# Chapter 1

# INTRODUCTION

## 1.1 Antifungal protein

Antifungal protein belongs to a class of small, basic and cysteine-rich peptide that exerts extremely multi-potent activity against human and plant-pathogenic fungi or yeast without affecting the viability of bacteria, plant and mammalian cells. All these antifungal proteins are secreted either extracellular or intracellular mode. Most reasonably, they are significantly related in regard to their structures, sizes, and basic characters.

Supported on their degrees of susceptibility, filamentous fungi are classified into sensitive species (e.g., *Aspergillus fumigatus*, *Aspergillus niger*, *Fusarium oxysporum*, and *Fusarium moniliforme*), moderately sensitive species (e.g., *Aspergillus nidulans*), and resistant species (e.g., *Penicillium chrysogenum*) **[1, 2].** Antifungal proteins are strings of amino acids that act on parts of the fungal cell. These proteins (typically 5-40 kDa) are cationic or anionic due to copiousness of arginine and lysine amino acids throughout the chain **[3]** and amphipathic (have both a hydrophilic and a hydrophobic region) and exhibit an undogmatic compass of activity **[4].** Since fungi and microbial surfaces are often negatively charged, they tend to attract the positively charged antifungal and antibacterial proteins.

A common characteristic observed for membrane-active peptides is their capability to disturb bilayer integrity, either by disruption or pore formation. The resulting openings in the lipid bilayer lead to the collapse of the trans-membrane electrochemical gradients and, therefore, can explain the cell-killing activities of these peptides" **[5].**

Antifungal protein make up a part of the innate immunity of most organisms and are often involved in the immune system's first line of defense when faced with an invader **[6].** It can be found in prokaryotic and eukaryotic organisms and in vertebrates and invertebrates **[4]** and are commonly grouped according to their second and third degree structures and compositions.

This refers to the spatial arrangement the peptide assumes as it assembles in the cell. Common groupings include **[4, 6-8]** amphipathic alpha helices deprived of cystine residues; beta pleats or alpha-beta mixed sheets with intramolecular disulfide bonds; rich in rare and/or modified amino acids; over-presentation of common amino acids, including proline, histidine, glycine; cyclic and open-cyclic with pairs of cystine residues.

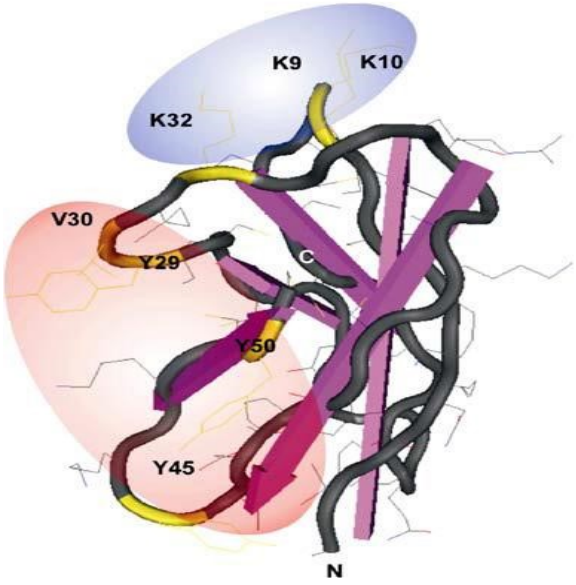


**Figure 1.1** Cartoon conformation of AFP **[9, 10],** the orientation of the β-strands is symbolized by arrows (image was drawn using Cn3D4.1 software). The amphipathic structure of AFP (PDB accession number P17737, with input sequence ATYNGKCYKKDNICKYKAQSGKTAICKCYVKKCPRDGAKCEFDSYKGKCYC) is defined by a cationic domain (K9, K10, K32) and a hydrophobic domain (Y29, V30, Y45, V50).

