecules and provide nutrients that can be readily utilized (Dignac et al. 1998). extracellular DNA (eDNA) plays an important role in biofilm establishment and integrity (Whitchurch et al. 2002; Sahu et al. 2012). Although, matrix polysaccharides and proteins confer a frame work, some enzyme activities are important for structural integrity and stability of biofilm (Sutherland 2001). The *C. albicans* biofilm architecture is affected by the surface properties of the substrate, environmental properties such as pH, shear stress, redox-

gradients, oxygen availability and exhibits a species specific structural heterogeneity (Kuhn et al. 2002; Stoodley et al. 2002; Mitchell et al. 2015). The composition of the matrix polymers varies significantly according to the biofilm growth phase (Mukherjee & Chandra 2004). Biofilm matrix in *Candida* aids in cell to cell communication, nutrient recycle and significantly contribute to high level of drug resistance (Al-Fattani & Douglas 2006).

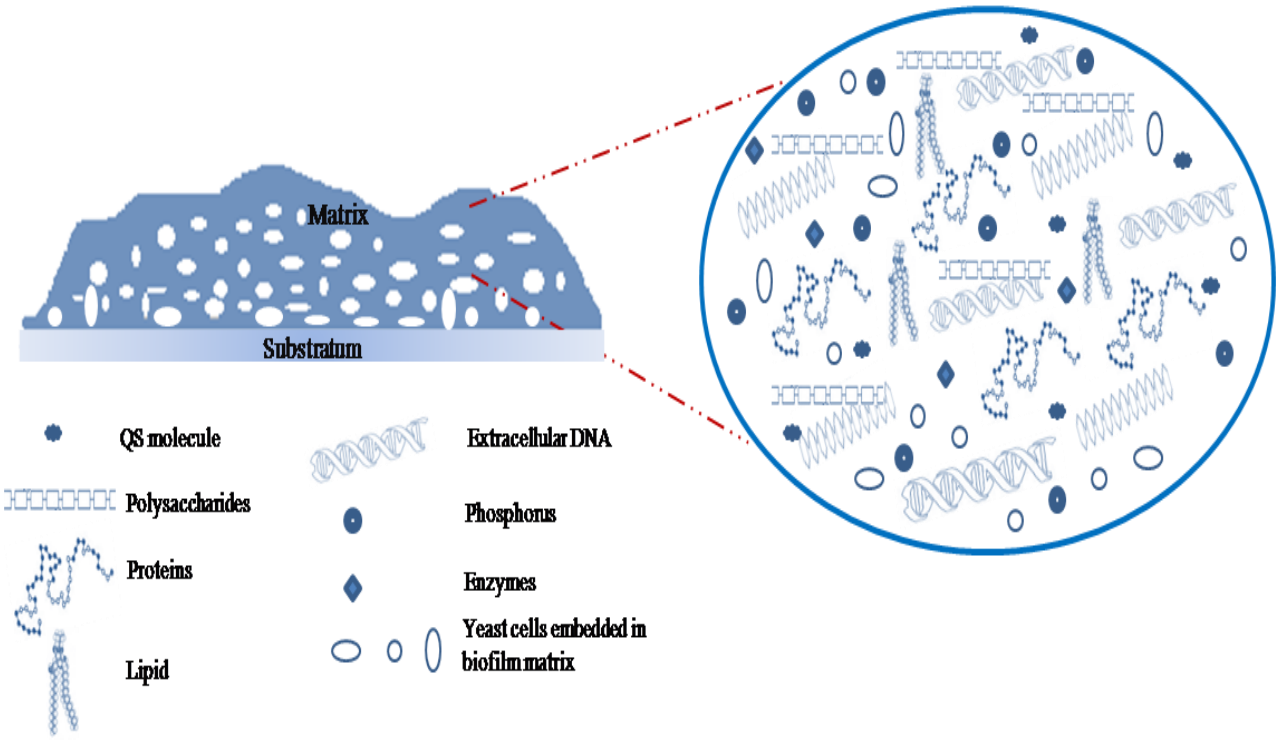


Figure 1.4 The extracellular matrix components within a biofilm. *C. albicans* biofilm cells are embedded in a self producing extracellular matrix that protects the cells from environmental changes. Main components of extracellular matrix are marked on the figure.

1.3 Virulence factors

Virulence factors in *C. albicans* facilitate adhesion, colonization and dissemination, assist in growth at different host niches and host environments (Mayer et al. 2013). They are considered as determinants of pathogenicity and their expression is niche specific. These virulence factors in *C. albicans* are briefly described here.

1.3.1 Adhesion

The adherence of *Candida* to biomaterial surfaces or host cells is a contributing factor for development of robust biofilms. Adhesion is regarded as one of the most important determinants of pathogenesis. It is a complex multifactorial process involving expression of several adhesins on a polymorphic cell surfaces. Hawser and Douglas (1994) reported a correlation between virulence of different *Candida* sp., and ability to form biofilm. Studies have shown several factors influence adherence step including growth medium composition, substratum properties (Donlan 2002). Enhanced production of surface manno-protein can increase adhesion to epithelial cell membranes (Cameron & Douglas 1996). Well studied adhesin is agglutinin-like sequence (ALS) that belongs to a family of eight members that encode glycosylphosphatidylinositol (GPI)-linked cell surface glycoproteins. Als1p, Als3p and Als5p take part in adhesion to human buccal epithelial cells (HBEC) and fibronectin, laminin, collagen, and endothelial cells (Hoyer 2001). The gene expression of ALS3 is upregulated during *in vitro* oral epithelial cell infection and *in vivo* vaginal infection (Wächtler et al. 2011). Als4p attaches to endothelial cells, whereas Als6p and Als9p bind to collagen and laminin. The role of Als7p is not clear and Als5p is required for cell aggregation (Karkowska-Kuleta et al. 2009; Filler et al. 2006).

Another 34 kDa adhesin called hyphal wall protein (Hwp1) found only on the cell walls of true hyphae and germ tube encodes a surface mannoprotein with its amino terminal sequences surface exposed and carboxyl terminus covalently linked via β-1,3 glucan to the cell wall (Sundstrom et al. 2002). Als3p and Hwp1 act as a complementary adhesins during biofilm formation (Nobile et al. 2008). Hwp1 are recognized as substrates for mammalian transglutaminase substrate (TGase) and form covalent bonding with HBEC. Disruption of HWP1 had shown reduced adherence and mortality in murine models (Staab et al. 1999)(Chaffin et al. 1998). The binding of *C. albicans* to extracellular matrix ligands such as fibrinogen, collagen, laminin, fibronectin is mediated by morphology independent proteins including, Ecm33 (GPI-linked), Mp65 (putative β-glucanase), Phr1 (β-1,3 glucanosyl tranferase), Int1p (integrin like proteins) Eap1 and Iff4 (Naglik et al. 2011). Transcriptional regulators Bcr1, Ace2, Snf5 and Arg81 are reported to be required for the adherence during biofilm development (Finkel et al. 2012).

1.3.2 Morphological transitions

The characteristic feature of *C. albicans* is its ability to switch between unicellular to filamentous morphological forms. Budding of *C. albicans* leads to formation of yeast cells,

hypha or filamentous forms arise from germ tubes and pseudo-hyphae results from the polarized cell division of budding yeast cells that have elongated without detachment from adjoining cells (Kumamoto & Vinces 2005). Hyphal formation is an important attribute associated with *C. albicans* biofilm (Sudbery et al. 2004). Polymorphism in *C. albicans* is often a response of cell to the changing environmental conditions and permits the cell to adapt to different host niches. *C. albicans* grow in predominantly yeast form under low pH (<5.5) whereas high pH (>6) induces hyphal growth (Odds 1988).

Other physiological conditions that affect morphology of *C. albicans* include starvation, carbon sources, serum, temperature, CO_2, amino acids and N-acetylglucosamine (Sudbery 2011). The quorum sensing molecules in *C. albicans* regulate morphogenesis by promoting yeast cell forms at high cell densities and hyphal forms at low cell density (Hall et al. 2011). The yeast to hyphal transition termed dimorphism is crucial for pathogenicity. The hyphal form is believed to be involved in invasion and yeast form primarily for dissemination. Lo et al. (1997) reported that mutants lacking filamentous or hyphae form were avirulent under *in vitro* conditions. Hwp1, Als3 and family of secreted aspartyl proteinases are hypha-associated proteins which encode virulence factors but are not involved in hyphal formation. These proteins are expressed during phagocytosis to kill macrophages and aid in escape of *Candida* cells from bloodstream (Hube 2004; Gow et al. 2002). During morphogenesis genes namely CHS2, CHS3, PHR1, HYR1, ECE1 are differentially expressed to regulate phase transitions (Calderone & Fonzi 2001).

1.3.3 Hydrolytic enzymes

1.3.3.1 Proteinases

Secreted aspartyl proteinases (SAPs) are an important virulence factors that hydrolyze human proteins such as hemoglobin, albumin, collagen, keratin, mucin, salivary lactoferin, fibronectin, secretory immunoglobulin A and interleukin 1β (Hube et al. 1997). SAPs degrade the host tissue barriers and acquire nutrition at the site of infection. SAP gene family includes ten proteins (SAPs 1-10) having similar function and characters but different molecular properties such as isoelectric point, molecular mass, pH for optimum enzyme activity (Mayer et al. 2013; Karkowska-Kuleta et al. 2009). Studies reported that SAPs 1-3 are expressed by yeast cells, SAPs 4-6 are expressed by hyphae and SAPs 9 and 10 are expressed by both forms (Albrecht et al. 2006; Naglik et al. 2004). SAP 7 was never detected *in vitro* while SAP 8 is connected with extensive penetration (Hube et al. 1997). SAPs are active across wide range of pH (2.0-7.0) and show varied levels of protein specificity (Tsai et al. 2013). This feature enables *C. albicans* to

uptake nitrogen at different tissues sites and aids in pathogenicity by adhesion of cells to tissues and their dissemination in blood. *In vivo* studies also affirmed the role of SAPs in tissue penetration and mutants lacking SAPs resulted in decreased virulence in murine models (Naglik et al. 2004; Hube & Naglik 2001). Increased expression of few SAP genes were observed in biofilm cells than planktonic cells (Naglik et al. 2008; Green et al. 2004). SAPs can degrade and inactivate the complement components and block the membrane attacking complex of the activated complement system (Gropp et al. 2009).

1.3.3.2 Phospholipases

C. albicans is the only *Candida* sp., that can produce high amounts of phospholipase and the secretion of extracellular phospholipases by *C. albicans* was first reported during 1960s (Vidotto et al. 1999; Costa et al. 1968; Werner 1966). Phospholipases are heterogeneous group of enzymes that has the ability to hydrolyze ester linkages of glycophospholipids and facilitate the tissue invasion (Ghannoum 2000). Enzyme secretion depends on the environmental stimuli, genetic patrimony and other factors (Samaranayake et al. 2006; Mukherjee et al. 2003). The phospholipases in *C. albicans* classified into phospholipase PLA, PLB, PLC and PLD on the basis of cleavage of ester bond in glycerophospholipids (Karkowska-Kuleta et al. 2009). All types of phospholipases comprise hydrolase activity, besides this PLB also possess lysophospholipase transacylase activity (Mukherjee et al. 2003). These enzymes are secreted at the periphery of yeast cell and hyphal tip either across the cell wall or remain attached to the yeast or hyphal walls. Phospholipases acts as a virulence factor in the pathogenesis of invasion of mucosal epithelia and hematogenous infections (Bhat et al. 2011). They are very active during tissue damage and high levels of phospholipases were produced in isolates from blood than commensal form of *C. albicans* (Ibrahim et al. 1995).

1.4 The cell wall

The cell wall of *C. albicans* is a dynamic bilayered organelle located external to the plasma membrane whose composition, thickness and organization can greatly differ depending on environmental conditions (Gow & Hube 2012). It accounts for 25-30 % of the cell dry weight and provides mechanical strength to withstand adverse conditions (Fleet 1991). The polymorphic nature of *C. albicans* is attributed to the plasticity of the cell wall structure, since changes in the cell wall determine the fungal architecture (Chaffin et al. 1998). In host-pathogen relationship, the cell wall is the external component that mediates the initial contact with the environment including other microorganisms and host. It promotes pathogenicity through cell- cell recognition, adhesion to host or inert surfaces and invasion (Calderone &

Clancy 2011). The cell wall of *C. albicans* is primarily composed of 80- 90% polysaccharides which are unique and differ from the cellulose rich plant cell wall (Chaffin et al. 1998). The cell wall components are strongly cross-linked to each other through covalent bonding (Fig. 1.5). It activates signal transduction pathways thus acts as a signaling center. It serves as a permeability barrier and protects the cell against dehydration (Gow & Hube 2012). Studying the cell wall and its dynamic architecture can potentially lead to the identification of the targets for the development of antifungal drugs.

1.4.1 Composition of the cell wall

The cell wall composition of *C. albicans* is similar to the well studied ascomycetous representative *Saccharomyces cerevisiae* (Klis et al. 2001). The fungal cell wall appears as layered structure under electron microscopy with outer fibrillar layer covering the inner homogenous layer. Three basic components represent the polysaccharides of the cell wall such as glucan, chitin and mannan. In addition, cell wall contains proteins (6-25%) which are found in covalent association with mannan (mannoproteins) and lipids (1-7%) (Cassone 1989).

1.4.1.1 The glucan component

Glucan is the major structural component constituting 50-60% dry weight of the cell wall. Glucans backbone is composed of polymer containing (1→3)- β-D-linked anhydroglucose repeat units and a side chain as β (1→6) glucan that cross links the components of the inner and outer walls (Klis et al. 2001; Lowman & Williams 2001). The β (1→3) glucan (65-90%) acts as a main structural component to which other cell wall constituents are attached covalently. The β (1→3) glucan molecules branches with the β (1→3) linked glucose residues at the 6^{th} position through hydrogen bonding to form a three dimensional structure that maintains the shape of the cell (Kopecká et al. 1974). The chain length of the β (1→3) glucan molecules varies according to the growth conditions. The cell wall shrinks and porosity decreases under hypotonic solutions (De Nobel et al. 1990). The soluble β (1→6) glucan has a high degree of polymerization thus are highly branched than β (1→3) glucan and this branching may function as an attachment sites for chitin chains (Magnelli et al. 2002).

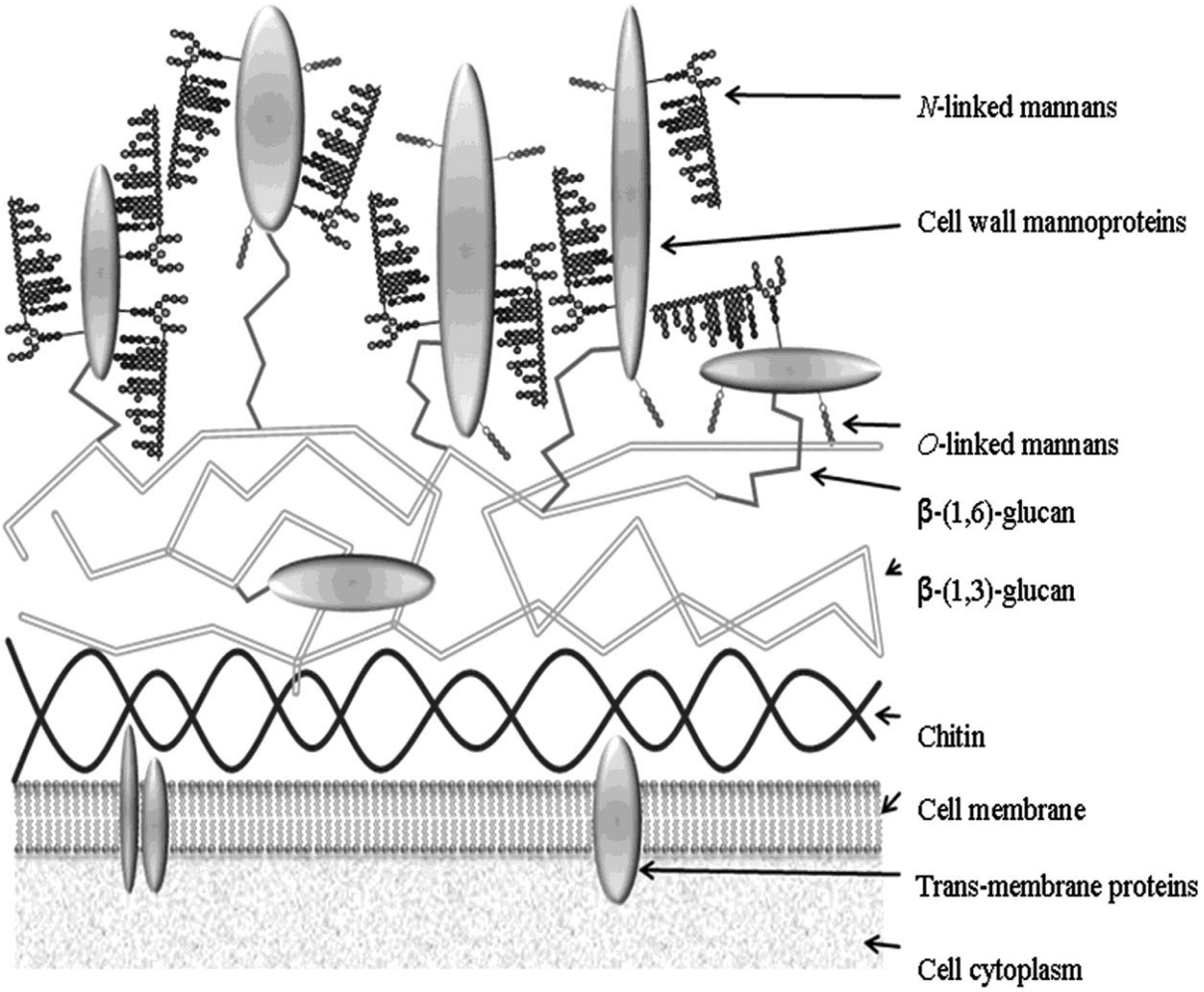


Figure 1.5 The cell wall of *C. albicans* The outer layer of cell wall is composed of glycoproteins with with O- and N-linked mannose polymers (mannans) covalently associated with proteins. The inner layer is comprises the skeletal polysaccharides β (1→3) glucan, β (1→6) glucan and chitin. The outer cell proteins are linked to inner wall framework by glycosylphosphatidylinositol (GPI) anchors via β (1→6) glucan (Figure taken from Grubb et al. 2008).

1.4.1.2 The chitin component

Chitin accounts for 1-2% of the cell wall dry weight, is a linear polymer of (1→4)- β- linked N-acetylglucosamine which is linked through inter-chain hydrogen bonding (Carotti et al. 2002) . Most of the chitin is found in the region of bud scars and a small fraction (< 10%) is covalently linked to β (1→3) glucan and β (1→6) glucan network. This complex cross-linkage forms a rigid skeleton and provides mechanical strength to the cell (Magnelli et al. 2002). Cell wall stress leads to hyper accumulation of chitin accounting for 20% of the cell wall dry weight (Kapteyn et al. 2000; Popolo et al. 2001).

1.4.1.3 Mannoproteins

Mannan, a polymer of phosphodiester-linked mannose sub-units is the only sugar found in *C. albicans* which is covalently attached to proteins hence, the named as mannoproteins (40% of cell wall by dry weight). This outer protein layer determines the cell surface properties such as immunogenicity, hydrophobicity, porosity and electrical charge. The cell wall proteins (CWPs) of *C. albicans* are attached to mannose polymers through N- linked (aspargine) and O-linked (threonine or serine) glycosidic bonds (Cutler 2001). The N- linked carbohydrate moiety is composed of chains of α (1→6) linked mannopyranosyl residues to which oligosaccharide side chain of mannopyranosyl residues with α (1→2), α (1→3), β (1→2), β (1→6) linkages are attached. In addition to this, phosphodiester linkage and α (1→6) branching is observed under acidic growth conditions. The O-linked side chains are usually 4-6 mannose residues in length and confer rigidity and spacer like properties (Jue & Lipke 2002). The phosphorylated carbohydrate side chains play a crucial role in water retention and protection against dehydration. The N-linked chains offer protection from the environment and porosity (De Nobel et al. 1990). Some of the cell wall proteins receive a modification called glycosylyphosphatidylinositol (GPI) anchor that serves to direct and localize proteins that contain C-terminal signal sequence to the cell wall and plasma membrane (Kapteyn et al. 2000). GPI-dependent cell wall proteins are most abundant cell wall proteins in *C. albicans.* The GPI proteins are rich in serine and threonine repeats and many are expressed under some growth conditions. Most of the cell wall proteins are integrated into the wall through covalent linkages between sugars present at the N- linked and O-linked sites and/or in the GPI anchor with those in the chitin and glucan polymers (Lipke & Ovalle 1998; Klis et al. 2009). Mannoproteins are the most potent immunomodulators that are able to regulate phagocytic cells, humoral and cell mediated immunity of the host immune system (Cassone 1989).

The composition of cell wall components in yeast and hyphal forms remains similar, although relative amounts of chitin, mannan and glucan vary according to the *C. albicans* growth pattern (Calderone & Braun 1991). Chitin is the first cell wall polymer to appear in regenerating protoplasts and hyphal cells contain similar chitin levels as yeast cells do (Elorza et al. 1987). Researchers reported decrease in branched β (1→6) glucan in yeast cells compared to filamentous forms and increase in mannan content in yeast forms (Adt et al. 2006).

1.4.2 Molecular organization of the cell wall

Transmission electron microscopy studies have revealed that the appearance of the cell wall layers varies according to the type of strain, growth conditions, morphology and phase of the

cell cycle (Osumi 1998). The outer cell wall layer is a dense microfibrillar network while inner layer is contiguous plasmalemma. The external protein layer is heavily phosphorylated and mannosylated thus binds to positively charged ions. The inner layer consists of β (1→3) glucan, β (1→6) glucan and chitin polysaccharides and appears to have extensive membrane invaginations that anchor cell wall to the plasma membrane (Klis et al. 2004). The different cell wall constituents concentrated in the inner layer interact with each other by hydrogen and hydrophobic bonds to give rise to the overall architecture of the cell wall. Besides this, mannoprotein present in the outer most layer associate covalently with β (1→6) glucan through a GPI anchor which in turn bind to β (1→3) glucan (Hamada et al. 1999). The branching of β (1→3) glucan through β (1→6) linkage creates nonreducing ends that may act as covalent attachment sites for either β (1→6) glucan or chitin polymer. A minor class of cell wall protein, Pir, linked to β (1→3) glucan network is dispersed over inner skeletal layer (Kapteyn et al. 2000). The outer layer forms a permeability barrier and limits the penetration of large molecules whereas inner layer appear to be porous and allows the movement of large molecules (De Nobel et al. 1990).

1.5 Host environmental factors

During colonisation, *C. albicans* is exposed to different environmental factors such as different nutrient resources, stresses, secretory factors of immune system imposed by the host and microbe derived environment (Hall 2015). These microenvironments are in constant state of flux to which *C. albicans* must activate suitable responses in order to survive under these environmental challenges. The environments encountered by *C. albicans* are niche specific and can alter the biofilm composition and properties which is a key player in causing infections.

1.5.1 Metabolic adaptation

In order to grow and survive in a wide range of host niches, pathogenic fungi depend on metabolic adaptability in addition to their virulence factors. This adaptation mediates effective assimilation of local nutrients in different host environments. *C. albicans* is a predominant fungal flora in gastrointestinal microbiome (Rosenbach et al. 2010). Mucosal surfaces in this region are considered highly competitive for nutrient acquisition due to the presence of other microorganisms. The blood stream rich in glucose (6-8 mM), amino acids and proteins act as nutrient supply for most of the pathogens inside the host. In contrast, necrotic tissues are depleted with nutrients and create starvation conditions due to phagocytosis by macrophage or neutrophil (Mayer et al. 2013). The carbon metabolic pathways glycolysis, gluconeogenesis as

well as glyoxylate cycle under stress response contribute to colonization and pathogenesis in a niche specific manner. *C. albicans* prefers to utilize glucose as a carbon and energy source through glycolysis. However, under hostile milieu in host such as inside macrophages, *C. albicans* switches from glycolysis to gluconeogenesis to assimilate alternative carbon sources like lactate, citrate, amino acids and glycerol (Desai et al. 2013). In addition, it also activates glyoxylate cycle as a result of starvation.

During systemic candidiasis, *C. albicans* disseminate to every organ in the host and secrete proteases to hydrolyze host proteins for nutrients under poor glucose conditions (Mayer et al. 2013). Thus, metabolic adaptation not only promotes growth and survival in *C. albicans* but also affects virulence. Several metabolic pathways are conserved between model yeast *Saccharomyces cerevisiae* and pathogenic *C. albicans* but the regulatory events of metabolism display significant differences (Askew et al. 2009). In *S. cerevisiae*, glucose concentration, growth rate and oxygen availability modulates the balance between fermentation and respiration (Gancedo 1998). Whereas, *C. albicans* classified as glucose-negative yeast undergoes respiration even in the presence of glucose (Niimi et al. 1988). The difference in central carbon metabolism between *S. cerevisiae* and *C. albicans* was revealed by a whole-genome comparison (Jones et al. 2004). They reported that many genes required for respiration and oxidative metabolism such as genes encoding for pyruvate dehydrogenase kinase, fatty acid catabolism and amino acid catabolic pathways were upregulated (Jones et al. 2004). These results were also supported by microarray data analysis (Ihmels et al. 2005). Interestingly, significant molecular rewiring and divergence have occurred between these two species and the regulatory network controlling central carbon metabolism is entirely distinct in *C. albicans* (Martchenko et al. 2007; Askew et al. 2009).

Morphogenesis has also been associated with genes involved in central carbon metabolism (Lan et al. 2002). During hyphal development, morphogenetic regulator Efg1 regulates glycolytic genes (Nantel et al. 2002). Glucose, one of the several stimuli for hyphal morphogenesis induces glycolytic genes during yeast to hyphal transition (Hudson et al. 2004; Maidan et al. 2005). Genome wide expression studies revealed that Efg1 regulates the expression of morphogenesis and metabolic genes by up-regulating glycolytic genes and repressing genes associated with oxidative metabolism (Doedt et al. 2004). It appears that metabolic adaptation and key virulence factors regulate in a coordinated fashion through complex signalling networks. The adenylyl cyclase PKA-Efg1 signalling pathway regulates carbon metabolism and morphogenesis, phenotypic switching and stress resistance (Doedt et al.

2004; Morschhäuser 2010). During evolution major molecular rewiring took place in regulation of carbon metabolism in *C. albicans* compared to *S. cerevisiae* (Natarajan et al. 2001; Tournu et al. 2005). For instance, in *C. albicans* glycolysis is induced by Gal4 and Tye7 but Gcr1 in *S. cerevisiae* (Askew et al. 2009; Ene et al. 2012). The cellular role of transcriptional networks of general amino acid metabolism responses and sugar sensing pathways have been conserved in these both yeasts. In *S. cerevisiae*, these responses are triggered by amino acid starvation stimulating biosynthetic pathways of all amino acids (Hinnebusch 1988). Besides this, other forms of cellular stress also trigger Gcn4 transcriptional response (Natarajan et al. 2001). Studies suggest that inactivation of Gcn4 significantly inhibited biofilm formation but does not attenuated *C. albicans* virulence in systemic candidiasis model (Brand et al. 2004; García-Sánchez et al. 2004). *C. albicans* gene expression studies by microarray analysis revealed that amino acid biosynthesis pathways were not affected by neutrophils exposure (Rubin-Bejerano et al. 2003). Recently it has been suggested that in response to neutrophils and nutrient limiting conditions, amino acid metabolism excretes ammonia which increases the surrounding pH thus stimulating morphogenesis (Vylkova et al. 2011).

1.5.2 Stress adaptation response

The ability of *C. albicans* to colonize different host niches and rapid adaptation to host derived as well as environmental stresses is the successful attribute behind its pathogenicity. Inside host, *C. albicans* is exposed to variety of stresses such as heat shock, pH, hypoxia, osmotic and oxidative stress. Several stress signaling pathways aid in adaptation to these stresses by generating robust stress responses which are closely linked to virulence of *C. albicans* (Brown et al. 2009). In model yeasts, *S. cerevisiae* and *Schizosaccharomyces pombe* stress signaling pathways are well characterized and induce stress responses by set of genes that are commonly expressed in response to all stresses (Ruis & Schüller 1995). Whereas, in *C. albicans* there are no genes that are induced in common to stresses and no-cross protection responses were detected (Enjalbert et al. 2003). Specific set of genes are induced in response to different stress stimuli which are regulated by specific signaling pathways (Enjalbert et al. 2003). The roles of key regulators that are evolutionarily conserved in *C. albicans* have diverged during the course of time (Fig. 1.6) (Butler et al. 2009; Nikolaou et al. 2009). This makes *C. albicans* relatively resistant to several stresses encountered inside warm-blooded host than model yeasts (Ramsdale et al. 2008).

