Isolation, Screening, and Molecular Analysis of Australian Freshwater Fungi for Antimicrobial Compounds

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List of Abbreviations

AAM	Antibiotic assay medium
AMR	Antimicrobial Resistance
C. albicans	Candida albicans
CDC	Centre for Disease Control and Prevention
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleotide triphosphate
DRBC	Dichloran Rose Bengal Chloramphenicol
E. coli	Escherichia coli
FQ	Fluoroquinolones
FTIR	Fourier Transform Infrared Spectroscopy
ITS	Internal Transcribed Spacer
LSU	Large Subunit
MRSA	Methicillin-resistant Staphylococcus aureus
$Na_2S_2O_3$	Sodium thiosulfate
NaClO	Sodium hypochlorite
NMR	Nuclear Magnetic Resonance
OD	Optical density

PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PYG	Peptone Yeast Glucose
Rf	Retention factor
RPB2	RNA Polymerase Subunit II
rRNA	Ribosomal ribonucleic acid
S. aureus	Staphylococcus aureus
TAE	Tris-Acetate-EDTA
TLC	Thin Layer Chromatography
TSA	Tryptone Soy Agar
TSB	Tryptone Soy Broth
Tween 20	Polysorbate 20
UV	Ultraviolet
ZOI	Zone of Inhibition

DECLARATION

I certify that this thesis does not include any material previously submitted for a degree or diploma at any university without proper acknowledgment and to the best of my knowledge and belief, it does not contain any material previously published or authored by another individual, except where due references are provided within the text.

Signed.....

Date.....06/05/2024.....

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ABSTRACT

Antibiotic resistance is a major global concern that has developed from overuse and misuse of antibiotics. This has resulted in significantly enhanced antimicrobial resistance (AMR). Pathogenic bacteria have developed resistance to antibiotics, causing a decline in the effectiveness of these medicines. As a result, new antibiotics must be discovered to combat AMR. Among various potential sources, freshwater fungi are regarded as one of the prospective sources for novel antimicrobial compounds. Freshwater fungi possess the capacity to generate a wide spectrum of secondary metabolites, several of which demonstrate potential pharmacological and industrial significance. Therefore, this study aims to discover novel antimicrobial compounds sourced from Australian freshwater fungi.

In this study, ten wood samples were collected from the Bridgwater River of Australia, and from that 56 fungi were isolated and forty-five were selected for the investigations including seven strains previously collected from the Flinders Ranges of Australia. Initially, the samples were examined on agar to assess the antimicrobial activity against bacteria as primary screening, and similarly on the solid-state selected as basmati rice for secondary screening. Furthermore, from five strains including, S64, S83, S93, S106, and FRW28A that showed significant antimicrobial activity, a scale-up process was conducted, followed by genomic extraction to isolate DNA from these cultures. This extracted DNA served as the template for subsequent PCR experiments to provide the template for PCR examination. The crude extract of compound S93 was analysed using Thin-layer chromatography on a silica gel plate. For future research, purification of antimicrobial compounds by column chromatography and structure elucidation can be performed.

Research plan

Hypothesis

This research hypothesizes that freshwater fungi can potentially provide novel antimicrobial compounds.

Aims of this project

The following aims were the focus of this research:

- Isolation of freshwater fungi from freshwater wood
- Screening of the isolated freshwater fungi
- Scaling -up the production of antimicrobial compounds from the selected fungi
- Molecular analysis of the selected fungi

CHAPTER 1 INTRODUCTION

1.1 Antibiotics

1.1.1 Discovery of antibiotics

The term ‘antibiotic’ originated from ‘antibiose,’ coined by Paul Vuillemin in 1890 to describe the antagonistic interaction between microorganisms, contrasting with symbiosis. Later, ‘antibiotic’ word described as naturally occurring secondary metabolites which produced by bacteria and fungi, capable of either inhibiting or killing other microorganisms (Nicolaou and Rigol, 2017). In 1947, Waksman established the definition of ‘antibiotic’ as a chemical compound produced by microorganisms, possessing the capability to inhibit the growth of and even to kill bacteria and other microorganisms (Waksman,1947). The discovery and production of antibiotics during the early twentieth century marked a significant milestone in medical history (Mohr, 2016). Antibiotics not only benefit individual patients by treating infections but also play a crucial role in reducing disease transmission within communities and leading to broader public health advantages (Cook and Wright, 2022). In addition to treating infectious diseases, antibiotics have helped numerous modern medical procedures that include, cancer therapy, cardiac surgery, and organ transplantation. Sir Alexander Fleming's discovery of penicillin in 1928 marked the inception of what came to be known as the ‘golden age’ of natural product antibiotic exploration, which reached its peak in the mid-1950s (Hutchings *et al.,* 2019). The identification of early antibiotics such as Salvarsan, Prontosil, and penicillin established a pattern for future drug discovery studies. The timeline from the 1950s to the 1970s represented a significant period in the discovery of novel antibiotic categories **Figure 1**. Subsequently, no new antibiotic classes have been identified (Aminov, 2010).

The evidence of tetracycline, an antibiotic, found in human skeletons from ancient Nubia and Egypt suggested the concept of using antibiotic-containing compounds in their diet like moldy bread to combat infections (Aminov, 2010). In the 1910s, Paul Ehrlich discovered that certain chemical dyes possibly kill selective bacteria, which helped lead to the development of the first modern antibiotic called salvarsan to treat syphilis disease (Hutchings *et al.*, 2019). In 1928, Alexander Fleming discovered that a penicillium Mold could produce a substance known as penicillin, which proved to be effective at killing *Staphylococcus* bacteria (Fleming, 1980). The 'Golden age' of antibiotic discovery, which was from the 1940s to the 1960s, was recognized by extensive research efforts to discover novel antibiotics (Muteeb *et al.*, 2023).

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Figure 1 The timeline illustrates the introduction of various antibiotic classes into medical use, categorized with assorted colours indicating their sources, including key dates regarding antibiotics provided at the bottom (Hutchings *et al.*, 2019).

1.1.2 Classification of antibiotics

Antibiotics are commonly classified based on numerous ways including molecular structure, mode of action, source or origin of activity, and spectrum of activity. Regarding spectrum, antibiotics are divided into a narrow spectrum which targets specific bacteria, and a broad spectrum, which impacts a wide range of Gram-positive and Gram-negative bacteria (Adzitey, 2015). The molecular structure serves as a dependable principle for categorizing antibiotics that are grouped into various classes including β-lactams, macrolides, aminoglycosides, sulphonamides, tetracyclines, and quinolones (Etebu and Arikekpar,

2016). The structure of antibiotics is linked to their diverse mechanisms of action. Primary targets of antibiotics within bacterial cells include protein synthesis, cell membrane function, cell wall synthesis, and nucleic acid synthesis. These processes play a key role in bacterial growth (Ullah and Ali, 2017). Antibiotics are classified based on their source, including natural compounds derived from microorganisms, semi-synthetic variants from natural products, and fully synthetic drugs. While natural antibiotics like benzylpenicillin and cephalosporins are associated with high toxicity, semi-synthetic antibiotics such as ampicillin and amikacin and others, offer improved therapeutic effects with lower toxicity levels (Pancu *et al.,* 2021).

1.2 The global antibiotic resistance crisis

1.2.1 Origin of antibiotic resistance

Antibiotic resistance is defined as the ability of microorganisms which includes bacteria, viruses, and a few parasites to resist the effects of antibiotics, designed to kill or inhibit them (Muteeb *et al.,* 2023). It has become a major concern to public health due to the low effectiveness of antibiotics caused by widespread antibiotic resistance (Orfali *et al.,* 2022). The antimicrobial resistance crisis is due to both the inappropriate use of current antibiotics and the limited availability of newer drugs (Aslam *et al.,* 2018). Multidrug resistance has rendered the treatment of diseases caused by both Gram-positive and Gram-negative bacteria increasingly challenging, making traditional antibiotic therapies ineffective (Velez and Sloand, 2016).

In 1930, Antibiotic resistance was initially observed with the introduction of sulphonamides, suggesting its presence in the natural environment during the pre-antibiotic era. However, there was no evidence available that indicated the presence of highly resistant pathogens

(Ghazala Muteeb *et al.*, 2023). The direct consequences of the antibiotic resistance process have become a significant worldwide threat in the 21st century (Sabtu *et al.*, 2015).

1.2.2 Historical review of antibiotic resistance

In the 1940s, indications of antibiotic resistance began to emerge. Sir Alexander Fleming who discovered penicillin was among the pioneers who warned about the possibility of penicillin resistance if it was not used adequately or for a sufficient duration (Aminov, 2010). In 1942, the initial instance of antibiotic resistance occurred with antibiotic penicillin with penicillin-resistant *Staphylococcus aureus* emerging. Methicillin was subsequently developed as a semi-synthetic antibiotic to combat penicillin-resistant *S. aureus*. However, methicillin-resistant *S. aureus* (MRSA) was identified in the same period as the drug introduction (Uddin *et al.*, 2021).

Enterobacteriaceae, which includes various strains of *Escherichia coli*, exhibit elevated levels of penicillin resistance, particularly to aminopenicillins. Between 1950 and 2001, around two-thirds of disease-causing *E. coli* strains in the US were resistant to ampicillin, with resistance rates continuing to increase (Lobanovska and Pilla, 2017). Further antibiotics that were discovered and the time when they became resistant are shown in **Figure 2**.

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Figure 2 Descriptive timeline of discovery and resistance of antibiotics (Dahal and Chaudhary, 2018).

The increase in multi/pan-drug resistant strains has led to higher rates of morbidity and mortality. Consequently, the ineffectiveness of antibiotic treatments against 'superbug' infections has contributed to the persistence and spread of multi-resistant species worldwide (Tanwar *et al.*, 2014).

1.2.3 Mechanisms of antibiotic resistance

Antibiotics primarily act on the biochemical and physiological processes of microbial cells, either inhibiting their growth or inducing death. Antibiotics like β-lactam target specific components of bacterial cell wall synthesis, such as transpeptidase, disrupting its function, leading to cell death. Similarly, glycopeptide antibiotics interfere with peptidoglycan precursor incorporation (Muteeb *et al.*, 2023) (**Figure 3**). Antibiotics that target protein synthesis include aminoglycosides, tetracycline, linezolid, chloramphenicol, and macrolides. Other antibiotics target cellular machinery and interfere with nucleic acid synthesis, such as rifampin and fluoroquinolones (FQ). Moreover, certain antibiotics have the capability to disrupt metabolic pathways and degrade the membrane matrix. These include sulfonamides, folic acid analogs, daptomycin, and polymyxins respectively (Chellat *et al.*, 2016).

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Figure 3 Different mechanisms of antibiotic resistance as antibiotics connect the cells of bacteria or microorganisms (Muteeb *et al.*, 2023).

1.2.4 Consequences of antibiotic resistance

It is suggested in 2019, antimicrobial resistance (AMR) contributed to approximately 4.95 million deaths worldwide. Among these, 1.27 million deaths were directly attributed to AMR, surpassing the death rates of HIV/AIDS and malaria (Thompson, 2022). The US Centre for Disease Control and Prevention (CDC) has estimated that over two million individuals in the US contract antibiotic-resistant infections annually, resulting in a minimum of 23,000 deaths attributed to these infections (Abadi *et al.,* 2019). In Europe annually, approximately 400,000 infections and 25,000 deaths occur due to the most common multidrug-resistant bacteria such as *S. aureus*, *E. coli*, *Enterococcus faecium*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* (Norrby *et al.,* 2009). Around 60%-70% of infections are attributed to staphylococcal strains, primarily *S. epidermidis* and *S. aureus*, with several incidents of methicillin-resistant *S. aureus* (MRSA) (Patel and Saiman, 2010).

Quantifying the economic consequences of antibiotic resistance is challenging due to several factors involved. Increased resistance levels result in higher expenses due to the necessity of using pricier antibiotics, specialized equipment, prolonged hospital stays, and additional isolation procedures for patients (Norrby *et al.,* 2009). According to the CDC, antimicrobial resistance (AMR) costs the US nearly $55 billion annually, about $20 billion in healthcare costs, and an additional $35 billion in societal losses from reduced productivity (Abadi *et al.,* 2019).

