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ISOLATION AND CHARACTERIZATION OF ANTIFUNGAL AGENTS PRODUCED BY WOOD DECAYING FUNGI FROM GHANA

A THESIS PRESENTED TO THE DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY
UNIVERSITY OF GHANA

BY

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFULMENT OF THE REQUIREMENT FOR THE AWARD OF M.PHIL BIOCHEMISTRY DEGREE

JULY 2014

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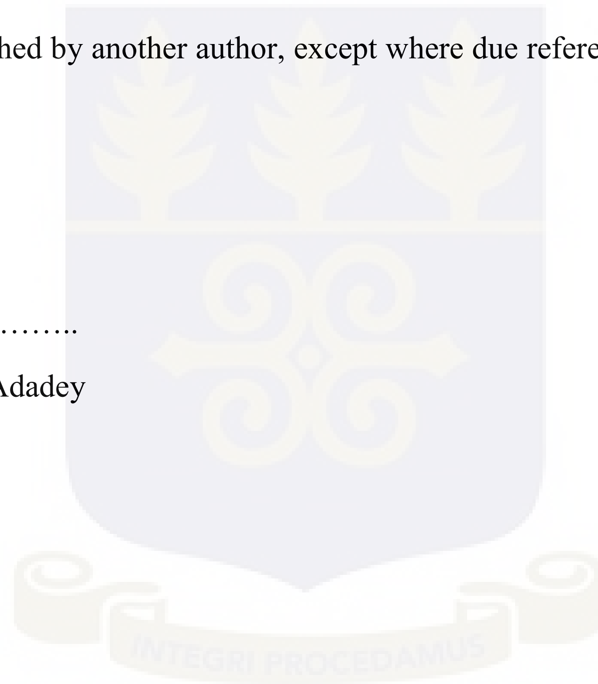
DECLARATION

I Samuel Mawuli Adadey (Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon-Accra) hereby declare that this thesis is the outcome of my own research project under the supervision of Dr. Patrick Kobina Arthur and with advice from Dr. Marian Nyako Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon-Accra). To the best of my knowledge, this thesis contains neither materials which been accepted for the award of any degree or any material previously published by another author, except where due reference is made in the text of the thesis.



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DEDICATION

I dedicate this work to all the past, present and future members of PAKARLab

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ACKNOWLEDGEMENT

I wish to express my profound gratitude to my principal supervisor Dr. Patrick Kobina Arthur for giving me the opportunity to discover the antifungal agents hidden in wood decaying fungi. The guidance, support and directions he gave were very instrumental to the success of this project not forgetting his financial support. He spent so much money, time and intellect on this work. I wish to thank him for his invaluable comments and critical evaluation, which built me up and greatly improved the quality of this work. Words cannot express my appreciation to him. This project would not have been a success without funds from DAAD, TWAS, NMIMR/Gates Foundation Fellowship and Grand Challenge Canada awarded to Dr. Patrick Kobina Arthur, from which I benefited.

I also wish to express my gratitude to Dr. Marian Nyako my co-supervisor for taking time off her busy schedule to review my work from time to time. I appreciate all the comments and contributions she made. I am most grateful to her for all the encouragement she gave me during my study.

I am highly indebted to all past and present members of PAKARLab for their support, especially Gloria Baaba Arkaifie, Precious Cramer and Arnold Abraham.

This project was kindled by the ideas and experiences obtained from the Cell Biology workshop (2011). I therefore wish to thank Dr. Gordon Awandare for giving me the opportunity to partake in the workshop. I am also grateful for all the support he gave me. I also wish to express my gratitude to Dr. Lydia Mosi for her support. I wish to express my appreciation to the teaching and non-teaching staff of the department of Biochemistry, Cell and Molecular Biology Department of university of Ghana.

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My warmest appreciation goes to Dr. Abidat Lawal and Aunty Doris for their motherly advice and support throughout this project work.

Finally, my heartfelt appreciation goes to my Mum and Dad, Vida and Paul and my sister Mawulorm for their financial, spiritual and moral support during my study and stay in the University of Ghana.

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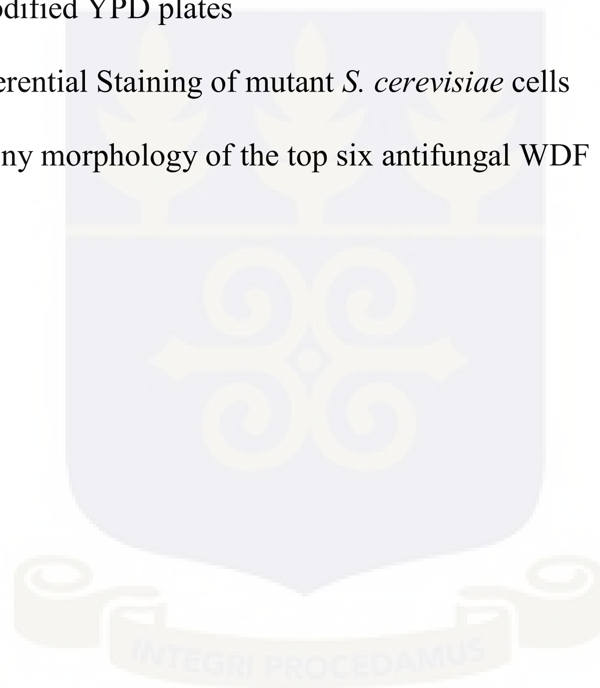

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LIST OF ABRBREVIATIONS

MEA	Malt Extract Agar
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSSA	Methicillin Susceptible *Staphylococcus aureus*
NB	Nutrient broth
PDB	Potato Dextrose Broth
PM	Plate Mycelia
SDA	Sabouraud Dextrose Agar
TLC	Thin Layer Chromatography
WDF	Wood Decaying Fungi
YPD	Yeast Peptone dextrose agar
YPMD	Yeast extract, Peptone, Malt extract and Dextrose broth

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ABSTRACT

Human systemic fungal infections have increased in the past decade due to the dramatic increase in immunocompromised patients hence the emergence of resistant fungal strains. It is therefore necessary to discover new antifungal compounds with novel mode of action. This study was aimed at isolating and characterizing antifungal agents from wood decaying fungi (WDF). The growth conditions for maximum production of bioactive agents from WDF were first tested. It was observed that mineral supplements from soil extract supported the growth and metabolite production of WDF. Media richness, culture volume and mineral supplementation were identified as the critical factors that influence the batch to batch consistency of metabolite production in WDF. Collections of 189 WDF were partially screened for their antifungal activity against *Candida albicans*. They were further screened against *Saccharomyces cerevisiae* in this project. Based on this screening, 33 of WDF were selected for mycelium isolation. The mycelia of 31 out of the 33 were successfully isolated on agar plates and stored called Plate Mycelium (PM). This served as a continuous fungal inoculum source. The 31 isolated WDF mycelia were then grown in broth and their metabolites were extracted with ethyl acetate. The antifungal activity of each WDF extracts was validated through a phenotypic assay screening using media conditions and mutant *S. cerevisaie* strains. In the chemical phenotypic array, the sensitivity of the *C. albicans* and *S. cerevisiae* cells to the WDF extracts was altered by chemical modifications in the YPD agar plates. This was used as a read out for validating the antifungal activities of the extracts. From the phenotypic screening, the best 6 WDF candidates with highly potent antifungal activity were selected. The mode of action of

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this top 6 WDF was examined by the antifungal activity pattern displayed against the mutant *S. cereviaise* cell.

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CHAPTER ONE

1.1. Introduction

Fungal infections also referred to as mycoses are caused by the growth of pathogenic fungi on the skin or within an animal including man. These fungal infections mostly results in disease conditions such as aspergillosis, candidiasis and cryptococcosis (Vyzantiadis *et al*., 2012). The mode of transmission of most mycoses is by the inhalation of fungal spores (Warris *et al*., 2001). The presence of fungal spores in the environment predisposes everyone to the disease. In addition, some strains of pathogenic fungi have gained resistance to some of the antifungal drugs especially the azoles (Nucci and Perfect, 2008). There is a crucial need therefore for the discovery of new, cost effective and efficient anti-fungal agents.

The general strategies for drug discovery can be classified into three categories. These strategies are chemically driven, biologically driven or a combination of both. The chemically driven approach involves the synthesis of compounds to target specific pathways of biological importance. However, in the biologically driven approach, crude extracts from plants and microbial cultures are screened for their biological activity. This is followed by the critical work of back tracking the active compounds from the 'hit'-extracts. In the use of this approach, a lot of experience is required to exclude both false positive and false negative results. Substantial effort is also required to get access to sufficient quantities of raw material for fractionation, structure elucidation and subsequent verification of biological activity. The complete process is highly time consuming (Koehn, 2008). The development of high through put assays now offers the opportunity to screen larger amounts of natural products within short times. In spite of all

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this, the approach to the discovery of pharmaceutical lead compounds appears to be changing drastically towards the biologically driven approach.

A lead compound has many desirable characteristics of a new drug and is used as a model for structural modification (Driggers *et al*., 2008). A Lead compound must be potent and have a specific target, but it does not need to possess the required biochemical specificity of a new drug. Compounds from natural source with novel mode of actions tend to be better leads compounds for the discovery of news drugs. They form a large number of anticancer and anti-infective agents on the market (Koehn and Carter, 2005). The top selling medicines on the Ghanaian market were derived from natural products. Aside the numerous Ghanaian herbal preparations, a typical example of top medicine from natural source is artemisinin which was isolated from sweet wormwood plant (Zhang *et al*., 2009).

Fungi are important in producing secondary metabolites (Schulz *et al*., 2002) which are useful in managing disease in humans and other animals (Zhong and Xiao, 2009). They may produce these metabolites as a response to an environmental influence in order to survive in that environment. Most microbial secondary metabolites are produced during the stationary phase of growth. Many microbial secondary metabolites are of high value for industries. This includes their use in the medical industry as antibiotics, antitumor agents and immunosuppressants. In the agrochemicals industry, they are used as pesticides, insecticides and antifeedants (Schulz *et al*., 2002).

Wood decaying fungi (WDF) are prolific producers of bioactive secondary metabolites (Kusari *et al*., 2012). Although the function of many of these metabolites remains

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unknown, few are discovered as drugs (antifungal and antibiotics), toxins or signal molecules (Zhong and Xiao, 2009). The secondary metabolites with antimicrobial activity are generally produced to enhance survival prospects of WDF in competitive niches. Some of these metabolites from WDF were clinically exploited as anti-fungal agents (Firáková *et al*., 2007).

The major challenge with the use of WDF in drug discovery is usually the screening of their secondary metabolites for bioactivity. This is partly due to the wide diversity; abundance of WDF as a source of natural products. Unlike plants, with predictable bioactivity based on their use in herbal medicine, the bioactivity of fungi cannot be predicted. This makes screening of active compounds from fungi more complicated. This implies that effective and fast screening methods are required to study and screen for antifungal agents from WDF.

Deletion mutant libraries of *Saccharomyces cerevisiae* are widely used as models for screening bioactive compounds (Hin-Yan-Tong and Boone, 2005). Often the haploid genome of this organism is used but in some few cases the diploid genome is used. Also *S. cerevisiae* has proven to be useful as a model to investigate the effects and mode of action of small molecules in eukaryotes. A typical example was the determination of the mode of action of the immunosuppressive drugs rapamycin and FK506 (Heitman *et al*., 1991). These yeast models allow for rapid and low-cost experiments and avoid the ethical questions inherent in the use of animals. In this study, the disc diffusion method combined with some yeast models was used for the screening of these anti-infectives from fungal sources.

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1.1.1. Problem statement

With the increasing number of immunocompromised cases, most clinicians have resorted to the use amphotericin B and fluconazole for the treatment of systemic fungal infection. Amphotericin B is a broad spectrum antifungal agent and it is been used with caution. This has resulted in the increased use of fluconazole leading to the emergence of resistant strains of pathogenic fungi such as *C. albicans* (Canuto and Rodero, 2002). Even after more than 100 years of research by pharmaceutical industries, most fungal infections cannot be effectively treated. This is due to the limited number of antifungal drugs on the market and high levels resistance developed by pathogenic fungi against the existing drugs (Cutler *et al*., 2007). This means that there is need for new drug entities with novel modes of action to enable therapeutic innovations. WDF could be a potential source of such drugs. It was hypothesized therefore that, wood decay fungi can produce secondary metabolites that are active against fungi.

1.1.2. Justification

Fungi have severed as a source of antibiotic, antifungal, antiviral and immunosuppressive compounds (Huang *et al*., 2001). Wood decaying fungi have a rich genome for the production of bioactive compounds with antifungal activity. Research by Wang *et al*., (2005) also proved that wood decaying fungi serve as a rich source of antifungal compounds. In their study, they isolated a new polyene from wood decaying fungi with an antifungal activity.

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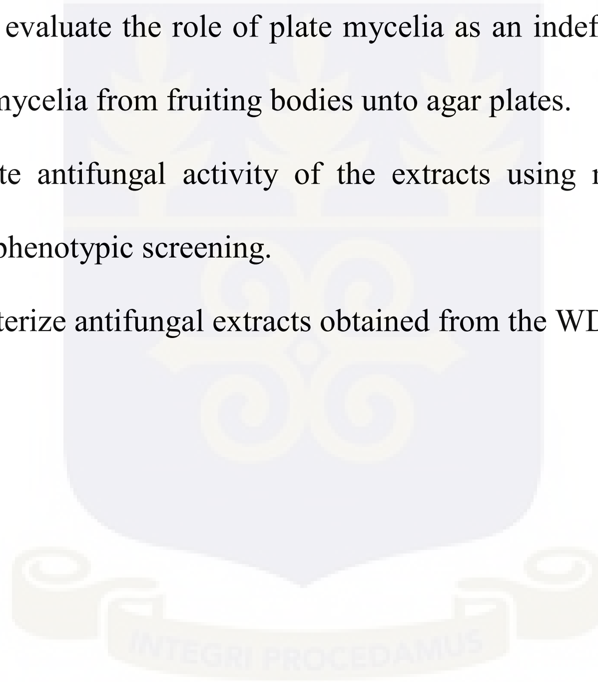
1.1.3. Objective of study

The main objective of the study was to isolate, and characterize bioactive agents, which could be active against fungi from wood decay fungi.

1.3.1. Specific objectives

1. To formulate growth medium conditions to improve production of bioactive compounds.
2. To screen for antifungal activity from WDF-extracts.
3. To obtain evaluate the role of plate mycelia as an indefinite inoculum source by isolating mycelia from fruiting bodies unto agar plates.
4. To validate antifungal activity of the extracts using mutant yeast strains and chemical phenotypic screening.
5. To characterize antifungal extracts obtained from the WDF cultures.



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CHAPTER TWO

LITERATURE REVIEW

2.1. Wood decaying fungi

An essential functional component of the forest ecosystems are wood decaying fungi which play a central role of recycling in the land ecosystem. However, their wood decaying activity results in about billion-euro losses (Lonsdale *et al*., 2008a). During the recycling process, wood decaying fungi uses enzymatic process to degrade cellulose, hemicellulose, lignin and other cell-wall components in the wood (Dashtban *et al*., 2010). These enzymes are extracellular oxidative enzymes (oxidoreductases).

2.1.1 Taxonomy of wood decaying fungi

Wood decaying fungi are classified under the kingdom fungi. Majority of these fungi belong to the phylum basidiomycota. Wood decaying fungi are usually classified into three main categories; white-rot, brown-rot and soft rot fungi based on the type of decay pattern they cause (Figure 2.1) (Schwarze, 2007). The most abundant wood decaying fungi in this category are soft rot fungi. They degrade wood extensively after their sexual stages by colonizing wood tissues. The brown rot fungi on the other hand decay wood to produce brown pigments. The white rot fungi form white “mat” on the surface of the wood they decay (Oh *et al*., 2003).

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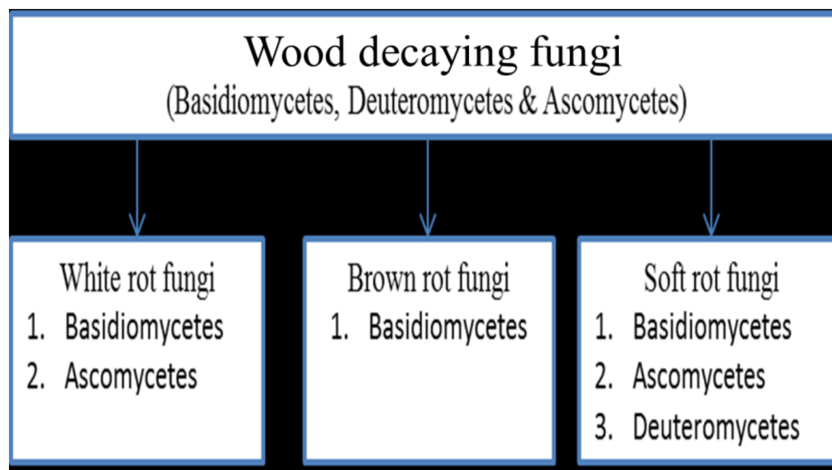

Figure 2.1: Classification of wood decaying fungi

(Adopted from Schwarze, 2007)

2.1.1.1. White rot fungi

The most common wood-rotting organisms are the white-rot basidiomycetes. They are characterized by their ability to degrade lignin, hemicelluloses, and cellulose to produce a cellulose-enriched white material. White rot-fungi are able to selectively degrade the wood either sequentially or simultaneously. The above attributes are used to characterize them. With the sequential degradation, the fungi species preferentially remove lignin from wood leaving pockets of white fungal colonized wood tissues. The degraded wood consists entirely of cellulose, which is broken down later. Simultaneous degradation involves the breakdown of lignin and cellulose simultaneously. An example of white rot fungus is *Pleurotus ostreatus* (Tuor *et al*., 1995).

2.1.1.2. Brown rot fungi

Brown rot-basidiomycetes on the other hand form the minority of wood-rotting basidiomycetes. They usually grow on soft wood. This group of fungi partially oxidizes lignin to a brown material consisting of the oxidized lignin. The oxidation of lignin precedes the degradation of the wood. This then becomes an important source of aromatic

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compounds for the forest soils. During the breakdown of the cellulose and hemicellulose, the fungi produce hydrogen peroxide which diffuses through the wood to degrade the wood (Hammel *et al*., 2002). The hydrogen peroxide degradation adds up to the brown discoloration and shrinking of the wood into cuboidal cracked pieces. Examples of brown rot fungi include *Fibroporia vaillantii* (mine fungus), and *Coniophora puteana* (cellar fungus), which may attack timber in buildings (Schwarze, 2007).

2.1.1.3. Soft rot fungi

The soft rot fungi sometimes degrade wood in a similar fashion as the brown rot fungi. However, the soft rot fungi depend on fixed nitrogen from the wood or the environment to produce their extracellular enzymes. The extracellular enzymes including cellulase are secreted to digest the wood. An example of soft rot-fungus is *Kretzschmaria deusta* (Hammel *et al*., 2002).

2.1.2. Life Cycle of wood decaying fungi

The fruiting body of wood decaying fungi produces spores, which are dispersed on maturity. The spores when landed on a wood/good substrate germinate to form hyphae, which grow into mycelia. The mycelia produce chemical signals which cause them to form fruiting bodies (Figure 2.2) (Lonsdale *et al*., 2008a).

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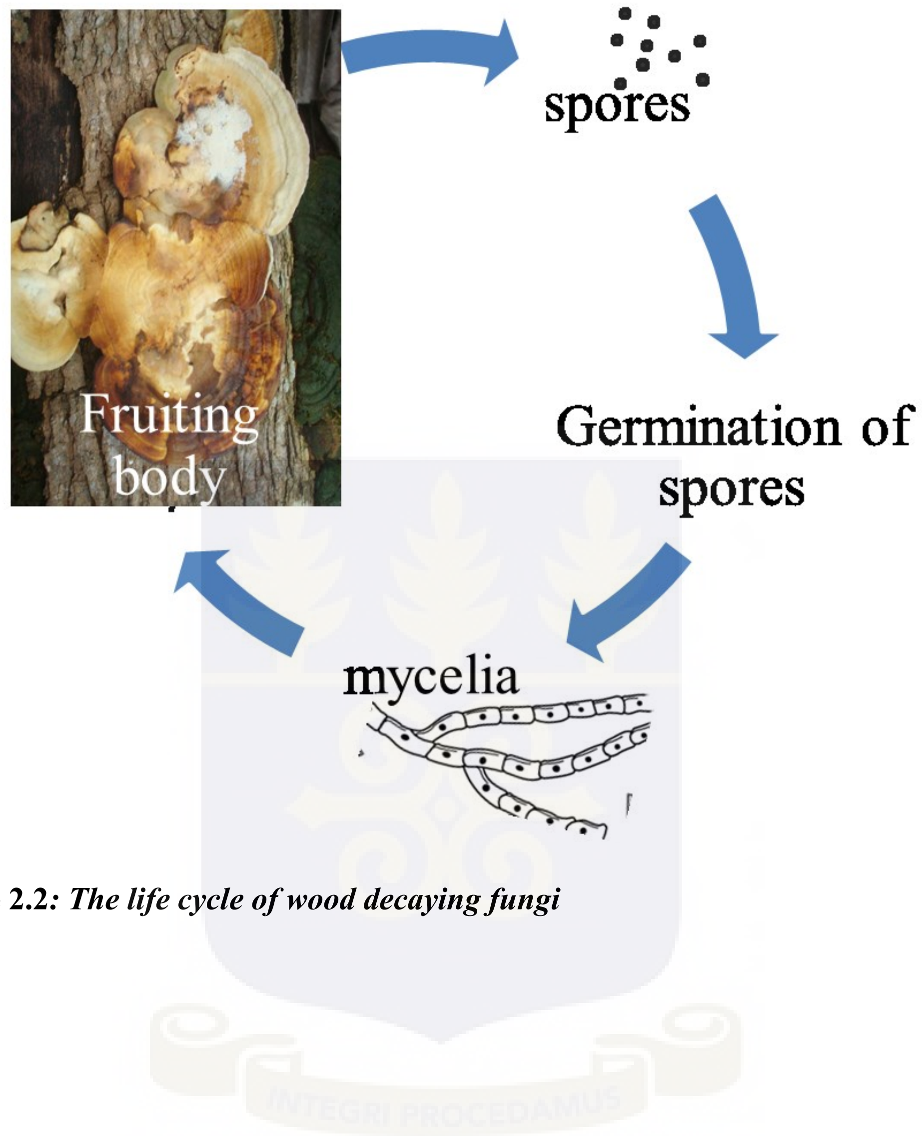

Figure 2.2*: The life cycle of wood decaying fungi*

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2.2. Bioactive compounds from microbial sources

Much of bioactive natural products from microbial source are produced by the secondary metabolism of the microorganisms (Cragg and Newman, 2013) hence are referred to as secondary metabolites. They are low-molecular-weight metabolites often with potent physiological activities. These molecules are not absolutely required for the survival of the microorganism (Brakhage and Schroeckh, 2011). Some of them serve as antibiotic which enhance the survival of the microorganisms. Penicillin and cephalosporin are well known antibiotics produced by fungi.

2.2.1. Bioactive compounds from fungi

Fungi are well noted for the production of diverse secondary metabolites through unique and unusual metabolic pathways. The products of these pathway are of industrial and pharmaceutical importance. In the pharmaceutical industries they are developed into drugs such as antibiotics, antifungals, anticancer drugs and immunosuppressant (Zjawiony, 2004).

In 1922, Harold Raistrick initiated the study of fungal secondary metabolites. He successfully characterized more than 200 metabolites from mould (Raistrick and Stickings, 1956). However, this study gained grounds in 1929, when Alexander Fleming discovered the antibacterial action of a 'mold juice' from *Penicillium notatum*. He named the biological active juice 'penicillin' (Rodriguez-Soiz *et al.*, 2005). The clinical efficacy of penicillin was established by the work done by Howard Florey and Ernst Chain a decade after the discovery of penicillin (Chain *et al*., 2005). To date, many other fungal molecules with cytotoxic, mutagenic, carcinogenic, teratogenic,

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immunosuppressive, enzyme inhibitory and other biological effects also have been found. The classes of fungal secondary metabolites are summarized below.

