Investigation of antifungal compounds against *Aspergillus* species from respiratory infections of immunocompromised patients

Submitted in fulfilment of the requirement for the Degree of Doctorate of Philosophy in Biotechnology in the Department of Biotechnology and Food Science, Durban University of Technology, Durban, South Africa

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DECLARATION

I hereby declare that this dissertation is my own, unaided work. It is being submitted for the award of Doctor of Philosophy in Biotechnology, to the Durban University of Technology, Department of Biotechnology and Food Science, Faculty of Applied Sciences, Durban, South Africa. It has not been submitted before for any degree or dissertation to any other institution.

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Dedicated to

My Father

(My unwavering guiding light)

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LIST OF ACRONYMS AND ABBREVIATIONS

ABPA : Allergic Bronchopulmonary Aspergillosis

AF : Aflatoxin

AIDS : Acquired immunodeficiency syndrome

CAF : Central Analytical Facilities

CAPA : COVID-19 associated pulmonary aspergillosis

CCR : carbon catabolite repression

CDC : Center for Disease Control

CF : Cystic Fibrosis

CGD : Chronic Granulomatus Disease

CNS : Central Nervous System

CPA : Chronic pulmonary aspergillosis

COPD : Chronic Obstructive Pulmonary Disease

COVID-19 : Corona virus disease of 2019

DMI : Dimethylase inhibitor

DMSO : Dimethyl sulfoxide

DNA : Deoxyribonucleic acid

ECDC : European Centre for Disease Prevention and Control

EDTA : Ethylenediaminetetraacetic acid

EGTA : Egtazic acid

ETP : Epipolythiodioxopipenazine

ESCMID : European Society of Clinical Microbiology and Infectious Diseases

F : Fumonisons

FF : Filamentous fungi

FAO : Food and Agriculture Organization

FDA : Food and Drug Administration

GT : Gliotoxin

HIV : Human immunodeficiency virus

IA : Invasive aspergillosis

ICU : Intensive care unit

ID : Identification

IDSA : Infectious Diseases Society of America

IFI : Invasive fungal infections

IS : intermediate susceptibility

IV : Intravenous

HPLC : High-performance liquid chromatography

HSCT : Hematopoietic stem-cell transplantation

IF : Inoculation fluid

ITS : Internal transcribe spacer

LCMS : Liquid chromatography-mass spectroscopy

LPS : Lipopolysaccharides

LTA : Lipoteichoic acid

MEA : Malt extract agar

MIC : Minimum inhibitory concentration

MTS : MIC Test Strip

NCR : Nitrogen catabolite repression

ND : Not detected

NGS : Next-generation sequencing

OD : Optical density

OT : Ochratoxins

PAMPS : Pathogen-associated molecular patterns

PATH : Prospective Antifungal Therapy

PCP : Pneumocystis pneumonia

PCR : Polymerase chain reaction

PDA : Potato dextrose agar

PG : Peptidoglycan

PM : Phenotypic microarray

POS : Posaconazole

PRR : Pattern recognition receptors

UV : Ultra violet

VRC : Voriconazole

R : Resistant

RPMI : Roswell Park Memorial Institute - medium

RNA : Ribonucleic acid

RP-HPLC : Reverse Phase High-Performance Liquid Chromatography

S : Susceptible

SBI : Sterol Biosynthesis Inhibitors

SOT : Solid Organ Transplant

SCT : Stem cell transplant

TLC : Thin layer chromatography

TLR : Toll-like receptors

TR : Tandem repeat

SDS : sodium lauryl sulphate

SNP : single nucleotide polymorphisms

WT : Wild -type

ZEN : Zearalenone

ABSTRACT

The rapid emergence of invasive fungal infections correlates with the increasing population of immunocompromised individuals, with many cases leading to death. The progressive increase in the incidence of *Aspergillus* isolates is even more severe due to clinical challenges in treating invasive respiratory infections in immunocompromised patients. Azoles are the drugs of choice for the prevention and treatment of *Aspergillus* infections however, azole resistance in *Aspergillus*-related infections is an increasing concern, especially against *Aspergillus fumigatus.* This situation is further complicated by the use of azoles in agriculture, as increasing resistance has been associated with increased use in agriculture. Rapid initiation in detection, diagnosis and appropriate antifungal therapy is needed to reduce mortality among individuals with invasive *Aspergillus*-related infections.

Improvements in all aspects of azole resistance management, from identification and antifungal therapy to the discovery of novel non-toxic drugs, impact clinical success. Hence, this study assessed the effect of routine and salvage drug therapy on *Aspergillus* respiratory infections and investigated the potential of new drugs in combination therapy in light of increasing antifungal resistance in the management of invasive fungal infections. *Aspergillus* species isolated from immunocompromised patients with respiratory infections at the Inkosi Albert Luthuli Hospital in Kwa-Zulu Natal were investigated. Conventional morphology identification methods were assessed for reliability and compared to molecular identification. In the search for reliable and rapid alternatives in fungal identification to impact diagnosis, phenotypic microarray (Biolog) for fungal species identification was explored. Phenotypic microarray (Biolog) was also used to analyse the isolates' nutritional patterns and drug sensitivity profiles to investigate the potential for new compounds in drug therapy. In addition, gliotoxin expression, which has been linked to increased pathogenesis, was quantified and analysed using high-performance liquid chromatography. This was investigated *in vitro* to correlate gliotoxin production as a possible virulence factor in azole resistance. The effect of pathogen-associated molecular patterns (PAMPS) in bacterial co-infection was also investigated, especially since the species were isolated from respiratory infections where co-infection is common.

Conventional morphological techniques were generally similar to 18S rRNA identification, assigning twenty-six *Aspergillus fumigatus* species, eight *A. niger* and two *A. flavus.* Biolog technology only identified isolates correctly up to genus level and had no significant similarity matches at species level due to the Biolog fungal database not having adequate reference clinical species however, an updated database makes this technology a good alternative when reliability and speed in identification are considered. Antifungal profiles showed that 6% of the 36 isolates were resistant to the routine azole voriconazole, with 61% having moderate susceptibility. All isolates resistant to the salvage therapy drug, posaconazole pose a serious concern. Significantly, *A.niger* was the only species resistant (25%) to voriconazole and has recently been reported as the species isolated from patients with COVID-19-associated pulmonary aspergillosis (CAPA). Mutations in the *cpy51A* gene representing common mechanisms for azole resistance in clinical *Aspergillus* isolates were highlighted in the azole susceptibility profile. Phenotypic microarray showed that 83% of the isolates were susceptible to the 24 new compounds. Berberine and blasticidin hydrochloride were selected and further investigated for combination drug therapy, considering their non-toxicity upon oral administration currently with thirty of the thirty-six isolates showing susceptibility to the new agents.

Carbon profiles demonstrated the consistent assimilation of alternate monosaccharides and disaccharides by all three *Aspergillus* species, accomplished by secreted enzymes which very likely contribute to nutrient acquisition during infection, impact persistent growth and survival to influence infection maintenance and disease progression. Of the twenty-six *A. fumigatus* isolates, 58% produced gliotoxin during the 48-hour incubation at 37℃. Forty per cent of those gliotoxin-producing isolates showed increased gliotoxin production after exposure to PAMPS, with the highest concentration of 2.6 mg/L and a mean of 0.6 mg/L. Compared to similar investigations, the gliotoxin concentrations for invasive aspergillosis were higher than for colonization in respiratory patients.

This study found that different identification strategies in azole resistance treatment management are required for both developed and resource-challenged situations to influence diagnosis. Drug resistance in azole monotherapy was demonstrated. Thus this study supports the search for new agents in drug regimen strategy and found potential in novel compounds, berberine and blasticidin hydrochloride, for combination therapy.

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Fungi are phylogenetically and functionally varied, ubiquitous components of nearly all ecosystems on Earth (Grossart *et al.* 2019). The pathogenic potential of fungi poses a substantial global threat to humans, plants, insects and animals and thus impacts varied ecosystems (Hernandez and Martinez 2018). These eukaryotic pathogens have infected and killed people over 1.5 million per year, yet the impact of fungi on human health is underrated (Brown, Denning and Levitz 2012; Fisher *et al.* 2020). Fungi present challenges to humanity due to their metabolic capacity and rapid ability in reaction to pressures to adapt, penetrate tissues and evade immune responses, as well as having a negative impact on human health through secondary metabolite formation of mycotoxins by filamentous fungi (Riquelme *et al.* 2018). This recognition that more attention and effort must be directed to research all areas of fungal biology led Nature Microbiology to push to end this neglect of fungi (Jermy 2017).

Invasive fungal diseases have emerged as a significant medical challenge worldwide. Systemic fungal infections having been rare in humans, until the 1950s with the start of the use of immunosuppressive agents like Corti-steroids, the development of cancer chemotherapies and the inception of catheters providing access for microbes to the body's interior (Bongomin *et al.* 2017; Friedman and Schwartz 2019). These infections are brought by the inhalation or injection of spores, activating commensal fungi that already exist on or within the body, or both. Fungi cause a majority of invasive fungal infections with low pathogenic potential; these infections primarily affect people with innate or acquired immunity deficiencies, such as those with HIV infection or severe influenza, as well as those who have received immunosuppressive cancer treatment (Fisher *et al.* 2020). The most common causes of fungal infections in human lungs are opportunistic fungi such as *Aspergillus* with invasive *Aspergillosis*, *Cryptococcus* with cryptococcosis, Pneumocystis with pneumonia and endemic fungi. Although these infections are uncommon in healthy individuals, they can cause life-threatening invasive illnesses in patients with compromised immune systems (Li, Lu and Meng 2019).

The progressive increase in the incidence of *Aspergillus* isolates poses a clinical challenge in treating invasive infection in immunocompromised patients (Friedman and Schwartz 2019). The severity and impact of fungal infections have resulted in the World Health Organization drawing up the first-ever list of fungal pathogens that are potential threats to human health. The pathogens are divided into critical, high and medium-priority categories, with *Aspergillus fumigatus* included in the critical group (WHO, 2022)

In resource-limiting settings common in African countries, invasive fungal infections remain understudied and underdiagnosed despite their high mortality rates compared with other infectious diseases (Bongomin *et al.* 2017). More than 200 000 aspergilli-related infections are reported annually (Rybak, Fortwendel and Rogers 2019). According to the Global AIDS Update in Geneva 2016 (HIV/AIDS 2019), fungal infections are frequent in South Africa and are mainly caused by the HIV/TB syndemic. In South Africa, fungal infections have a significant disease burden, negatively influencing public health. Thus, improved diagnostic and management procedures for fungal illnesses can help reduce morbidity and mortality associated with these diseases (Schwartz *et al.* 2019). The rise in immune-deficient patients due to cancer, ageing, organ transplants, AIDS, and other invasive surgical procedures impacts the rising prevalence of infections. An observational study of patients with invasive fungal infections in North America revealed that 81.8% of the *Aspergillus* species were isolated from pulmonary infections. Although a far more significant percentage of fungal species were *Candida* and *Cryptococcus,* the survival probability was lower in patients with *Aspergillus* than other fungal species (Marukutira *et al.* 2014).

The lack of effective diagnostic methods for quick identification is a significant limitation in tackling the high morbidity and mortality of fungal diseases. Microscopy and culture detection of fungal infections are insensitive and frequently negative in early infection when treatment is most impactful. Molecular techniques have emerged as standard approaches for microbiological infection (Blackwell *et al.* 2006). However, in developing countries, costs and a lack of infrastructure continues to be a disadvantage.

The prolonged use of antifungal compounds in human health and agriculture has altered the global microbiome, resulting in human drug-resistant fungal infections. Azole resistance in *Aspergillus* isolates further challenges this emergence. Azoles are the first-line therapies used to treat aspergillosis (Rivelli Zea and Toyotome 2022). However, the widespread proliferation of azole-resistant *A. fumigatus* isolates in medicine and the environment restricts antifungal

therapy choices and may be linked to an elevated death rate (Arastehfar *et al.* 2021). Emerging azole resistance in routine therapy against *Aspergillus* species is the clinical challenge in treating invasive fungal infection in immunocompromised patients. Although cpy51A mutations are known to be the most common cause of azole resistance, a new wave of azole-resistant isolates with wild-type cpy51A genotype calls into question the efficiency of current diagnostic techniques (Arastehfar *et al.* 2021). The drug tolerance along with liver and kidney toxicity of an antifungal agent, amphotericin B, demands a search for novel antifungal drugs to tackle azole-resistant *Aspergillus* species for the treatment and successful management of *Aspergillus*-related respiratory infections (Hamill 2013).

Fisher *et al.* (2020) emphasized the need for multidisciplinary research networks to understand the mechanisms that drive the genesis and spread of fungi that impact plants, animals, and people. To implement successful diagnostic approaches and develop novel strategies to prevent fungal diseases as a global health threat, the challenge of identifying mechanisms of fungal adaptation and interactions with hosts and other microbes, as well as understanding the evolution of resistance to fungicides and antifungals, must be addressed. Combining phenotypic and genetic profiles of these fungal species to mobilize such networks could help gain a better insight into the antifungal resistance mechanisms and their clinical impact. Chemical sensitivity profiles of *Aspergillus* species would show the current azole treatment impact and the potential of new compounds. Investigation of nutrient growth patterns may reveal metabolic adaptation of *Aspergillus* species that impacts infection. Virulence factors such as gliotoxin and the suggestion as a link to the species' pathogenesis could assist in managing and controlling *Aspergillus* infections (Lewis *et al.* 2005b; Vidal-García *et al.* 2018; Raffa and Keller 2019).

This pressing fungal threat to global health has prompted this study to determine the prevalence of *Aspergillus* species in respiratory infections in immunocompromised patients at the Inkosi Albert Luthuli Hospital in KwaZulu-Natal, South Africa. Conventional morphological techniques were carried out to test for reliability and compared to molecular identification. Routine and salvage therapeutic azoles were assessed for susceptibility and phenotypic microarray was used to investigate the potential of new compounds for the effective treatment of *Aspergillus* infections. The study analyzed nutrient growth patterns to correlate the *Aspergillus* species' persistent infection and examined gliotoxin expression as a virulence factor in its pathogenicity.

1.2. Literature Review

1.2.1. Fungi

Fungi including yeast and mould, are eukaryotic organisms classified in the Kingdom Fungi, with yeast being unicellular and mould multicellular organisms whose cell walls are made up of chitin. Moulds (filamentous fungi) grow as elongated, thread-like structures called hyphae that form mycelia. They reproduce by sexual and asexual means and produce spores (Mousavi *et al.* 2016). Fungi thrive in a wide range of habitats, aquatic and terrestrial, including extreme environments, in their role as decomposers of organic matter and nutrient recycling. Fungi acquire most of their nutrients by absorbing dissolved organic matter. Due to their lifestyle and degradative ability, humans have found benefits from using these microorganisms for millenniums (Mousavi *et al.* 2016).

Fungi have long been of human benefit in food production, as a leavening agent for bread and in the fermentation of numerous food products (Copetti 2019). They have been used to produce antibiotics as biological pesticides and their enzymes benefit industrially and detergents (Kour *et al.* 2019; Akhtar and Mannan 2020). However, specific species produce secondary metabolites like mycotoxins that are toxic to animals and humans. Fungal diseases result in crop losses, food spoilage impacts the human food supply and fungi have become significant human pathogens in opportunistic infections.

1.2.1.1. Fungal diseases

Although most fungi are harmless to humans, some cause severe diseases known as mycosis. When fungi reproduce and release spores into the environment, they can be inhaled or contacted directly, resulting in topical infections, usually skin, nail and internal infections, like lung infections. Although not a usual occurrence, fungi can penetrate the skin, affect organs and cause systemic infection. Some fungi don't usually cause infections in humans; however, they can become opportunistic pathogens and cause diseases in people with weakened immune systems. In immunocompromised patients, fungal infections are one of the most frequent complications, and endemic mycoses are the leading cause of morbidity and mortality in this population (Malcolm and Chin-Hong 2013). Fungi have been ranked by de Pauw (2011) as the ten most frequently isolated pathogens in febrile patients, with impaired immunity and filamentous fungi being mainly opportunistic pathogens, invading weakened natural defence. *Aspergillus* and C*andida* species represent the majority of fungi identified in

immunocompromised patients. *Aspergillus* species are the most commonly encountered filamentous fungi in infections in these immune-compromised populations (Rammaert *et al.* 2012; Lamps, Lai and Milner Jr 2014).

1.2.2. Ecology of *Aspergillus* species

Aspergillus species are found in various environments, including soil, crops, organic debris, animals, and humans (Samson *et al.* 2007; Barrs *et al.* 2013). The pathogenic *A. fumigatus, A. flavus, A. niger, A. terreus,* and *A. nidulans*, which share morphological similarities, are among the causative agents of aspergillosis (Sugui *et al.* 2015). *Aspergillus fumigatus* continues to be the principal cause of IA (invasive aspergillosis) even though the majority of these species are common in the environment (Raper and Fennell 1965), The fact that *A. fumigatus* is so common and is the primary clinical cause of aspergillosis is due in part to the fact that the average person inhales hundreds of airborne conidia each day (Latgé 1999). Due to its superior capacity to thrive and grow in a wider variety of environmental conditions than other aspergilli, *A. fumigatus* is more common than other aspergilli. Conidia released by conidiophores throughout the saprophytic cycle are easily disseminated in the environment. Conidia remain dormant until they come into contact with the environmental factors that trigger the activation of their metabolism. Figure 1.1 shows how conidia grow as they start to metabolically function, develop into hyphal filaments, mature into mycelium, and eventually produce conidia with conidiophores, which are fruiting structures.

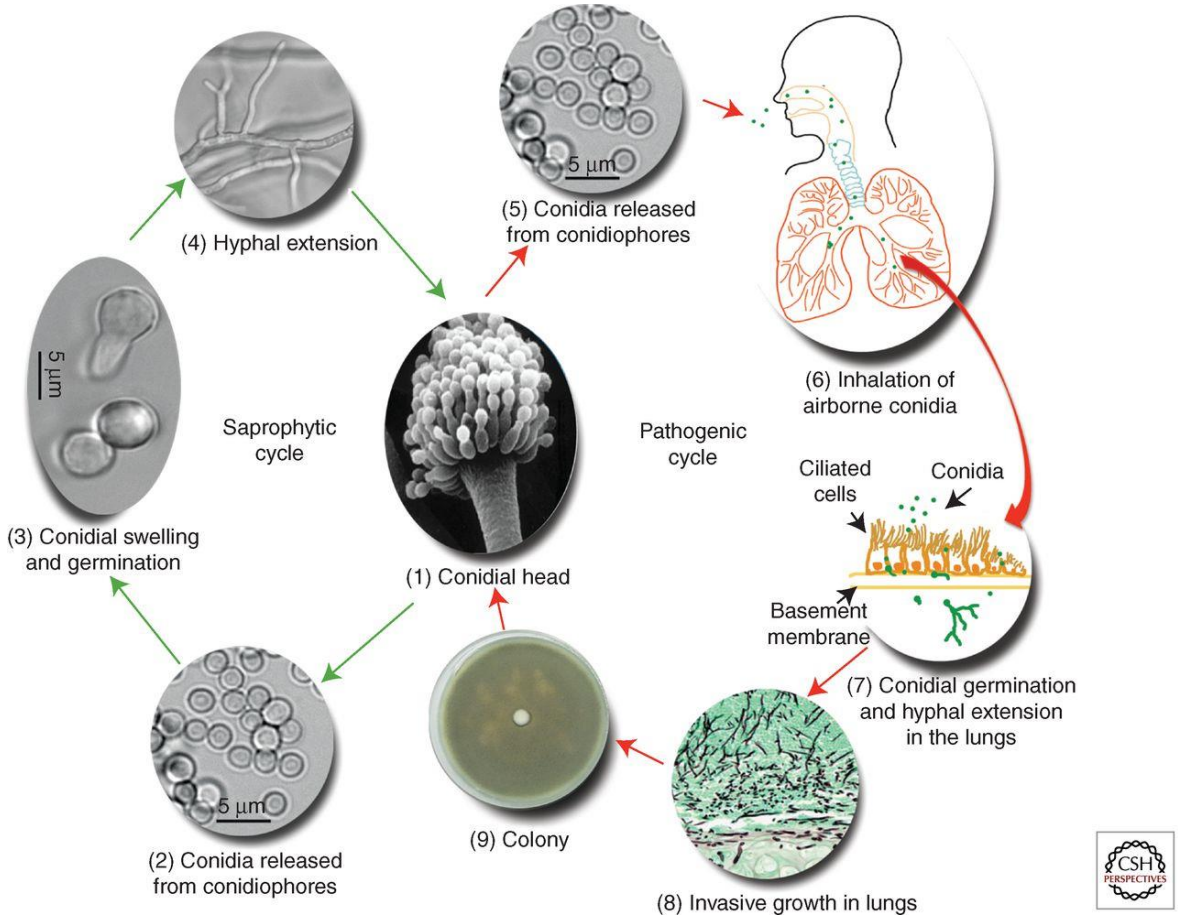

Figure 1.1: Saprophytic and pathogenic cycles of *A. fumigatus* (Sugui *et al.* 2015).

Since *A. fumigatus* grows best around 37°C, it can be isolated anywhere soil and decomposing plants reach temperatures of 12-65°C and a pH of 2.1-8.8 (Kozakiewicz and Smith 1994; Latgé 1999; Kwon-Chung and Sugui 2013). Additionally, the genome of *A. fumigatus* contains many genes that encode glycosyl hydrolases and extracellular proteinases. These enzymes help the organism grow successfully by breaking down plant cell polysaccharides and acquiring nitrogen by breaking down proteinaceous substrates (Tekaia and Latgé 2005; Abad 2010; Abad *et al.* 2010). *Aspergillus fumigatus* may grow on a range of substrates and can withstand stress brought on by extended dehydration or cold (Kozakiewicz and Smith 1994). Moreover, due to its well-known hydrophobicity, conidia of *A. fumigatus* disperse more easily than most other *aspergilli* (Bayry *et al.* 2012). Another element influencing the prevalence of *A. fumigatus* is its capacity to generate potent secondary metabolites and many efflux pumps that serve as defence mechanisms (Kwon-Chung and Sugui 2013).

1.2.2.1. Physiological adaptation to the environment

Pathogenic fungi have developed genetic mechanisms and molecular techniques to survive and cause disease in their hosts by controlling carbon metabolism (Matar *et al.* 2017). This is critical for the spread of infection by the filamentous fungus. Carbon catabolite repression (CCR) aids microorganisms in adapting their physiology to their surroundings. When a more easily accessible carbon source is in the media, CCR turns off some enzymes necessary to use less-favoured carbon sources (Ries *et al.* 2016). Therefore, CCR is a means in microorganisms for screening glucose. Besides regulating glucose uptake, CCR may also influence the survival of microorganisms by affecting virulence, adaptation, cellular communication and motility (Adnan *et al.* 2017).

1.2.3. *Aspergillus* species and their pathogenicity

A common saprophytic fungal genera called *Aspergillus* can be found in several ecological niches. The genus is distinguished by the development of flask-shaped or cylindrical phialides on the surface of a vesicle at the apex of a conidiophore, either in a single or multiple series (Raper and Fennell 1965). Conidia are globose, deciduous, oblong to elliptical, and have a variety of colours. In a monograph published in 1926, Thom and Church compiled all information about *Aspergillus* (Thom and Church 1926). Raper and Fennell continued this study in 1965, adding more numerous species and providing descriptions of those grouped into 18 informal groupings (Raper and Fennell 1965). The groupings were updated and allocated to 18 sections as a formal taxonomic rank in 1985 (Pitt and Samson 1990). The family Aspergillaceae now has roughly 250 species classified into 17 sections and this number will continue to rise as new species are discovered (Houbraken and Samson 2011). *Aspergillus* have a tremendously positive and negative influence on industrial applications and in the treatments of plant and human infections. *Aspergillus niger* is used in food and pharmaceutical industries because of its extensive enzymatic profile (Dagenais and Keller 2009).

The species that cause diseases in people are mainly *Fumigati*, *Flavi, Nigri, Terrei*, and *Nidulante*. The most common species that cause invasive illness are *A. fumigatus, A. flavus, A. niger,* and *A. terreus*. *A. fumigatus* is the most common causative organism in solid organ transplant (SOT) and hematopoietic stem cell transplant (HSCT) patients, as well as in leukemia patients (Pagano *et al.* 2010). The *Fumigati* group plays an important role in causing infectious diseases since it has not only *A. fumigatus* but also 11 other IA pathogenic species. They form a conidiophore with columnar conidial heads made of flask-shaped vesicles,

uniseriate phialides, and long chains of conidia. Conidia are hydrophobic, bluish-green to light green in hue, and 2.5-3.5 µm in size. It is estimated that *A. fumigatus* causes aspergillosis in about 200 000 persons yearly (Brown, Denning and Levitz 2012).

Species like *Aspergillus flavus* and *Aspergillus parasiticous* contaminate several crops with aflatoxin, a highly toxic carcinogen with immunosuppressive properties (Wild 2007), a common problem in developing countries. *Aspergillus nidulans* and *Aspergillus terreus* also cause human infections. Still, *Aspergillus fumigatus* is responsible for causing not only allergic diseases like asthma, allergic sinusitis and alveolitis but severe respiratory complications such as allergic bronchopulmonary aspergillosis (ABPA), aspergilloma and invasive aspergillosis (IA) in immunocompromised patients (Latgé 1999). Furthermore, *A. fumigatus* produces the secondary metabolite, gliotoxin, capable of altering host defence mechanisms through immunosuppression.

1.2.3.1. *Aspergillus niger*

Aspergillus niger (*A. niger*) grows more widely in nature than most other *Aspergillus* species due to its ability to grow on a wide range of substrates even those with limited nutrients. It frequently develops on moist walls in houses. *Aspergillus niger* is one of the most significant microorganisms in biotechnology due to its usage in bio-transformations and waste management as well as its remarkable potential for producing industrially important extracellular food enzymes and citric acid. According to molecular analysis, the only common species in the *Aspergillus* Section Nigri closely related to *A. niger* is *A. tubingensis* (Schuster *et al.* 2002). *Aspergillus niger* is not prominent in allergy or mycopathology compared to other filamentous fungi; nonetheless, a few lung infections in highly immunocompromised patients have been documented (Schuster et al. 2002). Its strains produce ochratoxin A however, even though only 3-10% of the strains proved positive under favourable conditions, new and unknown isolates should be screened for ochratoxin A before being used for the production of enzymes. (Schuster *et al.* 2002).

1.2.3.2. *Aspergillus flavus*

Aspergillus flavus (*A. flavus*) is a plant, animal, and insect pathogen that mainly infects immunocompromised individuals (Klich 2007). It is the second most common cause of invasive and non-invasive aspergillosis, as well as the species that cause mycoses in humans, after *A. fumigatus* (Stevens *et al.* 2000). This common soil fungus mostly affects oilseed crops

such as maize, peanuts, cottonseed, and tree nuts; it is a serious agricultural concern because it produces highly carcinogenic aflatoxins as secondary metabolites (Klich 2007). According to Toyotome *et al.* (2019) *A. flavus* is a significant plant pathogen that is extensively disseminated worldwide, nonetheless clinical isolates are not adequately explored, hence its virulence factors are primarily unknown.

1.2.3.3. *Aspergillus fumigatus*

Aspergillus fumigatus (A. fumigatus) is a saprophytic fungus prevalent in the environment (Latgé 2001). It is found naturally in soil and decaying organic materials, where it aids in nutrient recycling. It sporulates profusely, with each conidial head producing hundreds of conidia and releasing conidia up to 1 mm in size (2-3 µm) and remaining buoyant once in the air due to their small size. Humans inhale hundreds of conidia daily, yet reasonably healthy people are rarely harmed because of efficient elimination by innate immune systems. Until recently, *A. fumigatus* was thought to be more related to allergic responses than infections. Nevertheless, *A. fumigatus* has become the most common airborne fungal pathogen in the last two decades, generating severe and generally deadly invasive infections in immunocompromised hosts in affluent nations (Latgé and Chamilos 2019).

1.2.4. *Aspergillus* and respiratory infections

According to Chowdhary, Agarwal and Meis (2016), respiratory tract infections account for one-third of infectious disease-associated mortality globally, with over 4.3 million fatalities annually. Invasive pulmonary infections caused by *Aspergillus* species are identical to fungal infections of the respiratory system. Invasive aspergillosis (IA) is a dangerous opportunistic infection that primarily affects immunocompromised individuals, such as those suffering from chronic neutropenia or cancer (Meersseman *et al.* 2007; Walsh 2008).

Clinical manifestations of the illness range from an allergic reaction to an invasive lung infection and are impacted by a range of host-related factors (Figure 1.2). *Aspergillus fumigatus* is the most prevalent pathogenic *Aspergillus*, followed by *A. flavus*, *A. niger*, *A. terreus*, *A. nidulans*, alongside other morphologically similar species in the group of *A. Fumigati* (Sugui *et al*. 2015). Patients with weakened immune systems are more likely to get aspergillosis. Early diagnosis, species identification, and appropriate antifungal treatment are important for treating the condition, particularly in invasive pulmonary aspergillosis, which can progress rapidly.

Understanding the basic biology of *Aspergillus* species and the illnesses they cause is critical for future advancement in the field (Paulussen *et al.* 2017).

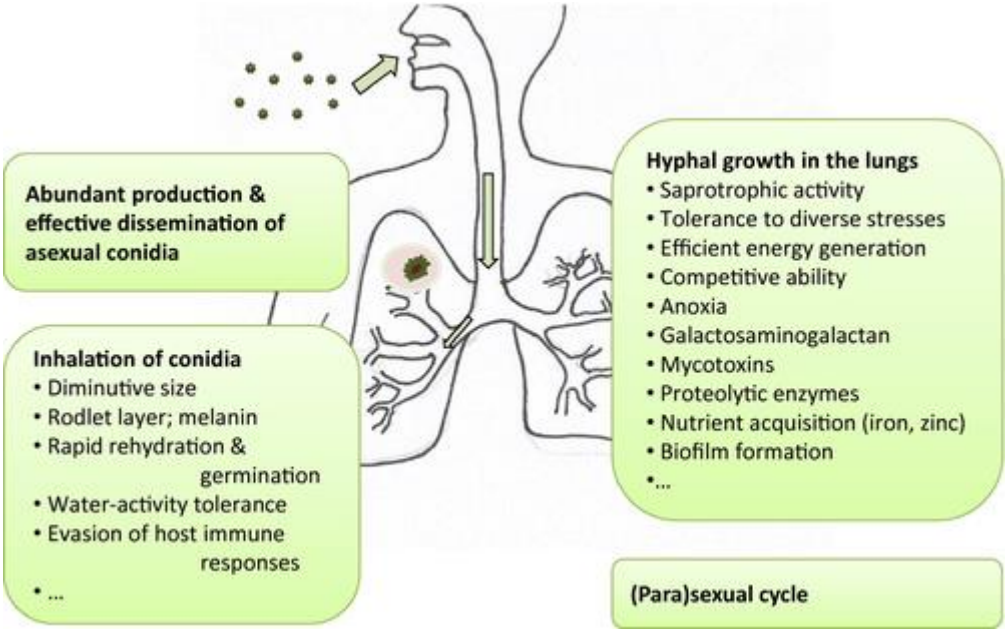

Figure 1.2: A range of *Aspergillus* and host-related factors that contribute to the success of *Aspergillus* species as potent pathogens (Paulussen *et al.* 2017).

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1.2.5. Clinical presentations

The immunological background of the infected individuals determines the kind of *Aspergillus* infection, with immunodeficient patients getting invasive illness and immunoreactive patients generating allergic disease. The most prevalent clinical symptoms are pulmonary, such as acute or chronic IA and allergic bronchopulmonary aspergillosis (ABPA). Acute or chronic sinusitis, cutaneous aspergillosis, aspergilloma, and cerebral aspergillosis are all prevalent *Aspergillus* infections. In some immunocompetent individuals, breathing conidia may result in non-pathogenic saprophytic colonization, but in immunocompromised patients, conidial acquisition generally results in the development of an invasive disease (Tomee and Van der Werf 2001). Immunocompromised individuals who have undergone HSCT, SOT, cancer patients, patients with different congenital immunological weaknesses such as chronic granulomatous disease (CGD), HIV, and patients treated for autoimmune illnesses bear a colossal burden of *Aspergillus* infections (Richardson 2005).

1.2.6. Invasive aspergillosis

Invasive aspergillosis (IA) is one of the most serious clinical invasive fungal diseases, with a significant case-fatality rate in immunocompromised patients (Table 1.1) (Sugui *et al.* 2015). Fresenius (1863) identified *Aspergillus fumigatus*, as the most important pathogenic species of the genus, and Virchow (1856) recognized the importance of aspergilli in human illness (Sugui *et al.* 2015). Virchow (1856)noted a connection between the aspergilli reported in animal illnesses and those seen in human diseases. Rankin found IA after observing extensive aspergillosis in an aplastic anaemia patient (Rankin 1953). Over the following 60 years, the number of cases of IA dramatically rose due to an increase in the number of immunocompromised patients, such as those with cancer, AIDS, SOT, and those receiving immunosuppressive therapies for underlying disorders (Denning 1998; Kontoyiannis and Bodey 2002; Marr *et al.* 2002b).

Table 1.1: Yearly estimates of incidences of invasive fungal infections (Pianalto and Alspaugh 2016)

Fungal disease	Estimated cases per year	Estimated mortality rates (% of infected)	Reference
Cryptococcosis	>1,000,000	20%–70%	(Park *et al.* 2009)
Candidiasis	>400,000	10%–75%	(Pfaller, Pappas and Wingard 2006)
Aspergillosis	>200,000	30%–95%	(Guinea *et al.* 2010; Brown, Denning and Levitz 2012; Garcia-Vidal *et al.* 2015)

Invasive aspergillosis is highly prevalent in developing countries; however, the epidemiology of the disease is relatively well documented in the developed world, but details are lacking in developing countries. Fungi thrive in the hot and humid climates of tropical and subtropical Asia, Africa, and South and Central America, which account for 85% of the world's population (Chakrabarti *et al.* 2011b). These continents have a limited medical infrastructure for the large numbers of poor and growing populations. The below-optimum hospital care practice in the economically deprived group and the modern medical intervention like transplantations in the prospering group have increased the number of invasive fungal diseases, including IA, in both

groups (Chakrabarti *et al.* 2011b). In a tertiary care hospital in North India, 2.4% of all autopsies (15,040 deaths autopsied over 26 years) revealed systemic fungal infections (Chakrabarti *et al.* 2011a). Invasive aspergillosis was detected in 49% of those positive fungal cases. Incidence of IA over six years from January 2000 to December 2005 in a tertiary care hospital in Thailand reports the lung involved in 68% of cases, followed by sinuses (17%) (Kiertiburanakul, Thibbadee and Santanirand 2007; Phikulsod, Suwannawiboon and Chayakulkeeree 2017). The major predisposing factors were neutropenia, chemotherapy and receiving corticosteroid therapy. *Aspergillus fumigatus* (67%) was the most frequently isolated species and 44 patients (47%) died due to aspergillosis with pulmonary infection being a significant predictive factor of death.

The factor that is concerning in developing countries, is when other illness conditions in critically ill patients often leave them susceptible to IA, and such cases were reported in India and China (Gupta *et al.* 2010). The critically ill elderly patients experiencing colonization with *Aspergillus* species with obstructive pulmonary disease are hospitalized, receive corticosteroids and multiple antibiotics, and become susceptible to IA. Patients with previous tuberculosis and consequent structural lung airway disease have been reported to acquire IA. Although many patients with tuberculosis are common in developing countries, still IA is infrequently reported. However, tuberculosis is a risk factor in 28% of patients in an autopsy series in Mumbai (Chakrabarti *et al.* 2011a).

Although *A. fumigatus* is implicated as the most common species in IA and other species such as *A. flavus*, *A. niger*, and *A. terreus* are less common pathogens in patients with IA, *A. flavus* has been isolated at a comparatively higher frequency from sino-orbital aspergillosis and *Aspergillus* eye infections, in developing countries (Chakrabarti *et al.* 2011b; Rudramurthy *et al.* 2019). Higher environmental contamination by *A. flavus* in developing countries is likely to lead to this increased *A. flavus* infections (Chakrabarti *et al.* 2011b). Although IA is under-reported or reported late in developing countries, the disease frequency is expected to be high (Chakrabarti *et al.* 2011b). Early diagnosis and appropriate antifungal therapy are needed to manage IA impacted by economic constraints effectively.

1.2.6.1. Coinfection of invasive pulmonary aspergillosis and COVID-19

Simultaneous infection caused by respiratory pathogens such as *Aspergillus* and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has become challenging in being associated with high morbidity and mortality in patients with COVID-19 (Lai *et al.* 2020; Lansbury *et al.* 2020). Clinicians are challenged by the SARS-CoV-2 pandemic and the rarely co-existing fungal infections caused by *Candida* species, *Cryptococcus* species, *Mucorales* species and *Aspergillus* species (Chen et al. 2020; Zhu et al. 2020). El-Baba, Gao and Soubani (2020) cautioned more attention to *Aspergillus* species as this can lead to severe complications like invasive pulmonary aspergillosis (IPA). *Aspergillus* was considered a rare cause of invasive pulmonary aspergillosis (IPA) before the COVID-19 pandemic, occurring mainly in immunocompromised patients (Fiema et al. 2022). Trovato et al. (2021) described IPA as a complication in patients with severe respiratory syndromes recently. They drew a correlation with COVID-19 pneumonia considering 38 cases of COVID-19-associated pulmonary aspergillosis reported in the literature by Mohamed, Rogers and Talento (2020) and Arastehfar et al. (2020).

1.2.7. Pathogenesis and virulence factors

One of the most relevant questions in understanding the pathobiology of *A. fumigatus* is identifying virulence determinants. This is complicated because of the redundancy of genes with the same functions, the pleiotropic effects of several genes, and complex enzymatic systems encoded by gene clusters. Moreover, as a successful saprophyte, *A. fumigatus* does not possess any particular virulence factors. According to the classical definition, "virulence factors," includes genes that affect virulence but are dispensable for normal growth, and only a few genes/molecules fulfil these requirements. Two of these factors are conidial melanin and gliotoxin. *A. fumigatus* does not require either of these two factors for growth; however, loss of conidial melanin rendered *A. fumigatus* nearly avirulent when inoculated intravenously in BALB/c mice (Tsai *et al.* 1998) and the absence of gliotoxin synthesis, virulence was reduced in a host immune-status dependent manner. Reduced virulence was observed in non-neutropenic mice immunosuppressed with hydrocortisone (Sugui *et al.* 2007; Spikes *et al.* 2008) but not in neutropenic mice immunosuppressed with cyclophosphamide and cortisone (Cramer Jr *et al.* 2006; Cramer 2006; Kupfahl *et al.* 2006). Melanin and gliotoxin are part of the fungal defence system to survive in an extreme environment, such as in soil where thousands of microorganisms cohabit. Such a defence system may protect aspergilli in the host

environment (Kwon-Chung and Sugui 2013). There is therefore a need to conduct studies that aim to induce or provoke metabolic responses from *A. fumigatus* during cultivation.

As an opportunistic pathogen, nearly all published *A. fumigatus* mutant strains defective in genes responsible for normal growth through proper hyphal extension exhibit a virulence defect in murine models. However, it is clear that not every *A. fumigatus* mutant strain with an *in vitro* growth defect displays a virulence defect. Like *in vitro* growth, lung fungal burden has been repeatedly directly correlated with virulence in the murine model of IA. Several of these genes can be considered virulence factors because they are crucial for the fungal establishment within the nutrient-limited host tissue. For instance, iron and zinc are primely important for *A. fumigatus* growth. Consequently, genes encoding proteins in acquiring these two metals are indispensable for *A. fumigatus* to cause disease (Moreno *et al.* 2007; Haas 2012; Moore 2013). The array of genes and molecules associated with *A. fumigatus* virulence can be classified based on their involvement in specific biological processes. Thermotolerance, cell-wall composition and maintenance, defence against host immune response, toxin production, and nutrient uptake during host invasion are some of the processes involved. The importance and the role of genes involved in these processes in *A. fumigatus* pathogenesis have been extensively reviewed (Askew 2008; Abad 2010; Sales-Campos *et al.* 2013).

In the absence of sufficient pulmonary defences, conidia establish themselves because of either immunosuppression that renders the human host neutropenic or cortisone injection to fight graft versus host disease, resulting in deficient phagocytic defence in the lung and adapts their physiology to overcome the stress imposed by the host environment. Because the optimum temperature for conidial germination and growth is between 37- 45°C, they germinate readily into hyphae by 6–8 h in mammalian tissue (McDonagh *et al.* 2008). During this early germination stage, *A. fumigatus* utilizes a highly coordinated gene expression program that allows for adaptation to nutrient deprivation, iron limitation, hypoxia, and other unfavourable growth conditions in the host environment (McDonagh *et al.* 2008). Excessive hyphal growth and dissemination follow in the profoundly neutropenic host, leading to an increased risk of IA resulting in thrombosis and haemorrhage (Hohl and Feldmesser 2007; Dagenais and Keller 2009).

1.2.7.1. Mycotoxins

Mycotoxins are small molecular weight compounds naturally produced by specific filamentous fungi as secondary metabolites and toxic to vertebrates in low concentrations upon ingestion, inhalation, or skin contact (Klich 2009). They cause infections of crops and thus enter the food chain, often resulting in adverse health effects in humans and animals. Mycotoxins are typically found in foods like cereal, grains, nuts and spices. They are ingested, inhaled or absorbed through the skin, causing diseases or death in humans and domestic animals, including birds (Pitt 2013) and occur primarily in hot and humid regions, favourable for the growth of moulds, with storage and environmental factors contributing to the production of mycotoxins (Zain 2011).

Contaminated feed also leads to diseased animals and the milk they produce, and subsequent human consumption results in mycotoxicoses, diseases caused by exposure to mycotoxins. Contamination of foods and feeds with mycotoxins is very significant as it impacts negatively on human health and economic losses, contaminating approximately 25% of agricultural products worldwide, according to the Food and Agriculture Organization of the United Nations (FAO CAST 2003)

Toxigenic moulds produce one or more of these toxic metabolites. Members of the genera *Aspergillus*, *Penicillium* and *Fusarium*, produce most of the major mycotoxins that significantly impact public health and agro-economy worldwide. They include aflatoxins (AF), ochratoxins (OT), zearalenone (ZEN) and fumonisins (F). Aflatoxins, found mainly in maize, peanuts and tree nuts, are produced by the genus *Aspergillus* with ochratoxins produced by *Penicillium* and *Aspergillus ochraceus,* found mainly in cereals, beans and roasted coffee. Zearalenone and fumonisins are produced by *Fusarium* species using corn, wheat, barley, oats and sorghum as substrates (Zain 2011). *Aspergillus* species also produce mycotoxin, gliotoxin, which was detected in culture filtrates of *A. fumigatus*, of both clinical and environmental strains (Kupfahl 2008).

1.2.7.2. Gliotoxins

Gliotoxin (GT) is an epipolythiodioxopiperazine (ETP) of molecular mass 326 Da and contains a disulfide bridge that can undergo repeated cleavage and reformation, resulting in potent intracellular redox activity (Choi *et al.* 2007). This class of secondary metabolites shows a diverse range of biological activities, having antimicrobial, antifungal and antiviral properties and also displays *in vitro* and *in vivo* immuno-modulating activity (Waring and Beavert 1996). This fungal metabolite is produced by several species, including *Aspergillus* (Richard, Peden and Williams 1994). Gliotoxin has also been found to cause apoptotic cell death in various tissues, especially cells of the immune system. It is the probable explanation for the selective immuno-toxicity of the toxin (Waring and Beavert 1996).

The structure of the ETP class of fungal products is characterized by the bridged disulphide piperazinedione six-membered ring that appears to be necessary for most of the biological properties of these compounds, such as potent immunosuppressive, genotoxic, cytotoxic and apoptotic effects (Nieminen 2002; Upperman 2003). Although there was much attention because of its antibacterial and antiviral properties, the *in vivo* toxicity of gliotoxin limited its potential use as a chemotherapeutic agent (Taylor 1971; Nouri 2015), however, its aetiology in causing diseases disease has stimulated interest in this class of toxins.

1.2.7.3. Gliotoxin and disease

Members of this ETP family of secondary metabolites have been associated with diseases attributed directly or indirectly to fungal infection (Waring and Beavert 1996). *A. fumigatus* produces several secondary metabolites during invasive hyphal growth (Latgé 2001). The ETP metabolite gliotoxin is abundantly produced by the species and exhibits a diverse array of biological effects on the immune system (Lewis *et al.* 2005a). Gliotoxin is one of the most toxic metabolites produced during the growth of specific species of the genus, with the toxicity attributed to the intermolecular disulfide bridge (Waring and Beavert 1996). Tomee (2000) suggested that with the immunosuppressive properties of gliotoxin exhibited and with the release of the mycotoxin by the hyphal form, gliotoxin *in situ* could aid evasion of fungal hyphae from the host's immune response and thereby contribute to the pathobiology of invasive aspergillosis.

Lewis *et al.* (2005b)proposed that the potential to produce immunosuppressive secondary metabolites might benefit the development and persistence of a saprophytic mould in people

while screening 103 *Aspergillus* isolates from cancer patients for gliotoxin. The investigation of the incidence and distribution of gliotoxin production among *Aspergillus* isolates recovered from individuals at risk for invasive aspergillosis would provide validation. This is also supported by Nucci *et al.* (2010) and is possibly why the immune system does not counteract the fungus. hypothesized that gliotoxin production is involved in the pathogenesis of IA and could be a marker of invasive infection with *A. fumigatus*. Based on the above and related findings, Cerqueira (2014) developed and validated an HPLC-MS/MS method for the potential early diagnosis of aspergillosis as an option for a diagnostic technique for aspergillosis.

When it comes to immunocompromised people, the diagnosis of aspergillosis is challenging since invasive diagnostic techniques are not always available and signs and symptoms are non-specific. Thus, high-risk patients are treated with an empirical therapeutic technique (Stevens 2002) and diagnosis is generally made by histology or culture (Wheat 2008). Similar disease symptoms, followed by delays under correct treatment, cause in some cases, death. Published conventional methods have drawbacks such as poor sensitivity and specificity, false positives, and lengthy confirmation periods (Pasqualotto and Denning 2005). In the weakened state of the patients, biopsies are not recommended. Enzyme immunoassay techniques against the galactomannan antigen show promise; however, the validity of polymerase chain reaction (PCR) in identifying this specific ailment must be determined (Wheat 2008).

1.2.7.4. The pathogenic species of *Aspergillus* that produce gliotoxin

The most common species of *Aspergillus* that cause invasive aspergillosis worldwide are *A. fumigatus, A. terreus, A. flavus and A. niger* (Kwon-Chung and Sugui 2009b; Sugui *et al.* 2015). Although all the species produce gliotoxin, not every strain is a gliotoxin producer (Lewis *et al.* 2005a). Several reports cite the frequency of toxin-producing strains isolated from the environment versus clinical specimens from patients. dos Santos, Dorner and Carreira (2003) reported that only 11% of the *A. fumigatus* strains isolated from mouldy silos on Terceira Island in the Azores were gliotoxin producers. In contrast, 93% of the *A. fumigatus* strains (n=40) were isolated between 1998 and 2003 from respiratory and tissue samples of cancer patients in MD. Anderson Cancer Center, Houston, Texas, USA, were gliotoxin producers compared to 75% and 25% of *A. niger* (n=9) and *A. terreus* (n=27) strains, respectively. Surprisingly, only 4% of the *A. flavus* (n=18) strains produced gliotoxin. Furthermore, the concentrations of gliotoxin produced by *A. fumigatus* were significantly higher than those of other species (Lewis *et al.* 2005a).

In the haematology unit at the University of Zagreb in Croatia, Kosalec and Pepeljnjak (2005) reported very different frequencies of gliotoxin-producing *A. fumigatus* strains recovered from immunocompromised patients with various diseases. Of the 50 clinical isolates, only 18% produced gliotoxin, while no environmental strains of an equivalent number screened were found to produce the toxin. Although the data may be insufficient, the results suggest that the frequency of gliotoxin-producing *A. fumigatus* strains varies depending on the geographic region and/or type of patients that yielded the fungal strains. It appears, however, that the frequency of finding gliotoxin-producing *A. fumigatus* strains is higher among clinical isolates than among environmental isolates.

1.2.7.5. Contribution of gliotoxin to virulence of *A. fumigatus*

The toxic and immunosuppressive characteristics of GT towards the host's immune effector cells (Stanzani *et al.* 2005; Orciuolo *et al.* 2007) imply a substantial role of this compound in fungal pathogenicity. Accordingly, GT was shown to be produced during the infection process and was detected in the lungs and sera of mice as well as in human lungs or sera of humans infected with *A. fumigatus* (Lewis *et al.* 2005a; Kupfahl *et al.* 2006). Previous studies claimed that environmental isolates of *A. fumigatus* rarely produce GT, in contrast to clinical isolates (Lewis 2005). However, it was recently demonstrated that the vast majority (>96 %) of both environmental and clinical isolates could produce GT (Kupfahl *et al.* 2008). Improvements in cultivation conditions and GT production detection limits could explain these studies' discrepancies. Initial studies to connect GT to virulence revealed that *A. fumigatus* strains producing high amounts of GT are more virulent than those with low GT production capacity (Reeves *et al.* 2004). When tested in neutropenic mice, i.e., when immune suppression was induced by treating animals with cyclophosphamide and cortisone acetate, the gliP and gliZ mutant strains revealed no altered virulence compared to those of animals with GT-producing wild-type strains (Bok *et al.* 2006; Cramer Jr *et al.* 2006; Cramer 2006; Kupfahl *et al.* 2006). By contrast, gliP deletion strains were attenuated in virulence when tested in mice only immunosuppressed by administration of cortisone acetate (Sugui *et al.* 2007; Spikes *et al.* 2008). Immune suppression using corticosteroids still enables neutrophils to be recruited to the site of infection. It was suggested that GT contributes to fungal pathogenicity by targeting primarily the activity of immune cells present in non-neutropenic mice, namely neutrophils or other phagocytes (Spikes *et al.* 2008). Therefore, it is conceivable that the nature of immune suppression decides on the contribution of GT to the establishment of infection with *A. fumigatus*.

1.2.7.6. Pathobiological significance of gliotoxin in *A. fumigatus*

Deletants of the gliP or gliZ gene showed no difference in morphology or growth rate compared to wild-type strains except that mutants produced no gliotoxin (Kupfahl *et al.* 2006; Sugui *et al.* 2007). These results were expected since, unlike primary metabolism, secondary metabolism, which includes gliotoxin biosynthesis, does not affect the organism's growth. Secondary metabolism associated with fungal development, such as polyketide synthesis, is commonly associated with the sporulation or pigmentation of spores (Tsai *et al.* 1999; Calvo *et al.* 2002). The deletion of gliP in Af293 caused the downregulation of gene expression in the 12-gene cluster. The addition of exogenous gliotoxin restored the gene expression level suggesting that gliotoxin regulates its production (Cramer Jr *et al.* 2006). *In vitro* analysis showed that culture filtrates from gliotoxin-negative mutants failed to inhibit oxidative burst in human neutrophils (Sugui *et al.* 2007), were unable to cause apoptosis or cell detachment in various mammalian cells (Bok *et al.* 2006; Sugui *et al.* 2007) and also failed to inhibit mast cell degranulation (Cramer Jr *et al.* 2006).

Invasive aspergillosis occurs most commonly in neutropenic patients. It has also increasingly been reported in allogeneic stem cell transplant (SCT) recipients treated with immunosuppressive regimens, most commonly corticosteroids, because of graft-versus-host disease (Kwon-Chung and Sugui 2009b). These patients are no longer neutropenic but susceptible to invasive fungal infection. Gliotoxin would play an important role in the pathogenesis of *A. fumigatus* in these patients.

1.2.7.7. Pathogen-associated molecule patterns (PAMPS)

In its protection against infection, the body detects the presence of microorganisms. Initially, the body accomplishes this by recognizing the molecules unique to groups of related microorganisms not associated with human cells. These unique microbial molecules are called pathogen-associated molecular patterns (PAMPS).

Through their long shared history, bacteria and fungi have developed receptor systems to detect each other’s presence (Svahn *et al.* 2014). Like all other organisms, fungi have receptors that perceive their surroundings to detect pathogen-associated molecular patterns (PAMPS) and induce immune responses (Kawai and Akira 2010). PAMPS are components of the bacterial cell wall and include lipopolysaccharide (LPS), peptidoglycan (PG), and lipoteichoic acid (LTA). Gram-negative bacteria have LPS and PG (Figure 1.3), while Gram-positive bacteria

are associated with PG and LTA (Figure 1.4). When such bacteria infect mammals, these molecules are released into the host, which senses them using pattern recognition receptors expressed on dendritic cells, macrophages and neutrophils (Blasius and Beutler 2010). Fungi, like other microorganisms, produce antimicrobial agents on a facultative basis to avoid self-toxicity and for resistance development (Svahn 2014). Thus, fungal metabolic pathways could be explored for new metabolites or increase the secretion of known ones to benefit drug discovery and production (Svahn *et al.* 2014).

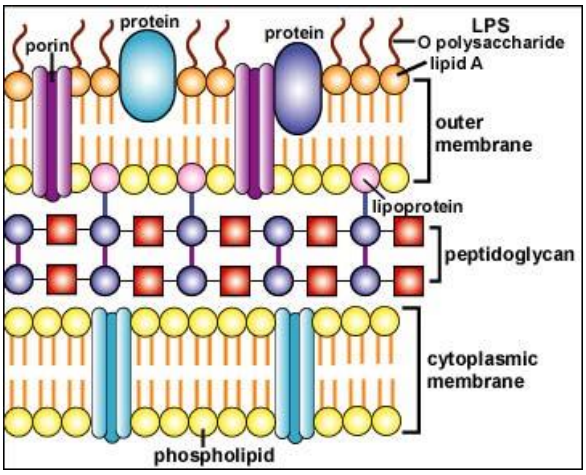

Figure 1.3: Structure of a Gram-Negative cell wall. The Gram-negative cell wall comprises a thin layer of peptidoglycan and an outer membrane consisting of molecules of phospholipids, lipopolysaccharides (LPS), lipoproteins and surface proteins. The lipopolysaccharide consists of lipid A and O polysaccharides (Kaiser, 2022).

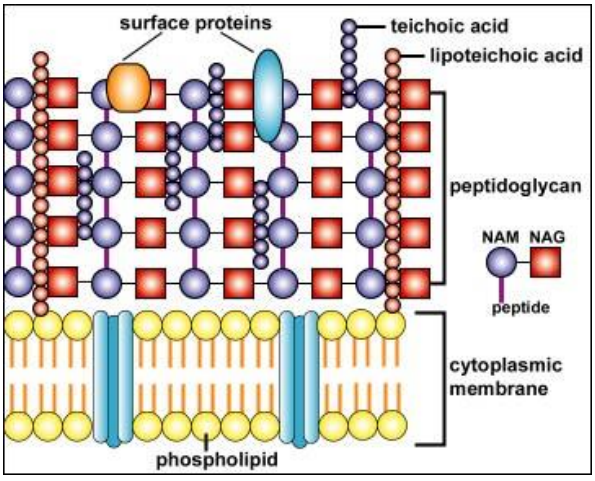

Figure 1.4: Structure of a Gram- positive cell wall. Gram-positive cell wall appears as a dense layer typically composed of numerous rows of peptidoglycan, molecules of lipoteichoic acid, wall teichoic acid and surface proteins (Kaiser, 2022).

Pathogen-associated molecular patterns are widely used to simulate the presence of bacteria and other pathogens in studies on mammalian immune systems. PAMPs are highly conserved molecular structures, typically essential components of the pathogen's cytoplasmic barrier, acting as general recognition targets for detecting their presence. The detection of PAMPs by the immune system of insects and mammals stimulates innate immune responses and thus prevents infections. Some molecules that have been classified as PAMPs include lipopolysaccharides (LPS) from Gram-negative bacteria, lipoteichoic acid (LTA) from Gram-positive bacteria, and peptidoglycan (PG) associated with both groups of bacteria. These bacterial cell wall and membrane components induce responses in mammalian hosts by binding to toll-like receptors (TLRs), a subfamily of pattern recognition receptors (PRRs). TLRs have been identified in a diverse range of animals but not in fungi. Although fungi seem to lack TLRs, it is essential to know if fungi have some mechanism for detecting and responding to common bacterial PAMPs. In studies on mammalian immune systems, PAMPs are widely used to simulate the presence of bacteria and other pathogens. These molecular structures, essential components of the pathogen's cytoplasmic barrier, act as recognition sites for detecting their presence.

Bacteria and fungi are two groups of microorganisms with a very long common history, and it is reasonable to assume that they have developed receptor systems to detect each other’s presence. Like all other organisms, fungi have receptors that perceive their surroundings, although only a few such receptors have yet been characterized. One such group of receptors in mammals is the Toll-like receptors, which detect pathogen-associated molecular patterns (PAMPs) and induce immune responses. PAMPs are various bacterial cell wall and cell membrane components and including lipopolysaccharide (LPS), peptidoglycan (PG), and lipoteichoic acid (LTA). Gram-negative bacteria are associated with LPS and PG, while Gram-positive bacteria are associated with PG and LTA. When such bacteria infect mammals, these molecules are released into the host, which senses them using pattern recognition receptors expressed on dendritic cells, macrophages, and neutrophils. No equivalent fungal system has yet been identified. There is a need therefore to conduct studies that aim to induce or provoke metabolic responses from *A. fumigatus* during cultivation.

1.2.8. Laboratory identification of *Aspergillus* species

1.2.8.1. Microscope and morphological techniques

Identification of *Aspergillus* species is based on a combination of biotypes and genotypes. A biotype involves cultural and morphological characteristics, whereas the sequences of specific genes define a genotype. Etiologic agents of aspergillosis are typically very distinct in their appearance; thus (Figure 1.5), the biotype (shape, size, and colour of the conidial head, thermotolerance, etc.) is a good indicator of their identity. For example, the blueish-green colour of *A. fumigatus* conidial heads is distinct from the yellowish-green, tan, dark green, or black conidial heads of *A. flavus*, *A. terreus*, *A. nidulans*, and *A. niger*, respectively, (Figures 1.6, 1.7 and 1.8). In instances in which differences in the colour of conidial heads are not clear, other characteristics can be helpful in species identification. For example, *A. nidulans* possesses deep green conidial heads, which might be mistaken for the blueish-green colour of *A. fumigatus*; *A. nidulans* conidia are produced by biseriate phialides. The presence of Hülle cells enclosing the cleistothecia produced by *A. nidulans* can also serve as a differential marker. Hülle cells are thick-walled, variously shaped cells that often encase cleistothecia (Raper and Fennell 1965). Conidial size can also aid in the identification of some species. *A. fumigatus*, *A. terreus*, and *A. nidulans* have conidia ranging from 2 to 3.5 µm in size, whereas conidia from *A. flavus* and *A. niger* are more significant (3–5 µm) (Raper and Fennell 1965). Although differences in biotype can often be sufficient to distinguish *A. fumigatus* from other aspergilli, identification should be confirmed by their genotype. Internal transcribed spacer (ITS) sequences and fungal sequences from the genes MCM7, TSR1, RPB1, and RPB2 are useful for species identification (Peterson 2008; Schmidt-Heydt *et al.* 2009). Thermotolerance is another key biotype that can aid in the differentiation of *A. fumigatus* from its sister species. Whereas *A. fumigatus* can grow at 50°C but not at 10°C, the sister species are generally able to grow at 10°C but not at 50°C (Sugui *et al.* 2010; Barrs *et al.* 2013).

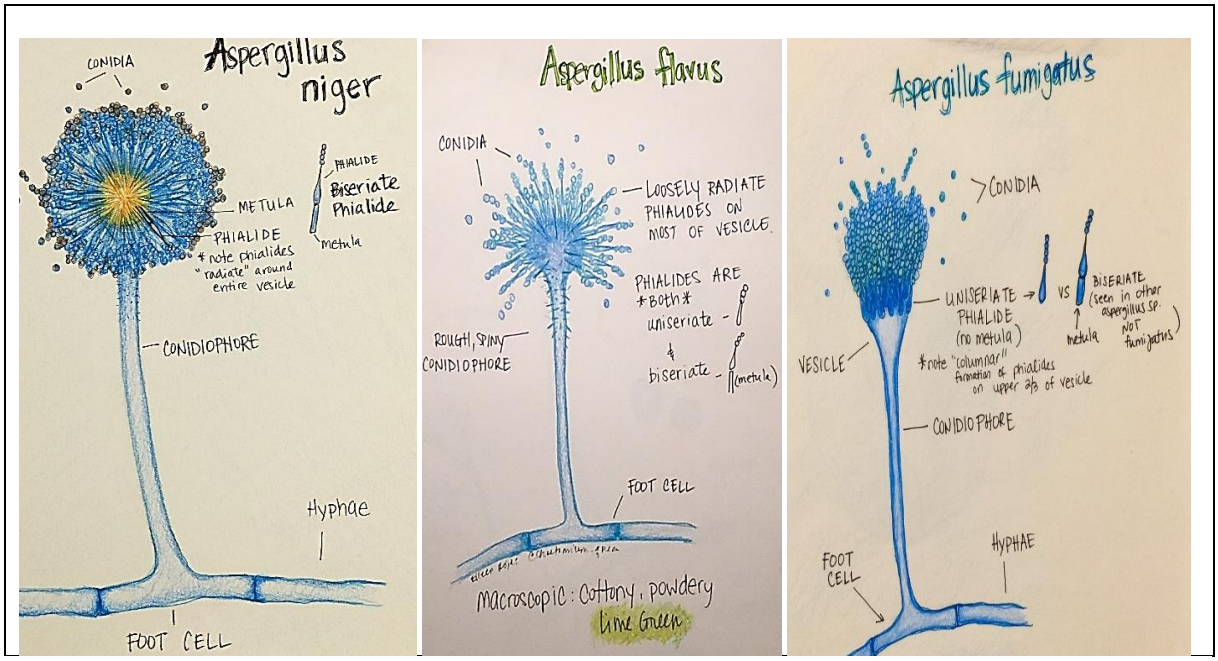

Figure 1.5: Illustrates typical morphological characteristics (under the microscope) used to identify and differentiate between the above *Aspergillus* species (https://za.pinterest.com)

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Figure 1.6: Electron micrograph (600x800) and photographic (474x479) capture of microscope and cultural morphology of *A. fumigatus* used for identification (Baniya 2022).

Figure 1.7: Photographic capture of microscope and cultural morphology of *A. flavus* used for identification (Baniya 2022).

Figure 1.8: Electron micrograph (800x800) and photographic capture of microscope and cultural morphology of *A. niger* used for identification (Baniya 2022).

1.2.8.2. DNA-based techniques

1.2.8.2.1. Ribosomal gene-based techniques

These methods evaluate DNA sequence variation in specific regions of the 18s ribosomal gene. Additionally, they can detect insertions and deletions in the genome. Fungal DNA is subject to PCR (polymerase chain reaction) using six to 20 bases primers. When a single primer is used, it hybridizes into homologous sequences throughout the genome. If the primer hybridizes into two sequences, one on each strand of the genome, which are separated by 3 kb or less (amplicon), the DNA between them will be amplified in the first reaction and extended beyond the point at which there is a cognate sequence on the opposite strand. Thus, in the next cycle, the primer will hybridize with the original DNA and the products of the first cycle. In the subsequent cycles, each amplicon is duplicated until the abundance of amplicons is such that primers continue to promote their synthesis exclusively. The main advantage of this technique is that it is rapid, easy and relatively inexpensive. PCR-based methods have been broadly used to compare isolates of several fungal species (Gil-Lamaignere *et al.* 2003).

1.2.8.2.2. DNA-fingerprinting

The development of DNA fingerprinting techniques has enabled the comparisons of genomes of the strains. The fingerprinting techniques depend on the assumption that comparisons of selected areas of the genome, named genetic markers, can be extrapolated to the complete genomes of the strains. The rationale for this is that, in clonal organisms, there is little or no chromosomal recombination and meiosis. Thus, chromosomes are transmitted from the parental organism to its offspring and the primary source of variability is a mutation, either point mutation or insertions and deletions of DNA fragments. Since those changes are transmitted to the new generations as a block, comparing enough genetic markers allows genetic relatedness to be detected. These techniques are reasonably rapid, economically feasible, and within the technological capabilities of most clinical, microbiological laboratories (Gil-Lamaignere *et al.* 2003).

1.2.8.2.3. DNA microarray genotyping

The DNA-microarray is a hybridization-based genotyping technique that simultaneously analyzes many polymorphisms. High-density microarrays (DNA chips) are created by attaching hundreds of thousands of oligonucleotides to a solid silicon surface in an ordered array. The DNA sample of interest is PCR amplified to incorporate fluorescently labelled nucleotides and then hybridized to the array. Each oligonucleotide in the high-density array acts as an allele-specific probe. Perfectly matched sequences hybridize more efficiently with their corresponding oligomers on the array, giving stronger fluorescent signals than mismatched probe-target combinations. The hybridization signals are quantified by high-resolution fluorescent scanning and computer software analysis. SNPs (single nucleotide polymorphisms), as well as insertions and deletions, can be identified by this method. Microarrays produce a considerable amount of sensitive and accurate data. They can analyze a vast number of polymorphisms at a time since hundreds of thousands of oligonucleotides can be arrayed on a microchip. The drawback of this technique is the high cost, making it unsuitable for large-scale experiments (Gil-Lamaignere *et al.* 2003).

1.2.8.2.4 DNA sequencing

Ideally, the most accurate way to compare two strains would be to compare the sequences of their genomes. Only DNA sequencing allows for the conclusion that two strains are genetically identical (Gil-Lamaignere *et al.* 2003). The best evaluation score is a highly probable relation with all other methods. Sequencing can assess the homology of discriminating bands generated by PCR.

1.2.9. Phenotypic-based methods

1.2.9.1. Phenotype characterization

Filamentous fungi have unique biochemical pathways to assimilate a vast array of simple and complex nutrients available to them and produce a variety of metabolites. The morphological and biochemical uniqueness are commonly used for identification, but differentiation of closely related cultures requires extensive phenotypic and genomic information or a database.

Atanasova and Druzhinina (2010a) reviewed the applications of phenotype microarrays (PMs) to conidial fungi. They highlighted the effective use of this system for the high-throughput analysis of global phenotypes and utilization of particular nutrients. Phenotype is the manifested attribute of an organism, the joint product of its genes and environment during

ontogeny. As such, phenotypes are the ultimate goal in strain improvement in biotechnology during the screening of fungi for new processes or products. Therefore, a semi-high-throughput and comprehensive phenotypic assay would significantly accelerate a functional genomic approach. Since macronutrients are the major determinants of the fungal phenotype, a comprehensive profiling system should ideally include the maximum number of various nutrient sources in a single assay. The nutrient sources would contain carbon, nitrogen, sulfur and phosphorous sources and have different pH and aeration conditions. Such a maximized system could be prepared in 5–7 microtiter plates, with each well containing a standard medium specific for one particular phenotype e.g., sucrose utilization and allowing for the quantitative analysis of growth and/or metabolism. Unfortunately, custom synthesis of this comprehensive phenotype analysis system is laborious and probably only feasible with laboratory robotics.

The Phenotype MicroArray (PM) system (Biolog Inc., Hayward, CA) offers a reasonable compromise. It has frequently been used to analyze bacteria and yeast mutant strains (Bochner, Gadzinski and Panomitros 2001). Seidl, Druzhinina and Kubicek (2006) applied carbon source PMs to soil fungi, thereby detecting species-diagnostic characters in a collection of *Trichoderma* (teleomorph *Hypocrea*) isolates from Southeast Asia. Tanzer *et al.* (2003) demonstrated how global nutrient utilization analysis could be used to elucidate the effects of either genetic alterations or chemical treatments on *Aspergillus nidulans*, *Aspergillus fumigatus*, *Magnaporthe grisea* and *Mycosphaerella graminicola*. The results presented in these studies demonstrate that PMs offer an ideal complement for the phenotypic characterization of knockout gene strains toward understanding genomic and proteomic data. Conidial fungi are capable of metabolizing a wide variety of nutrients. This characteristic has been exploited extensively to study metabolic pathways and their regulation in model fungi such as *Aspergillus nidulans* and *Neurospora crassa* (Atanasova and Druzhinina (2010b). Mchunu *et al.* (2013) found the application of Phenotypic Array as a tool of carbon utilization, a quick approach to studying and assessing filamentous fungi for specific activities. Singh (2009) used the filamentous fungal MicroPlate for substrate utilization, growth, secondary metabolite and antimicrobial profiles of some fungal cultures important to the drug discovery program.

1.2.10. Treatment for filamentous fungal infections

Currently, there are four major classes of antifungal drugs for the treatment of invasive fungal infections. When used as directed, these drugs can be highly effective, significantly preventing patient mortality. Until the 1940s, relatively few agents were available to treat systemic fungal infections. The development of polyene antifungals represented a significant advance in medical mycology. Although amphotericin B quickly became the mainstay of therapy for severe infections, its use was associated with infusion-related side effects and dose-limiting nephrotoxicity (Maertens 2004). The continued search for new and less toxic antifungals led to the azoles several decades later. The first available compound for the oral treatment of systemic fungal infections was released in the early 1980s. Ketoconazole was regarded as the drug of choice in nonlife-threatening endemic mycoses for almost a decade. The introduction of the first-generation triazoles represented a second significant advancement in treating fungal infections. Both fluconazole and itraconazole displayed a broader spectrum of antifungal activity than the imidazoles and had a markedly improved safety profile compared with amphotericin B and ketoconazole. Despite widespread use, however, these agents became subject to many clinically important limitations related to their suboptimal spectrum of activity, the development of resistance, and the induction of hazardous drug–drug interactions; they are less than optimal pharmacokinetics profile and toxicity.

Several analogues were developed to overcome these limitations. These so-called 'second-generation' triazoles, including voriconazole, posaconazole and ravuconazole, have greater potency and possess increased activity against resistant and emerging pathogens, particularly against *Aspergillus* spp. If the toxicity profile of these agents is comparable to or better than that of the first-generation triazoles and drug interactions remain manageable, then these compounds represent an actual expansion of our antifungal arsenal.

1.2.10.1. Polyenes: Amphotericin B and its derivatives

Amphotericin B and its newer lipid formulations are polyene antifungals that target the fungal plasma membrane. Recent models suggest that these drugs act as "sponges" that bind to and remove ergosterol from the plasma membrane, reducing membrane integrity (Anderson et al. 2014). Due to its mechanism of action, amphotericin B is a broad-spectrum drug and indicated for treating severe infections caused by *Candida* species, *Cryptococcus* species, Zygomycetes and as an alternative therapy for aspergillosis (Pianalto and Alspaugh 2016). Amphotericin B is cytocidal for most fungi. As amphotericin B is not highly bioavailable when administered

orally, only intravenous (IV) formulations are used clinically. However, amphotericin B can have severe side effects, such as nephrotoxicity, due to the off-target binding of host membranes, limiting its usage in patients with life-threatening infections (Deray 2002). Newer formulations of this drug, such as lipid-associated and liposomal formulations, demonstrate more selective fungal targeting and less host toxicity (Deray 2002).

1.2.10.2. Azoles and Triazoles

Antifungal agents in the azole class target the fungal plasma membrane by inhibiting the biosynthesis of ergosterol, a fungal plasma membrane component similar to cholesterol found in mammalian cell membranes. This occurs through the inhibition of the sterol 14α-demethylase (Cytochrome P450 51 or *cyp51*), catalyzing the final step in ergosterol biosynthesis (van den Bossche *et al*. 1983). The inhibition of this enzyme leads to defects in fungal plasma membrane integrity and cellular integrity. The most commonly used azoles for treating IFIs can be functionally divided between agents with primary activity against yeast-like fungi (yeast-active azoles) and those with expanded activity against fungi that often grow as moulds (mould-active azoles).

Fluconazole is the most widely-used yeast-active azole, and it is often very effective for treating infections caused by *Cryptococcus* and *Candida* species. Importantly, fluconazole resistance can present a significant clinical issue in systemic candidiasis: some *Candida* species, such as *C. krusei*, are intrinsically resistant to this drug, and others are often susceptible when the drug is administered at high concentrations. Therefore, precise species identification and targeted antifungal susceptibility testing for clinically-relevant isolates are crucial components of the care of patients with *Candida* IFIs.

The mould-active azoles include itraconazole, voriconazole, posaconazole and isavuconazole. In addition to retaining activity against *Candida* and *Cryptococcus* yeasts, these agents also inhibit many filamentous fungi. Itraconazole was the first available azole with significant activity against moulds, such as *Aspergillus fumigatus*. However, issues with bioavailability and toxicity limit its current use against IFIs. Two newer agents, voriconazole and posaconazole, are more widely used for these infections, especially in highly immunocompromised patients. Voriconazole has become the first-line antifungal drug for treating invasive aspergillosis caused by *Aspergillus fumigatus.* Comparative trials indicate that voriconazole is superior to many other antifungal agents against this infection (Sutton *et al.*

1999). Posaconazole is shown to prevent IFIs, especially in prolonged neutropenia after high-dose cancer chemotherapy. These drugs may greatly improve outcomes in patients with IFIs.

Isavuconazole (Cresemba®, Astellas Pharmaceuticals, Tokyo, Japan) is the recently approved triazole antifungal drug. It differs from other approved azoles in several clinically-relevant ways. First, it has expanded *in vitro* activity, including the Mucorales moulds (Zygomycetes), such as *Rhizopus*, *Muco*r and *Cunninghamella* species. Therefore, it may be an effective component of the complex, medical-surgical treatment of mucormycosis (Thompson and Wiederhold 2010). Additionally, the intravenous (IV) formulation of isavuconazole lacks cyclodextrin, a solubilizing agent used with other triazoles associated with nephrotoxicity in patients with renal insufficiency.

1.2.10.3. Echinocandins

The echinocandins represent the newest class of antifungals. Currently, three drugs from this class are approved for clinical usage: caspofungin, micafungin and anidulafungin. Echinocandins affect cell wall biosynthesis through the noncompetitive inhibition of β-1,3-glucan synthase (Taft, Stark and Selitrennikoff 1988). This enzyme is involved in the biosynthesis of one of the most abundant fungal cell wall components. Therefore, treatment with echinocandins leads to defects in fungal cell integrity. These drugs are primarily used to treat invasive candidiasis and as an alternative therapy for treating aspergillosis (Denning 2003). Echinocandins have low host toxicity and few drug interactions. However, they have no activity against *Cryptococcus* species and are decidedly poor agents for the treatment of endemic mycoses. Additionally, they are not orally bioavailable, likely due to their large molecular size, so they are only available in IV formulations.

1.2.10.4. Fluorocytosine

5-Fluorocytosine (Flucytosine) is a fluorinated pyrimidine analogue, which inhibits DNA and RNA synthesis by incorporating it into the growing nucleic acid chain, preventing further extension. This nucleic acid damage eventually leads to cellular defects in protein biosynthesis and cell division. This antifungal agent has been attributed to cytostatic effects and high resistance rates during monotherapy. Therefore, flucytosine is rarely used as a single agent to treat fungal infections. However, it has been shown in multiple clinical trials to be highly effective in combination with amphotericin B to treat cryptococcal meningitis (Bicanic *et al.* 2008; Dromer *et al.* 2008). Indeed, amphotericin B plus flucytosine is the first-line treatment

for *Cryptococcus* central nervous system (CNS) infections (Perfect *et al.* 2010). Flucytosine can also be used with other antifungals to treat *Candida* infections, though this is a less common practice. Adverse effects of flucytosine include bone marrow toxicity, especially in renal impairment. However, one of the limiting factors of this drug is its limited availability in countries with the highest incidence of cryptococcosis (Loyse *et al.* 2013). This, unfortunately, limits the effectiveness of cryptococcal meningitis therapy in those regions of the world in which it is most prevalent, likely increasing rates of mortality caused by this disease.

1.2.11. Development and impact of azoles

The Infectious Diseases Society of America recommends voriconazole as the primary therapy for IA. In developing countries, Amphotericin B and itraconazole are the first-line therapy in most patients due to the higher cost of anti-*Aspergillus* drugs. This was noted in a tertiary care hospital review in Thailand (Kiertiburanakul, Thibbadee and Santanirand 2007). The Brazillian study highlights a similar scenario for economic reasons but was forced to change to the first choice due to nephrotoxicity (55.6%) and failure (33%). The cost of the drugs seriously impacts the management of IA in developing countries. Voriconazole has an adequate response initially, but patients cannot complete the therapy due to costs. Immunocompetent patients respond well to amphotericin B and itraconazole. Still, immunosuppressed and neutropenic patients' response is not good, with mortality as high as 87% reported, possibly due to the delay in admission, diagnosis and start of antifungal therapy.

