Characterization of effects of silver nanoparticles on hyphal morphogenesis in an opportunistic fungal pathogen *Candida albicans*

to be submitted as Major Report in partial fulfillment of the requirement for the degree of

M. Tech. (BME)

Submitted by

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(2K14/BME/03)

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CERTIFICATE

This is to certify that the dissertation entitled **Characterization of effects of AgNPs on hyphal morphogenesis in an opportunistic fungal pathogen *Candida albicans*** submitted by **Anju Lata Singh**

(DTU/14/M.Tech/101) in the partial fulfillment of the requirements for the reward of the degree of Bachelor of Engineering, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by her under our guidance. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honoring of any other degree.

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I, Anju Lata Singh hereby declare that the dissertation entitled "**Characterization of silver nanoparticles on hyphal morphogenesis in an opportunistic fungal pathogen *candida albicans*"** submitted is in partial fulfillment of the requirement for the award of the degree of Master of Technology in Biomedical Engineering, Delhi Technological University. It is a record of original and independent research work done by me under the supervision and guidance of Dr. Asmita Das, Assistant Professor, Department of Biotechnology, Delhi Technological University, Delhi. The information and data enclosed in the dissertation is original and has not formed the basis of the award of any Degree/Diploma/Fellowship or other similar title to any candidate of the University/Institution.

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List of Abbreviations

AgNPs	Silver nanoparticles
ALS	Agglutinin like sequences
DHE	Dehydroergosterol
DNA	Deoxyribonucleic acid
ERG	Ergosterol gene
Erg	Ergosterol
HBEC	Human buccal epithelial cells
kDa	Kilodalton
MDR	Multi-drug resistance
ml	Millilitre
mM	Millimolar
NAG	N-acetyl glucosamine
nm	nanometer
OD	Optical density
RNA	Ribonucleic acid
rpm	Rotations per minute
YEPD	Yeast peptone dextrose (media)
YNB	Yeast nitrogen base

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Aims and Objectives of the Study

1. Synthesis of silver nanoparticles by chemical reduction method and characterize their physical properties.

2. To check the effect of AgNPs on the hyphal morphogenesis of *C. albicans* cells grown on two different media (spider media and N-Acetyl Glucosamine media).

3. To dissect the molecular mechanism of AgNPs effect on hyphal morphogenesis of *C. albicans* cells grown on two different media (spider media and N-Acetyl Glucosamine media).

Characterization of effects of silver nanoparticles on hyphal morphogenesis in an opportunistic fungal pathogen *Candida albicans*

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1. Abstract

Candida albicans, the polymorphic fungus, is a member of the normal human microbiome and a harmless commensal organism which emerged as major human pathogen and is able to cause life threatening diseases in immunocompromised persons. Several factors have been identified which contributes the pathogenic prospective to fungus including molecules mediate adhesion to invasion into host cells, phenotypic switching, transition from yeast to hyphal, contact sensing, thigmotropism and formation of biofilm. Their treatment is restricted due to appearance of multi drug resistance (MDR), emergence of drug resistance strains, high toxicity of drug and narrow range of the accessible drugs. In the effort to launch newer therapeutic strategies to setback MDR need to find novel determinants of drug resistance in *Candida albicans*. The antifungal drugs present nowadays are very few which is being used clinically. These are the reasons which restrict the clinician's therapeutic choices to the fungal pathogen *Candida albicans*. Silver has been recognized as a nontoxic, safe inorganic antifungal agent with various properties used for centuries. Silver demonstrates a very high potential in a wide range of biological applications, more particularly in the form of nanoparticles. In this study we are synthesizing the silver nanoparticles (AgNPs) by chemical reduction method and characterizing the effects of AgNPs on hyphal morphogenesis in *Candida albicans*.

CHAPTER 2

INTRODUCTION

2. Introduction

At cellular level fungi are closer to human than bacteria, they are (chemoheterotrophs) having chitinous cell wall (Sajjad *et al.,* 2010). On earth approximately 8.7 million eukaryotes are found in which 7% is fungi in terms of species they are about 611,000 of them only 600 species are pathogenic to human. Skin mild infection, cutaneous severe infection and systemic life threatening infection are caused by this group of fungi. *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigates* and *Histoplasma capsulatum* can cause systemic infection to life threatening diseases (Mayer *et al.,* 2013). *Candida albicans* is part of the common flora of the mucous membranes of the respiratory, gastrointestinal and female genital tracts of human body. *Candida* cause severe persistent infections in patients who are rigorously immune-compromised, have endured invasive clinical measures, or have experienced major trauma, and cure requires extensive stays in intensive care units. *Candida albicans* acquire fourth position reported by National Nosocomial Infections Surveillance System (Zheng *et al*., 2015; Sajjad *et al*., 2010).

Classification of *Candida albicans*:

Kingdom: **Fungi**

Division: **Ascomycota**

Class: **Saccharomycetes**

Order: **Saccharomycetales**

Family: **Saccharomycetaceae**

Genus: ***Candida***

species: ***albicans***

CHAPTER 3

REVIEW OF LITERATURE

3. Review of literature

3.1 *Candida albicans*

Candida spp. belongs to ascomycete's genus and is asexual, diploid with eight chromosomes. *C. albicans* is a commensal organism with no perfect sexual cycle which shows three different morphogenic phases of budding yeast, pseuodohyphal, and hyphal growth forms (Sajjad *et al.,* 2010; McCullough *et al*., 1996). *Candida* is generally commensal which is found in skin, respiratory path, mucous membrane, groin, urinary tract oral cavities, gastrointestinal tract, vaginal canal, vulva, and anus etc. (Panwar *et al*., 2016; Sajjad *et al.,* 2010). Normally 80% of human population has *Candida* as a part of their microflora. *Candida albicans* shows their pathogenic behaviour when a human being encounter by several immune suppressive drugs and various treatment and disease that leads to poor immunity (Barelle *et al.,* 2006; Odds *et al.,* 1988). The other *Candida* species are *C.tropicalis, C. parapsilosis, C. stellatoidea, C. glabrata, C. kyfer and C. krusei* gaining importance but among them *C. albicans* is most prevalent (Samaranayake *et al*., 1990; McCullough *et al*., 1996).

Acute, sub-acute, and chronic to life-threatening mycoses are stages of candidiasis opportunistic infections which infect mouth, throat, bronchi, lungs, skin, fingers, vagina, and gastrointestinal tract, or have systemic infection such as candidemia, endocarditis, and meningitis (Sajjad *et al.,* 2010). Predisposing or risk factors for candidiasis include young individual, AIDS, deficiency diabetes, burn patients, pregnancy, oral birth control, high fruit diets, steroids, organ transplant, prolonged antibiotic therapy, immunosuppressant, cancer treatments, heart surgery, genetic deficiency, endocrine, tuberculosis infections use of catheters, use of dirty needles etc (Barelle *et al.,* 2006; Odds *et al.,* 1988).

Even after treating with antifungal drugs, high crude mortality is found in systemic candidiasis where damage of the gastrointestinal mucosal layer and neutropenia cause disseminated candidiasis and further risk factors include central venous catheters where fungus have direct contact with bloodstream (Mayer *et al.,* 2013). Due to limitations of therapeutic options, availability of fewer broad spectrum antifungals and emergence of drug resistant strains in the biofilms-associated infection causes serious medical problems to treat systemic fungal infections (Barelle *et al.,* 2006; Odds *et al.,* 1988).

3.2 Antifungal drugs to treat *candidiasis*

Fungi and human hosts shows similarity because of this many drugs are not able to affect target thus there is need to develop antifungal drugs which must act specifically on fungi. Antifungal drugs which are in clinical use target the biosynthesis of ergosterol the major component of fungal cell membrane which is discriminated by cholesterol present in mammalin cells (White *et al*., 1998).

3.2.1 Flucytosine

Flucytosine or 5-fluorocytosine converted into 5-fluorouracil in target cell and thus works as an antifungal agent (Odds *et al*., 2003). Fluorouracil inhibits DNA synthesis by incorporating into RNA which results in premature chain termination, and thus affect on thymidylate synthase in DNA. But for this mechanism to take place target cell must have cytosine permease which internalize the flucytosine molecule followed by conversion of flucytosine into 5-fluorouracil by cytosine deaminase then taken as substrate for nucleic acid synthesis by uracil phosphoribosyl transferase (Odds *et al*., 2003).

3.2.2 Polyenes

Amphotericin B and nystatin are examples of polyenes, it binds to ergosterol rather than inhibiting the biosynthetic pathway resulted to form pores on membrane (Cannon *et al*., 2007). It disturb the membrane integrity and functionality thus cause the cellular components and cations such as K+ to leak out and destroy proton gradient which is fungicidal for *C. albicans* (Cannon *et al*., 2007, Shapiro *et al*., 2011)**.** Polyenes are heterocyclic amphipathic fungicidal molecule have both hydrophobic and hydrophilic sides which help it to bind ergosterol strongly forming lipid drug complex which intercalate with membrane and form membrane spanning channel (Ostrosky *et al*., 2010). Beside its broad fungicidal activity it is also thought that polyenes cause oxidative damage, host toxicity like renal dysfunction (Sanglard *et al.,* 2002; Shapiro *et al*., 2011).

