PROTEIN PROFILING OF *Candida albicans* PLANKTONIC AND BIOFILM CULTURES UPON EXPOSURE TO AUREOBASIDIN

KOMATHY A/P MUNUSAMY

FACULTY OF MEDICINE

UNIVERSITY OF MALAYA

KUALA LUMPUR

2019

PROTEIN PROFILING OF *Candida albicans* PLANKTONIC AND BIOFILM CULTURES UPON EXPOSURE TO AUREOBASIDIN

KOMATHY A/P MUNUSAMY

DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MEDICAL SCIENCE

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2019

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: Komathy a/p Munusamy

Matric No: MGN 130025

Name of Degree: Master of Medical Science

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

Protein profiling of *Candida albicans* planktonic and biofilm cultures upon exposure to Aureobasidin

Field of Study: Proteomics and Mycology

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date:

Subscribed and solemnly declared before,

Witness's Signature Date:

Name:

Designation:

UNIVERSITI MALAYA

PERAKUAN KEASLIAN PENULISAN

Nama: Komathy a/p Munusamy

No. Matrik: MGN 130025

Nama Ijazah: Master of Medical Science

Tajuk Kertas Projek/Laporan Penyelidikan/Disertasi/Tesis ("Hasil Kerja ini"):

Profil protein kultur planktonic dan biofilm *Candida albicans* selepas pendedahan kepada Aureobasidin

Bidang Penyelidikan: Proteomik dan Mikologi

Saya dengan sesungguhnya dan sebenarnya mengaku bahawa:

(1) Saya adalah satu-satunya pengarang/penulis Hasil Kerja ini;
(2) Hasil Kerja ini adalah asli;
(3) Apa-apa penggunaan mana-mana hasil kerja yang mengandungi hakcipta telah dilakukan secara urusan yang wajar dan bagi maksud yang dibenarkan dan apa-apa petikan, ekstrak, rujukan atau pengeluaran semula daripada atau kepada mana-mana hasil kerja yang mengandungi hakcipta telah dinyatakan dengan sejelasnya dan secukupnya dan satu pengiktirafan tajuk hasil kerja tersebut dan pengarang/penulisnya telah dilakukan di dalam Hasil Kerja ini;
(4) Saya tidak mempunyai apa-apa pengetahuan sebenar atau patut semunasabahnya tahu bahawa penghasilan Hasil Kerja ini melanggar suatu hakcipta hasil kerja yang lain;
(5) Saya dengan ini menyerahkan kesemua dan tiap-tiap hak yang terkandung di dalam hakcipta Hasil Kerja ini kepada Universiti Malaya ("UM") yang seterusnya mula dari sekarang adalah tuan punya kepada hakcipta di dalam Hasil Kerja ini dan apa-apa pengeluaran semula atau penggunaan dalam apa jua bentuk atau dengan apa juga cara sekalipun adalah dilarang tanpa terlebih dahulu mendapat kebenaran bertulis dari UM;
(6) Saya sedar sepenuhnya sekiranya dalam masa penghasilan Hasil Kerja ini saya telah melanggar suatu hakcipta hasil kerja yang lain sama ada dengan niat atau sebaliknya, saya boleh dikenakan tindakan undang-undang atau apa-apa tindakan lain sebagaimana yang diputuskan oleh UM.

Tandatangan Calon Tarikh:

Diperbuat dan sesungguhnya diakui di hadapan,

Tandatangan Saksi Tarikh:

Nama:

Jawatan:

PROTEIN PROFILING OF *CANDIDA ALBICANS* PLANKTONIC AND BIOFILM CULTURES UPON EXPOSURE TO AUREOBASIDIN

ABSTRACT

Candidiasis is a fungal infection leading to significant morbidity and mortality in immunocompromised patients. Aureobasidin (AbA), a cyclic depsipeptide antifungal drug, has been reported to be effective against *Candida albicans* but is yet to be used for therapy. Although AbA is presumed to target the fungal sphingolipid biosynthesis, the exact mechanism of action has yet to be defined. As a prerequisite to study the mechanism of action of this antifungal drug, this study investigated changes in the proteome of planktonic and biofilm cultures of *C. albicans* upon exposure to AbA. In this study, the whole cell extracts of biofilm and planktonic cultures of *C. albicans* strain SC5314 pre- and post-exposure to AbA were subjected to liquid chromatography mass spectrometry (LC-MS/MS) analysis. Using sequence homology search tool (SPIDER) of PEAKS software (ver. 7.5), proteins were identified based on the setting of at least one unique peptide matched and a significance (-10lgP) value of >20. The proteome prior to AbA exposure demonstrated a total of 203 and 533 proteins from the planktonic and biofilm cultures, respectively, with a false discovery rate of ≤1%. The biological process, molecular function, and subcellular localization of biofilm and planktonic proteins were annotated using Go Slim Mapper of the online *Candida* Genome Database. Classification of biological processes demonstrated greater differences in the number of biofilm proteins associated with the regulation of biological processes (n=65), organelle organization (n=57), transport (n=55), stress response (n=47), filamentous growth (n=30), chemical response (n=30), lipid metabolism (n=23) and carbohydrate metabolism (n=20) compared to planktonic culture. As for molecular function, no major difference was observed in both cultures,

with approximately 10-20% of the proteins are associated with hydrolase, transferase and protein binding activities. On subcellular localization, 68.7 and 66.4% proteins of planktonic and biofilm cultures were predicted to be localized in cytoplasm. A total of 293 and 374 proteins were annotated in the AbA-treated planktonic and biofilm cultures with sub-inhinbitory AbA concentration (1 µg/ml) for 2.5 hours. A lower percentage of proteins annotated for organelle organization in the AbA-treated planktonic culture (18.1%) as compared to the dimethyl sulfoxide (DMSO)-treated planktonic culture (23.2%). There was a 5% increase in the proteins annotated for translation in AbA-treated planktonic culture. AbA-treated biofilm culture has shown reduction in the percentages of proteins annotated for vesicle-mediated transport (from 8.4 to 5.3%) and filamentous growth (15.8 to 12.8%), and an increase in the percentage of proteins annotated for carbohydrate metabolic process (8.2 to 12.0%). The regulation of cellular amide metabolic process and actin cytoskeleton organization are two biological processes shared by AbA-treated planktonic and biofilm cultures. This study also reviewed some *C. albicans* proteins which are annotated for pathogenesis, biofilm formation, lipid metabolic process and filamentous growth in response to AbA. Further exploration of the drug-affected proteins may aid in the search for potential drug target in the treatment of candidiasis.

ABSTRAK

Kandidiasis ialah jangkitan kulat yang menyebabkan morbiditi dan mortaliti yang signifikan di kalangan pesakit yang berimuniti rendah. Aureobasidin (AbA) ialah ubat antikulat "cyclic depsipeptide" yang telah dilaporkan berkesan terhadap *Candida albicans* tetapi belum digunakan untuk terapi. Walaupun AbA menyasar biosintesis sphingolipid kulat, mekanisme tindakan yang sebenarnya masih belum diketahui. Sebagai prasyarat untuk menyelidik mekanisme ubat, perubahan proteome kultur planktonik dan biofilem *C. albicans* selepas pendedahan kepada AbA perlu dikenalpasti. Ekstrak keseluruhan daripada kultur biofilem dan planktonik *C. albicans* SC5314 telah diperolehi sebelum dan selepas pendedahan kepada AbA untuk analisa spektometer jisim–kromatografi cecair. Dengan menggunakan alat carian urutan homologi (SPIDER) perisian PEAKS (ver. 7.5), protein telah dikenalpasti berdasarkan penetapan sekurang-kurangnya dengan satu peptida unik yang berpadanan dengan nilai kepentingan (-10lgP) > 20. Proteome sebelum pendedahan kepada AbA menunjukkan sejumlah 203 dan 533 protein daripada kultur planktonik dan biofilem masing-masing, dengan "false discovery rate" ≤1 %. Proses biologi, fungsi molekul, dan penyetempatan subselular untuk protein-protein kultur biofilem dan planktonik telah dianotasi dengan menggunakan 'Go Slim Mapper' dari pangkalan data genom *Candida*. Pengelasan proses biologi menunjukkan perbezaan lebih besar dalam bilangan protein kultur biofilem yang berhubungkait dengan pengawalan proses biologi (n = 65), organisasi organel (n = 57) , pengangkutan (n = 55), gelakbalas stres (n = 47), pertumbuhan filamen (n = 30), gerakbalas kimia (n = 30), metabolisme lipid (n = 23) dan metabolisme karbohidrat (n = 20) berbanding kultur planktonik. Bagi fungsi molekul, tidak ada perbezaan ketara diperhatikan antara dua kultur. Secara anggaran, 10-20% protein daripada kedua-dua kultur telah dihubungkait dengan aktiviti hydrolase,

transferase dan pengikatan protein. Untuk penyempatan subselular, sejumlah 68.7% dan 66.4 % protein daripada kultur planktonik dan biofilem tertumpu dalam sitoplasma. Sejumlah 293 dan 374 protein telah dianotasi dalam kultur planktonik dan biofilem yang dirawat dengan AbA (1 µg/ml) selama 2.5 jam. Terdapat peratusan protein yang rendah yang dianotasi untuk organisasi organel dalam kultur planktonik yang dirawat dengan AbA (18.1%) berbanding dengan kultur planktonik yang dirawat dengan dimetil sulfoksida (DMSO, 23.2%). Terdapat peningkatan sebanyak 5% dalam protein yang dianotasi untuk translasi dalam kultur planktonik yang dirawat dengan AbA. Peningkatan dalam peratusan protein ini mungkin disebabkan oleh pengeluaran protein yang berkaitan dengan tekanan. Kultur biofilem yang diubati dengan AbA menunjukkan pengurangan peratusan protein yang dianotasi untuk pengangkutan diperantarakan vesikel (dari 8.4 hingga 5.3%) dan pertumbuhan filamen (15.8 hingga 12.8%), dan peningkatan dalam peratusan protein untuk proses metabolik karbohidrat (8.2 kepada 12.0%). Proses metabolik yang melibatkan amida selular dan organisasi aktin sitoskeleton adalah dua proses biologi yang dikongsi oleh kultur planktonik dan biofilem yang dirawat dengan AbA. Patogenesis, pembentukan biofilem, proses metabolik lipid, pertumbuhan filamen dan tindakbalas kimia adalah proses biologi penting yang dibincangkan dalam kajian ini. Protein yang ditemui untuk proses-proses ini dalam kedua-dua kultur planktonik dan biofilem selepas rawatan AbA adalah calon protein antifungal yang boleh dipelajari selanjutnya. Selain daripada menunjukkan kerumitan tindakbalas selular *C. albicans* terhadap AbA, penerokaan lanjut mengenai protein yang dipengaruhi oleh AbA boleh membantu dalam pencarian sasaran ubat yang berpotensi untuk rawatan kandidiasis.

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to my supervisors, Prof. Dr Tay Sun Tee and Prof. Dr Jamuna Vadivelu. Thank you for your continuous guidance, patience, and for being understanding especially during the most difficult times of the study. Without the guidance and persistent help, this dissertation would not have been possible.

My sincere gratitude goes to Dr Loke Mun Fai for all his help in LCMS experiments and for providing me with all the necessary facilities to complete the protein run. I am extremely grateful and indebted to him for his expertise, sincerity and valuable guidance extended to me. My deepest thanks to Miss Na Siang Ling from the Medical Microbiology Laboratory of the UMMC for collecting and providing the clinical isolates for this study.

I take this opportunity to record my sincere thanks to Dr. Vanitha, Dr. Kumutha and Dr. Anis Almaleki for their valuable guidance and encouragement throughout this research. I also thank my colleagues, Azadeh, Kho Kai Ling, Koh Fui Xian, Fatin Izzati, Nur Wahidah and Hou Siau Li for being very supportive until the completion of my bench work.

I wish to express my thanks to all the members of Department of Medical Microbiology for their help and support. Not forgetting my parents for their unceasing encouragement and support.

I also would like to extend my deepest gratitude to University of Malaya for supporting me with a scholarship (Skim Biasiswa Universiti Malaya). This study was supported by the Postgraduate Research Grant (PG045-2013B) and Fundamental Research Grant Scheme (FP035-2014A).

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
β	Beta
µ	Microgram
µm	Micrometer
1-DE	1-dimensional electrophoresis
2-D PAGE	Two-dimensional polyacrylamide gel electrophoresis
5-FC	5-Flucytosine
AbA	Aureobasidin A
ABC	ATP-binding cassette
ACN	Acetonitrile
AIDS	Acquired immunodeficiency syndrome
ATCC	American Type Culture Collection
CDC	Centre for Disease Control & Prevention
CGD	Candida Genome Database
CHIFNET	China Hospital Invasive Fungal Surveillance Net
CLSI	Clinical & Laboratory Standards Institute
CV	Crystal Violet
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
FEI	Field Electron and Ion
FESEM	Field Emission Scanning Electron Microscopic

GO	Gene Ontology
H_2O_2	Hydrogen peroxide
HPLC	High Performance Liquid Chromatography
i.e/ e.g	exempli gratia
IAA	Iodoacetamide
ICU	Intensive care unit
IPC	Inositolphosphorylceramide
ITS1	Internal transcribed spacer 1
LCMS	liquid chromatography mass spectrometry
MALDI-TOF MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometer
MIC	Minimum inhibitory concentration
NADH	Nicotinamide adenine dinucleotide hydrogenase
OD	Optical Density
PBS	Phosphate buffered saline
PEAKS	Proteomic Mass Spectrometry Software
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SAP	Secreted aspartyl proteinases
SD	Synthetic Dextrose medium
SDS-PAGE	Sodium Dodecyl Sulphate
SEM	Scanning electron microscope
sp.	Species (singular)
SPIDER	Sequence homology search tool
spp.	Species (plural)
SPSS	Statistical Package for the Social Sciences

TOF	Time-of-Flight
XICs	Extracted ion chromatograms
XTT	2,3-bis (2-methoxy-4- nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl] -2H-tetrazolium hydroxide)

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background of the study

Candida yeasts are members of normal flora of the mucocutaneous, gastrointestinal, and genitourinary areas in the healthy humans. The organisms can become opportunistic pathogens under certain conditions (Calderone & Gow, 2002) and cause a wide variety of infections, ranging from superficial thrush to dangerous invasive infections (Larriba *et al*., 2010). *Candida* spp. are among the five most common pathogenic agents of bloodstream infections in hospitalized patients globally (Pfaller & Diekema, 2007). Although the incidence of infections caused by non-albicans *Candida* species is escalating, *Candida albicans* remains as the most dominant species causing candidiasis, irrespective of the clinical settings and geographical locations (Sudbery *et al*., 2004).

Candidiasis includes mucosal and invasive candidiasis. Mucosal candidiasis is commonly known to affect the mucous membranes of the mouth and throat (oropharyngeal or oral candidiasis), esophagus (esophageal candidiasis) and vagina (vulvovaginitis). Invasive candidiasis, also known as systemic candidiasis or hematogenously disseminated candidiasis, involves the spread of the infection through bloodstream to various organs, such as the heart, brain, lungs, liver and kidneys. Pyrexia is normally present during a *Candida* infection. Invasive candidiasis often affects immunocompromised and immunodeficient patients undergoing hemodialysis, diabetes, persons with intravascular catheters, abdominal surgery and those under extended therapy with broad-spectrum corticosteroids or antibiotics. Elevation in antifungal resistance results in difficulties in treating and eradicating invasive candidiasis (Ellis, 2002).

A number of *C. albicans* virulence genes and proteins have been identified to contribute to the establishment of infection. Adherence to host cells, secretion of degradative enzymes, and morphology switching are among the factors contributing to the successful development of *Candida* infection. Yet, the mode of action of the respective virulence factors and the series of actions resulting in host invasion remains unknown (Lim *et al*., 2012).

Microorganisms exhibit different characteristics when they are grown either as free-floating cells (planktonic culture) or biofilm cells (Douglas, 2002). Biofilms are individual organisms that are embedded within a self-produced, slimy, and extracellular polymeric substance. Biofilm can be composed of microorganisms of different species or of the similar species (Adam *et al*., 2002; Davey & O'toole, 2000). The formation of biofilm benefits the microorganism by giving protection from the environment, metabolic co-operation, nutrient accessibility and acquirement of new genetic traits (Davey & O'toole, 2000; Douglas, 2003). Biofilms are extremely difficult to abolish and are the precursors for many infections. The establishment of *C. albicans* biofilm comprises of three developmental phases. The early phase involves the adherence of yeast cells to the device surface. The intermediate phase involves construction of matrix with morphology shift from yeast to hyphal forms. During the maturation phase, an increase in the surrounding material (matrix) forming on a three-dimensional structure is always observed (Chandra *et al*., 2001; Hawser & Douglas, 1994). The entirely matured *Candida* biofilms have a mixture of different morphological forms and a dense network of yeasts, hyphae, and pseudohyphae which are encased in a matrix carbohydrate, polysaccharides (Chandra *et al*., 2001), protein and unknown constituents (Kojic & Darouiche, 2004).

Candida biofilms are known to withstand host immune responses, and are significantly less susceptible to various antifungal agents, including amphotericin B (AMB), fluconazole, itraconazole, and ketoconazole compared to the planktonic (free-floating) cells (Bruckmann *et al*., 2000; Tan & Tay, 2013). Understanding the molecular basis underlying the resistance of biofilm-forming organisms towards antifungal agents is essential. The need for effective drugs for treatment of biofilm-associated infections has necessitated the search for new antifungal drug. Aureobasidin A (AbA) is a depsipeptide antibiotic produced by a black killer yeast, *Aureobasidium pullulans* (Naglik *et al*. 2003; Poikonen *et al*., 2003; Takesako *et al*., 1991) which targets at the fungal sphingolipid biosynthesis. It exhibits strong *in vitro* fungicidal activity against a variety of fungi including *C. albicans*, *Cryptococcus neoformans* and *Aspergillus spp.* (Naglik *et al*., 2003; Poikonen *et al*., 2003). It inhibits inositolphosphorylceramide (IPC) synthase, a fungal specific enzyme which is absent in humans. AbA is also known to arrest the growth of yeast cells through ceramide intoxication and deprivation of essential IPC in wild type cells (Cerantola *et al*., 2009) and inhibit the protein kinase activity of *Saccharomyces cerevisiae* (Roelants *et al*., 2010).

Many aspects of *C. albicans* have been studied using proteomic analysis, including its adaptive responses to salt, cadmium, peroxide, oxidative stress, macrophage exposure, fluconazole, itraconazole, and mulundocandin (Santana *et al*., 2013). Proteome analysis is necessary to determine which proteins have been conditionally and intensely expressed and whether any post-translational modifications are affected. Two or additional states of a cell or an organism (normal and disease state) can be compared and specific qualitative and quantitative protein changes could be identified (Luberto *et al*., 2001). Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) is capable of resolving over 1,800 proteins in a single gel (Nagiec *et al*.,

1997). Therefore, it is important as the principal means of proteomics research where multiple proteins are separated for comparable analysis and hundreds to thousands of gene products can be analyzed simultaneously (Luberto *et al*., 2001). Liquid chromatography mass spectrometry (LC/MS) is another approach of proteomic analysis which enhances protein profiling study and has provided significant impact to the fields of separation and automation, as well as sample introduction (ionization) technologies (Lyytikäinen *et al*., 2002). Today, LC/MS-based methodologies are vital in all the fundamental steps involved in the development of small-molecule therapeutics, including target identification, evaluation of compound identity/purity, synthesis, pharmacokinetics and drug breakdown, clinical assessment of activity or efficacy and quality control of a large proportion of drug substance (scale-up process, formulations) (Cheng *et al*., 2001).

This study was carried out in three phases. In the first phase of the study, *in vitro* susceptibility of the clinical isolates of various *Candida* spp. to AbA was determined. The inhibitory effects of AbA on planktonic and biofilm cells were investigated. All the *Candida* isolates were screened for the ability to develop biofilm using a crystal violet assay before subjected to *in vitro* susceptibility testing. In the second phase, the morphological alterations caused by AbA on planktonic and biofilm cells were investigated using electron microscopy. The proteome profiling of *C. albicans* SC5314 strain, pre- and post-exposure, to AbA were investigated using LCMS/MS approach to identify changes in protein expressed by both planktonic and biofilm cultures of *C. albicans* after exposure to AbA.

1.2 Objectives

Hence, the objectives of this study are:

1) To determine the *in vitro* susceptibility of clinical isolates of *Candida* spp. (for planktonic and biofilm-grown cells) and non-*Candida* spp. to Aureobasidin A (AbA)
2) To determine and compare the morphological changes of AbA-treated planktonic and biofilm cultures of *Candida* spp.
3) To determine the proteome profiling of *C. albicans* SC5314 strain, pre- and post-exposure, to AbA

CHAPTER 2: LITERATURE REVIEW

2.1 *Candida albicans* and candidiasis

Candida spp. are yeasts which commonly inhabit the mucus membrane of humans (Galagan *et al*., 2005; Jayatilake, 2011). They are members of the normal flora in the mucocutaneous, gastrointestinal, and genitourinary areas of healthy humans. However, *Candida* spp. are recognized as opportunistic pathogens which are prevalent in clinical settings (Larriba *et al*., 2010). The species is the sixth most common isolated nosocomial pathogen, especially from the urinary tract (Pfaller & Diekema, 2007) and the fourth most common cause of bloodstream infection (8%), leading to a mortality of 29% (Pfaller & Diekema, 2007). Globally, *Candida* spp. are among the five most common pathogenic agents of bloodstream infections in hospitalized patients (Pfaller & Diekema, 2007). There are more than 150 species of *Candida* spp. (Pfaller & Diekema, 2007), of which, *Candida albicans* is the most dominant species causing candidiasis irrespective of the clinical settings and geographical locations (Achkar & Fries, 2010). It is also the most virulent species contributing to a high mortality rate (Achkar & Fries, 2010).

The infections caused by *Candida* spp. range from superficial thrush to invasive candidiasis (Larriba *et al.*, 2010). Mucosal candidiasis commonly affects the mucous membrane of the mouth and throat (oropharyngeal or oral candidiasis), aesophagus (aesophageal candidiasis) and vagina (vulvovaginitis) (López-Martínez, 2010). Oral candidiasis is often described with adherent, white, confluent or painless discrete patches in the oral region. The infection has been associated with angular chelitis, impaired food intake and dysgeusia (Reinel *et al*., 2008). Centre for Disease Control and Prevention (CDC) and World Health Organization (WHO) have identified oral candidiasis as one of the major opportunistic infections (Ellepola & Samaranayake,

2000). Vulvovaginal candidiasis often presents in patients with large or small amount of vaginal discharge in whitish gray and thick form, accompanied with itching, burning, rash and pain during sex (Ellepola & Samaranayake, 2000).

Invasive candidiasis, also known as systemic candidiasis or hematogenously disseminated candidiasis, involves the spread of infection through bloodstream to various organs, such as the heart, brain, lungs, liver and kidneys. Pyrexia is normally present during infection. Invasive candidiasis often affects those immunocompromised and immunodeficient patients undergoing hemodialysis, diabetes, persons with intravascular catheters, abdominal surgery and those under extended therapy with broad-spectrum costicosteroids or antibiotics. Elevation in antifungal resistance results in difficulties in treating and eradicating invasive candidiasis (Ellis, 2002).

2.2 Non-albicans *Candida* species

Some studies reported that the incidence of *C. albicans* infections is reducing with the increase in the incidence of infections caused by non-albicans *Candida* spp. such as *C. tropicalis*, *C. glabrata*, *C. parapsilosis* etc. (Eggimann *et al*., 2003; Pfaller & Diekema, 2007; Bassetti *et al*., 2006; Chakrabarti *et al*., 2009; Colombo *et al*., 2007; Hasan *et al*., 2009). The epidemiology of non-albicans *Candida* spp. differs by region for unknown reasons (Pfaller & Diekema, 2007). Table 2.1 shows the relative frequency of various *Candida* species in candidemia reported by multicentre surveillance studies.

Table 2.1: Relative frequency of *Candida* spp in candidemia in multicenter surveillance studies (Falagas, Roussos & Vardakas 2010).

Country	Study design/ observation participating institutions/ time period (Ref.)	Total number of blood isolates	*C. albicans*	*C. glabrata*	*C. parapsilosis*	*C. tropicalis*
France	1) Prospective, 1998–2001, area of Lyon (covered by a group of hospitals) (Martin *et al.*, 2005)	198	49.5%	12.6%	12.1%	10.1%
Switzerland	1) Retrospective, 1997–2000, 17 university and tertiary care hospitals (original period 1991–2000) (Marchetti *et al*., 2004)	464	65–70%	15%	1–5%	5–9%
Finland	1) Prospective, 1999–2000, surveillance program (4 hospitals participating) (Lyytikäinen *et al*., 2002)	62	62.9%	6.5%	12.9%	2.3%
UK	1) Prospective, 2005–2006, 19 tertiary hospitals in Scotland (Odds *et al*., 2007)	300	52%	22.7%	11.7%	2%
USA	1) Prospective, 1995–2002, SCOPE nationwide surveillance study (Wisplinghoff *et al*., 2004)	1890	53.8%	18.8%	11.4%	11.2%
	2) Prospective, 1998–2002, 47 hospitals in 2 states (14 in Baltimore and 33 in Connecticut) (Hajjeh *et al*., 2004)	1143	45%	24%	13%	12%
	3) Prospective, 1998–2001, 16 hospitals in the state of Iowa (Diekema *et al*., 2002)	254	58%	20%	7%	11%
	4) Prospective, 1999–2003, 5 tertiary care hospitals from 4 countries (Godoy *et al*., 2003)	103 episodes of candidemia	42%	7.7%	21.3%	24.2%
	5) Prospective, 2003–2004, 11 tertiary care hospitals (Colombo *et al*., 2006)	711	40.9%	4.9%	20.5%	20.9%
	6) Prospective, 1995–2005, community-onset candidemia (Kung *et al*., 2007)	56 episodes of candidemia	39.7%	17.2%	15.5%	22.4%

A laboratory-based study in University of Malaya Medical Centre (UMMC) reported 16 *Candida* species isolated from patient samples (Ng *et al*., 2015). Generally, the most dominant *Candida* species isolated from patients from UMMC was *C. albicans* (Table 2.2). Among the non-albicans *Candida* species, *C. glabrata* was the most commonly detected, followed by *C. parapsilopsis*, *C. tropicalis* and *C. krusei*. Over the 14-year period, *C. albicans*, *C. parapsilopsis*, *C. tropicalis*, *C. glabrata*, and *C. krusei* constituted 99.50% of a total of 34220 isolates (Ng *et al.*, 2015). Various uncommon non-albicans *Candida* species were also detected from the clinical samples, including *C. rugosa*, *C. dubliniensis*, *C. guilliermondii*, *C. kefyr*, *C. lusitaniae*, *C. utilis*, *C. haemulonii*, *C. pelliculosa*, *C. ciferri* and *C. humicola*.

An increase in the number of *C. tropicalis* resistance to fluconazole has been reported over the past decade (Myoken *et al*., 2004; Kothavade *et al*., 2010). The ability of *C. tropicalis* to adhere, form biofilm and secrete aspartic proteinase enzyme has contributed to its virulence (Kothavade et al., 2010). Neonates admitted in intensive care unit (ICU) are more likely to acquire *C. tropicalis* through cross-contamination. Adults and children with haematological malignancies have a higher mortality rate due to candidiasis caused by *C. tropicalis*. In addition to weakened immunity, the high virulence and low azole susceptibility characteristics *C. tropicalis* have contributed towards the yeast being recognized as the leading cause of disseminated candidiasis in patients with haematological malignancies (Kothavade *et al*., 2010).

C. glabrata is a rising concern in causing mucosal infections at the clinical settings. It is associated with nearly 15% of all *Candida*-related systemic infections (Bethea *et al*., 2010; Kim & Sudbery, 2011; Lim *et al*., 2012; Pfaller & Diekema,

Table 2.2: *Candida* species isolated from clinical samples from year 2000 to 2013 in University Malaya Medical Centre (Ng *et al.*, 2015)

***Candida* spp.**	**Year**														**Total (%)**
	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	
C. albicans	890	1289	1311	1504	1620	1749	1611	2226	2175	2019	1633	1856	1734	1324	22941 (67.8)
C. tropicalis	119	167	183	247	244	218	235	258	335	255	222	236	206	237	3162 (9.3)
C. parapsilosis	124	245	189	345	251	321	410	220	286	283	259	229	285	245	3692 (10.9)
C. glabrata	107	171	210	221	293	318	271	362	350	370	325	305	396	330	4029 (11.9)
C. kefyr	1	0	0	1	0	1	0	0	0	0	0	3	3	1	10 (0.03)
Total	1241	1872	1893	2318	2388	2606	2527	3066	3146	2927	2439	2629	2624	2137	33834

2004; Sardi *et al*., 2013; Vincent *et al.,* 2009; Wisplinghoff *et al*., 2006). Patients with new acquisition of *C. glabrata* isolates experienced prolonged hospitalization with frequent use of antimicrobial compared to patients with no *Candida* exposure (Vazquez *et al*., 1998). The increase use of immunosuppressive therapy together with broad spectrum antifungal treatments have significantly increased the frequency of mucosal and bloodstream infections caused by *C. glabrata* (Lee *et al*., 2009). The incidence of candidemia caused by *C. glabrata* is higher in adults than in children, and even lower in neonates (Krcmery & Barnes, 2002). Unlike other species, *C. glabrata* lacks a number of virulence factors shown by other *Candida* species such as hyphal growth and ability to secrete proteases (Kaur *et al*., 2005).

Candida nivariensis and *Candida bracarensis* are phenotypically indistinguishable from *C. glabrata* (Alcoba-Flórez *et al*., 2005; Correia *et al*., 2006). *C. nivariensis* and *C. bracarensis* are small budding yeast cells which produced white colonies on CHROMagar with negative trehalose test results (Alcoba-Flórez *et al*., 2005; Bishop *et al*., 2008; Correia *et al*., 2006; Warren *et al*., 2010). *C. glabrata, C. nivariensis* and *C. bracarensis* can be identified by using multiplex PCR assay targeting the internal transcribed spacer 1 (ITS1) region, sequencing of the ITS and 26S rRNA gene regions, peptide nucleic acid fluorescence *in situ* hybridization method or restriction length polymorphism analysis (Bishop *et al*., 2008; Romeo *et al*., 2009; Warren *et al*., 2010).

C. parapsilosis complex is a major pathogen that persists in the hospital setting and can be nosocomially transmitted through hand carriage (Hernández-Castro *et al*., 2010). Within the complex, *Candida orthopsilosis* and *Candida metapsilosis* (Tavanti *et al*., 2005) are two novel species that are frequently misidentified

as *C. parapsilosis* based on culture-based phenotypic methods (Asadzadeh *et al*., 2015). According to a study by China Hospital Invasive Fungal Surveillance Net (CHIFNET), *C. parapsilosis* was the most common species causing candidemia (Wang *et al*., 2012a), and the second or third most common *Candida* spp. associated with invasive candidiasis in the United States of America (USA) (Hernández-Castro *et al*., 2010; Trofa *et al*., 2008). The reason behind the wide range pathogenicity of this species is not fully known. The ability of the yeast growing in total parenteral nutrition and forming biofilms on catheters and other implanted devices have been reported (Wang *et al*., 2012a).

C. krusei is an uncommon species reported in immunocompromised patients such as those with cancer (Abbas *et al*., 2000; Meurman*et al*., 2007), bone marrow transplantation (Fanci *et al*., 2005) or hematology patients with severe neutropenia (Conen *et al*., 2008). It has been known to be a multidrug-resistant fungal pathogen due to its inherent fluconazole-resistance and decreased susceptibility to 5- flucytosine, itraconazole and amphotericin B (Ellis, 2002; Pfaller *et al*., 2008).

2.3 Morphology and culture characteristics of *Candida* yeasts

Candida spp. can grow as blastopores (yeast), pseudohypha or as filamentous cells. True hyphae is formed by *C. albicans* and *C. dubliniensis* (Pfaller & Diekema, 2007) in the presence of serum at 37^{o}C. *Candida* pseudohyphae are extended yeast cells that stay attached to each other to form branches, but have constrictions at the septa in between the cells (Figure 2.1). Each pseudohypha appears with different thickness of unparallel sides with the width of $\geq$ 2 µm (Watkinson *et al*., 2015). At times, pseudohyphae physiology may represent yeasts more than hyphae, therefore, it is commonly assumed that pseudohyphae

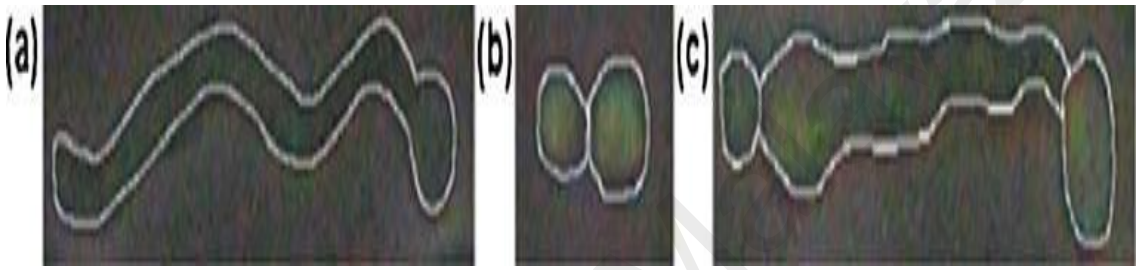


Figure 2.1: Illustration on different forms of *C. albicans* under a light microscope: (a) true hyphae having long filaments with parallel sides and no obvious constrictions between cells; (b) yeasts (also known as blastospores) are in ovoid shape and readily separated from each other during budding; (c) pseudohyphae resembling elongated yeast cells with wider, irregular sides and remain attached to each other at constricted septation sides (Lim *et al*., 2012).

could contribute to the pathogenicity and virulence (Calderone & Fonzi, 2001; Crampin *et al*., 2005).

Biofilm

Biofilms are widespread and complex communities of microorganisms which grow on any surface. The organisms are encased in exopolysaccharide matrix and can grow on medical devices as well as industrial and aquatic water systems (Jabra-Rizk *et al.*, 2004). Microorganisms exhibit different characteristics when they are growing in structured biofilm (Douglas, 2002). A biofilm could be built by a community of microorganisms of different species or of the similar species (Adam *et al*., 2002; Davey & O'toole, 2000). Formation of biofilm benefits the microorganism by giving protection from the environment, metabolic co-operation, nutrient accessibility and acquirement of new genetic traits (Davey & O'toole, 2000; Douglas, 2003). Biofilms are extremely difficult to abolish and are the precursors for many infections. Infections ranging from common urinary tract infections, catheter infection, dental plaque to more dangerous infections such as infections of heart valves and endocarditis can be caused by biofilms (Bachmann *et al*., 2002; Lewis, 2001). Immunosuppressed patients affected by cancer and HIV infection are often the most vulnerable to *Candida* infection.

Scanning electron microscope (SEM) analysis of *Candida* planktonic and biofilm cultures has been used as a promising tool to assess the antifungal activity of natural products and drugs. The death process of *C. albicans* has been investigated by evaluating the morphological and biophysical properties post exposure to flucytosine and amphotericin B (Kim *et al.*, 2011). In that study, *C. albicans* was treated at varying time periods (from 6 to 48 hours) and examined by SEM. The surface of *C. albicans* exposed to flucytosine

demonstrated shrinkage, membrane collapse, and development of holes, whereas the membranes of cells peeled off upon exposure to amphotericin B (Kim *et al*., 2011).

2.4 Laboratory diagnosis

Isolation of *Candida* from clinical samples is the primary method for the diagnosis of various forms of candidiasis. Automated blood culture systems have been used in the case of invasive candidiasis (Horvath *et al*., 2007), although it lacks sensitivity. Genotypic method to detect clinical samples based on fluorescent *in situ* hybridization (FISH) using a set of peptide nucleic acid (PNA) can be used for direct detection of *Candida* yeasts from blood culture bottles, thus, reducing the reporting time (Alexander *et al*., 2006; Forrest *et al*., 2006). Serodiagnostic tests such as latex agglutination commercial kits detect *Candida* antigen with 30 to 77 % sensitivity and 70-88% specificity (Laín *et al*., 2008), however; false positive results have been reported in the presence of rheumatoid factor and in patients with impaired renal function (Sendid *et al*., 2003; Sendid *et al*., 2002; White, Archer, & Barnes, 2005; Yera, Sendid, Francois, Camus, & Poulain, 2001). PCR for candidal DNA (McMullan *et al*., 2008) and assays for 1-3-D-ß-glucan (Kędzierska *et al*. 2007; Senn *et al*., 2008) have been promising. Lately, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been used for rapid identification of yeast from clinical specimens with great diagnostic accuracy and consistency (Marklein *et al*., 2009).

2.5 Virulence factors of *C. albicans*

Virulence is described as the capability of a microorganism to cause illness and is influenced by many factors (Lim *et al*., 2012). An advantage of the adaptability of *C. albicans* as a pathogen is on its ability to persist as normal flora at different sites of body tissues with the presence of environmental stresses (Calderone & Fonzi, 2001). A number

of virulence factors are associated with the establishment of infection by the opportunistic fungal pathogen, *C. albicans*. Adherence to host cells, secretion of degradative enzymes, and morphology switching are among the factors that contribute to the successful manifestation of the infection (Calderone & Fonzi, 2001). Yet, the mode of action of the respective virulence factor and the series of actions involved in the successful host invasion still remain unknown (Calderone & Fonzi, 2001; Lim *et al*., 2012). Table 2.3 shows some of the *Candida* virulence proteins or genes that have been identified.

Adherence

Adherence of *C. albicans* promotes extension of hyphal growth along the channels of a surface (Gow, 1997). Several authors referred this condition as 'filamentation under embedded condition', which means stimulation by contact alone can arouse hyphae formation (Brown Jr *et al*.1999). Adhesins are the biomolecules that support the attachment of *C. albicans* to host cells (Calderone & Fonzi, 2001). Several adhesins biomolecules such as Ala1p (Gaur *et al*.1999), Als1p (Fu *et al*., 1998), and Hwp1p (Staab *et al*., 1999) have been recognized. Lipids (Ghannoum *et al*., 1986), glycoprotein (Chaffin *et al*., 1998) and polysaccharides (Fukazawa & Kagaya, 1997) are cell superficial molecules which may function to enhance the attachment of *C. albicans*, and perhaps its dissemination in the host tissues.

Table 2.3: Virulent proteins/genes of *Candida* (Khan *et al.*, 2010)

Virulent proteins/genes	Cellular location	Function
Als1p and Als5p	Cell surface	Adherence of *C. albicans* to host cells or host-cell ligands
Hwp1p	Cell surface of hyphal- and germ tube	Binding of *C. albicans* hyphae to human buccal epithelial cells
MNT1	Membrane	Host recognition
Ste12p, Cph1p, Cdc2, Tup1p and Rbf1p, Chk1p and Cos1/Nik1	Pseudohyphae / hyphae	Morphogenesis
Egf1p	Germ tubes or hyphae	Formation of germ tubes or hyphae
Plb1p	Hyphal tips	Production of phospholipases
Saps	-	Tissue invasion (early invasion, extensive penetration and extensive hyphal growth)
OPA1	Opaque cells	Colonize the skin in a cutaneous model

Secretion of degradative enzymes

Secreted aspartyl proteinases (SAP) and phospholipases (PL) are two large families of secreted degradative enzymes that have been related with invasion of *Candida* yeasts into host cells. PL is known to have four subfamilies, i. e., PLA, PLB, PLC, and PLD. However, only PLB1 has been known to be essential for virulence (Naglik *et al*., 2003). PLB1 has been found at the edge of hyphae during host tissue invasion (Ghannoum, 2000). The SAP family comprises at least ten genes which are differentially expressed at different phases of infection (Monod *et al*., 1998). Additionally, haemolysin is another virulence factor leading to the pathogenesis of *Candida* in invasive candidiasis. Haemolysin production by *Candida* causes lysis of red blood cells which permits *Candida* to obtain iron from the host, enables hyphae invasion and establishes systemic infection in the host (Luo *et al*., 2001; Tsang *et al*., 2007).

Morphology switching/Morphogenesis

C. albicans has been known for its ability to undergo an alterable morpholological change over from single celled yeast (blastopore) to filamentous form (Brown, 2002; Calderone & Fonzi, 2001). The filamentous form of *C. albicans* comprises two discrete morphologies, i.e., hyphae and pseudohyphae. The shift from blastopores to filaments is accompanied by stimulation of many genes. Even though the blastopore-filament shift is needed for its virulence (Braun *et al*., 2000; Murad *et al*., 2001), the significance of having different morphological forms and the alterations in the gene expression is not clear.

2.6 Mechanism of *Candida* biofilm formation

Establishment of *C. albicans* biofilm comprises three developmental phases as shown in Figure 2.2. The early phase involves the adherence of yeast cells to the device surface. The intermediate phase involves construction of matrix with morphology shift from yeast to hyphal forms. During the maturation phase, there will be an increase in the surrounding material (matrix) forming a three-dimensional structures (Chandra *et al*., 2001; Hawser & Douglas, 1994). It has been reported that matured *Candida* biofilms have a blend of morphological forms and a dense network of yeasts, hyphae, and pseudohyphae encased in a matrix carbohydrate, polysaccharides (Chandra *et al*., 2001), protein and unknown constituents (Kojic & Darouiche, 2004).

2.7 Factors influencing the formation and structure of *Candida* biofilms

The architecture and formation of *Candida* biofilm is subjective to the nature of the surface in contact, *Candida* morphology shift from blastospores to filamentous cells, environmental factors, and the species of *Candida* involved. The chemical property of the contact surface affects the degree of biofilm formation (Hawser & Douglas, 1994). Biofilm formation is greater in latex compared to polyvinyl chloride but is noticeably decreased on pure silicone and polyurethane (Hawser & Douglas, 1994). The structure of *C. albicans* biofilm differ when the biofilm grows on cellulose filters compared when it is formed on catheter disk (Ellis, 2002). Hence, *Candida* biofilm may have specific contact-induced gene expression as reported by Douglas (2003).

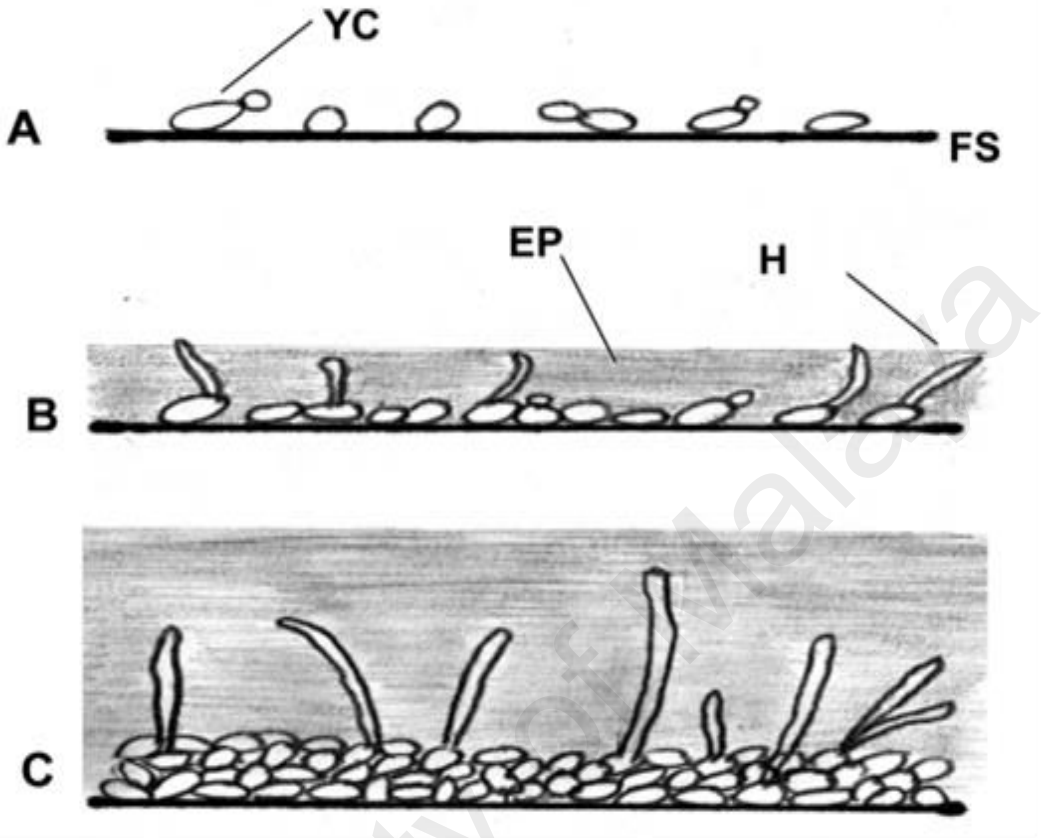


Figure 2.2: Illustration of the biofilm growth in *C. albicans*; A, early 0-11 hour; B, intermediate 12-30 hour; C, mature 38 – 72 hour; FS, flat surface; YC, yeast cell; H, hyphae; EP, exopolymeric matrix (Jabra-Rizk *et al*., 2004)

Candida biofilm formation has often been correlated with the cell surface hydrophobicity (Fanci *et al*., 2005). Biofilm on development can grow better in gentle shaking condition and the phenomenon has been observed *in vivo* (Mathé & Van Dijck, 2013). A few morphological forms of *C. albicans* influencing the biofilm formation have been reported (Sullivan *et al*., 1995). In a previous study, investigation of the biofilm formation of a wild-type mutant strain showed a separate two-layer biofilm, while a hypha-negative mutant formed only the basal layer, and the biofilm formed by yeast-negative mutant was easily detached from catheter disk because it only formed the outer layer. These studies suggest that dimorphism is essential for the pathogenicity of *C. albicans* and may be required for biofilm structure and architecture (Sullivan *et al*., 1995).

Many *Candida* spp. demonstrate abilities to form biofilm *in vitro* to different degrees. A study showed that the pathogenic *C. albicans* produced considerably more biofilm on polyvinyl chloride *in vitro* than C. *parapsilosis, C. glabrata* and *C. psudotropicalis* as determined using colorimetric/radioisotope assays and dry-weight measurement (Kuhn *et al*., 2002). A study by Kuhn *et al.* (2002) also found that the microscopic biofilm image of *C. albicans* is more complex than *C. parapsilosis* biofilm which were composed of clumped blastopores only (Kuhn *et al*., 2002). The variability in the biofilm formation among *Candida* spp. is known to be inconsistent (Kojic & Darouiche, 2004).

Several studies investigated the biofilm production of *C. albicans* obtained from sterile and non-sterile samples (Abbas *et al*., 2000; Fanci *et al*., 2005). *C. albicans* isolated from sterile sample was more capable of forming biofilm compared to those isolated from non-sterile sample, based on the dry weight measurement of biofilm (Kuhn *et al*., 2002). Lewis *et al*. (2001) found distinct variation within the biofilm formation of clones and

clonal lineages of *C. albicans* from different sources such as the vagina, oral cavity and environment. It has been reported that *Candida* may display extensive phenotypic diversity, possibly in association with its pathogenicity (Kojic & Darouiche, 2004).

2.8 Methods used for detection of biofilm

XTT (2,3-bis (2-methoxy-4- nitro-5-sulfophenyl)-5-[(phenylamino carbonyl]-2H-tetrazolium hydroxide) assay

Colorimetric assays are used to measure cellular viability and are an important aspect in the study of *Candida* biofilms. The 2,3-bis (2-methoxy-4- nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) assay has proved to be beneficial because the solubility of its formazan product in water simplifies the performance of the assay (Selvarangan *et al*., 2002). Kuhn *et al*. (2003) described the usage of XTT assay to study fungal biofilm development and drug resistance (Kuhn *et al*., 2003). XTT is transformed to a colored formazan in the occurrence of metabolic activity. The principal mechanism of the conversion of XTT to formazan involves enzyme such as mitochondrial succinate oxidase, flavoprotein oxidases and cytochrome P450 systems (Sullivan *et al*., 2004). As the formazan product is water soluble, it is easily measured in cellular supernatants. This particular property is crucial in biofilm research because it permits the study of intact biofilms and investigation of biofilm drug susceptibility without the interference of biofilm structure (Kuhn *et al*., 2003).

Crystal violet (CV) assay

Crystal violet assay is commonly used as a quick and cheap method for indirect quantification of adherent cells and the biofilm formed by *Candida* on flat surfaces. Crystal violet is a basic dye which binds to polysaccharides in the extracellular matrix and

negatively charged surface molecules (Warren *et al*., 2010). The stain is dissolved in sterile distilled water to take measurement of the absorbance. Isolate producing absorbance beyond certain cut-off point (e.g; $\geq$ 0.1) is considered as a biofilm-producing isolate (Reisner *et al*., 2006).

2.9 Classes of antifungal drugs for candidiasis

Currently available antifungal agents for the treatment of invasive candidiasis are categorized by their site of action in fungal cells (Table 2.4). Amphotericin B (Amp B) is a polyene with fungicidal property (Wang *et al*., 2012b). Among the available antifungal agents, it has the widest range of antifungal activity (Wang *et al*., 2012b). The polyene kill the fungal cells by binding to ergosterol, the main component of fungal membrane, and form channels to destruct the proton gradient in the cell, causing leakage of cytoplasmic content (Wang *et al*., 2012b). Amp B is given intravenously to treat systemic fungal infection (Asadzadeh *et al*., 2015). The serious side effect of Amp B is nephrotoxicity. To reduce the side effect, lipid preparations which have equivalent antifungal action but different pharmacologic and toxicologic properties are used (Pfaller *et al*., 2011).