1.3 Novel antibiotics

Exploring new ecological environments, such as the marine ecosystem, presents opportunities for discovering novel antimicrobial compounds (Hughes and Fenical, 2010; Rahman *et*

al., 2010). Another strategy involves extracting antimicrobial peptides and compounds from animals and plants (Hancock and Sahl, 2006). Additionally, mimicking natural lipopeptides found in bacteria and fungi holds promise for developing new antimicrobial agents (Makovitzki *et al.,* 2006). Utilizing metagenomics to explore the uncultivated portion of microbiota is another avenue for discovering antimicrobial diversity (MacNeil *et al.,* 2001). Lastly, the use of the synthetic routes pioneered during the early antibiotic era presents another potential strategy (Aminov, 2010).

1.4 Fungi: Potential source of novel antibiotics

1.4.1 Overview of fungi

Fungi belong to a large category of eukaryotic organisms that include microorganisms like yeasts and molds to the more commonly known mushrooms (Buckley, 2008). Fungi play a pivotal role in maintaining both environmental sustainability and biological health, as they possess one of the most extensive distributions among all living organisms on earth (Bills and Gloer, 2016). The majority of fungi are classified as mesophiles, flourishing in temperatures between 5°C to 35°C. Certain extremophilic fungi inhabit environments with lower temperatures, like glaciers at 0°C, while others demonstrate thermotolerance in environments ranging from 50°C to 60°C (Dix and Webster, 1995).

Fungi comprise a diverse, and heterogeneous group of organisms, inhabiting a wide range of habitats that can be found in environments ranging from the stratosphere to the depth of the Dead Sea, from Antarctic glaciers to scorching deserts, and from digestive systems of flies to the depths of oceanic sediments (Bhunjun *et al.,* 2022). Fungi, along with bacteria, play a crucial role in decomposing organic matter and releasing essential elements such as carbon, oxygen, nitrogen, and phosphorus into both soil and atmosphere. Furthermore, fungi are

indispensable in various domestic and industrial activities, including the production of bread, wine, beer, and selected cheese. Additionally, fungi are consumed as food; certain mushrooms, morels, and truffles, and mycoproteins sourced from specific fungal mycelia are employed to produce protein-rich foods (Hibbett *et al.,* 2007).

1.4.2 Classification of fungi

The number of fungal species in the world is in the range of 2.2 and 3.8 million species. Given the current acceptance of 120,000 species, it seems that only a small portion, between 3% to 8%, has been identified and classified (Hawksworth and Lücking, 2017). Fungal classification has undergone significant changes due to advancements in molecular techniques and new methodologies and the process of discovering and classifying fungi remains in a state of constant evolution (James *et al.,* 2020). For instance, James *et al.,* 2020 and Li *et al.,* 2021developed an extensive fungal tree, classifying 224 orders into 12 phyla. Whereas Wijayawardene *et al.*, (2022) proposed a fungal tree comprising 18 phyla. Based on present knowledge, Kingdom fungi can be categorized into nine phylum-level groups (**Figure 4**) that include, Opisthosporidia, Chytridiomycota, Neocallimastigomycota, Blastocladiomycota, Zoopagomycota, Mucoromycota, Glomeromycota, Basidiomycota and Ascomycota (Naranjo-Ortiz and Gabaldón, 2019).

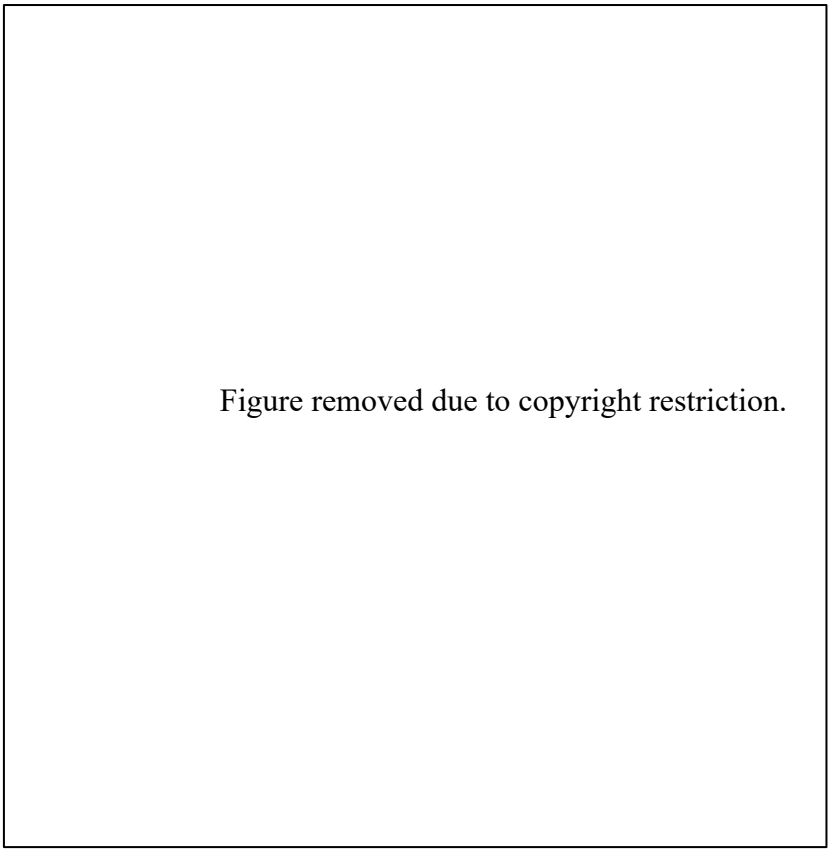

Figure 4 Phylogenetic tree of the fungi kingdom outlining nine phyla (Naranjo-Ortiz and Gabaldón, 2019).

Phylogenomics has transformed the understanding of fungal phylogeny, revealing a kingdom comprised of nine primary lineages including, Opisthosporidia, Chytridiomycota, Neocallimastigomycota, Blastocladiomycota, Zoopagomycota, Mucoromycota, Glomeromycota, Basidiomycota and Ascomycota (**Figure 5**). However, the phenotypic diversification alongside the emergence and proliferation of these lineages remains poorly understood (Smith and Donoghue, 2022).

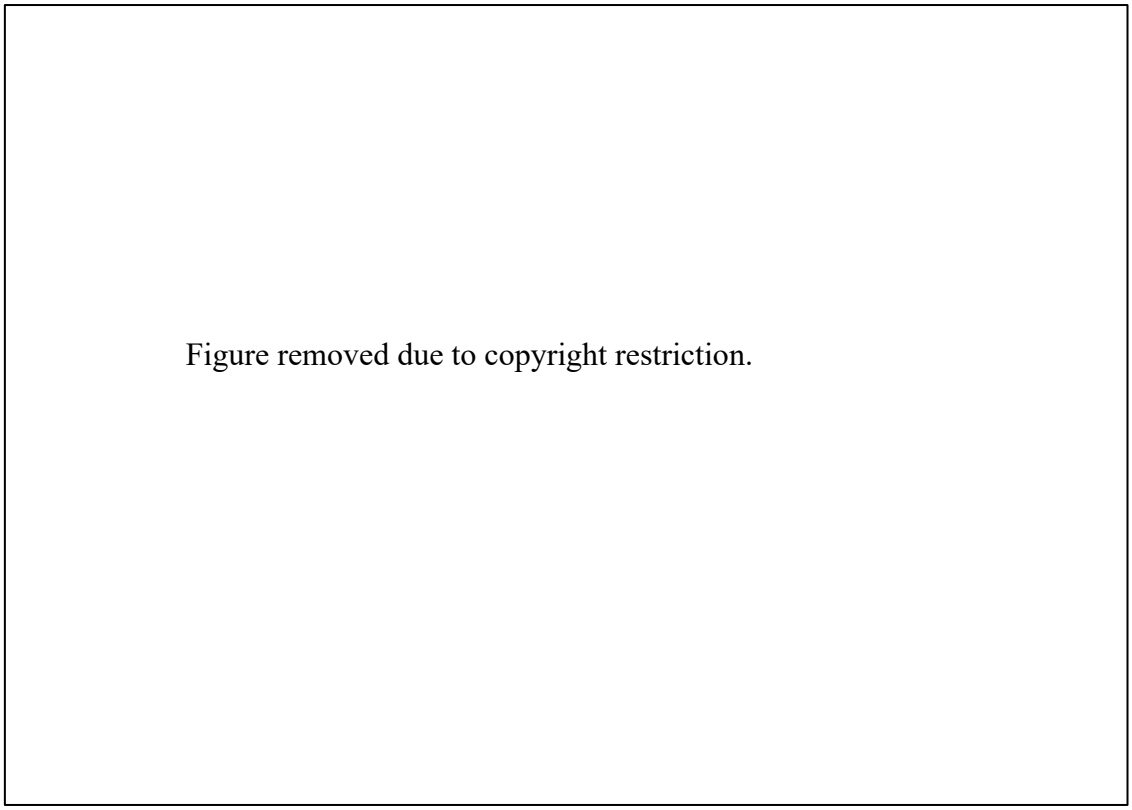

Figure 5 The evolutionary relationships among the nine primary fungal lineages (Smith and Donoghue, 2022).

Before the molecular era, species definitions primarily depended on morphological characteristics, occasionally supplemented by biochemical and cultural characteristics. However, the inclusion of molecular information has since facilitated a more precise refinement of species concepts (Hawksworth and Lücking, 2017). Since the introduction of DNA-based techniques for species identification, the annual rate of newly described taxa has increased from around 1000 to approximately 2000 (Bhunjun *et al.,* 2022). The nuclear ribosomal internal transcribed spacer (ITS) gene serves as a widely used marker gene, characterized by a well-defined barcode gap between inter- and intraspecific variation (Schoch *et al.,* 2012). However, the presence of intraspecific and intragenomic variability in the ITS region within certain fungal groups can complicate both the sequencing and classification

process. To address these complexities, additional markers such as the rRNA large subunit region (LSU) or RNA polymerase subunit II (RPB2) are often employed to enhance the accuracy and reliability of analyses (Tekpinar and Kalmer, 2019).

1.4.3 Bioactive compounds from fungi

Fungi are remarkable creators of essential natural compounds and play an essential role in global health by synthesizing a range of beneficial substances like enzymes, biofuels, acids, and diverse secondary metabolites, including antibiotics and anticancer drugs (Sanchez and Demain, 2017). Natural products from fungi are regarded as a significant source of new antibacterial and antifungal chemicals due to the diversity of fungal species that they contain, the abundance of secondary metabolites they produce, and advancements in their genetic breeding and fermentation techniques (Xu L *et al.,* 2015). The capability of fungi to biosynthesize beneficial compounds like penicillin (e.g., antibiotics) alongside harmful substances like aflatoxin (e.g., mycotoxins) has generated significant interest in exploring natural compounds (Gakuubi *et al.,* 2021). Numerous bioactive compounds are synthesized by microorganisms, of which 22,500 such substances identified to date. Among these, fungi contribute significantly to producing approximately 9000 of these bioactive compounds (Berdy 2005; Brakhage and Schroekh 2011).

Research reveals that filamentous fungi possess the genetic capability to produce a diverse array of 99% of fungal secondary metabolites (Berdy, 2005). Approximately 80% of endophytic fungi generate some form of bioactive compound. Endophytes offer diverse bioactive secondary metabolites, including alkaloids, benzopyranones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, xanthones, and more, with various applications like antimicrobials, antibiotics, agrochemicals, immunosuppressants and anti-parasitic (**Figure 6**) (Kumar *et al.,* 2014).