Fungal cell walls are dynamic structures that are very colonial and are obligated for maintaining the shape and the integrity of the cell. Factors such as polarized growth, environmental stimuli, and stress cause fungi to happening the composition and architecture of their cell walls **[11].**

**Table 1.1** Common cell wall constituents found in fungi (Gow & Gadd, 1995)

| Division | Fibrous | Gel-like Polymer |
|---|---|---|
| Basidiomycota | Chitin<br>β -(1-3), β-(1-6) Glucan | Xylomannoproteins<br>α (1-3) Glucan |
| Ascomycota | chitin<br>β -(1-3), β-(1-6) Glucan | Galactomannoproteins<br>α (1-3) Glucan |
| Zygomycota | Chitin<br>Chitosan | Polyglucuronic acid<br>Glucuronomannoproteins<br>Polyphosphate |
| Chytridiomycota | Chitin<br>Glucan | Glucan |

The constituents of cell walls are synthesized in the cytoplasm, linked in the walls at the hyphal tip, and polymerized and cross-linked in the wall matrix. Chitin and the glucans are synthesized at the plasma membrane by enzymes embedded in the membrane. Nucleotide sugar precursors are accepted from the cytoplasm, linked and passed to the wall.

Wall glycoproteins are synthesized in the endoplasmic reticulum, carried through the Golgi to the plasma membrane, where vesicles release the glycoprotein to the wall. Enzymes cross-linking fibrils in the wall are released through the plasma membrane.

Chitin, a polymer consisting of α-1,4-linked *N*-acetyl glucosamine (Glc-NAc) units, is one of the main structural components in fungal cell walls, and increased chitin biosynthesis is essential in the compensatory response to cell wall stress in yeasts and filamentous fungi **[12]**.

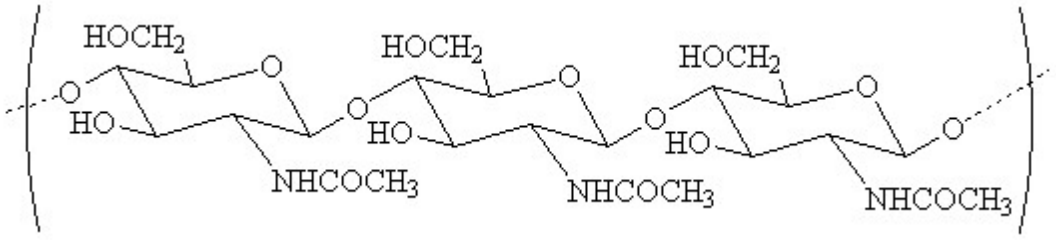


**Figure 1.2** Structure of chitin (N-acetyl-glucosamine)

## 1.2 The emerging world of fungal infection

The past two decades have seen an increasing number of virulent infectious diseases in natural populations and managed landscapes. In both animals and plants, an unprecedented number of fungal and fungal-like diseases have recently caused some of the most severe die-offs and extinctions ever witnessed in wild species, and are jeopardizing food security. It has been debatable that nascent fungal infections will cause increasing attrition of biodiversity, with wider implications for human and ecosystem health, unless steps are embezzled to limit biosecurity worldwide **[13, 14].**

Although the vast majority of fungal species affect plants and liable for operative efficient losses **[15].** In the historical duo of decades, fungal diseases of humans have become an increasing threat, especially for people transform an accelerative danger, especially for people who are immunologically compromised **[16].** Pathogenesis involves the interaction of two partners with input from the environment, a concept described as the “disease triangle” in plant pathology.

The “damage-response” concept developed for animal pathogens emphasizes that the outcome of an interaction is determined by the amount of damage incurred on the host **[17, 18].** Fungal diseases are considered “life threatening” to humans. At the same time, human health has benefited immensely from fungal-derived antibiotics, such as penicillin **[19-22].**

Indeed, fungi are indispensible to life on this planet through their ability to break down complex organic matter and recycle essential nutrients back into the environment **[23]**. Despite the extensive influence of fungi on economic well-being, as well as on human, animal, plant, and ecosystem health, the threats posed by emerging fungal pathogens are oftentimes ungratifying and poorly taken.

