Production of antimicrobials and antioxidants from filamentous fungi

NUI MAYNOOTH
Ollscoil na hÉireann Má Nuad

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Declaration of Authorship

This thesis has not been submitted in whole or in part, to this or any other university, for any degree and is, except where otherwise stated the original work of the author.

Signed: _____

                Helen Smith

Date: _____

Dedication

For Nanny Phyllis and Nana Mag

Missing you always xx

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Abstract

Filamentous fungi have proven throughout history to be a vast source of potential therapeutic activities. They are recognised as nutritious, highly palatable functional foods and are now widely accepted as an untapped source of potentially powerful natural products of pharmacological significance.

In the present study, ten species of filamentous fungi were explored on the basis of their curative potential. Submerged liquid fermentation (SLF) was employed and proved to be a promising method for the production of bioactive functional compounds. Crude aqueous mycelial biomass demonstrated a broad range of antibacterial activity against pathogenically significant bacteria. Supplementation with various carbon sources at 1 % (w/v) affected the biomass production of several species significantly (p < 0.05) and in some cases also had a significant impact on cellular composition. Additionally, depending on species specificity, cell wall composition was found to affect the interaction of microbes prior to infection.

Various extracts (crude, hot water and methanol) from the mycelium of each of the selected species were demonstrated as effective antioxidants. Additionally, total phenolic content positively attributed to the overall antioxidant capacity of the extracts. Bioassay-guided isolation and purification of extracts from cultured mycelia led to the identification of biologically active phenolic acids and compounds of lipid class. For effective utilisation of natural products or functional food components, quantitative information on the individual phenolic acids in each strain was generated. Compounds separated by TLC were extracted and analysed by LC/MS for fatty acid composition. The fatty acids; linoleic, palmitic, stearic and oleic acid were established as the main lipid metabolites of each fungal species. In addition, the effect of hot water and methanol extraction on fatty acid composition of the fungal cell was analysed by GC/MS. The fractionated extracts demonstrated that a combination of multiple chemical constituents yielded favourable biological activities.

Submerged fermentation of mycelium from ten species of filamentous fungi proved to be an effective cultivation method for the production of bioactive and nutritional functional compounds. This research has provided information which could benefit further research in the isolation and characterisation of active chemical components of natural origin.

Abbreviations

ABTS	2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
ABTS$^+$	2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) cation radical
AlCl$_3$.6H$_2$O	Aluminum Chloride Hexahydrate
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
CHCl$_3$	Chloroform
CUPRAC	Cupric Ion Reducing Activity
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-2-picrylhydrazyl
DW	Dry Weight
EDTA	Ethylenediaminetetraacetic Acid
F.D.A	Food and Drug Administration
FRAP	Ferric Reducing Antioxidant Power
GABA	γ-Aminobutyric acid
GC/MS	Gas Chromatography/Mass Spectrophotometry
GRAS	Generally Regarded As Safe
HCL	Hydrochloric Acid
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HWE	Hot Water Extract
K$_2$HPO$_4$	Dipotassium Phosphate
KH$_2$PO$_4$	Monopotassium Phosphate
KOH	Potassium Hydroxide
LC/MS	Liquid Chromatography/Mass Spectrophotometry
LDL	Low-Density Lipoprotein
MAE	Microwave Assisted Extraction
MgSO$_4$.7H$_2$O	Magnesium sulphate heptahydrate
MIC	Minimum Inhibitory Concentration
MOS	Mannanoligosaccharides
MTT	1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan
NADPH	Nicotinamide Adenine Dinucleotide Phosphate

NaOH	Sodium Hydroxide
nm	Nanometres
NP/PEG	Natural Products / Polyethylene Glycol
OD	Optical Density
ORAC	Oxygen Radical Absorption Capacity
PBS	Phosphate Bovine Serum
PDA	Potato Dextrose Agar
PLE	Pressurised Liquid Extraction
PSK	Polysaccharide K (Krestin)
PSP	Polysaccharide-peptide
R_f	Retention factor
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RPM	Revolutions per minute
SFE	Superficial Fluid Extraction
SLF	Submerged Liquid Fermentation
SPE	Solid-Phase Extraction
Spp.	Species
SSF	Solid State Fermentation
TEAC	Trolox Equivalent Antioxidant Capacity
TLC	Thin Layer Chromatography
TOF	Time of Flight
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
U.S.D.A	U.S. Department of Agriculture
UPLC	Ultra Performance Liquid Chromatography
UV	UltraViolet
v/v	Volume per volume
w/v	Weight per volume
YCW	Yeast Cell Wall
YPD	Yeast Potato Dextrose
ZOI	Zone of Inhibition

1.   Introduction

For thousands of years fungi have been recognised as nutritious, highly palatable functional foods in many societies and are now accepted as a valuable source for the development of medicines and nutraceuticals (Chang *et al.*, 1996; Wasser, 2002). Pharmacological and medicinal studies of fungi have shown that the Basidiomycete and Ascomycete divisions are an immense source of biologically active components, yet less than ten percent of all species have been described and even less have been tested for therapeutic significance (Lindequist *et al.*, 2005; Blackwell, 2011). It is clear that fungi represent a largely untapped source of potentially powerful new pharmaceutical products (Hawksworth, 1993; Wasser, 2002).

The following section examines the medicinal and antioxidant potential of several higher fungi from the Basidiomycota and Ascomycota division. The effect of the cultivation processes on mycelia generation and bioactive compound production will be explored and the future of drug discovery with filamentous fungi will also be discussed.

1.1   Filamentous fungi of therapeutic significance

1.1.1   History of therapeutic filamentous fungi

Most historical accounts personify fungi as "the foods of the Gods". The Romans describe how bolts of lightning cast to earth by Jupiter during thunder storms brought them to this planet (Alexopoulos *et al.*, 1996). In ancient Egypt, they were considered a gift from the God Osiris (Stephenson, 2010); while from the days of early civilisation, Asians considered their curative properties as an elixir of life (Mizuno *et al.*, 1995a). Over the centuries, a diverse range of fungi have evolved and with this, a practical knowledge of the use of fungi for consumption, medicinal purposes or natural poisons has developed. According to Schultes *et al.* (2001), consumption of *Psilocybe* species also known as "magic mushrooms" during the age of mankind may have caused conscious expansion in humans; ultimately projecting our brain and thought systems through evolution, setting us apart from animals. It is believed that in ancient times these properties influenced the integration of fungi into certain religions.

Many cultures, particularly Asian, originally identified certain mushrooms as having profound health promoting benefits (Hobbs, 1995). Since then, a large number

of mushroom derived compounds, both cellular components and secondary metabolites, have been shown to positively affect the immune system and are now being used to treat a variety of diseases. Various biomedical metabolites have been extracted from mushroom fruiting bodies, cultured mycelia and cultured broth from submerged cultivations (Mizuno *et al.*, 1995b; Hatvani, 2001; Gregori *et al.*, 2007).

A total of 126 therapeutic functions are thought to be produced by medicinal mushrooms (Wasser, 2011). Basidiomycetes as well as Ascomycetes comprise the vast majority of fungal diversity (Blackwell, 2011). Biologically, Basidiomycetes display a broad spectrum of pharmacological activities, showing anti-bacterial, anti-viral, antioxidant, anti-fungal, anti-inflammatory, antitumor and immunomodulating effects, as well as demonstrating a significant impact on cardiovascular disorders, in hypercholesterolemia and also diabetes (Gunde-Cimerman, 1999; Wasser *et al.*, 1999c; Zaidman *et al.*, 2005; Gregori *et al.*, 2007; Shamaki *et al.*, 2013). Similarly, Ascomycetes fungi are also recognised for their medicinal properties. Traditional Oriental medicine recognised these fungi for their diverse biological activities, including immunostimulatory, antitumor and hypoglycaemic activity (Sone *et al.*, 1985; Kiho *et al.*, 1993). The evolution of even the protective compounds which give fungi the ability to defend against predators in the wild is of immense interest from a pharmaceutical perspective.

Although curative features of Basidiomycota are mentioned in literature of Europe and North America, in depth investigation of pharmacological and medicinal characteristics of Basidiomycetes has only started recently (Lindequist *et al.*, 2005). In China, more than 100 mushroom species are used in traditional medicine from 270 registered mushroom species (Smith *et al.*, 2002). Commonly known edible mushrooms, which also have functional properties include; Auricula, Flammulina, Grifola, Hericium, Lentinus, Pleurotus and Tremella (Table 1.1).

Table 1.1　　Some important medicinal mushrooms
Adapted from (Stamets, 2000).

Species	Common name	Mushroom
Auricula auricularia	Wood Ears	
Trametes versicolor	Turkey Tail	Polypore
Flammulina velutipes	Enoki	Gilled
Ganoderma lucidum	Reishi	Polypore
Grifola frondosa	Maitake	Polypore
Hericium erinaceus	The Lion's Mane	
Lentinus edodes	Shiitake	Beech
Schizophyllum commune	Split Gill	Gilled
Tremella fuciformis	White Jelly	Jelly
Poria cocos	Wolfiporia extensa	
Pleurotus ostreatus	Oyster	

On the other hand, some edible mushrooms do not merit any medicinal value e.g. *Agaricus bisporus*, and some medicinal mushrooms such as Ganoderma and Coriolus are not edible (Chang, 1996), particularly Trametes and Inonotus which are mostly consumed in the form of extract, tea or powder (Wasser, 2002; Ferreira *et al.*, 2010). Some species of fungi are grown for their unique produce, such as pigment production. *Monascus* species are used for red rice wine, red soybean cheese and Anka (red rice) production (Lin *et al.*, 2008). In China, Japan, Korea, Taiwan and Southeast Asia, the main use of Monascus is in traditional medicine and as a food additive, usually in the form of a powdered extract. Of the twenty species recorded in published literature since the genus was first proposed in 1884, nine are internationally recognised; *M. pilosus, M. ruber, M. purpureus, M. eremophilus, M. pallens, M. sanguineus, M. lumisporas* and *M. argentinensis* (Shao *et al.*, 2011). Metabolic products from Monascus may be classified into four categories, namely; anticholesterol, anticancer, food colouring and essential fatty acids necessary for human health (Ahamed *et al.*, 2012).

1.1.2　Fungal diversity

Worldwide, fungi are extremely abundant and diversified. According to Hawksworth (1991) there are approximately 1.5 million species of fungi on Earth, of which 70 - 100,000 species are described (Hawksworth, 1991; Kirk *et al.*, 2011). Of these, 14,000 species are mushrooms, 5,000 of the macro fungi are edible and over 1,800 of these species are considered to have medicinal properties (Lindequist *et al.*, 2005; Kirk *et*

al., 2011). When taking into account the data acquired from molecular screening methods, recent estimates predict as many as 5.1 million species of fungi exist (O'Brien *et al.*, 2005; Bass *et al.*, 2011; Blackwell, 2011). Bioactive metabolite production for medicinal use in microscopic fungi has shown great potential; however, only a small proportion of the known investigated species have been studied for their pharmacological significance.

When considering the therapeutic effects of natural products, fungi are of particular interest because like us they are eukaryotic organisms and their metabolism is more closely related to us than that of prokaryotic bacteria. Animals and fungi share in common the same microbial pathogens, such as *Escherichia coli* and *Staphylococcus aureus*. Therefore as animals, we can exploit defence strategies used by fungi throughout evolution to fight microorganisms, making fungi our best source of antibiotics (Zjawiony, 2004). In fact, eukaryotic microbiologists have recently described a super kingdom named Opisthokonta that groups animalia and fungi together (Steenkamp *et al.*, 2006), with an ancient linkage estimated to be over 1 billion years old (Knoll, 1992).

In 1969, fungi were recognised as a very distinct and separate group of organisms and were classified into their own kingdom, separating them from the plant kingdom (Whittaker, 1969). There are a number of reasons as to why fungi and plants are not classed in the same kingdom: fungi are not photosynthetic; they differ in cell wall composition; body structure and modes of reproduction (Whittaker, 1969). Additionally, chitin is not found in higher plants as it is a cell wall component unique to fungi, crustaceans, insects and parasites. Finally, fungi commonly store glycogen, which is typical of animal cells but not of higher plants. Besides nutrition, a considerable distinction between plants and fungi is their behaviour; fungi lack chloroplasts and are heterotrophic organisms and so exploit their environment by absorbing organic materials and nutrients (Sharma, 2004). They play an important role in the decomposition of complex remains of dead organisms, which releases nutrients back into the soil and atmosphere for plant metabolism (Sharma, 2004).

The Kingdom Fungi is diversified into several divisions, these include; Basidiomycota (club fungi), Ascomycota (sac fungi), Blastocladiomycota, Chytridiomycota, and Zygomycota. Basidiomycetes are a highly evolved group of fungi, well known for their edible properties. According to Polishchuk *et al.* (2009), most research looking at fungi as a source of pharmacological importance has been

directed toward Basidiomycota and has mostly been focused on strains from Auricularia, Flammulina, Ganoderma, Grifola, Hericium, Lentinus (Lentinula), Pleurotus, Trametes, Coprinus, Laetiporus, Panus, Schizophyllum and Tramella. Although fungi from the Ascomycota kingdom are well established for having strong anti-hypercholesterolemic activity, there has been less research on the curative components of fungi from this division for other medicinal purposes. Together, Ascomycota and Basidiomycota form the subkingdom Dikarya, accounting for approximately 98% of all known fungal species (James *et al.*, 2006). Ascomycota represents the largest group of fungal phyla with over 64,000 species, while Basidiomycota contains about 30,000 described species (Ainsworth, 2008; Blackwell, 2011).

1.1.3   Structure and composition of fungal cells

The structure of the fungal cell itself has a significant outcome on the diverse medicinal application of the organism, and so it is important from a pharmacological perspective to understand this biologically significant structure-function relationship. Altogether, the fungal cell wall has a multifunctional role. It is involved in the regulation of morphogenesis, fungal reproduction, determination of the antigenic and adhesive characteristics, control of dimorphism, and transfer to the membrane and intracellular messengers (Feofilova, 2010).

The cell wall plays a fundamental role in the development and the integrity of the structural requirements for survival and proliferation. It is extremely protective, in that, it shields the fungus from abrasion, screens out poisons from the environment and by restricting the inflation of the cytoplasm, allows the cell to become highly pressurised (Osherov *et al.*, 2010). It also serves as a scaffold for proteins that protect the inner polysaccharide layers and provides a dynamic interface with its surroundings (Osherov *et al.*, 2010). However, despite much information being known about the protective role of the cell wall, there is little known about its complete biosynthesis, particularly among filamentous fungi (Esser *et al.*, 2006). The physical and biological properties of the fungal cell wall are determined by the composition and arrangement of their structural components, varying amongst species. (Fontaine *et al.*, 1997). It is these variations which led to the various taxonomic classifications between fungal species in the past (Roncero, 2002). As well as taxonomic differences, the dynamic

cell wall structure is subject to continuous change in response to culture conditions and environmental stresses, throughout the growth of the fungus (Latge, 2007).

Essentially, two types of components form fungal cell walls; fibrous polymers, which constitute the structural scaffold of the wall and gel-like polymers, which act as the interconnecting molecule. It is the relationship between these components which is responsible for the dynamic properties of the cell wall, required for its survival under diverse environmental conditions (Roncero, 2002).

1.1.3.1   Architecture of the cell wall

It has been suggested that the interrelationships between all of the major cell wall components of *Saccharomyces cerevisiae* are comparable to that of the fungal cell wall (Osherov *et al.*, 2010). In fact, most studies which look at the architecture of the fungal cell wall have been based on yeast models. Similar to *S. cerevisiae*, the fungal cell wall is complex, usually representing around 15 – 30 % of the cell dry weight (Valentin *et al.*, 1987; Nguyen *et al.*, 1998), with 80 – 90 % made up of polysaccharides (Bartnicki-Garcia, 1968; Nimrichter *et al.*, 2005). In general, the cell wall consists of an array of structural carbohydrate polymers interspersed with glycoproteins. Glucans, chitin and mannans are particularly abundant. The main structural components of the wall are; chitin, which is a linear polymer of the acetylated amino sugar *N*-acetylglucosamine, and the polysaccharide β-(1,3)-glucan. Mannoproteins are major components of the outer wall of fungal cells, especially yeasts; they are densely packed and limit wall permeability to solutes.

The innermost cell wall layer is composed of extremely robust chitin microfibrils (Figure 1.1). β-(1,3)-glucan fibrils, located in the inner part of the wall, are attached to chitin by glycosidic linkages. Chitin is crystalline and extraordinarily strong, more so than many artificial materials, due to the extensive hydrogen bonding along the chains when being formed (Roncero, 2002). Chitin is widely distributed in the fungal kingdom, occurring in Basidiomycetes, Ascomycetes and Phycomycetes (Peter, 2005). It is a structural component of mycelia, stalks, and spores (Peter, 2005). Aside from protein, glucan and mannan, chitin content varies from trace amounts to 45 % of the organic fraction (Roberts, 1992). In filamentous fungi, biosynthesis of chitin takes place in the growing hyphae. This polysaccharide is not only important for strength and rigidity of the structure but in some species it acts as a protective barrier; allowing a capsular polysaccharide on the outer surface of the wall to connect to the

outside environment, whilst the inner cell of the fungus is isolated from the surroundings (Roncero, 2002; Osherov *et al.*, 2010). According to Osherov *et al.* (2010), the cell wall of yeast contain around 2 – 3 % chitin, a much smaller quantity compared to filamentous fungi, which is recorded as having up to five times that amount. The most likely reason for this is due to the evolutionary pressures between the two organisms. Filamentous fungal growth forms at the hyphae tip and has the ability to penetrate hard surfaces with enormous turgor pressure, whereas yeast growth is largely isotropic and is usually confined to surfaces (Osherov *et al.*, 2010). In nature chitin does not occur in pure form; like other biopolymers it is bound predominantly by covalent bonds with other compounds, in fungi this is mainly β-glucans (Feofilova, 2010). Moving toward the outer cell surface, β-(1,3)-glucan forms the fibrous scaffold of the wall and is responsible for its mechanical strength; these fibrils are covalently bound to β-(1,6)-glucans. Glycosylphosphatidylinositol (GPI)-mannoproteins are either anchored in the lipid bilayer of the cell membrane, with a polypeptide chain extending out through the wall or are cleaved from their anchors and bound to β-(1,6)-glucans in the outer part of the wall. It is these heavily glycosylated mannoproteins which are responsible for cell-cell recognition events (Klis *et al.*, 2002). Another class of proteins characterised by internal repeats of amino acid sequences are known as 'Pir' cell wall proteins (Pir-CWPs) and are covalently linked to β-(1,3)-glucan through alkali-sensitive linkages (Figure 1.1) (Smits *et al.*, 1999; Carlile *et al.*, 2000).
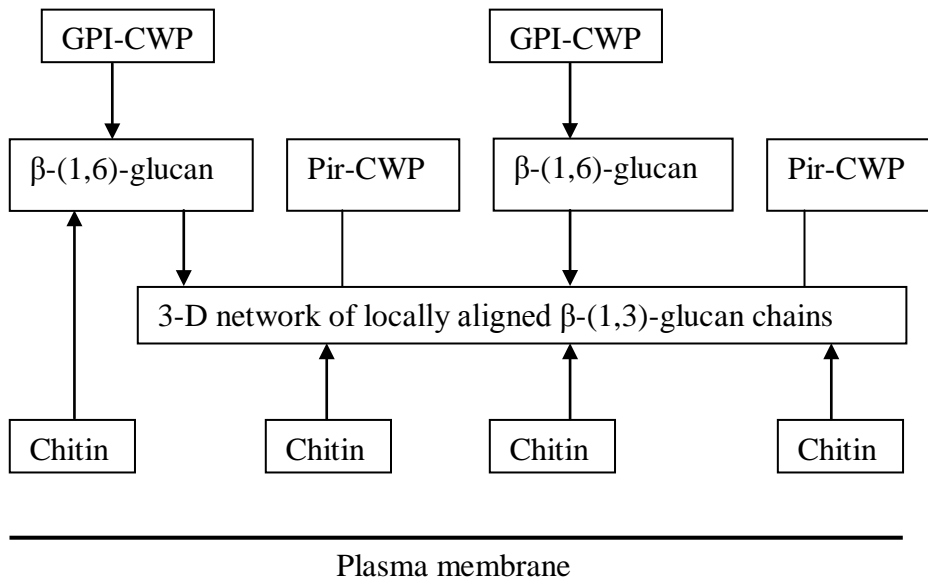
Figure 1.1   A molecular model of the major structural components of the fungal cell wall

Diagram adapted from Carlile *et al.* (1994) and Smits *et al.* (1999).
- Abbreviations: Pir-CWP, 'Pir' cell wall protein; GPI-CWP, glycosylphosphatidylinositol cell-wall protein.

Overall the cell wall forms a bilayered, dynamic and rigid structure that encompasses the entire cell. Its mechanical strength is largely based on an internal layer of moderately branched β-(1,3)-glucan molecules that form a three-dimensional network, kept together by hydrogen bonding between laterally associated chains (Whistler, 1973; Klis *et al.*, 2002).

While studies on *S. cerevisiae* provide an overview of the cell wall, diversity may exist in wall composition of different cell types in any fungal species and even when comparing strains within a species (Carlile *et al.*, 2000; Levitz, 2010). In addition to β-glucans, α-glucans have been seen in some (but not all) strains of *Histoplasma capsulatum*, chitosan in *Cryptococcus neoformans*, and galactomannans in species of *Aspergillus* (Banks *et al.*, 2005; Rappleye *et al.*, 2007). Melanin has also been found to be present in some fungal cell walls (Levitz, 2010). Variations in length and linkage type in mannans have also been seen (Levitz, 2010).

Fungi can survive extreme environments and the overall characteristics of the cell wall are a testament to the importance of each component. Organisation of each component which constitutes the whole structure is essential to the survival of the fungus. Fungal cells are also capable of transforming from viscoelastic state to a rigid cell and vice versa, to allow for functions which require changes of the cell wall such

as expansion, branching and mating (De *et al.*, 2001). These controlled alterations in the mechanical properties of the wall permit changes under high turgor pressure (Ruiz-Herrera, 2012). Differences in composition are important, as structure of the fungal cell wall is known to affect its biological activity (Rodrigues *et al.*, 2011).

1.1.4   Nutritional requirement, metabolism and development

All fungi are heterotrophic organisms and as such, obtain their energy and carbon supply from other organisms for metabolism and survival. Most fungi and yeast have basic nutritional needs and could survive alone once supplied with an aerobic environment, glucose, ammonium salts, inorganic ions and a few growth factors (Kavanagh, 2011). Carbon sources such as simple hexoses like glucose or complex polysaccharides such as starch and cellulose can be utilised by filamentous fungi for growth. The range of a species in the environment is likely to reflect the availability of carbon sources (Carlile *et al.*, 2000). In higher fungi, development takes place following uptake of nutrients from the environment by cellular components which stimulate septation and biomass.

The first stage of primary vegetation is the formation of branched filamentous structures of fungi known as hyphae, a mass of which is known as the mycelium. Prior to the development of the fruiting body (which is required for reproduction), fungi perform an important role in the decomposition of organic matter and have a fundamental role in nutrient intake and exchange. The mycelium (Figure 1.2) penetrate the substrate and continually interacts with the surrounding environment, breaking down waste products such as; nitrogen, carbon, plant and animal debris, converting them into assimilable nutrients. Mycelia have also shown the ability to break down pesticides, dioxin, chlorine and hydrocarbons which form the majority of heavy metal pollutants from industrial production (Stamets, 2011).

Initial growth and biomass production of fungi is dependent on the availability of primary metabolites (protein, carbohydrates, nucleic acids and lipids). These metabolites are associated with the rapid initial growth phase of the organism and maximum production occurs near the end of this phase (Kavanagh, 2011). This phase of growth is known as the exponential phase and is often termed primary metabolism.
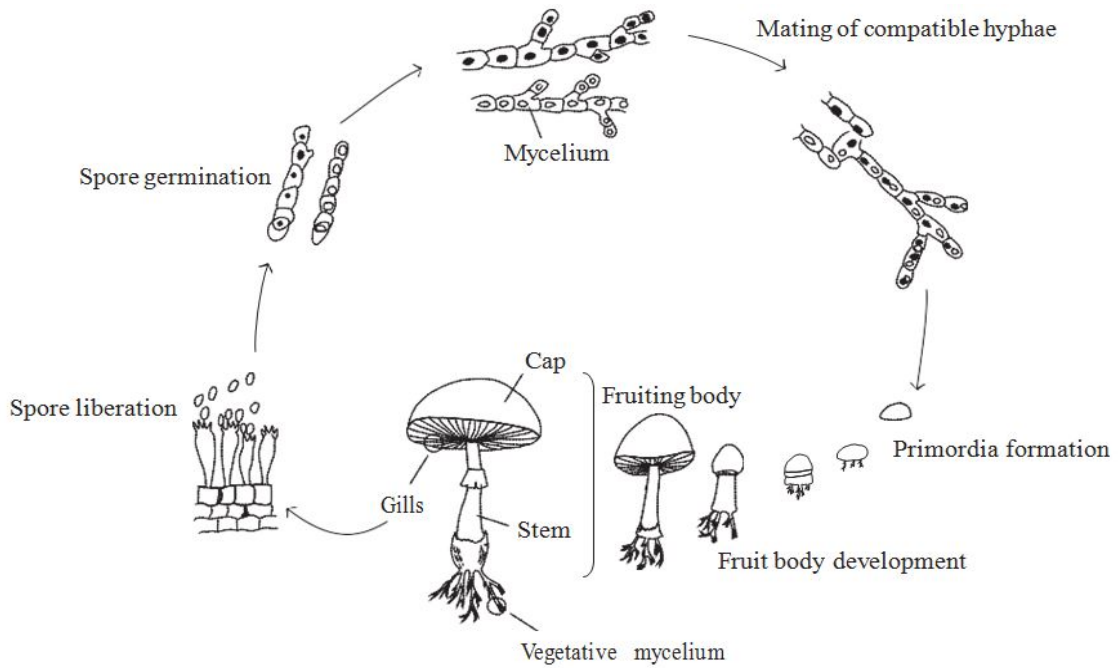
Figure 1.2   Diagrammatic representation of mushroom life cycle

Illustration taken from (Lull *et al.*, 2005).

Nutritional requirements are important in an industrial sense, with regard to cultivation and optimisation of growth conditions for increased commercial biomass or metabolite production (Section 1.5.2). Some primary metabolites are produced commercially, such as citric acids (food and soft drink manufacturing), ethanol (alcoholic drink production), enzymes (food processing) and amino acids, and vitamins (food supplements). When the supply of essential nutrients is exhausted, the specific growth rate of the culture enters the stationary phase, entering a stage of secondary metabolism. As fungi enter into the stationary phase, compounds (secondary metabolites) which are not required for active growth and are not essential for vegetative proliferation are produced. A range of secondary metabolites have been found to contain substantial biological activity and so a lot of research has focused on the production, isolation and extraction of these compounds (Mizuno, 1999).

One of the major benefits and advances of microbial technology in commercial scale operations is the ability to produce high-nutritional and pharmaceutical value biomass using abundant agro-industrial residues (Fazenda *et al.*, 2008; Akyuz *et al.*, 2009; Philippoussis *et al.*, 2011). Submerged liquid fermentation (SLF) has in recent years been utilised for this purpose. Mycelial biomass produced using SLF in high quantities for therapeutic downstream processes, such as isolation of bioactive

metabolites, may be generated in a short period of time. Simultaneous valorisation of important agro-industrial liquid residues such as waste glycerol, sugar cane and soya molasses, as well as olive-mill wastes or thin sillage may be used as a substrate medium for fungal mycelia or fruit body formation (Tang *et al.*, 2007; Diamantopoulou *et al.*, 2014). There is increased interest in the use of mycelial biomass as a possible foodstuff, not only because of its high nutritional value, but also due to its therapeutic potential, due to the presence of bioactive proteins, lipids, polyunsaturated fatty acids, sterols and polysaccharides (Dimou *et al.*, 2002; Pedneault *et al.*, 2008; Diamantopoulou *et al.*, 2012). Thereby, both intracellular and extracellular polysaccharides produced by SLF are of considerable industrial and economical significance. Intracellular carbohydrates are recognised as containing immunomodulating, antitumor and hypoglycaemic factors (Zhong *et al.*, 2004; Tang *et al.*, 2007), and extracellular polysaccharides are recognised as also having a range of biological activities (Wasser, 2002). Microscopic fungi have been shown to produce bioactive metabolites and with improved possibilities for genetic, pharmacological and chemical analysis, the therapeutic potential of fungi and fungal metabolites is promising (Lindequist *et al.*, 2005).

1.2   Secondary metabolites of filamentous fungi

Natural product chemistry is mainly attributed to a group of compounds known as secondary metabolites. Some of the most powerful secondary metabolites that have been developed into therapeutic agents are derived from filamentous fungi. Metabolites formed during primary metabolism are converted into secondary metabolites; which are not produced or essential during active growth (Mizuno *et al.*, 1995a; Zjawiony, 2004; Zhong *et al.*, 2009). Many of these fungal metabolites have shown activity against a number of diseases. Generally, immunomodulating secondary metabolites provide their bioactivity by stimulation of the host defences *via* several signal transduction pathways (Zhang *et al.*, 2007). These substances of therapeutic interest stem from five main metabolic sources (Zaidman *et al.*, 2005): amino-acid derived pathways; the shikimic acid pathway for the biosynthesis of aromatic amino acids; acetate-malonate pathway from acetyl coenzyme A; the mevalonic acid pathway from acetyl coenzyme A, which plays a role in the primary synthesis of sterols; and polysaccharides or peptidopolysaccharides (Wasser, 2011). The products of these pathways include pharmaceutically important products, such as the plant secondary

metabolites morphine, quinine and digitalis, and the fungal secondary metabolites penicillin, ergotrate, statins and cephalosporin. Some substances are also derived from these pathways such as aflatoxins, trichothecenes and ergot alkaloids which may be construed as toxic or pharmacologically useful (Keller *et al.*, 2005). The production of economically important metabolites such as antibiotics by microbial fermentation is one of the most important activities of the bioprocess industry. Keller *et al.* (2005) described secondary metabolites as bioactive substances (usually of low molecular weight), which are produced as families of related compounds at restricted parts of the life cycle, with production often correlated with a specific stage of morphological differentiation.

Since their discovery, secondary metabolites have demonstrated a range of bioactivities including antimicrobial activity towards bacteria, fungi, protozoa, parasites, insects and viruses, as well as antitumor activity. The following section describes the most significant secondary metabolites isolated from fungi.

1.2.1   Antibiotic production

The most well known secondary metabolites produced by fungi are β-lactam antibiotics, first discovered in 1928 by Sir Alexander Fleming. An isolate of *Penicillium* was discovered to produce a substance capable of killing gram-positive bacteria. Later this research led to the development of the antibiotic penicillin by Florey (1939) and opened the way for other antibiotic discoveries and therapeutic compounds from natural sources. This was the first discovery of extremely potent antimicrobial activity of fungi for use by humans. The discovery and subsequent development of antibiotics is one of the most important scientific breakthroughs of all time. Even now, penicillin remains among the most active and least toxic antibiotics (Demain *et al.*, 1999). Since the discovery of penicillin, several thousand antibiotics have been isolated from soil microorganisms, but these have been limited to only 50, most being too toxic to humans (Carlile *et al.*, 2000). Nonetheless, throughout evolution, various fungal species have developed compounds with strong health promoting abilities in humans, mostly through development of innate defence mechanisms against bacteria or other organisms which threaten the survival of the fungus in the wild. Natural products from fungi are our best source of antibiotics and by exploiting the protective strategies used by fungi to fight microorganisms, we can develop therapeutic strategies for pharmaceutical products.

The main use of antibiotics is for human therapeutic purposes, whilst prior to their EU-wide ban, the second largest use of antibiotics was as supplements in animal feed. These were commonly referred to as antibiotic growth promoters (AGPs). In Europe, a ban on the use of antibiotics as growth promoters in animal feed came into effect on January 1, 2006 (EuropeanCommission, 2005). Antibiotics had been added to animal feeds since 1946, when it was discovered that animals grew faster, put on more weight and illness was prevented, ultimately increasing profitability for meat producers. In the United States, the food and drug administration (F.D.A) reported that approximately 80 % of antibiotics produced are fed to animals and only 20 % are used to treat infections in humans (FoodandDrugAdministration, 2009). It is estimated that 75 % of antibiotics are not fully digested and eventually pass through the animal into the environment which has lead to the emergence of antibiotic resistance (Chee-Sanford *et al.*, 2009). The ban in Europe was a large step in combating the threat to human, animal and plant health posed by antimicrobial resistance. Notably, the use of natural products from some species of filamentous fungi has been seen to have strong antibacterial effects towards resistant bacterial strains (further discussed in Section 1.3.2).

1.2.2   Immunosuppressants

Cyclosporin A is a powerful immunosuppressant drug, relying heavily on fungal biotechnology. This compound has transformed transplant surgery by vastly reducing the risk of organ rejection. Produced by the fungus *Tolypocladium inflatum* from the Ascomycota division of fungi, it acts by inhibiting the production of interleukin-2 by T-lymphocytes, thereby inhibiting immune response produced naturally against transplanted organs (Carlile *et al.*, 2000; Kavanagh, 2011).

1.2.3   Statins

The most important group of fungal compounds responsible for cholesterol lowering properties are known commercially as statins and biologically as mevinic acids. Statins act by inhibiting the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which catalyses the reduction of HMG-CoA to mevalonate during the synthesis of cholesterol, thereby reducing the risk of hypercholesterolaemia and coronary heart disease (Shu, 1998; Quinn, 1999). Lovastatin and mevastatin are natural statins, while pravastatin is derived from the latter by biotransformation.

Simvastatin, the second leading statin in the market, is a lovastatin semisynthetic derivative (Barrios-Gonzalez *et al.*, 2010). A number of fungi produce a range of similar compounds. The first statin to be isolated was mevastatin from *Penicillium citrinum* (Endo *et al.*, 1976), followed by lovastatin from *Monascus ruber* (Manzoni *et al.*, 1999). Lovastatin from *Aspergillus terreus* is conventionally produced by submerged liquid fermentation (SLF) but can also be produced by solid state fermentation (SSF) (Barrios-Gonzalez *et al.*, 2010).

1.2.4   Alkaloids

Alkaloids are organic compounds with at least one nitrogen atom in a heterocyclic ring. The majority of indole alkaloids used in medicine are from Apocynaceae plant species, but they are also found in fungi, such as ergot (*Claviceps* spp.) and *Psilocybe* species (Hoffmann, 2003). Ergot refers to a group of fungi of the genus Claviceps. This fungus synthesises numerous secondary metabolites of pharmacological importance. The main groups of which have been characterised as; the lysergic-acid type, the clavinet type and the peptide alkaloids, with each group having different physiological properties (Keller *et al.*, 2005). Some alkaloids have been exploited for human use, such as a synthetically derived form of lysergic acid, an alkaloid produced by *paspali*, known commercially as lysergic acid diethylamide (LSD). Many alkaloids are currently used in medicine; such as the propanolamine of lysergic acid and ergometrine, produced from *Claviceps purpurea*. A wide variety of other medicinally important alkaloids have been produced through chemical modification of naturally occurring alkaloids (Carlile *et al.*, 2000).

1.2.5   Terpenes

There are several important terpenes synthesised by fungi including the aristolochenes, carotenoids, gibberellins, indole-diterpenes and trichothecenes (Keller *et al.*, 2005). All forms of terpenes can be linear or cyclic in structure, can be saturated or unsaturated and are composed of several isoprene units. Common classes of terpenes include monoterpenes, sesquiterpenes, diterpenes and carotenoids, classified according to the number of carbon atoms and are subsequently subdivided into; (i) volatile mono- and sesquiterpenes (essential oils) (C10 and C15) (ii) less volatile diterpenes (C20), (iii) non-volatile triterpenoids and sterols (C30), and (iv) carotenoid pigments (C40). A new terpenoid has recently been identified and named lucidone D (Liu *et al.*, 2013).

Sesquiterpenes are common fungal metabolites and they are particularly widespread among the Basidiomycetes. Triterpenes have exhibited strong activity against growing tumours *in vitro* and as a result, they are classified as potential anticancer agents (Lin *et al.*, 2003). Research is now looking at the production of terpenoids from other fungal species (Tang *et al.*, 2007).

In agriculture, two of the most recognised active compounds synthesised by fungi are strobilurins and gibberellins. Strobilurins are a class of antifungal antibiotics derived from *Strobilurus tenacellus*, a small mushroom found on buried pine cones. A synthetic form of this became a widespread agricultural fungicide in the 1990's (Carlile *et al.*, 2000). Gibberellins are an extremely potent plant hormone from the species *Gibberella fujikuroi*, but have also have been isolated from *Sphaceloma manihoticola*, *Neurospora crassa*, *Rhizobium phaseoli* and *Azospirillum lipoferum* (Kavanagh, 2011). They are diterpenoids with 19 or 20 carbon atoms, and like sterols and carotenoids, are biosynthesised from mevalonic acid (Carlile *et al.*, 2000). They are involved in the development and growth processes of plants and since their discovery, have been exploited to improve fruit size, malting of barley in brewing, and to control development and ripening of apples (Carlile *et al.*, 2000).

1.2.6   Polyketides

Fungal polyketides, the most abundant secondary metabolite, are a group of bioactive compounds which have applications as anti-cholesterol, anti-cancer and antibiotic agents. They are synthesised by a group of enzymes classified as type I, II or III polyketide synthases (PKSs) which are commonly found in microorganisms. *Monascus* spp. produce several well-known polyketides such as monacolin K, citrinin, and azaphilone pigments, of which monacolins are well known for their ability to control hyperlipidemia (Endo, 1979). Monacolin J, K, L and M are structural analogues of monacolin and are extremely effective compounds in lowering cholesterol (Endo *et al.*, 1985; Endo *et al.*, 1986; Akihisa *et al.*, 2005). As mentioned previously (Section 1.2.3), lovastatin isolated from species such as *Monascus ruber*, acts as a potent inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (Endo, 1979; Manzoni *et al.*, 1999; Akihisa *et al.*, 2005). It is also produced by higher Basidiomycetes, mainly by the fruiting bodies of Pleurotus*,* and has been isolated from *P. ostreatus, P. cornucopiae, P. eryngii* and *P. sapidus* (Wasser *et al.*, 1999c). Polyketides from Monascus are known to also form potentially toxic

metabolites, including; monascopyridines and the mycotoxin citrinin (Blanc *et al.*, 1995). Nonetheless, Monascus is widely regarded as a functional food because of its ability to produce monacolin K (antihyperholesterolemic agents), γ-aminobutyric acid (GABA) (hypotensive agent) and dimerumic acid (antioxidant) (Lin *et al.*, 2008).

1.2.6.1   *Pigment production*

Monascus is mostly well established for its pigment production, which are secondary metabolites synthesised from polyketides. There are six major pigments which can be classified according to colour; yellow pigments monascin ($C_{21}H_{26}O_5$) and ankaflavin ($C_{23}H_{30}O_5$), orange pigments monascorubin ($C_{23}H_{26}O_5$) and rubropunctatin ($C_{21}H_{22}O_5$) and the red pigments monascropunctamine ($C_{23}H_{27}NO_4$) and rubropuntamine ($C_{21}H_{23}NO_4$) (Jung *et al.*, 2003) (Figure 1.3). Besides Monascus being used as a traditional food colorant, pigments from this species have demonstrated strong bioactivity. Altogether, pigments have demonstrated antimicrobial (Kim *et al.*, 2006), tumour suppressive (Yasukawa *et al.*, 1994) and hyperlipidemic activities (Lee *et al.*, 2006). Furthermore, anti-hepatitis C virus (HCV) activity has recently been established by Monascus pigment derivatives (Sun *et al.*, 2012).
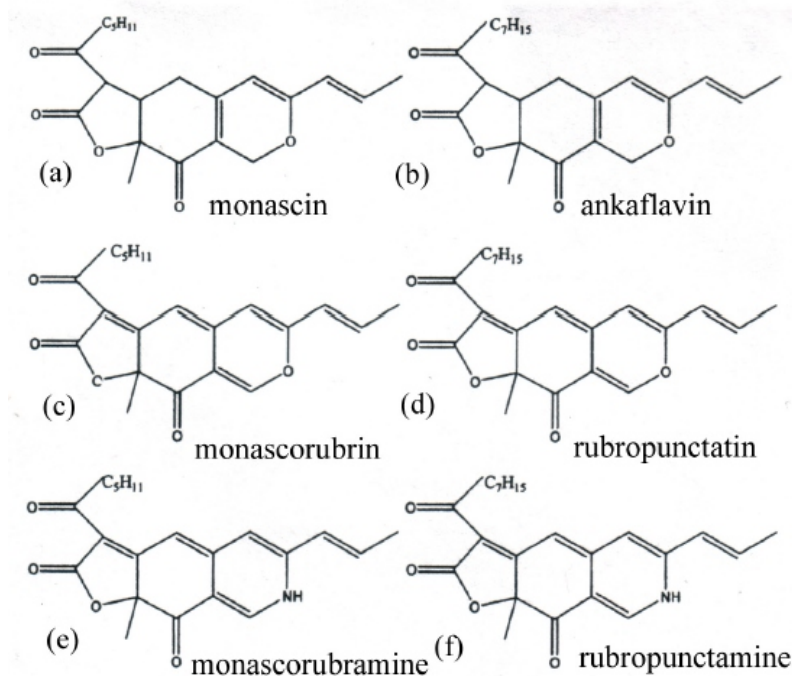
Figure 1.3   Chemical structure of Monascus pigments, (a)(b) yellow pigments, (c)(d) orange pigments, (e)(f) red pigments

Retrieved from Jung *et al.* (2003).

Monascorubin from *M. anka* and ankaflavin, a structural analogue of monascin have demonstrated inhibition of carcinogenesis in mice (Yasukawa *et al.*, 1994; Akihisa *et al.*, 2005). The pigments monascorubin and rubropunctatin (Figure 1.3, c and d, respectively) have also shown antimicrobial properties (Martinkova *et al.*, 1995). Other active constituents, such as compounds resembling statins in structure, unsaturated fatty acids, sterols and B-complex vitamins have also been produced by *Monascus* spp. (Wang *et al.*, 2007).

1.2.6.2   Citrinin

Citrinin is a secondary metabolite, first isolated from *Penicillium citrinum* in 1931 and later *Aspergillus* (Frisvad, 1986). It is synthesised by the polyketide pathway, through which many secondary metabolites are synthesised, particularly pigments. Wang *et al.* (2004b) later found that the production of citrinin is independent of pigment production. A nephrotoxic by-product of fermentation, citrinin is generally present in all red yeast rice products at various concentrations (Krejci *et al.*, 1996; Klimek *et al.*, 2009). With focus on medicinal products from *M. pilosus, M. purpureus* and *M. ruber*, standardisation of manufacturing practices and adequate labelling and analysis are required to ensure low concentrations of unwanted fermentation by-products (Wang *et al.*, 2004b; Klimek *et al.*, 2009). Industrially, care is required in the form of controlled fermentation conditions in order to produce large amounts of pigment or mevinolin with little or no citrinin. As citrinin is mostly produced during the stationary phase, by means of controlling fermentation rates this is a possibility (Leitão, 2011). Suitable standardised cultivation parameters is yet to be established, with strains, substrate, fermentation temperature, pH and moisture content important factors for the generation of metabolites. At this time, the statutory limit of citrinin content varies in different countries. In Japan, the substance must be lower than 200 µg kg^{-1} (The Ministry of Health and Welfare of Japan, 2000), in South Korea the limit is 50 µg kg^{-1} (Kim *et al.*, 2007) and the EU recommends no more than 100 µg kg^{-1} (Patakova, 2013). There is minimal evidence to link the citrinin content of fermented Monascus products with adverse medical effects, as there have seldom been reports of untoward health implications reported from populations who have consumed these products (Wang *et al.*, 2004b). Nevertheless this by-product is undesirable and as a consequence many beneficial health promoting products developed from Monascus are not recommended by health care professionals, highlighting the need for controlled fermentation

conditions in order to minimise citrinin content whilst maintaining high concentrations of desired active product.

1.2.7   Lipids

Lipids represent a broad group of naturally occurring compounds which differ widely in their chemical and physical properties, including; free fatty acids, steroids, sphingolipids, glycolipids, neutral lipids and phospholipids. They represent important structural and functional components of the fungal cell membrane and are the cells major energy source for metabolism. It is well known that excessive intake of saturated fatty acids (SFAs) may progress into health implications such as obesity, high cholesterol and cardiovascular diseases. However, there are a number of naturally occurring fatty acids which have positive effects on health, including; monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). Such compounds are known to have antioxidative, cancer inhibitive and cardioprotective properties (Tang *et al.*, 2011). In fungi, the major fatty acids that typically occur in membrane phospholipids and storage triacylglycerols are palmitic and stearic acids and their unsaturated derivatives palmitoleic, oleic, linoleic and linolenic acids (Suutari, 1995). Mycelial biomass produced using submerged cultures contains proteins and lipids and is principally composed of polyunsaturated fatty acids and polysaccharides (Dimou *et al.*, 2002; Pedneault *et al.*, 2008; Diamantopoulou *et al.*, 2012). The cellular fatty acid profile may be used for discriminatory purposes as different fungal strains vary in the relative concentration of specific fatty acids present (Stahl *et al.*, 1996). Notably, many fatty acids (lauric, linoleic, oleic and linolenic acid, amongst others) and their derivatives (aldehydes, acetate, ethyl esters, amide or substituted amides) are known to have demonstrated antibacterial activity against both gram-positive and gram-negative bacteria (Kabara *et al.*, 1972; Bergsson *et al.*, 2011). In addition, lipids exhibit antimicrobial properties and are capable of killing enveloped viruses, yeast, fungi and parasites (Bergsson *et al.*, 2011).

1.2.8   Other bioactive metabolites from filamentous fungi

Other important bioactive metabolites that have been isolated from Basidiomycetes and Ascomycetes include immunoactive compounds; lectins, polysaccharides, phenols and sterols (ergosterols). Metabolites which enhance or potentiate host resistance could be used to treat a variety of diseases, such as cancer or immunodeficiency related

diseases. The major immunomodulatory effects of these active compounds include mitogenicity and activation of immune cells. Consequently, it is from these main processes anticancer activity, suppression of autoimmune diseases and allergies have been exhibited (Lull *et al.*, 2005).

Lectins can be found in plants, animals, fungi and other microorganisms, however there has been little research conducted on lectins from fungi, particularly in submerged cultures (Wang *et al.*, 1998; Elisashvili, 2012). They are carbohydrate binding proteins of non-immune origin that have been implicated in cellular signalling, malignancy, host pathogen interactions, scavenging of glycoproteins from the circulatory system, cell-cell interactions in the immune system, differentiation and protein targeting to cellular compartments (Ashwell *et al.*, 1982; Springer *et al.*, 1991). The physiological role of these compounds in various organisms is particularly diverse; being involved in cell adhesion, recognition, and differentiation, transportation of sugars, and growth regulation (Elisashvili, 2012). They are used as a biochemical tool in targeted cancer therapy due to their characteristic sequences and can be used as specific binding moieties (She *et al.*, 1998; Wasser *et al.*, 1999a). Some fungal lectins were tested for inhibitory activity against human immunodeficiency virus (HIV). Lectins isolated from *Pleurotus citrinopileatus* inhibited HIV-1 reverse transcriptase (Li *et al.*, 2008). The possibility of inclusion of fungi or mushrooms as dietary components along with evidence that some lectins retain their biological activity after passage through the gastrointestinal tract, merits more research on fungal lectins (Wang *et al.*, 1998). These compounds have potential for manipulation for the production of pharmaceuticals and nutraceuticals in the future (Wang *et al.*, 1998).

Sterols are more varied amongst fungal species than was previously established (Weete *et al.*, 2010). These commercially important substances play a key role in the integrity of the cell membrane and are required for fungal growth (Tang *et al.*, 2007). Biosynthesis of sterol produces five dominant end-products, namely, cholesterol, ergosterol, 24-methyl cholesterol, 24-ethyl cholesterol and brassicasterol (Weete *et al.*, 2010). Ergosterol is a precursor of vitamin D, which, under ultraviolent radiation converts to cortisone and progestin, and is the most dominant sterol of Ascomycota and Basidiomycota. Commercial production of ergosterol is still reliant on either yeast fermentation or extraction from waste mycelium harvested from penicillin production (Tang *et al.*, 2007).

There is conclusive evidence that a large number of compounds derived from fungi, both cell wall components and secondary metabolites have shown the ability to affect a number of diseases, positively impacting health. A number of fungal metabolites have found their way into medical applications as natural products, starting material for pharmaceutical products or as lead structures for development of new therapeutic products. These biologically active metabolites include; polysaccharides, proteins and low molecular weight secondary metabolites such as phenolic compounds, polyketides, triterpenoids, steroids, alkaloids, nucleotides, lactones and fatty acids (Kim *et al.*, 1999; Wasser *et al.*, 1999b; Reshetnikov *et al.*, 2001; Lindequist *et al.*, 2005; Quang *et al.*, 2006; Wasser, 2010; Xu *et al.*, 2011; Elisashvili, 2012).

1.3   **Biomedicinal applications of filamentous fungi**

As discussed in Section 1.2, fungi are a well known source of biomedicinal metabolites that have a broad spectrum of pharmacological activities. Amongst many other health-promoting benefits, extracts from fungi and isolated compounds have been found to have antitumor, hypolipidemic, anti-inflammatory and antiviral activity. For thousands of years plants and fungi have been recognised as therapeutic agents, it is only in recent years that interest has focused on extracts and purified biologically active compounds from these therapeutic agents for the development of new effective drugs. Studies have shown *L. edodes*, *G. frondosa*, *A. bisporus* and *P. ostreatus* are sources of B-vitamins particularly niacin, flavin and pyridoxine (Solomko *et al.*, 1988); organic acids such as ascorbate, shikimate, malate and fumarate; carbohydrates such as the β-glucans; monoterpenoid and diterpenoid lipids; proteins such as hydrophobins and trace elements such as selenium (Dikeman *et al.*, 2005; Valentao *et al.*, 2005; Iwalokun *et al.*, 2007). Table 1.2 lists several biologically active products from various filamentous fungi and their preparation.

Table 1.2 **Biological activity of products of various filamentous fungi**

Adapted from Polishchuk *et al.* (2009) with modifications.

Species	Source	Preparation	Biological activity	Reference
Ganoderma lucidum	Fruit bodies	β-glucan	Antitumor, lipocholesteric effect	Wasser *et al.* (2005) Mizuno *et al.* (1995a)
	Mycelium	Ganoderan	Antitumor, antiviral	Lindequist *et al.* (2005)
Ganoderma pfeifferi	Mycelium	Ganomycin	Antibacterial	Mothana *et al.* (2000)
Grifola frondosa	Fruit bodies, mycelium, culture liquid	β-(1,3)-D-glucan	Antitumor, antiviral, antimicrobial activity	Kodama *et al.* (2002)
Tramella fuciformis	Fruit bodies	Aqueous extract	Reduction in thrombus size, decrease in blood pressure	Shen *et al.* (1990)
Tramella mesenterica	Culture liquid	Ethanolic extract	Increased immune response	Lindequist *et al.* (2005)
Trametes versicolor	Fruit bodies	PSP, PSK	Immunomodulation, antitumor activity	Hiroshi *et al.* (1993)
Pleurotus ostreatus	Fruit bodies	Lovastatin	Reduction in cholesterol	Zaidman *et al.* (2005)
	Mycelium	Ethanolic extract	Antibacterial, antifungal	Gerasimenya *et al.* (2002)
Lentinus edodes	Fruit bodies, mycelium	Lentinan	Antitumor, immunomodulating, antiviral	Wasser *et al.* (1999b)
	Culture liquid	Cortinelin	Antibiotic activity,	Hatvani (2001) Bianco (1981),
	Mycelial extract	Eritadenin heteroglycan-protein fraction	Reduction in cholesterol Antiviral activity	Pacumbaba *et al.* (1999) Suzuki *et al.* (1976) Hibino *et al.* (1994)
Schizophyllum commune	Culture liquid	Krestin, β-D-glycoprotein	Antitumor activity	Yanaki *et al.* (1983)
Agaricus blazei	Fruit bodies	Aqueous, ethanolic extract	Antiviral activity	Bruggemann *et al.* (2006), Faccin *et al.* (2007)
Inonotus hispidus	Hispolon, hispidin	Aqueous extract	Antiviral activity	Awadh *et al.* (2003)

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1.3.1   Bioactive compounds

Originating by popularity first in Japan with movement later towards China, Korea and most recently the U.S, a number of scientific studies have been published focusing on the production of medicinal bioactive substances from filamentous fungi (Mizuno, 1996; Mizuno, 1999; Wasser, 2011). Natural medicinal compounds exert beneficial health effects without known side-effects and often blends may be used for maximum benefit (Wasser *et al.*, 2000). These compounds, which merit nutritional and pharmaceutical properties, extractable either from the mycelium or the fruiting body of mushrooms are commonly referred to as "mushroom nutraceuticals". The majority of recent studies are focused on maximum production, utilisation and the mode of action of these functional molecules.

Extracellular and intracellular fungal lipids and polysaccharides produced by submerged liquid fermentation are important industrially and economically (Wasser, 2002). Major active compounds from filamentous fungi include low molecular weight compounds such as quinines, cerebrosides, isoflavones, catechols, amines, triacylglycerols, sesquiterpenes, steroids, organic germanium and selenium, as well as high molecular weight compounds such as homo- and hetero-polysaccharides, glycoproteins, glycopeptides, proteins, and RNA-protein complexes (Figure 1.4) (Ferreira *et al.*, 2010).

Section 1.2.1 discussed the discovery of the β-lactam antibiotic penicillin and its isolation from *Penicillium*; however, the production of antibiotic compounds from other divisions of fungi is less well documented (Miles *et al.*, 1997). Edible mushrooms from the genera Pleurotus, Lyophyllum, Flammulina and Grifola have been found to contain bioactive medicinal compounds and demonstrate therapeutic potential to human health (Ooi *et al.*, 2000; Ferreira *et al.*, 2010). Compounds from Trametes, Inonotus and Ganoderma have potent antitumor activity; however, they are not palatable fungi and so are mainly used in the form of extract, tea or powder (Wasser, 2002; Ferreira *et al.*, 2010).

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                          ┌─────────────────┐
                          │    Mushrooms    │
                          └─────────────────┘
              ┌───────────────────┴───────────────────┐
      ┌───────────────┐                        ┌───────────────┐
      │ LMW compounds │                        │ HMW compounds │
      └───────────────┘                        └───────────────┘

         ┌──────────────┐                        ┌──────────────┐
         │   Quinones   │                        │ Homoglucans  │
         └──────────────┘                        └──────────────┘
         ┌──────────────┐                        ┌──────────────┐
         │ Cerebrosides │                        │ Heteroglucans│
         └──────────────┘                        └──────────────┘
         ┌──────────────┐                        ┌──────────────┐
         │  Isoflavones │                        │    Glycans   │
         └──────────────┘                        └──────────────┘
         ┌──────────────┐                        ┌──────────────┐
         │   Catechols  │                        │ Glycoproteins│
         └──────────────┘                        └──────────────┘
         ┌──────────────┐                        ┌──────────────┐
         │    Amines    │                        │ Glycopeptides│
         └──────────────┘                        └──────────────┘
         ┌──────────────┐                        ┌──────────────┐
         │Triacylglycerols│                      │ Proteoglycans│
         └──────────────┘                        └──────────────┘
         ┌──────────────┐                        ┌──────────────┐
         │Sesquiterpenes│                        │   Proteins   │
         └──────────────┘                        └──────────────┘
         ┌──────────────┐                        ┌──────────────┐
         │   Steroids   │                        │ RNA-Protein  │
         └──────────────┘                        │   complex    │
         ┌──────────────┐                        └──────────────┘
         │Organic germanium│
         └──────────────┘
         ┌──────────────┐
         │   Selenium   │
         └──────────────┘
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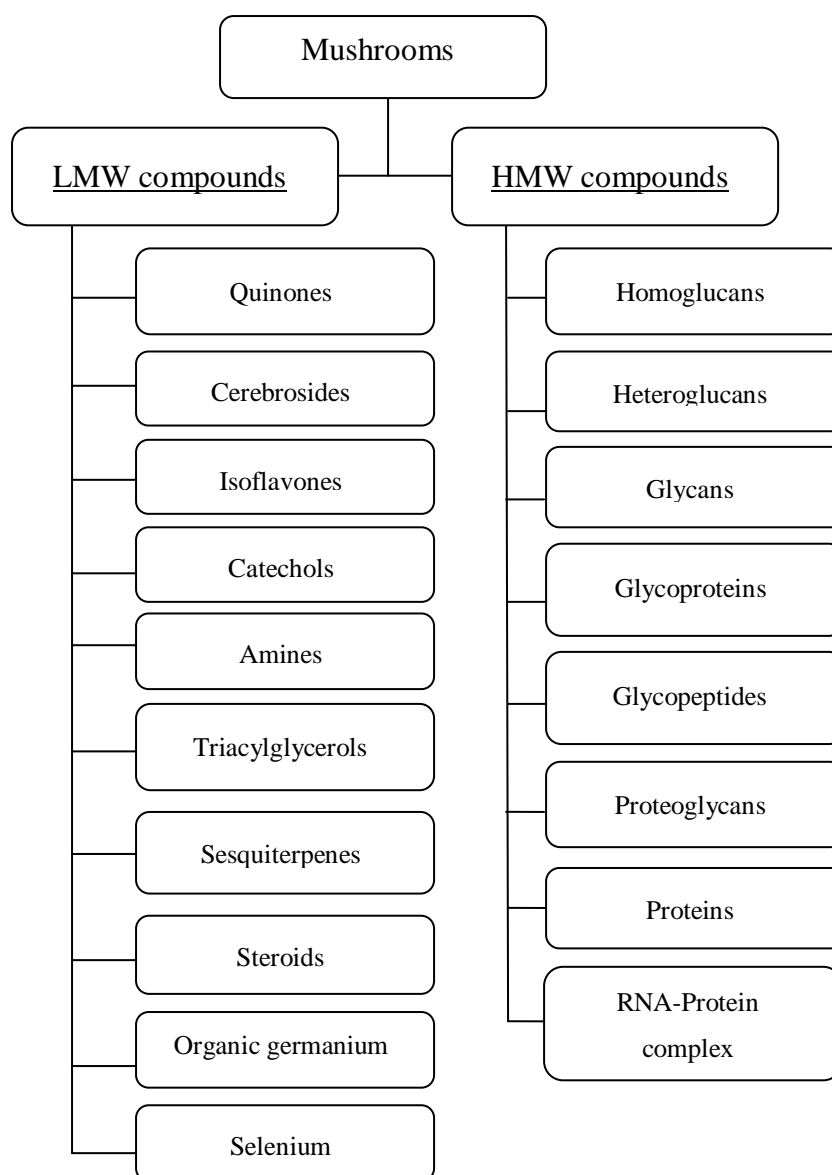
Figure 1.4   Low molecular weight and high molecular weight compounds with bioactivity found in mushrooms

Figure extracted from Ferreira *et al.* (2010).
- Abbreviations: LMW, low molecular weight; HMW, high molecular weight.

According to Suay *et al.* (2000), Basidiomycetes compare favourably with Ascomycetes as a source of biologically active natural products. During different developmental stages of fungi, these compounds undergo changes to their molecular structure and conformation which consequently affects their biological activity. At least 651 species and 7 infrastructure taxa representing 182 genera of hetero- and homobasidiomycetes mushrooms contain antitumor or immunostimulating metabolites (Wasser, 2002).

1.3.2   Antimicrobial compounds

As they exist in nature, mushrooms produce compounds of antibacterial and antifungal capacity to protect them from pathogenic insects, bacteria and protozoa. The harvesting and purification of these compounds has great pharmacological implications for human and animal medicine.

Since the discovery of antibiotics, multi-drug resistance to pathogenic bacteria has emerged due to exploitation and overuse (Lorenzen *et al.*, 1998; Monroe *et al.*, 2000; Donadio *et al.*, 2002). This antimicrobial resistance in both medicine and agriculture is now recognised by the World Health Organisation (WHO) as a continuous public health problem (Hearst *et al.*, 2009). As a consequence, there is an increasing need for alternative antimicrobial substances, effective against resistant pathogenic microorganisms, which would limit the use of conventional antibiotics. Epidemiological studies have indicated that protection against incidence and severity of a range of infectious diseases may be provided by an array of protective factors, including innate immune factors, secretory antibodies, lipids, carbohydrates, oligosaccharides, lysozyme, and lactoferrin, as well as components that have not yet been described (German *et al.*, 2002; Lonnerdal, 2004; Marks *et al.*, 2012). Research has found that a complex of natural components and traditional antibiotics may delay resistance-development. Marks *et al.* (2012) revealed that by combining a natural component of human milk with traditional antibiotics, antimicrobial activity was synergistically greater than when each of the active compounds were used individually. This adjuvant therapy in combination with antibiotics may extend the use of current treatment and potentially treat antibiotic-resistant strains of various pathogens.

The evolution, selection and spread of bacterial resistance to a wide range of antibiotics have made the development of novel strategies to prevent and treat bacterial infections crucial (Signoretto *et al.*, 2012). Fungal components have demonstrated *in vitro* antimicrobial activity against gram-positive and gram-negative bacteria, including several food-borne pathogenic bacterial strains (Venturini *et al.*, 2008), as well as yeasts and mycelial fungi, including dermatophytes and phytopathogens (Soboleva *et al.*, 2006; Hearst *et al.*, 2009; Jagadish *et al.*, 2009). In addition, many fungal species have demonstrated they are an alternative source of natural antimicrobial compounds, having demonstrated activity against bacterial, viral and

fungal pathogens which are resistant to current therapeutic agents (Wasser *et al.*, 1999b). Antimicrobial compounds isolated from different stages of the fungal cultivation process can be characterised by antibacterial, antifungal and antiviral activity (Polishchuk *et al.*, 2009).

1.3.2.1   Antibacterial activity

Experimentation into the antibacterial activity of fungi in the past has focused mostly on the isolation of compounds from the fruiting body, with a small proportion focusing on liquid cultivated mycelium. This is of great potential commercially with both the cultured mycelium and culture fluid found to possess antibacterial activity (Hatvani, 2001). Basidiomycota and Ascomycota have exhibited antibacterial activity toward a range of bacteria and over the years, research on various species has demonstrated that the boundaries of activity is strain specific (Polishchuk *et al.*, 2009).

Fungal cell wall components and biological activities have a well established structure-function relationship (as discussed in Section 1.1.3). A number of compounds, most of all polysaccharides from a number of fungi have exerted antibacterial action, such as; lentinan from the fruiting body of *L. edodes* (Jong *et al.*, 1991) and compounds from the cultured mycelium of *T. versicolor* (Hiroshi *et al.*, 1993). Mycelial extracts such as cortinelin (Table 1.2) from different strains of *L. edodes* have shown the ability to inhibit the growth of gram-positive and gram-negative bacteria (Hirasawa *et al.*, 1999; Polishchuk *et al.*, 2009). These mycelial extracts of *L. edodes* have demonstrated activity *in vitro* toward phytopathogenic bacterial and causative agents which affect foodstuffs and humans, such as; *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium* and *Staphylococcus aureus* (Hiroshi *et al.*, 1993; Pacumbaba *et al.*, 1999). Chitin with high molecular dimensions is immunologically inert; however, fungal polysaccharides with reduced dimensions were associated with stimulation of innate immunity and production of pro- and anti-inflammatory cytokines, illustrating that polysaccharides of identical composition but with varying dimensions may have different functions (Rodrigues *et al.*, 2011). Table 1.3 lists antimicrobial activities established from various filamentous fungi and their preparation.

Table 1.3   Antimicrobial activities of some filamentous fungi

Obtained from (Gregori *et al.*, 2007) with modifications.

Species	Preparation	Effective against	Reference
Ganoderma spp.	Methanolic extracts	*Bacillus subtilis*	Suay *et al.* (2000)
		Gram-positive bacteria; *B. cereus* and *Staphylococcus aureus*.	Smania *et al.* (2001)
		Gram-negative bacteria; *Pseudomonas aeruginosa* and *Escherichia coli*.	
Lentinula. edodes	Culture fluid	*Candida albicans*, *Streptococcus pyogenes*, *S. aureus* and *B. megaterium*	Hatvani (2001)
	Lentinan - aqueous and solvent extracts	*Streptococcus*, *Actinomyces* spp., *Lactobacillus* spp., *Prevotella* spp. and *Porphyromonas* spp. of bacteria.	Hirasawa *et al.* (1999)
Coprinus spp.	Coprinol isolated from culture fluid	Multi-drug resistant gram-positive bacteria	Johansson *et al.* (2001)
Pleurotus ostreatus	Crude extracts from culture broth	Gram-positive, gram-negative bacteria and *Aspergillus niger*	Gerasimenya *et al.* (2002)
	Hexane-dichloromethane extract containing *ρ*-anisaldehyde	*B. subtilis*, *P. aeruginosa*, *Aspergillus niger* and *Fusarium oxysporum*	Okamoto *et al.* (2002)
	Various extracts; two main unidentified compounds	*Bacillus* spp., *E. coli*, *Vibrio cholera* and *Salmonella typhi*	Periasamy (2005)
Pleurotus eryngii	Methanolic extracts	*Enterococcus faecium*, *S. aureus* and *B. subtilis*	Suay *et al.* (2000)
	Eryngin – an antifungal peptide	*Fusarium oxysporum* and *Mycosphaerella arachidicola*	Wang *et al.* (2004a)
Pleurotus sajor-caju	12 kDa ribonuclease	*F. oxysporun*, *M. arachidicola*, *P. aeruginosa* and *S. aureus*	Ngai *et al.* (2004)
Monascus spp.	Ethanol: n-hexane (1:10) extract-Monascidin A	*B. subtilis*, *P. aeruginosa* and *E. coli*.	Ferdes *et al.* (2009)
	Pigments: Rubropunctatin and monascorubin	*Aspergillus*, *Penicillium*, *Mucor*, *Fusarium*, *Alternaria* and *Botrytis*.	Patakova (2013)
	Citrinin	Gram-positive and gram-negative bacteria	Mazumder *et al.* (2002) Wong *et al.* (1981)
Grifola frondosa	Exopolysaccharide; D-fraction	Hepatitis B virus (HBV)	Gu *et al.* (2006)

A polysaccharide-rich fraction from Agaricus has also been shown to increase host resistance against some infectious agents through stimulation of microbicidal activity of macrophages (Martins *et al.*, 2008). Compounds such as the sesquiterpenoid hydroquinone ganomycin, obtained from the mycelia of *Ganoderma pfeifferi*, is capable of inhibiting the development of methicillin-resistant *S. aureus* and other bacteria (Mothana *et al.*, 2000). Other sesquiterpenes, velleral and isovelleral isolated from *Lactarius vellereus* have demonstrated strong antibacterial activity against *E. coli* and antifungal activity against *Candida utilis* (Sterner *et al.*, 1985).

Although there have been numerous reports on bioactive extracts and compounds relating to the antimicrobial action of higher Basidiomycetes, there has been far less research on the antibacterial action of Ascomycetes species. Antimicrobial activities of pigment derivatives were discovered in Monascus (Section 1.2.6.1); the non-specific biological activities (antimicrobial, antitumor or immunomodulative) of pigmented compounds are due to their reaction with amino-group containing compounds i.e. amino acids, proteins or nucleic acids (Patakova, 2013). Structurally, Monascus pigments belong to a group of fungal pigments known as azaphilones, which, through manifestation of different enzyme activities, exhibit their biological activity. This manifestation has exhibited antimicrobial, antiviral, antitumor, antioxidant and anti-inflammatory activity, amongst others (Patakova, 2013). There are more than 170 known azaphilones, produced by 23 different fungal genera, most of them exhibiting biological activity (Osmanova *et al.*, 2010). Citrinin has also been demonstrated to possess antimicrobial activity toward gram-positive and gram-negative bacteria (Wong *et al.*, 1981; Mazumder *et al.*, 2002). Additionally, diverse biological activities, including immunostimulatory, antitumor and hypoglycaemic action have been observed in *Cordeyceps militaris*, which also belongs to the Ascomycota division (Park *et al.*, 2002).

1.3.2.2 *Current antimicrobial strategies*

Strategies involved in the protection of an organism from pathogenic infectious agents, predominantly target; adhesion, colonisation, invasion and production of toxins. Antibacterial strategies targeting bacterial adhesion to substrates such as fungal components are considered a valuable alternative to traditional antibiotic therapy.

As well as the potential of functional natural bioactive compounds being used as health-promoting nutritious supplements, they have the additional benefit of combating the infection process at a very early stage without selecting for drug resistant cells (Signoretto *et al.*, 2012).

Structurally the main components of the fungal cell wall are glucan, chitin and mannan (as discussed in Section 1.1.3.1). Chitin predominates near the plasma membrane providing the cell wall with its enormous strength, and mannoproteins emanate from the cell surface and are involved in cell-cell recognition events. Mannanoligosaccharides (MOS) from yeast cell wall (YCW) have shown the ability to interact with bacteria *in vivo*, firstly; through binding of mannose residues with lectin components on the surface of enteric pathogens, and secondly; through stimulation of the immune system. Thereby, MOS are recognised as influencing selective nutrient utilisation by microbes, cell recognition and adhesion, and immune stimulation. The most common binding specificity with bacteria is mannose-containing receptors (Duguid *et al.*, 1966). As a consequence, a number of yeast cell wall MOS type products have been developed (for example Bio-MOS®, MOS 500 and Agri-MOS). MOS is not enzymatically digested in the small intestine; therefore, bacteria bound to MOS likely exit the intestine without attaching to the epithelium (Spring *et al.*, 2000).

Most isolates of Enterobactericeae, such as *E. coli*, *Klebsiella*, *Shigella* and *Salmonella*, attach to mannose receptors by means of type-1 fimbriae (Abraham *et al.*, 1988; Adlerberth, 2000; Becker *et al.*, 2008). Mannose containing receptor analogues have also demonstrated the ability to attach to type-1 fimbriae. These include; D-mannose or derivatives of the monomeric sugar (Aronson *et al.*, 1979; Oyofo *et al.*, 1989; Allen *et al.*, 1997), oligo- and polysaccharides, glycopeptides containing mannose (Fernandez *et al.*, 2002), complex sources of mannans (palm kernel extracts) (Allen *et al.*, 1997; Fernandez *et al.*, 2002) or yeast cell wall products (White *et al.*, 2002). When bacteria adhere to complementary receptors located in the host substrate, they avoid being passed through the gastrointestinal system, have access to nutrients, achieve colonisation of the surface and have the ability to deliver toxic agents to the host (Ofek *et al.*, 2003). Adherent status is key to bacterial survival and is a vital step in pathogenesis, highlighting that prevention of adhesion following exposure to the host substrate at an early stage would prevent onset of disease (Ofek *et al.*, 2003). Dietary fibres such as those from fungi may be alternative adhesion matrices because

of their carbohydrate nature and low digestibility, mimicking the host binding sites and allowing for competitive adherence.

As with many strategies to combat infectious pathogens *in vivo*, there are two issues which face anti-adhesion therapy. The first is the emergence of bacterial resistant anti-adhesion agents. However, with the binding of alternative adhesion matrices, the growth or death of the pathogen is not affected as it is with antibiotics. Therefore, it is possible that the resistant strain would be diluted with sensitive bacteria whose adhesion is inhibited and is shed from the host (Ofek *et al.*, 2003). The second issue is that most pathogens possess genes which encode more than one type of adhesin, or during the infection process, adhesion may encounter non-specific interactions other than just adhesin-receptor interactions under various conditions (Ofek *et al.*, 2003). It is likely for anti-adhesion strategies to be effective, an agent that has a broad spectrum of anti-adhesion activity (such as powerful carbohydrate inhibitors) or a cocktail of agents, specially assembled with each type of adhesin of the infecting pathogen, will have to be acquired (Ofek *et al.*, 2003).

1.3.2.3   Antifungal activity

Antifungal compounds have been found in extracts of *L. edodes* but generally very little has been reported on the use of Ascomycetes and Basidiomycetes as antifungal agents (Takazawa *et al.*, 1982; Mizuno *et al.*, 1995b). A compound known as lentin, isolated from the Basidiomycetous fruiting bodies of *L. edodes* has exerted potent antifungal activity. This novel protein inhibited mycelial growth in a variety of pathogenic fungal species including *Physalospora piricola*, *Botrytis cinerea* and *Mycosphaerella arachidicola* (Ngai *et al.*, 2003). Lentin also demonstrated restrictive action on HIV-1 reverse transcriptase and on the production of leukaemia cells (Ngai *et al.*, 2003). Antifungal proteins secreted by Ascomycetes have exhibited strong potential as anti-fungal agents (Marx, 2004). These proteins from Ascomycota look extremely promising as alternative antifungal agents for a number of reasons; at effective concentrations they do not exert toxic effects on plant and mammalian cells, do not provoke an inflammation effect, have high stability even in extreme environmental conditions and can interact synergistically with other drugs and antimicrobial peptides (Marx, 2004). Antifungal drugs currently on the market have demonstrated ineffectiveness or toxicity to immunocompromised patients, therefore the isolation of novel antifungal compounds are of particular interest.

1.3.2.4 Antiviral activity

Unlike bacterial infections which are treated by antibiotics, highly selective drugs are required to fight against viral infections. Antiviral effects are caused by the inhibition of viral enzymes, synthesis of viral nucleic acids or adsorption and uptake of viruses into mammalian cells (Polishchuk *et al.*, 2009). Polysaccharides of Basidiomycetes are capable of inhibiting the development of viral infections (Polishchuk *et al.*, 2009) and activity has been described for isolated compounds from fungi, particularly by smaller molecules, as well as whole extracts of mushrooms (Lindequist *et al.*, 2005). Immunostimulatory activity of polysaccharides or other complex molecules may cause an indirect antiviral effect (Brandt *et al.*, 2000).

Herpes simplex virus (HSV) and human immunodeficiency virus (HIV) are important viruses which have been tested with bioactive compounds from Basidiomycetes (Wasser *et al.*, 1999b). So far, low molecular weight compounds have demonstrated strong activity, inhibiting multiplication of HIV-1 (Kim et al. 1998) and HIV-2 proteases (El-Mekkawy *et al.*, 1998). As for high molecular weight compounds with antiviral activities; culture medium and water soluble lignin from *L. edodes* have both demonstrated antiviral efficiency (Tochikura *et al.*, 1988; Suzuki *et al.*, 1989). In addition, the proteoglycan PSP (polysaccharide-peptide) and PSK (polysaccharide-K), isolated from *T. versicolor*, has displayed antiviral activity against HIV and cytomegalovirus *in vitro* (Tochikura *et al.*, 1987). Overall there are numerous investigations which prove polysaccharides and glycoproteins from Basidiomycetes have antiviral activity in both humans and animals. Studies have also been successful in showing the inhibitory activity of lectins (described in Section 1.2.8) from Basidiomycetes to viruses of plants (Sun *et al.*, 2003).

Neuraminidase enzymes are glycoside hydrolase enzymes which are frequently used as drug targets for the prevention of influenza infection. The viral neuraminidases are antigenic determinants found on the surface of the influenza virus. Recently, a methanolic soluble fraction from *Ganoderma lucidum* demonstrated neuraminidase inhibitory activity against avian influenza virus (H5N2 strain) *in vivo* (Shamaki *et al.*, 2013). Hence, this fungal extract was established as a source of an anti-influenza viral agent, that could be used effectively to manage avian influenza infections (Shamaki *et al.*, 2013). It was suggested that neuraminidase inhibitory activity of this mushroom could have been attributed to detectable quantities of

flavonoids present in the methanol soluble fraction of the *Ganoderma lucidum* extract (Shamaki *et al.*, 2013). Flavonoids are well known secondary metabolites produced by fungi, which have been shown to exhibit other biologically important effects including; antioxidant, antibacterial, anti-inflammatory, antiallergic and vasodilatory actions (Cook *et al.*, 1996; Velioglu *et al.*, 1998).

1.3.3 Antitumor activity

Higher basidiomycetes were first identified for their antitumor effect by Lucas *et al.* (1957), having isolated a substance from *Boletus edulis* that showed significant inhibitory activity against sarcoma S-180 tumour cells. Since then there have been various low and high molecular weight secondary metabolites from fungi that have demonstrated antitumor activity. Fungal metabolites such as those discussed previously in Section 1.3.1, have demonstrated the ability to reduce tumour growth without having a negative effect on the host (Wasser *et al.*, 1999c; Ferreira *et al.*, 2010). Investigation of higher mushrooms revealed approximately 650 Basidiomycetes species (182 genera) with potential antitumor activity. In particular, those from the orders Auriculariales, Tremellales, and Polyporales, in addition to genera including Gasteromycetes and Agaricomycetidae (Polishchuk *et al.*, 2009). Over 270 recognised fungal species have been established to have specific immunotherapeutic properties, which directly effects anticancer function (Ooi *et al.*, 2000). Of these, 50 non-toxic fungi have yielded potential immunoceuticals in animal models and 6 species have been used in studies to treat human cancers (Kidd, 2000; Standish *et al.*, 2008).

1.3.3.1 Fungal polysaccharides and immune function

Polysaccharides are the most well known and most potent mushroom-derived substances with antitumor and immunomodulating activity (Zhang *et al.*, 2007). Many higher Basidiomycetes species, if not all, contain biologically active polysaccharides (Tang *et al.*, 2007). They have been shown to be activators of innate immunity and have also been demonstrated as regulators of virulence (Costa-de-Oliveira *et al.*, 2013).

Biologically active polysaccharides are typically located in the fungal cell wall, which consists mainly of chitin-glucan complexes (Section 1.1.3). They belong to a diverse class of macromolecules, in which monosaccharide polymers are connected by

glycosidic linkages. As a result their biological activity is largely defined by; the immune response of the host, their monosaccharide composition, glycosidic linkages, sequence of monosaccharides and the nature of the appended non-carbohydrate groups (Zhang *et al.*, 2007). Some are bound to protein or peptide residues such as polysaccharide-protein or -peptide complexes (Cui *et al.*, 2003). Generally, protein-linked glucans have greater immunomodulating action than corresponding glucans. Fungal glucans (α- and β-glucan), complex mannans, xylans and galactans, are well known immunoactive polysaccharides (Synytsya *et al.*, 2009; Rodrigues *et al.*, 2011). Some antitumor polysaccharides vary in their sugar composition and chemical structure; however, all have a relatively high molecular weight (Kim *et al.*, 1993; Wang *et al.*, 1993). Data from higher Basidiomycetes collected on polysaccharide composition reveal most differences are in the β-glucan fraction. Comprising of β-(1,3) linkages in the main chain with additional β-(1,6) branch points required for antitumor activity, these homopolysaccharides contain only glucose monomeric units, with potential origin dictating structure (Wasser, 2002; Ferreira *et al.*, 2010). Polysaccharides of identical composition but varying dimensions may have different functions (Da *et al.*, 2008). The biological activities of β-D-glucans is significantly affected by molecular weight, branching rate and form, water solubility and the number of substitutions, including ultrastructure (Adachi *et al.*, 2002; Wasser, 2002; Ferreira *et al.*, 2010). Zjawiony (2004) established higher molecular mass glucans, lower level of branching and greater water solubility correlated with greater antitumor activity. The β-glucans containing mainly β-(1,6) linkages have less activity (Wasser, 2002). Research has identified polysaccharides with varying sugars and some with alpha- rather than beta-glucans, proteins, terpenes and furans have all been implicated in various immune functions (Wasser, 2002; Synytsya *et al.*, 2009). In addition, changes in polysaccharide branching at various stages of mycelial development have also been shown to effect antitumor activity (Wang *et al.*, 1993; Adachi *et al.*, 2002).