Fungal cells might be exposed to changes in osmotic pressure and water balance during its colonization in skin, oral and kidney epithelium. Intracellular currents activated by host

immune cells might impose osmotic/cationic stresses (Steinberg et al. 2010). These stresses causes rapid water loss, cell size reduction and fall of turgor pressure (Kühn & Klipp 2012). Osmotic stress response activated by Hog1 (High osmolarity glycerol response) MAPK (mitogen activated protein kinase) pathway induces glycerol biosynthetic pathways (Enjalbert et al. 2003; Smith et al. 2010). This leads to accumulation of intracellular glycerol, regain of turgor pressure and growth resumption (Alonso-Monge et al. 2003; Smith et al. 2004; Wächtler et al. 2011). Hog1 is a highly conserved component of MAPK pathway that plays crucial role in osmotic stress adaptation, inactivation of which leads to attenuation of virulence (Cheetham et al. 2011). This pathway also mediates other cellular stresses, morphogenesis, cell wall biogenesis and metabolism in *C. albicans* (Smith et al. 2004; Eisman et al. 2006).

C. albicans is exposed to reactive oxygen species (ROS) and reactive nitrogen species (RNS) inside a phagolysosome (Mansour & Levitz 2002; Thorpe et al. 2004). Antioxidant enzymes such as catalase (Ctal1) superoxide dismutases (Sod1 and Sod 5), damage repair systems glutathione reductase-thoredoxin (GLR1 and TRX1) and glutathione synthetase (GCS1) are released in response to the oxidative stresses created by peroxide, superoxide and hydroxyl radicals (Chauhan et al. 2006; Brown et al. 2014). This response is mediated by AP-1 like transcription factor Cap1 (Alarco & Raymond 1999). Oxidative stress adaptation is crucial for *C. albicans* pathogenicity. The inactivation of oxidative stress response machinery and its components leads to attenuation of virulence and *C. albicans* cells become more sensitive to phagocytic killing (Fradin et al. 2005; Martchenko et al. 2007; Frohner et al. 2009).

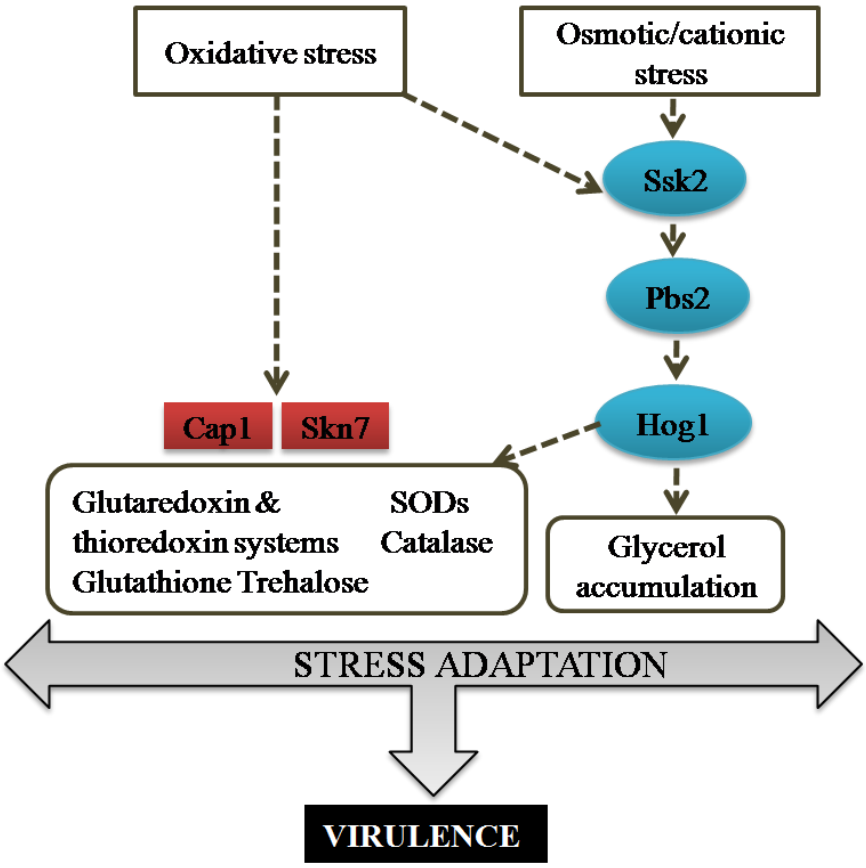


Figure 1.6 Conserved oxidative and osmotic stress regulators in *C. albicans*. Evolutionarily conserved mitogen-activated protein kinase (MAPK) signalling molecules (blue) and transcription factors (red) contribute to the regulation of stress functions in *C. albicans*. These pathways are perhaps operate in an integrated network but represented as linear pathways for simplicity Oxidative stress pathway: Cap1, AP-1 bZIP transcription factor; Skn7, putative response regulator; SODs, superoxide dismutases. Hog1 signalling pathway: Ssk2, MAPK kinase kinase (MAPKKK); Pbs2, MAPK kinase (MAPKK); Hog1, MAPK/stress-activated protein kinase (SAPK). (Figure adapted from Brown et al. 2014).

Metabolic and stress adaptation termed as 'fitness attributes' have undergone a evolutionary rewiring in *C. albicans* (Butler et al. 2009; Sandai et al. 2012). Metabolic adaptation affects stress response and cell wall architecture in *C. albicans* (Ene et al. 2012). Single cell profiling studies revealed the niche-specific induction of stress responses (Miramón et al. 2012).

1.5.3 Host immunity

The human immune system can recognize between the commensal and pathogenic *C. albicans* by pathogen associated molecular patterns (Cheng et al. 2012). A robust immune response

results in a cascade of host signaling events that eventually leads to destruction and clearance of *C. albicans* cells. The host innate immune system acts as the immediate primary line of defence against systemic fungal infections (Cheng et al. 2012). This process involves epithelial cells, complement system, antimicrobial peptides, dendritic cells, cellular response mediated by macrophages and neutrophils (Cheng et al. 2012; Jiménez-López & Lorenz 2013). Cell surface receptor that aids in recognition of *C. albicans* includes Toll-like receptors (TLR2 and TLR4), internal receptors (TLR9 and NLRP3) and C-type lectin receptors (Dectin-1, Dectin-2, DCSIGN, MR, Mincle and MBL). These receptors bind to sugars residues such as mannoproteins and β-1,3-glucans present on the cell surface of *C. albicans*. The release of proinflammatory mediators such as chemokines and cytokines induces the deployment of phagocytic cells at the site of infection (Hebecker et al. 2014). Dendritic cells acts as link between innate and adaptive immunity by processing and presenting *Candida* specific antigens (Hickey et al. 2011). T helper cells and antibodies from adaptive immune system also help in elimination of pathogenic *C. albicans* (Sallusto & Lanzavecchia 2002).

Epithelial cells are the first line of defence which act as passive physical barrier restricting the invasion of *Candida* to deeper tissues (Yan et al. 2013). Tight junctions formed by interepithelial cell connections maintains the epithelial integrity by sealing the gap between cell surface and the mucosal lamina propria thus preventing the interepithelial invasion of *C. albicans* (Yan et al. 2013). Intestinal and vaginal epithelial cells secrete mucus which blocks the direct contact of *C. albicans* with the epithelium (Yan et al. 2013; Cassone 2015). Inside oral cavity, saliva acts as chemical barrier by continuously flushing the dental and mucosal surfaces (Fig. 1.7). Saliva contains redundant amounts of antimicrobial agents such as histatins, lactoferrin, lysozyme, defensins, and cathelicidins which prevent adhesion of *Candida* to oral epithelial cells (Hibino et al. 2009).

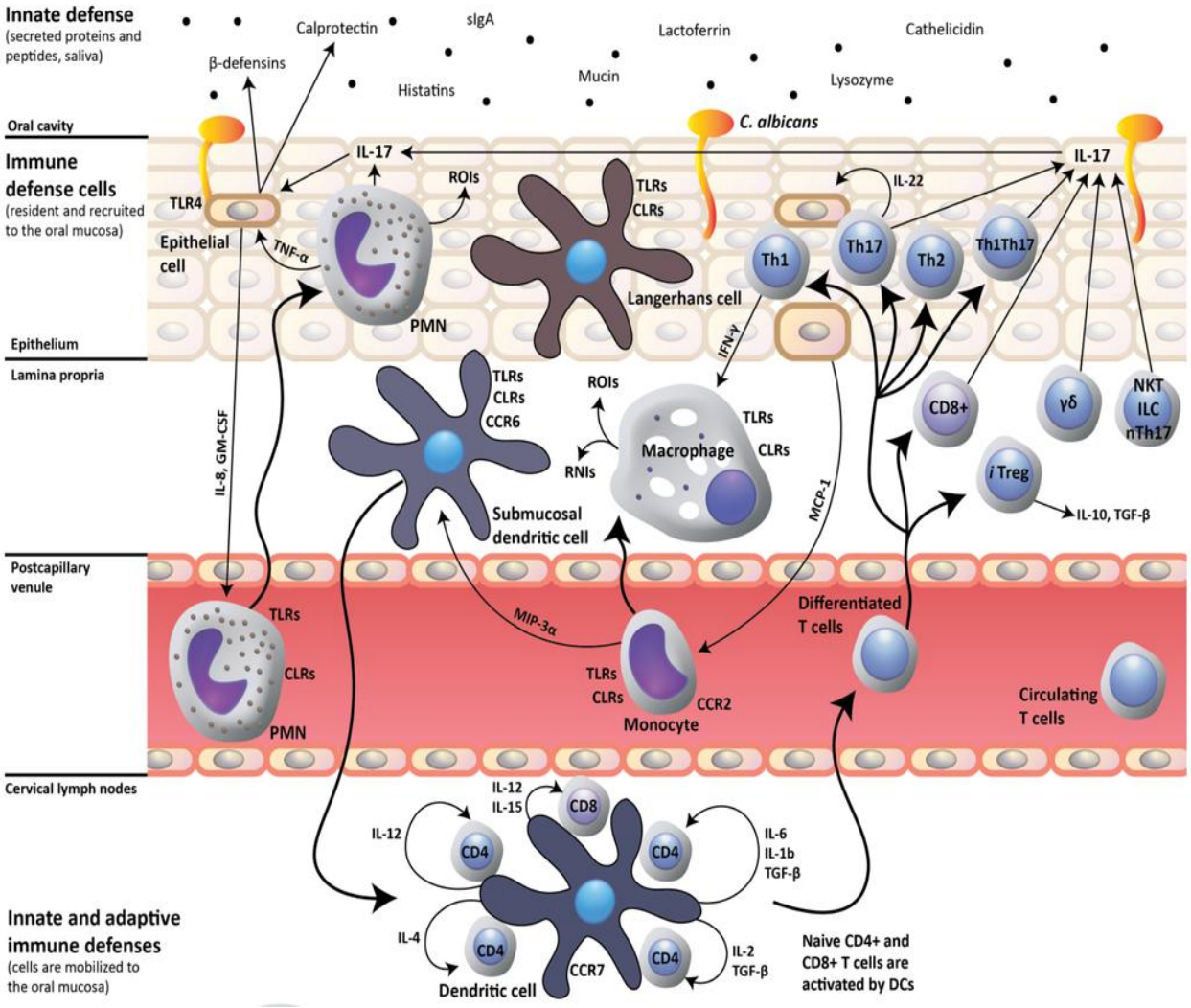


Figure 1.7 Host response to oral *C. albicans*. A robust protective host response to *C. albicans* oral infection is dependent on dendritic mediated induction of Th17 cell-mediated adaptive immunity which further upregulates the expression of mucosal antimicropial peptides (defensins, caprolectins), IL-8 and GM-CSF (trigger recruitment of neutrophils) by epitheilal cells. NKT cells, ILCs, nTh17 cells also particpate in mucosal host response by producing IL-17. C-type lectin receptors (CLRs); reactive oxygen intermediates (ROIs); reactive nitrogen intermediates (RNIs); toll-like receptors (TLRs). (Figure taken from de Repentigny et al. 2015).

Lysozyme is a cationic antimicrobial enzyme found in tissues, secretions such as saliva, tears, serum, breast milk as well as expressed by phagocytes and macrophages (Tenovuo et al. 1990). The fungicidal activity of lysozyme is likely to be mediated by hydrolysis of N- glycosidic bonds in *C. albicans* cell wall resulting in injury to cell membrane (Anil & Samaranayake

2002). Lactoferrin is also a bactericidal protein secretion of host that has diverse protective functions. Lactoferrin chelates iron, prevent adherence to the host surfaces, bind to the lipopolysaccharide and disrupts bacterial cell membranes (Che et al. 2009). It also prevents biofilm formation as reported in *Staphylococci* sp., (Singh et al. 2002). In cystic fibrosis patients, higher concentration of lactoferrin does not affect the planktonic growth of *P. aeruginosa* but inhibited adherence. Cleavage of lactoferrin by cathepsin B can result in loss of bactericidal and antibiofilm activity (Rogan et al. 2004).

Mucin is the gel-forming glycoproteins that are produced by mucous cells of glandular tissues and goblet cells of the gastro-intestinal tract. Salivary mucins are significant (approximately 26%) proportion of salivary proteins (Amerongen et al. 1995). Mucins provide lubrication on epithelial surfaces, respiratory, gastrointestinal and urogenital tracts as well as oral cavity (Van Klinken et al. 1997). Eleven specific human mucin (MUC1-MUC4, MUC5AC, MUC5B, MUC6-8 and MUC11-12) genes have been isolated to date (Porchet et al. 1991; Gum et al. 1994; Bobek et al. 1996; Williams et al. 1999). Salivary mucin MUC5B and MUC7 are the most abundant proteins in saliva (Shankar et al. 1997). Mucins are reported to prevent surface attachment and biofilm formation by promoting dispersion (Caldara et al. 2012). It helps in preventing infection by modulating the cellular processes associated to virulence (Nelson et al. 2007).

1.5.4 Polymicrobial interactions

Candida species exist in a mixed milieu with bacteria and other fungal species in oral as well as mucosal habitats such as gastrointestinal and vaginal tracts (Thein et al. 2009). Microbial interactions have been evolved through biofilm communities. Two common types of interaction are widely observed among subpopulations in mixed biofilm communities are commensalism and antagonism (McLean & Kakirde 2013). Fundamentally these interactions are far more complex than recognized. Apart from microbial factors, there are certain environmental factors which microbe's encounters in host niches (Thein et al. 2009). These include oxygen availability, nutrient conditions, and stresses which are likely to impact functionality and biomass spatial property of microbial cells. These factors can significantly alter the interactions between different species in mixed biofilm communities (Thein et al. 2009). For instance, *C. albicans* can modulate the adhesion and propagation of *C. krusei* in mixed biofilms (Thein et al. 2007). When *C. albicans* was co-incubated with *E. coli*, colonization of *Candida* was hindered whereas bacterial population occupied larger area of the substratum (Nair & Samaranayake 1996a). Thus, *Candida* sp., such as *C. albicans*, *C. tropicalis*, *C. krusei* and *C.*

glabrata either in combination or with other bacterial species such as *Staphylococcus* sp., *P. aeruginosa, Enterobacter* sp., and *Klebsiella pneumonia* are often associated with polymicrobial infections. Several researchers reported the bacterial-fungal interactions in implant associated infections involving catheters, endotracheal tubes, and mechanical heart valves, infections related to oral, gastrointestinal and vaginal mucosa (Sbordone & Bortolaia 2003; Doedt et al. 2004). *Candida* coexists with bacterial species *E. coli*, *S. aureus*, *Klebsiella* sp., *Pseudomonas*, *Streptococci* sp., in oral infections such as *Candida* associated denture stomatitis, angular stomatitis and periodontitis (Jenkinson & Douglas 2002). Researchers have shown the role of mixed biofilm which were more resistant to antimicrobial agents than biofilm formed by single species (van der Mei et al. 2000; Nelson et al. 2007). Klotz et al. (2007) reported that in blood stream infections, *Candida* sp., was commonly isolated with *S. aureus* (20%), *P. aeruginosa* (8%) and *E. coli* (4%).

In burn wounds, *C. albicans* almost never found co-existed with *P. aeruginosa* indicating the antagonistic interaction (Gupta et al. 2005). *Candida* sp., *Staphylococcus* and *Pseudomonas* sp., are the common pathogens found in ventilator-associated pneumonia (Adair et al. 1999). Antifungal treatment of respiratory infections caused by *Candida* colonization reduces the risk of Pseudomonas associated pneumonia (Ader et al. 2009). Co-infection of *Candida* may alter the host immune system ability to clear *Pseudomonas* causing pneumonia (Roux et al. 2009). In cystic fibrosis patients, *S. aureus* colonizes lungs of young children while in adults *P. aeruginosa* (80%) is the major colonizer (Hauser et al. 2011). Earlier, Valenza et al. (2008) reported that 78% of these patients are colonised by *C. albicans* along with *S. aureus* (63%) and *P. aeruginosa* (50%). *C. albicans* envelopes the *S. aureus* cells in an extracellular polymeric matrix which offers protection to it against antibiotics (Kart et al. 2014). This helps *S. aureus* to form biofilms in serum which typically *S. aureus* alone cannot form. Such mutualistic relationship resulted *C. albicans* and *S. aureus* in being the top three microorganisms co-isolated in nosocomial infections (Klotz et al. 2007).

S. aureus lacks the ability to invade host tissues; however, it seems to use *C. albicans* hyphae for tissue invasion in murine models (Peters et al. 2012). *Streptococcus gordonii* also share a synergistic interaction with *C. albicans* through hyphal formation and colonization of the oral mucosa (Bamford et al. 2009). Several adhesins of *C. albicans* namely, Als3p, Hwp1p and Eap1p play key role in attachment of *S. gordonii* (Nobbs et al. 2010).

1.6 Antifungal therapy

Antifungal drugs will select and remove pathogenic fungi with negligible toxicity to the host. These drugs available are classified according to their site of action in fungal cells into four main categories: polyenes, the azoles, the echinocandins and other miscellaneous classes of DNA analogue drugs such as 5-fluorocytosine (Ostrosky-Zeichner et al. 2011) (Fig. 1.8).

1.6.1 Antifungal drugs

1.6.1.1 Polyenes

The polyenes nystatin and amphotericin B are the broad-spectrum antifungal drugs available with both fungicidal and fungistatic activity (Andriole 1999; Wynn et al. 2003). Polyenes intercalate with the ergosterol present in the plasma membrane by creating membrane-spanning channels and affect the selective membrane permeability. This destroys the proton gradient and the membrane stability due to leakage of cytoplasmic contents (Bossche et al. 1994). In addition to this, amphotericin B induces oxidative damage to cell membrane (Ellis 2002). Amphotericin B is the drug of choice for invasive fungal infections whereas nystatin is used for superficial mycoses (Andriole 1999).

1.6.1.2 Azoles

The azole drugs include imidazoles (econazole, clotrimazole, fenticonazole, ketoconazole, and miconazole) and the traizoles (fluconazole, itraconazole, voriconazole and posaconazole). These azoles inhibit ergosterol biosynthesis by interacting with fungal cytochrome P450 enzyme lanosterol demethylase which converts 14α-demethylsterol to ergosterol (Ellepola & Samaranayake 2000; Lewis 2007). This disrupts plasma membrane synthesis by depleting ergosterol content in the cell membrane and thus halting the cell membrane functions. Along with this, imidazoles are also capable of lethal accumulation of hydrogen peroxide causing oxidative damage to fungal cells (Lewis 2007). Azoles are fungistatic and used in the treatment of tropical and systemic candidiasis.

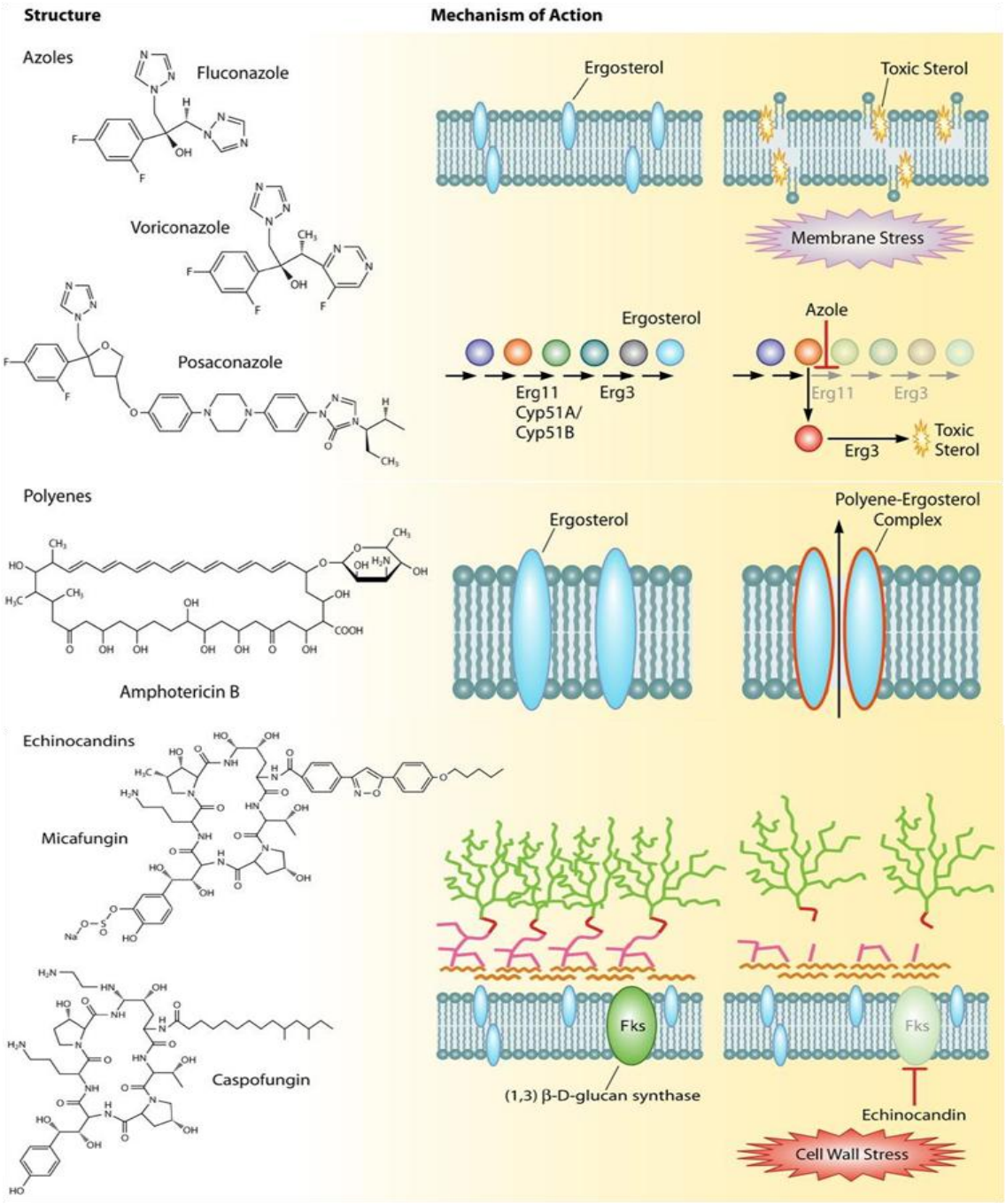


Figure 1.8 Mode of action of antifungal drugs. Azoles, polyenes, echinocandins and their targets from top to bottom (Figure taken from Shapiro et al. 2011).

1.6.1.3 Echinocandins

Echinocandins (caspofungin, anidulafungin and micafungin) and their analogues pneumocandins are lipopeptides that interrupt the synthesis of fungal cell wall component β

(1→3) glucan by inhibiting the enzyme β (1→3) glucan synthase that forms glucan polymers. The inhibition is effective, specific and results in osmotic lysis. Echinocandians are fungicidal and demonstrated new alternatives to fluconazole resistant *Candida* species (Denning 2002).

1.6.1.4 Nucleoside analogs

Nucleoside analogs such as flucytosine interfere with DNA and RNA synthesis in fungal cell. Flucytosine inhibits enzyme thymidylate synthetase, which is involved in nuclear division and DNA synthesis (Ellepola & Samaranayake 2000; Lewis 2007). This drug also inhibits protein synthesis leading to cell death. Flucytosine is fungistatic used in combination with amphotericin B to treat systemic mycoses (Wynn et al. 2003). Another nucleoside drug of bacterial origin is tunicamycin, produced by bacteria *Streptomyces clavuligerus* and *Streptomyces lysosuperficus*. It blocks the synthesis of *N*-linked oligosaccharide chains on mannoproteins. Tunicamycin inhibits *C. albicans* biofilm formation germ tube and mycelia formation (Pierce et al. 2009). Antibiotics such as nikkomycin and papulacandin damage fungal cell walls by inhibiting chitin synthases and β-glucan synthesis (Hector & Braun 1986).

1.6.2 Biofilm mediated antifungal drug resistance

It is now widely established that life in a sessile mode of growth confers a unique type of resistance in form of biofilm which is highly challenging for effective therapeutic choice (Bhattacharya et al. 2015). Hawser and Douglas (1994) demonstrated increased antifungal resistance of *Candida* biofilm. *Candida* in biofilm lifestyle exhibit 30 to 2000 times resistance to different antifungals than planktonic cells (Ramage et al. 2001b). The biofilm mediated antifungal resistance is multifactorial process and yet to be fully elucidated in fungal species. Due to quick consumption of nutrients by microbial cells at biofilm-fluid interface, nutrient limitation occurs at inner biofilm core. Waste products also accumulate at biofilm centre where exchange with external fluid is limited (Stewart & Franklin 2008). The heterogeneous population with different growth rates also contributes to antimicrobial sensitivity in biofilms. It has been observed that antimicrobial drugs are active against metabolically active cells than slow growers (Anwar & Costerton 1990). Studies on *Candida* biofilms reported that drug resistance increases with the progression of biofilm development (Chandra et al. 2001). In addition, mature biofilms were less susceptible to amphotericin B under nutrient limitation conditions (Baillie & Douglas 2000).

The exopolymeric matrix of *C. albicans* biofilm has been implicated in resistance to conventional antifungal agents (Desai et al. 2014). Matrix components reduce the penetration of antimicrobials by either chemical reaction with the compound or sorption. The constituents

of biofilm matrix such as polysaccharides, eDNA limits the entry of these antifungals. These components act an ion exchange resin and sequester positively charged antimicrobial substances (Hoyle et al. 1990; Gristina 1994). In *C. albicans* biofilm matrix, polysaccharide β (1→3) glucan contributes to the resistance by specifically binding to antifungal drug amphotericin B (Vediyappan et al. 2010). Nett et al. (2007) reported that treatment of biofilms with enzyme β (1→3) glucanase increased the susceptibility of biofilm cells to fluconazole and addition of β (1→3) glucan exogenously increased resistance to fluconazole in planktonic cells. Two drug efflux pumps that were studied in *C. albicans* planktonic cells are ATP binding cassette (ABC) transporter and major facilitator proteins encoded by CDR and MDR genes. The expression of genes encoding these pumps is up-regulated during different stages of biofilm development (Mukherjee & Chandra 2004). Adaptive resistance mechanisms considerably play a critical role in resistance to conventional antifungal drugs. Azole resistance is caused due to one of the several mechanisms such as mutations in the drug target, upregulation of multidrug transporters Mdr1, Cdr1, Cdr2 and alterations in the transcription factor Upc2, Efg1 (Calderone & Clancy 2011; Shapiro et al. 2011; Bink et al. 2012). Several stress reponse pathways mediated by calcineurin, PKA, PKC, TOR and Hsp90 also contribute for azole resistance (Cannon et al. 2007; Cowen & Steinbach 2008; Shapiro et al. 2011). Walker et al. (2008) reported the *C. albicans* cells acquired caspofungin tolerance by activation of cell wall salvage pathways and by increasing the chitin content in their cell walls. Drug susceptibility studies on mixed biofilms of bacterial and fungal species can affect the antifungal activity on biofilms (Jenkinson & Douglas 2002).

Persister cells are small population of reversible yeast cells which are adaptable to be in metabolically dormant state (Lewis 2007). These cells emerge from small colony variants and are highly resistant to antifungal drugs (LaFleur et al. 2006). They have a unique gene expression profile that turns on toxin-antitoxin operons and turns off metabolism related genes (Correia et al. 2006). Under stressful conditions, persister cells may survive by building reservoir cells that can re-grow and cause relapsing chronic infections (Stewart & Franklin 2008). Less is known about the role of persister cells in *C. albicans* biofilm development.

1.7 Developing novel therapeutics against *C. albicans* biofilm

The current treatment options for *C. albicans* biofilm related infections are very scarce due to the intrinsic augmented tolerance to antimycotics. As described above biofilm mediated antifungal resistance is a multifactorial phenomenon. Hence, there is an imperative need of intensive research in finding therapeutics against these biofilm infections. One approach is

gaining deeper knowledge in molecular mechanisms of biofilm formation to identify the novel targets for the design of antifungal drugs. Another approach is screening of the chemical libraries for the identification of active compounds that has biofilm disruption potential. Antifungal agents either plant or microbe derived is considered as significant sources of bioactive compounds for *C. albicans* biofilm eradication as discussed below.

1.7.1 Plant derived products

Despite several herbal products claimed to have antifungal activity, very few scientific studies have reported their efficacy (Havsteen 2002; Quiroga et al. 2006). These bioactive compounds from plant origin are mainly composed of polyphenols and flavonoids which are quite promising as antifungal agents however, the toxicity and the tolerable doses in patients need to be established. Essential oils are secondary metabolites synthesized by plants as a microbial defence also exhibits excellent anti-candidal activity (Agarwal et al. 2008b; Agarwal et al. 2010; Dohare et al. 2014; Kalia et al. 2015). The plant by-product terpenes contain a combination of several 5-carbon units called isoprenes have demonstrated antifungal activity by affecting morphology of *C. albicans* and induced fungal cell lysis (Tyagi & Malik 2010; Khan et al. 2013).

