The role of nitrogen sources and caffeine for growth of *Pleurotus ostreatus* (oyster mushroom)

By

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Originality Statement

This thesis is submitted to the University of Sydney in fulfilment of the requirement for the Doctor of Philosophy.

The work presented in the thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

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Presentations

Part of the research in this thesis has been presented in the following events:

- Poster at the Symposium Faculty of Agriculture and Environment, The University of Sydney, July 2014.
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Abstract

Waste products from coffee production are useful substrates for cultivation of *Pleurotus ostreatus* in coffee-producing countries as this species is relatively easy to grow, substrates are readily available and the mushrooms (or fruiting bodies) are a valuable source of nutrition and income. In developed countries, cultivation *P. ostreatus* on spent coffee grounds (SCG) from coffee consumption is a novel way to use this form of waste product in urban areas. It is not yet known if high mushroom yields can be achieved by growing *P. ostreatus* on SCG or if the caffeine content of residues can be substantially reduced by growing mushrooms for safe disposal of coffee waste. In addition, when mushrooms are grown on SCG it is not known how suitable they are for human consumption in terms of caffeine content. Thus, there is a need to understand the role of caffeine during the cultivation of oyster mushroom on SCG as caffeine utilization by this fungus has not yet been described.

The study presented here provides key information related to the fate of caffeine during cultivation of *P. ostreatus* on SCG. A wide range of nitrogen (N) sources (including caffeine) and extracts from fresh and spent coffee grounds were evaluated for their ability to support mycelial growth on solid agar and in liquid culture. Good biomass production was measured for a range of N forms, with inorganic N being the best source for growth. Caffeine also promoted good mycelial growth but only when provided in low concentrations. At higher concentrations, growth was inhibited.

Pleurotus ostreatus was cultivated on SCG-amended substrate to evaluate the effect of caffeine during the vegetative and reproductive growth stages. In two trials, *P. ostreatus* was grown in treatments ranging from pure SCG (SCG100) through to pure sawdust (sawdust100) and with mixtures of these substrates (i.e. SCG75+sawdust25, SCG50+sawdust50 and SCG25+sawdust75). During the vegetative phase of the laboratory-scale study, three of the four treatments became fully colonized (SCG100, SCG25+sawdust75 and SCG50+sawdust50) and of these treatments, only SCG100 and SCG25+sawdust75 developed mushrooms. Caffeine degradation by *P. ostreatus* occurred when grown on SCG (with and without sawdust) with detection of caffeine and its degradation products in the substrate and fruiting bodies. In a commercial-scale study, full colonization was observed for SCG25+sawdust75 and sawdust100 (control) and SCG50+sawdust50 was almost fully colonized, but fruiting bodies only developed on SCG25+sawdust75 and the control. Again, caffeine degradation occurred when *P. ostreatus* was grown on treatments including SCG and a decrease in caffeine content of the SCG was found. All of the compounds that have been previously described for fungal degradation of caffeine (xanthine, 7-methylxanthine, 3-methylxanthine, 1-methylxanthine, theobromine, paraxanthine, theophylline and caffeine) were detected and identified and a likely degradation pathway was suggested.

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List of Abbreviations

Ade – adenine

Ala – alanine

AM – arbuscular mycorrhizal

AmChl – ammonium chloride

AmNit – ammonium nitrate

AmTar – ammonium tartrate

ANOVA – analysis of variance

BE – biological efficiency

Caff – caffeine

CaNit – calcium nitrate

CDM – chemically-defined medium

DA – dextrose agar

ECM – ectomycorrhizal

FCG – fresh coffee grounds

FCG(A) – fresh coffee grounds (agar)

Gua – guanine

HPLC – high-performance liquid chromatography

IN– inorganic nitrogen

MEM – minimum essential medium

MM – minimal medium

ON – organic nitrogen

PAHs – polycyclic aromatic hydrocarbons

PDA – potato-dextrose agar

Phe–phenylalanine

RT – retention time

Saw – sawdust

SCG – spent coffee grounds

SCG(A) – spent coffee grounds (agar)

SMS – spent mushroom substrate

SOM – soil organic matter

TNT – trinitrotoluene

Trp –tryptophan

Tyr – tyrosine

UV – ultraviolet

WA – water agar

WDM – wood decomposer medium

YE – yeast extract

Chapter 1 The contrasting roles of naturally-occurring and cultivated fungi

1.1 Introduction

Fungi are ubiquitous organisms – we interact with them directly and indirectly on a daily basis. They are present in our natural and managed surroundings and the products that are derived from fungi or the processes in which fungi are involved are important for the functioning of modern human communities. This chapter firstly reviews the current knowledge about the roles of fungi in natural ecosystems (*in natura*). An understanding of the function of fungi in natural and managed systems will be used to interpret how and why certain fungi have been taken into cultivation. Secondly, the biology of cultivated fungi is investigated with the intentional growth of fungi to produce food, medicine and other products. Fungi may be grown under ambient conditions in limited- to moderately-managed forest or agriculture systems, for example, for the production of truffles or 'wild' harvested mushrooms. In other situations, fungi may be cultivated in highly controlled conditions for production of compounds for use in medicine or industry. Information from fungi growing in natural ecosystems and in cultivation can be used to inform studies done in the laboratory (*in vivo*). The review will finish with a discussion of *Pleurotus*, the genus of fungi which is the focus of the research component of this thesis.

1.2 Fungi in natural and managed ecosystems

It has been estimated that there are 1.5 million species of fungi worldwide (Blackwell 2011; Hawksworth 1991; 2001; Mueller & Schmidt 2007). Fungi can inhabit most types of

environments and climatic conditions and display a huge diversity in lifestyles. Fungi can interact with organisms in a number of ways ranging from commensalism and mutualism through to parasitism (Brundrett 2004). As heterotrophic organisms, they do not photosynthesize and must obtain carbon, nutrients and energy from external sources. Some strategies that have been developed by fungi to gain access to carbon include saprotrophism (Osono 2015), parasitism (Joop & Vilcinskas 2016), and mycorrhizal associations (Perotto *et al*. 2013). In natural and managed ecosystems, fungi have a key ecological role in nutrient cycling as decomposers (Perotto *et al*. 2013). Together with other soil microorganisms, fungi are responsible for converting carbon and nutrients held within dead material into forms that are more accessible to plants via decomposition (Azcon-Aguilar & Barea 2015; Lindahl & Tunlid 2015).

Saprotrophic fungi are critical in ecosystems as they 'feed' by absorption and acquire carbon and nutrients from dead organisms (e.g. wood and leaves of plants, animal bodies). As a result of enzymatic activity and death of their own biomass, fungi release compounds into the soil and make them available for use by other organisms. Saprotrophic fungi may be primary, secondary or tertiary decomposers according to the nature of the material they degrade (Osono 2015). Primary decomposers colonize wood, leaves and other carbon-containing substrates that have not been modified by other decomposing organisms. These fungi can access and metabolize large complex molecules (i.e. lignin, hemicellulose, polysaccharides) into forms of carbon that can be absorbed via hyphae (i.e. glucose) (Chang & Miles 2004). Such substrates usually have a relatively high carbon:nitrogen (C:N) ratio (e.g. plant litter has a C:N ratio of 71:1 (Mooshammer *et al*. (2014)). Secondary

decomposers take advantage of primary decomposer activity and access substrates with altered C:N ratios. Tertiary decomposers are involved towards the end of the decomposition process and use highly modified substrates such as decomposed litter and soil organic matter (SOM) with low C:N ratios that other decomposers cannot access (e.g. SOM has a C:N ratio of 17:1 (Mooshammer *et al*. (2014)). Thus, saprotrophic fungi are responsible for relocation of carbon and nutrients in the environment through different phases of decomposition (Gadd 2004).

There are many different types of saprotrophic fungi in natural ecosystems but only a small proportion are used in agricultural or production settings. For example, in nature many edible mushrooms are primary decomposers (i.e. several species from the genus *Pleurotus, Grifola frondosa*, *Lentinula edodes*, *Stropharia rugosoannulata*) (Stamets 2004). Other species, such as *Agaricus bisporus*, are secondary decomposers that can only access substrates after some degree of degradation has occurred, usually through composting (Kertesz & Thai 2018). Tertiary decomposers generally have limited potential for cultivation as they are typically very slow-growing. There are a few exceptions including some edible species of *Agaricus* and *Agrocybe* (Rahi *et al*. 2009; Stamets 2004).

Parasitic fungi live at the expense of their host. Depending on the degree of dependence by the host, parasites can be obligate or facultative. Obligate parasitic fungi obtain all of their nutrients from their host, while facultative fungi tend to obtain carbon, energy and nutrients from their hosts but can also fulfil their nutritional requirements through saprotrophic mode (Chang & Miles 2004; Ţura *et al.* 2016; Wrzosek *et al*. 2017). For example, the edible species, *Armillaria mellea*, is a parasitic fungus while its host is living but

can become saprotrophic if the host plant dies (Baumgartner *et al*. 2011). As can be expected from the nature of parasitic fungi, much research and management effort has been directed towards elimination of this type of fungi in an agricultural setting (e.g. Chakraborty *et al*. 1998; Palm 2001).

Fungi form mutualistic associations with organisms from most plant taxonomic groups (Blackwell 2011). Mutualism is a type of symbiosis where both organisms obtain some benefit. Lichens and mycorrhizal associations with plant roots are both mutualistic relationships. Lichens are an association between a unicellular green or cyanobacteria and a fungus and mycorrhizal associations form between higher plant roots and fungi. In both cases, the fungus acquires and supplies minerals and water to the host, while the plant or algae provides carbon via photosynthesis (Kendrick 2000; Smith & Read 2008). Arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) associations represent the two main types of fungal-plant associations. Many edible mushrooms are ECM fungi including species from the genera *Boletus*, *Lactarius*, *Suillus* and *Tuber* (Boa 2012). Arbuscular mycorrhizas are the most common associations between fungi and plants, forming partnerships with approximately 80% (at species level) and 92% (at family level) of plants (Blackwell 2011). When hyphae of AM fungi come in contact with roots, they penetrate outer epidermal cells of the root and grow into cortical cells where structures (arbuscules) suitable for absorption develop. In contrast, when hyphae of ECM fungi encounter new roots, they penetrate around the root and between cortical cells and develop a structure called a Hartig net (Smith & Read 2008).

As well as being involved in carbon cycling, fungi are also involved in mineral nutrient cycling in natural and managed systems. Nitrogen and phosphorous are mobilized by AM and ECM

fungi and are moved from soil to plants – an activity that is essential to sustain ecosystems (Courty *et al*. 2010; Miransari 2011). In forests, SOM is composed of molecules ranging in complexity. When SOM is degraded by AM and ECM fungi through secretion of extracellular enzymes, associated nutrients become available for use by other soil organisms (Ballhausen & de Boer 2016). Ectomycorrhizal fungi can utilize a variety of nitrogen-containing compounds (Itoo & Reshi 2014). Mineral dissolution, a complex process in which elements in mineral particles are released by solubilization into the soil, is also a very important contribution by mycorrhizal fungi (Gadd 2004; 2017). Another significant contribution from AM and ECM fungi is carbon translocation by their hyphal connection with other mycorrhizal fungi linking plants by forming mycorrhizal networks belowground (Courty *et al*. 2010).

Endophytic and epiphytic fungi associated with surfaces of living leaves are collectively referred to as phyllosphere fungi (Osono 2006; Whipps *et al*. 2008). In some instances, fungi isolated from surfaces of dead leaves that are in early stages of decomposition are also referred to as phyllosphere fungi (Hudson 1968; Osono *et al*. 2004) and, in these cases, are thought to be an important component of organic matter decomposition due to their capacity to release carbon, nitrogen and phosphorous (Saikkonen *et al*. 2015). These fungi are only part of the early stages of decomposition and their occurrence diminishes as the decomposition process proceeds (Osono 2006).

Fungi are also a source of food for many organisms ranging from bacteria through to animals. Many organisms feed directly on fruiting bodies. These include insects such as the fungus gnat and slugs (Keller & Snell 2002; Komonen 2003); nematodes (Ferris & Matute

2003), animals such as potoroos and squirrels (Johnson 1996; Trierveiler-Pereira *et al*. 2016; Vernes *et al*. 2015). Other organisms feed on fungal hyphae in the soil or woody substrates (e.g. nematodes, bacteria; Ballhausen & de Boer 2016). Bacteria have developed several strategies to obtain carbon and nutrients from fungi. For example, soil bacteria stimulate fungi to secrete metabolites from which they obtain nutrients, while endocellular bacteria absorb nutrients directly from fungal cytoplasm (Leveau & Preston 2008). Some fungal parasites such as *Trichoderma* may secrete enzymes to degrade fungal cell walls (Harman *et al*. 2004).

Soil can be greatly influenced by the presence of fungi. Fungi are integral for adding carbon to soil which can influence the composition of the plant community and, therefore, net primary production (Gadd 2004). Fungal hyphae can influence soil structure by binding soil particles and contributing to soil organic matter (Rillig & Mummey 2006). Fungi also have a synergistic effect on the interaction of plant roots with soil which include enhancing physical contact, improving water availability, stimulating release of compounds by roots to the soil and facilitating decomposition of roots (Rillig & Mummey 2006).

1.3 Exploitation of fungi

1.3.1 Fungi as a food source

For edible fungi, the part of the fungus that receives the greatest attention as a food source is the fruiting body, or mushroom. This structure represents the reproductive phase of the fungus. Reproduction takes place once the mycelium reaches a suitable state, which depends on genetic background and environmental conditions. The vegetative stage of the

fungus is composed of the mycelium – thread-like structures that grow through substrates including soil, compost and decayed wood (Chang 2009).

Fungi provide numerous benefits to humans. One important attribute is as a food source as they are rich in proteins, amino acids and vitamins (Chang 1996; Manzi *et al*. 1999) and fibre (Synytsya *et al*. 2008) and they contain essential microelements (Chang & Wasser 2012). Many of these compounds are needed by humans for general wellbeing and some are essential for prevention of disease (Chang & Miles 2004). Fungi can be sourced from naturally growing stocks (Arnolds 1995; Boa 2004) or, more readily, from cultivation. A few species of fungi are easy to cultivate (e.g. Oyster mushroom (*Pleurotus* spp.) and Button mushroom (*Agaricus bisporus*)) (Kurtzman Jr. 2010; Sánchez 2010) whereas other species, such as Burgundy truffles (*Tuber aestivum*), are difficult to cultivate (Stobbe *et al.* 2013) and are harvested from protected and managed forests. The commercial morel industry relies on wild harvest (Pilz *et al*. 2007) as morels are notoriously difficult to cultivate (Liu *et al*. 2018; Masaphy 2010). Estimates of economic return from wild harvested mushrooms are hard to make and they have different social impacts in different countries (Boa 2004; 2012).

It has been estimated that there are 7000 species of mushrooms with different degrees of edibility; 3000 species have been recorded as being edible, 200 species have been grown experimentally, 100 species are economically cultivated, 60 species have been commercially cultivated and only 10 species are cultivated on industrial scale (Chang & Miles 2004). It has been estimated that a total of more than 10 million tonnes of mushrooms were produced and harvested worldwide in 2014, with almost 70% grown in Asian countries and 23% in Europe (Food and Agriculture Organization 2018). Large-scale cultivation includes, in order

of world production, Shiitake (*Lentinula edodes*), Oyster mushroom (*Pleurotus* spp.), Wood-ear mushroom (*Auricularia*), Button mushroom (*Agaricus bisporus*) and *Flammulina velutipes* (Royse *et al*. 2017). Other fungal species cultivated on relatively smaller scales and for more localized markets include Black jelly fungus (*Auricularia polytricha*) (Irawati *et al*. 2012) and Wood blewit (*Clitocybe nuda*).

Fungi have a significant position in the manufactured food and beverage industry, with products including bread, cheese, soy sauce and fermented alcoholic drinks (Vaughan *et al.* 2005). The activity of yeasts makes wine and beer production possible, whereas other fungal interactions provide flavour and quality to food (Fleet 2003; Vaughan *et al.* 2005).

Edible fungi occur in two major taxonomic groups. The phylum Basidiomycota contains what is traditionally considered to be mushrooms, bracket fungi and boletes, while the Ascomycota includes truffles (*Tuber* spp.) and morels (*Morchella* spp.), but also yeasts. Basidiomycetes are filamentous fungi which reproduce sexually from haploid basidiospores formed in large multicellular fruiting bodies or mushrooms. Ascomycetes or sac fungi are filamentous fungi characterized by having an 'ascus', a sexual structure in which haploid ascospores are formed. Most asci are microscopic but in the case of truffles and morels, they are large fruiting bodies (Money 2016). Edible representatives of the Basidiomycota are either saprotrophic or mycorrhizal while edible Ascomycetes can be mycorrhizal (Chang & Miles 2004).

1.3.2 Fungi as medicine

Mushrooms have been consumed for hundreds of years due to their flavour and nutritional value but also for medicinal purposes, particularly in traditional oriental medicine (Ţura *et*

al. 2016). The term “mushroom nutraceutical” is a general term that refers to compounds that can be extracted from fruiting bodies and mycelium or from the culture supernatant and have a significant role in the industry of mushroom biotechnology (Carrasco-González *et al*. 2017; Chang & Buswell 1996). There is evidence to support the claim that consumption of mushroom nutriceuticals can improve health (Carrasco-González *et al*. 2017; Chang 2009; Chang & Wasser 2012; Ţura *et al.* 2016).

Secondary metabolites are compounds produced by living organisms that are not directly required for processes such as growth (Brakhage 2013; Fox & Howlett 2008; Frisvad *et al*. 2008). One of the main roles of secondary metabolites in fungi is for chemical communication and for defence from competitors and predators (Brakhage 2013; Fox & Howlett 2008; Frisvad *et al*. 2008). Extensive screening of secondary metabolites in fungi for their medicinal properties has been done since the discovery of penicillin in 1929 by Alexander Fleming (Moore & Chiu 2001). Active compounds have been found in fruiting bodies, cultured mycelia and supernatant (Mao *et al*. 2013; Ooi 2008; Wasser 2014). The industrial cultivation of fungi for medicinal products represents a particularly important alternative source of antimicrobials and compounds of interest have been obtained from many different fungi and by different means (Table 1.1). Reviews describing bioactive compounds in fungi are plentiful (e.g. Alves *et al*. 2013; Amirullaha *et al*. 2018; Bożena *et al*. 2018; Carrasco-González *et al*. 2017, He *et al*. 2017a, b; Jayachandran *et al*. 2017; Wang *et al*. 2017; Zhang *et al*. 2017; Zong *et al*. 2012).

An extraordinary amount of research has been done to investigate the usefulness of fungi in human health (Table 1.1). One of the greatest threats to human health is cancer and the

search for biologically-active compounds that could inhibit carcinogenic cells has included a range of fungi. Two potential modes of action have been identified in clinical trials: (1) direct toxicity to cancer cells and (2) indirect inhibition of the tumour through signals sent by the immune system (Ooi 2008).

Table 1.1 Recent research (since 2010) describing the use of fungal extracts obtained from mushrooms collected from natural habitats, commercially cultivated or grown in the laboratory with possible applications for human health.

Biological effect	Representative genera	References
Antimicrobial: inhibits pathogenic bacteria or fungal growth	*Anthracophyllum*, *Armillaria*, *Coprinus*, *Cordyceps*, *Grifola*, *Ganoderma*, *Lactarius*, *Hypholoma*, *Lentinula*, *Marasmius*, *Marasmiellus*, *Mycena, Pleurotus*, *Serpula*, *Stereum*, *Tephrocybe*, *Xerocomus*	Aqueveque *et al.* 2010; 2016; De Brujin *et al*. 2009; Casaril *et al.* 2011; Coleman *et al.* 2011; Nowacka *et al.* 2014; Smolskaitė *et al.* 2015; Vamanu 2012
Antiviral	*Lentinula*	Rincão *et al*. 2012;
Antioxidant: protect and prevent oxidative damage as a result of cellular metabolism	*Agaricus*, *Auricularia*, *Boletus*, *Flammulina*, *Ganoderma*, *Hypsizygus*, *Lentinula*, *Lentinus*, *Pleurotus, Suillus, Thelephora, Volvariella*	Anandhi *et al.* 2013; Alam *et al.* 2012; Carneiro *et al.* 2013; Chen *et al.* 2012; Ding *et al.* 2012; Dundar *et al.* 2013; Finimundy *et al.* 2013; Guo *et al.* 2012; He *et al.* 2012; Koutrotsios *et al*. 2017; Kozarski *et al.* 2012; Li & Shah 2016; Liu *et al.* 2013; Mao *et al.* 2013; Mishra *et al.* 2013; Patra *et al.* 2013; Reis *et al.* 2012; Tian *et al.* 2012; Vamanu 2012; Wang *et al.* 2013; Woldegiorgis *et al.* 2014
Inhibition of proliferation of cancer cells	*Lentinula*, *Pleurotus*	Finimundy *et al.* 2013; Yan & Chang 2012
Anti-inflammatory	*Craterellus*, *Lentinus*, *Pleurotus*	Gunawardena *et al*. 2014; Moro *et al*. 2012; Silveira *et al*. 2015
Hypoglycemic: reduces sugar levels in blood	*Agaricus*	De Silva *et al.* 2012; Jeong *et al.* 2010; Ravi *et al.* 2013
Hypocholesterolemic	*Agaricus*, *Pleurotus*	Anandhi *et al.* 2013; Jeong *et al.* 2010
Immunomodulation and immunostimulation	*Cordyceps*, *Ganoderma*, *Pleurotus*	Devi *et al.* 2013; Smiderle *et al.* 2013; Wang *et al.* 2013

1.3.3 Fungi in recycling – utilization of agro-industrial residues

Mushroom cultivation is the conversion of residual organic matter and lignocellulosic materials into a source of food (Sánchez 2004). This practice takes advantage of the role of fungi as a decomposer in natural ecosystems. Humans produce an enormous amount of lignocellulosic waste through agriculture and other practices that cannot be used directly for human consumption. Mushroom cultivation is considered to be an efficient alternative for recycling of agricultural wastes providing profit from low cost materials (Bisaria *et al.* 1997; Philippoussis 2009). Apart from providing a nutritious food supply from relatively cheap materials, there is great interest from an agricultural-waste recycling perspective (Philippoussis *et al*. 2001; Josephine 2014).

Mushrooms can be cultivated on more than 200 types of substrates (Poppe 2000). This is because fungi have the ability to produce a range of enzymes for biodegradation and assimilation of a multitude of organic substrates (Baldrian 2011; Elisashvili *et al.* 2008 Velázquez-Cedeño *et al.* 2002). For example, *Lentinula edodes*, *Pleurotus ostreatus* and *Ganoderma lucidum* have all been grown on vineyard and winery residues (Petre *et al.* 2012; Sánchez *et al.* 2002), on by-products from extraction of palm oil (Abd Razak *et al.* 2013; Neoh *et al*. 2015; Rahman *et al*. 2011), residues from olive oil production (Zervakis *et al.* 1996) and on remains from other agricultural products such tomato, sweet potato and basil (Nyochembeng *et al.* 2008).

Raw substrates for mushroom cultivation are primarily organic matter originating from agricultural or forestry practices (Kurtzman Jr. 2010). These can be wood, plant stems,

leaves, sawdust, crop straw or husks, cotton and coffee wastes (See Table 1.2). Some fungi (primary decomposers) can be cultivated on these substrates directly, however, when these materials need specific treatment such as composting before use as a substrate (i.e. for secondary decomposers), then mushroom cultivation will not only depend on substrate availability, but also on its ease of preparation (Stamets 2000). Substrates can be prepared in many ways including sterilization and pasteurization, fermentation and composting (Oseni *et al.* 2012).

The capacity of fungi to colonize waste substrates and to develop fruiting bodies depends on the activity of specific enzymes and on the composition of the substrate (Elisashvili *et al*. 2008). Wood, for example, is composed of lignin, cellulose and hemicelluloses and different fungal species have developed enzymatic pathways that allow degradation of wood and use as a carbon source (Ţura *et al.* 2016). For example, brown rot fungi are able to degrade cellulose and hemicelluloses and can modify lignin with demethylation reactions, but it is not able to degrade it (Arantes & Goodell 2014). White rot fungi can degrade lignin enzymatically via laccase, manganese peroxidase and lignin peroxidase (Elisashvili & Kachlishvili 2009; Knop *et al*. 2015). Enzyme production, including the type and amount of enzymes produced, varies throughout the life cycle of the crop during commercial production of *Pleurotus* spp. (Elisashvili *et al*. 2008; Salmones *et al*. 2005).