The incidence of fungal infections has augmented recently and is associated with the growing populations of vulnerable, immunocompromised individuals (e.g., people living with HIV/AIDS, recent organ transplant recipients) **[24].** These opportunistic fungal diseases include invasive aspergillosis and aspergilloma (*Aspergillus* spp.), invasive fusariosis (*Fusarium* spp.), Pneumocystis pneumonia (*Pneumocystis jirovecii*), and invasive candidiasis (*Candida* spp.) **[25, 26].**

In 1923 Christine Marie Berkhout named what we now call *Candida albicans*, for the white robe, *Toga candida*, worn by Roman senators and senatorial candidates **[27].** Fungal infections in general and Candida infections in particular, are important markers of innate or acquired immune dysfunction.

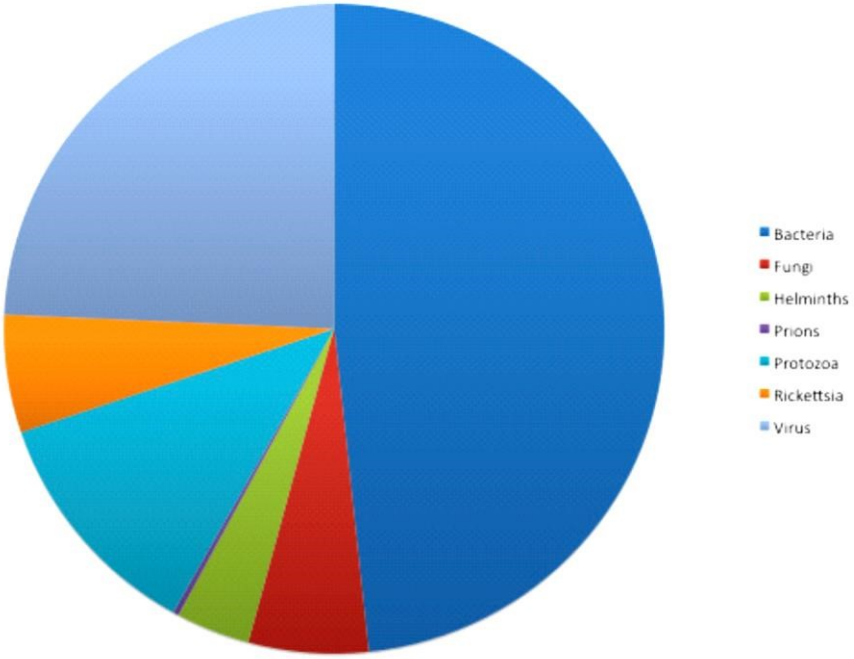


**Figure 1.3** Proportion of emerging infectious diseases caused by different taxonomic groups of pathogens **[28].**

### 1.3 Invasive Candidiasis (Indian context)

The most common yeast infection is the vaginal yeast infection, medically proverbial as vulvovaginal Candidiasis or vulvovaginitis. It is an inflammation that occurs in women when there is excessive growth of this yeast in the vagina. Almost 75 percent of women receive from this transmission at much quantity in their life experience. Candida infections are a rising grounds of rate in immunocompromised and critically ill patients.

Prolong life with immunosuppressive therapy for a variety of disorders and administer broad spectrum antibiotics increases the risk of fungal infections. An increasing incidence of invasive candidiasis reported from India **[29].**

Most of the grounds based guidelines for direction of systemic infections are based on participate of patients of piercing leukemia and withdraw bone marrow conveyance due to the high incidence of infections in this population. Normal amounts of Candida live in the mouth, stomach, and vagina, and do not cause infections. Candidiasis occurs when there is an overgrowth of Candida. Only when this number multiply rapidly it results in an infection due to its overgrowth and causes itching, burning sensation, feeling of soreness and pain **[30].**

Oral Candidiasis is another type of yeast infection. Commonly called thrush, this infection occurs as thick white and lacy looking patches on the tongue or palate. They look like curdled milk which does not wipe off. This can be painful and children stop eating and drinking, so a physician needs to be consulted. Infants can develop yeast infection as diaper rashes. In very rare cases the older can acquire an incident in their dentures or folds of regular peel under breasts. Vaginitis may become after a urinary tract infection, for which a direction of antibiotics is formal, which in transmute destroys the restrictive bacterium.