Despite implementing several preventive measures and antifungal chemoprophylaxis, physicians have witnessed an increased incidence of both mucosal and invasive fungal infections during the past two decades, the increase linked with progress in medical technology and novel therapeutic options and thus multifactorial (Marr *et al.* 2002a). The widespread use of quinolone prophylaxis in neutropenic cancer patients and the availability of broad-spectrum antibacterial agents have virtually eliminated early death due to bacterial sepsis, thereby setting the stage for fungal colonization and putting patients at risk for subsequent mycotic infections. Medical procedures have become more invasive and aggressive; the accompanying disruption of protective anatomical barriers as a result of indwelling catheters, therapy-induced mucositis, viral infections, and graft-versus-host disease, or following major abdominal surgery or associated with extensive burns allows fungi to reach normally sterile body sites (Maertens and Boogaerts 2000). In addition, the community of vulnerable patients is continuously expanding as a result of the spread of human immunodeficiency virus (HIV) infections and the increased use of (novel) immunosuppressive drugs in autoimmune disorders. Unfortunately, the

attributable mortality rate of (systemic) fungal infections remains high (Patterson *et al.* 2000). This may partly be explained by the difficulty of diagnosing these infections at an early stage of their development because definite proof often requires time-consuming and labour-intensive approaches that cannot always be achieved in these severely ill patients.

Progress in the development of new antifungals has lagged behind antibacterial research in fact, at least two factors can explain that. First, before the HIV era, fungal infections were believed to be too low to warrant aggressive research by the pharmaceutical industry. Second, the 'apparent' lack of a highly selective fungal target not present in other eukaryotic (including mammalian) cells precluded the development of new agents. Until recently, the arsenal available for treating systemic fungal infections was limited in number and consisted mainly of the polyene antibiotic amphotericin B, some azole derivatives, the allylamines–thiocarbamates and 5-flucytosine. Except for 5-flucytosine, all other agents acted by interfering with the structural or functional integrity of the fungal plasma membrane, either by physical disruption or by blocking the biosynthesis of membrane sterols. However, there has been an expansion of basic and clinical research in antifungal pharmacology, and many companies have launched new compounds, including several new azole compounds (Minamoto and Rosenberg 1997; Maertens and Boogaerts 2000).

1.2.12. Antifungal resistance in *Aspergillus* infections

The incidence of serious fungal infections is increasing rapidly, yet the rate of new drugs becoming available to treat them is slow. The limited therapeutic drugs are a challenge for clinicians because the available drugs are often toxic, expensive, difficult to administer, ineffective, or a combination of all four (Beardsley *et al.* 2018). Given this setting, the emergence of resistance is especially concerning. According to the editorial of National Microbiology (2017), the global incidence of fungal disease has increased dramatically in recent years. Current estimates suggest approximately 300 million life-threatening fungal infections annually, resulting in 1.6 million deaths (Revez *et al.* 2017). Health impacts worldwide include high morbidity, overall mortality of 30–80% and a multibillion-dollar annual economic burden and; with the limitations of current therapies, including one or more of toxicity, poor bioavailability and relative inefficacy, are now further amplified by the emergence and escalation of drug resistance (Beardsley *et al.* 2018).

In considering the impact of drug resistance in the clinical setting, it is important to note that resistance does not always equate with clinical failure as many factors contribute to clinical outcome, in particular, host factors such as an impaired immune system, other comorbidities, site of invasive fungal disease; therapeutic factors such as dosage regimen, compliance and drug toxicity; and ancillary factors such as source control all impact clinical outcomes (Beardsley *et al.* 2018). Nevertheless, there is evidence to suggest that rapid initiation of appropriate antifungal therapy reduces mortality in invasive candidiasis in critically ill and invasive aspergillosis in at-risk haematology patients (Colombo *et al.* 2017). Detection and characterization of drug resistance *in vitro* can assist clinicians in selecting the best antifungal treatments.

1.2.12.1. Azoles and resistance

Among several classes of antifungals, three main categories are used in humans: polyenes (amphotericin B), azoles (fluconazole, itraconazole, voriconazole, posaconazole and isavuconazole), and echinocandins (caspofungin, micafungin and anidulafungin) (Wiederhold *et al.* 2018). However, in agriculture, several other compounds, including triazoles and other sterol demethylation inhibitors, are used (Russell 2005; Cui *et al.* 2019).

Azole fungicides are widely used for crop protection and material preservation. They protect crops from diseases, ensure high yields and prevent fungal contamination of produce. It has been proposed that triazole resistance has been evoked in the environment and could be driven by the selective pressure of azole fungicides. As in the ECDC Technical report (2013), although evidence supporting this hypothesis is growing, the link between the environmental use of azole fungicides and the development of triazole resistance in *Aspergillus* species still needs more evidence to prompt action on their use in agriculture (Kleinkauf *et al.* 2013).

Antibiotic resistance is an ever-increasing threat to human health. In the case of *Aspergillus fumigatus*, the environmental fungus that also causes life-threatening infections in humans, antimicrobial resistance is suggested to arise from fungicide use in agriculture, as the chemicals used for plant protection are similar to the antifungals used clinically. However, removing azole fungicides from crop fields will threaten the global food supply by reducing yield (Barber *et al.* 2020). Barber *et al.* (2020) surveyed crop fields in Germany between 2016 and 2018, before and after fungicide application and found a low overall azole resistance rate among agricultural isolates, as well as a lack of genomic and population impact following fungicide application

and thus concluded that azole use on crops does not significantly contribute to resistance in *A. fumigatus*.

Fungi may be primarily resistant to the action of antifungal drugs (intrinsic resistance), or they may be initially susceptible but develop secondary mechanisms of resistance after exposure to antifungals and the selection of resistant clones (acquired resistance) (Perlin 2017). There are several mechanisms of resistance contributing to either intrinsic resistance (biofilm formation, cell wall impermeability) or acquired resistance (target site overexpression, pathway alterations), or both (target incompatibility, drug efflux systems, stress response) (Revie *et al.* 2018). Examples of intrinsic resistance include *Aspergillus* species resistant to fluconazole and examples of acquired resistance include *Aspergillus fumigatus* resistant to azoles. When exposed to antifungals such as azoles and echinocandins, fungal cells can develop antifungal resistance through random mutations or induced genome instability. The most important predisposing factor for acquired resistance is exposure to fungistatic antifungal agents, especially at sub-therapeutic concentrations (Shor and Perlin 2015).

1.2.12.2. Azole resistance in *Aspergillus*

Azoles are the first-line treatment for most infections caused by A*spergillus* species (Patterson *et al.* 2016). Globally, *A. fumigatus* is the most frequent cause of invasive aspergillosis, causing over 90% of such infections (Taccone *et al.* 2015), followed by *A. flavus, A.niger* and *A. terreus*. Distribution varies by geographic location; for example, *A. flavus* is isolated at least as frequently as *A. fumigatus* in subtropical and tropical regions (Chawla *et al.* 2013). Intrinsic resistance to azoles varies by *Aspergillus* species. They are all intrinsically resistant to fluconazole and ketoconazole, because of a naturally occurring amino acid substitution in 14-α sterol demethylase A, encoded by cyp*51A* (Leonardelli *et al.* 2016). However, they are generally considered intrinsically susceptible to other azoles. *Aspergillus fumigatus* is a species complex containing up to 63 members, many of which can only be distinguished by molecular methods (Frisvad and Larsen 2016). *Aspergillus fumigatus sensu stricto* is the most common species in the complex, accounting for approximately 95% of all isolates (Lamoth 2016). Although it is intrinsically susceptible to all *Aspergillus*-active azoles, some of its sibling species, including *Aspergillus lentulus, Aspergillus thermomutatus* and *Aspergillus udagawae*, have caused disease refractory to azole therapy (Lamoth 2016). Furthermore, within the *A. niger* species complex, itraconazole resistance has been observed in 25–50% of clinical

isolates, with some evidence of cross-resistance with voriconazole and posaconazole (Hagiwara *et al.* 2016). Both cases highlight the importance of molecular epidemiology.

1.2.12.3. Mechanisms of azole resistance in *Aspergillus*

Azoles inhibit the enzyme lanosterol 14α-demethylase, preventing the synthesis of ergosterol, a fungal cell-membrane component. In *Aspergillus*, the major causes of azole resistance are changes in this target enzyme (reduced drug–target affinity, upregulated production or both) or upregulation of efflux pumps (Eliopoulos, Perea and Patterson 2002).

The problem of acquired azole resistance in *A. fumigatus* has been well-described. Resistance developed following prolonged therapy has been recognized since the late 1980s (Beardsley *et al.* 2018). However, there has been growing interest in acquired resistance in environmental isolates of *A. fumigatus* over the past decade, which may have emerged through exposure to agricultural azoles. Such isolates have been associated with infection in azole-naive patients (Snelders *et al.* 2008; Snelders *et al.* 2009). Over 50% of azole-resistant clinical isolates shared common resistance mechanisms in a global surveillance study, with point mutations in *CYP51A* and variable tandem repeat insertions in the promoter region (TR34/L98H or TR46/Y121F/T289A). These mechanisms confer multi-azole resistance and have been frequently identified in environmental samples, (Figure 1.9) (Van der Linden *et al.* 2015). The mechanism of intrinsic azole resistance in the cryptic species described above is yet to be clarified.

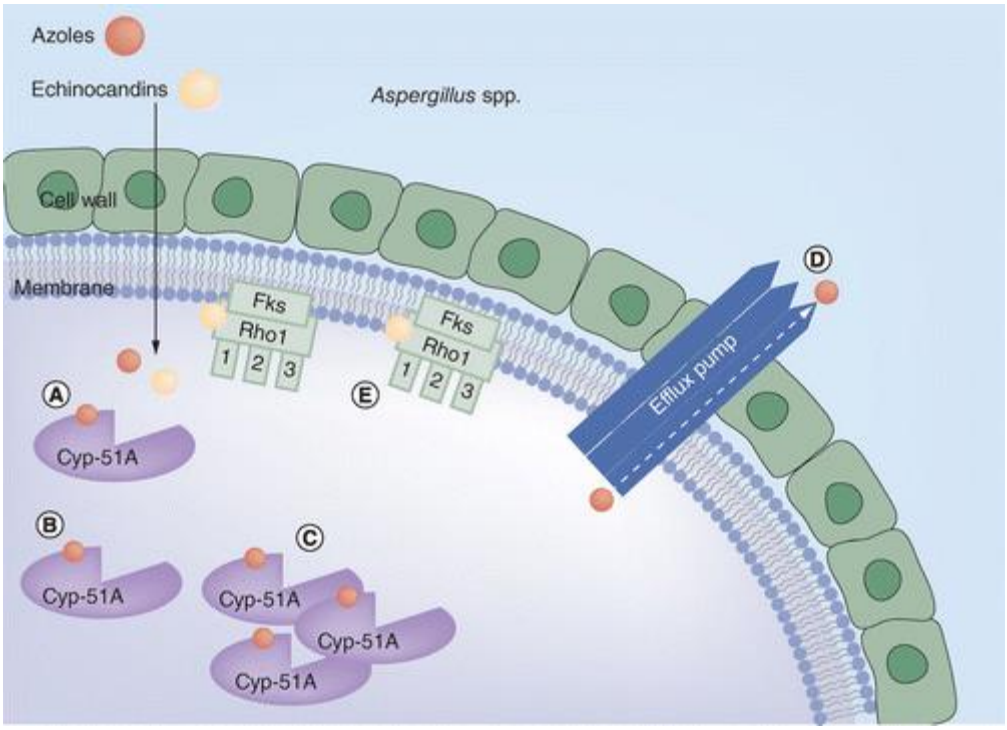


Figure 1.9 Overview of the major resistance mechanisms in *Aspergillus* species. (A) Shows normal binding of azoles (red) to 14-α-sterol demethylase (Cyp-51A); and normal binding of echinocandins (peach) to β-(1,3)-D-glucan synthase (Fks, including the three functional subunits Fks1, Fks2, and Fks3, and the regulatory subunit Rho1). (B) Shows reduced azole binding due to conformational changes in 14-α-sterol demethylase. (C) Shows reduced azole efficacy due to increased production of 14-α-sterol demethylase (+/- conformational changes). (D) Shows reduced azole efficacy due to increased efflux pump activity. (E) Shows conformational changes in the Fks1 subunit of β-(1,3)-D-glucan synthase leading to reduced echinocandin binding (Beardsley *et al.* 2018).

1.2.13. Management of drug-resistant *A. fumigatus* infections

The Infectious Diseases Society of America (IDSA) (2016) updated its guidelines for the management of invasive, chronic and allergic aspergillosis (Patterson *et al.* 2016). Azoles remain the preferred first-line therapy for treatment and prevention (Patterson *et al.* 2016). However, experimental evidence to guide the treatment of azole-resistant *A. fumigatus* infections is scarce and recommendations have not been included in the management guidelines. The choice of empiric antifungal therapy will be guided by the prevalence of azole resistance in a country, region or, preferably, in the treating institution. Where an elevated MIC to an azole is demonstrated by a validated test method, treatment with one or more alternative antifungal agents is recommended. As the number of effective drugs remains limited, regimens to extend azole effectiveness and overcome decreased susceptibility by optimizing drug exposure have also been explored (Seyedmousavi *et al.* 2014).

1.2.14. Azole use in medicine and the development of resistance through patient exposure

In hospitals, the increasing number of fungal infections causes a high morbidity and mortality rate in immunocompromised patients (Hagiwara 2016). This includes patients with haematological malignancy, pulmonary diseases, solid-organ or haematopoietic stem cell transplantation, and patients treated with corticosteroids (Verweij *et al.* 2009). Common fungal infections like chronic or acute invasive aspergillosis, allergic bronchopulmonary aspergillosis, and aspergilloma are caused by *Aspergillus* (Snelders *et al.* 2012). Patients develop these infections by inhaling airborne *Aspergillus* species conidia, which are normally eliminated by the immune system but can cause invasive diseases in immunocompromised individuals. Approximately 30 species of *Aspergillus* are involved in infections, but the most common species is *Aspergillus fumigatus* (Lelièvre *et al.* 2013).

To prevent and treat *Aspergillus* infections, four classes of antifungal drugs are used: pyrimidines, echinocandins, polyenes, and azoles (Hagiwara *et al.* 2016). Azoles are critical agents in the treatment and prophylaxis of aspergillosis (Lelièvre *et al.* 2013; Hagiwara *et al.* 2016). Itraconazole, voriconazole and posaconazole are the most widely used azole antifungal drugs. Voriconazole is prescribed for the primary treatment of aspergillosis, while posaconazole and itraconazole cause a reduction of invasive fungal infections in neutropenic patients with acute myeloid leukaemia and myelodysplastic syndrome (Verweij *et al.* 2009).

The increasing use of azoles in managing *Aspergillus* diseases led to resistance. Resistance is confined to the individual and no transmission between patients has been reported. Azole-resistant *A. fumigatus* strains were first reported in the late 1990s in patients from the United States who had received long-term itraconazole therapy (Lelièvre *et al.* 2013). Since then, many resistant strains have been discovered in Europe and elsewhere, especially in the Netherlands and the United Kingdom, where the prevalence reached 6 and 5%, respectively (Lelièvre *et al.* 2013). These resistances are mainly found in patients who received azole drugs for an extended period to treat chronic aspergillosis. Azole-resistant isolates are now identified in Norway, Spain, Belgium, Denmark, France, Germany, India, Iran, Portugal, Brazil, the Czech Republic, Turkey, Japan, Kuwait, Taiwan, Australia and China (Rivero-Menendez *et al.* 2016; Garcia-Rubio, Cuenca-Estrella and Mellado 2017).

1.2.15. Link between azole resistance in agriculture and clinic

Surprisingly, resistance to azoles is confined to patients undergoing azole therapy and in many azole naïve patients with no known prior exposure to azole drugs (Snelders *et al.* 2009). As inhalation of airborne *Aspergillus* spores is the typical route of infection for *Aspergillus* diseases, it has been hypothesized that the resistance could have been acquired from a common environmental source. For example, if azole-resistant *A. fumigatus* is present in our environment, patients could inhale resistant spores and subsequently develop azole-resistant diseases (Snelders *et al.* 2009; Brown, Denning and Levitz 2012).

Azoles are actively persistent for several months in many ecological niches, such as agricultural soil and aquatic environments. Azoles are commonly used in agriculture as fungicides and to preserve various materials such as wood. Hence, it was hypothesized that fungal exposure to azole compounds in the environment results in a cross-resistance to medical triazoles (Chowdhary *et al.* 2013). To validate this hypothesis, two studies sampled azole-resistant *A. fumigatus* strains from patients with previous exposure to azoles from the environment (flowerbeds, compost, leaves, plant seeds, soil samples of tea, paddy fields, hospital surroundings, and aerial samples of hospitals) (Chowdhary *et al.* 2013; Verweij *et al.* 2016) and different mechanisms of resistance were studied. Surprisingly, a combination of alterations previously described in patients was found not only in clinical samples but also in the environmental isolates. This includes mutations in the *cyp51A* gene (TR34/L98H, G448S and TR46/Y121F/T289A) essentially. In addition, a recent study highlighted that acquisition of mutation in the azole target by the environmental strains leads to cross-resistance between the azole antifungals in the environment and the clinic (Ren *et al.* 2017).

Among 144 soil samples treated with many azoles (epoxiconazole, tebuconazole, propiconazole, hexaconazole, metconazole). Ren *et al.* (2017) found that 5.8% of the analyzed samples were resistant to medical azoles. Two samples were resistant to voriconazole, with the most frequent mutations in the environmental samples (TR46/Y121F/T289A). One sample was resistant to itraconazole with mutation TR34/L98H/S297T/F495I. TR34/L98H was the most common mutation in environmental and clinical samples (Rivero-Menendez *et al.* 2016; Verweij *et al.* 2016). All *A. fumigatus* isolates with this mutation showed cross-resistance to medical and agricultural triazoles. Docking studies revealed that medical and agricultural triazoles have similar molecular structures and adopt similar conformations (Chowdhary *et al.* 2013). TR34/L98H mechanism was widespread in The Netherlands and is also found in other

European countries, the Middle East, Asia, Africa, Australia and, most recently, North and South America. A wide range of C*yp51A* mutations was reported in clinical and environmental isolates in Austria, Belgium, Denmark, France, Germany, Greece, Italy, The Netherlands, Poland, Portugal, Romania, Spain, Sweden, Turkey and the United Kingdom (Rivero-Menendez *et al.* 2016) Recently, azole resistance has also been found in Norway, Iran, Brazil, the Czech Republic, Japan, Kuwait and Taiwan (Garcia-Rubio, Cuenca-Estrella and Mellado 2017).

There is also a further need for surveillance, collection of precise information and extensive resistance monitoring programs in the agricultural field to investigate the size of the emerging problem of azole resistance. Research at the epidemiological level could indicate the geographic variations in resistance and facilitate the identification of the regions with a high incidence of resistance (Berger *et al.* 2017). Furthermore, research at the laboratory level should understand the conditions under which resistance mechanisms develop in the environment and which *Aspergillus* morphotype is most prone to develop means of resistance (Snelders *et al.* 2012).

Additionally, emerging resistance becomes a threat to millions of people across the globe because the same resistant strains of *A. fumigatus* observed in the environment are also found in the clinic. Since resistance is associated with treatment failure, it is necessary to optimize agricultural practices. In parallel, new, safe and effective classes of antifungals and compounds that inhibit the resistance mechanisms in fungi have to be developed in agriculture and clinic. Modification, combination, and repurposing of current antifungals and other FDA-approved drugs and seeking new sources of antifungal agents could serve as potential antifungal leads (Ngo, Garneau-Tsodikova and Green 2016).

Another issue of the extensive use of azoles in agriculture could be the emergence of resistance in non-environmental fungi. Non-ubiquitous fungi present in the human flora, such as *Candida* spp., are constantly in contact with environmental azoles. Indeed, this contact occurs inside the body by ingestion of azole-contaminated food, interaction with insects, or even by inhaling recently sprayed azoles on crops. However, this hypothesis requires further investigation. Resistance issues are confined to fungal, viral and bacterial pathogens. Finding new drugs and ways to control microbial pathogens and adapt the current strategies are a major concern for human health.

1.2.16. New antifungal agents for *Aspergillus*

The drug F901318, developed by F2G Ltd (Manchester, UK) (http://www.f2g.com/), is a promising antifungal agent entering Phase III clinical studies. It selectively inhibits the fungal dihydro-orotate dehydrogenase enzyme resulting in perturbed pyrimidine synthesis. *In vitro*, F901318 is highly active against azole-resistant *Aspergillus* isolates, including against isolates with cpy*51A* mutations known to confer azole resistance (Osherov and Kontoyiannis 2016). Other promising anti-*Aspergillus* agents include the Arylamine T-2307 (Toyoma Chemical Company, Tokyo, Japan), which selectively targets fungal mitochondria, E121010/APX001 (Amplyx Pharmaceuticals, CA, USA), which inhibits glycosylphosphatidylinositol-dependent anchoring of the fungal cell wall, and ASP2397 (Astellas Pharma, Tokyo, Japan) which interferes with fungal siderophore transport (Osherov and Kontoyiannis 2016).

At the same time, the development of diagnostic capacity for fungal infections is a priority with a recent review outlining a 'roadmap' calling for immediate access to the point of care testing for endemic fungal infections, followed by staged development of microscopy, culture and ultimately molecular techniques for low and middle-income countries (Cole *et al.* 2017). Next-generation sequencing is a new technology that can identify emerging threats whose value has been illustrated in identifying and tracking azole resistance in *A. fumigatus*. As molecular technologies evolve and their widespread use becomes more financially viable, molecular data can be integrated into clinical management guidelines.

1.2.17. Motivation for the study

Fungal infections cause a high disease burden in South Africa, impacting public health and improving strategies for diagnosing and managing fungal diseases can help address the morbidity and mortality associated with these conditions (Schwartz *et al*. 2019). This increasing incidence of infection is influenced by the growing number of immune-deficient cases related to cancer, old age, organ transplants, other invasive surgical procedures and AIDS. Opportunistic fungal infections impact fatal complications in immune-deficient cases, and *Aspergillus* species are the most frequently isolated moulds among these patients. Invasive aspergillosis carries a high fatality rate in immunocompromised patients. Although antifungal drugs exert activity against *Aspergillus* species, the pressure of pervasive antifungal use has the emerging clinical challenge of resistance (Friedman and Schwartz 2019). The increasing burden of azole resistance on a global scale limits the therapeutic options to treat aspergillosis

and patients with azole-resistant *Aspergillus* infections are at high risk for therapeutic failure (Denning and Perlin 2011; Arastehfar *et al.* 2021).

Antifungal resistance, virulence, and nutritional versatility all contribute to the multifaceted pathogenicity of *Aspergillus* (Ries *et al.* 2018). Among the many virulence traits of *Aspergillus*, nutrient acquisition and metabolism confers a significant advantage for growth during fungal infection under conditions of limited nutrient availability (Brock 2009; Blatzer and Latgé 2017). To further impact the virulence of *Aspergillus*, the species, in response to unfavourable environments during infection, activate fungal secondary metabolism to produce gliotoxin in order to increase survival chances (Hof and Kupfahl 2009).

Gliotoxin has been detected in the lungs and sera of humans infected with *A. fumigatus* (Cerquiera *et al*. 2014) and is suggested as a virulence determinant of the human pathogen (Scharf *et al.* 2015). In co-existence, pathogen-associated molecules are also known to induce the production of gliotoxin in *A. fumigatus* and give a possible explanation for the increased virulence of *A. fumigatus* during bacterial co-infection (Svahn *et al*. 2015).

Thus, in the dire need for improved management and control of respiratory-related *Aspergillus* infections, it is necessary to assess and compare conventional and molecular identification techniques and explore new techniques for more rapid detection. Susceptibility profiles of routine and salvage therapy azoles can then be analyzed and new compounds recovered and tested for antifungal potential in combination therapy to overcome the decreasing success of azole monotherapy. The nutritional versatility of *Aspergillus* and virulence factors like gliotoxin can equally be investigated for the pathogenicity of the human pathogen.

1.2.18. Aim and Objectives

1.2.18.1. Aim

To characterize and assess prevalent fungal species isolated from immunocompromised patients with respiratory infections using phenotypic microarray and genetic analysis.

1.2.18.2. Objectives

1. To identify prevalent fungal isolates from immunocompromised patients with respiratory infections by morphological features and 18S rRNA sequencing.
2. To analyze and compare growth and nutrient profiles of the prevalent fungal isolates using the phenotypic microarray system.
3. To determine and analyze the drug-resistant profiles of the fungal isolates on routinely used antifungal drugs and the potential of new compounds in antimicrobial drug panels (comprising 24 compounds) using a Biolog phenotypic microarray system.
4. To screen and quantify prevalent fungal isolates for gliotoxin production using HPLC as a possible virulence factor for resistance.
5. To identify the presence of genetic markers for antimicrobial compound resistance and gliotoxin production, using PCR and DNA sequencing and to relate this to phenotypic and resistance profiles.

CHAPTER TWO: IDENTIFICATION AND CHARACTERIZATION OF *ASPERGILLUS* ISOLATES FROM IMMUNOCOMPROMISED PATIENTS WITH RESPIRATORY INFECTIONS

2.1. Introduction

Fungi are opportunistic human pathogens that invade the body when the natural defence is severely weakened. Filamentous fungi and yeasts are among the ten most frequently isolated pathogens in intensive care unit patients (de Pauw, 2011). Fungal infections are a silent epidemic that continues to grow, impacting immunocompromised individuals hospitalised with severe diseases requiring surgical procedures or intensive care support (Wilcox, 2010). Fungal diseases, like invasive aspergillosis and candidiasis, are the most frequently occurring infections in immunocompromised individuals (Bassetti *et al.* 2015) and are the most common nosocomial fungal infections (Rudramurthy *et al.* 2019). There is a rapid emergence of invasive fungal infections (IFIs) with the increasing population of immunocompromised individuals. Invasive fungal infections are also the major cause of HIV-related mortality and account for 50% of AIDS-related deaths globally (Armstrong-James, Meintjes and Brown 2014). The fungal infection, invasive aspergillosis (IA), mainly affects immunocompromised individuals with a high incidence of intensive care unit patients (Taccone 2015). In the study by Taccone (2015) to investigate the epidemiology of IA in an immunocompromised population) (n=563), the study investigated patients from 30 ICUs in 8 countries (Belgium, France, Brazil, China, Spain, Greece, India and Portugal, found that the lung tissue was the most frequent site of infection (94%) and *A. fumigatus* the most commonly isolated species (92%). This study also reported that IA among critically ill patients was associated with high mortality and should be considered an emerging and devastating infectious disease in this population. The progressive increase in the incidence of *Aspergillus* isolates poses a clinical challenge in treating invasive infection in immunocompromised patients (Friedman and Schwartz, 2019).

The cause of morbidity and mortality in most human immunodeficiency virus (HIV) positive patients is not the virus itself but the opportunistic infections resulting from the decrease in immune response cd4 cell count, thus leading to an immunocompromised state (Kaur *et al.* 2017). The Centre for Disease Control and Prevention (CDC) also reported fungal diseases to be 20% of acquired immunodeficiency syndrome (AIDS) defining diseases. The common opportunistic mycosis is invasive aspergillosis, seen in advanced stages of AIDS, most commonly involving the lung. Thus, there is a need for early diagnosis to develop appropriate

and productive antifungal therapy, which necessitates the use of rapid and effective identification tools. A combination of modalities like microbiological, serological and molecular methods must be considered effective diagnosis as each technique may have specific limitations.

Fungal identification has been traditionally based on the organism's phenotype. However, phenotype-based identification can be subjective and lead to improper diagnostics and treatment. However, clinical diagnosis often relies on culture techniques and microscopic examination of positive cultures. Microscopy complicated by atypical phenotypes and lengthy colony growth incubation may also cause delays in treatment. Poor prognoses delay diagnosis and treatment, increasing patient morbidity and mortality (Wisplinghoff *et al.* 2004). The continual increase in opportunistic fungal infections and immunocompromised populations has necessitated the development of quick and precise fungal diagnosis technologies. Molecular techniques have emerged as standard approaches for microbiological infection (Blackwell *et al.* 2006). However, in developing countries, costs and lack of infrastructure continue to be a disadvantage. Technology for rapid and specific identification has however been developed and is now accessible. The Biolog advanced phenotypic technology (Biolog, USA) is one such technology that provides phenotypic information on the properties of strains for species-level identification. The Biolog Identification System can rapidly identify species of fungi from the system's reference laboratory using redox chemistry. The Biolog facilitates redox chemistry by utilizing several carbon compounds to provide varying biochemical characterizations, and this diverse set of tests enables the system to identify microorganisms.

This chapter examines the identification and characterisation of filamentous fungal isolates (36) from patients with respiratory infections at the Inkosi Albert Luthuli Hospital in Durban, Kwa-Zulu Natal. For preliminary genus identification, traditional culture and microscopy techniques were applied.

2.2. Methodology

2.2.1. Sample collection and culture maintenance

Thirty-six filamentous fungal samples were isolated from the upper respiratory tract of patients at the Inkosi Albert Luthuli Hospital between July 2015 and June 2016. The isolates were purified through consecutive sub-culturing and maintained on malt extract agar (MEA) and potato dextrose agar (PDA) plates (Merck), incubated at 37℃ 48 hours. The plates for routine maintenance were incubated at 37°C for 48 hours on slants and stored at 4°C for short-term use. Stock cultures were prepared using spore suspensions in 15% glycerol stored at -70°C.

2.2.2. Microscopic and culture morphology identification

Conventional culture and microscopic techniques were used for preliminary identification. Ten microlitres of the spore suspension were transferred onto malt extract plates (Merck) and incubated at 37°C for 72 hours. The mycelium was prepared and viewed in a wet mount under the light microscope (Nikon, YS100) for characteristic morphology at 48 hours of incubation. The plates were incubated further until sporulation and observed for expected growth, viz. and the colour of spores.

2.2.3. DNA extraction, PCR and DNA sequencing

2.2.3.1. DNA extraction, purification and quantification

For the identification of the species, DNA was extracted from cultures first grown on malt extract agar plates for 72 hours at 37°C. Thereafter two plaques were transferred to 100 ml malt extract broth and incubated at 37°C, 150 rpm for 18 hours. The mycelia were centrifuged, and the pellet was used to extract DNA using the protocol by Melo *et al.* (2006), with minor modifications. The frozen pellet (-70℃) of mycelia was re-suspended in 5 ml extraction buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and glass beads (0.2 mm) were added. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v) was added to the reaction tube, the suspension vortexed at high speed for 5 min and then centrifuged for 10 min at 10 000 g. The supernatant was transferred to a microcentrifuge tube, and DNA precipitated by adding two volumes of absolute ice-cold ethanol. The tube contents were mixed gently and incubated for 20 min at -70°C to precipitate nucleic acids. The sample was centrifuged at 4°C for 10 min at 150 rpm, the pellet re-suspended in 1 ml of Tris-EDTA, pH 8 with 10 µl RNAse (20 mg/ml) and incubated for 1 hour at 37°C without shaking to degrade contaminating RNA.

The DNA was precipitated by adding two volumes of absolute ethanol and gentle mixing, followed by re-incubation at -70°C. After centrifugation for 15 min, the DNA pellet was washed three times with 75% ethanol, then air-dried in a laminar flow cabinet and re-suspended in 500 µl of chromatography-quality sterile water. The genomic DNA was quantified using a NanoDrop 2000 analyzer (Thermoscientific) and analyzed by agarose gel electrophoresis before PCR.

2.2.3.2. PCR amplification

The PCR assay was performed with 5 µl of the extracted DNA sample in a total reaction volume of 50 µl consisting of 1.0 µl of each primer, 25 µl of Taq Readymix and 18 µl of ultrapure water. Thirty cycles of amplification were performed in a thermocycler (supplier) after the initial denaturation of DNA at 95°C for 5 min. Each cycle consisted of a denaturation step at 95°C for the 30 s, an annealing step at 58° C for 30 s and an extension step at 72°C for 1 min, with a final extension at 72°C for 5 min. After amplification, the products were stored at -20°C until use. Amplicons were separated by 0.8% agarose gel electrophoresis for confirmation.

2.2.3.3. Sequence analysis

The PCR products from successfully applied samples were purified using DNA Purification Kit for sequencing (Qiagen, USA) following the manufacturer's manual. Sequencing of PCR samples was performed by Inqaba Biotech (South Africa) on a Sanger Sequencing Platform. Sequence analysis (18S) was conducted on National Center for Biotechnology Information (NCBI) online at (http:// www.ncbi.nlm.nih.gov) using Nucleotide and Protein Blast functions to confirm identification (O'Leary *et al.* 2016).

2.2.4. Phenotypic characterization of fungal isolates

The Biolog FF panel was used for the identification of filamentous fungal isolates. Each plate of the FF panels contains 96 wells (95 tests and one control - water) (Figure 2.1). The isolates were inoculated (one plaque) on malt extract plates for 72 hours at 37°C. The conidia were then removed from the surface of the agar plates using a sterile swab by gently scraping off across the surface and transferred into the sterile inoculating fluid (FF-IF, Biolog USA). The turbidity of the suspension was measured (Biolog turbidimeter) until it reached a density of approximately 75% transmittance, according to the manufacturer's protocol (Biolog USA). A hundred µl of fungal inoculum were transferred into each well of the PM plates. Inoculated PM

plates were incubated at 37°C for 96 hours. Data was collected at zero hours and for eight, 12-hour intervals, reading on the Microlog station at 750 nm.

The Biolog FF microplate performs 95 discrete tests simultaneously (Figure 2.1) and gives a characteristic reaction pattern called a "fingerprint" (Figure 2.2). These fingerprint reaction patterns provide a vast amount of information about the metabolic properties of each fungus tested, along with a species-level identification (Figure 2.3). The FF database contains over 500 taxa of fungi from over 120 genera.

A1 Water	A2 Tween 80	A3 N-Acetyl-D-Galactosamine	A4 N-Acetyl-D-Glucosamine	A5 N-Acetyl-D-Mannosamine	A6 Adonitol	A7 Amygdalin	A8 D-Arabinose	A9 L-Arabinose	A10 D-Arabitol	A11 Arbutin	A12 D-Cellobiose
B1 α-Cyclodextrin	B2 β-Cyclodextrin	B3 Dextrin	B4 i-Erythritol	B5 D-Fructose	B6 L-Fucose	B7 D-Galactose	B8 D-Galacturonic Acid	B9 Gentiobiose	B10 D-Gluconic Acid	B11 D-Glucosamine	B12 α-D-Glucose
C1 Glucose-1-Phosphate	C2 Glucuronamide	C3 D-Glucuronic Acid	C4 Glycerol	C5 Glycogen	C6 m-Inositol	C7 2-Keto-D-Gluconic Acid	C8 α-D-Lactose	C9 Lactulose	C10 Maltitol	C11 Maltose	C12 Maltotriose
D1 D-Mannitol	D2 D-Mannose	D3 D-Melezitose	D4 D-Melibiose	D5 α-Methyl-D-Galactoside	D6 β-Methyl-D-Galactoside	D7 α-Methyl-D-Glucoside	D8 β-Methyl-D-Glucoside	D9 Palatinose	D10 D-Psicose	D11 D-Raffinose	D12 L-Rhamnose
E1 D-Ribose	E2 Salicin	E3 Sedoheptulosan	E4 D-Sorbitol	E5 L-Sorbose	E6 Stachyose	E7 Sucrose	E8 D-Tagatose	E9 D-Trehalose	E10 Turanose	E11 Xylitol	E12 D-Xylose
F1 γ-Amino-butyric Acid	F2 Bromosuccinic Acid	F3 Fumaric Acid	F4 β-Hydroxy-butyric Acid	F5 γ-Hydroxy-butyric Acid	F6 p-Hydroxyphenyl-acetic Acid	F7 α-Keto-glutaric Acid	F8 D-Lactic Acid Methyl Ester	F9 L-Lactic Acid	F10 D-Malic Acid	F11 L-Malic Acid	F12 Quinic Acid
G1 D-Saccharic Acid	G2 Sebacic Acid	G3 Succinamic Acid	G4 Succinic Acid	G5 Succinic Acid Mono-Methyl Ester	G6 N-Acetly-L-Glutamic Acid	G7 Alaninamide	G8 L-Alanine	G9 L-Alanyl-Glycine	G10 L-Asparagine	G11 L-Aspartic Acid	G12 L-Glutamic Acid
H1 Glycyl-L-Glutamic Acid	H2 L-Ornithine	H3 L-Phenylalanine	H4 L-Proline	H5 L-Pyroglutamic Acid	H6 L-Serine	H7 L-Threonine	H8 2-Amino Ethanol	H9 Putrescine	H10 Adenosine	H11 Uridine	H12 Adenosine-5'-Monophosphate

Figure 2.1: Carbon sources in FF microplate with 95 wells, containing water as a control (www.oxoid.com/pdf/biolog/FFMP.pdf)

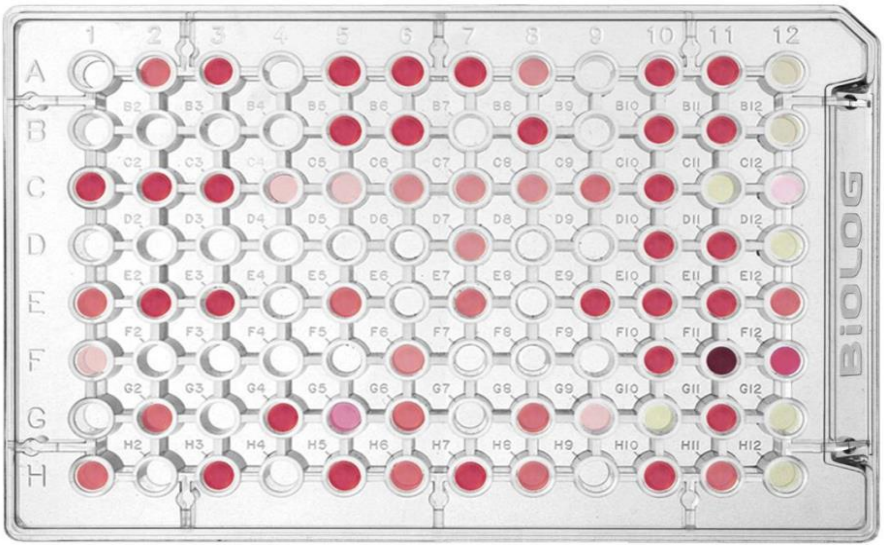


Figure 2.2: Biolog FF microplate showing colour reactions after incubation and growth (www.oxoid.com/pdf/biolog/FFMP.pdf)

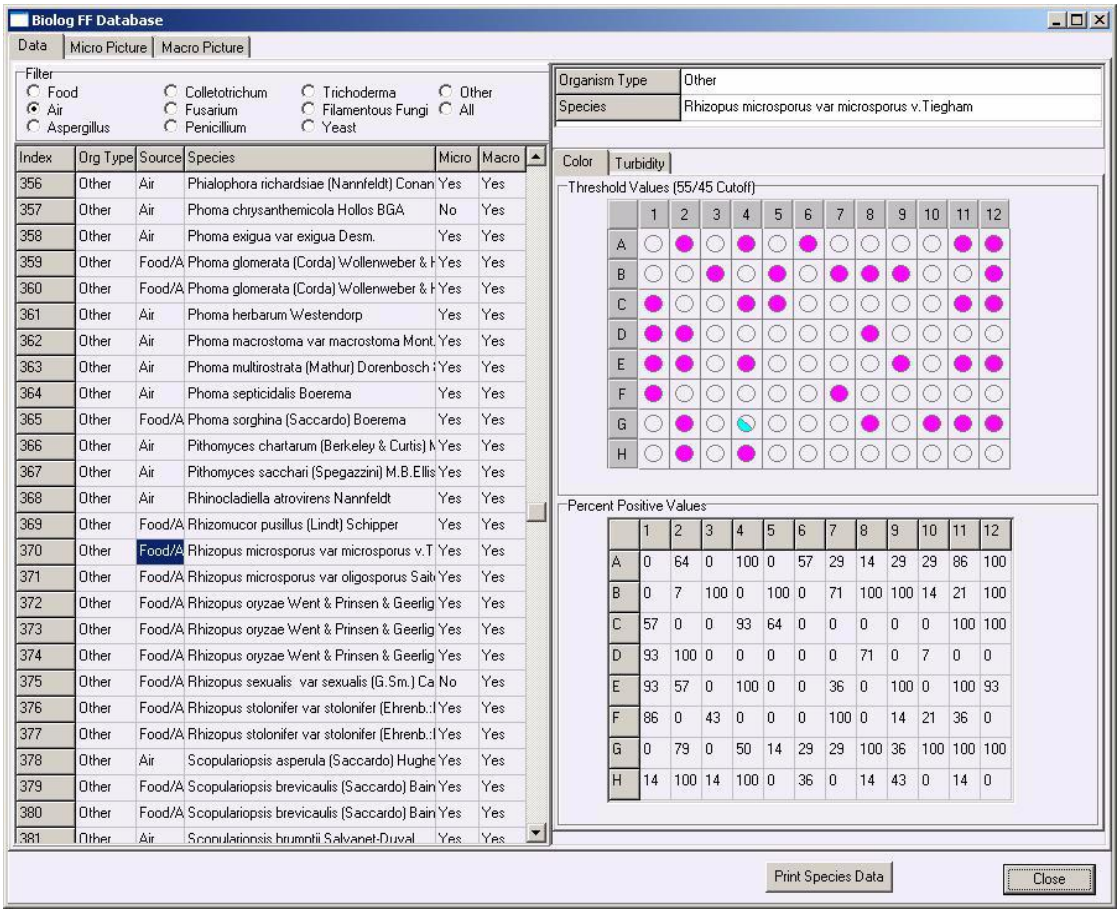


Figure 2.3: Biolog FF database. An example of colour results of turbidity and corresponding values to match species listed in the database.

2.2.5. Effect of temperature and media on growth

Half a millilitre of spore suspension (1 X 10^6) of each isolate was placed in the centre of the MEA and PDA plates prepared in duplicates for incubation at 30°C and 37°C respectively. Growth was observed and photographed at 24, 36, and 48 hours. Radial growth and sporulation were observed after each incubation period.

2.3. Results

Growth temperatures of 30 and 37ºC and growth media viz. malt extract agar and potato dextrose agar were explored for optimum growth and sporulation to support more efficient identification. Culture characterization was carried out after identifying isolates by culture and microscope morphology, as described in 2.3.2.1. Of the 36 isolates, 26 were identified as *A. fumigatus,* 8 as *A. niger* and 2 as *A. flavus* (Table 2.1).

Table 2.1: The isolate number and species identified by morphological characteristics and 18S rRNA.

Species	**Isolates**												
A. fumigatus	1	2	3	4	5	6	7	8	9	11	14	15	16
	17	19	20	23	24	26	27	28	29	31	34	35	36
A. niger	10	12	13	18	21	25	30	33					
A. flavus	22	32											

2.3.1. Culture characterization

All the isolates were cultured on two commonly used fungal media, MEA and PDA, at 30°C (environmental growth temperature) and 37°C (clinical growth temperature). Radial growth and sporulation were observed in all three *Aspergillus* species and they grew more rapidly on malt extract agar at 37°C. This was observed at 24, 36 and 48 hours; however, it was more pronounced at 36 and 48 hours. Sporulation by all isolates was observed after 36 hours of incubation on MEA but significantly less at 30°C compared to 37°C. After 48 hours of incubation, all three species showed considerably more growth and sporulation at 30°C and 37°C; however, there was no sporulation on PDA for *A. fumigatus*. (Figures 2.4-2.6), MEA at 37°C showed more rapid growth and sporulation in all species and thus was selected to be optimum for temperature and nutrient composition. The spores of each specific species displayed typical and distinct morphology in the colour of the respective spores, as described in Figure 2.7.

For the description of *Aspergillus fumigatus,* isolate 28 of the 26 *A. fumigatus* isolates was used for discussion. Isolate 28 shows the typical growth observed in this species, as seen in Figure 2.4. Photographs of other *A. fumigatus* strains can be seen in Appendix B. These isolates showed a much larger diameter in radial growth on MEA agar at 37℃ than on PDA. This is especially pronounced at 36 and 48-hour incubation and indicated more rapid culture growth of the species on MEA. At 48 hours, radial growth on PDA at 37℃ was good, but growth on MEA at 37℃ almost covered the plate medium. It was also noted that PDA does not support the sporulation of *A. fumigatus* even when the fungal/mycelial growth is optimal at 37℃.

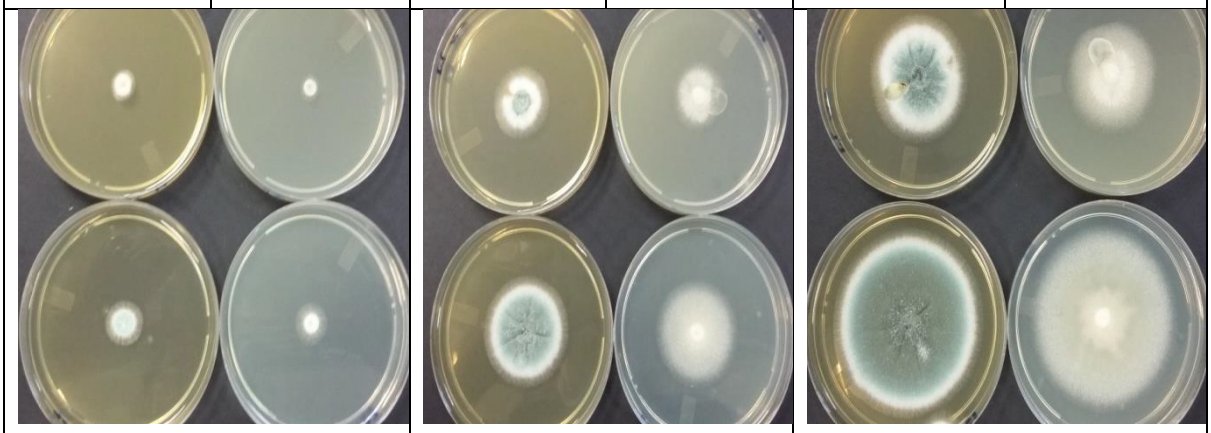
24h		**36h**		**48h**	
MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C
					
MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C

Figure 2.4: Growth and sporulation of *Aspergillus fumigatus* (28) on MEA and PDA after 24, 36 and 48 hours incubation at 30 and 37°C.

Isolate 18 of *A. niger* was selected from the eight *A. niger* strains to show the typical cultural growth of the species. *A. niger* species showed almost the same growth pattern on both MEA and PDA, based on radial growth evident from 36 hours of incubation in Figure 2.5. However, the sporulation rate is faster on MEA at both 30 and 37℃, slowed down on PDA at 30℃ and increased at 48 hours. Photographs of the remaining seven strains are in Appendix B.

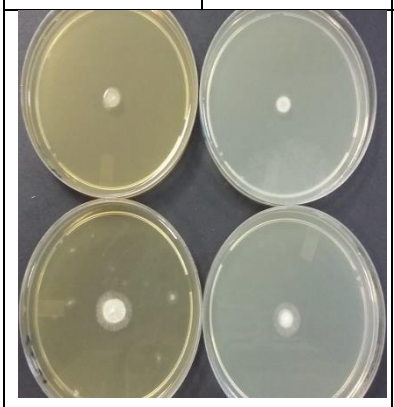
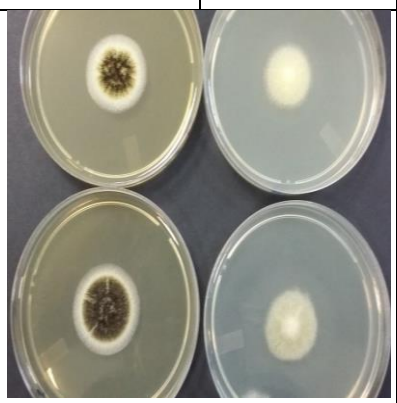
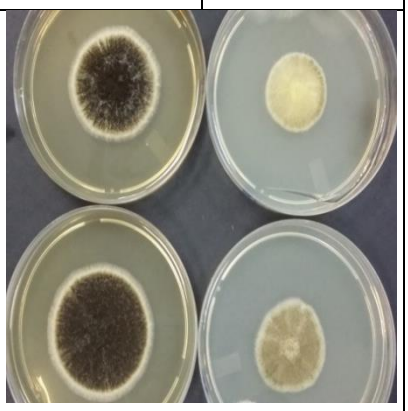
24h		**36h**		**48h**	
MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C
					
MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C

Figure 2.5: Growth and sporulation of *Aspergillus niger* (18) on MEA and PDA after 24, 36, and 48 hours of incubation at 30 and 37°C.

Aspergillus flavus isolate (22 and 32) showed typical growth characteristics. Radial growth of isolate 22 showed a similar growth rate at 30 and 37ºC on both MEA and PDA at 24, 36 and 48 hours, as seen in Figure 2.6. Sporulation in this species is much slower than in the *A. fumigatus* and *A. niger* species. Although MEA plates showed slight sporulation at 36 hours, sporulation is only clearly visible after 48 hours on MEA plates. Photographs of isolate 32 are in Appendix B.

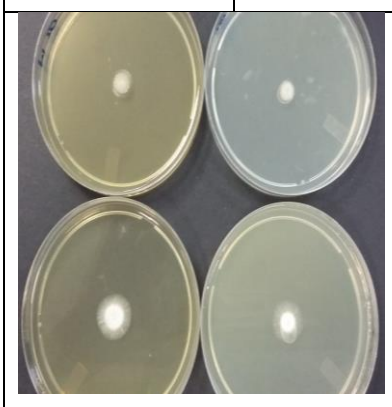
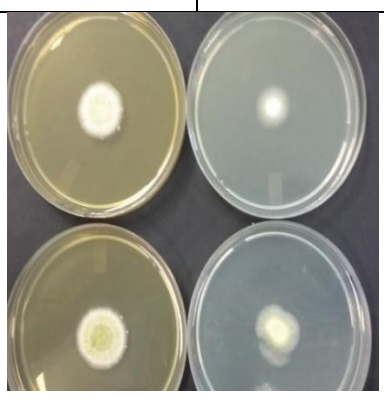
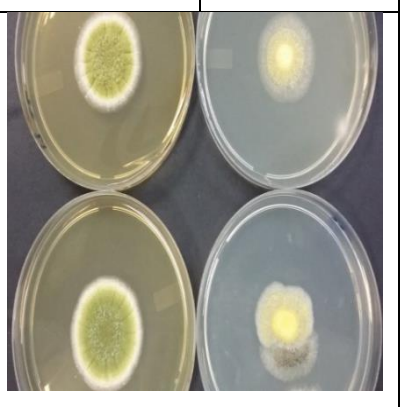
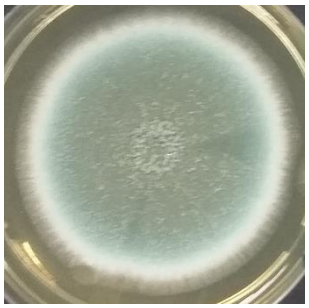
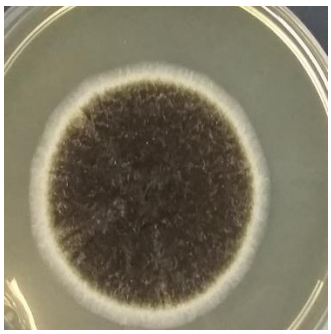
24h		36h		48h	
MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C
					
MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C

Figure 2.6: Growth and sporulation of *Aspergillus flavus* (22) on MEA and PDA after 24, 36, and 48 hours of incubation at 30 and 37°C.

2.3.2. Identification

2.3.2.1. Cultural and microscope

Traditional culture and microscope identification techniques were used to observe the typical features of the respective *Aspergillus* species, in order to characterize and identify each species (Figure 2.7). Culture morphology was observed at 48 hours of incubation, and then wet-mount preparations of the cultures were observed under the microscope. The sporulation colour was noted as a distinguishing cultural characteristic, and conidia and mycelial forms were observed under the microscope to enhance the identification of the fungal species. The 36 isolates were identified as *A. fumigatus* (36), *A. niger* (8) and *A. flavus* (2), as shown in Table 2.2.

	***Aspergillus* species on malt extract agar**	**Macroscopic observation**	**Microscopic observation**
A. fumigatus		White mycelial growth initially, with bottle green to greenish-grey conidia forming over with a velvety to cottony appearance. The reverse is white.	Conidiophores are smooth, and short to semi-long. Uniseriate and columnar conidial heads with the phialides are limited to the upper two-thirds of the vesicle and curve to the central axis to be roughly parallel. Conidia are spherical to ovoid (Afzal *et al.* 2013).
A. niger		Growth is initially white, then becomes black with conidial production. The reverse is white to pale yellow.	Smooth, thick-walled, hyaline conidiophores. Vesicles are globose and hyaline and covered with globose dark brown to black conidia (Afzal *et al.* 2013).
A. flavus		White mycelial growth initially, with olive to lime green conidia forming over with a	Conidiophores are uncoloured with globose to sub-globose vesicles, with phialides covering the

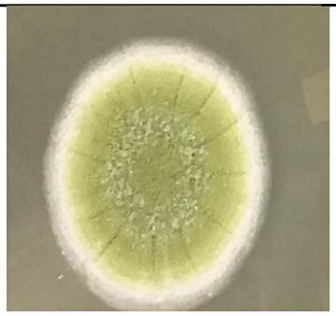
		woolly to cottony granular appearance. The reverse is creamy.	vesicle surface. Conidia are smooth, globose to sub-globose (Afzal *et al.* 2013).

Figure 2.7: The culture and microscopic features of *Aspergillus* species observed after 48 hours of incubation at 37ºC on malt extract agar.

2.3.2.2. Amplification for DNA-based identification (18S rRNA)

The isolates were cultured in malt extract broth and DNA was extracted from 18-hour cultures followed by PCR. The amplicons were analyzed using a 1.0 % agarose gel (Figure 2.8). The PCR product size for all the *Aspergillus* isolates was estimated to be around 600 base pairs, as seen in Figure 2.8, where selected representative isolates are shown from lanes 2 to 9.

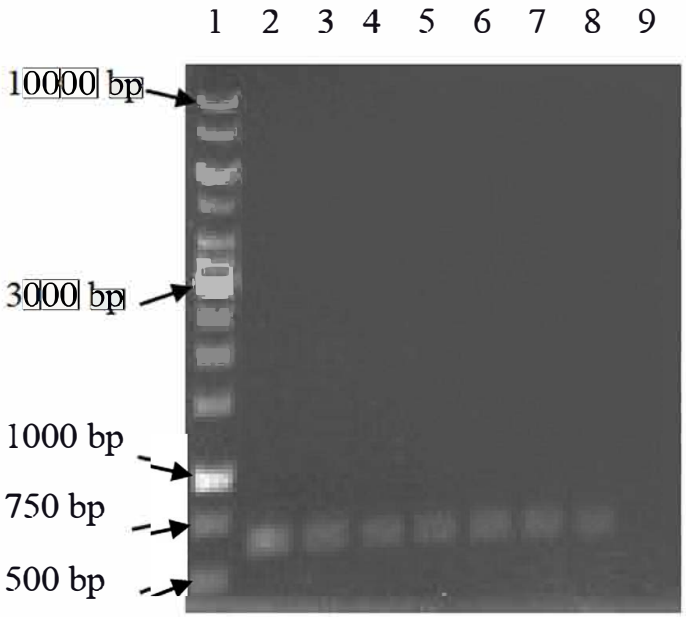


Figure 2.8: Amplification of *A. fumigatus, A. niger and A. flavus genes*. Lane 1:1 kb Plus DNA ladder, lanes 2-8: PCR products, 2-4 (*A. fumigatus*), lanes 5&6 (*A. niger*) and lanes 7&8 (*A. flavus*).

The blast results give E-values that describe the number of hits when searching a database. The E-value decreases exponentially as the score (bit score) of the match increases. A zero E-value indicates an exact match in a sequence and aligns with the maximum bit score. An E-value of one is not an exact match but close enough to be identified as a match. The blast results of the sequencing of isolates 2, 10 and 22 show close and exact matches shown in Table 2.2, displaying a typical blast result. The blast results of the remaining thirty-three isolates are in Appendix A, which have similar alignments. Sequencing results were used to draw a phylogenetic tree that revealed clustering within species and strains, as demonstrated in Figure 2.8.

Table 2.2: Blast results of isolates 2 (*A. fumigatus*), 10 (*A. niger*) and 22 (*A. flavus*) from the ITS sequencing report.

Isolate 2: *A. fumigatus*

Sequences producing significant alignments:		Score (Bits)	E Value
gi|386685951|gb|JQ776545.1|	Aspergillus fumigatus strain SGE5...	824	0E00
gi|386685951|gb|JQ776545.1|	Aspergillus fumigatus strain SGE5...	156	9E-34
gi|386685951|gb|JQ776545.1|	Aspergillus fumigatus strain SGE5...	135	3E-27
gi|386685951|gb|JQ776545.1|	Aspergillus fumigatus strain SGE5...	124	5E-24
gi|386685951|gb|JQ776545.1|	Aspergillus fumigatus strain SGE5...	72	3E-08
gi|386685951|gb|JQ776545.1|	Aspergillus fumigatus strain SGE5...	68	4E-07
gi|917553126|gb|KP881424.1|	Aspergillus fumigatus isolate FPZ...	822	0E00
gi|917553126|gb|KP881424.1|	Aspergillus fumigatus isolate FPZ...	124	5E-24
gi|917553126|gb|KP881424.1|	Aspergillus fumigatus isolate FPZ...	106	1E-18
gi|917553126|gb|KP881424.1|	Aspergillus fumigatus isolate FPZ...	100	6E-17

Isolate 10: *A. niger*

Sequences producing significant alignments:		Score (Bits)	E Value
gi|1074969850|gb|KT844472.1|	Aspergillus niger strain ASP sma...	1127	0E00
gi|1074969850|gb|KT844472.1|	Aspergillus niger strain ASP sma...	252	2E-62
gi|1074969850|gb|KT844472.1|	Aspergillus niger strain ASP sma...	109	1E-19
gi|632799235|gb|KJ432863.1|	Aspergillus niger isolate SS5 18S...	1121	0E00
gi|632799235|gb|KJ432863.1|	Aspergillus niger isolate SS5 18S...	138	2E-28
gi|632799235|gb|KJ432863.1|	Aspergillus niger isolate SS5 18S...	109	1E-19
gi|681957046|gb|KJ881376.1|	Aspergillus niger voucher MSR3 18...	1119	0E00
gi|681957046|gb|KJ881376.1|	Aspergillus niger voucher MSR3 18...	129	1E-25
gi|681957046|gb|KJ881376.1|	Aspergillus niger voucher MSR3 18...	109	1E-19
gi|681957047|gb|KJ881377.1|	Aspergillus niger voucher MSR4 in...	1109	0E00
gi|681957047|gb|KJ881377.1|	Aspergillus niger voucher MSR4 in...	176	1E-39

Isolate 22: *A.flavus*

Sequences producing significant alignments:	Score (Bits)	E Value
gi|1075210638|gb|KX531011.1| Aspergillus flavus var. flavus s...	1049	0E00
gi|817011865|gb|KP278181.1| Aspergillus flavus strain QRF354 ...	1049	0E00
gi|329458299|gb|HQ285545.1| Aspergillus flavus strain CJ-B4 1...	1049	0E00
gi|329458274|gb|HQ285520.1| Aspergillus flavus strain AD-B3 1...	1049	0E00
gi|969812107|dbj|LC105688.1| Aspergillus flavus genes for ITS...	1042	0E00
gi|595759065|gb|KC871017.1| Aspergillus sp. DX-SEL2 18S ribos...	1042	0E00
gi|1050758540|emb|LT604472.1| Aspergillus flavus genomic DNA ...	1040	0E00
gi|1044330086|gb|KU533844.1| Aspergillus sp. isolate TW4-2 18...	1040	0E00
gi|1022528166|gb|KU978916.1| Aspergillus flavus isolate TDPEF...	1040	0E00
gi|1002096737|gb|KT831451.1| Aspergillus sp. XMX001 18S ribos...	1040	0E00
gi|973966910|gb|KU504322.1| Aspergillus sp. BAB-5550 18S ribo...	1040	0E00

2.3.2.2.1. Phylogenetic analysis

The phylogenetic tree revealed branching within the species and strains; the *A. fumigatus* species falls into three branches from the same superfamily (Figure 2.9). Cluster 1, including isolates 8, 15, 20, 29, 34, 7, 9, 16 and 17 and Cluster 2, including isolates 23, 4, 31, 6, 35, 27, 1, 14, and 24, form two distinct groups. The remaining *A. fumigatus* isolates (5, 36, 3, 19, 2, 11, 26) form cluster 3, with smaller groupings within the cluster and seem connected to the *A. niger* branching. The *A. niger* species (10, 21, 25, 18, 33) form cluster 4 and *A. niger* isolates 12 and 13 form a sub-group within cluster 4. These isolates were identified as *A .niger/tubingensis* by 18s rRNA, while the remaining isolates were identified as *A. niger*. Isolates 22 and 32 (*A. flavus*) form cluster 5. Isolates 28 (*A. fumigatus*) and 30 (*A. niger*) do not fall into any of the clusters in the phylogenetic tree.

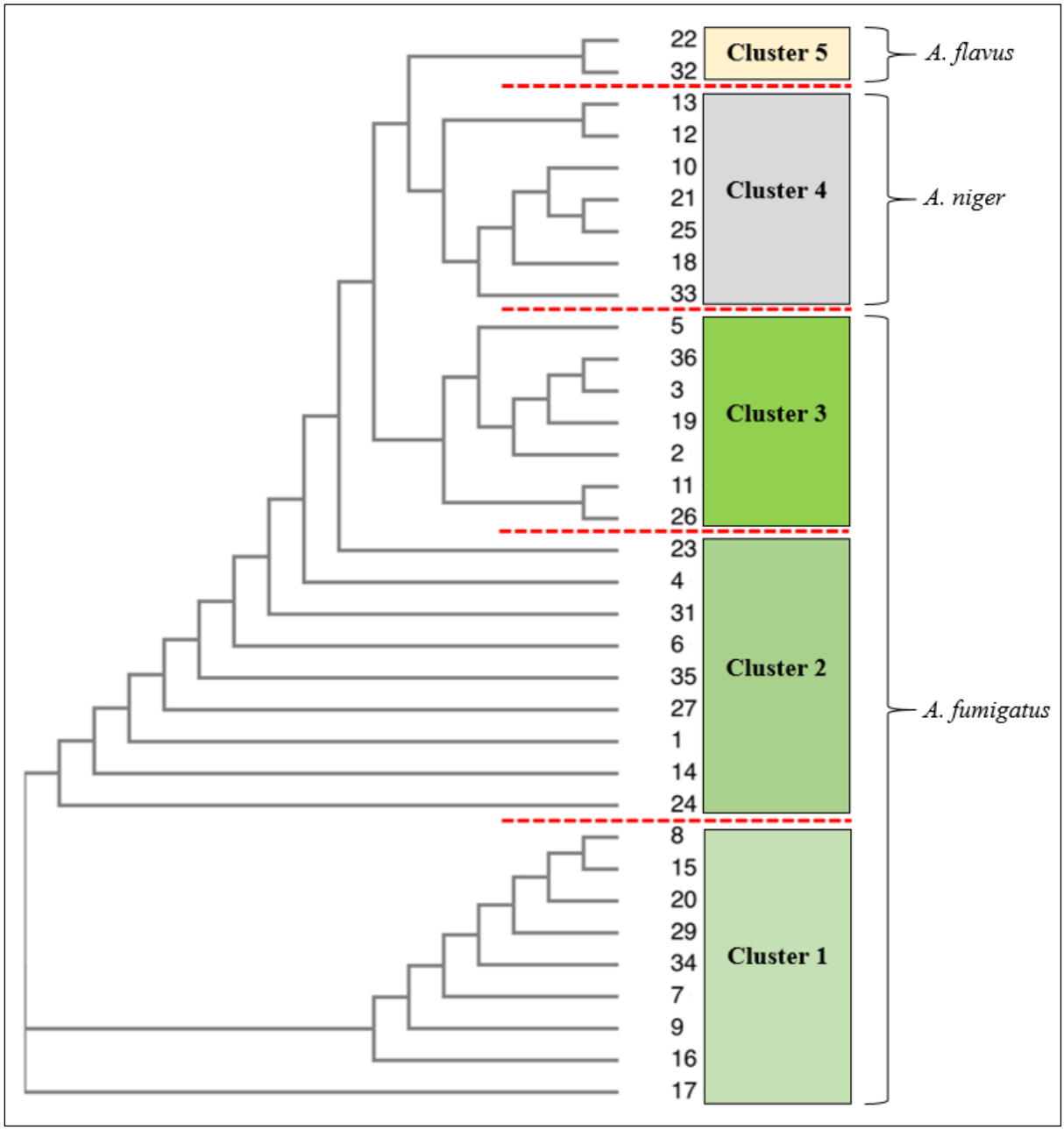


Figure 2.9: Phylogenetic tree showing clusters within isolates of *Aspergillus* species

2.3.2.3. Phenotypic identification using Biolog

This study explored a technology that makes use of metabolic activity with colour indication, thus screening for phenotypic characteristics to identify filamentous fungi, which deliver rapid and reliable results supported with relevant data on growth profiles. The system matched test strains with the strains in its data bank with similar biochemical profiles. The results of the Biolog are presented as a similarity (SM) value, of which 1.0 is 100% to confirm the identification of the four possibilities provided. Similarly, a 'No ID' was given for similarity values of < 0.5 and percentage match if between 0.5 and 0.9.

None of the 36 isolates had a 100% match to the species level using the Biolog MicroStation Identification system (Table 2.3); as per the Biolog protocol, comparing test species with the database species gave a similarity value of 1.0 as a match with confirmed species identification. Values between 0.5 and 0.9 provide a percentage identification of up to 99%. Values below 0.5 are species that were not identified as per the database of the Biolog Identification System. As an example, isolate 15 had a similarity value of 0.565 and three readings of 0.000 and thus a "no identification" confirmation, (Figure 2.10).

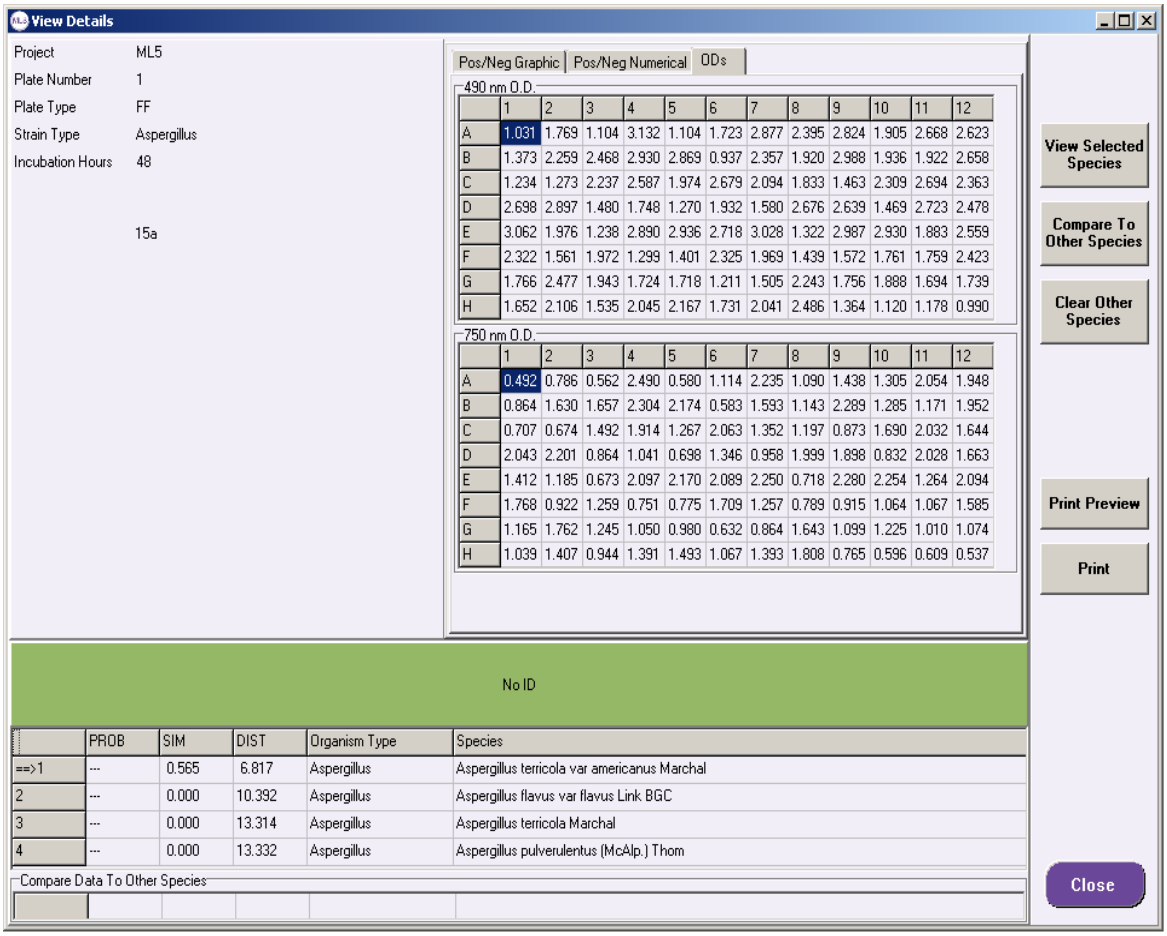


Figure 2.10: The FF plate for isolate 15 showing identification not confirmed at 48 hours. The data sheets of duplicate results for other isolates are in Appendix B.

Identification by the Biolog system matched most of the isolates to the genus level only, with similarity values of <0.1 and 1.0 being a full match. These matches were limited to strains carried in the Biolog database. Ten of the 26 *A. fumigatus* isolates had similarity values of < 0.1 and the remaining 16 had readings between 0.137 and 0.565, for a minimum of one and maximum of two species of the four possible matches. The species were *A. terricola* for 13 isolates and *N. ficheri*, *A. puniceus* and *A. flavus* for others. Isolate 36 was identified as *A. fumigatus* once only, but with a similarity value of 0.00. For the eight *A. niger* isolates, five of the eight isolates show one reading each as *A. niger* but with similarity values of < 0.090. *C. stromatoides, A. caespitosus, A. foetidus* and *A. brevipes* had similarity values between 0.130 and 0.187. The *A. flavus* strains had values of < 0.1, except for *A. terricola* (0.137 and 0.347). *A. flavus* is a possible species, but with similarity values of 0.000 and 0.002. Table 2.3 shows the confirmed results by morphological features and 18S rRNA against results obtained by the Biolog identification system. The Biolog identification of the isolates to species level was not successful.

Table 2.3: The identification results of the isolates by microscope and culture techniques, 18S rRNA and the Biolog Identification System. The colour key for species confirmed by 18S rRNA read as follows

Isolate	18S rRNA / Microscope and culture techniques	Biolog Identification (48 hr incubation)	Similarity 1.0 = 100%
1	*Aspergillus fumigatus*	*Neosartorya ficheri, Petromyces alliaceus, Aspergillus versicolor, Aspergillus clavatus, Aspergillus tamarii, Aspergillus parasiticus,*	< 0.1
2	*Aspergillus fumigatus*	*Neosartorya ficheri, Petromyces alliaceus, Aspergillus parasiticus, Aspergillus tamarii.*	< 0.1
3	*Aspergillus fumigatus*	*Neosartorya ficheri, Aspergillus tamarii, Aspergillus versicolor, Aspergillus puniceus, Petromyces alliaceus*	< 0.1
4	*Aspergillus fumigatus*	*Neosartorya ficheri, Petromyces alliaceus Aspergillus tamarii, Aspergillus versicolor, Aspergillus parasiticus*	< 0.1
5	*Aspergillus fumigatus*	*Neosartorya ficheri, Petromyces alliaceus Aspergillus tamarii, Aspergillus versicolor, Aspergillus parasiticus*	< 0.1
6	*Aspergillus fumigatus*	*Neosartorya ficheri, Petromyces alliaceus, Aspergillus puniceus, Aspergillus tamarii, Aspergillus parasiticus*	< 0.1
7	*Aspergillus fumigatus*	*Neosartorya ficheri, Petromyces alliaceus, Aspergillus tamarii, Aspergillus parasiticus, Aspergillus puniceus*	< 0.1
8	*Aspergillus fumigatus*	*Neosartorya ficheri, Petromyces alliaceus, Aspergillus tamarii, Aspergillus aureolatus, Aspergillus versicolor*	< 0.1
9	*Aspergillus fumigatus*	*Aspergillus parasiticus, Petromyces alliaceus, Neosartorya ficheri, Aspergillus tamarii, Chaetosartorya stomatoides.*	< 0.1
10	*Aspergillus niger*	*Aspergillus niger, Chaetosartorya stomatoides, Aspergillus ustus, Emericella variecola, Neosartorya ficheri*	< 0.1 *A. niger* = 0.006
11	*Aspergillus fumigatus*	*Petromyces alliaceus, Neosartorya ficheri, Aspergillus tamarii, Aspergillus parasiticus*	< 0.1
12	*Aspergillus niger*	*Aspergillus niger, Neosartorya ficheri, Aspergillus parasiticus, Aspergillus ustus, Chaetosartorya stomatoides, Aspergillus foetidus Emericella variecolor*	< 0.1 *A. niger* = 0.086 *C. stromatoides* = 0.138
13	*Aspergillus niger*	*Emericella striata, Emericella fruticulosa, Aspergillus wentii, Aspergillus phoenicis, Aspergillus brasiliensis, Emericella violacea, Aspergillus zonatus*	< 0.1
14	*Aspergillus fumigatus*	*Petromyces alliaceus, Neosartorya ficheri, Aspergillus tamarii, Aspergillus parasiticus, Aspergillus terricola, Aspergillus flavus*	< 0.1 *A. terricola* < 0.349

15	*Aspergillus fumigatus*	*Aspergillus terricola, Aspergillus flavus, Aspergillus pulverulentus, Chaetosartorya stomatoides, Aspergillus sydowii*	< 0.1 *A. terricola* = 0.565
16	*Aspergillus fumigatus*	*Aspergillus terricola, Aspergillus flavus, Aspergillus pulverulentus, Neosartorya ficheri, Aspergillus sydowii*	< 0.1 *A. terricola* = 0.371
17	*Aspergillus fumigatus*	*Aspergillus terricola, Aspergillus flavus, Neosartorya ficheri, Aspergillus sydowii*	< 0.1 *A. terricola* = 0.514
18	*Aspergillus niger*	*Aspergillus brasiliensis, Aspergillus kanagawaensis, Emericella striata, Aspergillus restrictus, Aspergillus zonatus, Emericella violacea*	< 0.1
19	*Aspergillus fumigatus*	*Aspergillus terricola, Aspergillus flavus, Neosartorya ficheri, Aspergillus versicolor, Chaetosartorya stomatoides*	< 0.1 *A. terricola* = 0.137
20	*Aspergillus fumigatus*	*Aspergillus puniceus, Chaetosartorya stomatoides, Aspergillus ustus, Aspergillus terricola, Aspergillus flavus, Aspergillus sydowii*	< 0.1 *A. puniceus* = 0.167
21	*Aspergillus niger*	*Aspergillus foetidus, Emericella variecolor, Aspergillus niger, Aspergillus brasiliensis, Emericella striata, Aspergillus ochraceus, Aspergillus carneus*	< 0.1 *A. foetidus* = 0.130 *A. niger* = 0.003
22	*Aspergillus flavus*	*Aspergillus terricola, Aspergillus flavus, Neosartorya ficheri, Aspergillus pulverulentus,*	< 0.1 *A. terricola* = 0.148 *A. flavus* = 0.002
23	*Aspergillus fumigatus*	*Aspergillus terricola, Aspergillus flavus, Chaetosartorya stomatoides, Aspergillus puniceus, Neosartorya ficheri, Aspergillus versicolor*	< 0.1 *A. terricola* = 0.407
24	*Aspergillus fumigatus*	*Aspergillus terricola, Aspergillus flavus, Neosartorya ficheri, Aspergillus puniceus Chaetosartorya stomatoides,*	< 0.1 *N. ficheri* = 0.186
25	*Aspergillus niger*	*Aspergillus foetidus, Aspergillus niger, Emericella variecolor, Aspergillus wentii, Aspergillus brevipes, Aspergillus carbonarius, Aspergillus brasiliensis, Chaetosartorya stomatoides*	< 0.1 *A. foetidus* = 0.116 *A. brevipes* = 0.145 *A. niger* = 0.015
26	*Aspergillus fumigatus*	*Aspergillus flavus, Neosartorya ficheri, Aspergillus terricola, Chaetosartorya stomatoides*	< 0.1 *A. flavus* = 0.350 *A. terricola* = 0.205
27	*Aspergillus fumigatus*	*Aspergillus terricola, Aspergillus flavus, Aspergillus puniceus, Aspergillus versicolor, Neosartorya ficheri, Aspergillus pulverulentus*	< 0.1 *A. terricola* = 0.240

28	*Aspergillus fumigatus*	*Aspergillus terricola, Aspergillus flavus, Aspergillus versicolor, Aspergillus pulverulentus, Neosartorya ficheri*	< 0.1 *A. terricola* = 0.332
29	*Aspergillus fumigatus*	*Aspergillus terricola, Aspergillus flavus, Aspergillus versicolor, Neosartorya ficheri, Aspergillus pulverulentus*	< 0.1 *A. terricola* = 0.238
30	*Aspergillus niger*	*Aspergillus niger, Emericella violacea, Aspergillus carbonarius, Aspergillus foetidus, Aspergillus brasiliensis, Emericella striata, Aspergillus zonatus, Aspergillus carbonarius*	< 0.1 *A. niger* = 0.008
31	*Aspergillus fumigatus*	*Aspergillus terricola, Aspergillus puniceus, Chaetosartorya stomatoides, Aspergillus flavus, Aspergillus ustus*	< 0.1 *A. terricola* = 0.296
32	*Aspergillus flavus*	*Aspergillus terricola, Aspergillus flavus, Neosartorya ficheri, Aspergillus pulverulentus, Aspergillus puniceus,*	< 0.1 *A. terricola* = 0.347 *A. flavus* =0.000
33	*Aspergillus niger*	*Aspergillus ustus, Aspergillus versicolor, Emericella violacea, Aspergillus sydowii, Aspergillus caespitosus, Aspergillus foetidus, Aspergillus variecolor*	< 0.1 *A. caespitosus* = 0.187
34	*Aspergillus fumigatus*	*Aspergillus terricola, Aspergillus flavus, Aspergillus puniceus, Neosartorya ficheri*	< 0.1 *A. flavus* = 0.162 *A. terricola* = 0.152
35	*Aspergillus fumigatus*	*Aspergillus terricola, Aspergillus flavus, Aspergillus pulverulentus, Chaetosartorya stomatoides, Aspergillus puniceus,*	< 0.1 *A. terricola* = 0.472
36	*Aspergillus fumigatus*	*Aspergillus puniceus, Neosartorya ficheri, Aspergillus ustus, Aspergillus flavus, Chaetosartorya stomatoides, Aspergillus fumigatus*	< 0.1 *A. puniceus* = 0.227 *N. ficheri* =0.247 *A. fumigatus* = 0.000

- *A. fumigatus*
- *A. niger*
- *A. flavus*

All thirty-six isolates from upper respiratory infections of immunocompromised patients were identified as *Aspergillus* species. Morphological techniques and 18S rRNA confirmed twenty-six isolates to be *A. fumigatus*, eight *A. niger* and two *A. flavus*, (Table 2.2). The results achieved by the conventional identification methods, viz. microscope and culture techniques, matched the identification confirmed by 18S rRNA. The Biolog identification system matched isolates to the genus level only. Seventy-two per cent of the *Aspergillus* isolates from the respiratory infections were *A. fumigatus*, with 22% *A. niger* and 6% *A. flavus*. *A. fumigatus* was the most prevalent *Aspergillus* species isolated from patients with upper respiratory infections.

