An Analysis of the Response of *Aspergillus fumigatus* and *Candida albicans* to Caspofungin

A thesis submitted to the National University of Ireland, Maynooth for the degree of Doctor of Philosophy

By

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October 2014

Publications

Publication from thesis

- **Eshwika A.**, Kelly J., Fallon JP. & Kavanagh K. (2013). Exposure of *Aspergillus fumigatus* to Caspofungin leads to leakage and *de novo* synthesis of gliotoxin. *Medical Mycology* 51, 121–127.

Publication from other work

- Coughlan C., Chotirmall S., Renwick J., Hassan T., Teck Boon Low**,** Bergsson G., **Eshwika A**., Bennett K., Dunne K., Greene C., Gunaratnam C., Kavanagh K., Logan P., Murphy P., Reeves EP. and McElvaney NG. (2012). Itraconazole up-regulates vitamin D receptor expression and reduces T-helper 2 responses in individuals with cystic fibrosis colonized with ***Aspergillus fumigatus*****.** *American Journal of Respiratory and Critical Care Medicine* 186: 999-1007

Poster presentation in conferences

- **Eshwika, A. and Kavanagh, K.** An analysis of the interaction of Caspofungin with the pulmonary pathogen *Aspergillus fumigatus*. Microbial Pathogenesis: the key to better therapies. Society for General Microbiology Irish division meeting in Institute of Technology, Tallaght. September 1/2 2011.

- **Eshwika, A., Kelly, J., Fallon, J. and Kavanagh, K.** Exposure of *Aspergillus fumigatus* to Caspofungin leads to leakage and de novo synthesis of gliotoxin. 5th Advances Against Aspergillosis conference in Istanbul, Turkey. January 26-28 2012,

- **Eshwika, A., and Kavanagh, K.** Induction of oxidative stress response in *Aspergillus fumigatus* following exposure to the antifungal drug, Caspofungin. Society for General Microbiology Irish Division Spring Conference in University College Dublin. March 21/22 2013.

- **Eshwika A., Smith A., Fallon JP, Kelly J. and Kavanagh K.,** Caspofungin induces protein release and an oxidative stress response in *Aspergillus fumigatus* Advances against Aspergillosis Conference in Madrid Spain. Feb 21/25 2014.

Oral presentations

- **Eshwika A**. (2010) Microbiological analysis of cellular response to Caspofungin. Departmental seminar
- **Eshwika A**. (2011) Analysis of the response of *Aspergillus fumigatus* to Caspofungin**.** Departmental seminar
- **Eshwika A**., (2012). The interaction of Caspofungin with the pulmonary pathogen *Aspergillus fumigatus*. Departmental seminar**.**
- **Eshwika A**., (2012). Exposure of *Aspergillus fumigatus* to Caspofungin leads to leakage and *de novo* synthesis of gliotoxin. Departmental seminar.
- **Eshwika A.**, (2013). Caspofungin induces protein release and an oxidative stress response in *Aspergillus fumigatus*. Departmental seminar.

Courses attend as part of structured PhD

- **Introduction to proteomics & mass spectrometry (BE5352) NICB, DCU**
- **Total Quality Management [MNGT] Course H2040 Section X33**
- **GMP 1 / Quality Assurance [QUAL] Course H1040**
- **GMP 2 / Quality Control [QUAL] Course H2040 Section EM2**
- **cGMP 3 / Regulatory Affairs [GMPR] Course H3041 Section EM2**
- **2012 Analytical Workshop [Centre For Microscopy and Analysis] TCD April 24th-26th**
- **Bacterial interactions at the host epithelium, ITT Dublin, 16th and 17th of May 2013**
- **Advanced Experimental Data Processing using Microsoft EXCEL®, 19-20 March 2013, DCU.**

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Signed: ……....

(Ahmed Mohamed Eshwika)

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Acknowledgements

I would like to thank Dr. Kevin Kavanagh for his supervision and guidance during the course of this work. His constructive patience, suggestions, comments, encouragement and advice throughout the project were invaluable, especially for support during the writing of this thesis.

I also would like to thank all the team in biology research group in Biology department, and the technical staff, especially for help in all my experimental work.

Also I would like to thank my colleagues and friends: in medical mycology lab for their invaluable help and encouragement.

Sincere thanks to my Mum, my wife, my kids, my brothers and sisters for giving me so much unconditionally and for their sacrifices and support when I needed them.

Finally, I gratefully acknowledge the financial support from the Ministry of Higher Education and Scientific Research of Libya.

Abstract

The filamentous fungus *Aspergillus fumigatus* is capable of causing a range of diseases in immunocompromised patients and is responsible for up to 3% of all hospital-based deaths in the EU. The yeast *Candida albicans* induces a range of diseases in immunocompromised patients. Caspofungin is a recently introduced antifungal compound that has good efficacy against these fungi with minimal side effects. The aim of the work presented here was to characterise the response of *A. fumigatus* and *C. albicans* to caspofungin. The results obtained demonstrated that caspofungin has potent anti-fungal activity and retards fungal growth but that the response of fungi to this drug is influenced by a variety of environmental factors (e.g. pH, serum, glucose). Exposure of *A. fumigatus* hyphae to this drug leads to the release of amino acids and gliotoxin. There is also an increase in internal levels of gliotoxin following exposure to the drug. Prolonged exposure to caspofungin leads to reduced growth but greatly elevated levels of secreted and internal gliotoxin. Since this toxin is highly immunosuppressive it is possible that *in vivo* elevated levels of this toxin may exacerbate the deterioration in the patient's condition following caspofungin therapy. *A. fumigatus* hyphae exposed to caspofungin demonstrated an increase in the expression of proteins associated with an oxidative response (e.g. catalase, antibiotic response protein) and in the activity of selected enzymes (catalase, glutathione reductase and superoxide dismutase). The results presented in this thesis demonstrate that caspofungin is a potent antifungal agent but that *A. fumigatus* responds to the drug by increasing the synthesis of gliotoxin and by inducing an oxidative stress response.

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Abbreviations

α	Alpha
β	Beta
ϒ	Gamma
µ	Micro
ABPA	Allergic Broncho-Pulmonary Aspergillosis
APS	Ammonium Persulphate
AMM	*Aspergillus* Minimal Media
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
BMT	Bone marrow transplant
^{o}C	Degree Centigrade
CF	Cystic Fibrosis
CFU	Colony Forming Unit
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CLSI	Clinical and Laboratory Standards Institute
CSF	Cerebral Spinal Fluid
DMSO	Dimethyl sulfoxide
DNA	Doxyribose Nucleic Acid
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetra Acetic acid
e.g	For Example
FBS	Fetal bovine Serum
FDA	Food and Drug Administration
g	Grams
hr	Hours
HCl	Hydrochloric Acid
IA	Invasive Aspergillosis

IAA	Iodoacetamide
IEF	Iso-electric focusing
IFI	Invasive Fungal Infection
Ig	Immunoglobulin
KDa	Kilodaltons
L	Litre
LC-MS	Liquid Chromatography Mass Spectroscopy
M	Molar
MEC	Minimal effective concentration
MIC	Minimal inhibitory concentration
MEM	Minimal Essential Media
Mg	Milligrams
Min	Minutes
ml	Milliliter
mM	Millimolar
MOPS	3-[N-morpholino]propanesulfonic acid]
mRNA	Messenger ribonucleic acid
MW	Molecular Weight
NADPH	Nicotinamide adenine dinucleotide phosphate
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffer Saline
PBST	Phosphate Buffer Saline Tween
PCR	Polymerase Chain Reaction
QC	Quality control
RNA	Ribonucleic Acid
rpm	Revolutions per minute
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
SDS	Sodium Dodecyl Sulphate

SEM	Scanning Electron Microscopy
solv	Solvent
Spp	Species
TBS	Tris Buffer Saline
TBST	Tris Buffer Saline tween
TCA	Trichloroacetic acid
TEMED	N,N.N',N'-Tetramethylethylenediamine
Temp	Temperature
TFA	Triflouroacetic acid
TLCK	n- α-P-tosyl-L-lysine chloromethylketone hydrochloride
U	Unit
U.T	Untreated
UV	Ultra violet
V	Volts
v/v	Volume per volume
w/v	weight per volume
YEPD	Yeast Extract-Peptone-D-Glucose

Chapter 1

INTRODUCTION

1.1 *Aspergillus fumigatus*

Aspergillus fumigatus is a fungus of the genus *Aspergillus*. *A. fumigatus* is a filamentous fungus producing bluish-green conidia (Figure 1.1), and is one of the most common *Aspergillus* species to cause disease in immunodeficient individuals. *A. fumigatus* is capable of growth at 37 °C (normal human body temperature), but can also grow at temperatures up to 50 °C, with conidia surviving up to 70 °C. *Aspergillus* conidia are released into the air from the colony of fungus and can be inhaled into the lungs. *Aspergillus* is a fungus whose spores are present everywhere in the air, but these are not normally harmful; typically these are quickly eliminated by the immune system in healthy individuals (O'Gorman *et al.,* 2008). However an individual with a weakened immune status may be susceptible to *Aspergillus* infection.

A. fumigatus is one of the major opportunistic fungal pathogens. It is associated with respiratory disorders such as hypersensitivity, allergic asthma, bronchopulmonary aspergillosis allergic type, and aspergilloma as well as invasive aspergillosis in immunocompromised individuals (Banerjee *et al,.* 1998). In immunocompromised individuals, such as AIDS or leukemia patients, and organ transplant recipients *A. fumigatus* is more likely to become pathogenic, causing a range of diseases generally termed aspergillosis. With the increase in the population of immunocompromised patients *A. fumigatus* has become the second most common opportunistic fungal pathogen of humans (Netea *et al.,* 2003).

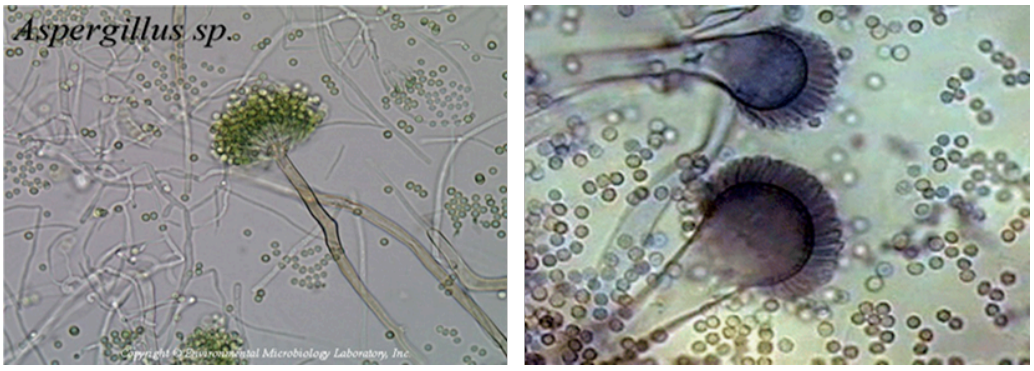


Figure 1.1 Images of *A. fumigatus* showing hyphae, conidiophore and conidia.

(Images from www.*Aspergillus*.org.uk)

1.2 *Aspergillus fumigatus* and the Environment

Previous studies have examined the prevalence of *A. fumigatus* in air samples. The concentration of viable aerosolised conidia varies depending on geographical location, climate, presence of decaying vegetation and other factors such as urbanisation and pollution (Vermani *et al.,* 2010; Guinea *et al.,* 2006). *Aspergillus* conidia were found everywhere in the air; however meteorological parameters such as temperature, rainfall and wind-speed affect the number of conidia in the air (Guinea *et al.,* 2006). The distribution of *A. fumigatus* conidia is not limited. Several cases of filamentous fungi including *A. fumigatus* have been reported in public water and hospital water systems, thus confirming the widespread distribution of conidia (Gottlich *et al.,* 2002). In immunocompetent populations, inhaled conidia are eliminated via alveolar macrophages that kill the conidia in an NADPH oxidase dependent manner and by polymorphonuclear leucocytes (PMNL) (Hohl and Feldmesser, 2007). *A. fumigatus* produces small hydrophobic conidia and each conidial head produces thousands of grey-green spores average size is between 2 – 3 μm in diameter which keeps them buoyant in the environment (Latge, 1999). *Aspergillus* conidia are estimated to be at concentrations of 1 – 100 conidia / m^3 which can bypass the mucociliary clearance mechanism upon inhalation (Denning *et al.,* 2002). Therefore, the fungus penetrate deep into the lung alveoli (Hohl and Feldmesser, 2007).

1.3 Diseases caused by *Aspergillus fumigatus*

1.3.1 Aspergillosis:

Aspergillosis is a group of diseases caused by fungus of the genus *Aspergillus* and usually occurs in patients with lung diseases or weakened immune systems. The diseases include invasive aspergillosis, allergic bronchopulmonary aspergillosis, and aspergilloma. Some individuals with very severe asthma may also be sensitised to fungi such as *Aspergillus* (Latge, 1999). Invasive fungal infection is a major cause of morbidity and mortality in immunocompromised patients. The mortality rate in allogeneic bone marrow transplant (BMT) recipients, who have invasive aspergillosis, is approximately 90% (Hovi *et al.,* 2000). There are several forms of aspergillosis including pulmonary aspergillosis, aspergilloma and pulmonary invasive aspergillosis. Despite the availability of new antifungal agents, the morbidity rate due to invasive aspergillosis has increased steadily in recent years (Blyth *et al.,* 2007).

1.3.2 Invasive Aspergillosis

Invasive fungal infections remain important life-threatening infections and they are a major cause of morbidity and mortality in immunocompromised patients. The incidence of invasive aspergillosis continues to increase, due to the rising number of patients undergoing bone marrow or solid organ transplantation or, corticosteroid treatment and those suffering from haematological malignancy or pulmonary disease (Maschmeyer *et al.,* 2007). In immunocompetent individuals, inhaled *Aspergillus* conidia are normally cleared by mucociliar transport and phagocytosis. However, if phagocytosis is impaired by the use of corticosteroids, *Aspergillus* conidia may germinate to form hyphae and invade the pulmonary tissue (Schneemann and Schaffner, 1999). Invasive aspergillosis is mainly caused by *A. fumigatus*, although other species such as *Aspergillus terreus*, *Aspergillus niger* and *Aspergillus flavus* can also cause invasive infection (Zmeili and Soubani, 2007). *A. fumigatus* hyphae may invade the surrounding pulmonary tissue and may then spread to other organs and tissues in the body (Dagenais and Keller, 2009).

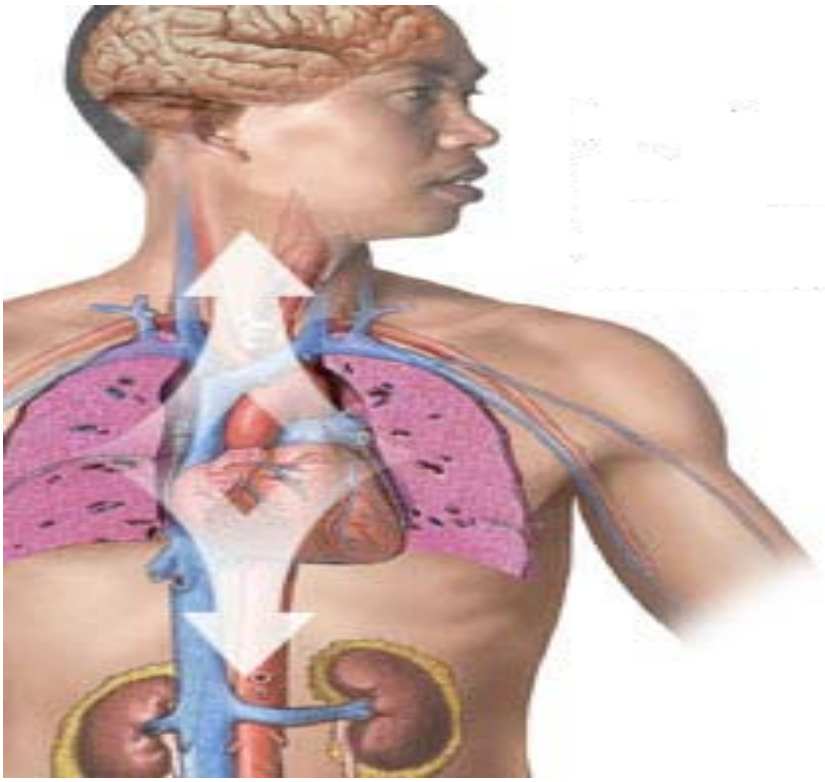


Figure 1.2 Invasive aspergillosis spreads to body organs via bloodstream

(http://www.scripps.org)

1.3.3 Aspergilloma

Inhalation of *Aspergillus* spores is the primary cause of aspergillosis. Aspergillosis may occur in different forms. A "pulmonary aspergilloma" occurs when aspergillosis develops in the sinuses or the lungs and forms a tangled ball of fungal mycelia and blood cells. Many fungal balls develop in areas of past lung disease or lung scarring such as tuberculosis or lung abscess. The *Aspergillus* spore settles in the lung cavity and grows free from interference because the immune system is impaired. As it multiplies it forms a ball, which incorporates dead tissue from the surrounding lung, mucus, and debris (Soubani *et al.,* 2002). Aspergilloma can be treated by surgical removal of fungal ball and this usually carries a high mortality rate (Latge, 1999).

Pulmonary aspergilloma is a type of aspergillosis and usually does not spread to other parts of the body. In some cases aspergillosis can become very aggressive and rapidly spreads throughout the lungs and often through the bloodstream to the brain, the heart, the liver and the kidneys. This is called “invasive aspergillosis” and this occurs in most patients with a weakened immune system.

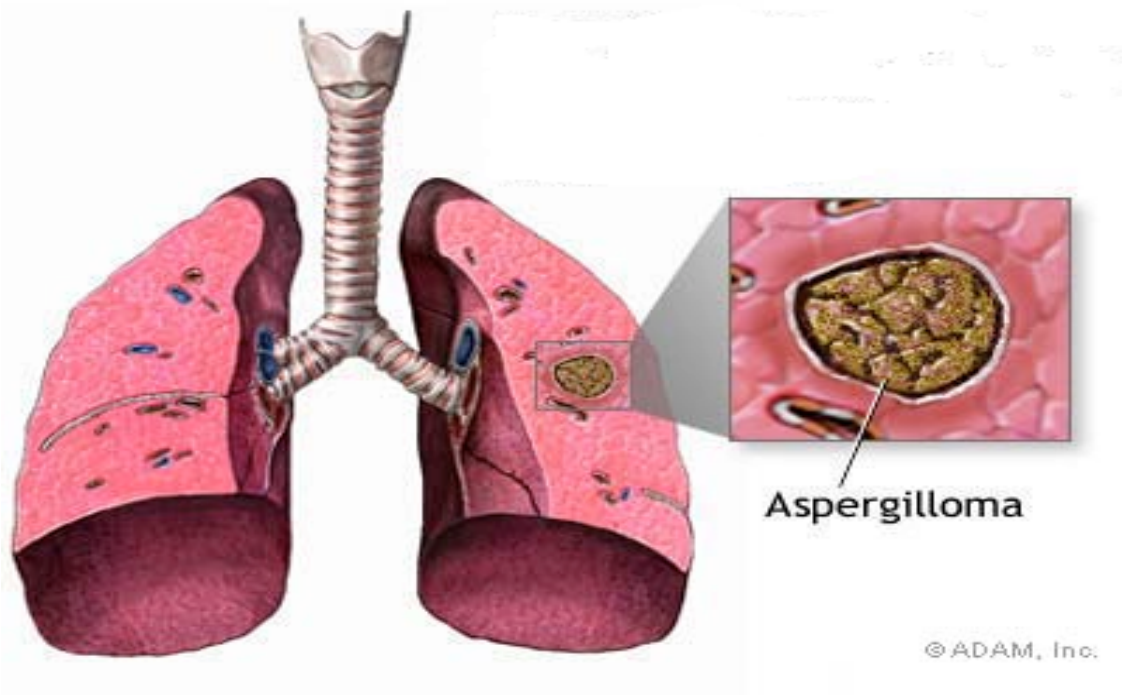


Figure 1. 3 Aspergilloma is a formation of fungal ball in lung tissue

(http://www.scripps.org)

1.3.4 Allergic bronchopulmonary *Aspergillosis* (ABPA)

Allergic bronchopulmonary type is an allergic reaction to the fungus that usually develops in people who already have problems in the lung (such as cystic fibrosis or asthma). It was reported that ABPA occurs in about 1% of *A. fumigatus*-sensitized asthmatics, while in case of cystic fibrosis (CF) patients the incidence varies from 10 to 35% of the case (Bouchara *et al.,* 1995). Allergic bronchopulmonary aspergillosis (ABPA)

is a hypersensitivity disease of the respiratory system mediated by an allergic late-phase inflammatory response to *A. fumigatus* antigens that occurs in approximately 1–2% of asthmatic and 7–10% of cystic fibrosis patients (Stevens *et al.,* 2000). The allergic inflammatory response in ABPA appears to be quantitatively greater than in *Aspergillus*-sensitive cystic fibrosis patients (Knutsen *et al.,* 2012).

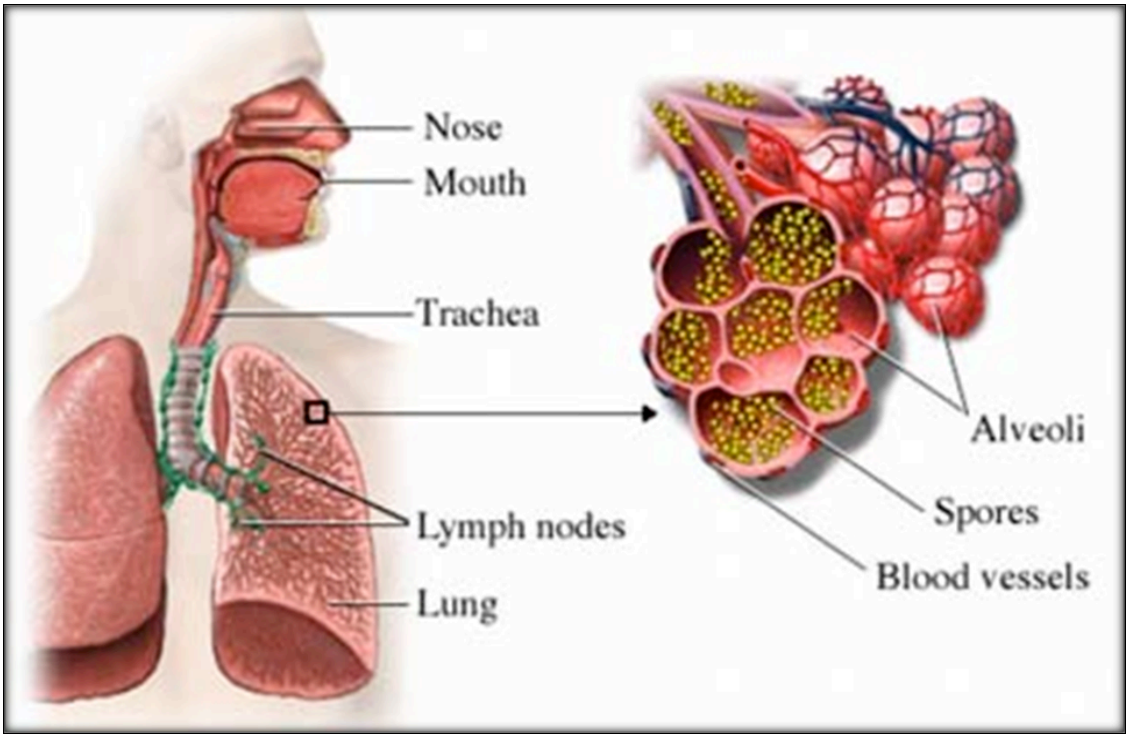


Figure 1.4 Allergic bronchopulmonary Aspergillosis (ABPA), develops an allergy to the spores of *Aspergillus*

(http://www.scripps.org)

1.3.5 *Aspergillus fumigatus* and virulence factors

Opportunistic fungal infections occur in susceptible patients who have weakened defense systems. Fungal-related characteristics such as dimorphism and phenotypic switching may play important roles in initiating and establishing infections by several fungi (Karkowska-Kuleta *et al.,* 2009). Casadevall, (2005) defined virulence factors as

pathogen determinants of the fungus which cause damage within the host. The ability of *A. fumigatus* to grow on host tissues is important in virulence. The respiratory tract is regarded as the main entrance to the body for conidia. *A. fumigatus* conidia are inhaled by all individuals and transit through the respiratory tract. However, these characteristics do not fully explain the pathogenicity of *A. fumigatus*.

Previously it has been demonstrated that some environmental strains are less virulent than their corresponding clinical strains and these observations support the view that *A. fumigatus* may have properties that protect it from the lung's defense mechanism (Maertens *et al.,* 2004). Such properties could ensure its survival in lung tissue and initiate allergic reactions followed by infection in appropriate hosts (Tomee *et al.,* 2001). A recent study showed that *A. fumigatus* has multi-genic virulence factors of pathogenicity (Wezensky and Cramer, 2011). *Aspergillus* virulence genes involved in different parameters include thermo-tolerance, cell wall composition, conidial surface, pigment biosynthesis, nutrient acquisition during infection, toxin production, signalling, metabolism and allergens (Hohl and Feldmesser, 2007; Abad *et al.,* 2010). Nierman *et al.,* (2005) suggested that the virulence of *A. fumigatus* may be supported by the number of secondary metabolism (SM) clusters and this may control secondary metabolism gene expression. Several groups of fungal SMs which are classed according to the class of enzyme involved in the biosynthesis process and listed as peptides, alkaloids, terpenes and polyketides (Keller *et al.,* 2005). *A. fumigatus* gene clusters responsible for the biosynthesis of epidithiodioxopiperazines (ETP) have been identified and they are responsible for the biosynthesis of gliotoxin (Sugui *et al.,* 2007; Spikes *et al.,* 2008).

1.4 Mycotoxins

Certain fungi and molds have the capability to form chemicals that are harmful when eaten by humans or animals. Mycotoxins are secondary metabolites produced by mycelial structure of the filamentous fungi, which are capable of causing illness and death in humans and other animals (Bhatnagar *et al.,* 2002). The growth of fungi on human and animal hosts produces the diseases called mycoses, while dietary, respiratory, dermal, and

other exposures to toxic fungal metabolites produce the diseases called mycotoxicosis (Bennet and Klich, 2003).

1.4.1 *A. fumigatus* and mycotoxins

Mycelia of *A. fumigatus* has been demonstrated to produce a range of secondary metabolites called mycotoxins with harmful actions on the host. These include gliotoxin, fumagillin, helvolic acid, fumitremorgin, fumigaclavine C, aureperone C, and restrictocin (Dagenais and Keller, 2009). *A. fumigatus* displays a number of virulence factors that may facilitate colonisation of the host tissue (Amitani *et al.,* 1995). One of these, gliotoxin ($C_{13}H_{14}N_2O_4S_2$, molecular weight 326.4) (Figure 1.5), displays immunosuppressive properties *in vivo* (Bennet and Klich, 2003). Gliotoxin has been detected in human tissue where it may assist fungal colonisation (Bok and Keller, 2004; Lewis *et al.,* 2005).

1.4.2 Gliotoxin

The production of gliotoxin by *A. fumigatus* strains is dependent upon culture conditions: previous studies pointed out different parameters that could affect *in vitro* gliotoxin biosynthesis (Kerzaona *et al.,* 2007). Several species of *Aspergillus* and other fungi produced gliotoxin both *in vitro* and *in vivo*. Gliotoxin produced by *A. fumigatus* has received wide attention because it is considered a virulence factor for this organism, as well as being present in the sera of immunocompromised patients with aspergillosis (Sugui *et al*, 2008; Bok *et al.,* 2005). In addition, gliotoxin has been implicated in the destruction of lung tissue in invasive aspergillosis (Sutton *et al.,* 1996).

Gliotoxin produced by *A. fumigatus* strains is a pipolythiodioxopiperazine immunosuppressive mycotoxin and is a cyclic non-ribosomal dipeptide (Figure 1.5) belonging to the epidithiodioxopiperazines (ETP). It demonstrates biological activities such as antimicrobial, antifungal, antiviral, genotoxic and immunosuppression (Nieminen *et al.,* 2002). Previous work demonstrated that gliotoxin inhibits NADPH oxidase assembly and suppresses the production of reactive oxygen species (ROS) and weakens phagocytosis function of neutrophil (Tsunawaki *et al.,* 2004; Orciuolo *et al.,* 2007). Previous work showed that all *A. fumigatus* strains do not necessarily produce gliotoxin

and confirmed that the ability to synthesize gliotoxin is discontinuously distributed among *Aspergillus* isolates (Boudra and Morgavi, 2005; Lewis *et al.,* 2005). Culture conditions have an effect on the production of gliotoxin by *A. fumigatus*. Kerzaon *et al.,* (2007) demonstrated that different parameters could affect the *in vitro* gliotoxin biosynthesis.

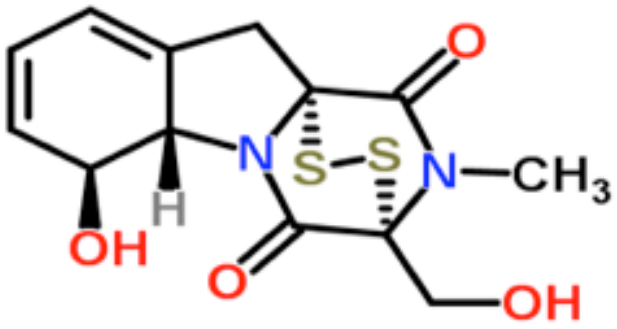


Figure 1.5 Chemical Structure of Gliotoxin.

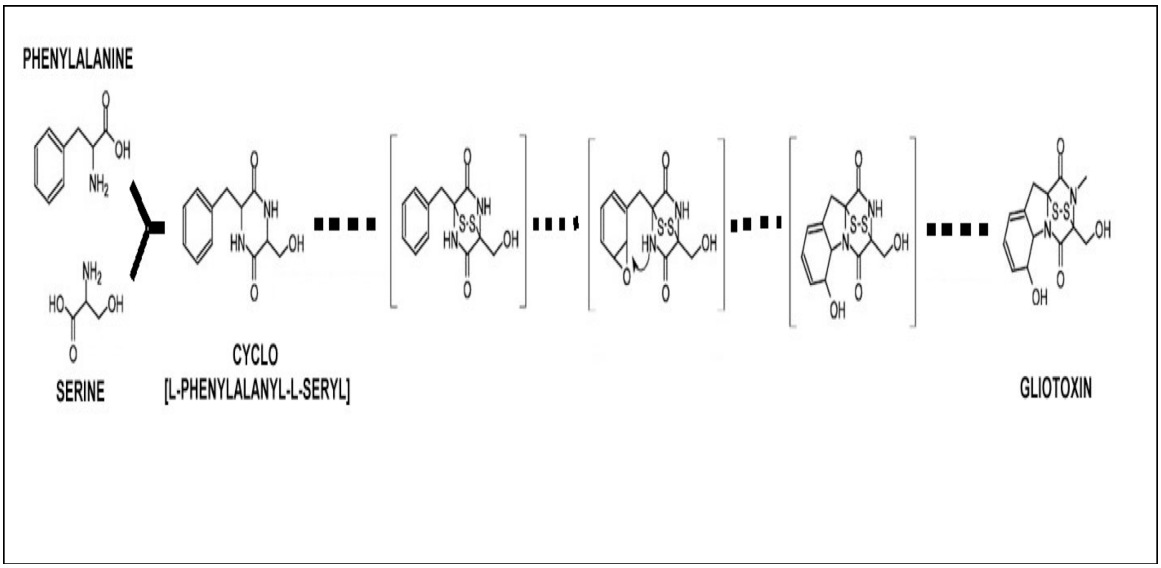


Figure 1.6 Proposed biosynthetic pathway for gliotoxin biosynthesis

(Balibar and Walsh, 2006).

Gene clusters responsible for biosynthesis and production of gliotoxin, have been identified in *A. fumigatus* (Gardiner and Howlett, 2005; Spikes *et al.,* 2008). Previous work confirmed the molecular formula of gliotoxin ($C_{13}H_{14}N_2O_4S_2$) and determined the activity of gliotoxin in inhibition of growth against some species of fungal and bacteria (Johnson *et al.,* 1943).

1.5 *Candida albicans*

Candida albicans is an opportunistic fungal pathogen and is a normal part of our gastrointestinal flora. *C. albicans* has the ability to colonize every human tissue, especially the mouth and vagina. The yeast may invade internal organs mostly in immunocompromized patients (Latge and Calderone, 2002). Depending on the *Candida* species, microscopically the colour of colony varies from cream-colured to yellowish and the texture may be smooth, pasty, glistening to dry (Eggimann *et al.,* 2003).

1.5.1 *Candida* Virulence and colonisation

C. albicans can convert from a benign commensal into a disease-causing pathogen, causing infections in the different human tissues and these can be defined in two broad categories; superficial mucocutaneous and systematic invasive. The morphogenetic conversions of *C. albicans* are undoubtedly important and the ability to reversibly switch between two forms (yeast and filamentous) is important for the pathogenicity of *C. albicans*. Several studies suggest that the ability to switch between the yeast and mycelial forms is one of the most important virulence factors of *C. albicans* (Chandra *et al.,* 2001). *C. albicans* has several properties which allow it recognize and adhere to the host. A number of these including the morphological transition mechanism between two forms, the expression of adhesins and invasins on the host surface, thigmotropism, the formation of biofilms, phenotypic switching and the secretion of hydrolytic enzymes (Haynes, 2001). *C. albicans* is able to adhere to different tissues within the human body thus facilitating the occupation of many host niches. Remarkably, host niches can have very different environments for growth such as pH, O_2 levels, temperature, and nutrient availability. Increased adherence to host tissue has been observed for the mycelial form and decreased adherence has been demonstrated by the non-germ tube form (Baillie and Douglas, 1999). This suggests that *C. albicans* has developed a large array of signaling and adaptation mechanisms in order to persist and proliferate, and is an effective colonizer of the human body which eventually causes disease (Ekkehard *et al.,* 2011). To colonize any surface, fungal cells must first adhere to the biomaterial surface. *Candida* cells initially attach to biomaterials and this is mediated by both non-specific factors (cell surface hydrophobicity and electrostatic forces) and also specific adhesins on the fungal surface recognizing ligands, such as serum proteins (fibrinogen and fibronectin) and other factors such as saliva (Chaffin *et al.,* 1998). It has been reported that *Candida* has the ability to grow in both aerobic and anaerobic conditions and possess adaptive mechanisms to survive in both situations (Sardi *et al.,* 2011). The adhesion genes may be activated by diverse environmental triggers such as carbon and/or nitrogen starvation, or changes in pH or ethanol levels, switching between non-adherence and adherence probably allows yeasts to adapt to stress (Sampermans *et al.,* 2005). Virulence factors of *C. albicans* can be induced

by environmental change and initiate signal transduction on the cell surface receptor to alter the transcription factors and modify the expression of several virulence genes (Haynes, 2001).

1.5.2 Biofilm formation by *C. albicans*

A positive correlation between biofilm formation and cell surface hydrophobicity has been demonstrated (Li *et al.,* 2003), and this may also be mediated by cell surface proteins which are encoded by members of the *ALS* family of adhesin-producing genes and *EAP1* (Green *et al.,* 2004). It is clear that morphogenesis of *C. albicans* plays an important role in the formation and development of biofilm (Baillie and Douglas, 1999). The populations of *C. albicans* cells were observed microscopically in the formation of biofilm. *Candida* yeast and germ tubes were attached to the mucosal surface and consisted of a mixture of closely associated yeast and hyphae surrounded by an extracellular matrix (Harriott *et al.,* 2010). The hyphal structure was demonstrated to be an essential element for providing the structural integrity and development of characteristic multilayered biofilm (Mayer *et al.,* 2013). *Candida* infections can be caused by the presence of medical implants that provide a chance for the development of a biofilm which is resistant to antifungal agents (Douglas, 2003). One feature of biofilms formed by *C. albicans* is the mixture of different morphological forms (Figure 1.7). Biofilm development on catheter discs was first examined by scanning electron microscopy, which showed that yeast cells initial attach to the surface followed by germ-tube formation after 3–6 hours and after incubation for up to 48 hours a fully mature biofilm was produced which consisted of a dense network of mycelia (Hawser and Douglas, 1994). In polymicrobial biofilms, bacteria are often found with *Candida* species *in vivo*, and inter species interactions take place in these adherent populations. For *in vitro* investigation of a mixed-species biofilm, catheter disc model systems have been used to investigate biofilms consisting of *C. albicans* and *Staphylococcus epidermidis*, the commonest agent of bacterial catheter-related infection (Adam *et al.,* 2002). *In vitro* resistance of *Candida* biofilms to antifungal agents was first reported in 1995 (Hawser and Douglas, 1995). Biofilms in *Candida albicans* have been reported on most medical devices and frequently occur on catheters, dentures, voice

prosthetics implants and artificial joints (Ramage *et al.,* 2006). Recent evidence suggests that biofilms also form on the mucosal surfaces of the mouth and vagina.

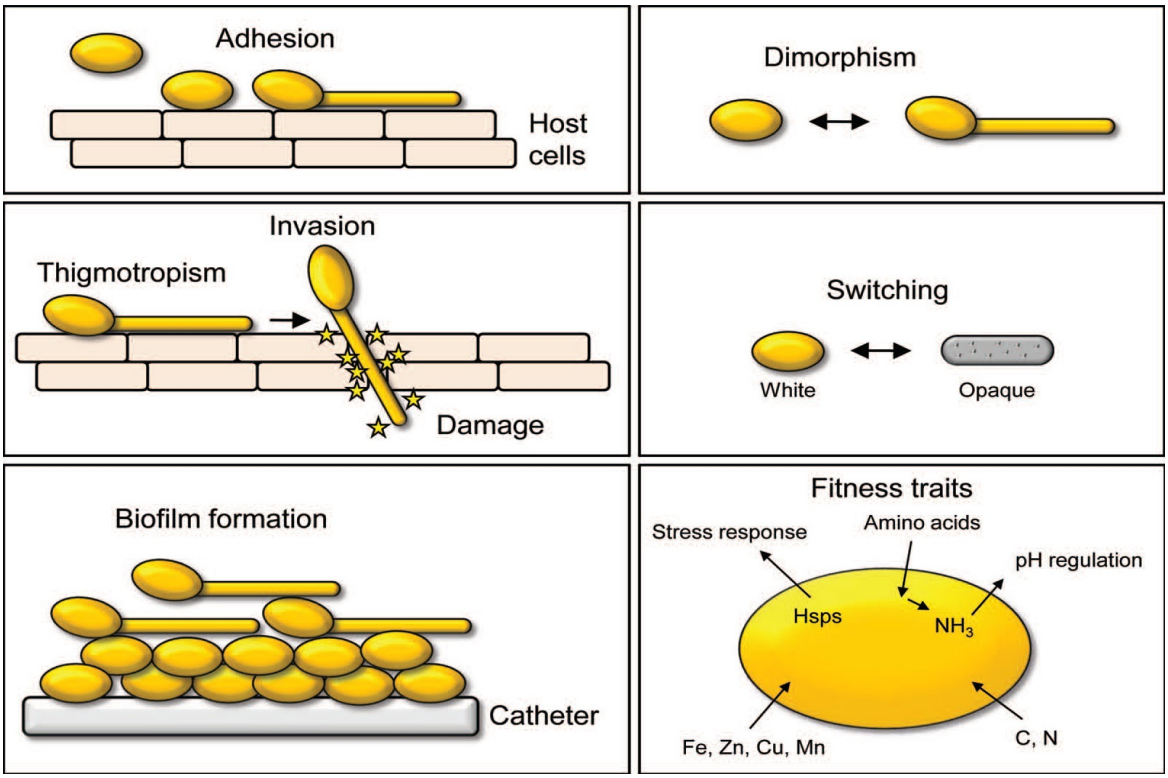


Figure 1.7 Adhesion, invasion, biofilm formation, dimorphism, switching and fitness traits (Mayer *et al.,* 2013)

Biofilms of *C. albicans* form when single cells attach and grow on the surface to form microcolonies, which then produce 3-D structure complex that merge together hyphae and an exopolymer matrix (Chandra *et al.,* 2001). Morphological biofilm is a heterogeneous component containing a mixture of yeast, hyphae and pseudohyphae forms (Hawser and Douglas, 1994).

1.5.3 Disease caused by *Candida*

Candidiasis is a disease caused by yeast of the genus *Candida.* Candidiasis is treatable in the healthy individual. However, candidiasis can be more difficult to treat in people with weakened immune systems due to such conditions as HIV/AIDS, or taking steroid medications or chemotherapy (Mayer *et al.,* 2013). In these cases, complications of disease may become life threatening. Impairment of immune system is the main cause of overgrowth of *Candida* cells. The immune system can be weakened by stress, illness and some medications. It is in such a state of weakness when harmless *Candida* yeast turns into an aggressive pathogens.

Superficial fungal infections of skin and mucous membranes are the most common types of skin infection caused by *Candida*. In certain cases, *Candida* infection of the skin has increased in prevalence in recent years, principally because of the increased numbers of patients who are immunocompromised. *C. albicans* infections can occur superficially and most cases of candidiasis can be treated with antifungal drugs once there is no predisposing factors such as immunosuppressive agent or antibiotic treatment (Haynes, 2001).

The first *Candida* sytemic infection was recorded in 1862. Since then, large numbers of cases have been documented with infection in internal organs such as respiratory tract, urinary tract, cardiovascular system, CNS, bones and liver (Odds, 1994). Approximately 90 % of HIV patients develop superficial candidiasis (Dorocka-Bobkowsk *et al.,* 2003) with high possibility of disseminated candidiasis leading to a number of deaths among the immunocompromised population (Lupetti *et al.,* 2003).

1.6 Antifungal agents

An antifungal agent is a drug that selectively eliminates fungal pathogens with minimal toxicity to the host. Treatment with antifungal agents has increased in the last 50 years, however, the incidence of serious fungal infections continues to rise and the rates of mortality are usually between 40 – 90 % in high-risk immuno-compromised populations (Dagenais and Keller, 2009). Several new antifungal drugs have been or will be licensed in

the next few years but despite of this the number of deaths due to invasive aspergillosis has increased (Mihai *et al.,* 2003). A clear understanding of the mechanisms responsible for defense against *Aspergillus* infection is required to develop strategies that boost the antifungal actions of the immune system. The three currently used antifungal agent classification and mechanism of action (Table 1.1)

The polyene and azole antifungal agents are focused on the cell envelope particularly on the fungal cell membrane sterol, ergosterol, and its biosynthesis. Targets elsewhere in the cell would be very useful in the fight against fungal pathogens (Frank *et al.,* 2003). The heat shock response, including major and minor heat shock proteins, has emerged as a promising antifungal target. Specifically, those heat shock proteins that are unique to fungi and (e.g. Hsp21) represent good candidates for specific drug targets (Mayer *et al.,* 2013). The mechanisms of biofilm drug tolerance and phenotypic heterogeneity may lead to the development of the first antifungal drugs capable of eradicating infection, salvaging medical devices, and preventing relapse (Michael, 2011).

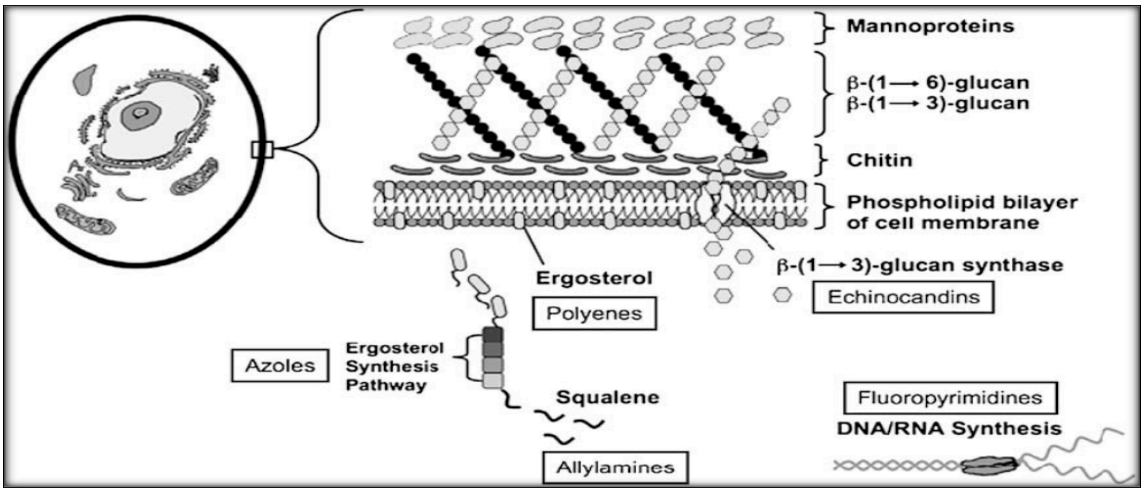


Figure 1.8 Targets of systemic antifungal agents.

[Thompson *et al.,* 2008]

Class	Examples	Mechanism of Action
Polyene	Amphotericin B, Nystatin	Binds ergosterol; creates pores in the cell membrane
Azole	Fluconazole, Voriconazole, Itraconazole,	Inhibition of 14-alpha lanosteroldemethylase; accumulation of 14-alpha-methyl 3,6 diol
Echinocandin	Caspofungin, Micafungin, Anidulafungin	Inhibition of cell wall glucan synthesis by beta-1,3 glucan synthase

Table 1.1 The main drug classifications and mechanism of action of some currently used antifungals agents.

1.6.1 Echinocandins

The echinocandins are a novel group of antifungal agents which have an unique mechanism of action that involves the inhibition of the synthesis of glucan in the cell wall via the enzyme 1,3-β glucan synthase (Figure 1.9). The echinocandin drugs are potent inhibitors of glucan synthase and are the first class of antifungal agents to target the fungal cell wall. The three principal drugs, caspofungin, micafungin and anidulafungin are highly serum protein bound (Wagner *et al.,* 2006), and display favorable pharmacokinetic and pharmacodynamic properties, as well as excellent safety profiles. Echinocandins have been introduced into clinical practice for systemic fungal infections in immuno-compromised patients. Although initially approved for salvage therapy for invasive aspergillosis, treatment regimens are still evolving. The echinocandin drugs are moderately fungistatic against *Aspergillus* (Perlin, 2007; Morris *et al.,* 2006). Echinocandins are poorly absorbed

when administered orally, however when administered by parenteral route they will reach most tissues and organs with concentrations sufficient to treat localized and systemic fungal infections (Wagner *et al.,* 2006). Caspofungin was the first echinocandin antifungal agent to gain FDA-approval for human use in the United States under trade name Cancidas, (Merck & Co. Inc.). It has excellent clinical activity against *Candida* and *Aspergillus*.

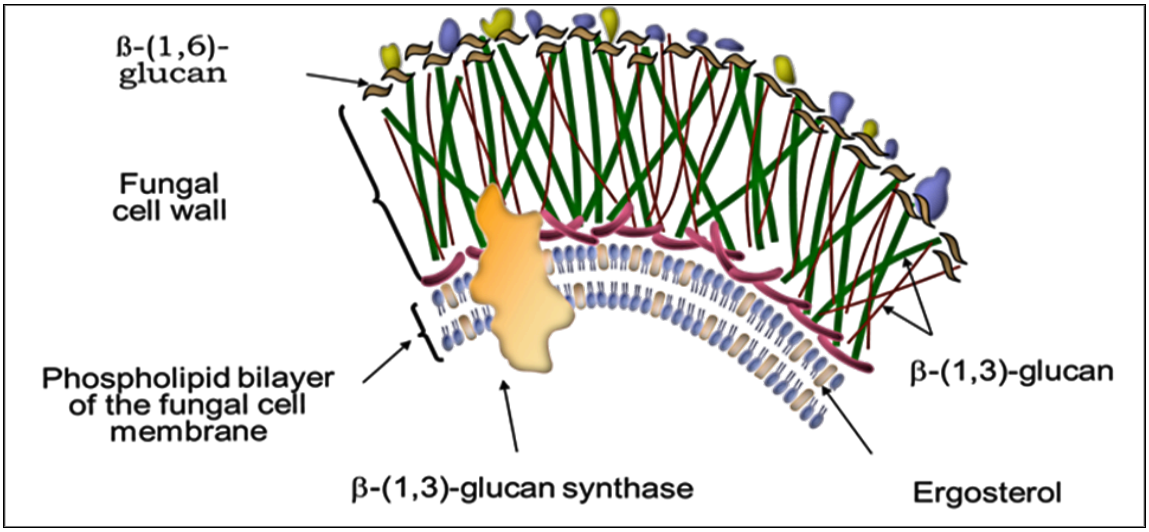


Figure 1.9 Schematic diagram of fungal Hyphae cell wall and membrane.

Echinocandins acting on the enzyme ß-1,3-glucan synthase leading to inhibit the synthesis of ß-1,3-glucan. [Kartsonis *et al.,*(2003)].

1.6.1.1 Caspofungin

Caspofungin is a semisynthetic lipopeptide (echinocandin) derived from a fermentation product of *Glarea lozoyensis*. Caspofungin shows activity against infections by *Aspergillus* and *Candida* (Kahn *et al.,* 2006). Caspofungin works by inhibiting the enzyme β (1,3)-D-Glucan synthase and thereby disturbing the integrity of the fungal cell wall (Baixench *et al.,* 2007). Caspofungin has an excellent safety profile with reduced

toxicity, compared to other licensed antifungal agents (Maertens *et al.,* 2004). It is used for treatment of acute invasive candidiasis and as salvage treatment for invasive aspergillosis (Denning *et al.,* 2003). Caspofungin has been shown to be active both *in vitro* and in clinical infections against most strains of *Candida* and in regions of active cell growth of the hyphae of *Aspergillus* species (Odds *et al.,* 2004). Caspofungin is the only echinocandin for which dosage reduction is recommended for patients with moderate to severe hepatic dysfunction. Caspofungin has few drug-drug interactions and clinician reports were promising to use caspofungin in combination with polyene and azoles antifungals due to the different mechanism of action (Eschenauer *et al.,* 2007). In the case of invasive aspergillosis caspofungin was successfully used especially in patients intolerant to other antifungal agent such as amphotericin B and fluconazole (Maertens *et al.,* 2004). In the structure formula of caspofungin (Figure 1.10) there is a long fatty acid side chain that may allow intercalation with bi-layer of the fungal cell membrane (Denning *et al.,* 2003), where it interacts with the enzyme ß-1,3-D-glucan synthase in fungal cell wall. There is a positive correlation between the clinical outcome *in vivo* failure and elevated *in vitro* MICs of caspofungin (Hernandez *et al.,* 2004). Resistance to echinocandins and especially caspofungin has been associated with mutation in the *FKS* genes or in *RHO1* (Letscher-Bru and Herbrecht, 2003).

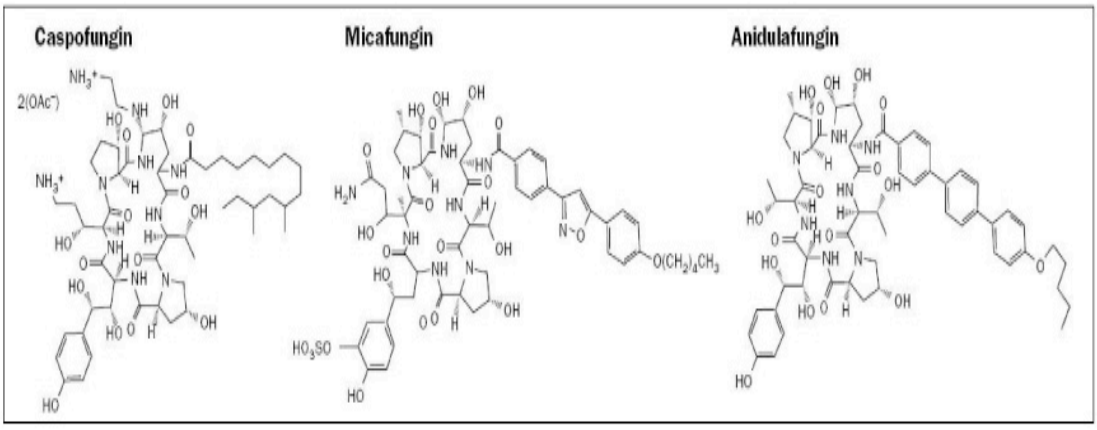


Figure 1.10 Structures of caspofungin, micafungin and anidulafungin

Non-growing *C. albicans* cells when exposed to caspofungin release a range of proteins which originate from different locations within the cell and these proteins may be strongly immunogenic to the host immune response and lead to increased inflammation (Kelly and Kavanagh, 2010). Echinocandins are active against most *Candida* species and caspofungin has an excellent safety profile and is reported to kill biofilms *in vitro* (Bachmann *et al.,* 2002). Fungicidal effect by echinocandins may be due to cell wall lysis and osmotic stresses, and may be largely dependent on media and growth conditions (Michael, 2011). The rapid activation of both pathways Cap1 and Hog pathways in *C. albicans* during exposure of cells to caspofungin may give the cell the ability to self-protect from the osmotic and oxidative stress induced by antifungal drug (Kelly *et al.,* 2009). *C. albicans* cells treated with caspofungin show increased expression of a number of genes associated with the oxidative stress response, including mitochondrial processing protease (*MAS1*) and catalase (*CAT1*) as well as elevated activity of glutathione reductase (*GLR*) and superoxide dismutase (*SOD*) (Kelly *et al.,* 2009).

1.6.2 Polyene antifungal

A polyene (e.g. Amphotericin B, Nystatin) is a molecule with multiple conjugated double bonds. The polyene antimycotics bind with ergosterol in the fungal cell membrane and this alters the cell membrane fluidity, thereby placing the membrane in a less fluid, more crystalline state. As a result, small organic molecules leak and this induces cell death (Walsh *et al.,* 2004). Animal cells contain cholesterol instead of ergosterol and so they are much less susceptible to the effects of polyene agents. However, at some stage of therapeutic doses, amphotericin B may bind to cholesterol, increasing the risk of adverse side effect such as nephrotoxicity (Denning, 2003). Amphotericin B (Figure 1.11) has a narrow therapeutic index due to the toxicity to some human organs. Nystatin can only be used topically. However amphotericin B is formulated as intravenous use, and only used for the treatment of life-threatening fungal infection (Dannaoui, 2000).

