MOLECULAR ANALYSIS OF *Candida* BIOFILMS

A THESIS

Submitted in fulfilment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

By

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JULY, 2003

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **MOLECULAR ANALYSIS OF CANDIDA BIOFILMS**, in fulfillment of the requirement for the award of the Degree of **Doctor of Philosophy** and submitted in the **Department of Biotechnology** of the Indian Institute of Technology Roorkee is an authentic record of my own work carried out during a period from January, 2001 to July, 2003 under the supervision of **Dr. Vikas Pruthi**.

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

Dated: 21 July, 2003

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

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ABSTRACT

The foremost task taken in the present study deals with the selection of *Candida* species which form biofilm on infected IMDs made up of various biocompatible materials (BCMs). 60% of the clinical samples obtained from the patients with infected indwelling medical devices (IMDs) such as urinary catheters and intra uterine devices showed high incidences of *Candida* infections. On microscopic examinations these clinically isolated *Candida* spp. appeared as ovoid, budding yeast, sometimes having mould like hyphae and occasionally with large refractile chalmydospores. Species identification of these strains were performed by both biochemical and microbiological procedures. CHROMagar, a selective media was used as a convenient way for rapid identification of *Candida* species adhering to medical implanted biocompatible materials. Green, blue, pale pink and pink color colonies were observed for *C. albicans*, *C. tropicalis*, *C. krusei* and *C. parapsilosis* respectively on this selective media. Among different clinical isolates of *Candida* species, clinical isolate no. AU7 (identified as *C. albicans*) showed maximum biofilm forming ability on BCM using MTP assay. Maximum dry weight (1.95 mg) for biofilm formed by *C. albicans* (AU7) was obtained after 48 h at 35°C. Health of the *C. albicans* biofilm was recorded using MTT assay which was based on reduction of this salt by mitochondrial dehydrogenase to violet color tetrazolium formazan product. Spectrophotometrically reading at 540 nm showed maximum biofilm formation by *C. albicans* (AU7) take place after 48 h. This correlates with increased cellular density in the biofilm as assessed by electron microscopy techniques (SEM). Data suggests that biofilm were highly metabolically active in its first

24h. However, as the *Candida* biofilm matures and become more complex (48 to 72 h), the metabolic activity reached its plateau reflecting high number of cells that constitute the mature biofilm. SEM analysis of *Candida albicans* biofilm topography formed on infected medical devices revealed a dense network of mono or multiplayer of cells embedded within the matrix of extracellular polymer material. Maximum colonization strength of *C. albicans* biofilm was observed at a pH 6.5 and temperature optimum of 35°C after 48h using galactose as the carbon source (50mM) in yeast nitrogen base medium. Results from our investigations on temporal development of *C. albicans* biofilms on polyvinylchloride (PVC) strip showed that biofilms formation on these materials used in manufacture of biocompatible devices progresses in three distinct development phases. Initially, (0-6h) majority of *C. albicans* cells were present as blastospores (yeast form) adhering to the surface of the strips. At 6-12h *C. albicans* communities appeared as thick tracks of fungal growth. The intermediate development phase (12-36h) was characterized by the emergence of predominantly noncellular materials, which appeared as hazy-like film covering the *C. albicans* microcolonies. During the maturation phase (36-72h) the amount of extracellular material increased with incubation time and completely encased within this period. Comparative analysis of *C. albicans* biofilm by static and shaking incubation showed slow shaker speed (5 rpm) to be optimum after 48 h at 35°C when measured using MTT assay. After UV irradiation (96 μJmm^{-2}) for 60s on *C. albicans* AU7 we were able to select a biofilm deficient mutant BDM, namely- *C. albicans bdm7* on the basis of its rough colonial morphology and its lack of ability to adhere to the MTP wells made up biocompatible material (PVC).

MTP assay at different time intervals on MTP well showed that maximum crystal violet colorization take places after 48 h of growth by *C. albicans* AU7 in comparison to its biofilm deficient mutant *bdm*7, which showed its incapability for the same. Optimum temperature, pH and NaCl concentration for biofilm deficient mutant *bdm*7 were found to be 35°C, 6.5 and 0.5%, respectively. Among different carbon sources tested galactose (50mM) was noticed to be most favorable one. Cell wall extracts of *C. albicans* (AU7 and its biofilm deficient mutant *bdm*7) obtained by treatment with 2-mercaptoethanol were separated by SDS-polyacrylamide gel electrophoresis and analyzed by western blotting suggests that when the above samples were treated with HRP labeled purified antirabbit IgG (anti-AU7 and anti-*bdm7*; 1:640 dilution) and developed for substrate color reaction (H_2O_2 and 4-chloro-1-napthol) a 58 KDa band as the major antigen present in *C. albicans* (AU7), which may be responsible for biofilm formation since it was absent in its biofilm deficient mutant form *bdm*7.

The *in vitro* activity of antifungal agents amphotericin B, nystatin, fluconazole and chlorhexidine against pre-formed *C. albicans* (AU7) and its biofilm deficient mutant (*bdm*7) were assessed using XTT- reduction assay revealed increased susceptibility of biofilm deficient mutant *bdm*7 (50% reduction in metabolic activity for the same antifungal at a concentration of 0.25, 1.0, 0.25, 8 µg/ml respectively) when compared with *C. albicans* AU7 (50% reduction in metabolic activity for the same antifungal at a concentration of 8, 16, >64, 128 µg/ml respectively).

ACKNOWLEDGEMENTS

Firstly, I praise to ***Almighty Allah*** for enabling me to complete this work, providing me healthy and peaceful life, and giving me a strong belief in him. I bow and pray to Him; let noble thoughts come to me to lighten my life.

It is my great pleasure to express my deepest sense of gratitude to my supervisor, **Dr. Vikas Pruthi**, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee for his guidance, valuable help, novel ideas and consistent reverence. My sincere thanks to **Dr. R.P. Singh**, Head of the Department, for providing me the necessary infrastructure for conducting this study. I would like to express my deep sense of gratitude to **Dr. B.M.J. Pereira**, **Dr. R. Prasad**, Professor **G.S. Randhawa**, Professor **Ritu Barthwal** Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee. Special thanks due to **Dr. Parul Pruthi** for the valuable guidance and unlimited help. My deep thanks are due **to Dr. Irshad Ahmed**, Department of Orthopedics, All India Institute of Medical Sciences, New Delhi.

I would like to express my deep sense of gratitude to **Dr. N.P. Gupta**, Professor and Head of the Department of Urology, All India Institute of Medical Sciences, New Delhi for his help in collecting the urinary catheter samples.

My sincere thanks are due to **Dr. Monish** and **Dr. Rajeev** in same above Department, **Dr. Shoeb**, laboratory of Medical Mycology, **Mr. Khalid**, Laboratory of Bacteriology, **Dr. Iqbal** (Surgeon), **Dr. Segal** (Surgeon),

Department of Surgery, All Indian Institute of Medical Science, New Delhi for their help in collection of the samples.

I would like to thank all the staff of **Department of Obstetrics and Gynecology, All India Institute of Medical Sciences, New Delhi** for their help in collection of the samples. Appreciation is expressed to **Dr. (Mrs.) Ghazala Khan** (gynecologist) and **Dr. Farman Khan**, 'and **Dr. (Miss) Shabana Firdaus**, (gynecologist) Fizzah Nursing Home, (Hospital) Roorkee, Dr. Sultan Ali for their help in collection of samples. Thanks are also due to **Dr. G. Singh**, Singh Laboratory, Roorkee for valuable help.

I am also thankful to **Dr. Ali Abdul Latif**, (PDF) Post Doctoral Fellow Life Sciences School, Jawaharlal Nehru University, New Delhi for his help in collection of the papers for my work. My great thanks are also due to **Mr. Sunil Sharma** and **Mr. Saini** Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

I would like to thank **Mrs. Rekha Sharma**, Institute Instrumentation Center, Roorkee, for helping me to use Scanning Electron Microscope.

My sincere thanks are due to **Dr. (Mrs.) Pritha Dutta** 'gynecologist', Indian Institute of Technology Roorkee Hospital, Roorkee for her help in collection of the intrauterine devices samples.

I would like to thank all of the staff of National Medical Library, New Delhi for their help in collection of the paper of this study. I am highly obliged to **Tarun** for his cooperation during the practical part of this study. I am also indebted to colleagues and my friends **Dr. Soma, Dr. Arshi, Dr. Abbas, Amit**, Nidhi, **Amod, Ajeet, Saumya** and **Anvita** for their assistance and suggestions during the progress of this work.

I cleared the hurdles in this study with less effort is because of the love, patience, encouragement, understanding and support rendered by my beloved wife **Majidah**, my sons **Mohammed**, **Mustafa**, **Ahmed**, **Mahmood** and **Abdul Hameed** and my daughters **Sarah** and **Meyssam**.

Special thanks are due to **Indian Council for Cultural Relations (ICCR) Government of India** for providing me research fellowship and the opportunity for pursuing research work at the Indian Institute of Technology Roorkee, Roorkee. I am deeply indebted to **the College of Medicine, University of Al-Anbar**, for sponsoring me for Ph.D. programme at Indian Institute of Technology Roorkee, Roorkee.

Finally, thanks are due to **Mr. Kapil Trivendra** and **Mr. Rajender Singh** (Times Computer) for their excellent typing and word processing.

(Abbas Ubaid Farhan Al-Janabi)

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1. Kulkarni Amod, P., **Al-Janabi, A.U.**, Pereria, B.M.J. and Pruthi, V. 2003. Biofilm susceptibility of alcholic seed extract of *Nigella sativa* Linn in : 2nd World Congress on Biotechnology Development of Herbal Medicine. February 20-22, 2003. Organized by: International Society for Herbal Medicine (I.S.H.M) and International Institute of Herbal Medicine (IIHM), Lucknow, India.

2. Pruthi, V.; **AL-Janabi, A.U.** and Pereira, B.M.J. 2003. Characterization of biofilm formed on Intrauterine devices. *Ind. J. Med. Microbiol.* **23**:(3).

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LIST OF IMPORTANT ABBREVIATIONS

AIIMS	:	All Indian Institute for Medical Science (Hospital)
BCM	:	Biocompatible material
BDM	:	Biofilm Deficient Mutant
bdm7	:	biofilm deficient mutant
C	:	*Candida*
CA	:	Chrom Agar
CLSM	:	Confocal Laser Scanning Microscopy
conc.	:	concentration
D.W	:	Distilled water
E	:	*Escherihia*
FNH	:	Fizzah Nursing Home (Hospital)
g	:	gram
h	:	hour
IITR	:	Indian Institute of Technology Roorkee (Hospital)
IMD	:	Indwelling Medical Devices
IUD	:	Intra uterine device
l	:	Litre
MIC	:	Minimum Inhibitory Concentration
min	:	minute
ml	:	millilitre
N	:	*Neisseria*
NC	:	Nitrocellulose
P	:	*Pseudomonas*
PBS	:	Phosphate buffer sulphate
PVC	:	Polyvinyl chloride
rpm	:	Round per minute
SDA	:	Sabouraud Dextrose agar
SDS	:	Sodium dodecyl sulphate
SEM	:	Scanning Electron Microscopy
Spp.	:	Species
Staph	:	*Staphylococcus*
UV	:	Ultra violet
vol	:	volume
wt	:	weight
YPD	:	Yeast Extract Peptone Dextrose
µg	:	microgram
µl	:	microlitre

Chapter 1

INTRODUCTION

Biofilm refers to microbial aggregates entrapped in a polymeric slime matrix, which adhere to different material surfaces in order to protect themselves against environmental stress. Microbial biofilms develop when microorganisms irreversibly adhere to a submerged surface and produce extracellular polymers that facilitate adhesion and provide a structural matrix. This surface may be inert, nonliving material or living tissue. Biofilm-associated microorganisms behave differently from planktonic (freely suspended) organisms with respect to growth rates and ability to resist antimicrobial treatment and therefore pose a public health problem. The microbial biofilms develop on or within indwelling medical devices (e.g., intrauterine devices, urinary catheters, endotracheal tubes, mechanical heart values, pacemakers, peritoneal dialysis catheters, prosthetic joints, voice prostheses, central venous catheters and contact lenses (Donlan, 2001). Until recently, biofilms were recognized mostly for their propensity to coat and corrode oil field pipelines or food processing instruments (Dexter, 1993; Costerton, 1995; Flemming, 1996; Fletcher, 1999). But within the past few years, there is mounting evidence to show that biofilm is one of the common cause of persistent and chronic microbial infections (Costerton *et al.*, 1999; Potera, 1999). The antibiotic resistance of biofilm forming microbes leads to growth and development of infections to an uncontrollable stage whereby the host defense system is of no console for preventing it further. Thus, biofilm formation could counter both: the defense mechanism and antibiotic therapy (Hoyle and Costerton, 1990; Costerton *et al.*, 1999; O'Toole *et al.*, 2000). The process of biofilm formation so far has been classified into different stages involving initial attachment to surfaces, formation of microcolonies on the surfaces and finally differentiation of microcolonies into exopolysaccharide-encased, mature biofilm (Marshall, 1992; Costerton *et al.*, 1994; Costerton *et al.*, 1995; O'Toole *et al.*, 2000). There have been

numerous attempts to understand the molecular interaction of biofilm development at these different stages but more information is needed to identify the mechanism behind the surface attachment chemistries that controls its formation (Marshall, 1994; Busscher *et al.*, 1997; Millsap *et al.*, 1999; Stickler, 1999; Kuchma and O'Toole, 2000). In the present study, to forge a link between molecular basis of infection and the ability to form biofilm, *Candida* species as a model system has been taken as little is known about fungal biofilm structures and its amenability to genetic approaches (Baillie and Douglas, 1999; Reynold and Fink, 2001).

These pathogenic fungi of the genus *Candida* can cause a variety of superficial (such as oral or vaginal thrush and chronic mucocutaneous candidasis) and deepseated mycoses (such as acute disseminated *Candida* septicemia) that are distributed world-wide (Odds, 1988). In recent years, *Candida* species, notably *Candida albicans,* have been identified as potentially lethal agents of hospital-acquired infection (Fridkin and Jarvus, 1996). The most recent surveys have shown *Candida* to be the fourth most commonly isolated bloodstream pathogen from hospitals, now surpassing gram negative rods in frequency (Pfaller *et al.,* 1998). Importantly, *Candida* species, such as *C. albicans* and *Candida parapsilosis* are the third leading cause of catheter-related infections, with the second highest colonization to infection rate and overall highest crude mortality (Raad, 1998; Crump and Collignon, 2000). Even with current antifungal therapy, mortality of patients with invasive candidiasis has been as high as 40% (Wey *et al.,* 1988). Almost invariably candidiasis is associated with indwelling medical devices (e.g., dental implants, catheters, heart valves, vascular bypass grafts, ocular lenses, artificial joints and central nervous system stunts), which act as substrates for biofilm growth.

An interesting feature of *C. albicans* is its ability to grow in two different ways; reproduction by budding, forming an ellipsoid bud, and in hyphal form, which can periodically fragment and give rise to new mycelia, or yeast-like forms. Transitions between the two phenotypes can be induced *in vitro* in response to several environmental cues such as pH or temperature, or different compounds such as N-acetylglucosamine or proline. In addition to the intrinsic biological interest of this dimorphism, its ability to switch between the yeast and the hyphal mode of growth has been implicated in its pathogenicity (Ryley and Ryley 1990; Cutler, 1991; Leberer *et al.,* 1997). In the light of the above, it is obvious that *C. albicans* is a major model of pathogenic yeast. Despite this, advances in the knowledge of the molecular genetics of *C. albicans* have proceeded very slowly if compared with those achieved in conventional yeasts. *C. albicans* lacks a sexual cycle and is a diploid organism, which has made it difficult to manipulate it genetically (Odds, 1988). Two decades ago, it began to become apparent that *C albicans* was a diploid organism and therefore it was very difficult to obtain mutant strains. However, in the following years many auxotrophic mutants were obtained and parasexual genetic methods to analyze them were developed. *C. albicans* molecular biology is mainly based on the molecular knowledge of *Saccharomyces cerevisiae* owing to their similarity and also because many *C. albicans* genes can be expressed in *Saccharomyces*, although not viceversa. Furthermore, *C. albicans* shows many peculiarities that do not have counterparts in *Saccharomyces,* such as its ability to grow in hyphal form (Odds, 1988) and its virulence (Cutler, 1991), thus encouraging researchers to develop genetic tools for its study. It is also of great importance to define molecular targets for the development of new antifungal agents (Georgopapadakou and Walsh, 1996). Keeping this in

view, following objectives were performed for investigating into the molecular mechanism of *Candida* biofilms:

1. Selection of *Candida* species which shows biofilms forming ability on various biocompatible material such as polyvinylchloride (PVC), polystyrenes, polypropylene plastic or borosilicate glass surfaces.
2. To study the growth (by dry weight, microtitre plate assay, colorometric assay or scanning electron microscopy) and effect of various physico-chemical parameters (effect of pH, temperature and various nutrients) that contributes to biofilms in *Candida* species.
3. To study molecular interaction between biofilms formed by *Candida* Species and different biocompatible material surfaces.
4. Attempts to find the mutants, incapable of forming biofilms, of the above selective *Candida* species by physical or chemical means.
5. Characterization of the above mutants.
6. To formulate strategies for the control and regulation of agents involve in *Candida* biofilms formation and to develop antifungal therapy for them.

Chapter 2

LITERATURE REVIEW

2.1 HISTORICAL PERSPECTIVE AND DEFINITION OF BIOFILM

Biofilms have been described in many systems since Van Leewenhoek examined the "animalcules" in the plaque on his own teeth in the seventeenth century, but the general theory of biofilm predominance was not promulgated until 1978 (Costerton *et al.*, 1978). The theory states that the majority of bacteria grow in matrix enclosed biofilm adherent to surfaces in all nutrient sufficient systems and that sessile bacteria cells differ profoundly from their planktonic (floating) counterparts. Costerton *et al.*, in 1987, stated that biofilm consists of single cells and microcolonies, all embedded in a highly hydrated, predominantly anionic exopolymer matrix. Characklis and Marshall, in 1990, went on to describe other defining aspects of biofilms, such as the characteristics of spatial and temporal heterogeneity and involvement of inorganic or organic substances held together in the biofilm matrix. Costerton and Lappin-Scott, in 1995, at the same time stated that adhesion triggered expression of genes controlling production of bacterial components necessary for adhesion and biofilm formation. They emphasized that specific genes transcribed during initial cell attachment regulates the process of biofilm formation. Thus, a new definition for biofilm can be defined as microbially derived sessile community characterized by cells that are irreversibly embedded to a substratum or interface or to each other, enclosed in a matrix of extracellular polymeric substances which these cells have produced, and exhibit an altered phenotype with respect to growth rate and genes transcription (Pulcini, 2001a, 2001b; Donlan and Costerton, 2002).

2.2 DEVELOPMENT OF BIOFILM

The structure of a mature biofilm community will vary with the location, the nature of the constituent organisms, surface composition, environmental factors, essential gene products and the availability of nutrients (Table –1).

Carpentier and Cerf, in 1993, showed that primary adhesion between microbes and abiotic surfaces is generally mediated by nonspecific (e.g., hydrophobic) interactions, whereas adhesion to living or devitalized tissue is accomplished through specific molecular (lectin, ligand, or adhesion) docking mechanisms. In this most basic form, bacterial adhesion (as a process distinct from but integral in biofilm formation) can be divided into two stages: the primary or docking stage and the secondary or locking phase (Marshall, 1985; Pearce *et al.*, 1995; An *et al.*, 2000). This process conjures up images of the Russia Soyuz shuttle docking with the Mir space station. Some researchers included an additional step in this process called surface conditioning to describe the interaction of the substratum with its environment (Gristina, 1987; Boland *et al.*, 2000). Conditioning occurs, for example, when a foreign body is placed in the bloodstream and the native surface is modified by the adsorption of water, albumin, lipid, extracellular matrix molecules, complement, fibronectin, inorganic salts, etc. Once a surface has been conditioned, its properties are permanently altered, so that the affinity of an organism for a native or a conditioned surface can be quite different.

Wimpenny *et al.*, in 1997, reported that developed biofilm can range from thick confluent layers of cells (dental plaque or urinary catheter biofilms) to dispersed microcolonies or stacks of cells protruding from a thin basal layer (biofilms that form on surfaces in oligotrophic natural waters). The biofilms produced by *P. aeruginosa* under conditions where the aqueous phase containing the nutrients is continuously flowing over the colonized surface have been used extensively as a model system for the study of biofilm development. In this case, the attached cells produce extracellular polysaccharide and migrate away from the surface slightly The cells then cluster together in pillar and

Table 1: Major Factors that Control Biofilm Development, Composition, and Structure

1.	Surface properties of substratum (e.g.. toughness, hydrophobicity)
2.	Surface properties of microorganisms
3.	Physiochemical conditions of the bulk liquid phase (temperature, pH, salinity, ions, organic matter).
4.	Concentration of available organic substances, measured as assimilable organic carbon (AOC), biodegradable dissolved organic carbon (BDOC) or biological oxygen demand (BOD).
5.	Morphology of microorganisms (e.g. filaments)
6.	Physiological activity of microorganisms
7.	Lysis of biofilms organisms
8.	Grazing by protozoa
9.	Activity of invertebrates
10.	Formation of gas bubbles in anoxic and anaerobic zone (e.g..N_2 ,CH_4)
11.	Continuous detachment of small particles (erosion) or sporadic detachment of larger fragments of biofilms (sloughing)
12.	Age of biofilm
13.	Adsorption of exogenous material from the bulk water phase
14.	Hydraulic conditions (flow rate, shear stress)
15.	Presence of antimicrobial agents

mushroom shaped structures. Water filled channels from between these microcolonies and are thought to constitute primitive circulatory systems, delivering nutrients to and removing waste products from the communities of cells in the microcolonies (Costerton *et al.*, 1995; Stickler *et al.*, 1999).

Characklis, in 1990, showed that development of biofilms can be divided into three phases of an overall sigmoidal growth curve (Figure-1):

(1) During the induction phase the "race for the surface" takes place, usually leading first to a conditioning film of traces of adsorbed organic macromolecules such as humic acids, proteins, and polysaccharides (Baier, 1980). Microorganisms are transported more slowly than macromolecules and reach the surface either by their own mobility or by Brownian motion. In flowing systems a laminar sublayer exists even under highly turbulent conditions, which prevents cell transport directly to the surface by advection. The induction phase is initiated by the reversible attachment of microbial cells to surfaces (primary adhesion). Some cells to are able to scavenge biodegradable material from surfaces without staying irreversibly adhered (Marshall *et al.*, 1994; Marshall, 1996). Sometimes, irreversible adhesion occurs with concomitant phenotypic changes such as increased EPS production (Kanbe *et al.*, 1991; Van Devivere and Kirchman, 1993; Davies *et al.*, 1993; 2000). How microorganisms sense contact to surface is still unclear. In the induction phase cells are described as living particles (Marshall *et al.*, 1971) and primary adhesion is attributed to weak physicochemical interactions such as electrostatic and dispersion interactions and hydrogen bonding (Van Loodsecht *et al.*, 1990). During this phase, accumulation of

cells on the surface is a function of adhesion of cells from the water phase and, thus, dependent on suspended cell concentration.

(2) The logarithmic phase with subsequent multiplication of the sessile bacteria results in the formation of single-species microcolonies with the daughter cells bound within the EPS matrix. In this phase biofilm accumulation is due to the growth of adhering cells and becomes independent from cell concentration in the water phase.

(3) The plateau phase is reached after further cell division and new recruitment of microorganisms from the bulk fluid phase, leading to a more or less continuous biofilm that finally consists of coalesced microcolonies and single cells embedded in a highly hydrated matrix of EPS and trapped macromolecules as well as particulate matter from the water phase (Costerton *et al.*, 1987; Korber *et al.*, 1995; Palmer and White, 1997). The level of the plateau is controlled by the mechanical stability of the biofilm towards shear forces and by the availability of nutrients. The external structure of biofilms has been described to be the result of a balance between growth processes and detachment mainly resulting from shear forces (Costerton, 1995; Van Loodsrecht *et al.*, 1995; Stoodley and Stoodley, 2001); under high shear forces stresses biofilms be come smoother and less susceptible to shear forces. The organisms can detach from the biofilm by means of different mechanisms such as erosion of cells and sloughing off of larger biofilm fragments (Rittmann, 1989) as well as actively leaving the biofilm by swarming or by change of cell surface hydrophobicity (Ascon-Cabera *et al.*, 1995). As the biofilm develops, microbial cells within the matrix will release chemical signals. These signal molecules may enable the microbial colonies to develop the characteristics of a more mature biofilm. A

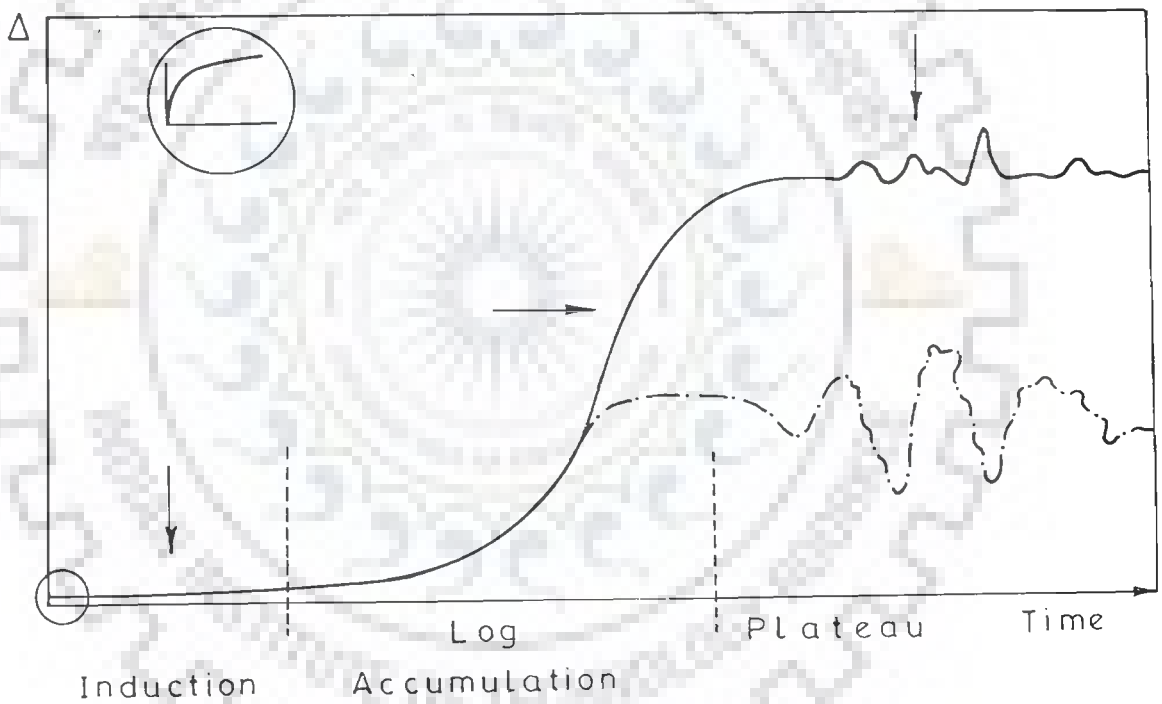


Fig. 1: Development of biofilms (Characklis, 1990)

number of bacterial species, both gram positive and gram negative, use these chemical signal molecules to coordinate activity (Parsek and Greenberg, 2000). The action of these signal molecules relies on a process called **quorum sensing**. In quorum sensing, the ability of the molecule to cause an action is dependent on its concentration within the environment. That concentration can increase only when there are a sufficient number of bacterial cells producing that particular signal. Probably some of the best-known quorum sensing systems is found in marine bacteria of the genus *Vibrio*. Species of this bacteria genus symbiotically colonize the light organs of certain fish or squid and will emit luminosity only when the population density has reached sufficient quorum density numbers (Ruby, 1996). Recently, researchers have isolated quorum sensing molecules produced by *P. aeruginosa* from the sputum of cystic fibrosis patients, suggesting that this is a biofilm disease of the lungs (Singh and Schaefer, 2000).