Many active fungal polysaccharides are classified as dietary fibers and depending on molecular structure and conformation they may present as soluble or insoluble dietary fibers during the digestion and absorption process by animals (Cheung *et al.*, 1998; Manzi *et al.*, 2000; Synytsya *et al.*, 2009). Of the polysaccharides typically found in fungi, only glycogen is digestible by human enzymes. Therefore β-glucans and mannangalactans isolated from Basidiomycetes are considered fibers (Smiderle *et al.*, 2012). Macrofungal dietary fibers are defined as

high molecular weight constituents which belong to β-glucans, chitin and heteropolysaccharides, and may represent up to 10 – 50 % of total dry matter (Wasser *et al.*, 1999b). It is their high structural variability which give polysaccharides the highest capacity for carrying biological information when compared to proteins and nucleic acids (Ooi *et al.*, 2000). Pleurotus is a well-known source of biologically active glucans, also known as pleurans. They are used as food supplements and operate like dietary fibre or as prebiotics (Synytsya *et al.*, 2009). β-D-glucans from Pleurotus have shown great immunomodulatory, antioxidant, anti-inflammatory and analgesic functions (Bobek *et al.*, 2001). Anti-cancer effects were demonstrated by low molecular weight α-glucans isolated from the mycelium of liquid cultivated *P. ostreatus* by induction of apoptosis, which resulted in inhibition of colon cancer cell proliferation (Lavi *et al.*, 2006). An α-(1,6)-glucan isolated from the fruiting bodies of *P. florida* demonstrated macrophage activation through release of nitric oxide (Rout *et al.*, 2005).

(a)   (b)
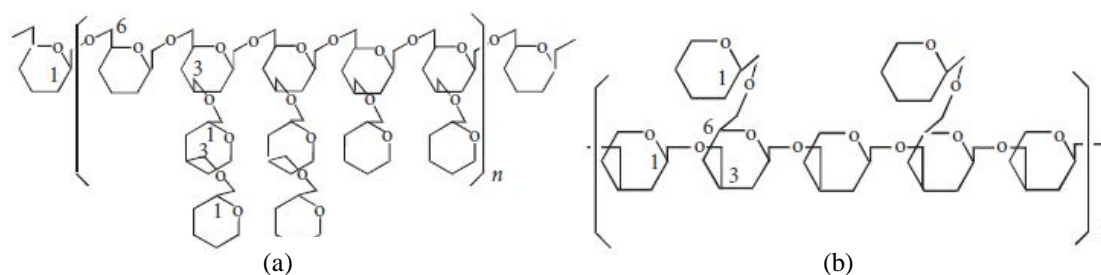
Figure 1.5   Repeating unit of immunomodulatory β-glucans

Repeating units of immunomodulatory β-glucans (a) from *Grifola frondosa* (D-fraction, MW: 1000 kD) and (b) from *L. edodes* (Lentinan, MW: 500 kD).
Adapted from Lull *et al.* (2005).

Lentinan from *L. edodes* (Figure 1.5) and Schizophyllan from *S. commune* are two of the best studied commercially available β-D-glucans. These and glucans from *Grifola frondosa*, known commercially as Grifolon D (Figure 1.5), have been shown to have strong immunomodulating and anticancer properties (Mizuno *et al.*, 1995a). Schizophyllan from the culture fluid of *S. commune* and Krestin (PSK) from the cultured mycelium of *T. versicolor* have been approved in some countries for the clinical treatment of cancer patients, passing phase 1, 2 and 3 of randomised clinical trials in stomach, colorectal, oesophageal, and breast cancer patients (Standish *et al.*, 2008; Polishchuk *et al.*, 2009; Asatiani *et al.*, 2010; Ferreira *et al.*, 2010). Lentinan and Schizophyllan are pure β-glucans, whereas PSK is a protein bound polysaccharide.

PSK is an aqueous extract, composed of 62 % polysaccharide and 38 % protein (Ikuzawa *et al.*, 1988). These active polysaccharides are widespread among higher Basidiomycetes, with potential for different strains from the same species having polysaccharides with different properties (Wasser, 2002). For example, proteoglucan Krestin isolated from *T. versicolor* CM-101 and the bioactive polysaccharide PSP has been isolated from the submerged culture of the same species (Cov-1). Differences were believed to be due to different protein molecules bound to the same polysaccharide component (Hiroshi *et al.*, 1993).

Lentinan, isolated from aqueous extracts of six edible fungi, inhibited the growth of tumours from sarcoma-180 cells implanted into mice by 72 – 92 % when tested compared to controls, with no notable side-effects (Ikekawa *et al.*, 1969; Mattila *et al.*, 2000). Promising data for host mediated antitumor and immunostimulatory activity from species of *Phellinus linteus, Flammulina velutipes* (Enokitake), *Hypsizygus marmoreus, Agaricus blazei* and others have also been described (Wasser, 2002). As well as antitumor activity, isolated β-glucans have been seen to lower total and LDL cholesterol levels (Kris-Etherton *et al.*, 2002).

The mode of action of antitumor fungal polysaccharides has been extensively studied (Mizuno, 1996; Wasser, 2002; Ferreira *et al.*, 2010). While they do not exert cytotoxic effects on tumour cells directly, they enhance host-mediated immunomodulatory responses, which has a negative effect on tumour growth in human cell lines (Fang *et al.*, 2002a). The polysaccharide β-(1,3)-glucan is known to inhibit tumour growth through stimulation of the immune system *via* activation of macrophages and through a balance of T helper cell populations, natural killer (NK) cells and cytokine production (Mizuno *et al.*, 1995a; Roupas *et al.*, 2012). For this reason, these immunopotentiators, or immunoinitiators have become known as Biological Response Modifiers (BRM).

Over the years, scientists have classified bioactive polysaccharides as beneficial for a number of reasons, (i) prevention of oncogenesis through digestion of mushrooms or mushroom extracts, (ii) direct antitumor activity against various allogeneic and syngeneic tumours, (iii) immunopotentiation activity against tumours in conjunction with chemotherapy, and (iv) preventative effects on tumour metastasis (Wasser, 2002). Unlike traditional antitumor therapies, treatment using macrofungal polysaccharides has proven to be relatively harmless to the host (Wasser *et al.*, 1999c). Consequently, these fungal polysaccharides are increasingly evaluated as adjuvant

cancer therapy compounds alongside conventional cancer treatments (Standish *et al.*, 2008; Roupas *et al.*, 2012).

Antitumor activity is mostly related to polysaccharides isolated from the fruiting body of mushrooms rather than those from the mycelia; also their activity has been shown to depend on composition and conditions of the nutrient medium (Polishchuk *et al.*, 2009). Bioactive polysaccharides are found in all parts of the mushroom and mycelium, and may also be secreted into the growth medium making fungi a valuable source of natural antitumor compounds when grown by submerged cultivation. It is theorised that as the density of mycelium increases to create the fruiting body of the fungi, the availability of β-glucans also increases.

1.3.4 Antihypertensive activity

Hypertension is an extremely important public health issue in the Western world, and many cases remain undiagnosed for many years. Cardiovascular disease, renal failure and diabetes all stem from high blood pressure, reduction of which by even small amounts has substantial health benefits.

Lovastatin (mevinolin), a low molecular weight compound, is a competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, a key enzyme in cholesterol metabolism that catalyses the reduction of HMG-CoA to mevalonate. Mature fruiting bodies of *P. ostreatus* have been recommended for consumption as a natural cholesterol-lowering functional food due to its high concentration of lovastatin (Wasser *et al.*, 1999c). Bobek *et al.* (1991a) demonstrated that addition of 2 % to 4 % (dry fruiting body) of *P. ostreatus,* to a hyperlipidemic diet, efficiently prevented serum cholesterol increase and ultimately reduced cholesterol content by more than 60 % in the liver. This activity was subsequently attributed to lovastatin, which is found in high quantities in the fruiting bodies of various cultures and extracts of *Pleurotus* spp. (Bobek *et al.*, 1991a; Bobek *et al.*, 1991b; Bobek *et al.*, 1993; Wasser *et al.*, 1999c).

Lovastatin is also produced by Monascus (Section 1.2.3). In the 1970's, monacolins were found to inhibit the synthesis of cholesterol (Section 1.2.6), the most effective of which was named monacolin K and is also known as lovastatin, mevinol, and mevacor (Akihisa *et al.*, 2005). Monacolin J, L and M are structural analogues of monacolin and were also found to have considerable cholesterol lowering ability (Endo *et al.*, 1985; Endo *et al.*, 1986). Trials using *M. purpureus* extracts significantly

reduced lipoprotein-cholesterol (LDL-C) by 27.7 %, total cholesterol by 21.5 %, triglycerides by 15.8 %, and apolipoprotein B by 26 % (Lin *et al.*, 2005). High-density lipoprotein-C (HDL-C) and apolipoprotein A-I levels were increased non-significantly by 0.9 % and 3.4 %, respectively (Lin *et al.*, 2005; Klimek *et al.*, 2009). Monacolins, in the form of β-hydroxyl acids, inhibit HMG-CoA reductase, a key enzyme in cholesterol biosynthesis in both animals and humans (Patakova, 2013).

Fukushima *et al.* (2000) demonstrated a reduction in plasma cholesterol level when fibre from *Agaricus bisporus* was supplemented in feed, through enhancement of the hepatic low density lipoprotein (LDL) receptor mRNA. In addition, similar cholesterol lowering effects were established in rats when fed fibre from other filamentous fungi such as *Lentinus edodes*, *Flammulina velutipe*. Additionally, *Grifola frondosa* has been shown to reduce serum cholesterol levels and reduce blood pressure in rats without impacting HDL levels (Kabir *et al.*, 1987; Adachi *et al.*, 1988). *T. versicolor* has also shown ability to lower serum cholesterol level in animals (Wasser *et al.*, 1999b), and Krestin decreased LDL cholesterol levels in hyperlipidemia cases (Tsukagoshi *et al.*, 1984). A glycoprotein isolated from the submerged cultivated mycelium of *T. versicolor* showed activity both *in vivo* and *in vitro* against hypertension and thrombosis. The protein inhibits blood platelet aggregation and is antihyperlipidemic and antiarrhythmic (Ikuzawa *et al.*, 1988).

In addition to demonstrating the ability to lower blood cholesterol, Pleurotus and Monascus have also demonstrated efficiency in lowering blood glucose levels in diabetics (Khatun *et al.*, 2007; Rajasekaran *et al.*, 2009). *Pleurotus sajor-caju* has been used as an extremely useful preventative of diabetes due to its low fat and high soluble fiber content (Kanagasabapathy *et al.*, 2012).

1.3.5   Concluding remarks

Overall, the spectrum of pharmacological activities demonstrated by many fungi is extremely broad. They have been shown to possess many biological functions including; antifungal, anti-inflammatory, antitumor, antiviral, antibacterial, antiparasitic, immunomodulatory, and hepatoprotective activity, as well as playing a large role in regulation of blood pressure and cardiovascular disorders, such as hypercholesterolemia and diabetes. Fungi are an alternative source of natural antimicrobial compounds having demonstrated activity toward bacterial, viral and fungal pathogens which in some cases are resistant to current therapeutic agents

(Wasser *et al.*, 1999b). Many fungal species have been shown to demonstrate more than one biological function. Fungal fruiting bodies are highly nutritious; low in fat, contain 19 – 35 % protein, are rich in carbohydrates (51 – 88 %, of which 4 – 20 % is dry weight) and are an immense source of a variety of minerals (Adejoye *et al.*, 2006). As well as demonstrating the ability to treat certain disease states, many fungi are not only a source of nutrition but have immense antioxidant power. The possibility of replacing synthetic antioxidants, whilst retaining strong bioactive ability, makes fungi of particular scientific interest.

1.4   Fungi as a source of natural antioxidants

1.4.1   Oxidative stress

For many living organisms oxidation is essential to produce energy to fuel biological processes (Yang *et al.*, 2002). During normal metabolic processes of aerobic cells, cellular damage is caused by free radicals, in the form of reactive nitrogen species (RNS) or primarily in the form of reactive oxygen species (ROS). Free radicals derived from molecular oxygen represent the most important class of radical species generated in living systems (Miller *et al.*, 1990). A free radical is defined as any atom or molecule possessing unpaired electrons in the outer orbit, they are generally unstable and very reactive (Gutteridge *et al.*, 2000). An imbalance in free radical production such as overproduction of ROS or loss of natural antioxidant defences is known as oxidative stress. Overproduction may lead to the oxidation of lipids, DNA or protein and is a major contributor to aging (Barja, 2004), degenerative diseases such as cancer (Valko *et al.*, 2006b), cardiovascular disease (Shah *et al.*, 2004), compromised immune function, inflammation and renal failure (Valko *et al.*, 2006a).

Oxidant signals interfere with the otherwise normal natural metabolic role of ROS in aerobic cells, such as; cell proliferation, differentiation and apoptosis. When such damage is caused, cell toxicity occurs leading to health implications (Suzuki *et al.*, 2010). Mitochondria are often the first target for free radical attack, as the lipid membrane is very vulnerable to degradation by ROS; this attack is known as lipid peroxidation. The interaction of ROS with molecules of lipidic nature, generate new radicals; superoxide anions ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydrogen radicals ($OH^{\cdot}$). These groups of radicals then interact with biological systems in a cytotoxic manner (Barros *et al.*, 2007c). The relationship between antioxidant defences and

ROS production is usually a representation of the degree of oxidative stress (Suzuki *et al.*, 2010). Various free radicals involved in oxidative stress are associated with different biological, chemical and physical properties, these include; hydroxyl, alkoxyl, peroxyl, superoxide, nitric oxide, sulphur- and nitrogen-centered radicals (Niki, 2010). There are several factors which influence the free radical scavenging capacity; the rate and number of radical molecules scavenged, the fate of antioxidant-derived radicals, interaction with other antioxidants, concentration, mobility in the environment, and the adsorption, distribution, retention and metabolism of the antioxidant compounds (Niki *et al.*, 2000; Noguchi *et al.*, 2000; Niki, 2010). When an active radical is scavenged by an antioxidant compound, a stable non-radical product is formed. At the same time the antioxidant yields one antioxidant-derived radical. The fate of this radical is also an important consideration of antioxidant efficiency (Niki *et al.*, 2000).

Antioxidants are crucial for the maintenance of normal cell function, health and well-being. They are compounds which prevent the initiation or propagation of oxidising chain reactions which in turn inhibits or delays oxidative damage related to aging and disease. Although animals have developed natural mechanisms to protect cells from free radical damage by neutralising them, the amount of antioxidant produced under normal conditions is not always sufficient. Fungi are a well-known source of antioxidants which can be used to prevent oxidative damage and as such, can limit their deleterious effects in humans and animals alike.

Other than the health promoting effects from consumption of natural antioxidants, prevention of oxidation is important in other areas also. In the food industry, oxidation affects the nutritional value of food and can cause rancidity or deterioration of colour, flavour and texture. Microbial spoilage is a huge problem for food manufacturers. Traditionally salt, sulphite and antimicrobial compounds have been used to inhibit growth of microorganisms in food products; however, this is not ideal. Additionally, consumption of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxyltoluene (BHT) are now known to have a negative impact on health (Branen, 1975; Larson, 1988). With increasing consumer awareness of food preparation, controversy has surrounded the use of artificial antioxidants such as BHA and BHT as preservatives, which has lead to particular interest in effective and safe preservatives from natural sources. A wide range of preservatives can be used alone or as a blend. A blend can produce a less expensive product without compromising efficiency. For example, tocopherols may be mixed

with another popular antioxidant, such as rosemary extract. The food preservation market is projected to reach €2 billion by 2018, though natural preservatives are expected to contribute to the smallest share of the market (MarketsandMarkets, 2014). The European market alone is believed to increase from €79 million, recorded in 2011 to €188 million by 2018. With consumer perception regarding the benefits of natural additives and health hazards associated with consumption of foods preserved chemically, natural preservatives, such as, antioxidants are in demand.

1.4.2   Natural antioxidants

Natural compounds such as ascorbic acid, vitamin E, carotenoids, flavonols and phenolic acids which are common to fungi, plants, citrus fruits and leafy vegetables possess the ability to scavenge free radicals in the human body. They play a key role in health maintenance and prevention of chronic and degenerative diseases such as atherosclerosis, carcinogenesis, neurodegenerative diseases, DNA damage and aging (Doughari, 2012).

There are two mechanisms of defence that have stemmed from an evolutionary response to the existence of oxygen radicals; classified as enzymatic and non-enzymatic. The major enzymatic antioxidant molecules are copper-zinc and manganese peroxidase, whereas the main non-enzymatic molecules are glutathione, and vitamins A, C, and E. The thiol glutathione is known for its efficient detoxification of free radicals as well as reactive oxygen and nitrogen species (Jones *et al.*, 2014). Antioxidants may be characterised by their mode of action in preventing oxidative damage, being classified as either preventative, scavenging, repair or *de novo* antioxidants (Lobo *et al.*, 2010). Antioxidants prevent the formation of ROS/RNS by reducing hydrogen peroxide and lipid hydroperoxidases, respectively, or by sequestering metal ions such as iron and copper (Niki, 2010). Scavenging antioxidants remove active species rapidly before they attack biologically essential molecules, such as, superoxide dismutase (SOD), which converts superoxide to hydrogen peroxide, while carotenoids scavenge singlet oxygen, physically or chemically (Lobo *et al.*, 2010; Niki, 2010). Repair enzymes act as antioxidants by repairing damage, clearing wastes or reconstituting lost function (Niki, 2010).

Phenolic compounds are aromatic hydroxylated compounds possessing at least one aromatic ring with one or more hydroxyl groups (Apak *et al.*, 2007a). By this means, a structure-function relationship exists between phenolic compounds; with their

antioxidant activity depending on the number and position of the hydroxyl groups and the nature of substitutions on the aromatic rings (Balasundram *et al.*, 2006). These compounds are common to vegetables, fruits and fungi and are important sources of bioactive substances (Apak *et al.*, 2007a). Phenolic acids are divided into two main groups, hydroxybenzoic acid and hydroxycinnamic acids, which are derivatives of the non-phenolic molecules benzoic and cinnamic acid, respectively (Ferreira *et al.*, 2009). The hydroxyl derivatives of benzoic acid, such as ρ-hydroxybenzoic, vanillic acid and syringic acid are thought to be less active antioxidants than caffeic, chlorogenic, ferulic, sinapic and ρ-coumaric acids (Larson, 1988). Hydroxybenzoic and hydroxycinnamic acid derivatives occur mostly in the bound form. Hydroxybenzoic acid is typically a component of a complex structure like lignin and hydrolysable tannins and hydroxycinnamic acid is mainly linked to cell wall structural components, such as cellulose, lignin and proteins, as well as associated to organic acids, such as tartaric or quinic acids (such as chlorogenic acids), through ester bonds (Liu, 2004).

Molecules such as simple phenolic acids, phenylpropanoids and flavonoids, as well as the highly polymerised molecules; lignin, melanin and tannins, accumulate naturally as end products from the shikimate and acetate pathways, with flavonoids representing the most common and widely distributed sub-group with antioxidant activity (Bravo, 1998; Liu, 2004; Ferreira *et al.*, 2009). There are over eight thousand naturally occurring phenolic compounds currently known (Balasundram *et al.*, 2006), their variation is seen structurally in the form of simple molecules to highly polymerised compounds (Kris-Etherton *et al.*, 2002). Over ten polyphenol classes have been established on the basis of chemical structure (Bravo, 1998).

Flavonoids are usually found in high quantities in many fruits, vegetables, plants and nuts (Liu, 2004). They are highly effective scavengers of free radicals, possessing the ability to protect from cardiovascular disease, DNA damage and tumour progression (Wright *et al.*, 2001).

Carotenoids are natural pigments, synthesised by plants responsible for the colouration of various fruits and vegetables and have been implicated in the prevention of numerous diseases. Structurally, they possess a 40 carbon poly-isoprenoid skeleton, with oxygen containing functional groups responsible for colour and antioxidant functions (Ferreira *et al.*, 2009). They are also regarded as dietary sources of vitamin A. Depending on the chain length and end groups attached, carotenoids act as

antioxidants by reacting with free radicals to become chain breaking radicals themselves in a lipid environment (Ferreira *et al.*, 2009).

Vitamin E is a liposoluble vitamin which plays an important role in the prevention of lipid peroxidation in the cell membrane. Vitamin E is made up of eight compounds; α, β, γ and δ-tocopherols and includes both tocopherol and tocotrienols (Kamal-Eldin *et al.*, 1996; Brigelius-Flohe. *et al.*, 1999). α-Tocopherol is the most active form of vitamin E in humans with high biological activity (Ferreira *et al.*, 2009). Vitamin E is a scavenger of free radicals and as such, has been implicated as having a protective role against disease (Burton *et al.*, 1990; Blokhina *et al.*, 2003) .

Vitamin C (ascorbic acid), present in several fungal species, has also demonstrated a protective role, and has been associated with having a positive effect against oxidative stress related diseases (Klein *et al.*, 1982). Vitamin C can react with peroxyl radicals prior to lipid peroxidation thus protecting the lipid membrane from oxidative damage (Davey *et al.*, 2000).

Together, vitamin E and vitamin C form a synergistic relationship. When vitamin E scavenges active free radicals it is converted to vitamin E radical, which is reduced back to its antioxidant form by vitamin C before the vitamin E radical attacks lipids to induce lipid peroxidation. Other antioxidants such as glutathione, ubiquinol and some phytochemicals have also demonstrated a synergistic relationship with vitamin E (Shi *et al.*, 1999; Mukai *et al.*, 2005; Nagaoka *et al.*, 2007). The relationship between these nutrients is important because alone, the concentration of each may not provide adequate protection against lipid peroxidation; however, by forming a cascade reaction, they have been demonstrated as playing an important role in disease prevention (Chew, 1995; Nagaoka *et al.*, 2007).

1.4.3   Origin of antioxidants

There are two categories of antioxidants; synthetic and natural. Synthetic antioxidants are generally compounds with phenolic structures of varying degrees of alkyl substitution which are created in laboratories, mainly for use in the preservation of foods. Conversely, natural antioxidants are obtained from plant or fungal sources; these include phenolic compounds (tocopherol, flavonoids, and phenolic acids), nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids, and amines) or carotenoids as well as ascorbic acid (Larson, 1988; Velioglu *et al.*, 1998). Synthetic antioxidants such as BHA and BHT have recently been found to cause carcinogenesis

in rodents, and by analogy also possibly in man (Ito *et al.*, 1985), highlighting the benefits of antioxidants from natural sources such as fungi.

Many enzymes and secondary compounds of higher fungi have been demonstrated *in vitro* to protect against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species. For example, ergothioneine, a naturally occurring amino acid has attracted much attention due to its antioxidant action in humans (Jones *et al.*, 2014). The main antioxidant polyphenolic compounds found in fungi are phenolic acids and flavonoids, followed by tocopherols, ascorbic acid and carotenoids. Flavonoids can be diversified into 13 classes, containing over 5000 compounds, the most common of which are flavones, flavonols, and their glycosides (Bravo, 1998; Kris-Etherton *et al.*, 2002). The mechanisms by which compounds exhibit their antioxidant activity include; (i) radical scavenging activity toward either reactive species (ROS or RNS) or toward lipid peroxidising radicals; generally proceeded *via* hydrogen atom transfer or electron donation, (ii) prevention of the transition metal-catalysed production of reactive species, through metal chelation, and (iii) interaction with other antioxidants, localisation, and mobility of the antioxidant at the microenvironment (Niki *et al.*, 2000; Apak *et al.*, 2007a). The involvement of the phenolic hydrogen in the radical reactions, the stability of the natural antioxidant radical formed and the chemical substitutions present on the structure are responsible for the overall efficiency of natural phenolic antioxidants (Hall, 2001). Overall, many studies have shown that natural antioxidants, such as fungal phenolics, may be extracted for use as functional ingredients in food.

1.4.4   Determination of antioxidant capacity

As discussed, antioxidants have different functions and carry out their protective role through a number of processes. Consequently, there is no universal method to accurately and quantitatively assess antioxidant capacity (Litescu *et al.*, 2010; Lobo *et al.*, 2010; Niki, 2010). Antioxidant activity is usually established in terms of the degree of antioxidant power based on colorimetric reactions, a measure of scavenging ability following the reduction of metal ions or radical scavenging capacity based on competitive methods.

Conjugated diene peroxides are formed when free radicals react with oxygen to form peroxide radicals which attack the lipid membrane. Conversely, the scavenging of free radicals or peroxide radicals prevents lipid peroxidation (Palacios *et al.*, 2011).

Many antioxidant activity assays are based on the prevention of lipid peroxidation, as this is the primary target for free radical damage. Assessment of this process involves a colorimetric assay which measures the degree of peroxidation. There are a number of different methods to measure reactivity of natural compounds towards radicals. Reactivity of antioxidant compounds to stable free radicals such as 2,2,-diphenyl-1-picrylhydrazyl (DPPH) is one such method. Reactivity of antioxidant compounds, their mixture, and natural and commercial products with 2,2,-diphenyl-1-picrylhydrazyl (DPPH) or 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) cation radical ($ABTS^+$), provides a stable colorimetric assay to establish antioxidant capacity (Niki, 2010). Free radicals react with oxygen species generated by an initiator, yielding peroxide radicals which subsequently attack lipids to form conjugated diene peroxides (Palacios *et al.*, 2011). Scavenging free radicals or peroxide radicals by an antioxidant substance leads to prevention of lipid oxidation in biological systems allowing measurement of antioxidant capacity (Palacios *et al.*, 2011). Another example is the inhibition reaction of a test compound in comparison to the reaction of a reference compound following generation of free radical species. Reactivity of natural fungal compounds to radicals may be analysed by a bleaching reaction of a reference compound e.g. strong visible adsorption of a carotenoid is reduced by reaction with radicals; the extent of suppression of bleaching is then a measure of the antioxidant reactivity (Niki *et al.*, 2000). The ferric reducing antioxidant power (FRAP) and cupric reducing antioxidant power (CUPRAC) assess reduction of metal ions due to a radical scavenging antioxidant and are also popular antioxidant capacity assays (Niki, 2010). Other methods for measurement of biological antioxidant capacity include induction of the autoxidation of linoleic acid or low-density lipoprotein (LDL) by Cu (II) or an azo initiator and control of the formation of conjugated diene peroxides (Palacios *et al.*, 2011).

Evaluation of radical scavenging capacity *in vitro* may be assessed by competition methods such as oxygen radical antioxidant capacity (ORAC) and trolox equivalent antioxidant capacity (TEAC) methods (also referred to as $ABTS^+$ radical scavenging assay) using a reference compound; however, these methods are not suitable for assessment of antioxidation capacity, such as efficiency of lipid peroxidation inhibition (Niki, 2010). Generally, antioxidation refers to how much or how long the compound or substance inhibits oxidation. Recently, the F.D.A removed the U.S Department of Agriculture (U.S.D.A) ORAC database for selected foods from

the National Diet Library (N.D.L.). Antioxidant capacity using this method was found to have no relevance to the effects of specific bioactive compounds, including polyphenols, on human health (U.S.D.A., 2010). These observations contribute to the importance of analysing a number of methods in order to accurately and quantitatively assess total antioxidant capacity.

1.4.5   Bioactivity of fungal antioxidant compounds

Fungi produce a variety of bioactive antioxidant secondary metabolites including phenolic compounds, polyketides, terpenes and steroids (Cui *et al.*, 2005; Schuemann *et al.*, 2009). Antioxidants, particularly flavonoids, have been shown to exhibit other biologically important effects including; antibacterial, antiviral, anti-inflammatory, antiallergic and vasodilatory actions (Cook *et al.*, 1996; Velioglu *et al.*, 1998). It is now well known that biological properties such as anticarcinogenicity and antiaging functions originate from this ability to prevent oxidation in cells (Cook *et al.*, 1996). Pleurotus and other higher Basidiomycetes have been recognised as antioxidant producers due to their phenolic, terpenoid and polysaccharide content (Valentao *et al.*, 2005). It is these bioactive compounds which also mediate other biological activities such as stimulation of interleukin-12 production, nitric oxide synthase activation, free-radical scavenging and iron chelating properties (Acharya *et al.*, 2005; Cui *et al.*, 2005; Iwalokun *et al.*, 2007).

Methanolic extracts of *M. purpureus* were found to possess strong radical scavenging and lipid peroxidation activity. The compound responsible was dihydromonacolin, a structural analogue of monacolin (Dhale *et al.*, 2007). Antioxidant activities from natural polysaccharides have also been reported. Unlike synthetic antioxidants, natural polysaccharides have low toxicity and therefore, they make ideal antioxidant agents. Recently, antioxidant activity of polysaccharides has demonstrated remarkable inhibition on lipid peroxidation *in vitro* (Tian *et al.*, 2013). By restoring systemic antioxidant defences, hepatic oxidative stress in mice was restored; this action was associated with a purified polysaccharide fraction (Tian *et al.*, 2013).

Structural features such as, glycosyl linkage, monosaccharide composition and configuration and in particular molecular weight are all known to affect the antioxidant activity of polysaccharides. Typically, polysaccharides with low molecular weight exhibit higher activity (Tian *et al.*, 2013). The uses of polyphenols (phenols, quinines,

flavonoids, tannins and coumarins) from natural sources have been suggested as alternatives to antimicrobial agents. Naturally occurring polyphenols inhibit the growth of microbes and in doing so, prevent microbial spoilage (rancidity or deterioration of colour, flavour and texture). Kao *et al.* (2010) demonstrated that the gram-positive bacteria *S. aureus* was susceptible to a reduction in growth rate due to the presence of a polyphenolic extract. It is believed that the antimicrobial action of polyphenolic compounds may be mediated by the ability of tannins to deplete metal ions through precipitation or by structural damage to the microorganism, since flavonoids can cause such damage by altering the permeability of the cell membrane. An antioxidant compound from fungi which demonstrates strong antimicrobial efficiency may be an attractive alternative source for food preservation as well as a natural functional food ingredient.

Antioxidants are critical for the maintenance of cellular processes and systemic health and well-being. This type of biological function is a fundamental property important for life. The amount of antioxidant produced by the body is not always adequate to protect cellular damage; meaning dietary supplementation of antioxidants is encouraged. As a natural source of antioxidants, combined with immense biological activity, fungi are excellent advocates for strong health promoting organisms.

1.5   Biomass generation from filamentous fungi

There are three main cultivation processes that exist for the generation of fungal biomass in industry, namely; submerged, solid state fermentations and static liquid culture. Submerged liquid fermentation (SLF) is the growth of a microorganism in a liquid medium agitated by shaking, stirring or sparging, as opposed to filamentous fungi in static liquid culture, which forms a mat of hyphae at the surface of the liquid. A typical submerged fermentation is composed of more than 95 % free water, whereas the water content retained within a solid mash in solid state fermentation often varies between 40 % and 80 % (Leib *et al.*, 2001). Solid state fermentation by definition, is the cultivation of a microorganism on a solid substrate at low moisture levels or water activities; however, the substrate must possess enough moisture to support growth and metabolism of the microorganism (Ali *et al.*, 2011). Commercial cultivation of medicinally important fungi has many benefits; mycelium can be kept for long periods and biochemical consistency may be checked over time, which is true for solid state, static and submerged fermentation. Each fermentation method has their advantages

and disadvantages; usually choice of fungal cultivation method is dependent on the application of the product formed following cultivation. Over the course of fungal evolution, different species adapted to various environments, meaning that today, fungi require different physical, chemical and nutritional conditions for optimum mycelia production.

Historically, research was more directed toward solid state fermentation (also known as the koji process), which was developed in Japan. As a greater amount of research has been done on enzyme and secondary metabolite production for SSF, there have been numerous reports of the advantages of SSF from an economical point of view; primarily as they are based on the nature of the substrate matrix, which is unique to SSF. Agricultural waste products, wood, straw or spent low-value raw materials may be used to produce high-value products. Recent research has focused on the use of lignocellulosic organic waste materials for either lignin degradation, animal feed or enzyme production (Gregori *et al.*, 2007). Low moisture levels reduce the risk of contamination and the substrate matrix may resemble the natural environment of the fungi, inducing specific enzyme synthesis. The main disadvantage of solid state fermentation in comparison to submerged liquid fermentation is the heterogeneous nature of the media, due to poor mixing characteristics, which results in an inability to control environmental parameters (pH, moisture, temperature and substrate concentration) within the fermentation system (Hoelker *et al.*, 2004; Ali *et al.*, 2011). Therefore, low thermal conductivity and uneven distribution of nutrients and moisture content are major issues of SSF. Section 1.5.1 discusses SLF and the predominant advantages and disadvantages of this method of cultivation.

1.5.1 Biomass production using submerged cultivation processes

Commercial production of mycelium using submerged culture was developed in the 1950's. Initially, very little research was carried out on mycelial biomass generation in higher fungi (Basidiomycetes) and much of the focus was on lower fungi for the economical production of various natural products (Fang *et al.*, 2002c). Mycelial production for development of commercial products was once mostly obtained through field-cultivation of the fungal fruiting body; however, now SLF is being used for the production of more consistent medicinal products (Smith *et al.*, 2002; Tang *et al.*, 2007). Mycelial biomass generated by SLF enables the production of more valuable medicinal compounds and has received increasing attention as it is viewed as a

promising alternative to the laborious and time-consuming extraction of field cultivated medicinal mushrooms. Overall, there are several advantages of liquid cultivation when comparing to solid state cultivation;

- Higher production of mycelium in a compact space with shorter time and little chance of contamination (Gregori *et al.*, 2007).
- The ability to control the growth environment (pH and temperature) which ultimately ensures standardised quality of the product formed (Chang, 2001; Elisashvili, 2012).
- Even distribution of nutrients required for growth throughout the culture medium.
- Less time required for biomass production and therefore, rapid product accumulation depending on the vessel (Yang *et al.*, 2003; Diamantopoulou *et al.*, 2014).
- Less labour intensive and high product rates may be achieved using large stir tank reactors or tower fermentors (Zhong *et al.*, 2004).
- The culture fluid may also provide a source of biological activity (Mizuno, 1996).

Ultimately, the cultivation of mycelia in liquid culture permits fully standardised production of biomass with high nutritional and metabolite value, whilst allowing predictable product composition (Elisashvili, 2012).

1.5.2 Factors that affect biomass generation and bioactive compound production in SLF

There are a number of factors that can affect the quality and quantity of biomass and bioactive compound production. The main areas which influence the growth of mycelia in submerged culture are the components of the culture medium (e.g. carbon, nitrogen and inorganic acids) and the fermentation parameters (e.g. temperature, pH, aeration, shear stress, and inoculation density) (Lin *et al.*, 1997). One of the major benefits of submerged liquid cultivation is the ability to influence the environment in order to maximise production or bioactivity of fungal biomass (Kim *et al.*, 2002). In submerged culture, mycelia may take different morphological forms i.e. they may grow as free mycelia or grow in pelleted form; depending on medium, inoculum size, physical environment and most importantly the species (Whitaker *et al.*, 1973; Sinha *et al.*, 2001a).

1.5.2.1   Culture medium

Most research on the cultivation of Pleurotus relating to SLF and SSF is linked to the manipulation of substrate composition and optimisation of culture parameters (Gregori *et al.*, 2007). Culture medium is a particularly important factor for growth in commercial cultivation of fungi as it provides the nutrients for mycelium propagation. Alteration of the basal medium with the addition of carbohydrate or nitrogen sources has been shown to effect biomass production and quality in various strains of fungi, demonstrating that different fungal species have different nutrient requirements (Hadar *et al.*, 1986; Bilay *et al.*, 2000; Adejoye *et al.*, 2006; Gregori *et al.*, 2007). Carbon source supplementation during growth has also been shown to influence polysaccharide production (Kim *et al.*, 2002; Duan *et al.*, 2013). Reports on *Ganoderma lucidum* have found that polysaccharide production is not affected by mycelia production (Sone *et al.*, 1985; Tang *et al.*, 2002). Therefore, biological components do not exhibit a parallel relationship with the optimal growth parameters for biomass production.

Research on the use of alternate materials to increase biomass and polysaccharide production, fatty acids (Yang *et al.*, 2000), plant oils (Hsieh *et al.*, 2008) and some surfactants (Nwanze *et al.*, 2005; Pi *et al.*, 2005; Hsieh *et al.*, 2008) have shown improvements in the production of mycelia and bioactive metabolites in liquid culture. For example, (Lin, 1973) was the first to demonstrate that different culture conditions of *Monascus* spp. affected pigment production. Over time, it was established that submerged cultures with 5 % polished rice powder as a carbon source, 0.5 % sodium nitrate as a nitrogen source, an initial pH of 6 and temperature of 32 ℃, gave higher pigment and dry weight production. In addition, supplemental alcohol (1 – 2 %, v/v) during incubation had a favourable effect on pigment production (Su *et al.*, 1976; Carels *et al.*, 1977).

1.5.2.2   pH

Culture pH does not only affect growth but also the production and activity of metabolites, either directly due to the effect of pH on cell permeability or indirectly by its effect on the availability of certain metals such as magnesium, phosphorus, iron, calcium and zinc. Fungi grow over a wide pH range, the optimum range of filamentous fungi is between pH 3.8 – 6, however; reasonable growth may be observed between pH 2 – 9. Yeast on the other hand have a narrower optimum pH range,

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between 4 and 5 when nutrient requirements are satisfied, but are still capable of growth between pH 2.5 – 8. Most bacteria grow optimally at a neutral pH, therefore a pH above or below this range allows for bacterial contamination to be minimised or prevented (Woods *et al.*, 1985).

On the whole, there is a lack of information on the effect of pH on fungal growth; however, this data would be of limited value as filamentous fungal metabolism can alter their external pH during their development. Additionally, during the submerged fermentation process, the pH may change due to substrate consumption and metabolite production. Secretion of organic acids such as oxalic acid will cause the pH to drop. On the other hand, the assimilation of organic acids will lead to an increase in pH; urea hydrolysis will result in alkalisation. An alkaline pH range has been observed to be more tolerable for *Aspergillus* species, while an acidic pH range appears to be more tolerable for *Penicillium* (Wheeler *et al.*, 1991).

Chen *et al.* (1993) noted that lower pH during growth of *Monascus* spp. in SLF promoted fungal growth and synthesis of ankaflavin pigment; however, the production of other pigments were unaffected by pH value. Different nitrogen sources supplemented in liquid culture have been observed to directly affect pH. For example, the pH of culture medium is around 6.5 when using yeast extract or nitrate as a nitrogen source, whilst when supplementing with ammonium nitrate as a nitrogen source, the pH can drop to 2.5, leading to differences in pigment formation (Lin *et al.*, 2008).

The recommended pH of the culture medium depends on the product and the species of fungi, whilst bearing in mind optimal conditions for growth. One of the advantages of submerged growth is the capacity to manage the growth environment during incubation, which ultimately aids the purity of the product formed.

1.5.2.3   *Temperature*

In the wild, fungi have adapted to survive in a range of temperatures, a demonstration of their extraordinary ability to adapt and survive. Environmental temperature is an important factor and strongly influences the growth of most fungi. The temperature range usually reported for fungal growth is broad (10 – 35 °C), with a few species capable of growth below or above this range (Kerry, 1990). Organisms can be divided according to their tolerance to temperature including psychrotolerant, psychrophilic, mesophilic, thermotolerant, thermophilic and extremophilic fungi. Optimal

temperature is species and strain dependant. From a commercial perspective, favourable conditions for maximum production are desirable and with even distribution throughout the substrate medium using SLF, this is attainable.

1.5.2.4   *Agitation and inoculation density*

Agitation of the culture medium evenly distributes dissolved air throughout the liquid culture vessel and is a very important environmental factor. As with any species of filamentous fungi grown in submerged liquid culture, shear stress caused by agitation due to excessive speeds will damage mycelia, whilst too slow can limit the transfer of oxygen evenly throughout the substrate medium. Both instances directly affect biomass production and secondary metabolite formation. The initial oxygen transfer rate and inoculation density in submerged culture is an important factor for cell growth and polysaccharide production for *P. ostreatus* as well as other species (Choi *et al.*, 2006a; Gregori *et al.*, 2007; Duan *et al.*, 2013). Marquez-Rocha *et al.* (1999) investigated the growth of *P. ostreatus* in stirred tank bioreactor and found a difference in agitation speed and aeration intensity positively or negatively influenced the growth rate and pellet size. Pellet size was reduced by 60 % and the specific growth rate decreased as agitation speed increased. Park *et al.* (2002) found similar results with *Cordyceps militaris* from the Ascomycota division. Morphological differences depending on inoculation density were observed; large inoculation density resulted in a small pellet size, whereas low inoculation density had the opposite effect (Fang, 2002a). Consequently, morphology is also an important factor in mycelial cultivation; smaller sized pellets result in higher specific growth rates (Marquez-Rocha *et al.*, 1999). These studies indicated that morphological changes in mycelial pellet formation act as a good indicator of cell activity for exo-biopolymer production (Park *et al.*, 2002). Aeration is known to positively influence mycelial growth in liquid media (Diamantopoulou *et al.*, 2014). Oxygen concentration has also been seen to affect biosynthesis of Monascus metabolites (Hajjaj *et al.*, 2000). When oxygen supply is limited, pigment and citrinin production occurs and both are biosynthesised as primary metabolites. However, when oxygen supply is in excess citrinin is produced as a secondary metabolite and pigment formation decreases dramatically (Lin *et al.*, 2008). It is believed this is due to metabolites produced in aerobic environments such as L-maltose, succinate, and dicarboxylic acid (Lin *et al.*, 2008).

1.5.3   Production of bioactive compounds and secondary metabolites by SLF

In the 1970's, researchers were able to extract and develop bioactive polysaccharides from the fruiting body, mycelium and culture medium of fungi grown in liquid cultures (Mizuno, 1996). Bioactive metabolites are often produced in small quantities; therefore, manipulation of growth environment for increased yield at the highest quality is an important factor for consideration. Bioactive compound production is not reflected in biomass generation (Sone *et al.*, 1985; Tang *et al.*, 2002); however, carbon source supplementation was observed to influence not only biomass generation, but also the production of polysaccharides (Kim *et al.*, 2002).

A number of bioactive metabolites of higher Basidiomycetes have been produced by submerged liquid cultivation. Biomass generated in controlled submerged cultivations has proven to have many advantages such as, the mycelia may be rapidly produced, early development of the fruiting body, and most importantly, culture medium can be manipulated to produce optimal quality and quantity of active products (Wasser *et al.*, 2000; Reshetnikov *et al.*, 2001; Ferreira *et al.*, 2009). These are also beneficial reasons for using fungi as a source of bioactive components over plants.

During the primary phase of growth and biomass production, as nutrients become exhausted, products and intermediate compounds accumulate in the mycelium and culture medium. Consequently, changes to nutrient content or specific environmental conditions for optimal biomass generation can be exploited for the commercial production of both primary and secondary fungal products. Metabolites such as polysaccharides, terpenoids, triterpenoids, diterpenoids, sesquiterpenoids and ergosterol (as discussed in Section 1.3.1) are typically produced using submerged liquid fermentation. Mycelial biomass generation and bioactive polysaccharide production in submerged culture depends heavily on growth parameters, growth timing and their nutritional requirements during metabolism and development (Kim *et al.*, 2002; Gregori *et al.*, 2007; Lin *et al.*, 2008).

According to Lindequist *et al.* (2005), 80 – 85 % of mushroom products with biological activity are isolated from mushroom fruiting bodies, which are collected from the wild or grown commercially, e.g. Lentinan or various products from *G. lucidum*. It is now known that bioactive substances are different depending on the stage of growth of the mushroom, indicating that compounds isolated from the fruiting

body may be different to those isolated from the mycelium of submerged cultures. This has prompted increasing interest in bioactive metabolites from fungal mycelia (Lorenzen *et al.*, 1998). Only around 15 % of all relevant products are based on extracts from mycelia e.g. PSK and PSP from *T. versicolor* and tremellastin from *Tramella mesenterica*. An even smaller percentage of products are currently isolated from culture filtrates e.g. Schizophyllan from *S. commune* and protein-bound polysaccharide complex from *Macrocybe lobayensis* (Lindequist *et al.*, 2005).

Extracellular polysaccharide formation in submerged culture is a common characteristic of higher Basidiomycetes, constituting roles in fungal adhesion to substrate immobilisation of extracellular enzymes, prevention of hyphal dehydration, storage of excess nutrients and lignin degradation (Elisashvili, 2012). Although biomass and exopolysaccharide formation is known to be independent of one another (Maziero *et al.*, 1999), Montoya *et al.* (2013) demonstrated that a change in nutrient composition of culture medium can affect growth yield. Extracellular polysaccharide production increased in submerged liquid culture when glucose, soy oil and yeast extract were added to the culture medium (Montoya *et al.*, 2013). Wang *et al.* (1995) reported that a change in pH of the liquid medium affected extracellular polysaccharide production, reporting that lowering the pH of the medium promoted extracellular and intracellular polysaccharide production in the *Ganoderma* species (Fang *et al.*, 2002b).

Various culturing parameters such as composition, temperature, nitrogen source, medium components and pH value, have been shown to affect the production of secondary metabolites from *Monascus* spp. (Sato *et al.*, 1935; McHan *et al.*, 1970). Miyake *et al.* (2005) also found that light frequency can effect secondary metabolite composition of Monascus pigments. This observation is most likely a circumstance of stress on development. It was demonstrated that red light at λ_{635nm} promoted the growth of red pigments and citrinin, whereas blue light at λ_{470nm} promoted the growth of GABA. Light also effects mycelial generation and spore formation of Monascus (Lin *et al.*, 2008).

As bioactive metabolites are typically generated in small quantities and production depends on the species and the growth parameters, optimisation of these factors is essential for maximum quantity and purity of secondary metabolite and mycelial biomass cultivation. Optimisation of the culture medium composition and physicochemical conditions of growth allows regulation of fungal metabolism and

attainment of standardised nutraceutical compounds in higher yield. Mycelia formed in controlled pure cultures by SLF is the best technique for obtaining consistent and safe mushroom products (Lull *et al.*, 2005).

1.5.4   Extraction and purification of compounds from fungi

Over the years, research has shown that filamentous fungi produce various bioactive compounds with a diverse range of biological activities. It has also demonstrated that the content and bioactivity of these compounds is heavily dependent on their preparation (Table 1.2 and 1.3). Concentrations of secondary or intermediate metabolites within the cell are generally low, particularly those required when determining metabolic pathways and biosynthesis of the cell. For metabolite extraction from mycelium, an activating or inhibitory effect is only detectable under certain conditions, such as neutral, acidic or basic pH, so choice of extraction procedure is an important process (Jernejc, 2004). It has also been noted that concentrations of metabolites present in fungal mycelia during fermentation vary with time (Jernejc, 2004). This highlights the significance of suitable extraction and purification steps as well as, downstream processing post-fermentation.

Isolation and separation of natural compounds is usually a long and tedious process due to the high density of molecular constituents which are present in fungal mycelia or mushroom fruiting bodies (Sticher, 2008). Combinations of various separation techniques are generally required for the isolation of compounds and these techniques depend heavily on the volatility and stability of the compounds (Sticher, 2008). Prior to purification or analysis, several sample preparations, pre-purification and clean-up extraction methods such as filtration, precipitation, thin layer chromatography (TLC) or solid phase extraction (SPE) may be required. These methods are beneficial in that, they allow for selective removal of interfering compounds and/or concentration of the desired active constituents.

In recent years, traditional extraction techniques such as Soxhlet extraction, maceration, percolation, and sonication have been optimised in order to reduce extraction time, amounts of organic solvent required, degradation of vulnerable compounds, increase efficiency and to minimise labour-intensive procedures. Some updated extraction procedures include superficial fluid extraction (SFE), pressurised liquid extraction (PLE), microwave-assisted extraction (MAE) and solid-phase extraction (SPE) (Sticher, 2008).

There are a number of considerations when choosing which extraction process to use. Factors such as the heat intolerance of the compounds are of prime importance. For instance, PLE and MAE are not suitable for heat-sensitive compounds as the sample is subjected to extreme heat and pressure during the extraction process. SFE with supercritical carbon dioxide allows for the extraction of heat sensitive compounds. For example, Barros *et al.* (2007a) reported that severe heat during the cooking process was extremely destructive to polyphenolic structures and as a result there was a decrease in antioxidant activity. Soxhlet and MAE are the more cost effective extraction procedures. PLE and MAE are best suited to the extraction of compounds of medium to high polarity compared to SFE. Application of superficial fluids (carbon dioxide) or organic solvents at high temperature and pressure drastically improves the speed of the overall procedure; from 6 – 24 hours (Soxhlet) to 10 – 45 minutes (SFE), and less than 30 minutes for PLE and MAE (Sticher, 2008).

Biologically active polysaccharides from fungi are structural components of the cell wall and as such the extraction method relies on cell wall structure (Zhang *et al.*, 2007). These bioactive polysaccharides are widely distributed among higher Basidiomycetes species; however, their practical application is dependant not only on their distinctive structural characteristics, but also the experimental conditions from which they are extracted (Wasser, 2002). The extraction of phenolic acids or flavonoids is usually carried out using polar solvent, such as hot water, methanol, ethanol, acetone or ethyl acetate, solely or a combination thereof (Robbins, 2003; Qian *et al.*, 2004; Chen *et al.*, 2012). Fractionation of the cell wall may be applied through a number of extraction and precipitation steps (Stamets, 2000). Hot water extraction was first developed by Mizuno (1996) and boasts a reliable method for polysaccharide extraction from mushroom fruiting bodies or cultured mycelia (Mizuno, 1996; Zhang *et al.*, 2007). Traditionally, hot water soluble fractions from mushrooms were used medicinally in the Far East, which is where knowledge and practice of the curative properties of fungi first originated (Hobbs, 1995). This extraction method breaks open the cell wall from the outer layer to the inner layer with mild-strong extraction conditions depending on pH and temperature. Generally successive extractions at a 100 ℃ temperature in water are used. Depending on the polysaccharide composition (i.e. molecular weight, branching degree and pattern of branches) they may be further purified using various techniques or a combination thereof, such as ethanol

precipitation, fractional precipitation, ion-exchange chromatography, gel filtration, and affinity chromatography (Zhang *et al.*, 2007).

Many studies are based on enhancing the efficiency of extraction of bioactive compounds from natural sources. Extraction conditions usually explored include particle size, solvent type, solvent concentration, solvent-to-solvent ratio, extraction temperature, extraction time and pH (Pinelo *et al.*, 2005; Chirinos *et al.*, 2007; Spigno *et al.*, 2007; Yim *et al.*, 2009). Solubility of the active compound is known to be an important contributor to biological activity. Compounds such as terpenoids present in fungi are known to be affected by potency of their functional groups and aqueous solubility (Knobloch *et al.*, 1989). In addition, anthocyanins, starches, tannins, saponins, terpenoids, polypeptides and lectins, are all known to exert bioactivity following component extraction with water alone (Kaul *et al.*, 1985; Cowan, 1999). When performing natural compound extraction using polar organic solvents such as methanol (highly polar); flavonoids, alkaloids, coumarins, fatty acids and triterpenes are commonly extracted (Cowan, 1999). In addition to these considerations, growth conditions and the growing stage of fungi is known to affect biological function, in association with the concentration of metabolites and the product formed (Jernejc, 2004; Ferreira *et al.*, 2009). Chemical modification methods such as Smith degradation, formolysis and carboxymethylation are often employed to improve clinical properties, water solubility and ability to permeate the stomach wall (Wasser, 2002). These are important considerations to take into account when extracting bioactive components.

The success of filamentous fungi for industrial production of biotechnological products is due to the metabolic versatility of this group of microorganisms (El-Enshasy, 2007). Characteristically, fungi with their diverse range of bioactive compounds and biological activity depend on cultivation and extraction technology for efficient production. The main commercial aspect to cultivation of filamentous fungi is the production of organic acids, antioxidants, fatty acids, polysaccharides, enzymes, plant growth regulators, alkaloids, pigments, mycotoxins and antibiotics (El-Enshasy, 2007). Proper knowledge and understanding of appropriate extraction techniques for isolation of biologically active compounds, as well as improved screening methods (high-throughput screening, genomics, and proteomics) will aid future applications of fungi for medicinal purposes and in doing so, advance our knowledge of cultivation processes directed toward biologically active products.

1.6   Application of fungi as a natural functional food source

1.6.1   Functional food

Now more than ever, consumers worldwide are more conscious of the food they eat. The science of nutrition has developed awareness between diet and disease (Saltmarsh *et al.*, 1998). A functional food is recognised as any food component which affects one or more identified function in the body in a positive manner (Thomas *et al.*, 1994). Medical and pharmaceutical sciences mostly aim to control or cure disease (Diplock *et al.*, 1998; Saris *et al.*, 1998), whereas the objective of functional food science is to maintain or improve gut health and possibly create conditions for disease prevention or reduction (Steinmetz *et al.*, 1991). While there are a range of names given to functional foods, the more widely accepted term dietary supplement (DS) was formally defined by the U.S food and drug administration as a supplement added to the diet with the aim of improving health (FoodandDrugAdministration, 1994).

The interest in foods which are fresh, natural with minimal processing are more popular now than ever and continue to gain more attention in the food and health industry. Therefore, there is now more pressure than ever on the food industry to remove chemical preservatives and this has fuelled further research into natural compounds which act as functional dietary antimicrobial and antioxidant agents. In 1989, the European Union (EU) and the European Economic Community (EEC), which govern strict laws on food safety, placed a ban on hormone treated meat and in 2006, an EU wide ban on antibiotics used in animal feed came into effect.

Numerous studies describe filamentous fungi as a functional food source, with reports suggesting that the consumption of extracts as dietary supplements may offer a novel, highly palatable, nutritious food source with added important health benefits. For example, Pleurotus could be considered as a functional food with natural cholesterol-lowering ability (Gunde-Cimerman, 1999). As with any natural product which has a history of curative properties, isolated compounds from mycelia which demonstrate good pharmacological activity cannot all be guaranteed as safe effective new medicines until they pass phase I, II and III clinical trials (Li *et al.*, 2011). A number of preparations of polysaccharides have been developed, mostly in Asia, for use as food additives and are widely used to improve general health and well-being. In Japan, these preparations are used as traditional medicine for general health improvement; prophylactics of malignant tumours, cancer treatment and prevention of

metastases. Current clinical investigations of preparations are now being used in conjunction with various therapies for cancer, HIV, hepatitis B and C, herpes treatment amongst others (Lee *et al.*, 2002; Gu *et al.*, 2006; Boh *et al.*, 2007; Faccin *et al.*, 2007; Rodrigues *et al.*, 2011; Lemieszek *et al.*, 2012). These trials demonstrated better quality of health compared to those who did not supplement their diet with polysaccharide preparations (Bobek *et al.*, 1991a).

1.6.2   Current production status

In general, more and more varieties of fungal species are being isolated and identified. The number of fungi being produced for edible and medicinal purposes is increasing. Total mushroom production worldwide has continued to increase over the last 50 years, from just over 340 thousand kilos in 1965 to 406 million kilos between 2012 and 2013, continuing an upward trend and topping sales of $1.1 billion (Chang, 1999; U.S.D.A., 2013). According to Chang *et al.* (1992), a mushroom is defined as 'a macrofungus with a distinctive fruiting body, which can be hypogeous or epigeous, large enough to be seen with the naked eye and to be picked by hand'. Both Basidiomycetes and some species of Ascomycetes belong to mushrooms, with mushrooms constituting at least 14,000 and perhaps as many as 22,000 known species (Lindequist *et al.*, 2005). Yet only ten percent are fully described as the number of individual mushroom species is estimated to be 140,000. Therefore, the proportion of investigations of known species is extremely low (Lindequist *et al.*, 2005). The main producer of edible mushrooms worldwide is China, producing about 64 % of the world's supply and over 85 % of the world's oyster mushrooms (*Pleurotus* spp.). Between 2001 to 2002, the U.S produced about 7 % of the world's total supply of mushrooms with *A. bisporus* accounting for over 90 % and Lentinula, Pleurotus, Grifola, Flammulina, Hypsizygus, Herium and Morchella the remaining contributors. It is expected that demand for oyster mushroom production will continue to increase, due to consumer awareness of their culinary palatability, relative ease of production and improved technology. Also, cultivation of each species more efficiently will mean price will decline and quality of the product formed will improve.

The data for functional foods such as cruciferous vegetables is more widely recognisable by the general public. Nevertheless, edible fungi are a highly palatable and nutritious source of functional food (Chang, 1996). In addition, they are now widely recognised in disease prevention and treatment, particularly with diseases such

as cancer, acquired immune deficiency syndrome (AIDS) and hypercholesterolemia, suggesting they deserve more serious investigation.

1.6.3   Natural product industry

Early medicines such as aspirin, digitoxin, morphine, quinine and pilocarpine were derived from clinical, chemical and pharmacological studies of natural products from plants, which were used as traditional drugs throughout the centuries (Butler, 2004). Over the years, recognition of the value of traditional medical systems, together with the demand for a modern natural solution to many of the world's health problems, has had a significant influence on the expansion of the natural product industry.

Many clinically useful drugs and numerous pharmaceutical grade products derived from fungi that play a major role in the treatment of human diseases have been obtained through the screening of natural products (Whittaker, 1969; Shu, 1998). Natural products such as antibiotics (penicillin, tetracycline and erythromycin), antiparasitics (avermectin), antimalarials (quinine, artemisinin), lipid control agents (lovastatin and analogs), immunosuppressants (cyclosporine, rapamycins), and anticancer drugs (taxol, doxorubicin) revolutionised medicine and most of the above mentioned drugs were discovered from components of fungi (Li *et al.*, 2011).

The efficiency and ability to identify and extract bioactive molecules from fungi has been extensively researched through recent decades, particularly in Japan, Korea and China and more recently the U.S. Since their discovery, certain medicinal fungi are now consumed in various forms, mostly as a powdered concentrate or extracts in hot water (Mizuno *et al.*, 1995b). Regular consumption of such concentrates is believed to enhance the immune response of the human body, increasing resistance to disease and in some cases, cause regression of the disease state (Jong *et al.*, 1991). β-glucans from fungi have been extensively subjected to safety testing during pre-clinical experiments. When applied to humans in phase 1 clinical trials, β-glucans derived from fungi and other sources showed remarkably few adverse reactions. No anaphylactic reactions and no effect on mutagenicity were found following haemolysis tests, blood coagulation and a range of other regulatory tests carried out. Since the development of synthetic medicinal chemistry, a decline was observed in the number of drugs based on natural products; however, between 2005 and 2007, thirteen natural products were approved in the U.S, with five of them being the first members of new classes (Li *et al.*, 2011).

In Western society, only defined chemical preparations can be prescribed by a medical doctor and are not provided by means of crude extract or nutraceuticals (Chen *et al.*, 1996). Most medicinal uses of fungi originate from historical use and some have not been adequately clinically tested. Most reports are on extracts and not the purified compounds. However, there are a number of justifications as to why natural products will continue to be major sources of new drugs in the future; (i) their incomparable structural diversity, (ii) their relatively small dimensions, and (iii) their 'drug-like' properties i.e. their ability to be absorbed and metabolised (Sticher, 2008).

1.6.4   The future of drug discovery with fungi

Two of the most substantial challenges facing drug discovery from natural sources are; the paradigm for drug discovery in the pharmaceutical industry and the difficulty in discovering natural product drug candidates (Li *et al.*, 2011). Extraction of bioactive compounds usually involves a complex mixture of fractionation steps, with only small quantities of the bioactive substance attainable. There is also the probability of duplication, in that; the result may be a known compound (Li *et al.*, 2011). The development of immunomodulating and anticancer pharmaceutical products from fungal polysaccharides (e.g., Lentinan, Schizophyllan and Krestin), are affected by the fact that high molecular weight polysaccharides (0.1 to 0.5 million Da) are used and excreted without digestion and absorption by humans (dietary fibers). Despite these concepts, there has been renewed interest in natural product research as a method for drug discovery. Although further improvements are required to compete with other methods and keep the pace with the ongoing changes in the drug discovery process (Goutam, 2011). The future for drug development from fungi depends on the development of low molecular weight compounds such as secondary metabolites, which target processes in the body that slow disease progression, or which aid treatment of disease (Zaidman *et al.*, 2005).

The majority of clinically useful drugs and numerous pharmaceutical grade products are derived from fungi (Wasser, 2002; Kirk *et al.*, 2011). However, only ten percent of mushroom species are described and even less have been tested for therapeutic significance (Lindequist *et al.*, 2005). The use of natural products has been the single most successful strategy for the discovery of new medicines and a multitude of studies prove that fungi represent enormous potential for production of powerful pharmaceutical products (Harvey, 2000; Butler, 2004). Substances such as taxol,

cyclosporins and statins are the foundation of modern pharmacotherapy (Goutam, 2011). Most medicinal fungi are viewed as a rapidly developing area of biotechnology for cancer and other treatments (Lindequist *et al.*, 2005; Tang *et al.*, 2007).

1.7 Overview

Evidence suggests that mycelial biomass and culture liquid of Basidiomycetes and Ascomycetes fungi grown using submerged fermentation are a substantial source of bioactive metabolites with a diverse range of biological activities. Biomedical activity is strongly species specific and of the 1.5 million fungal species on Earth, only around 1,800 have been described as having medicinal activity to date, illustrating the potential for new discoveries. Medicinal fungi demonstrate an immense and yet largely untapped source of accessible, natural compounds with a range of biological activities that may promote human and animal well-being (Elisashvili, 2012).

Submerged cultivation of fungi has proven to be a promising alternative for efficient production of mycelial biomass and secondary metabolites. Currently 80 – 85 % of medicinal mushroom products available are derived from the fungal fruiting body, cultivated mainly by SSF, with only 15 % of all products based on extracts from mycelia (Lindequist *et al.*, 2005). An even smaller percentage of products are derived from culture filtrates (Elisashvili, 2012). Previous research has shown that utilisation of appropriate cultivation parameters is vital for optimal production (biomass and secondary metabolites), particularly for the progression of industrial scale cultures for medicinal products.

In the present investigation, crude mycelial biomass extracts from ten filamentous fungi were tested for activity against a range of bacteria. These extracts were tested for antioxidant production and attempts were pursued to identify the active compounds using chromatographic techniques. The effect of carbon source supplementation on biomass production and the effects on fungal cell wall composition were explored. Consequently, the effects of cell wall composition in relation to gut health-promoting bacterial adhesion strategies were investigated.

Fungi are mainly produced for being extremely palatable foods, nonetheless with the additive and synergistic effects of other bioactive compounds, nutraceuticals from fungi may be a powerful instrument in maintaining and promoting health, longevity and life quality (Ferreira *et al.*, 2009). There is enormous potential for the

utilisation of nature as a source of novel bioactive compounds which have

immunomodulatory, antimicrobial and antioxidant properties.

1.8   Project Objectives

The aim of the present study was to examine several filamentous fungi; namely, *Grifola frondosa*, *Lentinula edodes*, *Pleurotus ostreatus* (*OYRM*1), *Monascus purpureus*, *Pleurotus ostreatus*, *Pleurotus ostreatus*, *Pleurotus citrinopileatus*, *Pleurotus eryngii*, *Pleurotus salmoneo-stramineus* and *Trametes versicolor* for their capacity to produce bioactive functioning components in submerged liquid culture. The objective was to firstly enhance the cultivation conditions for the production of fungal mycelial biomass and to examine how media composition and nutrient supplementation influenced biomass generation and cell wall composition.

By providing alternative matrices or dietary fibres to which bacteria may adhere, as opposed to epithelial cell receptors, the host naturally runs less risk of infection. It was believed that the carbohydrate nature and low digestibility of dietary fibre from fungi could make them ideal alternative adhesion matrices. Thereby, the mycelial extract was tested for the presence of bioactive secondary fungal metabolites in the form of carbohydrates of cellular composition with the aim of establishing the potential binding capacity for different species of bacteria. Further antimicrobial examination were directed toward the antibacterial potential of crude mycelial extracts from filamentous fungi against a broad range of gram-positive and gram-negative bacteria, with the aim of establishing qualitative inhibitory activity *via* microbial sensitivity using the agar well diffusion technique.

Fungi are well-known sources of powerful antioxidants; as such, an objective of the present investigation was to assess these particular species of filamentous fungi for their antioxidant production. Additionally in order to examine the effect of alternate extraction processes on isolation of these biological components, a hot water and solvent extract was employed. The final objective of the study was to attempt identification of the active agent(s) responsible for the observed biological activity. Highly sensitive and selective techniques allowed characterisation of the class of bioactive components.

An objective of this research was to demonstrate the potential bioactivities of these species, which could provide opportunities for the application of extracts as nutraceuticals and dietary supplement products for health promotion or as natural food preservatives.

2.   Materials and Methods

2.1   Materials

2.1.1   Chemicals, solvents and other reagents

All chemicals used were of a molecular biology grade or higher where appropriate.

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 4-hydroxybenzoic, α-tocopherol, acetic acid, acetone, acetonitrile, aluminium chloride ($AlCl_3$), ammonium acetate, ammonium dihydrogen phosphate, ammonium sulphate, ampicillin, β-carotene, benzoic acid, boron trifluoride (BF_3), bovine serum albumin (BSA), Bradford reagent, butylated hydroxytoluene (BHT), caffeic acid, calcium carbonate, (+)-catechin, chloramphenicol, chloroform, chlorogenic acid, cinnamic acid, citric acid, corn steep liquor, copper (II), α-cyano-4-hydroxycinnamic acid, D-(+)-fructose, D-(+)-galactose, D-(+)-glucose, D-(+)-maltose, D-(+)-mannose, D-(+)-trehalose, D-(+)-xylose, diphenylboryloxyethylamine, Dragendorff reagent, erythromycin, ethyl acetate, ethylenediaminetetraacetic acid (EDTA), fast blue salt (FBS), ferrous chloride, ferrozine, ferulic acid, flavone, Folin-Ciocalteu reagent, gallic acid, gentamicin, gentisic acid, glycerol, hexane, homogentisic acid, hydrochloric acid, kaempferol, linoleic acid, low viscosity carboxymethylcellulose (CMC), malt extract, methanol, mineral oil, myricetin, naringenin, neocuproine, ρ-coumaric acid, pectin, peptone (from soya), phosphate buffer, ρ-hydroxybiphenyl, polyethyleneglycol-4000, potassium ferric chloride, potassium hydroxide, potassium persulfate, potassium phosphate (monobasic and dibasic), protocatechuic acid, pyrogallol, quercetin, rutin, salicylic acid, saturated sodium carbonate, thiazolyl blue formazan (1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan) (MTT), TLC Alu foils (10 x 20 cm), TLC Alu foils (5 x 10 cm), sodium chloride, sodium hydroxide, sodium hydroxide, sodium phosphate (monobasic and dibasic), sodium sulphate, soluble starch, sterile filter disks (6 mm), sucrose, sulphuric acid, tannic acid, toluene, trichloroacetic acid (TCA), trolox, TWEEN 20, vanillic acid and vanillin were all supplied from Sigma-Aldrich, Arklow, Ireland.

Becton Dickinson (BD) Difco agar, brain heart infusion broth (BHI), deMan, Rogosa and Sharpe broth and agar (MRS), glucose buffered broth (YGB), Luria-Bertani broth (LB), nutrient broth (NB), peptone, potato dextrose agar (PDA), yeast extract, trypticase soy broth (TSB) and yeast malt (YM) broth were supplied by Unitech, Dublin, Ireland.

Buffered listeria broth and listeria selective agar were purchased from Oxoid Limited, Hampshire, United Kingdom (U.K.).

Dimethyl-sulfoxide, ethanol and formic acid and were supplied from Carbon Chemicals Group Limited, Dublin, Ireland.

Grifola frondosa, *Pleurotus ostreatus* (*OYRM*1) and *Lentinus edodes* were maintained by Alltech, Bioscience Centre, Dunboyne, Co. Meath, Ireland.

Campylobacter jejuni 4688, *Debaryomyces hansenii* 3428, *Escherichia coli* 10778, *Monascus purpureus* 1604, *Pasteurella multicida* 10454, *Pleurotus ostreatus* 1833, *Pleurotus citrinopileatus* 5341, *Pleurotus eryngii* 9619, *Pleurotus salmoneo-stramineus* 5338, *Staphylococcus aureus* 1104, *Staphylococcus epidermidis* 1798, *Streptococcus uberis* 700640, *Trametes versicolor* 3086 and *Yersinia enterocolitica* 11504 were supplied by DSMZ GmbH, Braunschweig, Germany.

Pleurotus ostreatus 32783 and *Salmonella enterica* 15480 were supplied by ATCC, American Type Culture Collection, VA, U.S.A.

Kluyveromyces fragilis 2415 was obtained from Alltech Inc., Nicholasville, Kentucky, U.S.A.

Saccharomyces cerevisiae 1026 and *Kluyveromyces lactis* 752 were supplied by NCYC, National Collection of Yeast Cultures, Institute of Food Research, Norwich, U.K.

Citrinobacter freundii 11490, *Enterococcus avium* 702691, *Enterococcus faecalis* 775, *Lactobacillus acidophilus* 702661, *Lactobacillus plantarum* 7220, *Lactococcus lactis*

6681, *Listeria monocytogenes* 13449, *Pediococcus acidilactici* 6990, *Pediococcus pentosaceus* 7837 and *Serratia marcescens* 1377 were supplied by NCIMB, National Collection of Industrial Food and Marine Bacteria, Scotland, U.K.

2.2 Methods

2.2.1 Growth and maintenance of fungi

2.2.1.1 *Storage and solid cultivation*

A mycelial suspension from stored agar slants was aseptically transferred to fresh agar medium and then incubated at 30 °C for 4 – 7 days until confluent growth was achieved. Mycelial plugs (8 mm) which were pierced out of the agar using an 8 mm-diameter cork borer (Sigma-Aldrich, Arklow, Ireland) were then used to subculture fresh agar plates. Agar slants of actively growing cultures were broken up and transferred to liquid culture flasks for submerged fermentation or stored at 4 °C for up to 3 months. For long term storage, frozen stocks of liquid culture (5 %, v/v) were prepared in 70 % glycerol and stored at -70 °C.

2.2.1.2 *Submerged liquid fermentation conditions*

Fungal mycelium was grown by submerged liquid fermentation (SLF) in 500 mL Erlenmeyer flasks under aseptic conditions. Yeast malt (YM) culture media (pH 6.10 ± 0.2) was prepared according to the manufacturer's specifications. Mushroom complete medium (MCM) (pH 6.92 ± 0.2) was prepared with glucose (20 g L^{-1}), KH_2PO_4 (0.46 g L^{-1}), K_2HPO_4 (1 g L^{-1}), $MgSO_4.7H_2O$ (0.5 g L^{-1}), peptone (2 g L^{-1}), and yeast extract (2 g L^{-1}). Malt extract broth with peptone medium (MEP) (pH 6.45 ± 0.2) consisted of malt extract (30 g L^{-1}) and peptone from soya (3 g L^{-1}). Yeast powder-soluble starch agar medium (YpSs) (pH 7.16 ± 0.2) was made up with K_2HPO_4 (1 g L^{-1}), $MgSO_4.7H_2O$ (0.5 g L^{-1}), soluble starch (15 g L^{-1}) and yeast extract (4 g L^{-1}). Flasks were autoclaved for 20 minutes at 105 °C. A starter culture was created by incubating either an actively growing fungal slant or a fungal slant from storage (4 °C) into liquid culture (200 mL) under aseptic conditions. Each species was incubated at 28 °C or the appropriate temperature recommended by the supplier. The fungus was allowed grow in the respective medium at 200 rpm for 4 – 7 days in a shaking incubator (Weiss-Gallenkamp, Loughborough, U.K.) until confluent growth was achieved. Seed cultures were prepared by placing aliquots of actively growing mycelia from starter cultures into fresh sterile medium for downstream experiments.

2.2.1.3 *Separation of mycelial biomass*

Mycelial biomass was separated from the culture medium by draining the culture through muslin cloth and rinsing with distilled water until the water ran clear (approximately 2 volumes), the biomass was collected and freeze-dried using an Alpha 1-4 LD plus freeze drying unit (Sigma, Osterode am Harz, Germany) and then ground to a fine powder (1.0 mm (no. 10) mesh) prior to further processing. The dry biomass was referred to as the crude extract or crude mycelial biomass throughout the investigation.

2.2.2 **Effect of fermentation conditions on biomass production**

2.2.2.1 *Enhancement of medium composition for biomass generation*

A starter culture was prepared in fresh sterile media (Section 2.2.1.2) according to the recommended growth conditions for mycelial production. On the fourth day, the seed culture was distributed into triplicate test flasks and diluted to 1 % (v/v). Test flasks contained 100 mL of MCM, YM and YpSs or MEP media. Fungal mycelium was grown for 4, 8 and 12 days. Triplicate flasks were prepared per analysis. Final pH was recorded after growth of the mycelia at 4, 8 and 12 days of growth. All test flasks were incubated at 25 ºC at 200 rpm, followed by collection and drying as described in Section 2.2.1.3.

2.2.2.2 *Carbohydrate supplementation*

A starter culture was prepared in fresh sterile media (Section 2.2.1.2) according to the optimal growth conditions for mycelial production (Section 2.2.2.1). On the fourth day, the seed culture was distributed into test flasks in triplicate and diluted to 1 % (v/v). Fresh sterile media (100 mL) was set up in 250 mL Erlenmeyer flasks which were supplemented with individual carbon sources at a concentration of 1 %, w/v. The carbon sources (1 %, w/v) tested were fructose, galactose, glucose, maltose, mannose, sucrose, trehalose and xylose. The liquid cultures were grown at 200 rpm, for 7 days following which the biomass was collected and dried as described in Section 2.2.1.3.

2.2.3 Compositional analysis of the fungal cell wall

2.2.3.1 *Acid hydrolysis and sample preparation*

Polysaccharides present in the dry mycelial biomass were hydrolysed to their component sugar monomers by sulphuric acid in a two-stage hydrolysis process. Dry biomass (300 ± 0.1 mg) was accurately weighed in triplicate and placed in a test tube. Following careful mixing, the biomass was hydrolysed with 3 mL of 72 % (v/v) sulphuric acid for 2 hours at 30 ºC, and mixed intermittently throughout the hydrolysis. Each hydrolysate was quantitatively transferred to its own serum bottle and diluted to a 4 % (v/v) sulphuric acid concentration by adding 84 mL deionised water. Each bottle was sealed using a crimper (Wheaton corporation, Millville, New Jersey) and autoclaved for 1 hour at 121 ºC. After completion of the cycle, the samples were allowed to cool at room temperature before removing the seals and stoppers. Aliquots of the hydrolysate (20 mL) were neutralised with calcium carbonate to a pH of between 5 and 6. The neutralised hydrolysate was filtered through a 0.2 µM filter (Chromafil ultra RC). A multi-component series of sugar calibration standards were prepared in 4 % (v/v) sulphuric acid and autoclaved for 1 hour at 121 ºC. Standards containing known concentrations of D-(+) glucose, D-(+) xylose, D-(+) galactose and D-(+) mannose were also prepared in 4 % (v/v) sulphuric acid and autoclaved for 1 hour at 121 ºC in pressure tubes.

2.2.3.2 *Compositional analysis by high performance liquid chromatography*

Separation by HPLC was performed using a Dionex DX500 chromatography system with a Waters 2410 refractor index detector. The mobile phase consisted of filtered, deionised water at a constant flow rate of 0.6 mL min^{-1} for 30 minutes. The column used for separation was a Bio-rad Aminex® HPX-87P column (300 x 7.8 mm), thermostatted at 80 ºC, whilst accompanying column guards were maintained at room temperature. Samples were analysed for glucose, mannose, galactose and xylose. The standards as prepared in Section 2.2.3.1 were filtered and used to quantify carbohydrate concentration.

2.2.3.3 *Chitin analysis*

Chitin content was determined by the method of Pessoni *et al.* (2005). Dry biomass (2.0 mg) was hydrolysed with 1 mL 6 M HCl at 90 ºC for 48 hours. The hydrolysate

was cooled to room temperature before being filtered through a glass microfiber filter (0.25 µm) and the flow-through was evaporated to dryness at 45 ºC under reduced pressure. The concentration of glucosamine hydrochloride in the hydrolysate was based on colorimetric determination as modified by Chen and Johnson (1983).

The dry hydrolysate was diluted with 1 mL deionised water (Nilsson *et al.*, 1998) and added to 0.25 mL 4 % acetylacetone solution (4 % (v/v) acetylacetone in 1.25 M sodium carbonate) and heated for 1 hour at 90 ºC. After cooling in a water bath (22 – 25 ºC), 2 mL of ethanol was added under shaking to dissolve the precipitate. Ehrlich reagent (0.25 mL; 1.6 g of N-N-dimethyl-ρ-aminobenzaldehyde in 60 mL of a 1:1 mixture of ethanol and concentrated HCl) was added and the absorbance was measured at λ_{530nm}. Chitin content, expressed as micrograms of glucosamine hydrochloride per mg dry weight of fungal cell wall was calculated from a standard curve of glucosamine hydrochloride in the range 5, 10, 15, 20 and 30 mg mL^{-1}.

2.2.4   Preparation of fungal extracts

2.2.4.1   *Hot water extraction*

Extraction was performed on a Sineo MD-S10 microwave digester (Elementec Ltd., Summerhill, Co. Meath, Ireland). Dry mycelial biomass (0.5 ± 0.05 g) was accurately weighed into the digester cylinders. After the addition of 10 mL deionised water, the mixture was heated at 100 °C for 20 minutes under reflux, and allowed to cool for 20 minutes. After this time, the filtrate was separated from the mycelial biomass by vacuum filtering using No. 42 (x 2) Whatman filter paper. The process was repeated twice. The combined filtrate was then freeze-dried and the dry weight recorded.

2.2.4.2   *Methanol extraction*

Freeze-dried mycelial biomass (5 g) was accurately weighed into a 100 mL sterilin and shaken overnight (18 – 24 hours) with 50 mL of methanol at room temperature. The extract was then filtered through Whatman paper No. 1 (x 2) by vacuum filtration. The residue was then resuspended in another 50 mL of methanol. The process was repeated twice. The combined methanol filtrate was transferred to a pre-weighted sterilin and the solvent was evaporated by applying a constant flow of air. Residual methanol was removed by vacuum pressure at 50 °C overnight in an oven and the dry weight recorded.

2.2.5   Antimicrobial activity

2.2.5.1   Inoculum preparation and storage

Frozen stocks of bacteria and yeast (5 % (v/v) in 70 % glycerol) were stored at -70 °C with the acquired medium. A single colony was isolated and transferred to approximately 25 mL of broth and grown overnight. OD at λ_{595nm} was adjusted accordingly by spectrophotometer (Shimadzu UV-1601PC). Working plates were prepared by transferring a single colony using spread plate technique to fresh agar and incubating overnight. Streaked plates were stored at 4 °C for up to six months.

2.2.5.2   Bacterial adhesion assay

The ability of fungal cell wall components to adhere to bacteria was tested using a miniaturised adhesion test described by Becker *et al.* (2007). Crude mycelial biomass (1 %, w/v) was suspended in 10 mM phosphate buffered saline (PBS). Samples were sonicated for three 30 second rounds and centrifuged at 2500 rpm for 5 minutes. For coating, 350 µL of the supernatant was added per well, in gamma sterilised 96-well microtitre plates (Starstedt) and plates were incubated overnight (16 – 18 hours) at 4 °C. The plate was washed with 350 µL 10 mM PBS buffer (pH 7.4) and then blocked with 350 µL 1 % (w/v) BSA at 4 °C for 1 hour. Non-coated wells were included as negative controls in each plate. The plate was washed with 350 µL 10 mM PBS twice, followed by the addition of bacteria (300 µL per well of an overnight culture diluted to 0.02 OD at λ_{595nm}). Bacteria were allowed to adhere at room temperature for 30 minutes. Subsequently, the wells were washed three times with 350 µL 10 mM PBS, to remove non-adherent bacteria. Liquid media (300 µL) was then added and the absorbance was read overnight at λ_{595nm} using a Biotek® Synergy HT microtitre plate reader (Biotek Instruments Inc., Vermont, U.S.A.), thermostatted to 37 ºC. The plate was shaken for 3 seconds prior to every reading at 15 minute intervals. All readings were carried out in triplicate. The initial OD was subtracted to obtain sigmoidal growth curves. An inverse relationship between cell densities and growth over time was tested.

   The growth of *E. coli* 10778 and *S. epidermidis* 1798 in microtitration plates coated with fungal cell wall components was measured in comparison to 1 % (w/v) BSA (control) and yeast cell wall (YCW) material (1 %, w/v). The kinetics of bacterial growth according to Groot *et al.* (1996) were examined and statistically

evaluated as described by Becker *et al.* (2007). The OD data obtained were processed by nonlinear regression analysis (Figure 2.1). The following sigmoidal equation was used to describe the kinetics of bacterial growth according to Groot *et al.* (1996);

$$[f_{OD}(t) = A / (1 + (B^c / t^c))]$$

where, *A* represented the optical density having reached the stationary phase, *B* was the time coordinate when half of the maximum yield was been reached, *c* was a constant representing the sharpness of the switching characteristics of the curve and *t* represented time.
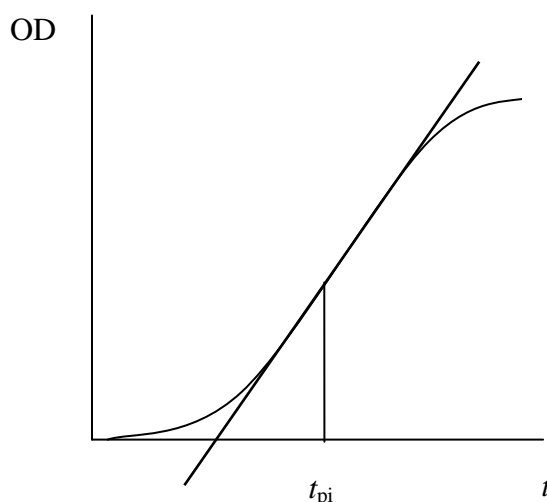
Figure 2.1   Sigmoidal growth curve representing growth parameters

Abbreviations: *t*, time; t_{pi}, time coordinate of point of inflection; OD, optical density.
Figure adapted from Becker *et al.* (2007).