Conventional therapy for the treatment and control of invasive aspergillosis still depends on the use of the polyene, amphotericin B and the azoles, itraconazole and

fluconazole (Canuto & Rodero, 2002; Ellis, 2002). Amphotericin B displays novel antifungal activity and functions by forming apertures in the cell membrane, by complexing the ergosterol in the cell membrane, each pore forming consists of an annulus of eight amphotericin B molecules linked hydrophobically to ergosterol (Abu-Salah, 1996). An alternative explanation suggests that the mode of action of amphotericin B involves an alteration in the permeability of the phospholipid bilayer to create pores leading to an increase in the fluidity of the membrane (Venegas *et al.* 2003).

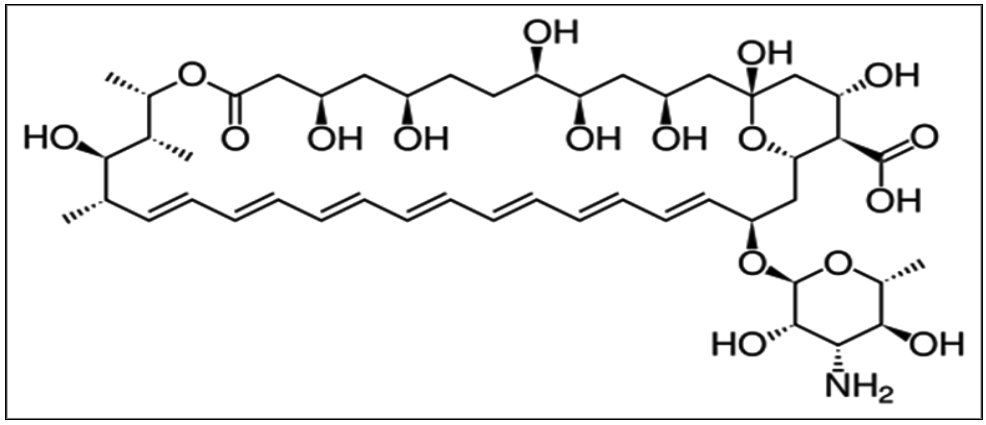
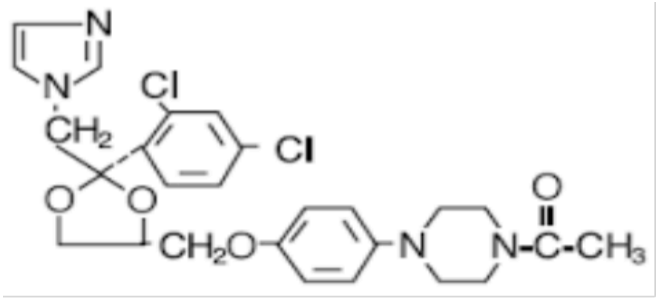


Figure 1.11 Structure of Amphotericin B.

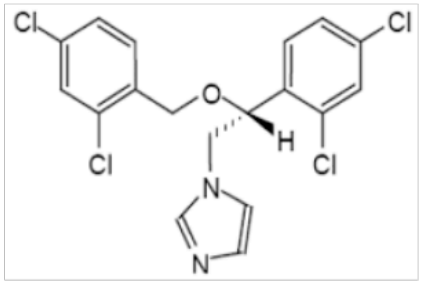
1.6.3 Azole antifungal

Azoles (Imidazoles and Triazoles) are the largest class of clinically used antifungal agents (Figure 1.12 and Figure 1.13). Their mode of action involves the inhibition of the 14α-demethylation of lanosterol in the pathway of ergosterol biosynthetic (Odds, 2003). The imidazole antifungals (e.g. miconazole, econazole, and fluconazole) are heterocyclic synthetic compounds that inhibit the enzyme lanosterol 14 α-demethylase; the enzyme necessary to convert lanosterol to ergosterol. The drugs block demethylation of the C-14 of lanosterol lead to depletion of ergosterol in fungal membrane. This disrupts the structure and many functions of fungal membrane leading to inhibition of fungal growth (Diaz-Guerra *et al.,* 2003). Azole antifungal agents have been used widely to treat superficial and systemic fungal infections. Miconazole was the first azole used to treat fungal

infections (Utz, 1980), has been discontinued because of failure in some cases. In treatment of candidemia fluconazole has been shown to be as effective as amphotericin B (Rex *et al.,* 1994). Voriconazole, posaconazole and ravuconazole are three triazoles that have broad spectrum of activity against fungi (Odds, 2003). In treatment of candidemia fluconazole has been shown to be active as amphotericin B with fewer side effects (Karthaus and Cornely, 2007). New triazoles have emerged: voriconazole, posaconazole and ravuconazole and each has broad spectrum of activity (Odds, 2003). The mechanism of resistance to azole antifungal agents in *C. albicans* have been elucidated and categorized as due to changes in the cell wall or plasma membrane, which lead to impaired azole uptake; alterations in the affinity of the drug target Erg11p due to target site mutagenesis and the increased efflux of drugs mediated by membrane transport proteins, deletion of these genes resulted in hypersensitivity to azoles (Sanglard and Bille, 2002).



Kitoconazole



Miconazole

Figure 1.12 Structures of Imidazoles

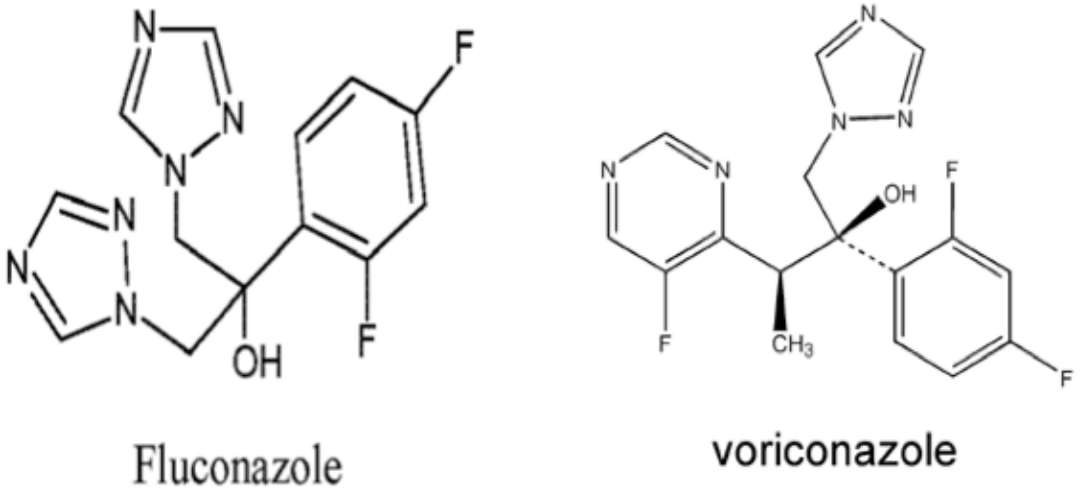


Figure 1.13 Structures of Triazoles

1.6.4 Pyrimidine analogues: 5-Fluorocytosine

5-Fluorocytosine is a fluorinated analogue of cytosine (Figure 1.14) that has antifungal activity resulting from the rapid conversion to 5-fluorouracil within target cells (Odds *et al.,* 2003). 5-FU inhibits fungal protein synthesis after incorporation into fungal RNA causing premature chain termination, and this inhibits DNA synthesis through inhibition of thymidylate synthetase. Antifungal resistance develops quickly to 5-FC therapy, so combination therapy with other agents has been reported to enhance the antifungal activity of amphotericin B (Siau and Kerridge. 1999), especially in anatomic sites where amphotericin B penetration is poor, such as cerebral spinal fluid (CSF) and heart valves (Smego *et al.,* 1984). One explanation for the synergism detected with amphotericin B plus 5-FC is that the membrane-permeabilizing effects of low concentrations of amphotericin B facilitate penetration of 5-FC to the cells (Vermes *et al.,* 2000).

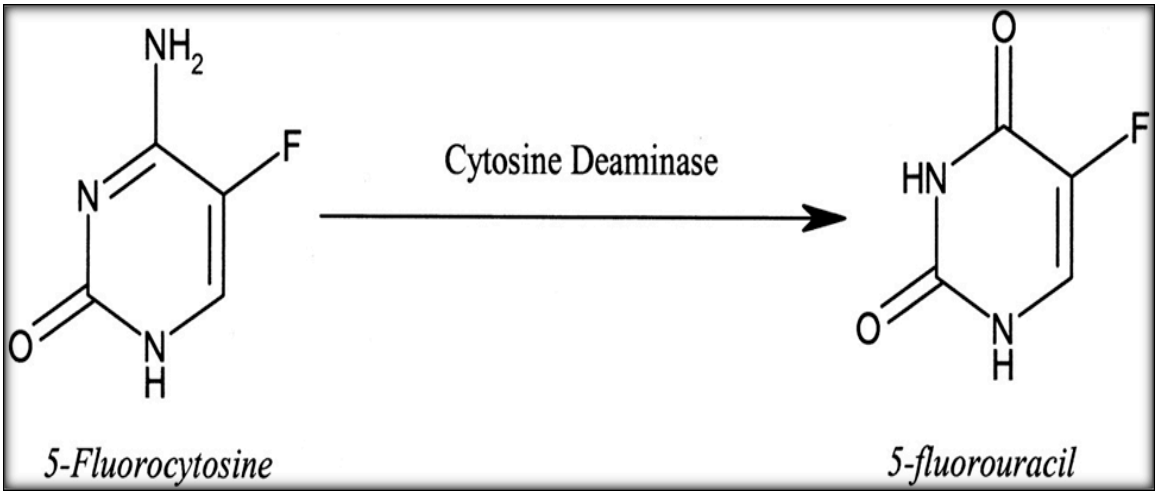


Figure 1.14 Structure of 5-Fluorocytosine

1.6.5 Combination antifungal therapy

The mortality rate among patients treated with polyene and azole antifungal agents has increased especially among immuno-compromised patients; therefore it is essential to find out new strategies for the treatment of invasive fungal infections. Echinocandins, polyenes, azoles and flucytosine, all belong to different pharmacological classes and possess distinct mechanisms of action (Georgopapadakou and Walsh, 1996). As different groups of antifungal drugs act on different targets, evaluation of their interactions is of potential interest for improving the strategies of treatments for invasive candidiasis and aspergillosis. Azoles act by inhibition of 14α-demethylase (Siau and Kerridge, 1999), flucytosine inhibits fungal RNA and DNA synthesis (Vermes, 2000), and polyenes binds to ergosterol in fungal cell membrane (Georgopapadakou and Walsh, 1996), whereas echinocandins inhibit the synthesis of beta-1,3-glucan (De-Lucca, 1999). The combination of amphotericin B or liposomal formula with caspofungin is still a safe and a suitable option (Arikan *et al.,* 2002) for the treatment of invasive fungal infections in immunocompromised individuals especially those with hematologic malignancies (Aliff *et al.,*2003; Arikan *et al.,* 2002), both *in vitro* and *in vivo* in animals (Luque *et al.,* 2003) showed no antagonism. Interactions between azole and echinocandins drugs deserve

consideration for the treatment of invasive aspergillosis and some *in vitro* testing has been conducted (Manavathu *et al.,* 2003) and their use in animal models of aspergillosis was investigated (Luque *et al.,* 2003).

1.7 Aims of study

1. To analyse the effect of culture conditions on the susceptibility of *C. albicans* and *A. fumigatus* to caspofungin.

2. To investigate the effect of caspofungin on the biosynthesis and release of gliotoxin from *A. fumigatus*.

3. To characterize the proteomic alterations occurring in *A. fumigatus* in response to caspofungin.

Chapter 2

MATERIALS AND METHODS

2.1 Strains used in this work

Clinical strain of *A. fumigatus* ATCC 26933, (MARYLAND USA) was used in this study. *C. albicans* MEN was a kind gift from Dr. D. Kerridge, Cambridge, UK.

2.2 Sterilization procedures

All growth culture media were sterilized by autoclaving prior to use at 121°C and 15 lb/sq.in for 15 minutes. Any liquid unsuitable for autoclaving was filter-sterilised using a pore size 0.2 μm (Sarstedt, Nümbrecht, Germany). All used liquid and solid cultures were autoclaved prior to disposal.

Laboratory equipment such as micro-centrifuge tubes and pipette tips were kept in a tied sealed container and sterilized by autoclaving at 121ºC for 15 minutes prior to use. RPMI1640 and MEM minimal essential media were supplemented with 5% (v/v) fetal bovine serum (Hyclone). Semi-solid agar media were allowed to cool until hand hot, (approximately 45-50ºC) and poured under sterile conditions using 9 cm petri-dishes. All work involving *A. fumigatus* was performed using a class II safety cabinet.

2.3 Chemical and reagent used in this study

All reagents used in this work were of the highest purity and were purchased from Sigma Aldrich Ltd, Somerset, U.K unless otherwise stated.

2.4 Preparation of Caspofungin acetate

Caspofungin (as acetate) [Merck & Co., Inc., Whitehouse Station, NJ., USA] powder was reconstituted in molecular grade water to obtain a stock solution of 1.0 mg/ml. Each 1 ml of solution was stored at -20 ^{o}C until required.

2.5 Statistical analysis of data

Results presented in this work are the mean of at least two independent determinations and the results are presented as the mean ± standard error. Experimental data were tested for statistical significance using a Students t-test. For all experimentation a p-value of ≤0.05 was considered statistically significant.

2.6 Culture media used in this work

2.6.1 Phosphate Buffered Saline.

Five tablets of PBS were completely dissolved in 500 mls deionised water and autoclaved at 121ºC for 15 minutes. PBS was stored at room temperature. For PBS-T, PBS was supplemented with 0.01% (v/v) Tween-80 (Merck), and was vortexed until mixed homogenously. PBS-T was sterilised by filtering through a 0.22 µm filter (Millipore), prior to use using 20 ml syringe.

2.6.2 YEPD [Yeast Extract peptone D-glucose]

YEPD broth was prepared by adding Glucose (2% w/v), Yeast extract (1% w/v), and bacteriological peptone (2% w/v), and completely dissolving in deionised water prior to autoclaving at 105ºC for 30 minutes.

For YEPD agar plates 2% (w/v) agar was added and autoclaved as described. In some cases antibiotic was added to the hand warm agar prior to pouring to avoid bacterial contamination. Solution of erythromycin in DMSO was added when the agar solution was hand warm at final concentration of a 0.05 mg/ml. Once in the agar solution the plates were spread as per normal and stored at 4ºC.

2.6.3 Antibiotic Culture medium 3

AB3 powder (17.5 gram) was dissolved in 1 litre of warm distilled water. When completely dissolved in the final volume it was sterilised by autoclaving at 121 ^{o}C for 15 minutes.

2.6.4 Malt Extract Agar [MEA]

Malt extract agar was prepared by adding 50 grams of MEA per one litre of deionised water, mixing and autoclaving at 121 ^{0}C for 15 minutes. Agar was poured into petri-dish once it was hand hot.

2.6.5 RPMI 1640

A single plastic bottle of sterile RPMI1640 Medium (1X) (Gibco^{R} Life technologies^{TM}) contained 500 ml.

2.6.6 Nutrient broth (NB)

NB (13 gram) powder was dissolved in 1 litre of distilled water. This was mixed well and sterilize by autoclaving at 121 ^{o}C for 15 minutes.

2.6.7 Czapek-Dox broth

Czapek-Dox broth powder (35 gram) was dissolved in one litre of deionized water, mixed well, and sterilised by autoclaving at 121 ^{o}C for 15 minutes.

2.6.8 Minimal Essential Media (MEM)

Sterile Minimum Essential Medium Eagle (Sigma) with Earles salts and $NaHCO_3$ without L-glutamine was delivered in 500 ml single bottle.

2.6.9 Sabouroud dextrose liquid medium

Sabouroud dextrose powder (30 gram) was dissolved in 1 litre of distilled water, mixed well, and sterilized by autoclaving at 121 ^{o}C for 15 minutes.

2.6.10 Liquid Culture of *A. fumigatus*

Minimum essential medium (MEM) and RPMI1640 supplemented with 5% (v/v) FBS or aspergillus minimal medium (AMM), antibiotic media (AB3), sabouroud dextrose, and Czapek sterile medium were inoculated in a sterile laminar flow hood with *A. fumigatus* conidial at 5 x 10^5 cells per ml of culture.

2.6.11 *Aspergillus* Trace Elements

$Na_2B_4O_7.7H_2O$ (0.04 g), $CuSO_4$.(5H2O) (0.7 g), $FeSO_4.7H_2O$ (1.2 g), $MnSO_4$ (0.7 g), NaMoO.$2H_2O$ (0.8 g) and $ZnSO_4.7H_2O$ (10 g) were added to 800 ml of distilled water and mixed to dissolve. The solution was made up to 1 L with distilled water and subsequently autoclaved at 121 ^oC for 15 minutes. The solution was aliquoted in 50 ml volumes and stored at -20ºC.

2.6.12 *Aspergillus* Salt Solution

KCl (26 g), $MgSO_4.7H_2O$ (26 g), KH_2PO_4 (76 g) and *Aspergillus* Trace elements (2.7.1) (50 ml) were added to 800 ml distilled water and dissolved completely. The solution was made up to 1 L with distilled water and autoclaved as before. The solution was stored at room temperature.

2.6.13 Ammonium Tartrate

Ammonium Tartrate (92 g) was completely dissolved in 1L of distilled water and autoclaved. The solution was stored at room temperature.

2.6.14 *Aspergillus* Minimal Medium (AMM)

Aspergillus minimal medium was prepared by adding Ammonium Tartrate (2.7.3), (10mls), *Aspergillus* Salt Solution (2.7.2), (20 mls), and Glucose, (10 g), to 800 mls of distilled water. pH of solution was adjusted to 6.8 and made up to 1 L using distilled water. The medium was distributed in final volumes, autoclaved at 121 ^oC for 15 minutes and stored at room temperature.

2.7 Microbial strains and culture conditions

2.7.1 *A. fumigatus* strains and culture conditions

Clinical strain of *A. fumigatus* ATCC 26933, (MARYLAND USA) was used in this study. Stocks of *A. fumigatus* were maintained on malt extract agar (MEA) [Oxoid Ltd] at 37°C and sub-cultured every 4 weeks. Culture was grown in different type of media for susceptibility assay, AMM, YEPD, NB, Czapek, and AB3. RPMI1640 (Gibco-BRL, Uxbridge, UK) with L-glutamine and MEM (SIGMA) were supplemented with 5% (v/v) Fetal bovine serum [HyClone], prior to the experiment.

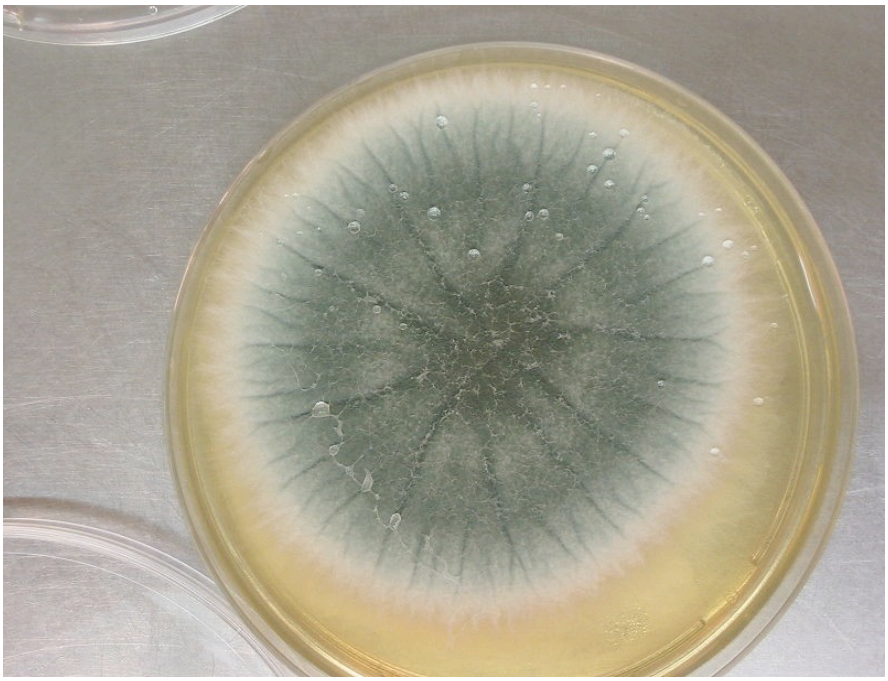


Figure 2.1 *Aspergillus fumigatus* on MEA agar plate has been incubation for 3 days at 37 ^{o}C.

2.7.2 Inoculation of *A. fumigatus*

Stocks of *A. fumigatus* spores were grown and maintained on Malt Extract Agar (Oxoid Ltd Basingstoke UK) at 37ºC in a thermally controlled incubator for 4 to 5 days.

Sterile medium was inoculated in a sterile laminar flow hood and *A. fumigatus* conidial inoculations of 5 x 10^7 cells per 100 ml culture were used.

2.7.3 Harvesting *A. fumigatus* conidia

Conidia of *A. fumigatus* were harvested in a safety cabinet Class II. 10 mls PBS-T [0.1% (v/v) Tween 80] was used to wash the plates. Conidia were harvested by centrifugation at (2,056 x g) for 5 minutes at room temperature on a Beckmann GS-6 bench centrifuge. The supernatant was removed and the conidial pellet was washed twice in sterile PBS to remove excess Tween 80 and re-suspended in sterile PBS. The concentration of conidia in the suspension was determined by counting with a Haemocytometer.

Concentration of cells in original prep:

No. cells counted in 5 square **X** 5 **X** Volume in which cells have been counted **X** (dilution factor)=

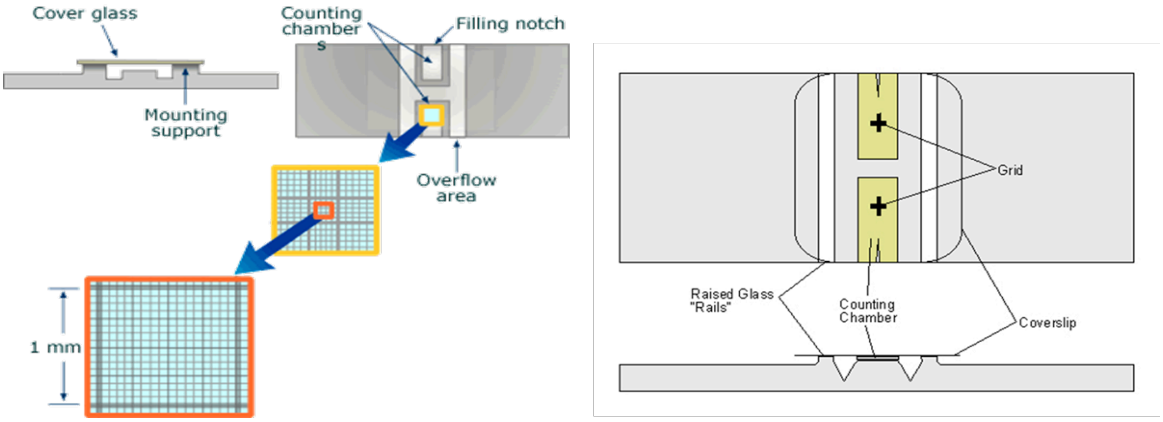


Figure 2.2 Images for Haemocytometer

2.7.4 *Candida albicans* culture

C. albicans MEN was a kind gift from Dr. D. Kerridge, (Cambridge, UK). An individual colony of cells was aseptically transferred to sterile YEPD broth (2.5.2) using a

sterile inoculating loop. The flask was re-plugged with cotton and incubated at 30ºC shaking at 200 rpm overnight. To obtain a suspension of cells, 50 ml culture was transferred to a sterile 50 ml tube and centrifuged at 500 x g for 5 minutes. The cellular pellet was washed twice in sterile PBS and the final cell concentration in a 10 ml volume of sterile PBS was ascertained by Haemocytometer.

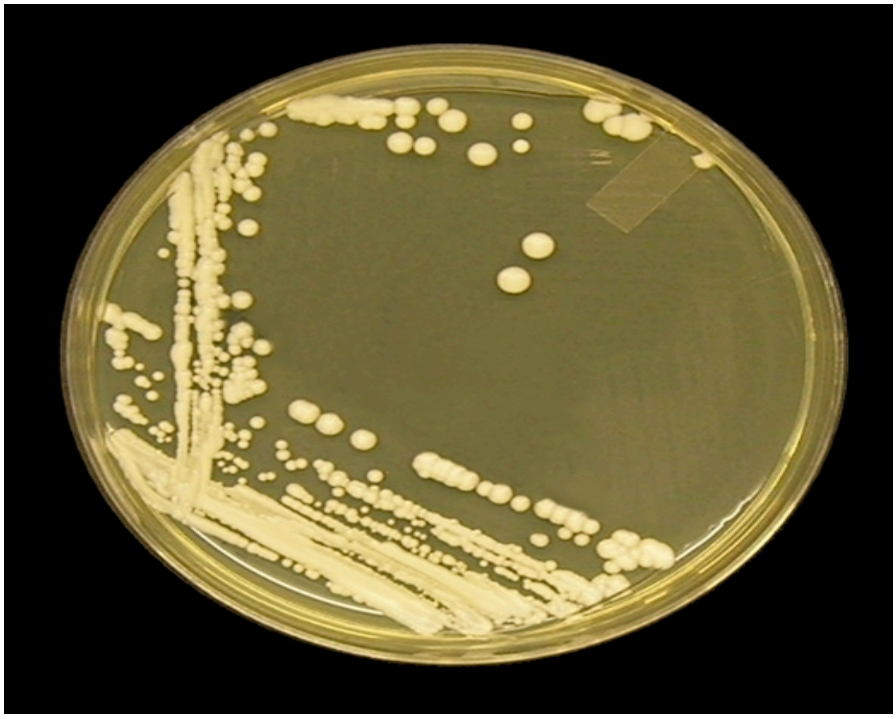


Figure 2.3 *Candida albicans* on YEPD agar plate has been grown at 30 ^{o}C for 48 hour

2.8 Analysis of the effect of caspofungin on the growth of *A. fumigatus*

RPMI1640 culture medium (25 ml) supplemented with 5% (v/v) fetal bovine serum (FBS) was inoculated initially with $5x10^{3}$ *A. fumigatus* conidia/ml. Four different concentrations of Caspofungin plus control were used in this experiment. Cultures were incubated at 37 ^{o}C on a rotary incubator, samples were taken every 24 hour, filtered using miracloth, and Dry hyphae mass was measured (Dry weight).

2.9 Susceptibility of *C. albicans* and *A. fumigatus* to caspofungin

C. albicans cells were grown to stationary phase in YEPD. Cells were counted on a Haemocytometer, and diluted to a density of 5 X 10^6 cells/ml. Cell suspensions (100 µl) were added to each well of a 96 well plate except row two was used control media. MIC50 and MIC90 were determined as the lowest concentration of caspofungin required to reduce growth by 50% and 90% relative to the growth of the control.

All experimental work for susceptibility testing of *A. fumigatus* to caspofungin was conducted according to the reference method for broth dilution antifungal susceptibility testing of filamentous fungi; Approved standard (M38-A2, 2008) with some modification. The Minimum Effective Concentration (MEC) is read as the lowest concentration of caspofungin that lead to the growth of small, rounded, compact hyphal forms as compared to the hyphal growth seen in the control *A. fumigatus*. To improve the reading all *Aspergillus* plates were shaken befor read the result. The optical density was recorded at 540 nm using a microplate reader (Synergy HT, Bio-Tek). MIC_{50} and MIC_{90} were calculated from the graph using Microsoft Excel sheet.

2.10 Effect of different types of culture media on the efficacy of caspofungin against *C. albicans* and *A. fumigatus*

C. albicans was used to test the activity of antifungal drug Caspofungin. AB3, YEPD and Sabouroud dextrose were used for *Candida* susceptibility test using 96 well plate microdilution assays against Caspofungin. All plates were incubated for 24 h at 30^{o}C. All plates were read using micro-plate reader (Synergy HT, Bio-Tek) to record the optical density at 540nm. MIC_{50} and MIC_{90} values were calculated using Microsoft Excel.

Caspofungin also was tested against *A. fumigatus* using 5 types of media and RPMI1640 was supplemented with 5% (v/v) FBS. AMM, CZOPEK, NB and AB3 media were prepared as describe in section (2.5). The 96 plates assay was used in toxicity assay

according to Reference method for broth dilution Antifungal susceptibility testing of filamentous fungi (M38-A2, 2008).

2.11 Effect of the different concentrations of glucose on Caspofungin activity.

All culture media used were supplemented with 1%, 2%, 5% and 7.5% (w/v) glucose. In this experiment Caspofungin was tested against *C. albicans* and *A. fumigatus*. The effect of glucose on the activity of the drug was studied by determining the MICs of drug in all cases.

2.12 Analysis of the activity of Caspofungin against *C. albicans* and *A. fumigatus* in media of different pH.

All media used were adjusted to PH 3, 4, 6, 7, 8 and 10 before sterilization. Caspofungin was tested against *C. albicans* and *A. fumigatus* at different pH value.

2.13 Analysis of the activity of caspofungin against *C. albicans* and *A. fumigatus* at different concentration of Bovine Serum Albumin (BSA).

Four concentrations of BSA were used to test the activity of Caspofungin against *C. albicans* and *A. fumigatus;* 25 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml. AB3 was used in this experiment to determine the growth rate over the different concentrations used. All plates were read and variable MICs were calculated.

2.14 To examine the effect of aeration on the susceptibility of *C. albicans* and *A. fumigatus* to caspofungin.

C. albicans was grown at 30 ^{o}C and *A. fumigatus* was grown at 37 ^{o}C, under static or shaker conditions to supply oxygen to help fungus grown aerobically. All plates were read at 540nm using plate reader to get the result and data were analysed using Microsoft Excel.

2.15 Effect of different carbon source on susceptibility of *C. albicans* and *A. fumigatus* to caspofungin.

Before this experiment different concentrations of glucose were used for susceptibility assay using AB3 media. In this experiment different carbon sources were used. Glucose, lactose, sucrose, maltose, galactose and glycerol were used at 2% (w/v) in AB3 media. All susceptibility tests were conducted and the percentage of growth under different conditions was plotted in graphs to facilitate calculation of MICs at each condition.

2.16 Morphological analysis of *A. fumigatus* when exposed to Caspofungin

A. fumigatus cultures RPMI1640 were exposed to Caspofungin for 48 hours at low and high concentrations (0.1 and 1.0 μg/ml) at 37 ^{o}C in an orbital incubator. Hyphae were collected and washed twice with PBS. 0.5 gram of *Aspergillus* hyphae were transferred aseptically to sterile universal tube and resuspended in 5 ml of sterile PBS. All samples were sent to CMA (Trinity College Dublin) for examination under electron-microscopy using air dry, critical point drying and freeze drying techniques.

2.17 Extraction and evaluation of Ergosterol from *A. fumigatus*

2.17.1 Ergosterol extraction

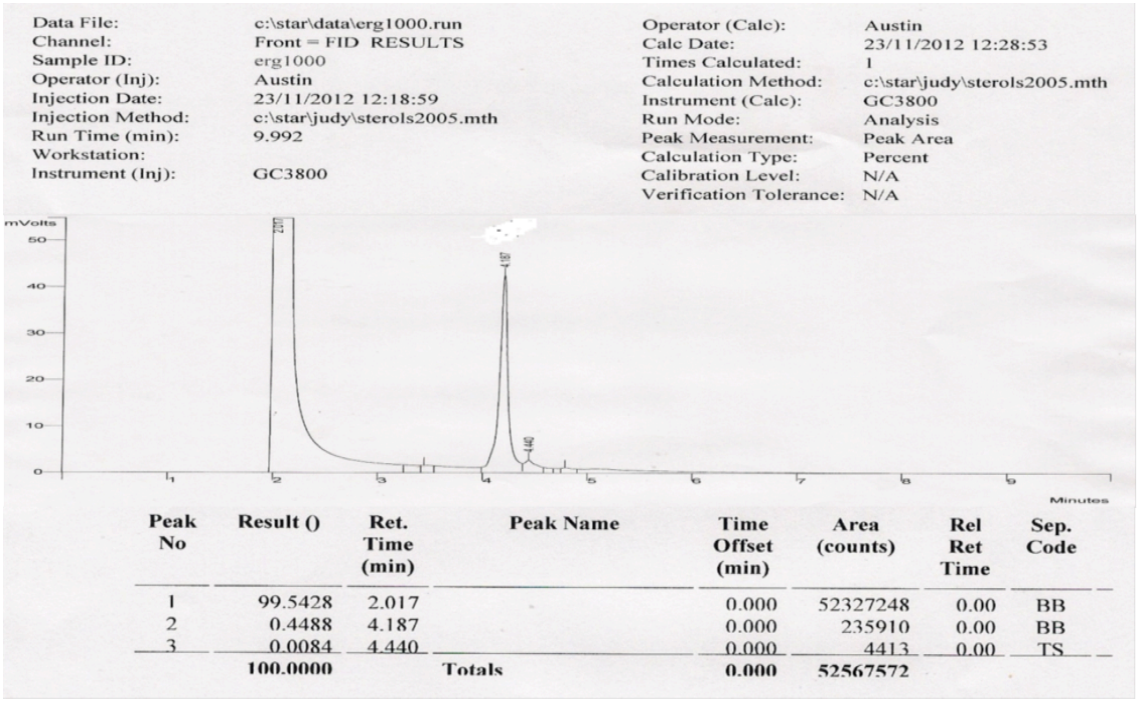
The Method of Arthington-Skaggs *et al*. (1999) was used to extract the sterol with slight modifications. One gram dry weight of each *A. fumigatus* hyphae sample was washed twice with sterile PBS. Hyphae were resuspended in 3 ml of a solution containing 20% (w/v) KOH (Sigma) and 60% (v/v) ethanol. Hyphae were broken up using spatula and vortexing. Samples were placed in a shaking water bath at (80 to 85 ^{0}C) for 2 hours. N-heptane (1.2 ml) was added to the solution then vortexed for 10 seconds. The top aqueous layer containing ergosterol was separated by centrifugation for 5 min at 3000 xg then collected using Pasteur pipette.

2.17.2 Ergosterol analysis

Before the analysis of ergosterol a few grains of anhydrous sodium sulfate (sigma) were added to the extract to remove excess water. Gas Chromatograph (Hewlett Packard 5890 Series II) was used to detect and quantify sterols (Figure 2.4). Different concentrations of ergosterol standard were prepared to calculate the standard curve (Figure 2.5).

2.17.3 Ergosterol retention time using gas chromatography (GC)

The fungal sterol content was quantified using Gas Chromatograph (Hewlett Packard 5890 Series II) with a flame ionization detector and a chromapack capillary column (Chromapack International BV, Middelburg, the Netherlands). Carried gas was N_2, injector and detector temperature were 320 ^{o}C. The standard was ergosterol (Sigma Aldrich).



Peak No	Result ()	Ret. Time (min)	Peak Name	Time Offset (min)	Area (counts)	Rel Ret Time	Sep. Code
1	99.5428	2.017		0.000	52327248	0.00	BB
2	0.4488	4.187		0.000	235910	0.00	BB
3	0.0084	4.440		0.000	4413	0.00	TS
	100.0000		Totals	0.000	52567572		

Figure 2.4 Peak at 4.187 minutes represent the 1 mg of standard Ergosterol

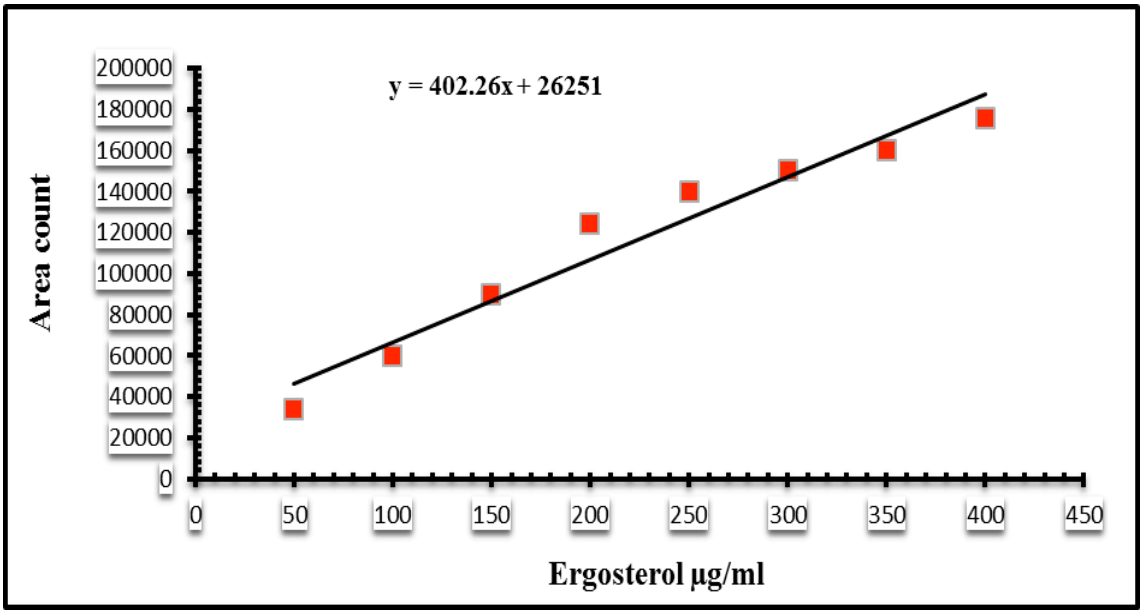


Figure 2.5 Standard curve of Ergosterol

2.18 RP-HPLC

High-performance liquid chromatography is a powerful tool in analysis. Reversed phase HPLC is the most commonly used form of HPLC. Different compounds have different retention times. For a particular compound, the retention time will vary depending on the pressure used (because that affects the flow rate of the solvent), the nature of the stationary phase (not only what material it is made of, but also particle size), the exact composition of the solvent and the temperature of the column.

2.19 Buffer used in (RP–HPLC).

2.19.1 Buffer A.

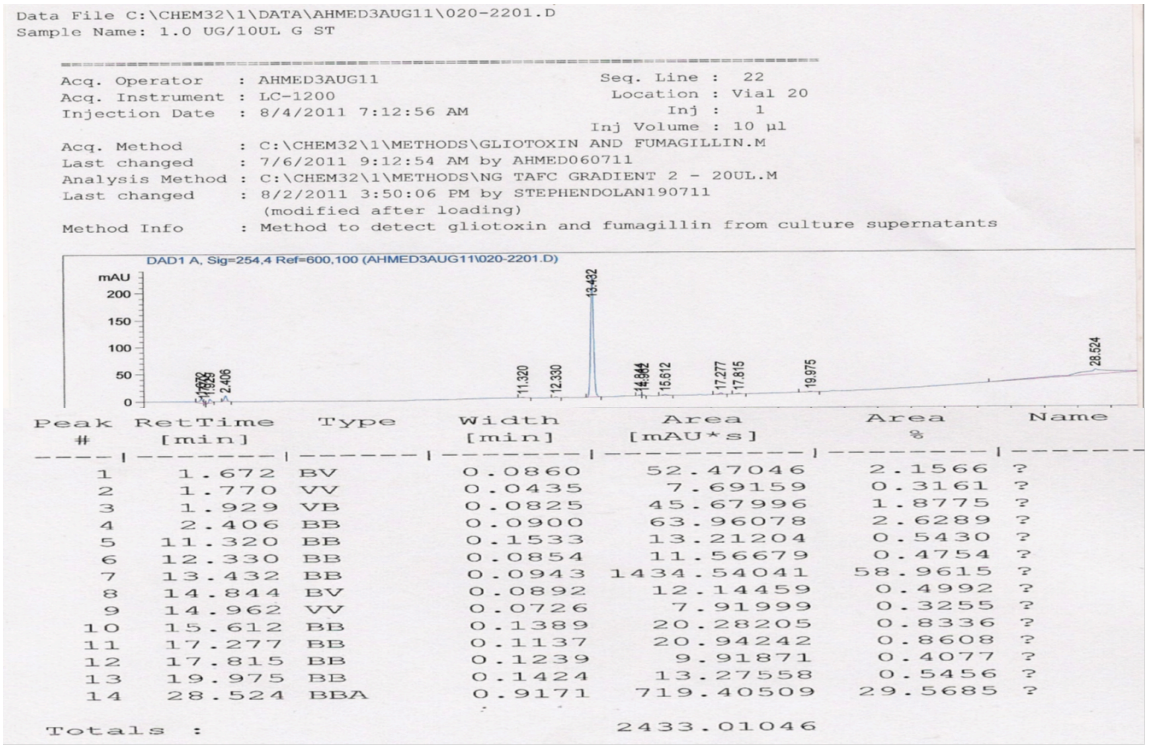
(1 L) HPLC grade water was placed in a darkened Duran bottle. 0.1% (v/v) Triflouroacetic acid was added and mixed thoroughly. This was made fresh on the day.

2.19.2 Buffer B.

HPLC grade Acetonitrile (1 L) was placed in a clear Duran bottle. Triflouroacetic acid 0.1% (v/v) was added to the bottle and contents of the bottle were mixed thoroughly. This was made fresh on the day.

2.20 Analysis of mycotoxins using RP-HPLC.

Organic extracts were analysed by reverse phase HPLC detect the compounds at multiple wavelength (254, 351, and 220 nm) on Agilent 1200 series using a C-18 RP-HPLC column at a flow rate of 1 ml/min. The concentration of gliotoxin in a given sample was ascertained by performing standards gliotoxin and diluted in HPLC-grade methanol.



Data File C:\CHEM32\1\DATA\AHMED3AUG11\020-2201.D
Sample Name: 1.0 UG/10UL G ST

Acq. Operator : AHMED3AUG11
Acq. Instrument : LC-1200
Injection Date : 8/4/2011 7:12:56 AM
Seq. Line : 22
Location : Vial 20
Inj : 1
Inj Volume : 10 µl
Acq. Method : C:\CHEM32\1\METHODS\GLIOTOXIN AND FUMAGILLIN.M
Last changed : 7/6/2011 9:12:54 AM by AHMED060711
Analysis Method : C:\CHEM32\1\METHODS\NG TAFC GRADIENT 2 - 20UL.M
Last changed : 8/2/2011 3:50:06 PM by STEPHENDOLAN190711
(modified after loading)
Method Info : Method to detect gliotoxin and fumagillin from culture supernatants

DAD1 A, Sig=254,4 Ref=600,100 (AHMED3AUG11\020-2201.D)

mAU
200
150
100
50
0
13.432
11.320
12.330
14.844
15.612
17.277
17.815
19.975
28.524
2.406

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Area %	Name
1	1.672	BV	0.0860	52.47046	2.1566	?
2	1.770	VV	0.0435	7.69159	0.3161	?
3	1.929	VB	0.0825	45.67996	1.8775	?
4	2.406	BB	0.0900	63.96078	2.6289	?
5	11.320	BB	0.1533	13.21204	0.5430	?
6	12.330	BB	0.0854	11.56679	0.4754	?
7	13.432	BB	0.0943	1434.54041	58.9615	?
8	14.844	BV	0.0892	12.14459	0.4992	?
9	14.962	VV	0.0726	7.91999	0.3255	?
10	15.612	BB	0.1389	20.28205	0.8336	?
11	17.277	BB	0.1137	20.94242	0.8608	?
12	17.815	BB	0.1239	9.91871	0.4077	?
13	19.975	BB	0.1424	13.27558	0.5456	?
14	28.524	BBA	0.9171	719.40509	29.5685	?
Totals :				2433.01046		

Figure 2.6 Detection of gliotoxin by RP-HPLC. Gliotoxin detection was performed at 254 nm with a retention time of approximately 13.432 minutes. Image shows gliotoxin detection of 1 µg/10µl.

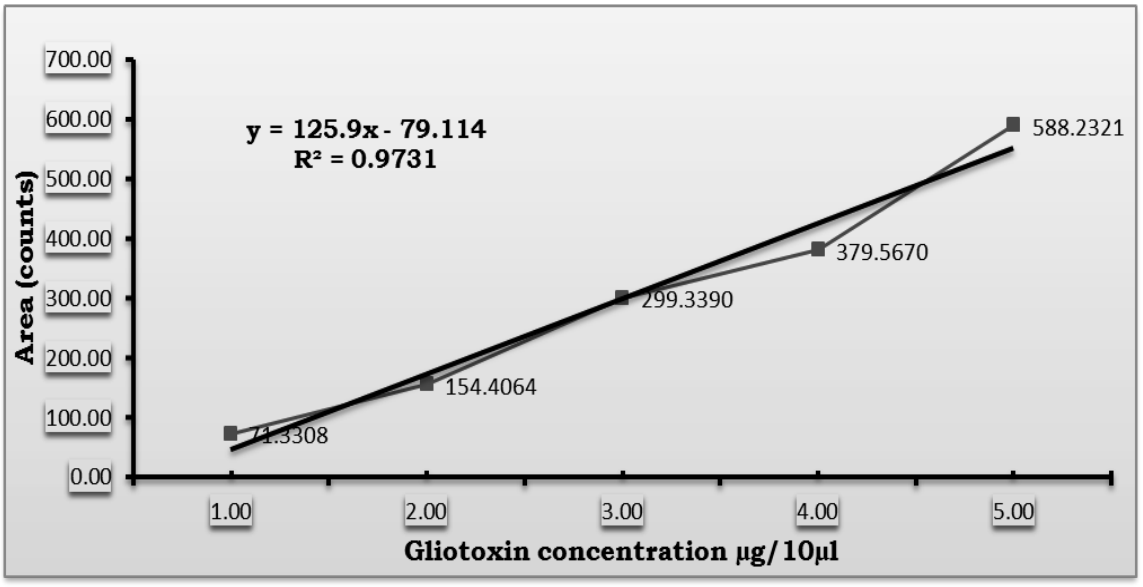


Figure 2.7 Standard curve of serial concentration of gliotoxin reference standard

2.20.1 Detection of Gliotoxin in sample

Gliotoxin detection was performed at 254 nm with a retention time of approximately 13.432 minutes (Figure 2.6).

2.20.2 Culture and extraction of Gliotoxin

Culture *A. fumigatus* were grown on MEA for 3 days at 37°C and the conidia were harvested using sterile 0.5% (v/v) Tween 80. The concentration of conidia was assessed using a Haemocytometer and adjusted to 5 x 10^5 conidia/ml in sterile PBS. This conidial suspension was used to inoculate 100ml of RPMI1640 supplemented with 5% FBS in a 250ml conical flask. The culture was incubated at 37°C in a shaking incubator at 200 rpm for 3 to 4 days. The fungal biomass was harvested by filtration through Miracloth filter. The filtrate was extracted with an equal amount of chloroform by shaking for 2 hr in 50 ml falcon tube at 25°C. The chloroform fractions were pooled and evaporated to dryness on a rotary evaporator at reduced pressure and at 50°C. The dried extracts were dissolved in 250µl HPLC grade methanol, and stored at -20°C until used for detection of gliotoxin by HPLC.

2.20.3 Analysis of the results of HPLC

HPLC system is composed of six basic modules (reservoir, pump, injector, column, detector, and data system) connected by appropriate tubing and fittings. Pure samples will generally only have one peak. The compounds separate as they are carried through the column at the left. As each band leaves the column, it passes through the detector. The display at the right shows the detector response as a function of time.

To prepare standards it is convenient to start with a 5 µg/10µl concentrated stock solution of gliotoxin. The largest amount that we need was 5µg, in a volume of 10µl. The calculations and the standard curve were shown in Figure (2.7).

2.20.4 Detection of gliotoxin in *A. fumigatus* samples grown in different type of culture media,

Different types of culture media were used in this experiment to study the ability of *A. fumigatus* to produce gliotoxin internally and externally. In this section four types of culture media were used: *Aspergillus* minimal media (AMM), Antibiotic media (AB3), RPMI1640 and Minimal essential media (MEM)

2.20.5 Evaluation of internal and external gliotoxin in *A. fumigatus* grown in different pH condition

Culture pH is one of the main factors which affect the growth of fungi. Variable ranges of pH were used to determine the amount of biosynthesis of gliotoxin over a broad range of pH value (3.5, 4.5, 5.5, 6.5 and 7.5).

2.20.6 Detection of Gliotoxin in *A. fumigatus* when grown in sub-inhibitory concentrations of Caspofungin.

A 96 hour old culture of *A. fumigatus* was grown in RPMI1640 at low and high concentrations of caspofungin (0.1 and 1.0 µg/ml) in presence of negative (PBS) and positive (DMSO) control. All four samples were prepared to determine the amount of gliotoxin internally and externally.

2.20.7 Preparation of extracts for detection of gliotoxin by reversed-phase HPLC.

A standard curve of peak value versus gliotoxin concentration was constructed using gliotoxin standards [0.1, 0.2, 0.5, 1.0 µg/10µl]. A volume of $5x10^5$ cells/ml *A. fumigatus* spore were inoculated in 100 ml of RPMI1640 (GIBCO). Caspofungin (0.01, 0.1 and 1.0 µg/ml) was added to the culture and the samples were removed at 24, 48, 72 and 96 hour.

2.20.8 Extraction of gliotoxin from *A. fumigatus* culture filtrate.

At each time interval the culture contents were filtered through miracloth (Calbiochem) and wet weights were recorded. Gliotoxin was extracted from the culture filtrate by mixing it with equal amounts of chloroform for 2 hour, separation of chloroform

layer, and extraction of gliotoxin by rotary evaporation at 55 ^{o}C. The dried extracts were dissolved in 250 µl of methanol and the level of gliotoxin determined using RP-HPLC.

2.20.9 Extraction of gliotoxin from hyphae of *A. fumigatus*

Intracellular gliotoxin was extracted from the hyphae of *A. fumigatus* as follows: one gram of hyphae was recovered from 100 ml of RPMI culture media by filtration. This was washed twice in PBS and ground to a fine powder under liquid nitrogen. The hyphae powders were re-suspended in 10 ml of 6 M HCl. Chloroform (35 ml) was added to 50 ml falcon tube and the mixture was stirred at room temp for 2 hours. Chloroform layer was separated and gliotoxin was extracted by evaporation of chloroform layer, the dried extracts were dissolved in 250 µl of methanol and the level of gliotoxin determined using RP-HPLC.

2.21 Evaluation of amino acid leakage from *A. fumigatus* exposed to caspofungin

A. fumigatus conidia were inoculated into 50 ml of RPMI 1640 to give a final concentration of 1X10^{5} conidia/ml and incubated at 37 ^{o}C for 96 hour. Hyphae were collected by filtering through miracloth (Calbiochem) and washed twice with 10 ml sterile PBS. The hyphal mass (2 g) was re-suspended in 25 ml PBS containing 0.5% (v/v) Dimethyl-sulfoxide (DMSO) [as positive control], or caspofungin (0.1 µg/ml or 1.0 µg/ml) and the cultures were incubated at 37 ^{o}C and 200 rpm for another 0.5, 2 or 4 hour. At each time point 750 µl of culture filtrate was removed and filtered through 0.45 µM syringe filters (Sarstedt). Free amino acids were measured at 570 nm using the ninhydrin colorimetric method described by Ghosh and Ghosh (1963). Supernatant (750 µl) was added to 250 µl of a ninhydrin solution (0.35 g ninhydrin in 100 ml molecular grade ethanol). This was heated to 95 ^{o}C for 5 minutes. The absorbance at 570 nm was recorded on a spectrophotometer (Shimadzu UVmini-1240) and expressed in terms of aspartic acid and glutamic acid (1:1), which were used as standards (Figure 2.8).

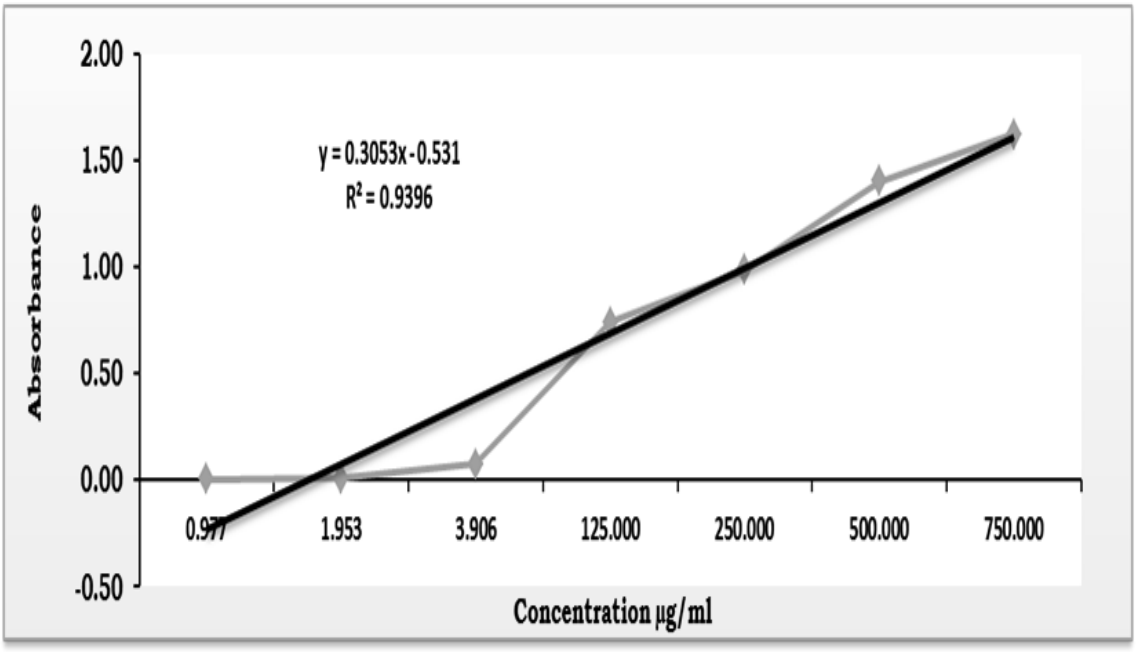


Figure 2.8 Standard curve of aspartic acid and glutamic acid.