There is an emerging industry for recycling of 'spent' or used substrate once mushroom production has finished (Sánchez 2010). Spent mushroom substrates (SMS) have been evaluated and recommended as a means of decreasing the impact of pathogens in vegetable production (Parada *et al.* 2012). It can be also used as substrate for cultivation of

other mushroom species (Gea *et al.* 2012; Mamiro & Royse 2008; Wang *et al.* 2015), as an animal food supply (Sanchez *et al.* 2002), soil fertilizer (Peregrina *et al*. 2012; Ribas *et al*. 2009), for bioremediation of fungicides and pesticides (Ahlawat *et al.* 2010; Marín-Benito *et al.* 2012) and as a source of energy (i.e. for production of biofuel) (Balan *et al.* 2008).

Biological efficiency (BE) is a term used in the mushroom industry to describe mushroom yield from various agricultural by-products. It is the relationship between the amount of dry substrate used and the fresh weight of mushroom produced (Stamets 2000). If the weight of the substrate used equals the weight of the mushrooms produced, then a BE of 100% has been achieved. Biological efficiencies as high as 115% have been achieved by *Agaricus bisporus* under certain growth conditions (Gea e*t al.* 2012). Species of *Pleurotus* vary considerably in their BE values depending upon substrate and conditions of growth (Table 1.2). This metric can allow useful comparisons among fungal species and strains, substrates and growing conditions.

Table 1.2 Indicative examples (since 2000) of agricultural and industrial residues used as substrates for *Pleurotus* cultivation, with the respective biological efficiency (BE) obtained. Biological efficiency is calculated as the ratio of fresh mushroom weight produced compared to the dry weight of the substrate used (Stamets 2000).

Species	Substrate (BE (%))	Reference
P. ostreatus	Paddy straw (83%), sesame straw (91%), viticulture waste (73%), cotton seed (74%), coffee pulp (125%), wheat cotton waste (116%), sunflower seed hull (112%), spent brewery grains (51%), wheat straw (59, 78, 98, 99%), straw, tree leaves (118%), olive mill waste (126%), tea waste (83%), paper (112%), cardboard (117%)	Curvetto *et al*. 2002; Elisashvili *et al*. 2008; Girmay *et al*. 2016; Gregori *et al*. 2007; Kurt & Buyukalaca 2010; Mandeel *et al*. 2005; Philippoussis *et al*. 2001; Velázquez-Cedeño *et al*. 2002; Yang *et al.* 2016; Zervakis *et al*. 2013
"P. saju-caju"	Paddy straw (75%), rice straw (138%)	Kurt & Buyukalaca 2010; Zhang *et al*. 2002
P. pulmonarius	Coffee pulp (138%), cotton waste (97%), wheat straw (123%)	Philippoussis *et al*. 2001; Velázquez-Cedeño *et al*. 2002
"P. florida"	Bean straw (89%), rice straw (90%), wheat straw (63%), paddy straw (125%)	Figueiró & Graciolli 2011; Sivagurunathan & Sivasankari 2015
P. eryngii	Wheat straw (89%)	Philippoussis *et al*. 2001
P. columbinus	Paper (101%), cardboard (135%)	Mandeel *et al*. 2005

1.3.4 Fungi in industry

Fungi are recognised as important biodegradation agents. Wood-inhabiting or white-rot fungi possess well-developed enzymatic pathways making them capable of metabolizing highly toxic compounds, particularly those produced during certain industrial processes (see review by Cameron *et al*., 2000). For example, the fungus *Phanerochaete chrysosporium* has been widely studied as degrader of certain compounds such as dyes from the textile industry and chlorinated compounds from the paper industry (Paszczynski & Crawford 1995). A diverse group of fungi can degrade polycyclic aromatic hydrocarbons (see review

by Cerniglia 1997). Species belonging to the fungal genera *Nematoloma*, *Pleurotus* and *Trametes* are capable of mineralization of several chloro-aromatic compounds as well as polycyclic aromatic hydrocarbons (PAHs) and trinitrotoluene (TNT) (Harms *et al*. 2011). Some of the mechanisms of degradation have been analyzed in detail and it has been concluded that a number of them can be explained by the low specificity of the fungal enzymes, which attack a broad range of substrates (Harms *et al*. 2011).

Mycotechnology is a term used to describe the use of fungi in biotechnology (Bennett 1998). *Mucor* is a genus comprising many well-studied species of fungi and several industrial applications make this fungus an extremely resource-rich organism. For example, *Mucor* is capable of producing ethanol from different carbon sources (Karimi & Zamani 2013; Lübbehüsen *et al*. 2004). In addition, *Mucor* is used for production of chitosan and polyunsaturated fatty acids, fermented foods for human consumption and as feed for fish cultivation (Arroyo *et al*. 2016; Rodriguez et al. 2004). In addition to these industrial applications, *Mucor* can also be used for treatment of wastewater and absorption of heavy metals (Karimi & Zamami 2013). The productivity and capabilities of species in this genus depend on the composition of the medium and morphology of the strain used (Morin-Sardin *et al*. 2017). The use of fungal biomass from *Ganoderma* spp. as a building material is a novel technology (Hebel *et*. *al* 2014).

1.3.5 Substrates for mushroom cultivation

Carbon and nitrogen are essential compounds for growth and, depending on the type of fungi, the substrate needs to have a particular C:N ratio. Button mushrooms, traditionally grown on composted straw and manure, have a high N requirement and needs a substrate

with a C:N ratio of close to 30:1 for optimum growth (Chang & Miles 2004). Oyster mushroom and Shiitake are saprotrophs and can be grown on wood-based substrates with C:N ratios of 30–97 (Kurt & Buyukalaca 2010) and 20–47 (Philippoussis *et al.* 2003), respectively. Although the C:N ratio refers to total values of carbon and nitrogen in the substrate, the ratio does not represent the absolute amount available for fungal growth (Chang & Miles 2004) as different fungi have different capabilities for substrate biodegradation (Mandeel *et al.* 2005).

Substrate selection and preparation will depend on the species of mushroom used and availability of resources for cultivation. Prior to fungal colonization, two different techniques are used to modify substrates: microbiological and physical. Microbiological processes include composting, a method by which microorganisms are involved in the breakdown of complex molecules to make them more accessible to the fungal mycelium (Chang 2009). Physical methods include heating, increasing the surface area of the substrates by changing the particle size (e.g. by milling) (Royse *et al.* 2004; Zhang *et al.* 2002) and addition of other materials that can alter the structure of the substrate (Membrillo *et al.* 2008; 2011).

Commercial production of mushrooms requires availability of low-cost substrates. For example, sawdust from wood processing and straw from agriculture residues are frequently used (Chang and Miles 2004; Kurtzman Jr. 2010; Stamets 2000). For cultivation of Button mushrooms, composted substrates are prepared with wheat straw and animal manure (e.g. horse, chicken). This species is a secondary decomposer with a lower capability for degradation of lignin and hemicelluloses than tertiary or saprotrophic fungi and must be grown on composted substrates (Sánchez 2010). Compost preparation can be done using a

number of different techniques with steps including composting (i.e. microbial composting, see Kertesz & Thai 2018), pasteurization (Stamets 2005, Chang & Miles 2004) and conditioning (Vajna *et al.* 2012). Saprotrophic species such as Oyster mushroom do not require such extensive substrate preparation for commercial production.

1.3.6 Coffee residues

A further agricultural waste stream that can be used for mushroom cultivation is coffee waste, but it has not yet been broadly used, and relatively little is known about its use. However, in coffee-producing countries it constitutes a readily available source of mushroom growth substrate, since more than 25 million small farmers worldwide rely on coffee production as a source of income (Murthy & Naidu 2012). More than 9 million tonnes of coffee beans were produced in 2017 (International Coffee Organization 2018), and the large amounts of wastes generated include coffee plant residues and coffee bean processing wastes (Cruz *et al*. 2012; Murthy & Naidu 2012; Mussatto *et al*. 2011; Rathinavelu & Graziosi 2005). Mushrooms have been successfully cultivated using some of these residues (e.g. pulp, husks) (Martínez-Carrera *et al.* 1990; Salmones *et al.* 2005; Savoie *et al.* 2007) and, in coffee-producing countries, can add value to the coffee production system as a food source, an additional source of income or a process for waste management. Many other uses of coffee wastes have been and are currently being investigated to decrease the amount of overall coffee waste, its negative effect on soil health and damage to human health (Fernandes *et al*. 2017; Murthy & Naidu 2012). Coffee by-products have been evaluated for a range of industrial applications, including food additives and pharmaceutical compounds (Cruz *et al*. 2014, López-Barrera *et al*. 2016; Machado *et al*. 2012; Mussatto *et al*. 2011; Petrik *et al*. 2014) and energy supply (Kang *et al*. 2017; Park *et al*. 2016; Woldesenbet *et al*.

2013; 2016). Since coffee residues contain resources that can potentially be used by microorganisms, their use as an animal feed supplement has been also investigated (Mazzafera *et al*. 2002; Murthy & Naidu 2012).

1.4 Growth and cultivation of *Pleurotus*

1.4.1 General biology

The name *Pleurotus* derives from Greek and Latin roots meaning "side-ear" and *ostreatus* represents "oyster-shaped". The genus *Pleurotus* is naturally distributed throughout temperate and tropical forests in the Northern Hemisphere including North America, Europe, Eastern Asia (Gao *et al*. 2018), the Middle East and Mexico, and in the Southern Hemisphere in Kenya (Otieno *et al*. 2015), Brazil (Menolli Jr. *et al*. 2014), Australia and New Zealand (Segedin *et al*. 1995; Vilgalys & Sun 1994; Zervakis *et al*. 2004). In its natural surroundings, *Pleurotus* grows on wood mainly from angiosperms (Vilgalys & Sun 1994), but also on non-woody substrates (Fernández-Fueyo *et al.* 2016).

Many species of *Pleurotus* are very important in the mushroom industry because of their flavour and gastronomic attributes, their nutritional value and medicinal applications. As a food, it is valuable source of proteins, carbohydrates, vitamins, mineral and essential amino acids (Cheung 2009). As with other cultivated mushrooms, *Pleurotus* can be grown on various agro-industrial wastes (Table 1.2), such as wheat straw, soybean straw and cotton stalks, maize cobs, plant steams (Stamets 2000; Kurtzman Jr. 2010).

The genus *Pleurotus* has been studied extensively and the potential of particular species has been examined through cultivation using a range of substrates and techniques (Gregori *et*

al. 2007; Da Carvalho *et al.* 2010; Sánchez 2010), for industrial applications (Cohen *et al.* 2002; Inácio *et al.* 2015*;* Knop *et al.* 2015), for degrading toxic materials, and bioremediation of soils (Baldrian 2008). The biology and physiology of *Pleurotus* has been investigated for many reasons but interest in this genus for food production is evident, particularly for species which are easy to cultivate and can take advantage of their capabilities as natural degraders (Chang & Miles 2004; Sánchez 2010; Stamets 2000).

1.5 Purpose of this research

In this thesis, a physiological assessment of *P. ostreatus* grown under laboratory and commercial mushroom farm conditions is presented. *Pleurotus* has been cultivated on many different agro-industrial wastes, including those from the coffee industry. In developing countries such as Latin America, Africa and Indonesia where coffee is grown, there is great potential to reduce this agricultural waste by using it as a substrate for mushroom cultivation while potentially providing additional income to coffee farmers. In developed countries, it is becoming fashionable to grow mushrooms on residues from coffee consumption. In either case, caffeine is an important component of the fungal substrate. Therefore, the aim of the research presented is to understand and clarify the role of caffeine during the cultivation of *P. ostreatus* on spent coffee grounds (SCG), as caffeine utilization by this fungus has not yet been described. If suitable yields can be achieved, there is considerable potential for high volume wastes produced by the coffee industry to be recycled for production of food and, if the caffeine content of residues can be reduced substantially by *P. ostreatus*, for safer disposal.

The objectives of the study are:

1. Evaluate the role of SCG and caffeine on *Pleurotus ostreatus* growth

2. Determine best nitrogen sources for *Pleurotus ostreatus* growth

3. Determine caffeine degradation during *Pleurotus ostreatus* cultivation on SCG

Chapter 2 Materials and methods

2.1 Chemicals used

Chemicals were sourced from Sigma-Aldrich (Castle Hill, NSW, Australia) and Univar (Ajax Chemicals, Australia), unless otherwise stated. Dichloroisocyanuric acid was purchased from ACROS Organics (ThermoFisher Scientific, Belgium), and agar was obtained from Gelita (Australia). Purity of chemicals was analytical grade unless otherwise noted. Nitrogen sources were 98.5–100% pure. Caffeine used in fungal growth studies and reversed-phase high-performance liquid chromatography (HPLC) analysis was 99% pure and methanol was HPLC grade.

2.2 Fungal strain

Pleurotus ostreatus strain PO13 (hereafter referred to as *P. ostreatus*) was kindly supplied by Dr Noel Arrold from Li Sun Exotic Mushrooms in Mittagong NSW, Australia. The strain was maintained on potato dextrose agar (PDA; Oxoid, United Kingdom), and incubated at 25 °C in constant dark.

2.3 Cultivation of *Pleurotus ostreatus*

For experiments using solid media, plates were inoculated with 0.5 x 0.5 cm square plugs of agar taken from the periphery of the colony containing 6-day old mycelium from a homologous culture of *P. ostreatus* (Fig. 2.1). Inoculated plates were incubated at 25 °C for 7

days. Transfer from the mother culture (stored at 5 °C) was done immediately before experimentation.

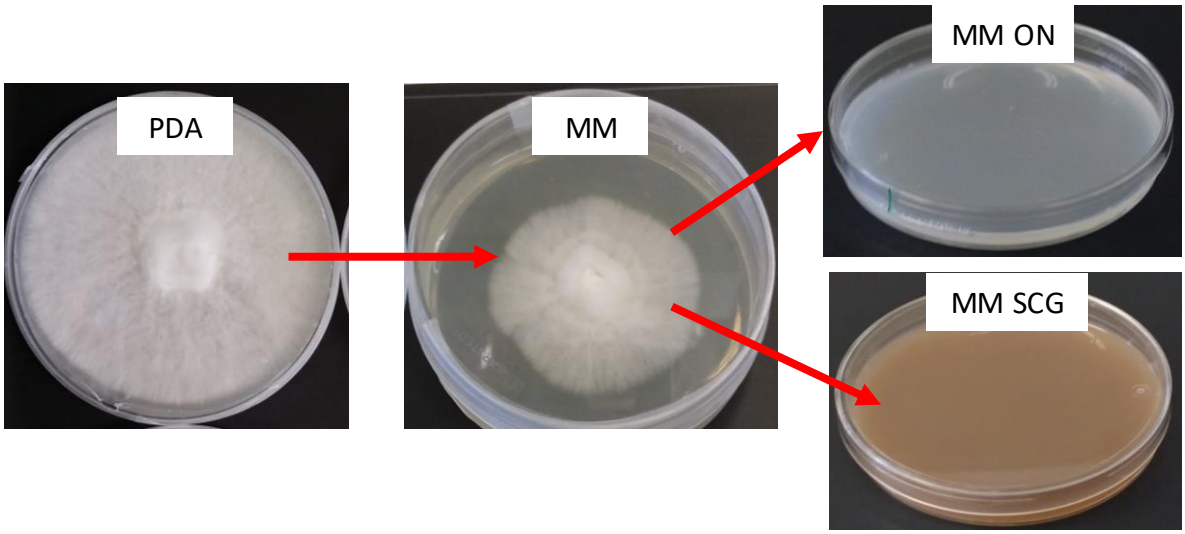

Figure 2.1 Inoculation scheme for *Pleurotus ostreatus* strain PO13 in agar culture. The strain was maintained on potato dextrose agar (PDA) and transferred to a homologous minimal medium (MM) prior to inoculation of treatments (e.g. organic nitrogen (ON), spent coffee grounds (SCG)).

For experiments using liquid media, flasks were inoculated with 2% (v/v) of 7-day old fungal culture suspension (Fig. 2.2a) previously washed twice with chemically defined medium (CDM; see Section 2.4.1.1) with no added nitrogen or carbon source to avoid any transfer of nutrients, and homogenized with an Ultra-Turrax blender at maximum speed for 10 s (TP 18-10, Janke & Kunkel KG, IKA WERK, Germany) (Wu *et al*. 2003). Flasks were incubated at 25 °C on a rotary shaker set at 150 rpm and in darkness for 14–25 days (Fig. 2.2b, c). Flasks were distributed randomly on the rotary shaker each time that they were accessed to account for possible variations within the chamber.

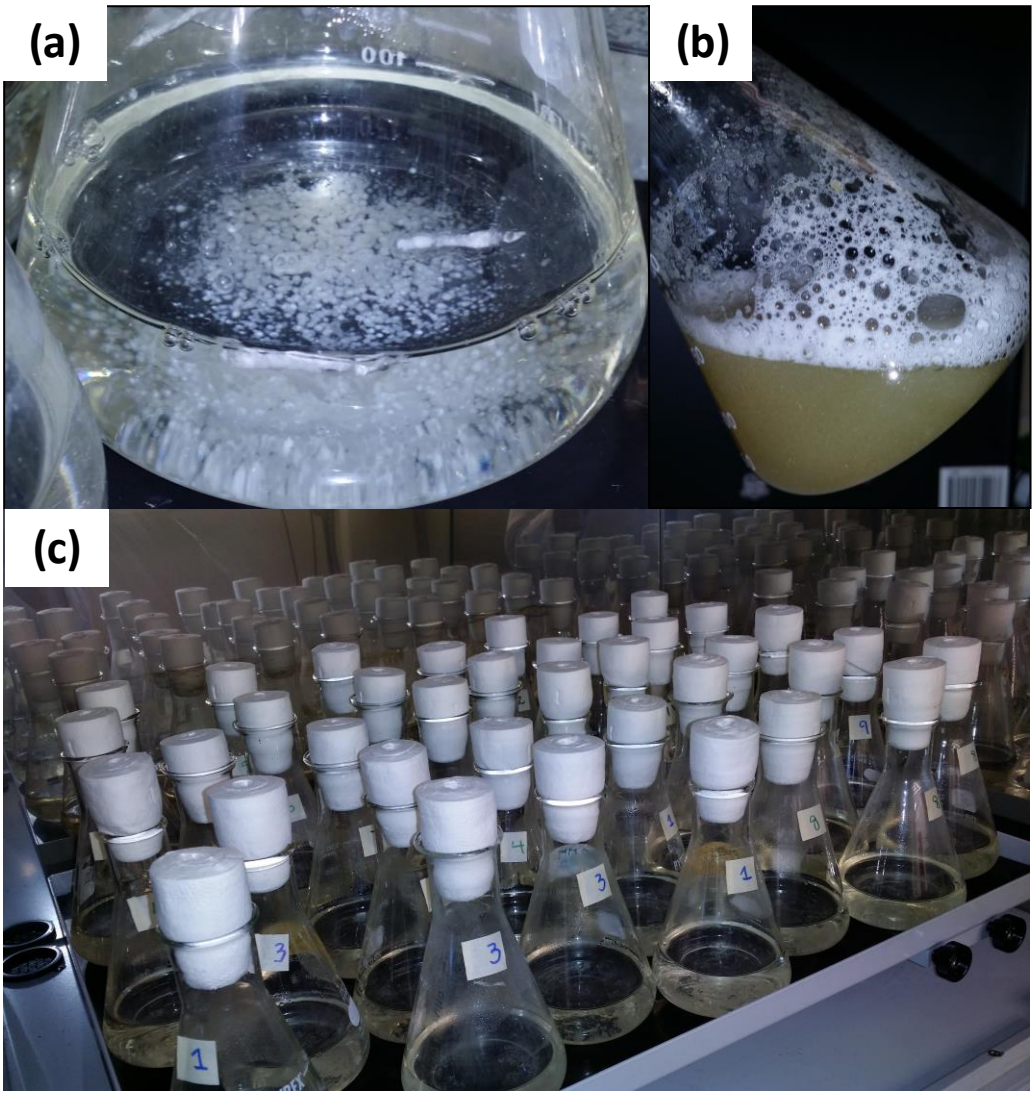

Figure 2.2 *Pleurotus ostreatus* strain PO13 in liquid medium (a) mycelium grown in chemically-defined medium, (b) homogenized inoculum (c) incubation chamber for liquid medium experiments.

For the first trial using solid substrate (spent coffee grounds (SCG) and sawdust), microboxes (Fig. 2.3a) were inoculated with a whole Petri dish of 6-day old mycelium grown on PDA, incubated at 25 °C in darkness for 28 days. After this period, microboxes that were fully colonized (Fig. 2.3b) were transferred to the Marsh Lawson Mushroom Research Unit where controlled temperature (16–18 °C) and humidity (80–85%) allowed fruiting body initiation.

For the second trial using solid substrate (SCG and sawdust), spawn was prepared by boiling rye grain for 25 min in water, allowed to drain for 30 min before adding 1% (w/w) lime and gypsum (Arrold, 2014). When the grain was cold, bottles were inoculated with 7-day old mycelium of *P*. *ostreatus* grown on PDA.

After preparing the substrate (Section 2.4.4), when substrates were cool enough to handle, they were inoculated with mycelium of *P*. *ostreatus* grown 21 days on rye (spawn).

2.4 Culture media

2.4.1 Liquid media

2.4.1.1 Chemically-defined medium

Chemically-defined medium was prepared according to Castanera *et al*. (2012) with a minor modification using ammonium chloride (NH_4Cl; 1.6 g L^{-1}) instead of ammonium tartrate ($(NH_4)_2C_4H_4O_6$). For some treatments, alternative nitrogen sources were added to the medium as required (see Tables 2.1, 2.2, 2.3). The basic CDM consisted of magnesium sulphate heptahydrate ($MgSO_4.7H_2O$; 0.5 g L^{-1}), potassium dihydrogen phosphate (KH_2PO_4; 1 g L^{-1}), potassium chloride (KCl; 0.5 g L^{-1}) and glucose ($C_6H_{12}O_6$; 10 g L^{-1}). Yeast extract (Biotech Grade, Biochemicals) (0.5 g L^{-1}) was added in some experiments to encourage mycelial growth (Table 2.2). A trace element solution was prepared using sodium tetraborate ($Na_2B_4O_7.H_2O$; 0.1 g L^{-1}), zinc sulfate ($ZnSO_4$; 0.07 g L^{-1}), copper sulfate pentahydrate ($CuSO_4.5H_2O$; 0.01 g L^{-1}), manganese sulfate ($MnSO_4.4H_2O$; 0.01 g L^{-1}), ferrous sulfate heptahydrate ($FeSO_4.7H_2O$; 0.05 g L^{-1}) and ammonium molybdate tetrahydrate

($(NH_4)_6$ Mo_7O_{24} $4H_2O$; 0.01 g L^{-1}). An aliquot (1 mL L^{-1}) of this solution was added to 1 L of CDM and mixed thoroughly.

Conical flasks (250 mL; n = 3 or n = 5 per treatment) were filled with 100 mL of medium, covered with non-absorbent cotton lids to facilitate air exchange and autoclaved at 121 °C for 20 min. N sources were filtered sterilized and added as required after sterilization of the media as describe below (Tables 2.1, 2.2, 2.3).

Table 2.1 Inorganic and organic nitrogen sources used with their respective concentration in the medium.