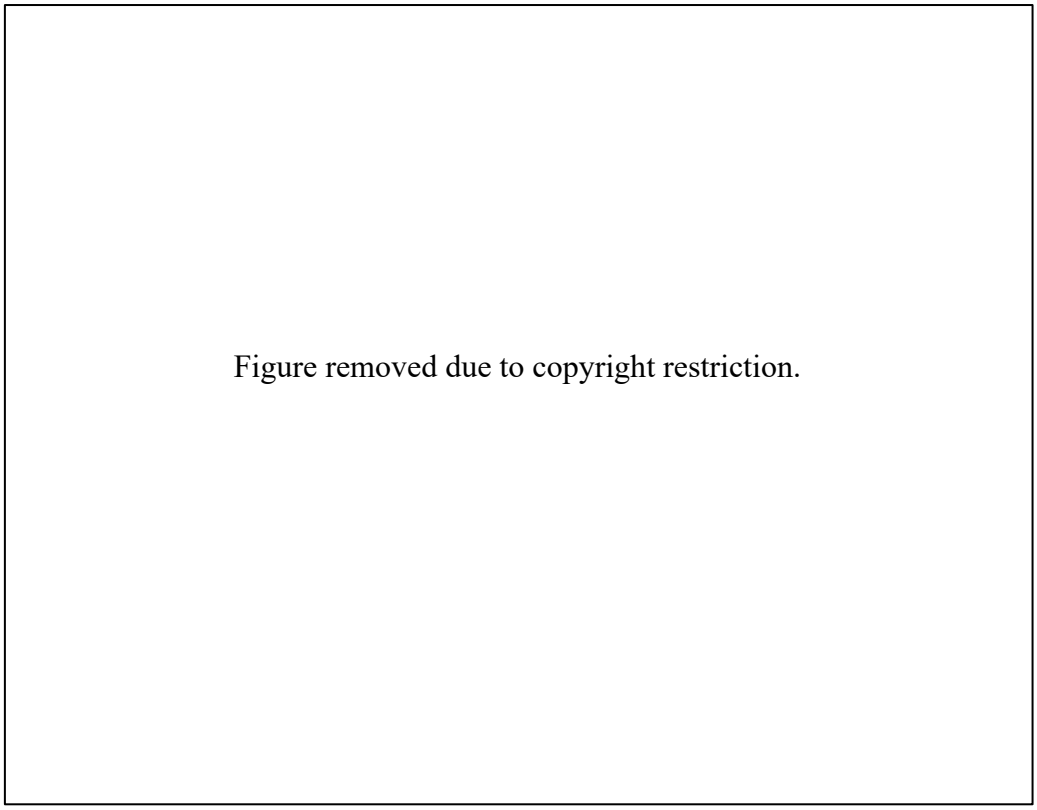

Figure 6 Bioactive compounds derived from fungal endophytes with their pharmacological relevance (Rai *et al.,* 2021).

1.4.4 Fungi as a source of antibiotics

An estimated 338 types of fungi are capable of producing antibiotics. Among them, soil fungi have contributed around 20% of the antibiotics that have been isolated (Rafiq *et al.,* 2018). Antibiotics sourced from fungi, particularly penicillins and cephalosporins are widely used. Additionally, griseofulvin, fusidic acid, and pleuromutilin, among others offer alternatives by their narrow spectrum activity, providing additional options (**Figure 7**) (Mishra *et al.,* 2024).

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Figure 7 Some of the well-known antibiotic structures are derived from fungi (Mishra *et al.*, 2024).

To address the drug-resistant strains, developing additional potential novel antibiotics is essential. **Table 1** provides a summary of antibiotics produced by fungi (Mishra *et al.*,2024).

Table 1 Overview of antibiotics derived from fungi with sources, examples, and spectrum of activity (Mishra *et al.*,2024).

Antibiotics	Source	Examples	References
Penicillins	Penicillium chrysogenum	Penicillin G acylase, aminopenicillins, natural penicillins, antipseudomonal penicillins	Elander (2003); Nigam and Singh (2014); Li et al. (2019), Nath et al. (2020)
Cephalosporins	Cephalosporum acremonium, Tolypocladium inflatum, Acremonium fusidioides	Cephalexin, cefuroxime, ceftazidime, cefepime, ceftaroline	Mishra et al. (2024)
Fusidic acid	Fusidium coccineum, Acremonium fusidiodes	–	Aly et al. (2011); Bouza (2009)
Pleuromutilin	Omphalina mutila (formerly Pleurotus mutilus)	Tiamulin, valnemulin, retapamulin, Lefamulin	Novak and Shlaes (2010); Chahine and Sucher (2020)
Griseofulvin	Penicillium griseofulvum, Penicillium nigricans, Penicillium urticae	Gris-peg	Hüttel (2020)
Fumagillin	Aspergillus fumigatus	Fumagilin-B	Wiemann et al. (2013), van den Heever et al. (2014), Guruceaga et al. (2019)
	Aspergillus eff	–	Saeedi et al. (2019), Siddiquee (2018)
		–	
		–	

1.5 Freshwater fungi

1.5.1 Overview of freshwater fungi

Freshwater fungi comprise species that, throughout their life cycle or a portion thereof, rely on freely available freshwater or utilize resources primarily aquatic or semi-aquatic in nature as a substrate (Thomas, 1996). Freshwater fungi play a significant ecological role within freshwater ecosystems globally, inhibiting lakes, rivers, streams, and waterholes, fulfilling roles as decomposers, endophytes, pathogens affecting both plant and animal life, and some form mycorrhizae (Fryar and Catcheside, 2023). In total, 3,870 species of freshwater fungi are documented and described with molecular characteristics (Calabon *et al.*, 2022). An approximate global estimate suggests there are around 20,000 species of freshwater fungi (Gessner & Van Ryckegem, 2003).

The diversity of freshwater fungi is notably extensive, including species from most of the phyla, with notable representation in Chytridiomycota, Blastocladiomycota, Ascomycota, and Basidiomycota, among others, within the fungal kingdom (Hawksworth, 2001). In the updated classification (**Table 2**), freshwater fungi are classified into thirteen phyla comprising 45 classes and among that a significant portion-2968 species distributed across 1,018 genera, all falling under the classification of Ascomycota (Calabon *et al.*, 2022).

Table 2 Distribution of freshwater fungi by phylum, indicating the number of species within each phylum (Calabon *et al.*, 2022).

Phylum	Number of Species
Ascomycota	
Chytridiomycota	
Rozellomycota	
Basidiomycota	
Blastocladiomycota	
Monoblepharomycota	
Mucoromycota	
Aphelidiomycota	
Entomophthoromycota	
Mortierellomycota	
Olpidiomycota	
Zoopagomycota	
Sanchytriomycota	

1.5.2 Freshwater fungi as a source of bioactive compounds

Since 1992, more than 280 freshwater fungal metabolites have been identified, with 199 of them discovered within the last decade. Freshwater fungi demonstrate the production of

diverse secondary metabolites, including, polyketides, phenylpropanoids, terpenoids, meroterpenoids, polypeptides, alkaloids, and monosaccharides (El-Elimat *et al.,* 2021). There are certain fungi found in freshwater environments that produce antimicrobial substances. (**Table 3**) (Canto *et al.,* 2022).

Table 3 Antimicrobial compounds produced by fungi within freshwater ecosystems (Canto *et al.,* 2022).

	Fungi species	Biological activity	References
Freshwater	*Massarina aquatica* J. Webster *Sporobolomyces roseus* Kluyver & C.B. Niel	Antifungal	Fisher and Anson (1983)
	Kirschsteiniothelia spp.	Antimicrobial	Poch et al. (1992)
	Helicoon richonis (Boud.) Linder	Antimicrobial	Adriaenssens et al. (1994)
	Anguillospora longissima (Sacc. & P. Syd.) Ingold *and A. crassa* Ingold	Antifungal	Harrigan et al. (1995)
	Massarina spp.	Antimicrobial	Oh et al. (2003)
	Clavariopsis aquatica De Wild. *Articulospora tetracladia* Ingold	Antimicrobial	Gulis and Stephanovich (1999)
	Chloridium spp. and *Sporoschisma mirabile* Berk & Broome	Antifungal	Oh, Kwon, Gloer, Marvanová , and Shearer (1999)

A large variety of secondary metabolites produced by freshwater fungi have been demonstrated to have potential uses in industry, agriculture, and medicine. New substances with antibacterial, antifungal, and anticancer effects may be found as a result of research on freshwater fungi (El-Elimat *et al.,* 2021). The discovery of novel compounds, addressing antibiotic resistance, biotechnological uses, ecological value, and economic rewards are all impacted by research on freshwater fungi for antimicrobial.

1.5.3 Current research in freshwater fungi

Researchers have been screening freshwater fungi for their capacity to create antibacterial chemicals. For instance, a study examined the existence of cultivable filamentous fungi in a highly contaminated waterway of Manaus City, Amazonas, Brazil, and their capacity to produce compounds with antibacterial action. The results demonstrate the viability of isolating fungal strains from contaminated areas that can produce metabolites that may one day replace existing multidrug-resistant bacterial treatments (Oliva *et al.,* 2022). The variety of fungi found in freshwater habitats, particularly in the Amazon, were examined if aquatic fungi are capable of producing substances with antibacterial activity. The Amazon has the potential to provide new antibacterial chemicals from freshwater fungi, but this potential is constrained by a lack of experts who are focused on this issue (Canto *et al.,* 2022). Vast areas of the world for freshwater fungi remain largely unexplored in comparison to terrestrial fungi, particularly in regions such as South America, Australia, Africa, and a significant part of Asia fungi (El-Elimat *et al.,* 2021).

In this study, freshwater fungi are screened for new compounds in research through a multi-step process. Initially, fungi are isolated and purified from freshwater sources. Cultures are then grown in optimized media to stimulate metabolite production. Extracts from these cultures are tested for bioactivity, and promising ones undergo separation using the thin-layer chromatography method. Finally, the chemical structure of the isolated compounds can be determined in the future.

CHAPTER 2 MATERIAL AND METHOD

2.1 Isolation of fungi from the freshwater environment

The isolation procedures were conducted under the direction and guidance of Dr. Sally Fryar, College of Science and Engineering, Flinders University.

2.1.1 Collection of Submerged wood samples

The deadwood samples were collected from Cox Creek in Bridgewater, South Australia. Partially decomposed wood pieces or branches with a thickness ranging from 1 to 3 cm were selected, preferably without bark. Ten samples were collected. The outer surface of the wood was carefully scraped using a serrated knife to remove ectophytic animals and algae. Following scraping, the wood was rinsed with sterile water and placed into individual zip-lock bags for 24 hours at room temperature before surface sterilization. A 1-liter solution of $Na_2S_2O_3$ 2% (w/v) was prepared by dissolving it in a 1000 mL flask filled with Milli-Q water. The solution was then autoclaved at 121°C for 90 minutes and next placed at room temperature. Similarly, a 1-liter solution of Tween 20 0.1% (w/v) was prepared by diluting it in a 1000 mL flask with Milli-Q water. This solution was also autoclaved at 121°C for 1 hour and 30 minutes and stored at room temperature. A 1-liter solution of NaClO 4% (w/v) was prepared by dissolving it in a 1000 mL flask with Milli-Q water and then stored at room temperature. Furthermore, a 0.5 g solution of chloramphenicol (100 mg/ml) was processed in a sterile 50 mL centrifuge tube and 5 mL of 70 % ethanol was added. This solution was stored at 4°C.

2.1.3 Surface sterilization of wood samples

The wood samples were cut into approximately 2 x 2 x 2 cubes and placed in a sterile 250 mL flask. Tween 20 0.1% (w/v) solution was poured into the flask until the samples were completely covered, and the flask was gently swirled for 1 minute. Afterward, the Tween 20 0.1% (w/v) solution was drained from the flask, and the samples were rinsed with sterile water solution for 30 seconds. Next, NaCIO 4% (v/v) solution was added to the flask until the samples were submerged, and the flask was swirled lightly for 15 seconds. The NaClO 4% (v/v) solution was then drained, and the samples were rinsed again with sterile water for 30 seconds. Following this, $Na_2S_2O_3$ 2% (w/v) solution was added to the flask until the samples were submerged, and the flask was swirled lightly for 15 seconds. After draining the $Na_2S_2O_3$ 2% (w/v) solution, the samples were rinsed 3 times with sterile water for 30 seconds each time (Le, 2021).

2.1.4 Preparation of Potato Dextrose Agar (PDA) and Dichloran Rose Bengal Chloramphenicol (DRBC) media

To prepare PDA and DRBC media, the respective powders were mixed with distilled water following the instructions provided by the manufacturer (as mentioned in **Table 6**). After autoclaving, 0.25 mL of filtered chloramphenicol (100 mg/ml) solution was added to each media while still hot, followed by swirling to ensure thorough mixing. The media were poured into sterile 9-cm Petri dishes, approximately 25 mL per dish. After allowing the Petri dishes to cool down, they were covered with cling wrap and stored at room temperature for future use.

2.1.5 Fungi Isolation in DRBC and PDA Plates

After the surface sterilization of the wood as mentioned in 2.1.3, then sterile scalpel was used to cut the wood into smaller pieces. Using a sterile tweezer, random wood pieces were carefully

picked and placed onto DRBC agar plates that had been previously prepared. The tweezer was sterilized using a bactizapper (by Sigma-Aldrich) and allowed to cool down to prevent contamination. Another wood piece was then selected and placed onto the same agar plate. This process was repeated for each plate and after placing the wood pieces, the plates were sealed with parafilm as well as placed inside a plastic seeable container box. The box was then incubated at 26°C. Starting from day 2, the plates were inspected daily for up to one month to observe fungal growth. After the detection of new growth on a plate, a sterile scalpel was used to carefully cut out the small amount of fungal growth. The subcultured fungus was then transferred onto fresh PDA plates for further cultivation and analysis.