The azoles (ketoconazole, clotrimazole and miconazole) target ergosterol but with different modes of action. The drugs interact with the fungal enzyme, lanosterol demethylase, which is responsible in the conversion of lanosterol to ergosterol in the fungal cell membrane. This inhibits the synthesis of ergosterol leading to exhaustion in the cell membrane (Wang *et al*., 2012b). Fluconazole has been reported as the most effective drug against vaginal and oropharyngeal candidiasis with very little side effects. It has also been suggested as a treatment for choice for neutropenic patients having bone marrow transplantation and for oropharyngeal candidiasis in HIV patients (López-Martínez, 2010). 5-Flucytosine (5-FC), a pyrimidine analogue, causes disruption of DNA and protein

Table 2.4: Mode of action of several antifungal agents (Spampinato & Leonardi, 2013)

Antifungal class	Mode of action	Drugs
Echinocandins	Inhibitors of (1,3)-β-D-glucan synthase	Caspofungin Micafungin Anidulafungin
Azoles	Inhibitors of lanosterol 14-α-demethylase	Voriconazole Posaconazole Miconazole Clotrimazole Ketoconazole Fluconazole Itraconazole
Polyenes	Binding ergosterol	Nystatin Amphotericin B
Nucleoside analogues Inhibitor of DNA/RNA synthesis	Inhibitor of DNA/RNA synthesis	Flucytosine
Allylamines	Inhibitors of squalene-epoxidase	Amorolfine Terbinafine Naftifine
Antibiotic	Interaction with β-tubulin	Griseofulvin
Thiocarbamates	Inhibitors of squalene-epoxidase	Tolnaftate Tolciclate

synthesis after its uptake into the fungal cell. Flucytosine is used in combination with Amp B to give optimum synergism effect to treat cryptococcal meningitis and endophtalmitis (Wang *et al*., 2012b).

Echinocandins (caspofungin, micafungin, and anidulafungin) are lipopeptide antifungal agents that inhibit the synthesis of fungal wall by β-1,3-glucan synthase (Jackson *et al*., 2007). The inhibition leads to reduced structural integrity in cell walls, leading to cell vulnerability to osmotic lysis (Grover, 2010). Most species of *Candida* are inhibited by all three agents (caspofungin, micafungin, and anidulafungin) in a concentration-dependent manner (Cappelletty & Eiselstein-McKitrick, 2007). All three agents have been accepted by the regulatory agency FDA for the treatment of aesophageal and invasive candidiases, including candidemia (Ostrosky-Zeichner *et al*., 2005).

2.10 Antifungal drug resistance

Understanding the molecular basis underlying the resistance of biofilm forming organisms towards antifungal agents is essential. The mechanisms for antifungal resistance have been summarized in Table 2.5. Minimal resistance to Amp B has been developed despite more than 30 years of clinical use. Some isolates of *C. krusei* and *C. glabrata* species resistant to Amp B have been reported (Kontoyiannis & Lewis, 2002), in addition to *C. lusitaniae* and *C. guilliermondii* (Pappas *et al*., 2004). Acquired resistance to Amp B is possibly due to a decrease or lack of ergosterol content in cell membranes. In fact, relatively low ergosterol content was noted in membranes of polyene-resistant *Candida* isolates compared to those of polyene-susceptible isolates. These deficiencies are perhaps consequences of the loss of functional mutations in the *ERG3* or *ERG6* genes which encode enzymes involved in ergosterol biosynthesis (Kontoyiannis & Lewis, 2002).

Table 2.5: Resistance basis of antifungal agents (Spampinato & Leonardi, 2013)

Antifungal class	Genetic basis for resistance	Functional basis for resistance
Azoles	Point mutations in *ERG11*	Decreased lanosterol 14-α-demethylase binding affinity for the drug
	Upregulation of *ERG11* by gene duplication and transcription factor regulation	Increased concentration of lanosterol 14-α-demethylase
	Point mutations in *ERG3*	Inactivation of C5 sterol desaturase leading to alterations in the ergosterol synthetic pathway
	Upregulation of *CDR1*/*CDR2* and *MDR1* by point mutations in *TAC1* and *MRR1* transcription factors	Upregulation of drug transporters
Polyenes	Point mutations in *ERG3* and *ERG6*	Decreased ergosterol content in cells
Echinocandins	Point mutations in *FKS1* and *FKS2*	Decreased glucan synthase processivity for the drug
Nucleoside analogues	Point mutations in *FCY1*	Inactivation of cytosine deaminase leading to alterations in the metabolism of 5-fluorocytosine
	Point mutations in *FUR1*	Inactivation of uracil phosphoribosyl transferase leading to alterations in the metabolism of 5-fluorocytosine
	Point mutations in *FCY2*	Inactivation of cytosine permease affecting drug uptake

Azole-resistant *C. albicans* is common in HIV-infected patients with oropharyngeal candidiasis (Skiest *et al*., 2007). An inherently reduced susceptibility to fluconazole has also been reported for non-*albicans Candida* species such as *C. glabrata*, *C. lusitaniae*, and *C. krusei* (Safdar *et al*., 2001; Vazquez *et al*., 2001). It has been postulated that variations in the structure of azoles are accountable for the cross-resistance patterns among *Candida* species (Cuenca-Estrella *et al*., 2006; Pfaller & Diekema, 2004; 2007). Azole resistance relies on an upregulation of two principal families of efflux pumps (Cannon *et al*., 2009), the specificity of the azole molecule and the source of energy used to pump out the drug varies in these transporters. The Cdr pumps belong to the superfamily of ATP-binding cassette (ABC) transporters and are capable of eliminating all azole antifungals. These pumps are encoded by *Candida* drug resistance 1 and 2 (*CDR1* and *CDR2*) genes in *C. albicans* (Cannon *et al*., 2009). The other pump is specific for fluconazole and a secondary transporter which uses proton gradient as a source of energy. This pump belongs to the major facilitator superfamily (MFS) transporters and is encoded by the *MDR1* gene in *C. albicans* (Cannon *et al*., 2009). Mutations in *TAC1* and *MRR1* transcription factors causes upregulation of *CDR1/CDR2* and *MDR1* (Coste *et al*., 2004; A. Coste *et al*., 2006). Other transporter genes have been reported to be upregulated in azole-resistant *C. glabrata* Cg*CDR1*, Cg*CDR2* (formerly named *PDH1*) and Cg*SNQ2* (another ABC transporter) (Bennett, Izumikawa, & Marr, 2004; Torelli *et al*., 2008) and *C. tropicalis* (CDR1-homologue) isolates (Vandeputte, Ferrari, & Coste, 2011). In *C. glabrata*, the Cg*PDR1* transcription factor regulates the Cg*CDR1*, Cg*SNQ2* and Cg*CDR2* genes (Vermitsky & Edlind, 2004; Vermitsky *et al*., 2006). Some studies have elucidated the function of Hsp90, a molecular chaperone for calcineurin in stimulating the rapid acquirement of fluconazole resistance by *C. albicans* (Kabir *et al*., 2012; López-Martínez, 2010).

Echinocandins are recommended as the first line drugs for invasive candidiasis.

Yet, reports of echinocandin resistance in patients affected by *C. albicans*, *C. tropicalis*, *C. glabrata* and *C. krusei* are increasing (Hernandez *et al*., 2004; Krogh-Madsen *et al*. , 2006; Pfaller *et al*., 2012). Intrinsic resistance of *C. parapsilosis, C. orthopsilosis*, *C.metapsilosis,* and *C. guilliermondii* to echinochandin has been reported (Cantón *et al*., 2006; Garcia-Effron *et al*., 2008). Echinocandin resistance is attributed to point mutations in the *FKS1* and/or *FKS2* genes (Achkar & Fries, 2010; Balashov *et al*., 2006) which encode the (1,3)-*β*-D-glucan synthase complex (Balashov *et al*., 2006).

Point mutations in the *FCY1* gene which encodes for the cytosine deaminase or *FUR1* gene which encodes for the uracil phosphoribosyl transferase enable *Candida* spp. to acquire resistance to flucytosine. These enzymes catalyze the conversion of 5-fluorouracil to 5-fluorouridine monophosphate and 5-fluorocytosine to 5-fluorouracil, respectively. The most frequently acquired resistance to flucytosine is based on point mutations in the *FUR1* gene. Several point mutations have been described in *C. albicans*, *C. glabrata*, and *C. lusitaniae* (Chapeland-Leclerc *et al*., 2005; Espinel-Ingroff, 2008; Pemán *et al*., 2009; Vandeputte *et al*., 2011).

2.11 Aureobasidin A (AbA) and cyclic peptide antibiotics

Aureobasidin A (AbA) is a new antifungal drug produced by the killer yeast *Aureobasidium pullulans* (R106) (Takesako *et al*., 1991). It is called as cyclic depsipeptide antibiotic due to its structure which is composed of eight L-form amino acids connected by one hydroxyl acid forming a ring structure. Several cyclic peptide antibiotics with antifungal activity such as valinomycin (Hernández-Castro *et al*., 2010), calophycin (Asadzadeh *et al*., 2008), syringomycin (Wang *et al*., 2012a) and aculeacin or echinocandin family (Kim *et al*., 2011; Watkinson *et al*., 2015) affect the cell membrane permeability and cause toxicity to the eukaryotic cells. AbA has a broader spectrum and greater effectiveness

to murine candidiasis than aculeacin or echinochandins (Naglik *et al*., 2003). It has potent fungicidal activity against a variety of fungi, including *Saccharomyces cerevisiae*, pathogenic fungi affecting humans such as *Candida* and *Cryptococcus* and protozoa (Takesako *et al*., 1993; Kuhn *et al*., 2003; Sonda *et al*. , 2005). The mode of action of AbA is blocking the activity of inositol phosphorylceramide (IPC) synthase, an essential enzyme for fungal sphingolipid biosynthesis that is absent in mammals (Cappelletty & Eiselstein-McKitrick, 2007; Jackson *et al*., 2007). The fact that AbA specifically targets IPC synthase of fungi makes it a particularly attractive compound for controlling opportunistic fungi infecting immuno-compromised patients, such as those with acquired immunodeficiency syndrome (AIDS), cancer, or organ transplants (Nagiec *et al*., 1997; Ostrosky-Zeichner *et al*., 2005). AbA was shown to have little toxicity to mice (Naglik *et al*., 2003).

Sphingolipids are ubiquitous components of plasma membranes. Their biosynthetic pathways are similar in all eukaryotic cells up to the formation of ceramide. The end points of sphingolipid synthesis in fungi are inositol phosphorylceramide (IPC) and its derivatives (McConville & Bacic, 1989). For these organisms, the enzyme that catalyzes the formation of IPC, Aur1, plays a pivotal role in the regulation of intracellular levels of sphingolipids and ceramide (Lester & Dickson, 1992). Figure 2.3 is a schematic representation of the synthetic pathway and site of action of AbA. IPC synthase is an essential enzyme in fungi, as its inhibition causes arrest in the cell cycle progression at G_1, followed by a loss of viability and alterations in the cytoskeleton (Cheng *et al*., 2001; Endo *et al*.,1997; Hashida-Okado *et al*., 1996; Hashida-Okado *et al*., , 1998; Takesako *et al*., 1991).

IPC and its mannosylated derivatives are the only complex sphingolipids of yeast. Their synthesis can be reduced by AbA, which specifically inhibits the IPC synthase (Aur1). AbA reportedly, by diminishing IPC levels, causes endoplasmic reticulum (ER)

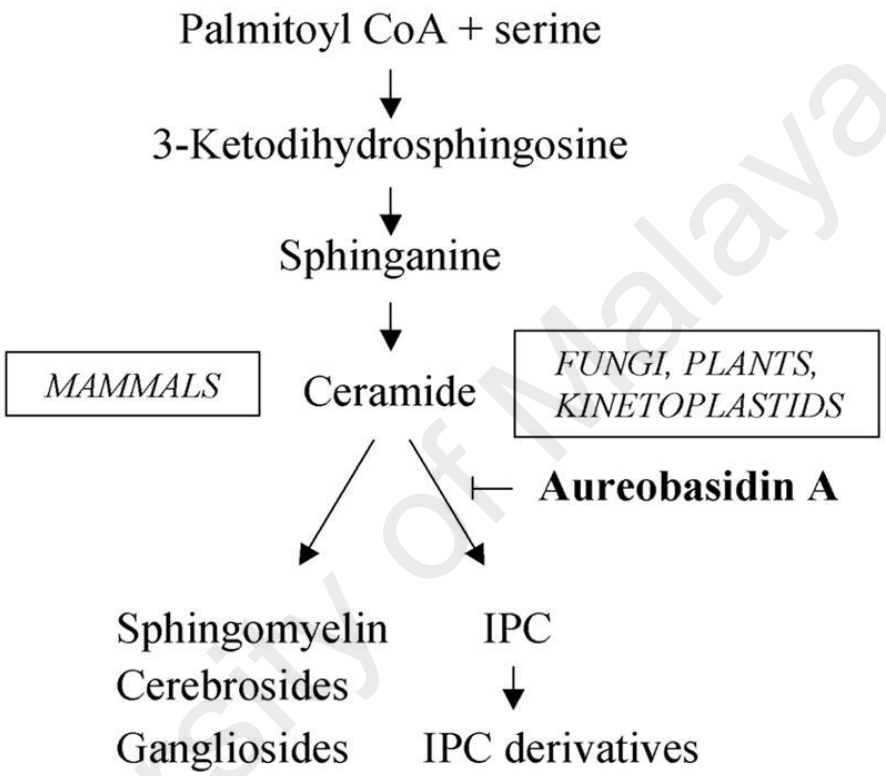


Figure 2.3: Sphingolipid synthesis in eukaryotic cells. Schematic representation of the synthetic pathways and site of action of the inhibitor aureobasidin A (Sonda *et al*., 2005)

stress, an increase in cytosolic calcium, reactive oxygen production, and mitochondrial damage leading to apoptosis (Voynova *et al*., 2015). Furthermore, a recent report showed that AbA causes the endoplasmic reticulum (ER) retention of glycosylphosphatidylinositol (GPI)-anchored proteins, an unfolded protein response, reactive oxygen species (ROS) production, mitochondrial cytochrome *c* release, and a metacaspase-mediated form of apoptosis that additionally is dependent on the concomitant increase of cytosolic Ca^{2+} concentrations, all through the reduction of IPCs rather than the accumulation of toxic ceramides, both of which are inevitable consequences of Aur1 depletion/inhibition (Kajiwara *et al*., 2012).

2.12 Protein profiling of *C. albicans*

The mechanisms that protect microorganisms in biofilms from antibiotics are still being elucidated. Currently, four mechanisms have been reported : (i) slow penetration of the antimicrobial agent into the biofilm, (ii) an altered chemical microenvironment within the biofilm leading to zones of slow or no growth, (iii) adaptive responses to environmental stress, and (iv) the existence of persister cells that are protected from all types of antimicrobial insult (Khakhina *et al*., 2015). As a prerequisite to study the mechanism of action, it is necessary to identify changes in the proteome of planktonic and biofilm cultures of *C. albicans* upon exposure to AbA.

The term “proteomics”, first introduced in 1995, is defined as the large-scale classification of the entire protein counterpart of a tissue, cell line or organism (Kontoyiannis & Lewis, 2002; Pappas *et al*., 2004). A more inclusive definition would be a combination of protein studies with analyses that have genetic readout such as mRNA analysis, genomics, and the yeast two-hybrid analysis (Skiest *et al*., 2007). The goal of proteomics is to obtain a more global and integrated view of biology by studying all the

proteins of a cell rather than each one individually. The proteome of a cell will reflect the immediate environment in which it is studied. In response to internal or external signals, proteins can be modified by posttranslational modifications, undergo translocations within the cell, or be synthesized or degraded. Therefore, investigation of the proteome of a cell is like taking a "snapshot" of the protein environment at any given time. Considering all the possibilities, it is likely that any given genome can potentially give rise to an unlimited number of proteomes (Vazquez *et al*., 2001). Many types of proteomics approaches with wide application to biology are available (Figure 2.4).

2.13 LC-MS for profiling of proteins

Liquid chromatography and mass spectrometry (LC/MS) have become technologies which are essential for protein identification and quantification, and have many applications in proteomics research. The components of a LC-MS system are consisted of the autosampler, the HPLC system, the ionization source which connects LC to MS and the mass spectrometer (Figure 2.5). All these components are under control of a single computer system. In order to interface HPLC with MS, there are some margins on the flow rate and mobile phases to be used. The classic reversed phase HPLC attached to MS uses some mixture of water and acetonitrile or methanol as options in the mobile phase. There are some restrictions on the mobile phase modifiers. The modifiers have to be volatile. The usage of mobile phase modifiers to the mobile phase is to enhance the chromatography of analytes of concern. The mobile phase modifiers are always chemicals such as ammonium acetate, acetic acid and formic acid.

There are several types of ionization sources that can be used as the crossing point between the HPLC eluent and the mass spectrometer. The commonly used ion sources are atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI). Both

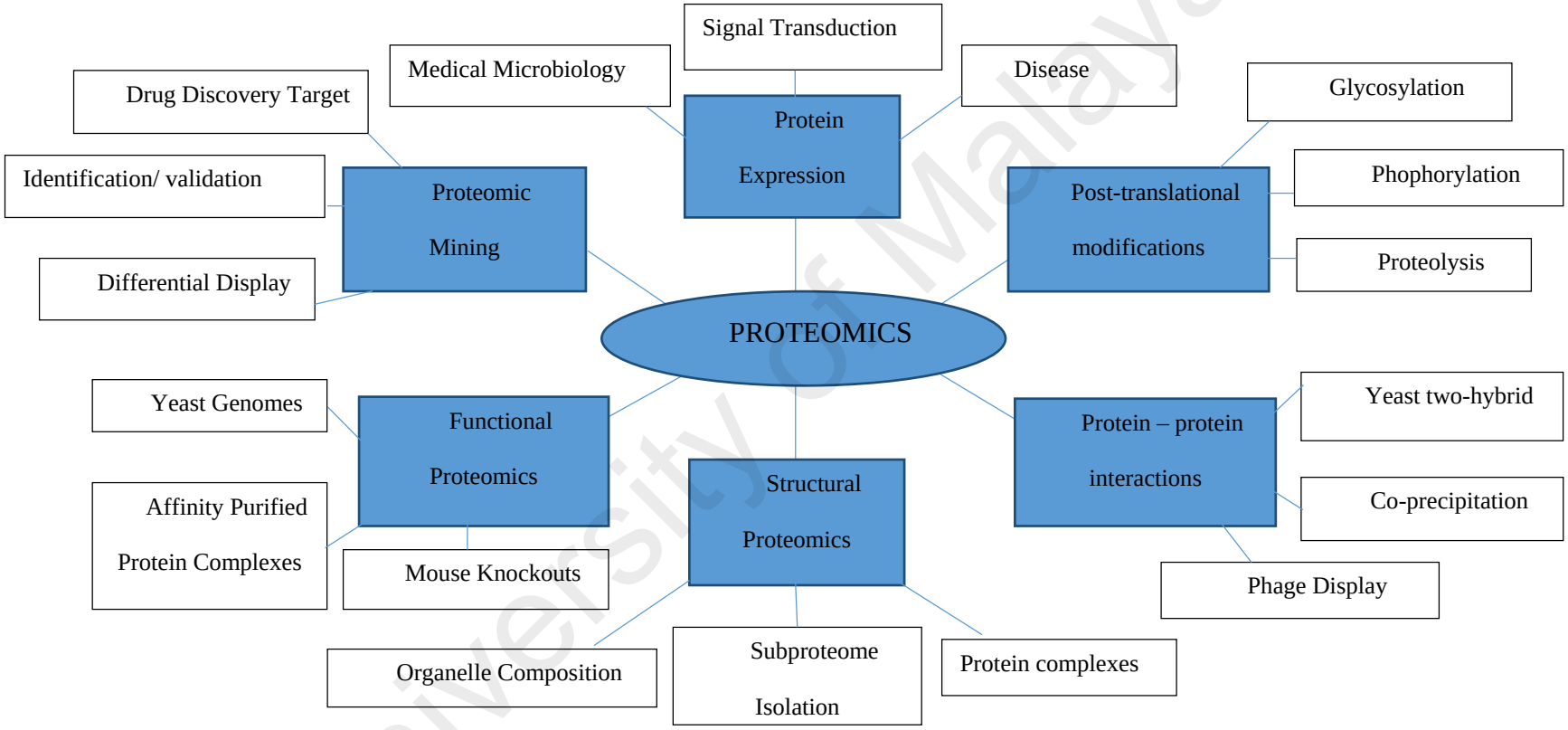


Figure 2.4: Types of proteomics and their applications to biology (Vazquez *et al*., 2001)

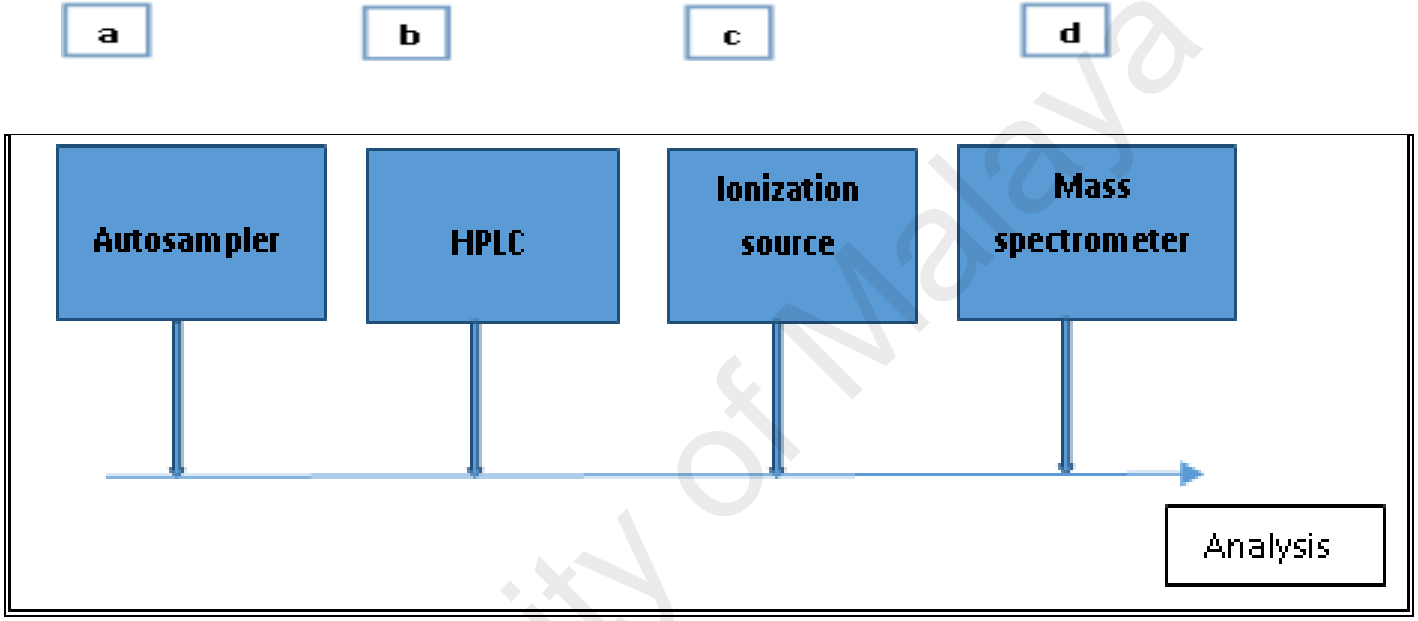


Figure 2.5: The components of LC-MS system. (a) Autosampler (loads the samples onto the HPLC); (b) HPLC; (c) ionization source; (d) Mass spectrometer (Burgers, 1998)

APCI and ESI are the standard equipment on mass spectrometers that are used for LC-MS applications. Both ESI and APCI measure the distribution, metabolism and excretion properties of the new chemical entities together with the pharmacokinetics parameters of the molecule (Burgers, 1998). There are many kinds of mass spectrometers available for interfacing with HPLC. The more common systems in use for HPLC-MS are the single quadrupole mass spectrometer. This system provides mass spectrum for each chromatographic peak produced from the LC column and analyses by the MS system. The second type of system is the time-off light (TOF) mass spectrometer. This system has added competency of providing a higher mass resolution spectrum from each constituent. The third system is the third quadrupole MS-MS system which is commonly used for bioanalytical experiment and metabolite identification assays (Franke *et al*., 2006; Li & Tian, 2016; Nakamura *et al*., 2016; Zarnowski *et al*., 2014). The fourth MS system is the ion-trap mass spectrometer which is proficient in producing MS information that are essential during structural interpretation assays (Franke *et al*., 2006; Nakamura *et al*., 2016).

Softwares used in LCMS analysis

PEAKS is an advanced software designed to derive amino acid sequences and detect proteins using tandem mass spectrometry data from all major mass spectrometry vendors (Pedrioli *et al*., 2004). PEAKS integrates *de novo* sequencing results into the database searching process for peptide/protein identification. This is performed by generating sequence tags which are used in conjunction with fragment ion mass matching to speed up the search, remove false positive matches, and find peptides with interesting sequence variations or modifications that would prevent them from being otherwise identified. The meta protein search tool, inChorus allows users to use multiple search engines (PEAKS,

Sequest, Mascot, X!Tandem and OMSSA) to have a broad range of sequence coverage and surge confidence (Bern *et al*., 2007).

SPIDER is another tool used to reconstruct the correct sequence using the *de novo* sequence and a homologous peptide (Bern *et al*., 2007). The list of proteins derived from SPIDER software stands higher chances of detection. SPIDER searches the database for homologous peptides, and attempt to merge these into protein hits as well. SPIDER does not provide information on the expression value, up-regulation or down-regulation of proteins. Hence, this is considered as qualitative analysis.

CGD database

The *C. albicans* genome was sequenced by the Stanford Genome Technology Center (SGTC). The Candida Genome Database (CGD; http://www.candidagenome.org) is a resource for information about *C. albicans* genomic sequence and the molecular biology of its encoded gene products. CGD is based on *Saccharomyces* Genome Database and is funded by the National Institute of Dental & Craniofacial Research at the US National Institutes of Health. CGD collects and organizes data from the biological literature concerning *C. albicans*, and provides tools for viewing, searching, analysing, and downloading these data which are updated daily. The comprehensive collection of sequence and literature data in CGD provides a unique opportunity to gain an overview of the state of characterization of the *C. albicans* genome and gene products at any given time (Costanzo *et al*., 2006).

Gene ontology (GO) is a major bioinformatics initiative to unify the representation of gene and gene product attributes across all species (Consortium, 2008). In the CGB database, the GO annotation state of the entire *C. albicans* genome is provided using GO Slim, a high-level subset of Gene Ontology terms that allows grouping of genes into

broad categories such as "DNA replication", "protein kinase activity", or "nucleus" tailored to *Candida* biology. GO Slim terms representing broad categories from a single aspect are along with the percentage of *C. albicans* SC5314 gene products annotated to a specific term that maps up the ontology to the GO Slim term. A gene product's biology is represented by three independent controlled, structured vocabularies such as biological process, molecular function, and celluler component (http://www.yeastgenome.org). Some gene products may be represented more than once, if they are annotated to one or more GO terms that map to more than one GO Slim term. Figure 2.6 shows the distribution of *C. albicans* gene by process, function, and component (as of Feb 25, 2017).

A) Distribution of Gene Products among Biological Process Categories

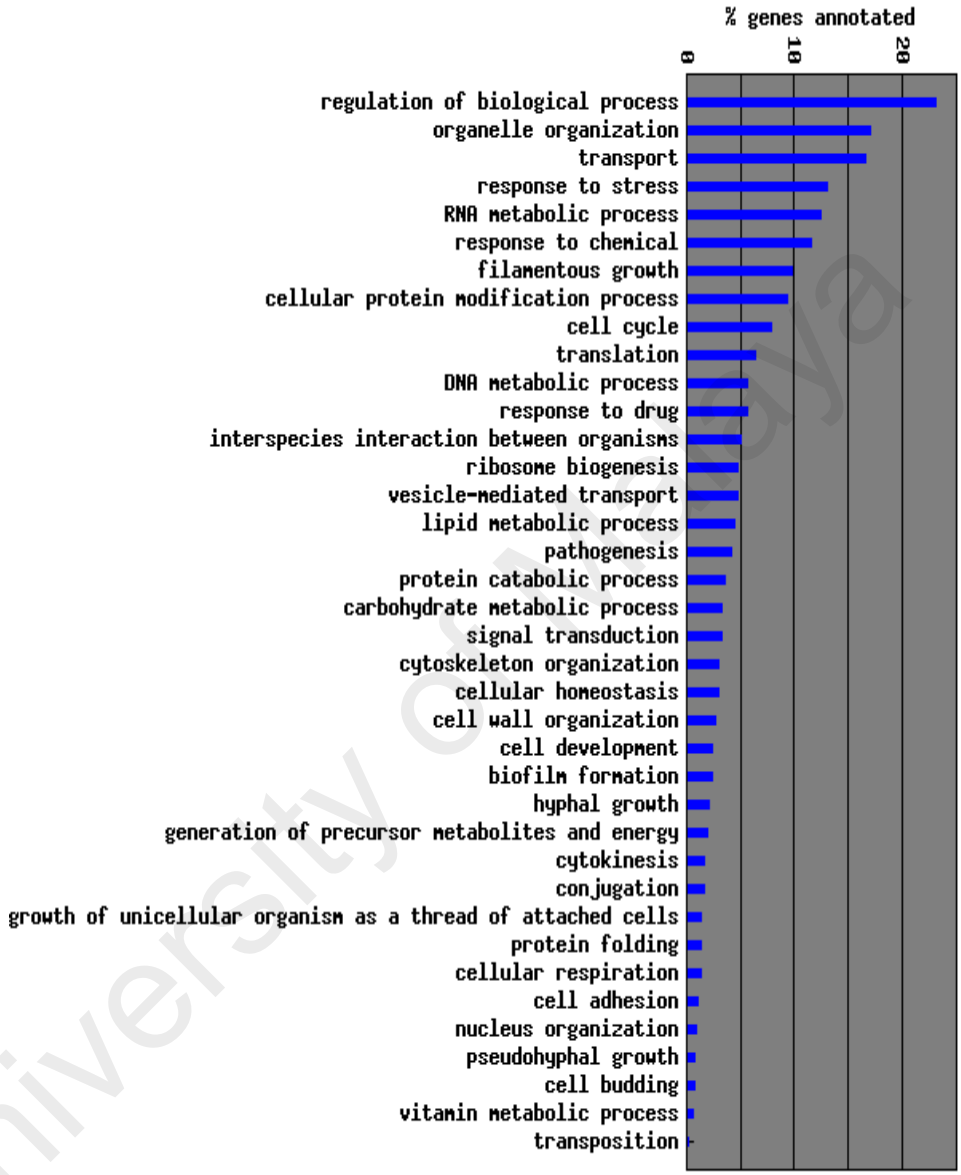
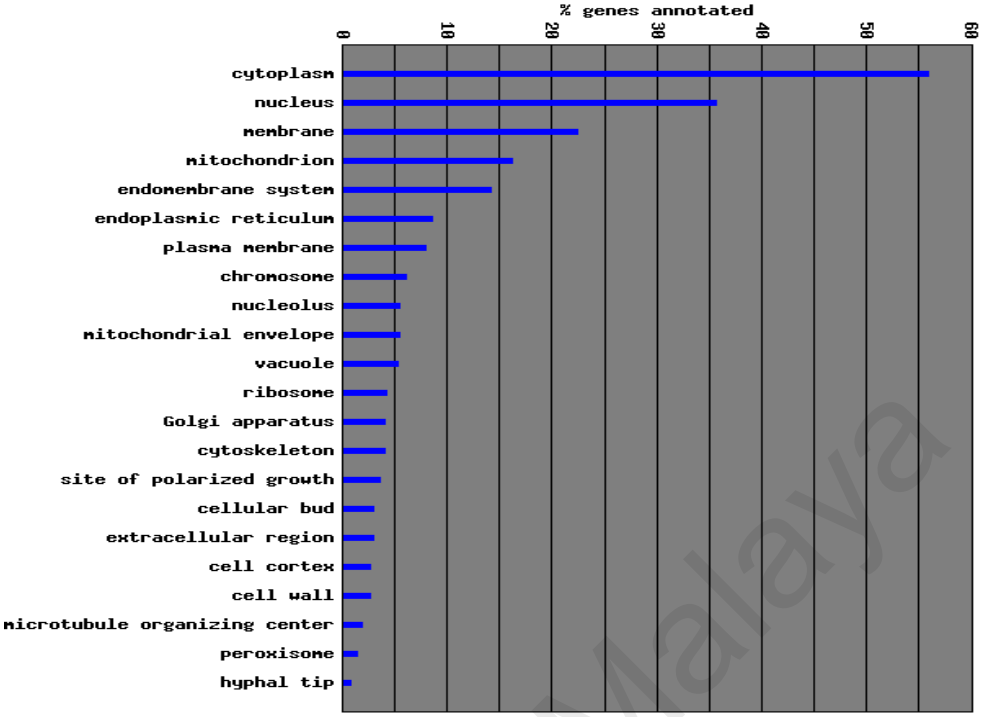


Figure 2.6: Distribution of *C. albicans* gene products by process, function, and component (as of Feb 25, 2017)

B) Distribution of Gene Products among Cellular Component Categories



C) Distribution of Gene Products among Molecular Function Categories

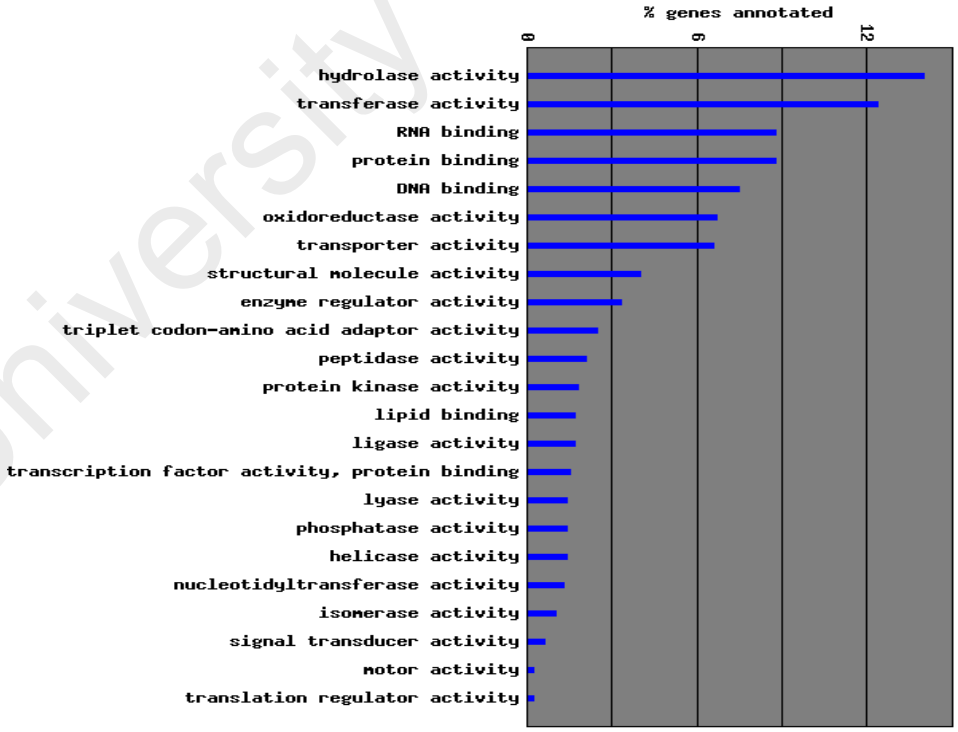


Figure 2.6: (continued)

CHAPTER 3: MATERIALS AND METHODS

3.1 Clinical isolates

A total of 118 *Candida* clinical isolates collected from the Medical Microbiology Diagnostic Laboratoty, University Malaya Medical Centre were used in this study. The isolates included in this study were: *C. albicans* (n=48), *C. tropicalis* (n=38), *C. parapsilosis* (n=16), *C. glabrata* (n=12), *C. nivariensis* (n=3), and *C. kefyr* (n=1). The *Candida* isolates were inclusive of invasive (n=50) and non- invasive (n=70) isolates. *Candida* yeast was freshly subcultured for 24 hours on Sabouraud's dextrose agar (SDA) or potato dextrose agar (PDA) (Appendix A).

3.2 Crystal violet (CV assay)

All isolates (n=118) were tested using a crystal violet (CV) assay (Reisner *et al*., 2006) to determine the ability of *Candida* isolates to form biofilm as well as to quantitate the biofilm biomass. The method for biofilm formation was adapted from a procedure described in a previous study (Kajiwara *et al*., 2012). Yeast colonies cultured on SDA at 37ºC for 24 h were suspended in 2 ml RPMI 1640 medium and adjusted to a final concentration of approximately 1 x 10^7 cells/ml (OD_{520} = 0.38) using a spectrophotometer (Genesys, USA). Biofilms were produced on sterilized, polystyrene, flattened-bottom, 96-well microtiter plates (Cat. No: 3596, Costar, USA). While 100 µl of the adjusted cell suspension was transferred to each well, no yeast cells were added to the final column of the microtiter (negative control). The attachment phase was performed at 37ºC for 1.5 h with constant shaking at 75 rpm. Unattached cells were then removed; the wells were washed three times with sterile phosphate buffered saline (PBS) and fresh RPMI 1640 medium was added into each well for further incubation at 37ºC for 24 h (with constant shaking at 75 rpm). Each isolate was tested twice on a microtiter plate.

Mature biofilms were stained with 200 µl of 0.1% (v/v) CV solution (Appendix A) for 15 to 20 min. After removal of the CV solution, the stained cells were washed three times with 200 µl of PBS. The CV stain was thoroughly dissolved in 200 µl of 96% ethanol (Appendix A). Following this, the absorbance measurement in each well was determined at 585nm (A_{585nm}) using an automated microplate reader (Epoch, BioTek, US). Each isolate was tested twice on a microtiter plate.

3.3 Preparation of stock and working solutions for aureobasidin A (AbA)

AbA stock solution was prepared by dissolving 10 mg of AbA powder (Clontech, USA) in 1 ml of DMSO (Fluka, USA) and stored at 4 ^{o}C prior to use. The working drug solution of AbA was prepared in RPMI medium buffered with MOPS (Appendix A) to the desired concentrations.

3.4 Antifungal susceptibility testing

Preparation of inoculum suspension

The inoculum suspension was prepared by suspending five yeast colonies of 1 mm in diameter from 24-hour cultures of *Candida* spp. in 2 ml of distilled water. The resulting suspension was vortexed for 15 seconds and adjusted to 75% to 85% transmittance at 520 nm, using a spectrophotometer (Genesys, USA), which was equivalent to 10^6 cells/ml of yeast. A stock suspension was prepared by adding 50 µl of the yeast suspension into a tube containing 4.95 ml distilled water (1:100 dilution). Prior inoculation into the antifungal drug plate, the stock suspension was diluted in 1:20 ratio with RPMI 1640 medium (Sigma, USA) buffered with 0.165 M morpholinepropanesulfonic acid (MOPS) (Sigma, USA) (Appendix A). The resulting suspension contained 5 x 10^2 to 2.5 x 10^3 yeast cells per ml.

Screening for antifungal activity of AbA on Candida spp.

The minimum inhibitory concentrations (MICs) for planktonic cells of various *Candida* species were determined using the microbroth dilution method as described by the Clinical and Laboratory Standards Institute (CLSI) M27-A3 document (CLSI, 2008). A working concentration of AbA (Clontech, USA) was prepared in RPMI medium (from a two-fold dilution of 16 to 0.0313 µg/ml) in a 96 well microtiter plate. A total of 100 µl yeast inoculum suspension was prepared as above method (preparation of inoculum suspension, pg 56) and mixed with various AbA solutions to prepare for the desired final concentrations (ranging from 8 to 0.0156 µg/ml) in the microtiter wells. The plate was then incubated at 37 ^{o}C for 48 h. A growth control (100 µl of yeast inoculum with 100 µl drug-free medium) and sterility control (200 µl drug-free medium) were included in each experiment. As recommended by CLSI, MICs were determined visually using an inverted reading mirror and was defined as the lowest concentration of AbA which inhibited the visible growth of the yeasts after incubation at 37 ^{0}C for 48 h. Each isolate was tested twice on a microtiter plate.

Assessment of the inhibitory effect of AbA on mature 24 hour mature biofilms of Candida isolates

The range of AbA (Clontech, USA) concentrations for the assay was prepared hundred times higher than the desired concentration. This is to overcome the drug dissolving problems as stated in CLSI M27-A3 (2008). A stock concentration was prepared by dissolving 12.8 mg of AbA in 1 ml of DMSO (12.8 mg/ml). Final concentrations of AbA ranging from 128 to 0.025 µg/ml were prepared and used to treat the 24 h mature biofilm of various isolates as described in section 3.2. Prior to drug exposure, the biofilm was washed twice with sterile PBS. A volume of 200 µl of AbA solution was added to four wells (quadruplicate) of a 96-well microtiter plate (3596,

Costar, USA). The plate was incubated at 37 ºC up to 72 h. a drug-free medium was used as negative control. A semiquantitative measure of the formed biofilm was determined using a XTT reduction assay as described by Jin *et al*. (2004) to measure the biofilm metabolic activity of *Candida* isolates. The biofilm MIC for *Candida* isolates was determined as the lowest concentration of AbA that caused a 50% reduction in the biofilm metabolic activity as compared with the metabolic activity of the biofilm without exposure to AbA.

Assessment of biofilm metabolic activity

The 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetra zolium-5-carboxanilide (XTT) reduction assay measures the mitochondrial cellular activity of the biofilms. XTT (Sigma Aldrich, USA) was prepared as a saturated solution at a concentration of 1 mg/ml in PBS (Appendix A). The solution was filter-sterilized using a 0.22µm-pore-size filter (Sartorium Stedium, Germany) and divided into 10 ml aliquots prior to storage at -80 ºC. Prior to each assay, a total of 20 ml of PBS was mixed with 1 ml of XTT (1mg/ml) and 200 µl of menadione solution (Appendix A). A stock solution of 0.4 mM of menadione solution was prepared in acetone. The resulting concentration of menadione in PBS is 1.6 µM. Two hundred microlitres of XTT-menadione-PBS (Appendix A) was then added to mature biofilms and to control wells to measure the background XTT level. The plates were then incubated in dark condition for 2 h at 37 ºC. After the incubation, 100 µl of the reaction solution was transferred into a new microtiter plate. XTT is converted to coloured formazan (colorimetric changes) in the presence of biofilm metabolic activity. The intensity of the formazan product in the supernatant was measured at optical density 492nm (A_{492nm}) using an automated microplate reader (Epoch, BioTek, US). The inhibitory effects of AbA on the metabolic activity of treated *C. albicans* biofilms were calculated using this formula:

XTT absorbance of growth control - XTT absorbance of treated biofilm x 100 %

XTT absorbance of growth control

3.5 Statistical analysis

All statistical analyses were performed using Statistical Package for the Social Sciences ver. 23.0 (SPSS Inc., Chicago, IL). Due to the small sample size, Mann Whitney test was used for comparison of the biofilm biomass, and metabolic activity between *Candida* species. A *p*-value of lesser than 0.05 was considered as statistically significant. The correlation analysis was performed with Pearson's bivariate correlation coefficicent to determine the strength and direction of the relationship between variables. A positive *r* value expresses positive relationship between the two variables.

3.6 Microscopic observation of planktonic and biofilm cultures

Bright field microscopy

Microscopic observation of mature biofilms was performed prior to CV staining. An inverted microscope (Zeiss, Axio, Germany) was used to capture the images of the *Candida* biofilm to confirm the results of CV assay. The images were captured at two hundred times magnification.

Field Emission Scanning Electron Microscopic (FESEM) analysis

The morphology of AbA-treated *C. albicans* biofilm and planktonic cells were observed using Field Emission Scanning Electron Microscope (FESEM). Yeast cells (planktonic/biofilm) were prepared on nucleopore membranes (diameter=13mm, pore size=0.2µm; Whatmann). The nucleopore membranes were sterilized by exposure to UV radiation for 15 min on both sides prior to inoculation. For preparation of mother inoculum, a loopful (size: 10µl) of *C. albicans* SC 5314 yeast colony suspended in 10ml

of SD minimal media (Appendix A) was grown to 18 h. The mother inoculum was diluted in 10 ml of fresh SD minimal medium in 1:20 ratio and let to grow for 15 to 16 h. The planktonic cells grown to mid-log phase were adjusted to 1 x 10^7 cells/ml using a haemocytometer. Aba concentrations of 0.25x MIC and 1x MIC were chosen to observe its antifungal effect. Untreated culture (1% DMSO in SD medium) was included as negative control. One milliliter of the cell suspensions was separated into three tubes respectively to be treated with AbA (0.25x MIC=0.5 µg/ml, and 1x MIC=2 µg/ml) for 2.5 h. One hundred microliters of an adjusted cell suspension (1 x 10^7 cells/ml) was inoculated onto the membrane. The membrane-supported yeast cells were transferred into a vial containing 4% (v/v) glutaraldehyde in cacodylate buffer and fixed overnight at 4 ^oC. Following a washing step in cacodylate buffer, the yeast cells on the membranes were fixed in 2% (w/v) osmium tetroxide for 1 h. After fixation, the samples were washed twice in sterile distilled water. After the washing step, the samples were dehydrated in a series of ethanol from 10% to 95% and twice in absolute ethanol. Dehydration step was continued in different ratios of ethanol: acetone mixtures (3:1, 1:1 and 1:3). Finally, a 100% acetone solution was added to the samples and incubated for 1 h with 3 changes of solution in between. Dehydrated samples were dried at critical point in CO_2 (CPD 7501, Polaron, UK). The nucleopore membranes were then mounted onto 0.5 inch aluminium specimen stubs and coated with gold in a sputtercoater (Biorad E5100 Series 11, USA).

The biofilm samples were prepared on nucleopore membranes resting on a flat bottom 24-well plate (SPL Life Sciences, Korea). The similar number (1 x 10^7 cells/ml) of yeast cells were inoculated and let to adhere for 1.5 h. The membrane was gently washed three times with 100 µl of PBS. New SD medium was replaced and the adherent cells were let to develop at 37 ^oC under minimal shaking condition. After 24 h of incubation, the mature biofilms were treated with AbA (0.25x MIC=8 µg/ml, and 1x

MIC=32 μg/ml) for 2.5 h. Similar dehydration, mounting and coating steps were performed. The stubs with sample were examined using a FEI Quanta 450 FEG (USA) Field Emission Scanning Electron Microscope (FESEM) at high vacuum condition (Working Distance= 9.6 mm, voltage=5 kV).

3.7 Determination of the growth curves for planktonic and biofilm cultures of *C. albicans*

Growth curve for planktonic culture (free-floating cells)

The growth curves for planktonic and biofilm cultures of *C. albicans* were generated using a temperature-controlled, automated microplate reader (Sunrise, Tecan Life Sciences, Switzerland). Briefly, a few colonies of a 24-hour-grown *C. albicans* SC5314 culture on a SDA plate was suspended in SD minimal medium (Appendix A) to a density of 10^6 cells/ml (equivalent to 75% transmittance), using a spectrophotometer (Genesys, USA) at $A_{520nm.}$ The cell suspension (200 μl) was then inoculated into four round bottom microtiter wells (Corning, USA). An equal volume of fresh SD minimal medium was also inoculated into another four different wells as an indication that the medium was free of contamination (negative control). A temperature-controlled, automated microplate reader (Sunrise, Tecan Life Sciences, Switzerland) was used to measure the absorbance of the increasing cell density. To assay the yeast growth, parameters such as absorbance, interval hour, number of cycle, shaking prior to reading, temperature control, evaluation defining were determined before the run. The proliferation of yeast cells was measured by determining the absorbance (A_{540nm}) of the cultures at every 2 h interval, up to 24 to 28 h. The plate was set to shake for three seconds before measurements were taken. The temperature was controlled in the range of 36 to 37 ^oC. Each isolate was tested twice on a microtiter plate.