Nitrogen source	Molecular weight (g mol^{-1})	Nitrogen (*units)	Nitrogen concentration (mM)	Chemical concentration (mM)
a. Solid agar cultures				
Adenine (Ade)	135.13	5	18.75	3.75
Alanine (Ala)	89.09	1	18.75	18.75
Ammonium chloride (AmChl)	53.49	1	18.75	18.75
Ammonium nitrate (AmNit)	80.04	2	18.75	9.38
Caffeine (Caff)	194.19	4	18.75	4.69
Calcium nitrate (CaNit)	164.09	2	18.75	9.38
Glutamic acid (Glu)	147.13	1	18.75	18.75
Glycine (Gly)	75.07	1	18.75	18.75
Guanine (Gua)	151.13	5	18.75	3.75
Histidine (His)	155.15	3	18.75	6.25
Phenylalanine (Phe)	165.20	1	18.75	18.75
Tryptophan (Try)	204.23	2	18.75	9.38
Tyrosine (Tyr)	182.20	1	18.75	18.75
Urea (Urea)	60.06	2	18.75	9.38
b. Liquid cultures				
Alanine (Ala)	89.09	1	10	10
Ammonium chloride (AmChl)	53.49	1	10	10
Ammonium nitrate (AmNit)	80.04	2	10	5
Caffeine (Caff)	194.19	4	10	2.5
Calcium nitrate (CaNit)	164.09	2	10	5
Glutamic acid (Glu)	147.13	1	10	10
Glycine (Gly)	75.07	1	10	10
Histidine (His)	155.15	3	10	3.33
Urea (Urea)	60.06	2	10	5

* Number of nitrogen atoms per molecule

Table 2.2 Details of treatments of ammonium chloride-N (AmChl-N) in combination with yeast extract (YE) for solid agar (a) and (b, c) liquid cultures.

Experiment	Ammonium chloride-N (mM)	Yeast extract (g L^{-1})
a. Solid agar cultures YE		
	10	0
	10	0.5
	10	1.0
	10	1.5
	10	2.0
	10	2.5
b. Liquid cultures YE		
	10	0
	10	0.020
	10	0.056
	10	0.170
	10	0.500
c. Liquid cultures AmChl-N		
	0	0
	0	0.5
	2	0.5
	4	0.5
	6	0.5
	8	0.5
	10	0.5
	20	0.5
	50	0.5
	100	0.5

Table 2.3 Treatments used to test the effect of caffeine on growth of *Pleurotus ostreatus* strain PO13 grown in solid media.

Treatment	PDA	MM	PDA	MM
Caffeine-N (mM)				
0	×	×	×	×
0.1	×	×	-	×
1.0	×	×	-	×
2.0	-	-	-	×
5.0	-	-	-	×
10	×	×	-	×
100	×	×	-	-
Extracts of fresh coffee grounds	Undiluted	Undiluted	-	Undiluted
	1:1 dilution	1:1 dilution	-	1:1 dilution
	1:10 dilution	1:10 dilution	-	1:10 dilution
	Undiluted, no PDA			
Extracts of spent coffee grounds	Undiluted	Undiluted	-	Undiluted
	1:1 dilution	1:1 dilution	-	1:1 dilution
	1:10 dilution	1:10 dilution	-	1:10 dilution
	Undiluted, no PDA			

Undiluted (FCG or SCG), 1:1 dilution (½ FCG or ½ SCG), 1:10 dilution (1/10 FCG or 1/10 SCG), undiluted no PDA: FCG (A), SCG (A). PDA – potato dextrose agar, MM – minimal medium, × – combination tested, - – combination not tested.

Table 2.4 Combination of nitrogen and carbon sources used with their respective concentration for liquid cultures experiments.

Caffeine-N (mM)	Yeast extract (g L^{-1})	Glucose-C (g L^{-1})
0	1	10
2	1	10
5	1	10
10	1	10
2	0.05	10
2	0.5	10
2	1	10
5	0.05	10
5	0.5	10
5	1	10
10	0.05	10
10	0.5	10
10	1	10

Caffeine-N – caffeine-nitrogen, Glucose-C – glucose-carbon.

Table 2.5 Controls used with their respective concentration of nitrogen, yeast extract and glucose for (a) solid agar, (b) liquid experiments.

(a) Controls for solid agar experiment cultures			
	Nitrogen (mM)	**Yeast extract (g L^{-1})**	**Glucose (g L^{-1})**
Positive (AmChl)	18.75	0	20
Negative (N free)	0	0	20
Water agar (WA)	0	0	20
Dextrose agar (DA)	0	0	20
(b) Controls for liquid experiment cultures			
Positive (AmChl)	18.75	0.5	10
Negative (N free)	0	0.5	10
Negative (N free, YE free)	0	0	10

AmChl – ammonium chloride, N – nitrogen, YE – yeast extract.

2.4.1.2 Wood decomposer medium

Liquid medium for wood decomposers was prepared according to Stamets (2000). To prepare wood decomposer medium (WDM), 40 g malt extract, 5 g dry sawdust (Mister Ply&Wood, Alexandria, NSW, Australia), 2 g yeast extract and 1 g calcium sulphate were mixed thoroughly in 1 L of distilled water, 100 mL of the medium was placed into 250 mL Erlenmeyer flasks and autoclaved at 121 °C for 20 min (Stamets 2000).

2.4.2 Solid agar media

2.4.2.1 Potato dextrose agar

Commercially-available dehydrated PDA was used (Oxoid, United Kingdom) and prepared following the manufacturers' instructions. For this, 39 g of dehydrated PDA was added to 1 L of distilled water, mixed thoroughly and autoclaved at 121 °C for 20 min.

2.4.2.2 Minimal medium

Nitrogen-free minimal medium (MM) was prepared according to Nayak *et al.* (2013) and was used exclusively for solid agar cultures. According to this method, potassium phosphate (KH_2PO_4; 3 g L^{-1}), sodium phosphate (Na_2HPO_4; 6 g L^{-1}), sodium chloride (NaCl; 5 g L^{-1}) and glucose ($C_6H_{12}O_6$; 2 g L^{-1}) were dissolved in distilled water. Magnesium sulphate ($MgSO_4$; 0.1 g L^{-1}) was dissolved separately and combined with the phosphate-glucose solution, which was diluted to 1 L and mixed thoroughly (Nayak *et al.* 2013). The medium was distributed evenly into five 200 mL storage bottles and 2% (w/v) agar was added, mixed thoroughly and autoclaved at 121 °C for 20 min. Nitrogen sources were filtered, sterilized and added to the

medium as required as described below (Tables 2.1, 2.2, 2.3). Experimental control treatments are described in Table 2.5.

2.4.2.3 Media with extracts from spent and fresh coffee grounds

An extract of fresh coffee grounds (FCG) was prepared as 1 L of double-strength espresso coffee using a commercially-available brand of coffee (Harris Coffee Black Label, Gordon, NSW, Australia) brewed in a coffee maker (Breville Cafe Roma, Australia). The unfiltered extract was used to prepare three FCG solutions (undiluted, 1:1, 1:10 FCG extract:distilled water). Dehydrated PDA (7.8 g) was added to 200 mL of each FCG solution, mixed thoroughly and autoclaved at 121 °C for 20 min.

An extract of spent coffee grounds (SCG) was prepared following the protocol described by Fan *et al*. (2000). Briefly, 40 g of SCG were boiled in approximately 1 L of distilled water for 1 h, with addition of more distilled water as required. The solution was filtered using Whatman filter paper (220 µm, No. 1) and diluted to 1 L with distilled water. Three solutions of SCG were prepared including undiluted extract, 1:1 and 1:10 (SCG extract:distilled water) dilutions. Dehydrated PDA (7.8 g) was added to 200 mL of each SCG solution, mixed thoroughly and autoclaved at 121 °C for 20 min.

2.4.2.4 Dextrose agar

Dextrose agar was prepared using 2% (w/v) agar and 2% (w/v) dextrose added to 200 mL distilled water, mixed thoroughly and autoclaved at 121 °C for 20 min.

2.4.2.5 Water agar

Water agar was prepared using 2% (w/v) agar. The agar was added to each bottle, which was then filled with 200 mL of distilled water and autoclaved at 121 °C for 20 min.

2.4.3 Spent coffee grounds and sawdust substrate – laboratory-scale trial

Bags containing SCG and sawdust were prepared as follows. Spent coffee grounds and sawdust (Mister Ply&Wood, Alexandria, NSW, Australia) were oven dried at 60 °C to constant weight. Different proportions of dried SCG and sawdust were weighed, mixed thoroughly (see Table 2.3) and adjusted to 65% (w/w) moisture content. The substrate mixture was autoclaved at 121 °C for 60 min. When substrates were cool enough to inoculate (the following day), they were placed into Microbox Micropropagation containers (Combiness, Belgium) fitted with an air exchange filter in the lid (base 125 x 65 mm, height 80 mm). The microboxes (n = 7 per treatment) were half-filled with substrate and the initial weight recorded for each treatment. One Petri dish of PDA with 6-day old mycelium of *P. ostreatus* was used to inoculate each microbox. Boxes were randomly placed in an incubator and held at 25 °C. Repeated sampling was done every 3 days starting from the time of full colonization (Fig. 2.3b, c). Samples of colonized substrate (from the substrate surface to bottom of the box) were taken using a sterilized brass core borer (10 mm diameter; Science Supply, Australia) (Fig. 2.3d, e) to analyze caffeine content and metabolites.

Table 2.6 Detailed treatments used to test fungal growth of *Pleurotus ostreatus* strain PO13 grown in mixture of substrates (w/w), spent coffee grounds (SCG) and sawdust. Substrates were dried to constant weight and rehydrated to 65% moisture content. Substrate combinations were 1 – SCG100, 2 – SCG25+sawdust75, 3 – SCG50+sawdust50, 4 – SCG75+sawdust25, 5 – sawdust100.

Treatment	Spent coffee grounds (g)	Sawdust (g)	Replicates	Final dry weight (g)	Water (mL)
1	0	50.0	7	350.0	228
2	25.0	37.5	7	437.5	284
3	50.0	25.0	7	525.0	341
4	75.0	12.5	7	612.5	398
5	100.0	0	7	700.0	455

2.4.4 Spent coffee grounds and sawdust substrate – commercial production-scale

Plastic bottles (volume of 1 L) containing SCG and sawdust were prepared as follows. A bulk sample of spent coffee grounds (approximately 80 kg; Campos Coffee Superior Blend) was donated by a local Sydney café. This substrate was used in the state it was obtained with no additional drying or adjustment of the moisture content and was used within 3 days of production (after being kept at 5 °C). Sawdust (saw milling residue from *Eucalyptus regnans*, Mountain ash) was donated by the mushroom producer where the trial was conducted (Dr Noel Arrold, LiSun Exotic Mushrooms, Mittagong, NSW). This substrate is regularly used by the producer.

Three different proportions of SCG and sawdust (25/75, 50/50, and 75/25; v/v) were mixed thoroughly, N supplement was added in the form of rice bran, also a small proportion of limestone was added to adjust initial pH of substrate (values are commercially confidential

information). Substrate was packed into 1 L bottles (approximately 800 g of substrate; n = 30 per treatment) and capped with a lid containing an air exchange filter. Treatments including pure sawdust and pure SCG (both n = 30) were prepared in the same way. Prepared bottles were autoclaved at 121 °C for 120 min. When substrates were cool enough to handle, they were inoculated with mycelium of *P*. *ostreatus* (see Section 2.3).

2.5 Measurement of fungal growth and biomass

2.5.1 Radial growth

Radial growth of fungal colonies was measured at 24 h intervals, as the average of two orthogonal measurements made using a ruler (0.1 cm intervals). Colony size was expressed as radius in mm, radial growth rate was expressed as mm day^{-1}.

2.5.2 Biomass determination in liquid culture

Fungal biomass in liquid culture was measured in 1 mL aliquots taken every 3–5 days from each flask. The number of samples taken in each experiment varied depending on when maximum fungus biomass was reached (14–25 days). Samples of fungal biomass were centrifuged for 5 min at 10,000 rpm and washed twice with deionised water. The supernatant was removed and stored for additional analysis (see Sections 2.6 and 2.7). Fungal biomass was dried to constant weight at 60° C, but the amounts recovered after drying were very small, introducing considerable weighing error. On the basis of this, it was decided to use wet weight of the mycelium instead. Wet weight was measured using an

analytical balance with an accuracy of 0.0001 g (Sartorius, A2005, Germany) (Fraatz *et al*. 2014). Fungal mycelium was expressed as mg and final biomass as mg mL^{-1}.

At the end of each experiment using liquid media any remaining fungal mycelium was collected by filtering the culture through sterile muslin (Jakubiak *et al*. 2014). Muslin was used after unsuccessful attempts to collect fungal biomass using Whatman filter paper, in which the mycelium adhered to the paper resulting in loss of biomass. With the cloth, the supernatant passed through and was easier to recover without excessive loss of fungal biomass. Once separated from the supernatant, biomass was washed twice with deionised water and weighed with an accuracy of 0.0001 g (Sartorius, A2005, Germany). Biomass samples were oven-dried at 60 °C for 72 h and final biomass was expressed as both wet and dry weight (mg mL^{-1}). Supernatant samples were stored at -20 °C in 1.8 mL Eppendorf tubes until needed for further analysis. Liquid samples were thawed at room temperature, centrifuged and filtered with sterile filters (0.22 µm) before analysis.

2.5.3 Fungal colonization of microboxes

Full colonization of microboxes with mycelium was visually assessed when observed from all possible orientations (Fig. 2.3c). One cross (+) indicated some colonization and five crosses (+++++) represented full colonization of the substrate. When full colonization was recorded, microboxes were transferred to appropriate conditions (i.e. 18 °C and 85% humidity) to induce fruiting.

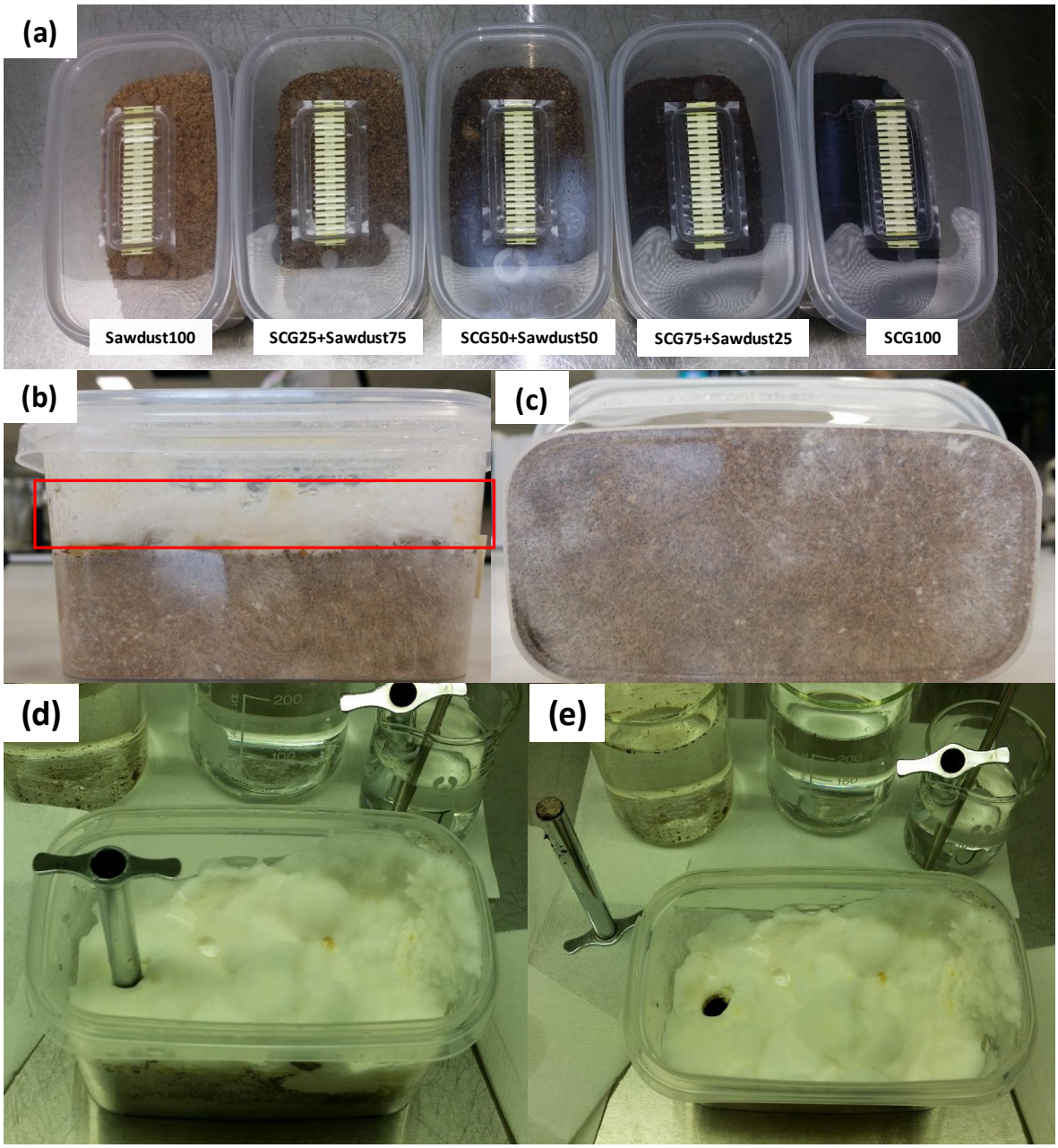

Figure 2.3 Microboxes with a plastic filter fitted in the lid for gas exchange; (a) substrates prior to inoculation, (b) thick white layer of mycelium indicating fully colonized substrate, (c) fully colonized substrate (+++++), (d, e) sampling of colonized substrate for analysis of methylxanthines. SCG – spent coffee grounds.

2.5.4 Fungal growth and sampling at commercial production-scale

Prepared bottles (see Section 2.3) were stored in an incubation growing room at 23 °C and 60% humidity (LiSun Exotic Mushrooms, Mittagong, NSW). Bottles were checked daily and any that showed signs of contamination were removed. Fungal colonization of bottles was assessed weekly using a visual scale (Fig. 2.4). After 4 weeks, colonized bottles were transferred to a cropping room at 15 °C and 85% humidity, where fruiting was induced.

Sacrificial sampling was done every 7 days throughout the cultivation period (60 days in total) for identification of caffeine and it potential degradation compounds and for measurement of moisture content and pH of the substrate. Each week, three bottles were removed from the growing room and the contents transferred to separate clear plastic bags, mixed by massaging the contents by hand and subsampled (approximately 100 g) for chemical analysis. These samples were oven-dried at 60 °C for 48 h (or until constant weight was achieved) and stored at room temperature until required for extraction (see Section 2.10.1).

Figure 2.4 Visual scale used for colonization during commercial cultivation experiment. A score of 1 represented minimal colonization, a score of 10 indicated full colonization of the substrate.

2.6 Moisture content

The moisture content of samples of substrate was determined by loss on drying. On the same day of sampling, approximately 5 g of fresh substrate was placed in a pre-weighed aluminium boat and oven-dried at 105 °C for 24 h. Moisture content was calculated as:

Moisture content (%) = [wet weight (g) - dry weight (g) /wet weight (g)] * 100

2.7 Total carbon and nitrogen in fungal biomass

Total carbon and nitrogen were determined by dry combustion (Elemental CN analyser, Germany) using glutamic acid as reference. Dried substrate samples were ground with a mortar and pestle and approximately 0.8 g of sample was used for analysis.

2.8 pH in liquid culture

The pH of all media was initially set at 5.5 using 1 M KOH, using a laboratory pH meter (Meter Lab, PHM 210, France). The pH of supernatant was measured at the conclusion of each experiment.

2.9 pH in substrate

The pH of substrate samples was measured on the same day of sampling by preparing a 1:10 dilution. For this, 1 g of fresh substrate was mixed with 10 mL of deionised water in a 15 mL Falcon tube, shaken on a vortex (Ratek, Victoria, Australia) for 1 min and centrifuged at 4.4 rpm for 5 min (Eppendorf AG, Hamburg, Germany). The pH of the solution was measured using a laboratory pH meter (Meter Lab, PHM 210, France).

2.9 Residual ammonium in culture supernatant

Residual ammonium was measured according to the method of Diatloff and Rengel (2001), modified for microtiter plate format according to Safianowicz (2013). For this method, an aliquot of 1 mL was taken from the culture medium, centrifuged and filtered. A 30 µl aliquot of sample was mixed with 40 µl of 2% (w/v) sodium salicylate ($C_7H_5NaO_3$), 0.05% sodium nitroprusside ($Na_2Fe(CN)_5NO.2H_2O$) dissolved in 2% (w/v) NaOH, and 30 µl of 0.04% (w/v) dichloroisocyanuric acid ($C_3Cl_2N_3NaO_3$) in 2% (w/v) NaOH. This solution was incubated for 20–30 min at room temperature. Dilutions of ammonium sulfate ($(NH_4)_2SO_4$) in the range of 0–100 µg mL^{-1} were used to make the standard curve. Absorbance at 620 nm was measured using a Synergy H1 spectrophotometer (BioTek, Vermont, United States).

2.10 Identification of caffeine

2.10.1 Caffeine extraction

Samples of substrate, colonized substrate and fruiting bodies were oven-dried at 60 °C for 48 h then ground with a mortar and pestle. An initial series of extraction tests with a range of different concentrations of methanol (0, 5, 10, 20 and 30%) were evaluated as extraction solvents (modified from Tfouni *et al*. (2014)) and 20% (v/v) methanol was chosen as extractant for further work as it provided the greatest amount of caffeine for all sample types. For this, 100 mg of dried sample was mixed with 10 mL of 20% methanol in a 15 mL Falcon tube, shaken on a vortex (Ratek, Victoria, Australia) for 30 s, extracted overnight at room temperature. The next day, samples were shaken on a vortex for 30 s and centrifuged at 4.4 rpm for 5 min (Eppendorf AG, Hamburg, Germany). Extracts were filtered before HPLC analysis.

2.10.2 Residual caffeine in culture supernatant

Caffeine in culture supernatants was analyzed using HPLC connected to an ultra-violet (UV) Dionex PDA-100 Photodiode Array Detector (Dionex, California, United States) and a P680 HPLC-pump. Caffeine and its potential degradation products (paraxanthine, theophylline, theobromine, 7-methylxanthine, 1-methylxanthine, 3-methylxanthine and xanthine) were separated using an Eclipse XDB-C18 column (4.6 x 150 mm, i.d. 5 µm) (Agilent, California, United States). Samples were thawed at room temperature, centrifuged and filtered (nylon 0.22 µm) before injection. The autosampler used was an ASI-100 automated sample

injector; the sample volume used was 10 µL. The mobile phase consisted of solvent A:water (100%) and solvent B:methanol (90%) with the following gradient: 0–10 min, 0–100% B; 10–20 min, 100% B; 20–23 min, 100-0% B to initial conditions with 1.0 mL min^{-1} flow rate. The separation was done at 25 °C (Thermostat column compartment TCC-100) and UV detection was measured at 275 nm. Individual calibration curves were built by plotting peak area versus standard concentrations for each compound and the best relationship was determined by a least-squares linear regression analysis (R^2). The software used to analyse the resulting chromatograms was Chromeleon Version 6.80 (Dionex, California, United States).

2.11 Statistical analysis

For all experiments involving radial colony growth, data were analysed using one-way ANOVA and a Tukeys post-hoc test using IBM SPSS Statistics 21. For all experiments involving changes in fungal biomass when grown in liquid culture, the effect of each treatment on biomass was evaluated using repeated measures ANOVA and Sphericity of data was tested using Mauchly's test using the IBM SPSS Statistics 21 package. For laboratory and commercial trials, mean ± standard deviation are presented.

Chapter 3 The role of caffeine in growth of oyster mushroom in axenic culture

3.1 Introduction

Coffee is an important agricultural product with more than 9 million tonnes produced in 2017 (ICO 2018). During coffee production, large quantities of waste are generated, from biomass produced during plant cultivation through to the brewing processing involved (Cruz *et al*. 2012; Murthy & Naidu 2012; Mussatto *et al*. 2011). Attempts to recycle production residues have been assessed for a variety of purposes with the major objectives of these studies being how to treat and dispose of coffee wastes safely due to adverse effects on soil, the environment and humans (Fernandes *et al*. 2017; Murthy & Naidu 2012). The recovery of useful by-products from coffee production, such as ethanol, enzymes and pigments are gaining interest, as is the use of coffee wastes as a source of compost (Murthy & Naidu 2012).