2.2.1.1. Polyketides

The most abundant and genetically well characterized fungal secondary metabolites are polyketides (Khaldi *et al*., 2010). The multi-domain protein type I polyketide synthases (related to eukaryotic fatty-acid synthases) catalyzes the synthesis of polyketides in fungi. The reaction involves the conjugation of acetyl coenzyme A and malonyl Coenzyme A to form carbon chain of varying lengths. Fungal polyketide synthases have the following domains, acyltransferase (AT) and acyl carrier (ACP) domains for polyketide synthesis and ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) domains for ketone reduction in fatty acids. However, reduction of the R-carbon in fatty acids is an optional event in polyketide synthesis. In fungi, the number of reduction, dehydration and cyclization reactions varies, resulting in a wide diversity of polyketides from fungi (Donadio and Katz, 1992). In *Aspergillus flavus* and *Aspergillus parasiticus* the type I polyketide gene is responsible for the synthesis of aflatoxin. An analog of this enzyme was found to be responsible for the production of antibiotic erythromycin A. in bacteria (Watanabe and Townsend, 2002).

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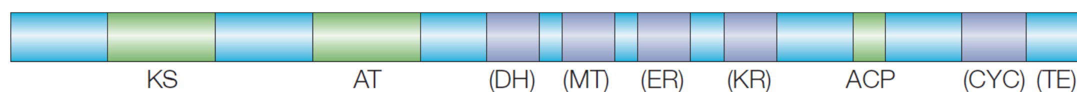


Figure 2. 3: Fungal polyketide synthase (PKS) domain structure.
Fungal polyketide synthase has the following domains, an acyl carrier (ACP) domain, acyltransferase (AT) domain, ketoacyl CoA synthase (KS) domain, ketoreductase (KR) domain, dehydratase (DH) domain, enoyl reductase (ER) domain is present, an unsaturated, methyltransferase (MT) domain and thioesterase (TE) domain. The minimal structure for the enzyme is KS–AT–ACP; the domains in the bracket are optional (Keller *et al*., 2005).

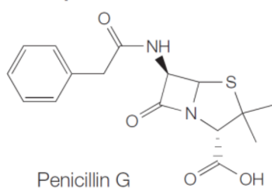
2.2.1.2. Terpenes

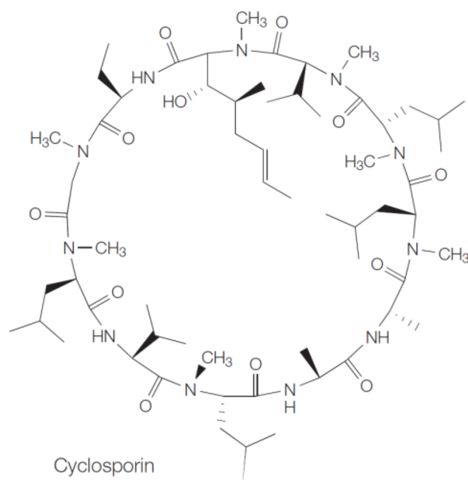
Plants are well known to produce terpenes, however, they are produced in fungi as well. Terpenes in fungi are synthesized by the conjugation of several isoprene units, which are modified in different ways. Terpenes may be cyclic or linear, saturated or unsaturated based on the modification offered by the fungal system producing them. They are classified as monoterpenes when formed from geranyl pyrophosphate, sesquiterpenes when synthesized from faresnylpyrophosphate and diterpenes and carotenoids when synthesized from geranylgeranyl pyrophosphate (Keller *et al*., 2005).

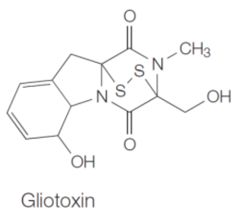
2.2.1.3. Indole alkaloids

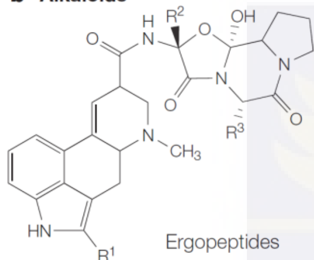
Amino acids such as tryptophan are precursors of indole alkaloids. They are usually conjugated to dimethylallyl pyrophosphate. Although the pathway for the synthesis of these compounds is not well elucidated, its committed step was found to be catalyzed by dimethylallyl tryptophan synthetase (DMATS) (Metzger *et al*., 2009).

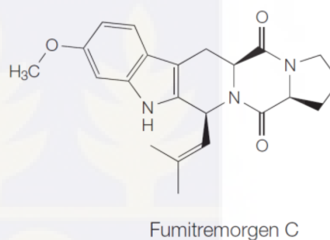
The committed step is followed by methylation of dimethylallyl tryptophan and a series of oxidation steps to produce the alkaloid. Typical examples of indole alkaloids are ergotamine, fumigaclavines and fumitremorgens (Metzger *et al*., 2009).

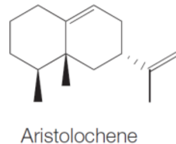


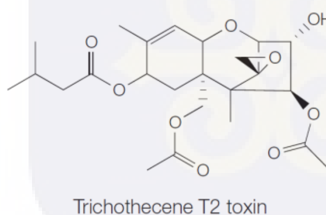


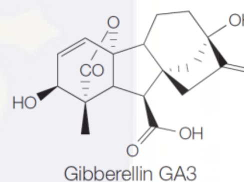


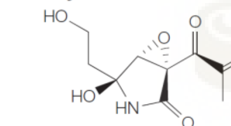


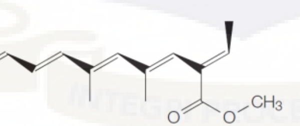


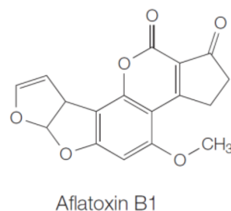












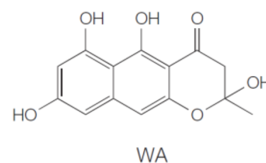


Figure 2.4: Major group of fungal secondary metabolites

(Keller et al., 2005)

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2.3. Fungal Metabolites as used as Drugs

2.3.1. Antibacterial Metabolites

Fungi are extremely useful organisms in the pharmaceutical industry. By using established metabolic pathways, some fungi produced unique and complex biomolecules with bacteriostatic or bactericidal activity (Wawrzyn *et al*., 2012). The main example is penicillin from the fungus *Penicillium chrysogenum* which was discovered in 1941 by Alexander Fleming as useful in treating infections caused by bacteria (Rodriguez-Soiz *et al*., 2005). Cephalosporins, beta lactam ring containing compound were produced by *Cephalosporium sp.*, hence the name. Just as penicillin, cephalosporins inhibit cell wall production in bacteria the basis for its use as antibiotics (Elander, 2003).

2.3.2. Immunosuppressing Metabolites

The primary metabolite for *Trichoderma polysporum* and *Cylindrocarpon lucidum* is cyclosporine A, which was proven to be a potent immunosuppressant. It is being widely used in bone marrow and organ transplant in humans. To suppress the immune system, it inhibits the activity of lymphocytes. Cyclosporin A binds tightly to a highly conserved protein (cyclophilin) and forms a complex. Cyclosporine A-cyclophilin complex binds calcineurin, a transcription factor responsible for T-cells activation. This binding prevents the proliferation of the T-cells subsequently preventing tissue rejection. Another immunosuppressant drugs from fungi are gliotoxins from *Aspergillus fumigatus*. Its effect on the immune system is by the suppression of the activity of macrophages. However, its mode of action is not well elucidated (Iida *et al*., 1999).

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2.3.3. Antifungal Metabolites

The application of chemically synthesized antifungal drugs is being replaced with naturally derived drugs (Didomenico, 1999). Most chemically synthesized drugs have biodegradation issues and usually persist in the environment or living organisms. However, the naturally derived drugs are easily degraded and this subsequently reduces the cumulative effect of the drug (Didomenico, 1999). In view of this, arundifungin, a potent antifungal drug was purified from fungal extracts obtained from a liquid culture of *Arthrinium arundinis*. Arundifungin had the potential of altering the morphology of *Aspergillus fumigatus*. It also inhibited the growth of *Candida* and *Aspergillus* by inhibiting glucan synthesis (Cabello *et al*., 2001).

2.4. Classification of antifungal drugs

Based on the mode of action of antifungal drugs, they are classified into five major categories, polyenes, azoles, allylamines and inhibitors of glucan synthesis.

Polyenes interact with ergosterol in the fungal cell membrane of fungi to be transported across the membrane. In the cell, they interact with the vacuoles and disrupt them leading to their leakage. The fungal cell eventually dies of vacuole damage (Young *et al*., 2003). An example of polyene is amphotericin B (Kang *et al*., 2013).

Azoles on the other hand, inhibit the synthesis of ergosterol by interacting with lanosterol-14α-demethylase. This enzyme catalyzes the committed step of ergosterol biosynthesis and is exclusive to fungi. Since ergosterol is a major component of the fungal membrane, inhibition of its synthesis compromises the integrity and fluidity of the

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membrane leading to the death of fungal cells (Song *et al*., 2004). Fluconazole and itraconazole are classified as azoles.

The third class of antifungal compounds inhibits ergosterol biosynthesis however; they are chemically and functionally distinct from the azoles (Ghannoum and Rice, 1999). They are known as allylamines with terbinafine as an example. The allylamines inhibit the growth of pathogenic fungi by inhibiting the oxidation of squalene by inhibiting squalene epoxidase in the ergosterol biosynthetic pathway (Onyewu *et al*., 2003).

The fourth class of antifungal compounds inhibit glucan synthesis by inhibiting β-(1,3)-glucan synthetase. Inhibition of glucan synthesis results in thickening of cell wall and the buds eventually fail to detach from the parent cells (Hochstenbach *et al*., 1998). The compounds in this group can be further classified into three groups; aculeacins, echinocandins, and papulacandins. The echinocandins are the only group that was extensively studied and pursued in clinical trials (Randhawa and Sharma, 2010; Sucher *et al*., 2009).

The last group of antifungal agents is nucleic acid inhibitors with 5-fluorocytosine (5FC), a fluorinated pyrimidine as the main example (Carrillo-Munoz et al., 2006). They are active against many types of yeast, including *Candida spp* and *Cryptococcus neoformans*. 5FC is converted in the fungi to 5-fluorouracil (5FU) which is further converted by UMP phosphorylase 5-fluorouradylic acid (FUMP). FUMP is phosphorylated and incorporated into RNA. This eventually interrupts protein synthesis in the fungi. In DNA synthesis, 5 5FU is converted to 5-fluorodeoxyuridine by thymidylate synthase and therefore interfering pyrimidine biosynthesis in fungi (Ghannoum and Rice, 1999).

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2.5. Impact of growth medium on fungal secondary metabolism

Fungi are well known to produce a wide variety of natural products that are of medicinal, agricultural and industrial importance. The growth and production of secondary metabolites by fungi can be greatly influenced by environmental, nutritional and genetic factors

2.5.1. Impact of pH on fungal secondary metabolism

Although most researchers have different views on the optimal pH for fungal growth, most fungi were found to grow better within the pH of 5 to 7 (Hung and Trappe, 1983). Some fungi especially aflatoxin producing fungi exhibit chemical and morphological change in response to pH (Cotty and Jaime-Garcia, 2007). In *Fusarium moniliforme,* pH of 5.5 increased biomass and pigment production however, pH of 7 reduced biomass and pigment production (Pradeep and Pradeep, 2013).

2.5.2. Impact of temperature on fungal secondary metabolism

In a study, increasing temperature reduced biomass and pigment production in *Fusarium moniliforme.* However, temperature of 28ºC supported maximum production of pigment and biomass in *F. moniliforme* (Pradeep and Pradeep, 2013).

2.5.3. Impact of Carbon and Nitrogen source on fungal secondary metabolism

The main sources of sugars for fungi are glucose, fructose, sucrose, and sorbitol. These sources support high fungal growth, sporulation, and toxin production. However, the complex sugars do not affect secondary metabolism (Lewis, 1991). The nitrogen source has varying effects on fungal secondary metabolism. Depending on the nitrogen source, fungal secondary metabolism can be repressed or tigered. In *A. parasiticus* nitrates as the

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only source of nitrogen reduces the production of aflatoxin and its intermediates while it enhances sterigmatocystin production in *A. nidulans.* In *Aspergillus spp*, the nitrogen source influences their developmental structures (Cotty and Jaime-Garcia, 2007).

2.5.4. Impact of mineral supplements on fungal secondary metabolism

Mineral elements are important for pigment production and secondary metabolism in fungi. Metal ions such as K^{+}, Mg^{2+} and Zn^{2+} are discovered to play important role in biomass and pigment production. They also influence the production of some secondary metabolites in *Fusarium moniliforme* (Pradeep and Pradeep, 2013).

2.6. Screening for bioactive compounds

One major challenge to natural product drug discovery is screening for bioactive compounds from natural sources (Grabley and Thiericke, 1999). To discover a hit compound, with for example antifungal activity, many compounds from natural libraries need to be screened. The traditional screening method usually used by most researchers is the disc or agar diffusion methods. These methods are easy to operate but they have the following drawbacks: The diffusion of the bioactive compounds in the agar is greatly influenced by the size of the molecules. Compounds with smaller size diffuse faster than the larger ones giving a false positive result (Gassner *et al*., 2007). This method does not allow for mass screen of compounds and it is also time consuming. This method however, is modified into a high throughput assay. In this assay, the compounds are prepared into 96 or 386 well plates and pin transferred unto ager plates to screen for active compounds by measuring the halos produced (Gassner *et al*., 2007).

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2.6.1 Use of mutant *S. cerevisiae* strains in screening for active compounds

For the study of basic eukaryotic biology, the budding yeast model is extensively used. The budding yeast model is also used to screen for bioactive small molecules. The ability of *S. cerevisiae* to exist as haploid and diploid makes it possible for them to be used in highthroughput robotic screening assays. The sensitivity of the deletion mutant strains enables the mode of action of the bioactive compounds to be predicted to some extent. The deletion mutants were used to screen marine sponge crude extracts which lead to the discovery of crambescidin 800 as a potent antifungal agent (Gassner *et al*., 2007). In another study, the deletion mutant strains were used to predict the mode of action of some chalcones. From the study it was shown that the chalcones may react with some proteins involved in cell separation (Lahtchev *et al*., 2008). Using yeast-based functional genomics technologies Bendaha *et al*., (2011) demonstrated that some new azoles operate via a mechanism of action distinct from existing azoles; thus inhibiting DNA repair in pathogenic fungi.

2.6.2. Chemical phenotypic modifiers as models for screening active compounds

The current hindrance to the discovery of new antifungal agents is the development of an *in vivo* model to screen natural and chemical libraries (Breger *et al*., 2007). Phenotypic modifiers therefore provide the opportunity to mimic physiological conditions in the laboratory. The phenotypic modifiers have the ability to influence the sensitivity of microorganisms to standard drugs and lead compounds (Golebiowski *et al*., 2003). This can be exploited to create chemical models, which will mimic physiological conditions for drug screening.

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Morphology switching is essential for the pathogenesis of *C.albicans*. Farnesol however prevents the germination of *C. albicans* into mycelia (Hornby *et al*., 2001) and serving as a tool for inhibiting the morphology switch. This can be used as a model to screen for compounds that target the non-filamentous state of the organism. Another molecule that interferes with morphology switching in yeast is N-acetylglucosamine (Huang *et al*., 2010). It acts in a like manner as farnesol and can also be used in a phenotypic screening.

In addition, some protein inhibitor such as cycloheximide and paramomycin influences the sensitivity of yeast to antifungal compounds (Vermitsky *et al*., 2006). In this study cycloheximide induced resistance to fluconazole in *S. cerevisiae* and this was used as a model for screening new antifungal agents.

2.7. Characterization of bioactive compounds

In natural product research, there is a need to characterize the bioactive natural product. Most researches do structural elucidation for characterization. Some also determine the physical and chemical properties of the active compounds. However, characterization goes beyond the scopes of the structure, physical and chemical properties. The bioactivity of the bioactive compounds in the presence of other molecules is another level of characterization. This is referred to as the phenotypic screening when the phenotypes of the test organisms are influenced by this compound-compound interaction.

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CHAPTER THREE

MATERIALS AND METHODS

3.1. Chemicals and Reagents

Solanum tuberosum (Irish potatoes) were obtained from Shoprite, Accra Mall-Ghana, Dextrose was purchased from "Pills and Tabs"-Legon, Ghana. Farnesol, ethyl alcohol, cycloserine, amphotericin B, fluconazole and cycloheximide were purchased from Sigma Aldrich – Gemany. Yeast extract, peptone, nutrient broth, Sabouraud dextrose agar, agar, malt Extract Agar and malt extract were purchased from Becton, Dickinson and company (BD) – USA.

The solvent, ethyl acetate, methanol, acetonitrile, petroleum ether, and ethanol used were purchased from Lab Aid, Ghana. All solvents used for the extraction and chromatography were of technical quality.

3.2. Strains of Microorganisms

Two different strains of yeast cells, *C. albicans* and *S. cerevisiae* and five mutants of *S. cerevisiae* were used for the antifungal assays. *Methicillin Susceptible Staphylococcus aureus (MSSA GGP 200), Methicillin Resistant Staphylococcus aureus (MRSA GGP E120), Mycobacterium smegmatis (M smeg ETHZ_2), Erythromycin Resistant Mycobacterium smegmatis (Ery M. smeg)* were used for the antibacterial and anti-mycobacterial assays.

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Table 1.1: Characteristics of the mutant *S. cerevisiae* cells

Mutant	Zygocity	Deficient protein	Description
Δerg2(hom)	Homozygote	Squalene epoxidase	catalyzes the epoxidation of squalene to 2,3-oxidosqualene; plays an essential role in the ergosterol-biosynthesis pathway
Δerg2(het)	Heterozygote	Squalene epoxidase	catalyzes the epoxidation of squalene to 2,3-oxidosqualene; plays an essential role in the ergosterol-biosynthesis pathway
Δeft1(het)	Heterozygote	Elongation factor 1	catalyzes ribosomal translocation during protein synthesis
Δeft2(het)	Heterozygote	Elongation factor 2	catalyzes ribosomal translocation during protein synthesis
Δyef3(het)	Heterozygote	Gamma subunit of translational elongation factor eEF1B	stimulates the binding of aminoacyl-tRNA (AA-tRNA) to ribosomes by releasing eEF1A (Tef1p/Tef2p) from the ribosomal complex

3.2. Collection of WDF Strains

WDFs were collected by former students of the laboratory. The criterion for identifying the WDF was that it must be attached to a decaying wood. They were collected after a clear photograph was taken of the WDF still attached to the decaying wood. The WDF were collected from the Ashanti, Northern, Central, Volta and Greater Accra Regions of Ghana. They were packed into tightly sealed containers and stored in cool dry place at room temperature until they were ready for culturing. The WDF were grouped based on their physical appearance and given codes like A1-A9, B1-B9 and C1-C9. From all over the major cities in Ghana, 189 WDF samples were collected.

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3.3. Preparation of media for growth and metabolite production

3.3.1. Potato dextrose broth

Irish Potatoes were purchased from the Accra mall and boiled. The potato outer skins were piled off. Potato dextrose broth (PDB) was prepared using 200g of blended potatoes and 20g of dextrose for each liter of broth. The weighed potato was boiled and blended to prepare slurry. The slurry with dextrose was autoclaved at 121°C for 60 minutes and allowed to cool to room temperature before inoculation.

3.3.2. Yeast extract-Peptone-Malt extract-Dextrose broth (YPMD)

Yeast extract (5g/L), peptone (5g/L), malt extract (5g/L) all obtained from Becton, Dickinson and Company (BD) – USA and dextrose (30g/L) from pills and tabs were weighed and mixed well. The suspension was dispensed into high density polyethylene bottles and autoclaved at 121°C for 60 minutes then allowed to cool to room temperature before inoculation.

3.3.3. Agar plates

Malt extract agar (MEA) and Sabouraud dextrose agar (SDA), were prepared by following the manufacturer's instruction.

3.4. Inoculation of liquid cultures

The fruiting bodies of the WDF were cut into pieces and surface-sterilized with isopropanol. The cutting and sterilization were done under aseptic conditions. The fungal pieces were added to the PBD/YPMD in their respective containers in a Lamina-flow biosafety cabinet. Mycelia from plate cultures or broth cultures were harvested into sterile containers and stored in the refrigerator. The harvested mycelia were used to

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inoculate PDB/YPMD in their respective containers. The fungal cultures were incubated at room temperature with daily swirling to allow aeration. Since ethyl acetate disintegrates the mycelia and disrupts the cell membrane leading to cell death, it was used to terminate the cultures and also extract the metabolites produced.

3.5. Inoculation of agar plates with wood decaying fungi

Fruiting bodies of the WDF were cut into pieces and surface sterilized with isopropanol under aseptic condition. The pieces were used to inoculate agar plates at different spots. Mycelia harvested from broth or preexisting agar plates were also used to inoculate the freshly prepared agar plates at different spots. The agar plates were incubated at room temperature and observed daily for 4 to 7 days.

3.6. Extraction of metabolites from liquid cultures

After growth of the WDF, the liquid cultures were inspected for contamination by microorganisms and equal volumes of ethyl acetate were added to the liquid cultures to stop their growth at the due dates. The solvent and the broth were in the ratio 1:1 for maximum extraction of the fungal metabolites as predetermined in the laboratory. The solvent was thoroughly mixed with the liquid cultures and allowed to stand for 24 hours at room temperature. The ethyl acetate phase was pumped off into a flask and concentrated using the rotatory evapourator. The concentrate was pipetted into vials and allowed to air dry. The dry weights of the extracts were taken and the crude extracts were dissolved in absolute methanol. The crude extracts in the vials were stored at 8°C in the refrigerator.

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3.7. Preparation of wood decaying fungi extract discs

Whatman filter papers were cut into 6 mm discs. The discs were sterilized by autoclaving them at 121°C for 15 min. The discs were placed in sterile 96 well plates and labeled. Exactly 10, 20 or 30 µL of the crude extracts were pipetted unto separate discs in succession and allowed to dry under aseptic conditions. Care was taken to ensure maximum absorption of the extract. The dried discs were stored at room temperature until they are ready to be used.

3.8. Thin layer chromatography (TLC) for the WDF extracts

Thin layer chromatography was performed on 60 F_{254} aluminum backed silica gel plates. Five (5µL) of crude fungal extracts were spotted unto the TLC plates 1 mm from the bottom. The spots were dried and developed in a pre-saturated glass TLC tank containing the developing solvent ethyl acetate, acetonitrile and petroleum ether 7:2:1 (Aboagye, 2011). The plates were allowed to air dry and viewed under short wave (254 nm) and long wave (365 nm) UV light. The plates were later sprayed with anisaldehyde reagent and viewed under long wave UV light.

3.9. Preparation of inoculum of fungal test organisms

The isolated fungal strains stored on agar slants were allowed to thaw to room temperature. Under sterile conditions, a loop was used to transfer fungal colonies into 50 mL of autoclaved sterile nutrient broth. The broth cultures were incubated at room temperature for 16-18 hours and the optical density (OD) determined. The starter culture was then used to inoculate another nutrient broth to obtain an OD of 0.1, which was incubated for 16-18 hours. The OD of the final culture was adjusted to OD of 0.7, which was then used for the sensitivity tests.

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3.10. Disc diffusion assay

The WDF extract discs were placed onto the surface of the inoculated agar plates and slightly pressed to ensure complete contact with the agar surface. Standard antifungal discs of fluconazole and amphotericin B were used as the positive control. Each plate was examined after 24 hours and the zones of inhibition of the various extracts and standard antifungal drugs were measured.

3.11. Exploration of the effect of mineral supplementation on metabolite production

Two selected WDFs (B7_new_BM and B7_old_PM) were cultured in potato dextrose broth (PDB), PDB modified with seawater and PDB modified with soil extracts as sources of mineral supplementation. Each mineral source was applied in two concentrations, 1x means low concentration and 5x high concentration (more details in sections 3.11.1 - 3.11.3). The WDF-cultures were incubated at room temperature for 6 weeks. The metabolites produced were extracted with equal volume of ethyl acetate (compared to the culture volume) and dried.

3.11.1. Preparation of soil extracted mineral supplement

Fresh garden soil, which was loamy-clay soil, was mixed with distilled water in the ratio 1:2 v/v. Thus to every 1ml of well parked soil, 2mls of water was added. The mixture was autoclaved and filtered through cotton wool twice. The filtrate was allowed to stand for 48 hours and re-autoclaved and filtered again to prepare the soil extract. For 1x and 5x concentrations, 12 ml and 60 ml of the soil extract were used in 200 ml PDB.