Polyphenolics, another bioactive compounds that are ubiquitously present in plants have been in use as traditional medicine since ages (Xu & Lee 2001). The chemical and structural similarities of these compounds with numerous biomoleules as well as their crucial role in plant-microbial interactions make them an attractive class of phyto-constituents for biological activity (Yi et al. 2008). Their widespread incidence, broad spectrum diversity and activity make them appropriate chemical scaffolds for novel therapeutic agents.

1.7.2 Microbial derived

Microbe derived surface active amphipathic compounds such as biosurfactants partition preferentially at the interface between two different phases in a heterogeneous system (Yu & Huang 2011). These compounds have gained significant commercial importance as compared to their synthetic chemical counterparts due to their tailor-made multifaceted diversity, eco-friendly nature, higher biodegradable ability, low toxicity, effectiveness at extreme pH and temperature suitability for large scale production and selectivity (Dusane et al. 2010). Biosurfactants have been emerged as a promising multipurpose ingredient, possessing emulsifying, anti-adhesive and antimicrobial activities. In recent decade, the use of biosurfactant as alternative therapeutics to control biofilms has been explored widely (Rivardo et al. 2009). In our study, biosurfactant di-rhamnolipid isolated from *P. aeruginosa* DSVP20

have shown to disrupt the *C. albicans* biofilm (Singh et al. 2013). Biosurfactants interfere in the microbial adhesion and desorption by modifying surface hydrophobicity of the substratum (Rodrigues et al. 2006). Thus, coating of biosurfactants on medical implants prior to implantation may be used as a preventive strategy against implant associated infections (Gan et al. 2002; Walencka et al. 2008).

1.7.3 Nano-drug delivery systems

Nano-drug delivery systems are evolving technologies for drug delivery using biodegradable and biocompatible polymers (Link et al. 2010). These delivery systems are designed to improve the therapeutic and pharmacological properties of drugs (Ansari et al. 2014; Sharma et al. 2016). Drug carriers in delivery systems are constructed and engineered with nanometer dimensions with enhanced bioavailability and less toxic side effects (Haley & Frenkel 2008). Despite broad range of applications of polyphenolic compounds and other plant metabolites, poor solubility in aqueous solutions remains critical in clinical efficiency and bioavailability (Soobrattee et al. 2005). To enhance the solubility and bioactivity of these compounds nano-encapsulation in polymeric nanoparticles/nanofibres is widely employed (Hosseini et al. 2013; Singh et al. 2015). In our investigation on anti-biofilm studies, polyphenolic compound quercetin was encapsulated with poly($_{D,L}$-lactide-*co*-glycolide)-poly(ε-caprolactone) nanofibers using an electrospinning technique (Vashisth et al. 2013). These quercetin encapsulated nanofibres were found to be effective against *C. albicans* biofilm.

1.8 Objectives of this study

As described in earlier sections, the pathogenicity of *C. albicans* is dependent on adaptation and the robust response that helps *C. albicans* to withstand the local environmental challenges be it imposed by the host or microbe derived. The main objective of the work carried out was to understand how *C. albicans* responds to different environmental factors it encounters during colonization and in particular how these environments affect the *C. albicans* biofilm, virulence factors and cell wall composition during the biofilm growth (Fig. 1.9).

The first aim of this work was to evaluate how different carbon sources affect surface associated growth in *C. albicans* and to describe the changes in virulence properties and structure of the cell wall component β-glucan (Chapter-2). The second aim was to better understand the adaptive response generated by *C. albicans* in the presence of oxidative and osmotic stresses and how these stresses affect the virulence factors and the cell wall

composition in biofilm cells (Chapter-3). Next environmental factor taken into consideration was innate immune secretory factors to which *C. albicans* is constantly exposed especially in oral milieu. The third main aim of the study was to evaluate the response of *C. albicans* in the presence of the innate immune secretory factors like mucin, lactoferrin, lysozyme and dATP (Chapter-4). The fourth main aim was to study the influence of microbe derived environment on *C. albicans* biofilm during co-culture conditions (Chapter-5). In addition to this, studies were also focussed on testing of phytochemical libraries for bioactive compounds having antifungal and antibiofilm activities (Chapter 6).

CHAPTER-2

2. Modulation of *Candida albicans* biofilm by different carbon sources

2.1 Background

The fungal pathogen *Candida albicans* thrive as a normal flora in the host microenvironment such as the oral cavity, skin, gastro-intestinal and urogenital tracts of healthy individuals (Calderone & Clancy 2011). The colonization of *C. albicans* remains benign in healthy individuals (Ruhnke 2002) However, in immunocompromised people *C. albicans* is an opportunistic pathogen as it colonises different host niches and medical implants by forming biofilm (Perlroth et al. 2007; Herwald & Kumamoto 2014). The commensal to pathogen shift of *C. albicans* requires an effective adaptation to the host environment. It has been observed that during infection, carbon play a central role in metabolism and critical for colonization in *C. albicans* (Brown et al. 2014). *C. albicans* need to assimilate locally available or alternative nutrients for their survival and multiplication in the dynamic environments.

It is likely that the locally available carbon sources can influence the fungal adhesion, biofilm formation and can trigger other crucial virulence factors including morphological transitions and secretion of hydrolytic enzymes during infection (Mayer et al. 2013). Moryl et al. (2014) also observed that composition and quantity of exopolymeric matrix also depend on carbon source in bacterial biofilm models. In *C. albicans*, cell wall plays a key role in cell robustness, morphology and adhesion to medical implants or host tissues (Gow & Hube 2012). Glucans (85-90% of the cell wall dry mass) are major structural components of the cell wall and they are also most important fungal pathogen-associated molecular pattern (PAMPs) (Fleet 1991; Klis 1994; Brown & Williams 2009). Presently, little information is available on how different carbon sources influence structure of β-glucans in *C. albicans* biofilm. Earlier reports were limited to studies on the effect of dietary sugars on *C. albicans* adhesion and biofilm formation (Jin et al. 2004; Abu-Elteen 2005). Based on the above facts, it is significant to study the impact of different carbon sources on virulence factors and β-glucan structure in *C. albicans* biofilm. Glucose, sucrose, arabinose and lactate were selected as carbons sources according to their physiological relevance at different host tissues as mentioned above. This is the first study that highlighted the role of arabinose in *C. albicans* biofilm to the best of our knowledge. The aim of this investigation was to study the impact of different carbon sources on biofilm

formation, virulence factors and β-glucan, an important cell wall component of *C. albicans*. These analyses will open up new avenues in understanding the influence of metabolic adaptation on *C. albicans* biofilm.

2.2 Materials and methods

2.2.1 Chemicals and media

2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT); Menadione; FITC-ConA were purchased from Sigma-Aldrich, USA. Yeast peptone dextrose (YPD) medium, yeast nitrogen base (YNB) and all other chemicals were obtained from Himedia, India.

2.2.2 Strain and growth conditions

C. albicans MTCC 227 (equivalent to reference strain ATCC 10231) was grown on YPD plate for 24 h at 37 ºC from its stock culture (Singh et al. 2013). A loop full of yeast cells were inoculated into YPD broth and incubated at 37 ºC under shaking conditions (120 rpm). The cells were harvested (5000×*g* for 10 min at 4 ºC) during mid-logarithmic phase (after 16-18 h incubation) and the pellet obtained was washed twice with phosphate buffered saline (PBS, pH 7.2). The cell pellet was suspended in YNB supplemented with 1% carbon source (glucose, arabinose, sucrose and lactate) and adjusted to a standard cell suspension of 1×10^7 cells mL^{-1} ($OD_{520\ nm}$ is equal to 0.25) by a spectrophotometer (Lasany, LI-2800 UV-vis Double beam, India).

2.2.3 Adhesion assay

The adhesion assay was performed by adding 100 µL of *C. albicans* standard cell suspensions (1×10^7 cells mL^{-1}) in YNB containing 1% carbon source (glucose, arabinose, sucrose and lactate) to sterile 96-well polystyrene microtiter plate (MTP) and incubated at 37 ºC for 2 h (Jin et al. 2004). Later, cell suspensions were aspirated washed twice with PBS to remove loosely bound cells and XTT assay was performed.

2.2.4 Biofilm formation

C. albicans biofilm formation experiment was performed as described earlier (Santana et al. 2013). 100 µL of *C. albicans* (1×10^7 cells mL^{-1}) in YNB medium were seeded into sterile 96-well polystyrene microtiter plate wells for 90 min at 37 ºC (adhesion phase). Afterwards, wells were washed twice with 200 µL of PBS to remove loosely adhered cells and 100 µL of YNB

supplemented with 1% carbon source (glucose, arabinose, sucrose and lactate) was added to wells. Plates were incubated at 37 ºC for 48 h or 72 h. The fresh growth medium was added after each 24 h period during 72 h biofilm development.

2.2.5 Quantification of biofilm

The metabolic activity of biofilm cells was quantified by XTT (2, 3-bis(2-Methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium salt) reduction assay. XTT is reduced to soluble tetrazolium formazan product by mitochondrial dehydrogenase of metabolically active cells in presence of an electron-coupling agent menadione (Hawser 1996). Either adherent cells (after 2 h in adhesion assay) or biofilm formed (after 48 h) in each well were washed thrice with sterile 200 µL of PBS to remove loosely adhered cells. XTT and menadione solutions were prepared as described earlier (Pierce et al. 2008). 100 µL of PBS, 48 µL of XTT (1 mg mL^{-1}) and 2 µL menadione (1mM) solution was added to each MTP wells. Plates were incubated in dark for 2 h at 37 ºC and the absorbance was measured at 492 nm by MTP reader (Spectra Max M2, Molecular Devices, USA). Biofilm was washed as described above and the scraped off carefully using a sterile scalpel from the MTP wells as described by Jin et al. (2004). The resulted suspensions were added to PBS and vortexed for 3 min to disrupt the cell aggregates. The cell suspensions were serially diluted in PBS and plated onto YPD agar plates, incubated at 37 ºC for 24 h and CFU mL^{-1} were quantified. Two parallel plates were used for XTT assay and CFU assay.

2.2.6 Isolation of matrix polysaccharides from C. albicans biofilm

The biofilm biomass of *C. albicans* grown in presence of carbon sources was harvested (10000×*g* 10 min) after 48 h and the obtained biofilm pellet was suspended in PBS. The polysaccharides in biofilm suspension were extracted by sonication (Q700 sonicator, QSonica, USA) at 35W in an ice bath up to five 30 seconds on ice to disrupt the clump of cells (Comte et al 2006). Proper care was taken not to disrupt the cell wall during sonication and disruption of aggregates was monitored by microscopy. The samples were centrifuged at 10000×*g* for 10 min at 4 ºC and the supernatant was collected for quantification of matrix polysaccharides. The total carbohydrate content was measured by phenol-sulphuric acid method (DuBois et al. 1956) with glucose as a standard. Briefly, 200 µL of supernatant solution containing matrix polymers was transferrd into a sterile glass tube and mixed with 200 µL of phenol (5% w/v) followed by 1 mL of conc. H_2SO_4. The solution was left undisturbed for 10 min at room temperature and then

incubated at 30 ºC for 30 min in a water bath. The absorbance at 485 nm was measured with a spectrophotometer (Lasany, LI-2800 UV-vis Double beam, India).

2.2.7 Confocal laser scanning electron microscopy (CLSM)

C. albicans biofilm was developed in presence of different carbon sources on sterile polystyrene discs (1 cm in diameter) placed in 12-well culture plate. After incubation (48 h), cells were washed with PBS and stained with 50 µg Fluorescein isothiocyanate-concanavalin A mL^{-1} (FITC-ConA, long-pass filter, excitation wavelength, 490 nm; emission wavelength, 525 nm; emits green fluorescence) for 1 h at 37 °C (Singh et al. 2013). FITC-Con A is a lectin that binds to terminal glucose and mannose residues of polysaccharide present in the biofilm matrix and cell walls and confers as a better tool to characterize exopolymeric substances (Lal et al. 2010; Jin et al. 2005; Sharon & Lis 1972). The samples were visualized using a Leica TCS SP5 confocal laser scanning electron microscope (Leica Microsystems, Germany) with 20x objective lens and a HyD hybrid detector. The interactive 3-D surface plot images were constructed through ImageJ software (version 1.50a).

2.2.8 Atomic force microscopy

Biofilm of *C. albicans* were developed in presence of carbon sources (glucose, lactate, sucrose and arabinose) for 48 h at 37 °C on sterile polystyrene discs (1 cm in diameter) in a 12-well culture plate. After incubation, medium was withdrawn and the discs were washed twice with PBS and dried. The images of biofilm formed on polystyrene discs were taken using a NTEGRA PRIMA system (NT-MDT). All images were recorded in a semi-contact mode regime using a sharpened SiN cantilever tip (NSG10S) with spring constant of 10 Nm^{-1}, amplitude range of 5-10 nm, tip radius 10 nm and cone angle of 22 º. Height and deflection images were acquired with a resonance frequency of 250 KHz. Data analysis was carried out using software called NOVA.

2.2.9 Assay of proteinase and phospholipase activity

The biofilm biomass obtained after 72 h of biofilm development was sonicated as described above and centrifuged at 10000×*g* for 10 min at 4 °C. The supernatant obtained was used for assay of extracellular enzymes. The proteinase activity was determined by mixing azocasein substrate (1% w/v) with supernatants thus making the final reaction volume to 1 mL and incubated at 37 ºC for 1 h (Santana et al. 2013). The reaction was stopped by adding 10% trichloroactetic acid and the mixture was centrifuged for 5 min at 10000×*g*. Subsequently, supernatants were mixed with 0.5 M NaOH and incubated for 15 min. The proteinase activity

was measured spectrophotometrically at 440 nm. The specific proteinase activity was defined as the amount of enzyme that elicited an increase of 0.001 units of absorbance per minute of reaction by biofilm biomass (OD at 492 nm). The phospholipase activity was determined as described (Taniguchi et al. 2009) by adding phosphatidylcholine substrate solution to biofilm supernatants, incubated for 1 h at 35 ºC and absorbance was read spectrophotometrically at 630 nm. The specific phospholipase activity was recorded as the absorbance shift per minute of reaction by biofilm biomass.

2.2.10 Effect of carbon sources on C. albicans morphogenesis

To examine the effect of different carbon sources on morphogenesis, experiments were performed at 37 ºC in YNB supplemented with 1% glucose, lactate, sucrose and arabinose. Briefly, medium was inoculated with yeast cells from overnight cultures and standardised to a density of 1×10^7 cells mL^{-1} followed by incubation under shaking conditions at 200 rpm. Cells were visualized by microscopy (EVOS-FL, Advance Microscopy Group, USA) after 4 h in transmittance mode and images were captured.

2.2.11 Isolation of glucan from C. albicans biofilm cells

Glucan was isolated from *C. albicans* biofilm grown in different carbon sources for 48 h by a modified method of Lowman et al. (2014). Briefly, biofilm suspensions were sonicated for 2 min as described above and harvested at 10000×*g* for 10 min at 4 °C. Biofilm cell pellets were suspended in PBS and β-glucan was extracted with 0.1 N NaOH for 15 min at 100 °C, followed by neutralization. The neutral residue was extracted by boiling with 0.1 N H_3PO_4 for 15 min at 100 °C and neutralized to pH 7.0. The water insoluble micro particulates of isolated glucan were lyophilized for further analysis.

2.2.12 Fourier transform infrared spectroscopy

The infrared spectra of the β-glucan samples were recorded using FT-IR spectrometer (Thermo Nicolet NEXUS, Maryland, USA) by KBr pellet technique and data was obtained with OMNIC software. The spectrum was taken between 500 cm^{-1} to 4000 cm^{-1} at a 4 cm^{-1} resolution on an average of 18 scans. Overlapping and hidden peaks often give broad peaks which are difficult to analyze and results in poorly resolved spectrum. These overlapping peaks due to absorptions of biomoleules present in cells and other intrinsic factors can be solved by mathematical methods (Lórenz-Fonfría & Padrós 2005). Fourier deconvolution, second derivative and curve fitting methods were used to analyze structural changes in spectral region with Peak Fit Version 4.12. Curve fitting was performed by non-linear regression method using Gaussian and

Lorentzian bands. The goodness of fit was estimated by the chi-square value; the lower the chi-square value better is the fit (Galichet et al. 2001; Adt et al. 2006).

2.2.13 Statistical analysis

Three independent experiments were conducted in duplicate and data was expressed as mean ± standard deviation. Data obtained were evaluated with analysis of variance (ANOVA) followed by *posthoc* Tukey's honestly significant different (HSD) test for pair-wise comparisons using XLSTAT statistical add-in software for Microsoft Excel© and OriginPro® 8. Letters on the histogram provides the graphical representation for posthoc pair-wise comparisons, (Tukey's HSD). Means sharing the same letter is not significantly different from each other. In all evaluations, *p* value less than or equal to 0.05 were considered significant.

2.3 Results

2.3.1 Carbon sources variably influence C. albicans adhesion and biofilm development

The adhesion and biofilm formation of *C. albicans* on to polystyrene MTP in the presence of carbon sources was determined by their metabolic activity. Glucose displayed the highest metabolic activity in adhesion assay ($p < 0.03$, Fig. 2.1a) and significant differences were observed among the other carbon sources ($p < 0.05$). During biofilm formation, the highest metabolic activity was observed in sucrose ($p < 0.001$) followed by glucose, arabinose and lactate (Fig. 2.1b). Statistically significant differences were observed between glucose and lactate/or arabinose ($p < 0.001$). However, no significant differences were observed between sucrose and glucose ($p > 0.05$). Similarly, lactate and arabinose were not different significantly ($p > 0.05$). These results were correlated with the viable cell counts represented as log CFU counts (10^5 dilution, $p < 0.05$) which were recorded highest in sucrose (8.36) grown cells compared to glucose (7.96), arabinose (6.83) and lactate (6.51) respectively.

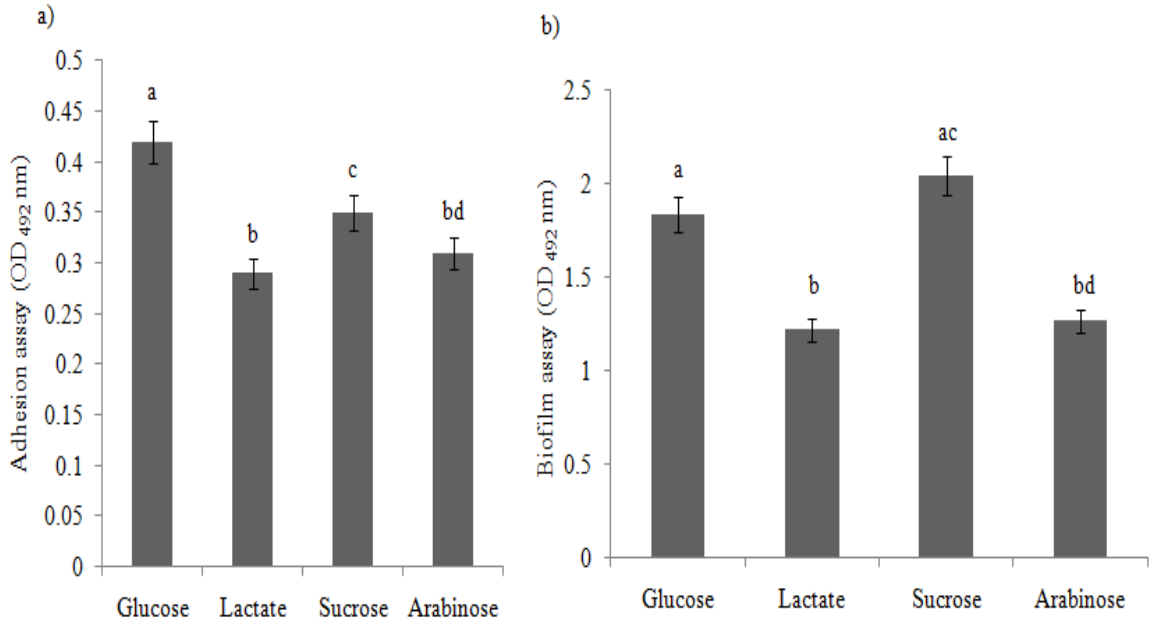


Figure 2.1 Adhesion and biofilm assay. a) Effect of different carbon sources on *C. albicans* a) adhesion after 2 h of incubation b) biofilm formation (48 h) in polystyrene plate. Data represent the means ± the SD of three independent measurements. Letters on the histogram provides the graphical representation for posthoc pair-wise comparisons, (Tukey's HSD, $p < 0.05$). Means sharing the same letter are not significantly different from each other.

2.3.2 Carbon sources modulate secretion of matrix polysaccharides

C. albicans biofilm matrix polysaccharides were evaluated and compared among different carbon sources (glucose, sucrose, arabinose and lactate) tested (Fig. 2.2a). The highest content of matrix polysaccharides were observed in sucrose followed by glucose ($p < 0.05$) and no significant difference was observed between lactate and arabinose ($p > 0.05$). The architecture of biofilm embedded in matrix polysaccharides was examined after 48 h using CLSM (Fig. 2.2b). The green fluorescence in the images was due to selective binding of FITC-ConA to glucose and mannose residues of the fungal polysaccharides present on the cell surface and biofilm matrix. Images revealed a heterogeneous structure of biofilm cells encased in a thick network of polysaccharides in matrix. No apparent difference in biofilm architecture and exopolysaccharide distribution was observed in glucose and sucrose grown cells. A similar observation was noted for lactate and arabinose in which exopolysaccharides were interspersed between biofilm cells.

2.3.3 Thickness and architecture of biofilm varies with the carbon source

The surface roughness and height of the biofilm formed in presence of carbon sources were analyzed by AFM (Fig. 2.3). The biofilm cells were observed as ridges and grooves due to varied production of exopolymeric matrix that projected out from the surrounding cells (Fig. 2.3a1-d1). The average roughness of biofilm formed in the presence of carbon sources were 21.75 nm (sucrose), 17.99 nm (glucose), 13.6 nm (arabinose) and 11.16 nm (lactate). The height of glucose and sucrose grown biofilm was 167.5 nm and 196 nm respectively. A significant variation in height and thickness were observed in arabinose (126 nm) and lactate (113 nm) as compared to sucrose. The three-dimensional structure of biofilm also exhibited significant differences in Z- axis value among carbon sources tested (Fig. 2.3a2-d2). The Z-axis values of 167 nm/div, 196 nm/div, 126 nm/div and 113 nm/div corresponds to glucose, sucrose, arabinose and lactate respectively.

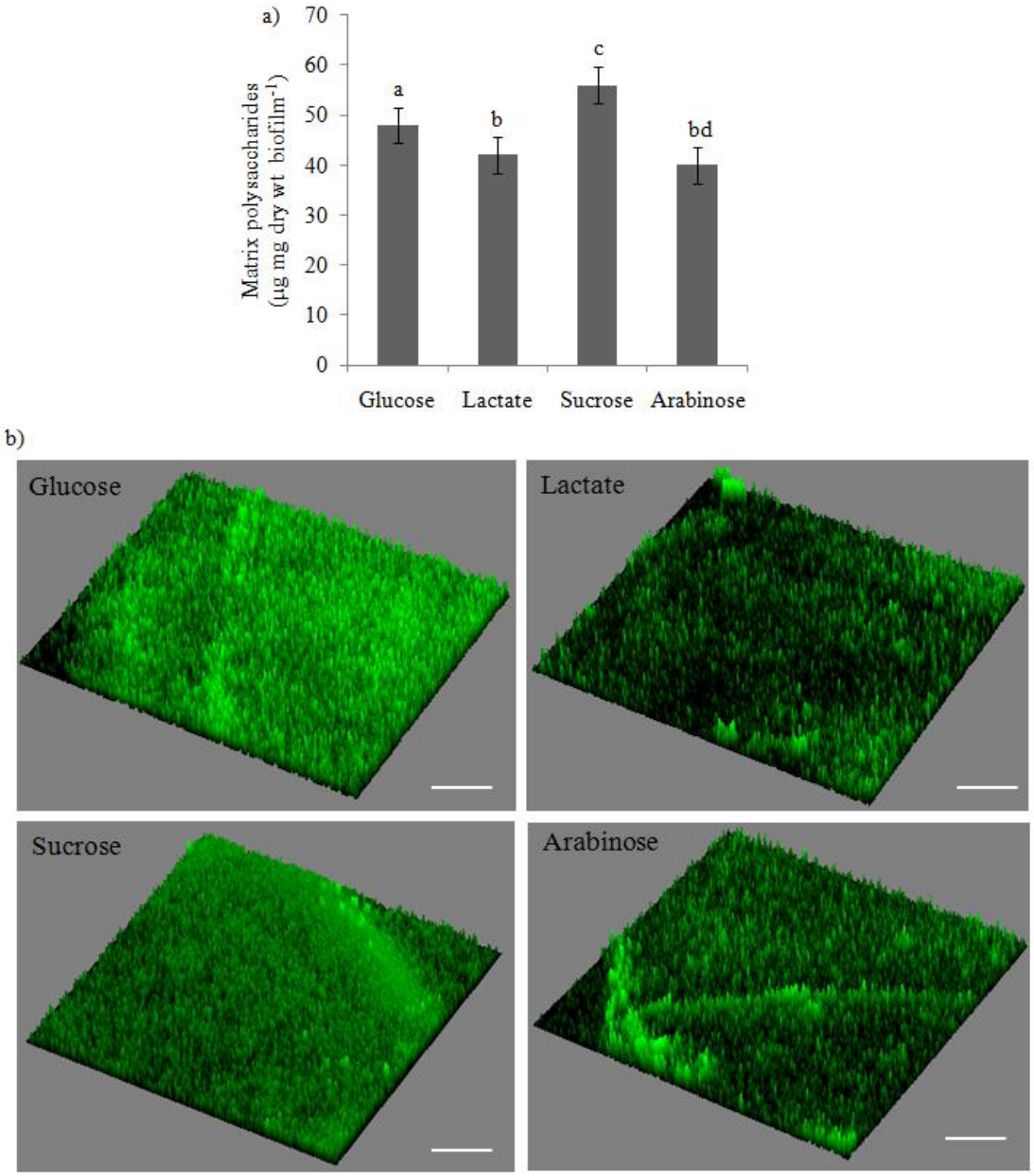


Figure 2.2 Influence of carbon sources on matrix polysaccharides secretion. a) Quantification of matrix polysaccharides secreted in *C. albicans* biofilm. Data represent the means ± the SD of three independent measurements. Letters on the histogram provides the graphical representation for posthoc pair-wise comparisons, (Tukey's HSD, $p < 0.05$). Means sharing the same letter are not significantly different from each other. b) Three dimensional CLSM images of *C. albicans* biofilm matrix stained with FITC-Con A. Images appear green in color due to selective binding of Con A to glucose and mannose residues of fungal polysaccharides in biofilm matrix and cell wall. Scale bar indicates 100 μm.

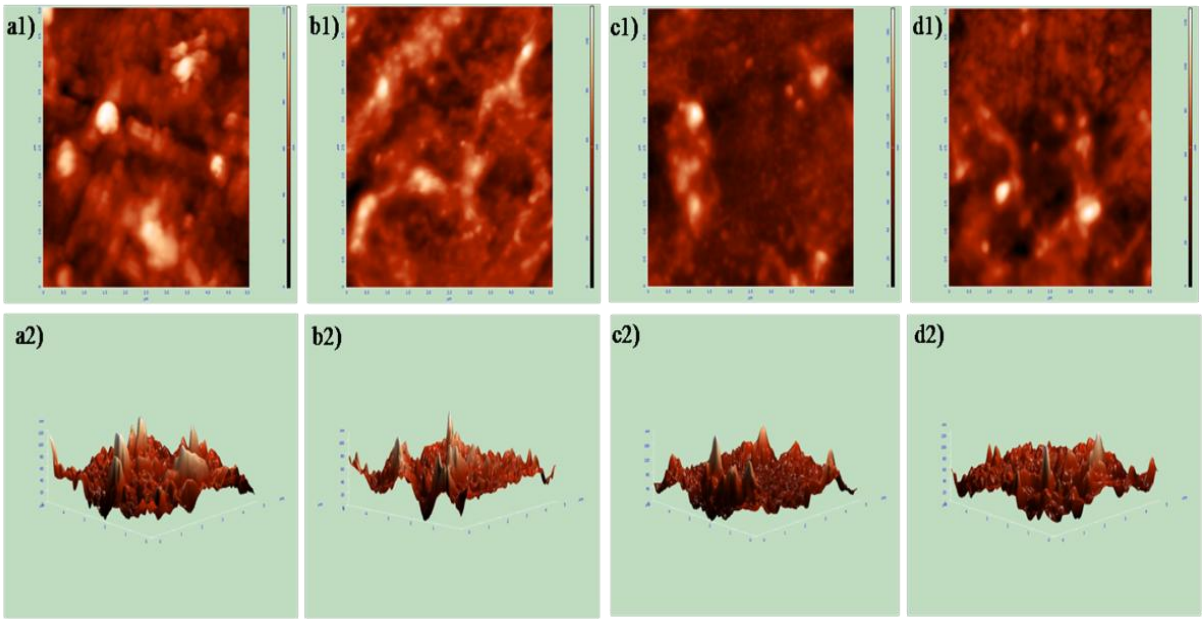


Figure 2.3 Atomic force microscopy. AFM micrographs of *C. albicans* biofilm on microtiter plates and their respective 3D images grown in presence of growth medium supplemented with a) glucose b) lactate c) sucrose and d) arabinose.