Coffee residues contain a range of compounds that can potentially be used by fungi (Table 3.1). Spent coffee grounds (SCG) are high in carbon (C) content in the form of hemicelluloses and lignin whereas the proportion of more readily accessible C in the form of cellulose is greater in pulp and husks (Table 3.1). Nitrogen (N) is present in the form of proteins and amino acids, and potassium (K), magnesium (Mg) and phosphorous (P) are the main minerals in SCG (Campos-Vega *et al.* 2015). Additionally, coffee residues contain microelements, minerals and vitamins (Mussatto *et al*. 2011). Other type of compounds such as chlorogenic acids, tannins and caffeine have also been reported to be part of industrial coffee residues (Table 3.1). Caffeine is one of the main compounds produced by

the coffee and tea industry. This compound and its sub-products are often used in food and pharmaceutical industries, with greatest human consumption in coffee and chocolate, but also in medicine (Summers *et al.* 2015).

Table 3.1 Chemical composition of coffee residues as a proportion (%) and concentration (mg g^{-1}).

Component	Coffee pulp (%)	Coffee husks (%)	Spent coffee grounds (%)	Reference
Total carbon	-	-	52.2	Caetano *et al*. 2012
Total nitrogen	-	-	2.1–2.3	Caetano *et al*. 2012; Cruz *et al.* 2012
Lignin	-	-	33.6	Caetano *et al*. 2012
Protein	10.0	10.4	13.3–15.4	Mussatto *et al.* 2011; Caetano *et al*. 2012; Cruz *et al.* 2012; Murthy & Naidu 2012; Vardon *et al.* 2013
Cellulose	63.0	43.0	8.6–13.8	Mussatto *et al.* 2011; Caetano *et al*. 2012
Hemicellulose	2.3	7.0	36.7	Mussatto *et al.* 2011; Murthy & Naidu 2012
Fiber	60.5	24.0	-	Murthy & Naidu 2012
Ash	1.9	-	1.43–1.9	Mussatto *et al.* 2011; Caetano *et al*. 2012; Cruz *et al.* 2012; Murthy & Naidu 2012; Vardon *et al.* 2013
Fat	-	-	12.5	Cruz *et al.* 2012
Tannins	6.0	-	0.02	Murthy & Naidu 2012
Chlorogenic acid	2.4	2.5	-	Murthy & Naidu 2012
Caffeine, microelements and minerals (mg g^{-1})				
Caffeine	1.5	-	3.8–5.6	Cruz *et al.* 2012; Murthy & Naidu 2012
Potassium	-	-	3.5–8.8	Mussatto *et al.* 2011; Cruz *et al.* 2012
Phosphorus	-	-	1.4–1.5	Mussatto *et al.* 2011; Cruz *et al.* 2012
Magnesium	-	-	1.3–2.2	Mussatto *et al.* 2011; Cruz *et al.* 2012
Calcium	-	-	3.7–0.7	Mussatto *et al.* 2011; Cruz *et al.* 2012
Aluminum	-	-	0.2	Mussatto *et al.* 2011
Iron	-	-	0.04–0.1	Mussatto *et al.* 2011; Cruz *et al.* 2012
Manganese	-	-	0.02–0.03	Mussatto *et al.* 2011; Cruz *et al.* 2012
Copper	-	-	2.5–3.2	Mussatto *et al.* 2011; Cruz *et al.* 2012
Zinc	-	-	0.01	Mussatto *et al.* 2011
Sodium	-	-	0.2	Cruz *et al.* 2012
Iron	-	-	0.04	Mussatto *et al.* 2011

Caffeine (1,3,7-trimethylxanthine) belongs to the family of purines. It commonly described as an alkaloid and occurs naturally in plants where it is synthesised for several purposes, particularly in young plants (Ashihara & Crozier 2001; Ashihara *et al*. 2008). There are two main hypotheses for the biosynthesis and accumulation of caffeine in plants: (i) it is accumulated in leaves, fruits and flowers to protect these organs from predators (herbivores such snails, slugs, beetles), and (ii) it is released into the soil from the seed coat during seed germination to inhibit germination of other seeds (Ashihara & Crozier 2001; Ashihara *et al*. 2008).

As an example of the potential toxicity of caffeine, it has been found to have an antimicrobial effect on different strains of the bacterium *Escherichia coli*, inhibiting growth of some strains and causing death of others (Gummadi *et al*. 2012; Sandlie *et al*. 1980). Both caffeine and SCG have been tested for the control of mosquitoes that transmit disease. For the mosquito *Aedes aegypti,* it was found that a concentration of 2 mg mL^{-1} caffeine killed all early-stage larvae. In addition, a water extraction made of SCG at 50 mg mL^{-1} was found to be the optimum concentration for eradication of advanced larvae stages, suggesting that this waste can be used as alternative to granular organophosphorus insecticides for control of these types of insects (Laranja *et al*. 2003).

3.1.1 Coffee waste for mushroom cultivation

Edible mushrooms have been cultivated using a variety of substrates including agricultural by-products. Coffee husks, pulp and SCG have all been used but as a substrate they differ in

structure and chemical composition (Cruz *et al*. 2012; Murthy & 2012; Mussatto *et al*. 2011). Research related to degradation of caffeine by fungi began with isolation of indigenous strains of *P*. *ostreatus* from decaying wood of native plants in Mexico and Latin America (Martínez-Carrera 1984). The use of coffee residues was reported again in the late 1980s when the same 10 strains of *P*. *ostreatus* were grown on coffee pulp mixed with barley straw (Martínez-Carrera *et al*. 1988). The main objective of this research was strain selection for further commercial cultivation. As this line of research developed, biological efficiencies (BE) (see Chapter 1) as high as 96% were achieved with one of the strains investigated (Martínez-Carrera *et al*. 1988). In a later study, optimization of growth was tested with substrate modification by adding sugar cane bagasse to coffee pulp to potentially increase N content. This resulted in a BE of greater than 96% compared to a BE of 65% when straw was used instead of coffee pulp (Martínez-Carrera *et al*. 1990).

Apart from early yield studies described above, there are limited studies that have investigated how caffeine can be used by edible mushrooms. In one of the few studies available, the cultivation of different strains of *P*. *ostreatus* were evaluated for genetic crossing and the effect of caffeine on mating was examined. Caffeine was not assimilated by the fungus and when caffeine was supplied at a concentration of 2 mg mL^{-1} and it was found to inhibit fungal growth (Martínez-Carrera *et al*. 1988). The authors hypothesized that caffeine was not taken up by hyphae as it is a relatively large molecule and fungi only absorb small molecular weight compounds. In another study, potato dextrose agar (PDA) supplemented with caffeine was used for screening strains of *Pleurotus* for their efficiency in degradation of caffeine. Mycelium could absorb between 0.06 and 13.9 mg g^{-1} caffeine,

but when caffeine concentrations in PDA were high (i.e. 2.5 mg mL^{-1}) the mycelium did not grow (Fan *et al*. 2003). Apart from these limited studies, there is little published information available to compare how much caffeine can be absorbed from coffee residues by fungal mycelium.

Studies using coffee husks as a substrate for cultivation of *Pleurotus* found a 27–40% decrease in caffeine (initial caffeine was in the range of 50–100 mg g^{-1} dry substrate) (Fan *et al*. 2000). When *Pleurotus pulmonarius* and *P*. *ostreatus* were cultivated on coffee pulp, they accumulated between 0.17–0.22% of the initial caffeine present in the substrate into their fruiting bodies (Salmones *et al*. 2005). When supplied with low levels of caffeine in coffee husks, "*P*. *sajor-caju*" absorbed 0.16 mg caffeine g^{-1} biomass in its fruiting body (Fan *et al*. 2003). In contrast, no caffeine was detected in fruiting bodies of *Lentinula edodes* (Shiitake) cultivated on SCG even though there was a decrease of 40% of the caffeine (equivalent to 110 mg g^{-1} dry substrate) present in the substrate (Fan *et al*. 2000). In comparison, cultivation of Enokitake (*Flammulina velutipes*) on the same substrate resulted in a modest 10% decrease in caffeine (from 65–58 mg g^{-1} dry substrate). However, no caffeine was detected in the fruiting body at the end of the cultivation and it was suggested that the fungus had the ability to degrade caffeine (Fan *et al*. 2001). Interestingly, caffeine has been found in the sclerotia of the parasitic fungus, *Claviceps sorghicola*, at a concentration of 0.33 mg g^{-1} and it has been identified as a fungal metabolite but is still unknown if the metabolic pathway to synthesise caffeine by this fungus is the same as in plants (Bogo & Mantle 2000).

3.1.2 Aims of this study

The use of coffee waste as a substrate for mushroom cultivation has the potential to become a large-scale recycling technology. Caffeine utilization by edible fungi is not yet fully understood and further investigation is needed to clarify the role of caffeine as a metabolite during cultivation of *P. ostreatus* on coffee residues. In this chapter, a laboratory-based assessment will be used to characterize the baseline for caffeine use by *Pleurotus ostreatus* as an aid to understand the basic physiology of this fungus when cultivated in the presence of caffeine.

The hypotheses being tested are:

1. The addition of caffeine as a sole N source has a negative effect on growth of *P. ostreatus* and, when provided at a certain concentration, will prevent fungal growth.

2. The combination of different N or C sources together with caffeine produces either a synergistic or antagonist effect on fungal growth.

3.2 Materials and methods

To determine the effect of caffeine from SCG on fungal growth in solid agar culture, minimal medium (MM), potato dextrose agar (PDA) were prepared. Media supplemented with caffeine (calculated as mM N), extracts of fresh coffee grounds (FCG) and SCG were prepared as described in Section 2.4. Statistical analyses included one-way ANOVA (radial growth rate, biomass, final pH) and repeated measures ANOVA (growth curves). The repeated measures ANOVA analyses were checked to meet the assumptions (independent observations, normality and sphericity) and, when required, appropriate corrections for

multiple comparisons were applied (if p >0.05, sphericity assumed, **if p <0.05, (i) Greenhouse-Geisser Epsilon <0.75, use Greenhouse-Geisser or multivariate results, or (ii) if Greenhouse-Geisser Epsilon >0.75, use Huynh-Feldt results) (Field 2013; Howel 2002).

3.3 Results

3.3.1 Effect of extracts from fresh and spent coffee grounds on fungal growth in solid agar culture

Mean daily rates of radial growth of mycelium of *P. ostreatus* grown on PDA and MM media with extracts of both FCG and SCG differed according to the media used (Figs. 3.1 and 3.2). Overall, there were statistically significant differences in radial growth rates of mycelium of *P. ostreatus* among SCG treatments when PDA was used (Fig. 3.1a; one-way ANOVA, $F_{(4,20)}$ = 388.059, p <0.001). Growth rates on PDA with added SCG extracts were similar to growth rates on the control PDA (without addition of coffee extracts or caffeine). This suggests that the compounds in the PDA in combination with SCG extract, including caffeine, were not inhibiting growth.

The slowest growth rate associated with SCG (3.5 ± 0.3 mm day^{-1}) was found with no PDA (SCG(A)) which was significantly different from the treatments with PDA added (one-way ANOVA, p <0.001). In contrast, use of ½ SCG (i.e. 1:1 dilution) resulted in only a slightly increased rate of mycelial growth (4.7 ± 0.1 mm day^{-1}). This made it clear that the fungus was not able to use the SCG as a complete nutrient source requiring the nutrients from PDA to achieve adequate growth (see example of thin and almost transparent mycelium in Fig. 3.3a).

When FCG was used in combination with PDA, fungal growth was strongly inhibited and there were considerable differences among rates of fungal growth depending on dilution (Fig. 3.1b; one-way ANOVA, $F_{(4,20)} = 1342.799$, $p < 0.001$). A comparable growth rate was achieved using 1/10 FCG and the control treatment (PDA only) (both were 5.7 ± 0.01 mm day^{-1}), whereas mycelial growth rate using FCG (A) and FCG was as little as 0.1 ± 0.01 and 1.6 ± 0.4 mm day^{-1}, respectively. Furthermore, when FCG was used with MM, the greatest rate of growth was found for 1/10 FCG and growth rates were comparable to the control MM (both were 4.1 ± 0.3 mm day^{-1}). Overall, there were statistically significant differences among treatments (Fig. 3.1d; one-way ANOVA, $F_{(3, 16)} = 414.502$, $p < 0.001$) which implies that one or more compounds present in FCG inhibited growth of this fungus.

The mycelial growth of *P. ostreatus* grown on extracts of SCG and FCG in combination with PDA and MM is shown in Fig. 3.2 (and see Fig. 3.3). As expected, when using PDA with the addition of extracts of SCG (Fig. 3.2a), faster and more consistent growth than in MM medium was observed. However, SCG with agar only (SCG (A)) was an exception as, in this treatment, the mycelium grew considerably slower than for other treatments. In all treatments with MM and SCG, mycelium grew at the same rate throughout the experimental period (Fig. 3.2c). The use of FCG extracts exhibited a similar pattern of growth when both PDA and MM were used (Fig. 3.2b, c), and this was particularly noticeable for the 1/10 FCG and ½ FCG treatments. In addition, when using FCG with MM, there was slow but steady growth in the 1/10 FCG treatment and it was comparable to the control treatment (MM only). Slow growth was measured when ½ FCG was used with MM and no growth was

measured when undiluted FCG and MM was used. This again suggests that compounds present in the FCG inhibit the growth of *P*. *ostreatus*.

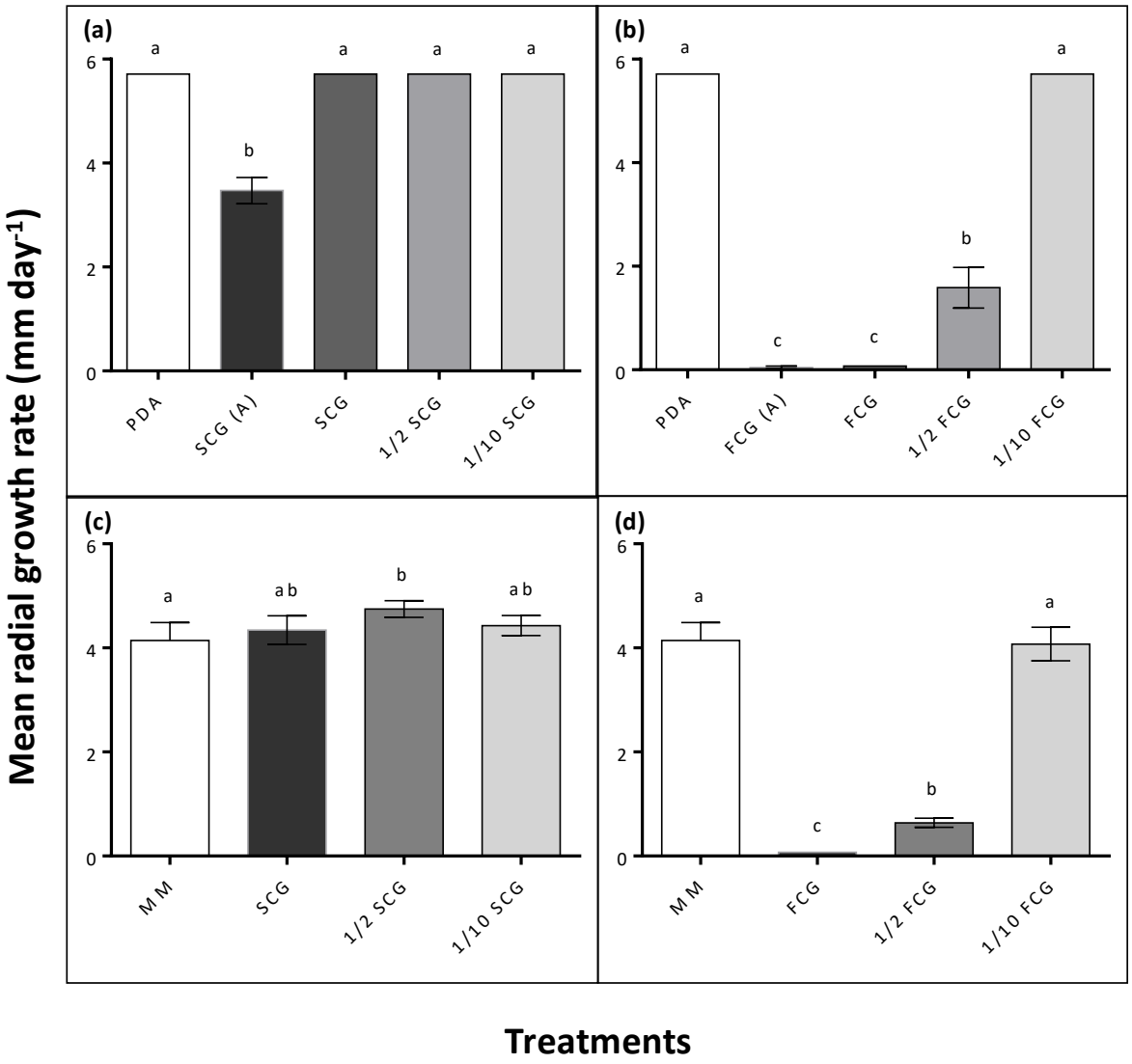

Figure 3.1 Mean rate of radial growth of mycelium of *Pleurotus ostreatus* strain PO13 grown on (a, b) potato dextrose agar (PDA), and (c, d) minimal medium (MM) supplemented with extracts of (a, c) spent coffee grounds (SCG), and (b, d) fresh coffee grounds (FCG) at three dilutions (undiluted, 1:1 and 1:10 extract:distilled water). Controls are PDA and MM, with no addition of extracts from coffee grounds. Additional alternative controls are SCG and FCG with agar only (SCG (A), FCG (A)). Bars represent mean values (n = 5) and error bars are standard deviation, lower case letters represent differences between means using one-way ANOVA and a Tukeys post-hoc test (p <0.05).

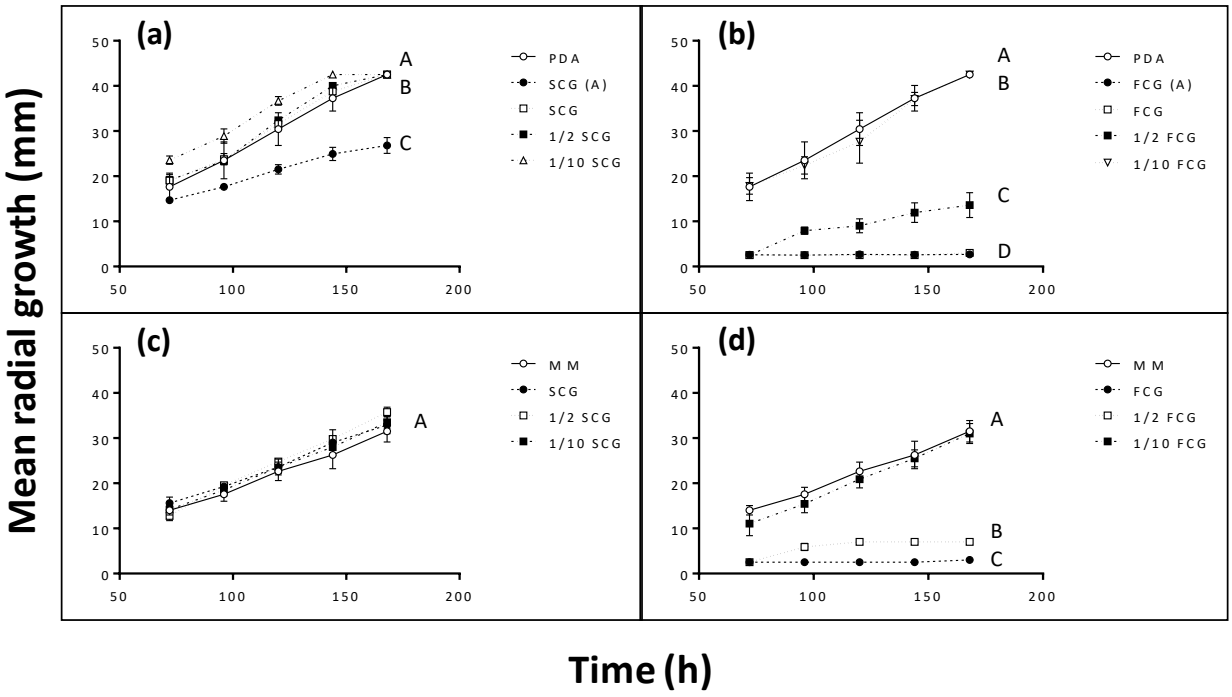

Figure 3.2 Radial growth of mycelium of *Pleurotus ostreatus* strain PO13 grown on (a, b) potato dextrose agar (PDA), and (c, d) minimal medium (MM) supplemented with extracts of (a, c) spent coffee grounds (SCG), and (b, d) fresh coffee grounds (FCG) at three dilutions (undiluted, 1:1 and 1:10 extract:distilled water). Controls are PDA and MM, with no addition of extracts from coffee grounds. Additional alternative controls are SCG and FCG with agar only (SCG (A), FCG (A)). Points represent mean values (n = 5) and error bars are standard deviation. Uppercase letters represent differences between means using repeated measures ANOVA and a Tukeys post-hoc test (p <0.05).

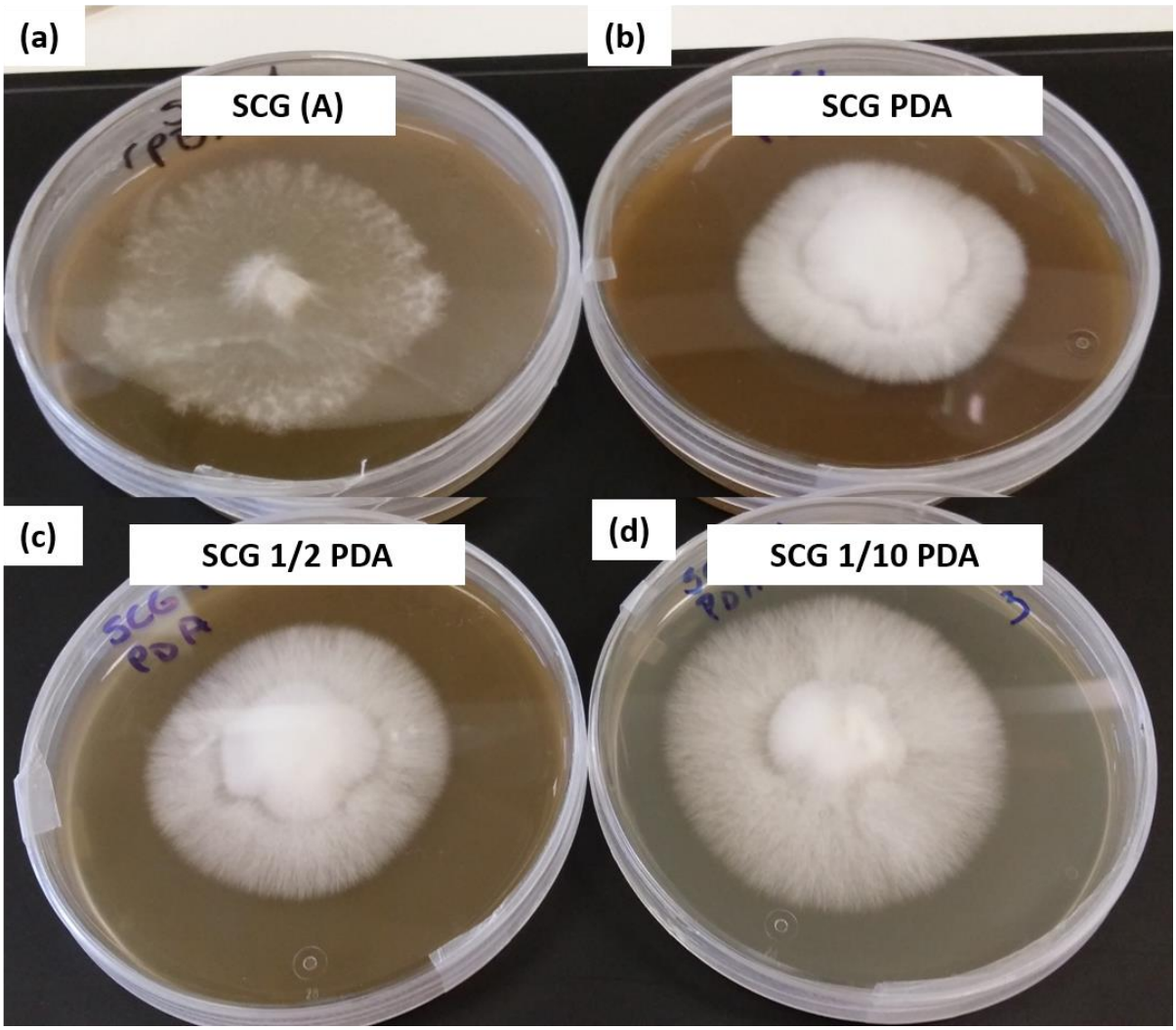

Figure 3.3 Mycelium of *Pleurotus ostreatus* strain PO13 grown on (a) spent coffee grounds with agar only (SCG) (A), (b) potato dextrose agar (PDA) with undiluted SCG, (c) PDA with ½ SCG, and (d) PDA with 1/10 SCG.