From the growth curves the following parameters were calculated for comparison between samples; (i) the time after incubation at which half of the maximum growth yield was reached (*B*) and the time coordinate of the point of inflection (t_{pi}) (Figure 2.1), in order to report the time-coordinate of a growth detection threshold when optical density reached 0.05 ($t_{OD = 0.05}$).

2.2.5.3   *Agar well diffusion*

A single colony of bacteria was inoculated in nutrient broth (25 mL) and incubated overnight for 24 hours at 37 °C. Soft agar was prepared by dissolving 8 g L^{-1} agar, and autoclaved at 121 °C for 20 minutes. It was cooled to 52 °C in a water bath and 300 µL of washed bacterial culture (0.1 OD at λ_{595nm}) was added to 8.7 mL soft agar. This

mixture was poured evenly onto the surface of previously solidified nutrient agar plates containing 30 mL solidified agar in petri dishes (150 x 15 mm). Nutrient medium was chosen according to strain recommendation in order to support sufficient diffusion of the test microorganism. The plate was rotated to ensure even distribution of the inoculated agar culture. After the plates with overlay had fully solidified, holes were pierced out of the agar using a 10 mm-diameter cork borer (Sigma-Aldrich, Arklow, Ireland). Exactly 150 µl of fungal biomass suspended in DMSO (0.1 g mL^{-1}) was added per well and vortexed until in suspension. The antibiotics ampicillin (1000 µg mL^{-1}), chloramphenicol (100 µg mL^{-1}) and erythromycin (94.6 µg mL^{-1}) were used as positive controls. The concentration of antibiotic used was determined as the concentration which exerted a suitable zone of inhibition to allow adequate fit relevant to the sample wells, per agar plate. Due to the hydrophobicity of the antibiotics in suspension only 60 µL of each were added to avoid flooding the well. An additional 60 µL mineral oil was added to the sample and control wells to prevent evaporation. The diameter of the zone of inhibition (ZOI) including the diameter of the well was recorded (mm) using a calliper; growth inhibition was measured as the diameter of inhibitory zone to the nearest 0.1 mm.

2.2.5.4 *Visibility of zones of inhibition (ZOI)*

When visibility of the ZOI was difficult thiazolyl blue formazan (MTT) spray was used (1 mg mL^{-1}). MTT is reduced from a purple complex by viable bacteria. Dead bacteria do not reduce MTT thereby enabling visibility of the zone where no growth occurred. The plate was sprayed with MTT and incubated (in the dark) at 37 °C for 30 minutes. The diameter of the zone of inhibition (ZOI) was recorded (mm) using a calliper; growth inhibition was measured as the diameter of inhibitory zone to the nearest 0.1 mm.

2.2.5.5 *Antiyeast activity*

The antiyeast activity of fungal extracts was tested *via* agar well diffusion (Section 2.2.5.3). A single colony of yeast was inoculated in YPD broth (30 mL) and incubated overnight for 48 hours at 28 ºC. Soft agar was prepared by dissolving 8 g L^{-1} agar, and autoclaved at 121 °C for 20 minutes. It was cooled to 52 °C in a water bath and 300 µL of washed yeast culture (0.1 OD at λ_{595nm}) was added to 8.7 mL soft agar. This mixture was poured evenly onto the surface of previously solidified PDA agar plates

containing 30 mL solidified agar in petri dishes (150 x 15 mm). The plate was rotated to ensure even distribution of the inoculated agar culture. After the plates with overlay had fully solidified, holes were pierced out of the agar using a 10 mm-diameter cork borer (Sigma-Aldrich, Arklow, Ireland). The fungal sample (150 µl) in DMSO (0.1 g mL^{-1}) was added per well and vortexed until in suspension. Nystatin (50 mg mL^{-1}) was use as the positive control. Plates were incubated overnight (18 – 24 hours) at 28 °C. The diameter of the zone of inhibition (ZOI) was recorded (mm) using a calliper; growth inhibition was measured as the diameter of inhibitory zone to the nearest 0.1 mm.

2.2.6   Antioxidant activity

2.2.6.1   β-carotene bleaching assay

Evaluation of antioxidants was performed using a method developed by Miller (1971). In this assay, antioxidant activity was measured by the ability of volatile organic compounds to inhibit conjugated diene hyperoxide formation from linoleic acid and β-carotene coupled oxidation in an emulsified aqueous system. The presence of antioxidants minimised the extent of β-carotene bleaching by neutralising the radicals.

An aliquot (1 mL) of crystallized β-carotene (0.2 mg mL^{-1} in chloroform) was dispensed into a round-bottom flask containing 25 µL of purified linoleic acid and 200 mg of TWEEN 20 emulsifier. The chloroform was removed using a Heidolph laborota 4000 rotary evaporator and 50 mL of oxygenated, distilled water (shaken at 500 rpm for 30 minutes) was added to the flask and shaken vigorously. Aliquots (200 µL) of each extract were added to 2.5 mL β-carotene/linoleic acid emulsion by pipetting into a series of spectrophotometer tubes with caps. A zero reading was taken at λ_{470nm} immediately after the addition of the emulsion to the antioxidant solution. Samples were capped and subjected to oxidation by placing in an oven for 3 hours at 50 °C. Antioxidant activity (%) was calculated using the following equation:

$$AA \% = 100 \times [1-(A_0 - A_t/ A_{00} - A_{0t})]$$

where, A_0 is the absorbance at the beginning of the incubation with the extract, A_t is the absorbance after 3 hours with the extract. A_{00} is the absorbance at the beginning of the incubation without extract and A_{0t} is the absorbance after 3 hours without the extract.

Samples (1 mg mL^{-1}) were read against a blank containing the emulsion minus β-carotene, i.e. 20 mg linoleic acid with 200 mg TWEEN mixed with 50 mL saturated H$_2$O (30 minutes). BHT and α-tocopherol (1 mg mL^{-1}) were tested as comparative positive controls. Each assay was repeated in triplicate and the average result and standard deviation calculated.

2.2.6.2   *ABTS radical cation decolourisation assay*

The free radical scavenging activity of fungal extracts was evaluated by ABTS radical cation decolorization assay. Radical scavenging activity was determined according to the method first reported by Miller *et al.* (1993) with slight modifications. ABTS was dissolved with water to give a 7 mM concentration stock solution. ABTS radical cation (ABTS$^+$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate 1:1 (v/v) and left in the dark at room temperature for 12 – 16 hours before use. ABTS$^+$ solution was diluted in 95 % ethanol to an absorbance between 0.7 and 0.75 at λ_{734nm}. The photometric assay was conducted with 180 µL of the ABTS reagent and 20 µL of the test samples. The optical density was measured at time zero. The radical scavenging activity of the fungal extracts was calculated using the following equation:

$$E = [(A_o - A_t) / A_o] \text{ x } 100$$

where, A_0 the absorbance of the negative control and A_t is the absorbance of the samples. Radical scavenging activity was expressed as the concentration that scavenged 50 % of the ABTS$^+$ radicals (EC$_{50}$). All determinations were carried out in triplicate. Trolox was used as a positive control.

2.2.6.3   *DPPH radical scavenging ability*

Scavenging effect was determined according to the method of Shimada *et al.* (1992). Each extract was mixed (4:1) with methanolic solution containing DPPH radicals, resulting in a final volume of 0.2 mM DPPH. This method was modified for hot water extracts and performed in a 96-well microtitre plate with 240 µL sample and 60 µL DPPH methanolic solution. The mixture was shaken vigorously and left to stand for 30 minutes in the dark, with absorbance then measured at λ_{517nm}. A blank methanolic solution containing DPPH radicals without test compound was used as a negative

control. BHT and α-tocopherol were used as comparative positive controls. The scavenging ability (%) was calculated using the following equation:

$$[(AbsControl - AbsSample) / AbsControl] \times 100$$

Radical scavenging activity was expressed as the concentration (mg mL^{-1}) that scavenged 50 % of the DPPH radicals (EC$_{50}$) and was obtained by interpolation from linear regression analysis of each extract (0.1 – 50 mg mL^{-1}).

2.2.6.4   *Determination of antioxidant activity using a reducing power assay*

Reducing power was determined according to the method of Oyaizu (1986), with slight modifications. Fungal extracts (200 µL) were mixed with 0.5 mL of 0.2 M phosphate buffer (pH 6.6) and 0.5 mL of 1 % (w/v) potassium ferricyanide. The reaction mixture was incubated at 50 °C for 20 minutes. After the addition of 0.5 mL of 10 % (w/v) trichloroacetic (TCA), the mixture was centrifuged at 1000 rpm for 10 minutes using a Heraeus multifuge (DJB labcare Ltd., Buckinghamshire, U.K.). An aliquot of supernatant (0.5 mL) was mixed with 0.5 mL of deionised water and 0.1 mL of 0.1 % (w/v) ferric chloride. Absorbance was measured at λ_{700nm} against a blank. Higher absorbance indicated greater reducing power. Tests were carried out in triplicate and the results were expressed as mean values ± standard deviations. Reducing power was expressed as the concentration (mg mL^{-1}) that reducing power reached 0.5 (EC$_{50}$). BHT and α-tocopherol were used as positive controls; deionised water was used a negative control.

2.2.6.5   *Cupric ion reducing antioxidant capacity (CUPRAC)*

Total antioxidant potential using Cu (II) as an oxidant was assessed using the CUPRAC assay (Apak *et al.*, 2008). To a test tube, 1.5 mL of the test mixture containing 10 mM of copper (II), 7.55 mM neocuproine and 1 M ammonium acetate buffer (pH 7.0) solutions were added. Following this, 0.5 mL of diluted fungal extracts at various concentrations was added to the reaction tubes to achieve a final volume of 2 mL. The tubes were incubated for 30 minutes at room temperature before the absorbance at λ_{450nm} was recorded against a blank. BHT and α-tocopherol were used as positive controls.

2.2.6.6   *Metal ion chelating ability*

The chelating effect on ferrous ions was determined according to the method of Dinis *et al.* (1994). Each extract (0.5 mL) was mixed with 1.85 mL methanol and 0.05 mL of 2 mM ferrous chloride. The reaction was initiated by the addition of 0.1 mL of 5 mM ferrozine. After 10 minutes the absorbance of the mixture was read at λ_{562nm} against a blank. A lower absorbance indicated a higher chelating power. Ethylenediaminetetraacetic acid (EDTA) was used as a standard compound. The effective concentration at which 50 % of the ferrous ions were chelated was calculated and obtained by interpolation from linear regression analysis of each extract and EDTA ($0.1 - 50$ mg mL^{-1}). The chelating activity was expressed as EC$_{50}$, the concentration (mg mL^{-1}) that chelated 50 % of Fe^{2+} ions.

2.2.7   **Determination of antioxidant compounds**

2.2.7.1   *Total phenolic content*

Total phenolic content of the fungal extracts was estimated using the Folin-Ciocalteu reagent method according to Singleton and Rossi with minor modification (Singleton 1985). Sample extracts (250 µL) were mixed with 250 µL of 10 % (v/v) Folin-Ciocalteu reagent, followed with the addition of 500 µL saturated sodium carbonate (10 %, w/v aqueous solution) after 2 minutes of incubation at room temperature. The mixture was placed in the dark for 1 hour. Absorbance was then measured at λ_{750nm}. The concentration of total phenols was calculated based on a calibration curve using gallic acid ranging from 10 to 100 µg mL^{-1}. The phenol content was expressed as gallic acid equivalent (GAE), which reflects the phenol content as the amount of gallic acid units in 1 gram of extract (mg GAE g^{-1}).

2.2.7.2   *Total flavonoid content*

Flavonoid content was measured according to the AlCl$_3$ method (Quettier-Deleu, Gressier *et al.,* 2000). Each extract (0.5 mL) at a concentration of 10 mg mL^{-1} was mixed with 1.0 mL of 2 (w/v) % AlCl$_3$.6H$_2$O methanolic solution and the absorbance was measured 10 minutes later at λ_{430nm}. Quantification of flavonoids was calculated on the basis on a calibration curve of quercetin ($0.01 - 50$ µg mL^{-1}) and is expressed as quercetin equivalent (QE), which reflects the flavonoid content as the amount of quercetin units in 1 gram of extract (mg QE g^{-1}).

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2.2.7.3 *Condensed tannin determination with vanillin-HCL*

Total condensed tannin content was determined according to the method of Broadhurst and Jones (1978) and Yim *et al.* (2009). Methanol and aqueous fungal extracts were dissolved in methanol and water, respectively. An aliquot of each extract (50 µL) was mixed with 1.5 mL of 4 % (w/v) vanillin in methanol, followed by the addition of 750 µL concentrated HCL. After mixing well, the mixture was allowed to stand at room temperature for 20 minutes in darkness. The blank was 4 % (v/v) concentrated HCL in methanol. The absorbance was read against a corresponding blank at λ_{500nm}. (+)-Catechin was used to prepare the standard curve (50 – 1000 µg mL^{-1}) and the results were expressed as catechin equivalent (CE), which reflects the tannin content as the amount of catechin in 1 gram of extract (mg CE g^{-1}).

2.2.8 Phytochemistry

2.2.8.1 *Thin layer chromatography*

Thin layer chromatography (TLC) was performed on silica gel, 60 F_{254}, Alu backed plates, 2 mm (10 cm x 20 cm) (Sigma-Aldrich Chemical Company, Arklow, Ireland). They were carefully scored using a scalpel and cut to the appropriate size. Volumes, in µL of the extract to be examined, were spotted onto the TLC plate approximately 2 cm from the bottom. The spots were dried under a warm current of air and the plate was developed in a solvent saturated glass TLC tank using an appropriate solvent system. Optimisation of the solvent system for different fractions was carried out. When the mobile phase reached approximately 1 cm from the top of the plate, the plate was removed from the TLC tank and allowed to dry. Plates were examined under visible light, UV-λ_{254nm} and UV-λ_{365nm}, with or without gentle heating. Compounds with native fluorescence were viewed as bright zones on a dark background under UV light. Absorbing compounds (fluorescence quenching) were detected as dark violet spots on a bright green background. The retention factor (R_f) was recorded for each compound in each methanol extract following TLC development, to allow direct comparison between fungal species. The R_f x 100 values (%) were calculated using the following equation:

$$[R_f\ (b)\ /\ R_f\ (a)\ (mm)]\ x\ 100$$

where, the R_f value for each isolate was the distance it had travelled from the origin (b) divided by the distance travelled by the solvent mobile phase (a).

2.2.8.2   *Identification of constituents*

For each type of detection reagent, two identical TLC plates were prepared alongside each other. The first plate was used as a reference whilst the second plate was subjected to spray reagents and detection. Following development of the plates, the R_f values (Section 2.2.8.1) were recorded and used for comparison.

Fast blue salt (FBS) reagent was used for the detection of flavonoids and phenolic compounds. A TLC plate was sprayed with 6 – 8 mL of a 0.5 % (w/v) solution of FBS in water, dried and then observed under visible light for the presence of red to brown zones, with or without warming.

Natural Products/Polyethylene Glycol (NP/PEG) was used for the detection of coumarins, arbitin drugs, bitter principles and flavonoids. Ten mL of 1 % (w/v) methanolic diphenylboryloxyethylamine followed by 8 mL of PEG (5 % (w/v) ethanolic polyethyleneglycol-4000) was sprayed onto the TLC plate and observed under UV light at λ_{365nm}. AlCl$_3$ solution (1 %, v/v) in ethanol was used to confirm the presence of flavonoids, which was indicated by yellow fluorescence. The appearance of blue spots revealed phenolic acids.

Freshly prepared potassium hydroxide (KOH) reagent was used for the detection of anthraquinones (red), anthrones (yellow) and coumarins (blue). The TLC plate was sprayed with 10 mL of a 10 % (w/v) KOH ethanolic solution, dried and then viewed under UV-λ_{365nm} or visible light, with or without warming.

For the detection of lipids, a plate was dipped in the 10 % (v/v) sulphuric acid solution, and placed in an oven (105 °C) for 30 minutes. This resulted in the charring of compounds which contained mainly lipids and allowed visualisation of compounds under normal light. However, this process is destructive to the constituents on the plate and cannot be used for further processing.

The presence of alkaloids was examined by spraying with Dragendorff reagent. The appearance of yellow to brown spots in visible light, immediately after spraying is indicative of the presence of alkaloids. Furthermore, the plate was sprayed with 0.01 % (v/v) fluorescein and dried in warm air. By this means, the presence of yellow zones is indicative of the presence of lipids.

Anisaldehyde-sulphuric acid (AS) reagent was used for the nonspecific detection of terpenes. The reagent consisted of anisaldehyde (0.5 mL) mixed with 10 mL glacial acetic acid, followed by methanol (85 mL) and concentrated sulphuric acid

(5 mL), in that order. The plate was sprayed with approximately 10 mL, warmed at 100 ℃ for 5 – 10 minutes and evaluated under visible light.

Iodine vapour was used for the detection of compounds with conjugated double bonds. A TLC plate was placed into the TLC tank, which had been previously saturated with iodine vapour by the addition of iodine crystals. The plate was removed and evaluated in visible light.

2.2.8.3   Bioautography

A single colony of bacteria was isolated and transferred to approximately 25 mL of broth and grown overnight. OD at λ_{595nm} was adjusted accordingly by spectrophotometer (Shimadzu UV-1601PC). Developed plates (Section 2.2.8.1) were cut to size and placed inside sterile petri dishes (150 x 15 mm). Agar was inoculated with an aliquot of the overnight bacterial culture, mixed thoroughly, and then poured over the TLC plate in the petri dish. The agar was spread uniformly over the plate to give an agar overlay thickness of approximately 1 mm. The plate was rotated slowly to remove air bubbles from the surface. After 30 minutes, the plates were inverted and incubated overnight at 37 °C. The plates were sprayed with MTT and reincubated for 30 minutes at 37 °C. The plates were then observed after approximately 7 days for spots that exhibited clear zones of microbial growth inhibition against a purple background of viable bacteria, in accordance with Section 2.2.5.4.

2.2.8.4   Antioxidant screening of compounds using bioautography

TLC was performed to screen for radical scavengers and antioxidants according to a method described by Cuendet *et al.* (1997). Developed plates (Section 2.2.8.1) were air dried for 30 seconds. The plate was dipped in DPPH solution (0.2 mg mL^{-1} in MeOH). The plate was allowed dry in darkness for 90 seconds at room temperature then for up to 1 hour at 50 ℃. Free radical scavenging zones were identified immediately as yellow areas against a violet background.

2.2.8.5   Extraction of compounds from TLC plates

TLC plates were developed as described in Section 2.2.8.1. Following visualisation of separated compounds; representative isolates were removed (according to their relative R_f value). Isolates were subjected to two successive overnight elution processes, in an attempt to purify and concentrate the constituents. Compounds were firstly extracted

overnight (16 – 24 hours) with diethyl ether (1 mL) at 500 rpm. Each isolate was then centrifuged at 14,000 rpm for 10 minutes. The supernatants were collected individually and placed in a new eppendorf and dried at 45 ºC for approximately 2 hours using an eppendorf concentrator plus/Vacufuge® plus. The pellet was resuspended in methanol and the process was repeated. The fractionated components of the extracted isolates were redissolved in methanol and analysed by LC/MS (Section 2.2.9.3).

2.2.9   Component extraction and identification

2.2.9.1   Solid phase extraction

Solid phase extraction was performed according to the method of Dopico-Garcia *et al.* (2007) with slight modifications. Freeze-dried mycelial biomass (0.5 g) was accurately weighed and mixed with 5 % methanol, pH 2. The mixture (25 mL) was placed in an ultrasonic bath (Ultrawave Ltd., Cardiff, U.K.) at 40 ºC for 20 minutes. The extracts were filtered and collected. This process was repeated 5 times per extract. The solution was passed through an Agilent Bond Elut C18 column (Apex Scientific, Co. Kildare, Ireland) previously conditioned with 30 mL of methanol and 70 mL acid water (pH 2 with HCL). Methanol (50 mL) was used as the elution solvent to recover phenolic compounds. The extracts were concentrated to dryness under reduced pressure (45 ºC) using an eppendorf concentrator plus/Vacufuge® plus and redissolved in 30 % methanol (1 mL) of which 10 µL were analysed by UPLC-DAD.

2.2.9.2   UPLC-DAD analysis of phenolic compounds

The phenolic extracts were analysed using an Agilent 1290 Infinity Ultra Pure Liquid Chromatography (UPLC) system, coupled a to photodiode array detector (UPLC-DAD). Separation was achieved on an Agilent Poroshell 120 EC-C18 column (3.0 x 50.0 mm, 2.7 micron) thermostatted at 28 ºC. The sample volume injection was 10 µL. A solvent system consisting of 0.2 % formic acid (solvent A) and methanol (solvent B) was used with the following gradients; 0 – 1 minute isocratic at 100 % A, following by a linear gradient for the subsequent conditions:

Table 2.1   UPLC-DAD solvent gradient levels

Time (min)	A (%)	B (%)
1.0 - 1.5	90	10
1.5 - 2.0	85	15
2.0 - 3.0	84	16
3.0 - 4.0	83	17
4.0 - 5.0	80	20
5.0 - 6.5	75	25
6.5 - 7.0	73	27
7.0 - 8.0	70	30
8.0 - 10.0	65	35
10.0 - 12.0	60	40
12.0 - 13.0	55	45
13.0 - 13.5	52	48
13.5 - 15.0	50	50
15.0 - 17.5	45	55
17.5 - 19.0	40	60
19.0 - 21.0	30	70
21.0 - 22.0	20	80
22.0 - 23.0	0	100
23.0 - 25.0	100	0

A linear gradient was applied at 23 – 25 minutes from 0 – 100 % A (Table 2.1). At 25.5 min the sample loop was rinsed and the gradient repeated. The flow rate was kept constant at 1 mL min^{-1}. Phenolic compounds were identified by comparison of retention times of standard materials and quantification was achieved by the absorbance recorded in the chromatograms relative to eighteen external standards, including; 4-hydroxybenzoic acid, caffeic acid, catechin, cyanocinnamic acid, ferulic acid, flavone, homogentisic acid, kaempferol, myricetin, naringenin, *ρ*-coumaric acid, *ρ*-hydroxybiphenyl acid, protocatechuic acid, pyrogallol, rutin, salicylic acid, vanillin and vanillin. Detection was performed with a multiple wavelenght UV detector, set at the following wavelengths; λ_{254nm}, λ_{265nm}, λ_{280nm} and λ_{320nm}. Chromatogram quantification was acheived using a calibration plot of external standards.

2.2.9.3   *Lipid analysis using liquid chromatography mass spectrometry (LC/MS)*

Fatty acid composition analysis was performed on an Agilent 6520 accurate-mass quadrupole time-of-flight (Q-ToF) LC/MS (Agilent Technologies Ireland Ltd., Cork, Ireland). The mobile phase used was 2 mM ammonium acetate (solvent A) and acetonitrile with 2 mM ammonium acetate (solvent B), with an injection volume of 10

µL. Separation was achieved on an Agilent Eclipse Plus C18 column (2.1 x 150 mm, 3.5 micron), thermostatted at room temperature. A slight stepwise gradient from 60 – 100 % solvent B was applied at a flow rate of 0.4 mL min^{-1} for 15 minutes. Electrospray mass spectra data were recorded on a negative ionisation mode for a mass range *m/z* 100 to *m/z* 1000. Identification was performed by comparing retention times and their masses with those of 38 authentic standards (Table 2.2).

Table 2.2   List of fatty acid standards

Fatty acid common name	Chemical formula	Carbon atom: Double bond (C:D)	Exact mass of neutral ion	Exact mass of negative ion [M-H]$^-$
Caprylic	$C_8H_{16}O_2$	08:0	144.115	143.108
Pelargonic	$C_9H_{18}O_2$	09:0	158.131	157.123
Capric	$C_{10}H_{20}O_2$	10:0	172.146	171.139
Undecylic	$C_{11}H_{22}O_2$	11:0	186.162	185.155
Lauric	$C_{12}H_{24}O_2$	12:0	200.178	199.170
Tridecylic	$C_{13}H_{26}O_2$	13:0	214.193	213.186
Myristic	$C_{14}H_{28}O_2$	14:0	228.209	227.202
Pentadecylic	$C_{15}H_{30}O_2$	15:0	242.225	241.217
Palmitic	$C_{16}H_{32}O_2$	16:0	256.240	255.233
Palmitoleic	$C_{16}H_{30}O_2$	16:1	254.225	253.217
Palmitelaidic	$C_{16}H_{30}O_2$	16:1	254.225	253.217
Heptadecanoic	$C_{17}H_{34}O_2$	17:0	270.256	269.249
Stearic	$C_{18}H_{36}O_2$	18:0	284.272	283.264
Oleic	$C_{18}H_{34}O_2$	18:1	282.256	281.249
Elaidic	$C_{18}H_{34}O_2$	18:1	282.256	281.249
Linoleic	$C_{18}H_{32}O_2$	18:2	280.241	279.233
Linolenic (α + γ)	$C_{18}H_{30}O_2$	18:3	278.225	277.217
Stearidonic	$C_{18}H_{28}O_2$	18:4	276.209	275.202
Nonadecylic	$C_{19}H_{38}O_2$	19:0	298.287	297.280
Arachidic	$C_{20}H_{40}O_2$	20:0	312.303	311.296
Gadoleic	$C_{20}H_{38}O_2$	20:1	310.287	309.280
Gondoic	$C_{20}H_{38}O_2$	20:1	310.287	309.280
Dihomolinoleic	$C_{20}H_{36}O_2$	20:2	308.272	307.264
Dihomolinolenic	$C_{20}H_{34}O_2$	20:3	306.256	305.249
Mead acid	$C_{20}H_{34}O_2$	20:3	306.256	305.249
Arachidonic	$C_{20}H_{32}O_2$	20:4	304.240	303.233
Eicosatetraenoic	$C_{20}H_{32}O_2$	20:4	304.240	303.233
EPA	$C_{20}H_{30}O_2$	20:5	302.247	301.217
Heneicosylic	$C_{21}H_{42}O_2$	21:0	326.319	325.311
Behenic	$C_{22}H_{44}O_2$	22:0	340.334	339.327
Eruic	$C_{22}H_{42}O_2$	22:1	338.319	337.311
Docosadienoic	$C_{22}H_{40}O_2$	22:2	336.303	335.296
Eranthic	$C_{22}H_{38}O_2$	22:3	334.287	333.280
Ardenic	$C_{22}H_{36}O_2$	22:4	332.272	331.264
DPA	$C_{22}H_{34}O_2$	22:5	330.256	329.249
DHA	$C_{22}H_{32}O_2$	22:6	328.240	327.233
Tricosylic	$C_{23}H_{46}O_2$	23:0	354.350	353.343
Lignoceric	$C_{24}H_{48}O_2$	24:0	368.365	367.358

2.2.9.4   *Lipid extraction prior to gas chromatography mass spectrometry (GC/MS)*

Dry biomass extracts (100 mg) and 25 mg of the internal standard (methyl behenate (C 22:0)) were accurately weighed into screw top test tubes (20 x 150 mm). Glass beads, toluene (1 mL) and NaOH (5 mL) were added to each tube. These were purged with nitrogen, capped, vortexed and heated at 100 – 110 ℃ using a heat block for 10 minutes. The tubes were allowed to cool at room temperature. Methanol (5 mL) was added to each tube and purged with nitrogen, capped, vortexed and heated at 100 – 110 ℃ using a heat block for 45 minutes. The tubes were allowed to cool at room temperature followed by the addition of hexane (5 mL) to each tube, mixing well for 5 – 10 seconds. Saturated NaCl (4 mL) was added to each tube, which was then vortexed until the solution was clearly mixed. A sample of the hexane layer was transferred to gas chromatography (GC) vials and analysed.

2.2.9.5   *Fatty acid identification and quantification from different extracts using gas chromatography mass spectrometry (GC/MS)*

Lipid mixtures (Section 2.2.9.4) were analysed on an Agilent 7890A gas chromatography system with a flame ionisation detector. The instrument was equipped with a Restek Famewax column (30 m x 0.25 mm ID X 0.25 µM). Helium was the carrier gas; column flow rate and total flow rate were 2 and 155 mL min^{-1}, respectively. Samples (1 µL) were injected into the GC autosampler at a split ratio of 75:1 (split flow 150 mL min^{-1}). Injector and detector temperature were 220 ℃ and 225 ℃, respectively. The following temperature program was used; 190 ℃ (0.25 min isothermal) to 225 ℃ (13 ℃ min^{-1}, 5 minutes isothermal) continuing to 228 ℃ (6 ℃ min^{-1}, 7 minutes isothermal) followed by 190 ℃ isothermal for 3 minutes. Fatty acids were identified by comparison of retention times of standard materials and quantification was achieved by the absorbance recorded in the chromatograms relative to nine external standards, including; capric (C10:0), lauric (C12:0), pentadecenoic (C15:0), palmitic (C16:0), palmitoleic (C16:1), heptadecanoic (C17:0), stearic (C18:0), oleic (C18:1) and linoleic (C18:2). Quantitative analyses were performed by the corrected area normalisation method with methyl behenate (C22:0) as the internal standard.

2.2.10 Statistical Analysis

Statistical analyses of results were performed using Minitab statistical software package version 16 (Coventry U.K.). One-way analysis of variance (ANOVA) and Tukey's multiple comparisons were carried out to test any significant differences among means, where the confidence level was set at 95 %. One-way analysis of variance (ANOVA) and Dunnett's comparisons were carried out to test any significant differences between each test mean and a control mean, where the confidence level was set at 95 %. Significant levels were defined using $p \leq 0.05$. For the correlations, the Minitab statistical software package version 16 (Coventry U.K.) was used to determine the Pearson's correlation coefficient (r) between the content of total phenols, flavonoids and condensed tannin, to the activity (%) of each antioxidant attribute. EC_{50} determinations were calculated using GraphPad PRISM, version 6.03 for Windows (GraphPad Software, California, U.S.A). The data was fitted by nonlinear regression to the variable slope dose-response curves, with a confidence interval of 95 %. Y was the percent activity and X was the corresponding Log [extract concentration]. The interpolated EC_{50} parameter is the absolute EC_{50}, and it is defined as the effective concentration giving an inhibition of 50 %.

2.2.11 Safety Information

For handling of hazardous or potentially dangerous solvents, the material safety data sheet (MSDS) was referred to, as provided by the supplier. It is advised to avoid contact with DMSO solutions containing toxic materials or materials with unknown toxicological properties. Dimethyl sulfoxide is readily absorbed through skin and may carry such materials into the body (Sigma-Aldrich, 2014).

3.   **Biomass generation using submerged liquid culture**

Evolutionary divergences have meant the vast majority of fungi have different growth requirements. As a consequence, optimisation of culture conditions is an essential process to obtain maximum biomass yield and secondary metabolite production. Submerged liquid fermentation (SLF) is the preferred alternative to the time-consuming and labour intensive solid state cultivation process. In addition, liquid cultivation is viewed as industrially efficient, as polysaccharides are not only produced by fungal mycelia but due to the nature of the cultivation they are also found in the culture liquid. There has been little research on comparison of mycelial production among different species of medicinal fungi for bioprocessing (Duan *et al.*, 2013). The effects of synthetic media formulation on submerged mycelial growth and final pH of various species of filamentous fungi in liquid shake flask cultures were examined in the present study. The objective of this aspect of research was to generate a large quantity of mycelial biomass from enhanced conditions for maximal growth of ten selected filamentous fungi for further downstream processing.

Nine Basidiomycetes from the genera; Grifola, Pleurotus, Lentinula and Trametes, in addition to one Ascomycete from the genera Monascus were cultivated in submerged flask cultures. The different species of filamentous fungi used for various analyses throughout this study included; *Grifola frondosa*, *Lentinula edodes*, *Pleurotus ostreatus* (*OYRM*1), *Monascus purpureus* 1604, *Pleurotus ostreatus* 1833, *Pleurotus ostreatus* 32783, *Pleurotus citrinopileatus* 5341, *Pleurotus eryngii* 9619, *Pleurotus salmoneo-stramineus* 5338 and *Trametes versicolor* 3086. These species were chosen based on their curative potential. The Basidiomycetes chosen in the present study have long been recognised as edible or therapeutic fungi and they are generally regarded as safe (GRAS) in accordance with the Food and Drug Administration (FDA). Currently, due to citrinin production by *Monascus* spp. in SLF (Section 1.2.6.2), only secondary products such as pigments from this species are GRAS to date. Pleurotus is one of the five main edible fungi cultivated globally. This higher fungi is known to have good developmental rates and is easily adaptable to different growth conditions in SLF (Hadar *et al.*, 1986), due largely to the production of a wide spectrum of enzymes able to digest a large number of materials (Das *et al.*, 2007). There is a large amount of information on the growth, cultivation and therapeutic activity of the Pleurotus genera.

Notably, evidence suggests closely related species within the same genus could necessitate different growth conditions and nutrient sources, hence; five different species from the Pleurotus genera were included in the present study. Each of the selected species was investigated based on their lack of information on bioactive compound production and the potential of each strain from a natural product perspective. *L. edodes*, *G. frondosa* and *T. versicolor* are well known for their medical significance however there is little or no information available on aspects of their antimicrobial potential, such as in terms of anti-adhesion capacity (Section 1.3.2.2). In addition, there are few reports of bioactivity in conjunction with antioxidant production for certain fungal species when grown in submerged culture.

Environmental parameters, including medium composition (particularly the nature and concentration of the carbon source) and morphological adaptations of mycelial formation are recognised as affecting not only the production of biomass, but also the generation of biologically active metabolites. Such metabolites include intracellular carbohydrates (polysaccharides; glycogen, pullulan and β-glucans) and disaccharides (trehalose, as well as extracellular polysaccharides), which are of great industrial and economical significance (Diamantopoulou *et al.*, 2014). This section reports the growth requirements for several filamentous fungi grown in shake flask cultures and the effect that growth parameters have on the cell wall composition. Several nutritional factors necessary for development were investigated to determine optimal conditions for growth of the mycelium. Firstly, a variety of media were investigated for their effect on mycelial biomass generation. Following this an investigation of the effect of various carbohydrate sources was carried out to examine which condition promoted mycelial biomass most efficiently. Finally, an assessment was performed on the effect of changes in nutrient composition of the liquid cultivation medium on the constituents of the fungal cell wall, with any subsequent effect toward antibacterial anti-adhesion strategies further examined in Section 4.1.

3.1   Culture optimisation for improved biomass production

3.1.1   Effect of growth media on fungal biomass and pH over time

The aim of this research was to understand the growth preferences of the different fungal species on defined medium, with the goal of obtaining large amounts of biomass for downstream analysis of biological activity. The optimal media was subsequently used to investigate other parameters, such as the effect of nutrient source on biomass production and fungal cell wall constituents. Three liquid cultivation substrates were assessed per species, including; mushroom complete medium (MCM), yeast malt (YM) medium, and either malt peptone extract (MEP) or yeast-starch agar medium (YpSs). The cultivation media chosen for this study were either previously used for the cultivation of higher fungi (Kim *et al.*, 2002), or were recommended by the supplier DSMZ (Germany). YpSs media was recommended for the growth of *Monascus purpureus* 1604. Therefore, *M. purpureus* was investigated with MCM, YM and YpSs exclusively. Table 3.1 displays the effect of different media on biomass production and pH of the selected species.

Relevant literature has shown that alterations in pH of the culture media during submerged growth of various fungi is a consequence of the utilisation and exhaustion of compounds in the medium (Griffin, 1994). Accordingly, the pH of the culture media during fungal development is important from an industrial perspective, particularly depending on the application of the product formed following cultivation. Many bioprocesses are based on the ability of several fungi to convert sugars to acids. Citric and gluconic acid are the largest organic acids produced commercially by fungal bioprocesses through fermentation of glucose and sucrose by *Aspergillus niger*. During cultivation, the conversion of sugar to acids naturally decreases the pH of the culture medium. Fungi may not have evolved tight regulation on acid production due to the availability of excess sugars in their environment for metabolism; thereby when grown on synthetic media with available carbohydrates, fungi proceed to convert sugar to acid which subsequently leads to the cessation of growth (Magnuson *et al.*, 2004). This generally happens when the pH of the media reaches a low acidic level (~pH 3.8). This process may also be a defence mechanism, as the acidification process will prevent the growth of pathogenic microorganisms, considering that almost all bacteria species and many fungi will not grow below a pH of 3 (Woods *et al.*, 1985).

Table 3.1   Effect of growth media on fungal biomass and pH over time

Fungi	Medium	4 d DCW (g)	4 d Final pH[1]	8 d DCW (g)	8 d Final pH	12 d DCW (g)	12 d Final pH
Grifola frondosa	MCM[a]	0.02 ± 0.02	5.44 ± 0.55	0.03 ± 0.01	4.52 ± 0.77	0.01 ± 0.01	5.37 ± 0.42
	MEP[b]	0.05 ± 0.01	4.80 ± 0.13	0.07 ± 0.03	3.98 ± 0.51	0.07 ± 0.03	3.71 ± 0.28
	YM[c]	0.02 ± 0.01	4.74 ± 0.35	0.06 ± 0.03	3.88 ± 0.73	0.04 ± 0.03	4.15 ± 0.48
Lentinula edodes	MCM	0.01 ± 0.01	6.38 ± 0.06	0.03 ± 0.02	4.02 ± 0.53	**0.04 ± 0.01**[*]	4.17 ± 0.54
	MEP	0.05 ± 0.02	4.84 ± 0.30	0.09 ± 0.02	3.30 ± 0.17	**0.11 ± 0.00**[*]	3.25 ± 0.01
	YM	0.03 ± 0.02	4.81 ± 0.32	**0.09 ± 0.01**[*]	3.49 ± 0.09	**0.08 ± 0.01**[*]	3.47 ± 0.08
Monascus purpureus	MCM	0.75 ± 0.07	6.32 ± 0.40	0.53 ± 0.05	8.37 ± 0.06	0.42 ± 0.10	8.28 ± 0.14
	YpSs[d]	0.35 ± 0.02	8.76 ± 0.06	0.15 ± 0.03	8.62 ± 0.03	0.17 ± 0.00	8.48 ± 0.08
	YM	0.57 ± 0.03	8.41 ± 0.06	0.33 ± 0.02	8.45 ± 0.14	0.36 ± 0.09	8.20 ± 0.08
*OYRM*1	MCM	0.06 ± 0.02	7.10 ± 0.02	**0.40 ± 0.02**[*]	7.94 ± 0.04	0.31 ± 0.06	7.73 ± 0.09
	MEP	0.17 ± 0.19	7.76 ± 0.06	0.13 ± 0.02	8.27 ± 0.18	0.17 ± 0.02	8.25 ± 0.02
	YM	0.10 ± 0.11	6.34 ± 0.06	0.15 ± 0.13	6.64 ± 0.27	0.18 ± 0.05	7.55 ± 0.06
Pleurotus ostreatus 1833	MCM	0.09 ± 0.03	6.67 ± 0.02	**0.79 ± 0.11**[*]	5.66 ± 0.12	**0.70 ± 0.15**[*]	6.22 ± 0.37
	MEP	0.13 ± 0.05	7.37 ± 0.21	**0.43 ± 0.09**[*]	7.89 ± 0.16	**0.33 ± 0.03**[*]	7.40 ± 0.01
	YM	0.03 ± 0.02	6.07 ± 0.12	**0.10 ± 0.01**[*]	6.25 ± 0.04	**0.40 ± 0.07**[*]	7.51 ± 0.18
Pleurotus ostreatus 32783	MCM	0.09 ± 0.05	6.46 ± 0.07	**0.41 ± 0.05**[*]	7.40 ± 0.08	**0.62 ± 0.11**[*]	5.71 ± 0.18
	MEP	0.06 ± 0.01	7.32 ± 0.30	**0.26 ± 0.04**[*]	5.69 ± 0.06	**0.31 ± 0.01**[*]	5.47 ± 0.13
	YM	0.09 ± 0.06	6.18 ± 0.16	**0.31 ± 0.05**[*]	7.41 ± 0.10	**0.35 ± 0.02**[*]	7.49 ± 0.14
Pleurotus citrinopileatus	MCM	0.98 ± 0.05	5.48 ± 0.09	0.91 ± 0.07	4.50 ± 1.00	0.77 ± 0.09	6.73 ± 0.87
	MEP	0.48 ± 0.06	5.03 ± 0.33	**0.71 ± 0.06**[*]	6.33 ± 0.01	**0.80 ± 0.03**[*]	6.01 ± 0.48
	YM	0.70 ± 0.07	5.06 ± 0.83	0.85 ± 0.39	7.42 ± 0.01	0.29 ± 0.03	7.96 ± 0.02
Pleurotus eryngii	MCM	0.33 ± 0.00	6.49 ± 0.06	**0.83 ± 0.03**[*]	4.68 ± 0.06	**0.75 ± 0.04**[*]	5.74 ± 0.22
	MEP	0.33 ± 0.02	6.85 ± 0.20	**0.49 ± 0.04**[*]	4.85 ± 0.42	**0.49 ± 0.02**[*]	6.32 ± 0.50
	YM	0.51 ± 0.07	6.39 ± 0.06	**1.32 ± 0.07**[*]	5.12 ± 0.65	0.57 ± 0.16	8.08 ± 0.09
Pleurotus salmoneo-stramineus	MCM	1.64 ± 0.04	5.30 ± 0.04	1.20 ± 0.08	5.94 ± 0.46	1.05 ± 0.10	6.69 ± 0.39
	MEP	0.82 ± 0.02	3.74 ± 0.05	**0.97 ± 0.02**[*]	6.15 ± 0.99	**1.18 ± 0.13**[*]	5.18 ± 0.44
	YM	1.83 ± 0.03	6.87 ± 0.11	1.45 ± 0.16	8.03 ± 0.08	0.92 ± 0.13	8.04 ± 0.04
Trametes versicolor	MCM	0.37 ± 0.03	6.38 ± 0.02	**0.74 ± 0.08**[*]	5.58 ± 0.29	**0.82 ± 0.04**[*]	5.34 ± 0.07
	MEP	0.38 ± 0.03	6.27 ± 0.29	**0.50 ± 0.05**[*]	6.03 ± 0.02	**0.61 ± 0.03**[*]	6.27 ± 0.31
	YM	0.35 ± 0.02	6.14 ± 0.09	**0.63 ± 0.04**[*]	6.98 ± 0.47	**0.58 ± 0.07**[*]	5.56 ± 0.60

Results displayed are a representation of the mean ± SD (n = 3) of triplicate cultivations per time point. [1]Final pH was calculated after growth of the mycelia on day 4, 8 and 12 in 100 mL culture medium within 250 mL shaker flasks; [a]Initial pH of MCM, 6.92; [b]Initial pH of MEP, 6.45; [c]Initial pH of YM, 6.10; [d]Initial pH of YpSs, 7.16. The probability values were determined using a Dunnett's test to evaluate significance, with a confidence level of 95 %. An asterisk [*] denotes a significant increase ($p \leq 0.05$) in biomass production compared to minimum time required for growth (4 days).

- Abbreviations: DCW; Dry cell weight (g); MCM, mushroom complete medium; MEP, malt peptone extract medium; YM, yeast malt medium. *OYRM*1, *Pleurotus ostreatus*.

The mean mycelial dry weight and pH of ten species of filamentous fungi as affected by substrate medium in submerged liquid culture is depicted in Table 3.1. Culture pH of *L. edodes* and *G. frondosa* grown on MCM, MEP and YM became acidic on day eight of growth. The pH of the medium remained low for *L. edodes* and *G. frondosa* cultures only; other low pH levels observed such as day eight cultures of *P. citrinopileatus* grown on MCM and *P. eryngii* grown on MCM and MEP increased slightly following twelve days of continual growth. This observation may be due to the slow consumption of nutrients in the medium which would coincide with the low yield of biomass produced by *L. edodes* and *G. frondosa*. It is also likely that depending on the fungi various acidic or basic compounds are produced over time which naturally alters the pH of the media accordingly. It has previously been reported that for some species optimal culture pH for biomass and polysaccharide production in submerged culture was observed with an acidic pH medium (Cho *et al.*, 2006; Pokhrel *et al.*, 2007). This observation was made for *G. frondosa, L. edodes, P. 1833, P. 32783* and *T. versicolor* which produced the most biomass in more acidic pH cultures compared to others. An increase of pH with decreased production of biomass may have been due to exhaustion of nutrients prior to the end of the twelve day growth cycle, since the utilisation of nutrients such as carbon or nitrogen, may lead to adverse effects on growth. The pH of other species generally remained within the range of the initial pH (Table 3.1). This may have been due to the presence of buffering salts in the medium or poor utilisation of media components. Yeast-starch agar medium (YpSs) is recommended for the cultivation of *M. purpureus*, this medium is usually favourable to thermophilic fungi as it is based on salts, soluble starch and yeast extract (Cooney *et al.*, 1964). Initial pH of the substrate medium in SLF is also an important factor, known to affect cell membrane function, uptake of various nutrients and product biosynthesis (Duan *et al.*, 2013).

In some instances the biomass reached its maximum production on day eight, followed by a reduction of growth on day twelve. Apart from *L. edodes* grown on MCM and MEP, cultures of *G. frondosa* and *L. edodes* grown in YM media, ceased growth following eight days cultivation at low pH levels. *L. edodes* grown on MCM and MEP produced another 0.01 g and 0.06 g of biomass following an additional four days of growth at an acidic pH level (day eight – day twelve), respectively. This slight increase may be due to nutrients which were still available for metabolism and the

different requirements of different species for various nutrients. For this reason, investigation of the nutritional requirements depending on the species is important.

Of the three different liquid culture media used per strain, MCM produced the best results for the majority of species analysed. Notably, for maximum biomass production of each species, the optimal media was different to the recommended media (Table 3.2). MCM medium was the optimum substrate for *M. purpureus, P. 1833, P. 32783, OYRM*1, *P. citrinopileatus* and *T. versicolor*, resulting in maximum mycelial biomass yield in the shortest period of time possible. Mycelia generation was positively influenced over time by a significant amount when grown in MCM medium for *P. 1833*, *OYRM*1, *P. 32783*, *P. eryngii* and *T. versicolor* ($p \leq 0.05$). Generally, this media significantly improved biomass generation over time of each of the selected species, with the exception of *M. purpureus*, *P. citrinopileatus* and *P. salmoneo-stramineus.*

YM broth significantly improved growth of *L. edodes*, *P. 1833*, *P. 32783*, *P. eryngii* and *T. versicolor* ($p \leq 0.05$) over eight to twelve days. Continual incubation decreased biomass production of *P. salmoneo-stramineus* in YM media after four days of growth. In addition, the mycelial growth of *P. citrinopileatus* and *L. edodes* increased until day eight followed by a reduction in biomass on day twelve. However, *P. salmoneo-stramineus* demonstrated maximum biomass generation after four days and *P. eryngii* demonstrated maximum biomass generation after eight days when grown in YM medium (Table 3.1).

MEP medium significantly improved the overall mycelia production of *L. edodes*, *P. 1833*, *P. 32783*, *P. citrinopileatus*, *P. eryngii*, *P. salmoneo-stramineus* and *T. versicolor* over time ($p \leq 0.05$); however, after eight days of growth *P. 1833* biomass dry weight decreased slightly. When cultivated with MEP medium, *G. frondosa* and *L. edodes* demonstrated maximum biomass generation after eight and twelve days, respectively. The medium had no significant impact on *G. frondosa* or *OYRM*1 production over time.

Morphology of filamentous fungi in submerged cultures is said to play an important role in its metabolism during cultivation. During submerged cultivation, fungal growth may take different forms, such as compact pellets of hyphae or homogenous suspensions of dispersed mycelia, usually depending on the species. Morphological differences are usually related to growth kinetics and physiological differences (El-Enshasy, 2007). In the present study, each Basidiomycetes species

displayed spherical pelleted formation (small, medium or large, depending on the fungus). In contrast, *M. purpureus* displayed formation of looser masses of hyphae (filamentous) in agitated flasks during the entire cultivation period. Morphological structure formations are known to depend on adaptation of the organisms in liquid media (Diamantopoulou *et al.*, 2014). Pellet morphological characteristics have been found to be an indicator of fermentation productivity (Sinha *et al.*, 2001b). On the whole, different media sources did not affect morphological characteristics of each species. From the results, the optimal medium (basal medium) observed for each species compared to the recommended growth conditions was obtained (Table 3.2).

Table 3.2   Overview of optimal conditions for generation of mycelial biomass

Fungal spp.	Strain ID[a]	Recommended		Optimal biomass growth conditions	
		Medium	Temp (° C)	Medium	Time
Grifola frondosa	*Grif. Fr.*[1]	-	-	MEP	8 days
Lentinula edodes	*Shiitake*[1]	-	-	MEP	12 days
Monascus purpureus	1604[2]	YpSs	28	MCM	4 days
Pleurotus ostreatus	*OYRM*1[1]	YM	30	MCM	8 days
Pleurotus ostreatus	1833[2]	MEP	25	MCM	8 days
Pleurotus ostreatus	32783[3]	YM	24	MCM	12 days
Pleurotus citrinopileatus	5341[2]	MEP	25	MCM	4 days
Pleurotus eryngii	9619[2]	MEP	25	YM	8 days
Pleurotus salmoneo-stramineus	5338[2]	MEP	25	YM	4 days
Trametes versicolor	3086[2]	MEP	24	MCM	12 days

Active fungal culture (1 %, w/v) grown with YM, MCM, MEP or YpSs medium, accordingly. Species were grown at 28 ºC or according to source recommendation, at 200 rpm for 4, 8 and 12 days as outlined in Section 2.2.2.1. [a]Strain reference ID, according to DSMZ & ATTC. [1]In-house laboratory strain. [2]DSMZ, German Collection of Microorganisms and Cell Cultures, Germany. [3]American Type Culture Collection, LGC Standards, UK.

As glucose is the main carbohydrate source of MCM (2 %, w/v glucose), it is believed the presence of this carbon source at a higher concentration, provided the majority of structural and energy requirements for mycelial growth at an early stage compared to the other media employed. Potentially, this is the reason that MCM demonstrated maximum biomass production for *M. purpureus*, *OYRM*1, *P. 1833*, *P. 32783*, *P. citrinopileatus* and *T. versicolor* after only four days cultivation, in comparison to YM and MEP media (Table 3.1). Glucose is a commonly consumed sugar source for most species of fungi. The favourability of most fungi toward glucose is perhaps due to the common availability of this sugar, in cellulose, starches, and other carbohydrates naturally (Griffin, 1994). It is evident from the data collected that optimisation of cultivation parameters in SLF maintains a degree of species specificity.

The basal medium and optimal time conducted for maximum biomass production were subsequently used for downstream investigations of antimicrobial and antioxidant potential.

3.1.2   Effect of nutrient supplementation on pH and biomass production

Fungi are heterotrophic organisms and as such they rely on obtaining their energy and carbon supply for metabolism from other organisms. Most research on the cultivation of Pleurotus using submerged liquid and solid state fermentation is related to manipulation of substrate composition and optimisation of culture parameters (Gregori *et al.*, 2007). As a consequence of there being very little information available on the effect of nutrient supplementation with *Monascus purpureus* in SLF, the effect of nutrient supplementation on biomass production and pH of the culture medium was preliminarily examined with this species (Figure 3.1). Further work in this section was then continued with *Grifola frondosa*, *Lentinula edodes*, *Pleurotus ostreatus* (*OYRM*1), *Pleurotus ostreatus* 1833, *Pleurotus ostreatus* 32783, *Pleurotus citrinopileatus* 5341, *Pleurotus eryngii* 9619, *Pleurotus salmoneo-stramineus* 5338 and *Trametes versicolor* 3086.

Prior to this investigation, a short optimisation experiment testing 1 – 5 % (w/v) supplemental glucose in MCM media was grown for four days (data not shown). The results indicated that an additional 1 % (w/v) glucose produced the largest quantity of mycelia in this time period. This concentration of supplemental glucose was the optimum quantity of carbon source for maximum biomass production, bringing the total glucose content of this substrate media to 3 % (w/v). Consequently, supplementation with 1 % (w/v) was deemed sufficient to determine any influence on biomass production with the basal media for downstream investigations. The effect of supplementation with various monosaccharides, including; fructose, galactose, glucose, mannose and xylose, and the disaccharides; maltose, sucrose and trehalose in comparison to non-supplemented cultures was examined. Glucose is a widely accepted supplementation source in various fungal cultivations; serving as an energy source and possessing nutritional requirements favoured by most species, as was indicated in Section 3.1.1. This monosaccharide is an easily utilisable sugar, most likely due to its availability naturally in cellulose, starches, and other carbohydrates (Griffin, 1994). Fructose, mannose and galactose are also commonly consumed sugars by fungi.

A significant difference in biomass production was revealed under certain conditions (Figure 3.1). Most nutrient sources added to the medium increased production, these included; fructose, galactose, mannose, corn steep liquor and xylose which significantly influenced mycelia production ($p \leq 0.05$) compared to the non-supplemented (NS) basal medium. Galactose significantly increased mycelial biomass generation the most after four days (1.15 g), compared to the basal medium without supplementation (0.75 g). Sucrose restricted the production of *M. purpureus*, generating 0.32 g mycelial dry weight by completion of cultivation (4 days). Different species of fungi have different growth requirements and as a consequence have different consumption profiles for various nutrients. In this case it is evident that sucrose, a non-reducing sugar was not suitable for optimal biomass generation of *M. purpureus*. According to Porras-Arboleda *et al.* (2009) additional sucrose may affect catabolic repression of cellular secondary metabolism negatively impacting growth.
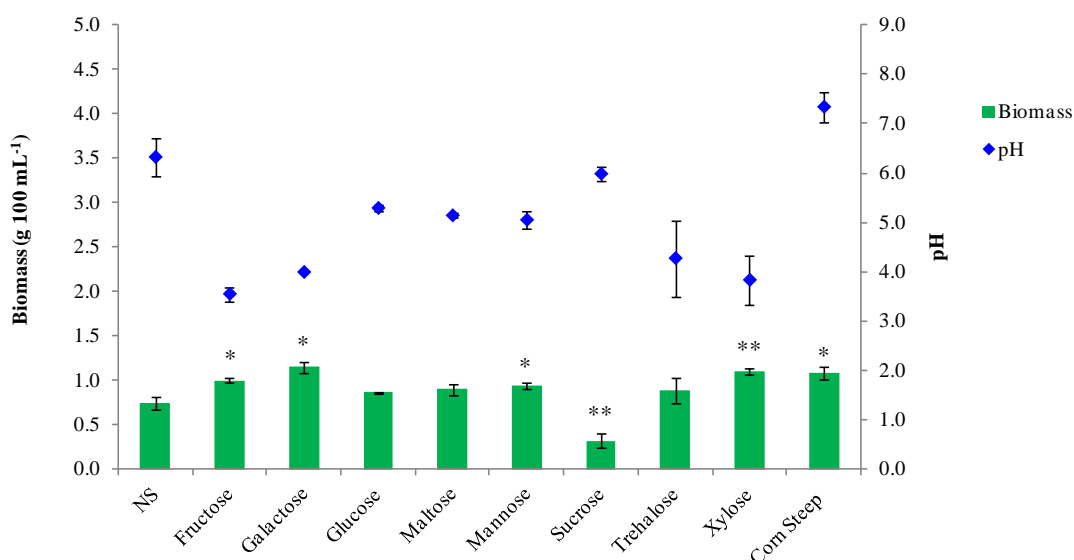
Figure 3.1   Effect of nutrient source on *Monascus purpureus* biomass and pH in SLF

M. purpureus was grown for a 4 day period in MCM medium as previously optimised (Table 3.2). Results displayed are representative of the mean of triplicate cultivations, with standard deviation represented by error bars. Initial pH of MCM was 7.64. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control (NS), with a confidence level of 95 %. A significant difference compared to non-supplemented cultures is denoted by; [*], $p \leq 0.05$ and [**], $p \leq 0.01$.
- Abbreviations: NS, non-supplemented culture.

Generally, supplemental carbon resulted in a more acidic culture compared to the non-supplemented control. In addition, apart from sucrose, a greater yield of mycelia was produced (Figure 3.1). This observation has been previously observed with other fungal species (Cho *et al.*, 2006; Pokhrel *et al.*, 2007). Galactose, xylose

and fructose resulted in a final pH of 3.99, 3.83 and 3.55, respectively. As mentioned, sucrose suppressed mycelial growth compared to the other sugars which is most likely to be due to catabolic repression and not the influence of culture pH. As can be appreciated from Figure 3.1, the final pH of sucrose (pH 6.32) was close to the initial pH and that of the non-supplemented control.

Corn steep liquor, a commonly used source of organic nitrogen, was tested as a preliminary indicator of the effect of additional nitrogen on mycelia production compared to carbon supplementation. The opposite effect was shown for corn steep liquor; having produced 1.09 g of dry mycelia this culture had a final pH of 7.34 (Figure 3.1), a significant increase in production ($p \leq 0.01$) compared to non-supplemented cultures. As a result, the effect of nitrogen source supplementation on biomass production compared to non-supplemented cultures was briefly examined with selected fungal species of Pleurotus, Trametes and Grifola (results not shown). The nitrogen sources tested were peptone, yeast extract and ammonium sulphate. Additional nitrogenous sources during cultivation at concentrations of 1 % (w/v) demonstrated poor morphological characteristics, with concentration above 5 % (w/v) completely retarding growth. Extremely poor morphological characteristics were observed as the concentration was increased (1 – 5 %, w/v); pellets were particularly small, completely discoloured (mostly dark brown or black), illustrating poor viability subsequently restricting growth. Cultures supplemented with ammonium sulphate produced less biomass than non-supplemented cultures. Peptone generated only slightly more biomass of *P. citrinopileatus*, *P. 1833* and *P. 32783* at 1 % (w/v). Although slight increases in the concentration of certain nitrogen sources influenced biomass yield for some species, the morphological modifications were not ideal. Nutrient sources; yeast extract, malt extract, starch, peptone, magnesium and phosphate ions were already incorporated into the cultivation medium (Section 2.2.1.2). Therefore with consideration of the known quantity of nitrogen already contained in the media composition (MCM, MEP, YM and YpSs), cultivation was deemed sufficient without additional nitrogenous supplementation, due to the poor morphological implications. Most importantly, it has also previously been suggested by Adrio *et al.* (2003) that nitrogen sources favourable for growth may negatively affect many secondary metabolite pathways. Accordingly, biomass produced with the addition of 1 % (w/v) supplemental carbon was deemed as being adequate to proceed with further examination of biological activity. This quantity of additional sugar was

also more economical and did not negatively impact morphology of the mycelium. With previous work having demonstrated that manipulation of substrate composition and optimisation of culture parameters affected cultivation of *Pleurotus* species (Gregori *et al.*, 2007; Papaspyridi *et al.*, 2010) and having demonstrated that nutrient supplementation affected biomass production of *M. purpureus* cultures, further work in this section was continued with each of the selected species (Figure 3.2 a-j).
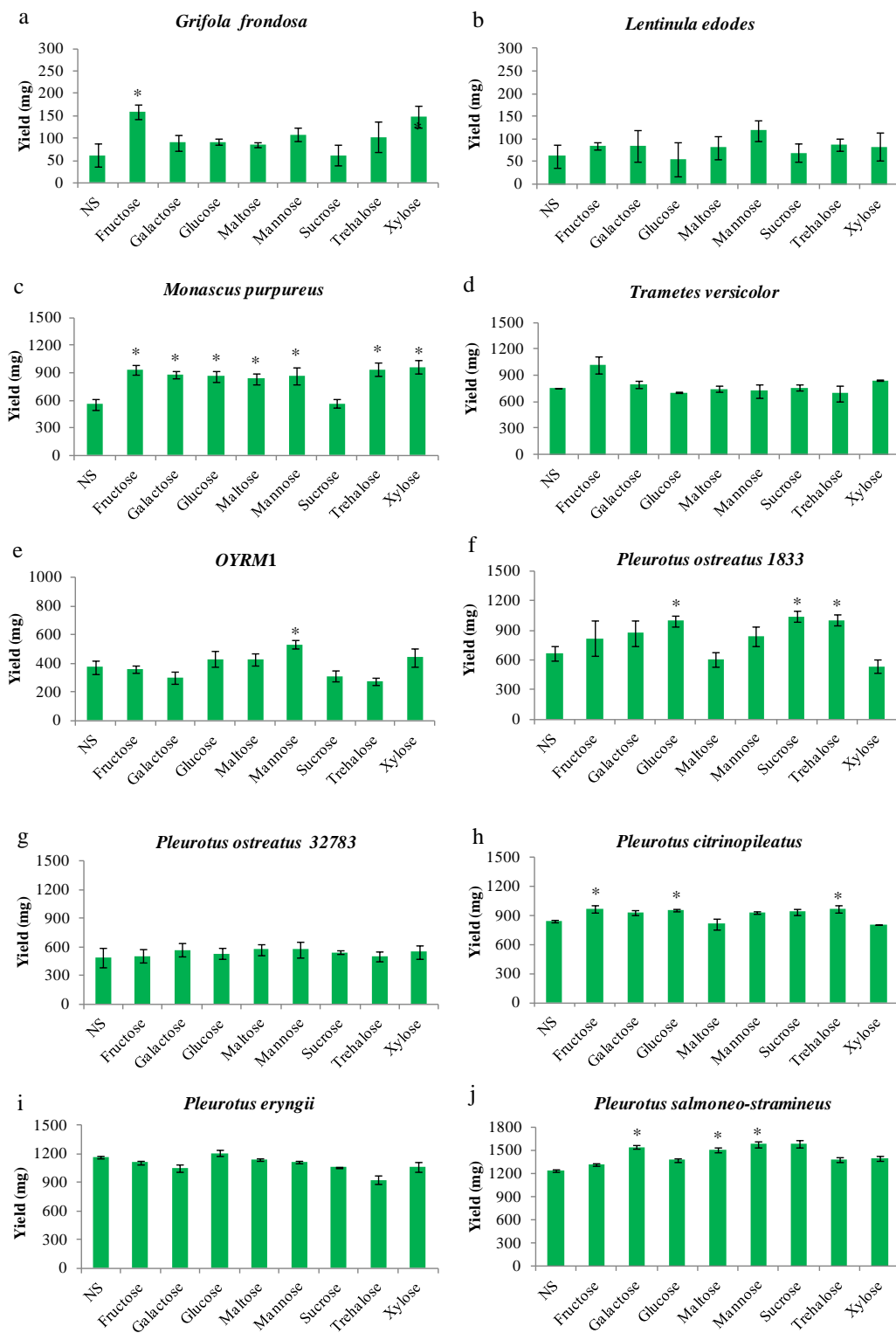
Figure 3.2 (a-j) Effect of carbon source supplementation on mycelial biomass production in SLF

Cultures were grown for seven days and supplemented with 1 % (w/v) carbon source with medium previously observed for optimal biomass yield (Table 3.2). Results plotted are a representation of the mean of triplicate flasks with the standard deviation of each treatment represented by error bars. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control (NS), with a confidence level of 95 %. An asterisk [*] denotes a significant positive impact ($p \leq 0.05$) in biomass production compared to the non-supplemented culture.
- Abbreviations: NS, Non-supplemented culture; *OYRM*1, *Pleurotus ostreatus*.

Figure 3.2 displays the individual graphical representation of each fungal species and the effect of single sugar source supplementation on biomass production in SLF. By supplementing the basal medium, an increase in the quantity of biomass produced, although not all statistically significant, was observed for most fungal strains. Cultures were allowed to grow for a period of seven days to allow for uniform analysis of the low-producing strains *L. edodes* and *G. frondosa* with the other species. Similar to preliminary examinations with *M. purpureus* (Figure 3.1), certain carbon sources had a significant impact on the growth of some fungi in submerged culture (Figure 3.2 a-j). The biomass of both *L. edodes* and *G. frondosa* increased with nutrient supplementation (Figure 3.2 a, b), which is noteworthy as these strains were previously noted to have a low yield of biomass (Table 3.1). Even so, *L. edodes* produced the least biomass in the time period tested compared to the other species, creating a maximum of 120 mg of dry biomass following one week cultivation (Figure 3.2 b). Nonetheless, all sources apart from glucose positively impacted the production of *L. edodes*. Likewise, biomass of *G. frondosa* significantly increased with the addition of fructose and xylose to the medium ($p \leq 0.05$) (Figure 3.2 a). With the exception of sucrose, biomass generation of *M. purpureus* was significantly enhanced ($p \leq 0.05$) with all nutrient additions (Figure 3.2 c). A negative impact on biomass production of *M. purpureus* due to supplemental sucrose was also previously made during preliminary investigations (Figure 3.1) and is believed to be associated with catabolic repression of cellular secondary metabolism (Porras-Arboleda *et al.*, 2009). Similarly, in sucrose-containing medium of other fungal species, biomass generation was restricted with supplementation of sucrose, although the impact was not significant. With exception, the biomass yield of *P. 1833* in sucrose-containing medium was significantly increased ($p \leq 0.01$), confirming that the optimal culture parameters for mycelia generation are species specific. Generally, cultures of *G. frondosa, L. edodes, P. 32783*, *OYRM*1, *P. salmoneo-stramineus* and *T. versicolor* produced similar quantities of mycelia with additional single sugar source compared to the non-supplemented cultures.

Growth of *T. versicolor* was mostly affected by the addition of fructose; increasing production by 57 %, and least by sucrose; only increasing production by 2 %. Glucose, maltose, mannose and trehalose restricted growth of *T. versicolor* in submerged culture (Figure 3.2 d). The *Pleurotus* spp. was the highest producing strain owing to the ability of this species to easily adapt to cultivation on defined media.

*OYRM*1 was significantly affected by addition of mannose ($p \leq 0.05$) in a positive manner, however; seven days cultivation inclusive of additional 1 % (w/v) fructose, galactose, sucrose and trehalose did not increase biomass production compared to the non-supplemented control (Figure 3.2 e). Nonetheless, when grown with glucose, maltose, mannose and xylose, yield increased 20 – 70 %. *Pleurotus 1833* produced 22 – 54 % more biomass than unsupplemented cultures with additional carbon (Figure 3.2 f). However, maltose and xylose restricted biomass production by 9 and 20 %, respectively. Although no significant observation on biomass development was made, production of *P. 32783* was increased with each supplementation source (2 – 13 %) (Figure 3.2 g). *P. citrinopileatus* production increased significantly ($p \leq 0.05$) with the addition of glucose and trehalose to the culture medium (Figure 3.2 h). Supplemental maltose and xylose to the fungus *P. citrinopileatus* had a similar affect to *P. 1833*, in that the minimum biomass yield was generated. Growth of *P. eryngii* was not affected significantly with any compositional change tested (Figure 3.2 i). Similarly, this observation was made for *P. 32783*, *L. edodes* and *T. versicolor*. Altogether, supplementation positively impacted biomass production of *P. salmoneo-stramineus*. Biomass yield of *P. salmoneo-stramineus* was beneficially affected by galactose, mannose and maltose supplementation, in that a significant increase in biomass yield was noted ($p \leq 0.05$) (Figure 3.2 j).

The results demonstrated that a change in nutrient composition affected biomass production of *G. frondosa, M. purpureus, OYRM*1, *P. 1833, P. citrinopileatus* and *P. salmoneo-stramineus* significantly ($p \leq 0.05$). On the contrary, cultures of *L. edodes*, *P. 32783, P. eryngii* and *T. versicolor* were unaffected. Taking into account each of the carbon sources tested, the best mycelial yield for *L. edodes, OYRM*1*, P. 32783* and *P. salmoneo-stramineus* was recorded in media that contained mannose. The results correspond with other studies which state that depending on the species, biomass production may vary according to carbohydrate source (Smiderle *et al.*, 2012). Nonetheless, the data indicated that the fungi were capable of using each of the carbon sources tested for the generation of biomass.

3.2   Effect of carbon supplementation on fungal cell wall composition

Composition and arrangement of structural components of the fungal cell wall is implicated in both physical and biological properties (Fontaine *et al.*, 1997). Functional responsibilities include; protection, shape, strength, cell-cell interactions, and contact with the environment (Pessoni *et al.*, 2005). The cell wall of filamentous fungi encompasses an assortment of cross-linked fibres (polysaccharides; glucan and chitin) and matrix components, combined primarily with proteins and mannans which hold the fibres together (as described in Section 1.1.3). Usually, the main fibrous component of the cell wall is glucan (a polymer of glucose), followed by chitin (a polymer of *N*-acetylglucosamine; a derivative of glucose) which is prevalent near the plasma membrane providing the cell wall with its enormous strength. Along with mannan, these components are the main constituents of the fungal cell wall and have been found in all medically relevant fungi studied to date. Mannans are chains of up to several hundred mannose units which are linked to fungal proteins *via* N- or O-linkages (Levitz, 2010). Proteins are present in the cell wall; mostly associated with hetero- and homopolymers of sugars (Fontaine *et al.*, 1997). Fungal cell wall components may be divided into structural components such as chitin, β-(1,3)-(1,6)-glucans and β-(1,4)-glucan (cellulose); as well as intrastructural components (the matrix) which include mannoproteins and α-(1,3)-glucan (Sietsma *et al.*, 1977). According to Osherov *et al.* (2010), chitin usually composes 10 to 15 % of the total cell wall dry weight in filamentous fungi, making up the bulk polysaccharide content. This biopolymer is widespread in nature and is similar in some physicochemical properties to cellulose (Feofilova, 2010).

Relevant literature has shown that changes in culture composition may influence yield, sporulation, colony morphology and chemical components such as cellular polysaccharides of mycelium, and that these changes are also dependant on the species (Kim *et al.*, 2002; Pessoni *et al.*, 2005; Smiderle *et al.*, 2012; Duan *et al.*, 2013). Generally, research in the area of secondary metabolite production is focused on optimising the culture medium in submerged liquid fermentation, with the aim of improving productivity of the mycelium and the production of polysaccharides (Gregori *et al.*, 2007).

The aim of this research was to obtain quantitative data on the chemical constituents of the selected fungi and to examine if the addition of 1 % (w/v) carbon source to the basal cultivation medium affected the cellular composition of the cell wall. Table 3.3 lists the biomass yield, the total cellular glucose, mannose, galactose, xylose and chitin content of ten species of filamentous fungi of known edible or therapeutic significance following cultivation with various additional sugars to the liquid culture. Compositional analysis of the cell wall was performed following acid hydrolysis and was analysed by HPLC as described in Section 2.2.3.

Table 3.3   Compositional analysis of fungal cell wall components following supplementation with various carbon sources

	Supplemental Carbon	Glucose % (w/v)	Mannose % (w/v)	Galactose % (w/v)	Xylose % (w/v)	Chitin % (w/v)	BM Yield (g 100 mL^{-1})
Grifola frondosa	NS	28.99 ± 6.74	7.67 ± 1.72	1.82 ± 0.15	6.10 ± 0.24	14.42 ± 1.37	0.06 ± 0.03
	Fructose	26.54 ± 0.96	3.74 ± 1.68	1.62 ± 0.42	4.18 ± 1.88	13.84 ± 3.00	**0.16 ± 0.02***
	Galactose	32.06 ± 0.29	4.99 ± 0.24	**12.26 ± 0.06***	**3.56 ± 0.03***	**11.49 ± 0.90***	0.09± 0.02
	Glucose	31.13 ± 4.64	5.79 ± 1.96	1.50 ± 0.10	5.78 ± 1.93	11.75 ± 2.09	0.09 ± 0.01
	Maltose	22.05 ± 0.28	10.92 ± 0.35	2.16 ± 0.07	**8.85 ± 0.32***	11.72 ± 3.31	0.09 ± 0.01
	Mannose	27.30 ± 0.28	11.79 ± 0.00	**1.33 ± 0.13***	**3.63 ± 0.43***	**12.09 ± 0.59***	0.11 ± 0.01
	Sucrose	40.00 ± 1.67	4.50 ± 2.87	2.39 ± 0.49	5.03 ± 3.21	14.28 ± 1.84	0.06 ± 0.02
	Trehalose	30.81 ± 0.30	4.31 ± 0.59	2.32 ± 0.34	**3.28 ± 0.03***	**11.87 ± 0.80***	0.10 ± 0.03
	Xylose	28.43 ± 4.27	5.78 ± 1.76	1.14 ± 0.65	**17.89 ± 0.55***	14.08 ± 1.82	**0.15 ± 0.02***
Lentinula edodes	NS	38.58 ± 0.11	5.50 ± 1.13	1.46 ± 0.82	4.81 ± 0.11	11.89 ± 0.67	0.06 ± 0.03
	Fructose	39.14 ± 0.04	4.26 ± 0.16	1.51 ± 0.09	**3.18 ± 0.07***	**14.40 ± 0.73***	0.08 ± 0.01
	Galactose	**40.40 ± 0.04***	4.16 ± 0.07	**11.07 ± 0.05***	*nd*	10.08 ± 1.05	0.08 ± 0.03
	Glucose	**53.98 ± 0.23***	4.30 ± 0.18	3.41 ± 0.09	*nd*	14.74 ± 0.26	0.05 ± 0.04
	Maltose	**47.10 ± 0.21***	6.26 ± 0.32	1.93 ± 0.11	*nd*	11.10 ± 0.85	0.08 ± 0.03
	Mannose	32.10 ± 8.79	**9.85 ± 1.62***	0.84 ± 0.44	*nd*	14.87 ± 1.31	0.12 ± 0.02
	Sucrose	**47.53 ± 0.13***	4.37 ± 0.12	1.10 ± 0.04	*nd*	9.70 ± 1.00	0.07 ± 0.02
	Trehalose	**47.40 ± 0.22***	4.51 ± 0.16	1.18 ± 0.06	*nd*	**16.79 ± 1.99***	0.09 ± 0.01
	Xylose	**31.56 ± 0.13***	4.77 ± 0.16	0.58 ± 0.05	**6.10 ± 0.21***	13.65 ± 0.90	0.08 ± 0.03
Monascus purpureus	NS	34.98 ± 1.04	10.80 ± 0.29	6.03 ± 1.55	*nd*	19.48 ± 0.93	0.56 ± 0.01
	Fructose	31.75 ± 0.91	11.40 ± 0.32	4.89 ± 2.98	0.38 ± 0.33	16.57 ± 1.03	**0.94 ± 0.05***
	Galactose	34.21 ± 4.44	13.70 ± 5.01	7.22 ± 3.73	1.28 ± 0.25	**11.85 ± 1.35***	**0.88 ± 0.04***
	Glucose	34.82 ± 0.13	14.11 ± 4.31	8.18 ± 2.81	*nd*	**13.07 ± 5.21***	**0.86 ± 0.06***
	Maltose	40.78 ± 0.75	11.14 ± 0.26	6.28 ± 0.29	*nd*	**13.68 ± 1.88***	**0.83 ± 0.06***
	Mannose	40.43 ± 0.84	11.35 ± 0.11	7.10 ± 0.13	0.41 ± 0.22	**15.53 ± 2.24***	**0.86 ± 0.09***
	Sucrose	34.22 ± 1.27	10.85 ± 0.14	5.86 ± 1.43	*nd*	18.76 ± 0.32	0.57 ± 0.05
	Trehalose	36.01 ± 1.10	11.44 ± 0.36	8.41 ± 0.77	0.80 ± 0.35	**15.77 ± 1.71***	**0.94 ± 0.07***
	Xylose	38.12 ± 0.75	11.29 ± 0.18	6.18 ± 0.47	0.43 ± 0.24	**12.04 ± 3.15***	**0.97 ± 0.08***
OYRM1	NS	30.44 ± 0.68	12.36 ± 1.76	2.28 ± 0.18	*nd*	10.82 ± 1.85	0.37 ± 0.05
	Fructose	30.30 ± 2.32	12.53 ± 0.80	1.02 ± 0.24	*nd*	8.15 ± 0.81	0.36 ± 0.03
	Galactose	30.07 ± 0.61	10.73 ± 0.08	**16.98 ± 0.36***	*nd*	7.70 ± 0.26	0.30 ± 0.04
	Glucose	35.87 ± 6.60	14.16 ± 3.88	2.00 ± 0.15	*nd*	9.78 ± 1.49	0.43 ± 0.05
	Maltose	30.56 ± 0.39	11.98 ± 0.56	2.24 ± 0.08	*nd*	9.56 ± 1.02	0.43 ± 0.04
	Mannose	**35.44 ± 0.92***	11.59 ± 1.91	1.70 ± 0.45	*nd*	9.39 ± 1.18	**0.53 ±0.03***
	Sucrose	30.58 ± 0.61	11.63 ± 0.31	1.98 ± 0.37	*nd*	**15.16 ± 1.18***	0.31 ± 0.04
	Trehalose	28.49 ± 1.30	10.56 ± 1.28	1.99 ± 0.19	*nd*	15.40 ± 2.24	0.27 ± 0.02
	Xylose	33.12 ± 2.30	11.28 ± 0.24	**1.54 ± 0.03***	*nd*	8.98 ± 1.16	0.44 ± 0.06

Fungi cultivated with 1 % (w/v) carbon source under conditions previously optimised for optimal biomass yield (Table 3.2). Results displayed are a representative of the mean of triplicate cultivations ± sum of standard deviation. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, with a confidence level of 95 %. An asterisk [*] denotes a significant change ($p \leq 0.05$) in the composition compared to the non-supplemented control sample.

- Chitin content expressed as the % total dry weight (w/v) of fungal cell wall and was calculated from a standard curve of glucosamine hydrochloride (µg mL^{-1}).

- Abbreviations: BM, Biomass; NS, non-supplemented control; *nd*, not detected; *OYRM*1, *Pleurotus ostreatus*.