2.22 Whole cell protein extraction from *A. fumigatus* ATCC 26933 mycelium grown in MEM liquid culture media

A culture of *A. fumigatus* was grown in MEM liquid culture media for 96 hour at 37 ^{o}C in an orbital incubator. A 100 ml volume of the culture medium was filtered through 2 layers of Mira-cloth and squeezed to dry. Hyphae were left 5 minutes to dry on filter paper. A certain weight of mycelia mass was added to 50 ml falcon tube and Snap frozen in liquid nitrogen to break-up by agitation. Frozen mycelium (1 gram) was placed into an autoclaved mortar. Liquid nitrogen was added and material ground into a fine powder. Four ml of protein extraction buffer [0.4 M NaCl, 10mM Tris HCl, 2 mM EDTA] 1 µl per 1ml of buffer protease inhibitor were added and ground until a viscous liquid consistency was attained. The viscous liquid was centrifuged at 2500 x g, 4 ^{o}C for 5 min. The supernatant was retained and pellet was discarded.

2.23 Protein Leakage from *A. fumigatus* Hyphae

2.23.1 Evaluation of protein leakage from *A. fumigatus* exposed to caspofungin

Conidia of *A. fumigatus* were inoculated as described in section (2.7.2). Wet Hyphae mass (1.5 gram) was resuspended in 25 ml of PBS and incubated with drug in presence of positive control. The quantity of protein release from the wet hyphae mass was determined for each sample using the Bradford assay (Bio-Rad), with BSA (Sigma Aldrich) as reference standard (Figure 2.9).

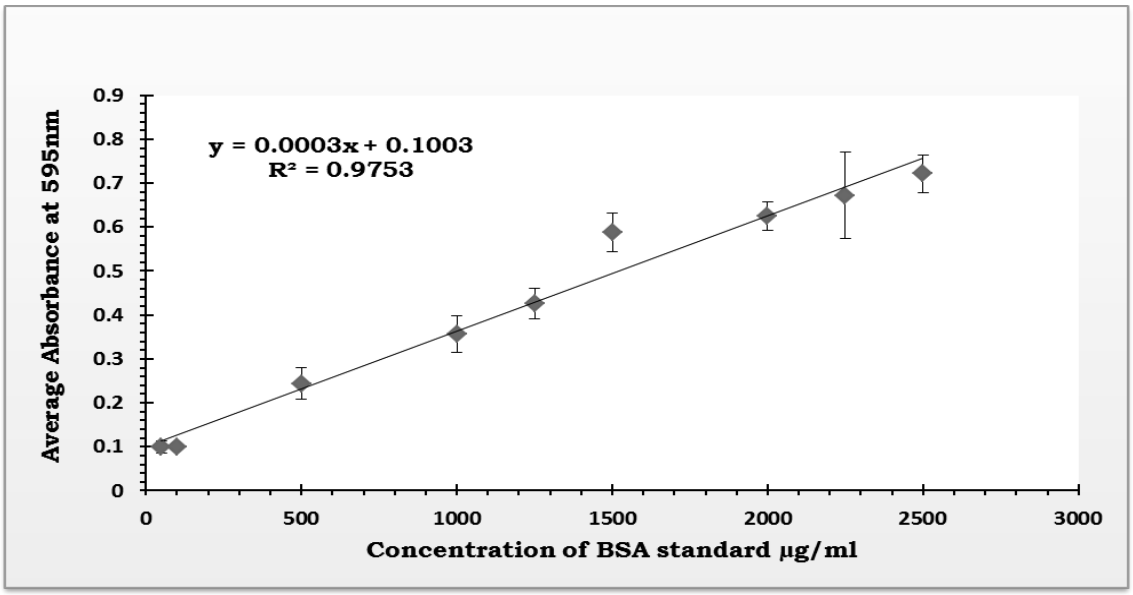


Figure 2.9: Standard curve of Bovine serum albumin used to determine unknown protein concentrations

2.23.2 Extraction and Evaluation of protein released from *A. fumigatus*

Conidia of *A. fumigatus* were inoculated in 100 ml RPMI1640 media supplemented with (5%) (v/v); FBS, to densty of $5x10^6$ cell/ml and incubated for 96 hours at 37 ^oC. cells were then harvested and washed twice with sterile PBS. Cell pellets were collected using Mira-cloth; all samples were weighed and adjusted to 1.5 g (wet weight). Cells were re-suspended in either PBS (5 ml), DMSO (5% (v/v); 5 ml) or caspofungin (0.1 or 1.0 µg/ml; 5 ml) and incubated at 37°C for 4 hours. Hyphae were harvested and the supernatants were placed on ice with protease inhibitors (100 µl pepstatin A (1 mg/ml; Sigma-Aldrich), 100 µl aprotinin (1 mg/ml; Sigma-Aldrich) and 100 µl phenylmethanesulfonyl fluoride (PMSF; 1 mM; Sigma-Aldrich)). Samples were diluted 1 in 10 in 100% (v/v) Trichloroacetic acid (TCA; dissolved in sterile distilled water; Merck) and placed at 4°C overnight to precipitate protein. Samples were centrifuged at 17,950 x g for 30 minutes at 4°C (Eppendorf centrifuge 5417R). Protein pellets were re-suspended in ice-cold acetone (300 µl; Sigma-Aldrich) and held at -20°C for at least 1 hour to further precipitate protein. Protein was centrifuged at 17,950 x g for 30 minutes at 4°C (Eppendorf centrifuge 5417R) and the acetone was discarded.

2.23.3 Assessment of protein leakage from *A. fumigatus* using the Bradford assay

Bovine Serum Albumin standards (5 - 60 µg ml-1), were made in 800 µl deionised water and 200 µl Biorad Bradford protein assay reagent (Bio-Rad Munich Germany) and read on a spectrophotometer at 595 nm (Eppendorf Biophotometer). A standard curve was prepared. Bradford protein assay reagent was prepared by diluting the stock 1/5 using deionised water. Protein samples were added (20 µl) to 980 µl of Bradford protein assay reagent. The samples were inverted, allowed to stand for 5 minutes and read spectrophotometrically.

2.24 Solutions and buffers used for 1D and 2D SDS-PAGE

2.24.1 Coomassie Blue Stain Solution:

Brilliant Blue R 2 g

Methanol 450 ml

Acetic acid 100 ml

Bring to 1 L Distilled water

2.24.2 Coomassie Destain Solution:

Acetic acid 100 ml

Methanol 200 ml

Distilled water 700 ml

2.24.3 IEF Equilibration (Reducing) Buffer

30% (v/v) Glycerol (150 ml)

2% (w/v) SDS (10 g)

6M Urea (180.1 g)

50mM Tris HCl (3.94 g)

Up to 500ml distilled water adjust pH to 6.8

Aliquot into 20ml (in 50 ml falcon tubes)

Store at -20 ^{o}C

When using, add 2% DTT (0.4 g), for reducing

(2.5% IAA (0.5 g) + few grains of Bromophenol Blue) for alkylation

2.24.4 IEF Buffer

8 M Urea (48 g)

1% (v/v) Triton X100 (1 ml)

4% (w/v) Chaps (4 g)

10 mM Tris HCl (0.158 g)

2 M Thiourea (15.22 g)

Bring up to 100 mls of sterile distilled water

Aliquot into 2 ml samples and stored at -20 ^{o}C

When using, add 65 mM DTT (0.2 g) and 0.8% (v/v) Ampholytes (2 µl)

2.24.5 Running Buffer 10X = Electrode Buffer

Trizma base	30 g
Glycine	144 g
SDS (sodium lauryl sulphate)	10 g

Add distilled water to make up 1 L,

pH should be 8.9

Stored at room temp

1:10 Dilution in distilled water = 1X

2.24.6 1% Agarose (for sealing strips):

1 g Agarose

100 ml 10X Running Buffer

Few grain of Bromophenol Blue

Heat until boiling and sets when cool

2.24.7 200 mM EDTA Solution:

EDTA (14.89 g)

Make up to 200ml with Distilled water

Adjust pH to 7.2

Dissolved with NaOH tablets and autoclaved

2.24.8 5X Sample Buffer for 1-D SDS–PAGE.

SDS	2.0 g
DTT	1.54 g

Take 2 ml of (0.2 % (w/v) Bromophenol blue (0.1 g) + 50% (w/v) Sucrose (25 g) make up to 50 ml H2O)

3 M Tris HCl (pH 6.7)	2.1 ml
200 mM EDTA (pH 7)	0.5 ml

Add deionized water- make up to 20 ml

Aliquot to 500 microliter

Store at -20 ^{o}C

2.24.9 Tris–HCl 1.5 M

Tris – HCl (1.5 M) was prepared by dissolving 11.8 g Trizma Base (Tris Base) in 50 ml deionised water and adjusted to pH 8.9. Following pH adjustment 1.5 M Tris – HCl was filter sterilised through a 0.22 μM cellulose filter (Millipore) and stored at 4ºC.

2.24.10 Tris – HCl 0.5 M

0.5 M Tris-HCl was prepared by dissolving 3.94 g Trizma Base in 50 mls deionised water and adjusted to pH 6.8. Following pH adjustment 0.5 M Tris– HCl was filter sterilised through a 0.22 μM filter (Millipore) and stored at 4ºC.

2.24.11 Ammonium Persulphate (APS) 10%

APS (10% w/v) was prepared by adding 0.05 g APS into 0.5 ml of deionised water and vortexing to dissolve completely. APS (10% w/v) was prepared fresh every day and kept on ice when not in use.

2.24.12 Sodium Dodecyl Sulphate (SDS) 10% w/v

Sodium Dodecyl Sulphate (SDS), (10% w/v) was prepared by adding 10 g SDS to 100 mls of deionised water and left to stir until all SDS had been solubilised. The solution was stored at room temperature after filtration through a 0.22 μM filter.

2.25 Preparation of SDS–PAGE minigels

All glass plates were washed in warm soapy water and cleaned thoroughly with 70% ethanol prior to use in SDS–PAGE minigels, (Mini-Protean II gel casting apparatus). A 12.5% acrylamide gel was normally used in experimental procedure. The leakage protein dimensional gels were: (8 cm x 6.5 cm x 1 mm). The separating gel separates the proteins from each other depending on size therefore aiding visualisation. All gels were used within 24 hours of setting and were allowed to polymerise for at least one hour prior to being loaded. Solution component of separating gel 12.50%, 1.5 M Tris-HCl 3 mls, Deionised water 3.8 mls, 30% Acrylamide 5 mls 10% v/v SDS 120 µl, 10% v/v APS 75 µl, TEMED 3 µl. These volumes were sufficient to make 3 minigels.

2.25.1 Loading and voltages of 1-D SDS-PAGE

Protein was extracted from *A. fumigatus* hyphae (grown with and without Caspofungin) using method described in Section (2.28). Bradford method was used to calculate the concentration of protein as described in Section (2.29.2) and equal concentrations were loaded into the wells of separating gel and run at 50 Volts for 10 minutes followed by 80 Volts for 120 minutes on (Bio-Rad, CA, USA). Gels were stained with Coomassie Blue solution for 5 hours and destained with Coomassie Destaining solution for 8 hours.

2.25.2 Preparation and analysis of 2-D SDS-PAGE

Protein was extracted from *A. fumigatus* cultures as described in section (2.28). Using the Bradford method, a solution of 250 µg/ml protein was prepared and then precipitated by adding three times the volume of ice cold acetone and incubated overnight at -20 ^{o}C. The precipitated protein was collected by centrifugation (12,400 xg for 30 minutes at 4 ^{o}C (Eppendorf centrifuge 5417R). The acetone was removed and the pellet was allowed to air dry. The dried precipitated protein was resuspended in 250 µl IEF buffer (2.30.4) with 0.8% (v/v) IPG buffer, pH 3-10 (G.E. Healthcare BioSciences AB, Uppsala, Sweeden;) and few grain of bromophenol blue. The solution was applied to a 13 cm Immobiline^{TM} DryStrip pH 3-10 (G.E. Healthcare) and iso-electric focusing was

performed on an Ettan IPGphor II (Amersham Biosciences, NJ, USA) system using the following program.

1. Step and Hold	50 Volts	12 hours
2. Step and Hold	250 Volts	15 minutes
3. Gradient	8000 Volts	5 hours
4. Step and Hold	8000 Volts	8 hours

Following IEF IPG strips were frozen at -80ºC or were transferred to equilibration. Strips were initially equilibrated in 5 ml reducing equilibration buffer for 15 minutes. Strips were transferred to the 5 ml alkylation buffer for 15 minutes. Following equilibration strips were rinsed in 1X electrode running buffer. Strips were placed on top of SDS-PAGE gels and sealed with 1% w/v agarose sealing solution. The top of the gel was sealed with molten cooled sealing solution and allowed to set. The second dimension of protein separation was achieved by placing the gels in ProteanXi-II vertical electrophoresis cells as per manufacturer's instructions. The inner chamber was filled to the top with 5X electrode running buffer while the outer chamber was filled with 2X running buffer. Gels were initially electrophoresed for 1 hour at 50 volts and at 80 volts for the remaining time. Gels were monitored regularly to assess the level of electrophoresis and were transferred to a staining dish of Coomassie Blue solution and destained with destaining solution, until the blue background was removed.

2.26 In gel trypsin digestion and bioinformatics analysis of LC/MS results.

The method of Shevchenko *et al.,* (2006) was used in processing of bands and spots for LC-MS analysis with slight modifications. LC/MS grade Acetonitrile100 % (v/v) was used to wash tubes and pipette tips. Following this 30-minute incubation period 500µls of Acetonitrile was added for 10 minutes shaking at room temperature. Samples were centrifuged at 20,000 xg for 10 minutes and the supernatant was removed. Gel pieces were placed in 40 µl of 1:1 ammonium bicarbonate (100 mM): acetonitrile (100 % (v/v) and incubated with occasional vortexing for one hour. The supernatant was removed and 40 µl of 100% (v/v) Acetonitrile was placed over gel pieces and incubated until gel became

white and shriveled. Acetonitrile was removed and gel pieces were covered with 500 µl 50mM Ammonium Bicarbonate for 5 minutes. An equal volume of 100% (v/v) Acetonitrile was added and the pieces were incubated for a further 15 minutes. Supernatant was removed and pieces were again covered with acetonitrile until gel became white and shrinked. Trypsin (20 µg-Promega) was reconstituted in 100 µl of reconstitution buffer (10 mM Ammonium Bicarbonate containing 10% Acetonitrile). This was aliquoted into 10 x10 µL aliquots and to each of these 500 µl of 50 mM Ammonium Bicarbonate was added. The trypsin solution (50 µl) was added to each gel piece. This was incubated at 4°C for 1 hour and at 37°C overnight. For peptide recovery samples were centrifuged at 20,000xg for 10 minutes and supernatant was transferred to a fresh 1.5ml tube. Extraction buffer (1:2 5% Formic Acid (v/v): Acetonitrile) was added to the gel pieces (50 µl) and incubated at 37°C for further 15 minutes. Samples were centrifuged at 20,000 xg for 10 minutes and added to the supernatant. Extracts were dried in a vacuum centrifuge overnight. Dried peptides were resuspended in 20 µl of 0.1% Formic Acid and sonicated for 2 minutes. Samples were filter sterilized and supernatants were added to mass spectroscopy vials (Agilent Technologies, USA). Samples were analysed on a 6340 Ion Trap LC/MS spectrometer (Agilent Technologies) using BSA as external reference standards. Resulting data were analysed using the mascot search engine, (www.matrixscience.com). The mass error tolerance was 1 Da allowing for a maximum of no more than 2 missed cleavages. Verification of protein sequences was confirmed by blasting the protein sequence on the Uniprot, (www.uniprot.org), and NCBI, (www.ncbi.nlm.nih.gov), databases. Identified proteins are listed by their gi number as accessed through www.ncbi.nlm.nih.gov.

2.27 Whole cell protein extraction for enzymatic assay of *A. fumigatus*

A. fumigatus hyphae (96 hr culture, 1 g) were exposed to caspofungin (0.1 µg/ml) for 15, 30 or 60 minutes or to hydrogen peroxide (0.5 mM) for 15 minutes. At each time point hyphae were harvested, washed in PBS and resuspended in 8 ml of lysing buffer (4

ml Tris-HCl (100 mM, pH 7.5); 4 ml EDTA (1mM, Sigma-Aldrich); 100 µl Pepstatin A (1 mg/ml, added fresh); 100 µl Aprotinin (1 mg/ml, added fresh); 100 µl PMSF (1 mM, added fresh) and 5 mM DTT (added fresh)). Cells were disrupted by vigorous vortexing on ice with glass beads (4 g, size: 425-600 µm, Sigma-Aldrich). Cellular debris and glass beads were removed by centrifugation (250 x g for 5 minutes at 4°C, Eppendorf centrifuge 5417R) and the supernatant was used for evaluation of enzymatic activity.

2.28 Assessment of Enzymatic activity

2.28.1 Superoxide dismutase activity (SOD) Assay

Superoxide dismutase activity was measured using a SOD Assay Kit (Fluka Biochemika) in accordance with the manufacturer's instructions, using a concentration of 1 µg/µl of protein extract as described in (R. Rowan *et al*, 2010). The SOD activity was calculated by a tetrazolium salt that produce a water-soluble formazon dye upon reduction with a superoxide anion. The absorbance at 450 nm was read using a microplate reader (Synergy HT, Bio-Tek) and the following formula was employed to determine SOD activity;

$$\text{SOD Activity} = \frac{[(\text{Ablank1} - \text{Ablank3}) - (\text{Asample} - \text{Ablank2})]}{[(\text{Ablank1} - \text{Ablank3})]} \times 100$$

2.28.2 Glutathione reductase activity (GLR) Assay

For analysis of the glutathione reductase activity of cells the method described by Foster and Hess (1980) was used. It is based upon the reduction of glutathione by NADPH in presence of glutathione reductase. The absorbance were read at 340 nm for 2 minutes at 20 second intervals (Cary varian UV-Visible Spectrophotometer). The GLR activity was calculated using the following equation:

GLR (units/ µl) =	**(Rate of change of sample – Rate of change of blank)**	
	6.22 mM/cm X Concentration of protein (µg/µl)	

2.28.3 Catalase activity Assay

Catalase activity was measured as described by Larsen and White with slight modifications. *A. fumigatus* hyphae of 96 old culture was used. Caspofungin 0.1 µg/ml or H_2O_2 (0.5 mM, 15 min) was added to the medium and the culture grown for a further ½, 2 or 4 hours. Protein was extracted as in section 2.28 and used immediately. A 100 µl of protein extract (7.1 mg/ml) was added to 1.8 ml of 17 mM H_2O_2 in sterile tube. Mixture was left to rest at room temperature for 15 min then suspension was centrifuged at 10,000 xg (Eppendorf centrifuge 5417R) for 1 min to stop reaction. The supernatant was placed in a clean quaetz cuvette. The absorbance at 240 nm was obtained on a Beckman DU640 spectrophotometer. A blank consisted of 17 mM H_2O_2.

Chapter 3

INTERACTION OF ENVIRONMENTAL FACTORS WITH THE ANTIFUNGAL ACTIVITY OF CASPOFUNGIN AGAINST *C. ALBICANS* AND *A. FUMIGATUS*

3.1 Characterisation of factors affecting the response of *C. albicans* to caspofungin

Microdilution assays were performed using various concentrations of caspofungin (0.02 to 10 µg/ml) in order to establish the response of the fungus to the drug when grown under different culture conditions. The method employed is described in section 2.9. The microdilution assay employed 5 x 10^6 *C. albicans* cells/ml and was performed under static conditions in a total volume of 200 µl per well in a 96 well plate as recommended by the CLSI. For each experiment the growth in the presence of caspofungin was calculated and MIC_{50} and MIC_{90} values were determined using Microsoft excel 2010.

3.1.1 Effect of culture media on susceptibility of *C. albicans* to caspofungin

In this experiment five different types of culture media were employed in order to establish whether the culture medium affected the susceptibility of *C. albicans* to caspofungin. The susceptibility assay was performed using Antibiotic medium 3, Sabourud dextrose broth, YEPD broth, RPMI1640 and Minimal essential media (MEM) and the assay was performed as described previously. After 24 hour of incubation at 30 ^oC

all the plates were read on the microplate reader (Synergy HT, Bio-Tek) at 450nm, the absorbances were recorded and the growth was expressed as a percentage of the control (Figure 3.1).

It is clear from the results that caspofungin inhibits the growth of *C. albicans* in all types of media but high MIC_{90} values (7.5 and 5.0 μg/ml) were recorded in RPMI1640 and YEPD media (Table 3.1). It will be noted that there is a three-fold (0.15 – 0.45 μg/ml) difference in the MIC_{50} calculated in the different growth media. There is a big variation in the response of *C. albicans* to 0.05 and 1.25 μg/ml caspofungin when grown in the different media.

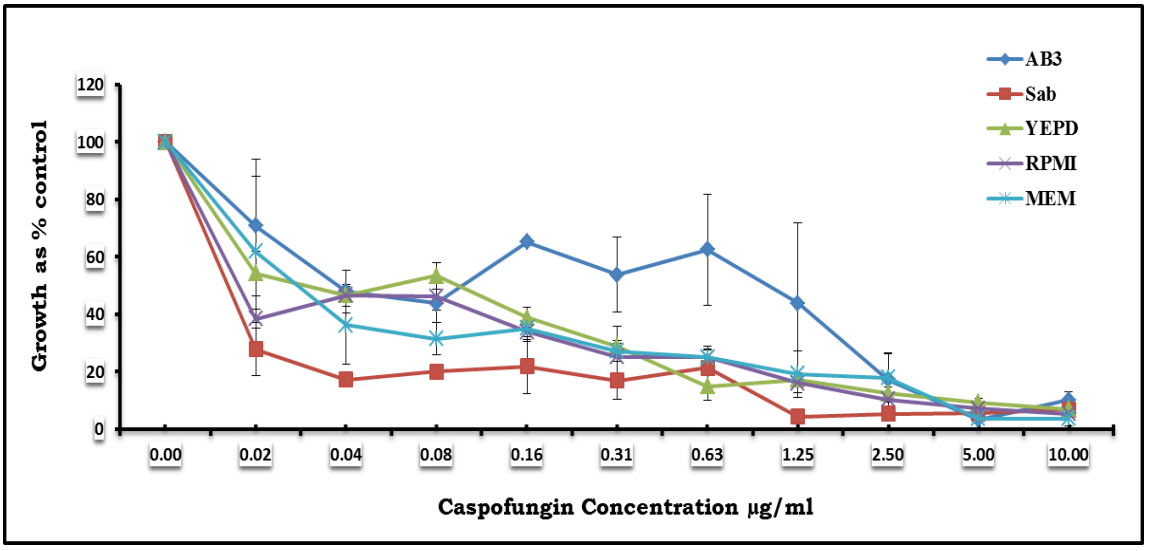


Figure 3.1 The effect of culture media on the susceptibility of *C. albicans* to Caspofungin.

The assay was performed as in section 2.10. All values are expressed as a percentage of the relevant control and are the average of three independent experiments.

Media used	MIC $_{50}$ µg /ml	MIC $_{90}$ µg/ml
Antibiotic medium 3	0.045	3.50
Sabourud Dextrose broth	0.015	1.25
YEPD broth	0.030	7.50
RPMI1640	0.015	5.00
MEM	0.035	4.25

Table 3.1 MIC_{50} and MIC_{90} values obtained in each culture medium.
Caspofungin concentrations required to inhibit the *C. albicans* growth by 50% and 90% were determined relative to control culture.

3.1.2 Effect of BSA on the susceptibility of *C. albicans* to caspofungin

The effect of protein on the activities of echinocandins has been evaluated against *C. albicans*. The relatively unpredictable nature of the effect of serum on antifungal activity has been demonstrated. A previous study described the effect of protein binding on the activities of caspofungin and observed that serum modestly affects the MICs of caspofungin (Odabasi *et al.,* 2007).

Bovine serum albumin (25, 50, 100 and 200 µg/ml) was added to medium prior to inoculation with *C. albicans* cells. BSA was added after AB3 media had been sterilized by autoclaving because it readily coagulates with heat sterilisation. The method is described in section 2.13. The results demonstrated that the presence of BSA buffers the effect of caspofungin on *C. albicans* (Figure 3.2). For example at a caspofungin concentration of 10 µg/ml the culture supplemented with 25 µg/ml BSA showed approximately 15% growth but the culture supplemented with 50, 100 or 200 µg/ml BSA showed approximately 40% growth of *C. albicans* cultures.

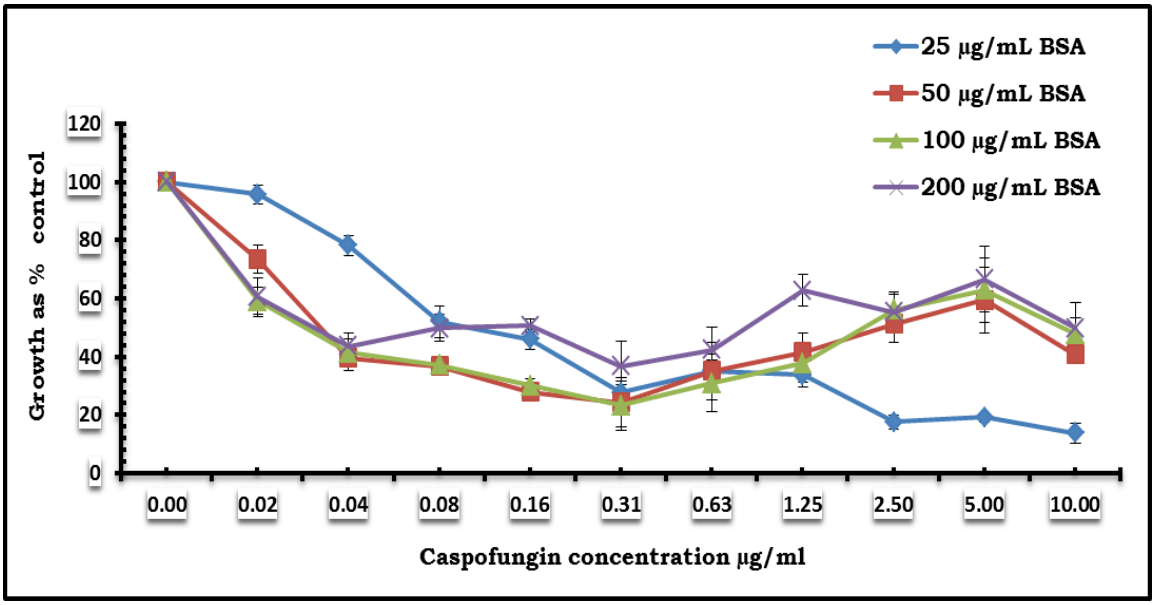


Figure 3.2 The effect of bovine serum albumin (BSA) on the susceptibility of *C. albicans* to caspofungin using AB3 media.

Average of three independent experiments.

Assay	MIC $_{50}$ µg /ml	MIC $_{90}$ µg /ml
25 µg/ml BSA	0.080	10
50 µg/ml BSA	0.035	> 10
100 µg/ml BSA	0.025	> 10
200 µg/ml BSA	0.030	> 10

Table 3.2 MIC_{50} and MIC_{90} values obtained from cultures supplemented with different concentrations of serum.

Caspofungin concentration required to inhibit the *C. albicans* growth by 50% and 90% were determined relative to control culture.

3.1.3 Effect of Glucose on the susceptibility of *C. albicans* to Caspofungin

Glucose supplementation of media has a significant influence on the growth of *C. albicans*. The activity of the antifungal agent miconazole against *C. albicans* is affected by increasing concentrations of glucose (Rodaki *et al.,*2009). Rodaki *et al.,*(2009) demonstrated the induction of a number of genes associated with the oxidative stress response in *C. albicans* under high glucose culture conditions, which contributed to the increased resistance to Miconazole. To investigate whether increasing glucose concentrations affected the response of *C. albicans* to caspofungin, AB3 medium was supplemented with different concentrations of glucose prior to a microdilution assay as in section 2.11. The results demonstrate that the greatest growth of *C. albicans* over the caspofungin range 0.02 – 1.25 μg/ml occurred in those cultures supplemented with 2, 5 or 7.5% glucose. Table 3.3 shows a significant increase in MIC_{50} and MIC_{90} values of caspofungin, when cells were grown in glucose supplemented medium.

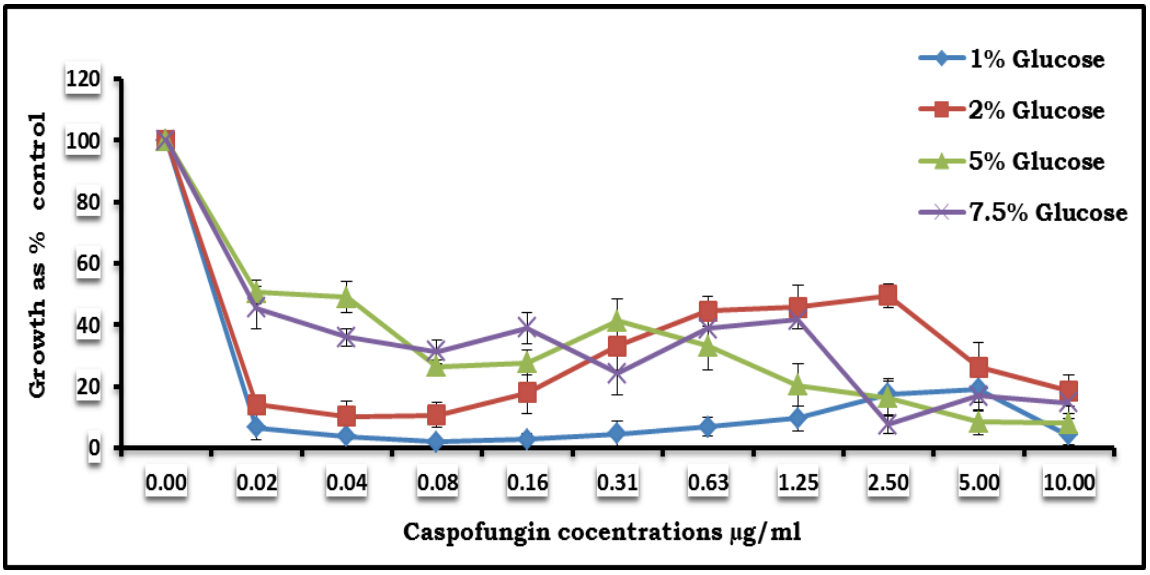


Figure 3.3 The effect of different glucose concentrations on the susceptibility of *C. albicans* to Caspofungin.

Average of three independent experiments.

% Glucose	MIC $_{50}$ µg/ml	MIC $_{90}$ µg /ml
1 %	0.010	0.020
2 %	0.015	0.050
5 %	0.040	5.000
7.5 %	0.020	2.500

Table 3.3 MIC_{50} and MIC_{90} values from each graph of varying percentage of glucose in AB3 media were determined.

Caspofungin concentration required to inhibit the *C. albicans* growth by 50% and 90% were determined relative to control culture.

3.1.4 Effect of different carbon source on susceptibility of *C. albicans* to caspofungin

In this experiment different carbon sources were employed to establish whether these had an effect on the susceptibility of *C. albicans* to caspofungin. AB3 medium was supplemented with 2% (w/v) lactose, glycerol, sucrose, galactose, maltose or glucose and used to determine the effect of carbon source on the susceptibility of *C. albicans* to caspofungin. *C. albicans* was grown to stationary phase with 2 % of each sugar and *C. albicans* (5 x 10^6 cells/ml) were inoculated in 96 well plates containing serial dilution of caspofungin in range from 0.02 to 10 μg/ml in the same culture medium. Plates were incubated at 30^{o}C for 24 hours. The MIC_{50} and MIC_{90} for each experiment were determined. The results revealed that cultures grown in medium supplemented with galactose or sucrose showed the highest MIC_{90} values (Figure 3.4).

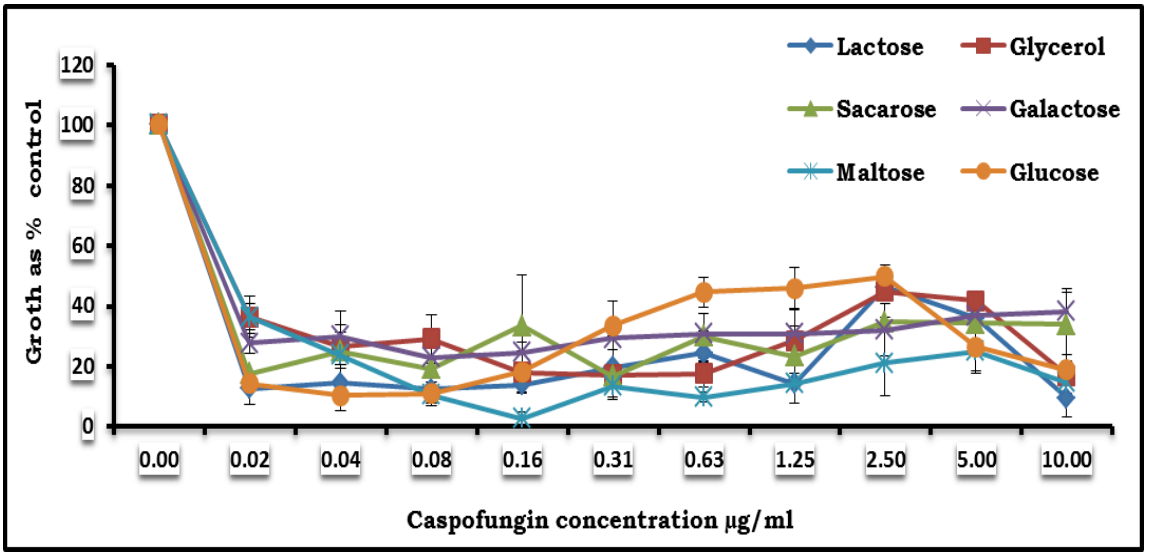


Figure 3.4 The effect of varying carbon source on the susceptibility of *C. albicans* to caspofungin.

AB3 media was supplemented with different carbon sources and used in a micro-dilution susceptibility assay to determine the effect of carbon source on the antifungal activity of caspofungin. Average of three independent experiments.

Type of Carbon used	MIC $_{50}$ µg/ml	MIC $_{90}$ µg/ml
Lactose	0.010	9.00
Glycerol	0.015	> 10
Sucrose	0.010	> 10
Galactose	0.012	> 10
Maltose	0.015	0.10
Glucose	0.015	0.050

Table 3.4 Determination of MIC_{50} and MIC_{90} values from graph of each experiment of AB3 media supplemented with 2% of different carbon source.
Caspofungin concentration required to inhibit the *C. albicans* growth by 50% and 90% were determined relative to control culture.

3.1.5 Effect of pH on the susceptibility of *C. albicans* to caspofungin

The pH range 5 to 7 was recommended by Merck Research Laboratories for use in caspofungin toxicity assays (Ripeau *et al.,* 2002). Cultures of *C. albicans* were grown in AB3 media to stationary phase at 30 ^{o}C and 200 rpm. Cells (5 x 10^{6} cells/ml) were inoculated into a 96 well plates containing AB3 media of varying pH (3, 5, 7 or 8) in serial dilutions of caspofungin (0.02 to 10.0 μg/ml) and incubated at 30^{o}C for 24 hours. The optical density (OD) was recorded at 450 nm as described in section 2.12.

The MIC value of caspofungin required to inhibit the growth of *C. albicans* by 90% or 50% relative to the growth of the control was determined. From Figure 3.5 it can be seen that the maximum susceptibility of *C. albicans* to 0.02 – 0.63 μg/ml caspofungin occurred at pH 5. In contrast high growth of *C. albicans* was present in medium adjusted to pH 3, 7 or 8 over the same caspofungin range.

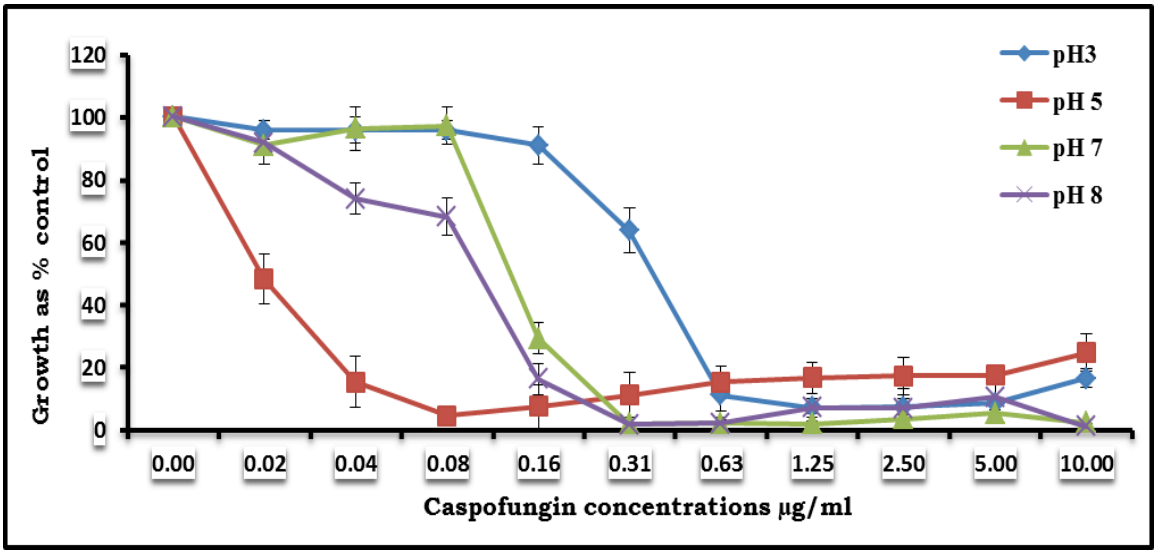


Figure 3.5 The effect of medium pH on the antifungal activity of caspofungin.

C. albicans was grown to stationary phase in AB3 media of varying pH and subjected to a micro-dilution susceptibility assay to determine the effect of pH on the antifungal activity of caspofungin. Average of three independent experiments.

Medium pH	MIC $_{50}$ µg/ml	MIC $_{90}$ µg/ml
pH 3	0.315	1.250
pH 5	0.019	0.075
pH 7	0.125	0.250
pH 8	0.100	0.215

Table 3.5 Determination of MIC_{50} and MIC_{90} values from each graph of varying pH. The lowest concentration of caspofungin required to inhibit growth of *C. albicans* by 50% and 90% relative to the growth of the control.

3.1.6 Summary

The results presented here indicate that the susceptibility of *C. albicans* to caspofungin is affected by a variety of factors including the type of medium used in the assay, the nature of the carbon source, the glucose content, the pH and the protein content of the culture medium.

3.2 The effect of environmental conditions on protein expression in *C. albicans*.

C. albicans must adapt to, and thrive in, a broad range of environmental conditions especially when confronted with environmental stresses such as low or high pH. Previous studies have identified more than 500 genes that are differentially regulated in response to pH and also contribute to adaptation to environmental pH (Davis *et al.,* 2003). The aim of the work described here was to analyse the changes in the expression of proteins when *C. albicans* was exposed to different environmental conditions, which might contribute to the altered susceptibility of *C. albicans* to caspofungin. It is recognized that these conditions (e.g. pH, protein concentration, glucose concentration) might also affect the activity of caspofungin but this analysis was confined to assessing if these factors affected the ability of *C. albicans* to tolerate exposure to the drug.

Cells of *C. albicans* were cultured in AB3 media and grown to the stationary phase. Whole cell protein was extracted as described in section 2.27. Table 3.6 shows the proteins identified following 1-D electrophoresis of protein samples from *C. albicans* cells incubated under different environmental conditions. Protein bands were excised, digested and identified by LC-MS. For identification, MASCOT analysis was used and scores greater than 62 were accepted. Table 3.6 illustrates all the peptides identified by LC-MS and the molecular function of each protein. In total, 21 spots were successfully identified and these were found to be up-regulated or down-regulated under different environmental condition. Densitometry was subsequently carried out on bands corresponding to proteins of interest to investigate the relative changes (increase or decrease) in expression of the

identified protein. The function and information about these proteins was determined using the UniProt data bank available at http://www.uniprot.org and / or the InterPro website available at http://www.ebi.ac.uk/interpro/ unless otherwise stated.

Band	Score	Name of protein	PI Value	Sequences	Accession #	Nominal mass (Mr)	Seq Cavarage	Source
1A	691	Hypothetical protein CaO19.8025	5.54	10(7)	XP_711912	47202	29%	*Candida albicans SC5314*
2A	471	Glyceraldehyde-3-phosphate dehydrogenase	6.61	8(4)	XP_719909	35811	34%	*Candida albicans SC5314*
3A	222	Eno1 enolase, partial	5.25	5(1)	XP_003866483	42216	15%	*Candida orthopsilosis Co 90-125*
4A	242	Enolase 1 OS	5.54	7(1)	Q59QC3	47202	25%	*Candida albicans SC5314*
5A	59	Pyruvate decarboxylase, putative	5.39	1(1)	XP_821342	62645	3%	*Candida dubliniensis CD36*
6A	282	29 kDa IgE-binging protein	4.79	7(1)	AAN11300	25934	27%	*Candida albicans*
7A	200	Cyclophilin type peptidyl-prolyl cis-trans isomerase	7.74	3(1)	XP_721313	17680	14%	*Candida albicans SC5314*
8A	227	Phosphoglycerate mutase	5.91	6(1)	EMG50907	27411	29%	*Candida maltosa Xu316*
9A	236	Alcohol dehydrogenase I	8.44	8(0)	EEQ46516	46598	31%	*Candida albicans WO-1*
10A	164	Adenosylhomocysteinase	5.35	4(1)	EMG46558	49532	9%	*Candida maltosa Xu316*
11A	145	Fructose-bisphosphate aldolase, partial	5.43	2(1)	EMG48311	39332	9%	*Candida maltosa Xu316*
12A	148	Likely mitochondrial malate dehydrogenase	5.73	2(2)	XP_722820	34823	10%	*Candida albicans SC5314*
13A	124	Fructose-1,6-bisphosphate aldolase, putative	5.69	2(1)	XP_002419830	39374	12%	*Candida dubliniensis CD36*
14A	95	NADH-cytochrome b5 reductase precursor, putative	8.26	2(1)	XP_002420980	33589	14%	*Candida dubliniensis CD36*
15A	184	ATP synthase beta chain, mitochondrial precursor	4.83	4(0)	EEQ45430	44512	18%	*Candida albicans WO-1*
16A	134	Potential oxidoreductase	6.04	3(1)	XP_720744	37611	16%	*Candida albicans SC5314*
17A	65	Enolase	5.26	1(1)	XP_001561265	47196	3%	*Botryotinia fuckeliana B05.10*
18A	302	Heat shock protein SSA4	5.06	8(2)	EEQ41915	69426	17%	*Candida albicans WO-1*
19A	271	Elongation factor 1-gamma 1	8.44	5(3)	EEQ44636	47077	14%	*Candida albicans WO-1*
20A	81	Conserved hypothetical protein	6.04	1(1)	EEQ46322	37584	5%	*Candida albicans WO-1*

Table 3.6 Identities of proteins observed to be altered in intensity following 1-D electrophoresis of samples from *C. albicans* cells incubated under different culture condition.

Bands showing changed intensity were excised, digested and identified by LC/MS as described. For identification a MASCOT score greater than 62 was used.

3.2.1 Effect of varying pH on protein expression in *C. albicans*

C. albicans was incubated in AB3 media of varying pH (3, 5, 7 and 9) and grown for 48 hour as described in section 2.7.2. One gram of cells (wet weight) was harvested and washed twice with PBS and the whole cell protein was extracted as in section 2.22. Protein concentration was evaluated using Bradford protein assay as described in section 2.23.3. One dimensional gel analysis of proteins was used as described in section 2.25.1. Equal concentrations of protein from cells grown in media of different pH were loaded into the wells of a 12.5% separating gel with a 5% stacking gel. Proteins were run for 10 min at 40 Volts followed by 120 minutes at 80 Volts. A staining solution of Coomassie Blue was used to stain the gels for 5 hours followed by destaining with destaining solution. The SDS-PAGE gel presented in Figure 3.6 shows the separation of proteins from *C. albicans* cultured in media of pH of 3, 5, 7 or 9. Proteins separated from different samples appeared to be either up-regulated or down-regulated across the pH range. The variation in protein expression may play a role in the susceptibility and response of *C. albicans* to a variety of conditions. In relation to pH 7, some proteins were up-regulated such as band 4A (Table 3.7) the Enolase 1 OS at pH 3, 5 and 9. Glyceraldehyde-3-phosphate dehydrogenase (Band 2A) at pH 9 was increased by 1.7 fold in expression. In addition to the role in glycolysis and an immunogenic protein, it also is involved in host interaction as evident by its binding to the extracellular matrix protein plasminogen (Kelly & Kavanagh, 2010). Enolase 1 OS (Band 4A) and hypothetical protein CaO19.8025 showed a change in expression at acidic pH 3 of 1.4 and 1.6 fold respectively and at pH 9 these proteins show 1.3 and 0.9 fold increase. Hypothetical protein CaO19.8025 (Band 1A, Table 3.7). At acidic pH values, *C. albicans* showed an increase in tolerance to caspofungin concentration range (0.02 to 0.6 μg/ml) (Figure 3.6). This correlates to the increase in the expression of hypothetical protein CaO19.8025 that may play role in the stress response.

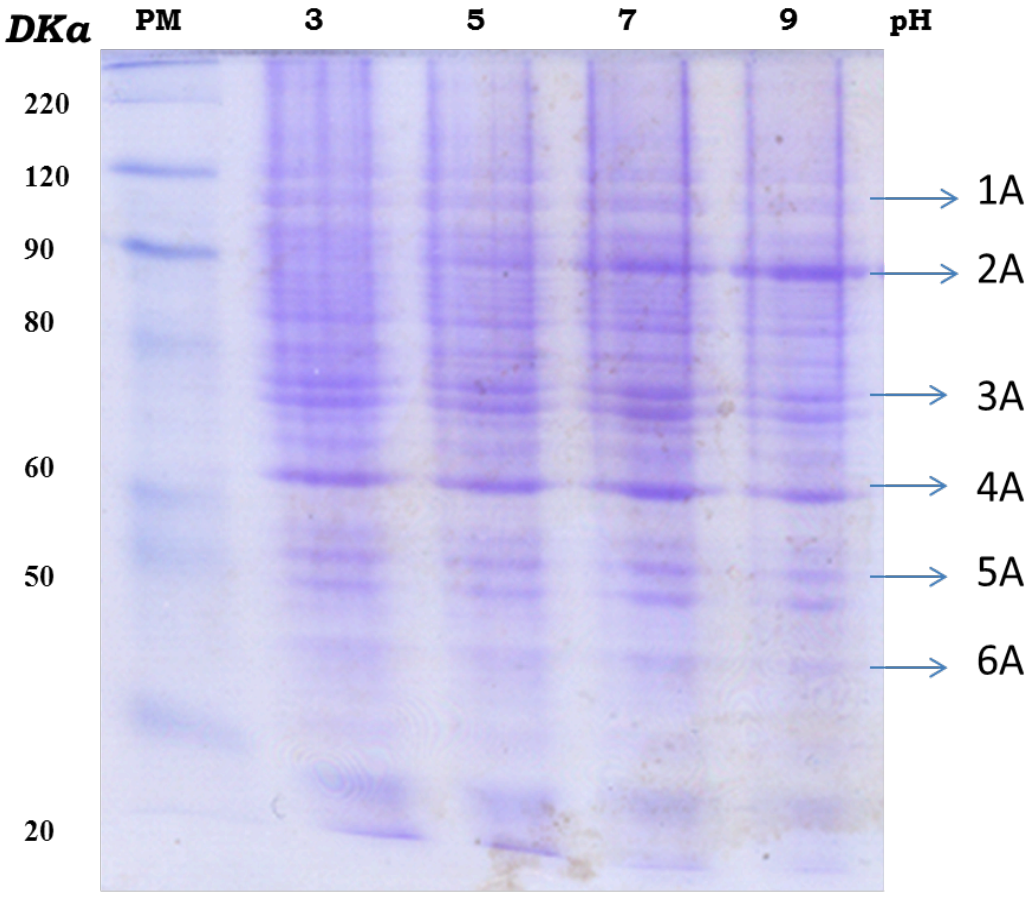


Figure 3.6 Coomassie stained SDS-PAGE gel (12.5%) of separated proteins from *C. albicans* inoculated in media of varying pH.

C. albicans was cultured in AB3 media of varying pH from 3 to 9. Protein was extracted from stationary phase cultures and 20 µg proteins were resolved by 1D SDS-PAGE.

Band	Name of protein	Fold Increase				Molecular function
		pH 7	pH 3	pH 5	pH 9	
1A	Hypothetical protein CaO19.8025	1	1.6	1.2	0.9	Annotation remark
2A	Glyceraldehyde-3-phosphate dehydrogenase	1	0.8	0.9	1.7	Glycolytic enzyme; changes with drug exposure
3A	Eno1 enolase, partial	1	0.8	1.2	1.1	Glycolysis
4A	Enolase 1 OS	1	1.4	1.5	1.3	Glycolysis
5A	Pyruvate decarboxylase, putative	1	0.9	1	0.9	Catalytic activity, magnesium ion binding
6A	29 kDa IgE-binging protein	1	0.7	0.9	1.2	Oxidative stress via Cap1

Table 3.7 Identity and fold change in expression of excised proteins from *C. albicans* cultured in media of varying pH.

Identified protein by LC-MS and molecular function was determined using the UniProt data bank. Average of two independent experiments.

3.2.2 Effect of BSA on protein expression in *C. albicans*

A previous study suggested that in *C. albicans* the cell wall could be associated with mediating the adhesion of fungal cells to serum, thus involved in contributing to the attachment of the microorganism to host tissues and to the dissemination of *Candida* infection (Gozalbo *et al.,* 1998). Other studies demonstrated an elevation of the MIC_{50} of caspofungin against some *Candida* spp, including *C. albicans* in the presence of human serum albumin (Paderu *et al.,* 2007; Odabasi *et al.,* 2007). A previous study suggested that the activity of caspofungin was reduced in presence of serum albumin due to the direct interference on the drug as a result of protein binding (Paderu *et al.,* 2007).

C. albicans was grown for 48 hours in AB3 media containing different BSA concentrations. Protein was extracted as described in section 2.22 and resolved by 1D SDS-PAGE. Protein bands which appeared to be either increased or decreased in expression were analysed and fold changes in intensity were recorded. The image of the SDS-PAGE gel in Figure 3.7 shows the separation of protein from *C. albicans* grown in AB3 medium supplemented with varying BSA concentrations. In comparison to the control, an up-regulation of protein appears at high concentrations of BSA. Six excised proteins from *C. albicans* grown in media of varying BSA concentrations were successfully identified by LC-MS. It was found that the intensity of protein expression increased especially when 100 µg/ml and 200 µg/ml of BSA were applied to culture media.

Band 7A (Table 3.8) was identified as cyclophilin type peptidyl-prolyl cis-trans isomerase, and the densitometry result showed the expression of this protein was up-regulated at high levels of BSA, with a 1.5 and 1.3 fold increase recorded at 100 µg/ml and 200 µg/ml of BSA respectively. Phosphoglycerate mutase and Adenosylhomocysteinase were identified from Band 8A and 10A and showed an up-regulation and down-regulation of protein expression of 2.4 and 0.8 fold, respectively.

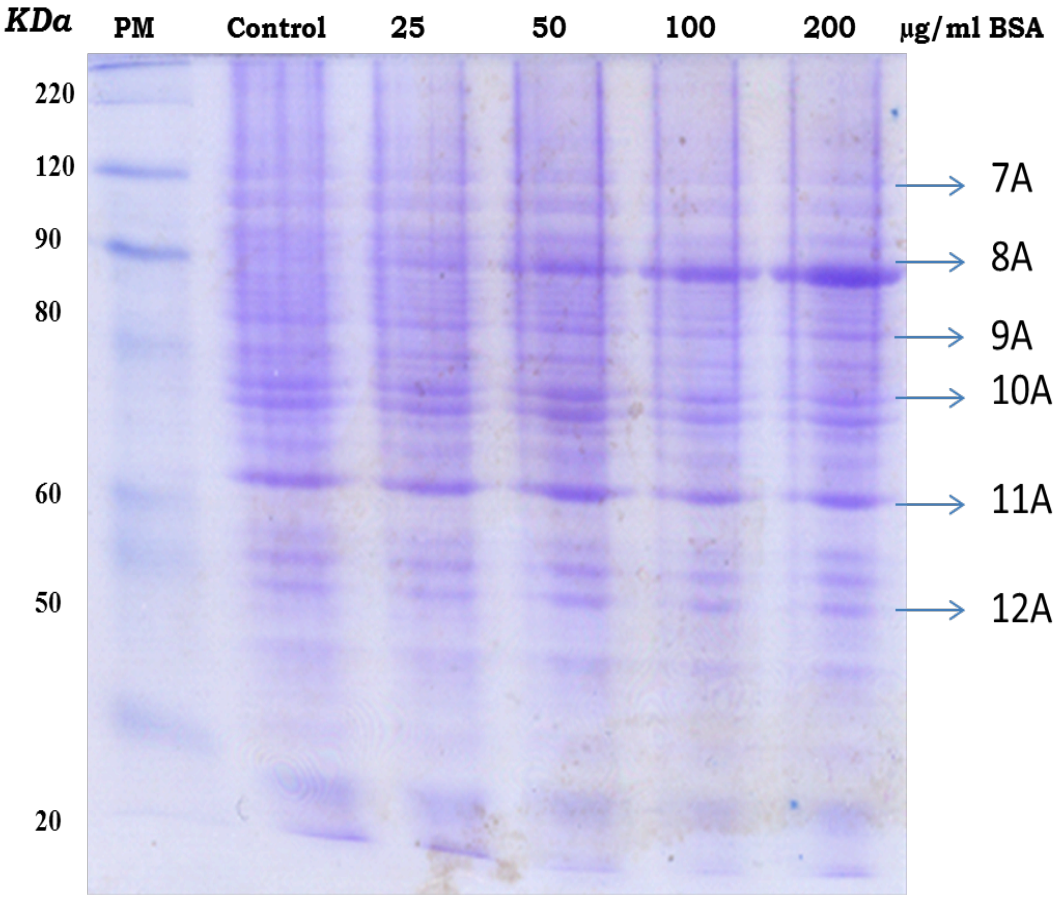


Figure: 3.7 Coomassie stained SDS-PAGE gel (12.5%) of separated proteins from *C. albicans* incubated in AB3 media supplemented with different concentrations of BSA.