2.3 QUANTITATIVE ANALYSIS OF BIOFILM

Numbers or biomass of attached bacteria can be measured by destructively sampling test surfaces and evaluating them by microscopy, biochemical analysis of biomass components, radiolabeling cells, or applying molecular probes. Alternatively, attachment numbers and rates can be evaluated in real time by on-line computer analysis. Most often, attached cell numbers have been determined by bright field or fluorescent microscopy (Walker and Keevil, 1994) combined with staining of cells (Fletcher, 1976) or polymers (Allison and Sutherland, 1984). Besides this, phase contrast, differential interference contrast (Walker *et al.*, 1994), and interference reflection microscopy (Fletcher, 1988) have been used

to visualize attached cells. Cells can be stained with standard stains, such as crystal violet, or with fluorochromes, e.g. acridine orange (Fletcher, 1979; Meyer-Reil, 1978; Paul, 1982), 4'6-diamidino-2-phenylindole (DAPI) (Huang *et al.*, 1995; Porter and Feig, 1980), Hoechst dyes 33258 or 33342 (Paul, 1982), or 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC) (Huang *et al.*, 1998; Rodriguez, *et al.*, 1992). Combinations of stains can also make estimates made of relative activities of cells. For example, information on activity of attached *Klebsiella pneumoniae* was provided by intracellular accumulation of CTC, a soluble redox indicator reduced by respiring bacteria to fluorescent CTC formazan crystals, and staining by rhodamine 123 (Rh 123), which can be incorporated into bacteria in relation to proton motive force (Yu and McFeters, 1994).

Numerous cell constituents have been used as biochemical markers of attached cell biomass. These methods vary in their specificity and sensitivity, and the amount of biomass present is often a major criterion when selecting an appropriate analysis. Valuable information on viable biomass can be obtained by quantitative analysis of total phospholipid ester linked fatty acids (PLFA), which occur in the intact membrane of viable cells (White *et al.*, 1979). After death, the phosphate group is hydrolyzed, leaving diglyceride with the same signature fatty acids as the phospholipids (Nickels *et al.*, 1979; Findlay and White, 1983a; Bakke *et al.*, 2001) or triglyceride in microeukaryotes (Gehron and White, 1982), when quantified in relationship to PLFA. An increase in specific trans monoenoic PLFA, compared to *cis* isomers, is believed to an indication of physiological stress (Guckert *et al.*, 1986; Heipieper *et al.*, 1992).

Biomass of attached cells has been indirectly measured by extracting ATP and quantifying it by the luciferin/ luciferase reaction (Harber *et al.*, 1983). DNA can be

quantified by fluorometry with Hoechst staining (Paul and Loeb, 1983). Gram-negative bacteria can be measured as lipopolysaccharide using the *Limulus* amoebocyte lysate (Dexter *et al.*, 1975). Other, less commonly used markers are lipid A of lipopolysaccharide (Parker *et al.*, 1982), teichoic acids in gram-positive bacteria (Findlay *et al.*, 1983b), and muramic acid (McEldowney and Fletcher, 1987). Immunochemical techniques may be used for specific organisms, such as enzyme linked lectinsorbent assay for measurement of *Staphylococcus epidermidis* biofilms. This method is based on a phosphatase labeled wheat germ agglutinin, which bound with GlcNAc β-1, 4n, a component of the extracellular biofilm polymer (Thomas *et al.*, 1997). Sensitive and accurate determination of attached cell biomass can be achieved by prelabeling cells with a radioisotope and precalibrating radioactivity with some other measure of biomass, such as direct or viable counts. Effective substrates for incorporation of radioisotopes have been ^{14}C- or ^{3}H-leucine (McEldowney and Flectcher, 1987) or ^{14}C-acetate (Mittelman *et al.*, 1992).

Attempts made to identify the surface characteristics of microbes that determine adhesiveness have shown the roles of electrostatic charge and surface energy interactions, including hydrophobicity (Rosenberg and Kjelleberg, 1986; Van Loosdrecht *et al.*, 1987). Hydrophobic interaction chromatography (Mafu *et al.*, 1991) and electrostatic interaction chromatography provide relative values of cell hydrophobicity and surface charge, respectively.

For real-time computer analysis, a variety of flow chambers and apparatus (Table-2) have been employed (Duxbury *et al.*,, 1980; Kjellberg *et al.*, 1982; Sjollema *et al.*, 1988; Busscher and Vander Mei, 1995). These range from chambers machined for laminar flow (Mittelman *et al.*, 1992; Mittelman *et al.*, 1993; Wienck and Fletcher, 1997), to capillary

tubes (Rutter and Leech, 1980), to chambers constructed from coverslips and microscope slides (Caldwell and Lawrence, 1986). Some flow chambers, such as the radial flow reactor, have been designed to control, measure and modify shear stresses on developing biofilms (Mittelman *et al.*, 1990). Such controlled studies have demonstrated that biofilm characteristics can be altered by shear stresses; for example, higher C:N ratios and total fatty acids in *Pseudomonas atlantica* biofilms were associated with higher shear stresses (Mittelman *et al.*, 1990).

By interfacing microscopic imaging with computer analysis, numerical data on attached cell numbers, area coverage, and biovolume of attached cells can be accurately measured over time (Moller *et al.*, 1996; Caldwell and Germida, 1985; Caldwell and Lawrence, 1986; Lawrence *et al.*, 1987). By capturing successive images, such as at 2-min intervals Wiencek and Fletcher, in 1995 and 1997, showed data that can be analyzed to determine growth pattern of organism on surface as well as rates of deposition, rates of desorption and residence time of cells (Gow *et al.*, 1994).

2.4 BIOFILM ON INFECTED INDWELLING MEDICAL DEVICES (IMDs)

Biofilms on indwelling medical devices may be composed of gram positive (*Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis*, and *Streptococcus viridans)*; or gram negative bacteria (*Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis*, and *Pseudomonas aeruginosa)* or yeasts (Donlan, 2001). These organisms may originate from the skin of patients or health care workers, tap water to which entry ports are exposed, or other sources in the environment. Biofilms may be composed of a single species or multiple species, depending on the device and its duration of use in the patient.

Table 2 : Methods used for measurement of biofilms

Method	Basic protocol	Advantage	Limitations	References
Roll plate	Roll the catheter tip over the surface of a blood agar plate	Easy to use	Examines only catheter outer surface, inaccurate	Murga *et al.*, 2001
Vortex, then viable count	Catheter section in PBS is vortexed then cultured on different media	Measures intraluminal and extraluminal biofilm	Recovery efficiency unknown	Tenny *et al.*, 1986
Sonicate, vortex, then viable count	Catheter section in TSB, sonicate then vortex, then culture on blood agar	Measures intraluminal and extraluminal biofilm	Recovery efficiency unknown	Sherertz *et al.*, 1990
Sonicate, vortex, homogenize, then viable count	Catheter section in PBS sonicated/ vortexed repeatedly, then homogenize and culture on blood agar	Recovery efficiency determined	Measures intraluminal biofilm only	Donlan *et al.*, 2001
Acridine orange direct staining	Following roll-plate method, catheter section is stained with acridine orange	Allows direct examination of catheter	Method does not allow quantification	Zufferey *et al.*, 1988
Endoluminal brush	Brush is introduced into the implanted catheter, removed, placed into PBS, sonicated and plated	Allows direct examination of catheter	Effect of procedure on patient and recovery efficiency unknown	Kite *et al.*, 1997
Alginate swab	Swab introduced into the implanted catheter, removed, then streaked over a blood agar plate	Allows examination of indwelling catheter	Effect of procedure on patient and recovery efficiency unknown	Cercendo, 1990

Table 2 methods that have been used for measurement of biofilms on catheters. Urinary catheter biofilms may initially be composed of single species, but longer exposures inevitably lead to multispecies biofilms (Stickler, 1996). A distinguishing characteristic of biofilms is the presence of extracellular polymeric substances, primarily polysaccharides, surrounding and encasing the cells. These polysaccharides, which have been visualized by scanning electron microscopy, appear either as thin strands connecting the cells to the surface and one another or as sheets of amorphous material on a surface (Hawser *et al.*, 1998). Most biofilm volume is actually composed of this extracellular polymeric substance rather than cells, a fact that has been confirmed by ruthenium red staining and transmission electron microscopy (Jones *et al.*, 1969). This biofilm matrix may act as a filter, entrapping minerals (Stickler, 1996), or host produced serum components (Braunwald, 1997). A number of apparatuses have been developed for growing and testing biofilms as listed in Table 3. Anwar *et al.* 1992 showed that biofilms are both tenacious and highly resistant to antimicrobial treatment. They reported that treatment with levels of tobramycin far in excess of the MIC reduced biofilm cell counts for *P. aeruginosa* by approximately 2 logs, while the same dosage provided a >8-log decrease in planktonic cells of this organism.

2.5 BIOFILM FORMATION BY FUNGAL PATHOGENS

Virulence is a complex interrelationship between the infecting organism and the host, pathogenesis involves interaction (and sometimes modification) of factors on both sides. This is particularly true of fungal pathogenesis. Fungal pathogens can be divided into two general classes, primary pathogens and opportunistic pathogens (Chander, 1996; Baillie and Douglas, 1998a; Odds, 1988; Baillie and Douglas, 1999a; Baillie and Douglas, 2000; Doggett, 2000; Chandra *et al.*, 2001a; Andes, 2003). Fungi in the former class usually have an environmental

reservoir and infect individuals who have either been exposed to a large dose or who are immunologically naïve to the fungus. Opportunistic pathogens take advantage of debilitated or immunocompromised hosts to cause infection. They may have an environmental reservoir (e.g., *Cryptococcus neoformans*, *Aspergillus fumigatus*) or exist as commensals in healthy organisms (e.g. *Candida* species). The mechanism of fungal pathogenesis are much less well understood than are those of bacterial pathogens. In contrast to bacteria, few fungi are professional pathogens. Fungal pathogenic mechanisms tend to be highly complex, arising in large part from adaptations of preexisting characteristics of the organisms nonparasitic lifestyles. In the past few years, genetic approaches have elucidated many fungal virulence factors, and increasing knowledge of host reactions has also clarified much about fungal disease. The literature on fungal pathogenesis has grown correspondingly (Kauffman *et al.*, 1983; Klotz, 1990; Frey *et al.*, 1990a, 1990b; Kerridge, 1993; Georgopapadakou and Walsh, 1996; Chander, 1996; Freydiere *et al.*, 2000; Chandra *et al.*, 2001a). Opportunistic human fungal pathogens have become increasingly important over the past 20 years, paradoxically because the success of modern medical practice has led to the survival of debilitated and immunosuppressed patients. Such patients are highly susceptible to infections by opportunistic pathogens such as *Candida* species, *Aspergillus* species and the zygomycetes (Sobel *et al.*, 1982; Chandra *et al.*, 2001a; Chandra *et al.*, 2001b; Kuhn, 2002a; Nakai *et al.*, 2003). In these cases at least, the genetic composition of the pathogen population may be different at the end of the infection compared with the beginning. Whether there are corresponding pathogen-specific changes (which would have to be phenotypic rather than genotypic) in the host is unclear, although the standard immunological response pathways are

Table 3: Apparatus that have been used for growing and testing biofilms

Apparatus	Organism(s) tested	Flow dynamics	Substratum	Method for removing and quantifying biofilm	References
Modified Robbins device	*Pseudomonas pseudomallei*	Batch/ mixing	Silastic disks	Method of removal not given; viable count	Vorachit *et al.*, 1993
Calgary biofilm device	*P. aeruginosa, S. aureus, E. coli*	Batch/ mixing	Plastic pegs	Sonicate peg, then viable count	Ceri *et al.*, 1999
Disk reactor	Gram negative bacteria	Batch/ mixing	Teflon coupons	Sonicate, vortex, homogenize, then viable or direct count	Donlan *et al.*, 1999
CDC biofilm reactor	Gram negative bacteria	Continuous/ open system	Needleless connectors (plastic)	Sonicate, vortex, homogenize, then viable or direct count	Murga *et al.*, 2001
Perfused biofilm fermentor	*Candida albicans*	Continuous/ open system	Cellulose acetate filters	Shake in sterile water, then viable count	Baillie and Douglas, 1998a
Model bladder	Gram negative bacteria	Continuous/ open system	Urinary catheters	Direct examination by SEM or TEM of by chemical analysis	Stickler *et al*, 1999

of course induced by infection and play important roles in both resistance and pathogenesis (Odds, 1988; Magee *et al.*, 1988; Georgopapadakou and Walsh, 1996).

2.6 TAXONOMY AND MORPHOLOGICAL CHARACTERISTICS OF CANDIDA SPECIES

2.6.1 Taxonomy

The genus *Candida* comprised an array of yeast species unified mainly by the absence of any sexual form. This method of classification has created a somewhat confusing taxonomy in which most species ultimately are assigned to at least two genera (by sexual and nonsexual forms). Many attempts have been made to define natural relationships among *Candida* species, but these have been generally confounded by the great heterogeneity within the group (Kurtzman and Phaff, 1987; Barns *et al.*, 1991). The artificiality of the classification scheme has manifested itself in the partial failure of biochemical and genetic tests to define coherent taxa (Kurtzman and Phaff, 1987). Several members of the genus are associated with significatnt human disease, including the leading yeast pathogen, *Candida albicans* (Rippon, 1988).

A few researchers have turned to nucleic acid analyses to resolve relationships within the genus (Nakase, 1971; Segal and Eylan, 1974; Segal and Eylan, 1982; Magee *et al.*, 1987), analysis of DNA sequences in particular has been proposed as an objective method for such assessment (Odds, 1988). The advantages of rRNA sequencing for phylogentic studies have been discussed (Fox *et al.*, 1980; Woese, 1987). Use of such macromolecular data allows determination of relationships independent of morphological and physiological criteria, criteria which, among the *Candida* species and relatives in particular, have yielded often confusing and contradictory classification systems. Although rRNA sequences have been

widely used to elucidate bacterial evolutionary relationships (Woese, 1987), only a few rRNA sequences of fungal species have been determined, mostly for 5S rRNA (Blanz and Gottschalk, 1984; Walker, 1985; Walters and Erdmann, 1988); and some for 18S rRNA (Sogin *et al.*, 1986; Dams *et al.*, 1988). Barns *et al.*, 1991 sequenced cellular rRNA or cloned 18S genes of 10 of the most commonly isolated pathogenic *Candida(s)* as well as *Hansenula polymorpha* and *Aspergillus fumigatus*. These sequences, together with those of *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* var. lactis were used to explore evolutionary relationships amongst the speciies. Their results indicate that *C. albicans*, *Candida tropicalis*, group, *C. parapsilosis*, and *C. viswanathii* form a highly interrelated group, whereas the sequence of the other significant yeast pathogen, *Torulopsis glabrata*, is associated with a second distinct phylogenetic grouping the tree (Fig. 2).

2.6.2 Morphological Characteristics of *Candida* Species

The germ tube test is the standard laboratory method for identifying *Candida* species (Taschdjian *et al.*, 1960). The test involves the induction of hyphal outgrowths (germ-tubes) from yeast cultured in serum for 2-4 h at 37°C. Approximately 95% of *C. albicans* isolates produce germ tubes (Perry and Miller, 1988), a property also shared by *C. stellatoidea* and *C. dubliniensis* (Sullivan and Coleman, 1998). Rare isolates of *C. tropicalis* were also reported to be capable of germ tube formation (Martin, 1979). Chlamydospore production is primarily associated with *C. albicans* but again is found with *C. stellatoidea* and rare isolates of *C. tropicalis* (Hasenclever and Mitchell, 1961). *Candida dubliniensis* also develops numerous chlamydospores, which tend to occur in pairs or triplets attached to laterally branching pseudohyphae (Sullivan and Coleman, 1998). Carbohydrate assimilation profiles for *Candida* species can be obtained by examining zones of candidal growth around discs or wells impregnated with various sugars on basal agars (Di Menna,

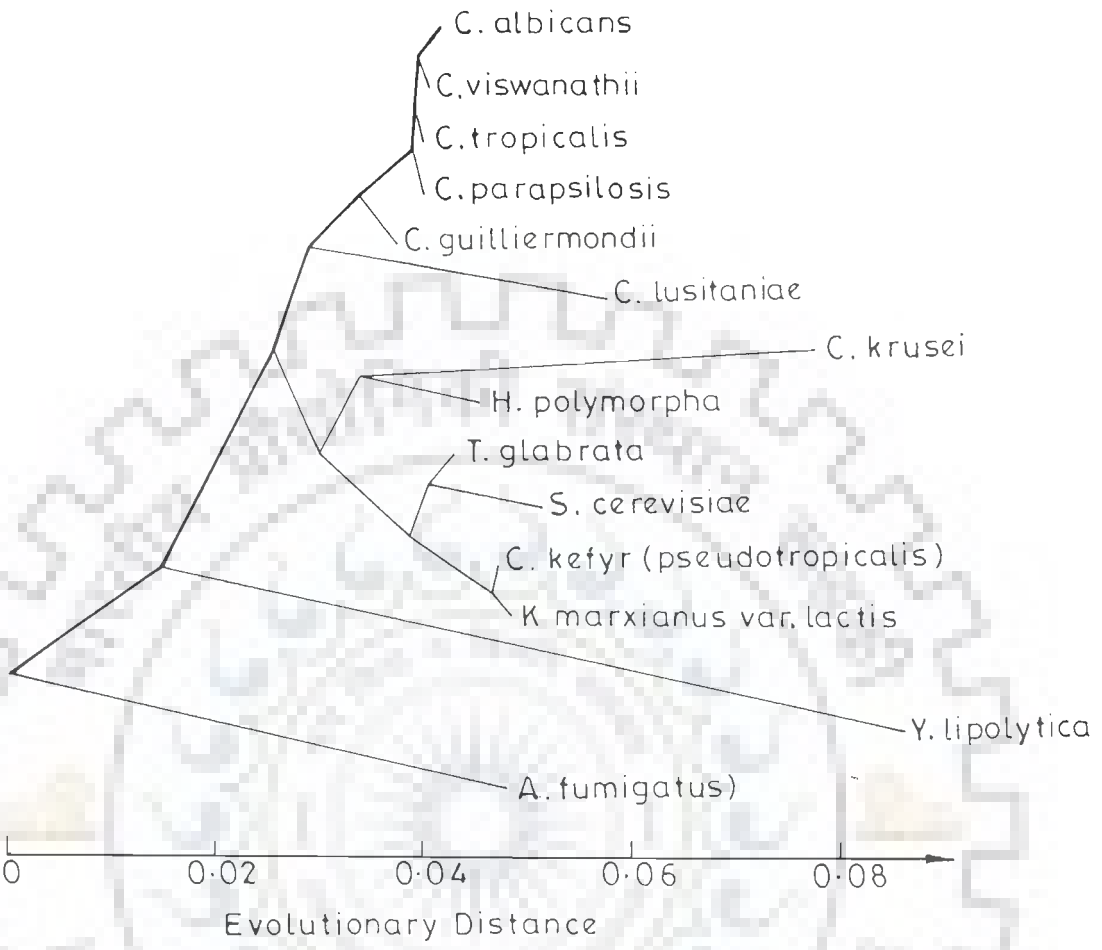


Fig. 2: Evolutionary tree for members of the genus *Candida* and relative based on the evolutionary distance (Barns *et al.*, 1991)

1955). The API-20 C and API-32 C (bioMérieux, Basingstoke, UK) systems consist of identification strips based on carbohydrate assimilation that provide an identification after 24-72 h incubation, and permit the identification of 24 and 38 types of *Candida* species, respectively. Since the API-20 C is 96-99% as accurate as conventional procedures, it is frequently used as a reference method for *Candida* identification (Buesching *et al.*, 1979). Unfortunately, isolates of *C. dubliniensis*, have atypical assimilation profiles that are not currently incorporated into the analytical profile indices for the API systems (Salkin *et al.*, 1998) Table 4.

In a recent evaluation of the RapID system (Kitch *et al.*, 1996), 94.1% of 304 clinically significant yeasts were correctly identified to species level. The Candifast system (International Mycoplasma, Toulon, France) identifies *Candida* based on sugar fermentation reactions, urease production and resistance to actidone (Theunissen and Thompson, 1993). The Microring YT (MYT; Medical Wire & Equipment Co. Ltd., Corsham, UK) examines the ability of *Candida* to grown in the vicinity of filter paper discs impregnated with six separate chemicals. Although the MYT is simple to use, evaluations have been unfavourble due to the limited database which is restricted to 18 yeast species (Ridgway and Allen, 1991; McGowan and Mortensen, 1993). Consequently the definitive identification of isolates using the MYT often requires supplementary tests. The Iatron *Candida* Check system (Iatron Laboratories, Tokyo, Japan) identifies *Candida* by slide agglutination of yeast cells with specific antisera. A battery of 10 antisera is available, enabling the identification of eight *Candida* species. Serotyping of *C. albicans* into two antigenic groups, A and B, (Hasenclaver and Mitchell, 1961) is also possible, although discrepancy with other antisera has been reported (Brawner, 1991). An additional identification method for *C. albicans* based on serology is the Bichrolatex albicans test (Fumouze Diagnostics, Asieres, France) that relies on latex particle

agglutination of *C. albicans* (cultured on primary culture media) using a *C. albicans* specific monoclonal antibody (Quindos *et al.*, 1997).

2.7 DEVELOPMENT OF C. ALBICANS BIOFILM ON INFECTED IMDs

Recent data from the US National Nosocomial Infections Surveillance system ranked *Candida albicans* as the fourth most common cause of bloodstream infection, behind coagulase-negative *Staphylococci*, *Staphylococcus aureus* and *Enterococci*. Superficial *Candida* infections associated with implanted devices are much less serious but can be troublesome and are encountered very frequently. The commonest is probably denture stomatitis, which is *Candida* infection of the oral mucosa that is promoted by a close fitting upper denture. A mixed species biofilm is formed on the surface of the acrylic denture contains large numbers of bacteria, particularly *streptococci*, in addition to yeasts (Budlz-Jorgensenn, 1990). Silicone rubber voice prostheses, which are fitted in the laryngectomized patients, are also subject to contamination by polymicrobial biofilms containing *Candida* spp.

Biofilms are the most common mode of bacterial growth in nature and are also important in clinical infections, especially due to the high antibiotic resistance associated with them (Bagge *et al.*, 2000; Chandra *et al.*, 2001b). In contrast to the extensive literature describing bacterial biofilms (O'Toole *et al.*, 2000; O'Toole *et al.*, 1999; Watnick and Kotler, 2000) little attention has been paid to medically relevant fungal biofilms (Doggett *et al.*, 2000).

Transplantation procedures, immunosuppression, the use of chronic indwelling devices, and prolonged intensive care unit stays have increased the prevalence of fungal

Table 4 : Commercial test systems for the identification of *Candida* species

System	Basis of test
CHROMagar® *Candida*	Chromogenic substrate based agar medium
Albicans ID	Chromogenic substrate based agar medium
Candichrom	Chromogenic substrate based agar medium
Fluroplate	Flurogenic substrate based agar medium
API-20C AUX	Carbohydrate assimilation
MiniTek	Carbohydrate assimilation
Microring YT	Chemical sensitivity
Candifast	Biochemical profiling
RapID yeast plus	Biochemical profiling
Iatron Candida Check	Serology
AMS-YBC	Biochemical profiling
Abbott MS-2	Biochemical profiling
Abbott Quantum II	Biochemical profiling

diseases. Fungi most commonly associated with such disease episodes are in the genus *Candida*, most notably *Candida albicans*, which causes both superficial and systemic disease. Even with current antifungal therapy, mortality of patients with invasive candidiasis can be as high as 40% (Wey *et al.*, 1988; Ptaller, 1998). Candidiasis is usually associated with indwelling medical devices (e.g., dental implants, catheters, heart valves, vascular bypass grafts, ocular lenses, artificial joints and central nervous system shunts), which can act as substrates for biofilm growth (Plate-1). In a multicenter study of 427 consecutive patients with candidemia, the mortality rate for patients with catheter related candidemia was found be 41% (Nguyen *et al.*, 1995). Forty percent of patients with microbial colonization of intravenous catheters develop occult fungemia, with consequences ranging from focal diseases to sever sepsis and death (Anaissie *et al.*, 1998; Nguyen *et al.*, 1995). The tenacity with which *Candida* infects indwelling biomedical devices necessitates their removal to effect a cure. Biofilm formation is also critical in the development of denture stomatitis, a superficial form of candidiasis that affects 65% of edentulous individuals (Budtz-Jorgensen, 1990; Budtz-Jorgensen, 1991).

2.8 ULTRASTRUCTURE OF CANDIDA BIOFILM

Several techniques for microscopy examination of biofilms *in situ* on the substrate supporting their growth have been used. These have included transmission electron microscopy (TEM), scanning electron microscopy (SEM), environmental scanning electron microscopy (ESEM), episcopic differential interference contrast microscopy (DIC) with and without fluorescence, Hoffman modulation contrast microscopy (HMC), atomic force microscopy (AFM) and scanning confocal laser microscopy (SCLM) (Thomas *et al.*, 1983;

Ray and Payne, 1988; Mϕller et al, 1996; Chandra *et al.*, 2001a). Cross-sections prepared for TEM analysis gave useful information about the spatial relationships of microorganisms within the biofilm matrix, whilst SEM enabled the surface topology of the biofilms to be examined at a high magnification (Hawser *et al.*, 1998; Chandra *et al.*, 2001a). The preparation required for TEM and SEM, may however, result in the inclusion of artifacts. ESEM and AFM allow direct visualization to intact hydrated specimens at high magnification. AFM images may be rotated and manipulated to provide accurate measurement of individual microorganisms with relative ease. SCLM was used to investigate not only the presence and the ability of the biofilm consortium but also biofilm/substrate interactions (Mϕller *et al.*, 1996). Light microscopy techniques, although unable to reproduce the high magnification of the methods described above, are still of importance in the examination of intact biofilms (Walker and Keevil, 1994; Surman *et al.*, 1996). HMC allows the *in situ* examination of biofilms, a clear image is produced without refracts. DIC may be used to examine biofilms on opaque surfaces and if used in conjunction with fluorescent vital stains can be used to assess the viability of the microbial population Table -5. Hawser and Douglas, 1994 showed mixture of morphological forms of *C. albicans* using Scanning Electron Microscopy (SEM). Baillie and Douglas, in 1999b, further investigated the role of morphogenesis in overall biofilm structure, using scanning electron microscopy. They compared wild types strains of *C. albicans* with two morphological mutants incapable of yeast and hyphal growth, respectively.

On a different type of surface (cellulose fibres), biofilms of wild type *C. albicans* consisted exclusively of yeast cells and the bilayer structure was absent (Baillie and Douglas, 1998b; Baillie and Douglas, 1999b), suggesting that biofilm architecture is

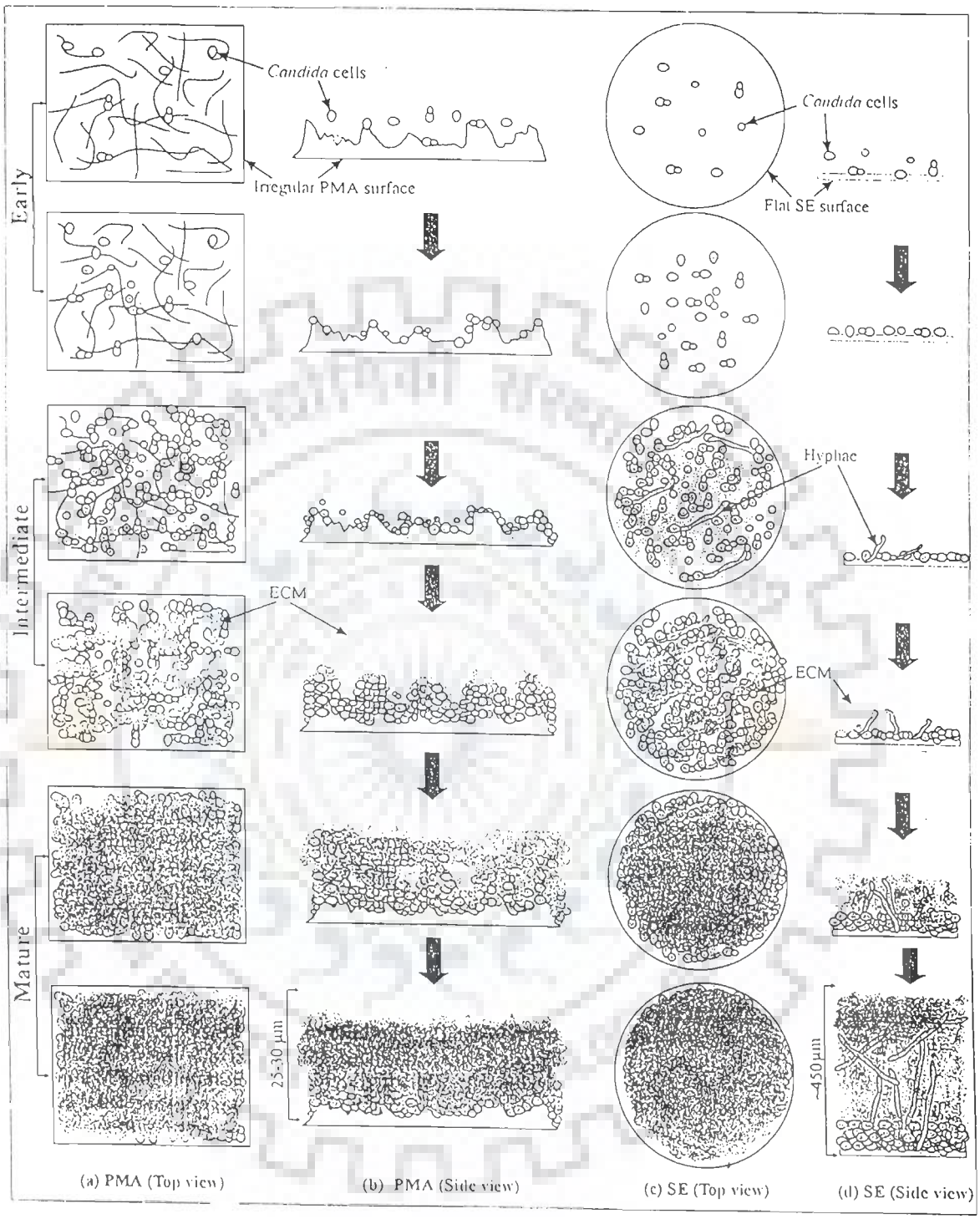


Plate 1: **Schematic representation of biofilm development in *C. albicans*. (a and b) Biofilm grown on polymethylmethacrylate (PMA) strips. (c and d). Biofilm grown on silicone elastomer (SE) disks. Panels a and c represent the substrate seen from the top, while panels c and d show the view from the sides of PMA strip and SE disk, respectively. ECM, extracellular material (Chandra *et al.*, 2001a)**

Table 5 : A summary of the features of microscopes used

Technique	Possible in vivo examination	Generation of artifacts	Special features
Hoffman	Yes	Low	Motility/ real time video
DIC	Yes	Medium	Real time good surface topography
DIC + fluorescence	Yes	Medium	Use of specific fluorochromes
SEM	No	High	Topography of complex shapes
ESEM	No	Medium	Topography analysis
TEM	No	High	Cross sectional internal detail
AFM	Yes	Low	Mechanical properties 3D, topography
Confocal	Yes	Low	Optical thin sections 3-D quantification

dependent on highly specific contact-induced gene expression. Recent CLSM studies suggest that biofilms of both *C. albicans* and *Candida dublineiensis* have similar 3-D structures consisting of microcolonies surrounded by water channels (Ramage *et al.*, 2001a; Chandra, 2001a; Keinanen *et al.*, 2002).