2.2 Subculture of freshwater fungi cultures

After 7 days of inoculation on PDA plates, fungi had grown and were prepared for subculture into two different media: Potato Dextrose Agar (PDA) and Peptone Yeast Glucose (PYG). A total of 56 samples were to be sub-cultured along with 7 fungi samples obtained from the Flinders Ranges (FR) by Dr. Sally Frayer.

Table 4 A list containing all 56 fungal samples collected from Bridgewater and selected for subculture.

S12	S15	S21	S22	S23	S25	S27	S31	S32	S34
S35	S36	S37	S38	S41	S42	S44	S45	S46	S47
S48	S61	S62	S63	S64	S65	S66	S71	S72	S74
S75	S77	S79	S710	S81	S82	S83	S84	S85	S86
S87	S88	S91	S93	S94	S95	S98	S99	S101	S102
S103	S104	S105	S106	S107	S108				

Table 5 List of 7 fungal samples that were collected from the Flinders Ranges.

FRW2A	FRW20A	FRW28A	FRW29A
FRW39A	FRW41A	FRW45A	

2.3 Maintenance of freshwater fungi

Fungal culture maintenance involved the inoculation onto PDA media. The inoculated cultures were then incubated at 26 °C for 7-14 days characterized by rapid growth and next stored at 4 °C for future utilization. Details regarding the media employed for both maintenance and experimental procedures are explained in **Table 6**.

Table 6 The media utilized for the study were purchased from The Bacto Laboratories Pty Ltd located in Mt Pritchard, NSW, 2170, Australia.

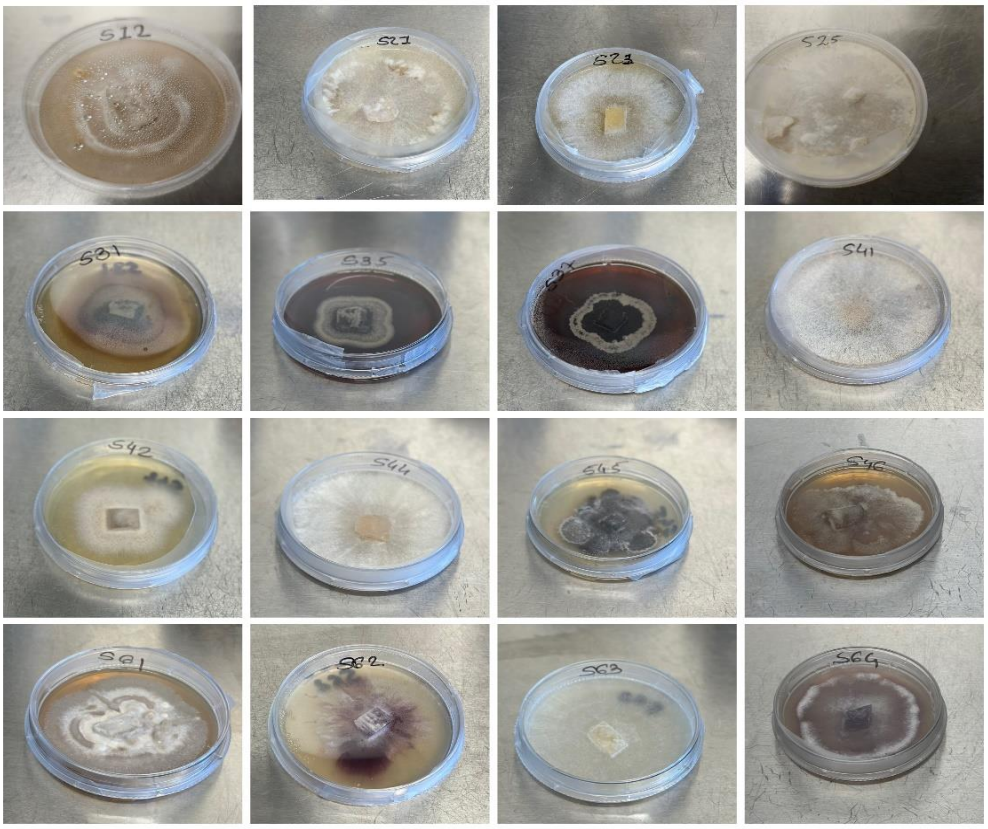
Medium	Composition
Potato Dextrose Agar (PDA)(Difo-BD)	PDB medium supplied with agar 15g/L
Peptone Yeast Glucose (PYG) Agar	Yeast Extract 5g/L; Peptone 10g/L; Glucose 20g/L; Agar 15g/L
Antibiotic assay medium (AAM)	Peptone 6 g/L; Tryptone 6 g/L; Yeast extract 3 g/L; 'Lab-Lemco' powder 1.5 g/L; Glucose 1 g/L; Agar 11.5 g/L in deionized water
Tryptone Soy Broth (TSB)	Tryptone 17 g/L; Enzymatic digest of soya bean 3 g/L; NaCl 5

	g/L; K2HPO4 2.5 g/L; Glucose 2.5 g/L in demineralized water
Tryptone Soy Agar (TSA)	TSB medium with agar 15 g/L
Dichloran Rose Bengal Chloramphenicol (DRBC)	Peptone 5g/L; Glucose 10.0g/L; Potassium dihydrogen phosphate 1.0g/L; Magnesium sulphate 0.5/L; Dichloran 0.002g/L; Rose-Bengal 0.025g/L; Agar 15.0g/L in demineralized water

After subculture, all the fungi were examined for their morphological characteristics, to remove common fungi and contaminants. A subset of 45 fungi were selected for further experiments.

Table 7 The table below displays the selected 45 strains of freshwater fungi for further experiments including 6 strains previously isolated by Dr. Sally Fryar from flinders ranges.

S12	S21	S23	S25	S31	S35	S37	S41	S42
S44	S45	S46	S61	S62	S63	S64	S65	S66
S71	S72	S74	S75	S77	S710	S81	S83	S85
S86	S87	S91	S93	S95	S98	S101	S102	S103
S105	S106	S108	FRW2A	FRW28A	FRW29A	FRW39A	FRW41A	FRW45A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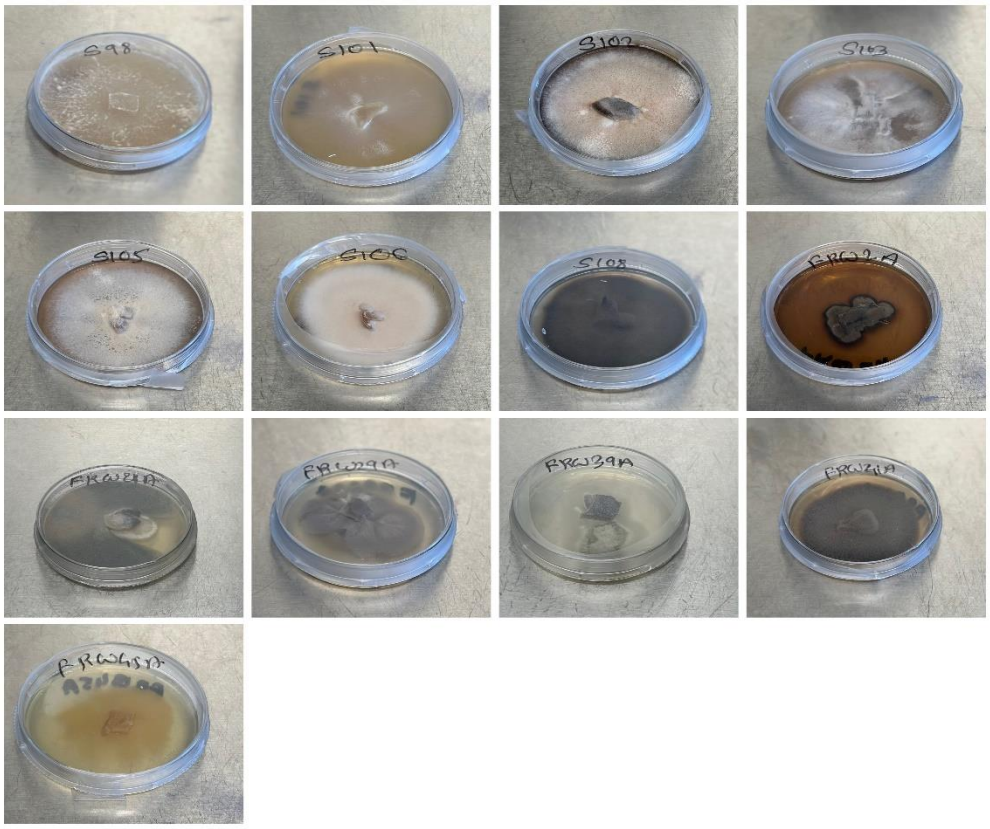


Figure 8 Photographs of 45 freshwater fungi with each strain name specified on the lid of the petri dish.

2.4 Primary screening of freshwater fungi

For further primary as well as secondary screening 45 well-grown strains were selected and sub-cultured on both Potato Dextrose Agar (PDA) and Potato Yeast Extract Glucose (PYG) media.

2.4.1 Methanol extraction

Methanol extraction was carried out on all 84 fungal samples on Potato Dextrose Agar (PDA) and Potato Yeast Extract Glucose (PYG) medium. 5 ml of methanol was transferred to15 ml

centrifuge tube with a 2x2cm piece of agar containing a well-grown fungal culture. The centrifuge tube was then placed on a shaker operating at a speed of 120 revolutions per minute (rpm) for at least 1 hour. After that, the methanol extract was stored at room temperature until further experiment.

2.4.2 Preparation of tested organisms

Three test microorganisms as described in **Table 8** were selected in the research study to assay for antimicrobial activity of freshwater fungi. These microorganisms were sourced from the Department of Microbiology and Infectious Diseases at Flinders Medical Centre. These microorganisms were cultivated on Tryptic Soy Agar (TSA) with the streak plate technique and then incubated at 37°C for 24 hours. After the incubation, cultures were refrigerated at 4°C for storage purposes.

Table 8 Three test organisms comprising yeast, Gram-positive bacteria, and Gram-negative bacteria were selected for the antimicrobial activity of the fungal extracts.

Classification	Test Organism
Yeast	*Candida albicans*
Gram-negative Bacteria	*Escherichia coli*
Gram-positive Bacteria	*Staphylococcus aureus*

2.4.3 Antibiotic activity assay by agar well diffusion method

The antimicrobial activity of fungal crude extracts was performed using the Agar well diffusion method (Perez *et al.,* 1990). Firstly, a loopful of test bacteria was inoculated into 10ml of sterile Tryptic Soy Broth (TSB) at 37°C for 24 hours. Afterward, from the overnight culture, 1 ml was transferred into fresh 5ml TSB and incubated for 2 hours at 37°C. Following this, the bacterial inoculum was diluted to an optical density (OD) of 0.2 at 600nm for both Gram-negative and Gram-positive bacteria as well as to an OD of 0.6 at 600nm for yeast. The diluted bacterial inoculum was then transferred into freshly prepared sterile Antibiotic Assay Medium (AAM) at inoculum percentages of 1% (v/v) for *S. aureus* and *E. coli*, and 4% (v/v) for *C. albicans*. Furthermore, 25ml of the seeded medium was poured into each 9cm diameter sterile petri plate and allowed to solidify. Wells were then created using a sterile 5mm diameter cork borer, with ten wells per petri plate. Each well was loaded with 50 microliters of the test extracts or solutions, along with 50 µl of positive and negative controls. Positive controls included Colistin (400 µg/ml) - Gram-negative bacteria, Vancomycin (400 µg/ml) - Gram-positive bacteria, and Nystatin (400 µg/ml) for yeast. Methanol was used as the negative control. Plates were left until the complete diffusion of all solutions into the agar and after that, they were sealed with the use of parafilm and then incubated at 37°C for 24 hours. The Zone of Inhibition (ZOI, Diameter) was measured after the incubation period (Le, 2021).

2.5 Secondary screening of freshwater fungi

During this phase, fungi were cultivated on cooked basmati rice as a solid substrate (purchased from Woolworths, Australia). The antimicrobial activity of each strain following solid fermentation on various substrates was assessed. The following procedures were involved to accomplish the secondary screening of freshwater fungi.