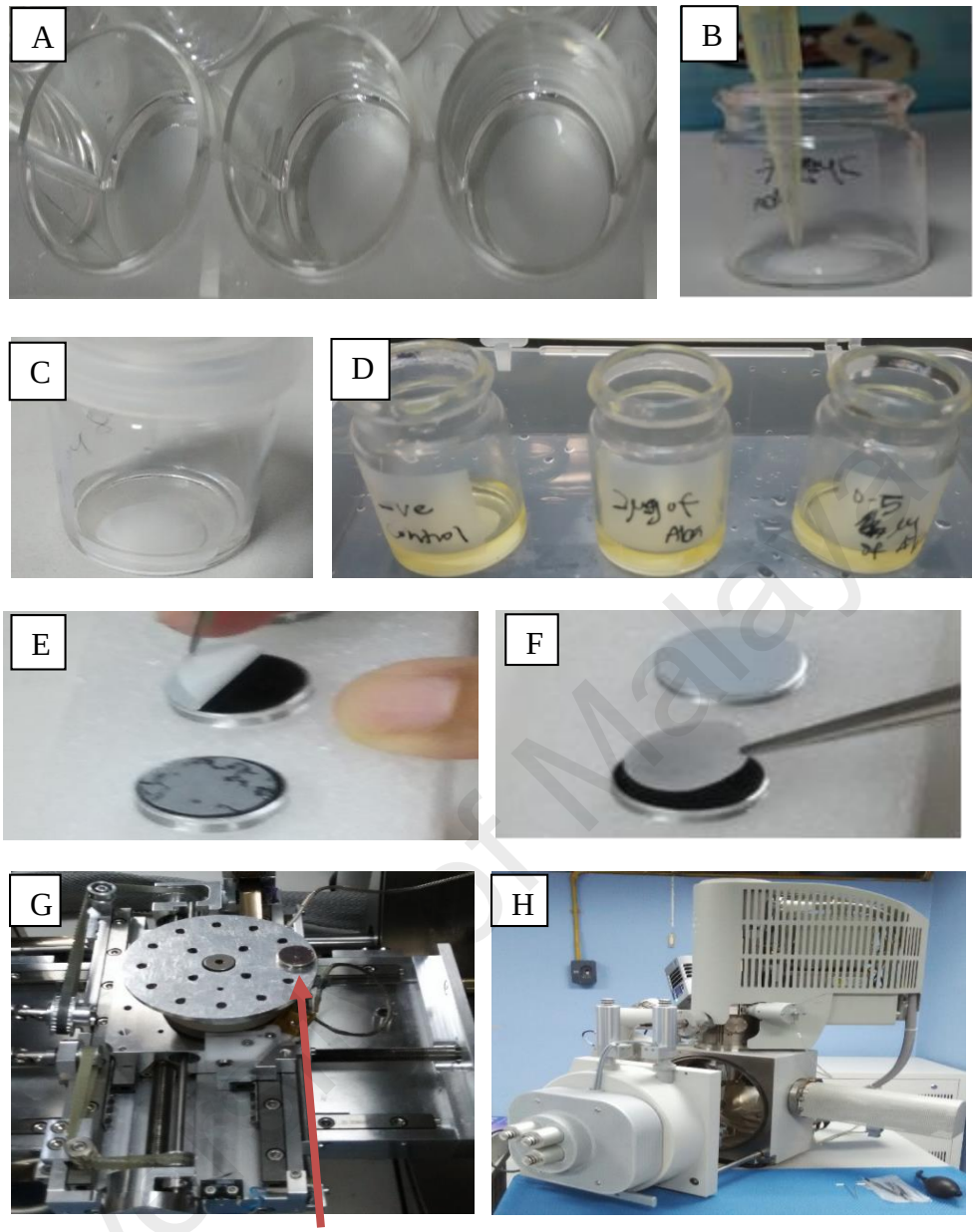


Figure 3.1: Work flow on sample preparation for FESEM; (A) Inoculation of suspension cells on nucleopore membranes in a 24- well plate for the development of biofilm; (B) Inoculation of yeast suspension on nucleopore membranes for the attachment of planktonic (free-floating) cells; C) Fixation of cells in 4% of glutaraldehyde; (D) Fixation of cells in 2% of Osmium tetroxide; (E,F) Mounting of nucleopore membrane on aluminium stubs; (G) Aluminium stubs in viewing platform of FEI Quanta 450 FEG; (H) FEI Quanta 450 FEG Field Emission Scanning Electron Microscope (FESEM).

Growth curve for biofilm culture

Similar method was used to generate the growth curve for *Candida* biofilm cultures. Following the same steps, a few colonies of a 24-hour-grown *C. albicans* SC5314 cultured on a SDA plate was diluted in SD minimal medium to achieve a cell density of 10^7 cells/ml (equivalent to an OD reading of 0.38 at A_{520nm}). The cell suspension (200 µl) was then inoculated into four flat bottom microtiter wells (Cat. No: 3596, Costar, USA). After 1.5 hours of adhesion period, the non-adherent cells were aspirated. Two hundred microliters of SD minimal medium were then added into the wells. The development of the adherent cells was monitored to generate the growth curve for biofilm cultures at A_{540nm}. Similar to the planktonic cultures, this set of experiment was accompanied with a negative control (uninoculated growth medium) as well. The same parameters were set on an automated microplate reader (Sunrise, Tecan Life Sciences, Switzerland) to generate the biofilm growth curve, for 48 to 50 hours. Each isolate was tested twice on a microtiter plate.

Growth curves of C. albicans on treatment with AbA

The growth profiles of planktonic and biofilm cells of *C. albicans* SC5314 were assessed in a microtiter well plate. The absorbances of the planktonic and biofilm cells were followed as stated above by the respective culture. The planktonic cultures of *C. albicans* were exposed to different concentrations of AbA (2µg, 1µg and 0.5µg/ml) during the mid-log growth phase. The cells actively grow and are viable during the mid-log growth phase. The biofilm cultures of *C. albicans* were exposed to 32 µg, 16 µg and 8 µg/ml of AbA during the mid-log growth phase. Dimethyl sulfoxide (200 µl of yeast cells treated with 1 % DMSO) and drug-free control (200 µl of yeast cells) were included in the experiment. The yeast growth was measured every 2 hours interval until the cultures reached the plateau stage. Each growth curve obtained was compared with the

negative controls. The assay was repeated on three different days (three "biological replicates).

3.8 Survival assay

Planktonic culture

C. albicans SC5314 strain was cultivated similarly as described in the growth curve experiment for planktonic culture. The experiment was conducted during the mid-log growth phase (OD=1.0, Hoehamer *et al*., 2014). The planktonic culture was treated with inhibitory (2 µg/ml) and sub-inhibitory concentrations of AbA (0.5 µg/ml and 1 µg/ml) at early mid-log phase (12^{th} hour). Prior to drug exposure (10^{th} hour), the yeast cells were diluted 100x in PBS solution serially (from 10^{-1} to 10^{-6}). Ten microlitres of the yeast cells were plated onto SDA or PDA by spreading the inoculum using a spreader. Plating was performed at every two hours interval after exposure to AbA. The assay was repeated on three different days (three "biological replicates).

Biofilm culture

Biofilm cultures were developed similarly as described in the growth curve experiment for biofilm culture in a 24-well microtiter plate (Cat. No: 3596, Costar, USA). The entire experiment was conducted during the mid-log phase. The biofilm cultures were developed to mature stage (for 24 h). The mature biofilm cultures were treated with inhibitory (MIC, 32 µg/ml) and sub-inhibitory concentrations of AbA (16 µg/ml and 8 µg/ml) during mid-log phase. Post to drug exposure at every 2 h, the biofilm cells were scraped off from the well and diluted 100x in PBS solution serially (from $10^{-}1$ to 10^{-3}). Ten microlitres of biofilm cells were plated onto SDA by spreading the inoculum using a spreader. Plating was performed at every two hours interval after

exposure to AbA. The experiment was conducted in triplicate. The assay was repeated on three different days (three "biological replicates).

3.9 Cultivation of yeast for SDS-PAGE analysis (1-DE)

For the cultivation of planktonic culture, *C. albicans* SC5314 strain was streaked onto a SDA plate from a glycerol stock culture and incubated at 37 ºC for 24 h. A loopful of the yeast colony was inoculated aseptically into 20 ml of SD minimal medium (Appendix A, Hoehamer *et al*., 2014) in a 50 ml falcon tube. The cells were cultivated with rotary shaking (200 rpm) at 37 ºC until it reached the mid-log phase (12 to 18 h). Depending on the end volume needed, the mother inoculum was further diluted at a ratio of 1:20. For instance, if the required end volume is 200ml, 10ml of the mother inoculum was added to 190 ml of SD minimal medium. To ensure the purity of the culture, the culture was spreaded onto blood agar and the growth was examined the next day.

3.10 Preparation of protein lysates (protein extraction) for SDS-PAGE (1-DE)

Protein lysates of *C. albicans* SC 5314 was extracted using a sonicator (Omni International, USA). The yeast was cultivated as described in section 3.9. The culture grown to mid-log phase was harvested by centrifugation (13,000 rpm, 15 min, 4 ºC). The pellet was washed twice with cold PBS. The culture pellet was kept in ice throughout the protein extraction. The pellet was suspended in cold lysis buffer (Appendix A) at a ratio of 1:5. The lysis buffer contained 40mM Tris-HCl (Promega, pH 9.0, 20 mM DTT (GE Healthcare, UK), 4% (v/v) Triton X-100 (Sigma, USA), 1 mM EDTA and 2 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma, USA) (Appendix A). Two mililiters of the suspended pellet was subjected to mechanical lysis using a sonicator (Omni international, USA). The power and pulse magnitude of the sonicator was adjusted to 20 to 30 Watt and 50 percent, respectively. The falcon tube that has culture is placed into a small beaker or container containing ice during the protein extraction. The cells were

disrupted for 30 s and let to rest for 30 s. This step was repeated for 10 times. Cell debris was removed by centrifugation (13000 x g, 15 min, 4^{o} C), and the supernatant, representing the total lysates, was pipetted into 1.5 ml tubes (Eppendorf, Sigma, USA).

3.11 Concentration of extracted protein lysates using spin concentrator

One milliliter of the extracted protein lysates was aliquoted into 1.5 ml tubes (Eppendorf, Sigma, USA) and placed in a refrigerated centrivap concentrator (Labconco, USA) to concentrate the protein lysates. Vacuum pump and temperature controller were switched on prior to the concentration process. The temperature was adjusted to 4^{o} C. The concentration process was stopped after 4 h. The concentrated protein lysates were pooled according to the sample.

3.12 Clean-up of protein samples for SDS-PAGE (1-DE)

A 2-D clean-up kit (GE Healthcare, UK) was used to purify protein samples prior to SDS-PAGE electrophoresis. The procedure was performed as described by the manufacturer. All steps were carried out in an ice bucket unless otherwise specified. Three milliliters of the protein samples were transferred into 15 ml Falcon tubes.

For each volume of a sample, 3 volumes of precipitant (provided by the kit) were added. The content was mixed well by vortexing or inversion. The tubes containing the mixture of sample and precipitant were incubated on ice (4-5^{o} C) for 15 min. For each original volume of the sample, 3 volumes of co-precipitant were added to the mixture of protein and precipitant. The content was again mixed by vortexing. The tubes were centrifuged at 8000 x g for 10 min. The tubes were removed from the centrifuge as soon as the centrifugation was completed. A small pellet visible after the centrifugation was rapidly preceded to the next step to avoid resuspension or diffusion of the pellet. The supernatant was removed as much as possible by decanting or careful pipetting. Without the pellet being disturbed, the tubes were carefully repositioned as before with the pellet

facing outward. The tubes were centrifuged again for at least 1 min to bring any remaining liquid to the bottom of the tubes. A pipette tip was used to remove the remaining supernatant. No liquid was visible in the tubes. Co-precipitant (provided by the kit) was added 3 to 4 times to the size of the pellet. The tubes were carefully repositioned in the centrifuge as before. The tubes were centrifuged again for 5 min. Pipette tip was used to remove and discard the wash. Distilled or de-ionized water was pipetted on top of each pellet to cover the pellet. The tubes were vortexed for several seconds.

The pellets were then dispersed in sterile distilled water. One mililiter of pre-chilled (at least 1 h at -20^{o} C) wash buffer (provided by the kit) was added to the mixture. A 5 µl of wash additive (regardless of the original sample volume) was added. The tubes were vortexed until the pellet was fully dispersed. The tubes were incubated at -20^{o} C for at least 30 min and vortexed for 20 to 30 s once every 10 minutes. The tubes were centrifuged at 8000 x g for 10 m. The supernatant was carefully removed and discarded. A white pellet was visible. The pellet was allowed to air dry briefly (no more than 5 min).

3.13 Protein quantitation (2-D quant kit) for SDS-PAGE (1-DE)

Prior to performing the assay, an appropriate volume of working color reagent was prepared by mixing 100 parts of color reagent A (provided by the 2-D quant kit, GE Healthcare, UK) with 1 part color reagent B (provided by the 2-D quant kit, GE healthcare, UK). A standard curve was prepared according to Table 3.1 using 2 mg/ml bovine serum albumin (BSA) standard solution provided in the kit. A series of standard test solutions was prepared according to Table 3.1. Tubes containing 0, 10, 20, 30, 40 and 50 µg of protein samples were prepared. A volume of 500 µl of the precipitant

Table 3.1: Preparation of standard curve for protein quantitation

Tube number	**1**	**2**	**3**	**4**	**5**	**6**
Volume of 2 mg/ml BSA standard solution (µl)	0	5	10	15	20	25
Protein quantity (µg)	0	10	20	30	40	50

(provided by the 2-D quant kit, GE Healthcare, UK) was added to each tube including the standard curve tubes. The tubes were vortexed briefly and incubated 2 to 3 min at room temperature. A volume of 500 µl of co-precipitant (provided by the 2-D quant kit, GE Healthcare, UK) was then added to each tube and mixed briefly by vortexing or inversion. The tubes were centrifuged at 10 000 x g for 5 min. The tubes were removed from the centrifuge as soon as the centrifugation step was complete. A small pellet was visible. The supernatants were decanted.

The following step was proceeded rapidly to avoid resuspension or dispersion of the pellets. The tubes were carefully repositioned in the microcentrifuge as before, with the cap-hinge and pellet facing outward. The tubes were centrifuged again to bring any remaining liquid to the bottom of the tube with a brief pulse. There should not be any visible liquid remaining in the tubes. One hundred microliters of copper solution (provided by the 2-D quant kit, GE Healthcare, UK) and 400 µl of distilled water was added to each tube. The mixture was vortexed briefly to dissolve the precipitated protein. One mililiter of working color reagent was added to each tube and mixed instantaneously by inversion to introduce the reagent as rapidly as possible. The tubes were incubated at room temperature for 15 to 20 min. The absorbance of each sample and standard were measured at 480 nm using water as the reference. The absorbance should be read within 40 min after the addition of the working color reagent. A standard curve was generated by plotting the absorbance of the standards against the protein samples. The standard curve was used to determine the protein concentration of the samples.

3.14 SDS-PAGE (1-DE) analysis

Preparation of 10% polyacrylamide Gel

Five milliliters of 10% polyacrylamide gel (resolving gel) solution (Appendix A) was poured until it filled 4 cm of the sandwich plate [(glass plate: 7.3 cm x 10.2 cm,

spacer glass plate: 8.3 cm x 10.2 cm)] (Biorad, CA, USA). Water was added to overlay the gel and to prevent the exposure of the gel to air. The resolving gel was allowed to polymerize for at least 30 min. After the gel had polymerized, the overlay was rinsed off. Two milliliters of stacking gel solution (Appendix A) were then pipetted onto the resolving gel. A comb was inserted into the sandwich by avoiding bubbles and the gel was allowed to polymerize for at least 1 hour

Preparation of protein samples for SDS-PAGE

Protein sample (20 µg) was mixed with 10 µl of 2x solubilizing buffer (Appendix A), consisting of 4x stacking gel buffer (pH 6.8), 10% SDS (Merck, USA), 20% (v/v) glycerol (Amersham, USA), 0.2 M DTT (GE Healthcare, UK), 0.02 % bromophenol blue (Amersham Pharmasia BioTech, Uppsala, Sweden), in a test tube and boiled at 95^{o}C for 5-10 min. After boiling, the tube was kept in ice until use.

Electrophoresis

Electrophoresis of the protein samples was performed according to Laemlli (1970) using a Bio-Rad Mini Protean II System. The comb was removed after the inner tank was filled with SDS cathodal buffer (Appendix A) [0.025 M Tris-base (Amersham, USA); 0.192 M glycine (Amresco, USA); 0.1 % (w/v) SDS (Merck, Germany)]. Twenty micrograms of each sample was slowly loaded into each well that had been filled with SDS cathodal buffer. Broad range prestained protein markers (New England, BioLabs) (MBP-β- galactosidae 175,000 Da, MBP-paramyosin 83,000 Da, glutamic dehydrogenase 62,000 Da, lysozyme 16,500 Da, and aprotinin 6,500 Da) were included for electrophoresis in each gel. First, the protein samples were electrophoresed at 15 mA for 30 minutes and subsequently at 30 mA for 1 hour or until the dye reached the bottom of the gel. Each gel was stained with Coomassie blue for visualization of protein bands.

Staining of SDS-PAGE gels (Coomassie Blue Staining)

Coomassie blue staining solution was prepared using Coomassie brilliant blue tablets (GE Healthcare, UK) (Appendix A). The gels were stained for at least 1 hour and destained with 10% acetic acid (Appendix A) until the bands were visible or the gel background was clear.

3.15 Total protein isolation for LCMS analysis

Cell counting

Maximum input amount for each lysis tube is 10^9 yeast cells. The yeast cells were stained with Trypan blue (Sigma, US) and counted using a haemocytometer. Equal volume of Trypan blue (Sigma, US) was added to a desired volume of yeast cells (1:1 ratio) for staining purpose.

Protein extraction for LCMS analysis

Protein extraction was carried out using a PROTEOSPIN detergent-free total protein isolation kit (Norgen, Canada). An input of $2.7x10^8$ (planktonic) and $1.7\ x10^9$ (biofilm) yeast cells were used for extraction. Biofilm cells were scraped off from the culture flask after the removal of the suspension cells. The culture with a density of $2.7x10^8$ (planktonic) and $1.7\ x10^9$ (biofilm) yeast cells were harvested by centrifugation at 14000 x g for 5 minutes. The supernatant was completely discarded and the cells were resuspended in 50 µl of the lysis solution (provided by the kit). The resuspended cells were added to a lysis tube (provided by the kit). The lysis tube was vortexed vigorously for 2 minutes and centrifuged at 14000 x g for 2 minutes. A filter column was placed into an elution tube (provided by the kit). The liquid content was transferred from the lysis tube (provided by the kit) into the filter column. The samples were filtered through the filter column by centrifugation (14000 x g for 1 minute) to ensure that the entire sample

has passed through into the elution tube. Each sample was stored at -20 ^{o}C to avoid protein degradation. The protease inhibitor (provided by the kit) were added to the isolated proteins, mixed well and stored at -20 ^{o}C.

Alkylation, reduction, and trypsin digestion

The lysates were then treated with 10 mM dithiothreitol (DTT; GE Healthcare, UK) (Appendix A) at 37 ^{o}C for 1 hour and alkylated with 55 mM iodoacetamide (IAA; GE Healthcare, UK) (Appendix A). The volume of DTT or IAA were prepared respectively based on the volume of concentrated sample (example provided in Appendix A). The proteins in the sample were digested in the ratio of 1:50 (trypsin: protein) with MS grade porcine trypsin (Calbiochem, Germany) at 37 ^{o}C for overnight. The samples were desalted using a Pierce C-18 spin column (Thermo Scientific) or Zip-Tip C-18 Cartridge column (Millipore, Billerica, MA, USA) and completely dried in a refrigerated CentriVap centrifugal vacuum concentrator (Labconco, USA) before mass spectrometry analysis.

3.16 Purification and concentration of peptides for LCMS analysis

Desalting with Pierce C-18 spin column

The porous C18 reversed-phase resin (Thermo Scientific, USA) was firstly activated by wetting with 200 µl of 50% acetonitrile (ACN). The column was centrifuged for 3 to 5 minutes and the step was repeated. The resin was equilibrated in 200 µl of 5% acetonitrile (ACN) and 0.5% trifluoroacetic acid (TFA) loaded into the column and centrifuged for 5 minutes. The step was repeated. For sample binding, 10 to 150 µl of each sample was added to the wetted resin. The column was centrifuged for 14, 000 x g for 3 to 5 minutes and the step was repeated. The nest step involved washing of the resin with 200 µl of 0.5% TFA in 5% of ACN. The column was centrifuged for 5 minutes and

the step was repeated twice. The sample was eluted using 20 µl of 70% ACN. The samples were air-dried and resuspended for MS analysis.

Purification and concentration of Peptides by Zip-Tip C18

Seven tubes (A - G) with different protein contents were prepared prior to the purification and concentration of the samples. Tube A contained 100 µl acetonitrile (ACN) (HPLC Grade) which was used as a wetting solution. Tube B contained 1000 µl water (HPLC Grade) which functioned as a cleaning solution. Tube C contained 10 to 20 µl of sample solution. Tube D contained 500 µl of formic acid for desalting of the sample. Tube E contained 100 µl of 0.5 % formic acid in water: acetonitrile (1:1 ratio, v/v) solution which was used as the first extraction solution. Tube F contained 100 µl of acetonitrile, which was used as the second extraction solution. Tube G was a clean empty 200 µl tube.

The Zip-Tip column (Millipore, Billerica, MA, USA) was attached to a 20 µl micropipette. The volume setting was adjusted to 7 µl. ACN in tube A was withdrawn carefully through the Zip-Tip column and then, while the tip of the Zip-Tip was still immersed in the ACN, the ACN was pipetted out carefully, taking precaution to prevent introducing air bubbles into the Zip-Tip column. This step was repeated 7 to 8 times. Finally, the ACN was pipetted out slowly and the tip of the Zip-Tip column was immersed into the water (Tube B) while the plunger was still down. Seven microliters of water were withdrawn slowly through the Zip-Tip column, taking care not to introduce air bubbles into the Zip-Tip column. This step was repeated carefully at least 10 times to ensure that all ACN had been washed away. The water was pipetted and while the plunger was still down, the tip of the Zip-Tip column was moved into the sample solution (Tube C). The Zip-Tip column was filled with the sample solution and slowly pushed into the tube (Tube C) carefully. The step was repeated carefully 10 times to ensure that

most of the peptides were retained on the Zip-Tip column. The Zip-Tip column was then washed with 0.5% formic acid (Tube D) solution at least 10 times to perform desalting and washing of the peptides in the same fashion as the steps above. While the plunger was still down, the Zip-Tip column was transferred to the tube containing the extraction solution (Tube E) after the wash solution was pipetted out. The Zip-Tip column was slowly filled with the extraction solution and waited for 20 seconds for complete extraction. The extraction solution was pipetted (Extract 1) into the empty tube G, and, while the plunger was still down, the Zip-Tip column was moved to the tube containing the ACN. The ACN was slowly withdrawn through the Zip-Tip. After waiting for 10 seconds, the solution (Extract 2) was pipetted into the tube G containing the extract 1. The combined extracts (Tube G) was submitted for MS analysis or stored in a freezer (-20 °C) until required for analysis.

3.17 Protein quantitation with Nano Spectrophotometer

The sampling arm of a nano spectrophotometer (Malcom, Japan) was raised and the sample (0-2 µL) was pipetted onto the lower measurement pedestal. The sampling arm was lowered and a spectral measurement was initiated using the E-Spect analysis software. The sample column was automatically drawn between the upper and lower pedestals and the measurement was made. When the measurement was complete, the sampling arm was raised and the sample was wiped from both the upper and lower pedestals using a dry, lint-free laboratory wipe. Simple wiping prevents sample carryover in subsequent measurements for samples varying by more than 1000 fold in concentration.

3.18 LC-MS/MS Analysis

Tryptic peptides prepared after purification and concentration with Zip-Tip C18 were analyzed using a 1260 Infinity HPLC-Chip/MS System (Agilent, USA). The

HPLC-Chip was the Large Capacity C18 Chip (G4240-6210), which comprised a 160 nL enrichment column and a 150 mm analytical column. HPLC-grade water (0.1% formic acid) and acetonitrile (0.1% formic acid) were used as mobile phases A and B respectively. HPLC-grade Acetonitrile was obtained from Friendmann Schmidt (Australia), water from Mili-Q water purification system and formic acid from Sigma (USA). The Nanoflow gradient (%B) used was: 3% at 0 min, 3% at 5 min, 35% at 60 min, 40% at 67 min, 60% at 85 min, 60% at 95 min, 3% at 105 minutes; stop time: 120 minutes; nanopump flow rate: 0.3 µl/min; capillary pump gradient; 3% B isocratic; capillary pump flow: 2.5 µl; chip value position; enrichment at 95 min. An Agilent 6540 Accurate-Mass Q-TOF LC/MS System operated in positive ion mode was used for mass detection, applying the following settings: capillary voltage: 1850 V; drying gas flow: 5 L/min; drying gas temperature: 250 ^{o}C; fragmenter: 175 V; skimmer: 65 V; acquisition mode: autoMS/MS; scan range: 125 – 1700 m/z (MS/MS), 50 – 1700 m/z (MS/MS); acquisition rate: 15 spectra/s (MS), 12 spectra/s (MS/MS); isolation width (MS/MS): narrow (~ 1.3 m/z); collision energy: -4.8 (offset) + 3.6 (slope); max. precursors/cycle: 15; active exclusion: enabled, exclude after one spectrum, release after 0.25 min; charge state preference ; 2, 3 and 0637 >3, sorted by abundance only; reference mass: 299.294457 and 1221.990637 m/z from continuous addition of trace amounts of methyl stearate and HP-1221 calibrant respectively into the ionization region. Reference mass correction was enabled. *De novo* sequencing was conducted with PEAKS Studio 7.5 (Bioinformatics Solutions Inc., Canada) with default parameters, except that: i) parent mass error tolerance was 1.5 Da, ii) fragment mass error tolerance was 0.5 Da, iii) trypsin as digestion enzyme, iv) carbamidomethylation (+57.02 Da, C) as fixed modification, v) oxidation (+15.99 Da, M) as variable modification, vi) maximum variable post-translation modification allowed per peptide was three. Total volume of injection was 2 to 3 µl of sample.

Protein analysis with PEAKS software (v 7.5)

Sequence homology search tool (SPIDER) software of the PEAKS (v 7.5) software was used to detect all the proteins that were expressed by *Candida* biofilm and planktonic cultures. The overall picture of the protein expression of *C. albicans* SC5314 planktonic and biofilm cultures were obtained using the selection criteria of at least one unique peptide match for protein identification, significance (-10lgP) value of >20 for protein identification and false discovery rate of ≤1%.

3.19 Gene Ontology (GO) Slim Mapper maps annotation

The GO Slim Mapper maps can be used for annotations of a group of genes to more general terms and/or to bin them into broad categories, ie. GO Slim Terms. First, a species should be selected for genes in query set. Secondly, gene/ORF names must be entered in the given space. The GO Slim Term (the respective biological process, molecular functions, subcellular localization) must be chosen at step 3. Upon clicking search, all the biological processes, molecular functions or subcellular localization annotated to each genes/proteins will appear.

CHAPTER 4: RESULT

4.1 Growth characteristics of *C. albicans* SC5314 / ATCC MYA 2876

C. albicans SC5314 strain formed smooth, white to creamy colonies on SDA after 24 hours of incubation at 37ºC (Figure 4.1). Gram-staining of the broth culture (mid-log phase) of *C. albicans* SC5314 demonstrated spherical to subspherical budding yeast-like cells, also known as blastoconidia. Additionally, yeast cells with elongated hyphae were observed (Figure 4.2).

4.2 *In vitro* susceptibilities of *Candida* planktonic and biofilm cultures to AbA

The AbA planktonic and biofilm MICs of 118 *Candida* isolates investigated in this study are shown in Table 4.1. The MICs for the isolates ranged from 0.25 to ≥ 8 µg/ml, with the MIC50 and MIC90 being 1 and 4 µg/ml, respectively. Eleven (22.4%) of 49 isolates of *C. albicans*, two of 38 (5.3%) isolates of *C. tropicalis*, 1 of 16 (6.3 %) isolates of *C. parapsilosis* and 1 of 12 *C. glabrata* isolates had MICs of ≥ 4 µg/ml. Except for three *C. nivariensis* isolates exhibiting higher MICs (ranging from 4 – ≥ 8 µg/ml); the MICs for majority of non-albicans *Candida* spp. were < 4 µg/ml (Table 4.1). *C. albicans* isolates demonstrated significantly higher planktonic MICs than *C. tropicalis* (p=0.000) and *C. parapsilosis* (p=0.006) when analysed with Mann- Whitney test.

Figure 4.1: Appearance of *C. albicans* on Sabouraud's dextrose agar (SDA)

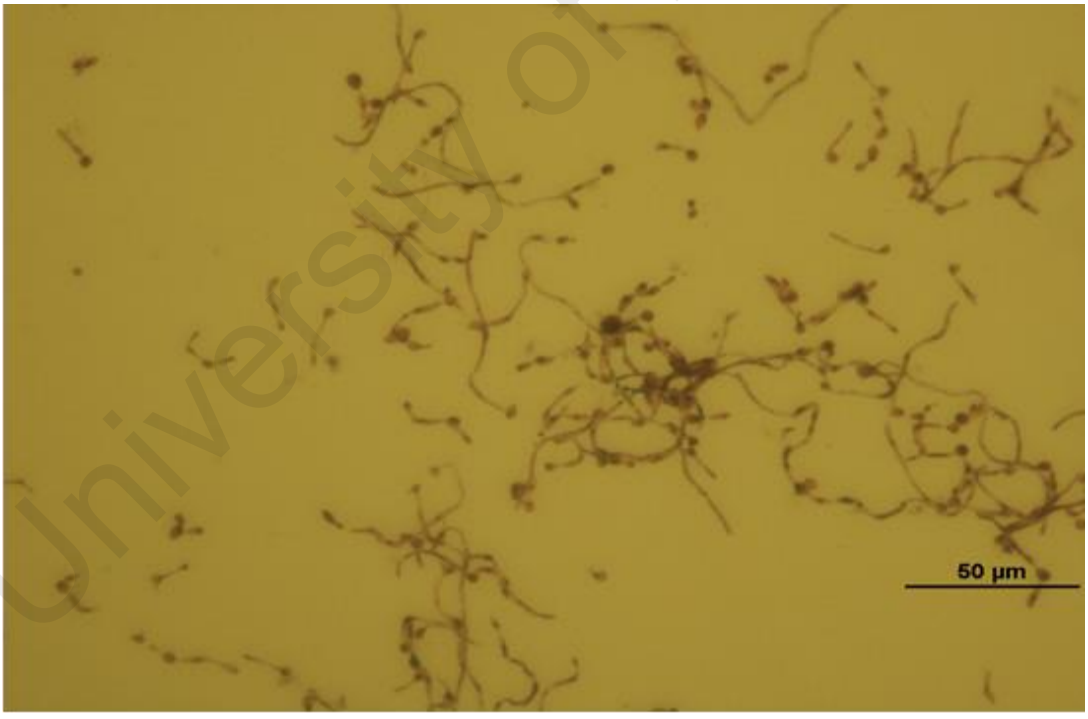


Figure 4.2: Microscopic observation of Gram-stained *C. albicans* SC5314 strain in SD broth medium (mid-log phase) after 24 hours of incubation (Magnification; x100)

Table 4.1: *In vitro* susceptibilities of *Candida* planktonic and biofilm cultures to AbA

Yeast isolates (n=118)	Planktonic MIC (µg/ml)			Biofilm MIC (µg/ml)*		
	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}
C. albicans (n=48)	0.5 - ≥8	2	4	1 - ≥128	32	≥ 128
Non-albicans *Candida spp.* (n=70)						
C. parapsilosis (n=16)	0.5 – 4	1	2	2 - ≥ 128	≥ 128	≥ 128
C. tropicalis (n= 38)	0.25 – ≥ 8	1	2	0.5 - ≥128	32	≥ 128
C. glabrata (n= 12)	1 – 4	1	2	ND	ND	ND
C. nivariensis (n=3)	4 – ≥8	ND	ND	ND	ND	ND
C. kefyr (n=1)	1	ND	ND	ND	ND	ND
***Candida* isolates (n=118)**	**0.25-⩾8**	**1**	**4**	**0.5-⩾128**	**32**	**⩾128**

Footnote:

- The biofilm MICs were determined for 10 isolates of *C. albicans*, 17 isolates of *C. tropicalis* and 8 isolates of *C. parapsilosis*
- Planktonic MIC: The lowest concentration of AbA which inhibits the visible growth of the yeast after incubation at 37 ^{o}C for 48 hours.
- Biofilm MIC: The lowest concentration of AbA which causes 50% reduction in the metabolic activity.
- MIC50 and MIC90 values were only calculated for *Candida* species with five or more isolates.
- ND: Not determined

Figure 4.3 shows the images of AbA antifungal susceptibility testing on various ATCC reference strains (SC5314, ATCC90028, ATCC 6258, ATCC 22019, ATCC 3290, ATCC 3080). Clear well (as pointed by red arrow) demonstrated the complete inhibition of the yeast growth by AbA. *Candida krusei* ATCC 6258, *C. rugosa* ATCC 3290 and *C. tropicalis* ATCC 3080 demonstrated low planktonic MICs in the range of 0.5-4 µg/ml.

The biofilm MICs for 35 biofilm-producing (as determined using CV assay; Figure 4.4) *Candida* isolates ranged from 1 to ≥128 µg/ml, with the MIC50 and MIC90 being 32 and ≥128 µg/ml, respectively (Table 4.1). The biofilm MIC50 of *C. albicans* and *C. tropicalis* were 32 µg/ml, respectively (Table 4.1). *C. parapsilosis* exhibited much higher MIC50 at 128 µg/ml (Table 4.1). Five out of 11 *C. albicans* isolates, 6 out of 17 *C. tropicalis* isolates and 6 out of 8 *C. parapsilosis* exhibited high biofilm MICs (≥128 µg/ml).

4.3 Screening of biofilm-producing *Candida* isolates and their morphovariants

Biofilm-producing *Candida* isolates were identified using the crystal violet (CV) assay as described in section 3.4. The cut-off point for CV assay was OD ≥0.1 as indicated by Reisner *et al*. (2006). A total of 35 (29.7%; Figure 4.4) of 118 *Candida* isolates were regarded as biofilm producers in this study, of which, majority were isolates of *C. parapsilosis* (8/12 isolates, 66.7 %), followed by *C. tropicalis* (17/38 isolates, 44.7 %), and *C. albicans* (10/48 isolates, 20.8%) (Table 4.4).

Interstrain and interspecies variability in biofilm growth was observed and the images were captured microscopically after staining with crystal violet. Figure 4.5, 4.6 and 4.7 shows *Candida* isolates with biofilm composed of mainly yeast/blastospores

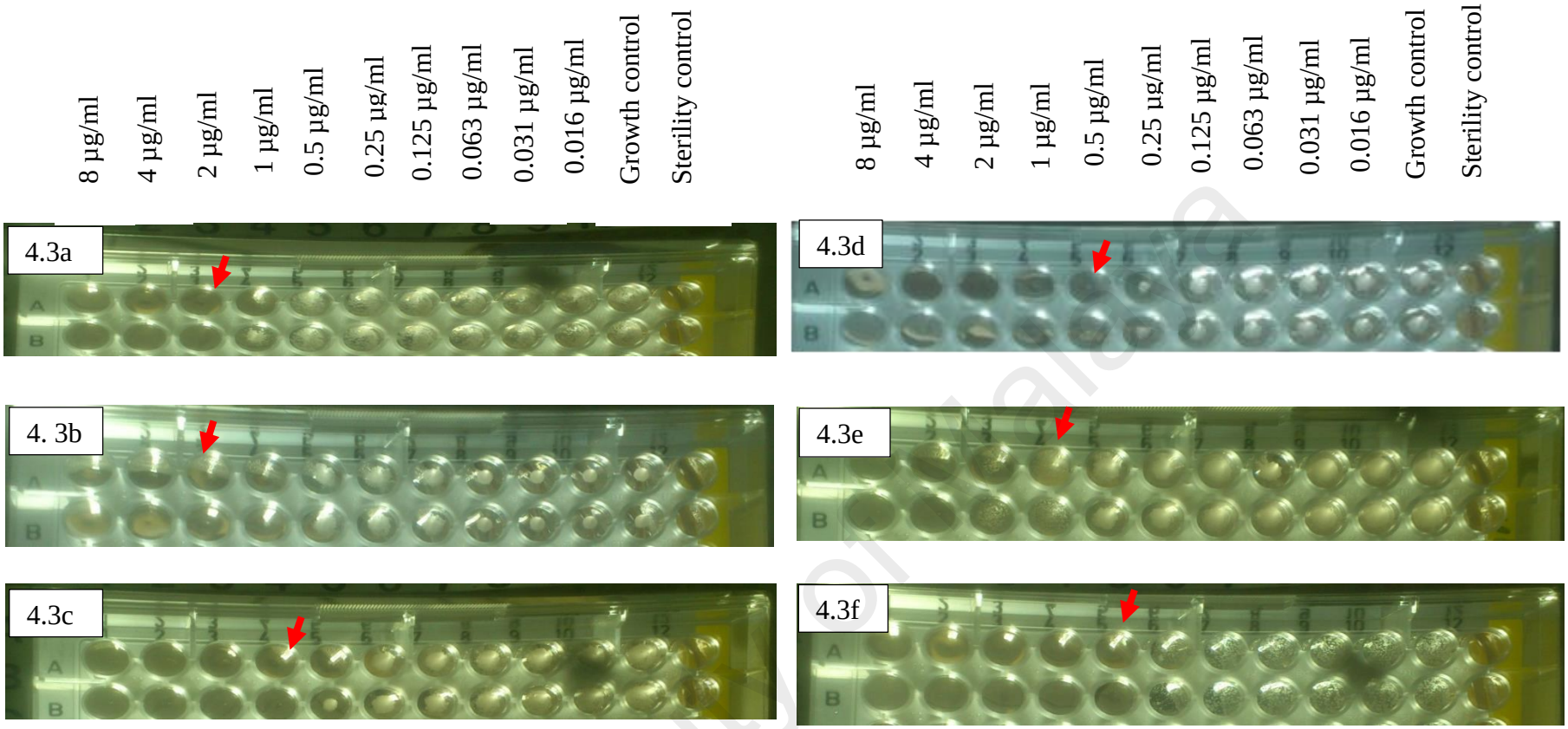


Figure 4.3: Arrow in red showing complete inhibition of *Candida* spp. by AbA after 48 hours of incubation; 4.3a) *C. albicans* SC5314, MIC: 2 µg/ml; 4.3b) *C. albicans* ATCC 90028, MIC:1 µg/ml; 4.3c) *C. krusei* ATCC 6258, MIC: 1 µg/ml; 4.3d) *C. parapsilosis* ATCC 22019, MIC: 1 µg/ml; 4.3e) *C. rugosa* strain 3290, MIC: 4 µg/ml; 4.3f) *C. tropicalis* strain 3080, MIC: 0.5 µg/ml. The concentration of AbA in each well is shown on top of the image.

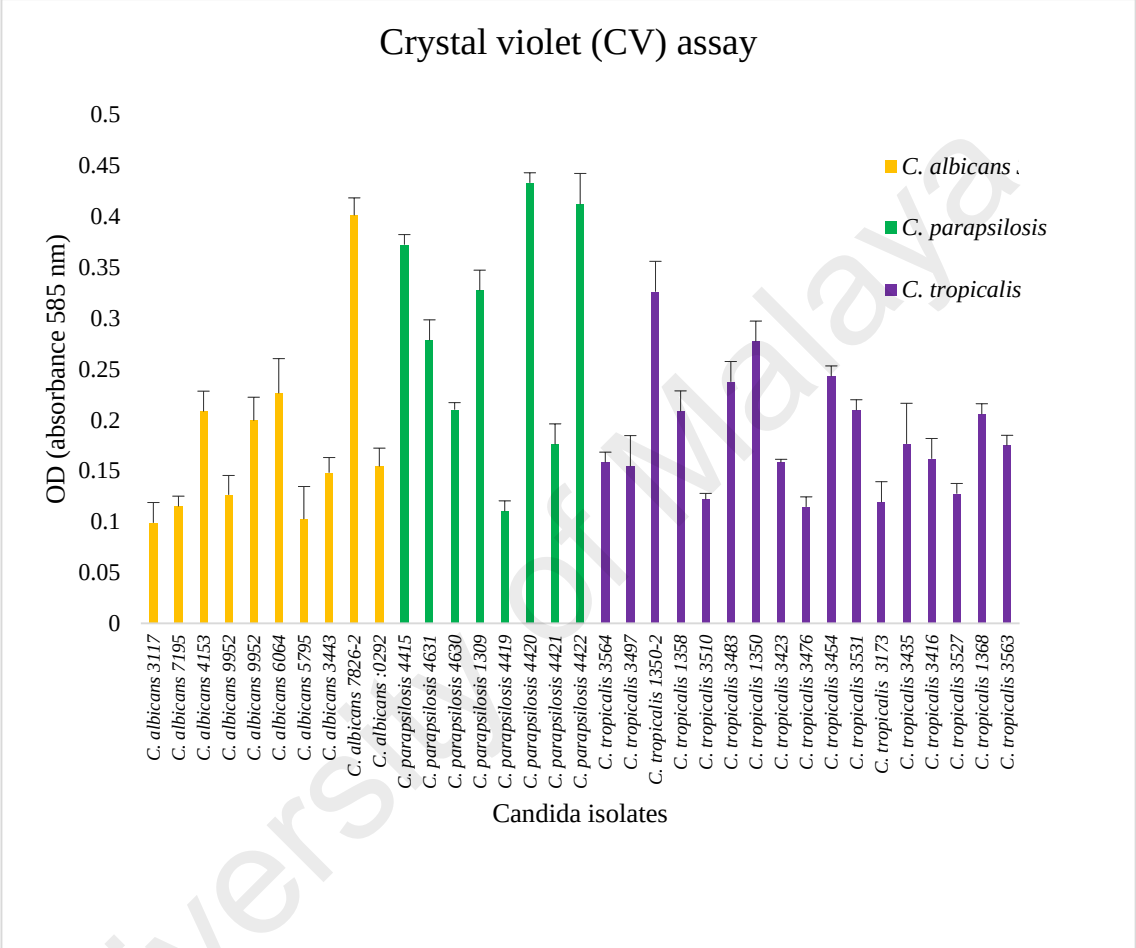


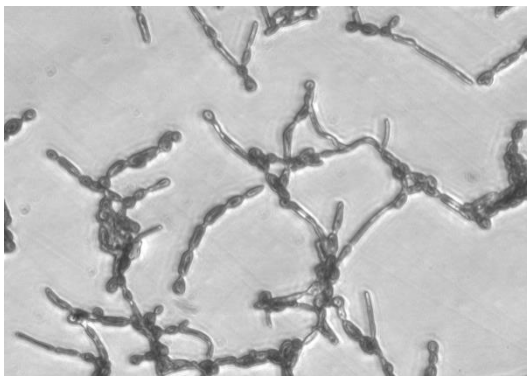
Figure 4.4: OD readings of 35 isolates of biofilm-producing *Candida* isolates (OD > 0.1) as determined by Crystal violet assay.

with or without pseudohyphae while Figure 4.8 and 4.9 shows *Candida* isolates with biofilm predominantly composed of a dense network of cells and hyphae. Amongst 35 biofilm-producing isolates, 17 (48.6%) demonstrated only yeast/blastospores or pseudohyphae, while the remaining 18 (51.4%) isolates exhibited a dense network of biofilms consisting mainly of filamentous cells. For *C. glabrata* and *C. nivariensis* only yeast cells were observed (Figure 4.7).

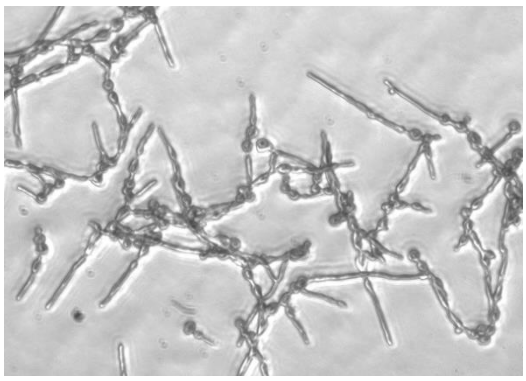
Table 4.2 shows the results obtained from the quantitation of biofilm biomass and metabolic activity. Although the biofilm biomass was highest in *C. parapsilosis* (Table 4.2), no significant difference was noted when comparison was made to *C. albicans* and *C. tropicalis* ($p>0.05$). The biofilm metabolic activity of *C. parapsilosis* was significantly higher compared to *C. albicans* ($p=0.014$) and *C. tropicalis* ($p=0.012$) (Table 4.2). Although higher biofilm biomass was noted in the blood isolates of *C. parapsilosis* and *C. tropicalis* as compared to the isolates obtained from nonsterile sites, the differences are not significant ($p=0.441$ and $p=0.182$, respectively). There was no significant relationship between biofilm MICs with *Candida* species and biofilm morphology ($p>0.05$). No correlation was found between *Candida* biofilm MICs with the biofilm biomass ($r^2=0.808$) or metabolic activity ($r^2=0.089$).