2.4. Discussion

According to the 2003 American Society for Microbiology (ASM) Benchmarking Survey, 89% of laboratories performing mycological testing employ cultural techniques, 16% use serologic testing, and less than 5% use molecular diagnostics (McClenny 2005). This has recently evolved as more molecular testing tools have become available; nevertheless, phenotypic identification utilizing culture and microscopic techniques still serves a function and adds value in many circumstances, as illustrated by Aslam *et al.* (2017) and Lücking *et al.* (2020). Thus, improving morphological characteristics identification in stained smears of the specimen and growth rate can improve the laboratory contribution to rapid diagnosis without the capacity and infrastructure for molecular testing.

As shown in this study, media choice for growth could assist the phenotypic identification of *Aspergillus* species. The MEA supported faster growth of *A. fumigatus*, *A. niger* and *A. flavus* than PDA (Tables 2.4-2.6). MEA has a nutrient composition of 30% malt extract and 3% peptone, and PDA has 4% potato infusion and 20% glucose (Wu, Su and Ho 2000; Jaiswal and Kumar 2011). The combination of carbohydrate and protein sources seems to impact growth and population, as evident in MEA (Figs 2.4 -2.6). There were no issues of slow sporulation, unusual conidiophore formation, and atypical clinical aspergilli manifesting, which was observed by Balajee *et al.* (2007). The results are in conjunction with those of other authors who report that *A. fumigatus* has a rapid growth rate similar to other species like *A. flavus, A. niger, A. nidulans and A. terreus* that are significant in invasive aspergillosis (Hope, Walsh and Denning 2005). McClenny (2005) highlighted that although molecular methods continue to improve and become more rapidly available, microscopic and culture techniques remain commonly used and essential tools for identifying *Aspergillus* species.

All isolates showed faster growth rates at 37°C than at 30°C, demonstrating how this genus has adapted to body temperature and challenges infection control. Conidia as reproductive structures play a crucial role in the distribution and survival of fungi (Wyatt, Wösten and Dijksterhuis 2013) and *Aspergillus* conidia are relatively stress-resistant cells that survive environmental stresses such as drought and high temperatures (Jørgensen *et al.* 2011). The rapid growth and sporulation of clinical isolates support faster conidial germination in pathogenic species within 48 hours and should be considered in infection control. Araujo and Rodrigues (2004b) also showed that germination rates differed significantly among the most common pathogenic *Aspergillus* species, with *A. fumigatus* having the highest germination rate

and raised the concern of faster onset of tissue invasion due to faster germination and development of hyphae. The study also found that species with low pathogenicity demonstrated an optimal temperature for growth of around 30°C. According to Araujo and Rodrigues (2004b), environmental variables such as temperature influence the selection and promotion of pathogenic *Aspergillus* species, as is the case during the development of deep-seated infections. *A. fumigatus* is the most adaptive species to extreme changes in environmental circumstances and hence the most common species in respiratory illnesses.

Invasive infections by filamentous fungal species remain a significant cause of mortality in immunocompromised patients and those undergoing invasive procedures (Slavin *et al.* 2015; Bassetti and Bouza 2017). Mortality remains remarkably high, reaching 60% in ICU when admissions are delayed in case of invasive fungal infections (Mokart *et al.* 2016). Rapid identification of fungal isolates from clinical samples is essential, given their innate difficulty with treatment and the increasing number of emerging pathogenic species. Identification and diagnosing fungal infections pose many problems, including a decline in the expertise needed for identifying fungi and, at times, access to the specialised equipment required for specific fungal identification.

These problems are further compounded by the fact that patients with fungal infections are often immunosuppressed, which predisposes them to infections from both common and rarely-seen fungi (Wickes and Wiederhold 2018). These challenges centre on the need to quickly and accurately identify the infecting species from a growing list of fungal pathogens recovered from various patient types and clinical specimens. Time to diagnosis is one of the most critical risk factors for mortality from many systemic mycoses (Inácio *et al.* 2022). Thus, rapid diagnosis is a critical component of patient care. Unfortunately, phenotypic and biochemical identification methods are time-consuming, which has created an increasing demand for new molecular diagnostic methods for fungal identification. Thus, the identification of fungal pathogens has increasingly become dependent on modern molecular techniques, mainly based on PCR amplification of conserved regions of the genome and sequencing of the resulting PCR products.

It is estimated that more than a billion people worldwide have fungal infections (Bongomin *et al.* 2017), up to 30% being of an invasive nature (Denning 2017). Systemic mycoses continue to be a public health threat. Yet, over the past decades, considerable attention has been paid to improving the treatment and prevention of bacterial respiratory infections (Schwarz *et al.* 2018), which has led to a marked increase in life expectancy (Langan, Kotsimbos and Peleg 2015). However, microorganisms other than bacteria, including fungal species, populate the respiratory system, causing respiratory illnesses and reducing life expectancy, but have not received the same level of attention. This is conceivable because fungal infections, particularly filamentous fungal infections, appear insignificant compared to bacterial infections. However, fungal infections significantly impact mortality and morbidity in immunocompromised individuals and are further challenged by an ever-increasing number of immunosuppressed adults (Harpaz, Dahl and Dooling 2016; Azoulay *et al.* 2017).

This study used the traditional methods of culture and microscopic examination of morphological features and 18S rRNA, as well as a high throughput phenotypic/biochemical identification system to identify the clinical isolates. Traditional methods for diagnosing aspergillosis and other mycoses are supplemented by molecular and immunologic approaches (Arastehfar *et al.* 2019). Although molecular methods continue to improve and become more readily available, microscopy and culture techniques remain the primary laboratory tools for initial identification. Molecular and immunological tests may promise a more rapid and accurate laboratory diagnosis of aspergillosis; however, microscopy and culture are still common and essential tools (McClenny 2005). Molecular identification has gained reliability and confidence in results to earn the status of ‘gold standard’ (Borman *et al.* 2008). Still, the cost and affordability are factors that impact access to this technique of identification and thus, other phenotypic and serologic techniques can be used purposefully (Arastehfar *et al.* 2019).

The lack of experience and equipment required to quickly diagnose an infectious fungus, particularly one that is usually uncommon, is a concern in medical mycology. This is primarily a concern in developing countries where the disease burden is much higher. The solution to this problem is to improve diagnostic platforms to identify infecting fungi quicker and with better accuracy and precision, but in the search for new technologies of diagnostic value, training in mycology for fungal identification based on morphological and phenotypic characteristics is further depleted. In this study, the molecular identification results concurred with the preliminary conventional phenotypic identification. Conventional identification proved successful; however, this was a small number of isolates compared to the vast diversity

of fungal species that are capable of causing diseases in humans. Thus, clinical mycologists competent in identification are in high demand and should not be disregarded. This identification method serves a purpose when the cost or availability of resources is challenging in considering other diagnostic assays, such as molecular techniques. Molecular approaches may appear expensive, and phenotypic identification may raise reliability issues, yet both may be utilized purposefully and reliably.

Molecular methods for fungal identification using 18S rRNA and sequencing of conserved regions of the fungal genome allow unambiguous identification of filamentous fungal species that cannot be identified with classical mycological techniques (Tekpinar and Kalmer 2019). Species identification based on conventional phenotypic methods is often time-consuming and is hindered by the unstable and subjective nature of phenotypic characteristics, which are readily influenced by culture conditions (Borman *et al.* 2008). Conversely, molecular methods are frequently more rapid than phenotypic approaches and can discriminate between fungi that fail to produce distinctive morphological features.

However, over-reliance on molecular techniques is a concern in cases where there is no accepted rule on the number of nucleotide differences in rRNA genes required to define different species or genera. Also, doubt in the accuracy of the true identities of a proportion of sequences submitted to public, searchable databases is another challenge (Borman *et al.* 2008). Capacity and the availability of equipment and software required for molecular techniques pose further challenges in developing countries (Oladipo *et al.* 2020). In these circumstances, identification becomes reliant on affordable phenotypic methods, a means rather than a choice and thus cannot be written off with the development and success of new technologies. In this study, all thirty-six isolates' phenotypic data correlated with the results of molecular identification and thus, this correlation between the results of molecular techniques and phenotypic features emphasizes the success and potential of the different identification techniques.

This study also explored the relatively new technology from Biolog in the quest for a reliable and rapid alternative in fungal identification, especially where the identification of human pathogens is essential. The Biolog is a broad-based rapid identification system designed for filamentous fungi and yeasts, including species from the genera *Aspergillus*. The filamentous fungi database contains over 400 taxa of fungi from 120 genera and thus provides one of the largest databases of any kit-based identification system for filamentous fungi. The database

includes clinically significant, allergenic and mycotoxigenic fungi. The Biolog system is a modern technology claiming reliable results with supporting data (Mackie *et al.* 2014).

However, in this study, the Biolog system's species identification did not match the species level identification achieved using 18S rRNA, which however correlated at the genus level with the conventional culture and microscope identification. This is likely due to the database not including the clinical *Aspergillus* strains isolated at the time of this study. Although the Biolog database carries clinically related *Aspergillus* species, the database, most likely at the time of use, did not have closely associated clinical species with the same biochemical pattern and thus revealed low and insignificant similarity readings. The Biolog system could have since been vastly increased to include more clinical and environmental strains. Most isolates were matched but only at the genus level, which is not useful for clinical settings (Table 2.3). The limitation or drawback in this system for identifying the isolates was evident in this study that the system is only good as its reference database for effective use and assumes a fixed biochemical pattern which is not in biological systems. This system and the procedure for species identification are relatively simple and rapid and would have been of value had the database included more relevant clinical *Aspergillus* species and thus serves the purpose of reliable identification in a short period. However, the Biolog system with an expanded database can provide another option for identifying filamentous fungi where molecular methods capacity is unavailable.

This study investigated different identification techniques to enable the effective diagnosis of respiratory infections and subsequent treatment. Each of the identification techniques used could be applied under different conditions. Classical mycological procedures based on phenotypic traits should continue to develop expertise to enable correct identification where molecular techniques and other modern equipment-based techniques are not available. However, molecular techniques like 18S rRNA here proved reliable and consistent but require specialized and advanced equipment and software that are sometimes not accessible in the developing world. Cost becomes a factor in considering isolates could be sent externally for sequencing. The Biolog system is a simple and quick identification technique that relies on a database; nevertheless, the system must be able to incorporate new species into the diagnostic capabilities and be continuously updated.

CHAPTER THREE: GROWTH AND NUTRIENT PROFILE ANALYSIS OF *ASPERGILLUS* ISOLATES

3.1. Introduction

The rise in the number of immune-compromised individuals, primarily among HIV/AIDS patients and advances in medical treatment, such as organ transplants and chemotherapies that suppress the immune system, has increased the number of patients susceptible to invasive fungal diseases (Beattie *et al.* 2017). The most common filamentous fungi isolated from patients with such infections are those belonging to the genus *Aspergillus,* and more specifically, the environmental mould *Aspergillus fumigatus*, the causative agent of invasive aspergillosis (IA) (Beattie *et al.* 2017). The mortality from IA remains high, thus making the need to understand *Aspergillus* pathogenesis mechanisms to uncover new therapeutic targets and strategies. It has been hypothesized that metabolic flexibility contributes to *Aspergillus* species becoming virulent. The microbes employ mechanisms to regulate and optimize nutrient acquisition and metabolism to survive in different environments, including human tissue (Brock 2009; Amich and Krappmann 2012). Growth profiles revealing patterns of nutrient acquisition would shed light on this theory which impacts infection maintenance and progression of the disease by *Aspergillus* species.

Filamentous fungi possess many unique biochemical pathways to assimilate a vast array of available substrates to produce a variety of metabolites (Singh 2009). They can utilise a battery of carbon sources, as in the case of *A. niger*, using different enzymes that degrade a vast diversity of organic compounds (Schuster *et al.* 2002). HAMAD *et al.* (2014) explored sugar uptake in the filamentous fungus *A. niger*, as a sole carbon source. They vary in their ability to use the supplied carbon sources, showing higher affinity to less complex carbon sources like glucose and maltose. Also, Mogensen *et al.* (2006) found that *Aspergillus nidulans* could adjust its carbon catabolism depending on available substrates. When grown on substrates containing metabolically favourable carbon sources (such as glucose) and less favourable types (such as ethanol), *A. nidulans* could upregulate or repress certain genes to be able to utilise the less favourable carbon sources using a mechanism known as carbon catabolite repression (CCR) or carbon repression. Adnan *et al.* (2017) explain that the CCR mechanism allows for preferential utilization of an energy-efficient and readily available carbon source over relatively less easily accessible carbon sources. This mechanism helps microorganisms to obtain the maximum amount of energy efficiently to keep pace with their metabolism.

In certain pathogenic organisms like *A. fumigatus*, the organism's pathogenicity and its ability to continue the progression of the disease within the host is based on CCR and helps in the metabolic plasticity of the fungus that allows it to adapt to changes in nutrient availability and nutrient depletion, including adapting to hypoxic conditions (Beattie *et al.* 2017). Wu *et al.* (2020) found that aspergilli are initially more selective but can use a wide range of carbon sources if necessary and may have evolved to favour adaptation to the environmental niches the fungi occupy.

Thus, this study analysed the carbon profile of the *Aspergillus* isolates isolated from patients with upper respiratory infections to relate growth and nutrient patterns to their adaptability to the maintenance and progression of diseases. Conventional nutrient analysis involving numerous biochemical tests is highly time-consuming in preparation and sometimes requires complex procedures. The Biolog Phenotypic Microarray (PM) system allowed for the testing of fungi against many different carbon sources simultaneously (Pinzari *et al.* 2016). This advanced new technology can generate a vast amount of relevant data with reliable results. The Biolog PM system is a technique that scans and measures the energy metabolism pathways present in a wide range of cell types, including fungi. The metabolic pathway activities are assayed with a colourimetric reagent that measures redox energy produced by a cell oxidizing a carbon source (Bochner, Gadzinski and Panomitros 2001).

The Biolog microplates for filamentous fungi (FF) rely on the iodonitrophenyl tetrazolium redox dye, which can detect respiration (NADH formation) on sole carbon sources with minimal supplementation. In the FF plate, 95 wells contain the individual substrates and the dye, while one well (control well) contains the dye alone (Figure 3.1). The content of the wells is dehydrated; the medium is rehydrated when the spore sample in an aqueous solution is added. The principle of this assay is, in fact, the use of a tetrazolium dye (tetrazolium violet), which is reduced by the action of succinate dehydrogenase, enabling the quantification of respiration. Reduction of this dye results in the formation of varied purple colouration with maximum absorbance at 490 nm for plates and is used to deduce the assimilation of the compounds into the cell. Additionally, optical density at 750 nm is used to measure cell density (Tanzer *et al.* 2003) and more accurately quantifies growth based on mycelial production (Tanzer *et al.* 2003; Atanasova and Druzhinina 2010b).

Phenotypic microarray technology was developed to provide scientists with a simple and efficient way to test nearly 2000 phenotypes of a microbial cell and gain a comprehensive overview of pathway functions in a single experiment. The phenotypic assays are designed from a physiological perspective to survey, *in vivo*, the function of diverse pathways, including metabolic and regulatory pathways. Included in the tests are basic cellular nutritional pathways for C, N, P, and S metabolism, pH growth range and regulation of pH control, sensitivity to NaCl and various other ions, as well as chemical agents that disrupt various biological pathways (Bochner, Gadzinski and Panomitros 2001; Mchunu *et al.* 2013; Martín *et al.* 2019). Phenotypic microarray technology allows over 1000 cellular traits to be measured simultaneously and accurately (Borglin *et al.* 2012). The PM technology FF MicroPlate is specifically designed for testing carbon utilization in filamentous fungi and yeast, including species from the genus *Aspergillus.*

This study focuses on using phenotypic microarray to establish the ability of the 36 *Aspergillus* isolates (from patients with respiratory infections from the Inkosi Albert Luthuli Hospital in Durban, Kwa-Zulu Natal. to assimilate and grow different carbon sources. Monosaccharides and disaccharides were selected for in-depth analysis as the literature reveals the foremost degradation of the respective sugars by *Aspergillus* species and also produces more significant growth than amino acid derivatives and sugar alcohols.

3.2. Methodology

3.2.1. Sample preparation

The thirty-six filamentous fungal samples isolated from infected respiratory patients from the Inkosi Albert Luthuli Hospital between July 2015 and June 2016 were cultured and maintained as described in Chapter 2, Section 2.2.1(Chapter 2). Isolates were grown on malt extract agar plates (MEA) (Merck) and incubated at 37°C for 72 hours. Spore suspensions were prepared using sterile distilled water and stored at -70℃.

3.2.2. Phenotypic profile: Biolog

The isolates were first inoculated (one plaque) on malt extract plates for 72 hours at 37°C. The spores were then removed from the surface of the agar plates using a sterile swab by gently rubbing across the surface and transferred into the sterile inoculating fluid (FF-IF, Biolog). The turbidity of the suspension was measured (Biolog turbidimeter) until it reached a density of approximately 75% transmittance, according to the manufacturer's (Biolog) protocol. The Biolog FF panel was used to evaluate the carbon profile of filamentous fungal isolates. Each plate of the FF panels contains 96 wells (95 tests and one water as a control) with a different compound (Figure 3.1). One- hundred microlitres of fungal inoculum were transferred into each well of duplicate PM plates. The inoculated PM plates were first taken from zero hours before incubation of PM plates at 37°C for 96 hours. Data were collected at 12-hour intervals for the 96-hour incubation and results were read on the Microlog.

The Biolog FF microplate performs 95 individual tests simultaneously on the compounds shown in Figure 3.1. Figure 3.2 shows colour development in the FF microplate after incubation which indicates substrate intake measured at 450 nm and growth measured at 750 nm using the Microstation. The microplate readings give a characteristic "fingerprint" reaction pattern (Figure 3.3). The fingerprint reaction patterns provide a vast amount of information about the metabolic properties of each fungus tested.

A1 Water	A2 Tween 80	A3 N-Acetyl-D-Galactosamine	A4 N-Acetyl-D-Glucosamine	A5 N-Acetyl-D-Mannosamine	A6 Adonitol	A7 Amygdalin	A8 D-Arabinose	A9 L-Arabinose	A10 D-Arabitol	A11 Arbutin	A12 D-Cellobiose
B1 α-Cyclodextrin	B2 β-Cyclodextrin	B3 Dextrin	B4 i-Erythritol	B5 D-Fructose	B6 L-Fucose	B7 D-Galactose	B8 D-Galacturonic Acid	B9 Gentiobiose	B10 D-Gluconic Acid	B11 D-Glucosamine	B12 α-D-Glucose
C1 Glucose-1-Phosphate	C2 Glucuronamide	C3 D-Glucuronic Acid	C4 Glycerol	C5 Glycogen	C6 m-Inositol	C7 2-Keto-D-Gluconic Acid	C8 α-D-Lactose	C9 Lactulose	C10 Maltitol	C11 Maltose	C12 Maltotriose
D1 D-Mannitol	D2 D-Mannose	D3 D-Melezitose	D4 D-Melibiose	D5 α-Methyl-D-Galactoside	D6 β-Methyl-D-Galactoside	D7 α-Methyl-D-Glucoside	D8 β-Methyl-D-Glucoside	D9 Palatinose	D10 D-Psicose	D11 D-Raffinose	D12 L-Rhamnose
E1 D-Ribose	E2 Salicin	E3 Sedoheptulosan	E4 D-Sorbitol	E5 L-Sorbose	E6 Stachyose	E7 Sucrose	E8 D-Tagatose	E9 D-Trehalose	E10 Turanose	E11 Xylitol	E12 D-Xylose
F1 γ-Amino-butyric Acid	F2 Bromosuccinic Acid	F3 Fumaric Acid	F4 β-Hydroxy-butyric Acid	F5 γ-Hydroxy-butyric Acid	F6 p-Hydroxyphenyl-acetic Acid	F7 α-Keto-glutaric Acid	F8 D-Lactic Acid Methyl Ester	F9 L-Lactic Acid	F10 D-Malic Acid	F11 L-Malic Acid	F12 Quinic Acid
G1 D-Saccharic Acid	G2 Sebacic Acid	G3 Succinamic Acid	G4 Succinic Acid	G5 Succinic Acid Mono-Methyl Ester	G6 N-Acetly-L-Glutamic Acid	G7 Alaninamide	G8 L-Alanine	G9 L-Alanyl-Glycine	G10 L-Asparagine	G11 L-Aspartic Acid	G12 L-Glutamic Acid
H1 Glycyl-L-Glutamic Acid	H2 L-Ornithine	H3 L-Phenylalanine	H4 L-Proline	H5 L-Pyroglutamic Acid	H6 L-Serine	H7 L-Threonine	H8 2-Amino Ethanol	H9 Putrescine	H10 Adenosine	H11 Uridine	H12 Adenosine-5'-Monophosphate

Figure 3.1 Carbon Sources in FF microplate (www.oxoid.com/pdf/biolog/FFMP.pdf)

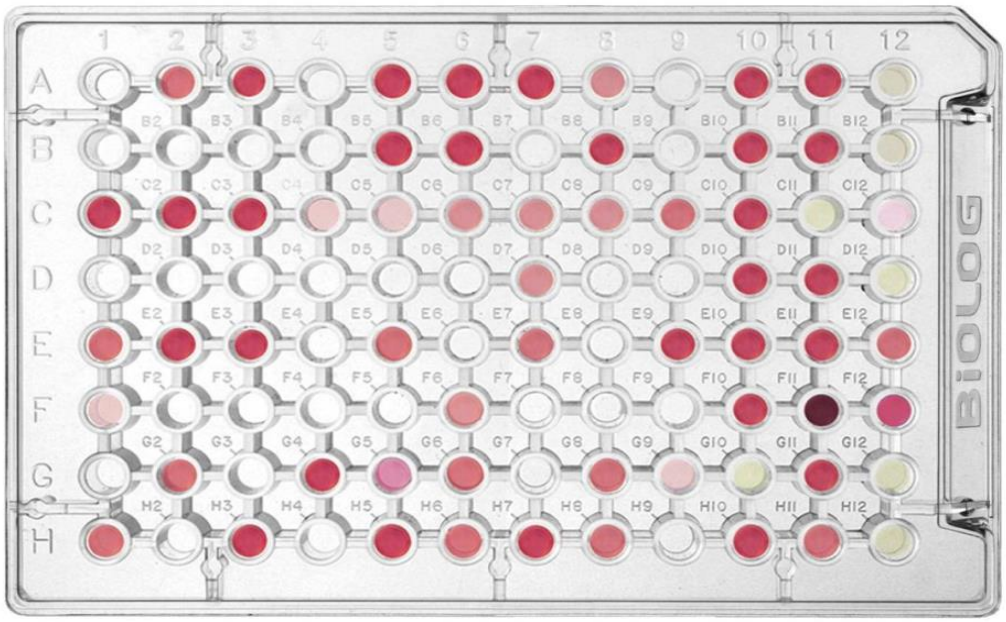


Figure 3.2: Biolog FF microplate showing colour reactions after incubation and growth (www.oxoid.com/pdf/biolog/FFMP.pdf)

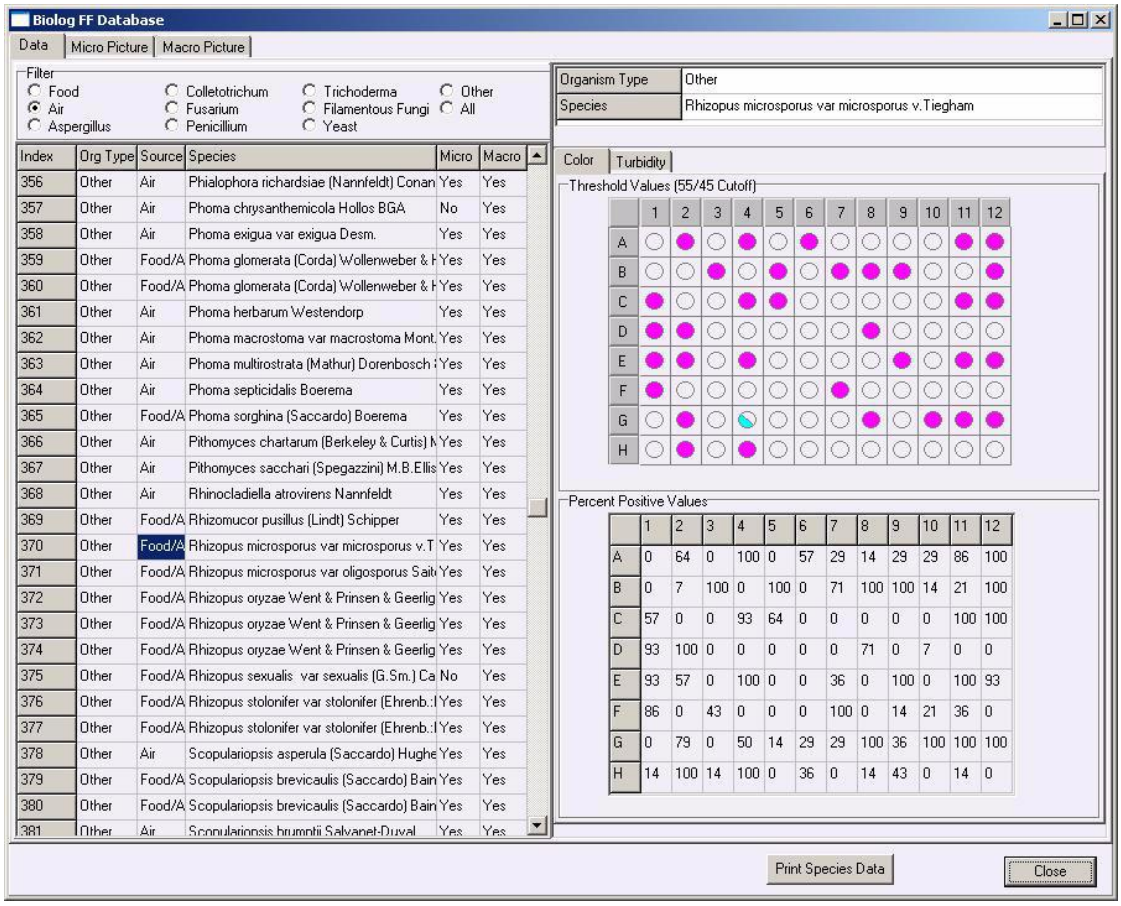


Figure 3.3: The colour change in the FF microplate as shown on the top right under Turbidity with the corresponding absorbance (750 nm) reading at the bottom right under Percent Positive Value.

3.3. Results

3.3.1. Carbon profile

The carbon profile of the *Aspergillus* isolates was assessed using the Biolog FF microplate to analyse the patterns of nutritional acquisition and utilisation for the growth of the clinical isolates. The test panels contained 95 compounds, each with one empty well, where water was added as a control. The assimilation of the compounds is recorded when the colour of the dye changes once inside the cell and the growth is measured at an absorbance of 750 nm as a function of turbidity. The results were grouped for monosaccharide and disaccharide compounds. The other compounds (amino acid derivatives and sugar alcohols) in the panel were not used for detailed analysis due to the low assimilation and growth induction, however, they are reported in the appendix section.

Most monosaccharides and disaccharides had considerably good absorbance, measured at around 2.0. The majority of isolates went into the stationary phase at this stage after reaching this level which could be a measure of substrate exhaustion. The absorbance of the control was consistent. The growth of most isolates reached the stationary phase between 48 and 60 hours. However, a few isolates were still in the log phase with certain compounds after 72 hours.

3.3.2. Monosaccharide

3.3.2.1. *Aspergillus fumigatus*

Most compounds with the log phase of the *A. fumigatus* isolates began at around 12 hours of growth for monosaccharides, reaching optimal growth by 48 hours, and then entering the stationary phase (Figures 3.4 and 3.5). This was the normal trend for all *A. fumigatus* isolates (Appendix C for the growth profiles of the remaining isolates). The monosaccharides, D-glucosamine, N-acetyl-D-galactosamine, Sedoheptulosan, D-fructose and D-tagatose consistently showed absorbance above 2.0 between 36 and 48 hours. This confirms the preferred compounds of *A. fumigatus* as optimum assimilation was recorded as early as 36 hours in some instances. N-acetyl-D-galactosamine, an amide derivative of glucose and D-glucosamine, an amino sugar synthesised from glucose and glutamine, both are absorbed within 24 hours and reaffirm glucose as being the substrate rapidly used up by *Aspergillus* species before other carbons. The monosaccharides β – methyl -D-galactoside, α – methyl – D- glucoside and L-sorbose showed absorbance close to 2.0 and above between 48 and 60 hours. L-fucose consistently showed a sharp increase in absorbance from 48 to 72 hours, with

absorbance readings reaching close to 2.5. This continued use of alternate monosaccharides after the preferred substrates ensures the initial establishment of infection and then disease progression.

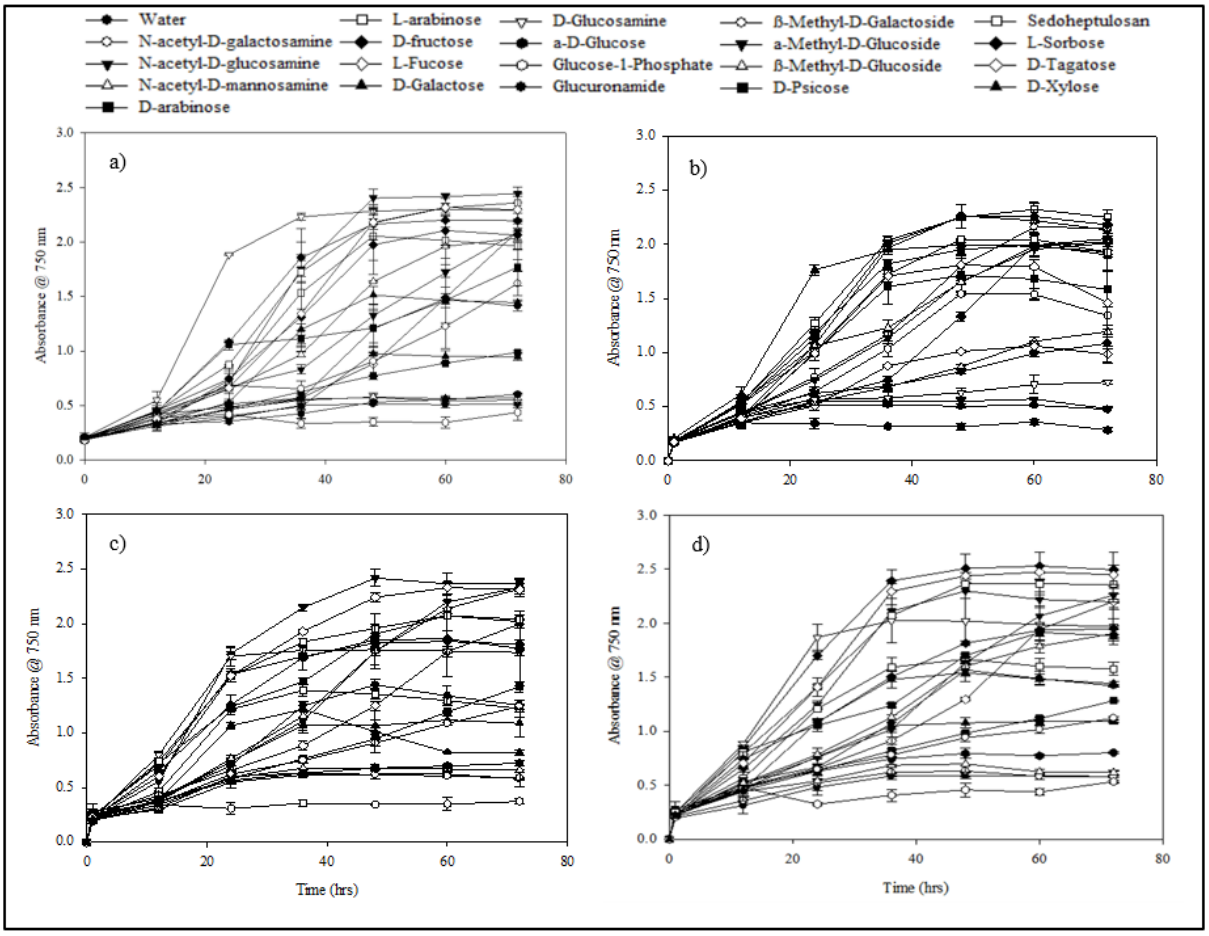


Figure 3.4: Growth of *A. fumigatus* (isolates 1-4) [a-d] in monosaccharide sugars. The growth was measured in absorbance of 750 nm for 72 hours at 12 hours intervals (n=26).

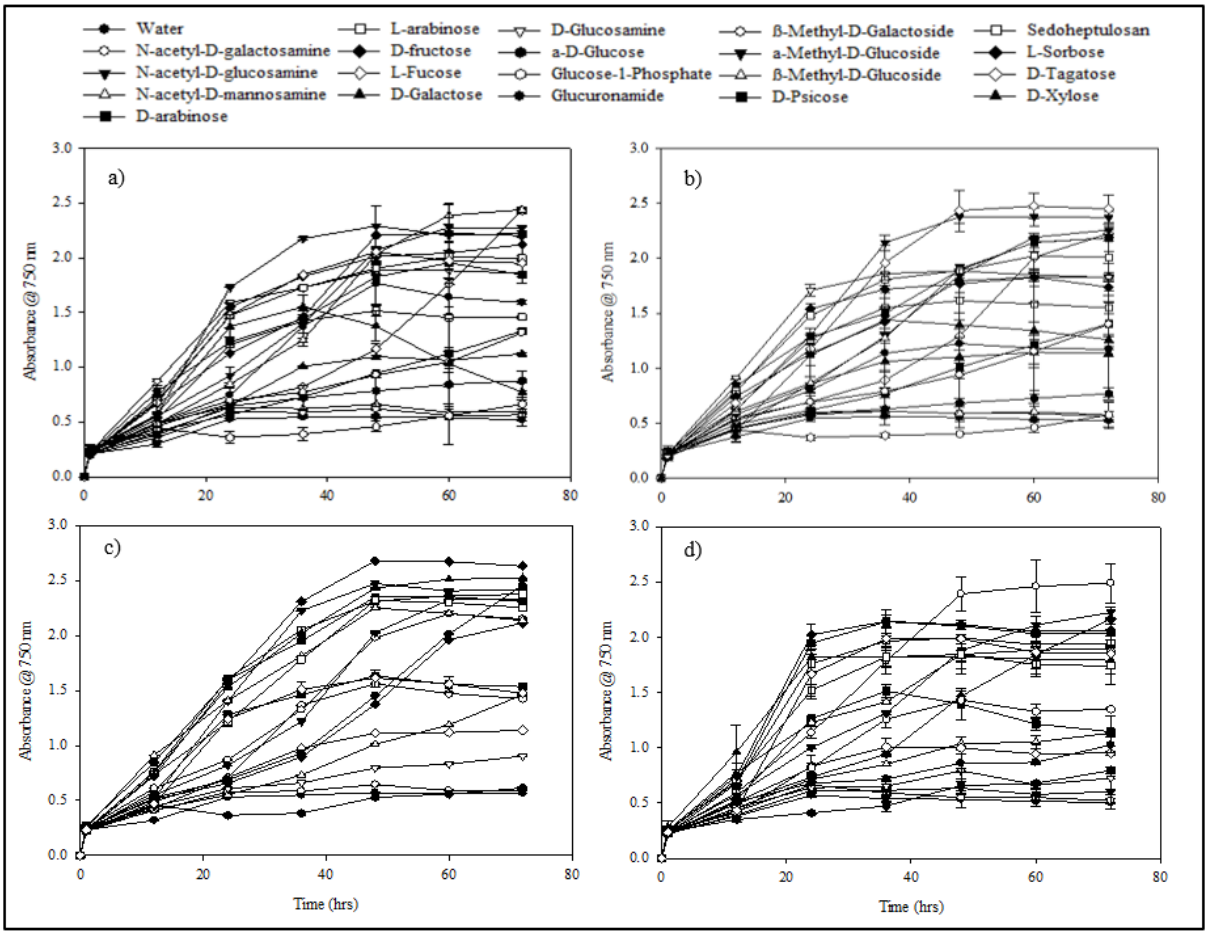


Figure 3.5: Growth of *A. fumigatus* (isolates 5-8) [a-d] in monosaccharide sugars. The growth was measured in absorbance of 750 nm for 72 hours at 12 hours intervals (n=26).

3.3.2.2. *Aspergillus niger*

The log phase for the *A. niger* isolates commenced at about 24 hours of growth for monosaccharides and reached optimum growth by 48 hours; however, absorbance by the monosaccharides was above 2.0 only for D-fructose consistently, and D-glucosamine for 50% of the isolates (n=8) (Figures 3.6 and 3.7). Sedoheptulosan, α-methyl-D-glucoside, L-arabinose, and D-psicose, revealed absorbance between 1.5 and just over 2.0 but mostly by 60 hours. D-tagatose showed increased absorbance from 36 hours but was incredibly steep from 48 to 60 hours. *A. niger* also prefers glucose-based substrates such as D-glucosamine (synthesised from glucose) and D-Fructose (glucose isomer). However, the *A.niger* isolates have not used monosaccharides as rapidly as *A. fumigatus* species, with a 12-hour delay in the commencement of the log phase with *A. niger* species. This species also assimilates other monosaccharides viz. Sedoheptulosan, α-methyl-D-glucoside, L-arabinose, D-psicose and D tagatose into the stationary phase and possibly the reason for establishing infection.

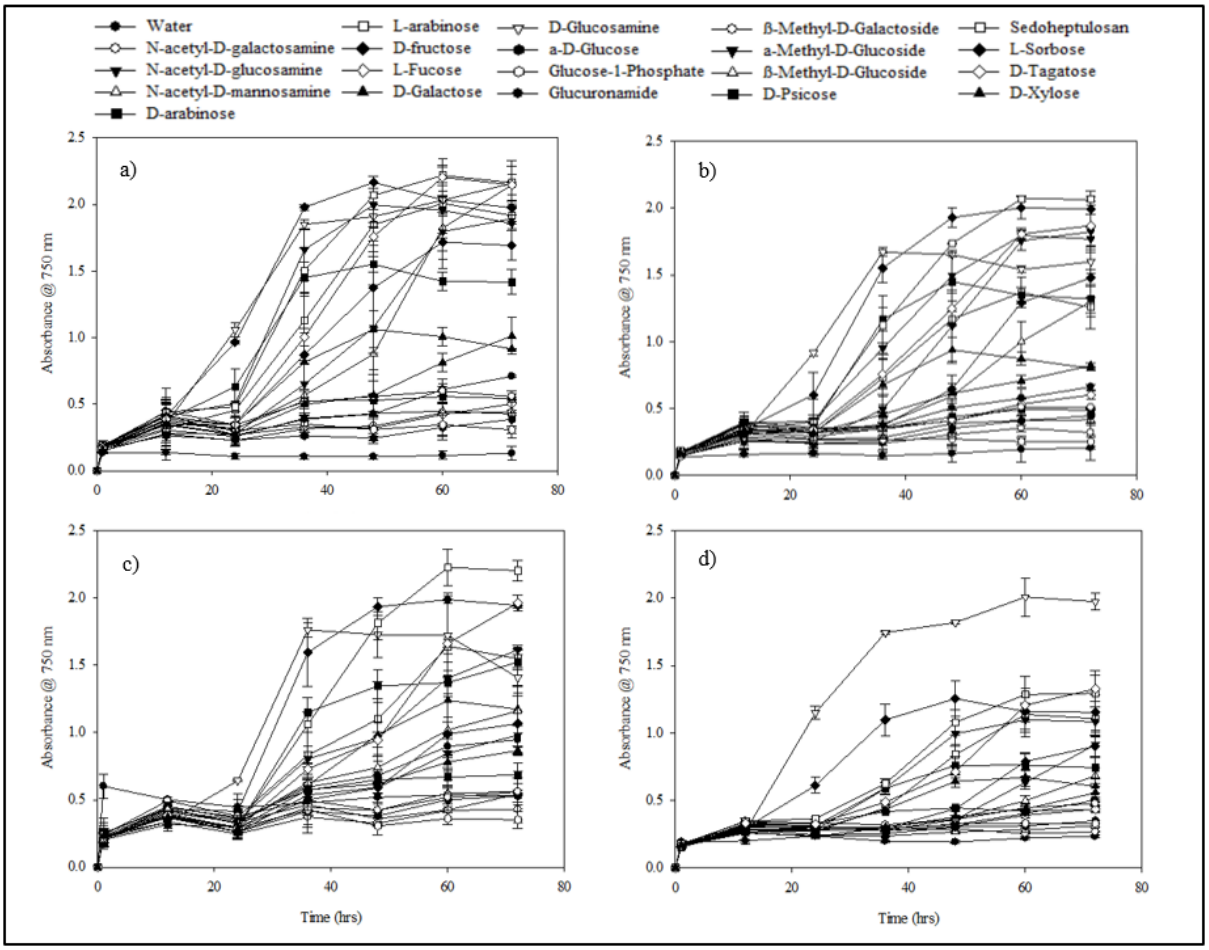


Figure 3.6: Growth of *A. niger* (isolates10, 12, 13, 18) [a-d] in monosaccharide sugars. The growth was measured in absorbance of 750 nm for 72 hours at 12 hours intervals (n=8).

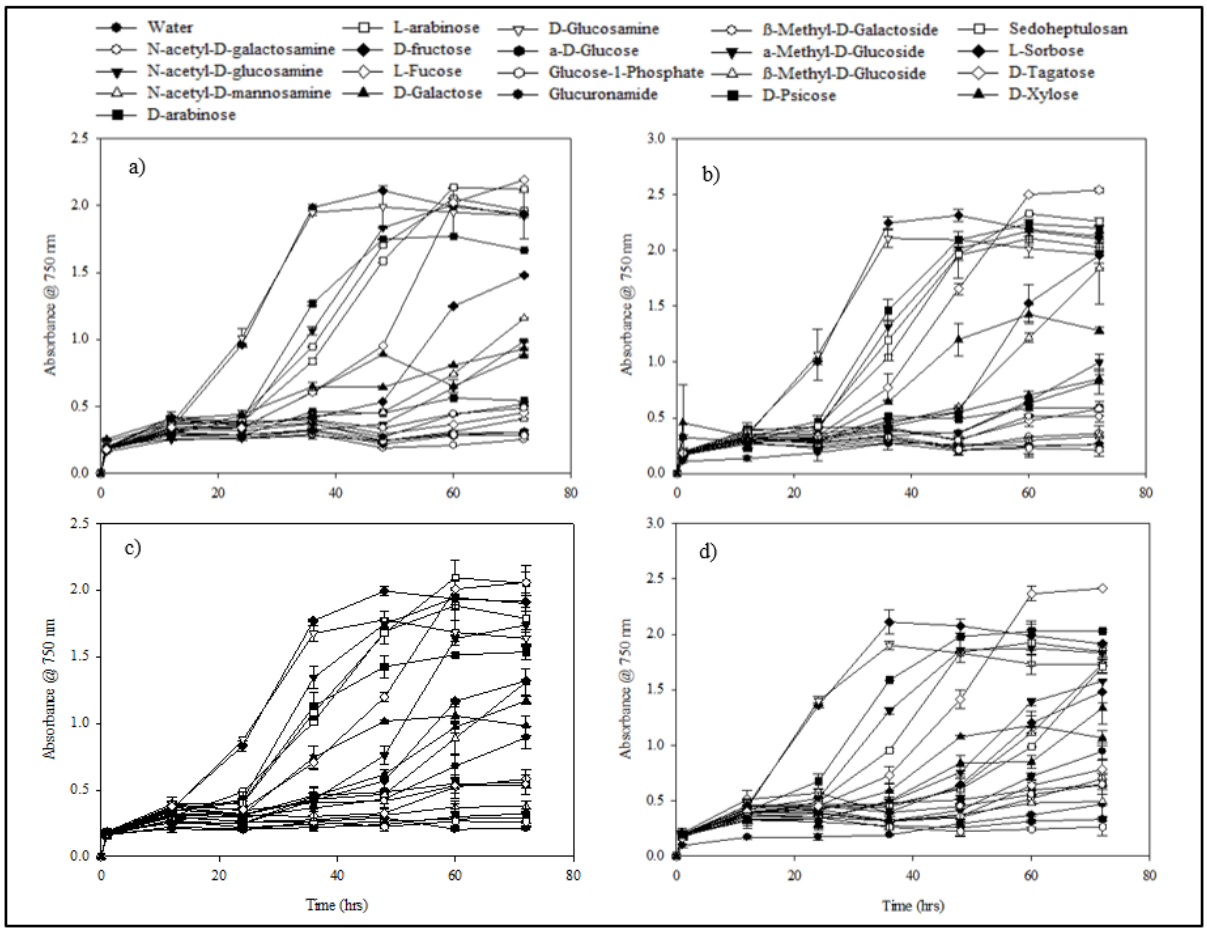


Figure 3.7: Growth of *A. niger* (isolates 21, 25, 30, 33) [a-d] in monosaccharide sugars. The growth was measured in absorbance of 750 nm for 72 hours at 12 hours intervals (n=8).

3.3.2.3. *Aspergillus flavus*

The log phase in *A. flavus* isolates (22 and 32 shown here) commenced at about 12 hours of growth and reached optimum growth by 48 hours. The monosaccharides, sedoheptulosan, D-psicose and β – methyl-D- glucoside, were common in both isolates, with absorbance between 1.5 and 2.09 by 48 hours. The log phase is comparable to *A. fumigatus* species, but the monosaccharides initially assimilated by the two species are not common to both species. The two *A. flavus* isolates, although having differences in absorbance, show consistent assimilation of the monosaccharides resulting in all compounds reaching the stationary phase at 72 hours. There are no late surges in the absorbance of monosaccharides, as seen with *A. flavus* assimilation of disaccharides and with *A. fumigatus* and *A. niger* for both monosaccharide and disaccharide assimilation.

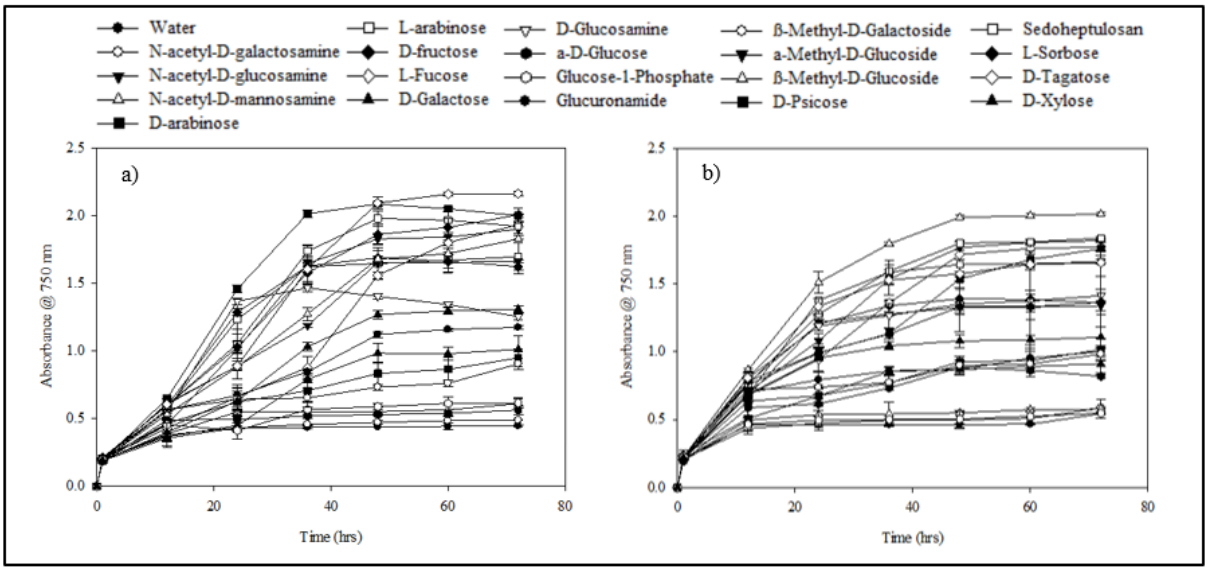


Figure 3.8: Growth of *A. flavus* (isolates 22 and 32) [a-d] in monosaccharide sugars. The growth was measured in absorbance of 750 nm for 72 hours at 12 hours intervals (n=2).

3.3.3. Disaccharide

3.3.3.1. *Aspergillus fumigatus*

The disaccharide assimilation growth profile of *A. fumigatus* isolates was similar to that of the monosaccharide assimilation growth profile of the species+, generally commencing around 12 hours of growth for disaccharides; however, with a slightly lower absorbance than monosaccharides reaching maximal growth at 48 hours **(**Figure 3.9 and 3.10). This was the typical pattern for all *A. fumigatus* isolates for the remaining isolates (Appendix C). Trehalose showed an absorbance of over 2.0 by 48 hours, followed by sucrose, maltose, gentiobiose, turanose, cellobiose and α-D- lactose showing absorbance close to 2.0 and above from 48 to 72 hours. Most disaccharides assimilation was comparable to monosaccharides by *A. fumigatus* strains with absorbance above 2.0 by 48 hours. This ability to assimilate a wide range of substrates reaffirms this species' persistence in colonizing and causing infection. Turanose and α-D-lactose, after a 12-hour lag phase, displayed surges in absorbance between 36 hours and as late as 60 hours, while most disaccharides reached the stationary phase by 48 hours. These compounds were still rising in absorbance after 72 hours. Isolate 1(a) and 5(a) had a sharp increase in absorbance at 60 hours for α-D-lactose and turanose, respectively, 12 hours after the commencement of the stationary phase for the majority of the compounds. This demonstrates that *A. fumigatus* also can and does shift to alternate carbon sources in circumstances of depleting nutrients.

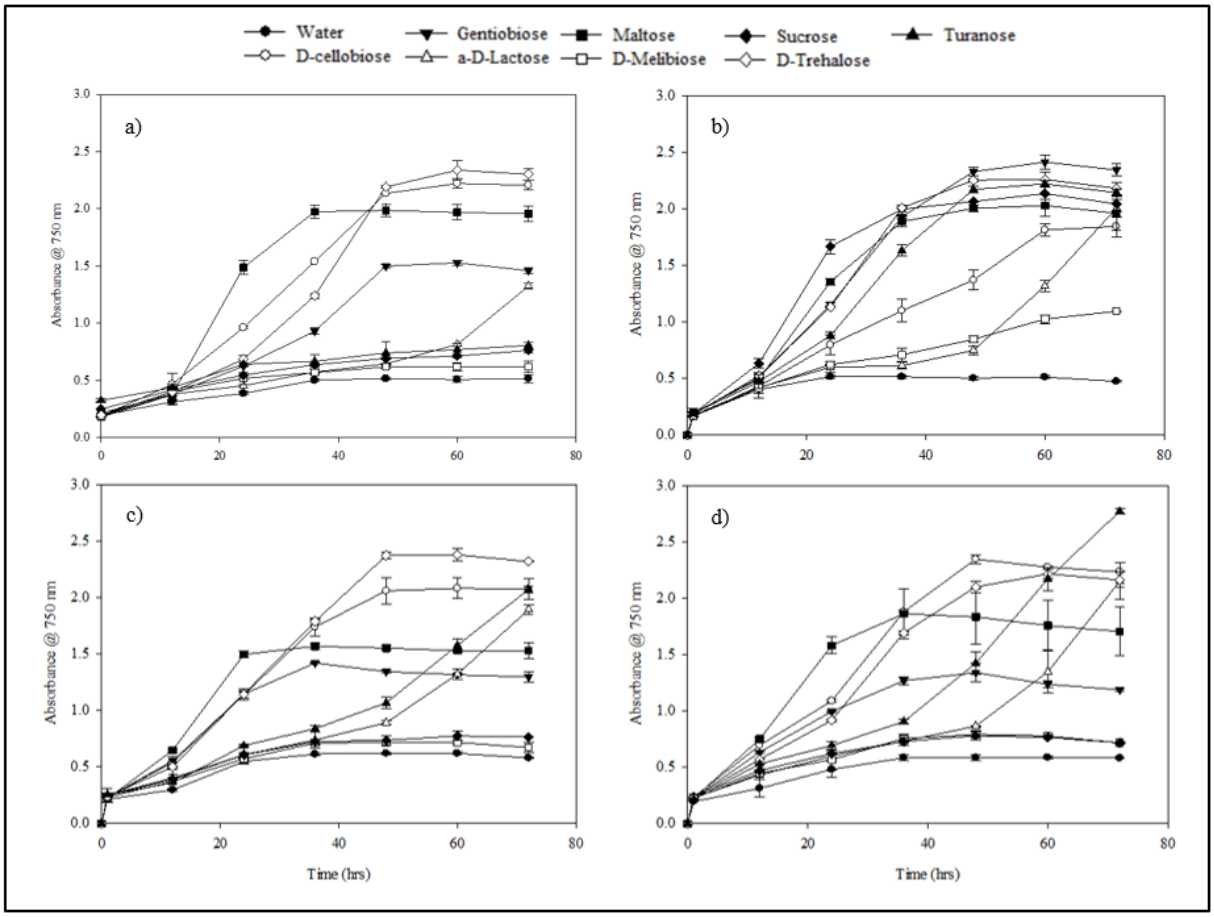


Figure 3.9: Growth of *A. fumigatus* (isolates 1-4) [a-d] in disaccharide sugars. The growth was measured in absorbance of 750 nm for 72 hours at 12 hours intervals (n=26).

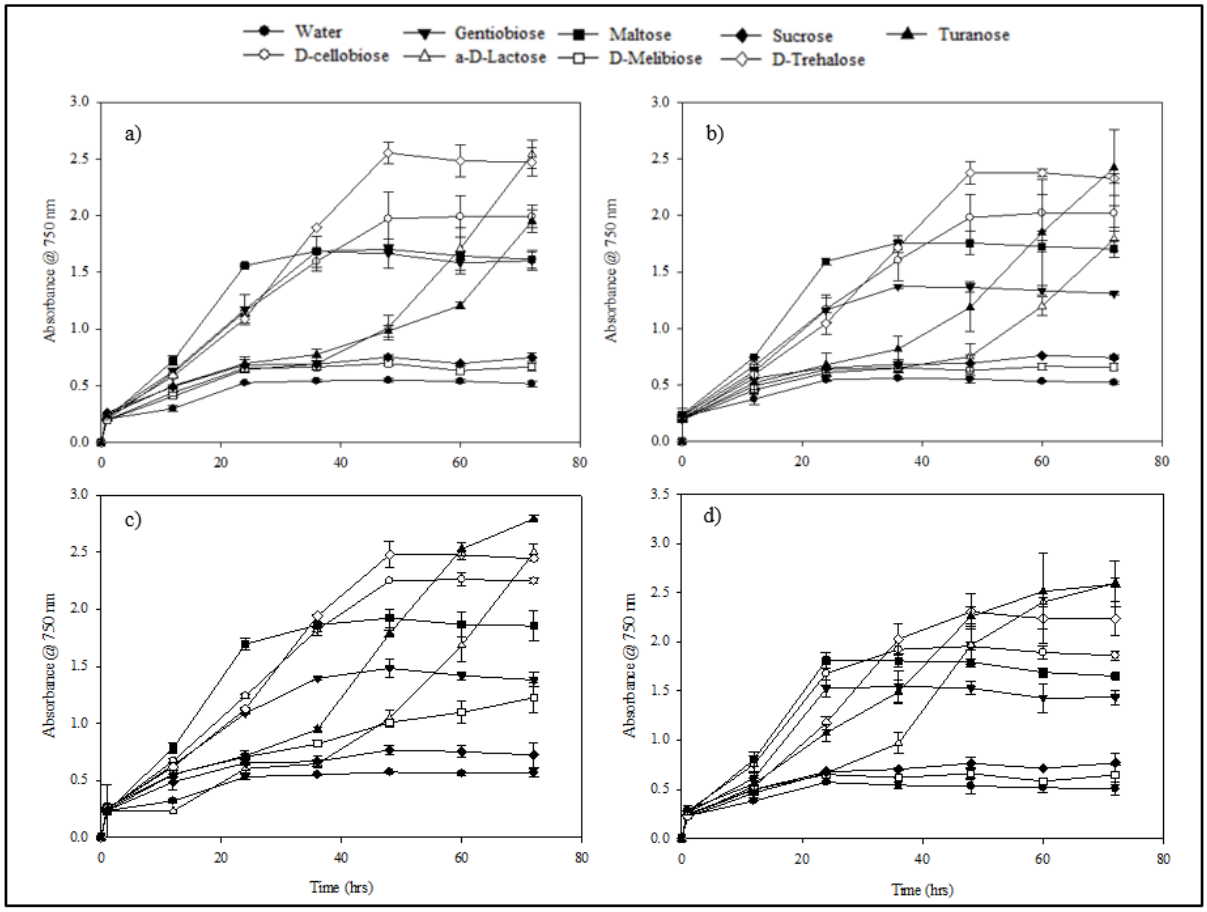


Figure 3.10: Growth of *A. fumigatus* (isolates 5-8) [a-d] in disaccharide sugars. The growth was measured in absorbance of 750 nm for 72 hours at 12 hours intervals (n=26).

3.3.3.2. *Aspergillus niger*

The log phase of the *A. niger* isolates commenced at about 24 hours of growth for disaccharides and reached optimum growth by 60 hours (Figures 3.11 and 3.12). Maltose, D-cellobiose and turanose showed absorbances between 1.0 and just over 2.0 from 36 to 72 hours. Assimilation of disaccharides by the *A. niger* isolates was over a prolonged log phase that was not steady or consistent build-up to the stationary phase. Turanose and gentiobiose showed sharp increases in absorbance from 48 hours for isolates 13, 18, 21 25,30 and 33 and D-trehalose for isolates 10 and 12 at 60 hours, which was 24 and 36 hours, respectively, from the end of a long lag phase of 24 hours. The absorbance values of these disaccharides were at 1.0 and below at 48 hours and reached 2.0 and above by 72 hours of growth for isolates 10, 21, 25, 30 and 33. The absorbance was doubled in 24 hours by these isolates. There is no particular commencement of the stationary phase by the *A. niger* isolates with a very wide absorbance range; however, for *A. fumigatus*, the stationary phase is seen at 48 hours with the majority of the compound

absorbance values above 1.0. For *A. flavus*, the stationary phase started at 48 hours as well, but only 50% of the compounds have absorbance values of 1.0 or more at this stage. When *A. niger* assimilation of monosaccharides and disaccharides is compared, the growth pattern is very consistent for the monosaccharides and not so for the disaccharides. There is a distinct stationary phase starting at 60 hours and levelling up to 72 hours with no sharp rise in absorbance as with disaccharides.

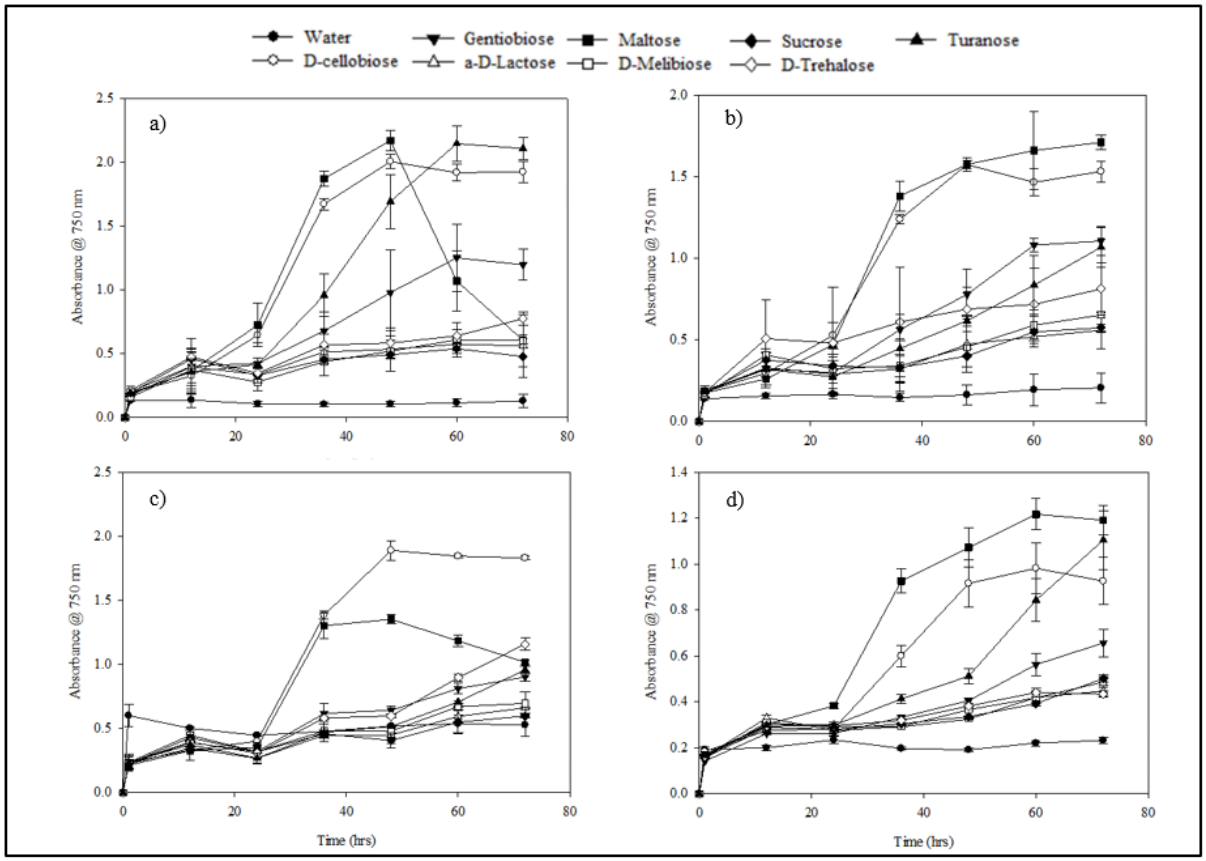


Figure 3.11: Growth of *A. niger* (isolates 10, 12, 13, 18) [a-d] in disaccharide sugars. The growth was measured in absorbance of 750 nm for 72 hours at 12 hours intervals (n=8).

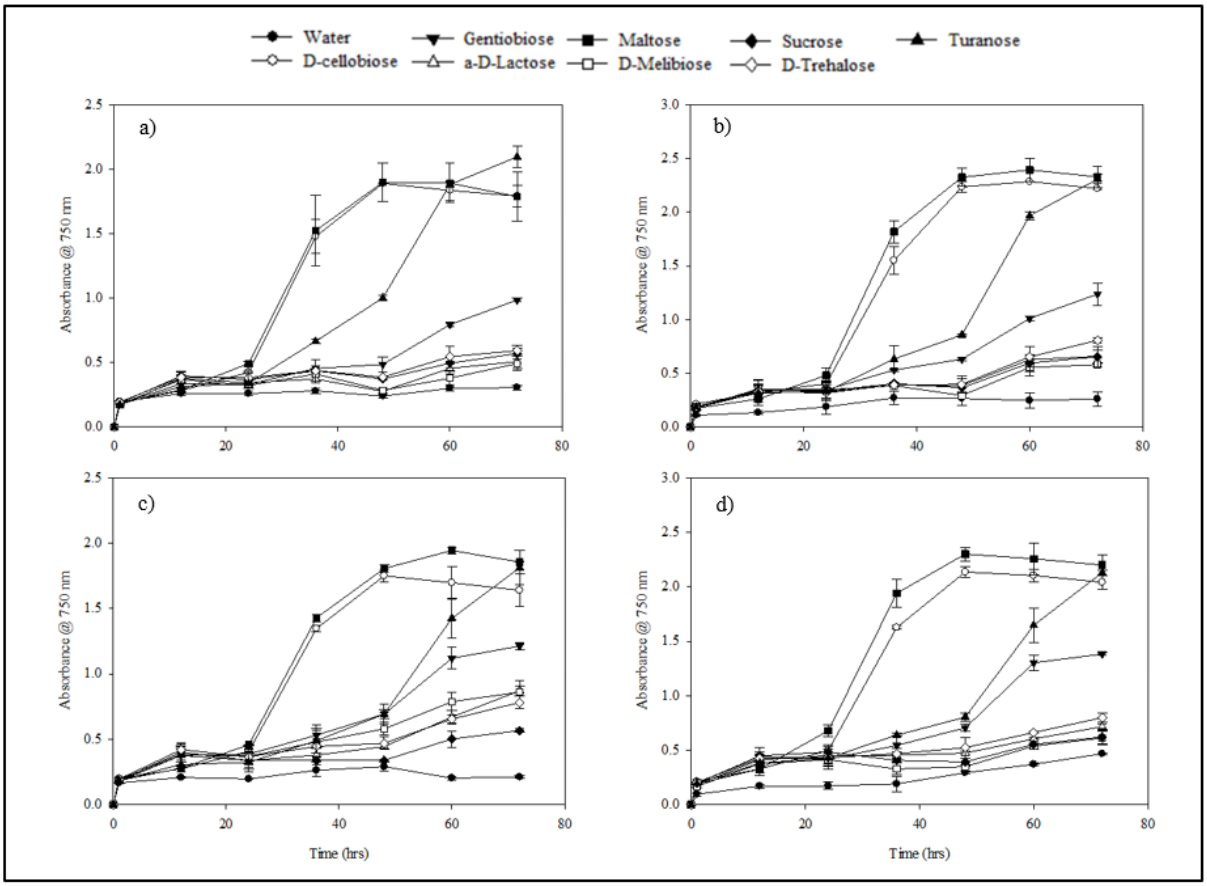


Figure 3.12; Growth of *A. niger* (isolates 21, 25, 30, 33) [a-d] in disaccharide sugars. The growth was measured in absorbance of 750 nm for 72 hours at 12 hours intervals (n=8).

3.3.3.3. *Aspergillus flavus*

The log phase of the *A. flavus* isolates commenced at about 12 hours of growth for disaccharides and reached optimum growth by 48 hours (Figure 3.13). Maltose had higher absorbance in both isolates, 1.78 by 36 hours for isolate 22 and 1.63 by 72 hours for isolate 32. D-cellobiose, turanose, D- trehalose and gentiobiose revealed absorbances between 1.5 and 1.8 by 36 hours. For isolate 22, α-D- Lactose showed a steep increase in absorbance from 1.4 at 36 hours reaching 2.4 at 72 hours. There was also an increase in absorbance in the case of turanose for isolate 22 at 60 hours; however, a far smaller increase in comparison with α-D- lactose. Other than these two compounds, both isolates plateaued by 72 hours. Optimum growth by both isolates was also reached at 48 hours for monosaccharide and disaccharide assimilation.

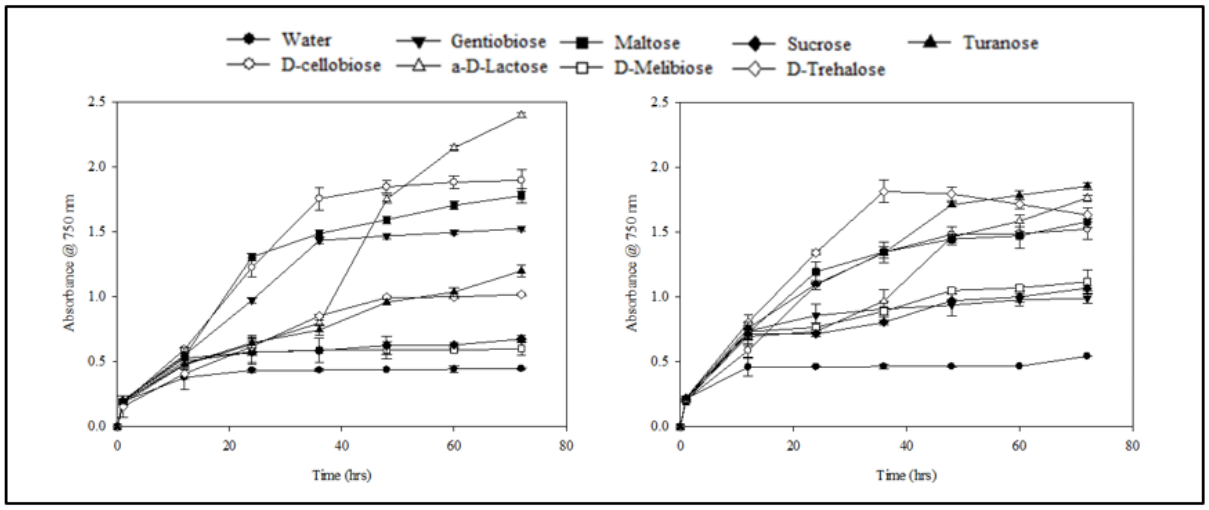


Figure 3.13: Growth of *A. flavus* (isolates 22 and 32) in disaccharide sugars. The growth was measured in absorbance of 750 nm for 72 hours at 12 hours intervals (n=2).

3.4. Discussion

The nutritional requirements for carbon sources are of fundamental importance for fungal growth in synthesizing the cell components like the cell wall, proteins, and nucleic acids, reserved as food material and energy sources for oxidation (Mohammad *et al.* 2011). Thus, carbon utilization profiles are important to understanding fungal physiology during growth and are a valuable reference for selecting the best carbon sources in medium development and diagnosis as well as understanding environments that support the pathogen survival (Mohammad *et al.* 2011). All three *Aspergillus* species could use an array of monosaccharides, being the simplest sugars, absorbed them rapidly and utilizing these sugars as building blocks for rapid growth at conducive temperatures and in the case of clinical strains impacting infection similar to human body temperature. Ries *et al.* (2019) also noted that the use of preferred carbon by *A. fumigatus* provided quick energy and material for niche colonisation and survival.

The growth profiles show overall rapid growth when utilizing monosaccharides and disaccharides, consistent with other literature reports. Mohammad *et al.* (2011) found that *A. niger* used carbon sources to produce much faster growth also in non-carbohydrate carbon sources of gluconeogenic (mainly amino acids) carbon sources and polymers and thus concluding that *Aspergillus* diversity of carbon utilization might be due to structural variation and configuration of molecules from various carbon compounds as compounds in the gluconeogenic group have more complex structural molecules compared to the carbohydrate group and resulted in faster growth.

The monosaccharides, D- glucosamine, N-acetyl-D-galactosamine, sedoheptulosan, D-fructose, and D-tagatose had absorbance values above 2.0 between 36 and 48 hours for *A. fumigatus* strains, (Figures 3.4 and 3.5).β – methyl -D-galactoside, α – methyl – D- glucoside and L-sorbose, showed absorbance values close to 2 and above between 48 and 60 hours. These monosaccharides make up about 50% of the monosaccharides analysed in this study and show high absorbance by *A. fumigatus* strains. L-fucose consistently showed a steep increase in absorbance from 48 to 72 hours, with absorbance readings reaching close to 2.5. The surge of absorbance of L-fucose later in the growth cycle extends the log phase resulting in more growth. This explains the high growth rate of growth of *A. fumigatus* cultures within 48 hours and up to 60 hours. This assimilation of preferred carbon sources viz. monosaccharides and disaccharides, confirms the metabolic flexibility of *A. fumigatus.* The consistent assimilation

of alternate monosaccharides for up to 60 hours can impact the persistence of infections initiated by this species in the human body due to their wide range of availability of monosaccharides. This has been attributed to the array of hydrolytic enzymes that *Aspergillus* is known to possess (De Assis *et al.* 2015). In the study by Ghazaei (2017), hydrolytic enzymes, such as serine and aspartic protease, phospholipases, metalloproteinase and dipeptidyl peptidases, were considered factors contributing to the pathogenesis and infections caused by *A. fumigatus*. Richie *et al.* (2009) reported that filamentous fungi rely heavily on the pathway for the production and secretion of extracellular hydrolytic enzymes needed to support growth on polymeric substrates. This hypothesis is very likely as the high assimilation of alternate monosaccharides and disaccharides by all three *Aspergillus* species demonstrated in this study can contribute to nutrient acquisition during infection. This shift to alternate monosaccharides and disaccharides late in the growth phase can be seen in *A. fumigatus, A. niger* and *A. flavus,* (Figures 3.4 to 3.7 and 3.9 to 3.13).

De Assis *et al.* (2015) detailed the involvement of carbon source utilization protein kinase A (PKA) as necessary in coordinating primary metabolism, carbon catabolic repression and fungal growth. In *A. fumigatus*, PKA activity increased in the presence of glucose compared to glycerol (Fuller and Rhodes 2012). Deleting the *pkaC1/pkaC2* genes in *A. fumigatus* renders the fungus unable to grow on glucose, further supporting a role for PKA in glucose metabolism (Fuller *et al.* 2011). De Assis *et al.* (2015) further demonstrated that in the absence of this protein kinase, the status of the cell is directed to carbon starvation resulting in increased hydrolytic enzyme production. These enzymes then seem to facilitate the metabolism of alternate carbon sources, as observed here 72 hours into the growth phase, with L-fucose, turanose and α-D-lactose for *A. fumigatus*, turanose, gentiobiose and D-trehalose for *A. niger* and α-D-lactose for *A. flavus* resulting in an extended growth of *Aspergillus* species and thus persistent infection (Figures 3.9 to 3.13). The production of the enzymes by *Aspergillus* species may allow for this shift in its metabolism to utilise alternative carbon sources to survive even in depleting nutrient status and in the case of these isolates, in immunocompromised individuals.

The exponential phase in this study for *A. niger* strains commenced at about 24 hours of growth for monosaccharides, approximately 12 hours later than for *A. fumigatus* strains, and reached optimum growth by 48 hours. However, absorbance by the monosaccharides was consistently above 2.0 only for D-fructose. This was the same for D-glucosamine for 50% of the isolates (Figures 3.6 and 3.7). Other monosaccharides, sedoheptulosan, α-methyl-D-glucoside, L-

arabinose and D-psicose, were used up, slowly plateauing around 60 hours after inoculation. D-Tagatose increased absorbance from 36 hours, getting more rapid from 48 to 60 hours. A similar pattern is observed for *A. niger* and *A. fumigatus*, except for D-tagatose, which had an elongated growth cycle and late assimilation resulting in an extended log phase and more biomass (Chroumpi *et al.* 2021).

Disaccharide assimilation by *A. fumigatus* strains, with an absorbance of 2.0 and above at 48 hours, is much later than for monosaccharide assimilation (Figures 3.9 and 3.10). Perhaps disaccharides with the glycosidic bond joining the two monosaccharides result in larger molecules and, consequently, slower assimilation by the isolates. Enzymes are presumably required to break the larger molecule down into smaller saccharide units first (Hobbie *et al.* 2003) occurring even at high utilization, where the absorbance of 2.0 is rarely reached and even if it does, it is much later in the growth cycle. This is also true for *A. niger* and *A. flavus* strains with absorbance below 2.0 and also occurring later in the growth phase.

All the *Aspergillus* isolates showed the ability to use a diverse range of carbon sources, revealing their versatility. Thus, they can thrive in most environments and possibly adapt in limiting settings to cause infection. The carbon profile of *A. fumigatus* suggests that this could also be one of the tools on how this pathogen persists and thrives in limiting environments by the rapid assimilation of various carbon sources to cause infection and persist during infection. The results are in agreement with those of Matar *et al.* (2017) who found that pathogenic fungi develop genetic mechanisms and molecular strategies to survive unpredictable scenarios and establish effective disease conditions in their hosts. Regulation of carbon metabolism is vital for disease establishment by filamentous fungi, deduced from the findings of this study, carbon catabolite repression and up-regulation help microorganisms adapt their physiology to the environment with balancing selections for rates of reprogramming (New *et al.* 2014; Ries *et al.* 2016). CCR switches off certain enzymes required to use less-favoured carbon sources when a more readily available carbon source is present in the medium (Ries *et al.* 2016). Therefore, CCR may also influence the survival of microorganisms by affecting virulence and adaptation.