2.5.1 Seeding of fungi on PYG broth medium

A 250 ml volume of Peptone Yeast Extract Glucose (PYG) broth medium was prepared in a 500 ml Scott bottle, followed by aliquoting 5 ml into each 15 ml centrifuge tube, which was labelled with respective fungal codes. With the sterilized tip of a scalpel, approximately 10mm x 10mm sections of agar from the margin of the mycelium were excised. These fungal culture fragments were then carefully transferred into the liquid medium. The culture-containing tubes were placed inside a shaker incubator and agitated at a speed of 100 rpm at a temperature of 26°C. The cultures were allowed to grow for 6 days before being seeded to other cultures.

2.5.2 Fungi Cultivation on solid substrates in blue pods containers

For further secondary screening of freshwater fungi, cultivation on a solid substrate, basmati rice was chosen as that can support fungal growth and antibiotic production. Approximately 55 grams of basmati rice were transferred into small aluminium foil trays measuring 31 cm x 20 cm x 5 cm (Armada foil trays, purchased from Woolworth, Australia). After that, tap water was added to the substrate surface evenly. The tray was then covered with double-layered foil, autoclaved, and allowed to cool down for 24 hours. Following this, 110 ml of tap water containing 0.5 grams (w/v) of dissolved yeast extract was added to the autoclaved substrate tray and again tray underwent another cycle of wet autoclaving. After cooling it down, the substrate was transferred into dry-autoclaved sterile 60 ml containers (Polypropylene, 54x44mm, with blue screw caps assembled and flat bottom base) with a height of 1 cm layer and labelled with the respective fungi code. Afterward, 2 ml of seed cultures that were prepared in 2.5.1 were evenly inoculated into the substrate containers. The containers were loosely capped to ensure sufficient air supply and then incubated at 26 °C for 21 days for the next procedure.

At 7, 14, and 21 days during the incubation period, a 1cm cubic piece of hyphae and substrate from the centre of the mycelium were transferred into sterile 15-ml centrifuge tubes and in each tube, 2 ml of methanol was added. The tubes were placed in an orbital shaker at 120 rpm for 2 hours. Next, the antimicrobial activity of the methanol extracts was measured against *E. coli* and *S. aureus*, as outlined in section 2.4.3 (Le, 2021).

2.6 Scale-up fermentation of selected strains and extraction of the antimicrobial compound

The fungi strains exhibiting significant antimicrobial activity during solid-substrate fermentation in the secondary screening, as described in section 2.5, were cultivated on a large-scale using basmati rice as the solid substrate. A total of five fungi strains were selected based on the activity in secondary screening. For each strain, ten substrate trays were prepared adding 200 gram rice a tray as the methodology outlined in Subsection 2.5. For each strain, a 50 ml volume of Peptone Yeast Extract Glucose (PYG) broth medium was prepared in a 250 ml glass flask, equivalent to 10 ml for each rice tray as delineated in subsection 2.5. The prepared cultures were then incubated for durations of 7 and 17 days.

After conducting the agar well diffusion method as described in section 2.4.3, the one fungal strain that produced an extract showing notable antimicrobial activity was chosen for additional processing. To proceed, the leftover contents of five culture trays of fungus were gathered and transferred into 1000ml conical flasks, and 400mL of methanol was added to each flask for extraction purposes. The flasks were then placed on an orbital shaker set at 100 rpm for 2 hours. After this time, the methanol extract was collected from each flask using filter paper for filtration purposes and stored at 4°C for further agar well diffusion method to check the activity of solvent against *E. coli* and *S. aureus* (Le, 2021).

2.7 Thin Layer Chromatography (TLC)

In this study, thin-layer chromatography (TLC) was used as a method to detect the compound within the methanol extract solutions (Santiago and Strobel, 2013) TLC silica gel 60 F254 plates, with 10 cm in height was used as the stationary phase for compound separation as well as a singular solvent system containing chloroform and methanol in a ratio of 9:1 as the mobile phase.

A total of 4 microliters and 6 microliters of the same extract were precisely loaded onto the TLC plate on situated points approximately 1 cm from the bottom edge. The plate was placed into a closed chamber saturated with a specific mobile phase. In which, the compound underwent separation based on their individual properties. The process was stopped once the solvent front reached approximately 5 mm from the top edge of the plate.

This plate was then observed under a UV lamp with 254 nm and 365 nm UV light. Specific consideration was given to spots on the plate corresponding to the compound potentially possessing antimicrobial properties. Afterward, the bioautography test was conducted against bacteria such as *E. coli* and *S. aureus* to verify these properties (Le, 2021).

2.8 Methanol extract evaporation

Methanol extracts derived from solid substrates from subsection 2.6 were subjected to use in a rotary evaporator, effectively removing the methanol, and resulting in an aqueous residue.

The water bath was turned on at the temperature of 40 °C, the chiller of the rotary evaporator was activated and approximately 200 ml of the methanol extract was added to the round bottom flask. The flask was then attached to the rotary evaporator and lowered it down into a hot water bath until the methanol surface touched the water surface. After setting up this, the vacuum pump was turned on set to 218mmBar which is ideal for methanol extract solution. After

methanol evaporated, the rotator and vacuum pump were turned off the flask was lifted out and the water bath and chiller were turned off. The water residue from the flask was poured into a glass beaker for further use (Le, 2021).

2.9 PCR-Based Strain Differentiation Using ITS Primers

Identification of strains was to be achieved by sequencing the Internal Transcribed Spacer (ITS) region. Genomic DNA was extracted from the disrupted sample utilizing the DNeasy Plant Mini Kit (QIAGEN CAT. No. 69104) as stated by the manufacturer's instructions. The primers ITS5 (5' - GGAAGTAAAAGTCGTAACAAGG - 3') and ITS4 (5' – TCCTCCGCTTATTGATATGC – 3') (Bellemain *et al.,* 2010) were used to amplify the Internal Transcribed Spacer (ITS) sequence via polymerase chain reaction (PCR).

Table 9 Each PCR tube contained a 50-µL reaction mixture comprising the following components (Sigma Aldrich, 2024).

Component	Volume (µL)
Standard Taq Buffer	5
10Mm dNTPs	1
Hot Start Taq DNA Polymerase	0.25
10µM of each primer	1.5
DNA template	2
Sterile water	40.25

The amplification was processed using an Eppendorf Mastercycler nexus- PCR Thermal Cycler, 30 cycles of three stages including, as a first denaturation at 95 °C for 1 minute.

Secondly, annealing at 52 °C for 50 seconds, and lastly, extension at 72 °C for 1 minute including a final elongation step at 72 °C for 10 minutes (Le, 2021).

2.9.1 Agarose gel electrophoresis

This experiment has been performed with Bio-Rad PowerPac basic electrophoresis power supply equipment. The 0.6 grams agarose powder for 60 ml buffer was dissolved in 1% TAE buffer and the solution was heated for 1 minute. After cooling it down, 5 microliters of gel red were added and swirled into the solution before pouring it into a mold equipped with a comb to form sample wells. The gel was left to solidify at room temperature and then placed in an electrophoresis tank filled with 1% TAE buffer until it matched the maximum volume. Afterward, DNA samples of 5 microliters each were mixed with gel loading dye (Gel loading purple (6x)) to enhance their density and to provide visible bands. Ladder (Quick load DNA ladder) and samples were loaded into the wells. The gel tank was then operated with 100 V for 60-90 minutes. After finishing the gel electrophoresis, the gel was visualized using a gel documentation system (Steward, 2022).

CHAPTER 3 RESULTS

3.1 Isolation of freshwater fungi

The freshwater wood samples from Cox Creek, Bridgewater, South Australia were collected and subjected to surface sterilization using $Na_2S_2O_3$ 2%, Tween 20 0.1%, NaCIO 4%, and inoculated in DRBC. After 6 days of incubation period, the presence of hyphae was noted (**Figure 9**). Out of the 10 wood samples collected, fungal growth was observed from 9 samples. In some wood samples, multiple types of fungi were identified within a single wood sample.

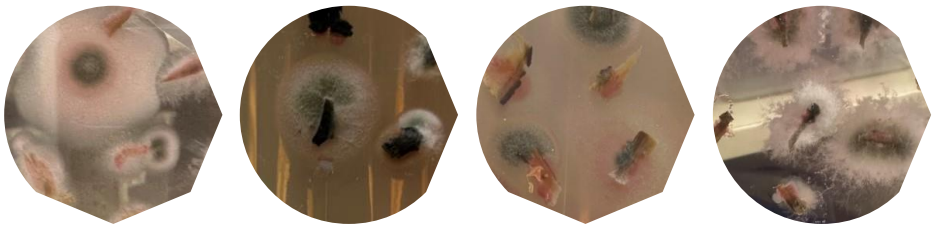
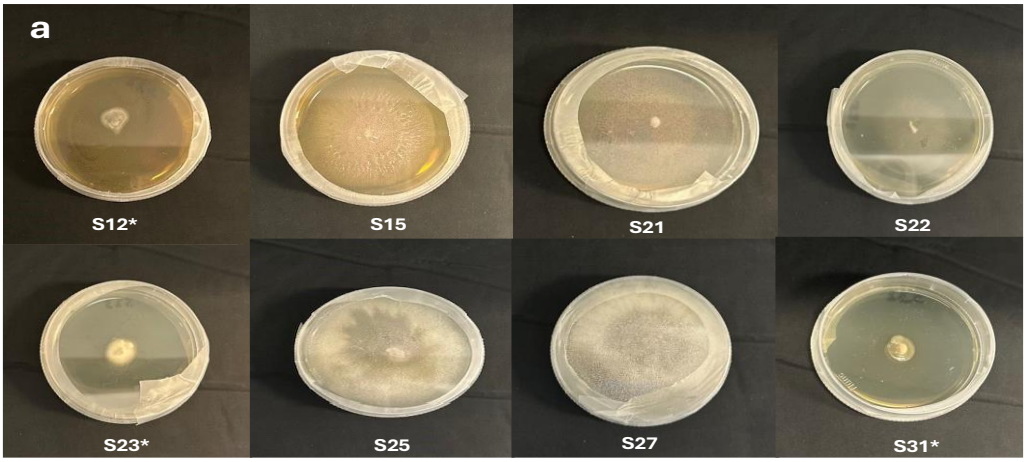


Figure 9 Observation of freshwater fungi growth from freshwater wood samples on DRBC media.