Such studies have also confirmed the yeast hyphal bilayer structure of *C. albicans* microcolonies when biofilms are grown on plastic surfaces (Kuhn *et al.*, 2002b). The spectroscopic techniques such as Raman microscopy (Suci *et al.*, 2001) failed to reveal complex architectural features. Instead, the biofilms, which were grown on a germanium surface, appeared to take the form of relatively homogeneous planer sheets (Lindberg *et al.*, 2001). Matrix polymers of bacterial biofilms are primarily exopolysaccharides and many are negatively charged. Smaller amounts of proteins, nucleic acids and various other components can also be present. However, much of the biofilm matrix (up to 97%) is water (Sutherland, 2001). The matrix of *C. albicans* biofilms has been isolated and its composition compared with that of extracellular polymeric material obtained from culture supernatants of planktonically grown organisms (Baillie and Douglas, 2000). Both preparations contained carbohydrate, protein, phosphorus and hexosamine but the matrix had significantly less carbohydrate (41%) and protein (5%). It also had a higher proportion of glucose (16%) than mannose, and contained galactose, suggesting that it might possess components unique to biofilms (Baillie and Douglas, 2000).

2.9 GENETICS OF CANDIDA BIOFILM

Our knowledge on the molecular genetics of *C. albicans* has proceeded very slowly as compared with conventional yeasts (Magge, 1988; Ghannonum and Abu-Elteen, 1991;

Ausubel *et al.*, 1999). This may due to lacks a sexual cycle in *C. albicans* and its diploid nature, which has made it difficult to manipulate it genetically (Odds, 1988). Twenty years ago, it began to become apparent that *C. albicans* was a diploid organism and therefore it was very difficult to obtain mutant strains. However, in the following years many auxotrophic mutants were obtained and parasexual genetic methods to analyze them were developed. Different mutagenic agents have been used to obtain *C. albicans* mutants. The most common are UV irradiation methyl-nitro-nitrosoguanidine, ethylmethane-sulfonate and nitrous acid (Kakar and Magee, 1982; Poulter *et al.*, 1982; Sarachek and Bish, 1976; Chiang and Burrows, 2003). Several mutant enrichment procedures for auxotrophs have been developed, such as protocols based on amphotericin B treatment (Poulter *et al.*, 1982), inositol starvation and folate pathway inhibitors (Corner and Poulter, 1989). In addition, morphological mutants have been isolated by using differential filtration enrichment techniques (Pomes *et al.*, 1985; Cannon, 1986; Gil *et al.*, 1990). With these procedures, a wide range of mutants has been isolated (Poulter, 1990). Although molecular protocols have now replaced classical mutant isolation in *C. albicans*, the former approach may still provide a source of host strains for direct cloning in *C. albicans* (Goshorn *et al.*, 1992; Negredo *et al.*, 1997). The first experiments allowing the introduction of DNA into *C. albicans* were achieved by homologous integration at the ADE2 locus in the genome of the hOG300 strain (Kurtz *et al.*, 1986). Although integrative transformation is very useful for introducing stable traits or for disrupting genes in *C. albicans*, an auto replicative system is necessary for library screening and DNA recovery. Due to the lack of natural plasmids and to the inability of 2-micron sequences able to promote autonomous replication in *C. albicans* (called ARS, for Autonomously Replicating Sequences). A major advance in *C. albicans* genetic manipulation

was achieved using disruption strategy used in *S. cerevisiae*. The method involves the use of *C. albicans URA3* flanked by *Salmonella typhimurium hisG* genes to provide flanking recombination regions. Recently, three important *C. albicans* genes have been cloned : *TUPI, EFG1,* and *CLA4* (Poulter, 1990; Stoldt *et al.*, 1997; Ramage *et al.*, 2002c; Lewis *et al.*, 2002b). They reported that all of them are important regulators for *C. albicans* morphogenesis. TUPI, a transcriptional repressor, controls filament formation in *C. albicans*. *CLA4* encodes a member of the Ste20p family of serine/threonine protein kinases.

The product encoded by *EFG1* is a member of a conserved class of bHLH (basic helix-loop-helix) proteins that regulate morphogenetic processes in fungi. This protein plays a dual role as a transcriptional activator and repressor, whose balanced activity is essential for the yeast, pseudohyphal and hyphal morphogenetic stages of *C. albicans* (Stoldt *et al.*, 1997). Double mutants *efgl* and *cphl* fail to produce germ tubes and hyphae when tested in mouse model (Loo *et al.*, 2000). Their experiments suggested that the dimorphic transition was an important requisite for biofilm development (Elorza *et al.*, 1985). Ramage, in 2002c, assessed the ability of five mutant strains, with defined defects in genes involved in filamentation (Δ*cphl*, Δ*efgl*, Δ*cphl*/Δ*efgl*, *Dhst7* and *Dcst20*), to form biofilms in comparison to wild type strains (Gaur and Klotz, 1997).

Although cell wall components such as chitin, β-glucan, and lipid play roles in the adhesion of *C. albicans* proteins and mannoproteins are unquestionably the major mediators of adhesion (Klotz *et al.*, 1983, 1993, 1994; Ghannoum *et al.*, 1986; Klotz and Smith, 1991, 1992, 1995; Bendel and Hostetter, 1993; López-Ribot *et al.*, 1995; Saporito-Irwin *et al.*, 1995; Glee *et al.*, 1996; Olson *et al.*, 1996). Early studies in the mid 1980s that sparked interest in the host ligands bound by *C. albicans* and the proteins that mediates such

interactions. Bonali *et al.*, 1987b reported binding of human fibrinogen to the surface of germ tube and mycelium of *C. albicans* (Bouali *et al.*, 1987a) whereas Heidenreich and Dierich, 1985 showed binding of complement fragments iC3b and C3b to the *Candida* species. Since, the additional host ligands and some of the fungal binding proteins or receptors for these ligands have been identified. The most relevant features of candidal cell wall adhesions and receptors for host ligands are summarized in Table 6.

2.10 ANTIFUNGAL SUSCEPTIBILITY OF CANDIDA BIOFILM

One of main consequences of the biofilm mode of growth is the increased resistance to antimicrobial therapy, which is the main reason why biofilm-associated infections are frequently refractory to conventional antibiotic therapy (Goldway *et al.*, 1995; Hawser and Douglas, 1995; Gilbert *et al.*; 1997; Baillie and Douglas, 1999a; Costerton *et al.*, 1999). Antifungal susceptibility testing represents a means of predicting therapeutic concentrations of antifungal drugs used to treat a variety of *Candida* infections (Ghannoum *et al.*, 1996; Ghannoum, 1997). It was not until recently that the National Committee for Clinical Laboratory Standards (NCCLS) published its guidelines for a standardized broth macro- and microdilution assay for *in vitro* testing of antifungal susceptibilities (National Committee for Clinical Laboratory Standards, 1997). NCCLS guidelines use free suspended planktonic cells for *in vitro* susceptibility testing. However, sessile cells from biofilms are phenotypically distinct from their planktonic counterparts and the associated with an increased resistance phenotype (Hawser and Douglas, 1995; Hawser, 1996; Baillie and Douglas, 1998a; Baillie and Douglas, 1998b; Baillie and Douglas, 1999). Consequently, for suspected biofilm-related infections, NCCLS standardized testing does not provide an accurate *in vitro-in vivo* correlation. When bacteria exist in the biofilm form they are 10-1000 times more resistant to

Table 6 : Adhesins and binding proteins (Source: Chaffin *et al.*, 1998)

Adhesin-ligand interaction	Location	Comments	References
Laminin binding proteins			
P37 (37 kDa), 67 kDa	Yeast cell, hyphal cell wall	67 KDa only yeast cell wall; p37 of yeast but not hyphal cells binds laminin; reacts with human high affinity receptor; p37ubiquitinated, collagen like domains	Lopez-Ribot *et al.*, 1994; Sepulveda *et al.*, 1995; Sepulveda *et al.*, 1996
68, 62, 60 kDa	Hyphae > yeast cells	Multifunctional binding; fibrinogen, plastic, laminin, C3d	Bouali *et al.*, 1987a; Bouchora *et al.*, 1990
Multiple species		Ligand affinity blot of yeast cell wasll extract	Jakab *et al.*, 1993
Viltronectin binding protein			
30 kDa	Yeast cells	Ligand affinity blot will cell wall extract	Jakab *et al.*, 1993; Limper and Standing, 1994; Silva *et al.*, 1995
Protein-sugar			
Fucose binding protein	Yeast cells, hyphae?	Buccal, vaginal epithelial cell adhesin, strain variation	Brassart *et al.*, 1991; Cameron and Douglas, 1996; Critchley and Douglas, 1987; McCourtie and Douglas, 1985; Tosh and Douglas, 1992
> 15.7 kDa			Cameron and Douglas, 1996; Tosh and Douglas, 1992
GlcNAc binding protein	Yeast cells, hyphae ?	Buccal, vaginal epithelial cell adhesin; strain variation	Brassart *et al.*, 1991; Cameron and Douglas, 1996; Critchley and Douglas, 1987, McCourtie and Douglas, 1981, 1985
190 kDa	Yeast cells	Cell wall extracts of galactose but not glucose grown cells; not known if is the same protein in reference 345	Enache *et al.*, 1996
Protein-plastic			
Plastic binding proteins		Binding to many plastics environmental influences	Hawser and Douglas, 1994; Kennedy *et al.*, 1989; Kerridge, 1993; Samaranayake and MacFarlane, 1980
60, 68, 200, > 200 kDa	Hyphae	60, 68 kDa may be multifunctional binding fibrinogen, laminin, C3d	Bouali *et al.*, 1987a; 1987b Tronchin *et al.*, 1988
30 kDa	Yeast cells, hyphae	Bind polystyrene and epithelial cells	Barki *et al.*, 1993; 1994

antibiotics that are planktonic cells (Lewis, 2002; Donlan and Costerton, 2002). Corresponding resistance of *Candida* biofilms to antifungal agents was first demonstrated in 1995 (Hawser and Douglas, 1995). All clinically important antifungal agents amphotericin B, fluoconazole, flucytosine, itraconazole and ketoconazole agents showed much less active against *C. albicans* biofilms on PVC discs than against planktonic cells. Drug concentrations required to reduce metabolic activity by 50% were five to eight times higher for biofilms than for planktonic cells, and 30-2000 times higher than the corresponding minimum inhibitory concentrations (MICs) (Baillie and Douglas, 1999a; Baillie and Douglas, 1998a; Ramage *et al.*, 2001b; Chandra *et al.*, 2001b). Scanning electron microscopy revealed that the biofilm structure remained intact at amphotericin B concentration of 11 times of MIC (Ramage *et al.*, 2002a; Ramage *et al.*, 2002b). Biofilms of non-*C. albicans* species, such as *C. tropiclalis* and *C. parapsilosis*, were also drug resistant (Hawser and Douglas, 1995). Subsequent studies have demonstrated drug resistance for *Candida* biofilms grown on cellulose (Baillie and Douglas, 1998a; Baillie and Douglas, 1999a) polystyrene (Ramage *et al.*, 2001a; Ramage *et al.*, 2001b) silicone elastomer (Chandra *et al.*, 2001a), polyurethane (Lewis *et al.*, 2002) and denture acrylic (Chandra *et al.*, 2001b). Recently, however, it has been reported that some of the newer antifungal agents are active against *Candida* biofilms (Douglas, 2002; Douglas, 2003). Although biofilms of *C. albicans* and *C. parapsilosis* were clearly resistant to two new triazoles (voriconazole and revuconazole), there appeared to be some antibiofilm activity with lipid formulations of amphotericin B and two echinocandins-caspofungin and micafungin (Kuhn *et al.*, 2002b). The efficacy of caspofungin against *C. albicans* biofilms *in vitro* has now been confirmed by other workers (Bachmann *et al.*, 2002; Ramage *et al.*, 2002b). Caspofungin is the first antifungal agent to be licensed that inhibits the synthesis of β 1,3-glucan, the major structural component of *Candida* cell walls; glucan

synthesis might prove to be a particularly effective target for biofilms if, as seems possible from analytical data (Sturtvant and Calderone, 1997; Baillie and Douglas, 2000), the biofilm matrix also contains this polysaccharide. These intriguing recent findings could lead to important developments in the treatment of fungal infections due to implants.

Chapter 3

MATERIALS AND METHODS

3.1 MATERIAL

3.1.1 Indwelling medical devices (IMDs)

Indwelling medical devices (IMDs)	
Intra uterine device (Model: 200B)	SMB Corporation of India, Mumbai, India
Urinary catheters	TTK Maersk Medical Ltd., Aurangabad, India

3.1.2 Clinical Isolates from Infected Indwelling Medical Device (IMDs)

Clinical isolates obtained from patients with infected IMDs used in this study is listed in Table – 7.

Table – 7 : Clinical isolates from patients with infected IMDs

Source				Clinical isolates No.
Device	*Site*	*Hospital*	*Ward*	
Intra uterine device	Uterus	AIIMS	Gynecology	AU6, AU7, AU10, AU11, AU12, AU15, AU20, AU24
Intra uterine device	Uterus	IITR	Gynecology	AU25, AU26, AU28
Intra uterine device	Uterus	FNH	Gynecology	AU8, AU9
Urinary catheter	Urethra (bladder)	AIIMS	Urology	AU1, AU2, AU3, AU4, AU5, AU13, AU14, AU16, AU17, AU18, AU19, AU21, AU22, AU23, AU27, AU29, AU30

3.1.3 Culture media and its composition

3.1.3.1 Sabouraud Dextrose Agar (Oxoid-England)

Constituents	Amount (g/ L)
Peptone	10.0
Dextrose	40.0
Agar	15.0

Distilled water was added to make up to 1 liter volume, pH 5.6

3.1.3.2 Blood Agar (Oxoid-England)

Constituents	Amount (g/ L)
Beaf heart, infusion form	500
Tryptose	10.0
NaCl	5.0
Agar	15.0

Distilled water was added to make up to 1 liter volume.

3.1.2.3 Yeast Extract Peptone Dextrose Agar (Oxoid-England)

Constituents	Amount (g/ L)
Yeast extract	0.3
Peptone bacteriological	10.0
Dextrose	20.0
Agar	15.0

Distilled water was added to make up to 1 liter volume, pH 7.0

3.1.2.4 Nutrient Agar (Oxoid-England)

Constituents	Amount (g/ L)
Beef extract	3.0
Tryptone	5.0
Agar	15.0

Distilled water was added to make up to 1 liter volume, pH 7.0

3.1.3.5 Corn Meal Agar (Oxoid-England)

Constituents	Amount (g/ L)
Corn meal	60.0
Agar	15.0

Distilled water was added to make up to 1 liter volume, pH 6.0

3.1.3.6 Potato Dextrose Agar (PDA) (Himedia-India)

Constituents	Amount (g/ L)
Potato (peeled)	200.0
Dextrose	20.0
Agar	15.0

Distilled water was added to make up to 1 liter volume, pH 5.6 ± 0.2

3.1.3.7 Rice Agar (Bayer-Germany)

Constituents	Amount (g/ L)
Rice	20.0
Agar	20.0

Distilled water was added to make up to 1 liter volume.

3.1.3.8 Luria-Bertani Agar (Himedia-India)

Constituents	Amount (g/ L)
Tryptone	10.0
Yeast extract	5.0
NaCl	10.0

Distilled water was added to make up to 1 liter volume.

3.1.3.9 CHROMagar (HiCrome Candida Agar, Himedia-India)

Constituents	Amount (g/ L)
Peptic digest of animal tissue	15.00
Dipotassium hydrogen phosphate	1.00
Chromogenic mixture	11.22
Chloramphenicol	0.50
Agar	15.00

Distilled water was added to make up to 1 liter volume, pH 6.3 ± 0.2

3.1.3.10 Yeast Fermentation Agar (Himedia-India)

Constituents	Amount (g/ L)
Peptone	10.0
Sodium chloride	5.0
Beef extract	3.0
Sodium hydroxide (1N NaOH)	1.0

Distilled water was added to make up to 1 liter volume.

3.1.3.11 Yeast Nitrogen Base Agar (Himedia-India)

Constituents	Amount (g/ L)
Glucose	40.0
Peptone	5.0
Yeast extract	2.5
$(NH_4)_2 SO_4$	6.0
KH_2PO_4	2.0
$CaCl_2.2H_2O$	0.34
$MgSO_4.7H_2O$	0.5
Sodium lactate (60%)	6.0

Distilled water was added to make up to 1 liter volume.

3.1.3.12 Yeast Nitrogen Base (YNB) Agar

Constituents	Amount (g/ L)
Peptone	5.0
Yeast extract	2.5
$(NH_4)_2 SO_4$	6.0
KH_2PO_4	2.0
$CaCl_2.2H_2O$	0.34
$MgSO_4.7H_2O$	0.5
Sodium lactate (60%)	6.0

Distilled water was added to make up to 1 liter volume.

3.1.3.13 *Amino acids synthetic medium for C. albicans (Lee et al., 1975)*

Chemical	Grams
$(NH_4)_2SO_4$	5.0
$(MgSO_4).7H_2O$	0.2
K_2HPO_4 (Anhydrous)	2.5
NaCl	5.0
Glucose	12.5
L-amino acids	0.5
Alanine	1.3
Leucine	1.0
Lysine	0.1
Methionine	0.0714
Ornithine	0.5
Phenylalanine	0.5
Proline	0.5
Threonine	0.5
Biotin	0.001

Distilled water to 1 liter volume. Combine ingredients and autoclave at 110° C to 20 min.

3.1.3.14 RPMI-1640 Medium (Rosewell Park Memorial Institute; Hyclon Utah)

Constituent	g/ L water	Constituent	g/ L water
L-arginine (free base)	0.200	Biotin	0.0002
L-aspargine (anhydrous)	0.050	D-pantothenic	0.00025
L-aspartic acid	0.020	Choline Chloride	0.003
L-cystine 2HCI	0.0652	Folic Acid	0.001
L-glutamic acid	0.020	Myo-Inositol	0.035
L-glutamine	0.300	Niacinamide	0.002
Glycine	0.010	PABA	0.001
L-histidine (free base)	0.015	Pyridoxine HCI	0.001
L-hydroxyproline	0.020	Riboflavin	0.0002
L-isoleucine	0.050	Thiamine HCI	0.001
L-leucine	0.050	Vitamin B_{12}	0.000005
L-lysine-HCI	0.040	Calcium nitrate H_2O	0.100
L-methionine	0.015	Potassium chloride	0.400
L-phenylalanine	0.015	Magnesium sulfate	0.04884
L-proline	0.020	(anhydrous)	
L-serine	0.030	Sodium chloride	6.000
L-threonine	0.020	Sodium phosphate, dibasic	0.800
L-tryptophan	0.005	(anhydrous)	
L-tyrosine 2 Na	0.02883	D-glucose	2.00
L-valine	0.020	Glutathione, reduced	0.001
		Phenol red, Na	0.0053

3.1.4 Chemicals, Reagents, and Diagnostic Kits

All the chemicals used were obtained from commercial sources and were of analytical grade. Sources for some of the fine chemicals used in this study have been listed below.

- 3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT); 2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT); 3-(N-Morpholino) propanesulfonic acid] (MOPS) buffer; Sodium dodecyl sulfate (SDS); Menadione **(Sigma, USA)**.
- Potassium hydroxide, Tris-buffer, Dextrose (anhydrous); Phenol, **(Ranbaxy, India)**
- Agar-Agar (Bacteriological grade); Toluene, Sucrose, Ethyl acetate, Sodium chloride, Potassium phosphate dibasic anhydrous, Magnesium Sulfate, Peptone, Dimethyl sulfoxide (DMSO), Methanol, Ethanol, **(Qualigens, India)**
- Tetra methylene diamine (TEMED); Ammonium Sulphate, Ammonium Persulfite (APS); Acetic acid glacial, Glutaraldehyde **(SISCO Research Lab., India)**
- Mercaptoethanol **(Spectrochem, India)**
- Acrylamide; Bis-acrylamide **(GENEI, Bangalore, India)**
- Antifungal drugs Amphotericin B; Nystatin; Chlorhexidine **(Sigma,USA)** and Fluconazole **(Pizer Pharmaceuticals Group, USA)**
- Stains- Lactophenol Cotton Blue and Gram Stain **(HiMedia, India)**
- Total protein estimation kit **(Span Diagnostic Ltd, Surat, India)**
- IgG purification kit, HRP labeling kit and Western blot development kit for rabbit antibody **(GENEI, Bangalore, India)**.

3.1.5 Instruments and equipments

Apparatus	Company
Laminar Air Flow	ACCO, India
Homogenizer	Remi motors, Mumbai, India
Gel electrophoresis	Hoefer Scientific Instruments, Sanfranciscs, USA
Western blot	Hoefer Scientific Instruments, Sanfranciscs, USA
Centrifuge	SIGMA 1-15 K
Sensitive balance (Model Precisa 220 M)	Precisa Instrument, Switzerland
Sensitive balance	Samson, India
Scanning Electron Microscopy (SEM) (Model LEO 435 VP)	Leo Electron Microscope Ltd., England
Digital pH meter (Model Cyberscan 510)	Eutech Instrument, Singapore
Autoclave	Vikrant Scientific Ltd., India
Incubator	Colton
Light microscope (Model: Reichert)	Austria
Light microscope with camera	Leica, Germany
Orbital shaker incubator	Gallen Kamp, USA
Haemocytometer	Neubauer, Germany
Water bath	Branson 2200, USA
Gel documentation and analysis system	Alpha Infotech Corporation
Water purification system	PURELAB Plus UV/UF
Ultrasonicator	Misonix, USA
96 well microtitre plates	Greiner Labotechnik
Millipore filter	Millipore
UV/VIS Spectrophotometer	Perkin Elmer
ELISA (Model: Σ960)	Metertech Inc.

3.2 Methods

3.2.1 Maintenance of clinical isolates

Clinical isolates obtained from patients suffering from infected IDMs as listed in Table-7 were maintained on slopes of Luria-Bertani (LB) agar and Sabouraud dextrose agar (Oxoid-England) for bacterial and fungal isolates respectively. The isolates were subcultured monthly. After every 2 months, cultures were replaced by new ones freshly grown from freeze-dried stocks.

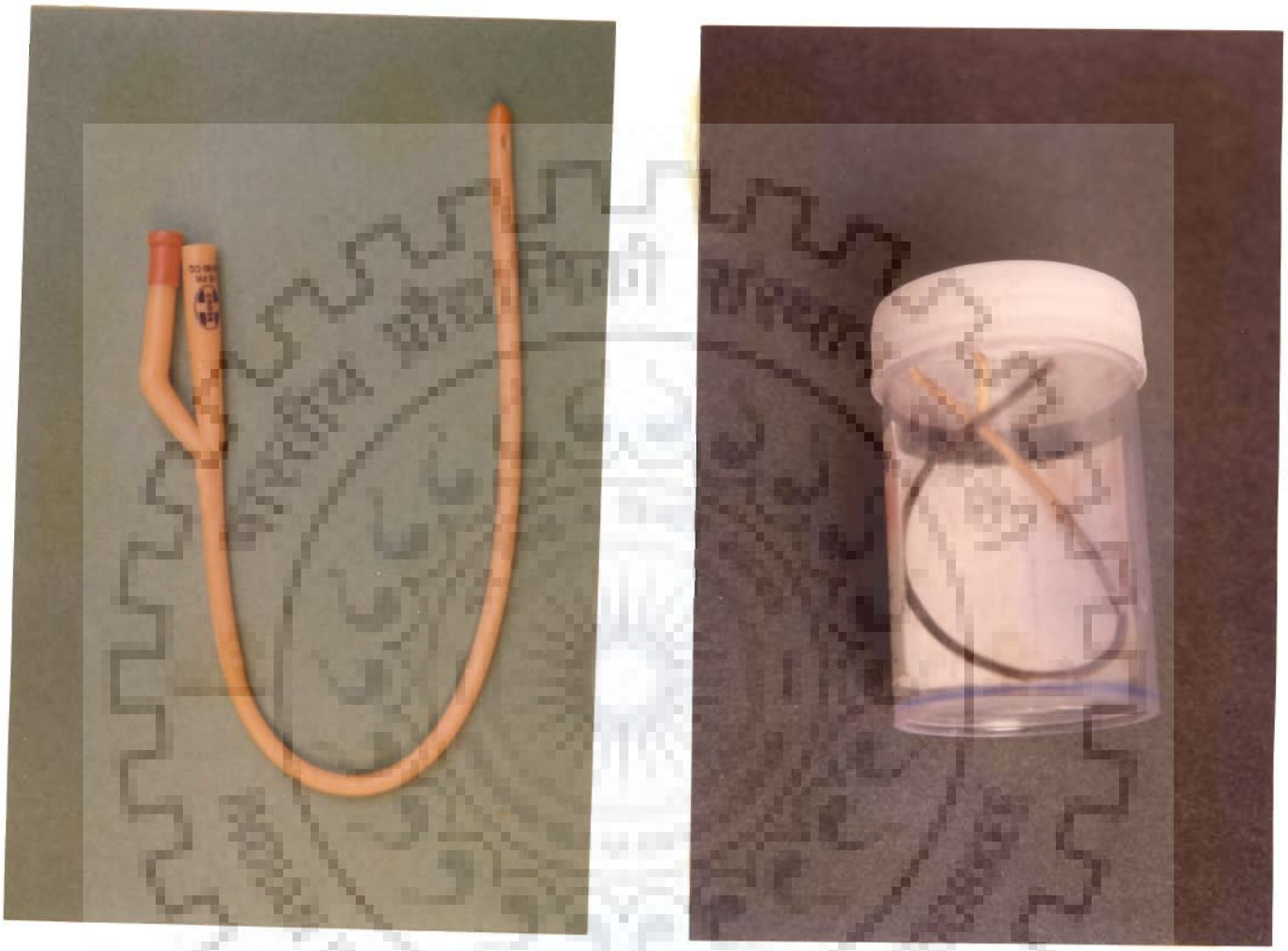
3.2.2 Isolation of biofilm forming clinical isolates from IMDs

3.2.2.1 Urinary catheters

Discs of catheter material (surface area, 0.5 cm^2) were cut from catheters (Plate –2) and were placed in a 10 ml of 0.15 M PBS, pH 7.2 that contained 0.1% Tween-80. It was then sonicated in an ultrasonic cleaner waterbath (Branson 2200, USA) for 30 min at room temperature to detach adherent microorganisms. The microbial suspension was vortexed vigorously for 15 s to break up clumps. Ten fold serial dilutions of each suspension were plated on 5% blood agar base (Oxoid-England) using spread plate technique, incubated at 30°C for 18 h. All microbial strains so obtained were maintained on slants of Luria-Bertani (LB) agar medium and sub-cultured monthly. After every 2 months, cultures were replaced from freeze-dried stocks.