3.3.2 Effect of caffeine on fungal growth in solid agar culture

From the results of the previous experiment, it was hypothesized that compounds present in FCG may have inhibited the growth of *P. ostreatus* – caffeine being the most likely compound. Thus, caffeine was tested to evaluate its effect on fungal growth on solid agar culture. A range of known concentrations (0.1–100 mM of caffeine-N) were added to PDA or

MM, while control treatments had no added caffeine. Treatments were prepared as described in Section 2.4. Mean daily rates of radial growth of mycelium of *P. ostreatus* on either PDA or MM with different concentrations of caffeine were similar (Fig. 3.4a, b). In general, hyphal growth with low concentrations of caffeine-N (0.1–1.0 mM) were comparable with the control, whereas additions of 10 mM of caffeine-N reduced fungal growth, and it was completely inhibited with addition of 100 mM caffeine.

The best rates of hyphal growth were achieved when *P*. *ostreatus* was grown on MM with low concentrations of caffeine-N (0.1 mM: 4.8 ± 0.2 mm day^{-1}; 1.0 mM: 4.6 ± 0.3 mm day^{-1}) (Fig. 3.4b, c). When *P*. *ostreatus* was grown on PDA, the greatest rates of radial growth were also achieved with 0.1 mM and 1.0 mM of caffeine-N (both 5.0 ± 0.1 mm day^{-1}) and these rates were comparable to the mycelium growth on the control PDA (5.0 ± 0.01 mm day^{-1}). This was not a surprising result as PDA is a nutrient-rich medium and the observed growth of mycelium on the agar plates was white and thick (Fig. 3.4b, c, d).

Overall, there were statistically significant differences among radial growth rates of mycelium when grown on PDA with the addition of caffeine-N (Fig. 4a; one-way ANOVA, $F_{(4,20)} = 781.775$, $p < 0.001$). A Tukeys post-hoc test ($p < 0.05$) revealed that radial growth rate was significantly decreased when 10 mM N was added (1.36 ± 0.42 mm day^{-1}, $p < 0.001$). Furthermore, there were statistically significant differences in radial growth rates of mycelium when grown on MM with the addition of caffeine-N (Fig. 3.4b; one-way ANOVA, $F_{(4,20)} = 465.270$, $p < 0.001$). Radial growth rate was not significantly different after adding 0.1 and 1.0 mM caffeine-N (4.6 ± 0.2 mm day^{-1}, $p = 0.149$, 4.6 ± 0.3 mm day^{-1}; Tukeys post-hoc

test, p = 0.051). In comparison, the rate of radial growth was significantly lower when 10 mM caffeine-N was added (1.4 ± 0.3 mm day^{-1}, p <0.001).

Different patterns of growth were found when using PDA and MM in combination with the same concentrations of caffeine-N (Fig. 3.5a, b). When caffeine-N was used at 10 mM in combination with PDA, no growth was observed until after 120 h of incubation, whereas when 10 mM was used in combination with MM, mycelium growth occurred through the incubation period. This indicated a possible interaction between components in the medium and caffeine on fungal growth.

At the beginning of the experimental period, for treatments using MM without added caffeine (Control) and treatments with low concentrations of caffeine, 0.1 and 1.0 mM caffeine-N, mycelium initially grew equally well, but after 144 h, growth of the control treatment increased at a faster rate than observed for other treatments (Fig. 3.4b). When using MM with concentrations of caffeine-N ranging from 0.1 to 10 mM (Fig. 3.5c), the treatment without caffeine (Control) and the two treatments with the lowest caffeine content (0.1 and 1.0 mM caffeine-N) continued to grow at the same rate throughout the experimental period. The addition of 2 and 5 mM of caffeine-N to MM supported less and slower fungal growth. Different morphology of mycelial growth was observed for the media used. Using caffeine in combination with PDA (Fig. 3.6a), white cotton like mycelium was produced, suggesting a positive nutrient effect from PDA, as expected from this type of medium. While when caffeine in combination with MM, light clear mycelium grew, most likely a result of lack of nutrients in the medium (Fig. 3.6b).

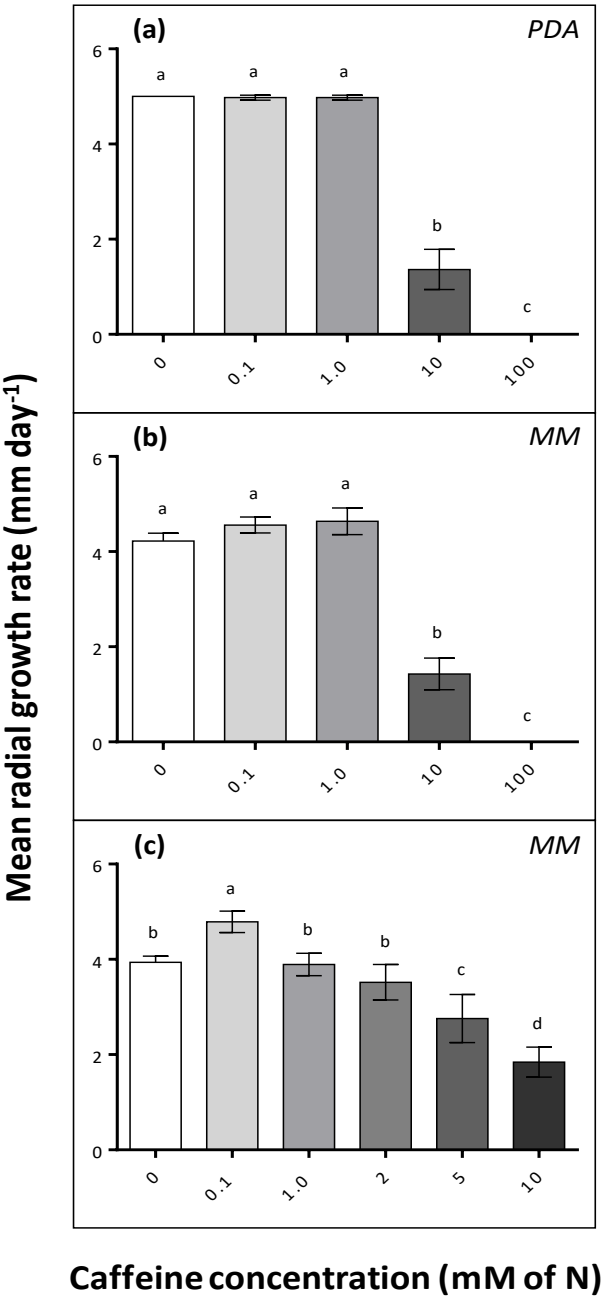

Figure 3.4 Mean rate of radial growth of mycelium of *Pleurotus ostreatus* strain PO13 grown on (a) potato dextrose agar (PDA) and (b, c) minimal medium (MM) supplemented with different concentrations of nitrogen (N) supplied as caffeine-N (0.1–100 mM). Controls are PDA and MM, both with no addition of caffeine. Bars represent mean values (n = 5) and error bars are standard deviation, lower case letters represent differences between means using one-way ANOVA and Tukeys post-hoc test (p <0.05).

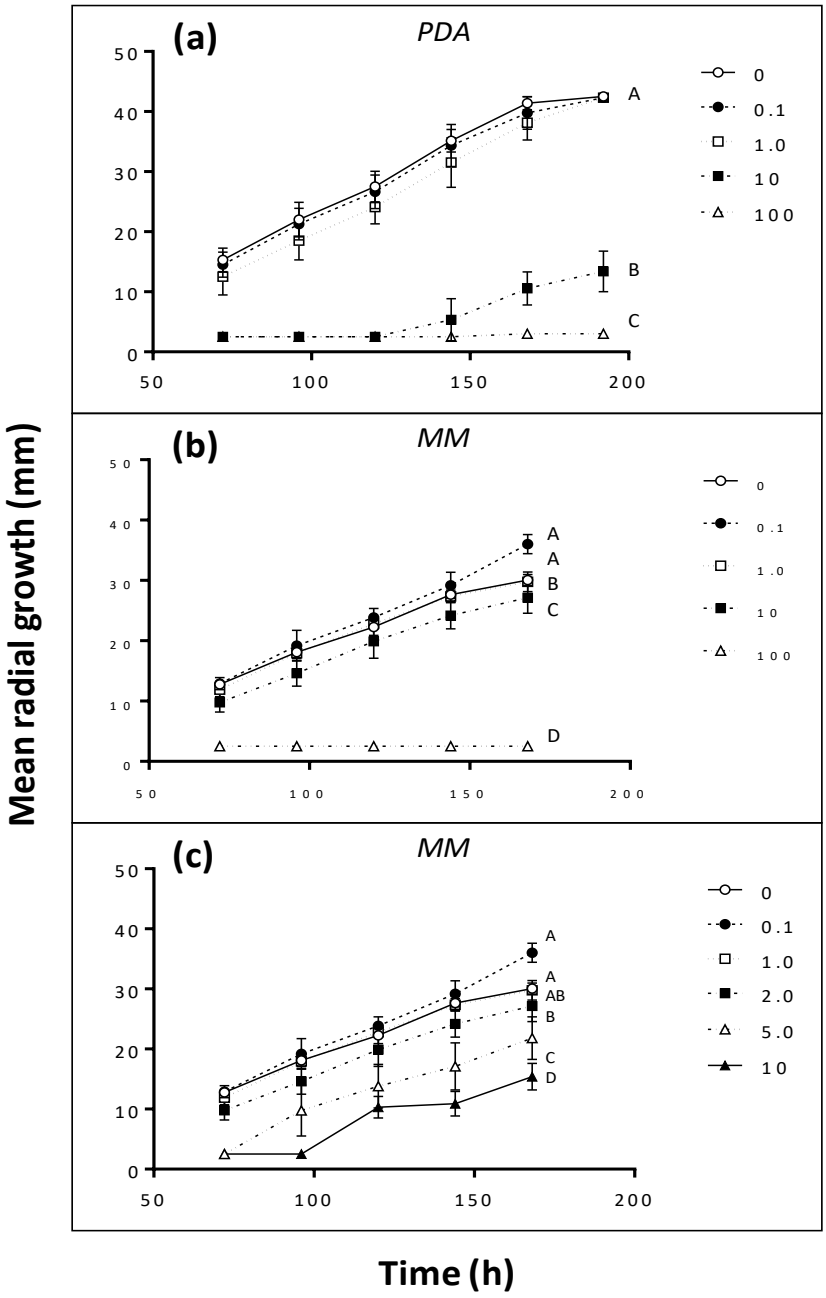

Figure 3.5 Radial growth of mycelium of *Pleurotus ostreatus* strain PO13 grown on (a) potato dextrose agar (PDA) and (b, c) minimal medium (MM) supplemented with different concentrations of nitrogen (N) supplied as caffeine-N (0.1–100 mM). Controls are PDA and MM, both with no addition of caffeine. Points represent mean values (n = 5) and error bars are standard deviation. Uppercase letters represent differences between means using repeated measures ANOVA and a Tukeys post-hoc test (p <0.05).

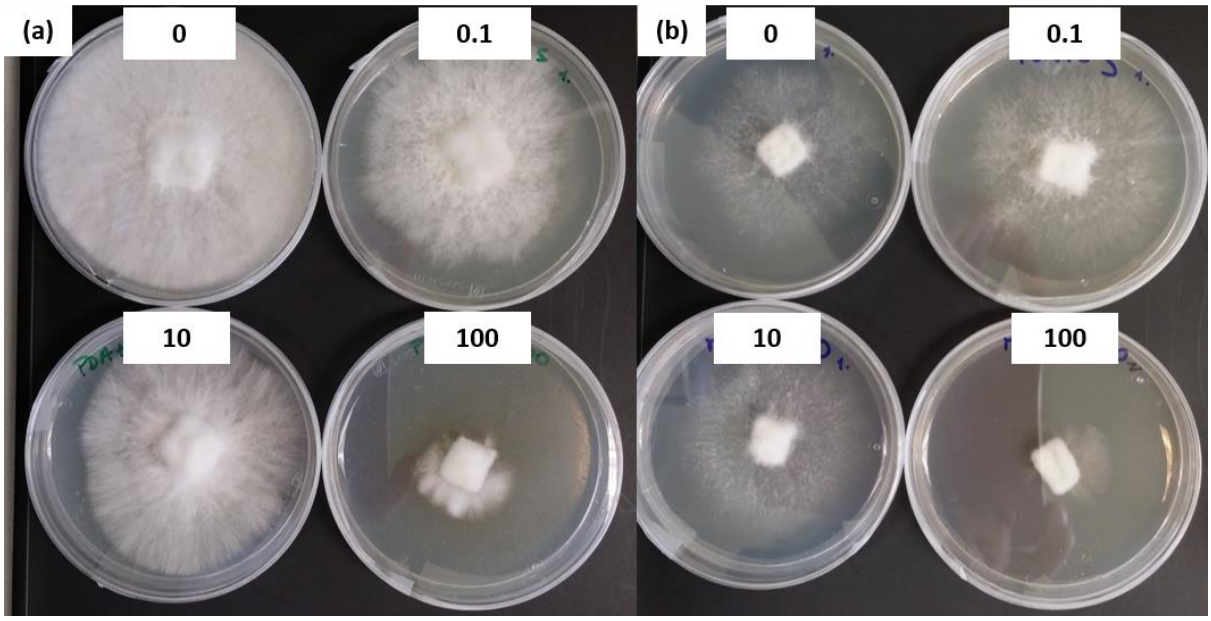

Figure 3.6 Mycelium of *Pleurotus ostreatus* strain PO13 supplemented with nitrogen (N) supplied as caffeine-N (0–100 mM) grown on (a) potato dextrose agar (PDA), and (b) minimal medium (MM). Controls are PDA and MM with no caffeine-N added, represented with a zero (0).

3.3.3 Effect of caffeine on fungal growth in liquid culture

From the previous experiments, it was hypothesized that an interaction between *P. ostreatus* and caffeine grown with different media affect fungal growth. Therefore, the effect of caffeine concentration on fungal growth was evaluated using a chemically-defined medium (CDM) prepared as described in Section 2.4. This medium is mineral based with caffeine as the N source (expressed as mM N) and glucose as C source (10 g L^{-1}). The combination of experiments used to determine the importance of C and N supply from caffeine and other compounds in the medium is also described in Section 2.4. Unless stated otherwise, yeast extract was added to the chemically-defined medium (CDM) in all experiments described below.

Regardless of the concentration of caffeine-N, mycelium began growing quickly in all liquid media. However, as time passed, there was a noticeable demarcation between growth in the lowest concentration of caffeine-N and growth in the rest of the treatments. The lowest concentration of caffeine-N maintained growth similar to the control (no caffeine added), and, as the concentration of caffeine-N increased, the fungus continued growing but at a slower rate (Fig. 3.7). When caffeine-N was added at the equivalent of 2 and 5 mM, the final biomass was comparable with the control treatment (no caffeine-N added to the medium). The lowest final biomass was recorded when 10 mM caffeine-N was used (235 ± 9 mg mL^{-1}). No significant differences were found among the final pH values of the culture supernatants (Table 3.2).

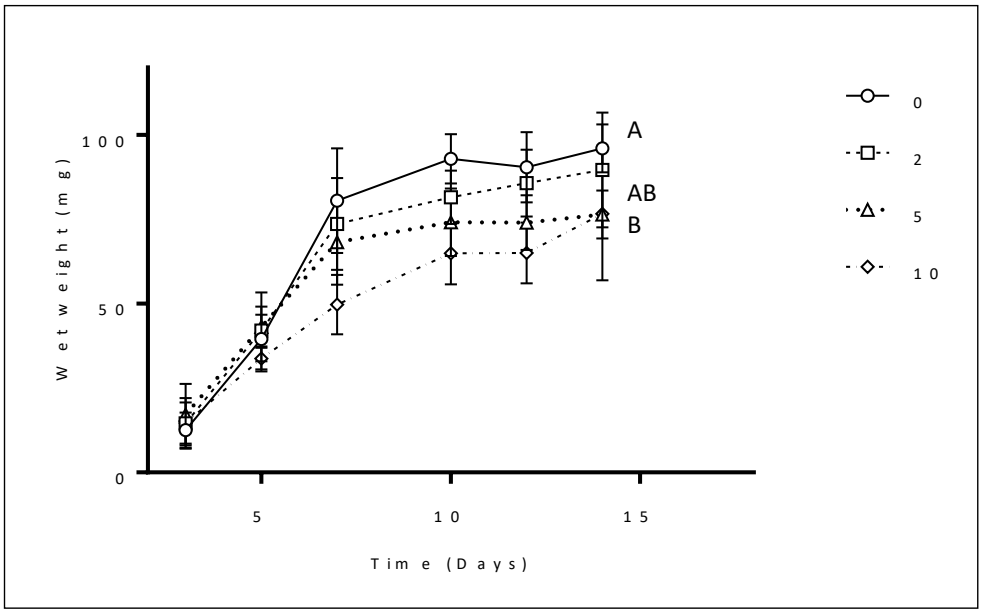

Figure 3.7 Mycelium of *Pleurotus ostreatus* strain PO13 grown in chemically-defined medium (CDM) (volume 100 mL) supplemented with nitrogen (N) supplied as caffeine-N (2, 5, 10 mM) and 1 g L^{-1} yeast extract. Points represent mean values (n = 3) and error bars are standard deviation. Uppercase letters represent differences between means using repeated measures ANOVA and a Tukeys post-hoc test (p <0.05).

Table 3.2 Final wet weight and fungal biomass, final pH and volume of the supernatant of *Pleurotus ostreatus* strain PO13 grown in chemically-defined medium (CDM) supplemented with low concentration of nitrogen (N) supplied as caffeine (0–10 mM) and yeast extract (YE) (1 g L^{-1}). Data represent mean values (n = 5) and standard deviation, letters represent differences between means using one-way ANOVA and a Tukeys post-hoc test (p <0.05).

Caffeine-N (mM N)	pH	Final wet weight (mg)	Final volume (mL)	Final biomass (mg mL^{-1})
1 g L^{-1} YE				
0	5.36 ± 0.04 (a)	18708 ± 1406 (A)	60 ± 1 (*a*)	314 ± 40 (*A*)
2	5.41 ± 0.03 (a)	18072 ± 794 (A)	62 ± 2 (*a*)	291 ± 20 (*A*)
5	5.35 ± 0.06 (a)	19578 ± 908 (A)	62 ± 1 (*a*)	317 ± 20 (*A*)
10	5.38 ± 0.02 (a)	15855 ± 679 (B)	67 ± 1 (*b*)	235 ± 9 (*B*)

3.3.4 Effect of caffeine and yeast extract on fungal growth in liquid culture

To evaluate the effect of caffeine concentration on fungal growth, CDM was prepared as described in Section 2.4. Unless stated otherwise, yeast extract was added to the CDM in all experiments described below. The concentration of caffeine from the supernatant was measured by HPLC as described in Section 2.10.

Increasing the concentration of caffeine-N resulted in a reduction of hyphal biomass. In comparison, increasing the concentration of yeast extract resulted in a considerable increase in biomass. A constant and steady growth pattern was supported when 0.05 g L^{-1} yeast extract was used in combination with caffeine-N at 2 and 5 mM (Fig. 3.8a). However, when the final biomass was measured, maximum biomass was achieved using 5 mM caffeine-N in combination with 0.05 g L^{-1} yeast extract (492 ± 24 mg mL^{-1}) (Table 3.3).

When the concentration of yeast extract was increased 10-fold (i.e. 0.5 g L^{-1}), enhancement of fungal growth was evident. With this level of yeast extract, the lowest and highest concentration of caffeine-N (2 and 10 mM) afforded the greatest mycelium growth. Considerably different amounts of biomass were produced by the end of the experimental period (2 mM caffeine-N: 491 ± 25 mg mL^{-1}; 5 mM caffeine-N: 205 ± 46 mg mL^{-1}). Additionally, increasing the concentration of caffeine-N to 10 mM also produced considerable amounts of final biomass (Table 3.3).

The greatest biomass production was achieved by increasing the concentration of yeast extract to 1.0 g L^{-1}. This suggests that when a higher concentration of yeast extract was used, high biomass production was attributable to the C source (glucose 10 g L^{-1}) and/or to

components in the yeast extract, and not to low or high caffeine concentration in the medium.

No caffeine consumption was detected as there were no changes in caffeine concentration in the supernatant of the cultures (Table 3.4). It is suggested the fungus most likely utilized the nitrogen from the yeast extract and it did not take up caffeine from the culture medium.

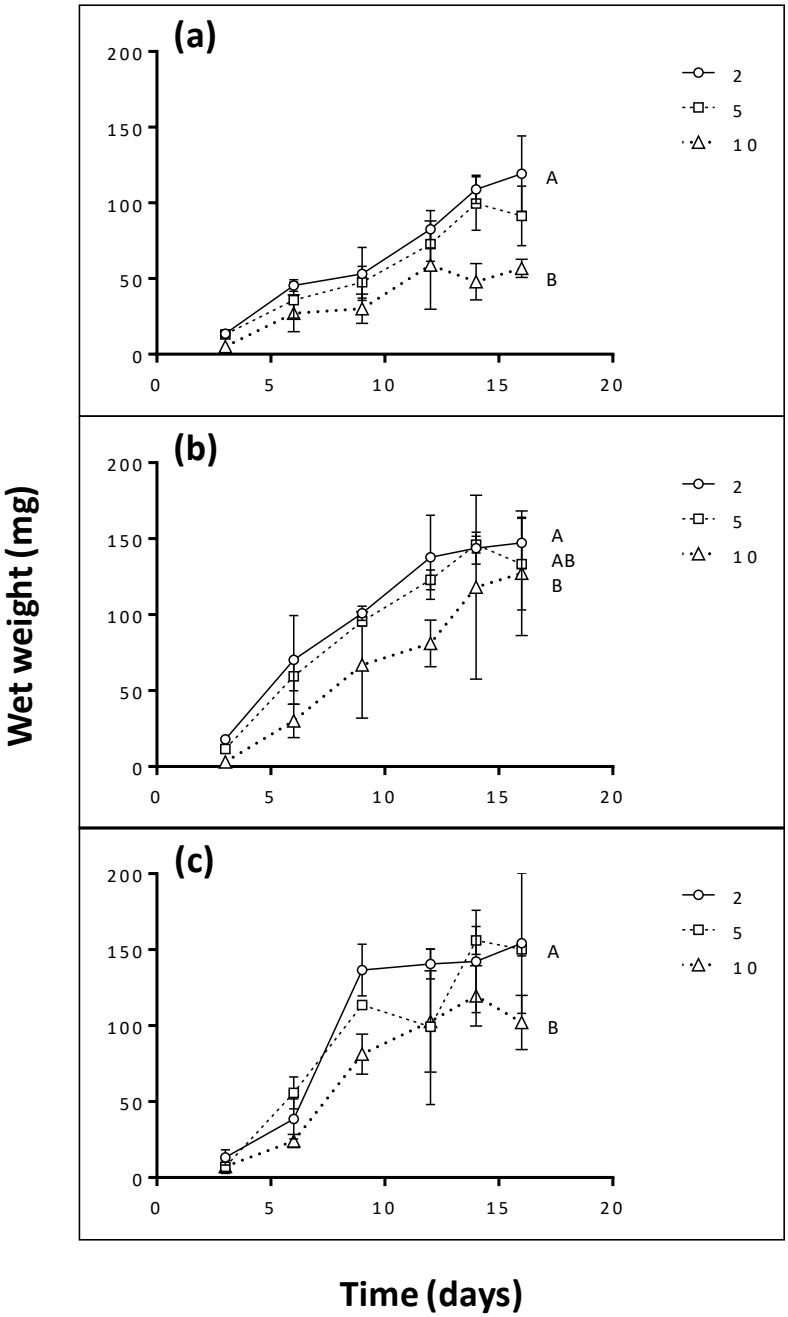

Figure 3.8 Mycelium of *Pleurotus ostreatus* strain PO13 grown in chemically-defined medium (CDM) (volume 100 mL) supplemented with nitrogen (N) supplied as caffeine-N (2, 5, 10 mM) and (a) 0.05 g L^{-1}, (b) 0.5 g L^{-1}, and (c) 1.0 g L^{-1} of yeast extract. Points represent mean values (n = 3) and error bars are standard deviation. Uppercase letters represent differences between means using repeated measures ANOVA and a Tukeys post-hoc test (p <0.05).