Furthermore, fungal culture was transferred to PDA media plates, where fungal growth was evident by the 7th day (**Figure 10**). This rapid proliferation of PDA media indicates mostly fast-growing characteristics of the isolated fungi.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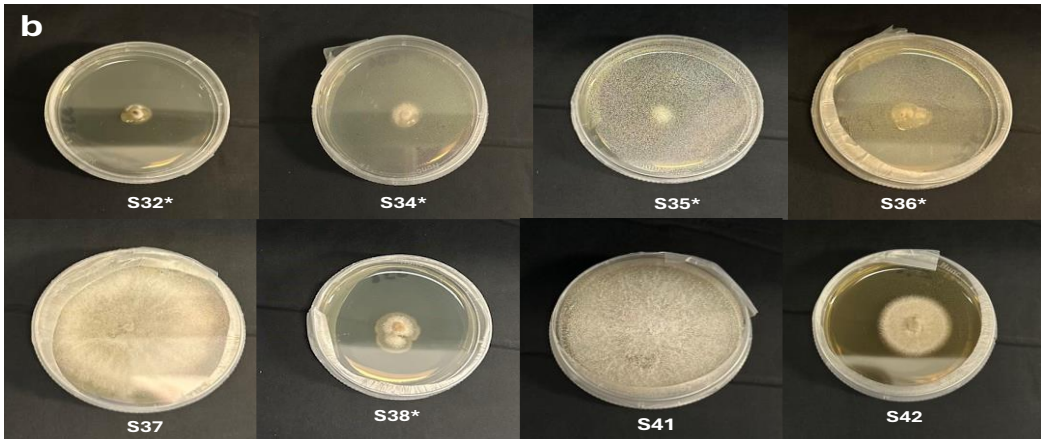
S23*
S25
S27
S31*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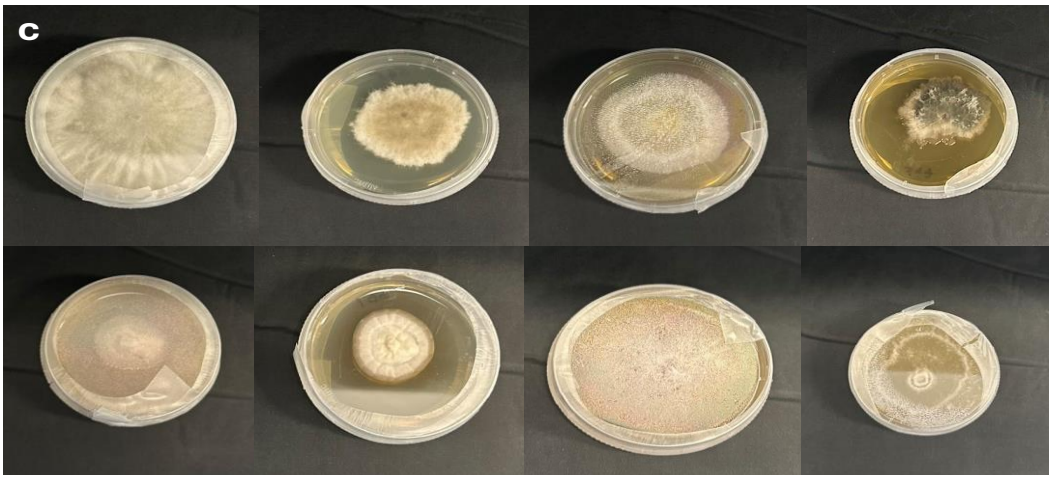
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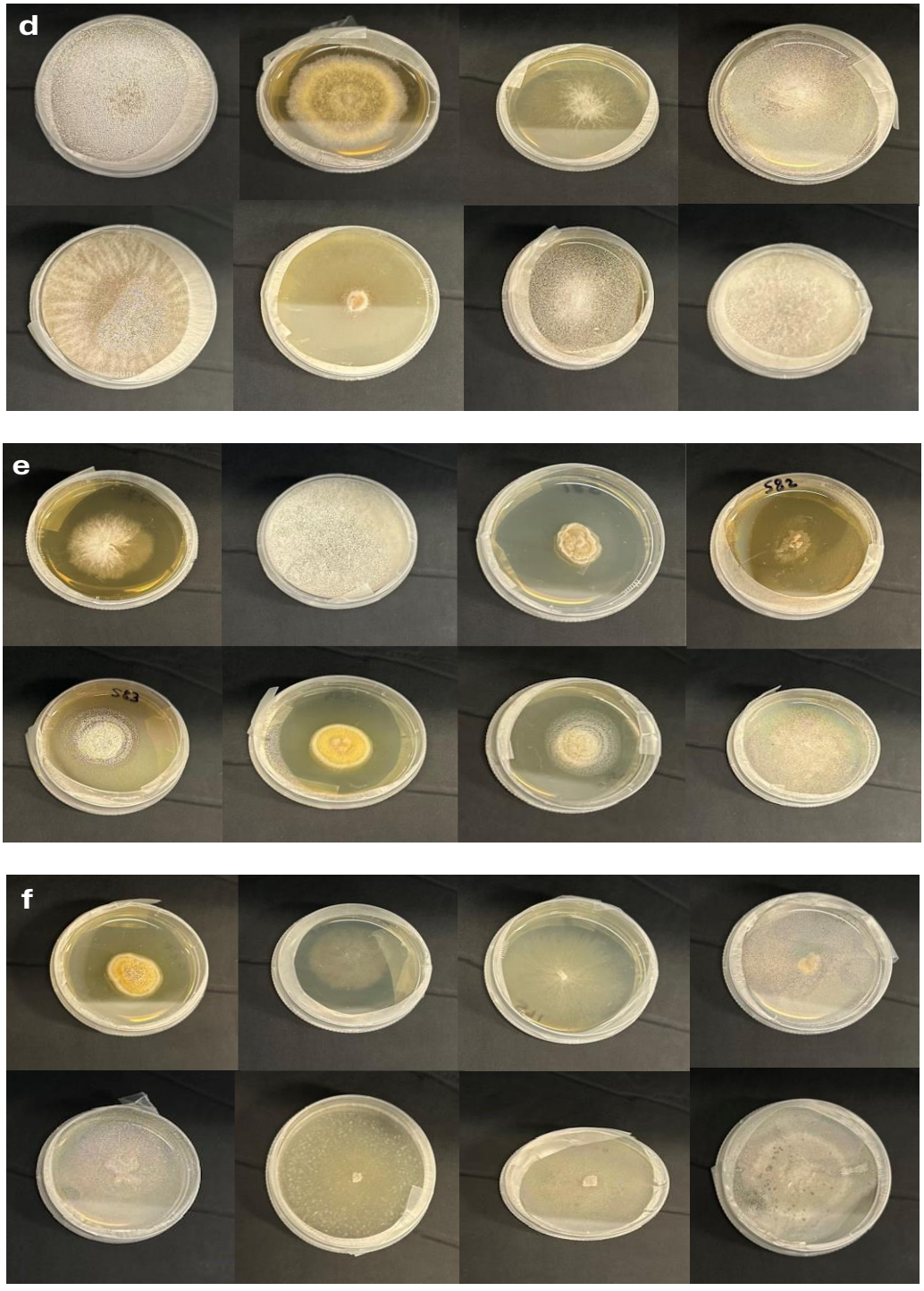
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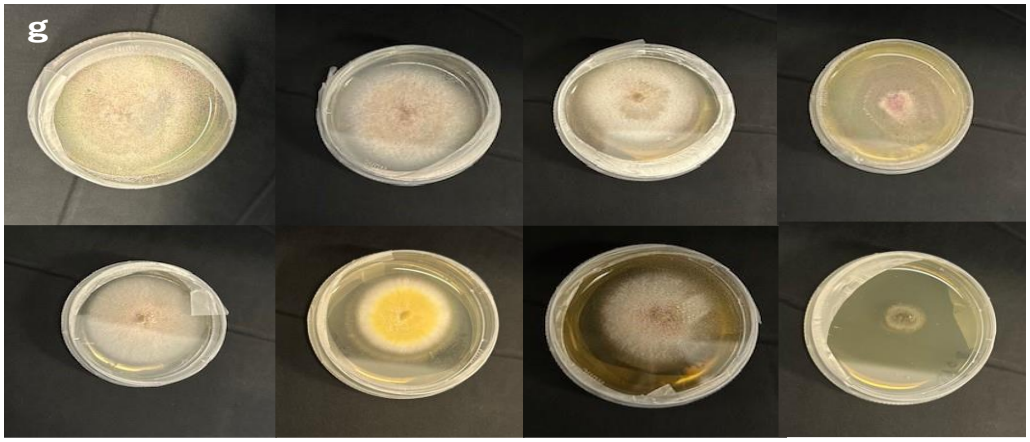
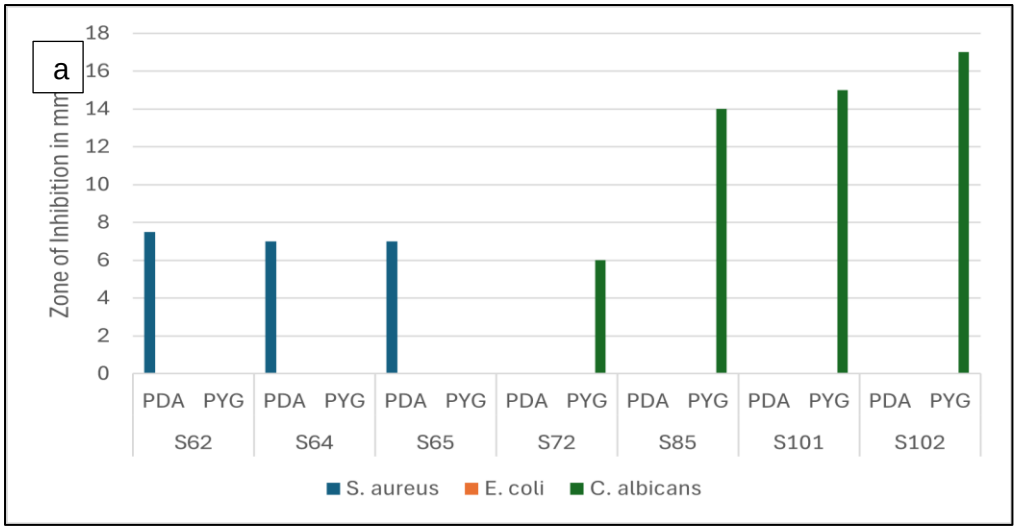


Figure 10 Photographs of 56 freshwater fungi (a-g) cultured on PDA medium in 9-cm plates. The cultures marked with an asterisk (*) were slow-growing strains and others were medium to fast-growing strains, the photos were taken on day 7th after the inoculation.

3.2 Primary screening of freshwater fungi

This study aimed to assess the potential of Australian freshwater fungi as a novel source of antibiotics with a random primary screening method. Fungi were cultivated on various media and examined for antimicrobial properties. This approach was selected for its simplicity and ability to reveal various forms of activity. **Figure 11** illustrates the antimicrobial activity of the active strains.



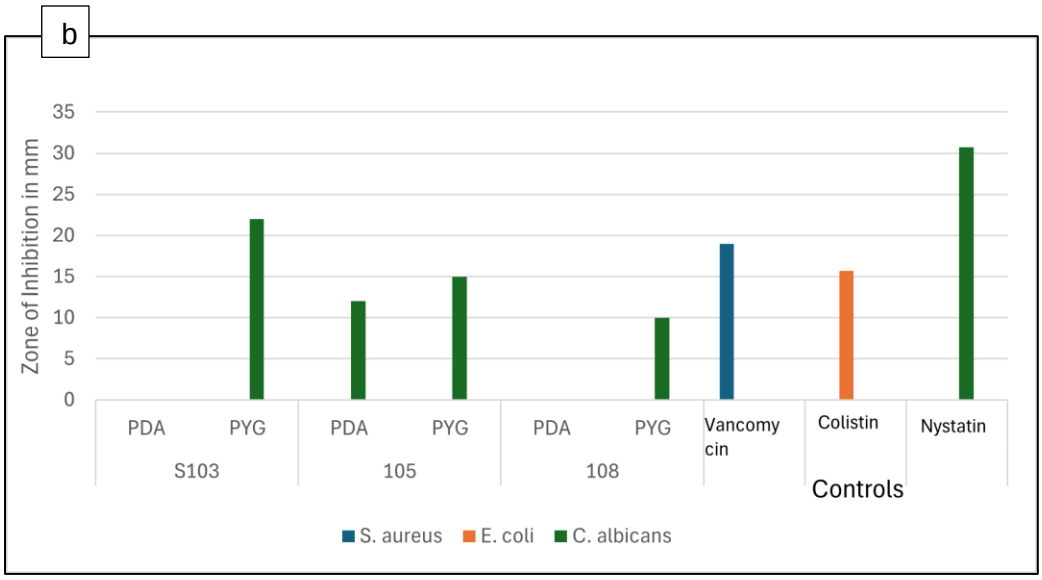


Figure 11 a and b represent the antimicrobial activity of fungal strains against *S. aureus*, *E. coli*, and *C. albicans* cultivated on two distinct types of agar media. Controls included vancomycin, colistin, and nystatin (at 400 µg/mL).

Among the 45 fungal strains screened, only 10 demonstrated antimicrobial activity, representing 22.22% of the total screened group. **Table 10** Presents the results of primary screening involving 45 strains of freshwater fungi cultivated on PDA and PYG agar against *E. coli*, *S. aureus*, and *C. albicans*.

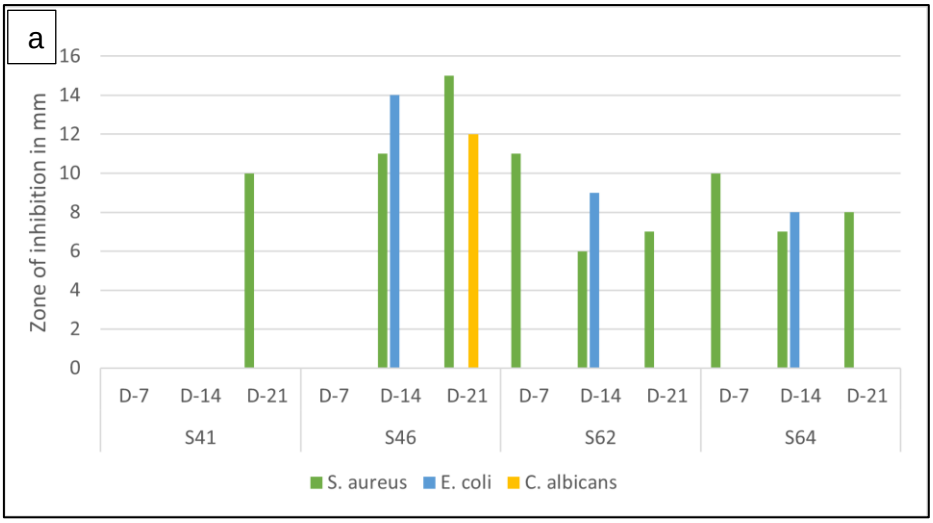
Table 10 Results of the primary screening of 45 strains of freshwater fungi including 6 strains previously isolated by Dr. Sally Fryar. 10 strains highlighted in blue exhibited antimicrobial activity, whereas the remaining 35 strains showed no activity.