Table 3.3   **Compositional analysis of fungal cell wall components following supplementation with various carbon sources (cont.)**

	Supplemental Carbon	Glucose % (w/v)	Mannose % (w/v)	Galactose % (w/v)	Xylose % (w/v)	Chitin % (w/v)	BM Yield (g 100 mL^{-1})
Pleurotus ostreatus 1833	NS	39.87 ± 1.85	3.12 ± 0.36	0.64 ± 0.30	3.01 ± 0.02	14.20 ± 1.56	0.67 ± 0.07
	Fructose	37.92 ± 0.26	3.15 + 0.41	0.61 ± 0.40	3.01 ± 0.02	14.82 ± 1.53	0.82 ± 0.18
	Galactose	**33.89 ± 0.55***	3.26 ± 0.00	**7.86 ± 0.41***	2.98 ± 0.02	12.76 ± 0.91	0.87 ± 0.13
	Glucose	40.42 ± 4.44	3.46 ± 0.12	3.18 ± 3.98	3.00 ± 0.02	14.37 ± 2.18	**1.00 ± 0.05***
	Maltose	42.95 + 7.39	3.29 ± 0.28	0.33 ± 0.00	3.05 ± 0.04	**10.39 ± 0.48***	0.61 ± 0.08
	Mannose	37.25 ± 0.62	**9.36 ± 0.07***	0.33 ± 0.00	2.98 ± 0.02	12.81 ± 0.71	0.84 ± 0.10
	Sucrose	37.82 + 5.81	3.43 ± 0.27	0.33 ± 0.00	3.05 ± 0.04	14.58 ± 0.91	**1.04 ± 0.06***
	Trehalose	44.35 ± 1.97	2.71 + 0.06	0.33 ± 0.01	3.03 ± 0.06	13.67 ± 2.09	**1.00 ± 0.05***
	Xylose	**31.25 + 3.51***	2.79 + 0.15	0.34 ± 0.02	**6.45 ± 0.25***	10.72 ± 2.29	0.53 ± 0.07
Pleurotus ostreatus 32783	NS	35.15 + 6.21	3.07 ± 0.08	0.67 ± 0.10	*nd*	11.14 ± 1.33	0.49 ± 0.10
	Fructose	27.98 + 4.85	3.16 ± 0.09	0.67 ± 0.05	*nd*	12.08 ± 1.01	0.51 ± 0.07
	Galactose	30.35 + 5.68	3.06 ± 0.04	**3.01 ± 0.19***	*nd*	10.03 ± 0.83	0.57 ± 0.07
	Glucose	31.23 + 4.74	3.21 ± 0.11	0.65 ± 0.10	*nd*	11.81 ± 1.55	0.53± 0.06
	Maltose	36.41 + 6.95	3.90 ± 0.48	0.77 ± 0.26	*nd*	11.37 ± 0.77	0.58 ± 0.06
	Mannose	36.64 + 2.04	**5.16 ± 0.10***	0.95 ± 0.13	*nd*	10.26 ± 3.91	0.88 ± 0.08
	Sucrose	38.82 + 0.83	**4.06 ± 0.24***	0.67 ± 0.95	0.77 ± 0.54	9.78 ± 3.72	0.54 ± 0.01
	Trehalose	36.37 ± 4.32	**3.74 ± 0.14***	*nd*	*nd*	11.28 ± 0.95	0.50 ± 0.05
	Xylose	36.27 ± 0.58	**3.74 ± 0.06***	1.67 ± 0.01	4.37 ± 0.36	11.77 ± 2.73	0.55 ± 0.07
Pleurotus citrinopileatus	NS	39.50 ± 0.43	9.86 ± 0.10	2.70 ± 1.05	*nd*	11.07 ± 0.81	0.84 ± 0.09
	Fructose	**36.28 ± 1.08***	9.94 ± 0.28	2.87 ± 0.17	*nd*	11.21 ± 2.29	**0.97± 0.03***
	Galactose	**33.95 ± 0.98***	10.09 ± 0.03	**8.05 ± 0.09***	*nd*	9.39 ± 0.58	0.93 ± 0.02
	Glucose	**43.83 ± 1.33***	10.00 ± 0.16	2.15 ± 0.30	*nd*	8.95 ± 1.81	**0.96 ± 0.01***
	Maltose	40.44 ± 1.32	**10.64 ± 0.15***	2.77 ± 0.49	*nd*	8.88 ± 2.03	0.81 ± 0.05
	Mannose	37.43 ± 1.20	**12.31 ± 0.26***	2.52 ± 0.05	*nd*	8.52 ± 1.58	0.93 ± 0.01
	Sucrose	40.04 ± 2.24	10.01 ± 0.07	2.51 ± 0.27	*nd*	**9.08 ± 1.31***	0.94 ± 0.03
	Trehalose	40.19 ± 0.52	10.04 ± 0.07	2.12 ± 0.18	*nd*	10.10 ± 1.82	**0.97 ± 0.04***
	Xylose	36.93 ± 2.80	10.07 ±0.13	1.77 ± 0.42	*nd*	8.66 ± 1.96	0.81 ± 0.00
Pleurotus eryngii	NS	18.86 ± 1.75	4.34 ± 0.77	0.75 ± 0.40	0.83 ± 0.59	16.41 ± 3.21	1.16 ± 0.04
	Fructose	20.52 ± 0.39	3.14 ± 0.07	0.68 ± 0.07	0.65± 0.47	15.64 ± 3.50	1.11 ± 0.02
	Galactose	**28.24 ± 1.70***	3.79 ± 0.10	**5.60 ± 0.32***	0.77 ± 0.55	15.76 ± 1.00	1.05 ± 0.03
	Glucose	21.54 ± 0.52	3.28 ± 0.29	0.79 ± 0.30	0.14 ± 000	14.81 ± 1.48	1.21 ± 0.03
	Maltose	38.83 ± 0.88	4.01 ± 0.23	1.46 ± 0.35	1.29 ± 0.01	12.70 ± 0.78	1.13 ± 0.01
	Mannose	22.78 ± 1.12	3.95 ± 0.89	0.68 ± 0.05	0.32 ± 0.30	15.73 ± 4.22	1.11 ± 0.01
	Sucrose	**28.73 ± 3.28***	3.94 ± 0.21	0.98 ± 0.03	1.04 ± 0.04	16.65 ± 1.41	1.06 ± 0.00
	Trehalose	30.86 ± 0.26	3.89 ± 0.14	1.22 ± 0.29	0.54 ± 0.69	14.47 ± 1.92	0.93 ± 0.05
	Xylose	22.21 ± 2.24	3.91 ± 0.03	0.48 ± 0.12	**3.70 ± 0.59***	13.71 ± 1.33	1.06 ± 0.05

Fungi cultivated with 1 % (w/v) carbon source under conditions previously optimised for optimal biomass yield (Table 3.2). Results displayed are a representative of the mean of triplicate cultivations ± sum of standard deviation. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, with a confidence level of 95 %. An asterisk [*] denotes a significant change ($p \leq 0.05$) in the composition compared to the non-supplemented control sample.

- Chitin content expressed as the % total dry weight (w/v) of fungal cell wall and was calculated from a standard curve of glucosamine hydrochloride (µg mL^{-1}).

- Abbreviations: BM, Biomass; NS, non-supplemented control; *nd*, not detected.

Table 3.3   Compositional analysis of fungal cell wall components following supplementation with various carbon sources (cont.)

		Cell wall composition				
Supplemental Carbon	**Glucose % (w/v)**	**Mannose % (w/v)**	**Galactose % (w/v)**	**Xylose % (w/v)**	**Chitin % (w/v)**	**BM Yield (g 100 mL^{-1})**
Pleurotus salmoneo-stramineus NS	28.61 ± 0.43	10.56 + 0.35	2.30 ± 0.17	*nd*	17.99 ± 2.31	1.24 ± 0.03
Fructose	23.10 ± 2.21	10.35 ± 0.31	1.41 ± 1.13	*nd*	**13.06 ± 2.25***	1.32 ± 0.02
Galactose	**25.93 ± 0.34***	10.27 + 0.13	**13.82 ± 0.40***	*nd*	14.42 ± 3.12	**1.54 ± 0.03***
Glucose	33.63 + 8.44	10.52 + 0.37	2.25 ± 0.64	*nd*	16.23 ± 1.95	1.38 ± 0.02
Maltose	**34.51 ± 0.57***	10.42 ± 0.45	3.67 ± 0.04	*nd*	15.40 ± 2.09	1.51 ± 0.03*
Mannose	**38.16 + 0.36***	10.68 + 0.28	2.53 ± 0.21	*nd*	**14.20 ± 1.51***	**1.58 ± 0.04***
Sucrose	**34.85 ± 0.77***	10.07 + 0.14	**3.20 ± 0.28***	0.76 ± 0.03	**12.79 ± 2.21***	1.58 ± 0.05
Trehalose	29.17 ± 4.24	9.56 ± 1.41	**1.43 ± 0.10***	*nd*	17.64 ± 2.61	1.38 ± 0.03
Xylose	**32.64 ± 0.12***	10.02 + 0.06	**2.87 ± 0.15***	5.47 ± 0.05*	14.46 ± 1.75	1.39 ± 0.03
Trametes versicolor NS	31.81 ± 2.11	4.34 ± 0.12	0.73 ± 0.02	2.07 ± 0.15	11.78 ± 2.60	0.75 ± 0.00
Fructose	34.10 ± 0.01	4.69 ± 0.36	1.13 ± 0.38	2.17 ± 0.02	9.30 ± 1.03	1.02 ± 0.10
Galactose	23.66 ± 1.97	4.24 ± 0.64	**5.94 ± 0.75***	2.64 ± 1.21	11.37 ± 1.83	0.80 ± 0.04
Glucose	23.82 ± 5.92	**3.31 ± 0.12***	0.67 ± 0.07	**3.84 ± 0.22***	11.22 ± 1.77	0.70 ± 0.01
Maltose	27.85 ± 1.06	**3.47 ± 0.15***	1.41 ± 0.36	**3.90 ± 0.17***	11.07 ± 1.92	0.75 ± 0.03
Mannose	**23.03 ± 2.22***	**7.56 ± 0.84***	0.60 ± 0.38	1.60 ± 0.53	10.86 ± 2.83	0.72 ± 0.07
Sucrose	27.88 ± 1.05	**4.91 ± 0.20***	**0.82 ± 0.01***	2.00 ± 0.02	10.39 ± 1.56	0.76 ± 0.03
Trehalose	40.26 ± 0.87	4.59 ± 0.10	0.78 ±0.03	2.30 ± 0.09	15.84 ± 1.56	0.70 ± 0.09
Xylose	32.85 ± 0.40	4.03 ± 0.17	**0.20 ± 0.02***	**8.33 ± 0.28***	9.93 ± 1.52	0.84 ± 0.01

Fungi cultivated with 1 % (w/v) carbon source under conditions previously optimised for optimal biomass yield (Table 3.2). Results displayed are a representative of the mean of triplicate cultivations ± sum of standard deviation. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the control, with a confidence level of 95 %. An asterisk [*] denotes a significant change ($p \leq 0.05$) in the composition compared to the non-supplemented control sample.
- Chitin content expressed as the % total dry weight (w/v) of fungal cell wall and was calculated from a standard curve of glucosamine hydrochloride (μg mL^{-1}).
- Abbreviations: BM, Biomass; NS, non-supplemented control; *nd*, not detected.

The cell wall composition of mycelia from *G. frondosa*, *L. edodes*, *Pleurotus* spp., *M. purpureus* and *T. versicolor* indicated that glucose was the predominant monosaccharide constituent, followed by mannose (Table 3.3). In agreement with Smiderle *et al.* (2012), who also tested the effect of additional sugar sources in liquid cultivation on cell wall composition, glucose was the major monosaccharide detected in the mycelium. In the present investigation, the total glucose concentration of the cell wall varied, depending on the species and represented 22 – 54 % (w/v) of the total monosaccharide composition regardless of the supplemental sugar source. The monosaccharide cell wall composition included glucose, mannose and in most cases galactose and xylose for each strain. Monosaccharides galactose and xylose were mostly present as minor constituents or completely absent (Table 3.3).

The data suggests that the cell wall composition of each of the selected filamentous fungi varied depending on each species and strain. In addition, each

fungal species exploited the various carbon sources for biomass production and chitin generation differently (Table 3.3). It has been previously observed that composition of cell wall components may be affected by carbohydrate concentration of the medium; however, there was no trend detected between polysaccharide composition of each species and the supplemental carbon sources in the present investigation. Although it could be expected that in glucose supplemented media the mycelium would reach its maximum value of glucan content earlier than disaccharide containing media, certain sugars may be utilised earlier and with a higher rate than glucose during fungal growth. Papaspyridi *et al.* (2010) demonstrated that depending on the species certain sugars may be more readily metabolised for cellular energy production. In the study by Papaspyridi *et al.* (2010), xylose was determined as the best from the viewpoint of mycelial growth, even though glucose stimulated a similar level of biomass yield. In the present investigation, the incidence of certain sugars being utilised more readily by the fungus compared to glucose containing media was evident for *G. frondosa*, *M. purpureus*, *P. 1833*, *P. 32783*, *P. eryngii*, *P. salmoneo-stramineus* and *T. versicolor* (Table 3.3). This phenomenon may be attributed to a special consumption profile of some fungi, as was previously suggested with *Pleurotus ostreatus* and was associated with high efficiency during uptake of disaccharides by use of effective sugar transporters within its metabolic mechanism (Papaspyridi *et al.*, 2010). Thereby, the present study demonstrates primary indications of similar observations with *G. frondosa*, *M. purpureus* and *T. versicolor*. Relevant literature has also shown sugar composition of the media to be implicated in the resulting molecular weight of extracellular polysaccharides, suggesting that other carbohydrate sources apart from glucose be used for biosynthesis of extracellular polysaccharides since glucose is a main sugar component of the cell wall (Porras-Arboleda *et al.*, 2009; Elisashvili, 2012).

The present data agrees with previous reports, which state that the effect of different complex media during growth strongly influences cellular composition and subsequently mycelial growth (Kim *et al.*, 2002). In general, where glycosyl content of the fungal mycelium was reduced by certain sugar sources compared to the non-supplemented medium, the biomass yield was increased (Table 3.3). Galactose and xylose content were influenced considerably following addition of these nutrients (1 %, w/v) to the growth medium of several fungal species (Table 3.3), even after the biomass was appropriately washed following separation from culture liquid prior to

drying (Section 2.2.1.3). On the contrary, the cell wall glycosyl composition of *M. purpureus* was not significantly affected by the nature of any of the carbon sources tested, although biomass generation of this species was significantly influenced ($p \leq$ 0.05) (Table 3.3). This observation indicated that like data reported by Maziero *et al.* (1999), sugar availability and utilisation may affect biomass generation and not polysaccharide production depending on the nature of the fungi.

On the whole, each species in this investigation demonstrated that cell wall polysaccharide production is not affected by mycelial biomass production (Sone *et al.*, 1985; Zhong *et al.*, 2004). No significant correlations were observed between monosaccharide composition and biomass production of any of the species tested. In other words, additional carbon sources can be utilised to improve production yield of polysaccharide content; however, good mycelial growth is not a determining factor for high polysaccharide yield. Similar results were observed in other investigations with *Pleurotus pulmonarius* and *Phellinus linteus* (El-Dein *et al.*, 2004).

Total glucan and chitin components represented on average 35 – 58 % (w/v) of the total wall dry weight of each species examined. Generally, most fungi have considerable amounts of chitin within their cell wall structure (Bartnicki-Garcia, 1968). As can be seen from the strains employed in this investigation, chitin proves a major contributor to the overall composition of the cell wall (Table 3.3). Chitin and glucan, the main fungal polysaccharides, are linked *via* an α-(1-4)-glycosidic bond (Bartnicki-Garcia, 1968; Nwe *et al.*, 2008). In fungal mycelia, chitin may be isolated as free or covalently bound in a chitin-glucan complex. The rigid cross-linked network of chitin-glucan chains is responsible for the cells rigidity however this causes difficulty for extraction of intact chitin and glucan. Total chitin content in the present investigation was quantified according to a method adapted by Pessoni *et al.* (2005), which was originally developed by Chen *et al.* (1983). Chen *et al.* (1983) evaluated chromogen interference (by neutral polysaccharides and amino acids in hydrolysates), sensitivity, colour stability, and hydrolysis conditions for maximum release of glucosamine from fungal cell walls. One of the objectives of this research was to examine if differences in sugar content during cultivation significantly affected the chitin concentration of the cell wall compared to non-supplemented cultures. Research has shown this indigestible fibre constituent is an immune supporting component from many medicinal fungal species, having been isolated from both fruiting body and fungal mycelia. The total chitin content varied from 8 – 19.5 % (w/v) of the cell wall

dry weight between strains (Table 3.3). A high concentration of chitin was observed in the biomass of *P. 32783*, *M. purpureus* and *P. eryngii*. Supplemental carbon source (1 %, w/v) had a significantly positive impact on the chitin content of *L. edodes* and *OYRM*1 cultures. The total chitin content of *P. 32783, P. eryngii* and *T. versicolor* was not affected significantly by any changes in medium composition.

Relevant literature has found that differences in glycosyl composition and chitin content may be attributed to nutrient utilisation, morphological characteristics, stress signals (pH and temperature) or the stage and condition of growth from which the dry mycelia was extracted (Roberts, 1992; Ruiz-Herrera, 2012). Cell wall chitin of *L. edodes* and *P. salmoneo-stramineus* was significantly influenced by the presence of additional fructose to the growth medium ($p \le 0.05$). Additional sucrose in the medium significantly affected the production of chitin within *P. salmoneo-stramineus, P. citrinopileatus* and *OYRM*1 ($p \le 0.05$) biomass; by restricting the cellular chitin content of *P. salmoneo-stramineus* and *P. citrinopileatus* and improving chitin content of *OYRM*1. With the exception of sucrose, the chitin content of *M. purpureus* was significantly reduced by addition of all carbon sources tested ($p \le 0.05$). Therefore, the results suggest that chitin content may vary depending on micronutrient composition of the culture medium. A significant correlation ($p \le 0.05$) between chitin content and biomass generation was observed for non-supplemented, galactose, glucose and mannose supplemented cultures. This observation may be due to the relationship of this constituent as a structural analogue in the mycelial biomass.

The results propose that the ratio between the main cell wall polysaccharides (chitin and glucan) were susceptible to change not only during ontogeny of mycelia in submerged culture, but also depending on the composition of the cultivation medium and is species specific. A screening of 56 higher Basidiomycetes species, conducted by Maziero *et al.* (1999), concluded that there was no relationship between biomass and exopolysaccharide production and almost all strains tested produced different quantities of cellular polysaccharides. Due to the individuality of fungal species and strains within a species, they suggested that proper kinetic profiles on polymer production be carried out on each individual strain prior to growth. As the present investigation was not progressive, a linear relationship between chitin and biomass could not be determined, only the effect of sugar composition in the media on cell wall carbohydrate content at a particular time point was analysed. In the present investigation, as no correlation was determined between monosaccharide composition

and biomass production, it is suggested compositional analysis was dependent on the stage of growth of each individual fungi, whereas, chitin being a structural unit was somewhat related to the biomass generation of certain species.

3.3   General Conclusions

Altogether, there are few reports in published literature for quantitative comparison of the major cell wall constituents of the filamentous fungi analysed. The results of the present study provide information on the growth parameters of ten species of fungi under submerged cultivation conditions, as well as outlining effects on cell wall composition. Extensive studies have reported growth of pure cultures by submerged liquid the most efficient technique for production of consistent and safe natural products from fungi (Wasser *et al.*, 2000; Reshetnikov *et al.*, 2001; Lull *et al.*, 2005; Papaspyridi *et al.*, 2012). Although absorption of high molecular weight polysaccharides following oral administration is unlikely, there is potential for direct interaction with the host leading to stimulation of the immune system (Lull *et al.*, 2005), as well as interaction with microbes prior to infection (Section 4.1).

Cultivation of *Pleurotus* spp., *M. purpureus*, *T. versicolor*, *L. edodes* and *G. frondosa* by means of submerged liquid cultivation is noteworthy, due to the short time taken to obtain the mycelium. The conversion of various carbon sources into mycelial mass by each fungus was analysed. The data indicated that production of biomass and cell wall constituents were affected by several environmental parameters, particularly medium composition i.e. nature and concentration of the carbon source. Like previous reports, there was no significant relationship established between biomass production and polysaccharide composition.

On the whole, it is clear that depending on the goal of the cultivation process, critical evaluation of the physical and chemical parameters is essential. The results show that optimal production of mycelial biomass and cell wall constituents by *Pleurotus* spp, *M. purpureus*, *T. versicolor*, *L. edodes* and *G. frondosa* are highly dependent on the strain, growth parameters and nutritional factors. Specific optimisation of culture composition and physiological conditions unique to each strain would benefit the regulation of fungal metabolism for downstream isolation of standardised nutraceutical substances in higher yield (Lull *et al.*, 2005).

4.   Antimicrobial activity of filamentous fungi

4.1   Pathogen binding capacity of fungal components

Bacterial infections are responsible for a broad spectrum of animal and human illnesses and diseases worldwide. As a consequence there is a growing need for the development of novel strategies to prevent and treat bacterial infections, particularly since the evolution of bacterial resistance to a wide range of traditional antibiotics (Signoretto *et al.*, 2012).

The majority of infectious diseases are initiated by the adhesion of pathogenic organisms to cells and mucosal surfaces (Ofek *et al.*, 1994), which subsequently form a colony or biofilm (Boland *et al.*, 2000). Attachment of the pathogen to host tissue is required so it can access nutrients for proliferation and subsequently deliver its toxins. Without this mechanism, pathogens would otherwise be swept away from potential sites of infection by the body's normal cleansing mechanisms. The search for naturally derived bioactive substances which target bacterial adhesion, colonisation, invasion and production of toxins are considered a valuable complement to antibiotic therapy. Ultimately, bacterial adherence to host epithelial cells *in vivo* is a significant step in the progression of infection and disease (Sharon *et al.*, 2001). By providing compatible dietary fibre to which bacteria may attach, as opposed to epithelial cells, the host is less likely to contract gastrointestinal infections and subsequently the risk of the transmission of human-pathogens *via* the food-chain is reduced (Becker *et al.*, 2007).

Adherence of pathogens to epithelial cells is mainly mediated by lectins located on the surface of the infectious pathogenic organisms, which bind to complementary carbohydrate constituents of glycoproteins or glycolipids present on host cell surfaces (Sharon, 2006). The carbohydrate nature and low digestibility of dietary fibres from fungi make them ideal alternative adhesion matrices (Becker *et al.*, 2007). Research has demonstrated that many saccharides have the ability to prevent infection from different bacteria in a variety of animals (Ofek *et al.*, 2003). The most characterised bacterial surface lectins are products of enterobacterial strains, mainly mannose-specific type-1 fimbriae of *E. coli*. Type-1 fimbriae enable bacteria to bind to D-mannose residues attached to cell surfaces. These are expressed by a large number of *E. coli* strains and have been found in greater than 95 % of *E. coli* isolates from

intestinal and extraintestinal infections (Sokurenko *et al.*, 1998). *In vitro* studies have shown that 80 % of *Salmonella typhimurium* and 67 % of *Salmonella enteritidis* possess fimbriae which attach to mannose receptors (Duguid *et al.*, 1966).

The objective of this aspect of research was to examine the potential of fungal cell wall components as preventatives in initial adherence of bacteria and as such, demonstrate their potential as natural health-promoting components. Agglutination of *Escherichia coli* 10778 and *Staphylococcus epidermidis* 1798 with fungal cell wall material was assessed. These two bacterial species were selected because of their described capacity for adherence in previously published literature.

Having established the effect of different carbohydrate supplements (1 %, w/v) on biomass production and fungal cell wall composition, differences in antimicrobial capacity in the form of anti-adhesion therapy was examined. In order to efficiently test fungal cell components as adhesion matrices of bacterium, examination of the overall growth phase was assessed (as outlined in Section 2.2.5.2). Alternatively Sokurenko *et al.* (1995) compared growth curves of bacteria at a particular time coordinate i.e. following 2 – 3 hours incubation; this method requires examining growth during the exponential phase. By examination of the overall growth phase, results obtained provide information on the growth of the bacteria over time. According to Becker *et al.* (2007) this method is less likely to miss relevant information.

Table 4.1 and 4.2 provide information on the capacity of fungal components to bind to bacteria *in vitro*, presenting the detection times of growth as the duration (hours) needed for the cultures to reach an OD of 0.05 at λ_{595nm}, calculated as described in Section 2.2.5.2. The times of detection of growth were used as a measure of adhering cell number i.e. the greater the number of attached bacterial cells to fungal components presented on the microplate, the shorter the detection time of its subsequent growth in liquid medium (Becker *et al.*, 2007; Becker *et al.*, 2008).

Table 4.1   **Detection times of bacterial growth $t_{0.05}$ (h) for *E. coli* 10778 in microtitration plates coated with fungal cell wall components**

Culture condition (1 %, w/v)*	*Grifola frondosa*	*Lentinula edodes*	*Monascus purpureus*	*OYRM1*	*Pleurotus ostreatus 1833*	*Pleurotus ostreatus 32783*	*Pleurotus citrinopileatus*	*Pleurotus eryngii*	*Pleurotus salmoneo-str.*	*Trametes versicolor*
NS	5.41 ± 0.52[cd]	5.98 ± 0.34[c]	4.30 ± 0.15[b]	3.71 ± 0.19[c]	6.53 ± 0.40[b]	5.46 ± 0.18[b]	5.17 ± 0.29[b]	4.50 ± 0.03[bcd]	5.75 ± 0.21[b]	3.61 ± 0.31[ab]
Fructose*	6.86 ± 0.27[ab]	5.99 ± 0.14[c]	4.24 ± 0.23[b]	4.17 ± 0.12[b]	6.91 ± 0.30[b]	5.54 ± 0.24[ab]	5.23 ± 0.24[b]	4.49 ± 0.02[bcd]	5.63 ± 0.30[b]	3.78 ± 0.08[ab]
Galactose*	**4.86 ± 0.32[d]**	**5.93 ± 0.18[c]**	**3.98 ± 0.14[b]**	3.89 ± 0.09[bc]	6.64 ± 0.91[b]	5.49 ± 0.25[b]	5.02 ± 0.40[b]	4.51 ± 0.10[bcd]	5.86 ±0.17[b]	3.72 ± 0.04[ab]
Glucose*	4.93 ± 0.38[d]	6.16 ± 0.29[c]	4.36 ± 0.06[b]	4.01 ± 0.02[bc]	7.14 ± 0.08[b]	5.67 ± 0.20[ab]	**4.59 ± 0.74[b]**	**4.32 ± 0.11[d]**	**5.52 ± 0.35[b]**	3.94 ± 0.02[ab]
Maltose*	5.20 ± 0.78[cd]	6.03 ± 0.22[c]	4.27 ± 0.03[b]	**3.78 ± 0.10[bc]**	6.96 ± 0.24[b]	5.34 ± 0.30[b]	4.64 ± 0.52[b]	4.44 ± 0.15[bc]	5.76 ± 0.28[b]	3.96 ± 0.07[ab]
Mannose*	5.45 ± 0.26[bcd]	6.31 ± 0.09[bc]	5.57 ± 0.73[a]	3.92 ± 0.13[bc]	6.75 ± 0.04[b]	5.42 ± 0.29[b]	4.88 ± 0.88[b]	4.69 ± 0.05[bc]	5.71 ± 0.09[b]	3.84 ± 0.41[ab]
Sucrose*	6.46 ± 0.85[abc]	6.30 ± 0.07[bc]	4.28 ± 0.15[b]	4.05 ± 0.15[bc]	**6.27 ± 0.32[b]**	5.46 ± 0.15[b]	5.33 ± 0.37[b]	4.68 ± 0.11[bc]	5.63 ±0.15[b]	4.05 ± 0.12[a]
Trehalose*	5.63 ± 0.32[abcd]	6.50± 0.14[bc]	4.53 ± 0.11[ab]	3.91 ± 0.28[bc]	6.87 ± 0.28[b]	5.54 ± 0.62[ab]	5.13 ± 0.61[b]	4.45 ± 0.29[bcd]	5.77 ± 0.29[b]	3.67 ± 0.29[ab]
Xylose*	5.30 ± 0.44[cd]	6.24 ± 0.21[bc]	4.59 ± 0.28[ab]	3.96 ± 0.07[bc]	6.68 ± 0.23[b]	**5.13 ± 0.13[b]**	5.10 ± 0.33[b]	4.71 ± 0.08[bc]	6.05 ± 0.19[ab]	**3.42 ± 0.12[b]**
YCW	6.05 ± 0.47[abcd]	6.87 ± 0.13[b]	4.19 ± 0.24[b]	3.90 ± 0.07[bc]	6.63 ± 0.22[b]	5.32 ± 0.18[b]	4.64 ± 0.29[b]	4.80 ± 0.03[b]	5.66 ± 0.20[b]	4.13 ± 0.29[a]
Control	**6.89 ± 0.52[a]**	**8.08 ± 0.36[a]**	**5.59 ± 0.86[a]**	**5.46 ± 0.23[a]**	**10.94 ± 0.61[a]**	**6.31 ± 0.07[a]**	**6.98 ± 0.78[a]**	**6.39 ± 0.11[a]**	**6.71 ± 0.12[a]**	**3.90 ± 0.09[ab]**
SEM	0.23	0.28	0.13	0.12	0.16	0.10	0.21	0.14	0.08	0.05

Results displayed are representative of the mean of triplicate determinations ± sum of standard deviation. Means within a column represent the fungal species grown in submerged liquid flask cultures under various conditions i.e. 1 % (w/v) carbon source supplementation. Conditions with the shortest $t_{0.05}$ (h) bound the most bacterial cells; Control, BSA (1 %, w/v). Detection time means marked by different letters within a column are significantly different ($p \leq 0.05$, ANOVA, Tukey-HSD), with alphabetical order of letters (a-d) representative of the least binding capacity to the best, respectively.

- Abbreviations: *E. coli*, *Escherichia coli*; NS, Non-supplemented cultures; SEM, Standard error of means; YCW, yeast cell wall material; *OYRM*1, *Pleurotus ostreatus*; *Pleurotus salmoneo-str.*, *Pleurotus salmoneo-stramineus.*

Table 4.2   **Detection times of bacterial growth $t_{0.05}$ (h) for *S. epidermidis* 1798 in microtitration plates coated with fungal cell wall components**

Culture condition (1 %, w/v)*	*Grifola frondosa*	*Lentinula edodes*	*Monascus purpureus*	*OYRM*1	*Pleurotus ostreatus 1833*	*Pleurotus ostreatus 32783*	*Pleurotus citrinopileatus*	*Pleurotus eryngii*	*Pleurotus salmoneo-str.*	*Trametes versicolor*
NS	5.99 ± 0.33^{ab}	4.77 ± 0.23^{cd}	4.65 ± 0.05^{ab}	5.37 ± 0.11^{b}	10.78 ± 1.09^{a}	10.41 ± 0.78^{ab}	9.20 ± 0.36^{ab}	5.49 ± 0.08^{ab}	8.38 ± 0.93^{ab}	4.39 ± 0.12^{bc}
Fructose*	6.53 ± 0.37^{ab}	5.67 ± 0.27^{bc}	4.53 ± 0.09^{b}	4.88 ± 0.26^{c}	10.53 ± 2.00^{a}	9.55 ± 0.91^{b}	9.62 ± 0.65^{ab}	5.50 ± 0.06^{ab}	9.84 ± 0.68^{ab}	$\mathbf{3.84 \pm 0.08^{d}}$
Galactose*	$\mathbf{5.58 \pm 0.17^{b}}$	5.15 ± 0.46^{bcd}	$\mathbf{4.12 \pm 0.11^{b}}$	4.89 ± 0.12^{c}	9.69 ± 0.65^{a}	10.89 ± 0.69^{ab}	9.54 ± 0.70^{ab}	$\mathbf{4.96 \pm 0.10^{c}}$	9.28 ± 0.57^{ab}	3.86 ± 0.19^{d}
Glucose*	5.78 ± 0.28^{b}	5.07 ± 0.03^{bcd}	4.45 ± 0.12^{b}	4.80 ± 0.18^{c}	9.61 ± 0.17^{a}	9.24 ± 0.68^{b}	9.45 ± 0.70^{ab}	$\mathbf{4.96 \pm 0.11^{c}}$	8.27 ± 0.14^{ab}	3.90 ± 0.08^{d}
Maltose*	6.32 ± 0.23^{ab}	5.22 ± 0.08^{bcd}	4.27 ± 0.14^{b}	4.76 ± 0.15^{c}	10.27 ± 1.63^{a}	$\mathbf{9.09 \pm 0.68^{b}}$	9.79 ± 0.39^{a}	5.19 ± 0.19^{bc}	8.36 ± 0.33^{ab}	4.16 ± 0.18^{cd}
Mannose*	6.24 ± 0.44^{ab}	$\mathbf{4.52 \pm 0.86^{d}}$	5.80 ± 0.76^{a}	4.86 ± 0.16^{c}	9.96 ± 0.36^{a}	10.22 ± 0.63^{ab}	9.56 ± 0.37^{ab}	5.73 ± 0.09^{a}	10.03 ± 1.18^{a}	4.46 ± 0.12^{bc}
Sucrose*	6.38 ± 0.72^{ab}	5.93 ± 0.18^{b}	4.94 ± 0.22^{ab}	5.05 ± 0.10^{bc}	10.68 ± 1.47^{a}	10.00 ± 0.48^{ab}	9.36 ± 0.31^{ab}	5.61 ± 0.30^{ab}	9.57 ± 1.84^{ab}	4.63 ± 0.05^{ab}
Trehalose*	5.78 ± 0.13^{b}	5.65 ± 0.11^{bc}	5.24 ± 0.21^{ab}	4.99 ± 0.06^{bc}	9.71 ± 0.04^{a}	10.83 ± 0.48^{ab}	9.73 ± 0.44^{a}	5.89 ± 0.30^{a}	9.55 ± 1.25^{ab}	4.39 ± 0.11^{bc}
Xylose*	5.70 ± 0.08^{b}	5.87 ± 0.20^{b}	5.17 ± 0.21^{ab}	$\mathbf{4.74 \pm 0.27^{c}}$	10.02 ± 0.58^{a}	10.44 ± 1.45^{ab}	9.35 ± 0.21^{ab}	5.82 ± 0.09^{a}	9.22 ± 0.36^{ab}	4.51 ± 0.11^{abc}
YCW	5.79 ± 0.25^{ab}	5.61 ± 0.18^{bc}	4.51 ± 1.03^{b}	5.10 ± 0.08^{bc}	$\mathbf{9.19 \pm 0.03^{a}}$	10.57 ± 0.64^{ab}	$\mathbf{8.18 \pm 0.40^{bc}}$	5.84 ± 0.10^{a}	$\mathbf{8.24 \pm 0.25^{ab}}$	4.67 ± 0.12^{ab}
Control	$\mathbf{6.78 \pm 0.23^{a}}$	$\mathbf{8.02 \pm 0.54^{a}}$	$\mathbf{4.82 \pm 0.14^{ab}}$	$\mathbf{6.21 \pm 0.14^{a}}$	$\mathbf{10.47 \pm 1.25^{a}}$	$\mathbf{12.38 \pm 1.76^{a}}$	$\mathbf{7.19 \pm 0.71^{c}}$	$\mathbf{5.79 \pm 0.16^{a}}$	$\mathbf{7.37 \pm 0.15^{b}}$	$\mathbf{4.88 \pm 0.16^{a}}$
SEM	0.23	0.28	0.13	0.12	0.16	0.10	0.21	0.14	0.08	0.05

Results displayed are representative of the mean of triplicate determinations ± sum of standard deviation. Means within a column represent the fungal species grown in submerged liquid flask cultures under various conditions i.e. 1 % (w/v) carbon source supplementation. Conditions with the shortest $t_{0.05}$ (h) bound the most bacterial cells; Control, BSA (1 %, w/v). Detection time means marked by different letters within a column are significantly different ($p \leq 0.05$, ANOVA, Tukey-HSD), with alphabetical order of letters (a-d) representative of the least binding capacity to the best, respectively.

- Abbreviations: *S. epidermidis*, *Staphylococcus epidermidis*; NS, Non-supplemented cultures; SEM, Standard error of means; YCW, yeast cell wall material; *OYRM*1, *Pleurotus ostreatus*; *Pleurotus salmoneo-str.*, *Pleurotus salmoneo-stramineus*.

Bovine serum albumin (BSA), a purely proteinaceous product was used as a negative binding matrix (Sokurenko *et al.*, 1995). Yeast cell wall (YCW) material containing mannanoligosaccharides (MOS) obtained from Alltech® (Ireland) was selected as a reference material, as it was previously established as a positive binding matrix (as discussed in Section 1.3.2.2) (Newman *et al.*, 1993; Jaques *et al.*, 1994). Like fungi, the main components of the yeast cell wall are glucan, mannan and chitin (Section 1.1.3). It is recognised that the mannan fraction of the cell wall is involved in cellular adhesion, whilst the glucan portion is recognised by the immune system; both components being particularly abundant in the fungal cell wall. Relevant literature has demonstrated that polysaccharides derived from the fungal cell wall function as regulators of virulence or activators of the innate immune system. α- and β-Glucans, as well as complex mannans are well established immunoactive polysaccharides produced by fungi (Rodrigues *et al.*, 2011). The generation of an immunological response is an important factor of antimicrobial activity. In addition, there is a lack of information available on not only the interaction of the bacterial species tested in this investigation with adhesion capacity *in vitro*, but also use of fungi as alternative adhesion matrices as an initial point of prevention in general.

Fungal cellular components demonstrated that they may successfully serve as alternative binding matrices for *E. coli* (Table 4.1). Consequently, these components may effectively prevent infection from this microorganism. It is appreciable from the data that fungal cellular components adhered to this bacterium more effectively than YCW containing mannan-oligosaccharides (MOS) (Table 4.1). With the exception of *P. 1833*, *P. citrinopileatus* and *P. salmoneo-stramineus*, the same observation was made for *S. epidermidis* (Table 4.2). *G. frondosa*, *L. edodes*, *M. purpureus*, *OYRM*1, *P. 32783*, *P. eryngii* and *T. versicolor* components bound to *S. epidermidis* more effectively compared to YCW material (Table 4.2). The data revealed a difference in binding capability depending on the selected fungal species.

Bacterial adhesion is mediated by surface lectins that bind to complementary sugars on the host cells. *S. epidermidis* was most susceptible to adherence with *G. frondosa*, *M. purpureus* and *P. eryngii* components having been cultivated with supplemental galactose (1 %, w/v) (Table 4.2). The same is true for the adherence susceptibility of *G. frondosa*, *L. edodes* and *M. purpureus* components to *E. coli* (Table 4.1). Therefore, a degree of sugar specificity of bacterial lectins was established with a small number of fungi. Sugar specific lectins are widely distributed

among Eubacteria and have been studied for many years. Although galactose supplementation positively influenced monosaccharide composition in terms of glucose and galactose content for the majority of fungi (Table 3.3), there was no significant correlation observed with this sugar source and biological anti-adhesion capacity. It is known that the basic structure of a polysaccharide and its subsequent biological activity can vary depending on a number of factors, which may be the case here. These include its monosaccharide composition, configuration and position of glycosidic linkages, sequence of monosaccharides, as well as the nature, number and location of appended non-carbohydrate groups (Chen *et al.*, 2007; Silva *et al.*, 2012). These results agree with previous research which state that a high degree of structural complexity is associated with more potent immunomodulatory effects (Wasser, 2002; Silva *et al.*, 2012). Notably, the data suggests that a high degree of structural complexity is also associated with adherence capacity of alternative carbohydrate constituents from fungi. On the whole, these observations propose that individual carbon supplementations are favourable for biomass generation, polysaccharide production and potentially biological activity.

It is known that changes in the nutrient composition of bacterial growth medium influences metabolic activity and subsequently affects the content of the cell wall (Ellwood *et al.*, 1972). Adherence capacity of the pathogen to epithelial cells can be altered with this regard (Ofek *et al.*, 2003). However, much less attention has been given to changes in the growth conditions of alternative binding matrices from natural sources such as from fungi and their interaction with pathogens. As aforementioned, carbohydrates are well recognised by bacterial lectins and as a result, these polysaccharides possess the ability to block the binding of the bacteria to animal cells *in vitro* and *in vivo* (Sharon *et al.*, 2001). Adherence of fimbriae from gram-negative bacteria and the proteinaceous partners of gram-positive bacteria to host epithelium is essential for establishing infection (Ohlsen *et al.*, 2009). The main carbohydrate constituents of each fungal species were previously outlined in Table 3.3.

In general, the data revealed that depending on the fungal species, additional sugar source during development of mycelial biomass may influence antimicrobial capacity toward bacterial lectins. This action takes place *via* type-1 fimbriae of *E. coli* and cell bound protein receptors of *Staphylococcus*. All ten filamentous fungi demonstrated binding capacity towards gram-negative *E. coli 10778* compared to the control (Table 4.1). *P. citrinopileatus* and *P. salmoneo-stramineus* did not adhere to *S.*

epidermidis, however; YCW material, having previously been established as a positive binding matrix did not effectively adhere to this species of bacteria either (Table 4.2). Therefore, it is possible that non-adherence was elucidated as a result of the bacterial binding capacity. Other causes may have influenced adhesion, such as the presence of multiple bacterial adhesins and variability in adhesin-receptor interactions, which would depend on the stage of growth of the bacterium. *S. epidermidis* has a vast array of surface proteins also known as microbial surface components which recognise adhesive matrix molecules, with potential to interact with matrix proteins (Otto, 2009). *S. epidermidis* is well-known as a biofilm producing bacteria. Clinical and animal studies show the importance of this characteristic to its virulence (Christensen *et al.*, 1982; Deighton *et al.*, 1990; Mack *et al.*, 1996). Extracellular polysaccharides, as well as surface proteins, are known to contribute to initial adherence of bacteria and biofilm formation. The polysaccharide intercellular adhesin (PIA), a homoglycan composed of β-1,6-linked *N*-acetylglucosamine residues, is a major functional factor of biofilm accumulation of this opportunistic pathogen (Otto, 2008; Spiliopoulou *et al.*, 2012). This polymer is different from other poly-*N*-acetylglucosamine polymers found in nature (such as chitin) because of its β-1,6 linkage (Mack *et al.*, 1996). The replacement of β-1,4 by β-1,6 linkages renders the overall structure of the polysaccharide of *S. epidermidis* less compact and less rigid (Mack *et al.*, 1996). According to Otto (2009), this association has recently been detected in other microorganisms such as *Yersinia pestis* and *E. coli*. The most commonly known binding protein is a fibrinogen-binding protein which belongs to the serine/aspartate (SD) repeat family of which three variations are present in most strains of *S. epidermidis* (Otto, 2009). There are numerous studies about the different mechanisms and virulence factors which can influence initial attachment of *S. epidermidis*, however; there are no reports to date which examine the prevention of attachment using alternative binding matrices *in vitro*. With the knowledge that competence for primary attachment is an inherent characteristic of most *S. epidermidis* strains (Mack *et al.*, 1996), this antimicrobial strategy may be of particular interest for infections *in vivo*. Also, this species of bacteria served as a model for other proposed forms of bacterial adhesion capacity, alongside the reported type-1 fimbriae previously associated with anti-adhesion therapies. Altogether, by testing two different bacterial species it was predicted that both pathogens would have different binding specifications and as such; fungal cell wall constituents would demonstrate results

based on the characteristics of each bacterium. The data also illustrates that bacterial species specificity is an important consideration for development of novel adhesion agents and gut health.

Although medium supplementation demonstrated an effect on mycelial biomass production, it was unknown what effect supplementation during biomass production would have on availability of cellular components with capacity for bacterial attachment. In the present investigation, crude mycelial biomass extracts were tested for the presence of bioactive secondary fungal metabolites in the form of carbohydrates of cellular composition. Biological activity was found to be influenced by compositional breakdown depending on the fungal and bacterial species. Bioactive component production relating to or depending on the stage of growth of the organism could also affect biological activity. Studies have shown accumulation of compounds (like intracellular polysaccharides or storage lipids in the case of oleaginous microorganisms) is a secondary anabolic activity occurring after exhaustion of a nutrient other than carbon from the culture medium (Tang *et al.*, 2008; Papanikolaou *et al.*, 2011). This suggests that cellular bioactivity may also relate to secondary metabolite production following exhaustion of nutrients in the growth medium, which would associate biological activity of fungal components to a stage of mycelial production and metabolite extraction. This may account for differences in the data between species, as cellular component composition is related to not only the stage of growth but has been implicated as being heavily species specific.

The results obtained in this study show promising efficiency for the use of fungal components in anti-adhesion therapies; nonetheless, there are certain criteria which would have to be followed before a sufficient therapy could suffice, mostly depending on the bacterium tested. Further investigation on a molecular level would aid understanding of the binding mechanisms between both bacteria and the fungal cell wall constituents in this regard. It is recognised that growth of fungi with various carbohydrate supplements not only affected the biomass generation of each species (Section 3.1) but also affected the adherence ability of fungal cell wall components; most likely, owing to differences of carbohydrate constituents which lead to alterations in the interaction with bacterial surface lectins. A major benefit of the use of anti-adhesive agents is that they are not bactericidal; therefore, are unlikely that strain resistance to such agents will arise, particularly at the rate at which strains become resistant to antibiotics (Ofek *et al.*, 2003; Sharon, 2006). The future of anti-adhesion

strategies will rely on knowledge of the bacterial surface components such as lectins and the particular factors affecting their expression during the course of natural infection (Sharon *et al.*, 2001). In addition, better knowledge of the properties of the specific adhesins and the targeted receptors, together with the use of a combination of targeted inhibitor compounds (Ofek *et al.*, 2003; Signoretto *et al.*, 2012).

4.1.1 General discussion

Fungi are a source of dietary fibre and have high abundance of cellular carbohydrate polysaccharides; this concept along with their low digestibility, make them ideal alternative binding components for bacteria. The analogous results of fungal cell wall components and YCW material as a positive binding matrix are significant. In addition, under certain parameters some species were more efficient than YCW material. Overall, differences in bacterial proliferation due to variation in composition of the fungal cells is noteworthy; the results suggest that, in addition to host factors, compositional structure of binding matrices are an important factor in pathogenesis. The data also illustrated that cell wall constituents of fungi through modifications of culture medium may positively influence binding moieties responsible for adherence capability to both gram-positive and gram-negative bacterium. On the whole, fungal cell wall components are capable of effectively adhering to bacteria *in vitro* and in some instances, were as effective as yeast cell wall material.

4.2   Examination of antimicrobial activity

The systematic screening of products from natural sources is routine in the search for new biologically active substances (Salvat *et al.*, 2001). A number of species of fungi have been known to demonstrate a range of antimicrobial properties. For this reason, the aim of the present study was to perform an assessment of the antimicrobial potential of selected fungal species against highly relevant industrial and agricultural pathogens, including yeast. Crude aqueous mycelial extracts from liquid cultivated mycelium of several fungi were investigated.

There are a number of established methods used for the assessment of antimicrobial activity of naturally derived antimicrobials. Efficiency of an antimicrobial agent usually requires determination of its minimum inhibitory concentration (MIC) by either broth dilution or agar diffusion methods (agar well diffusion and disk diffusion). The MIC of an organism is known as the minimum

amount of antimicrobial agent required to inhibit the growth of the test organism. However, MIC determinations using agar well diffusion require a certain degree of hydrophilicity (Al-Bakri *et al.*, 2007). Therefore, when considering antimicrobial activity of insoluble substances, such as crude fungal extracts, broth dilution and disk diffusion methods are unsuitable. Additionally, broth dilution determination of MIC using organic solvents has concentration dependant toxicity toward most, if not all bacteria. In most studies, agar well diffusion is generally used for determination of sensitivity and MIC determinations are usually carried out by the broth dilution method (Sharma *et al.*, 2011). The size of the ZOI resulting from agar well diffusion tests relates to the diffusion rate of the antimicrobial compound(s), the degree of sensitivity of the microorganism, and the growth rate of the bacterium.

4.2.1   Screening of antimicrobial activity of filamentous fungi

The bacteriostatic nature of the crude mycelial extracts was tested against a range of gram-positive and gram-negative bacteria, including some environmental, industrial and clinical important pathogenic species. There are extensive reports of the antimicrobial properties of *G. frondosa*, *L. edodes* and *T. versicolor* (Takazawa *et al.*, 1982; Hirasawa *et al.*, 1999; Hatvani, 2001; Kodama *et al.*, 2002; Alves *et al.*, 2012). A number of polysaccharides and exopolysaccharides from these particular species have been developed for pharmaceutical application. Polysaccharides and particularly β-glucans, including Krestin (PSK) and polysaccharide peptide (PSP) from *T. versicolor*; Lentinan, isolated from *L. edodes*; Schizophyllan (SPG) from *S. commune*; Befungin from *Inonotus obliquus*; D-fraction from *G. frondosa*, amongst others, now have common commercial application (Wasser, 2011). The (1,3)-branched (1,6)-β-glucan also known as D-fraction from *G. frondosa* and lentinan from *L. edodes* are isolated polysaccharides recognised as biological response modifiers (BRMs) (Adachi *et al.*, 1988; Nanba *et al.*, 1988; Kodama *et al.*, 2002). Exopolysaccharide PSK from *T. versicolor* is well documented for its bioactivity. In addition, a highly water soluble, low cytotoxic polysaccharide-peptide (PSP) isolated from *T. versicolor* has been proposed as an anti-viral agent inhibiting replication of human immunodeficiency virus (HIV). The polysaccharide D-fraction from *G. frondosa* has demonstrated effectiveness against HIV infections and has also shown interaction with immune functions which subsequently positively affect antitumor function (Kodama *et al.*, 2002). The antimicrobial activities from *G. frondosa*, *L. edodes* and *T. versicolor* are

reported to be from polysaccharides or exopolysaccharides isolated from mycelia following growth in submerged cultures. Therefore, since the majority of compounds found to be most active in these species of fungi have current commercial application and with focus toward novel natural components from fungi of which there is little information, the investigation of antimicrobial sensitivity was focused on seven strains of selected filamentous fungi. In addition, similar to the components from *G. frondosa*, *L. edodes* and *T. versicolor* which are produced commercially, the present investigation was directed toward antimicrobial activity of mycelial biomass grown in submerged culture. Although previous work has suggested *Pleurotus* species as having antimicrobial properties, the majority of studies are focused on activity of extracts from its fruiting body portion, otherwise known as the oyster mushroom (Hearst *et al.*, 2009) and not extracts of mycelia following submerged cultivation. Studies suggest that there can be variation between fungal mycelium and mushroom fruiting body in terms of bioactive compound isolation (Lorenzen *et al.*, 1998; Lindequist *et al.*, 2005).

The diverse nature of organisms means they respond differently to different solvents; particularly at varying concentrations. Ethanol, methanol and DMSO are the most commonly used experimental solvents for analysis of antibacterial activity. Dimethyl-sulfoxide (DMSO) is a highly polar, stable substance and is a commonly used solvent in antimicrobial testing as most organic molecules, carbohydrates, peptides, inorganic salts and gases are soluble in this solvent (Balakin *et al.*, 2004). Acetone, methanol and ethanol are also frequently used solvents, particularly for antifungal testing. Ethanol and DMSO are preferred since they are miscible with water. However, it has been established that these solvents may exhibit a mutagenic effect or cause membrane damage in test organisms (Parasher *et al.*, 1978; Hakura *et al.*, 1993). Antimicrobial activity due to experimental substances such as certain organic solvents may lead to misinterpretation of the data (Salvat *et al.*, 2001), particularly with substances of low antimicrobial activity (Al-Bakri *et al.*, 2007). Previous studies have observed concentration dependent effects against various organisms such as dermatophytes, bacteria and fungi (Randhawa, 2006; Rekha *et al.*, 2006; Sharma *et al.*, 2011). Additional studies have shown that low concentrations of DMSO do not have any serious biological effect and therefore are appropriate for use in various bioassays (Balakin *et al.*, 2004). In addition, Eloff *et al.* (2007) demonstrated that acetone and DMSO are the safest solvents to use in fungal

bioassays. They verified that when ethanol or methanol are used in determining antifungal activity, it was possible to have false positive results, which may explain why the majority of literature investigating antimicrobial effects of fungal extracts use DMSO. This solvent was also recorded in the literature as being the optimal solvent for agar well diffusion experiments due to its use as an appropriate emulsifier throughout agar. In fact, DMSO has established low activity by every route of administration (oral, inhalation and dermal) and also has low environmental toxicity (Balakin *et al.*, 2004). Having investigated the effects of DMSO on cell viability, Al-Bakri *et al.* (2007) indicated that 7.8 % (v/v) DMSO concentration was the optimum concentration at which no significant reduction in viable cell count of the tested microbial strains was detected compared to negative controls (no DMSO). Therefore, in order to detect a potential antimicrobial effect based on tested fungal extracts, the concentration of DMSO used was 7.8 % (v/v). This concentration was deemed sufficient to assess antimicrobial efficiency of natural products (Al-Bakri *et al.*, 2007).

Crude aqueous extracts (0.1 g mL^{-1}) suspended in DMSO, were tested for inhibition of microbial growth *via* agar well diffusion, as outlined in Section 2.2.5.3. The test organisms included a range of gram-positive and gram-negative bacteria. The gram-negative bacteria examined were *Citrinobacter freundii*, *E. coli* 7134, *E. coli* 10778, *E. coli* 8879, *Pasteurella multocida*, *Salmonella enterica*, *Serratia marcescens*, and *Yersinia enterocolitica*. The gram-positive bacteria tested were; *Enterococcus avium*, *Enterococcus faecalis*, *Lactobacillus acidophilus*, *Lactococcus lactis*, *Listeria monocytogenes*, *Pediococcus pentosaceus*, *Pediococcus acidilactici*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus uberis*.

Table 4.3   **Antibacterial activity of several filamentous fungi against Gram-negative bacteria in terms of zone of inhibition (mm)[a]**

	Monascus	*Pleurotus*						*Neg. Control*[b]	*Ab. Control*[c]
	purpureus	*OYRM*1	*1833*	*32783*	*citrino.*	*eryngii*	*salm-str.*		
***E. coli* 8879**	18.33 ± 0.76	19.17 ± 0.76	18.25 ± 0.25	19.25 ± 2.75	18.33 ± 0.58	20.58 ± 2.67	20.00 ± 1.00	-	25.32[+] ± 0.63
***E. coli* 10778**	19.56 ± 0.69	19.21 ± 0.53	19.36 ± 0.85	19.39 ± 0.54	18.21 ± 0.85	18.63 ± 0.88	19.58 ± 0.63	-	29.81[+] ± 0.73
Salmonella enterica	20.00 ± 1.00	20.75 ± 0.90	17.75 ± 0.25	19.67 ± 1.53	17.67 ± 1.15	19.33 ± 1.04	19.83 ± 1.44	-	26.90[+] ± 0.36
Serratia marcescens	16.17 ± 0.29	17.67 ± 0.58	15.00 ± 0.00	15.67 ± 0.58	17.67 ± 0.58	16.83 ± 0.76	16.83 ± 1.04	-	21.17[+] ± 2.75
Pasteurella multicida	18.50 ± 1.30	17.25 ± 0.25	**19.58* ± 1.01**	16.92 ± 0.88	17.58 ± 0.88	18.17 ± 2.02	**19.42* ± 3.36**	-	22.39[26][++] ± 0.98
Yersinia enterocolitica	18.25 ± 1.80	14.75 ± 3.21	16.92 ± 4.40	17.50 ± 2.18	17.75 ± 0.43	16.50 ± 3.04	17.50 ± 4.87	-	38.67[++] ± 2.52
Citrinobacter freundii	15.67 ± 0.76	15.67 ± 0.58	15.00 ± 0.00	16.67 ± 1.15	17.67 ± 0.58	16.00 ± 1.00	17.00 ± 1.00	-	42.33[+++] ± 3.06
***E. coli* 7134**	22.17 ± 2.57	19.83 ± 0.29	17.71 ± 0.41	19.00 ± 1.00	20.33 ± 0.58	18.00 ± 1.00	18.00 ± 1.73	-	54.29[+++] ± 4.23

*(Row label on left margin: **Gram-negative bacteria**)*

Data is representative of the mean zone of inhibition (mm) following inoculation with gram-negative bacteria ± SD of triplicate experiments. The probability values were determined using Dunnett's test to evaluate any significance of samples to the antibiotic control mean, with a confidence level of 95 %. An asterisk [*] and indication in bold denotes no significant difference between inhibitory action of the test sample and antibiotic control. [a]Based on the diameter of the well (10 mm), a zone of inhibition > 11 mm was considered active. [b]Negative control (DMSO), a zone of inhibition < 11 mm denoted by [-]. If a double zone of inhibition was observed; the larger second zone was a zone of incomplete inhibition, indicated by the value in parentheses [ ]. [c]Antibiotic (Ab) control; [+]Ampicillin (1000 µg mL^{-1}), [++]Chloramphenicol (100 µg mL^{-1}), [+++]Erythromycin (94.6 µg mL^{-1}). *Pasteurella multicida* 10454 and *Yersinia enterocolitica* 11504 plates were incubated at 25 °C for 48 – 72 hours; all other bacterial cultures were incubated at 37 °C for 24 hours.

- Abbreviations: *E. coli*, *Escherichia coli*; *OYRM*1, *Pleurotus ostreatus*; *Pleurotus citrino.*, *Pleurotus citrinopileatus*; *Pleurotus salm.-str.*, *Pleurotus salmoneo-stramineus*.

Table 4.4   Antibacterial activity of several filamentous fungi against Gram-positive bacteria in terms of zone of inhibition (mm)[a]

		Monascus	*Pleurotus*						*Neg. Control*[b]	*Ab. Control*[c]
		purpureus	*OYRM*1	*1833*	*32783*	*citrino.*	*eryngii*	*salm-str.*		
Gram-positive bacteria	*E. faecalis*	15.00 ± 1.73	16.83 ± 0.76	16.17 ± 0.29	16.00 ± 1.50	16.33 ± 0.30	17.33 ± 0.58	16.67 ± 0.58	-	21.2[28][+] ± 0.41
	Lactobacillus acidophilus	**20.00*** **± 0.00**	**18.33*** **± 0.29**	17.21 ± 0.47	**19.38*** **± 2.30**	**19.08*** **± 2.32**	**19.92*** **± 1.29**	**20.08*** **± 2.67**	-	22.28[+] ± 1.95
	Lactococcus lactis	**19.83**** **± 1.44**	**16.58*** **± 1.15**	**16.33*** **± 0.58**	**18.21*** **± 1.29**	**17.00*** **± 2.20**	**16.92*** **± 1.30**	**17.33*** **± 1.53**	-	16.83[+] ± 1.20
	Listeria mono-cytogenes	**20.08*** **± 0.38**	**18.33*** **± 0.58**	**19.00*** **± 0.75**	**19.63*** **± 0.29**	**19.33*** **± 1.01**	**19.08*** **± 0.29**	**21.17*** **± 1.04**	-	24.00[+] ± 0.19
	Pediococcus acidilactici	**22.00**** **± 3.00**	16.58 ± 0.58	14.17 ± 0.95	15.83 ± 0.38	16.58 ± 1.51	16.92 ± 0.52	**18.92**** **± 1.01**	-	14.33[+] ± 2.31
	Pediococcus pentosaceus	**19.00*** **± 0.43**	17.04 ± 1.54	17.17 ± 0.79	**19.50*** **± 0.00**	**18.17*** **± 2.15**	**19.25*** **± 0.72**	**19.58*** **± 0.88**	-	21.00[+] ± 0.5
	S. uberis	*NI*[d]	15.9[19] ± 0.50[0]	18.6[22] ± 1.13[0]	15.9[21] ± 1.70[1]	16.8[0] ± 0.25[0]	15.3[21] ± 1.18[3]	18.75[2] ± 3.25[1]	-	42.0[0][+] ± 3.28[0]
	E. avium	15.75 ± 0.35	16.67 ± 2.08	19.75 ± 0.35	15.00 ± 0.00	15.33 ± 0.29	15.67 ± 0.58	17.67 ± 0.58	-	45.67[++] ± 1.53
	S. aureus	13.84 ± 0.23	14.50 ± 0.71	14.42 ± 1.53	17.00 ± 2.65	14.17 ± 1.89	17.50 ± 0.47	14.29 ± 0.06	-	39.08[41][+++] ± 2.16
	S. epidermidis	12.50 ± 0.71	12.67 ± 0.47	12.67 ± 0.10	13.2[16] ± 0.21	12.4[16] ± 0.59	14.42 ± 0.59	12.38 ± 0.53	-	35.25[38][+++] ± 0.90

Data is representative of the mean zone of inhibition (mm) following inoculation with gram-positive bacteria ± SD of triplicate experiments. The probability values were determined using Dunnett's test to evaluate any significance of samples to the antibiotic control mean, with a confidence level of 95 %. An asterisk [*] and indication in bold denotes no significant difference between inhibitory action of test sample and antibiotic control, extracts which were significantly different to antibiotic control, by way of enhanced inhibitory action are denoted by [**]. [a]Based on the diameter of the well (10 mm), a zone of inhibition > 11 mm was considered active. [b]Negative control (DMSO), a zone of inhibition < 11 mm denoted by [-]. [c]Antibiotic (Ab) control; [+]Ampicillin (1000 µg mL^{-1}), [++]Chloramphenicol (100 µg mL^{-1}), [+++]Erythromycin (94.6 µg mL^{-1}). [d][*NI*], No inhibition observed. If a double zone of inhibition was observed; the larger second zone was a zone of incomplete inhibition, indicated by the value in parentheses [ ]. *Listeria monocytogenes* 13449 was incubated at 30 °C for 24 hours; all other bacterial cultures were incubated at 37 °C for 24 hours, unless otherwise stated.
- Abbreviations; *E. faecalis*, *Enterococcus faecalis*; *L. monocytogenes*, *Listeria monocytogenes*; *S. uberis*, *Streptococcus uberis*; *E. avium*, *Enterococcus avium*; *S. aureus*, *Staphylococcus aureus*; *S. epidermidis*, *Staphylococcus epidermidis*; *OYRM*1, *Pleurotus ostreatus*; *Pleurotus citrino.*, *Pleurotus citrinopileatus*; *Pleurotus salm.-str.*, *Pleurotus salmoneo-stramineus*.

In this study, ten gram-positive and eight gram-negative bacteria were used to screen the antibacterial activities of crude mycelial extracts from seven fungal species. Each fungal species (1 %, w/v) displayed antimicrobial activity, with specific extracts performing better than certain concentrations of selected antibiotics in the agar

diffusion test (Table 4.3 and 4.4). To aid visualisation of zones of inhibition, some plates were sprayed with MTT, as previously outlined in Section 2.2.5.4. MTT is reduced to a purple complex formazan by viable bacteria. Non-viable bacteria do not reduce MTT, thereby enabling clear zones of inhibition (Marston *et al.*, 1999).

As well as clinically significant pathogens, many of the bacteria tested including; *Streptococcus uberis*, *Listeria monocytogenes*, *Pasteurella multicida*, *Staphylococcus aureus*, *Escherichia coli*, *Yersinia enterocolitica* and *Salmonella enterica*, present a constant challenge in today's agricultural environment. *Streptococcus uberis* for instance is a contagious environmental pathogen currently posing a potential threat, even to well managed dairy herds. Like *Staphylococcus epidermidis*, this pathogen has implications in mastitis and is the most common mastitis-causing pathogen in England, Wales and New Zealand (Bradley *et al.*, 2007). In Ireland, since the 1980's, staphylococcal isolates accounted for up to 66 % of mastitis cases, whilst streptococcal isolates accounted for 20 % (Barrett *et al.*, 2005). *Pasteurella multocida* is a significant causal agent of bovine respiratory disease, which is one of the most, if not the biggest killer of adult cattle. Presence of these pathogens in the animal feed industry illustrates the importance of strategies to prevent infection, preferably by natural means.

In general, the most susceptible gram-negative bacteria to fungal inhibitory action were *E. coli* spp., *Pasteurella multicida* 10454 and *Salmonella enterica* 15480 (Table 4.3). *M. purpureus*, *P. citrinopileatus* and *P. eryngii* showed on average the greatest inhibition of gram-negative microbial growth. The most susceptible gram-positive bacteria to fungal inhibitory action were *Lactobacillus acidophilus*, *Lactobacillus lactis*, *Listeria monocytogenes* and *Pediococcus pentosaceus* (Table 4.4). *M. purpureus*, *OYRM*1, *P. 32783*, *P. citrinopileatus* and *P. salmoneo-stramineus* extracts were most effective against *Listeria monocytogenes* and at 1 % (w/v) extracts were close to the inhibitory action of the antibiotic control ampicillin (1000 µg mL^{-1}). *M. purpureus*, *P. eryngii* and *P. salmoneo-stramineus* displayed on average the greatest inhibition of gram-positive microbial growth.

Overall, a broad spectrum of antimicrobial activity was observed. In addition, aqueous extracts from *P. 1833* and *P. salmoneo-stramineus* demonstrated significantly similar inhibition of the bacteria *Pasteurella multicida* when compared to chloramphenicol (100 µg mL^{-1}) (Table 4.3). Similarly, extracts from the majority of fungal species demonstrated antimicrobial activity against *Lactobacillus acidophilus*,

Lactococcus lactis, *Listeria monocytogenes* and *Pediococcus pentosaceus* statistically similar to the relevant antibiotic controls. With *M. purpureus* and *P. salmoneo-stramineus* extracts demonstrating significantly more inhibitory action against *Lactococcus lactis* and *Pediococcus acidilactici* compared to ampicillin (1000 µg mL) (Table 4.4). Generally, the gram-positive bacteria were more susceptible to inhibition from antimicrobial action than the gram-negative bacteria. Previous research has reported that a greater resistance from gram-negative bacteria is due to the complexity of the cell wall in comparison to gram-positive bacteria (Al-Bakri *et al.*, 2007; Ohlsen *et al.*, 2009; Alves *et al.*, 2012).

The outer wall and membrane of gram-positive bacteria is different to gram-negative bacteria in that, the cell wall and inner cell membrane of gram-positive bacteria is thick and consists mainly of peptidoglycan. While the cell walls of gram-negative bacteria consist of an inner cell membrane, a peptidoglycan layer and a thick layer of lipid-polysaccharide complex, which altogether plays a large role in resistance of these microorganisms (Opal *et al.*, 1999). The greater resistance of gram-negative bacteria is most likely to be a consequence of this outer membrane, which may act as a barrier to many environmental substances, such as antibiotics. In addition, differences in inhibition by the same fungal extract is believed to be due to inherent differences between the various bacterial species, such as; unique cell envelope composition and non-susceptible proteins, or the development of resistance and genetic exchange, resulting in different reactions to the same bactericidal causing agents (Cloete, 2003).

Antimicrobial agents are often categorised according to their principal mode of action. The mode of antibiotic action is usually related to interferences in synthesis of the cell wall (e.g. β-lactam and glycopeptide agents), modification of plasmatic membrane permeability or interference in nucleic acid or protein synthesis (Tenover, 2006). Ultimately, the bioactive compound must firstly bind to the bacterial surface and then traverse the cell wall (gram-positive) or the outer membrane (gram-negative) to reach its site of action at the cytoplasmic membrane or cytoplasm (Cloete, 2003). Within gram-positive bacteria species, antimicrobial substances can damage the cell wall and cytoplasmic membrane, which ultimately leads to leakage of the cytoplasm and its coagulation (Kalemba *et al.*, 2003). As aforementioned, significant differences are implicated in the outer membrane and unique periplasmic space found solely in gram-negative bacteria (Nikaido, 1994). Usually resistance of novel antibiotic agents is linked to their hydrophilic outer membrane surface which is rich in

lipopolysaccharide molecules, presenting a barrier to the penetration of numerous antibiotic molecules (Nikaido, 1994). Korber *et al.* (1997) demonstrated that bacteria which grow as adherent biofilms are significantly more resistant toward antimicrobial agents. Biofilm producing bacteria such as *S. epidermidis* have inherent resistance, causing limited diffusion of active agents not only through the biofilm matrix but also *via* interaction with the biofilm matrix (Cloete, 2003). It has been suggested that complete saturation of the biofilm with its thick glycocalyx is needed for microbial death, and as a consequence more time is required for the antimicrobial agent to contact cells that remain attached at the deepest portion of the biofilm (Cloete, 2003). Although antibacterial sensitivity was observed following incubation of extracts from each species, the antibacterial action was found to be least effective against *S. epidermidis* (Table 4.4). Possible limited diffusion of bioactive compounds through the biofilm matrix and the 24 hour time period for growth may represent its lack of activity compared to the other species and antibiotic control, as visualised by agar well diffusion. Another possibility of low activity may be a consequence of too low a concentration of the antimicrobial substance; bactericides often act bacteriostatically and may be only bactericidal at higher concentrations (Cloete, 2003). Nonetheless, the results of this study show that a respectable degree of activity was observed against both gram-positive and gram-negative bacteria (Table 4.3 and 4.4), which is indicative of broad spectrum antimicrobial compounds. In addition, previous results (Section 4.1) demonstrated that fungal cell wall components from these species were capable of serving as effective alternative binding matrices to *E. coli* and *S. epidermidis in vitro*, due to their high abundance of cellular carbohydrate polysaccharides.

Less polar compounds diffuse through agar at a slower rate, consequently, the active molecule polarity and the agar medium polarity may also contribute to the ZOI (Pimentel *et al.*, 2013). Thereby, antimicrobial action or efficiency of inhibition of bacterial growth is dependent on mode of action of the active compound within the extract, the cellular constituents of the pathogenic microorganism itself and the rate of diffusion of the microbe through the medium. The observation of incomplete inhibition, indicated by the value in parentheses [ ] in Table 4.4, highlights the influence of agar diffusion. This observation demonstrates that inhibition of each bacterium is largely dependent on the ability of the active agent to diffuse through the agar. This occurrence was mostly evident against *Streptococcus uberis*. Overall, incorporation of agar well diffusion methodology demonstrated that antimicrobial

compounds exhibit different degrees of inhibition toward different microbes depending on the antibiotic nature of the compounds themselves. *M. purpureus* did not reduce *Streptococcus uberis* growth and inhibition of *Staphylococcus epidermidis* was minimal in comparison to other bacteria. Nonetheless, the data revealed antibiotic substances from metabolite producing strains were present in aqueous extracts of each fungal species.

This broad spectrum of antimicrobial activity is believed to be attributed to the presence of secondary metabolites in the fungal mycelia, as a variety of phytochemicals from plants and fungi alike are known to exert antimicrobial activity against clinically significant infectious microorganisms (Tsuchiya *et al.*, 1996; Silva *et al.*, 2012). It is well known that secondary metabolites are produced by fungi during various stages of growth and serve as the survival functions for the organism producing them. A common feature of secondary metabolites is that most have established bioactivity, with many from fungi being studied for pharmacological or toxic effect towards humans and animals (Teichert *et al.*, 2011). There are a number of compounds that may be responsible for the inhibitory action of mycelial extracts (Table 4.3 and 4.4), these include; polyphenols (phenols, quinines, flavonoids, tannins and coumarins), terpenoids, alkaloids, lectins and polypeptides, all of which are known to be present in fungi. Some of the best studied metabolites are polyketides, peptides, terpenes and alkaloids (Teichert *et al.*, 2011). Fungal lectins, lactones, terpenoids, alkaloids, antimicrobial compounds, and metal chelating agents are implicated in immune function, therefore these compounds are particularly important from a therapeutic perspective (Wasser, 2011). Some secondary metabolites are responsible for texture, flavour and pigmentation of the mushroom fruiting body, as is the case for *M. purpureus*. The nature of the agar well diffusion method and the bacteriostatic nature observed suggest bioactivity may be due to an extracellular antimicrobial agent; similar to the polysaccharides or exopolysaccharides from *G. frondosa*, *L. edodes* and *T. versicolor*, which, as discussed, have current pharmaceutical application. Glucan, which was established as the predominant constituent of the fungal cell wall (Section 3.4) has proven antimicrobial activity. The ability of this polysaccharide to solubilise in water is an important characteristic of the biological activity of this particular compound. It is known that certain factors such as, molecular weight, chain length, number of chains on the main chain, linkages, and ionisation by acid are associated with solubility and pharmaceutical activity (Wasser, 2002; Ohno, 2005; Zhang *et al.*,

2007; Wasser, 2011). The exact mechanism of intestinal absorption is uncertain but it is believed orally administered insoluble β-glucans are degraded into smaller bioactive oligomers after ingestion (Lehmann *et al.*, 2000). There is potential for direct interaction of these constituents with the host leading to stimulation of the immune system (Lull *et al.*, 2005), as well as interaction with microbes prior to infection (Section 4.1).

In addition, various extraction solvents have been implicated in the isolation of bioactive metabolites and differences in activity may be based on the experimental solvent used during extraction. The bioactivity observed for mycelial extracts in the present investigation (Table 4.3 and 4.4) was exerted from crude aqueous extracts suspended in DMSO. According to Cowan (1999), anthocyanins, starches, tannins, saponins, terpenoids, polypeptides and lectins may exert bioactivity following component extraction with water alone (Kaul *et al.*, 1985). However, biological activity due to the presence of tannins or terpenoids are more often identified by extraction with less polar solvents i.e. ethanol or methanol extraction (Cowan, 1999). It is also worth noting, carbohydrates, peptides and most organic molecules are soluble in DMSO (Balakin *et al.*, 2004; Sharma *et al.*, 2011). Therefore, when taking into account the utilisation of the aqueous mycelial extracts and the solubility of particular compounds in DMSO, also with consideration of the most likely compounds present in each fungal species, the antimicrobial activity observed here was believed to be attributed to compounds of phenolic or lipid structure. This observation is looked into in further detail in Chapters 5 and 6. In certain circumstances, having performed initial screening using crude aqueous or alcoholic extractions, various organic extraction methods may follow; within which different components may be further extracted and isolated depending on the compound of interest and the solvent used. Notably, antibiotic activity of fungal carbohydrates is dependent on application i.e. normally β-glucans generate immunomodulatory effect in the host, which in turn affects disease state, unlike compounds which directly affect microbial growth stimuli. Exopolysaccharides are implicated in prevention of infection (Section 4.1), as opposed to direct cytotoxicity. Further isolation of the bioactive compounds responsible for positive restriction of microbial growth is detailed in Chapter 6.

In summary, the results suggest potential usefulness of various fungal extracts as antibacterial agents. The majority of fungal extracts demonstrated strong bacteriostatic and significant bactericidal power *in vitro* against 18 gram-positive and

gram-negative species of bacteria in comparison to their antibiotic control. Thus, the data revealed antibiotic substances from metabolite producing strains were present in aqueous extracts of fungi. In this study, inhibition of pathogenic microorganisms of agricultural and industrial importance was reported. Fungal extracts demonstrated activity against pathogenic microorganisms associated with nosocomial infections (*Staphylococcus epidermidis*, *Staphylococcus aureus*, *Citrinobacter freundii* and *Serratia marcescens*) as well as, microbes associated with negative implications to the agriculture industry (*Listeria monocytogenes*, *Pasteurella multicida* and *Streptococcus uberis*). Research relating to the antibacterial activity of compounds against these pathogens are of particular significance as many of these microorganisms are problematic to both animal and human health (Alves *et al.*, 2012). Like other bioactive compounds isolated from *G. frondosa*, *T. versicolor* and *L. edodes*, the data proposes that those from *M. purpureus* and *Pleurotus* spp. are not only an important source of dietary fibre which could be utilised as dietary supplements, but also could have medicinal application as an antimicrobial agent.

4.2.2 Antiyeast activity

Contaminant pathogenic yeast is a general concern in the animal health and nutrition industry. In the present investigation, dry mycelial biomass (1 %, w/v) from selected fungal strains were extracted according to Section 2.2.1.3 and tested for their ability to inhibit growth of yeast. Four species of yeast were examined; *Saccharomyces cerevisiae*, *Kluyveromyces fragilis*, *Kluyveromyces lactis* and *Debaryomyces hansenii*. *Debaryomyces hansenii* is a species particularly prevalent in the agricultural industry.

Table 4.5   Antiyeast activity of several filamentous fungi by agar well diffusion

Fungi	S. cerevisiae	%[1]	K. fragilis	%[1]	K. lactis	%[1]	D. hansenii	%[1]
M. purpureus	17.08 ± 0.00^b	63	14.83 ± 0.71^{bc}	55	15.00 ± 0.00^b	56	14.92 ± 0.72^b	54
OYRM1	14.13 ± 0.35^b	52	16.54 ± 0.00^b	61	16.67 ± 0.35^b	62	15.67 ± 0.71^b	57
P. 1833	17.08 ± 0.71^b	63	13.17 ± 0.00^c	49	16.17 ± 0.00^b	60	16.25 ± 0.35^b	59
P. 32783	16.33 ± 0.00^b	60	15.58 ± 0.70^{bc}	58	15.92 ± 0.35^b	59	16.83 ± 0.00^b	61
P. citrinopileatus	21.50 ± 0.35^b	80	14.08 ± 0.00^{bc}	52	15.17 ± 0.70^b	56	15.83 ± 0.00^b	58
P. eryngii	15.08 ± 0.76^b	56	15.75 ± 0.00^{bc}	58	17.08 ± 0.71^b	63	16.38 ± 0.35^b	60
P. salm-stramineus	15.83 ± 0.00^b	59	15.25 ± 0.71^{bc}	57	15.67 ± 0.71^b	58	16.85 ± 0.00^b	61
Control[2]	27.00 ± 1.00^a	100	26.94 ± 0.72^a	100	26.92 ± 0.72^a	100	27.42 ± 0.38^a	100

A zone of inhibition (mm) > 11 mm was considered active. Data are expressed as means $\pm$ SD of triplicate experiments. Means in the same column with different letters were significantly different ($p \leq 0.05$, ANOVA, Tukey-HSD). %[1]; Percentage inhibition based on corresponding control; Control[2]; Nystatin (50 mg mL^{-1}).
- Abbreviations: *OYRM1*[b], *Pleurotus* (*P.*) *ostreatus*; *P. salm-stramineus*, *Pleurotus salmoneo-stramineus*; *S. cerevisiae*, *Saccharomyces cerevisiae*; *K. fragilis*, *Kluyveromyces fragilis*; *K. lactis*, *Kluyveromyces lactis* and *D. hansenii*, *Debaryomyces hansenii*.

All species of fungi demonstrated antiyeast activity (Table 4.5). *P. citrinopileatus* showed particularly good inhibitory action (80 %) toward *S. cerevisiae* compared to the control. Each fungus inhibited the growth of *K. fragilis* to a similar extent, showing good activity. The same observation was made for *K. lactis* and *D. hansenii*, with *P. eryngii* and *P. salmoneo-stramineus* demonstrating the strongest activity, respectively. The results demonstrate the potential for fungal extracts to be used against pathogenic yeast contamination. As expected, the positive control applied to each plate showed strong uniformity. The observation of similar activity toward the various species of yeast may be a consequence of the same active compound(s) present in each fungal extract.

In summary, the objective of this investigation was to assess potential microbiological antiyeast activity from several species of filamentous fungi. As there is evidence to suggest both Basidiomycetes and Ascomycetes have antimicrobial properties, a select number of species were examined. This pilot scale investigation of antiyeast function, revealed activity toward a highly pathogenic strain of yeast. Further development in the form of isolation and development is necessary to identify the bioactive component(s). Following on from this, suitable pharmaceutical delivery systems should be explored to allow concentrated extracts to be prepared and delivered optimally, rather than crude ingestion of raw materials, which could promote resistance of the organism (Hearst *et al.*, 2009). On review of the literature, there appears to be little or no reports on the inhibitory effects of mycelial extracts of the fungi tested, grown in submerged liquid culture against the variety of yeast species tested and therefore, few comparisons could be made. Data from this investigation could aid further work in this area of fungal nutriceuticals.

4.3   General Conclusions

As stated by Cowan (1999), water soluble compounds; polysaccharides, polypeptides and various lectins, are commonly more effective as inhibitors of pathogen adsorption and would not be identified in screening techniques commonly used for antimicrobial analysis such as agar well diffusion. For this reason, biological activity of filamentous fungi was examined in terms of both anti-adhesion capacity and agar well diffusion sensitivity.

Pathogen binding capacity of fungal cell components was significant; with data indicating that fungal cell wall components were capable of effectively adhering to

bacteria *in vitro* and in some instances, were as effective as the reference standard (yeast cell wall material). The results propose that fungal cellular components (high molecular weight polysaccharides) can be used as a pathogen preventative measure by acting as alternative binding matrices through direct interaction with the microbial cell *in vitro*. Differences in efficiency of adherence due to variation in composition of the fungal cell is noteworthy; the results suggest that, in addition to host factors, compositional structure of binding matrices is an important factor in pathogenesis. The data illustrated that cell wall constituents of fungi through modification of the culture medium may positively influence binding moieties responsible for adherence capability to both gram-positive and gram-negative bacteria.

Agar well diffusion antimicrobial analysis established different degrees of microbial sensitivity due to species specificity. Relevant literature has reported that prior to any antimicrobial analysis, evaluation of the degree of toxicity from solvents such as DMSO toward both fungi and bacteria alike is important due to the mutagenic nature and possibility of membrane damage of the test organism. Apart from a direct effect on growth cytomorphology, reproduction and metabolic activity may be affected by some solvents. Not only should the basis of the method used be dependent on the nature of the test organism but other parameters should be tested besides a single criterion. Zone of inhibition analysis shows the degree of sensitivity or differentiation of activity in comparison to other parameters of a test compound; however, studies suggest this method is not suitable for minimum inhibitory concentration (MIC) of a compound, even though an inverse relationship may be suggested.

Mastitis, bovine respiratory disease and other agricultural illnesses due to opportunistic, contagious pathogens are a general concern to the agricultural industry. Illness can have a significant impact on the welfare and productivity of the dairy cow, as well as a subsequent economical consequence. The high prevalence of these pathogens in the animal feed industry illustrates the importance of alternative natural strategies to prevent infection. With the emergence of antibiotic resistance over the past decades, antimicrobials of natural origin have generated a considerable amount of global interest. Multiple drug resistant organisms present a new challenge to animal and human medicine. Hence, development of novel antimicrobial agents to reduce threat of further resistance is now widely accepted as a necessity. Application of antibacterial properties, with low cytotoxicity to animalia hosts, underscores the

usefulness of natural sources, particularly fungi for new medicines (Tamokou *et al.*, 2009).