3.2.2.2 Intra uterine devices

The thread that is attached to keep the device in place was found to harbor microbes, which were screened and characterized as described below. Briefly, infected thread pieces (0.5 cm) from the devices (Plate-3) were placed in a 10 ml vol. of 0.15 M PBS that contained 0.1% Tween-80 and sonicated in an ultrasonic cleaner waterbath (Branson 2200, USA) for



A B

Plate 2: A. Sterile urinary catheter
B. Infected urinary catheter

A

B

Plate 3: A. Sterile interauterine device (IUD)
B. Infected IUD

30 min at room temperature to detach adherent microorganisms as described in section 3.2.2.1.

3.2.3 Identification of clinical isolates

The strains were identified upto species level by standard tests listed in Bergey's Manual of Systematic Bacteriology (Washington *et al.*, 1981;Krieg and Holt, 1984) and Cowan and Steel (1974).

3.2.3.1 Morphological tests

For identification of medical important fungal strain germ tube formation studies were performed as described by Odds 1979, 1988. Chlamydospore formations in clinical fungal isolates were studied using corn meal agar media (Williams and Lewis, 2000; Silverman *et al.*, 1990). To check the formation of germ tubes in these clinical isolates standard laboratory method of Hedden and Buck (1980) was followed. Briefly, the test involves the induction of hyphal outgrowths (germ-tubes) from fungal isolates grown in serum for 2-4 h at 37°C.

3.2.3.2 Biochemical tests

The fermentation of carbohydrates (sugars) test was performed by adding 0.2 ml (5 drops) of fungal suspension in saline equivalent to each fermentation tubes (glucose, maltose, sucrose and lactose). The tubes were then incubated at 37°C for 48 hours. Carbohydrate fermentation tests were useful for supplementing carbohydrate assimilation tests results in the definitive identification of fungal species recovered from clinical isolates. Carbohydrate assimilation profile for *Candida* species was obtained as per the procedure given by Di Menna, 1995. Briefly, Candidal growth around the discs impregnated with various sugars on basal agar was examined for zone formation after 48 hours.

3.2.4 Selection of *Candida* species Showing Biofilm forming Ability on Various Biocompatible Materials (BCMs)

A differential medium, CHROMagar for the isolation of clinically important yeasts was investigated. The culture medium and colony colour reading were prepared according to the manufacturer's recommendation. This differentiation was easily visible due to the different colours of the colonies. This study corroborates the usefulness of this medium for the rapid identification of the main species of *Candida* isolated from clinical material.

3.2.5 Quantitative analysis of *Candida* biofilm

3.2.5.1 Culture preparation

Culture preparation for *C. albicans* was done according to the protocol given by Baillie and Douglas, 1999a. Briefly, *C. albicans* was grown in a liquid medium consisting of yeast nitrogen base (Difco) containing an appropriate carbon source such as 50mM glucose. Batches of medium (20 ml, in 100 ml Erlenmeyer flasks) were inoculated from fresh culture slopes and incubated at 37°C in an orbital shaker at 60 rpm. Almost all *Candida* species and strains grow extensively in the budding yeast phase under these conditions. Cells were harvested after 24 h and washed twice in 0.15 M phosphate buffered saline (PBS), pH 7.2. Before use in biofilm assays, all washed cell suspensions were standardized to an optical density of 0.8 at 520 nm.

3.2.5.2 Microtitre Plate (MTP) Assay

Biofilm formation was monitored by the ability of cells to adhere to the wells of microlitre plate (MTP) made of PVC (Greiner Labotechnik). The biofilm forming microorganisms recovered from infected IMDs were grown in LB medium and samples were drawn at 12 h intervals. 100 µl (1:100 diluted LB broth) of these samples were inoculated in

MTP and incubated at 30°C for 10 h. MTP wells were then rinsed thoroughly thrice with 0.15 M PBS to remove free floating microbes. 100 µl of a 1% solution of crystal violet (CV) was then added to each well (this dye stains the cells but not the PVC) and the plates were incubated at room temperature for 15 min. Excess of stain was removed by rinsing with distilled water. The stain that was taken up by biofilm forming microbes was extracted twice in 200 µl aliquots of 95% ethanol, 100 µl of which was transferred to a new MTP and the absorbance was determined in a plate reader at 600 nm (Metertech Microplate Reader Model 960).

3.2.5.3 Determination of dry weights

Microbes formed on infected IMDs were first washed gently with 5.0 ml of 0.15M PBS, pH 7.2 to remove non-biofilm cells. Biofilm forming microbes were then scraped from the device with a sterile scalpel and vortexed gently from 3 minutes. The device was washed in 0.15 M PBS (5 ml) to remove any remaining cells. All organisms were collected on preweighed cellulose nitrate filter (0.45 µm pore size, 25 mm diameter) and given three washes with PBS (5 ml). The filter was dried overnight at 80°C and the dry weight was determined in triplicate.

3.2.5.4 Comparative analysis of Candida biofilm by static and shaking incubation

Biofilms were grown on small disks (surface area, 0.5 cm^2) cut from PVC catheters as described previously (Hawser and Douglas, 1994). Briefly, the disks were placed in wells of 24 well tissue culture plates and standardized cell suspension (80 µl) was applied to the surface of each one. After incubation for 1 h at 37°C (adhesion period), non-adherent organisms were removed by washing with 0.15 M PBS. The disks were then incubated in the wells for 48 h at 37°C submerged in growth medium (biofilm formation). In other

experiments, biofilms were formed on catheter disks incubated with shaking (2-60 rpm) instead of statically. After the 1-h adhesion period (static incubation) non-adherent organisms were removed by washing and before each disk was then transferred to an Erlenmeyer flask (100 ml) containing 20 ml of growth medium and shaking at the appropriate speed for 48 h at 37°C. In control experiments, disks without cells were incubated with or without shaking.

3.2.5.5 Tetrazolium reduction assay

The tetrazolium salt, 3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) was used in an assay modified from that described by (Mosomann, 1983). After biofilm formation on MTP, 50 µl of MTT solution (a stock containing 5 mg of MTT per ml of PBS, diluted 1:5 in prewarmed 0.15 M PBS prior to application) was added to each well. After incubation for 5 h at 37°C, the medium plus MTT was removed, and the wells were washed three times with 2 ml of 0.15 M PBS, pH 7.2 to remove all traces of MTT. Dimethyl sulfoxide (1 ml) was then added to solubilize the MTT formazan product. MTT formazan formation was measured at 540 nm using (Perkin Elmer Lambda Bio-40) UV-VIS spectrophotometer. Control contained media plus MTT to determine background formazan values. All assays were carried out 5 times in triplicate

3.2.5.6 Scanning Electron Microscopy (SEM)

SEM was performed in accordance with procedure given by Hawser and Douglas, 1994. Briefly, biofilm of *C. albicans* formed on polyvinyl chloride (PVC) strips were fixed with 2.5% (v/v) glutaraldehyde in 0.15 M PBS for 1 h at room temperature. They were then treated with 1% (w/v) uranyl acetate for 1 h, and washed again with 3 ml distilled water. The samples were dehydrated with the series of ethanol solutions (Loba Chemie India) which ranged in 10% increased from 30% (v/v) ethanol in distilled water to 50%, 80%, 90% and

100%. All samples were dried to critical point by Polaron critical point drier, coated with gold with a polar on coater, and viewed under SEM, Leo 435, England.

3.2.6 Effect of physico-chemical parameters on *Candida albicans* biofilms

3.2.6.1 Effect of Temperature

YPD media (200 ml) was made and sterilized by autoclaving at 121°C for 15 min. After sterilization media was inoculated with standardized cell suspension (1 ml of suspension containing $3.2x10^6$ cells/ ml in YPD medium) and was incubated in orbital shaker incubator at 5 rpm and 37°C. Biofilm forming cells were obtained at different time intervals (0, 12, 24, 36, 48, 60 and 72 h) from YPD media. Biofilm was formed by pipetting candidal cells suspension (100 µl/ well) into wells of microtiter plates and incubating it overnight at different temperatures (4, 15, 25, 35 and 45°C). After biofilm formation, the medium was aspirated and non-adherent cells were removed by thoroughly washing the biofilms, thrice with 0.15 M PBS, pH 7.2. 50 µl of MTT solution was then added to each well and the plates were incubated for 5 h at 37°C as mentioned in section 3.2.5.5. MTT formazan product formation was measured at 540 nm by using (Perkin Elmer Lambda Bio-40) UV-VIS spectrophotometer. Dry weight estimation of biofilm formed was done as described in section 3.2.5.3.

3.2.6.2 Effect of pH

YPD media (100 ml) of different pH gradient (4.5, 5.5, 6.5, 7.5 and 8.5) was made and sterilized. After sterilization, media was inoculated with standardized cell suspension (1 ml of suspension containing $3.2x10^6$ cells/ ml in YPD medium) and was incubated in orbital shaker incubator at 5 rpm and 37°C. Biofilm forming cells were obtained at different time intervals (0, 12, 24, 36, 48, 60 and 72 h) from cultures grown at different pH values. Biofilms

were formed by pipetting candidal cell suspension (100 µl/well) into selected wells of microtiter plates and incubated overnight 37°C. After biofilm formation, the medium was aspirated and non-adherent cells were removed by thoroughly washing the biofilms, thrice with 0.15 M PBS, pH 7.2. 50 µl of MTT solution was then added to each well and the plates were incubated for 5 h at 37°C as mentioned in section 3.2.5.5. Dry weight estimation of biofilm formed was done as described in section 3.2.5.3.

3.2.6.3. Effect of Osmolarity

YPD media (100 ml) of varying NaCl concentration gradient (0.5, 2.0, 5.0, 10.0 and 15%) was made and sterilize by the autoclaving at 121°C for 15 min. After sterilization, media was inoculated with standardized cell suspension (1 ml of suspension containing $3.2x10^6$ cells/ ml in YPD medium) and was incubated in orbital shaker incubator at 5 rpm and 37°C. Biofilm forming cells were obtained at different time intervals (0, 12, 24, 36, 48, 60 and 72 h) from cultures grown at different NaCl concentrations. Biofilms were formed by pipetting candidal cell suspension (100 ml/ well) into selected wells of microtitre plates and incubated at 37°C for overnight. After biofilm formation, dry weight estimation and MTT formazan product formation was done following the methodology given in section 3.2.5.3 and 3.2.5.5, respectively.

3.2.6.4 Effect of different carbon sources

YNB agar media (100 ml) containing 50 mM of varying carbon sources (glucose, galactose, lactose, maltose, sucrose and raffinose) were made and sterilize by the autoclaving at 110°C for 20 min. After sterilization, media was inoculated with standardized cell suspension (1 ml of suspension containing $3.2x10^6$ cells/ ml in YPD medium) and was incubated in orbital shaker incubator at 5 rpm and 37°C. Biofilm forming cells were obtained

at different time intervals (0, 12, 24, 36, 48, 60 and 72 h) from cultures grown on varying carbon sources. Biofilms were formed by pipetting candidal cell suspension (100 ml/ well) into selected wells of microtiter plates and incubated at 37°C for overnight. After biofilm formation, dry weight estimation and MTT formazan product formation was done following the methodology given in section 3.2.5.3 and 3.2.5.5 respectively.

3.2.7 Preparation of biofilm deficient mutants of *Candida albicans*

3.2.7.1 UV mutagenesis

Cells grown for 48 h were suspended in sterile 0.15 M PBS, pH 7.2 and appropriate dilutions were plated on YEPD agar to give a final count of 2 x 10^2 to 2 x 10^3 c.f.u. per plate, depending on the UV dose to be used. The plated were irradiated in the dark, with a UV atom 70 UV lamp for periods of 20 to 120s at a dose rate of 1.6 μJ $mm^{-2}s^{-1}$ measured with a UV intensity meter (Ultraviolet Products). The irradiated plates and unirradiated control plates were incubated at 37°C for 48 h.

3.2.7.2 Characterization of biofilm deficient mutants

Effect of temperature, pH, osmolarity and different carbon sources at varying time intervals on biofilm deficient mutant of *C. albicans* (*bdm7)* were studied using the protocols as described in section 3.2.6.1, 3.2.6.2, 3.2.6.3, and 3.2.6.4 respectively.

3.2.8 Extraction of *Candida albicans* cell wall protein of wild type and *bdm7*

C. albicans (AU7) and its biofilm deficient mutant (*bdm7)* obtained as describe in section 3.2.7.1 were used in this experiment for extraction of cell wall proteins. The cultures were grown in the minimal medium supplemented with amino acids as mentioned in 3.1.2.12 in an orbital shaker (200rpm) at 37°C for 48 h.

Cells from *C. albicans* (AU7) its biofilm deficient mutant (*bdm7)* were collected by centrifugation and extracted of cell wall proteins was preformed according to Casanova and Chaffin, 1991 and Chaffin *et al.* 1995. Cells collected were resuspended in cold water and were again broken by agitation with glass beads (0.45 mm) using homogenizer (Braun-FRG). Obtained cell walls were again centrifuged and washed extensively with cold distilled water (Cassone *et al.*, 1989; Chaffin *et al.*, 1988; Casanova *et al.*, 1992; Chaffin, 1996). Finally the isolated cell walls was extracted by boiling in SDS-βME and the SDS was removed by extraction with solvent containing acetone-triethylamine-acetic acid-water in the ratio 85:5:5:5 (Konigsberg and Henderson, 1983).

3.2.9 Gel electrophoresis and western blot analysis

3.2.9.1 SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed essentially as described by Laemmli (1970) and modified by Casanova *et al.* (1989) using 5-15% linear gradient gels (14 cm wide by 12-13 cm) with a ratio of acrylamide to bis-acrylamide of 29.2:0.8. Electrophoresis was performed at a constant voltage of 55 V. Prestained molecular mass standards (GENEI, Bangalore, India) included the following: Myosin, (205 KDa); Phosphorydrase b (97 KDa); Bovine serum albumin (66 KDa); Ovalbumin (43 KDa); Carbonic anhydrase (29 KDa); Soyabean trypsin inhibitor (20.1 KDa); Lysozyme (14.3 KDa); Aprotinin (6.5 KDa); Insulin (3 KDa).

a) Preparation of stock solution of gel electrophoresis (SDS-PAGE)

1. Acrylamide-bis-acrylamide (29.2:0.8); is prepared by dissolving 29.2 g of acrylamide and 0.8 g bisacrylamide in a total volume of 100 ml water. The solution was filtered through Whatman No. 1 filter paper, and stored at 4°C in a dark bottle.
2. TEMED; used as supplied. It is stable in undiluted solution at 4°C in a dark bottle.

3. Ammonium persulphate (1.5%, w/v); 0.15 g of ammonium persulphate is dissolved in 10 ml water. This solution is unstable and should be made fresh just before use.

4. SDS (10%, w/v); prepared by dissolving 10 g SDS in water to 100 ml. The solution should be both clear and colourless.

b) Procedure of SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Assemble the plates for casting gel. Clamp the assembly to hold together tightly or fix in a gel casting apparatus. Ensure the assembly is leak proof by filling water between the plates. The plates should be clean and free of detergents. Silicon grease can be applied to spacer to make a good, water tight seal. The comb should be cleaned with water an ethanol.

2. Add 500 µl of water to the APS vial.

3. Mix 70 µl of APS with 7 ml of separating gel mix.

4. Pour the gel solution between the plates till the level is below 3-4 cm form the top of notched plate.

5. After the gel has set (about 20-30 min), wash the top of the separating gel with distilled water and drain off the water completely.

6. Mix 30 µl of APS solution with 3 ml of stacking gel mix and pour directly onto the polymerized separating gel. Insert an appropriate comb into the gel solution carefully without trapping any bubbles till about 1 cm above the separating gel. The stacking gel will set in approximately 10 min.

7. Suspend 100 µl of cell wall extracted protein samples from *C. albicans* (AU7) and its biofilm deficient mutant (*bdm7)* in 50 µl of distilled water, add 50 µl of sample loading buffer and heat at 85°C for 10 min.

8. After the stacking gel has set, carefully remove the comb. Wash the wells immediately with distilled water to remove non-polymerized acrylamide. Straighten the teeth of the stacking gel, if necessary, and fix to a appropriate PAGE apparatus with Tris-glycine buffer in the bottom reservoir. Any bubbles caught between the plates at the bottom of the gel can be removed by squirting Tris-glycine buffer through a syringe with a bent needle. Add Tris-glycine buffer to top reservoir.

9. Load 100 µl of sample supernatant in duplicates into of the wells. The samples can be conveniently loaded using either a microliter syringe (washed by pipetting in the bottom reservoir buffer between each sample) or a micropipetter fitted with a long narrow up.

10. Start electrophoresis at 50 – 150 V.

11. When the dye front comes out of the bottom of the gel, turn off the power pack.

c) *Gel Staining with* Coomassie *Brilliant Blue R-250* according to Merril, 1990

1. Prepare the staining solution : 0.1% Comassie Brilliant Blue R-250 (w/v) in 40% methanol (v/v), 10% acetic acid (v/v). Filter the staining solution after the dye has dissolved. The staining solution is reusable. Store it at room temperature.

2. Soak the gel in an excess of staining solution for 30 min.

3. Destain with a large excess of 40% methanol, 10% acetic acid. Change the destaining solution several times, until the background has been satisfactory remove.

5. Permanent fixation is obtained by incubating the gel in 40% methanol (v/v), 10% trichloroacetic acid (w/v) for 1 h before it is immersed in the staining solution.

d) Molecular Weight Determination

According to Lambin, 1978; Laue and David, 1990 molecular weights of proteins are determined by comparison of their mobilities with those of several marker proteins of known molecular weight. After the gel has been run, but before it has been stained, mark the position of the Bromphenol Blue tracking dye to identify the leading edge of the electrophoretic ion front. This can be done by cutting notches in the edges of the gel or by inserting a needle soaked in India ink into the gel at the dye front. After staining, measure the migration distances of each protein (markers and unknowns) from the top of the resolving gel. divide the migration distance of each protein by the distance traveled by the tracking dye. The normalized migration distances so obtained are called the relative mobilities of the proteins (relative to the dye front) and conventionally denoted as R_f. Construct a (semilogarithmic) plot of the logarithms of the molecular weights of the protein standards as functions of the R_f values. Unknown molecular weights can be estimated by linear regression analysis or interpolations from the curves of log M_r vs R_f.

3.2.9.2 Preparation of antisera

a) Immunization Schedule

New Zealand White rabbits were immunized weekly by subcutaneous injection of $2.5x10^8$ Formalin treated (0.5%) mycelial-phase cells from *C. albicans* (AU7) and its biofilm deficient mutant (*bdm7)* separately in 0.5 ml of Freund complete adjuvant as per the protocol of Walker, 1996. Seven days after their thirds immunization of the rabbits were bled for serum collection. 1 g serum fraction was separated from the crude serum by precipitation with 40% ammonium sulfate for both *C. albicans* (AU7) and its biofilm deficient mutant (*bdm7)*. The precipitate was recovered by centrifugation (22000x*g*, 15 min) and dissolved

with 10 mM Tris-HCl buffer, pH 7.4 and dialyzed against the same buffer containing 1 mM sodium azide individually for *C. albicans* (AU7) and its biofilm deficient mutant (*bdm7*). The dialyzed fluid was used as antiserum (anti-AU7 and anti-*bdm*7) for further experiments.

b) Titration of antibody

10 ml of both antisera (anti-AU7 and anti-*bdm*7) were collected separately and titrated with antigenic samples from crude cell wall extract of *C. albicans* (AU7) and its biofilm deficient mutant (*bdm7*) as per the procedure given below:

(a) The reagents and the samples were brought to room temperature

(b) Serial dilution of both antigenic samples were prepared (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:216, 1:512 and 1:1024) in 0.9% NaCl from the stock solution (2 mg/ml).

(c) 100 µl from the each serial dilution was added along with positive (crude suspension from cell wall extracts of *C. albicans* AU7 and its biofilm deficient mutant *bdm*7) and negative (normal saline) control in the wells of MTP

(d) To the MTP wells 100 µl of antisera (anti-AU7 and anti-*bdm*7) was further added and mixed thoroughly.

(e) The plates were incubated for 20 min and the minimum dilution showing agglutination was used for further experiments.

c) IgG purification

The IgG fraction was purified by affinity chromatography using IgG purification kit (GENEI, Bangalore, India). Briefly, kit contained Protein A bound gel matrix packed in a biocompatible and inert polystyrene column.

Kit contents

1. Oxidation reaction tubes, each containing 2 mg HRP and oxidizing agent	2 Nos.
2. Coupling reaction tube each containing the required reagents	2 Nos.
3. Reductant solution	0.08 ml
4. Quench buffer	0.2 ml
5. Desalting column of size 10 ml	1 No.
6. Phosphate buffered saline 10 x conc.	50 ml
7. Stabilizer	3 ml

Procedure

- Add 0.2 ml doubled distilled water in oxidation reaction tube, mix for 1 h at 20-35° C
- Add purified IgG solution, 10-20 nmole in 0.5-1.0 ml at pH 9-9.2 to coupling reaction tube mix and add immediately oxidized HRP. Mix end to end (preferably use a rotor) for 2 hours.
- Add 10 µl reductant solution and mix for 15min.
- Add 20 µl quench solution and mix for 15 min.
- Pass through the desalting column immediately.
- Equilibrate the desalting column with 1X PBS.
- Break open the tip of coupling reaction tube and allow the content to drain over the desalting column. Add 1 ml of PBS in the coupling reaction tube and allow to drain over the column.
- Pass PBS over the desalting column and collect the HRP colored fraction containing the conjugated labeled antibody.
- Wash the column with 50 ml of PBS and store.

Kit Contents

1. Protein A CL Agarose column		1 No.
2. Equilibration buffer	25 X concentrate	60 ml
3. Elution buffer	5 X concentrate	25 ml
4. Storage buffer	25 X concentrate	10 ml
5. Neutralizing buffer		4 ml

Procedure

1. Pass 10ml of 1X equilibration buffer through the column.
2. Mix the antisera (anti-AU7 and anti-*bdm*7) with equal volume of 1X equilibration buffer and centrifuge for clarity.
3. Load the equilibrated immunesera on column at a rate of <0.25 ml/ minute.
4. Pass 25 ml of 1X equilibration buffer and discard.
5. Pass 0.5 ml 1X elution buffer and discard.
6. Pass 5 ml 1X elution buffer and collect the eluate in tubes containing neutralizing buffer (25 µl/ml). This contains IgG. Dialyze the eluted IgG against the required buffer.
7. To continue purification go to step 1 or pass 10 ml of 1X storage buffer.
8. Store the column and stock reagents at 4 to 8°C.

d) Labeling of antibody (HRP Labelling Kit, GENEI, Bangalore, India)

The purified IgG antibodies of *C. albicans* (AU7) and its biofilm deficient mutant (*bdm7)* were labeled by HRP Labelling Kit (GENEI, India) as per the protocol given below:

- Add 1 ml of the stabilizer in the conjugate, aliquot and store at –20°C.
- Titration of labeled antibody was redetermined by the method followed in section 3.2.9.2 (b).

3.2.9.3 Western blotting

After SDS-PAGE, cell wall extracted proteins of *C. albicans* (AU7) and its biofilm deficient mutant (*bdm7)* were transferred to nitrocellulose paper using a semi-dry electroblotter. The transfer was accomplished at 0.8 mA cm^2 for 4h. The filter was subsequently blocked for 1 h at 37°C in blocking reagent containing 3% w/v bovine serum albumin (BSA) in 0.01 M Tris (pH 7.4) and 0.05 M NaCl (TBS). The filter was rinsed with TBS and incubated with labeled antibodies: anti-AU7 and anti-*bdm7* raised in section 3.2.9.1 (d). Then filter was washed for 3-5 times with wash buffer and immersed in 10 ml of substrate solution with gentle shaking. Colored bands so obtained after 5-10 min. were kept in dark so as to prevent it from fading (Dunbar and Schwoebel, 1990).

Western blotting kit contents (GENEI, India).

1.	Wash buffer	25x concentrate	10 ml
2.	Blocking buffer	5x concentrate	10 ml
3.	Assay buffer	10x concentrate	10 ml
4.	HRP labeled purified anti rabbit IgG (anti-AU7 and anti *bdm7*)		2 ml
5.	Substrate for ALP		10 ml

3.2.10 Antifungal susceptibility to *C. albicans* biofilms

3.2.10.1 Effect of Amphotericin B, Nystatin, Fluconazole and Chlorhexidine

Following biofilm formation by *C. albicans* (AU7) its biofilm deficient mutant (*bdm7)* on MTP, growth medium was removed and MTP wells were washed with 0.15 M

PBS, pH 7.2. YNB broth (4ml) containing different concentrations of an antifungal agent was then added to each well. Antifungal concentrations used ranged from 1-64 μg/ml for nystatin, amphotericin B, and fluconazole and from 8-256 μg/ml for chlorhexidine. The plates were incubated for 48 h at 37°C and washed in PBS; biofilm activity was determined by means of the XTT assay as given by Hawser *et al.*, 1998. Briefly, XTT was prepared in a saturated solution at 0.5 g/L in Ringer's Lactate. The solution was filter sterilized through a 0.22 μm pore size filter, aliquoted and stored at –70° C prior to each assay. An aliquot of stock XTT was thawed, and menadione (Sigma; 10 mM prepared in acetone) was added to a final concentration of 1 mM. 100 μl aliquot of the XTT menadione solution was then added to each prewashed biofilm and to the control well (for the measurement of background XTT-reduction level). The plates were then incubated in the dark for up to 2 h at 37°C. Colorimetric change in the XTT reduction assay indicate direct correlation of the metabolic activity of the biofilm that was measured in a MTP reader (Metertech Microplate Reader Model 960) at 490 nm. The antifungal concentration which caused 50% reduction in metabolic activity of *C. albicans* biofilm compared with control (incubated in the absence of drug) was determined according to the procedure of Hawser and Douglas, 1995; Hawser *et al.*, 1998.

Chapter 4

RESULTS

4.1 ISOLATION AND IDENTIFICATION OF CLINICAL ISOLATES FROM INFECTED INDEWLLING MEDICAL DEVICES (IMDs)

Table-8 shows identity and incidence percentage of microbes in 30 clinical isolates obtained from the patients with infected indwelling medical devices (IMDs). Results obtained indicate high incidences of *Candida* infection (60%) on these IMDs (*Candida albicans* -33.33%, *C. tropicalis* - 16.66%, *C. parapsilosis* - 6.66% and *C. krusei* - 3.33%). On microscopic examination these clinically isolated *Candida* spp. appeared as gram positive (plate-4A), ovoid, budding yeast, sometimes having mould like hyphae (plate-4B) and occasionally with large refractile chlamydospores (plate-4C). Identification of *Candida* species was performed by both microbiological (Table-9) and biochemical procedures (Table-10). Germ tube formation by *C. albicans* after 3h, 6h and 9h time intervals respectively at 37°C is shown in plate-5.

4.2 SELECTION OF CANDIDA SPECIES SHOWING BIOFILM ABILITY ON VARIOUS BIOCOMPATIBLE MATERIALS (BCMs)

Selection of different *Candida* species isolated from patients with infected IMDs is shown in Figure-3. Clinical isolates of *Candida* species: *C. albicans*, *C. tropicalis*, *C. krusei* and *C. parapsilosis* adhering to infected IMDs made up of BCMs appeared as green, blue, pale pink and pink colour colonies respectively on selective media, CHROMagar (Plate-6). Result shown in Figure-4 indicates that among different *Candida* spp. clinical isolate no. AU7 identified as *C. albicans* showed maximum biofilm forming ability on BCM when tested using MTP well assay.