Hayer, Stratford and Archer (2013) presented evidence to suggest a two-step germination process in *A. niger* conidia: a trigger step that precedes and is separate from the uptake and metabolism of certain external sugars that support the continued outgrowth of the spore. The trigger interaction induces the mobilization of D-trehalose, leading to conidial swelling and the activation of metabolic pathways required to sustain conidial outgrowth. In this study, however,

some isolated had absorbance below 1.0 for D-trehalose in *A. niger* strains except for isolate 13 (Figures 3.6 and 3.7). However, these *A. niger* isolates showed increased absorbance values of D-trehalose from 48 hours of growth. The log phase of the *A. niger* isolates starts at 24 hours and 24 hours later, the increased assimilation of D-trehalose likely sustains the conidial outgrowth.

Interestingly, this study reveals all *A. fumigatus* isolates showed increased absorbance of D-trehalose from the start of the log phase at 12 hours and further increased from 24 hours to reaching high absorbance at 48 hours (Figures 3.4 and 3.5). This assimilation of D-trehalose by *A. fumigatus* has the same pattern as *A. niger* but occurred much earlier (12 hours) in the growth phase and at higher absorbances than for *A. niger* isolates. These results may point to a two-step germination process in *A. fumigatus* conidia with higher absorbance of D-trehalose, resulting in the abundant sporulation of the species. But it also supports the suggestion that *A. fumagatus* is more adaptable by causing infection with higher prevalence than other *Aspergillus* species.

Opportunistic fungal infections have become a significant concern globally, as they are predicted to lead to more deaths annually than malaria and tuberculosis (Denning and Bromley 2015). Of the few hundred identified species in the *Aspergillus* genus (Visagie *et al.* 2014; Namvar *et al.* 2015), *A. fumigatus* is the primary and most common causative agent of opportunistic infectious diseases in humans (Dagenais and Keller 2009). Pathogenicity of *Aspergillus fumigatus* is a multifactorial trait encompassing a series of survival and fitness-enhancing factors determining virulence. Nutrient acquisition and subsequent metabolic processes are crucial for initial host colonisation and promote invasion and long-term survival within the host. In addition to sustaining growth and cellular biosynthetic processes, carbon and nitrogen metabolism also affect fungal traits such as enzyme secretion and cell wall integrity, which are essential pathogenicity determinants (Anderson *et al.* 2014).

The Biolog System allowed for a relatively simple technique using phenotypic microarray to screen a large number of carbon sources and is a valuable tool for analysing growth profiles for relevant investigations. Results presented in this chapter suggest that *Aspergillus* species can utilise a diverse range of carbon sources and have broad substrate assimilation to influence infection maintenance and disease progression. Further data analyses may reveal additional evidence of factors supporting fungal fitness and disease progression.

CHAPTER FOUR: POTENTIAL ANTIFUNGAL AGENTS AGAINST RESPIRATORY INFECTIONS

4.1. Introduction

The rising incidence of invasive fungal infections has unfortunately coincided with a significant increase in azole resistance in pathogenic *Aspergillus* species (Enoch *et al*. 2017). Yet, this has not attracted significant attention, although these life-threatening infections occur among the most vulnerable populations. Additionally, drug resistance in *Aspergillus* infections is catastrophic among these groups with chronic lung diseases or immunosuppression, which also has grown recently (Verweij 2016). These fungal pathogens have emerged as a leading cause among individuals with underlying health conditions or undergoing immunosuppressive treatments, with an estimated 1.5 million human mortalities annually (Brown, Denning and Levitz 2012). Despite this, fungal infections have not been a priority among health risks relative to other classes of infectious diseases. Yet, fungal infections that seriously compromise human health are spiralling and with an ever-increasing global mortality rate (Brown, Denning and Levitz 2012; Fisher 2018).

The most frequently implicated filamentous fungal species is *A. fumigatus,* which is estimated to cause over 200 000 cases of invasive aspergillosis annually with a mortality rate often exceeding 50%, followed by *A. flavus, A. niger*, *A. terreus* and *A. nidulans* (Brown, Denning and Levitz 2012). Among the pathogenic aspergilli, *A. fumigatus* is one of the most ubiquitous fungal species in the environment (Sugui *et al.* 2015). Therefore, concerted efforts are needed to diagnose and treat A*spergillus*-related infections. This proved to be more challenging, with a concurrent rise in azole resistance being widely identified in many *Aspergillus* and other fungal species (Lestrade *et al.* 2019).

Effective antifungal therapy is crucial in treating diseases among patients with a compromised immune system, especially in cases of invasive pulmonary aspergillosis, which often advances very rapidly. Currently, there are three classes of antifungal drugs to treat fungal infections, which are (1) azoles that target ergosterol biosynthesis, (2) echinocandins that inhibit fungal cell wall biosynthesis and (3) polyenes that bind to ergosterol in the fungal cell membrane leading to cell lysis (Perfect 2017; Robbins, Caplan and Cowen 2017). Recently, azoles have been recommended as the first choice of prophylaxis and in treating *Aspergillus* diseases (Patterson *et al.* 2016). Amphotericin B was the drug of choice for many years to treat fungal

infections. Still, due to adverse side effects, identification of other antifungal drugs is necessary and azole antifungals emerged as the preferred drugs against a broad range of fungi (Price *et al.* 2015). The most widely used azole clinical settings are voriconazole, posaconazole and itraconazole and their modified derivatives. Voriconazole displays wide-spectrum activities on fungi from all clinically important pathogenic groups. In a clinical trial, voriconazole was established as the treatment of choice against IA with significantly better efficacy, improved survival, and less toxicity compared to conventional amphotericin B (Lat and Thompson 2011). Itraconazole and its hydroxylated analogue, posaconazole, are broad-spectrum triazoles approved by the Food and Drug Administration with broad-spectrum activity against opportunistic and endemic fungal pathogens and are highly active against *Aspergillus* species. However, azole resistance is an increasing concern among *Aspergillus* species that cause these infections, especially *Aspergillus fumigatus* (Resendiz Sharpe *et al.* 2018).

This challenge is further complicated by the use of azoles in agriculture, as increasing levels of azole resistance have been associated or linked with increased use in agriculture over the last decade (Lelièvre *et al.* 2013; Hagiwara 2016). It has been suggested that *Aspergillus* acquire cross-resistance to medical azoles through exposure to fungicides in the environment that induce resistance to medical triazoles (Chowdhary *et al.* 2013; Berger *et al.* 2017). Cui *et al.* (2019) and Trovato *et al.* (2018) also reported an increase in azole resistance with the widespread use of related agricultural demethylase inhibitors of clinical and environmental isolates. Thus, monitoring the prevalence of azole resistance and its mechanism is important.

Resistance is usually due to genetic alterations that may be stable or transitory in the genome of implicated strains (Perlin 2015). Azoles are part of the class of compounds that target the 14-α sterol demethylase enzyme, also known as demethylation inhibitors (DMI) and are used not only in clinical settings but also in the agricultural sector to control plant pathogens (Garcia-Rubio *et al.* 2021). *Aspergillus* azole resistance, specifically in the case of *A. fumigatus*, can be acquired through selective pressure (1) clinically during repeated and prolonged exposure to azole therapy or (2) via environmental exposure due to the use in agriculture (Mortensen et al. 2011). In both likely situations, isolates with different resistance mechanisms and azole susceptibility profiles can be selected. However, the acquired resistance mechanism to azoles is likely due to mutations in the cyp51A gene (Garcia-Rubio, Cuenca-Estrella and Mellado 2017). The target molecule of azoles is *cpy51*, a lanosterol 14- ∞ - demethylase involved in the biosynthesis of ergosterol, essential fungal membrane lipid and ergosterol deficiency (Alcazar-Fuoli *et al.* 2008). The most widely reported and frequent mutations in the coding sequence of

the cyp51A gene that has led to azole resistance are on tandem-repeat (TR) insertions in the promoter region with mutations TR34 which usually co-occurs with a substitution of leucine to histidine at position 98 (L98H) of the coding region. There is another lower occurring insertion at the promoter region known as TR46. Isolates with these mutations can infect immunocompromised patients, limiting their treatment options and leading to serious disease progression and complications (Geddes-McAlister and Shapiro 2019). Although the number of cases of azole-resistant *A. fumigatus* recovered from clinical samples is still limited, azole resistance continues to spread and increase globally, reducing the effectiveness of azole in aspergillosis treatment, thus, the increased mortality rate associated with it (Verweij *et al.* 2016).

Fungal infections remain challenging for clinicians as the number of infections increases with the growing population of immunocompromised patients (Sienkiewicz 2016). The continued efforts to identify new antifungal drugs that are effective and safe like combination therapy are critical. Therefore, this chapter assessed the effectiveness of voriconazole and posaconazole currently used for the treatment of 36 *Aspergillus* isolates from patients with respiratory infections and known resistant mechanisms for azoles. Also, testing of potential novel antifungal compounds was conducted.

4.2. Methodology

4.2.1. Minimum inhibitory concentration (MIC) of voriconazole and posaconazole

Minimum inhibitory concentration (MIC) was employed to assess the susceptibility of organisms to the effectiveness of azoles used for infection treatment. Isolate maintenance was carried out as described in Section 2.2.1(Chapter 2). The 36 *Aspergillus* species were tested against current therapeutic drugs, voriconazole and posaconazole, using the Liofilchem MIC Test Strip (MTS), with a concentration range of 0.002 to 32 µg/ml. Half a millilitre of spore suspensions ($1 X 10^6$) was transferred and spread on malt extract agar plates. Azole MIC strips were applied to the inoculated agar surface per the manufacturer's instructions (Liofilchem). Inhibition was assessed and recorded at 24 and 48 hours incubation at 37°C on MEA plates to determine susceptibility and resistance to the azoles.

The Liofilchem MIC Test Strip uses a gradient test to determine the MIC of microorganisms to identify resistance patterns. The MIC test strips are impregnated with a predetermined concentration gradient of the antibiotic. When the MIC strip was applied to the inoculated agar surface, the pre-impregnated exponential gradient of the antimicrobial agent was immediately transferred to the agar matrix. After the appropriate incubation period, a symmetrical inhibition ellipse centred along the strip was formed. The MIC was read directly from the scale in terms of ug/ml at the point where the edge of the inhibition ellipse intersects the strip.

The susceptibility of the isolates to the azoles was tested and analysed as per the manufacturer's protocol (Liofilchem 2015). The isolates were scored as follows:
(i) Voriconazole: MIC – susceptible ≤ 0.25 µg/ml; moderately susceptible > 0.25 – 2.0 µg/ml; resistant >2.0 µg/ml
(ii) Posaconazole: MIC - susceptible ≤ 0.06 µg/ml ; moderately susceptible > 0.06 – 0.25 µg/ml; resistant >0.25 µg/ml

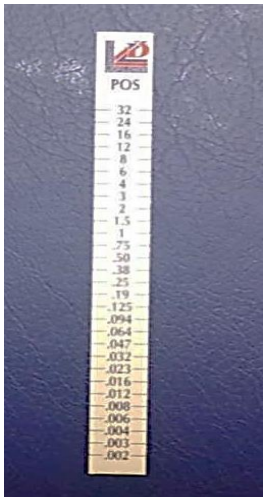


Figure 4.1: Liofilchem MIC test strip with 0.002-32 µg/ml posaconazoleand voriconazole.

4.2.2. Phenotypic microarray of antifungal compounds

An array plate containing 24 control compounds of varying concentrations was used to identify possible alternative chemicals as novel fungal control agents. The phenotype microarray technology is described in Section 2.2.4 (Chapter 2); however, a different panel plate was used for control agents testing. The PM 24C panel was chosen as it contains compounds that can be used to control fungi. The panel consisted of 24 antimicrobial chemicals of unknown concentrations in 96 wells (Biolog, USA, Figure 4.2). The 24 compounds in the Biolog chemical sensitivity panel PM 24C plate (catalogue no 12224) varied from azoles, fungicides, antibiotics, and food preservatives to antimicrobial agents.

A1 Apramycin Sulfate	A2 Apramycin Sulfate	A3 Apramycin Sulfate	A4 Apramycin Sulfate	A5 Aminacrine	A6 Aminacrine	A7 Aminacrine	A8 Aminacrine	A9 Zaragozic acid A	A10 Zaragozic acid A	A11 Zaragozic acid A	A12 Zaragozic acid A
B1 Blasticidin hydrochloride	B2 Blasticidin hydrochloride	B3 Blasticidin hydrochloride	B4 Blasticidin hydrochloride	B5 Thioridazine hydrochloride	B6 Thioridazine hydrochloride	B7 Thioridazine hydrochloride	B8 Thioridazine hydrochloride	B9 Sodium Benzoate	B10 Sodium Benzoate	B11 Sodium Benzoate	B12 Sodium Benzoate
C1 Chlortetracycline hydrochloride	C2 Chlortetracycline hydrochloride	C3 Chlortetracycline hydrochloride	C4 Chlortetracycline hydrochloride	C5 Sodium metasilicate	C6 Sodium metasilicate	C7 Sodium metasilicate	C8 Sodium metasilicate	C9 Pentamidine Isethionate	C10 Pentamidine Isethionate	C11 Pentamidine Isethionate	C12 Pentamidine Isethionate
D1 6-Azauracil	D2 6-Azauracil	D3 6-Azauracil	D4 6-Azauracil	D5 Potassium Chromate	D6 Potassium Chromate	D7 Potassium Chromate	D8 Potassium Chromate	D9 Thialysine	D10 Thialysine	D11 Thialysine	D12 Thialysine
E1 Berberine	E2 Berberine	E3 Berberine	E4 Berberine	E5 EGTA	E6 EGTA	E7 EGTA	E8 EGTA	E9 Sodium Pyrophosphate Decahydrate	E10 Sodium Pyrophosphate Decahydrate	E11 Sodium Pyrophosphate Decahydrate	E12 Sodium Pyrophosphate Decahydrate
F1 Isoniazid	F2 Isoniazid	F3 Isoniazid	F4 Isoniazid	F5 Methyl Viologen Dichloride hydrate	F6 Methyl Viologen Dichloride hydrate	F7 Methyl Viologen Dichloride hydrate	F8 Methyl Viologen Dichloride hydrate	F9 Sodium Fluoride	F10 Sodium Fluoride	F11 Sodium Fluoride	F12 Sodium Fluoride
G1 Cisplatin	G2 Cisplatin	G3 Cisplatin	G4 Cisplatin	G5 Aluminum sulfate	G6 Aluminum sulfate	G7 Aluminum sulfate	G8 Aluminum sulfate	G9 Fluconazole	G10 Fluconazole	G11 Fluconazole	G12 Fluconazole
H1 Propiconazole	H2 Propiconazole	H3 Propiconazole	H4 Propiconazole	H5 Tamoxifen	H6 Tamoxifen	H7 Tamoxifen	H8 Tamoxifen	H9 Miconazole Nitrate	H10 Miconazole Nitrate	H11 Miconazole Nitrate	H12 Miconazole Nitrate

Figure 4.2: Chemical sensitivity panel PM 24C with 24 chemical agents at four concentrations.

The isolates were first inoculated on malt extract plates for 72 hours at 37°C for spore formation. The spores were then removed from the surface of the agar plates using a sterile swab by gently rubbing across the surface and then transferred into the sterile inoculating fluid (Biolog, USA). The turbidity of the suspension was measured using a Biolog turbidimeter and a density of approximately 75% transmittance was used as recommended by the manufacturer's protocol. One-hundred microliters of fungal inoculum were transferred into each well of the PM plates, followed by incubation at 37°C for 96 hours. Absorbance data (750 nm) was collected at 24-hour intervals over 96 hours, using the Microlog station to read the panels and capture data.

4.2.4. Genes associated with resistance

4.2.4.1. DNA isolation and PCR amplification

Cultures were grown on malt extract agar plates for three days at 37°C. Two plaques were transferred to 100 ml malt extract broth and incubated at 37°C, 150 rpm for 18 hours. The mycelia were centrifuged, and the pellet was used to extract DNA. DNA was isolated as described in Section 2.2.3 (Chapter 2), followed by PCR amplification using specific primers (Table 4.1) following the protocol described in Section 2.2.3.1 (Chapter 2) (Nabili *et al*., 2016), then analysed for associated genes to identify genetic markers for antifungal resistance. MUSCLE (multiple sequence comparison by log expectation) was used for multiple protein sequence alignment for amino acids of the wild-type (WT) cyp51A gene (Edgar 2004).

Table 4.1: Primers used in PCR amplification of genes

Forward Primers	Reverse Primers
P-A5-TCTCTGCACGCAAAGAAGAAC	P-A7-TCATATGTTGCTCAGCGG
CYP1-L-CACCCTCCCTGTGTCTCCT	CYP1-R-AGCCTTGAAAGTTCGGTGAA
CYP2-L-CATGTGCCACTTATTGAGAAGG	CYP2-R-CCTTGCGCATGATAGAGTGA
CYP3-L TTCCTCCGCTCCAGTACAAG	CYP3-R CCTTTGAAGTCCTCGATGGT

4.3. Results

4.3.1. Antifungal analysis: Voriconazole and Posaconazole

In this study, azole-impregnated strips were used to assess the susceptibility/resistance of the clinical isolates to known azole therapeutics. The MIC of voriconazole and posaconazole on all *Aspergillus* species were recorded as explained in 4.2.1. The results are reported in Table 4.2 for *A. fumigatus*, Table 4.3 for *A. niger* and Table 4.4 for *A. flavus*. Two isolates of each species are selected to represent typical reactions on the MIC of voriconazole and posaconazole at 48 hours of incubation, Figures 4.3-4.6. Other isolates are presented in Appendix D.

Isolates 14 and 28 for example (*A. fumigatus*), had MIC of 0.25 µg/ml for voriconazole (susceptible ≤ 0.25 µg/ml) and 12.0 µg/ml and 8.0 µg/ml (resistant >0.25 µg/ml), respectively, for posaconazole as can be seen in Table 4.2. This represents the trend for the results of the *A. fumigatus* strains that were susceptible to voriconazole and high resistance well past the minimum concentration of 0.25µg/ml to posaconazole. The 26 *A. fumigatus* isolates recorded the lowest effective concentration from 0.19 µg/ml to 1.5 µg/ml for voriconazole and from 1.0 µg/ml to 16.0 µg/ml for posaconazole.

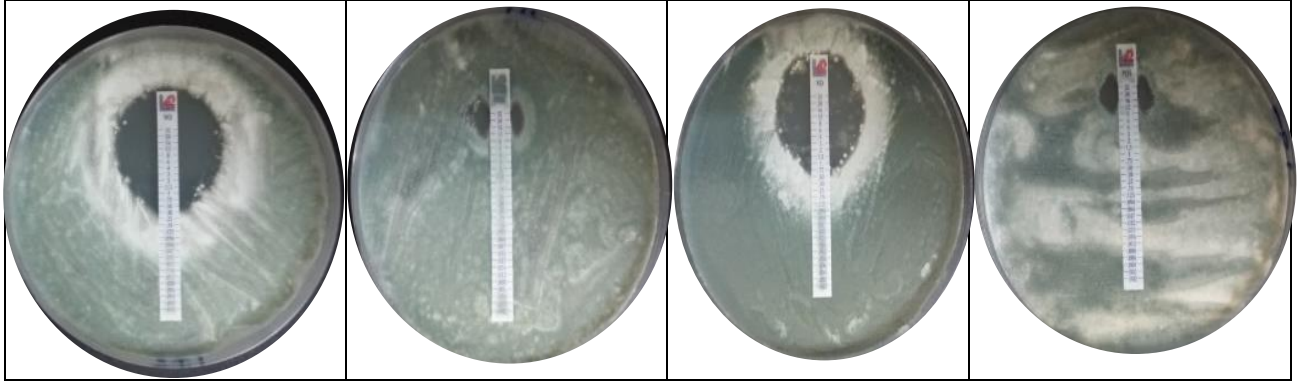
***A. fumigatus* (14)**		***A. fumigatus*(28)**	
Voriconazole	**Posaconazole**	**Voriconazole**	**Posaconazole**
			

Figure 4.3: Effect of voriconazole and posaconazole on the growth of *Aspergillus fumigatus* at 48 hours. MIC Test Strip with azole concentration from 0.002-32 µg/ml.

Aspergillus niger isolates 18 and 25 were susceptible (1.0 µg/ml) to voriconazole in the intermediate range with the azole being more effective than posaconazole that had a MIC of 8.0 µg/ml (Figure 4.4). Higher voriconazole concentrations resulted in much larger inhibition zones (34 and 39 mm) than those of posaconazole. For isolate 18, 12 mm was achieved with a MIC of 16 µg/ml and 14 mm at a MIC of 24 µg/ml (Table 4.3).

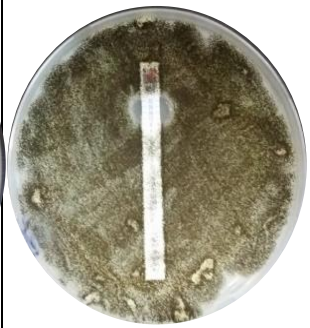
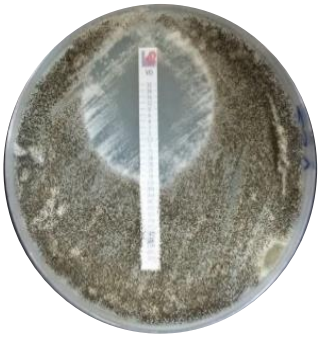
***A. niger* (18)**		***A. niger* (25)**	
Voriconazole	**Posaconazole**	**Voriconazole**	**Posaconazole**
			

Figure 4.4: Effect of voriconazole and posaconazole on the growth of *Aspergillus niger* at 48 hours. MIC Test Strip with azole concentration from 0.002-32µg/ml.

Voriconazole was more effective on *Aspergillus flavus* than posaconazole for isolates 22 and 32 (Figure 4.5). MICs (0.5 and 1.5 µg/ml) were higher than for *A. fumigatus* but much lower than for *A. niger*. Like *A. niger*, higher voriconazole concentrations (18 and 12 µg/ml) resulted in much larger inhibition zones (39 and 31 mm) but not for posaconazole. Twenty-four µg/ml of posaconazole resulted in only 21 and 18 mm inhibition zones for both *A. flavus* isolates 22 and 32 (Table 4.4).

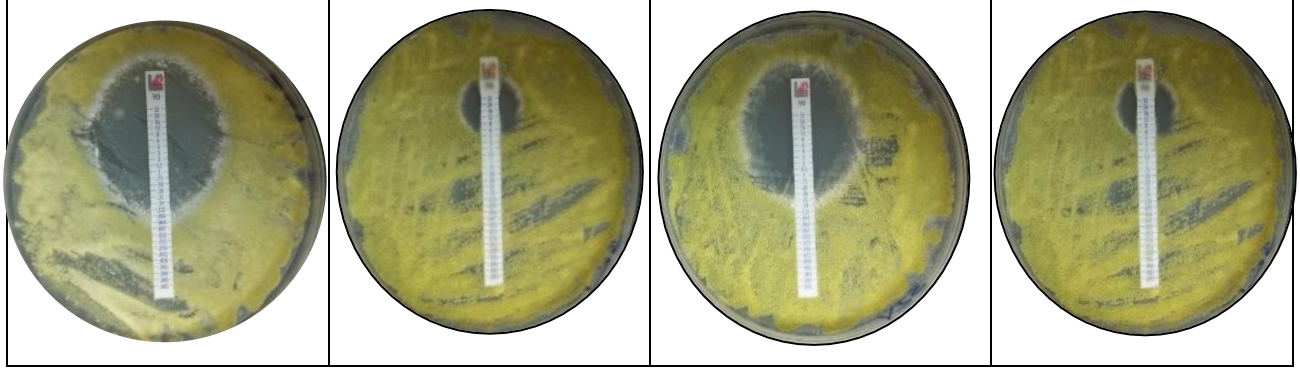
A. flavus (22)		*A. flavus* (32)	
Voriconazole	**Posaconazole**	**Voriconazole**	**Posaconazole**
			

Figure 4.5: Effect of voriconazole and posconazole on the growth of *Aspergillus flavus* at 48 hours. MIC Test Strip with azole concentration from 0.002-32 µg/ml.

The results of voriconazole and posaconazole effectiveness against *A. fumigatus* reveal 11 isolates susceptible (≤0.25 µm) to voriconazole (green) and 15 isolates with intermediate susceptibility (>0.25-2.0 µm), highlighted in yellow. All 26 isolates were resistant (>2.0 µm) to posaconazole (blue), and recorded resistance between 1.0 -16.0 µg/ml. For each *A. fumigatus* isolate, the lowest effective concentration and the concentration producing the most significant inhibition in millimetres, are shown in Table 4.2. The most significant inhibition zones of 34 mm for *A. fumigatus*, resulted from 16 µg/ml concentration, as seen with isolates 4, 5 and 34, shaded grey. The 26 *A. fumigatus* isolates recorded the lowest effective concentration from 0.19-1.5 µg/ml for voriconazole resulting in 42% susceptible isolates and 58% with intermediate susceptibility.

Table 4.2: Azole effect on *A fumigatus*, at the lowest effective concentration and the concentration that produced the most significant inhibition in millimetres after 48 hours of incubation at 37°C.

		Voriconazole		Posaconazole	
Isolate No.	**Species**	**Lowest effective concentration (µg/ml)**	**Concentration (µg/ml) for the largest inhibition zone (mm)**	**Lowest effective concentration (µg/ml)**	**Concentration (µg/ml) for the largest inhibition zone (mm)**
1	*A. fumigatus*	0.19	12.0 - 29	16.0	24.0 - 10
2	*A. fumigatus*	0.38	12.0 - 30	16.0	24.0 - 10
3	*A. fumigatus*	0.38	12.0 - 31	12.0	24.0 - 11
4	*A. fumigatus*	0.25	16.0 - 34	12.0	24.0 - 12
5	*A. fumigatus*	0.19	16.0 - 34	12.0	24.0 - 12
6	*A. fumigatus*	0.38	12.0 - 28	12.0	24-0 - 13
7	*A. fumigatus*	0.38	12.0 - 27	12.0	24.0 - 13
8	*A. fumigatus*	0.50	12.0 - 28	16.0	24.0 - 12
9	*A. fumigatus*	0.38	12.0 - 29	12.0	24-0- -12
11	*A. fumigatus*	0.38	12.0 - 31	12.0	24.0 - 17
14	*A. fumigatus*	0.25	12.0 - 29	12.0	24.0 - 15
15	*A. fumigatus*	0.50	12.0- 28	8.0	16.0 - 13
16	*A. fumigatus*	0.38	12.0 - 29	8.0	24.0 -17
17	*A. fumigatus*	0.19	12.0 - 32	12.0	24.0 - 12
19	*A. fumigatus*	0.38	12.0 - 30	6.0	16.0 - 15
20	*A. fumigatus*	0.25	12.0 - 29	8.0	24.0 - 17
23	*A. fumigatus*	0.50	12.0- 38	8.0	16.0 - 16
24	*A. fumigatus*	0.25	8..0 - 29	8.0	16.0 - 16
26	*A. fumigatus*	0.50	23.0- 28	6.0	16.0 - 17
27	*A. fumigatus*	0.75	12.0 -29	8.0	24.0 - 16
28	*A. fumigatus*	0.25	12.0 - 30	8.0	16.0 - 12
29	*A. fumigatus*	0.25	12.0 - 32	7.5	12.0 - 24
31	*A. fumigatus*	0.25	12.0 - 32	8.0	24.0 - 19
34	*A. fumigatus*	0.38	16.0 - 34	6.0	16.0 - 17
35	*A. fumigatus*	1.5	16.0 - 31	8.0	24.0 - 15
36	*A. fumigatus*	0.19	12.0 - 30	1.0	16.0 - 21

Voriconazole: MIC - susceptible ≤ 0.25 µg/ml; moderately susceptible > 0.25 – 2. µg/ml 0 ; resistant >2.0 µg/ml

Posaconazole: MIC - susceptible ≤ 0.06 µg/ml; moderately susceptible > 0.06 – 0.25 µg/ml ; resistant >0.25 µg/ml

Voriconazole effectiveness against *A. niger* showed 12.5% susceptibility (≤0.25µm), 62.5% intermediate susceptibility (>0.25-2.0 µm) and 25% resistance (>2.0 µm) to voriconazole. Of the eight *A. niger* isolates, one was sensitive (green), five showed intermediate sensitivity (yellow) and two were resistant (blue) to voriconazole. Isolates 12 and 30 are resistant strains. All isolates were resistant to posaconazole, recording resistance at 8.0 µg/ml and 12.0 µg/ml. Table 4.3 shows the lowest effective concentration, i.e., MIC against each isolate and the concentration that produced the largest inhibition. A. *niger* isolates revealed larger inhibition zones at higher concentrations of the azole. Isolate 21 and 33 had over 40 mm inhibition at 8 µg/ml (shaded grey). As seen, *in vitro,* voriconazole was more effective on *A. niger* isolates than *A. fumigatus* isolates at higher concentrations (Table 4.3 and 4.2).

Table 4.3: Azole effect on *A. niger* at the lowest effective concentration and the concentration that produced the most significant inhibition in millimetres after 48 hours of incubation 37°C.

		Voriconazole		**Posaconazole**	
Isolate No	**Species**	**Lowest effective concentration (µg/ml)**	**Concentration (µg/ml) for the largest inhibition zone (mm)**	**Lowest effective concentration (µg/ml)**	**Concentration (µg/ml) for the largest inhibition zone (mm)**
10	*Aspergillus niger*	0.19	12.0 - 39	8.0	16.0 - 13
12	*Aspergillus niger/tubingensis*	2.0	16.0 - 16	12.0	32.0 - 16
13	*Aspergillus niger/tubingensis*	1.5	12.0 - 21	12.0	16.0 - 13
18	*Aspergillus niger*	1.0	12.0 - 34	8.0	16.0 - 12
21	*Aspergillus niger/welwitschiae*	1.0	8.0 - 40	8.0	16.0 - 14
25	*Aspergillus niger*	1.0	8.0 - 39	8.0	24.0 - 14
30	*Aspergillus niger*	2.0	12.0 - 39	8.0	24.0 - 17
33	*Aspergillus niger*	1.5	8.0 - 43	12.0	34.0 - 13

Voriconazole: MIC - susceptible ≤ 0.25 µg/ml; moderately susceptible > 0.25 – 2. µg/ml 0; resistant >2.0 µg/ml

Posaconazole: MIC - susceptible ≤ 0.06 µg/ml; moderately susceptible > 0.06 – 0.25 µg/ml; resistant >0.25 µg/ml

A. flavus isolates 22 and 32 showed moderate susceptibility (>0.25-2.0 µl/ml) against voriconazole and were resistant (>2.0 µl/ml) against posaconazole., as seen in Table 4.4. Isolate 22 was moderately susceptible with one-third of the concentration (0.5 µl/ml) compared to isolate 32 (1.5 µl/mg), against voriconazole.

Table 4.4: Azole effect on *A. flavus*, at the lowest effective concentration and the concentration that produced the largest inhibition in millimetres after 48-hour incubation at 37°C.

		Voriconazole		Posaconazole	
Isolate No	**Species**	**Lowest effective concentration (µg/ml)**	**Concentration (µg/ml) for the largest inhibition zone (mm)**	**Lowest effective concentration µg)**	**Concentration (µg/ml) for the largest inhibition zone (mm)**
22	*Aspergillus flavus*	0.5	8.0 - 39	6.0	24.0 - 21
32	*Aspergillus flavus*	1.5	12.0 - 31	6.0	24.0 - 18

Voriconazole: MIC - susceptible ≤ 0.25 µg/ml; moderately susceptible > 0.25 – 2. µg/ml 0; resistant >2.0 µg/ml

Posaconazole: MIC - susceptible ≤ 0.06 µg/ml; moderately susceptible > 0.06 – 0.25 µg/ml; resistant >0.25 µg/ml

4.3.1.1. Antifungal analysis

The overall effect of voriconazole and posaconazole on the *Aspergillus* isolates is represented in Figure 4.6. Thirty-three per cent of all species showed susceptibility (≤ 0.25 µg/ml) to voriconazole and 61% had intermediate susceptibility (0.25 – 2.0 µg/ml). Six per cent of the isolates were resistant to voriconazole meanwhile all three *Aspergillus* species were resistant to posaconazole. The 6% resistance to voriconazole was from the *A. niger* strains, with *A. fumigatus* and *A. flavus* showing no resistance. Of the 33% susceptible strains (MIC ≤ 0.25µl/mlm), 30% were *A. fumigatus* strains and 3% were *A. niger* strains.

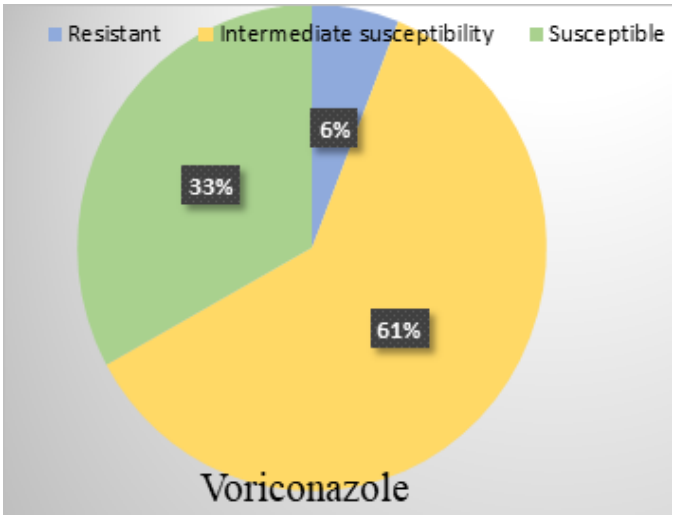


Figure 4.6: Effect of Voriconazole on *Aspergillus* isolates after 48-hour incubation at 37 ℃ (n=36). 61% (22 isolates); 33% (12 isolates); 6% (2 isolates).

Voriconazole: MIC - susceptible ≤ 0.25 µg/ml; moderately susceptible > 0.25 – 2. µg/ml 0; resistant >2.0 µg/ml
Posaconazole: MIC - susceptible ≤ 0.06 µg/ml; moderately susceptible > 0.06 – 0.25 µg/ml; resistant >0.25 µg/ml

The susceptibility and resistance of each *Aspergillus* species to voriconazole and posaconazole are displayed in Figure 4.7. Only *A. niger* isolates showed resistance to voriconazole. Resistance was seen in 25% of *A. niger* isolates. All *A. fumigatus* and *A. flavus* strains were susceptible to voriconazole. However, 42% of *A. fumigatus* strains were susceptible (≤ 0.25µl/ml) and 58% showed intermediate susceptibility (> 0.25-2.0 µl/ml).

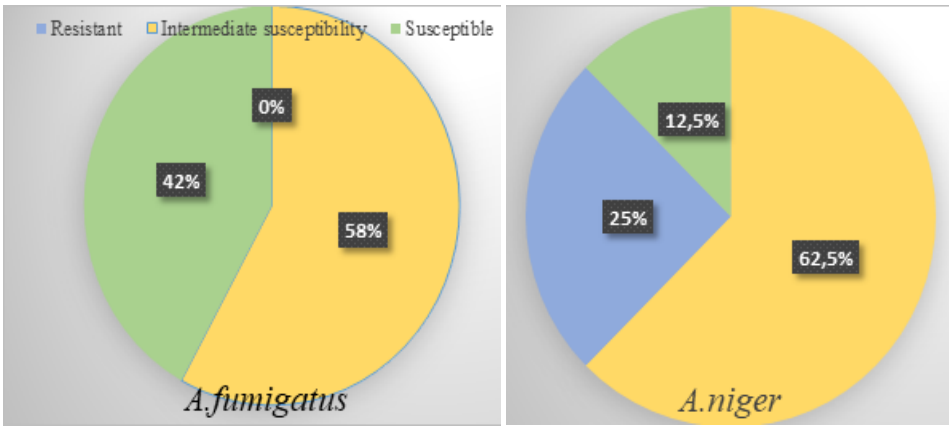


Figure 4.7 Effect of voriconazole on *A. fumigatus* and *A. niger* isolates after 48-hour incubation at 37℃.

Voriconazole: MIC - susceptible ≤ 0.25 µg/ml; moderately susceptible > 0.25 – 2. µg/ml 0; resistant >2.0 µg/ml
Posaconazole: MIC - susceptible ≤ 0.06 µg/ml; moderately susceptible > 0.06 – 0.25 µg/ml; resistant >0.25 µg/ml

4.3.2. Phenotypic microarray of antifungal compounds.

Twenty-four compounds (four wells each) made up the 96-well panel of antifungal compounds tested on the isolates. Growth data was captured and recorded using the Biolog Microlog system at 24-hr intervals over a 96-hr period at 750 nm. However, 48-hour readings were selected for analysis for isolates to be compared for their chemical response (Figure 4.9). Analysis of the response of the 36 *Aspergillus* isolates in the presence of 24 potential antifungal agents was visualized using a heatmap yellow indicates compound resistance and red indicates susceptible compounds.

Significant resistance to most compounds by *A. fumigatus* isolates (8, 9 and 15) and *A. niger* isolates (10, 12 and 18) is seen in Figure 4.8. The three *A. fumigatus* isolates were resistant to all 24 compounds while the three *A. niger* isolates were resistant to more than 50% of the 24 compounds. The resistant *A. fumigatus* (isolate 8) had the highest growth reading of 2.475 nm absorbance displaying the highest resistance to 6-Azauracil. The remaining thirty isolates were generally susceptible to all 24 compounds. Of these susceptible isolates, 63% of the *A. niger* strains (n = 8), 88% of the *A. fumigatus* strains (n = 26), and the *A. flavus* strains had varying susceptibilities to the 24 compounds with absorbance generally below 0.50 nm (Appendix E), showing a significant percentage of susceptibility and potential for antifungal activity.

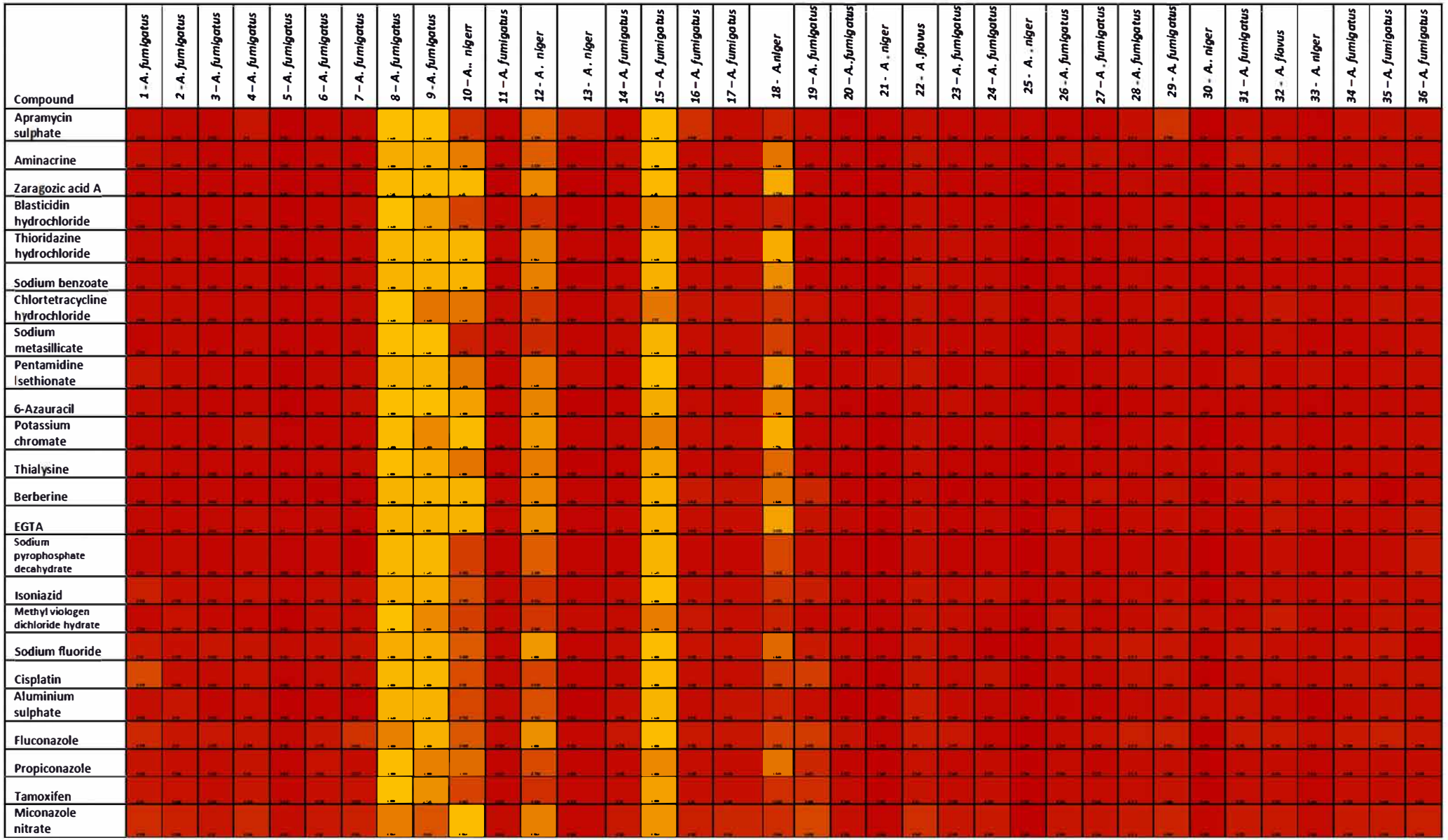


Figure 4.8: Absorbance readings taken at 750nm after 48hr of incubation of isolates in a growth medium incorporated with the 24 compounds. Red indicates susceptibility and yellow indicates the resistance of isolates to the potential antifungal agents.

4.3.3. Azole resistance genetic marker

A comparison of the cyp51A gene using the MUSCLE multiple sequence alignment tool (https://www.ebi.ac.uk/Tools/msa/muscle/) to detect mutation marker TR34/98, a major marker of azole genetic resistance, were conducted. The leucine-to-histidine mutation is at position 98. Histidine (H) in position 98 is usually associated with *A. fumigatus* strains becoming resistant. This sequence alignment tool was used to determine if the mutation was present in the isolates and ascertained if the mutation created resistance to azoles.

The amino acid alignment revealed that 42% (11 strains) contained the tr34/98 mutation, while the rest (58%, 15 strains) contained the wild-type *cyp51A* gene. Although 15 isolated had wild-type genes, only 31% were susceptible to voriconazole and 27% with intermediate susceptibility. Of the eleven mutated *A. fumigatus* isolates, 73% showed intermediate susceptibility (≤0.25 μm) to voriconazole and three strains (27%) remained susceptible even though the strains recorded mutations. The wild-type *A. fumigatus* strains showed intermediate susceptibility (>0.25-2.0 μm) but at higher concentrations between 0.50 -1.50 mm for six of the isolates (8, 15, 23, 26, 27 and 35) (Table 4.2). The wild-type and mutated strains of all three species showed resistance to posaconazole. The sample size was not large enough to ascertain a trend of resistance in the isolates due to mutations. The cyp51A gene was not determined for *A. niger* and *A. flavus* isolates.

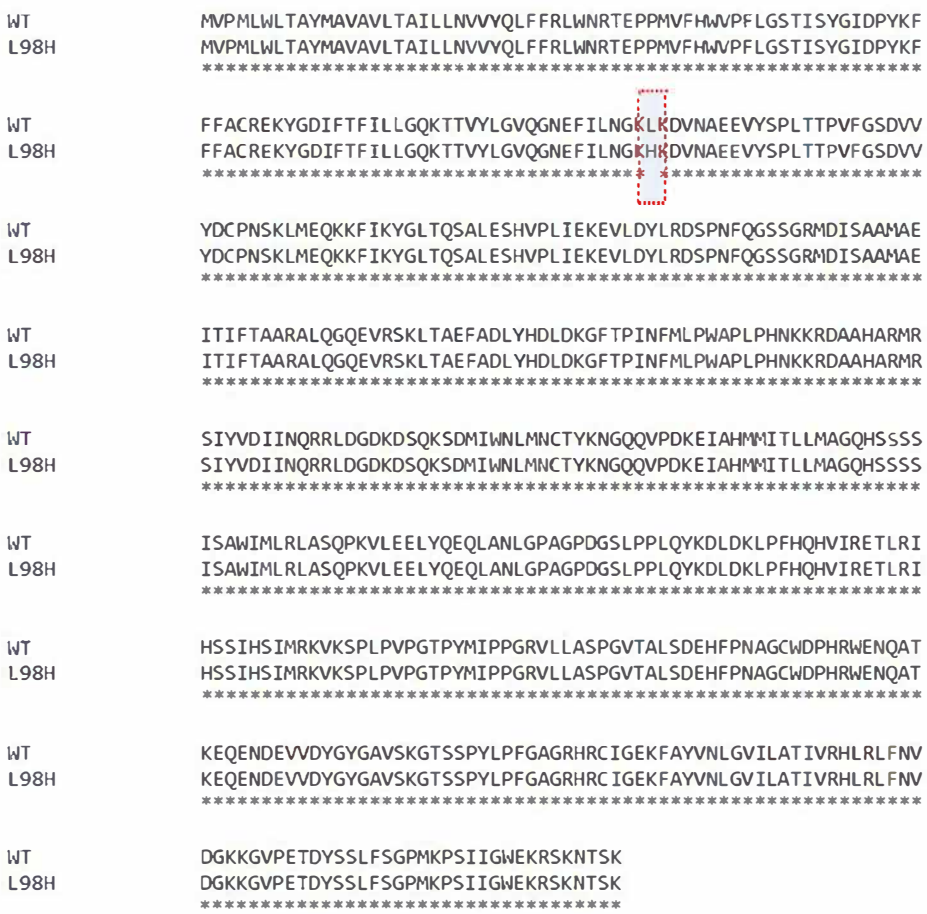


Figure 4.9: Amino Acid alignment of the wild-type (WT) cyp51A gene vs. the leucine to histidine mutation at position 98 using MUSCLE Tool (EMBL)

Table 4.5: Minimum inhibitory concentration of voriconazole (VRC) against *Aspergillus fumigatus* and posaconazole (POS) and mutations in the cyp51A gene.

Isolate no.	**Species**	**Resistant/susceptible/ intermediate susceptibility (R/S/IS)**		***cyp51A* Mutations**
		VRC	POS	
1	*A. fumigatus*	S	R	WT
2	*A. fumigatus*	IS	R	L98H
3	*A. fumigatus*	IS	R	L98H
4	*A. fumigatus*	S	R	L98H
5	*A. fumigatus*	S	R	WT
6	*A. fumigatus*	IS	R	L98H

Isolate no.	Species	Resistant/susceptible/ intermediate susceptibility (R/S/IS)		*cyp51A* Mutations
		VRC	POS	
7	*A. fumigatus*	IS	R	L98H
8	*A. fumigatus*	IS	R	WT
9	*A. fumigatus*	IS	R	L98H
10	*A. niger*	S	R	ND
11	*A. fumigatus*	IS	R	WT
12	*A. niger*	R	R	ND
13	*A. niger*	IS	R	ND
14	*A. fumigatus*	S	R	WT
15	*A. fumigatus*	IS	R	L98H
16	*A. fumigatus*	IS	R	WT
17	*A. fumigatus*	S	R	WT
18	*A. niger*	IS	R	ND
19	*A. fumigatus*	IS	R	WT
20	*A. fumigatus*	S	R	L98H
21	*A. niger*	IS	R	ND
22	*A. flavus*	IS	R	ND
23	*A. fumigatus*	IS	R	WT
24	*A. fumigatus*	S	R	L98H
25	*A. niger*	IS	R	ND
26	*A. fumigatus*	IS	R	WT
27	*A. fumigatus*	IS	R	L98H
28	*A. fumigatus*	S	R	WT
29	*A. fumigatus*	S	R	WT
30	*A. niger*	R	R	ND
31	*A. fumigatus*	S	R	WT
32	*A. flavus*	IS	R	ND
33	*A. fumigatus*	IS	R	WT
34	*A. fumigatus*	IS	R	L98H
35	*A. fumigatus*	IS	R	WT
36	*A. fumigatus*	S	R	WT

WT: wild-type
ND : not determined
VRC: voriconazole
POS: posaconazole

Isolate no.	Species	Resistant/susceptible/ intermediate susceptibility (R/S/IS)		*cyp51A* Mutations
		VRC	POS	

The phylogenetic analysis clustered *A. fumigatus* into three branches (Clusters 1, 2 and 3). The *A. niger* species (10, 21, 25, 18, 33) formed their cluster (4) and isolates 12 and 13 formed a sub-group within cluster 4. Isolates 22 and 32 (*A. flavus*) form cluster 5. Sixty-seven per cent of *A. fumigatus* isolates in cluster 1 show intermediate susceptibility to voriconazole, 44% in cluster 2 and 71% in cluster 3. Cluster 4 (*A. niger* isolates) reveals low susceptibility (14.3%) to voriconazole. Fourteen per cent of the isolates were resistant and 71, 4% showed intermediate susceptibility. Both *A. flavus* isolates in cluster 5 showed intermediate susceptibility. The leucine to histidine mutation at position 98 (L98H) is revealed in 56% (7, 9, 15, 20, 34) of the isolates in cluster 1, 44% (4, 6, 24, 27) of isolates in cluster 2 and 29% of isolates in cluster 3 (2 and 3).

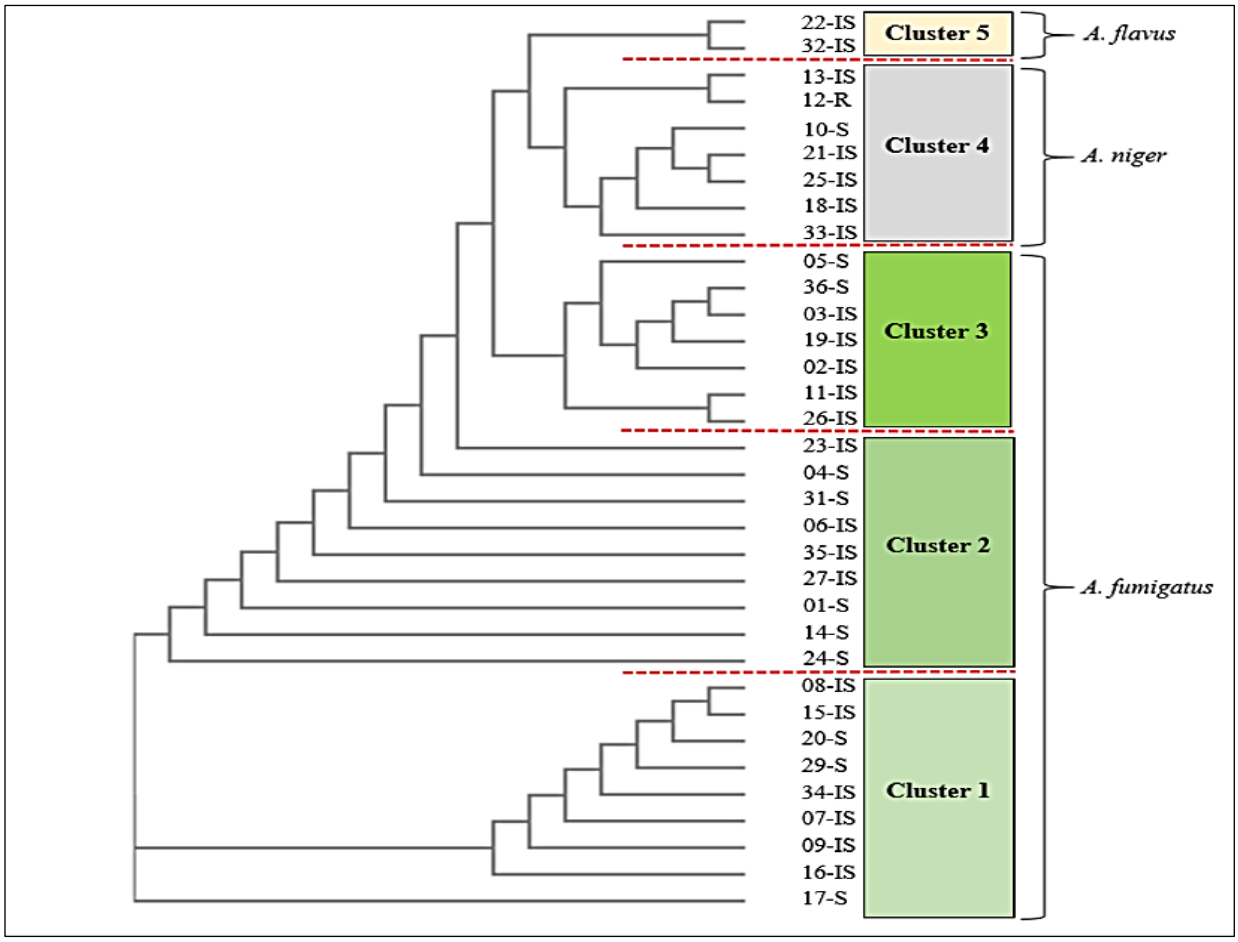


Figure 4.10: Phylogenetic tree showing clusters within isolates of *Aspergillus* species and isolates susceptibility to voriconazole (S-susceptible; IS-intermediate susceptibility; R-resistant)

4.4. Discussion

The 2017 global estimates indicated that more than 3 million cases of chronic pulmonary aspergillosis (CPA) and 250 000 cases of invasive aspergillosis (IA) occur annually with increasing mortality rates. (Bongomin *et al.* 2017). Recently, *A. fumigatus* has been included on the watch list in the CDC publication Antibiotic Resistance Threats in the United States, 2019 due to the worldwide emergence of azole resistance (Garcia-Rubio *et al.* 2021). This fungal infection increases heavily impacts the population of immunocompromised patients; thus, there is a need for more effective drug therapy. This chapter aimed to address the increasing azole resistance towards respiratory infections by assessing the effectiveness of routine and salvage therapy azoles voriconazole and posaconazole. Also, resistant isolates were further investigated for the presence of *cpy51A* mutations associated with azole resistance in pathogenic *Aspergillus* species.

Voriconazole and posaconazole are broad-spectrum triazole antifungal agents used to treat invasive fungal infections (Sienkiewicz 2016). Voriconazole is used for routine therapy and posaconazole as salvage therapy on *Aspergillus* infections (Jenks and Hoenigl 2018); however, voriconazole is the first-line agent for the treatment of IA and resistance to these agents is emerging (Perlin 2017). The *Aspergillus* isolates in this study recorded 6% resistance and showed 61% intermediate/weakened susceptibility to voriconazole, with 33% susceptible isolates. All thirty-six isolates were resistant to posaconazole (Figure 4.6). These results raise concerns as azoles have improved the treatment of IA and decreased mortality rates (Jenks and Hoenigl 2018). Therefore, due to emerging resistance, more efforts are needed to further investigate solutions to decrease mortality and morbidity. In a single-centre study in Brazil, Zoran *et al.* (2018) investigated resistant profiles for *Aspergillus* isolates, of which *A. fumigatus* represented 74% of the 228 isolates, showing high MIC values to at least one azole, although none showed complete resistance. Van De Veerdonk *et al.* (2017) found triazole resistance in 16% of patients with influenza-associated IA and the detection of resistance to voriconazole was highly associated with treatment failure. In this study, not many isolates of *A. fumigatus* were resistant to voriconazole; however, more than half (58%) showed moderate susceptibility (> 0.25 – 2.0 ug/ml). Thus *Aspergillus* susceptibility to voriconazole reported from all of the above investigations, as a first-line therapy azole, requires vigilance for triazole-resistant

strains, especially since *A. fumigatus* is responsible for over 90% of cases of invasive aspergillosis (Araujo and Rodrigues 2004a; Jenks and Hoenigl 2018).

In this study, twenty-five per cent of the *A. niger* isolates were resistant to voriconazole (Figure 4.7) and showed increased resistance to voriconazole (1.0 – 1.5 ug/ml) (Table 4.3) as well, when compared to *A. fumigatus* and *A. flavus*. This is especially significant because the only species resistant to voriconazole and posaconazole in this study are two *A. niger* strains (isolates 12 and 30). Person *et al.* (2010) reported *A. niger* in the lung histopathology of a chronic obstructive pulmonary disease patient. In that study after 4 weeks of voriconazole therapy, chest scans showed a significant progression of the infection, although *A. niger*, unlike *A. fumigatus* and *A. flavus*, were less commonly considered a cause of invasive aspergillosis (IA). The virulent nature of *A. niger* is widely confirmed but is lower when compared to other *Aspergillus* species and can produce severe pulmonary disease. Still, it is rarely reported as a cause of invasive aspergillosis (Person *et al.* 2010). Trovato *et al.* (2021) also described a case of invasive pulmonary aspergillosis by *A. niger* in a patient with COVID-19 pneumonia and acute respiratory distress syndrome. Invasive pulmonary aspergillosis, a complication in patients with severe respiratory syndromes, recently showed a correlation with COVID-19 pneumonia, and the clinical characteristics of COVID-19-associated pulmonary aspergillosis (CAPA) have been described. Unfortunately, infections by the *Aspergillus* genus are often diagnosed in post-mortems because of diagnostic delays and a rapid worsening of respiratory conditions. This developing azole resistance in *A. niger* strains is also evident in cluster analysis in Figure 4.10 where the isolates recorded 14.3% resistance and 71.4% intermediate susceptibility to voriconazole. *A. niger* isolates show only 14.3 % susceptibility to voriconazole and 100% resistance to posaconazole.

It is well documented that *A. fumigatus* is the most commonly isolated species from respiratory infections (Jenks and Hoenigl 2018), with azole resistance recorded as 1.5% in the United States (Berkow *et al.* 2018) and resistance extending beyond Europe to other continents (Resendiz Sharpe *et al.* 2018). *A. flavus*, the second most common cause of aspergillosis (Pappas *et al.* 2010), has shown low resistance rates (Pfaller *et al.* 2015; Sharma *et al.* 2018), although more in tropical and semi-arid climates. However, it has to be noted that *A. niger* made up 14.3% of *Aspergillus* species recovered from pulmonary aspergillosis infections in patients (n=71) with HIV in Northern India (Kaur *et al.* 2017), in this study, 22% of the isolates from respiratory infections (n = 36), collected between July 2015 and June 2016 from the

Inkosi Albert Luthuli Hospital in Kwa-Zulu Natal. Although no data for the HIV status of patients were available for this study, many of these isolates could be from HIV-positive patients due to the region this hospital serves. It is significant to mention that *A. niger* is emerging in respiratory infections and recording lower susceptibility, as found herein with 25% resistance to Voriconazole and 100% resistance to Posaconazole. Although *A. fumigatus* is the most commonly isolated species from respiratory infections and increasing azole resistance (Arendrup *et al.* 2017) towards *A. niger,* may be the new challenge in not so distant future if not monitored.

The resistance of all isolates to posaconazole is a serious concern as the survival of immune-compromised patients with invasive aspergillosis depends on the availability of multiple options for antifungal drugs (Herbrecht *et al.* 2002). Thus, the resistance observed with posaconazole threatens the effectiveness of therapy. Rodriguez-Goncer *et al.* (2018) reported that first-line therapies for CPA (itraconazole and voriconazole) are often curtailed due to toxicity or the development of drug resistance and posaconazole is usually the alternative for these patients. However, in this study, all three species of respiratory-related *Aspergillus* isolates showed resistance to posaconazole (Figure 4.6). Resistance against posaconazole is described as any growth above the concentration of 0.25 µg/ml and *A. fumigatus* isolates in this study recorded growth at much higher concentrations (6.0 – 16.0 µg/ml) (Table 4.2), for *A. niger* between 8.0-12.0 µg/ml (Table 4.3) and 6.0 µg/ml for *A. flavus* (Table 4.4) which raise serious concerns. Although voriconazole remains the standard of care and is recommended by international guidelines for the primary treatment of invasive aspergillosis, concerns exist about acute toxicities (Patterson *et al.* 2016; Ullmann *et al.* 2018) and posaconazole is thus the alternative. Maertens *et al.* (2021) attempted to assess posaconazole for the primary treatment of invasive aspergillosis and supported that using posaconazole is a first-line treatment for the condition The Etest method for the antifungal susceptibility testing has recorded over 90% success with triazole testing with sharp inhibition ellipses and easy interpretation, however the FDA has not approved any antifungal strip for use in clinical mould testing(Espinel-Ingroff, Cantón and Pemán 2012).

Ashu *et al.* (2017), in genetic analysis of the global population of *A. fumigatus*, to understand the molecular epidemiology of the pathogen to impact effective control, found *A. fumigatus* isolates belonging to different gene clusters. The triazole-susceptible and triazole-resistant populations showed other structures consistent with antifungal drug pressure. The study results suggested that global populations of *A. fumigatus* are also shaped by antifungal drug selection

that drives the clonal expansion of genotypes resistant to multiple triazole drugs. Interestingly, in Table 4.2 (n=26), 38% of *A. fumigatus* strains show susceptibility for voriconazole at 0.38µg/ml, 7% at 0.25µg/ml and 4% each at 0.19 and 0.5 µg/ml. This could very likely be *A. fumigatus* genetic clusters in operation. Table 4.3 shows the same pattern of clusters for *A. niger*.

As discussed in Section 2.1(Chapter 2), the rapid growth of these clinical isolates at 37℃ and 30℃ suggests adapting the species from environmental isolates to human pathogens. This is further possible evidence that resistant isolates emerge due to the widespread use of agricultural azole antifungals (Verweij *et al.* 2009) and adaptation to temperature changes. Azoles are commonly used for crop protection or material preservation with some fungicides found to have a molecular structure very similar to that of medical triazoles (Verweij 2009; Snelders *et al.* 2012). The fungus is believed to develop mutations that confer resistance to fungicides due to the molecule's structural similarity with the medical triazoles that become inactive (Seyedmousavi *et al.* 2014). Mutations in the *cpy51A* gene represent common mechanisms for azole resistance in clinical *Aspergillus* isolates.

Garcia-Rubio *et al.* (2021) reported that azole drugs are the largest group of 14-α sterol demethylation inhibitor fungicides used in agriculture and clinical practice. As plant-pathogenic moulds share their natural environment with fungi that cause opportunistic infections in humans, both are exposed to demethylase inhibitor (DMI) fungicides, including imidazole and triazole drugs. As a result, several species have become resistant to this drug class (Garcia-Rubio *et al.* 2021). With the global increase in the use of DMI fungicides, a rise in the number of *A. fumigatus* azole-resistant isolates has been reported (Garcia-Rubio, Cuenca-Estrella and Mellado 2017). Azole drugs act by inhibiting the activity of *cyp51* enzymes, the azole target. Many filamentous fungi, particularly ascomycetes, harbour *cyp51* paralogous genes encoding these enzymes (Becher and Wirsel 2012). In *A. fumigatus*, the azole target 14-α sterol demethylase is encoded by two paralogous genes (*cyp51A* and *cyp51B*), with *cyp51* mutations resulting in acquired azole resistance, most often *cyp51A*; thus, any cost associated with a change in the protein might be eluded by the other wild-type paralogs with unchanged enzyme activity (Hawkins *et al.* 2014).

Two main mechanisms of azole resistance have been identified, (i) mutations in the *cyp51* target resulting in decreased enzyme affinity for inhibitors and (ii) overexpression of the *cyp51* target gene caused by insertions in the predicted promoter regions. Both azole resistance

mechanisms appear in different *cyp51* combinations resulting in various azole susceptibility profiles (Berger *et al.* 2017; Garcia-Rubio, Cuenca-Estrella and Mellado 2017). In *A. fumigatus*, the different susceptibility profiles depend on the specific *cyp51A* amino acid substitution (Camps *et al.* 2012), as is the case in this study with the mutations involving amino acids, histidine and leucine (Figure 4.9). Also, *A. fumigatus* strains with promoter integrations (tandem repeat [TR]) and *cyp51A* point mutations (TR_{34}/L98H, TR_{34}/L98H/S297T/F495I, TR_{46}/Y121F/T289A, and TR_{53}) normally show a multi-azole resistance phenotype (van der Linden *et al.* 2013). TR_{34}/L98H mutation is recorded in isolates in this study as it is the major mutation associated with azole resistance (Table 4.5) and compared to the azole MIC analysis for the 26 *A. fumigatus* strains and emerging resistance to voriconazole. Garcia-Rubio *et al.* (2021) found the more frequent *A. fumigatus* mechanism of azole resistance involves the overexpression of the *cyp51A* gene, at times together with mutations (TR_{34}/L98H,) and is associated with the environmental route and the extended use of DMI fungicides in crop protection (Berger *et al.* 2017). In this study, the 26 *A. fumigatus* isolates were resistant to posaconazole but did not show resistance to voriconazole; however, 73% of the 11 mutated strains showed weakened susceptibility (≤0.25 µm) to Voriconazole as can be seen in Table 4.5. These strains could develop resistance, noting that their susceptibility was at higher concentrations, between 0.50 to 1.50 µm, for six isolates (8, 15, 23, 26, 27 and 35) (Table 4.2). The *cpy51A* likely impacts emerging resistance as 42% of the wild-type isolates also showed intermediate susceptibility.

There is also a strong appreciation that stress responses promote drug adaptation which does not necessarily lead to clinical failure but can lead to higher-level resistance and diminished clinical response by the emergence of stable genetic alterations that confer drug resistance (Perlin 2015). Brown and Goldman (2016) observed that although most fungi have evolved to thrive at ambient temperatures, fungal adaptability to stress experienced within the human host is a prerequisite for the pathogen's survival and virulence mechanisms. *Aspergillus fumigatus* considered a mesophile displays optimal growth at 37°C and is thermotolerant up to 50°C (Robert and Casadevall 2009), showing the capacity of *A. fumigatus* to sense and respond to stressors and is thus fundamental to pathogenesis and antifungal tolerance. Despite the increased frequency of life-threatening diseases, a limited number of antifungal drugs are available and with *cpy51A* gene mutations impacting mechanisms for azole resistance in *Aspergillus* isolates together with the adaptations for fungal survival within the host, there is an urgent need for new strategies to combat fungal infections and to develop novel combination

antifungal therapies. Exploring novel, non-toxic agents that could have effects in combination or alone, becomes an urgent need.

Exposure of the *Aspergillus* isolates to the potential antifungal agents using the Biolog antimicrobial panel resulted in 83% susceptible isolates, as seen in Figures 4.2 and 4.8. Three *Aspergillus fumigatus* (8, 9 and 15) strains and three *Aspergillus niger* (10, 12 and 18) strains, showed resistance to the compounds (making up 17%). The *A. fumigatus* isolates revealed stronger resistance than the *A. niger* isolates, as shown by the yellow colour on the heat map. Spore inoculation of the wells in the panels was approximately 75% transmittance; thus, the number of spores could not be the same for every isolate. This is likely the reason for the slightly erratic pattern within the concentrations based on the absorbance readings for specific compounds. All absorbance values for susceptible isolates were below the absorbance of 0.5 nm, with most compounds showing a strong control of fungal growth (< 0.30 nm). The significant number of isolates susceptible to the 24 tested compounds (although in varying degrees of susceptibility) shows promise in the quest for new alternative therapies against fungal infections treatment that could reduce mortality rates as high as 90% in invasive fungal infections due to limited treatment (Wambaugh *et al.* 2020). When analysing the six strains that showed resistance to the compounds explored with antifungal potential, it is noted that three *A. fumigatus* strains (8, 9 and 15) are part of cluster 1 in the phylogenic tree and possibly share a common genetic make-up resulting in resistance, Figure 4.10.

Invasive fungal infections are a growing problem in severely immunocompromised patients and mortality rates from invasive fungal infections remain unacceptably high. Treatment of patients with a compromised immune system is complicated further by delayed diagnosis, antifungal resistance and toxicity of antifungal agents. The steady increase in the population of immunocompromised individuals and the increased resistance to fungal pathogens urges the need for antifungal therapeutics. Antifungal agents that isolates were susceptible to in this investigation could be explored for combination therapy, as monotherapy using azoles has led to resistance after initial success (Maertens *et al.* 2021). Wambaugh *et al.* (2020) tested a combination of drugs with fluconazole for therapy, revealing synergistic and antagonistic drug interactions in treating systemic fungal infections in mice. This study suggested that to boost efficiency and combat resistance in infections, combine antifungal treatments that could potentially lead to the elimination of the pathogen rather than slow its progression. Antifungal

drugs are usually fungistatic of fungal cells rather than fungicidal therefore, patients remain on treatment for extended periods (Wambaugh *et al.* 2020). This is a serious challenge with the weakened state among immunocompromised individuals, as the inherent toxicity of the treatment compounds further pressurizes their immune system.

Interestingly, fluconazole was more effective in combination with 40 other tested compounds, doubling the survival rate of mice with severe fungal infections when fluconazole was paired with dicyclomine in an *in vivo* trial. These combination treatments could be applied or tested with other azoles in infections showing resistance to azoles alone. Compounds in this investigation that isolates were susceptible to, could be paired with azoles to treat fungal infections effectively.

In this study, berberine and blasticidin hydrochloride from the compounds that were assessed were also part of the Wambaugh *et al.* (2020) investigation with fluconazole, resulting in an antagonistic and synergistic effect, respectively. Berberine is a plant extract that reduces blood sugar levels (Vuddanda, Chakraborty and Singh 2010; Zhang *et al.* 2010). Both compounds have low-toxic at therapeutic concentrations and therefore are suitable candidates for trials in combination therapy, with azoles showing toxicity or resistance in monotherapy. Except for the six resistant *isolates*, all the remaining (30) isolates were susceptible to berberine and blasticidin hydrochloride. The potential of these compounds could be investigated further for their effect on *Aspergillus* respiratory infections. Blasticidin hydrochloride is a gene selection antibiotic effective in eucaryotic cells, inhibiting the peptide bond of ribosomes (Powers *et al.* 2021). The increased therapeutic range and exploring effective new compounds in combination therapy is important, which could reduce the resistance rate and increase potency (Robbins, Caplan and Cowen 2017; Revie *et al.* 2018). Zhang *et al.* (2021) found berberine to be a safe drug due to the low absorption rate of berberine and the results showed that *cyp*51 in the gut microbiota could bind stably with berberine and the addition of voriconazole (a specific inhibitor of *cyp*51) slowed down the metabolism of berberine, which prevented the production of the demethylated metabolites thalifendine and berberrubine.

Sodium metasilicate, 6-azauracil and sodium benzoate are significant as they are all antifungal compounds in fungicides to prevent agricultural plants from fungal invasion. In our analysis, 83% of the isolates were susceptible to these 3 compounds (Figure 4.8). These compounds provide the opportunity for testing their effect on these clinical strains to be explored, as sodium

metasilicate is already used effectively in fungicides, insecticides and detergents. However, since they are not used for human therapy, toxicity needs to be determined for these compounds. With broad-spectrum antibiotic activity, three other compounds, aminacrine, apramycin sulphate, and chlortetracycline hydrochloride, were effective against 83% of the isolates. They could be analysed further for their potential. Aminacrine is already used as a human therapeutic to treat topical infection caused by fungi (Küng, Fürnkranz and Walochnik 2019).

A. fumigatus is resistant to the three following azoles, fluconazole, propiconazole and miconazole and shows high resistance to fluconazole. Propiconazole is a fungicide that is highly effective in reducing postharvest sour rot of citrus and has favourable toxicological characteristics as a post-harvest treatment (McKay, Förster and Adaskaveg 2012; Zhang *et al.* 2015). Miconazole and fluconazole are used to treat yeast infections; fluconazole is used as a preventative agent for fungal infection, i.e., candidiasis in individuals with a weak immune system caused by HIV progressed to AIDS. This is where combination therapy would play a life-saving role of traditional azoles and new compounds as boosters, as shown by Wambaugh *et al.* (2020).

The resistant strains make up 17% of the isolates assessed for sensitivity in the presence of the 24 potential antifungal agents, including those against *A. niger* (isolate 12) (Figure 4.8) which is also resistant to voriconazole and posaconazole (Table.4.3). The three resistant *A. fumigatus* strains (9, 10 and 15) (Figure 4.8) are resistant to posaconazole however they are still susceptible to voriconazole (Table 4.2). These results show a growing concern where salvage therapy azoles are also becoming ineffective during therapy. The strong inhibitory effect of non-toxic agents, berberine and blasticidin hydrochloride, generally not used for fungal treatment explored in this study, provide great potential for a new candidate for both individual and combination therapy in the treatment of aspergillosis infections.

CHAPTER FIVE: GLIOTOXIN AS A VIRULENCE FACTOR IN THE PATHOGENESIS OF *ASPERGILLUS* INFECTIONS

5.1. Introduction

Filamentous fungi produce different mycotoxins, including aflatoxin, ochratoxin, fumonisin, zearalenone, citrinin, patulin and gliotoxin, as a survival mechanism in various environments (Arias *et al.* 2018). Aflatoxin is the most documented of all mycotoxins and is detected in most agricultural commodities. They can be acutely toxic, carcinogenic, mutagenic, teratogenic and immunosuppressive to most mammals (Smith 2020). *Aspergillus* species, a human pathogen, produce gliotoxin. *A. fumigatus* is the main genus responsible for the dominant expression of gliotoxins. Gliotoxin (GT), an epipolythiodioxopiperazine (ETP) class of toxin, is a potent immunosuppressive mycotoxin and has been suspected as one of the virulence determinants among various secondary metabolites produced by the species (Kwon-Chung and Sugui 2009). It is known to alter the immune response due to its ability to inhibit immune function, exert toxic effects on phagocytic cells and T-lymphocytes at low concentrations *in vitro* and induce apoptotic cell death in macrophages (Hof and Kupfahl 2009; Nouri *et al.* 2015; Pena 2015). Its immunosuppressive activity may facilitate fungal persistence and colonization of tissues and thus is considered a virulence factor in human and animal aspergillosis (Carberry *et al.* 2012; Hussein, Sulaiman and Hashim 2017).

The frequency of gliotoxin-producing *A. fumigatus* strains is higher among clinical isolates than environmental isolates (Kosalec and Pepeljnjak, 2005). Pathogenic species of the genus *Aspergillus* often infect severely immunocompromised patients as well as those with more moderate levels of immunosuppression, such as patients with chronic obstructive pulmonary disease (COPD) or critically ill intensive care patients, in whom it can cause severe invasive infections with high mortality rates (Kousha, Tadi and Soubani 2011). Among *Aspergillus* species, *A. fumigatus*, which produces high amounts of gliotoxin, colonizes the cystic fibrosis (CF) airways and may significantly impact the microbiome composition with potential clinical implications (Reece *et al.* 2018). This has a dual effect on the individuals infected with fungal disease plus the impact of toxification.

Over 90% of the *A. fumigatus* strains isolated from invasive aspergillosis cases in tertiary-care cancer centres express gliotoxin (Lewis *et al.* 2005a). In a study that correlated gliotoxin production and virulence, Reeves *et al.* (2004) found that gliotoxin was produced by over 95% of pathogenic isolates of *A. fumigatus*. Therefore, as invasive aspergillosis becomes increasingly prevalent and the population of immunocompromised patients increases, the presence of gliotoxin makes disease progression and treatment complex and complicated (Bok 2006; Cramer 2006). Kwon-Chung and Sugui (2009a) confirmed that the specific gene cluster was responsible for the biosynthesis of gliotoxin and *gliP* or *gliZ* genes essential for gliotoxin expression (Cramer 2006; Kupfahl *et al.* 2006; Sugui 2007). When the gliP gene, which encodes the essential non-ribosomal peptide synthetase of the gliotoxin biosynthetic gene cluster, is deleted from *A. fumigatus*, the mutant strain does not biosynthesize gliotoxin and exhibits attenuated virulence in mouse/mice models (Sugui 2007; Spikes *et al.* 2008). Similarly, the deletion of laeA, a positive regulator of several *A. fumigatus* secondary metabolites, including gliotoxin, had a negative effect on virulence (Perrin *et al.* 2007). These findings suggest that gliotoxin contributes to increased *A. fumigatus* virulence (Raffa and Keller 2019). This could open new investigations where gliotoxin is considered a specific biomarker for *Aspergillus* infections (Vidal-García *et al.* 2018).

Aspergillus fumigatus contributing virulence to IA is likely not due to a single factor but rather a combination of interactions of various molecules and biological properties of the fungus (Bok 2006). Growth characteristics such as its high spore concentration in the air and its faster growth relative to any other airborne fungi at 40°C contribute to its virulence (Tekaia and Latgé 2005; Bok 2006). Ghazaei (2017) adds that fungal virulence depends on factors like the immune status of patients and biological features of fungi, as in the case of *A. fumigatus* and its thermotolerance in thriving at temperatures up to 55°C and thus contribute to its virulence. Arias *et al.* (2018) reported that gliotoxin is the better-characterized virulence factor, for it can generate reactive oxygen species (ROS) due to the disulfide bridge present in its structure.