Table 4.2: Association of biofilm growth, biomass, metabolic activity with susceptibility of *Candida* isolates to AbA

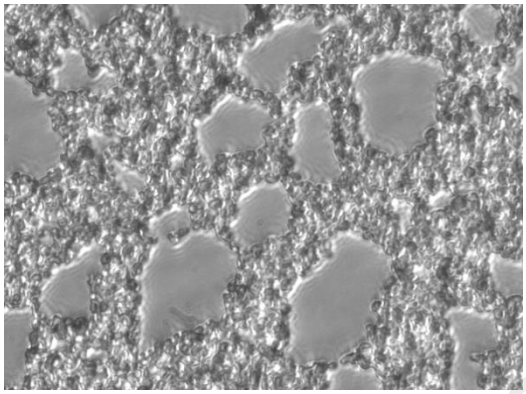
Biofilm growth morphology	***Candida* species (n=35)**	**OD readings**		**AbA susceptibility**
		Biofilm biomass	Biofilm metabolic activity	(MIC range, µg/ml)
Predominantly yeast/pseudohyphae (n=17, 48.6%)	*C. albicans* (n= 6)	0.162 ± 0.06	0.510 ±0.15	1 - ≥ 128
	C. tropicalis (n= 7)	0.212 ± 0.07	0.705 ± 0.09	1 - ≥ 128
	C. parapsilosis (n= 4)	0.297 ± 0.07	0.909 ± 0.11	2 - ≥ 128
Average		0.224 ± 0.07	0.707 ± 0.20	
Compact with filamentous form (n=18, 51.4%)	*C. albicans* (n= 4)	0.202 ± 0.14	0.787 ± 0.32	4 - ≥ 128
	C. tropicalis (n= 10)	0.169 ± 0.04	0.728 ± 0.13	0.5 - ≥ 128
	C. parapsilosis (n= 4)	0.283 ± 0.16	0.774 ± 0.15	32 - ≥ 128
Average		0.217 ± 0.06	0.763 ± 0.03	



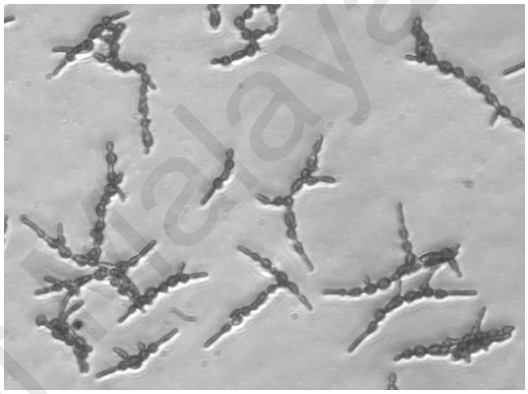
C. albicans isolate 7195



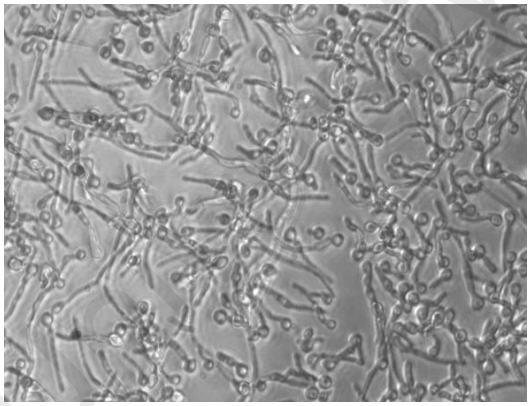
C. albicans isolate 3117



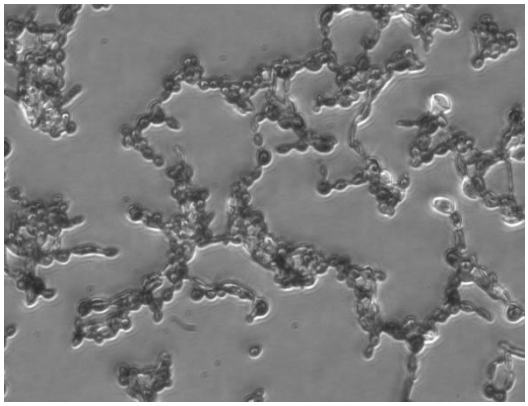
C. albicans isolate 0452



C. albicans isolate 9952

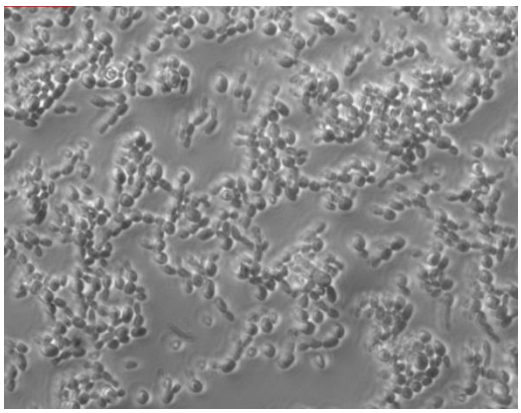


C. albicans ATCC MYA 2876 /SC 5314

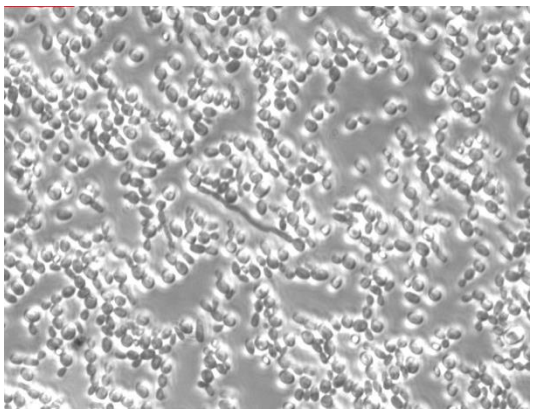


C. albicans isolate 4153

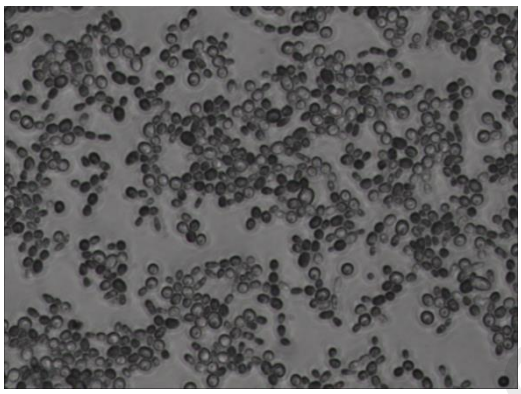
Figure 4.5: Microscopic images of *C. albicans* isolates demonstrating yeast/blastospores with pseudohyphae (Magnification: x 200)



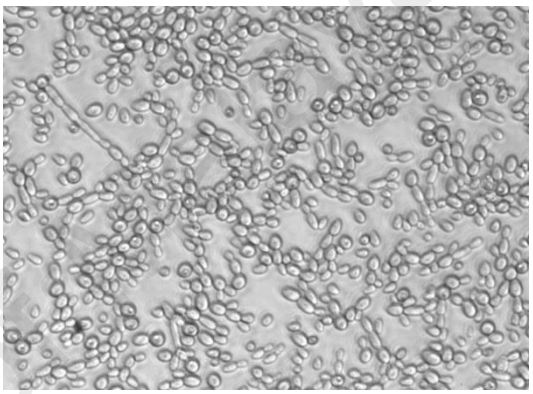
C. tropicalis isolate 3510



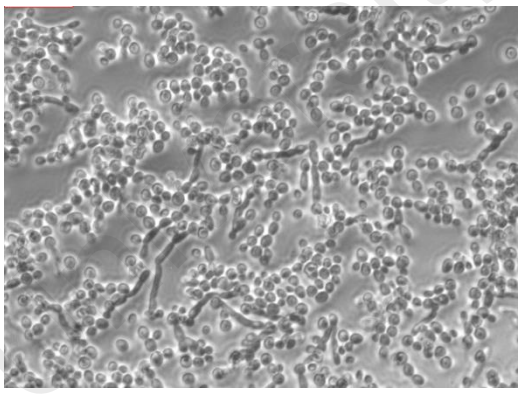
C. tropicalis isolate 3497



C. tropicalis isolate 3833

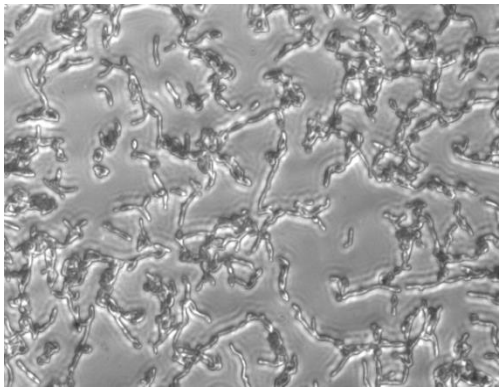


C. tropicalis isolate 3564

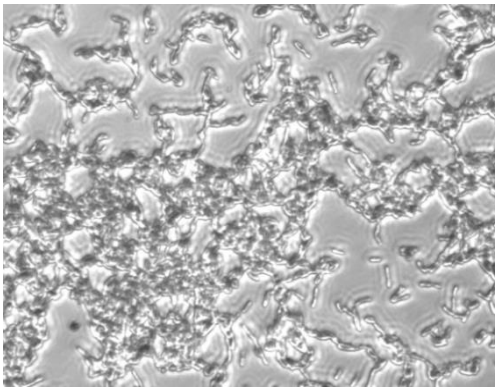


C. tropicalis isolate 3483

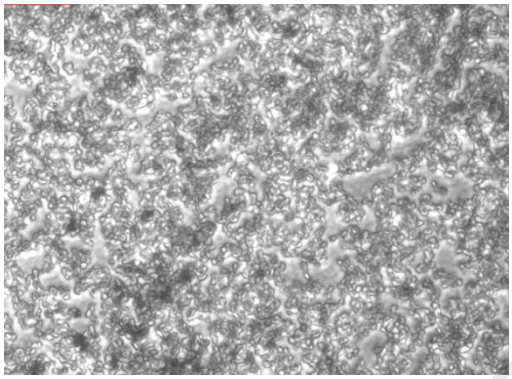
Figure 4.6: Microscopic images of *C. tropicalis* isolates demonstrating yeast/blastospores with or without pseudohyphae (Magnification: x 200).



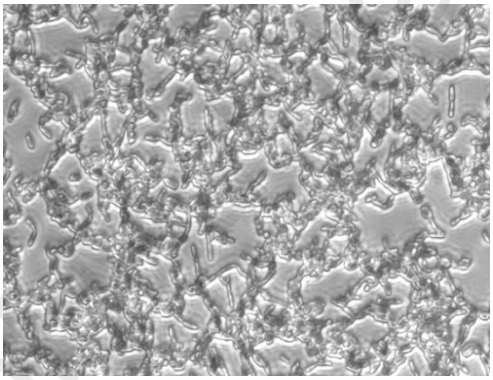
C. parapsilosis isolate 4631



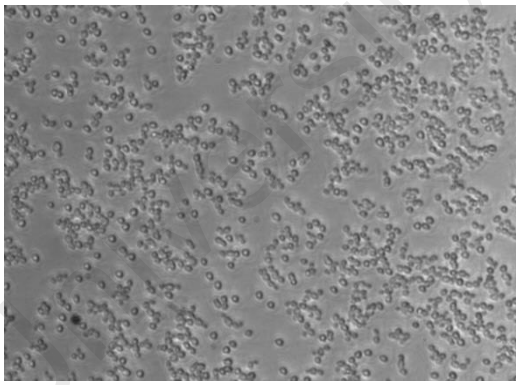
C. parapsilosis isolate 4630



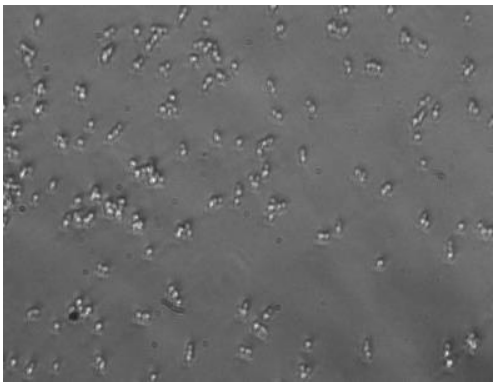
C. parapsilosis isolate 4415



C. parapsilosis isolate 1309

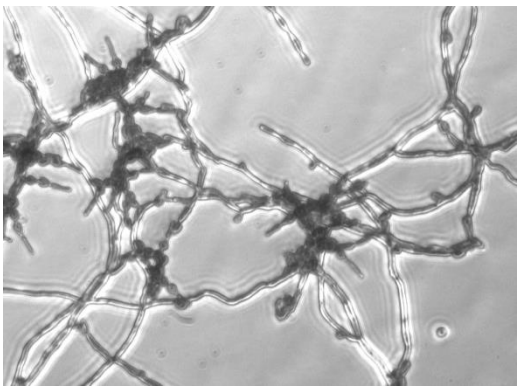


C. nivariensis isolate 139

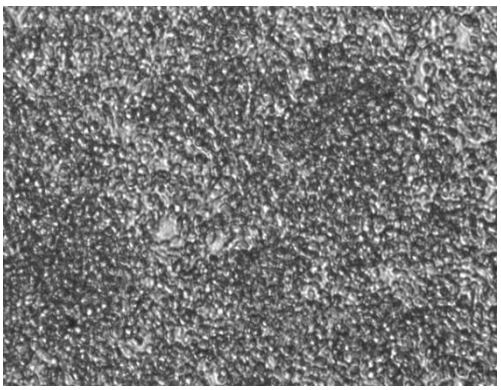


C. glabrata isolate 140

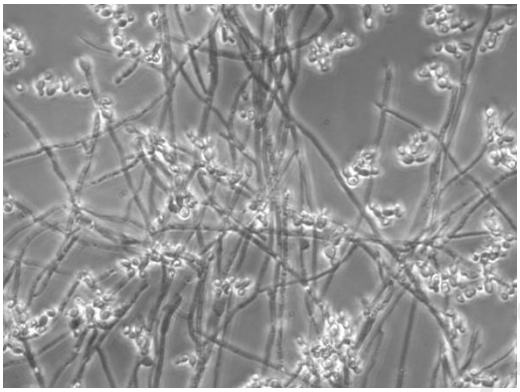
Figure 4.7: Microscopic images of *C. parapsilosis* demonstrating yeast/blastospores or pseudohyphae. *C. nivariensis* and *C. glabrata* isolates showing yeast/blastospores only (Magnification: x 200).



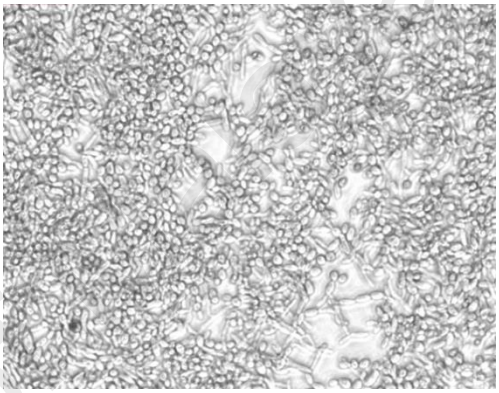
C. albicans isolate 5795



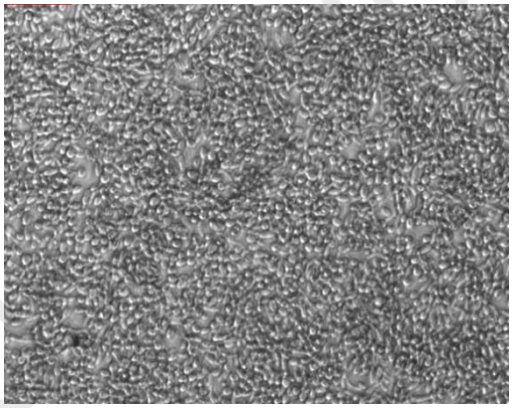
C. albicans isolate 7826-2



C. parapsilosis isolate 3184

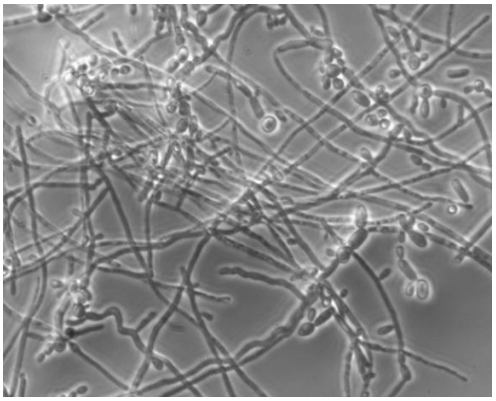


C. parapsilosis isolate 3231

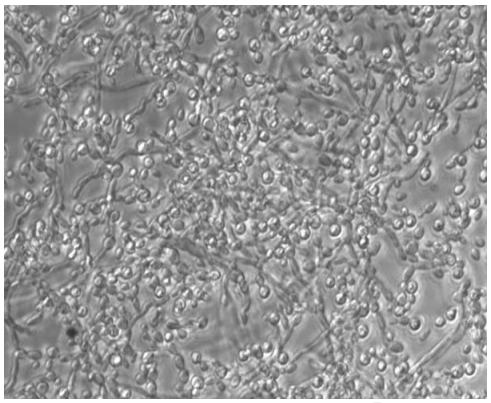


C. parapsilosis isolate 3080

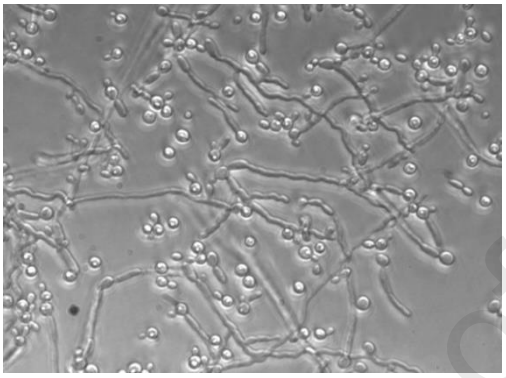
Figure 4.8: Microscopic images of *C. albicans* and *C. parapsilosis* isolates demonstrating a dense network of yeast/blastospores and hyphae (Magnification: x 200).



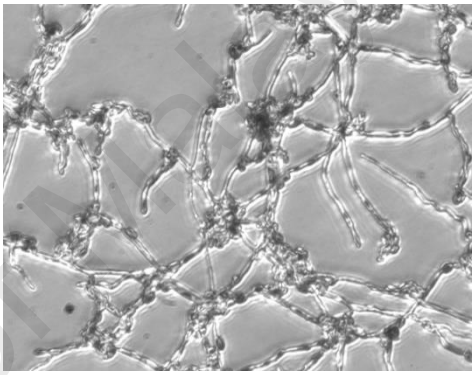
C. tropicalis isolate 3423



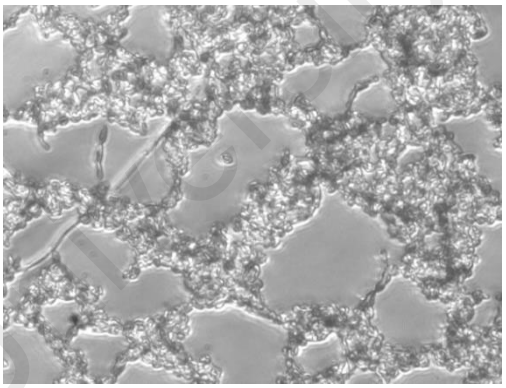
C. tropicalis isolate 3531



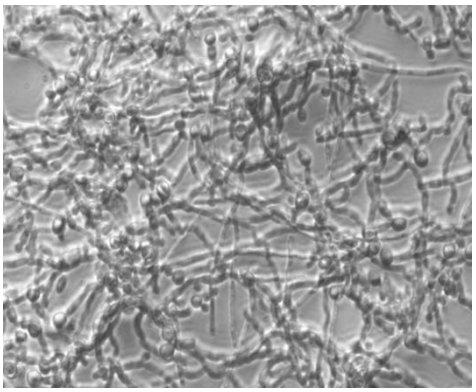
C. tropicalis isolate 3563



C. tropicalis isolate 1368



C. tropicalis isolate 1350



C. tropicalis isolate 3454

Figure 4.9: Microscopic images of *C. tropicalis* isolates demonstrating a dense network of cells and hyphae (Magnification: x 200).

4.4 Effect of species, biofilm growth morphology, biomass, and metabolic activity on AbA MIC50s

Table 4.3 shows the effect of AbA MIC50s on *Candida* species, biofilm growth morphology, biomass, and metabolic activity. The biofilm-producing *Candida* isolates were further categorized into isolates demonstrating low or high biofilm biomass or metabolic activity. *C. parapsilosis* showed the highest biofilm susceptibility (MIC50s ≥ 128 µg/ml) compared to *C. albicans* (32 µg/ml) and *C. tropicalis* (32 µg/ml). *Candida* isolates with filamentous growth, higher biomass and metabolic activity demonstrated lower AbA MIC50 readings (32 µg/ml), compared to those exhibiting yeast growth, and lower biomass and metabolic activity, ≥128 µg/ml (Table 4.3). The cut-off ODs were determined based on the ODs of the 18th isolate when the isolates were arranged according to the OD readings in ascending order.

4.5 Field emission scanning electron microscope (FESEM) analysis

FESEM analysis on planktonic cultures

Figure 4.10 shows the FESEM analysis of the planktonic cultures of *C. albicans* SC 5314 strain upon exposure to different AbA concentrations. Figure A1, A2 and A3 are images of the untreated and AbA-treated (0.25x MIC and 1x MIC) planktonic culture after 18 hours of incubation under shaking condition. The figures show the presence of mother cells and the detached daughter cells. Budding and elongation of hyphae were observed. Treatment of AbA (of any concentration) did not show major impact on the morphology or growth of mother cells.

An increase in the size of *C. albicans* SC 5314 yeast cells was observed upon exposure to increased concentration of AbA (0.25x MIC, 0.5 µg/ml and 1x MIC (2 µg/ml). To confirm this finding, the diameter of 60 yeast cells was measured and the average size of the yeast cells is shown in Table 4.4. Unpaired sample t-test revealed

Table 4.3: Effect of species, biofilm growth, morphology, biomass, and metabolic activity on AbA MIC_{50}s

***Candida* species**	**Biofilm AbA susceptibility (MIC_{50}, µg/ml)**	**Biofilm AbA susceptibility (range, µg/ml)**
***Candida* species**		
C. albicans (n=10)	32	1 - ≥ 128
C. parapsilosis (n=8)	≥ 128	2 - ≥ 128
C. tropicalis (n=17)	32	0.5 - ≥ 128
Biofilm morphology		
Predominantly yeast growth (n=17)	≥128	1 - ≥ 128
Predominantly filamentous growth (n=18)	32	0.5 - ≥ 128
Biofilm biomass		
Low (n=17)	≥ 128	0.5 - ≥ 128
High (n=18) *	32	1 - ≥ 128
Biofilm metabolic activity		
Low (n=17)	≥ 128	0.5 - ≥ 128
High (n=18) #	32	1 - ≥ 128

*OD reading >0.175; #OD reading >0.71

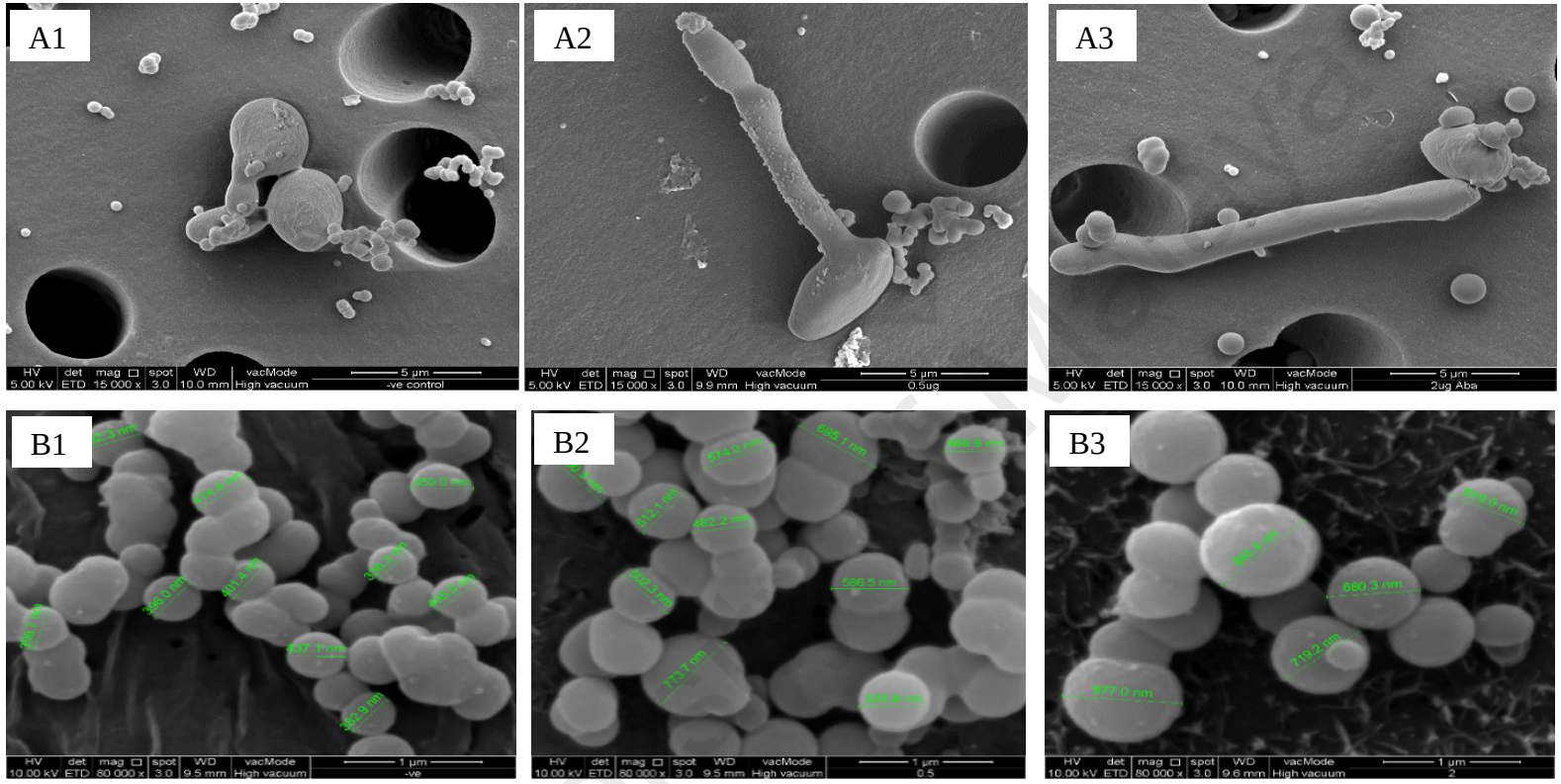


Figure 4.10: FESEM analysis of *C. albicans* SC 5314 planktonic cultures showing mother cells in A1, A2 and A3 (Magnification: x15000) and daughter cells (blastospores) in B1, B2 and B3 (Magnification: x80000). A1 and B1: Untreated planktonic culture; A2 and B2: Planktonic culture treated with 0.25x MIC (0.5 µg/ml); A3 and B3: Planktonic culture treated with 1x MIC (2 µg/ml)

Table 4.4: Diameters of yeast cells in *C. albicans* SC5314 planktonic cultures (blastospores) before and after AbA treatment

Sample group	Average diameter of yeast cell (µm) Magnification x8000	P value
Negative control	1.26 ± 0.17	Reference
AbA (0.5 µg/ml)	1.72 ± 0.31	0.054
AbA (2 µg/ml)	1.98 ± 0.44	0.014

significant difference in the diameter of the yeast cells for yeast cells treated at 1x MIC when compared to the untreated cells ($p<0.05$). Although an increase in the average diameter of the yeast cells was observed, there was no significant difference exhibited by the yeast cells treated with 0.5 µg/ml AbA (0.25x MIC) ($p>0.05$), as compared to the negative control (SD medium only) (Figure 4.11).

FESEM analysis on biofilm cultures

Figure 4.12 shows the FESEM images of *C. albicans* SC5314 biofilm cultures upon exposure to different AbA concentrations. Yeasts and filaments with well-defined and smooth surfaces were observed for untreated cells (Figure 4.12A), while AbA treated cells displayed rough and distorted cell surfaces (Figure 4.12C). In one of the images, filament detachment was observed in the biofilm cultures treated with 8 µg/ml AbA (Figure 4.12B). Only scanty yeast cells were observed in the biofilm culture exposed to 32 µg/ml AbA (Figure 4.12C). The yeast and filaments of AbA-treated cells appears to have smaller diameters compared to the untreated cells. To confirm this finding, the diameter of 10 cells and 10 filaments were measured and averaged as shown in Table 4.5. Unpaired sample t-test was used to determine if there was significant difference in the diameter of the treated and untreated fungal structures. Although a decrease in the average diameter of the filaments was observed, there was no significant difference exhibited by the filaments upon exposure to 8 and 32 µg/ml AbA (0.25x and 1x MIC) ($p>0.05$), as compared to the untreated control (Figure 4.13).

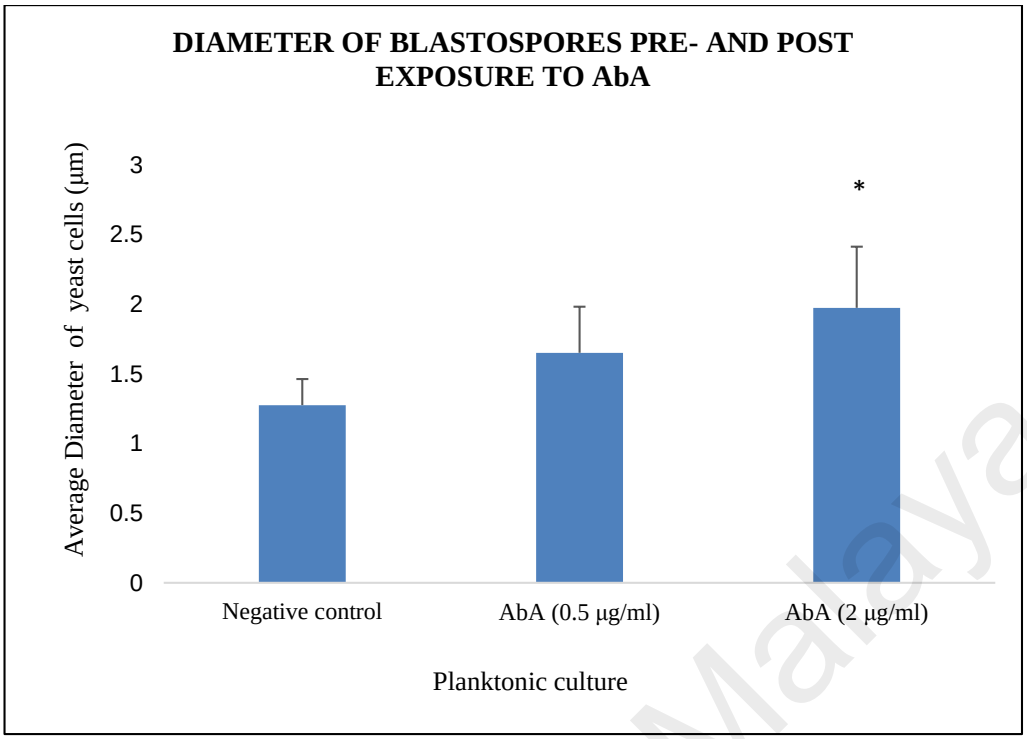


Figure 4.11: The diameter of yeast cells in planktonic cultures. The average diameter of yeast cells upon exposure to AbA. Compared to negative control (untreated), statistically significant differences in the diameter of yeast cells upon exposure to 2 µg/ml AbA was noted (* indicates significant value by unpaired sample t-test), as shown in Table 4.4.

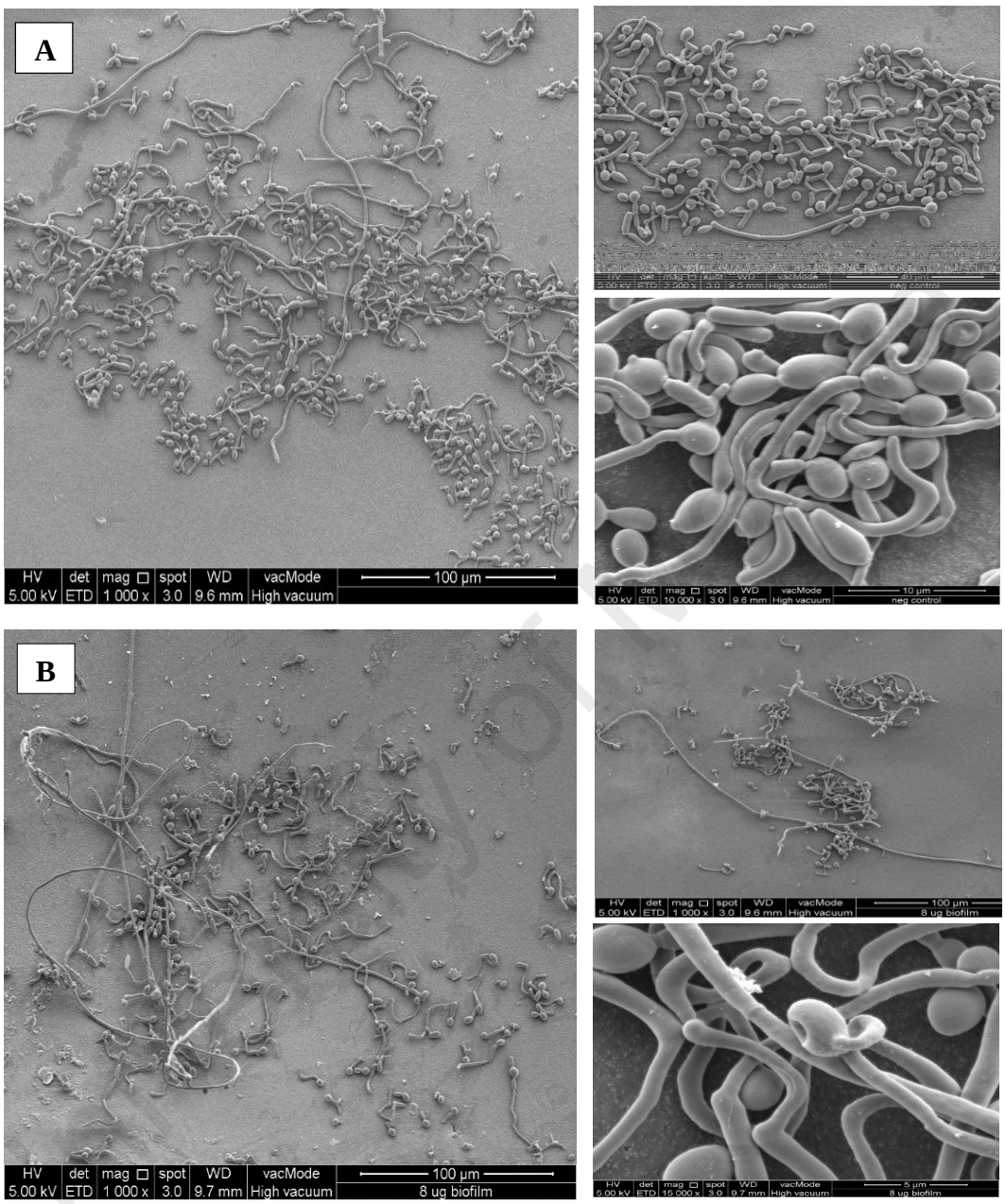


Figure 4.12: FESEM analysis of *C.albicans* SC5314 biofilm cultures; A: untreated; B: treated with 0.25x MIC (8 µg/ml) AbA; C: treated with 1x MIC (32 µg/ml) AbA.

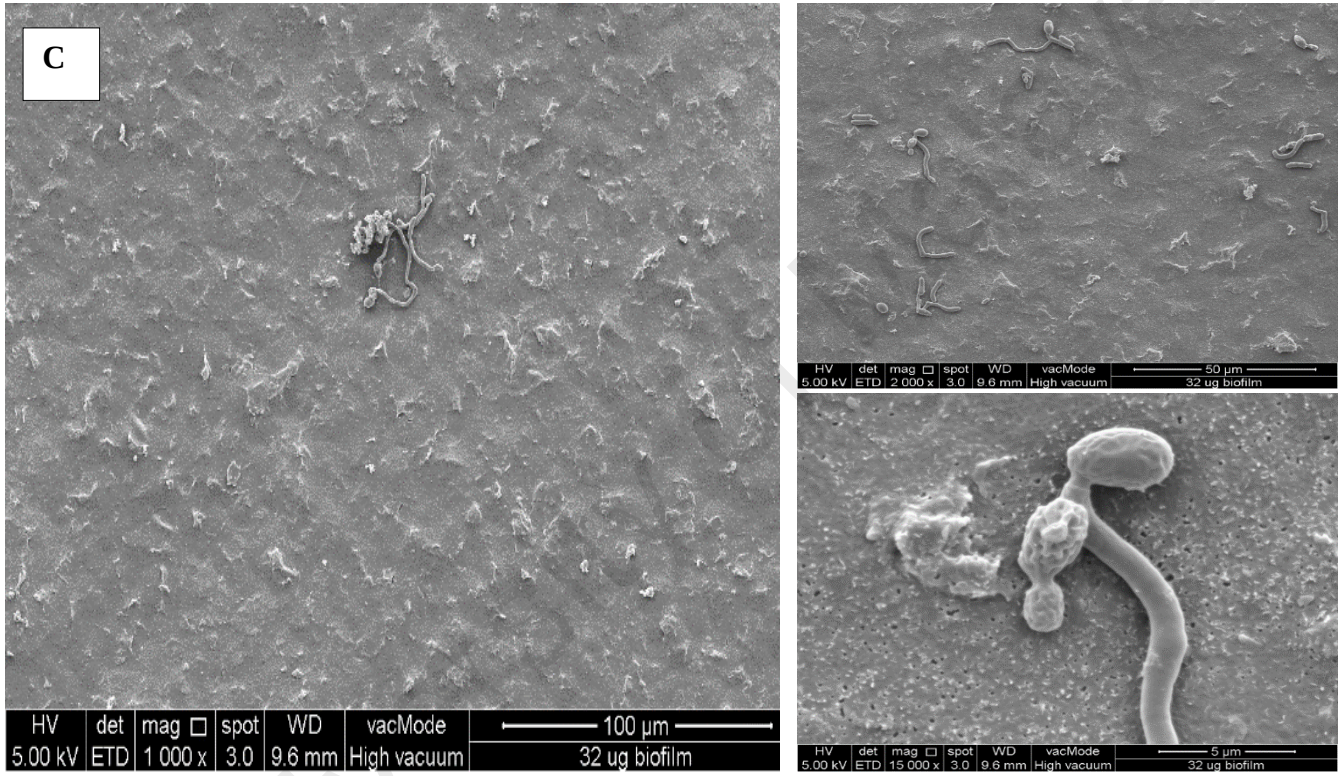


Figure 4.12: (continued)

Table 4.5: Diameter of yeast cells and filaments in biofilm cultures before and after treatment with AbA

Sample group	Average diameter of yeast cells (µm) (n=10)	P value	Average diameter of yeast filaments (µm) (n=10)	P value
Negative control (0 µg/ml)	3.22 ± 0.55		1.66 ± 0.29	
AbA (8 µg/ml)	3.0 ± 0.22	0.68	0.91 ± 0.42	0.293
Aba (32 µg/ml)	2.5 ± 0.33	0.58	1.13 ±0.25	0.940

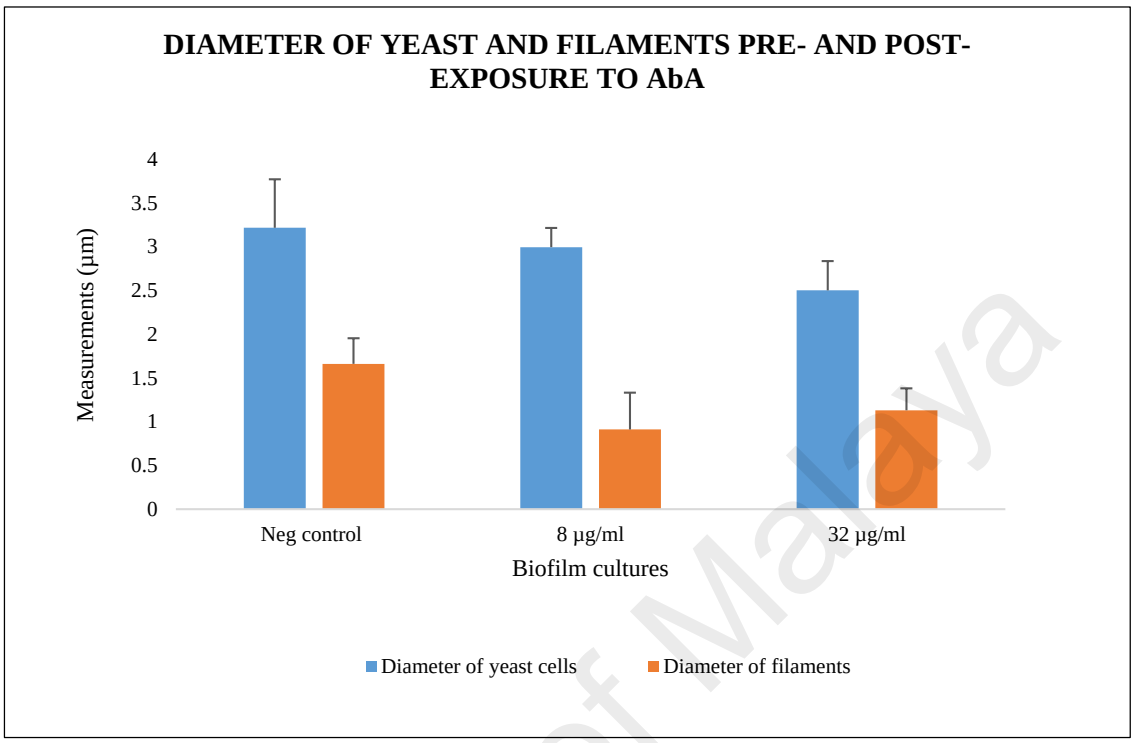


Figure 4.13: Diameter of the yeast cells and filaments in biofilm cultures. Statistically significant differences (by unpaired t-test) between the samples are not observed.

4.6 Growth curves for planktonic and biofilm cultures *C. albicans* SC5314

Figure 4.14 shows the growth curves generated for *C. albicans* SC5314 planktonic and biofilm cultures in this study. The planktonic cultures reached the mid-log growth phase between 10-18 hours and the stationary phase at around 20-24 hours. The biofilm cultures did not show a drastic increase in the growth development, as compared to the planktonic cultures. Based on the growth curve displayed in Figure 4.14, the mid-log phase of the *Candida* biofilm culture was estimated to be between 16-30 hours, and the stationary phase was estimated to be around 40-52 hours.

Growth curves of planktonic and biofilm cultures of Candida upon AbA treatment

Figure 4.15 shows the growth curves of *C. albicans* SC5314 planktonic cultures after exposure to different concentrations of AbA. In this study, planktonic cultures of *Candida* were exposed to 1x, 0.5x and 0.25x MIC (equivalent to MIC: 2 µg/ml, 1 µg/ml and 0.5 µg/ml, respectively) of AbA during the mid-log growth phase. The growth of the cultures after exposure to AbA was observed until it reached the plateau phase. The absorbance of the planktonic cultures treated at 0.5x (1 µg/ml) and 0.25x MIC (0.5 µg/ml) reduced by 17.7 and 19.5 %, respectively, as compared to the negative control at 16th hour (Figure 4.15).

C. albicans planktonic culture treated at 1x MIC of AbA exhibited very minimal reduction in the growth level (as referred by a 0.9 % reduction in OD reading) compared to those treated with 0.5x and 0.25x MICs of AbA (Figure 4.15). The presence of DMSO in the planktonic culture showed little effect on planktonic culture, with a 0.9 % reduction in the OD reading after 16th hours of incubation. Figure 4.16 shows the growth curves of *Candida* biofilm cultures upon AbA treatment. Biofilm culture of *Candida* was exposed to

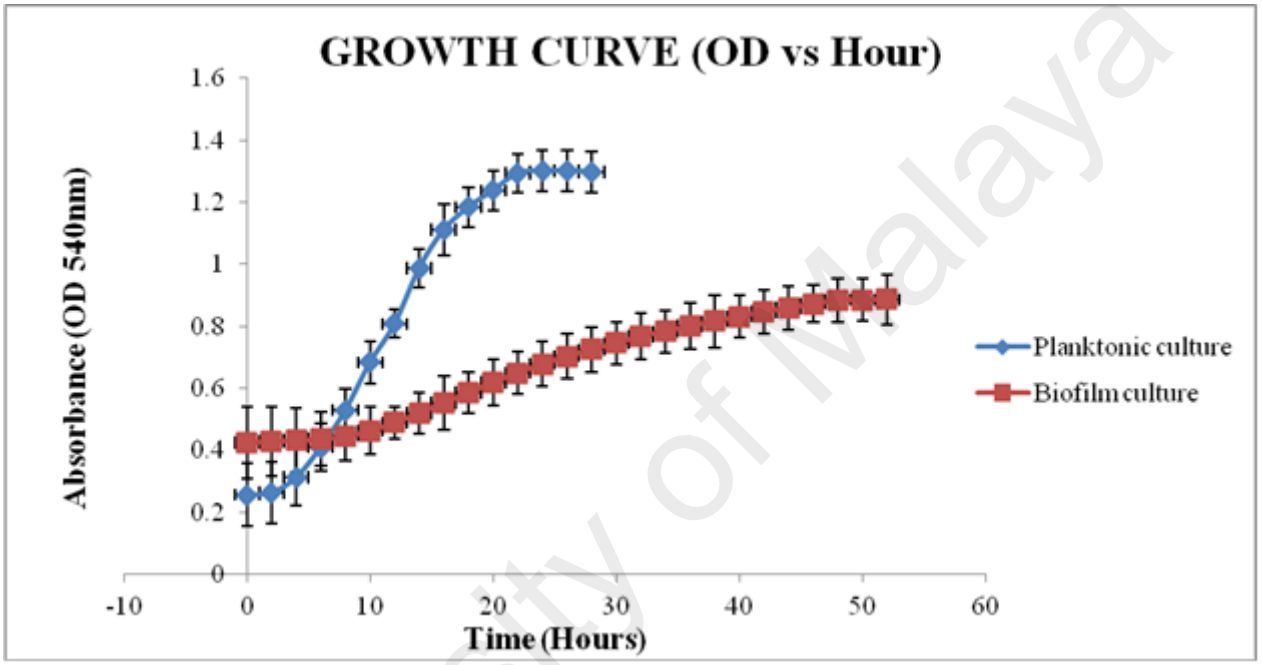


Figure 4.14: Growth curves of *C. albicans* SC5314 planktonic and biofilm cultures. The OD readings were determined using a microplate reader at 540 nm

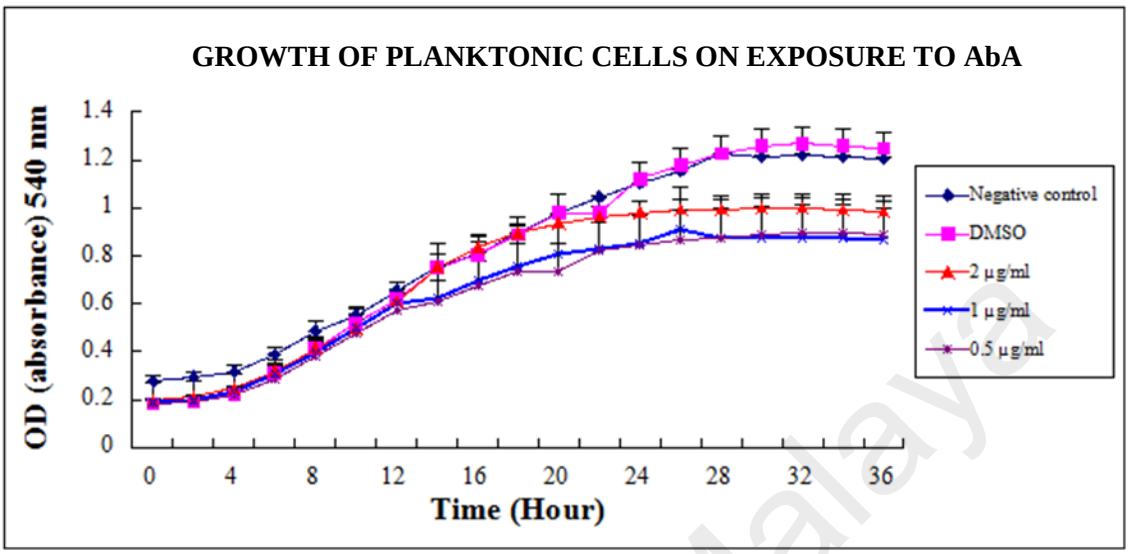


Figure 4.15: Growth curves of *C. albicans* SC5314 planktonic cultures upon exposure to different AbA concentrations.

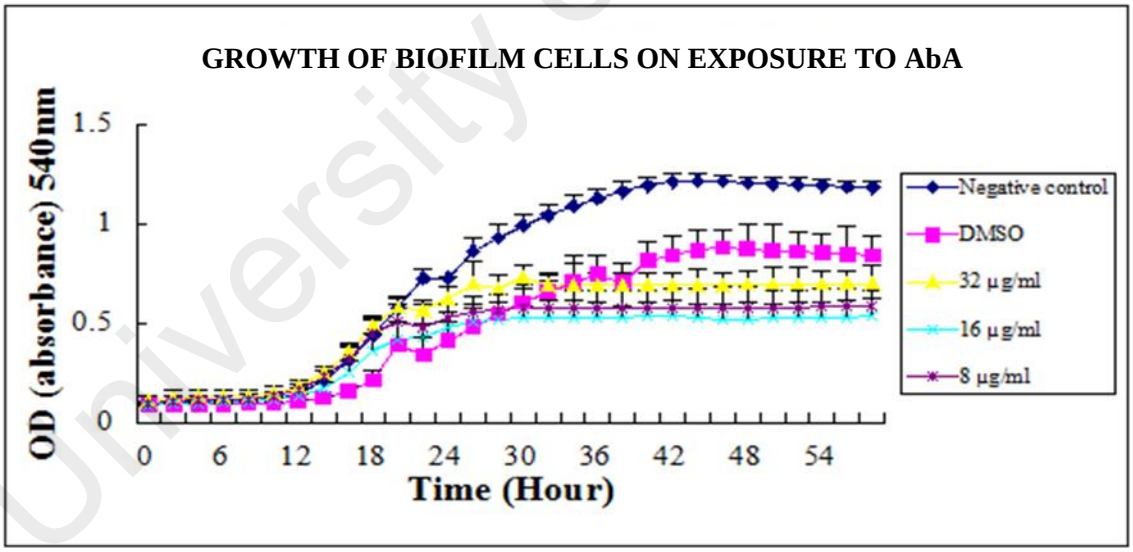


Figure 4.16: Growth curves of *C. albicans* SC5314 biofilm cultures upon exposure to different AbA concentrations.

AbA to 1x, 0.5x and 0.25x of its biofilm MIC (equivalent to MIC: 32 µg/ml, 16 µg/ml and 8 µg/ml, respectively) after the mid-log phase. Biofilm cultures responded differently when exposed to DMSO. A reduction in the absorbance (approximately 50%) was seen in DMSO-treated biofilm culture compared to untreated culture at the 20th hour (Figure 4.4). Similar to planktonic cultures, biofilm cultures treated with a high AbA concentration (32 µg/ml; 1x MIC) demonstrated lower level of growth reduction (22.5%) compared to biofilm cultures treated at 0.5 (41.3%) and 0.25x MIC (34.9%) (Figure 4.16).

4.7 Effect of AbA on the planktonic and biofilm cultures (survival assay)

A survival assay was conducted to demonstrate the effect of AbA on the planktonic and biofilm cultures by determining the viable cell counts of the yeast cells after exposure to 1 µg/ml AbA for 2.5 hours. The results showed that the colony counts for AbA-treated planktonic and biofilm culture fell below the untreated ones throughout the experiment. However, the colony counts for AbA-treated planktonic culture increased after the 8th hour of exposure whereas for the biofilm cultures, the colony counts of treated biofilm culture increased after the 6th hour of exposure (Figure 4.17).

4.8 Quantitation of protein extracted from planktonic and biofilm cultures for liquid chromatography mass spectrometry (LCMS) analysis

The concentrations of the protein extract of planktonic and biofilm cultures (from 20 ml of cultures obtained from treated and untreated planktonic and biofilm cultures) are shown in Table 4.6.