Protein was extracted from stationary phase *C. albicans* culture grown in medium supplemented with different BSA concentrations. 20 µg of protein from each sample was loaded into each well of the gel.

Band	Name of protein	Fold Increase				Molecular function
		25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml	
7A	Cyclophilin peptidyl-prolyl cis-trans isomerase	1	1.7	1.5	1.3	Accelerate the folding of proteins
8A	Phosphoglycerate mutase	1	1.4	1.6	2.4	Isomerase, Interconversion as the primer
9A	Alcohol dehydrogenase I	1	1.5	1.1	1.4	Oxidoreductase, Allergen
10A	Adenosylhomocysteinase	1	1.7	0.9	0.8	Control of methylations, competitive inhibitor
11A	Fructose-bisphosphate aldolase, partial	1	1.5	0.9	1.3	Glycolysis, lyase
12A	Likely mitochondrial malate dehydrogenase	1	1.3	0.8	1.4	Oxidoreductase

Table 3.8 Band ID of excised proteins from *C. albicans* cultured in media of varying BSA. Identified protein was carried out by LC-MS and molecular function was determined using the UniProt data bank. Control = 0 µg/ml BSA.

Average of two independent experiments

3.2.3 Effect of Glucose on the protein expression of *C. albicans*

A number of studies have reported that a high level of glucose resulted in the induction of a number of genes in *C. albicans* involved in the osmotic and oxidative stress response (Rodaki *et al.,* 2009). As a result, an increase in the resistance of *C. albicans* to miconazole was observed. Interestingly Kelly *et al.,* (2009) demonstrated that caspofungin caused an oxidative and osmotic stress response in *C. albicans*.

C. albicans was grown in AB3 medium containing different BSA concentrations. Protein was extracted as described in section 2.29 and resolved by 1D SDS-PAGE. Protein bands were found to be either increased or decreased in expression and changes were recorded. The image of the SDS-PAGE gel in Figure 3.8 shows the separation of protein from *C. albicans* grown in AB3 medium supplemented with varying glucose concentrations. In comparison to the control an up-regulation of protein appears at high levels of glucose. Eight excised proteins from *C. albicans* grown in media of varying glucose concentration were successfully identified by LC-MS. Table 3.9 shows the down-regulation of proteins at higher glucose concentrations in comparison to 1% glucose (Lane 1).

Densitometry recorded the highest increase in expression of protein band 14A and 17A (Table 3.9) which represent NADH-cytochrome b5 reductase and enolase; these proteins demonstrated 1.8 fold increases in expression at 10% glucose in comparison to *C. albicans* grown at 1% glucose level. A heat shock protein SSA4 (Band 18A) showed an increase in expression of 1.7 fold in cells grown at the 10% glucose level.

At low glucose concentrations, *C. albicans* was most susceptible to caspofungin. Previous results demonstrated that a number of genes involved in *C. albicans* response to osmotic and oxidative stress were induced at high levels of glucose (Rodaki *et al.,*2009). Therefore, pre-exposure of *C. albicans* to high glucose levels result in an up-regulation of stress response genes prior to drug treatment which confers an increased degree of protection (Rodaki *et al.,* 2009). Consequently this may contribute to an increased level of tolerance to antifungal activity of caspofungin. In addition, 1D SDS PAGE analysis showed an increase in expression of some proteins (NADH-cytochrome b5 reductase

precursor and conserved hypothetical protein) involved in the stress response at higher glucose concentrations compared to 1% supplemented media.

3.2.4 Summary

The result presented here show that culture conditions affect protein expression in *C. albicans*. Some of the proteins that increase in expression e.g (alcohol dehydrogenase and hypothetical protein) could alter the susceptibility of *C. albicans* to caspofungin.

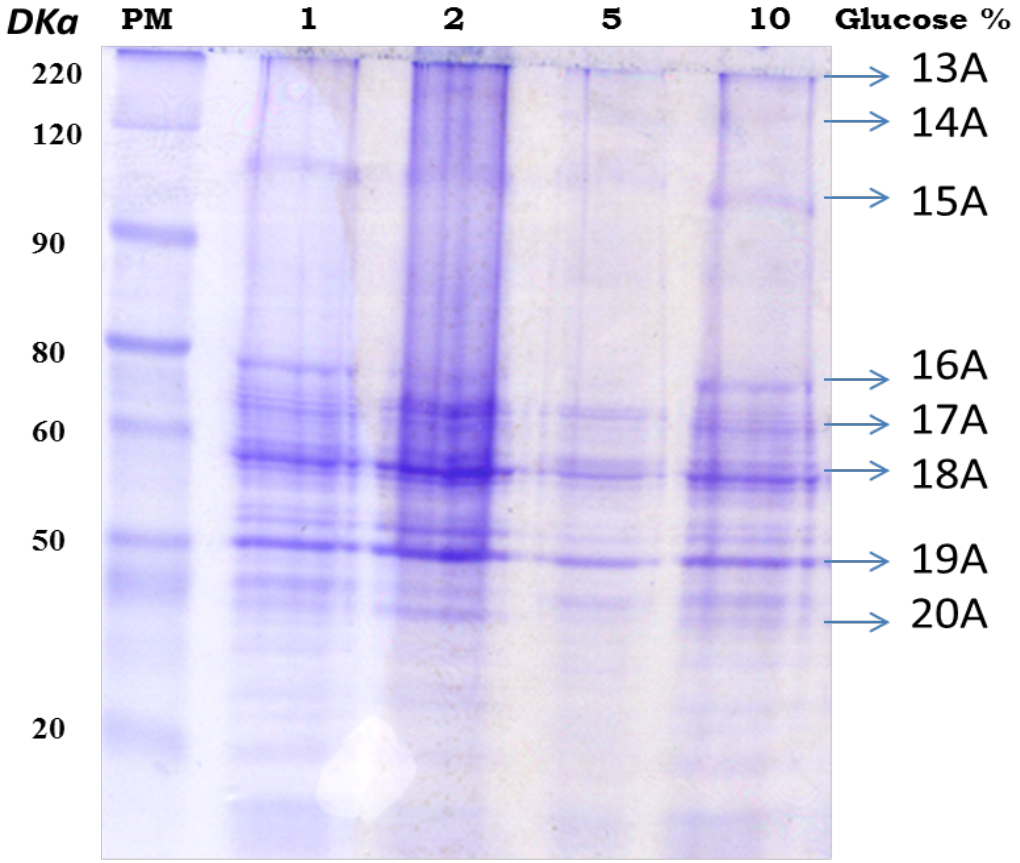


Figure 3.8 Coomassie stained SDS-PAGE gel (12.5%) of separated proteins from *C. albicans* incubated in AB3 media of varying level of glucose.

C. albicans was cultured in AB3 media containing different glucose concentrations, protein was extracted from stationary phase culture of each sample of varying glucose concentrations. 20 µg of protein from each sample was loaded into each well of the gel

Band	Name of protein	Fold Increase				Molecular function
		1% G	2% G	5% G	10% G	
13A	Fructose-1,6-bisphosphate aldolase, putative	1	1.9	0.8	1.3	Catalyzes the aldol, Lyase
14A	NADH-cytochrome b5 reductase precursor	1	2.3	1.7	1.8	Oxidoreductase
15A	ATP synthase beta chain, mitochondrial precursor	1	1.2	0.8	1.2	Hydrolase
16A	Potential oxidoreductase	1	0.8	0.7	1.3	Flavin adenine dinucleotide binding
17A	Enolase	1	2.5	0.9	1.8	Metabolic pathway via the GAL83
18A	Heat shock protein SSA4	1	2.9	0.8	1.7	Protein folding, ATP binding
19A	Elongation factor 1-gamma 1	1	2.6	0.9	1.4	Translation elongation factor activity
20A	Conserved hypothetical protein	1	1.8	1.1	1.3	Oxidoreductase

Table 3.9 Band ID of excised proteins from *C. albicans* cultured in media containing different glucose concentrations.

Identified protein by LC-MS and molecular function was determined using the UniProt data bank. Average of two independent experiments

3.3 Effect of environmental conditions on the catalase activity of *C. albicans*

Catalase exhibits antioxidant properties and is essential in maintaining the redox balance of the cell and has previously been implicated in the increased tolerance of *C. albicans* to caspofungin (Kelly *et al.,* 2009). The presence of catalase in a microbial or tissue sample can be tested by adding a volume of hydrogen peroxide and observing the reaction. The formation of oxygen bubbles indicates a positive result. Catalase has a very high specific activity, which produces a detectable response. Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long. It contains four porphyrin heme (iron) groups that allow the enzyme to react with the hydrogen peroxide. The optimum pH for human catalase is approximately 7 (Maehly *et al.,* 1954). The reaction of catalase in the decomposition of living tissue:

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

Cultures of *C. albicans* were grown under the following conditions: Chance in glucose concentration, change in pH and change in the concentration of serum (BSA)

In this experiment catalase activity was measured. Cells were exposed to different environmental condition and catalase activity was assessed as described in section 2.39.3. Cultures of *C. albicans* supplemented with 10 % glucose showed an increase in the activity of catalase, induced a one and half fold increase in catalase activity (Figure 3.9a) while 200 µg/ml BSA induced an two-fold increase in activity (Figure 3.9b). Exposure of *C. albicans* cells to pH showed a significant increase in activity of catalase, which resulted in a three-fold increase in catalase activity (Figure 3.9c).

The result shows a large increase in the activity of catalase under conditions such as a high glucose level, high concentration of BSA and at acidic pH, which give to tolerance of caspofungin.

3.3.1 Summary

The enzymatic activity of catalase was found to be significantly increased in *C. albicans* cells upon different environmental conditions especially at acidic pH

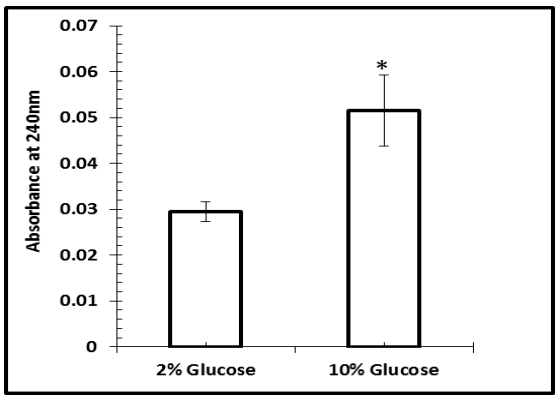


Figure 3.9a Effect of glucose on the catalase activity of *C. albicans*

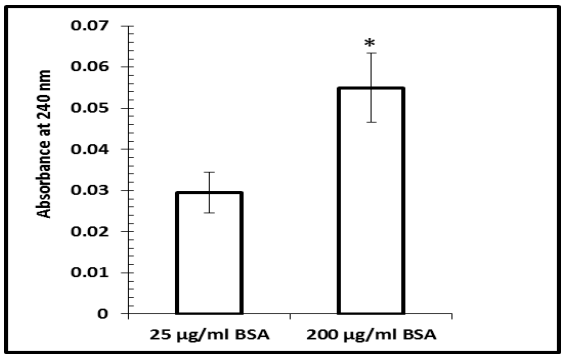


Figure 3.9b Effect of BSA on the catalase activity of *C. albicans*

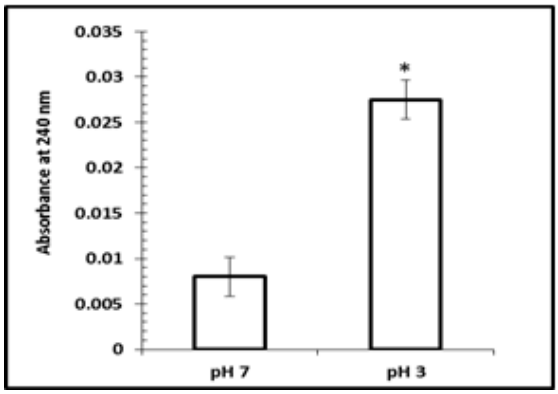


Figure 3.9c Effect of pH on the catalase activity of *C. albicans*

3.4 Effect of culture condition on the susceptibility of *A. fumigatus* to caspofungin.

Microdilution assays to determine the activity of caspofungin against *A. fumigatus* were conducted according to the reference method for broth dilution antifungal susceptibility testing of filamentous fungi, (M38-A2, 2008). The concentration range of caspofungin was 0.02 to 10 μg/ml in order to establish the response of the fungus to the drug when grown under different culture conditions.

3.4.1 Effect of culture media on susceptibility of *A. fumigatus* to caspofungin

Caspofungin was tested against *A. fumigatus* in different types of culture media as described in section 2.10 in order to establish whether the culture medium affected the susceptibility of *A. fumigatus* to the drug. The assay was performed using Antibiotic medium 3, Sabourud dextrose broth, YEPD broth, RPMI1640, nutrient broth (NB), COZPT, *Aspergillus* minimal media (AMM) and Minimal essential media (MEM) as described previously. All the plates were incubated at 37^{o}C for 48 hours. Plates were read on the microplate reader (Synergy HT, Bio-Tek) at 450nm, the absorbances were recorded and the growth was expressed as a percentage of the control (Figure 3.10).

Result presented here (Figure 3.10) show that caspofungin inhibits the growth of *A. fumigatus* in all types of media but high MIC_{90} values were recorded (Table 3.10). There is a big variation in the response of *A. fumigatus* to 0.02 - 1.25μg/ml caspofungin depending on the type of medium used in the assay.

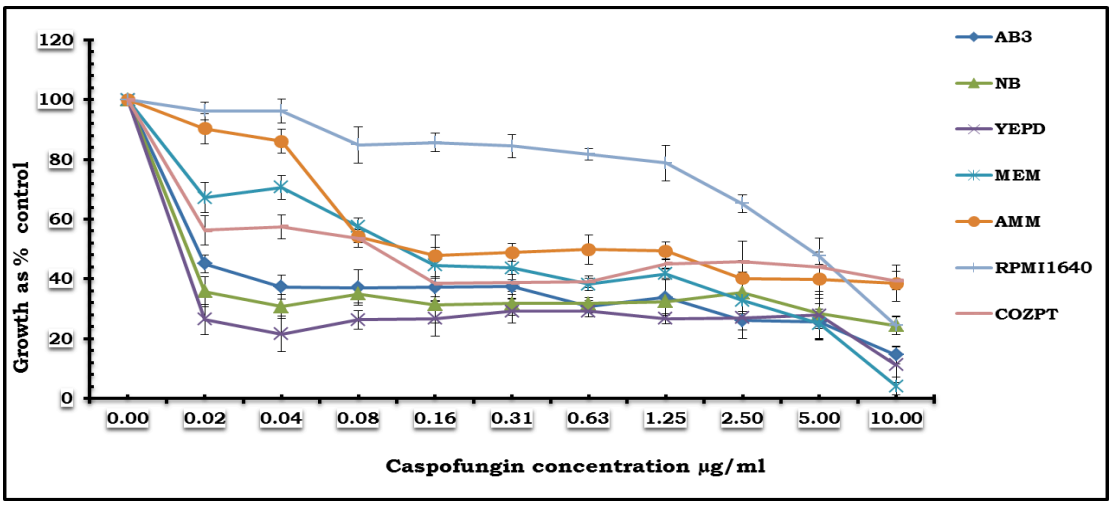


Figure 3.10 The effect of culture medium on the susceptibility of *A. fumigatus* to caspofungin.

The assay was performed as in section 2.10. All values are expressed as a percentage of the relevant control and are the average of three independent experiments.

Media type	MIC $_{50}$ µg/ml	MIC $_{90}$ µg/ml
AB3	0.020	> 10
NB	0.015	> 10
YEPD	0.010	10
MEM	0.125	9.00
AMM	0.085	> 10
$RPMI_{1640}$	5.500	> 10
COZPT	0.100	> 10

Table 3.10 Determination of MIC_{50} and MIC_{90} values from each graph of different media type. Caspofungin concentrations required to inhibit *A. fumigatus* growth by 50% and 90% were determined relative to control culture.

3.4.2 Effect of BSA on the susceptibility of *A. fumigatus*

Protein binding alters the activities of caspofungin and previous work observed that serum affects the susceptibility of *A. fumigatus* and *C. albicans* to caspofungin (Odabasi *et al.,* 2007). In this experiment 25, 50, 100 and 200 µg/ml Bovine serum albumin was added to RPMI1640 medium prior to inoculation with *A. fumigatus* conidia. Toxicity assays were performed as described in section 2.10. The results presented here demonstrated that BSA buffers the effect of caspofungin on *A. fumigatus* (Figure 3.11). High concentrations of BSA lead to an increase in the growth of *A. fumigatus* in the presence of caspofungin from 0.6 – 10 µg/ml. Table 3.11 show an MIC_{90} was increased according to the amount of BSA used in the media.

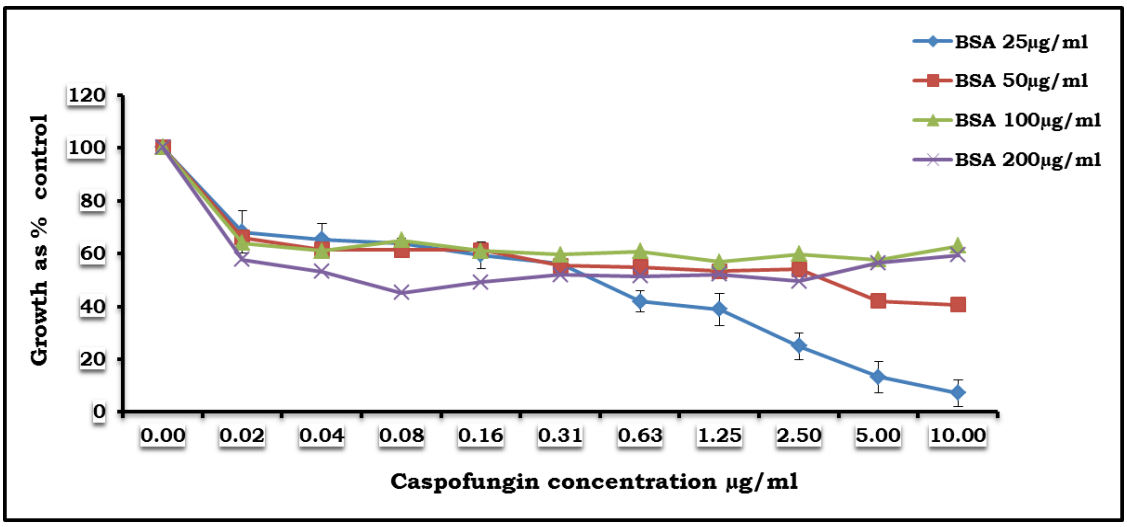


Figure 3.11 The effect of bovine serum albumin (BSA) on the susceptibility of *A. fumigatus* to Caspofungin using RPMI1640

BSA Concentrations	MIC $_{50}$ µg/ml	MIC $_{90}$ µg/ml
25 µg/ml BSA	0.45	7.5
50 µg/ml BSA	7.50	> 10
100 µg/ml BSA	> 10	> 10
200 µg/ml BSA	0.06	> 10

Table 3.11 Determination of MIC_{50} and MIC_{90} values from each graph of different concentrations of serum.

Caspofungin concentration required to inhibit the *A. fumigatus* growth by 50% and 90% were determined relative to control culture.

3.4.3 Effect of Glucose and FBS on the susceptibility of *A. fumigatus* to caspofungin using AMM

Glucose supplementation of media has a significant influence on the growth of *A. fumigatus* and is associated with organic acid production (Kubicek *et al.,* 1994). Serum contains iron-binding proteins, which inhibit the growth of most pathogenic microorganisms, including some fungi. In contrast Gifford *et al.,* (2002) indicated that serum does not inhibit the growth of *A. fumigatus* and that the nutrients in serum result in high levels of proteinase secretion, potentially increasing the invasiveness of this species.

The objective of this experiment was to assess the effect of combining glucose and serum on the susceptibility of *A. fumigatus* to caspofungin. *Aspergillus* minimal media was supplemented with 2% w/v glucose and/or fetal bovine serum (5% w/v) (FBS), and the response of *A. fumigatus* to caspofungin was determined as described in section 2.11.2. The results presented here (Figure 3.12) show increased growth of *A. fumigatus* in the presence of caspofungin when the medium contains glucose, serum or a combination of glucose and serum. Glucose and serum increased the tolerance of *A. fumigatus* to caspofungin. Table 3.12 show that the MIC_{50} values of caspofungin were elevated by presence of serum and glucose in AMM.

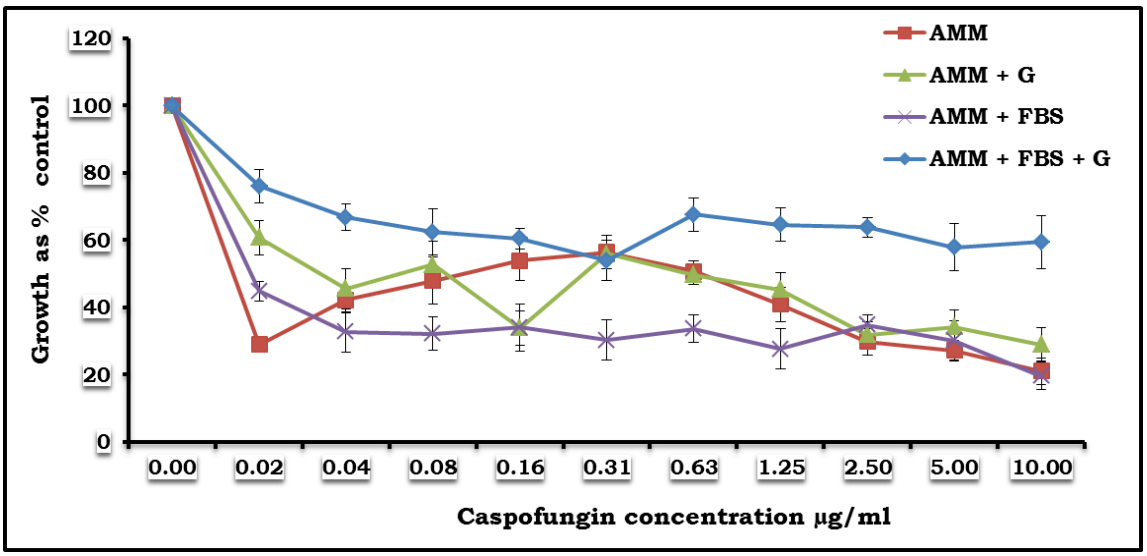


Figure 3.12 The effect of glucose and serum on the susceptibility of *A. fumigatus* to caspofungin using *Aspergillus* Minimal Medium (AMM).

The results are the average of three independent experiments.

Culture condition	MIC $_{50}$ µg/ml	MIC $_{90}$ µg/ml
AMM	0.015	> 10
AMM + G	0.035	> 10
AMM + FBS	0.025	> 10
AMM + FBS + G	> 10	> 10

Table 3.12 Determination of MIC_{50} and MIC_{90} values from each graph of glucose and serum culture conditions.

Caspofungin concentrations required to inhibit the *A. fumigatus* growth by 50% and 90% were determined relative to control culture.

3.4.4 Effect of Glucose and FBS on the susceptibility of *A. fumigatus* to caspofungin using MEM

In this experiment minimal essential media was used to determine the susceptibility of *A. fumigatus* to caspofungin in presence of glucose and FBS. Glucose (2% w/v) and 5% (w/v) serum were added to the media. The result show that the addition of glucose and combination of glucose and serum increased the MIC value of the drug over the range of 0.08 - 10 μg/ml as shown in Figure 3.13. Table 3.13 demonstrates that glucose supplemented media results in a large increase in the MIC_{50} value of caspofungin compared to the control.

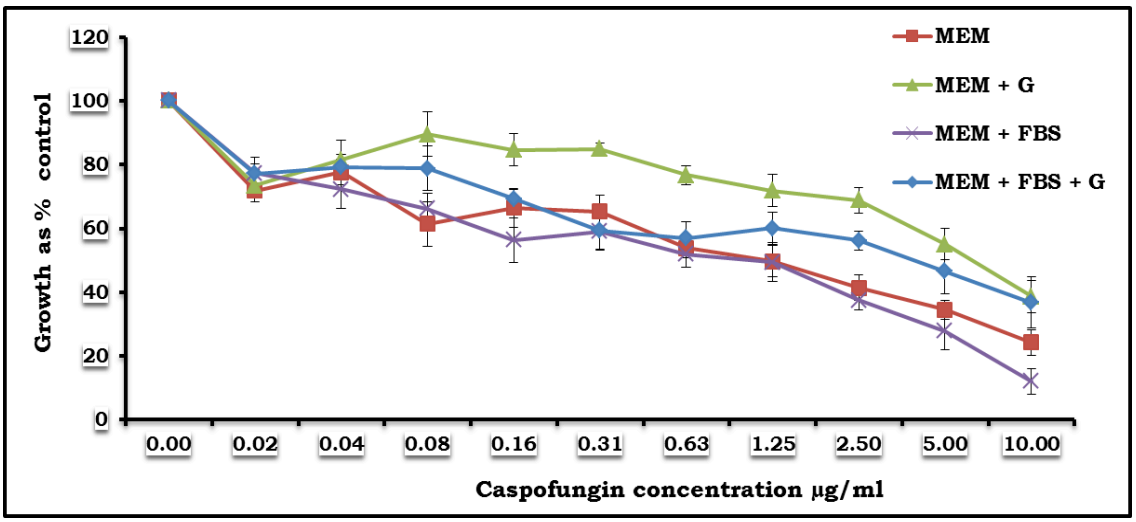


Figure 3.13 The effect of glucose and serum on the susceptibility of *A. fumigatus* to caspofungin using Minimal Essential Media (MEM)

MEM media was supplemented with glucose and FBS prior micro-dilution susceptibility assay to determine the effect of glucose and serum on the antifungal activity of caspofungin. Average of three independent experiments.

Culture condition	MIC $_{50}$ µg/ml	MIC $_{90}$ µg/ml
MEM	1.250	> 10
MEM + G	7.500	> 10
MEM + FBS	1.250	10
MEM + FBS + G	6.500	> 10

Table 3.13 Determination of MIC_{50} and MIC_{90} values from each graph of culture was supplemented with glucose and serum.

3.4.5 Effect of Glucose and FBS on the susceptibility of *A. fumigatus* to caspofungin using AB3

Antibiotics 3 media (AB3) was used to conduct this experiment. Media was supplemented with 2% (w/v) glucose and 5% (w/v) serum. Susceptibility assay was performed as described in section 2.9. The same observation was recorded and the MIC value was elevated in presence of glucose and in case of combination of glucose and serum (Figure 3.14) in tested media. Table 3.14 show the significant increase in MIC when medium is supplemented with glucose.

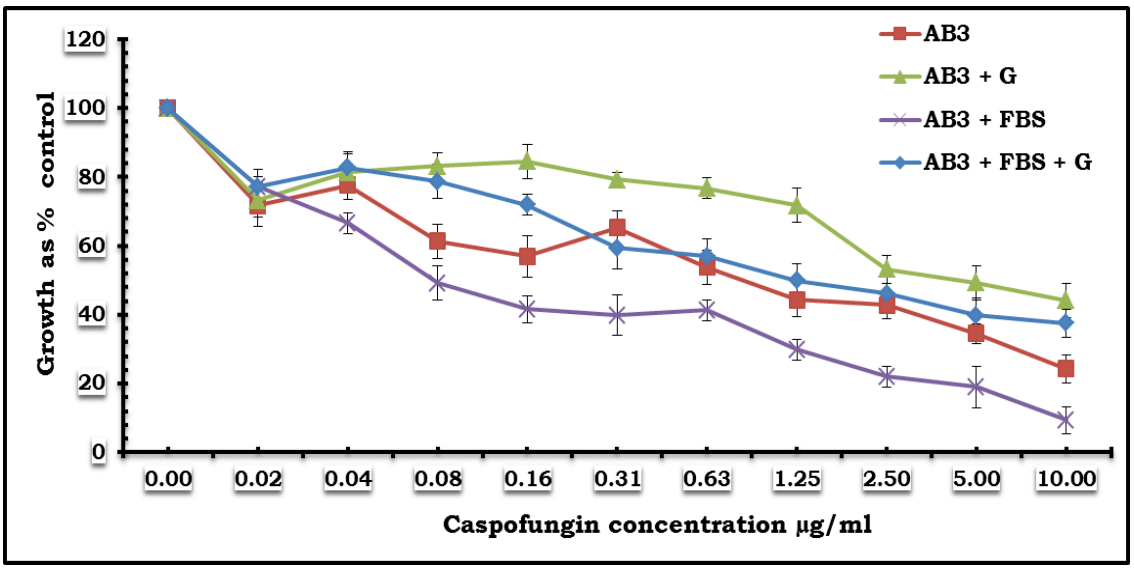


Figure 3.14 The effect of glucose and serum on the susceptibility of *A. fumigatus* to caspofungin using Antibiotic medium 3 (AB3).

Results are the average of three independent experiments.

Culture condition	MIC $_{50}$ µg/ml	MIC $_{90}$ µg/ml
AB3	1.250	> 10
AB3 + G	7.500	> 10
AB3 + FBS	0.080	9.5
AB3 + FBS + G	1.000	> 10

Table 3.14 MIC_{50} and MIC_{90} values, which calculated from the equation on each graph when culture of AB3 was supplemented with glucose and serum.

Caspofungin concentration required to inhibit the *C. albicans* growth by 50% and 90% were determined relative to control culture.

3.4.6 Effect of different carbon sources on the susceptibility of *A. fumigatus* to caspofungin

The survival of all microbes depends upon their ability to respond to environmental challenges. The nature of the carbon source significantly influences the resistance of *A. fumigatus* and other pathogenic fungi to environmental stresses and antifungal drugs (Askew *et al.,* 2008). Changes in carbon source also altered the virulence of *C. albicans* in models of systemic candidiasis and vaginitis, confirming the importance of alternative carbon sources within host niches during *C. albicans* infections (Ene *et al.,* 2012). A previous study has shown that growth on alternative carbon sources, such as lactate; strongly influence the resistance of *C. albicans* to antifungal drugs (Ene *et al.,* 2012).

RPMI1640 medium was supplemented with 2% (w/v) lactose, glycerol, sucrose, galactose, maltose or glucose and used to determine the effect of carbon source on the susceptibility of *A. fumigatus* to caspofungin. *A. fumigatus* conidia were grown in RPMI1640 with 2 % (w/v) of each sugar and *Aspergillus* conidia 5 x 10^5/ml were inoculated in 96 well plates containing serial dilution of caspofungin in range from 0.02 to 10µg/ml in the same culture medium. Plates were incubated at 37^{o}C for 48 hour. The MIC_{50} and MIC_{90} for each experiment were determined. The results revealed that cultures grown in medium supplemented with galactose or glycerol showed the highest MIC_{90} values Table 3.15

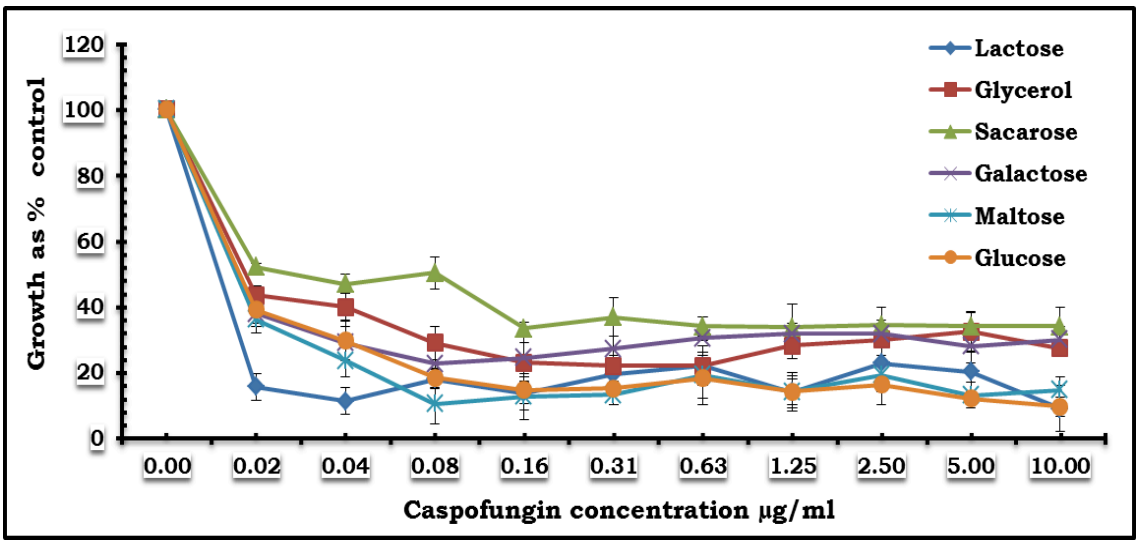


Figure 3.15 The effect of different carbon source on the susceptibility of *A. fumigatus* to caspofungin using RPMI1640.

RPMI1640 medium was supplemented with different carbon sources and used in a micro-dilution susceptibility assay to determine the effect of carbon source on the antifungal activity of caspofungin. The results are the average of three independent experiments.

Carbon sources	MIC $_{50}$ µg/ml	MIC $_{90}$ µg/ml
Lactose	0.010	0.040
Glycerol	0.015	> 10
Sacarose	0.020	> 10
Galactose	0.015	10
Maltose	0.015	> 10
Glucose	0.015	9.2

Table 3.15 MIC_{50} and MIC_{90} values, which calculated from the equation on each graph when culture was supplemented with different carbon sources.

Caspofungin concentration required to inhibit the *C. albicans* growth by 50% and 90% were determined relative to control culture.

3.4.7 Effect of pH on the susceptibility of *A. fumigatus* to caspofungin

The pH range recommended by Merck Research Laboratories for use of caspofungin in toxicity assays is 5 – 7 (Ripeau *et al.,* 2002). The aim of this experiment was to ascertain how the susceptibility of *A. fumigatus* to caspofungin varied under basic and acidic conditions. *A. fumigatus* conidia (5 x 10^5/ml) were inoculated into a 96 well plates in the presence of RPMI 1640 media at pH 3, 4, 6, 7, 8.5 or 10. Conidia were exposed to caspofungin (0.02 to 10.0 μg/ml) and incubated for 48 hour at 37^{o}C. The optical density (OD) was recorded at 450nm as described in section 2.11.3.

The MIC value of caspofungin required to inhibit the growth of *A. fumigatus* by 90% or 50% relative to the growth of the control was determined. From Figure 3.16 it can be seen that the maximum susceptibility of *A. fumigatus* to caspofungin over the concentration range 0.31 – 10.0 μg/ml occurred at pH 6 and pH 7. In contrast high growth of *A. fumigatus* was present in medium adjusted to pH 3, 4 or 10 over the same caspofungin range.

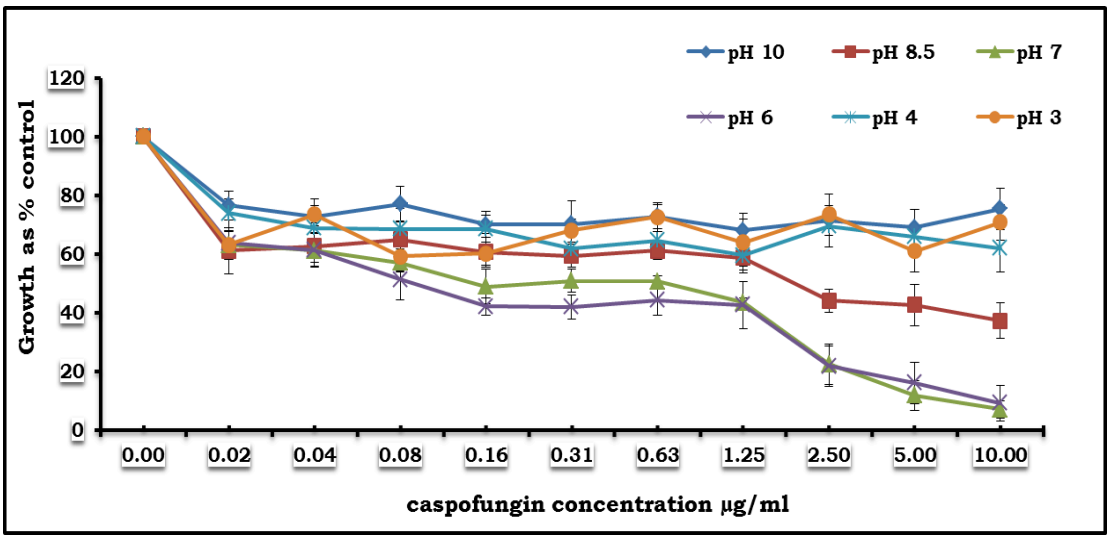


Figure 3.16 The effect of pH on the susceptibility of *A. fumigatus* to caspofungin.

A. fumigatus was grown in AB3 media of varying pH and subjected to a microdilution susceptibility assay to determine the effect of pH on the susceptibility of *A. fumigatus* to caspofungin. The results are the average of three independent experiments.

pH of culture media	MIC $_{50}$ µg/ml	MIC $_{90}$ µg/ml
pH 3	> 10	> 10
pH 4	> 10	> 10
pH 6	0.10	9.0
pH 7	0.16	7.5
pH 8.5	2.00	> 10
pH 10	> 10	> 10

Table 3.16 MIC_{50} and MIC_{90} values from each graph of varying pH.
The lowest concentration of caspofungin required to inhibit growth of *A. fumigatus* by 50% and 90% relative to the growth of the control.

3.4.8 Summary

The results presented here indicate that the susceptibility of *A. fumigatus* to caspofungin is affected by a variety of factors including the type of medium used in the assay, the nature of the carbon source, the % glucose in the medium, the pH and the protein content of the culture medium.

3.5 The effect of environmental conditions on protein expression of *A. fumigatus*.

The ability of *A. fumigatus* to thrive in a wide range of environmental condition is one of its virulence factors. *A. fumigatus* can adapt to environmental stresses such as nutrition, pH, and oxygen limitation. McDonagh *et al.,* (2008) demonstrated fungal adaptation to the mammalian lung during invasive aspergillosis. Selected genes were differentially expressed *in vivo* compared to laboratory media and correlated with genes expressed under alkaline conditions *in vitro* (McDonagh *et al.,* 2008).

The aim of the work described here was to examine whether the altered susceptibility of *A. fumigatus* to caspofungin recorded in different environmental conditions correlated with changes in protein expression of *A. fumigatus*.

Conidia of *A. fumigatus* (5 x 10^5cells/ml) were inoculated in culture media as described in section 2.7.4. Whole cell protein was extracted as described in section 2.22. Table 3.17 shows the proteins identified following 1-D electrophoresis of protein samples from *A. fumigatus* incubated under different environmental conditions. Protein bands were excised, digested and identified by LC-MS. All the peptides identified by LC-MS are in Table 3.17. In total 19 peptides were successfully identified and these were found to be up-regulated or down-regulated under different environmental condition. Densitometry was subsequently carried out on bands corresponding to proteins of interest to investigate the relative changes in expression of the identified protein.

Band	Score	Name of protein	PI	Seq	Accession #	Nominal mass	Seq Cav	Source
1B	60	46 kda IgE-binding antigen	5.11	1(1)	AAB21854	3618	50%	*Candida albicans*
2B	141	catalase	5.67	3(1)	XP_001273665	80098	5%	*Aspergillus clavatus NRRL 1*
3B	85	IgE-binding protein	4.43	1(1)	CAA12162	19413	11%	*Aspergillus fumigatus*
4B	65	L10A ribosomal protein	9.77	1(1)	CAB56219	24623	5%	*Candida albicans*
5B	70	FG-GAP repeat protein	5.53	2(0)	XP_750162	33739	6%	*Aspergillus fumigatus Af293*
6B	442	Secreted dipeptidyl peptidase	5.58	8(2)	AAB67282	79667	17%	*Aspergillus fumigatus*
7B	422	Catalase	5.50	12(1)	AAB71223	79987	21%	*Aspergillus fumigatus*
8B	261	Secreted dipeptidyl peptidase DppV	5.59	6(1)	XP_755237	79754	9%	*Aspergillus fumigatus Af293*
9B	282	Cobalamin-independent methionine synthase MetH/D	6.33	6(2)	XP_752090	87072	10%	*Aspergillus fumigatus Af293*
10B	287	Mycelial catalase Cat1	5.5	7(2)	XP_748550	79919	10%	*Aspergillus fumigatus Af293*
11B	166	Likely cytosolic ribosomal protein S4	9.96	4(0)	XP_718444	27295	13%	*Candida albicans SC5314*
11B	226	Potential mitochondrial inner membrane ATP/ADP translocator	9.62	6(1)	XP_716829	32908	27%	*Candida albicans SC5314*
12B	313	IgE-binding protein	4.43	6(2)	CAA12162	19413	41%	*Aspergillus fumigatus*
13B	194	P-loop containing nucleoside triphosphate hydrolase protein	6.37	4(1)	EGV66030	94420	6%	*Candida tenuis ATCC 10573*
14B	119	Plasma membrane ATPase	4.84	3(0)	EEQ44146	98186	4%	*Candida albicans WO-1*
15B	233	Translation elongation factor EF-1 alpha	9.11	5(0)	XP_710148	50419	12%	*Aspergillus oryzae*
16B	391	Alcohol dehydrogenase I	8.44	11(1)	EEQ46516	46598	32%	*Candida albicans WO-1*
17B	179	Hypothetical protein CANTEDRAFT_105339	5.46	4(1)	EGV64126	35806	15%	*Candida tenuis ATCC 10573*
18B	358	Malate dehydrogenase, NAD-dependent	9.08	8(1)	XP_748936	35876	32%	*Aspergillus fumigatus Af293*
19B	207	Likely thioredoxin peroxidase	4.98	5(1)	XP_716082	21962	27%	*Candida albicans SC5314*

Table 3.17 Identities of proteins observed to be altered in intensity following 1-D electrophoresis of samples from *A. fumigatus* incubated under different conditions.

Protein bands were excised, digested and identified as described. For identification a MASCOT score lower limit of 62 was used.

3.5.1 Effect of varying pH on protein expression in *A. fumigatus*

Conidia of *A. fumigatus* were incubated in RPMI1640 media of varying pH (3, 5, 7 or 9) and grown for 72 hours at 37 ^{o}C. One gram of cells (wet weight) was harvested and washed twice with PBS and the whole cell protein was extracted as in section 2.22. One dimensional gel analysis of proteins was used as described in section 2.25.1. Equal concentrations of protein from cells grown in media of different pH were loaded into the wells of a 12.5% separating gel with a 5% stacking gel. Proteins were run for 10 min at 40 Volts followed by 120 minutes at 80 Volts. The SDS-PAGE gel is presented in Figure 3.17 and shows the separation of proteins from *A. fumigatus* cultured in media of pH of 3, 5, 7 or 9. Proteins separated from different samples appeared to be either up-regulated or down-regulated across the pH range. The variation in protein expression may play a role in the susceptibility and response of *A. fumigatus* to a variety of conditions. Compared to the expression at pH 7 some proteins were up-regulated at other pH values (Table 3.18). Catalase was increased by 2.1 fold in expression at pH 3 and up-regulated by 1.5 fold at pH 9. In addition to the role as an oxido-reductase, catalase is also involved in the stress response. An increase in the activity of catalase may contribute to the increase in the tolerance of *A. fumigatus* to caspofungin. Kelly *et al.,* (2009) demonstrated that the induction of catalase activity in *C. albicans* HOG dependent manner in response to stress caused by caspofungin action. Secreted dipeptidyl peptidase showed a change in expression at pH 3 of 0.9 fold and at alkaline pH9 of 0.7 fold. IgG-GAP repeat protein is down regulated at acidic and alkaline pH which decreased in intensity by 0.7 and 0.9 for pH 3 and pH 9 respectively. FG-GAP repeat protein was up-regulated at pH 3 but it showed a 2.1 fold increase and at pH 9 decreased by 0.9 fold.

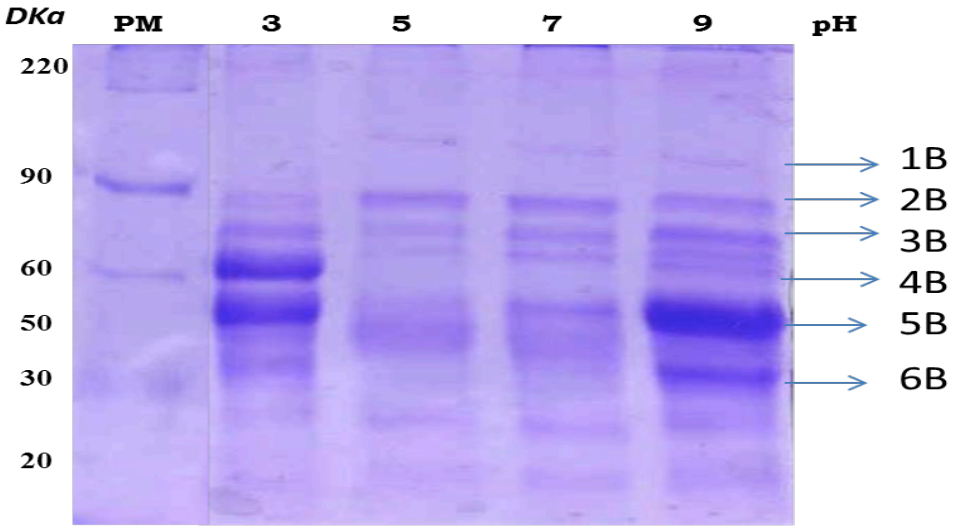


Figure 3.17 Coomassie stained SDS-PAGE gel (12.5%) of separated proteins from *A. fumigatus* inoculated in media of varying pH.

A. fumigatus was cultured in RPMI1640 media of varying pH from 3 to 9. Protein was extracted from 72hr old *Aspergillus* cultures and 20 µg proteins were resolved by 1D SDS-PAGE.

Band	Name of protein	Fold Increase				Molecular function
		pH 7	pH 3	pH 5	pH 9	
1B	46 kda IgE-binding antigen	1	1.3	1.2	0.7	Transcript induced
2B	Catalase	1	2.1	1.9	1.5	Oxidoreductase, Peroxidase
3B	IgE-binding protein	1	0.7	0.9	0.9	Evidence at transcript level
4B	L10A ribosomal protein	1	1.2	1.3	1.1	RNA binding
5B	FG-GAP repeat protein	1	2.1	0.7	0.8	Calcium ion binding
6B	Secreted dipeptidyl peptidase	1	0.9	0.5	0.7	Contributes to pathogenicity

Table 3.18 Identity and fold change in expression of excised proteins from *A. fumigatus* cultured in media of varying pH.

Identified protein by LC-MS and molecular function was determined using the UniProt data bank.

3.5.2 Effect of BSA on protein expression in *A. fumigatus*

The purpose of this experiment was to examine if there were any changes in the protein expression of *A. fumigatus* when grown in different concentrations of BSA, which may contribute to the increased level of *Aspergillus* tolerance to caspofungin at high serum level. Previous studies demonstrated an elevation of the MIC of caspofungin against some *Aspergillus* and *Candida* species in the presence of human serum albumin (Paderu *et al.,* 2004; Odabasi *et al.,* 2007). *A. fumigatus* was grown for 72 hours in RPMI1640 culture media containing different concentrations of bovine serum albumin (BSA). Whole cell protein was extracted as described in section 2.27 and resolved by 1D SDS-PAGE. Protein bands which appeared to be either increased or decreased in expression were analysed and fold changes in intensity were recorded.

One-dimensional SDS-PAGE gel in Figure 3.18 shows the separation of protein from *A. fumigatus* grown in RPMI1640 culture medium supplemented with varying BSA concentrations. Protein up-regulation was apparent at high concentrations of BSA. Seven excised proteins from *A. fumigatus* were successfully identified by LC-MS. It was found that the intensity of protein expression increased especially when 100 µg/ml and 200 µg/ml of BSA were applied to culture media. *A. fumigatus* produces three active catalases; one is produced by conidia, and two are produced by mycelia. The mycelial catalase Cat1p was studied previously; studies have shown that the mycelium of *A. fumigatus* produces two mycelial catalases, one that is monofunctional and one that is a bifunctional catalase-peroxidase (Hearn *et al.,* 1992). At high serum level, band 10B showed an increase in intensity of bands. At 100 and 200 µg/ml exposure to BSA was shown an increase in expression to 2.6 and 3.2 fold respectively. The protein synthesis inhibitor IgE-binding protein was up-regulated when the culture of *A. fumigatus* was supplemented with 50 and 100 µg/ml BSA, by 2.1 and 2.7 fold respectively.

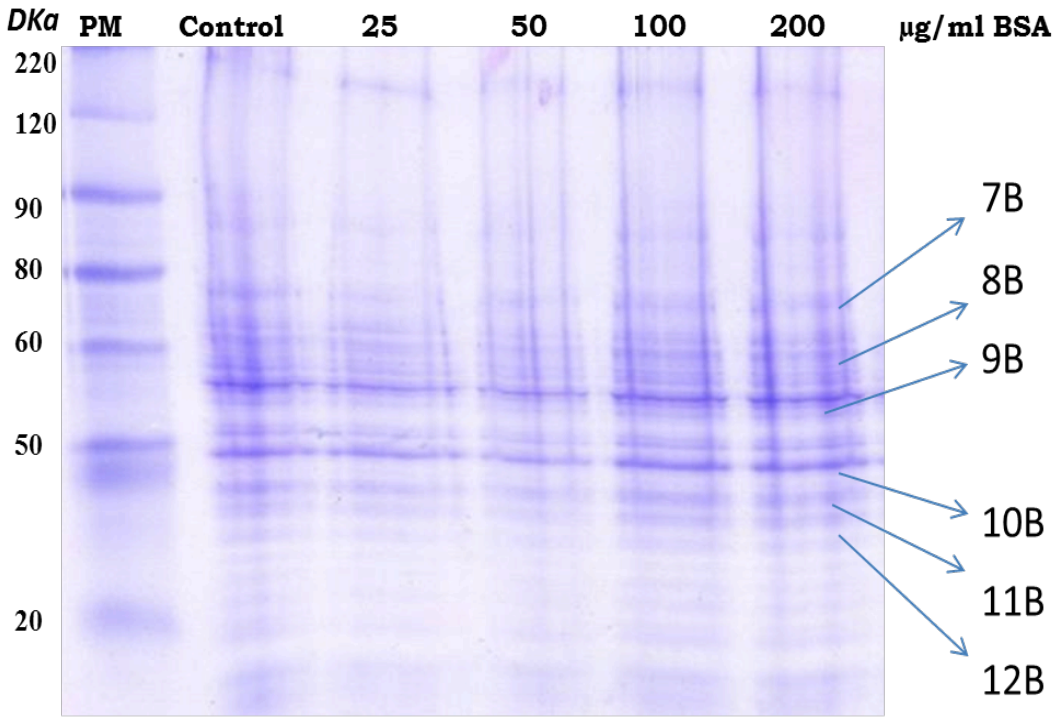


Figure 3.18 Coomassie stained SDS-PAGE gel (12.5%) of separated proteins from *A. fumigatus* incubated in RPMI1640 media supplemented with different concentrations of BSA.

Protein was extracted from *A. fumigatus* culture grown in medium supplemented with different BSA concentrations. 20 µg of protein from each sample was loaded into each well of the gel.

Band	Name of protein	Fold Increase				Molecular function
		25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml	
7B	Catalase	1	3.2	3.8	2.9	Immunoreactive protein
8B	Secreted dipeptidyl peptidase DppV	1	2.8	3.7	3.1	Hydrolase, Protease
9B	Cobalamin-independent methionine synthase MetH/D	1	3.2	3.5	2.9	Methyltransferase activity
10B	Mycelial catalase Cat1	1	1.4	2.6	3.2	Heme binding, metal ion binding
11B	Likely cytosolic ribosomal protein S4	1	2.4	2.6	3.5	RNA binding
11B	Potential mitochondrial inner membrane ATP/ADP translocator	1	2.4	2.6	3.5	Transporter activity
12B	IgE-binding protein	1	2.1	2.7	1.1	Protein synthesis inhibitor

Table 3.19 Band ID of excised proteins from *A. fumigatus* cultured in RPMI1640 media containing different BSA concentrations.

Identified protein by LC-MS and molecular function was determined using the UniProt data bank.

3.5.3 Effect of Glucose on the protein expression of *A. fumigatus*

The aim of this experiment was to establish whether glucose supplementation of culture medium altered the protein expression of *A. fumigatus* and might contribute to the elevated tolerance to caspofungin evident in these culture media. Cultures of *A. fumigatus* were grown in RPMI1640 medium containing different glucose concentrations. Protein was extracted as described in section 2.27 and resolved by 1D SDS-PAGE. Protein bands were analysed by densitometry to be either increased or decreased in expression. The image of the SDS-PAGE gel in Figure 3.19 shows the separation of protein from *A. fumigatus* grown in medium supplemented with varying glucose concentrations. In comparison to the control an up-regulation of protein appears at high levels of glucose. Eight excised proteins from *A. fumigatus* grown in media of varying glucose concentration were successfully identified by LC-MS.

Band 14B (Plasma membrane ATPase) was identified and the results show an up-regulated in protein expression at 5% and 10% glucose level by 2.5 and 1.7 fold respectively in comparison to cells grown in 1% glucose media. The expression of Band 16B (Alcohol dehydrogenase 1) was up-regulated 1.9 and 2.3 fold in 5% and 10% glucose media respectively in compared to cells grown in 1% glucose medium.