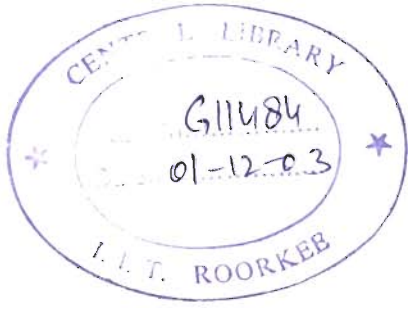


Table-8: Incidence percentage and identification clinical isolates from infected IMDs

Clinical isolates	IMDs	Isolate identity	% Incidence
AU6, AU7, AU11, AU24, AU15	Intra uterine device	*Candida albicans*	16.66
AU14, AU18 AU19, AU21, AU17	Urinary catheter	*Candida albicans*	16.66
AU5, AU13, AU16	Urinary catheter	*Candida tropicalis*	10.00
AU8 , AU12	Intra uterine device	*Candida tropicalis*	6.66
AU2, AU4	Urinary catheter	*Candida parapsilosis*	6.66
AU1	Urinary catheter	*Candida krusei*	3.33
AU10, AU20, AU25	Intra uterine device	*Staphylococcus aureus*	10.00
AU3	Urinary catheter	*Staphylococcus aureus*	3.33
AU29	Urinary catheter	*Staphylococcus epidermidis*	3.33
AU9, AU28	Intra uterine device	*Escherichia coli*	6.66
AU23, AU27	Urinary catheter	*Escherichia coli*	6.66
AU26	Intra uterine device	*Neisseria gonorrhoeae*	3.33
AU22, AU30	Urinary catheter	*Pseudomonas aeruginosa*	6.66

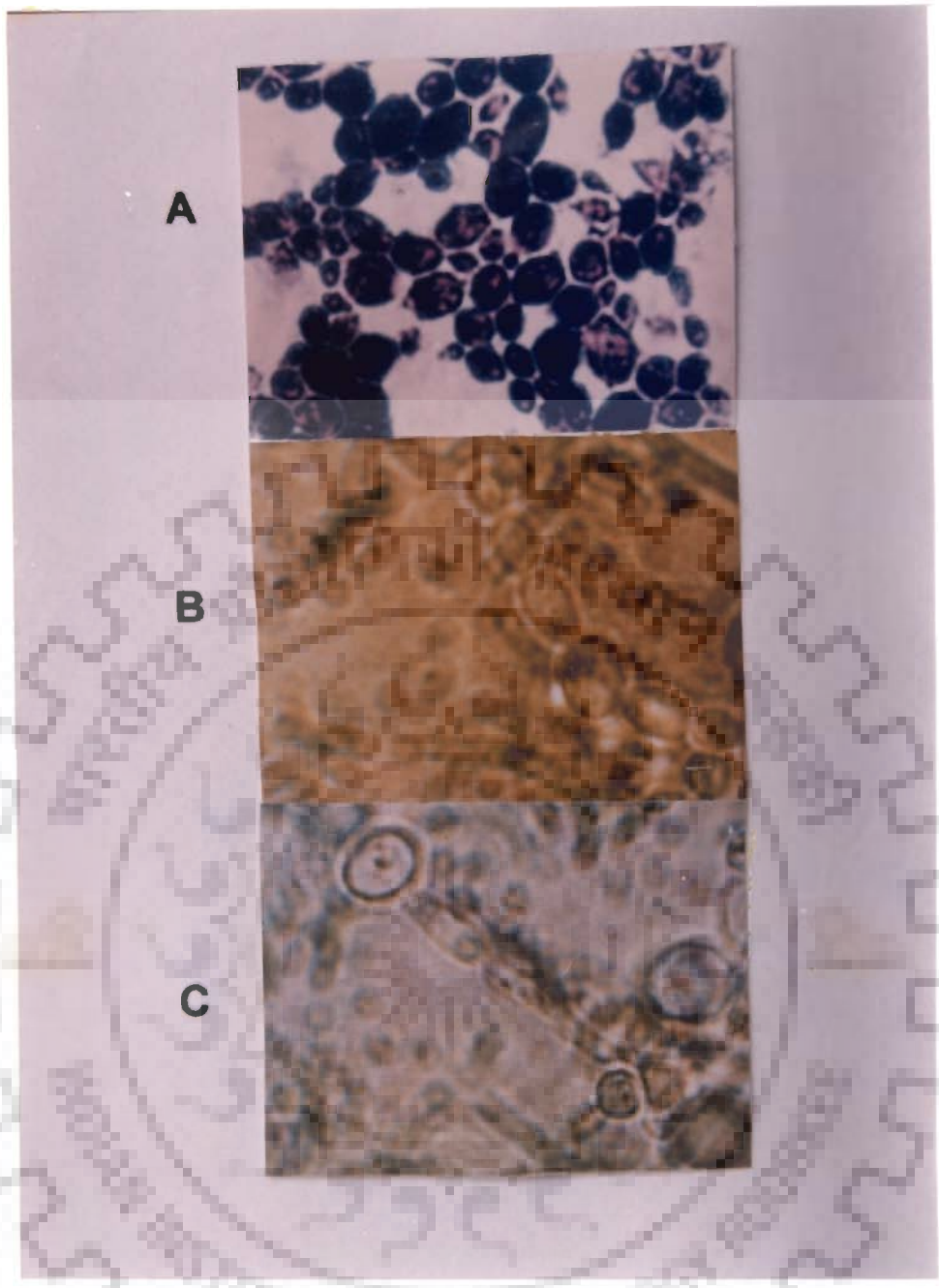


Plate 4: Morphological tests
A. Gram staining of *Candida albicans* (100x)
B. Budding of *C. albicans* (100x)
C. Chlamydospore of *C. albicans* (100x)

Table-9 : Identification of *Candida* spp.

Candida spp.	Morphological examination (Macroscopic)	Microscopic examination	Growth on surface on SDB	Chalamydospores formation on corn meal agar	Germ tube formation
C. albicans	Creamy, bright, smooth colonies	Cells : Cocci to oval shape	–	+	+
C. tropicalis	Creamy to whitish crush colonies	Oval to short oval cells	+	–	–
C. parapsilosis	Yellowish gray colonies	Elongated oval cells to cylinder	+++	–	–
C. krusei	Creamy to yellow bright, smooth colonies	Oval elongate cells or short	–	–	–

Table-10 : The biochemical tests based on fermentation and assimilation by different *Candida* spp.

Candida spp.	Fermentation				Assimilation			
	G	M	S	L	G	M	S	L
C. albicans	A^+G^+	A^+G^+	A^+G^-	–	+	+	+	–
C. tropicalis	A^+G^+	A^+G^+	A^+G^+	–	+	+	+	–
C. parapsilosis	A^+G^+	–	–	–	+	+	+	–
C. krusei	A^+G^+	–	–	–	+	–	–	–

G : Glucose
S : Sucrose
L : Lactose
M: Maltose

+ : Positive
– : Negative
A : Acid Reaction
G : Gas production

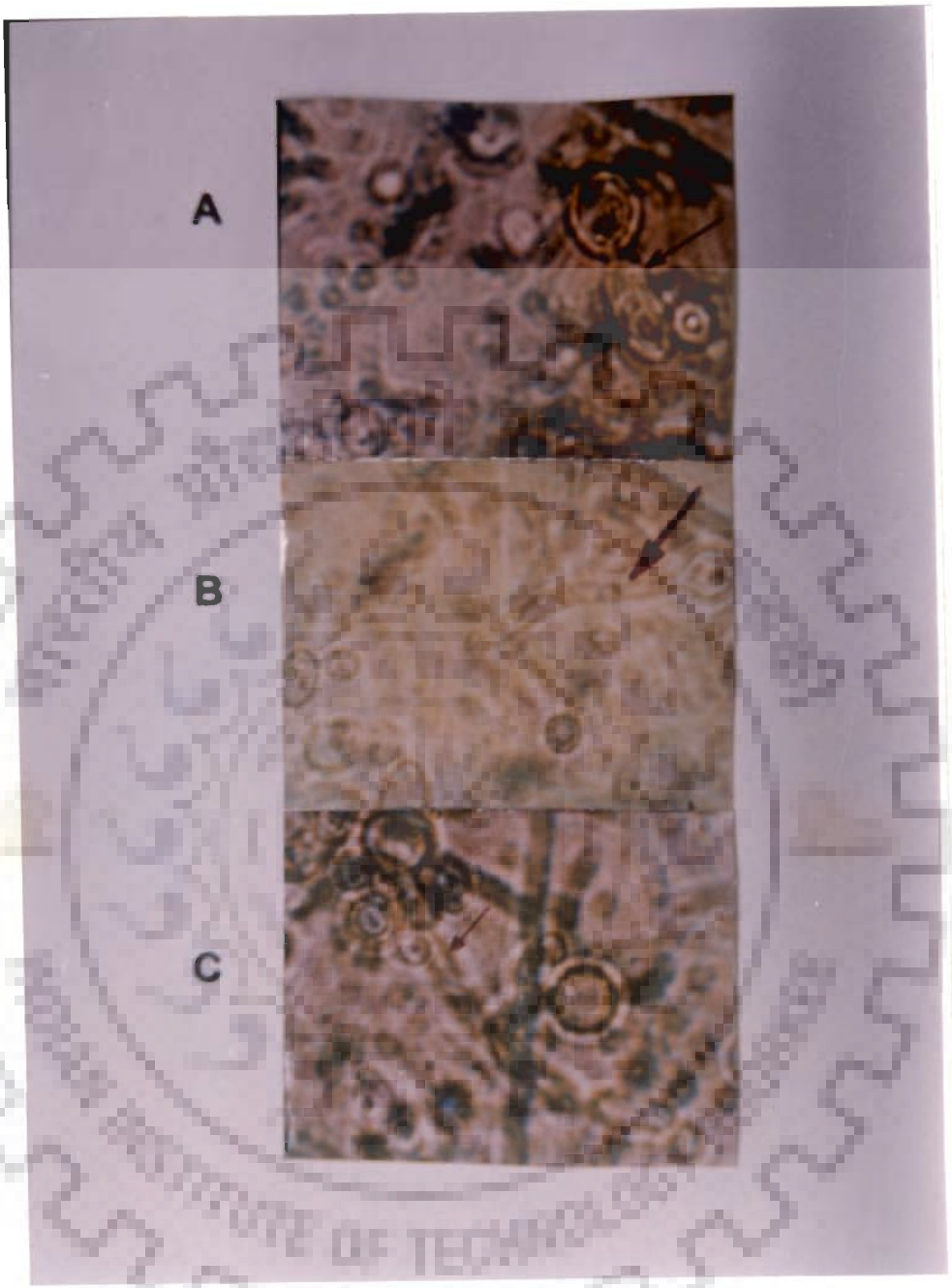


Plate 5: Germ tube formation by *C. albicans* at 37°C after
A) 3 h B) 6 h C) 9 h
Magnification:100x

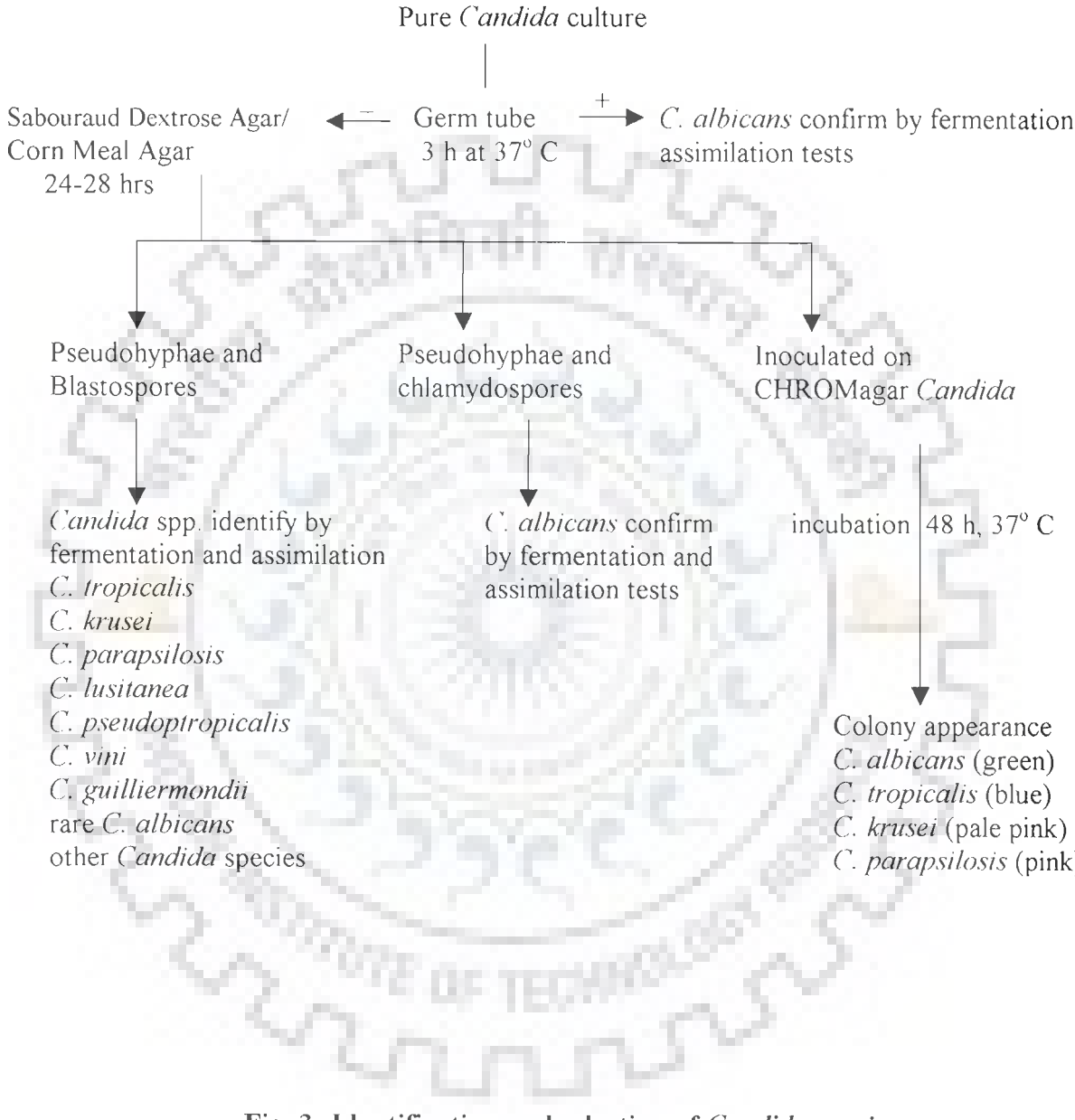


Fig. 3. Identification and selection of *Candida* species

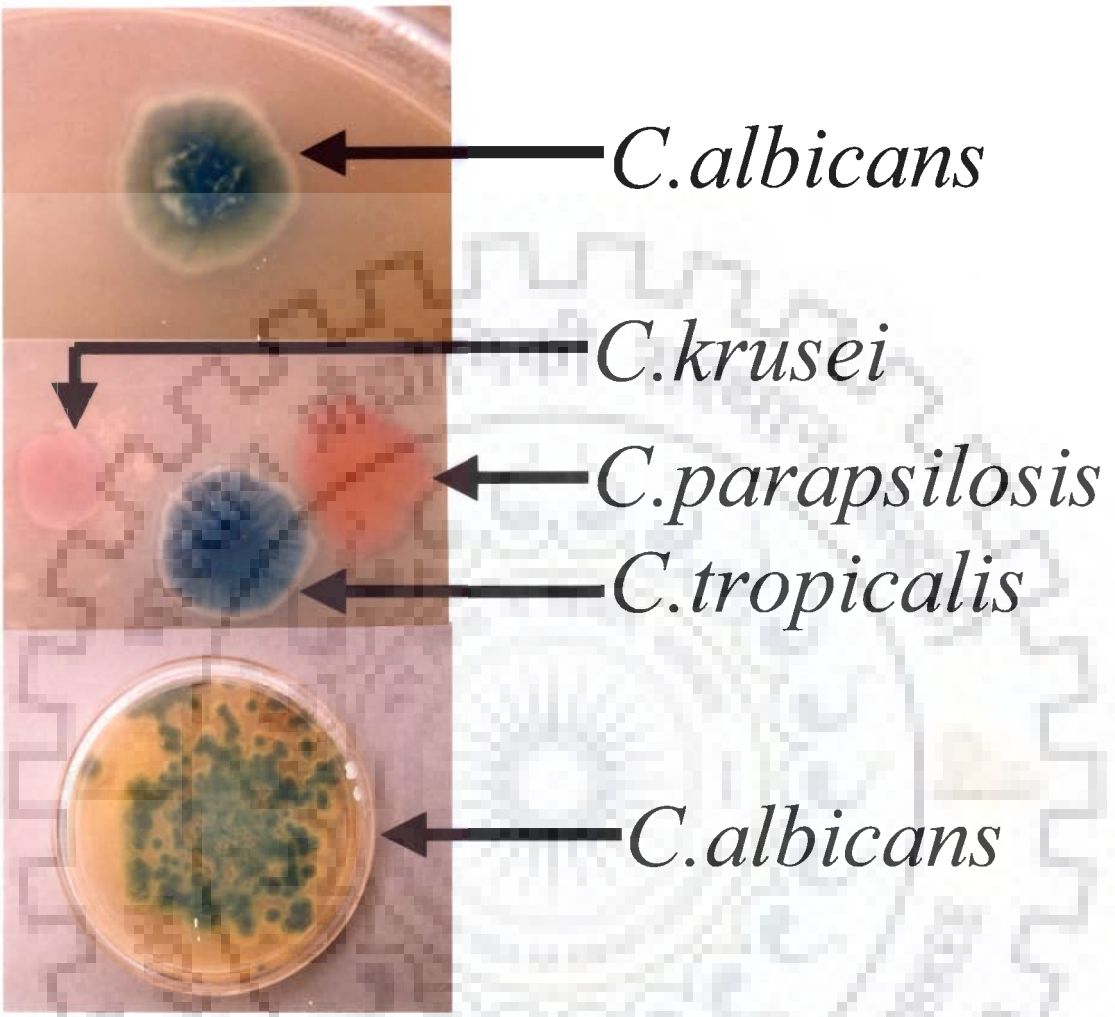


Plate 6: Selection of different *Candida* spp. using CHROMagar media

A. *C. albicans* (green color colony)

B. *C. tropicalis* (blue color colony),
C. krusei (pale pink color colony),
C. parapsilosis (pink color colony)

C. *C. albicans* (green color colonies)

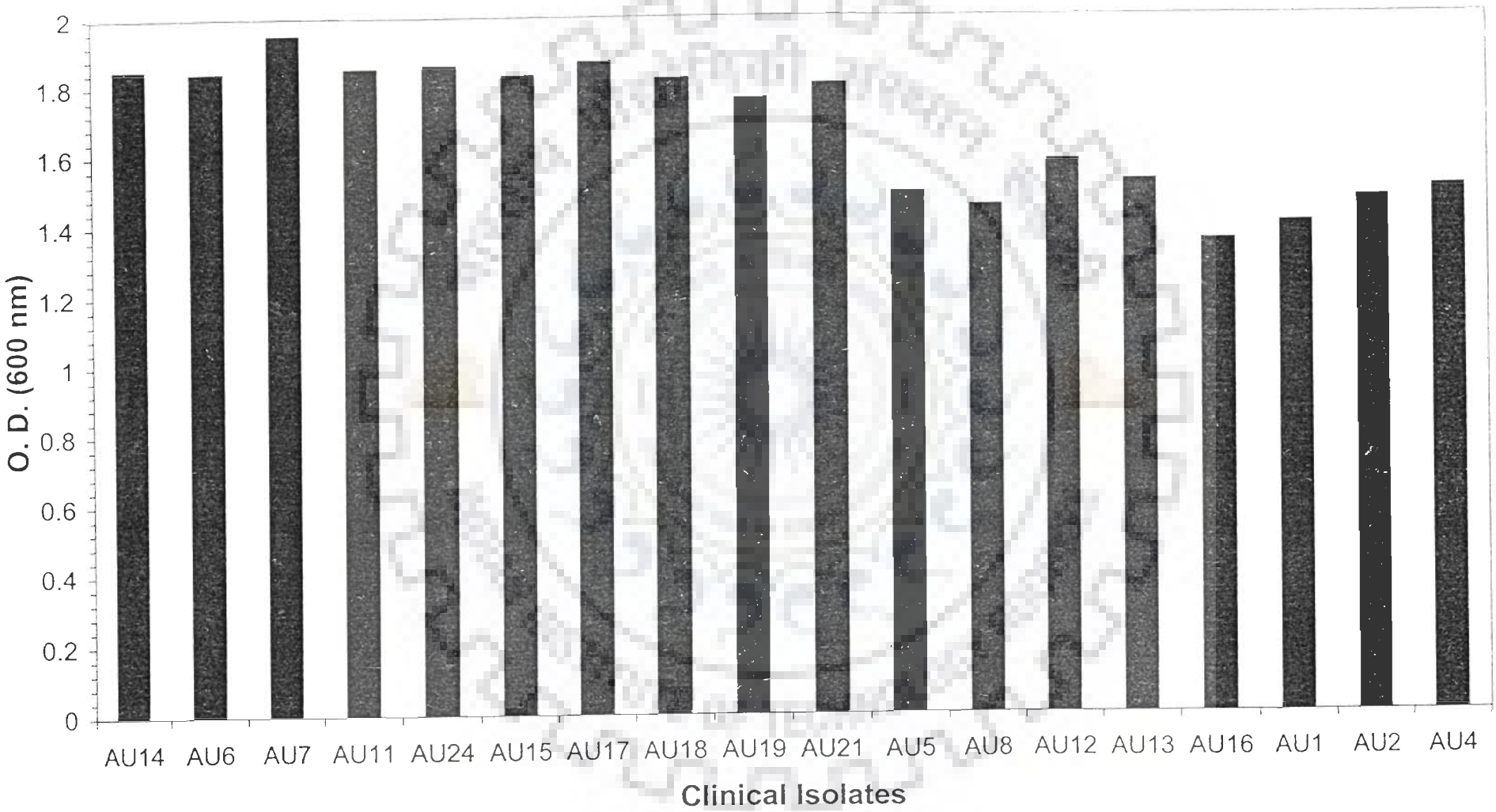


Fig. 4 : Selection of biofilm forming clinical isolates

4.3 QUANTITATIVE ANALYSIS OF CANDIDA BIOFILMS

4.3.1 Microtitre plate assay (MTP)

Results shown in Figure-5 indicate that maximum adherence by *Candida albicans* (clinical isolate no. AU7) to the 96 wells MTP take place after 48 h at 35°C.

4.3.2 Dry weight estimation

Quantitative measurements of *C. albicans* (AU7) biofilm growth on IMDs were made by dry weight estimation. Results shown in Figure-6 indicated that maximum dry weight (1.95 mg) was obtained after 48 h at 35°C.

4.3.3 Comparative analysis of *Candida* biofilm by static and shaking incubation

Data obtained by scanning electron microscopy of biofilm formed by *C. albicans* (AU7) incubated statically for 48 h at 35°C is shown in plate-7A. Results depicts a dense network of yeasts, germ tubes, pseudohyphae, with small amounts of extracellular polymeric material present on the surface of some of these cells. By contrast, incubation of *C. albicans* infected IMDs (catheter disks) with gentle shaking showed marked increase in the synthesis of extracellular matrix material. Slow shaker speeds (2 -5 rpm) produced biofilms consisting of mixture of yeasts and pseudohyphae as before (Plate- 7B), but the cells were surrounded by a thick extracellular matrix. At higher speeds (10-30 rpm) biofilm cells were largely hidden under an extensive canopy of matrix material. Quantitative measurement results shows that maximum MTT formazan product formation by *C. albicans* (AU7) takes place at slow shaker speed (5 rpm) after 48 h at 35°C (Table-11).

4.3.4 Tetrazolium Reduction Assay

Tetrazolium salt, 3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) was used in colorimetric determination of *C. albicans* biofilm formation. The salt reduced by mitochondrial dehydrogenase to violet color tetrazolium formazan product was

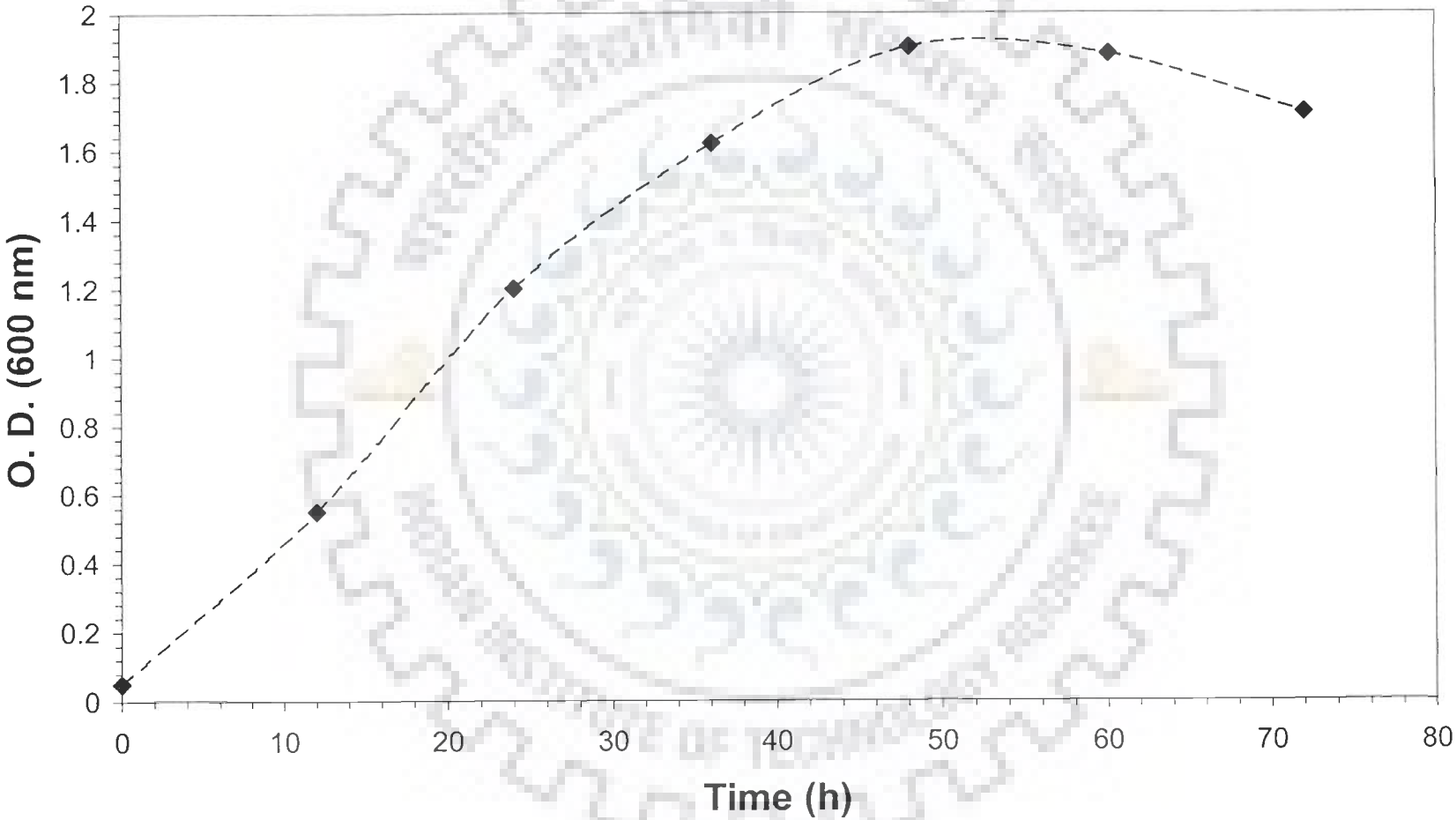


Fig. 5 : Adherence of *C. albicans* (clinical isolated No. AU7) to MTP at different time intervals