To conclude, bioassay of aqueous extracts from fungal mycelia grown in submerged cultures revealed qualitative inhibitory activity against a range of pathogenically important bacteria particular to the agricultural industry. In the present investigation, crude mycelial biomass extracts were tested for the presence of bioactive secondary fungal metabolites in the form of carbohydrates of cellular composition. This study has shown that the crude extracts of *G. frondosa*, *L. edodes*, *M. purpureus*, *P. ostreatus*, *P. 1833*, *Pl. 32783*, *P. eryngii*, *Pl. citrinopileatus*, *P. salmoneo-stramineus* and *T. versicolor* have *in vitro* antimicrobial activities and has provided information which could lead to further research in the area of biological activity toward a range of pathogens of clinical significance. The results of anti-adhesion examination *in vitro* and agar well diffusion as well as other reports in the literature, demonstrate that differences in antimicrobial activity may arise from cell wall composition, biochemical constituents, physical and chemical differences between solvents used in biomass extraction and the test microorganism.

As a result of antimicrobial efficiency, interest was directed toward the isolation and identification of active components from fungal mycelial extracts. Evaluation of possible synergism of antioxidant activity was assessed (Chapter 5) prior to downstream identification of components with biological activity (Chapter 6).

5.   Antioxidant activity of fungal extracts

Antioxidant activity is one of the most important bioactivities known in higher Basidiomycetes (Elisashvili, 2012). Extensive epidemiology studies have demonstrated a variety of natural foods to be sources of multiple antioxidants which are strongly associated with reduced disease risk (Liu, 2004; Ferreira *et al.*, 2009). For years, research has focused on the antioxidant activity of the fungal fruiting body, as this stage of fungal growth is widely accepted as a nutritional food source. With recent research demonstrating that extracts of fungi possess important biological activities; there is increasing interest in the antioxidant potential of fungi at various stages of their development. In addition, there has been little research focused on antioxidant production by submerged cultures of medicinal fungi. Studies have demonstrated potent antimicrobial, antioxidant and antitumor function of numerous fungal species are associated with the presence of β-glucans, lipids and protein constituents in aqueous and alcoholic extracts (Iwalokun *et al.*, 2007). In addition, ergothioneine, a naturally occurring amino acid has attracted much attention due to its antioxidant action in humans and its production by fungi *via* methylation of histidine (Jones *et al.*, 2014). Fungal lectins, lactones, terpenoids, alkaloids, antimicrobial compounds, and metal chelating agents are important from a therapeutic perspective as they are implicated in stimulating immune function (Wasser, 2011). For this reason, aqueous mycelial extracts were examined for further biological activity in the form of antioxidant function, based on hot water and methanol extraction.

The objective of this research was to provide more information on secondary metabolite production in the mycelial biomass following cultivation in submerged liquid medium. Antioxidant assessment is based on a number of methods depending on the expected mechanisms and dynamics of the antioxidant action. As previously described in Section 1.4.4, activity may be based on colorimetric reactions, measure of scavenging ability following reduction of metal ions or radical scavenging capacity based on competitive methods. Due to mechanistic differences, antioxidants may be classified by their functionality as preventative, scavenging and repair and *de novo* antioxidants (Niki, 2010). Generally, antioxidant efficiency is dependent on the type of oxidant; for example, α-tocopherol is a potent radical scavenger of oxidants, however it has poor activity against lipid peroxidation (Niki *et al.*, 2000). Likewise,

carotenoids being poor radical scavenging antioxidants are potent inhibitors of the oxidation induced by singlet oxygen (Niki *et al.*, 2000). Oxidisable substrates include a variety of possible compounds including, lipids, proteins, DNA, and carbohydrates (Becker 2004). Therefore, depending on the mode of action by the selection of assays employed, details of the active component(s) may be generated. In the present investigation, antioxidant capacity was measured (as detailed in Section 2.2.6) in terms of; β-carotene bleaching (Section 5.2.1), radical scavenging activity using DPPH and ABTS$^+$ antioxidant methods (Section 5.2.2), assessment of metal ions using reducing power (FRAP) and cupric ion reducing capacity (CUPRAC) (Section 5.2.3), and chelating ability against ferrous ions (Section 5.2.4). Halliwell *et al.* (1989) defines antioxidants as "any substance that when present in low concentrations compared to those of an oxidisable substrate significantly delays or prevents oxidation of that substance". This observation does not limit antioxidant activity to a definite group of chemical compounds nor refer to any specific mechanism of action (Becker *et al.*, 2004). Due to differential use in the literature of the term 'antioxidant', and to prevent inconsistency and confusion in this investigation, the term 'antioxidant' was used to describe overall antioxidant activity depending on the particular method of analysis.

BHT (2, 6-di-tert-butyl-4-methoxyphenol) and α-tocopherol were employed as reference standards. BHT, a chemically synthesised phenolic antioxidant is commonly used in food stuffs to prevent spoilage. As potential carcinogenic substances, the use of synthetic BHT and BHA (2-tert-butyl-4-methoxyphenol) has been restricted in recent years, causing increased interest in antioxidant substances of natural origin (Branen, 1975). The most active form of vitamin E in humans; α-tocopherol, is generally used as a positive control in most antioxidant evaluations (Ferreira *et al.*, 2009). BHT was employed in the β-carotene bleaching and CUPRAC assays. A solution of α-tocopherol (vitamin E) is known to be a potent antioxidant even at low concentrations (Lambert *et al.*, 1996). α-Tocopherol was incorporated into the β-carotene bleaching, DPPH radical scavenging, reducing power, CUPRAC and chelating assays. Standard curves of trolox, EDTA and gallic acid, which are also known as potent antioxidants, were sufficient as reference standards in the ABTS$^+$, chelating and phenol assay. Synthetic antioxidants such as BHA and BHT only tend to have one mode of action, i.e. *via* free radical scavenging and are not able to sequester metal ions through the metal catalysed route (Tsao *et al.*, 2004). Later in this section, the total content of the suspected main compounds from *Pleurotus* spp., *G. frondosa*,

M. purpureus and *T. versicolor* responsible for antioxidative capacity were explored (Section 5.3).

5.1   Extraction process and recovery

Extraction is the first step in the isolation of pure biologically active compounds. When extracting compounds from fungi or any living source, the type of solvent, the extraction process and cultivation characteristics affect the type of compound extracted (Section 1.5.4). Extraction conditions are a major concern for enhancement of efficiency in order to obtain the highest yields of antioxidative compounds from natural sources (Yim *et al.*, 2009). When exploring natural products for new bioactive compounds, fractionation is usually required using solvents of varying polarity (Cowan, 1999; Dinan *et al.*, 2001). With this knowledge, concentrations of pure compounds from crude aqueous extracts of fungal mycelia produced by submerged fermentation were subjected to different extraction conditions. The two extraction processes used in the present investigation, included; a microwave assisted (MA) extraction process, also named hot water (HW) extraction, and a solvent extraction in the form of methanol. Lyophilised biomass (also referred to as the crude extract) was included in antioxidant investigation for direct comparison of efficiency.

Table 5.1   Extraction recovery (%) from filtrate

Fungi	% Recovery[a]	
	Methanol extract	**Hot water extract**
Grifola frondosa	30.61 ± 2.89	58.28 ± 8.00
Lentinula edodes	33.01 ± 4.88	56.80 ± 4.42
Monascus purpureus	5.97 ± 2.19	40.30 ± 6.55
*OYRM*1	23.57 ± 3.39	56.76 ± 8.43
Pleurotus ostreatus 1833	33.93 ± 2.10	50.55 ± 3.36
Pleurotus ostreatus 32783	27.49 ± 8.05	56.23 ± 3.23
Pleurotus citrinopileatus	20.57 ± 8.20	51.15 ± 0.53
Pleurotus eryngii	33.15 ± 2.31	54.96 ± 1.45
Pleurotus salmoneo-stramineus	19.81 ± 6.11	57.72 ± 9.47
Trametes versicolor	30.60 ± 2.79	42.02 ± 6.76

Data represents the mean ± standard deviation (n = 3) of recovered dry extract following hot water and methanol extraction from crude mycelial biomass (5 %, w/v). [a]Total (%) dry weight recovered following extraction.
- Abbreviations: *OYRM*1, *Pleurotus ostreatus*.

Table 5.1 represents the extraction yields, expressed as the percentage of sample on the basis of dry weight (DW) recovered. The extraction processes employed provided a good source of comparison between the crude mycelial extract (intact cell wall) and the microwave-assisted hot water extract (filtrate), where

polysaccharides and other metabolites have been extracted from the cell. As discussed in Section 3.2, the fungal cell wall contains polysaccharides which are known for their immune supporting function, this is a prerequisite of both the mycelium and fruiting body of fungi. A hot water extraction with successive boiling steps was performed in order to obtain extracts containing compounds of high molecular weight such as polysaccharides and low molecular weight compounds such as phenols (Vaz *et al.*, 2011). Both types of compounds are known to have important biological activity (Ferreira *et al.*, 2010). Previous research found aqueous extracts using hot water performed on both fruiting body and mycelium of *Ganoderma* species resulted in the extraction of many proteins, lectins and polysaccharides (Kawagishi *et al.*, 1996; Bao *et al.*, 2002). Extraction of polysaccharides from the fungal cell is an important procedure for their application in biological processes, which although beyond the scope of this investigation, could be precipitated from aqueous suspension of mycelia by the use of ethanol (Whistler *et al.*, 1965; Lee *et al.*, 2007b). Hot water extraction is the most widely used technique for polysaccharide extraction and is the only validated method to effectively dissolve indigestible fibre (chitin), allowing the fibre to be removed from the extract when the water is removed (Ong *et al.*, 2003). This process concentrates the polysaccharides to the effective levels identified in published research. The extraction of polysaccharides from cultured mycelium by hot water extraction has increased in recent years due to its success in isolating cellular components. Many publications are based on the optimisation of different hot water extraction methods. Prasad *et al.* (2010) employed ultrasound technology combined with high pressure to extract bioactive compounds from plant tissue and as result effectively determined the optimal extraction conditions by response surface technology. Methods of extraction in general are associated with long extraction times and high temperatures. Microwave assisted extraction permitted improvement of extraction of bioactive compounds in combination with an accelerated extraction process.

The extraction yield obtained for methanol fractions was considerably lower than the yield recovered for water soluble fractions for all ten selected species (Table 5.1). Compared to previous reports the quantity recovered following hot water extraction (40 – 58 %) was quite high. In a study by Huang *et al.* (2010) using hot water extraction, less than 10 % dry weight was recovered. The recovery (%) of *M. purpureus* was substantially less than the other species with both extraction processes.

This is believed to be associated with the characteristic morphological differences of this species compared to the others. When performing natural compound extraction using polar organic solvents such as methanol; flavonoids, alkaloids, coumarins, fatty acids and triterpenes are commonly extracted (Cowan, 1999). Compounds such as terpenoids present in fungi are known to be affected by potency of their functional groups and aqueous solubility (Knobloch *et al.*, 1989). Furthermore, anthocyanins, starches, tannins, saponins, terpenoids, polypeptides and lectins, are known to exert bioactivity following component extraction with water alone (Kaul *et al.*, 1985; Cowan, 1999).

5.2   Assessment of antioxidant capacity for various fungal extracts

5.2.1   Antioxidant capacity by β-carotene/linoleic acid bleaching

The β-carotene bleaching method of determination of antioxidant capacity is a colorimetric assay based on the ability of volatile organic compounds to inhibit the conjugated diene hydroperoxide formation from linoleic acid and β-carotene coupled oxidation in an emulsified aqueous system (Lopes-Lutz *et al.*, 2008). Antioxidants donate hydrogen atoms to quench the radicals and thus reduce the degree of β-carotene bleaching caused by free radicals which decolorise the β-carotene emulsion. The degree of decolourisation is a measure of lipid peroxidation caused by the presence of free radicals, resulting in a measure of biological antioxidant capacity. Inhibition of linoleic acid oxidation is an important issue in food processing and preservation (Oke *et al.*, 2011). Besides giving rise to mutagenic lipid epoxides, hydroperoxides, alkoxyl and peroxyl radicals, lipid peroxidation is also implicated in food deterioration, affecting colour, flavour, texture and nutritional value (Gutteridge *et al.*, 2000). This method is useful for the evaluation of antioxidant capacity of compounds in emulsions, as food systems usually comprise of multiple phases where lipids and water coexist with some emulsifiers (Abdullah *et al.*, 2012). Table 5.2 shows the antioxidant capacity of crude, hot water and methanol extracts with the coupled oxidation of β-carotene and linoleic acid. The fungal extracts and reference standards were tested at 1 mg mL^{-1} for a direct comparison of activity. Controls were included with each set of analysis as a consequence of the complex β-carotene reagent preparation.

Table 5.2   Antioxidant activity (%) of fungal extracts measured by β-carotene bleaching

Fungi	Crude extract	Hot water extract	Methanol extract
Grifola frondosa	84.09 ± 2.37[a]	34.83 ± 1.57[c]	65.22 ± 2.90[b]
Lentinula edodes	80.77 ± 1.70[a]	22.07 ± 1.31[b]	76.56 ± 0.85[a]
Monascus purpureus	80.45 ± 2.83[a]	60.13 ± 3.95[b]	**83.94 ± 1.22[a]***
*OYRM*1	24.94 ± 1.36[c]	63.37 ± 2.67[b]	77.41 ± 1.30[a]
Pleurotus ostreatus 1833	71.33 ± 3.53[a]	50.82 ± 3.74[b]	63.49 ± 2.84[a]
Pleurotus ostreatus 32783	48.93 ± 3.11[b]	**65.98 ± 2.63[b]**	83.13 ± 1.45[a]*
Pleurotus citrinopileatus	**84.40 ± 1.39[a]**	56.80 ± 2.01[b]	53.60 ± 4.64[b]
Pleurotus eryngii	38.57 ± 0.63[c]	62.65 ± 2.19[a]	55.22 ± 0.81[b]
Pleurotus salmoneo-stramineus	33.93 ± 0.08[b]	57.73 ± 2.54[a]	60.11 ± 4.72[a]
Trametes versicolor	35.59 ± 6.08[b]	59.71 ± 4.49[a]	60.22 ± 0.76[a]
BHT	96.47 ± 1.30[a]*	92.17 ± 1.09[b]*	93.66 ± 0.25[b]*
α-tocopherol	96.68 ± 1.48[a]**	90.53 ± 0.22[b]**	90.05 ± 0.09[b]**

Data is representative of mean ± SD of triplicate measurements. Both the fungal extracts and reference standards were measured at a concentration of 1 mg mL^{-1}. The strain with the lowest EC_{50} value per extract is indicated in bold. Means in the same column which were not significantly different to the reference standards α-tocopherol and BHT are denoted by [*] and [**], respectively ($p \leq 0.05$, ANOVA, Tukey-HSD). For the same fungi between different extraction processes, means with different letters (a,b,c) were significantly different ($p \leq 0.05$, ANOVA, Tukey-HSD), in the order of highest (a) to lowest (c) activity.
- Abbreviations: BHT, Butylated hydroxyltoluene; *OYRM*1, *Pleurotus ostreatus.*

The antioxidant activity (%) assayed (Table 5.2) shows the ability of the various extracts to inhibit the peroxidation of linoleic acid (Lee *et al.*, 2007a). The observed measure of activity using this method of analysis may be due to the reducing of hydroperoxide, inactivation of free radicals, complexation with metal ions, or a combination thereof (Taylor *et al.*, 1980; Lee *et al.*, 2007a). Differences in the level of oxidation from the different extraction methods employed are believed to be due to the presence of different quantities of antioxidant molecules isolated, depending on the species. With regard to the crude, hot water and methanolic extracts, *P. citrinopileatus* (84 %), *P. 32783* (66 %) and *M. purpureus* (84 %) had the highest antioxidant activity, relative to BHT and α-tocopherol (Table 5.2). A study by Taylor *et al.* (1980) found that cysteine exhibited better protection of a linoleate emulsion than BHA, BHT and α-tocopherol against oxidation by haemoglobin. Lee *et al.* (2007a) stated that mycelium of *P. citrinopileatus* containing 9.11 mg g^{-1} of free cysteine was steadily soluble in water and consequently was believed to have contributed to the antioxidant activity of cold and hot water extracts. This observation may be true of the extracts in the present study also. Despite being beyond the scope of this project, it would be interesting to measure the amino acid content of the mycelia at various stages of submerged

fermentation, with reference to antioxidant activity. Variation between oxidation ability of the extracts may also be due to possible deviations of phenolic, ascorbic acid and β-carotene content in response to the aging process of the particular fungi. Although conditions were determined for optimal biomass yield, some secondary metabolites are not produced or are not essential during active growth. Therefore, similar to the effect on antimicrobial compounds, the stage of growth of the individual species is also a factor with respect to variations in antioxidant activity (Barros *et al.*, 2007b). In addition, there are a number of studies which illustrate the effect of processing and growth conditions on antioxidant potential (Ferreira *et al.*, 2009). In particular, high temperature processes are known to cause changes in the extractability of bound polyphenolic and flavonoid compounds due to destruction of the cell wall, these compounds may be released more easily compared to those of raw materials (Choi *et al.*, 2006b). This is believed to attribute to the lower bleaching inhibition seen by the hot water extracts of some filamentous species (Table 5.2). The higher antioxidant activity of the hot extract of *OYRM*1, *P. 32783*, *P. eryngii*, *P. salmoneo-stramineus* and *T. versicolor* illustrates the retention of antioxidant activity from intracellular constituents using this method of extraction. Most capacity was retained in the crude extract of *G. frondosa*, *L. edodes*, *M. purpureus*, *P. 1833* and *P. citrinopileatus*. This observation suggests that further extraction of the biomass may be detrimental to β-carotene bleaching activity of these species. The methanol extraction process was most successful in retaining the overall content of antioxidant compounds across the range of species tested. Methanol extracts from *M. purpureus*, *OYRM*1, *P. 32783*, *P. eryngii* and *T. versicolor* resulted in the highest inhibition of linoleic acid oxidation. Altogether, the methanol extracts restricted oxidation by at least 53 %. The antioxidant activity of *M. purpureus* and *P. 32783* were statistically similar ($p \leq 0.05$) to that of α-tocopherol at the same concentration (Table 5.2).

As mentioned in Section 5.1, biological activity may be affected by the solubility of active compounds. The measure of activity can be associated with various natural compounds following consideration of the solvent concentration (Cowan, 1999). For this reason, the alcoholic solvent extract, in this case methanol, was subjected to different concentrations of water (100 %) and methanol (30 and 100 %). Aqueous methanol (30 %) was chosen to test the efficiency of solubilisation of antioxidant compounds from fungal extracts. Consequently, the solubility of the

extracted antioxidant compounds from the selected species in terms of β-carotene bleaching capacity was explored (Figure 5.1).
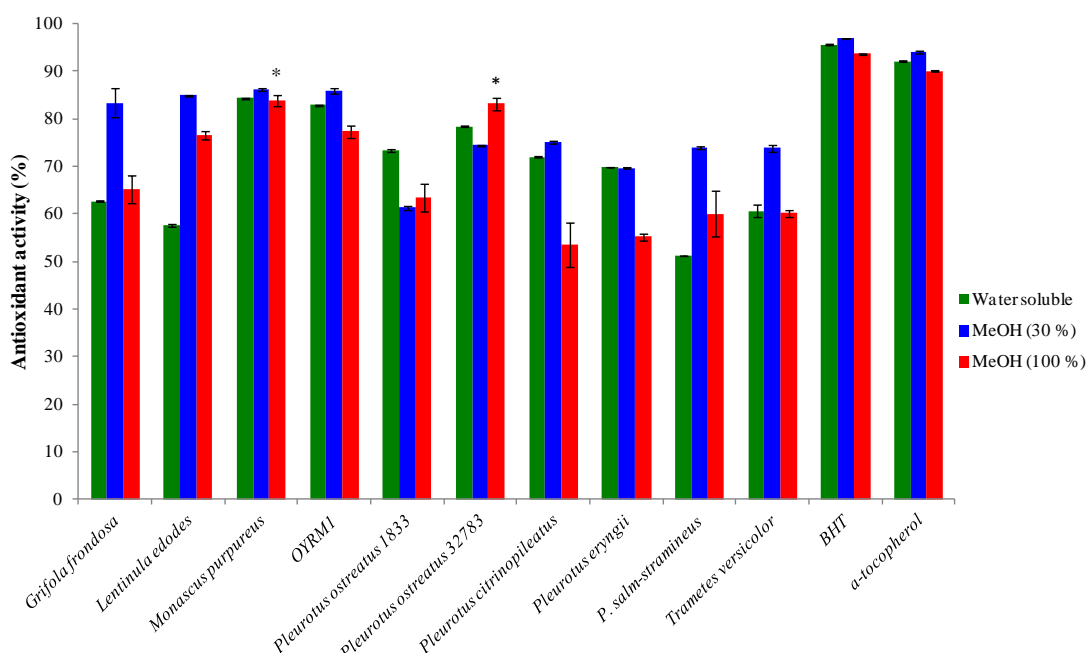
Figure 5.1   Antioxidant activity of the methanolic extracts assayed by the β-carotene bleaching method

Results plotted are a representation of the mean of triplicate experiments, with the standard deviation of each treatment represented by error bars. Means that were not significantly different to the reference standards α-tocopherol and BHT are denoted by (*) and (**), respectively ($p \leq 0.05$, ANOVA, Tukey-HSD).
- Abbreviations: *P. salm-stramineus, Pleurotus salmoneo-stramineus*; *OYRM*1, *Pleurotus ostreatus*.

Figure 5.1 displays the percentage oxidation by the methanol extract (1 mg mL^{-1}) of each fungal strain. There was little variation in carotene destruction of the methanol extract with different solutions. As expected, 30 % methanol yielded the highest percentage of oxidation for *G. frondosa*, *L. edodes*, *M. purpureus*, *OYRM*1, *Pl citrinopileatus*, *P. salmoneo-stramineus* and *T. versicolor* (Figure 5.1). The results from the water extract and 30 % (v/v) solvent concentration indicated a higher percentage oxidation compared to the 100 % methanol extracts of *OYRM*1, *P. citrinopileatus*, *P. eryngii* and *T. versicolor* (Figure 5.1); suggesting that compounds from these species might present a moderately polar profile. Altogether, the data revealed evidence that various extracts from the different species of fungi were capable of effectively reducing the extent of β-carotene destruction by neutralising the linoleate free radical to a high degree.

5.2.2 Radical scavenging activity of various fungi as determined by ABTS$^+$ and DPPH methods

Radical scavenging action is dependent on both the reactivity and the concentration of the antioxidant (Apak *et al.*, 2007b). According to Niki (2010), the capacity of an antioxidant compound for scavenging free radicals should be assessed by two factors; the rate of scavenging radicals and the number of radicals each antioxidant molecule can scavenge. Both factors can be measured by reaction with a stable reference free radical such as DPPH and ABTS$^+$ (Niki, 2010), as outlined in Section 5.2.2.1 and 5.2.2.2, respectively. The ABTS and DPPH assays measure the ability of the antioxidants to scavenge free radicals prior to them attacking biologically essential molecules through donation of a hydrogen atom (Niki, 2010). The concentration of an antioxidant or the time needed to reach a 50 % decrease of initial DPPH/ABTS$^+$ is known as the EC$_{50}$ value expressed as mg of extract per mL. This was used throughout this section (Section 5.2.2) to enable comparisons of efficiency between extraction processes with regard to isolation of radical scavenging compounds. Due to extensive research in the literature and previously established reports of antioxidant activity in some of the species incorporated throughout this study, assessment of DPPH radical scavenging activity was based on species, having been grown by SLF and extracted separately by either hot water or methanol; of which there is little information. Therefore, preliminary investigations proceeded with the analysis of the *Pleurotus* spp. and *M. purpureus* which were examined in Section 4.2 for antimicrobial activity.

5.2.2.1 *DPPH radical scavenging ability of extracts*

DPPH (2,2-diphenyl-2-picrylhydrazyl) free radical scavenging is a common method of determining antioxidant capacity. Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid peroxidation, by way of reduction of methanolic DPPH solution in the presence of a hydrogen donating compound (antioxidant) (Vamanu, 2012). The resulting decolourisation upon absorption of hydrogen from the antioxidant is stoichiometric with respect to the degree of reduction and the remaining DPPH, measured after a certain time and corresponds inversely to the radical scavenging activity of the antioxidant. α-Tocopherol was used as a reference standard in this assay. This compound being a powerful and commonly used antioxidant is particularly well known as a scavenger of free radicals (Vamanu, 2012).

This assay was assessed spectrophotometry (Liang *et al.*, 2009) using a modified version developed on the microplate as outlined in Section 2.2.6.3 for increased efficiency and reproducibility.
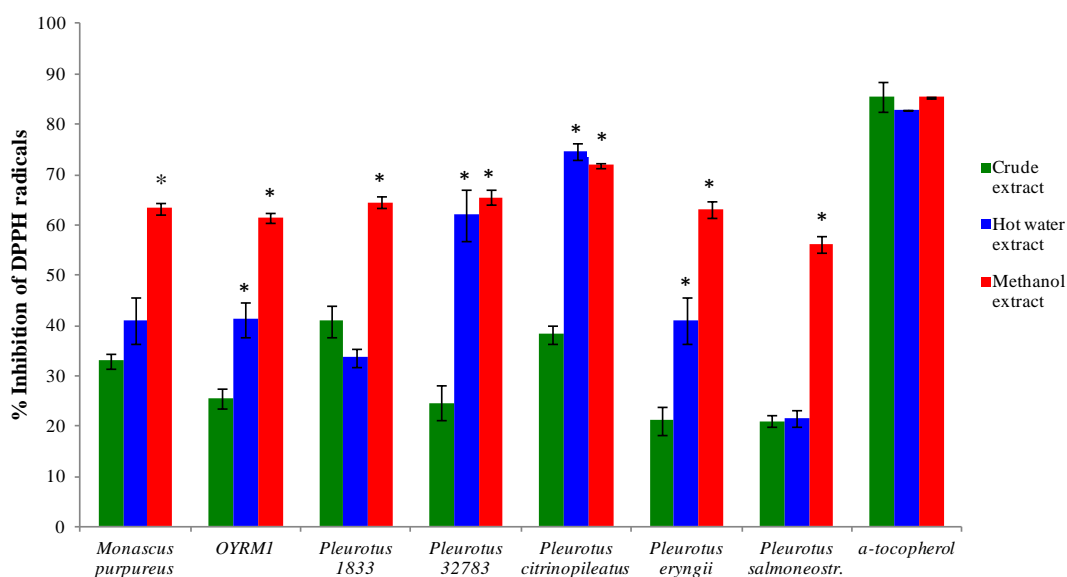
Figure 5.2 **Radical scavenging activity (%) of extracts (10 mg mL^{-1}) against DPPH radicals**

Results plotted are the mean activity (%) of extracts (10 mg mL^{-1}) with the standard deviation of each treatment represented by error bars (n = 3) compared to positive control α-tocopherol (1 mg mL^{-1}). An asterisk [*] denotes a significant positive impact ($p \leq 0.05$) in scavenging activity compared to the crude extract (ANOVA, Tukey-HSD).
- Abbreviations: *OYRM*1, *Pleurotus ostreatus*.

As can be seen in Figure 5.2, the various extraction processes (crude (aqueous), hot water, and methanol (100 %, v/v)) were shown to scavenge the stable DPPH radical to different degrees. The crude extract of *P. 1833* scavenged the highest percentage of DPPH radicals (41 %) compared to the other fungal crude extracts. Nonetheless, the crude extract from the majority of fungi at 10 mg mL^{-1} did not show maximum relative activity (Figure 5.2). The hot water and methanol extract of *P. citrinopileatus* demonstrated the highest scavenging activity (75 and 72 %, respectively), similar to the value of α-tocopherol at 1 mg mL^{-1} (84 %). The scavenging ability of hot water extracts on DPPH radicals demonstrated moderate to high activity (22 – 75 %) at 10 mg mL^{-1} (Figure 5.2). At the same concentration, the methanol extracts had scavenged 56 – 71 % free radicals. The methanol extract significantly increased the DPPH radical scavenging effectiveness ($p \leq 0.05$) compared to the crude extract, for all species tested (Figure 5.2). In addition, the hot water extract significantly increased the DPPH radical scavenging effectiveness ($p \leq 0.05$)

compared to the crude extract, for *OYRM*1, *P. 32783*, *P. citrinopileatus* and *P. eryngii* (Figure 5.2).

In Table 5.3 the extract concentrations (mg mL^{-1}) required to scavenge 50 % of initial DPPH, due to reduction of radicals by different fungal extracts is illustrated. Radical scavenging activity in the form of EC$_{50}$ values were obtained by interpolation from linear regression analysis and were calculated from the total percentage activity per extract against extract concentration.

Table 5.3   Scavenging activity (EC$_{50}$ mg mL^{-1}) of fungal extracts on DPPH radicals

Fungi	Crude extract	Hot water extract	Methanol extract
Monascus purpureus	>50	16.68 ± 0.01	3.56 ± 0.16
*OYRM*1	>50	12.57 ± 0.01	**2.99 ± 0.35**
Pleurotus 1833	>50	10.26 ± 0.01	3.89 ± 0.22
Pleurotus 32783	>50	**8.98 ± 0.00**	3.01 ± 0.17
Pleurotus citrinopileatus	>50	9.92 ± 0.01	4.07 ± 0.02
Pleurotus eryngii	>50	9.54 ± 0.04	3.21 ± 0.40
P. salmoneo-stramineus	>50	13.61 ± 005	3.01 ± 0.34
α-tocopherol		0.05 ± 0.01	

Data are expressed as means ± SD (n = 3) of upper and lower limit of dose interpolation curve, with 95 % confidence. The strain with the lowest EC$_{50}$ value per extract indicated in bold.
- Abbreviations: *P. salmoneo-stramineus, Pleurotus salmoneo-stramineus*; *OYRM*1, *Pleurotus ostreatus*.

The scavenging effect of the crude, hot water and methanol extracts increased with increasing concentration (0.1 – 50 mg mL^{-1}). A considerable difference in scavenging activity was observed between extraction processes (Table 5.3). The EC$_{50}$ (mg mL^{-1}) for the crude extracts of each fungus could not be attained as the concentration at which 50 % of the DPPH radicals were scavenged was greater than the maximum concentration examined (50 mg mL^{-1}). Determination of the EC$_{50}$ (mg mL^{-1}) values revealed that the scavenging activity of the methanol extracts was considerably more effective than the water extracts (Table 5.3). Therefore, the methanol extraction process was the most efficient extraction process for DPPH free radical scavenging activity. Figure 5.3 displays the ability of fungal methanol extracts (mg mL^{-1}) to react rapidly with DPPH radicals and reduce most of them compared to α-tocopherol. As can be seen, the radical scavenging activity increased with increasing concentration up to 5 – 10 mg mL^{-1}. At this concentration 56 – 72 % of the DPPH radicals were scavenged (Figure 5.3).
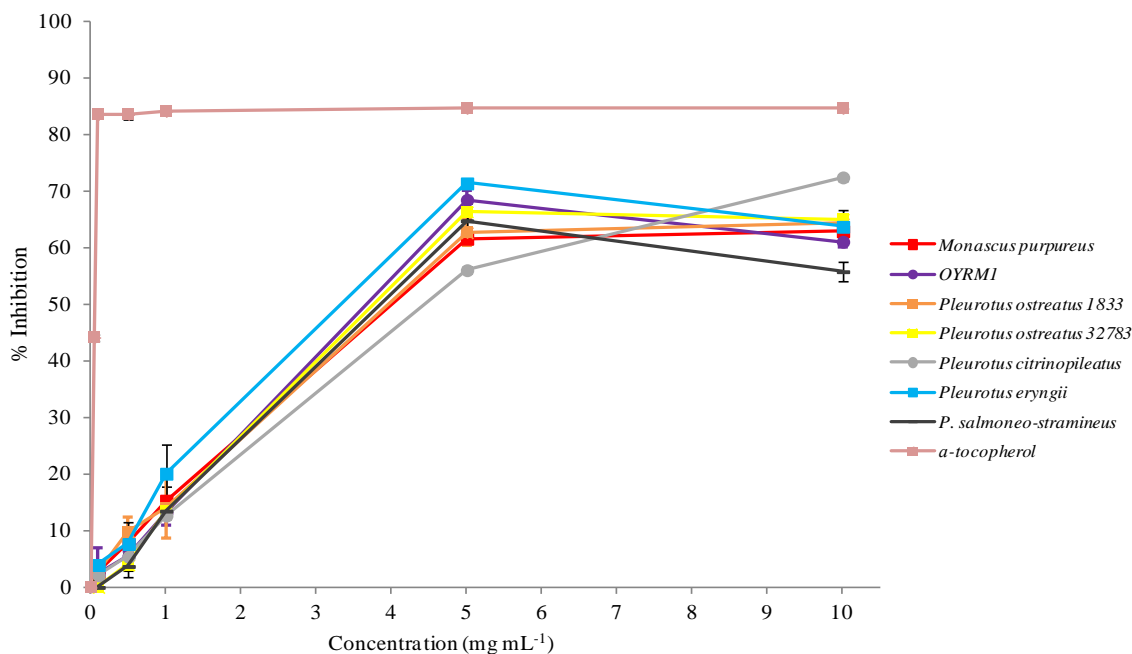
Figure 5.3　DPPH activity (%) of methanol extracts from fungi

Results plotted are a representation of the means ± SD (n = 3) of the radical scavenging activity of fungal extracts, with the standard deviation of each treatment represented by error bars.

- Abbreviations: *P. salmoneo-stramineus, Pleurotus salmoneo-stramineus*; *OYRM*1, *Pleurotus ostreatus*.

On the whole, the fungal extracts proved to be effective radical scavenging antioxidants. The data indicated that the extraction process is an extremely important factor in the isolation of scavenging compounds. In addition, differences in effectiveness between different fungal species were shown. According to the established EC_{50} value (Table 5.3), the methanol extract of each species showed the highest rate of scavenging activity compared to the hot water and crude extracts. Research has found that the DPPH method measures only compounds which are soluble in organic solvents, which is likely to be the reason why the methanol extract of each species showed the highest rate of scavenging activity compared to the hot water and crude extracts. Thereby the better ability of the methanol extract is due to the better extraction of hydrogen donating components by organic solvents (Arnao, 2000; Lee *et al.*, 2007a), in this case, methanol. The data demonstrated similar results to the findings of the ethanol extract from the mycelium of *P. citrinopileatus* by Lee *et al.* (2007a), where the scavenging ability of DPPH radicals was attributed to the presence of the essential amino acid L-tryptophan. Although beyond the scope of this investigation, this may be true of the extracts in the present study also. As can be seen in Figure 5.3, at 10 mg mL^{-1}, *Pleurotus citrinopileatus* demonstrated the highest DPPH scavenging activity.

The EC_{50} values (Table 5.3) provide information on the rate of radical scavenging and the number of radical molecules scavenged by different fungal species. The efficiency of fungal extracts to scavenge free radicals, as demonstrated by the DPPH method, is significant as consequently there is less risk of attack on biologically essential molecules (Niki *et al.*, 2000). Following the successful demonstration of the ability of fungal extracts to scavenge free radicals from species of which there is little information in the literature, further analysis of radical scavenging efficiency proceeded with the incorporation of *G. frondosa*, *T. versicolor* and *L. edodes* using the $ABTS^+$ radical scavenging activity assay. One of the most significant advantages of the ABTS method of antioxidant capacity is the possibility of multiple media usage since the reagent is soluble in both aqueous and organic solvent media. This allows for the determination of both hydrophilic and lipophilic antioxidant capacities of food extracts and physiological fluids (Apak *et al.*, 2007b). Aspects of antioxidant determination of which the DPPH assay does not allow.

5.2.2.2   The $ABTS^+$ radical scavenging activity assay

The 2,2'-azonobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay is a common method used for quantification of antioxidant activity. This competitive method of antioxidant evaluation is based on radical scavenging capacity of the fungal extracts as measured by electron transfer. The solubility and stability of ABTS as well as the reactions which generate it are well established (Arnao, 2000; Becker *et al.*, 2004). In this assay, the antioxidant capacity is quantified by the reduction of the coloured cation radical $ABTS^+$. ABTS is converted to its radical cation by addition of sodium persulfate. This radical cation is blue in colour and absorbs light at λ_{734nm}. During this reaction, the blue $ABTS^+$ radical cation is converted back to its colourless neutral form and is monitored spectrophotometrically. The reactivity of the various antioxidants was compared to the reactivity of Trolox, a water-soluble vitamin E analogue. Table 5.4 displays the relative capacity for scavenging radicals of the different fungal extracts in terms of their EC_{50} value. Similar to the β-carotene method of analysis (Section 5.2.1), the methanol extract was subjected to different concentrations of water (100 %) and methanol (30 and 100 %), to obtain a measure of activity based on the solubility of the scavenging compounds. Thereby, the polarity of the extracted antioxidant compounds from the selected species in terms of $ABTS^+$ radical scavenging activity was considered.

Table 5.4   Scavenging activity (EC_{50} mg mL^{-1}) of fungal extracts on ABTS^{+} radicals

Fungi	Crude extract	Hot water extract	Methanol extract		
			MeOH:H$_2$O (0:100)	MeOH:H$_2$O (100:0)	MeOH:H$_2$O (30:70)
Grifola frondosa	25.95 ± 0.03^{w}	14.79 ± 0.04^{x}	13.88 ± 0.30^{x}	8.43 ± 0.04^{y}	8.59 ± 0.04^{y}
Lentinula edodes	33.18 ± 0.16^{w}	13.77 ± 0.04^{x}	11.83 ± 0.01^{x}	9.90 ± 0.03^{x}	9.59 ± 0.04^{x}
Monascus purpureus	19.79 ± 0.13^{w}	4.63 ± 0.12^{x}	$\mathbf{2.83 \pm 0.06^{x}*}$	$\mathbf{1.84 \pm 0.05^{x}*}$	$\mathbf{2.77 \pm 0.24^{x}*}$
*OYRM*1	12.23 ± 0.06^{w}	7.67 ± 0.07^{w}	7.92 ± 0.03^{w}	3.41 ± 0.07^{x}	2.94 ± 0.11^{x}
Pleurotus ostreatus 1833	$\mathbf{10.16 \pm 0.06^{wx}*}$	7.38 ± 0.03^{x}	15.52 ± 0.03^{w}	8.90 ± 0.04^{wx}	7.19 ± 0.14^{x}
Pleurotus ostreatus 32783	15.12 ± 0.02^{w}	3.95 ± 0.04^{xy}	7.81 ± 0.03^{wx}	2.72 ± 0.08^{y}	3.68 ± 0.19^{xy}
Pleurotus citrinopileatus	13.04 ± 0.05^{w}	6.21 ± 0.07^{x}	10.88 ± 0.03^{w}	2.62 ± 0.06^{y}	$\mathbf{2.62 \pm 0.07^{y}*}$
Pleurotus eryngii	13.07 ± 0.03^{w}	5.36 ± 0.05^{x}	15.85 ± 0.03^{w}	6.25 ± 0.04^{x}	5.07 ± 0.13^{x}
P. salmoneo-stramineus	23.90 ± 0.09^{w}	7.67 ± 0.07^{x}	6.02 ± 0.03^{x}	7.71 ± 0.03^{x}	7.31 ± 0.04^{x}
Trametes versicolor	24.51 ± 0.03^{w}	$\mathbf{2.34 \pm 0.06^{z}*}$	5.57 ± 0.04^{y}	8.22 ± 0.03^{x}	8.46 ± 0.04^{x}
Trolox			0.02 ± 0.04		

Data are expressed as means $\pm$ SD (n = 3) of upper and lower limit of dose interpolation curve, with 95 % confidence. For the same fungi with different extraction processes, means with different letters (w,x,y,z) were significantly different ($p \leq 0.05$, ANOVA, Tukey-HSD), with alphabetical order of letters (w-z) representative of the lowest to highest scavenging activity (EC_{50} mg mL^{-1}). The strain with the lowest EC_{50} value per extract is indicated in bold. For the same extraction process, means denoted with an asterisk [*] were significantly lower (more active) than the other species of fungi ($p \leq 0.05$, ANOVA, Tukey-HSD).
- Abbreviations: *P. salmoneo-stramineus*, *Pleurotus salmoneo-stramineus*; *OYRM*1, *Pleurotus ostreatus*.

The data indicated that like the β-carotene bleaching assay (Table 5.2) and the DPPH radical scavenging activity (Table 5.3), the extraction process is an extremely important factor in the isolation of antioxidant compounds. In agreement with the data obtained following the β-carotene assay, the methanol extracts generally demonstrated the greatest activity proceeded by the hot water and crude extracts. It may be considered that for these selected species (Table 5.4), both the alcoholic and aqueous extracts gave rise to the presence of significant amounts of antioxidant compounds, as they show the ability to react with ABTS^{+} radicals. The crude extract of *P. 1833*, the hot water extract of *T. versicolor* and the methanolic extract of *M. purpureus* and *P. citrinopileatus* were significantly more effective at scavenging ABTS^{+} radicals, as denoted by an asterisk [*] in Table 5.4. The ABTS^{+} radical cation is reactive towards most antioxidants including phenolics, thiols and vitamin C. In Section 5.3, the total phenol content was measured and correlated with ABTS antioxidant capacity in order to identify compounds produced by these fungi responsible for the observed radical scavenging activity.

5.2.3   Antioxidant assessment by reduction of metal ions

A potent radical scavenging antioxidant often acts as a potent reductant of metal ions. The FRAP and the cupric reducing antioxidant capacity (CUPRAC) measure the

ability of the antioxidant to reduce ferric Fe (III) and cupric Cu (II) ions to their respective lower valency state (Niki, 2010). The ability of the extracts to donate electrons serves as a significant indicator of its potential antioxidant activity (Jayakumar *et al.*, 2009). The ABTS and DPPH assays use decolourisation to measure antioxidant reaction, whereas in the FRAP and CUPRAC method, there is an increase in absorbance at the pre-specified wavelength as the antioxidant reacts with the chromogenic reagent (i.e. the lower valencies of iron and copper, namely Fe (II) and Cu (I), and form charge-transfer complexes with the ligands, respectively) (Apak *et al.*, 2007b). The ABTS (which may also be recognised as the Trolox equivalent antioxidant capacity assay (TEAC)), CUPRAC, DPPH and FRAP methods assessed in this study are classified as electron transfer assays, each using different chromogenic redox reactions with different standard potentials (Apak *et al.*, 2007b).

5.2.3.1   *The ferric reducing antioxidant power (FRAP)*

The ferric reducing antioxidant power (FRAP) is based on a similar principle to the ABTS assay. Although the reducing power of a test compound or sample is not generally related to its radical scavenging capability, it is an important parameter of antioxidants (Apak *et al.*, 2007b). This assay monitors the presence of reductants which exert antioxidant action by breaking the free radical chain through donation of hydrogen atoms (Jayakumar *et al.*, 2009). In the presence of antioxidants, the Fe^{3+}-ferricyanide complex is reduced to the ferrous form Fe^{2+} which can then be monitored by measuring the formation of Perl's Prussian blue at λ_{700nm}. The ability of the fungal extracts and relevant controls to donate electrons is shown in Table 5.5, which displays the effective concentration (mg mL^{-1}) at which the absorbance of the fungal extracts was 0.5 for reducing power. The positive controls tested included BHT and α-tocopherol, which at 1 mg mL^{-1} their reducing power reached 1.11 ± 0.05 and 1.68 ± 0.08, respectively.

Table 5.5   Reducing power (EC$_{50}$ mg mL^{-1}) of fungal extracts

Fungi	Crude extract	Hot water extract	Methanol extract
Grifola frondosa	37.78 ± 0.01^{x}	15.11 ± 0.09^{x}	5.01 ± 0.15^{y}
Lentinula edodes	47.51 ± 0.11^{x}	13.20 ± 0.08^{y}	7.08 ± 0.14^{y}
Monascus purpureus	24.83 ± 0.21^{x}	10.80 ± 0.06^{xy}	4.22 ± 0.08^{y}
*OYRM*1	13.96 ± 0.08^{x}	5.43 ± 0.05^{y}	2.96 ± 0.07^{y}
Pleurotus ostreatus 1833	$\mathbf{6.78 \pm 0.05^{x*}}$	7.51 ± 0.14^{x}	2.93 ± 0.07^{y}
Pleurotus ostreatus 32783	21.93 ± 0.18^{x}	4.61 ± 0.04^{y}	$\mathbf{2.44 \pm 0.04^{y*}}$
Pleurotus citrinopileatus	18.77 ± 0.06^{x}	5.99 ± 0.07^{y}	2.88 ± 0.02^{z}
Pleurotus eryngii	8.53 ± 0.06^{x}	6.70 ± 0.04^{xy}	4.06 ± 0.07^{y}
P. salmoneo-stramineus	14.07 ± 0.04^{x}	5.71 ± 0.11^{y}	2.97 ± 0.02^{y}
Trametes versicolor	43.30 ± 0.01^{x}	$\mathbf{3.56 \pm 0.05^{y*}}$	3.42 ± 0.14^{y}
α-tocopherol		0.56 ± 0.03	

EC$_{50}$ (mg mL^{-1}) is representative of the effective concentration at which the absorbance was 0.5 for reducing power. Data are expressed as means $\pm$ SD (n = 3) of upper and lower limit of dose interpolation curve, with 95 % confidence. The strain with the lowest EC$_{50}$ value per extract is indicated in bold. Means with different letters (x,y,z) within a row are significantly different ($p \leq 0.05$, ANOVA, Tukey-HSD). For the same extraction process, means denoted with an asterisk [*] were significantly lower (more active) than the other species of fungi ($p \leq 0.05$, ANOVA, Tukey-HSD).
- Abbreviations: *P. salmoneo-stramineus, Pleurotus salmoneo-stramineus*; *OYRM*1, *Pleurotus ostreatus*.

The reducing power of fungi is directly associated to the hydrogen donating ability of the antioxidant compounds (Shimada *et al.*, 1992; Jayakumar *et al.*, 2009). From the data obtained, it was established that each species of fungi possessed reduction capacity. The results demonstrated significant ($p \leq 0.05$) differences in reducing power between crude, hot water and methanol extracts for each fungus (Table 5.5). Generally, with the exception of *P. 1833*, the methanol and hot water extracts demonstrated the strongest reducing power. The crude extract of this particular strain of *Pleurotus ostreatus* also demonstrated the strongest scavenging activity on DPPH radicals (Figure 5.2). This observation was also observed for this species for its effect on ABTS^{+} radicals. Notably, the DPPH method measures only compounds which are soluble in organic solvents and the ABTS^{+} radical cation is reactive towards most water soluble antioxidants including phenolics, thiols and vitamin C. As DPPH and FRAP methods assess electron transfer ability directly, this species is assumed to have greater hydrogen donating components which are soluble in water, as well as possibly having its hydrogen donating ability reduced following hot water extraction. The hot water extract of *T. versicolor* had the lowest effective concentration at which the absorbance was 0.5 for reducing power (Table 5.5), comparable to the ABTS^{+} radical scavenging assay, of which the hot water extract of this species had the most scavenging activity of ABTS^{+} radicals (Table 5.4). These observations confirm a

relationship between both methods, which is believed to be attributed to the shared mode of analysis associated with these assays, in that they are based on electron transfer by hydrogen donating compounds. Altogether, each type of extract demonstrated good reducing capacity for each of the fungal species tested. The methanol extraction process positively impacted the reducing power ability compared to the crude mycelial extract by a significant degree ($p \leq 0.05$) for each of the selected fungal species (Table 5.5). The crude extract of *P. 1833*, the hot water extract of *T. versicolor* and the methanolic extract of *P. 32783* were significantly more effective reducing agents, as denoted by an asterisk [*] in Table 5.5. Figure 5.4 shows the reducing power of fungal crude, hot water and methanol extracts as a function of increasing concentration.
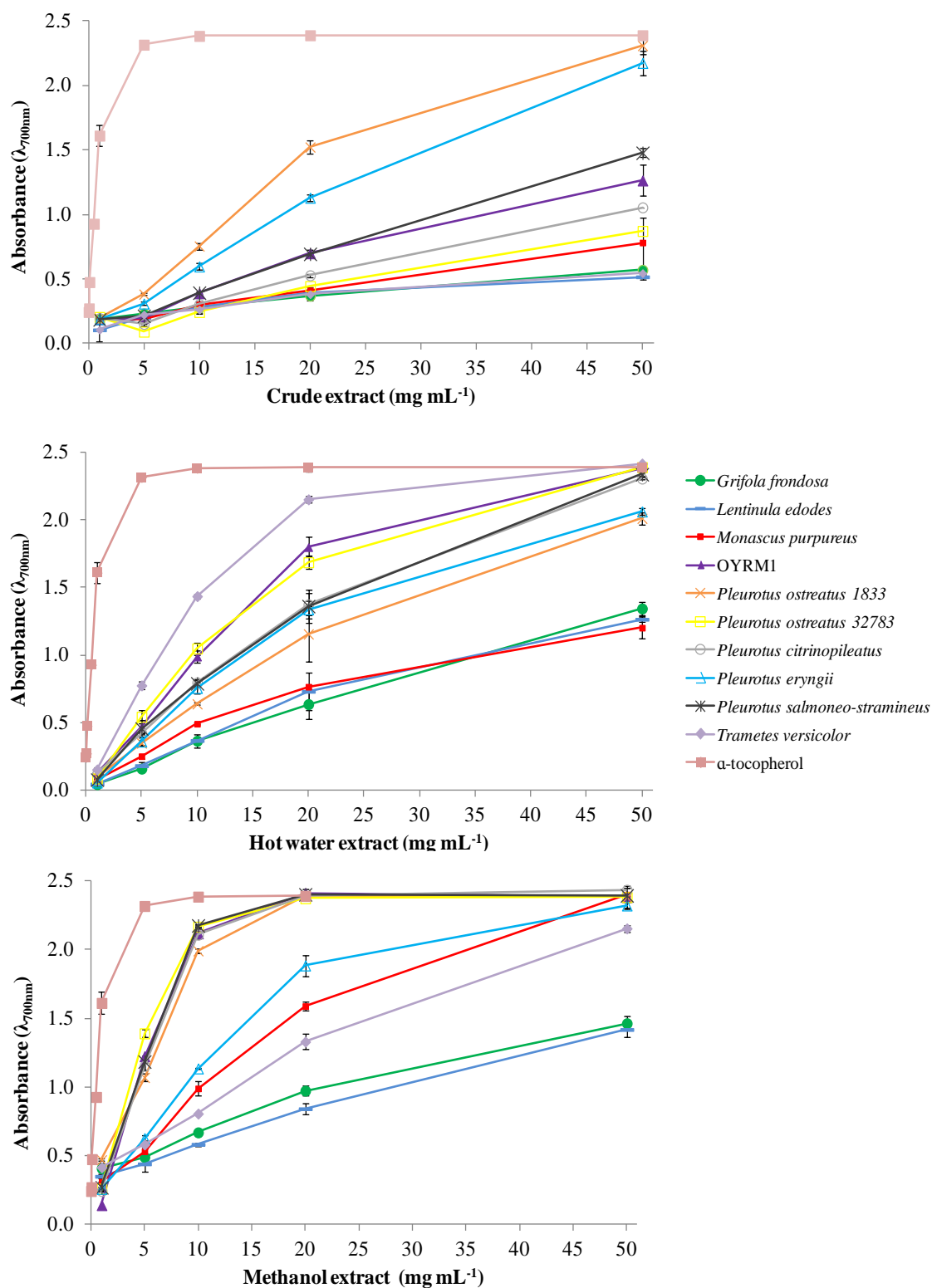
Figure 5.4   Reducing power of crude, hot water and methanol extracts

Results plotted are a representation of the means ± SD (n = 3) with the standard deviation of each treatment represented by error bars.

- Abbreviations: *OYRM*1, *Pleurotus ostreatus*.

As can be seen, the fungal extracts showed variable reducing capacity with each extraction process. This is believed to be attributed to the different antioxidant compounds (and concentration of compounds) isolated with either type of extraction (hot water and methanol). That being said, both *G. frondosa* and *L. edodes* exhibited relatively low reducing power for each type of extract, suggesting differences may also be a result of species specificity (Figure 5.4).

It is evident that the reducing power of the fungal extracts demonstrated a dose-related effect (Figure 5.4). At higher concentrations (20 – 50 mg mL^{-1}) the reducing power of various extracts was most like the control α-tocopherol (1 mg mL^{-1}) for most fungal species, as is demonstrated by a plateau effect at a high concentration. The moderate to high efficiency of the hot water extracts of *OYRM*1, *P. 32783*, *P. citrinopileatus*, *P. salmoneo-stramineus* and *T. versicolor* (1 – 50 mg mL^{-1}) is believed to be attributed to the use of the microwave extraction process under controlled conditions (as described in Section 2.2.4.1). These extraction conditions are assumed to have contributed to extraction efficiency consequently increasing the recovery of biologically active compounds compared to other investigations. At 1 mg mL^{-1} the mycelial hot water extract of *P. eryngii* grown by SLF demonstrated a reducing power of 0.057 ± 0.004. Abdullah *et al.* (2012) demonstrated a hot water extract of *P. eryngii* at 1 mg mL^{-1} to be 0.165 ± 0.041, however; this study was based on the fungal fruiting body. This highlights that there is considerable differences in effectiveness of biologically active compounds at various stages of growth.

At 50 mg mL^{-1}, the crude extract of *P. 1833*, the hot water extract of *T. versicolor* and the methanol extract of *P. 32783* had the strongest reducing capacity of each extraction process, as they reached a plateau at this concentration. This may be attributed to the aqueous extracts having a higher polyphenol content and the methanol extract having a higher content of reductones such as ascorbic acid, which react with free radicals to stabilise and block radical chain reactions (Ferreira *et al.*, 2007; Vamanu, 2012).

Overall, it was evident that the antioxidants produced from the various *Pleurotus* spp., *G. frondosa*, *M. purpureus* and *T. versicolor* were potent reducing agents compared to reference controls. That being said, the use of ferric ions to monitor antioxidant activity can lead to an underestimation of the antioxidant potential as some antioxidants such as thiols are not detected using this method. As such, the FRAP method is recommended for use in conjunction with other methods (Prior *et al.*,

2005). Although the data demonstrates the presence of these antioxidants using the FRAP assay, it does not provide information on whether the antioxidants measured have any biological role. Antioxidants detected by FRAP are limited to water soluble compounds, measuring only the hydrophilic antioxidants and carotenoids have no ferric reducing ability (Pulido *et al.*, 2000; Apak *et al.*, 2007b). Considering the methanol extract contained the most active FRAP, the data suggests that a high quantity of antioxidants with electron transfer ability or hydrogen donating components, soluble in organic solvents are present in this extract.

5.2.3.2   *Cupric ion reducing antioxidant capacity (CUPRAC)*

The CUPRAC assay utilises copper(II)-neocuproine (Cu(II)-Nc) reagent as the chromogenic oxidising agent (Abdullah *et al.*, 2012). It is based on the measurement of absorbance at λ_{450nm} by the formation of a stable complex between neocuproine and copper (I). There are a number of advantages of this method for the determination of antioxidant capacity. There is no risk of overestimation (under basic conditions) or underestimation (under acidic conditions) of total antioxidant capacity as the optimal pH of this method is close to the physiological pH (Apak *et al.*, 2007b). Similar to other electron transfer assays, the antioxidant capacity is assumed to be equal to the reducing capacity of the extracts (Huang *et al.*, 2005). This method may be classed as advantageous over FRAP since the redox chemistry of copper (II) should have faster kinetics (Apak *et al.*, 2007b). Also, the CUPRAC method is capable of assaying both hydrophilic and lipophilic antioxidants, as the bis(neocuproine)copper (I) cation chromophore is soluble both in water and organic media (Apak *et al.*, 2007b). Absorbance values for fungal extracts and reference standards were measured at a concentration of 10 mg mL^{-1} for direct comparison of CUPRAC activity (Figure 5.5).
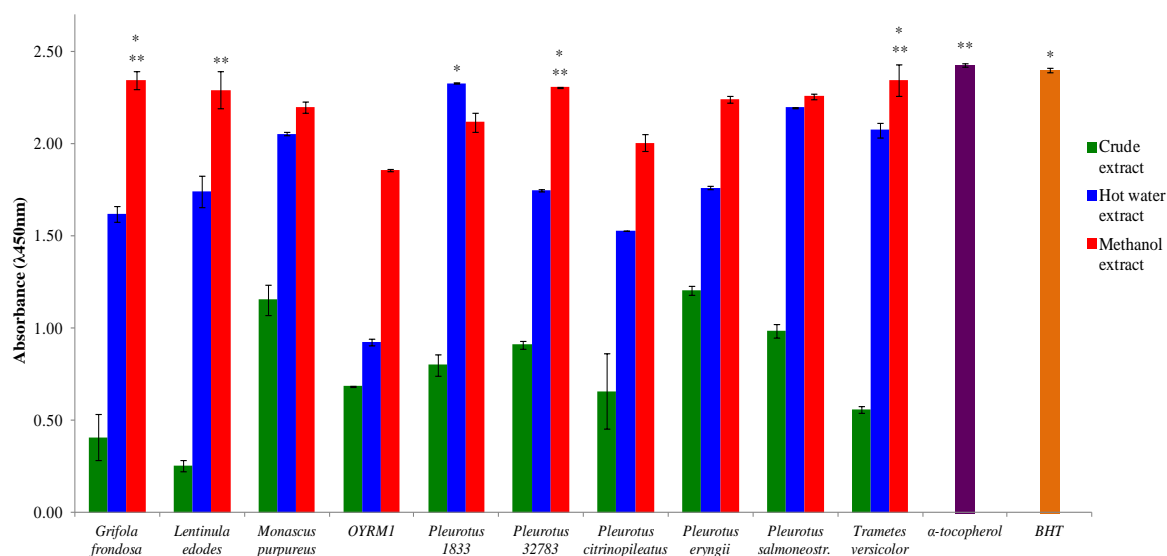
Figure 5.5   CUPRAC of various fungal extracts

Results plotted are a representation of mean ± standard deviations of triplicate experiments, with the standard deviation of each treatment represented by error bars. Probability values were determined using a Dunnett's test to evaluate the significance of each sample to the antioxidant control, where the confidence level was set at 95 %. No significant difference between test sample (10 mg mL^{-1}) and α-tocopherol or BHT (10 mg mL^{-1}) is denoted by (*) and (**), respectively.
- Abbreviations: *OYRM*1, *Pleurotus ostreatus*; BHT, Butylated hydroxyltoluene.

At 10 mg mL^{-1} the methanol extract showed the highest CUPRAC compared to the other extracts for the majority of filamentous fungal species. As illustrated in Figure 5.5, the hot water extract of *P. 1833* had higher CUPRAC (2.327 ± 0.005) than the methanol extract (2.115 ± 0.053). Although both extracts of this species demonstrated good antioxidant activity, the hot water extract had a higher capacity to reduce cupric ions compared to the methanol extract. In previous analysis (Table 5.5), the crude extract of this species demonstrated the strongest reducing power and ABTS$^+$ scavenging activity compared to the other species. In addition, the crude extract (10 mg mL^{-1}) had the highest capacity to scavenge DPPH radicals compared to the other species also. Altogether, the CUPRAC (10 mg mL^{-1}) of the methanol extracts ranged from 1.854 ± 0.008 (*OYRM1*) to 2.343 ± 0.048 (*G. frondosa*), 0.925 ± 0.017 (*OYRM1*) to 2.327 ± 0.005 (*P. 1833*) for the hot water extracts and 0.254 ± 0.029 (*L. edodes*) to 1.203 ± 0.026 (*P. eryngii*) for the crude extracts. Methanol extracts of *G. frondosa*, *P. 32783* and *T. versicolor* (10 mg mL^{-1}) were significantly similar ($p \leq 0.05$) to α-tocopherol (1 mg mL^{-1}) (Figure 5.5). Also, methanol extracts of *G. frondosa*, *L. edodes*, *P. 32783*, and *T. versicolor* were equivalent ($p \leq 0.05$) to BHT (1 mg mL^{-1}) (Figure 5.5). Table 5.6 shows the CUPRAC absorbance values of the different

filamentous fungi following different extraction processes at various concentrations compared to the controls which were tested at a concentration of 1 mg mL^{-1}.

Table 5.6   CUPRAC (λ_{450nm}) of selected fungal crude, hot water and methanol extracts

	Crude extract concentration (mg mL^{-1})				
	0.1	**0.5**	**1.0**	**5.0**	**10.0**
Grifola frondosa	0.014 ± 0.016	0.097 ± 0.047	0.123 ± 0.032	0.357 ± 0.019	0.408 ± 0.123
Lentinula edodes	0.017 ± 0.015	0.057 ± 0.060	0.105 ± 0.061	0.185 ± 0.022	0.254 ± 0.029
Monascus purpureus	0.036 ± 0.001	0.057 ± 0.003	0.057 ± 0.003	0.838 ± 0.063	1.153 ± 0.081
*OYRM*1	0.056 ± 0.001	0.121 ± 0.006	0.126 ± 0.006	0.472 ± 0.026	0.684 ± 0.004
P. ostreatus 1833	0.065 ± 0.006	0.201 ± 0.010	0.546 ± 0.004	0.718 ± 0.013	0.799 ± 0.057
P. ostreatus 32733	0.025 ± 0.001	0.060 ± 0.011	0.113 ± 0.002	0.548 ± 0.015	0.910 ± 0.021
P. citrinopileatus	0.013 ± 0.001	0.026 ± 0.011	0.029 ± 0.003	0.791 ± 0.001	0.867 ± 0.025
P. eryngii	0.016 ± 0.000	0.081 ± 0.001	0.060 ± 0.005	0.661 ± 0.107	1.203 ± 0.026
P. salmoneo-str.	0.036 ± 0.001	0.057 ± 0.003	0.057 ± 0.003	0.838 ± 0.063	0.983 ± 0.036
Trametes versicolor	0.030 ± 0.021	0.092 ± 0.066	0.187 ± 0.005	0.680 ± 0.089	0.557 ± 0.018
α-tocopherol			**2.442 ± 0.022[y]**		
BHT			**2.425 ± 0.019[x]**		

Data is representative of mean ± standard deviation (n = 3) of triplicate experiments. CUPRAC of reference standards (α-tocopherol and BHT) was measured at a concentration of 1 mg mL^{-1}. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the antioxidant controls, where the confidence level was set at 95 %. No significant difference between CUPRAC of test sample and α-tocopherol control is denoted by ([x]) and no significant difference between CUPRAC of test sample and BHT control is denoted by ([y]), indicated in bold.
- Abbreviations: *P.*, *Pleurotus*; *OYRM*1, *Pleurotus ostreatus*; *P. salmoneo-str.*, *Pleurotus salmoneo-stramineus*; BHT, Butylated hydroxyltoluene.

	Hot water extract concentration (mg mL^{-1})				
	0.1	**0.5**	**1.0**	**5.0**	**10.0**
Grifola frondosa	0.084 ± 0.014	0.194 ± 0.071	0.316 ± 0.086	1.097 ± 0.570	1.594 ± 0.050
Lentinula edodes	0.078 ± 0.002	0.191 ± 0.001	0.371 ± 0.013	0.982 ± 0.006	1.743 ± 0.060
Monascus purpureus	0.070 ± 0.004	0.176 ± 0.002	0.286 ± 0.007	1.309 ± 0.009	2.061 ± 0.018
*OYRM*1	0.085 ± 0.094	0.119 ± 0.006	0.208 ± 0.004	0.372 ± 0.002	0.927 ± 0.012
P. ostreatus 1833	0.103 ± 0.014	0.290 ± 0.092	0.205 ± 0.001	1.722 ± 0.010	**2.325 ± 0.004[y]**
P. ostreatus 32733	0.029 ± 0.000	0.112 ± 0.001	0.154 ± 0.028	0.643 ± 0.018	1.220 ± 0.015
P. citrinopileatus	0.036 ± 0.001	0.112 ± 0.008	0.205 ± 0.003	0.963 ± 0.012	1.527 ± 0.010
P. eryngii	0.081 ± 0.002	0.141 ± 0.001	0.255 ± 0.003	1.078 ± 0.009	1.752 ± 0.014
P. salmoneo-str.	0.093 ± 0.002	0.174 ± 0.003	0.299 ± 0.006	1.351 ± 0.018	2.191 ± 0.003
Trametes versicolor	0.060 ± 0.028	0.135 ± 0.026	0.228 ± 0.054	1.009 ± 0.254	2.082 ± 0.033
α-tocopherol			**2.432 ± 0.017[y]**		
BHT			**2.382 ± 0.000[x]**		

Data is representative of mean ± standard deviation (n = 3) of triplicate experiments. CUPRAC of reference standards (α-tocopherol and BHT) was measured at a concentration of 1 mg mL^{-1}. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the antioxidant controls, where the confidence level was set at 95 %. No significant difference between CUPRAC of test sample and α-tocopherol control is denoted by ([x]) and no significant difference between CUPRAC of test sample and BHT control is denoted by ([y]), indicated in bold.
- Abbreviations: *P.*, *Pleurotus*; *OYRM*1, *Pleurotus ostreatus*; *P. salmoneo-str.*, *Pleurotus salmoneo-stramineus*; BHT, Butylated hydroxyltoluene.

	Methanol extract concentration (mg mL^{-1})				
	0.1	**0.5**	**1.0**	**5.0**	**10.0**
Grifola frondosa	0.028 ± 0.012	0.677 ± 0.104	0.689 ± 0.031	1.220 ± 0.318	**2.343 ± 0.048^{xy}**
Lentinula edodes	0.049 ± 0.050	0.703 ± 0.125	0.693 ± 0.033	1.235 ± 0.320	**2.288 ± 0.101^{y}**
Monascus purpureus	0.713 ± 0.262	0.554 ± 0.028	0.732 ± 0.042	1.650 ± 0.031	2.196 ± 0.029
*OYRM*1	0.672 ± 0.177	0.646 ± 0.194	0.664 ± 0.197	0.899 ± 0.044	1.854 ± 0.008
P. ostreatus 1833	0.723 ± 0.152	0.803 ± 0.099	0.667 ± 0.086	1.097 ± 0.068	2.115 ± 0.053
P. ostreatus 32733	0.651 ± 0.067	0.810 ± 0.124	0.710 ± 0.070	1.294 ± 0.111	**2.302 ± 0.002^{xy}**
P. citrinopileatus	0.643 ± 0.255	0.603 ± 0.208	0.687 ± 0.055	1.058 ± 0.074	2.002 ± 0.047
P. eryngii	0.618 ± 0.082	0.675 ± 0.181	0.750 ± 0.134	1.313 ± 0.106	2.237 ± 0.018
P. salmoneo-str.	0.810 ± 0.124	0.586 ± 0.074	0.846 ± 0.055	1.330 ± 0.051	2.254 ± 0.017
Trametes versicolor	0.011 ± 0.053	0.669 ± 0.088	0.748 ± 0.061	1.249 ± 0.111	**2.342 ± 0.088^{xy}**
α-tocopherol			**2.412 ± 0.022^{y}**		
BHT			**2.396 ± 0.002^{x}**		

Data is representative of mean ± standard deviation (n = 3) of triplicate experiments. CUPRAC of reference standards (α-tocopherol and BHT) was measured at a concentration of 1 mg mL^{-1}. The probability values were determined using a Dunnett's test to evaluate the significance of the samples to the antioxidant controls, where the confidence level was set at 95 %. No significant difference between CUPRAC of test sample and α-tocopherol control is denoted by (^{x}) and no significant difference between CUPRAC of test sample and BHT control is denoted by (^{y}), indicated in bold.
- Abbreviations: *P.*, *Pleurotus*; *OYRM*1, *Pleurotus ostreatus*; *P. salmoneo-str.*, *Pleurotus salmoneo-stramineus*; BHT, Butylated hydroxyltoluene.

As expressed in Table 5.6, the CUPRAC of each fungal extract was dependant on the species and extraction process. As expected, the commercial antioxidant controls BHT, as well as α-tocopherol (1 mg mL^{-1}) demonstrated more pronounced CUPRAC than the majority fungal extracts. However, at 10 mg mL^{-1} the hot water and methanol extracts produced CUPRAC comparable to the positive antioxidant controls (Table 5.6). The hot water extract of *P. 1833* was equivalent to BHT ($p \geq$ 0.05). In addition, the methanol extracts of *G. frondosa*, *P. 32783* and *T. versicolor* (10 mg mL^{-1}) were significantly similar ($p \geq 0.05$) to both α-tocopherol and BHT (1 mg mL^{-1}), which is noteworthy (Table 5.6).

Overall, with exception of the hot water extract of *P. 1833*, the data revealed that the methanol extract had the highest CUPRAC in each filamentous fungal species tested. The utilisation of CUPRAC in this investigation contributed to the evaluation of antioxidant potential of these species. This is the first report on the evaluation of antioxidant activity of the mycelium of these fungi, using these extraction techniques tested by the CUPRAC method. Altogether, the demonstration of antioxidant capacity of fungal extracts using the CUPRAC method has multiple advantages over other electron transfer methods, namely; the selection of working pH at physiological pH (opposed to the FRAP method, which works at acidic pH), applicability to both hydrophilic and lipophilic antioxidants (unlike DPPH), completion of the redox

reactions for most common flavonoids (unlike FRAP) and the capability to assay -SH bearing antioxidants (unlike FRAP) (Apak *et al.*, 2007b). Although the FRAP assay is commonly used as a measure of reducing capacity, this method does not necessarily correlate with actual antioxidant capacity (Frankel *et al.*, 2000; Becker *et al.*, 2004). In addition, evaluation of CUPRAC is highly recommended when evaluating antioxidant capacity of natural extracts rich in phenolic antioxidants (Apak *et al.*, 2007b). The methanol extract of *P. 32783* (10 mg mL^{-1}) which was statistically similar ($p \geq 0.05$) to both α-tocopherol and BHT (1 mg mL^{-1}) also had the lowest EC$_{50}$ value ($p \leq 0.05$) for FRAP (Table 5.5). Additionally, the FRAP of the crude (water soluble) extract of *P. 1833*, demonstrated the lowest EC$_{50}$ value, respectively. The hot water extract of this species demonstrated the best CUPRAC compared to the other fungi, and was statistically similar ($p \geq 0.05$) to α-tocopherol. This association between different methods of analysis suggests that antioxidant capacity was a not a response to flavonoids or an –SH bearing antioxidant and was most likely due to electron transfer ability. With consideration of both ABTS and CUPRAC methods, which measure both hydrophilic and lipophilic antioxidant capacities, the crude and hot water extract of *P. 1833* was the most effective species compared to the others, respectively. The relationship with antioxidant capacity is most likely due to radical scavenging ability *via* electron transfer.

5.2.4   Chelating ability against ferrous ions

Reaction with a stable free radical is not the only mechanism by which an antioxidant may exert its activity; these alternative antioxidants do not convert free radicals to more stable products but instead, slow the rate of oxidation by several different mechanisms (Končić *et al.*, 2011). One such mechanism of action of these secondary antioxidants is the chelation of pro-oxidant metals. Transition metals (iron, copper, chromium, cobalt, vanadium, cadmium, arsenic and nickel) are known to be involved in the initiation and propagation of lipid peroxidation (Končić *et al.*, 2011; Oke *et al.*, 2011). Ferrous ions play an important role as catalysts in free radical reactions. Consequently, the chelation of metals by certain compounds decreases their pro-oxidant effect by reducing their redox potentials and stabilising the oxidised form of the metal (Končić *et al.*, 2011), thereby; these chelating agents serve as secondary antioxidants. Chelating compounds may also sterically hinder formation of the metal hydroperoxide complex (Končić *et al.*, 2011).

The chelating activity at various concentrations of the different fungal extracts was examined and compared to a chelating standard, EDTA. Activity was determined using the ferrozine assay, as previously outlined in Section 2.2.6.6, which distinguishes the ability of the extracts to inhibit the complex formation of ferrozine with ferrous ions. Table 5.7 displays the effective concentrations (mg mL^{-1}) at which 50 % of ferrous ions were chelated.

Table 5.7   Chelating effect (EC$_{50}$ mg mL^{-1}) of fungal extracts

Fungi	Crude extract	Hot water extract	Methanol extract
Grifola frondosa	4.57 ± 0.91^{xa}	$\mathbf{0.64 \pm 0.10^{ya}}$	$\mathbf{4.09 \pm 1.15^{xyb}}$
Lentinula edodes	3.65 ± 1.05^{xa}	0.70 ± 0.15^{xa}	4.24 ± 1.07^{xb}
Monascus purpureus	1.22 ± 1.46^{xya}	0.66 ± 0.25^{ya}	4.67 ± 0.76^{xb}
OYRM1	1.13 ± 0.69^{ya}	1.47 ± 1.06^{ya}	7.60 ± 0.76^{xab}
Pleurotus ostreatus 1833	1.16 ± 0.86^{ya}	0.72 ± 0.16^{ya}	5.84 ± 0.90^{xab}
Pleurotus ostreatus 32783	$\mathbf{1.12 \pm 0.90^{ya}}$	0.87 ± 0.16^{ya}	6.18 ± 0.68^{xab}
Pleurotus citrinopileatus	1.27 ± 1.19^{xa}	1.11 ± 0.51^{xa}	4.31 ± 1.16^{xb}
Pleurotus eryngii	1.39 ± 1.20^{xa}	2.60 ± 1.17^{xa}	6.20 ± 0.77^{xab}
Pleurotus salmoneo-stramineus	1.39 ± 0.56^{ya}	0.81 ± 0.34^{ya}	9.56 ± 1.35^{xa}
Trametes versicolor	1.34 ± 0.66^{ya}	0.79 ± 0.34^{ya}	4.84 ± 0.72^{xab}
EDTA		0.07 ± 0.02^{c}	

EC$_{50}$ (mg mL^{-1}) is representative of the effective concentration at which ferrous ions were chelated by 50 %. Data are expressed as means $\pm$ SD (n = 3) of upper and lower limit of dose interpolation curve, with 95 % confidence. The strain with the lowest EC$_{50}$ value per extract is indicated in bold. Means with different letters (x,y,z) within a row are significantly different ($p \le 0.05$, ANOVA, Tukey-HSD). Means with different letters (a-e) within a column are significantly different ($p \le 0.05$, ANOVA, Tukey-HSD).
- Abbreviations: *OYRM*1, *Pleurotus ostreatus*; EDTA, ethylenediaminetetraacetic acid.

The data shows that the chelating ability of extracts was moderate to high (Table 5.7). A significant difference ($p \le 0.05$) in the presence of chelating antioxidants following extraction was observed for some species. According to their EC$_{50}$ value (mg mL^{-1}), the crude extract of *P. 32783*, as well as the hot water and methanol extract of *G. frondosa* had the most chelating power of the species analysed (Table 5.7).

The fungal extracts and reference standard EDTA demonstrated interference with the formation of ferrous and ferrozine complex. Interestingly, the water extracts (mostly microwave assisted hot water extracts) showed the highest ferrous iron chelating activity, preceded by the methanol extracts which exhibited weaker chelating effect for each fungal species, suggesting higher solubility and chelation of the metal ions in aqueous solution. That being said, both the methanol and water extracts demonstrated iron binding capacity, suggesting that total antioxidant activity was

related to the capacity to chelate metal ions. Figure 5.6 shows the chelating ability of crude, hot water and methanol fungal extracts as a function of their concentration. As presented in Figure 5.6, the metal chelating activity increased with increasing concentrations, demonstrating that fungal extracts have effective chelating activity and capture ferrous ion before ferrozine.
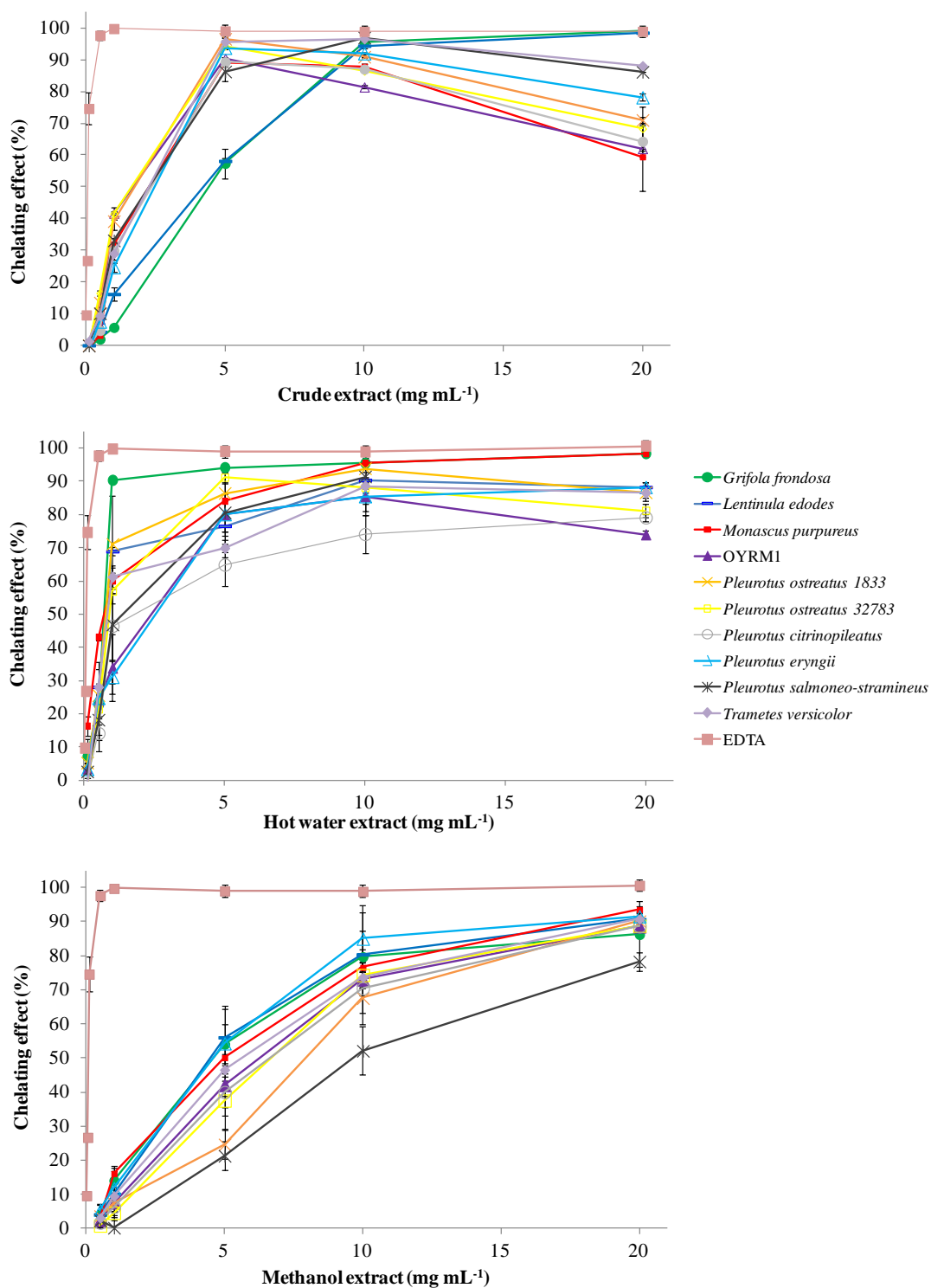
Figure 5.6   Chelating effect of crude, hot water and methanol extracts

Results plotted are a representation of the means ± SD (n = 3), with the standard deviation of each treatment represented by error bars.

- Abbreviations: *OYRM*1, *Pleurotus ostreatus*; EDTA, ethylenediaminetetraacetic acid.

At 10 mg mL^{-1} the chelating effect of crude fungal extracts ranged from 86 (*P. 32783*) to 97 % (*P. salmoneo-stramineus*), hot water extracts ranged from 74 (*P. citrinopileatus*) to 95 % (*G. frondosa*) and methanol extract ranged from 52 (*P. salmoneo-stramineus*) to 85 % (*P. eryngii*). EDTA showed excellent chelating ability, of 99 % at a concentration as low as 1 mg mL^{-1}.

This investigation demonstrated that fungal mycelial biomass as a crude extract or following various extraction processes can effectively slow the rate of oxidation by chelation of pro-oxidant metals. As ferrous ions are the most effective pro-oxidants in food systems, the high ferrous-ion chelating abilities of the various extracts from the mycelium of each of the species tested would be beneficial if formulated into foods (Mau *et al.*, 2004). It was observed that the hot water extract of *G. frondosa*, *L. edodes*, *M. purpureus*, *P. 1833*, *P. 32783*, *P. citrinopileatus*, *P. salmoneo-stramineus* and *T. versicolor* attributed to a greater chelating effect compared to the crude mycelia and methanol extracts, thereby; this method of extraction allowed the isolation of compounds responsible for chelation of metal ions more effectively. On the contrary, the crude extract of *OYRM*1 and *P. eryngii* had the highest chelating effect; indicative of the water extracts more effectively isolating compounds responsible for slowing the progression of oxidation caused by metal ions. It is possible that the high activity associated with the fungal antioxidants is a consequence of a high manifestation of phenolic compounds in the extracted fungal mycelia. Phenolic compounds can act as antioxidants in many ways and in relevant literature, have been found to chelate to metal ions, prevent radical formation and improve the antioxidant endogenous system (Al-Azzawie *et al.*, 2006). This is significant as metal ions are proposed as the catalysts for the initial formation of reactive oxygen species.