3.2.3 Azoles

Imidazoles and triazoles are heterocyclic nitrogen-containing compounds which are most widely used as antifungal (Cowen *et al*., 2008; Ostrosky *et al*., 2010). Fluconazole, itraconazole, voriconazole, and posaconazole are triazoles currently, available for clinical use (Shapiro *et al*., 2011). Lanosterol demethylase or cytochrome P450 which is product of *ERG11* in *C. albicans* is responsible for ergosterol biosynthesis is the target of azole drugs (Shapiro *et al*., 2011). Toxic sterol pathway intermediate are accumulated by inhibiting ERG11 which results in growth inhibition (Akins *et al.,* 2005; Sanglard *et al.,* 2002). In *Candida* species, azoles generally acts in fungistatic manner which evolve into drug resistance by showing strong directional selection on surviving population (Anderson, J. B., 2005; Cowen *et al*., 2008).

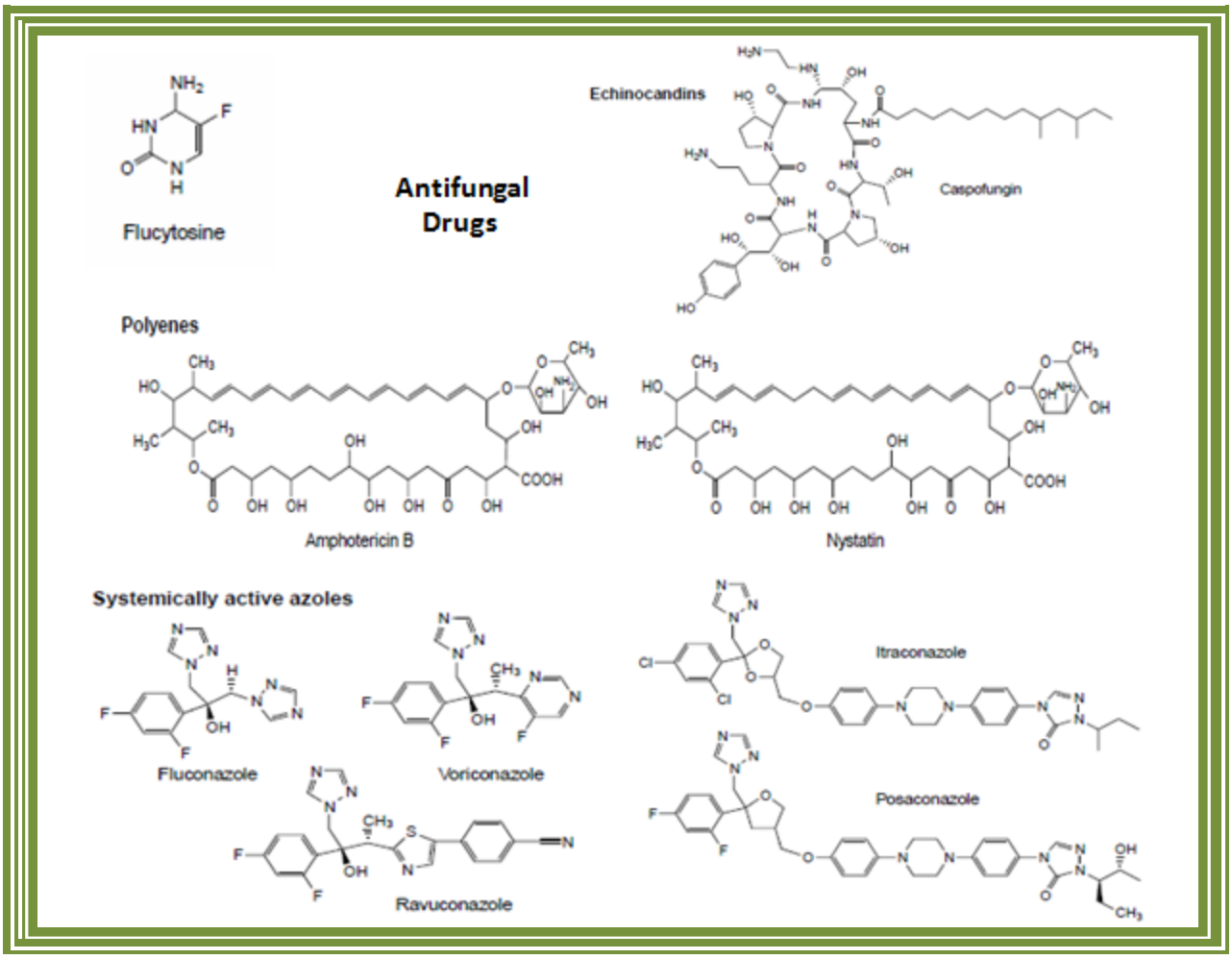

Figure 3.1 Structure of antifungal agent used clinically such as Pyrimidine-flucytosine; echinocandin- caspofungin; Polyenes- Amphotericin B, Nystatin; Azoles- fluconazole, voriconazole, itraconazole, ravuconazole, posaconazole.

3.2.4 Echinocandins

Echinocandins are new class of antifungal currently having three drugs caspofungin, micafungin, and anidulafungin which contains large lipopeptide molecules (Shapiro *et al*., 2011). Echinocandins inhibit (1, 3)-β-D-glucan synthase noncompetitively which play role in cell wall synthesis of fungi (Denning *et al.*, 2003). This mechanism cause severe stress on fungal cell wall and it also lost their cell wall integrity. This shows good fungicidal activity against yeast such as *Candida* which is alternative to azole. This drug is impressively safe in usage because it targets

specifically to the components of fungi only (Ostrosky *et al*., 2010; Shapiro *et al*., 2011). Their resistance mechanism in long term studies is not present due to its short duration usage (Shapiro *et al*., 2011).

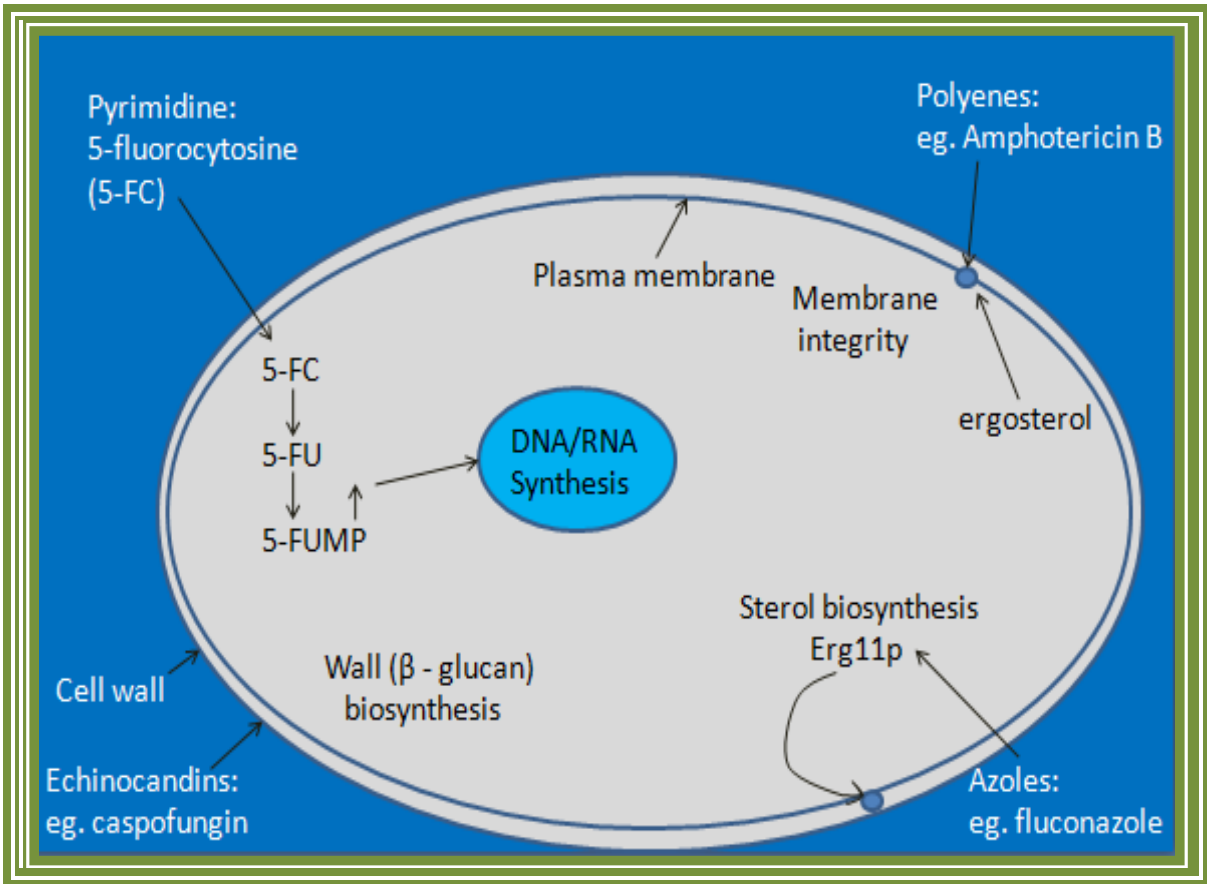

Figure 3.2 Mode of action of various antifungal agents

3.3 Drug resistance

Drug resistance is the mechanism where effectiveness of the drug is reduced. Disease-causing microorganisms such as bacteria, viruses, fungi or parasites show resistance towards drugs. Globally the main cause of microbial diseases is their rapidly evolving resistance towards antimicrobials (Shapiro *et al*., 2011). Since fungi shows close evolutionary relation with human host, it is able to minimize the drug targets which kill the pathogen selectively (Shapiro *et al*., 2011). Although depending on variety of fungal species, show different intrinsic level of drug

resistance but specific resistance mechanisms is also shown within a species (Shapiro *et al*., 2011).