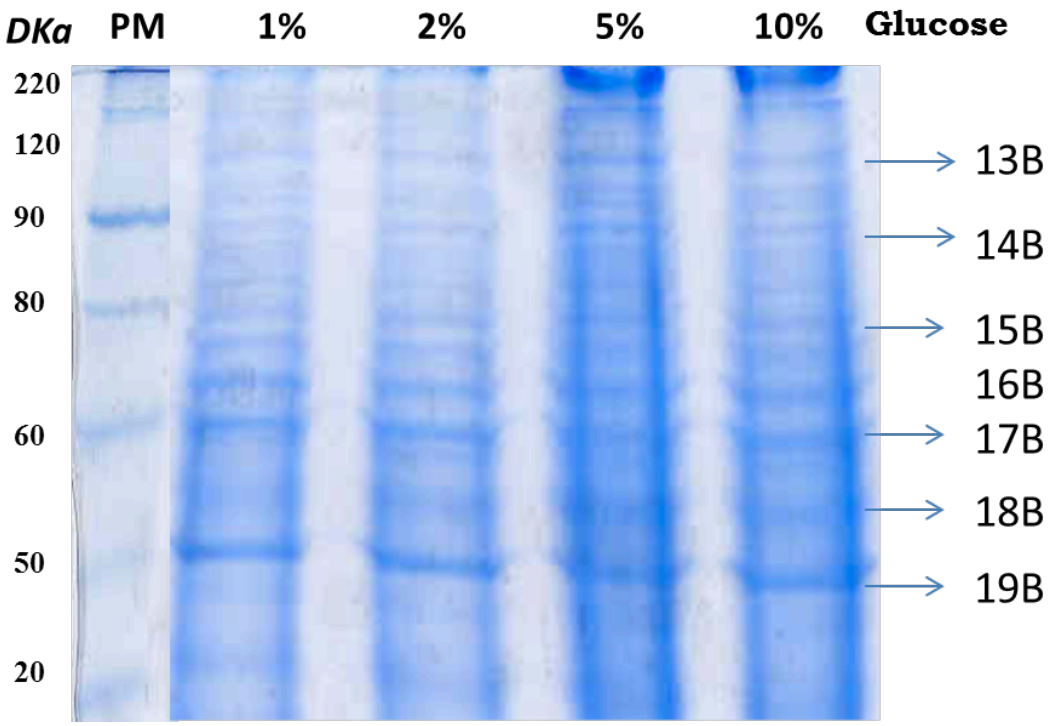


Figure 3.19 Coomassie stained SDS-PAGE gel (12.5%) of separated proteins from *A. fumigatus* incubated in RPMI1640 culture media of varying level of glucose.

A. fumigatus was cultured in media of varying glucose; protein was extracted from culture of each sample of varying glucose concentrations. 20 µg of protein from each sample was loaded into each well of the gel.

Band	Name of protein	Fold Increase				Molecular function
		1% G	2% G	5% G	10% G	
13B	P-loop containing nucleoside triphosphate hydrolase protein	1	1.4	2.7	2.1	Nucleoside-triphosphatase activity
14B	Plasma membrane ATPase	1	1.2	2.5	1.7	ATP binding
15B	Translation elongation factor EF-1 alpha	1	1.7	2.9	2.4	Elongation factor
16B	Alcohol dehydrogenase I	1	0.9	1.9	2.3	Oxidoreductase, Allergen
17B	Hypothetical protein CANTEDRAFT_105339	1	1.5	2.2	2.7	DNA repair
18B	Malate dehydrogenase, NAD-dependent	1	1.9	3.1	2.9	L-malate dehydrogenase activity
19B	Likely thioredoxin peroxidase	1	2.4	3.4	3.1	Thioredoxin peroxidase activity

Table 3.20 Band ID of excised proteins from *A. fumigatus* cultured in media of varying glucose. Identified protein by LC-MS and molecular function was determined using the UniProt data bank.

3.6 Effect of environmental conditions on the catalase activity of *A. fumigatus*

Previous work demonstrated an increase in the activity in catalase when *C. albicans* and *A. fumigatus* were exposed to caspofungin (Kelly *et al.,* 2009). *A. fumigatus* conidial and mycelial catalases protect the fungus against hydrogen peroxide *in vitro*. However, both mycelial catalases, Cat1p and Cat2p, are involved in the degradation of hydrogen peroxide to detoxifying cells *in vitro* and transiently protected the fungus against oxidative stress (Paris *et al.,* 2003). The aim of the work presented here was to establish whether different culture conditions induced an increase in catalase activity that might contribute to the increased tolerance to caspofungin.

For the determination of catalase activity protein extracts from cells grown under different conditions were used. Proteins were extracted as described in section 2.27. In a sterile tube, the fresh protein (100 µl, 7 mg/ml) was added to 17 mM H_2O_2. The mixture was vortexed and left at room temperature for 20 min to allow for reaction, after this time, the suspension was centrifuged at high speed for one minute to stop the reaction. The supernatant was removed and placed in a clean quartz cuvette. The absorbance was read at 240 nm using (UV-2550, UV-VIS spectrophotometer SHIMADZU). Solution of 17 mM of H_2O_2 was used as a blank. Catalase exhibits antioxidant properties and is essential in maintaining the redox balance of the cell. Exposure of *A. fumigatus* to specific environmental conditions results in a significant rise in the activity of catalase.

Results demonstrate that catalase activity is changed according to the growth condition of *A. fumigatus*. At 10 % glucose the catalase activity was increased significantly compared to that in 2% glucose supplemented media (Figure 3.20a), the activity of catalase was increased when the media was supplemented with 200 µg/ml compared to 25 µg/ml BSA Figure 3.20b, and also at the acidic pH 3 catalase was increased in activity by two fold compared to neutral pH 7 (Figure 3.20c).

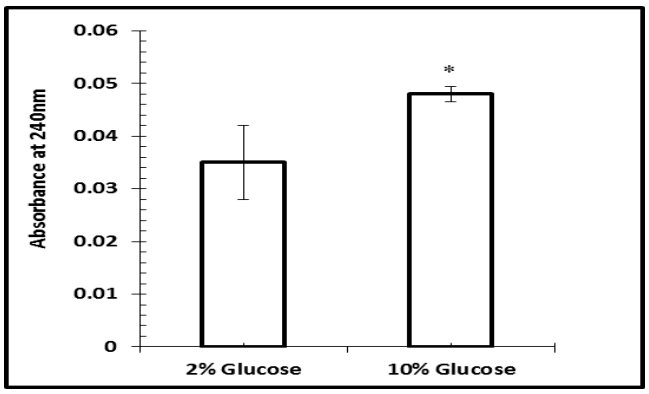


Figure 3.20a Catalase activity of *A. fumigatus* grown in the presence of 2 or 10% w/v glucose

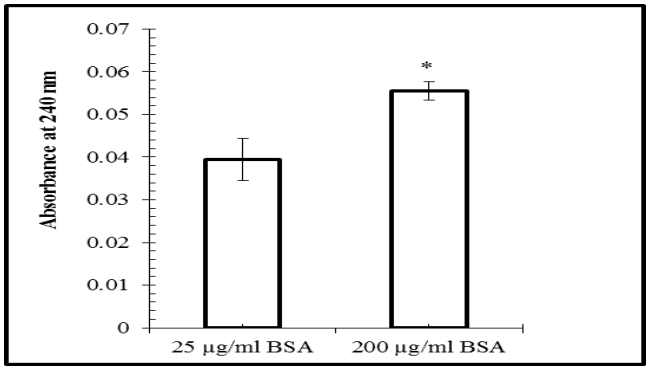


Figure 3.20b Catalase activity of *A. fumigatus* grown in medium supplemented with different BSA concentrations.

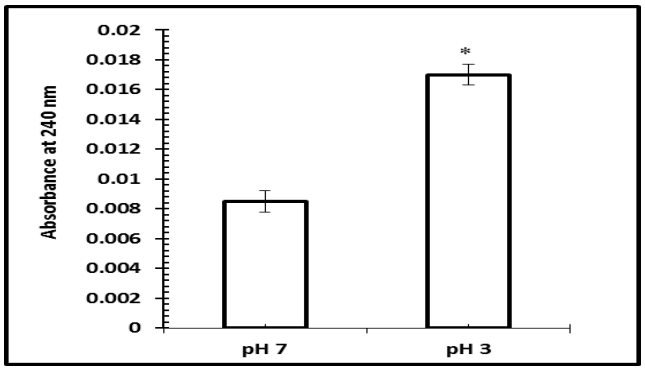


Figure 3.20c Catalase activity of *A. fumigatus* grown at pH 3 or 7.

3.7 Summary

The results presented here indicate that culture conditions affect protein expression in *A. fumigatus*. Some of the proteins that increase in expression e.g (mycelial catalase Cat1, IgE-binding protein and plasma membrane ATPase), could alter the tolerance of *A. fumigatus* to caspofungin.

The enzymatic activity of the catalase was increased in *A. fumigatus* at some conditions, at high level of glucose (10% w/v) and serum (200 µg/ml), and also at the acidic pH 3.0 (Figure 3.21a,b & c).

3.8 Effect of caspofungin on the germination of *A. fumigatus* conidia under different environmental condition.

A. fumigatus is an opportunistic fungal pathogen of various immunocompromised populations. It has the ability to form filaments within the lungs, and producing dense mycelial balls, which make it difficult to treat. The aim of this work was to determine the activity of caspofungin on germination of *A. fumigatus* conidia grown under different environmental conditions. Germination of *A. fumigatus* conidia is the most crucial step in the development of *Aspergillus* as hyphae represent the invasive form (Manavathu *et al.,* 1999). Germination was assessed morphologically using microscopic examination of 50 µl of *A. fumigatus* culture from microdilution plates containing different concentrations of caspofungin in media with specific alterations, including serum, pH or glucose, after 24 hours. The effect of caspofungin on the germination ability of *A. fumigatus* at 0.2, 5.0 and 10 µg/ml was examined.

3.8.1 Effect of culture conditions on germination of *A. fumigatus* in caspofungin.

The germination ability of *A. fumigatus* spores was determined at different concentration of caspofungin in medium supplemented with FBS. *A. fumigatus* was cultured in media supplemented with 2.5, 5.0 and 10.0 µg/ml FBS prior to microdilution of caspofungin in 96 plates. Mature spores were collected by gently pipetting 50 µl of culture

after 24 hour of incubation at 37^{o}C. The purpose of this study was to determine the effect of FBS on the activity of caspofungin against *A. fumigatus* conidia, and morphological change in conidial germination. Previous studies showed that caspofungin alone demonstrated no significant inhibition of hyphal growth, whereas the addition of 5% human serum to the inactive drug, increased the activity of caspofungin. This study suggests that human serum acts synergistically with caspofungin to enhance its inhibitory activity *in vitro* against *A. fumigatus* (Manavathu *et al.,* 1999).

Studies have looked for evidence that FBS adversely affect the activity of caspofungin. In this study we examined the effect of various levels of FBS on the activity of caspofungin against *A. fumigatus* by microscopic examination of germ-tubes and hyphae. This interaction was studied under a variety of conditions.

At high serum concentrations germination and growth of hyphae were observed but, in contrast, at low serum concentrations germination was inhibited at 5 and 10 μg/ml caspofungin (Figure 3.21). In medium supplemented with high levels of glucose germination and hyphal development were observed at 5 and 10 μg/ml caspofungin compared to medium supplemented with 2% glucose (Figure 3.22). Conidia incubated in medium of pH 3 and 9 showed higher level of germination at 0.2 and 5 μg/ml caspofungin compared to the pH 7.0 (Figure 3.23). These results show that culture conditions affect the germination rate of *A. fumigatus* in the presence of caspofungin

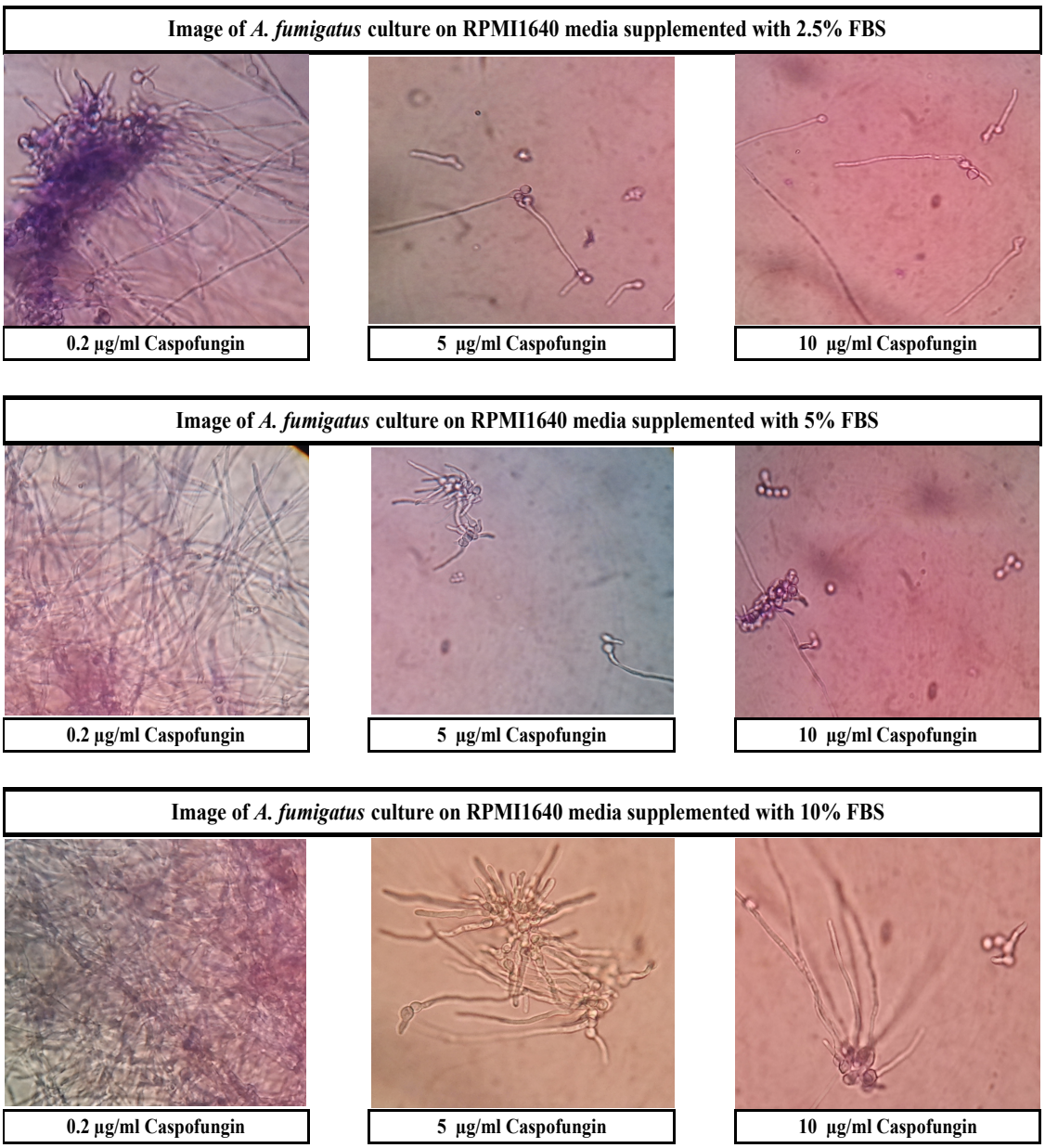


Figure 3.21 Effect of caspofungin on germination of *A. fumigatus* conidia exposed to varius FBS.

Aspergillus culture grown in RPMI1640 with (2.5, 5 and 10% FBS) for 24 hour at 37 ^{o}C. Image taken at concentration 0.2, 5, and 10 µg/ml caspofungin (magnification X40)

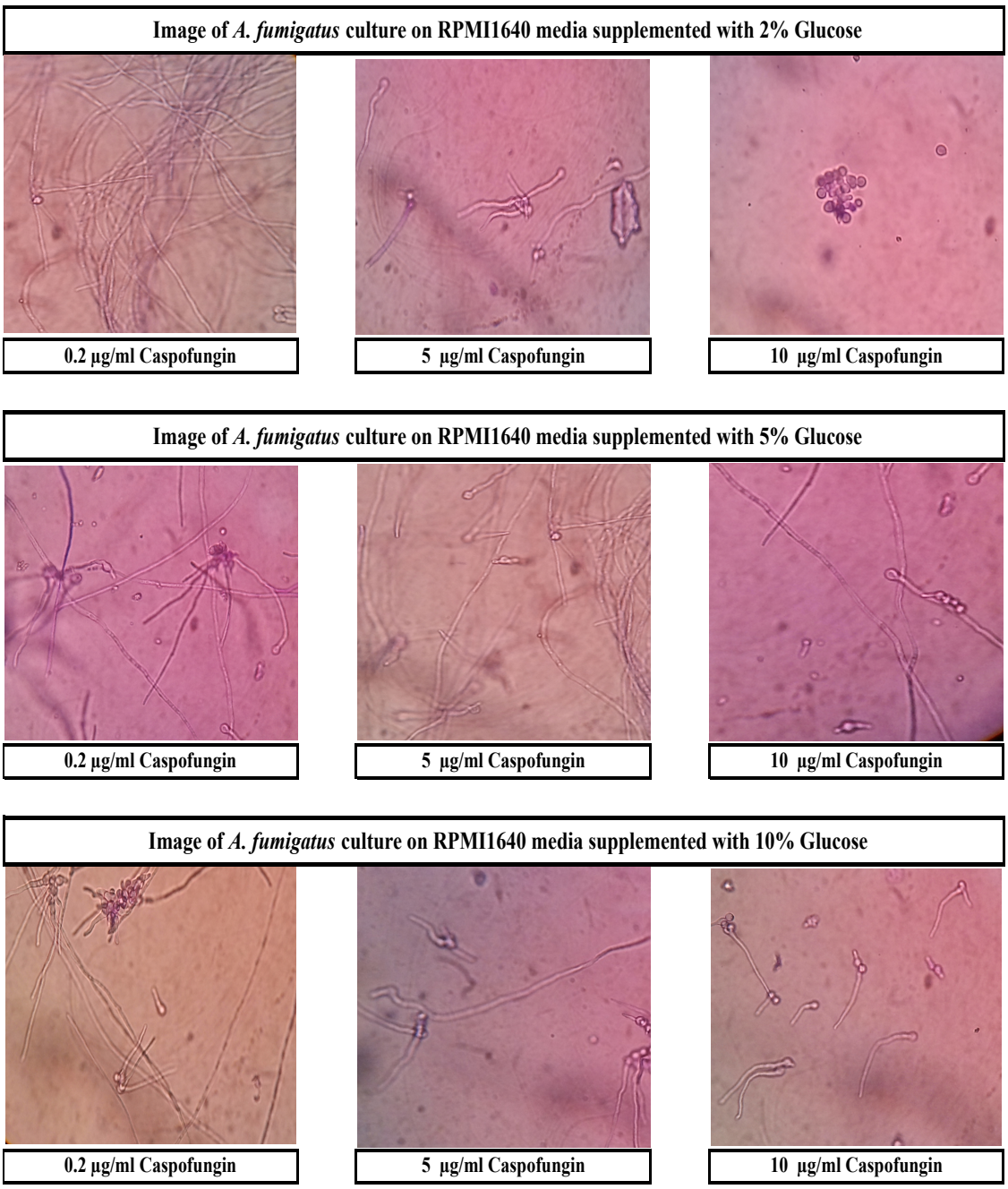


Figure 3.22 Effect of caspofungin on germination of *A. fumigatus* conidia exposed to varius glucose level.

Aspergillus culture grown in RPMI1640 with (2%, 5% and 10%) glucose for 24 hour at 37 ^{o}C. Image taken at concentration 0.2, 5, and 10 µg/ml caspofungin. (magnification X40)

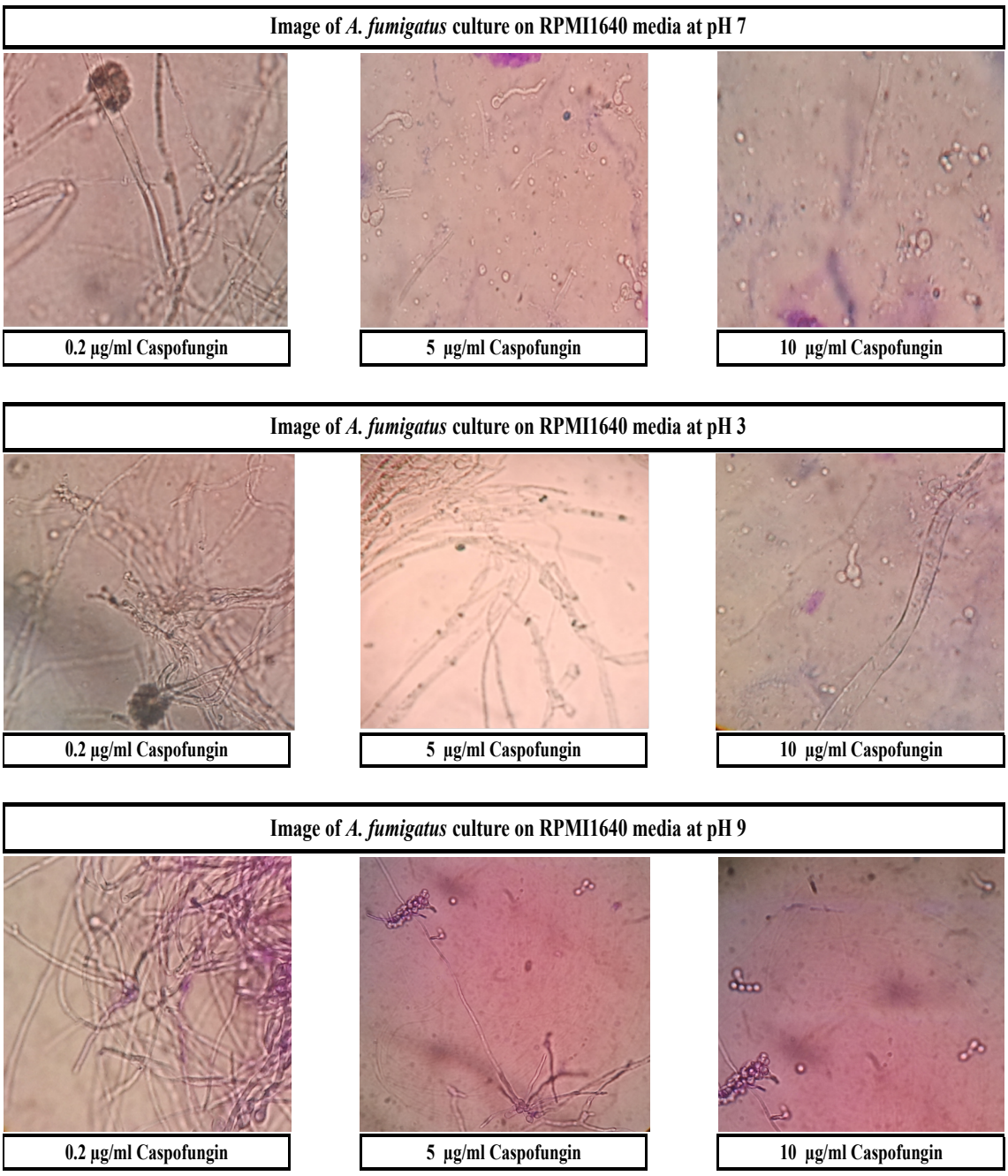


Figure 3.23 Effect of caspofungin on germination of *A. fumigatus* conidia at varius pH.

Aspergillus culture grown in RPMI1640 with different pH value (3, 7 and 9) for 24 hour at 37 ^{o}C. Image taken at concentration 0.2, 5, and 10 µg/ml caspofungin. (magnification X40)

3.9 Summary

The result presented here indicate the increase in glucose, change pH and also high serum levels that elevate the germination and growth rate of *A. fumigatus* even in presence of 5 or 10 μg/ml caspofungin

3.10 Discussion

The primary goal of the work presented in this Chapter was to characterise the effect of environmental factors on the susceptibility of *C. albicans* and *A. fumigatus* to caspofungin. The effect of glucose, serum and pH on the susceptibility of each fungus to caspofungin was examined and an alteration in the expression of proteins induced by these conditions was characterized. The increased level of tolerance of both *C. albicans* and *A. fumigatus* to caspofungin is evident in this study by the elevated growth of both organisms in presence of caspofungin. The results in this Chapter may explain the paradoxical effect of echinocandin antifungal drugs (Steven *et al.,* 2006). This phenomenon has been demonstrated *in vitro* with echinocandins against *Candida* and *Aspergillus* species (Wiederhold 2009). The increase in tolerance of fungal cells may be correlated with an increase in cell wall chitin even at high doses of caspofungin (Steven *et al.,* 2006). Elevated levels of chitin may substitute for the reduction of ß-(1, 3) D glucan which may enable the cells to increase their tolerance to caspofungin activity.

Caspofungin is a member of the echinocandins and was first licensed for clinical used in the United States in 2001 (Kartsonis *et al.,* 2003). This group of antifungals target ß-(1,3)-glucan synthesis, which is essential for the structural integrity of the fungal cell wall. Caspofungin has an excellent safety profile and limited side effect (Maertens *et al.,* 2004). In this work we looked at the antifungal activity of caspofungin on *A. fumigatus* by the broth microdilution toxicity assay. Due to the fact that there is no specific and standardized susceptibility methods for the ß-(1,3)glucan synthesis inhibitors (Deresinski and Stevens, 2003) and there is a variation in MIC value (Odds *et al.,* 2004) the reference method for broth dilution antifungal susceptibility testing of filamentous fungi was used in this study (M38-A2, 2008). Determination of the actual MIC of caspofungin appears to be

difficult because of the paradoxical effect of caspofungin at certain concentrations (Stevens *et al.,* 2004), which allowed the growth of *A. fumigatus* at 37 ^{o}C. This phenomenon has been previously characterised as an increase in the growth rate of *C. albicans* at concentrations above MIC and some researchers demonstrated that this effect occurs when echinocandins are used against *Candida* and *Aspergillus* species (Wiederhold, 2009; Chamilos *et al.,* 2007). Although the explanation of this phenomenon is not clearly defined, a number of studies support the hypothesis that the protection of fungal cells is associated with an increase in chitin at high echinocandin concentrations as a dramatic increase in cell wall chitin has been shown to correlate with an increase in tolerance (Chamilos *et al.,* 2007; Stevens *et al.,* 2006). From the results presented here it can be deduced that environmental conditions affect the susceptibility of *A. fumigatus* and *C. albicans* to caspofungin over the range of 0.1 to 10.0 µg/ml caspofungin as shown in section 3.1 and 3.4 of the results. On the other hand caspofungin may induce the cell to remodel the cell wall as a result of an increase in chitin content which may substitute for the reduction of ß-1,3 D glucan that leading to an increase in the tolerance of cell to drug activity. This phenomenon may not be restricted to *in vitro* studies but may be of clinical relevance in the *in vivo* setting.

Investigation of the effect of culture conditions on protein expression of *C. albicans* lead to the discovery of differentially expressed proteins. From the 1D SDS PAGE gel analysis, it was evident that the expression of some proteins (e.g. catalase and IgE-binding protein) was increased while the expression of others was down regulated. One of the important virulence factors of *C. albicans* is the ability to thrive in a wide range of environmental conditions, thereby supporting it as a successful opportunistic pathogen (Calderone and Fonzi, 2001). The results presented here show that *C. albicans* is capable of growing in the diverse range of culture conditions. It is important to note that changes in environmental conditions may have a direct effect on caspofungin activity and the degree of susceptibility of *C. albicans*. Environmental conditions also altered the protein profile of cells, with various sized proteins up or down-regulated within a variety of conditions. This suggests that the *C. albicans* and *A. fumigatus* may respond to the growth condition and attempt to protect themselves by altering various biosynthetic pathway and in so doing alter their susceptibility to caspofungin.

Low environmental pH has been shown to affect the antifungal action of azoles against *C. albicans*, and an increase in MIC of fluconazole in an acidic environment has been demonstrated (Marr *et al.,* 1999). From the result in section 3.1.5 and 3.4.7 we can speculate that the pH of the environment is important for the *in vitro* response of *C. albicans* and *A. fumigatus* to caspofungin. Analysis of the protein expression of *C. albicans* and *A. fumigatus* cultured at various pH levels allowed us to assess the change in protein expression which may have contributed to the altered susceptibility of these organisms to caspofungin. From data analysis of 1D SDS-PAGE gel (Figure 3.1.5) there was an apparent decrease in expression of a number of *C. albicans* proteins in acidic (e.g. pyruvate decarboxylase and IgE-binding protein) and basic (e.g. hypothetical protein) environment in comparison to the proteins visualised at the pH 7. LC-MS facilitated the identification of proteins of interest from the 1D gel shown in Figure 3.6 and of the proteins successfully identified, glyceraldehyde-3-phosphate dehydrogenase and enolase 1 OS were of particular interest. Increase in the expression of these proteins compared to that at neutral pH correlated with increase in tolerance of *C. albicans* to caspofungin. Interestingly glyceraldehyde-3-phosphate dehydrogenase is involved in cell wall biogenesis as well as having other function in glycolysis, adherence and modulation of immune system (Kelly & Kavanagh, 2010). Enolase is involved in the glycolytic pathway as a vital component, catalyzing the dehydration of 2-phosphoglycerate to create phosphoenolpyruvate (Sundstrom and Aliaga, 1992). Enolase is predominantly found in the cytoplasm and may also be found in cell wall as glucan associated cell wall protein (Pitarch *et al.,* 2002). Proteins extracted from *A. fumigatus* and analysed using 1D SDS-PAGE are shown in Figure 3.18. Results show that an increase in protein expression compared to that at pH7 was correlated with increase in tolerance of *A. fumigatus* to caspofungin (Figure 3.16). One protein (FG-GAP repeat protein) showed an up-regulation (2.1 fold increase) at pH 3. Other identified *A. fumigatus* protein e.g. 46 kda IgE-binding antigen, catalase and L10A ribosomal protein gave similar results and showed shown increased expression at acidic and basic pH. The increase tolerance of *A. fumigatus* to caspofungin may be due to elevated expression of certain proteins due to the change in environmental pH.

Rodaki *et al.,* (2009) demonstrated the induction of a number of genes when *C. albicans* was grown at high levels of glucose and these genes were involved in combating osmotic and oxidative stress. As a result of this an increase in the tolerance of *C. albicans* to azole antifungal drugs was observed. Caspofungin is known to cause oxidative and osmotic stress in *C. albicans* (Kelly *et al.,* 2009). A number of proteins were altered in expression in *C. albicans* when grown in elevated concentrations of glucose. A range of proteins were identified by LC-MS, some showed altered expression as shown in Table 3.20. Malate dehydrogenase, NAD-dependent and thioredoxin peroxidase showed an increase in protein expression at 10% glucose by increase to 2.9 and 3.1 fold respectively compared to 1% glucose supplemented media. This alteration in the protein expression may be important in detoxifying the cell at different environmental conditions.

There was a strong increase in expression of proteins when the culture serum concentration was increased; an increase in expression of particular proteins may contribute to the decrease in susceptibility of *C. albicans* and *A. fumigatus* to caspofungin. A number of identified *A. fumigatus* proteins showed a change in expression when the serum level was altered e.g Catalase increase in expression by 3.8 and 2.9 fold in case of 100 and 200 µg/ml BSA supplemented culture respectively. The proteins Secreted dipeptidyl peptidase DppV and Cobalamin-independent methionine synthase Met H/D were up-regulated by 3.7 and 3.5 fold respectively when cultures were supplemented with 100ug/ml BSA. Mycelial catalase Cat1 was increased in expression by 3.2 fold when 200µg/ml BSA was applied to the culture media. The potential mitochondrial inner membrane ATP/ADP translocator and IgE - binding protein were increased in expression by 2.6 and 2.7 fold at 100 µg/ml BSA respectively. A similar result was shown in the case of *C. albicans* when the cells were grown in different serum level e.g Cyclophilin type peptidyl-prolyl cis-trans isomerase and Phosphoglycerate mutase were increased in expression by 1.3 and 2.4 fold at 200ug/ml BSA. Alcohol dehydrogenase 1 and Fructose-bisphosphate aldolase, were increased in expression by 1.4 and 1.3 fold at 200 µg/ml BSA. The mitochondrial protein identified is likely mitochondrial malate dehydrogenase wich showed an increased in expression by 1.4 fold at 200 µg/ml BSA. Some of these proteins may help the cell to combat oxidative stress and thus withstand the effects of caspofungin.

Differences in germination of *A. fumigatus* conidia were found in a variety of carbon sources, including glycerol, acetate, sorbitol, and lactose (Xue *et al.,* 2004). Spore swelling is dependent upon an external supply of carbon compounds. Germination at a fixed incubation time is much reduced by lack of nutrients and is influenced more by the level of external carbon than by that of external nitrogen compounds (Carmbell, 1971). In this study we examined the effect of various levels of FBS on the activity of caspofungin against *A. fumigatus* using a microscopic examination of germ-tubes and hyphae formation. Under a variety of conditions we found a change in the germination rate. At low serum concentrations germination is inhibited at 5 and 10 μg/ml caspofungin as shown in (Figure 3.22), in contrast, at high serum concentrations there is an increase in the germination rate. Similar results were obtained when cultures of *A. fumigatus* were supplemented with high levels of glucose (Figure 3.24).

The results presented here demonstrate that environmental conditions e.g. high glucose level (10%), high serum concentration (200 μg/ml) and acidic pH all increased the activity of catalase, glutathione reductase (GLR) and superoxide dismutase (SOD) in *C. albicans* and *A. fumigatus*. These enzymes may allow the fungus tolerate the osmotic and oxidative effects of caspofungin.

Acidic pH, high serum level and high glucose level lead to an increase the enzymatic activity of *C. albicans* and *A. fumigatus*, which may correlate to the tolerance of these organisms to caspofungin activity. The results presented in this Chapter demonstrate that a variety of environmental conditions alter the susceptibility of *C. albicans* and *A. fumigatus* to caspofungin. These conditions may also alter the activity of caspofungin but this work shows that environment-induced alterations in protein expression, enzymatic activity and germination have the capacity to reduce the sensitivity of these fungi to caspofungin.

Chapter 4

ANALYSIS OF THE EFFECT OF CASPOFUNGIN ON THE LEAKAGE OF INTRACELLULAR CONTENTS FROM *ASPERGILLUS FUMIGATUS*

4.1 Introduction

Caspofungin shows excellent *in vitro* and *in vivo* activity against *Candida* and *Aspergillus* species (Maertens *et al.,* 2004; Zaoutis *et al.,* 2009). It has an excellent safety, it is as effective as, and usually better tolerated than, liposomal amphotericin B (Maertens *et al.,* 2004). Caspofungin was the first member of the echinocandins to be licensed for use, and functions by inhibiting the synthesis of β -1,3-D-glucan, an essential component of the fungal cell wall (Deresinski *et al.,* 2003). It is unlike polyenes and azoles that target ergosterol or the ergosterol biosynthetic pathway, respectively. Caspofungin mediated disruption of glucan synthesis leads to the formation of an osmotically fragile aberrant cell wall and osmotic lysis of the cell at high concentrations due to a long fatty acid side chain that may allow intercalation in the bi-layer of the fungal cell membrane (Deresinski *et al.,* 2003; Denning, 2003). *A. fumigatus* is an important fungal pathogen, particularly affecting those with pre-existing pulmonary malfunction (e.g., asthma, cystic fibrosis), disease (e.g., cancer, tuberculosis, chronic granulomatous disease), or undergoing immunosuppressive therapy prior to organ transplantation (Brookman and Denning, 2000). The fungus can induce a variety of diseases including allergic and invasive aspergillosis (Denning, 1996). *A. fumigatus* displays a number of virulence factors that may facilitate tissue colonization and persistence in the host (Amitani *et al.,*1995; Rementeria *et al.,*2005). One of these, gliotoxin (C_{13} H_{14} N_2 O_4 S_2), is an epipolythiodioxopiperazine toxin (Waring and Beaver

1996), which displays immunosuppressive properties *in vivo* (Sutton *et al.,*1995). Gliotoxin is capable of inhibiting macrophage function and may alter the immune response to *Aspergillus* as it can induce apoptotic cell death in macrophages (Waring and Beaver 1996; Sutton *et al.,* 1995), and disrupt the function of neutrophils (Tsunawaki *et al.,* 2004). In addition, gliotoxin has been implicated in the destruction of lung parenchyma in invasive aspergillosis (Sutton *et al.,* 1995) and the penetration of blood vessels in angio-invasive aspergillosis (Fraser, 1993). Caspofungin has previously been shown to induce amino acid and protein leakage from growth-arrested stationary phase *C. albicans* cells indicating that inhibition of glucan biosynthesis may not be the only mode of action exhibited by this drug (Kelly and Kavanagh, 2010). It was postulated that caspofungin could have a secondary effect in altering membrane permeability. The aim of the work presented here was to establish if exposure of *A. fumigatus* to caspofungin induced leakage of intracellular amino acids, protein and gliotoxin and whether the fungus responded to this by synthesizing elevated levels of toxin intra-cellularly. The immunosuppressive effects of gliotoxin are well established (Sutton *et al.,* 1995; Tsunawaki *et al.,* 2004) and any therapy that inadvertently induced the increased biosynthesis and release of this toxin could exacerbate inflammation and tissue damage at the site of infection.

4.2 Analysis of amino acid leakage from *A. fumigatus*

A. fumigatus hyphae were assessed for amino acid leakage following caspofungin treatment. Free amino acids were measured using the Ninhydrin method as described previously in section 2.26. All results expressed are in term of aspartic acid and glutamic acid, which were used as standards.

4.2.1 Effect of DMSO on the amino acid leakage from *A. fumigatus* hyphae

A. fumigatus conidia were inoculated into 50 ml of RPMI1640 and grown for 96 hours as described in section 2.26. Hyphae were collected by filtering and washed as previously described. The hyphal mass (2 g) was re-suspended in 25 ml PBS containing 0.5% v/v Dimethyl sulfoxide (DMSO), cultures were incubated at 37 °C and 200 rpm for another 4 hours. At 4-hour time point 750 µl of culture filtrate was removed and filtered

through 0.45 μM syringe filters (Sarstedt). Free amino acids were measured using the Ninhydrin colorimetric method (Reeves *et al.,* 2004). The results show a significant increase (P = 0.02) in the amount of free amino acids when the culture was supplemented with DMSO compared to control as shown in Figure 4.1.

4.2.2 Effect of culture media on the amino acid leakage

Four different types of culture media (Antibiotic medium 3, *Aspergillus* minimal media, Nutrient broth and RPMI1640) were used to determine the amount of amino acid, which leaked from *A. fumigatus* hyphae at three different time point (0.5, 2 and 4 hours) of incubation at 37 ^{o}C, and 200 rpm. Ninety six hour old culture hyphae were collected using miracloth (Calbiochem) to remove the *A. fumigatus* hyphae, one gram of dry hyphae was washed twice with PBS, and resuspended in 25 ml PBS. At each time point 750 μl of culture filtrate were removed and filtered through 0.45 μM syringe filters (Sarstedt). Free amino acids were measured using the ninhydrin colorimetric method as previously described. Results in Figure 4.2 show that different media affect the amount of amino acid released from *A. fumigatus* hyphae, as the highest release of amino acid occurred when *A. fumigatus* was grown in RPMI1640 culture media for 4 hour.

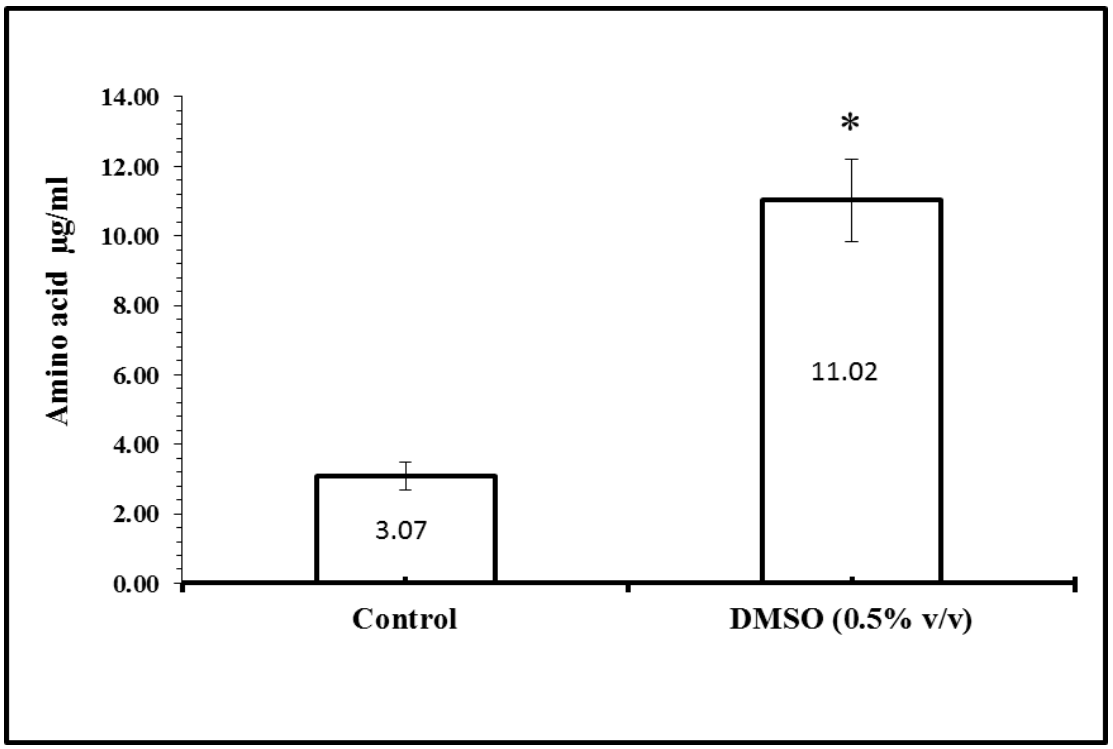


Figure 4.1 Determination the effect of DMSO on amino acid leakage from *A. fumigatus* hyphae.

96 hour old hyphae were used to assess the amount of amino acid that escaped from the cells. * p = 0.02

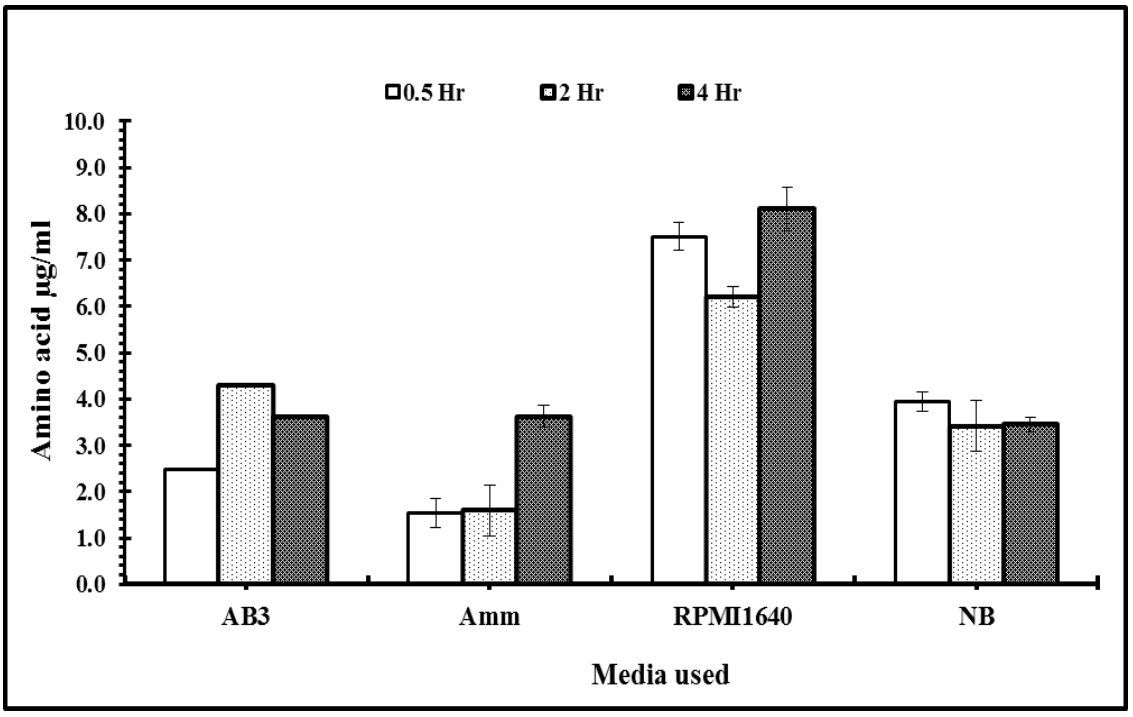


Figure 4.2 Four different types of culture media were used to determine the amount of amino acid leakage.

Amino acid released from *A. fumigatus* hyphae for 0.5, 2 and 4 hours incubation at 37 ^{o}C and 200rpm.

4.2.3 Effect of low and high dose of caspofungin on amino acid leakage

Caspofungin has previously been shown to induce amino acid and protein leakage from stationary phase *C. albicans* (Kelly and Kavanagh, 2010). *A. fumigatus* culture was grown for 96 hours as described in section 2.21. Two gram of hyphae were re-suspended in 25 ml PBS and exposed to 0.5% v/v DMSO (as a positive control), or caspofungin (0.1 µg/ml or 1.0 µg/ml) for 4 hour. The amount of amino acid in the supernatant was measured as described previously. The result in Figure 4.3 shows highest amount of amino acid was detected when the hyphae were exposed to 1.0 µg/ml caspofungin

4.2.4 Effect of the caspofungin dose and incubation time on the amino acid leakage

Three different concentrations of caspofungin were used on *A. fumigatus* hyphae which were grown for 24, 48, 72 and 96 hour in RPMI1640. Amino acid leakage was measured for all samples. Results show a proportional increase in the amount of amino acid leakage with the increase in caspofungin concentration, the highest level of amino acid was detected in 72 hour old culture exposed to 1.0 µg/ml caspofungin as shown in Figure 4.4.

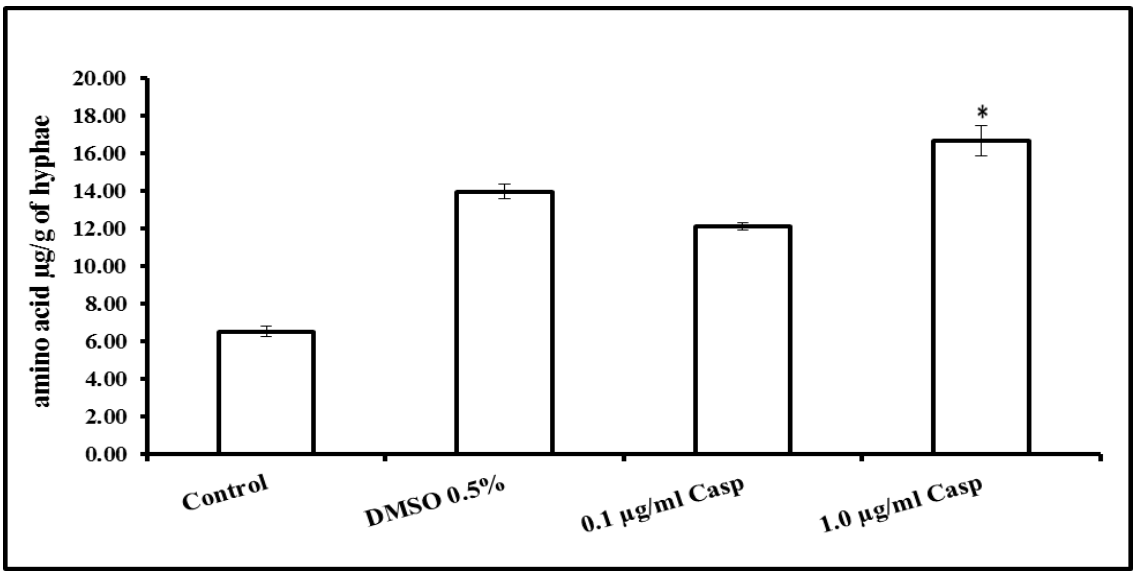


Figure 4.3 The effect of caspofungin on amino acid leakage from the hyphae of *A. fumigatus*.

Four hours incubation with 0.1 and 1.0 µg/ml caspofungin, 0.5 % v/v DMSO as a positive control. Average of three independent experiments. *P = 0.03

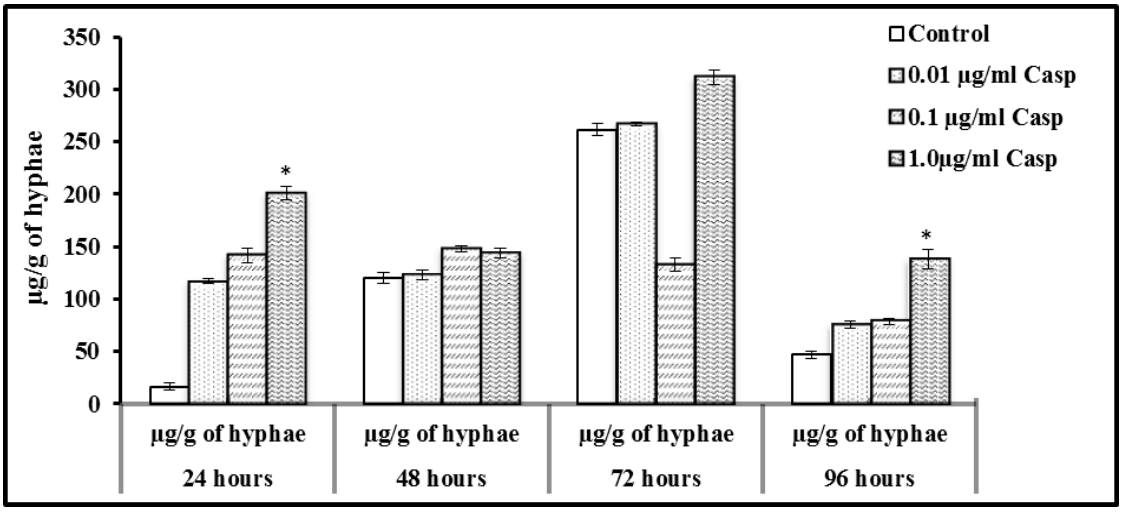


Figure 4.4 Amino acids leakage from *A. fumigatus* hyphae at various incubation times with three different concentrations of caspofungin compared to control.

*P = 0.025,

4.2.5 The effect of time on the amino acid release from the *A. fumigatus* hyphae in presence of drug

Further investigations of amino acids leakage from the *A. fumigatus* hyphae were conducted using low and high doses of caspofungin at three time points (1/2, 2 and 4 hours). The release of intracellular amino acid was investigated as described previously. In Figure 4.5 the results indicate the variable amount of amino acid leakage detected when the cultures were exposed to drugs at different times. Results show an increase in the release of amino acid when hyphae were treated with drugs particularly at 4 hours, the highest amount of amino acid detected was 5.5 µg/ml at a dose of 1.0 µg/ml caspofungin.

4.3 Analysis of protein leakage from *A. fumigatus*

Caspofungin is widely used in the treatment of systemic fungal infections and has the advantage of being highly effective but of negligible toxicity to humans (Denning, 2003). Previous work established that exposure of *A. fumigatus* to the polyene antifungal agent Amphotericin B lead to leaking of intracellular content including proteins (Reeves *et al.,* 2004). To determine the quantity of protein released from the *A. fumigatus* hyphae, samples were assayed using the Bradford assay (Bio-Rad) as previously described in section 2.23, with BSA (Sigma Aldrich) as standard.

4.3.1 Examination of the effect of DMSO on protein Leakage from *A. fumigatus*

Stationary phase (96 hour) *A. fumigatus* hyphae were chosen for this analysis since glucan synthesis would be low in a stationary phase cultures. Figure 4.6 shows the amount of protein released into the media at indicated time. Results indicate that the amount of protein released increased as the exposure of hyphae to DMSO increased compared to the control.

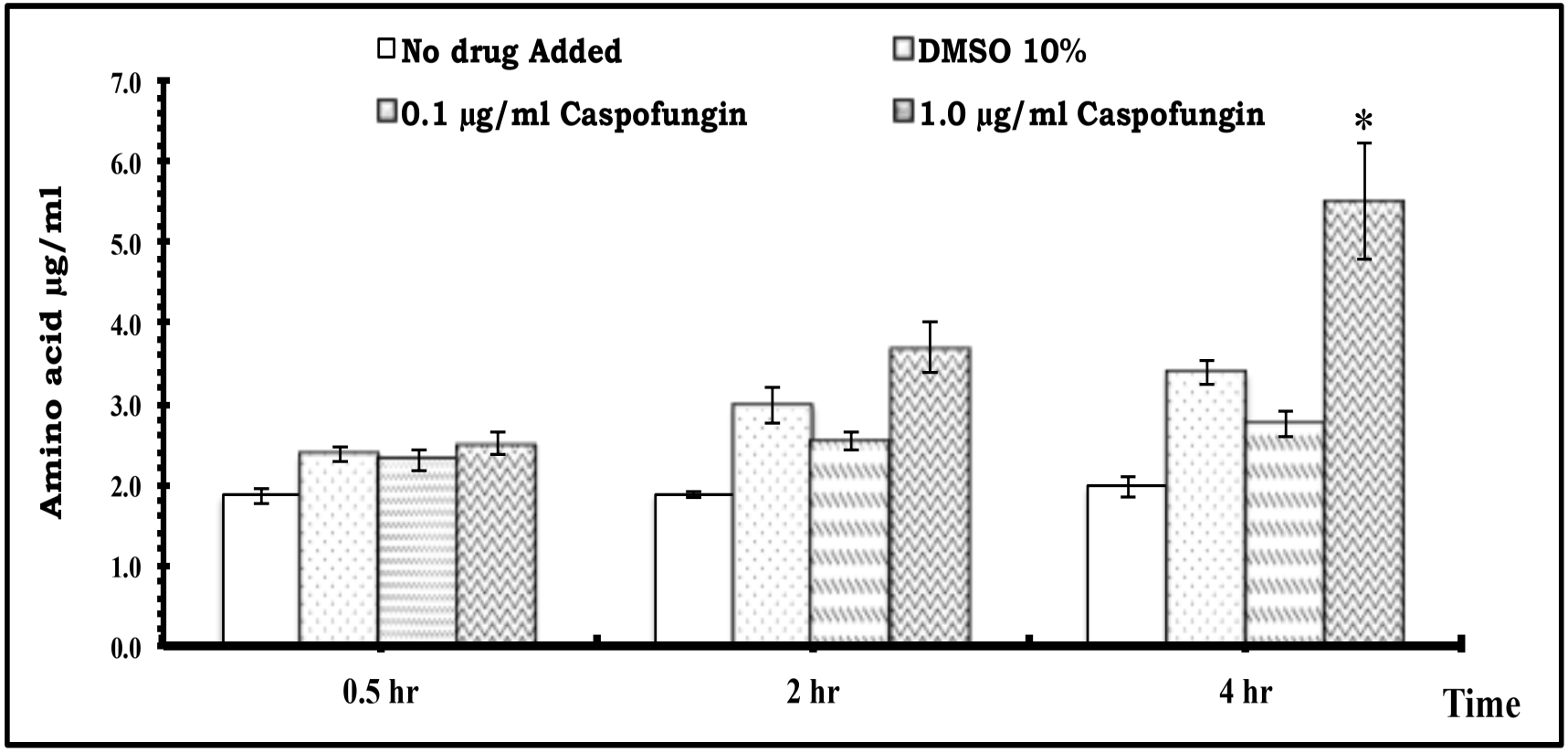


Figure 4.5 Amino acid leaked from *A. fumigatus* hyphae when exposed to Caspofungin for 0.5, 2 and 4 hour at 37 ^{o}C and 200rpm. DMSO used as a positive control. *P = 0.05

4.3.2 Effect of caspofungin on protein leakage from *A. fumigatus*

A. fumigatus conidia were inoculated into 50 ml of RPM1640 plus 5% (v/v) FBS, the culture was incubated and hyphae were harvested as described in section 2.23. Hyphae (1.5g) were re-suspended in either PBS (5 ml), DMSO (0.5% (v/v); 5 ml) or caspofungin (0.1 or 1.0 µg/ml; 5 ml) and incubated at 37 °C for 240 minutes. To determine the quantity of protein released from the hyphal mass, supernatants at 0, 30, 120, and 240 minutes were assayed for their protein content using a Bradford assay. The result is shown in Figure 4.7. The amount of protein released was increased when the culture was treated with DMSO and different doses of caspofungin at three time points.