5.2.5   Overview of the antioxidant capacity of fungal extracts

The radical scavenging, metal chelating and antioxidant activities of several species of filamentous fungi were investigated by a number of assay systems. Consequently, these assays allowed for comparison of the effect of various extraction processes on antioxidant capacity. The effectiveness of antioxidant properties is inversely correlated with EC$_{50}$ value. Therefore, the antioxidant properties of the various species and differences between extraction processes are summarised in Table 5.8, for ABTS$^+$ radical scavenging activity, reducing power (FRAP) and chelating ability. A more detailed overview of antioxidant capacity is discussed later in this chapter.

Table 5.8   **EC$_{50}$ values (mg mL^{-1}) of fungal extracts in ABTS$^{+(i)}$, reducing power $^{(ii)}$ and chelating ability assays $^{(iii)}$**

		Grifola frondosa	*Lentinula edodes*	*Monascus purpureus*	*OYRM*1	*Pleurotus 1833*	*Pleurotus 32783*	*Pleurotus citrinopileatus*	*Pleurotus eryngii*	*Pleurotus salmoneo-str.*	*Trametes versicolor*	**Control^d**
ABTS radical scavenging (EC$_{50}$^{a})	**Crude extract**	25.95 ± 0.03^{xab}	33.18 ± 0.16^{xa}	19.79 ± 0.13^{xabcde}	12.23 ± 0.06^{xde}	**10.16 ± 0.06^{xye}**	15.12 ± 0.02^{xbcde}	13.04 ± 0.05^{xcde}	13.07 ± 0.03^{xcde}	23.90 ± 0.09^{xabcd}	24.51 ± 0.03^{xabc}	0.02 ± 0.04^{f+}
	Hot water extract	14.79 ± 0.04^{ya}	13.77 ± 0.04^{ya}	4.63 ± 0.12^{ybc}	7.67 ± 0.07^{xb}	7.38 ± 0.03^{yb}	3.95 ± 0.04^{yzcd}	6.21 ± 0.07^{ybc}	5.36 ± 0.05^{ybc}	7.67 ± 0.07^{yb}	**2.34 ± 0.06^{zd}**	
	Methanol extract	8.43 ± 0.04^{zab}	9.90 ± 0.03^{ya}	**1.84 ± 0.05^{yd}**	3.41 ± 0.07^{yc}	8.90 ± 0.04^{xyab}	2.72 ± 0.08^{zcd}	2.62 ± 0.06^{zcd}	6.25 ± 0.04^{yb}	7.71 ± 0.03^{yab}	8.22 ± 0.03^{yab}	
Reducing power (EC$_{50}$^{b})	**Crude extract**	37.78 ± 0.01^{xa}	47.51 ± 0.11^{xa}	24.83 ± 0.21^{xab}	13.96 ± 0.08^{xbcd}	**6.78 ± 0.05^{xd}**	21.93 ± 0.18^{xab}	18.77 ± 0.06^{xabc}	8.53 ± 0.06^{xcd}	14.07 ± 0.04^{xbcd}	43.30 ± 0.01^{xa}	0.56 ± 0.03^{f++}
	Hot water extract	15.11 ± 0.09^{xa}	13.20 ± 0.08^{yab}	10.80 ± 0.06^{xyabc}	5.43 ± 0.05^{ycde}	7.51 ± 0.14^{xbcd}	4.61 ± 0.04^{yde}	5.99 ± 0.07^{ycde}	6.70 ± 0.04^{xybcde}	5.71 ± 0.11^{ycde}	**3.56 ± 0.05^{ye}**	
	Methanol extract	5.01 ± 0.15^{ybcd}	7.08 ± 0.14^{yabcd}	4.22 ± 0.08^{yabcd}	2.96 ± 0.07^{ya}	2.93 ± 0.07^{ycd}	**2.44 ± 0.04^{yabc}**	2.88 ± 0.02^{zab}	4.06 ± 0.07^{yabcd}	2.97 ± 0.02^{yabc}	3.42 ± 0.14^{yd}	
Chelating ability (EC$_{50}$^{c})	**Crude extract**	4.57 ± 0.91^{xa}	3.65 ± 1.05^{xa}	1.22 ± 1.46^{xya}	1.13 ± 0.69^{ya}	1.16 ± 0.86^{ya}	**1.12 ± 0.90^{xya}**	1.27 ± 0.19^{xa}	1.39 ± 0.20^{xa}	1.39 ± 0.56^{ya}	1.34 ± 0.66^{ya}	0.07 ± 0.02^{c+++}
	Hot water extract	**0.64 ± 0.10^{ya}**	0.70 ± 0.15^{xa}	0.66 ± 0.25^{ya}	1.47 ± 1.06^{ya}	0.72 ± 0.16^{ya}	0.87 ± 0.16^{ya}	1.11 ± 0.51^{xa}	2.60 ± 0.17^{xa}	0.81 ± 0.34^{ya}	0.79 ± 0.34^{ya}	
	Methanol extract	**4.09 ± 1.15^{xb}**	4.24 ± 1.07^{xb}	4.67 ± 0.76^{xb}	7.60 ± 0.76^{xab}	5.84 ± 0.90^{xab}	6.18 ± 0.68^{xab}	4.31 ± 0.16^{xb}	6.20 ± 0.77^{xab}	9.56 ± 1.35^{xa}	4.84 ± 0.72^{xab}	

Data are expressed as means ± SD (n = 3) of upper and lower limit of dose interpolation curve, with 95 % confidence. $^{(i)}$EC$_{50}$ (mg mL^{-1}), effective concentration at which 50 % of ABTS$^+$ radicals are scavenged. $^{(ii)}$EC$_{50}$ (mg mL^{-1}), effective concentration at which the absorbance is 0.5. $^{(iii)}$EC$_{50}$ (mg mL^{-1}), effective concentration at which 50 % of Fe^{2+} ions were chelated. For the same extraction process, means with different letters (a-f) within a row are significantly different ($p \leq 0.05$, ANOVA, Tukey-HSD) according to the corresponding method of analysis. Means with different letters (x,y,z) within a column, according to each individual method are significantly different ($p \leq 0.05$, ANOVA, Tukey-HSD). Control^d; $^+$, Trolox; $^{++}$, α-tocopherol; $^{+++}$, EDTA.
- Abbreviations: *OYRM*1, *Pleurotus ostreatus*; *Pleurotus salmoneo-str.*, *Pleurotus salmoneo-stramineus*.

The inhibition of β-carotene bleaching was considered moderate to high, with activity of a number of extracts being close to that of BHT and α-tocopherol (1 mg mL^{-1}), demonstrating that these antioxidants worked well in an emulsion. By this means, various extracts demonstrated pronounced antioxidant activity in the prevention of heat-induced oxidation of linoleic acid (Section 5.2.1).

Scavenging effects on DPPH radicals increased with increasing concentration and were moderate to high based on the methanol extract. The ABTS assay which assessed antioxidant capacity *in vitro* by competitive method for evaluation of radical scavenging revealed that the methanol extract had the highest scavenging activity. In addition, similar to the β-carotene assay, the results reveal the antioxidant compounds present in the methanol extract might present a moderately polar profile. Notably, unlike the β-carotene bleaching assay and the measure of chelating ability, the ABTS method is not suitable for the assessment of antioxidant capacity in the form of efficiency of lipid peroxidation inhibition (Niki, 2010).

Reducing powers were excellent and increased with increasing concentration. All extracts demonstrated hydrogen donating ability, identifying the presence of antioxidants. The methanol and hot water extracts demonstrated the strongest reducing power; with the methanol extract positively influencing the reducing capacity by a significant degree ($p \leq 0.05$) compared to the crude mycelial extract of each of the selected species.

At 10 mg mL^{-1}, the methanol extract showed the highest CUPRAC for the majority of species, with the methanol extract of *G. frondosa*, *P. 32783* and *T. versicolor* equivalent ($p \leq 0.05$) to BHT. In addition, the activity of the hot water extract of *P. 1833* and the methanol extracts of *G. frondosa*, *L. edodes, P. 32783* and *T. versicolor* were equivalent ($p \leq 0.05$) to α-tocopherol (1 mg mL^{-1}). The demonstration of antioxidant capacity using the CUPRAC method was incorporated to complement the electron transfer methods examined; namely, the reducing power assay. This assay boasts a working pH similar to physiological pH, has the capability to assay -SH bearing antioxidants (thiol group) and is applicable to both hydrophilic and lipophilic antioxidants; therefore, is highly recommended for evaluation of antioxidant potential of natural extracts rich in phenolic compounds.

Metal ions are proposed as the catalysts for the initial formation of reactive oxidative species. In the present investigation, fungal extracts demonstrated that they have the ability to interfere with the formation of ferrous and ferrozine complex; with

water extracts demonstrating chelating ability most similar to the reference standard EDTA. Hence, water extracts, in particular the microwave assisted hot water extract was more effective for isolation of compounds responsible for slowing the progression of oxidation caused by metal ions.

Overall, various extracts from *G. frondosa*, *L. edodes*, *OYRM*1, *M. purpureus*, *P. 1833*, *P. 32783*, *P. eryngii*, *P. citrinopileatus*, *P. salmoneo-stramineus* and *T. versicolor* possessed moderate to high antioxidant activity, in terms of; antioxidant capacity, scavenging ability on DPPH and ABTS$^+$ radicals, reducing power and chelating ability. Similar to other fungal species in relevant literature, the species tested in the present study were excellent radical scavengers, with activity similar to that of BHT and α-tocopherol. In general, the methanol extract was the most effective extraction process for isolation of radical scavenging antioxidants, with high FRAP and CUPRAC, through hydrogen donating components. Conversely, this extract demonstrated weak chelating ability. The fungal crude and hot water extracts were more successful in chelation of ferrous ions for the majority of species. Production of antioxidants was species specific for each assay examined and different fungi exhibited considerable differences in antioxidant isolation following hot water or methanol extraction. These differences were believed to be attributed to the different phenolic compositions of the considered extracts.

There are a number of studies which state that processing and growth conditions along with various stages of ontogenesis may affect antioxidant potential (Ferreira *et al.*, 2009). The present study provides information on the antioxidant production of ten filamentous fungi grown by submerged cultivation under optimised conditions for biomass generation (Table 3.2), following different extraction processes in a comparative manner. The results confirm the possibility of the development of a new source of natural antioxidants. Thereby, in addition to their dietary therapeutic potential (Chapter 4), the species used throughout the present investigation have demonstrated they may function as a source of possible protective agents in animal diets to help reduce oxidative damage.

5.3   Antioxidant capacities of extracts in relation to total phenolics and other compounds

In this section, some of the most important antioxidant compounds known to be present in fungi were quantified, including the polyphenolic compounds; phenolic acids,

flavonoids and condensed tannins. These compounds were believed to have had a major contribution to the antioxidant activity observed in Section 5.2. Polyphenols represent a diverse class of compounds including flavonoids (i.e. flavones, flavonols, flavanones, flavononols, chalcones and flavan-3-ols), lignins, tocopherols, tannins and phenolic acids (Shukla *et al.*, 1997). Other antioxidant compounds such as carotenoids, alkaloids and polyketide derivatives are also known to be present in fungi. Hence, total antioxidant capacity may have incorporated the activity of other fungal antioxidants, including carotenoids, alkaloids and vitamins. Any quantified compound which didn't demonstrate a relationship with antioxidant capacities of fungal extracts is believed to be a response of the possible presence of other aforementioned compounds in higher abundance. In a study by Lee *et al.* (2007b) ascorbic acid was detected in small amounts, with β-carotene and tocopherols not being detected in the water extracts of *Pleurotus citrinopileatus* due to their fat-soluble nature. In addition, ascorbic acid and β-carotene were either not present or present in small quantities in a study by Tsai *et al.* (2009), which tested ethanolic and hot water extracts of *Clitocybe maxima*, *Pleurotus ferulae* and *Pleurotus ostreatus*. In order to obtain more information about the nature of the antioxidant compounds in each of the selected fungi and in order to support the results obtained in Section 5.2, the quantity of the main antioxidant compounds believed to be present were obtained and correlated with $ABTS^{+}$ radical scavenging activity, reducing power and chelating ability.

It has been reported that the process of lyophilisation, retains and preserves the highest quantities of biologically active compounds (Ferreira *et al.*, 2007; Jayakumar *et al.*, 2009). Lyophilised extracts from Pleurotus mycelia has been seen to possess antioxidant and reducing activity higher than other commercial mushrooms (Vamanu, 2012). In the present study, three extraction conditions, namely; crude, hot water and methanol, were selected to determine their effects on antioxidant capacity (Section 5.2), as well as, the isolation of antioxidant compounds. The EC_{50} value of *P. 1833*, *P. citrinopileatus*, *G. frondosa* and *L. edodes* revealed these species retained more β-carotene bleaching activity in the crude extract than the hot water and methanol extracts from these species (Table 5.2). In addition, the crude extracts from *OYRM*1 and *P. eryngii*, had the lowest EC_{50} value compared to hot water and methanol extracts for chelating ability, with the crude extract from *M. purpureus*, *P. 1833*, *P. 32783*, *P. citrinopileatus*, *P. salmoneo-stramineus* and *T. versicolor* demonstrating better activity than the methanol extract (Table 5.7).

5.3.1   Total phenolic content of fungal extracts in relation to antioxidant capacity

Fungal mycelium contains a substantial amount of vitamins, fibres, phenolic compounds and carotenoids. Thereby determination of antioxidant capacity in various studies has focused on these compounds (Jayakumar *et al.*, 2009). Phenolic compounds represent the main antioxidant components derived from fungi. Whilst providing protection from predators and pathogens, these compounds play an important role in growth and reproduction, contributing towards the colour and sensory characteristics of fungi. The ability of organisms such as plants and fungi to prevent oxidation is believed to be possibly attributed to the organisms ability to defend against microbial attacks and make food unpalatable to predators (Apak *et al.*, 2007b).

With the physiological importance of phenolic compounds, as well as their role in antioxidant capacity, the phenolic content of each fungal extract was estimated using the Folin-Ciocalteu method. The total phenolic content was determined using a standard curve of gallic acid and was expressed as gallic acid equivalents (GAE) (Section 2.2.7.1). The concentration of estimated phenolic antioxidant compounds in each sample was expressed in milligrams of phenol per gram (mg GAE g^{-1}) of dry extract for each sample.

As the solubility of certain compounds such as phenols and tannins are known to be an important contributor to biological activity and the measure of activity can depend the solvent concentration, the alcoholic solvent extract, in this case methanol, was subjected to different concentrations of water (100 %) and methanol (30 and 100 %). Consequently, the solubility of the extracted antioxidant compounds from the selected species in terms of phenolic and tannin content was estimated and their relationship with antioxidant capacity in terms of $ABTS^{+}$ radical scavenging activity was explored.

Table 5.9   Total phenolic content (mg GAE g^{-1}) of various extracts of fungi

Fungi	Crude extract	Hot water extract	Methanol extract MeOH:H$_2$O (0:100)	MeOH:H$_2$O (100:0)	MeOH:H$_2$O (30:70)
Grifola frondosa	2.31 ± 0.50[c]	9.33 ± 0.26[a]	4.76 ± 0.08[b]	8.13 ± 1.99[a]	7.53 ± 0.04[a]
Lentinula edodes	0.93 ± 0.37[e]	9.09 ± 0.16[a]	4.15 ± 0.21[d]	5.73 ± 0.04[c]	6.93 ± 0.08[b]
Monascus purpureus	1.98 ± 0.49[c]	1.19 ± 0.03[d]	5.41 ± 0.05[a]	3.02 ± 0.01[b]	5.69 ± 0.02[a]
*OYRM*1	3.40 ± 0.57[c]	5.64 ± 1.84[bc]	4.37 ± 0.79[c]	7.45 ± 0.62[ab]	7.82 ± 0.26[a]
Pleurotus ostreatus 1833	4.82 ± 0.86[ab]	0.44 ± 0.10[c]	1.12 ± 0.19[c]	4.02 ± 0.50[b]	5.51 ± 0.17[a]
Pleurotus ostreatus 32783	3.66 ± 0.43[cd]	5.01 ± 1.24[c]	2.97 ± 0.08[d]	9.29 ± 0.45[a]	7.99 ± 0.05[b]
Pleurotus citrinopileatus	4.26 ± 0.99[b]	2.55 ± 0.25[b]	2.94 ± 0.16[b]	8.54 ± 1.33[a]	7.97 ± 0.06[a]
Pleurotus eryngii	4.12 ± 1.94[ab]	1.63 ± 0.24[bc]	0.99 ± 0.16[c]	5.69 ± 1.24[a]	5.32 ± 0.11[a]
Pleurotus salmoneo-str.	4.14 ± 0.27[a]	4.12 ± 1.95[a]	5.03 ± 0.07[a]	5.72 ± 0.68[a]	5.03 ± 0.18[a]
Trametes versicolor	1.90 ± 0.15[b]	7.34 ± 2.31[a]	7.48 ± 0.30[a]	7.70 ± 1.47[a]	4.35 ± 0.04[b]

Results displayed are a representation of triplicate quantifications per extract. Total phenol content is based on a calibration curve of gallic acid (10 – 100 µg mL^{-1}), expressed as milligram Gallic Acid Equivalents (GAE)/g extract; $r^2 = 0.983$. For the same fungi with different extraction processes, means with different letters were significantly different ($p \le 0.05$, ANOVA, Tukey-HSD), with alphabetical order of letters (a-e) representative of the highest to lowest phenolic content, respectively.
- Abbreviations: *OYRM*1, *Pleurotus ostreatus*; *Pleurotus salmoneo-str.*, *Pleurotus salmoneo-stramineus*.

Analysis of the data (Table 5.9) indicates that total phenolic content of the various extracts was dependant on the extraction process and shows the solubility of the phenolic compounds isolated following the methanol extraction process. The crude extract of *G. frondosa*, *L. edodes* and *T. versicolor* had a significantly lower phenolic content than the corresponding hot water and methanol extracts ($p \le 0.05$). As briefly mentioned in Section 5.1, this method of extraction with boiling water is capable of extracting compounds of high molecular weight such as polysaccharides and low molecular weight compounds such as phenols (Vaz *et al.*, 2011). Phenolic content in hot water extracts of *G. frondosa* and *L. edodes* was higher than that of methanol extracts of the same species. This observation is thought to be associated with the previously observed low ABTS$^+$ scavenging, CUPRAC, reducing power and chelating ability compared to the other fungi. The highest phenolic content was obtained from the methanol extracts of *M. purpureus*, *OYRM*1, *P. 1833*, *P. 32783*, *P. citrinopileatus*, *P. eryngii*, *P. salmoneo-stramineus* and *T. versicolor*. As previously observed by Cheung *et al.* (2003), higher extraction yields of phenolic compounds were obtained with an increase in polarity of the solvent, implying that most of the soluble components in fungi may be high in polarity. As such, a correlation between the polarity of the extraction solvent and phenolic content has been reported by several authors (Cheung *et al.*, 2003; Puttaraju *et al.*, 2006; Rajauria *et al.*, 2013). In general, the water extracts

and the methanol extract dissolved in 100 % water, demonstrated a lower quantity of phenolic compounds in comparison to the methanol extract dissolved in methanol (Table 5.9). With methanol being a highly polar solvent, the degree of extraction of phenolic compounds could be expected to be similar to that of the polar water extract, suggesting that differences in total content of phenols is mainly focused towards the extraction process utilised and individual differences between the range of species.

As phenolic compounds represent the main antioxidant components produced by fungi, it was assumed that the phenolic content of the mycelial extracts contributed significantly to the observed antioxidant activity. Several comprehensive works have been done on the effects of phenolic compounds, as well as, their association with total antioxidants. The bioactivity of phenolics may be associated with their ability to chelate metals, inhibit lipoxygenase and scavenge free radicals (Decker, 1997). These hydrophilic antioxidant compounds are secondary metabolites which have not only been found to be excellent antioxidants, but also are synergists that are not mutagenic. Phenols such as BHT and gallate are known as effective antioxidants (Lee *et al.*, 2007b), with BHT being used as a reference standard in many antioxidant assays. Both have been found to possess scavenging abilities of free radicals and chelating abilities on ferrous ions (Lee *et al.*, 2007b). For this reason, total phenolic content was correlated with radical scavenging activity, previously determined using the ABTS assay (Section 5.2.2.2). Total phenolic content of the crude, hot water and methanol extracts were also examined for their relationship with reducing power and chelating ability, summarised later in this chapter (Table 5.16). Table 5.10 displays the Pearson's correlation coefficient (*r*) between total phenolic content and ABTS antioxidant capacity.

Table 5.10 Correlation between antioxidant activity[a] and total phenolic content

| Fungi | Crude extract | Hot water extract | Methanol extract | | |
			MeOH:H$_2$O (0:100)	MeOH:H$_2$O (100:0)	MeOH:H$_2$O (30:70)
Grifola frondosa	0.994*	0.950	0.993*	0.984*	0.999*
Lentinula edodes	0.916	0.942	0.994*	0.999*	0.995*
Monascus purpureus	0.998*	0.962*	0.967*	0.932	0.966*
*OYRM*1	0.996*	0.998*	0.997*	0.997*	0.951
Pleurotus ostreatus 1833	0.996*	0.970*	1.000*	0.983*	0.883
Pleurotus ostreatus 32783	0.998*	0.977*	0.999*	0.952	0.943
Pleurotus citrinopileatus	0.995*	0.992*	0.999*	0.982*	0.965*
Pleurotus eryngii	0.997*	0.995*	0.999*	0.999*	0.977*
Pleurotus salmoneo-stramineus	1.000*	0.992*	0.994*	0.994*	0.988*
Trametes versicolor	0.995*	0.960*	0.979*	0.993*	0.992*

Data is representative of the correlation coefficient (r) between ABTS$^+$ radical scavenging activity (%) and total phenol content of filamentous fungi (1 – 20 mg mL^{-1}). All correlations were significant ($p \leq 0.05$), correlations with 99 % confidence ($p \leq 0.01$) are denoted with an asterisk [*]. [a]Antioxidant activity in terms of ABTS$^+$ radical scavenging activity.
- Abbreviations: *OYRM*1, *Pleurotus ostreatus*.

As discussed in Section 5.2.2, free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation. The methods of scavenging DPPH or ABTS$^+$ radicals can be used to evaluate the antioxidant activity of specific compounds or extracts in a short time (Cheung *et al.*, 2003). The results obtained show a significant correlation ($p \leq 0.01$) between total phenolic content and ABTS$^+$ radical scavenging capacity for the majority of fungal extracts (Table 5.10). With exception of *L. edodes*, a particularly significant correlation ($p \leq 0.01$) was observed for the crude extracts of each of the ten filamentous fungi (Table 5.10). The correlation coefficient was significant with at least 95 % confidence for all fungal extracts, indicating a strong correlation between antioxidant activity and phenolic content. Consequently, the data suggests that the reductive potential of the fungal extracts was mainly due to phenolic compounds. This observation was in accordance with many findings, testing different fungal sources, which report total phenolic content is strongly associated with free radical scavenging activity (Puttaraju *et al.*, 2006; Yang *et al.*, 2006). In addition, previous reports of a strong correlation between phenolics and reducing power ability of several mushrooms has been seen (Puttaraju *et al.*, 2006). As this correlation is attributed to the reductive potential of the fungal extracts, it may be proposed that high free radical scavenging activity, reducing power ability and CUPRAC may also be associated with high phenolic content in accordance with the antioxidant capacity

(Table 5.16). As such, higher content of total phenols in each of the ten filamentous species would be expected to attribute to greater antioxidant function.

Estimation of total phenolic content using the Folin-Ciocalteu phenol assay is based on hydroxyl groups and their place in the molecules. It is now known that this method is sensitive for monohydric phenols, polyphenols, flavonoids and tannins, and as such is not exclusive to phenolics (Palacios *et al.*, 2011). This may lead to an overestimation of phenolic content since other readily oxidised substances, such as sugars, ascorbic acid and amino acids may interfere with the colorimetric reagent (Palacios *et al.*, 2011). Nonetheless, despite its tendency to overestimate the level of phenolics, Folin-Ciocalteu is a widely employed method prior to the quantitative measurement of phenolic compounds using HPLC (Ferreira *et al.*, 2009). It is these aspects which increase the importance of the determination of phenolic compounds by more sophisticated methods (Ferreira *et al.*, 2009). As a result of the strong correlation between phenolic content and antioxidant capacity and due to possible discrepancies in phenolic content using the Folin-Ciocalteu method as a result of interfering components, an individual qualitative and quantitative profile of phenolic compounds was obtained by UPLC later in this study (Section 6.2.1).

Antimicrobial activity established in Chapter 4 was thought to be attributed to compounds of phenolic or lipid structure, due to the solubility of particular compounds from aqueous mycelial extracts in DMSO. The observed antioxidant capacity of extracts is expected to be associated to some degree of bioactivity. The mechanism of action believed to be responsible for restriction of bacterial growth is similar for phenolics, flavonoids, tannins, catechins and quinones (Cowan, 1999). Surface-exposed adhesins, cell wall polypeptides, and membrane bound enzymes are possible targets in the microbial cell, with some compounds such as quinones, capable of rendering substrates unavailable to the microorganism (Cowan, 1999). Simple phenolics consisting of a single substituted phenolic ring are known to be bioactive, such as; cinnamic and caffeic acids. In addition, hydroxylated phenols, catechols and pyrogallol have shown toxicity to microorganisms, with evidence that increased hydroxylation leads to increased toxicity (Geissman, 1963). Some reports show that the more highly oxidised phenols have greater inhibitory action (Urs *et al.*, 1975; Scalbert, 1991). The mechanisms responsible for phenolic toxicity include enzyme inhibition by the oxidised compounds *via* reaction with sulfhydryl groups or more nonspecific interactions with the proteins (Mason *et al.*, 1987). Flavonoids are believed to exert antimicrobial

activity through their ability to complex with extracellular and soluble proteins, and to complex with bacterial cell walls, resulting in effectiveness against a wide range of microorganisms (Cowan, 1999). The phenolic compounds which have a greater ability to dissolve fats i.e. are more lipophilic, may be more inclined to disrupt microbial membranes (Tsuchiya *et al.*, 1996), which may have been the predominant cause of action during agar well antimicrobial examination in Section 4.2. Quercetin, naringin, hesperetin, and catechin, which have strong antibacterial association, have also demonstrated activity toward a number of viruses *in vitro* (Kaul *et al.*, 1985). Tannins are also presented by fungi, with condensed tannin being a derivative of flavonoid monomers. Tannins have been associated with stimulation of phagocytic cells, host-mediated tumour activity, and a wide range of anti-infective actions (Haslam, 1996). Their mode of action is believed to be their ability to inactivate microbial adhesins, enzymes and cell envelope transport proteins amongst others, similar to that of other natural compounds (Scalbert, 1991; Haslam, 1996). Terpenoids have established activity toward bacteria, fungi and protozoa (Cowan, 1999). Although their mechanism of action is not fully understood it is believed to be due to interference of the cell membrane due to lipophilic compounds (similar to the mechanism of flavonoids), the potency of their functional groups and their aqueous solubility (Knobloch *et al.*, 1989). Mechanisms such as inhibition of electron transport; protein translocation; phosphorylation and other enzymatic reactions take place at the phospholipid bilayer, resulting in antimicrobial action directly (Knobloch *et al.*, 1989). Peptides were first reported to have antimicrobial activity in 1942 (Balls *et al.*, 1942), now these compounds are known to partake in cellular metabolism, development, pathogenicity and stress responses (Teichert *et al.*, 2011). Specifically, nonribosomal peptides represent a superior group of fungal secondary metabolites, second only to polyketides (Teichert *et al.*, 2011). These compounds represent a small selection of metabolites from fungi which have been known in multiple instances to be active toward a variety of microorganisms. General differences in the antimicrobial effect by the group of fungi tested may not only be due to the inherent characteristics of the bacterial cell wall as discussed (Section 4.2.1), but may also be due to phytochemical differences among the fungal species.

The possible health benefits derived from dietary phenolic compounds depends on their absorption and metabolism (Parr *et al.*, 2000); hence, structural specifications, such as their conjugation with other phenols, degree of glycosylation/acylation,

molecular size and solubility are important factors for consideration (Bravo, 1998). Notably, the bioavailability of phenolic compounds can also be affected by differences in cell wall structures, glycoside location in cells and binding moieties of phenolic compounds within food matrices (Hollman *et al.*, 1997). Differences in total antioxidant capacity established in the methods used in the present investigation may also be attributed to a combinational effect of different phenolic compounds (Puttaraju *et al.*, 2006).

5.3.2   Relationship between antioxidant activities, as determined by $ABTS^+$, FRAP and chelating assays, and total phenolics, flavonoids and condensed tannin content

Although there is a large degree of structural diversity in the range of phenolic compounds that exist in nature, several main classes have been characterised (Balasundram *et al.*, 2006). With regard to these classes, phenolic acids, flavonoids and tannins are considered the main dietary phenolic compounds (Balasundram *et al.*, 2006). In order to obtain more information about the fungi used throughout this study and with a view to rationalising the antioxidant potential of the extracts in terms of their polyphenolic constituents, Pearson correlation coefficients were calculated between $ABTS^+$ and total phenolic, flavonoid and condensed tannin content. A summary of correlations between total polyphenolic content and FRAP and chelating assays is summarised later in this chapter (Table 5.16).

The most important polyphenolic compounds believed to be present in the selected fungal species were estimated as described in Section 2.2.7. Flavonoids constitute the largest group of phenolics, accounting for over half of the eight thousand naturally occurring phenolic compounds (Balasundram *et al.*, 2006). These polyphenolic compounds represent a special class of low molecular weight compounds consisting of fifteen carbon atoms, arranged in a C_6-C_3-C_6 configuration and two aromatic rings linked by three carbons cyclised with oxygen (Balasundram *et al.*, 2006; Apak *et al.*, 2007b). Flavonoids differ with respect to the degree of unsaturation and oxidation of the three-carbon segment (Apak *et al.*, 2007b). Variations in substitution patterns result in the major flavonoid classes, i.e. flavonols, flavones, flavanones, flavonols (catechins), isoflavones, flavanonols, and anthocyanidins, of which flavones and flavonols are most widely occurring and structurally diverse (Balasundram *et al.*, 2006). In the present investigation, flavonoids occurring in the fungal extracts were

examined by a conversion of red complexes with aluminium chloride in alkaline media as described in Section 2.2.7.2. With this method, flavone derivatives give no colour with aluminium chloride therefore this method is highly selective for flavonoids (Turner, 1952; Balbaa *et al.*, 1974; Palacios *et al.*, 2011). Total flavonoid content of the hot water and methanol extracts in comparison to the lyophilised biomass of the selected fungal species is displayed in Table 5.11.

Table 5.11   Total flavonoid content (mg QE g^{-1}) of various extracts

Fungi	Crude extract	Hot water extract	Methanol extract MeOH:H$_2$O (0:100)	MeOH:H$_2$O (100:0)
Grifola frondosa	0.16 ± 0.01^c	0.38 ± 0.02^b	1.26 ± 0.07^a	0.37 ± 0.03^b
Lentinula edodes	0.05 ± 0.06^c	0.33 ± 0.04^b	1.52 ± 0.03^a	0.27 ± 0.05^b
Monascus purpureus	0.22 ± 0.02^c	1.43 ± 0.24^a	1.71 ± 0.10^a	0.64 ± 0.02^b
OYRM1	0.06 ± 0.00^b	2.17 ± 0.72^a	2.43 ± 0.04^a	0.85 ± 0.03^b
Pleurotus ostreatus 1833	0.05 ± 0.00^d	2.83 ± 0.18^a	1.53 ± 0.08^b	0.84 ± 0.03^c
Pleurotus ostreatus 32783	0.07 ± 0.00^d	1.08 ± 0.13^b	2.84 ± 0.11^a	0.74 ± 0.02^c
Pleurotus citrinopileatus	0.07 ± 0.01^d	2.40 ± 0.12^a	1.72 ± 0.07^b	0.50 ± 0.02^c
Pleurotus eryngii	0.37 ± 0.02^d	1.69 ± 0.08^b	2.40 ± 0.06^a	0.73 ± 0.03^c
Pleurotus salmoneo-stramineus	0.07 ± 0.00^c	3.90 ± 0.23^a	1.23 ± 0.15^b	1.22 ± 0.03^b
Trametes versicolor	0.02 ± 0.00^b	0.87 ± 0.06^a	0.77 ± 0.08^a	0.84 ± 0.03^a

Results displayed are a representation of triplicate quantifications per extract. Total flavonoid content is based on a calibration curve of quercetin (0.01 – 50 µg mL^{-1}), expressed as mg quercetin equivalents (QE)/g of extract; $r^2 = 0.999$. For the same fungi with different extraction processes, means with different letters were significantly different ($p \leq 0.05$, ANOVA, Tukey-HSD), with alphabetical order of letters (a-e) representative of the highest to lowest tannin content, respectively.
- Abbreviations: *OYRM*1, *Pleurotus ostreatus*.

The flavonoid content of the crude extracts was significantly lower than the other extracts ($p \leq 0.05$), demonstrating that further extraction processes were successful in concentrating these water soluble polyphenolic compounds. The highest quantity of flavonoids were retained in the hot water and the methanol extract when solubilised in water (100 %). Any significant association of antioxidant activity to total flavonoid content is noted in Table 5.12.

Table 5.12 Correlation between antioxidant activity[a] and total flavonoid content

Fungi	Crude extract	Hot water extract	Methanol extract[x]
Grifola frondosa	0.969*	0.838	0.949
Lentinula edodes	0.649	0.663	0.934
Monascus purpureus	0.949	0.848	0.947
*OYRM*1	0.901	0.926	0.975*
Pleurotus ostreatus 1833	0.930	0.889	0.979*
Pleurotus ostreatus 32783	0.976*	0.627	0.979*
Pleurotus citrinopileatus	0.877	0.996**	0.901
Pleurotus eryngii	0.904	0.956*	0.966*
Pleurotus salmoneo-stramineus	0.894	0.935	0.981*
Trametes versicolor	0.902	0.531	0.972*

Data is representative of the correlation coefficient (r) between $ABTS^+$ radical scavenging activity (%) and the total flavonoid content of filamentous fungi ($1-20$ mg mL^{-1}). Significant correlation, $p \leq 0.05$ and $p \leq 0.01$ is denoted with an asterisk [*] and [**], respectively. [a]Antioxidant activity in terms of $ABTS^+$ radical scavenging activity.
[x]Methanol extract (100:0, MeOH:H$_2$O)
- Abbreviations: *OYRM*1, *Pleurotus ostreatus*.

High flavonoid content should not be assumed to be a prerequisite for high antioxidant capacity, as the aluminium chloride (AlCl$_3$) colorimetric method of flavonoid determination does not measure for flavonoids which are absent of the characteristic chelating functional groups for Al binding (Apak *et al.*, 2007b). As is appreciable from Table 5.12, few extracts demonstrated a correlation between flavonoid content and $ABTS^+$ radical scavenging activity. Studies have shown low correlation between both ABTS or CUPRAC results and total flavonoids to be a result of the nature of the measurement technique (Park *et al.*, 2006). Nonetheless, poor correlation may also be due to species specificity for flavonoid production.

Tannins are relatively high molecular weight compounds which represent the third important group of phenolics (Balasundram *et al.*, 2006). These polyphenolic compounds are broadly categorised into two major groups namely, hydrolysable and condensed tannins. The hydrolysable tannins are esters of carboxylic acids and sugars that are easily hydrolysed to give benzoic acid derivatives and sugars. Hydrolysed tannins work both in the feed and animal digestive system, where they; act as natural preservatives, bind to protein and prolong feed stability as well as, slow passage of feed, reduce diarrhoea and increase growth rates. However, the consumption of feeds containing high levels of hydrolysable tannins can potentially cause liver and kidney toxicity (Makkar *et al.*, 2007). Condensed tannins, or proanthocyanidins, consist of oligomers of two or more flavan-3-ols, such as catechin, epicatechin that are linked

through C-C bonds (Apak *et al.*, 2007b). The condensed tannins are a group of polymers based on a hydroxylated C-15 flavonoid monomer unit. Tannins have a very high affinity for dietary proteins and carbohydrates, as well as enzymes, and encompass the ability to form complexes (Balasundram *et al.*, 2006). A concern with regards to phenolic compounds has been their ability to reduce the digestibility of proteins, either by direct precipitation or by inhibition of enzyme activity (Ferguson, 2001). Condensed tannins bind to protein and protect them from degradation by rumen microbes (Iqbal *et al.*, 2007). Protection of dietary protein from rumen degradation can increase protein availability or absorption in the small intestine, consequently improving gastrointestinal health (Iqbal *et al.*, 2007). Condensed tannins have also shown the ability to reduce the absorption of minerals such as iron and copper (Balasundram *et al.*, 2006). Nevertheless, phenolic compounds may exert their antioxidant activity by chelation of these metals also (Bravo, 1998). The ingestion of condensed tannins decreases nutrient utilisation with protein being affected to a great extent, and decreases feed intake. Tannin consumption has shown *in vivo* antioxidant activity, reducing lipid peroxidation, combating lymphocyte DNA damage and has a beneficial effect on the liver of pigs that were under oxidative stress induced with high polyunsaturated fatty acid (PUFA) intake (Riedl *et al.*, 2002).

Altogether, a dose dependant manner of tannin (condensed and hydrolysable) supplementation is imperative in order to avoid negative nutritional effects. Nevertheless, studies suggest that tannins can have beneficial effects on digestion and animal performance when they are incorporated into animal diets. Table 5.13 shows the total condensed tannin content following different mycelial extraction conditions. The solubility of tannins is known to affect their biological function. As such, measure of activity can depend on the solvent concentration; therefore, the methanol extract was subjected to different concentrations of water (100 %) and methanol (30 and 100 %). Consequently, the polarity of the extracted tannin compounds was explored. The total condensed tannin content was determined using a standard curve of gallic acid and was expressed in milligrams of catechin per gram (mg CE g^{-1}) of dry extract, where the (+)-catechin standard curve was prepared as previously outlined in Section 2.2.7.3.

Table 5.13   **Total condensed tannin content (mg CE g^{-1}) of various extracts**

Fungi	Crude extract	Hot water extract	Methanol extract MeOH:H$_2$O (0:100)	MeOH:H$_2$O (100:0)	MeOH:H$_2$O (30:70)
Grifola frondosa	1.94 ± 0.17^e	4.80 ± 0.25^b	6.97 ± 0.17^a	2.61 ± 0.00^d	4.19 ± 0.14^c
Lentinula edodes	1.77 ± 0.14^d	5.11 ± 0.29^a	3.19 ± 0.14^b	2.40 ± 0.00^c	3.11 ± 0.14^b
Monascus purpureus	2.28 ± 0.03^b	10.52 ± 1.08^a	**10.86 ± 0.29^a**	3.23 ± 0.14^b	11.19 ± 0.14^a
*OYRM*1	2.39 ± 0.43^d	8.80 ± 0.17^a	3.36 ± 0.14^c	3.19 ± 0.14^c	4.55 ± 0.14^b
Pleurotus ostreatus 1833	2.19 ± 0.14^c	10.75 ± 0.61^a	2.59 ± 0.16^c	6.90 ± 0.53^b	11.48 ± 0.06^a
Pleurotus ostreatus 32783	5.53 ± 0.38^b	6.01 ± 0.20^b	4.19 ± 0.29^b	5.19 ± 1.75^b	9.94 ± 0.14^a
Pleurotus citrinopileatus	**8.86 ± 0.14^c**	**18.83 ± 0.32^a**	4.78 ± 0.00^d	**11.53 ± 1.38^b**	**19.15 ± 0.53^a**
Pleurotus eryngii	2.78 ± 0.00^c	6.86 ± 1.10^a	2.64 ± 0.13^c	5.00 ± 0.10^c	3.19 ± 0.29^b
Pleurotus salmoneo-str.	4.03 ± 1.41^{cd}	15.94 ± 0.38^a	3.28 ± 0.00^d	5.28 ± 0.52^c	7.39 ± 0.45^b
Trametes versicolor	2.44 ± 0.29^c	6.00 ± 0.54^a	5.11 ± 1.66^{ab}	3.32 ± 0.29^{bc}	5.34 ± 0.22^{ab}

Results displayed are a representation of triplicate quantifications per extract. Total condensed tannin content is based on a calibration curve of (+)-catechin (50 – 1000 µg mL^{-1}), expressed as mg catechin equivalents (CE)/g extract; $r^2 = 0.996$. For the same fungi with different extraction processes, means with different letters were significantly different ($p \leq 0.05$, ANOVA, Tukey-HSD), with alphabetical order of letters (a-e) representative of the highest to lowest tannin content, respectively.
- Abbreviations: *OYRM*1, *Pleurotus ostreatus*; *Pleurotus salmoneo-str.*, *Pleurotus salmoneo-stramineus*.

In general, no particular extraction process was optimal for extraction of condensed tannin with the species tested. Though, the crude extract contained the minimal amount of tannin in comparison to the other extraction processes. The total condensed tannin content was species specific. *Pleurotus citrinopileatus* had the highest condensed tannin content of the crude, hot water and methanol (30 % and 100 %, v/v) extracts compared to the other species (Table 5.13). Variation between extracts is also believed to be attributed to the location of compounds within the fungal cell and the polarity of the compounds depending on the extraction process. The hot water extract of a number of fungi contained the highest quantity of condensed tannin; this is most likely due to the water soluble nature of these compounds. This observation demonstrates that the high temperature conditions of consecutive extraction steps during hot water extraction allowed effective isolation of this compound.

The crude extract of *G. frondosa*, *L. edodes*, *M. purpureus*, *OYRM*1, *P. 1833* and *T. versicolor*, and the water soluble methanol extract (100 % water) of *P. 32783*, *P. citrinopileatus*, *P. eryngii* and *P. salmoneo-stramineus* contained significantly less condensed tannin compared to other extracts (Table 5.13). The water soluble methanol extract of *G. frondosa* had the highest quantity of condensed tannin of this species, as did the hot water extract of *L. edodes*, *OYRM*1, *P. eryngii*, *P. salmoneo-stramineus* and *T. versicolor*. *P. 1833*, *P. 32783* and *P. citrinopileatus* suspended in 30 % methanol (v/v) contained in the highest quantity of solubilised tannins of each extract from these

species. Together with the hot water extract of *P. 1833* and *P. citrinopileatus*, these extracts were significantly higher than other extracts ($p \leq 0.05$).

In the previous section (Section 5.3.1), the antioxidant capacity of the various selected fungi were positively attributed to their phenolic content. To examine if these compounds could be responsible for antioxidant potential, the total condensed tannin content was also examined for correlation with radical scavenging activity, previously determined using the ABTS$^+$ assay.

Table 5.14   Correlation between antioxidant activity[a] and total tannin content

			Methanol extract		
Fungi	**Crude extract**	**Hot water extract**	**MeOH:H$_2$O (0:100)**	**MeOH:H$_2$O (100:0)**	**MeOH:H$_2$O (30:70)**
Grifola frondosa	0.882	0.857	0.998**	0.993**	0.995**
Lentinula edodes	0.980*	0.730	0.993**	0.974*	0.980*
Monascus purpureus	0.972*	0.734	0.781	0.983*	0.706
*OYRM*1	0.990**	0.803	0.915	0.996**	0.821
Pleurotus ostreatus 1833	0.983*	0.744	0.951*	0.967*	0.984*
Pleurotus ostreatus 32783	0.962*	0.777	0.891	0.777	0.776
Pleurotus citrinopileatus	0.992**	0.813	0.963*	0.944	0.863
Pleurotus eryngii	0.973*	0.863	0.968*	0.978*	0.953*
Pleurotus salmoneo-stramineus	0.883	0.775	0.944	0.998**	0.974*
Trametes versicolor	0.912	0.626	0.963*	0.953*	0.992**

Data is representative of the correlation coefficient (*r*) between ABTS$^+$ radical scavenging activity (%) and total condensed tannin content of filamentous fungi (1 – 20 mg mL^{-1}). Significant correlation, $p \leq 0.05$ and $p \leq 0.01$ is denoted with an asterisk [*] and [**], respectively. [a]Antioxidant activity in terms of ABTS$^+$ radical scavenging activity.
- Abbreviations: *OYRM*1, *Pleurotus ostreatus*.

In general, a significant correlation ($p \leq 0.05$) was observed for the crude and methanol extracts with the tannin content from the majority of species (Table 5.14). The data revealed that condensed tannin content positively influenced the antioxidant radical scavenging activity for some species. In general, a high degree of correlation is evidenced in Table 5.14; however, there is a lack of significant correlation between certain fungal extracts. Particularly between the ABTS$^+$ radical scavenging activity (%) and total condensed tannin of the hot water extracts. This observation demonstrates that a combinational effect of different phenolic compounds is likely to have attributed to the overall antioxidant capacities. Antioxidant activity may not always correlate with high quantities of phenolics; therefore, when evaluating the antioxidant potential of natural extracts, information must be provided on both the antioxidant activity and phenolic content (Kähkönen *et al.*, 1999).

The total phenolic, flavonoid and condensed tannin content is summarised for the efficiency of the different extraction processes in Table 5.15. This overview

illustrates any significant difference in content following the various extraction methods compared to the lyophilised crude mycelial extract. Any significant correlation of phenolic, flavonoid and condensed tannin content to ABTS$^+$ scavenging is also noted.

Table 5.15 Overview of antioxidant constituents present in various extracts of mycelium

Fungi	Extract	Total phenol[a] mgGAE g^{-1}	Total flavonoid[b] mgQE g^{-1}	Total tannin[c] mgCE g^{-1}
Grifola frondosa	Crude	2.31 ± 0.50^b	0.16 ± 0.01^a	1.97 ± 0.17^c
	Hot water	9.33 ± 0.26^{a*}	0.38 ± 0.02^{a*}	4.80 ± 0.25^{a*}
	Methanol	8.13 ± 1.99^{a*}	0.37 ± 0.03^{b*}	2.61 ± 0.00^{b*}
Lentinula edodes	Crude	0.93 ± 0.37^c	0.05 ± 0.06^b	1.77 ± 0.14^c
	Hot water	9.09 ± 0.16^{a*}	0.33 ± 0.04^{a*}	5.11 ± 0.29^{a*}
	Methanol	5.73 ± 0.04^{b*}	0.27 ± 0.05^{a*}	2.40 ± 0.00^{b*}
Monascus purpureus	Crude	1.98 ± 0.49^b	0.22 ± 0.02^c	2.28 ± 0.03^b
	Hot water	1.19 ± 0.03^{b*}	1.43 ± 0.24^{a*}	10.52 ± 1.08^{a*}
	Methanol	3.02 ± 0.01^{a*}	0.64 ± 0.02^{b*}	3.23 ± 0.14^b
*OYRM*1	Crude	3.40 ± 0.57^b	0.06 ± 0.00^b	2.39 ± 0.43^c
	Hot water	5.60 ± 1.84^{ab}	2.17 ± 0.72^{a*}	8.80 ± 0.17^{a*}
	Methanol	7.45 ± 0.62^{a*}	0.85 ± 0.03^b	3.19 ± 0.14^{b*}
Pleurotus ostreatus 1833	Crude	4.82 ± 0.86^a	0.05 ± 0.00^c	2.14 ± 0.13^c
	Hot water	0.44 ± 0.10^{b*}	2.83 ± 0.18^{a*}	10.75 ± 0.61^{a*}
	Methanol	4.02 ± 0.50^a	0.84 ± 0.03^{b*}	6.90 ± 0.53^{b*}
Pleurotus ostreatus 32783	Crude	3.66 ± 0.43^b	0.07 ± 0.00^c	5.53 ± 0.38^a
	Hot water	5.01 ± 1.24^b	1.08 ± 0.13^{a*}	6.01 ± 0.20^a
	Methanol	9.29 ± 0.45^{a*}	0.74 ± 0.02^{b*}	5.19 ± 1.75^a
Pleurotus citrinopileatus	Crude	4.26 ± 0.99^b	0.07 ± 0.01^c	8.86 ± 0.14^c
	Hot water	2.55 ± 0.25^b	2.40 ± 0.12^{a*}	18.83 ± 0.32^{a*}
	Methanol	8.54 ± 1.33^{a*}	0.50 ± 0.02^{b*}	11.53 ± 1.38^{b*}
Pleurotus eryngii	Crude	4.12 ± 1.94^{ab}	0.37 ± 0.02^c	2.78 ± 0.00^b
	Hot water	1.63 ± 0.24^b	1.69 ± 0.08^{a*}	6.86 ± 1.10^{a*}
	Methanol	5.69 ± 1.24^a	0.73 ± 0.03^{b*}	3.19 ± 0.29^b
Pleurotus salmoneo-stramineus	Crude	4.14 ± 0.27^a	0.07 ± 0.00^c	4.03 ± 1.41^b
	Hot water	4.12 ± 1.95^a	3.90 ± 0.23^{a*}	15.94 ± 0.38^{a*}
	Methanol	5.72 ± 0.68^a	1.22 ± 0.03^{b*}	5.28 ± 0.52^b
Trametes versicolor	Crude	1.90 ± 0.15^b	0.02 ± 0.00^b	2.44 ± 0.29^b
	Hot water	7.34 ± 2.31^{a*}	0.87 ± 0.06^{a*}	6.00 ± 0.54^{a*}
	Methanol	7.70 ± 1.47^{a*}	0.84 ± 0.03^{a*}	3.32 ± 0.29^b

Results displayed are a representation of triplicate quantifications per extract. [a]Total phenolic content based on calibration curve of gallic acid, expressed as milligram gallic acid equivalents (GAE) per gram of dry extract; $r^2 = 0.983$. [b]Flavonoid content expressed as milligram quercetin equivalents (QE) per gram of dry extract; $r^2 = 0.999$. [c]Total condensed tannin content based on calibration curve of (+)-catechin, expressed as milligram catechin equivalents (CE) per gram of dry extract; $r^2 = 0.996$. For the same fungi with different extraction processes, means with different letters were significantly different ($p \leq 0.05$, ANOVA, Tukey-HSD), with alphabetical order of letters (a-c) representative of the highest to lowest content. The Dunnett's test was used to evaluate the significance of compound isolation following hot water and methanol extraction in comparison to the crude extract, confidence level was set to 95 %. An asterisk [*] denotes a significant change in the content of a particular compound in comparison to the crude mycelial biomass extract.
- Abbreviations: *OYRM*1, *Pleurotus ostreatus*.

A significantly higher content of phenols was obtained following extraction of *G. frondosa*, *L. edodes*, *M. purpureus*, *OYRM*1, *P. 32783*, *P. citrinopileatus* and *T. versicolor* compared to the crude mycelium. A higher yield of total phenols is usually obtained using water or solvents of high polarity during extraction, such as, water, methanol and ethanol (Yim *et al.*, 2009). A higher yield of phenolic compounds were retained following methanol extraction for *M. purpureus*, *OYRM*1, *P. 32783*, *P. citrinopileatus*, *P. eryngii*, *P. salmoneo-stramineus* and *T. versicolor*. Apart from *P. 32783*, a significantly higher content ($p \leq 0.05$) of condensed tannin was retained in the hot water extract of each species examined (Table 5.15). Apart from the methanol extract of *OYRM*1, both extraction processes tested (methanol and hot water) significantly influenced the total flavonoid content of each species of fungi ($p \leq 0.05$). Therefore, the extraction methods tested had a significant positive impact on the extraction of flavonoids (Table 5.11). As shown in Table 5.11, the flavonoids isolated were mostly water-soluble compounds as the highest content was observed in hot water extracts (Table 5.15) or the methanol extract when solubilised in water (Table 5.11). Regardless of whether the flavonoids which were extracted by the methanol extraction process were dissolved in water or methanol, the methanol and hot water extraction method significantly ($p \leq 0.05$) improved total flavonoid isolation from each species.

It is recognised that further developmental stages of fungal growth can contain more antioxidative compounds than the mycelium; however, growth by submerged liquid fermentation represents a fast, industrial suitable method of cultivation for the production of antioxidant compounds. Table 5.15 demonstrates that extraction conditions can significantly improve efficiency in order to obtain high yields of particular antioxidant compounds. A similar quantity of phenols was obtained from the mycelium of *G. frondosa* (2.31 mg GAE/g^{-1}) when compared to a study by Mau *et al.* (2004), which measured the phenolic content of the mycelium of this species (1.59 mg g^{-1}) using the Folin-Ciocalteu reagent method also. They established phenols to be the major antioxidant component found in the methanolic extracts from the mycelia of *G. frondosa*, as is the case here (Table 5.15). A strong correlation between the total phenol content and radical scavenging activity was observed for all the species tested demonstrating these compounds to be responsible for the majority of antioxidant capacity. Overall, the extraction processes in the form of hot water and/or methanol positively influenced the concentration of antioxidant compounds and in doing so influenced antioxidant activity of *G. frondosa*, *L. edodes*, *OYRM*1, *M. purpureus*, *P.*

1833, *P. 32783*, *P. eryngii*, *P. citrinopileatus*, *P. salmoneo-stramineus* and *T. versicolor.* Table 5.16 gives an overview of the antioxidant contribution of the selected fungal species.

Table 5.16   Summary of antioxidant activity of fungal extracts in relation to total phenolic[x], flavonoid[y] and condensed tannin[z] content

Antioxidant activity	*Grifola frondosa*	*Lentinula edodes*	*Monascus purpureus*	*OYRM*1	*Pleurotus 1833*	*Pleurotus 32783*	*Pleurotus citrinopileatus*	*Pleurotus eryngii*	*Pleurotus salmoneo-str.*	*Trametes versicolor*	Control[d]
Crude extract											
β-carotene	84.09 ± 1.15	80.77 ± 0.95	80.45 ± 1.37	24.21 ± 0.60	71.33 ± 1.72	48.93 ± 0.44	84.40 ± 0.78	38.57 ± 0.06	22.13 ± 0.08	35.59 ± 0.05	96.47 ± 1.30[++]
CUPRAC	0.41 ± 0.12	0.25 ± 0.03	1.15 ± 0.08	0.68 ± 0.00	0.80 ± 0.06	0.91 ± 0.02	0.87 ± 0.03	1.20 ± 0.03	0.98 ± 0.04	0.56 ± 0.02	2.44 ± 0.02[++]
ABTS activity (EC$_{50}$[a])	25.95 ± 0.03[x]	33.18 ± 0.16[xz]	19.79 ± 0.13[xz]	12.23 ± 0.06[xz]	10.16 ± 0.06[xz]	15.12±0.02[xyz]	13.04 ± 0.05[xz]	13.07 ± 0.03[xz]	23.90 ± 0.09[x]	24.51 ± 0.03[x]	0.02 ± 0.04[+]
Reducing Power (EC$_{50}$[b])	37.78 ± 0.01[x]	47.51 ± 0.11[x]	24.83±0.21[xyz]	13.96 ± 0.08[x]	6.78 ± 0.05[xyz]	21.93 ± 0.18	18.77 ± 0.06[y]	8.53 ± 0.06[yz]	14.07 ± 0.04[yz]	43.30 ± 0.01[x]	0.56 ± 0.03[++]
Chelating ability (EC$_{50}$[c])	4.57 ± 0.09	3.65 ± 0.13[x]	1.22 ± 0.45	1.13 ± 0.23	1.16 ± 0.26	1.12 ± 0.29	1.27 ± 0.30	1.39 ± 0.29	1.39 ± 0.16	1.34 ± 0.18	0.07 ± 0.02[+++]
Total phenols[x]	2.31 ± 0.50	0.93 ± 0.37	1.98 ± 0.49	3.40 ± 0.57	4.82 ± 0.86	3.66 ± 0.43	4.26 ± 0.99	4.12 ± 1.94	4.14 ± 0.27	1.90 ± 0.15	-
Total flavonoids[y]	0.16 ± 0.01	0.05 ± 0.06	0.22 ± 0.02	0.06 ± 0.00	0.05 ± 0.00	0.07 ± 0.00	0.07 ± 0.01	0.37 ± 0.02	0.07 ± 0.00	0.02 ± 0.00	-
Total condensed tannins[z]	1.97 ± 0.17	1.77 ± 0.14	2.28 ± 0.03	2.39 ± 0.43	2.14 ± 0.13	5.53 ± 0.38	8.86 ± 0.14	2.78 ± 0.00	4.03 ± 1.41	2.44 ± 0.29	-
Hot water extract											
β-carotene	34.83 ± 1.57	22.07 ± 1.31	60.13 ± 3.12	63.37 ± 2.67	50.82 ± 3.74	65.98 ± 2.08	56.80 ±1.59	62.65 ± 1.73	57.73 ± 2.01	59.71 ± 3.55	94.69 ± 3.57[++]
CUPRAC	1.59 ± 0.05	1.74 ± 0.06	2.06 ± 0.02	0.93 ± 0.01	2.33 ± 0.00	1.22 ± 0.02	1.53 ± 0.01	1.75 ± 0.01	2.19 ± 0.00	2.08 ± 0.03	2.43 ± 0.02[++]
ABTS activity (EC$_{50}$[a])	14.79 ± 0.04[x]	13.77 ± 0.04	4.63 ± 0.12[xy]	7.67 ± 0.07[xy]	7.38 ± 0.03[xy]	3.95 ± 0.04[x]	6.21 ± 0.07[xy]	5.36 ± 0.05[x]	7.67 ± 0.07[x]	2.34 ± 0.06[x]	0.02 ± 0.04[+]
Reducing Power (EC$_{50}$[b])	15.11 ± 0.09[xz]	13.20 ± 0.08[xz]	10.80±0.06[xyz]	5.43 ± 0.05[yz]	7.51 ± 0.14[xyz]	4.61 ± 0.04[xz]	5.99 ± 0.07[yz]	6.70 ± 0.04[xyz]	5.71 ±0.11[yz]	3.56 ± 0.05[xz]	0.56 ± 0.03[++]
Chelating ability (EC$_{50}$[c])	0.64 ± 0.07[xz]	0.70 ± 0.09	0.66 ± 0.17[xy]	1.47 ± 0.27[y]	0.72 ± 0.10	0.87 ± 0.08	1.11 ± 0.18[xy]	2.60 ± 0.19	0.81 ± 0.17[xz]	0.79 ± 0.17	0.07 ± 0.02[+++]
Total phenols[x]	9.33 ± 0.26	9.09 ± 0.16	1.19 ± 0.03	5.60 ± 1.84	0.44 ± 0.10	5.01 ± 1.24	2.55 ± 0.25	1.63 ± 0.24	4.12 ± 1.95	7.34 ± 2.31	-
Total flavonoids[y]	0.38 ± 0.02	0.33 ± 0.04	1.43 ± 0.24	2.17 ± 0.72	2.83 ± 0.18	1.08 ± 0.13	2.40 ± 0.12	1.69 ± 0.08	3.90 ± 0.23	0.87 ± 0.06	-
Total condensed tannins[z]	4.80 ± 0.25	5.11 ± 0.29	10.52 ± 1.08	8.80 ± 0.17	10.75 ± 0.61	6.01 ± 0.20	18.83 ± 0.32	6.86 ± 1.10	15.94 ± 0.38	6.00 ± 0.54	-
Methanol extract											
β-carotene	65.22 ± 1.80	76.61 ± 1.06	83.94 ± 0.70	77.41 ± 0.75	63.49 ± 1.64	83.13 ± 0.84	53.60 ± 2.88	53.72 ± 0.05	59.73 ± 0.70	63.94 ± 0.05	93.93 ± 1.10[++]
CUPRAC	2.34 ± 0.045	2.29 ± 0.10	2.20 ± 0.03	1.85 ± 0.01	2.12 ± 0.05	2.30 ± 0.00	2.00 ± 0.05	2.24 ± 0.02	2.25 ± 0.05	2.34 ± 0.09	2.41 ± 0.02[++]
ABTS activity (EC$_{50}$[a])	8.43 ± 0.04[xyz]	9.90 ± 0.03[xyz]	1.84 ± 0.05[xz]	3.41 ± 0.07[xyz]	8.90 ± 0.04[xyz]	2.72 ± 0.08[x]	2.62 ± 0.06[x]	6.25 ± 0.04[xyz]	7.71 ± 0.03[xyz]	8.22 ± 0.03[xyz]	0.02 ± 0.04[+]
Reducing power (EC$_{50}$[b])	5.01 ± 0.15[xyz]	7.08 ± 0.14[xyz]	4.22 ± 0.08[xyz]	2.96 ± 0.07	2.93 ± 0.07[z]	2.44 ± 0.04	2.88 ± 0.02[z]	4.06 ± 0.07[xyz]	2.97 ± 0.02	3.42 ± 0.14[xy]	0.56 ± 0.03[++]
Chelating ability (EC$_{50}$[c])	4.09 ± 0.12	4.24 ± 0.11	4.67 ± 0.07	7.60 ± 0.04	5.84 ± 0.07[z]	6.18 ± 0.05[x]	4.31 ± 0.12[x]	6.20 ± 0.05	9.56 ± 0.06[xz]	4.84 ± 0.07[z]	0.07 ± 0.02[+++]
Total phenols[x]	8.13 ± 1.99	5.73 ± 0.04	3.02 ± 0.01	7.45 ± 0.62	4.02 ± 0.50	9.29 ± 0.45	8.54 ± 1.33	5.69 ± 1.24	5.72 ± 0.68	7.70 ± 1.47	-
Total flavonoids[y]	0.37 ± 0.03	0.27 ± 0.05	0.64 ± 0.02	0.85 ± 0.03	0.84 ± 0.03	0.74 ± 0.02	0.50 ± 0.02	0.73 ± 0.03	1.22 ± 0.03	0.84 ± 0.03	-
Total condensed tannins[z]	2.61 ± 0.00	2.40 ± 0.00	3.23 ± 0.14	3.19 ± 0.14	6.90 ± 0.53	5.19 ± 1.75	11.53 ± 1.38	3.19 ± 0.29	5.28 ± 0.52	3.32 ± 0.29	-

[a]Total phenolic content based on calibration curve of gallic acid, expressed as milligram gallic acid equivalents (GAE) per gram of dry extract; $r^2 = 0.983$. [b]Flavonoid content expressed as milligram quercetin equivalents (QE) per gram of dry extract; $r^2 = 0.999$. [c]Total condensed tannin content based on calibration curve of catechin, expressed as milligram catechin equivalents (CE) per gram of dry extract; $r^2 = 0.996$.
β-carotene of selected fungal extracts (1 mg mL^{-1}) based on % bleaching inhibition. CUPRAC of selected fungal extracts (10 mg mL^{-1}) based on absorbance (λ_{450nm}). EC$_{50}$ values [a,b,c] (mg mL^{-1}) of fungal extracts in terms of ABTS$^+$ scavenging, reducing power and chelating ability assays, data are expressed as means ± SD (n = 3) of upper and lower limit of dose interpolation curve, with 95 % confidence. [a]EC$_{50}$ (mg mL^{-1}) is representative of the effective concentration at which 50 % of ABTS$^+$ radicals are scavenged. [b]EC$_{50}$ (mg mL^{-1}) is representative of the effective concentration at which the absorbance was 0.5. [c]EC$_{50}$ (mg mL^{-1}) is representative of the effective concentration at which 50 % of Fe^{2+} ions were chelated. Control[d]: [+]Trolox; [++] α-tocopherol; [+++]EDTA. Significant correlation of AA in terms of ABTS$^+$ activity, reducing power and chelating ability to phenolic, flavonoid and condensed tannin content denoted by x, y and z, where confidence level was set to 95 %, respectively.
-Abbreviations: *Pl., Pleurotus*; *OYRM*1, *Pleurotus ostreatus*; *Peurotus salmoneo-str.*, *Pleurotus salmoneo-stramineus*.

As can be appreciated from Table 5.16, the total phenolic content extracted by the crude, hot water and methanol extraction processes, positively contributed to the ABTS$^+$ scavenging ability and reducing power potential. The total phenolic content of the crude extracts from *G. frondosa*, *L. edodes*, *M. purpureus*, *OYRM*1, *P. 1833*, *T. versicolor*, as well as, hot water extracts from *G. frondosa*, *L. edodes*, *M. purpureus*, *P. 1833*, *P. 32783*, *P. eryngii*, *T. versicolor* and methanol extracts of *G. frondosa*, *L. edodes*, *M. purpureus*, *P. eryngii* and *T. versicolor* showed significant correlation ($p \leq$ 0.05) to reducing power (Table 5.16). Apart from *L. edodes*, the phenolic content of each crude extract did not contribute to the chelating ability of fungi. However, the phenolic content of the hot water extract from *G. frondosa*, *M. purpureus*, *P. citrinopileatus*, *P. salmoneo-stramineus* and the methanol extract from *P. 32783*, *P. citrinopileatus* and *P. salmoneo-stramineus* showed significant correlation ($p \leq 0.05$) to chelating ability. In general, observations made from Table 5.16 emphasise that antioxidant activity of various extracts may be due to the reduction of hydroperoxide, inactivation of free radicals, complexation with metal ions, or a combination thereof (Lee *et al.*, 2007b).

It was observed that the overall total condensed tannin content of the crude, hot water and methanol extracts of each species positively contributed to the reducing power activity, particularly following hot water extraction; by which a significant correlation was revealed between content and the reducing power ($p \leq 0.05$) (Table 5.16). This is due to the water soluble nature of tannins and the efficiency of this extraction process in isolating them from the fungal cell. The tannin content extracted from the crude extract of each species did not contribute to the chelating ability of fungi. The correlation coefficient range observed was 0.261 for *P. 1833* to 0.811 for *T. versicolor*. By using the microwave assisted hot water extraction process, the tannin content of *G. frondosa* and *P. salmoneo-stramineus* significantly correlated ($p \leq 0.05$) to chelating ability and when extracted using methanol, *P. 1833*, *P. salmoneo-stramineus* and *T. versicolor* correlated to chelating ability also. These observations reveal that different compounds are extracted following different extraction procedures and these compounds have different affinities for different oxidative processes, most likely due to their polarity. In general, the antioxidant content of the crude extract showed poor contribution to chelating power, which is unexpected as the chelating power of crude and hot water extracts was high. This indicates that the chelating ability of fungi may be due to a different type of water soluble compound, for example; polysaccharides

from fungi have recently been reported to have antioxidative capabilities (Zeng *et al.*, 2010). Due to the considerable contribution of the water soluble phenolic compounds, it is possible that the poor correlation of both crude and hot water extracts be attributed to the fast reduction in ions, effectively causing a misinterpretation of their EC_{50} values. This is because calculation of the EC_{50} value for each species is based on a specific concentration of extract. As can be seen in Figure 5.6, at low concentrations of extract a high rate of chelation (%) was observed and increased with increasing concentration. In addition, at low concentrations of mycelial extract, the majority of metal ions were chelated in comparison to the methanol extracts (Figure 5.6).

According to the data obtained, phenols, flavonoids and condensed tannin were found to be effective antioxidants in different *in vitro* assays including reducing power, $ABTS^+$ radical scavenging and metal chelating activities when compared to standard antioxidant compounds such as BHT, Trolox, EDTA and α-tocopherol. Based on the results obtained, the fungal extracts show great potential for minimising or preventing lipid oxidation in food products, retarding the formation of toxic oxidation products, maintaining nutritional quality and prolonging the shelf life of foods and pharmaceuticals.

5.4   General Conclusions

For a more reliable assessment of antioxidant activity, selected fungal species were evaluated using a range of antioxidant tests. The crude, hot water and methanol extracts from mycelium of each of the selected species were effective as antioxidants. The results concluded that the antioxidant activity of fungal extracts, with stronger inhibition of reducing power, scavenging ability and chelating ability was concentration dependant. Methanolic extracts were typically more effective in antioxidant properties, except for chelation of metal ions. It appeared no one fungal species was optimal for antioxidant production as maximal activity from each assay was achieved with different fungi. Unfortunately, it can be difficult to compare the antioxidant capacity between different laboratories for a number of reasons; namely, different protocols, together with a wide variety of methods to determine antioxidant capacity, various methods of extraction, the various stages of ontogenesis (mycelium or fruiting body) and the variety of solvents which may be utilised; resulting in different yields. The data revealed that different extraction conditions affect antioxidant activities through their effect on biologically active compounds. That is, depending on the process of extraction,

different yields of antioxidant compounds were recovered. In addition, solvent selection during extraction leads to the extraction of a range of different compounds possibly with varying biological properties (Rajauria *et al.*, 2013).

In the previous section (Section 5.3) the antioxidant capacity of the various species of selected fungi was positively attributed to their phenolic content. The data indicated that a possible mechanism of antioxidant activity of the fungal extracts was through scavenging of free radicals generated during lipid peroxidation, most likely through hydrogen donating ability. This observation has been extensively reported (Barros *et al.*, 2007c; Barros *et al.*, 2008; Kim *et al.*, 2008). The antioxidant activities of polyphenols, as well as their metal chelating abilities were attributed to their redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers (Vaz *et al.*, 2011). Other components which were not quantified in this instance are also believed to have contributed to the antioxidant activity of fungal extracts. However, phenolic, flavonoid and tannin compounds made a significant contribution to the antioxidant activity of each species. In the present study, it was observed that the yield of phenolic acids from fungi can vary using different proportion mixtures of water and methanol. Variations in antioxidant activity between extracts may be attributed to aqueous extracts having high polyphenol content and the methanol extract having higher carotenoid and ascorbic acid content (Oke *et al.*, 2011). Compounds associated with antioxidant activity include ascorbic acid, β-carotene and tocopherols (α, β and γ) (Ferreira *et al.*, 2009); however, due to the heat sensitive characteristics of ascorbic acid as well as the fat-soluble nature of tocopherols and β-carotene, it is unlikely these compounds were extracted into hot water extracts (Lee *et al.*, 2007b). It is possible that the total antioxidant capacity observed may be due to a different type of compound with reducing capabilities; for example, polysaccharides from fungi have recently been reported for antioxidative effect (Zeng *et al.*, 2010). The production of darker red pigmentation of *M. purpureus* with increasing concentration was concurrent with an increase of antioxidant potential. This observation is believed to be due to secondary metabolite production of this fungus in the form of phenolic compounds and pigment production derived from polyketides. Dimerumic acid was previously established as having inhibiting NADPH- and iron (II)-dependant lipid peroxidation of rat liver microsomes (Aniya *et al.*, 2000).

In the present investigation, a number of assay systems were performed in an attempt to elucidate the mode of action of the antioxidants produced in mycelial

biomass using submerged liquid cultivation. The effect of different extraction processes on antioxidant content and capacity was also examined. The results indicated that when antioxidant activity determination was carried out by a single method, the antioxidant potential could be under or overestimated. The reaction conditions of the different antioxidant assays may have a great influence on the efficiency of antioxidants produced from the various fungi. From the three biomass extracts examined, it was concluded that the overall antioxidant capacity of *G. frondosa*, *M. purpureus*, *Pleurotus* spp., *L. edodes* and *T. versicolor* was attributed mainly to their phenolic content.

It is appreciable that the various antioxidants produced in the mycelium during submerged liquid cultivation are radical scavengers and primary chain-breaking antioxidants. The results revealed powerful natural antioxidant potential with different modes of action. Altogether, many fungi contain different antioxidants such as phenolic compounds, tocopherols, ascorbic acid, and carotenoids which could be specifically extracted for the purpose of being used as functional ingredients against diseases related to oxidative stress (Ferreira *et al.*, 2009). The results suggest that the filamentous fungi investigated in the present study may be used as a potential source of natural antioxidants for supplementation purposes or in the development of nutraceuticals. Combined with strong bactericidal properties (Chapter 4), these fungi show promising activity for further application. Submerged cultivation of *G. frondosa*, *M. purpureus*, *Pleurotus* spp., *L. edodes* and *T. versicolor* has proven to be an appropriate method for the production of natural antioxidants, which could potentially be used to partially substitute synthetic antioxidants which are currently used, such as BHT.

6.   Identification of active fungal constituents

Combining extraction and separation techniques for the isolation of pure, pharmacologically active constituents from fungi can be a long and tedious process (Hostettmann, 1999; Sticher, 2008). The localisation of constituents with biological activity using various chemical screening methods can enable elimination of unnecessary separation procedures, which would have downstream expenditure implications (Marston *et al.*, 1999).

The objective of the present research was to identify an active agent responsible for the biological activity previously established from ten selected filamentous fungi. Preliminary separation and identification of bioactive compounds was performed using thin layer chromatography (TLC). This technique is a well-known method for screening natural product constituents. It is one of the simplest and most inexpensive methods for this purpose, that can produce reproducible results easily and requires little equipment (Marston *et al.*, 1999). TLC is a liquid-solid absorption technique; that is, the mobile phase ascends the thin layer of the stationary phase by capillary action. Incorporation of bioassays may then allow for the localisation of active constituents from natural sources, followed by further isolation and purification (Marston *et al.*, 1999). In general, the detection of active constituents from extracts depends on both the biological activity of the extract and the sensitivity of the techniques used. High-performance liquid chromatography (HPLC) provides efficient separation of metabolites, this technique boasts sensitivity of detection, selectivity and quantification (Marston *et al.*, 1999). Also, HPLC coupled to mass spectrometry (LC/MS) is a widely used technology as it is one of the most sensitive methods for determination of molecular mass and structure of a compound (Hostettmann, 1999). These highly sensitive and selective techniques provide structural information and allow characterisation of metabolites in complex mixtures.

6.1   Isolation of bioactive constituents using TLC

Detection of compounds by TLC depends on chemical structure, natural colour and fluorescence under ultraviolet light at wavelengths of λ_{254nm} or λ_{365nm}. It is common practice when using TLC to apply selective or universal chemical reagents to assist in the identification of different types of components within a mixture.

To allow greater selectivity, secondary metabolites from fungi are generally purified and identified using TLC or column chromatography in conjunction with HPLC or GC/MS techniques. In the present investigation, analysis was then preceded by isolation and identification with antimicrobial testing. An advantage of the relative simplicity of these methods is that each stage of the extraction, fractionation and separation procedures may be monitored as the active material is purified. In the present study, a methanolic extract which was hypothesised to contain a broad assortment of metabolites was separated using TLC. The compound class was then determined using various detection reagents, with subsequent testing of the separated compounds for antibacterial activity. This allowed for specific extraction of the partially isolated active compounds from the TLC plate, in order to obtain information about the main active metabolites *via* LS/MS technology.

6.1.1   TLC method development

In order to separate and isolate individual compounds for downstream identification and examination of antibacterial activity, a number of experimental parameters were tested. They involved determining a suitable solvent system, optimal development time, concentration and quantity of the extract applied, along with determination of a suitable mobile phase. The retention factor (R_f) is a term used to measure the chromatographic differences of samples examined by TLC. The R_f value for a component is the distance it has travelled from the origin, divided by the distance travelled by the solvent mobile phase. Silica gel retains more polar compounds, therefore; non-polar compounds are eluted first. The more polar the component, the lower the R_f value and the less polar the component, the higher the R_f value. The intensity and size of the developed spot (component) on the plate is generally an indication of the concentration of that component in the mixture; allowing estimation of the relative concentration of between the various constituents.

TLC was performed as outlined in Section 2.2.8.1. The first step was to determine the solubility of the sample. Different solvent systems were examined for optimal separation of components from different species. The solvent systems in which the dry methanol extracts were resuspended included, chloroform:acetone:methanol:water, hexane:ethyl acetate, and chloroform:methanol, in varying ratios. The most suitable solvent system, yielding good separation of the chemical components from each fungal species was established as

chloroform:acetone:methanol:water (20:20:10:1, v/v/v/v). As water is one of the most strongly adsorbed solvents, the presence of a small quantity of water in the solvent system can greatly increase its eluting power (Ault, 1998). It is evident that different solvent systems which test combinations of solvents result different levels of separation of a particular mixture (Ault, 1998).

TLC plates were spotted with increasing volumes (1 to 5 µL) of the methanol extract (100 mg mL^{-1}). In the previous chapter, the methanol extract positively influenced the concentration of antioxidant compounds and in doing so influenced antioxidant activity of *G. frondosa*, *L. edodes*, *OYRM*1, *M. purpureus*, *P. 1833*, *P. 32783*, *P. eryngii*, *P. citrinopileatus*, *P. salmoneo-stramineus* and *T. versicolor*. It was determined that 1 µL of the methanol extract (100 mg mL^{-1}) applied to the TLC plate was sufficient for separation and visualisation. Higher concentrations lead to smearing as a result of overloading of components that were present in higher concentration.

The next step in the development of a suitable chromatographic system was selection of an appropriate mobile phase. TLC mobile phase systems usually contain a polar solvent and a chromatographically dissimilar less-polar solvent. The resolution of the compound to be visualised depends on the affinity between the sample, solvent and support. Modification of the polar solvent may result in a change in the resolution of the compound, whilst modification of the less-polar solvent results in a change in the R_f of the sample. Ideally the desired mobile phase would have the greatest solubility, to increase affinity for the sample on the stationary phase and to allow efficient separation and elution throughout the stationary phase. Initially, the two mobile phases examined were chloroform:hexane:ethyl acetate (95:5:5, v/v/v) and hexane:ethyl acetate (1:1, v/v). Hexane:ethyl acetate (1:1, v/v) was established as the most suitable mobile phase. This mixture resulted better visibility than the other mobile phase examined and yielded good separation of the different compounds within the extracts from the various species of fungi. For demonstrative purposes, Figure 6.1 exemplifies the separation of *M. purpureus* under optimised conditions for efficient separation and visualisation.
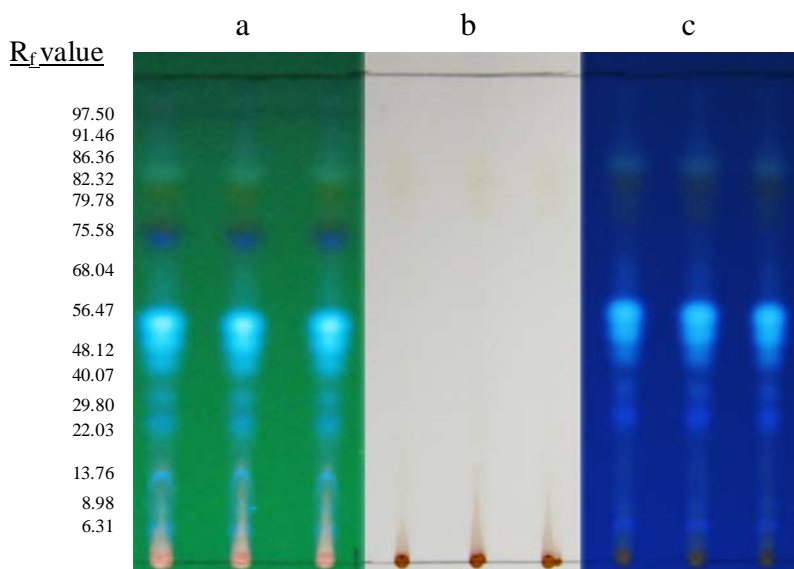
Figure 6.1   TLC of the methanol extract of *Monascus purpureus*

The methanol extract (1 µL) of *M. purpureus* (100 mg mL^{-1}) was spotted onto the TLC plate in triplicate. Sample was suspended in CHCl$_3$:acetone:MeOH:H$_2$O (20:20:10:1, v/v/v/v) (100 mg mL^{-1}). The plate was developed in hexane:ethyl acetate (1:1, v/v), with components visualised under λ_{254nm}[a], visible light[b] and λ_{365nm}[c]. R$_f$ values (R$_f$ x 100) are displayed to the left.

The mobile phase hexane:ethyl acetate (1:1, v/v) resulted in the separation of at least fifteen components in the methanol extract of *M. purpureus*. Fewer components were present in *Pleurotus* spp., *L. edodes*, *G. frondosa* and *T. versicolor* (results not shown), making *M. purpureus* the most diverse species of each of the selected fungi in the present investigation. This species also contained the most highly concentrated components, as florescence detected *via* UV light following TLC development was quite strong in comparison to the other species. In addition, due to the varying intensities of the different components observed under UV light, it was evident that the methanol extract contained components of varying concentration. This observation was common to the other species also. It is possible that different concentrations of the active component may have attributed to the overall efficiency of antioxidant and/or antibacterial activity previously observed for these species.