In nature, fungal toxins are produced as a competitive arsenal against other microorganisms, including bacteria. These interactions may also contribute to increased pathogenicity, which could significantly impact the treatment of these pathogens in patients with CF (Reece *et al.* 2018). Through their long common history, bacteria and fungi have developed receptor systems to detect each other's presence (Svahn *et al.* 2014). Reece *et al.* (2018) showed that these co-

infecting microbes contribute to an altered inflammatory response that could aid evasion of the immune system and chronic colonization resulting in detrimental effects on a CF patient's health status. *P. aeruginosa* was identified in 54.1% of CF patients with persistent *A. fumigatus* infection and these co-colonized patients showed decreased lung function compared with patients clear of both pathogens (Amin *et al.* 2010). Fungi may detect the presence of bacteria by the metabolites they release into the environment and the most studied are known as pathogen-associated molecular patterns (PAMPS) (Kawai and Akira 2010). PAMPs are highly conserved molecular structures, typically essential components of the pathogens' cytoplasmic barrier, acting as general recognition targets for detecting their presence (Ray *et al.* 2013). Molecules that have been classified as PAMPs include lipopolysaccharides (LPS) from Gram-negative bacteria, lipoteichoic acid (LTA) from Gram-positive bacteria, and peptidoglycan (PG) associated with both gram-positive and gram-negative bacteria. Svahn (2014) suggested that the possibility of increased virulence of *A. fumigatus* during co-infection may be due to the induction of gliotoxin by bacteria-associated molecules such as PAMPS.

In vitro, it has been demonstrated that adding PAMPs to cultures of *A. fumigatus* increased their secretion of gliotoxin by up to 65%, thus further showing the correlation between the presence of PAMPs and the production of gliotoxin (Svahn *et al.* 2014). While the condition of the host immune response is the most determining factor in the development of aspergillosis, the presence of bacteria-associated molecules can increase the fungal toxin production and thereby increase its virulence. This further explains the increase in virulence of *A. fumigatus* during bacterial co-infection, which is important for the transition from colonization to invasiveness in pulmonary disease (Svahn *et al.* 2014).

This study aimed to determine gliotoxin expression in thirty-six *Aspergillus* species isolated from patients with respiratory infections from the Inkosi Albert Luthuli Hospital in Durban, Kwa-Zulu Natal and assess if bacteria-associated molecules, PAMPS (lipopolysaccharide, peptidoglycan and lipoteichoic acid) play any role in the expression of gliotoxin.

5.2. Methodology

5.2.1. Sample collection

Thirty-six filamentous fungal samples isolated from respiratory patients from the Inkosi Albert Luthuli Hospital between July 2015 and June 2016 were grown on Malt Extract Agar plates (MEA) (Merck, Germany) and incubated at 37°C for 48 hours. Cultures were prepared using spore suspensions in saline water and stored at -70℃. Dilutions (10^6 /ml) were prepared for gliotoxin analysis.

5.2.2. Preparation of gliotoxin expression and PAMPS induction

One plaque of each *Aspergillus* isolate was transferred to duplicate sterile 50 ml falcon tubes containing 10 ml of RPMI medium (Sigma Aldrich) and incubated at 37°C for 72 hours at 150 rpm as described by Svahn *et al.* (2014).

A further set of duplicate tubes were prepared as described above, containing 300 ul of each PAMPS mix (LPS, LTA and PG) (Sigma Aldrich) at a 100 ug/ml concentration resuspended in dimethyl sulfoxide (DMSO) (Sigma Aldrich). Control cultures were treated with 300 µl of DMSO solution only. After incubation, the falcon tubes were centrifuged at 10 000 g for 10 min to separate solid material and the filtrate was decanted into sterile falcon tubes and stored at -80°C for analysis.

5.2.2.1. Preparation of Gliotoxin standards

The gliotoxin standard was obtained through a commercial source (Sigma Aldrich) The standards were prepared for use by dissolution in 50% methanol acidified with 0.15 M formic acids. Standards were kept at -70ºC until use. Table 5.1 shows the dilutions prepared from a 10 mg/L stock solution.

Table 5.1: Gliotoxin standards at different dilutions used for RP-HPLC

Gliotoxin concentration (mg)	**The volume of 50% methanol acidified with 0.15 M formic acid (**mL)	**Final Concentration of gliotoxin (mg/l)**
5.00	1000	5.00
1.00	1000	1.00
0.50	1000	0.50
0.25	1000	0.25
0.10	1000	0.10
0.05	1000	0.05

5.2.2.2. Preparation of concentration of PAMPS

The PAMPS, lipopolysaccharide (LPS), peptidoglycan (PG) and lipoteichoic acid (LPS) were obtained from Sigma-Aldrich and isolated from *Escherichia coli* (LPS) or *Staphylococcus aureus* (LTA and PG). PAMPS were prepared in dimethyl sulfoxide (DMSO) at a concentration of 100 µg/ml.

5.2.2.3. Extraction of gliotoxin

Extraction of gliotoxin followed the procedure developed by Central Analytical Facilities (CAF), LC-MS Unit at Stellenbosch University. One millilitre of the filtrate (sample) was transferred into an Eppendorf tube and one millilitre of the solvent (25% methanol and 75% MilliQ water) was added and microfuged at 13 000 g for 5 minutes. One millilitre of the top layer was transferred to analysis vials for the gliotoxin analysis.

5.2.3. Thin-layer chromatography (TLC)

The Gliotoxin standard (Sigma Aldrich) was prepared for use by dissolution in 50% methanol acidified with 0.15 M formic acids. Standards were kept at -70ºC until use (Table 1). Gliotoxin was detected using TLC plates as per Nouri *et al*. (2015). Twenty microliters of the test samples and the Gliotoxin standard (Sigma) were spotted on TLC plates, Silica gel 60 F_{254} 20 X 20 cm (Merck) in a methyl chloride: methanol (97:3 v/v) solvent system as the mobile phase. The plates sprayed using 5% silver nitrate in ethanol: water (95:5 v/v) were air-dried and gliotoxin was visualized under UV light at a wavelength of 366 nm.

5.2.4. High-performance liquid chromatography (HPLC) analysis

High-performance liquid chromatography analysis for the quantification of gliotoxin was carried out by Central Analytical Facilities (CAF), LC-MS Unit at Stellenbosch University. A Waters UPLC BEH C_{18} column (2.1 x 100 mm) was used with a 20 µl injection volume of the standard and each test sample. The flow rate was set at one ml/min and the UV-900 detector was operated at a wavelength of 254 nm with a run time of 6 minutes. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) under gradient elution conditions.

5.2.5. Sequencing analysis for gliotoxin genes

5.2.5.1. DNA isolation

Cultures were grown on malt extract agar plates for 72 hours at 37°C. Two plaques each were transferred to 100 ml malt extract broth and incubated at 37°C, for 18 hours at 150 rpm. The mycelia were centrifuged for 10 min at 5000g and DNA was extracted from the pellet using Melo *et al.* (2006) protocol, with minor modifications as described in Section 2.2.3 (Chapter Two). The genomic DNA was quantified using a NanoDrop 2000 analyzer (Thermo Scientific) and analysed by agarose gel electrophoresis before PCR (Hansen *et al.* 2007).

5.2.5.2. PCR amplification

The PCR assay was performed with 5 µl of the test sample in a total reaction volume of 50 µl consisting of 1.0 µl of each primer (F :) and (R :), 25 µl of Taq/Readymix and 18 µl of ultrapure water. Thirty cycles of amplification were performed in a thermocycler with each cycle consisting of a denaturation step at 95°C for 30 seconds, an annealing step at 58° C for 30 seconds and an extension step at 72° C for 1 min, with a final extension at 72°C for 10 min. After amplification, the products were stored at -20°C until use.

Table 5.2: Primers associated with gliotoxin production

Primer	Sequence
gliP-1F	5’-GACCTCTGCCAGCTTTTTGA-3’
gliP-1R	5’-GGGATGATCGAAGATGTTGC-3’
gliZ-GZINTF	5’AAGGGCCGGTAGTCTACCTCTTC3’
gliZ-GZINTR	5’ CGATCTGGTAGCTGCCCAGCTGGAAG3

5.2.5.3. Blast analysis

The PCR products from successfully applied samples were purified using DNA Purification Kit for sequencing (Qiagen, USA) following the manufacturer's manual. Sequencing of PCR samples was performed by Inqaba Biotech (South Africa) on a Sanger Sequencing Platform. Sequence analysis was conducted on National Center Biotechnology Information (NCBI) online at (http:// www.ncbi.nlm.nih.govusing Nucleotide and Protein Blast function to confirm if they match any synthetic gliotoxin genes.

5.3. Results

5.3.1. Gliotoxin expression

Preliminary screening of all thirty-six *Aspergillus* isolates was performed after incubating the cultures at 37℃ for 48 hours. Extraction for gliotoxin was followed by HPLC analysis for gliotoxin expression (Figure 5.1). As expected, gliotoxin was detected only in *A. fumigatus* isolates (Table 5.3). Gliotoxin was detected in 58% of the 26 *A. fumigatus* isolates. The concentrations of gliotoxin detected were between 0.12 and 2.6 mg/ml, with a mean of 0.60 mg/l. However, a large number (42%) of the *A. fumigatus* isolates did not produce gliotoxin (Table 5.3).

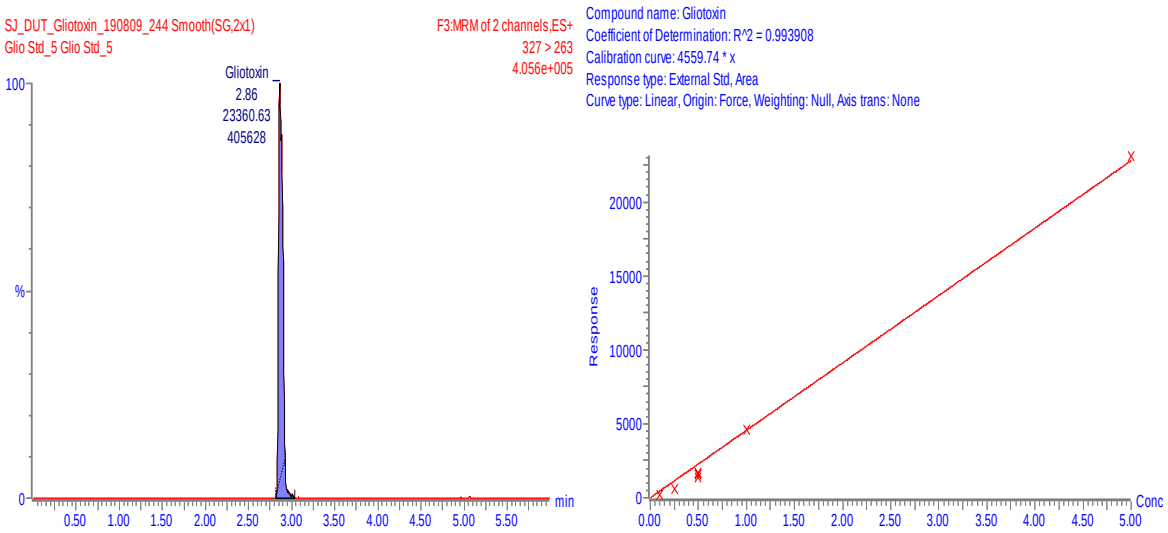


Figure 5.1: Chromatograms and calibration curves (limit of quantification (LOQ) for gliotoxin is 0.1 mg/L).

Table 5.3: Gliotoxin (mg/l) expression in the 36 *Aspergillus* isolates after 48-hour incubation at 37℃.

Isolate	**Gliotoxin (mg/l)**			
	sample 1	**sample 2**	**Average**	**SD**
A. fumigatus	0.47	0.35	0.41	0.08
A. fumigatus	0.58	0.54	0.56	0.03
A. fumigatus	0.42	0.45	0.44	0.02
A. fumigatus	ND	ND		
A. fumigatus	ND	ND		
A. fumigatus	0.23	0.21	0.22	0.01
A. fumigatus	0.43	0.29	0.36	0.10
A. fumigatus	0.33	0.30	0.32	0.02
A. fumigatus	0.41	0.33	0.37	0.06
A. fumigatus	0.27	0.25	0.26	0.01
A. fumigatus	0.14	0.10	0.12	0.03
A. fumigatus	ND	ND		
A. fumigatus	ND	ND		
A. fumigatus	ND	ND		
A. fumigatus	ND	ND		
A. fumigatus	ND	ND		
A. fumigatus	0.15	0.25	0.2	0.07

A. fumigatus	ND	ND		
A. fumigatus	ND	ND		
A. fumigatus	1.35	1.12	1.24	0.16
A. fumigatus	0.90	0.94	0.92	0.03
A. fumigatus	2.81	2.39	2.6	0.30
A. fumigatus	ND	ND		
A. fumigatus	0.25	0.24	0.25	0.01
A. fumigatus	ND	ND		
A. fumigatus	0.66	0.76	0.71	0.07
A. niger	ND	ND		
A. niger	ND	ND		
A. niger	ND	ND		
A. niger	ND	ND		
A. niger	ND	ND		
A . niger	ND	ND		
A. niger	ND	ND		
A. niger	ND	ND		
A. flavus	ND	ND		
A. flavus	ND	ND		
			0.60 (mean)	

*ND – not detected

*SD – standard deviation

5.3.2. Induction of gliotoxin by bacteria-associated molecules

The 36 isolates were exposed to the same conditions of nutrient content, duration of incubation and temperature of incubation with PAMPS and without PAMPS (lipopolysaccharides, peptidoglycan, and lipoteichoic acid). Control cultures to detect gliotoxin production were the isolates for each species exposed to PAMPS (DMSO only and no PAMPS).

Treatment of cultures with PAMPS showed that of 58% of *A. fumigatus* strains that produced gliotoxin before exposure to PAMPS, 40 % of the isolates showed an increase in gliotoxin concentration (between 18 and 88%) after exposure to PAMPS and 20% showed a decrease in concentration (between 28 and 52%). Interestingly, in isolates 4 and 5 gliotoxins were detected after exposure to PAMPS, but no gliotoxin was detected in the preliminary screening. No

gliotoxin was detected in the remaining isolates (40%), although gliotoxin was detected in the isolates before exposure to PAMPS.

Table 5.4: HPLC of gliotoxin (mg/L) in *A. fumigatus* isolates before and after exposure to PAMPS (lipopolysaccharides (LPS), peptidoglycan (PG), lipoteichoic acid (LTA)).

	Gliotoxin (mg/l) no PAMPS		**Average**	**Gliotoxin (mg/l) with PAMPS**		**Average**	**% increase (↑) decrease (↓)**
Blank	ND	ND		ND	ND		
Control:	1.12	1.10	1.11	ND	ND		
A.				(DMSO only)	(DMSO only)		
fumigatus							
culture only							
1	0.47	0.35	0.41	ND	ND		
2	0.58	0.54	0.56	ND	ND		
3	0.42	0.45	0.44	0.20	0.22	0.21	↓ (52%)
4	ND	ND	0.0	0.18	0.20	0.19	↑ (100%)
5	ND	ND	0.0	1.82	1.71	1.77	↑ (100%)
6	0.23	0.21	0.22	0.49	0.43	0.46	↑ (52%)
7	0.43	0.29	0.36	ND	ND		
8	0.33	0.30	0.32	2.59	2.61	2.6	↑ (88%)
9	0.41	0.33	0.37	0.19	0.25	0.22	↓ (41%)
11	0.27	0.25	0.26	ND	ND		
14	0.14	0.10	0.12	ND	ND		
23	0.15	0.25	0.2	ND	ND		
24	ND	ND		ND	ND		
26	ND	ND		ND	ND		
27	1.35	1.12	1.24	0.86	0.91	0.89	↓ (28%)
28	0.90	0.94	0.92	1.03	1.20	1.12	↑ (18%)
29	2.81	2.39	2.6	ND	ND	0.0	
31	ND	ND		ND	ND		
34	0.25	0.24	0.25	ND	ND	0.0	
35	ND	ND		ND	ND		
36	0.66	0.76	0.71	0.83	0.91	0.87	↑ (18%)

Gliotoxin ND in isolates 15 to 20 with and without PAMPS

Of the twenty-six *A. fumigatus* isolates, 58% produced gliotoxin during the 48-hour incubation at 37℃. Forty percent of those gliotoxin-producing isolates showed an increase in gliotoxin production after exposure to PAMPS while 20 % showed a decrease in gliotoxin concentration. HPLC results confirmed that all eight *A. niger* and *A. flavus* isolates did not produce gliotoxin before and after exposure to PAMPS.

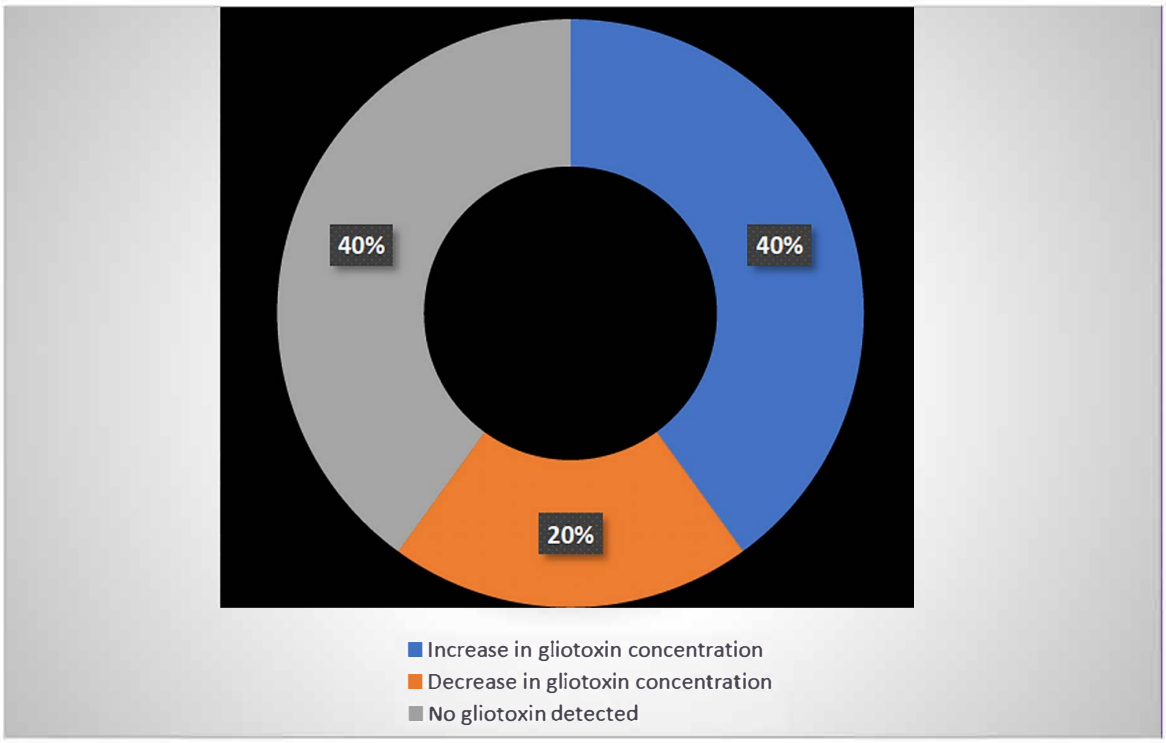


Figure 5.2: Percentage fluctuation of gliotoxin concentration in *A. fumigatus* isolates after exposure to PAMPS at a growth temperature of 37°C for 48 hours.

5.3.3. Gene markers for gliotoxin

Sequence analysis conducted to confirm the matching of gliotoxin genes revealed the results in Table 5.5. The gliP/gliZ genes were not detected in isolates 7, 14 and 34, although there was gliotoxin expression. No gliotoxin was detected in isolates 5 and 19; however, the gliP/gliZ genes were detected in those isolates.

Table 5.5: Detection of gliotoxin and gliP/gliZ genes in the isolates.

Isolate	Gliotoxin detected	gliP/gliZ detected
1 - *A. fumigatus*	+	+
2 - *A. fumigatus*	+	+
3 - *A. fumigatus*	+	+
4 - *A. fumigatus*	**ND**	**ND**
5 - *A. fumigatus*	**ND**	+
6 - *A. fumigatus*	+	+
7 - *A. fumigatus*	+	-
8 - *A. fumigatus*	+	+
9 - *A. fumigatus*	+	+

11 - *A. fumigatus*	+	+
14 - *A. fumigatus*	+	-
15 - *A. fumigatus*	**ND**	**ND**
16 - *A. fumigatus*	**ND**	**ND**
17 - *A. fumigatus*	**ND**	**ND**
19 - *A. fumigatus*	**ND**	+
20 - *A. fumigatus*	**ND**	**ND**
23 - *A. fumigatus*	+	+
24 - *A. fumigatus*	**ND**	**ND**
26- *A. fumigatus*	**ND**	**ND**
27 - *A. fumigatus*	+	+
28 - *A. fumigatus*	+	+
29 - *A. f umigatus*	+	+
31 - *A. fumigatus*	**ND**	**ND**
34 - *A. fumigatus*	+	-
35 - *A. fumigatus*	**ND**	**ND**
36 - *A. fumigatus*	+	+

*ND not detected

5.4. Discussion

The genus, *Aspergillus*, is significant in the pharmaceutical and food industry and the respective industries have benefitted from the biological activity of the secondary metabolites that the fungi synthesize (Arias *et al.* 2018). However, *Aspergillus* has many adverse effects on humans and crops. *A. fumigatus* is not only an ubiquitous environmental fungus but a human pathogen. It is an important pathogenic fungus in clinical settings that can cause diseases like invasive aspergillosis, a highly prevalent fungal infection with a high mortality rate (Wang 2014). *A. fumigatus* produces a few mycotoxins, including gliotoxin, which has been shown to have toxic effects on phagocytic cells and T-lymphocytes at low concentrations *in vitro* (Malekinejad *et al.* 2013). Additionally, *in vivo,* studies have suggested that it impairs the host's defence mechanism during intoxication. Thus, it has been proposed as a virulence factor for IA due to its cytotoxic, genotoxic and apoptotic properties (Bok 2006; Hof and Kupfahl 2009).

Vidal-García *et al.* (2018) reported that for more than a decade, the invasive aspergillosis biomarker, galactomannan, was developed and used in combination with clinical signs and symptoms in the diagnosis of this infectious disease. However, recent diagnostic approaches have been developed with increased accuracy, like PCR-based methods and lateral flow devices to detect an *Aspergillus*-derived protein (White *et al.* 2015). Despite these advances, IA management continues to be challenging due to the increase in at-risk populations, the diversity of the clinical presentation and the lack of standardized diagnostic methods (Lamoth and Calandra 2017). Thus, the quest to accurately diagnose *Aspergillus* infections continues, hence the need to understand new biomarkers with a clear understanding of their limitations and strengths (Maertens *et al.* 2016; Mercier and Maertens 2017) and their role as virulence factors in IA management.

In this chapter, all thirty-six *Aspergillus* species were tested for their ability to produce gliotoxin. Results in Table 5.3 showed that 58% of the clinical *A. fumigatus* isolates produced gliotoxin, supporting Hof and Kupfahl (2009) findings that gliotoxin is produced during infection and thus functions as a virulence factor. Spikes *et al.* (2008) also concluded that gliotoxin production by *A. fumigatus* contributes to virulence in immunosuppressed hosts.

There was no gliotoxin detected in cultures of *A. niger* and *A. flavus.* This was expected as literature shows that gliotoxin is typically produced by *A. fumigatus*. Fifty-eight per cent of the *A. fumigatus* isolates produced gliotoxin at concentrations between 0.12 mg/L and 2.6 mg/L. Kupfahl (2008) found a much lower frequency of gliotoxin-producing isolates among other *Aspergillus* species (*A. niger*, *A. flavus, A. terreus*) than *A. fumigatus*, with a maximum of 0.0021 mg/L gliotoxin concentration produced by clinical *A. fumigatus* strains. In this study, *A. fumigatus* isolate 29 revealed a gliotoxin concentration of 2.6 mg/L and a minimum concentration of 0.12 mg/L in isolate 14. It is interesting to consider the impact, with the mean gliotoxin concentration of 0.60 mg/L in this study, especially with Kupfahl *et al.* (2008) noting a trend towards higher gliotoxin concentrations in isolates of patients that experienced invasive aspergillosis than those that experienced *Aspergillus* colonization. Lewis *et al.* (2005a) also reported higher gliotoxin concentrations (< 0.25mg/L) that induced apoptosis in macrophages. Although this study has a comparatively smaller sample size, it adds significant information on gliotoxin production by clinical isolates and the impacts as a virulent factor of the infection (Reeves *et al.* 2004).

Reeves *et al.* (2004) studied the correlation between gliotoxin production and virulence of *A. fumigatus* on the *Galleria mellonella* larvae. They found that *A. fumigatus* strains that produced higher amounts of gliotoxin were more virulent (produced higher larvae mortality) than those with lower toxin production. This evidence supports gliotoxin as a virulence factor for invasive aspergillosis, although a defined and focused study with sound statistics is required for further confirmation.

Watanabe *et al.* (2004) reported that the composition of nutrients, aeration and temperatures influence gliotoxin production and, thus, differences in the frequency of gliotoxin production in environmental and clinical strains. Alonso *et al.* (2016) found gliotoxin production similar in clinical and environmental strains. The study found environmental strains of *A. fumigatus* were able to grow and produce gliotoxin under physiological conditions similar to those in the lung, demonstrating the ability to adapt to the humid environment of the lungs (Pollmächer and Figge 2015).

To a large extent, the effect of bacteria-associated molecules (lipopolysaccharides, peptidoglycan and lipoteichoic acid) with their pathogen-associated molecular patterns induced an increase in gliotoxin secretion by *A. fumigatus*. Results showed that adding PAMPS to *A. fumigatus in vitro* cultured isolates increased their secretion by up to 65% after a selected incubation period. This study showed that 40% of the gliotoxin-producing isolates demonstrated increases between 18% and 52% after exposure to PAMPS over a 72-hour incubation (Table 5.4). Two isolates (4 and 5) produced detectable levels of gliotoxin after exposure to PAMPS, even though the initial screening showed no gliotoxin production. These findings are supported by Svahn *et al.* (2014). There was an increase in the virulence of *A. fumigatus* during bacterial co-infection and colonization by large numbers of Gram-negative and Gram-positive bacteria in COPD patients. These microorganisms release large quantities of LPS, PG and LTA, suggesting that co-infection provokes the mould to produce more mycotoxins. The decrease in gliotoxin production in 20% of the gliotoxin-producing isolates could reflect differences in detection techniques and culture conditions used for assessing gliotoxin production (Belkacemi 1999; Watanabe *et al.* 2004).

All genes involved in gliotoxin biosynthesis are organized in a cluster within the *A. fumigatus* genome (Patron *et al.* 2007) and the cluster comprises 13 genes (Schrettl *et al.* 2010). The gliotoxin biosynthesis genes are mainly regulated by the zinc finger transcription factor GliZ, a putative transcriptional regulator gene (Bok 2006) and the largest gene in the cluster, *gliP* codes for a non-ribosomal peptide synthetase which catalyzes the first steps of the gliotoxin biosynthesis (Balibar and Walsh 2006). The gliP and gliZ genes were detected in 80% of the isolates that produced gliotoxin, as seen in Table 5.5, and thus confirmed the matching gliotoxin genes. However, for isolates 5 and 19 (Table 5.5), the gliP and gliZ genes were detected, but no gliotoxin was produced. A possible explanation is that other genes that make up the 13 cluster gene for gliotoxin biosynthesis (Schrettl *et al.* 2010) were deficient and biosynthesis was not completed. The gliP and gliP genes were not detected for isolates 7, 14 and 34, but those isolates produced gliotoxin.

Bok (2006) reported that GliZ, a transcriptional regulator of gliotoxin biosynthesis, contributes to *Aspergillus fumigatus* virulence. This investigation identified the gliotoxin gene cluster that identified the genes (gli genes), including gliZ, involved in gliotoxin production. Replacement of gliZ with a marker gene resulted in no detectable gliotoxin production and loss of gene expression of other gliZ cluster genes. The placement of multiple copies of gliZ in the genome increased gliotoxin production.

Wang (2014) analyzed the function of this gene and provided new insights into gliotoxin metabolism in *A. fumigatus*, in that gliA has essential functions in the export of gliotoxin and the self-protection of the fungus from gliotoxin, thereby playing a critical role in virulence. The study suggests that control of the expression of gliA and its related products may facilitate the development of a new strategy for the supportive management of aspergillosis.

GT exerts a broad spectrum of immunosuppressive effects and is present in the sera of patients with IA (Lim *et al.* 2021). One possible mechanism of GT is the suppressive effect on host immune cells: inhibition of cytokine production, apoptosis of immune cells, and reduced activity of cytotoxic T-cells (Kupfahl *et al.* 2006; Arias *et al.* 2018). The gene ZafA regulates GT production, the activation of which is dependent on the availability of zinc and the *AflkhA* strain produced increased amounts of GT and showed increased transcription of GT cluster genes and *zafA* gene, suggesting that LkhA negatively regulates GT production by controlling transcription of GT cluster genes and *zafA* gene in *A. fumigatus* (Lim *et al*. 2021)

Fifty-eight percent of the *A. fumigatus* isolates in this study produced gliotoxin, supporting gliotoxin as a virulence factor in the pathogenesis of *Aspergillus* infections and possibly further indicating the potential of gliotoxin as a biomarker for invasive aspergillosis infections. PAMPS increased gliotoxin production by 52 to 100% in 50 % of the isolates. This is especially significant in respiratory infections, with commonly co-occurring bacterial and *Aspergillus* infections.

CHAPTER SIX: GENERAL DISCUSSION

Fungal diseases, such as aspergillosis, influence the mortality of immunocompromised individuals with respiratory infections. The editorial in National Microbiology (2017) highlighted the issue of fungal infection neglect, and estimated the global prevalence of life-threatening fungal infections to be over 300 million per year, resulting in 1.6 million fatalities (Beardsley et al. 2018). Alarmingly, severe fungal infections are on the rise, and new drugs to treat them are getting into the market at an unacceptably slow rate. As a result, if fatality rates continue to rise or remain stable, infection management from detection and diagnosis through therapy should be addressed.

Aspergillus species are linked to major invasive fungal infections and allergy disorders, with a significant percentage linked to respiratory infections, contributing to total fatality rates of 30-80%. Azole resistance in *Aspergillus* is one of the most challenging clinical mycology concerns. Due to the widespread rise of azole resistance, *A. fumigatus* has been included in the 2019 CDC publication Antibiotic Resistance Threats in the United States(Garcia-Rubio *et al.* 2021). The limitations of conventional therapy, such as toxicity, low bioavailability, and relative inefficacy, are exacerbated by drug resistance formation and spread. Given this context, the emergence of resistance is very concerning.

Many factors contribute to the progression of infection, thus, the clinical outcomes. Host factors such as compromised immune systems, additional comorbidities, invasive disease sites, and treatment factors such as dose regimen, compliance, and drug toxicity all have an influence. Nonetheless, rapid initiation of effective antifungal therapy dramatically decreases mortality in invasive aspergillosis, making identification and characterization of drug resistance in vitro critical in guiding physicians in selecting the optimum antifungal treatment (Columbo et. al. 2017). Clinical success in managing azole resistance is determined by advancements in all aspects, from detection and antifungal therapy to the discovery of novel, non-toxic medicines. As a result, the purpose of this study was to analyze the effect of regular and salvage drug therapy on *Aspergillus* species related to respiratory infections, as well as to investigate the Conventional and molecular identification methods employed and technology like the phenotypic microarray explored for more rapid identification to impact diagnosis and therapy. A phenotypic microarray was also employed for antifungal drug screening, substituting the labour-intensive biochemical assays used in normal analysis to assess the *Aspergillus* species' carbon nutrition profile and better understand resistance patterns.

Denning *et al.* (2020) reported that the prevalence of chronic obstructive pulmonary disease (COPD) is much higher than previously estimated, and mortality may be higher because not all diagnoses of IA are likely made. This calls for improved rapid diagnosis of IA in COPD hospital admissions using culture and non-culture-based techniques to reduce mortality. Invasive and chronic aspergillosis was detected in COPD patients admitted to the hospital (Hammond *et al.* 2020). Positive *Aspergillus* cultures are identified in up to 20% of patients on admission, with 22-67% indicating invasive aspergillosis and the remaining colonization (Guinea *et al.* 2010; Xu *et al.* 2012). This study evaluated the conventional methods of culture and microscopic examination of morphological features, 18S rRNA, and a high throughput phenotypic/biochemical identification system in identifying clinical isolates to address this aspect of identification and diagnosis in the management of IA. Although molecular technologies are becoming more accessible, microscopy and culture techniques remain the principal laboratory tools for first identification. Molecular identification has gained reliability and confidence in results to earn the status of 'gold standard' (Borman *et al.* 2008). However, cost and affordability are factors, and thus phenotypic identification using culture and microscopy still serves its purpose and value in many settings, as demonstrated in this study, where phenotypic identification matched identification by 18S rRNA and proving to be reliable where molecular techniques are not an option due to cost. Of the 36 isolates, 26 were identified as *A. fumigatus*, eight *A. niger* and two *A. flavus* using culture and microscopy and DNA identification validated this.

In this investigation, conventional identification was effective; nevertheless, the number of isolates was small compared to the vast diversity of fungal species capable of causing human diseases. Isolating fungi that grow close together is a complex and time-consuming process when working with large numbers and contributes to diagnostic delays. This is a concern in developing countries where the disease burden is much higher. The solution to this problem is to improve diagnostic platforms so that infecting fungi can be identified faster and with greater accuracy and precision. However, in the search for new diagnostic technologies, training in mycology for fungal identification based on morphologic and phenotypic characteristics is further reduced.

When looking for accurate and fast alternatives in fungal identification, especially when detecting human pathogens affects diagnosis, the phenotypic microarray for fungal species identification is a suitable option. The Biolog system used in this study is a relatively new technology that provides valuable extra results with a large quantity of support data. This supporting data is a benefit of the Biolog system over molecular identification, which has gained confidence in the dependability of the results but provides no supporting data. However, the Biolog system's species identification did not match the species level identification established using 18S rRNA, which corresponded with conventional culture and microscopy identification in this investigation. Although the Biolog database contains clinically related *Aspergillus* species, the database most likely did not contain closely correlated clinical species with the same biochemical profile at the time of usage, resulting in low and insignificant similarity readings. Most isolates were matched, but only at the genus level, which is ineffective in clinical settings (Table 2.3). This system's restriction or disadvantage for recognizing isolates is only as good as its reference database for effective utilization. This technique and procedure for species identification are relatively simple and quick, and they would have been useful if the database included more clinically important *Aspergillus* species. However, with its extended database, the Biolog system may give an alternative for detecting filamentous fungi where molecular approaches are ineffective.

Each identification technique investigated in this study could be used in different conditions depending on the financial support and skills available. Classical mycological procedures based on phenotypic traits should continue to develop expertise to enable correct identification where molecular techniques and other modern equipment-based techniques are not available. However, molecular techniques, although reliable and consistent require specialized and advanced equipment and software and are, therefore, sometimes not accessible in the developing world. Cost becomes a factor in considering isolates to be sent externally for sequencing. The Biolog system is a simple and quick identification technique that relies on a database; nevertheless, the system must be able to incorporate new species into the diagnostic capabilities that should be continuously updated.

Beardsley (2018) argued that because routine diagnostic laboratories in low- and middle-income countries cannot perform routine species identification, clinicians must rely on epidemiology and susceptibility patterns from reference laboratories to guide treatment decisions. He urged a priority call for the development of diagnostic capacity for fungal infections. Thus, improving recognition of morphological features of opportunistic fungi in

stained smears of the specimen, maximizing the growth rate and production of conidia by *Aspergillus* species in culture, and recognizing atypical variants of common aspergilli can all improve the laboratory's contribution to rapid diagnosis. This study found that growth media containing a high concentration of reusable sugars and protein produced rapid mycelial growth and sporulation at 37°C for cultural phenotypic identification of *Aspergillus* species and recognition of typical morphological features under the microscope. The fast growth of these clinical isolates at 37°C, equivalent to body temperature, indicates the species' perilous ease of adaption from environmental isolates growing optimally at 30°C, to human infections.

For management, it is also crucial to understand why these *Aspergillus* pathogens show persistent growth in infected tissue; consequently, growth profiling becomes important. The conventional way for obtaining this data is via biochemical and physiological assessment; however, it is laborious and time-consuming. In a microarray system, the Biolog allows cells to be evaluated for nutrient use. This approach is relatively fast and reliable and provides a substantial number of support data to add value to the nutritional profiles. The Biolog System is a relatively simple technique of screening a large number of carbon sources using phenotypic microarray to evaluate and analyze growth profiles for relevant investigations, in this case, the persistence in infection and pathogenesis of *Aspergillus* species in respiratory infections.

The carbon profiles of all *Aspergillus* isolates revealed their capacity to use a diverse range of carbon sources, demonstrating their versatility and adaptability, allowing them to thrive in most environments and possibly adapt in limiting settings to cause infection. The carbon profile of *A. fumigatus* gives information on how this persistent human pathogen may thrive in limiting environments by rapidly absorbing various carbon sources to establish infection or for persistent infection. These observations are consistent with the findings of Matar *et al.* (2017), that pathogenic fungi have developed genetic mechanisms and molecular strategies to withstand unpredictable conditions and establish effective disease conditions in their hosts. Regulation of carbon metabolism is vital for disease establishment by filamentous fungi. As seen here, carbon catabolite repression (CCR) and up-regulation help microorganisms adapt their physiology to the environment by balancing selections for rates of reprogramming (New *et al.* 2014; Ries *et al.* 2016). When there is a more easily accessible carbon source in the media, CCR turns off some enzymes necessary to use less-favoured carbon sources (Ries *et al.* 2016). As a result, CCR may alter microbe survival by influencing virulence and adaptability.

A. fumigatus strains consistently showed a steep increase in absorbance from 48 to 72 hours, with absorbance readings approaching 2.5. One such example is the surge in absorbance caused by fucose later in the growth cycle, which extends the log phase with additional growth production; explaining *A. fumigatus* prolific growth within 48 to 60 hours. This assimilation of preferred carbon sources, monosaccharides and disaccharides, confirms *A. fumigatus* metabolic flexibility. The preferred or favoured monosaccharides are absorbed late in the growth phase, influencing the persistence of this species infection. This could be attributable to the range of hydrolytic enzymes found in *Aspergillus* (De Assis *et al.* 2015). Ghazaei (2017) emphasized the role of hydrolytic enzymes in *A. fumigatus* pathogenesis and infection, while Richie *et al.* (2009) demonstrated that filamentous fungi rely heavily on the secretory pathway for the production and secretion of extracellular hydrolytic enzymes required for growth on polymeric substrates. The results of this investigation showed that all three *Aspergillus* species consistently assimilate alternative monosaccharides and disaccharides, and enzymes could contribute to nutrient intake during infection and impact persistent growth and survival. This shift to alternate monosaccharides and disaccharides late in the growth phase can be seen in *A. fumigatus, A. niger* and *A. flavus,* (Figures 3.4 to 3.7 and 3.9 to 3.13). The findings reported in Chapter 3 imply that *Aspergillus* species may use a wide variety of carbon sources and have extensive substrate absorption to impact infection maintenance and disease progression.

Despite the difficulties in detecting *Aspergillus* causing respiratory infections, the most concerning factor remains azole resistance in *Aspergillus*. These life-threatening diseases strike the most vulnerable patient populations. These fungi have emerged as a major cause in people who have underlying medical conditions or are undergoing immunosuppressive therapy (Verweij *et al.* 2016). The increased prevalence of invasive fungal infections has coincided with a significant increase in azole resistance in *Aspergillus* (Enoch *et al*. 2017), and fungal infections are spiralling, with an estimated 1.5 million human mortalities yearly (Brown, Denning and Levitz 2012; Fisher *et al.* 2018). Effective antifungal therapy is critical in treating the disease in patients with compromised immune systems, particularly in cases of invasive pulmonary aspergillosis, which can progress quickly. As a result, this study evaluated the efficacy of voriconazole and posaconazole, which are presently employed in the normal treatment of *Aspergillus* infections in respiratory patients, as well as the potential of new antifungal agents in azole resistance. Voriconazole and posaconazole are broad-spectrum triazole antifungal medicines used to treat invasive fungal infections (Sienkiewicz 2016). Voriconazole is used for routine therapy and is the first-line agent for the treatment of IA (Perlin

2017) and posaconazole as salvage therapy for *Aspergillus* infections (Jenks and Hoenigl 2018).

The investigation reported in Chapter 4 shows that 6% of the *Aspergillus* isolates were resistant to the standard drug voriconazole, whereas 61% exhibited moderate sensitivity (Figure 4.6). Alarmingly, all isolates were resistant to the salvage therapy drug posaconazole. These findings are concerning because azoles have previously improved the treatment of IA and reduced mortality rates (Jenks and Hoenigl 2018). As a result of growing resistance, further techniques for controlling mortality and morbidity must be studied. Van De Veerdonk *et al.* (2017) discovered triazole resistance in 16% of patients with influenza-related IA, and voriconazole resistance was substantially linked with treatment failure. Although not many *A. fumigatus* isolates were resistant to voriconazole in this investigation, more than half (58%) exhibited moderate sensitivity (> 0.25 - 2.0 ug/ml) (Figure 4.7). Thus *Aspergillus* susceptibility to voriconazole reported in the above investigations, as a first-line therapy azole, requires vigilance for triazole-resistant strains, especially since *A. fumigatus* is responsible for over 90% of cases of invasive aspergillosis (Araujo and Rodrigues 2004b; Jenks and Hoenigl 2018).

Twenty-five per cent of the *A. niger* isolates were resistant to voriconazole (Figure 4.7) and showed more resistance to voriconazole than *A. niger and A. flavus*. This is especially significant because the only isolates resistant to voriconazole and posaconazole in this study were two *A. niger* strains (isolates 12 and 30) (Table 4.3). It is well documented and reported that *A. fumigatus* is the most commonly isolated species from respiratory infections (Jenks and Hoenigl 2018), with azole resistance recorded as 1.5% in the United States (Berkow *et al.* 2018) and resistance extending beyond Europe to other continents (Resendiz Sharpe *et al.* 2018). However, it has to be noted that *A. niger* made up 14.3% of *Aspergillus* species recovered from pulmonary aspergillosis-infected patients (*n*=71) with HIV in Northern India (Kaur *et al.* 2017) and in this current study, 22% of the isolates from respiratory infections (*n* = 36), collected between July 2015 and June 2016. It is thus significant that *A. niger* is emerging in respiratory infections and recording high resistance, as noted here with 25% resistance to voriconazole and 100% resistance to posaconazole. Although *A. fumigatus* is the most commonly isolated species from respiratory infections and increasing azole resistance (Arendrup *et al.* 2017), *Aspergillus niger* may pose a more significant challenge in the not-so-distant future if not monitored and effective measures put in place. The resistance of all isolates to posaconazole is a serious concern as the survival rate among immune-compromised patients

with invasive aspergillosis depends on the availability of multiple options for antifungal drugs (Herbrecht *et al.* 2002). Thus, the resistance observed with posaconazole threatens the overall effectiveness of the therapy strategy.

Recently, Trovato *et al.* (2021) also described a case of invasive pulmonary aspergillosis caused by *A. niger* in a patient with COVID-19 pneumonia and acute respiratory distress syndrome. Invasive pulmonary aspergillosis, a complication in patients with severe respiratory syndromes, recently showed a correlation with COVID-19 pneumonia, and the clinical characteristics of COVID-19-associated pulmonary aspergillosis (CAPA) have been described.

These coinfections between severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and respiratory pathogens such as *Aspergillus* have added a new complication and are associated with high morbidity and mortality in patients with COVID-19 (Lai *et al.* 2020; Lansbury *et al.* 2020). Data from the incidence of IPA in Italian ICUs was 0.2% in 2013. Still, studies during the COVID-19 pandemic in Europe have reported high rates of COVID-19-associated IPA, with a prevalence ranging from 20% to 35% and an increase of IPA in patients with COVID-19 admitted to ICUs was reported (15.1%) (Montagna *et al.* 2013; Arastehfar *et al.* 2020). El-Baba, Gao and Soubani (2020) urge more attention to *Aspergillus* due to the severity of the complications such as invasive pulmonary aspergillosis (IPA). Thus, it is a huge step forward that the World Health Organization has drawn up the first-ever list of fungal pathogens that are potential threats to human health. The pathogens are divided into critical, high and medium-priority categories, with *Aspergillus fumigatus* included in the critical group. Trovato *et al.* (2021) suggest that even immunocompetent patients with COVID-19 infection should be screened early for microbiological colonization before admission to critical wards, such as ICUs because colonization data allow careful monitoring of the patient and preventing invasive infections, especially in cases on ventilator support.

Aspergillus resistance associated with adapting environmental isolates to human pathogens must be considered in the emergence of this resistance due to the widespread use of agricultural azoles (Verweij 2009). Azoles are commonly used for crop protection and some fungicides have a molecular structure similar to that of medical triazoles (Snelders *et al.* 2012). Thus it has been suggested that mutations that confer resistance to fungicides can offer resistance to medical azoles due to the molecule's structural similarity (Seyedmousavi *et al.* 2014). Plant-pathogenic moulds that cause opportunistic infections in humans are exposed to demethylase inhibitor (DMI) fungicides, including imidazole and triazole drugs. As a result, a loss of

efficacy occurred for this drug class in several species. With the global increase in the use of DMI fungicides, a rise in the number of *A. fumigatus* azole-resistant isolates has been reported (Garcia-Rubio *et al.* 2021). The azole drugs act by inhibiting the activity of *cyp51* enzymes, the azole target. Mutations in the *cpy51A* gene represent common mechanisms for azole resistance in clinical *Aspergillus* isolates (Becher and Wirsel 2012). In this study, of the eleven *A. fumigatus* isolates that were susceptible (≤0.25 µm) to voriconazole, eight isolates (73%) were the wild type and only three of those susceptible strains remained susceptible even though the strains recorded mutations. The other fifteen *A. fumigatus* strains showed moderate susceptibility (>0.25-2.0 µm) and at higher concentrations between 0.50 to 1.50 µm, for six of the isolates. Isolates, 15 and 27 were mutated strains and could be developing resistance, due to the mutation (Table 4.5). Therefore it seems that maybe different genetic mutations offer resistance to azole than the tr34/96 tested here. Mutations at positions G54, M220, and G448 of the *cyp51A* gene have been reported to be mutation hot spots and were observed in patients with chronic aspergillosis treated with azole antifungals in the long term (Meis *et al.* 2016; Garcia-Rubio, Cuenca-Estrella and Mellado 2017). Other less common point mutations (G138C and F219I) were found in patients receiving long-term treatment with azole antifungals (Garcia-Rubio, Cuenca-Estrella and Mellado 2017; Jeanvoine *et al.* 2017).

In light of emerging antifungal resistance, in an attempt to increase the therapeutic range, effective alternative compounds were explored to improve therapeutic options like combination therapy, as suggested by Revie *et al.* (2018) and Robbins, Caplan and Cowen (2017), to reduce the rate of resistance and increase potency. This antifungal phenotypic study was achieved by analyzing the sensitivity profile of the isolates in a microarray system (Biolog). The profile results show the resistant compounds in yellow and susceptibility in red on the heat map (Figure 4.8). Exposure of the *Aspergillus* isolates to the potential antifungal agents showed that 83% of the isolates were susceptible to the 24 compounds. The large number of isolates being susceptible to the 24 compounds although in varying degrees of susceptibility, shows promise in the quest to find new therapies for fungal resistance that could reduce mortality rates that are as high as 90% in invasive fungal infections due to limited treatment (Wambaugh *et al.* 2020). However, the agents must be considered for toxicity after oral administration.

Two compounds, berberine and blasticidin hydrochloride, from the agents assessed and reported in Chapter 4 were effective against thirty of the isolates. This is promising because this could offer an alternative, or azoles could be assessed for combination therapy with either or both of these agents as a potential option, seeing that azole monotherapy has led to resistance after initial success. Berberine and blasticidin hydrochloride from the compounds assessed were also part of the Wambaugh *et al.* (2020) investigation with fluconazole, resulting in an antagonistic and synergistic effect, respectively. Berberine is a plant extract that reduces blood sugar levels (Vuddanda, Chakraborty and Singh 2010; Zhang *et al.* 2010). Both compounds have low toxicities at therapeutic concentrations and may be suitable candidates for trials in combination therapy, with azoles showing toxicity or resistance in monotherapy. Blasticidin hydrochloride is a gene selection antibiotic effective in eucaryotic cells, inhibiting the peptide bond of ribosomes (Powers *et al.* 2021). Increasing the therapeutic range and exploring effective alternative compounds in combination therapy is important and could reduce the resistance rate and increase potency (Robbins, Caplan and Cowen 2017; Revie *et al.* 2018).

IA management continues to be challenging due to the population at risk, the diversity of the clinical presentation and the lack of a gold standard to diagnose related infections (Lamouth and Calandra, 2017). Missed diagnoses mean that patients are overtreated with antibacterial agents, corticosteroids and anti-TB drugs, resulting in continuing illness and often death (Denning 2021). Vidal-García *et al.* (2018) reported the invasive aspergillosis biomarker, galactomannan used in combination with clinical signs and symptoms in the diagnosis of this infectious disease and diagnostic approaches with increased accuracy, like PCR-based methods and lateral flow devices to detect *Aspergillus*-derived protein (White *et al.* 2015) with diagnoses still slow processes. Thus, the quest to accurately diagnose this challenging infection continues and realizes a need for biomarkers and understanding their role as virulence factors in IA management (Mercier and Maertens, 2017). *A. fumigatus* produces gliotoxin, which has been shown to have toxic effects on phagocytic cells and T-lymphocytes at low concentrations *in vitro* (Malekinejad *et al.* 2013). Additionally, *in vivo,* studies have suggested that it impairs the host defence mechanism during infection. Thus, it has been proposed as a virulence factor for IA due to its cytotoxic, genotoxic and apoptotic properties (Bok 2006; Hof and Kupfahl 2009).

All 36 *Aspergillus* species were isolated in the clinical setting from patients with respiratory-related infections. The clinical isolates were gliotoxin-producing (Table 5.3), supporting Hof and Kupfahl (2009) in finding that gliotoxin is produced during infection and thus functions as a virulence factor. Spikes *et al.* (2008) also concluded that gliotoxin production by *A. fumigatus* contributes to virulence in immunosuppressed hosts. As the results in Chapter 5 reveal, fifty-eight per cent of the *A. fumigatus* isolates produced between 0.12 mg/l and 2.6 mg/l with a mean gliotoxin concentration of 0.60 mg/l. This compares significantly high to the mean concentration of 0.005 mg/l in the study (n=100) by Kupfahl (2008), which was reported to have significantly influenced cytotoxicity. Fifty-eight per cent of the *A. fumigatus* isolates in this study produced gliotoxin, supporting gliotoxin as a virulence factor in the pathogenesis of *Aspergillus* infections and possibly further indicating the potential of gliotoxin as a biomarker for invasive aspergillosis infections.

The question of whether bacteria-associated molecules (lipopolysaccharides, peptidoglycan and lipoteichoic acid), with their pathogen-associated molecular patterns (PAMPS), induced gliotoxin secretion by *A. fumigatus* revealed a correlation between the presence of PAMPS and the secretion of gliotoxin (Svahn *et al.* 2014). This study showed that 40% of the gliotoxin-producing isolates demonstrated increases between 18 and 52% after exposure to PAMPS over a 72-hour incubation (Table 5.4). These results collaborated with those of Svahn *et al.* (2014) in a possible explanation for the increased virulence of *A. fumigatus* during bacterial co-infection and the colonization by large numbers of Gram-negative and Gram-positive bacteria in COPD patients, which produce and release large quantities of LPS, PG and LTA to propose the theory that bacterial infection possibly aggravates the mould co-infection and is vital in the transition from colonization to invasiveness in pulmonary diseases. However, these results advocate further investigation to act on the suppression or prevention of pulmonary bacterial colonization in high-risk patients contracting aspergillosis (Svahn *et al.* 2014).

This study showed that different identification techniques in azole resistance therapy management serve needs in developed and resource-challenging situations, impacting diagnosis. Azole monotherapy has resulted in drug resistance after initial success. This study supports the search for new agents in drug regimen strategy and suggests new compounds (berberine and blasticidin hydrochloride) for potential in combination therapy.

CHAPTER SEVEN: CONCLUSION AND RECOMMENDATIONS

These studies highlight the relevance of conventional and molecular identification techniques for filamentous fungus in clinical settings, each serving a function and purpose. Each identification technique had a function and could be used in various situations based on available resources and skills. In the rising demand for other efficient identification methods to improve the diagnosis and treatment of respiratory infections, phenotypic microarray was also introduced as a quick and effective identification tool. Carbon profiles of all of the *Aspergillus* isolates demonstrate their capacity to utilize a diverse range of carbon sources, demonstrating their flexibility and adaptability. As a result, they thrive in most environments and may adapt to cause infection in limited circumstances.

The antimicrobial profiles of the isolates show that all isolates had 6% resistance to routine therapy voriconazole and 100% resistance to salvage therapy posaconazole. Resistance to posaconazole is especially concerning since the survival probability of immune-compromised patients with invasive aspergillosis is contingent on the availability of various antifungal drug options. *A. niger* isolates demonstrated 25% resistance to voriconazole, raising concerns about developing resistance in this species. Invasive pulmonary aspergillosis has recently been linked to COVID-19 pneumonia, prompting a call for immunocompetent patients with COVID-19 infection to be screened for microbiological colonization before admission to critical care units, such as ICUs because colonization data allows for careful monitoring of patients and prevention of invasive infections.

The Biolog microarray system facilitated the exploration of new agents and the identification of two new compounds, berberine and blasticidin hydrochloride, showing potential in combination therapy of azoles due to growing resistance in azole monotherapy. Gliotoxin was analyzed for its role as a virulence factor and biomarker in respiratory infections, with 58% of the *A. fumigatus* isolates having been found to produce gliotoxin. The increased levels of gliotoxin in the 40% gliotoxin-producing isolates may be associated with the increased virulence of *A. fumigatus* during bacterial coinfection in the presence of PAMPS.

In profiling *Aspergillus* species isolated from patients with a respiratory infection, a limitation in this study is that a larger sample size of isolates from respiratory patients would have been ideal for providing representative reporting. More importantly, if permission was granted to acquire and report on patient details relevant to the isolates and results, the correlation with virulence and pathogenesis of the infection could be confirmed more emphatically. Patients' sensitivity to routine and salvage therapy drugs could have been related to establishing a clear *in vivo* antifungal profile to make inferences on azole resistance. Further data analyses may reveal additional evidence of factors supporting fungal fitness and disease progression.

Another constraint for this investigation was the availability of just one panel of antifungal agents for the drug profile of isolates on novel antimicrobial drugs. More panels may even be developed in future with different agents that have been studied and tested using the microarray technology for successful novel agents. An updated Biolog database containing relevant clinical strains would have demonstrated the system's effectiveness as a fast and accurate detection tool for filamentous fungus.

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APPENDICES

Appendix A: 18S rRNA sequences of the respiratory isolates not represented in Chapter 2

>10
GAGTGCGGGTCCTTTGGGCCCAACCTCCCATCCGTGTCTATTGTACCCTGTTGCTTCGGCGGGCCCG
CCGCTTGTCGGCCGCCGGGGGGGCGCCTCTGCCCCCCGGGCCCGTGCCCGCCGGAGACCCCAACAC
GAACACTGTCTGAAAGCGTGCAGTCTGAGTTGATTGAATGCAATCAGTTAAAACTTTCAACAATGGA
TCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCA
GTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGGCATGCCTGTCCGAGC
GTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGTCGCCGTCCCCCTCTCCGGGGGGGACGGGCCC
GAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACATGCTCTGTAGGA
TTGGCCGGCGCCTGCCGACGTTTTCCAACCATTCTTCCCAGGTTGCTCGCGGATCGGGTAGGGATAC
>11
GAGTGAGGGCCCTCTGGGTCCAACCTCCCACCCGTGTCTATCGTACCTTGTTGCTTCGGCGGGCCCG
CCGTTTCGACGGCCGCCGGGGAGGCCCTGCGCCCCCGGGCCCGCGCCCGCCGAAGACCCCAACATG
AACGCTGTTCTGAAAGTATGCAGTCTGAGTTGATTATCGTAATCAGTTAAAACTTTCAACAACGGATC
TCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
TGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCG
TCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCCCCCGTCCCCCTCTCCCGGGGGACGGGCCCG
AAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCTGCTCTGTAGGCC
CGGCCGGCGCCAGCCGACACCCAACTTTATTTTTTAAAGGTTGACCTCGGATCAGGTAG
>12
GTGCGGGTCCTTTGGGCCCAACCTCCCATCCGTGTCTATTATACCCTGTTGCTTCGGCGGGCCCGCCG
CTTGTCGGCCGCCGGGGGGGCGCCTTTGCCCCCCGGGCCCGTGCCCGCCGGAGACCCCAACACGAA
CACTGTCTGAAAGCGTGCAGTCTGAGTTGATTGAATGCAATCAGTTAAAACTTTCAACAATGGATCT
CTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGT
GAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGT
CATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGTCGCCGTCCCCCTCTCCGGGGGGGACGGGCCCGA
AAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACATGCTCTGTAGGATT
GGCCGGCGCCTGCCGACGTTTTCCAACCATTTTTTCCGGGTTGACCTCGGATCAGGTAGGGAT
>13
CCGAGTGCGGGTCCTTTGGGCCCAACCTCCCATCCGTGTCTATTATACCCTGTTGCTTCGGCGGGCCC
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Appendix B: Media and temperature results for Chapter 2

Aspergillus fumigatus:

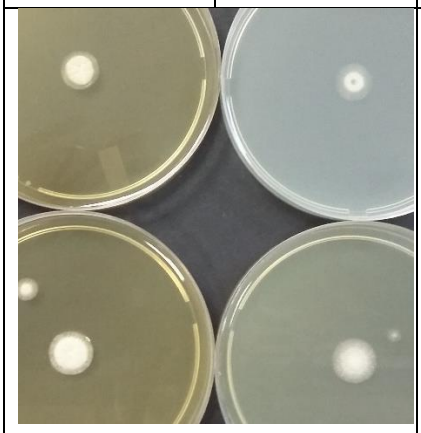
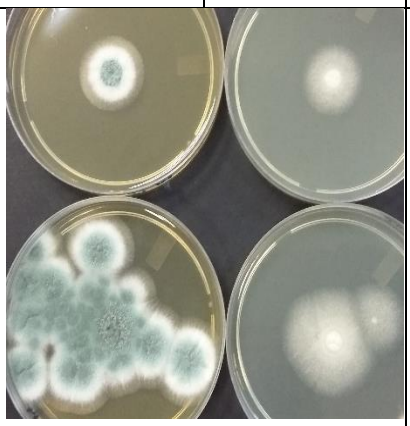
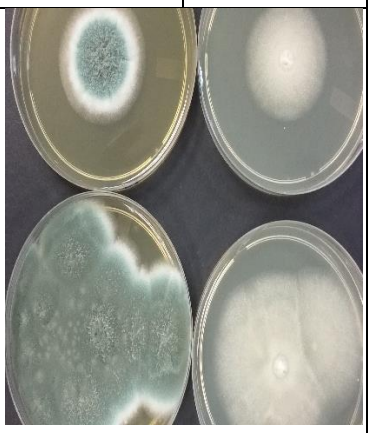
24h		36h		48h	
MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C
					
MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C

Figure B1 Growth and sporulation of *Aspergillus fumigatus* (14) on MEA and PDA after 24, 36, and 48 hours of incubation at 30°C and 37°C.

24h		36h		48h	
MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C
					
MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C

Figure B2 Growth and sporulation of *Aspergillus fumigatus* (17) on MEA and PDA after 24, 36, and 48 hours of incubation at 30°C and 37°C.

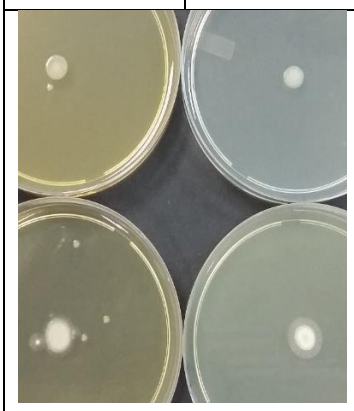
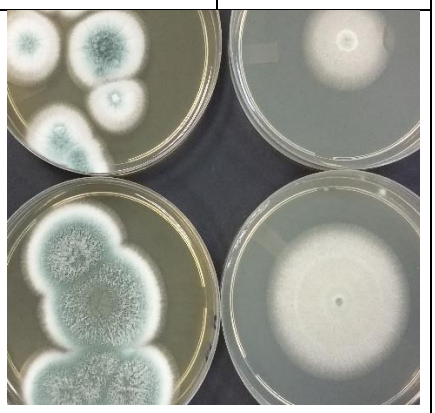
24h		36h		48h	
MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C
					
MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C

Figure B3 Growth and sporulation of *Aspergillus fumigatus* (19) on MEA and PDA after 24, 36, and 48 hours of incubation at 30°C and 37°C.

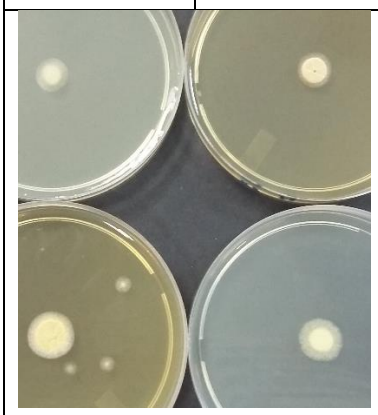
24h		36h		48h	
PDA 30°C	MEA 30°C	MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C
					
MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C

Figure B4 Growth and sporulation of *Aspergillus fumigatus* (20) on MEA and PDA after 24, 36, and 48 hours of incubation at 30°C and 37°C.

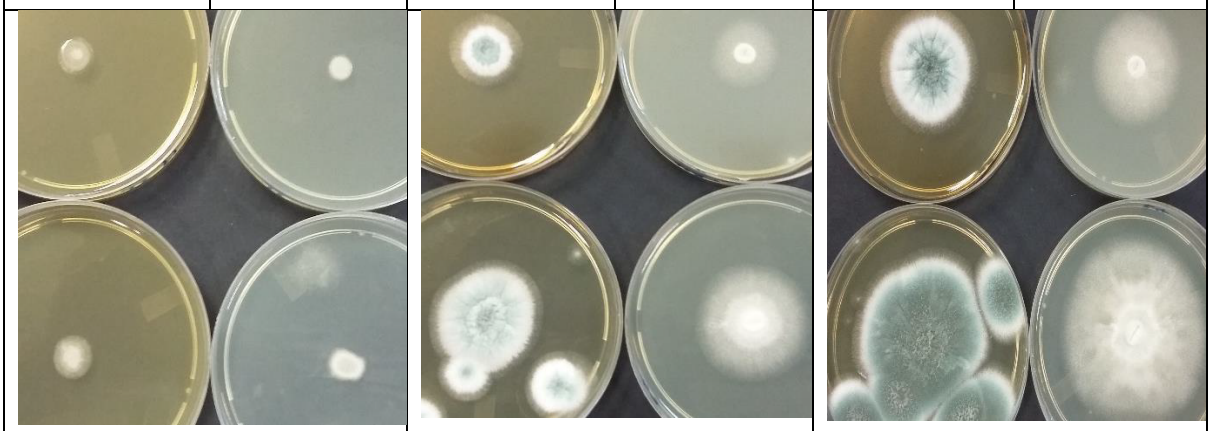
24h		36h		48h	
MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C
					
MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C

Figure B5 Growth and sporulation of *Aspergillus fumigatus* (24) on MEA and PDA after 24, 36, and 48 hours of incubation at 30°C and 37°C.

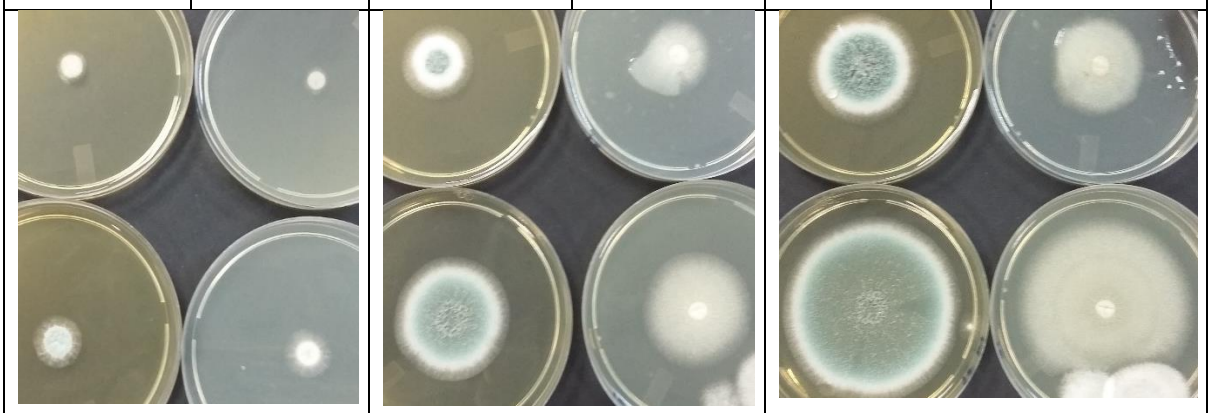
24h		36h		48h	
MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C
					
MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C

Figure B6 Growth and sporulation of *Aspergillus fumigatus* (31) on MEA and PDA after 24, 36, and 48 hours of incubation at 30°C and 37°C.

***A.niger*:**

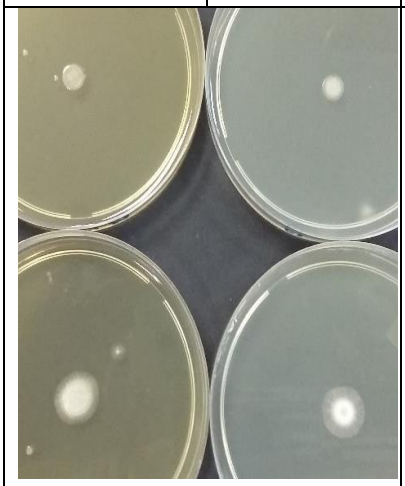
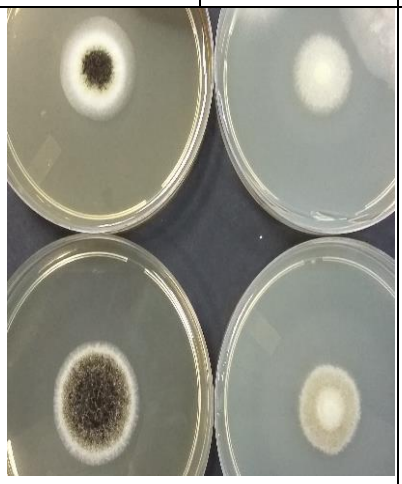
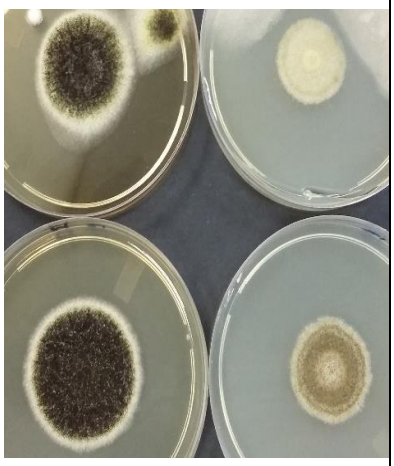
24h		**36h**		**48h**	
MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C
					
MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C

Figure B7 Growth and sporulation of *Aspergillus niger* (21) on MEA and PDA after 24, 36, and 48 hours of incubation at 30°C and 37°C.

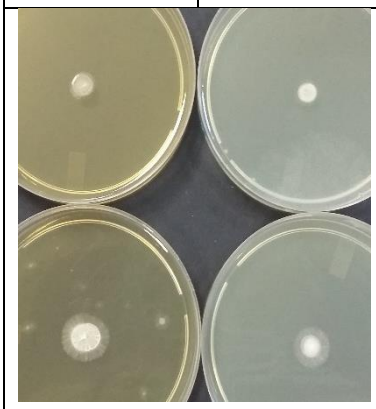
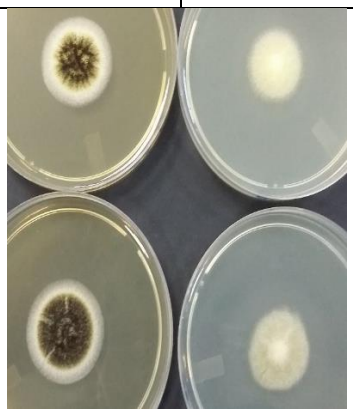
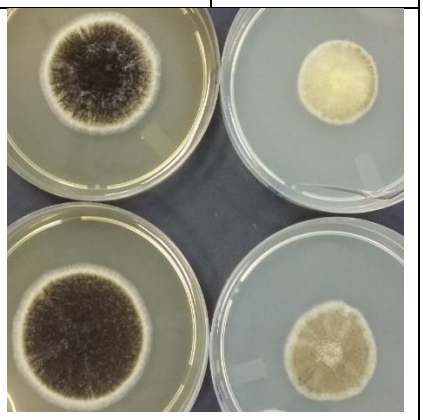
24h		**36h**		**48h**	
MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C
					
MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C

Figure B8 Growth and sporulation of *Aspergillus niger* (25) on MEA and PDA after 24, 36, and 48 hours of incubation at 30°C and 37°C.

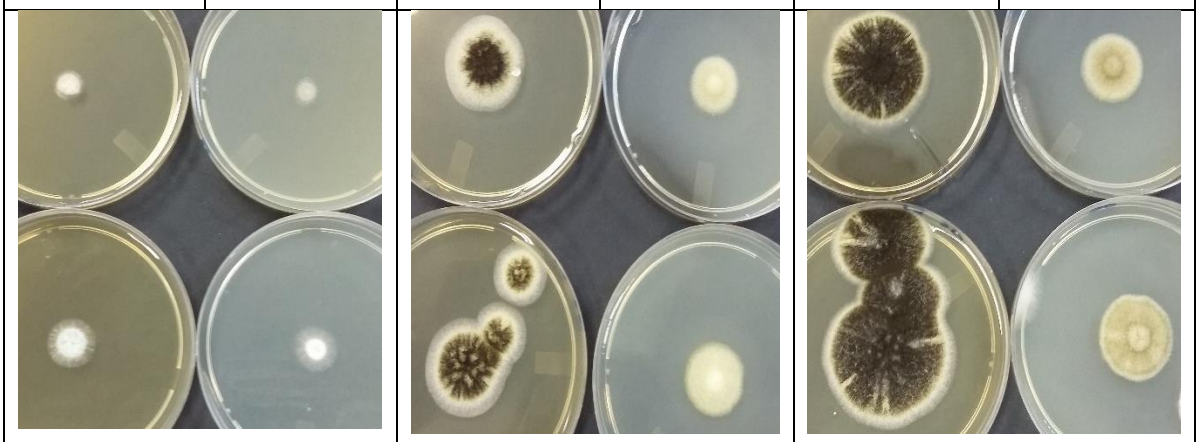
24h		36h		48h	
MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C
					
MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C

Figure B9 Growth and sporulation of *Aspergillus niger* (30) on MEA and PDA after 24, 36, and 48 hours of incubation at 30°C and 37°C.

Aspergillus flavus:

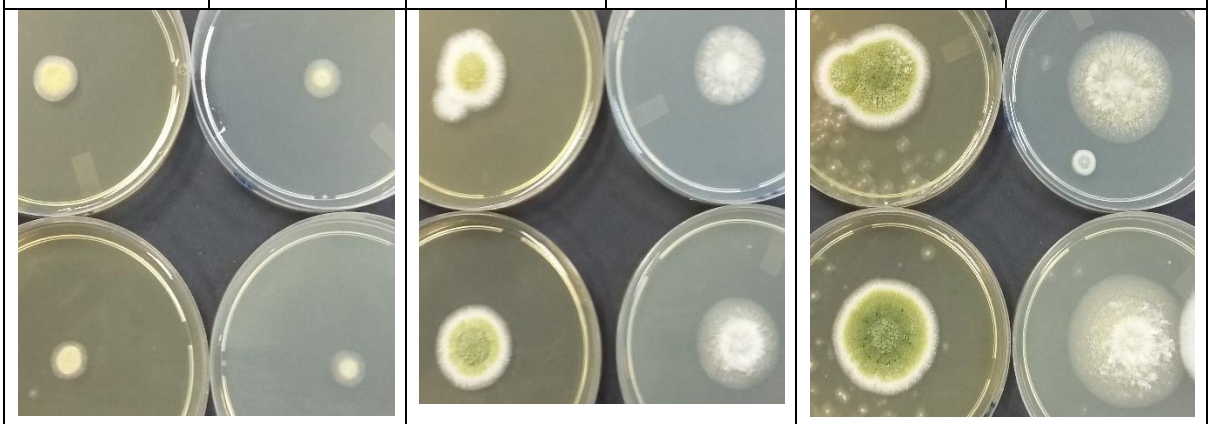
24h		36h		48h	
MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C	MEA 30°C	PDA 30°C
					
MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C	MEA 37°C	PDA 37°C

Figure B10 Growth and sporulation of *Aspergillus flavus* (32) on MEA and PDA after 24, 36, and 48 hours of incubation at 30°C and 37°C.

Appendix B: Biolog identification using FF plate - for Chapter 2

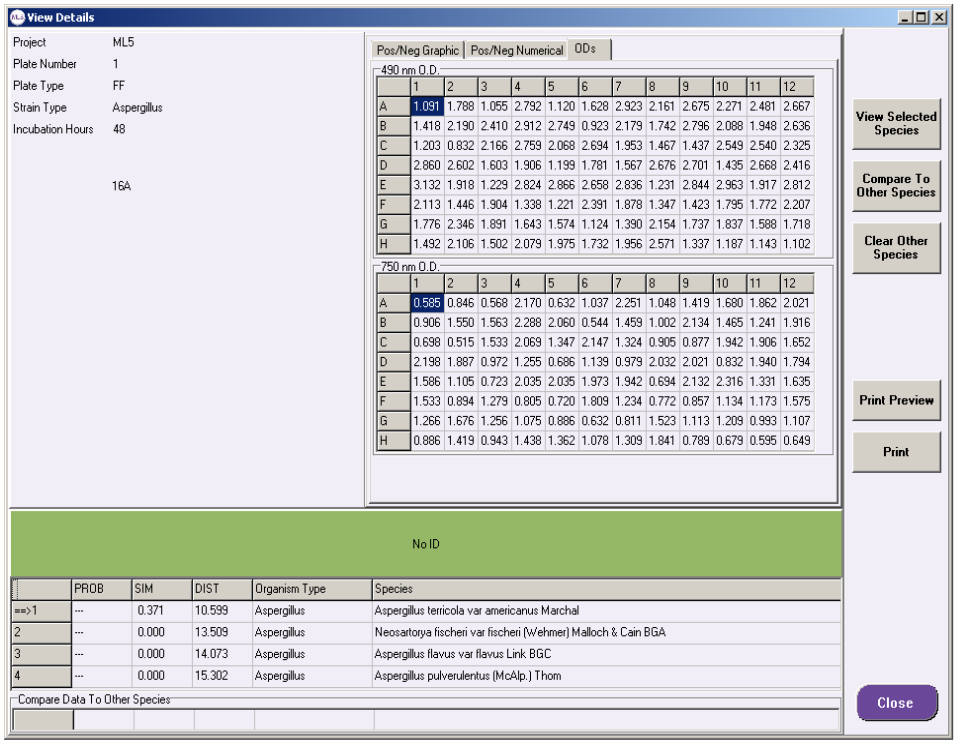


Figure B1. Result of FF plate for isolate 16A showed identification not confirmed at 48 hours of growth (*A. fumigatus*).

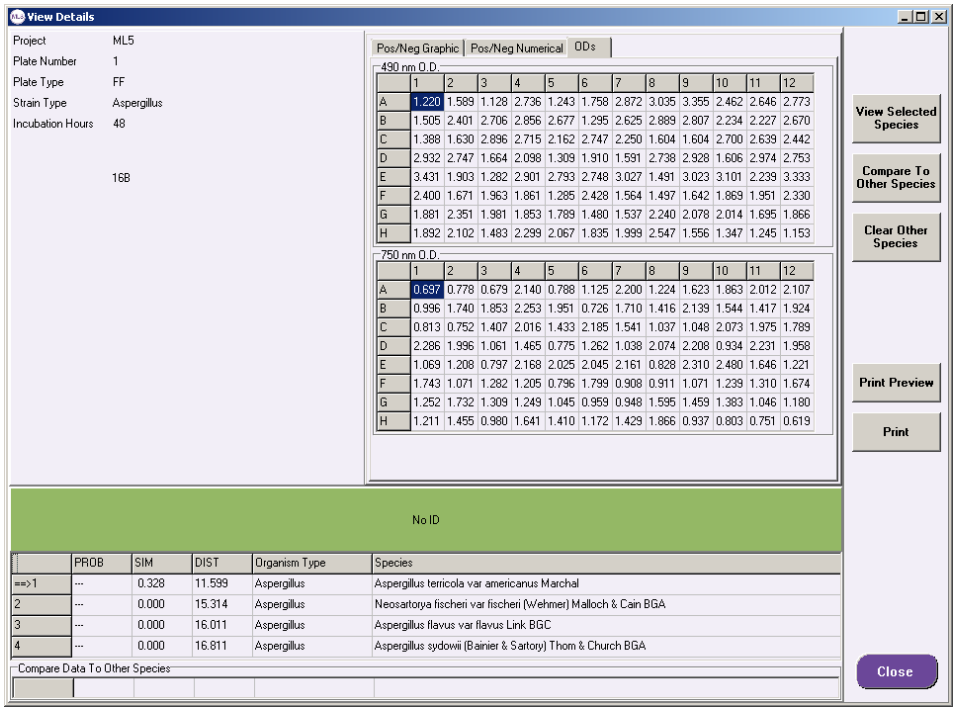


Figure B2. Result of FF plate for isolate 16B showed identification not confirmed at 48 hours of growth (*A. fumigatus*).