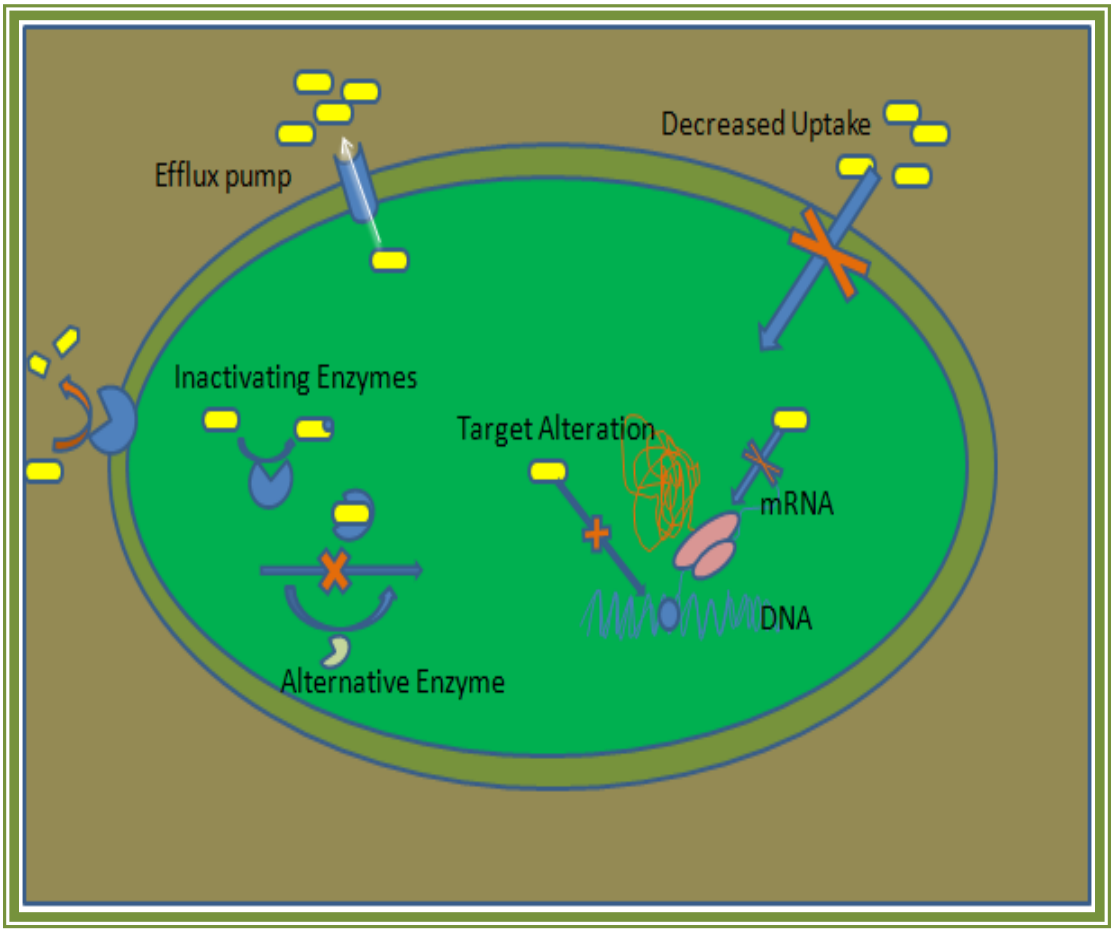

Figure 3.3 Drug resistance mechanisms of *Candida albicans* (Li *et al.*, 2009).

Resistance can be of two types primary microbial resistance which is intrinsic, found in fungi naturally have no prior access to drugs and secondary microbial resistance is species already exposed to antifungal agents and susceptible which altered due to various conditions (Kanafani *et al*., 2008).

Drug resistance from a clinical perspective is the failure to remove infection despite having appropriate drug therapy. This failure depends on many factors which can be related to

the pathogen, host, or antifungal agent (Kanafani *et al*., 2008, Shapiro *et al*., 2011). Drug resistances also evolve due to tolerance which allows dividing cells to respond to selection which developed due to drug (Shapiro *et al*., 2011).

3.4 Drug resistance mechanisms in *Candida albicans*

C. albicans are showing multiple drug resistance towards azole drugs and attain resistance towards unrelated compounds (Franz *et al*., 1998; White *et al*., 1998; White *et al*., 2002). *Candida albicans* shows various mechanisms to develop MDR some of which include the following:

3.4.1 Drug efflux pumps overexpression

Overexpression of efflux pumps such as Major Facilitator Superfamily transporters like *MDR1*, *FLU1*, *BENr* (Ben-Yaacov *et al.,* 1994) and ATP-Binding Cassette Superfamily of transporters these include *CDR1* and *CDR2* (White *et al.,* 1998).

3.4.2 Molecular Alterations of the drug target

lanosterol 14-α demethylase is the target enzyme of the azoles. *ERG11* which encodes in fungus *C. albicans.* Gene amplification, coding region point mutated, mitotic recombination, conversion of gene are genetic alterations found in ERG11 (White *et al*., 1997). Mutation of arginine to lysine at position 467 in ERG11 is found in the clinical isolate of azole (White *et al*., 1997). In some clinical isolates of *C. albicans* some point mutations are found to which lead to alter drug resistance but completely nonfunctional ERG11 is not found. Increase in the number of gene copies called gene amplification show resistance towards target enzyme by overexpression in ERG11 (White *et al*., 1997).

3.4.3 Alterations in other ERG genes of the ergosterol biosynthetic pathway

The acquisition of drug resistance in ergosterol biosynthesis pathway analysis of sterol will help to correlate. 14α methyl fecosterol and 14α methyl-ergosta-8,24(28)-dien-3β,6α-diol the toxic sterol intermediate accumulation is found in azole treated *C. albicans* results from a defective lanosterol demethylase the main azole target enzyme (Kelly *et al*., 1997). Sterol Δ5, 6-desaturase which is encoded by the gene, *ERG3* is adapted by pathogens to prevent this toxic sterol accumulation.

3.4.4 Alteration in facilitated drug diffusion or membrane permeability

Altered drug accumulation inside the cells, one of the common mechanisms in drug resistance is having defect in drug import inside cells which may be due to plasma membrane sterol composition. Lack of fluidity of plasma membrane arise when 14a-methyl sterol a component of other sterol eliminate or reduce ergosterol component of membrane which create difficulty to enter azole drug (Kelly *et al*., 1997). Main factors responsible for alteration in permeability are alteration in the membrane ergosterol and sphingolipid interactions, genes disruption of ergosterol or sphingolipid biosynthetic pathways, iron depletion, morphogenetic transcription factors which include *EFG1*, *SSK1*, etc. (Prasad *et al*., 2010, Neeraj *et al*., 2007, Mukhopadhyay *et al*., 2006, Hameed *et al*., 2008).

3.4.5 Molecular alterations in Glucan synthase gene FKS1

FKS1 activity has been linked to triazole resistance is linked to FKS1 in *Candida* biofilms. Antibiotics amphotericin B, anidulafungin, and flucytosine is effect on biofilm is related to FKS1 expression where its reduction in expression render biofilms more susceptible and its increased expression leads to enhanced resistance (Nett *et al*., 2010). Echinocandin drugs resistance is related with amino acid substitutions in two "hotspot" regions of *FKS1* (Niimi *et al*.,

2010). In wide variety *Candida* spp the *FKS1*-mediated resistance mechanism is found and responsible for intrinsic resistance of some species (Niimi *et al*., 2010).

3.4.6 Molecular alterations in uracil phosphoribosyltransferase gene (FUR1)

Genetic mutation responsible for 5-Fluorocytosine (5FC) resistance is the single nucleotide change in the uracil phosphoribosyltransferase gene (*FUR1*) of *C. albicans* from cytosine to thymine at position 301 (Dodgson *et al*., 2004).

3.5 Pathogenesis and virulence in *C. albicans*

C. albicans an opportunistic pathogen utilize several kinds of virulence factors which regulates expression of certain genes and their products as virulence factors to produce disease. Commonly virulence factors present in *C. albicans* are briefly described here.

3.5.1 Adhesion

While taking birth child is first exposed to fungus *C. albicans* when passing through the birth canal then it colonizes the buccal cavity, and upper and lower parts of the gastrointestinal there it becomes commensal (Khan and Gyanchandani 1998; Claderon and Fonzi 2001).

Adhesins are utilized to stick firmly the *Candida* cells to host tissue, this is expressed on the surface of morphogenetically changing cell, but adherence is enhanced in *Candida* by the formation of biofilm. Agglutinin-like sequences (ALS) are adhesins belong to seven glycosylated proteins family. Hyphae cell surface shows Als1p, Als3p and Als5p (Ala1p) which binds to human buccal epithelial cells (HBEC), laminin, collagen, fibronectin and endothelial surface (Hawser and Douglas 1994; Hoyer 2001). Als6p bind to collagen and Als9p bind to laminin, endothelial cells binds Als4p, and for cell aggregation Als5p is required, but role of Als7p is not clear (Filler *et al*., 2006; Kuleta *et al*., 2009). Mannoprotein is encoded by Hwp1adhesin molecule on outer surface of hyphal wall.