2.3.4 Carbon sources modulate hydrolytic enzymes and morphology

The specific activity of proteinase after 72 h was highest in lactate grown *C. albicans* followed by arabinose, glucose and sucrose grown cells as shown in Figure 2.4a. The specific phospholipase activity was highest in biofilm developed on arabinose ($p < 0.001$) followed by glucose and lactate (Fig. 2.4b). No significant difference was observed in specific phospholipase activity between glucose and lactate grown cells. Significant differences were observed between sucrose and lactate or arabinose in both virulence enzyme assays ($p < 0.05$). Filamentation in *C. albicans* was microscopically observed after 4 h. Despite analogous physio-chemical parameters employed (incubation time, temperature and rpm), filamentation in *C. albicans* grown on different carbon sources were observed (Fig. 2.4c). Images depicted germ tubes in glucose, hyphal and pseudo-hyphal forms in lactate and arabinose. While predominantly yeast and chains of yeast forms were observed in sucrose grown cells.

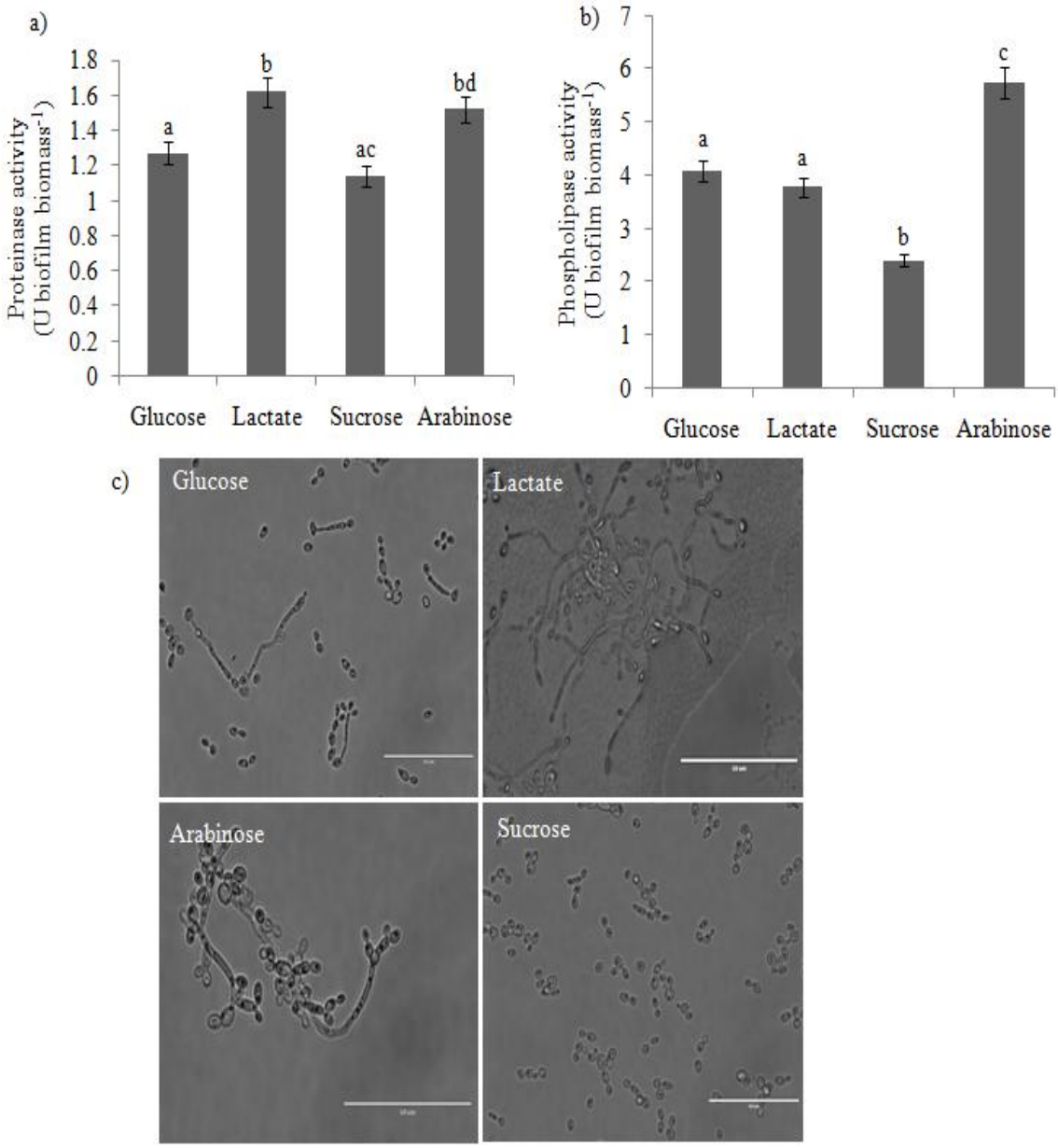


Figure 2.4 Test for virulence factors. a) The specific activity proteinase and b) the specific activity of phospholipase in *C. albicans* biofilm. Data represent the means ± the SD of three independent measurements. Letters on the histogram provides the graphical representation for posthoc pair-wise comparisons, (Tukey's HSD, $p < 0.05$). Means sharing the same letter are not significantly different from each other. c) Microscopic images of morphogenesis in presence of different carbon sources. Scale bar indicates 50 μm.

2.3.5 FTIR analysis revealed structural changes in cell wall component β-glucan

FT-IR spectrum of β-glucan from *C. albicans* biofilm grown in presence of different carbon sources were studied in the absorption region of 600 cm^{-1}-4000 cm^{-1}. It was noted that spectra obtained exhibited slightly different spectral profile in each carbon source tested (Fig. 2.5). To assess these spectral changes which might be due to structural modifications, curve fitting method was used. The theoretical spectrum was calculated in the region 850 cm^{-1} to 1350 cm^{-1} that represents functional groups corresponding to component structures of glucans and their content (Fig. 2.6). This region also reflects the absorption of polysaccharides. The corresponding band areas and their assignments were detailed in Table 2.1. The characteristic β-glucan band at 892 cm^{-1} was found to be same in all carbon sources investigated. The bands identified at 998 cm^{-1} and 1145 cm^{-1} that were characteristic to β(1→6) and β(1→3) glucans was observed in all the spectra. The other functional groups were recognized by bands at wave numbers 1639 cm^{-1} (C=O stretching) between 2323 cm^{-1} to 2345 cm^{-1} (O=C=O stretching) and 3400 cm^{-1} (-OH vibrational stretch) corresponding to polysaccharides.

The positions of most of the bands do not vary much in arabinose and sucrose grown cells but the absorption intensity of β(1→6) and β(1→3) glucan was highest in arabinose which was also found similar to glucose (Fig. 2.6a,c,d). There was an absorption intensity decrease of the band at 998 cm^{-1} in lactate and up field 3 cm^{-1}shift of band from 1241 cm^{-1} to 1238 cm^{-1} (δ CH + δ OH in plane). Other bands observed at 1050 cm^{-1} (CO and CC vibrational stretching), 1162 cm^{-1} (vibrational stretching of CO) and 1335 cm^{-1} (bending vibrations of CCH and OCH) have significant band area in lactate which were absent in other carbon sources tested (Fig. 2.6b). The two bands identified at 998 cm^{-1} and 1145 cm^{-1} assigned to β(1→6) and β(1→3) glucan were used for band area evaluation. From the calculations, β(1→6) to β(1→3) glucan ratio was less in lactate grown cells (1.15) compared to glucose (1.73), sucrose (1.62) and arabinose (2.85) respectively. The β(1→6) to β(1→3) glucan ratio in arabinose reflects its absorption intensity which was found to be highest among all carbon sources tested (Fig. 2.6c).

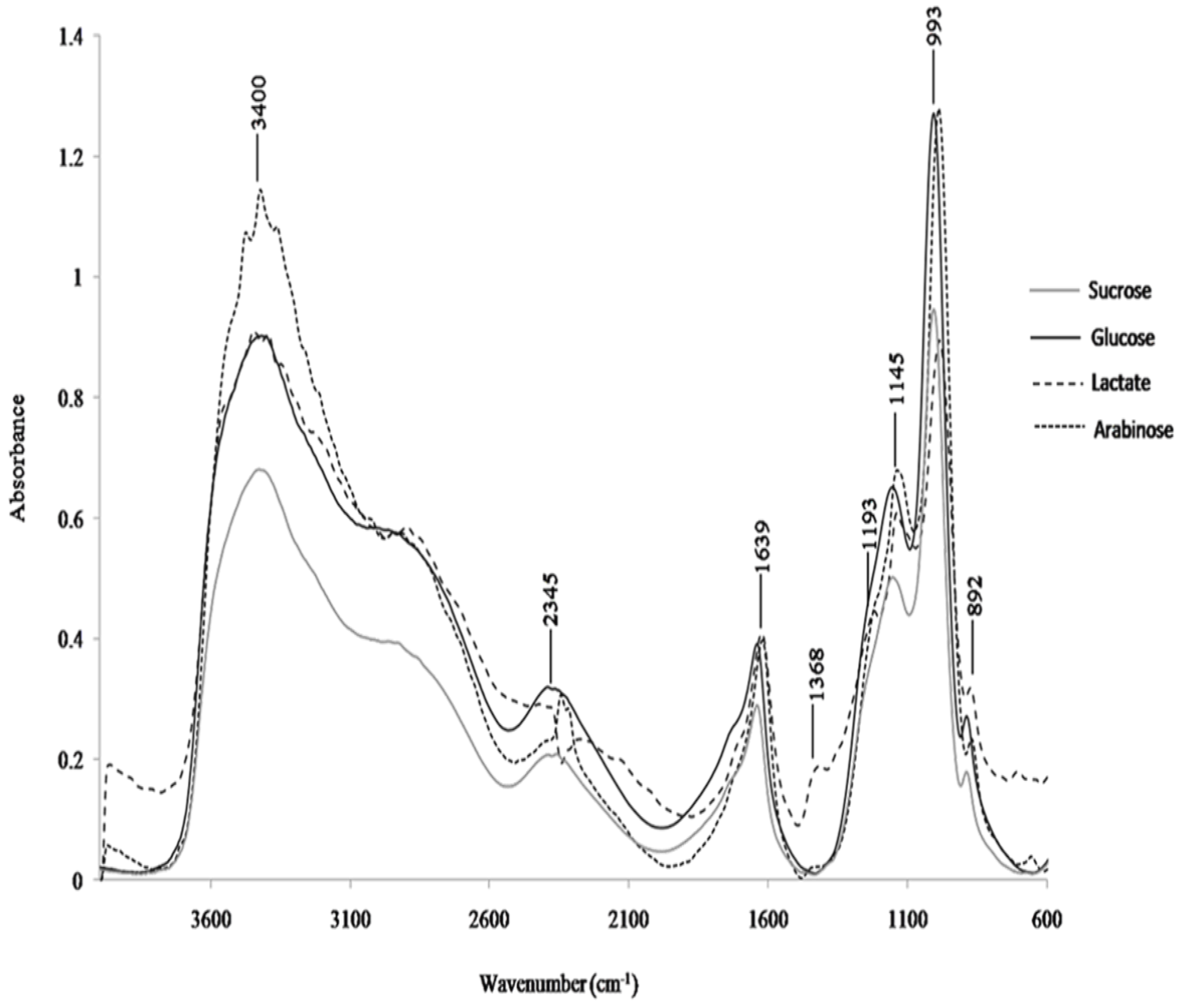


Figure 2.5 FT-IR analysis. Normalised FT-IR spectrum of β-glucans isolated from cell wall of *C. albicans* biofilm grown on different carbons sources.

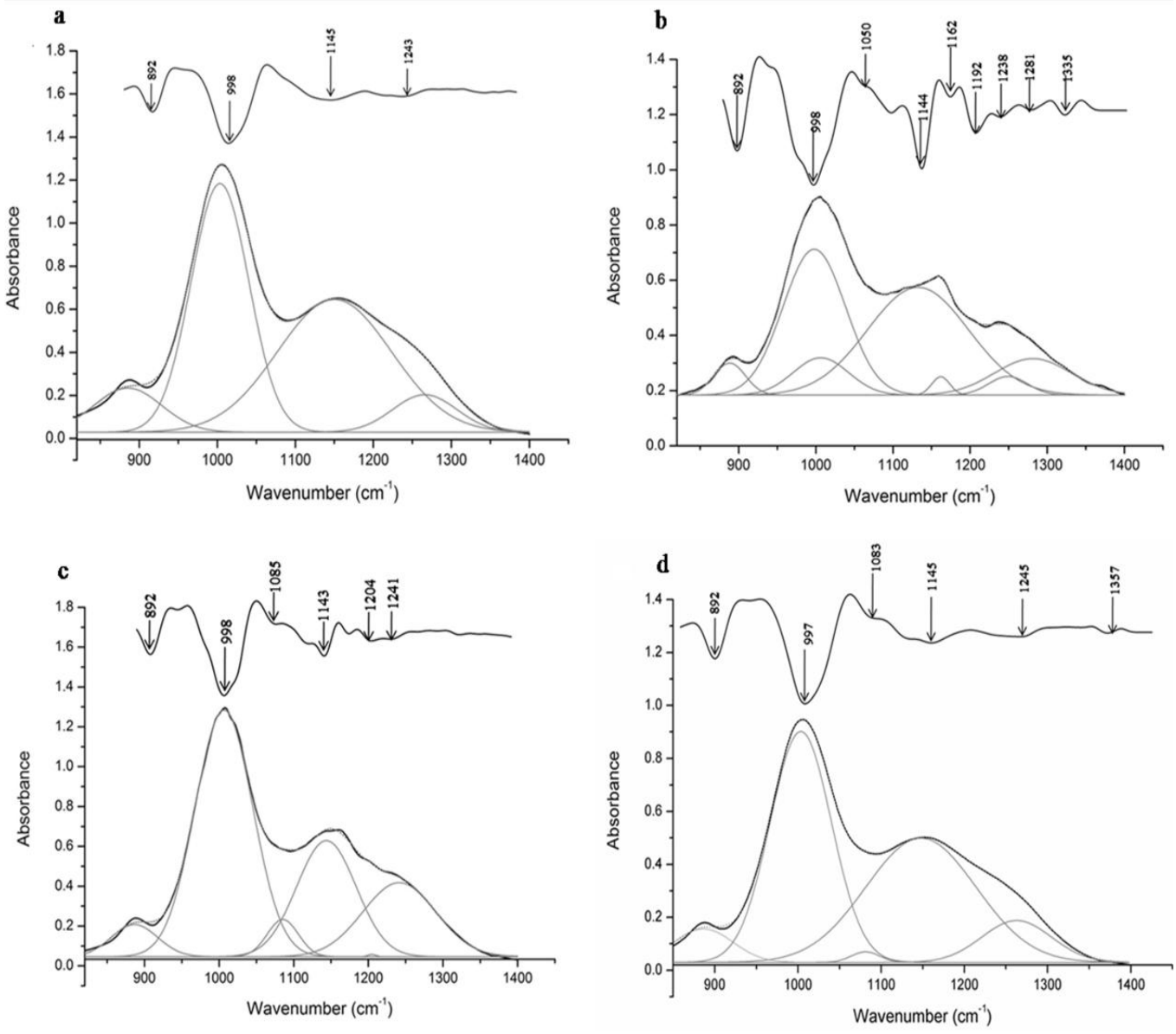


Figure 2.6 Determination of structural modification in β-glucan. Curve-fitting of β-glucan FT-IR spectra in the frequency range of 850 cm^{-1}-1350 cm^{-1} a) glucose b) lactate c) sucrose and d) arabinose. Black and grey lines represent the experimental and calculated spectra. Second derivative spectra are displayed above each spectrum with band positions used for calculation. χ^2 values for curve-fitting were in the range of 10^{-6} to 10^{-5}.

	Band Position (cm^{-1})	Band Area (%)	Assignment
Glucose	892	9.3	β-glucan
	998	53.5	β (1→6)glucan
	1145	30.2	β (1→3)glucan
	1243	6.8	δ CH + δ OH in plane
Sucrose	892	8.9	β-glucan
	998	48.8	β (1→6)glucan
	1083	5.8	β (1→3)glucan
	1145	30.0	β (1→3)glucan
	1245	6.5	δ CH + δ OH in plane
Arabinose	892	8.5	β-glucan
	997	57.0	β (1→6)glucan
	1085	5.0	β (1→3)glucan
	1143	20.0	β (1→3)glucan
	1204	0.1	δ CH + δ OH in plane
	1241	9.2	δ CH + δ OH in plane
Lactate	892	9.1	β-glucan
	997	31.8	β (1→6)glucan
	1050	9.35	ν CO + ν CC
	1144	27.6	β (1→3)glucan
	1162	4.5	ν CO
	1238	6.6	δ CH + δ OH in plane
	1335	10.8	δ CCH + δ OCH

Table 2.1 Quantitative assessment of curve-fitting bands of the FT-IR spectra obtained from *C. albicans* β-glucans in the frequency range of 850 cm^{-1}-1350 cm^{-1}.

2.4 Discussion

C. albicans has evolved adaptive tactics which are driven by the needs to assimilate nutrients, colonise, multiply and survive in different host niches. The impact of these plans contributes to design of structural and pathogenicity platform as biofilm, which provide them an armoury to evade antifungals as well as host defence system. Carbon sources possibly act as a key player in *C. albicans* biofilm formation, virulence factors and cell wall structure which might enhance

pathogenicity. When the effect of different carbon sources on adhesion were tested, YNB medium supplemented with glucose resulted in higher Candidal adhesion to the polystyrene plates. In subsequent stages of biofilm, higher degree of biofilm formation was seen in sucrose grown *C. albicans* cells. Given the effect of these dietary carbohydrates glucose and sucrose on the adherence and biofilm formation in vitro, these commonly consumed carbohydrates are important in the pathogenesis of *C. albicans* in the oral cavity (Jin et al. 2004).

The host niches are multifarious, dynamic and often glucose limited. For instance, glucose levels in bloodstream are at 0.06-0.1% and 0.5% in vaginal secretions (Owen & Katz 1999; Barelle et al. 2006). Lactate, a carboxylic acid present in vaginal mucosa is a physiologically relevant carbon source required for *Candida* proliferation in the gastrointestinal tract (Ueno et al. 2011). On the other hand, arabinose and its polyol product arabitol were reported in association with vulvovaginitis and invasive candidiasis (Kiehn et al. 1979; Horowitz et al. 1984; Wong et al. 1993). The ability to use and assimilate carbon sources like lactate and arabinose indicate the effective adaptation of *C. albicans* to the available nutrients. These observations are in agreement with the ability of *C. albicans* to rapidly tune its metabolism and the expression of key metabolic functions in a niche-specific manner (Brown et al. 2007).

Biofilm matrix consists of polysaccharides, proteins, and signalling molecules that help in nutrient channelling and act as a barrier for the diffusion of antifungals (Moryl et al. 2014). Biochemical analysis revealed that in presence of sucrose, *C. albicans* produced highest amount of polysaccharides. CLSM images demonstrated the distribution of exopolysaccharides in *C. albicans* biofilm matrix. AFM studies revealed that biofilm thickness and roughness varied significantly among the carbon sources tested. The irregular ridges and grooves due to exopolymeric secretion increased the height of the biofilm.

In *C. albicans*, metabolic adaptation also alter key virulence factors such as yeast to hyphal transitions, proteinases and phospholipases which aid in tissue invasion and modulate host immune response (Vidotto et al. 2004; D'Eça Júnior et al. 2011). The expression of certain proteinase and phospholipase genes are regulated in response to carbon sources, pH of the medium and correlated with the site of infection (Ghannoum 2000; Naglik et al. 2003; D'Eça Júnior et al. 2011). Glucose a commonly available carbon source can stimulate hyphal morphogenesis at low concentration of 0.1% (mimics glucose concentration in blood) and higher concentration (2%) results in few hyphae (Maidan et al. 2005; Sabina & Brown 2009). In this study, germ tubes were observed in 1% glucose growth medium similar to the reports of Buu and Chen (2014). Growth of *C. albicans* on lactate induced hyphal forms and displayed the highest proteinase activity. Studies on hyphal morphogenesis reveal that hyphal forms elicit

the production of hypha-associated secreted aspartyl proteinases (Felk et al. 2002; Buu & Chen 2014). Ene et al. (2012) stated that growth on alternative carbon sources like lactate increased the virulence potential in *in-vivo* models of systemic candidiasis and vaginal infection. Arabinose grown cells induced pseudo-hyphal forms and exhibited the highest phospholipase activity. A correlation study between filamentation and high phospholipase activity in oral isolates suggests that these virulence factors are necessary for colonization and infection as phospholipase activity is particularly concentrated in hyphal tips (Vidotto et al. 1999). Enjalbert and Whiteway (2005) reported hyphal forms when overnight cultures were transferred to a fresh medium. This response was transient and hyphal cells produced budded cells after 3h. Similarly, chains of yeast (pseudomycelia) were visualized in sucrose grown cells along with yeast forms which might be due to cells undergoing reversible morphological transition phase. However, it is not clear why pseudomycelial forms were not observed in *C. albicans* grown on other carbon sources which also had similar cultural conditions. These results indicates that adaptation of *C. albicans* to grow on the carbon sources available or alternative carbon sources influences the virulence and thereby its ability to colonize different anatomical sites.

Glucan backbone is composed of polymer containing (1→3)- β-D-linked anhydroglucose repeat units and a side chain as β(1→6) glucan that cross links the components of the inner and outer walls (Klis et al. 2001; Lowman & Williams 2001). The bands observed in FT-IR spectra at different wavelengths corresponding to β-glucan were in good agreement with previous works (Galichet et al. 2001; Adt et al. 2006; Ibrahim et al. 2006; Plata et al. 2013). Present study results indicate that different carbon sources influence glucan structure variably in *C. albicans* primarily affecting the cross-linkage. The reduction of band intensity of β(1→6) and β(1→6) to β(1→3) ratio could indicate that lactate triggered a decrease in cross-linkage. The cross-linkage among glucan chains confers to the rigidity of cell wall. These results are in concordance with earlier reports stating that carbon adaptation in *C. albicans* can bring changes in cross-linking among cell wall biopolymers and biophysical properties (Aguilar-Uscanga & Francois 2003). Researchers also demonstrated that the thickness of the inner layer of cell wall decreased when cells were grown on lactate (Ene et al. 2012). Our findings evidently illustrate that carbon adaptation in *C. albicans* strongly influence the virulence properties such as biofilm development, filamentation, hydrolytic enzymes secretion and alter the cell wall glucan structure thus making *C. albicans* fit to survive in diverse host niches. This modulation of *C. albicans* virulence by carbon adaptation might affect host immune response thereby promoting pathogenesis of biofilm related infections.

CHAPTER-3

3. Impact of oxidative and osmotic stresses on *Candida albicans* biofilm formation

3.1 Background

C. albicans is the most common pathogen associated with fungal biofilm infections related to implanted medical devices. The ability of it to form biofilm on both biotic and abiotic surfaces has an important clinical repercussion with increasing mortality rates (Kojic & Darouiche 2004). During infection, *C. albicans* encounters a range of stresses from the host environment across diverse niches. Discrepancy in cellular osmo-homeostasis leads to rapid water loss, cell size reduction and fall of turgor pressure in *C. albicans* (Klipp et al. 2005; Kühn & Klipp 2012). *C. albicans* also get exposed to oxidative stress during infection in form of reactive oxygen species (ROS) released by polymorphonuclear leukocytes (PMNLs) and macrophages (Mavor et al. 2005). The effective adaptation to stresses is an essential feature to medically important pathogens like *C. albicans* (Martinez & Casadevall 2007). Several studies reported that stress responses in *C. albicans* have been evolutionarily tuned in comparison to *Saccharomyces cerevisiae* and molecular rewiring takes place according to the types and intensities of stresses that *C. albicans* come across during infection (Enjalbert et al. 2003; Brown et al. 2014). Inactivation of signalling pathways or genes associated with robust stress response attenuates virulence factors. This multifactorial virulence process includes surface adhesion, biofilm formation, morphogenesis and secretion of extracellular enzymes such as proteinases and phospholipases (Nicholls et al. 2011; Mayer et al. 2013). Additionally, virulence relies on *C. albicans* cell wall, a complex and dynamic structure containing glucan, mannan and chitin, which are crucial for colonization at different niches in a host. Among them, mannan helps with adhesion of *Candida* to the mammalian cells as the first step of infection, which induces production of cytokines and may act as a virulence factor (Miyakawa et al. 1992; Jouault et al. 1994; Trinel et al. 2002). Stress adaptation is crucial for *C. albicans* virulence as it increases the survival of this pathogen (Arana et al. 2007; Patterson et al. 2013). Though, much is known about the stress signalling pathways and the powerful responses against different stresses (Enjalbert et al. 2003; Ullmann et al. 2004; Fradin et al. 2005), little is known about how stress conditions influence biofilm formation. It is important to study biofilm, since the cell phenotypes within biofilm demonstrate reduced susceptibility to antifungals and cells of the immune system. Moreover, the influence of different stress

conditions on the cell wall composition and other virulence factors in biofilm provides the general physical mechanism that controls the pathogenicity of *C. albicans* biofilms. The link between stress and biofilm formation was studied to some extent in eubacteria, particularly in *Staphylococcus aureus* and *Streptococcus mutans* where osmotic and acid/oxidative stress induced biofilm formation (Rachid et al. 2000; Wen et al. 2005). This relationship is yet to be empathized clearly in *C. albicans* as there is no paradigm for stress induction, therefore, stress response varies according to the stress condition. Thus, this study investigated the influence of oxidative and osmotic stresses on *C. albicans* biofilm formation and matrix composition. Osmotic and oxidative stresses were focussed in this work since they contribute to fungal virulence and antifungal susceptibility. Importantly, this study highlights how oxidative and osmotic stresses may affect virulence factors and the cell wall components such as chitin and mannan.

3.2 Materials and methods

3.2.1 Strain and growth conditions

C. albicans MTCC 227 (equivalent to reference strain ATCC 10231) was grown on YPD plate (1% peptone, 1% yeast extract, 2% dextrose, 1.5% agar) for 24 h at 37 ºC. A loop full of yeast cells were inoculated into YPD broth and incubated at 37 ºC under shaking conditions (120 rpm). The cells were harvested (5000×*g* for 10 min at 4 ºC) during mid-logarithmic phase (after 16-18 h incubation) and the pellet obtained was washed twice with phosphate buffered saline (PBS, pH 7.2). The cell pellet was suspended in RPMI-1640 (Sigma-Aldrich, USA) supplemented with 2% glucose and buffered with 0.165 mM morpholinepropanesulfonic acid (MOPS). The suspension was standardised to a final concentration of 1×10^7 cells mL^{-1} (0.25 OD at 520 nm) by a spectrophotometer (Lasany, LI-2800 UV-visible Double beam, India).

3.2.2 Biofilm formation

C. albicans biofilm formation experiment was performed as described earlier (Jin et al. 2004; Santana et al. 2013). The cell suspension of 100 µL *C. albicans* (1×10^7 cells mL^{-1}) in RPMI-1640 medium was seeded into sterile 24-well polystyrene microtiter plate (MTP) wells for 90 min at 37 ºC (adhesion phase). Afterwards, the wells were washed twice with PBS (pH 7.2) to remove loosely adhered cells followed by addition of RPMI-1640 medium containing 5 mM H_2O_2 (oxidative stress) and 2 M NaCl (osmotic stress) into separate wells. Plates were then incubated at 37 ºC up to 72 h and growth was monitored at different time intervals (6, 12, 24, 48, 72 h).

3.2.3 Quantification of biofilms

The *C. albicans* biofilm formed in each well was washed thrice after specified intervals (6, 12, 24, 48, 72 h) with sterile PBS to remove loosely adhered cells and XTT assay was performed as described in Chapter 2 (sub-section 2.2.5).

3.2.4 Scanning electron microscopy

To observe *C. albicans* biofilm cells morphology and architecture, scanning electron microscopy was performed. *C. albicans* biofilm was developed in the presence of oxidative and osmotic stress on sterile polystyrene discs (1 cm in diameter) placed in 12-well culture plate at 37 °C. After 48 h of incubation, biofilm was washed twice with PBS and fixed with 2.5% glutaraldehyde for 2 h. The cells were then dehydrated in series of ethanol concentrations (35, 50, 70, 90 and 100%) for 10 min incubation. All samples were then dried to a critical point in the Polaron critical point dryer, mounted on to SEM stubs, sputter coated with gold. Specimens were observed with a SE microscope (Carl Zeiss, Quanta 200F Model, Netherlands) at a voltage of 15 kV and magnification from 1000 to 5000 X.