4.3.3 Summary

These result show that large amounts of amino acids and protein escaped from the cells when hyphae were exposed to DMSO and caspofungin at different time point.

4.4 Analysis of the effect of caspofungin on biosynthesis and release of gliotoxin from *A. fumigatus*

A. fumigatus is known to produce various immunosuppressive mycotoxins including gliotoxin and the high concentration of oxygen in the lung may provide optimal conditions for the production of gliotoxin by *A. fumigatus*. *A. fumigatus* has been shown to produce a range of secondary metabolites/mycotoxins with different harmful effects. These include gliotoxin, fumagillin, helvolic acid, fumitremorgin, fumigaclavine C, aureperone C, restrictocin (Kamei and Wanatabe, 2005; Dagenais and Keller, 2009). Gliotoxin is an epipolythiodioxopiperazine (ETP) type toxin, which is characterised by a disulphide bridge (Fox and Howlett, 2008), and is the best-characterised mycotoxin and most potent secondary metabolite produced by *A. fumigatus* (Kwon-Chung and Sugui, 2009). Spikes *et al.,* (2008) demonstrated that gene clusters responsible for the biosynthesis of ETP type toxin have been identified in *A. fumigatus,* which is responsible for the production of gliotoxin.

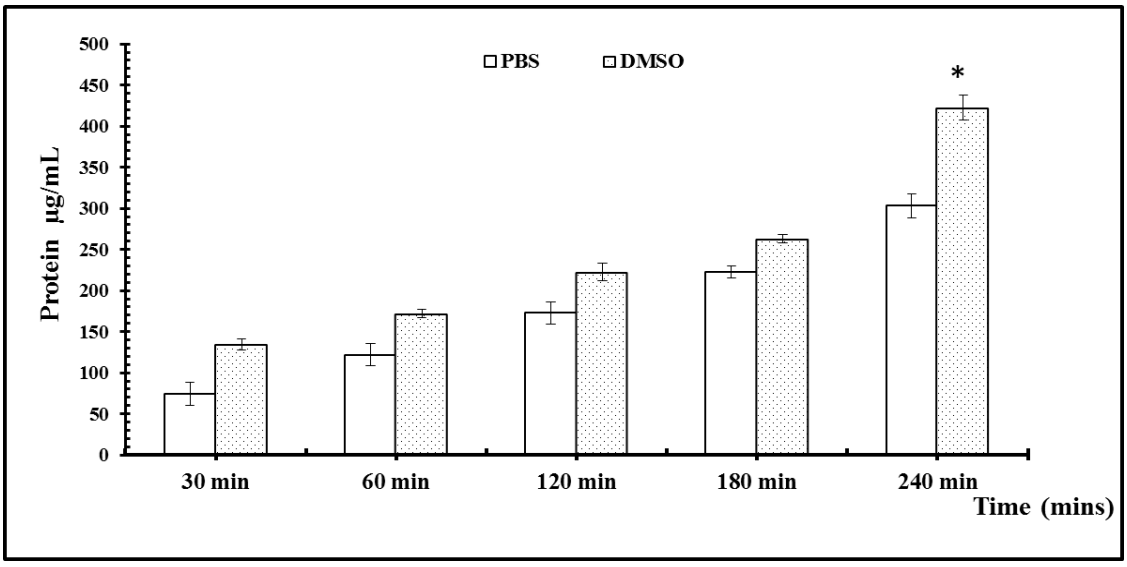


Figure 4.6 Protein leakage from *A. fumigatus* at different time points for control and DMSO treated hyphae.

P = 0.02

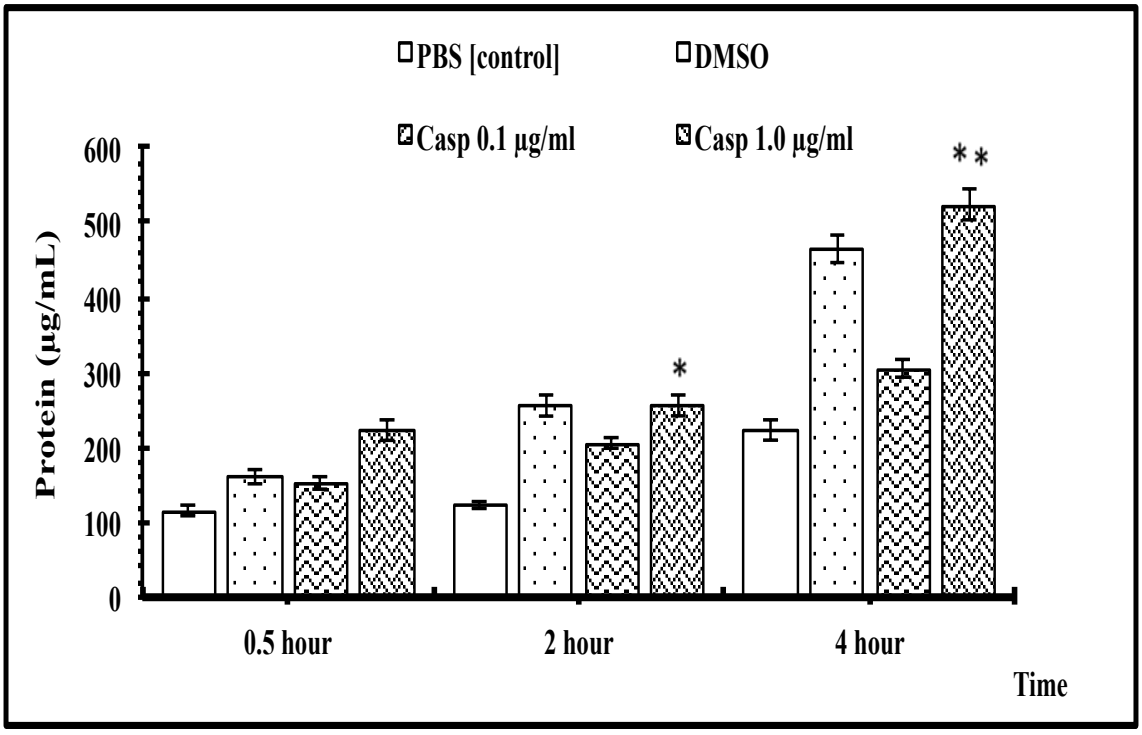


Figure 4.7 Amount of protein leaking (µg/ml) from *A. fumigatus* exposed to 0.1 and 1.0 µg/ml caspofungin as determined using standard curve of BSA at three different times 0.5, 2 and 4 hour.

*P = 0.023, **P = 0.05

4.4.1 Effect of different type of culture media on the growth of *A. fumigatus*

Sterile flasks containing 100 ml of RPMI1640, AB3, AMM and NB were inoculated with *A. fumigatus* conidia as described in section 2.18 to give a concentration of $1x10^5$conidia/ml and incubated at 37 °C and 200 rpm for 96 hour. After 96 hours the contents were filtered and squeeze through Miracloth filters (Calbiochem). Dry weights were recorded for each type of culture media. Results show that when AB3 and RPMI1640 were used to culture the *A. fumigatus,* a high growth rate was achieved at ninety-six hour incubation and gave 2.8 and 2.3 gram per 100 mls, respectively.

4.4.2 Effect of caspofungin on the Growth of *A. fumigatus* using RPMI1640

MEA plates containing sporulating *A. fumigatus* colonies were washed with 10 ml of 0.01% (v/v) Tween 80 (Merck) in Phosphate Buffered Saline (PBS, pH 7.2; Sigma Aldrich) to isolate conidia. The latter were washed twice in sterile PBS, harvested by centrifugation (1,500 g, 5 min in a Beckman GS-6 centrifuge) and enumerated using a haemocytometer. *A. fumigatus* conidia were inoculated in RPMI1640 as described in section 2.8. Cultures were supplemented with caspofungin (0.01, 0.1 or 1.0 μg/ml) for various times (24, 48, 72 and 96 hours).

It was previously determined in section 3.4 that a concentration of 0.1 μg/ml caspofungin inhibited growth of *A. fumigatus* by approximately 50%. A growth curve was constructed of fungal biomass versus incubation time. Results show the *A. fumigatus* growth was inhibited by 50% at a concentration of 0.1 μg/ml caspofungin and 80% at concentration of 1.0 μg/ml caspofungin at 72-hour incubation time (Figure 4.9).

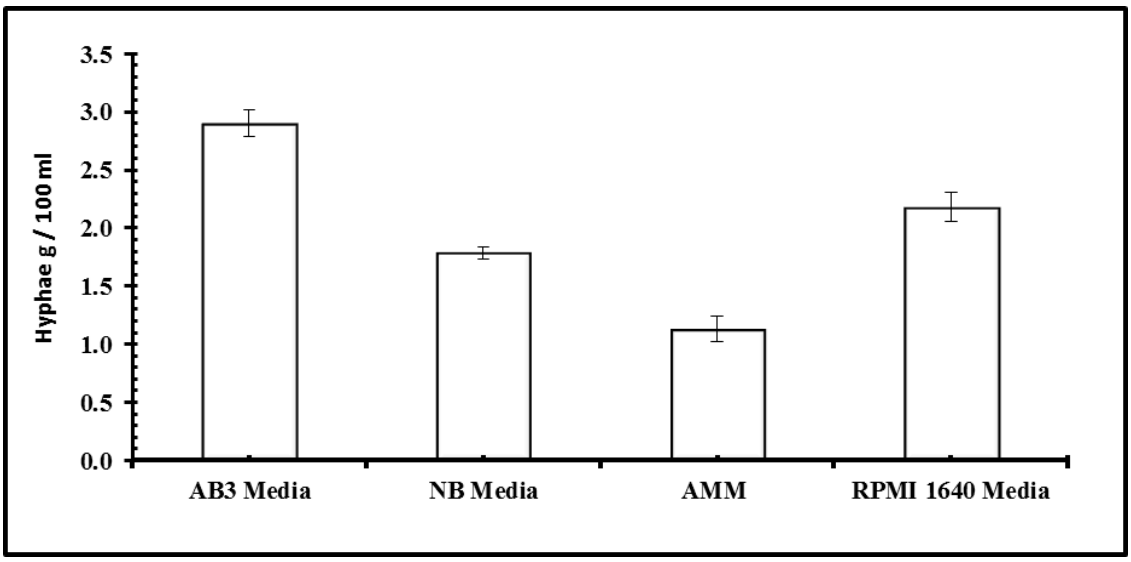


Figure 4.8 Determination of hyphal weight (gram) after 96-hour incubation at 37°C, 200rpm, and using four types of culture media.

250 ml conical flasks were used and the total volume was 100 ml for each medium.

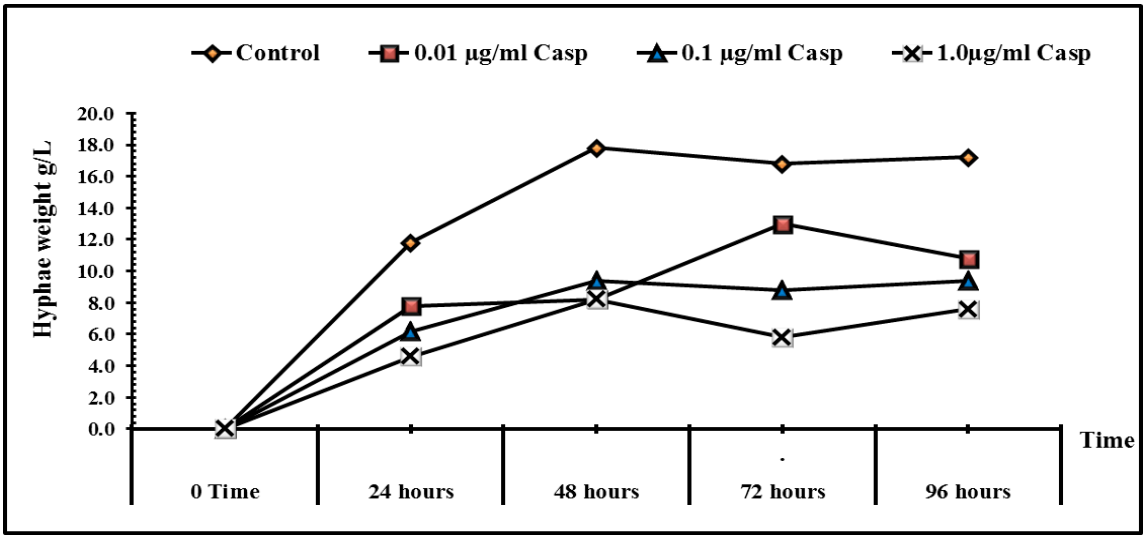


Figure 4.9 Hyphae weight at different incubation times with three different Caspofungin concentration using RPMI1640 culture media.

4.4.3 The effect of environmental conditions on the biosynthesis and release of gliotoxin

Cultures of *A. fumigatus* (72-hour-old) were used in this experiment. *Aspergillus* hyphae or culture contents from different environmental conditions were filtered through miracloth (Calbiochem), weights were recorded and gliotoxin was extracted from the culture filtrate by mixing with an equal amount of chloroform (Sigma-Aldrich) for 2 hour. The extraction of gliotoxin from the chloroform layer was accomplished by rotary evaporation at 55 °C. The dried extracts were dissolved in 250 µl of molecular-grade methanol (Sigma-Aldrich) and the level of gliotoxin determined by Reverse phase high performance liquid chromatography (RP-HPLC).

4.4.3.1 The effect of Glucose on Gliotoxin biosynthesis and release from *A. fumigatus* hyphae

RPMI1640 culture media was used in this experiment. A range of glucose concentrations were used to determine the effect of glucose on the gliotoxin release from the *A. fumigatus* hyphae. Cultures were supplemented with different amounts of glucose (0.5, 1.0, 2.5 or 5.0 %), cultures were incubated as described in section 2.18, gliotoxin concentration from each condition was determined using RP-HPLC. Results were recorded and presented in Figure 4.10. Results show the highest amount of gliotoxin release was in 2.5% glucose supplemented culture media and the high at value for intracellular gliotoxin was recorded in 1.0% glucose supplemented culture media.

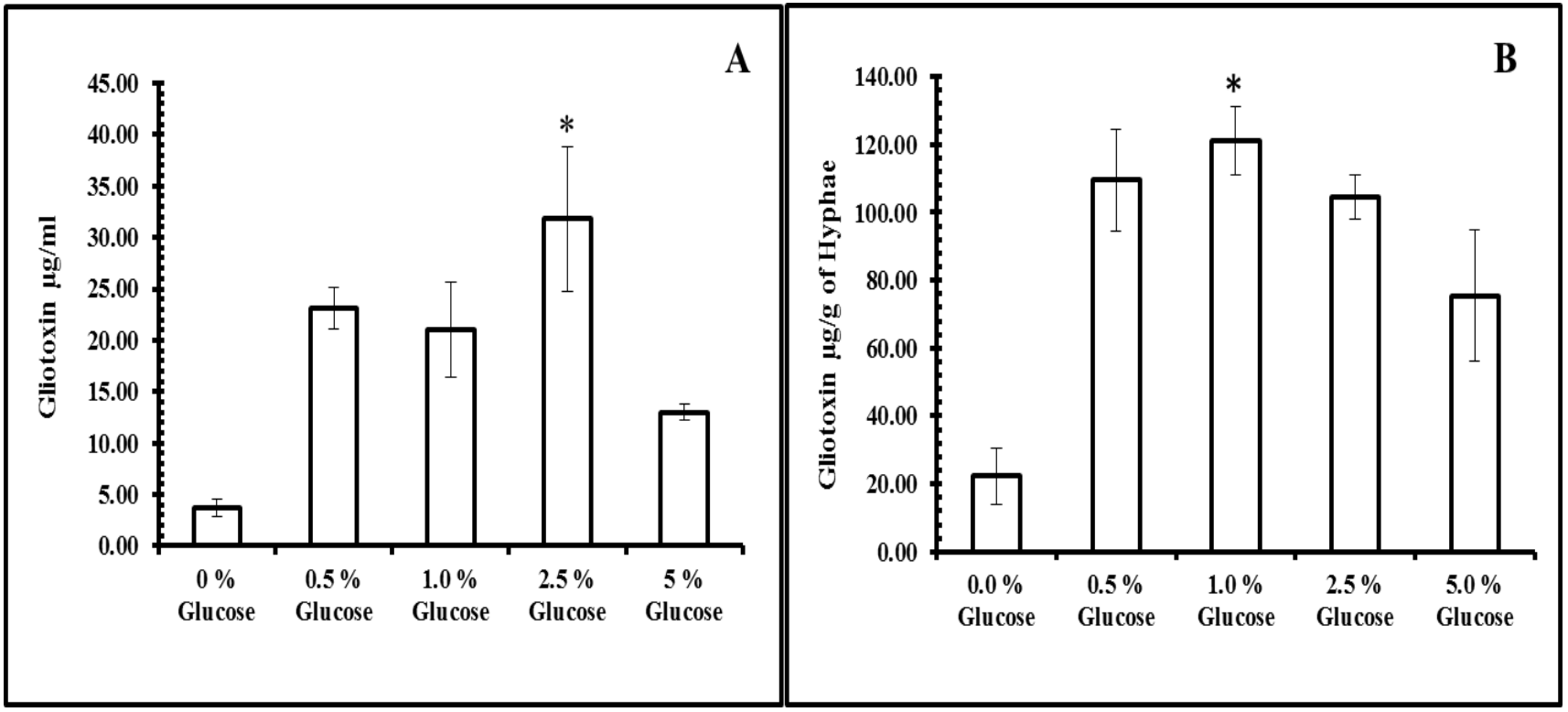


Figure 4.10 Effect of glucose on the extracellular (A) and intracellular (B) gliotoxin, *A. fumigatus* was grown on RPMI1640 for 72 hour in different concentrations of glucose.

P = 0.025

4.4.3.2 Effect of Fetal bovine serum (FBS) on the biosynthesis and release of Gliotoxin from *A. fumigatus* hyphae

RPMI1640 medium was supplemented with FBS (1.0, 2.5, 5.0 or 10 %) and all cultures were incubated as described in section 2.20.2. Gliotoxin concentration was determined as described in section 2.20.7. Further experiments were conducted in MEM using 10, 20 and 30 % FBS compared to optimal condition (pH 7, 2% glucose and 5% FBS). The HPLC results were recorded and the results plotted are shown in Figures 4.11 and 4.12. Results in Figure 4.11 indicate that the 10% FBS supplemented cultures show the highest amount of extracellular gliotoxin and 5% FBS medium gave the high amount of intracellular gliotoxin. The result in Figure 4.12 show the highest amount of internal and external gliotoxin was observed for hyphae grown in 20% of FBS medium.

4.4.3.3 Effect of pH on gliotoxin biosynthesis and release from *A. fumigatus* hyphae

Media of different pH (3.5, 4.5, 5.5, 6.5 or 7.5) were used and values were adjusted for the culture media before conidia were inoculated. *A. fumigatus* culture conditions were maintained as described in section 2.18. Gliotoxin release from the hyphae was determined using RP-HPLC; the results are presented in Figure 4.13. Results show that the media with pH value of 7.5 gave the highest amount of gliotoxin biosynthesis and release from the hyphae and the lowest amount was recorded at pH 3.5.

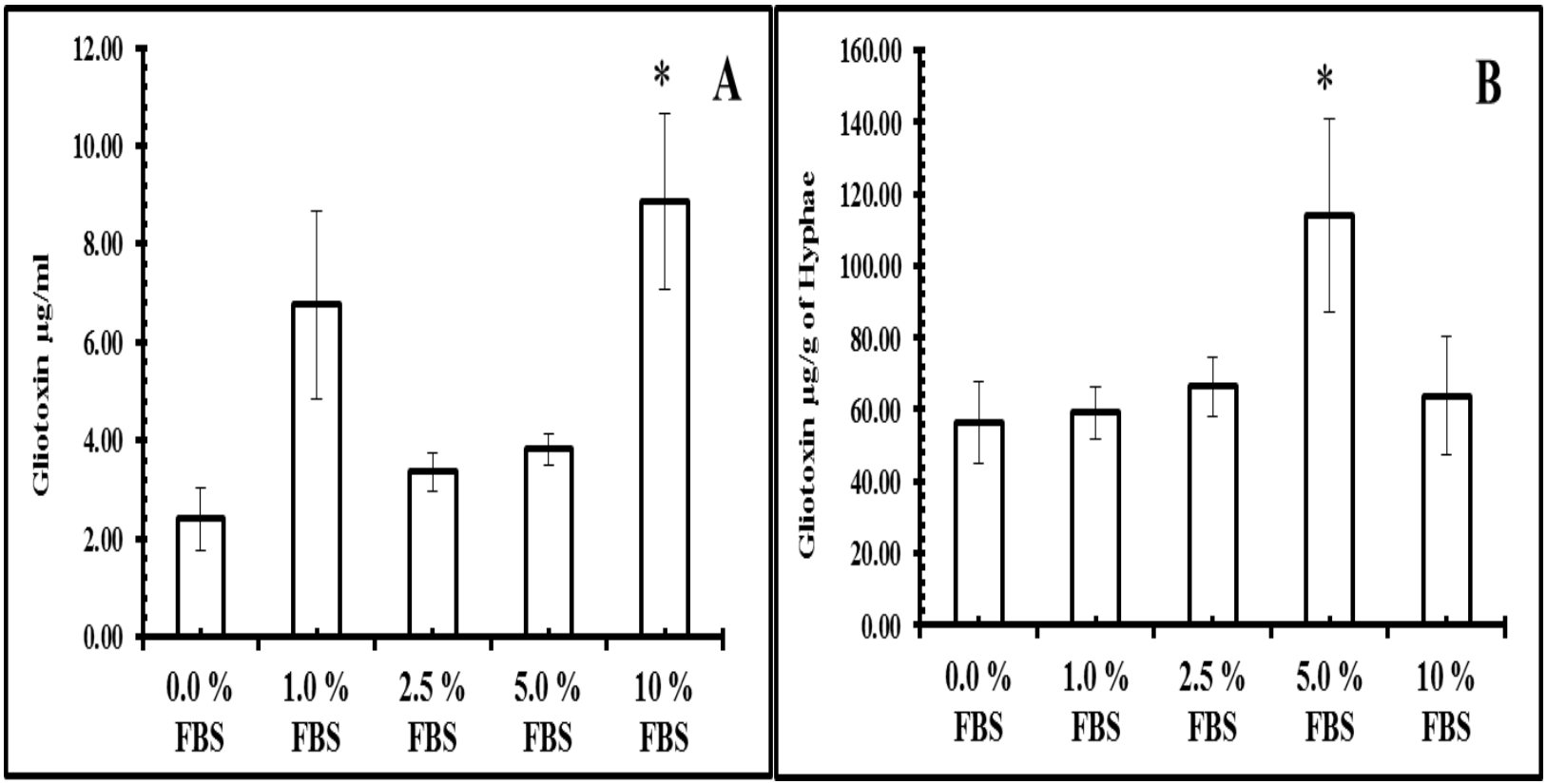


Figure 4.11 Effect of FBS on the external (A) and internal (B) gliotoxin, *A. fumigatus* was grown in RPMI1640 for 72 hour at different concentration of FBS. P = 0.05

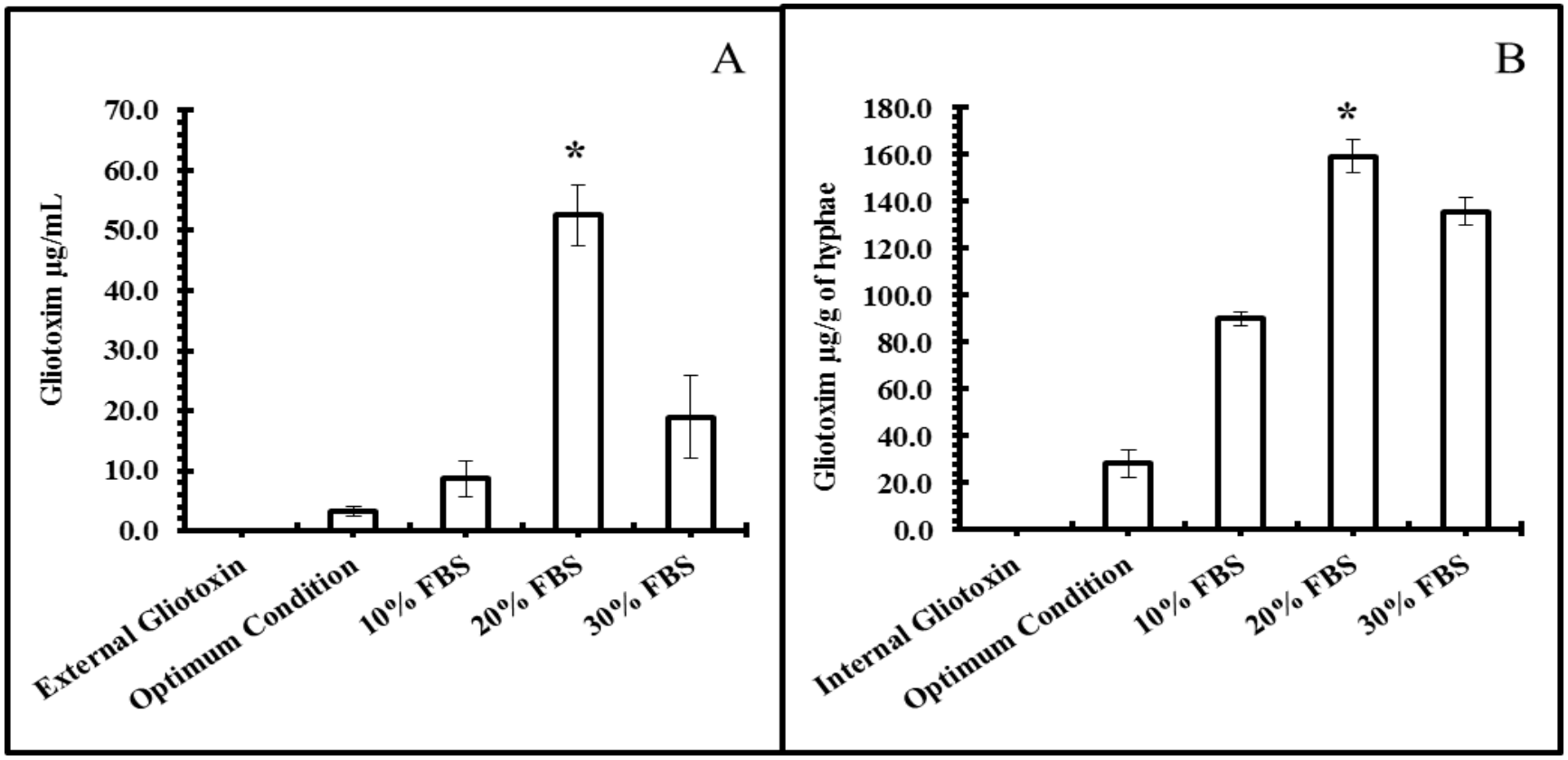


Figure 4.12 Effect of three different concentrations of FBS on the release (A) and biosynthesis (B) of gliotoxin from *A. fumigatus*, using MEM. The optimal condition (pH 7, glucose 2% and 5% FBS). P = 0.023

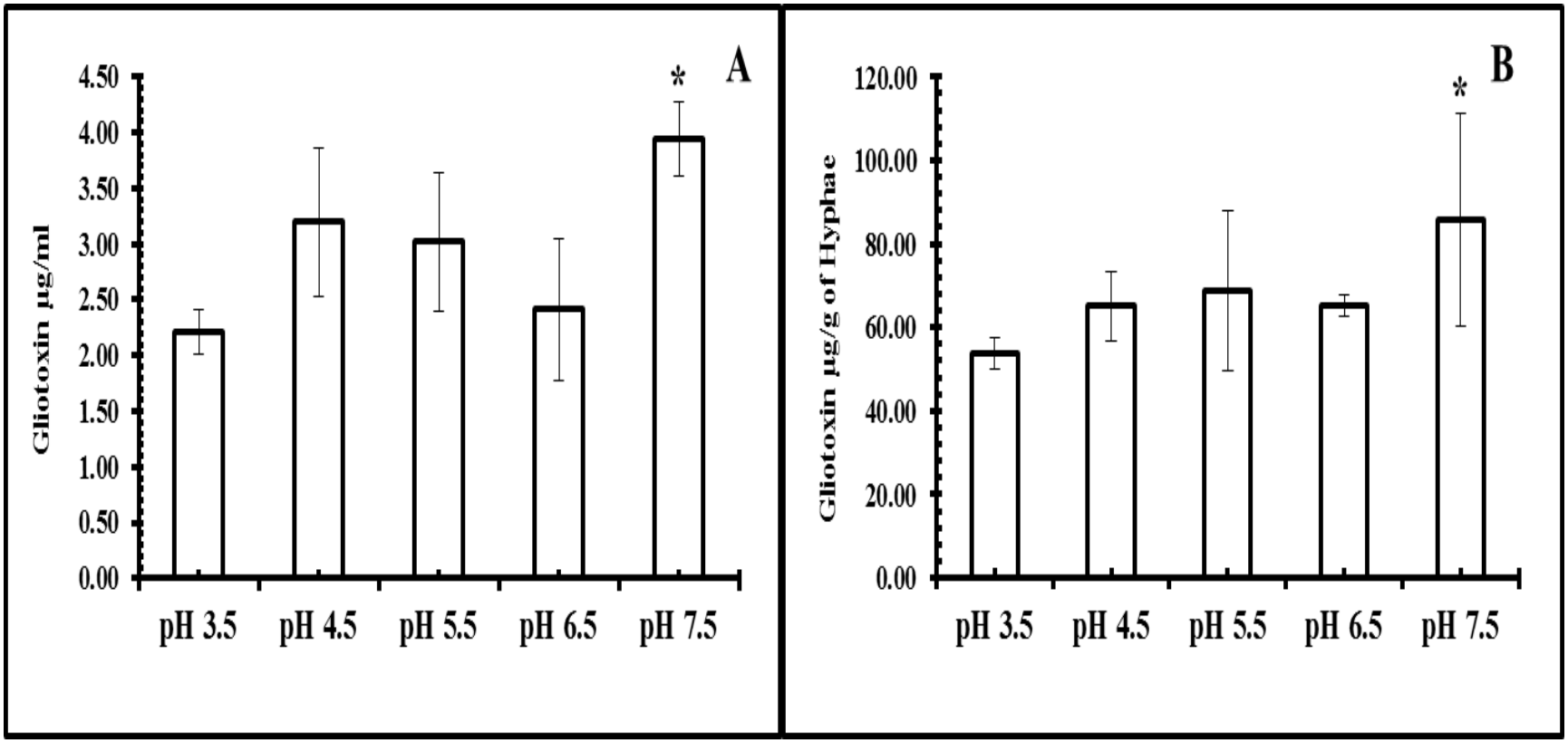


Figure 4.13 Effect of pH on the internal and external concentration of gliotoxin. *A. fumigatus* was grown on RPMI1640 for 72 hour at different pH values. P = 0.05

4.4.3.4 Effect of aeration on gliotoxin release from and biosynthesis in *A. fumigatus*

In order to study the effect of aeration on gliotoxin release from *A. fumigatus,* hyphae were incubated at 25 ^{o}C, 30 ^{o}C and 37 ^{o}C under static or shaking condition to provide an oxygen supply to the culture. After 72 hours of incubation, all samples were taken and *A. fumigatus* filtrates were used to extract the gliotoxin released from the hyphae by mixing with equal volums of chloroform. The gliotoxin concentration in each sample was determined using RP-HPLC and the results are plotted in Figures 4.14 and 4.15. The results show the highest amount detected in *Aspergillus* filtrate media and intracellular gliotoxin at 37 ^{o}C and 200 rpm which show 6.5 (±1.1) µg/ml and 180 (±12.1) µg/g of hyphae. For the 25 ^{o}C incubation there was not enough growth for internal and external gliotoxin concentration to be measured.

4.4.3.5 Determination of the effect of incubation time on gliotoxin production using 4 types of culture media

To determine the effect of incubation time on the gliotoxin release from *A. fumigatus* hyphae, four types of culture media were used in this experiment. *A. fumigatus* conidia were inoculated into each medium as described in section 2.20.4. Gliotoxin was extracted from culture filtrate using chloroform. Results are expressed as µg/ml.

The level of gliotoxin released by *A. fumigatus* was detected in culture filtrate after 24, 48, 72, and 96 hour incubation at 37 ^{o}C, 200 rpm and measured using RP-HPLC. Results show different types of culture media gave different amounts of gliotoxin release, Figure 4.16 and 4.17 show highest values were recorded at 72 hour with RPMI1640 and AMM, but in 96 hour old culture greater values were recorded when the MEM and AMM were used as shown in Figure 4.18 and 4.19.

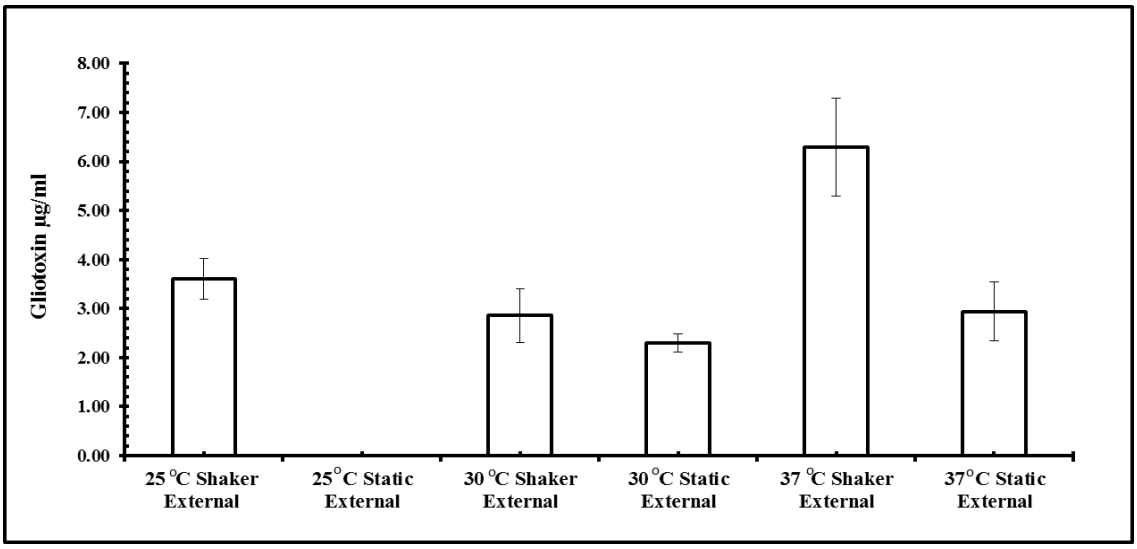


Figure 4.14 Effect of aeration on the release of gliotoxin from *A. fumigatus.*

Cells were grown on RPMI1640 for 72 hour at different aeration [Shaker, static,] and temperature conditions [25, 30 and 37 ^{o}C].

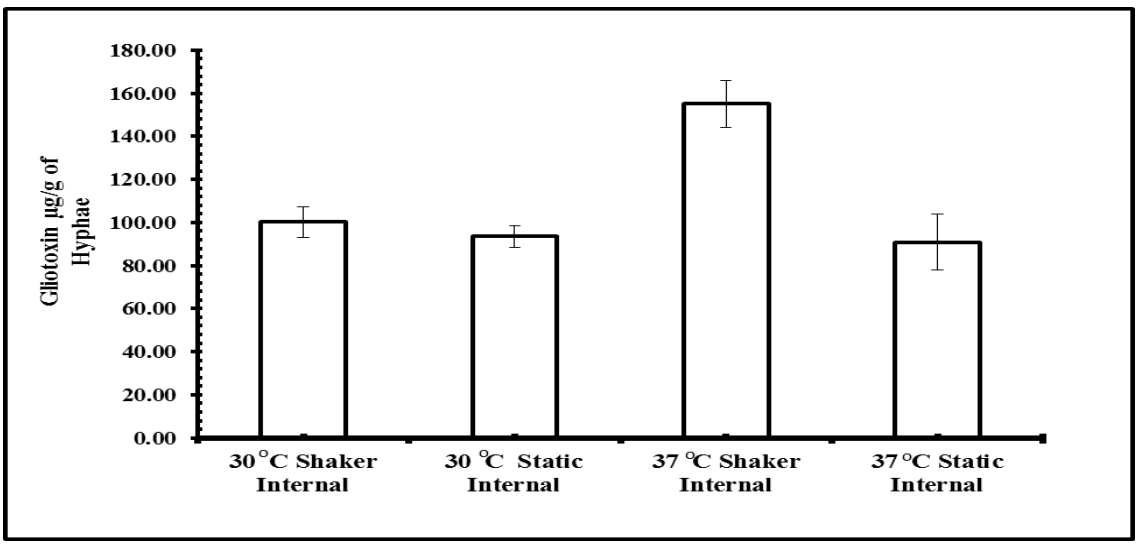


Figure 4.15 Effect of aeration on the internal gliotoxin, *A. fumigatus*

Conidia were grown on RPMI1640 after 72-hour incubation at different aeration [Shaker, static,] and temperature conditions [30 and 37 ^{o}C].

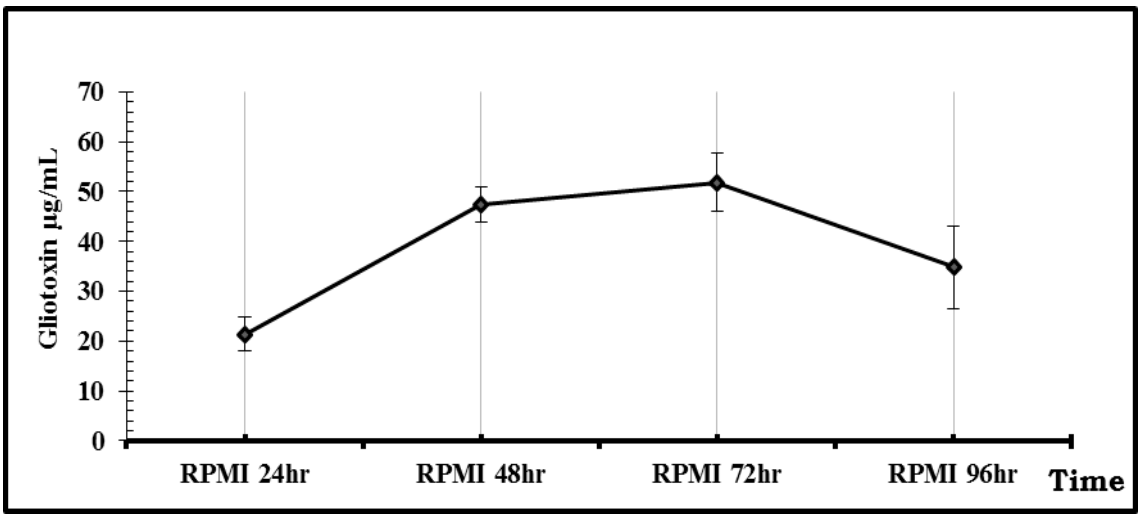


Figure 4.16 Gliotoxin production at 24, 48, 72 and 96 hour when the *A. fumigatus* conidia were grown in RPMI1640 supplemented with 5% FBS at 37 ^{o}C and 200rpm.

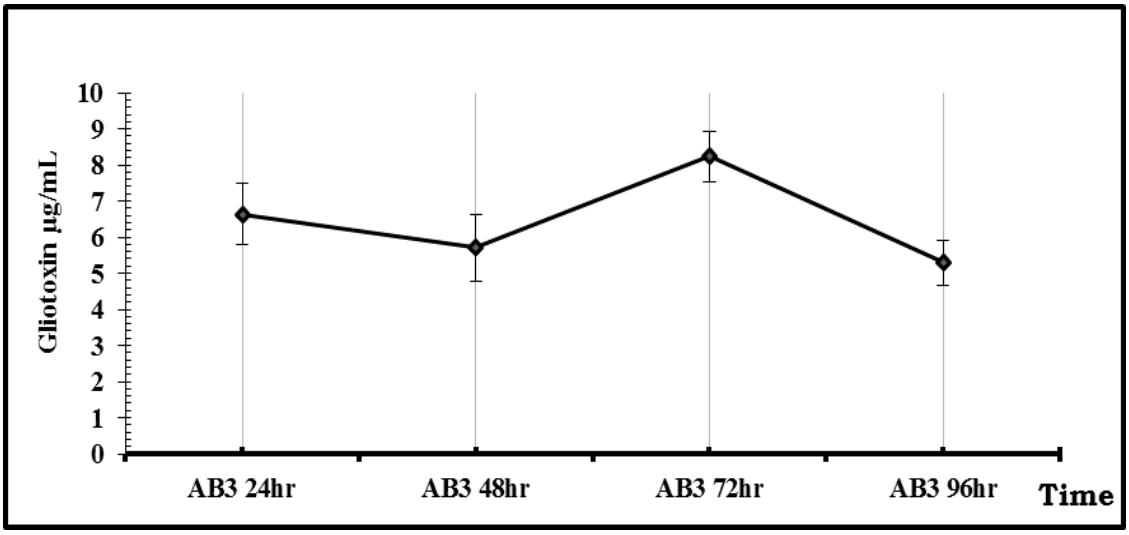


Figure 4.17 Gliotoxin production at 24, 48, 72 and 96 hour when the *A. fumigatus* conidia were grown in AB3 at 37 ^{o}C and 200rpm.

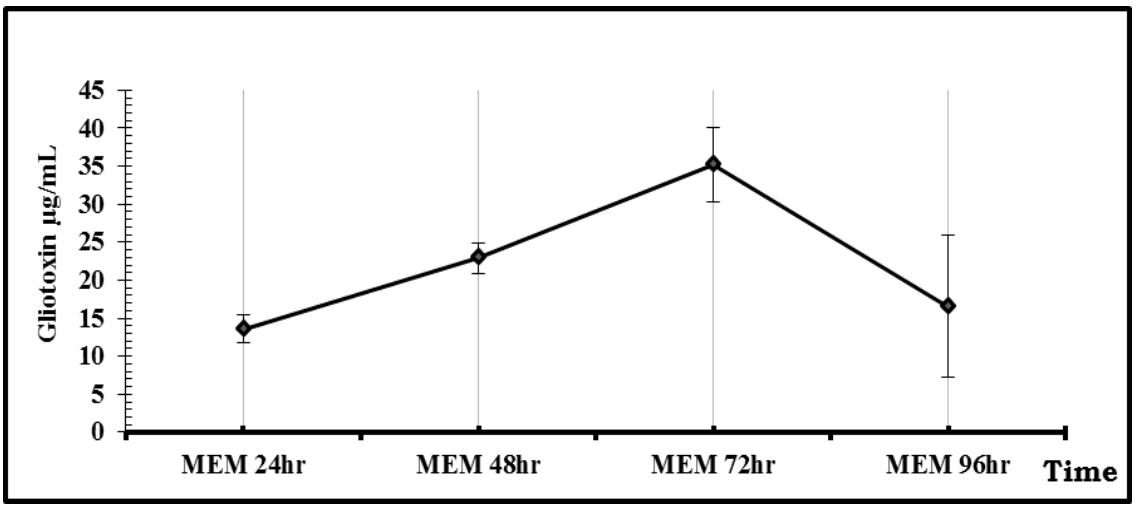


Figure 4.18 Gliotoxin production at 24, 48, 72 and 96 hour when the *A. fumigatus* conidia were grown in MEM supplemented with 5% FBS at 37 ^{o}C and 200rpm.

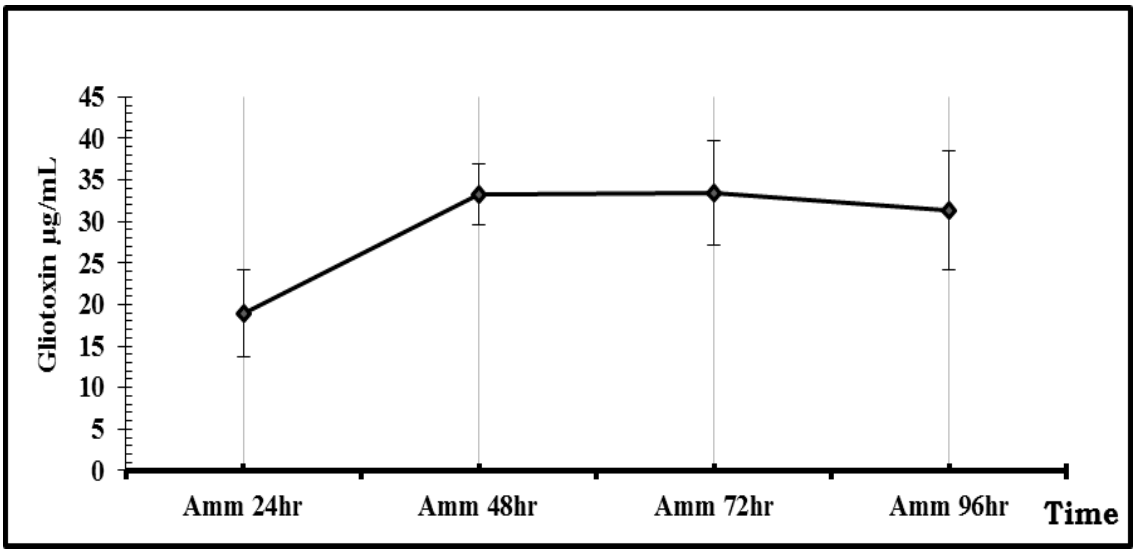


Figure 4.19 Gliotoxin released at 24, 48, 72 and 96 hour when the *A. fumigatus* conidia were grown in AMM at 37 ^{o}C and 200rpm.

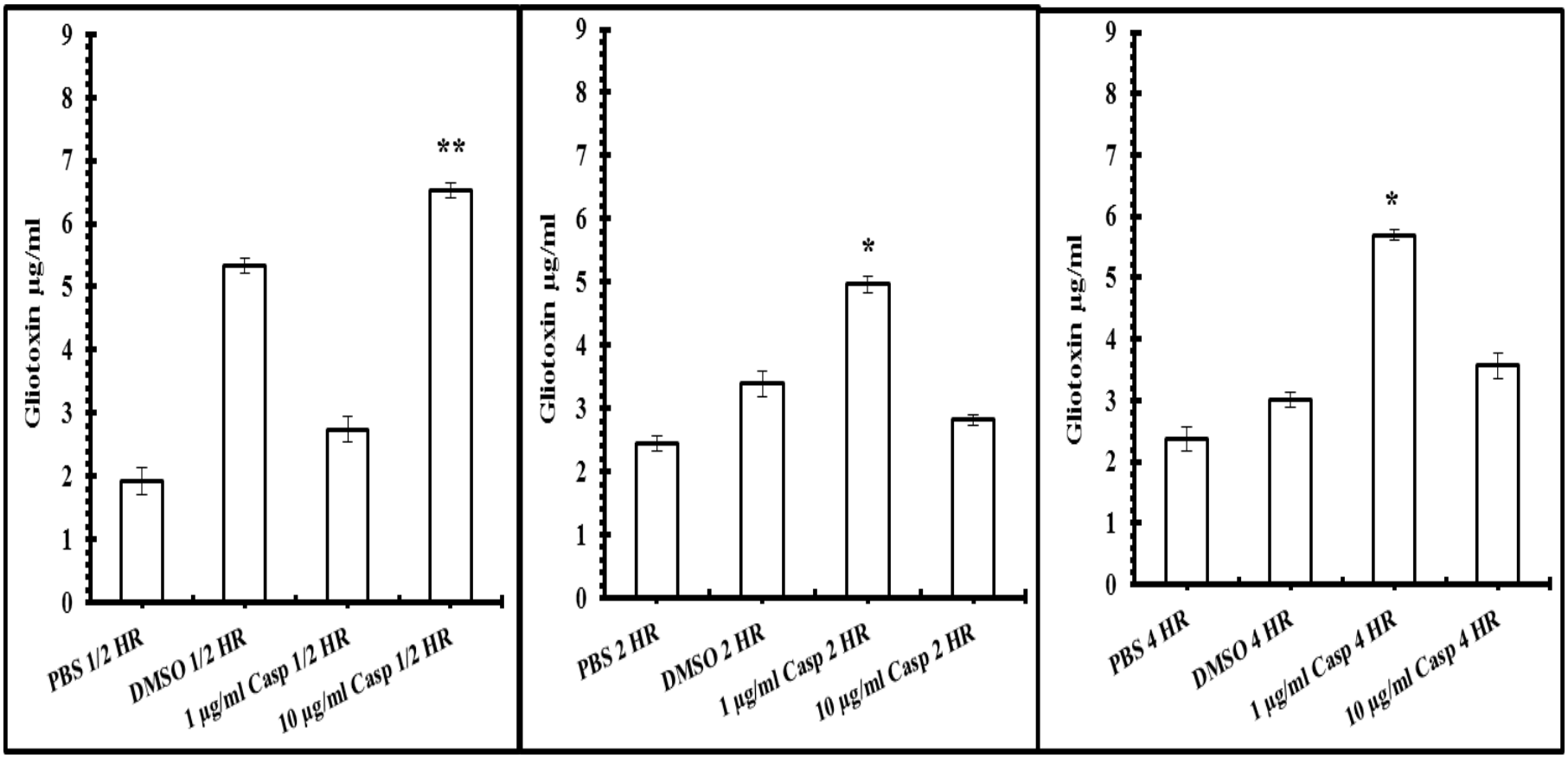


Figure 4.20 Effect of caspofungin on gliotoxin release, from *A. fumigatus* (96 hour old).

Culture treated with low (1.0 µg/ml) and high (10 µg/ml) doses of Caspofungin, at 0.5, 2 and 4 hour using one gram hyphae mass of (96 hour old culture). *$P < 0.05$, **$P = 0.02$

4.4.3.6 Effect of caspofungin on the gliotoxin production by *A. fumigatus.*

Ninety six hour old hyphae were resuspended in low (1.0 μg/ml) or high (10 μg/ml) doses of caspofungin for 0.5, 2 and 4 hour (Figure 4.20). After each time the supernatants were collected and the gliotoxin was extracted as described in section 2.18. Results show the highest concentration of gliotoxin recorded with high dose of caspofungin at 0.5 hour other results show an increase in the amount of gliotoxin when the cells were exposed to drug for 4 hour using RPMI1640 and MEM (Figure 4.21).

4.4.4 Effect of different doses of caspofungin on the amount of Gliotoxin detected at different time of incubation.

Cultures of *A. fumigatus* were grown at 37 °C for 96 hour, harvested, washed and resuspended in PBS supplemented with caspofungin (0.01, 0.1 or 1.0 μg/ml). The release of gliotoxin was quantified by RP-HPLC after 4-hour exposure to the drug or DMSO. The results (Figure 4.22 and Figure 4.23) indicate that the culture supplemented with 1.0 μg/ml caspofungin released a significantly greater amount of gliotoxin than the untreated control ($P \leq 0.03$). Results indicate that exposure of stationary phase *A. fumigatus* hyphae to caspofungin leads to increased leakage of gliotoxin.

4.4.4.1 Effect of long term exposure (24 hour) to caspofungin on gliotoxin release from *A. fumigatus*

Exposure of cultures of *A. fumigatus* to caspofungin for 24 hours leads to increased internal and external concentrations of gliotoxin. Cultures of *A. fumigatus* were grown in RPMI medium for 96 hour at 37 °C at which time caspofungin was added to cultures to give final concentrations of 0.1 or 1.0 μg/ml. The cultures were then incubated for a further 24-hour in RPMI1640 medium at which time the internal and external gliotoxin concentrations were ascertained as described. The results reveal an increase in the external gliotoxin concentration in cultures that were supplemented with caspofungin. The culture supplemented with 1.0 μg/ml caspofungin had a significantly higher level of gliotoxin in culture medium at 8 h (8.29 ± 0.08 μg/ml), 16 h (6.09 - 0.5 μg/ml) and 24 h (6.78 ± 0.29

μg/ml) (Figure 4.22A) compared to the relevant controls. In addition, the intracellular concentration of gliotoxin at 24 hour also increased in the cultures supplemented with 0.1 or 1.0 μ g/ml caspofungin relative to the control (Figure 4.22B). These results indicate that incubation of stationary phase cultures of *A. fumigatus* with caspofungin for 24 hour leads to increased extracellular and intracellular levels of gliotoxin.