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3.11.2. Preparation of mineral salt supplement

The salts listed in Table 3.2 were weighed and dissolved in distilled water to obtain the concentrations indicated. The solution was used to prepare the 1 liter PDB, which gave the 1x concentration. The 5x concentration was prepared with 5 times the concentrations indicated in Table 3.1.

Table 3. 2: Concentration of mineral salts used in preparing 1x mineral salt supplement

Mineral salt	Concentration
Ferrous sulphate	0.04 g/l
Calcium chloride	0.01 g/l
Magnesium sulphate	0.08 g/l
Sodium Chloride	5.00 g/l
Dipotasium ortho phosphate	5.00 g/l

3.11.3. Preparation of sea water mineral supplement

The 1x concentration of PDB broth was prepared using 200 ml of seawater to 800 ml of distilled water per a liter of PDB broth. For the 5x concentration only seawater was used to prepare the PDB.

3.12. The batch-to-batch consistency assay of wood decaying fungi metabolite production

PDB, YPMD and PDB modified with 2x mineral salt liquid cultures were inoculated with the WDF. Five replicates of two volumes (100 ml and 400 ml) were prepared for each media type. The cultures were allowed to grow for 9 weeks. The metabolites were extracted with an equal volume of ethyl acetate for maximum extraction. The extracts

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were evaporated to dryness using the rotary evaporator at 40 ºC, the weight of dried extracts were obtained and reconstituted in 1 ml of absolute methanol.

3.13. Antifungal assay

The antifungal activity was determined by measuring the susceptibility of *C. albicans* and *S. cerevisiae* to antifungal drugs and the WDF extracts. The thawed stock of inoculum on slants were used to inoculate 50ml of autoclaved nutrient broth and allowed to grow for 12 hours. The 16-18 hour cultures were used to inoculate new 50ml nutrient broth to obtain an OD of 0.01 and also allowed to grow for 12 hours. The OD of the cells was adjusted to 0.7 then used to spread on yeast extract, peptone and dextrose agar (YPD) plates. Using the disc diffusion method, the sensitivity of the test organisms on the plates was determined. The zones of inhibition were measured as the diameter of the halo around each disc including the diameter of the disc. Fluconazole and amphotericin B were used as the positive controls and cycloserine was used as the negative control.

3.14. Phenotypic screening of plate mycelia extracts

The phenotypic screening was done with chemical phenotypic modifiers and the mutant *S.cerevisiae* cells. For the chemical modified phenotypic screening, YPD agar plates were modified with farnesol (0.056 mg/ml), phenyl ethyl alcohol (0.15 mg/ml), cycloserine (0.103 mg/ml), N-acetyl glucosamine (2.2 mg/ml), cycloheximide (0.25mg/ml) and 3-O-methylglucose (1.9 mg/ml). The phenotypic modifiers were selected based on their ability to induce or resist morphology switch in the yeast cells. The modified plates were used in a sensitivity test by employing the disc diffusion method. The sensitivity of the test organisms (*C. albicans* and *S. cerevisiae*) to the extracts was determined by measuring the zones of inhibition of the extracts and control drugs. The second

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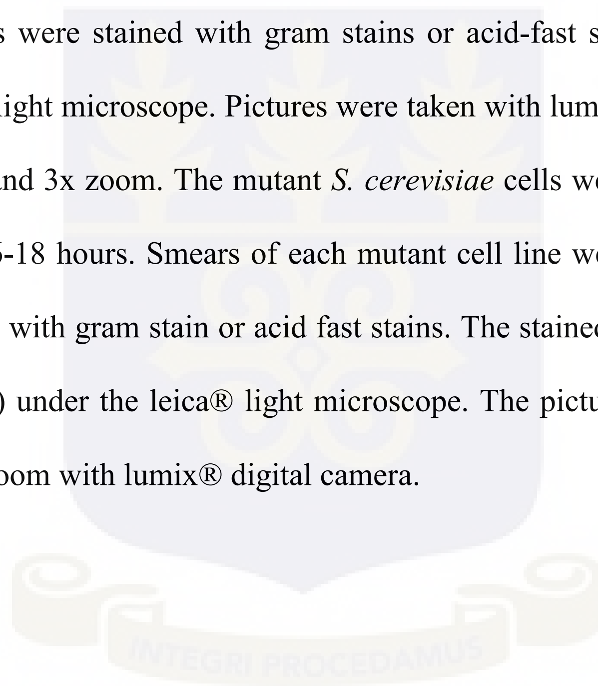
phenotypic screening was done with the mutant *S. cerevisiae* cells on YPD agar plates. The sensitivity of the mutant *S. cerevisiae* cells to the extracts and control drugs was also determined by measuring the zones of inhibition.

3.15. Morphological characterization of *C. albicans* and *S. cerevisiae*

C. albicans and *S. cerevisiae* cells were cultured on YPD agar plates with the modification stated in the phenotypic screening above. The cells were allowed to grow for 16-18 hours. A smear of cells on microscopic slide was prepared from each modified plate. The smears were stained with gram stains or acid-fast stain and viewed at 100x under the leica® light microscope. Pictures were taken with lumix® digital camera at full view (1x zoom) and 3x zoom. The mutant *S. cerevisiae* cells were also cultured on YPD agar plates for 16-18 hours. Smears of each mutant cell line were made on microscopic slides and stained with gram stain or acid fast stains. The stained cells were viewed at oil immersion (100x) under the leica® light microscope. The pictures were acquired at full view and 100% zoom with lumix® digital camera.



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CHAPTER FOUR

RESULTS

4.1. The effect of mineral supplementation on metabolite production

To explore if mineral supplementation could induce the production of antifungal agents, two WDF (B7_new_BM and B7_old_PM), which were noted not to produce antifungal activity, were used in this study. The culture medium was modified with mineral salt, seawater and soil extract. From the results (Figure 4.1), it was observed that the WDF extracts from the modified PDB and unmodified PDB cultures had no activity against *C. albicans* and *S. cerevisiae*.

From figure 4.1, it was observed that PDB modified with soil extract improved the production of bioactive compounds compared with the unmodified PDB. However the yields of crude extracts from the soil extract cultures were lower than the unmodified PDB (Figure 4.2). This was evident when B7_new_BM extracts from the soil extract modified PDB had high and consistent activity against *M. smegmatis* and MSSA compared to the unmodified PDB. Seawater extract treatment at low concentration (1x) also improved the activity of the extracts of B7_new_BM against *M. smegmatis* and MSSA. However, the *M. smegmatis* activity was absent in the 5x seawater concentration (Figure 4.1). From this it was apparent that, PDB modified with seawater extracts had higher but inconsistent bioactivity against the test organism.

The B7_old_PM fungal extracts made it possible for the differences between the 1x and 5x concentrations, which were not obvious in the B7_new_BM extracts to be examined. The 5x seawater extracts of B7_old _PM produced activity in only one of the duplicates,

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which proved that the 1x concentration gave a consistent result compared to the 5x concentrations. Comparing the effect of mineral supplementation on the activity of B7_old_PM, seawater and soil extract supplements improved the activity of the extracts against *M. smegmatis* and MSSA compared to the unmodified media, which had no activity. However, PDB modified with soil extract produced a consisted activity against MSSA compared to seawater modified PDB extract which did not produce consistent activity against *M. smegmatis* and MSSA (Figure 4.1). The yield of the B7_new_BM extracts from the soil extract modified PDB was lower than the unmodified PDB (Figure 4.2). The weight of the unmodified PDB cultures did not reflect in their activity, which implies the media modification increased the production of some specific molecules, which may be responsible for the recorded bioactivity.

The effect of mineral supplementation on metabolite production was examined on TLC plates. The plates were spotted with 5 µL of each WDF extract. On the chromatograms, some prominent bands were observed in the extracts from soil extract, mineral salt and sea water supplementations which were faint or absent in the unmodified PDB cultures (Figures 4.3 and 4.4).

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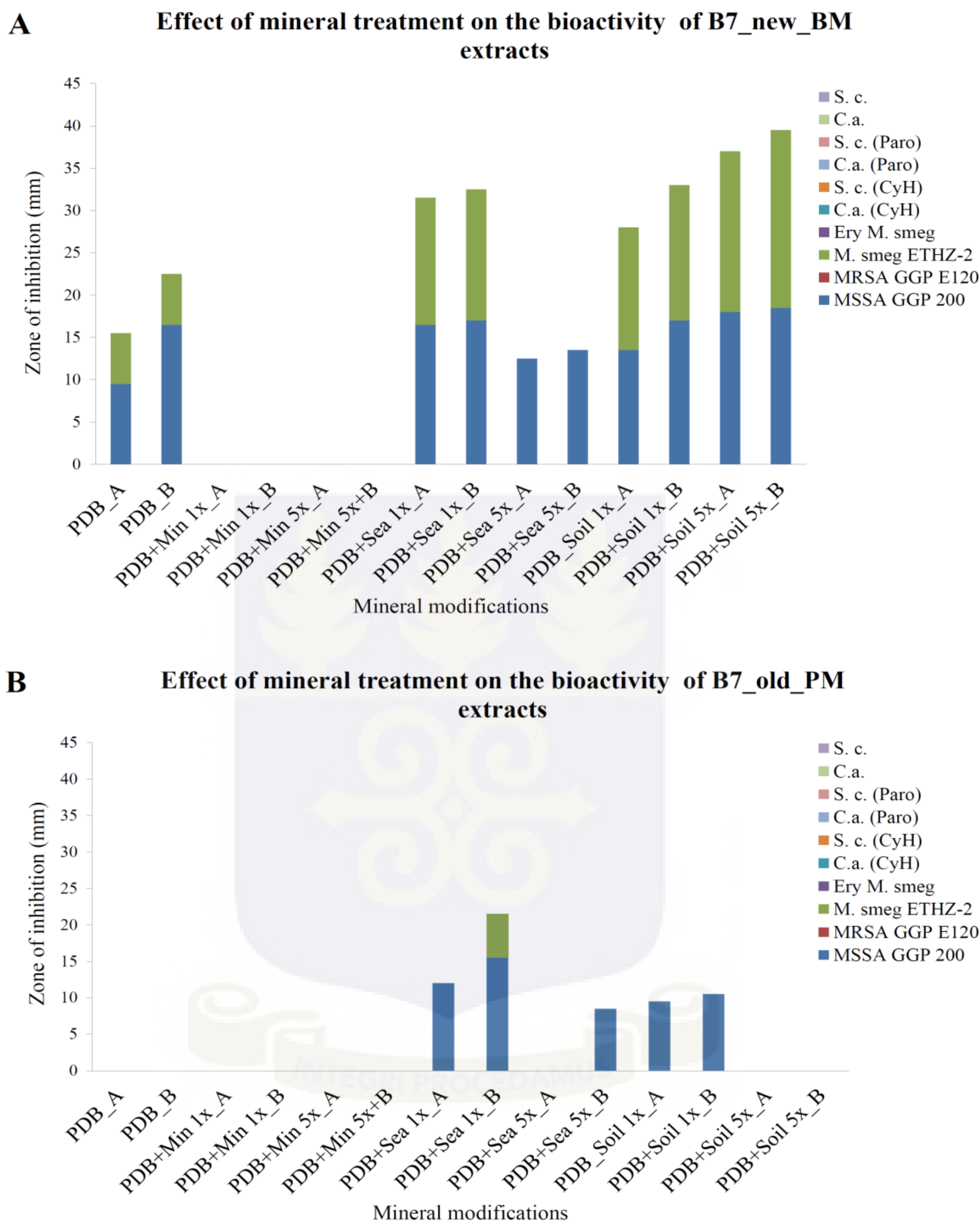

Figure 4.1: Disc Diffusion based analysis of bioactive compounds produced by WDF cultured in PDB with mineral supplementation. *The Extract from these cultures were tested against Methicillin Susceptible Staphylococcus aureus (MSSA GGP 200), Methicillin Resistant Staphylococcus aureus (MRSA GGP E120), Mycobacterium smegmatis (M smeg ETHZ_2), Erythromycin Resistant Mycobacterium smegmatis (Ery M. smeg) C. albicans with cycloheximide treatment (C.a. CyH), S. cerevisiae with cycloheximide treatment (S.c. CyH), C. albicans and S. cerevisiae.* ***(A)*** *B7_new_BM and* ***(B)*** *B7_old_PM were cultured in 500 ml PDB modified with defined mineral salts (Min), sea water (Sea) or soil extract (Soil) at 1x and 5x concentrations in a 1 litre plastic bottle.*

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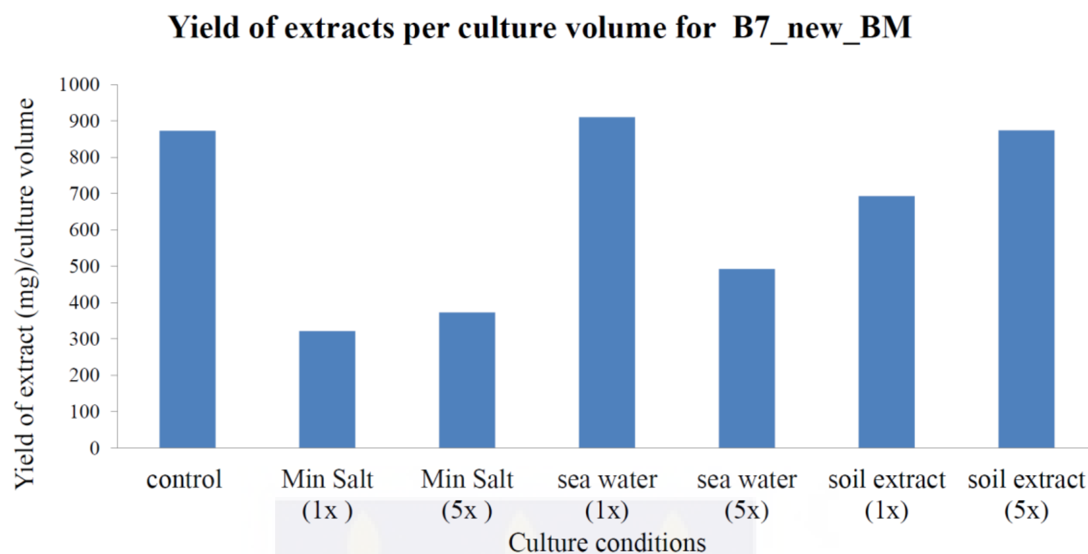
A



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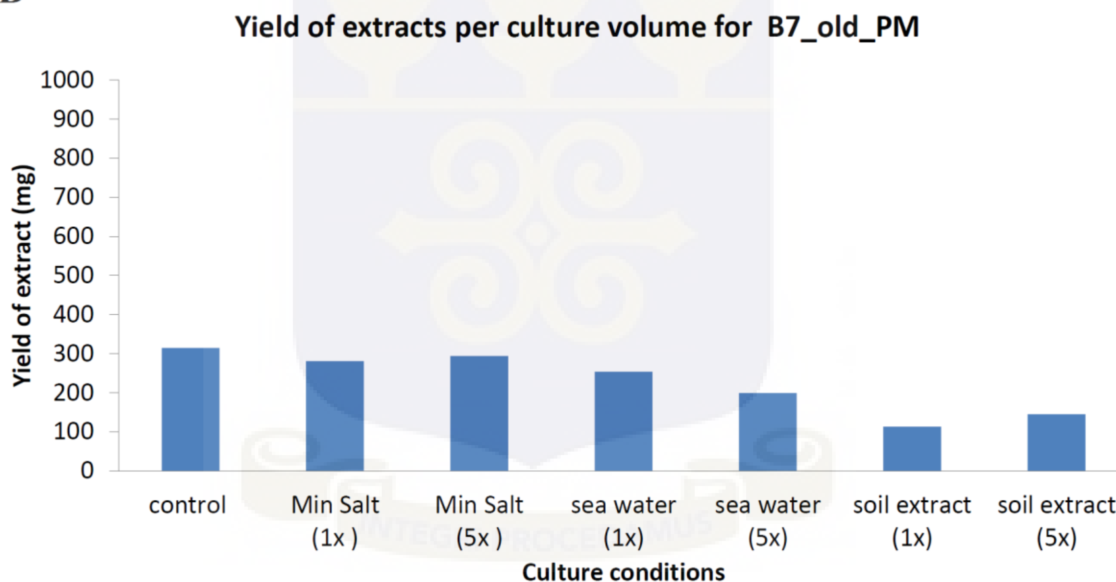

Figure 4.2: The yields of WDF metabolites by WDF cultured in PDB with mineral supplementation *(**A**) The yield of B7_new_BM extracts when extracted with equal volumes of solvent per culture medium. (**B**) The yield of B7_old_PM extracts when extracted with equal volumes of solvent per culture medium. The WDF were cultured in PDB modified with 1x and 5x concentrations of the mineral supplements.*

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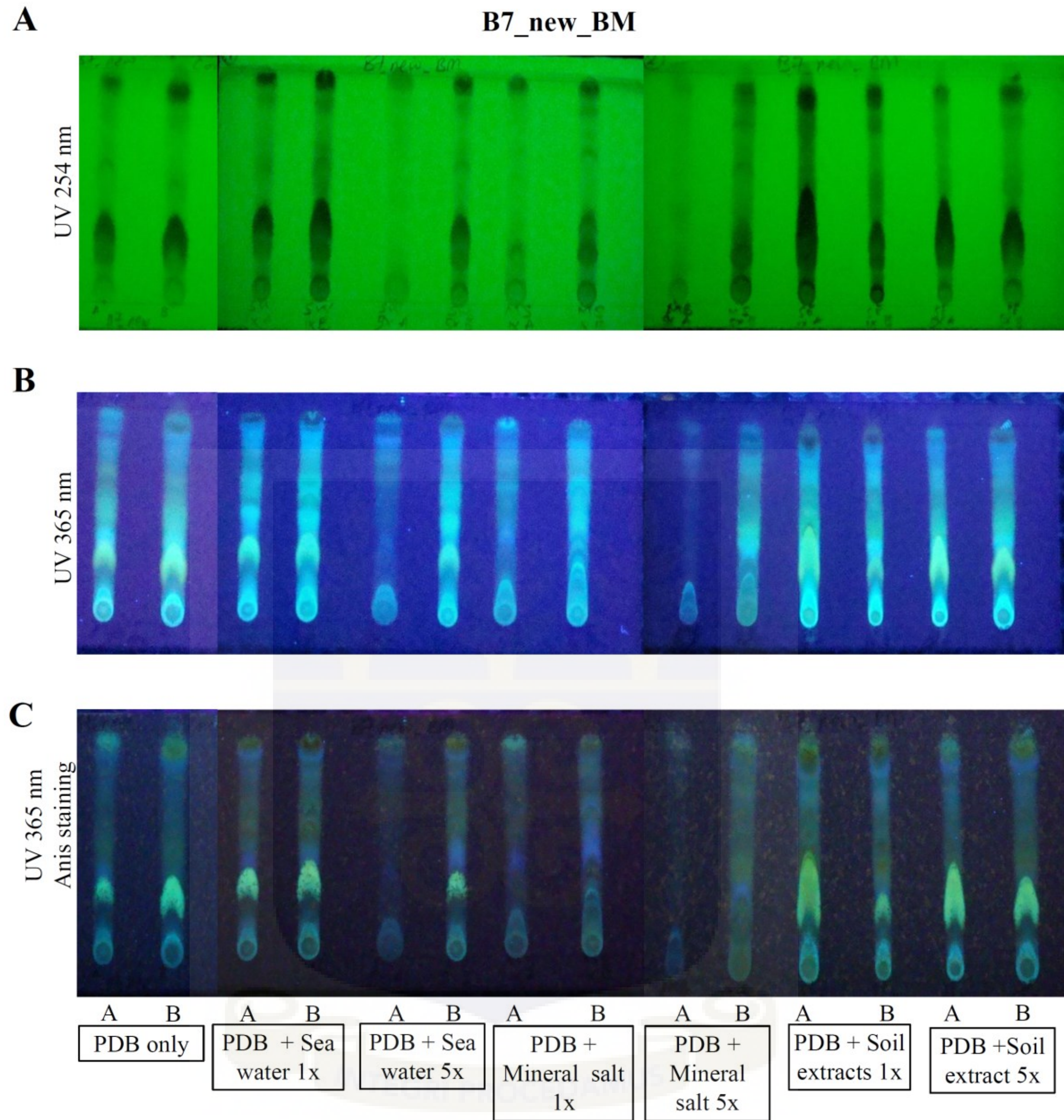

Figure 4.3: TLC analysis of the metabolites produced by B7_new_BM cultured in PDB with mineral supplementation. *The WDF coded B7_new_BM was cultures in soil extract, seawater and mineral salt modified PDB. The figure displays the picture of the TLC plates viewed under **(A)** UV 254 nm **(B)** UV 365 nm and **(C)** anisaldehyde stains at 365 nm.*

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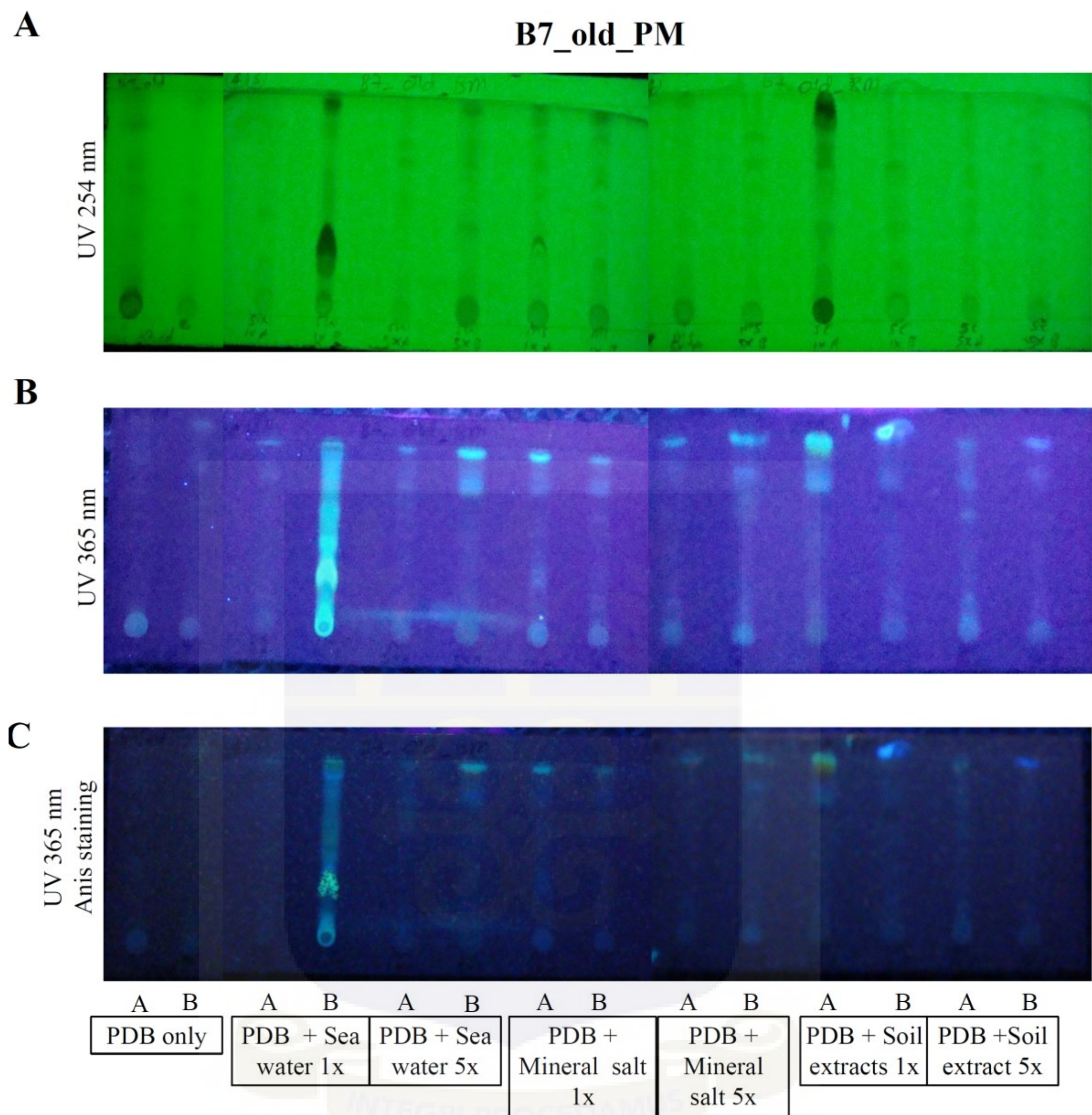

***Figure 4.4: TLC analysis of the metabolites produced of B7_old_PM cultured in PDB with mineral supplementation**. The WDF coded B7_new_BM was cultured in soil extract, seawater and mineral salt modified PDB. The figure displays the picture of the TLC plates viewed under **(A)** UV 254 nm **(B)** UV 365 nm and **(C)** anisaldehyde stains at 365 nm.*

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4.2. Critical factors for determining batch-to-batch consistency of wood decaying fungi metabolite production

One major challenge faced in the production of bioactive compounds is batch-to-batch consistency of the products: thus, products from each batch appear to be different. To explore effect culture conditions on the batch-to-batch consistency of WDF extracts produced, the first variable to be tested was aeration of the cultures. To do this, two different culture volumes (100ml and 400 ml) and three media types were used to study the effect of aeration and media richness on the metabolite production by WDF. The media types were a combination of Yeast extract, Peptone, Malt extract, Dextrose broth (YPMD) that represents enriched media; standard media (PDB) and PDB modified mineral salt at 2x concentration. Each condition was set up in 5 replicates.