3.2.5 Extraction of matrix polymers from C. albicans biofilms

The *C. albicans* biofilm formed in the presence of oxidative and osmotic stress was harvested (10000×*g* 10 min) after 48 h and the obtained biofilm pellet was suspended in PBS. For matrix polymer extraction, biofilm suspension was sonicated (Q700 sonicator, QSonica, USA) at 35W in an ice bath up to 2 min (five 30s cycles with 1 min cooling on ice) as described earlier (Comte et al. 2006; Pemmaraju et al. 2016). The samples were centrifuged at 10000×*g* for 10 min at 4 °C and the supernatant was collected for quantification of matrix polymers. The total carbohydrate content was measured by phenol-sulphuric acid method with glucose as a standard (DuBois et al. 1956). Briefly, 200 µL of supernatant solution containing EPS was transferred into a sterile glass tube and mixed with 200 µL of phenol (5% w/v) followed by 1 mL of conc. H_2SO_4. The solution was left undisturbed for 10 min at room temperature and then incubated at 30 °C for 30 min in a water bath. The absorbance at 485 nm was measured with a spectrophotometer (Lasany, LI-2800 UV-visible Double beam, Haryana, India). The total amount of protein was quantified by a microplate procedure with Micro BCA (bicinchoninic acid) protein assay reagent kit (Thermo Fisher Scientific, Pierce Biotechnology, IL, USA) with bovine serum albumin as a standard. The absorbance was read at 562 nm by microtiter plate reader (SpectraMax M2, Molecular Devices, CA, USA). The total extracellular DNA (eDNA) content was determined by Allesen-Holm method by mixing 0.25 M NaCl to the supernatant

solution and the eDNA was precipitated by adding 2:1 volume of ethanol (Allesen-Holm et al. 2006). The precipitated eDNA was dissolved in TE buffer and the eDNA concentration was measured spectrophotometrically (OD_{260}/OD_{280}). The ratio OD_{260}/OD_{280} of eDNA sample within the range of 1.8-1.9 was considered as pure.

3.2.6 Assay of proteinase and phospholipase activity

The *C. albicans* biofilm from oxidative and osmotic stress treatment was sonicated after 72 h of incubation and centrifuged at 10000×*g* for 10 min at 4 °C. The supernatant obtained was used for assay of extracellular enzymes as described in Chapter 2 (sub-section 2.2.9).

3.2.7 Reactive oxygen species accumulation and catalase activity

The level of intracellular reactive oxygen species (ROS) and catalase activity in *C. albicans* biofilm due to oxidative stress (5 mM H_20_2) was determined using a modified protocol of Jakubowski et al. 2000. Briefly, biofilm cells were collected by centrifugation (at specified intervals) and suspended in PBS (pH 7.2). 10 µM of 2′,7′- dichlorodihydrofluorescein diacetate (DCFDA, Sigma-Aldrich, USA) and 1 mg mL^{-1} of propidium iodide (PI, Sigma-Aldrich, USA) was added to tubes containing biofilm cells and incubated at 37 ºC for 30 min in the dark. DCFDA is a fluorogenic dye freely permeable across cell membranes and measures the ROS activity within the cell. PI was used to monitor the cell lysis as it binds to eDNA or DNA of cells with compromised cell membrane (Ma et al. 2009). For ROS measurements, cells were harvested (10000×*g* for 10 min at 4 °C) after incubation, suspended in PBS and cells were broken with glass beads (0.5 mm) using a cell disruptor (Constant Systems, England). The cell homogenates were sedimented and the fluorescence of DCFDA in the supernatant was measured using fluorescence spectrophotometer (Fluorolog-3 LS55, Horiba Jobin Yvon Spex®, USA) at 485 nm excitation wavelength and emission wavelength at 520 nm respectively. The fluorescence of PI in the cell suspension was calculated at an excitation wavelength 543 nm and emission wavelength at 617 nm. Alternatively, cells were also examined by fluorescence microscope (EVOS-FL, Advance Microscopy Group, USA). For catalase activity measurements, biofilm cells were centrifuged (10000×*g* for 10 min at 4 °C), suspended in PBS (pH 7.2). Cells were subjected to lysis with glass beads (0.5 mm) using cell disruptor (Constant Systems, England) and cell debris was removed by centrifugation. The aqueous solution of H_2O_2 in PBS was added quickly to the supernatant and the rate of decrease in absorbance at 240 nm was measured. One unit of catalase activity corresponded to the amount of enzyme that decomposes 1 μmole of H_2O_2 per minute of reaction at 37 °C.

3.2.8 Isolation and purification of cell walls for chitin quantification

C. albicans biofilm cell walls were isolated and purified using modified procedure of de Groot et al. (2004). The biofilm obtained from oxidative and osmotic stress treatment after 48 h was centrifuged (10000×g for 10 min at 4 °C) and the cell pellet was suspended in PBS (pH 7.2). Cells were disrupted using cell disruptor (Constant Systems, England) with glass beads (0.5 mm) in the presence of a protease inhibitor cocktail. The complete cell breakage was examined by microscope. The cells were washed thrice with 1 M NaCl and then washed twice with MilliQ-water and extracted for 10 min at 100 °C in an appropriate volume of SDS-Mer extraction buffer (150 mM NaCl, 20 g/l SDS, 100 mM EDTA, 100 mM β-mercaptoethanol, 50 mM Tris-HCl, pH 7.8). These SDS-Mer treated cells were washed thrice with MilliQ-water and lyophilized overnight.

3.2.9 Quantification of chitin

Chitin content in the cell wall of each sample was determined as described earlier by Heilmann et al. (2013). Freeze-dried cell walls were suspended in tubes containing 100 µL of 1 M NaOH and boiled for 10 min. The insoluble cell wall components were hydrolyzed in 1mL of 6 M HCl for 18 h at 100 °C. After hydrolysis, the liquid was evaporated under a stream of nitrogen and the pellet was suspended in MilliQ- water. Then samples were mixed with equal volumes of 1.5 M Na_2CO_3 in 4% acetylacetone and boiled for 20 min. The tubes were allowed to cool at room temperature and 700 µL of 96% ethanol and 100 µL of a *p*-dimethyl-aminobenzaldehyde solution (1.6 g in 30 mL of concentrated HCl and 30 mL of 96% ethanol) was added to incubate for 1 h at room temperature. The absorbance at 520 nm was measured spectrophotometrically and compared to a standard of glucosamine.

3.2.10 Structural studies of the cell wall component mannan isolated from C. albicans biofilm cells

C. albicans biofilm from stress treatments was centrifuged (10000×g for 10 min at 4 °C) after 48 h of incubation and the cell pellet was suspended in PBS (pH 7.2). Mannans were extracted from the cells as described previously (Sen et al. 2011) with water at 120 ºC in an autoclave for 3 h. The supernatants were recovered after centrifugation and treated with equal volume of Fehling's reagent which forms copper-mannan precipitate complex. The copper-mannan complex was dissolved in 3 N HCl and further precipitated drop-wise in Methanol: Acetic acid (6:1) solvent. The precipitate was dried, dissolved in water, purified and then lyophilized till further use. The purified mannan obtained from above mentioned procedure was analyzed

using a Fourier transform infrared (FT-IR) spectrometer (Thermo Nicolet NEXUS, Maryland, USA) by KBr pellet technique. The spectrum was taken in the frequency range of 500 cm^{-1} to 4000 cm^{-1} at a 4 cm^{-1} resolution. Each final spectrum was the average of 40 scans and data was obtained with OMNIC software. Nuclear magnetic resonance (NMR) spectroscopic analysis of purified mannans was performed with Bruker Avance 500 MHz spectrometer equipped with Z-gradient triple resonance probe (TXI). All ^{1}H NMR spectra were recorded for a solution sample of mannan in 600 µL of D_2O at 35 °C using pulse program zg30 with spectral width of 20 ppm and 256 scans. 0.1 µL of 0.1 M 3-(trimethylsilyl) propionic-2, 2, 3, 3-d_4 acid sodium salt (TSP) was used as internal reference. All spectra were processed and analyzed using Topsin (version 1.3, Bruker) program package.

3.2.11 Statistical analysis

Three independent experiments were conducted in duplicate and data was expressed as mean ± standard deviation. Data obtained were evaluated by analysis of variance (ANOVA) followed by post hoc Tukey's honestly significant difference (HSD) test for pair-wise comparisons using XLSTAT statistical add-in software for Microsoft Excel© and OriginPro® 8. Letters on the histogram provides the graphical representation for post hoc pair-wise comparisons, (Tukey's HSD). Means sharing the same letter is not significantly different from each other. In all evaluations, p value less than or equal to 0.05 were considered significant.

3.3 Results

3.3.1 Osmotic and oxidative stress increases biofilm formation and matrix production

A time course study (6, 12, 24, 48, 72 h) of metabolic activity of *C. albicans* biofilm cells developed in the presence of oxidative and osmotic stress revealed significant differences ($p < 0.01$) in biofilm formation compared to control (Fig. 3.1a,b). XTT assay demonstrated that the osmotic stress induced biofilm formation in *C. albicans* with gradual increase in metabolic activity. Data showed that under oxidative stress the initial growth of *C. albicans* was slow but a well-established biofilm was formed after 24 h. In control experiments, *C. albicans* adhered to polystyrene plate with lower metabolic activity compared to both stress conditions. Data revealed that under the influence of osmotic stress, *C. albicans* secreted highest amount of polysaccharides and protein (Fig. 3.2a, b). Significant difference in polysaccharide content was observed in osmotic stress treated samples ($p < 0.001$). However, no significant difference was observed between control (12 µg mg^{-1} biomass) and oxidative stress condition (15 µg mg^{-1} biomass) during total protein content estimation while in the presence of osmotic stress the total

protein content was found to be 18 µg mg^{-1} biomass. Interestingly, eDNA content was highest under oxidative stress treatment as compared to control. The architecture of *C. albicans* biofilm topology visualized under SEM depicted extensive biofilm with an amorphous extracellular matrix enclosing yeast cells and germ tubes in the presence of osmotic stress compared to oxidative stress and control (Fig. 3.3).

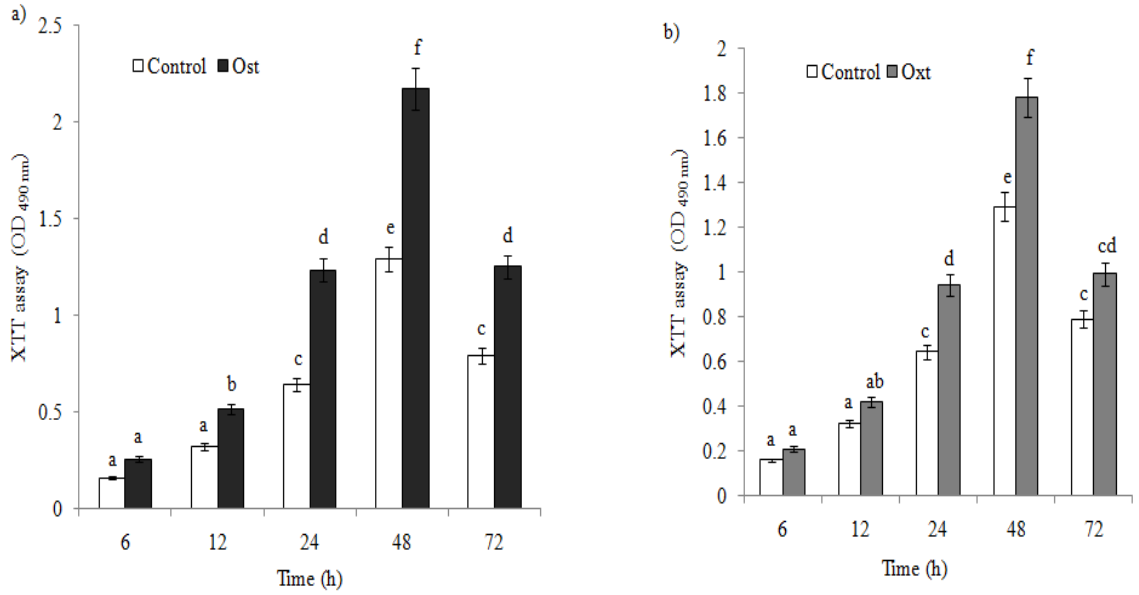


Figure 3.1 Effect of osmotic and oxidative stress on *C. albicans* biofilm formation. Metabolic activity of *C. albicans* biofilms developed in the presence of a) osmotic stress (Ost) b) oxidative stress (Oxt). Data represent the means ± standard deviations of three independent experiments. Letters on the histogram provides the graphical representation for posthoc pair-wise comparisons, (Tukey's HSD, $p < 0.05$). Means sharing the same letter are not significantly different from each other.

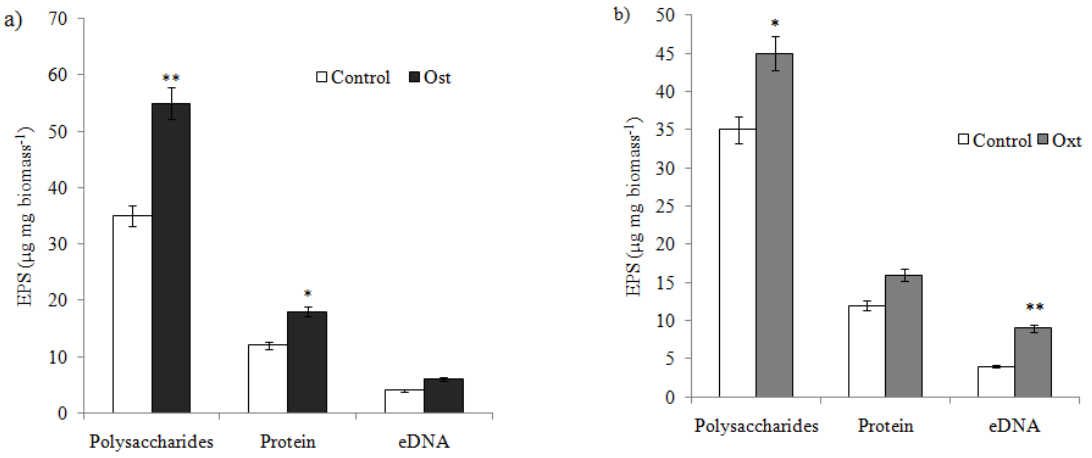


Figure 3.2 The influence of osmotic and oxidative stress on matrix polymers production in *C. albicans* biofilm. Quantification of extracellular polymeric substances (EPS) secreted in *C. albicans* biofilm under a) osmotic stress (Ost) b) oxidative stress (Oxt). Data represent the means ± standard deviations of three independent experiments (**p<0.001, * p<0.05).

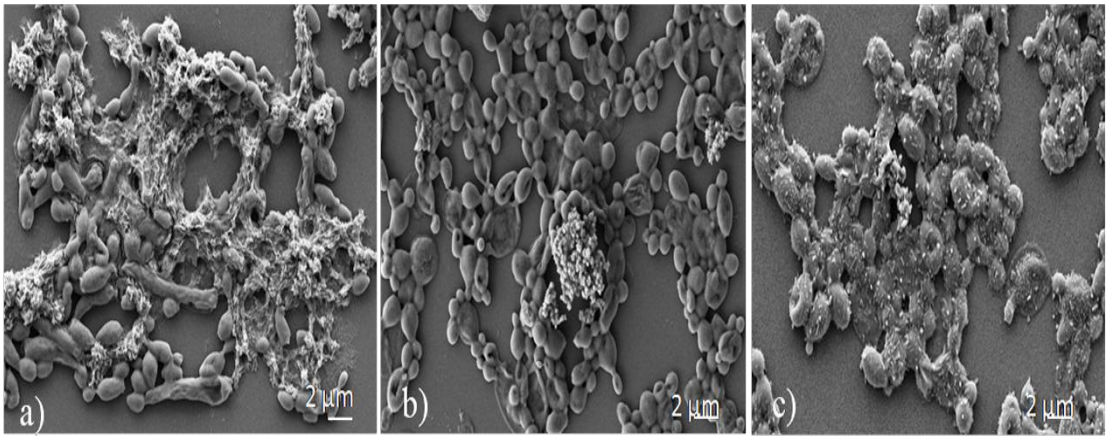


Figure 3.3 Scanning electron microscopy. Micrographic images of *C. albicans* biofilm developed on a) Osmotic stress b) Oxidative stress c) Control. Images were captured at 5000 X magnification and bar represents 2 µm.

3.3.2 Stress conditions regulates virulence traits and alters chitin levels

Significant differences were observed in phospholipase and proteinase activity of *C. albicans* biofilm grown under stress conditions (oxidative and osmotic) as compared to control (Fig. 3.4a, b). The level of intracellular ROS accumulated in the presence of 5 mM H_2O_2 was recorded highest at 4 h and found to decrease gradually over time, reaching minimum levels after 48 h of *C. albicans* biofilm development (Fig. 3.5a). Results showed that ROS levels in control remained stable during the time course. ROS accumulation demonstrated a damaging effect on cells as the fluorescence intensity of PI that binds to DNA of dead cells increased substantially (Fig. 3.5b). Fluorescence microscopic images also demonstrated green (ROS) and red fluorescence (dead cells) in cells exposed to oxidative stress (Fig. 3.5c). The level of catalase activity observed to be higher at 4 h in biofilm under oxidative stress and decreased over time (Fig. 3.5d). The catalase activity remained steady throughout biofilm development in control. Chitin content 56.21 µg mg^{-1} and 39.6 µg mg^{-1} of the cell wall was recorded from *C. albicans* grown under oxidative and osmotic stress conditions respectively as compared to 27.3 µg mg^{-1} of the cell wall control (without stress) after 48 h.

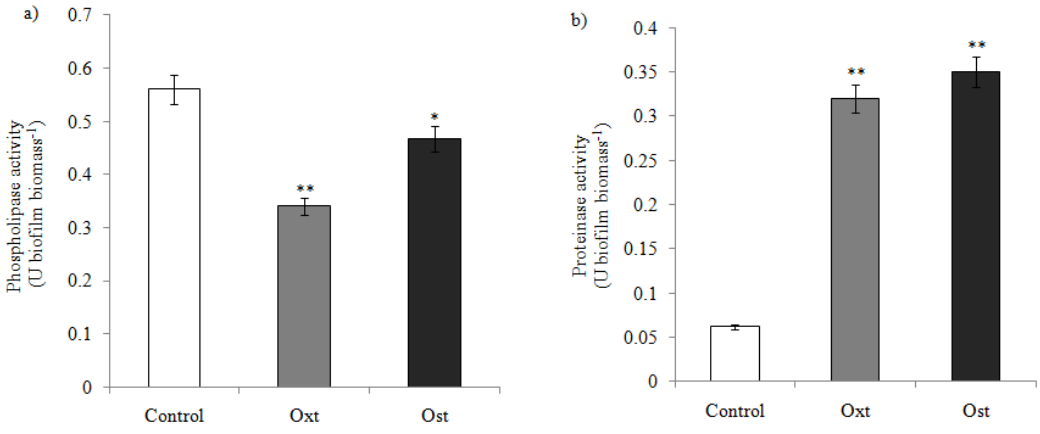


Figure 3.4 Assay of virulence factors. a) The specific activity of enzyme phospholipase in *C. albicans* biofilms was estimated at 630 nm after 72 h. b) The secreted aspartyl proteinase activity in *C. albicans* biofilms was measured at 440 nm after 72 h. Data represent means ± standard deviations of three independent experiments (**p<0.001, * p<0.05).

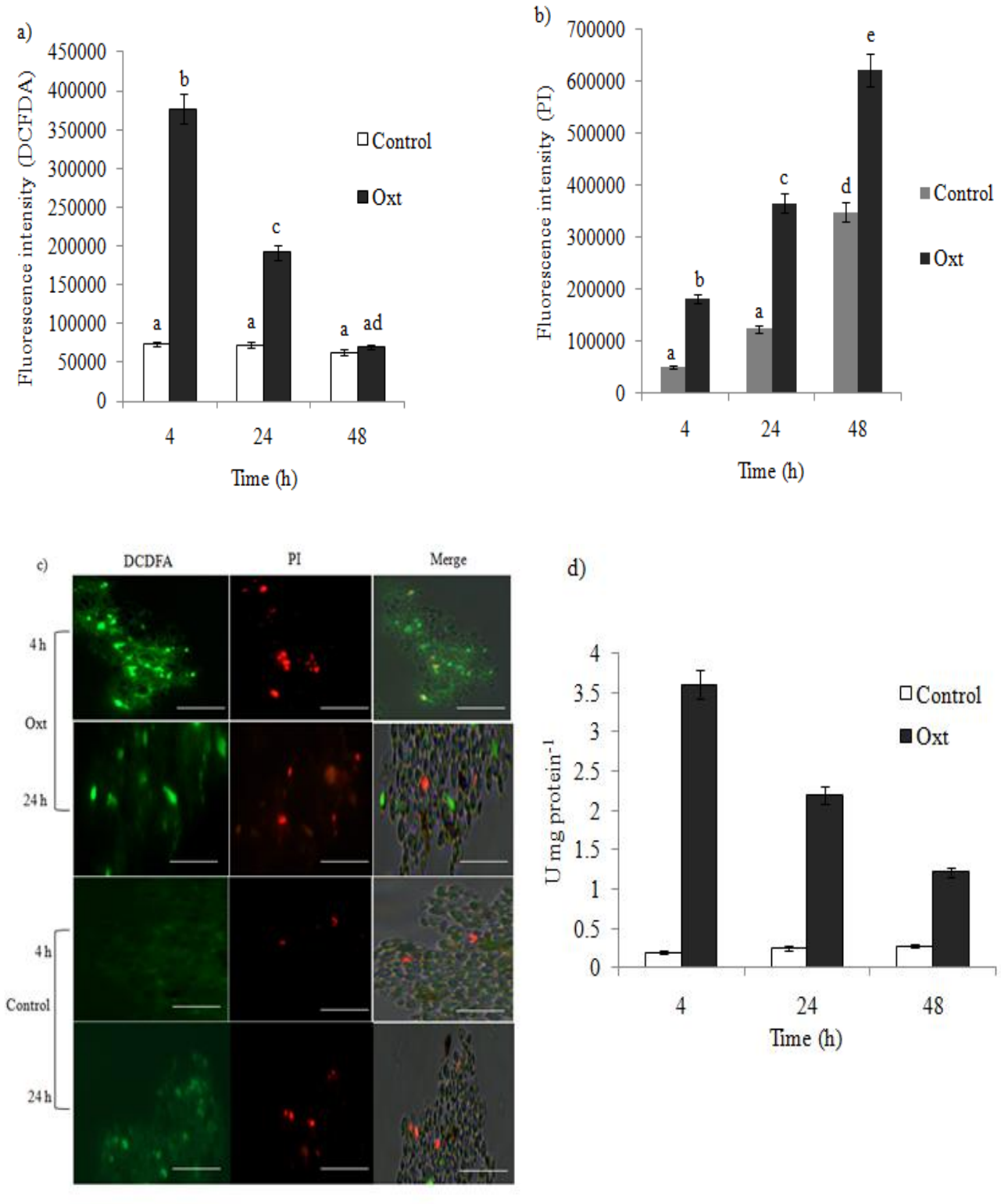


Figure 3.5 Level of oxidative stress (Oxt). a) Intracellular ROS accumulation b) Fluorescence intensity of PI c) Fluorescence microscopic images of *C. albicans* biofilm cells (4 h and 24 h) stained for detection of ROS (green), cell lysis (bright red) and eDNA (diffuse red) d) Catalase activity.

3.3.3 Spectral analysis of stress response revealed structural changes in mannan

The FT-IR spectral profile of *C. albicans* cell wall component mannan isolated from biofilm grown under the influence of both oxidative and osmotic stress was observed in the frequency range of 500-4000 cm^{-1} (Fig. 3.6a-c). The spectral vibration between 900 cm^{-1} to 1200 cm^{-1} corresponding to polysaccharides revealed the structural changes in mannan (Fig. 3.6d). The band maximum was observed in a very packed spectral region between 1200 and 1000 cm^{-1} region, which was dominated by ring vibrations and stretching vibrations of (C-OH) side groups. The oxidative and osmotic stress treated samples showed band maximum at 1133 cm^{-1} and 1128 cm^{-1} corresponding to (C-OH) stretching vibrations. The infrared bands at lower frequencies 1051-1039 cm^{-1} represents the glycosidic linkage *ν*(C-O-C). The high absorption intensity band at lower frequency 3435 cm^{-1} represents the ν(OH) stretching. In control, significant difference in absorption intensity of mannan spectra was observed when compared to spectra of both stress conditions. The absorption intensity of a characteristic band of mannan at 1045 cm^{-1} (C-O-C) was higher in control as compared to both oxidative and osmotic stress conditions. The spectral shape with diminished bands at about 1066 and 1008 cm^{-1} were observed in oxidative stress. The absorption bands in anomeric region at 834 cm^{-1} was assigned to α-linkage and 898 cm^{-1} for β-linkage. In oxidative stress conditions, α-linked mannose units were predominant. The intensity of β-linked mannose units was slightly increased than α-linked units under the influence of osmotic stress. Similar results were also observed in the ^{1}H NMR spectrum of oxidative and osmotic treated *C. albicans* mannan (Fig. 3.7). The peaks observed between 3.2 ppm-4.5 ppm indicates signal from H2, H3, H4 and H5 protons of mannose moiety. The H1 proton gave signals between 4.9 ppm-5.6 ppm (α- anomeric region) and 4.7 ppm-4.9 ppm (β- anomeric region). In oxidative stress conditions, peaks observed at 4.94 ppm, 5.23 ppm, 5.13 ppm suggests the existence of α-linked mannose moieties (Fig. 3.7a). The peaks observed at 5.35 ppm and 4.91 ppm in osmotic stress treated mannan were assigned to α-linked and latter to β- linked mannose residues (Fig. 3.7b). Control showed signals at 5.53 ppm, 4.89 ppm and 5.12 ppm. The two former signals correspond to β-linked mannose residues and the later signal to the α-linked mannose moieties. The signal at 5.53 ppm in control corresponds to β-1,2 branched mannose units attached to through phosphodiester moiety which was absent in both oxidative and osmotic stress conditions (Fig. 3.7c).