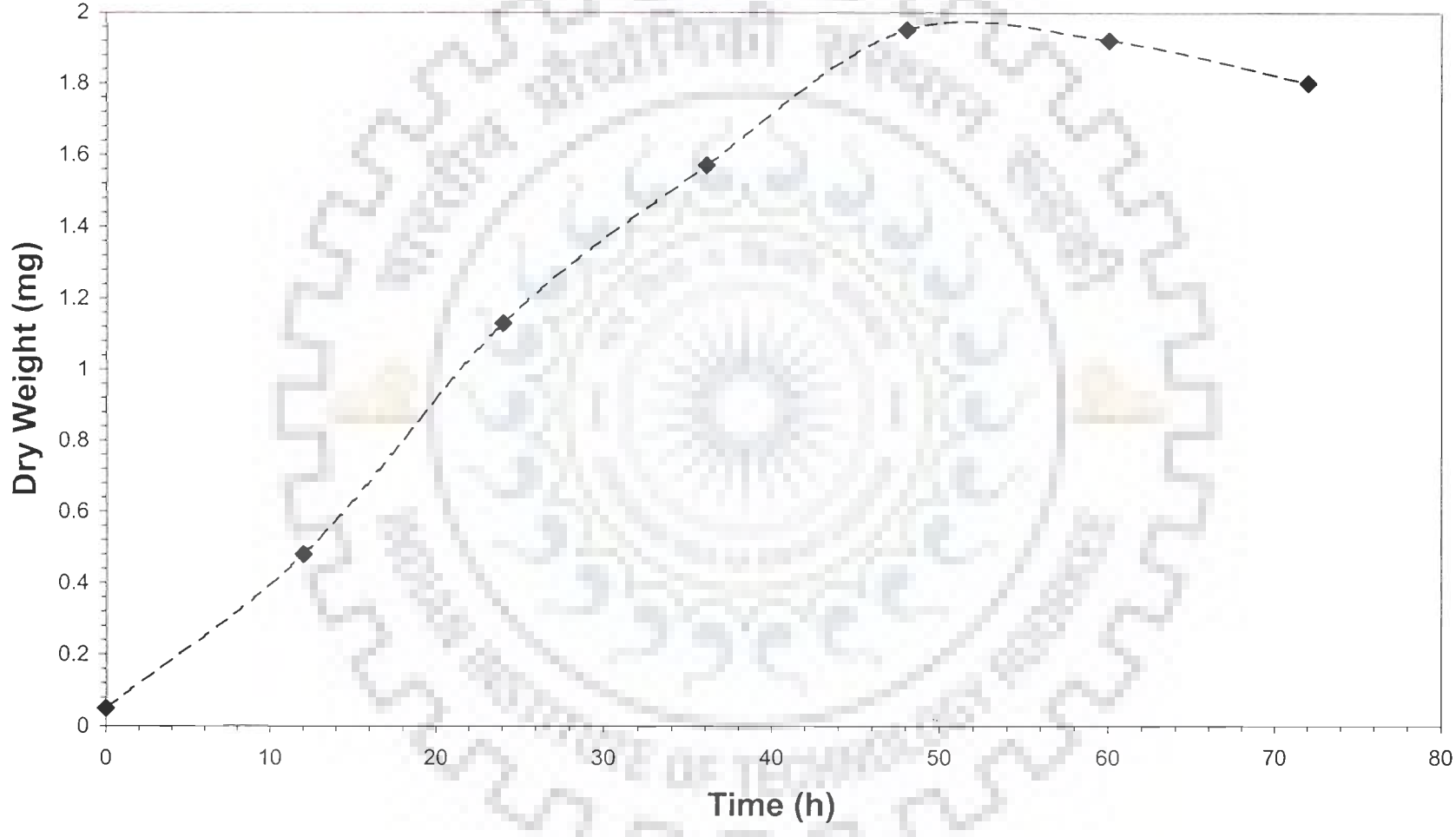
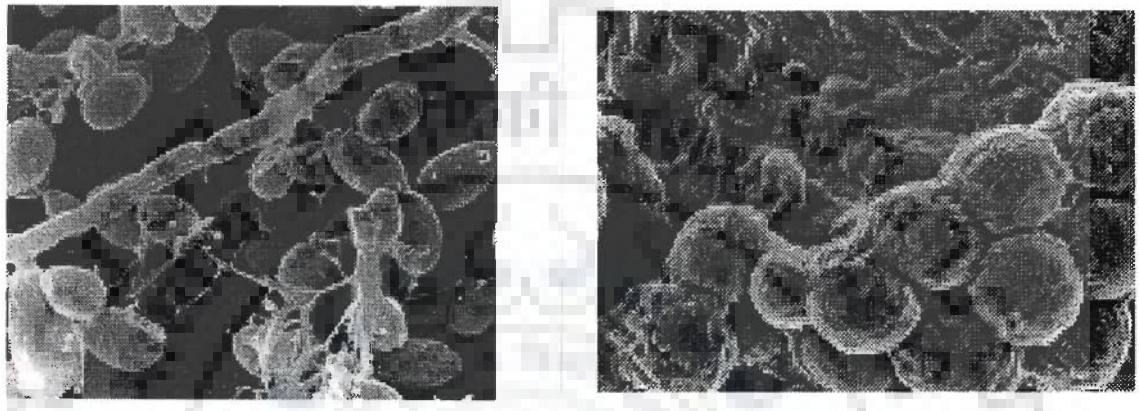


Fig. 6: Dry weight estimation of *C. albicans* (AU7) biofilm at different time intervals



A B

Plate 7: Scanning electron microscopy of biofilm formed by *C. albicans* (AU7) on catheter disks. Biofilm incubated (A) statically (B) gentle shaking (5rpm)

Table-11 : **Quantitative measurements of 48 h biofilm of *C. albicans* (AU7) incubated statically or with shaking**

Shaking speed (rpm)	Dry weight (mg)	MTT formazan formation (O.D. 540 nm)
0	1.90	1.10
2	1.92	1.12
5	1.95	1.36
10	2.21	1.01
20	2.44	1.01
30	2.40	1.00
45	2.38	0.75

determined spectrophotometrically (O.D. 540 nm). Results showed maximum biofilm formation by *C. albicans* takes place after 48 h (Figure-7).

4.3.5 Scanning Electron Microscopy (SEM)

Scanning electron microscopy demonstrated that after 48 h, *C. albicans* (AU7) biofilms consisted of a dense network of yeasts, germ tubes, pseudohyphae, and hyphae. Extracellular polymeric material was also visible on the surfaces of some of these morphological forms (Plate-8). Results from our investigations on development of *C. albicans* biofilms on polyvinylchloride (PVC) strips showed that biofilms formation on this material used in manufacture of biocompatible devices progresses in three distinct development phases. Initially, (0-6h) majority of *C. albicans* cells were present as blastospores (yeast form) adhering to the surface of the strips (Plate-9A). At 6-12h *C. albicans* communities appeared as thick tracks of fungal growth. The intermediate development phase (12-36h) was characterized by the emergence of predominantly noncellular materials, which appeared as hazy-like film covering the *C. albicans* microcolonies (Plate-9B). During the maturation phase (36-72h) the amount of extracellular material increased with incubation time and completely encased the cells (Plate-9C).

4.4 EFFECT OF VARIOUS PHYSICO-CHEMICAL PARAMETERS ON CANDIDA BIOFILMS

4.4.1 Effect of temperature

Figure-8 shows the effect of temperature (4, 15, 25, 35 and 45°C) on *C. albicans* (AU7) biofilm grown at different time intervals (0, 12, 24, 36, 48, 60 and 72 h). Results obtained by tetrazolium salt reduction assay (MTT assay) indicates that maximum biofilm formation take place at 35°C after 48 h. Maximum dry weight 1.85 mg, was also recorded at this temperature (35°C).

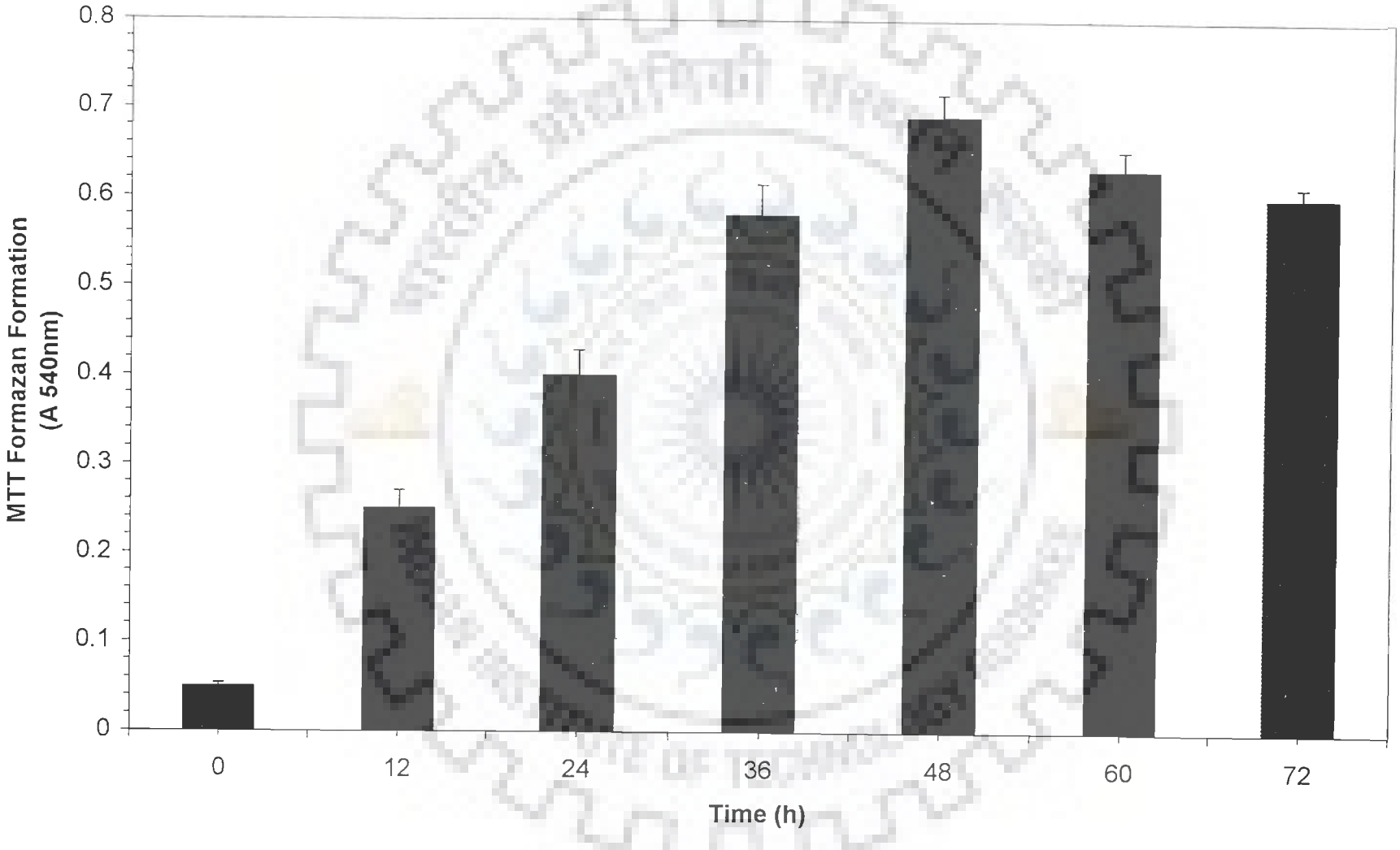


Fig. 7: *C. albicans* (AU7) biofilm formation on MTP wells as determined by the colorimetric MTT-reduction assay

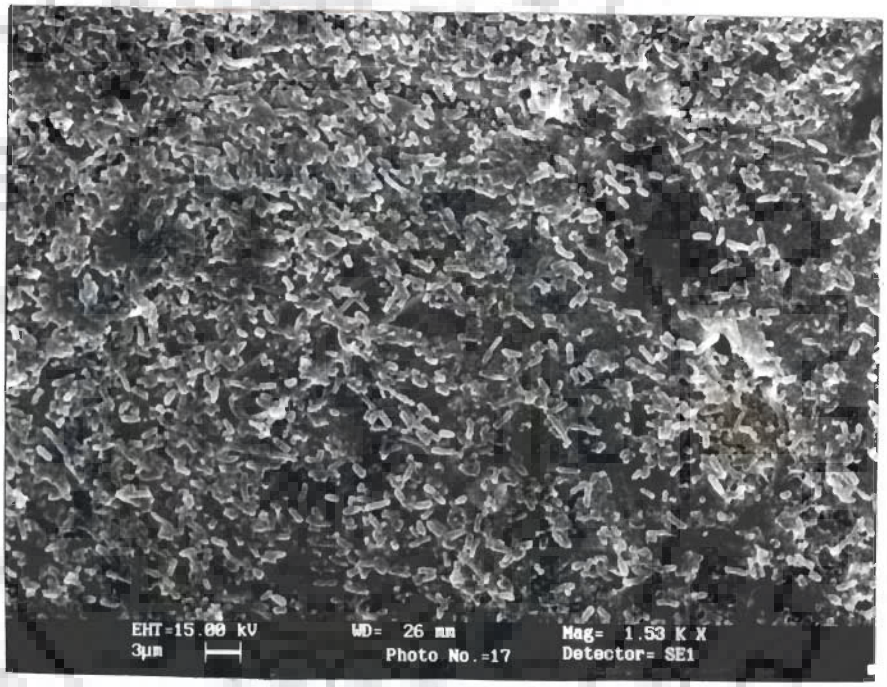


Plate 8: Scanning electron microscopy of *C. albicans* (AU7) biofilm on infected IMD

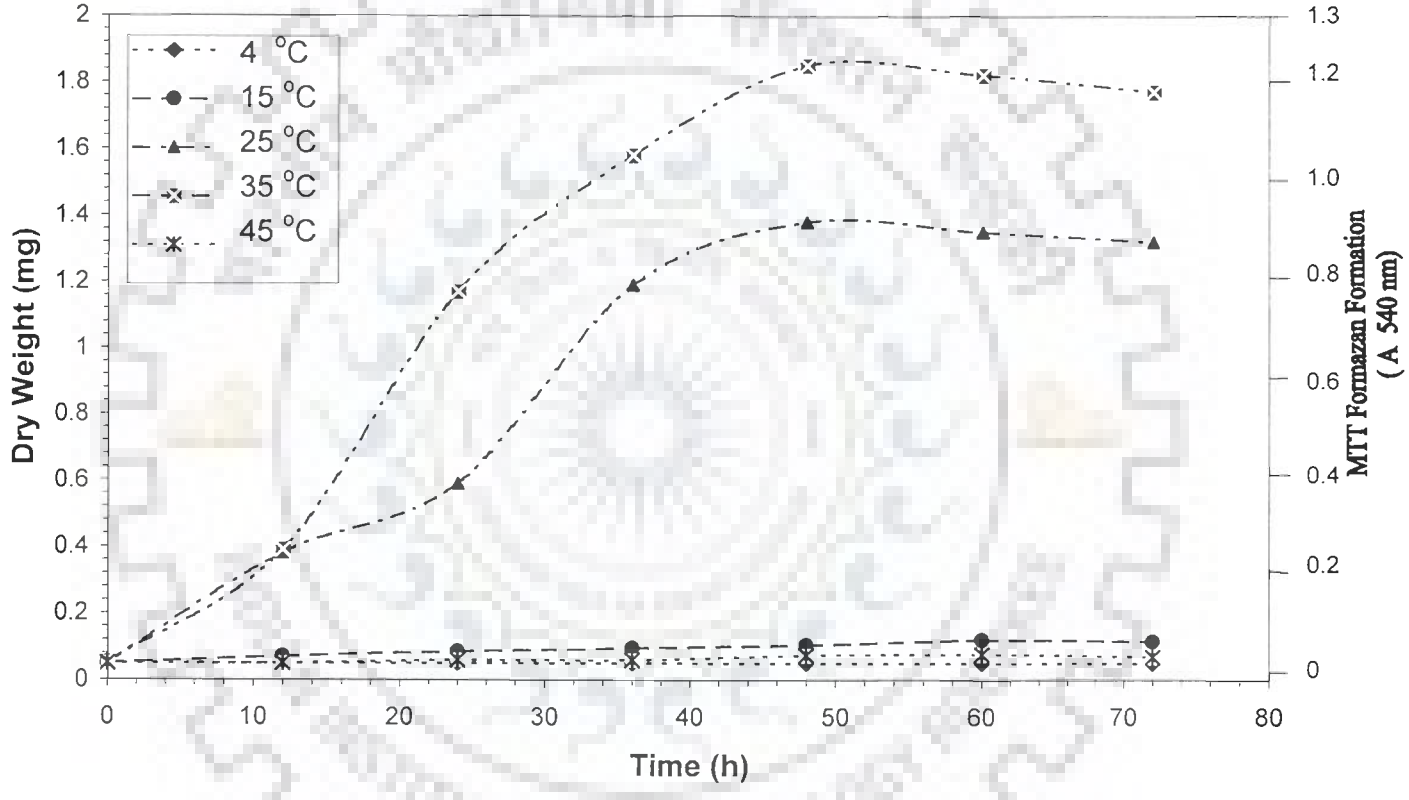


Fig. 8: Effect of temperature on *C. albicans* (AU7) grown at different time intervals

4.4.2 Effect of pH

Data obtained from effect of different pH ranges (4.5, 5.5, 6.5, 7.5 and 8.5) on *C. albicans* (AU7) biofilm at different time intervals (0, 12, 24, 36, 48, 60 and 72 h) indicates that maximum biofilm formation by MTT assay take place at pH 6.5 after 48 h. Maximum value of dry weight (1.3 mg) was also noticed at pH 6.5 (Figure-9).

4.4.3 Effect of osmolarity

Effect of varying NaCl concentration gradient (0.5, 2.0, 5.0, 10.0 and 15%) on *C. albicans* (AU7) biofilm grown at different time intervals (0, 12, 24, 36, 48, 60 and 72 h) is shown in Figure-10. Results obtained by MTT assay indicate that 0.5% NaCl concentration at 48 h is optimum for the biofilm growth. Maximum dry weight (1.2 mg) was also recorded at 0.5% NaCl concentration.

4.4.4 Effect of different carbon sources

Result presented in Figure-11 shows the effect of different carbon sources (glucose, galactose, lactose, maltose, sucrose and raffinose) on the *C. albicans* (AU7) biofilm. MTT assay shows that the maximum biofilm formation take place using galactose followed by glucose as the carbon source after 48 h. Maximum dry weight 1.98 mg and 1.0 mg respectively, were recorded using galactose and glucose as carbon source (Figure-11).

4.5 MUTAGENESIS STUDIES FOR BIOFILM DEFICIENT MUTANTS (BDMs) OF CANDIDA ALBICANS

4.5.1 UV mutagenesis

After UV irradiation (96 μJmm^{-2}) for 60s on *C. albicans* (AU7) we were able to obtain 18 rough colonial morphological variants from the *Candida* cells having smooth colony morphology (Table-12) and selected a biofilm deficient mutant (BDM), namely-

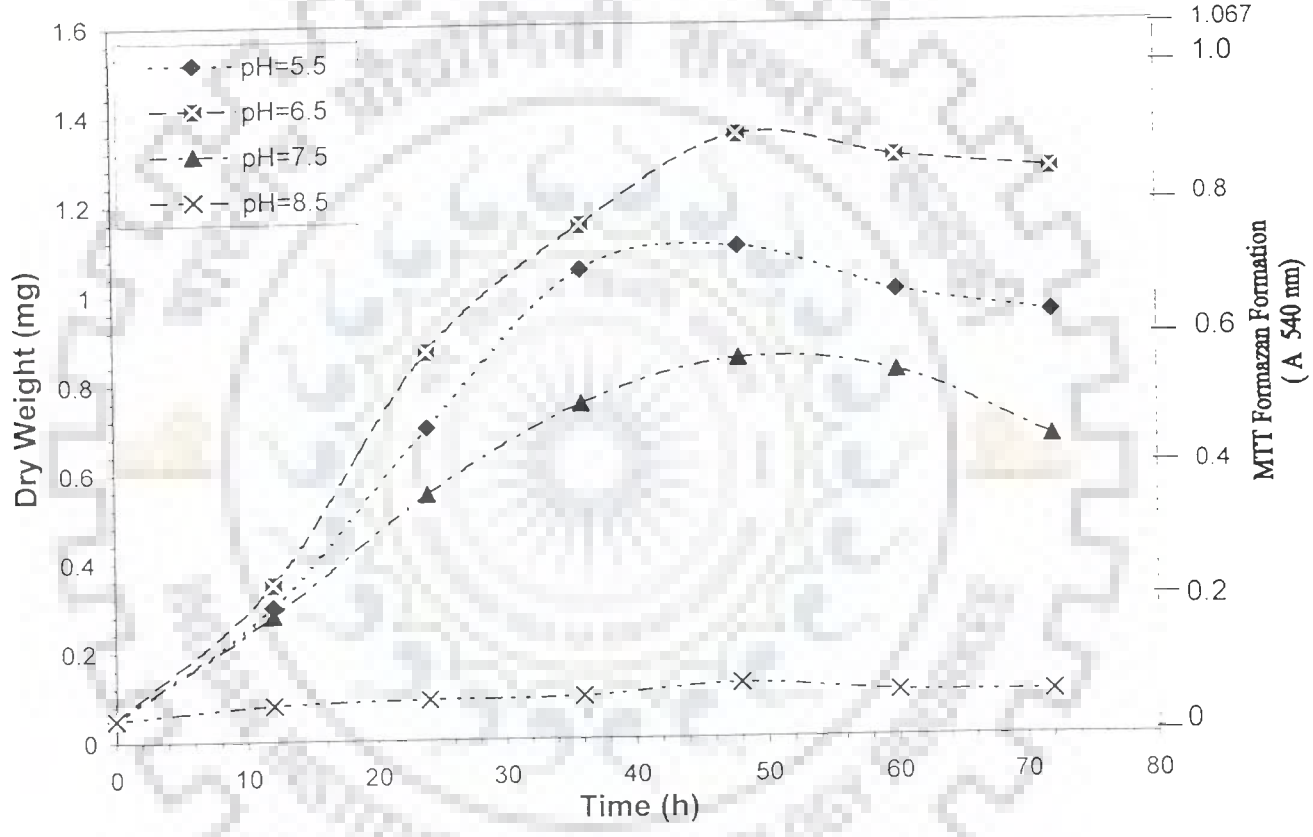


Fig. 9 : Effect of pH on *C. albicans* (AU7) grown at different time intervals

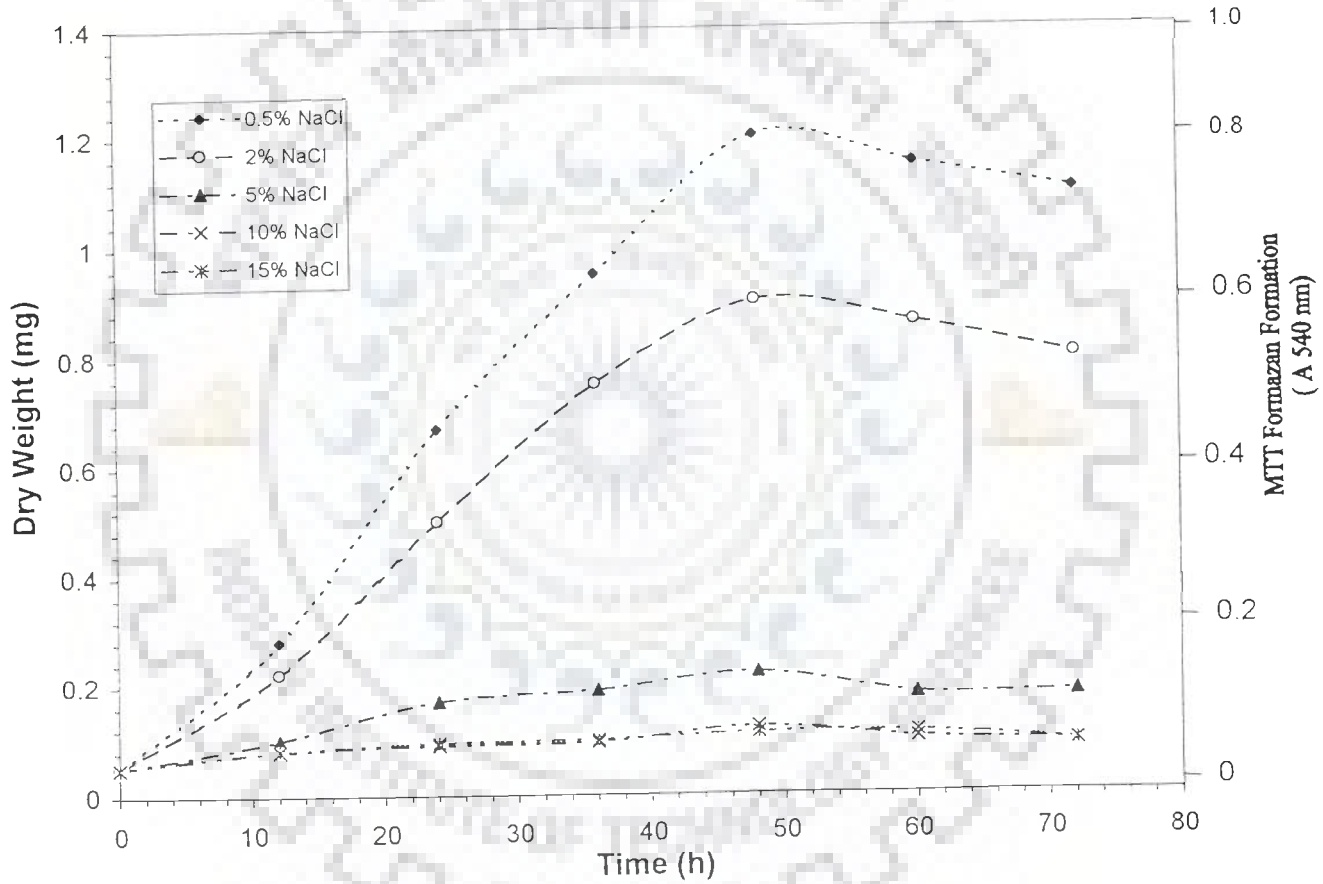


Fig. 10: Effect of osmolarity on *C. albicans* (AU7) grown at different time intervals

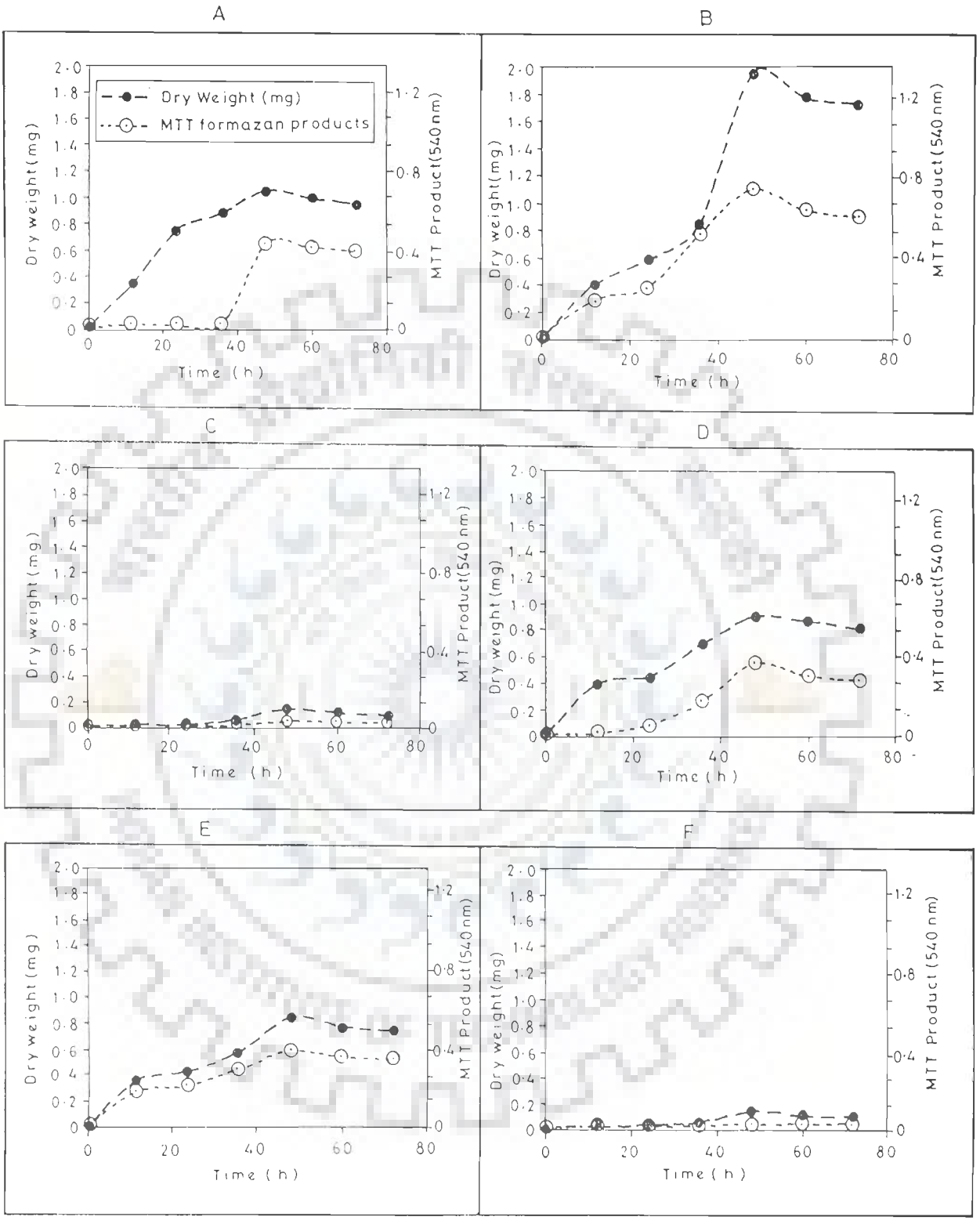


Fig. 11: Effect of different carbon sources on *C. albicans* (AU7) grown at different time intervals

(A) glucose (B) Galactose (C) Lactose (D) Maltose (E) Sucrose and (F) Rafinose

Table 12. **Frequency of rough colony mutants induced by UV treatment of *C. albicans* (AU7)**

UV dose ($\mu J\ mm^{-2}$)	No. of colonies screened	Rough colonies		Percentage survival
		Total	Percentage	
0	10000	0	0	100
32	1985	4	0.2	76
64	1436	6	0.4	28
96	380	8	2.1	12

C. albicans (*bdm7*) on the basis of its rough colonial morphology (Plate-10) and its lack of ability to adhere to the MTP wells made up of biocompatible material (PVC). MTP assay at different time intervals shows that maximum crystal violet colorization take places after 48 h of growth by *C. albicans* (AU7) in comparison to its biofilm deficient mutant (*bdm7*), which showed its incapability for the same (Plate-11).