As a result, yeast thrives multifold and causes irritation to the walls of the vagina. The Candida infection also takes place when immune suppressive drugs are taken by a patient. Fungal infections are progressively being recognized as a cause of the utmost mortality noted in ICUs across the world. Data from India is meager as we have very few good diagnostic mycology laboratories, and many clinicians are still not aware of the emerging trends in diagnosis & Management of Fungal Disease. Invasive candidiasis is emerged as the most common opportunistic mycosis; while Invasive aspergillosis may be the second contender. Invasive zygomycosis is an important concern as the world's highest number of cases of this disease is reported from India in patients with uncontrolled diabetes mellitus **[31].**

Emergence of fungal *rhinosinusitis, penicilliosis marneffei* and *zygomycosis* due to *Apophysomyces elegans* is unique in the Indian scenario. Among HIV infected patients Candidiasis (41.7 %), Cryptococcosis (10.0 %), Pneumocystinosis (8.3%), Aspergillosis (8.3%) and Histoplasmosis were commonly diagnosed **[32]**.

A novel agent of fungemia, *Candida auris*, is reported as having been sensed in India **[33].** Preliminary analysis of data collected by Post Graduate Institute of Medical Education and Research Chandigarh showed seven out of every 1,000 patients admitted in ICUs across India are affected with fungal infection. The data was composed from 27 hospital ICUs across the country (11 government hospitals and 16 private hospitals). At 39.55 per 1,000 patients, Global Hospitals of Hyderabad recorded the highest rank of infection. Delhi's Safdarjung Hospital was second worst with 32.75 people per every 1,000 affected with fungal infection **(PGIMER-WHO, 2014 report).** Nearly 10 per cent of farmers and agricultural workers going feat sightless can be attributed to fungal infection.

This is because while working in the field, tips of wood or straw enter their eyes and cause infection by touching the cornea and the damage in such cases cannot be reversed as people in rural India usually visit a health facility very late after the onset of disease. Due to tropical climate, South Asian country has a high incidence of fungal infections. For example, infection from yeast, which is a type of fungus, is found in 0.8 out of every 1,000 patients in the US; the incidence is 0.2 and 0.9 per 1,000 patients in Europe and Australia respectively. In India, 1-12 cases out of every 1,000 patients are found to be affected with yeast infection. Apart from climate, there are other factors too.

India has an extensive confine of grouping from economically weaker sections and malnutrition makes children statesman immature to the infections. Prevalence of quacks with little awareness

of fungal infections is another cause for higher incidence of cases. Although various governments recognized body (Fungal Infection Study Forum) is working on a proposal to spread awareness programme about the infections, its effects and keep a stock of antifungal drugs at district health centers at any instant to avoid the infection.

### 1.4 Antifungal drugs spectrum and toxicity

Fungal infections range from superficial conditions of the skin (e.g. ringworm and athlete's foot) and nails (onychomycoses) to disseminated life threatening diseases. Serious invasive fungal infections caused by Candida spp., *Cryptococcus neoformans*, *Aspergillus* spp., *Pneumocystis carinii* and *Histoplasma capsulatum*, represent an increasing threat to human health. The prevalence of these systemic fungal infections has increased significantly during the past decade **[34, 35].**

Major factors responsible for this dramatic rise include greater use of broad-spectrum antibiotics, marked increases in the numbers of immune-compromised persons (AIDS, cancer and transplant patients), the use of central venous catheters, and an aging patient population **[36].** Until the 1970s, fungal infections were advised mostly treatable and the claim for new medicines to treat them was very small. Before this period, antifungal chemotherapy included only two kinds of compounds: potassium iodide, competent in the treatment of sporotrichosis; and two useful polyenes, nystatin and amphotericin B, which were introduced in the 1950s. Until the development of flucytosine (1964), there was little progress until the development of the azole drugs in the early 1970s. Thus, only a limited number of antifungal agents (polyenes and azoles plus the recently introduced Cancidas) are currently purchasable for the treatment of life-threatening fungal infections.