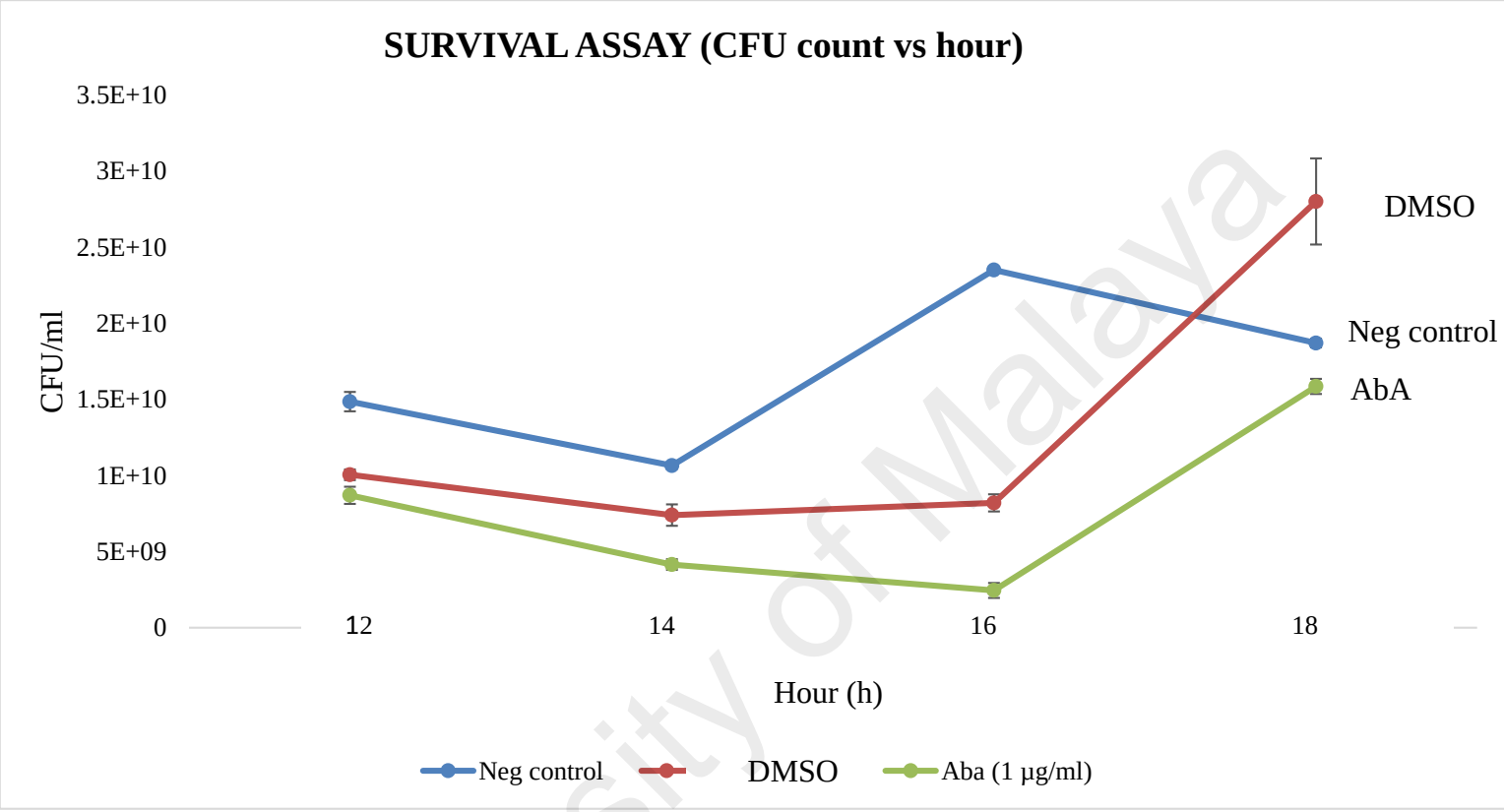


Figure 4.17: Colony counts of *C. albicans* planktonic cultures before (12th hour) and after exposure (14 -18th) to AbA (1 µg/ml=0.5x MIC). The AbA exposure was performed during the mid-log phase after 12 hours of incubation of the planktonic culture.

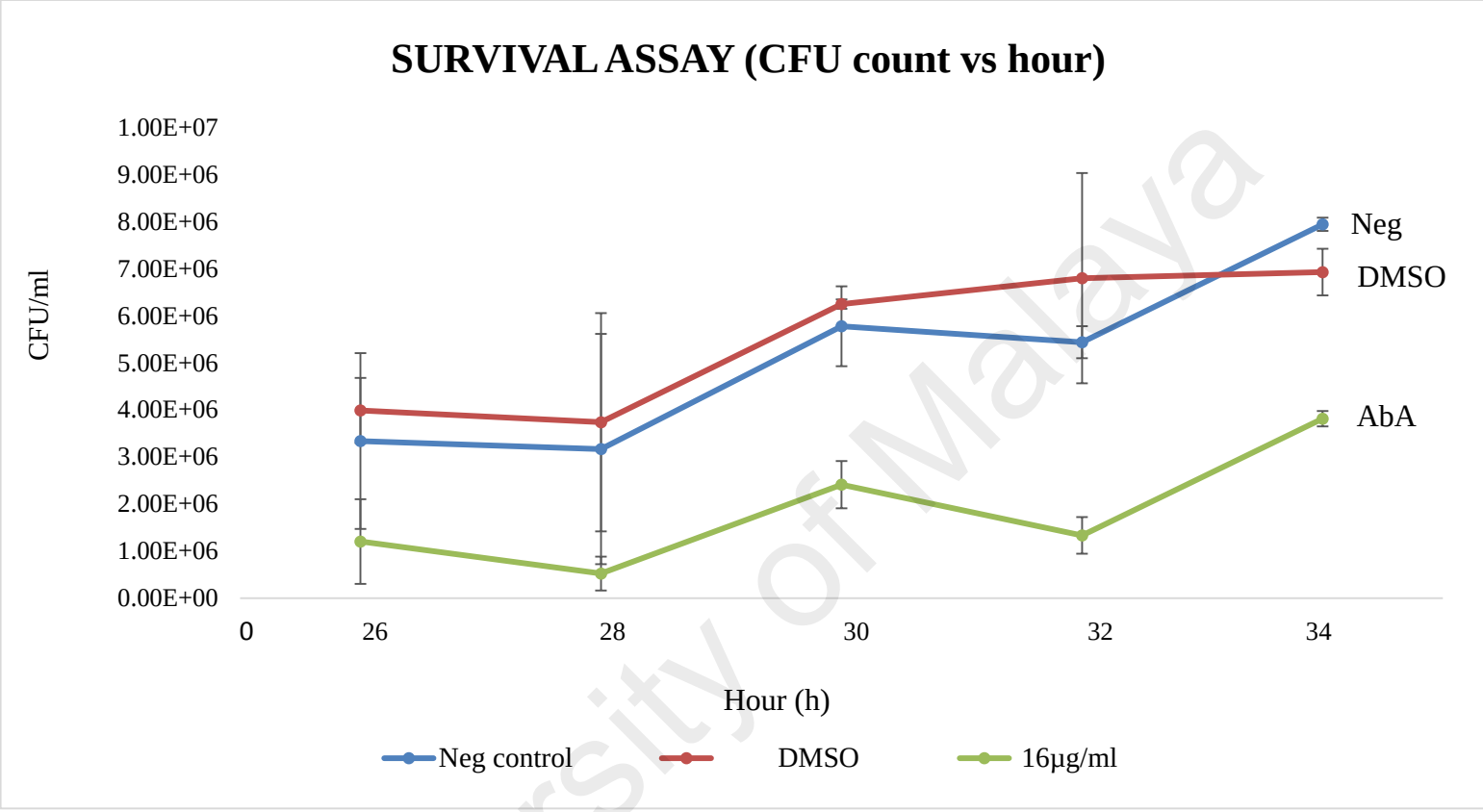


Figure 4.18: Colony counts of *C. albicans* biofilm cultures before (24th hour) and after exposure (26th -34th) to AbA (16 µg/ml=0.5x MIC). The AbA exposure was performed during the mid-log phase after 24th of incubation of the biofilm culture.

Table 4.6: Protein concentrations of planktonic and biofilm cultures

Protein Samples	Average concentration (mg/ml)
Planktonic Culture	
Negative control	1.062
DMSO	0.873
AbA (1µg/ml)	0.960
AmpB (0.5µg/ml)	0.866
Biofilm culture	
Negative control	1.316
DMSO	1.265
AbA (1µg/ml)	1.466

4.9 SDS-PAGE analysis of proteins extracts obtained from planktonic and biofilm cultures

Figure 4.19 shows the separation of the protein extracts obtained from *C. albicans* SC5314 planktonic and biofilm cultures using SDS-PAGE analysis. The amount of protein samples loaded per lane was 20 µg. Only minimal difference was observed in the expression of planktonic and biofilm proteins. The major difference was noted for protein fragments between 15 to 75 kDa of the planktonic and biofilm cultures, as indicated by arrows.

4.10 LCMS/MS analysis

4.10.1 Analysis of protein profiles of planktonic and biofilm cultures pre- and post-treatment to AbA

Protein expression of *C. albicans* SC5314 planktonic and biofilm cultures obtained from the LCMS/MS analysis was analysed using the selection criteria of at least one unique peptide match for protein identification, significance (-10lgP) value of >20 for protein identification and false discovery rate of ≥1%. A total of 533 and 203 proteins, respectively, were demonstrated in this study (Table 4.7). Following treatment with DMSO or AbA, planktonic cultures demonstrated an increase from 203 to 344 proteins when treated with DMSO and from 203 to 359 with AbA (Table 4.7). In contrast, biofilm cultures demonstrated a reduction from 533 to 465 on treatment with DMSO and from 533 to 464 with AbA.

Upon treatment with 1 µg/ml of AbA, *C. albicans* planktonic culture was found to have higher increase in total number of annotated proteins (by 4.4 %) as compared to,DMSO-treated planktonic culture. In contrast, biofilm culture showed a slight reduction

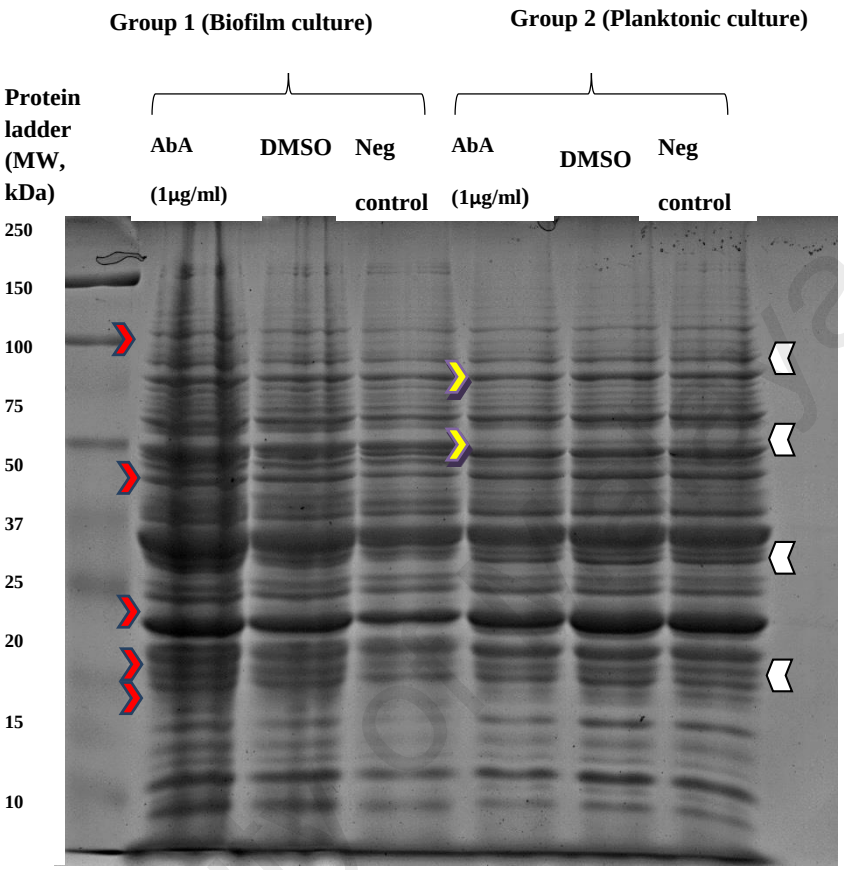


Figure 4.19: SDS-PAGE analysis of protein samples obtained from planktonic and biofilm cultures of *C. albicans* SC5314 strain. (Red arrow: Indicates difference within the biofilm samples; white arrow: indicates the difference within the planktonic samples; yellow arrows: indicates the difference between the cultures).

Table 4.7: Total proteins detected/annotated from different cultures of *C. albicans* SC5314

Total proteins detected		
Samples	**Planktonic**	**Biofilm**
Negative control	203	533
DMSO	344 (100%)	465 (100%)
Aba (1 µg/ml)	359 (+4.4%)	464 (-0.2%)
Total proteins annonated		
Negative control	163	414
DMSO	276	368
Aba (1 µg/ml)	293	373

in the total number of protein annotated (by 0.2%) when treated with AbA as compared to DMSO-treated biofilm culture.

4.10.2 LC-MS qualitative analysis of biofilm and planktonic proteins (untreated)

The GO Slim Terms such as molecular function, biological process, and subcellular localization of each protein were determined using the GO Slim Mapper of the online *Candida* Genome Database (CGD, http://www.*Candida*genome.org/). The various biological processes, molecular functions and subcellular localizations that are associated to common and unique proteins of *Candida* biofilm culture were presented in comparison to the planktonic culture.

Biological processes

A total of 414 proteins were annotated from *C. albicans* biofilm culture for biological processes, molecular functions and subcellular localization, whereas 163 proteins were annotated from the planktonic culture. The number and percentage distribution of different proteins identified in the biological process of the biofilm and planktonic cultures of *C. albicans* SC5314 are presented in Table 4.8. A total of 40 GO Terms were identified for biological processes of both planktonic and biofilm cultures. The top five biological process identified in both planktonic and biofilm cultures were associated with regulation of biological process, response to stress, organelle organization, transport and response to chemical (Table 4.8). Many more proteins were annotated for the biofilm culture as compared to the planktonic culture, for instance, higher number of proteins were associated with the regulation of biological processes (n=65), organelle organization (n=57), transport (n=55), stress response (n=47), filamentous growth (n=30), chemical response (n=30), lipid

Table 4.8: Comparison of biological processes of *Candida* cultures using the GO Slim Mapper maps annotations.

No.	GoID	Go Term	Planktonic culture (untreated), n=163		Biofilm culture (untreated), n=414		Difference (%) (x-y)
			No. protein	% of protein (x)	No. protein	% of protein (y)	
1	50789	regulation of biological process	50	30.7%	115	27.8%	2.90%
2	6950	response to stress	38	23.3%	85	20.5%	2.80%
3	6996	organelle organization	35	21.5%	92	22.2%	-0.70%
4	6810	Transport	29	17.8%	84	20.3%	-2.50%
5	42221	response to chemical	28	17.2%	58	14.0%	3.20%*
6	16070	RNA metabolic process	26	16%	49	11.8%	4.20%*
7	7049	cell cycle	22	13.5%	45	10.9%	2.60%
8	30447	filamentous growth	21	12.9%	51	12.3%	0.60%
9	5975	carbohydrate metabolic process	19	11.7%	39	9.4%	2.30%
10	8150	biological_process	18	11.0%	64	15.5%	-4.50%*
11	Other	Other	15	9.2%	38	10.2%	-1.00%
12	64 64	cellular protein modification process	14	8.6%	41	9.9%	-1.30%
13	42254	ribosome biogenesis	13	8%	20	4.8%	3.20%*
14	6259	DNA metabolic process	12	7.4%	40	9.7%	-2.30%
15	44419	interspecies interaction between organisms	10	6.1%	20	4.8%	1.30%
16	6091	generation of precursor metabolites and energy	9	5.5%	17	4.1%	1.40%
17	7010	cytoskeleton organization	9	5.5%	15	3.6%	1.90%
18	7165	signal transduction	9	5.5%	17	4.1%	1.40%
19	42493	response to drug	9	5.5%	24	5.8%	-0.30%
20	746	Conjugation	8	4.9%	12	2.9%	2.00%
21	6629	lipid metabolic process	8	4.9%	31	7.5%	-2.60%
22	48468	cell development	8	4.9%	9	2.2%	2.70%
23	9405	Pathogenesis	7	4.3%	25	6.0%	-1.70%
24	71555	cell wall organization	7	4.3%	10	2.4%	1.90%
25	6457	protein folding	6	3.7%	12	2.9%	0.80%
26	6412	Translation	5	3.1%	18	4.3%	-1.20%
27	16192	vesicle-mediated transport	5	3.1%	28	6.8%	-3.70%*
28	30448	hyphal growth	5	3.1%	10	2.4%	0.70%
29	42710	biofilm formation	5	3.1%	12	2.9%	0.20%

Table 4.8, continued

30	910	Cytokinesis	4	2.5%	9	2.2%	0.30%
31	6997	nucleus organization	4	2.5%	7	1.7%	0.80%
32	7114	cell budding	3	1.8%	6	1.4%	0.40%
33	70783	growth of unicellular organism as a thread of attached cells	3	1.8%	6	1.4%	0.40%
34	7124	pseudohyphal growth	2	1.2%	4	1%	0.20%
35	19725	cellular homeostasis	2	1.2%	16	3.9%	-2.70%
36	30163	protein catabolic process	2	1.2%	12	2.9%	-1.70%
37	45333	cellular respiration	2	1.2%	6	1.4%	-0.20%
38	6766	vitamin metabolic process	1	0.6%	0	0%	0.60%
39	7155	cell adhesion	1	0.6%	5	1.2%	-0.60%
40	32196	Transposition	0	0%	1	0.2%	-0.20%

Footnote: (*) / boxed row indicating ≥ 3 difference, difference of the percentages of protein (x and y) were generated from the search in the CGD.

metabolism (n=23) and carbohydrate metabolism (n=20) in the biofilm culture (Table 4.8).

Higher (greater than 3%) percentages of proteins were annotated for response to chemical (3.2%), RNA metabolic process (4.2%) and ribosome biogenesis (3.2%) in planktonic culture. On the other hand, higher (greater than 3%) percentages of proteins annotated for biological process (4.5%) and vesicle-mediated transport (3.7%) are noted for biofilm culture (Table 4.8).

Cellular components

The number and percentage distribution of proteins annotated for various cellular components in biofilm and planktonic cultures of *C. albicans* SC5314 are shown in Table 4.9. Classification of proteins based on subcellular localization did not show much difference between the two cultures with the identification of only 26 Go Term. A total of 68.7 and 66.4% proteins annotated from planktonic and biofilm cultures were predicted to be localized in cytoplasm, respectively (Table 4.9). The top five cellular components annotated for the planktonic and biofilm cultures include cytoplasm, nucleus, membrane, mitochondrion and plasma membrane. Greater difference in the percentage of proteins (>3%) were noted for several cellular components in the planktonic culture, including nucleus (5.6%), membrane (3.2%), plasma membrane (4.1%) and cell wall (6.1%). As for the biofilm culture, higher percentages of proteins were annotated for endoplasmic reticulum (3.3%) and endomembrane system (7.3%), as compared to the planktonic culture.

Molecular functions

The top five molecular functions with the highest number of proteins annotated for the planktonic and biofilm cultures are hydrolase activity, transferase activity, protein binding, oxidoreductase activity and DNA binding activity. There were no differences

Table 4.9: Comparison of cellular components of *Candida* cultures using the GO Slim Mapper maps annotations.

No.	GoID	Go Term	Planktonic culture (untreated), n=163		Biofilm culture (untreated), n=414		Difference (%) (x-y)
			No. proteins	% of proteins (x)	No. proteins	% of proteins (y)	
1	5737	Cytoplasm	112	68.7%	275	66.4%	2.3
2	5634	Nucleus	69	42.3%	152	36.7%	5.6*
3	16020	Membrane	50	30.7%	114	27.5%	3.2*
4	5739	Mitochondrion	35	21.5%	93	22.5%	-1
5	5886	plasma membrane	30	18.4%	59	14.3%	4.1*
6	5618	cell wall	25	15.3%	38	9.2%	6.1*
7	5575	cellular_component	23	14.1%	65	15.7%	-1.6
8	5773	Vacuole	15	9.2%	26	6.3%	2.9
9	12505	endomembrane system	14	8.6%	66	15.9%	-7.3*
10	5576	extracellular region	11	6.7%	21	5.1%	1.6
11	5694	Chromosome	11	6.7%	40	9.7%	-3
12	5730	Nucleolus	11	6.7%	17	4.1%	2.6
13	5794	Golgi apparatus	9	5.5%	26	6.3%	-0.8
14	5856	Cytoskeleton	9	5.5%	17	4.1%	1.4
15	30427	site of polarized growth	9	5.5%	22	5.3%	0.2
16	5740	mitochondrial envelope	7	4.3%	22	5.3%	-1
17	5840	Ribosome	7	4.3%	17	4.1%	0.2
18	5938	cell cortex	7	4.3%	16	3.9%	0.4
19	5777	Peroxisome	6	3.7%	15	3.6%	0.1
20	5815	microtubule organizing center	5	3.1%	7	1.7%	1.4
21	5933	cellular bud	5	3.1%	20	4.8%	-1.7
22	5783	endoplasmic reticulum	4	2.5%	24	5.8%	-3.3*
23	16023	cytoplasmic, membrane-bounded vesicle	3	1.8%	6	1.4%	0.4
24	1411	hyphal tip	2	1.2%	6	1.4%	-0.2

Footnote: (*) / boxed row indicating ≥ 3% difference, difference of the percentages of protein (x and y) were generated from the search in the CGD.

observed between the two cultures (Table 4.10) in terms of the total number of proteins classified by molecular function, except that a higher percentage (>3%) of proteins was annotated for the lyase activity (GOID:16829) of the planktonic culture as compared to biofilm culture.

4.10.3 Proteins associated with the regulation of biological processes for planktonic and biofilm cultures

The common GO terms which are significant in both cultures were identified using the GO Term Finder in the CGB database. This is also aimed to discover the proteins that were in common. The GO terms search was performed using a default settings and hits with p-value of ≤ 0.1. A sum of 208 and 146 GO Terms were associated to the biofilm and planktonic cultures, respectively (Figure 4.20). A total of 105 GO Terms were common to both cultures, while, 41 were specific to the planktonic culture and 103 to the biofilm culture (Figure 4.20).

Of the 41 GO Terms specifically detected in the planktonic culture, majority were associated with metabolic process. A total of 21 (42%) of the proteins were associated with nucleobase-containing compound metabolic process, followed by growth (n=14, 28.0%), single organism reproductive process (n=9, 18.0%), establishment or maintenance of cell polarity (n=8, 16.0%) and sexual reproduction (n=8, 16.0%).

GO terms (n=103) that are specifically associated with the biofilm culture are shown in Table 4.12. Most of the GO Terms were related to regulation of biological process. Majority of the proteins were associated with the regulation of biosynthetic process (n=62, 53.9%), regulation of macromolecule biosynthetic process (n=59, 51.3%), followed by regulation of gene expression (n=54, 47%), regulation of nucleobase-containing compound metabolic process (n=52, 45.2%), regulation of RNA metabolic process (n=46, 40.0%),

Table 4.10: Comparison of molecular functions of *Candida* cultures using the GO Slim Mapper maps annotations.

No.	GoID	Go Term	Planktonic culture (untreated), n=163		Biofilm culture (untreated), n=414		Difference (%) (x-y)
			No. protein	% of protein (x)	No. protein	% of protein (y)	
1	16787	hydrolase activity	37	22.7%	88	21.3%	1.4
2	3674	molecular_function	33	20.2%	81	19.6%	0.6
3	16740	transferase activity	24	14.7%	67	16.2%	-1.5
4	5515	protein binding	22	13.5%	58	14.0%	-0.5
5	16491	oxidoreductase activity	18	11.0%	41	9.9%	1.1
6	3677	DNA binding	14	8.6%	43	10.4%	-1.8
7	3723	RNA binding	14	8.6%	38	9.2%	-0.6
8	5215	transporter activity	10	6.1%	26	6.3%	-0.2
9	16829	lyase activity	10	6.1%	12	2.9%	3.2*
10	4386	helicase activity	7	4.3%	17	4.1%	0.2
11	5198	structural molecule activity	7	4.3%	14	3.4%	0.9
12	other	Other	7	4.3%	24	5.8%	-1.5
13	30234	enzyme regulator activity	6	3.7%	18	4.3%	-0.6
14	988	transcription factor activity, protein binding	5	3.1%	3	0.7%	2.4
15	16853	isomerase activity	5	3.1%	10	2.4%	0.7
16	4672	protein kinase activity	4	2.5%	8	1.9%	0.6
17	8233	peptidase activity	4	2.5%	12	2.9%	-0.4
18	8289	lipid binding	4	2.5%	10	2.4%	0.1
19	16874	ligase activity	3	1.8%	6	1.4%	0.4
20	3774	motor activity	2	1.2%	3	0.7%	0.5
21	4871	signal transducer activity	1	0.6%	5	1.2%	-0.6
22	16779	nucleotidyltransferase activity	1	0.6%	7	1.7%	-1.1
23	16791	phosphatase activity	1	0.6%	3	0.7%	-0.1
24	not_yet_annotated	not_yet_annotated	1	0.6%	3	0.7%	-0.1

Footnote: (*) / boxed row indicating ≥ 3%difference, difference of the percentages of protein (x and y) were generated from the search in the CGD.

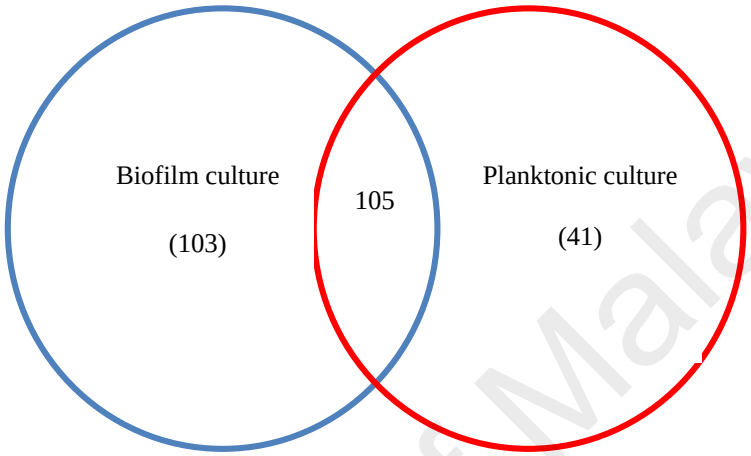


Figure 4.20: GO Terms associated with regulation of biological process of biofilm and planktonic cultures.

Table 4.11: GO terms (n=41) associated specifically with planktonic culture.

No.	GOID	Go Term	Cluster frequency
1	65007	biological regulation	50 out of 50 genes, 100.0%
2	6139	nucleobase-containing compound metabolic process	21 out of 50 genes, 42.0%
3	40007	Growth	14 out of 50 genes, 28.0%
4	44702	single organism reproductive process	9 out of 50 genes, 18.0%
5	7163	establishment or maintenance of cell polarity	8 out of 50 genes, 16.0%
6	19953	sexual reproduction	8 out of 50 genes, 16.0%
7	32787	monocarboxylic acid metabolic process	7 out of 50 genes, 14.0%
8	72521	purine-containing compound metabolic process	7 out of 50 genes, 14.0%
9	44723	single-organism carbohydrate metabolic process	7 out of 50 genes, 14.0%
10	46034	ATP metabolic process	6 out of 50 genes, 12.0%
11	45930	negative regulation of mitotic cell cycle	6 out of 50 genes, 12.0%
12	9123	nucleoside monophosphate metabolic process	6 out of 50 genes, 12.0%
13	9141	nucleoside triphosphate metabolic process	6 out of 50 genes, 12.0%
14	6733	oxidoreduction coenzyme metabolic process	6 out of 50 genes, 12.0%
15	44093	positive regulation of molecular function	6 out of 50 genes, 12.0%
16	9126	purine nucleoside monophosphate metabolic process	6 out of 50 genes, 12.0%
17	9144	purine nucleoside triphosphate metabolic process	6 out of 50 genes, 12.0%
18	6163	purine nucleotide metabolic process	6 out of 50 genes, 12.0%
19	9167	purine ribonucleoside monophosphate metabolic process	6 out of 50 genes, 12.0%
20	9205	purine ribonucleoside triphosphate metabolic process	6 out of 50 genes, 12.0%
21	9150	purine ribonucleotide metabolic process	6 out of 50 genes, 12.0%
22	1901987	regulation of cell cycle phase transition	6 out of 50 genes, 12.0%
23	1901990	regulation of mitotic cell cycle phase transition	6 out of 50 genes, 12.0%
24	9161	ribonucleoside monophosphate metabolic process	6 out of 50 genes, 12.0%
25	9199	ribonucleoside triphosphate metabolic process	6 out of 50 genes, 12.0%
26	9259	ribonucleotide metabolic process	6 out of 50 genes, 12.0%
27	22603	regulation of anatomical structure morphogenesis	5 out of 50 genes, 10.0%
28	46578	regulation of Ras protein signal transduction	4 out of 50 genes, 8.0%
29	6094	Gluconeogenesis	3 out of 50 genes, 6.0%
30	19319	hexose biosynthetic process	3 out of 50 genes, 6.0%
31	46785	microtubule polymerization	3 out of 50 genes, 6.0%
32	46364	monosaccharide biosynthetic process	3 out of 50 genes, 6.0%
33	61621	canonical glycolysis	2 out of 50 genes, 4.0%
34	71266	'de novo' L-methionine biosynthetic process	2 out of 50 genes, 4.0%
35	6007	glucose catabolic process	2 out of 50 genes, 4.0%
36	61718	glucose catabolic process to pyruvate	2 out of 50 genes, 4.0%
37	61615	glycolytic process through fructose-6-phosphate	2 out of 50 genes, 4.0%
38	61620	glycolytic process through glucose-6-phosphate	2 out of 50 genes, 4.0%
39	19280	L-methionine biosynthetic process from homoserine via O-acetyl-L-homoserine and cystathionine	2 out of 50 genes, 4.0%
40	19279	L-methionine biosynthetic process from L-homoserine via cystathionine	2 out of 50 genes, 4.0%
41	6735	NADH regeneration	2 out of 50 genes, 4.0%

Table 4.12: GO terms (n=103) associated specifically to the biofilm culture.

No.	GOID	Go Term	Cluster frequency
1	65007	biological regulation	115 out of 115 genes, 100.0%
2	9889	regulation of biosynthetic process	62 out of 115 genes, 53.9%
3	31326	regulation of cellular biosynthetic process	62 out of 115 genes, 53.9%
4	10556	regulation of macromolecule biosynthetic process	59 out of 115 genes, 51.3%
5	2000112	regulation of cellular macromolecule biosynthetic process	57 out of 115 genes, 49.6%
6	10468	regulation of gene expression	54 out of 115 genes, 47.0%
7	19219	regulation of nucleobase-containing compound metabolic process	52 out of 115 genes, 45.2%
8	48522	positive regulation of cellular process	46 out of 115 genes, 40.0%
9	51252	regulation of RNA metabolic process	46 out of 115 genes, 40.0%
10	1903506	regulation of nucleic acid-templated transcription	43 out of 115 genes, 37.4%
11	2001141	regulation of RNA biosynthetic process	43 out of 115 genes, 37.4%
12	6355	regulation of transcription, DNA-templated	43 out of 115 genes, 37.4%
13	51179	Localization	41 out of 115 genes, 35.7%
14	31325	positive regulation of cellular metabolic process	38 out of 115 genes, 33.0%
15	9893	positive regulation of metabolic process	38 out of 115 genes, 33.0%
16	10604	positive regulation of macromolecule metabolic process	35 out of 115 genes, 30.4%
17	40007	Growth	33 out of 115 genes, 28.7%
18	51641	cellular localization	30 out of 115 genes, 26.1%
19	51173	positive regulation of nitrogen compound metabolic process	30 out of 115 genes, 26.1%
20	9891	positive regulation of biosynthetic process	29 out of 115 genes, 25.2%
21	31328	positive regulation of cellular biosynthetic process	29 out of 115 genes, 25.2%
22	42221	response to chemical	29 out of 115 genes, 25.2%
23	22607	cellular component assembly	28 out of 115 genes, 24.3%
24	33036	macromolecule localization	28 out of 115 genes, 24.3%
25	10557	positive regulation of macromolecule biosynthetic process	28 out of 115 genes, 24.3%
26	9607	response to biotic stimulus	28 out of 115 genes, 24.3%
27	70887	cellular response to chemical stimulus	27 out of 115 genes, 23.5%
28	10628	positive regulation of gene expression	27 out of 115 genes, 23.5%
29	6357	regulation of transcription from RNA polymerase II promoter	27 out of 115 genes, 23.5%
30	45935	positive regulation of nucleobase-containing compound metabolic process	26 out of 115 genes, 22.6%
31	70727	cellular macromolecule localization	25 out of 115 genes, 21.7%
32	51254	positive regulation of RNA metabolic process	25 out of 115 genes, 21.7%
33	8104	protein localization	25 out of 115 genes, 21.7%
34	1902589	single-organism organelle organization	25 out of 115 genes, 21.7%
35	34613	cellular protein localization	23 out of 115 genes, 20.0%
36	1903508	positive regulation of nucleic acid-templated transcription	23 out of 115 genes, 20.0%
37	1902680	positive regulation of RNA biosynthetic process	23 out of 115 genes, 20.0%
38	45893	positive regulation of transcription, DNA-templated	23 out of 115 genes, 20.0%
39	51246	regulation of protein metabolic process	23 out of 115 genes, 20.0%
40	32268	regulation of cellular protein metabolic process	22 out of 115 genes, 19.1%

Table 4.12, continued

41	71496	cellular response to external stimulus	20 out of 115 genes, 17.4%
42	31668	cellular response to extracellular stimulus	20 out of 115 genes, 17.4%
43	45944	positive regulation of transcription from RNA polymerase II promoter	20 out of 115 genes, 17.4%
44	9991	response to extracellular stimulus	20 out of 115 genes, 17.4%
45	31667	response to nutrient levels	20 out of 115 genes, 17.4%
46	31669	cellular response to nutrient levels	19 out of 115 genes, 16.5%
47	6259	DNA metabolic process	19 out of 115 genes, 16.5%
48	42594	response to starvation	19 out of 115 genes, 16.5%
49	9267	cellular response to starvation	18 out of 115 genes, 15.7%
50	36170	filamentous growth of a population of unicellular organisms in response to starvation	17 out of 115 genes, 14.8%
51	40029	regulation of gene expression, epigenetic	17 out of 115 genes, 14.8%
52	10564	regulation of cell cycle process	16 out of 115 genes, 13.9%
53	44087	regulation of cellular component biogenesis	16 out of 115 genes, 13.9%
54	6342	chromatin silencing	15 out of 115 genes, 13.0%
55	16458	gene silencing	15 out of 115 genes, 13.0%
56	45814	negative regulation of gene expression, epigenetic	15 out of 115 genes, 13.0%
57	9405	Pathogenesis	15 out of 115 genes, 13.0%
58	7059	chromosome segregation	14 out of 115 genes, 12.2%
59	7010	cytoskeleton organization	13 out of 115 genes, 11.3%
60	51130	positive regulation of cellular component organization	13 out of 115 genes, 11.3%
61	65009	regulation of molecular function	13 out of 115 genes, 11.3%
62	19953	sexual reproduction	13 out of 115 genes, 11.3%
63	7067	mitotic nuclear division	12 out of 115 genes, 10.4%
64	70	mitotic sister chromatid segregation	12 out of 115 genes, 10.4%
65	51129	negative regulation of cellular component organization	12 out of 115 genes, 10.4%
66	98813	nuclear chromosome segregation	12 out of 115 genes, 10.4%
67	10608	posttranscriptional regulation of gene expression	12 out of 115 genes, 10.4%
68	32505	reproduction of a single-celled organism	12 out of 115 genes, 10.4%
69	819	sister chromatid segregation	12 out of 115 genes, 10.4%
70	35556	intracellular signal transduction	11 out of 115 genes, 9.6%
71	10639	negative regulation of organelle organization	11 out of 115 genes, 9.6%
72	34248	regulation of cellular amide metabolic process	11 out of 115 genes, 9.6%
73	19220	regulation of phosphate metabolic process	11 out of 115 genes, 9.6%
74	51174	regulation of phosphorus metabolic process	11 out of 115 genes, 9.6%
75	31399	regulation of protein modification process	11 out of 115 genes, 9.6%
76	6417	regulation of translation	11 out of 115 genes, 9.6%
77	30036	actin cytoskeleton organization	10 out of 115 genes, 8.7%
79	30029	actin filament-based process	10 out of 115 genes, 8.7%
80	32269	negative regulation of cellular protein metabolic process	9 out of 115 genes, 7.8%
81	51248	negative of cellular protein metabolic process	9 out of 115 genes, 7.8%
82	32270	positive regulation of cellular protein metabolic process	9 out of 115 genes, 7.8%
83	51247	positive regulation of protein metabolic process	9 out of 115 genes, 7.8%
84	50793	regulation of developmental process	9 out of 115 genes, 7.8%

Table 4.12, continued

85	32784	regulation of DNA-templated transcription, elongation	9 out of 115 genes, 7.8%
86	44089	positive regulation of cellular component biogenesis	8 out of 115 genes, 7.0%
87	32786	positive regulation of DNA-templated transcription, elongation	8 out of 115 genes, 7.0%
88	43254	regulation of protein complex assembly	8 out of 115 genes, 7.0%
89	30466	chromatin silencing at silent mating-type cassette	7 out of 115 genes, 6.1%
90	32968	positive regulation of transcription elongation from RNA polymerase II promoter	7 out of 115 genes, 6.1%
91	90066	regulation of anatomical structure size	7 out of 115 genes, 6.1%
92	32535	regulation of cellular component size	7 out of 115 genes, 6.1%
93	1902275	regulation of chromatin organization	7 out of 115 genes, 6.1%
94	34243	regulation of transcription elongation from RNA polymerase II promoter	7 out of 115 genes, 6.1%
95	7064	mitotic sister chromatid cohesion	6 out of 115 genes, 5.2%
96	31334	positive regulation of protein complex assembly	6 out of 115 genes, 5.2%
97	32956	regulation of actin cytoskeleton organization	6 out of 115 genes, 5.2%
98	32970	regulation of actin filament-based process	6 out of 115 genes, 5.2%
99	30261	chromosome condensation	5 out of 115 genes, 4.3%
100	45727	positive regulation of translation	5 out of 115 genes, 4.3%
101	1903338	regulation of cell wall organization or biogenesis	5 out of 115 genes, 4.3%
102	70828	heterochromatin organization	4 out of 115 genes, 3.5%
103	7076	mitotic chromosome condensation	4 out of 115 genes, 3.5%

growth (n=33, 28.7%), response to biotic stimulus (n=28, 24.3%), and filamentous growth of a population of unicellular organisms in response to starvation (n=17, 14.8%).

GO Terms (n=105) that are identified in both planktonic and biofilm cultures are presented in Table 4.13. Higher percentages of proteins were annotated for regulation of biological process, single-organism cellular process and response to stimulus/stress and cellular component organization of biogenesis (Table 4.13). Greater difference (>10%) were noted in the percentage of proteins annotated for cell cycle (12.3%), regulation of response to stimulus/stress (12.3%) in planktonic culture compared to the biofilm culture. On the other hand, greater difference (>10%) were noted in proteins annotated for regulation of cellular metabolic process (26.1%), regulation of macromolecule metabolic process (23.7%) and nitrogen compound (21.7%) in the biofilm culture as compared to the planktonic culture, as shown in Table 4.13.

4.10.4 Protein profiles of planktonic and biofilm cultures upon exposure to DMSO

In this set of experiment, since DMSO was used to prepare AbA stock solution, the protein profiles generated by DMSO was also investigated. Both planktonic and biofilm cultures were exposed to 1% of DMSO. For comparison studies, the same selection criteria of at least one unique peptide match for protein identification, significance (-10lgP) value of >20 for protein identification and false discovery rate of ≤1% was maintained. A total of 465 and 344 proteins were identified from the DMSO-treated biofilm and planktonic cultures, respectively. Both the cultures responded differently on DMSO treatment and there was a reduction (from 533 to 465) in the total proteins of biofilm culture (Table 4.7). In contrast, an increase in the total proteins (from 203 to 344) was observed in planktonic culture (Table 4.7). The percentage of peptide coverage ranged from 0% to 99% with the mean of 7% ± 12.5 for biofilm culture and 12% ± 19.7 for planktonic culture, respectively.

Table 4.13: GO terms (n=105) common in planktonic and biofilm cultures (only the top 15% of processes are shown).

No.	GoID	GO term	Planktonic culture (untreated), n=50		Biofilm culture (untreated), n=115		Difference (%) (x-y)
			No. protein	% of protein (x)	No. protein	% of protein (y)	
1	50789	regulation of biological process	49	98.00%	114	99.10%	-1.1
2	9987	cellular process	47	94.00%	103	89.60%	4.4
3	44699	single-organism process	41	82.00%	89	77.40%	4.6
4	50794	regulation of cellular process	40	80.00%	99	86.10%	-6.1
5	44763	single-organism cellular process	34	68.00%	73	63.50%	4.5
6	50896	response to stimulus	32	64.00%	74	64.30%	-0.3
7	71840	cellular component organization or biogenesis	30	60.00%	69	60.00%	0
8	51716	cellular response to stimulus	28	56.00%	64	55.70%	0.3
9	16043	cellular component organization	27	54.00%	63	54.80%	-0.8
10	6996	organelle organization	25	50.00%	54	47.00%	3
11	6950	response to stress	21	42.00%	48	41.70%	0.3
12	33554	cellular response to stress	21	42.00%	46	40.00%	2
13	19222	regulation of metabolic process	20	40.00%	76	66.10%	-26.1*
14	48518	positive regulation of biological process	20	40.00%	58	50.40%	-10.4*
15	31323	regulation of cellular metabolic process	19	38.00%	74	64.30%	-26.3*
16	48519	negative regulation of biological process	19	38.00%	51	44.30%	-6.3
17	51704	multi-organism process	19	38.00%	36	31.30%	6.7
18	60255	regulation of macromolecule metabolic process	19	38.00%	71	61.70%	-23.7*
19	65008	regulation of biological quality	19	38.00%	41	35.70%	2.3
20	80090	regulation of primary metabolic process	19	38.00%	73	63.50%	-25.5*
21	44085	cellular component biogenesis	18	36.00%	35	30.40%	5.6
22	7049	cell cycle	17	34.00%	25	21.70%	12.3*
23	22402	cell cycle process	17	34.00%	25	21.70%	12.3*
24	48523	negative regulation of cellular process	17	34.00%	47	40.90%	-6.9
25	48583	regulation of response to stimulus	17	34.00%	25	21.70%	12.3*

Table 4.13, continued

26	51171	regulation of nitrogen compound metabolic process	17	34.00%	64	55.70%	-21.7*
27	51276	chromosome organization	17	34.00%	30	26.10%	7.9
28	51128	regulation of cellular component organization	16	32.00%	33	28.70%	3.3
29	7154	cell communication	15	30.00%	33	28.70%	1.3
30	3	Reproduction	14	28.00%	22	19.10%	8.9
31	9605	response to external stimulus	14	28.00%	29	25.20%	2.8
32	30447	filamentous growth	14	28.00%	33	28.70%	-0.7
33	22414	reproductive process	13	26.00%	20	17.40%	8.6
34	44182	filamentous growth of a population of unicellular organisms	13	26.00%	29	25.20%	0.8
35	44419	interspecies interactionbetween organisms	13	26.00%	26	22.60%	3.4
36	278	mitotic cell cycle	12	24.00%	18	15.70%	8.3
37	9892	negative regulation of metabolic process	12	24.00%	37	32.20%	-8.2
38	10605	negative regulation of macromolecule metabolic process	12	24.00%	35	30.40%	-6.4
39	10629	negative regulation of gene expression	12	24.00%	30	26.10%	-2.1
40	80134	regulation of response to stress	12	24.00%	15	13.00%	11*
41	1903047	mitotic cell cycle process	12	24.00%	18	15.70%	8.3
42	6325	chromatin organization	11	22.00%	22	19.10%	2.9
43	9890	negative regulation of biosynthetic process biosynthetic process	11	22.00%	32	27.80%	-5.8
44	10558	negative regulation of macromolecule	11	22.00%	28	24.30%	-2.3
45	31324	negative regulation of cellular metabolic process	11	22.00%	34	29.6%	-7.6%
46	31327	negative regulation of cellular biosynthetic process	11	22.00%	32	27.80%	-5.8
47	33043	regulation of organelle organization	11	22.00%	22	19.10%	2.9
48	51172	negative regulation of nitrogen compound metabolic process	11	22.00%	33	28.70%	-6.7
49	2000113	negative regulation of cellular macromolecule biosynthetic process	11	22.00%	28	24.30%	-2.3

Table 4.13, continued

50	45892	negative regulation of transcription, DNA-templated	10	20.00%	23	20.00%	0
51	45934	negative regulation of nucleobase-containing compound metabolic process	10	20.00%	25	21.70%	-1.7
52	48584	positive regulation of response to stimulus	10	20.00%	15	13.00%	7
53	51253	negative regulation of RNA metabolic process	10	20.00%	23	20.00%	0
54	51726	regulation of cell cycle	10	20.00%	19	16.50%	3.5
55	1902679	negative regulation of RNA biosynthetic process	10	20.00%	23	20.00%	0
56	1903507	negative regulation of nucleic acid-templated transcription	10	20.00%	23	20.00%	0

Footnote: (*) indicating > 10% difference, difference of the percentages of protein (x and y) were generated from the search in the CGD.

The molecular function, biological process, and subcellular localization of each protein were determined by using CGD as well.

Upon exposure of DMSO to planktonic culture, there were higher percentages of proteins annotated for cell cycle (3.7%), response to chemical (3.1%) and ribosome biogenesis (3.3%) (Table 4.14). The other proteins did not show much change. DMSO-treated biofilm culture also showed higher percentages of proteins annotated for filamentous growth (3.5%), response to chemical (3.4%) and regulation of biological process (3.2%) (Table 4.15). On the other hand, a reduction in the proteins annotated for biological process (4.4%) was noted in the DMSO-treated biofilm culture. Protein changes in other biological processes were less than 3% (Table 4.15).

4.10.5 Comparison of protein profiles of DMSO-treated biofilm and planktonic cultures

The common and specific proteins annotated in the selected biological process include those associated with regulation of biological process, organelle organization, response to stress, pathogenesis, biofilm formation, lipid metabolic process, filamentous growth and chemical response are shown in Table 4.16 (for DMSO-treated cultures) and Table 4.17 (for AbA-treated cultures).

4.10.6 Comparison of protein profiles of DMSO-treated and AbA- treated planktonic and biofilm cultures

A comparison of proteins annotated from DMSO and AbA-treated planktonic cultures demonstrated a decrease in the percentage of proteins annotated for organelle organization (5.1%) in *C. albicans* planktonic culture and an increase in proteins annotated for translation (5.0%) upon exposure to AbA (Table 4.18). Table 4.19 shows the

Table 4.14: Comparison of proteins identified in the DMSO-treated planktonic culture of *C. albicans* SC5314.

No.	GOID	GO term	Planktonic culture (untreated), n=163		Planktonic culture (DMSO), n=276		Difference (%) (x-y)
			No. protein	% protein (x)	No. protein	% protein (y)	
1	50789	regulation of biological process	50	30.70%	87	31.50%	-0.8
2	6950	response to stress	38	23.30%	63	22.80%	0.5
3	6996	organelle organization	35	21.50%	64	23.20%	-1.7
4	6810	Transport	29	17.80%	49	17.80%	0
5	42221	response to chemical	28	17.20%	39	14.10%	+3.1*
6	16070	RNA metabolic process	26	16%	41	14.90%	1.1
7	7049	cell cycle	22	13.50%	27	9.80%	+3.7*
8	30447	filamentous growth	21	12.90%	36	13.00%	-0.1
9	5975	carbohydrate metabolic process	19	11.70%	25	9.10%	2.6
10	8150	biological_process	18	11.00%	36	13.00%	-2
11	Other	other	15	9.20%	21	7.60%	1.6
12	6464	cellular protein modification process	14	8.60%	25	9.10%	-0.5
13	42254	ribosome biogenesis	13	8%	13	4.70%	+3.3*
14	6259	DNA metabolic process	12	7.40%	21	7.60%	-0.2
15	44419	interspecies interaction between organisms	10	6.10%	12	4.30%	1.8
16	6091	generation of precursor metabolites and energy	9	5.50%	12	4.30%	1.2
17	7010	cytoskeleton organization	9	5.50%	11	4%	1.5
18	7165	signal transduction	9	5.50%	12	4.30%	1.2
19	42493	response to drug	9	5.50%	14	5.10%	0.4
20	746	conjugation	8	4.90%	7	2.50%	2.4
21	6629	lipid metabolic process	8	4.90%	15	5.40%	-0.5
23	9405	pathogenesis	7	4.30%	12	4.30%	0
24	71555	cell wall organization	7	4.30%	4	1.40%	2.9
25	6457	protein folding	6	3.70%	6	2.20%	1.5
26	6412	translation	5	3.10%	7	2.50%	0.6
27	16192	vesicle-mediated transport	5	3.10%	16	5.80%	-2.7
28	30448	hyphal growth	5	3.10%	5	1.80%	1.3
29	42710	biofilm formation	5	3.10%	6	2.20%	0.9

Table 4.14, continued

30	910	cytokinesis	4	2.50%	2	0.70%	1.8
31	6997	nucleus organization	4	2.50%	5	1.80%	0.7
32	7114	cell budding	3	1.80%	0	0%	1.8
33	70783	growth of unicellular organism as a thread of attached cells	3	1.80%	4	1.40%	0.4
34	7124	pseudohyphal growth	2	1.20%	0	0%	1.2
35	19725	cellular homeostasis	2	1.20%	11	4%	-2.8
36	30163	protein catabolic process	2	1.20%	11	4%	-2.8
37	45333	cellular respiration	2	1.20%	4	1.40%	-0.2
38	6766	vitamin metabolic process	1	0.60%	0	0%	0.6
39	7155	cell adhesion	1	0.60%	4	1.40%	-0.8
40	not_yet_annotated	not_yet_annotated	1	0.60%	5	1.80%	-1.2
41	32196	transposition	0	0%	0	0%	0

Footnote: (*) / boxed rows indicating ≥ 3% difference, difference of the percentages of protein (x and y) were generated from the search in the CGD.

Table 4.15: Comparison of proteins identified in the DMSO-treated biofilm cultures of *C. albicans* SC5314.