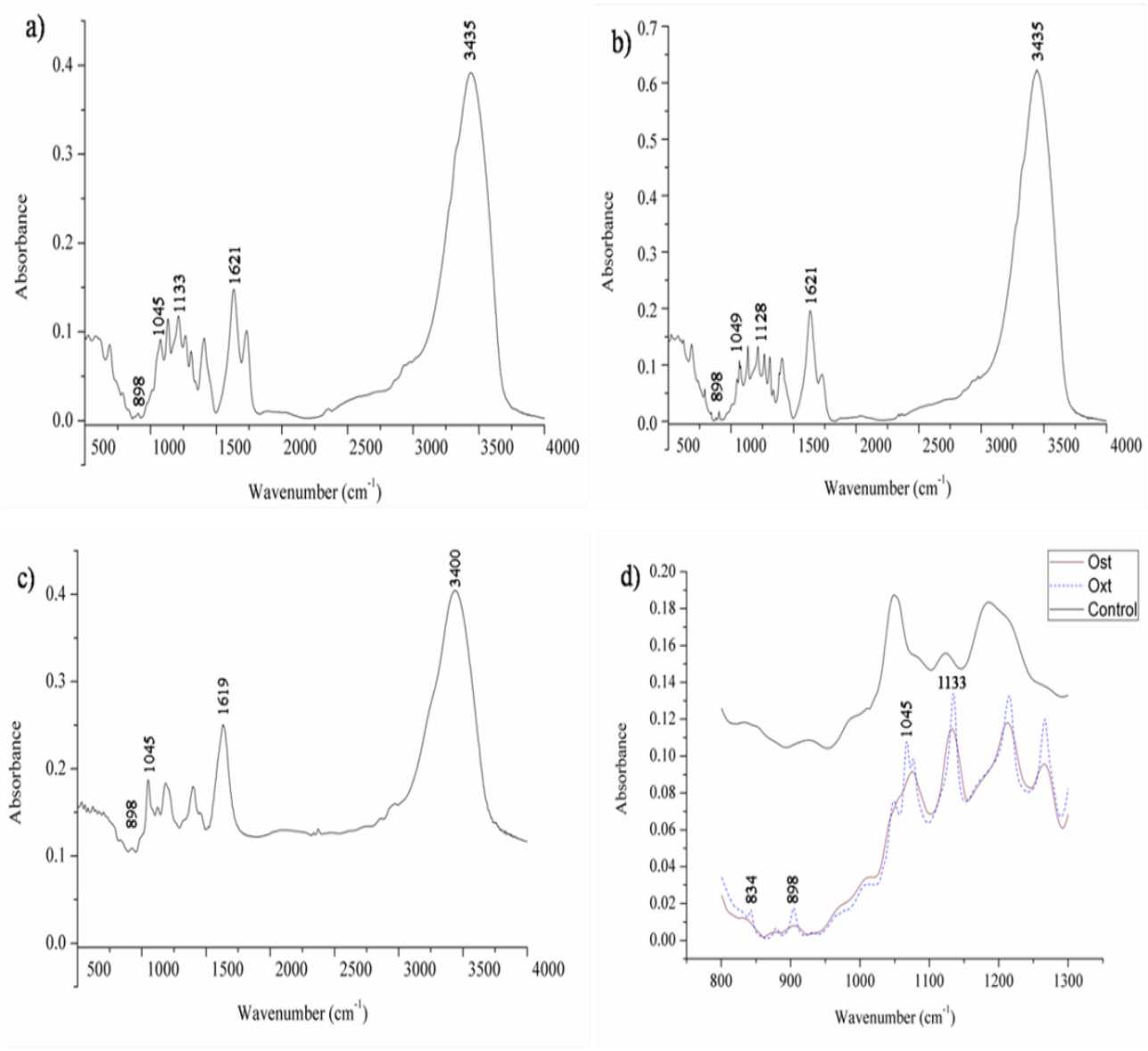
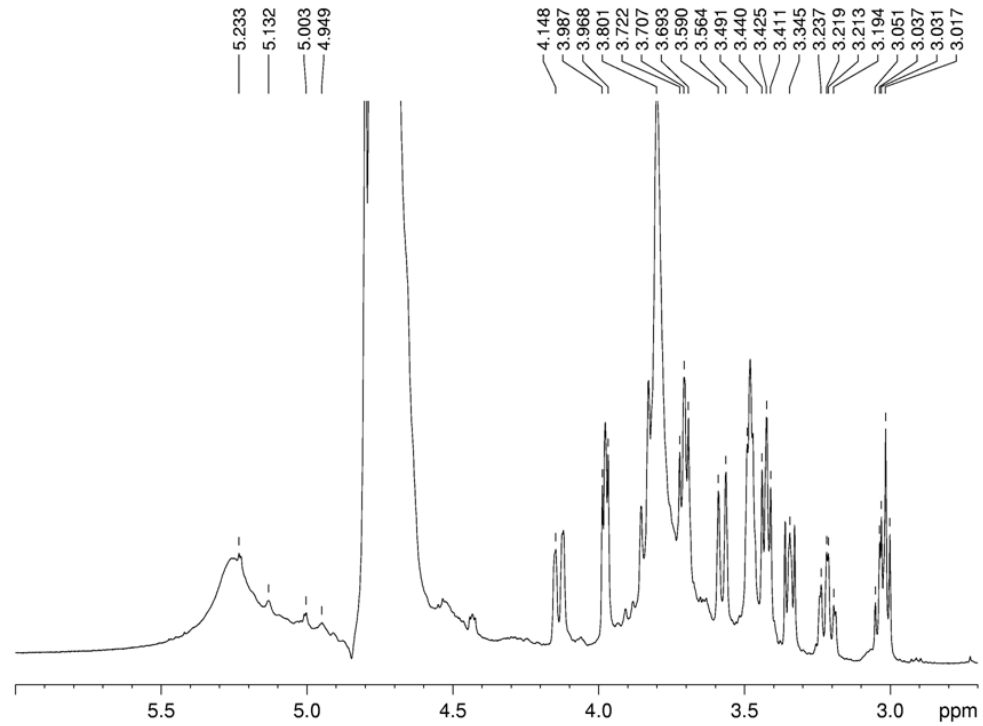


Figure 3.6 FT-IR spectra of mannan. The cell wall component mannan was isolated from *C. albicans* biofilm cells grown under a) Osmotic stress (Ost) b) Oxidative stress (Oxt) c) Control and d) Spectra in the frequency region of 800-1300 cm^{-1} that corresponds to polysaccharides.

a)


5.233
5.132
5.003
4.949
4.148
3.987
3.968
3.801
3.722
3.707
3.693
3.590
3.564
3.491
3.440
3.425
3.411
3.345
3.237
3.219
3.213
3.194
3.051
3.037
3.031
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5.5
5.0
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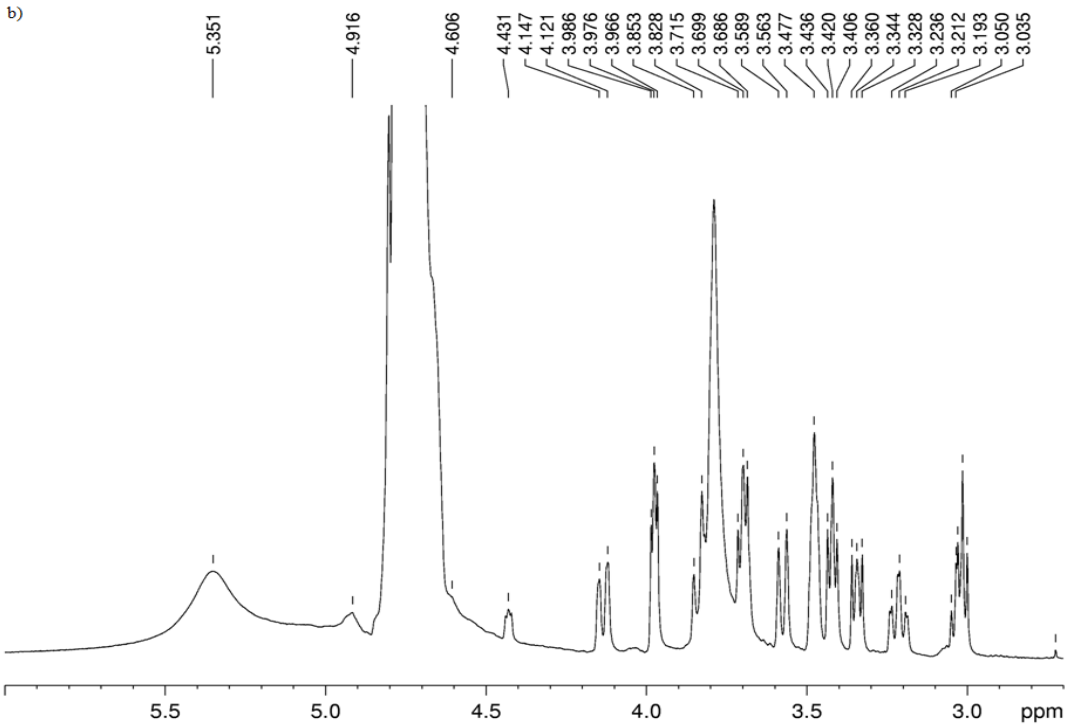
3.0
ppm

b)


5.351
4.916
4.606
4.431
4.147
4.121
3.986
3.976
3.966
3.853
3.828
3.715
3.699
3.686
3.589
3.563
3.477
3.436
3.420
3.406
3.360
3.344
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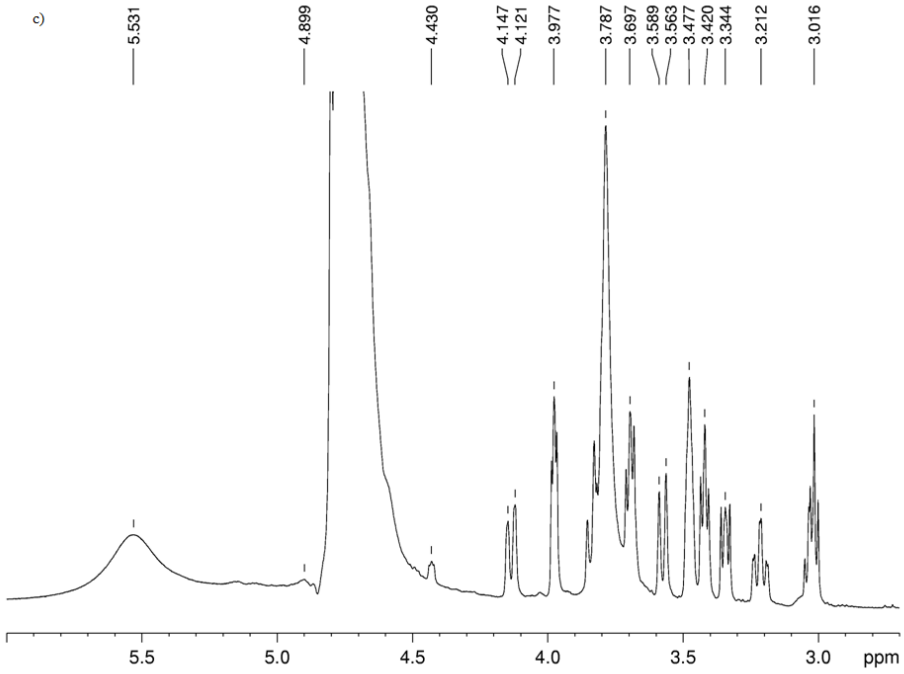

Figure 3.7 ^{1}H NMR spectra of mannan isolated from the cell wall of *C. albicans* biofilm cells. a) Oxidative stress b) Osmotic stress c) Control.

3.4 Discussion

The robust stress response of *C. albicans* contributes to its survival and virulence by facilitating the adaptation to changing host environmental factors. The conditions for oxidative and osmotic stress were optimised by investigating the *C. albicans* growth under different conditions of NaCl and H_2O_2 according to the previous investigations (Li et al. 2004; Koyama et al. 2009). This study highlighted the cellular response of *C. albicans* and adaptive strategies to combat these stresses. Significant observations were recorded in secretion of virulence factors (proteinase and phospholipase) and content of the cell wall components chitin and mannan. Data demonstrated that exogenous stress conditions, H_2O_2 and NaCl increased *C. albicans* biofilm formation and promoted surface-associated sessile behaviour with rich extracellular polymeric matrix. The response of *C. albicans* to the stresses indicates that adverse environmental conditions induce biofilm formation with maximum secretion of exopolymeric substances to protect the cells. These results are in consistence with the earlier reports of biofilm formation in the presence of stress conditions observed in bacterial biofilm models (Zhang et al. 2007; Villa et al. 2012; Moryl et al. 2014). Results obtained suggested that *C. albicans* responded to osmotic stress by producing maximum polysaccharide and protein

secretion into matrix, whereas H_2O_2 induced sub-lethal oxidative stress and enhanced eDNA content during *C. albicans* biofilm formation.

In this investigation, oxidative stress (5 mM H_2O_2) generated high levels of ROS and cell lysis at early stages of biofilm formation followed by its significant decline. These results were correlated with release of eDNA in *C. albicans* biofilm under oxidative stress compared to control while catalase activity decreased substantially in these cells. Phagocytic cells of the innate immune system are the first line of defence against microbial infections generating ROS to destroy the pathogens (Arana et al. 2007). To counter this, biofilm formation is one of the immune evasion strategies adopted by *C. albicans* (Xie et al. 2012). Although cell lysis is supposed to be disadvantageous for individual cell, but in biofilm communities it is believed to be an essential process as it leads to release of DNA into the extracellular medium (Čáp et al. 2012). Thus, the release of eDNA to ROS might be an adaptation of *C. albicans* to oxidative stress as it maintains biofilm integrity. In addition, the results indicated that tolerance of *C. albicans* to oxidative stress increased with the biofilm development and not with the scavenging activity of catalase *per se*. This adaptive mechanism to survive in the presence of H_2O_2/ROS generated due to oxidative stress is not surprising for highly successful opportunistic pathogen like *C. albicans*. Recent literature on biofilm also suggests that eDNA production happens because of cell lysis via H_2O_2 generation (Das & Manefield 2012). For successful tissue colonization, invasion and dissemination inside the host, extracellular hydrolytic enzymes are required for *C. albicans* (Ghannoum 2000; Naglik et al. 2003). Results showed that cells exposed to oxidative stress produced less extracellular phospholipase that effectively degrades cell membrane components, but proteinase production required for tissue invasion exceeded that of control. Fekete et al. (2007) reported the high aspartic proteinase production and low extracellular phospholipase in *C. albicans* AF06 mutant (oxidative tolerant) than its wild-type *C. albicans* ATCC 14053. Similarly, this investigation showed a decreased extracellular phospholipase production in *C. albicans* biofilm formed under osmotic stress. These results are in line with the previous findings which indicated significant reduction in enzyme activities due to an adaptive stress response (Lórenz-Fonfría & Padrós 2005; Fradin et al. 2005). The cell wall of *C. albicans* is dynamic and its response to changes in the environment plays a crucial role in host-pathogen interactions. Many stresses affect the membrane composition and fluidity, which could in turn affect the cell wall composition. Thus, the elevated chitin levels may be more likely a potential resistance mechanism or tolerance against deleterious effects of oxidative stress. Fungi respond to damaged cell walls by increasing the chitin levels to strengthen the weakened cell wall and to maintain the cell wall

integrity (Heilmann et al. 2013). Structural analysis by FT-IR and NMR revealed that the content of mannan was decreased under both osmotic and oxidative stress conditions. Besides this, the β-linked mannose units were also reduced in cells exposed to stress. These findings corroborate with the previous observation that osmotic and oxidative stresses induce structural changes in the β-1,2-linked mannose side chains (Koyama et al. 2009). Mannan is known to be a significant component of *C. albicans* with potential virulence and antigenic properties (Nelson et al. 1991). Data suggests that the adaptability of *C. albicans* to oxidative and osmotic stresses by alteration in mannan structure might alter the antigenic variability useful for identification of the species. This may lead to delay in rapid and early diagnosis of *C. albicans* mediated infections. Even though the changes identified in mannan oligosaccharide sub-units were subtle, these alterations might increase resistance to cell-mediated immunity and persistence of infection.

In conclusion, the obtained results highlight the response by *C. albicans* towards oxidative and osmotic stress promoted surface-associated growth by biofilm genesis, increased levels of polysaccharides and eDNA in the extracellular matrix. Increase in chitin levels and decrease in virulence traits accompanied with the changes in the mannan structure might affect the innate immune recognition during host-pathogen interaction. These findings suggest that addressing the gap areas in stress inducible biofilm formation could aid in identification of potential targets for the development of novel antifungal therapies.

CHAPTER-4

4. Innate immune secretory factors affect *Candida albicans* biofilm formation

4.1 Background

Oropharyngeal candidiasis is the most common infection seen in immunocompromised people such as HIV patients and individuals with systemic diseases (Powderly et al. 1999). In HIV individuals, salivary gland secretion is severely impaired which contributes to recurrent oral infections. The suppression of the local defences leads to invasive colonization of opportunistic *C. albicans* and other pathogenic microorganisms (Samaranayake & MacFarlane 1990). In human host, a constant flow of saliva is a prime source of many antimicrobial substances such as lactoferrin, lysozyme, salivary peroxidase, antimicrobial peptides, immunoglobulin (secretory IgA) and salivary mucin (Tenovuo et al. 1990). These innate immune defences regulate the quality and quantity of microflora present on mucosal surfaces. Lactoferrin is an iron binding protein present in granules of PMNLs and various exocrine secretions including saliva that has antimicrobial activity (Gupta & Satyanarayana 2002; Anil & Samaranayake 2002). Lysozyme is secreted into saliva by the gingival tissue and gingival the crevicular fluid of salivary glands (Tenovuo et al. 1990). *In vitro* studies reported the antibacterial and anti-candidal activity of lysozyme (Iacono et al. 1983; Iacono et al. 1985). Mucin is the main glycoprotein of mucus lining the inner cavities of trachea, stomach and intestine (Loomis et al. 1987). Salivary mucins demonstrated antibacterial and antifungal activities by suppressing cellular processes associated to virulence (Kavanaugh et al. 2014). *N*-acetylglucosamine (GlcNAc) which is an inducer of morphogenesis in *C. albicans* is present covalently linked to mucin (Cassone et al. 1985). Earlier it has been reported that oral bacteria secrete enzyme *N*-acetyl-D-glucosaminidase that catalyses the breakdown of GlcNAc from the oligosaccharide portion of glycoproteins (Beighton et al. 1988). It is reported that *S. mutans* ferments GlcNAc found freely in saliva and several oral bacteria also utilizes GlcNAc as carbon or nitrogen source under starvation conditions (Homer & Beighton 1992).

In addition to the innate immune factors, many host molecules potentiate cell-to-cell communication as danger signals in response to tissue damage (Dando & Roper 2009). Adenosine triphosphate (ATP) a decisive enzyme cofactor in cellular metabolism and a reservoir of the intracellular energy also signal ‘danger’ as a powerful extracellular messenger

(eATP) via purinergic signaling (Bours et al. 2006). ATP can be included into the restricted family of damage-associated molecular patterns (DAMPs) that signal danger to the immune system. eATP with purinergic receptors on immune cells induce inflammation, intracellular pathogen killing by apoptosis, cell adhesion and chemo attraction in response to tissue injury or stress (Cruz et al. 2007). The role of eATP as a host derived signal and its protective advantage to eukaryotic host has been well established (Burnstock 2006; Bayliss et al. 2014). In addition, eATP has also shown to stimulate biofilm formation in bacterial strains namely *S. aureus*, *A. baumannii*, *E. coli* and *S. maltophilia* (Xi & Wu 2010). There is no data reported to the best of our knowledge about how opportunistic yeast *C. albicans* responds to the danger signal dATP/ATP. It is believed that there exists a dynamic interplay between the activation of immune responses and the ability of the pathogen to modulate these responses. Here, we investigate the response of *C. albicans* to the innate immune secretory factors such as mucin, lactoferrin, lysozyme and dATP during biofilm formation.

4.2 Materials and Methods

4.2.1 Strain and growth conditions

C. albicans MTCC 227 (equivalent to reference strain ATCC 10231) was grown on YPD plate (1% peptone, 1% yeast extract, 2% dextrose, 1.5% agar) for 24 h at 37 ºC as elaborated earlier in Chapter-3 (sub-section 3.2.1).

4.2.2 Biofilm formation and quantification

A stock solution of 1000 µM dNTP's (dATP, dCTP, DTTP, dGTP) were prepared in sterile PBS (pH 7.2), stored at 4 ºC and used within a week. For biofilm formation, 100 µL of the standard cell suspensions of *C. albicans* (1×10^7 cells mL^{-1}) in RPMI-1640 medium was seeded into presterilised 96-well polystyrene microtiter plate wells for 90 min at 37 ºC (adhesion phase). Afterwards, wells were washed twice with sterile PBS to remove loosely adhered cells. RPMI-1640 medium supplemented with different concentrations of dATP/ and other dNTP's (100-500 µM) were added to wells. Plates were incubated at 37 ºC up to 48 h. After incubation, biofilm cells were carefully washed twice with PBS without disrupting the biofilm integrity. Biofilm formation was quantified by XTT reduction assay as described earlier in Chapter-2 (sub-section 2.2.5).

4.2.3 Extraction and quantification of polysaccharides and eDNA from C. albicans biofilms

The total polysaccharides content of *C. albicans* biofilm was measured by phenol-sulphuric acid method with glucose as a standard (DuBois et al. 1956) as briefed earlier in Chapter-3 (sub-section 3.2.5). To determine the total eDNA content, protocol of Allesen-Holm et al. 2006 was followed as described in Chapter-3 (sub-section 3.2.5).

4.2.4 Scanning electron microscopy

To observe *C. albicans* biofilm morphology and architecture, scanning electron microscopy was performed by placing a small drop of suspension from *C. albicans* biofilm developed in the presence of 500 µM eATP after 48 h of incubation on a sterile glass surface. The sample fixation was done as described earlier in Chapter-3 (sub-section 3.2.4).

4.2.5 Measurement of ROS in dATP treated biofilms

The level of intracellular ROS generated in the presence of dATP was determined by fluorescent probe 2′,7′- dichlorodihydrofluorescein diacetate (DCFDA) and propidium iodide (PI) using fluorescence spectrometer (Fluorolog, Horiba Jobin Yvon). *C. albicans* cells were incubated with dATP (500 µM) up to 4 h and the fluorescent intensity was measured as described earlier in Chapter-3, (sub-section 3.2.7). The viability of cells treated with dATP were assessed by diluting the cells (10^5) in series and plated on YPD medium followed by incubation at 37 ºC for 24 h.

4.2.6 Effect of lactoferrin and lysozyme

A stock solution of 10 mg mL^{-1} Hen-egg-white lysozyme (Himedia, India) and Lactoferrin (Sigma, India) were prepared in sterile distilled water separately, stored at 4 ºC and consumed with one week. *C. albicans* MTCC 227 was grown in RPMI-1640 medium containing different concentrations (0-8 mg mL^{-1}) of the above stock solutions at 37 ºC for 24 h in 96-well microtiter plates separately. In addition to this, the effect of lactoferrin and lysozyme were also tested on the 24 h grown preformed *C. albicans* biofilm.

4.2.7 Effect of mucin

Mucin type III from porcine stomach (Himedia, India) was dissolved as per manufacturer's instructions. GlcNAc was dissolved in water, autoclaved before each experiment and used. To study the effect of mucin and GlcNAc on *C. albicans* biofilm formation and extracellular hydrolytic enzymes secretion, 1% mucin and 1% GlcNAc solution were added separately to

wells containing standard suspension of *C. albicans* (100 µL of 1×10^7 cells mL^{-1}) in YNB medium and incubated up to 72 h at 37 ºC with periodic replacement of growth medium.

4.2.8 Statistical analysis

Three independent experiments were conducted in duplicate and data was expressed as mean ± standard deviation. Data obtained were evaluated with Student's t-test using XLSTAT statistical add-on software for Microsoft Excel©. In all evaluations, *p* value less than or equal to 0.05 were considered statistically significant.

4.3 Results

4.3.1 eATP stimulates biofilm formation in C. albicans

The effect of eATP on *C. albicans* biofilm formation was recorded using polystyrene MTP by XTT reduction assay. Xi and Wu (2010) reported that physiological concentration (400 µM) of eATP stimulates biofilm formation in bacterial models, which is in line with the eATP concentration tested in the present study. Results demonstrated that the metabolic activity of *C. albicans* in biofilms was significantly increased at 500 µM eATP concentration while, the ATP hydrolyzing enzyme apyrase reduced the biofilm development (Fig. 4.1a). These results demonstrate that addition of eATP stimulates biofilm formation in dose dependent manner. It was observed that the extracellular polysaccharide secretion was enhanced in the presence of eATP (Fig. 4.1b). SEM images revealed that eATP at 500 µM concentration promoted cell aggregation and extracellular matrix deposition (Fig. 4.1c).

4.3.2 Effect of other extracellular nucleotides on C. albicans biofilm

To determine whether the observed increase in biofilm formation was specific to eATP or not, the effect of dCTP, dTTP and dGTP on C. albicans biofilm formation was also tested. Interestingly, no stimulatory effect on biofilm formation was observed from nucleotides dCTP and dGTP while dTTP showed marginal increase in biofilm formation as compared to control (Fig. 4.2).

4.3.3 eATP induces biofilm formation through eDNA release

To test the effect of eATP on eDNA release, *C. albicans* was cultured in the presence of different concentrations (100-500 µM) of eATP for 48 h on MTPs. *C. albicans* significantly released more eDNA in the presence of eATP (Fig.4.3). These results also indicate a dose dependent relationship between eATP concentration and eDNA release into biofilm matrix.

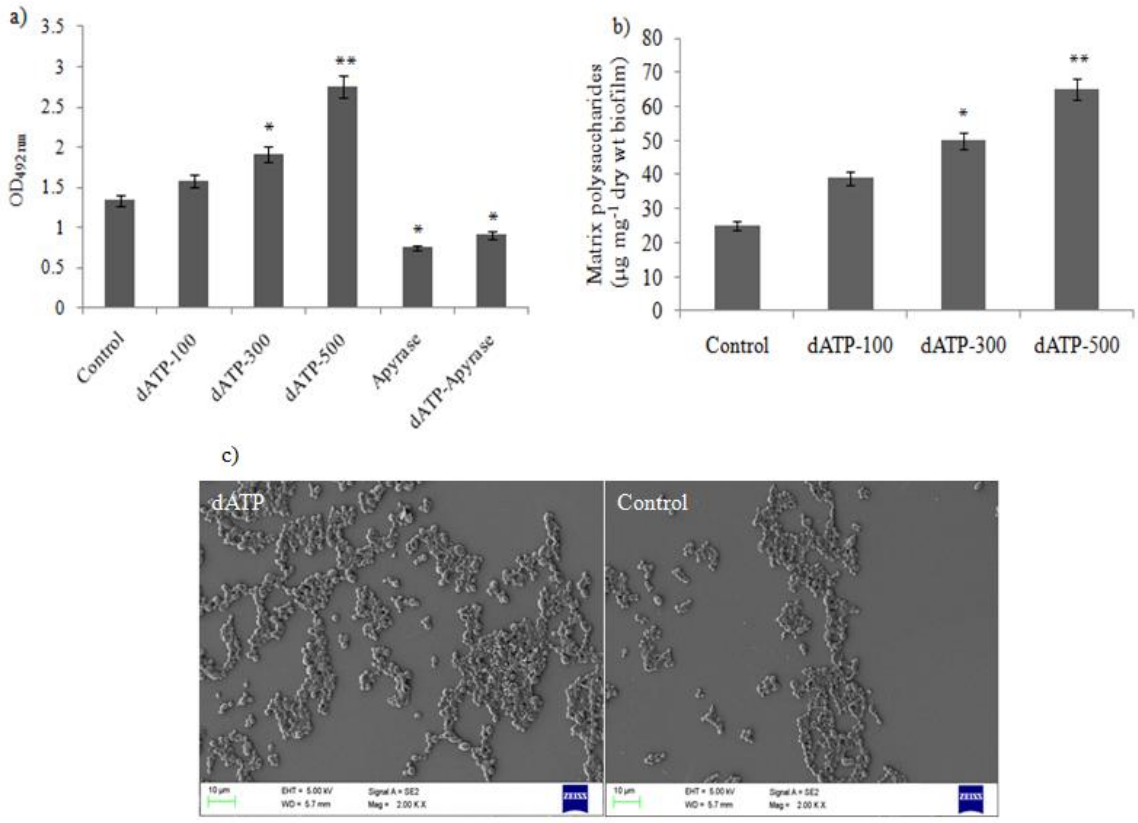

Figure 4.1 Quantification of biofilm developed by *C. albicans*. a) Biofilms developed were examined after 48 h of incubation in RPMI-1640 medium supplemented with different test agents: without any test agent (control); dATP (different concentration 100-500 µM); Apyrase (200 mU mL^{-1}); dATP (500 µM) and apyrase (200 mU mL^{-1}). Biofilms were quantified by XTT reduction assay. b) Quantification of matrix polysaccharides secreted in *C. albicans* biofilm. Data represent the means ± the SD of three independent measurements (Student's t-test **$p<0.001$; *$p<0.05$ compared to control). c) Scanning electron microscopic images of *C. albicans* biofilm developed in the presence of eATP/dATP at 500 µM concentration and without dATP (control).

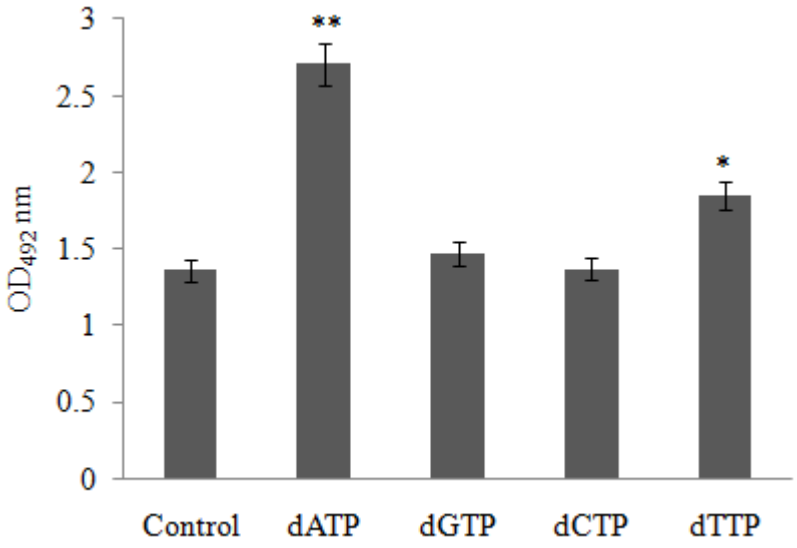

Figure 4.2 The effect of other dNTPs on *C. albicans* biofilm formation. *C. albicans* biofilms developed in the presence of 500 µM dGTP, dTTP, dCTP were quantified after 48 h of incubation by XTT reduction assay. Data represent the means ± the SD of three independent measurements (Student's t-test **p<0.001; *p<0.05 compared to control).

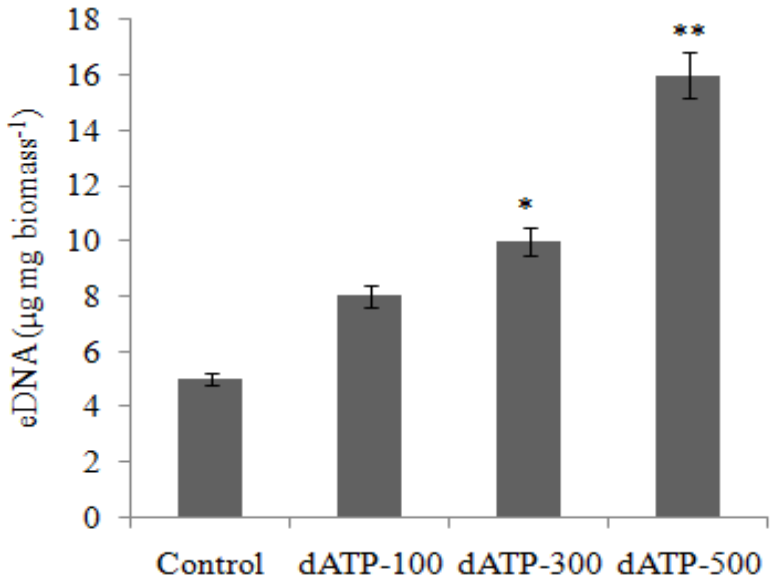

Figure 4.3 Influence of dATP on extracellular DNA production. Quantification of eDNA secreted in *C. albicans* biofilm. Data represent the means ± the SD of three independent measurements (Student's t-test **p<0.001; *p<0.05 compared to control).