These antifungal agents pretending some limitations, specified as the evidential nephrotoxicity of amphotericin B **[35]** and emerging resistivity to the azoles **[37].** Despite several recent improvements, such as lipid formulations of polyenes with lower toxicity and new triazoles (voriconazole, rovuconazole and pasaconazole) with a wider spectrum of action, including activity against some azole-resistant isolates **[38].**

The exercise of new antifungal agents, preferably naturally occurring with novel mechanisms of action, is therefore, an urgent medical essential. The era of systemic antifungal chemotherapy effectively began with the introduction of amphotericin B-deoxycholate in 1958 by Squibb Laboratories, after complete attempts to develop orally bioavailable formulations of more than 200 polyene macrolide antibiotics produced by the soil actinomycete *Streptomyces* **[39].** Although amphotericin B was to become the criterion ideal treatment for sensible fungal infections for more than 40 years, infusion-related harmful effects and dose-limiting nephrotoxicity prompted the continuing hunting for equally effective but less toxic alternatives that could be administered both intravenously and orally. This agent emerged as the desirable polyene over the more toxic agent in this class, nystatin.

Nystatin has since been relegated to topical and localized therapy because of its unfavorable adverse effect profile. In 1979, the first systemic azole antifungal agent, ketoconazole, was introduced **[40]**. Azole agents exert their antifungal activity by blocking the demethylation of lanosterol, thereby inhibiting ergosterol synthesis. Ketoconazole was followed chronologically by fluconazole, itraconazole, and voriconazole **[41].**

Four another antifungal agents were investigated and developed: posaconazole, which has been submitted for US Food and Drug Administration, ravuconazole, BAL8557, and albaconazole. This goal was not realized until more than 3 decades later with the introduction of fluconazole in

1990. Unlike amphotericin B and the earlier imidazole antifungal agents (miconazole, ketoconazole), fluconazole possessed excellent oral bioavailability; foreseeable collinear pharmacokinetics with comprehensive distribution into many tissues, including the cerebral spinal fluid and vitreous chamber of the eye; and a much lower risk of drug interactions and toxicity in critically ill patients compared with earlier azoles **[42].**

Fluconazole was also effective for the treatment of oropharyngeal candidiasis in patients with AIDS; notwithstanding, resistance could be problematic in patients receiving prolonged treatment who had declining CD4+ cell counts **[43].** The polyene agents hold their antifungal activity via binding to ergosterol in the fungal cell membrane (**Figure 1.1**). This disrupts cell permeability and results in rapid cell death.

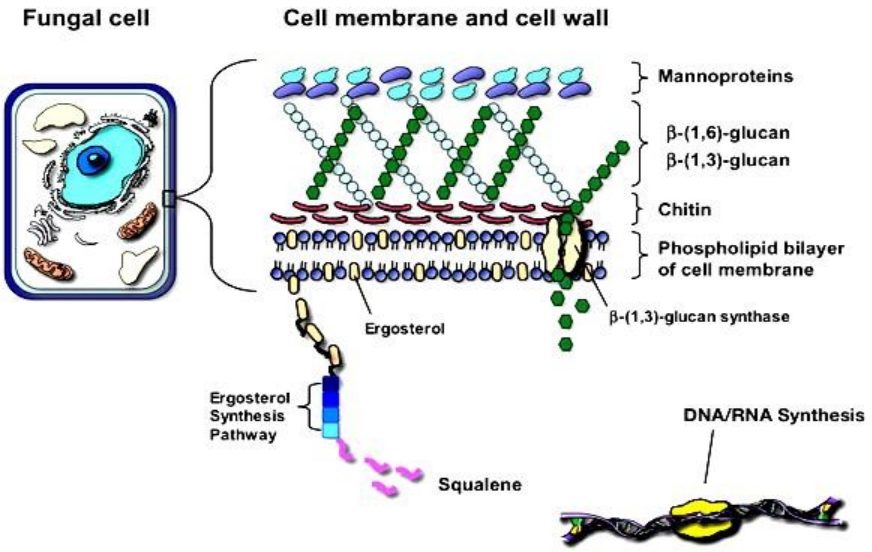


**Figure 1.4** Targets of systemic antifungal agents **[44]**

However, the lack of activity against opportunistic molds (ie, *Aspergillus*, *Mucorales*, and *Fusarium* species) and intrinsic resistance among some *candida* species (eg, *Candida glabrata*, *Candida crusei*) created a need for broader-spectrum alternatives.