No.	GOID	GO term	Biofilm culture (untreated), n=414		Biofilm culture (DMSO), n=368		Difference (%) (x-y)
			No. protein	% protein (x)	No. protein	% protein (y)	
1	50789	regulation of biological process	115	27.80%	114	31%	-3.2*
2	6996	organelle organization	92	22.20%	78	21.20%	1
3	6950	response to stress	85	20.50%	77	20.90%	-0.4
4	6810	transport	84	20.30%	83	22.60%	-2.3
5	8150	biological_process	64	15.50%	41	11.10%	4.4*
6	42221	response to chemical	58	14.00%	64	17.40%	-3.4*
7	30447	filamentous growth	51	12.30%	58	15.80%	-3.5*
8	16070	RNA metabolic process	49	11.80%	52	14.10%	-2.3
9	7049	cell cycle	45	10.90%	41	11.10%	-0.2
10	6464	cellular protein modification process	41	9.90%	39	10.60%	-0.7
11	6259	DNA metabolic process	40	9.70%	33	9%	0.7
12	5975	carbohydrate metabolic process	39	9.40%	30	8.20%	1.2
13	other	other	35	8.50%	31	8.40%	0.1
14	6629	lipid metabolic process	31	7.50%	23	6.30%	1.2
15	16192	vesicle-mediated transport	28	6.80%	31	8.40%	-1.6
16	9405	pathogenesis	25	6.00%	22	6%	0
17	42493	response to drug	24	5.80%	23	6.30%	-0.5
18	42254	ribosome biogenesis	20	4.80%	18	4.90%	-0.1
19	44419	interspecies interaction between organisms	20	4.80%	16	4.30%	0.5
20	6412	translation	18	4.30%	18	4.90%	-0.6
21	6091	generation of precursor metabolites and energy	17	4.10%	22	6%	-1.9
22	7165	signal transduction	17	4.10%	18	4.90%	-0.8
23	19725	cellular homeostasis	16	3.90%	17	4.60%	-0.7
24	7010	cytoskeleton organization	15	3.60%	13	3.50%	0.1
25	746	conjugation	12	2.90%	14	3.80%	-0.9
26	6457	protein folding	12	2.90%	17	4.60%	-1.7
27	30163	protein catabolic process	12	2.90%	8	2.20%	0.7

Table 4.15, continued

28	42710	biofilm formation	12	2.90%	12	3.30%	-0.4
29	30448	hyphal growth	10	2.40%	14	3.80%	1.4
30	71555	cell wall organization	10	2.40%	7	1.90%	0.5
31	910	cytokinesis	9	2.20%	9	2.40%	-0.2
32	48468	cell development	9	2.20%	15	4.10%	-1.9
33	6997	nucleus organization	7	1.70%	8	2.20%	-0.5
34	7114	cell budding	6	1.40%	5	1.40%	0
35	45333	cellular respiration	6	1.40%	8	2.20%	-0.8
36	70783	growth of unicellular organism as a thread of attached cells	6	1.40%	8	2.20%	-0.8
37	7155	cell adhesion	5	1.20%	5	1.40%	-0.2
38	7124	pseudohyphal growth	4	1%	5	1.40%	-0.4
39	not_yet_annotated	not_yet_annotated	4	1%	4	1.10%	-0.1
40	32196	transposition	1	0.20%	0	0%	0.2
41	6766	vitamin metabolic process	0	0%	1	0.30%	-0.3

Footnote: (*) indicating ≥ 3% difference, difference of the percentages of protein (x and y) were generated from the search in the CGD.

Table 4.16: Categorization of proteins identified in the DMSO-treated planktonic and biofilm cultures of *C. albicans* SC5314

Categories	Common in biofilm and planktonic cultures	Only identified in biofilm culture	Only identified in planktonic culture
Regulation of biological process	23	91	64
	AHP1, BRO1, C1_04510W_A, CR_04720C_A, CR_06740W_A, DYN1, ENO1, FAB1, FBA1, HSP90, IRA2, MET6, OFR1, PDI1, PGK1, PRX1, SLN1, SPT5, TDH3, TFS1, TPI1, YCG1, ZCF5	ABP1, ADH1, AGO1, AHR1, ANB1, APL2, ASC1, BFR1, BMH1, BNI1, C1_05750C_A, C1_07340W_A, C2_00350W_A, C2_00360C_A, C2_03780C_A, C2_04380C_A, C2_04500W_A, C2_05220C_A, C2_05720C_A, C2_08380C_A, C2_08860W_A, C3_02920W_A, C3_07770C_AC4_04920W_A, C5_02750C_A, C6_00850W_A, CDC19, CDC39, CDC5, CET1, CHK1, CLA4, CMD1, CR_01170W_A, CR_02430C_A, CR_06680C_A, CR_09230C_A, CTF1, CYP1, EFT2, ERG1, FGR27, HAL9, HSP12, HST3, HTA1, HTA2, HXK2, IQG1, KOG1, LRG1, LTE1, LYS2, MBF1, MBP1, MEC1, MSB1, MYO2, NAM7, NIK1, ORC1, PES1, PHO87, PIL1, PST3, PTC6, RBP1, RFC2, RIM15, RLI1, RPP1A, SBP1, SEC7, SGS1, SNF2, SNT1, SOK1, SPA2, SRB9, SSC1, SUV3, TOP2, TRA1, TRX1, TSA1, TUS1, UGA32, UTP5, VPS33, YBL053, YCF1	A(PC)1, ARC40, ARG83, (BC)K1, BEM2, C1_06630W_A, C1_06910C_A, C1_07260C_A, C2_01650W_A, C3_02420C_A, C3_07680W_A, C4_01300W_A, C4_02960W_A, C5_04640C_A, C6_01850W_A, C6_02430W_A, C6_03300C_A, C6_03320W_A, C7_02670W_A, CDC47, CDC48, CR_02930W_A, CR_06090W_A, DCK1, DOT1, ECE1, EFB1, FUN12, GCN1, GIN1, HST1, KAR9, KEM1, LPD1, MP65, NPR1, PET127, PEX6, PIF1, PSY2, PUF3, PUT3, RAD50, RAD9, REG1, RGD3, SFL2, SIZ1, SPT20, SST2, SWI1, SWI4, SWI6, SWR1, TSC2, TTR1, USO5, VPS34, ZCF13, ZCF14, ZCF15, ZCF27, ZCF39, ZRT2
Organelle organization	16	63	49
	BRO1, C5_03140C_A, CR_04720C_A, CR_06740W_A, DYN1, FAB1, HSP60, HSP90, IFM1, ILV5, KAR2, MDN1, MYO1, MYO2, TEL1, YCG1	ABP1, BFR1, BNI1, C1_03330C_A, C1_05750C_A, C1_07340W_A, C2_05220C_A, C3_02020W_A, C3_07230W_A, C3_07770C_A, C4_02340W_A, C4_04920W_A, C5_02590C_A, C5_02750C_A, C6_00340C_A, C6_01350W_A, C6_02630C_A, CDC5, CLA4, CMD1, CYP1, DNM1, GSG1, HSP104, HST3, HTA1, HTA2, IQG1, LRG1, MEC1, MEF2, MSK1, MYO2, NAM7, NOP1, NUP188, ORC1, PEX12, PGA63, PST3, PTC6, RBP1, RET2, RFC2, RLI1, RPO41, SEC5, SEC7, SEC8, SGS1, SNF2, SNT1, SPA2, SSC1, SUV3, TIF4631, TOP2, TRA1, TRX1, UBI3, VPS33, YBL053, YCF1	A(PC)1, ARC40, (BC)K1, BEM2, BRN1, C1_04190C_A, C1_06630W_A, C1_07260C_A, C2_01650W_A, C2_01720C_A, C3_07680W_A, C4_00420C_A, C4_01300W_A, C6_00540W_A, C6_01850W_A, C6_02430W_A, C6_03300C_A, C7_02670W_A, CDC13, CDC47, CDC48, CR_02930W_A, CR_06090W_A, CSI2, DCK1, DOT1, DRS1, FUN12, GIN1, HST1, KAR9, KEM1, MTG1, PET127, PEX5, PEX6, PIF1, POM152, PUF3, PUT3, RAD50, REG1, RGD3, SPT20, SWI1, SWR1, USO5, VPH1, VPS34
Response to stress	22	55	41
	AHP1, CIP1, CR_04720C_A, DYN1, FAB1, GLX3, GND1, HSP21, HSP60, HSP90, IRA2, KAR2, MET6, MMS22, PMA1, PRX1, SLN1, SPT5, TEL1, TPI1, YNK1, ZWF1	AHR1, ASC1, BMH1, BNI1, BNI4, C1_05750C_A, C1_13270W_A, C2_00350W_A, C2_03780C_A, C2_05220C_A, C2_08380C_A, C4_03170W_A, C6_00850W_A, CDC19, CDC39, CDC5, CLA4, DDR48, ERG1, FGR27, FGR28,	APN1, ARC40, (BC)K1, C1_04190C_A, C1_06630W_A, C1_07260C_A, C3_00940W_A, C4_02960W_A, C4_06020C_A, C6_00540W_A, C6_03320W_A, C7_02670W_A, CDC47,

Table 4.16, continued

Response to stress		GAD1, HPR5, HSP104, HSP12, HST3, HTA2, KOG1, MEC1, MLT1, MYO2, NAM7, NIK1, NUP188, PES1, PIL1, PLD1, POL1, RAD2, REV3, RFC1, RFC2, RIM15, RLI1, RNR1, SBP1, SGS1, SMC5, SNF2, SNT1, TOP2, TRA1, TRX1, TSA1, YBL053	CDC48, CDR2, CSI2, DCK1, DCK2, DOT1, FGR47, FGR6, FGR6-3, GIN1, KEM1, MCR1, MP65, NMA111, NPR1, PIF1, PSY2, RAD50, RAD9, REG1, SPT20, SWI1, SWI4, SWI6, TSC2, TTR1, VPS34, ZCF13
Pathogenesis	2	10	20
	HSP21, HSP90	AHR1, ASC1, BMH1, BNI1, CHK1, CLA4, CSH1, CTF1, HSP104, ICL1, INP51, LEU2, MBF1, MLT1, NIK1, PES1, PLD1, RIM13, SNF2, SPA2	ECE1, HEX1, KEM1, MP65, NMT1, SFL2, SPF1, SWI1, TTR1, VPS34
Biofilm formation	1	11	5
	YWP1	ADH1, AHR1, CHK1, CSA1, CSH1, FGR27, HSP104, IFD6, QDR2, SRB9, SUV3	ECE1, KEM1, MP65, SPF1, ZCF39
Lipid metabolic process	4	19	11
	ERG10, FAB1, FAS1, INO1	ACH1, C2_05720C_A, C2_05980C_A, C3_00400C_A, C6_03240W_A, CMD1, CTF1, ECM39, ERG1, GPI1, GPI8, HST3, ICL1, INP51, LPT1, PEL1, PLD1, SAH1, SCT2	ACC1, C1_04190C_A, C1_08860C_A, C5_05000C_A, CRC1, MCR1, NMA111, PEX5, PEX6, SCS7, VPS34
Filamentous growth	12	58	24
		AHR1, ASC1, BMH1, BNI1, BNI4, C1_13270W_A, C6_03240W_A, CDC19, CDC39, CDC5, CHK1, CLA4, CMD1, CR_10290C_A, DDR48, DNM1, DYN1, ENO1, ERG1, FAB1, FGR27, FGR28, GFA1, HAL9, HPR5, HSP21, HSP90, HST3, INP51, MLT1, MYO2, NAM7, NIK1, PDA1, PES1, PGI1, PLD1, RIM13, RIM15, RNR1, SEC7, SGS1, SLN1, SMC5, SNF2, SNT1, SPA2, SPT5, SRB9, SSO2, SUV3, TOP2, TPI1, TRX1, TSA1, UGA32, YNK1, ZCF5	ARC40, ARG83, C3_07680W_A, CSI2, DCK1, DCK2, FGR43, FGR47, FGR6, FGR6-1, FGR6-3, KEM1, LPD1, MP65, RAD9, SFL2, SWI1, TSC2, USO5, VPS34, ZCF13, ZCF14, ZCF27, ZCF39
Chemical response	18	48	21
	AHP1, BRO1, C1_10820C_A, C4_02960W_A, CIP1, FAB1, FAS1, GLX3, GND1, HSP21, HSP90, IRA2, KAR2, PRX1, SLN1, SSA2, UTP22, ZWF1	ACH1, ADO1, AGO1, AHR1, APL2, ASC1, BNI1, C1_05750C_A, C1_13270W_A, C2_00360C_A, C2_03780C_A, C2_08860W_A, C3_07230W_A, C4_02960W_A, C4_03110W_A, C6_00850W_A, CDC39, CET1, CHK1, CLA4, CMD1, CR_09230C_A, DDR48, EFT2, ERG1, GAD1, GFA1, HSP104, NIK1, NOP14, OPT6, PES1, PLD1, PRT1, QDR2, RBP1, RIM15, RLI1, RPN8, SEC7, SPA2, SRB9, SUV3, TRK1, TRX1, TSA1, UTP5, YCF1	(BC)K1, C1_04190C_A, C3_00940W_A, C6_02430W_A, C6_03320W_A, C7_00780W_A, C7_02670W_A, CDC48, CDR2, DRS1, KEM1, MCR1, RAD50, REG1, SEC20, SPT20, SST2, SWI1, TSC2, TTR1, VPS34

Table 4.17: **Categorization of proteins identified in the AbA-treated planktonic and biofilm cultures of *C. albicans* SC5314**

Categories	Common in biofilm and planktonic	Only identified in biofilm	Only identified in planktonic	Total genes identified in each culture
Regulation of biological process	30	78	60	Biofilm: 108 out of 374 genes (28.9%) Planktonic: 90 out of 293 genes(30.7%)
	ABP1, ADH1, AHP1, ASC1, BLM3, BNI1, C1_05380C_A, C6_03320W_A, CDC19, CDC37, CDC68, CHK1, CYP1, EFB1, EFT2, ENO1, FBA1,HSP12, HSP90, LPD1, MET6, PDI1, PGK1, PIL1, PRX1, SSB1, TDH3, TPI1, TRA1, TSA1	ANB1, ARC18, ARC35, BMH1, BRE1, BUB3, C1_02240W_A, C1_03360W_A, C1_12350W_A, C1_13010W_A, C2_10760C_A, C3_02490C_A, C3_02920W_A, C3_04740C_A, C3_06150W_A, C3_07680W_A, C4_05340W_A, C4_06810C_A, C5_02900W_A, C5_04640C_A, C6_00850W_A, C6_01920C_A, C6_02430W_A, C6_03180C_A, C7_03480W_A, C7_04240C_A, CAS4, CAT8, CCH1, CDC15, CDC46, CDC48, CDH1, CR_01170W_A, CR_01420W_A, CR_06960W_A, CTA3, CTA4, CTA7, DCK1, DEF1, DOA4, DYN1, ESP1, FHL1, GIS2, GLR1, GST2, HAL9, HTA1, HTA2, HXK2, IRS4, KAR3, LSP1, MYO2, OFR1, orf19.3835, PET127, PEX1, PEX6, PST3, RBP1, SEC7, SSC1, SSD1, SSK2, STN1, TFS1, TOR1, TRX1, TSC11, TTR1, ULP3, UTP5, VPS34, YCG1, ZDS1	ACT1, BFR1, BUD14, C1_00570C_A, C1_01590C_A, C1_01820C_A, C1_03370W_A, C1_14240W_A, C2_00350W_A, C2_02530W_A, C2_05220C_A, C2_08860W_A, C3_00380C_A, C4_02850W_A, C5_04920C_A, C7_00410C_A, CAF16, CCN1, CHL4, CR_01720W_A, CRN1, DOT1, ECE1, GCN2, HAP43, HMT1, HYM1, INO2, IRA2, IRE1, KEM1, LAP3, LRG1, MCM1, MED14, MLP1, MP65, MTLALPHA2, PBS2, PDE2, PFY1, POR1, PRA1, RAD3, RDI1, RPP1A, RPP2B, SIN3, SKP1, SPT20, SRB9, TRY5, URA2, YCF1, ZCF14, ZCF26, ZCF30, ZCF6, ZNC1, ZRT2	
Organelle organization	15	75	38	
	ABP1, BNI1, C1_05380C_A, C1_07470C_A, CDC37, CDC68, CYP1, HSP60, HSP90, ILV5, MYO1, RAD16, ABP1, REX2, TRA1, UBI3	ACS2, ARC18, ARC35, BRE1, C1_02240W_A, C1_03360W_A, C1_13060C_A, C2_00570W_A, C2_10760C_A, C3_00420W_A, C3_01510W_A, C3_02490C_A, C3_03820C_A, C3_04540C_A, C3_04740C_A, C3_06440W_A, C3_07680W_A, C4_01790W_A, C4_02340W_A, C4_06810C_A, C5_02900W_A, C5_03140C_A, C6_02430W_A, CDC15, CDC46, CDC48, CR_01790C_A, CR_06960W_A, CR_07910C_A, CR_10230W_A, CTF18, DCK1, DOA4, DYN1, ESP1, HSP104, HSP78, HTA1, HTA2, KAR2, KAR3, MDH1, MDN1, MIA40, MSH6, MSK1, MTO1, MYO2, PET127, PEX1, PEX6, PST3, RBP1, RIA1, RIM2, RPL12, RPL5, SEC7, SEC8, SHE9, SHP1, SPO1, SPO72, SSC1, STN1, TAZ1, TOM1, TOR1, TRX1, TSC11, ULP3, USO1, VPS34, YCG1, ZDS1	ACS1, ACT1, BFR1, BNR1, BUD14, C1_00430W_A, C1_01590C_A, C1_14240W_A, C2_00830C_A, C2_05220C_A, C3_00380C_A, C6_00390W_A, C6_01350W_A, CHL4, CR_01720W_A, CRN1, DOT1, KEM1, LRG1, MAK21, MLH1, MLP1, MSH3, MTG1, NAM2, NOP1, PBS2, PDS5, PFY1, POR1, RAD3, RDI1, RPP0, SIN3, SKP1, SPT20, VPS1, YCF1	

Table 4.17, continued

Response to stress	31	49	28	Biofilm: 108 out of 374 genes (28.9%) Planktonic: 90 out of 293 genes (30.7%)
	AHP1, ASC1, BNI1, C1_05380C_A, C6_03320W_A, CDC19, CDC37, CIP1, ECM4, GAD1, GLX3, GND1, GPH1, GRE3, HSP12, HSP21, HSP60, HSP90, MET6, MMS22, PIL1, PMA1, POL1, PRX1, RAD16, SSB1, TPI1, TRA1, TSA1, YNK1, ZWF1	APN2, BMH1, BRE1, C3_02490C_A, C3_02760C_A, C3_04740C_A, C6_00850W_A, CAS4, CCH1, CCT8, CDC46, CDC48, CR_01790C_A, CR_06530W_A, CR_07910C_A, CTA3, CTF18, DCK1, DCK2, DDR48, DYN1, FGR51, GIS2, GLR1, GST2, HAL21, HGT12, HPR5, HSP104, HSP78, HTA2, IRS4, KAR2, KAR3, KRE5, LSP1, MCR1, MSH6, MYO2, PET9, PLD1, SHP1, SOD5, SSK2, TRX1, TSC11, TTR1, UBI4, VPS34	ACT1, C1_11120C_A, C1_14240W_A, C2_00350W_A, C2_05220C_A, CDR2, DOT1, FGR38, GCN2, HAP43, HEM13, HYM1, IRA2, IRE1, KEM1, MLH1, MP65, MSH3, PBS2, PDE2, PDS5, PHR1, PYC2, RAD3, RDI1, SIN3, SMC6, SPT20	
Pathogenesis	7	14	10	
	ASC1, BNI1, CHK1, CSH1, HSP21, HSP90, LEU2	BMH1, FAS2, GLR1, HSP104, ICL1, INP51, IRS4, PLD1, SLK19, SOD5, SSD1, TTR1, UBI4, VPS34	ECE1, HAP43, INT1, IRE1, KEM1, MP65, MTLALPHA2, PDE2, PHR1, ZCF6	
Biofilm formation	4	9	11	
	C1_05380C_A, CHK1, CSH1, ADH1	CAS4, CSA1, DEF1, FAS2, HSP104, IFD6, PGA10, RBT5, YWP1	ECE1, KEM1, MP65, PDE2, PHR1, PIKA, PIKALPHA, SRB9, TRY5, VPS1, ZNC1	
Lipid metabolic process	4	22	8	
	ERG20, INO1, SAH1, ACC1	ACH1, C3_01510W_A, CWH43, ECM39, ERG10, FAS2, FET3, GDE1, HAL21, HAL22, ICL1, INP51, MCR1, MLS1, PEX1, PEX6, PLD1, POX1-3, SPO1, TAZ1, TSC11, VPS34	ALG6, C5_05360C_A, C6_03040C_A, FAS1, GPI1, HMG1, PIKA, PXP2	
Filamentous growth	14	34	25	
	ASC1, BNI1, CDC19, CHK1, ECM4, ENO1, HSP21, HSP90, LPD1, PGI1, SSB1, TPI1, TSA1, YNK1	BMH1, BRE1, C3_02760C_A, C3_07680W_A, C7_03480W_A, CAS4, CAT8, CCH1, CCT8, CTA4, DCK1, DCK2, DDR48, DEF1, DYN1, FGR51, GFA1, HAL9, HGT12, HPR5, INP51, IRS4, KAR3, KRE5, MYO2, PLD1, PMI1, SEC7, SHP1, STN1, TRX1, TSC11, UBI4, VPS34	ACT1, C1_11120C_A, C1_14040W_A, C3_00380C_A, C6_00390W_A, CCN1, CR_10290C_A, FGR38, FGR43, GCN2, HYM1, INT1, KEM1, MCM1, MP65,PBS2, PDE2, PHR1, RDI1, SIN3, SRB9, VPS1, YTA6,ZCF14, ZCF30	
Chemical response	19	45	28	
	ADO1, AHP1, ASC1, BNI1, C6_03320W_A, CDC37, CHK1, CIP1, EFT2, GAD1, GLX3, GND1, GRE3, HSP21, HSP90, PRX1, SSA2, TSA1, ZWF1	ACH1, APN2, ARC18, ARC35, C1_12350W_A, C3_03260W_A, C6_00850W_A, C6_01920C_A, C6_02430W_A, CAS4, CAT8, CCH1, CCT8, CDC48, CDH1, CR_06530W_A, CTA3, CTA4, DDR48, DOA4, ESP1, GDE1, GFA1, GIS2, GLR1, GST2, HGT12, HSP104, KAP120, KAR2, KRE5, MCR1, PLD1, RBP1, SEC7, SHP1, SOD5, SSK2, SSY5, TOR1, TRX1, TTR1, UTP5, VPS34, YUH2	ABD1, ACT1, C1_01590C_A, C2_06230W_A, C2_08860W_A, C5_00570W_A, CAR2, CCN1, CDR2, CR_10640W_A, DAK2, FAS1, FRS1, GCN2, HEM13, HMG1, HYM1, IRA2, IRE1, KEM1, LAP3, MAK21, PDE2, PHR1, SPT20, SRB9, UTP22, YCF1	

Table 4.18: Comparison of proteins annotated from DMSO and AbA-planktonic cultures.

No.	GOID	GO term	Planktonic culture (DMSO) (n=276)		Planktonic culture (AbA) (n=293)		Percentage difference (x-y)
			No. protein	% protein (x)	No. protein	% protein (y)	
1	50789	regulation of biological process	87	31.5%	90	30.7%	0.8
2	6996	organelle organization	64	23.2%	53	18.1%	5.1*
3	6950	response to stress	63	22.8%	59	20.1%	2.7
4	6810	Transport	49	17.8%	49	16.7%	1.1
5	16070	RNA metabolic process	41	14.9%	40	13.7%	1.2
6	42221	response to chemical	39	14.1%	47	16.0%	-1.9
7	8150	biological_process	36	13.0%	33	11.3%	1.7
8	30447	filamentous growth	36	13.0%	39	13.3%	-0.3
9	7049	cell cycle	27	9.8%	24	8.2%	1.6
10	5975	carbohydrate metabolic process	25	9.1%	33	11.3%	-2.2
11	6464	cellular protein modification process	25	9.1%	34	11.6%	-2.5
12	6259	DNA metabolic process	21	7.6%	22	7.5%	0.1
13	other	Other	21	7.6%	34	11.6%	-4
14	16192	vesicle-mediated transport	16	5.8%	9	3.1%	2.7
15	6629	lipid metabolic process	15	5.4%	12	4.1%	1.3
16	42493	response to drug	14	5.1%	21	7.2%	-2.1
17	42254	ribosome biogenesis	13	4.7%	15	5.1%	-0.4
18	6091	generation of precursor metabolites and energy	12	4.3%	20	6.8%	-2.5
19	7165	signal transduction	12	4.3%	11	3.8%	0.5
20	9405	pathogenesis	12	4.3%	17	5.8%	-1.5
21	44419	interspecies interaction between organisms	12	4.3%	18	6.1%	-1.8
22	7010	cytoskeleton organization	11	4%	14	4.8%	-0.8
23	19725	cellular homeostasis	11	4%	17	5.8%	-1.8
24	30163	protein catabolic process	11	4%	7	2.4%	1.6
25	746	Conjugation	7	2.5%	8	2.7%	-0.2
26	6412	Translation	7	2.5%	22	7.5%	-5
27	6457	protein folding	6	2.2%	11	3.8%	-1.6
28	42710	biofilm formation	6	2.2%	15	5.1%	-2.9
29	6997	nucleus organization	5	1.8%	4	1.4%	0.4
30	30448	hyphal growth	5	1.8%	10	3.4%	-1.6
31	not_yet_annotated	not_yet_annotated	5	1.8%	1	0.3%	1.5

Table 4.18, continued

32	7155	cell adhesion	4	1.4%	8	2.7%	-1.3
33	45333	cellular respiration	4	1.4%	5	1.7%	-0.3
34	70783	growth of unicellular organism as a thread of attached cells	4	1.4%	5	1.7%	-0.3
35	71555	cell wall organization	4	1.4%	8	2.7%	-1.3
36	48468	cell development	3	1.1%	2	0.7%	0.4
37	910	Cytokinesis	2	0.7%	7	2.4%	-1.7
38	7114	cell budding	0	0%	4	1.4%	-1.4
39	7124	pseudohyphal growth	0	0%	1	0.3%	-0.3
40	32196	transposition	0	0%	0	0%	0

Footnote: (*) / boxed row indicating ≥ 3% difference, difference of the percentages of protein (x and y) were generated from the search in the CGD.

Table 4.19: Comparison of 41 GO Term profiles of AbA-treated biofilm culture as compared to those treated with DMSO.

No.	GOID	GO term	Biofilm DMSO (n=368)		Biofilm AbA (n=374)		Difference in percentage (x-y)
			No. protein	% protein (x)	No. protein	% protein (y)	
1	50789	regulation of biological process	114	31%	108	28.90%	2.10%
2	6810	Transport	83	22.60%	77	20.60%	2.00%
3	6996	organelle organization	78	21.20%	90	24.10%	-2.90%
4	6950	response to stress	77	20.90%	80	21.40%	-0.50%
5	42221	response to chemical	64	17.40%	64	17.10%	0.30%
6	30447	filamentous growth	58	15.80%	48	12.80%	3.00%*
7	16070	RNA metabolic process	52	14.10%	42	11.20%	2.90%
8	7049	cell cycle	41	11.10%	35	9.40%	1.70%
9	8150	biological_process	41	11.10%	38	10.20%	0.90%
10	6464	cellular protein modification process	39	10.60%	41	11%	-0.40%
11	6259	DNA metabolic process	33	9%	24	6.40%	2.60%
12	16192	vesicle-mediated transport	31	8.40%	20	5.30%	3.10%*
13	Other	Other	31	8.40%	38	10.20%	-1.80%
14	5975	carbohydrate metabolic process	30	8.20%	45	12.00%	-3.80%*
15	6629	lipid metabolic process	23	6.30%	26	7%	-0.70%
16	42493	response to drug	23	6.30%	23	6.10%	0.20%
17	6091	generation of precursor metabolites and energy	22	6%	28	7.50%	-1.50%
18	9405	pathogenesis	22	6%	21	5.60%	0.40%
19	6412	Translation	18	4.90%	18	4.80%	0.10%
20	7165	signal transduction	18	4.90%	14	3.70%	1.20%
21	42254	ribosome biogenesis	18	4.90%	19	5.10%	-0.20%
22	6457	protein folding	17	4.60%	19	5.10%	-0.50%
23	19725	cellular homeostasis	17	4.60%	24	6.40%	-1.80%*
24	44419	interspecies interaction between organisms	16	4.30%	20	5.30%	-1.00%
25	48468	cell development	15	4.10%	6	1.60%	2.50%
26	746	Conjugation	14	3.80%	8	2.10%	1.70%
27	30448	hyphal growth	14	3.80%	9	2.40%	1.40%
28	7010	cytoskeleton organization	13	3.50%	16	4.30%	-0.80%
29	42710	biofilm formation	12	3.30%	13	3.50%	-0.20%

Table 4.19, continued

30	910	Cytokinesis	9	2.40%	5	1.30%	1.10%
31	6997	nucleus organization	8	2.20%	7	1.90%	0.30%
32	30163	protein catabolic process	8	2.20%	16	4.30%	-2.10%
33	45333	cellular respiration	8	2.20%	10	2.70%	-0.50%
34	70783	growth of unicellular organism as a thread of attached cells	8	2.20%	5	1.30%	0.90%
35	71555	cell wall organization	7	1.90%	12	3.20%	-1.30%
36	7114	cell budding	5	1.40%	3	0.80%	0.60%
37	7124	pseudohyphal growth	5	1.40%	1	0.30%	1.10%
38	7155	cell adhesion	5	1.40%	6	1.60%	-0.20%
39	not_yet_annotated	not_yet_annotated	4	1.10%	2	0.50%	0.60%
40	6766	vitamin metabolic process	1	0.30%	0	0%	0.30%
41	32196	transposition	0	0%	0	0%	0.00%

Footnote: (*) / boxed row indicating ≥ 3% difference, difference of the percentages of protein (x and y) were generated from the search in the CGD.

comparison of GO Term profiles of AbA-treated biofilm culture as compared to those treated with DMSO. A reduction in the percentage of proteins annotated for vesicle-mediated transport (3.1%), and filamentous growth (3.0%) was noted, while an increase in the percentage of proteins annotated in carbohydrate metabolic process (3.8%) was noted.

4.10.7 GO Terms specifically related to AbA-treated planktonic and biofilm culture

GO Term profiles of AbA-treated planktonic (Table 4.18) and biofilm cultures (Table 4.19) for regulation of biological process indicated that both cultures respond differently to AbA treatment. Only five GO Terms were common to both AbA-treated cultures, i.e., regulation of translation, actin cytoskeleton organization, regulation of actin cytoskeleton organization, regulation of cellular amide metabolic process, and regulation of actin filament-based process (Table 4.20). The regulation of translation (GO:0006417) refers to any process that modulates the frequency, rate or extent of the chemical reactions and pathways resulting in the formation of proteins by the translation of mRNA (CGB database), while the regulation of actin cytoskeleton organization refers to any process that modulates the frequency, rate or extent of the formation, arrangement of constituent parts, or disassembly of cytoskeletal structures comprising actin filaments and their associated proteins. The regulation of cellular amide metabolic process refers to any process that modulates the frequency, rate or extent of the chemical reactions and pathways involving amides. There were five GO Terms identified specifically in the AbA-treated planktonic culture, including nucleobase-containing compound metabolic process (n=32, 35.6%), cellular protein modification process (n=21, 23.3%), reproduction (n=18, 20.0%), negative regulation of cellular macromolecule biosynthetic process (n=16, 17.8%) and phosphorylation (n=15, 16.7%) (Table 4.21). The five GO Terms specific to AbA-treated biofilm cultures are metabolic process (n=71, 65.7%), cellular component biogenesis

Table 4.20: GO Terms shared by AbA-treated planktonic and biofilm cultures

No.	GOID	Protein sample	GO Term	No. protein (n=90)	% protein	Corrected P-value	False discovery rate
1	6417	Shared-(PC)	regulation of translation	8	8.9%	0.01922	0.02%
2	30036	Shared-(PC)	actin cytoskeleton organization	8	8.9%	0.02085	0.02%
3	34248	Shared-(PC)	regulation of cellular amide metabolic process	8	8.9%	0.03321	0.01%
4	6417	Shared-(BC)	regulation of translation	9	8.3%	0.01177	0.00%
5	34248	Shared-(BC)	regulation of cellular amide metabolic process	9	8.3%	0.0217	0.03%
6	30036	Shared-(BC)	actin cytoskeleton organization	8	7.4%	0.09334	0.07%
7	32956	Shared-(PC)	regulation of actin cytoskeleton organization	6	6.7%	0.01614	0.00%
8	32970	Shared-(PC)	regulation of actin filament-based process	6	6.7%	0.01614	0.00%
9	32956	Shared-(BC)	regulation of actin cytoskeleton organization	6	5.6%	0.05475	0.07%
10	32970	Shared-(BC)	regulation of actin filament-based process	6	5.6%	0.05475	0.07%

Table 4.21: Identification of specific biological processes associated to AbA-treated planktonic cultures (Biological processes with > 10% protein annotation are presented)

No.	GOID	Protein sample	GO Term (n=90)	No. protein	% protein	Corrected P-value	False discovery rate
1	6139	AbA-(PC)	nucleobase-containing compound metabolic process	32	35.6%	0.07841	0.04%
2	6464	AbA-(PC)	cellular protein modification process	21	23.3%	0.02636	0.01%
3	36211	AbA-(PC)	protein modification process	21	23.3%	0.02636	0.01%
4	37219	AbA-(PC)	Reproduction	18	20.0%	0.00583	0.00%
5	22414	AbA-(PC)	reproductive process	17	18.9%	0.0041	0.00%
6	32268	AbA-(PC)	regulation of cellular protein metabolic process	17	18.9%	6.92E-06	0.00%
7	51246	AbA-(PC)	regulation of protein metabolic process	17	18.9%	1.22E-05	0.00%
8	2000113	AbA-(PC)	negative regulation of cellular macromolecule biosynthetic process	16	17.8%	0.00098	0.00%
9	16310	AbA-(PC)	Phosphorylation	15	16.7%	4.07E-05	0.00%
10	31668	AbA-(PC)	cellular response to extracellular stimulus	14	15.6%	0.02275	0.02%
11	44764	AbA-(PC)	multi-organism cellular process	14	15.6%	0.00227	0.00%
12	71496	AbA-(PC)	cellular response to external stimulus	14	15.6%	0.02749	0.01%
13	31669	AbA-(PC)	cellular response to nutrient levels	13	14.4%	0.08131	0.04%
14	71216	AbA-(PC)	cellular response to biotic stimulus	13	14.4%	0.0211	0.02%
15	7010	AbA-(PC)	cytoskeleton organization	11	12.2%	0.02966	0.01%
16	44087	AbA-(PC)	regulation of cellular component biogenesis	11	12.2%	0.00152	0.00%
17	65009	AbA-(PC)	regulation of molecular function	11	12.2%	0.05001	0.01%
18	19220	AbA-(PC)	regulation of phosphate metabolic process	10	11.1%	0.01581	0.00%
19	50790	AbA-(PC)	regulation of catalytic activity	10	11.1%	0.06913	0.04%
20	51174	AbA-(PC)	regulation of phosphorus metabolic process	10	11.1%	0.01581	0.00%
21	6468	AbA-(PC)	protein phosphorylation	9	10.0%	0.0818	0.04%
22	9266	AbA-(PC)	response to temperature stimulus	9	10.0%	0.01632	0.00%
23	42710	AbA-(PC)	biofilm formation	9	10.0%	0.09119	0.04%
24	44010	AbA-(PC)	single-species biofilm formation	9	10.0%	0.05835	0.01%
25	90605	AbA-(PC)	submerged biofilm formation	9	10.0%	0.05192	0.01%
26	90609	AbA-(PC)	single-species submerged biofilm formation	9	10.0%	0.0434	0.01%

Table 4.22: Identification of specific biological processes specifically associated to AbA-treated biofilm cultures (Biological processes with > 10% protein annotation are presented).

No.	GOID	Protein sample	GO Term (n=108)	No. protein	% protein	Corrected P-value	False discovery rate
1	8152	AbA-(BC)	metabolic process	71	65.7%	0.06432	0.07%
2	44085	AbA-(BC)	cellular component biogenesis	33	30.6%	0.00264	0.00%
3	42592	AbA-(BC)	homeostatic process	19	17.6%	4.42E-05	0.00%
4	51130	AbA-(BC)	positive regulation of cellular component organization	18	16.7%	3.70E-10	0.00%
5	40029	AbA-(BC)	regulation of gene expression, epigenetic	15	13.9%	4.51E-06	0.00%
6	1903508	AbA-(BC)	positive regulation of nucleic acid-templated transcription	15	13.9%	0.03162	0.05%
7	6351	AbA-(BC)	transcription, DNA-templated	14	13.0%	0.01797	0.01%
8	32774	AbA-(BC)	RNA biosynthetic process	14	13.0%	0.02077	0.03%
9	97659	AbA-(BC)	nucleic acid-templated transcription	14	13.0%	0.01797	0.01%
10	6342	AbA-(BC)	chromatin silencing	13	12.0%	6.80E-05	0.00%
11	33044	AbA-(BC)	regulation of chromosome organization	13	12.0%	7.48E-05	0.00%
12	45814	AbA-(BC)	negative regulation of gene expression, epigenetic	13	12.0%	6.80E-05	0.00%
13	10638	AbA-(BC)	positive regulation of organelle organization	12	11.1%	1.13E-05	0.00%
14	19725	AbA-(BC)	cellular h2 omeostasis	12	11.1%	0.03918	0.07%
15	71103	AbA-(BC)	DNA conformation change	12	11.1%	1.89E-05	0.00%
16	71824	AbA-(BC)	protein-DNA complex subunit organization	12	11.1%	0.00233	0.00%
17	6323	AbA-(BC)	DNA packaging	11	10.2%	2.70E-06	0.00%
18	6979	AbA-(BC)	response to oxidative stress	11	10.2%	0.02449	0.03%
19	7163	AbA-(BC)	establishment or maintenance of cell polarity	11	10.2%	0.01364	0.00%
20	32787	AbA-(BC)	monocarboxylic acid metabolic process	11	10.2%	0.01779	0.01%
21	34599	AbA-(BC)	cellular response to oxidative stress	11	10.2%	0.00837	0.00%
22	51129	AbA-(BC)	negative regulation of cellular component organization	11	10.2%	0.00363	0.00%

(n=33, 30.6%), homeostatic process (n=19, 17.6%), positive regulation of cellular component organization (n=18, 16.7%), and regulation of gene expression and epigenetic (n=15, 13.9%) (Table 4.22).

4.10.8 Identification of AbA-induced proteins

The DMSO-treated cultures serve as negative controls for the analysis of the protein profiles of AbA-treated cultures. This is because DMSO was used to dissolve AbA which is insoluble in water. Proteins of several important biological processes identified specifically in the AbA treated planktonic and biofilm cultures are shown in Table 4.23. Identification of AbA-induced proteins associated with pathogenesis (n=5), biofilm formation (n=4), lipid metabolic process (n=3), filamentous growth (n=10) and chemical response (n=12) could be useful for future investigation of antifungal targets and virulence studies. After comparison with the proteins expressed by DMSO-treated cultures, the additional proteins that were found on exposure to AbA are known to be induced by the antifungal drug.

Table 4.23: Proteins identified specifically in the AbA-treated planktonic and biofilm cultures of *C. albicans* SC5314.

Categories	Specific proteins expressed in both planktonic and biofilm cultures upon exposure to AbA	Proteins detected in both planktonic and biofilm cultures upon exposure to DMSO	Proteins detected in both planktonic and biofilm cultures upon exposure to AbA
Regulation of biological process	20 ABP1, ADH1, ASC1, BLM3, BNI1, C1_05380C_A, C6_03320W_A, CDC19, CDC37, CDC68, CHK1, CYP1, EFB1, EFT2, HSP12, LPD1, PIL1, SSB1, TRA1, TSA1	23 AHP1, BRO1, C1_04510W_A, CR_04720C_A, CR_06740W_A, DYN1, ENO1, FAB1, FBA1, HSP90, IRA2, MET6, OFR1, PDI1, PGK1, PRX1, SLN1, SPT5, TDH3, TFS1, TPI1, YCG1, ZCF5	30 ABP1, ADH1, AHP1, ASC1, BLM3, BNI1, C1_05380C_A, C6_03320W_A, CDC19, CDC37, CDC68, CHK1, CYP1, EFB1, EFT2, ENO1, FBA1, HSP12, HSP90, LPD1, MET6, PDI1, PGK1, PIL1, PRX1, SSB1, TDH3, TPI1, TRA1, TSA1
Organelle organization	11 ABP1, BNI1, C1_05380C_A, C1_07470C_A, CDC37, CDC68, CYP1, RAD16, REX2, TRA1, UBI3	16 BRO1, C5_03140C_A, CR_04720C_A, CR_06740W_A, DYN1, FAB1, HSP60, HSP90, IFM1, ILV5, KAR2, MDN1, MYO1, MYO2, TEL1, YCG1	16 ABP1, BNI1, C1_05380C_A, C1_07470C_A, CDC37, CDC68, CYP1, HSP60, HSP90, ILV5, MYO1, RAD16, ABP1, REX2, TRA1, UBI3
Response to stress	17 ASC1, BNI1, C1_05380C_A, C6_03320W_A, CDC19, CDC37, ECM4, GAD1, GPH1, GRE3, HSP12, PIL1, POL1, RAD16, SSB1, TRA1, TSA1	22 AHP1, CIP1, CR_04720C_A, DYN1, FAB1, GLX3, GND1, HSP21, HSP60, HSP90, IRA2, KAR2, MET6, MMS22, PMA1, PRX1, SLN1, SPT5, TEL1, TPI1, YNK1, ZWF1	31 AHP1, ASC1, BNI1, C1_05380C_A, C6_03320W_A, CDC19, CDC37, CIP1, ECM4, GAD1, GLX3, GND1, GPH1, GRE3, HSP12, HSP21, HSP60, HSP90, MET6, MMS22, PIL1, PMA1, POL1, PRX1, RAD16, SSB1, TPI1, TRA1, TSA1, YNK1, ZWF1
Pathogenesis	5 LPD1, BNI1, CHK1, CSH1, LEU2	2 HSP21, HSP90	7 LPD1, BNI1, CHK1, CSH1, HSP21, HSP90, LEU2
Biofilm formation	4 C1_05380C_A, CHK1, CSH1, ADH1	1 YWP1	4 C1_05380C_A, CHK1, CSH1, ADH1
Lipid metabolic process	3 ERG20, SAH1, ACC1	4 ERG10, FAB1, FAS1, INO1	4 ERG20, INO1, SAH1, ACC1

Table 4.23, continued

Categories	Specific proteins expressed in both planktonic and biofilm cultures upon exposure to AbA	Proteins detected in both planktonic and biofilm cultures upon exposure to DMSO	Proteins detected in both planktonic and biofilm cultures upon exposure to AbA
Filamentous growth	10 ASC1, BNI1, CDC19, CHK1, ECM4, LPD1, PGI1, SSB1, TPI1, TSA1	12 CR_10290C_A, DYN1, ENO1, FAB1, HSP21, HSP90, PGI1, SLN1, SPT5, TPI1, YNK1, ZCF5	14 ASC1, BNI1, CDC19, CHK1, ECM4, ENO1, HSP21, HSP90, LPD1, PGI1, SSB1, TPI1, TSA1, YNK1
Chemical response	12 ADO1, AHP1, ASC1, BNI1, C6_03320W_A, CDC37, CHK1, EFT2, GAD1, GRE3, PRX1, TSA1	18 AHP1, BRO1, C1_10820C_A, C4_02960W_A, CIP1, FAB1, FAS1, GLX3, GND1, HSP21, HSP90, IRA2, KAR2, PRX1, SLN1, SSA2, UTP22, ZWF1	19 ADO1, AHP1, ASC1, BNI1, C6_03320W_A, CDC37, CHK1, CIP1, EFT2, GAD1, GLX3, GND1, GRE3, HSP21, HSP90, PRX1, SSA2, TSA1, ZWF1

CHAPTER 5: DISCUSSION

5.1 Overview of the study

This study was carried out in three phases. In the first phase of the study, *in vitro* susceptibility of the clinical isolates of various *Candida* spp. to AbA was evaluated. The antifungal effects of AbA on planktonic and biofilm cultures were studied. All the *Candida* isolates were then screened for the ability to develop biofilm using a crystal violet assay. The biofilm-forming strains were subjected to *in vitro* susceptibility testing. In the second phase, the morphological alterations caused by AbA on planktonic and biofilm cultures were investigated using field emission scanning electron microscopy. The diameters of the untreated and treated cells were compared. The changes in the cellular responses of planktonic and biofilm cells of *C. albicans* SC5314 were investigated using LCMS/MS approach to identify changes in the protein expression of both cultures after exposure to AbA.

5.2 *In vitro* susceptibility of clinical isolates of *Candida* spp. (for planktonic and biofilm-grown cells) and non- *Candida* spp. to Aureobasidin A (Aba)

Antifungal susceptibility testing

C. albicans isolates apparently had two times higher planktonic MIC_{50} (2 µg/ml) and MIC_{90} (4 µg/ml) as compared to the non-albicans *Candida* isolates (Table 4.1). Overall, the AbA planktonic and biofilm MICs of *Candida* yeasts in this study were higher than those reported in a previous study (Tan & Tay, 2013). This is probably due to the inclusion of a larger number of *Candida* isolates for testing in this study. Additionally, the increase in the isolates exhibiting higher planktonic MICs (> 1 µg/ml), as compared to the previous study (Tan & Tay, 2013), could be due to the variation in drug preparation or differences in the cellular response of *Candida* spp. to AbA.

The AbA biofilm MICs for all *Candida* isolates in this study were at least two folds higher as compared with their respective planktonic MICs. *C. parapsilosis* had the highest biofilm MIC50s, compared to *C. albicans* and *C. tropicalis* in this study (Table 4.1). The reduced susceptibility of *Candida* biofilm cultures compared to planktonic cultures to several antifungal drugs has been related with various mechanisms of resistance, such as expression of resistance genes, efflux pumps, and the existence of 'persister' cells (Raad *et al*., 2008). *Candida* efflux pumps have shown to contribute to drug resistance during the early phase of biofilm growth, while their role in resistance in mature biofilms appears to be minimal (Ramage, Bachmann, Patterson, Wickes, & López-Ribot, 2002; Mukherjee, Chandra, Kuhn, & Ghannoum, 2003). Persister cells are subset of cells that lie deep in a biofilm and exhibit tolerance to multiple drug classes, including amphotericin B, azoles and chlorhexidine (Taff, Mitchell, Edward, & Andes, 2013). The mechanism of *C. albicans* persister cell transition remains unclear. Some results show the possibility that the transition to a persister cell involves changes in both cell membrane and cell wall. Transcriptional analysis of the persister cells shows differential regulation of genes involved in both ergosterol (ERG1 and ERG25) and β-1,6 glucan (SKN1 and KRE1) pathways (Khot *et al*., 2006).

Biofilm growth morphology, biomass, and metabolic activity on AbA susceptibility

The effects of biofilm growth morphology, biomass, and metabolic activity on AbA susceptibility are shown in Table 4.3. *Candida* isolates exhibiting filamentous growth, high biomass and high metabolic activity demonstrated at least fourfold lower MIC_{50} (32 µg/ml), compared to those exhibiting yeast growth, lower biomass and metabolic activity (≥128 µg/ml). In a recent study, Marcos-Zambrano *et al.* (2014) reported higher susceptibility of *Candida* isolates with high biofilm metabolic activity to micafungin. The authors explained that since a high degree of fungal wall biosynthesis was involved in biofilm formation, this may cause the yeasts to become

more susceptible to the antifungal drug (Marcos-Zambrano, Escribano, Bouza, & Guinea, 2014). The similar explanation may apply to this study, as higher susceptibility to AbA was noted in biofilm cultures exhibiting high metabolic activity (Table 4.2). The high metabolic activity in *Candida* biofilm cultures is often correlated with filamentous growth of *Candida* biofilm (and thus higher biomass as compared to yeast growth). Hence, it is not surprising that higher susceptibility to AbA was also noted in biofilm cultures exhibiting filamentous growth with high biomass (Table 4.3).

Parameters for antifungal testing

Determination of a suitable concentration of AbA for testing is essential as the effect of the antifungal drug on protein expression may not be detectable if the drug concentration is too low. The yeasts may be killed if the drug concentration is higher than its MIC. A 0.5× minimum inhibitory concentration (MIC) was chosen for testing in this study. This drug concentration is also used in other previous investigations using different classes of antifungal drugs (Zhang *et al*., 2002; De Backer *et al*., 2001). A minimal medium such as synthetic dextrose (SD) medium was used in the experiments as nutrient-rich media might compensate for the inhibitory effects of the drug and obscured detection of some gene expression responses. Two independent "biological replicates" were included in the experimental design of this study.