6.1.2   Screening of compound class using TLC

TLC in combination with staining reagents is a reliable method for analysing organic compounds without chromophores (Johnsson *et al.*, 2007). With few reports of the use of spray reagents for the identification of secondary metabolites from fungi, those used for chemical identification of phytochemical constituents in plants were applied. Extracts were subjected to silica gel TLC as described in Section 2.2.8.1. When

excited with UV-λ_{254nm}, absorbing compounds diminish (or quench) the uniform layer of fluorescence and are detected as dark violet spots on a bright green background. Fluorescence quenching compounds include mostly those with aromatic rings, conjugated double bonds and some unsaturated compounds. This type of detection, also known as natural fluorescence is non-destructive to the component and so is suitable for downstream active compound analysis using bioassay. Some compounds also emit a distinctive blue or yellow fluorescence under UV light. This type of visualisation was established in some of the extracts at UV-λ_{254nm}. Most of the compounds present in *M. purpureus* fluoresced light purple or white under UV light. Reaction of separated compounds following exposure to UV light contributed to the characterisation of individual compounds within the sample. This allowed the comparison of separated compounds between each fungal species. R_f values of components were determined and compared to the R_f values following detection with universal and specific TLC detection reagents. Tables 6.1 – 6.5 summarise the constituent class of separated components, as detected by a number of reagents following TLC development.

Throughout this section, the different constituents detected in each of the compounds (spots) visualised under different UV wavelengths are grouped together for discussion according to their R_f values. Therefore although isolates are grouped within the same R_f value does not imply that these components were the same between each species, but rather had similar polarity. Detection reagents were used for an indication of the class of constituents within each separated isolate. A combination of UV visibility and detection reagents was used to compare and contrast components between each species of fungi. A total of fifteen compounds were separated from *M. purpureus*, twelve compounds from *P. citrinopileatus*, ten compounds from *OYRM*1, *P. 1833*, *P. 32783* and *P. salmoneo-stramineus*, seven compounds from *G. frondosa*, eight compounds from *L. edodes* and eight compounds from *T. versicolor*. For discussion, the Pleurotus strains were grouped together, detailing which constituents were identified according to each individual species. In total fifteen different components were separated in this Basidiomycetes fungi.

Table 6.1   Determination of the class of constituents from *Grifola frondosa* using TLC detection reagents

| # | R_f x 100 | UV visibility | | Constituents | | | | | |
		λ_{254nm}	λ_{365nm}	Phenol	Flavonoid	Lipid	Terpenoid	Anthra-quinone/ Coumarin	Cdbs
1	**6.31 ± 0.96**	purple	white	-	-	-	-	-	-
2	8.98 ± 0.71	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
3	**13.76 ± 1.99**	NV	white	-	-	-	-	-	-
4	22.03 ± 2.16	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
5	**29.80 ± 1.67**	purple	NV	-	-	+	-	-	-
6	40.07 ± 6.89	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
7	48.12 ± 0.75	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
8	**56.47 ± 2.89**	NV	white	+	-	-	-	-	-
9	68.04 ± 2.12	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
10	**75.58 ± 1.84**	purple	white	-	-	-	+	-	+
11	79.78 ± 1.17	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
12	82.32 ± 0.83	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
13	86.36 ± 0.21	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
14	**91.46 ± 2.93**	white	white	+	+	+	-	-	-
15	**97.50 ± 0.74**	white	white	+	-	+	+	-	+

Data indicates regions of positive detection (+) according to R_f value of separated zones; positive and negative reaction to spray reagents are denoted by (+) and (-), respectively. Separation was performed using mobile phase hexane:ethyl acetate (1:1, v/v). Fast blue salt (FBS) was used for the detection of phenolic compounds; NP/PEG in conjunction with $AlCl_3$ was used for the detection of flavonoids; Lipids were detected with 10 % (w/v) sulphuric acid; Terpenoid structures were detected with anisaldehyde-sulphuric acid (AS) reagent; potassium hydroxide (KOH) reagent was used for detection of anthraquinones (red), anthrones (yellow) and coumarins (blue) and iodine vapour was used for the detection of compounds with conjugated double bonds.
- Abbreviations: *nd*, not detected; Cdbs, Conjugated double bonds.

Table 6.2   Determination of the class of constituents from *Lentinula edodes* using TLC detection reagents

| # | R_f x 100 | UV visibility | | Constituents | | | | | |
		λ_{254nm}	λ_{365nm}	Phenol	Flavonoid	Lipid	Terpenoid	Anthra-quinone/ Coumarin	Cdbs
1	**6.31 ± 0.96**	purple	white	-	-	-	-	-	-
2	8.98 ± 0.71	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
3	**13.76 ± 1.99**	NV	white	-	-	-	-	-	-
4	22.03 ± 2.16	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
5	**29.80 ± 1.67**	purple	NV	-	-	-	-	-	-
6	40.07 ± 6.89	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
7	48.12 ± 0.75	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
8	**56.47 ± 2.89**	NV	white	+	-	-	-	-	-
9	**68.04 ± 2.12**	purple	NV	-	-	-	-	-	-
10	**75.58 ± 1.84**	purple	white	-	-	+	+	-	+
11	79.78 ± 1.17	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
12	82.32 ± 0.83	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
13	**86.36 ± 0.21**	white	white	-	-	+	-	-	-
14	**91.46 ± 2.93**	NV	white	+	+	+	-	-	-
15	97.50 ± 0.74	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*

Data indicates regions of positive detection (+) according to R_f value of separated zones; positive and negative reaction to spray reagents are denoted by (+) and (-), respectively. Separation was performed using mobile phase hexane:ethyl acetate (1:1, v/v). Fast blue salt (FBS) was used for the detection of phenolic compounds; NP/PEG in conjunction with $AlCl_3$ was used for the detection of flavonoids; Lipids were detected with 10 % (w/v) sulphuric acid; Terpenoid structures were detected with anisaldehyde-sulphuric acid (AS) reagent; potassium hydroxide (KOH) reagent was used for detection of anthraquinones (red), anthrones (yellow) and coumarins (blue) and iodine vapour was used for the detection of compounds with conjugated double bonds.
- Abbreviations: *nd*, not detected; Cdbs, Conjugated double bonds.

Table 6.3   Determination of the class of constituents from *Monascus purpureus* using TLC detection reagents

#	R_f x 100	λ_{254nm}	λ_{365nm}	Phenol	Flavonoid	Lipid	Terpenoid	Anthra-quinone/ Coumarin	Cdbs
1	6.31 ± 0.96	purple	purple	+	+	-	-	+	+
2	8.98 ± 0.71	orange	orange	+	-	-	-	+	-
3	13.76 ± 1.99	orange	white	-	-	-	+	+	+
4	22.03 ± 2.16	white	orange	-	-	-	-	-	-
5	29.80 ± 1.67	purple	purple	+	-	-	+	+	+
6	40.07 ± 6.89	white	white	-	+	+	+	-	-
7	48.12 ± 0.75	white	white	+	+	-	-	+	-
8	56.47 ± 2.89	white	white	+	+	-	-	+	-
9	68.04 ± 2.12	purple	green	+	-	-	+	+	+
10	75.58 ± 1.84	NV	purple	-	-	-	+	-	+
11	79.78 ± 1.17	purple	NV	-	-	+	-	+	+
12	82.32 ± 0.83	orange	orange	+	+	+	-	+	-
13	86.36 ± 0.21	white	white	+	+	+	+	-	+
14	91.46 ± 2.93	white	yellow	+	+	+	+	+	+
15	97.50 ± 0.74	NV	yellow	+	-	+	+	-	+

Data indicates regions of positive detection (+) according to R_f value of separated zones; positive and negative reaction to spray reagents are denoted by (+) and (-), respectively. Separation was performed using mobile phase hexane:ethyl acetate (1:1, v/v). Fast blue salt (FBS) was used for the detection of phenolic compounds; NP/PEG in conjunction with $AlCl_3$ was used for the detection of flavonoids; Lipids were detected with 10 % (w/v) sulphuric acid; Terpenoid structures were detected with anisaldehyde-sulphuric acid (AS) reagent; potassium hydroxide (KOH) reagent was used for detection of anthraquinones (red), anthrones (yellow) and coumarins (blue) and iodine vapour was used for the detection of compounds with conjugated double bonds.
- Abbreviations: *nd*, not detected; Cdbs, Conjugated double bonds.

Table 6.4   Determination of the class of constituents from Pleurotus* using TLC detection reagents

#	R_f x 100	λ_{254nm}	λ_{365nm}	Phenol	Flavonoid	Lipid	Terpenoid	Anthra-quinone/ Coumarin	Cdbs
1	6.31 ± 0.96	purple	white	+[cd]	-	-	-	-	-
2	8.98 ± 0.71	NV	purple	+[ad]	-	-	-	-	-
3	13.76 ± 1.99	white	white	-	-	-	-	+[ab]	+[ab]
4	22.03 ± 2.16	purple	white	+[df]	-	-	-	-	-
5	29.80 ± 1.67	purple	purple	+[bdf]	-	+[abcdef]	-	-	-
6	40.07 ± 6.89	purple	NV	-	-	+[abcdef]	+[df]	-	-
7	48.12 ± 0.75	purple	white	+[d]	-	-	-	-	-
8	56.47 ± 2.89	NV	white	+[df]	-	-	-	-	-
9	68.04 ± 2.12	purple	NV	-	-	-	+[abcdef]	-	+[abcdef]
10	75.58 ± 1.84	purple	white	-	-	-	+[d]	-	+[abcdef]
11	79.78 ± 1.17	NV	white	-	-	+[abcdef]	-	+[ab]	+[ab]
12	82.32 ± 0.83	NV	white	+[abcdef]	-	+[abcdef]	-	-	-
13	86.36 ± 0.21	purple	NV	-	-	-	-	-	-
14	91.46 ± 2.93	white	white	+[abcd]	+[abcdef]	+[abcdef]	-	+[ab]	+[ab]
15	97.50 ± 0.74	purple	white	+[f]	-	+[abcdef]	+[bcef]	-	+[abcdef]

Data indicates regions of positive detection (+) according to R_f value of separated zones; positive and negative reaction to spray reagents are denoted by (+) and (-), respectively. Separation was performed using mobile phase hexane:ethyl acetate (1:1, v/v). Fast blue salt (FBS) was used for the detection of phenolic compounds; NP/PEG in conjunction with $AlCl_3$ was used for the detection of flavonoids; Lipids were detected with 10 % (w/v) sulphuric acid; Terpenoid structures were detected with anisaldehyde-sulphuric acid (AS) reagent; potassium hydroxide (KOH) reagent was used for detection of anthraquinones (red), anthrones (yellow) and coumarins (blue) and iodine vapour was used for the detection of compounds with conjugated double bonds. *Relative to each *Pleurotus* species used throughout the present investigation; *OYRM*1[a], *P. 1833*[b], *P. 32783*[c], *P. eryngii*[d], *P. citrinopileatus*[e] and *P. salmoneo-stramineus*[f].
- Abbreviations: *nd*, not detected; Cdbs, Conjugated double bonds.

Table 6.5   Determination of the class of constituents from *Trametes versicolor* using TLC detection reagents

#	R_f x 100	λ_{254nm}	λ_{365nm}	Phenol	Flavonoid	Lipid	Terpenoid	Anthra-quinone/ Coumarin	Cdbs
		UV visibility				**Constituents**			
1	6.31 ± 0.96	purple	white	-	-	-	-	-	-
2	8.98 ± 0.71	nd	nd	nd	nd	nd	nd	nd	nd
3	13.76 ± 1.99	NV	white	-	-	-	-	-	-
4	22.03 ± 2.16	purple	white	+	-	-	-	-	-
5	29.80 ± 1.67	nd	nd	nd	nd	nd	nd	nd	nd
6	40.07 ± 6.89	nd	nd	nd	nd	nd	nd	nd	nd
7	48.12 ± 0.75	nd	nd	nd	nd	nd	nd	nd	nd
8	56.47 ± 2.89	NV	white	-	-	-	-	-	-
9	68.04 ± 2.12	nd	nd	nd	nd	nd	nd	nd	nd
10	75.58 ± 1.84	purple	white	-	-	-	+	-	+
11	79.78 ± 1.17	nd	nd	nd	nd	nd	nd	nd	nd
12	82.32 ± 0.83	NV	white	+	-	-	+	-	-
13	86.36 ± 0.21	nd	nd	nd	nd	nd	nd	nd	nd
14	91.46 ± 2.93	white	white	+	+	+	-	-	-
15	97.50 ± 0.74	purple	NV	-	-	+	+	-	+

Data indicates regions of positive detection (+) according to R_f value of separated zones; positive and negative reaction to spray reagents are denoted by (+) and (-), respectively. Separation was performed using mobile phase hexane:ethyl acetate (1:1, v/v). Fast blue salt (FBS) was used for the detection of phenolic compounds; NP/PEG in conjunction with $AlCl_3$ was used for the detection of flavonoids; Lipids were detected with 10 % (w/v) sulphuric acid; Terpenoid structures were detected with anisaldehyde-sulphuric acid (AS) reagent; potassium hydroxide (KOH) reagent was used for detection of anthraquinones (red), anthrones (yellow) and coumarins (blue) and iodine vapour was used for the detection of compounds with conjugated double bonds.
- Abbreviations: *nd*, not detected; Cdbs, Conjugated double bonds.

Phenols can be detected by a number of developing reagents. Phenolic compounds were confirmed by the appearance of red to brown zones in visible light after spraying with fast blue salt (FBS) reagent. They are visualised as dark spots (purple) as they absorb in short UV wavelength.

The data revealed that isolate number 3 (R_f value; 13.76 ± 1.99), 10 (R_f value; 75.58 ± 1.84) and 11 (R_f value; 79.78 ± 1.17) in each species of which these isolates were detected, did not react positively to FBS detection reagent, indicative of the absence of phenolic constituents in these regions (Table 6.1 – 6.5). Isolate number 3 is believed to be the same compound in *G. frondosa*, *L. edodes* and *T. versicolor*, due to the similar characteristics when visualised under UV wavelength. However, detection reagents employed were unsuccessful in detecting of the type of component(s) present, which suggests that the concentration of the compound was too low for detection or the possible components tested were absent from this region. Region number 11 was not detected in *G. frondosa*, *L. edodes* or *T. versicolor*. The data revealed that some compounds may be present in fungi of the same genus at different concentrations. For instance, isolate number 12 from Pleurotus reacted positively for phenolic compounds in each of the species from this genera tested. However, of the other regions

containing natural constituents from Pleurotus which reacted positively to FBS reagent, detection was only illustrated in some species (Table 6.4). This was believed to be due to the varying concentrations of phenolic components between the various species, as was also illustrated by the total phenolic content, established earlier in Section 5.3.1 (Table 5.9). This observation is assumed to be true for the other types of components. A correlation between the polarity of the extraction solvent and phenolic content has been reported by several authors (Cheung *et al.*, 2003; Puttaraju *et al.*, 2006; Rajauria *et al.*, 2013). This indicated that each of the Pleurotus species may have contained the same phenolic constituents at region 12. Although the most polar constituent of *M. purpureus* and Pleurotus extracts (R_f value; 6.31 ± 0.96) reacted positively for phenolics when tested with FBS, it is believed that the same compound was present in *G. frondosa*, *L. edodes* and *T. versicolor*, due to the similar characteristics when visualised under UV wavelength. This observation suggests that the concentration of the compound in this region was too low for detection. The same observation was also made for constituents within the R_f region of 56.47 (Table 6.1 – 6.5). Thereby as these species demonstrated relatively high phenolic content in the methanol extract (100 %) (Table 5.9), a different phenolic component within the extract with similar polarity is believed to have attributed to the associated antioxidant activity of the phenolic content.

Flavonoids and coumarins are phenolic structures. Preparations of physiologically active constituents such as flavonoids have been used for centuries to treat human disease (Cushnie *et al.*, 2005). This class of natural product is an increasing focus of anti-infective research. The isolation and identification of flavonoids have been associated with antifungal, antiviral and antibacterial activity (Cushnie *et al.*, 2005). NP/PEG is generally used for the detection of coumarins, arbitin drugs, bitter principles and flavonoids. This reagent intensifies the fluorescence of native coumarins at UV-λ_{365nm}. In the present investigation, use of this reagent confirmed the presence of flavonoid structures in each of the selected fungi; seven compounds of this nature were detected in *M. purpureus* extracts (Table 6.3). As established in Section 5.3.2, water soluble flavonoids were predominantly present in each fungus. This is likely to attribute to the weak fluorescence demonstrated by the methanol extract when developed by TLC. TLC plates were also subjected to analysis using aluminium chloride ($AlCl_3$) which reacts with flavonoids allowing visualisation at UV-λ_{365nm}. Isolate number 14 demonstrated a strong reaction to this reagent. Under

UV light, galloyl esters and gallo tannins appear as violet fluorescence, which may be enhanced on fumigation with ammonia vapour. Reaction with $AlCl_3$ solution was indicated by yellow fluorescence indicating the presence of flavonoids; with blue spots revealing phenolic acids. Isolate number 14 appeared suggestive of a phenolic acid and was found to be common to each species. This observation was confirmed by a positive reaction in this region to FBS reagent.

Ten isolates which were separated from *M. purpureus* reacted with KOH (Table 6.3), which is indicative of the presence of anthraquinones (red), anthrones (yellow) or coumarins (blue). With the exception of isolate number 9 and 14, respectively, each of the visible regions appeared red. *M. purpureus* is well known for its pigment production. Anthraquinones are a group of pigments of quinoid nature often found in fungi (Gessler *et al.*, 2013). These naturally occurring constituents are known to exert colour to the mycelium of microscopic fungi, fruiting bodies of macroscopic fungi, and lichens (Gessler *et al.*, 2013). These phenolic compounds are widespread in the kingdom of fungi and may exhibit a broad range of biological activity, including bacteriostatic, fungicidal, antiviral, herbicidal and insecticidal activity (Gessler *et al.*, 2013). Isolate number 9 and 14 from *M. purpureus*, as well as, 3, 11 and 14 from both *OYRM*1 and *P. 1833*, also displayed a positive reaction with KOH reagent. This reaction was demonstrated by the appearance of blue zones under UV wavelength and therefore was indicative of the presence of coumarins.

The presence of alkaloids is indicated by the appearance of yellow to brown spots in visible light immediately after spraying with Dragendorff reagent. Furthermore, in this instance the plate was sprayed with 0.01 % (v/v) fluorescein which is an indicator to the presence of lipids. Detection of regions that demonstrated a positive reaction to these reagents was unsuccessful. However, the spot of application appeared yellow to orange in visible light after spraying with Dragendorff reagent, as well as, fluorescing bright blue at UV-λ_{365nm}, indicative to the presence of alkaloids. This observation may be a consequence of this type of compound present at low concentration.

In order to establish the presence of lipids, developed TLC plates were submerged (as opposed to sprayed) in 10 % (v/v) sulphuric acid solution and placed in an oven (105 °C) for 30 minutes, as described in Section 2.2.8.2. Six compounds (mostly less polar components) from *M. purpureus* tested positive for the presence of

lipids (Table 6.3). These constituents were common to most of the other fungi tested also.

Anisaldehyde-sulphuric acid (AS) reagent was applied for the non-specific detection of terpenoids. This reagent is sensitive to most functional groups and tends to be insensitive toward alkenes and aromatic compounds, unless other functional groups are present. Some components exhibited distinct colours (violet/blue-green) with these reagents, positively revealing the presence of terpenoid molecules within the extracts. Reaction with AS was strong, particularly with *M. purpureus*, indicating that eight components were of terpenoid structure (Table 6.3). The presence of this type of compound within the extracts is not unusual as terpenes along with lactones, amino acids and carbohydrates determine the aroma and flavour characteristics of both the fruiting body and mycelial biomass (Tsai *et al.*, 2009). As there is no sensitive universal reagent for the detection of terpenoids, the use of iodine vapour was tested to yield more information about the structure of the main compound. Iodine has a high affinity for both saturated and aromatic compounds. The majority of natural terpenoids have cyclic structures with one or more functional groups (e.g. hydroxyl, carbonyl) (Harborne, 1998); therefore, their conjugated double bond systems appear as yellow zones in visible light when exposed to iodine vapour (Fuchs *et al.*, 2011). Most of the components which tested positive for terpenoid structures displayed this reaction when exposed to the vapour, confirming that they were natural compounds that contained conjugated double bonds (Table 6.1 – 6.5). This confirmed the positive reaction observed with the universal detection reagent, anisaldehyde-sulphuric acid. Isolate number 15, in the R_f region of 97.50, which tested positive for AS reagent from *M. purpureus* was common to *G. frondosa*, *P. 1833*, *P. 32783*, *P. citrinopileatus*, *P. eryngii*, *P. salmoneo-stramineus* and *T. versicolor*. In addition, these compounds also reacted positively to iodine vapour revealing natural terpenes with conjugated double bonds. Vanillin, a phenolic aldehyde, is an organic compound with the molecular formula $C_8H_8O_3$. Therefore, phenolic units can also be encountered in terpenoids (as a functional group) and may display positive reactions with some of the detection reagents that detect phenolic compounds. In addition, the colour reactions observed for phenolics were less intense than those for terpenoids, which may suggest that they were a substituent attached to the main terpenoid compound. Enormous structural diversity is associated with terpenoids, which is almost matched by their functional variability (Ashour *et al.*, 2010).

Overall, the data confirmed that the majority of components were of phenolic or lipid structure. Information obtained following this section contributed to their quantitative identification using UPLC, GC/MS and LC/MS.

6.1.3   Preliminary isolation and identification of bioactive compounds

The objective of this aspect of research was to establish if separated compounds were associated with antibacterial activity. This would also complement information about the antimicrobial activity previously established by agar well diffusion (Section 4.2.1). Having established the compound class using universal and specific TLC detection reagents, TLC with a bioassay *in situ* and comparison of R_f values with reference chromatograms, allowed for localisation of biologically active constituents within the complex matrix. In this section, the separated components were assessed for antibacterial activity against various gram-positive and gram-negative bacteria using the bioautography agar over-lay method.

Bioautography is a simple and convenient method of applying target-directed isolation of active pure compounds (Marston *et al.*, 1999; Ejikeme *et al.*, 2010). There are three methods of bioautography; contact, direct and agar-overlay (Marston *et al.*, 1999; Horvath *et al.*, 2002). The agar over-lay method is a hybrid of contact and direct techniques and is applicable to a broad spectrum of microorganisms (Hostettmann, 1999). Zones of inhibition (ZOI) are formed where antimicrobial agents are present. In the present study, the agar over-lay method was used in conjunction with tetrazolium salts (MTT) to help localise and measure zones of bacterial inhibition (as described in Section 2.2.5.4). Using this method, the active compounds are transferred from the stationary phase to the agar layer by diffusion. MTT converts the dehydrogenase of living microorganisms into intensely coloured formazan, aiding visualisation of antimicrobial agents (Choma *et al.*, 2011). Non-viable bacteria do not reduce MTT, thereby enabling clear zones of inhibition for viable bacteria (Marston *et al.*, 1999). This technique was previously observed by Saxena *et al.* (1995); whereby, the agar-overlay method was used in conjunction with MTT for visualisation of inhibitory zones of various microorganisms, including *Staphylococcus aureus*.

Figure 6.2 illustrates results of bioautographic analysis, following separation of active compounds by TLC. The bacterial species tested were gram-positive *Escherichia coli* 10778 and gram-negative *Staphylococcus epidermidis* 1798, both

bacteria demonstrated sensitivity to fungal crude mycelial extracts when examined using agar well diffusion (Section 4.2.1; Table 4.3 and Table 4.4).
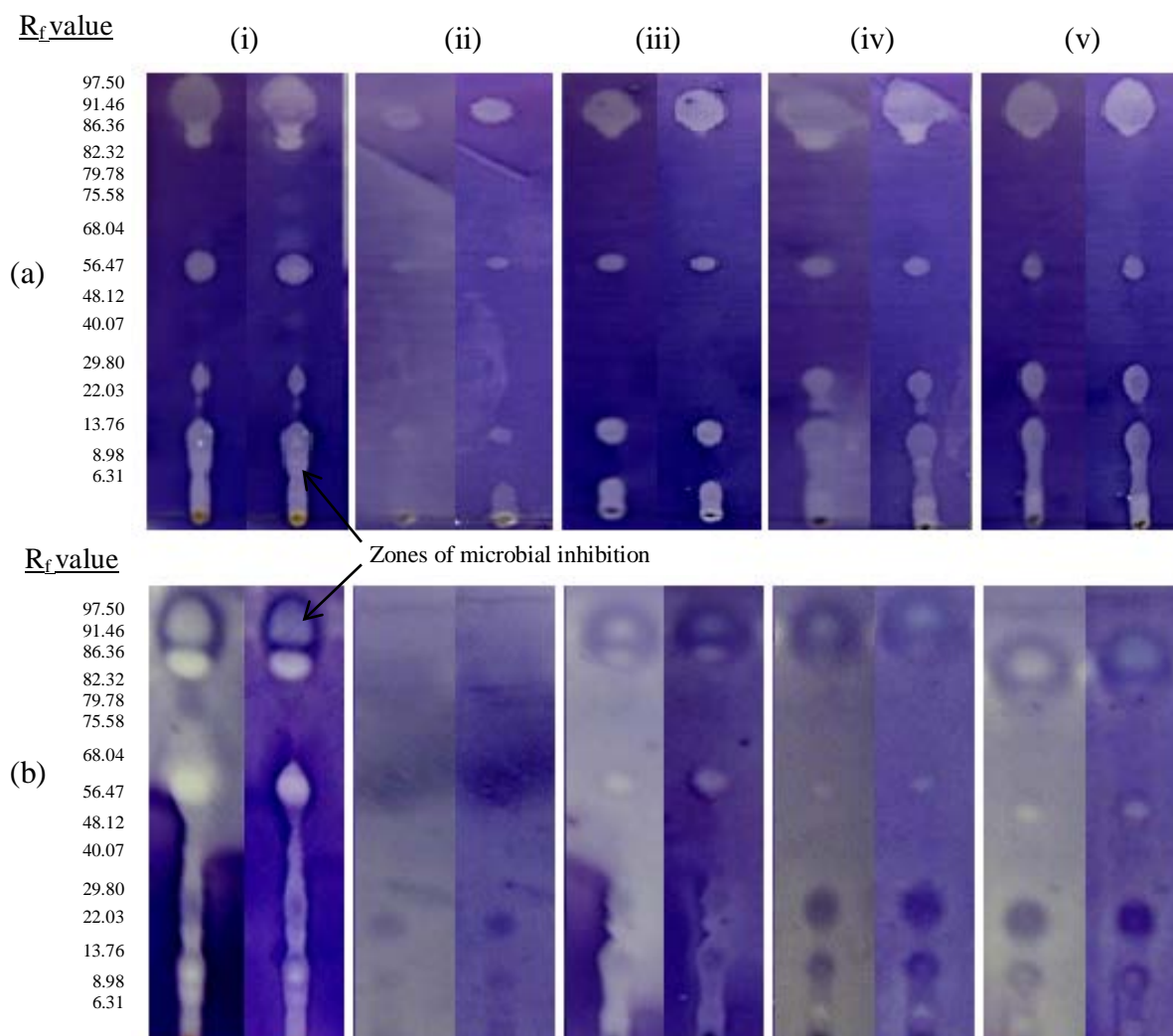
Figure 6.2   Zone of inhibition analysis of methanol extracts against (a) *E. coli* 10778 and (b) *S. epidermidis* 1798 using bioautography

Figure is demonstrative of bioautographic analysis; results are displayed in duplicate from the following filamentous species; *Monascus purpureus* (i), *OYRM*1 (ii), *Pleurotus 1833* (iii), *Pleurotus citrinopileatus* (iv), *Pleurotus eryngii* (v). Separation of methanol extract (100 mg mL^{-1}) was performed using mobile phase hexane:ethyl acetate (1:1, v/v). R_f values (R_f x 100) are displayed to the left. Clear zones of microbial growth inhibition are demonstrated against a purple background of viable bacteria grown on solidified nutrient agar.

Figure 6.3 illustrates a comparative bioautographic profile of six fungal strains against *E. coli* 10778.

R$_f$ value
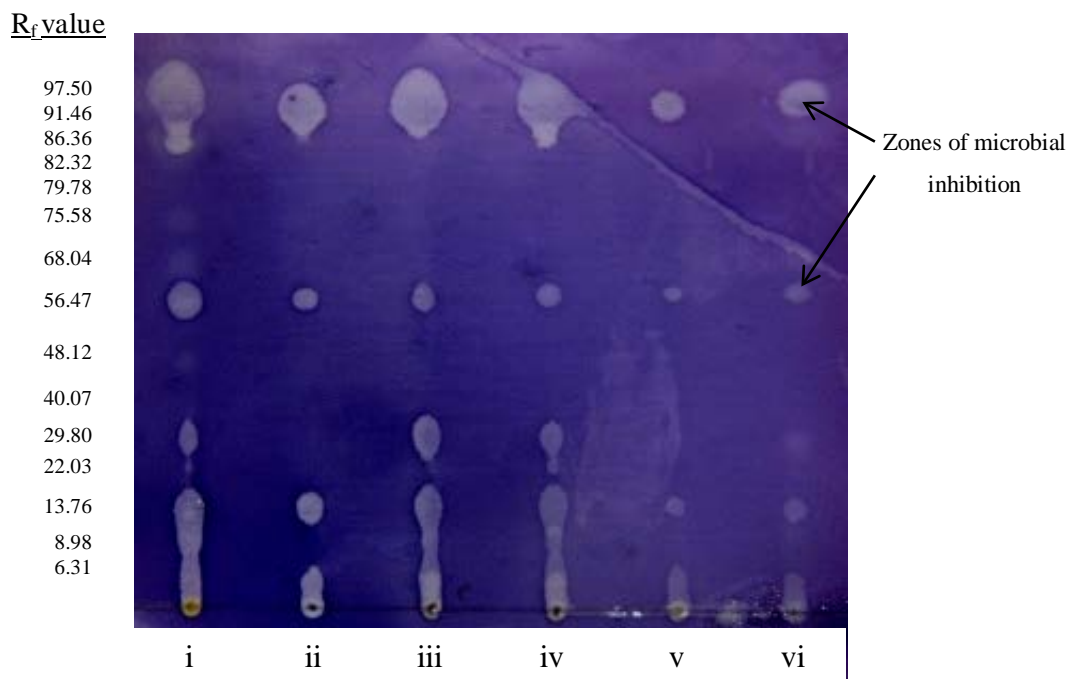
Figure 6.3   Zone of inhibition analysis against *E. coli* 10778 using bioautography
Results are demonstrative of the bioautographic analysis results from the following filamentous species; *Monascus purpureus* [i], *Pleurotus 1833* [ii], *Pleurotus eryngii* [iii], *Pleurotus citrinopileatus* [v], *OYRM*1 [v] and *Pleurotus 32783* [vi]. Antibacterial activity established as zones of inhibition against *E. coli* 10778. Separation of methanol extract (100 mg mL^{-1}) was performed using mobile phase hexane:ethyl acetate (1:1, v/v). R$_f$ values (R$_f$ x 100) are displayed to the left. Clear zones of microbial growth inhibition are demonstrated against a purple background of viable bacteria grown on solidified nutrient agar.

Figure 6.3 demonstrates the differences in the antibacterial activity of the separated regions of each fungal species against *E. coli* 10778. As can be appreciated, many of the active compounds were common to each species or different active constituents were of similar polarity. This observation was also made when determining the compound class in the previous section (Section 6.1.2). Four distinguishable active compounds displayed in Figure 6.3 i.e. R$_f$ values; 6.31, 13.76, 56.47 and 91.46 were common to *M. purpureus* and each *Pleurotus* species. Figures 6.2 and 6.3 illustrate that the majority of non-polar compounds (which elute first) from *M. purpureus*, *P. eryngii* and *P. citrinopileatus* were active against *E. coli* 10778. Each species demonstrated different intensities of antibacterial activity for each constituent region. This may be due to a high concentration of an active agent or the presence of multiple antibacterial components. This observation was also evident for *P. salmoneo-stramineus*, *G. frondosa*, *L. edodes* and *T. versicolor*. Previously (Section 4.2.1), different fungi exerted only slightly different degrees of antibacterial action, as was indicated by their zone of inhibition using agar well diffusion. *M. purpureus* demonstrated the strongest activity against *E. coli* 10778, which may be indicative of

the quantity of isolated active components (Figure 6.3). Conversely, the separated components of *OYRM*1 and *P. 32783* (Figure 6.3) demonstrated weaker activity in comparison to the other species, yet demonstrated strong inhibition of microbial growth by agar well diffusion. Therefore, the data suggests that biological activity of the whole extract may be not only due to one constituent but combination thereof. The combined action of multiple constituents may work synergistically or additively. Junio *et al.* (2011) found optimal activity against *S. aureus* using a combination of three flavonoids synergistically, in order to enhance the antimicrobial activity against *S. aureus*. Individually they possessed no inherent activity against *S. aureus*; therefore, they could have been missed using traditional bioactivity directed fractionation (Junio *et al.*, 2011). Downstream separation and identification of active constituents was established with extracts from each of the ten selected filamentous fungi used throughout this study, using the R_f value of these compounds in conjunction with their established antibacterial ability.

     M. purpureus contained the most diverse range of compounds; additionally, this species also had the most heavily concentrated compounds following separation and visualisation by TLC. This was represented by the intensity of the separated components. Isolates with similar R_f values and characteristics when visualised under UV light were identified in each of the different species of fungi. According to their relative R_f values, some of the constituents visualised in the different higher Basidiomycetes species were also present in the *M. purpureus* species. Therefore, identification of particular compound class prior to high-throughput identification using UPLC and GC/MS focused on this strain (Table 6.6).

     Table 6.6 summarises the various classes of compounds from *M. purpureus* and displays the components which were bioactive against *E. coli* 10778. *M. purpureus*, as well as, *G. frondosa*, *L. edodes* and Pleurotu*s* were further assessed for components demonstrating antibacterial action against *Streptococcus uberis* 700640, *Yersinia enterocolitica* 11504, *Staphylococcus aureus* 1604, *S. epidermis* 1798, *Pasteurella multicida* 10454, *E. coli* 10778 and *E. coli* 8879 later in this chapter (Tables 6.7 – 6.11). Each of these bacteria previously displayed sensitivity to the crude fungal extract using agar well diffusion (Section 4.2.1). In Section 4.1, fungal cell wall components showed capacity to bind to *S. epidermis* 1798 and *E. coli* 10778 *in vitro*. *Pasteurella multicida* and *Streptococcus uberis* are strongly associated with negative implications to the agriculture industry.

Table 6.6   **Preliminary isolation of constituents from *Monascus purpureus* according to R_f value in relation to antimicrobial activity against *E. coli* 10778**

	Isolate number[*]														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
R_f x 100	6.31 ± 0.96	8.98 ± 0.71	13.76 ± 1.99	22.03 ± 2.16	29.80 ± 1.68	40.07 ± 6.89	48.12 ± 0.75	56.47 ± 2.89	68.04 ± 2.12	75.58 ± 1.84	79.78 ± 1.17	82.32 ± 0.83	86.36 ± 0.21	91.46 ± 2.93	97.50 ± 0.74
UV-λ_{254nm}	V	V	V	V	V	V	V	V	V	/	V	V	V	V	/
UV-λ_{365nm}	V	V	V	V	V	V	V	V	V	V	/	V	V	V	V
Conjugated db bonds[a]	+	-	+	-	+	-	-	-	+	+	+	-	+	+	+
Lipids[b]	-	-	-	-	-	+	-	-	-	-	+	+	+	+	+
Terpenoids[c]	-	-	+	-	+	+	-	-	+	+	-	-	+	+	+
Phenolics[d]	+	+	-	-	+	-	+	+	+	-	-	+	+	+	+
Flavonoids[e]	+	-	-	-	-	+	+	+	-	-	-	+	+	+	-
Anthraquinones[f]	+	+	+	-	+	-	+	+	+	-	+	+	-	+	-
Activity[g]	**Y**	**Y**	**Y**	**Y**	**Y**	**N**	**Y**	**Y**	**N**	**Y**	**N**	**Y**	**Y**	**Y**	**Y**

Data is representative of triplicate determinations using various spray reagents for compound class identification of the methanol extract (100 mg mL^{-1}) from *Monascus purpureus* (1 µL). Mobile phase, hexane:ethyl acetate (1:1, v/v). Isolate number[*], (/) not visible at this wavelength; (V) visible detection; (-) no change or colour reaction; (+) positive reaction to reagent. [a]When exposed to iodine vapour, yellow zones were observed if the component contained conjugated double bonds; [b]Lipid constituents were visualised when the plate was submerged in 10 % sulphuric acid solution; [c]Terpenoid structures were observed with reaction to AS reagent, terpene constituents which also reacted with iodine vapour are representative of natural terpenoid structures with one or more functional group; [d]Phenolic compounds were detected with FBS spray reagent; [e]Flavonoids were detected using NP/PEG; [f]Anthraquinones or coumarin constituents were detected using KOH reagent; [g]Antibacterial activity tested using the agar over-lay bioautography technique, positive and negative inhibition of *E. coli* 10778 denoted by (Y) and (N), respectively.

Studies have shown that antimicrobial activity of mushrooms screened against gram-positive and gram-negative bacteria and fungi correlate with the amount of phenols, flavonoids, ascorbic acid, β-carotene, and lycopene present at various stages of the developing mushroom fruiting body, including development of mycelium (Barros *et al.*, 2007b). Phenolic acids, flavonoids and coumarins from fungi are known to exhibit antibacterial activity. The active compounds numbered 1, 2, 5, 7, 8, 12, 13, 14 and 15 (Table 6.6) gave positive colour reactions to FBS reagent, indicating the presence of phenolics. Conversely, isolate number 3, 4 and 10 did not react with the FBS spray reagent, suggesting these active compounds were not phenolic structures or structures which contained phenolic units. In addition, isolate number 9 reacted positively for phenolic structure and did not inhibit the growth of this species of bacteria (Table 6.6).

Some active compounds (1, 2, 3, 5, 7, 8, 10, 12, 13 and 14) reacted with KOH and/or NP/PEG, indicating that these compounds may be coumarin, flavonoid or anthraquinone constituents (Table 6.6). However, these compounds were established as active in the presence of other constituents of phenolic or lipid structure. Therefore, association of activity to a particular compound was difficult. There are some characteristics of the extracts to support the classification of the active components into the class of terpenoids. Terpenoids are lipid soluble and often difficult to characterise on a micro scale level since all (except carotenoids) are colourless (Harborne, 1998). These compounds represent a large class of secondary metabolites which may have pharmacological activities stemming from their role in natural defensive strategies (Ashour *et al.*, 2010). As mentioned previously (Section 6.1.2), their structure is extremely diverse, with different carbons and a large assortment of functional groups. Their classification is based on the number of isoprenoid units within the structure (Ashour *et al.*, 2010). The majority of terpenes that have been developed in the medicinal field are of plant origin. However, many terpenes from fungi have been found to possess antibacterial activity (Ghosh, 2013). Sesquiterpenes (three isoprenoid units) from the mycelial culture of two Basidiomycetes fungi, *Flammulina velutipes* and *Resupinatus leightonii*, have been shown to exhibit antibacterial activity toward a range of bacteria (Eilbert *et al.*, 2000; Ishikawa *et al.*, 2001). Although there is little information on the mode of action of the antimicrobial agents from fungi, it is known that compounds such as terpenoids, lectins and polysaccharides act on the bacterial cytoplasmic membrane (Lin *et al.*, 1984; Yang *et al.*, 2002; Urzúa *et al.*, 2008). Active compounds 3, 5, 10, 13, 14 and 15 reacted positively for the presence of terpenoids with

functional groups attached (Table 6.6). In addition, active lipid isolates numbered 12, 13, 14 and 15 demonstrated antibacterial action against *E. coli* 10778. Again, these compounds were established as active in the presence of other constituents of phenolic, terpenoid or lipid structure. As a result, definite association of activity of a particular compound requires further fractionation and biological analysis. A number of techniques or a combination thereof, such as ethanol precipitation, fractional precipitation, ion-exchange chromatography, gel filtration, and affinity chromatography are generally used for the intention of purification (Zhang *et al.*, 2007). Nonetheless, the primary aim of this section was to aid identification of the class of active compound for downstream analysis. The objective was to direct identification and detection of active constituents prior to analysis of the mycelial biomass by UPLC and GC/MS, and for that, TLC successfully associated compound class to active zones of interest.

TLC provided a good basis for preliminary identification of compound class in the fungal methanol extracts. This data lead to a better understanding to the type of constituents present and allowed for an association of active agents to particular compound structures. Universal and specific detection reagents, combined with the localisation of the active components using bioautographic analysis, revealed that the active constituents were that of terpenoid, lipid and/or phenolic structures. Furthermore, it was observed that some of the components which were terpenoid type constituents contained conjugated double bond systems (Table 6.6). Therefore, some active components of *M. purpureus* were possibly terpenoid constituents, with phenolic groups attached due to the presence of conjugated double bonds, particularly for compound numbers 5, 10, 13, 14 and 15 (Table 6.6). These isolates were typically non-polar and agreed with the R_f values of separated components which were visualised as having the most activity in comparison to the other isolated active regions (Figure 6.2 and 6.3).

6.1.4   Bioactivity of isolated compounds from several filamentous fungi

TLC was used for the biological testing of each species of filamentous fungi used throughout this investigation. The technique was also used as a means of establishing an association between antioxidant components of interest and antibacterial activity. Naturally derived antioxidants such as flavonoids, terpenoids and steroids have received considerable attention recently due to their diverse pharmacological properties. In Section 5.2, the fungal extracts were shown to contain substantial antioxidant activity.

Each TLC separated fraction was further tested for their antioxidant activity (DPPH radical-scavenging). DPPH solution was used to identify free radical scavenging zones, as outlined in Section 2.2.8.4, whilst also providing information on the polarity of the separated antioxidant compounds. DPPH transformed the conditioned plate purple, allowing the presence of antioxidants to be visualised by white spots on a purple background (Hostettmann, 1999), essentially acting as a qualitative indicator of free radical scavenging activity.

The following tables (6.7 – 6.11) outline the active areas of interest from each genre of fungi, according to R_f regions, as determined using bioautography agar over-lay. Using TLC, a total of fourteen active compounds were separated from *M. purpureus*, ten from *Pleurotus* spp., seven from *G. frondosa*, eight from *L. edodes* and six from *T. versicolor*.

Table 6.7 **Antibacterial and antioxidant compounds from *Grifola frondosa***

#	R_f x100	AA[1]	i	ii	iii	iv	v
			Antibacterial activity[2]				
1	**6.31 ± 0.96**	**Y**	-	-	**Y**	**Y**	-
2	8.98 ± 0.71	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
3	**13.76 ± 1.99**	-	-	**Y**	-	**Y**	**Y**
4	22.03 ± 2.16	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
5	**29.80 ± 1.67**	-	-	-	-	**Y**	**Y**
6	40.07 ± 6.89	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
7	48.12 ± 0.75	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
8	**56.47 ± 2.89**	-	-	-	**Y**	-	-
9	68.04 ± 2.12	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
10	**75.58 ± 1.84**	**Y**	-	-	**Y**	**Y**	**Y**
11	79.78 ± 1.17	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
12	82.32 ± 0.83	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
13	86.74 ± 0.68	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
14	**91.46 ± 2.93**	**Y**	-	**Y**	-	-	**Y**
15	**97.50 ± 0.74**	-	-	**Y**	**Y**	-	**Y**

Separation was performed using mobile phase hexane:ethyl acetate (1:1, v/v). [1]AA, Antioxidant activity detected in terms of DPPH$^+$ scavenging activity; [2]Antibacterial activity of separated compounds established by agar over-lay bioautography; i, *E. coli* 8879; ii; *E. coli* 10778; iii, *Staphylococcus aureus* 1604; iv, *Staphylococcus epidermis* 1798; v, *Salmonella enterica* 15480. Positive and negative inhibition of the microorganism is denoted by (Y) and (-), respectively.
- Abbreviations: AA, Antioxidant activity; *nd*, not detected; NV: not visible.

Table 6.8   Antibacterial and antioxidant compounds from *Lentinula edodes*

#	R_f x100	AA[1]	Antibacterial activity[2]				
			i	ii	iii	iv	v
1	**6.31 ± 0.96**	**Y**	-	-	**Y**	-	-
2	8.98 ± 0.71	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
3	**13.76 ± 1.99**	**-**	-	-	-	**Y**	-
4	22.03 ± 2.16	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
5	**29.80 ± 1.67**	**-**	-	-	-	-	**Y**
6	40.07 ± 6.89	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
7	48.12 ± 0.75	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
8	**56.47 ± 2.89**	**-**	-	-	-	-	-
9	**68.04 ± 2.12**	**Y**	-	-	-	-	-
10	**75.58 ± 1.84**	**-**	-	-	**Y**	**Y**	**Y**
11	79.78 ± 1.17	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
12	82.32 ± 0.83	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
13	**86.74 ± 0.68**	**-**	-	-	**Y**	**Y**	-
14	**91.46 ± 2.93**	**-**	-	**Y**	-	**Y**	**Y**
15	97.50 ± 0.74	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*

Separation was performed using mobile phase hexane:ethyl acetate (1:1, v/v). [1]AA, Antioxidant activity detected in terms of DPPH[+] scavenging activity; [2]Antibacterial activity of separated compounds established by agar over-lay bioautography; i, *E. coli* 8879, ii; *E. coli* 10778; iii, *Staphylococcus aureus* 1604; iv, *Staphylococcus epidermis* 1798; v, *Salmonella enterica* 15480. Positive and negative inhibition of the microorganism is denoted by (Y) and (-), respectively.
- Abbreviations: AA, Antioxidant activity; *nd*, not detected; NV: not visible.

Table 6.9   Antibacterial and antioxidant compounds from *Monascus purpureus* 1604

#	R_f x 100	AA[1]	Antibacterial activity[2]				
			i	ii	iii	iv	v
1	**6.31 ± 0.96**	**Y**	-	**Y**	**Y**	**Y**	**Y**
2	**8.98 ± 0.71**	**Y**	-	**Y**	**Y**	**Y**	**Y**
3	**13.76 ± 1.99**	**Y**	**Y**	**Y**	-	**Y**	**Y**
4	**22.03 ± 2.16**	**-**	-	**Y**	**Y**	**Y**	-
5	**29.80 ± 1.67**	**-**	-	**Y**	-	**Y**	**Y**
6	**40.07 ± 6.89**	**-**	-	-	-	**Y**	**Y**
7	**48.12 ± 0.75**	**-**	**Y**	**Y**	**Y**	**Y**	**Y**
8	**56.47 ± 2.89**	**-**	-	**Y**	**Y**	**Y**	**Y**
9	**68.04 ± 2.12**	**Y**	-	-	-	-	-
10	**75.58 ± 1.84**	**-**	**Y**	**Y**	**Y**	-	**Y**
11	**79.78 ± 1.17**	**-**	-	-	-	-	**Y**
12	**82.32 ± 0.83**	**-**	**Y**	**Y**	**Y**	-	-
13	**86.74 ± 0.68**	**Y**	-	**Y**	**Y**	**Y**	-
14	**91.46 ± 2.93**	**Y**	**Y**	**Y**	-	-	**Y**
15	**97.50 ± 0.74**	**-**	**Y**	**Y**	**Y**	**Y**	**Y**

Separation was performed using mobile phase hexane:ethyl acetate (1:1, v/v). [1]AA, Antioxidant activity detected in terms of DPPH[+] scavenging activity; [2]Antibacterial activity of separated compounds established by agar over-lay bioautography; i, *E. coli* 8879, ii; *E. coli* 10778; iii, *Staphylococcus aureus* 1604; iv, *Staphylococcus epidermis* 1798; v, *Salmonella enterica* 15480. Positive and negative inhibition of the microorganism is denoted by (Y) and (-), respectively.
- Abbreviations: AA, Antioxidant activity; NV: not visible.

Table 6.10 Antibacterial and antioxidant compounds from *Pleurotus** spp.

#	R_f x 100	AA[1]	Antibacterial activity[2]				
			i	ii	iii	iv	v
1	6.31 ± 0.96	Y	Y	Y	-	Y	Y
2	8.98 ± 0.71	-	-	-	-	-	-
3	13.76 ± 1.99	-	Y	Y	Y	Y	Y
4	22.03 ± 2.16	-	-	Y	-	Y	-
5	29.80 ± 1.67	-	-	Y	-	-	-
6	40.07 ± 6.89	Y	-	-	-	-	-
7	48.12 ± 0.75	-	-	-	-	-	-
8	56.47 ± 2.89	-	Y	Y	Y	Y	Y
9	68.04 ± 2.12	Y	Y	-	Y	-	-
10	75.58 ± 1.84	-	-	-	Y	-	Y
11	79.78 ± 1.17	-	Y	-	-	-	Y
12	82.32 ± 0.83	-	-	-	-	-	-
13	86.74 ± 0.68	-	-	-	-	-	-
14	91.46 ± 2.93	-	-	Y	-	-	Y
15	97.50 ± 0.74	-	Y	Y	Y	Y	Y

Separation was performed using mobile phase hexane:ethyl acetate (1:1, v/v). [1]AA, Antioxidant activity detected in terms of DPPH[+] scavenging activity; [2]Antibacterial activity of separated compounds established by agar over-lay bioautography; i, *E. coli* 8879, ii; *E. coli* 10778; iii, *Staphylococcus aureus* 1604; iv, *Staphylococcus epidermis* 1798; v, *Salmonella enterica* 15480. Positive and negative inhibition of the microorganism is denoted by (Y) and (-), respectively. *Relative to each *Pleurotus* species used throughout the present investigation (*OYRM*1, *P. 1833*, *P. 32783*, *P. eryngii*, *P. citrinopileatus* and *P. salmoneo-stramineus*) unless otherwise stated.
- Abbreviations: AA, Antioxidant activity; *nd*, not detected; NV: not visible.

Table 6.11 Antibacterial and antioxidant compounds from *Trametes versicolor*

#	R_f x100	AA[1]	Antibacterial activity[2]				
			i	i	i	i	v
1	**6.31 ± 0.96**	Y	-	-	-	Y	-
2	8.98 ± 0.71	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
3	**13.76 ± 1.99**	-	-	Y	Y	Y	Y
4	**22.03 ± 2.16**	-	-	-	-	-	-
5	29.80 ± 1.67	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
6	40.07 ± 6.89	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
7	48.12 ± 0.75	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
8	**56.47 ± 2.89**	-	-	-	-	Y	-
9	68.04 ± 2.12	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
10	**75.58 ± 1.84**	Y	Y	Y	Y	Y	Y
11	79.78 ± 1.17	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
12	**82.32 ± 0.83**	-	-	-	-	-	-
13	86.74 ± 0.68	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
14	**91.46 ± 2.93**	-	-	-	-	Y	-
15	**97.50 ± 0.74**	-	-	-	-	Y	Y

Separation was performed using mobile phase hexane:ethyl acetate (1:1, v/v). [1]AA, Antioxidant activity detected in terms of DPPH[+] scavenging activity; [2]Antibacterial activity of separated compounds established by agar over-lay bioautography; i, *E. coli* 8879, ii; *E. coli* 10778; iii, *Staphylococcus aureus* 1604; iv, *Staphylococcus epidermis* 1798; v, *Salmonella enterica* 15480. Positive and negative inhibition of the microorganism is denoted by (Y) and (-), respectively.
- Abbreviations: AA, Antioxidant activity; *nd*, not detected; NV: not visible.

A total of seven constituents from *G. frondosa* were separated using TLC (Table 6.7). Isolate number 1 was common to each of the selected fungi; this R_f region

exhibited scavenging ability against DPPH radicals, establishing antioxidant activity for this compound (Table 6.7 – 6.11). This may suggest components having similar structure being present within this region. This is not surprising as when tested with *Pleurotus* spp. and *M. purpureus* this region demonstrated a positive reaction to FBS detection reagent, which indicated the presence of a phenolic structure (Table 6.3 and 6.4, respectively). Depending on the fungal species, this isolate demonstrated antibacterial activity against each bacterium tested, which may be indicative of different concentrations of the same active agent in this region (Table 6.7 – 6.11). Isolate number 3 (R_f value; 13.76), was common to *M. purpureus*, *Pleurotus* spp., *G. frondosa*, *L. edodes* and *T. versicolor*. This region reacted similarly under UV light in *G. frondosa*, *L. edodes* and *T. versicolor* species (Table 6.1, 6.2 and 6.5, respectively). However, *Pleurotus* spp. and *M. purpureus* constituents in this region, which reacted positively for anthraquinones, terpenoids, conjugated double bonds and phenolics, displayed different characteristics when visualised under UV light. Therefore, the constituents in this region were likely to be of a different class. Isolate number 4 (R_f value; 22.03) was unique to *Pleurotus* spp., *M. purpureus* and *T. versicolor*. This compound reacted positively for phenolic constituents (Table 6.4 and 6.5) and demonstrated biological activity against *E. coli* 10778 and *Staphylococcus* spp. (Table 6.9 and 6.10); however, no resistance of growth was recorded with *T. versicolor* (Table 6.11). This is indicative of a different component in the same R_f region with similar structure or lower concentration of the same constituents; too low for detection. Isolate number 14 (R_f value; 91.46) of *G. frondosa*, *M. purpureus*, *T. versicolor* and *Pleurotus* spp. demonstrated constituents of native fluorescence; visible at both UV-λ_{254nm} and UV-λ_{365nm}; however, the same region was visible only at UV-λ_{365nm} in *L. edodes*. This fraction of *G. frondosa*, *L. edodes*, *Pleurotus* spp. and *T. versicolor* did not inhibit the growth of *E. coli* 8879 (Table 6.7, 6.8, 6.10 and 6.11, respectively), nor did this fraction inhibit the growth of *S. aureus*. This is believed to be attributed to a possible level of resistance or too low a concentration of the antibacterial agent, as these species of fungi demonstrated good activity against the other bacteria tested.

A total of eight compounds from *L. edodes* were separated using TLC (Table 6.8). Isolate number 9 (R_f value; 68.04) of this species (Table 6.2), displayed similar characteristics to the constituents of this region present in *Pleurotus* spp., reacting similarly under UV light (Table 6.4). The constituents of this region from *M. purpureus* (Table 6.9) were visible under both UV wavelengths; indicative of a different

compound, in comparison to *G. frondosa* and *Pleurotus* species. Extract constituent number 13 (R_f value; 86.36) from *L .edodes*, demonstrated similar characteristics under UV wavelength as *M. purpureus*. This R_f region demonstrated a positive reaction to a number of constituents i.e. those of lipid, terpenoid and phenolic nature (Table 6.3). This compound from *L. edodes* was active toward both *Staphylococcus aureus* 1604 and *Staphylococcus epidermis* 1798. In general, the compounds separated from *L. edodes* were less active toward *E. coli* compared to *G. frondosa*, *Pleurotus* spp., *T versicolor* and *M. purpureus* (Table 6.8). This observation highlights a major limitation of bioautography in that the number of biologically active components exhibiting activity could be underestimated if the concentration of the active component is low. Additionally, applying too high a concentration of extract can result in smearing due to overloading of components present in higher concentration.

Isolate number 3 from *M. purpureus* reacted positively toward AS, iodine and KOH detection reagents, indicative of terpenoids, conjugated double bonds and anthraquinones, respectively (Table 6.3). Bioautographic analysis revealed this region also displayed scavenging ability for $DPPH^+$ radicals, which could be associated with either lipids or phenolic constituents, as is the case here. Many of the constituents of *M. purpureus* were identified as phenolics through their reaction with FBS reagent, including; isolate number 1, 2, 5, 7, 8, 9, 12 13, 14 and 15 (Table 6.3). As shown in Table 6.9, not all of these compounds demonstrated antioxidant activity. This observation may be associated with the presence of phenolic constituents with poor or low scavenging activity toward $DPPH^+$ radicals. As described in Chapter 5, antioxidant assessment is usually based on a number of methods depending on the expected mechanisms and dynamics of the antioxidant action. In addition, the DPPH scavenging method for detection of scavenging ability does not allow for the determination of both hydrophilic and lipophilic antioxidant capacities (Apak *et al.*, 2007b). Nonetheless, as a primary indication of antioxidant association with isolates of antimicrobial capacity, this reagent in conjunction with TLC was successful. The results obtained in Section 5.3.2 demonstrated a significant correlation ($p \leq 0.01$) between total phenolic content and $ABTS^+$ radical scavenging capacity for the majority of fungal extracts (Table 5.10). As this correlation is attributed to the reductive potential of the fungal extracts, it may be proposed that free radical scavenging activity, reducing power ability and CUPRAC may also be associated with high phenolic content in accordance with the antioxidant capacity (Table 5.16). Isolates numbered 2, 7 and 12 were unique to *M. purpureus*,

Pleurotus spp. or *T. versicolor*, these compounds were previously identified as having phenolic, anthraquinone, flavonoid or lipid structure; with 7 and 12 from *M. purpureus* active against *E. coli* 10778 (Table 6.9). The biotechnological potential of *M. purpureus* in terms of production of phenolic compounds capable of inhibiting both pathogenic bacteria is noteworthy. Pigments from *M. purpureus* have a history of biological activity (as described in Section 1.2.6.1), which could explain why some compounds were not detected in the other species of fungi. In general, a broad spectrum of antibacterial activity from a variety of different separated components was observed. A definite phenolic constituent (number 7) with antimicrobial activity was produced by *M. purpureus* (Table 6.3 and 6.9). A similar constituent was also detected in *P. eryngii* (Table 6.4 & 6.10); however this constituent (number 7) did not exert any antibacterial activity, suggesting a low concentration of a similar component or a component unique to *M. purpureus* active in this region.

Altogether a total of fifteen compounds from *Pleurotus* species were separated using TLC. This included *OYRM*1, *P. 1833*, *P. 32783*, *P. citrinopileatus*, *P. salmoneo-stramineus*. As can be appreciated from Table 6.10, each compound demonstrated differential activity. Phenolic compounds detected in *Pleurotus* spp. included 1, 2, 4, 5, 7, 8, 12, 14 and 15 (Table 6.4). Isolate number 12 was common to each *Pleurotus* species and tested positive for phenolic components using the detection reagent FBS (Table 6.4). This fraction demonstrated activity toward *E. coli* 10778 and *S. epidermidis*. Isolate number 5 from *Pleurotus* spp. (also present in *M. purpureus*), demonstrated appreciable biological activity against *E. coli* 10778, *S. epidermidis* and *Salmonella*, this region reacted positively for lipid or phenolic type structures (Table 6.3 & 6.4).

A total of eight compounds from *T. versicolor* were separated using TLC (Table 6.11). Isolates number 3 and 10 demonstrated a broad range of activity, with number 10 also displaying the ability to scavenge free radicals (Table 6.11). Association of a particular compound class of isolate number 3 (R_f value; 13.76) from *G. frondosa*, *L. edodes* and *T. versicolor* was too low for detection. Although a compound of similar polarity was identified in *Pleurotus* spp. and *M. purpureus* extracts, due to different characteristics when visualised under UV light, the constituents were assumed to be of a different class. Isolate number 10, displayed similar characteristics under UV wavelength to the other Basidiomycetes species tested, revealing evidence for the presence of terpenoid or lipid structure with conjugated double bonds attached. These

observations demonstrate the possibility of different concentrations or structure of an active agent within different species.

The least polar compound in each fungus (R_f value; 97.50) was illustrative of different constituents between species. This active region reacted positively with most detection reagents suggesting non-polar lipid, terpenoid or phenolic constituents, or a combination thereof. Altogether, bioautographic analysis of the components determined that antibacterial activity was due to the presence of both polar and non-polar compounds within each extract. Different observations using UV light of the same separated region (R_f value) demonstrated that different compounds between species may elute at the same time. In addition, different concentrations of the bioactive molecule were observed between the various species of fungi. Relevant research has shown that different solvents influence the ability of the bioactive molecule to different degrees of antimicrobial effect (Sukanya *et al.*, 2011). Therefore, differences in antimicrobial activity between spots/regions of the same R_f value is potentially due to differences in constituents, the solubility of the active compounds particular to specific solvents and the concentration of the active component particular to the species of fungi. Nonetheless, bioautography was successfully used as an indicator of biological activity. The presence of antioxidant components were indicated by a positive reaction to DPPH reagent. This is an important result as it demonstrated that biological activity (antibacterial and antioxidant) may be due, to a primary constituent or synergistic effect of a combination thereof.

6.1.5   General discussion

The components of the methanol extract from fungi were separated by TLC and assessed based on their visibility under natural, UV-λ_{254nm} and UV-λ_{365nm} light. TLC provided a good basis for preliminary identification of the compound class, present in the fungal methanol extracts and as a result, a better understanding of the type of compounds present within each species of fungi was obtained.

Separated compounds demonstrating antibacterial activity against a range of bacteria were investigated using the bioautography agar over-lay method. The class of components in the fungal mycelial extract was based on fluorescence at UV- λ_{254nm} and UV-λ_{365nm}, in conjunction with universal or specific detection spray reagents. A comparison of R_f values with reference chromatograms and bioautographic results provided information about the nature of the antibacterial constituents. This

information allowed association of active components in the methanol extracts to a particular compound structure(s). Universal and specific detection reagents, combined with the localisation of the active components using bioautographic analysis, revealed that the active constituents were that of terpenoid, lipid and/or phenolic acid structure. Further, it was observed that the components which were terpenoid type structures contained conjugated double bond systems. The active components of *M. purpureus* which were possibly terpenoid compounds, showed evidence of attachment of phenolic groups, particularly for compounds number 14 and 15 (Table 6.6). On this basis, bioautography successfully provided information about both the bioactivity and structure of the analytes.

Preliminary separation of components using TLC has some advantages over other chromatographic techniques, in that, complex mixtures can be analysed whilst allowing analysis of individual components. However, compounds with similar structures can be difficult to separate using TLC, regardless of the optimised developing system, as they migrate together and separate similarly. This observation was made for many of the separated regions in the present investigation. Nonetheless, a growing interest in an effect-directed analysis has lead to the success of TLC with microbial detection (Choma *et al.*, 2011).

In general, a preliminary identification of some of the major active compounds found in methanol extracts from *M. purpureus*, *G. frondosa*, *L. edodes*, *Pleurotus* spp. and *T. versicolor* was performed. Information provided was used to contribute to the quantitative identification of specific active components. In order to further determine the structures of the active constituents, whole extracts were subject to UPLC and GC/MS analysis for quantitative identification of phenolic and lipid compounds. The active separated isolates (R_f regions) were individually extracted and analysed by LC/MS for lipid type derivatives. By selective ion monitoring in LC/MS, specific target molecules which were found to exhibit biological and antioxidant activity were determined.

6.2   Bioactive compound analysis

6.2.1   UPLC-DAD analysis of phenolic compounds

For effective utilisation of medicinal natural products or functional food components, qualitative and quantitative information on individual phenolic acids that were present

in each strain was obtained. In Section 6.1.3, a number of separated components from the methanol extract of each species were established as phenolic constituents, with potential antibacterial activity toward a range of bacteria. The aim of this aspect of research was to investigate phenolic metabolite production, as well as to quantitatively identify natural phenolic compounds by ultra high performance liquid chromatography coupled to photodiode array detector (UPLC-DAD).

Among the biologically active components of fungi, phenols have attracted much attention due to their antioxidant, anti-inflammation or antitumor properties (Palacios *et al.*, 2011). Phenolic compounds can be classified as simple phenols or phenolic acids, such as gallic, benzoic, syringic and chlorogenic acids. Polyphenols can be classified into many subgroups, including flavonoids and tannins (Kim *et al.*, 2008). Natural phenolic compounds accumulate as end-products from the shikimate and acetate pathways and can present as relatively simple molecules to highly polymerised compounds (Ferreira *et al.*, 2009). Previously, polyphenolic content was evaluated by means of Folin-Ciocalteu assay and was demonstrated to have a strong correlation with antioxidant capacity (Section 5.3). However, it is well known that this approach can suffer from interference since the reagent (mixture of phosphotungstic acid and phosphomolybdic acid) also reacts with other non-phenolic reducing compounds e.g. ascorbic acid, some sugars and amino acids, which can lead to overestimation of content (Ferreira *et al.*, 2009). In addition, individual phenolic compounds can show markedly different antioxidant effects as a result of synergism, antagonism, co-antioxidation and the presence of oxidation retarders (Becker *et al.*, 2004).

HPLC is an analytical tool for the separation of complex mixtures containing metabolic intermediates. Mixtures of phenolic acids may contain considerable flavonoid components as the two are often found in a common matrix (Banwart *et al.*, 1985). Hence, for effective simultaneous separation of phenolic acids and flavonoids, a solvent gradient was employed, to ensure each compound was properly identified. In addition, for effective separation of compounds that may have similar retention times (coelution) under specific gradient analysis, measurement of the absorbance simultaneously at different wavelengths was applied (Banwart *et al.*, 1985). Prior to analysis by UPLC, solid phase extraction (SPE) was used to selectively remove interfering compounds and concentrate of the compounds of interest. Thereby, SPE allowed for the efficient extraction and purification of phenolic compounds (Table 6.12). Experimentally, identification of compounds was carried out by comparing

retention times with those of eighteen authentic standards, as described in Section

2.2.9.2. The total phenol profile of each fungal species is detailed in Table 6.12.

Table 6.12   Identification and quantification of phenolic compounds

Common Name	Chemical Name	*Grifola frondosa*	*Lentinula edodes*	*OYRM1*	*Monascus purpureus*	*Pleurotus 1833*	*Pleurotus 32783*	*Pleurotus citrino.*	*Pleurotus eryngii*	*Pleurotus salmoneo stramineus*	*Trametes versicolor*
						Concentration (mg g^{-1})					
Benzoic acids											
4-hydroxybenzoic	4-hydroxybenzoic acid	0.05[c]	0.01[c]	0.05[c]	*nd*	0.02[c]	0.02[c]	0.08[c]	0.01[c]	0.02[c]	≤ 0.01[c]
Protocatechuic acid	3,4-dihydroxybenzoic acid	0.41[d]	0.91[a]	4.87[a]	0.02[b]	2.74[a]	3.37[d]	12.03[a]	18.70[a]	6.90[a]	1.44[a]
Salicylic acid	2-hydroxybenzoic acid	1.68[c]	1.20[c]	2.33[c]	*nd*	1.99[c]	1.93[c]	1.80[c]	5.34[c]	0.53[c]	0.49[c]
Vanillic acid	4-hydroxy-3-methoxybenzoic acid	0.86[b]	1.98[b]	17.30[b]	**10.87[b]**	5.58[b]	**19.92[b]**	1.16[a]	4.61[b]	**15.19[b]**	8.39[b]
Benzaldehydes											
Vanillin	4-hydroxy-3-methoxybenzaldehyde	0.02[d]	*nd*	0.02[a]	0.06[a]	0.02[b]	0.03[a]	0.03[c]	0.02[a]	0.03[d]	0.01[a]
Phenols											
Pyrogallol	1,2,3-benzenetriol	**17.16[c]**	**17.06[b]**	11.02[a]	2.03[a]	5.63[c]	3.25[a]	**20.61[a]**	4.95[c]	14.78[a]	8.11[c]
ρ-hydroxybiphenyl	4-phenylphenol	*nd*	*nd*	0.30[c]	0.05[a]	0.81[c]	0.31[c]	1.48[c]	0.21[c]	0.37[c]	**19.98[c]**
Cinnamic acids											
Caffeic acid	3,4-dihydroxycinnamic acid	0.16[c]	0.13[a]	0.25[a]	0.06[a]	0.23[a]	0.08[b]	0.90[b]	0.29[c]	0.28[a]	0.19[a]
Ferulic acid	4-hydroxy-3-methoxycinnamic acid	2.09[b]	1.89[b]	12.16[b]	2.52[b]	4.87[b]	6.14[b]	14.26[b]	0.99[b]	13.00[b]	3.69[b]
ρ-coumaric	4-hydroxycinnamic acid	2.21[a]	2.75[a]	3.35[a]	1.60[a]	3.87[a]	3.39[a]	14.17[a]	4.42[a]	4.13[a]	1.70[a]
α-cyano-4 -hydroxycinnamic acid	3-phenyl-2-propenoic acid	10.16[c]	3.06[a]	**27.83[a]**	2.62[a]	**23.42[c]**	5.99[c]	12.22[a]	5.49[a]	9.77[a]	1.53[a]
Flavanones											
Naringenin	4',5,7-trihydroxyflavanone	0.19[d]	0.97[a]	3.45[a]	*nd*	2.11[a]	7.38[a]	12.55[a]	6.88[a]	2.75[b]	3.85[a]
Catechin	3,3',4',5,7-pentahydroxyflavanone	3.53[a]	5.70[a]	0.09[a]	4.17[a]	0.02[c]	*nd*	1.05[a]	*nd*	0.30[a]	0.04[a]
Flavones											
Flavone	2-phenyl-4*H*-1-benzopyran-4-one	*nd*	0.44[b]	0.84[b]	0.79[b]	0.63[b]	0.49[a]	4.80[b]	0.85[c]	0.67[b]	0.59[b]
Kaempferol	3,4',5,7-tetrahydroxyflavone	*nd*	0.12[c]	0.14[c]	0.80[c]	0.13[a]	0.13[c]	0.28[c]	0.16[a]	0.45[a]	0.31[c]
Myricetin	3,3',4',5,5',7-hexahydroxyflavone	0.19[c]	0.24[c]	0.15[c]	0.06[c]	0.17[c]	0.09[c]	0.32[c]	0.28[a]	0.12[c]	0.17[a]
Flavone glycosides											
Rutin	Quercetin-3-rutinoside	4.21[c]	10.34[c]	0.40[a]	5.97[c]	0.15[a]	2.71[c]	3.54[c]	**35.73[c]**	0.14[a]	1.51[c]
Other standards:											
Homogentisic acid	2,5-dihydroxyphenylacetic acid	1.76[a]	0.15[a]	3.05[a]	0.23[a]	3.15[a]	2.27[b]	0.49[a]	0.88[a]	1.63[b]	2.04[a]
Total phenolic content (mg g^{-1})*		**44.68**	**46.95**	**87.60**	**31.85**	**55.54**	**57.50**	**101.77**	**89.81**	**71.06**	**54.04**

Data is representative of the phenolic compounds present in ten selected fungal species using UPLC-DAD analysis. Concentration (mg g^{-1} dry extract)* is based on maximum UV detection at wavelengths of λ_{254nm}[a], λ_{265nm}[b], λ_{280nm}[c], and λ_{320nm}[d]. *Data is representative of the total concentration of phenols as detected using authentic standards. The main phenolic acid of each species is indicated in bold.

- Abbreviations: *OYRM*1, *Pleurotus ostreatus*; *Pleurotus citrino.*, *Pleurotus citrinopileatus*; *nd*, not detected.

Comparison of relative retention times is the most reliable index for compound identification (Banwart *et al.*, 1985). Various phenolic acids were identified by comparing the retention times and absorption spectra of peaks with reference standards; selected on the basis of their probability of being present in the selected filamentous species. As aforementioned, due to the suspected large number of compounds of interest and in an effort to improve the probability of proper compound identification, a selection of wavelengths were reported for detection, including; λ_{254nm}, λ_{265nm}, λ_{280nm} and λ_{320nm}. For example, many phenolics absorb between λ_{270nm} and λ_{290nm} (Tsimogiannis *et al.*, 2007). 4-hydroxybenzoic and salicylic acid were detected at λ_{254nm} only; other compounds demonstrated differences in maximum absorbance and thus detection, depending on compound and the particular species of fungi. It is evident from the data obtained that some compounds absorb differently at one wavelength than they do at another, signifying that measuring the absorbance of the sample simultaneously at different wavelengths increases the accuracy of quantification.

It is appreciable from the data presented in Table 6.12 that a number of different constituents made up the total concentration of phenolics in each fungus. Different species contained different phenolic compounds in varying numbers, ranging from fifteen to eighteen, from a total of eighteen different compounds analysed. This observation indicates that antioxidant activity may be correlated with synergism among the different polyphenols, flavonoids and phenolic acids occurring in the various species of fungi (Palacios *et al.*, 2011). Previously, *G. frondosa* and *L. edodes* demonstrated lower antioxidant effect in comparison to the other species, as was illustrated by their EC_{50} values noted in Table 5.8. Apart from *M. purpureus*, the total content of phenolics for these species was lower than the others tested; *G. frondosa* contained 44.68 mg g^{-1}, while the total phenolic concentration of *L. edodes* was 46.95 mg g^{-1} (Table 6.12). This observation was also true for the phenolic content of the crude extracts when tested by Folin-Ciocalteu; *M. purpureus*, *T. versicolor*, *G. frondosa* and *L. edodes* contained the least phenol content (mgGAE g^{-1}) (Table 5.15). Conversely, these species demonstrated relatively high phenolic content following hot water and methanol extraction (Table 5.15), which illustrates the significance of extraction conditions for enhancement of efficiency for high yields of antioxidative compounds from natural sources (Yim *et al.*, 2009). The main phenolic compound found in *G. frondosa* was pyrogallol and α-cyano-4-hydroxycinnamic acid, while the

main phenolic constituents of *L. edodes* were pyrogallol and rutin (Table 6.12). The crude, hot water and methanol extracts from both of these species demonstrated low antioxidant efficiencies in comparison to the other species analysed. The total phenolic content of *G. frondosa* and *L. edodes* had strong correlation coefficients, in relation to antioxidant activity (ABTS$^+$ radical scavenging activity) (Table 5.10). This observation suggested the phenolic content was responsible for the antioxidant capacity of these species. The data from Table 6.12 suggests that the phenolic composition of either species was quite similar; thereby, the antioxidant action of these species is likely to be contributed to a similar mechanism of antioxidant action. The phenolic composition of *Pleurotus* spp. is comparable to those in the literature (Kim *et al.*, 2008; Palacios *et al.*, 2011). This is the first report of phenolic compositional data from *M. purpureus*, *L. edodes*, *G. frondosa* and *T. versicolor* methanol extracts from biomass cultivated in submerged culture. It is appreciated that specific and characteristic composition of each species is likely to be associated with the environmental factors of their growth conditions.

The total phenolic content in each of the filamentous fungi ranged from 31.85 mg g^{-1} to 101.77 mg g^{-1}. Nine phenolic compounds, namely, myricetin, rutin, protocatechuic, vanillic, caffeic, ferulic, ρ-coumaric, ɑ-cyano-4-hydroxycinnamic and homogentisic acids were dominant among all ten fungal species (Table 6.12). *P. 32783* and *P. eryngii* did not show evidence for the presence of catechin. With this exception, every phenolic compound tested was detected in each *Pleurotus* species. The benzoic acids, salicylic acid and 4-hydroxybenzoic acid, in addition to the flavonoid naringenin, were not detected in *M. purpureus*. Vanillin and ρ-hydroxybiphenyl were not detected in *L. edodes*, and *G. frondosa* did not contain flavone, kaempferol or ρ-hydroxybiphenyl (Table 6.12). Similar to Kim *et al.* (2008), homogentisic, protocatechuic, catechin, myricetin and certain cinnamic acids were detected in extracts of *Pleurotus ostreatus* (*OYRM*1) and *P. eryngii*; however, much greater concentrations were detected from the extracts in the present study (Table 6.12). This is potentially attributed to numerous factors; analysis in said study was based on the fruiting body not mycelium, as is the case here, there was no solid phase extraction prior to analysis and efficiency of detection parameters of the UPLC were different to those performed in this investigation.

G. frondosa and *M. purpureus* contained fifteen phenolic compounds, while *OYRM*1, *P. 1833*, *P. citrinopileatus*, *P. salmoneo-stramineus* and *T. versicolor*

contained all eighteen (Table 6.12). *P. citrinopileatus* had the largest concentration of phenolic compounds (101.77 mg g^{-1}) detected by UPLC, while *M. purpureus* contained the least (31.85 mg g^{-1}). Previous results using the Folin-Ciocalteu method indicated that the methanol extract of *Pl. 32783* (9.29 mg GAE g^{-1}) followed by *P. citrinopileatus* (8.54 mg GAE g^{-1}) contained the highest quantity of phenolic compounds and *M. purpureus* had lowest (3.02 mg GAE g^{-1}) compared to the other species tested (Section 5.3.2, Table 5.15). Although similarities were observed, this observation demonstrates that while the main peaks of the chromatogram from the various extracts were associated with relevant reference standards, some unidentified peaks may have attributed to the total phenolic content and consequently the antioxidant capacity of the extracts, particularly *P. 32783*.

The benzoic compound, 4-hydroxybenzoic acid, was detected at trace levels in each of the fungi tested (Table 6.12). This compound constitutes a common phenolic secondary metabolite of higher fungi; with confirmation of its presence in *P. ostreatus* reported for the first time only recently (Papaspyridi *et al.*, 2012). Hydroxybenzoic acid derivatives are typically a component of a complex structure like lignins and hydrolysable tannins, occurring in the bound form (Ferreira *et al.*, 2009). Similarly, hydroxycinnamic acid derivatives are mainly present in the bound form, but, are linked to cell wall structural components, such as cellulose, lignin, and proteins (Ferreira *et al.*, 2009). They may also be associated with organic acids, such as tartaric acid or quinic acids (i.e. chlorogenic acids), through ester bonds (Liu, 2004).

Caffeic acid has been described in diverse species such as *Cantharellus cibarius*, *Agaricus bisporus*, as well as, *Lentinus edodes* and *Pleurotus ostreatus*, as described in the present investigation (Table 6.12). The components rutin and caffeic acid have demonstrated antimicrobial and anti-inflammatory activity (Fernandez *et al.*, 1998; Oke *et al.*, 2011). Antioxidant effects of caffeic acid include antilipid peroxidation, radical scavenging and antioxidation of LDL (Oke *et al.*, 2011). This constituent was identified in small quantities in each of the fungi tested (Table 6.12). The same observation could be said for catechin; a constituent which is known to have versatile biological effects such as anticancer, anti-allergy and antioxidant activities (Kondo *et al.*, 2000; Oke *et al.*, 2011). Rutin was detected as the major phenolic component of *P. eryngii*. Conversely, in a study by Kim *et al.* (2008) rutin was not detected in extracts of Pleurotus; however, these observations were based on extracts from the mushroom fruiting bodies and different conditions of analysis were

employed, such as, no SPE procedure and different liquid chromatography parameters. This illustrates the significance of preliminary extraction and purification procedures, sensitivity of methods involved in qualitative and quantitative detection and possible influences of various growth stages on phenol production.

There are few studies concerning the individual profiles of phenolic compounds in therapeutic and edible fungi (Palacios *et al.*, 2011). Production of individual phenolics can depend on growth conditions, stage of growth and largely, the individual species of fungi, as was the case here. In addition, the efficiency of natural phenolic antioxidants is largely dependent on the involvement of the phenolic hydrogen in radical reactions and its chemical substitutions (Hall, 2001; Ferreira *et al.*, 2009).

Active extracts having demonstrated antimicrobial and antioxidant activity were partitioned using TLC and through the use of detection reagents were suggested as lipid, terpenoid or phenolic constituents or a combination thereof. In the previous chapter, phenolic compounds demonstrated a strong correlation to antioxidant efficiency. TLC demonstrated each species as having a broad spectrum of antibacterial activity, particularly *M. purpureus* which contained the most diverse range of constituents compared to the other species. Additionally, antioxidant activity of this species demonstrated a strong correlation with its phenolic content ($r = 0.955$). Analysis of the individual phenolic profiles of each fungus (Table 6.12) revealed that *M. purpureus* had the lowest total phenolic content of the species tested, as was detected using UPLC and Folin-Ciocalteu methods. TLC and bioautography (Section 6.1), indicated the main active agent to be of phenolic or lipid structure. Altogether, data from TLC, detection reagents, bioautography and UPLC analysis suggested that biological activity of fungal mycelial extracts was most likely due to a combination of constituents, with the phenolic fraction mainly associated with relevant antioxidant capacity. Thereby, a fatty acid composition profile for each species was generated using LC/MS.

6.2.2   Fatty acid analysis of active isolates separated using TLC

Liquid chromatography-tandem mass spectrophotometry (LC/MS) was used to identify the individual separated active constituents of lipid structure from ten filamentous fungi. There is now increasing interest in lipids, particularly in fatty acid composition, being applied for physiological, chemotaxonomy, and intrageneric differentiation, as

well as studies of human nutrition (Pedneault *et al.*, 2007). Much of the interest has been focused on organisms such as bacteria, algae, fungi and flowering plants (Stahl *et al.*, 1996; Dimou *et al.*, 2002; Parikh *et al.*, 2005).