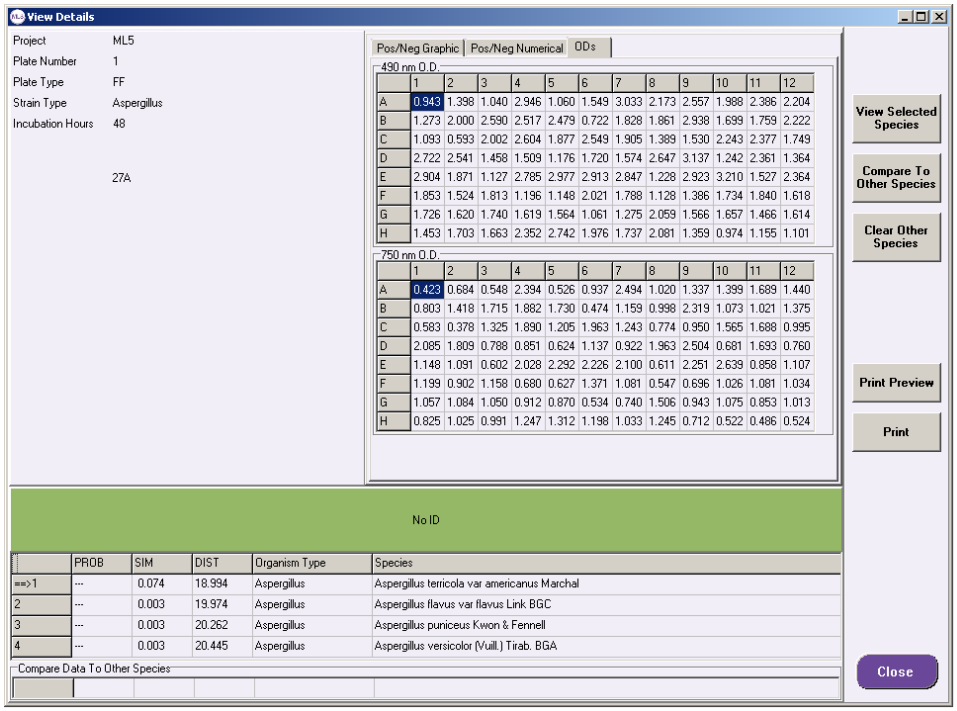


Figure B3. Result of FF plate for isolate 27A showed identification not confirmed at 48 hours of growth (*A. fumigatus*).

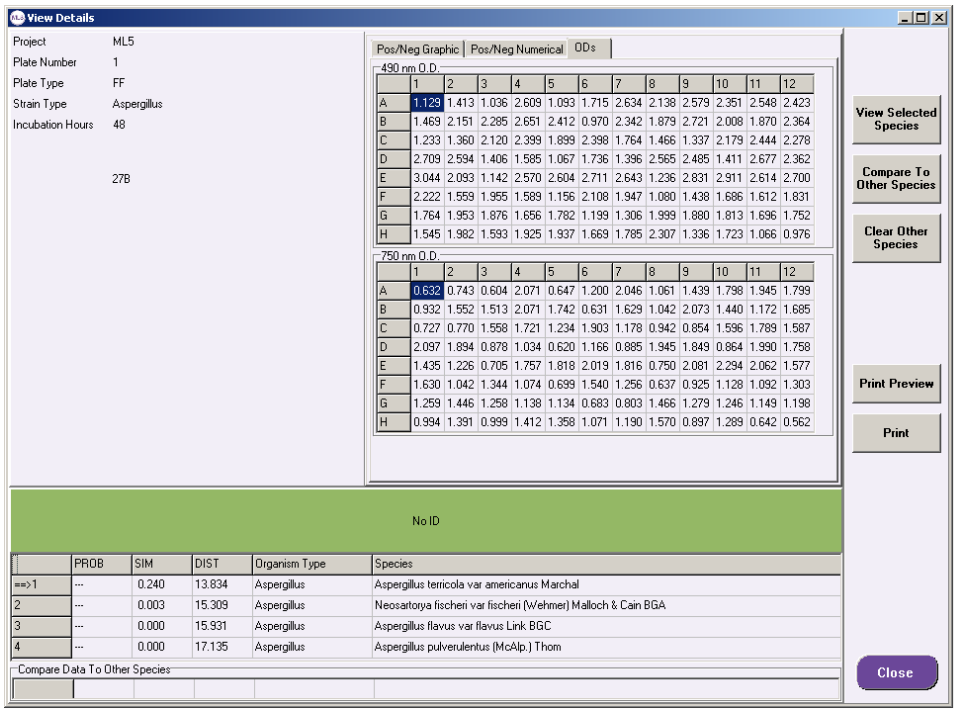


Figure B4. Result of FF plate for isolate 27B showing identification not confirmed at 48 hours of growth (*A. fumigatus*).

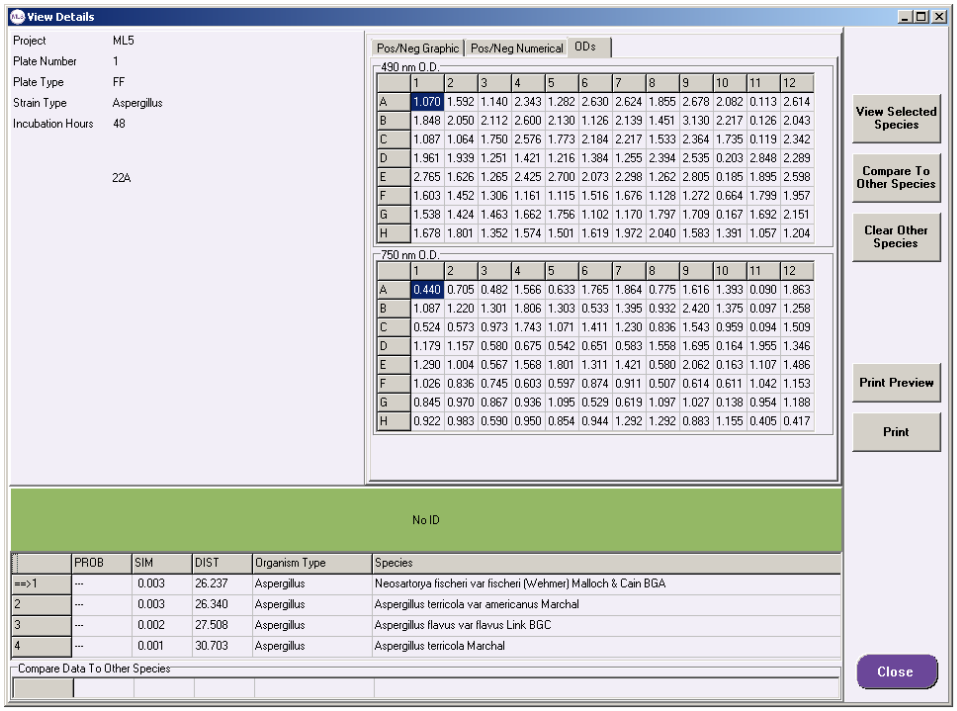


Figure B5. Result of FF plate for isolate 22A showed identification not confirmed at 48 hours of growth (*A. flavus*).

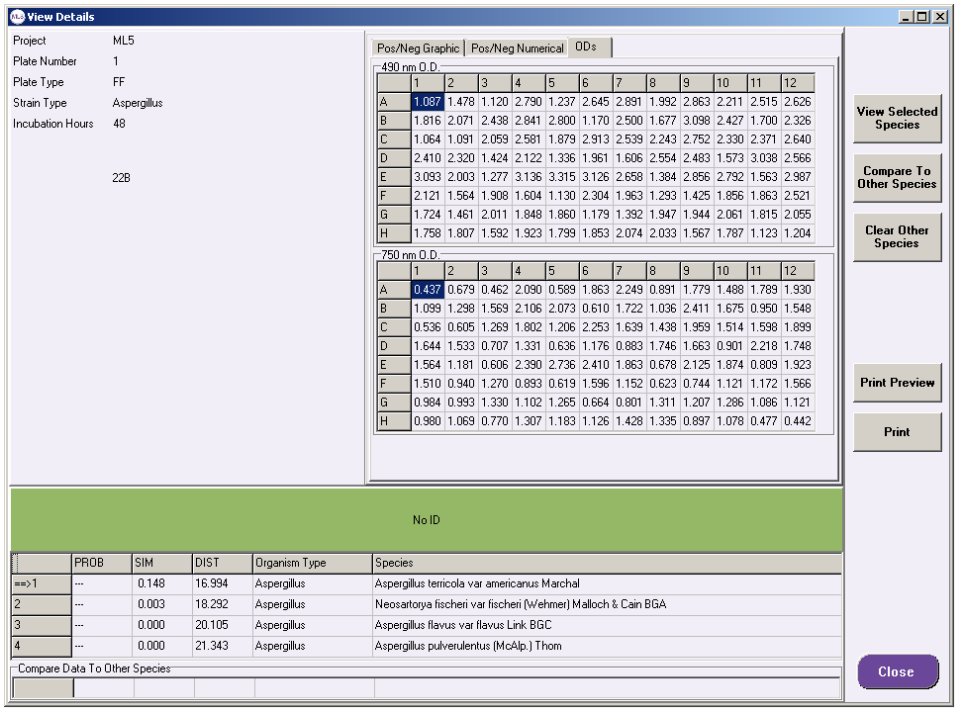


Figure B6. Result of FF plate for isolate 22B showed identification not confirmed at 48 hours of growth (*A .flavus*).

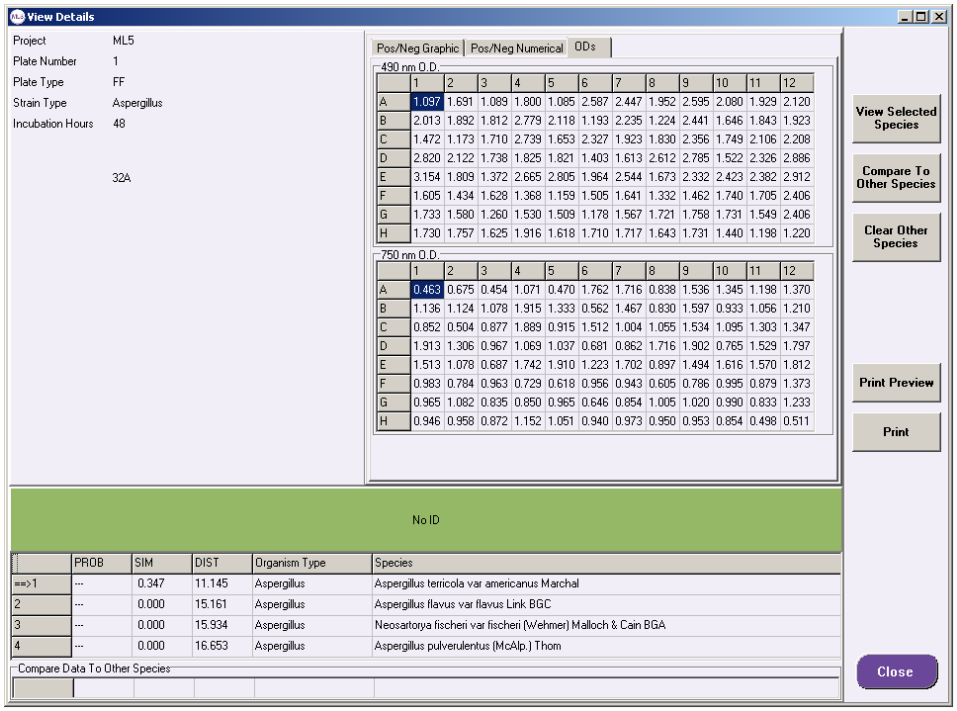


Figure B7. Result of FF plate for isolate 32A showed identification not confirmed at 48 hours of growth (*A. flavus*).

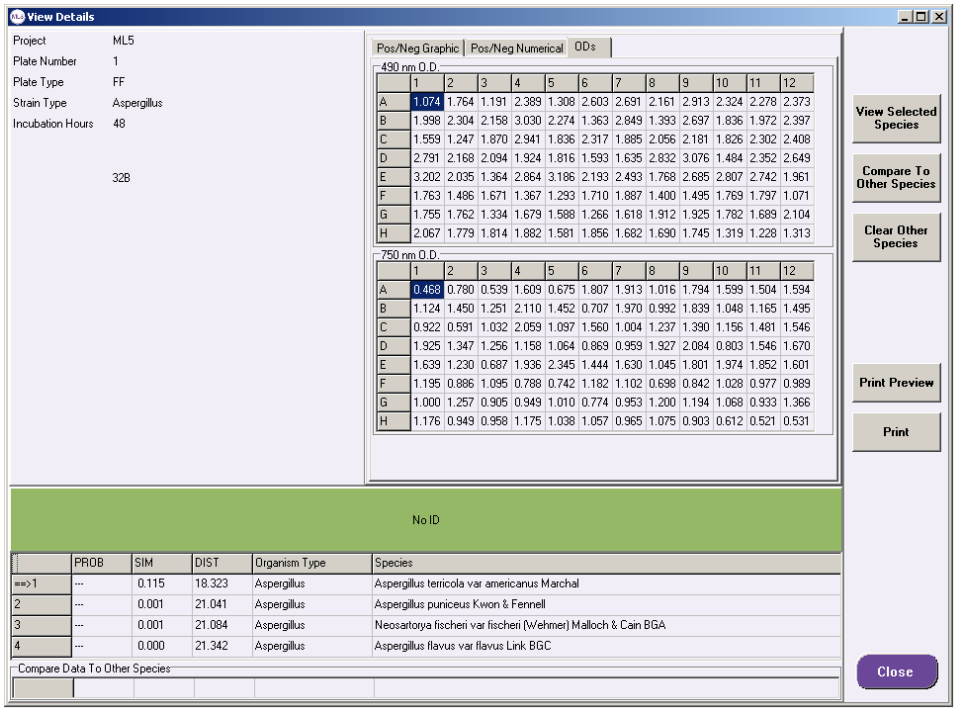


Figure B8. Result of FF plate for isolate 32B showed identification not confirmed at 48 hours of growth (*A. flavus*).

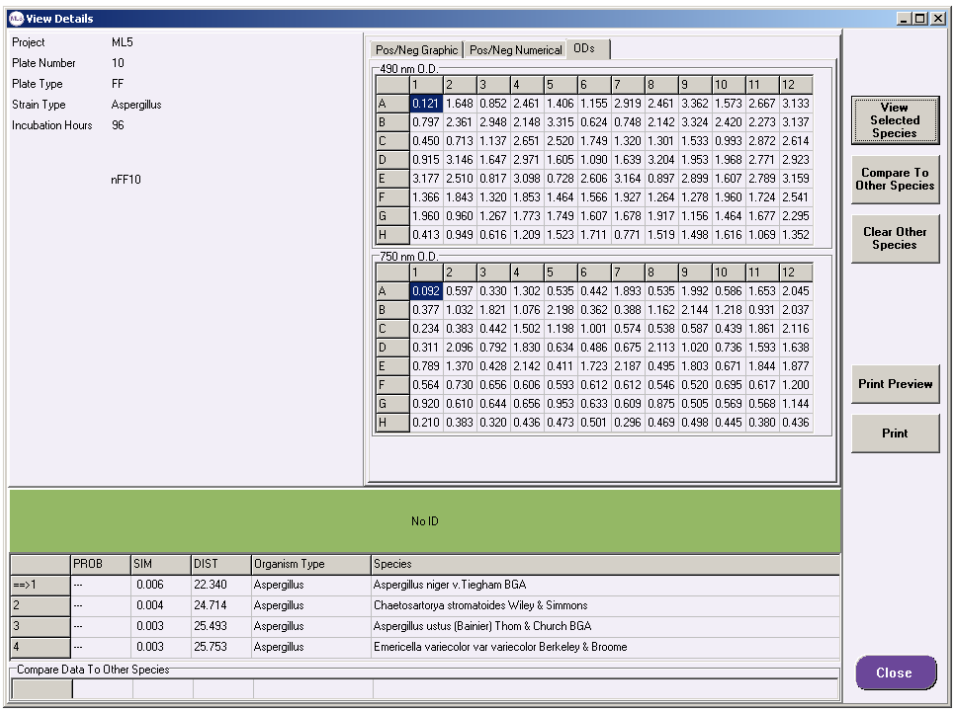


Figure B9. Result of FF plate for isolate 10 showed identification not confirmed at 48 hours of growth (*A. niger*).

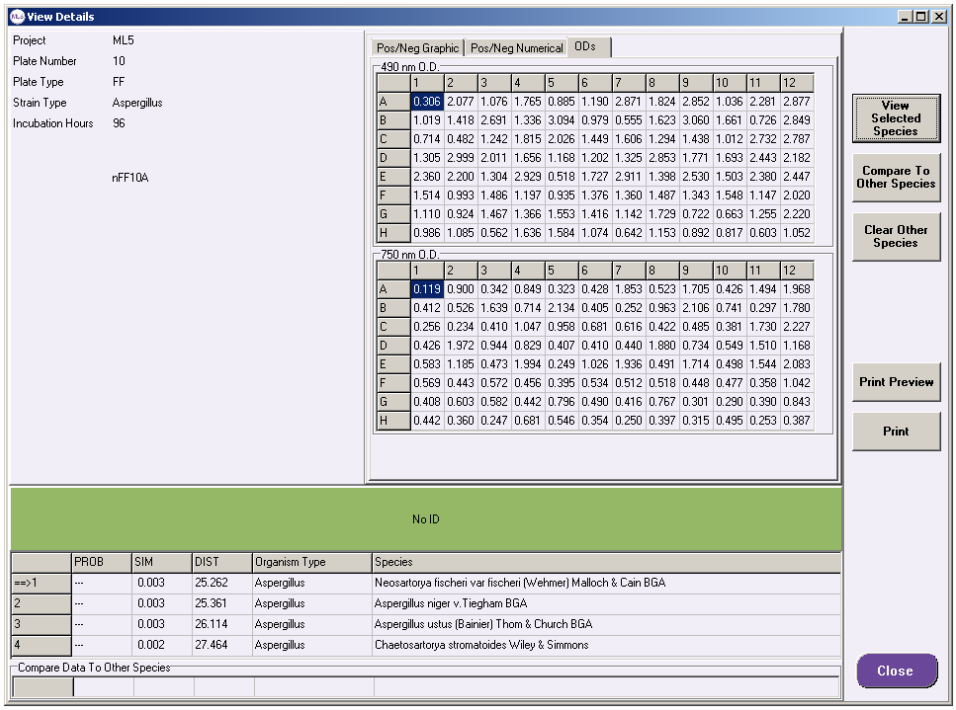


Figure B10. Result of FF plate for isolate 10A showed identification not confirmed at 48 hours of growth (*A. niger*).

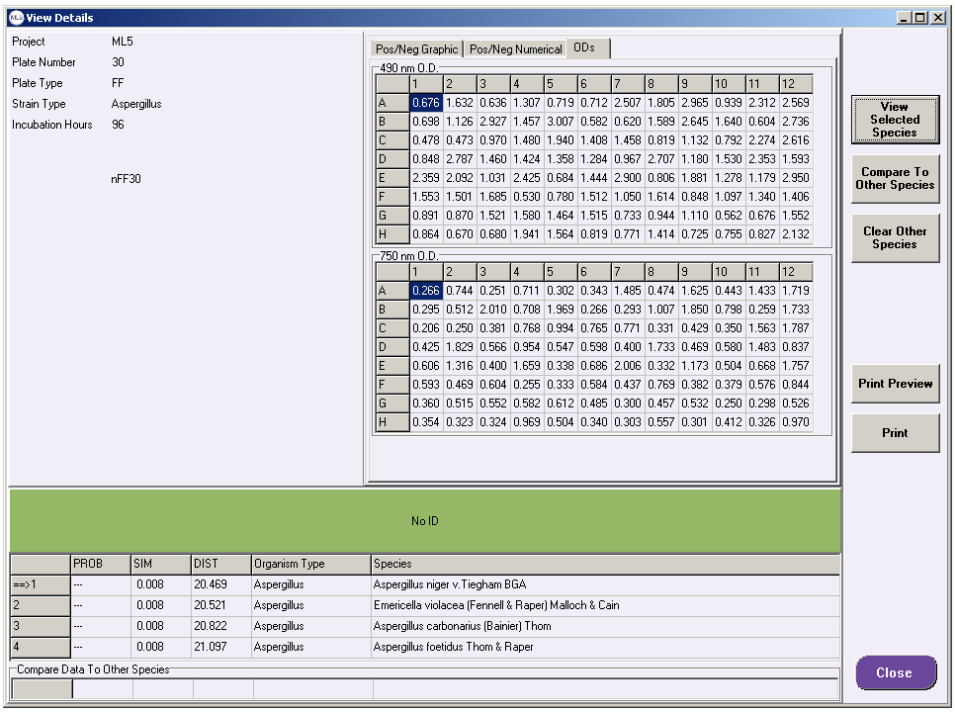


Figure B11. Result of FF plate for isolate 30 showed identification not confirmed at 48 hours of growth.

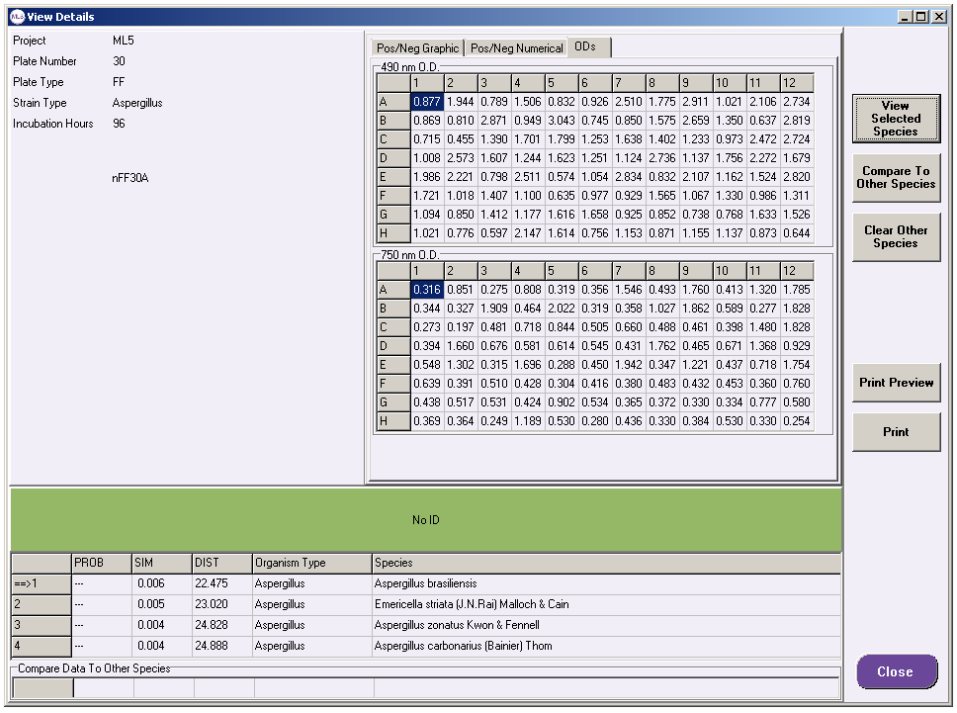


Figure B12. Result of FF plate for isolate 30A showed identification not confirmed at 48 hours of growth (*A. niger*).

Appendix C:

Growth profiles (monosaccharides) for Chapter 3.

Monosaccharides: Absorbance readings of isolates during 72-hour incubation (FF plate -Biolog). Data used to represent growth profiles of isolates in Chapter 3.

A. fumigatus (1)								
Monosaccharide:	**0**	**1**	**12**	**24**	**36**	**48**	**60**	**72**
water	0,00	0,190	0,310	0,390	0,500	0,520	0,510	0,520
N-acetyl-D-galactosamine	0,00	0,190	0,310	0,460	0,560	0,570	0,550	0,550
N-acetyl-D-glucosamine	0,00	0,190	0,330	0,710	1,750	2,400	2,420	2,440
N-acetyl-D-mannosamine	0,00	0,190	0,340	0,470	0,550	0,580	0,560	0,570
D-arabinose	0,00	0,190	0,330	0,470	0,630	0,770	0,890	0,990
L-arabinose	0,00	0,180	0,350	0,740	1,530	2,060	2,100	1,960
D-fructose	0,00	0,200	0,440	1,080	1,860	2,170	2,200	2,200
L-Fucose	0,00	0,200	0,370	0,510	0,570	0,880	1,490	2,100
D-Galactose	0,00	0,190	0,350	0,400	0,490	0,970	0,950	0,950
D-Glucosamine	0,00	0,200	0,550	1,880	2,230	2,280	2,300	2,290
α-D-Glucose	0,00	0,190	0,330	0,360	0,430	0,530	0,550	0,600
Glucose-1-Phosphate	0,00	0,210	0,390	0,420	0,330	0,350	0,350	0,440
Glucuronamide	0,00	0,180	0,420	0,490	0,560	1,200	1,480	1,410
β-Methyl-D-Galactoside	0,00	0,190	0,420	0,690	0,650	0,910	1,230	1,620
α-Methyl-D-Glucoside	0,00	0,210	0,440	0,670	0,830	1,320	1,720	2,100
β-Methyl-D-Glucoside	0,00	0,200	0,410	0,650	0,970	1,640	1,960	2,040
D-Psicose	0,00	0,210	0,440	1,060	1,110	1,210	1,470	1,770
Sedoheptulosan	0,00	0,190	0,450	0,870	1,730	2,180	2,320	2,360
L-Sorbose	0,00	0,200	0,450	0,740	1,310	1,970	2,110	2,060
D-Tagatose	0,00	0,180	0,380	0,660	1,340	2,180	2,320	2,300
D-Xylose	0,00	0,200	0,330	0,530	1,200	1,510	1,470	1,440

FF: A.fumigatus (2)	Monosaccharide														
	0	1		12		24		36		48		60		72	
		Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD
water	0,00	0,168	0,007	0,404	0,074	0,517	0,013	0,517	0,011	0,504	0,017	0,513	0,013	0,476	0,009
N-acetyl-D-glucosamine	0,00	0,165	0,001	0,412	0,052	1,065	0,022	1,965	0,064	2,264	0,110	2,221	0,155	2,134	0,192
N-acetyl-D-mannosamine	0,00	0,169	0,002	0,354	0,049	0,553	0,004	0,551	0,012	0,549	0,025	0,571	0,015	0,480	0,015
D-arabinose	0,00	0,178	0,022	0,359	0,032	0,542	0,009	0,682	0,011	0,868	0,016	1,105	0,037	1,194	0,021
L-arabinose	0,00	0,172	0,020	0,378	0,007	0,999	0,078	1,609	0,164	1,714	0,154	1,679	0,185	1,583	0,163
D-fructose	0,00	0,184	0,004	0,533	0,074	1,271	0,056	2,038	0,035	2,250	0,024	2,324	0,069	2,250	0,071
L-Fucose	0,00	0,182	0,003	0,392	0,065	0,577	0,000	0,743	0,040	1,334	0,045	1,990	0,045	2,053	0,098
D-Galactose	0,00	0,177	0,005	0,348	0,058	0,508	0,045	0,877	0,120	1,011	0,015	1,061	0,046	0,985	0,071
D-Galacturonic Acid	0,00	0,189	0,022	0,507	0,047	1,153	0,015	1,930	0,011	2,328	0,034	2,413	0,064	2,343	0,054
α-D-Glucose	0,00	0,174	0,010	0,447	0,008	0,565	0,052	0,582	0,047	0,629	0,045	0,704	0,082	0,728	0,018
Glucose-1-Phosphate	0,00	0,168	0,015	0,351	0,006	0,345	0,051	0,318	0,001	0,315	0,028	0,359	0,023	0,284	0,019
Glucuronamide	0,00	0,170	0,004	0,401	0,022	0,640	0,023	1,036	0,069	1,545	0,018	1,538	0,051	1,341	0,084
β-Methyl-D-Galactoside	0,00	0,190	0,030	0,449	0,013	0,623	0,028	0,689	0,039	0,822	0,022	0,991	0,024	1,085	0,056
α-Methyl-D-Glucoside	0,00	0,190	0,025	0,438	0,030	0,773	0,085	1,167	0,136	1,799	0,100	2,159	0,060	2,152	0,045
β-Methyl-D-Glucoside	0,00	0,188	0,023	0,435	0,025	0,745	0,001	1,135	0,053	1,660	0,023	1,956	0,066	2,011	0,063
D-Psicose	0,00	0,167	0,009	0,535	0,055	1,059	0,030	1,229	0,073	1,651	0,103	1,985	0,018	2,024	0,042
Sedoheptulosan	0,00	0,172	0,011	0,546	0,024	1,187	0,007	1,820	0,035	1,951	0,054	1,997	0,116	1,901	0,144
L-Sorbose	0,00	0,169	0,008	0,520	0,020	1,009	0,008	1,725	0,006	2,046	0,006	2,045	0,042	1,928	0,049
D-Tagatose	0,00	0,185	0,026	0,524	0,016	1,131	0,008	2,007	0,030	2,253	0,014	2,259	0,064	2,181	0,054
D-Xylose	0,00	0,172	0,004	0,391	0,074	0,990	0,062	1,707	0,049	1,809	0,099	1,793	0,187	1,459	0,553

FF: *A.fumigatus* (3)	Monosaccharide															
	0	1		12		24		36		48		60		72		
		Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD	
water	0,00	0,215	0,025	0,298	0,010	0,549	0,013	0,615	0,009	0,621	0,006	0,621	0,013	0,581	0,006	
N-acetyl-D-galactosamine	0,00	0,202	0,002	0,314	0,052	0,601	0,022	0,633	0,043	0,618	0,043	0,604	0,013	0,588	0,080	
N-acetyl-D-glucosamine	0,00	0,214	0,012	0,558	0,067	1,727	0,050	2,149	0,033	2,418	0,078	2,366	0,096	2,366	0,040	
N-acetyl-D-mannosamine	0,00	0,204	0,008	0,341	0,047	0,586	0,031	0,677	0,043	0,681	0,029	0,670	0,012	0,660	0,091	
D-arabinose	0,00	0,254	0,028	0,373	0,002	0,581	0,056	0,763	0,018	0,945	0,002	1,192	0,005	1,423	0,050	
L-arabinose	0,00	0,217	0,013	0,462	0,005	1,217	0,019	1,389	0,057	1,356	0,050	1,296	0,024	1,223	0,076	
D-fructose	0,00	0,245	0,028	0,703	0,066	1,538	0,046	1,701	0,033	1,822	0,062	1,838	0,093	1,812	0,043	
L-Fucose	0,00	0,246	0,011	0,427	0,023	0,657	0,005	0,885	0,043	1,250	0,042	1,744	0,045	1,999	0,040	
D-Galactose	0,00	0,225	0,026	0,375	0,008	0,739	0,035	1,071	0,076	1,066	0,050	1,117	0,041	1,087	0,125	
D-Galacturonic Acid	0,00	0,220	0,004	0,566	0,007	1,421	0,090	1,930	0,017	2,436	0,083	2,522	0,098	2,517	0,071	
α-D-Glucose	0,00	0,191	0,023	0,371	0,089	0,565	0,074	0,633	0,026	0,678	0,008	0,697	0,011	0,723	0,030	
Glucose-1-Phosphate	0,00	0,201	0,001	0,343	0,049	0,306	0,055	0,356	0,028	0,344	0,004	0,347	0,058	0,373	0,026	
Glucuronamide	0,00	0,213	0,008	0,390	0,018	0,703	0,040	1,249	0,044	1,441	0,045	1,339	0,092	1,254	0,004	
β-Methyl-D-Galactoside	0,00	0,255	0,052	0,360	0,030	0,621	0,013	0,748	0,026	0,907	0,032	1,085	0,008	1,245	0,059	
α-Methyl-D-Glucoside	0,00	0,279	0,070	0,400	0,004	0,735	0,004	1,109	0,037	1,746	0,044	2,200	0,061	2,330	0,085	
β-Methyl-D-Glucoside	0,00	0,236	0,023	0,380	0,047	0,764	0,030	1,156	0,131	1,746	0,164	2,141	0,005	2,321	0,046	
D-Psicose	0,00	0,234	0,025	0,722	0,014	1,238	0,018	1,469	0,031	1,905	0,091	2,073	0,016	2,019	0,056	
Sedoheptulosan	0,00	0,236	0,006	0,740	0,024	1,521	0,047	1,833	0,033	1,958	0,010	2,074	0,017	2,041	0,016	
L-Sorbose	0,00	0,217	0,020	0,652	0,030	1,258	0,094	1,689	0,112	1,855	0,232	1,860	0,350	1,773	0,345	
D-Tagatose	0,00	0,228	0,040	0,602	0,021	1,523	0,019	1,930	0,015	2,242	0,045	2,330	0,052	2,313	0,022	
D-Xylose	0,00	0,207	0,011	0,385	0,039	1,066	0,033	1,217	0,001	1,011	0,189	0,823	0,009	0,819	0,028	

FF: *A.fumigatus* (4)	Monosaccharide															
	0	1		12		24		36		48		60		72		
		Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD	
N-acetyl-D-galactosamine	0,00	0,214	0,004	0,357	0,021	0,523	0,036	0,620	0,041	0,631	0,048	0,583	0,028	0,579	0,048	
N-acetyl-D-glucosamine	0,00	0,220	0,008	0,650	0,003	1,243	0,037	2,111	0,060	2,300	0,074	2,220	0,071	2,198	0,074	
N-acetyl-D-mannosamine	0,00	0,229	0,023	0,441	0,042	0,539	0,091	0,685	0,078	0,698	0,074	0,626	0,019	0,618	0,023	
D-arabinose	0,00	0,276	0,069	0,483	0,008	0,637	0,039	0,820	0,013	0,982	0,005	1,119	0,018	1,283	0,103	
L-arabinose	0,00	0,237	0,010	0,565	0,060	1,208	0,001	1,594	0,093	1,668	0,112	1,602	0,074	1,577	0,061	
D-fructose	0,00	0,236	0,004	0,848	0,013	1,702	0,035	2,393	0,105	2,508	0,130	2,530	0,130	2,496	0,157	
L-Fucose	0,00	0,224	0,011	0,490	0,013	0,656	0,007	0,912	0,031	1,296	0,018	1,942	0,063	2,204	0,167	
D-Galactose	0,00	0,247	0,021	0,471	0,008	0,765	0,076	1,051	0,037	1,079	0,046	1,090	0,033	1,090	0,008	
D-Galacturonic Acid	0,00	0,234	0,002	0,690	0,031	1,314	0,049	2,198	0,088	2,558	0,091	2,618	0,086	2,591	0,089	
α-D-Glucose	0,00	0,221	0,008	0,452	0,092	0,630	0,040	0,745	0,053	0,795	0,045	0,773	0,012	0,802	0,010	
Glucose-1-Phosphate	0,00	0,218	0,013	0,479	0,006	0,325	0,011	0,408	0,056	0,458	0,063	0,438	0,029	0,534	0,011	
Glucuronamide	0,00	0,214	0,001	0,535	0,040	0,667	0,033	1,072	0,196	1,567	0,028	1,487	0,064	1,421	0,011	
β-Methyl-D-Galactoside	0,00	0,230	0,010	0,471	0,016	0,646	0,006	0,780	0,022	0,940	0,034	1,016	0,036	1,121	0,012	
α-Methyl-D-Glucoside	0,00	0,247	0,004	0,508	0,015	0,759	0,006	1,016	0,030	1,636	0,013	2,067	0,000	2,261	0,066	
β-Methyl-D-Glucoside	0,00	0,218	0,001	0,520	0,025	0,784	0,153	1,130	0,243	1,604	0,089	1,787	0,061	1,906	0,071	
D-Psicose	0,00	0,213	0,001	0,810	0,058	1,053	0,056	1,239	0,144	1,702	0,134	1,915	0,076	1,886	0,115	
Sedoheptulosan	0,00	0,248	0,018	0,801	0,016	1,410	0,022	2,071	0,020	2,365	0,071	2,364	0,097	2,362	0,138	
L-Sorbose	0,00	0,223	0,021	0,743	0,064	1,083	0,216	1,504	0,472	1,815	0,250	1,934	0,144	1,950	0,094	
D-Tagatose	0,00	0,209	0,011	0,706	0,045	1,408	0,083	2,296	0,067	2,439	0,030	2,474	0,065	2,449	0,088	
D-Xylose	0,00	0,219	0,001	0,463	0,043	1,083	0,038	1,479	0,081	1,542	0,086	1,483	0,049	1,442	0,022	

FF: *A.fumigatus* (5)		Monosaccharide													
	0	1		12		24		36		48		60		72	
		Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD
water	0,00	0,208	0,001	0,299	0,025	0,526	0,008	0,544	0,010	0,549	0,018	0,541	0,015	0,518	0,518
N-acetyl-D-galactosamine	0,00	0,202	0,001	0,359	0,033	0,594	0,056	0,586	0,016	0,621	0,010	0,560	0,035	0,565	0,565
N-acetyl-D-glucosamine	0,00	0,243	0,036	0,569	0,019	1,731	0,001	2,178	0,008	2,294	0,011	2,209	0,010	2,226	2,226
N-acetyl-D-mannosamine	0,00	0,211	0,011	0,400	0,030	0,634	0,045	0,623	0,001	0,662	0,009	0,588	0,057	0,600	0,600
D-arabinose	0,00	0,250	0,039	0,414	0,006	0,568	0,030	0,728	0,026	0,946	0,010	1,126	0,060	1,332	1,332
L-arabinose	0,00	0,223	0,008	0,525	0,040	1,207	0,030	1,427	0,026	1,517	0,044	1,457	0,016	1,459	1,459
L-Fucose	0,00	0,201	0,004	0,463	0,008	0,670	0,037	0,822	0,001	1,166	0,054	1,762	0,203	2,434	2,434
D-Galactose	0,00	0,224	0,003	0,381	0,016	0,654	0,006	1,010	0,006	1,097	0,018	1,065	0,071	1,123	1,123
D-Galacturonic Acid	0,00	0,214	0,009	0,642	0,012	1,319	0,004	1,784	0,005	2,173	0,031	2,307	0,064	2,341	2,341
α-D-Glucose	0,00	0,199	0,001	0,484	0,047	0,646	0,046	0,722	0,107	0,785	0,139	0,846	0,179	0,878	0,878
Glucose-1-Phosphate	0,00	0,209	0,005	0,445	0,008	0,359	0,055	0,390	0,058	0,459	0,049	0,558	0,264	0,663	0,663
Glucuronamide	0,00	0,221	0,007	0,495	0,044	0,747	0,063	1,375	0,183	1,770	0,196	1,643	0,161	1,594	1,594
β-Methyl-D-Galactoside	0,00	0,234	0,001	0,487	0,004	0,698	0,001	0,773	0,001	0,929	0,027	1,049	0,095	1,316	1,316
α-Methyl-D-Glucoside	0,00	0,232	0,012	0,552	0,040	0,924	0,072	1,400	0,088	2,073	0,032	2,278	0,033	2,271	2,271
β-Methyl-D-Glucoside	0,00	0,225	0,011	0,484	0,001	0,847	0,011	1,250	0,017	2,034	0,004	2,391	0,098	2,442	2,442
D-Psicose	0,00	0,219	0,024	0,777	0,053	1,233	0,046	1,443	0,037	1,828	0,021	1,957	0,189	1,847	1,847
Sedoheptulosan	0,00	0,217	0,001	0,692	0,035	1,475	0,028	1,731	0,014	1,906	0,064	2,012	0,057	2,000	2,000
L-Sorbose	0,00	0,202	0,002	0,664	0,010	1,127	0,007	1,461	0,062	2,208	0,265	2,228	0,279	2,205	2,205
D-Tagatose	0,00	0,212	0,003	0,677	0,021	1,475	0,111	1,849	0,011	2,057	0,046	1,975	0,087	1,954	1,954
D-Xylose	0,00	0,209	0,001	0,572	0,212	1,373	0,110	1,554	0,109	1,378	0,136	1,033	0,410	0,775	0,775

FF: *A.fumigatus* (6)	Monosaccharide														
	0	1		12		24		36		48		60		72	
		Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD
water	0,00	0,218	0,011	0,375	0,047	0,544	0,010	0,560	0,011	0,552	0,032	0,533	0,004	0,522	0,011
N-acetyl-D-galactosamine	0,00	0,199	0,008	0,452	0,057	0,601	0,010	0,609	0,040	0,589	0,059	0,587	0,004	0,562	0,042
N-acetyl-D-glucosamine	0,00	0,192	0,009	0,617	0,015	1,301	0,001	2,142	0,070	2,379	0,059	2,380	0,081	2,372	0,060
N-acetyl-D-mannosamine	0,00	0,191	0,002	0,452	0,123	0,576	0,063	0,609	0,125	0,589	0,134	0,603	0,078	0,575	0,112
D-arabinose	0,00	0,221	0,037	0,490	0,115	0,618	0,052	0,768	0,001	1,008	0,065	1,213	0,013	1,404	0,097
L-arabinose	0,00	0,242	0,051	0,620	0,129	1,264	0,101	1,552	0,133	1,615	0,177	1,585	0,276	1,549	0,245
D-fructose	0,00	0,187	0,006	0,782	0,030	1,540	0,046	1,720	0,083	1,771	0,122	1,824	0,081	1,832	0,124
L-Fucose	0,00	0,217	0,002	0,528	0,052	0,694	0,078	0,890	0,075	1,293	0,064	2,010	0,170	2,228	0,036
D-Galactose	0,00	0,222	0,001	0,454	0,092	0,858	0,274	1,062	0,078	1,104	0,058	1,145	0,107	1,137	0,031
D-Galacturonic Acid	0,00	0,188	0,004	0,649	0,006	1,237	0,161	2,055	0,127	2,450	0,141	2,546	0,090	2,540	0,110
α-D-Glucose	0,00	0,220	0,033	0,446	0,027	0,585	0,018	0,628	0,016	0,682	0,004	0,725	0,071	0,769	0,052
Glucose-1-Phosphate	0,00	0,203	0,006	0,439	0,006	0,369	0,025	0,387	0,021	0,402	0,001	0,460	0,044	0,576	0,124
Glucuronamide	0,00	0,227	0,037	0,536	0,069	0,800	0,015	1,137	0,403	1,223	0,554	1,183	0,414	1,174	0,432
β-Methyl-D-Galactoside	0,00	0,209	0,040	0,544	0,091	0,690	0,057	0,788	0,033	0,938	0,032	1,152	0,146	1,400	0,199
α-Methyl-D-Glucoside	0,00	0,220	0,027	0,580	0,047	0,842	0,023	1,293	0,036	1,848	0,030	2,192	0,005	2,259	0,039
β-Methyl-D-Glucoside	0,00	0,205	0,006	0,606	0,165	0,864	0,059	1,274	0,041	1,904	0,039	2,145	0,085	2,187	0,128
D-Psicose	0,00	0,210	0,051	0,838	0,059	1,285	0,021	1,499	0,049	1,894	0,011	2,147	0,048	2,186	0,003
Sedoheptulosan	0,00	0,228	0,037	0,789	0,065	1,478	0,018	1,807	0,052	1,891	0,043	2,022	0,144	2,008	0,153
L-Sorbose	0,00	0,197	0,006	0,741	0,055	1,134	0,003	1,423	0,058	1,797	0,105	1,830	0,040	1,738	0,073
D-Tagatose	0,00	0,213	0,038	0,681	0,076	1,174	0,035	1,961	0,156	2,435	0,186	2,477	0,116	2,451	0,126
D-Xylose	0,00	0,227	0,021	0,434	0,008	1,121	0,102	1,441	0,121	1,395	0,112	1,344	0,077	1,259	0,030

A. niger (10)	Monosaccharides							
	0	1	12	24	36	48	60	72
water	0,00	0,132	0,137	0,108	0,105	0,106	0,115	0,131
N-acetyl-D-galactosamine	0,00	0,151	0,276	0,228	0,316	0,336	0,436	0,438
N-acetyl-D-glucosamine	0,00	0,151	0,279	0,277	0,651	1,076	1,796	1,893
N-acetyl-D-mannosamine	0,00	0,162	0,305	0,257	0,394	0,429	0,449	0,427
D-arabinose	0,00	0,173	0,376	0,317	0,526	0,529	0,557	0,544
L-arabinose	0,00	0,177	0,317	0,455	1,130	1,849	2,008	1,918
D-fructose	0,00	0,162	0,413	0,966	1,978	2,166	2,035	1,975
L-Fucose	0,00	0,174	0,344	0,269	0,331	0,320	0,422	0,504
D-Galactose	0,00	0,168	0,345	0,352	0,815	1,063	1,007	0,915
D-Galacturonic Acid	0,00	0,167	0,351	0,552	1,667	2,125	2,142	2,049
α-D-Glucose	0,00	0,166	0,264	0,231	0,264	0,245	0,326	0,386
Glucose-1-Phosphate	0,00	0,168	0,380	0,302	0,352	0,309	0,346	0,307
Glucuronamide	0,00	0,194	0,422	0,266	0,386	0,426	0,611	0,710
β-Methyl-D-Galactoside	0,00	0,181	0,446	0,345	0,516	0,558	0,597	0,558
α-Methyl-D-Glucoside	0,00	0,190	0,412	0,506	1,660	1,997	1,956	1,857
β-Methyl-D-Glucoside	0,00	0,168	0,361	0,314	0,567	0,877	1,824	2,147
D-Psicose	0,00	0,183	0,379	0,632	1,449	1,552	1,422	1,418
Sedoheptulosan	0,00	0,189	0,442	0,481	1,504	2,068	2,218	2,164
L-Sorbose	0,00	0,183	0,408	0,343	0,870	1,375	1,717	1,692
D-Tagatose	0,00	0,174	0,388	0,347	1,003	1,759	2,205	2,147
D-Xylose	0,00	0,156	0,350	0,276	0,497	0,567	0,813	1,012

FF: *A.niger* (12)	Monosaccharide							
	0	**1**	**12**	**24**	**36**	**48**	**60**	**72**
water	0,00	0,139	0,1565	0,166	0,1465	0,163	0,1945	0,2055
N-acetyl-D-galactosamine	0,00	0,1445	0,276	0,2415	0,24	0,3055	0,354	0,321
N-acetyl-D-glucosamine	0,00	0,153	0,2585	0,254	0,494	1,118	1,757	1,8235
N-acetyl-D-mannosamine	0,00	0,146	0,316	0,2715	0,282	0,3885	0,4115	0,415
D-arabinose	0,00	0,1715	0,329	0,291	0,351	0,4395	0,4935	0,489
L-arabinose	0,00	0,18	0,3525	0,3145	0,7305	1,1705	1,3695	1,2575
D-fructose	0,00	0,161	0,347	0,602	1,5525	1,9305	2,0035	1,9935
L-Fucose	0,00	0,156	0,378	0,349	0,3585	0,408	0,5345	0,6015
D-Galactose	0,00	0,161	0,3215	0,321	0,678	0,938	0,8735	0,8005
D-Galacturonic Acid	0,00	0,1635	0,3555	0,439	1,0275	1,5435	1,8875	1,877
α-D-Glucose	0,00	0,147	0,256	0,2395	0,254	0,3525	0,41	0,4455
Glucose-1-Phosphate	0,00	0,142	0,2905	0,267	0,267	0,272	0,254	0,2515
Glucuronamide	0,00	0,174	0,4	0,359	0,3595	0,504	0,5775	0,662
β-Methyl-D-Galactoside	0,00	0,1765	0,3805	0,3195	0,3475	0,4115	0,511	0,5085
α-Methyl-D-Glucoside	0,00	0,1745	0,391	0,407	0,953	1,4955	1,7965	1,7685
β-Methyl-D-Glucoside	0,00	0,168	0,3815	0,323	0,382	0,593	1,0005	1,3025
D-Psicose	0,00	0,163	0,3035	0,3685	1,1665	1,451	1,3485	1,3205
Sedoheptulosan	0,00	0,1815	0,3535	0,399	1,1225	1,7375	2,0705	2,0685
L-Sorbose	0,00	0,1695	0,386	0,3145	0,3725	0,647	1,2935	1,479
D-Tagatose	0,00	0,169	0,3435	0,341	0,757	1,2485	1,8065	1,87
D-Xylose	0,00	0,1655	0,3395	0,3175	0,4575	0,614	0,7075	0,8225

FF:*A.niger* (13)		Monosaccharide						
	0	**1**	**12**	**24**	**36**	**48**	**60**	**72**
water	0,00	0,602	0,502	0,447	0,478	0,520	0,537	0,529
N-acetyl-D-galactosamine	0,00	0,215	0,337	0,247	0,373	0,327	0,422	0,430
N-acetyl-D-glucosamine	0,00	0,212	0,381	0,261	0,537	0,593	0,846	0,981
N-acetyl-D-mannosamine	0,00	0,210	0,376	0,266	0,443	0,423	0,549	0,562
D-arabinose	0,00	0,248	0,452	0,360	0,573	0,650	0,670	0,686
L-arabinose	0,00	0,249	0,441	0,373	0,832	1,099	1,642	1,550
D-fructose	0,00	0,215	0,435	0,415	1,596	1,936	1,988	1,945
L-Fucose	0,00	0,214	0,408	0,333	0,465	0,360	0,426	0,544
D-Galactose	0,00	0,214	0,409	0,334	0,610	0,983	1,240	1,174
D-Galacturonic Acid	0,00	0,207	0,364	0,343	1,097	1,572	1,822	1,747
α-D-Glucose	0,00	0,231	0,368	0,262	0,416	0,381	0,502	0,530
Glucose-1-Phosphate	0,00	0,209	0,398	0,292	0,425	0,307	0,362	0,354
Glucuronamide	0,00	0,256	0,433	0,332	0,595	0,681	0,897	0,948
β-Methyl-D-Galactoside	0,00	0,240	0,494	0,379	0,496	0,424	0,519	0,561
α-Methyl-D-Glucoside	0,00	0,220	0,420	0,351	0,805	0,960	1,406	1,615
β-Methyl-D-Glucoside	0,00	0,232	0,430	0,324	0,627	0,741	1,019	1,162
D-Psicose	0,00	0,204	0,318	0,301	1,150	1,347	1,371	1,525
Sedoheptulosan	0,00	0,213	0,383	0,299	1,061	1,814	2,227	2,204
L-Sorbose	0,00	0,223	0,381	0,296	0,501	0,590	0,990	1,067
D-Tagatose	0,00	0,215	0,387	0,289	0,731	0,947	1,663	1,963
D-Xylose	0,00	0,230	0,373	0,289	0,577	0,618	0,782	0,866

FF:750nm *A.niger* (18)		Monosaccaharide						
	0	**1**	**12**	**24**	**36**	**48**	**60**	**72**
water	0,00	0,193	0,200	0,235	0,198	0,192	0,221	0,233
N-acetyl-D-galactosamine	0,00	0,149	0,326	0,302	0,280	0,290	0,254	0,267
N-acetyl-D-glucosamine	0,00	0,163	0,313	0,278	0,281	0,357	0,633	0,914
N-acetyl-D-mannosamine	0,00	0,161	0,257	0,238	0,237	0,266	0,280	0,312
D-arabinose	0,00	0,180	0,319	0,329	0,418	0,444	0,420	0,435
L-arabinose	0,00	0,156	0,268	0,270	0,443	0,839	1,138	1,107
D-fructose	0,00	0,156	0,296	0,611	1,098	1,255	1,160	1,154
L-Fucose	0,00	0,156	0,289	0,278	0,278	0,308	0,390	0,436
D-Galactose	0,00	0,162	0,310	0,285	0,433	0,642	0,669	0,605
D-Galacturonic Acid	0,00	0,161	0,288	0,304	0,641	1,081	1,295	1,292
α-D-Glucose	0,00	0,157	0,175	0,244	0,253	0,303	0,311	0,349
Glucose-1-Phosphate	0,00	0,167	0,345	0,298	0,290	0,324	0,330	0,327
Glucuronamide	0,00	0,153	0,286	0,289	0,293	0,321	0,403	0,504
β-Methyl-D-Galactoside	0,00	0,171	0,347	0,332	0,319	0,358	0,445	0,477
α-Methyl-D-Glucoside	0,00	0,163	0,310	0,313	0,591	0,995	1,098	1,084
β-Methyl-D-Glucoside	0,00	0,153	0,305	0,287	0,303	0,362	0,497	0,683
D-Psicose	0,00	0,156	0,277	0,280	0,589	0,760	0,760	0,744
Sedoheptulosan	0,00	0,177	0,343	0,367	0,625	1,079	1,285	1,295
L-Sorbose	0,00	0,166	0,297	0,302	0,294	0,437	0,789	0,902
D-Tagatose	0,00	0,163	0,329	0,325	0,487	0,712	1,207	1,327
D-Xylose	0,00	0,164	0,259	0,234	0,298	0,378	0,436	0,557

FF:750nm A.niger (21)		**Monosaccharide**						
	0	**1**	**12**	**24**	**36**	**48**	**60**	**72**
water	0	0,198	0,259	0,2595	0,2795	0,24	0,2995	0,306
N-acetyl-D-galactosamine	0	0,1795	0,2845	0,259	0,2915	0,189	0,2085	0,252
N-acetyl-D-glucosamine	0	0,1615	0,2505	0,259	0,3235	0,3675	0,638	0,9845
N-acetyl-D-mannosamine	0	0,1745	0,286	0,285	0,3255	0,2415	0,311	0,4085
D-arabinose	0	0,185	0,299	0,2805	0,4615	0,45	0,562	0,542
L-arabinose	0	0,1875	0,316	0,4285	0,9455	1,708	2,055	1,9625
D-fructose	0	0,1765	0,3365	0,963	1,986	2,113	1,9915	1,9385
L-Fucose	0	0,1875	0,362	0,39	0,3935	0,2925	0,365	0,452
D-Galactose	0	0,174	0,3175	0,3395	0,605	0,8885	0,6515	0,88
D-Galacturonic Acid	0	0,1835	0,335	0,4245	1,456	2,1015	2,271	2,1865
α-D-Glucose	0	0,1835	0,3065	0,284	0,314	0,219	0,282	0,31
Glucose-1-Phosphate	0	0,184	0,344	0,344	0,375	0,243	0,2885	0,282
Glucuronamide	0	0,17	0,331	0,316	0,37	0,269	0,4395	0,5165
β-Methyl-D-Galactoside	0	0,1865	0,4205	0,3855	0,3985	0,3375	0,4435	0,487
α-Methyl-D-Glucoside	0	0,1645	0,3775	0,368	1,065	1,8355	2,008	1,9205
β-Methyl-D-Glucoside	0	0,1905	0,3965	0,355	0,428	0,456	0,7415	1,1575
D-Psicose	0	0,173	0,2985	0,3975	1,2675	1,7495	1,772	1,667
Sedoheptulosan	0	0,188	0,395	0,3815	0,8365	1,588	2,137	2,123
L-Sorbose	0	0,1955	0,414	0,3615	0,4145	0,534	1,2485	1,481
D-Tagatose	0	0,1785	0,3445	0,3395	0,6055	0,953	2,023	2,1925
D-Xylose	0	0,255	0,408	0,4385	0,647	0,6415	0,807	0,9295

FF:750nm *A.niger* (25)								
	0	**1**	**12**	**24**	**36**	**48**	**60**	**72**
water	0,00	0,110	0,135	0,188	0,272	0,266	0,248	0,260
N-acetyl-D-galactosamine	0,00	0,169	0,266	0,244	0,305	0,234	0,327	0,360
N-acetyl-D-glucosamine	0,00	0,181	0,307	0,280	0,345	0,364	0,650	0,995
N-acetyl-D-mannosamine	0,00	0,167	0,292	0,284	0,339	0,238	0,297	0,335
D-arabinose	0,00	0,163	0,281	0,296	0,516	0,490	0,590	0,587
L-arabinose	0,00	0,174	0,293	0,427	1,039	1,957	2,108	2,031
D-fructose	0,00	0,176	0,367	1,006	2,247	2,315	2,190	2,127
L-Fucose	0,00	0,169	0,298	0,324	0,395	0,306	0,473	0,581
D-Galactose	0,00	0,182	0,318	0,314	0,643	1,200	1,428	1,284
D-Galacturonic Acid	0,00	0,176	0,335	0,453	1,578	2,351	2,663	2,621
α-D-Glucose	0,00	0,327	0,280	0,227	0,274	0,205	0,241	0,263
Glucose-1-Phosphate	0,00	0,194	0,311	0,268	0,323	0,216	0,228	0,214
Glucuronamide	0,00	0,177	0,319	0,301	0,380	0,362	0,629	0,825
β-Methyl-D-Galactoside	0,00	0,191	0,391	0,415	0,413	0,296	0,514	0,510
α-Methyl-D-Glucoside	0,00	0,173	0,338	0,390	1,315	2,016	2,175	2,106
β-Methyl-D-Glucoside	0,00	0,194	0,359	0,352	0,445	0,594	1,219	1,841
D-Psicose	0,00	0,175	0,330	0,467	1,462	2,095	2,239	2,201
Sedoheptulosan	0,00	0,195	0,384	0,422	1,196	1,963	2,330	2,261
L-Sorbose	0,00	0,182	0,336	0,322	0,421	0,551	1,528	1,957
D-Tagatose	0,00	0,183	0,332	0,335	0,770	1,655	2,501	2,542
D-Xylose	0,00	0,456	0,333	0,281	0,472	0,553	0,704	0,848

FF:750nm *A.niger* (30)	Monosaccharide							
	0	**1**	**12**	**24**	**36**	**48**	**60**	**72**
water	0,00	0,167	0,208	0,198	0,263	0,291	0,204	0,212
N-acetyl-D-galactosamine	0,00	0,179	0,268	0,219	0,241	0,263	0,283	0,290
N-acetyl-D-glucosamine	0,00	0,169	0,269	0,243	0,425	0,760	1,638	1,741
N-acetyl-D-mannosamine	0,00	0,170	0,303	0,260	0,276	0,311	0,371	0,375
D-arabinose	0,00	0,158	0,277	0,310	0,459	0,484	0,548	0,553
L-arabinose	0,00	0,185	0,301	0,484	1,011	1,693	1,886	1,790
D-fructose	0,00	0,184	0,363	0,833	1,774	1,996	1,937	1,916
L-Fucose	0,00	0,169	0,323	0,309	0,300	0,326	0,524	0,584
D-Galactose	0,00	0,177	0,352	0,318	0,745	1,017	1,057	0,981
D-Galacturonic Acid	0,00	0,169	0,308	0,443	1,135	1,856	2,237	2,186
α-D-Glucose	0,00	0,160	0,223	0,204	0,218	0,240	0,299	0,321
Glucose-1-Phosphate	0,00	0,173	0,296	0,262	0,260	0,224	0,265	0,262
Glucuronamide	0,00	0,177	0,314	0,294	0,366	0,431	0,679	0,894
β-Methyl-D-Galactoside	0,00	0,186	0,393	0,346	0,406	0,416	0,532	0,540
α-Methyl-D-Glucoside	0,00	0,175	0,343	0,425	1,346	1,748	1,945	1,903
β-Methyl-D-Glucoside	0,00	0,180	0,354	0,312	0,429	0,467	0,889	1,312
D-Psicose	0,00	0,170	0,338	0,411	1,131	1,426	1,516	1,537
Sedoheptulosan	0,00	0,185	0,394	0,400	1,082	1,678	2,097	2,053
L-Sorbose	0,00	0,181	0,371	0,318	0,423	0,568	1,167	1,321
D-Tagatose	0,00	0,177	0,377	0,353	0,704	1,197	2,011	2,061
D-Xylose	0,00	0,178	0,253	0,247	0,452	0,616	0,971	1,171

FF:750nm *A.nige* r (33)	Monosaccahride							
	0	**1**	**12**	**24**	**36**	**48**	**60**	**72**
water	0	0,2935	0,3705	0,468	0,1905	0,172	0,171	0,2045
N-acetyl-D-galactosamine	0	0,205	0,3575	0,384	0,3135	0,3485	0,519	0,6975
N-acetyl-D-glucosamine	0	0,1935	0,338	0,331	0,489	0,756	1,39	1,5785
N-acetyl-D-mannosamine	0	0,171	0,359	0,351	0,318	0,3735	0,478	0,4925
D-arabinose	0	0,1935	0,3985	0,432	0,4785	0,508	0,5965	0,644
L-arabinose	0	0,2	0,3625	0,4905	0,9545	1,8395	1,9265	1,845
D-fructose	0	0,1785	0,459	1,361	2,1125	2,077	1,989	1,916
L-Fucose	0	0,184	0,388	0,42	0,3205	0,359	0,637	0,783
D-Galactose	0	0,182	0,398	0,4095	0,5875	1,0755	1,1785	1,0655
D-Galacturonic Acid	0	0,188	0,3915	0,5645	1,3175	2,204	2,535	2,5015
α-D-Glucose	0	0,193	0,325	0,3085	0,2745	0,255	0,3135	0,332
Glucose-1-Phosphate	0	0,1925	0,3755	0,3495	0,2605	0,222	0,2415	0,2625
Glucuronamide	0	0,189	0,389	0,3925	0,3175	0,4145	0,7205	0,9455
β-Methyl-D-Galactoside	0	0,207	0,512	0,5685	0,3915	0,449	0,556	0,6435
α-Methyl-D-Glucoside	0	0,2005	0,4215	0,5205	1,311	1,8585	1,8765	1,834
β-Methyl-D-Glucoside	0	0,19	0,4275	0,448	0,443	0,62	1,1115	1,7345
D-Psicose	0	0,187	0,407	0,675	1,5885	1,981	2,028	2,031
Sedoheptulosan	0	0,191	0,4565	0,469	0,4615	0,604	0,984	1,711
L-Sorbose	0	0,198	0,4525	0,4505	0,397	0,6435	1,2015	1,4825
D-Tagatose	0	0,1665	0,4005	0,4515	0,7295	1,4145	2,367	2,417
D-Xylose	0	0,18	0,322	0,3325	0,498	0,84	1,3355	0,85

A. flavus (22)		**Monosaccharide**					
	0	**1**	**12**	**24**	**36**	**48**	**60**	**72**
water	0,00	0,193	0,380	0,436	0,447	0,439	0,444	0,434
N-acetyl-D-galactosamine	0,00	0,189	0,357	0,488	0,485	0,472	0,458	0,432
N-acetyl-D-glucosamine	0,00	0,191	0,454	1,015	1,646	1,828	1,841	1,900
N-acetyl-D-mannosamine	0,00	0,191	0,388	0,550	0,565	0,611	0,552	0,549
D-arabinose	0,00	0,204	0,466	0,622	0,707	0,833	0,864	0,949
L-arabinose	0,00	0,185	0,572	1,233	1,646	1,698	1,670	1,624
D-fructose	0,00	0,214	0,596	1,286	1,628	1,688	1,663	1,623
L-Fucose	0,00	0,204	0,429	0,660	0,879	1,559	1,800	1,934
D-Galactose	0,00	0,200	0,368	0,450	0,791	0,984	0,980	1,012
D-Glucosamine	0,00	0,193	0,554	1,368	1,466	1,403	1,344	1,254
α-D-Glucose	0,00	0,186	0,490	0,495	0,518	0,530	0,539	0,563
Glucose-1-Phosphate	0,00	0,213	0,455	0,412	0,568	0,589	0,612	0,616
Glucuronamide	0,00	0,204	0,565	0,671	0,847	1,121	1,159	1,175
β-Methyl-D-Galactoside	0,00	0,210	0,566	0,643	0,653	0,733	0,761	0,905
α-Methyl-D-Glucoside	0,00	0,189	0,613	0,888	1,188	1,652	1,653	1,664
β-Methyl-D-Glucoside	0,00	0,195	0,533	0,883	1,273	1,679	1,720	1,828
D-Psicose	0,00	0,190	0,649	1,459	2,015	2,087	2,051	2,001
Sedoheptulosan	0,00	0,206	0,598	1,052	1,738	1,979	1,966	1,921
L-Sorbose	0,00	0,205	0,598	1,027	1,578	1,861	1,913	2,006
D-Tagatose	0,00	0,199	0,606	0,889	1,606	2,094	2,159	2,162
D-Xylose	0,00	0,193	0,398	0,632	1,031	1,268	1,297	1,310

***A.flavus* 32 :Monosaccharide**								
	0	**1**	**12**	**24**	**36**	**48**	**60**	**72**
water	0,00	0,216	0,545	0,467	0,461	0,466	0,464	0,462
N-acetyl-D-galactosamine	0,00	0,208	0,586	0,510	0,496	0,497	0,474	0,436
N-acetyl-D-glucosamine	0,00	0,204	0,677	1,217	1,281	1,340	1,332	1,328
N-acetyl-D-mannosamine	0,00	0,207	0,579	0,538	0,551	0,573	0,542	0,497
D-arabinose	0,00	0,214	0,637	0,679	0,771	0,927	0,935	1,017
L-arabinose	0,00	0,213	0,686	1,382	1,589	1,665	1,645	1,651
D-fructose	0,00	0,223	0,773	1,207	1,340	1,393	1,387	1,363
L-Fucose	0,00	0,216	0,680	0,945	1,362	1,719	1,762	1,776
D-Galactose	0,00	0,241	0,509	0,674	0,856	0,911	0,894	0,870
D-Galacturonic Acid	0,00	0,215	0,738	1,142	1,409	1,718	1,817	1,828
α-D-Glucose	0,00	0,193	0,588	0,617	0,735	0,887	0,864	0,821
Glucose-1-Phosphate	0,00	0,200	0,497	0,466	0,501	0,548	0,525	0,505
Glucuronamide	0,00	0,215	0,700	0,860	1,003	0,955	0,871	0,795
β-Methyl-D-Galactoside	0,00	0,224	0,739	0,723	0,773	0,911	0,900	0,985
α-Methyl-D-Glucoside	0,00	0,199	0,706	1,082	1,538	1,822	1,808	1,770
β-Methyl-D-Glucoside	0,00	0,223	0,867	1,513	1,798	1,993	2,006	2,016
D-Psicose	0,00	0,203	0,758	0,991	1,135	1,538	1,682	1,762
Sedoheptulosan	0,00	0,228	0,764	1,278	1,596	1,839	1,811	1,802
L-Sorbose	0,00	0,221	0,785	0,995	1,132	1,334	1,333	1,366
D-Tagatose	0,00	0,227	0,810	1,337	1,657	1,648	1,577	1,531
D-Xylose	0,00	0,218	0,688	0,956	1,046	1,089	1,082	1,108

Growth profiles (disaccharides) for Chapter 3.

Disaccharides: Absorbance readings of isolates during 72-hour incubation (FF plate - Biolog). Data used to represent growth profiles of isolates in Chapter 3.

A.fumigatus (1)							
Disaccharide	**0Hr**	**12Hr**	**24Hr**	**36Hr**	**48Hr**	**60Hr**	**72Hr**
water	**0,19**	**0,31**	**0,39**	**0,5**	**0,52**	**0,5**	**0,5**
D-cellobiose	**0,19**	**0,47**	**0,96**	**1,54**	**2,14**	**2,2**	**2,2**
Gentiobiose	**0,21**	**0,38**	**0,62**	**0,93**	**1,5**	**1,5**	**1,5**
α-D-Lactose	**0,19**	**0,39**	**0,45**	**0,57**	**0,65**	**0,8**	**1,3**
Maltose	**0,18**	**0,38**	**0,49**	**1,97**	**1,99**	**0,6**	**2,0**
D-Melibiose	**0,19**	**0,4**	**0,52**	**0,57**	**0,62**	**0,6**	**0,6**
Sucrose	**0,25**	**0,42**	**0,55**	**0,64**	**0,69**	**0,7**	**0,8**
D-Trehalose	**0,2**	**0,4**	**0,69**	**1,24**	**2,19**	**2,3**	**2,3**
Turanose	**0,33**	**0,44**	**0,64**	**0,67**	**0,74**	**0,8**	**0,8**

FF: A.fumigatus (2)	Disaccharide														
	0	1		12		24		36		48		60		72	
		Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD
water	0,00	0,168	0,007	0,404	0,074	0,517	0,013	0,517	0,011	0,504	0,017	0,513	0,013	0,476	0,009
D-cellobiose	0,00	0,164	0,003	0,432	0,066	0,797	0,085	1,100	0,100	1,370	0,086	1,813	0,057	1,844	0,095
Gentiobiose	0,00	0,189	0,022	0,507	0,047	1,153	0,015	1,930	0,011	2,328	0,034	2,413	0,064	2,343	0,054
α-D-Lactose	0,00	0,177	0,013	0,421	0,010	0,599	0,051	0,614	0,025	0,749	0,040	1,318	0,049	2,005	0,078
Maltose	0,00	0,195	0,040	0,495	0,005	1,355	0,027	1,887	0,044	2,004	0,029	2,030	0,095	1,961	0,148
D-Melibiose	0,00	0,165	0,009	0,418	0,048	0,624	0,003	0,710	0,058	0,846	0,028	1,025	0,038	1,095	0,001
Sucrose	0,00	0,190	0,013	0,633	0,042	1,666	0,062	1,997	0,021	2,064	0,001	2,135	0,054	2,044	0,070
D-Trehalose	0,00	0,185	0,026	0,524	0,016	1,131	0,008	2,007	0,030	2,253	0,014	2,259	0,064	2,181	0,054
Turanose	0,00	0,188	0,017	0,473	0,062	0,878	0,028	1,631	0,050	2,172	0,028	2,221	0,032	2,143	0,033

FF: *A.fumigatus* (3)	Disaccharide														
	0	1		12		24		36		48		60		72	
		Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD
water	0,00	0,215	0,025	0,298	0,010	0,549	0,013	0,615	0,009	0,621	0,006	0,621	0,013	0,581	0,006
D-cellobiose	0,00	0,233	0,037	0,541	0,004	1,138	0,028	1,739	0,083	2,060	0,118	2,081	0,091	2,075	0,093
Gentiobiose	0,00	0,231	0,006	0,554	0,040	1,145	0,048	1,420	0,003	1,345	0,012	1,317	0,011	1,297	0,047
α-D-Lactose	0,00	0,253	0,020	0,402	0,004	0,608	0,080	0,740	0,069	0,892	0,174	1,322	0,453	1,893	0,385
Maltose	0,00	0,220	0,006	0,649	0,013	1,498	0,026	1,569	0,025	1,553	0,022	1,530	0,015	1,529	0,071
D-Melibiose	0,00	0,233	0,013	0,373	0,060	0,573	0,001	0,712	0,015	0,718	0,003	0,718	0,019	0,672	0,045
Sucrose	0,00	0,250	0,063	0,391	0,086	0,607	0,023	0,726	0,021	0,738	0,040	0,775	0,043	0,765	0,001
D-Trehalose	0,00	0,240	0,022	0,503	0,008	1,144	0,031	1,788	0,025	2,371	0,028	2,378	0,053	2,319	0,004
Turanose	0,00	0,253	0,009	0,361	0,054	0,689	0,013	0,839	0,031	1,069	0,050	1,577	0,054	2,071	0,033

FF: *A.fumigatus* (4)	Disaccharide															
	0	1		12		24		36		48		60		72	
		Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD
water	0	0,196	0,004	0,313	0,078	0,482	0,071	0,586	0,029	0,587	0,023	0,588	0,011	0,581	0,008
D-cellobiose	0,00	0,236	0,003	0,695	0,041	1,090	0,009	1,881	0,008	2,348	0,037	2,277	0,004	2,235	0,006
Gentiobiose	0,00	0,227	0,006	0,634	0,006	0,996	0,008	1,268	0,037	1,341	0,081	1,237	0,028	1,188	0,008
α-D-Lactose	0,00	0,229	0,008	0,433	0,040	0,601	0,013	0,732	0,004	0,866	0,006	1,348	0,190	2,153	0,164
Maltose	0,00	0,222	0,018	0,751	0,018	1,581	0,074	1,862	0,219	1,835	0,246	1,759	0,227	1,705	0,216
D-Melibiose	0,00	0,225	0,011	0,448	0,015	0,568	0,088	0,756	0,037	0,792	0,033	0,779	0,016	0,717	0,011
Sucrose	0,00	0,238	0,004	0,472	0,004	0,622	0,014	0,730	0,040	0,781	0,040	0,766	0,001	0,718	0,005
D-Trehalose	0,00	0,233	0,026	0,571	0,002	0,917	0,011	1,694	0,017	2,100	0,051	2,217	0,044	2,163	0,066
Turanose	0,00	0,222	0,002	0,531	0,008	0,694	0,029	0,906	0,020	1,425	0,100	2,171	0,102	2,772	0,025

FF: *A.fumigatus* (5)	Disaccharide														
	0	1		12		24		36		48		60		72	
		Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD
water	0,00	0,208	0,001	0,299	0,025	0,526	0,008	0,544	0,010	0,549	0,018	0,541	0,015	0,518	0,518
D-cellobiose	0,00	0,204	0,004	0,611	0,037	1,155	0,041	1,596	0,087	1,972	0,232	1,992	0,185	1,990	1,990
Gentiobiose	0,00	0,241	0,013	0,627	0,033	1,171	0,131	1,685	0,137	1,665	0,128	1,584	0,100	1,601	1,601
α-D-Lactose	0,00	0,199	0,005	0,407	0,008	0,642	0,009	0,693	0,020	1,014	0,110	1,707	0,185	2,539	2,539
Maltose	0,00	0,211	0,008	0,722	0,044	1,559	0,010	1,686	0,020	1,703	0,031	1,645	0,032	1,614	1,614
D-Melibiose	0,00	0,200	0,007	0,440	0,015	0,654	0,037	0,665	0,027	0,698	0,025	0,633	0,038	0,669	0,669
Sucrose	0,00	0,263	0,002	0,492	0,016	0,685	0,069	0,691	0,054	0,752	0,023	0,696	0,008	0,749	0,749
D-Trehalose	0,00	0,234	0,011	0,584	0,006	1,090	0,033	1,897	0,001	2,552	0,093	2,482	0,138	2,472	2,472
Turanose	0,00	0,237	0,009	0,500	0,062	0,698	0,037	0,775	0,051	0,982	0,050	1,207	0,027	1,950	1,950

FF: *A.fumigatus* (6)	Disaccharide														
	0	0		12		24		36		48		60		72	
		Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD	Avr	SD
water	0	0,218	0,011	0,375	0,047	0,544	0,010	0,560	0,011	0,552	0,032	0,533	0,004	0,522	0,011
D-cellobiose	0,00	0,228	0,033	0,669	0,076	1,171	0,124	1,603	0,180	1,981	0,206	2,022	0,164	2,021	0,158
Gentiobiose	0,00	0,224	0,010	0,617	0,029	1,163	0,108	1,371	0,018	1,364	0,046	1,336	0,042	1,308	0,011
α-D-Lactose	0,00	0,193	0,008	0,456	0,004	0,608	0,024	0,649	0,033	0,752	0,115	1,197	0,082	1,790	0,106
Maltose	0,00	0,236	0,057	0,746	0,006	1,593	0,031	1,758	0,059	1,756	0,104	1,726	0,051	1,704	0,074
D-Melibiose	0,00	0,197	0,003	0,491	0,018	0,637	0,001	0,655	0,048	0,628	0,074	0,663	0,028	0,659	0,029
Sucrose	0,00	0,210	0,006	0,559	0,098	0,651	0,017	0,681	0,042	0,692	0,071	0,760	0,004	0,743	0,023
D-Trehalose	0,00	0,198	0,010	0,594	0,088	1,045	0,096	1,719	0,050	2,377	0,098	2,378	0,032	2,327	0,039
Turanose	0,00	0,199	0,033	0,518	0,105	0,678	0,104	0,817	0,111	1,186	0,209	1,849	0,467	2,423	0,336

A. niger (10)			Disaccharide					
	0	**1**	**12**	**24**	**36**	**48**	**60**	**72**
water	0,00	0,132	0,137	0,108	0,105	0,106	0,115	0,131
D-cellobiose	0,00	0,195	0,326	0,646	1,674	2,007	1,920	1,925
Gentiobiose	0,00	0,203	0,402	0,425	0,680	0,980	1,252	1,198
α-D-Lactose	0,00	0,175	0,398	0,338	0,514	0,536	0,574	0,564
Maltose	0,00	0,183	0,367	0,726	1,873	2,172	1,071	0,606
D-Melibiose	0,00	0,160	0,375	0,279	0,439	0,521	0,610	0,603
Sucrose	0,00	0,195	0,461	0,330	0,454	0,493	0,541	0,479
D-Trehalose	0,00	0,213	0,476	0,347	0,569	0,585	0,641	0,776
Turanose	0,00	0,195	0,358	0,416	0,960	1,694	2,148	2,109

FF:750nm *A.niger* (12)	**Disaccharide**							
	0	**1**	**12**	**24**	**36**	**48**	**60**	**72**
water	0,00	0,139	0,157	0,166	0,147	0,163	0,195	0,206
D-cellobiose	0,00	0,182	0,297	0,528	1,239	1,574	1,466	1,533
Gentiobiose	0,00	0,191	0,322	0,300	0,563	0,779	1,081	1,106
α-D-Lactose	0,00	0,181	0,330	0,291	0,322	0,474	0,519	0,559
Maltose	0,00	0,176	0,261	0,472	1,382	1,575	1,661	1,711
D-Melibiose	0,00	0,163	0,408	0,324	0,341	0,457	0,590	0,653
Sucrose	0,00	0,175	0,377	0,341	0,330	0,402	0,549	0,575
D-Trehalose	0,00	0,176	0,507	0,481	0,609	0,689	0,718	0,813
Turanose	0,00	0,182	0,320	0,274	0,447	0,618	0,835	1,069

FF:750nm A.niger (13)								
	0	**1**	**12**	**24**	**36**	**48**	**60**	**72**
water	0,00	0,602	0,502	0,447	0,478	0,520	0,537	0,529
D-cellobiose	0,00	0,237	0,332	0,402	1,385	1,891	1,846	1,832
Gentiobiose	0,00	0,226	0,390	0,321	0,618	0,644	0,812	0,903
α-D-Lactose	0,00	0,225	0,346	0,268	0,449	0,449	0,595	0,662
Maltose	0,00	0,213	0,328	0,351	1,303	1,353	1,184	1,018
D-Melibiose	0,00	0,236	0,448	0,320	0,482	0,480	0,671	0,697
Sucrose	0,00	0,226	0,429	0,319	0,458	0,407	0,550	0,598
D-Trehalose	0,00	0,235	0,395	0,309	0,582	0,599	0,897	1,160
Turanose	0,00	0,235	0,374	0,269	0,471	0,517	0,708	0,960

FF:750nm A.niger (18)			Disaccharide					
	0	1	12	24	36	48	60	72
water	0,00	0,193	0,200	0,235	0,198	0,192	0,221	0,233
D-cellobiose	0,00	0,184	0,275	0,284	0,601	0,916	0,982	0,927
Gentiobiose	0,00	0,142	0,262	0,263	0,333	0,406	0,563	0,657
α-D-Lactose	0,00	0,161	0,331	0,277	0,292	0,325	0,420	0,448
Maltose	0,00	0,171	0,302	0,383	0,927	1,073	1,218	1,192
D-Melibiose	0,00	0,158	0,311	0,291	0,294	0,366	0,418	0,487
Sucrose	0,00	0,156	0,289	0,280	0,305	0,336	0,392	0,503
D-Trehalose	0,00	0,163	0,294	0,296	0,320	0,382	0,441	0,434
Turanose	0,00	0,166	0,299	0,299	0,413	0,513	0,843	1,105

FF:750nm A.niger (21)								
	0	**1**	**12**	**24**	**36**	**48**	**60**	**72**
water	0,00	0,198	0,259	0,260	0,280	0,240	0,300	0,306
D-cellobiose	0,00	0,188	0,283	0,424	1,477	1,890	1,836	1,791
Gentiobiose	0,00	0,180	0,336	0,329	0,454	0,486	0,794	0,986
α-D-Lactose	0,00	0,167	0,378	0,343	0,373	0,277	0,455	0,508
Maltose	0,00	0,187	0,289	0,492	1,524	1,900	1,893	1,788
D-Melibiose	0,00	0,176	0,369	0,323	0,411	0,285	0,380	0,493
Sucrose	0,00	0,186	0,398	0,368	0,436	0,373	0,496	0,570
D-Trehalose	0,00	0,195	0,384	0,382	0,438	0,387	0,546	0,593
Turanose	0,00	0,175	0,314	0,339	0,666	1,001	1,882	2,096

FF:750nm A.niger (25)	Disaccaharide							
	0	**1**	**12**	**24**	**36**	**48**	**60**	**72**
water	0,00	0,110	0,135	0,188	0,272	0,266	0,248	0,260
D-cellobiose	0,00	0,214	0,322	0,403	1,551	2,236	2,286	2,221
Gentiobiose	0,00	0,189	0,345	0,346	0,531	0,631	1,011	1,238
α-D-Lactose	0,00	0,178	0,322	0,328	0,390	0,386	0,627	0,659
Maltose	0,00	0,175	0,261	0,481	1,820	2,326	2,395	2,329
D-Melibiose	0,00	0,180	0,354	0,347	0,385	0,293	0,555	0,581
Sucrose	0,00	0,258	0,344	0,335	0,405	0,365	0,595	0,655
D-Trehalose	0,00	0,187	0,331	0,317	0,394	0,397	0,654	0,809
Turanose	0,00	0,186	0,318	0,329	0,633	0,857	1,968	2,308

FF:750nm A.niger (30)	**Disaccharide**							
	0	**1**	**12**	**24**	**36**	**48**	**60**	**72**
water	0,00	0,167	0,208	0,198	0,263	0,291	0,204	0,212
D-cellobiose	0,00	0,199	0,275	0,408	1,348	1,752	1,698	1,641
Gentiobiose	0,00	0,178	0,377	0,387	0,532	0,694	1,120	1,214
α-D-Lactose	0,00	0,184	0,377	0,339	0,380	0,445	0,671	0,872
Maltose	0,00	0,186	0,279	0,458	1,429	1,808	1,945	1,857
D-Melibiose	0,00	0,192	0,386	0,370	0,480	0,581	0,788	0,861
Sucrose	0,00	0,187	0,401	0,339	0,340	0,340	0,503	0,568
D-Trehalose	0,00	0,195	0,229	0,367	0,445	0,471	0,656	0,782
Turanose	0,00	0,187	0,308	0,324	0,489	0,693	1,427	1,813

FF:750nm A.niger (33)	Disaccahride							
	0	**1**	**12**	**24**	**36**	**48**	**60**	**72**
water	0,00	0,294	0,371	0,468	0,191	0,172	0,171	0,245
D-cellobiose	0,00	0,212	0,329	0,497	1,626	2,137	2,104	2,042
Gentiobiose	0,00	0,200	0,442	0,417	0,544	0,706	1,303	1,384
α-D-Lactose	0,00	0,184	0,413	0,453	0,455	0,474	0,604	0,716
Maltose	0,00	0,190	0,328	0,679	1,942	2,301	2,258	2,200
D-Melibiose	0,00	0,168	0,377	0,413	0,327	0,350	0,538	0,613
Sucrose	0,00	0,195	0,448	0,485	0,406	0,393	0,554	0,621
D-Trehalose	0,00	0,205	0,424	0,432	0,469	0,524	0,663	0,799
Turanose	0,00	0,195	0,382	0,429	0,638	0,804	1,648	2,131

A. flavus **(22)**		**Disaccharide**						
	0	1	12	24	36	48	60	72
water	0	0,193	0,3795	0,436	0,447	0,4385	0,4435	0,4335
D-cellobiose	0	0,1855	0,5965	1,2265	1,882	1,8965	1,846	1,7555
Gentiobiose	0	0,2025	0,5525	0,974	1,4955	1,525	1,4685	1,433
α-D-Lactose	0	0,182	0,4775	0,6345	0,798	1,751	2,1455	2,3965
Maltose	0	0,192	0,543	1,487	1,7785	1,704	1,5935	1,3075
D-Melibiose	0	0,19	0,5045	0,589	0,589	0,589	0,6015	0,573
Sucrose	0	0,2	0,5275	0,5745	0,586	0,629	0,6265	0,675
D-Trehalose	0	0,155	0,409	0,617	0,8535	1,0185	0,999	0,9945
Turanose	0	0,196	0,483	0,6465	0,745	0,958	1,037	1,199

A.flavus 32 :Disaccharide								
	0	**1**	**12**	**24**	**36**	**48**	**60**	**72**
water	0	0,216	0,5445	0,4665	0,4605	0,4655	0,464	0,4615
D-cellobiose	0	0,208	0,5895	1,093	1,3455	1,482	1,489	1,523
Gentiobiose	0	0,2165	0,7375	0,9375	0,9785	0,9905	0,9075	0,858
α-D-Lactose	0	0,213	0,696	0,7335	0,97	1,462	1,585	1,763
Maltose	0	0,196	0,724	1,194	1,3475	1,4465	1,4725	1,581
D-Melibiose	0	0,215	0,7325	0,7665	0,89	1,0505	1,0705	1,117
Sucrose	0	0,2205	0,7165	0,716	0,8045	0,971	1,0015	1,069
D-Trehalose	0	0,221	0,8035	1,3425	1,8135	1,795	1,715	1,6335
Turanose	0	0,2225	0,76	1,0995	1,3395	1,711	1,7845	1,8545

Growth profiles for Chapter 3.