3.5.2 Phenotypic Switching

By affecting several parameters of phenotypic and metabolic with virulence traits like SAP gene regulation *Candida* shows pleitropism type phenotypic switching which allows it to adapt to a host environment during infection (Soll 1992; Soll 2002). Smooth, rough, stippled, star, wrinkle, hat, and fuzzy type morphological variation was shown by *C. albicans* and occurs spontaneously in stress, which changes cell surface behavior, colony appearance, and metabolic, biochemical and molecular attributes and during infection becomes virulent and effective (Soll 2002; Odds *et al*., 2006).

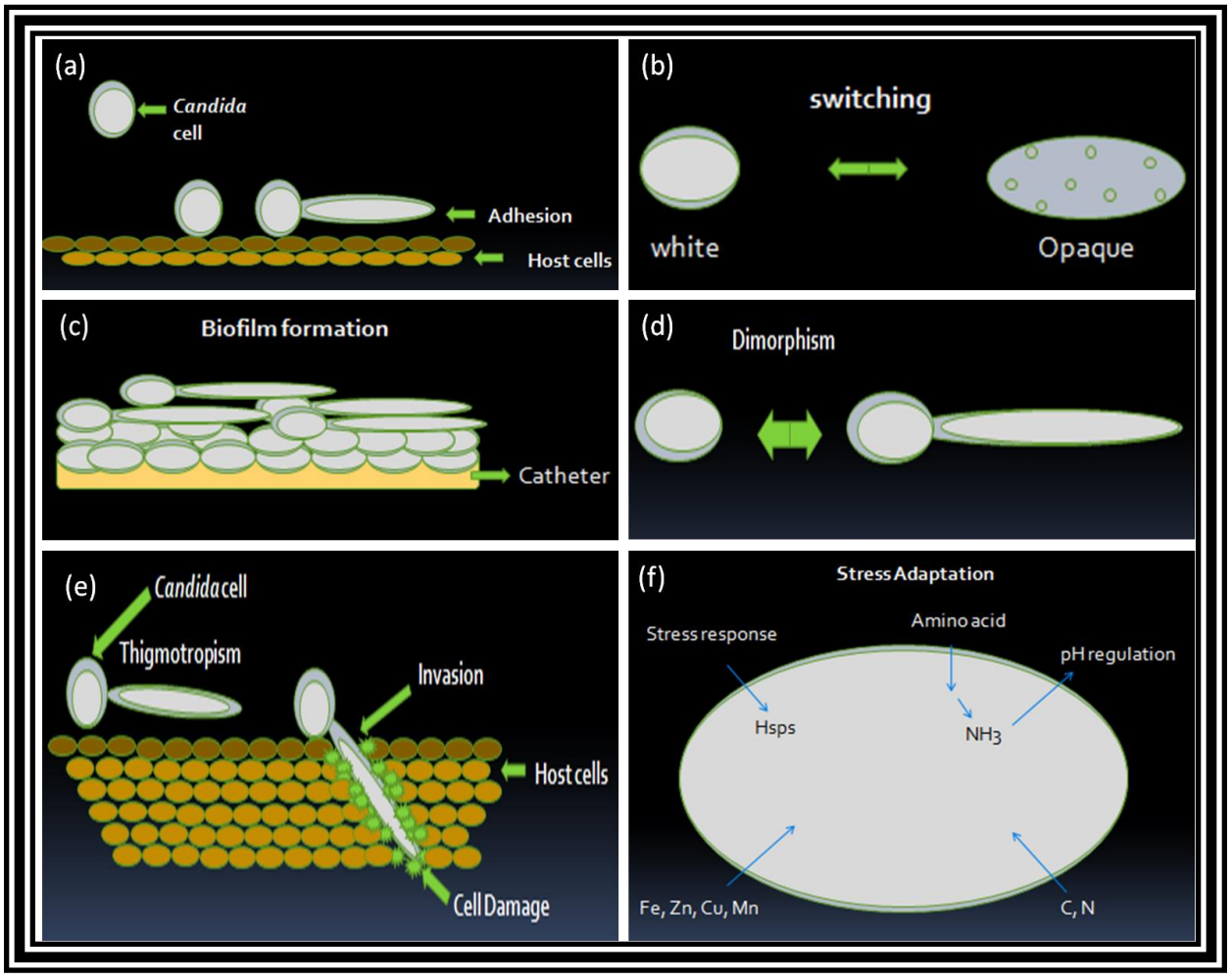

Figure 3.5 Pathogenesis and virulence in *C. albicans*: a) Adhesions; b) Phenotypic plasticity (switching) c) Biofilm pathogenesis d) Di morphism; e) Invasin and thigmotropism f) Stress adaptation.

3.5.3 Phospholipases

Invasion into tissue by *Candida* cells it is important to hydrolyze glycophospholipids ester linkages and this was done by phospholipases. Phospholipase A, B, C, and D are classified on the basis of ester bond they cleave, all of them possess hydrolase activity but lysophospholipase transacylase activities in addition was shown by PLB thus from phospholipids it release fatty acids and from lysophospholipids the remaining fatty acid produce phospholipids by transferring a free fatty acid to lysophospholipids. In candidiasis during tissue invasion only PLB1, a 84 kDa glycoprotein which was isolated from hyphal tip is required for virulence in a murine model (Ghannoum 2000; Yang 2003; Theiss *et al*., 2006).

3.5.4 Proteinases

At the infection site to degrade the tissue barriers and obtain nutrition is important and is done by secretion of proteinases by pathogen. *Candida* secreted aspartyl proteinases (SAPs) which hydrolyze many proteins such as albumin, keratin, hemoglobin, collagen, fibronectin, laminin, mucin, salivary lactoferin, cystatin A, interleukin1b, and Immunoglobulin A (Hube *et al*., 1998).

3.5.5 Biofilm Formation

Structured microbial communities which are attached to a surface and self-produced extracellular matrix is surrounded consist of biofilms (Costerton *et al*., 1995; Höfs *et al*., 2016). Both biotic which include mucosal cell surfaces and abiotic surfaces such as catheters and dentures form biofilms in *C. albicans* (Fanning and Mitchell, 2012).

There are four stages involved in the process of biofilm formation which are cell wall-mediated adherence of yeast cells to the substrate; proliferation yeast cells into a thin layer; development of both pseudohyphal and hyphal cells causes maturation of the biofilm and

extracellular matrix material secretion or accumulation and, finally colonization at different niches within the host by dispersing yeast cells from the mature biofilm (Chandra *et al*., 2001; Blankenship and Mitchell, 2006; Uppuluri *et al*., 2010; Kaneko *et al*., 2013). *Candida* cells are highly resistant to antifungal compounds within a biofilm developed on a catheter (Ramage *et al*., 2006; Fanning and Mitchell, 2012; Höfs *et al*., 2016).

3.5.6 Morphogenesis

C. albicans have a striking feature that it shows various morphological forms which include budding yeast which is a unicellular form, a pseudohyphae form and a true hyphal form with parallel side walls. When *C. albicans* transform into filamentous form (pseudohyphae or hyphae) from unicellular yeast form is defined as morphogenesis. In pseudohyphae the daughter bud elongates and form septum and remain attached to the mother cell, thus filaments have elongated cells with constrictions at the septa. Pseudohyphae superficially resemble hyphae because the elongation of buds is so extreme thus, both forms pseudohyphae and hyphae together called as 'filamentous' (Sudbery *et al*., 2004). Nutrients, 37°C–40°C temperature, 5.5% CO2 concentration, near-neutral pH, and presence of N-acetyl-D-glucosamine, some amino acids, serum and biotin are responsible for morphogenesis and the opposite condition like low temperature, absence of serum, acidic pH, and higher concentration of glucose reverse the hyphal form to yeast form (Corner and Magee 1997; Eckert *et al*., 2007).

Normally yeast cells form smooth, white dome-shaped colonies though, at low frequency, 3153A strain of *C. albicans* can instinctively and reversibly convert into various colony shapes i.e. star, ring, stipple, hat, irregular wrinkle and fuzzy) in which the colonies are poised of a mixture of yeast and filamentous cells. The ability to switch between different morphological forms such as yeast, hyphal and pseudohyphal is considered necessary for its virulence. The

yeast form is suited for disseminate in the bloodstream whereas invasion are caused by both hyphae and pseudohyphae. In laboratory they invade the agar substratum thus; this property could show infection during early stage and promote tissue penetration. Hyphae are unfailingly induced from unbudded yeast cells by the addition of serum and a growth temperature of 37°C (Sudbery *et al*., 2004).

3.6 Nanoparticles

Nanoparticles are a class of materials with at least one dimension in the nanoscale length (<100 nm). As the size of material changes and approaches to nanoscale size the physio-chemical properties of nanomaterials has changed significantly. Environmental remediation, biomedical devices, cosmetics, and renewable energies are some fields where novel properties of NPs have been exploited (De M, *et al.*, 2008; Lu A-H *et al.*, 2007; Tran *et al.*, 2013).

3.7 Silver Nanoparticles

Silver nanoparticles (AgNPs or nanosilver) due to their unique physical, chemical and biological properties have attracted lot of attention (Sharma *et al.*, 2009). High electrical and thermal conductivity, surface-enhanced Raman scattering, non linear optical behavior chemical stability and catalytic activity are some physico-chemical properties of silver nanoparticles have helped to use in various fields inks, electronics, photonics, biosensing, imaging techniques, medicine, antimicrobial applications and microelectronics (Krutyakov *et al.*, 2008; Monteiro *et al.*, 2009; Wei *et al*., 2015). In regular domestic needs such as plastics, soaps, antiseptic sprays, catheters, pastes, bandages, textiles, food storage containers, silver nanoparticles are being used because of it well known broad spectrum of antimicrobial property (Ahmed *et al.*, 2010; García-Barrasa *et al.*, 2011).