Itraconazole (1992) was a partial solution to the limitations of fluconazole because the drug had improved activity against endemic fungi and *Aspergillus* species.

But the oral dosing formulations were plagued by erratic absorption **[40]** or unfavorable gastrointestinal (GI) effects **[45]** that qualified its powerfulness in cancer patients with mucositis or nausea and vomiting **[46].**

The echinocandins represent the newest class of antifungals. Caspofungin was released in 2001. This was followed by micafungin in 2005 and anidulafungin. The mechanism of activity of the echinocandins is inhibition of the production of glucan, an essential component in the fungal cell wall **[40].** The spectrum of activity is therefore modest to pathogens that rely on these glucans polymers and is less broad than the spectrums of the polyene or azole agents. The echinocandins exhibit fungicidal expression against many *Candida* species, making this drug class a coveted alternative to the azole agents, which exhibit exclusively static activity against yeasts **[41, 47].**

The commencement of the broader-spectrum triazoles voriconazole (2002) and posaconazole (2006) transformed the management of invasive mold infections in gravely immunocompromised patients. Voriconazole was shown to be more effective than conventional amphotericin B for the treatment of invasive aspergillosis **[48]** and is a useful agent for fusariosis **[49]** whereas posaconazole had a spectrum of activity that included not only *Aspergillus* and *Fusarium* species but also many *Mucorales* **[50, 51]**.

Both agents could be administered orally, paving the way for their use not only for the treatment of suspected or documented mildew infections but also as prophylaxis in severely immunocompromised patients **[52-54].** Unfortunately, the broader spectrum of activity with triazole antifungal agents comes at the disbursement of increased pharmacokinetic variability

and risk of dose-response interactions. Newer triazoles currently under investigation (ie, isavuconazole) appear to have a spectrum of activity similar to voriconazole and posaconazole, with inferior pharmacokinetic variances and drug interactions **[55].**

Efforts under way to reformulate the posaconazole suspension into better oral and intravenous dosage forms could address many of the drug's pharmacokinetic shortcomings. The final milestone of antifungal drug discovery in the 20th century was the identification and development of echinocandins antifungal agents.

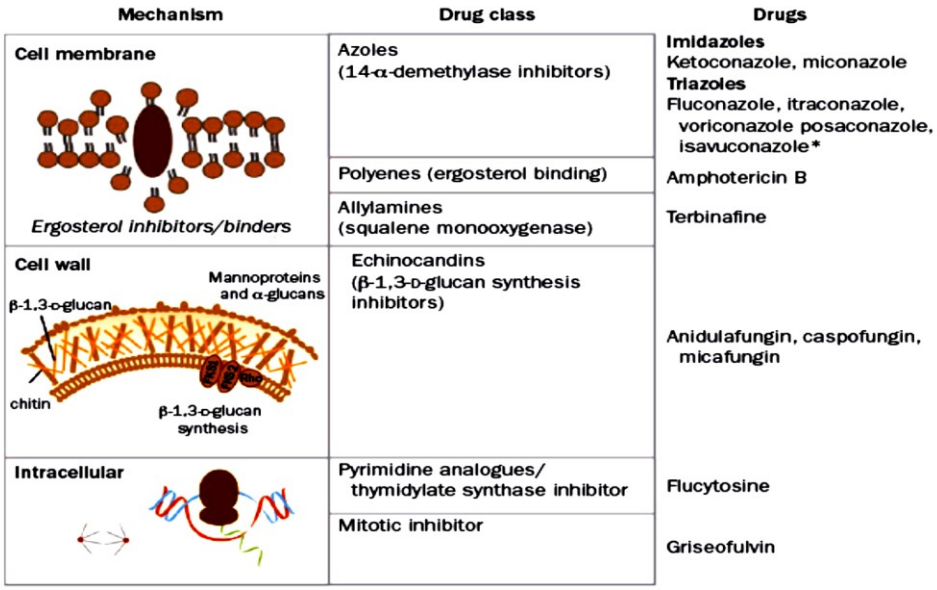


**Figure 1.5** Sites of action and mechanisms of systemic antifungal agents. Glucan synthase complex are the putative target binding site of echinocandins **[44]**