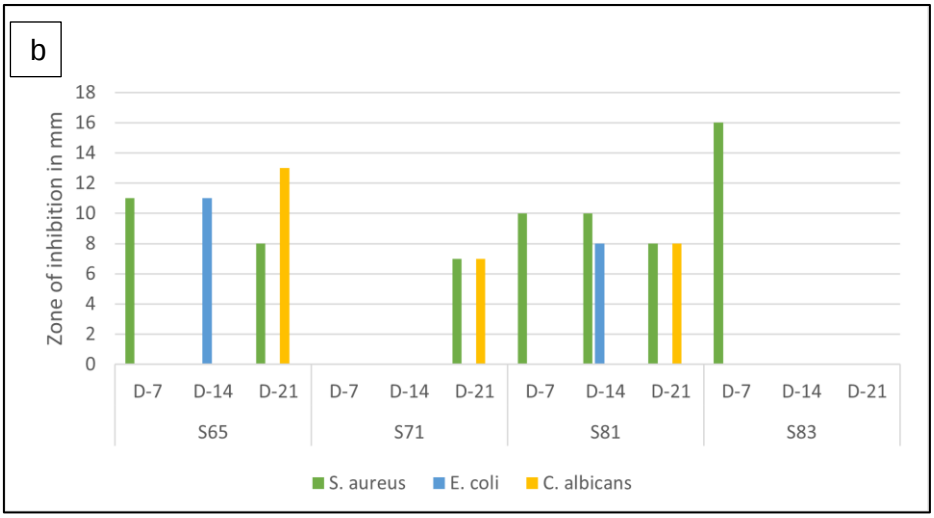
S12	S21	S23	S25	S31	S35	S37	S41	S42
S44	S45	S46	S61	S62	S63	S64	S65	S66
S71	S72	S74	S75	S77	S710	S81	S83	S85
S86	S87	S91	S93	S95	S98	S101	S102	S103
S105	S106	S108	FRW2A	FRW28A	FRW29A	FRW39A	FRW41A	FRW45A

Notably, none of the strains exhibited activity against all three tested pathogens: *S. aureus, E. coli*, and *C. albicans*, and also no strain showed activity against *E. coli*. Moreover, the majority of active strains produced antimicrobial activity when grown on PYG agar media.

3.3 Secondary screening of freshwater fungi

In the study of antimicrobial activity produced by growth on solid-state fermentation using basmati rice as a substrate, 45 fungal strains were screened, including 6 strains previously isolated by Dr. Sally Fryar. **Figure 12** provides a quantitative analysis of the activity of each strain on a solid substrate medium (basmati rice), on day 7, day 14, and day 21 of the incubation period.





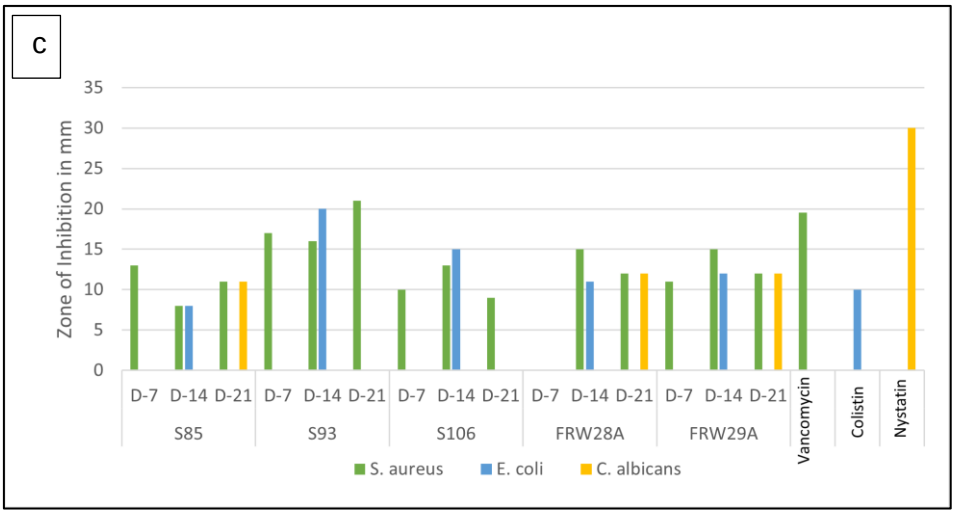


Figure 12 a, b, and **c** represent the antimicrobial activity of freshwater fungi grown on basmati rice at day, 7, 14, and 21 of the incubation period against *S. aureus*, *E. coli*, and *C. albicans*, including Vancomycin, colistin, and nystatin as controls.

Among these strains, 13 fungal strains exhibited antimicrobial activity, representing 28.89% of the total strains screened (**Table 11**).

Table 11 The table presents the outcomes of the secondary screening of 45 strains of freshwater fungi on a solid substrate. 13 strains, highlighted in blue, demonstrated antimicrobial activity. Whereas the remaining 32 strains displayed no activity.

S12	S21	S23	S25	S31	S35	S37	S41	S42
S44	S45	S46	S61	S62	S63	S64	S65	S66
S71	S72	S74	S75	S77	S710	S81	S83	S85
S86	S87	S91	S93	S95	S98	S101	S102	S103
S105	S106	S108	FRW2A	FRW28A	FRW29A	FRW39A	FRW41A	FRW45A

From the secondary screening, 6 strains exhibited activity against all *S. aureus*, *E. coli*, and *C. albicans*. Additionally, 8 strains displayed consistent activity across all incubation periods of day 7, 14, and 21. Based on the outcomes of the secondary screening, 5 fungal strains were chosen for further experimentation. These strains include S64, S83, S93, S106, and FRW28A, exhibiting notable activity against *S. aureus*.

3.4 Scale-up fermentation and antimicrobial compound extraction from selected strains

Five fungal strains – S64, S83, S93, S106, and FRW28A were selected due to their activity observed in the secondary screening. These strains were cultivated on a large- scale utilizing basmati rice as the solid substrate. A total of 50 trays were used, each strain was grown in ten substrate trays with 200 grams of rice per tray. From each strain, four sample trays of rice

cultures containing fungal growth were chosen for testing against *S. aureus* and *E. coli*, with an incubation period of day 7 and day 17. All 5 strains exhibited antimicrobial activity against *S. aureus*. **Figure 13** illustrates the data obtained from the results.

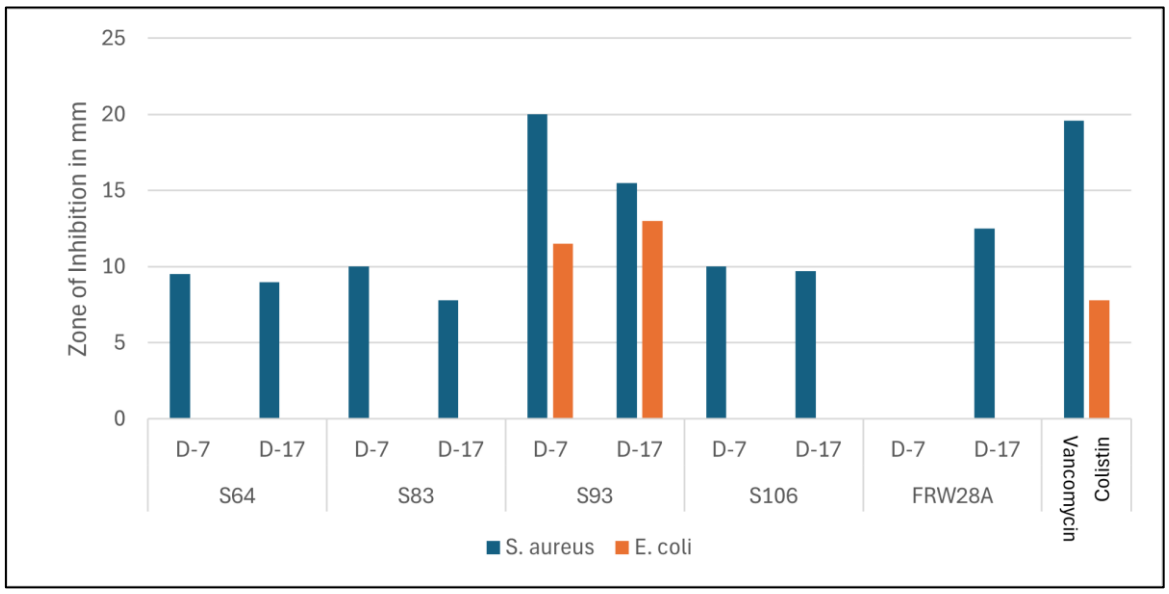


Figure 13 Antimicrobial activity of selected strains cultivated on basmati rice at day 7 and day 17 of the incubation period against *S. aureus* and *E. coli* with controls vancomycin and colistin.

In the results, all selected fungal strains exhibited antimicrobial activity against *S. aureus* during both the 7-day and 17-day incubation periods, with one exception. FRW28A demonstrated activity only on the 17th day of incubation. Furthermore, among the tested strains, only S93 displayed antimicrobial activity against both *S. aureus* and *E. coli* in contrast, the remaining strains were not active against *E. coli*. For strain S93, the leftover solid-state fermentation trays were harvested and each was subjected to methanol extraction. After filtration of this methanol-extracted flask, a total of 1.6 L of the extract was obtained for further investigation. The methanol extract was then condensed using a rotary evaporator

to remove methanol, leaving only the aqueous residue. However, attempts to further purify these extracts using column chromatography were unsuccessful.

3. 5 Separation of the selected extract using thin-layer chromatography

The TLC analysis of the chosen extract S93 with loading 4 microliters and 6 microliters was conducted on silica gel plates using the Chloroform: Methanol (9:1) solvent system. Extract S93 exhibited two distinct bands that were significantly visible under UV light. Upon performing bioautography, inhibition of zones against both *S. aureus* and *E. coli* was recorded (**Figure 14**). While additional bands were also visualized under UV light during TLC analysis of the S93 extract, the zone of inhibition observed in the bioautography was notably lighter or not present in comparison to the other two. Rf values of both antimicrobial compound bands of S93 extract in TLC analysis using Chloroform: Methanol (9:1) solvent system was 0.7.

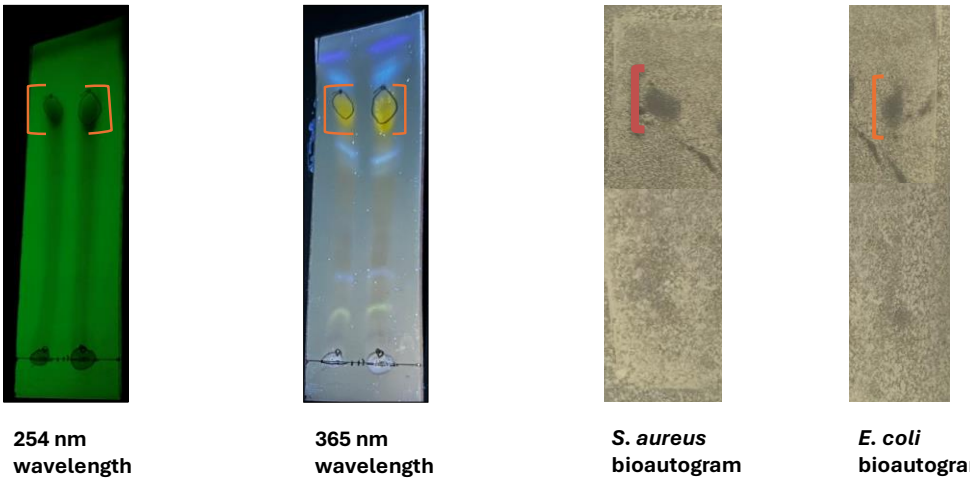


Figure 14 TLC separation of S93 extract on silica gel using Chloroform: Methanol (9:1) solvent system. Visualization under UV light at 254nm and 365nm, presents two

antimicrobial compounds with corresponding inhibition zones against *S. aureus* and *E. coli*. The brackets indicate antimicrobial compounds under UV light.