The experiments with AgNPs have gained attention because of the positive outcomes when it was used to treat cancer. It is also used in various therapeutic applications and it is necessary to expand their biomedical applications by controlling the morphological and physicochemical features. Nanosilver technologies now appear in a variety of manufacturing processes and end products (Wei *et al*., 2015). Chemical, physical, and biological methods are being used nowadays to synthesize silver nanostructures (Wei *et al*., 2015). In diagnosing and imaging, an optical property of silver nanostructures is important which result from their unique interaction with light. In cancer cell killing by radiation treatment AgNPs are used to enhance the effect (Wei *et al*., 2015).

3.7.1 Properties of AgNPs

To determine their biological role in interaction and their impacts physicochemical properties such as shape, surface charge, size (surface area), coating, agglomeration, and dissolution rate of AgNPs are important. Because of their small size they have large surface area thus show maximum toxic potential (Johnston, 2010). Shape of AgNPs dramatically affects chemical and physical properties. Biomedical utilize various silver nanostructures such as silver spherical nanoparticles, nano-rods, nano-wires, nano-cubes and nano-plates (Rycenga et al., 2011). Interaction of AgNPs with biological systems largely depends on the different surface charges present on their coatings (Powers et al., 2011). Agglomeration occurs with most engineered nanoparticles and also occurs within the cytoplasm, culture media and nuclei of HepG2 cells. Surface oxidation of AgNPs i.e. dissolution leads to the production of silver ions depends on the surface, size, and chemical properties also affected by the surrounding media (Wei *et al*., 2015).

CHAPTER 4

MATERIALS & METHODS

4. Materials and Methods

4.1 Chemicals and reagents

All the media and chemicals were obtained from HI-MEDIA (Mumbai, India) and Fisher Scientific (Mumbai, India). All the solvents used for experiments were of analytical grade and obtained from Fisher Scientific (Mumbai, India).

4.2 Strains and growth media

Wild type *Candida albicans* strain (Fonzi *et al.,* 1993) has been used for this study. The fungal strain was maintained on liquid broth (YEPD medium) containing 2% (w/v) dextrose, 1% (w/v) yeast extract and 2% (w/v) peptone. In addition, 2.5% (w/v) agar was used for the preparation of solid media. Strains were preserved in 15 % (v/v) glycerol at -80 ºC. Cells were revived on YEPD plates from frozen glycerol stocks and maintained at 4°C. Liquid media cell cultures were grown at 30 °C with continuous shaking of 140-150 rpm for 14-16 h and these exponentially growing cells were used for all the experiments.

4.3 Preparation of silver nanoparticles by chemical reduction method

Silver nanoparticles were prepared by a combination of two methods including seed preparation and nanoparticle preparation. Silver seed has been prepared by rapid injection of 0.5ml of 10mM sodium borohydrate containing the solution 0.5ml of 10mM silver nitrate and 20ml of 1mM sodium citrate. The solution was stirred well for 5 min and kept for ageing for 90 minutes. The silver nanoparticles have been prepared by reducing silver nitrate and aqueous solution of sodium citrate near boiling point temperature. The 100ml aqueous solution of 1mM silver nitrate was heated to near boiling temperature, then 3ml of silver seed and sodium citrate were added in such a way that the final concentration of sodium citrate would become 1mM. The solution was heated till it became greenish yellow in color and allowed to cool at room

temperature. Particles were separated by centrifugation at around 40000 rpm. The thus obtained nanoparticles were washed with deionized water thrice to remove impurities and freeze dried for long term storage (Sukdeb *et al.*, 2007).

4.4 Characterization of AgNPs by UV-Vis spectroscopy

Silver nanoparticles prepared by chemical reduction method were characterized by UV-Vis spectroscopy. The absorption spectrum of AgNPs was obtained by filling the diluted solution of nanoparticles into the quartz cuvette and then measures the absorption at scan mode from 200nm to 600nm in (Perkin almer L35) UV-Vis spectroscopy.

4.5 Drug susceptibility assay

4.5.1 Spot assay

In this assay, cells from overnight grown YEPD agar plate were suspended in 0.9% saline and the OD_{600} of the suspension was adjusted to 0.1. Then, 5 µL of fivefold serial dilutions of each yeast culture was spotted onto YEPD plates in the absence (Growth control) and presence of the silver nanoparticles. Growth differences were recorded after incubation of the plates for 48 hrs at 30°C (Mukhopadhyay *et al.*, 2004).

4.6 Morphogenic studies on spider media

Studies on hyphal morphogenesis of *C.albicans* cells were carried out in spider media (T.Prasad, 2005). It consists of 1% nutrient broth, 1% Mannitol and 0.2% K_2HPO_4 and pH of media was adjusted to 7.2 using 1N HCl. The induced cells were inoculated in spider media and allowed to grow at 37ºC. Cells were taken out at frequent intervals and checked for hyphal growth. AgNp with two different concentrations 20µg/ml and 40µg/ml were added in the media to check the inhibition on hyphal morphogenesis. Images were taken at confocal microscope *FluoView™ fv1000* at different interval.

4.7 Morphogenic studies on N–acetyl glucosamine media

Studies on hyphal morphogenesis of *C. albicans* cells were carried out in N- acetyl glucosamine media. It consists of 0.45% Sodium Chloride (NaCl), 0.335% Yeast Nitrogen Base (YNB) without amino acid and 2.5mM N-acetyl glucosamine. The induced cells were inoculated in N-acetyl glucosamine media and allowed to grow at 37ºC. Cells were taken out at frequent intervals and checked for hyphal growth. AgNp with two different concentrations 20µg/ml and 40µg/ml were added in the media to check the inhibition of hyphal morphogenesis. The cells were allowed to grow at 37 ºC and images were captured using confocal microscope *FluoView™ fv1000*.

4.8 Sterol estimation in spider and N-acetyl glucosamine media

Sterols were extracted as described earlier in (T Prasad, 2010) with slight modifications from cells grown overnight at 30°C in the absence (growth control) and presence of AgNp from two different media (spider media and N-acetyl glucosamine media). The hyphal induced cells along with AgNp treated were maintained separately. Cell pellets were re-suspended in 2.5 ml methanol, 1.5 ml potassium hydroxide (60% w/v) and 1 ml pyrogallol dissolved in methanol (0.5% w/v). The obtained suspension was kept for refluxing at 80°C for 2 hrs and allowed to cool. The sterol was extracted with the help of *n*-heptane and extraction was repeated by adding known volume of *n*-heptane two to three times. The extracted sterols indicated four-peak spectral absorption patterns which are produced by ergosterol and 24(28)-dehydroergosterol (24 (28) -DHE) contents. Both ergosterol and 24 (28) -DHE absorb at 281.5 nm ($A_{281.5nm}$), whereas only 24(28)-DHE absorbs at 230 nm (A_{230nm}).

Ergosterol content was calculated as a percentage of the wet weight of the cells employing the following equations:

$$\textbf{\% Ergosterol + \% 24(28)-DHE} = [(A_{281.5nm}/290) \times F] / \textbf{pellet weight;}$$

$$\textbf{\% 24(28)-DHE} = [(A_{230nm}/518) \times F] / \textbf{pellet weight and}$$

$$\textbf{\% Ergosterol} = [\textbf{\% ergosterol + \% 24(28) DHE}] - \textbf{\% 24(28) DHE,}$$

where, A is absorbance, F is the factor for dilution in hexane, 290 and 518 are the E values (in percent per centimeter) determined for crystalline ergosterol and 24(28) DHE, respectively.

CHAPTER 5

RESULTS AND DISCUSSION

5. Results and Discussion

5.1 Characterization of AgNPs by UV-Vis spectroscopy

UV- Visible spectroscopy is the most widely used technique to characterize the metal nanoparticles by monitoring their optical absorption spectra. AgNPs absorption spectrum was taken in a quartz cuvette at full scan mode from 200nm to 600nm. Since, metallic nanoparticles have characteristic optical absorption spectra in the UV-Vis region. A single strong peak was obtained at 407nm in the absorption spectrum (Fig 5.1).