As shown in Figure 4.15 and 4.16, low concentrations (0.5x and 0.25x MIC) of AbA appeared to demonstrate higher levels of antifungal activity to planktonic and biofilm cultures as compared to the use of a higher concentration (1x MIC) of AbA. The finding in this study showed that 1X MIC of AbA was unable to reduce the growth level of planktonic and biofilm culture as effectively as those compared to 0.5x and 0.25x MIC of AbA. Referring to Figure 4.15, the planktonic culture treated at 1x MIC continued to grow more actively (demonstrated by the OD reading) compared to the culture treated at 0.5x and 0.25x MIC. The growth of the yeast cells was reduced as

soon as both the cultures were exposed to 0.5x and 0.25x MIC of AbA. The same results were observed repetitively. Further experiment is warranted to identify the underlying reason of this condition. The prominent growth inhibition of biofilm cells could be due to the sensitivity of the biofilm cells towards AbA as compared to the planktonic cells (Figure 4.15 and 4.16). The overall effect of DMSO on planktonic culture was not as prominent as compared to the biofilm culture. Only minimal reduction in growth (approximately 0.9% by absorbance) of planktonic culture was observed upon exposure to 1% DMSO. In contrast, a major reduction in the growth (approximately 50% by absorbance) of DMSO-treated biofilm culture was observed when compared to untreated culture at 20th hour (Figure 4.16).

The atypical results in this assay demanded for some modification in the method. Therefore, culture plating was used in survival assay instead of the absorbance measurement. In this study, plating method provided viable counts for *Candida* cultures pre- and post-exposure to drug (Figure 4.17 and 4.18). Figure 4.17 and 4.18 demonstrate the growth of the planktonic and biofilm cells upon treatment with AbA during the mid-log phase. As 50% growth reduction was seen within 2 to 4 hours after exposure to drug (Figure 4.17 and 4.18), this study has selected 2.5 - 3 hours as the time for drug exposure for subsequent studies.

5.3 Determination and comparison of the morphological changes of AbA-treated planktonic and biofilm cultures of *Candida* spp.

Field emission scanning electron microscope (FESEM) analysis on Candida planktonic and biofilm culture

Morphological changes of yeast cells in the presence of antifungal drugs have been reported by Rueda *et al*. (2014). The morphological changes such as enlarged size, abnormal septa, and absence of filamentation were seen in *Candida* yeasts when exposed to caspofungin (Rueda *et al*., 2014).

In this study, the increase in the size of blastoconidia in Figure 4.10 (B3) was found to be significant with *Candida* planktonic culture upon exposure to 2 µg/ml AbA (Table 4.4), whereas complete elimination of filamentous cells and morphological alterations (rough and distorted cell surfaces) were noted from *C. albicans* biofilm culture treated with 32 µg/ml AbA. The exact mechanism behind the shrinkage of the treated filaments ($p>0.05$) is not known. AbA susceptibility has been related with lipid rafts which are important for *Candida* biofilm formation (Mukherjee *et al*., 2003). Lipid rafts are known to be the polarization of sterol- and sphingolipid-enriched domains and it has been linked to morphogenesis and cell movement in diverse cell types (Martin & Konopka, 2004). Phosphatidylinositol (PI), a critical component of lipid rafts has been shown to contribute to antifungal resistance (Mukherjee *et al*., 2003). Interestingly, FESEM analysis of *C. albicans* biofilm culture in this study corroborated with the antifungal susceptibility results. Antifungal effects were seen when 8 µg/ml (0.25x MIC) and 32 µg/ml (1x MIC) were used in susceptibility testings and FESEM analysis. The detachment of *C. albicans* biofilm and almost complete elimination of filaments (some yeast cells left) are suggestive of the AbA antibiofilm or inhibitory effect.

5.4 Investigation of the cellular responses of *C. albicans*, pre- and post-exposure to AbA using liquid chromatography mass spectrometry (LCMS) analysis

5.4.1 SDS-PAGE

SDS-PAGE analysis showed different intensity of protein bands (ranging from 15 to 75 kDa) present in the samples extracted from *C. albicans* biofilm culture, as compared to those prepared from the planktonic cultures. The high intensity of the protein bands could be due to the upregulation of proteins in response to the presence of AbA. As SDS-PAGE profiles were not able to reveal the identity of the proteins

involved, LCMS was used to provide detailed insight on the protein profiles of AbA-treated planktonic and biofilm cultures in this study.

5.4.2 Protein profiles of *C. albicans* planktonic and biofilm cultures using LCMS analysis

LCMS analysis is capable to identify a large number of proteins in comparison to SDS-PAGE and 2D gel electrophoresis (as reported in other studies). The protein profiles of 6 samples extracted from *C. albicans* SC5314 (untreated, DMSO- and AbA-in both treated planktonic and biofilm cultures) were described in this study. Overall, a large number of proteins with different roles and functions were annotated in *C. albicans* biofilm cultures compared to the planktonic cultures. The difference in the profiles of cell surface proteins of *C. albicans* planktonic and biofilm cells has also been reported by Gil-Bona *et al*. (2016) using LCMS analysis. In their study, the investigators identified a total of 943 proteins, of which 438 proteins were from yeast form and 928 from hyphae form (approximately two times higher than that in the yeast cells). The number of proteins annotated from the untreated biofilm culture in this study was also approximately two times higher than those annotated from the planktonic culture (Table 4.7). The findings in this study are thus in support of a previous study that biofilm culture is a more complex entity as compared to its planktonic counterpart (Stoodley *et al*., 2002).

5.4.3 LCMS analysis for proteins from untreated cultures

In this study, a total of 414 proteins in the biofilm culture and 163 proteins in the planktonic culture were annotated using gene ontology in accordance to biological processes, molecular functions and subcellular localization (Table 4.8 - 4.10), using Candida genome database (http://www.candidagenome.org/).

Comparison in biological processes

A total of 249 biological processes were annotated from *C. albicans* planktonic and biofilm cultures, of which 41 and 103 were specific to planktonic and biofilm cultures (Figure 4.20) and 105 biological processes were shared by both cultures. The percentage of proteins annotated for cell cycle, regulation of response to stress/stimulus, and regulation of nitrogen compound metabolic process within the cultures varied (ranging from 11 to 26.3%) in both cultures (Table 4.13).

The biofilm culture exhibited a higher percentage (61.7%) of proteins for regulation of macromolecule metabolic process, as compared to the planktonic culture (38.0%) (Table 4.13). In a comprehensive analysis of *C. albicans* biofilm matrix, Zarnowski *et al*. (2014) reported a composition of protein (55%), carbohydrate (25%), lipid (15%), and nucleic acid (5%). Three individual polysaccharides and matrix lipids consisting of neutral glycerolipids (89.1%), polar glycerolipids (10.4%), and sphingolipids (0.5%) were also reported. In addition, examination for matrix nucleic acid had also revealed DNA, primarily, noncoding sequences. The proteomic analysis of functional matrix revealed 458 distinct activities (Zarnowski *et al*., 2014). In this study, the complexity of *C. albicans* biofilm culture was reflected by the higher number of proteins (n=103) annotated with biological processes than those of planktonic culture (n=41) (Table 4.11 and Table 4.12). It is possible that the higher number of biological processes observed in *C. albicans* biofilm culture in this study are needed to maintain structural complexity and to keep the survival of yeast cells in the biofilm matrix.

Of the 41 biological processes specific to planktonic culture in this study (Table 4.11), many (n=17, 41.5%) are associated with metabolic processes such as nucleobase-containing compound (42%), monocarboxylic acid (14%), purine-containing compound (14%), single-organism carbohydrate (14%), ATP (12%), nucleoside monophosphate (12%), nucleoside triphosphate (12%) etc. In contrast, 62 (51.3%, total = 103)

biological processes specific to biofilm culture are associated with biological processes that involves regulation (including for biosynthetic process [53.9%], macromolecule biosynthetic process [51.3%], gene expression [47%], nucleobase-containing compound metabolic [45.2%] etc.).

Higher percentages of proteins are annotated in the planktonic culture (Table 4.8), particularly for RNA metabolic process (16% vs 11.8%) and ribosome biogenesis (8% vs 4.8%). According to Candida genome database (CGD), RNA metabolic process (GO: 0016070) refers to cellular chemical reactions and pathways involving RNA, ribonucleic acid, one of the two main types of nucleic acid, while ribosome biogenesis (GO: 0042254) refers to a cellular process that results in the biosynthesis of constituent macromolecules, assembly, and arrangement of constituent parts of ribosome subunits; includes transport to the sites of protein synthesis. The enrichment of these proteins reflects the active growth process that was undergoing in the planktonic culture.

Li and Tian (2016) reported high cell density and close proximity of cells in biofilm cultures of microorganisms. Organisms interact with each other in the biofilm cultures and develop complex interactions that can be either competitive or cooperative. Competition between species is a well-recognized ecological force to drive microbial metabolism, diversity and evolution, while cooperative activities are recognized to play important roles in microbial physiology and ecology. The overall function, biomass, diversity and pathogenesis of biofilm culture could be affected by these microbial interactions. Additionally, different dynamic states and the up-regulation and down-regulation of distinct sets of genes have been reported in bacterial planktonic and biofilm cultures (Nakamura *et al*., 2016). For instance, genes involved in iron-sulfur metabolism, lipid metabolism, amino acid and carbohydrate transport, biosynthesis of secondary metabolites, stress response and efflux system components are up-regulated during biofilm formation (Guilhen *et al*., 2016, Rumbo-Feal e*t al*., 2013, Nakamura *et*

al., 2016) whereas DNA repair genes are down regulated in biofilm cells (Nakamura *et al*., 2016). Additionally cells dispersed from biofilms are transcriptionally closer to their parent cells than to planktonic cells and display specific phenotypes with a high adaptive ability allowing the colonization of new environments (Guilhen *et al*., 2016). However, biofilm cells and newly dispersed cells also differ; for example, overexpression or higher response was observed in the planktonic growth mode (Guilhen *et al*., 2016; Nakamura *et al*., 2016).

In this study, besides a high percentage of proteins annotated for regulation of biological process (30.7% in planktonic culture vs 27.8% in biofilm culture), the percentage of protein annotated for vesicle-mediated transport (3.1% in planktonic culture vs 6.8% in biofilm culture) was higher in the biofilm culture (Table 4.8). This is also supported by a higher percentage of proteins (15.9% in biofilm culture vs 8.6% in planktonic culture) annotated for endoplasmic system (when subcellular localization of the proteins was analyzed) in the *C. albicans* biofilm culture investigated in this study (Table 4.9). Vesicle-mediated transport (GO:0016192) is a cellular transport process in which transported substances are moved in membrane-bounded vesicles and transported substances are enclosed in the vesicle lumen or located in the vesicle membrane. The process begins with a step that directs a substance to the forming vesicle, which includes vesicle budding and coating. Vesicles are then targeted to, and fuse with, an acceptor membrane (CGD).

Environmental stress has been reported to affect protein transport into the vacuole of *C. albicans* (Franke *et al*., 2006). Additionally, the mode of repression of inositol phosphorylceramide synthase (IPC), the target of AbA, has been reported to affect vesicular traffic between Golgi apparatus, endosomes, and vacuole which are critical for cell survival in IPC-repressed cells (Endo *et al*., 1997). This process may

induce signals for the activation of virulence signaling pathways and the start of the pathogenic life cycle (Franke *et al*., 2006).

It was noted in this study that the percentage of proteins annotated to cellular homeostasis of the untreated biofilm culture was higher than that of planktonic culture (3.9% in biofilm culture as compared to 1.2% in the planktonic culture) (Table 4.8). According to Candida genome database, cellular homeostasis (GO:0019725) refers to any process involved in the maintenance of an internal steady state at the level of the cell. Homeostasis results from a dynamic balance of microbial–microbial and microbial–host interactions and when the homeostasis is disturbed in a community, the self-regulatory mechanisms may restore the previous homeostasis status in the community, by balancing cooperative and competitive activities in microbial communities.

In this study, only two proteins (AHP1 and PMA1) were annotated for cellular homeostasis in the untreated planktonic culture, while 16 were annotated in the untreated biofilm culture (Appendix C, Table 1, pg 210). This could be explained by the diverse microbial composition that keeps biofilm stable over time.

Comparison in subcellular localization of the proteins

The results in this study demonstrated higher percentages of proteins in planktonic culture were associated with the nucleus, plasma membrane and cell wall of *C. albicans* (Table 4.9), whereas, a majority of proteins from biofilm culture were also associated with the endomembrane system. The variation in the protein expression patterns of both forms of cultures may be due to the differences in the morphology and proliferation of *C. albicans* in different culture conditions. Rapid proliferation of yeast cells in the planktonic culture was reflected in this study with the observation of diverse protein changes in the nucleic acid, plasma membrane and cell wall. These changes are

probably required for biological processes such as RNA metabolic process, response to chemical, and ribosome biogenesis (Table 4.8). Since the biofilm cells were isolated during mature stage (after 24 hours of incubation), the proliferation of the sessile cells might not be as active as those of the suspension cells in the planktonic culture. However, as protein synthesis and transport system are still required to keep the biofilm cells functioning, active protein transport system is a necessity. This was demonstrated by the observation of high percentages of proteins annotated for vesicle-mediated transport and endomembrane system in *C. albicans* biofilm culture in this study (Table 4.8), whereby the endomembrane system and endoplasmic reticulum are believed to be the main cellular components involved in these biological processes.

Comparison in molecular functions of proteins

Major difference was not observed in the molecular functions of the biofilm and planktonic cultures except for a slightly higher percentage (by 3.2%, Table 4.10) of proteins annotated with lyase activity (GOID:16829) in the planktonic culture. The lyase activity is known to involve the catalysis of the cleavage of C-C, C-O, C-N and other bonds by other means apart from hydrolysis or oxidation. Lyase activity can also take place by conversely adding a group to a double bond. A total of 5 proteins i.e., ENO1 (Enolase 1), GLX3 (Glutathione-independent glyoxalase), FBA1 (Fructose-bisphosphate aldolase), MET15 (O-acetylhomoserine aminocarboxypropyltransferase), and PDC11 (Pyruvate decarboxylase) were annotated with lyase activity in both untreated cultures of planktonic and biofilm (Appendix C, Table 1, pg 210). The functions of the proteins are shown in Table 1, Appendix E (pg 216).

5.5 DMSO treatment of biofilm and planktonic cultures

In this set of experiment, the protein profiles of DMSO-treated planktonic and biofilm cultures served as the background for identification of proteins specifically

induced by AbA. DMSO was used for preparation of AbA stock solution. It is a highly polar and stable substance with exceptional solvent property for antifungal drugs (Randhawa, 2008). DMSO 2% has been reported to significantly inhibit the growth of *Candida* species by broth dilution method, while 1% and below had insignificant effect (Randhawa, 2008; Rodríguez-Tudela *et al*., 2001). In this study the concentration of DMSO in the AbA working solutions (1 μg/ml) was 1%. The number of proteins detected in DMSO and AbA-treated cultures in this study was higher as compared to untreated cultures (Table 4.7), suggesting some effects of DMSO on *C. albicans* growth (for both planktonic and biofilm cultures). Hence, to understand the true effect of AbA on *C. albicans* cultures, a comparison was made between DMSO and AbA-treated *Candida* cultures.

5.6 Cellular response of *Candida* cultures to AbA

Effects of AbA on planktonic culture

In this study, a total of 293 and 374 proteins are annotated in the AbA-treated planktonic and biofilm cultures (Table 4.7). One of the distinct finding is the lower percentage of proteins annotated for organelle organization in the AbA-treated planktonic culture (18.1%) as compared to that of the DMSO-treated planktonic culture (23.2%) (Table 4.18). Organelle organization (GO:0006996) refers to a process that is carried out at the cellular level for the assembly, arrangement of constituent parts, or disassembly of an organelle within a cell (Candida Genome Database), which includes the nucleus, mitochondria, plastids, vacuoles, vesicles, ribosomes and the cytoskeleton, but excluding the plasma membrane.

The finding in this study shows that a total of 42 proteins annotated for organelle organization are found to be specific to AbA-treated planktonic culture (Appendix D, Table 1, pg 213). A search against CGB database shows that 29 (64.4%) of the proteins

are associated with the regulation of biological process and 16 (35.6%) are associated with response to stress (Appendix D, Table 1, pg 213). The 8 proteins (DOT1, HSP60, HSP90, ILV5, KEM1, MTG1, MYO1 and SPT20) annotated for organelle organisation in both AbA- treated planktonic and biofilm cultures and their functions are shown in Table 2 in Appendix E (pg 217).

Another finding observed in this study is the 5% increase in the proteins annotated for translation in AbA-treated planktonic culture (Table 4.18). The increase in the percentage of these proteins could be due to the production of proteins associated with stress, as also evidenced by the predominance of biological processes associated with nucleobase-containing compound metabolic process (35.6% proteins annotated) cellular protein modification process and protein modification process (23.3%) and reproductive process (18.9%) (Table 4.21).

Effects of AbA on Candida biofilm culture

It was observed in this study that the exposure to AbA caused a reduction in the percentage of proteins annotated for vesicle-mediated transport (from 8.4 to 5.3%) and filamentous growth (from 15.8 to 12.8%) and an increase in the percentage of proteins annotated for carbohydrate metabolic process (from 8.2 to 12%) and cellular homeostasis (from 4.6 to 16.4%) in the biofilm culture (Table 4.19). The importance of vesicle-mediated has been discussed under *comparison in biological proceses* (pg 147). AbA might affect the biological process involved in the vesicle-mediated transport as the number/percentage of proteins annotated for this process were reduced into almost half ([from 8.4 to 5.3%] , Table 4.19). The percentage of protein annotated for vesicle-mediated transport was reduced to 5.3% in AbA-treated biofilm culture in this study (Table 4.19). One of the putative vesicle transport protein, Vac1, has a role in protein transport to the vacuole, and has been identified as a novel and interesting target for antifungal drugs (Franke *et al*., 2006). However, this protein was not

annotated in this study. Instead, phosphatidylinositol (PI) 3-kinase (Vps34) of *C. albicans* was annotated in the planktonic culture (DMSO-treated) and biofilm culture (untreated and AbA-treated) in this study (Appendix C, Table 1, pg 210). VPS34 is a key enzyme of the vacuolar protein transport, and is required for *Candida* virulence (Franke *et al*., 2006). The defect/mutation in this gene (as demonstrated through the use of a *vps34* null mutant strain) has been related to *Candida* avirulence in a mouse model of systemic candidiasis, and its inability to form hyphae on different solid media, delayed yeast-to-hyphae transition in liquid media, hyperfilamentation under microaerophilic/embedded conditions, hypersensitivity to high temperature and hyperosmotic stress, and reduced adherence to human cells (Bruckmann *et al*., 2000). Besides VPS34, all the proteins that were annotated with vesicle-mediated transport are shown in Appendix C, Table 1, pg 210.

Similarly, the percentage of proteins annotated for filamentous growth was reduced from 15.8 to 12.8% (Table 4.19) as compared to the DMSO-treated cultures in this study. A total of 26 proteins annotated for filamentous growth, i.e, BRE1, C3_02760C_A, C3_07680W_A, C7_03480W_A, CAS4, SHP1CAT8, CCH1, CCT8, CTA4, DCK1, DCK2, DEF1, ECM4, FGR51, HGT12, IRS4, KAR3, KRE5, LPD1, PMI1, SSB1, STN1, TSC11, UBI4, and VPS34 were annotated in *C. albicans* biofilm culture upon exposure to AbA treatment in this study. When these proteins were analysed using Slim mapper of the CGD database, majority of the proteins were associated with response to stress (69.2%) and regulation of biological process (61.5%) (Appendix D, Table 2, pg 214).

Carbohydrates constitute an important component of biofilms (Branda *et al*., 2005). The carbohydrate metabolic process (GOID: 5975) refers to the chemical reactions and pathways involving carbohydrates, any organic compounds based of the general formula Cx(H2O)y (Candida genome database). This includes the formation of

carbohydrate derivatives by the addition of a carbohydrate residue to another molecule. Carbohydrates are the primary source of metabolic carbon for most organisms, and are used for generating energy and producing biomolecules. Many of the proteins annotated for carbohydrate metabolic process are amongst those proteins involved in carbohydrate metabolism (Figure 5.1) (Santana *et al*., 2013). According to Askew *et al*., (2009), most sugars are converted to glucose 6-phosphate or fructose 6-phosphate before entering the glycolytic pathway. These hexose phosphates will then be converted into key metabolite pyruvate for production of ATP and NADH (Figure 5.1) through glycolysis which is critical for carbon assimilation

In this study, the percentage of proteins annotated for carbohydrate metabolic process has increased from 8.2 to 12.0% (Appendix C [pg 210]) in the AbA-treated biofilm culture as compared to the DMSO-treated biofilm culture. The 20 proteins (AMS1, C1_14060W_A, C5_01230C_A, C5_04940W_A, GDB1, GDE1, GLC3, GPH1, GRE3, GSL2, KAR2, KRE5, MDH1, MLS1, MNN21, PFK2, PFK26, PGM2, PMI1, and SHP1) annotated for carbohydrate metabolic process were found to be linked to biological process for generation of precursor metabolites and energy (35.0%), response to chemical (25%), response to stress (25%) and transport (15%), as shown in Appendix D, Table 3, pg 215.

Two proteins, AHP1 and PMAI were annotated with the function of cellular homeostasis in AbA-treated planktonic and biofilm cultures. AHP1 (alkyl hydroperoxide reductase) is an immunogenic protein that is induced by fluconazole, but suppressed by amphotericin B, and caspofungin (CGD database). It is regulated by Ssk1/Nrg1/Tup1/Ssn6/Hog1 and is flow model biofilm induced. PMA1 (Plasma membrane H(+)-ATPase) is highly expressed and comprises 20-40% of total plasma membrane protein. Similar to AHP1, it is induced with the presence of fluconazole, but repressed by the presence of caspofungin. Similar to the vesicle-mediated transport, all

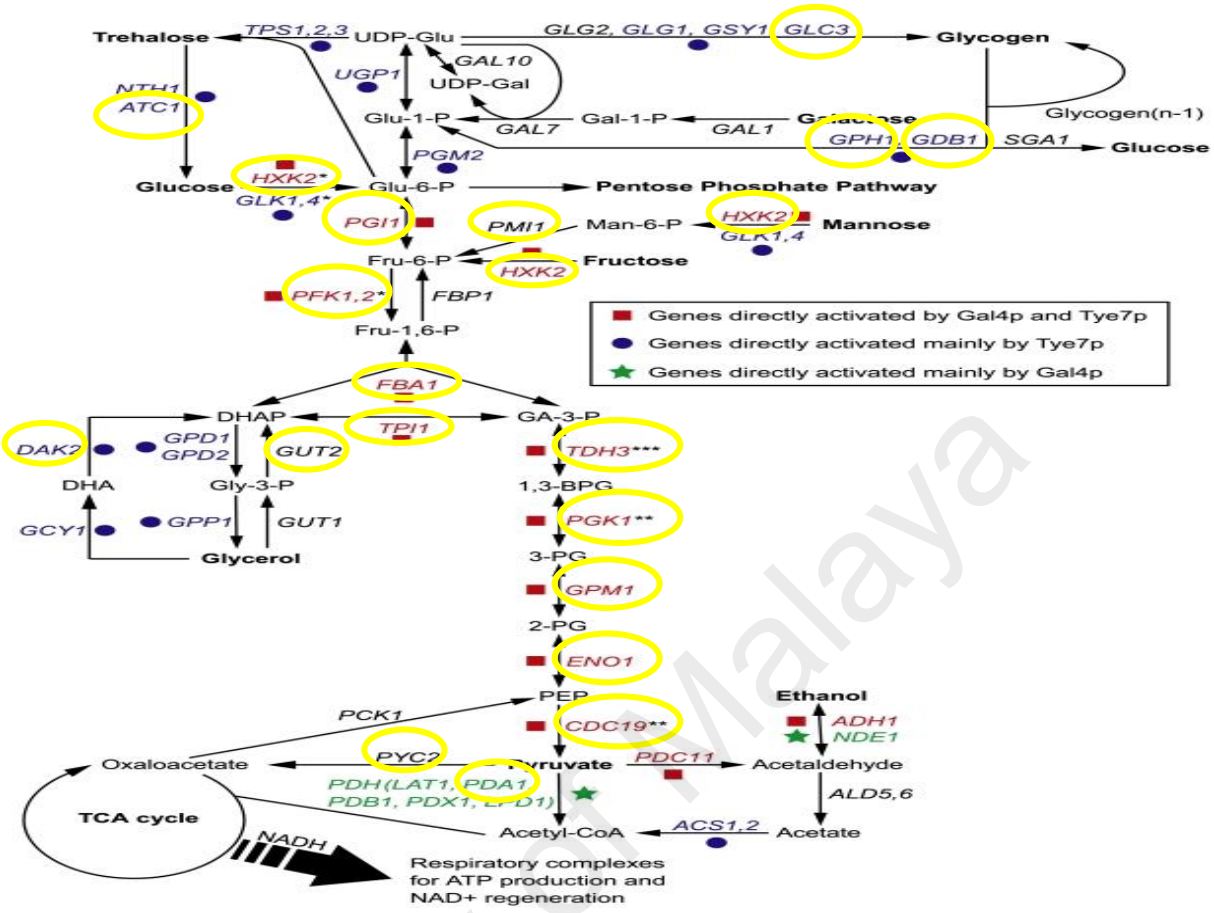


Figure 5.1: The central metabolic pathways of *S. cerevisiae* yeast (Askew et al., 2009). The enzyme names are for *C. albicans* but some have not been directly characterized and are annotated based on *S. cerevisiae* homology. For simplicity, ‘‘*’’ represents reactions requiring ATP, ‘‘’’ represents reactions producing ATP, and ‘‘***’’ represents reactions generating NADH. These symbols are given for reactions of the glycolytic pathway only (Askew *et al.*, 2009). Those proteins that are annotated in this study are highlighted in yellow (refer to carbohydrate metabolic process, Appendix C, pg 210).**

the proteins annotated to cellular homeostasis were shown in Appendix C, Table 1, pg 210.

5.7 Effect of AbA on regulation of cellular amide and actin cytoskeleton organization of *C. albicans* planktonic and biofilm cultures

Among the biological processes annotated in AbA-treated planktonic and biofilm cultures, the regulation of cellular amide and actin cytoskeleton organization are shared by both cultures, suggesting that they are important in the cellular responses of *C. albicans* to AbA (Table 4.20). Endo *et al*. (1997) reported that the end points of sphingolipid synthesis in fungi are inositol phosphorylceramide (IPC) and its derivatives (McConville & Bacic, 1989). IPC synthase is the enzyme that catalyzes the formation of IPC which is important in the regulation of intracellular levels of sphingolipids and ceramide (Lester & Dickson, 1992). Upon exposure to AbA treatment, it is expected that the total synthesis of sphingolipids will decrease while the ceramide will increase. The lipid synthetic pathway is blocked by ceramide. In yeast, as well as in mammalian cells, the increased levels of ceramide have been linked to cell cycle arrest and apoptosis (Nagiec *et al*., 1997). Therefore, the increased relative levels of cellular ceramide could induce cell death and account for the inhibition of total sphingolipid synthesis. Additionally, it has also been reported that IPC synthesis is required for the proper organization of filamentous yeast cells (Cheng *et al*., 2001). Both accumulation of ceramide and/or the lack of a lipid inhibited by aureobasidin A is believed to cause cell death in *C. albicans.*

The regulation of actin cytoskeleton organization refers to any process that modulates the frequency, rate or extent of the formation, arrangement of constituent parts, or disassembly of cytoskeletal structures comprising actin filaments and their associated proteins (http://www.ebi.ac.uk). According to Gross and Kinzy (2007), actin organization in the *S. cerevisiae* yeast produces two major types of filament-based

structures such as patches and cables. These provide the structural basis for cell morphology, polarity, and endocytosis (Gross & Kinzy, 2007). The effect of AbA on the regulation of actin cytoskeleton organization would probably lead to disorientation in the yeast cell formation. This could also explain the distorted cell morphology of biofilm cultures observed in FESEM analysis of this study (Figure 4.12).

The increase in the translation upon exposure to AbA seems to support the finding of Kajiwara *et al*. (2012) who elucidated the reduction of complex sphingolipids caused by AbA might result in endoplasmic reticulum (ER) retention of GPI-anchored proteins, ER stress, an unfolded-protein response, reactive oxygen species (ROS) production, mitochondrial cytochrome *c* release, and a metacaspase-mediated form of apoptosis that additionally is dependent on the concomitant increase of cytosolic Ca^{2+} concentrations (Kajiwara *et al*., 2012). The ER stress has been evidenced by an increase in the vesicle-mediated transport as discussed earlier in this study. The accumulation of ROS appears to be involved in the mechanism of action for azole drugs (Bink *et al*., 2011). ROS productions exist in AbA-treated biofilm culture. The reactive oxygen species metabolic process refers to the chemical reactions and pathways (GOID term: 72593) involving a reactive oxygen species, any molecules or ions formed by the incomplete one-electron reduction of oxygen. They contribute to the microbicidal activity of phagocytes, regulation of signal transduction and gene expression, and the oxidative damage to biopolymers (Voynova *et al*., 2015).

In this study, 5 proteins (TTR1, SOD5, TSA1, C6_00850W_A and TRX1) were annotated for reactive oxygen species metabolic process (GO ID: 72593) in the AbA-treated biofilm cultures (Appendix E, Table 3, pg 218). Sun *et al*. (2013) elucidated that TSA1 plays an important role in intracellular redox homeostasis. TSA1 appears to function primarily as an antioxidant in protecting both the cytosol and actively translating ribosomes against endogenous ROS (reactive oxygen species), but shifts

towards its chaperone function in response to oxidative stress conditions (Trotter *et al*.,2008). *C. albicans* expresses SOD5 (superoxide dismutase 5), a cell wall protein related to Cu/Zn SODs, to combat the host immune response. SOD5-like molecules are present in many fungal pathogens and appear to be specialized for the metal and oxidative challenges presented by the host immune system (Gleason *et al*., 2014). TSA1, TTRI and TRX1 are some of the proteins involved in the antioxidant systems mediate hydrogen peroxide (H_2O_2) detoxification in *C. albicans.* TTR1, a glutaredoxin, involves in the glutathione system that repairs oxidatively damaged protein thiols. The thioredoxin system detoxifies H2O2 via peroxiredoxin (Tsa1), which uses the reductant thioredoxin (TRX1) (Komalapriya *et al*., 2015).

5.8 Analysis of *C. albicans* proteins annotated to pathogenesis, biofilm formation, lipid metabolic process, filamentous growth and chemical response

In this study, *C. albicans* proteins that were annotated for pathogenesis, biofilm formation, lipid metabolic process, filamentous growth and chemical response were also investigated.

5.8.1 Pathogenesis

It has been reported that *C. albicans* is able to grow in different morphological forms. The changes in the morphological forms and the ability to switch between yeast and hypha are essential for the virulence of *Candida* (Kojic & Darouiche, 2004; Martins *et al*., 2010; Robbins *et al*., 2011). The yeast form is essential for dissemination of *Candida* infection to different host niches *via* the bloodstream. The hyphal form is invasive and enables the organism to evade phagocytic cells (Donlan, 2001; O'toole, 2003).

A total of 25 (6.0%) and 7 (4.3%) of the proteins from untreated biofilm and planktonic culture were associated with pathogenesis (Appendix B). HSP 90 and HSP

21 were two common proteins identified from both DMSO- treated planktonic and biofilm cultures. HSP21 is a small heat shock protein which plays a role in stress response and virulence of *C. albicans*. This protein is fluconazole-downregulated and induced in cyr1 or ras1 mutant. HSP90 is an assential chaperone which regulates several signal transduction pathways and temperature-induced morphogenesis. This protein is activated by heat shock and stress. It localizes to surface of hyphae, not yeast cells and also mediates echinocandin and biofilm azole resistance (CGD database).

Upon AbA treatment, these proteins became undetectable or most likely down-regulated, instead, 5 other proteins were detected or upregulated after AbA treatment in both planktonic and biofilm cultures. These proteins are ASC1, BNI1, CHK1, CSH1 and LEU2 (Table 4.23). ASC1 (Guanine nucleotide-binding protein subunit beta-like protein) is a 40S ribosomal subunit similar to the G-beta subunits. ASC1 promotes glucose or N starvation- induced filamentation. It is repressed in stationary phase and required for virulence in mice. BNI1, also called formin, has a role in hyphal cytoskeletal polarity. BNI1 goes through synthetic lethality if Bnr1p and Bni1p are absent. CHK1 is histidine kinase which is involved in 2-component signaling, cell wall synthesis and hyphal growth defect. CHK1 is bcr1-induced protein in biofilm. CSH1 is aldo-keto reductase which has a role in fibronectin adhesion, cell surface hydrophobicity and is regulated by temperature. It is also associated to azole resistance. LEU2 is isopropyl malate dehydrogenase which is involved in leucine biosynthesis (CGD Database).

5.8.2 Biofilm formation

A total of 12 (2.9%) and 5 (3.1%) proteins from biofilm (n=414) and planktonic cultures (n=163) were annotated for biofilm formation (Appendix B), respectively, of which 10 and 3 were specific to biofilm and planktonic cultures, respectively. Only one protein (YWP1) was detected when both the cultures were treated with DMSO (Table

4.23). YWP1 is secreted yeast wall protein which has a possible role in the dispersal of *C. albicans* in host. Mutation of this protein increases adhesion and biofilm formation. Four proteins (C1_05380C_A, CHK1, CSH1, ADH1) were specifically induced by AbA (Table 4.17) in both cultures. C1_05380C_A orthologs have telomeric DNA binding activity. ADH1 is alcohol dehydrogenase which oxidizes ethanol to acetaldehyde. It is localised at yeast cell surface. It is immunogenic in humans or mice, and induced by fluconazole and farnesol (CGD Database). CHK1 and CSH1 genes have been described to be associated with pathogenesis (section 5.8.1), because biofilm formation is one of the pathogenicity features of *Candida*.

5.8.3 Lipid metabolic process

A total of 31 and 8 proteins from biofilm and planktonic cultures were annotated for their association in lipid metabolic process (Appendix B). Three proteins i,e., INO1 (Inositol-1-phosphate synthase), ERG10 (Acetyl-CoA acetyltransferase) and ACH1 (Acetyl-coA hydrolase) were common for both DMSO-treated planktonic and biofilm cultures. INO1 is an upstream inositol/choline regulatory element which is repressed by farnesol in biofilm or by caspofungin. ERG10 has role in ergosterol biosynthesis and soluble in hyphae. The change in protein abundance is associated with azole resistance. The expression of this protein is also induced by fluconazole or ketoconazole. ACH1 is involved in acetate utilization. This protein is soluble in hyphae, antigenic in human and a stationary phase-enriched protein. It is specifically induced on polystyrene adherence and if exposed to farnesol and ketoconazole. While INO1 was still found on exposure to AbA, three other proteins (ERG20, SAH1, ACC1) were annotated in the AbA-treated cultures. ERG20 (putative farnesyl pyrophosphate synthetase) which is involved in the isoprenoid and sterol biosynthesis was annotated in both AbA-treated cultures, suggesting that it may have important role in drug response (CGD database). S-adenosyl-L-homocysteine hydrolase (SAH1) is involved in sulfur amino acid

metabolism, antigenic in human and involved in fluconazole-induced expression. This protein is repressed in amino acid starvation (3-AT) (reference needed/refer to Candida genome database). ACC1 (Acetyl-COA-carboxylase) is regulated by Efg1 (Enhanced filamentous growth protein 1) and repressed by amphotericin B and caspofungin.

5.8.4 Filamentous growth

Fifty one and 21 proteins of biofilm and planktonic cultures were annotated for filamentous growth (Appendix B) with 9 proteins also annotated in both DMSO-treated cultures (ADR1, CDC19, DCK1, DYN1, ENO1, HSP21, HSP90, TPI1, YNK1) (Appendix B). All the proteins have a role in biofilm formation, either causing a repression or an induction in biofilm formation. The functions of HSP21 and HSP90 have been explained in section 5.8.1. ADR1 (Transcriptional regulator ADR1) is C2H2 transcription factor, and the ortholog of *S. cerevisiae* Adr1. The transposon mutation of this gene affects filamentous growth. ENO1 (Enolase 1) was found in lyase and carbohydrate metabolic activity in both the treated and untreated conditions. It is essential in glycolysis and gluconeogenesis. Its expression in both the cultures on DMSO-treatment could be due to its protective role to survive in the energy deprivation condition. DCK1 (putative guanine nucleotide exchange factor) is required for embedded filamentous growth. It activates Rac1 and has a DOCKER catalytic domain which acts as Rac1 activator during *C. albicans* filamentous growth (Hope *et al*., 2008) . It is also similar to adjacent DCK2 and to *S. cerevisiae* Ylr422wp. CDC19 is pyruvate kinase at the yeast cell surface. It is regulated by Gcn4/Hog1/GlcNAc and induced by adherence to Hap43/polystyrene. The protein is also repressed by phagocytosis/farnesol. Its role in hyphal growth explains the annotation to molecular function of filamentous growth. The protein is commonly found in stationary phase. DYN1 (Dynein heavy chain) is a motor protein that moves itself to microtubule minus end. It is required for yeast cell separation, spindle positioning, nuclear migration and hyphal growth and

supports the development and growth of biofilm. DYN1 is regulated by Mig1 (Regulatory protein MIG1) and Hap43 (Cell wall protein IFF6). TPI1 (triose-phosphate isomerase) is present in exponential and stationary growth phase of yeast. The mutation of this gene affects filamentation. YNK1 (Nucleoside diphosphate kinase/NDP kinase) is a soluble protein in hyphae. It is also induced by flucytosine and in biofilm condition (CGD Database).

Upon AbA treatment, these proteins were not detectable in both cultures, except for CDC19. Instead, other proteins (ASC1, BNI1, CHK1, ECM4, LPD1, PGI1, SSB1, TPI1, TSA1) were annotated (Table 4.23). The presence of ASC1 was noted in both the molecular functions of pathogenesis and filamentous growth. This could be explained by justifying filamentous growth as one of the pathogenecity factor of *Candida*. BNI1 seems to have additional function in filamentous growth as explained in section 5.8. During filamentous growth, BNI1 is involved in cell-cycle regulated localization to the site of polarized growth and bud neck. It also goes through minor localization at septum. The presence of CHK1 in pathogenesis, biofilm formation and filamentous growth clearly shows that this protein is associated with the virulence of *Candida*. ECM1 (cytoplasmic glutathione S-transferase), SSB1 (heat shock protein) and TSA1 [TSA/alkyl hydroperoxide peroxidase C (Ahpc) family protein] are proteins involved in stress response. Exposure to AbA could have trigerred these proteins to be expressed. SSB1 (ribosome-associated molecular chaperone SSB1) belongs to HSP70 family. It is present at yeast cell surface but not hyphae. TSA1 can be found at hyphal surface, yeast-form nucleus, cytoplasm and in biofilm. It is also expressed in peroxide-induced oxidative stress. LPD1 (putative dihydrolipoamide dehydrogenase) is soluble in hyphae. This protein is present in exponential and stationary phase of yeast cultures. TPI1 (triosephosphate isomerase) and PGI1 (glucose-6-phosphate isomerase) are involved in

glucose pathway which explains their need during the glucose deprivation under stress condition.

5.8.5 Chemical response of *C. albicans*

This study found a higher number of biofilm proteins (n=58) responding to chemical as compared to planktonic culture (n=28) (Appendix B). There were 47 and 17 proteins annotated specifically for biofilm and planktonic cultures. Eleven (ACH1, ADR1, AHP1, CIP1, GLX3, GND1, HSP21, HSP90, KAR2, RBP1, SSA2) are common proteins which were annotated in both the DMSO-treated cultures (Appendix B). The functions of some of these proteins (ACH1, HSP21, ADR1, and AHP1) have been explained in section 5.8.3 (ACH1), section 5.8.4 (ADR1), section 5.8.1 (HSP21, HSP 90) and section 5.6 (effects of AbA on *Candida* biofilm culture, AHP1).

5.9 Limitation and future studies

One of the ways in which *C. albicans* adjust to changes in their environment is by altering protein expression patterns. Thus, measurement of changes in protein expression (protein profiling) upon exposure to a drug can help to determine how drugs work in cells and organisms. Few limitations of this study are the lack of quantitative data on the protein expression and RNA transcriptomic data. The use of Candida genome database to illustrate the proteins identified have enabled further understanding on the mechanisms of action of AbA. The LCMS technology is a powerful tool to measure protein expression on a large scale, allowing simultaneous measurement of changes in the expression of thousands of proteins. The study of protein composition of yeast and hypha morphologies may assist us in finding novel therapeutic targets.

Since it is now possible to investigate protein expression changes in response to various experimental conditions, future study should include the use of deletion mutants for *C. albicans* to facilitate the validation of novel hypotheses generated from this study,

for instance, proteins that are associated with the pathogenesis, lipid metabolic process, response to stress and etc. Further studies are required to understand the complexity of *C. albicans* growing in biofilm state in order to find new drug targets and biomarkers for *Candida* device-associated infections.

Some proteins, such as secreted aspartyl proteinase (SAP) and phospholipase B (PLB) families were not detected in this study. These proteins are hydrolytic enzymes which enable the organism to break down proteins for nutrition, however their relative contribution to *C. albicans* pathogenicity is not clear (Correia *et al*., 2010; Gil-Bona *et al*., 2015; Lermann & Morschhäuser, 2008; Mayer *et al*., 2013; Naglik *et al*., 2003; Schaller *et al*., 2005). Proteins such as Als2, Cht2, Crh11, Ihd1, Pir1 and Sap9-10 which were described as GPI-anchored proteins were also not detected in this study (Richard & Plaine, 2007). The absence of these proteins could be related to the ways protein samples were prepared for LCMS analysis.

In this study, some proteins were detected exclusively in *C. albicans* planktonic or biofilm cultures (Appendix B). Although the detection of the proteins exclusively in one of the morphological forms may suggest their specific role in either planktonic or biofilm cultures, the data has to be interpreted cautiously as other factors such as pH and media can also regulate their expression (Gil-Bona *et al*., 2016). The effects of environmental pH on *C. albicans* morphology and its ability to respond to stress have been documented (Davis, 2009). Additionally, it has also been reported by Gil-Bona *et al*. (2016) that as proteins have a pH optimum for activity, the media conditions may have an effect on the functionality of secreted and surface exposed proteins.

The antifungal effects of AbA against *Candida* biofilm cultures should be further explored at the molecular (gene) levels since little is known about its mechanism of action. The mechanism behind the higher susceptibility to AbA of *Candida* biofilm

cultures exhibiting filamentous form, and higher biomass/metabolic activity should be further explored.

CHAPTER 6: CONCLUSION

The results in this study demonstrated that there is variability in the biofilm growth characteristics of *Candida* spp. Two different morphovariants showing predominantly yeasts cells and filamentous growth was observed in all the *Candida* species tested. Since these morphologies were consistently observed for each isolate, further investigation is warranted to understand the genetic and expression profiles of different biofilm morphovariants of *Candida* isolates. As compared to the planktonic cultures, AbA appears to be less active against *Candida* biofilms. Additionally, higher susceptibility to AbA was noted in *Candida* biofilm cultures exhibiting filamentous form, higher biomass and metabolic activity in this study. The FESEM analysis in this study showed interesting findings with the observation of enlargement of blastospores (planktonic cells) in response to AbA. Further work is required to find the underlying reasons for this phenomenon. The antifungal effects of AbA against *Candida* biofilms cultures were further explored using LCMS approach.

In general, the LCMS analysis in this showed that *C. albicans* biofilm culture is expressing more proteins compared to planktonic culture in many biological processes, thus justifying the complexity biofilm culture of *C. albicans*. Both planktonic and biofilm cultures showed different cellular and metabolic responses to AbA as exhibited by the proteins annotated for each culture. The regulation of cellular amide and actin cytoskeleton organisation are two biological processes affected by AbA which are shared by both AbA-treated planktonic and biofilm cultures. The finding in this study provides a basis for future research on the use of AbA as an alternative drug for treatment of biofilm-associated candidiasis. Further exploration of the drug-affected proteins may aid in the search for potential drug target for the treatment of candidiasis.

REFERENCES

Abbas, J., Bodey, G. P., Hanna, H. A., Mardani, M., Girgawy, E., Abi-Said, D., ... & Raad, I. (2000). *Candida krusei* fungemia: an escalating serious infection in immunocompromised patients. *Archives of Internal Medicine*,160(17), 2659-2664.

Achkar, J. M., & Fries, B. C. (2010). Candida infections of the genitourinary tract. *Clinical Microbiology Reviews*, 23(2), 253-273.

Adam, B., Baillie, G. S., & Douglas, L. J. (2002). Mixed species biofilms of *Candida albicans* and *Staphylococcus epidermidis*. *Journal of Medical Microbiology*, 51(4), 344-349.

Alcoba-Flórez, J., Méndez-Álvarez, S., Cano, J., Guarro, J., Pérez-Roth, E., & del Pilar Arévalo, M. (2005). Phenotypic and molecular characterization of *Candida nivariensis* sp. nov., a possible new opportunistic fungus. *Journal of Clinical Microbiology*, 43(8), 4107-4111.

Alexander, B. D., Ashley, E. D., Reller, L. B., & Reed, S. D. (2006). Cost savings with implementation of PNA FISH testing for identification of *Candida albicans* in blood cultures. *Diagnostic Microbiology and Infectious Disease*,54(4), 277-282.

Anderson, M. S., Yarger, J. G., Burck, C. L., & Poulter, C. D. (1989). Farnesyl diphosphate synthetase. Molecular cloning, sequence, and expression of an essential gene from *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*, 264(32), 19176-19184.

Asadzadeh, M., Ahmad, S., Hagen, F., Meis, J. F., Al-Sweih, N., & Khan, Z. (2015). Simple, low-cost detection of Candida parapsilosis complex isolates and molecular fingerprinting of *Candida orthopsilosis* strains in Kuwait by ITS region sequencing and amplified fragment length polymorphism analysis.*PloS One*, 10(11), e0142880.

Asadzadeh, M., Al-Sweih, N. A., Ahmad, S., & Khan, Z. U. (2008). Antifungal susceptibility of clinical *Candida parapsilosis* isolates in Kuwait. *Mycoses*,51(4), 318-323.

Askew, C., Sellam, A., Epp, E., Hogues, H., Mullick, A., Nantel, A., & Whiteway, M. (2009). Transcriptional regulation of carbohydrate metabolism in the human pathogen *Candida albicans*. *PLoS Pathology*, 5(10), e1000612.

Bachmann, S. P., VandeWalle, K., Ramage, G., Patterson, T. F., Wickes, B. L., Graybill, J. R., & López-Ribot, J. L. (2002). *In vitro* activity of caspofungin against *Candida albicans* biofilms. *Antimicrobial Agents and Chemotherapy*, 46(11), 3591-3596.

Balashov, S. V., Park, S., & Perlin, D. S. (2006). Assessing resistance to the echinocandin antifungal drug caspofungin in *Candida albicans* by profiling mutations in FKS1. *Antimicrobial Agents and Chemotherapy*, 50(6), 2058-2063.

Bassetti, M., Righi, E., Costa, A., Fasce, R., Molinari, M. P., Rosso, R., ... & Viscoli, C. (2006). Epidemiological trends in nosocomial candidemia in intensive care. *BMC Infectious Diseases*, 6(1), 21.

Bennett, J. E., Izumikawa, K., & Marr, K. A. (2004). Mechanism of increased fluconazole resistance in *Candida glabrata* during prophylaxis. *Antimicrobial Agents and Chemotherapy*, 48(5), 1773-1777.

Bern, M., Cai, Y., & Goldberg, D. (2007). Lookup peaks: a hybrid of de novo sequencing and database search for protein identification by tandem mass spectrometry. *Analytical Chemistry*, 79(4), 1393-1400.

Bethea, E. K., Carver, B. J., Montedonico, A. E., & Reynolds, T. B. (2010). The inositol regulon controls viability in *Candida glabrata*. *Microbiology*,156(2), 452-462.

Bink, A., Vandenbosch, D., Coenye, T., Nelis, H., Cammue, B. P., & Thevissen, K. (2011). Superoxide dismutases are involved in *Candida albicans* biofilm persistence against miconazole. *Antimicrobial Agents and Chemotherapy*, 55(9), 4033-4037.

Bishop, J. A., Chase, N., Lee, R., Kurtzman, C. P., & Merz, W. G. (2008a). Production of white colonies on CHROMagar *Candida* medium by members of the *Candida glabrata* clade and other species with overlapping phenotypic traits. *Journal of Clinical Microbiology*, 46(10), 3498-3500.

Bishop, J. A., Chase, N., Magill, S. S., Kurtzman, C. P., Fiandaca, M. J., & Merz, W. G. (2008b). *Candida bracarensis* detected among isolates of *Candida glabrata* by peptide nucleic acid fluorescence *in situ* hybridization: susceptibility data and documentation of presumed infection. *Journal of Clinical Microbiology*, 46(2), 443-446.

Branda, S. S., Vik, Å., Friedman, L., & Kolter, R. (2005). Biofilms: the matrix revisited. *Trends in Microbiology*, 13(1), 20-26.

Braun, B. R., Head, W. S., Wang, M. X., & Johnson, A. D. (2000). Identification and characterization of TUP1-regulated genes in *Candida albicans*. *Genetics*, 156(1), 31-44.

Brown Jr, D. H., Giusani, A. D., Chen, X., & Kumamoto, C. A. (1999). Filamentous growth of *Candida albicans* in response to physical environmental cues and its regulation by the unique CZF1 gene. *Molecular Microbiology*, 34(4), 651-662.

Brown, A. J. (2002). Morphogenetic signaling pathways in *Candida albicans*.*Candida* and candidiasis, 95-106.