3.6 Species identification of selected fungal strains

The identification of selected fungal species, including S64, S83, S93, and S106 with selected controls, FRW28A, and FRW29A, was conducted using the DNA primers ITS5 (5' - GGAAGTAAAAGTCGTAACAAGG - 3') and ITS4 (5' – TCCTCCGCTTATTGATATGC – 3'). Initially, Nanodrop One by Thermo Scientific was used to assess the concentration and purity of the extracted DNA samples before performing PCR. To improve the DNA quality, adjustments were made to the extraction protocol with reagent concentrations. However, the DNA yield was found to be insufficient. Subsequently, agarose gel electrophoresis was performed to visualize the extracted DNA fragments. In this experiment, bands corresponding to the DNA of S83, S93, and S106 were successfully observed under the GelDoc Go imaging system (by BIO-RAD), indicating successful DNA extraction for these strains. Based on this, the DNA-extracted samples failed to meet the necessary criteria for molecular identification, characterized by inadequate DNA concentration and impaired purity.

CHAPTER 4 DISCUSSION

4.1 Isolation of freshwater fungi

The isolation of fungi from freshwater wood samples collected from Cox Creek in Bridgewater, South Australia, sheds light on the fungal diversity present in this ecosystem. DRBC media was chosen to inoculate the wood samples due to its ability to enhance fungal discovery and suitability for detecting a wide range of fungal taxa commonly associated with wood substrates (Sharebiology, 2022). This led to the observation of hyphal growth after a 6-day incubation period. Subsequent transfer of fungal cultures to PDA media plates resulted in rapid proliferation, with most fungi displaying visible growth by the 7^{th} day of incubation. During the subculture process, out of the 75 PDA plates inoculated with fungi, 9 plates of fungi were found to have a similar share of characterization, and the other 9 plates were bacterial contaminated indicating potential issues with contamination may arise from improper handling techniques. The rapid growth of the 56 fungi on PDA plates suggests that the isolated fungi possess fast-growing characteristics. The specific taxa isolated may exhibit unique characteristics. Hence, further morphological, and molecular characteristics are needed to fully understand their diversity and ecological roles.

4. 2 Primary screening of freshwater fungi

The primary screening aimed to preliminarily isolate potential antimicrobial compound-producing fungi from a group of 45 strains of Australian freshwater fungi. Each strain underwent cultivation on two agar media, PDA and PYG, followed by screening for antimicrobial activity using the method of antibacterial activity assay as it determines whether fungi produce compounds that have the potential to inhibit the growth of bacteria

(Armengol *et al.*, 2021). Among the 10 strains that displayed activity (**Figure 11**), none demonstrated antimicrobial activity against all tested pathogens: *S. aureus, E. coli,* and *C. albicans*. This indicates that while certain strains may possess antimicrobial properties, their effectiveness may be limited to specific types of pathogens.

The antimicrobial activity of the screened strains was categorized based on the diameter of the zone of inhibition observed in the agar diffusion assay. Strains were classified into four groups: High activity (16-20mm), intermediate activity (11-15nm), low activity (8-10mm), and weak activity (less than 8mm). Upon evaluation, only strains S103 and S105 demonstrated high antimicrobial activity against *C. albicans*. Strains S101 and S105 exhibited intermediate activity against *C. albicans,* while strain S85 showed intermediate activity against *S. aureus*. Strain S108 displayed a low activity level, whereas strains S62, S64, S65, and S72 exhibited weak activity against *S. aureus*.

For media, Potato Dextrose Agar (PDA) was selected for its rich composition for the growth of a wide spectrum of fungi and Peptone Yeast Glucose Agar (PYG) for providing a nutrient-rich environment for fungal cultivation (Tsao, 1970). The comparison between PYG and PDA media revealed notable differences in antimicrobial activity against the tested pathogens. Specifically, a higher proportion of strains cultivated on PYG media (7 strains) exhibited antimicrobial activity than those grown on PDA media (4 strains). Strain S105 displayed antimicrobial activity against pathogens in PYG and PDA media, suggesting consistent activity across different growth conditions. These findings suggest that the composition of the growth media may influence the antimicrobial activity of the screened fungal strains (Brown, 1923). Despite the unidentified nature of these freshwater fungi, it is anticipated that these may contain unique metabolic pathways and novel bioactive compounds.

4.3 Secondary screening of freshwater fungi

A total of 45 stains of freshwater fungi were screened for their antimicrobial activity using solid-substrate fermentation, in which 13 freshwater fungi strains displayed antimicrobial activity (**Figure 12**). Basmati rice was utilized as the solid-state media to employ the natural habitat of fungi, proving a cost-effective and readily available substrate compared to artificial laboratory-based media. (VanderMolen *et al.*, 2013). Previous studies have investigated the effectiveness of rice as a growth substrate for the production of antimicrobial secondary metabolites across various organisms, including fungi (Lizardi-Jimenez and Hernandez-Martinez, 2017; VanderMolen et al., 2013). Additionally, this method demonstrates scalability, facilitating the production of significant quantities of selected fungi compounds for subsequent experiments, particularly structure elucidation (Lizardi-Jimenez and Hernandez-Martinez, 2017).

In contrast to primary screening, an increase in the number of strains demonstrating antimicrobial activity was observed with significant variations. Notably, only 4 strains, including S62, S64, S65, and S85 were consistent between both screenings. All 13 strains exhibited antimicrobial activity against *S. aureus*, while 10 strains displayed activity against *E. coli*, and 7 strains were active against *C. albicans*. These finding highlights the dynamic nature of antimicrobial production among the screened strains and suggests potential variations in their antimicrobial spectra.

On the 7th day of screening, 9 out of the total 13 strains exhibited antimicrobial activity. By the 14th day, the number of active strains increased to 10, and on the 21st day of screening all strains, except for strain S83, displayed antimicrobial activity. This indicates the progressive trend in antimicrobial production over time. Throughout all incubation periods of the screening, strain S93 consistently displayed the highest zone of inhibition compared to the

other strains, with its antimicrobial activity notably increasing by day 21. This finding shows the potential significance of strain S93 as a promising source of potent antimicrobial agents.

4.4 Scale-up process of selected freshwater fungi

The Scale-up fermentation of freshwater fungi for antimicrobial compound production was investigated, focusing on 5 selected strains: S64, S83, S93, S106, and FRE28A, based on their observed activity from secondary screening. These strains were cultivated on a large-scale using basmati rice trays as the solid substrate against *S. aureus* and *E. coli*, with incubation periods of day 7 and day 17. All five fungal strains exhibited antimicrobial activity against *S. aureus* during both the 7-day and 17-day incubation periods, except the FRW28A strain which was active on the 17^{th} day only. This highlights their consistent antimicrobial effect over time. Furthermore, among the tested strains in this experiment, the S93 strain displayed antimicrobial activity against both *S. aureus* and *E. coli* with a notable increase in zone of inhibition compared to other tested strains, indicating its broad-spectrum effectiveness against bacterial pathogens. For strain S93, leftover solid-state fermentation trays underwent harvesting, with five culture trays of fungus subsequently subjected to methanol extraction using 400ml of methanol in a 1000ml flask. Following filtration of the methanol-extracted flask, a total of 1.6L of the extract was obtained for the next investigation purpose.

Moreover, the scalability of solid fermentation allows for the production of antimicrobial compounds on a large scale, which provides advantages for potential industrial applications (Wang *et al.*, 2023).

4.5 Separation of the selected extract using thin-layer chromatography

The process of Thin Layer Chromatography (TLC) served as a pivotal step in characterizing and separating the bioactive compounds within the methanol crude extract (Sheeba *et al.*, 2019). The TLC profiles of the extract notably revealed the presence of antimicrobial compounds within the S93 extract strain. However, need to acknowledge that due to the utilization of only one strain for the TLC method and a singular solvent system, Chloroform: Methanol (9:1), the comparative analysis of the extract was somewhat limited. Despite this limitation, the bioautogram analysis yielded insightful results, demonstrating activity against both *S. aureus* and *E. coli* when the TLC plate was tested.

This finding suggests that the bioactive compound present in the extract S93 extract possesses antimicrobial properties against these bacterial strains. Further research incorporating a broader range of strains and the solvent system could offer additional insights into the diversity of antimicrobial compounds present in methanol crude extract.

4.6 Species identification of selected strains

The molecular identification of selected fungal strains, including S64, S83, S93, and S106, along with controls FRW28A and FRW29A, was attempted using the DNA primers ITS5 and ITS4. Initially, the concentration and purity of the extracted DNA samples were assessed. However, the DNA yield was found to be insufficient, indicating challenges in the DNA extraction process.

Furthermore, agarose gel electrophoresis was employed to visualize the extracted DNA fragments, and bands corresponding to S83, S93, and S106 were successfully observed. Despite successful visualization of DNA bands, DNA-extracted samples failed to meet the necessary criteria for molecular identification. This was characterized by inadequate DNA concentration and impaired purity of the samples. These challenges may arise due to various

factors such as incomplete cell lysis during DNA extraction, DNA degradation during handling, or the presence of PCR inhibitors in the extracted DNA samples.

4.7 Conclusion

This study aimed to validate the hypothesis that novel strains of freshwater fungi have the potential to produce antimicrobial compounds, with previous findings of bioactive secondary metabolites from freshwater fungi. The study was structured around four main aims, isolation of freshwater fungi from freshwater wood, screening of isolated fungi for antimicrobial activity, scale-up process for production of antimicrobial compounds from selected fungi, and molecular identification of the selected freshwater fungi. To achieve this, initially, a total of 56 freshwater fungi were isolated from Bridgwater River, South Australia. In primary screening, conducted on PDA and PYG agar, 22.22% of the strains displayed antimicrobial activity. Subsequent secondary screening with solid-state fermentation on basmati rice revealed antimicrobial activity in 28.89% of the screened freshwater fungi. Five strains (S64, S83, S93, S106, and FRW28A) showing promising activity in screenings underwent large-scale fermentation. Due to time constraints, only strain S93 was chosen for the thin-layer chromatography (TLC) experiment and for further. Molecular identification was attempted for four strains including, S64, S83, S93, and S106. However, challenges prevented further identification. Furthermore, Purification and structural elucidation of the compounds will be essential to validate the study. The successful isolation of active strains with promising antimicrobial activity levels underscores the validity of exploring freshwater fungi as a source of novel antimicrobial compounds.

4.8 Challenges of the study

During the study, several challenges were identified. The methanol extract underwent condensation using a rotary evaporator to eliminate methanol, intending to obtain only aqueous residue. However, this process encountered challenges, and the desired outcome was not achieved. This process led to a pause in progressing further with the purification of the antimicrobial compound through column chromatography experiment. Another is particularly concerning the incubation periods required for the growth of fungi and for screening antimicrobial activity in freshwater fungi. Scale-up fermentation posed difficulties in maintaining 50 rice trays for solid-state fermentation. Additionally, molecular identification of strains proved challenging, requiring multiple DNA extraction methods before employing the final one, yet yielded insufficient DNA concentration for further sequencing and identification. This resulted in the inability to assign fungal names or genera. Furthermore, the time limit constraint of the nine-month research duration, with the large number of 56 fungal strains to work with, presented significant limitations. However, despite these challenges, all s6 strains were successfully screened, one promising strain underwent scale-up fermentation, and four strains were subjected to molecular identification.

4.9 Future directions

A critical future step for this study involves purifying antimicrobial compounds through column chromatography to isolate and obtain highly pure compounds. A silica gel column will be prepared, and the evaporated extract compound will be dissolved in chloroform and loaded onto the column. Compounds will be eluted using an appropriate solvent system, and fractions will be collected, the presence and purity of antimicrobial compounds in each fraction will be assessed using thin-layer chromatography (TLC) and contact bioautogram analysis. Potential fractions containing purified antimicrobial compounds will be evaporated

and stored at -20 °C for further experiments. Additionally, the elucidation structure of these purified compounds will be essential in advancing the research objectives. The determination of the compound's structure will involve utilizing NMR spectroscopy, mass spectroscopy, and FTIR spectroscopy. Another important future direction involves conducting molecular identification of the 56 strains with additional PCR methods, which may potentially reveal novel freshwater fungi species also it will be an opportunity to develop novel antibiotics targeting antibiotic-resistant bacteria. Further research should explore additional freshwater fungi from various geographic locations to identify potential novel compounds for addressing antibiotic resistance.

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