Absorbance readings were taken at 750nm after 48 hours of incubation of isolates in a growth medium. Duplicate readings at 24-hour intervals up to 72 hours were used to draw monosaccharide and disaccharide growth profiles.

48 hours: isolates 1 - 9									750nm									
COMPOUND	1A	1B	2A	2B	3A	3B	4A	4B	5A	5B	6A	6B	7A	7B	8A	8B	9A	9B
water	0 523	0 509	0 516	0 492	0 616	0 625	0 603	0 57	0 562	0 536	0 529	0 574	0 569	0 575	0 588	0 48	0 562	0 54
tween 80	0 66	0 749	0 679	0 762	0 739	0 773	0 819	0 919	0 801	0 734	0 687	0 738	0 839	0 837	0 732	0 874	0 731	0 85
N-acetyl-D-galactosamine	0 549	0 596	0 475	0 525	0 648	0 587	0 665	0 597	0 614	0 628	0 547	0 631	0 673	0 616	0 537	0 567	0 501	0 547
N-acetyl-D-glucosamine	2 46	2 346	2 186	2 341	2 362	2 473	2 352	2 247	2 286	2 301	2 421	2 337	2 463	2 482	2 413	2 508	2 425	2 419
N-acetyl-D-mannosamine	0 552	0 604	0 567	0 531	0 701	0 66	0 75	0 645	0 668	0 655	0 494	0 684	0 641	0 667	0 73	0 542	0 498	0 568
adonitol	1 546	0 789	0 925	0 893	0 901	0 943	0 868	0 819	1 017	1 08	0 899	0 935	1 055	1 065	0 953	0 952	0 82	0 801
amygdalin	2 08	2 01	2 197	2 248	2 462	2 407	2 238	2 026	2 33	2 546	2 499	2 3	2 336	2 337	2 095	2 118	2 445	2 581
D-arabinose	0 778	0 769	0 879	0 857	0 946	0 943	0 985	0 978	0 953	0 939	0 962	1 054	0 949	1 072	1 082	0 999	0 928	0 952
L-arabinose	2 214	1 897	1 605	1 823	1 391	1 32	1 747	1 589	1 486	1 548	1 74	1 489	1 538	1 731	1 506	1 29	1 771	1 799
D-arabitol	0 96	0 8	1 668	1 462	1 469	1 358	1 224	1 066	2 04	2 209	1 361	1 242	1 417	1 478	2 383	2 505	1 168	1 194
arbutin	1 369	1 284	1 43	1 309	1 828	1 82	2 037	1 695	1 784	1 807	1 828	1 672	2 073	1 914	2 055	2 051	1 753	1 906
D-cellobiose	2 136	2 135	2 077	2 168	2 143	1 976	2 374	2 322	1 808	2 136	1 835	2 127	2 25	2 25	1 929	1 984	2 334	2 355
α- cyclodextrin	0 888	0 894	0 839	0 887	0 875	1 09	1 007	0 869	0 895	0 986	0 887	0 941	0 912	0 962	0 951	0 945	0 818	0 867
β- cyclodextrin	1 236	1 224	1 385	1 457	1 365	1 452	1 592	1 458	1 619	1 587	1 288	1 745	1 73	1 7	1 81	1 739	1 698	1 691
dextrin	1 54	1 6	1 671	1 731	1 511	1 538	1 692	1 552	1 922	2 047	1 648	1 791	1 82	1 821	1 772	1 623	1 71	1 772
I- erythritol	2 048	1 621	2 127	2 122	1 929	1 993	2 141	2 05	2 116	2 078	2 05	2 11	2 284	2 317	2 376	2 39	2 222	2 21
D-fructose	2 129	2 201	2 233	2 267	1 778	1 866	2 6	2 416	2 058	1 971	1 684	1 857	2 281	2 343	1 999	1 996	2 13	2 392
L-fructose	0 496	0 526	0 527	0 473	0 54	0 56	0 577	0 51	0 608	0 592	0 551	0 576	0 617	0 63	0 632	0 579	0 492	0 563
L-Fucose	0 96	0 806	1 365	1 302	1 28	1 22	1 308	1 283	1 204	1 128	1 338	1 247	1 346	1 402	1 5	1 436	1 14	1 261
D-Galactose	0 981	0 969	1	1 021	1 101	1 03	1 111	1 046	1 11	1 084	1 145	1 063	1 081	1 143	0 994	1 005	0 983	1 051
D-Galacturonic Acid	2 074	2 034	2 304	2 352	2 377	2 495	2 622	2 494	2 195	2 151	2 35	2 549	2 409	2 455	2 043	1 967	2 425	2 47
Gentiobiose	1 618	1 38	1 603	1 657	1 336	1 353	1 398	1 283	1 574	1 755	1 331	1 396	1 428	1 538	1 483	1 576	1 294	1 258
D-Gluconic Acid	0 808	0 676	1 001	0 942	1 111	1 064	1 09	1 007	1 146	1 142	1 243	1 112	1 223	1 268	1 303	1 382	1 251	1 245
D-Glucosamine	2 264	2 302	2 014	1 976	1 751	1 774	2 166	1 874	1 902	1 871	1 88	1 891	2 174	2 151	1 794	1 867	2 278	2 138
α-D-Glucose	0 536	0 526	0 597	0 661	0 683	0 672	0 827	0 763	0 883	0 686	0 685	0 679	0 807	0 786	0 795	0 609	0 659	0 667
Glucose-1-Phosphate	0 321	0 379	0 334	0 295	0 341	0 347	0 502	0 413	0 493	0 424	0 401	0 402	0 52	0 538	0 735	0 582	0 393	0 424
Glucuronamide	1 317	1 092	1 532	1 557	1 409	1 473	1 586	1 547	1 631	1 908	0 831	1 614	1 568	1 555	1 424	1 445	1 543	1 558
D-Glucronic Acid	1 199	0 97	1 67	1 612	1 69	1 888	1 621	1 655	1 809	1 993	1 678	1 677	1 915	1 764	2 394	2 455	1 706	1 676
Glycerol	0 988	0 981	1 199	1 256	1 155	1 197	1 107	1 1	1 46	1 514	1 324	1 285	1 363	1 347	1 477	1 445	1 336	1 453
Glycogen	1 968	1 927	2 163	2 145	1 966	2 111	2 154	1 954	2 177	2 214	2 194	2 184	2 296	2 297	2 175	2 132	2 178	2 305
m-Inositol	1 314	1 461	1 348	1 492	1 272	1 12	1 345	1 071	1 278	1 296	1 28	1 324	1 419	1 41	1 359	1 513	1 069	1 182
2-Keto-D-Gluconic Acid	0 679	0 636	0 777	0 721	0 945	0 861	0 906	0 764	1 129	0 985	0 773	0 836	1 077	0 975	2 031	2 074	0 861	1 021
α-D-Lactobiose	0 648	0 642	0 818	0 701	1 015	0 769	0 87	0 862	1 091	0 936	0 67	0 833	1 093	0 991	2 119	1 811	0 929	1 02
Lactulose	1 443	1 236	1 364	1 324	1 409	1 338	1 425	1 263	1 906	1 825	1 736	1 437	1 566	1 667	1 249	1 28	1 647	1 543
Maltitol	1 948	2 202	1 983	2 024	1 639	1 746	2 514	2 115	1 739	1 757	1 778	1 888	1 908	2 025	1 981	1 975	2 074	2 171
Maltose	1 948	2 026	1 807	1 783	1 568	1 537	2 009	1 661	1 725	1 681	1 682	1 829	1 979	1 868	1 814	1 776	1 93	1 788
Maltotriose	1 248	1 067	2 269	2 361	1 509	1 467	1 685	1 52	1 92	2 303	1 547	1 713	1 825	1 556	2 611	2 591	1 564	1 545
D-Mannitol	2 162	2 248	1 735	2 006	1 859	1 79	2 57	2 259	2 144	1 911	1 962	2 038	2 39	2 34	1 82	1 882	1 91	2 248
D-Mannose	0 945	1 088	0 732	0 753	0 733	0 784	0 902	0 867	0 81	0 806	0 812	0 834	0 97	1 015	0 813	0 717	0 828	0 861
D-Melezitose	0 781	0 796	0 866	0 826	1 136	0 898	1 074	0 933	1 105	1 248	1 086	0 932	0 996	1 02	1 27	1 316	1	0 895
D-Melibiose	0 627	0 611	0 669	0 584	0 72	0 716	0 815	0 768	0 68	0 716	0 576	0 68	0 675	0 813	0 723	0 597	0 521	0 64
α-Methyl-D-Galactoside	0 841	0 852	1 364	1 264	1 279	1 171	1 383	1 037	1 622	1 402	1 346	1 253	1 527	1 375	1 31	1 216	1 393	1 351
β-Methyl-D-Galactoside	0 788	1 023	0 837	0 806	0 929	0 884	0 964	0 916	0 948	0 91	0 96	0 915	0 961	1 011	0 932	0 792	0 755	0 86
α-Methyl-D-Glucoside	1 393	1 255	1 728	1 869	1 777	1 715	1 645	1 626	2 05	2 095	1 826	1 869	1 948	2 01	2 287	2 503	1 941	1 656
β-Methyl-D-Glucoside	1 641	1 634	1 643	1 676	1 862	1 63	1 667	1 541	2 037	2 031	1 876	1 931	1 99	2 063	1 952	1 815	1 968	1 99
Palatinose	0 733	0 727	0 755	0 746	0 825	0 88	0 79	0 773	0 836	0 792	0 791	0 766	0 871	0 879	0 84	0 849	0 719	0 766
D-Psicose	1 334	1 089	1 724	1 578	1 841	1 969	1 796	1 607	1 813	1 842	1 886	1 902	2 416	2 086	1 939	1 756	2 128	2 103
D-Raffinose	1 775	1 873	1 824	1 763	1 638	1 737	2 017	1 819	1 518	1 679	1 75	1 93	1 987	1 883	1 873	2 052	1 908	1 89
L-Rhamnose	1 552	1 433	1 338	1 373	1 008	0 684	1 614	1 631	1 178	1 465	0 73	1 37	1 535	1 54	1 311	1 225	1 699	1 638
D-Ribose	1 21	1 101	1 204	1 205	0 872	0 801	1 394	1 233	1 116	1 17	1 168	1 158	1 288	1 37	1 135	1 028	1 28	1 229
Salicin	0 609	0 624	0 686	0 655	0 682	0 675	0 877	0 779	0 699	0 741	0 669	0 707	0 77	0 765	0 764	0 676	0 628	0 643
Sedoheptulosan	2 089	2 263	1 913	1 989	1 965	1 951	2 415	2 314	1 951	1 861	1 921	1 86	2 396	2 306	2 087	2 123	2 221	2 261
D-Sorbitol	2 1	2 292	1 992	1 951	1 98	2 02	2 337	2 147	1 811	1 741	1 691	1 891	2 286	2 235	2 169	2 227	2 027	2 168
L-Sorbose	2 168	1 781	2 042	2 05	1 691	2 019	1 638	1 992	2 02	2 395	1 722	1 871	2 296	2 343	1 975	1 721	2 15	1 872
Stachyose	2 048	2 05	2 065	2 063	1 766	1 748	2 587	2 514	1 919	1 897	1 916	1 949	2 409	2 462	1 914	1 86	2 315	2 566
Sucrose	0 62	0 765	0 716	0 603	0 709	0 766	0 809	0 753	0 736	0 768	0 641	0 742	0 739	0 794	0 843	0 687	0 578	0 687
D-Tagatose	2 296	2 066	2 243	2 263	2 21	2 274	2 46	2 418	2 089	2 024	2 303	2 566	2 68	2 678	2 088	2 147	2 567	2 505
D-Trehalose	2 111	2 271	2 191	2 152	2 351	2 391	2 136	2 064	2 618	2 486	2 308	2 446	2 397	2 564	2 18	2 434	2 483	2 484
Turanose	0 808	0 673	0 881	0 769	1 104	1 033	1 354	1 496	1 017	0 946	1 334	1 038	1 769	1 808	1 885	2 633	1 325	1 286
Xylitol	1 693	1 733	1 773	1 838	1 521	1 448	1 881	1 55	1 744	1 605	1 154	1 607	1 845	1 987	1 527	1 52	1 947	1 877
D-Xylose	1 571	1 457	1 739	1 879	1 144	0 877	1 602	1 481	1 281	1 474	1 315	1 474	1 614	1 545	1 956	2 019	1 821	1 899
y-Amino-butyric Acid	0 573	0 548	0 831	0 791	0 858	0 851	0 958	0 867	0 858	0 857	0 84	0 884	0 827	0 971	0 915	0 806	0 834	0 932
Bromosuccinic Acid	0 954	0 881	1 238	1 128	1 314	1 284	1 334	1 128	1 221	1 284	1 076	1 249	1 415	1 371	1 218	1 292	1 24	1 259
Fumaric Acid	0 61	0 74	0 941	0 692	1 161	0 897	0 908	0 933	1 062	0 946	0 9	0 819	0 725	0 858	1 009	0 716	0 631	1 037
y-Hydroxy-butyric Acid	0 747	0 721	0 714	0 628	0 783	0 833	0 869	0 83	0 803	0 796	0 581	0 777	0 75	0 808	0 812	0 682	0 649	0 751
p-Hyroxyphenyl-acetic Acid	0 847	0 874	1 642	1 53	1 801	1 707	1 825	1 704	1 579	1 635	1 437	1 575	1 665	1 725	2 041	1 989	1 658	1 505
α-Keto-glutaric Acid	0 942	0 878	1 124	1 109	1 14	1 148	1 31	1 121	1 314	1 281	0 576	1 091	1 23	1 248	1 172	1 093	1 12	1 195
D-Lactic Acid Methyl Ester	0 706	0 782	0 725	0 701	0 758	0 769	0 861	0 871	0 845	0 777	0 668	0 774	0 842	0 827	0 777	0 604	0 648	0 77
L-Lactic Acid	0 769	0 659	0 807	0 746	0 787	0 844	0 899	0 853	0 948	0 824	0 835	0 849	0 923	0 997	0 947	0 712	0 77	0 816
D-Malic Acid	0 859	0 734	0 944	0 848	0 889	0 856	0 903	0 747	1 07	0 978	1 128	0 938	1 036	0 986	0 941	0 813	0 858	1 025
L-Malic Acid	1 239	0 754	1 027	1 006	0 935	0 926	0 966	0 852	1 126	1 022	1 07	0 879	0 983	1 164	1 092	1 053	0 993	1 135
Quinic Acid	1 781	1 725	1 655	1 722	1 518	1 513	1 511	1 422	1 679	1 442	1 585	1 775	1 553	1 53	1 575	1 47	1 551	1 346
D-Saccharic Acid	0 726	0 549	1 04	1 131	0 997	0 892	1 152	1 063	1 135	1 125	0 965	1 124	1 097	1 099	1 064	1 037	1 102	1 199
Sebacic Acid	0 644	0 716	1 349	1 268	1 966	1 711	2 084	1 803	1 684	1 877	1 614	1 797	1 943	1 959	1 888	1 959	1 868	1 661
Succinamic Acid	1 07	0 844	1 2	1 278	1 18	1 309	1 256	1 181	1 264	1 371	1 149	1 289	1 272	1 256	1 397	1 308	1 295	1 165
Saccinic Acid	0 907	0 808	1 06	1 068	1 021	1 098	1 102	1 037	1 139	1 092	0 954	1 134	1 039	1 15	1 054	1 059	1 207	1 008
Succinic Acid Mono-Methy Ester	0 814	0 882	0 836	0 871	0 915	0 974	1 105	0 903	1 034	1 033	0 791	0 999	0 897	1 012	0 758	0 865	0 838	0 856
N-Acetyl-L-Glutamic Acid	0 55	0 643	0 613	0 544	0 727	0 672	0 822	0 674	0 712	0 656	0 619	0 638	0 64	0 784	0 725	0 577	0 6	0 581
Alaninamide	0 703	0 714	0 763	0 599	0 851	0 795	0 83	0 82	0 79	0 828	0 684	0 832	0 764	0 853	0 775	0 718	0 642	0 715
L-Alanine	1 352	1 413	1 571	1 513	1 494	1 573	1 534	1 291	1 809	1 761	1 644	1 547	1 575	1 587	1 537	1 501	1 509	1 496
L-Alanyl-Glycine	0 936	0 662	1 162	1 054	1 015	0 914	0 934	0 948	1 127	1 209	1 017	0 887	1 194	1 232	1 318	1 252	1 072	1 003
L-Asparagine	1 213	1 054	1 298	1 107	1 192	1 295	1 225	1 233	1 391	1 258	1 207	1 15	1 293	1 315	1 132	1 114	1 057	1 047
L-Aspartic Acid	1 108	1 334	0 988	0 996	0 971	0 972	1 085	0 949	1 033	1 014	0 942	0 917	1 035	1 011	0 933	0 969	0 856	0 985
L-Glutamic acid	1 203	1 098	1 162	1 157	1 033	1 057	1 023	0 972	1 114	1 06	1 057	1 169	1 102	1 104	0 854	0 777	1 004	1 043
Glycyl-L-Glutamic Acid	0 732	0 595	0 898	0 878	0 942	0 988	0 888	0 876	0 525	0 811	1 353	0 852	1 073	0 976	0 715	0 702	1 081	0 925
L-Omithine	1 289	1 314	1 518	1 478	1 34	1 267	1 444	1 296	1 499	1 551	1 515	1 469	1 411	1 487	1 574	1 723	1 485	1 461
L-Phenylalanine	0 683	0 579	0 871	1 227	1 248	1 135	1 086	0 902	0 922	0 923	0 934	0 921	1 029	1 099	1 452	1 403	0 824	0 983
I-Proline	1 223	1 234	1 244	1 261	1 398	1 29	1 488	1 236	1 424	1 433	1 647	1 511	1 347	1 353	1 748	1 768	1 22	1 3
L-Pyroglutamic Acid	0 827	0 742	1 301	1 159	1 465	1 438	1 316	1 258	1 465	1 3	0 101	1 331	1 326	1 43	1 608	1 563	1 292	1 27
L-Serine	1 024	1 04	1 12	1 096	1 02	1 029	1 166	1 103	1 085	1 129	1 015	1 013	1 083	1 145	1 09	0 982	0 957	1 224
L-Threonine	1 158	0 964	1 158	1 187	1 132	1 109	1 262	1 204	1 33	1 268	1 416	1 247	1 344	1 327	1 216	1 152	1 288	0 668
2-Amino Ethanol	1 174	0 85	1 266	1 708	1 702	1 708	1 823	1 649	1 874	1 857	1 862	1 815	1 753	1 776	1 579	1 595	1 873	1 185
Putrescine	0 59	0 524	0 737	0 688	0 808	0 741	0 893	0 792	0 925	0 854	0 721	0 76	0 777	0 895	0 808	1 738	0 682	1 042
Adenosine	0 609	0 371	0 586	0 499	0 718	0 688	0 796	0 627	0 668	0 715	0 647	0 643	0 672	0 775	0 753	0 852	0 534	0 493
Uridine	0 525	0 525	0 595	0 522	0 646	0 647	0 719	0 601	0 592	0 554	0 569	0 648	0 639	0 698	0 627	0 593	0 499	0 507
Adenosine-5'-Monophosphate	0 457	0 593	0 436	0 438	0 6	0 574	0 609	0 524	0 533	0 562	0 514	0 586	0 545	0 542	0 573	0 485	0 508	0 477

Figure C Isolates 1-9.

48 hours: isolates 10 - 18	*A.niger*	*A.niger*			*A.niger*	*A.niger*	*A.niger*	*A.niger*									*A.niger*	*A.niger*
	10A	10B	11A	11B	12A	12B	13A	13B	14A	14B	15A	15B	16A	16B	17A	17B	18A	18B
COMPOUND	0,092	0,119	0,51	0,496	0,206	0,12	0,591	0,449	0,532	0,549	0,514	0,492	0,697	0,585	0,59	0,557	0,132	0,251
water	0,597	0,9	0,784	0,695	0,74	0,751	0,682	0,909	0,798	0,791	0,735	0,786	0,778	0,846	0,786	0,816	0,277	0,796
tween 80	0,33	0,342	0,582	0,498	0,39	0,221	0,318	0,335	0,528	0,552	0,545	0,562	0,679	0,568	0,556	0,818	0,397	0,182
N-acetyl-D-galactosamine	1,302	0,849	2,29	2,202	1,307	0,929	0,621	0,564	2,301	2,313	2,516	2,49	2,14	2,17	2,227	2,341	0,39	0,324
N-acetyl-D-glucosamine	0,535	0,323	0,582	0,539	0,457	0,32	0,467	0,379	0,545	0,567	0,537	0,58	0,788	0,632	0,608	0,71	0,307	0,225
N-acetyl-D-mannosamine	0,442	0,428	0,824	0,839	0,487	0,392	0,416	0,428	0,933	0,88	0,924	1,114	1,125	1,037	0,91	0,961	0,31	0,238
adonitol	1,893	1,853	2,047	2,061	1,316	1,154	1,592	1,41	2,141	2,088	2,167	2,235	2,2	2,251	2,355	2,315	0,324	1,523
amygdalin	0,535	0,523	1,115	1,001	0,474	0,405	0,479	0,821	0,888	0,918	1,021	1,09	1,224	1,048	0,944	1,087	0,386	0,502
D-arabinose	1,992	1,705	1,967	1,767	1,132	1,209	1,295	0,903	1,214	1,399	1,339	1,438	1,623	1,419	1,679	1,742	0,272	1,405
L-arabinose	0,586	0,426	1,447	1,465	0,609	0,555	0,62	0,43	1,35	1,298	1,355	1,305	1,863	1,68	1,339	1,372	0,271	0,365
D-arabitol	1,653	1,494	1,722	1,462	1,151	0,861	1,46	1,242	1,82	1,661	1,958	2,054	2,012	1,862	1,982	2,039	0,255	0,939
arbutin	2,045	1,968	2,051	1,961	1,555	1,593	1,945	1,837	1,696	2,069	2,053	1,948	2,107	2,021	1,835	2,395	0,194	1,638
D-cellobiose	0,377	0,412	0,855	0,829	0,461	0,353	0,523	0,422	0,772	0,799	0,8	0,864	0,996	0,906	0,864	0,931	0,259	0,356
α- cyclodextrin	1,032	0,526	1,747	1,536	0,595	0,458	0,531	0,378	1,505	1,388	1,496	1,63	1,74	1,55	1,447	1,757	0,301	0,213
β- cyclodextrin	1,821	1,639	1,737	1,664	1,493	1,384	1,648	1,653	1,491	1,472	1,689	1,657	1,853	1,563	1,605	1,794	0,34	2,086
dextrin	1,076	0,714	2,325	2,209	0,734	0,639	0,594	0,615	1,858	1,878	2,238	2,304	2,253	2,288	2,092	2,258	0,308	0,37
I- erythritol	2,198	2,134	2,238	2,125	1,98	1,881	1,891	1,98	1,743	1,748	1,991	2,174	1,951	2,06	2,133	2,134	0,304	2,206
D-fructose	0,362	0,405	0,633	0,619	0,37	0,301	0,358	0,315	0,444	0,618	0,497	0,583	0,726	0,544	0,569	0,594	0,333	0,294
L-fructose	0,388	0,252	1,563	1,196	0,467	0,349	0,445	0,275	1,136	1,17	1,508	1,593	1,71	1,459	1,233	1,566	0,3	0,316
L-Fucose	1,162	0,963	1,269	0,978	0,868	1,008	1,174	0,792	0,894	1,011	1,003	1,143	1,416	1,002	1,05	0,586	0,312	0,971
D-Galactose	2,144	2,106	2,235	2,218	1,535	1,552	1,723	1,421	2,399	2,221	2,217	2,289	2,139	2,134	2,486	2,572	0,299	1,862
D-Galacturonic Acid	1,218	0,741	1,667	1,504	0,887	0,671	0,62	0,667	1,185	1,419	1,304	1,285	1,544	1,465	1,341	1,481	0,313	0,498
Gentiobiose	0,931	0,297	1,114	1,087	0,694	0,352	0,302	0,328	0,893	0,846	1,105	1,171	1,417	1,241	1,111	1,113	0,272	0,24
D-Gluconic Acid	2,037	1,78	1,946	1,796	1,666	1,639	1,846	1,61	1,65	1,688	1,991	1,952	1,924	1,916	1,86	1,871	0,181	1,829
D-Glucosamine	0,234	0,256	0,732	0,67	0,453	0,252	0,385	0,376	0,595	0,59	0,54	0,707	0,813	0,698	0,759	0,684	0,325	0,28
α-D-Glucose	0,383	0,234	0,756	0,665	0,301	0,243	0,304	0,31	0,346	0,406	0,548	0,674	0,752	0,515	0,353	0,425	0,384	0,264
Glucose-1-Phosphate	0,442	0,41	1,534	1,484	0,545	0,463	0,779	0,583	1,301	1,493	1,56	1,492	1,407	1,533	1,417	1,551	0,36	0,282
Glucuronamide	1,502	1,047	1,89	1,823	0,9	0,635	0,68	0,717	1,582	1,719	1,805	1,914	2,016	2,069	1,607	1,97	0,443	0,68
D-Glucronic Acid	1,198	0,958	1,127	1,205	0,911	0,74	0,956	0,836	1,225	1,235	1,257	1,267	1,433	1,347	1,304	1,234	0,312	0,776
Glycerol	1,001	0,681	2,138	2,149	0,832	0,568	0,744	0,823	1,984	1,989	1,938	2,063	2,185	2,147	2,284	2,358	0,331	0,699
Glycogen	0,574	0,616	1,442	1,377	0,565	0,823	0,579	0,574	1,137	1,307	1,276	1,352	1,541	1,324	1,268	1,413	0,32	0,538
m-Inositol	0,538	0 422	0,392	0,861	0,529	0,481	0,437	0,458	0,925	0,925	1,192	1,197	1,037	0,905	0,871	1,086	0,342	0,43
2-Keto-D-Gluconic Acid	0,587	0,485	0,912	0,861	0,595	0,352	0,402	0,496	0,953	0,834	1,09	0,873	1,048	0,877	1,152	1,121	0,308	0,341
α-D-Lactobiose	0,439	0,381	1,758	1,562	0,49	0,419	0,391	0,415	1,669	1,723	1,717	1,69	2,073	1,942	1,713	1,718	0,483	0,28
Lactulose	1,861	1,73	2,002	2,005	1,727	1,759	1,806	1,812	1,64	1,795	1,906	2,032	1,975	1,906	2,09	2,037	0,493	1,323
Maltitol	2,116	2,227	1,796	1,632	1,606	1,544	1,594	1,112	0,717	1,538	1,663	1,644	1,789	1,652	1,782	1,882	0,471	1,675
Maltose	0,311	0,426	2,066	2,162	0,85	0,723	0,55	0,447	2,167	1,912	1,89	2,043	2,286	2,198	1,913	1,886	0,405	0,314
Maltotriose	2,096	1,972	2,173	1,982	1,803	1,596	1,447	1,37	1,697	1,739	1,844	2,201	1,996	1,887	1,956	2,012	0,308	1,888
D-Mannitol	0,792	0,944	0,836	0,744	1,748	1,594	1,278	1,965	0,77	0,839	0,8	0,864	1,061	0,972	0,902	0,884	0,355	0,574
D-Mannose	1,83	0,829	0,966	0,894	1,214	0,831	0,811	0,594	0,847	0,882	0,966	1,041	1,465	1,255	0,964	1,08	0,35	0,439
D-Melezitose	0,634	0,407	0,707	0,626	0,449	0,464	0,517	0,443	0,52	0,692	0,605	0,698	0,775	0,686	0,618	0,687	0,33	0,402
D-Melibiose	0,486	0,41	1,171	1,128	0,493	0,555	0,474	0,476	1,347	1,295	1,389	1,346	1,262	1,139	1,502	1,483	0,352	0,403
α-Methyl-D-Galactoside	0,675	0,44	1,002	0,888	0,505	0,318	0,458	0,389	0,803	0,878	0,943	0,958	1,038	0,979	0,838	0,86	0,345	0,371
β-Methyl-D-Galactoside	2,113	1,88	1,815	1,771	1,632	1,359	0,875	1,045	1,767	1,862	2,091	1,999	2,074	2,032	1,976	2,137	0,337	1,652
α-Methyl-D-Glucoside	1,02	0,734	1,846	1,837	0,705	0,481	0,776	0,706	2,021	2,114	1,974	1,898	2,208	2,021	1,742	1,83	0,321	0,402
β-Methyl-D-Glucoside	0,736	0,549	0,744	0,697	0,852	0,554	0,587	0,64	0,798	0,862	0,743	0,832	0,934	0,832	0,732	0,967	0,324	0,646
Palatinose	1,593	1,51	1,797	1,773	1,608	1,294	1,434	1,259	1,632	2,014	2,016	2,028	2,231	1,94	1,826	1,894	0,288	1,231
D-Psicose	1,638	1,168	1,884	1,833	1,864	0,884	0,997	0,733	1,466	1,728	1,686	1,663	1,958	1,794	1,894	2,165	0,084	0,806
D-Raffinose	0,789	0,583	1,955	1,72	0,598	0,495	0,629	0,494	1,249	1,17	1,502	1,412	1,069	1,586	1,534	1,574	0,332	0,522
L-Rhamnose	1,37	1,185	1,352	1,188	1,068	0,85	1,242	0,982	1,117	1,238	1,1	1,185	1,208	1,105	1,319	1,876	0,443	1,119
D-Ribose	0,428	0,473	0,728	0,557	0,426	0,297	0,429	0,561	0,501	0,679	0,561	0,673	0,797	0,723	0,661	0,76	0,345	0,292
Salicin	2,142	1,994	2,206	2,028	1,734	1,741	1,728	1,899	1,735	1,8	1,985	2,097	2,168	2,035	2,165	2,294	0,422	1,735
Sedoheptulosan	0,411	0,249	2,219	1,993	0,409	0,327	0,349	0,427	1,976	1,901	1,909	2,17	2,025	2,035	2,017	2,12	0,324	0,356
D-Sorbitol	1,723	1,026	1,91	1,946	0,674	0,62	0,536	0,643	2,197	2,084	2,097	2,089	2,045	1,973	2,237	2,315	0,318	0,556
L-Sorbose	2,187	1,936	2,136	2,129	2,02	1,983	2,169	2,07	1,744	1,748	2,061	2,25	2,161	1,942	2,1	2,041	0,356	2,081
Stachyose	0,495	0,491	0,467	0,653	0,449	0,354	0,364	0,449	0,541	0,713	0,669	0,718	0,828	0,694	0,627	0,841	0,363	0,309
Sucrose	1,803	1,714	2,423	2,345	1,333	1,164	0,987	0,906	2,405	2,607	2,223	2,28	2,31	2,132	2,492	2,535	0,41	1,013
D-Tagatose	0,671	0,498	2,237	2,106	0,593	0,785	0,695	0,503	2,272	2,522	2,299	2,254	2,48	2,316	2,045	2,319	0,358	0,406
D-Trehalose	1,844	1,544	1,38	1,077	0,595	0,641	0,445	0,588	1,302	0,862	1,361	1,264	1,646	1,331	0,915	0,963	0,272	0,753
Turanose	1,877	2,083	1,927	1,532	1,192	1,34	0,861	1,256	1,676	1,606	1,556	2,094	1,221	1,635	1,628	1,843	0,14	1,779
Xylitol	0,564	0,569	1,659	1,615	0,621	0,607	0,609	0,627	1,418	1,306	1,48	1,768	1,743	1,533	1,652	1,681	0,303	0,452
D-Xylose	0,73	0,443	0,97	0,836	0,606	0,307	0,516	0,303	0,82	0,893	0,826	0,922	1,071	0,894	0,886	1,08	0,327	0,274
y-Amino-butyric Acid	0,656	0,572	1,247	1,288	0,615	0,524	0,417	0,491	1,068	1,164	1,313	1,259	1,282	1,279	1,26	1,341	0,329	0,468
Bromosuccinic Acid	0,606	0,456	0,993	0,635	0,601	0,346	0,512	0,458	0,571	0,818	0,748	0,751	1,205	0,805	1,06	0,796	0,341	0,285
Fumaric Acid	0,593	0,395	0,801	0,736	0,496	0,294	0,462	0,381	0,578	0,804	0,655	0,775	0,796	0,72	0,709	0,839	0,473	0,328
y-Hydroxy-butyric Acid	0,612	0,534	1,552	1,59	0,479	0,426	0,428	0,43	1,457	1,5	1,645	1,709	1,799	1,809	1,605	1,664	0,468	0,457
p-Hyroxyphenyl-acetic Acid	0,612	0,512	1,273	1,209	0,591	0,314	0,482	0,471	1,151	1,21	1,217	1,257	0,908	1,234	1,229	1,23	0,441	0,278
α-Keto-glutaric Acid	0,546	0,518	0,699	0,707	0,452	0,466	0,488	0,557	0,613	0,801	0,738	0,789	0,911	0,772	0,741	0,917	0,456	0,527
D-Lactic Acid Methyl Ester	0,52	0,448	1,07	0,817	0,493	0,389	0,482	0,44	0,765	0,852	0,845	0,915	1,071	0,857	0,829	0,88	0,333	0,516
L-Lactic Acid	0,695	0,477	1,177	1,07	0,51	0,388	0,5	0,459	1,057	1,098	0,977	1,064	1,239	1,134	1,04	1,33	0,315	0,402
D-Malic Acid	0,617	0,358	1,2	1,096	0,609	0,468	0,542	0,438	0,981	1,121	1,007	1,067	1,31	1,173	1,058	1,07	0,251	0,41
L-Malic Acid	1,2	1,042	1,43	1,461	1,364	1,101	1,44	1,072	1,355	1,403	1,569	1,585	1,674	1,575	1,528	1,555	0,203	0,883
Quinic Acid	0,92	0,408	1,25	1,267	0,79	0,453	0,762	0,369	1,24	1,119	1,074	1,165	1,252	1,266	1,247	1,335	0,278	0,187
D-Saccharic Acid	0,61	0,603	1,602	1,569	0,958	0,414	0,496	0,667	1,525	1,484	1,753	1,762	1,732	1,676	1,657	1,807	0,313	0,585
Sebacic Acid	0,644	0,582	1,219	1,219	0,564	0,499	0,469	0,422	1,073	1,19	1,268	1,245	1,309	1,256	1,249	1,51	0,299	0,512
Succinamic Acid	0,656	0,442	1,259	0,975	0,735	0,478	0,547	0,477	0,98	1,052	1,001	1,05	1,249	1,075	1,045	1,425	0,314	0,436
Saccinic Acid	0,953	0,796	0,996	0,849	0,856	0,912	0,716	0,652	0,819	0,903	0,983	0,98	1,045	0,886	0,985	0,962	0,326	0,809
Succinic Acid Mono-Methy Est	0,633	0,49	0,734	0,577	0,614	0,46	0,479	0,417	0,521	0,58	0,588	0,632	0,959	0,632	0,632	0,636	0,395	0,433
N-Acetyl-L-Glutamic Acid	0,609	0,416	0,534	0,756	0,451	0,284	0,374	0,449	0,689	0,72	0,833	0,864	0,948	0,811	0,737	0,768	0,411	0,296
Alaninamide	0,875	0,767	1,379	1,295	0,596	0,66	0,87	1,201	1,431	1,485	1,493	1,643	1,595	1,523	1,487	1,667	0,344	0,544
L-Alanine	0,505	0,301	1,143	1,078	0,437	0,289	0,525	0,487	1,068	1,171	1,058	1,099	1,459	1,113	1,142	1,257	0,321	0,236
L-Alanyl-Glycine	0,569	0,29	1,105	0,982	0,42	0,409	0,497	0,481	0,167	1,114	1,147	1,225	1,383	1,209	1,142	1,319	0,289	0,297
L-Asparagine	0,568	0,39	1,064	0,904	0,629	0,404	0,448	0,387	0,968	0,986	0,957	1,01	1,046	0,993	0,96	1,226	0,275	0,317
L-Aspartic Acid	1,144	0,843	1,037	0,972	0,961	1,003	1,057	0,93	1,073	1,15	1,014	1,074	1,18	1,107	1,15	1,047	0,176	0,685
L-Glutamic acid	0,21	0,442	0,956	0,994	0,638	0,197	0,629	0,427	1,036	0,815	0,983	1,039	1,211	0,886	1,016	0,896	0,202	0,26
Glycyl-L-Glutamic Acid	0,383	0,36	1,488	1,393	0,458	0,451	0,481	0,448	1,309	1,236	1,259	1,407	1,455	1,419	1,392	1,664	0,266	0,201
L-Ornithine	0,32	0,247	1,291	0,82	0,437	0,285	0,337	0,266	0,615	0,984	0,84	0,944	0,98	0,943	1,091	1,347	0,271	0,225
L-Phenylalanine	0,436	0,681	1,419	1,348	0,775	0,828	0,8	0,891	1,281	1,266	1,304	1,391	1,641	1,438	1,276	1,446	0,278	1,264
I-Proline	0,473	0,546	1,356	1,238	0,564	0,468	0,417	0,563	1,318	1,281	1,368	1,493	1,41	1,362	1,417	1,535	0,281	0,496
L-Pyroglutamic Acid	0,501	0,354	1,363	1,142	0,568	0,27	0,536	0,469	1,091	1,05	0,975	1,067	1,172	1,078	1,078	1,13	0,291	0,294
L-Serine	0,296	0,25	1,064	1,133	0,324	0,314	0,377	0,23	1,133	1,136	1,21	1,393	1,429	1,309	1,154	1,554	0,258	0,218
L-Threonine	0,469	0,397	1,802	1,597	0,5	0,562	0,341	0,359	1,787	1,542	1,776	1,808	1,866	1,841	1,69	1,784	0,251	0,387
2-Amino Ethanol	0,498	0,315	0,956	0,735	0,475	0,275	0,38	0,386	0,711	0,608	0,657	0,765	0,937	0,789	0,715	0,726	0,252	0,229
Putrescine	0,445	0,495	0,686	0,603	1,037	0,703	0,483	0,636	0,591	0,563	0,501	0,596	0,803	0,679	0,767	0,717	0,238	0,66
Adenosine	0,38	0,253	0,595	0,482	0,517	0,246	0,365	0,265	0,496	0,507	0,471	0,609	0,751	0,595	0,581	0,62	0,206	0,197
Uridine	0,436	0,387	0,541	0,506	0,44	0,308	0,369	0,358	0,996	0,507	0,483	0,537	0,619	0,649	0,565	0,542	0,16	0,18
Adenosine-5'-Monophosphate	0,436	0,387	0,541	0,506	0,44	0,308	0,369	0,358	0,996	0,507	0,483	0,537	0,619	0,649	0,565	0,542	0,16	0,18

Figure C Isolates 10-18

48 hours: isolates 19 - 27	19A	19B	20A	20B	21A	21B	22A	22B	23A	23A	24A	24B	25A	25B	26A	26B	27A	27B
	0,53	0,579	0,596	0,566	0,138	0,342	0,437	0,44	0,506	0,554	0,548	0,495	0,31	0,221	0,594	0,644	0,632	0,423
COMPOUND	0,619	0,558	0,713	0,713	0,6	0,863	0,679	0,705	0,628	0,724	0,647	0,621	0,664	0,813	0,69	0,777	0,743	0,684
water	0,558	0,749	0,628	0,606	0,194	0,184	0,462	0,482	0,596	0,669	0,551	0,523	0,261	0,206	0,569	0,684	0,604	0,548
tween 80	1,441	2,025	2,332	2,294	0,374	0,361	2,09	1,566	2,048	2,064	2,068	1,994	0,353	0,374	2,205	2,326	2,071	2,394
N-acetyl-D-galactosamine	0,527	0,733	0,727	0,615	0,229	0,254	0,589	0,633	0,62	0,674	0,653	0,536	0,244	0,232	0,617	0,631	0,647	0,526
N-acetyl-D-glucosamine	1,521	1,761	1,184	1,201	0,364	0,299	1,863	1,765	1,043	0,981	0,804	0,865	0,268	0,322	0,772	0,858	1,2	0,937
N-acetyl-D-mannosamine	1,715	0,095	1,889	2,256	1,872	1,866	2,249	1,864	2,195	2,071	2,136	1,981	1,961	2,042	1,972	1,887	2,046	2,494
adonitol	1,016	1,169	1,077	1,127	0,452	0,448	0,891	0,775	1,027	1,043	0,887	0,898	0,504	0,475	1,047	1,036	1,061	1,02
amygdalin	1,297	1,581	1,367	1,23	1,787	1,629	1,779	1,616	1,273	1,28	1,266	1,207	1,99	1,923	1,268	1,289	1,439	1,337
D-arabinose	1,541	1,754	1,302	1,398	0,432	0,294	1,488	1,393	1,469	1,276	1,194	1,259	0,328	0,411	1,252	1,357	1,798	1,399
L-arabinose	1,886	2,083	1,932	1,951	1,219	1,478	1,789	0,09	1,653	1,778	1,864	1,737	1,72	1,678	1,704	1,69	1,945	1,689
D-arabitol	1,887	2,095	1,78	1,731	1,892	1,888	1,93	1,863	1,318	1,501	1,91	1,548	2,199	2,273	1,8	1,757	1,799	1,44
arbutin	0,752	0,852	0,912	0,876	0,316	0,539	1,099	1,087	0,844	0,82	0,832	0,847	0,375	0,309	0,841	0,851	0,932	0,803
D-cellobiose	1,257	1,526	1,577	1,761	0,296	0,285	1,298	1,22	1,56	1,535	1,504	1,454	0,401	0,265	1,67	1,562	1,552	1,418
α- cyclodextrin	1,429	1,789	1,662	1,817	1,788	1,843	1,569	1,301	1,434	1,451	1,508	1,657	2,149	2,186	1,744	1,758	1,513	1,715
β- cyclodextrin	1,889	2,084	2,029	2,069	0,478	0,554	2,106	1,806	1,952	1,979	1,963	2,027	0,574	0,554	2,078	2,196	2,071	1,882
dextrin	1,63	1,889	1,714	1,742	2,082	2,144	2,073	1,303	1,849	1,797	1,83	1,807	2,353	2,276	2,155	2,003	1,742	1,73
I- erythritol	0,446	0,735	0,595	0,654	0,313	0,244	0,61	0,533	0,575	0,6	0,48	0,473	0,25	0,32	0,547	0,586	0,631	0,474
D-fructose	1,426	0,338	1,536	1,608	0,317	0,268	1,722	1,395	1,26	1,246	1,32	1,191	0,297	0,315	1,537	1,438	1,629	1,159
L-fructose	0,876	1,108	1,032	1,076	0,884	0,893	1,036	0,932	1,072	1,068	1,046	0,91	1,305	1,095	1,033	1,18	1,042	0,998
L-Fucose	2,075	2,281	2,042	2,094	2,002	2,201	2,411	2,42	2,146	2,199	2,223	2,077	2,317	2,384	2,013	2,019	2,073	2,319
D-Galactose	1,046	1,357	1,295	1,273	0,445	0,527	1,675	1,375	1,147	1,218	1,239	1,132	0,541	0,721	1,119	1,259	1,44	1,073
D-Galacturonic Acid	1,191	1,519	1,1	1,096	0,251	0,282	0,95	0,097	1,102	1,035	0,977	1,388	0,33	0,346	1,094	1,301	1,172	1,021
Gentiobiose	1,616	1,899	1,691	1,648	2,1	1,882	1,548	1,258	1,499	1,493	1,675	1,37	2,149	2,045	1,619	1,543	1,685	1,375
D-Gluconic Acid	0,661	0,769	0,669	0,704	0,191	0,247	0,536	0,524	0,633	0,633	0,569	0,599	0,234	0,175	0,724	0,74	0,727	0,583
D-Glucosamine	0,369	0,596	0,511	0,645	0,28	0,206	0,605	0,573	0,604	0,53	0,414	0,411	0,245	0,186	0,546	0,474	0,77	0,378
α-D-Glucose	1,498	1,73	1,476	1,532	0,273	0,265	1,269	0,973	1,403	1,395	1,402	1,438	0,351	0,372	1,319	1,305	1,558	1,325
Glucose-1-Phosphate	1,774	2,05	1,68	1,675	1,095	0,873	1,802	1,743	1,582	1,665	1,549	1,538	0,818	0,993	1,904	1,93	1,721	1,89
Glucuronamide	1,155	1,377	1,169	1,173	0,868	0,87	1,206	1,071	1,193	1,167	1,026	1,039	0,752	0,805	1,212	1,136	1,234	1,205
D-Glucronic Acid	1,728	2,214	1,804	1,966	0,583	0,505	2,253	1,411	2,002	1,983	1,919	1,826	0,508	0,638	1,81	1,909	1,903	1,963
Glycerol	0,774	1,067	1,147	1,09	0,392	0,484	1,639	1,23	1,332	1,218	1,181	1,377	0,535	0,573	1,22	1,044	1,178	1,243
Glycogen	0,873	1,046	1,657	1,374	0,234	0,316	1,438	0,836	0,92	0,814	0,691	0,566	0,284	0,327	0,774	0,736	0,942	0,774
m-Inositol	0,688	0,986	1,093	1,329	0,271	0,283	1,959	1,543	0,958	0,897	0,631	0,617	0,34	0,432	0,755	0,742	0,854	0,95
2-Keto-D-Gluconic Acid	1,807	1,985	1,804	1,865	0,24	0,321	1,514	0,959	1,625	1,569	1,263	1,172	0,342	0,34	1,245	1,361	1,596	1,565
α-D-Lactobiose	1,736	2,003	1,67	1,734	0,759	1,446	1,598	0,094	1,707	1,743	1,782	1,65	1,496	1,676	1,684	1,83	1,789	1,688
Lactulose	1,573	1,717	1,494	1,536	1,794	2,005	1,899	1,509	1,179	1,427	1,42	1,107	2,266	2,385	1,651	1,532	1,587	0,995
Maltitol	1,737	1,864	1,808	1,756	0,438	0,486	1,644	1,179	2,027	1,987	1,852	1,819	0,401	0,123	1,803	1,687	2,097	2,085
Maltose	1,634	1,843	1,625	1,729	1,992	1,983	1,533	1,157	1,689	1,703	1,821	1,674	2,112	2,006	2,105	2,005	1,894	1,809
Maltotriose	0,777	1,072	0,868	0,834	0,377	0,487	0,707	0,58	0,809	0,797	0,739	0,71	0,531	0,602	0,777	0,736	0,878	0,788
D-Mannitol	1,401	1,625	0,931	0,912	0,43	0,577	1,331	0,675	1,012	0,891	0,771	0,825	0,953	0,522	0,93	0,783	1,034	0,851
D-Mannose	0,624	0,911	0,71	0,748	0,302	0,268	0,636	0,542	0,682	0,714	0,674	0,64	0,277	0,309	0,698	0,766	0,62	0,624
D-Melezitose	1,243	1,523	1,556	1,552	0,336	0,316	1,176	0,651	1,086	1,289	0,915	0,952	0,341	0,331	0,95	0,985	1,166	1,137
D-Melibiose	0,804	1,058	1,027	1,082	0,321	0,354	0,883	0,583	0,933	1,087	0,905	0,731	0,306	0,286	0,899	0,853	0,885	0,922
α-Methyl-D-Galactoside	1,803	2,123	2,071	2,015	1,812	1,859	1,746	1,558	1,908	1,978	1,71	1,654	2,024	2,008	1,7	1,733	1,945	1,963
β-Methyl-D-Galactoside	1,991	2,371	2,068	2,123	0,502	0,41	1,663	1,695	2,121	1,985	1,727	1,763	0,709	0,479	1,873	1,97	1,849	2,504
α-Methyl-D-Glucoside	0,723	0,924	0,761	0,795	0,598	0,584	0,901	0,164	0,8	0,822	0,716	0,662	0,739	0,705	0,716	0,795	0,864	0,681
β-Methyl-D-Glucoside	1,913	2,27	1,837	1,807	1,721	1,778	2,218	1,955	1,827	1,828	1,83	1,706	2,11	2,079	1,516	1,64	1,99	1,693
Palatinose	1,623	1,757	1,418	1,338	0,799	0,906	1,748	1,346	1,109	1,386	1,19	0,657	0,765	0,992	1,504	1,483	1,758	0,76
D-Psicose	1,184	1,308	1,299	1,303	0,644	0,649	1,564	1,29	1,406	1,478	1,314	1,257	0,692	0,489	1,633	1,454	1,435	1,148
D-Raffinose	1,088	1,052	1,225	1,341	1,008	0,988	1,181	1,004	1,191	1,281	1,273	1,216	0,994	0,969	1,132	1,12	1,226	1,091
L-Rhamnose	0,62	0,921	0,652	0,716	0,309	0,281	0,606	0,567	0,681	0,683	0,639	0,609	0,26	0,359	0,699	0,703	0,705	0,602
D-Ribose	1,732	1,893	1,921	1,95	1,473	1,703	2,39	1,568	1,939	1,869	1,893	1,749	1,815	2,11	2,296	2,24	1,757	2,028
Salicin	1,477	1,856	1,82	1,908	0,311	0,303	2,736	1,801	1,962	1,914	1,953	1,922	0,291	0,276	2,077	2,138	1,818	2,292
Sedoheptulosan	1,861	2,093	2,312	2,171	0,532	0,536	2,41	1,311	2,1	2,08	2,009	1,93	0,577	0,525	2,041	1,859	2,019	2,226
D-Sorbitol	1,641	1,894	1,723	1,867	2,105	1,991	1,863	1,421	1,857	1,716	1,799	1,643	2,311	2,19	2,169	2,011	1,816	2,1
L-Sorbose	0,695	0,881	0,84	0,797	0,452	0,293	0,678	0,58	0,747	0,747	0,782	0,58	0,397	0,333	0,791	0,732	0,75	0,611
Stachyose	1,777	2,005	2,229	2,049	0,91	0,996	2,125	2,062	2,04	2,202	1,905	1,851	1,619	1,691	2,215	2,01	2,081	2,251
Sucrose	2,414	2,655	2,392	2,403	0,387	0,387	1,874	0,163	2,22	2,247	2,267	2,008	0,342	0,452	2,204	2,375	2,294	2,639
D-Tagatose	2,226	2,881	2,111	2,364	0,988	1,014	0,809	1,107	1,395	1,468	1,56	1,546	0,849	0,865	1,964	1,922	2,062	0,858
D-Trehalose	1,311	1,619	1,47	1,436	1,905	1,831	1,923	1,486	1,229	1,399	1,115	1,155	2,06	2,111	1,381	1,365	1,577	1,107
Turanose	1,578	1,701	1,467	1,541	0,566	0,717	1,51	1,026	1,633	1,643	1,525	1,354	0,586	0,52	1,417	1,589	1,63	1,199
Xylitol	0,831	0,95	0,903	0,971	0,339	0,357	0,94	0,836	0,995	0,996	0,889	0,861	0,39	0,334	0,948	1,028	1,042	0,902
D-Xylose	1,043	1,383	1,203	1,31	0,532	0,496	1,27	0,745	1,187	1,229	1,102	1,047	0,57	0,498	1,35	1,317	1,344	1,158
γ-Amino-butyric Acid	0,948	1,047	0,951	1,088	0,345	0,546	0,893	0,603	0,68	1,027	0,8	0,758	0,298	0,277	0,736	0,764	1,074	0,68
Bromosuccinic Acid	0,604	0,94	0,775	0,731	0,296	0,296	0,619	0,597	0,691	0,753	0,642	0,669	0,359	0,28	0,741	0,755	0,699	0,627
Fumaric Acid	1,495	1,754	1,816	1,728	0,506	0,408	1,596	0,874	1,604	1,652	1,491	1,49	0,535	0,474	1,605	1,537	1,54	1,371
γ-Hydroxy-butyric Acid	1,081	1,433	1,204	1,175	0,272	0,282	1,152	0,911	1,145	1,191	1,055	0,978	0,306	0,302	1,064	1,036	1,256	1,081
p-Hyroxyphenyl-acetic Acid	0,607	0,9	0,705	0,655	0,269	0,5	0,623	0,507	0,721	0,656	0,759	0,563	0,341	0,472	0,675	0,622	0,637	0,547
α-Keto-glutaric Acid	0,765	1,026	0,912	0,94	0,315	0,303	0,744	0,614	0,885	0,862	0,73	0,681	0,534	0,498	0,857	0,83	0,925	0,696
D-Lactic Acid Methyl Ester	1,093	1,3	1,135	1,197	0,243	0,286	1,121	0,611	1,213	1,141	0,954	0,845	0,279	0,404	1,083	1,041	1,128	1,026
L-Lactic Acid	0,978	1,389	1,328	1,138	0,415	0,283	1,172	1,042	1,045	1,22	0,982	0,993	0,38	0,42	0,91	0,897	1,092	1,081
D-Malic Acid	1,216	1,424	1,336	1,395	0,647	0,456	1,566	1,153	1,405	1,45	1,226	1,134	0,617	0,659	1,069	1,094	1,303	1,034
L-Malic Acid	1,11	1,336	1,064	1,125	0,187	0,562	0,984	0,845	1,295	1,263	1,051	1,047	0,321	0,19	1,035	1,019	1,259	1,057
Quinic Acid	1,562	1,903	1,361	1,59	0,704	0,436	0,993	0,97	1,327	1,321	1,404	1,327	0,545	0,611	1,679	1,25	1,446	1,084
D-Saccharic Acid	1,273	1,501	1,27	1,17	0,522	0,549	1,33	0,867	1,107	1,234	1,107	1,087	0,554	0,524	1,27	1,222	1,258	1,05
Sebacic Acid	0,922	1,159	1,011	0,951	0,438	0,477	1,102	0,936	1,04	1,138	0,958	0,967	0,52	0,502	0,92	0,91	1,138	0,912
Succinamic Acid	0,898	1,094	1,148	1,06	0,593	0,971	1,265	1,095	1,003	1,016	0,914	0,934	0,543	1,023	0,805	0,932	1,134	0,87
Saccinic Acid	0,578	0,838	0,769	0,691	0,417	0,498	0,664	0,529	0,653	0,716	0,564	0,504	0,463	0,492	0,589	0,579	0,683	0,534
Succinic Acid Mono-Methy Est	0,771	1,013	1,002	0,899	0,231	0,252	0,801	0,619	0,801	0,908	0,745	0,669	0,296	0,311	0,818	0,782	0,803	0,74
N-Acetyl-L-Glutamic Acid	1,348	1,654	1,343	1,43	0,724	0,697	1,311	1,097	1,442	1,424	1,342	1,176	0,739	0,822	1,349	1,315	1,466	1,506
Alaninamide	1,021	1,31	0,952	1,253	0,233	0,246	1,207	1,027	1,104	1,022	0,95	0,853	0,342	0,306	0,931	0,889	1,279	0,943
L-Alanine	0,978	1,288	1,138	1,182	0,261	0,289	1,286	0,138	1,17	1,174	0,989	1,016	0,317	0,257	1,008	0,979	1,246	1,075
L-Alanyl-Glycine	0,868	1,145	0,893	0,981	0,263	0,253	1,086	0,954	1,015	0,973	0,937	0,86	0,33	0,296	0,843	0,783	1,149	0,853
L-Asparagine	1,031	1,175	1,165	0,977	0,306	0,522	1,121	1,188	1,085	1,207	1,462	0,999	0,671	0,642	1,009	0,899	1,198	1,013
L-Aspartic Acid	0,882	0,924	0,878	0,913	0,091	0,479	0,98	0,922	1,127	1,11	0,773	0,8	0,204	0,162	0,301	0,754	0,994	0,825
L-Glutamic acid	1,385	1,46	1,26	1,267	0,161	0,279	1,069	0,983	1,254	1,382	1,284	1,388	0,406	0,358	0,587	1,46	1,391	1,025
Glycyl-L-Glutamic Acid	0,951	1,047	1,121	1,098	0,246	0,615	0,77	0,59	1,106	1,083	0,997	1,095	0,327	0,356	1,027	1,537	0,999	0,991
L-Ornithine	1,549	1,817	1,407	1,392	0,531	0,692	1,307	0,95	1,334	1,373	1,295	1,392	1,319	1,431	1,564	1,412	1,412	1,247
L-Phenylalanine	1,154	1,369	1,508	1,463	0,501	0,539	1,183	0,854	1,306	1,355	1,242	1,374	0,51	0,481	1,268	1,264	1,358	1,312
l-Proline	1,152	1,168	1,024	1,083	0,228	0,348	1,126	0,944	1,136	1,118	1,086	0,957	0,393	0,392	1,079	1,057	1,071	1,198
L-Pyroglutamic Acid	1,389	1,42	1,291	1,23	0,241	0,238	1,428	1,292	1,163	1,206	1,114	1,047	0,247	0,255	1,289	1,262	1,19	1,033
L-Serine	1,587	1,87	1,758	1,693	0,452	0,409	1,335	1,292	1,395	1,44	1,387	1,404	0,427	0,377	1,159	1,237	1,57	1,245
L-Threonine	0,63	1,007	0,836	0,845	0,292	0,282	0,897	0,883	0,715	0,789	0,722	0,688	0,289	0,353	0,84	0,804	0,897	0,712
2-Amino Ethanol	0,503	0,721	0,737	0,639	0,68	0,547	1,078	1,155	0,689	0,735	0,529	0,536	0,284	0,62	0,442	0,61	1,289	0,522
Putrescine	0,512	0,655	0,579	0,617	0,194	0,198	0,477	0,405	0,533	0,523	0,571	0,674	0,235	0,218	0,579	0,566	0,642	0,486
Adenosine	0,906	0,512	0,563	0,62	0,272	0,179	0,442	0,417	0,537	0,528	0,508	0,421	0,208	0,219	0,566	0,571	0,562	0,524
Uridine	0,512	0,655	0,579	0,617	0,194	0,198	0,477	0,405	0,533	0,523	0,571	0,674	0,235	0,218	0,579	0,566	0,642	0,486
Adenosine-5'-Monophosphate	0,906	0,512	0,563	0,62	0,272	0,179	0,442	0,417	0,537	0,528	0,508	0,421	0,208	0,219	0,566	0,571	0,562	0,524

Figure C Isolates 19-27

48 hours: isolates 28 - 36																		
					A.niger	*A.niger*			*A.flavus*	*A.flavus*	*A.niger*	*A.niger*						
COMPOUND	**28A**	**28B**	**29A**	**29B**	**30A**	**30B**	**31A**	**31B**	**32A**	**32B**	**33A**	**33B**	**34A**	**34B**	**35A**	**35B**	**36A**	**36B**
water	0, 83	**D2:BU13**	0,547	0,576	0,266	0,316	0,56	0,607	0,468	0,463	0,091	0,253	0,614	0,642	0,606	0,524	0,551	0,501
tween 80	0,707	0,711	0,651	0,733	0,744	0,851	0,711	0,726	0,78	0,675	0,51	0,801	0,79	0,761	0,836	0,798	0,668	0,651
N-acetyl-D-galactosamine	0,599	0,535	0,602	0,589	0,251	0,275	0,563	0,671	0,539	0,454	0,485	0,212	0,662	0,687	0,655	0,608	0,465	0,522
N-acetyl-D-glucosamine	1,801	2,305	2,362	2,5	0,711	0,808	2,406	2,456	1,609	1,071	0,833	0,679	2,267	2,309	2,578	2,632	2,484	2,453
N-acetyl-D-mannosamine	0,576	0,528	0,637	0,617	0,302	0,319	0,565	0,671	0,675	0,47	0,439	0,308	0,711	0,712	0,699	0,644	0,474	0,549
adonitol	1,104	0,869	1,026	0,958	0,343	0,356	0,719	0,817	1,807	1,762	0,473	0,559	1,014	1,066	0,906	0,926	0,685	0,775
amygdalin	1,978	2,409	2,1	2,22	1,485	1,546	2,359	2,196	1,913	1,716	1,88	1,885	2,248	2,417	2,558	2,663	2,222	2,063
D-arabinose	1,067	0,937	1,053	1,031	0,474	0,493	0,868	0,941	1,016	0,838	0,531	0,485	1,099	1,096	0,981	0,933	0,735	0,791
L-arabinose	1,362	1,251	1,525	1,591	1,625	1,76	1,382	1,466	1,794	1,536	1,834	1,845	1,549	1,567	1,586	1,483	1,486	1,31
D-arabitol	1,534	1,399	1,201	1,169	0,443	0,413	1,436	1,364	1,599	1,345	0,456	0,329	1,546	1,659	1,349	1,304	1,151	1,133
arbutin	1,825	1,811	1,705	1,694	1,433	1,32	1,969	1,85	1,504	1,198	1,224	1,483	1,892	1,959	1,998	1,882	1,942	1,864
D-cellobiose	1,531	1,795	1,741	1,655	1,719	1,785	1,918	1,813	1,594	1,37	2,102	2,171	1,968	1,814	1,966	1,596	1,876	1,535
α- cyclodextrin	0,876	0,768	0,854	0,785	0,295	0,344	0,724	0,709	1,124	1,136	0,381	0,333	0,897	0,961	0,914	0,87	0,717	0,734
β- cyclodextrin	1,636	1,32	1,718	1,618	0,512	0,327	1,827	1,74	1,45	1,124	0,83	0,682	1,649	1,687	1,688	1,554	1,554	1,468
dextrin	1,669	1,545	1,82	1,623	2,01	1,909	1,708	1,751	1,251	1,078	2,061	2,207	1,75	1,936	1,793	1,724	1,535	1,574
I- erythritol	1,98	1,838	2,093	2	0,708	0,464	2,53	2,5	2,11	1,915	0,683	0,492	2,266	2,238	2,598	2,492	2,222	2,188
D-fructose	1,729	1,695	1,906	1,948	1,969	2,022	2,056	2,001	1,452	1,333	2,032	2,122	1,957	1,906	2,417	2,373	2,319	2,395
L-fructose	0,567	0,412	0,602	0,572	0,266	0,319	0,609	0,637	0,707	0,562	0,341	0,314	0,618	0,711	0,694	0,633	0,428	0,432
L-Fucose	1,701	1,115	1,341	1,36	0,293	0,358	1,612	1,758	1,97	1,467	0,423	0,295	1,583	1,605	1,536	1,555	0,98	1,104
D-Galactose	1,011	0,872	1,13	0,987	1,007	1,027	1,022	1,094	0,992	0,83	1,065	1,086	1,118	1,178	1,161	1,02	0,995	1,072
D-Galacturonic Acid	2,077	2,241	1,996	1,931	1,85	1,862	2,348	2,23	1,839	1,597	2,163	2,245	2,253	2,328	2,308	2,142	2,581	2,48
Gentiobiose	1,225	1,006	1,261	1,209	0,798	0,589	1,229	1,378	1,048	0,933	0,729	0,682	1,337	1,382	1,513	1,5	1,328	1,432
D-Gluconic Acid	1,162	1,035	1,034	1,236	0,259	0,277	1,189	1,101	1,165	1,056	0,433	0,29	1,285	1,25	1,036	0,984	0,947	1,028
D-Glucosamine	1,535	1,686	1,764	1,807	1,733	1,828	1,89	1,647	1,495	1,21	1,828	1,838	1,832	1,896	1,805	1,674	1,855	1,791
α-D-Glucose	0,708	0,595	0,69	0,737	0,206	0,273	0,709	0,704	0,922	0,852	0,31	0,2	0,714	0,761	0,751	0,671	0,655	0,605
Glucose-1-Phosphate	0,734	0,305	0,612	0,617	0,25	0,197	0,468	0,527	0,591	0,504	0,248	0,196	0,661	0,589	0,606	0,55	0,414	0,414
Glucuronamide	1,588	1,284	1,496	1,481	0,381	0,481	1,574	1,464	1,032	0,877	0,479	0,35	1,46	1,437	1,515	1,584	1,368	1,418
D-Glucronic Acid	1,72	1,592	1,801	2,041	0,768	0,718	1,982	2,15	2,059	1,889	0,732	0,614	2,053	1,867	2,12	2,182	2,008	1,821
Glycerol	1,318	1,191	1,193	1,22	0,994	0,844	1,328	1,357	1,097	0,915	0,893	0,91	1,388	1,269	1,284	1,284	1,234	1,158
Glycogen	2,019	1,951	2,13	2,216	0,765	0,505	2,28	2,376	1,56	1,512	0,835	0,745	2,004	1,968	2,236	2,228	1,98	2,181
m-Inositol	1,124	1,145	1,249	1,271	0,771	0,66	1,173	1,245	1,004	1,004	0,507	1,152	1,293	1,199	1,35	1,303	1,074	1,229
2-Keto-D-Gluconic Acid	1,014	0,862	0,887	0,909	0,331	0,488	1,165	1,18	1,237	1,055	0,49	0,468	0,94	0,896	1,102	1,133	0,611	0,732
α-D-Lactobiose	0,977	0,698	0,844	0,881	0,429	0,461	1,075	1,108	1,39	1,534	0,6	0,348	0,841	0,805	1,01	1,017	0,572	0,626
Lactulose	1,547	1,695	1,632	1,653	0,35	0,398	1,914	1,815	1,156	1,095	0,405	0,325	1,892	1,754	1,984	1,904	1,452	1,521
Maltitol	1,7	1,681	2	2,032	1,563	1,48	2,337	2,235	1,481	1,303	1,385	1,471	1,801	1,926	2,17	1,98	2,087	1,866
Maltose	1,491	1,458	1,596	1,61	1,787	1,828	1,983	1,515	1,546	1,347	2,257	2,345	1,754	1,663	1,717	1,617	1,718	1,51
Maltotriose	2,224	2,111	1,767	1,759	0,425	0,394	2,088	2,03	1,925	1,913	0,395	0,344	1,856	1,868	2,198	2,139	2,349	2,308
D-Mannitol	1,776	1,765	1,98	1,784	1,829	1,66	2,146	2,076	1,347	1,306	1,995	2,023	1,974	1,97	2,281	2,2	2,298	2,372
D-Mannose	0,823	0,698	0,829	0,83	0,566	0,676	0,884	0,894	1,256	0,967	0,51	0,834	0,87	0,815	1,024	0,965	0,696	0,825
D-Melezitose	1,002	0,802	0,912	0,908	0,954	0,581	1,121	1,146	1,158	1,069	0,581	0,413	1,68	0,977	1,246	1,228	0,591	0,748
D-Melibiose	0,678	0,542	0,7	0,761	0,547	0,614	0,745	0,683	1,064	1,037	0,349	0,35	0,893	0,724	0,824	0,787	0,585	0,68
α-Methyl-D-Galactoside	1,363	1,172	1,165	1,211	0,598	0,545	1,283	1,297	0,869	0,681	0,633	0,442	1,101	1,099	1,317	1,257	0,884	1,019
β-Methyl-D-Galactoside	0,922	0,882	1,026	0,924	0,4	0,431	0,971	0,971	0,959	0,862	0,466	0,432	0,998	0,987	1,089	1,042	0,751	0,88
α-Methyl-D-Glucoside	1,977	1,973	1,921	1,903	1,733	1,762	2,175	2,069	1,927	1,716	1,778	1,939	1,921	1,965	2,109	2,085	1,64	1,71
β-Methyl-D-Glucoside	1,992	2,25	1,901	1,876	0,469	0,465	2,165	1,971	2,084	1,902	0,691	0,549	1,892	1,986	2,248	2,174	1,656	1,762
Palatinose	0,743	0,684	0,776	0,744	0,58	0,671	0,679	0,815	0,803	0,765	0,776	0,72	0,797	0,846	0,891	0,818	0,652	0,645
D-Psicose	1,826	1,968	1,561	1,668	1,483	1,368	1,979	1,938	1,546	1,529	1,989	1,973	1,884	1,863	2,099	2,147	1,44	1,334
D-Raffinose	1,439	1,355	1,342	1,601	0,837	0,929	1,922	1,202	1,67	1,797	1,119	0,979	1,714	1,678	1,879	1,898	1,726	1,423
L-Rhamnose	1,358	1,231	1,546	1,576	0,606	0,548	1,656	1,613	1,639	1,513	0,567	0,731	1,597	1,421	1,595	1,811	1,465	1,424
D-Ribose	1,175	1,155	1,345	1,326	1,316	1,302	1,297	1,191	1,23	1,078	0,958	0,873	1,35	1,156	1,371	1,227	1,1	1,137
Salicin	0,711	0,541	0,745	0,75	0,4	0,315	0,676	0,693	0,687	0,687	0,424	0,299	0,799	0,75	0,836	0,837	0,598	0,672
Sedoheptulosan	1,708	1,91	1,99	2,007	1,659	1,696	2,247	2,055	1,936	1,742	0,626	0,582	2,129	2,105	2,158	2,024	2,332	2,312
D-Sorbitol	1,869	1,969	2,15	2,225	0,338	0,288	2,452	2,397	2,345	1,91	0,345	1,302	2,188	2,114	2,272	2,193	2,446	2,422
L-Sorbose	1,991	2,17	2,268	2,214	0,686	0,45	2,444	2,404	1,444	1,223	0,506	0,781	2,157	2,303	2,294	2,362	1,14	1,536
Stachyose	1,712	1,894	2,183	2,163	2,006	1,942	2,511	2,217	1,63	1,702	2,057	2,098	2,125	2,168	2,315	2,327	2,365	2,313
Sucrose	0,73	0,622	0,806	0,789	0,332	0,347	0,792	0,776	1,045	0,897	0,414	0,372	0,845	0,835	0,886	0,826	0,647	0,668
D-Tagatose	2,169	2,493	2,194	2,174	1,173	1,221	2,521	2,553	1,801	1,494	1,355	1,474	2,27	2,332	2,585	2,496	2,329	2,406
D-Trehalose	2,29	2,497	2,425	2,386	0,504	0,437	2,53	2,357	1,974	1,616	0,592	0,455	2,469	2,469	2,61	2,65	2,36	2,402
Turanose	2,365	0,88	1,599	1,526	0,668	0,718	2,056	2,262	1,852	1,57	0,779	0,829	1,371	1,484	1,721	1,951	2,215	2,015
Xylitol	1,282	1,39	1,503	1,743	1,757	1,754	1,785	0,932	1,601	1,812	1,821	1,955	1,731	1,758	1,691	1,721	1,977	1,612
D-Xylose	1,625	1,529	1,638	1,667	0,593	0,639	1,934	1,877	1,195	0,983	0,79	0,89	1,635	1,746	1,576	1,653	1,739	1,742
y-Amino-butyric Acid	0,983	0,812	0,937	1	0,469	0,391	0,991	1,014	0,886	0,784	0,436	0,337	1,054	1,012	1,115	1,126	0,851	0,91
Bromosuccinic Acid	1,207	1,131	1,244	1,232	0,604	0,51	1,251	1,36	1,095	0,963	0,549	0,554	1,252	1,342	1,334	1,213	1,336	1,126
Fumaric Acid	0,762	0,696	1,143	0,764	0,255	0,428	1,116	1,021	0,788	0,729	0,43	0,405	0,956	1,053	0,947	1,003	0,664	1,007
y-Hydroxy-butyric Acid	0,671	0,588	0,793	0,694	0,333	0,304	0,711	0,818	0,742	0,618	0,481	0,276	0,84	0,786	0,834	0,772	0,604	0,669
p-Hyroxyphenyl-acetic Acid	1,573	1,617	1,535	1,392	0,584	0,416	1,923	1,891	1,182	0,956	0,518	0,387	1,832	1,975	1,649	1,658	1,379	1,469
α-Keto-glutaric Acid	1,137	1,045	1,133	1,091	0,437	0,38	1,314	1,24	1,102	0,943	0,371	0,319	1,152	1,134	1,243	1,259	1,041	1,14
D-Lactic Acid Methyl Ester	0,665	0,533	0,769	0,698	0,769	0,483	0,743	0,701	0,698	0,605	0,39	0,548	0,841	0,818	0,767	0,776	0,645	0,612
L-Lactic Acid	0,861	0,708	0,862	0,847	0,382	0,432	0,9	0,841	0,842	0,786	0,498	0,446	0,932	0,949	0,915	0,939	0,762	0,722
D-Malic Acid	1,111	1,02	1,104	0,996	0,379	0,453	1,158	1,248	1,028	0,995	0,437	0,372	0,954	1,144	1,188	1,178	0,988	1,022
L-Malic Acid	1,162	1,005	1,074	1,034	0,576	0,36	1,147	1,247	0,977	0,879	0,351	0,459	1,071	1,188	1,161	1,145	0,955	0,989
Quinic Acid	1,064	1,494	0,492	1,513	0,844	0,76	1,46	1,242	0,989	1,373	0,725	0,599	1,395	1,519	1,681	1,68	1,645	1,489
D-Saccharic Acid	1,194	1,1	1,241	1,223	0,36	0,438	1,155	1,282	1	0,965	0,303	0,408	1,192	1,261	1,262	1,364	0,537	0,514
Sebacic Acid	1,456	1,263	1,202	1,464	0,515	0,517	1,742	1,425	1,257	1,082	0,369	0,438	1,603	1,522	1,652	1,707	1,544	1,449
Succinamic Acid	1,361	1,088	1,206	1,236	0,552	0,531	1,404	1,401	0,905	0,835	0,647	0,532	1,284	1,293	1,27	1,275	1,283	1,299
Saccinic Acid	0,989	0,991	1,119	1,067	0,582	0,424	1,089	1,127	0,949	0,85	0,514	0,533	1,041	1,093	1,171	1,158	0,963	1,04
Succinic Acid Mono-Methy Este	1,073	0,904	1,064	1,012	0,612	0,902	1,111	1,081	1,01	0,965	0,784	1,061	1,093	1,05	1,177	1,117	1,043	1,077
N-Acetyl-L-Glutamic Acid	0,651	0,522	0,752	0,666	0,485	0,534	0,699	0,706	0,774	0,646	0,553	0,409	0,715	0,762	0,693	0,749	0,532	0,617
Alaninamide	0,798	0,66	0,9	0,83	0,3	0,365	0,897	0,903	0,953	0,854	0,485	0,361	0,871	0,975	0,915	0,873	0,739	0,795
L-Alanine	1,348	1,405	1,535	1,517	0,457	0,372	1,75	1,679	1,2	1,005	0,892	0,85	1,495	1,582	1,655	1,608	1,717	1,699
L-Alanyl-Glycine	1,198	1,169	1,476	1,396	0,532	0,33	1,114	0,944	1,194	1,02	0,33	0,324	0,957	1,07	0,988	1,034	0,813	0,839
L-Asparagine	1,019	1,08	1,176	1,125	0,25	0,334	1,228	1,239	1,068	0,99	0,345	0,275	1,161	1,202	1,355	1,267	1,163	1,186
L-Aspartic Acid	0,956	0,841	0,982	0,988	0,298	0,777	0,907	0,936	0,933	0,833	0,426	0,434	0,909	0,985	1,084	1,093	0,967	0,932
L-Glutamic acid	1,023	1,026	1,051	1,167	0,526	0,58	1,058	0,966	1,366	1,233	0,656	0,755	1,06	1,154	1,123	1,137	1,149	1,355
Glycyl-L-Glutamic Acid	1,053	0,795	0,908	0,846	0,354	0,369	0,684	0,755	1,176	0,946	0,215	0,251	0,81	0,819	0,85	0,843	0,701	0,732
L-Ornithine	1,434	1,213	1,278	1,328	0,323	0,364	1,375	1,395	0,949	0,958	0,303	0,335	1,344	1,353	1,477	1,378	1,25	1,25
L-Phenylalanine	1,064	0,989	0,996	1,047	0,324	0,249	1	1,228	0,958	0,872	0,467	0,604	0,784	0,748	0,948	0,976	0,877	0,93
l-Proline	1,436	1,288	1,389	1,45	0,969	1,189	1,462	1,609	1,175	1,152	1,294	1,19	1,457	1,533	1,418	1,397	1,295	1,221
L-Pyroglutamic Acid	1,43	1,294	1,425	1,406	0,504	0,53	1,521	1,878	1,038	1,051	0,585	0,591	1,373	1,396	1,327	1,323	1,297	1,312
L-Serine	0,978	0,877	1,074	1,028	0,34	0,28	1,041	0,989	1,057	0,94	0,488	0,359	1,093	1,145	1,242	1,216	1,275	1,172
L-Threonine	1,277	1,124	1,224	1,262	0,303	0,436	1,271	1,178	0,965	0,973	0,288	0,224	1,276	1,345	1,354	1,345	1,294	1,265
2-Amino Ethanol	1,638	0,091	1,429	1,554	0,557	0,33	1,694	1,507	1,075	0,95	0,489	0,375	1,745	1,777	1,696	1,528	1,583	1,604
Putrescine	0,769	0,652	0,806	0,753	0,301	0,384	0,646	0,604	0,903	0,953	0,263	0,253	0,796	0,873	0,769	0,8	0,84	0,894
Adenosine	0,69	0,815	0,657	0,755	0,412	0,53	0,718	0,922	0,612	0,854	0,556	0,418	0,767	0,867	0,697	0,698	0,629	0,63
Uridine	0,536	0,512	0,587	0,585	0,326	0,33	0,56	0,581	0,521	0,498	0,231	0,265	0,622	0,717	0,578	0,603	0,522	0,548
Adenosine-5'-Monophosphate	0,526	0,481	0,559	0,577	0,97	0,254	0,472	0,513	0,531	0,511	0,238	0,239	0,568	0,675	0,586	0,591	0,456	0,655

Figure C Isolates 28-36

Appendix D: Effect of voriconazole and posaconazole on the growth of isolates for Chapter 4

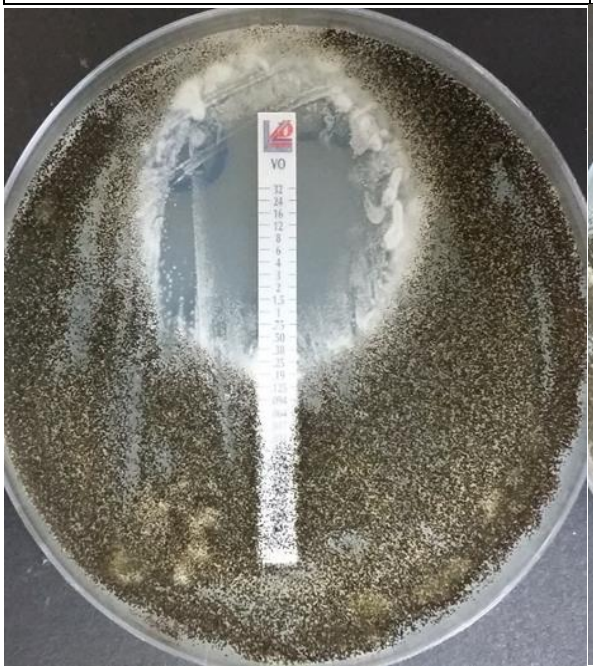
A. niger (10)	
voriconazole	**posaconazole**
	

Figure D1 Effect of voriconazole and posaconazole on *Aspergillus niger* at 48 hours. MIC Test Strip with azole concentration from 0.002-32µg/ml.