The results (Figure 4.5) revealed that 100 ml liquid broth in a 500 ml culture vessel (good aeration) promoted WDF growth hence increasing metabolite production compared to the 400ml in 500 ml culture vessel, which represented poor aeration. From the chromatogram, the 100 ml cultures gave more consistent band patterns across all the conditions compared to the 400 ml cultures (Figure 4.7). However, there was no significant difference in the yield of the extracts produced by both the highly aerated and the poorly aerated cultures.

To evaluate the effect of the culture aeration on the production of bioactive compounds, the extracts were tested against *Methicillin Susceptible Staphylococcus aureus (MSSA GGP 200), Methicillin Resistant Staphylococcus aureus (MRSA GGP E120), Mycobacterium smegmatis (M smeg ETHZ_2), Erythromycin Resistant Mycobacterium smegmatis (Ery M. smeg), C. albicans with cycloheximide treatment (C.a. CyH), S.*

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cerevisiae with cycloheximide treatment (S.c. CyH), C. albicans and S. cerevisiae. From these activities, B7_new_BM extracts from the poorly aerated cultures (high volume) had higher zones of inhibition compared the highly aerated cultures (low volume). However, the activities of these low volume culture extracts against the test organisms were not consistent among the five replicates of poorly aerated cultures (Figure 4.5A). Similar observation was made with the B7_old_PM culture with the exception of the remarkable observation made with the 400 ml PDB only cultures. These extracts obtained from the five replicates had very consistent activities against four of the test organisms (Figure 4.5).

The effect of media richness was determined across the two aeration volumes used. From the chromatogram, it was revealed that the poor media (PDB) produced consistent bands pattern compared to the rich media (YPMD) (Figure 4.7A and C). The mineral enriched PDB produced the most inconsistent band pattern (Figure 4.7B). Although the PDB only cultures produced the highest relative yield, the yield within the five replicates of the PDB cultures was not consistent. Similarly the YPMD and PDB enriched with mineral salt culture did not produce consistent yield within the 5 replicates. Mineral salt enrichment of PDB had low yields compared to the PDB only cultures

From the bioactivity data (Figure 4.5), it was observed that the enriched media (YPMD) produced consistent activity against the test organisms compared to the other media types, which were however mainly active against MSSA GGP 200 and M. smeg ETHZ_2. The replicates of B7_old_PM cultured in PDB 400 ml produced consistent activity against four of the test organisms.

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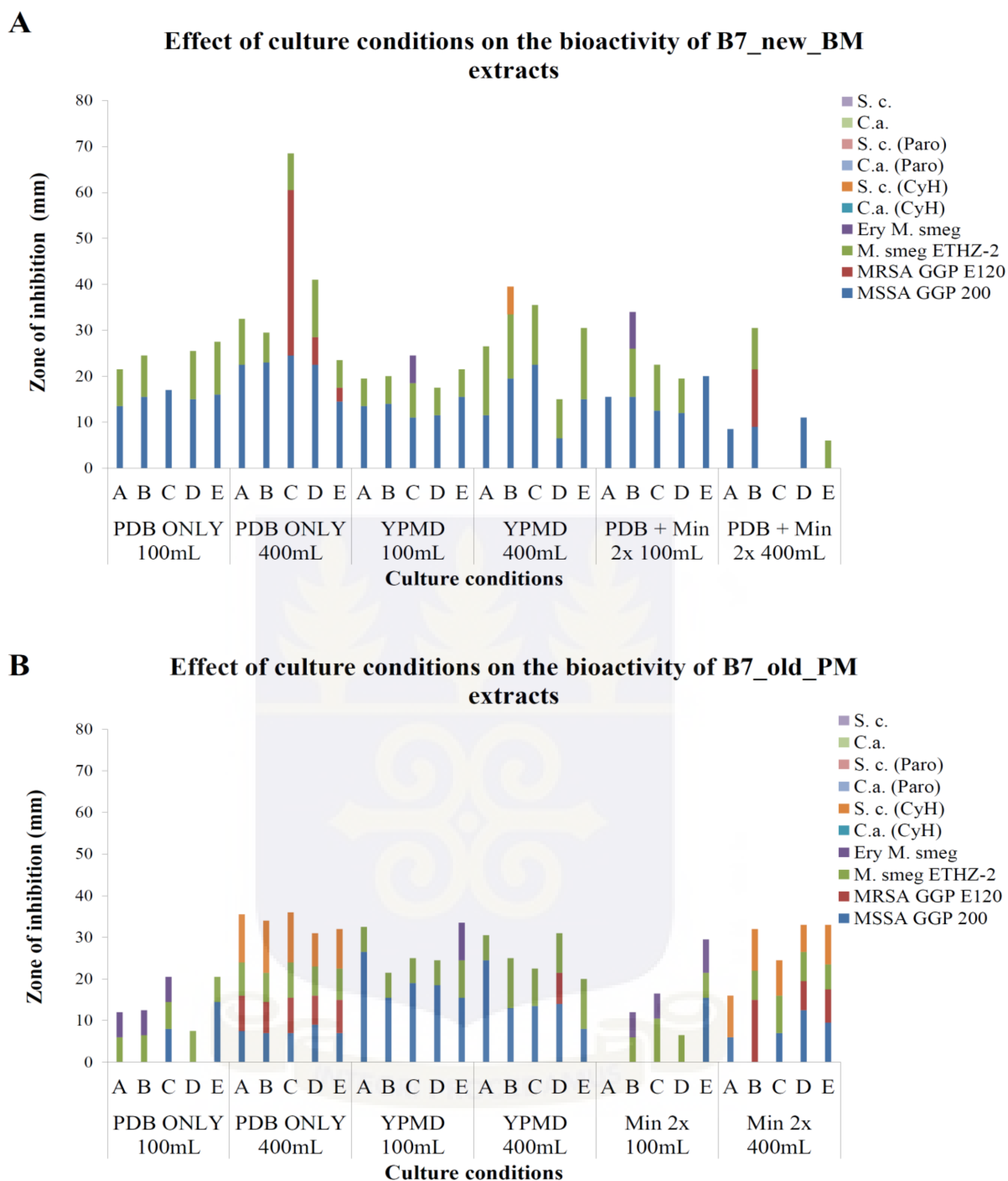

Figure 4.5: Disc Diffusion based analysis of bioactive compounds produced by WDF cultured in 100 ml and 400 ml of three different media formulations. *Two selected WDF (A) B7_new_BM and (B) B7_old_PM were cultured in 100/400ml of PDB modified with mineral salt, 100/400 ml of YPMD and 100/400ml of unmodified PDB. Extracts from the various cultures were tested against Methicillin Susceptible Staphylococcus aureus (MSSA GGP 200), Methicillin Resistant Staphylococcus aureus (MRSA GGP E120), Mycobacterium smegmatis (M smeg ETHZ_2), Erythromycin Resistant Mycobacterium smegmatis (Ery M. smeg), C. albicans with cycloheximide treatment (C.a. CyH), S. cerevisiae with cycloheximide treatment (S.c. CyH), C. albicans and S. cerevisiae.*

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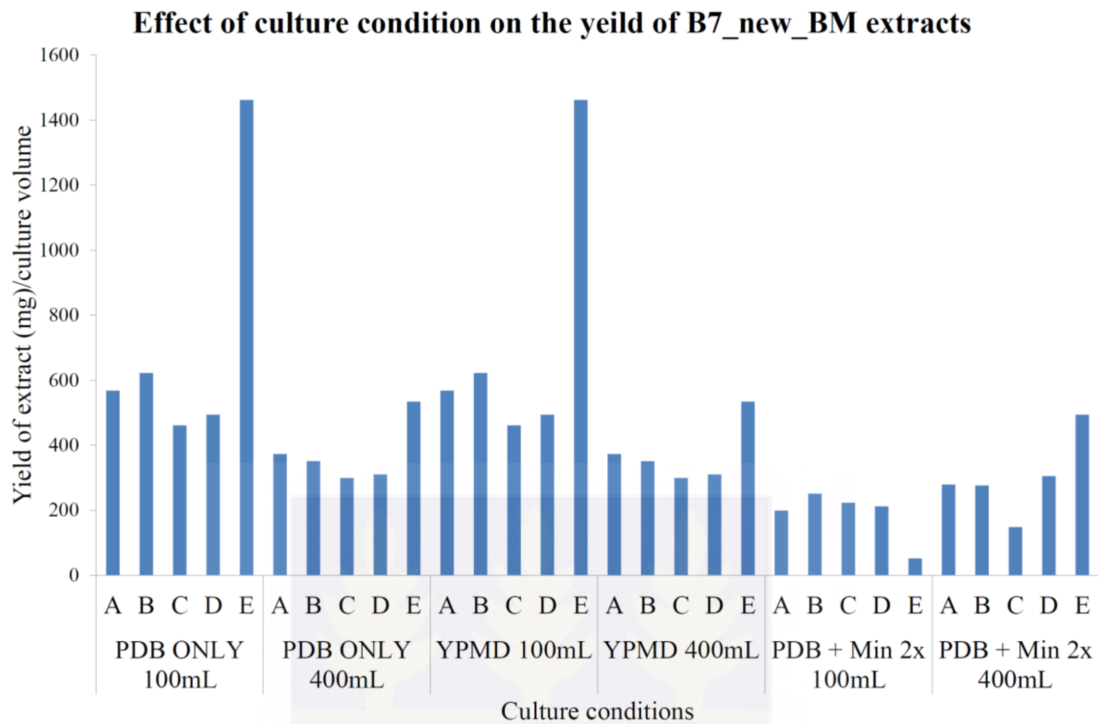

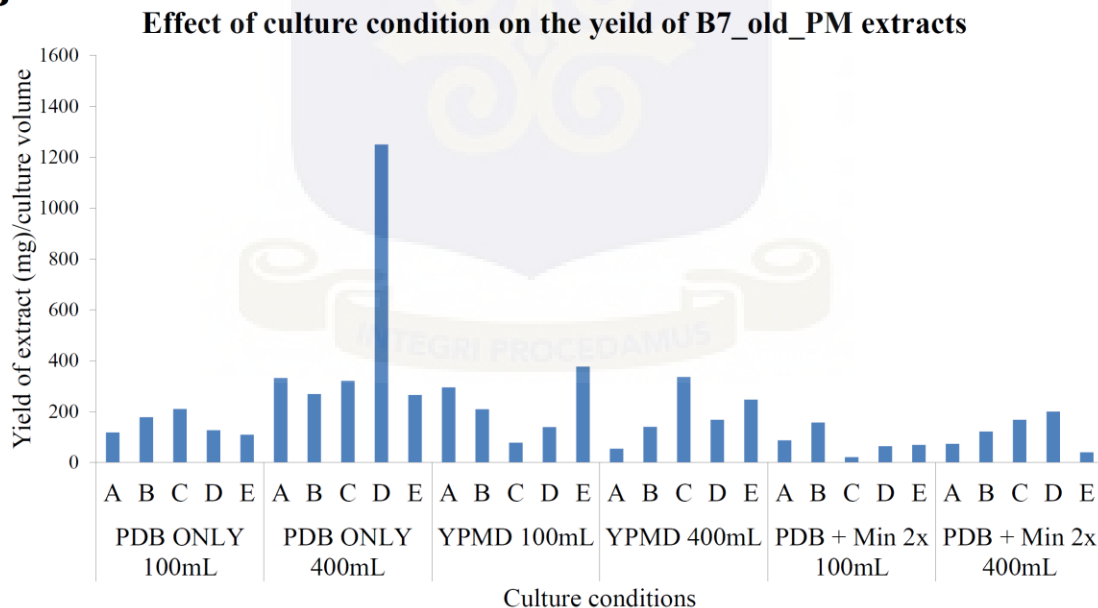

Figure 4.6: The yield of crude extracts from WDF cultured in 100 ml and 400 ml of three different media formulations. *The WDF were cultured in PDB only, PDB modified with 2x concentrations of the mineral supplements and YPMD (A) The yield of B7_new_BM extracts prepared with equal volumes of solvent per culture medium. (B) The yield of B7_old_PM extracts prepared with equal volumes of solvent per culture medium.*

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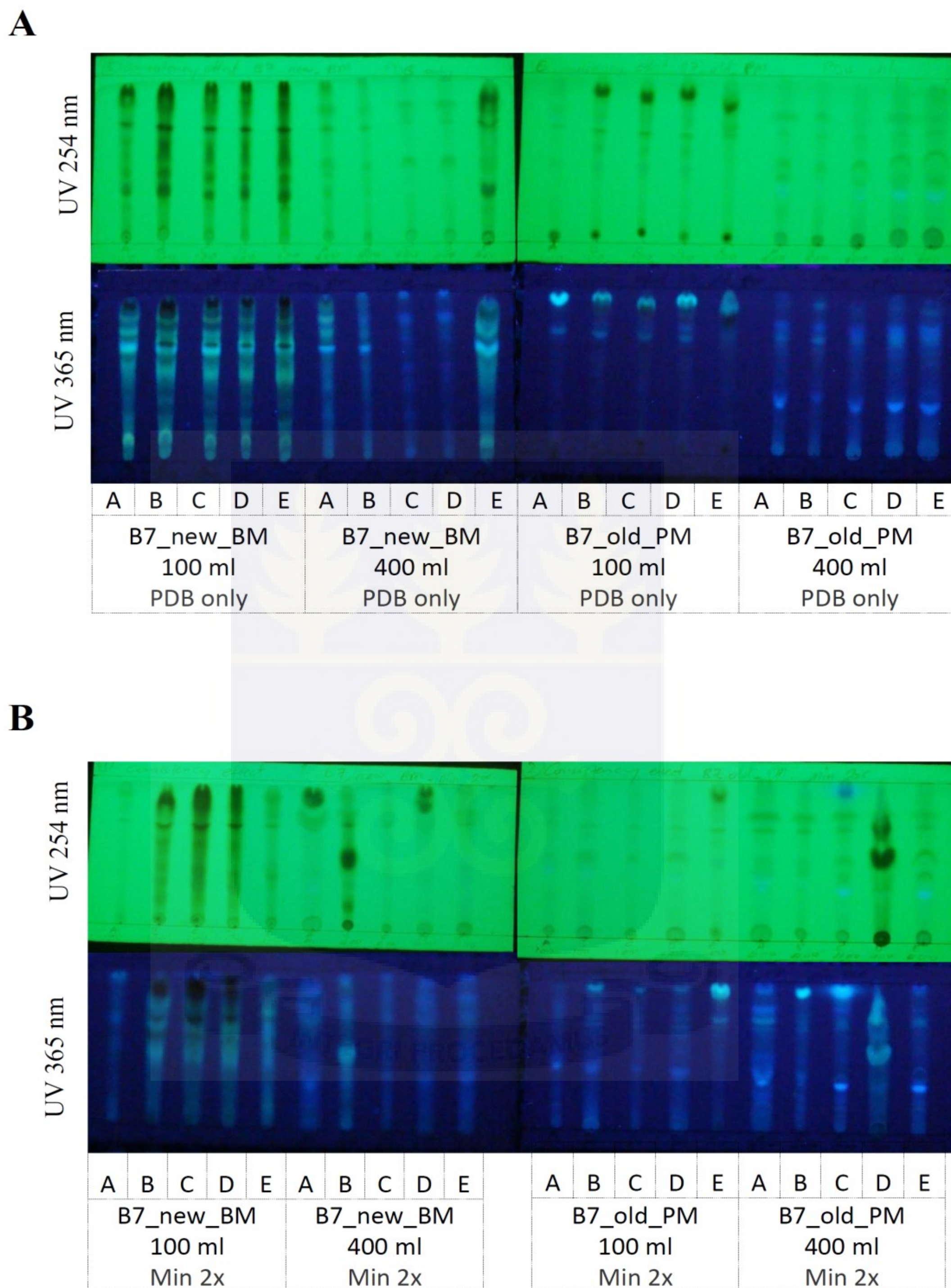

Figure 4.7: TLC analysis of extracts from WDF cultured in 100 ml and 400 ml of three different media formulations. ***(A)*** *TLC plates of WDF extracts from Potato Dextrose Broth (PDB only cultures)* ***(B)*** *TLC plates of WDF extracts from PDB enriched with mineral salt 2x cultures* ***(C)*** *TLC plate of WDF extracts from Yeast extract, Peptone, Malt extract, Dextrose broth (YPMD) cultures.*

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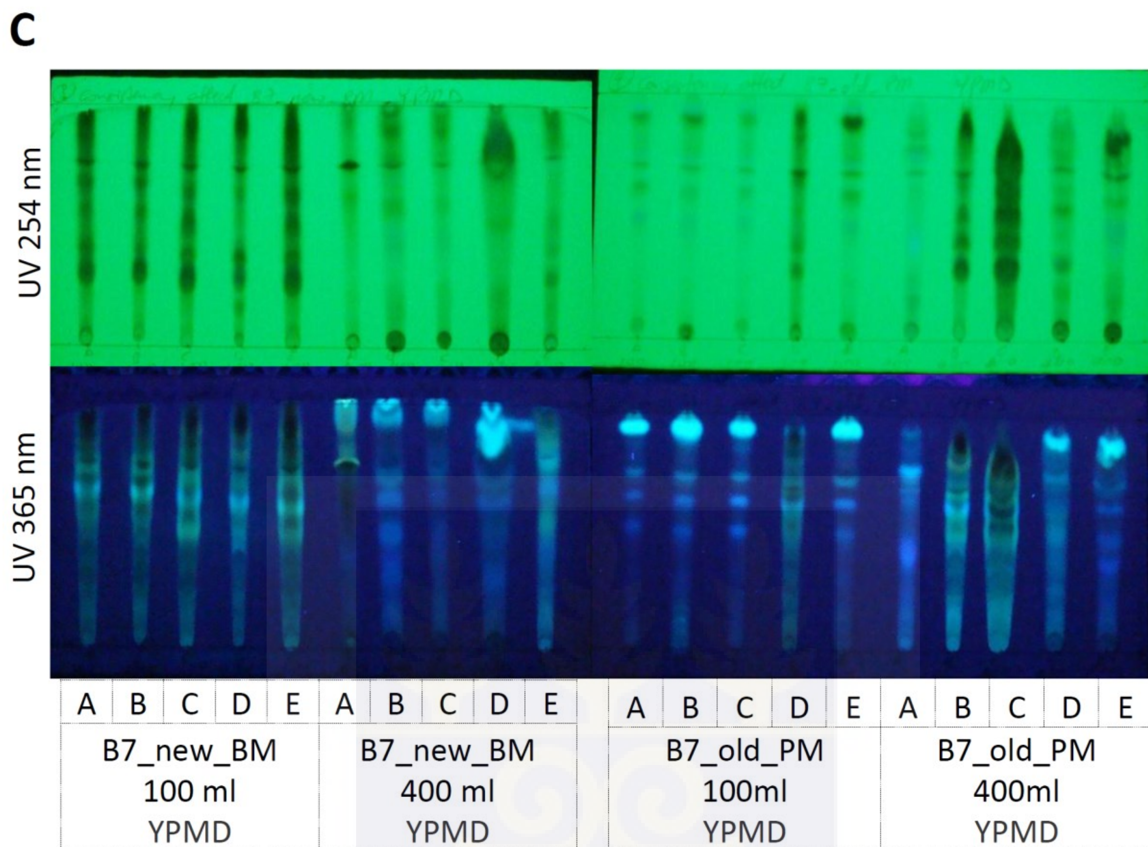

Figure 4.7C: TLC analysis of extracts from WDF cultured in 100 ml and 400 ml of three different media formulations. ***(C)*** *TLC plate of WDF extracts from Yeast extract, Peptone, Malt extract, Dextrose broth (YPMD) cultures.*

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4.3. Screening of WDF for their antifungal activity

After collecting 189 WDF fruiting bodies, there was a need to screen them for their antifungal activity and select the best candidates. The disc diffusion method was employed to screen by previous students of the laboratory for the antifungal properties of the WDF collected. To do this, the 189 WDF collected were cultured in 500ml of potato dextrose broth (PDB) and their metabolites were extracted using ethyl acetate after the cultures matured. The maturity of the cultures was determined by colour change and rate of growth (increase of mycelia mass). The ethyl acetate extracts were tested against *C. albicans*. From the primary screen, 31% of the crude extracts had antifungal activity against *C. albicans*. The primary screen uncovered some extracts, which had significant zones of inhibition against *C. albicans.* Noteworthy was J2, which had an average zone of inhibition of 27 mm (Figure 4.8). Based on the antifungal activity of the WDF, 33 of them were selected for further analysis as the start point of this project. Although two of the WDF (B8 and H4) did not show any anti-candida activity during the primary screening, they were selected based on their profound anti-mycobacterial activity. They might not have shown antifungal activity due to low metabolite production. When these WDF were cultured and screened against *C. albicans* and *S, cerevisiae* for the second time, metabolite production was improved hence, they showed antifungal activity.

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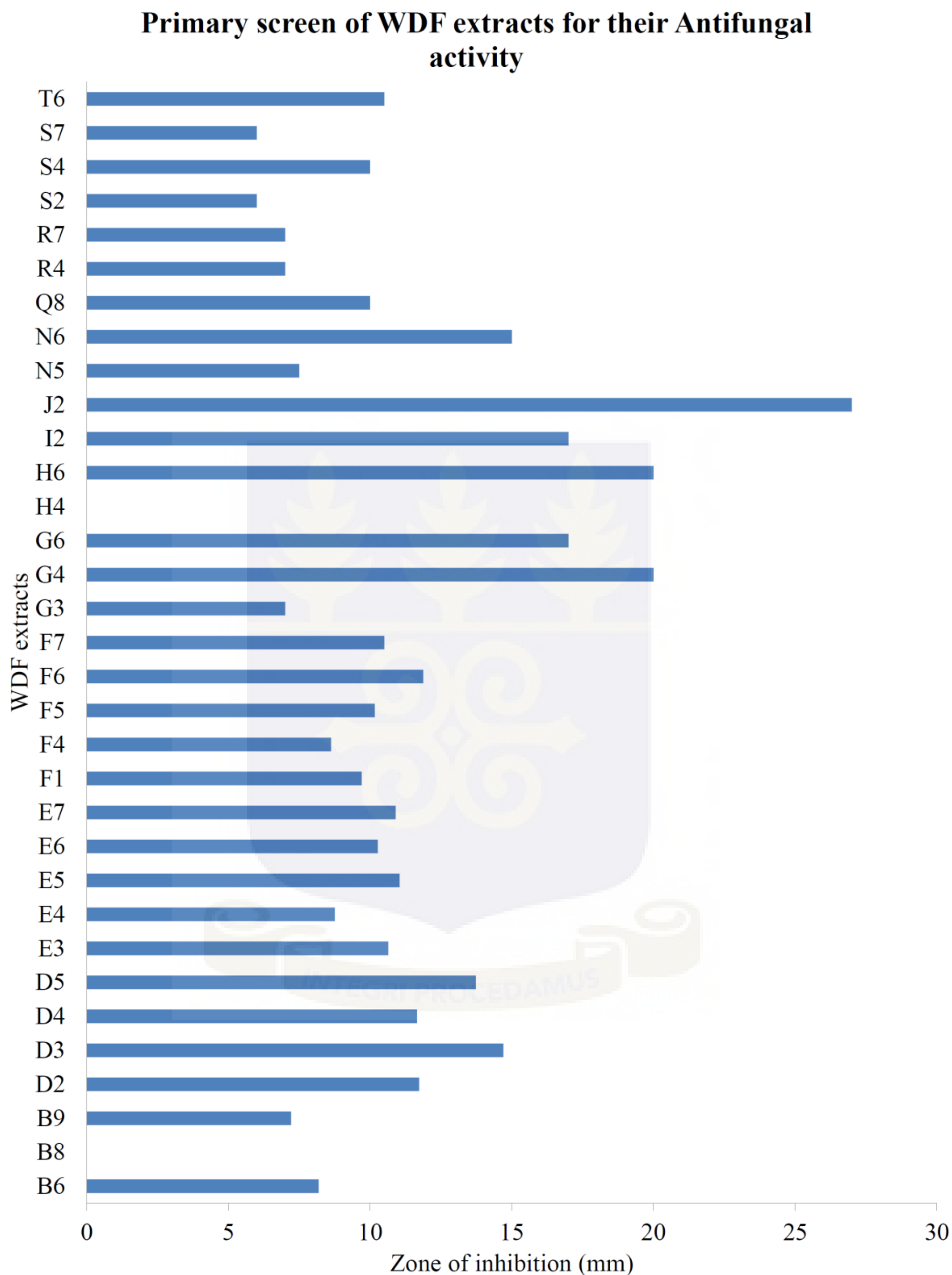

Figure 4.8: primary screen of 33 selected wood decaying fungi. *Ethyl acetate extracts of 33 WDF cultured in 500ml PDB for four weeks were tested against C. albicans. The chart represents the average zones of inhibition of the WDF extracts.*

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4.5. Mycelia Isolation from the wood decaying fungi

There was the need to find a constant and continuous source of fungal inoculum for the production of bioactive compounds in the laboratory. The fruiting body of the WDF does not provide a continuous source of inoculum, since it is difficult to find the exact same samples in the environment. Therefore it was essential to isolate and store mycelia from fruiting bodies to serve as a continuous and constant source of inoculum.