Table 3.3 Final pH and volume of the supernatant and wet weight and biomass of *Pleurotus ostreatus* strain PO13 grown in chemically-defined medium supplemented with nitrogen (N) supplied as caffeine-N (2, 5, 10 mM) and yeast extract (YE) (0.05, 0.5, 1.0 g L^{-1}). Data represent mean values (n = 5) and standard deviation, letters represent significant differences between means using one-way ANOVA and a Tukeys post-hoc test (p <0.05).

Caffeine-N (mM N)	pH	Wet weight (mg)	Final volume (mL)	*Final biomass (mg mL^{-1})
0.05 g L^{-1} YE				
2	5.39 ± 0.06 (a)	16122 ± 240 (A)	71 ± 1 (*a*)	226 ± 5 (*A*)
5	5.30 ± 0.01 (ab)	39519 ± 1521 (B)	80 ± 2 (*a*)	492 ± 24 (*B*)
10	5.22 ± 0.03 (b)	8941 ± 606 (C)	81 ± 9 (*a*)	112 ± 15 (*C*)
0.5 g L^{-1} YE				
2	5.16 ± 0.03 (a)	33072 ± 1511 (A)	67 ± 1 (*a*)	491 ± 25 (*A*)
5	4.96 ± 0.01 (b)	12999 ± 2514 (B)	64 ± 3 (*a*)	205 ± 46 (*B*)
10	5.00 ± 0.05 (b)	36015 ± 3669 (A)	67 ± 5 (*a*)	545 ± 100 (*A*)
1.0 g L^{-1} YE				
2	5.08 ± 0.03 (a)	31408 ± 1141 (A)	61 ± 2 (*a*)	515 ± 17 (*A*)
5	4.90 ± 0.04 (b)	32292 ± 6261 (AB)	60 ± 1 (*a*)	541 ± 104 (*A*)
10	4.92 ± 0.06 (b)	40896 ± 570 (B)	68 ± 36 (*b*)	603 ± 37 (*A*)

* Values were obtained after 16 days of continuous growth with no repetitive sampling.

Table 3.4 Values of caffeine-N concentration (mM) of the supernatant of *Pleurotus ostreatus* strain PO13 mycelium grown in chemically-defined medium supplemented with nitrogen (N) supplied as caffeine-N (2, 5, 10 mM) and yeast extract (YE) (0.05, 0.5, 1.0 g L^{-1}). Data represent mean values (n = 3) and standard deviation.

Caffeine-N (mM)	Initial caffeine-N (mM)	Final caffeine-N (mM)
0.05 g L^{-1} YE		
2	0.91 ± 0.05	1.17 ± 0.17
5	6.72 ± 0.23	6.97 ± 0.27
10	9.98 ± 0.29	10.28 ± 0.77
0.5 g L^{-1} YE		
2	1.05 ± 0.06	1.28 ± 0.25
5	6.67 ± 0.03	7.11 ± 0.13
10	9.41 ± 0.04	9.80 ± 0.03
1.0 g L^{-1} YE		
2	1.05 ± 0.03	1.06 ± 0.09
5	6.48 ± 0.11	6.79 ± 0.30
10	9.33 ± 0.10	9.78 ± 0.22

3.4 Discussion

Caffeine was found to have an inhibitory effect on the growth of *P*. *ostreatus* when supplied at concentrations greater than 10 mM N. This is not surprising as caffeine has been found to limit the development and growth of many organisms from diverse taxonomic groups (see Table 2 in Kim *et al*. 2010 and references therein). In the present investigation, this effect was also found when coffee extracts were added to different types of media.

Most of the available research on caffeine degradation has been done for Ascomycota and Zygomycota fungi, while there is very little research using Basidiomycota fungi. For example, in an investigation screening fungal strains to evaluate detoxification of coffee husks, the fungus *Phanerochaete chrysosporium* produced far less biomass per plate compared to *Rhizopus arrhizus* and *Aspergillus* sp. (Brand *et al*. 2000). In the same study, the growth rate

of *Rhizopus* sp. was 10-fold greater than measured for *P. ostreatus* in the current investigation.

There was no evidence that caffeine-N was utilized by *P. ostreatus* when grown on liquid medium. However, there was evidence of an interaction between the media and caffeine in their effect on fungal growth. For example, when yeast extract and caffeine were used to grow *P. ostreatus* in liquid media the fungus grew slowly when supplied with low amounts of yeast extract and caffeine, but when grown with higher amounts of yeast extract it grew proportionally faster than would be expected (See Fig. 3.6). This might suggest that compounds present in the yeast extract (e.g. vitamins, amino acids) have a greater effect on fungal growth when caffeine is also present in the culture medium.

For several strains of *Pseudomonas,* it has been found that specific components from yeast extract might be responsible for enhancing not only biomass production but also production of particular enzymes used to degrade environmental pollutants (Li *et al.* 2011). Yeast extract is an essential component of culture medium that facilitates growth of many microorganisms in industrial applications, for example, during the production of compounds for the food industry (Gorret *et al.* 2001; Lo *et. al*. 1997; Olmos-Dichara *et al.* 1997). Alternative sources of energy for hydrogen photoproduction have been evaluated by using yeast extract in the growth medium to stimulate the process (Hakobyan *et al.* 2012). In the present investigation, a minimal concentration of yeast extract (0.5 g L^{-1}) was required in a defined growing medium for *P. ostreatus* strain PO13 to enable at least some mycelia growth. Some fungi, particularly those used for mushroom cultivation, are generally adapted to selected growing conditions (Foulongne-Oriol *et al*. 2014; Menolli & Paccola-

Meirelles 2010). The strain used in the present investigation (*P*. *ostreatus* strain PO13) might have been growth-limited by presence of one or more components (e.g. vitamins, amino acids, cofactors) in the yeast extract.

In this study, caffeine degradation by *P. ostreatus* was not observed when C was supplied as glucose (10 g L^{-1}) and N was supplied in the form of caffeine. Since there are limited reports for utilization of caffeine by fungi, few examples can be used for direct comparison with this study. Apart from a report that *Phanerochaete chrysosporium* can degrade caffeine (Brand *et al*. 2000), there is no other information available for caffeine degradation by Basidiomycota fungi. However, more information is available for degradation of caffeine by bacteria. For example, a recent investigation showed that some C sources (sucrose and galactose) favoured degradation of caffeine by the bacterium, *Leifsonia*, whereas others such as cellulose negatively affected caffeine degradation (Ibrahim *et al.* 2016). In another investigation using *Penicillium verrucosum,* it was found that the fungus could not degrade caffeine in presence of other N sources, and caffeine degradation occurred only when no other N source was present in the medium (Roussos *et al*. 1994). It has also been found that the addition of N can affect caffeine degradation by other fungi such *Aspergillus tamarii* (Hakil *et al*. 1999) and different strains of *Aspergillus* spp. and *Penicillium* spp. (Roussos *et al*. 1995). The fungus *Paeocilomyces gunnii* was found to be able to degrade caffeine by means of *N*-demethylation (Zheng *et al*. 2016). Because yeast extract was required in the liquid medium for growth of *P*. *ostreatus* the presence of an alternative source of N may explain why there was no degradation of caffeine.

In this investigation, *P. ostreatus* was maintained on PDA and a homologous medium was prepared prior to the commencement of each growth experiment. In other studies, fungal strains were maintained on media with a coffee infusion or coffee extract prior to inoculation (Hakil *et al.* 1999; Tagliari *et al.* 2003). This might have led to the situation where the fungal strain has become adapted to the growth conditions used prior to experimentation which could have activated specific enzymes to degrade molecules present in the growth medium or substrate. Fungi have evolved to respond to environmental changes by modifying their metabolism, not only for nutrient deficiencies, but also for a range of environmental stresses such as light, gases and temperature (Bahn *et al.* 2007). It is possible that the fungal strain used in this study might not have had sufficient time or have reached minimum trigger conditions to adjust to the conditions to grow and degrade caffeine under the conditions tested. This could be evaluated by growing and maintaining the strain in a caffeine-containing medium (i.e. SCG extract-agar, or low caffeine-N) prior to transfer onto the medium with caffeine as N or as an alternative C source.

3.5 Conclusion

When used as a sole source of N for growth, caffeine at concentrations below 10 mM N did not inhibit growth of *P. ostreatus* strain PO13 to a substantial degree. In addition, this particular strain of *P. ostreatus* did not take up or degrade caffeine from the culture medium if more easily accessible sources of C and N were available.

The utilization of residues from the coffee industry to produce mushrooms is an attractive approach to recycling of wastes. This study showed how mycelial growth of *P. ostreatus* and

biomass production was achieved when different combinations of N- and C-containing compounds were supplied in a complex medium (i.e. coffee extracts with PDA) and how the growth of *P*. *ostreatus* strain PO13 was inhibited in a medium containing caffeine. From this perspective, *P*. *ostreatus* may not be useful for detoxification of coffee waste, but from the results presented, this fungus is able to grow in the presence of caffeine and SCG and could be an alternative substrate for mushroom production. The growth of this species in the presence of alternative inorganic and organic sources of N will be investigated in Chapter 4.

Chapter 4 Assimilation of different nitrogen sources by oyster mushroom

4.1 Introduction

Nitrogen (N) is an essential element for all living organisms. Fungi have evolved to utilize a large variety of N and carbon (C) sources including living and dead plant tissues (Stamets 2005). They can make use of many forms of N, and when there is an abundance or shortage of N, they are able to modify their metabolism accordingly (Todd 2016). In nature, organic N comprises the dominant form of available N. Organic N sources can also be used, and amino acids can serve both as N and C source for fungi (Chalot & Brun 1998). When environmental conditions are not favorable for growth, fungi can also store and recycle N (Shoji & Craven 2011). Fungal metabolic systems regulate expression of genes for N uptake according to their needs (Todd 2016). The genes for uptake and metabolism of alternative N sources are repressed when a preferred or more easily metabolized type of N, such as ammonium, is present in the growing medium (Todd 2016).

Nitrogen uptake and utilization by fungi has been studied using a range of different laboratory-based methods. For example, for cultivation and production purposes, *Pleurotus ostreatus* can be grown on wheat-based agar cultures to produce a high quality and inexpensive spawn (Sainos *et al*. 2006). To optimize spawn production, two strains of *P. ostreatus* were used to assess the effect of inorganic N and substrate particle size on protein and enzymatic production using sugar cane bagasse (Membrillo *et al*. 2008). Using this type of substrate, the geometrical ratio and size of the fibres of sugar cane bagasse was found to affect enzyme production and the optimum particle size differed between the two strains (Membrillo *et al*. 2008).

Some of the parameters required for cultivation of *P*. *tuber-regium* have been evaluated using liquid media as the use of this type of medium readily allows modification of C and N sources to optimize biomass production (Wu *et al*. 2003). Using a similar system, the effect of C:N ratio on production of dietary fiber by *P*. *tuber-regium* was determined by Wu *et al*. (2004). *Pleurotus saju-caju* has also been grown in submerged culture for production of enzymes using different C and N sources (Bettin *et al*. 2009) as the use of liquid medium is particularly well suited for growth of these species. Another example, a study investigating the effect of different C and N sources together with growth inducers, used liquid culture of the morel, *Morchella crassipes* (Kanwal & Reddy 2011). Maximum biomass was achieved using a mineral salt broth with glucose, but fructose and mannitol as a C source also promoted production of fungal biomass. When N sources were varied, sodium nitrate and peptone resulted in comparable amounts of biomass production (Kanwal & Reddy 2011).

As a preliminary step for cultivation of the edible mushroom, *Psathyerella atroumbonata*, a study using liquid media prepared with a variety of C and N sources was used to elucidate the nutritional requirements of this species. A medium containing glucose as the C source and yeast extract as N source was chosen for C:N determination experiments and a C:N ratio of 2:3 was found for optimal growth (Jonathan & Fasidi 2001).The effect of N deficiency in liquid medium was also assessed for another edible mushroom, *Trametes versicolor*. It was revealed that the ubiquitin/proteasome system was affected by the deficiency of N (Staszczak 2008).

The type and concentration of N sources affects enzymatic activities in Basidiomycota fungi (Elisashvili *et al*. 2001; Membrillo *et al*. 2008). To degrade lignin efficiently, white rot fungi

require a medium with limited N (Todd 2016). In medicinal applications, for the production of antitumor polysaccharides by *Pleurotus*, the best N source was found to be yeast extract while for biomass production corn steep liquor was most effective (Gern *et al*. 2008).

Possible enzymatic pathways responsible for N metabolism have been identified for *P. ostreatus* (Mikes *et al*. 1994) and the key enzyme responsible, glutamine synthetase, has been characterized (Zofall *et al*. 1996). Similar investigations have manipulated N availability to optimize the production of dietary fiber (Papaspyridi *et al*. 2010), and laccase and peroxidase enzyme production by three species of *Pleurotus* (Stajić *et al*. 2006). It has been reported that *P. ostreatus* can grow well under a range of C:N in the culture medium as a result of its adaptative capabilities (D'Agostine *et al*. 2011).

4.1.1 Aims of this study

A variety of techniques have been used to assess fungal growth to provide insights into fungal biology and physiology for practical applications such as commercial cultivation of mushrooms for consumption and mass production of pharmaceuticals. As fungal growth depends on nutrients present in the growth medium, an understanding of the ways in which the composition of the growth medium affects hyphal growth and physiology is essential. By manipulating the composition of media in solid and liquid culture, this study aims to determine the effect of different forms of N, including caffeine, on mycelial growth of *P. ostreatus*. The hypotheses being tested are:

1. The type and form of N compounds available (inorganic or organic sources including caffeine) will affect mycelium growth and development differently when added to a culture medium.

2. With addition of caffeine to a culture medium at below-inhibitory concentrations, the fungus can use caffeine as a source of N for growth.

4.2 Materials and methods

Methods used in the experiments presented in this chapter are described in Section 2.4. (see Table 2.4 for detailed concentration of C and caffeine-N). Statistical analyses included one-way ANOVA (radial growth rate, biomass, final pH) and repeated measures ANOVA (growth curves, biomass and pH). The repeated measures ANOVA analyses were checked to meet the assumptions (independent observations, normality and sphericity) and, when required, appropriate corrections for multiple comparisons were applied (e.g. if p >0.05, use sphericity assumed, if p <0.05, (i) Greenhouse-Geisser Epsilon <0.75, use Greenhouse-Geisser or multivariate results, or (ii) if Greenhouse-Geisser Epsilon >0.75, use Huynh-Feldt results) (Field 2013; Howell 2002).

The details of experimental conditions are provided in Section 2.4 and in Table 4.1 below (i.e. for experiments using agar and liquid culture comparing fungal growth with different N sources). For ease of presentation, urea, an organic N source, has been grouped with inorganic N sources because of its low molecular weight.

In experiments presented in Section 4.3.1, two vitamin mixtures were added (1 mL per 100 mL of culture medium): (i) minimum essential medium (MEM) composed of choline chloride, D-calcium pantothenate, colic acid, nicotinamide, pyridoxal hydrochloride, thiamine hydrochloride (each at a concentration of 100 mg L^{-1}), riboflavin (10 mg L^{-1}), i-inositol (200 mg L^{-1}) and sodium chloride (8,500 mg L^{-1}); (ii) STABA vitamin solution (myo-inositol (100 mg L^{-1}), thiamine hydrochloride, D-biotin, choline chloride, calcium pantothenate, (each at a concentration of 1 mg L^{-1}) pyridoxine hydrochloride, niacinamide (each at a concentration of 2 mg L^{-1}), riboflavin, aminobenzoic acid, folic acid (each at a concentration of 0.5 mg L^{-1}), cyanocobalamin (vitamin B12; at a concentration of 0.0015 mg L^{-1}). The nucleotide β-adenosine was used at a concentration 0.05 mg mL^{-1}.

Table 4.1 Inorganic and organic nitrogen sources used with their respective concentration in culture medium.

Nitrogen source	Molecular weight (g mol^{-1})	Nitrogen (*units)	Nitrogen concentration (mM)	Chemical concentration (mM)**
Agar culture				
Adenine (Ade)	135.13	5	18.75	3.75
Alanine (Ala)	89.09	1	18.75	18.75
Ammonium chloride (AmChl)	53.49	1	18.75	18.75
Ammonium nitrate (AmNit)	80.04	2	18.75	9.38
Caffeine (Caff)	194.19	4	18.75	4.69
Calcium nitrate (CaNit)	164.09	2	18.75	9.38
Glutamic acid (Glu)	147.13	1	18.75	18.75
Glycine (Gly)	75.07	1	18.75	18.75
Guanine (Gua)	151.13	5	18.75	3.75
Histidine (His)	155.15	3	18.75	6.25
Phenylalanine (Phe)	165.20	1	18.75	18.75
Tryptophan (Try)	204.23	2	18.75	9.38
Tyrosine (Tyr)	182.20	1	18.75	18.75
Urea (Urea)	60.06	2	18.75	9.38
Liquid culture				
Alanine (Ala)	89.09	1	10	10
Ammonium chloride (AmChl)	53.49	1	10	10
Ammonium nitrate (AmNit)	80.04	2	10	5
Caffeine (Caff)	194.19	4	10	2.5
Calcium nitrate (CaNit)	164.09	2	10	5
Glutamic acid (Glu)	147.13	1	10	10
Glycine (Gly)	75.07	1	10	10
Histidine (His)	155.15	3	10	3.33
Urea (Urea)	60.06	2	10	5

* Number of atoms per molecule

** Chemical concentration in the medium: the total compound present in the medium, based on nitrogen (N) concentration calculated as concentration of N required in the medium/atoms of N per molecule. This assumes that all N atoms in the molecule are equally available to the growing fungus.

4.3 Results

4.3.1 Optimizing liquid growth medium for biomass production of *Pleurotus ostreatus*

In several initial experiments, *Pleurotus ostreatus* strain PO13 did not grow well with ammonium chloride as the sole N source in the chemically-defined medium (CDM) used for liquid culture (data not shown). It was hypothesized that growth might be limited by a growth factor so the effects of adding yeast extract, two types of vitamin mixtures (Minimum Essential Medium (MEM) and STABA) and β-adenosine (a promoter of fungal growth; Domodon *et al*. 2004; Zhang *et al*. 2010) were examined individually and in combination with N in the form of ammonium chloride or ammonium tartrate.

Ammonium chloride (10 mM N) in combination with yeast extract (0.5 g L^{-1}) promoted better growth of *P*. *ostreatus* than the other additives tested, whereas poor growth was observed when vitamins (MEM or STABA) or β-adenosine were used (Fig 4.1a). A similar pattern of growth was recorded when ammonium tartrate (10 mM N) was used, albeit with slightly slower mycelial growth than for addition of ammonium chloride (Fig. 4.1b). The culture supernatant showed a marked fall in pH in the first few days of cultivation for both N sources tested, indicating a relationship between pH and observed biomass production (Fig. 4.1c). From these results, ammonium chloride was chosen as the most suitable source of N and yeast extract was selected as a suitable growth stimulant for further experiments. In the following experiment, *P*. *ostreatus* strain PO13 grew best with addition of yeast extract (0.5 g L^{-1}) to the medium to overcome deficiencies in growth observed using CDM medium alone. Yeast extract was not included in the calculations for C:N in the medium as it was used as growth stimulant, thus C:N was calculated from N and C added to the medium.

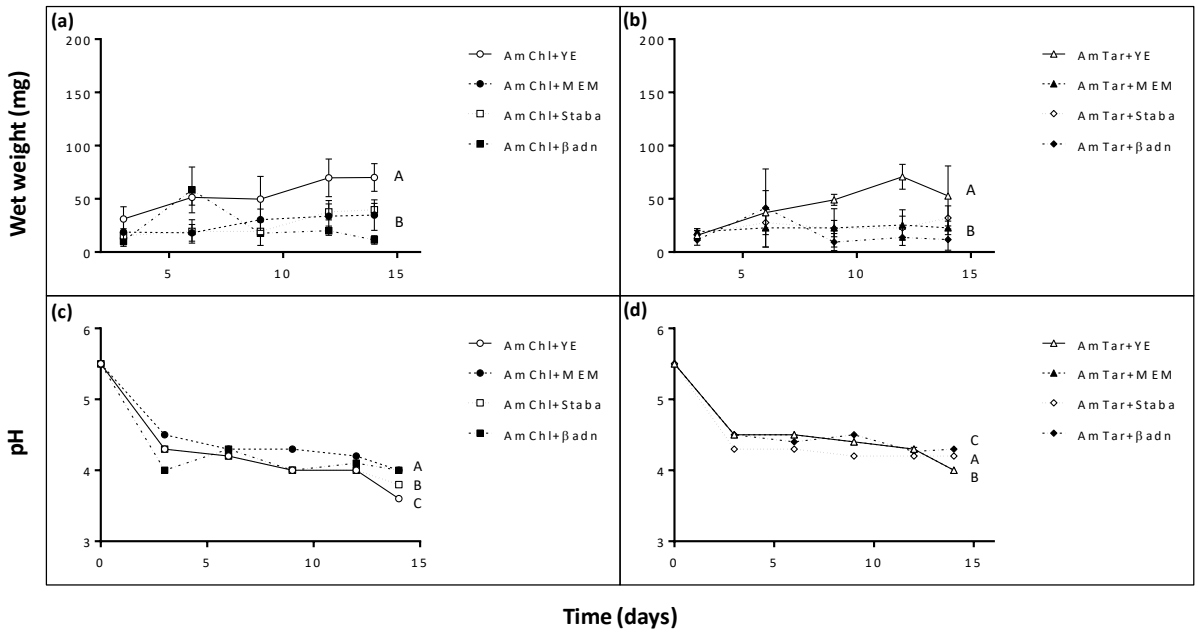

Figure 4.1 Biomass production and pH of supernatant of *Pleurotus ostreatus* strain PO13 grown in chemically-defined medium (CDM) (culture volume 100 mL) supplemented with 10 mM nitrogen (N) supplied as (a, b) ammonium chloride (AmChl) and (c, d) ammonium tartrate (AmTar). Points represent mean values (n = 3) and error bars are one standard deviation. Uppercase letters represent differences assessed using repeated measures ANOVA and Tukeys post-hoc test (p <0.05). YE – yeast extract, MEM – Minimum Essential Medium, Staba – STABA vitamin solution, βadn – β-adenosine.

4.3.2 Yeast extract as enhancer of fungal growth on agar plates and in liquid culture

The effect of adding yeast extract was evaluated at different concentrations using different types of media (agar and liquid culture). Unless stated otherwise, yeast extract (YE) was added to the chemically-defined medium (CDM) in all experiments described below. Statistically significant differences were found for the radial growth rates of *P. ostreatus* mycelium among yeast extract treatments using agar (Fig. 4.2a; one-way ANOVA, Tukeys post-hoc test, p = 0.001). Mean radial growth rate of mycelium grown on agar MM

supplemented with different concentrations of yeast extract was greatest for 2.0 g L^{-1} (5.36 ± 0.25 mm day^{-1}) and was significantly greater than for 0.5 g L^{-1} yeast extract (3.51 ± 0.76 mm day^{-1}) (one-way ANOVA, Tukeys post-hoc test, p = 0.021) and no added yeast extract (2.50 ± 0.01 mm day^{-1}) (one-way ANOVA, Tukeys post-hoc test (p <0.05), p = 0.001). Minimal growth was observed in the absence of yeast extract. Furthermore, steady mycelial growth was achieved by adding yeast extract to agar medium (Fig. 4.2b), and the increasing effect on growth with increasing concentration of yeast extract is evident.