A. niger (12)	
voriconazole	**posaconazole**

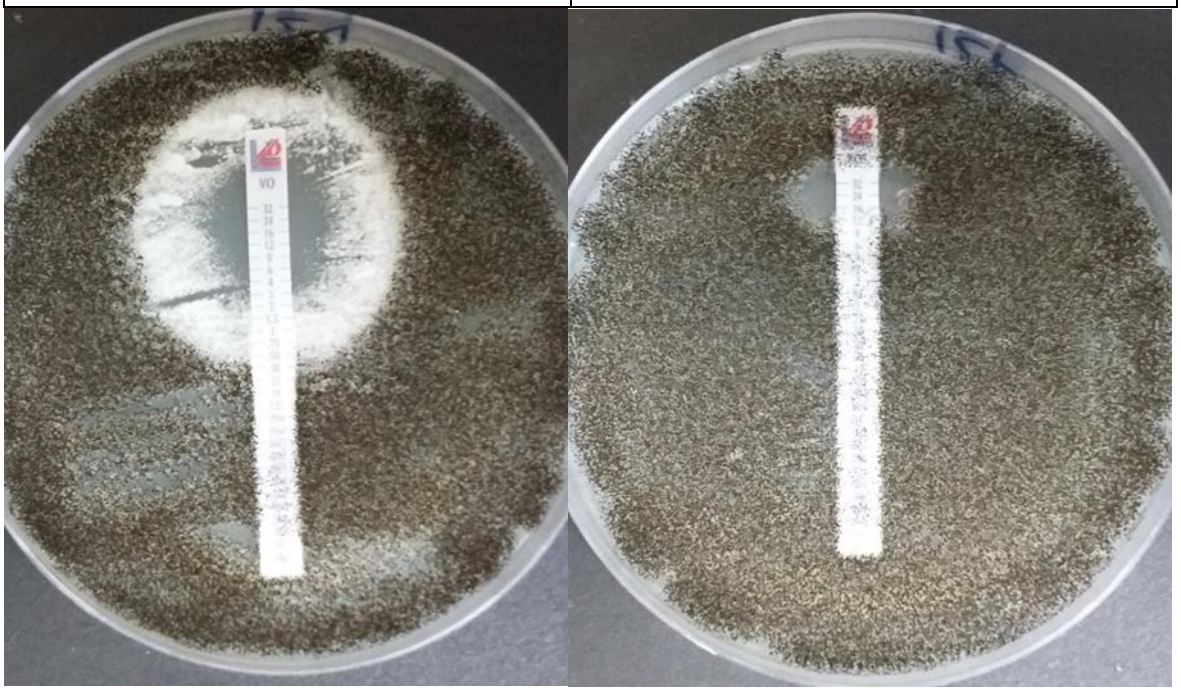


Figure D2 Effect of voriconazole and posaconazole on the growth of *Aspergillus niger* at 48 hours. MIC Test Strip with azole concentration from 0.002-32µg/ml.

A. niger (13)	
voriconazole	**posaconazole**

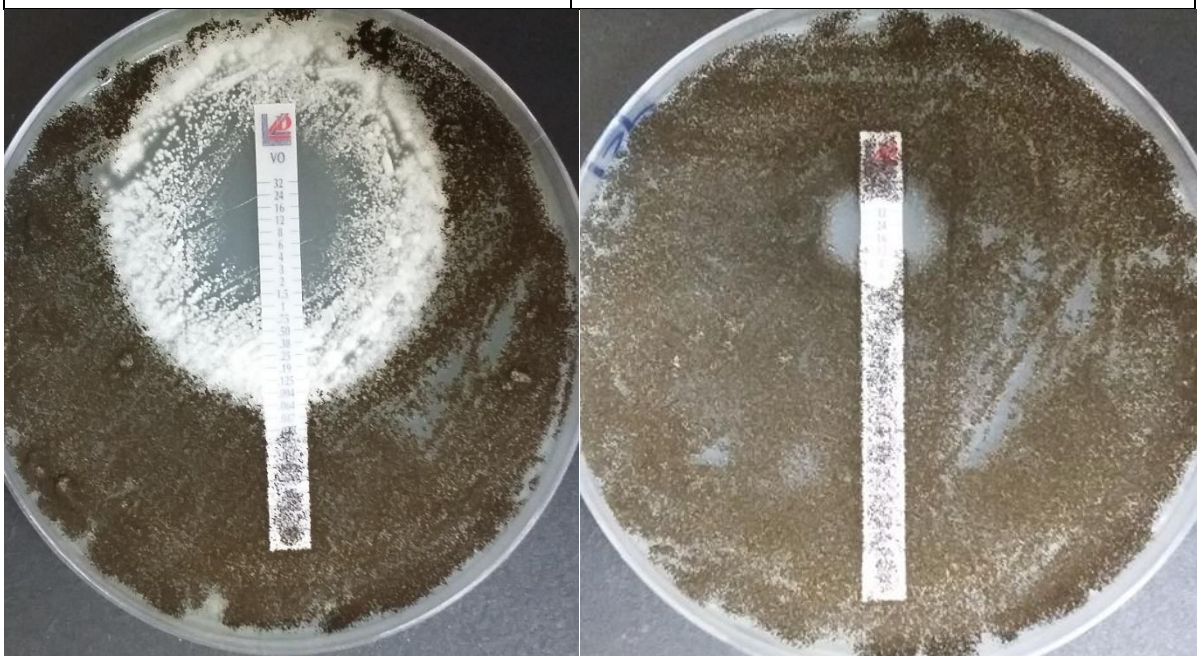


Figure D3 Effect of voriconazole and posaconazole on the growth of *Aspergillus niger* at 48 hours. MIC Test Strip with azole concentration from 0.002-32µg/ml.

A. niger (21)	
voriconazole	**posaconazole**

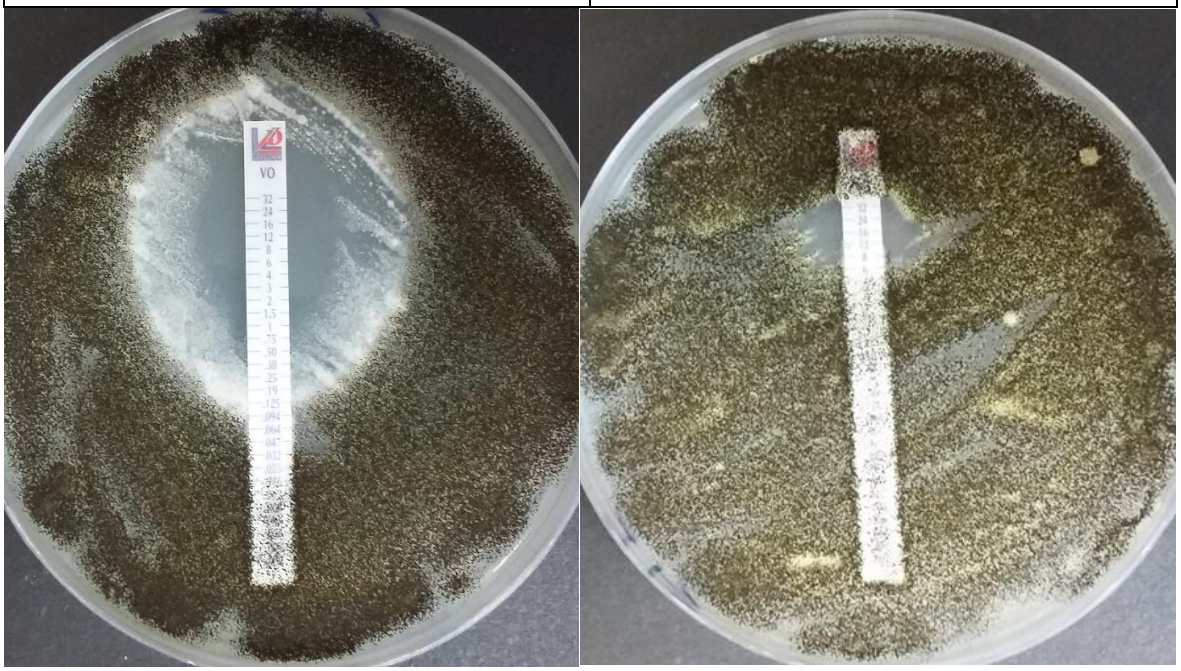


Figure D4 Effect of voriconazole and posaconazole on the growth of *Aspergillus niger* at 48 hours. MIC Test Strip with azole concentration from 0.002-32µg/ml.

A. niger (30)	
voriconazole	**posaconazole**

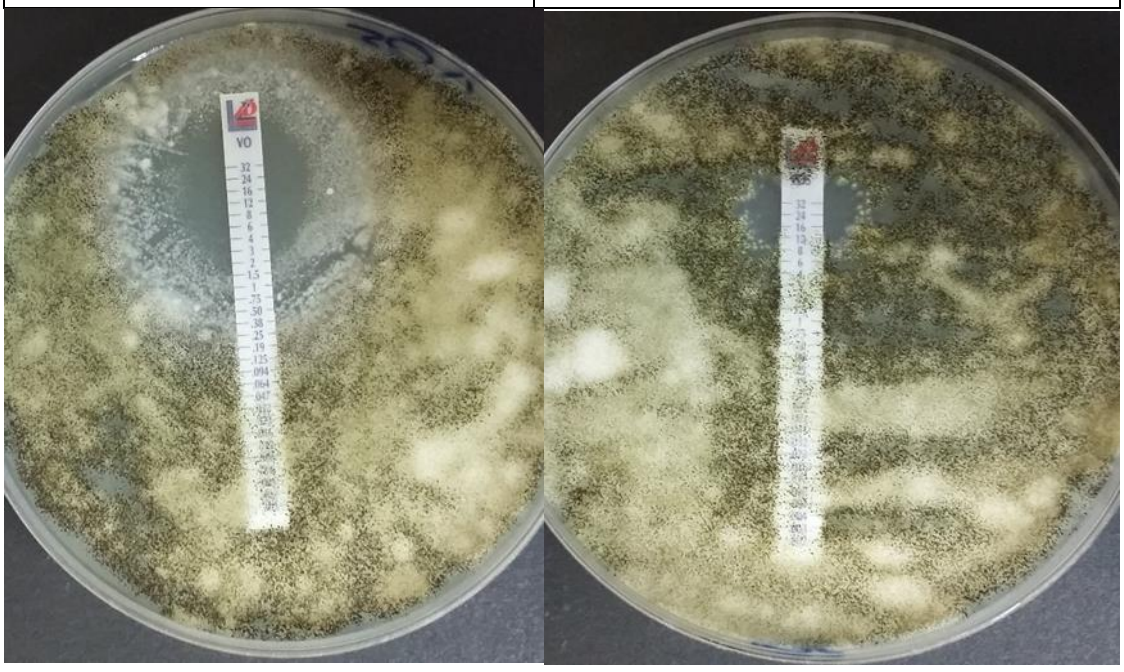


Figure D5 Effect of voriconazole and posaconazole on the growth of *Aspergillus niger* at 48 hours. MIC Test Strip with azole concentration from 0.002-32µg/ml.

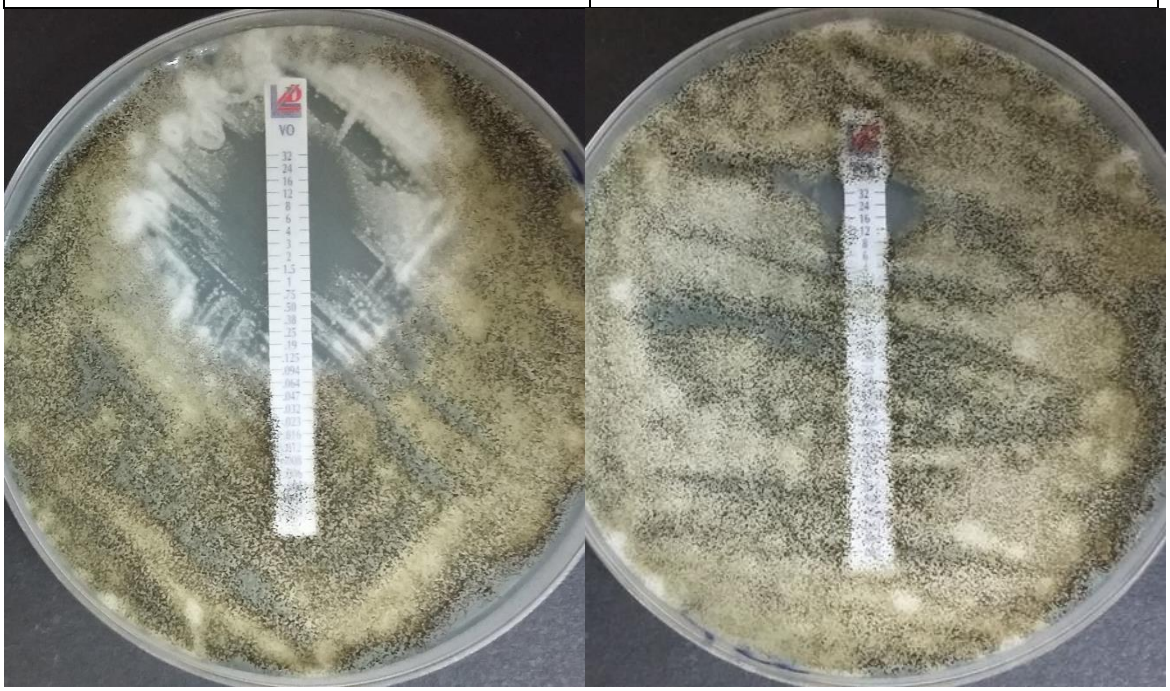
***A. niger* (33)**	
voriconazole	**posaconazole**
	

Figure D6 Effect of voriconazole and posaconazole on the growth of *Aspergillus niger* at 48 hours. MIC Test Strip with azole concentration from 0.002-32µg/ml.

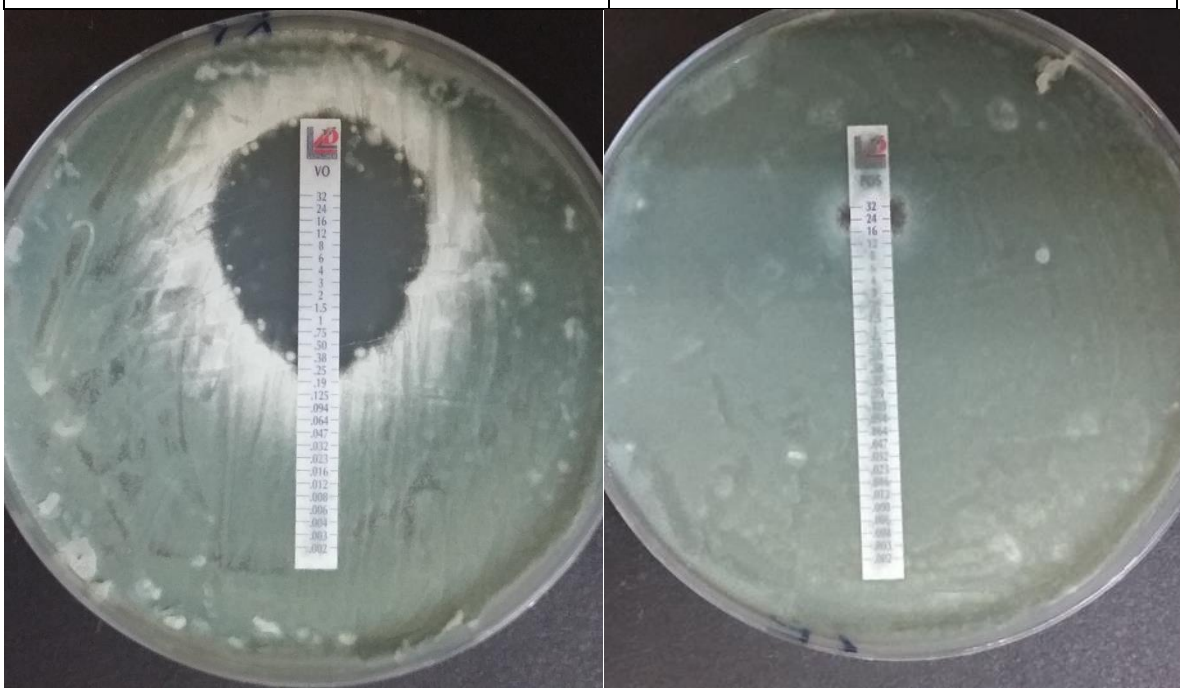
***A. fumigatus* (1)**	
voriconazole	**posaconazole**
	

Figure D7 Effect of voriconazole and posaconazole on the growth of *Aspergillus fumigatus* at 48 hours. MIC Test Strip with azole concentration from 0.002-32µg/ml.

***A. fumigatus* (3)**	
voriconazole	**posaconazole**

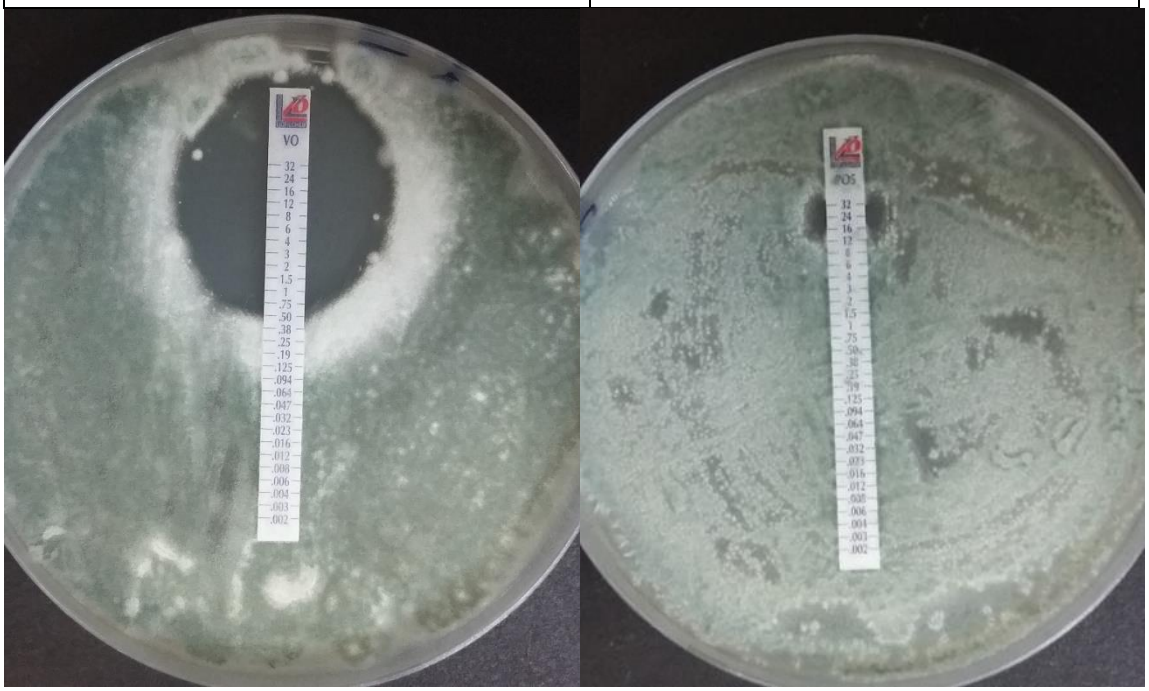


Figure D8 Effect of voriconazole and posaconazole on the growth of *Aspergillus fumigatus* at 48 hours. MIC Test Strip with azole concentration from 0.002-32µg/ml.

***A. fumigatus* (5)**	
voriconazole	**posaconazole**

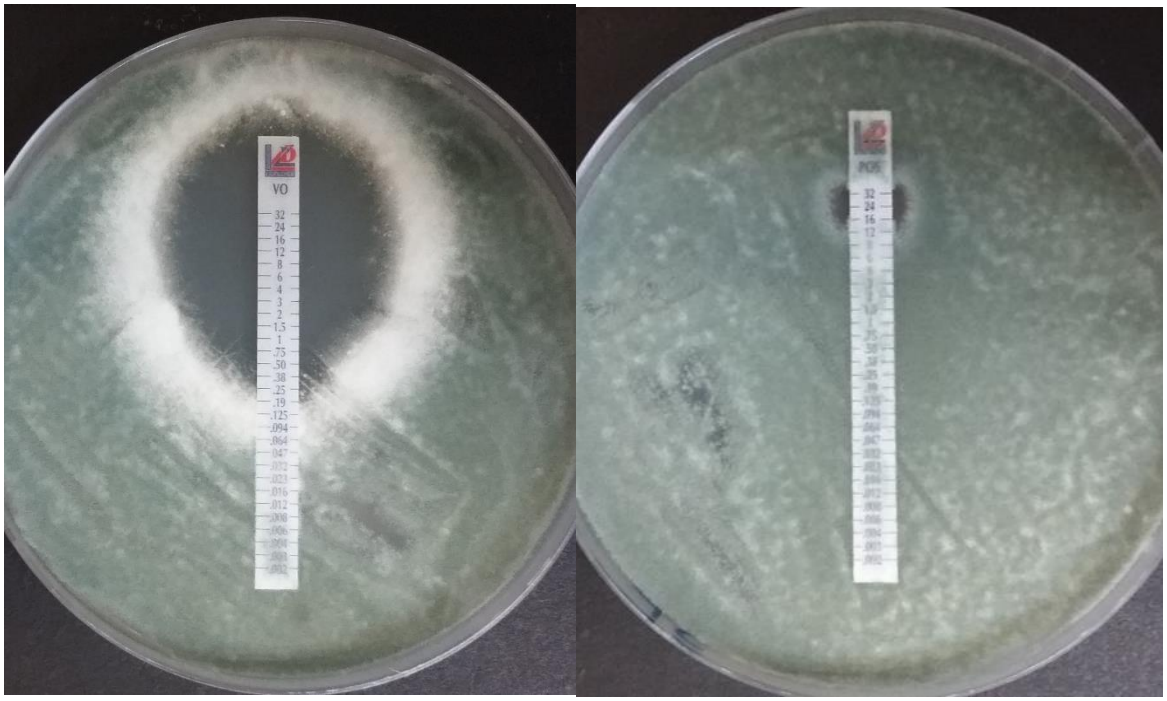


Figure D9 Effect of voriconazole and posaconazole on the growth of *Aspergillus fumigatus* at 48 hours. MIC Test Strip with azole concentration from 0.002-32µg/ml.

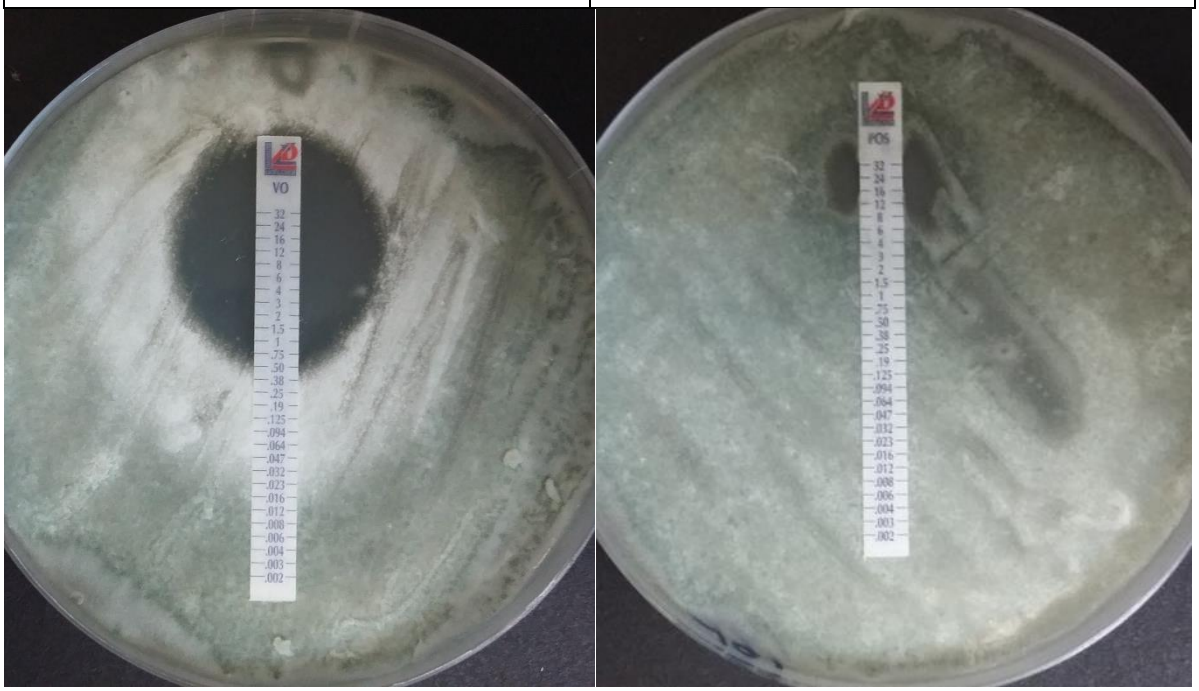
***A. fumigatus* (16)**	
voriconazole	**posaconazole**
	

Figure D10 Effect of voriconazole and posaconazole on the growth of *Aspergillus fumigatus* at 48 hours. MIC Test Strip with azole concentration from 0.002-32µg/ml.

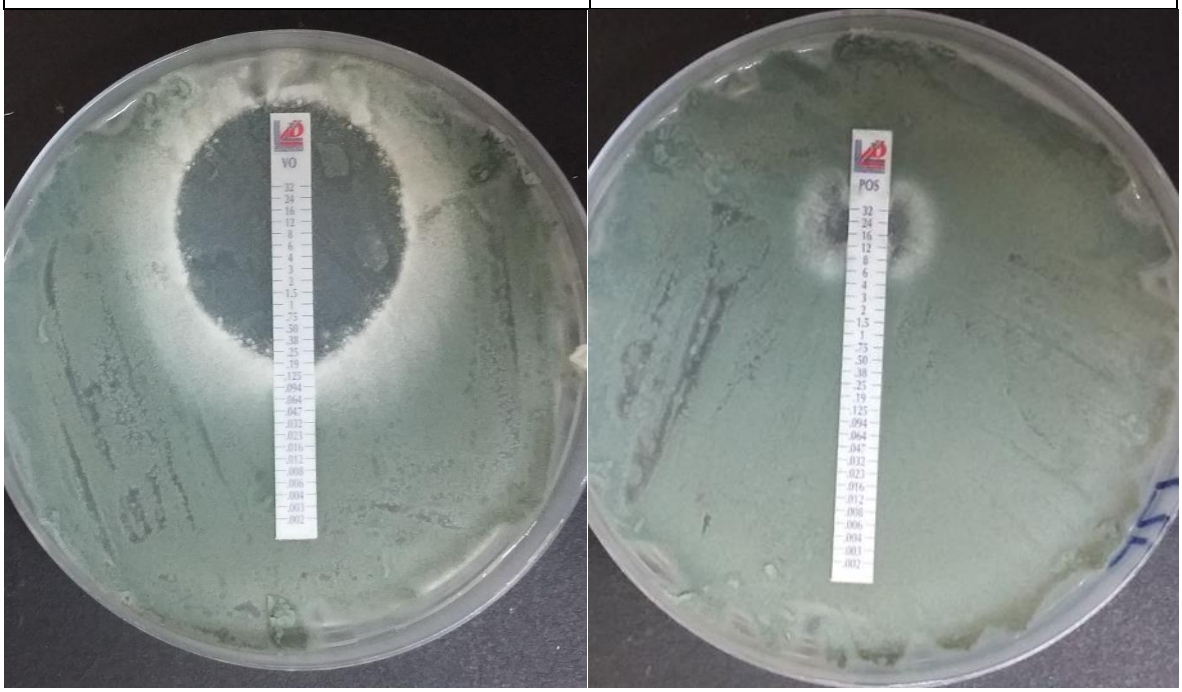
***A. fumigatus* (17)**	
voriconazole	**posaconazole**
	

Figure D12 Effect of voriconazole and posaconazole on the growth of *Aspergillus fumigatus* at 48 hours. MIC Test Strip with azole concentration from 0.002-32µg/ml.

***A. fumigatus* (20)**	
voriconazole	**posaconazole**

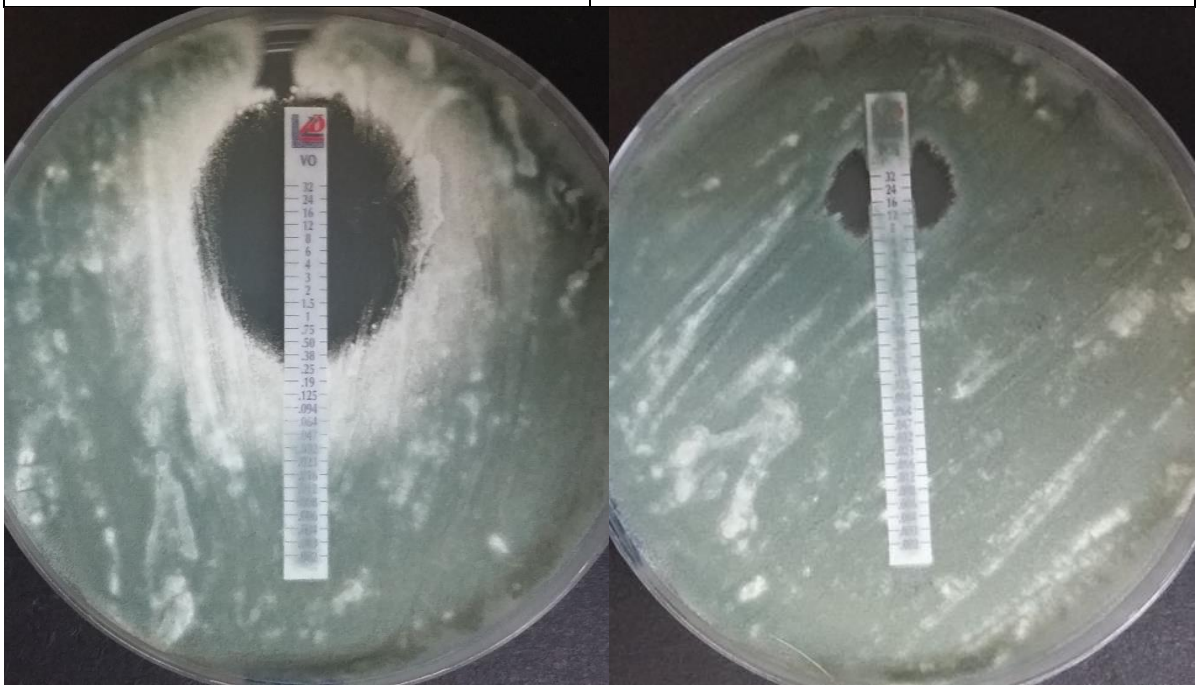


Figure D13 Effect of voriconazole and posaconazole on the growth of *Aspergillus fumigatus* at 48 hours. MIC Test Strip with azole concentration from 0.002-32µg/ml.

***A. fumigatus* (26)**	
voriconazole	**posaconazole**

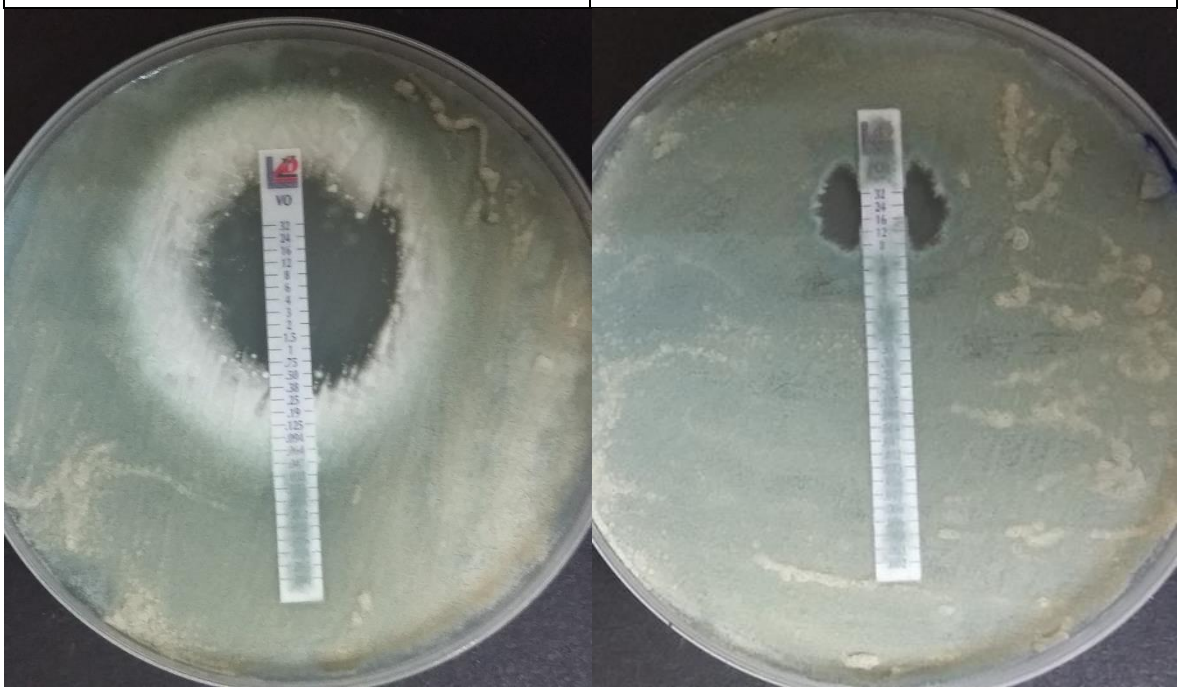


Figure D14 Effect of voriconazole and posaconazole on the growth of *Aspergillus fumigatus* at 48 hours. MIC Test Strip with azole concentration from 0.002-32µg/ml.

***A. fumigatus* (27)**	
voriconazole	**posaconazole**

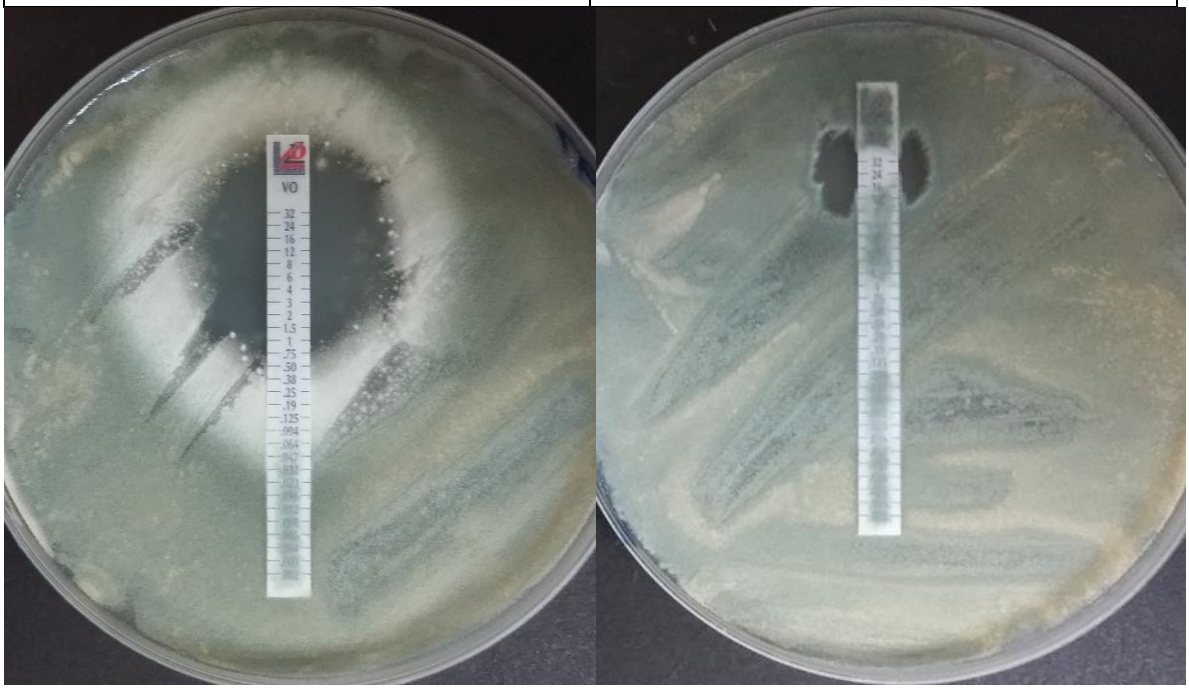


Figure D15 Effect of voriconazole and posaconazole on the growth of *Aspergillus fumigatus* at 48 hours. MIC Test Strip with azole concentration from 0.002-32µg/ml.

***A. fumigatus* (29)**	
voriconazole	**posaconazole**

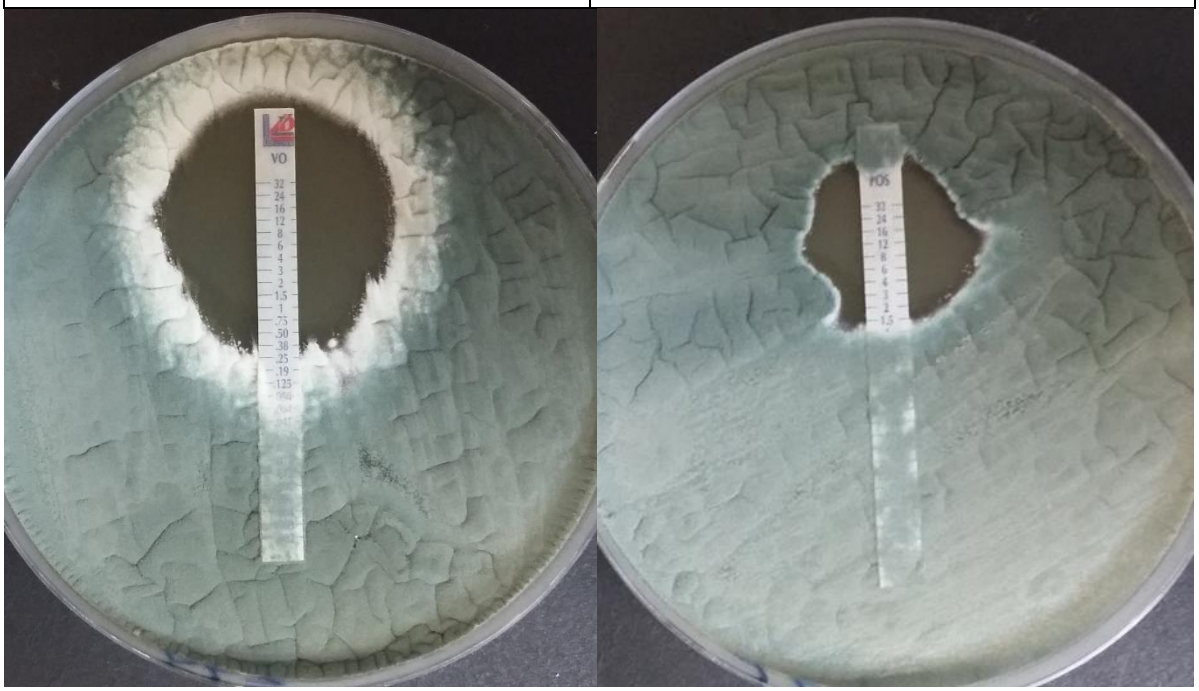


Figure D16 Effect of voriconazole and posaconazole on the growth of *Aspergillus fumigatus* at 48 hours. MIC Test Strip with azole concentration from 0.002-32µg/ml.

***A. fumigatus* (31)**	
voriconazole	**posaconazole**

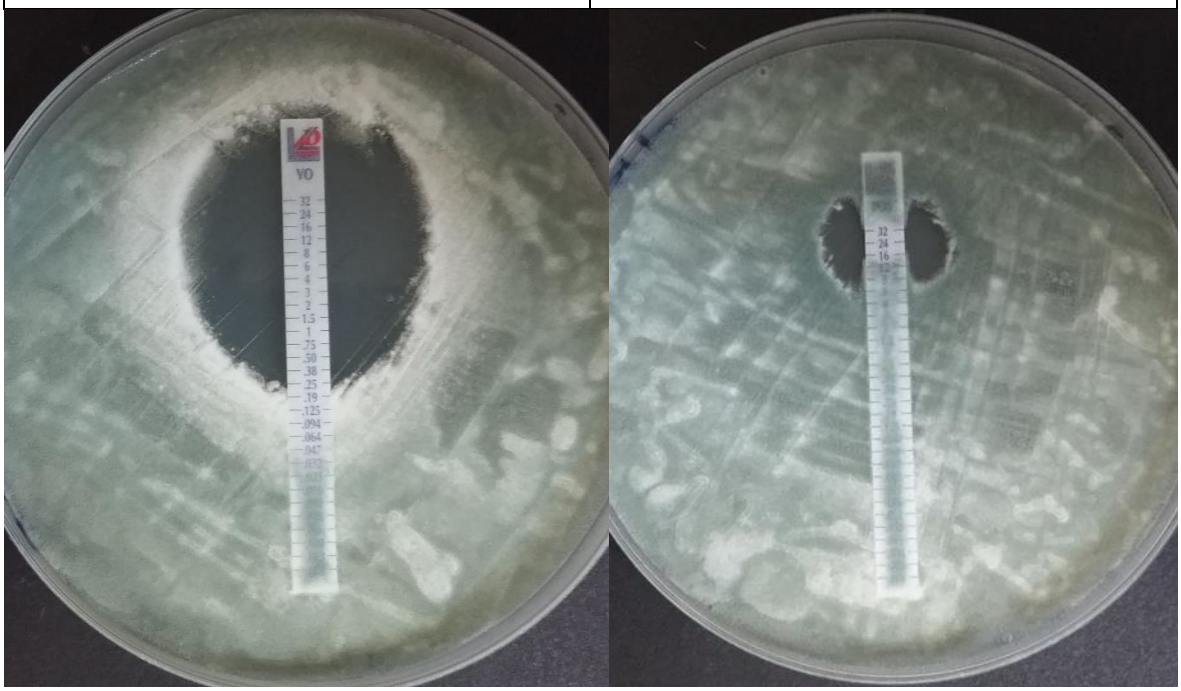


Figure D17 Effect of voriconazole and posaconazole on the growth of *Aspergillus fumigatus* at 48 hours. MIC Test Strip with azole concentration from 0.002-32µg/ml.

***A. fumigatus* (34)**	
voriconazole	**posaconazole**

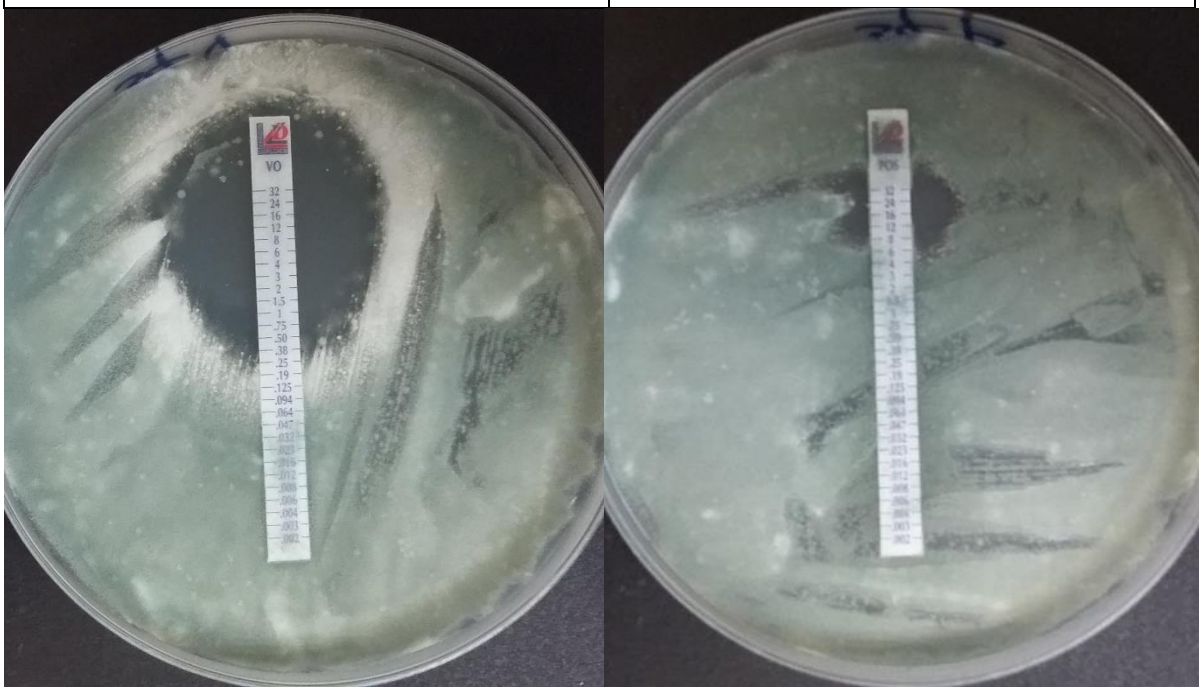


Figure D18 Effect of voriconazole and posaconazole on the growth of *Aspergillus fumigatus* at 48 hours. MIC Test Strip with azole concentration from 0.002-32µg/ml.

***A. fumigatus* (35)**	
voriconazole	**posaconazole**

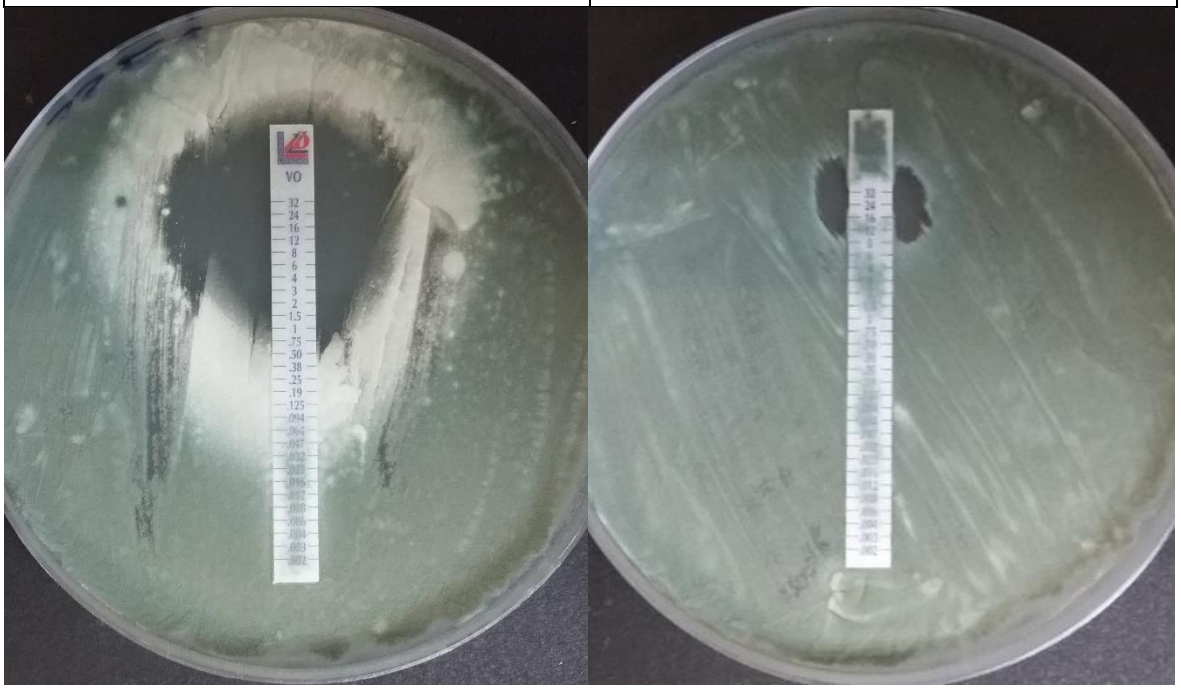


Figure D19 Effect of voriconazole and posaconazole on the growth of *Aspergillus fumigatus* at 48 hours. MIC Test Strip with azole concentration from 0.002-32µg/ml.

***A. fumigatus* (36)**	
voriconazole	**posaconazole**

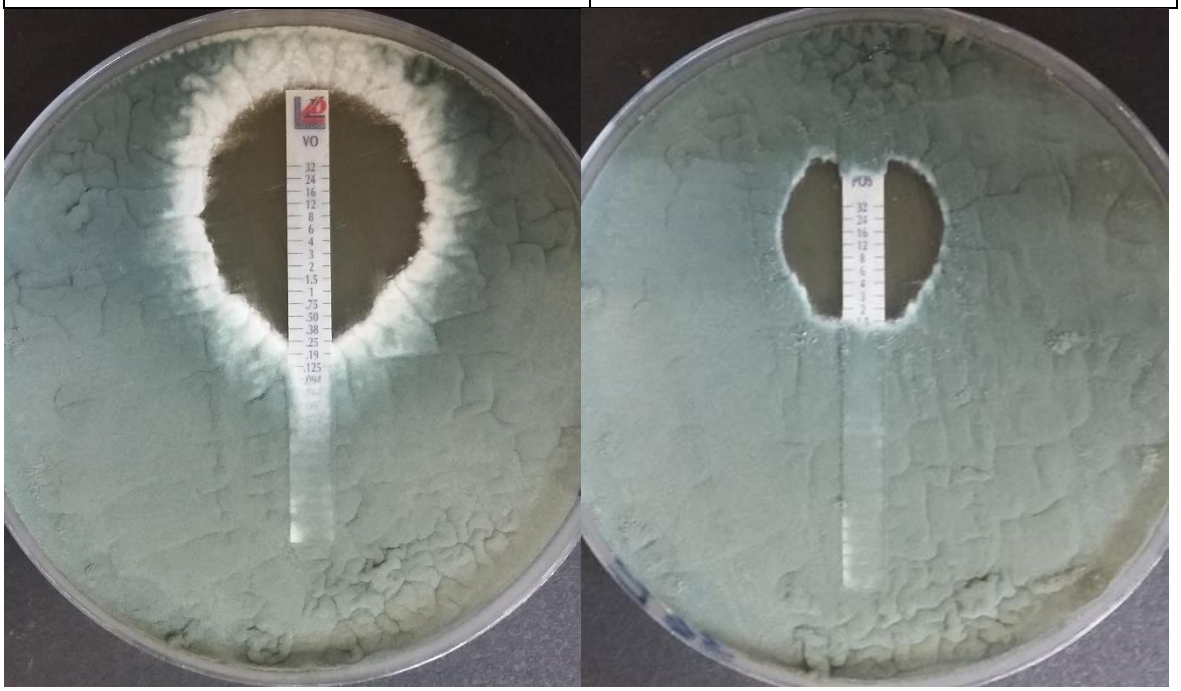
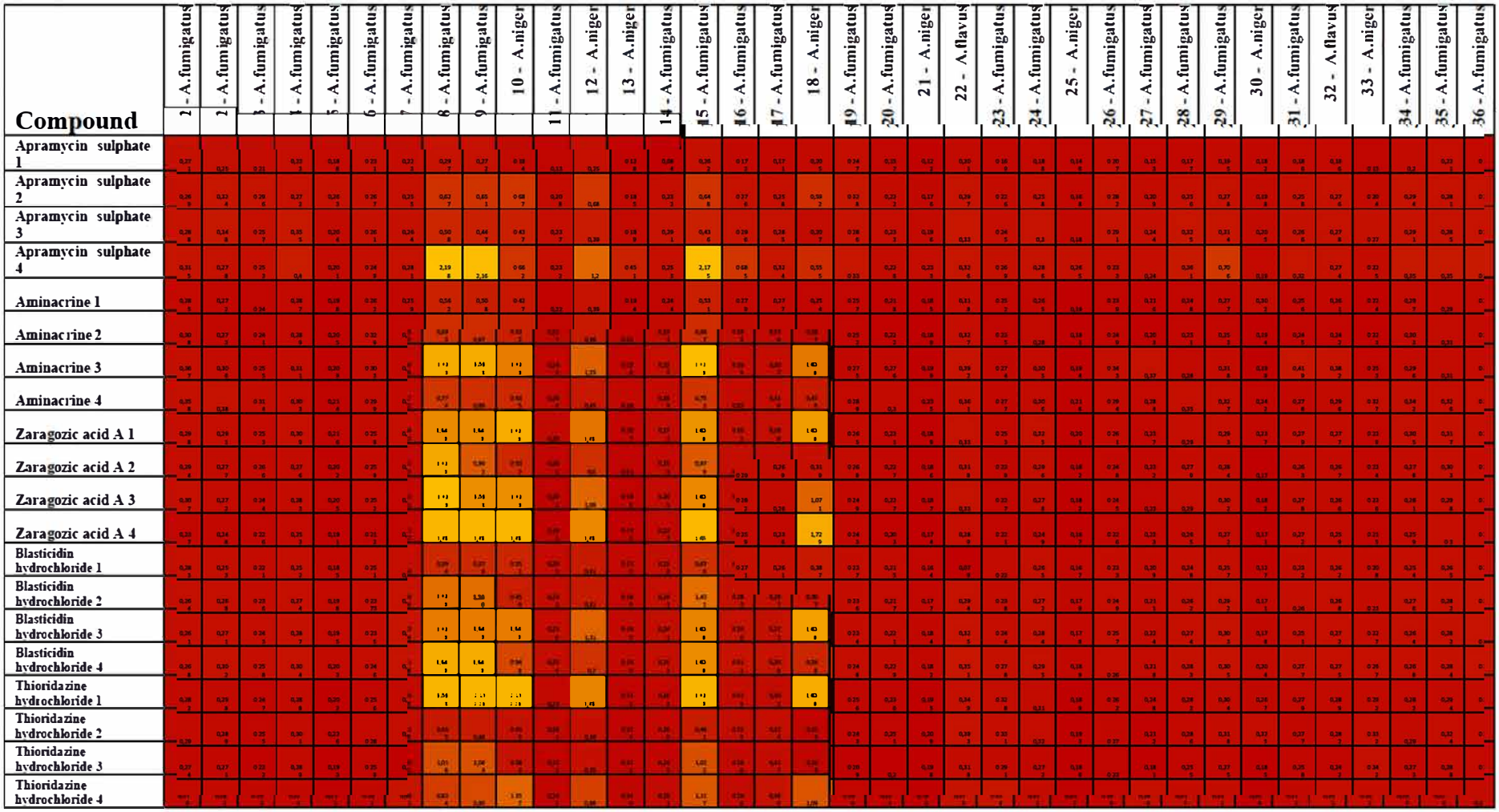
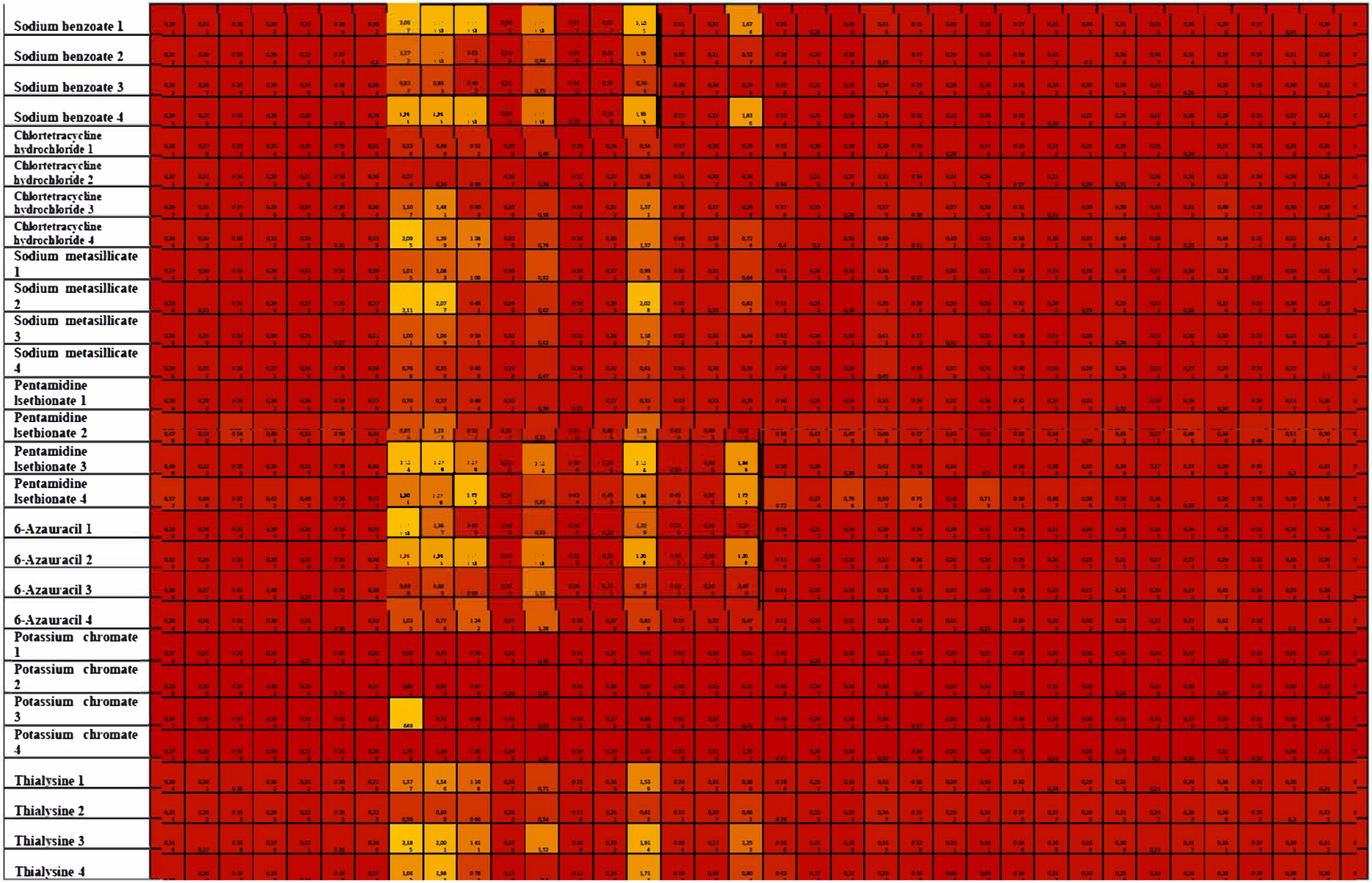
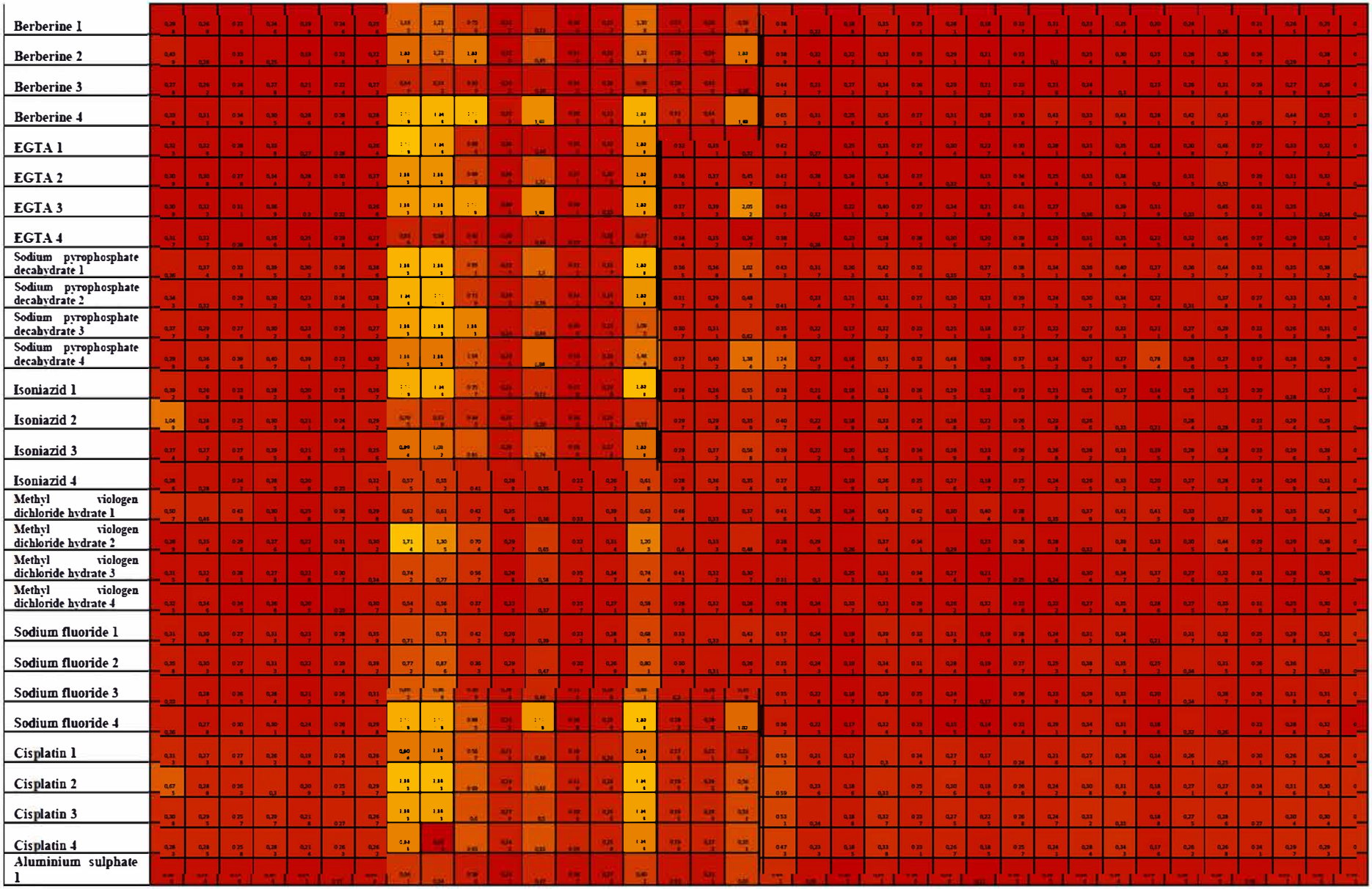


Figure D20 Effect of voriconazole and posaconazole on the growth of *Aspergillus fumigatus* at 48 hours. MIC Test Strip with azole concentration from 0.002-32µg/ml.

Appendix E: Heat map of absorbance readings of the isolates at all four concentrations of the 24 compounds.




Sodium benzoate 1
Sodium benzoate 2
Sodium benzoate 3
Sodium benzoate 4
Chlortetracycline hydrochloride 1
Chlortetracycline hydrochloride 2
Chlortetracycline hydrochloride 3
Chlortetracycline hydrochloride 4
Sodium metasillicate 1
Sodium metasillicate 2
Sodium metasillicate 3
Sodium metasillicate 4
Pentamidine Isethionate 1
Pentamidine Isethionate 2
Pentamidine Isethionate 3
Pentamidine Isethionate 4
6-Azauracil 1
6-Azauracil 2
6-Azauracil 3
6-Azauracil 4
Potassium chromate 1
Potassium chromate 2
Potassium chromate 3
Potassium chromate 4
Thialysine 1
Thialysine 2
Thialysine 3
Thialysine 4


Berberine 1
Berberine 2
Berberine 3
Berberine 4
EGTA 1
EGTA 2
EGTA 3
EGTA 4
Sodium pyrophosphate decahydrate 1
Sodium pyrophosphate decahydrate 2
Sodium pyrophosphate decahydrate 3
Sodium pyrophosphate decahydrate 4
Isoniazid 1
Isoniazid 2
Isoniazid 3
Isoniazid 4
Methyl viologen dichloride hydrate 1
Methyl viologen dichloride hydrate 2
Methyl viologen dichloride hydrate 3
Methyl viologen dichloride hydrate 4
Sodium fluoride 1
Sodium fluoride 2
Sodium fluoride 3
Sodium fluoride 4
Cisplatin 1
Cisplatin 2
Cisplatin 3
Cisplatin 4
Aluminium sulphate 1

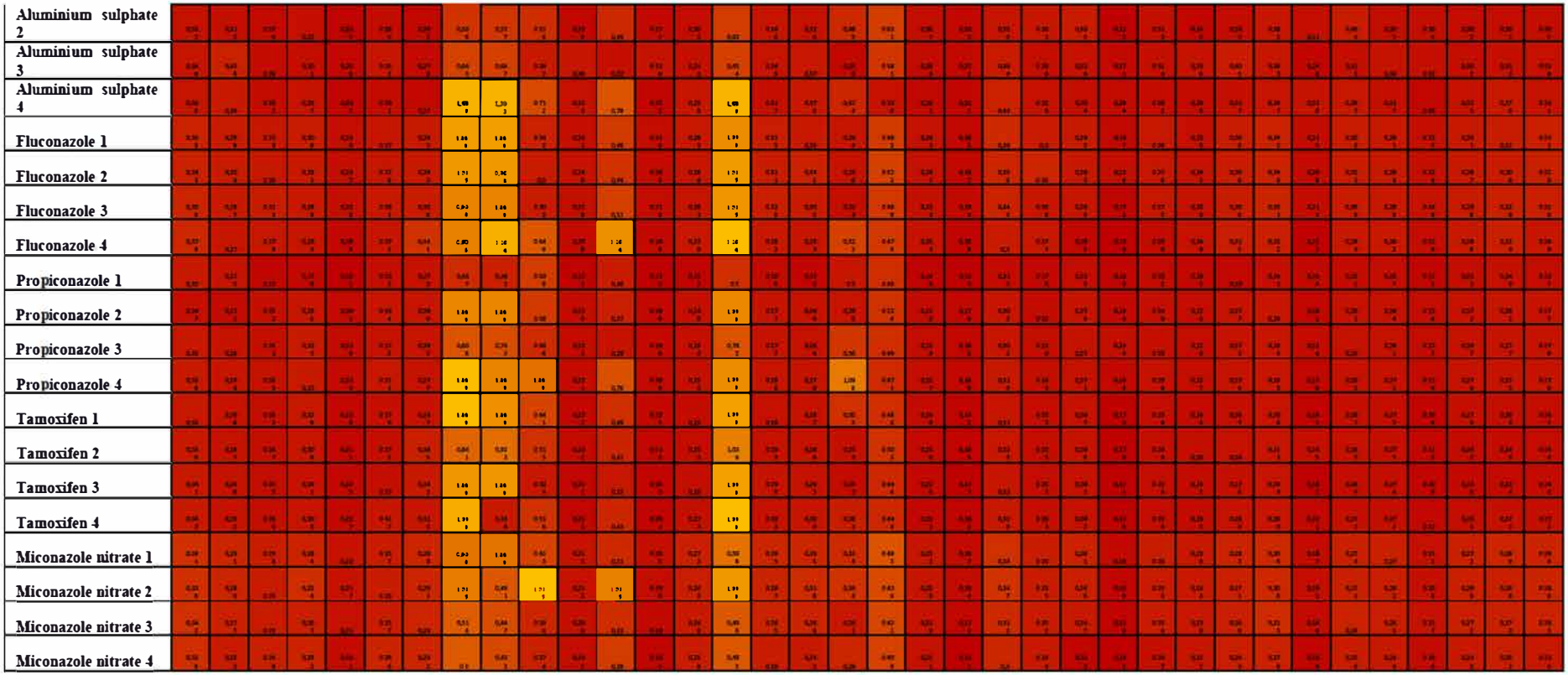


Figure E1 Shows absorbance readings taken at 750nm after 48hr of incubation of isolates in a growth medium incorporated with the 24 compounds in four different concentrations. Red indicates susceptibility and yellow indicates the resistance of isolates to the potential antifungal agents.