4.5.1. Isolation of plate mycelia (PM) from fruiting body

To obtain a continuous source of inoculum, the 33 WDF selected from the primary screening were cultured on agar plates (Table 4.2). Out of the 33 fruiting bodies that were grown on Sabouraud Dextrose Agar (SDA), 27 germinated. The 6, which did not germinate, were further cultured on Malt Extract Agar (MEA) since the first media did not support their growth. Out of the 6 WDF, 4 germinated on the MEA plates. Each plate was inoculated at 3 different points with pieces of the fruiting body. On the SDA plates, it was observed that 12 fruiting bodies germinated from all 3 points while 8 and 7 fruiting bodies germinated from 1 and 2 points of inoculation respectively (Table 4.1). Three fruiting bodies germinated from all 3 inoculation points on the MEA plates while one geminated from two inoculation points. All mycelia that germinated from both SDA and MEA plate were cultured on freshly prepared MEA plates and stored. In all, mycelia were isolated from 31 WDF fruiting bodies out of the 33-selected WDF onto agar plates. These were referred to as Plate Mycelia (PM) and were stored in the refrigerator at 8ºC.

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Table 4.1: Growth of 33 WDF fruiting bodies on agar plates

	Number of WDF that grew on agar plate		
Number of inoculation points that geminated	Sabouraud Dextrose Agar (SDA) n=33	Malt Extract Agar (MEA) n=6	Total number of WDF
1	8	0	8
2	7	1	8
3	12	3	15
Total	27	4	31

4.5.2. Isolation of plate mycelia (PM) from liquid cultures

The main purpose of isolating plate mycelia was to obtain a continuous and reliable source of fungal inoculum. It was therefore necessary to ensure that the mycelia isolated were distinct from each other and consistent in both morphology and bioactivity. To do this, the 31 plate mycelia (PM) were used to inoculate PDB (200 ml) in duplicates giving a total of 62 liquid cultures which were allowed to grow for 13 weeks. Their mycelia harvested and used to inoculate MEA plates from which were obtained the next generation of plate mycelia PM1. The morphology and colour of the PM1 were compared to that of the PM. It was observed that 76% had similar morphology and colour on both PM and PM1 plates, 15% were not similar and 9% WDF did not germinate at all (Figure 4.9). For testing the antifungal activity of the PM1, extracts of the 62 liquid cultures were treated as unique isolate despite the 76% similarity observed.

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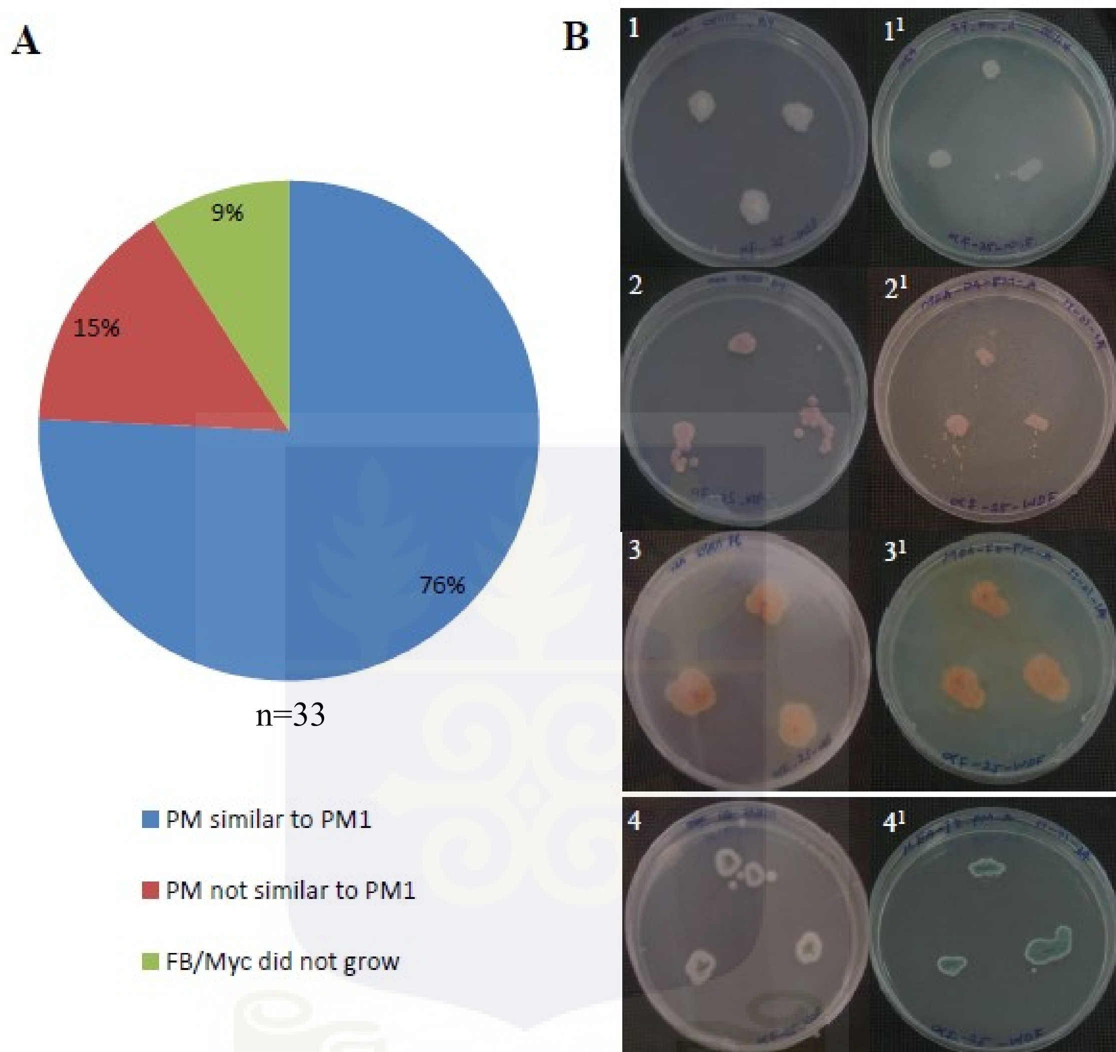

Figure 4.9: Mycelia isolated from 33 selected wood decaying fungi. *Fruiting bodies of WDF were cultured on plates and mycelia obtained (PM) were used to inoculate potato dextrose broth (PDB). Mycelia harvested from the broth cultures were used to inoculate malt extract agar plates (MEA).* ***(A)*** *Percentage similarity between PM (mycelia obtained from fruiting bodies grown on plates) and PM1 (broth mycelia grown on plate.* ***(B)*** *A representation PM and PM1 on MEA plates. Plates 1 – 3 and $1^1$to 3^1shows similarity between PM and PM1 while plates 4 and 4^1shows isolates which were not similar*

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4.5.3. Thin layer chromatography of the 62 wood decaying fungi

To establish that distinct isolates were obtained from the mycelium isolation experiments, metabolites were extracted with ethyl acetate from the 62 liquid cultures and dried using a rotatory evaporator at 40ºC. The WDF extracts were spotted on TLC plates and developed with ethyl acetate, acetonitrile and petroleum ether 7:2:1. From the chromatogram, it was observed that, the band patterns for the replicates of each fungus were similar except for 3 out of the 31 WDF which did not show similar band patterns with their duplicates (Figure 4.10 and Figure 4.11). The mycelia isolated from each WDF were distinct.

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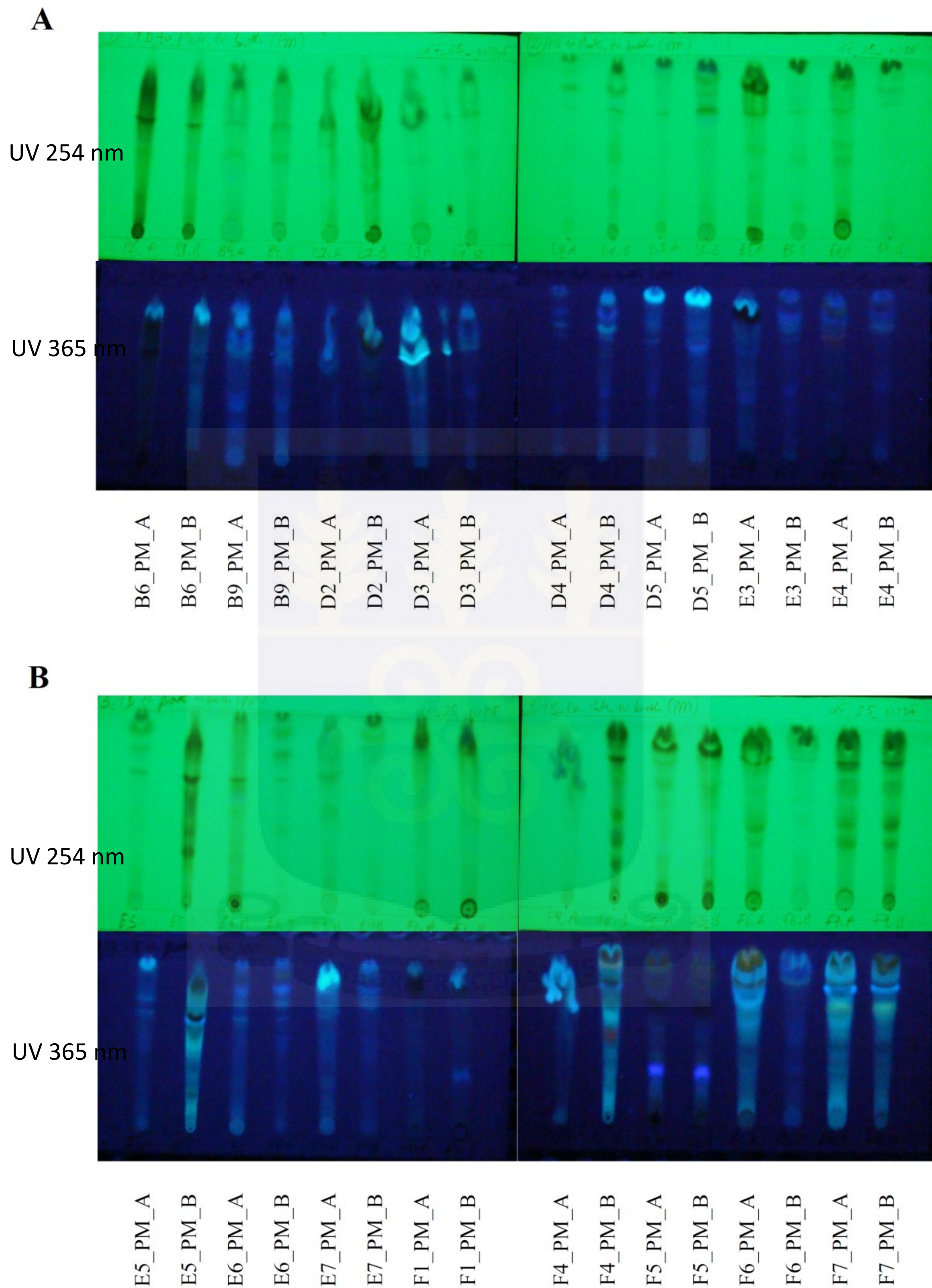

Figure 4.10: TLC analysis of extracts of the 62 WDF Plate Mycelia #1*. The TLC plates display the band pattern of the first 32 WDF of the 62 liquid cultures. The codes A and B are duplicates of each fungus.*

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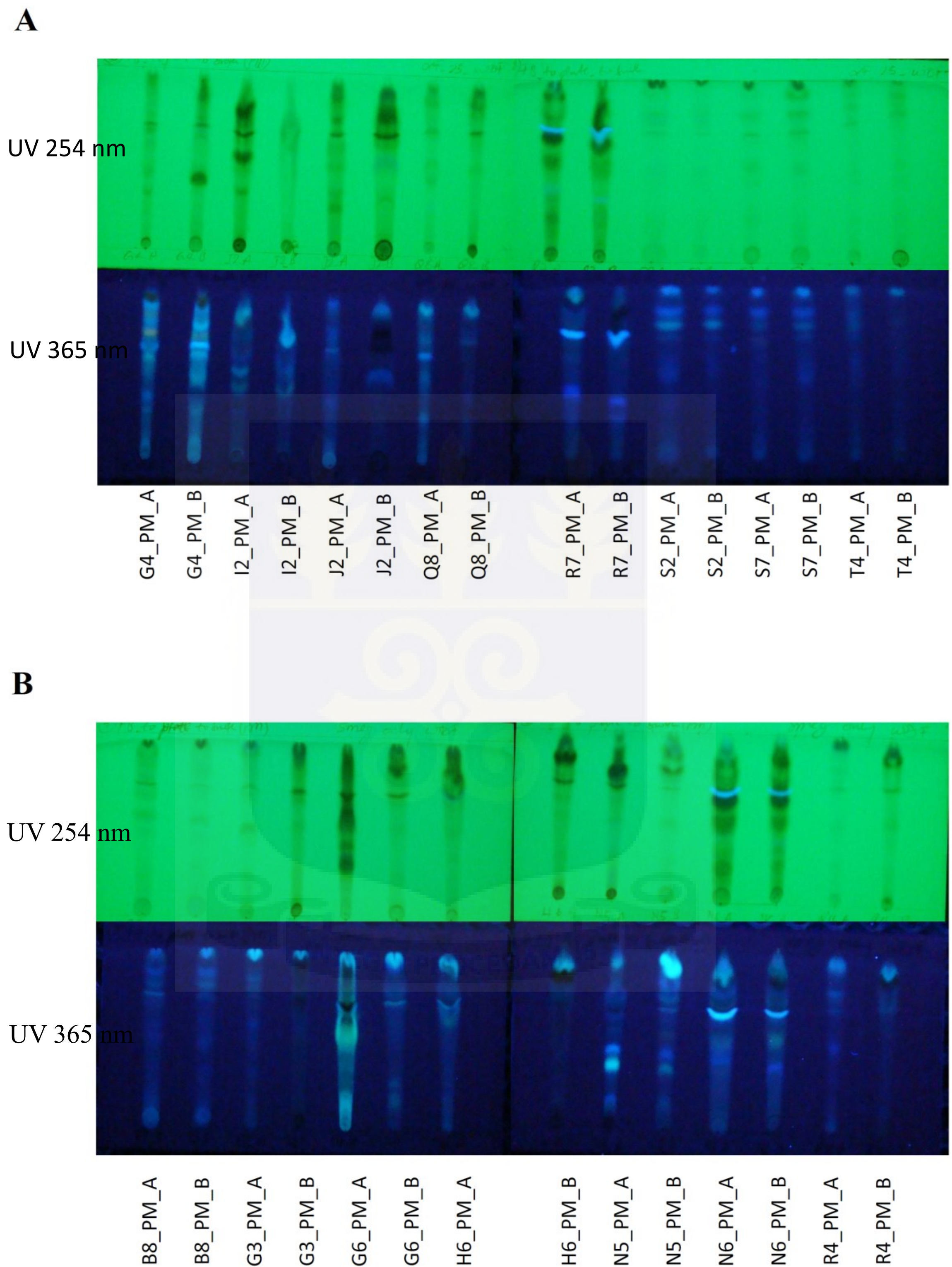

***Figure 4.11: TLC analysis of extracts of the 62 WDF Plate Mycelia #2**. The TLC plates display the band pattern of the last 30 WDF of the 62 liquid cultures. The codes A and B are duplicates of each fungus.*

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4.5.4: Determination of the antifungal activity of the 62 plate mycelia extracts

To determine the antifungal activity of the 62 WDF extracts in comparison to the activity obtained from the primary screen, the crude extracts from the 62 WDF broth cultures were tested against *C. albicans* and *S. cerevisiae*. From this assay, 23 of the WDF extracts were active against at least one of the two organisms, *C. albicans* and *S. cerevisiae* (Figure 4.12). These activities were measured using 30 ul, which makes up 1/30th v/v of the total extract from a 200 ml culture. Comparing the antifungal activity with the duplicates, 8 WDF extracts out of the 23 showed similar levels of activity among their duplicates (A and B) against *C. albicans*. The remaining 7 WDF were different in the activities of the duplicates. When they were tested against *S. cerevisiae* however, 4 WDF extracts out of the 23 showed activities, which were replicated in their duplicates. In total, 15 out of the 31 WDF from the primary screen maintained their anti-candida activity after the mycelia isolation compared to the primary screen results.

The extracts H6_PM_A and R7_PM_B had zones of 16.5 mm and 17 mm against *S. cerevisiae*, which were higher than the standard drug, fluconazole (10 µg) that had a zone of 15 mm (Figure 4.12B). However, none of the extracts had zone of inhibition higher than that of fluconazole (25 mm) when tested against *C. albicans*, G6_PM_B and H6_PM_B had zones of 17 mm and 20 mm, which were relatively higher (Figure 4.12 A).

To test whether paromomycin and cycloheximide increased the sensitivity of the test organisms to the WDF extracts, which do not show any antifungal activities in an unmodified growth plate. Seven of PM extracts without antifungal activity during the secondary screen when retested against *C. albicans* and *S. cerevisiae* on YPD agar plates

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modified with 50 μg/ml paromomycin and 50 μg/ml cycloheximide showed activity (Figure 4.13).

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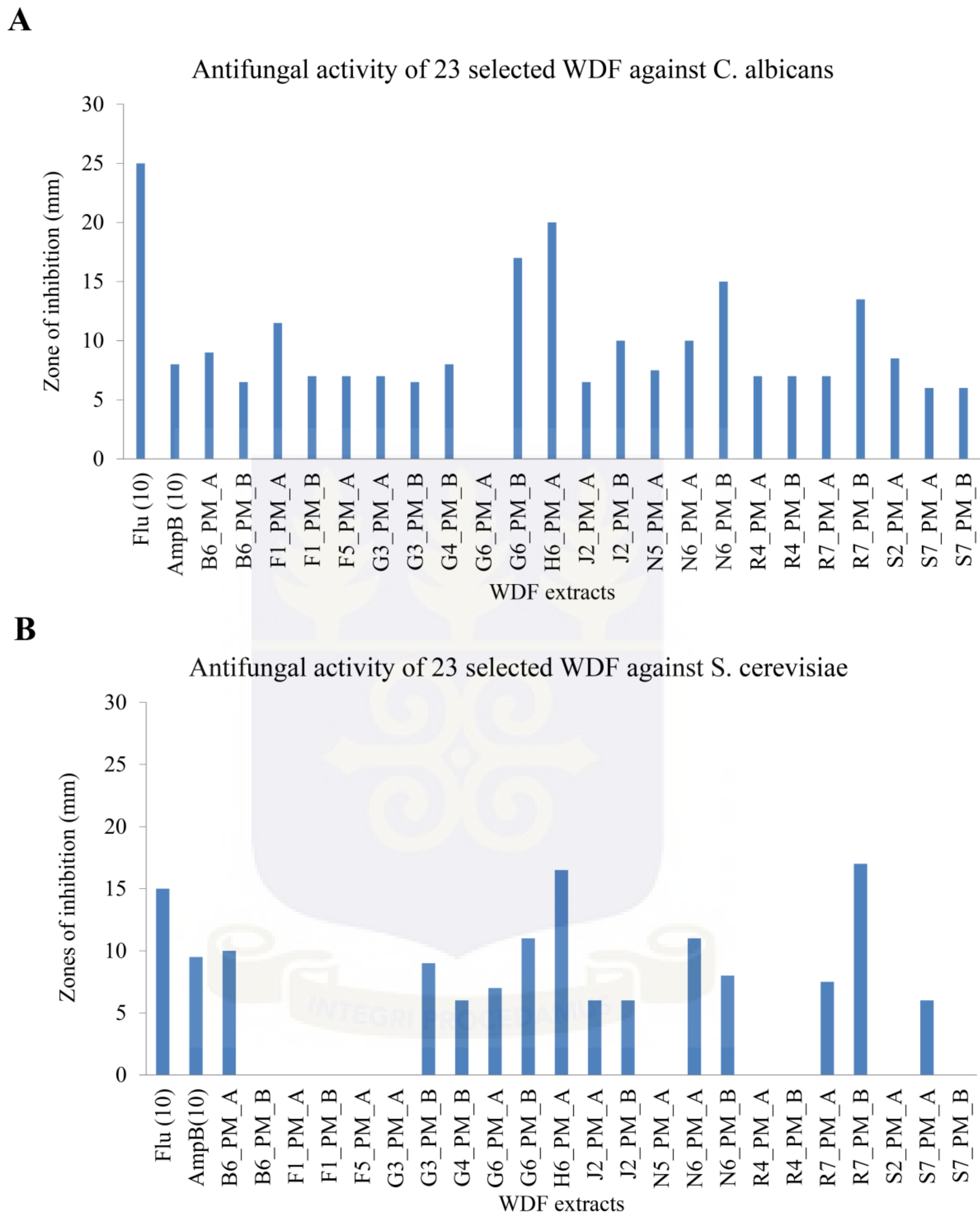

***Figure 4.12: Secondary screening of 23 out of 62 plate mycelia extracts**. Ethyl acetate extracts of 62 WDF were tested against (A) C. albicans and (B) S. cerevisiae. The above chart displays the average zones of inhibitions of the 23 WDF extracts which showed activity against either one of the organisms.*

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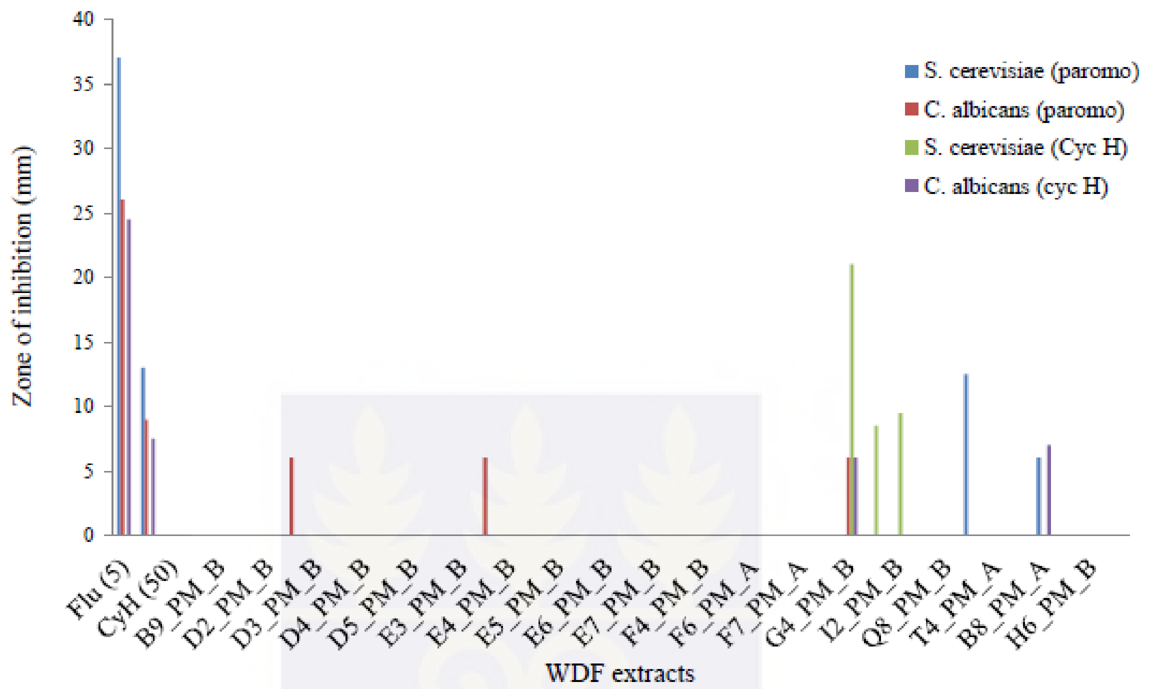

Figure 4.13: Antifungal activity of previously inactive plate mycelia extracts on modified YPD media. *The 49 out of 62 PM extracts without antifungal activity in the secondary screen were retested against C. albicans and S.cerevisiae on YPD agar plates modified with either 50ug/ml paromomycin (Paro) or 50ug/ml cycloheximide (CycH).*

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4.6 Phenotypic screening of PM wood decaying fungal extracts

To validate the antifungal activity and also select the most promising extracts with potential novel mode of actions from the 23 selected WDF extracts, the extracts were taken through a phenotypic screening which involved the use of chemically modified media and mutant yeast cells.