Echinocandins are semisynthetic lipopeptides that inhibit synthesis of β-1, 3-d-glucan in susceptible fungi, leading to damage of the fungal cell wall. Because a glucans rich cell wall is a target not found in mammalian cells, these agents were predicted to be effective antifungal agents with very little collateral toxicity in mammalian cells, a prediction that has been proven true in clinical trials of patients with invasive Candidiasis **[56-58]** and aspergillosis **[59].**

Nonetheless, echinocandins still lack activity against some common opportunistic yeasts (*Cryptococcus* species) and less common molds (ie, *Fusarium, Scedosporium*, and *Mucorales*) that often meliorate as breakthrough infections in gravely immunocompromised patients.

Therefore, although significant progress has been achieved since the dawn of systemic antifungal therapy in the 1950s, the current antifungal armamentarium is far from perfect. No single antifungal agent is appropriate for all patients for a given mycosis because of patient-specific co-morbid conditions, hypersensitivities, risk of drug interactions, immune-suppression, site of infection, and risk of infection with more intrinsically antifungal-resistant pathogens. This article reviews key aspects of the clinical pharmacology of older vs. newer antifungal agents, with a particular emphasis on pharmacokinetic issues that arise with newer agents and emerging data on toxicity with longer-term therapy.

An additional concern related to the increasing number of antifungal drugs is the rapid increase in expenditures associated with their use. Many institutions throughout the United States are struggling with the increased financial burden related to the prescribing of these antifungal drugs **[60].**

The newer, expanded-spectrum of antifungal agents have been shown to have both static and cidal activity against a wide spectrum of yeasts **([61, 62],** moulds and other dimorphs as well as enhanced activity against *Candida* species.

## 1.5 Benefits of antifungal proteins

### 1.5.1 Genetic improvement of crop with increased resistance

More work has been relinquished to an act supported on generation of transgenic plants expressing antifungal proteins to achieve significant reduction of disease symptoms (for example

**[63, 64].** Expression of osmotin genes in transgenic potato has resulted in multiple resistances such as to *Phytophthora infestanse*, a fungus that is known as the late-blight pathogen on potato **[65].** Almost all PR families have been expressed in transgenic plants and these plants have become more resistant to diseases **[66**].

Chitinases and β-1, 3- glucanases are the most cativating antifungal proteins due to their strong *in vitro* activity. Transgenic plants over expressing chitinases or β-1, 3- glucanases had been demonstrated with enhanced resistance to fungal pathogens and a synergistic benefit were observed when both transgenes were present **[67].** Due to the potential of broad-spectrum resistance from use of barley chitinase with antifungal activity, the chitinase gene could be used to enhance fungal resistance in crop plants such as tobacco, rice, clover and tea **[68].**

### 1.5.2 Novel agrichemical

Application of crop protection chemicals or agrichemicals including those with antifungal properties is a inferior agricultural effectuation. This is, however, also recognized as unsustainable and could have many unfavorable environmental impacts **[69].** Novel antifungal agents that are safer and solon are environmentally friendly are desirable control measures **[63,70].** Application of an antifungal protein from *Aspergillus giganteus* on rice leaves has been successful to control infection by *Magnaporthe grisea* [**71**].

Pre-incubation of tomato roots with an antifungal protein from *A. giganteus* protected tomato plants from infection by *Fusarium oxysporum* **[72].** A protease inhibitor isolated from potato sprout reduced fungal lesion on tobacco leaves caused by *Botrytis cinerea* **[73, 74].** These few studies using antifungal proteins from microbial and plant suggest the efficacy of exogenous application of AFPs for curb of fungal infections in plants.