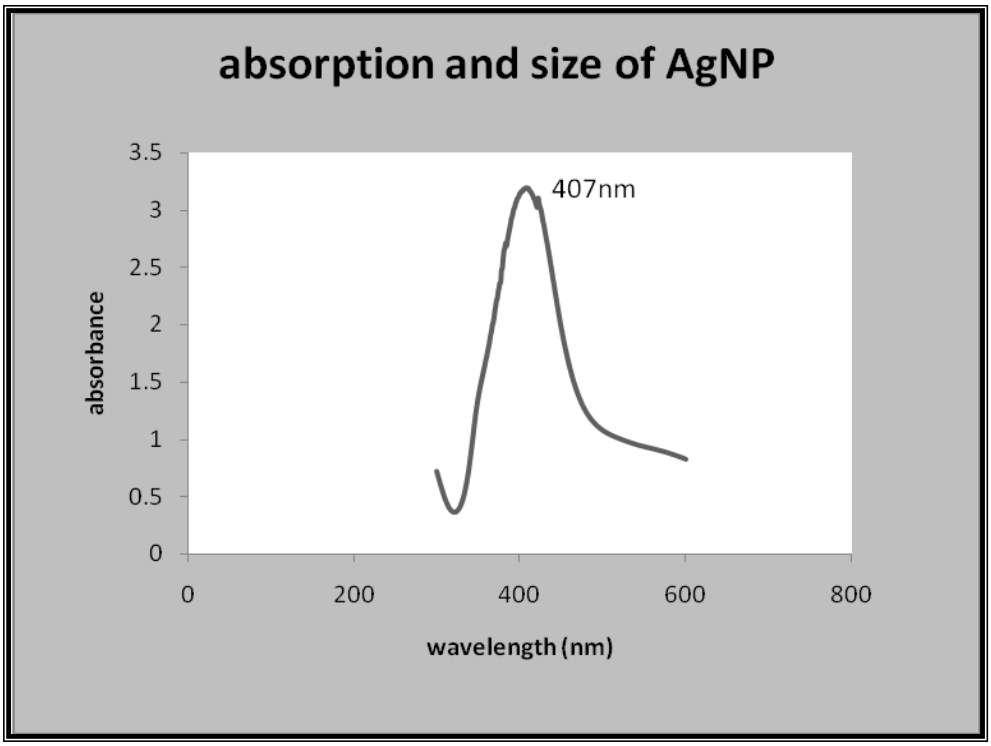

Figure 5.1 UV-Vis absorption spectra of colloid solution of chemically synthesized silver nanoparticles.

Surface plasmon resonance (SPR) is responsible for optical absorption of metal nanoparticles which usually shifts to longer wavelength with size of particles. Plasmon absorption of silver nanoparticles is dependent on size of nanoparticles, dielectric medium and

surface absorbed species. Single SPR band is most predominant in spherical nanoparticles according to Mie`s theory whereas anisotropic particles could rise two or more SPR band. The results obtained confirmed that the prepared nanoparticles were spherical in shape (Chitte *et al*., 2012; Sukdeb *et al.*, 2007).

5.2 *Candida albicans* cells showed sigmoid growth pattern

Cells were grown on YEPD growth media. Growth of *Candida* cells were monitored by measuring OD at 600nm. The obtained values were plotted against time (Fig. 5.2). *Candida albicans* cells showed the characteristic sigmoid pattern of growth. The mid-exponential time was noted for further experiments.

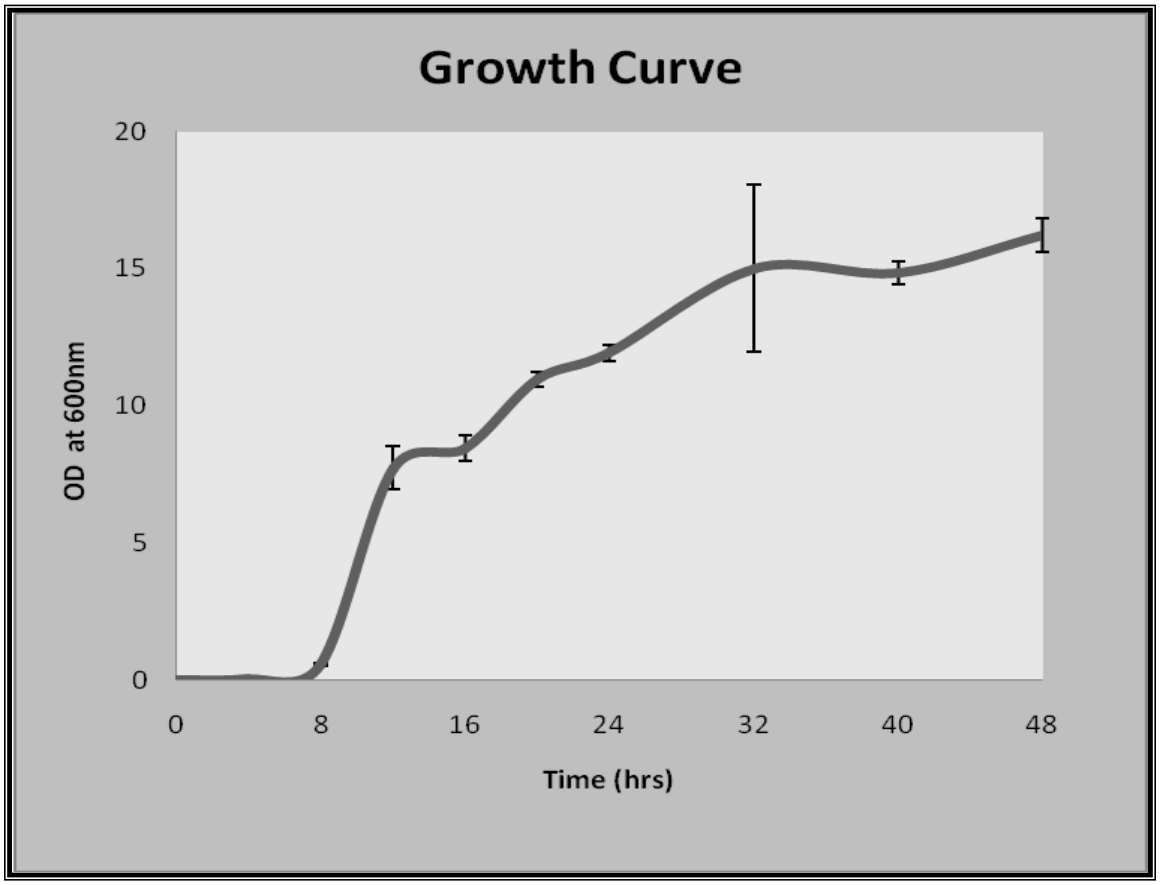

Figure 5.2 Growth curve of *Candida albicans* with respect to Time.

5.3 Spot assay validated the anti-*Candida* effects obtained by determination of the minimum inhibitory concentration of AgNps

Spot assay was used to evaluate the drug susceptibilities of *C. albicans* cells on solid media in presence and absence of AgNPs. The serially fivefold diluted cell suspension were spotted on the agar plates in the presence of AgNPs and growth was recorded after 48hrs incubation. The obtained results confirmed the susceptibility of *Candida* cells towards AgNPs. Spot assay helped to determine the minimum inhibitory concentration of AgNPs (Fig. 5.3). Non-treated plates were maintained separately.

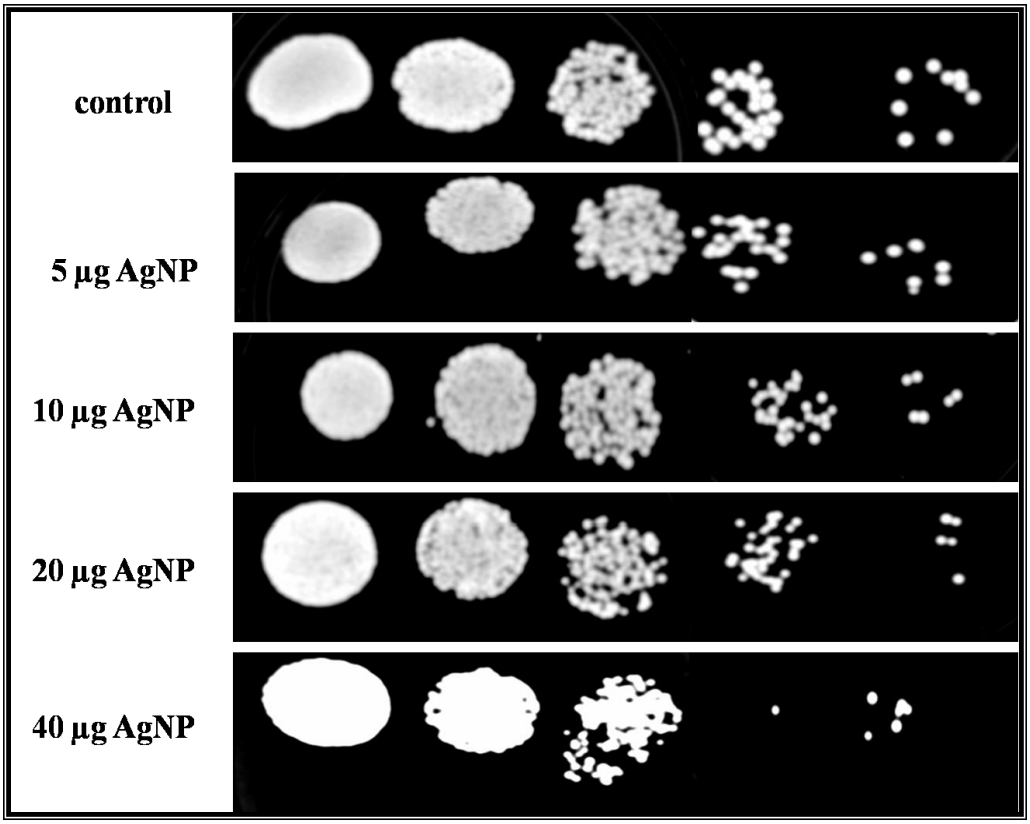

Figure 5.3 Spot assay of *Candida albicans* showed inhibition in growth in the presence of various concentrations of AgNPs and control (non-treated) were maintained separately.

5.4 AgNps on spider media showed inhibition on hyphal morphogenesis

This study helped to explore the effect of AgNPs on hyphal development in different growth media. Spot assay results of *Candida* cells helped to find the AgNPs minimum inhibitory concentration. The induced *Candida* cells were grown on spider media and incubated at 37ºC in the presence of different concentration of AgNPs. Cells were taken at various time intervals and viewed under confocal microscope. The obtained results showed the inhibition of hyphal morphogenesis upon AgNPs treatment (Fig.5.4). The hyphal development could be prevented completely by increasing concentration of AgNPs.