4.4.4.2 Effect of short term exposure (4 hour) to caspofungin on gliotoxin released from *A. fumigatus*

Cultures of *A. fumigatus* were grown for 96 hour in RPMI1640 medium at 37 °C. Cultures were supplemented with caspofungin (0.01, 0.1 or 1.0 μg/ml) for 4 hour. After this time hyphae were harvested, washed, and resuspended in the same volume of fresh RPMI medium as previously. The concentration of gliotoxin in the culture medium and within the hyphae (intracellular) was assessed for up to 48 hour after the short-term (4 hour) exposure to caspofungin. Cultures exposed to 1.0 μg/ml caspofungin had significantly higher gliotoxin concentrations in the medium at 24 hour (7.94 ± 1.88 μg/ml) and 48 h (11.19 ± 0.57 μg/ml) post-exposure to caspofungin compared to the relevant control (Figure 4.23A). Internal concentrations of gliotoxin were also measured, as described, 48 hour post-exposure to caspofungin (Figure 4.23B). Internal gliotoxin levels were significantly higher (224.8 μg/g of hyphae) in the cultures that had been exposed to 1 μg/ml caspofungin for 4 hour compared to the control. These results reveal that short-term (4 hour) exposure to caspofungin has the ability to potentiate the production of gliotoxin when cells are placed in fresh medium.

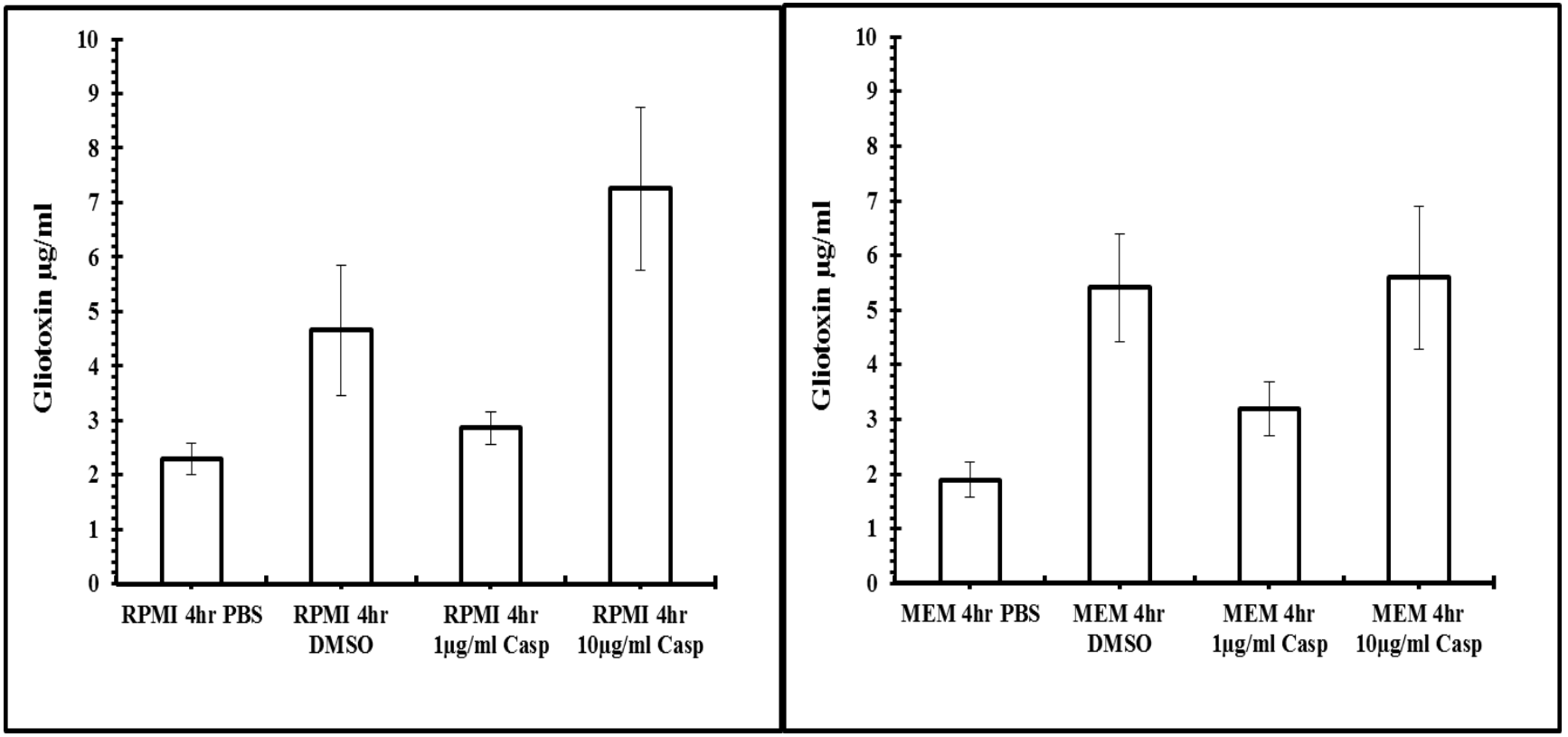


Figure 4.21 Effect of caspofungin on gliotoxin production after short-term exposure of 96 old cultures.

1 g of Hyphae (96 old) was exposed to low (1.0 µg/ml) and high (10 µg/ml) doses of Caspofungin for 4 hours using MEM and RPMI1640 supplemented with 5% FBS. 0.5 % DMSO is the positive control.

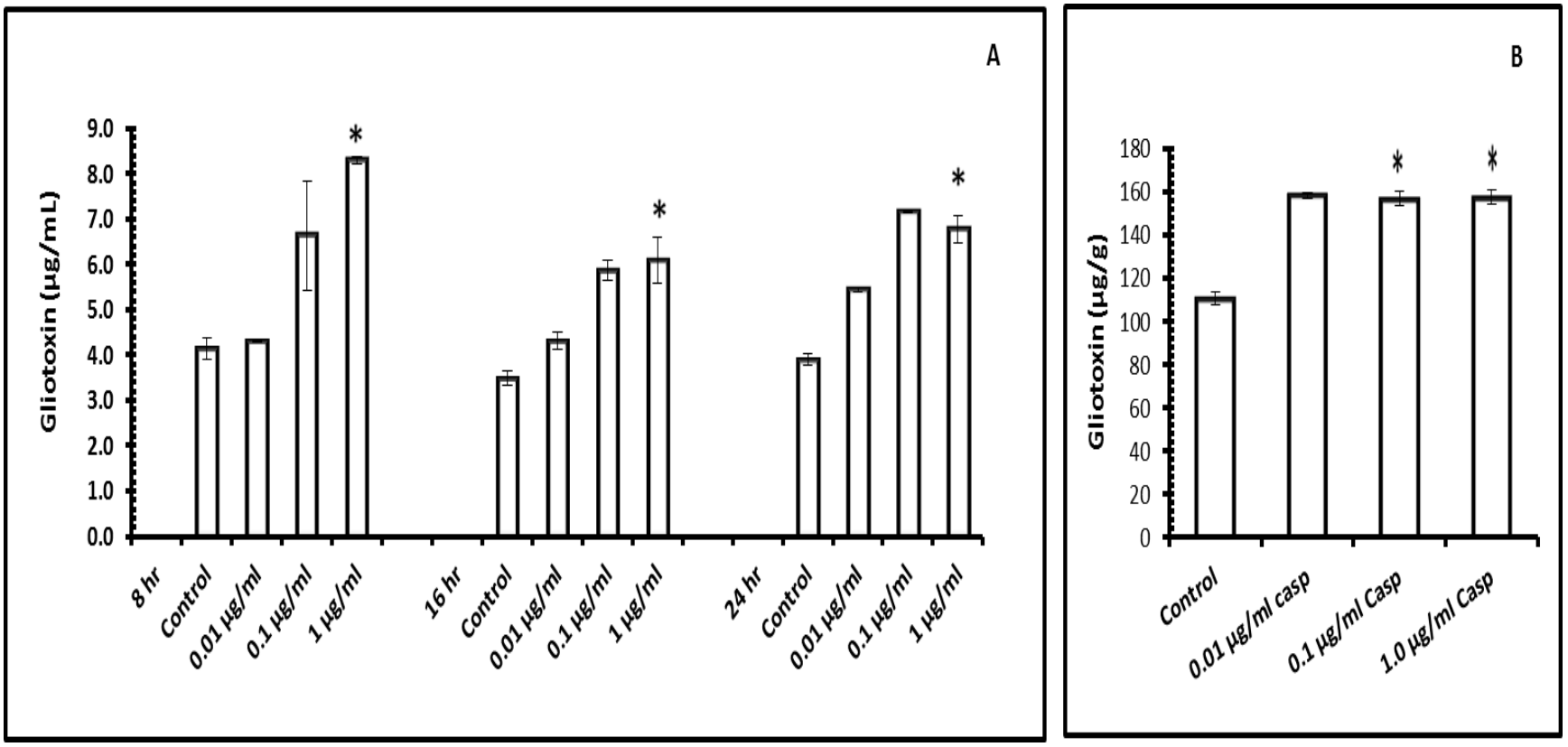


Figure 4.22 Effect of long-term caspofungin exposure (24 hour) on gliotoxin release and biosynthesis in *A. fumigatus*.

Extracellular and intracellular gliotoxin levels in 96-hour-old cultures after exposure to caspofungin for 24 hours. Gliotoxin release was measured at 8, 16 and 24 hours after addition of caspofungin to cultures (A). The intracellular concentration of gliotoxin was measured at 24 hours (B). Statistically significant difference compared to control indicated by * $P \leq 0.03$.

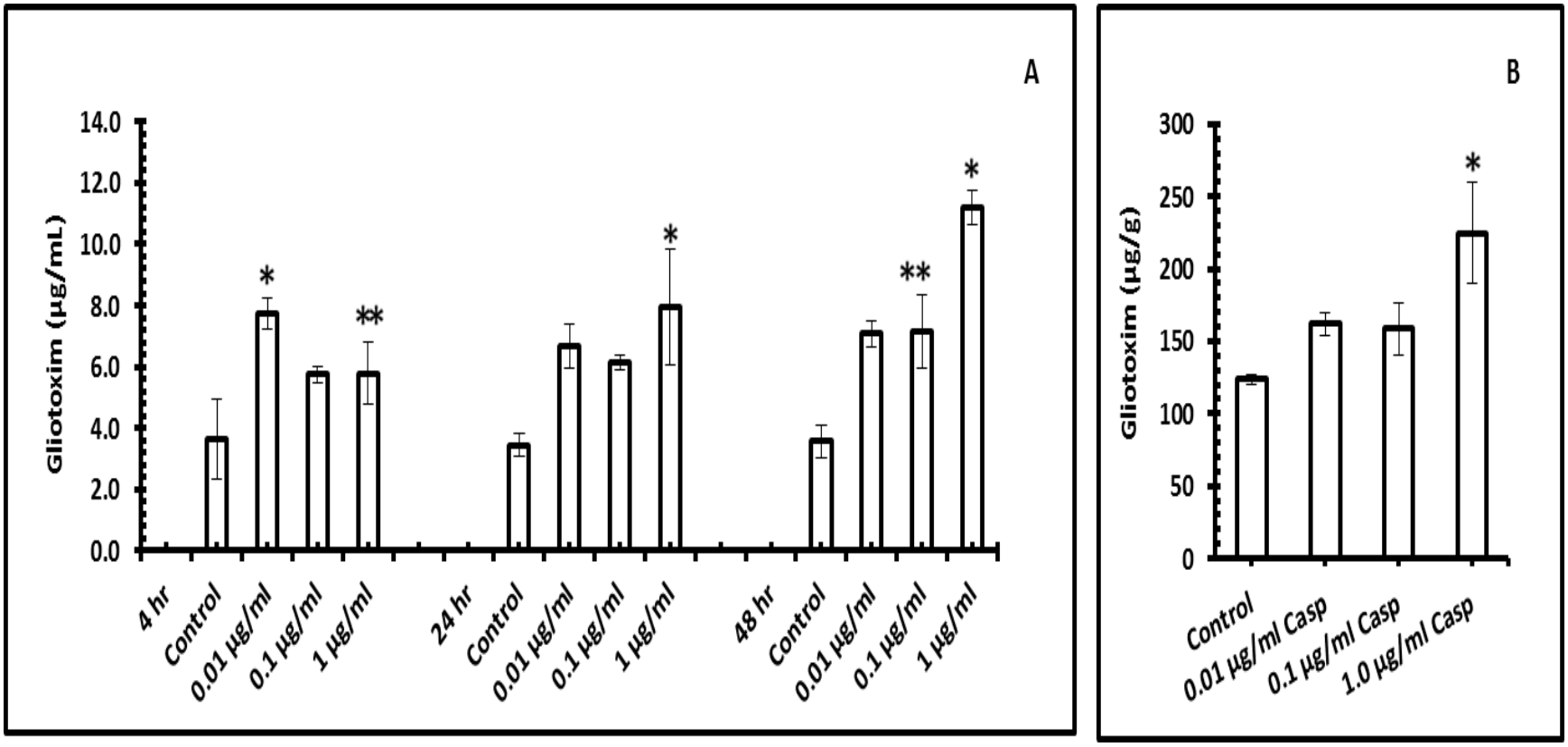


Figure 4.23 Effect of short-term caspofungin exposure (4 hour) on gliotoxin release and biosynthesis in *A. fumigatus*.

Extracellular gliotoxin concentration (A) and internal concentration of gliotoxin (B) in *A. fumigatus* 4, 24 and 48 hours after exposure of hyphae to caspofungin for 4 hours was measured. The intracellular concentration of gliotoxin was measured at 48 hours. Statistically significant difference compared to control indicated by * P ≤0.04. * * Indicates P ≤ 0.023.

4.4.5 Effect of Caspofungin (0.01, 0.1 and 1.0 µg/ml) on extracellular and intracellular gliotoxin levels when hyphae were exposed to drug for 4 hours.

Cultures of *A. fumigatus* were grown in RPMI medium for 96 hour at 37 ° C at which time caspofungin was added to cultures to give final concentrations of 0.01, 0.1 or 1.0 µg/ml. The cultures were incubated for a further 24 hour in RPMI1640 medium at which time the internal and external gliotoxin concentrations were ascertained as described previously. The results reveal an increase in the external gliotoxin concentration in cultures that were supplemented with caspofungin. The culture supplemented with 1.0 µg/ml caspofungin had a significantly higher level of gliotoxin in culture medium compared to the relevant controls as indicated in Figure 4.24 ($P = 0.02$). In addition, the intracellular concentration of gliotoxin at 24 hour also increased in the cultures supplemented with 0.1 or 1.0 µg/ml caspofungin relative to the control. These results indicate that incubation of stationary phase cultures of *A. fumigatus* with caspofungin for 24 hour leads to increased extracellular and intracellular levels of gliotoxin.

4.4.6 Summary

Exposure of *A. fumigatus* hyphae to caspofungin show increased in the biosynthesis and release of gliotoxin

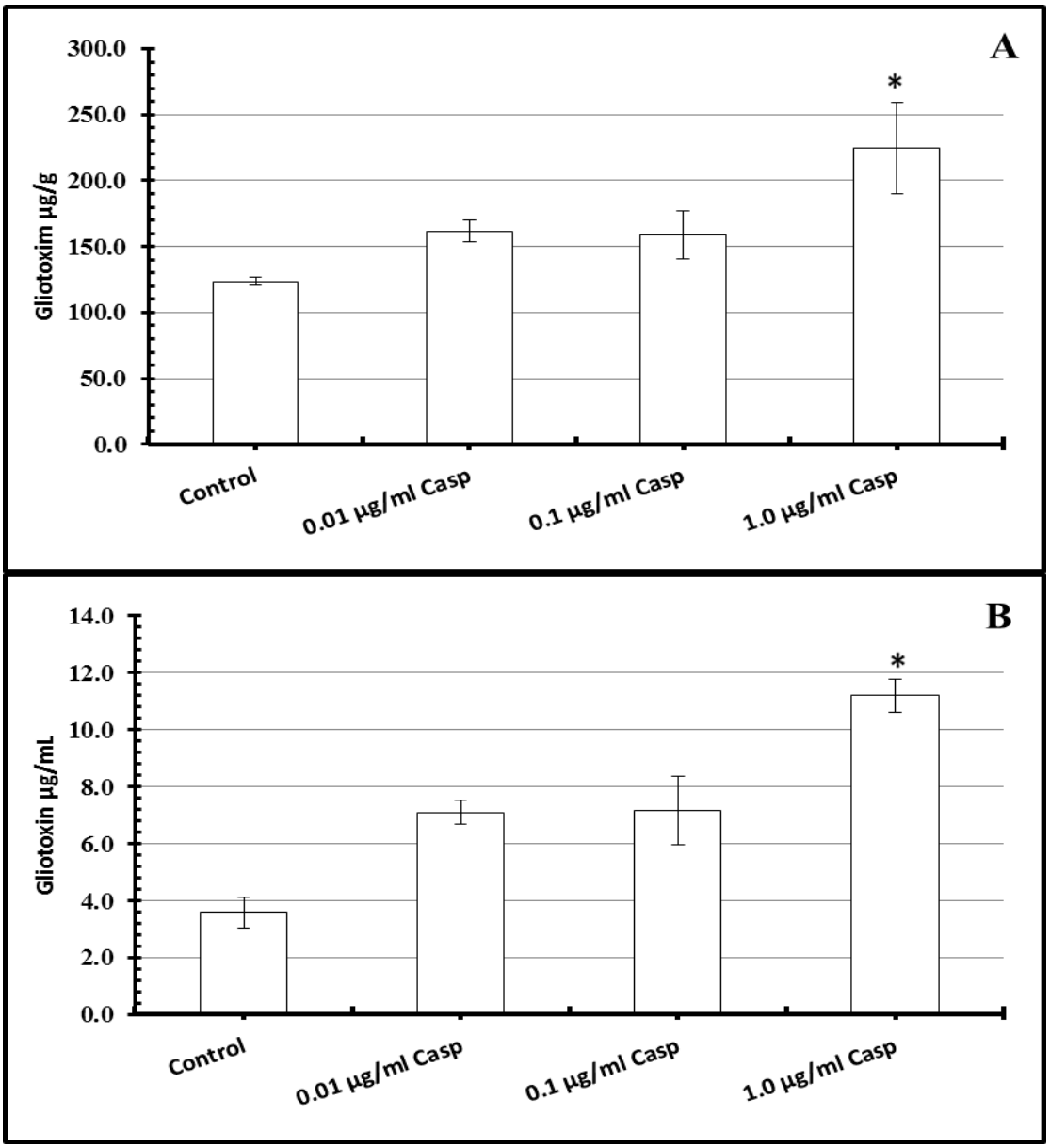


Figure 4.24 Effect of caspofungin on gliotoxin biosynthesis (A) and release (B) from *A. fumigatus*

Hyphae (1.0 gram) of 96 old cultures were exposed to three different concentration of caspofungin for 4 hours. Gliotoxin was extracted, intracellular and extracellular gliotoxin were detected using RP-HPLC.

4.5 Ergosterol Extraction from *A. fumigatus*

The amount of ergosterol present in the cell membrane of fungi is important for the action of polyene and azole antifungal drugs. Polyenes demonstrate antifungal activity by binding to ergosterol leading to leakage of metabolites, which may lead to cell death (Abu Salah, 1996). Azoles disrupt ergosterol biosynthesis leading to the formation of toxic sterol intermediates, which may prove lethal to fungal cells (White *et al.,* 1998). The ergosterol content of *A. fumigatus* exposed to caspofungin was analysed in order to establish if the drug affected cell membrane integrity or structure.

4.5.1 Effect of caspofungin on the ergosterol biosynthesis of *A. fumigatus*

Sterols were extracted using the technique by Arthington-Skaggs *et al.* (1999) with some modification as described in section 2.13. Incubation times of 24, 48 and 72 were used (Figure 4.25), and different concentrations of caspofungin were used (Figure 4.26). Miconazole was used as a positive control (Figure 4.26). Two gram dry weight of hyphae were collected for each sample. Results show an increase in ergosterol biosynthesis at 0.1 and 1.0 μg/ml caspofungin relative to control culture (Figure 4.25). Figure 4.26 shows a significant increase in ergosterol level over three caspofungin concentrations range used compared to control ($P = 0.05$).

4.5.2 Summary

Caspofungin increased the biosynthesis of ergosterol in *A. fumigatus.* Increased ergosterol may be produced to stabilise the fungal cell membrane.

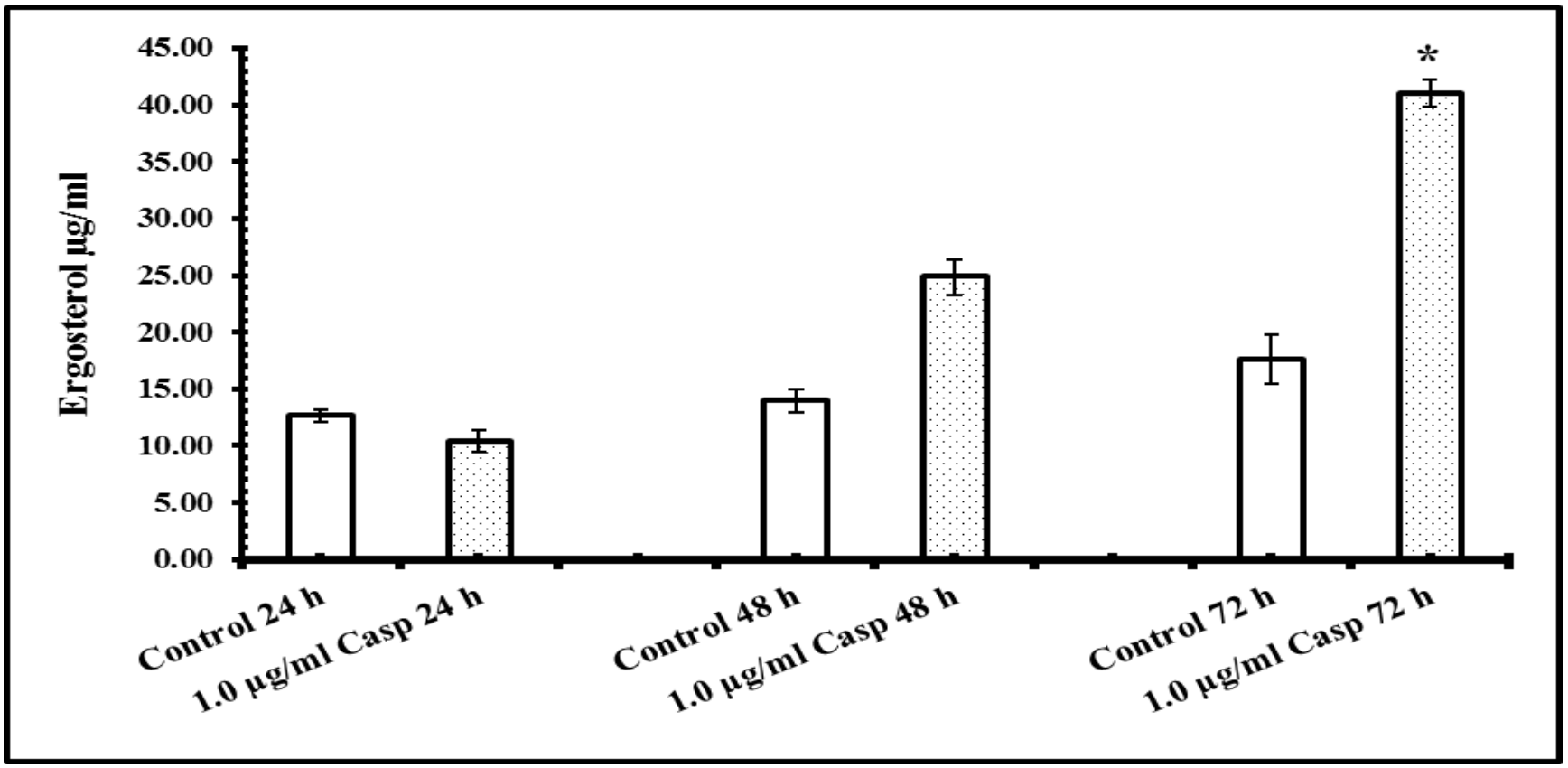


Figure 4.25 Detection of ergosterol in *A. fumigatus* using Gas chromatography. Samples were exposed to 1.0 µg/ml Caspofungin for 24, 48 and 72 hour.

P=0.02

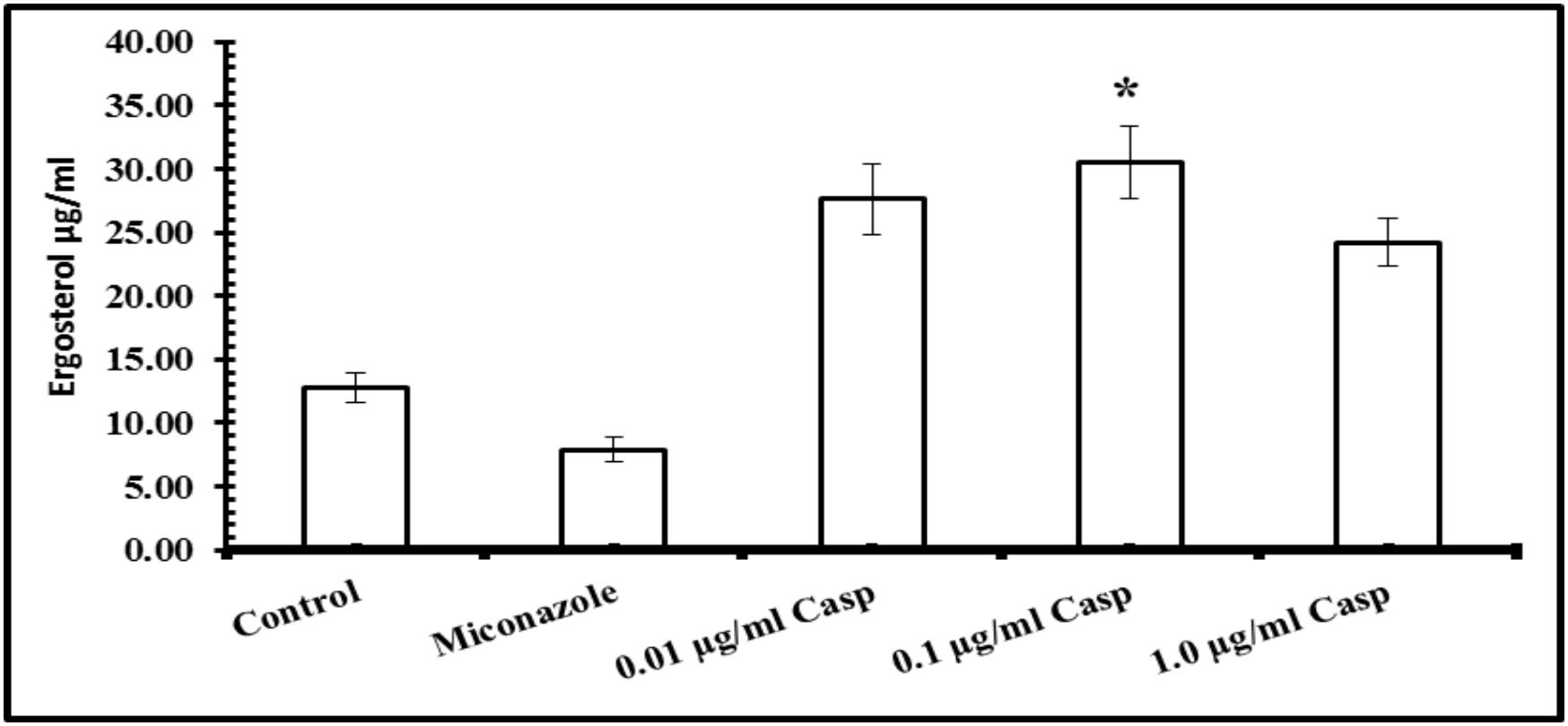


Figure 4.26 Detection of ergosterol in *A. fumigatus* using Gas chromatography, 72 hour old RPMI1640 culture.

Samples were exposed to 0.01, 0.1 and 1.0 µg/ml Caspofungin for 4 hour exposure.

P=0.05

4.6 Analysis of the *A. fumigatus* hyphal wall by Scanning Electron Microscopy following exposure to caspofungin.

A. fumigatus hyphae were exposed to low (0.1 μg/ml) or high (1.0 μg/ml) doses of caspofungin for 48 hours. Hyphae were harvested and were prepared for scanning electron microscopy (SEM) as described in section 2.16. Three different sample preparation techniques were employed (Air drying, Freeze drying and Critical point drying) to ensure no artifacts due to the drying process were observed. Samples that were processed by the Air-drying technique (Figure 4.27) or Freeze-drying technique (Figure 4.28) demonstrated no alteration in the morphology of the hyphae following exposure to caspofungin. The sample prepared using the critical point drying technique (Figure 4.29) demonstrated some change in the hyphae shape as the hyphae surface appeared smoother and possibly thinner than the control especially in those hyphae exposed to 1.0 μg/ml caspofungin. These results demonstrated the lack of gross morphological changes to the hyphae of *A. fumigatus* following exposure to caspofungin. In particular no evidence for cell lysis was detected nor was the presence of deformed hyphae observed in any of the caspofungin treated samples.

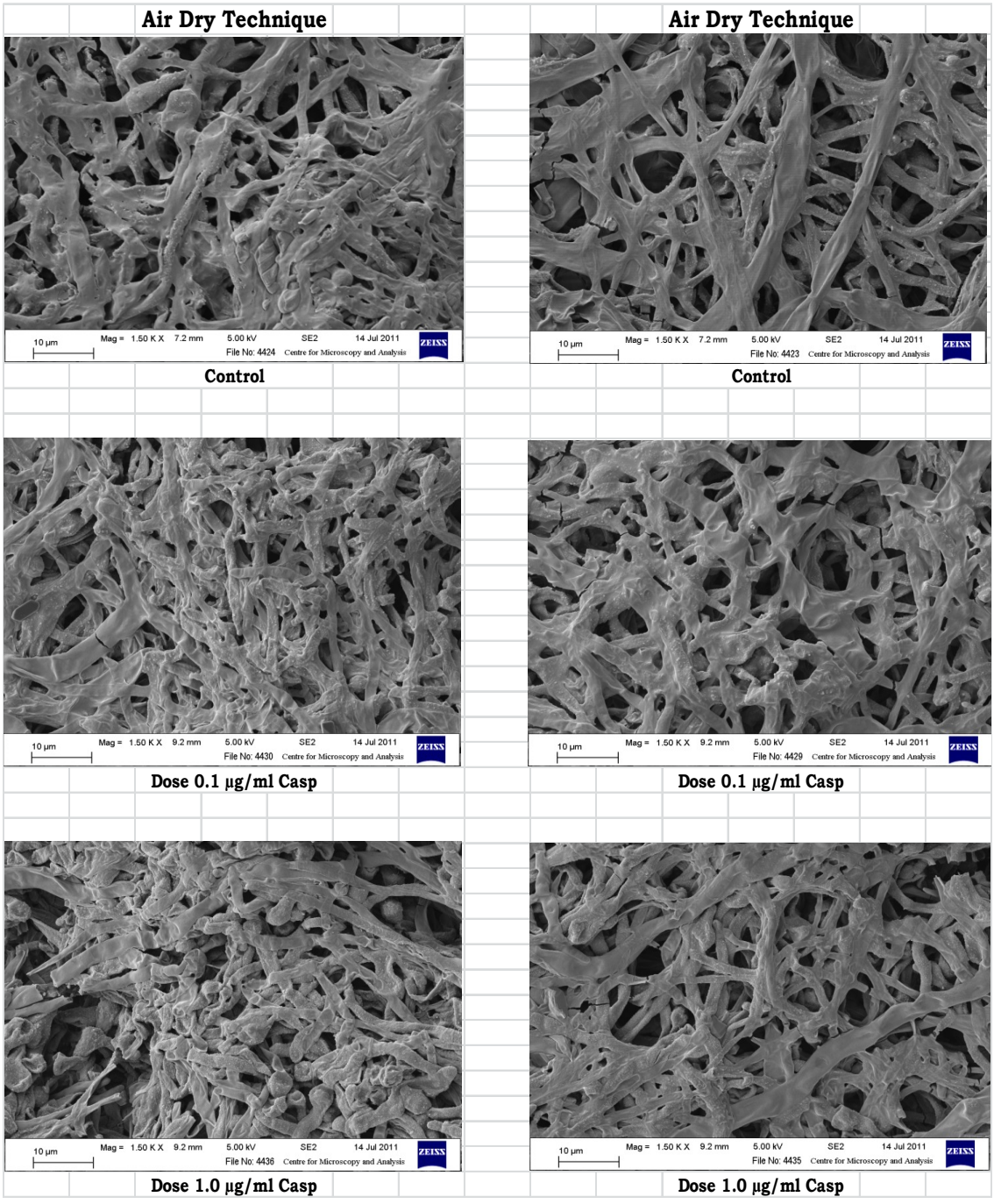


Figure 4.27: Image of *Aspergillus fumigatus* hyphae when treated with 0.1 and 1.0 µg/ml caspofungin using air-dry technique at magnification power 1.5 KX.

Result taken in duplicate.

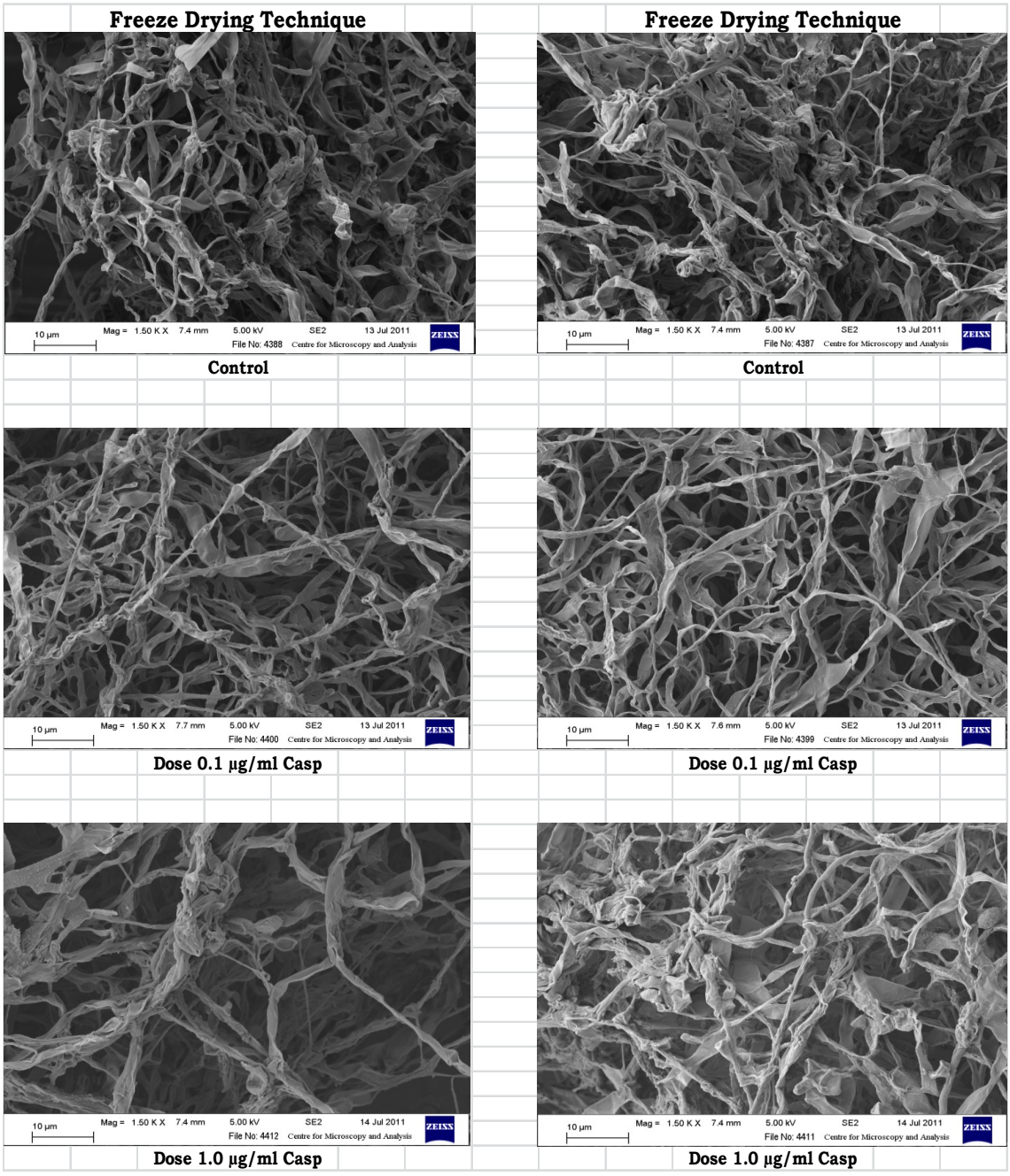


Figure 4.28 Image of *Aspergillus fumigatus* hyphae when treated with 0.1 and 1.0 µg/ml caspofungin using freeze drying technique at magnification power 1.5 KX.

Result taken in duplicate.

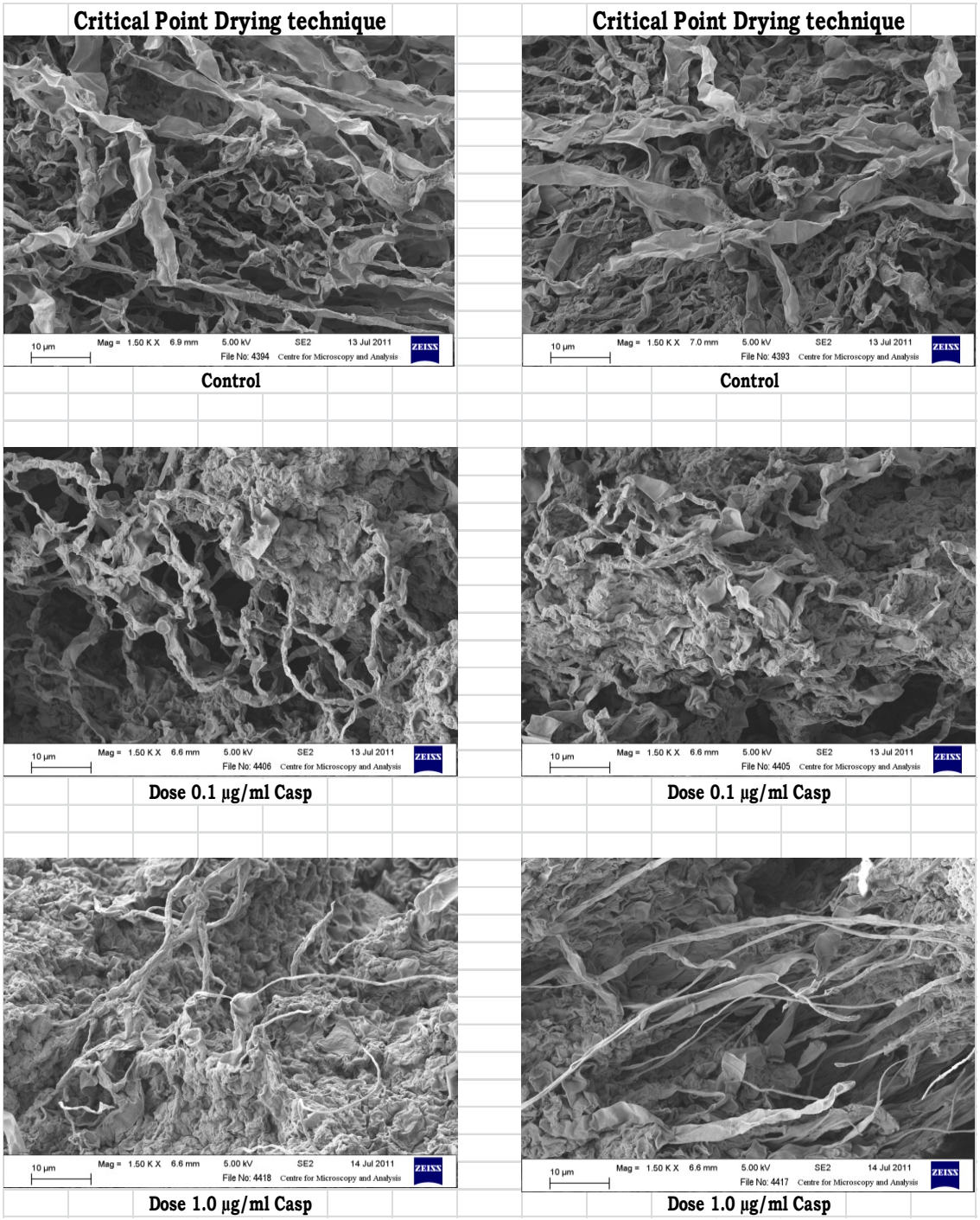


Figure 4.29 Image of *Aspergillus fumigatus* hyphae when treated with 0.1 and 1.0 µg/ml caspofungin using Critical point drying technique at magnification power 1.5 KX.

Result taken in duplicate.

4.7 Analysis of the proteins released by *A. fumigatus* following exposure to caspofungin

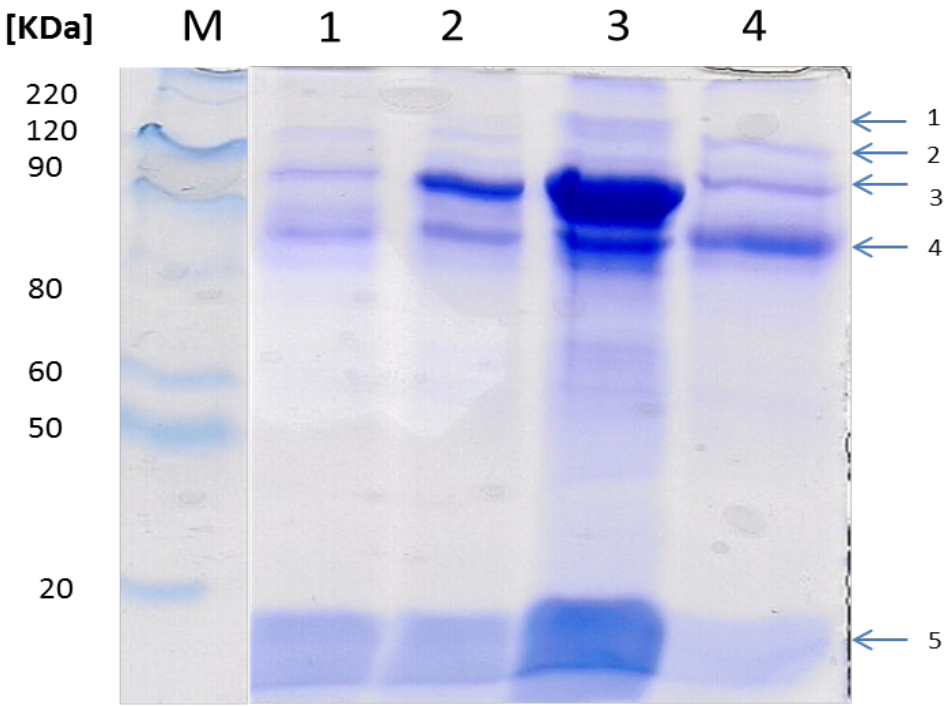
In order to ascertain whether caspofungin induced the release of proteins from *A. fumigatus,* hyphae were suspended in PBS, exposed to 0.1 and 1.0 μg/ml caspofungin or to 5% (v/v) DMSO for 4 hours and the released constituents were precipitated as described in section 2.28.1. The protein which was released from hyphae by 240 min was collected and placed on ice with protease inhibitors as described in section 2.28.2

4.7.1 One-dimensional SDS-PAGE analysis of released protein

Proteins were resolved on a 1-D SDS PAGE gel and stained with Coomassie blue (Figure 4.30). It was evident that several proteins were released from the control cells during the 4-hour incubation period. Cells treated with DMSO and caspofungin released more proteins. When hyphae were exposed to caspofungin, increased release of protein was evident when compared to the control. It was noted that the pattern of proteins released from DMSO and caspofungin-treated cells differed in that there was a greater number of larger proteins released when cells were treated with the DMSO. Released proteins ranged in size from 40 to 220 kDa. There was a significant increase in the abundance of band 1 (IgE-binding protein), 2 (aldehyde dehydrogenase) a cytoplasmic protein, 3 (alpha-1-antiproteinase precursor), 4 (secreted dipeptidyl peptidase), and 5 (ATPB_NEUCR ATP synthase beta chain, mitochondrial precursor) also showed increased abundance when hyphae were exposed to 0.1 μg/ml caspofungin for 240 minutes (Table 4.1). A number of the released proteins are associated with the cytoplasm e.g band 2 (aldehyde dehydrogenase), and could be released due to the activity of caspofungin on cell wall. However, mitochondrial protein band 5 (ATPB_NEUCR ATP synthase beta chain, mitochondrial precursor) may be a result of the increased permeability of the cell wall.

4.7.2 Effect of Caspofungin on the protein leakage from *A. fumigatus* as assessed by Two Dimensional SDS-PAGE analysis

Two–dimensional SDS-PAGE was employed in order to further separate the released proteins and thus facilitate their identification by LC-MS. Cells were exposed to drugs for 4 hours and the released proteins were collected, separated by 2-D electrophoresis and stained with Coomassie blue (Figure 4.31). It is obvious that caspofungin induced the release of a number proteins and a change in spot intensity was observed as shown in the Figure 4.31. Selected spots were excised, washed, trypsin digested and analyzed on LC-MS as described previously in section 2.33. Twelve peptide spots were successfully identified. The spot intensity changed following the treatment of cells with caspofungin. There was a significant increase in the abundance of spots 2 (14-3-family protein ArtA), 4 (Triosephosphate isomerase), 6 (methyltransferase Sir-like), 7 (Allergen Asp F3), 8 (mitochondrial peroxiredoxin Prx1), 9 (6-phosphogluconolactonase), 10 (EEef1-beta subunit) and 12 (cobalamin-independent methionine synthase) (Table 4.2). A number of proteins (spots 6, 7, 8, 10, 11, 12) also showed increased abundance when hyphae were exposed to 0.1 μg /ml caspofungin for 240 minutes (Table 4.2). A number of the released proteins were associated with the cell wall e.g. spot 5 (endochitanase), and spot 7 (Asp F3)) and may have been released due to the perturbation in the cell wall structure associated with the activity of caspofungin. However, a number of proteins are normally located within the cell, e.g. spot 4 (Triosephosphate isomerase), spot 6 (methyltransferase Sir-like), and spot A8 (mitochondrial peroxiredoxin Prx1) and their release from treated hyphae may be a result of the increased permeability of the cell wall.



Lane number	Leakage proteins from *Aspergillus fumigatus*
M	Molecular weight marker
1	Control
2	DMSO
3	1.0 µg/ml Casp
4	0.1 µg/ml Casp

Figure 4.30 Visualization by 1 D SDS-BAGE of released proteins from *A. fumigatus* following exposure to caspofungin.

Hyphae were exposed to caspofungin or DMSO and released proteins were precipitated and separated by SDS-PAGE.

Data analysis for 1 Dimensional Leakage protein									
Band	Identified protein	Accession No	Score	Coverage [%]	Source	Fold Increase			
						PBS	DMSO	1.0 µg/ml	0.1 µg/ml
1	IgE-binding protein	CAA12162	72	11%	*Aspergillus fumigatus*	1	1.28	3.6	1.3
2	aldehyde dehydrogenase	XP_746831	276	20%	*Aspergillus fumigatus*	1	1.28	3.6	1.4
3	alpha-1-antiproteinase precursor	NP_776307 XP_615250	175	5%	*Aspergillus fumigatus*	1	0.75	1.9	0.7
4	secreted dipeptidyl peptidase	AAB67282	397	14%	*Aspergillus fumigatus*	1	2.8	6.9	1.2
5	ATPB_NEUCR ATP synthase beta chain, mitochondrial precursor	XP_659919	202	10%	Aspergillus nidulans FGSC A4	1	1.1	2.4	1.5

Table 4.1 Identified leaked peptides from *A. fumigatus* plus fold increase in response to 0.1 and 1.0 µg/ml caspofungin.
Bands were excised, trypsin digested and analysed using LC-MS. The identity of bands was analysed to assess fold change in intensity.

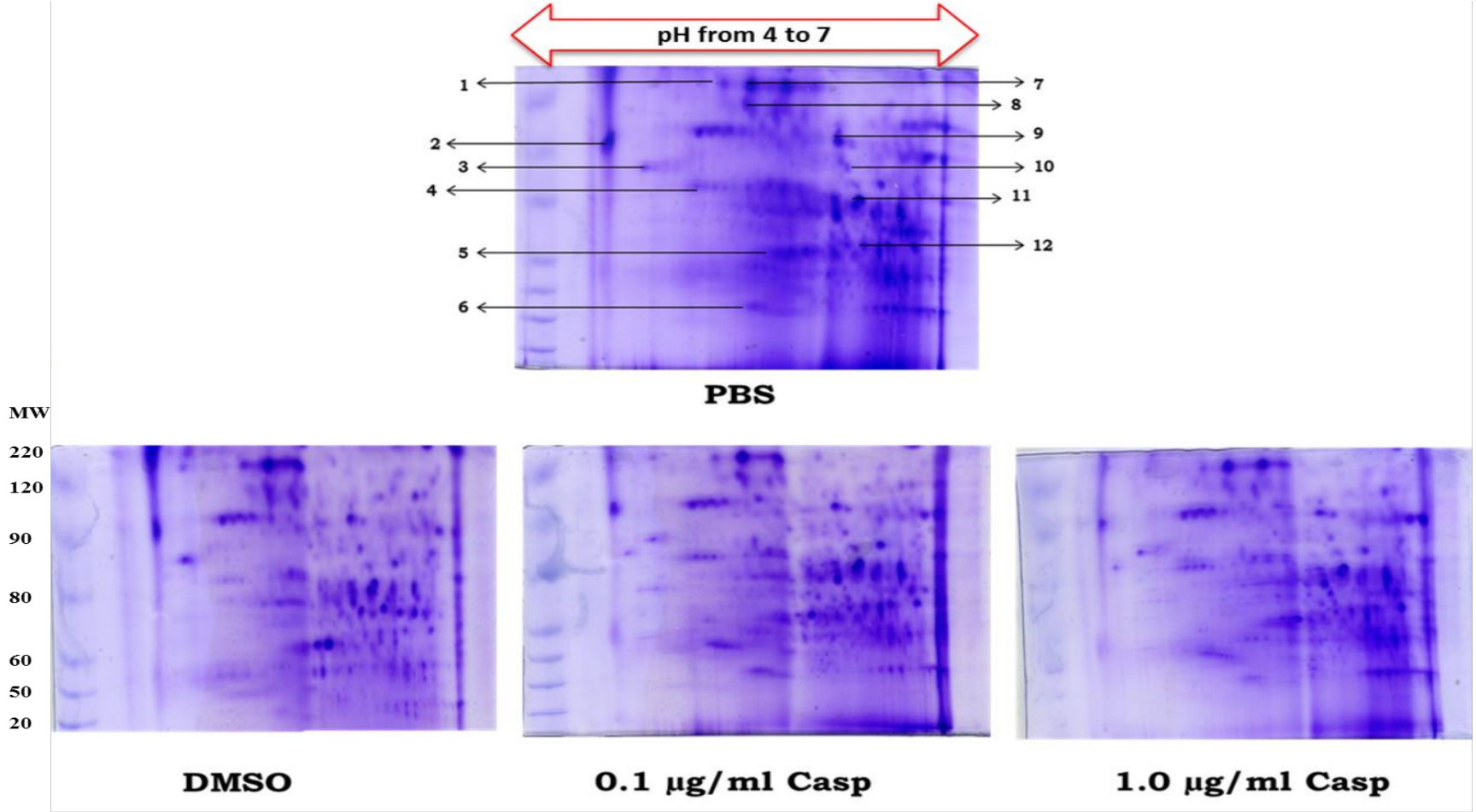


Figure 4.31 Analysis of protein leakage from caspofungin treated *A. fumigatus*

Data analysis for 2 D Leakage protein										
Spot	Score	Identified protein	Accession No	Coverage [%]	Source	Protein Size [Kda]	Fold Increase in abandance			
							PBS	0.5% DMSO	0.1 µg/ml Casp	1.0 µg/ml Casp
1	245	Conserved hypothetical protein	XP_749979	29%	*Aspergillus fumigatus Af293*	80	1.0	1.3	1.2	2.3
2	731	14-3-3 family protein ArtA [H_2O_2 induced]	XP_749464	68%	*Aspergillus fumigatus Af293*	58	1.0	3.6	1.3	3.8
3	453	Conserved hypothetical protein [CAP1 dependent & H_2O_2 induced]	EDP52196	44%	*Aspergillus fumigatus Af294*	80	1.0	1.1	1.1	2.0
4	784	Triosephosphate isomerase	XP_753309	42%	*Aspergillus fumigatus Af296*	80	1.0	1.3	1.1	3.8
5	533	Endochitosanase	ABZ88800	44%	*Aspergillus sp. CJ22-326*	90	1.0	1.2	1.3	1.3
6	701	Methyltransferase SirN-like	XP_747150	62%	*Aspergillus fumigatus Af293*	72	1.0	1.9	1.4	2.0
7	723	Allergen Asp F3 [Gliotoxin & H_2O_2 induced]	XP_747849	88%	*Aspergillus fumigatus Af293*	175	1.0	2.0	1.6	3.2
8	541	Mitochondrial peroxiredoxin Prx1	XP_751969	79%	*Aspergillus fumigatus Af294*	85	1.0	1.3	1.4	1.7
9	870	6-phosphogluconolactonase	XP_001481696	65%	*Aspergillus fumigatus Af295*	76	1.0	1.2	1.3	2.1
10	684	Eukaryotic translation elongation factor 1 subunit Eef1-beta, putative	XP_752484	85%	*Aspergillus fumigatus Af296*	60	1.0	2.5	2.2	2.7
11	1348	Molecular chaperone and allergen Mod-E/Hsp90/Hsp1	XP_747926	42%	*Aspergillus fumigatus Af297*	39	1.0	1.3	2.7	2.1
12	1332	cobalamin-independent methionine synthase MetH/D	XP_752090	46%	*Aspergillus fumigatus Af293*	28	1.0	2.2	4.6	4.5

Table 4.2 List of identified proteins, using LC-MS, leaking from the *A. fumigatus* hyphae when the culture was exposed to 0.1 and 1.0 µg/ml caspofungin.