Liquid culture is a useful technique for investigating nutritional requirements of fungi because it allows easy manipulation of media composition and evaluation of fungal growth, as shown previously (Chapter 3). However, in preliminary experiments, liquid culture of *P. ostreatus* in liquid MM proved unsuccessful (data not shown), therefore a different medium had to be adopted. To test the effect of adding yeast extract as growth factor source in liquid culture, a chemically-defined medium (CDM), was prepared as described in Section 2.4. A range of concentrations of yeast extract was added as selected from the above experiment (0–0.5 g L^{-1}). In liquid medium, increasing the concentration of yeast extract also resulted in greater growth where mycelium production was measured as biomass (Fig. 4.3). There were statistically significant differences in fungal growth among treatments when wet weight was measured (Fig. 4.3a; one-way ANOVA, Tukeys post-hoc test, p <0.001), with the highest yeast extract concentration (0.5 g L^{-1}) yielding the greatest fungal biomass. Similarly, when fungal biomass was calculated on a volume basis (mg mL^{-1}), there were statistically significant differences among treatments (Fig. 4.3c; one-way ANOVA, Tukeys post-hoc test, p <0.001). When 0.5 g L^{-1} yeast extract was used, mycelium growth of *P. ostreatus* was

enhanced (52 ± 1 mg mL^{-1}) and was 10-fold greater compared to the negative control (no yeast extract added; 5 ± 1 mg mL^{-1}). This concentration of yeast extract was deemed to be sufficient for providing resources for fungal growth and it was chosen for use in further experiments.

The initial pH was adjusted to 5.5 for all treatments, but it was found that the final pH of the liquid cultures changed depending on the amount of yeast extract added (Table 4.2). With increasing concentrations of yeast extract, pH increased significantly (one-way ANOVA, Tukeys post-hoc test, p <0.001).

Residual ammonium decreased significantly as the concentration of yeast extract increased (one-way ANOVA, Tukeys post-hoc test, p <0.001). Maximum residual ammonium was measured when no yeast extract was used. Although maximum biomass was achieved using 0.5 g L^{-1} yeast extract, C:N of the biomass was comparable with the other treatments. There were no statistically significant differences for C:N in the final fungal biomass (Table 4.2; one-way ANOVA, Tukeys post-hoc test, p = 0.028).

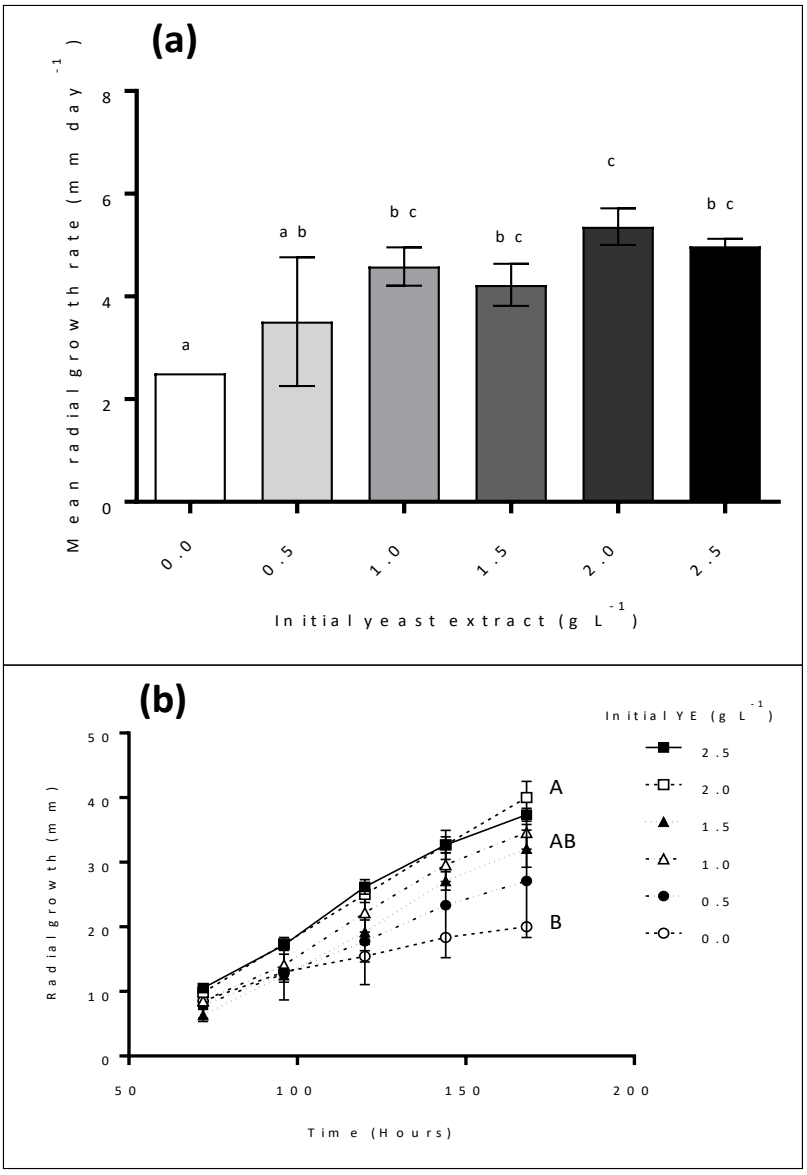

Figure 4.2 Increase in (a) radial growth rate and (b) radial growth of *Pleurotus ostreatus* strain PO13 mycelium grown on minimal medium (MM) supplemented with yeast extract (YE) (0–2.5 g L^{-1}). For (a) bars represent mean values (n = 3) and error bars are standard deviation; in (b) points represent mean values (n = 3) and error bars are one standard deviation. Lowercase letters represent differences among means using one-way ANOVA and Tukeys post-hoc test (p <0.05). Uppercase letters represent differences among means using repeated measures ANOVA and Tukeys post-hoc test (p <0.05).

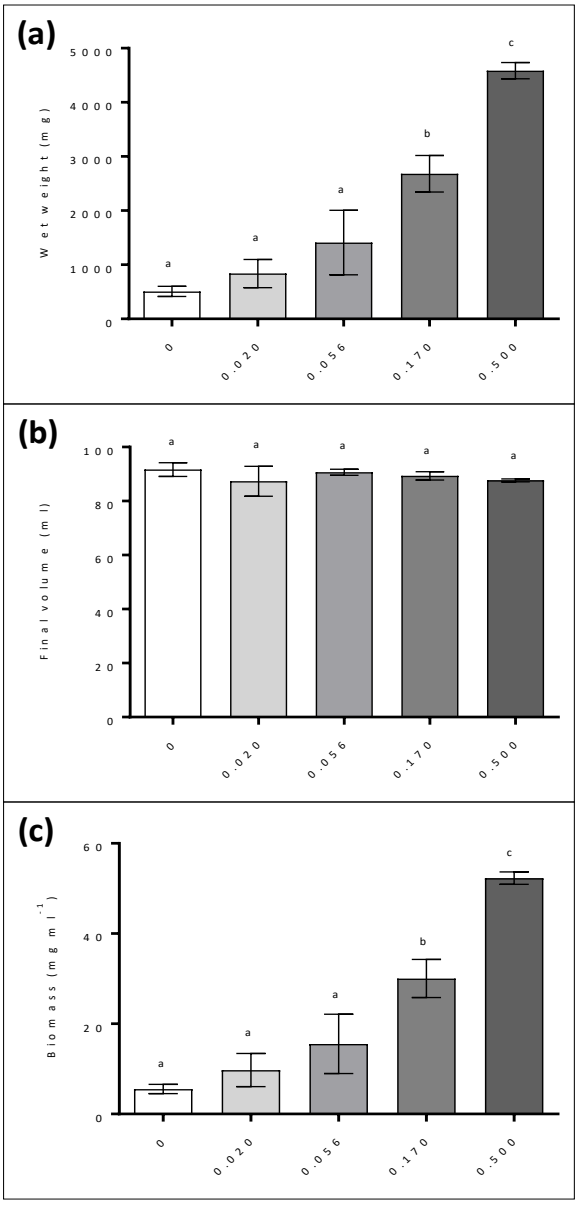

Figure 4.3 Final (a) wet weight, (b) culture volume and (c) biomass of *Pleurotus ostreatus* strain PO13 mycelium grown in chemically-defined medium (CDM) supplemented with different amount of yeast extract (0–2.5 g L^{-1}) and low concentration of nitrogen (N) (10 mM) in the form of ammonium chloride. Bars represent mean values (n = 3) and error bars are standard deviation. Lowercase letters represent differences among means using one-way ANOVA and a Tukeys post-hoc test (p <0.05).

Table 4.2 Final fungal biomass (wet weight) and carbon:nitrogen (C:N) ratio of mycelium, final pH and residual ammonium of the supernatant of *Pleurotus ostreatus* strain PO13 grown in chemically-defined medium supplemented with different concentrations of yeast extract (0–0.5 g L^{-1}) and low concentration of N (10 mM) in the form of ammonium chloride. Data represent mean values (n = 3) and standard deviation, letters represent differences among means using one-way ANOVA and a Tukeys post-hoc test (p <0.05).

Initial yeast extract (g L^{-1})	C:N ratio in the medium	Biomass (mg mL^{-1})	Final pH	Biomass C:N ratio	Residual ammonium (µM)
0	33.3	6 ± 1 (a)	4.59 ± 0.48 (A)	13 ± 2 (*ab*)	2.46 ± 0.33 (*B*)
0.020	33.3	10 ± 4 (a)	4.90 ± 0.11 (AB)	12 ± 1 (*a*)	2.32 ± 0.27 (*B*)
0.056	33.3	16 ± 7 (a)	5.47 ± 0.09 (BC)	17 ± 2 (*b*)	2.22 ± 0.10 (*B*)
0.170	33.3	30 ± 4 (b)	5.52 ± 0.05 (BC)	14 ± 2 (*ab*)	1.67 ± 0.05 (*A*)
0.500	33.3	52 ± 1 (c)	5.83 ± 0.14 (C)	12 ± 1 (*ab*)	1.22 ± 0.04 (*A*)

4.3.3 Effect of nitrogen sources on fungal growth in solid culture

To assess the effect of a range of N sources on fungal growth on agar culture, MM and solid media with equivalent concentrations but different sources of N were prepared as described in Section 2.4.

The radial growth rate of *P. ostreatus* ranged from 0.14 mm day^{-1} to more than 4.50 mm day^{-1} (Fig. 4.4). The greatest growth rate of mycelium in solid culture was measured when 0.1 mM N was supplied in the form of caffeine-N (4.79 ± 0.23 mm day^{-1}; Fig. 4.4c). This suggests that caffeine at this concentration does not inhibit growth, and the fungus utilized the N from the caffeine.

When inorganic/low molecular weight N sources were used, there were statistically significant differences among treatments (one-way ANOVA, Tukeys post-hoc test, p = 0.001). Ammonium nitrate (AmNit) produced the lowest growth rate (3.09 ± 0.45 mm day^{-1}, p = 0.001) compared to the two other N sources used (calcium nitrate and urea) and ammonium chloride, all at concentrations of 18.75 mM (Fig. 4.4a).

When provided with organic N sources, there were statistically significant differences among treatments (one-way ANOVA, Tukeys post-hoc test, p <0.001). When N was supplied as a simple form of organic N (amino acid), the fungus grew significantly faster with addition of glycine or glutamic acid (3.65 ± 0.43 and 3.16 ± 0.39 mm day^{-1}, respectively; Fig. 4.4b). An example of mycelium growth with glycine is shown in Figure 4.6c, dense white mycelium was considered as an indicator of good fungal growth. The slowest growth rate was found for addition of N in the form of alanine (0.14 ± 0.01 mm day^{-1}; Fig. 4.4b) which was statistically different compared to the growth associated with other amino acids (one-way ANOVA, Tukeys post-hoc test, p <0.001). The growth of *P. ostreatus* with N available as ammonium chloride (18.75 mM) resulted in slow radial growth (3.94 ± 0.13 mm day^{-1}). When grown under N-free conditions, radial growth rates were also low (3.11 ± 0.39 mm day^{-1}) compared to N supplied as 0.1 mM caffeine-N (Fig. 4.4c).

In addition, variability of fungal growth rate on media supplemented with different N sources was observed. For most of the inorganic N sources, fungal growth was found to be consistent over time; however, when ammonium chloride was supplied, fungal growth started slowly, then accelerated over time and plateaued to be equivalent to growth associated with calcium nitrate and urea by the end of the measurement period (Fig. 4.5a).

In contrast, marked differentiation in daily growth rate occurred when organic N sources were used (Fig. 4.5b). Equivalent growth of mycelium was found when glycine and glutamic acid were supplied in agar culture, but radial growth for alanine was considerably lower than for ammonium chloride and other N sources over time (Fig. 4.5b; repeated measures ANOVA, F = 617.607, p <0.001).

Rapid radial growth of *P*. *ostreatus* was found when caffeine-N (0.1 mM) was supplied in agar culture and was significantly different from the other treatments (Fig. 4.5c; repeated measures ANOVA, F = 1501.698, df = 4, p <0.001). The time × treatment interaction of radial growth was also significantly different among treatments (F = 36.809, df = 12, p <0.001). A Tukeys post-hoc test revealed that mycelial growth resulting when caffeine-N was supplied at 1.0 mM was not significantly different from ammonium chloride. These results suggest that caffeine could be a good N source; however, when it was used at higher concentrations, growth was reduced, presumably due to an inhibitory effect.

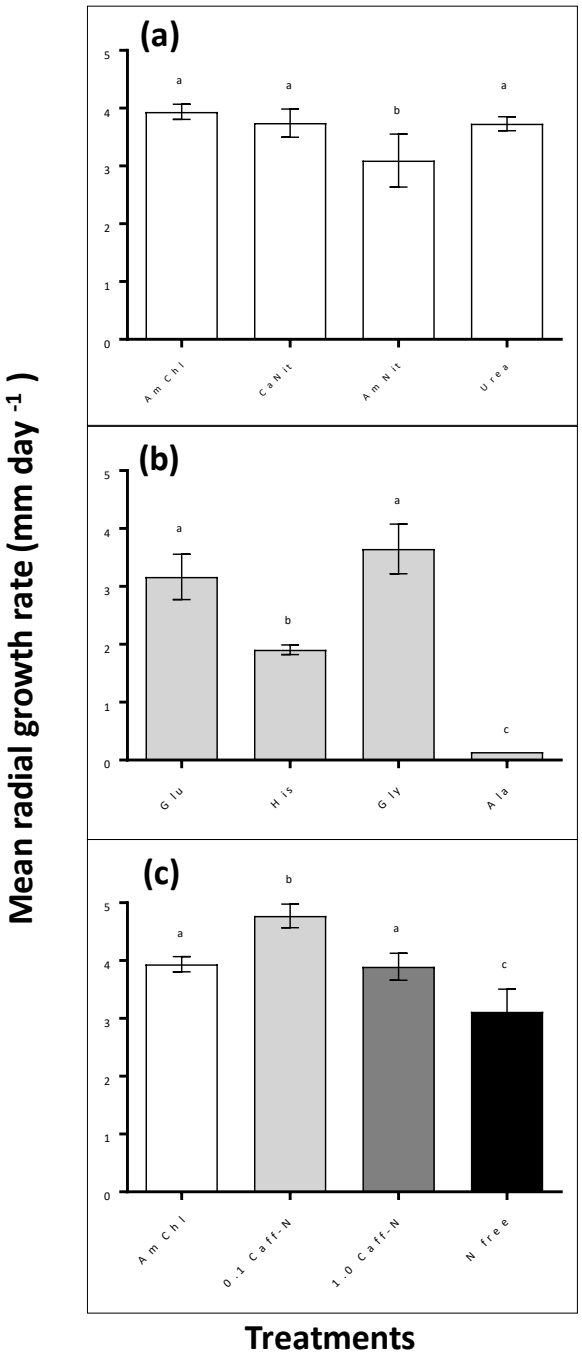

Figure 4.4 Rate of radial growth of *Pleurotus ostreatus* strain PO13 mycelium grown on minimal medium supplemented with nitrogen (N) at 18.75 mM as (a) ammonium chloride (AmChl), calcium nitrate (CaNit), ammonium nitrate (AmNit) and urea, (b) glutamic acid (Glu), histidine (His), glycine (Gly) and alanine (Ala), and (c) two concentrations of caffeine-N (0.1 and 1.0 mM, caff-N), ammonium chloride and N free. Bars represent mean values (n = 5) and error bars are standard deviation. Lowercase letters represent differences among means using one-way ANOVA and a Tukeys post-hoc test (p <0.05).

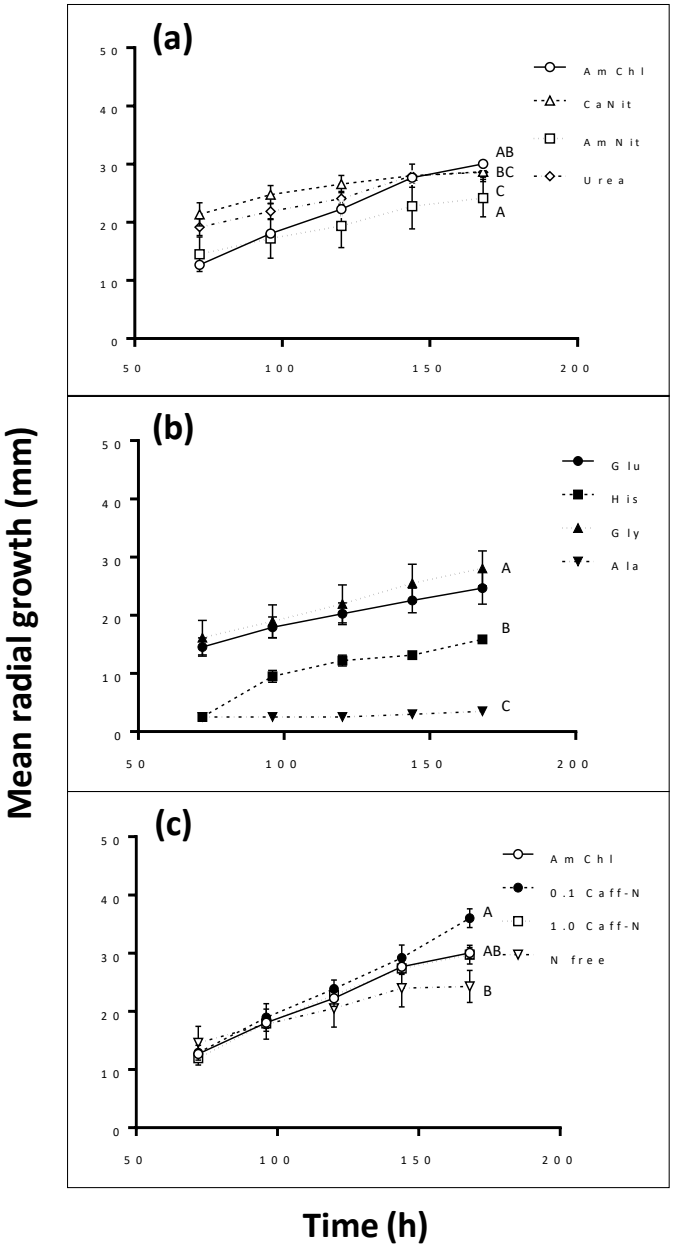

Figure 4.5 Radial growth of mycelium of *Pleurotus ostreatus* strain PO13 grown on minimal medium supplemented with nitrogen (N) at 18.75 mM as (a) ammonium chloride (AmChl), calcium nitrate (CaNit), ammonium nitrate (AmNit) and urea, (b) glutamic acid (Glu), histidine (His), glycine (Gly) and alanine (Ala), and (c) two concentrations of caffeine-N (0.1 and 1.0 mM N, caff-N), ammonium chloride and N free. Points represent mean values (n = 5) and error bars are standard deviation. Uppercase letters represent differences among means using repeated measures ANOVA and a Tukeys post-hoc test (p <0.05).

Mycelial growth was observed regardless of the medium composition, either as thinner and transparent mycelia (Fig. 4.6a, b) or as dense and white, cotton-like mycelia (Fig. 4.6c, d). Hyphal extension was similar on water agar (WA) and N-free MM conditions (Fig. 4.6b).

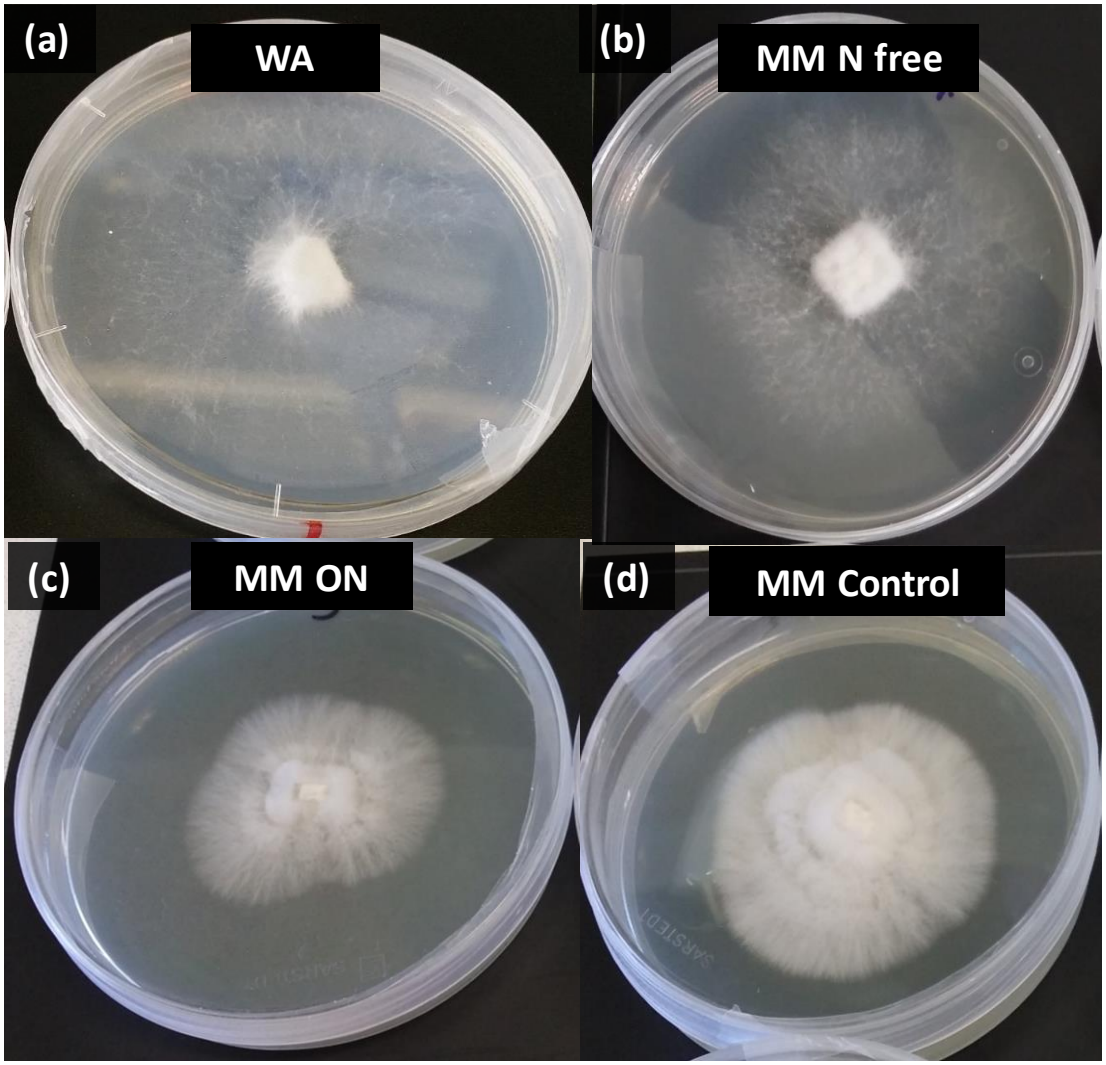

Figure 4.6 Mycelium of *Pleurotus ostreatus* strain PO13 grown on (a) water agar (WA), (b) nitrogen free minimal medium (MM N free), (c) MM with organic N supplied as glycine at 18.5 mM N (MM ON), and (d) MM with inorganic N supplied as ammonium chloride at 18.5 mM N (MM Control).