In the first phenotypic screening, the phenotype of *C. albicans* and *S. cerevisiae* was altered chemically by culturing them on YPD agar plates modified with farnesol (Far), phenyl ethyl alcohol (PEA), cycloserine (Cys), N-acetyl glucosamine (NAG), cycloheximide (CyH) and 3-O-methylglucose (3-oMG). These different conditions had an effect on the sensitivity of the organisms.

The chemical modifications in the YPD agar plates produced interesting results with cycloheximide increasing the sensitivity of both *C. albicans* and *S. cerevisiae* to the extracts compared to the unmodified YPD agar plates (Figure 4.14). *S. cerevisiae* was resistant to fluconazole and amphotericin B in the presence of cycloheximide but sensitive to majority of the extracts tested (Figure 4.14B). Six of the PM WDF extracts had remarkable activities across majority of the conditions against *S. cerevisiae* and 5 of the 6 extracts also had significant anti-candida activity (Table 4.2).

In the second phenotypic screening, mutant *S. cerevisiae* cells with deficiency in ergosterol biosynthetic pathway, or the elongation step of protein synthesis were used. The properties of the mutant strains are summarized in Table 4.3. The mutations offered different phenotypes with different sensitivity to the standard drugs and the extracts. In this assay, a varying pattern of activities was displayed when the 23 PM WDF selected

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extracts were screened against the mutant *S. cerevisiae* cells. Among the mutant strains, Δerg (het) emerged the most sensitive strain. It was sensitive to 10 of the 23 WDF extracts. Comparing the cumulative antifungal activity across all the mutant cells, H6_PM_A and G6_PM_B had zones of inhibition higher than that of the standard antifungal drugs (fluconazole and amphotericn B) (Figure 4.15A).

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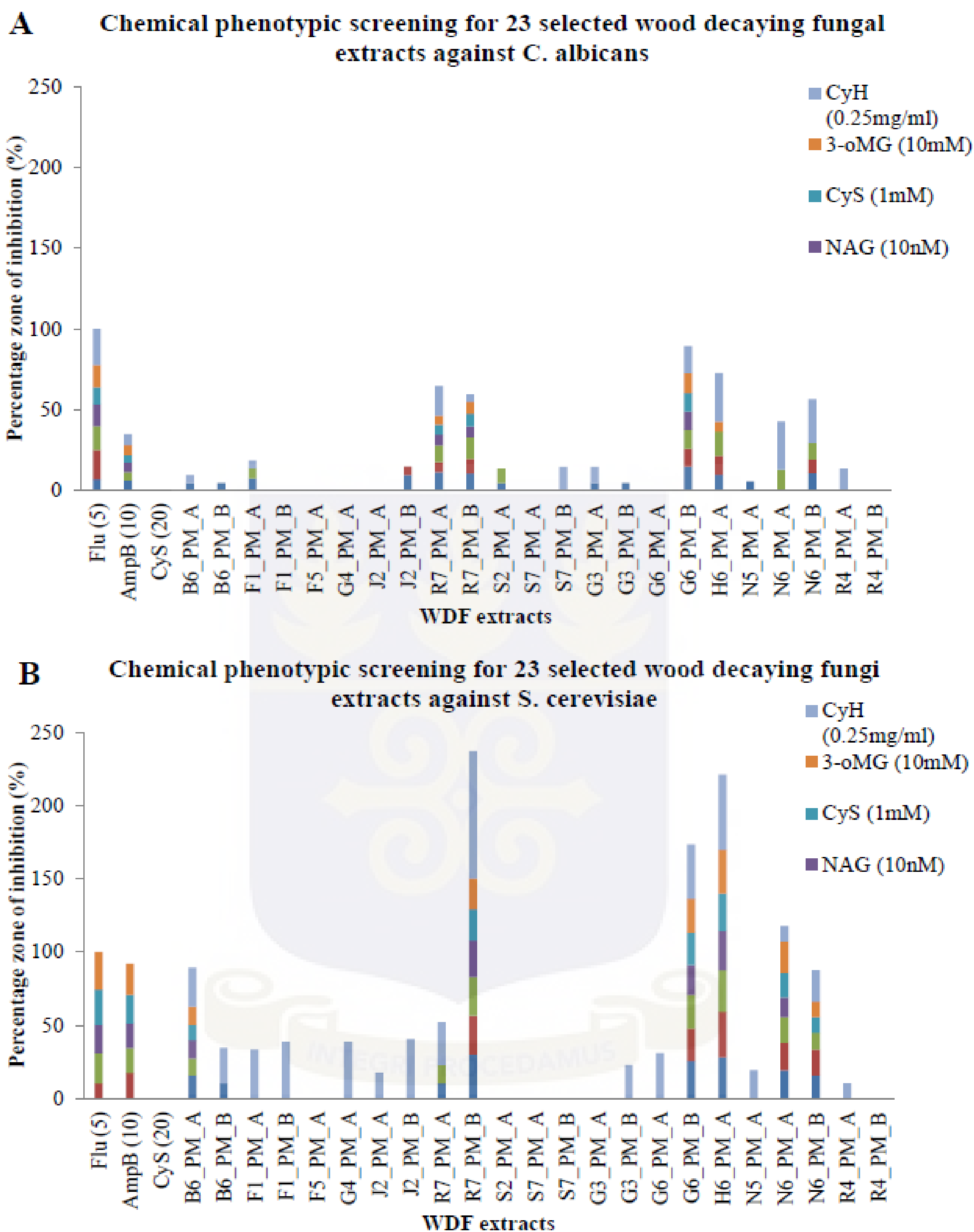

Figure 4.14: Chemical phenotypic screening of 23 selected wood decaying fungi extracts: *The 23 WDF extracts were tested against (A) C. albicans and (B) S. cerevisiae on YPD agar plates modified with farnesol (Far), phenyl ethyl alcohol (PEA), Cycloserine (Cys), N-acetyl glucosamine (NAG), Cycloheximide (CyH) and 3-O-methylglucose (3-oMG). For each assay, 0.5 units (1 ml supernatant/culture equivalent) of each extract were used. The test organisms and the extract discs were incubated for 24 hours at room temperature and the zones of inhibition measured. The graphs show the percentage zones of inhibition of the WDF extracts compared to fluconazole (flu (5)).*

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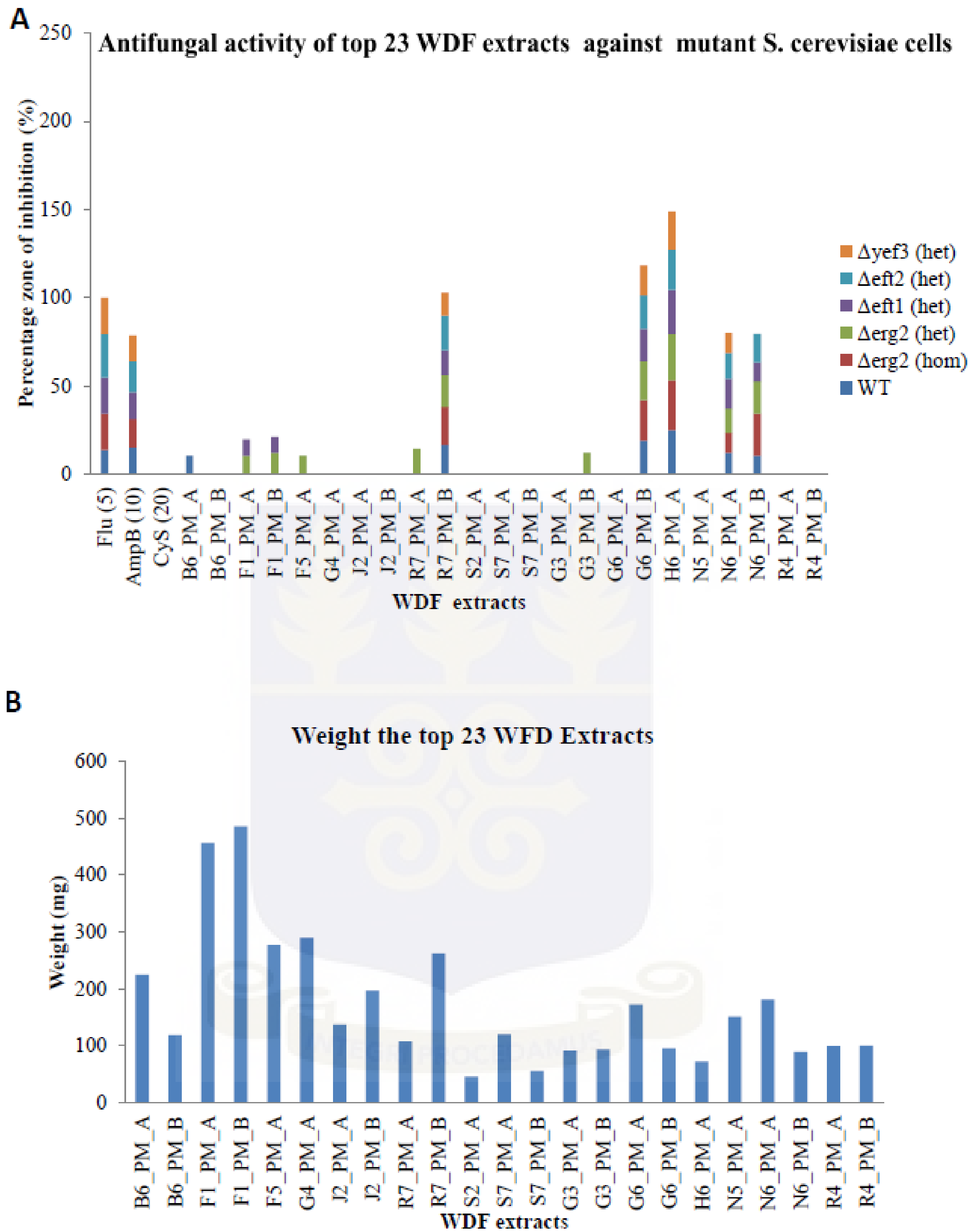

Figure4.15: The yield and antifungal activity of top 23 wood decay fungi extracts: ***(A)*** *The first 23 WDF extracts were tested against mutant S. cerevisiae cells on different YPD agar plates. Exactly, 0.5 units (1 ml supernatant/culture equivalent) of each extract were used. For each assay, 0.5 units (1 ml supernatant/culture equivalent) of each extract were used. The test organisms and the extract discs were incubated for 24 hours at room temperature and the zones of inhibition measured. The graphs show the percentage zones of inhibition of the WDF extracts compared to fluconazole (flu (5)).* ***(B)*** *The dry weight of the top 23 WDF extract per culture volume.*

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To select the top six WDF extracts with antifungal activity, the activity of all the 23 WDF extracts were analysed across all the chemical conditions and mutant strains used in the phenotypic assays. The WDF extracts were ranked based on their total zone of inhibition Six of the extracts were active against all the mutant *S. cerevisiae* cells (Table 4.2)

From this ranking the top 6 candidates were selected (Table 4.3).

Table 4. 2: Ranking of WDF extracts based on their total antifungal activity

		Zone of inhibition (mm) of the WDF extracts			
Rank	Top 6 WDF extracts	Chemical phenotypic screening against *C. albicans*	Chemical phenotypic screening against *S. cerevisiae*	Phenotypic screening using mutant *S. cerevisiae*	Total zone of inhibition (mm)
1	H6_PM_A	110	141.5	97.5	349
2	G6_PM_B	129.5	110.5	77.5	317.5
3	R7_PM_B	86.5	145	67.5	299
4	N6_PM_A	67.5	74.5	52.5	194.5
5	N6_PM_B	82	56.5	52	190.5
6	R7_PM_A	92.5	29.5	9.5	131.5
7	B6_PM_A	18	57.5	7	82.5
8	F1_PM_A	31.5	19	13	63.5
9	J2_PM_B	25	23	0	48
10	F1_PM_B	0	22	14	36
11	N5_PM_A	22.5	11	0	33.5
12	G3_PM_B	6	13	8	27
13	B6_PM_B	6	19.5	0	25.5
14	S2_PM_A	24	0	0	24
15	R4_PM_A	17	6	0	23
16	G4_PM_A	0	22	0	22
17	S7_PM_B	18.5	0	0	18.5
18	G3_PM_A	18.5	0	0	18.5
19	G6_PM_A	0	17.5	0	17.5
20	J2_PM_A	0	10	0	10
21	F5_PM_A	0	0	7	7
22	S7_PM_A	0	0	0	0
23	R4_PM_B	0	0	0	0

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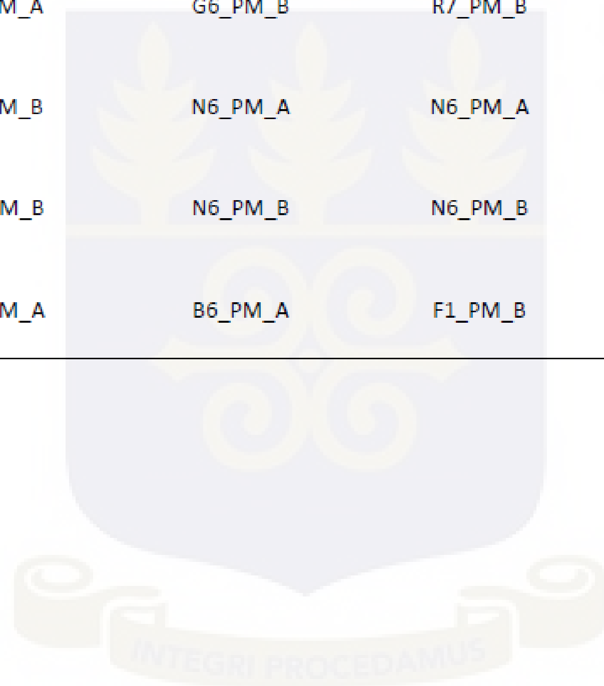
Table 4.3: Top 6 most activity antifungal extracts from each phenotypic array

Top 6 extracts from each screen			
Chemical phenotypic screening against *C. albicans*	Chemical phenotypic screening against *S. cerevisiae*	Phenotypic screening using mutant *S. cerevisiae*	**Top 6 WDF extracts selected**
G6_PM_B	R7_PM_B	H6_PM_A	**H6_PM_A**
H6_PM_A	H6_PM_A	G6_PM_B	**G6_PM_B**
R7_PM_A	G6_PM_B	R7_PM_B	**R7_PM_B**
R7_PM_B	N6_PM_A	N6_PM_A	**N6_PM_A**
N6_PM_B	N6_PM_B	N6_PM_B	**N6_PM_B**
N6_PM_A	B6_PM_A	F1_PM_B	**R7_PM_A**



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4.7: Morphological characterization of *C. albicans* and *S. cerevisiae*

To determine the effect of the chemical modifications on the morphology and staining properties of *C. albicans and S. cerevisiae*, the organisms were cultured on the modified YPD agar plates and taken through acid fast and gram stains.

The cycloheximide treatment changed the morphology of the *S. cerevisiae* cells from spherical to rod-like shape. This may account for the increase in drug sensitivity of the *S. cerevisiae* cells to the extracts. N-acetyl glucosamine induced an increase in the cell elongation compared to the cells grown on the unmodified YPM agar plates. Most of the cells retained the primary carbol fuchsin acid fast stain across all the conditions.

The morphology of *C. albicans* was not changed in the presence of any of the chemically modified media. However the chemical modifications changed the ability of the *C. albicans* to pick up the stains. *C. albicans* grown on the unmodified media retained the primary stain (carbol fushen) however, when grown on cycloheximide the cells picked up the secondary stain (methylene blue) (Figure 4.16A). N-acetyl glucosamine also had similar effect on the *C. albicans* cells just as cycloheximide (Figure 4.16A). The 3-O-methylglucose (3-oMG) treated *C. albicans* cells were unable to pick up both stains giving them a faint look. The media modifications had no significant effect on the gram-staining pattern of the *C. albicans* cells. It was also noted that the *C. albicans* cells did not have any major change in cell structure due to the chemical modifications (Figure 4.16B).

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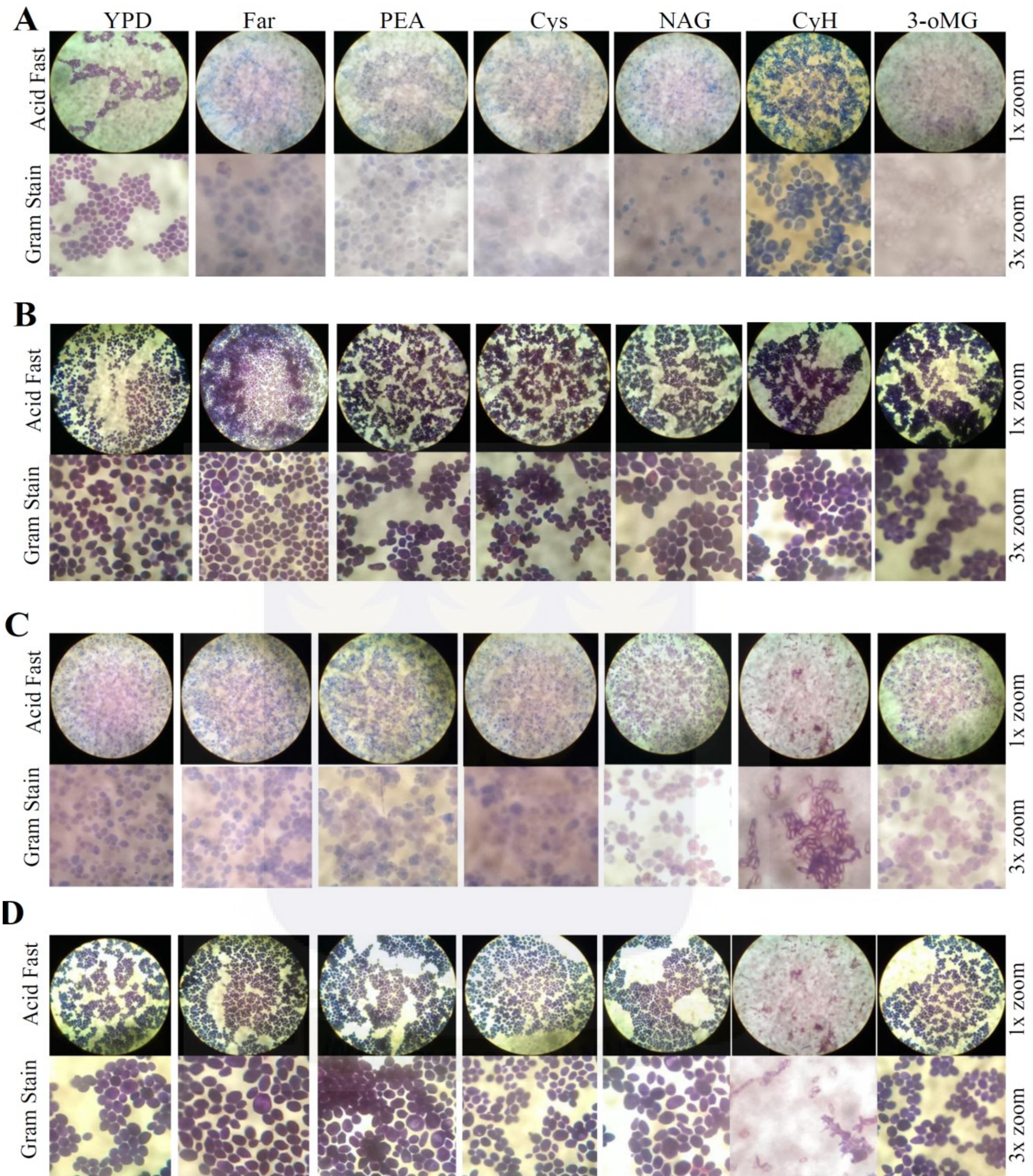

Figure 4.16: Differential Staining of C. albicans and S. cerevisiae cells grown on chemically modified YPD plates*. The C. albicans and S. cerevisiae cells were grown on yeast peptone dextrose agar (YPD), YPD modified with farnesol (Far), phenyl ethyl alcohol (PEA), Cycloserine (Cys), N-acetyl glucosamine (NAG), Cycloheximide (CyH) and 3-O-methylglucose (3-oMG). (A) Acid fast stains of C. albicans cells (B) Gram stains of C. albicans cells (C) Acid fast stains of S. cerevisiae cells (D) Gram stains of S. cerevisiae cells. The stained cells were viewed under the light microscope.*

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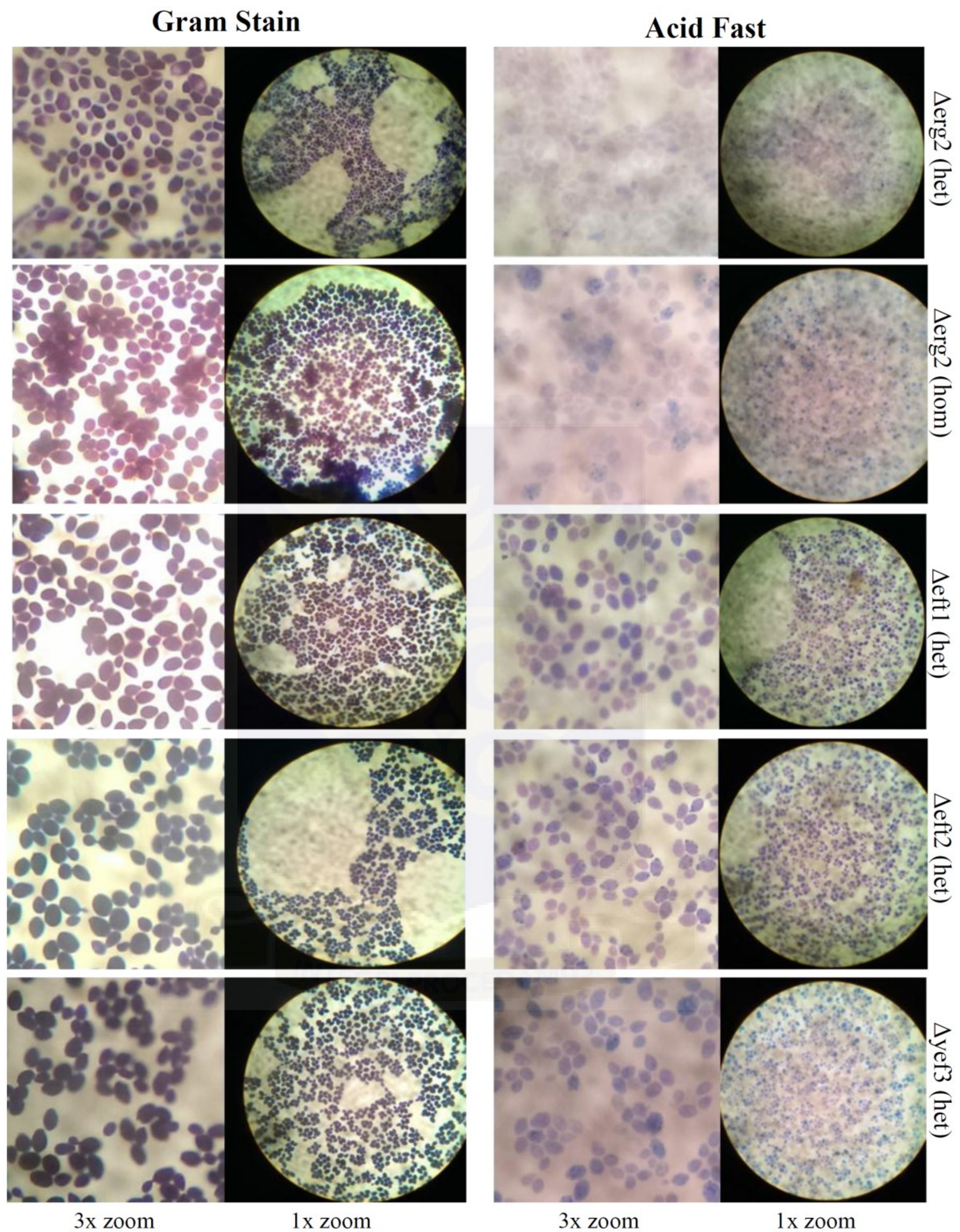

Figure 4.17: Differential Staining of mutant S. cerevisiae cells. *The mutant S. cerevisiae cells were grown on yeast peptone dextrose agar (YPD) and stained with* ***(A)*** *acid fast stains* ***(B)*** *Gram stain. The stained cells were viewed under 100x magnification of the light microscope.*

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4.8: Morphological characterization of mutant *S. cerevisiae* cells

In order to examine the effect of the mutation on the morphology of the mutant cells, gram stain and acid-fast stain of the cells were observed under the light microscope. The regular spherical shape of the *S. cerevisiae* cells was maintained. However, there were some differences between them; comparing the wild type to the mutant *S. cerevisiae* cells, Δeft1 (het), Δeft2 (het) and Δyef3 (het) appeared to be elongated. However Δerg2 (het) was more elongated with smaller cells which looked like new buds (Figure 4.17).