The cell walls of filamentous fungi contain the structural macromolecules, chitin and cellulose together with many other polysaccharides, and specific amounts of protein and lipids. They contain an appreciable quantity of lipids, with bound lipids generally being present in two to three times greater abundance than the freely extractable lipids (Harwood *et al.*, 1984). Triacylglycerols, sterols, sterol esters and phospholipids are usually major components. Fatty acids are major constituents of both phospholipids (membrane compounds) and neutral lipids (storage compounds) (Suutari, 1995; Olsson *et al.*, 2000; Pedneault *et al.*, 2007). α-Linolenic acid is strongly related to flavour in certain mushrooms, as it is the precursor to 1-octen-3-ol, or the 'alcohol' of fungi, thus, is the principal aromatic compound in most mushrooms (Cheung, 2008).

Compounds were subjected two successive overnight elutions from the silica. Firstly, the non-polar solvent diethyl ether was used for the extraction of neutral lipids (storage lipids), followed by methanol, in order to disrupt hydrogen bonds or electrostatic forces of membrane-associated lipids (Dobush *et al.*, 1985). According to Tringali (2003), MS is one of the most sensitive methods of molecular analysis. It has the potential to provide information on the molecular weight, as well as the structure of the analytes. Natural extracts, such as those from fungi, represent complex mixtures of metabolites, having various physiochemical properties. LC/MS allows high power mass separation and as a result, allows very good selectivity for identification of various metabolites (Tringali, 2003).

LC/MS profiles of the partially purified fractions (isolates) were compared to bioactivity data (Section 6.1.4) and were used to identify potential bioactive components of each of the ten filamentous fungal species. Spots were fractionated (separated and eluted from the silica individually following TLC development, as outlined in Section 2.2.8.5) and a fatty acid profile based on 38 authentic standards was generated. This allowed association of biological activity to a particular class or constituent type based on the R_f value of the spot of interest.

LC/MS allowed for small molecule analysis (Section 2.2.9.3), which ultimately assisted the determination of constituents within the fungal extracts. Linoleic, palmitic, oleic and stearic acid were among the most abundant fatty acids established

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in the methanol extracts (Table 6.13 – 6.17). In fungi, the major fatty acids that typically occur in membrane phospholipids and storage triacylglycerols are palmitic and stearic acids and their unsaturated derivatives palmitoleic, oleic, linoleic and linolenic acids (Suutari, 1995), most of which were also established in each of the species examined (Table 6.18). Traditionally, lipids can be defined as fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds (Fuchs *et al.*, 2011). Essential fatty acids, linoleic and α-linolenic acids are of particular interest in terms of human nutrition, as they are essential for basal metabolism. In addition, long-chain polyunsaturated fatty acids have been shown to have positive implications in human health (Diplock *et al.*, 1998). As a result, the fatty acid content of edible mushrooms is of growing interest.

Tables 6.13 – 6.17 display the major fatty acid constituents per extracted isolate according to their R_f value (1 – 15) for each species. It can be appreciated from the data that a number of different constituents made up the total concentration of fatty acids in each isolated region. Altogether, a total of 19 different fatty acid constituents were detected in the methanol extract of *M. purpureus*, namely; pelargonic, lauric, myristic, pentadecylic, palmitic, palmitoleic/palmitelaidic, heptadecanoic, stearic, oleic/elaidic, linoleic, arachidic, gadoleic, arachidonic/eicosatetraenoic, nonadecylic, dihomolinoleic, EPA, DPA, DHA and tricosylic acids (Table 6.13).

Table 6.13   Constituent analysis of separated components from the *Monascus purpureus* methanol extract following TLC and spot extraction

#	R_f x 100*	Fatty acid (%)[1]								Component detection[2]					
		Myristic	Pentadecylic	Palmitic	Palmitoleic/ Palmitelaidic	Hepta-decanoic	Stearic	Oleic/ Elaidic	Linoleic	Phenol	Flavonoid	Lipid	Terpenoid	Anthra-quinone	Cdbs
1	6.31 ± 0.96[bcde]	8.51	2.56	23.18	4.71	4.71	**45.80**	7.93	1.17	+	+	-	-	+	+
2	8.98 ± 0.71[bcde]	6.41	1.93	20.80	4.12	4.12	**52.49**	6.52	2.30	+	-	-	-	+	-
3	13.76 ± 1.99[abde]	6.96	2.30	23.41	4.51	4.73	**45.46**	8.17	2.05	-	-	-	+	+	+
4	22.03 ± 2.16[bcd]	6.36	2.11	22.69	3.72	4.41	**50.74**	6.43	1.58	-	-	-	-	-	-
5	29.80 ± 1.67[bde]	8.39	2.44	24.99	4.14	5.60	**42.70**	7.41	1.29	+	-	-	+	+	+
6	40.07 ± 6.89[de]	8.39	2.44	24.99	4.14	5.60	**42.70**	7.41	1.29	-	+	+	+	-	-
7	48.12 ± 0.75[acde]	8.22	2.91	24.10	4.17	6.03	**41.41**	8.89	1.22	+	+	-	-	+	-
8	56.47 ± 2.89[bcde]	6.78	2.22	24.07	2.90	4.51	**51.48**	7.20	0.84	+	+	-	-	+	-
9	68.04 ± 2.12	*na*	*na*	*na*	*na*	*na*	*na*	*na*	*na*	+	-	-	+	+	+
10	75.58 ± 1.84[abce]	16.30	3.73	**37.70**	6.33	4.71	22.90	7.25	1.09	-	-	-	+	-	+
11	79.78 ± 1.17[e]	4.19	1.38	18.59	3.08	1.55	5.91	4.51	**59.89**	-	-	+	-	+	+
12	82.32 ± 0.83[abc]	1.81	0.65	15.08	1.36	0.66	3.70	6.50	**69.56**	+	+	+	-	+	-
13	86.74 ± 0.68[bcd]	1.30	0.50	20.80	0.32	1.62	11.19	24.22	**39.76**	+	+	+	+	-	+
14	91.46 ± 2.93[abe]	0.76	0.38	19.83	0.71	1.26	6.30	19.03	**51.09**	+	+	+	+	+	+
15	97.50 ± 0.74[abcde]	11.87	3.15	**29.47**	4.30	2.41	12.24	11.46	25.11	+	-	+	+	-	+

Separation of methanol extract (100 mg mL^{-1}) was performed using mobile phase hexane:ethyl acetate (1:1, v/v). *Antibacterial activity of separated isolates established by agar over-lay bioautography; [a], *E. coli* 8879; [b], *E. coli* 10778; [c], *Staphylococcus aureus* 1604; [d], *Staphylococcus epidermis* 1798; [e], *Salmonella enterica* 15480. [1]Data is representative of the relative % area of total lipids detected, identified by comparing their retention times and characteristic MS spectral data with those of authentic standards using LC/MS. The main fatty acid constituent of each isolate is indicated in bold. Pelargonic, lauric, arachidic, gadoleic, nonadecylic, dihomolinoleic, arachidonic, EPA, DPA, DHA and tricosylic (≤ 1 %.) were also detected. [2]Component detection using TLC was determined using various detection reagents. Data indicates regions of positive and negative reaction to spray reagent are denoted by (+) and (-), respectively. Fast blue salt (FBS) was used for the detection of flavonoids and phenolic compounds; Lipids were detected with 10 % (w/v) sulphuric acid; Terpenoid structures were detected with anisaldehyde-sulphuric acid (AS) reagent; potassium hydroxide (KOH) reagent was used for detection of anthraquinones (red), anthrones (yellow) and coumarins (blue) and iodine vapour was used for the detection of compounds with conjugated double bonds. The major fatty acid of each isolate is indicated in bold.
- Abbreviations: *na*, not active.

Of the fifteen isolates which were separated and isolated using TLC, fourteen were determined as active using bioautography (Table 6.9). Isolates 1 – 8 predominantly contained stearic acid; followed by palmitic acid and myristic or oleic acids (Table 6.13). Conversely, palmitic, stearic and myristic were the predominant fatty acids detected in isolate number 10. Isolates 11, 12 and 15 were primarily made up of linoleic and palmitic acid; while, oleic and linoleic acid were the main constituents detected in isolate number 13 (Table 6.13). Finally, isolate number 14 was shown to mostly contain linoleic, palmitic and oleic acid. Notably, the data reveals that a number of different constituents made up the total concentration of active components in each isolate (1 – 15). LC/MS analysis revealed fatty acid constituents did not elute at a specific retention time, but instead migrated through the development system (hexane:ethyl acetate, 1:1, v:v) according to polarity. Therefore, it is likely that a number of constituents were responsible for bacterial inhibition. Additionally, bioactivity may depend on the concentration of the active component as well as resistance of the bacterial species itself. Isolate number 4 did not react with any of the detection reagents, yet maintained antibacterial action and a diverse fatty acid profile. This suggests either a degree of non-specific determination of phenolic and lipid components using detection reagents (Section 6.1.2), the concentration of the compound was too low for detection and/or the components tested were absent from this region. It may also be an indication that antibacterial action was a consequence of a synergistic relationship with other components such as polyphenols, flavonoids and phenolic acids in the various species of fungi. Altogether, a broad range of activity was determined from the majority of separated isolates, with isolate number 15 yielding the most diverse antimicrobial action, *via* inhibition of *E. coli* 8879, *E. coli* 10778, *Staphylococcus aureus* 1604, *Staphylococcus epidermis* 1798 and *Salmonella enterica* 15480 (Table 6.13). Similar to the results of *M. purpureus*, the methanol extracts of *G. frondosa* contained a number of fatty acid constituents (Table 6.14).

Table 6.14 Constituent analysis of separated components from the *Grifola frondosa* methanol extract following TLC and spot extraction

#	R_f x 100*	Myristic	Pentadecylic	Palmitic	Palmitoleic/ Palmitelaidic	Hepta-decanoic	Stearic	Oleic/ Elaidic	Linoleic	Phenol	Flavonoid	Lipid	Terpenoid	Anthra-quinone	Cdbs
1	6.31 ± 0.96[cd]	16.56	0.21	**41.12**	1.03	0.25	38.41	1.70	0.59	-	-	-	-	-	-
2	8.98 ± 0.71	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
3	13.76 ± 1.99[bde]	14.79	0.78	**39.68**	1.03	0.31	34.35	1.97	6.00	-	-	-	-	-	-
4	22.03 ± 2.16	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
5	29.80 ± 1.67[de]	15.93	0.57	**40.75**	0.73	0.24	36.78	1.81	0.24	-	-	+	-	-	*nd*
6	40.07 ± 6.89	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
7	48.12 ± 0.75	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
8	56.47 ± 2.89[c]	4.68	0.83	36.17	0.50	0.69	15.53	2.30	**39.11**	+	-	-	-	-	-
9	68.04 ± 2.12	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
10	75.58 ± 1.84[cde]	16.48	0.76	**41.61**	0.49	0.31	36.66	1.66	1.09	-	-	-	+	-	+
11	79.78 ± 1.17	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
12	82.32 ± 0.83	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
13	86.74 ± 0.68	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
14	91.46 ± 2.93 [be]	0.18	0.55	**40.92**	0.49	0.18	37.80	1.84	0.87	+	+	+	-	-	-
15	97.50 ± 0.74[bce]	15.27	0.53	**41.34**	0.55	0.16	37.74	1.87	1.96	+	+	+	+	-	+

Separation of methanol extract (100 mg mL^{-1}) was performed using mobile phase hexane:ethyl acetate (1:1, v/v). *Antibacterial activity of separated isolates established by agar over-lay bioautography; [a], *E. coli* 8879; [b], *E. coli* 10778; [c], *Staphylococcus aureus* 1604; [d], *Staphylococcus epidermis* 1798; [e], *Salmonella enterica* 15480. [1]Data is representative of the relative % area of total lipids detected, identified by comparing their retention times and characteristic MS spectral data with those of authentic standards using LC/MS. The main fatty acid constituent of each isolate is indicated in bold. Caprylic, pelargonic and lauric (≤ 1 %.) were also detected. [2]Component detection using TLC was determined using various detection reagents. Data indicates regions of positive and negative reaction to spray reagent are denoted by (+) and (-), respectively. Fast blue salt (FBS) was used for the detection of flavonoids and phenolic compounds; Lipids were detected with 10 % (w/v) sulphuric acid; Terpenoid structures were detected with anisaldehyde-sulphuric acid (AS) reagent; potassium hydroxide (KOH) reagent was used for detection of anthraquinones (red), anthrones (yellow) and coumarins (blue) and iodine vapour was used for the detection of compounds with conjugated double bonds. The major fatty acid of each isolate is indicated in bold.
- Abbreviations: *nd*, not detected.

Table 6.14 displays the main fatty acid constituents of *G. frondosa*. A total of
11 different fatty acids were detected in the separated active regions from the methanol
extract (Table 6.14). They included caprylic, myristic, pentadecyclic, palmitic,
palmitoleic/palmitelaidic, heptadecanoic, stearic, oleic/elaidic and linoleic acids (Table
6.14). Pelargonic and lauric were also detected but were only present ($\leq$ 1 %) in
extracted regions numbered 3, 5, 8, 10 or 15. All 7 isolates which were separated and
isolated using TLC were determined as active using bioautography (Table 6.7). The
predominant fatty acid of each isolate from this species of fungi was palmitic acid or
linoleic acid. A strong presence of palmitic acid was detected in each of the extracted
regions. Altogether antibacterial activity of the extracted regions was demonstrated
against *E. coli* 10778, *S. epidermidis*, *S. aureus* and *Salmonella enterica* (Table 6.14).
Table 6.15 displays the main fatty acid constituents of *L. edodes* according to polarity.

Table 6.15   Constituent analysis of separated components from the *Lentinula edodes* methanol extract following TLC and spot extraction

#	R_f x 100*	Myristic	Pentadecylic	Palmitic	Palmitoleic/ Palmitelaidic	Hepta-decanoic	Stearic	Oleic/ Elaidic	Linoleic	Phenol	Flavonoid	Lipid	Terpenoid	Anthra-quinone	Cdbs
				Fatty acid (%)[1]						Component detection[2]					
1	6.31 ± 0.96^{c}	16.00	0.56	**42.39**	0.50	0.00	38.00	1.83	0.46	-	-	-	-	-	-
2	8.98 ± 0.71	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
3	13.76 ± 1.99^{d}	19.32	0.51	**40.26**	1.26	0.27	35.73	1.91	0.46	-	-	-	-	-	-
4	22.03 ± 2.16	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
5	29.80 ± 1.67^{e}	17.90	0.56	**41.89**	0.99	0.00	36.64	1.69	0.34	-	-	-	-	-	-
6	40.07 ± 6.89	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
7	48.12 ± 0.75	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
8	56.47 ± 2.89	*na*	*na*	*na*	*na*	*na*	*na*	*na*	*na*	+	-	-	-	-	-
9	68.04 ± 2.12	*na*	*na*	*na*	*na*	*na*	*na*	*na*	*na*	-	-	-	-	-	-
10	75.58 ± 1.84^{cde}	18.13	0.65	28.12	1.17	0.65	12.94	3.11	**50.01**	-	-	+	+	-	+
11	79.78 ± 1.17	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
12	82.32 ± 0.83	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
13	86.74 ± 0.68^{cd}	14.97	0.41	**41.70**	1.14	0.26	36.62	2.37	1.33	-	-	+	-	-	-
14	91.46 ± 2.93^{bde}	14.59	0.42	**41.63**	1.43	0.28	35.93	2.47	2.77	+	+	+	-	-	-
15	97.50 ± 0.74	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*

Separation of methanol extract (100 mg mL^{-1}) was performed using mobile phase hexane:ethyl acetate (1:1, v/v). *Antibacterial activity of separated isolates established by agar over-lay bioautography; [a], *E. coli* 8879; [b], *E. coli* 10778; [c], *Staphylococcus aureus* 1604; [d], *Staphylococcus epidermis* 1798; [e], *Salmonella enterica* 15480. [1]Data is representative of the relative % area of total lipids detected, identified by comparing their retention times and characteristic MS spectral data with those of authentic standards using LC/MS. The main fatty acid constituent of each isolate is indicated in bold. Caprylic, pelargonic, undecylic, lauric and linolenic (α + γ) (≤ 1 %.) were also detected. [2]Component detection using TLC was determined using various detection reagents. Data indicates regions of positive and negative reaction to spray reagent are denoted by (+) and (-), respectively. Fast blue salt (FBS) was used for the detection of flavonoids and phenolic compounds; Lipids were detected with 10 % (w/v) sulphuric acid; Terpenoid structures were detected with anisaldehyde-sulphuric acid (AS) reagent; potassium hydroxide (KOH) reagent was used for detection of anthraquinones (red), anthrones (yellow) and coumarins (blue) and iodine vapour was used for the detection of compounds with conjugated double bonds. The major fatty acid of each isolate is indicated in bold.
- Abbreviations: *nd*, not detected.

A total of 13 different fatty acids were detected from the separated regions of the methanol extract of *L. edodes*. They included caprylic, pelargonic, undecyclic, lauric, myristic, pentadecyclic, palmitic, palmitoleic/palmitelaidic, heptadecanoic, stearic, oleic/elaidic, linoleic and linolenic (α + γ) acids (Table 6.15). Of the eight isolates separated using TLC demonstrated, six demonstrated antibacterial activity against bacteria using bioautography (Table 6.8). With the exception of isolate number 10, the main fatty acid constituent of each isolate from this species of fungi was palmitic acid. Similar to *G. frondosa*, this extracted region demonstrated antibacterial activity against *E. coli* 10778, *S. aureus*, *S. epidermidis* and *Salmonella enterica* (Table 6.8 & 6.9). In addition, a high percentage of myristic, palmitic and stearic was detected and this region also tested positive for the presence of terpenoids in both species (Table 6.1 & 6.2). Table 6.16 displays the main fatty acid constituents of Pleurotus.

Table 6.16 Constituent analysis of separated components from the *Pleurotus*[†] methanol extract following TLC and spot extraction

#	R_f x 100*	Myristic	Pentadecylic	Palmitic	Palmitoleic/ Palmitelaidic	Hepta- decanoic	Stearic	Oleic/ Elaidic	Linoleic	Phenol	Flavonoid	Lipid	Terpenoid	Anthra- quinone	Cdbs
				Fatty acid (%)[(i)]						Component detection[(ii)]					
1	6.31 ± 0.96[abde]	14.46	0.88	**40.79**	1.94	0.00	36.69	3.92	1.31	+[3,4]	-	-	-	-	-
2	8.98 ± 0.71	*na*	*na*	*na*	*na*	*na*	*na*	*na*	*na*	+[3,4]	-	-	-	-	-
3	13.76 ± 1.99[abcde]	17.18	0.67	**41.32**	1.29	0.00	36.43	2.49	0.62	-	-	-	-	+[2]	+[1,2]
4	22.03 ± 2.16[bd]	17.33	0.79	**42.38**	1.97	0.00	33.59	3.04	0.91	+[4,6]	-	-	-	-	-
5	29.80 ± 1.67[b]	18.25	0.97	**39.81**	2.39	0.00	34.81	2.74	0.80	+[2,4,6]	-	+	-	-	-
6	40.07 ± 6.89	*na*	*na*	*na*	*na*	*na*	*na*	*na*	*na*	-	-	+	+[46]	-	-
7	48.12 ± 0.75	*na*	*na*	*na*	*na*	*na*	*na*	*na*	*na*	+[4]	-	-	-	-	-
8	56.47 ± 2.89[abcde]	4.45	1.57	21.41	1.89	0.59	9.80	5.06	**55.00**	+[4,6]	-	-	-	-	-
9	68.04 ± 2.12[ac]	14.14	1.62	**33.73**	2.81	1.21	26.47	6.77	12.76	-	-	-	+	-	+
10	75.58 ± 1.84[ce]	18.03	0.84	**41.02**	1.52	0.00	34.58	2.59	0.73	-	-	-	+[6]	-	+
11	79.78 ± 1.17[ae]	16.54	1.71	**40.21**	3.13	1.06	31.96	3.27	1.57	-	-	+	-	+[2]	+[1,2]
12	82.32 ± 0.83	*na*	*na*	*na*	*na*	*na*	*na*	*na*	*na*	+	-	+	-	-	-
13	86.74 ± 0.68	*na*	*na*	*na*	*na*	*na*	*na*	*na*	*na*	-	-	-	-	-	-
14	91.46 ± 2.93[be]	18.06	1.23	**39.90**	2.84	0.00	32.87	3.33	1.13	+[1,2,3,4]	+	+	-	+[2]	+[1,2]
15	97.50 ± 0.74[abcde]	16.68	1.22	**39.59**	3.07	1.16	31.28	3.54	2.74	+[6]	+	+	+	-	+

Separation of methanol extract (100 mg mL^{-1}) was performed using mobile phase hexane:ethyl acetate (1:1, v/v). *Antibacterial activity of separated isolates established by agar over-lay bioautography; [a], *E. coli* 8879; [b], *E. coli* 10778; [c], *Staphylococcus aureus* 1604; [d], *Staphylococcus epidermis* 1798; [e], *Salmonella enterica* 15480. [(i)]Data is representative of the relative % area of total lipids detected, identified by comparing their retention times and characteristic MS spectral data with those of authentic standards using LC/MS. The main fatty acid constituent of each isolate is indicated in bold. Caprylic, pelargonic and nonadecylic (≤ 1 %.) were also detected. [(ii)]Component detection using TLC was determined using various detection reagents. Relative to each *Pleurotus* species used throughout the investigation, unless otherwise stated; *OYRM*1[1], *P. 1833*[2], *P. 32783*[3], *P. eryngii*[4], *P. citrinopileatus*[5] and *P. salmoneo-stramineus*[6]. Data indicates regions of positive and negative reaction to spray reagent denoted by (+) and (-), respectively. Fast blue salt (FBS) was used for the detection of flavonoids and phenolic compounds; Lipids were detected with 10 % (w/v) sulphuric acid; Terpenoid structures were detected with anisaldehyde-sulphuric acid (AS) reagent; potassium hydroxide (KOH) reagent was used for detection of anthraquinones (red), anthrones (yellow) and coumarins (blue) and iodine vapour was used for the detection of compounds with conjugated double bonds. The major fatty acid of each isolate is indicated in bold.
- Abbreviations: *na*, not active. [†]*Pleurotus 1833*, *Pleurotus 32783*, *OYRM*1, *Pleurotus citrinopileatus*, *P. eryngii* and *P. salmoneo-stramineus*.

A total of 11 different fatty acids were detected from the separated active regions of the methanol extract of Pleurotus. They included caprylic, pelargonic, myristic, pentadecyclic, palmitic, palmitoleic/palmitelaidic, heptadecanoic, stearic, oleic/elaidic, linoleic and nonadecylic acids (Table 6.16). Of the fifteen isolates which were separated and isolated using TLC, ten were determined as active using bioautography (Table 6.10). Isolates 1 – 5 and 9 – 15 primarily contained palmitic acid; followed by stearic acid and myristic acid (Table 6.16). Linoleic, palmitic and stearic acid were the predominant fatty acids detected in isolate number 8, respectively. The main constituents of this fungus were in agreement with Papaspyridi *et al.* (2012). Similar to the other species of fungi, the data reveals that a number of different constituents made up the total concentration of active components in each isolate. Isolates 3, 8 and 15 were demonstrative of diverse antimicrobial action (Table 6.16). Table 6.17 displays the main fatty acid constituents of *T. versicolor*.

Table 6.17 Constituent analysis of separated components from the *Trametes versicolor* methanol extract following TLC and spot extraction.

#	R_f x 100*	Myristic	Pentadecylic	Palmitic	Palmitoleic/ Palmitelaidic	Hepta-decanoic	Stearic	Oleic/ Elaidic	Linoleic	Phenol	Flavonoid	Lipid	Terpenoid	Anthra-quinone	Cdbs
1	6.31 ± 0.96^d	15.35	0.31	**43.87**	1.33	0.17	36.42	1.89	0.48	-	-	-	-	-	-
2	8.98 ± 0.71	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
3	13.76 ± 1.99^{bcde}	19.75	0.25	**39.57**	1.14	0.21	36.84	1.77	0.12	-	-	-	-	-	-
4	22.03 ± 2.16	na	na	na	na	na	na	na	na	+	-	-	-	-	-
5	29.80 ± 1.67	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
6	40.07 ± 6.89	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
7	48.12 ± 0.75	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
8	56.47 ± 2.89^d	17.22	0.28	**42.04**	1.25	0.19	36.60	1.84	0.33	-	-	-	-	-	-
9	68.04 ± 2.12	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
10	75.58 ± 1.84^{abcde}	4.72	2.28	30.81	3.19	0.50	12.63	5.68	**39.47**	-	-	-	+	-	+
11	79.78 ± 1.17	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
12	82.32 ± 0.83	na	na	na	na	na	na	na	na	+	-	-	+	-	-
13	86.74 ± 0.68	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
14	91.46 ± 2.93^d	14.20	1.14	**41.82**	1.78	0.33	34.16	4.05	1.86	+	+	+	-	-	-
15	97.50 ± 0.74^{de}	13.41	0.55	**42.34**	1.67	0.34	36.56	2.81	1.87	-	+	+	+	-	+

Separation of methanol extract (100 mg mL^{-1}) was performed using mobile phase hexane:ethyl acetate (1:1, v/v). *Antibacterial activity of separated isolates established by agar over-lay bioautography; [a], *E. coli* 8879; [b], *E. coli* 10778; [c], *Staphylococcus aureus* 1604; [d], *Staphylococcus epidermis* 1798; [e], *Salmonella enterica* 15480. [1]Data is representative of the relative % area of total lipids detected, identified by comparing their retention times and characteristic MS spectral data with those of authentic standards using LC/MS. The main fatty acid constituent of each isolate is indicated in bold. Caprylic, pelargonic, undecylic, lauric and linolenic ($\alpha + \gamma$) (≤ 1 %.) were also detected. [2]Component detection detection using TLC was determined using various detection reagents. Data indicates regions of positive and negative reaction to spray reagent are denoted by (+) and (-), respectively. Fast blue salt (FBS) was used for the detection of flavonoids and phenolic compounds; Lipids were detected with 10 % (w/v) sulphuric acid; Terpenoid structures were detected with anisaldehyde-sulphuric acid (AS) reagent; potassium hydroxide (KOH) reagent was used for detection of anthraquinones (red), anthrones (yellow) and coumarins (blue) and iodine vapour was used for the detection of compounds with conjugated double bonds. The major fatty acid of each isolate is indicated in bold.
- Abbreviations: *nd*, not detected; *na*, not active.

A total of eight different fatty acids were detected from the separated regions of the methanol extract of *T. versicolor*. They included caprylic, pelargonic, undecyclic, myristic, pentadecyclic, palmitic, palmitoleic/palmitelaidic, heptadecanoic, stearic, oleic/elaidic, linoleic and linolenic ($\alpha + \gamma$) acids (Table 6.17). Of the eight isolates which were separated and isolated using TLC, six were determined as active using bioautography (Table 6.11). With exception of isolate number 10, the main fatty acid constituent of each isolate from this species was palmitic acid. Similar to isolate number 10 of *G. frondosa* and *L. edodes*, and isolate number 8 of *P. ostreatus*, of which the predominant fatty acid was linoleic acid, microbial inhibition was demonstrated against *Staphylococcus aureus* 1604, *Staphylococcus epidermis* 1798 and *Salmonella enterica* 15480 (Table 6.14 – 6.17). In *P. ostreatus* and *T. versicolor*, this fatty acid was associated with the greatest antimicrobial diversity (Table 6.16 – 6.17). By comparison, other spots may have contained a greater quantity of other fatty acids but had lower antimicrobial diversity.

Relevant literature has associated several important relationships between structure and bacteriostatic action of fatty acids. Kabara *et al.* (1972) proposed that addition of a *cis* double bond increased the inhibition of gram-positive bacteria and addition of a second double bond further improved the toxicity of compounds. Conversely, a third double bond was ineffective. It was revealed that only *cis* forms of unsaturated acids were bacteriostatic. Activity was dependent on the free carboxyl group, as bactericidal activity of the fatty acid decreased with ester formation (Kabara *et al.*, 1972). Due to the diverse fatty acid profile of each isolate, in conjunction with positive reaction for phenolics, flavonoids, terpenoids and/or conjugated double bonds, it is appreciable that antimicrobial activity was due to a synergistic effect of a range of structurally diverse fatty acids.

Altogether, twenty-four different fatty acids were identified by comparing their retention times and characteristic MS spectral data with those of authentic standards (Section 2.2.9.3; Table 2.2). Table 6.18 summarises the detected fatty acids of each species tested in accordance with the standards used for identification. Linoleic acid was the main compound identified in all of the fungal extracts. Fatty acids; oleic, stearic and palmitic acid were also present in high quantities, depending on the species. Isolated compounds from *Pleurotus* spp., *G. frondosa*, *L. edodes*, *M. purpureus* and *T. versicolor* contained large quantities of linoleic, palmitic, stearic and oleic acid, whereas fatty acids possessing undesirable technological properties, e.g. α-linolenic

acid were absent or encountered in insignificant quantities in extracts of *L. edodes* and *T. versicolor* (Table 6.15 and 6.17, respectively). The composition of mycelial fatty acids have been observed to vary greatly depending on growth conditions such as nutritional factors, oxygen, and temperature (Suutari, 1995; Pedneault *et al.*, 2007). Having identified fatty acids as the most abundant lipid component of the extracts, the effect of hot water and methanol extraction process on the fatty acid composition of cellular lipids is detailed later in this chapter (Section 6.2.3). Table 6.18 summarises the fatty acid constituents detected in the various species of fungi following development, extraction and elution using TLC and detection by LC/MS.

Table 6.18　Summary of peak assignments of methanol extract

Fatty acids[a]	Chemical formula	Exact mass of negative ion [M-H]⁻	Exact mass of neutral ion	Carbon atom: Double bond (C:D)	Detected in[b]
Caprylic	$C_8H_{16}O_2$	143.108	144.115	08:0	L, G, P, T
Pelargonic	$C_9H_{16}O_2$	157.123	158.131	09:0	L, G, M, P, T
Undecylic	$C_{11}H_{20}O_2$	185.155	186.162	11:0	L, T
Lauric	$C_{12}H_{20}O_2$	199.170	200.178	12:0	L, G, M
Myristic	$C_{14}H_{28}O_2$	227.202	228.209	14:0	L, G, M, P, T
Pentadecylic	$C_{15}H_{30}O_2$	241.217	242.225	15:0	L, G, M, P, T
Palmitic	$C_{16}H_{32}O_2$	255.233	256.240	16:0	L, G, M, P, T
Palmitoleic/ Palmitelaidic	$C_{16}H_{30}O_2$	253.217	254.225	16:1	L, G, M, P, T
Heptadecanoic	$C_{16}H_{30}O_2$	269.249	270.256	17:0	L, G, M, P, T
Stearic	$C_{17}H_{34}O_2$	283.264	284.272	18:0	L, G, M, P, T
Oleic/Elaidic	$C_{18}H_{36}O_2$	281.249	282.256	18:1	L, G, M, P,T
Linoleic	$C_{18}H_{34}O_2$	279.233	280.241	18:2	L, G, M, P,T
Linolenic (α + γ)	$C_{18}H_{34}O_2$	277.217	278.225	18:3	L, T
Nonadecylic	$C_{18}H_{30}O_2$	297.280	298.287	19:0	M, P
Arachidic	$C_{18}H_{28}O_2$	311.296	312.303	20:0	M
Gadoleic	$C_{19}H_{38}O_2$	309.280	310.287	20:1	M
DihomoLinoleic	$C_{20}H_{38}O_2$	307.264	308.272	20:2	M
Arachidonic/ Eicosatetraenoic	$C_{20}H_{34}O_2$	303.233	304.240	20:4	M
EPA	$C_{20}H_{34}O_2$	301.217	302.247	20:5	M
DPA	$C_{22}H_{40}O_2$	329.249	330.256	22:5	M
DHA	$C_{22}H_{38}O_2$	327.233	328.240	22:6	M
Tricosylic	$C_{22}H_{36}O_2$	353.343	354.350	23:0	M

[a]Identification confirmed using authentic standards. [b]M., *M. purpureus*; P., *P. ostreatus*; G., *G. frondosa*; L., *L. edodes*; T., *T. versicolor*.

In consideration of the possible presence of one or more hydroxyl and/or carboxylic acid groups, the tentative mass spectrum for compounds was acquired in negative ionisation mode ([M-H]⁻). By this technique, the diagnostic fragmentation patterns of the compounds during collision induced dissociation (CID) elucidated structural information of the compounds analysed. The results of accurate mass measurements fitted well with the elemental composition of the compounds. The combination of accurate mass measurement to determine the elemental composition and liquid chromatography to separate isomeric compounds provided a powerful tool for identification of lipid diversity in each of the ten species of filamentous fungi.

In general, isolates were discussed in terms of activity with association of the compound class identified using TLC methods. However; owing to the partially purified nature of the isolates and the limited number of organisms tested, interpretation of structure-function relationship was difficult. It was established that biological activity observed in each extracted isolates of interest was most likely a result of a multi-factorial combination of attributes.

LC/MS analysis of individual TLC separated isolates indicated that the fatty acid components migrated through the TLC development system. This is believed to be associated with the mobile phase used, resulting in the separation and elution of different lipid classes (cholesterol and cholesterol esters, triglycerides, free fatty acids, diacylglycerols, along with complex lipids, such as phospholipids and glycosphingolipids) at different retention times, according to polarity. In fungi, the major fatty acids that typically occur in membrane phospholipids and storage triacylglycerols are palmitic and stearic acids and their unsaturated derivatives palmitoleic, oleic, linoleic and linolenic acids (Suutari, 1995), most of which demonstrated a strong presence in each of the fungal extracts in the present investigation (Table 6.18). It is evident that the mixture of fatty acids in each fungal isolate was complex. Generally extraction of lipids is difficult, as many are associated in the original biological material with other compounds (Fuchs *et al.*, 2011). For efficient separation of the individual homologues using this type of chromatographic support, saponification or derivatisation of individual fatty acids is required prior to analysis. The occurrence of lipid mixtures of homologues that have different side chain lengths and unsaturation implies the need for a two-step derivatisation procedure (Kozubek *et al.*, 1999). The best practical method for efficient separation of fatty acids with double bonds prior to development for quantitative determination using TLC

involves separation on silver ion TLC plates (Ag-TLC), in conjunction with GC determination of the fatty acyl compositions of the individual lipid classes (Fuchs *et al.*, 2011). Ag-TLC is still the most powerful method to separate *cis-* and *trans-* isomeric fatty acids, and to isolate specific functions in quantities suitable for downstream analysis (Fuchs *et al.*, 2011). Although TLC separates acids or esters with additional polar groups; this method did not appear to separate acids or esters with differing chain length or degree of unsaturation.

According to the R$_f$ values, isolates which indicated a strong presence of fatty acid components also demonstrated a positive reaction to phenolic or flavonoid constituents. It is well known that polyphenols (phenols, quinines, flavonoids, tannins and coumarins) from natural sources have been suggested as alternatives to antimicrobial agents (Ponnusha *et al.*, 2011). Naturally occurring polyphenols have previously been associated with the management of microbial pathogens in food (Shetty *et al.*, 1998) and crops (Mandavia *et al.*, 2003). By demonstrating the ability to inhibit the growth of microbes, they prevent microbial spoilage (rancidity or deterioration of colour, flavour and texture). Kao *et al.* (2010) demonstrated that the gram-positive bacterium *S. aureus* was susceptible to a reduction in growth rate due to the presence of a polyphenolic extract. This may be the case in the present investigation also. It is believed that the antimicrobial action of polyphenolic compounds may be mediated by the ability of tannins to deplete metal ions through precipitation or by structural damage to the microorganism, since flavonoids can cause such damage by altering the permeability of the cell membrane. An antioxidant compound from fungi which demonstrates strong antimicrobial efficiency may be an attractive alternative source for food preservation as well as a natural functional food ingredient. Although phenolic content was positively attributed to antioxidant activity (Section 5.3.1), it was unknown whether biological activity was associated with either phenols or lipids, or a combination of protein-phenol and/or lipid-phenol complexes. Similar to other medicines derived from natural sources; the combined action of multiple constituents may work synergistically or additively. At present, there is little information available on the synergy of compounds in relation to antibacterial activity (Vaquero *et al.*, 2011). Certain phenols and fatty acids are expected to have similar polarity and as a result this would lead to co-elution by TLC, consequently having similar capacity to cross the cell membrane (Vaquero *et al.*, 2011). As aforementioned (Section 6.1.3), Junio *et al.* (2011) found activity against *Staphylococcus aureus* using

a combination of three flavonoids synergistically, whilst individually the flavonoids possessed no inherent antimicrobial activity against *Staphylococcus aureus*; therefore, they could have been missed using traditional bioactivity directed fractionation (Junio *et al.*, 2011). In a study by Vaquero *et al.* (2011) synergistic effects were also observed for various other phenolic compounds against *Listeria monocytogenes*. Additionally, mixtures of protocatechuic and caffeic acids were found to exert an antagonistic effect, with the most effective antilisterial mixture of flavonoids determined as quercetin and rutin (Vaquero *et al.*, 2011). In the present investigation, *Staphylococcus aureus* and *Listeria monocytogenes* demonstrated microbial sensitivity to fungal components in Section 4.2.1.

Apart from their important physiological and biochemical roles, phenols and polyphenols, as well as their derivatives may be defined as any substance that possess an aromatic ring bearing a hydroxyl substituent and functional derivatives (Kozubek *et al.*, 1999). Additionally, the efficiency of phenolic compounds may be associated with factors such as number of hydroxyl groups and the site of binding, as well as the number of hydroxyls in the aromatic ring. As previously mentioned, molecules such as simple phenolic acids, phenylpropanoids and flavonoids, as well as the highly polymerised molecules; lignin, melanin and tannins, accumulate naturally as end products from the shikimate and polyketide (acetate) pathways, with flavonoids representing the most common and widely distributed sub-group with antioxidant activity (Bravo, 1998; Liu, 2004; Ferreira *et al.*, 2009). Phenols have been found in conjugate forms bearing a sugar or lipidic moiety (Correia *et al.*, 2004; Takahashi *et al.*, 2010). Phenolic lipids or long chain phenols are amphiphilic in nature due to the non-isoprenoid side chains attached to the hydroxybenzene ring and are also believed to be derived from the polyketide pathway (Kozubek *et al.*, 1999). Consequently, these compounds can be incorporated into erythrocytes and liposomal membranes. Being relatively uncommon, these non-isoprenoid lipids can be considered for simplicity as fatty acids, with the carboxyl group replaced by the hydroxybenzene ring (Kozubek *et al.*, 1999; Stasiuk *et al.*, 2010). Chemically, these phenolic lipid compounds are derivatives of mono- and dihydroxyphenols, namely catechol, resorcinol, and hydroquinone (Stasiuk *et al.*, 2010). The content of catechin was previously established in Section 6.2.1 and although not detected in *P. 32783* and *P. eryngii*, other species contained up to 5.7 mg per gram of dry extract (*L. edodes*). This suggests that possible phenolic lipids present may have derived from another

compound such as hydroquinones for some or all species. Lipid composition is also known to affect membrane proteins, in that, slight changes of the lipid composition may strongly affect their functionality (Fuchs *et al.*, 2011). Research has demonstrated that conjugation of phenols exists in many forms. Hydrophobic aliphatic moieties of lipids interact with the non-polar regions of proteins, particularly, valine, leucine and isoleucine. Additionally, the acidic phosphate groups of lipids strongly interact with metal ions normally bound to proteins (Fuchs *et al.*, 2011). Antimicrobial activity of phenolic lipids is associated with their interaction with proteins and/or membrane-disturbing properties (Stasiuk *et al.*, 2010). Increasing interest in phenolic lipids, from pure and industrial chemistry to agricultural, nutritional and biochemical sciences, has led to interest in the bioactivity of such components (Stasiuk *et al.*, 2010).

Fatty acids in association with other individual isolate constituents were identified in the present investigation, demonstrated antibacterial activity against a range of bacteria. As discussed in Section 1.2.7, lauric, linoleic, oleic and linolenic acid, amongst others, including their derivatives (aldehydes, acetate, ethyl esters, amide or substituted amides) are known to have demonstrated strong bacteriostatic activity against both gram-positive and gram-negative bacteria (Kabara *et al.*, 1972; Bergsson *et al.*, 2011). In addition, lipids exhibit antimicrobial properties capable of killing enveloped viruses, yeast, fungi and parasites (Bergsson *et al.*, 2011). Previous literature has associated several important relationships between structure and bacteriostatic action of fatty acids (Kabara *et al.*, 1972). Relevant research has reported that addition of a *cis* double bond increased the inhibition of gram-positive bacteria and addition of a second double bond further improved the toxicity of compounds (Kabara *et al.*, 1972). It was observed that only *cis* forms of unsaturated acids were bacteriostatic and activity was dependent on the free carboxyl group, as bactericidal activity of the fatty acid decreased with ester formation (Kabara *et al.*, 1972). This may have been associated with the activity observed in the present investigation, however further purification would be required in order to determine this concept. Ag-TLC is capable of *cis-* and *trans*-isomeric fatty acid separation suitable for downstream analysis (Fuchs *et al.*, 2011). Additionally, enzymatic hydrolysis of phenolic glycosidic linkages *via* β-glucosidase, which is produced naturally by a broad spectrum of organisms including bacteria, fungi and plants, may increase the concentration of free phenolics and other components, and thus enhance nutraceutical

activity of such compounds (Zheng *et al.*, 2000; Murashima *et al.*, 2002; Correia *et al.*, 2004).

LC/MS analysis of individual isolates also emphasised the non-specific nature of lipid detection, using detection reagents in conjunction with TLC analysis (Section 6.1.2). For non-specific determination of lipids (Section 2.2.8.2), the TLC plate was treated with 10 % (v/v) sulphuric acid reagent, and heated at high temperature to degrade any organic species to carbon. As a result, charring is included in what is termed a universal reagent (Wall *et al.*, 2005). Whilst TLC did contribute to the identity of individual isolates/active components and different derivatives, it did not however do so efficiently in a complex mixture consisting of homologues with different side chain length and unsaturation. Although some constituents of lipid structure were determined in Section 6.1.2, characteristic functional groups such as amino or carbohydrate residues were not detected using this staining reagent, resulting in underestimation of lipid components. Nonetheless, a growing interest in an effect-directed analysis has lead to the success of TLC with microbial detection, and this chromatographic method is more efficient than HPLC, where post column derivatisation is normally more difficult (Fuchs *et al.*, 2011).

Overall, the components obtained following the methanol extraction process may be regarded as functional food ingredients or as constituents of great interest to the pharmaceutical industry, having demonstrated strong antibacterial, anti-adhesive and antioxidant functionalities in the present investigation. In particular, the methanol extract of *M. purpureus* was found to isolate the highest concentration of phenols (mgGAE g^{-1}) (Section 5.3.1), demonstrated the highest antioxidant activity in terms of inhibition of linoleic acid oxidation (Section 5.2.1), was significantly more effective at scavenging $ABTS^+$ radicals (Section 5.2.2.1) and had the strongest reducing capacity, in comparison to its hot water and crude extract. This species contained the most diverse range of compounds and contained the most heavily concentrated compounds following separation and visualisation by TLC. In addition, *M. purpureus* from the Ascomycota division of fungi had a more diverse fatty acid composition (Table 6.13), however contained the lowest quantity of phenolic compounds when analysed by UPLC (Section 6.2.1). Yet interestingly the majority of isolates demonstrated antibacterial activity as determined by bioautography (Table 6.9). This reveals a strong antimicrobial action of the phenolic constituents of this species or a strong synergistic capacity with the other constituents as previously discussed.

The detection of linoleic, oleic, stearic and palmitic acid in the mycelium produced by submerged liquid fermentation and identified in the methanol extract, indicated that this bioprocess did not prevent the production or isolation of these predominant fatty acids (Pedneault *et al.*, 2007; Papaspyridi *et al.*, 2012). Similarly, submerged fermentation and the solid phase extraction process did not prevent the production or isolation of bioactive phenolic compounds. A similar observation was also made by Tan *et al.* (2004), whereby, the submerged fermentation and methanol extraction process did not prevent the production or isolation of bioactive phenolic compounds, respectively. The effect of differing extraction procedures for isolation of bioactive fatty acid components is examined in the following section (Section 6.2.3). A consequence of certain extraction procedures would be expected as environmental growth factors are known to affect lipid content and composition of living organisms, including fungi (Pedneault *et al.*, 2007). In addition, depending on the cellular location of the active components, extraction conditions may negatively impact isolation due to destruction of the cell wall. The extractability of bound polyphenolic and flavonoid compounds, release more easily compared to those of raw materials with certain conditions such as extreme heat (Choi *et al.*, 2006b). In the present investigation, differences in antioxidant capacity in relation to phenolic content of various extracts from the same species were previously observed (Table 5.15). Nonetheless, the formation and production of bioactive metabolites such as fatty acids, phenolic metabolites, alkaloids, terpenoids, flavonoids and anthraquinones have been established in the mycelium, under optimised cultivation conditions for optimal biomass yield. Thus, submerged fermentation of the mycelium of several species of filamentous fungi proved to be a promising alternative to SSF for production of bioactive and nutritional functional compounds on an industrial scale (Papaspyridi *et al.*, 2012).

6.2.3 Fatty acid identification and quantification from different extracts using gas chromatography mass spectrometry (GC/MS)

Fungal mycelial mass produced using submerged cultures is predominately made up of lipids in the form of polyunsaturated fatty acids, and polysaccharides (Dimou *et al.*, 2002; Pedneault *et al.*, 2008; Diamantopoulou *et al.*, 2012). Lipids are important cell constituents which have functional properties as either structural components or as carbon and energy reserves (Arora, 2003). Although mammals use fat as a storage

form of energy, they are unable to synthesise specific lipid molecules such as polyunsaturated fatty acids (PUFAs). For this reason, they are required to obtain these compounds from exogenous producers i.e. plants, fungi or microorganisms (Arora, 2003). It is often necessary to modify essential fatty acid content in animal nutrition to ensure adequate intake. Fungi, being metabolically versatile organisms, are a natural dietary source of such compounds.

The cellular fatty acid profile can be used for discriminatory purposes of different fungal species (Stahl *et al.*, 1996; Dimou *et al.*, 2002). Fungal species and strains within a species differ in the number and kinds of fatty acids present and in relative concentration of each type (Stahl *et al.*, 1996). Current knowledge of fatty acid composition from filamentous fungi is poor, and mostly based on Basidiomycota analysis (Dimou *et al.*, 2002). Similar to carbohydrate compositional structure, changes to cultivation conditions are known to affect cellular lipid profile of the biomass. Lipid accumulation is triggered at stressed conditions, such as high carbon-to-nitrogen ratio and temperature (Konova *et al.*, 2009). Also, agitation, temperature and time have been seen to affect lipid concentration of mycelium grown by submerged cultures (Stahl *et al.*, 1996; Smiderle *et al.*, 2012).

Polysaccharides and lipids synthesised during early stages of growth are subject to degradation as fermentation proceeds (Diamantopoulou *et al.*, 2014). In a similar fashion different extraction processes may also affect biologically active compounds. In the present study, ten filamentous fungi from the Ascomycota and Basidiomycota division were examined by GC/MS with regard to individual intracellular (endopolysaccharides and lipids) and extracellular (exopolysaccharides) compounds produced in liquid cultured mycelium following the different extraction processes (Section 2.2.4). The results for fatty acid composition, total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) of three extracts from the fungal mycelial biomass of each of the selected species are displayed in Table 6.19. Up to nine fatty acids were detected and quantified using GC/MS as outlined in Section 2.2.9.5.

Table 6.19 Fatty acid composition of cellular lipids produced by ten filamentous fungi in submerged culture

	Extract	Fatty acid (% of total fatty acid content)[a]					$\sum$SFA[b]	$\sum$MUFA[c]	$\sum$PUFA[d]
		C16:0	C18:0	C18:1	C18:2	Others*			
Grifola frondosa	Crude	**18.0**	*nd*	*nd*	**82.0**	*nd*	**18.0**	*nd*	**82.0**
	HW	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
	MeOH	**17.9**	*nd*	**4.4**	**77.6**	*nd*	**17.9**	**4.4**	**77.6**
Lentinula edodes	Crude	**17.3**	*nd*	*nd*	**82.7**	*nd*	**17.3**	*nd*	**82.7**
	HW	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
	MeOH	**16.7**	*nd*	**6.3**	**77.0**	*nd*	**16.7**	**6.3**	**77.0**
Monascus purpureus	Crude	**19.5**	**4.6**	**17.1**	**58.0**	**0.7**[1]	**24.8**	**17.1**	**58.0**
	HW	**20.3**	**13.5**	**29.4**	**32.6**	**4.1**[1]	**37.9**	**29.4**	**32.6**
	MeOH	**19.9**	**3.4**	**16.3**	**58.7**	**1.4**[1,5]	**24.7**	**16.3**	**58.7**
OYRM1	Crude	**13.4**	*nd*	**8.1**	**77.0**	**1.6**	**15.0**	**8.1**	**77.0**
	HW	*nd*	*nd*	*nd*	**100.0**	*nd*	*nd*	*nd*	**100.0**
	MeOH	**13.7**	*nd*	**7.9**	**74.6**	**3.8**	**17.5**	**7.9**	**74.6**
Pleurotus 1833	Crude	**15.3**	*nd*	**7.6**	**77.1**	*nd*	**15.3**	**7.6**	**77.1**
	HW	*nd*	*nd*	*nd*	**83.0**	**17.0**[2]	**17.0**	*nd*	**83.0**
	MeOH	**12.8**	*nd*	**8.4**	**78.8**	*nd*	**12.8**	**8.4**	**78.8**
Pleurotus 32783	Crude	**13.3**	*nd*	**7.4**	**75.9**	**3.4**[1]	**16.7**	**7.4**	**75.9**
	HW	*nd*	*nd*	*nd*	**75.7**	**24.3**[1]	**24.3**	*nd*	**75.7**
	MeOH	**12.1**	**2.5**	**6.1**	**76.1**	**3.2**[3,4]	**16.4**	**7.5**	**76.1**
Pleurotus citrinopileatus	Crude	**18.1**	*nd*	*nd*	**81.9**	*nd*	**18.1**	*nd*	**81.9**
	HW	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
	MeOH	**15.3**	**2.5**	**7.6**	**73.0**	**1.5**[3]	**19.3**	**7.6**	**73.0**
Pleurotus eryngii	Crude	**12.9**	*nd*	**19.7**	**67.5**	*nd*	**12.9**	**19.7**	**67.5**
	HW	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
	MeOH	**13.1**	**2.6**	**14.7**	**69.7**	*nd*	**15.7**	**14.7**	**69.7**
Pleurotus salmoneo stramineus	Crude	**16.3**	*nd*	**8.8**	**74.9**	*nd*	**16.3**	**8.8**	**74.9**
	HW	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
	MeOH	**18.3**	**2.8**	**7.7**	**68.7**	*nd*	**21.1**	**7.7**	**68.7**
Trametes versicolor	Crude	**21.0**	*nd*	**6.2**	**72.8**	*nd*	**21.0**	**6.2**	**72.8**
	HW	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
	MeOH	**17.1**	*nd*	**8.4**	**73.9**	**2.3**[3]	**19.4**	**8.4**	**73.9**

Fatty acid composition of cellular lipids following different extraction procedures. [a]Total fatty acid (% w/w) of C16:0, Palmitic acid; C18:0, Stearic acid; C18:1, Oleic acid; C18:2, Linoleic acid; Others*, sum of total Capric (10:0)[1], Lauric (12:0)[2], Pentadecanoic (15:0)[3], Palmitoleic (16:1)[4] and Heptadecanoic (17:0)[5]. [b]$\sum$SFA, total saturated fatty acid content; [c]$\sum$MUFA, total monounsaturated fatty acid content; [d]$\sum$PUFA, total polyunsaturated fatty acid content.
- Abbreviations: HW, hot water, MeOH, methanol; *nd*, not detected; *OYRM*1, *Pleurotus ostreatus*.

Extracts from *Pleurotus* spp., *G. frondosa*, *L. edodes*, *M. purpureus* and *T. versicolor* contained large quantities of linoleic acid. The results are in agreement with

Diamantopoulou *et al.* (2014), where unsaturated fatty acids were the predominant fatty acids making up more than 80 % of the total fatty acid content (Table 6.19). As was observed in the individual isolates separated by TLC (Table 6.13 – 6.17), palmitic acid (C16:0), oleic acid (C18:1) and linoleic acid (C18:2) were present in each fungus. GC/MS analysis of the extracts revealed linoleic acid accounted for the majority of fatty acids present in each sample; representing up to 83 % (w/w) of the total fatty acid concentration of the crude extracts, 100 % (w/w) of the total fatty acid content of hot water extracts and 79 % (w/w) of the total fatty acid content of the methanol extracts. Other fatty acids examined, namely pentadecanoic acid (C15:0), palmitoleic acid (C16:1) and heptadecanoic acid (C17:0) are commonly reported in fungi, usually representing less than 5 % (w/w) of the total fatty acid content, as was the case here (Table 6.19). The relative concentration of an individual fatty acid ranged from 4.4 % to 100 % (w/w) of the total fatty acid content.

Overall, linoleic acid was the main fatty acid found in the fungal extracts followed by palmitic and oleic acid. Previously (Section 6.2.2), fatty acid content was estimated following separation of individual components according to polarity using TLC and analysed by LC/MS, resulting in a similar profile; i.e. linoleic, palmitic, oleic and stearic acid were among the most abundant fatty acids established in the extracts. *Monascus purpureus* contained the most diverse range of fatty acids which included linoleic (58.7 %, w/w), palmitic (19.9 %, w/w), oleic (16.3 %, w/w), stearic (3.4 %, w/w), capric (0.7 %, w/w) and heptadecanoic (0.7 %, w/w) acid (Table 6.19). This coincides with the TLC results for this species; as a range of unknown compounds were visualised under UV light. The methanol extract had higher fatty acid content (215.1 mg g^{-1}) than the hot water extract (10.7 mg g^{-1}), certain fatty acids were retained better in the hot water extract such as oleic (29.4 %, w/w), palmitic (20.3 %, w/w), stearic (13.5 %, w/w) and capric (4.1 %, w/w) acid. No fatty acids were detected in the hot water extract of *P. citrinopileatus, P. eryngii, P. salmoneo-stramineus* or *Trametes versicolor.* MAE methods are not suitable for heat-sensitive compounds as they are subjected to extreme heat and pressure during the extraction process. However, this observation is most likely associated with the extraction of hydrophobic components with hydrophilic liquid and species specificity of cellular lipid content. Thus, as expected this investigation demonstrated that hot water extraction processes are preferred for extracting hydrophilic compounds and not lipophilic components. Nonetheless, research has shown that under appropriate conditions, pressurised water

can stabilise even the most non-polar, hydrophobic organic compounds. The dielectric constant of water can be adjusted as a function of temperature, allowing water to dissolve a variety of organic solutes, as a value is obtained similar to that presented by some organic solvents like ethanol or methanol (Joana Gil-Chávez *et al.*, 2013). The methanol extract yielded the greatest fatty acid content (mg g^{-1}) in the order; *Monascus purpureus > Trametes versicolor > OYRM*1 *> Pleurotus 32783 > Pleurotus salmoneo-stramineus > Pleurotus eryngii > Pleurotus citrinopileatus > Pleurotus 1833.* Followed by the crude biomass fatty acid content in the order; *Monascus purpureus > Pleurotus eryngii > OYRM*1 *> Pleurotus 32783 > Trametes versicolor > Pleurotus salmoneo-stramineus > Pleurotus citrinopileatus > Pleurotus 1833.*

The concentration of palmitic, oleic and linoleic acid in the present study were similar to the quantities detected by Stahl *et al.* (1996), for species of *L. edodes* (18.06, 7.42 and 76.54 % (w/w), respectively) and *T. versicolor* (16.63, 20.87 and 55.59 % (w/w), respectively). In the present study, the concentration of palmitic acid in extracts of *L. edodes* was 17.3 % (w/w) and 16.7 % (w/w), in the crude and methanol extract, respectively. The concentration of oleic acid found in the methanol extract was 6.3 % (w/w), while the concentration of linoleic acid was 82.7 % (w/w) and 77.0 % (w/w) in the crude and methanol extract, respectively. Similarly, the concentration of palmitic acid found in extracts of *T. versicolor* was 21.0 % (w/w) and 17.1 % (w/w), in the crude and methanol extract, respectively. In addition, the concentration of oleic acid was 6.2 % (w/w) and 8.4 % (w/w) in the crude and methanol extract, respectively. Whilst the concentration of linoleic acid found in extracts was 72.8 % (w/w) and 73.9 % (w/w) in the crude and methanol extract, respectively. It was reported by Stahl *et al.* (1996) that species of fungi from different phyla had the most disparate fatty acid compositions, contributing to the ability to characterise between different filamentous fungal species and strains. A similar observation was made from the data obtained in this study; although the fungi in the present investigation are within the same subphylum (Ascomycetes and Basidiomycetes); *M. purpureus* from the Ascomycota division of fungi had a more diverse fatty acid composition. This species had less linoleic acid, more stearic acid and in most cases more palmitic and oleic content than the other Basidiomycetes species examined (Table 6.19). This example of unique fatty acid signature is used for strain differentiation, analysis of which has mostly been focused toward Basidiomycetes (Stahl *et al.*, 1996; Dimou *et al.*, 2002). That being said, Stahl *et al.* (1996) revealed that Dikaryotic fungi were composed of the fewest

different fatty acids of any other taxonomic group (usually between five and ten out of twenty-one analysed). In this present study, the number of different fatty acids detected ranged between three and six, of the nine examined by GC/MS; with *M. purpureus* and *P. 32733* producing the most (Table 6.19). LC/MS which allowed for small molecule analysis of isolated active constituents of interest (Section 6.2.2), detected 19 fatty acid constituents in *M. purpureus*, 13 in *L. edodes*, 11 in *G. frondosa* and *P. ostreatus*, and 8 in *T. versicolor* from a total of 38 authentic standards tested.

Other fatty acids, namely, capric (C10:0), lauric (C12:0), pentadecanoic (C15:0), palmitoleic (C16:1) and heptadecanoic (C17:0) were detected; however, with exception of *P. 1833* and *P. 32783* they represented less than 5 % (w/w) of the total fatty acid content (Table 6.19). Capric (C10:0) was detected in the hot water extract of *P. 1833* (17.0 %, w/w) while, lauric was detected in the hot water extract of *P. 32783* (24.3 %, w/w). Unlike the major fatty acids identified in the different mycelial extracts, these fatty acids were affected by the nature of this extraction processes. This is likely as to why these fatty acids were not detected in the methanol extracts of this species by LC/MS having been separated into isolates (Table 6.16). Similar to the effect on phenolic components, many environmental factors may affect lipid content in living organisms including fungi (Suutari, 1995; Pedneault *et al.*, 2007). In addition, various stages of growth and different species of fungi can vary in their lipid profile. There is also evidence to suggest that temperature during cultivation and age of the culture can affect fatty acid composition (Stahl *et al.*, 1996). These are important parameters for consideration for optimal production of an active natural product from filamentous fungi. Previous reports suggested that lipids inhibit bacterial growth, creating more favourable conditions for spore conservation and germination (Feofilova, 2010). Generally, diets that are low in fat and calorific intake are recommended to people with high blood cholesterol, in conjunction with oils that contain high linoleic and oleic acid levels, this is used as a preventative measure for atherosclerosis (Vaz *et al.*, 2011). Mushrooms or mycelial extracts in the form of tea or powder are known to be rich in these fatty acids (Table 6.19).

Overall, *Monascus purpureus* contained the highest fatty acid concentration. Bioautography demonstrated this species of fungi to be highly active towards both gram-positive and gram-negative bacteria. The data above (Table 6.19) in conjunction with TLC, bioautography and UPLC-DAD analysis of phenolic compounds provides evidence that a degree of biological activity of the extracts may be associated with the

fatty acid content of the biomass. The data suggests mycelial biomass produces biologically active lipids, terpenoids and/or phenolic constituents. Analysis revealed that the antibacterial components of the filamentous fungal species examined were most likely associated with a combined action of multiple constituents of phenolic and/or lipid structure which may work synergistically or additively.

6.3   General Conclusions

In conclusion, the findings of this section are demonstrated to be valuable, as the established cultivation and extraction process of the selected filamentous fungi has led to the formulation and production of known bioactive metabolites such as phenols and fatty acids occurring in the mycelium. Submerged fermentation proved to be a promising alternative for production of bioactive and nutritionally functional compounds on an industrial scale. This type of fermentation is faster, easily controlled, more efficient and is of more interest in food and drug industry processes as opposed to the established field-cultivation of fruit-bodies (Papaspyridi *et al.*, 2012).

Biological assays and chemical screening methods are important tools in the separation of biologically active metabolites from natural sources. Putative chemical identification (compound class) of antibacterial components in the methanol extract from the mycelium of *Pleurotus* spp., *G. frondosa*, *M. purpureus*, *T. versicolor* was performed. This was achieved using an agar-overlay bioautographic technique. TLC with a bioassay *in situ* was a successful tool in localising the active constituents, particularly as antibacterial activity was demonstrated against a range of bacteria. Bioautography was used as an indicator of biological activity and not a quantitative measure of activity.

The major constituents of the fungal cultures were investigated using LC/MS, UPLC and GC/MS. Preliminary identification detected phenolics (e.g. phenolic acids and their derivatives, flavonoids) and volatile and aliphatic compounds. The phytochemical studies revealed the presence of multiple constituents including phenolic acids, flavonoids, lipids and compounds of terpenoid structure as having biological activity. Bioautography and LC/MS analysis confirmed the presence of different compounds within the separated isolates of each species. All the active compounds demonstrated good activity individually against gram-positive and gram-negative bacteria.

Some studies suggest species may be differentiated from one another on the basis of fatty acid composition (Stahl *et al.*, 1996). Many species contained the same fatty acids but had different quantities in their relative amounts of each; some species differed in both the kind of fatty acids produced and in the amount (Table 6.19). The data revealed that the extraction process had an enormous impact on the isolation of potentially biologically active fatty acids and as a consequence is an important factor in retaining bioactive compounds of interest in the future. Based on existing literature, the compounds present in the methanol extracts from each of the selected fungal species may be regarded as functional food ingredients or as constituents of considerable interest to the pharmaceutical industry, as they may exhibit numerous health benefits. With exception of the hot water extraction process, the presence of linoleic, oleic, stearic and palmitic acid in the mycelium produced by submerged liquid cultivation demonstrates that the established bioprocess does not prevent the production and consequently the elution of these predominant fatty acids. Similarly, submerged liquid fermentation did not prevent the production of phenolic compounds which had a strong association with antioxidant activity (Section 5.3). It is evident that cultivation conditions may be a source of optimisation, in order to influence the production of active components of interest. For example, a change in temperature and the age of culture as well as extraction methods have been shown to affect fatty acid composition (Stahl *et al.*, 1996).

This study shows that extracts isolated from ten filamentous fungi can be a potential antioxidant and antibacterial resource. The fractionated extracts demonstrated that a combination of multiple chemical constituents yielded beneficial biological activities.

7.   Conclusions

Many species of fungi are recognised as medicinal as a result of their established pharmacological activity and because they contain substances that can be used for therapeutic purposes or as precursors for the synthesis of new drugs. It is suggested that fungi may be used directly in the diet to promote health, taking advantage of the additive and synergistic effects of the bioactive compounds that may be present (Ferreira *et al.*, 2009). Nutraceuticals are considered a powerful instrument in maintaining and promoting health, longevity and quality of life. Filamentous fungi are being increasingly investigated for their biological activity, which has lead to different applications in pharmaceuticals, biotechnology and food preservatives. Pharmacological and medicinal studies of fungi have shown that the Basidiomycota and Ascomycota divisions are an immense source of biologically active components, yet only ten percent of all species have been described and even less have been tested for therapeutic significance (Lindequist *et al.*, 2005). It is clear that fungi represent a largely untapped source of useful compounds and as such many potentially powerful new pharmaceutical products await discovery (Hawksworth, 1993; Wasser, 2002; Marx, 2004; Kirk *et al.*, 2011).

An assessment of filamentous fungi as a source of novel antimicrobial and antioxidant components was investigated. Species, for which there is little information documented in the literature, were assessed for their potential to produce antioxidant and antimicrobial compounds using submerged fermentation techniques. Submerged fermentation is a biotechnology tool capable of supplying industrially relevant metabolites, on a large scale and in a short period of time (Smiderle *et al.*, 2012). The vegetative phase is represented through mycelial growth, whereas the fruiting body represents the reproductive phase of the life cycle of Dikarya fungi. The contrasting stage of growth is an important consideration with regard to metabolite production. Hence, differences in isolation and identification of metabolites of bioactive nature are observed, this allows directed development of bioactive metabolites. In the present investigation, mycelium was grown under favorable controlled conditions for optimum biomass generation. Each of the selected species was investigated based on the potential of each strain from a natural product perspective. In addition, there are few reports of bioactivity in conjunction with antioxidant production from fungi grown by

submerged cultivation. The most effective media composition for maximum biomass production of each species was established as different to the recommended media. In addition, the results demonstrated that depending on the species, biomass production may vary according to supplemental carbohydrate source. Nonetheless, each species was capable of using each of the carbon sources tested for the generation of biomass.

In order to explain the factors which influence chemical composition of the cultured mycelium, as well as the production of components for the food industry, biomass studies are necessary (Smiderle *et al.*, 2012). Fungi are excellent sources of dietary fibre. The cell wall is composed mainly of chitin, hemicellulose, mannan and β-glucan. These constituents can assist intestinal motility and increase stool bulk, decreasing absorption of harmful toxic carcinogenic substances, leading to lower incidence of colorectal cancer (Manzi *et al.*, 2000; Smiderle *et al.*, 2012). In the present study, a quantitative comparison of the major cell wall constituents of ten filamentous fungi was performed and differences in cellular composition following growth with various nutrient additives to the media were investigated. The data revealed that depending on the species of fungi, the yield of mycelial biomass and its chemical composition may vary with supplemental carbohydrate. Although no correlation was established between biomass production and polysaccharide production, the data revealed that certain environmental parameters affected both the cellular composition and biomass production for various species differently. Chitin was a major contributor to the overall composition of the cell wall; differences in sugar content during cultivation significantly affected the chitin concentration of the cell wall of *G. frondosa*, *L. edodes*, *M. purpureus*, *P. ostreatus*, *P. citrinopileatus* and *P. salmoneo-stramineus* compared to non-supplemented cultures. Research has shown this indigestible fibre constituent is an immune supporting component from many medicinal fungal species. A high concentration of chitin was observed in the biomass of *P. 32783*, *M. purpureus* and *P. eryngii*. Supplemental carbon source (1 %, w/v) had a significantly positive impact on the chitin content of *L. edodes* and *OYRM*1 mycelia. Hence, the data demonstrated that suitable growth conditions for particular species can direct fungal cellular composition. The total chitin content of *P. 32783, P. eryngii* and *T. versicolor* was not affected significantly by any changes in medium composition.

The present investigation indicated the presence of antimicrobial and antioxidant activities in a number of filamentous fungi, which could provide opportunities for the application of fungal extracts as natural food preservatives or as

nutraceuticals in food and dietary supplement products for health promotion. Fungi, containing various phenolic compounds have shown potential for application as functional foods or as medicinal products. In recent years, much attention has been focused on the use of natural preservatives to enhance the quality, safety and stability of food products, such as those from fungi, algae and plants. In the present investigation, crude, hot water and methanolic extracts from the mycelium of each of the selected species were effective antioxidants. Aside from radical scavenging action, polyphenolic compounds inhibited oxidation by a variety of mechanisms. Antioxidant activity of the extracts was concentration dependant, with stronger inhibition of reducing power, scavenging ability and chelating ability occurring at higher concentrations. Due to their antioxidant and possible health promoting role in the diet, the use of phenolics has increased greatly in the food industry (Rajauria *et al.*, 2013). This study confirmed that phenolic content made a significant contribution to antioxidant function and explained the relationship between phenolic compounds and antioxidant activities. Naturally occurring polyphenols have previously been associated with the management of microbial pathogens in food (Shetty *et al.*, 1998) and crops (Mandavia *et al.*, 2003). Having additionally demonstrated the ability of the various species to inhibit the growth of microbes, the extracts demonstrated potential to prevent microbial spoilage (rancidity or deterioration of colour, flavour and texture). Notably, the total phytochemical content (phenol, flavonoid and condensed tannin) and their activities were affected by solvent polarity and the extraction process. The cultivation and extraction processes enabled the production and isolation of natural antioxidants, which could potentially be used to partially substitute synthetic antioxidants such as BHT.

The fight against antibiotic resistance is one of the most significant challenges to public health of our time. The inevitable development of resistance following the introduction of novel antibiotics has led to an urgent need for antibacterial drugs with new mechanisms of action that are not susceptible to existing resistance mechanisms (Marks *et al.*, 2012). Fungal polysaccharides have been associated with stimulation of innate immunity and production of pro- and anti-inflammatory cytokines (Rodrigues *et al.*, 2011). The present investigation suggests that there is potential for not only direct interaction with the host through stimulation of an immunological response but also *via* attachment to microbes prior to infection. This observation was proposed in the present study with fungal cellular components from these species for the first time.

Characterisation of efficient alternative-adhesion materials from fungi that are beneficial for the health of man and animals is an interesting prospective for nutraceuticals from fungi. The data revealed that modification of the growth medium may positively influence the antimicrobial effects against both gram-positive and gram-negative bacteria. Furthermore, aqueous mycelial extracts demonstrated qualitative inhibitory action toward a range of pathogenically significant bacteria, relevant to the Irish agricultural industry. Generally, a broad range of activities was observed. The majority of fungal extracts demonstrated strong bacteriostatic and significant bactericidal power *in vitro* against eighteen gram-positive and gram-negative species of bacteria in comparison to their antibiotic control. Thus, the data revealed antibiotic substances from metabolite producing strains were present in aqueous extracts of *M. purpureus*, *P. ostreatus*, *P. ostreatus* 1833, *P. ostreatus* 32783, *P. citrinopileatus*, *P. eryngii*, *P. salmoneo-stramineus*, *G. frondosa*, *L. edodes* and *T. versicolor*.

Diseases such as mastitis and bovine respiratory disease which are caused by opportunistic, contagious pathogens are a major concern of the agricultural industry. These illnesses can have a significant impact on the welfare and productivity of the dairy cow. The high prevalence of these pathogens in the animal feed industry illustrates the importance of alternative natural strategies to prevent infection. In this study, inhibition of pathogenic microorganisms of agricultural and industrial importance was reported. Fungal extracts demonstrated activity against pathogenic microorganisms associated with negative implications to the agriculture industry (*Listeria monocytogenes*, *Pasteurella multicida* and *Streptococcus uberis*) as well as, nosocomial infections (*Staphylococcus epidermidis*, *Staphylococcus aureus*, *Citrinobacter freundii* and *Serratia marcescens*). Research relating to the antibacterial activity of compounds against these pathogens are of particular significance as many of these microorganisms are problematic to both animal and human health (Alves *et al.*, 2012). Results can be considered as promising, in view of the development of new mycodrugs for the fight against microbial infections. Application of antibacterial properties, with low cytotoxicity, underscores the usefulness of natural sources, particularly fungi for new medicines (Tamokou *et al.*, 2009).

The extraction method proved critical for the recovery of antioxidant and bioactive metabolites. It is essential that the nature of bioactive components should be considered in order to achieve good extraction efficiency (Tsao *et al.*, 2004). The

solubility of the active constituent in the extraction solvent and the polarity of the solvent were affected by lipophilicity or hydrophilicity. In this investigation active constituents were extracted *via* hot water and methanol extraction. Hot water extraction generally isolates compounds of high molecular weight such as polysaccharides and low molecular weight compounds such as phenols (Vaz *et al.*, 2011). When performing natural compound extraction using polar organic solvents such as methanol; flavonoids, alkaloids, coumarins, fatty acids and triterpenes are commonly extracted (Cowan, 1999). In addition, anthocyanins, starches, tannins, saponins, terpenoids, polypeptides and lectins, are known to exert bioactivity following component extraction with water alone (Kaul *et al.*, 1985; Cowan, 1999). The crude mycelial aqueous suspension of biomass demonstrated strong antibacterial activity against a range of bacteria, using agar well diffusion. In addition, the crude extracts from all ten filamentous fungal species demonstrated the ability to efficiently adhere to a range of bacteria *in vitro*. Generally, the crude, hot water and methanol extracts from each species demonstrated differences in their antioxidant activity. The data indicated that different compounds were likely to have been extracted following different extraction procedures and these compounds have different affinities for different oxidative processes, most likely due to their polarity. According to the data obtained, phenols, flavonoids and condensed tannin were found to be effective antioxidants in different *in vitro* assays including reducing power, $ABTS^+$ radical scavenging and metal chelating activities when compared to standard antioxidant compounds such as BHT, Trolox, EDTA and α-tocopherol. The fatty acid composition of each fungus was also implicated by the extraction process, with *Monascus purpureus* containing the highest fatty acid concentration of the species which were tested. Lipid content of each species was mainly composed of unsaturated fatty acids, particularly linoleic acid. TLC and bioautography demonstrated each species of fungi to contain a diverse number of isolated components and was highly active towards both gram-positive and gram-negative bacteria. The data suggested the formation and production of bioactive metabolites such as fatty acids, phenolic metabolites, alkaloids, terpenoids, flavonoids and anthraquinones present in the mycelium, under optimised cultivation conditions for optimal yield. Antibacterial activity was associated with both polar and less-polar compounds within each species as a result of separation and identification of active isolates using bioautographic analysis. However, according to the phenolic profile generated following solid phase

extraction and UPLC, this species contained the least quantity of phenolic constituents compared to the other filamentous fungi. Although phenolic content was positively attributed to antioxidant activity, association of biological activity due to either phenols or lipids was difficult. In addition, analysis using universal and specific detection reagents suggested the presence of lipid, terpenoid or phenolic constituents, or a combination thereof. In conjunction with bioautography and LC/MS analysis, evidence suggested that a degree of biological activity may have been strongly associated with the fatty acid content of the separated components. Individual isolates which indicated a strong presence of fatty acid components also demonstrated a positive reaction to phenolic or flavonoid constituents. Thereby, a combination of chromatographic analysis with a bioassay *in situ* revealed that biological activity was most likely correlated with a combined action of multiple constituents of different polyphenols, flavonoids, phenolic acids and/or lipids occurring in each of the various species of fungi investigated. These may have worked synergistically or additively. An antioxidant compound from fungi which demonstrates strong antimicrobial efficiency in this instance is an attractive alternative source for food preservation as well as a natural functional food ingredient.

The goal of functional foods, nutraceuticals and dietary supplements is to improve quality of life, enhance health status and possibly increase life-span, while maintaining overall health. The information gathered in the present study is considered useful for the further development of selected fungal cultivation processes on an industrial scale, particularly for enhanced bioactive metabolite production. Altogether, components from ten species of filamentous fungi were shown to possess strong antimicrobial and antioxidant activity. The findings suggest that there is a potential to use fungal components from the species analysed, to act as antioxidants and antimicrobials in feed, which would enhance their quality and nutritive value. UPLC analysis revealed that the selected filamentous fungi produced different quantities of antioxidant phenolic compounds. In addition, their antimicrobial properties would have promising applications in enhancing food safety. There is great potential of the established bioprocess, including the extraction techniques, to be used for the purification of active components. In addition, there is evidence to suggest, that by using different growth conditions, the metabolic profile of the active components may be improved.

Results from this investigation demonstrate that fungal secondary metabolites constitute a wide variety of compounds which have the potential in agricultural, pharmaceutical and industrial contexts, which is an advantage from a fermentation perspective as process residue is reduced. Uses include food colourings, preservatives or feed additives as a source of lipids, antimicrobial and antioxidant secondary metabolites, as well as other metabolites such as, polysaccharides. Nonetheless, there is still much to learn about antioxidant action *in vivo*, including synergistic and inhibitory roles, uptake, biotransformation, and tissue distribution (Becker *et al.*, 2004). In recent decades, research has shown that potentiation (or antagonism) provided by multiple pharmacological targets converging in parallel biochemical pathways may be overlooked. Ultimately, effective research into the mechanisms of action of phytomedicines will need to account for the possibility, indeed the probability, of synergistic activity between multiple constituents (Cseke *et al.*, 2006).

The data demonstrated that bioactive metabolites from several filamentous fungi encompass the ability to be produced industrially by submerged cultivation and revealed it is possible to enhance phenolic and lipid profile when growing fungi, through modified cultivation conditions, thus influencing nutritional value. High extract yield previously obtained from culture filtrate using submerged liquid fermentation is believed to be due to the large amount of small, water-soluble components (Lee *et al.*, 2007a). In addition, studies have shown that Basidiomycetes have been cultivated using different substrates and/or waste mixtures (Chang, 2001; Akyuz *et al.*, 2009; Philippoussis *et al.*, 2011). This may be a strong source of further analysis, further minimising process residue waste. Therefore, bioactive component production on defined nutrient media such as industrial wastes demonstrates commercial efficiency of production and cultivation (Lee *et al.*, 2007a). Further scope for investigation, may be the examination of industrial wastes as a growth substrate or media, and analysis of bioactive metabolite production in biomass and culture fluid of liquid cultivations. Success on a commercial scale depends on the cost compared with existing technology and economic advantages (Tang *et al.*, 2007). Nonetheless, submerged liquid fermentation has been reported as the most efficient method for the commercial production of consistent and safe natural products (Wasser *et al.*, 2000; Reshetnikov *et al.*, 2001; Lull *et al.*, 2005; Papaspyridi *et al.*, 2012). The crude biomass extract from *M. purpureus*, *P. ostreatus*, *P. ostreatus* 1833, *P. ostreatus* 32783, *P. citrinopileatus*, *P. eryngii*, *P. salmoneo-stramineus*, *G. frondosa*, *L. edodes*

and *T. versicolor*, as well as isolated components from fractionated extracts were revealed as a source of development for antimicrobials.

Due to complexity of extracts from natural sources it can be difficult to separate active constituents of interest, as was observed in this study. Multiple bioactive compounds, which are closely related, pose difficulties for effective separation and can possibly impede isolation procedures. In the present investigation, the biological activity was concentrated in particular fractions. Bioactive metabolites are typically generated in small quantities and production depends on the species and growth parameters. Thereby, enhancement of these factors is essential for maximum quantity and purity of secondary metabolite and mycelial biomass cultivation. Additionally, the results of this study have demonstrated the significance of suitable extraction methods in the downstream isolation of active compounds.

Prior to purification or analysis, several sample preparations, pre-purification and clean-up extraction methods such as filtration, precipitation or thin layer chromatography techniques may be applied. Likewise consideration of purification steps as well as, downstream processing post-fermentation is essential. For example, depending on the polysaccharide composition (i.e. molecular weight, branching degree and pattern of branches) they may be further purified using various techniques or a combination thereof, such as ethanol precipitation, fractional precipitation, ion-exchange chromatography, gel filtration, and affinity chromatography. Following successful separation of extracted components based on differences in their physicochemical properties. This in turn leads to bioactive compound isolation, in conjunction with pure compound screening, structural characterisation of the bioactive components and pharmacological and toxicological testing. The next stage of analysis usually through biological testing in cell-based assays would be to apply the extracts to clinical studies demonstrating antioxidant uptake, protection and evidence of downstream events in suitable cell models. Following isolation and identification of active compounds, suitable pharmaceutical delivery systems could be explored. With emphasis on optimal preparation and delivery of low concentrated extracts. The efficiency of any resulting treatment regimen should subsequently be proven with well-designed randomised control trials (Hearst *et al.*, 2009). Although the present study has investigated potential uses of fungal components from a natural product perspective, further research into the production, purification and optimisation of these

biologically active components is required to fully realise the potential these active components have to offer.

In conclusion, the findings of this study are demonstrated as valuable. The established fermentation process led to the production of bioactive metabolites of fungal origin, produced by species of which there is a lack of documented research. Active metabolites were produced under optimised conditions for efficient biomass production. Submerged fermentation of the mycelium proved to be a promising alternative for the production of bioactive and nutritional functional compounds, from a commercial aspect. This method of cultivation is now gaining more interest in the food and drug industry, in comparison to the established field cultivation of fruit bodies (Papaspyridi *et al.*, 2012). Natural products, either isolated as pure products or used as standardised fungal extracts, provide limitless opportunities for novel additives and drug treatments because of their unmatched range of biochemical diversity. Fungal extracts demonstrated *in vitro* antioxidant and antimicrobial activities, and has provided information which could lead to further research in the area of isolation and characterisation of active biochemical components.

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