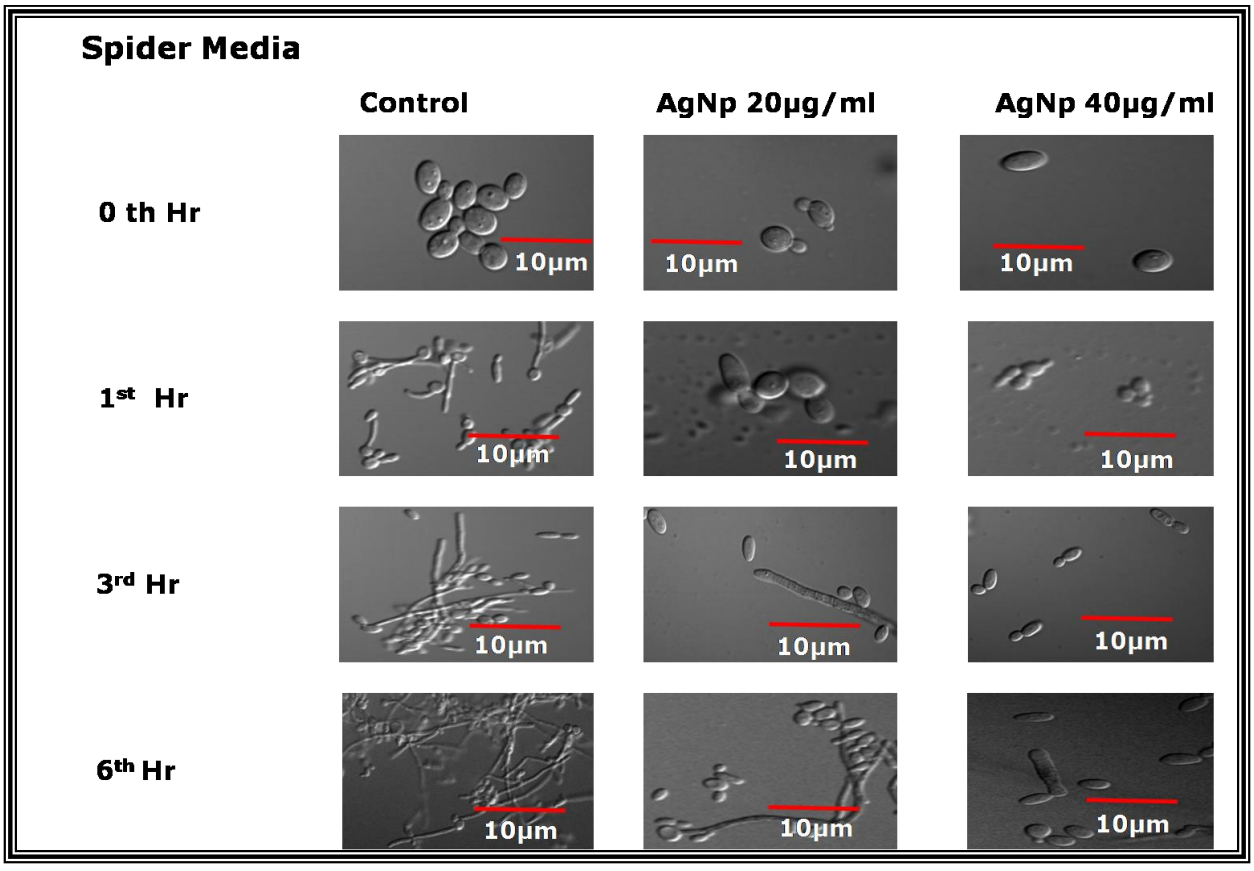

Figure 5.4 Hyphal morphogenesis on spider media with presence of AgNPs of different concentrations. Non-treated (control) cells were maintained separately. The images were captured by confocal microscope *FluoView™ FV1000*.

5.5 AgNPs on N-acetyl glucosamine supplemented media showed inhibition on hyphal morphogenesis

The induced *Candida* cells were grown on N-acetyl glucosamine supplemented media and incubated at 37 ℃ in the presence of different concentration of AgNPs. Cells were taken at various time intervals and viewed under confocal microscope. The obtained results were showed the inhibition of hyphal morphogenesis upon AgNPs treatment (Fig. 5.5). The lower concentration of AgNPs could inhibit the hyphal morphogenesis by only 50%. But at higher concentration, AgNPs were able to inhibit 80% - 90% of hyphal morphogenesis.

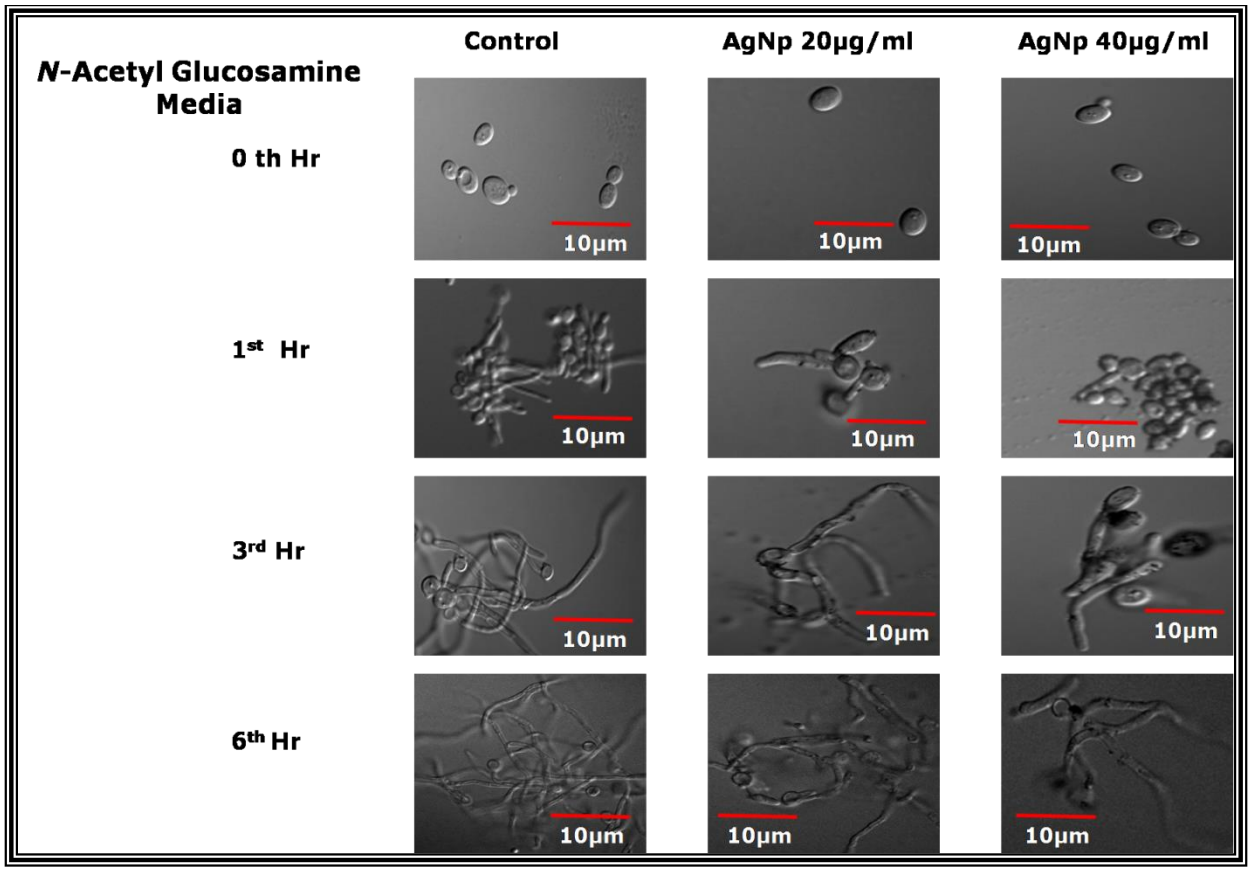

Figure 5.5 Hyphal morphogenesis on N-Acetyl glucosamine supplemented media with presence of AgNps of different concentrations. Non-treated (control) cells were maintained separately. The images were captured by confocal microscope *FluoView™ FV1000.*

5.6 Marked reduction in levels of ergosterol was observed in cells after treatment with AgNPs

The ability of AgNPs in the inhibition of hyphal morphogenesis was well illustrated in the susceptibility assay. But the molecular mechanism behind the AgNPs inhibition on hyphal morphogenesis was not well known. This study was an effort to elucidate the role of sterol on hyphal morphogenesis. Ergosterol is the main component of cell membrane which is involved in membrane homeostasis. Induced cells were grown on spider media and incubated at 37 ºC to induce hyphal cells. Cells were harvested and the sterols were extracted. The unique four peak absorption pattern between 200 nm to 400 nm indicates the presence of both ergosterol and dihydroergosterol (DHE) at 281.5 nm and only DHE at 230 nm. Therefore, the amount of ergosterol can be determined by subtracting the absorption of only DHE from total ergosterol and DHE content (Arthington-Skaggs *et al*., 1990). Cells treated with different concentration of AgNPs showed significant reduction in sterol level (Fig. 5.6). When compared to control, 20µg and 40µg of AgNPs showed 9% and 19% reduction in spider media.