DMSO is the positive control

4.7.3 Summary

Protein was released from *A. fumigatus* hyphae throughout the incubation period but the greatest release occurred from hyphae exposed to 1.0 μg/ml caspofungin for 240 minutes. Protein was also released from hyphae exposed to 0.1μg/ml caspofungin and the level of release was comparable to that induced by exposure to DMSO which was employed as a positive control (Reeves *et al.,* 2004). Proteins were identified from different locations within the cell. e.g. cell wall, cytoplasm and mitochondria as shown in Table 4.1.

4.8 Discussion

Treatment of aspergillosis has previously centered on the use of the polyene antifungal (liposomal amphotericin B), however its limited efficacy and financial cost has limited its use. Furthermore the nephrotoxic effects have been reported with studies demonstrating high renal failure in patients (Bates *et al.,* 2001). In recent years the use of caspofungin has been demonstrated as a combination therapy with azole antifungals. Caspofungin inhibits hyphal growth by targeting cell wall glucan synthesis and demonstrates limited toxicity thus highlighting utility in antifungal treatment (Patterson, 2006; Sable *et al.,* 2008). Its low toxicity makes it a more attractive antifungal agent for therapy than amphotericin B, which has characterized toxic side effects. The results presented in this Chapter demonstrate that exposure of *A. fumigatus* to caspofungin results in increased membrane permeability as measured by the elevated release of intracellular content (amino acids, protein and gliotoxin).

Leaking of amino acid was reported previously when *A. fumigatus* hyphae were exposed to polyene antifungal Amphotericin B (Reeves *et al.,* 2004). In this work the leaking of amino acid was significantly increased due to the effect of caspofungin on the *A. fumigatus* hyphae. This effect was confirmed by using DMSO as a positive control at different times of exposure. However *A. fumigatus* is known to produce various immunosuppressive mycotoxins including gliotoxin (Watanabe *et al.,* 2003). Part of the ability of *A. fumigatus* to colonise tissue is achieved through the action of gliotoxin

(Reeves *et al.,* 2006). The release of immunosuppressive agents from *A. fumigatus* following exposure to caspofungin could indicate a general increase in cell permeability thus suggesting the need for the cell to counteract the elevated permeability of the cell. The results presented in this work indicate that caspofungin is capable of inhibiting the growth of *A. fumigatus* but that cultures exposed to this drug for short (4 hours) or long (96 hours) periods demonstrated increased secretion of gliotoxin and elevated levels of internal biosynthesis. Gliotoxin is a potent suppressor of the local and systemic immune response and increased production by caspofungin-treated cultures could have a negative effect on the immune response of the patient.

Exposure of *A. fumigatus* to caspofungin leads to enhanced internal and external gliotoxin concentrations up to 48 h after the beginning of the exposure period as shown in Figure 4.23A and 4.23B. Interestingly exposure of growing cultures of *A. fumigatus* to caspofungin results in reduced biomass but the production of gliotoxin is comparable to that in control cultures (Figure 4.22A and 4.22B). While gliotoxin has well established cytotoxic properties (Kwon-Chung and Sugui 2009), its primary function may be to act as an antioxidant within the fungal cell and so protect it from the effects of exogenous and endogenous oxidants (Schrettl *et al.,* 2010). The activation of the Cap and Hog pathways occurs in *C. albicans* when exposed to caspofungin indicating the drug induces oxidative and osmotic stress within the cells (Kelly *et al.,* 2009). The increased level of gliotoxin within *A. fumigatus* following exposure to caspofungin as evident by this study may be an attempt by the cell to restore the redox balance disrupted by exposure to caspofungin, or by the loss of toxin from within the cell. While gliotoxin may represent a means by which *A. fumigatus* regulates its environment, the net effect of increased biosynthesis and release following exposure to caspofungin is the possibility of enhanced tissue damage and inflammation at the site of infection. Previous work established that exposure of growth arrested stationary phase *C. albicans* cells to caspofungin induced the release of amino acids and a variety of intracellular peptides (Kelly and Kavanagh, 2010). Caspofungin inhibits glucan biosynthesis, which results in disruption of fungal cell wall synthesis and ultimately cell lysis when high concentrations are employed (Deresinski and Stevins 2003). In the results presented here, and in those of the earlier work (Kelly and Kavanagh 2010), stationary phase cultures of *A. fumigatus* or *C. albicans* were exposed to

caspofungin so *de novo* wall formation would not have been significant. Consequently, it is possible that in addition to inhibiting glucan biosynthesis, caspofungin may have a secondary effect on membrane permeability and thus facilitate the leakage of amino acids, protein and toxins from fungal cells. Depletion of the intracellular gliotoxin concentration may stimulate increased biosynthesis of the toxin to replenish the lost gliotoxin in order to restore the redox balance within the cell (Schrettl *et al.,* 2010). This phenomenon of caspofungin-induced gliotoxin leakage and *de novo* biosynthesis has the potential to contribute to elevated levels of pulmonary damage and immunosuppression (Tsunawaki *et al.,* 2004). It is possible that administration of caspofungin to patients may lead to eventual fungal cell death but that before this is achieved internal gliotoxin would be released from cells and the cells could have the opportunity to commence synthesizing more toxins to replenish that which is lost into surrounding tissue. Previous work that examined the response of *C. albicans* to amphotericin B illustrated the ability of cells to recover from antifungal therapy even though they appeared incapable of replicating in culture thus highlighting the possibility of continued cell survival in the presence of fungicidal concentrations of drug (Liao *et al.,* 1999). This finding illustrates the possibility of continued gliotoxin biosynthesis in the presence of caspofungin concentrations that would inhibit fungal cell growth.

Analysis of the changes in the release of proteins following exposure of *A. fumigatus* to 0.1 µg/ml caspofungin indicated the increased abundance of a number of proteins. It was of interest to investigate if larger molecules could escape through this altered membrane and protein leakage was assessed via one-dimensional and two-dimensional electrophoresis. It was determined that caspofungin caused the escape of both large and small proteins from *A. fumigatus*. Five and twelve proteins were positively identified from one-dimensional and two-dimensional gels respectively and the leakage of all of these was increased upon exposure to low and high doses of caspofungin. Proteins come from different locations within the cell some of proteins originated from the cytosol as triosephosphate isomerase, allergen Asp F3 6-phosphogluconolactonase and aldehyde dehydrogenase, mitochondrial protein e.g mitochondrial peroxiredoxin Prx1 and ATPB_NEUCR ATP synthase beta chain, mitochondrial precursor. Cell wall protein such

as endochitosanase and nuclear proteins such as molecular chaperone allergen mod-E/Hsp90/Hsp1were leaked.

Ergosterol is the principle sterol in fungi, which is similar to cholesterol in mammalian membrane but with significant difference in structure (Parks and Casey, 1995). Ergosterol plays a part in membrane integrity and permeability (Abu-Salah 1996). In contrast to the control, exposure of hyphae to caspofungin resulted in an increase in ergosterol content. Any change in membrane structure or function of the membrane may result in the loss of selective permeability of the membrane (Ghosh and Ghosh, 1963). Altered cell membrane may lead to leaking of intracellular content includes the large molecules which escaped through these pores.

This Chapter demonstrates that caspofungin exposure increase the release of amino acids, proteins and gliotoxin. Biosynthesis of gliotoxin was also enhanced and increased production. Caspofungin causes an increase in ergosterol biosynthesis. Protein leakage was increased due to the effect of caspofungin and leaked proteins came from different locations within the cell.

Chapter 5

ANALYSIS OF THE PROTEOMIC RESPONSE OF *ASPERGILLUS FUMIGATUS* TO CASPOFUNGIN

5.1 Introduction

Caspofungin is a member of the newest class of antifungal drugs and inhibits the biosynthesis of ß-1,3,-D-glucan, which is the principal cell wall component (Kahn *et al.,*2006). Caspofungin shows a high level of efficacy against *A. fumigatus* (Pfaller *et al.,* 2009) and there are limited reports of clinical resistance (Perlin, 2009). Exposure to sub-inhibitory concentrations of caspofungin also leads to the unmasking of ß-glucan in the cell wall of *C. albicans* (Wheeler *et al.,* 2006, 2008) which may provoke an enhanced immune response and possibly lead to increased cell-mediated killing of the fungus in the body. Analysis of the proteomic changes that occur in *A. fumigatus* following exposure to caspofungin revealed decreased expression of proteins associated with the mitochondrial hypoxia response (Cagas *et al.,* 2011). In addition decreased expression of Asp F1 was also observed. While echinocandins are effective antifungal agents (Walsh *et al.,* 2004), they also demonstrate negligible toxicity to humans (Denning, 2003).

The aim of the work presented in this Chapter was to analyse the response of *A. fumigatus* hyphae to caspofungin in order to determine whether the fungus could mount a protective response against the effects of this antifungal agent. The proteomic response of *A. fumigatus* to caspofungin was evaluated by LC-MS. The fold change in relative abundance of selected proteins following exposure to caspofungin at sub MIC (1.0 μg/ml) was determined.

5.2 Examination of the effect of caspofungin on the protein profile of *A. fumigatus*

5.2.1 One-dimensional SDS-PAGE protein profile of *A. fumigatus* exposed to caspofungin

A. fumigatus cultures (96 hour old) were exposed to various concentrations of caspofungin for 4 hours and whole cell protein was extracted as described in section 2.22. This was resolved by one dimensional gel electrophoresis as in section 2.25.1 and stained with coomassie blue stain. The protein profile of *A. fumigatus* is illustrated in Figure 5.1. It is evident from this gel that caspofungin treatment of cells caused some differences in the *A. fumigatus* protein profile. For example proteins between the molecular weight 90 and 50 KDa appeared to be up-regulated and appeared to be increased in intensity upon treatment with (0.1 and 1.0 μg/ml) caspofungin. Table 5.1 shows proteins altered in expression in *A. fumigatus* treated with caspofungin. Band 3, identified as fructose-bisphosphate aldolase showed a 2.9 fold increase at 1.0 μg/ml caspofungin. Alcohol dehydrogenase (band 4) was up-regulated by 3.7 fold at 1.0 μg/ml caspofungin and 2.8 fold at 0.1 μg/ml. Both fructose-bisphosphate aldolase and alcohol dehydrogenase are cell wall proteins (Pitarch *et al.,* 2002) characterised as highly immunogenic in nature and the elevated release of these immunogenic proteins could lead to a stronger immune response and possibly inflamation during infections (Swoboda *et al.,* 1993; Kelly and Kavanagh, 2010). Prevous studies have demonstrated fructose-biphosphate aldolase to be immunogenic (Pitarch *et al.,* 2004). The intensity of protein in band 5 (Mn superoxide dismutase) was increased by 2.1 fold at the same concentration of caspofungin. Superoxide anions and peroxides play a direct role in killing of *A. fumigatus* in an immunocompromised host and proteins including stress response proteins such as Mn-superoxide dismutase were increased in expression when *Aspergillus* hyphae were exposed to caspofungin (Cagas *et al.,* 2011).

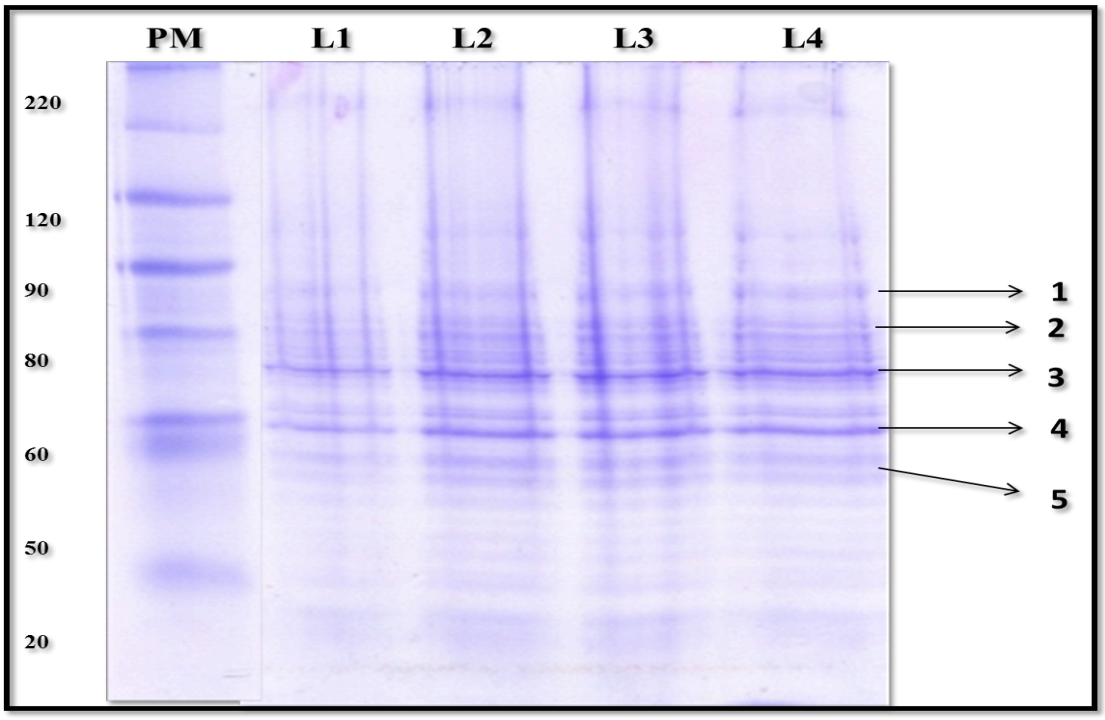


Figure 5.1 One-dimensional protein analysis of caspofungin treated *A. fumigatus*.

(Hyphae of 96 hour old culture were used), DMSO is positive control, and caspofungin concentration of 0.1 and 1.0 µg/ml were used. PM protein marker, L1 Control, L2 DMSO, L3 0.1 µg/ml Caspofungin, L4 1.0 µg/ml caspofungin

1 Dimensional SDS-PAGE internal protein												
Band	Score	Name of protein	PI	Sequences	Accession #	Seq Cov	Source	Molecular function	Fold Change			
									PBS	DMSO	0.1 µg/ml	1.0 µg/ml
1	99	secreted dipeptidyl peptidase	5.58	3 (0)	AAB67282	7%	*Aspergillus fumigatus*	metabolism of dipeptides	1	2.1	1.2	1.8
2	123	Catalase R	5.44	2	P55303	2%	*Aspergillus fumigatus*	Detoxification	1	3.1	1.1	1.9
3	200	fructose-bisphosphate aldolase	5.73	3	XP_001263297	10%	*Aspergillus fumigatus*	gluconeogenesis	1	3.7	2.7	2.9
4	677	alcohol dehydrogenase	7.04	13	XP_746830	61%	*Aspergillus fumigatus*	Oxidoreductase	1	3.9	2.8	3.7
5	86	Mn superoxide dismutase MnSOD	7.14	2	XP_752824	14%	*Aspergillus fumigatus*	Dismutase	1	1.7	1.1	2.1

Table 5.1 Identified peptide bands from *A. fumigatus* plus fold change related to control.

Excised bands were removed, trypsin-digested and analysed using LC-MS.

5.2.2 Effect of Caspofungin on the proteome of *A. fumigatus* as assessed by two-dimensional SDS-PAGE analysis

Following the observation of changes to the 1-D protein profile of caspofungin treated hyphae, it was decide to investigate these changes by 2-D analysis. Cultures were grown for 96 hours at 37 ^{o}C, hyphae were harvested and washed with PBS as described. Hyphae (1.5g) were exposed to caspofungin (0.1 or 1.0 μg/ml) for 4 hours. Hyphae were harvested, washed with PBS and ground to a fine powder using a pestle and mortar by temporary freezing in liquid nitrogen. Protein extraction buffer was added. Proteins were separated by pH at range from 3 to 10 then by molecular weight as described in section 2.25 and the acrylamide gels were stained with coomassie blue. Figure 5.2 and 5.3 demonstrate 2-D protein profile of *A. fumigatus* with and without exposure to caspofungin. It was evident that the expression of protein was changed between control and caspofungin treated samples, some proteins were up-regulated when sample were treated with drug and some were down-regulated. Progenesis analysis was performed on a selection of spots in order to ascertain the fold change

Proteins showing alteration in intensity were excised and identified by LC/MS. A wide range of proteins demonstrated altered expression following exposure of *A. fumigatus* to caspofungin (Table 5.2). Strong identities were recorded for nineteen proteins. A range of proteins showed homology to proteins involved in stress responses e.g. spot B18 (catalase) was up-regulated by 1.3 and 1.4 fold at 0.1 and 1.0 μg/ml caspofungin respectively. Spot B2 (CipC-like antibiotic response protein), was up-regulated by 4.7 fold at 0.1 μg/ml caspofungin. CipC-like antibiotic response protein is located in cytoplasm and involved as a virulence factor in some fungal strains, and increased release of immunogenic proteins may lead to stronger immune response (Cobos *et al.,* 2010; Kelly and Kavanagh, 2010). Spot B15 (Aspartic endopeptidase Pep2) was down-regulated by 0.8 fold at both caspofungin concentrations. Aspartic endopeptidase pep2 is an aspartic proteinases which are a group of proteolytic enzymes. Aspartic acid proteases depend on aspartic acid residues for their catalytic activity, but on the conidial surface may play an important role in the processing of allergens causing fungal infections such as aspergillosis (Jehangir and Ahmed, 2013). Spot B16 (Vacuolar protease A) was decreased by 0.8 and 0.9 at 0.1 and 1.0 μg/ml caspofungin respectively, and showed homology to proteins associated with the virulence of *A. fumigatus.* Spot B19 (molecular chaperone Hsp70), was up-regulated by 2 fold at 0.1 μg/ml caspofungin

and spot B12 (thioredoxin reductase) showed 1.2 fold increase at both concentrations. Both showed homology to proteins involved in stress responses. Elevated expression of these proteins display antigenic properties of *A. fumigatus* (Diaz-Arevalo *et al.,* 2012; Carberry *et al.,* 2012).

A number of spots showed homology to proteins associated with the virulence of *A. fumigatus* (e.g. spot B1 (18kDa antigen), spot B3 (allergen Asp F3)), and with cell homeostasis (e.g. spot B7 (glyceraldehyde 3-phosphate), spot B9 (translation elongation factor), and spot B17 (ATP synthase F1). Protein spot B1 (18 kDa antigen) and spot B3 (allergen Asp F3) which are associated with virulence were also increased in abundance. Cagas *et al.,* (2011) noted an increase in the secretion of Asp F3 (3.5 fold) from susceptible *A. fumigatus* strains exposed to caspofungin. This allergen is a thioredoxin peroxidase and is increased in expression in cells experiencing oxidative stress as a result of exposure to hydrogen peroxide (Lessing *et al.,* 2007; Cagas *et al.,* 2011). The increased expression of a variety of proteins involved in the oxidative stress response e.g. catalase (spot B18), antibiotic response protein (spot B2), virulence e.g. Asp F3 (spot B3), 18kDa antigen (spot B1) and homeostasis e.g. glyceraldehyde 3-phosphate (spot B7), translation elongation factor (spot B9), ATP synthase F1 (spot B17).

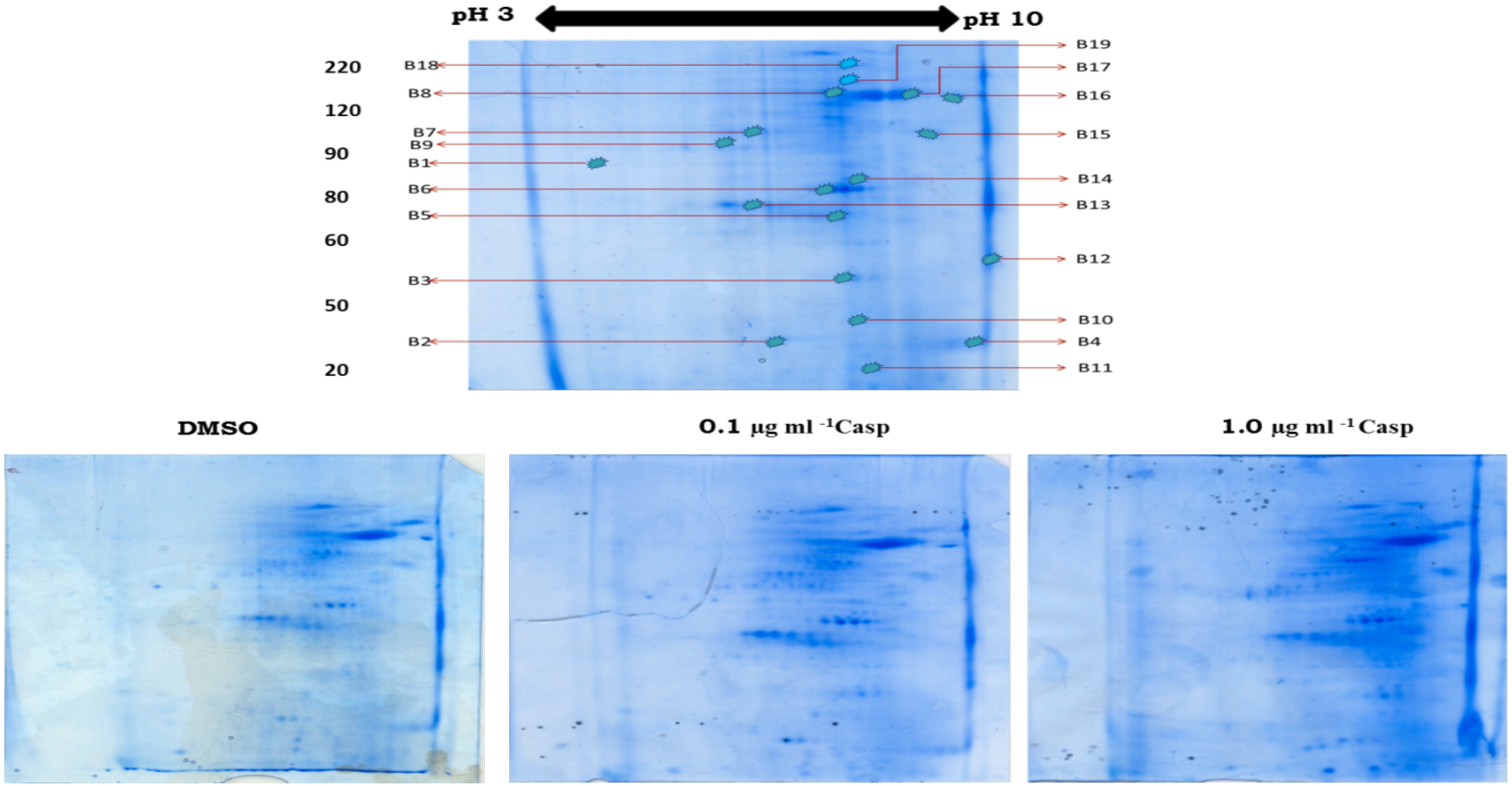


Figure 5.2 Identified spots chosen for fold change analysis from two-dimensional gels from *A. fumigatus*.

Spots were analysed and the fold changes were calculated using Progenesis SameSpots^{TM}.

Spot	Score	Name of protein	Accession No	Seq. Cov	Source	Molecular function	Fold Increase			
							PBS	0.5% DMSO	0.1 μg ml^{-1} Casp	1.0 μg ml^{-1} Casp
B1	84	18-kDa antigen	CAA41217	11%	*A. fumigatus*	Fungal type ribonuclease (Cytotoxin)	1.0	2.1	1.8	1.5
B2	270	CipC-like antibiotic response protein	XP_753706	40%	*A. fumigatus Af293*	CipC-like antibiotic response protein	1.0	1.3	4.7	0.8
B3	278	Allergen Asp F3	XP_747849	48%	*A. fumigatus Af293*	Oxidoreductase activity	1.0	1.5	1.4	1.8
B4	175	Chitosanase, partial	AAD26111	26%	*A. fumigatus*	Chitosanase activity	1.0	1.7	1.9	0.9
B5	148	Dienelactone hydrolase family protein	XP_751152	10%	*A. fumigatus Af293*	Hydrolase activity	1.0	1.4	1.5	1.5
B6	276	FG-GAP repeat protein	XP_750162	30%	*A. fumigatus Af293*	Calcium ion binding	1.0	0.6	0.7	0.8
B7	250	Glyceraldehyde 3-phosphate dehydrogenase	XP_748238	27%	*A. fumigatus Af293*	Oxidation-reduction process	1.0	0.7	1.2	1.4
B8	57	Fructosyl amino acid oxidase	XP_747733	12%	*A. fumigatus Af293*	FAD dependent oxidoreductase	1.0	1.5	1.2	0.8
B9	219	Translation elongation factor 1-alpha	ABF50913	19%	*Zygozyma suomiensis*	Promotes the GTP-dependent binding	1.0	1.0	1.1	1.3
B10	84	Actin-depolymerizing factor, putative	XP_002420345	21%	*C. dubliniensis*	Polypeptide binding	1.0	1.0	2.0	2.1
B11	68	ATP synthase delta chain,	XP_750060	9%	*A. fumigatus Af293*	ATP synthase mitochondrial precursor	1.0	1.5	1.8	1.2
B12	88	Thioredoxin reductase GliT	XP_750863	10%	*A. fumigatus Af293*	Posttranslational , protein turnover	1.0	1.1	1.2	1.2
B13	73	Short chain dehydrogenase (AtsC)	XP_748339	6%	*A. fumigatus Af293*	NADP binding site [chemical binding]	1.0	0.9	1.1	1.3
B14	130	GliN	AAW03301	11%	*A. fumigatus*	Methyltransferase domain	1.0	0.9	1.8	1.3
B15	148	Aspartic endopeptidase Pep2	XP_754479	11%	*A. fumigatus Af293*	Eukaryotic aspartyl protease	1.0	0.9	0.8	0.8
B16	90	Vacuolar protease A	XP_001399855	11%	*Aspergillus niger*	Fungal Proteinase A , aspartic proteinase	1.0	3.0	0.8	0.9
B17	291	ATP synthase F1, beta subunit	XP_753589	17%	*A. fumigatus Af293*	F1 ATP synthase beta subunit	1.0	1.3	1.2	1.4
B18	724	Catalase	AAB71223	24%	*A. fumigatus*	Inorganic ion transport and metabolism	1.0	1.9	1.3	1.4
B19	322	Molecular chaperone Hsp70	XP_750490	13%	*A. fumigatus Af293*	Nucleotide binding site [chemical binding]"	1.0	0.9	2.0	1.0

Table 5.2 Identified peptide spots from *A. fumigatus* plus fold change in response to caspofungin.

Excised spots were removed, trypsin-digested and protein identified using LC-MS. Spots were analysed using Progenesis SameSpots^{TM} to determine the fold change related to control.

5.2.3 Summary

The results presented here indicate that when *A. fumigatus* is exposed to caspofungin there is increased expression of a number of proteins associated with virulence (e.g. 18kDa antigen and allergen Asp F3) and the oxidative stress response (e.g catalase, superoxide dismutase).

5.3 Investigation into the effect of caspofungin on the activity of enzymes involved with the oxidative stress response

In this section the effect of exposure to caspofungin on the activity of a range of detoxifying enzymes of *A. fumigatus* was examined. *A. fumigatus* hyphae (96 hr culture, 1 g) were exposed to caspofungin (0.1 μg/ml) for 15, 30 or 60 minutes or to hydrogen peroxide (0.5 mM) as described in section 2.28. At each time point hyphae were harvested, washed in PBS and resuspended in 8 ml of lysing buffer as described previously. Supernatant was used for evaluation of enzymatic activity.

5.3.1 Analysis of Catalase activity in caspofungin treated *A. fumigatus* cultures

Microbiological resistance to antifungal polyenes may be associated with phenotypic switching, such as increased catalase activity with decreased susceptibility to oxidative damage (Chamilos *et al.,* 2007; Paris *et al.,* 2003). Catalase activity was measured as described (Larsen & White, 1995) with slight modifications as detailed previously (Section 2.28.3). Exposure of *A. fumigatus* to 0.1 μg/ml caspofungin for 30 minutes induced a six-fold increase in catalase activity while exposure to the drug for 60 minutes induced a seven-fold increase in activity (Figure 5.3). Exposure of hyphae to hydrogen peroxide for 15 minutes resulted in a four-fold increase in catalase activity. Catalase demonstrates anti-oxidant properties and is essential in maintaining the redox balance of the cell.

5.3.2 Detection of glutathione reductase (GLR) activity in caspofungin treated *A. fumigatus* cultures

Glutathione reductase (GLR) activity was measured in caspofungin and hydrogen peroxide treated *A. fumigatus* as described in section 2.28.2. Exposure of *A. fumigatus*

to 0.1µg/ml caspofungin for 15 – 60 minutes resulted in a two fold increase in GLR activity ($p < 0.02$). GLR is essential for the recycling of oxidized glutathione to its reduced form and plays a key role in detoxifying the cell. The results (Figure 5.4) demonstrate the elevated activity of GLR following treatment with the drug at all timepoints used in comparison to the control sample. Results show a significant increased in GLR activity occurred especially after 30 minutes, increasing from a value of 3.15E-05 units/ml (± 3.3) in the control to 7.21E-05 units/ml (±3.1) units/ml in caspofungin treated cells. Hydrogen peroxide was found to be a strong inducer of GLR activity with levels rising to 7.35E-05 (± 3.6) units/ml after 30 minutes.

5.3.3 Detection of superoxide dismutase (SOD) activity in caspofungin treated *A. fumigatus* cultures

SOD is an anti-oxidant enzyme that catalyses the dismutation of superoxide radicals into dioxygen and hydrogen peroxide. The transcription of many genes including *SOD1* and *SOD5* were involved in detoxification of oxidative species, were up-regulated in the presence of human neutrophils (Fradin *et al.,* 2005). Superoxide dismutase (SOD) activity was measured using a SOD Assay Kit (Fluka Biochemika) in accordance with the manufacturer's instructions, using a concentration of 1.0 µg/µl of protein extract as described (Foster & Hess, 1980). Exposure of *A. fumigatus* to caspofungin lead to elevated activity of SOD at all timepoints compared to the control. SOD activity was increased significantly ($p = 0.05$) when *A. fumigatus* was exposed to hydrogen peroxide. SOD activity was increased by approximately two fold following exposure of *A. fumigatus* to caspofungin for 15 – 60 minutes. The highest value was recorded after 30 minutes with activity rising from 42.0 (± 2.1) % in the control to 84 (± 3.2) % in drug treated cells (Figure 5.5). Hydrogen peroxide treated cells elevated the SOD activity to 86 (± 5.2) % compared to control.

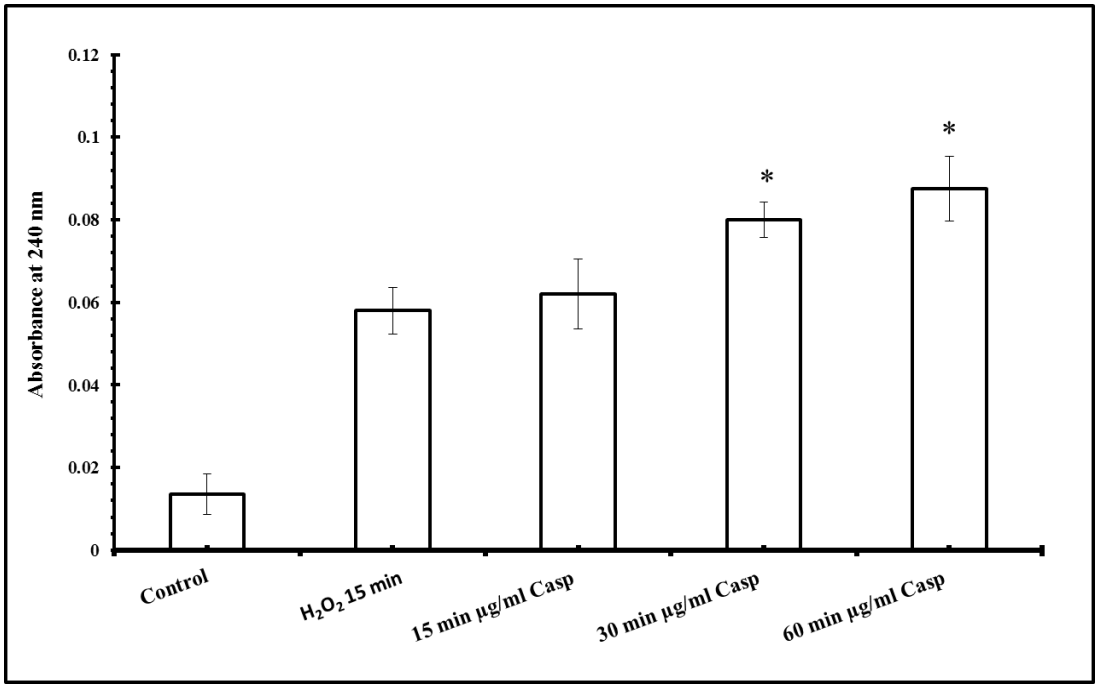


Figure: 5.3 Catalase activity in caspofungin treated *A. fumigatus* hyphae compared to control. Hydrogen peroxide is the positive control.

Hyphae were exposed to 0.5 mM hydrogen peroxide for 15 minutes or 1.0 µg/ml caspofungin for 15, 30 or 60 minutes. Indicates a p value = 0.05

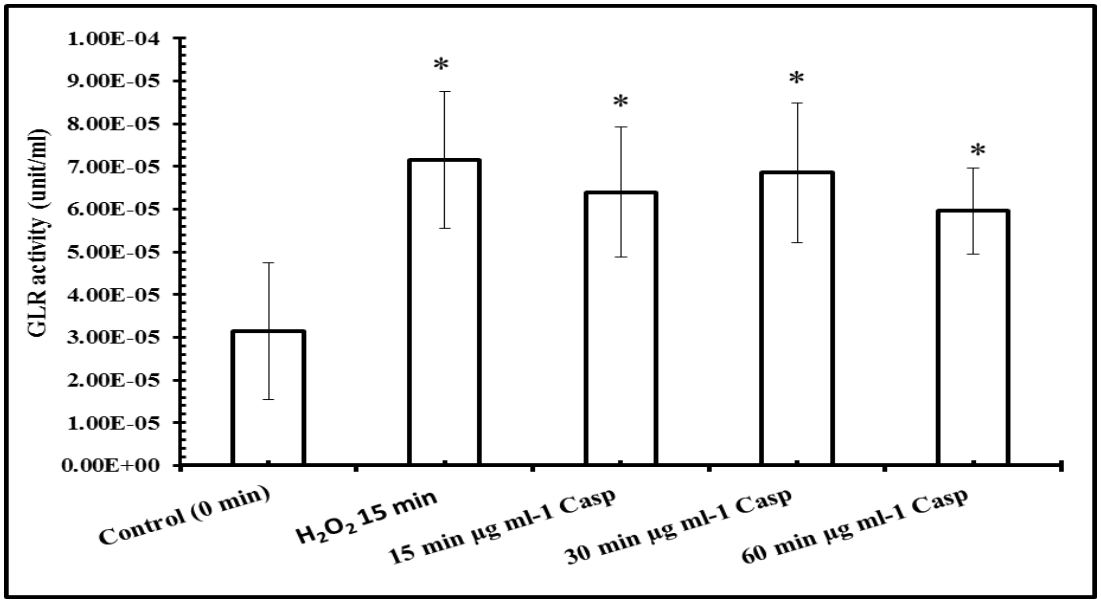


Figure 5.4 Glutathione reductase activity in caspofungin treated *A. fumigatus*. Hydrogen peroxide is the positive control.

Hyphae were exposed to 0.5 mM hydrogen peroxide for 15 minutes or 1.0 µg/ml caspofungin for 15, 30 or 60 minutes. Indicates a ($p < 0.02$)

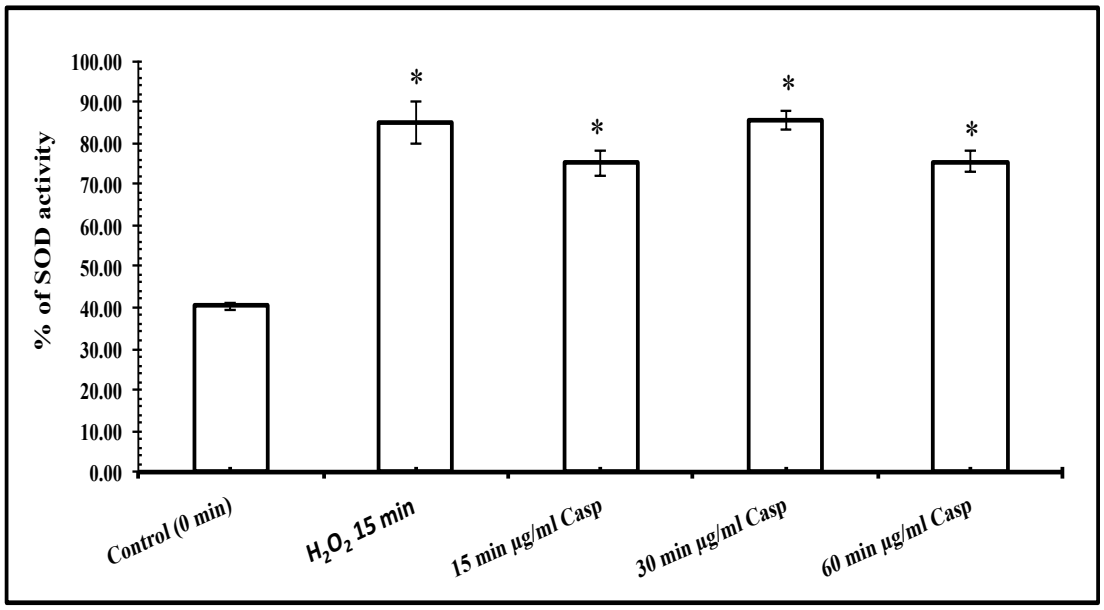


Figure 5.5 Superoxide dismutase activity in caspofungin treated *A. fumigatus*

Hyphae were exposed to 0.5 mM hydrogen peroxide for 15 minutes or 1.0 µg/ml caspofungin for 15, 30 or 60 minutes. Indicates a p value = 0.05

5.4 Discussion

Caspofungin is widely used in the treatment of systemic fungal infections and has the advantage of being highly effective but of negligible toxicity to humans (Denning, 2003). The aim of the work presented here was to establish how *A. fumigatus* responded to caspofungin and to determine whether the fungus mounted a response aimed at minimizing the effect(s) of the drug. Previous work established that exposure of *C. albicans* to caspofungin lead to the induction of oxidative and osmotic stress responses as evidenced by the activation of the Cap and Hog pathways (Kelly *et al.,* 2009).

The release of protein from *A. fumigatus* following exposure to caspofungin (section 4.3.2) could indicate a general increase in cell permeability thus suggesting the need for the cell to counteract the elevated permeability of the cell. Analysis of the changes in the expression of proteins following exposure of *A. fumigatus* to 0.1 μg/ml caspofungin indicated the increased expression of a number of proteins associated with the oxidative stress response including spot B18 (catalase), spot B19 (Hsp70), and spot 12 (thioredoxin reductase). Protein spot B1 (18 kDa antigen) and spot B3 (allergen Asp F3) which are associated with virulence were also increased in abundance. Cagas *el al*., (2011) noted an increase in the secretion of Asp F3 (3.5 fold) from susceptible *A. fumigatus* strains exposed to caspofungin. This allergen is a thioredoxin peroxidase and is increased in expression in cells experiencing oxidative stress as a result of exposure to hydrogen peroxide (Lessing *et al.,* 2007). Exposure of *A. fumigatus* to caspofungin resulted in the elevated activity of catalase, glutathione reductase and superoxide dismutase, which are associated with the oxidative stress response. Increased activity of these enzymes has also been observed in *C. albicans* exposed to caspofungin or hydrogen peroxide (Kelly *et al.,* 2009).

While caspofungin is a highly effective antifungal agent and has proven clinical efficacy, the response of fungal cells to it may not be fully determined. The primary action of caspofungin lies in the inhibition of glucan synthase and the consequent disruption of cell wall formation in fungi. However it has been established that exposure of *C. albicans* to this agent leads to increased chitin content (Walker *et al.,* 2008) and the induction of a number of genes associated with the PKC pathway in *C. albicans* leading to alterations in cell wall architecture (Munro *et al.,* 2007). In

addition, caspofungin has been shown to unmask glucan in the cell wall and thus may lead to a potentially elevated immune response (Wheeler *et al.,* 2008).

The results presented here indicate that exposure of *A. fumigatus* to caspofungin leads to the induction of an oxidative stress response as indicated by the increased expression and activity of a number of stress–response proteins and enzymes. Previous work demonstrated the enhanced leakage and *de novo* biosynthesis of gliotoxin from caspofungin-treated *A. fumigatus* (Chapter 4). Elevated production of this toxin by *A. fumigatus* following its amphotericin B- or caspofungin-mediated release (Reeves *et al.,* 2004; chapter 4) may be associated with restoring the redox balance within the fungus (Schrettl *et al.,* 2010). Cagas *et al.,* (2011) have demonstrated altered expression of a range of proteins in *A. fumigatus* following exposure to caspofungin. The work presented here demonstrates the increased expression of a number of protein associated with the oxidative response and the elevated activity of enzymes associated with this response.

While the primary mode of action of caspofungin is the inhibition of glucan biosynthesis (Denning, 2003) this work and that of others (Walker *et al.,* 2008; Wheeler *et al.,* 2008; Stevens *et al.,* 2006; Kelly *et al.,* 2009; Cagas *et al.,* 2011) demonstrate other effects including the stimulation of chitin synthesis and the induction of an oxidative stress response as the fungal cell attempts to counteract the direct and indirect effects of the antifungal.

Chapter 6

GENERAL DISCUSION

Aspergillus is the second most common pathogenic fungus after *Candida* and is responsible for up to 30 % of all fungal infections in cancer patients. However *A. fumigatus* and other species have emerged as important causes of morbidity and mortality in immunocompromised individuals (Fluckiger *et al.,* 2006). *A. fumigatus* is recognised as an important cause of life-threatening fungal infections in the immunocompromised population. This expanding population is composed of patients with prolonged neutropenia, HIV infection, and patients who have undergone hematopoietic stem cell transplantation (HSCT) and/or lung transplantation (Walsh *et al.,* 2004). The major forms of aspergillosis are invasive aspergillosis, aspergilloma, and allergic forms of aspergillosis (ABPA). Invasive aspergillosis currently constitutes the most common cause of mortality in pneumonia patients and is an important cause of opportunistic respiratory and disseminated infection in other immunocompromised populations (Dagenais and Keller, 2009). Furthermore, *Aspergillus* species also produce a wide range of chronic, saprophytic, and allergic conditions. Invasive aspergillosis (IA) is one of the most difficult infections to treat, with high mortality rates approaching 90% in the most severely immunocompromised patients.

Antifungal therapy remains the mainstay of treatment for invasive aspergillosis. The main groups of drugs used to treat aspergillosis are azoles, (voriconazole, itraconazole, posaconazole), polyene (amphotericin B) and echinocandins, (caspofungin, micafungin). Caspofungin is recommended for use in patients with invasive aspergillosis that is refractory to or intolerant of azole and polyene drugs.

The nephrotoxicity is the main side effect of polyene antifungal drugs. Amphotericin B have been documented and the demonstrated rates of renal failure as high as 30% in patients. The use of itraconazole, voriconazole and posaconazole has been evaluated with voriconazole proving to be the recommended therapy for most

immunocompromised patients to reduce the nephrotoxicity side effect and increase efficacy of drug (Steinbach and Stevens, 2003). The use of the echinocandin drugs caspofungin, micafungin, and anidulafungin has also been demonstrated in combination therapy with azole antifungals. The echinocandins are effective at inhibiting hyphal growth and have limited toxicity thus highlighting their utility in antifungal treatment (Sable *et al.,* 2008). Caspofungin is an echinocandin antifungal compound, first licensed for use by FDA in the United States in 2001 (Kartsonis *et al.,* 2003). Caspofungin is indicated for the treatment of invasive aspergillosis in patients who are intolerant of amphotericin B, lipid formulations of amphotericin B and/or azoles. It is the first representative of glucan synthesis inhibitors, a new class of antifungal agents that inhibit the synthesis of beta (1,3)-D-glucan, an integral polymer part of the fungal cell wall. Caspofungin is a sterile powder of lyophilised product for intravenous infusion that contains 70 mg caspofungin; the recommended dose is 70 mg as a single loading dose on Day 1, followed by 50 mg daily thereafter.

This study looked at the antifungal activity of caspofungin on *A. fumigatus*. It is difficult to determine the actual MIC for caspofungin as the paradoxical effect of caspofungin recorded when caspofungin was used at concentrations above MIC on the growth of *C. albicans* (Stevens *et al.,* 2004). This phenomenon has been clearly observed in this study especially when using different types of culture media for susceptibility testing. The reasons for this effect are still unclear. This effect has been observed elsewhere, where high growth was recorded after caspofungin treatment (Kyselgof *et al.,* 2007). This phenomenon may indicate that caspofungin may not kill the cells, but prevent them from actively dividing. Morphological alterations of hyphae were observed using electron microscopy after exposure to caspofungin (Section 4.6 / Figure 4.29). The unique mechanism of action of the echinocandins results in a lack of cross-resistance with azoles and polyenes and the spectrum of activity includes *Aspergillus* and *Candida* species. Development of resistance to caspofungin is a rare event. In recent years the over-use of antifungal drugs induced the emergence of azole resistance as a result of mutations in the *cyp51A* gene however it is now believed that some other genes may be responsible for the resistance (Verweij *et al.,* 2009). It was reported that reduced susceptibility of clinical and laboratory *C. albicans* strains to caspofungin is linked with mutations in FKS1 at codon 645 in which serine is replaced by proline, tyrosine, or phenylalanine (Balashov *et al.,* 2005). It have been

demonstrated that caspofungin resistance can occur at a high frequency following mutation in the *FKS1* gene of *C. albicans* and most prominently to alterations of serine 645 of Fks1p (Sergey *et al.,* 2006). Previous studies indicate that neither target site mutations, nor changes in target gene expression are present in *A. fumigatus*. Instead, other groups found that results indicate that the molecular mechanism underlying reduced susceptibility to caspofungin in the *A. fumigatus* strains is novel, possibly due to remodelling of the cell wall components (Gardiner *et al.,* 2005).

The advantages of caspofungin versus fluconazole include the expanded spectrum of activity against *Candida* and *Aspergillus*, and their decreased potential for drug interactions. Potential disadvantages include their higher cost, lack of oral formulations and lack of activity against some emerging pathogens such as *Scedosporium, Fusarium*, and *zygomyces* (Gregory *et al.,* 2007).

Previous work demonstrated that non-growing *C. albicans* cells are susceptible to the effect of caspofungin and it mediated the release of proteins which could lead to a stronger immune response *in vivo* (Kelly and Kavanagh, 2010). In addition to hampering cell wall synthesis, caspofungin may also interfere with the permeability of the fungal cell wall (Kelly and Kavanagh, 2010). Analysis of the proteomic changes that occur in *A. fumigatus* following exposure to caspofungin revealed decreased expression of proteins associated with the mitochondrial hypoxia response (Cagas *et al.,* 2011). In addition decreased expression of Asp F1 was also observed.

The experiments described in Chapter three investigated the effect of environmental factors (e.g. pH, glucose, protein and serum concentration) on the growth and susceptibility to caspofungin of *A. fumigatus* and *C. albicans*. As caspofungin interferes with cell wall synthesis it was postulated that only actively growing cells would be susceptible to drug treatment. However previous work established that caspofungin was effective against growth arrested cells of *C. albicans* despite the reduction in the activity of β 1,3 glucan synthase (Kelly & Kavanagh, 2009). It is clear from this study that environmental factors affect the antifungal activity of caspofungin and the response of the fungi to this drug. The results presented here can lead to another conclusion that the *in vitro* result may translate *in vivo* and may be of clinical relevance. Caspofungin may not be as effective as other antifungal agents in the treatment of vulvovaginal candidiasis (VVD), in women where the pH is within

range of 4 to 5 (Fidel & Sobel, 1996). Another condition is commonly associated with elevated fungal infection is diabetes mellitus which is associated with higher blood glucose level (Belazi *et al.,* 2005). Caspofungin may also display a reduction in activity in acidic environment such as the stomach in cases of *Candida* infection. Change in the protein profile within different environmental conditions give further understanding of the effect of protein on the increased tolerance of *C. albicans* and *A. fumigatus* to caspofungin.

Mycotoxins production has been identified as a key factor in the modulation of the host immune response during pathogenic fungal infection. *A. fumigatus* has been demonstrated to produce a range of secondary metabolites including mycotoxins with different harmful effects. These include gliotoxin, fumagillin, helvolic acid, fumitremorgin, fumigaclavine C, aureperone C, restrictocin (Dagenais and Keller, 2009). *A. fumigatus* produces a variety of secondary metabolites and enzymes which are associated with growth and persistence within pulmonary tissue (Bennet and Klich, 2003; Ben-Ami *et al.,* 2010). As a result of this an analysis of the production of gliotoxin within caspofungin-treated hyphae was necessary to facilitate an improved understanding of *A. fumigatus* pathogenesis *in vivo*. It has been demonstrated that gliotoxin was produced in significant quantities *in vitro* and reached peak production after exposure to Amphotericin B (Reeves *et al.,* 2004). It was previously established that exposure of growth arrested stationary phase *C. albicans* cells to caspofungin induced the release of amino acids and a variety of intracellular peptides (Kelly and Kavanagh, 2010). In the results presented in Chapter four stationary phase cultures of *A. fumigatus* were exposed to caspofungin so *de novo* wall formation would not have been a significant occurrence. Caspofungin induced leakage of gliotoxin and the fungus responded to this by synthesizing elevated levels of toxin intra-cellularly. Caspofungin caused an increased release of gliotoxin. The immunosuppressive effects of gliotoxin are well established (Tsunawaki *et al.,* 2004) and any therapy that inadvertently induced the increased biosynthesis and release of this toxin could exacerbate inflammation and tissue damage at the site of infection. Caspofungin also increased the biosynthesis of ergosterol, this mechanism may protect the cell when the cell wall is targeted with drugs (section 4.5.1). Consequently, it is possible that in addition to inhibiting glucan biosynthesis, caspofungin may have a secondary effect on membrane permeability and thus facilitate the leakage of amino acids, protein and toxins from

fungal cells. Depletion of the intracellular gliotoxin may stimulate increased biosynthesis of the toxin to replenish the lost gliotoxin in order to restore the redox balance within the cell (Schrettl *et al.,* 2010). This phenomenon of caspofungin-induced gliotoxin leakage and biosynthesis has the potential to contribute to elevated levels of pulmonary damage and immunosuppression (KwonChung and Sugui, 2009; Tsunawaki *et al.,* 2004). It is possible that administration of caspofungin to patients may lead to eventual fungal cell death but that before this is achieved, internal gliotoxin would be released from cells and the cells could have the opportunity to commence synthesizing more gliotoxin to replenish that was lost into surrounding tissue. Previous work that examined the response of *C. albicans* to amphotericin B illustrated the ability of cells to recover from antifungal therapy even though they appeared incapable of replicating in culture (Liao *et al.,* 1999).

The results presented in Chapter five indicate that exposure of *A. fumigatus* to caspofungin leads to the induction of an oxidative stress response as indicated by the increased abundance and activity of a number of stress response proteins and enzymes. In addition, caspofungin has been shown to unmask glucan in the cell wall and thus may lead to a potentially elevated immune response (Wheeler *et al.,* 2008). Exposure of *A. fumigatus* to caspofungin resulted in the elevated activity of catalase, glutathione reductase and superoxide dismutase which are associated with the oxidative stress response. Increased activity of these enzymes has also been observed in *C. albicans* exposed to caspofungin or hydrogen peroxide (Kelly *et al.,* 2009).

It can be concluded from this work that caspofungin results in the activation of an oxidative stress response in *A. fumigatus*. As shown in a previous study (Kelly and Kavanagh, 2010), caspofungin caused leakage of intracellular component from *C. albicans* and increased in ergosterol level of the membrane (Chapter 4, Figure 4.26) it may lead to changes in the membrane permeability or cell wall organization, which possibly results in the oxidative stress within the cell. Interestingly, another conclusion that can be made is that caspofungin resulted in the leakage of proteins from different locations within the cell including cell wall, cytoplasm and mitochondria.

C. albicans and *A. fumigatus* were capable of growing in the diverse range of culture conditions but the environmental factors affect the antifungal activity of caspofungin or the response of the cells to this drug. The data presented in this study

show that *in vitro* results may translate to *in vivo* and may lead to a significant clinical relevance for some diseases e.g diabetes, vulvovaginal candidiasis and hyper acidosis. As caspofungin interferes with cell wall synthesis, it is not surprising to see escape of proteins from a range of location within the cell and caspofungin also alter the protein profile of cells. This suggests that cell may attempt to protect itself by altering various biosynthetic pathways.

The results presented in this study indicate that environmental factors play a significant role in altering the susceptibility of *A. fumigatus* and *C. albicans* to caspofungin. Future work might examine whether patients displaying high blood glucose levels (e.g. diabetes mellitus) display altered susceptibility to caspofungin as a result of altered fungal metabolism. In particular, due to the development of cross-resistance, the various modes of action of caspofungin may be used as combination therapy with polyenes or azoles and could lead to a synergistic effect. Future work might also examine the mechanism of caspofungin-induced *in vivo* leakage of gliotoxin from *A. fumigatus*.

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