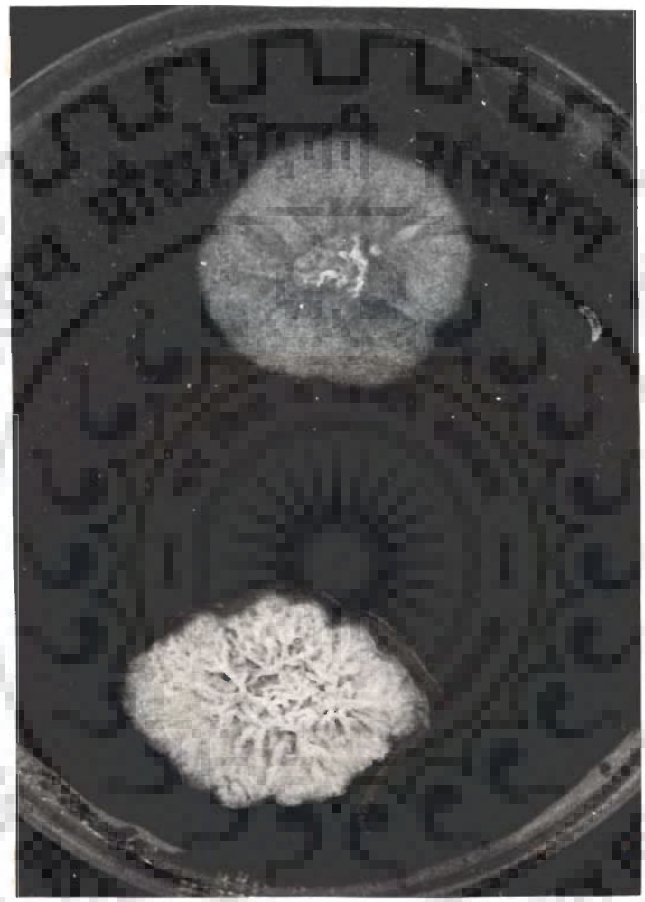
4.5.2 Characterization of BDM

Effect of varying temperatures (4, 15, 25, 35 and 45°C) and different pH ranges (4.5, 5.5, 6.5, 7.5 and 8.5) of *C. albicans* biofilm deficient mutant (*bdm7*) at different time intervals (0, 12, 24, 36, 48, 60 and 72 h) are shown in Figure-12 and 13, respectively. Results show optimum temperature and pH for biofilm deficient mutant (*bdm7*) to be 35°C and 6.5, respectively. MTT formazan formation along with dry weight estimation of the above mutant (*bdm7*) at varying NaCl concentrations (0.5, 2.0, 5.0, 10.0 and 15%) shows 0.5% NaCl to be optimum (Figure-14). Among different carbon sources (glucose, galactose, lactose, maltose, sucrose and raffinose) studied for the above mutant (*bdm7*) maximum dry weight and MTT formazan formation was recorded using galactose as a carbon source (Figure-15).

Comparative analysis of biofilm formation by *C. albicans* (AU7) and its biofilm deficient mutant (*bdm7*) carried out using MTT assay at different time intervals. Results show maximum violet colorization (formazan product formation) takes place after 48 h of growth by *C. albicans* (AU7) in comparison to biofilm deficient mutant (*bdm7*) when measured spectrophotometrically at 540 nm (Figure – 16).

4.6 GEL ELECTROPHORESIS AND WESTERN BLOT ANALYSIS

Electrophoretic mobility of protein standards along with cell wall components of *C. albicans* (AU7) and its biofilm deficient mutant (*bdm7*) released by βME treatment were analyzed by SDS-PAGE (Plate –12A).



C. albicans (AU7)

C. albicans (*bdm*7)

Plate 10: **Comparative colony morphology between *C. albicans* (AU7) and its biofilm deficient mutant *(bdm*7).**

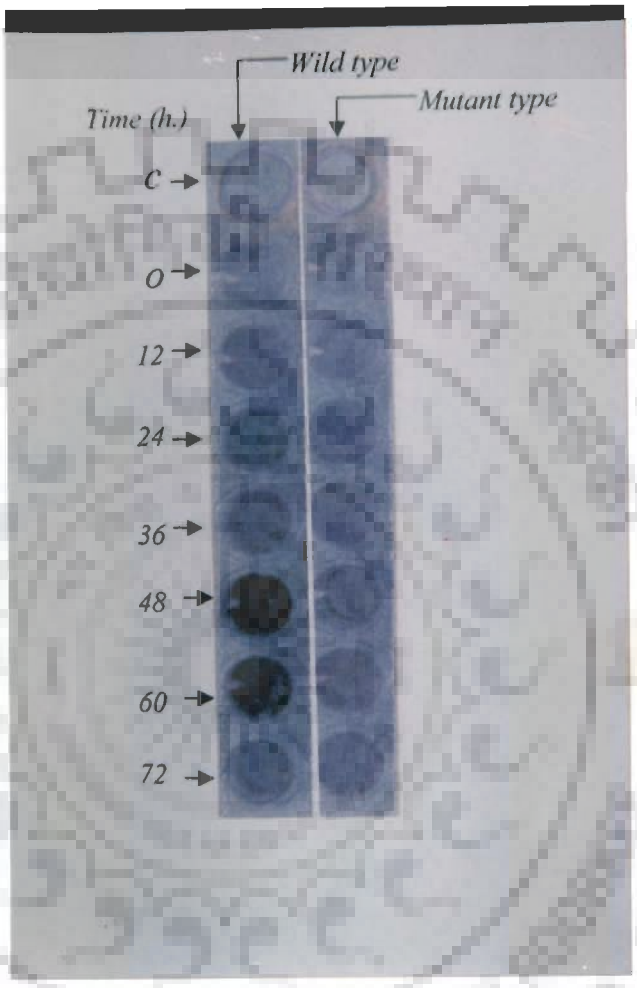


Plate 11: Biofilm formation by *C. albicans* (AU7) and its mutant (*bdm*7) on the MTP wells

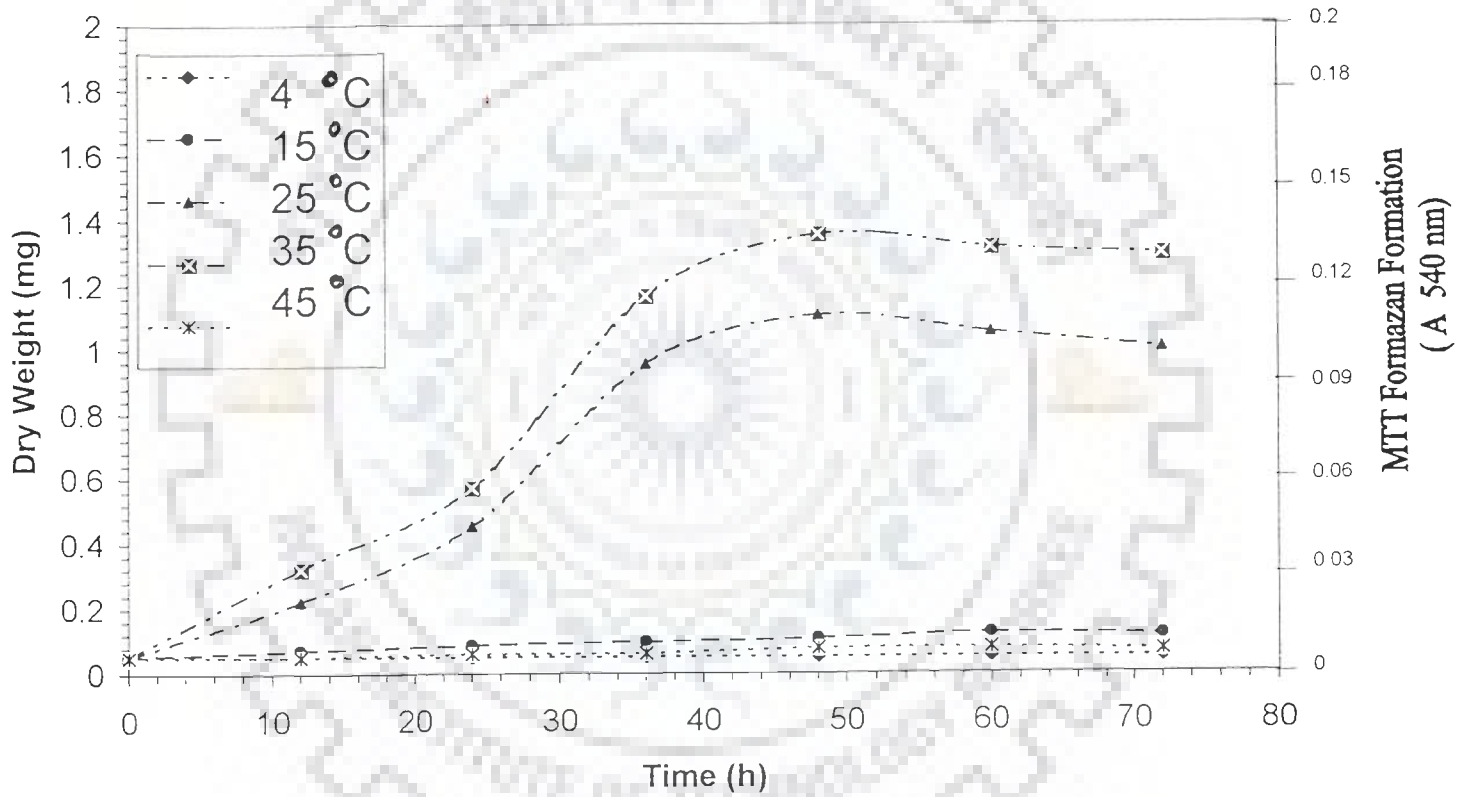


Fig. 12: Effect of temperature on *C. albicans* mutant (*bdm*7) grown at different time intervals

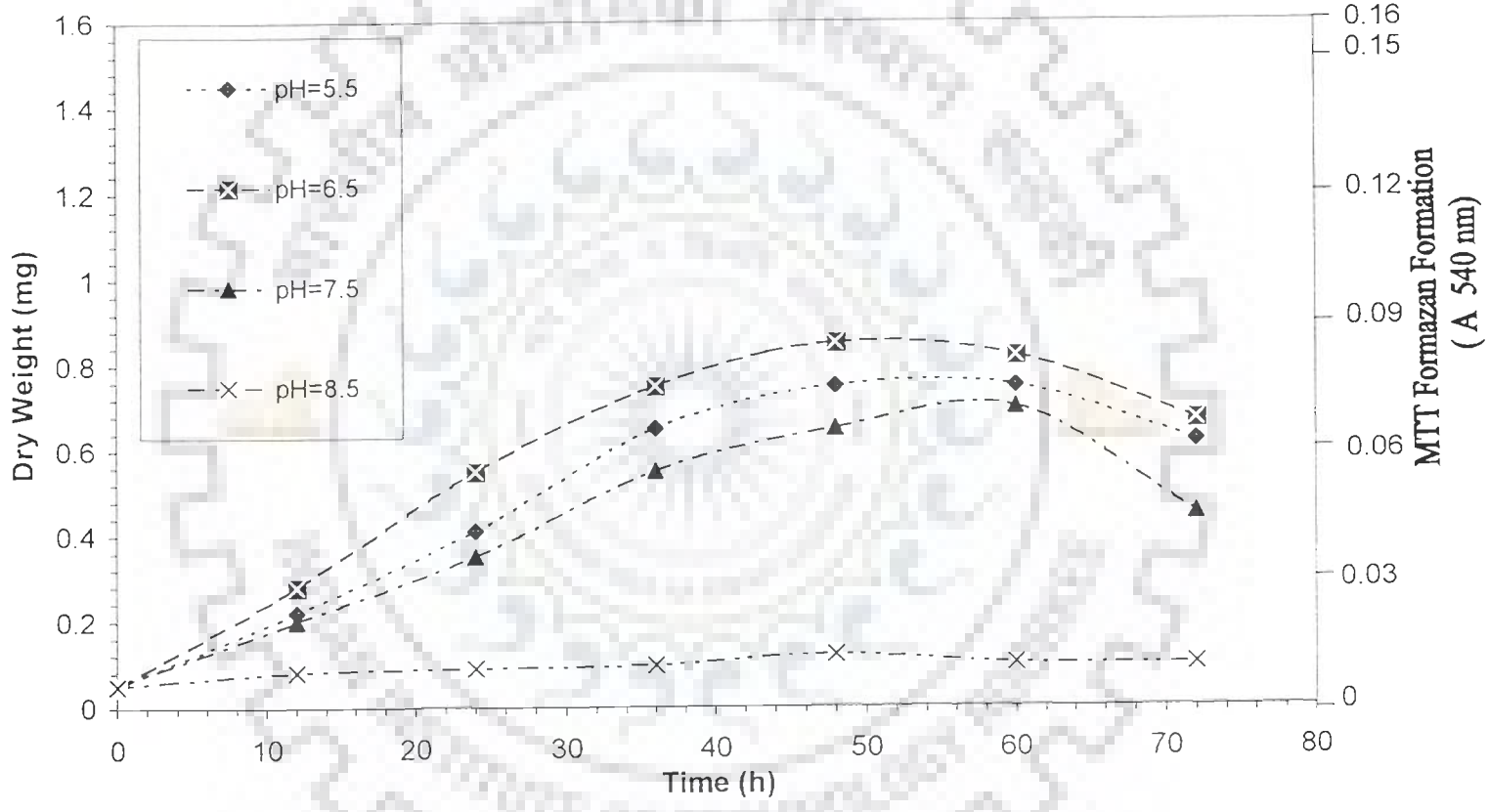


Fig. 13: Effect of pH on *C. albicans* mutant *(bdm7)* grown at different time intervals

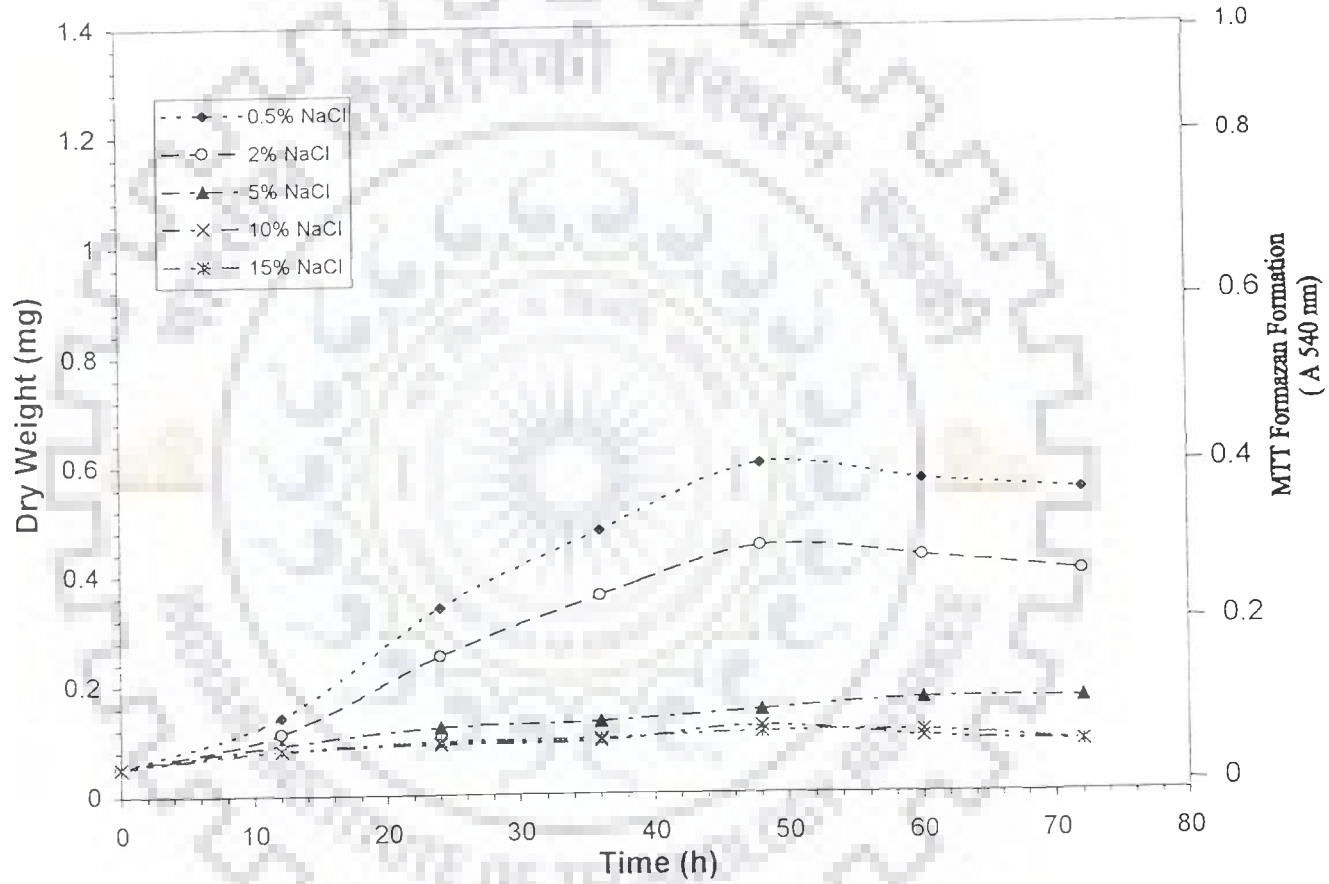


Fig. 14: Effect of osmolarity on *C. albicans* mutant *(bdm7)* grown at different time intervals.

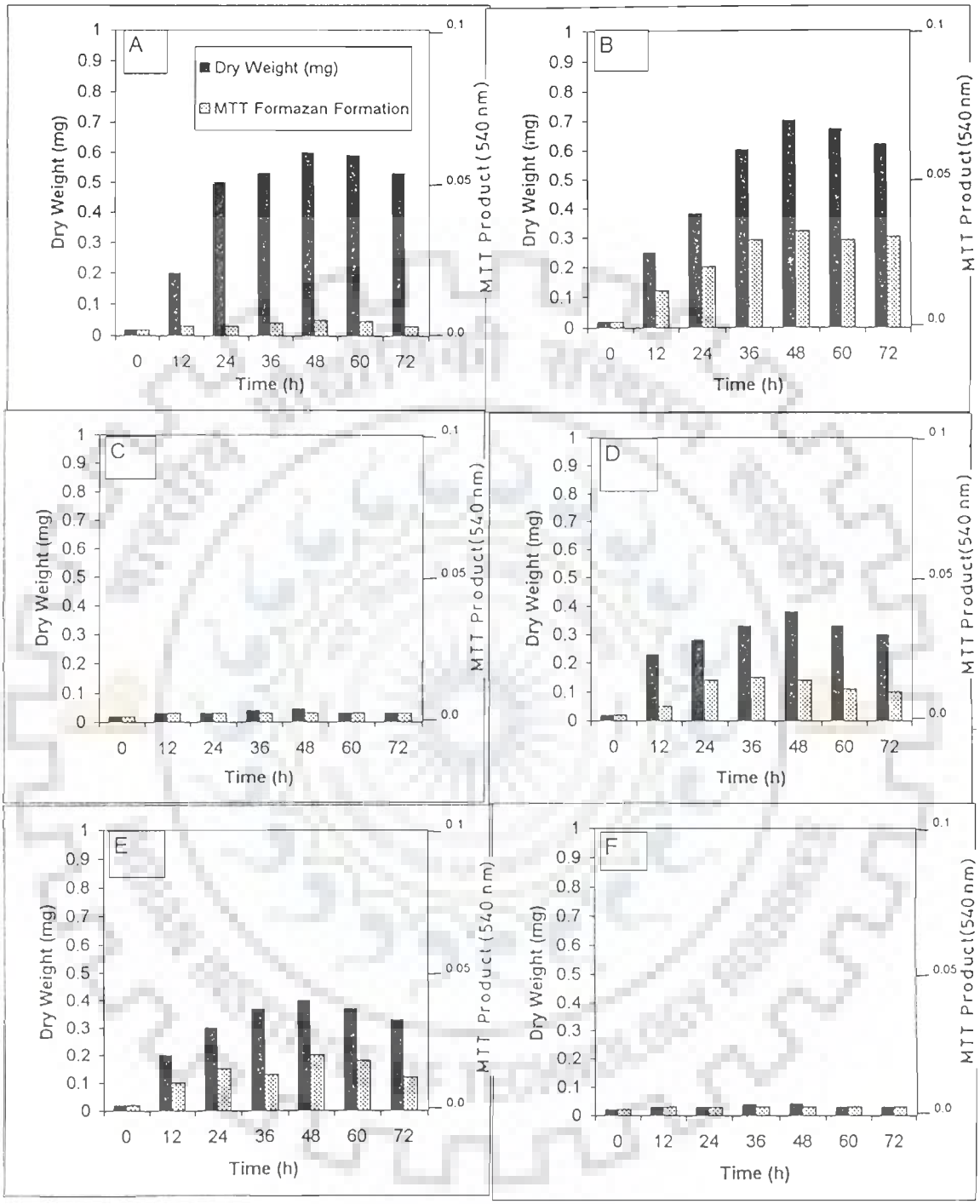


Fig. 15 Effect of different carbons sources on *C. albicans* mutant *(bdm7)* grown at different time intervals

(A) Glucose (B) Galactose (C) Lactose (D) Maltose (E) Sucrose (F) Raffinose

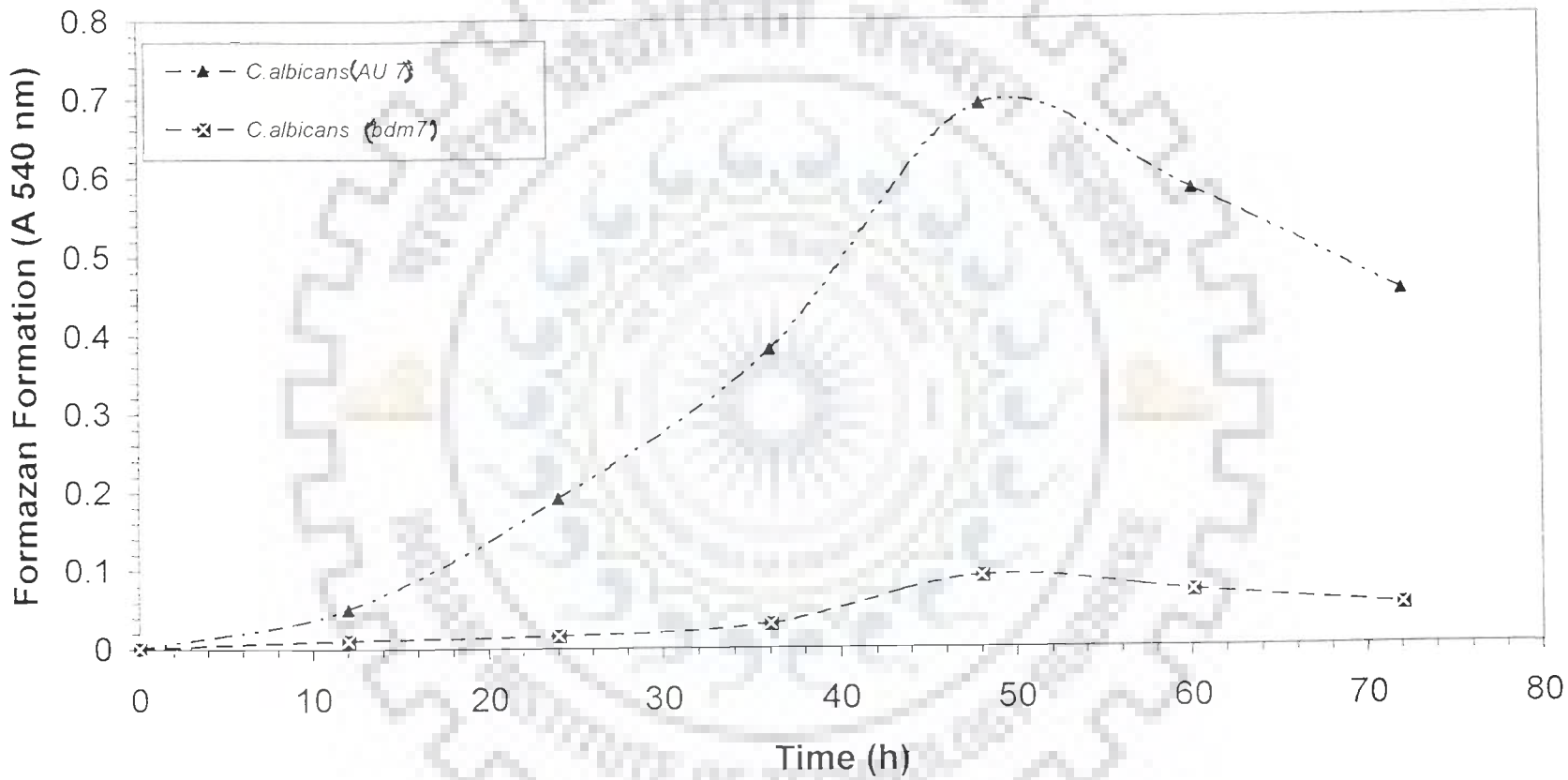


Fig. 16: Comparative analysis of biofilm formation between *C. albicans* (AU7) and its biofilm deficient mutant (*bdm*7) using MTT assay

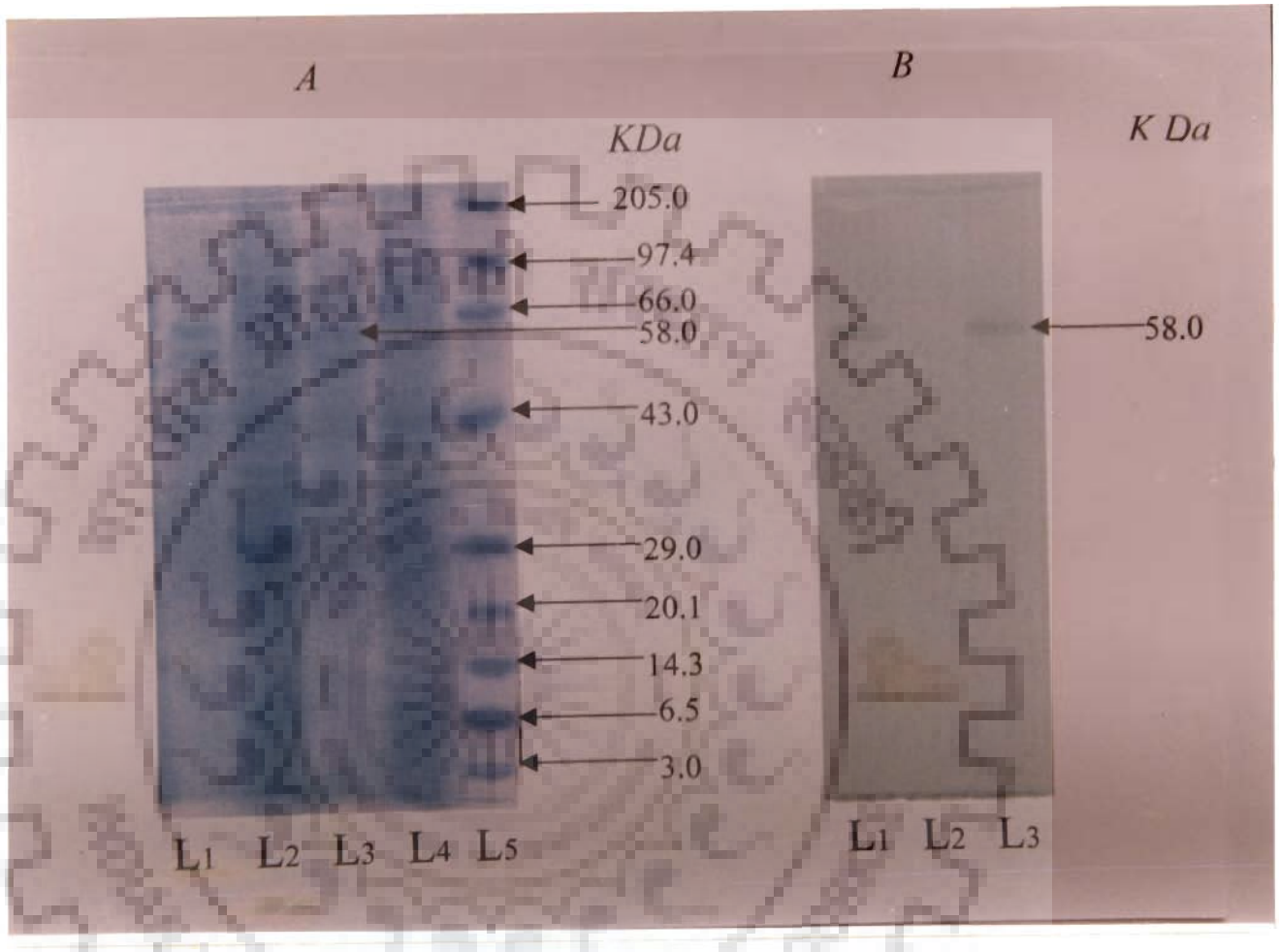


Plate 12: A) SDS-PAGE analysis of the cell wall components of *C. albicans* (AU7) and its biofilm deficient mutant (*bdm*7) released by βME treatment. Lanes 1 and 3 samples from AU7; Lanes 2 and 4 samples from *bdm*7. The electrophoretic mobilities of protein standards run in parallel (from bottom to top 3, 6.5, 14.3, 20.1, 29, 43, 66, 97.4, 205 KDa) are indicated in Lane5

B) Western blot of cell wall components of *C. albicans* (AU7) and its biofilm deficient mutant (*bdm*7) released by βME extract reacted with HRP labeled purified antirabbit IgG (anti-AU7 and anti-*bdm7*; 1:640 dilution) and detected with substrate color reaction. The arrow points towards a 58 KDa band that was the major antigen present in *C. albicans* (AU7) for biofilm formation

Molecular weight of the unknown protein was determined by plotting a semi logarithmic plot of the molecular weights of known protein standards as function of the R_f value (Figure-17). Results indicate that 58 kDa protein band present in the cell wall extract of *C. albicans* (AU7) which was absent in the cell wall extract of biofilm deficient mutant (*bdm*7).