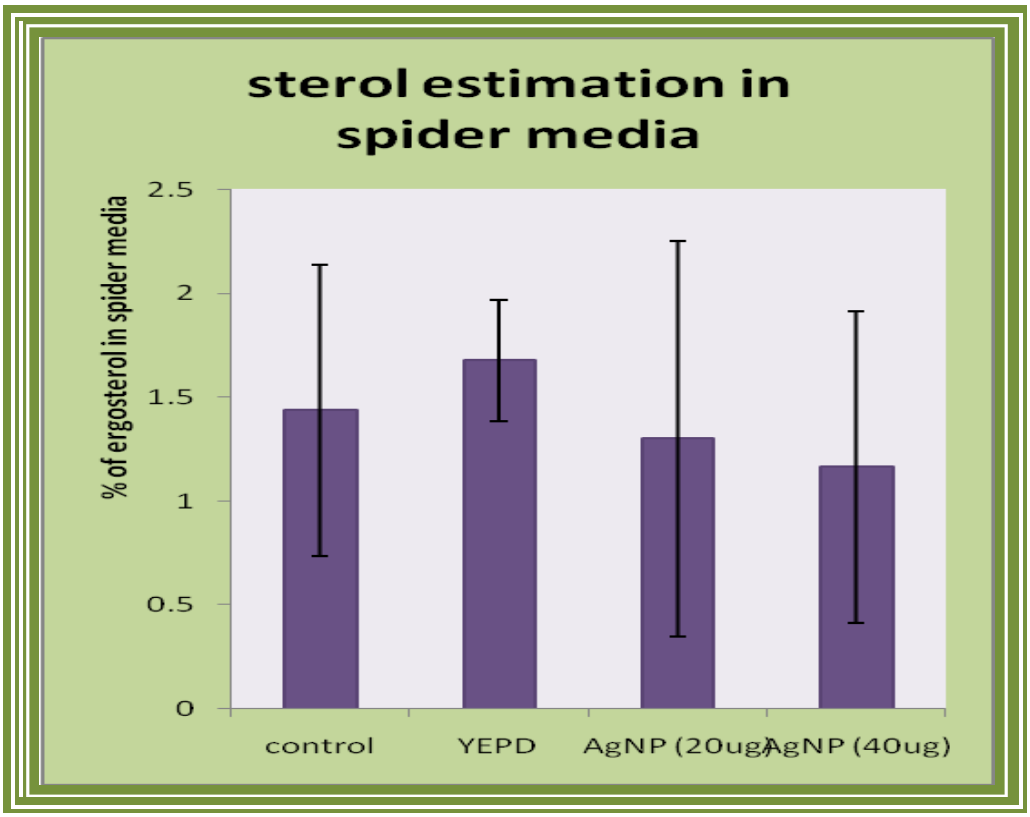

Figure 5.6 Sterol estimation of untreated (control) cells and cells treated with different concentration of AgNPs in spider media.

5.7 Marked reduction in levels of ergosterol was observed in cells after treatment with AgNPs

This study was an effort to elucidate the role of sterol on hyphal morphogenesis. Ergosterol is the main component of cell membrane which is involved in membrane homeostasis. Induced cells were grown on N-Acetyl glucosamine supplemented media and incubated at 37 ℃ to induce hyphal cells. Cells were harvested and extracted the sterols. The unique four peak absorption pattern between 200 nm to 400 nm indicates the presence of both ergosterol and dihydroergosterol (DHE) at 281.5 nm and only DHE at 230nm. Therefore, the amount of ergosterol can be determined by subtracting the absorption of only DHE from total ergosterol and DHE content (Arthington-Skaggs *et al*., 1990). Cells treated with different concentration of

AgNPs showed significant reduction in sterol level (Fig. 5.7). When compared to control, 20µg and 40µg of AgNPs showed 29% and 50% reduction in N-acetyl glucosamine supplemented media.

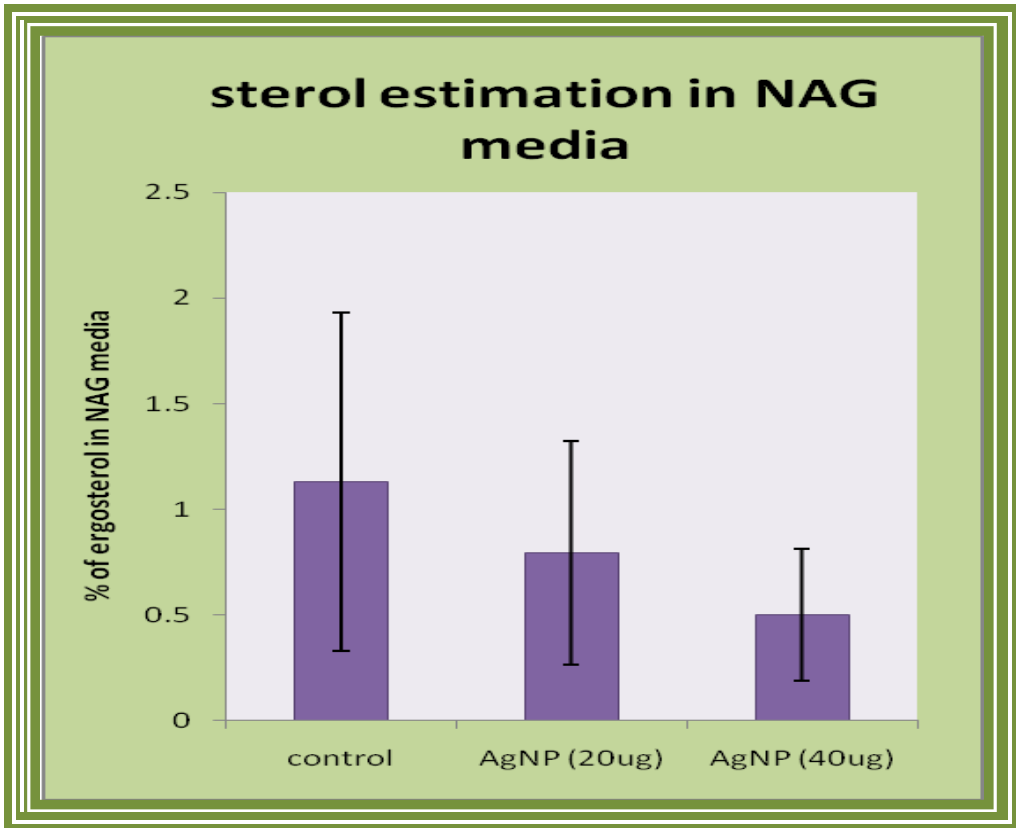

Figure 5.7 Sterol estimation of untreated (control) cells and cells treated with different concentration of AgNps in N-Acetyl Glucosamine supplemented media.

CHAPTER 6

CONCLUSIONS

6. Conclusions

AgNPs were prepared by chemical reduction method and characterized by UV – Visible spectroscopy. The synthesized nanoparticles have shown a single sharp peak at absorption of 407nm. Spot assay helped to find the minimum inhibitory concentration of AgNPs in *C. albicans* cells. Hyphal morphogenesis was completely inhibited in the presence of AgNPs in as low as 20μg to 40μg concentrations. AgNPs showed hyphal inhibition on both spider and N-acetyl glucosamine supplemented media. Furthermore, the sterol content was analyzed to dissect the molecular mechanism behind the AgNPs inhibition on hyphal morphogenesis. Significant reductions in sterol content were observed in AgNPs treated cells grown in spider and N-acetyl glucosamine supplemented media. The obtained results were compared with non-treated (control) cells. Ergosterol is the main sterol present in membrane and maintains the stability of membrane, thus by altering their sterol content it may inhibit the hyphal morphogenesis of *C. albicans* towards AgNPs. Further studies are underway to decipher the activity of AgNPs and to elucidate the molecular level mechanism of action involed which control its inhibition. Thus, it appears from the results that the AgNPs used for this study have clinical relevance since they are effective against hyphal morphogenesis because of the opportunistic *Candida* infection in immunocompromised hosts such as cancer, transplant, burned, AIDS patients etc. It is evident from the results that the AgNPs used for this study are also effective in interfering with the bio-synthetic pathway of ergosterol. It is important to find that AgNPs other cellular targets which appear to have the potential to contribute to development of new therapeutic strategies for clinical applications. Further investigations are underway.

CHAPTER 7

FUTURE PERSPECTIVES

7. Future Perspectives

AgNPs can effectively inhibit the hyphal morphogenesis of *C. albicans* which has been considered as the most pathogenic, invasive and life threatening form for patients who have weak immune system. Thus silver nanoparticles can be used to treat patients and prevent the development of worse conditions. Since *Candida albicans* is commensal in human body, thus is also important for body, AgNPs do not kill them; they only inhibit its pathogenic hyphal form. Synthesis of silver nanoparticles can also be done by green synthesis methods instead of chemical reduction methods to remove toxicity and check the effecs of hyphal morphogenesis and ergosterol content of *Candida albicans*. Silver nanoparticles in addition can be combined with other drugs to treat patients by synergistic effects or combinatorial therapy. Further studies can be executed to find the transcription level inhibiting the hyphal morphogenesis of *Candida albicans* due to the presence of AgNPs. Silver nanoparticles can be applied further to be used as an important antifungal agent to treat patients who are severely immunocompromised with fungal infections such as candidiasis mainly caused by *C. albicans*. Thus clinical applications could be approached for further investigations. Drug delivery system can be used to enhance the effect and specificity of AgNPs. Further AgNPs can also be used to check its effects on other fungal diseases.

CHAPTER 8

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