4.9. Characterization of top 6 antifungal WDF

To examine the morphology of the top 6 antifungal compound producing WDF, MEA plates were inoculated with the fungi and allowed to grow for 3 days. Well defined photographs of the WDF on the plate were taken. Close examination of the morphology of R7_PM_A showed that it had brown mycelia, which grew into the agar. R7_PM_B had dark mycelia with whitish mycelia at its edges, which grew on the surface of the agar. G6_PM_B had cream coloured mycelia, which grew on the surface of the agar. The mycelia remain compacted and grew out of the agar. The morphology of H6_PM_A was quite different from the others. It had fluffy looking mycelia, which grew on the surface of the agar. Both N6_PM_A and N6_PM_B had thick mass of mycelia which grew on the agar. The thick mass of mycelia was surrounded by fluffy looking mycelia, which grew into the agar (Figure 4.18).

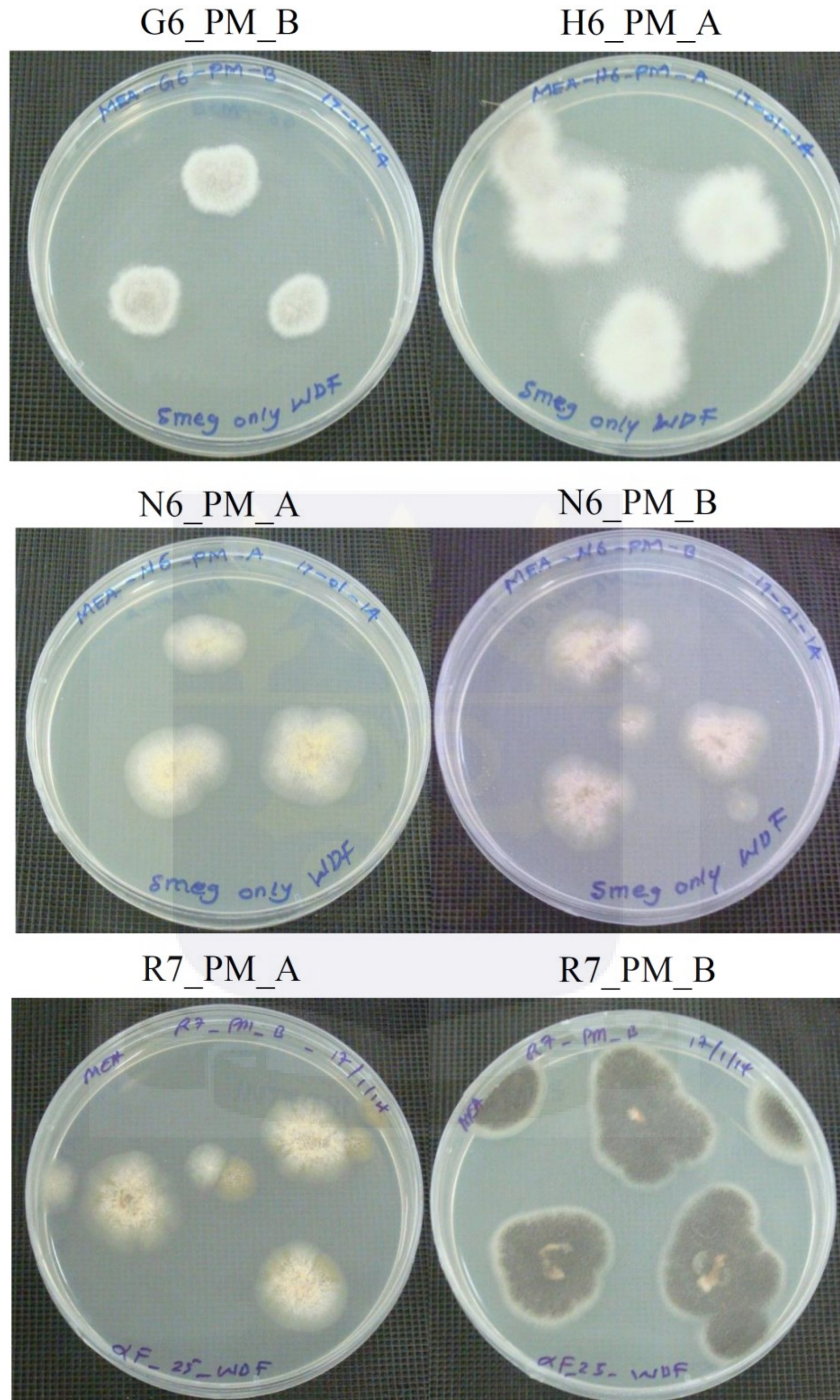

Figure 4.18: Colony morphology of the top six antifungal WDF. *The top 6 WDF were cultured on MEA plates and their plate morphology examined.*

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CHAPTER FIVE

DISCUSSION

Previous work done by Aboagye (2011), showed the potential of Wood Decaying Fungi (WDF) to produce antifungal agents. From his work, 54 WDF were collected and screened, out of which 31% had activity against *C. albicans.* Thus, this project sought to isolate and characterize antifungal compounds from Wood Decaying Fungi from Ghana. The WDF were cultured in potato dextrose broth (PDB) and their metabolites extracted with ethyl acetate. The extracts were studied for their antifungal properties.

5.1. The best source for obtaining mineral supplementation for WDF cultures

Secondary metabolism in fungi has become a promising source of novel bioactive compounds (Sakai *et al*., 2012). However the routine culturing of WDF in our laboratory demonstrated that the normal media for culturing WDF, (potato dextrose broth) does not fully support the production of these metabolites. It was found that, the genes responsible for the production of these metabolites were silent under standard laboratory conditions (Sakai *et al*., 2012). To explore if mineral supplementation could induce the production of antifungal agents, two WDF (B7_new_BM and B7_old_PM), which were found not to produce antifungal activity.

The standard media potato dextrose broth (PDB) for culturing WDF does not provide the WDF with the entire minerals required for their growth and maximum production of bioactive metabolites (Sharma and Sharma, 2011). In view of this, well-defined mineral salts, soil extract, and seawater were used as sources of mineral for the enrichment of the PDB.

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From the chromatogram of the WDF extracts obtained from the modified PDB cultures gave bright and dark thick bands, which were faint in the unmodified PDB. This was remarkable because the mineral enrichments appeared to be influencing the production of some specific compounds. Out of the mineral sources screened, soil extract had the most consistent band patterns on the chromatogram. Although soil extract modified PDB gave lower yield of the crude, it enhanced the bioactivity of the extracts obtained when tested against the selected organisms (*Methicillin Susceptible Staphylococcus aureus (MSSA GGP 200), Methicillin Resistant Staphylococcus aureus (MRSA GGP E120), Mycobacterium smegmatis (M smeg ETHZ_2), Erythromycin Resistant Mycobacterium smegmatis (Ery M. smeg) C. albicans with cycloheximide treatment (C.a. CyH), S. cerevisiae with cycloheximide treatment (S.c. CyH), C. albicans and S. cerevisiae).*

The yield of the soil extract relative to its bioactivity demonstrated a 10-fold increase in activity compared to the unmodified media. In a similar work by Pradeep and Pradeep, (2013), KH_2PO_4 modified PDB increased pigment and biomass production in four *Fusarium moniliforme* isolates. Soil naturally is rich in potassium, which may exist as phosphates. These minerals may be responsible for the increase in the production of bioactive compounds from the WDF. In all, soil extract supplementation may serve as a better source of minerals, which supported bioactive compound production in the WDF compared to seawater supplementation and the defined mineral salt supplementation.

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5.2 Media quality is critical in maintaining batch-to-batch consistency of WDF metabolite production

In the laboratory setting, there were several factors that influenced the batch to batch consistency of fermentation products (Valentinotti *et al*., 2003). In the previous experiment (Figure 4.1), some level of inconsistency was observed from the extracts obtained from sea water modified PDB. It was therefore necessary to examine the critical conditions that influence batch-to-batch variations in the fermentation products of WDF.

The first factor considered was the aeration of the culture. The two models used were 100 ml liquid in 500 ml high-density polyethylene bottles, which represented good aeration and 400 ml liquid broth in 500 ml bottles, which represented poor aeration. Each culture volume had 5 replicates and the consistence within 5 replicates was examined. The 100 ml cultures gave 4: 5 aeration to culture ratio while the 400 ml gave1 1: 5 ratio. The cultures were agitated once daily by swirling. Aeration within the 100 ml cultures was more efficient than the 400 ml cultures.

From the results the 400 ml cultures had high yield compared to the 100 ml cultures. The abundance of nutrient in the 400 ml cultures compared to the 100 ml cultures may be the reason for the increase in yield. The band patterns of the replicates on the chromatogram revealed that the 100 ml volume supports the production of consistent band patterns. When the extracts were tested against different microorganisms, the 400 ml cultures had higher zones of inhibition compared to the 100 ml cultures. This may be due to the higher yield of extracts obtained from these cultures. Nonetheless, the replicates of the 100 ml cultures were consistent inhibitory activity against the test organisms. To obtain

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consistent production of metabolites from WDF, the liquid cultures needed to be well aerated.

Submerged fermentation of mushroom was reported to be a good method for increasing the production of bioactive metabolites (Mshandete and Mgonja, 2009). Fungi in submerged cultures depend on dissolved oxygen in the media which when used up induces anaerobic respiration in the fungus (Asaff *et al.*, 2009). It is therefore worth noting that the high yield and inhibitory activity by the poor aerated cultures might be linked to the anaerobic fermentation of the WDF. The bioactive compounds may be produced through anaerobic fermentation pathways. For some the WDF submerged cultures, the mycelia carpets floated on the media while others were submerged within the media, creating uniform anaerobic condition among the replicates. This might be the cause of the high level of inconsistency among the replicates of the 400 ml cultures.

The second factor considered was richness of the culture. Potato dextrose broth (PDB) was used as the standard nutrient media while yeast extract, peptone, malt extract and dextrose (YPMD) broth was used as a model for enriched nutrient media. The PDB was fortified with mineral salts to determine the effect of mineral salt on the consistency and growth of the WDF. Each condition had 5 replicates and the consistency within 5 replicates was examined.

From the chromatograms obtained, a high level of consistency was observed with the YPMD cultures especially the 100 ml cultures. The 100 ml cultures were more properly aerated than the 400 ml cultures hence it supported the growth of the WDF. In addition, the YPMD cultures produced consistent inhibitory activity against two of the test

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organisms used compared to the PDB and PDB with mineral salt enrichment. A remarkable consistent inhibition was observed with the extracts obtained from the B7_old_PM cultures, which was not expected. On the contrary, a higher level of inconsistency was observed for the B7_new_BM PDB only cultures. YPMD contained a rich protein source (peptone) and the malt and yeast extracts provided the other essential nutrients, which enhanced the growth of the WDF and improved the quality of the metabolites they produced. This was consistent with the work done by other scientist were it was reported that rich protein and glucose growth medium was required for the production of bioactive molecules (Pradeep and Pradeep, 2013).

5.3 The need for primary screening of wood decaying fungi

Wood decaying fungi are essential in decaying wood in the forest. In spite of their destructive and recycling ability (Lonsdale *et al*., 2008b), they also produce metabolites that can serve as leads for new drugs. In present study, 189 WDF were collected and screened for their antifungal properties. From this screen 33 were selected for their antifungal property. This was consistent with other studies where natural libraries consisting of many compounds or extracts were screened for their antifungal properties and only a few hits were obtained (Breger *et al*., 2007; Golebiowski *et al*., 2003; Rakotonirainy and Lavidrine, 2005). Most of the crude extracts from these WDF had low antifungal activity. The active compounds in the crude extracts may be in low concentrations or inhibited by other compounds that may be present in the crude extracts.

5.4 The usefulness of mycelia isolation as a source of inoculum

The primary screen for antifungal agents from wood decaying fungi (WDF) was carried out from vegetative part (fruiting bodies) of the WDF cultured in Potato Dextrose Broth

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(PDB). However, the fruiting bodies of the WDF picked from the forest were in limited supply and several attempts to regrow them on different forms of wooden substrates proved futile. To obtain a constant and continuous source of fungal inoculum for the production of bioactive compounds in the laboratory, mycelia from the WDF grown on agar plates were resorted to. The 33 WDF selected from the primary screen were cultured on agar plates to obtain mycelia for future propagation. A similar approach was used by Kauserud *et al*., (2008) to overcome the challenge of getting a continuous source of fungal inoculum.

5.4.1. The potential of plate mycelium of wood decaying fungi fruiting body

A modified method of De Jong *et al*., (1992), was used to isolate mycelium from the fruiting bodies. Pieces of the fruiting bodies were placed at four inoculation points on the agar plates. The ability of the fruiting bodies to grow on the agar plates depends on the culture medium and viability of the spores in the fruiting bodies (Barrasa *et al*., 2009). The 33 fruiting bodies collected were propagated on Sabouraud Dextrose Agar (SDA). Twelve fruiting bodies germinated from all the three points of inoculation. However, 7 out of the 33 germinated from two inoculation points and 8 germinated from only one inoculation point. This observation could be due to the long period of storage (between 1 to 2 years) which might have caused a reduction in the viability of the fungal spores. SDA did not support the growth of 6 of the fruiting bodies. This may be due to the fact that these 6 fungi may require different nutritional supplement other than that provided by SDA. Malt extract agar (MEA), a richer medium (Skaar and Stenwig, 1996) was used to enhance the germination of the remaining 6 fruiting bodies. On the MEA 3 of the fruiting bodies germinated from all three-inoculation points while 1 germinated from 2

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points. Two fruiting bodies however did not germinate on any of the media used. In all, 31 WDF fruiting bodies germinated and were cultured on MEA plates for storage.

5.4.2. Plate mycelium purification by re-isolation from broth cultures of newly isolated plate mycelium

To ensure that the mycelia isolated were distinct and reproducible in morphology and bioactivity, the 31 mycelia stored on plates were propagated in liquid PBD in duplicates giving a total of 62 broths. Mycelia from these broths were harvested and cultured on agar plates (PM1) and their morphology was compared to that obtained from fruiting bodies on plates (PM). Since it was the same fungal species propagated on plate and broth, 76% similarity was observed between the PM and PM1. However, 15% of the PM1 were not similar to their PM counterparts. One of the broth mycelia did not grow on the agar plates and this may because they were adapted to growing only in liquid cultures. The mycelium placed in the broth may not have germinated or may germinate and perish during the culturing period without producing spores (Kües and Liu, 2000).

5.4.3: The antifungal potential of extracts from plate mycelium of WDF

From the study, it was observed that 23 of the extracts from plate mycelia of WDF had remarkable antifungal activity. The inability of the others to show activity against *C. albicans* and *S. cerevisiae* may be due to low production of the bioactive compounds after the mycelial isolation as seen in a similar work by Elisashvili, (2012) where the production of lectin by mycelia was reduced in submerged cultures compared to the fruiting bodies. Their inability to produce antifungal compound may also be due to sub-optimum laboratory culture conditions which may not correspond to their natural habitat (Collado *et al*., 2008). In order to determine whether the extracts without antifungal

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activity had not lost their activity entirely, they were retested against the *C. albicans* and *S. cerevisiae* in the presence of paromomycin and cycloheximide. Paromomycin and cycloheximide are protein inhibitors that increased the sensitivity of *S. cerevisiae* during the phenotypic screening experiment (Section 5.3). In the presence of the protein inhibitor, 7 of the WDFs showed antifungal activity against *C. albicans* and *S. cerevisiae.* This result points to the fact that these extracts might still have their antifungal property but may be present in low quantities. This might possible explain the absence of antifungal activity in this extracts. Work done by Elisashvili, 2012 supported the fact that some metabolites of medicinal value are produced in lower quantities in submerged mycelia cultures compared to the fruiting body cultures.

From the study it was observed that 8 WDF extracts out of the 23 showed similar levels of activity among their duplicates (A and B) when tested against *C. albicans*. When against *S. cerevisiae*, 4 WDF extracts out of the 23 showed similar levels of activity among the duplicates (A and B). In total, 15 out of the 31 WDF (48.4%) from the primary screen retained their anti-candida activity after the mycelia isolation. These data did not correspond to the data obtained from the plate morphology comparison in which the PM1 had 76% similarity with their PM isolates. Although only 48.4% of the WDF retained their primary anti-candida activity, the mycelia isolation cannot be said to be an inappropriate approach for obtaining a continuous source of fungal inoculum, because distinct WDF isolates were obtained from this approach. In addition, unlike the fruiting body cultures, the antifungal activity obtained after the mycelia isolation had a high level of reproducibility. The 23 out the 62 extracts with antifungal activity were selected as individual extracts for phenotypic screening to validate their antifungal activity.

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5.5 Developing a technique for predicting the potential of antifungal extracts yielding new chemical entities

Some frequently used antifungal drugs on the clinic have become ineffective due to the development of resistance against them. There is an urgent need for the discovery of lead compounds for antifungal drug development (Nucci and Perfect, 2008). These leads must have mode of action different from that of the present antifungal drugs on the market in order to avoid the development of cross-resistance against them. In the light of these, phenotypic screens were developed in our laboratory to predict the potential of antifungal extracts to produce new chemical entities. The screening assays used to identify these compounds were a slight modification of the classic disc diffusion screening method. From the phenotypic screen, each WDF extract generated a pattern of activity against the test organisms under different chemical conditions. The sensitivity of the test organisms to the extracts in the presence of the chemical modifications was used to predict the presence of molecules with novel modes of action. In addition to the novelty of the WDF extracts, the phenotypic screening assay offered us the chance to reduce redundancy among the extracts selected; extracts with similar modes of action may consist of the same or similar active compounds.

The antifungal activity of the 23 selected PM WDF extracts was validated through two phenotypic screening assays. Firstly, the extracts were taken through a chemical phenotypic screening then through another phenotypic screening using mutant strains of *S. cerevisiae*. This was done to select the best six antifungal extracts with novel modes of action.

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Kvitek *et al*., (2008) have suggested that the phenotype expressed by *C. albicans* and *S. cerevisiae* is influenced by environmental factors such as glucose levels. These factors trigger the expression of genes responsible for producing compounds that cause the morphology switching in these organisms. *C. albicans* was the first eukaryotic organism to have been discovered to produce farnesol which causes morphology change in response to a serum factor (Nickerson *et al*., 2006). The morphology switches due to farnesol cause the organism to switch between its pathogenic and non-pathogenic forms. It was therefore of interest to examine the effect of the morphology switching induced by farnesol and other compounds that act in the same way (cycloheximide, N-acetyl glucosamine and phenyl ethyl alcohol) on the sensitivity of *C. albicans* and *S. cerevisiae* to the WDF extracts and standard antifungal drugs. Two other compounds, cycloserine and 3-O-methylglucose (3-oMG) which are known to influence the sensitivity of yeast cells to antifungal drugs (Akache and Turcotte, 2002) but do not trigger morphology switching were also employed in the chemical phenotypic screening. The sensitivity of *C. albicans* and *S. cerevisiae* to the 23 WDF extracts on the chemically modified plates were used as a scoring matrix to rank the extracts.

The present study demonstrated that, cycloheximide increased the sensitivity of *C. albicans* and *S. cerevisiae* to the fungal extracts. It changed the morphology of *S. cerevisiae* from spherical to rod-like forms. Cycloheximide is a known protein synthesis inhibitor (Baliga *et al*., 1969). The inhibition of protein synthesis in the *S. cerevisiae* cells may be responsible for increasing the susceptibility of these cells to the WDF extracts. The sensitivity of the *S. cerevisiae* cells to fluconazole and amphotericin B was reduced in the presence of cycloheximide. Fluconazole being an azole exerts its antifungal

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activity through the inhibition of lanosterol-14-demethylase (Carrillo-Munoz *et al*., 2006) and amphotericin B on the other hand interacts with ergosterol in the membrane to be transported to the vacuole where it creates pores to leak its components leading to cell death (Baginski and Czub, 2009). Since cycloheximide is a protein synthesis inhibitor, it might reduce ergosterol biosynthesis and therefore increase resistance of the cells to amphotericin B. In addition, morphological switch induced by cycloheximide causes biofilm formation which was likely to reduce the uptake of the antifungal drugs by the cells (Uwamahoro and Traven, 2010). Unlike *S. cerevisiae*, cycloheximide increased the sensitivity of the *C. albicans* to the standard antifungal drugs.

The budding yeast *S. cerevisiae* is a powerful model system for screening and the identification of bioactive small molecules (Gassner *et al*., 2007). In the present study, two major mutants of *S. cerevisiae* were used. They had deficiency in ergosterol biosynthesis or protein synthesis. Comparing the mutant cells, Δerg (het) was the most sensitive strain to extracts of the WDF. However, it was sensitive to only 10 out of the 23 WDF extracts. Matching the activity of the extracts together with all the mutant strains, 6 WDF extracts stood out as the top six antifungal extracts. Ranking the WDF extracts based on the total activity across all the chemical modification assays and the mutant *S. cerevisiae* assay, H6_PM_A, G6_PM_B, R7_PM_B, N6_PM_A, N6_PM_B and R7_PM_A were found to be the top 6 potent extracts. Each of the six selected WDF had a different pattern of activity showing that each may contain a distinct compound with a distinct modes of action.