4.3.4 eATP mediated ROS accumulation and cell lysis

A fluorescence spectroscopic assay for ROS quantification using a fluorescent probe DCHCF revealed ROS generation in the presence of 500 µM eATP (Fig. 4.4a). To investigate the relation between ROS generation and cell lysis, florescent dye propidium iodide that binds to DNA of cells with compromised cell membrane or eDNA was used. The cell viability (%) was also monitored (Fig. 4.4b). The fluorescence intensity of the PI decreased over time than the control reflecting the cell lysis in eATP treated *C. albicans* (Fig. 4.4c).

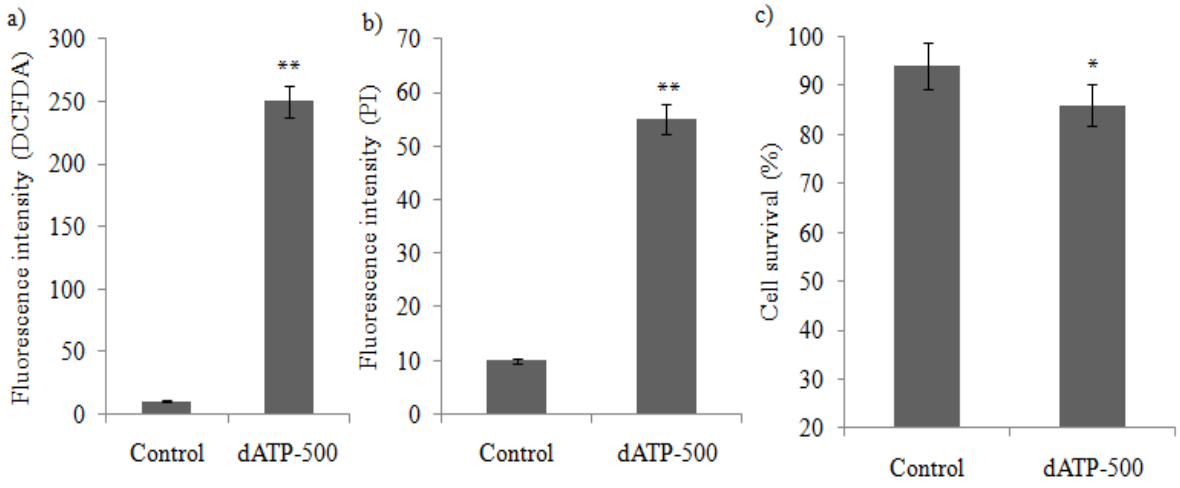

Figure 4.4 ROS accumulation and cell lysis in the presence of eATP/dATP. a) Intracellular ROS accumulation b) Fluorescence intensity of PI c) Cell survival (%) of *C. albicans* (4 h). Data represent the means ± the SD of three independent measurements (Student's t-test **p<0.001; *p<0.05 compared to control).

4.3.5 Lactoferrin and lysozyme affects biofilm formation

Lactoferrin and lysozyme are known for their antifungal activity on *C. albicans*. To test their effect on biofilm formation, *C. albicans* cells were grown in the presence of different concentrations of both these compounds separately. Biofilm formation was suppressed at concentration ≥0.25 mg mL^{-1} lysozyme and ≥0.5 mg mL^{-1} lactoferrin respectively (Fig. 4.5a). A preformed biofilm of *C. albicans* was disrupted only at higher concentrations of lactoferrin and lysozyme (≥2 mg mL^{-1}), while lower concentrations (≤0.65 mg mL^{-1}) of both test agents did not showed any inhibitory activity on biofilm growth (Fig. 4.5b).

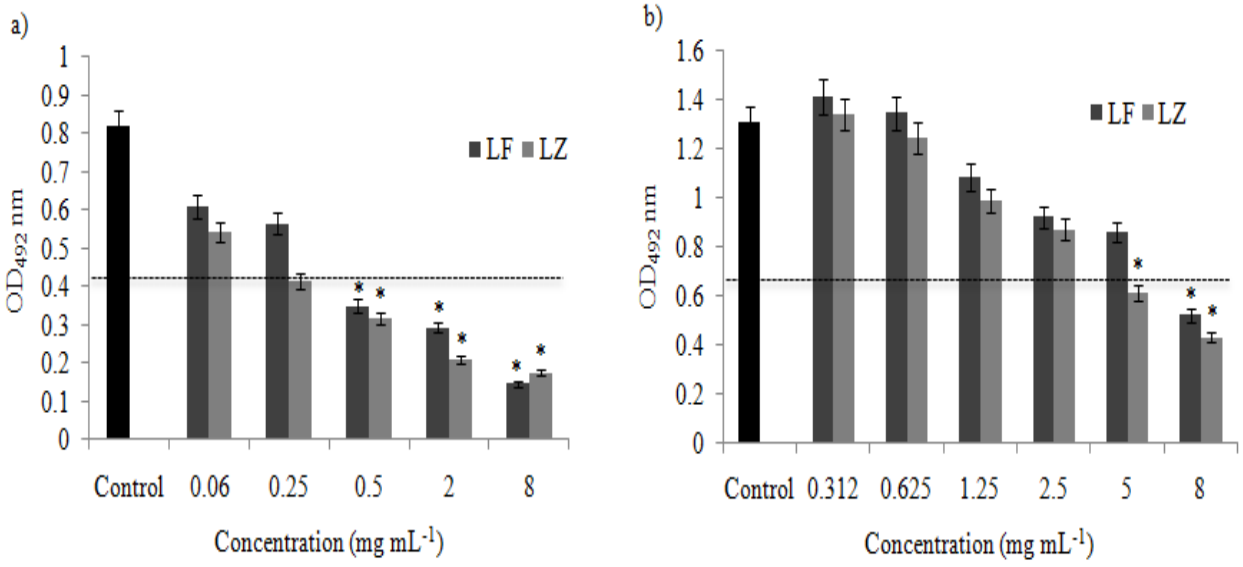

Figure 4.5 Effect of different concentration of lactoferrin (LF) and lysozyme (LZ) on *C. albicans.* a) Biofilm formation b) preformed biofilm (24 h) in polystyrene plate. Data represent the means ± the SD of three independent measurements (Student's t-test **p<0.001; *p<0.05 compared to control).

4.3.6 Mucin suppress biofilm formation and virulence traits in C. albicans

The effect of mucin and GlcNAc on *C. albicans* biofilm formation was evaluated by XTT reduction assay after 48 h of incubation. In the presence of mucin biofilm formation by *C. albicans* was reduced compared to control (Fig. 4.6). On the other hand, *C. albicans* utilized GlcNAc and formed well adhered biofilms in polystyrene wells. Microscopic visualization of cells adhered after 4 h of incubation depicted that mucin relatively reduced the cell attachment to polystyrene surface than GlcNAc (Fig.4.7). Data from extracellular phospholipase and proteinase activity measurements suggests that mucin suppressed the respective enzyme activities compared to control and GlcNAc (Fig.4.8).

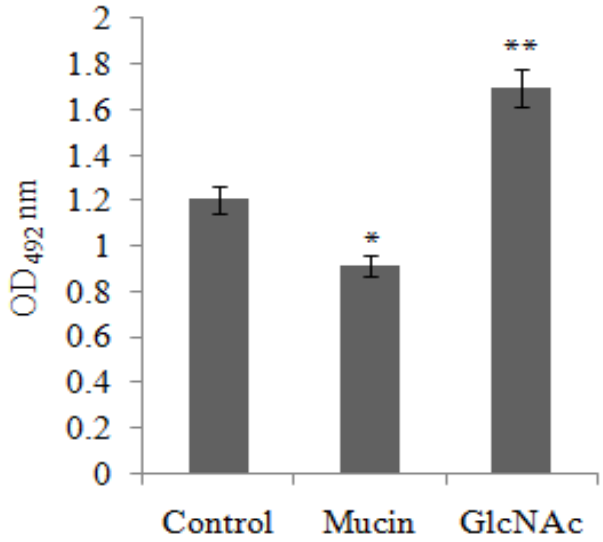

Figure 4.6 Effect of mucin on *C. albicans.* Cells were incubated in the presence of mucin and GlcNAc in a MTP and the biofilm developed after 48 h was quantified by XTT reduction assay. Data represent the means ± the SD of three independent measurements (Student's t-test **p<0.001; *p<0.05 compared to control).

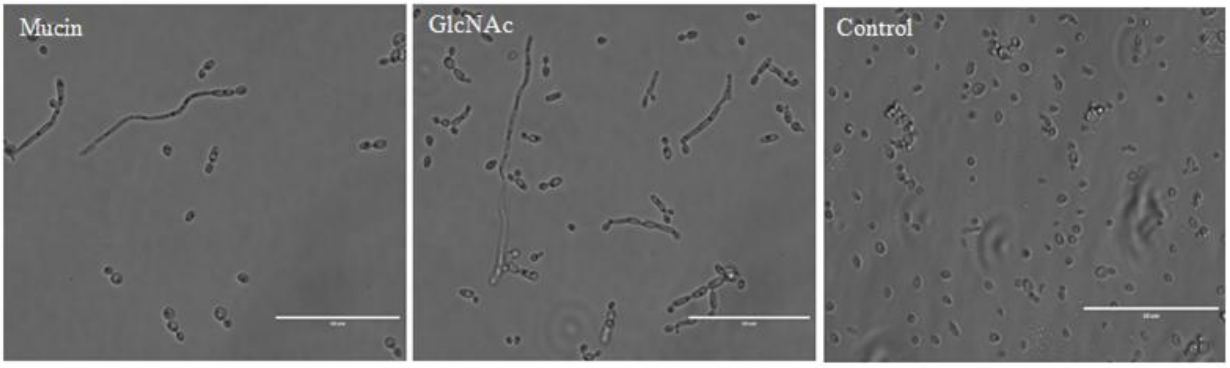

Figure 4.7 Microscopic images of *C. albicans*. Effect of mucin on adhesion of *C. albicans* to MTP was observed by microscopy after 4h of incubation. Images were captured at 60 X magnification and bar represents 50 µm.

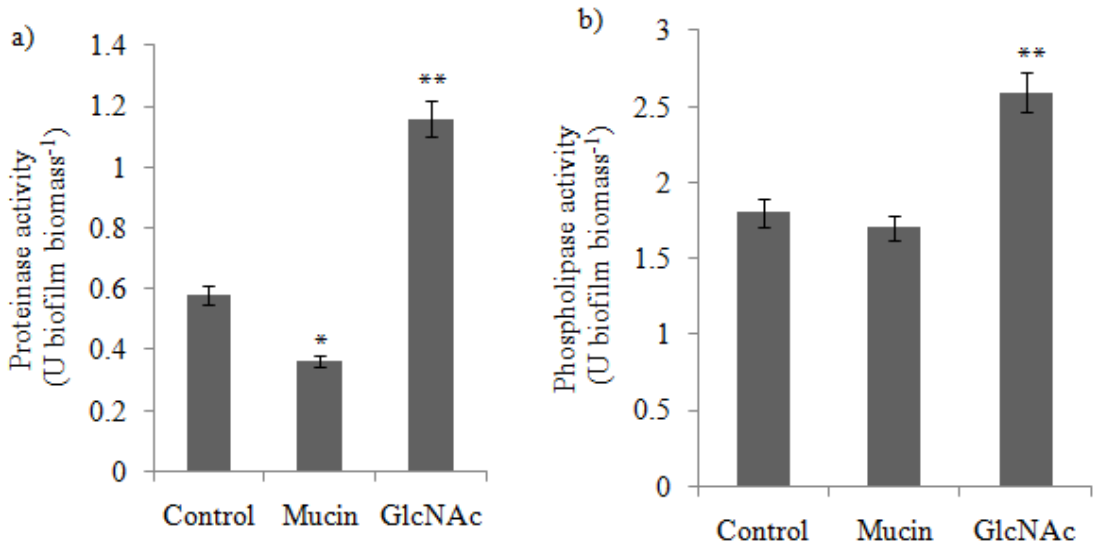

Figure 4.8 Specific activity of extracellular enzymes. a) proteinase activity b) phospholipase in *C. albicans* biofilms after 72 h. Data represent the means ± the SD of three independent measurements (Student's t-test **p<0.001; *p<0.05 compared to control).

4.4 Discussion

C. albicans is a commensal colonizer of mucosal tissues in healthy individuals however, in immunosuppressed individuals, it causes systemic infections. Invading *C. albicans* encounter a complex and intricate host defence system in form of immune cells, cellular receptors, signalling pathways and effector molecules. These defence machineries recognise potential invasive forms of *C. albicans* and eliminate them.

During mucosal infections, there is a high possibility that *C. albicans* damage epithelial cells on mucosal surface. eATP is a signalling molecule which is released (5-10 mM) in response to injury by epithelial cells to alert the host defence system (Di Virgilio 2005). The released eATP is involved in activation of inflammatory cells, chemo-attraction, cell adhesion and killing of intracellular pathogens by apoptotic stimuli in host macrophages (Cruz et al. 2007). Thus, it is likely that *C. albicans* is exposed to high levels of eATP during infection process. In this study, we investigated the response of *C. albicans* in the presence of eATP stimuli under *in vitro* conditions. In *C. albicans*, exogenous addition of ATP/dATP stimulated biofilm formation. Apyrase, an enzyme that catalyses ATP hydrolysis to generate AMP and inorganic phosphate

showed significant decreased in biofilm formation. eATP (500 µM) showed increased polysaccharide secretion which acts as a scaffold to hold biofilm cells. Further, tests found that dATP induces eDNA release into biofilms and observed a link between dATP concentration and amount of eDNA released in a dose dependent manner. In the presence of other dNTPs (dGTP, dCTP and dTTP) similar trend in biofilm formation and eDNA release was not observed. eDNA release occurs via cell lysis of bacterial or fungal subpopulations (Das & Manefield 2012). eATP has been shown to stimulate ROS generation in macrophages and microglia (Parvathenani et al. 2003; Cruz et al. 2007; Noguchi et al. 2008). Based on these facts, attempts were made to validate our hypothesis that eATP through the ROS induces eDNA release in *C. albicans* biofilm. The increase in ROS levels coincided with the cell lysis and cell viability.

Despite cellular lysis, the release of DNA into extracellular matrix is demonstrated to offer greater biofilm stability. Contrary to this, the robust antioxidant enzyme machinery like catalase, glutathione peroxidase, superoxide dismutase reduces the deleterious effect of ROS generated which in turn are responsible for the viable population of biofilm cells.

Oral cavity is a unique niche that *C. albicans* colonizes to causes oral candidiasis in immunocompromised patients (Powderly et al. 1999). Saliva contains various antimicrobial peptides and enzymes that modulate *C. albicans* growth as well as mediate fungicidal activity (Tenovuo et al. 1990). Hence, in the present study the effects of lactoferrin and lysozyme on *C. albicans* biofilm in vitro were explored. It has been reported that lactoferrin inhibits biofilm formation by iron chelating mechanism and cause extracellular leakage of proteins in yeast cells (Nikawa et al. 1993). The antifungal activity of lysozyme may be due to the enzymatic hydrolysis of N-glycosidic bonds that connect polysaccharides and proteins in cell wall as well as damage to enzymatic machinery together may cause subsequent injury to the cell (Anil & Samaranayake 2002). In this study, lactoferrin and lysozyme inhibited the biofilm formation of *C. albicans* in a dose dependent manner. However, similar inhibitory effect of lactoferrin and lysozyme was not observed while treating preformed *C. albicans* biofilms. The higher concentration (> 5 mg mL^{-1}) of lactoferrin and lysozyme were proved to be effective in inhibiting preformed biofilms. This shielding effect to lactoferrin and lysozyme could be due the presence of extracellular matrix of *C. albicans* biofilm which is rich in polysaccharides, proteins and other biomoleules. These extracellular polymers are well recognised to form a barrier in reducing the diffusion of antimicrobial agents (Al-Fattani & Douglas 2006).

Another host derived important salivary glycoprotein and a major component of mucus layer is mucin. These glycopolymers are emerging as important regulators of microbial colonization

and microbial virulence (Caldara et al. 2012). The present study showed that mucin suppressed the *C. albicans* biofilm formation which is a major virulence factor. Further work demonstrated reduction in phospholipase and proteinase activity in the presence of 1% mucin. It was reported that in *C. albicans* genes related to adhesion, biofilm formation and filamentation were down-regulated in the presence of 0.5% mucin (Kavanaugh et al. 2014). *C. albicans* utilized GlcNAc efficiently as a carbon source and formed well-established biofilms on polystyrene MTP. *C. albicans* can uptake GlcNAc and metabolise it further inside the cell due to the presence of Ngt1 transporter in the plasma membrane (Alvarez & Konopka 2007). This transporter is specific for GlcNAc as other sugars cannot compete for the transport. It is likely that under nutrient-limiting conditions *C. albicans* can metabolize GlcNAc either that is freely available in the saliva or by catabolising the glycoproteins like mucin. Also in the presence of GlcNAc, the specific activity of extracellular enzymes namely phospholipase and proteinase was recorded highest than mucin. It is known that GlcNAc induce morphological switching in *C. albicans* from yeast form to filamentous hyphal or pseudohyphal cells (Sudbery et al. 2004). As hyphal specific genes and SAP genes are regulated in a coordinated fashion the expression of one these virulence factor can influence the other (Naglik et al. 2003). The presence of less number of cells attached to polystyrene surface indicate that mucin suppress the cell surface attachment and biofilm formation. These results further confirm the role of mucin as a therapeutic agent in limiting the surface attached communities. Although it appears to be minor factor, the ability of *C. albicans* to utilize GlcNAc and promote the virulence traits indicates the need to consider these factors strongly while studying the complex interactions of oral community.

CHAPTER-5

5. Influence of bacteria on *Candida albicans* biofilm formation

5.1 Background

Microorganisms in their natural environments co-exist as multi species biofilms where cell to cell communication and intercellular interaction plays a key role in their survival (Lynch & Robertson 2008; Moons et al. 2009). These interactions can be synergistic where the presence of one microorganism provides a niche for other microorganism which can give rise to infections (Wargo & Hogan 2006). Synergistic coexistence of *C. albicans* and bacterial pathogen *S. aureus* in dual species microbial biofilm revealed a unique biofilm architecture and differential protein expression related to virulence factors in *S. aureus* (Peters et al. 2010). This interaction also promoted vancomycin resistance in *S. aureus* biofilms (Harriott & Noverr 2009). *S. aureus* and *C. albicans* dual species biofilm is medically important as these two species are currently most commonly isolated blood stream pathogens ranking under top four in nosocomial patients (Perlroth et al. 2007). On the other hand, interaction between *C. albicans* and *P. aeruginosa* was reported to be antagonistic as *P. aeruginosa* kills *C. albicans* by forming dense biofilms on hyphae (Nseir et al. 2007). *C. albicans* and *P. aeruginosa* are part of mucosal flora that causes serious infections. Signalling molecules that regulate *P. aeruginosa* virulence inhibits *C. albicans* hyphal development (Shiner et al. 2005). They are often co-isolated from cystic fibrosis infections and burn wounds (McAlester et al. 2008). Other pathogenic bacterium *E. coli* causes infections in immunocompromised and hospitalized patients is often associated with *C. albicans* (Bandara et al. 2010). Earlier, studies reported that *E. coli* modulates adhesion of *C. albicans* to different biotic and abiotic surfaces (Nair & Samaranayake 1996a; Nair & Samaranayake 1996b).

Polymicrobial diseases caused by co-aggregation of bacteria and fungi are increasing alarmingly (Peters et al. 2010). Limited studies have focussed on deciphering the complex inter-kingdom interactions in polymicrobial biofilms, particularly between pathogenic fungi and bacteria. Pathogenic yeast and bacterial cells compete for adhesion sites. During this process, they secrete quorum sensing molecules which can affect the adhesion of other colonising organism and subsequent biofilm formation (Hogan & Kolter 2002). Little is known about the influence of bacterial population on *C. albicans* biofilm formation. Biofilm formation is the major virulence factor in the pathogenicity of *C. albicans* as biofilms are highly resistant to antifungal therapy and host defences (Seneviratne et al. 2008). Hence, in this study the effect

of bacterial strains namely *S. aureus*, *P. aeruginosa* and *E. coli* on *in vitro* biofilm formation of *C. albicans* was evaluated.

5.2 Materials & methods

5.2.1 Strains and culture preparation

C. albicans and bacterial strains (*S. aureus* MTCC 3160, *E. coli* MTCC 4315 and *P. aeruginosa* DSVP20) were sub-cultured on YPD medium (1% peptone, 1% yeast extract, 2% dextrose, 2% agar) and Luria-Bertani (LB) medium (1% tryptone, 1% NaCl, 5% yeast extract, 2% agar) respectively for 24 h at 37 ºC. A loopful of these overnight grown cultures were inoculated into YPD broth and LB broth respectively and incubated at 37 ºC under shaking conditions (120 rpm) for 18 h. After incubation, the resulting cells were harvested by centrifugation (5000×*g*) for 10 min at 4 ºC, and washed thrice with PBS (pH 7.2). The cell pellets were suspended in RPMI-1640 medium and adjusted to standard suspension of 1×10^7 cells mL^{-1} (0.1 OD at 600 nm) by a spectrophotometer (Lasany, LI-2800 UV-visible Double beam, India).

5.2.2 Biofilm growth

Pre-sterilized polystyrene, flat bottom 96-well MTP were used to study biofilm formation as described earlier with some modifications (Jin et al. 2004). For growth of mixed species biofilms, *C. albicans* (100 µL) was added to 100 µL each of *S. aureus, P. aeruginosa* and *E. coli* cell suspension separately in wells of MTP. Cell suspensions were mixed thoroughly and incubated for 90 min at 37 °C to promote adhesion. Similarly, 100 µL of diluted monospecies *C. albicans* or bacterial strains were added to each well and marked as control. Following adhesion phase, the wells were washed carefully thrice with PBS to remove non-adherent cells and 100 µL of RPMI-1640 medium was added to each well. Plates were incubated at 37 ºC for 48 h. After incubation, wells were washed carefully thrice with PBS without disrupting the biofilm integrity.

5.2.3 Crystal violet assay

The biofilm biomass of monospecies and mixed species were quantified by crystal violet staining method as described earlier (Peters et al. 2010). Each well was washed thrice with sterile PBS to remove non-adherent cells and 0.1% crystal violet was added to each well for 15 min. Stain was washed several times with distilled water and the crystal violet bound to cells

was resolubulized in 95% ethanol. The absorbance was measured at 590 nm using a microtiter plate reader (Spectra Max M2, Molecular Devices, USA).

5.2.4 Viable cell measurements

Biofilms formed after 48 h were washed using sterile PBS (pH 7.2) and carefully scraped off from the well by a sterile scalpel and then transferred to 1 mL of PBS solution (Jin et al. 2004). The resultant suspension was vortexed for 3 min to disrupt the aggregates. Serial dilutions were performed in sterile PBS and cells were inoculated on to YPD agar medium containing 2 µg mL^{-1} ampicillin for *C. albicans* enumeration. For bacterial enumeration LB agar medium supplemented with 2 µg mL^{-1} amphotericin B was used. Plates were incubated at 37 ºC for 24 h and the resultant CFU mL^{-1} was calculated.

5.2.5 Assay of proteinase and phospholipase activity

The biofilm biomass obtained after 72 h of growth on MTP from both mono and mixed species was used for determination of proteinase and phospholipase activity. The assay was performed by mixing azocasein substrate for proteinase and phosphatidylcholine substrate for phospholipase activities respectively as described in Chapter-2 (sub-section 2.2.9).

5.2.6 Statistical analysis

Three independent experiments were conducted in triplicate and the data was expressed as mean ± standard deviation. Significant differences between two groups were evaluated by Student's t-test and *p* value less than or equal to 0.05 were considered significant.

5.3 Results

The interactions between fungal and bacterial cells have important physiological and medical implications, but the influence of bacteria on *C. albicans* biofilm formation is not well-established. The biofilm formation of *C. albicans* in mono and mixed species were compared after 48 h of incubation. The biomass of mixed species biofilm was greater when *C. albicans* and *S. aureus* were grown together than the biomass of either *C. albicans* or *S. aureus* biofilms grown independently (Fig 5.1). On the other hand, biofilm biomass was suppressed in *P. aeruginosa* - *C. albicans* mixed species biofilm. Significant increase in biofilm biomass data was observed in *E. coli* - *C. albicans* co-cultured biofilms. Cell counts of *C. albicans* and bacteria in mono and mixed biofilms were calculated by CFU assay which also differentiates between the viable fungal and bacterial cells in biofilms (Fig 5.2). The viability of *C. albicans* in *C. albicans* - *S. aureus* mixed biofilms was not statistically different from mono species

biofilms. Cell numbers of viable *S. aureus* increased dramatically in co-cultures with *C. albicans*. The growth of *C. albicans* was significantly reduced in mixed species biofilm with *P. aeruginosa*. While *P. aeruginosa* cell counts remained unaffected in the presence of *C. albicans*. In the presence of *E. coli*, the viability of *C. albicans* was reduced while significant increase in *E. coli* growth was observed in mixed species biofilm with *C. albicans*. The specific activity of *C. albicans* proteinases and phospholipases differed between mono and mixed biofilms (Fig 5.3). Maximum proteinase activity was observed in *C. albicans* - *S. aureus* mixed biofilms compared to *C. albicans* monospecies biofilms. While, specific activity of phospholipase was similar in both mono and mixed biofilms. In *P. aeruginosa* - *C. albicans* biofilm the specific activity of proteinases and phospholipases were significantly reduced. Similarly, in *E. coli* - *C. albicans* mixed biofilm, the specific activity of both enzymes were decreased relative to the *C. albicans* biofilms.

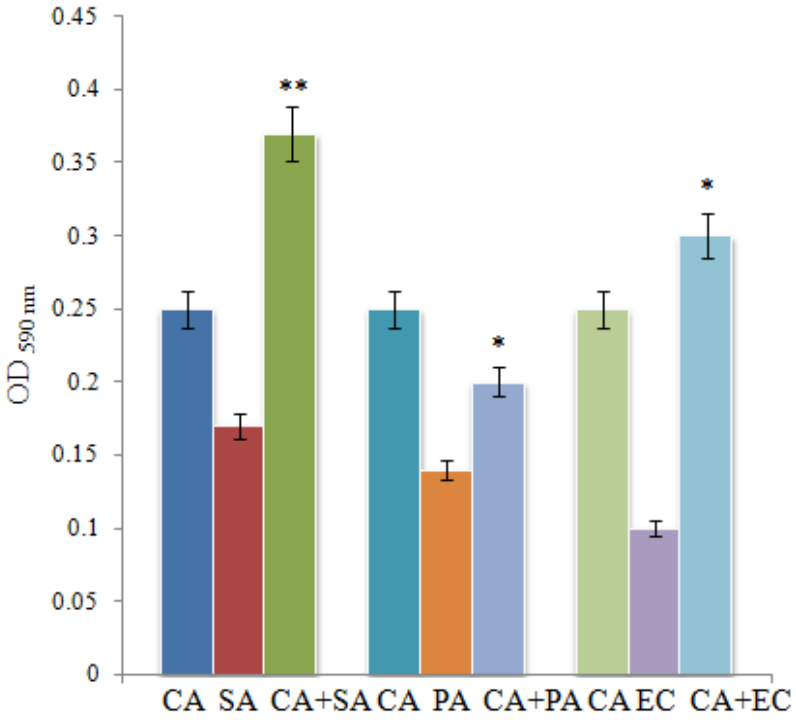

Figure 5.1 Biomass quantification of mono and mixed species biofilm. The effect of co-culture of bacteria *S. aureus* (SA), *P. aeruginosa* (PA), *E. coli* (EC) and *C. albicans* (CA) on biofilm formation. Mono and mixed species biofilms were grown on polystyrene plates for 48 h and biofilm biomass was quantified by CV assay. Data represent the means ± the SD of three independent measurements. $p<0.05$ was considered statistically significant (*$p<0.05$, **$p<0.01$).

Test microorganisms	**Mean CFU counts (±SD)**
C. albicans	25.89±6.60
C. albicans + *S. aureus*	21.22±5.92
S. aureus	119.89±86.60
S. aureus + *C. albicans*	189.11±50.02
C. albicans	26.76±3.05
C. albicans + *P. aeruginosa*	13.60±2.17
P. aeruginosa	157.33±35.71
P. aeruginosa + *C. albicans*	112.00±48.73
C. albicans	27.67±5.86
C. albicans + *E. coli*	18.60±6.85
E. coli	90.67±56.78
E. coli + *C. albicans*	140.22±69.67

Table 5.1 Viable counts of mono and mixed species biofilm. The mean CFU counts of mono (*C. albicans* or bacteria) and mixed species (*C. albicans* and bacteria) biofilms. Data represent the means ± the SD of three independent measurements.