### 1.5.3 Food bio-preservatives

Contamination of bread and different baked products with fungi kingdom including varied Penicillium species is oftentimes an undesirable regulating to the shelf-life of these goods. Addition of chemical preservatives to baked goods is a common relevant solution to this problem **[75].** However, there is an increasing demand for preservatives from natural sources. Amaranthus seed has been investigated as ingredients for baked goods including use in making gluten-free bread **[76].**

Moreover, a recent study has shown that water-soluble extracts from amaranthus seed has potent antifungal activity against *Penicillium roqueforti*, a fungus isolated from contaminated bread **[75].** This fungus is a major food spoilage fungus and is somewhat resistant to chemical antifungal preservatives. For more effective control, a higher dosage of chemical preservatives might have to be used with implications for human health. When extracts of amaranthus seed were incorporated into bread making mixture and the baked bread stored under "typical conditions" (inside a polyethylene bag at $28^0$C), fungal contamination of the bread without the added extracts showed up after 7 days but that of the bread with the added extract was greatly reduced or delayed until the end of 3 weeks of storage [**76**].

The inclusion of water-soluble extracts of amaranthus seed did not seem to alter color or affect sensory property of the bread but had added some desirable nutritional and baking qualities to the bread. Thus, there were some novels AFPs as well as other desirable seed proteins present in amaranthus seed extracts. In another study, oat seed extracts were found to be effective in preventing the formation of *Penicillium roqueforti* colonies on rye bread [**77]**.

## 1.6 Thesis aim and objective

Developing reliable and cost-saving methods, which allow preclinical selection of new antifungal protein drug candidates with original mechanism, is required to improve candidiasis therapy. Combinatorial approaches including growth kinetics and cell morphology of the fungi seem promising in order to acquire a global insight into the biological processes mediated in submerged fermentation.

In this thesis, several new aspects of the statistical methods were investigated to establish a new modified culture media for enhanced antifungal protein production. First, we examined OFAT variations induced by preliminary screening of most influential factors (culture pH, temperature, slant age, inoculum volume, agitation and C/N, $K^+/Ca^{2+}$, $Mg^{2+}/Na^+$ ratio) could be related to the submerged fermentation of *Aspergillus giganteus* MTCC 8408. Second, we compared these factors among basal media using various Taguchi statistical DOE orthogonal array. Third, we attempted to isolate and purify antifungal protein based on pure ammonium sulphate fractionation followed by ion exchange and gel chromatography. Fourth, we attempted to interpret *in vitro* assay and minimum inhibitory concentration (MIC) determination.

We also investigated *in vitro* antibiofilm activity using various microscopic techniques viz., confocal microscopy, scanning electron microscopy (SEM) and atomic force microscopy (AFM), in order to evaluate ultrastructural changes to interpret whether the ultrastructural variations due to the treatment could reflect the modification in the cell induced by the antifungal protein.

We attempted to obtain more precise information about molecular characterization using SDS-PAGE, ESI-MS, ATR-FTIR and MALDI-TOF.

FTIR spectroscopy provides a global fingerprint of the antifungal protein but it remains generally difficult to assess the molecular origin of the spectral signatures. FTIR spectrum could allow the recognition of the various secondary structures (α-helix or β-pleated sheaths) in a mixture. MALDI-MS study would allow the Mascot algorithm for protein identification by peptide mass fingerprinting. The comparison of sequence of peptides matched with potential peptide candidate in antifungal protein would evidence sequence coverage that revealed a partial sequence of the examined protein.

We investigated i*n silico* study of antifungal protein to identify cysteines bonding state variations with the possibility of disulfide bridges, hydropathy value of protein, amino acid composition.

Finally, several mathematical models have been investigated to elucidate the interdependence of morphological properties attributed to filamentous growth in fermented broths and kinetics phenomena related to better bioprocess strategy to optimize the operating conditions for process improvement.

The main objectives of our work is to present the implementation of better design of experiment (DOE) to develop antifungal protein with enhanced activity and to evaluate proteins in vitro for potential MICs. Thus, our work has been divided into five components;

- Growth and Production media design
- Isolation and purification of antifungal protein
- In vitro Application and MICs determination
- Molecular characterization of antifungal protein
- Growth Modeling and kinetic study