Western blot of the cell wall extracts of the above samples were treated with HRP labeled purified antirabbit IgG (anti-AU7 and anti-*bdm7*; 1:640 dilution). It was further developed for substrate color reaction (H_2O_2 and 4-chloro-1-napthol) as shown in Plate-12B. Result indicates a 58 KDa band as the major antigen present in *C. albicans* (AU7), which may be responsible for biofilm formation since it was absent in its biofilm deficient mutant (*bdm*7).

4.7 ANTIFUNGAL SUSCEPTIBILITY TO CANDIDA ALBICANS BIOFILMS

The *in vitro* activity of antifungal agents amphotericin B, nystatin, fluconazole and chlorhexidine against pre-formed *C. albicans* (AU7) and its biofilm deficient mutant (*bdm*7) were assessed using XTT- reduction assay (Figure-18). Results obtained revealed increased susceptibility of biofilm deficient mutant *bdm*7 (50% reduction in metabolic activity for the same antifungal at a concentration of 0.25, 1.0, 0.25, 8 µg/ml respectively) when compared with *C. albicans* AU7 (50% reduction in metabolic activity for the same antifungal at a concentration of 8, 16, >64, 128 µg/ml respectively).

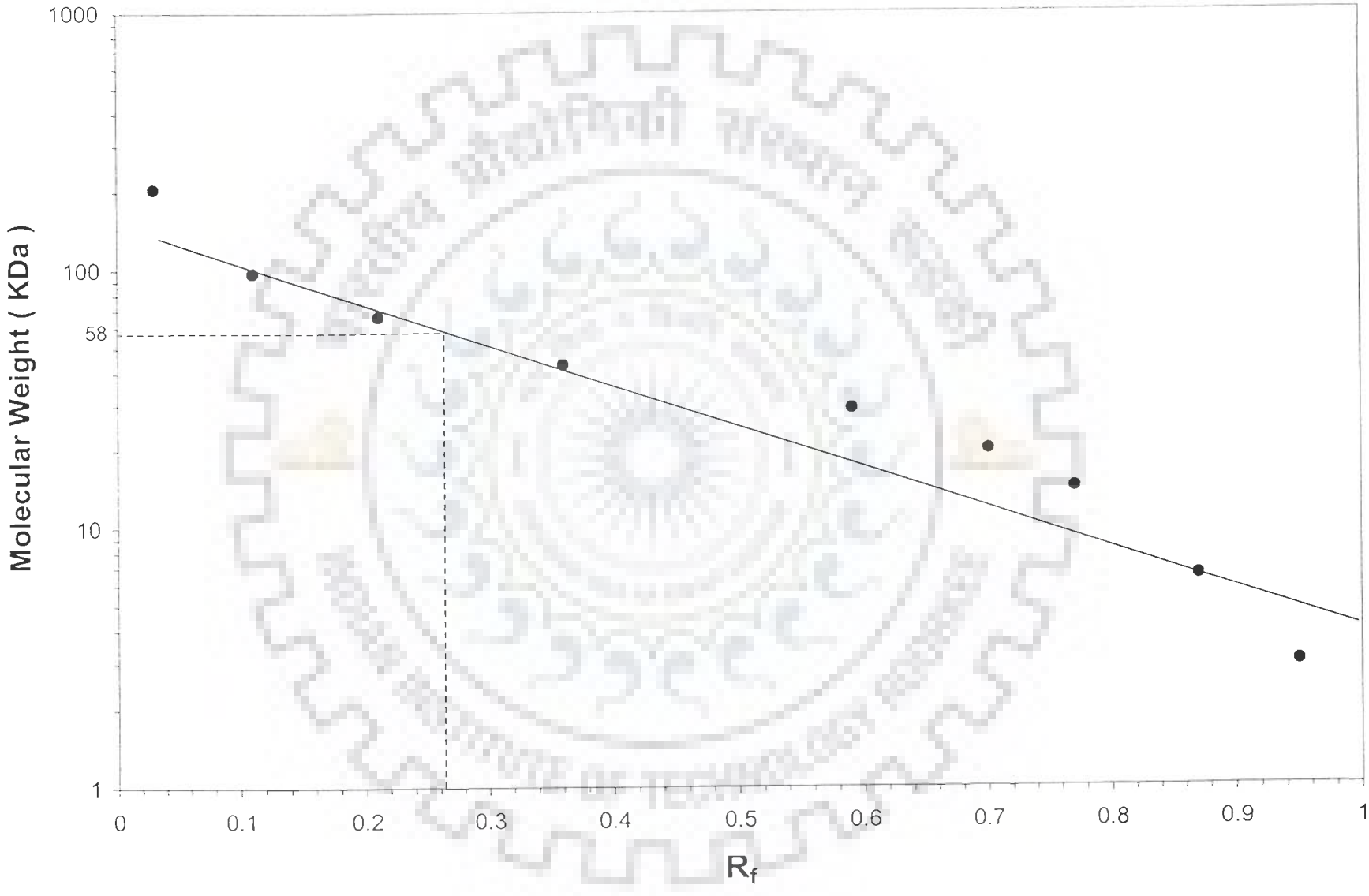


Fig. 17: Estimation of molecular of protein weight by linear regression analysis

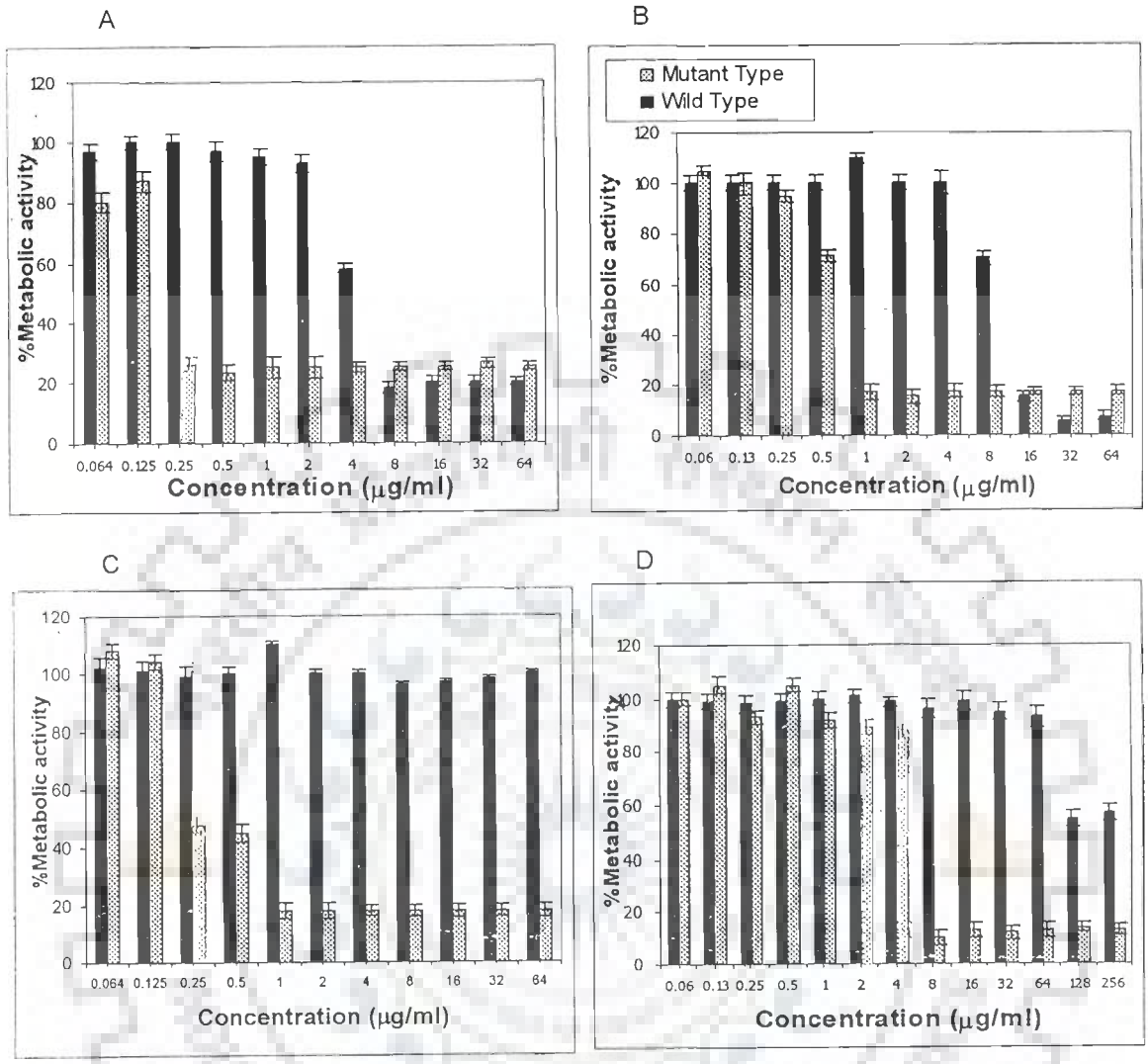


Fig. 18: Susceptibility of *C. albicans* (AU7 and *bdm*7) to different concentrations of (A) Amphotericin B, (B) Nystatin, (C) Fluconazole, and (D) Chlorhexidine. Metabolic activity was normalize to control without drugs which was taken as 100%. Results are from a single experiment performed in four replicated wells

Chapter 5

DISCUSSION

Our perception of microorganisms as unicellular life forms is primarily based on its free floating (planktonic) mode of growth. Since microorganisms can be diluted to a single cell and studied in liquid culture, this mode of growth has overwhelmingly predominated in the study of microbial physiology and pathogenesis (Ghigo, 2003). However, the majority of microbes remain attached to surfaces within a structured biofilm ecosystem as sessile organisms (McCourtie and Douglas, 1985; Miyake *et al.*, 1986; Klotz, 1987; Ray and Payne, 1988; Klotz, 1990; Lee *et al.*, 1996; Ramage, 2001a). But within the past few years, there is mounting evidence to show that biofilm is one of the common cause of persistent and chronic microbial infections (Costerton *et al.*, 1999; Potera, 1999). The antibiotic resistance of biofilm forming microbes leads to growth and development of infections to an uncontrollable stage whereby the host defense system is of no console for preventing its further development. Thus, biofilm formation could counter both: the host defense mechanism and antibiotic therapy (Costerton *et al.*, 1999; Hoyle and Costerton, 1990; O'Toole *et al.*, 2000). In the most recent surveys, *Candida* has been shown to be the third most commonly isolated bloodstream pathogen, now surpassing Gram-negative rods in frequency (Hazen and Glee, 1995; Holmes *et al.*, 1995; Khadori and Yassien, 1995; Hogan *et al.*, 1996; Donlan, 2001; Adam *et al.*, 2002; Dunne, 2002; Douglas, 2002, 2003). Strikingly, yeasts (mainly *C. albicans*) are the third leading cause of catheter-related infections, with the second highest colonization to infection rate and the overall highest crude mortality (Hawser and Douglas, 1994; Kite, 1997; Raad, 1998; Stickler, 1999; Crump and Colliognon; 2000; Lewis, 2002a). Moreover, the increase in *Candida* infections in the past decades has almost paralleled the increase and widespread use of a broad range of medical implant devices, mainly in populations with impaired host defenses. Biomaterials such as stents, shunts, prostheses

(voice, heart valve, knee, etc), implants (lens, breast, denture, etc.) entotracheal tubes, pacemakers, and various types of catheters, have been shown to support colonization and biofilm formation by *Candida*. These devices provide the necessary surfaces for biofilm formation and are currently responsible for a significant percentage of clinical candidiasis.

In our studies we have chosen Intrauterine devices (IUDs) as Indwelling Medical Devices (IMDs) that is one of the most popular contraceptive methods used by women of Asian and Africa countries. This tiny IUDs that are fitted inside the uterus are available in several shapes and sizes and are made up of a variety of materials ranging from copper to plastic (Newton, 1982; Marrie and Costerton, 1983; Thomas *et al.*, 1983; Lee *et al.*, 1983; Jacques, *et al.*, 1986; Lewis, 1988; Motghare and Purwar, 2002). Although studies have shown that IUDs effectively prevent fertilization, provide high degree of sexual satisfaction and are cost effective (Newton, 1982), they are known is be associated with a risk of pelvic infection, heavier periods, menstrual cramps (Lee *et al.*, 1983; Thomas *et al.*, 1983; Allison and Sutherland, 1984; Wolf and Kreiger, 1986; Lewis, 1998; An and Friedman, 1997; Hatcher *et al.*, 1997; Tunney, 1999) and above all complication associated with colonization of microbes on these implanted devices (Arendorf and Walker, 1979; Lee *et al.*, 1983; Buldtz-Jorgensen, 1991; Bendel and Hostetter, 1993; Bendel *et al.*, 1995; An *et al.*, 2000). In our studies it was found that 60% of clinical samples were positive for culturable *Candida* spp. from the patient infected with IUDs. Marrie and Costerton, 1983 also isolated *Lactobacillus plantarum, S. epidermidis, Corynebacterium* spp., group B streptococci, *Micrococcus* spp., *Candida albicans, S. aureus* and *Enterococcus* spp. Tatum *et al.*, 1975 proposed that the tail of IUD could act as a wick to allow bacteria to travel by capillary action and enter the endometrial cavity. Besides this urinary catheters, the tubular, latex, or

silicone devices that are inserted through the urethra into the bladder to measure urine output, collect urine during surgery, prevent urinary retention, or control urinary incontinence were also taken as clinical sample for our studies. Kaye and Hessen, 1994 showed that the microbes attached to the catheter and develop the biofilm originate from one of several sources : (i) organisms are introduced into the urethra or bladder as the catheter is inserted, (ii) organisms gain entry through the sheath of exudates that surrounds the catheter, or (iii) organisms travel intraluminally from the inside of the tubing or collection bag. In our study it was found out that the *Candida* infection percentage of patient undergoing urinary catheterization was as high as 43%.

The introduction of commercially available identification systems has greatly reduced laboratory time involved in the identification of candidal isolates (Taschdjian *et al.*, 1960; Buesching *et al*; 1979; Cannon, 1986; Beighton *et al.*, 1995; Calderone *et al.* 1998; Warren and Hazen, 1999; Calvi *et al.*, 2001). The commercial systems presently available are based on growth or enzyme production profiles. Growth systems are relatively slow, typically requiring 72 h incubation, whilst enzyme systems can potentially provide results within a few hours (Samarnayake, 1980; McCourtie and Douglas, 1981; Mosmann, 1983; Fletcher, 1990; Ener and Douglas, 1992; Mio *et al.*, 1996; Kirkpatrick *et al.*, 2000; O'Toole, 2003). Our initial finding shows that on selective media, CHROMagar clinical isolates of *Candida* species- *C. albicans*, *C. tropicalis*, *C. krusei* and *C. parapsilosis* adhering to infected IMDs made up of BCMs appeared as green, blue, pale pink and pink colour colonies respectively. Data obtained showed *C. albicans* was the most frequently isolated yeast from clinical samples on this selective media which rely on the formation of colored colonies in which the coloration is species specific. Perry and Miller, 1988 also in their work, have demonstrated

that *C. albicans* produces β-N-acetylgalactosaminidase, which enabled chromogenic or fluorogenic hexosaminidase substrates to be incorporated directly into the growth medium of *C. albicans* isolates to be identified directly on primary isolation. Odds, 1988 also took the direct identification of yeasts from clinical samples, they too reported the use of a differential and selective medium (CHROMagar) that permitted the presumptive identification of *C. albicans*, other *Candida* spp., and isolates of related genera.

A microtiter plate based method was successfully used in this study to select for biofilm forming *Candida* strains among the clinical isolates. After biofilm formation cells were stained with modified crystal violet solution and measured spectrophotometrically. Our data indicates that maximum biofilm formation by *Candida albicans* (clinical isolate no. AU7) to the 96 wells MTP take place after 48 h at 35°C.

Scanning electron microscopy demonstrated that after 48 h, *C. albicans* biofilms consisted of a dense network of yeasts, germ tubes, pseudohyphae, and hyphae. Extracellular polymeric material was also visible on the surfaces of some of these morphological forms. Results from our investigations on development of *C. albicans* biofilms on polyvinylchloride (PVC) strips showed that biofilms formation on this material used in manufacture of biocompatible devices progresses in three distinct development phases. Initially, (0-6h) majority of *C. albicans* cells were present as blastospores (yeast form) adhering to the surface of the strips. At 6-12h *C. albicans* communities appeared as thick tracks of fungal growth. The intermediate development phase (12-36h) was characterized by the emergence of predominantly noncellular materials, which appeared as hazy-like film covering the *C. albicans* microcolonies. During the maturation phase (36-72h) the amount of extracellular material increased with incubation time and completely encased the cells. Baillie and Douglas, 1999b also studied the role of morphogenesis in overall biofilm structure using

scanning electron microscopy. Their investigation showed comparison of biofilms produced by wile-type strains of *C. albicans* to those formed by two morphological mutants incapable of yeast and hyphal growth, respectively (Bailey *et al.*, 1996; Liu *et al.*, 1997). Baillie and Douglas (1998a, 1998b), reported that on a different type of surface (cellulose fibres), biofilms of wild type *C. albicans* consisted exclusively of yeast cells and the bilayer structure was absent, suggesting that biofilm architecture is dependent on highly specific contact-induced gene expression (Baillie and Douglas, 1998a).

Marginal increased amounts of matrix are formed when biofilms of *C. albicans* are incubated with gentle shaking (5 rpm), instead of statically. Under these conditions the microorganisms can be almost hidden by the enveloping matrix. Other researchers (Hawser *et al.*, 1998; Baillie and Douglas, 1998a) also showed that matrix production was similarly increased when conventional flow systems such as the perfused biofilm fermenter were used.

The number of microbes that attach to the surfaces and their rates of attachment depend not only on the species, but also on the strain and on its physiological phenotype (Fridkin and Jarvis, 1996; Gale *et al.*, 1996). A range of environmental factors can influence adhesion properties, either through their effect on the physiology of the organism or on the physiochemistry of the adhesion interaction. Researchers have found out not only must test organisms be selected on the basis of their being representative of the environment under study but attention must be paid to whether adhesion phenotypes have been altered through laboratory selection or growth conditions (White, 1979; Fletcher, 1988; Glustafson *et al.*, 1991; Brassart *et al.*, 1991; Barki *et al.*, 1994; Yu *et al.*, 1994; Lòpez-Ribot and Chaffin, 1994; Marsh and Bradshaw, 1995; Navarro-Garcia *et al.*, 1995; Maki *et al.*, 1997; Gonzalez *et al.*, 1997; Baillie and Douglas, 1998a; Boland *et al.*, 2000; Huang and Shih, 2000; Li *et al.*,

2003; Ramage *et al.*, 2002c; Banning *et al.*, 2003; Tavares *et al.*, 2003). Attachment properties can be affected by nutrient sources, concentration and flux. Factors that influence extracellular polymer production can also influence adhesion. Carbons source, carbon nitrogen ratio, carbon flux, or nutrient concentration may influence polymer production or adhesive ability in various ways, depending on the organism. Attached microbes produce an exopolysaccharide matrix that can act as a protective polymer for the cells embedded within. As the biofilm grows and thickens, it begins to develop into a heterogeneous matrix interspersed with channels that allow nutrients and oxygen to penetrate into the depths of even the thickest biofilms. Researchers have shown that the cells within the biofilm matrix exhibit differences in physiology depending on their location (Guckert *et al.*, 1986; Marshall, 1996; Jordan, 1999; Cote *et al.*, 2000; Willis *et al.*, 2000). This concept of spatial heterogeneity within a biofilm has been applied to oxygen limitations (from aerobic to anaerobic), pH, nutrients, and rates of growth (Huang *et al.*, 1998). Within a thick biofilm, there are various microniches that allow for numerous types of metabolic processes to take place.

Our investigations to determine optimum physiological and biochemical conditions for *C. albicans* (AU7) showed temperature (35°C), pH (6.5), NaCl concentration (0.5%) and galactose (50 mM) are best suited for biofilm process. Although there have been numerous attempts to identify fundamental processes that control attachment by different workers, a wealth of information has illustrated the high degree of diversity among microbial adhesion strategies (Sarachek and Bish, 1976; Ghannoum and Abu-Elteen, 1991; Dexter *et al.*, 1993; Marshall, 1996; Waters *et al.*, 1997; Xu *et al.*, 1998; Williams *et al.*, 1998; Lindberg *et al.*, 2001).

A rapid, inexpensive, easy to use, accurate, and reproducible methodology for testing of *Candida* biofilms based on the use of conventional 96-well microtiter plates coupled to a colorimeteric method was used in this study (Hawser, 1996; Baillie and Douglas, 1999a; Mosamann, 1983; Pitts *et al.*, 2003). The cleavage of Tetrazolium salt, 3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) has several desirable properties for assaying cell survival and proliferation. MTT is cleaved by all living, metabolically active cells but not by dead cells. An advantage of this colorimetric assay is that substrate does not interfere with measurement of the product. This allows the assay to be read with minimum steps, which increases the speed of the assay and helps to minimize variability between samples. During experiments we observed that the final stages of the assay (adding the MTT, reading the plate and printing the data) take much less time that setting up the assay (mixing cells and growth factor dilutions). Findings showed that the maximum biofilm formation by *C. albicans* takes place after 48 h.

Using the above biofilm forming clinically isolated strains AU7 we have further isolated biofilm deficient mutant by UV mutagenesis. After UV treatment of *Candida* cells having smooth colony morphology we obtained 18 rough colonial morphological variants. Out of which a biofilm deficient mutant (*bdm7*) because of its lack of ability to adhere to the MTP wells made up of biocompatible material (PVC) was selected. Reynolds and Fink, 2001 in their study on baker's yeast (*Saccharomyces cerevisiae)* have also showed attachment to plastic and mat formation require Flo11p, a member of a large family of fungal cell surface glycoprotein involved in adherence. They further reported that the formation of mats and spokes, like adherence to plastic, in *S. cerevisiae* was sensitive to glucose concentration. The

FLO11 genes in also required for filamentous growth, a morphological switch from the yeast form to multicellular pseudohyphae (invasive chains of elongated cells) that is induced by the starvation condition. O'Toole and Kolter, 1998 named transposon mutants defective for the initiation of biofilm formation in *Pseudomonas fluorescens* as 'Surface Attachment Defective' (*sad*) mutants. Molecular analysis of *sad* mutants revealed that the ClpP protein (a component of the cytoplasmic Clp protease participates in biofilm formation in this organism. They further suggested that biofilm formation could proceed via multiple, convergent signaling pathways, which are regulated by various environmental signals.

Optimum conditions required for growth of the BDM does not varied much from its wild type strains. However, when comparative analysis of biofilm formation by *C. albicans* AU7 and its biofilm deficient mutant *bdm*7 was carried out using MTT assay at different time intervals. Maximum violet colorization (formazan product formation) takes place after 48 h of growth by *C. albicans* (AU7) in comparison to biofilm deficient mutant *bdm*7 that fails and demonstrates the same.

In our studies electrophoretic mobility of protein standards along with cell wall components of *C. albicans* (AU7) and its biofilm deficient mutant (*bdm*7) released by βME treatment were analyzed by SDS-PAGE similar to extraction procedure followed by Casanova and Chaffin, 1991. They too obtained cell extract by boiling with β-mercaptoethanol and SDS.

We determine the molecular weight of the unknown protein by plotting a semi logarithmic plot of the molecular weights of known protein standards as function of the R_f value. Our investigation showed that 58 kDa protein band present in the cell wall extract of *C. albicans* AU7 which was absent in the cell wall extract of biofilm deficient mutant *bdm*7.

Western blot of the cell wall extracts of the above samples were treated with HRP labeled purified antirabbit IgG (Edwards *et al.*, 1986; Dunbar and Schwoebel, 1990) (anti-AU7 and anti-*bdm7*; 1:640 dilution). It was further developed for substrate color reaction (H_2O_2 and 4-chloro-1-napthol). A prominent 58 KDa band as the major antigen present in *C. albicans* (AU7), was noticed which may be responsible for biofilm formation. Casanova *et al.*, 1982 studied on the adhering ability of cell surface mannoproteins form *C. albicans* and showed similar size protein responsible for adherence (Ghannoum and Abu-Elteen, 1991). Ponton and Jones, 1986 in their studies have demonstrated dithiothreitol extract from the outer cell wall layers of *Candida* contained a complex array of polysaccharides, glycoproteins, and proteins. The proteins contributed to a latticework stabilized by covalent bonds that was important in determining the porosity of the outer cell wall layers, however, its biofilm ability is still to be decipher.

To test *in vitro* activity of antifungal agents amphotericin B, nystatin, fluconazole and chlorhexidine against pre-formed *C. albicans* (AU7) and its biofilm deficient mutant (*bdm7*) XTT- reduction assay was used. This simple, inexpensive, rapid and accurate assay relies on the measurement of metabolic activities of the sessile cells growing within the biofilm and is based on the reduction of 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) to yield a water-soluble formazan colored product. Because of its water solubility, the XTT-reduction assay can be easily quantified without additional steps such as centrifugation, addition of lysis buffer, solubilization, removal of medium and sonication (Hawser and Douglas, 1995; Baillie and Douglas, 1999a; Bakki, *et al.*, 2001; Suci and Tyler, 2002; Rogers and Barker, 2003, Andes *et al.*, 2003; Liao *et al.*, 2003). Our results obtained revealed increased susceptibility of biofilm deficient mutant *bdm7* (50% reduction in

metabolic activity for the same antifungal at a concentration of 0.25, 1.0, 0.25, 8 μg/ml respectively) when compared with *C. albicans* AU7 (50% reduction in metabolic activity for the same antifungal at a concentration of 8, 16, >64, 128 μg/ml respectively). These results are in agreement with the observations of Hawser and Douglas, 1995, who showed that *C. albicans*, growing in catheter-associated biofilms was resistant to antifungals. Unlike the M27-A technique (National Committee for Clinical Laboratory Standards, 1997), which involves turbidometric measurements of cells grown in suspension. XTT based method is suitable for determining the antifungal susceptibility of biofilm-grown cells. In this regard, the tetrazolium based assays have been successfully used to determine antifungal susceptibility of *Candida* biofilms. The mechanism contributing to increased antifungal resistance of biofilm grown *C. albicans* have not been investigated including low growth rate (Rex, 1997; Baillie and Douglas, 1998a; Lewis, 2001; Ramage, 2001b, 2002; Sobel *et al.*, 2003), phenotypic changes resulting from nutrient limitation (Baillie and Douglas, 1998b) and a possible protective effect of the matrix material (Baillie and Douglas, 2000). Interestingly, candidal cells resuspended from biofilms were more resistant than planktonic organisms, but were less resistant than cells in intact biofilms (Baillie and Douglas, 1998a). A better understanding of *Candida* biofilms may lead to the development of novel therapeutic approaches for the treatment.

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