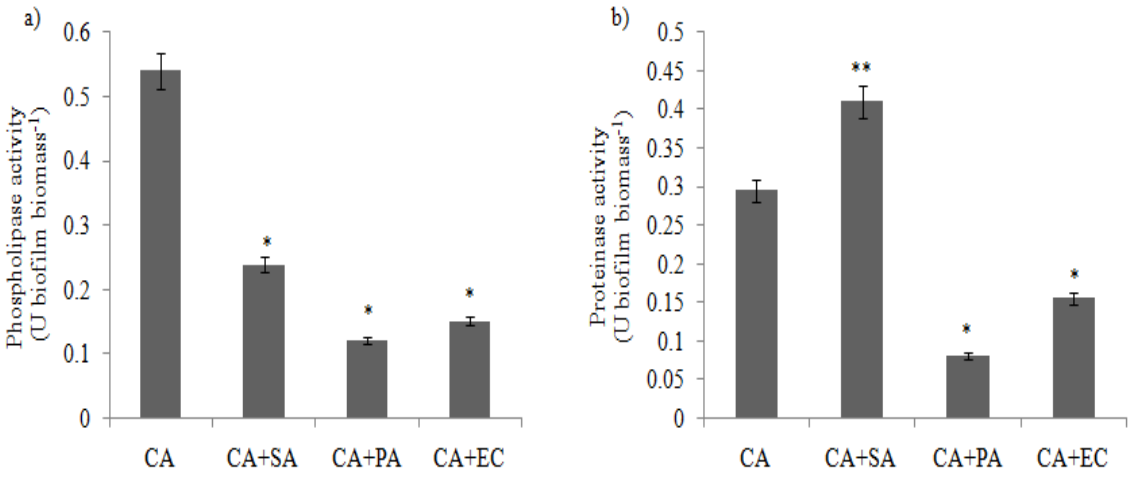

Figure 5.2 Test for virulence factors. a) The specific activity proteinase and b) the specific activity of phospholipase in *C. albicans* mono and mixed species biofilm. Data represent the means ± the SD of three independent measurements. $p<0.05$ was considered statistically significant (*$p<0.05$, **$p<0.01$).

5.4 Discussion

The architecture of biofilm is a complex phenomenon involving multiple microbial species colonizing to both biotic and abiotic surfaces. Recently, it has been noted that the majority of the nosocomial infections are due to mixed biofilms involving both bacteria and fungi (Peters et al. 2012). The interaction between bacteria and fungi need to be elucidated as these may have an impact on biofilm forming ability and its functionality. Bacteria and fungi may co-aggregate with each other and promote biofilm formation. They may also compete with each other which results in reduction of biofilm formation. This study was aimed to evaluate the role of bacteria in affecting *C. albicans* biofilm formation. The biofilm of each mono and mixed species were developed in polystyrene MTP. Data showed significant increase in biofilm biomass in the presence of *S. aureus* and *E. coli*. However, the viability of *C. albicans* was reduced in the presence of bacteria *E. coli* and *P. aeruginosa* compared to the biofilm formed when *C. albicans* was incubated alone. The co-incubation of *C. albicans* with *S. aureus* does not have any detrimental effects on *C. albicans* growth as measured by cell counts. Together with *C. albicans, S. aureus* is responsible for increasing the number of nosocomial infections (20%) as they are often co-associated with polymicrobial infections (Klotz et al. 2007). As expected the growth of *S. aureus* was significantly increased in the presence of *C. albicans*. The reduction in

the cell number of *C. albicans* was observed more in the presence of *P. aeruginosa*. These reports are in consistent with the earlier reports that *P. aeruginosa* suppress filamentation in *C. albicans* resulting in inhibition of biofilm formation (Hogan & Kolter 2002). It is likely that *P. aeruginosa* secretes or releases several key compounds that inhibit growth of *C. albicans*. Besides this, that quorum sensing molecule of *P. aeruginosa* 3-oxo-C-12 homoserine lactone inhibits *C. albicans* filamentation (Hogan & Kolter 2002). In addition to this, biosurfactant rhamnolipid derived from *P. aeruginosa* also reported to disrupt *C. albicans* biofilm formation (Singh et al. 2013). *P. aeruginosa* has a special affinity to hyphal elements as yeast form of *C. albicans* is generally not affected (Shiner et al. 2005). Enterobacterium *E. coli* is a significant component of human microbiome which often causes infections in hospitalized and immunocompromised patients (Thein et al. 2006; Kumar et al. 2015). Earlier, studies have found that *E. coli* enhance adhesion of *C. albicans* to mucosal epithelia and no inhibitory effect on adhesion to polystyrene surfaces (Seneviratne et al. 2008). The present data showed that viability of *C. albicans* was decreased in the presence of *E. coli*. These results are in agreement with the significant inhibition of *C. albicans* biofilm formation at higher concentrations of *E. coli* (Thein et al. 2006). The inhibiting action of *E. coli* was also reported by Park et al. (2014) in which density of *E. coli* - *C. albicans* biofilms were decreased compared to *C. albicans* monospecies biofilm. Proteinases and phospholipases are extracellular hydrolytic enzymes which helps *C. albicans* in tissue invasion by degradation of host cell molecules and membranes (Hube & Naglik 2001). The specific activities of these enzymes were significantly decreased in mixed biofilms when tested on bacterial strains used in this study. Researchers has earlier reported the significant decrease in expression of *C. albicans* virulence related genes required for adhesion and hyphal morphogenesis such as ALS3, ECE1, SAP5 and HWP1 in the presence of different bacteria strains (Park et al. 2014).

CHAPTER-6

6. Biofilm prevention and control strategies- *C. albicans* biofilm inhibition by synergistic action of terpenes and fluconazole

6.1 Background

The uprising of *Candida* biofilm infections in the last decades are almost in line with the increased use of a broad range of medically implanted devices in patients with impaired host defences. *C. albicans* is a foremost human fungal pathogen allied with colonisation and biofilm formation on the surfaces of medical devices (Douglas 2003). Due to its versatility it can behave as a commensal organism in several anatomically distinct sites which can pose a major problem from a clinical point of view, resulting in infections (Eraso et al. 2006). Furthermore, in recent years there has been mounting concern about the rising pervasiveness of infections caused by yeasts that are resistant to normally used antifungal drugs. The efficacy of the majority of antifungal agents is greatly reduced if yeasts are in a biofilm as opposed to the planktonic state. Of particular concern is that biofilms display increased resistance to antifungal therapy which can cause failure of implant devices, and serve as a reservoir or source for future continuing infections (Alexander & Perfect 1997; Kojic & Darouiche 2004). Hence, there is a need to identify new methods of preventing and treating biofilms to improve treatment of established infections and to limit further development of drug resistance. The new therapeutic strategies using natural products, of which essential oils are of immense importance due to their use in reducing oral infections (Singh et al. 2012). These oils are complex mixtures of volatile compounds known for their *in vitro* and/or *in vivo* antifungal properties (Tampieri et al. 2005; Marcos-Arias et al. 2011). The strong antifungal activity of some major components of essential oils, i.e. terpenes, has been described in several studies (Hammer et al. 2003; He et al. 2007; Mansouri & Darouiche 2008; Barchiesi et al. 2009). Hence, the present investigation evaluates the synergistic action of terpenes (eugenol, menthol and thymol) with fluconazole (FLA) on *C. albicans* with an aim to inhibit its biofilm formation. The search for new antifungal agents effective against biofilm has important clinical implications that may affect the outcome of patients suffering from these difficult-to-treat infections.

6.2 Materials and methods

6.2.1 Fungal strain and growth conditions

C. albicans MTCC 227 used in the present investigation was cultured in yeast peptone dextrose broth (YPD) medium and incubated for 24 h at 35 °C with agitation (120 rpm). Cells were harvested, washed twice in 0.1 M phosphate-buffered saline (PBS, pH 7.2) and adjusted to 1×10^7 cells mL^{-1} in PBS for biofilm formation (Singh et al. 2012). All growth media chemicals used in the present investigation were purchased from Himedia and Sigma, India.

6.2.2 Minimum inhibitory concentration (MIC) of terpenes

The terpenes (eugenol, menthol and thymol) were prepared as a stock solution of 16 % (v/v) in Roswell Park Memorial Institute medium (RPMI-1640) and 0.1 % (v/v) Tween 80. The MICs of the terpenes against planktonic *C. albicans* were determined by Clinical and Laboratory Standards Institute (CLSI M27-A3) broth micro-dilution method (Wayne 2010). An overnight grown culture of *C. albicans* was diluted to a final concentration of 2.5×10^3 cells mL^{-1} in RPMI 1640-MOPS medium and inoculated into commercially available, presterilised, polystyrene, flat-bottomed 96-well microtiter plate (MTP). Terpenes concentrations ranging from 0.01 %-8 % (v/v) were added to the 96-well MTP wells and incubated for 48 h at 35 °C. The growth in presence of terpenes was estimated using the MTP reader at optical density 530 nm after incubation.

6.2.3 Biofilm formation of C. albicans

The effects of terpenes on biofilm cell viability were established by measurement of cell metabolic activity of *C. albicans* biofilm in 96-well MTP. A 200 µL aliquot of *C. albicans* cell suspension containing 1×10^7 blastospores mL^{-1} in RPMI 1640-MOPS medium was added in MTP wells and incubated at 35 °C for 90 min for adhesion. The medium was then aspirated from the wells and washed with sterilized PBS to remove loosely adhered cells. Further, RPMI 1640-MOPS medium (200 µL) was added to each MTP wells and incubated for 24 h at 35 °C to obtain biofilm. After that, the growth medium was carefully removed by aspiration without disrupting the integrity of biofilm, washed thrice with PBS (pH 7.2) to remove non-adherent cells (Singh et al. 2012).

6.2.4 Effect of terpenes on C. albicans biofilms

The plate with the seeded biofilm was used subsequently to determine the effect of terpenes on the metabolic activity of the biofilm. The final concentration of terpenes used in this

experiment was in the range of 0.01 % to 8 % (v/v). Controls plates of seeded biofilm without terpenes were also included in each experiment. After incubation at 35 °C for 24 h, the treated biofilm were washed thrice with PBS and XTT assay was performed.

6.2.5 Evaluation of the effect of terpenes on biofilm development

Metabolic activity was assessed using the XTT tetrazolium salt reduction, assay as described earlier in Chapter 2 (sub-section 2.2.5). The sessile minimum inhibitory concentration ($SMIC_{90}$) was recorded as the lowest concentration resulting in a 90 % reduction in absorbance when compared with control biofilm.

6.2.6 Assessment of drug synergy against C. albicans biofilm

The effects of terpenes and fluconazole (FLA) on biofilm cell viability were established by measurement of cell metabolic activity of *C. albicans* biofilm in a 96-well MTP (Wei et al. 2011). Briefly, 200 µL aliquot of a suspension of *C. albicans* cells in RPMI 1640-MOPS (1×10^7 blastospores mL^{-1}) was added in wells for biofilm formation. After incubation at 35 °C for 24 h, the growth medium was carefully removed by aspiration without disruption of the integrity of the biofilm, and the formed biofilms were carefully washed thrice with PBS (pH 7.2) to remove non-adherent cells. The plate with the seeded biofilms was used to determine the effect of terpenes with FLA (terpenes/FLA) on the metabolic activity of the biofilm by a checkerboard micro-dilution assay. The final concentrations of terpenes ranged from 0.01 % to 8 % (v/v), and for FLA, from 0.01 to 0.5 mg mL^{-1}. After incubation at 35 °C for 24 h, the terpenes and antifungal agents were removed, and the treated biofilm was washed three times with PBS and XTT reduction assay was performed. The fractional inhibitory concentration (FIC) defined as the ratio of the MIC of an agent used in combination to the MIC of the agent used alone was calculated using the formula:

FIC = MIC (A combination)/MIC (A alone) + MIC (B combination)/MIC (B alone).

A- MIC value of individual terpenes in % (v/v) when combined with FLA

B- MIC value of FLA in mg mL^{-1} when combined with individual terpenes.

ΣFIC < 0.5 indicates synergy; 0.5–4.0, indifference; and >4, antagonism (ΣFIC, the sum of individual FICs).

6.2.7 Adherence assay

The effect of the terpenes/FLA on *C. albicans* adhesion was estimated by adding 200 µL of *Candida* l cells (1×10^7 blastospores mL^{-1} in RPMI 1640-MOPS medium) to pre-sterilized polystyrene 96-well plate which were then treated with terpenes along with the FLA (0.5 mg mL^{-1}) for 4 h at 37 °C. The media was aspirated after incubation and the wells were washed with sterilized PBS to remove loosely adhered cells. The plates were read at 492 nm by performing the XTT reduction assay. The results were expressed in terms of percent cell viability compared to terpenes/FLA-untreated wells, which were used as control.

6.2.8 Scanning electron microscopy (SEM)

The effect of thymol and FLA on *C. albicans* biofilm in comparison to control formed on polystyrene coupons were visualized by SEM. Briefly, *C. albicans* biofilm formed on polystyrene surface, were fixed with 2.5% (v/v) glutaraldehyde in PBS (0.1M, pH 7.2) for 2 h at room temperature. They were then treated with 1% (w/v) uranyl acetate for 1 h, and washed with distilled water. The samples were dehydrated with ethanol series (30%, 50%, 70%, 90% and 100%). All samples were dried to a critical point by Polaron critical point drier, coated with gold and viewed under SEM (Leo435, England).

6.2.9 Statistical analysis

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

6.3 Results

6.3.1 MIC of terpenes

The MIC done using CLSI M27-A3 broth micro-dilution method showed antifungal activities at a concentration of 0.12 % (v/v) for both thymol and eugenol as compared to 0.25 % (v/v) for menthol respectively, against *C. albicans* MTCC 227 (Fig 6.1).

6.3.2 Effect of terpenes on C. albicans biofilms

The effect of terpenes (eugenol, thymol and menthol) on metabolic activity of preformed *C. albicans* biofilm cells was evaluated using XTT reduction assay in 96-well MTP. Data obtained from different concentration of terpenes mediated disruption of pre-formed *C. albicans* biofilm

for 24 h at 35 ℃ revealed that reduction of *C. albicans* biofilm took place in a dose dependent manner. Data also showed thymol and eugenol were more effective at lower concentrations of ≥ 1.0 % (v/v) than menthol (Fig 6.2).

6.3.3 Assessment of drug synergy against C. albicans biofilm

Synergistic studies using checkerboard micro-dilution assay showed a fractional inhibitory concentration index (ΣFIC=0.31) between thymol/FLA followed by eugenol/FLA (Σ FIC=0.37) and menthol/FLA (Σ FIC<0.5) against pre-formed *C. albicans* biofilms (Table 6.1). Thymol with fluconazole showed highest synergy in reduction of biofilm formation than eugenol and

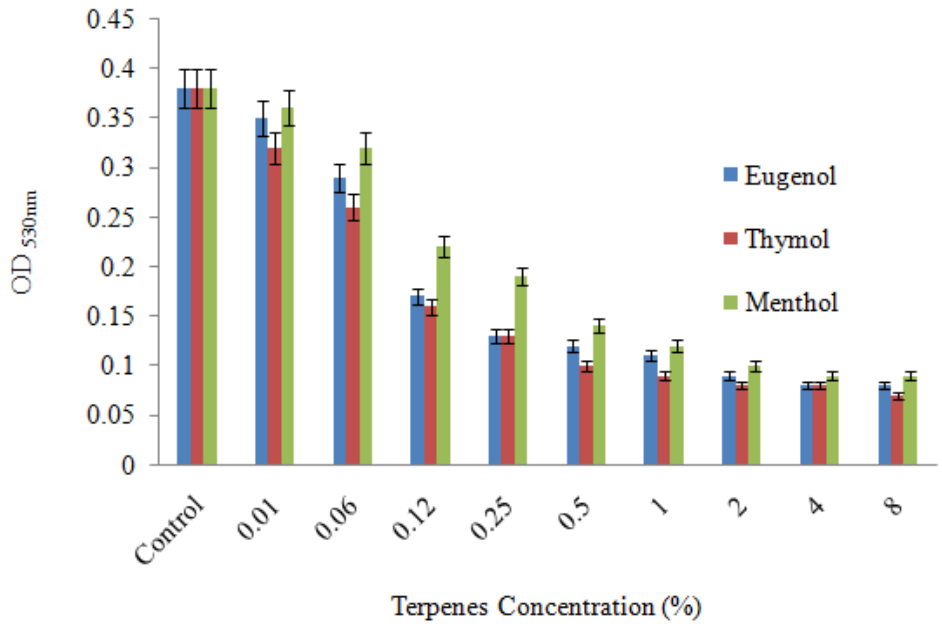

Figure 6.1 MIC of terpenes (eugenol, thymol and menthol) against *C. albicans*.

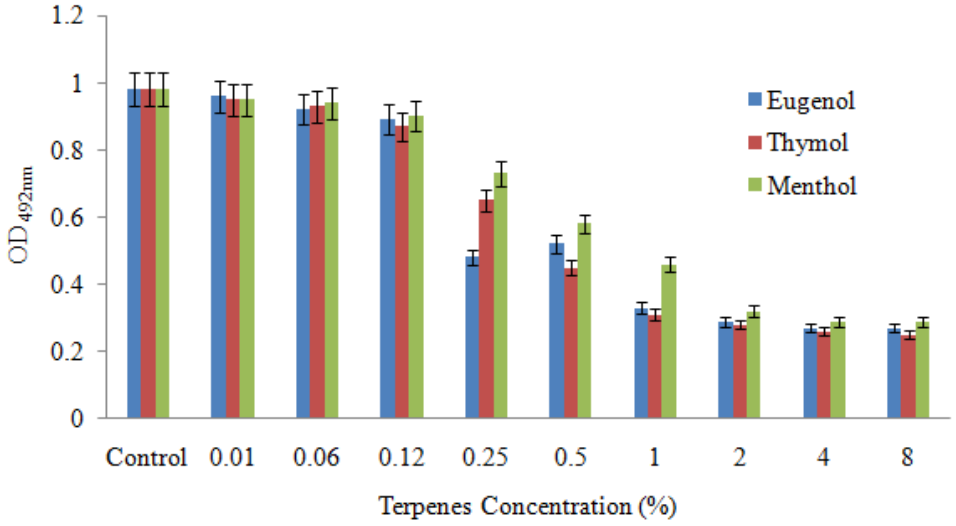

Figure 6.2 Effect of different concentrations of terpenes (eugenol, thymol and menthol) on metabolic activity of pre-formed *C. albicans* biofilm.

menthol, which was not observed when their activities were observed independently. The results demonstrated that synergistic effects can also be observed in cells that have already grown in a biofilm, which are typically the most difficult to treat.

S. No.	Agent	$SMIC_{90}$ (%)		Synergistic activity (ΣFIC)
		Alone	Combo	
1	Eugenol	2.0	0.25	0.37
2	Thymol	1.0	0.06	0.31
3	Menthol	4.0	1.0	0.5

Table 6.1 Synergistic activity (ΣFIC) of terpenes/fluconazole. The table shows the $SMIC_{90}$ (%) results of terpenes alone and in combination (combo) with fluconazole.

6.3.4 Adherence assay

In vitro studies on effect of terpenes/FLA on *Candida* l cell adhesion after 4 h showed it to be concentration dependent (Fig 6.3). Data showed 30% viability of *C. albicans* cells after 2 h of treatment with 0.05 % (v/v) thymol/FLA.

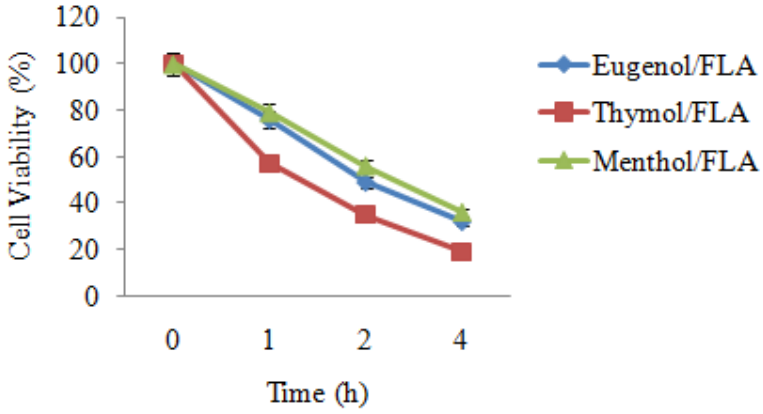

Figure 6.3 Synergistic effect of terpenes/fluconazole on adhesion of *C. albicans* to polystyrene plates.

6.3.5 Scanning electron microscopy (SEM)

The effect of thymol/FLA at 0.05 % (v/v)/0.5 mg mL^{-1} on *C. albicans* biofilm visualized by SEM micrographs showed reduction in cell number and disruption of *C. albicans* cells compared to the control (Fig 6.4 A and B).

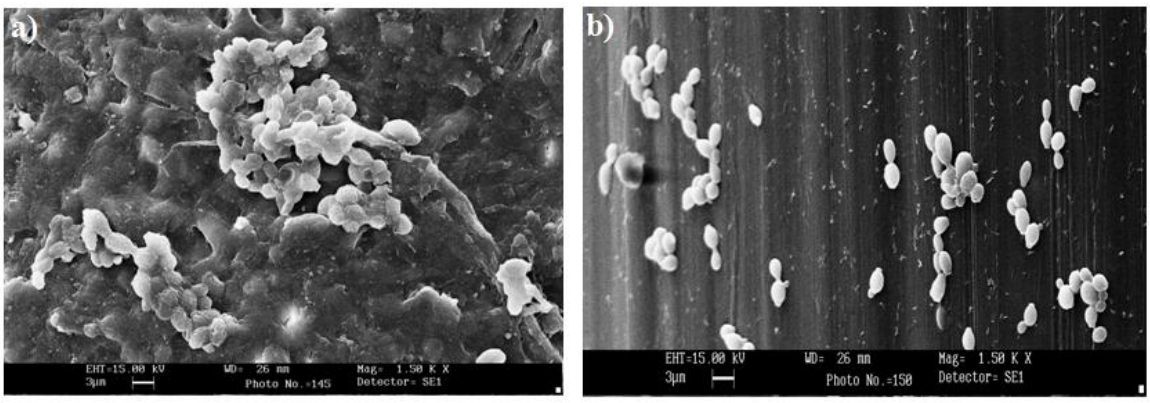

Figure 6.4 SEM micrographs of *C. albicans.* a) control and b)thymol/fluconazole treated of pre-formed *C. albicans* biofilm.

6.4 Discussion

Increased use of antifungal agents, corresponding to susceptible individuals has resulted in the emergence of multidrug-resistant *Candida* strains. The present study demonstrated that

synergistic activity of terpenes with fluconazole, which exerted strong inhibitory effect against *C. albicans* biofilms. These results were in agreement with our earlier data depicting the usefulness of essential oils in complex natural mixtures extracted from several aromatic plants having antimicrobial activities (Agarwal et al. 2008b). These results suggested the potential benefit of terpenes in treating immunocompromised individuals infected with *C. albicans*. Terpenes are made from combinations of several 5-carbon-base units called isoprene. The monoterpenes are formed from the coupling of two isoprene units. They are the most representative molecules constituting 90% of the essential oils and allow a great variety of structures. Among the monoterpenes, menthol, terpinen-4-ol and μ-terpineol are monocyclic alcohols; while carvacrol and thymol are phenols. The aromatic compounds such as eugenol, derived from phenylpropane, occur less frequently than the terpenes (Hammer et al. 2000; Pauli 2006; Palmeira-de-Oliveira et al. 2009).

The antifungal properties are correlated with the ability of terpenes to pass through the fungal cell wall and position between fatty acid chains of lipid bilayers, disrupting lipid packaging and altering the structure of the cell membrane (Ahmad et al. 2011). While evaluating the antifungal properties of terpenes, the method for determining MIC is important, for this reason, CLSI reference method for antifungal susceptibility testing was used in the current study and fluconazole was considered as control. The metabolic activity of *C. albicans* in biofilm was assessed using the tetrazolium (XTT), assay which based upon the reduction of XTT tetrazolium to the tetrazolium formazan product by mitochondrially active *C. albicans* in the presence of menadione, an electron-coupling agent (Hawser 1996). Among three terpenes tested, thymol exerted strongest effect towards both the planktonic and biofilm phase of *C. albicans*. However, its effectiveness against *C. albicans* biofilm mode was achieved at threefold higher of $SMIC_{90}$ value, than the MIC for its planktonic counterparts, which could be due to the resistant form of fungal growth. Earlier, researchers demonstrated by scanning electron microscopy, that thymol affected the envelope of planktonic *C. albicans* (Braga et al. 2008). The present study also revealed that, inhibition of biofilm formation was observed when thymol and fluconazole were supplemented together. The $SMIC_{90}$ of FLA was found to be 0.5 mg mL^{-1} when combined with thymol as compared to 2.0 mg mL^{-1} when tested alone. This can be related to synergistic antimicrobial action of FLA and thymol which may be due to inhibition of ergosterol biosynthesis and alternation in permeability and membrane fluidity causing degradation of cell wall and variable effects like disruption of cytoplasm membrane, leakage of cell contents (Ahmad et al. 2011). Based on the growth inhibitory effects of thymol with fluconazole in the broth micro-dilution assay, it is reasonable to assume that the effect of

the combination on biofilm formation is related to an effect on *C. albicans* cell growth rather than an effect on cell adhesion. Fluconazole belongs to the azole antifungals, which inhibit biosynthesis of ergosterol, a crucial component of fungal cell membranes, leading to permeability changes. The inhibition of ergosterol biosynthesis also results in the accumulation of toxic methylated sterol intermediates and subsequently, arrests fungal cell growth (De Nollin & Borgers 1975; Bailey et al. 1990). Even though the mode of synergic action is not clear at this time, a similar mechanism may exist in the thymol with fluconazole combination against biofilm cells. Menthol has also been observed with the antifungal activity against *C. albicans*. In agreement with our observations, other authors have found that eugenol exhibited anti-*Candida* activity against fluconazole-resistant *Candida* isolates (Chami et al. 2004; Ahmad et al. 2010). Similar results related to the inhibitory action of terpenic derivatives on *C. albicans* biofilm also pointed out the reduction in biofilm activity (Dalleau et al. 2008). The potent antibiofilm activities of these naturally occurring active principles might convert them into promising alternatives for the treatment of *Candida* -associated infections as they are relatively safe, and their side effects are minor and self-limiting (Bakkali et al. 2008; Traboulsi et al. 2008). Thus, our results collectively indicate that terpenes, especially thymol, along with fluconazole can be a better medication for *C. albicans* biofilm related infections.

Conclusion and future directions

The work carried out in this investigation was to understand how *C. albicans* responds to different environmental factors it encounters during colonization and in particular the effect of these factors on *C. albicans* biofilm. Initially, the role of different carbon sources and stresses on *C. albicans* biofilm formation, matrix polymers secretion and its biofilm architecture were studied. The effect of these environmental factors on virulence traits (proteinases and phospholipases) and cell wall components during biofilm growth were also monitored. Apart from this, the investigation was done to study how *C. albicans* responds to innate immune secretory factors (mucin, lactoferrin, lysozyme, dATP) and other co-infecting microorganisms (*S. aureus*, *E. coli* and *P. aeruginosa*).

The results presented signify that carbon sources influence *C. albicans* biofilm development, modulate virulence factors and structural organization of the cell wall component β-glucan. Microscopic studies showed that there exists a morphological plasticity of *C. albicans* to switch between yeast and hyphal forms which has a major influence on its virulence. The ability of *C. albicans* to survive the osmotic and oxidative stresses generated by the host immune system is essential for virulence. Several stress responsive signalling pathways and the evolutionary rewiring that took place in *C. albicans* has been well established. Despite this, little is known about how *C. albicans* adapts to these stresses which affects its virulence. The results discussed indicate that stresses promote surface associated growth in *C. albicans* with dense extracellular matrix to protect the cells within from harsh environmental conditions. In addition, data also showed that these stresses can cause alterations in the content of *C. albicans* cell wall components mainly chitin and mannan which are the key players in cell robustness and its adhesion to biotic and abiotic surfaces.

Biofilm formation and high levels of extracellular DNA into matrix by *C. albicans* in response to host danger signal eATP is likely to provide a protective advantage for this opportunistic pathogen. This might significantly impact the development of *C. albicans* infection process. However, this area of research needs a complete exploration of adaptation process and pathogenesis of *C. albicans* in the presence of host derived molecules.

Association of microbial communities is a complex process and the influence of one on each other might results in modulation of biofilm formation. The findings presented collectively

indicate that bacteria alter *C. albicans* biofilm formation and virulence factors. The synergistic interaction during polymicrobial infections along with the increase in virulence factors may be partially responsible for high infection rate. In this study, attempts were made to decipher the bacterial and fungal relationship during biofilm formation. Their interactions at molecular level can elucidate the differential expression and regulation of several genes involved in metabolism and virulence that are essential for survival of pathogenic microorganisms.

Overall, the work presented in this thesis adds to our understanding on *C. albicans* biofilm formation in response to different environmental challenges. Biofilm formation and alteration in virulence factors to a range of host and microbe derived environments are likely to contribute to the successful colonisation of this opportunistic pathogen. Further, work is required to understand the adaptive responses and the complex biofilm formation. Moreover, microbial interactions that interfere in the process of *C. albicans* biofilm development might aid in the identification of drug targets for therapeutic purposes. The characterization of quorum sensing molecules and plant derived compounds that are inhibitory to *C. albicans* and their mechanism of action would ease the discovery process of novel antifungal agents.

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