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5.6. Morphology of *C. albicans* and *S. cerevisiae* in the presence of phenotypic modifiers

Morphological switching is essential for the pathogenesis of *C. albicans.* There are some chemical factors in the blood which are responsible for this switching (Uwamahoro and Traven, 2010). Chemicals such as cycloheximide and farnesol are also used to induce morphological switching in yeast cells (Nickerson *et al*., 2006). In the present study, YPD agar plates were modified with chemicals, which interfere with the sensitivity of yeast cells to antifungal agents. The cells from the modified plates were stained with either gram stain or acid-fast stain. Examination of the morphology of the stained cells, showed that cycloheximide switched the morphology of *S. cerevisiae* cells from the spherical shape to a rod-like shape. The rods looked like the pseudo-filamentous form of *S. cerevisiae*, which made the *S. cerevisiae* more susceptible to the WDF extracts. Other compounds such as farnesol and phenyl ethyl alcohol inhibited filament formation in the *S. cerevisiae* cells as expected. As described by Jensen *et al*., 2006, these chemical modifications induced frequent budding of the *S. cerevisiae* cells compared to the untreated cells

In order to examine the effect of the mutations on the morphology of the *S. cerevisiae*, the mutant strains were cultured on YPD agar plates and stained with gram stain or acid fast stains. The mutations however did not have any significant effect on the shape of the cells. The mutant cells had similar cell shape as the wild type cells. It was also observed that ergosterol deficient mutant cells Δerg2 (het) and Δerg2 (hom) had more buds per field than the wild type. Nonetheless, the other mutant cells also had a number of buds but not as much as the ergosterol deficient cells. The bud concentration per field indicates

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the rate of cell division. Although the ergosterol mutation favoured rapid cell division it increased the sensitivity of the mutants to the WDF extracts. Similar observations were made with ergosterol mutant *Trichoderma harzianum* species. The *T. harzianum* cells with the partial silencing of the erg1 gene gave rise to transformants with a higher level of sensitivity to terbinafine (Cardoza *et al*., 2006).

5.7. The top 6 wood decaying fungi with antifungal properties

Yeast is widely used as a model organism for investigating the mode of action for many drug entities (Simon and Bedalov, 2004). The sensitivity of yeast mutants to drug entities is used to predict its action in the cell. From the present study, the yeast mutants were found to have different levels of sensitivity to the top six WDF extracts. This was used to characterize the WDF extracts and also predict the modes of action of the active components in these crude extracts.

The erg 2 gene has been implicated in ergosterol biosynthesis in yeast cells (Soustre *et al*., 2000). The present study showed that, crude extract from R7_PM_A was only active against Δerg 2 (het). The absence of this activity in the wild type *S. cerevisiae* therefore indicated that inhibition of ergosterol biosynthetic pathway might be responsible for its antifungal action. Unlike R7_PM_A, three of the six selected extracts (G6_PM_B, H6_PM_A and R7_PM_B) were less specific. They had antifungal activity against all the mutant cells signifying that these crude extracts had more than one bioactive molecule giving the different activities observed. Individually, R7_PM_B was found to produce extracts with higher activity against Δerg2 (hom), Δerg2 (het) and Δeft2 (het) compared to the wild type. Since the Δeft2 (het) mutants were partially deficient in protein synthesis (Perentesis *et al*., 1992), it was evident that this crude extract might interfere with

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ergosterol biosynthesis or protein synthesis in the yeast cells. Similar observations were made for N6_PM_B extract. It was active against all the mutant cells except Δeft1. Although H6_PM_A extract had activity against all the mutant strains, these activities were low compared to the wild type with the exception of the activity observed against Δerg2. This signifies that the extract produced by H6_PM_B might be an inhibitor of the ergosterol biosynthetic pathway.

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CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

The culture conditions for the production of bioactive metabolites were optimized. It was observed that supplementing PDB with minerals from the soil improved growth and metabolite production of the WDF. The study showed that aeration, mineral supplementation and media richness were as critical factors that influence the batch-to-batch consistency of metabolites produced by WDF.

A total of 189 wood decaying fungi (WDF) were screened for their antifungal properties of which 33 were selected for mycelia isolation. Mycelia were isolated from 31 out of the 33 selected wood decaying fungi fruiting bodies on to SDA or MEA plates. Two fruiting bodies however failed to germinate on any of the agar plates.

The viability and metabolite producing ability of the 31 WDF isolated mycelia was examined by culturing them in PDB. Each of the 31 WDF isolated mycelia yielded 2 sets of mycelia from the broth cultures, which amounted to 62 isolated mycelia. The TLC band patterns of the extracts from the liquid cultures proved that distinct WDF isolates were obtained. The plate morphology of the mycelia isolated onto agar plates from the liquid cultures was compared with the mycelia isolated from the corresponding fruiting bodies. The results demonstrated that, mycelia can be isolated from fruiting bodies onto agar plates and used as a continuous source of inoculum. Propagation of the plate mycelia in liquid cultures had no significant effect on the plate morphology of the WDF.

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Although there was a high level of distinction among the mycelia isolated and high similarity between the duplicates from the same fruiting body, each of the 62 isolated mycelia was considered as a distinct inoculum source. After the secondary screening, 23 out of the 62 WDF extracts retained their antifungal activity. Seven of the extracts, which had no activity in the secondary screen, had activity when retested in the presence of cycloheximide or paramomycin. This revealed that these isolates may not lose their antifungal property completely and may have produced the antifungal agents in low levels.

The antifungal activity of the 23 WDF extracts were validated by screening them against mutant *S. cereviaise* alongside wild type *S. cereviaise* and *C. albicans* in a chemical phenotypic assay. From these assays the top six WDF active extracts were selected. The top six WDF extracts had antifungal activity against *S. cerevisiae* in the presence of cycloheximide while this activity was absent in fluconazole (a standard azole). This suggested that the WDF extracts might contain compounds with different cellular targets other than that of fluconazole. From the study, it was deduced that the WDF extracts might inhibit ergosterol biosynthesis or protein elongation in the fungi to elicit their antifungal activity. These observations would have been conclusive if fractions or pure compounds isolated of the crude extracts produced similar results.

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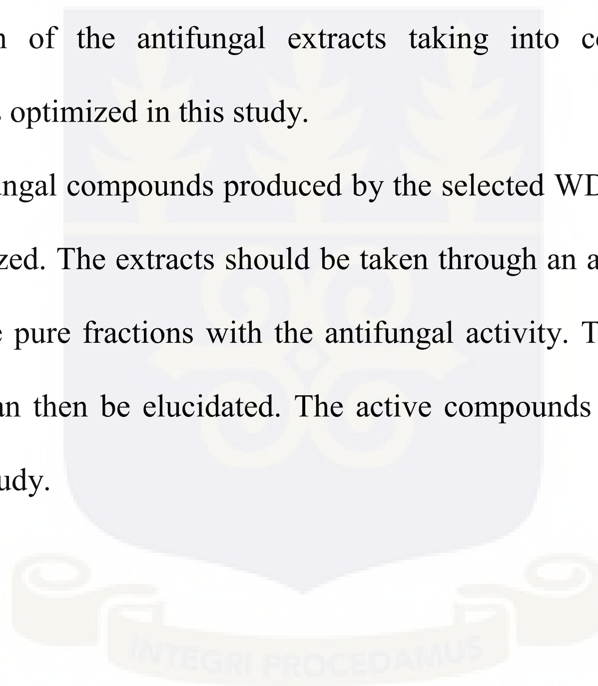
5.1. Recommendations



1. Mycelia should be isolated from WDF fruiting bodies cultured directly in liquid media to compare their morphology and antifungal activity with their plate mycelia (PM).
2. The extracts without antifungal activity should be tested against the test organisms on a wider array of chemical modification to amplify their activity.
3. The top six WDF isolates should be cultured in large quantities to scale up the production of the antifungal extracts taking into consideration the culture conditions optimized in this study.
4. The antifungal compounds produced by the selected WDF should be isolated and characterized. The extracts should be taken through an activity-guided fraction to isolate the pure fractions with the antifungal activity. The structures of the pure isolates can then be elucidated. The active compounds should be taken through toxicity study.

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APPENDIX

Table A 1: Antifungal activity of 33 selected wood decaying fungi**.**

Extract	Zone of inhibition (mm)	Extract	Zone of inhibition (mm)
B6	8.18	G3	7
B8	0	G4	20
B9	7.21	G6	17
D2	11.73	H4	0
D3	14.7	H6	20
D4	11.65	I2	17
D5	13.73	J2	27
E3	10.64	N5	7.5
E4	8.75	N6	15
E5	11.04	Q8	10
E6	10.27	R4	7
E7	10.9	R7	7
F1	9.7	S2	6
F4	8.62	S4	10
F5	10.16	S7	6
F6	11.87	T6	10.5
F7	10.5		

The ethyl acetate extracts of 33 WDF cultured in 500ml PDB for four weeks were tested against C. albicans. The table represents the average zones of inhibition of the WDF extracts (Figure 4.1).

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Table A 2: Antifungal activity of 62 plate mycelia extracts against *C. albicans* and *S. cerevisiae*

	Zone of inhibition (mm)				
Extract	***C. albicans***	***S. cerevisiae***	**Extract**	***C. albicans***	***S. cerevisiae***
B6_PM_A	9	10	F6_PM_B	0	0
B6_PM_B	6.5	0	F7_PM_A	0	0
B8_PM_A	0	0	F7_PM_B	0	0
B8_PM_B	0	0	G3_PM_A	7	0
B9_PM_A	0	0	G3_PM_B	6.5	9
B9_PM_B	0	0	G4_PM_A	0	0
D2_PM_A	0	0	G4_PM_B	8	6
D2_PM_B	0	0	G6_PM_A	0	7
D3_PM_A	0	0	G6_PM_B	17	11
D3_PM_B	0	0	H6_PM_A	20	16.5
D4_PM_A	0	0	H6_PM_B	0	0
D4_PM_B	0	0	I2_PM_A	0	0
D5_PM_ B	0	0	I2_PM_B	0	0
D5_PM_A	0	0	J2_PM_A	6.5	6
E3_PM_A	0	0	J2_PM_B	10	6
E3_PM_B	0	0	N5_PM_A	7.5	0
E4_PM_A	0	0	N5_PM_B	0	0
E4_PM_B	0	0	N6_PM_A	10	11
E5_PM_A	0	0	N6_PM_B	15	8
E5_PM_B	0	0	Q8_PM_A	0	0
E6_PM_A	0	0	Q8_PM_B	0	0
E6_PM_B	0	0	R4_PM_A	7	0
E7_PM_A	0	0	R4_PM_B	7	0
E7_PM_B	0	0	R7_PM_A	7	7.5
F1_PM_A	11.5	0	R7_PM_B	13.5	17
F1_PM_B	7	0	S2_PM_A	8.5	0
F4_PM_A	0	0	S2_PM_B	0	0
F4_PM_B	0	0	S7_PM_A	6	6
F5_PM_A	7	0	S7_PM_B	6	0
F5_PM_B	0	0	T4_PM_A	0	0
F6_PM_A	0	0	T4_PM_B	0	0

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Table A 3: Antifungal activity of previously inactive plate mycelia extracts on modified media.

Extracts	*S. cerevisiae (paramo)*	*C. albicans (paramo)*	*S. cerevisiae (Cyc H)*	*C. albicans (cyc H)*
Flu (5)	*37*	*26*	*0*	*24.5*
AmpB (10)	*13*	*9*	*0*	*7.5*
CyH (50)	*0*	*0*	*0*	*0*
B9_PM_A	0	0	0	0
B9_PM_B	0	0	0	0
D2_PM_A	0	0	0	0
D2_PM_B	0	0	0	0
D3_PM_A	0	6	0	0
D3_PM_B	0	0	0	0
D4_PM_A	0	0	0	0
D4_PM_B	0	0	0	0
D5_PM_A	0	0	0	0
D5_PM_B	0	0	0	0
E3_PM_A	0	0	0	0
E3_PM_B	0	0	0	0
E4_PM_A	0	6	0	0
E4_PM_B	0	0	0	0
E5_PM_A	0	0	0	0
E5_PM_B	0	0	0	0
E6_PM_A	0	0	0	0
E6_PM_B	0	0	0	0
E7_PM_A	0	0	0	0
E7_PM_B	0	0	0	0
F4_PM_A	0	0	0	0
F4_PM_B	0	0	0	0
F5_PM_B	0	0	0	0
F6_PM_A	0	0	0	0
F6_PM_B	0	0	0	0
F7_PM_A	0	0	0	0
F7_PM_B	0	0	0	0
G4_PM_B	0	6	21	6
I2_PM_A	0	0	8.5	0
I2_PM_B	0	0	9.5	0
Q8_PM_A	0	0	0	0
Q8_PM_B	0	0	0	0
S2_PM_B	12.5	0	0	0
T4_PM_A	0	0	0	0
T4_PM_B	0	0	0	0
B8_PM_A	6	0	0	7
B8_PM_B	0	0	0	0
H6_PM_B	0	0	0	0
N5_PM_B	0	0	0	0

The 49 out of 62 PM extracts without antifungal activity in the secondary screen were retested against C. albicans and S.cerevisiae on YPD agar plates modified with either 50ug/ml paromomycin (Paro) or 50ug/ml cycloheximide (CycH)

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Table A 4: Chemical phenotypic screening of 23 selected wood decaying against C. albicans

	Zones of inhibition (mm)						
Extract	YPD	Far (25uM)	PEA (0.15mg/ml)	NAG (10nM)	CyS (1mM)	3-oMG (10mM)	CyH (0.25mg/ml)
Flu (5)	9	23	19	17	13.5	17.5	28.5
AmpB (10)	8	0	6.5	7.5	6	8	8.5
CyS (20)	0	0	0	0	0	0	0
B6_PM_A	6	0	0	0	0	0	6
B6_PM_B	6	0	0	0	0	0	0
F1_PM_A	9.5	0	8	0	0	0	6
F1_PM_B	0	0	0	0	0	0	0
F5_PM_A	0	0	0	0	0	0	0
G4_PM_A	0	0	0	0	0	0	0
J2_PM_A	0	0	0	0	0	0	0
J2_PM_B	12	7	0	0	0	0	0
R7_PM_A	14.5	8	13	8.5	8	7	23.5
R7_PM_B	13.5	11.5	17	8.5	10	9.5	6
S2_PM_A	6	0	11	0	0	0	0
S7_PM_A	0	0	0	0	0	0	0
S7_PM_B	0	0	0	0	0	0	18.5
G3_PM_A	6	0	0	0	0	0	12.5
G3_PM_B	6	0	0	0	0	0	0
G6_PM_A	0	0	0	0	0	0	0
G6_PM_B	19	14	15	14.5	14.5	15.5	21.5
H6_PM_A	12.5	15	19	0	0	8	38
N5_PM_A	7	0	0	0	0	0	0
N6_PM_A	0	0	16.5	0	0	0	38
N6_PM_B	14	10.5	13	0	0	0	34.5
R4_PM_A	0	0	0	0	0	0	17
R4_PM_B	0	0	0	0	0	0	0

For each assay, 0.5 units (1 ml supernatant/culture equivalent) of each extract were used. The plates were modified with farnesol (Far), phenyl ethyl alcohol (PEA), Cycloserine (Cys), N-acetyl glucosamine (NAG), Cycloheximide (CyH) and 3-O-methylglucose (3-oMG)

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Table A 5: Chemical phenotypic screening of 23 selected wood decaying fungi extracts against *S. cerevisiae*

	Zones of inhibition (mm)							
	YPD (10uL)	YPD	Far (25uM)	PEA (0.15mg/ml)	NAG (10nM)	CyS (1mM)	3-oMG (10mM)	CyH (0.25mg/ml)
Flu (5)	9		6	11.5	11	13.5	14.5	0
AmpB (10)	10		10	9.5	9.5	11	12	0
CyS (20)	0		0	0	0	0	0	0
B6_PM_A	7	9	0	6.5	7	6	7	15
B6_PM_B	0	6	0	0	0	0	0	13.5
F1_PM_A	0	0	0	0	0	0	0	19
F1_PM_B	0	0	0	0	0	0	0	22
F5_PM_A	0	0	0	0	0	0	0	0
G4_PM_A	0	0	0	0	0	0	0	22
J2_PM_A	0	0	0	0	0	0	0	10
J2_PM_B	0	0	0	0	0	0	0	23
R7_PM_A	0	6	0	7	0	0	0	16.5
R7_PM_B	11	17	15	15	14	12	12	49
S2_PM_A	0	0	0	0	0	0	0	0
S7_PM_A	0	0	0	0	0	0	0	0
S7_PM_B	0	0	0	0	0	0	0	0
G3_PM_A	0	0	0	0	0	0	0	0
G3_PM_B	0	0	0	0	0	0	0	13
G6_PM_A	0	0	0	0	0	0	0	17.5
G6_PM_B	12.5	14.5	12.5	13	11.5	12.5	13	21
H6_PM_A	16.5	16	17.5	16	15	14.5	17	29
N5_PM_A	0	0	0	0	0	0	0	11
N6_PM_A	8	11	10.5	10	7.5	9.5	12	6
N6_PM_B	7	9	10	6.5	0	6	6	12
R4_PM_A	0	0	0	0	0	0	0	6
R4_PM_B	0	0	0	0	0	0	0	0

For each assay, 0.5 units (1 ml supernatant/culture equivalent) of each extract were used. The plates were modified with farnesol (Far), phenyl ethyl alcohol (PEA), Cycloserine (Cys), N-acetyl glucosamine (NAG), Cycloheximide (CyH) and 3-O-methylglucose (3-oMG).

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Table A 6: Phenotypic screening of 23 selected wood decaying fungi extracts against the mutant *S. cerevisiae* strains

	Zones of inhibition (mm)					
	WT	Δerg2 (hom)	Δerg2 (het)	Δeft1 (het)	Δeft2 (het)	Δyef3 (het)
Flu (5)	9	13.5		13.5	16	13.5
AmpB (10)	10	10.5		10	11.5	9.5
CyS (20)	0	0		0	0	0
B6_PM_A	7	0	0	0	0	0
B6_PM_B	0	0	0	0	0	0
F1_PM_A	0	0	7	6	0	0
F1_PM_B	0	0	8	6	0	0
F5_PM_A	0	0	7	0	0	0
G4_PM_A	0	0	0	0	0	0
J2_PM_A	0	0	0	0	0	0
J2_PM_B	0	0	0	0	0	0
R7_PM_A	0	0	9.5	0	0	0
R7_PM_B	11	14	12	9	13	8.5
S2_PM_A	0	0	0	0	0	0
S7_PM_A	0	0	0	0	0	0
S7_PM_B	0	0	0	0	0	0
G3_PM_A	0	0	0	0	0	0
G3_PM_B	0	0	8	0	0	0
G6_PM_A	0	0	0	0	0	0
G6_PM_B	12.5	15	14.5	12	12.5	11
H6_PM_A	16.5	18.5	17	16.5	15	14
N5_PM_A	0	0	0	0	0	0
N6_PM_A	8	7.5	9	11	9.5	7.5
N6_PM_B	7	15.5	12	7	10.5	0
R4_PM_A	0	0	0	0	0	0
R4_PM_B	0	0	0	0	0	0

For each assay, 0.5 units (1 ml supernatant/culture equivalent) of each extract were used.

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Table A 7: The anti- *Methicillin Susceptible Staphylococcus aureus (MSSA)* of wood decaying fungi cultured in different mineral supplemented potato dextrose broth

Condition	Zone of inhibition (mm)	
	B7_new_BM	B7_old_PM
Control A	9.5	0
Control B	16.5	0
Min 1x A	0	0
Min 1x B	0	0
Min 5x A	0	0
Min 5x B	0	0
Sea 1x A	16.5	12
Sea 1x B	17	15.5
Sea 5x A	12.5	0
Sea 5x B	13.5	8.5
Soil 1x A	13.5	9.5
Soil 1x B	17	10.5
Soil 5x A	18	0
Soil 5x B	18.5	0

The extracts of B7_new_BM and B7_old_PM cultured in defined mineral salt (Min), sea water (Sea) and soil extract (Soil) modified media at 1x and 5x concentrations were tested against Methicillin Susceptible Staphylococcus aureus (MSSA).

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Table A 8: Effect of culture conditions on the consistency and production of anti-*Methicillin Susceptible Staphylococcus aureus (*MSSA) compounds by wood decaying extracts

	Replicates	Zone of inhibition (mm)	
Conditions		B7_new_BM	B7_old_PM
PDB ONLY 100mL	A	13.5	0
	B	15.5	0
	C	17	8
	D	15	0
PDB ONLY 400mL	E	16	14.5
	A	22.5	7.5
	B	23	7
	C	24.5	7
	D	22.5	9
YPMD 100mL	E	14.5	7
	A	13.5	26.5
	B	14	15.5
	C	11	19
	D	11.5	18.5
YPMD 400mL	E	15.5	15.5
	A	11.5	24.5
	B	19.5	13
	C	22.5	13.5
	D	6.5	14
Min 2x 100mL	E	15	8
	A	15.5	0
	B	15.5	0
	C	12.5	0
	D	12	0
Min 2x 400mL	E	20	15.5
	A	8.5	6
	B	9	0
	C	0	7
	D	11	12.5
	E	0	9.5

Two selected WDF (B7_new_BM and B7_old_PM) were cultured in 100/400ml of PDB modified with mineral salt, 100/400 ml of YPMD and 100/400ml of unmodified PDB. Extracts from the various cultures were tested against Methicillin Susceptible Staphylococcus aureus (MRSA) and their zones of inhibition measured.

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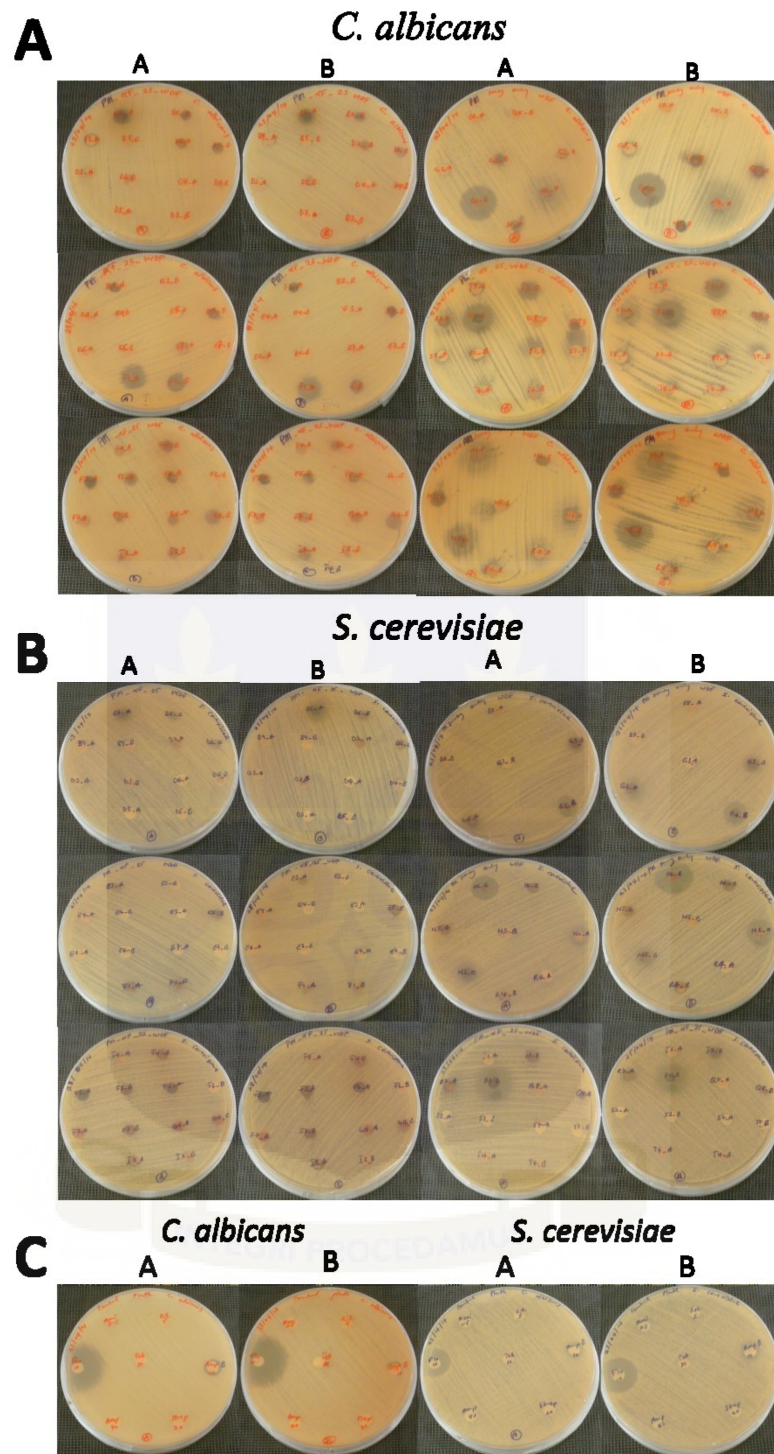

Figure A 1: *Antifungal activity of the 62 plate mycelia extracts.*

(A) *Antifungal activity of the extracts against C. albicans* ***(B)*** *Antifungal activity of the extracts against S. cerevisiae* ***(C)*** *Antifungal activity of control antibiotics and antifungal drugs against C. albicans and S. cerevisiae*