Bruckmann, A., Künkel, W., Härtl, A., Wetzker, R., & Eck, R. (2000). A phosphatidylinositol 3-kinase of *Candida albicans* influences adhesion, filamentous growth and virulence. *Microbiology*, 146(11), 2755-2764.

Burgers, P. M. (1998). Eukaryotic DNA polymerases in DNA replication and DNA repair. *Chromosoma*, 107(4), 218-227.

Calderone, R. A., & Fonzi, W. A. (2001). Virulence factors of *Candida albicans*. *Trends in Microbiology*, 9(7), 327-335.

Calderone, R. A., & Gow, N. A. (2002). Host recognition by *Candida* species.*Candida and candidiasis*. ASM Press, Washington, DC, 67-86.

Cannon, R. D., Lamping, E., Holmes, A. R., Niimi, K., Baret, P. V., Keniya, M. V., ... & Monk, B. C. (2009). Efflux-mediated antifungal drug resistance.*Clinical Microbiology Reviews*, 22(2), 291-321.

Cantón, E., Pemán, J., Sastre, M., Romero, M., & Espinel-Ingroff, A. (2006). Killing kinetics of caspofungin, micafungin, and amphotericin B against *Candida guilliermondii*. *Antimicrobial Agents and Chemotherapy*, 50(8), 2829-2832.

Cappelletty, D., & Eiselstein-McKitrick, K. (2007). The echinocandins.Pharmacotherapy: *The Journal of Human Pharmacology and Drug Therapy*,27(3), 369-388.

Cerantola, V., Guillas, I., Roubaty, C., Vionnet, C., Uldry, D., Knudsen, J., & Conzelmann, A. (2009). Aureobasidin A arrests growth of yeast cells through both ceramide intoxication and deprivation of essential inositolphosphorylceramides. Molecular microbiology,71(6),1523-1537.

Chaffin, W. L., López-Ribot, J. L., Casanova, M., Gozalbo, D., & Martínez, J. P. (1998). Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. *Microbiology and Molecular Biology Reviews*, 62(1), 130-180.

Chakrabarti, A., Chatterjee, S. S., Rao, K. L. N., Zameer, M. M., Shivaprakash, M. R., Singhi, S., ... & Varma, S. C. (2009). Recent experience with fungaemia: change in species distribution and azole resistance. *Scandinavian Journal of Infectious Diseases*, 41(4), 275-284.

Chandra, J., Kuhn, D. M., Mukherjee, P. K., Hoyer, L. L., McCormick, T., & Ghannoum, M. A. (2001). Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *Journal of Bacteriology*, 183(18), 5385-5394.

Chapeland-Leclerc, F., Bouchoux, J., Goumar, A., Chastin, C., Villard, J., & Noël, T. (2005). Inactivation of the FCY2 gene encoding purine-cytosine permease promotes cross-resistance to flucytosine and fluconazole in *Candida lusitaniae*. *Antimicrobial Agents and Chemotherapy*, 49(8), 3101-3108.

Cheng, J., Park, T. S., Fischl, A. S., & Xiang, S. Y. (2001). Cell cycle progression and cell polarity require sphingolipid biosynthesis in *Aspergillus nidulans*. *Molecular and Cellular Biology*, 21(18), 6198-6209.

Clinical and Laboratory Standards Institute . Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast; Approved Standard-Third Edition. CLSI document M27-A3. Wayne: Clinical and Laboratory Standards Institute; 2008.

Colombo, A. L., Guimaraes, T., Silva, L. R., de Almeida Monfardini, L. P., Cunha, A. K. B., Rady, P., ... & Rosas, R. C. (2007). Prospective observational study of candidemia in Sao Paulo, Brazil: incidence rate, epidemiology, and predictors of mortality. *Infection Control & Hospital Epidemiology*, 28(05), 570-576.

Colombo, A. L., Nucci, M., Park, B. J., Nouér, S. A., Arthington-Skaggs, B., da Matta, D. A., ... & Morgan, J. (2006). Epidemiology of candidemia in Brazil: a nationwide sentinel surveillance of candidemia in eleven medical centers. *Journal of Clinical Microbiology*, 44(8), 2816-2823.

Conen, A., Weisser, M., Orasch Jörg, C., Battegay, M., Trampuz, A., Frei, R., ... & Flückiger, U. (2008). *Candida krusei*–a serious complication in patients with hematological malignancies: successful treatment with caspofungin. *Transplant Infectious Disease*, 10(1), 66-70.

Consortium, G. O. (2008). The gene ontology project in 2008. Nucleic acids research, 36(suppl 1), D440-D444.

Correia, A., Lermann, U., Teixeira, L., Cerca, F., Botelho, S., da Costa, R. M. G., ... & Pais, C. (2010). Limited role of secreted aspartyl proteinases Sap1 to Sap6 in *Candida albicans* virulence and host immune response in murine hematogenously disseminated candidiasis. *Infection and Immunity*, 78(11), 4839-4849.

Correia, A., Sampaio, P., James, S., & Pais, C. (2006). Candida bracarensis sp. nov., a novel anamorphic yeast species phenotypically similar to *Candida glabrata*. International journal of systematic and evolutionary microbiology,56(1), 313-317.

Costanzo, M. C., Arnaud, M. B., Skrzypek, M. S., Binkley, G., Lane, C., Miyasato, S. R., & Sherlock, G. (2006). The Candida Genome Database: facilitating research on *Candida albicans* molecular biology. *FEMS yeast research*, *6*(5), 671-684.

Coste, A. T., Karababa, M., Ischer, F., Bille, J., & Sanglard, D. (2004). TAC1, transcriptional activator of CDR genes, is a new transcription factor involved in the regulation of *Candida albicans* ABC transporters CDR1 and CDR2.*Eukaryotic Cell*, 3(6), 1639-1652.

Coste, A., Turner, V., Ischer, F., Morschhäuser, J., Forche, A., Selmecki, A., ... & Sanglard, D. (2006). A mutation in Tac1p, a transcription factor regulating CDR1 and CDR2, is coupled with loss of heterozygosity at chromosome 5 to mediate antifungal resistance in *Candida albicans*.*Genetics*, 172(4), 2139-2156.

Crampin, H., Finley, K., Gerami-Nejad, M., Gale, C., Berman, J., & Sudbery, P. (2005). *Candida albicans* hyphae have a Spitzenkörper that is distinct from the polarisome found in yeast and pseudohyphae. *Journal of Cell Science*,118(13), 2935-2947.

Cuenca-Estrella, M., Gomez-Lopez, A., Mellado, E., Buitrago, M. J., Monzon, A., & Rodriguez-Tudela, J. L. (2006). Head-to-head comparison of the activities of currently available antifungal agents against 3,378 Spanish clinical isolates of yeasts and filamentous fungi. *Antimicrobial Agents and Chemotherapy*, 50(3), 917-921.

Davey, M. E., & O'toole, G. A. (2000). Microbial biofilms: from ecology to molecular genetics. *Microbiology and Molecular Biology Reviews*, 64(4), 847-867.

Davis, D. A. (2009). How human pathogenic fungi sense and adapt to pH: the link to virulence. *Current Opinion in Microbiology*, 12(4), 365-370.

De Backer, M. D., Ilyina, T., Ma, X. J., Vandoninck, S., Luyten, W. H., & Bossche, H. V. (2001). Genomic profiling of the response of *Candida albicans* to itraconazole treatment using a DNA microarray. *Antimicrobial Agents and Chemotherapy*, 45(6), 1660-1670.

Diekema, D. J., Messer, S. A., Brueggemann, A. B., Coffman, S. L., Doern, G. V., Herwaldt, L. A., & Pfaller, M. A. (2002). Epidemiology of candidemia: 3-year results from the emerging infections and the epidemiology of Iowa organisms study. *Journal of Clinical Microbiology*, 40(4), 1298-1302.

Donlan, R. M. (2001). Biofilm formation: a clinically relevant microbiological process. *Clinical Infectious Diseases*, 33(8), 1387-1392.

Douglas, L. J. (2002). Medical importance of biofilms in *Candida* infections.*Revista Iberoamericana de Micologia*, 19(3), 139-143.

Douglas, L. J. (2003). *Candida* biofilms and their role in infection. *Trends in Microbiology*, 11(1), 30-36.

Eggimann, P., Garbino, J., & Pittet, D. (2003). Epidemiology of Candida species infections in critically ill non-immunosuppressed patients. The Lancet infectious diseases, 3(11), 685-702.

Ellepola, A. N., & Samaranayake, L. P. (2000). Antimycotic agents in oral candidosis: an overview: 1. Clinical variants. *Dental Update*, 27(3), 111-116.

Ellis, D. (2002). Amphotericin B: spectrum and resistance. *Journal of Antimicrobial Chemotherapy*, 49(suppl 1), 7-10.

Endo, M., Takesako, K., Kato, I., & Yamaguchi, H. (1997). Fungicidal action of aureobasidin A, a cyclic depsipeptide antifungal antibiotic, against *Saccharomyces cerevisiae*. *Antimicrobial Agents and Chemotherapy*, 41(3), 672-676.

Espinel-Ingroff, A. (2008). Mechanisms of resistance to antifungal agents: yeasts and filamentous fungi. *Revista Iberoamericana de Micología*, 25(2), 101.

Falagas, M. E., Roussos, N., & Vardakas, K. Z. (2010). Relative frequency of albicans and the various non-albicans Candida spp among candidemia isolates from inpatients in

various parts of the world: a systematic review.*International Journal of Infectious Diseases*, *14*(11), e954-e966.

Fanci, R., Guidi, S., Bonolis, M., & Bosi, A. (2005). *Candida krusei* fungemia in an unrelated allogeneic hematopoietic stem cell transplant patient successfully treated with Caspofungin. *Bone Marrow Transplantation*, 35(12), 1215-1215.

Forrest, G. N., Mankes, K., Jabra-Rizk, M. A., Weekes, E., Johnson, J. K., Lincalis, D. P., & Venezia, R. A. (2006). Peptide nucleic acid fluorescence in situ hybridization-based identification of *Candida albicans* and its impact on mortality and antifungal therapy costs. *Journal of Clinical Microbiology*, 44(9), 3381-3383.

Franke, K., Nguyen, M., Härtl, A., Dahse, H. M., Vogl, G., Würzner, R., ... & Eck, R. (2006). The vesicle transport protein Vac1p is required for virulence of Candida albicans. Microbiology, 152(10), 3111-3121.

Fu, Y., Rieg, G., Fonzi, W. A., Belanger, P. H., Edwards, J. E., & Filler, S. G. (1998). Expression of the *Candida albicans* GeneALS1 in *Saccharomyces cerevisiae* Induces Adherence to Endothelial and Epithelial Cells. *Infection and Immunity*, 66(4), 1783-1786.

Fukazawa, Y., & Kagaya, K. (1997). Molecular bases of adhesion of *Candida albicans*. *Journal of Medical and Veterinary Mycology*, 35(2), 87-99.

Galagan, J. E., Henn, M. R., Ma, L. J., Cuomo, C. A., & Birren, B. (2005). Genomics of the fungal kingdom: insights into eukaryotic biology. *Genome Research*, 15(12), 1620-1631.

Garcia-Effron, G., Katiyar, S. K., Park, S., Edlind, T. D., & Perlin, D. S. (2008). A naturally occurring proline-to-alanine amino acid change in Fks1p in *Candida parapsilosis*, *Candida orthopsilosis*, and *Candida metapsilosis* accounts for reduced echinocandin susceptibility. *Antimicrobial Agents and Chemotherapy*, 52(7), 2305-2312.

Gaur, N. K., Klotz, S. A., & Henderson, R. L. (1999). Overexpression of the *Candida albicans* ALA1 gene in *Saccharomyces cerevisiae* results in aggregation following attachment of yeast cells to extracellular matrix proteins, adherence properties similar to those of *Candida albicans*. *Infection and Immunity*, 67(11), 6040-6047.

Ghannoum, M. A. (2000). Potential role of phospholipases in virulence and fungal pathogenesis. *Clinical Microbiology Reviews*, 13(1), 122-143.

Ghannoum, M. A., Burns, G. R., Elteen, K. A., & Radwan, S. S. (1986). Experimental evidence for the role of lipids in adherence of *Candida* spp. to human buccal epithelial cells. *Infection and Immunity*, 54(1), 189-193.

Gil-Bona, A., Parra-Giraldo, C. M., Hernáez, M. L., Reales-Calderon, J. A., Solis, N. V., Filler, S. G., ... & Gil, C. (2015). *Candida albicans* cell shaving uncovers new proteins involved in cell wall integrity, yeast to hypha transition, stress response and host–pathogen interaction. *Journal of Proteomics*, 127, 340-351.

Gil-Bona, A., Reales-Calderon, J. A., Parra-Giraldo, C. M., Martinez-Lopez, R., Monteoliva, L., & Gil, C. (2016). The Cell Wall Protein Ecm33 of *Candida albicans* is Involved in Chronological Life Span, Morphogenesis, Cell Wall Regeneration, Stress Tolerance, and Host–Cell Interaction. *Frontiers in Microbiology*, 7, 64.

Gleason, J. E., Galaleldeen, A., Peterson, R. L., Taylor, A. B., Holloway, S. P., Waninger-Saroni, J., … & Culottaa, V. C. (2014). *Candida albicans* SOD5 represents the prototype of an unprecedented class of Cu-only superoxide dismutases required for pathogen defense. *Proc Natl Acad Sci USA*, 111(16), 5866–5871.

Godoy, P., Tiraboschi, I. N., Severo, L. C., Bustamante, B., Calvo, B., Almeida, L. P. D., ... & Colombo, A. L. (2003). Species distribution and antifungal susceptibility profile of *Candida* spp. bloodstream isolates from Latin American hospitals. *Memórias do Instituto Oswaldo Cruz*, 98(3), 401-405.

Gow, N. A. (1997). Germ tube growth of *Candida albicans*. *Current topics in Medical Mycology*, 8(1-2), 43-55.

Gross, S. R., & Kinzy, T. G. (2007). Improper organization of the actin cytoskeleton affects protein synthesis at initiation. *Molecular and Cellular Biology*, *27*(5), 1974-1989.

Grover, N. D. (2010). Echinocandins: A ray of hope in antifungal drug therapy.*Indian Journal of Pharmacology*, 42(6), 422.

Guilhen, C., Charbonnel, N., Parisot, N., Gueguen, N., Iltis, A., Forestier, C., & Balestrino, D. (2016). Transcriptional profiling of Klebsiella pneumoniae defines signatures for planktonic, sessile and biofilm-dispersed cells. *BMC Genomics*, 17(1), 237.

Hajjeh, R. A., Sofair, A. N., Harrison, L. H., Lyon, G. M., Arthington-Skaggs, B. A., Mirza, S. A., . . . Ciblak, M. A. (2004). Incidence of bloodstream infections due to

Candida species and in vitro susceptibilities of isolates collected from 1998 to 2000 in a population-based active surveillance program. Journal of clinical microbiology, 42(4), 1519-1527.

Hasan, F., Xess, I., Wang, X., Jain, N., & Fries, B. C. (2009). Biofilm formation in clinical *Candida* isolates and its association with virulence. *Microbes and Infection*, 11(8), 753-761.

Hashida-Okado, T., Ogawa, A., Endo, M., Yasumoto, R., Takesako, K., & Kato, I. (1996). AUR1, a novel gene conferring aureobasidin resistance on *Saccharomyces cerevisiae*: a study of defective morphologies in Aur1p-depleted cells. *Molecular and General Genetics MGG*, 251(2), 236-244.

Hashida-Okado, T., Yasumoto, R., Endo, M., Takesako, K., & Kato, I. (1998). Isolation and characterization of the aureobasidin A-resistant gene, aur1R, on *Schizosaccharomyces pombe*: roles of Aur1p+ in cell morphogenesis. *Current Genetics*, 33(1), 38-45.

Hawser, S. P., & Douglas, L. J. (1994). Biofilm formation by *Candida* species on the surface of catheter materials *in vitro*. *Infection and Immunity*, 62(3), 915-921.

Hernandez, S., López-Ribot, J. L., Najvar, L. K., McCarthy, D. I., Bocanegra, R., & Graybill, J. R. (2004). Caspofungin resistance in *Candida albicans*: correlating clinical outcome with laboratory susceptibility testing of three isogenic isolates serially obtained from a patient with progressive *Candida esophagitis*. *Antimicrobial Agents and Chemotherapy*, 48(4), 1382-1383.

Hernández-Castro, R., Arroyo-Escalante, S., Carrillo-Casas, E. M., Moncada-Barrón, D., Álvarez-Verona, E., Hernández-Delgado, L., ... & Lavalle-Villalobos, A. (2010). Outbreak of *Candida parapsilosis* in a neonatal intensive care unit: a health care workers source. *European Journal of Pediatrics*,169(7), 783-787.

Hoehamer, C.F., Cummings, E.D., Hilliard, G.M., Rogers, P.D., Changes in the Proteome of *Candida albicans* in Response to Azole, Polyene, and Echinocandin Antifungal Agents, *Antimicrobial Agents and Chemotherapy*, 54(5), 1655–1664.

Horvath, L. L., George, B. J., & Hospenthal, D. R. (2007). Detection of fifteen species of *Candida* in an automated blood culture system. *Journal of Clinical Microbiology*, 45(9), 3062-3064.

Jabra-Rizk, M. A., Falkler, W. A., & Meiller, T. F. (2004). Fungal biofilms and drug resistance. *Emerging Infectious Diseases*, 10(1), 14.

Jackson, B. E., Wilhelmus, K. R., & Mitchell, B. M. (2007). Genetically regulated filamentation contributes to *Candida albicans* virulence during corneal infection. *Microbial Pathogenesis*, 42(2), 88-93.

Jayatilake, J. A. M. S. (2011). A review of the ultrastructural features of superficial candidiasis. *Mycopathologia*, 171(4), 235-250.

Jin, Y., Samaranayake, L.P., Samaranayake, Y., & Yip, H.K. (2004). Biofilm formation of *Candida albicans* is variably affected by saliva and dietary sugars. *Arch Oral Biol*, 49(10), 789-798. doi: 10.1016/j.archoralbio.2004.04.011.

Kabir, M. A., Hussain, M. A., & Ahmad, Z. (2012). *Candida albicans*: a model organism for studying fungal pathogens. *ISRN Microbiology*, 2012.

Kajiwara, K., Muneoka, T., Watanabe, Y., Karashima, T., Kitagaki, H., & Funato, K. (2012). Perturbation of sphingolipid metabolism induces endoplasmic reticulum stress-mediated mitochondrial apoptosis in budding yeast. *Molecular Microbiology*, 86(5), 1246-1261.

Kaur, R., Domergue, R., Zupancic, M. L., & Cormack, B. P. (2005). A yeast by any other name: *Candida glabrata* and its interaction with the host. *Current Opinion in Microbiology*, 8(4), 378-384.

Kędzierska, A., Kochan, P., Pietrzyk, A., & Kędzierska, J. (2007). Current status of fungal cell wall components in the immunodiagnostics of invasive fungal infections in humans: galactomannan, mannan and (1→ 3)-β-D-glucan antigens. *European Journal of Clinical Microbiology & Infectious Diseases*,26(11), 755-766.

Khakhina, S., Johnson, S. S., Manoharlal, R., Russo, S. B., Blugeon, C., Lemoine, S., ... & Moye-Rowley, W. S. (2015). Control of plasma membrane permeability by ABC transporters. *Eukaryotic Cell*, 14(5), 442-453.

Khot, P. D., Suci, P. A., Miller, R. L., Nelson, R. D., & Tyler, B. J. (2006). A small subpopulation of blastospores in *Candida albicans* biofilms exhibit resistance to amphotericin B associated with differential regulation of ergosterol and β-1, 6-glucan pathway genes. *Antimicrobial Agents and Chemotherapy*, 50(11), 3708-3716.

Kim, J., & Sudbery, P. (2011). *Candida albicans*, a major human fungal pathogen. *The Journal of Microbiology*, 49(2), 171.

Kim, K. S., Kim, Y. S., Han, I., Kim, M. H., Jung, M. H., & Park, H. K. (2011). Quantitative and qualitative analyses of the cell death process in *Candida albicans* treated by antifungal agents. *PLoS ONE*, 6(12), e28176.

Kojic, E. M., & Darouiche, R. O. (2004). Candida infections of medical devices. *Clinical Microbiology Reviews*, 17(2), 255-267.

Komalapriya, C., Kaloriti, D., Tillmann, A. T., Yin, Z., Herrero-de-Dios, C., Jacobsen, M, D., … & Romano M, C., (2015). Integrative Model of Oxidative Stress Adaptation in the Fungal Pathogen *Candida albicans*. *PLoS ONE*, 10(9): e0137750.

Kontoyiannis, D. P., & Lewis, R. E. (2002). Antifungal drug resistance of pathogenic fungi. *The Lancet*, 359(9312), 1135-1144.

Kothavade, R. J., Kura, M. M., Valand, A. G., & Panthaki, M. H. (2010). *Candida tropicalis*: its prevalence, pathogenicity and increasing resistance to fluconazole. *Journal of Medical Microbiology*, 59(8), 873-880.

Krcmery, V., & Barnes, A. J. (2002). Non-*albicans Candida* spp. causing fungaemia: pathogenicity and antifungal resistance. *Journal of Hospital Infection*, 50(4), 243-260.

Krogh-Madsen, M., Arendrup, M. C., Heslet, L., & Knudsen, J. D. (2006). Amphotericin B and caspofungin resistance in *Candida glabrata* isolates recovered from a critically ill patient. *Clinical Infectious Diseases*, 42(7), 938-944.

Kuhn, D. M., Balkis, M., Chandra, J., Mukherjee, P. K., & Ghannoum, M. A. (2003). Uses and limitations of the XTT assay in studies of Candida growth and metabolism. Journal of Clinical Microbiology, 41(1), 506-508.

Kuhn, D. M., Chandra, J., Mukherjee, P. K., & Ghannoum, M. A. (2002). Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. *Infection and Immunity*, 70(2), 878-888.

Kung, H. C., Wang, J. L., Chang, S. C., Wang, J. T., Sun, H. Y., Hsueh, P. R., & Chen, Y. C. (2007). Community-onset candidemia at a university hospital, 1995-2005. *Journal of Microbiology, Immunology, and Infection= Wei Mian Yu Gan Ran Za Zhi*, 40(4), 355-363.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227(5259), 680-685.

Laín, A., Elguezabal, N., Moragues, M. D., García-Ruiz, J. C., Del Palacio, A., & Pontón, J. (2008). Contribution of serum biomarkers to the diagnosis of invasive candidiasis. *Expert Review of Molecular Diagnostics*, 8(3), 315-325.

Larriba, G., Rubio Coque, J. J., & Andaluz, E. (2010). *Candida albicans* molecular biology reaches its maturity. *International Microbiology*, 3(4), 247-252.

Lee, I., Fishman, N. O., Zaoutis, T. E., Morales, K. H., Weiner, M. G., Synnestvedt, M., ... & Lautenbach, E. (2009). Risk factors for fluconazole-resistant *Candida glabrata* bloodstream infections. *Archives of Internal Medicine*, 169(4), 379-383.

Lermann, U., & Morschhäuser, J. (2008). Secreted aspartic proteases are not required for invasion of reconstituted human epithelia by *Candida albicans*.*Microbiology*, 154(11), 3281-3295.

Lester, R. L., & Dickson, R. C. (1992). Sphingolipids with inositolphosphate-containing head groups. *Advances in Lipid Research*, 26, 253-274.

Lewis, K. (2001). Riddle of biofilm resistance. *Antimicrobial Agents and Chemotherapy*, 45(4), 999-1007.

Li, Y. H., & Tian, X. L. (2016). Microbial Interactions in Biofilms: Impacts on Homeostasis and Pathogenesis. *In Microbial Biofilms-Importance and Applications*. InTech.

Lim, C. Y., Rosli, R., Seow, H. F., & Chong, P. P. (2012). *Candida* and invasive candidiasis: back to basics. *European Journal of Clinical Microbiology & Infectious Diseases*, 31(1), 21-31.

López-Martínez, R. (2010). Candidosis, a new challenge. *Clinics in Dermatology*, 28(2), 178-184.

Luberto, C., Toffaletti, D. L., Wills, E. A., Tucker, S. C., Casadevall, A., Perfect, J. R., ... & Del Poeta, M. (2001). Roles for inositol-phosphoryl ceramide synthase 1 (IPC1) in pathogenesis of *C. neoformans.Genes & Development*, 15(2), 201-212.

Luo, G., Samaranayake, L. P., & Yau, J. Y. (2001). Candida species exhibit differential *in vitro* hemolytic activities. *Journal of Clinical Microbiology*, 39(8), 2971-2974.

Lyytikäinen, O., Lumio, J., Sarkkinen, H., Kolho, E., Kostiala, A., & Ruutu, P. (2002). Nosocomial bloodstream infections in Finnish hospitals during 1999–2000. *Clinical Infectious Diseases*, 35(2), e14-e19.

Marchetti, O., Bille, J., Fluckiger, U., Eggimann, P., Ruef, C., Garbino, J., ... & Pittet, D. (2004). Epidemiology of candidemia in Swiss tertiary care hospitals: secular trends, 1991–2000. *Clinical Infectious Diseases*, 38(3), 311-320.

Marcos-Zambrano, L. J., Escribano, P., Bouza, E., & Guinea, J. (2014). Production of biofilm by *Candida* and non-*Candida* spp. isolates causing fungemia: comparison of biomass production and metabolic activity and development of cut-off points. *International Journal of Medical Microbiology*,304(8), 1192-1198.

Marklein, G., Josten, M., Klanke, U., Müller, E., Horre, R., Maier, T., ... & Sahl, H. G. (2009). Matrix-assisted laser desorption ionization-time of flight mass spectrometry for fast and reliable identification of clinical yeast isolates.*Journal of Clinical Microbiology*, 47(9), 2912-2917.

Martin, D., Persat, F., Piens, M. A., & Picot, S. (2005). *Candida* species distribution in bloodstream cultures in Lyon, France, 1998–2001. *European Journal of Clinical Microbiology and Infectious Diseases*, 24(5), 329-333.

Martin, S. W., & Konopka, J. B. (2004). Lipid raft polarization contributes to hyphal growth in *Candida albicans*. *Eukaryotic Cell*, 3(3), 675-684.

Martins, M., Uppuluri, P., Thomas, D. P., Cleary, I. A., Henriques, M., Lopez-Ribot, J. L., & Oliveira, R. (2010). Presence of extracellular DNA in the *Candida albicans* biofilm matrix and its contribution to biofilms.*Mycopathologia*, 169(5), 323-331.

Mathé, L., & Van Dijck, P. (2013). Recent insights into *Candida albicans* biofilm resistance mechanisms. *Current Genetics*, 59(4), 251-264.

Mayer, F. L., Wilson, D., & Hube, B. (2013). *Candida albicans* pathogenicity mechanisms. *Virulence*, 4(2), 119-128.

McConville, M. J., & Bacic, A. (1989). A family of glycoinositol phospholipids from *Leishmania* major. Isolation, characterization, and antigenicity. *Journal of Biological Chemistry*, 264(2), 757-766.

McMullan, R., Metwally, L., Coyle, P. V., Hedderwick, S., McCloskey, B., O'neill, H. J., ... & Hay, R. J. (2008). A prospective clinical trial of a real-time polymerase chain reaction assay for the diagnosis of candidemia in nonneutropenic, critically ill adults. *Clinical Infectious Diseases*, 46(6), 890-896.

Meurman, J. H., Siikala, E., Richardson, M., & Rautemaa, R. (2007). Non-*Candida albicans Candida* yeasts of the oral cavity. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*, 1(1), 719-731.

Monod, M., Hube, B., Hess, D., & Sanglard, D. (1998). Differential regulation of SAP8 and SAPS, which encode two new members of the secreted aspartic proteinase family in *Candida albicans*. *Microbiology*, 144(10), 2731-2737.

Mukherjee, P. K., Chandra, J., Kuhn, D. M., & Ghannoum, M. A. (2003). Mechanism of fluconazole resistance in *Candida albicans* biofilms: phase-specific role of efflux pumps and membrane sterols. *Infection and Immunity*,71(8), 4333-4340.

Murad, A. M. A., Leng, P., Straffon, M., Wishart, J., Macaskill, S., MacCallum, D., ... & d'Enfert, C. (2001). NRG1 represses yeast–hypha morphogenesis and hypha-specific gene expression in *Candida albicans*. *The EMBO Journal*, 20(17), 4742-4752.

Myoken, Y., Kyo, T. I., Fujihara, M., Sugata, T., & Mikami, Y. (2004). Clinical significance of breakthrough fungemia caused by azole-resistant *Candida tropicalis* in patients with hematologic malignancies. *Haematologica*, 89(3), 378-380.

Nagiec, M. M., Nagiec, E. E., Baltisberger, J. A., Wells, G. B., Lester, R. L., & Dickson, R. C. (1997). Sphingolipid synthesis as a target for antifungal drugs complementation of the inositol phosphorylceramide synthase defect in a mutant strain of *Saccharomyces cerevisiae* by the AUR1 gene. *Journal of Biological Chemistry*, 272(15), 9809-9817.

Naglik, J. R., Challacombe, S. J., & Hube, B. (2003). *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiology and Molecular Biology Reviews*, 67(3), 400-428.

Nakamura, Y., Yamamoto, N., Kino, Y., Yamamoto, N., Kamei, S., Mori, H., ... & Nakashima, N. (2016). Establishment of a multi-species biofilm model and metatranscriptomic analysis of biofilm and planktonic cell communities. *Applied Microbiology and Biotechnology*, 100(16), 7263-7279.

Ng, K. P., Kuan, C. S., Kaur, H., Na, S. L., Atiya, N., & Velayuthan, R. D. (2015). Candida species epidemiology 2000–2013: a laboratory-based report.*Tropical Medicine & International Health*, 20(11), 1447-1453.

Odds, F. C., Hanson, M. F., Davidson, A. D., Jacobsen, M. D., Wright, P., Whyte, J. A., ... & Jones, B. L. (2007). One year prospective survey of *Candida* bloodstream infections in Scotland. *Journal of Medical Microbiology*,56(8), 1066-1075.

Ostrosky-Zeichner, L., Kontoyiannis, D., Raffalli, J., Mullane, K. M., Vazquez, J., Anaissie, E. J., ... & Lau, W. (2005). International, open-label, noncomparative, clinical trial of micafungin alone and in combination for treatment of newly diagnosed and refractory candidemia. European *Journal of Clinical Microbiology and Infectious Diseases*, 24(10), 654-661.

O'toole, G. A. (2003). To build a biofilm. *Journal of Bacteriology*, 185(9), 2687-2689.

Pappas, P. G., Rex, J. H., Sobel, J. D., Filler, S. G., Dismukes, W. E., Walsh, T. J., & Edwards, J. E. (2004). Guidelines for treatment of candidiasis. *Clinical Infectious Diseases*, 38(2), 161-189.

Pedrioli, P. G., Eng, J. K., Hubley, R., Vogelzang, M., Deutsch, E. W., Raught, B., ... & Cheung, K. (2004). A common open representation of mass spectrometry data and its application to proteomics research. *Nature Biotechnology*, 22(11), 1459-1466.

Pemán, J., Cantón, E., & Espinel-Ingroff, A. (2009). Antifungal drug resistance mechanisms. *Expert Review of Anti-Infective Therapy*, 7(4), 453-460.

Pfaller, M. A., & Diekema, D. J. (2004). Twelve years of fluconazole in clinical practice: global trends in species distribution and fluconazole susceptibility of bloodstream isolates of *Candida*. *Clinical Microbiology and Infection*, 10(s1), 11-23.

Pfaller, M. A., & Diekema, D. J. (2007). Epidemiology of invasive candidiasis: a persistent public health problem. *Clinical Microbiology Reviews*, 20(1), 133-163.

Pfaller, M. A., Castanheira, M., Lockhart, S. R., Ahlquist, A. M., Messer, S. A., & Jones, R. N. (2012). Frequency of decreased susceptibility and resistance to echinocandins among fluconazole-resistant bloodstream isolates of *Candida glabrata*. *Journal of Clinical Microbiology*, 50(4), 1199-1203.

Pfaller, M. A., Diekema, D. J., Gibbs, D. L., Newell, V. A., Nagy, E., Dobiasova, S., ... & Global Antifungal Surveillance Group. (2008). *Candida krusei*, a multidrug-resistant opportunistic fungal pathogen: geographic and temporal trends from the ARTEMIS DISK Antifungal Surveillance Program, 2001 to 2005. *Journal of Clinical Microbiology*, 46(2), 515-521.

Pfaller, M. A., Moet, G. J., Messer, S. A., Jones, R. N., & Castanheira, M. (2011). *Candida* bloodstream infections: comparison of species distributions and antifungal resistance patterns in community-onset and nosocomial isolates in the SENTRY Antimicrobial Surveillance Program, 2008-2009.*Antimicrobial Agents and Chemotherapy*, 55(2), 561-566.

Poikonen, E., Lyytikainen, O., Anttila, V. J., & Ruutu, P. (2003). Candidemia in Finland, 1995-1999. *Emerging Infectious Diseases*, 9(8), 985-990.

Raad, I. I., Hachem, R. Y., Hanna, H. A., Fang, X., Jiang, Y., Dvorak, T., ... & Kontoyiannis, D. P. (2008). Role of ethylene diamine tetra-acetic acid (EDTA) in catheter lock solutions: EDTA enhances the antifungal activity of amphotericin B lipid complex against *Candida* embedded in biofilm.*International Journal of Antimicrobial Agents*, 32(6), 515-518.

Ramage, G., Bachmann, S., Patterson, T. F., Wickes, B. L., & López-Ribot, J. L. (2002). Investigation of multidrug efflux pumps in relation to fluconazole resistance in *Candida albicans* biofilms. *Journal of Antimicrobial Chemotherapy*, 49(6), 973-980.

Randhawa, M. A. (2008). Dimethyl Sulfoxide (DMSO) Inhibits the Germination of *Candida albicans* and the Arthrospores of *Trichophyton mentagrophytes*.*Nippon Ishinkin Gakkai Zasshi*, 49(2), 125-128.

Reinel, D., Plettenberg, A., Seebacher, C., Abeck, D., Brasch, J., Cornely, O., ... & Hipler, U. C. (2008). Orale Candidose. JDDG: *Journal der Deutschen Dermatologischen Gesellschaft*, 6(7), 593-597.

Reisner, A., Krogfelt, K. A., Klein, B. M., Zechner, E. L., & Molin, S. (2006). *In vitro* biofilm formation of commensal and pathogenic Escherichia coli strains: impact of environmental and genetic factors. *Journal of Bacteriology*,188(10), 3572-3581.

Richard, M. L., & Plaine, A. (2007). Comprehensive analysis of glycosylphosphatidylinositol-anchored proteins in *Candida albicans*.*Eukaryotic Cell*, 6(2), 119-133.

Robbins, N., Uppuluri, P., Nett, J., Rajendran, R., Ramage, G., Lopez-Ribot, J. L., ... & Cowen, L. E. (2011). Hsp90 governs dispersion and drug resistance of fungal biofilms. *PLoS Pathology*, 7(9), e1002257.

Rodríguez, A., Herrero, P., & Moreno, F. (2001). The hexokinase 2 protein regulates the expression of the GLK1, HXK1 and HXK2 genes of *Saccharomyces cerevisiae*. *Biochemical Journal*, 355(3), 625-631.

Rodríguez-Tudela, J. L., Cuenca-Estrella, M., Díaz-Guerra, T. M., & Mellado, E. (2001). Standardization of antifungal susceptibility variables for a semiautomated methodology. *Journal of Clinical Microbiology*, 39(7), 2513-2517.

Roelants, F. M., Baltz, A. G., Trott, A. E., Fereres, S., & Thorner, J. (2010). A protein kinase network regulates the function of aminophospholipid flippases. Proceedings of the National Academy of Sciences, 107(1), 34-39.

Romeo, O., Scordino, F., Pernice, I., Passo, C. L., & Criseo, G. (2009). A multiplex PCR protocol for rapid identification of *Candida glabrata* and its phylogenetically related species *Candida nivariensis* and *Candida bracarensis*. *Journal of Microbiological Methods*, 79(1), 117-120.

Rueda, C., Cuenca-Estrella, M., & Zaragoza, O. (2014). Paradoxical growth of *Candida albicans* in the presence of caspofungin is associated with multiple cell wall rearrangements and decreased virulence. *Antimicrobial Agents and Chemotherapy*, 58(2), 1071-1083.

Rumbo-Feal, S., Gómez, M. J., Gayoso, C., Álvarez-Fraga, L., Cabral, M. P., Aransay, A. M., ... & Bou, G. (2013). Whole transcriptome analysis of Acinetobacter baumannii assessed by RNA-sequencing reveals different mRNA expression profiles in biofilm compared to planktonic cells. *PLoS One*,8(8), e72968.

Safdar, A., Van Rhee, F., Henslee-Downey, J. P., Singhal, S., & Mehta, J. (2001). *Candida glabrata* and *Candida krusei* fungemia after high-risk allogeneic marrow transplantation: no adverse effect of low-dose fluconazole prophylaxis on incidence and outcome. Bone marrow transplantation, 28(9), 873.

Santana, I. L., Gonçalves, L. M., de Vasconcellos, A. A., da Silva, W. J., Cury, J. A., & Cury, A. A. D. B. (2013). Dietary carbohydrates modulate *Candida albicans* biofilm development on the denture surface. *PloS One*, 8(5), e64645.

Sardi, J. C. O., Scorzoni, L., Bernardi, T., Fusco-Almeida, A. M., & Giannini, M. M. (2013). *Candida* species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *Journal of Medical Microbiology*, 62(1), 10-24.

Schaller, M., Borelli, C., Korting, H. C., & Hube, B. (2005). Hydrolytic enzymes as virulence factors of *Candida albicans*. *Mycoses*, 48(6), 365-377.

Selvarangan, R., Limaye, A. P., & Cookson, B. T. (2002). Rapid identification and differentiation of *Candida albicans* and *Candida dubliniensis* by capillary-based amplification and fluorescent probe hybridization. *Journal of Clinical Microbiology*, 40(11), 4308-4312.

Sendid, B., Caillot, D., Baccouch-Humbert, B., Klingspor, L., Grandjean, M., Bonnin, A., & Poulain, D. (2003). Contribution of the Platelia *Candida*-specific antibody and antigen tests to early diagnosis of systemic *Candida tropicalis* infection in neutropenic adults. *Journal of Clinical Microbiology*, 41(10), 4551-4558.

Sendid, B., Poirot, J. L., Tabouret, M., Bonnin, A., Caillot, D., Camus, D., & Poulain, D. (2002). Combined detection of mannanaemia and anti-mannan antibodies as a strategy for the diagnosis of systemic infection caused by pathogenic Candida species. *Journal of Medical Microbiology*, 51(5), 433-442.

Senn, L., Robinson, J. O., Schmidt, S., Knaup, M., Asahi, N., Satomura, S., ... & Marchetti, O. (2008). 1, 3-β-D-glucan antigenemia for early diagnosis of invasive fungal infections in neutropenic patients with acute leukemia. *Clinical Infectious Diseases*, 46(6), 878-885.

Skiest, D. J., Vazquez, J. A., Anstead, G. M., Graybill, J. R., Reynes, J., Ward, D., ... & Isaacs, R. (2007). Posaconazole for the treatment of azole-refractory oropharyngeal and esophageal candidiasis in subjects with HIV infection. *Clinical Infectious Diseases*, 44(4), 607-614.

Sonda, S., Sala, G., Ghidoni, R., Hemphill, A., & Pieters, J. (2005). Inhibitory effect of aureobasidin A on Toxoplasma gondii. Antimicrobial agents and chemotherapy, 49(5), 1794-1801.

Spampinato, C., & Leonardi, D. (2013). *Candida* infections, causes, targets, and resistance mechanisms: traditional and alternative antifungal agents. *BioMed Research International*, 2013.

Staab, J. F., Bradway, S. D., Fidel, P. L., & Sundstrom, P. (1999). Adhesive and mammalian transglutaminase substrate properties of *Candida albicans* Hwp1. *Science*, 283(5407), 1535-1538.

Stoodley, P., Sauer, K., Davies, D. G., & Costerton, J. W. (2002). Biofilms as complex differentiated communities. *Annual Reviews in Microbiology*, 56(1), 187-209.

Sudbery, P., Gow, N., & Berman, J. (2004). The distinct morphogenic states of *Candida albicans*. *Trends in Microbiology*, 12(7), 317-324.

Sullivan, D. J., Moran, G. P., Pinjon, E., Al-Mosaid, A., Stokes, C., Vaughan, C., & Coleman, D. C. (2004). Comparison of the epidemiology, drug resistance mechanisms, and virulence of *Candida dubliniensis* and *Candida albicans*. *FEMS yeast research*, 4(4-5), 369-376.

Sullivan, D. J., Westerneng, T. J., Haynes, K. A., Bennett, D. E., & Coleman, D. C. (1995). *Candida dubliniensis* sp. nov.: phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals. *Microbiology*, *141*(7), 1507-1521.

Sun, X., Lu, H., Jiang, Y., & Cao, Y. (2013). CaIPF19998 reduces drug susceptibility by enhancing the ability of biofilm formation and regulating redox homeostasis in *Candida albicans*. *Current Microbiology*, *67*(3), 322-326.

Taff, H. T., Mitchell, K. F., Edward, J. A., & Andes, D. R. (2013). Mechanisms of *Candida* biofilm drug resistance. *Future Microbiology*, *8*(10), 1325-1337.

Takesako, K., Ikai, K., Haruna, F., Endo, M., Shimanaka, K., Sono, E., ... & Yamaguchi, H. (1991). Aureobasidins, new antifungal antibiotics. Taxonomy, fermentation, isolation, and properties. *The Journal of Antibiotics*, *44*(9), 919-924.

Takesako, K.,Kuroda, H., Inoue, T., Haruna, F., Yoshikawa, Y., Kato, I., …& Yamaguchi, H. (1993). Biological properties of aureobasidin A, a cyclic depsipeptide antifungal antibiotic. *The Journal of* Antibiotics, 44(9), 1414-1420.

Tan, H. W., & Tay, S. T. (2013). The inhibitory effects of aureobasidin A on *Candida* planktonic and biofilm cells. *Mycoses*, *56*(2), 150-156.

Tavanti, A., Davidson, A. D., Gow, N. A., Maiden, M. C., & Odds, F. C. (2005). Candida orthopsilosis and *Candida metapsilosis* spp. nov. to replace *Candida parapsilosis* groups II and III. *Journal of Clinical Microbiology*, *43*(1), 284-292.

Torelli, R., Posteraro, B., Ferrari, S., La Sorda, M., Fadda, G., Sanglard, D., & Sanguinetti, M. (2008). The ATP-binding cassette transporter–encoding gene CgSNQ2 is contributing to the CgPDR1-dependent azole resistance of *Candida glabrata*. *Molecular Microbiology*, *68*(1), 186-201.

Trofa, D., Gácser, A., & Nosanchuk, J. D. (2008). *Candida parapsilosis*, an emerging fungal pathogen. *Clinical Microbiology Reviews*, *21*(4), 606-625.

Trotter, E. W., Rand, J. D., Vickerstaff, J., & Grant, C. M. (2008). The yeast Tsa1 peroxiredoxin is a ribosome-associated antioxidant. *Biochemical Journal*, *412*(1), 73-80.

Tsang, C. S. P., Chu, F. C. S., Leung, W. K., Jin, L. J., Samaranayake, L. P., & Siu, S. C. (2007). Phospholipase, proteinase and haemolytic activities of *Candida albicans* isolated from oral cavities of patients with type 2 diabetes mellitus. *Journal of Medical Microbiology*, *56*(10), 1393-1398.

Vandeputte, P., Ferrari, S., & Coste, A. T. (2011). Antifungal resistance and new strategies to control fungal infections. *International Journal of Microbiology*, 2012.

Vandeputte, P., Pineau, L., Larcher, G., Noel, T., Brèthes, D., Chabasse, D., & Bouchara, J. P. (2011). Molecular mechanisms of resistance to 5-fluorocytosine in laboratory mutants of *Candida glabrata*. *Mycopathologia*,*171*(1), 11-21.

Vazquez, J. A., Dembry, L. M., Sanchez, V., Vazquez, M. A., Sobel, J. D., Dmuchowski, C., & Zervos, M. J. (1998). Nosocomial *Candida glabrata* colonization: an epidemiologic study. *Journal of Clinical Microbiology*, *36*(2), 421-426.

Vazquez, J. A., Peng, G., Sobel, J. D., Steele-Moore, L., Schuman, P., Holloway, W., & Neaton, J. D. (2001). Evolution of Antifungal Susceptibility among *Candida* Species Isolates Recovered from Human Immunodeficiency Virus—Infected Women Receiving Fluconazole Prophylaxis. *Clinical Infectious Diseases*, *33*(7), 1069-1075.

Vermitsky, J. P., & Edlind, T. D. (2004). Azole resistance in Candida glabrata: coordinate upregulation of multidrug transporters and evidence for a Pdr1-like transcription factor. *Antimicrobial Agents and Chemotherapy*,*48*(10), 3773-3781.

Vermitsky, J. P., Earhart, K. D., Smith, W. L., Homayouni, R., Edlind, T. D., & Rogers, P. D. (2006). Pdr1 regulates multidrug resistance in Candida glabrata: gene disruption and genome-wide expression studies. *Molecular Microbiology*, *61*(3), 704-722.

Vincent, J. L., Rello, J., Marshall, J., Silva, E., Anzueto, A., Martin, C. D., ... & Reinhart, K. (2009). International study of the prevalence and outcomes of infection in intensive care units. *Jama*, *302*(21), 2323-2329.

Voynova, N. S., Roubaty, C., Vazquez, H. M., Mallela, S. K., Ejsing, C. S., & Conzelmann, A. (2015). Saccharomyces cerevisiae is dependent on vesicular traffic between the Golgi apparatus and the vacuole when inositolphosphorylceramide synthase Aur1 is inactivated. *Eukaryotic Cell*,*14*(12), 1203-1216.

Wang, H., Xiao, M., Chen, S. C. A., Kong, F., Sun, Z. Y., Liao, K., ... & Hu, Z. D. (2012). *In vitro* susceptibilities of yeast species to fluconazole and voriconazole as determined by the 2010 National China Hospital Invasive Fungal Surveillance Net (CHIF-NET) study. *Journal of Clinical Microbiology*,*50*(12), 3952-3959.

Wang, H., Xiao, M., Chen, S. C., Kong, F., Sun, Z. Y., Liao, K., ... & Hu, Z. D. (2012). Results from the National China Hospital Invasive Fungal Surveillance Net (CHIF-NET) Study, 2010: Yeast Species and *In vitro* Susceptibilities to Fluconazole and Voriconazole. *Journal of Clinical Microbiology*, JCM-01130.

Warren, T. A., McTaggart, L., Richardson, S. E., & Zhang, S. X. (2010). *Candida bracarensis* bloodstream infection in an immunocompromised patient. *Journal of Clinical Microbiology*, *48*(12), 4677-4679.

Watkinson, S. C., Boddy, L., & Money, N. (2015). *The Fungi*. Academic Press.

White, P. L., Archer, A. E., & Barnes, R. A. (2005). Comparison of non-culture-based methods for detection of systemic fungal infections, with an emphasis on invasive Candida infections. *Journal of Clinical Microbiology*,*43*(5), 2181-2187.

Wisplinghoff, H., Bischoff, T., Tallent, S. M., Seifert, H., Wenzel, R. P., & Edmond, M. B. (2004). Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study.*Clinical Infectious Diseases*, *39*(3), 309-317.

Wisplinghoff, H., Seifert, H., Wenzel, R. P., & Edmond, M. B. (2006). Inflammatory response and clinical course of adult patients with nosocomial bloodstream

infections caused by *Candida* spp. *Clinical Microbiology and Infection*, *12*(2), 170-177.

Yera, H., Sendid, B., Francois, N., Camus, D., & Poulain, D. (2001). Contribution of serological tests and blood culture to the early diagnosis of systemic candidiasis. *European Journal of Clinical Microbiology & Infectious Diseases*, 20(12), 864-870.

Zarnowski, R., Westler, W. M., Lacmbouh, G. A., Marita, J. M., Bothe, J. R., Bernhardt, J., ... & Ntambi, J. M. (2014). Novel entries in a fungal biofilm matrix encyclopedia. *MBio*, *5*(4), e01333-14.

Zhang, L., Zhang, Y., Zhou, Y., An, S., Zhou, Y., & Cheng, J. (2002). Response of gene expression in Saccharomyces cerevisiae to amphotericin B and nystatin measured by microarrays. *Journal of Antimicrobial Chemotherapy*, 49(6), 905-915.

LIST OF PUBLICATIONS AND PAPERS PRESENTED

PUBLICATION

Munusamy, K., Vadivelu, J., Tay, ST. (2018). A study on *Candida* biofilm growth characteristics and its susceptibility to aureobasidin A. Revista Iberoamericana de Micologia. 35(2), 68-72.

ATTENDED CONFERENCE

INFECTIONS, 2015

P1021-15 Susceptibility of planktonic and biofilm cultures of *Candida* spp. upon exposure to Aureobasidin A and premilimary investigation of the drug effets on yeast growth

Munusamy K, Vellasamy KM, Mariappan V, Vadivelu J, Tay ST