4.3.4 Effect of aromatic nitrogen sources on fungal growth in solid culture

In the caffeine molecule, N is part of the aromatic structure, which impacts accessibility and therefore utilization by microorganisms. To investigate the effect of position of the N atoms in the molecule (aromatic or aliphatic N), a range of aromatic N sources was tested (adenine, phenylalanine, guanine, tryptophan, tyrosine, caffeine) for impact on fungal growth on agar culture. MM was used, and N sources were added as described in Section 2.4.

Overall, aromatic N sources did not support fungal growth as well as inorganic or simple organic N sources did (Fig. 4.7) and statistically significant differences were found among treatments (one-way ANOVA, Tukeys post-hoc test, p <0.001). The greatest growth rate for *P. ostreatus* was measured when an inorganic form of N (ammonium chloride, control) was added to MM agar (3.53 ± 0.26 mm day^{-1}; Fig. 4.7a; one-way ANOVA, Tukeys post-hoc test, p <0.001). Adenine, phenylalanine and guanine all promoted rates of growth which were significantly lower than for ammonium chloride, but greater than fungal growth on agar supplied with caffeine (Fig. 4.7a; one-way ANOVA, Tukeys post-hoc test, p <0.002). Interestingly, adenine and guanine both have N positioned in the aromatic ring and in the amine group, whereas phenylalanine only has N as an aliphatic amino group. The slowest growth rate was recorded with tryptophan as N source (Fig. 4.7a; 0.39 ± 0.14 mm day^{-1}). This amino acid also has one N in the aromatic ring while the other N is in the form of an amino group. In addition, a significantly faster radial growth was measured when inorganic N (ammonium chloride) was used as N source in comparison with aromatic N compounds

(Fig. 4.7b; repeated measures ANOVA, $F_{(4,112)} = 911.151$, $p < 0.001$). From these results, there was no clear relationship between growth rates and the chemical structure of the N sources. In addition, for all the organic N sources tested (except tyrosine), an inverse relationship with solubility in water was observed: the more soluble N sources such as tryptophan and caffeine produced slower growth, compared to less soluble ones like adenine, guanine and phenylalanine.

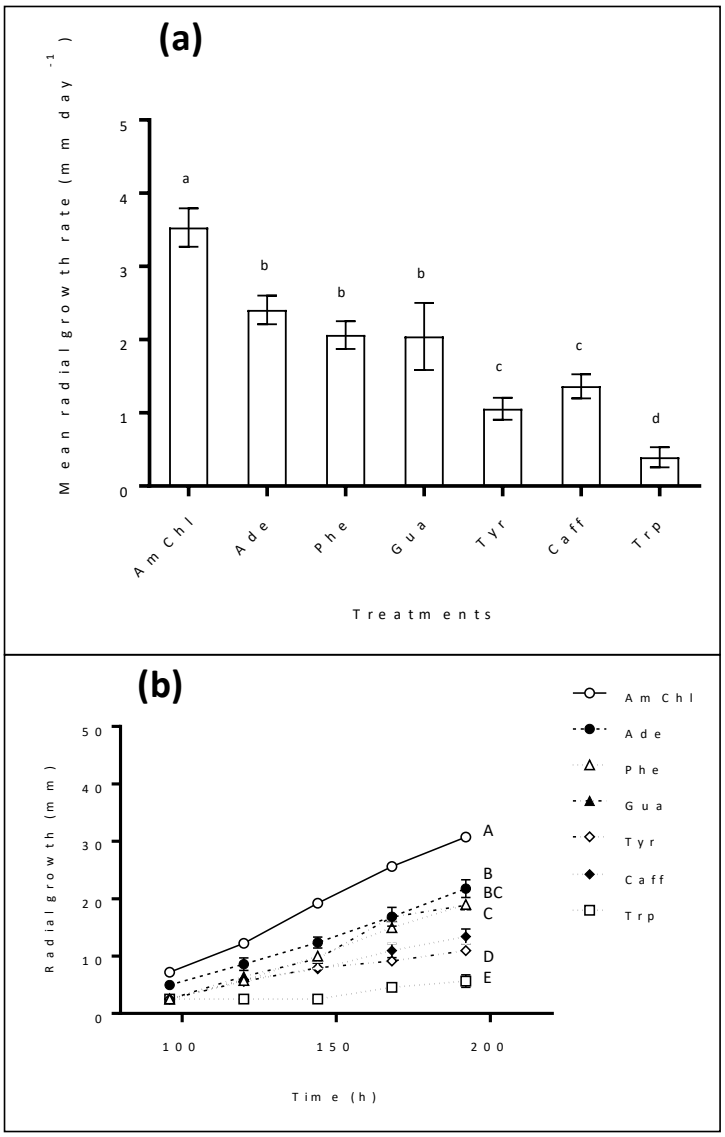

Figure 4.7 (a) Radial growth rate and (b) daily increase in radial growth of *Pleurotus ostreatus* strain PO13 mycelium grown on minimal medium (MM) supplemented with aromatic nitrogen sources (18.75 mM N equivalent): adenine (Ade), phenylalanine (Phe), guanine (Gua), tryptophan (Trp), tyrosine (Tyr), caffeine (Caff). The control is ammonium chloride-N (18.75 mM, AmChl). In (a) bars represent mean values (n = 5) and error bars are standard deviation. Lowercase letters represent differences among means using one-way ANOVA and a Tukeys post-hoc test (p <0.05). In (b) points represent mean values (n = 5) and error bars are standard deviation. Uppercase letters represent differences among means using repeated measures ANOVA and a Tukeys post-hoc test (p <0.05).

4.3.5 Effect of caffeine and nitrogen on fungal growth in liquid culture

To evaluate the effect of different N sources on fungal growth in liquid culture, a modified CDM with yeast extract (0.5 g L^{-1}) was used, and N sources were added as described in Section 2.4.

The best growth of *P. ostreatus,* together with greatest production of fungal biomass, was achieved when inorganic N (or urea) was provided (Fig. 4.8a, Table 4.3). The combination of inorganic N as ammonium chloride with caffeine-N resulted in slow but steady mycelium growth suggesting growth inhibition by caffeine-N at this concentration (10 mM N) (Fig. 4.8c). Maximum fungal growth was reached on day 14 for both ammonium nitrate and ammonium chloride but it was reached later (day 22) for calcium nitrate (CaNit; Fig. 4.8a). An apparent mycelial lysis occurred at the end of the experimental period, with a biomass decrease observed after 14 days for most N sources (including for wood decomposer medium (WDM) used as a visual control; Fig. 4.8d). Similar growth was achieved using simple organic N sources, but maximum growth was achieved later than for those containing ammonium chloride and nitrate (day 16; Fig. 4.8b). The greatest final biomass was achieved when *P. ostreatus* was grown with calcium nitrate (96 ± 59 mg mL^{-1}; Table 4.4). Fungal biomass produced during growth with different forms of inorganic N were not significantly different (one-way ANOVA, Tukeys post-hoc test, p = 0.195). In contrast, when grown on media with organic forms of N, there were statistically significant differences among treatments (one-way ANOVA, Tukeys post-hoc test, p = 0.001). Glycine as the N

source produced the maximum biomass (94 ± 22 mg mL^{-1}) and histidine produced the least amount of biomass (13 ± 2 mg mL^{-1}) (Table 4.3).

When *P. ostreatus* was grown with inorganic N sources, the pH of the liquid culture decreased, whereas using organic forms of N increased the pH (Table 4.3). Using two times more N in liquid culture did not produce an increase in fungal biomass, as indicated by growth in culture with 10 mM of ammonium chloride (inorganic N treatments) (see Fig. 4.9a) compared to 18.75 mM ammonium chloride, and compared to 20 mM from the interaction treatment (ammonium chloride and caffeine, each at 10 mM N) suggesting that saturating amounts of N (above 10 mM) for growth were supplied for this fungus in this type of medium (Table 4.3).

When *P. ostreatus* was grown in a different liquid medium (i.e. WDM), the greatest production of biomass was achieved (945 ± 286 mg mL^{-1}). This medium contains malt extract, sawdust, yeast extract and calcium sulphate (see Section 2.4 for preparation of WDM). It is important to mention that growth achieved using WDM was not included in the statistical analyses as its composition is very different from the composition of the medium used for other treatments and was used exclusively as an indicator of fungal growth (Figs. 4.8d and 4.9b).

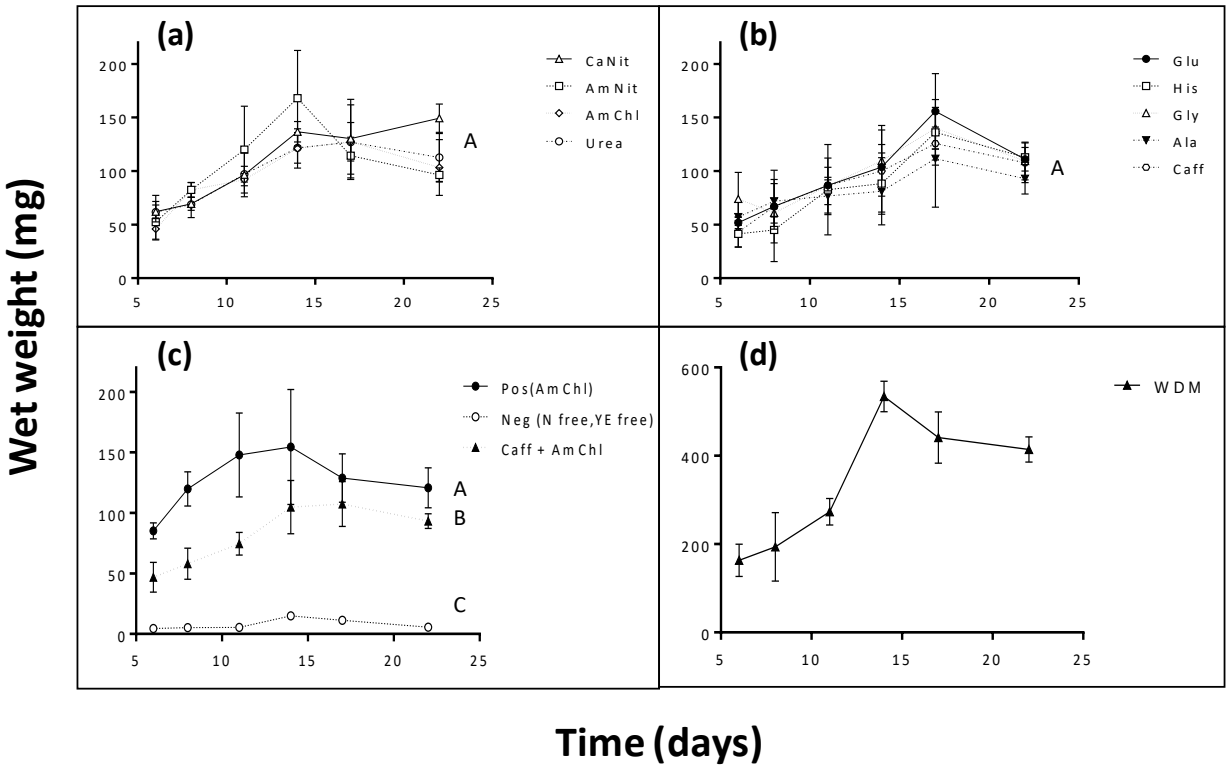

Figure 4.8 Biomass production of *Pleurotus ostreatus* strain PO13 grown in chemically-defined medium supplemented with 10 mM nitrogen (N) supplied as (a) calcium nitrate (CaNit), ammonium nitrate (AmNit), ammonium chloride (AmChl), (b) glutamic acid (Glu), histidine (His), glycine (Gly), alanine (Ala), urea and caffeine (Caff). In (c) alternative controls (positive (Pos); AmChl-N 18.75 mM N) and negative (N free), and a combination of two N sources (Caff+AmChl: 10 mM caffeine-N and 10 mM ammonium chloride) were used. In (d) wood decomposer medium (WDM) was used. Points represent mean values (n = 3) and error bars are standard deviation. Uppercase letters represent differences among means using repeated measures ANOVA and a Tukeys post-hoc test (p <0.05).

Table 4.3 Final fungal biomass and carbon:nitrogen (C:N) ratio; final pH of the supernatant of *Pleurotus ostreatus* strain PO13 mycelium grown on chemically-defined medium with low concentration (10 mM) of a range of N sources. Data was collected after 22 days of growth. Data represent mean values (n = 3) and standard deviation. Letters in lowercase, uppercase and italics indicate differences among means using one-way ANOVA and a Tukeys post-hoc test (p <0.05) for groups of treatments analyzed separately.

Nitrogen source	C:N in the medium	Biomass (mg mL^{-1})	pH	Biomass C:N
Calcium nitrate	33.3	96 ± 59 (a)	4.41 ± 0.61(a)	14 ± 3 (a)
Ammonium nitrate	33.3	31 ± 25 (a)	3.70 ± 0.08 (a)	11 ± 0.5 (a)
Urea	33.3	61 ± 7 (a)	6.96 ± 0.08 (b)	12 ± 1 (a)
Ammonium chloride	33.3	87 ± 31 (a)	3.60 ± 0.19 (a)	12 ± 0.5 (a)
Glutamic acid	33.3	34 ± 3 (A)	6.51 ± 0.61(AB)	12 ± 0.2 (AB)
Histidine	33.3	13 ± 2 (A)	6.48 ± 0.01 (A)	10 ± 0.4 (A)
Glycine	33.3	94 ± 22 (B)	6.82 ± 0.04 (B)	10 ± 0.5 (A)
Alanine	33.3	38 ± 27 (A)	7.02 ± 0.04 (AB)	11 ± 1 (AB)
Caffeine	33.3	32 ± 5 (A)	5.35 ± 0.06 (C)	14 ± 2.9 (B)
Ammonium chloride*	18.0	78 ± 13 (*a*)	3.48 ± 0.17 (*a*)	8 ± 0.7 (*a*)
Interaction AmChl+Caff**	16.7	66 ± 41 (*a*)	5.50 ± 0.16 (*b*)	10 ± 1.7 (*b*)
N free	-	0.6 ± 0.1 (*b*)	5.30 ± 0.01 (*b*)	18 ± 0.0 (*c*)
Wood decomposer medium		945 ± 286	6.62 ± 0.68	17 ± 1.7

***** Ammonium chloride at 18.75 mM of N

** Ammonium chloride and caffeine, each at 10 mM of N

AmChl+Caff – ammonium chloride + caffeine

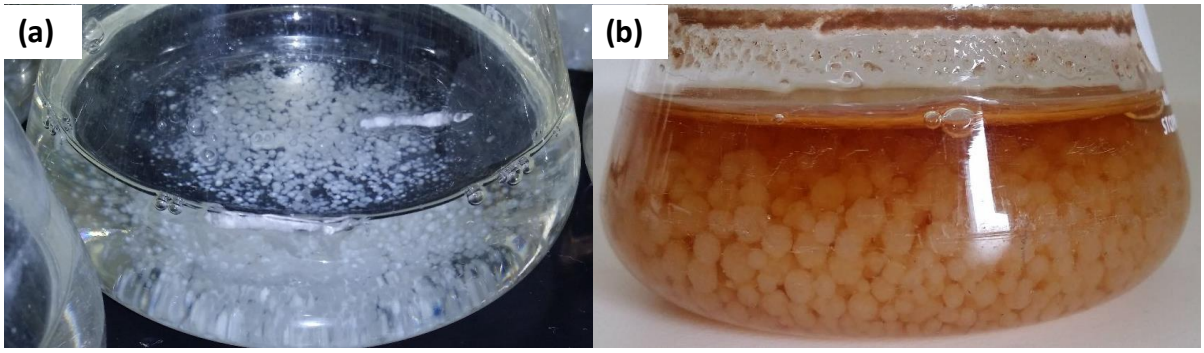

Figure 4.9 Biomass of *Pleurotus ostreatus* strain PO13 grown in (a) chemically-defined medium supplemented with 10 mM N as ammonium chloride, (b) wood decomposer medium.

4.3.6 Effect of nitrogen concentration on fungal growth in liquid culture

From previous experiments using CDM, ammonium chloride proved to be the best N source for *P. ostreatus* growth. To evaluate the concentration of N on fungal growth in liquid culture, CDM modified with yeast extract (0.5 g L^{-1}) was used and different concentrations of N in the form of ammonium chloride were added as described in Section 2.4.

The minimum amount of N that was non-limiting to support growth for *P. ostreatus* in this type of medium was 2 mM (Fig. 4.10). Maximum biomass was produced with 2 and 4 mM of N (101 ± 13 and 107 ± 7 mg mL^{-1}, respectively) whereas lowest biomass was produced when 100 mM N was used (71 ± 19 mg mL^{-1}) (excluding the negative control which contained no added N). There were statistically significant differences in biomass production among treatments (one-way ANOVA, Tukeys post-hoc test, p <0.001).

The lowest concentrations of ammonium chloride-N (≤10 mM) produced highest residual ammonium, whereas for treatments using higher concentrations of N, smaller amounts of residual ammonium were measured in the culture supernatant after final biomass was measured (Table 4.4). This suggests efficient uptake of N from the medium by the fungus, as the residual ammonium measured was a very low proportion of the initial N added. However, this does not necessarily mean more efficient utilization. Additionally, after 14 days of cultivation, the pH of the culture decreased for all treatments, even when no N was supplied for fungal growth (Table 4.4). Overall, there was a significant difference in pH among treatments (one-way ANOVA, Tukeys post-hoc test, p <0.001) but for treatments in the range of 2–100 mM N, the pH was comparable (one-way ANOVA, Tukeys post-hoc test, p = 0.060). The C:N ratio of the mycelium was found to be low when the highest concentrations of N were supplied (Table 4.4) and differences among mean C:N values were significantly different (one-way ANOVA, $F_{(9, 20)}$ = 24.809, p <0.001), suggesting that at higher input concentrations, N may be being stored but not converted to greater biomass because of limiting C supply. When N free medium was used (with yeast extract at 0.5 g L^{-1}), very low biomass production was measured.

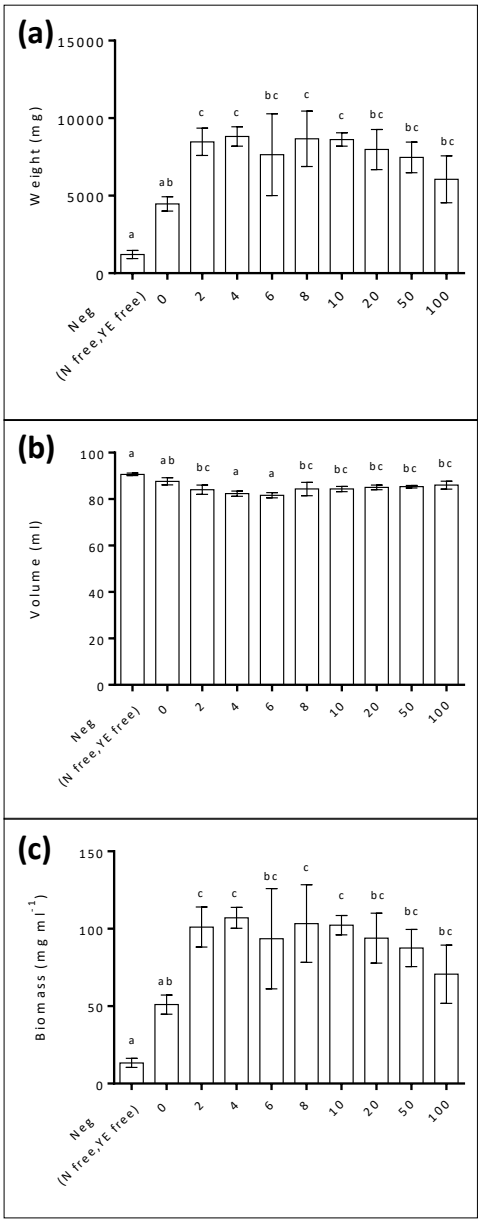

Figure 4.10 Final (a) wet weight, (b) residual culture volume, and (c) biomass of *Pleurotus ostreatus* strain PO13 mycelium grown in chemically-defined medium with low concentration of nitrogen (N) (0–100 mM) in the form of ammonium chloride. An additional negative control was included without any form of nitrogen (N) or yeast extract (Neg (N free, YE free). Bars represent mean values (n = 3) and error bars are one standard deviation. Letters represent differences among means using one-way ANOVA and a Tukeys post-hoc test (p <0.05).

Table 4.4. Final fungal biomass and carbon:nitrogen (C:N) ratio; final pH and residual ammonium of the supernatant of the mycelium of *Pleurotus ostreatus* strain PO13 with low concentration of N (0–100 mM) in the form of ammonium chloride. An additional negative control was included without any form of nitrogen or yeast extract (Neg (N free, YE free). Data represent mean values (n = 3) and standard deviation. Letters represent statistical differences among means using one-way ANOVA and a Tukeys post-hoc test (p <0.05).

Initial concentration of N (mM)	Medium C:N	Biomass (mg mL^{-1})	pH	Biomass C:N	Residual ammonium (µM)
0	-	51 ± 6 (ab)	4.43 ± 0.23 (C)	12 ± 0.2 (*a*)	12.8 ± 1.7 (*A*)
2	166.5	101 ± 13 (c)	3.75 ± 0.03 (AB)	13 ± 1 (*a*)	341.7 ± 87.3 (*D*)
4	83.3	107 ± 7 (c)	3.68 ± 0.16 (AB)	12 ± 1 (*a*)	316.9 ± 89.0 (*D*)
6	55.5	94 ± 32 (bc)	3.67 ± 0.26 (AB)	12 ± 1 (*a*)	281.9 ± 81.5 (*CD*)
8	41.6	103 ± 25 (c)	3.50 ± 0.11 (AB)	12 ± 1 (*ab*)	159.7 ± 39.3 (*BC*)
10	33.3	102 ± 6 (c)	3.64 ± 0.11 (AB)	11 ± 1 (*ab*)	103.6 ± 37.9 (*AB*)
20	16.7	94 ± 16 (bc)	3.46 ± 0.04 (AB)	9 ± 0.1 (*bc*)	16.7 ± 3.0 (*AB*)
50	6.7	88 ± 12 (bc)	3.51 ± 0.06 (AB)	8 ± 0.4 (*cd*)	16.9 ± 2.4 (*AB*)
100	3.3	71 ±19 (bc)	3.38 ± 0.12 (A)	6 ± 0.4 (*d*)	2.2 ± 1.7 (*A*)
Negative*	-	13 ± 3 (a)	3.85 ± 0.02 (A)	12 ± 0.3 (*a*)	60.8 ± 2.5 (*B*)

* N free, YE free

4.3.7 Effect of phosphate buffer in liquid medium

The experiments described above revealed a rapid decrease in pH during growth of *P. ostreatus*, coupled with a decrease in growth (Fig. 4.1.). To test whether the change in pH had an adverse effect on fungal growth, the buffering capacity of the CDM medium was varied by changing phosphate concentration.

For cultures using ammonium chloride as N source, use of 25 mM phosphate buffer resulted in slightly more biomass production (Fig. 4.11a), but when pH was measured, equivalent pH values to 7.5 mM phosphate buffer were recorded. With 50 mM of phosphate buffer, the pH value was slightly higher than for the other treatments (Fig. 4.11c). When ammonium tartrate was used as an alternative N source, 25 and 50 mM phosphate buffer was associated with greater production of fungal biomass, however there were no differences in pH among buffer concentrations tested. These data suggest that another type of buffer might be required to maintain pH for a longer period and therefore achieve higher fungal biomass.

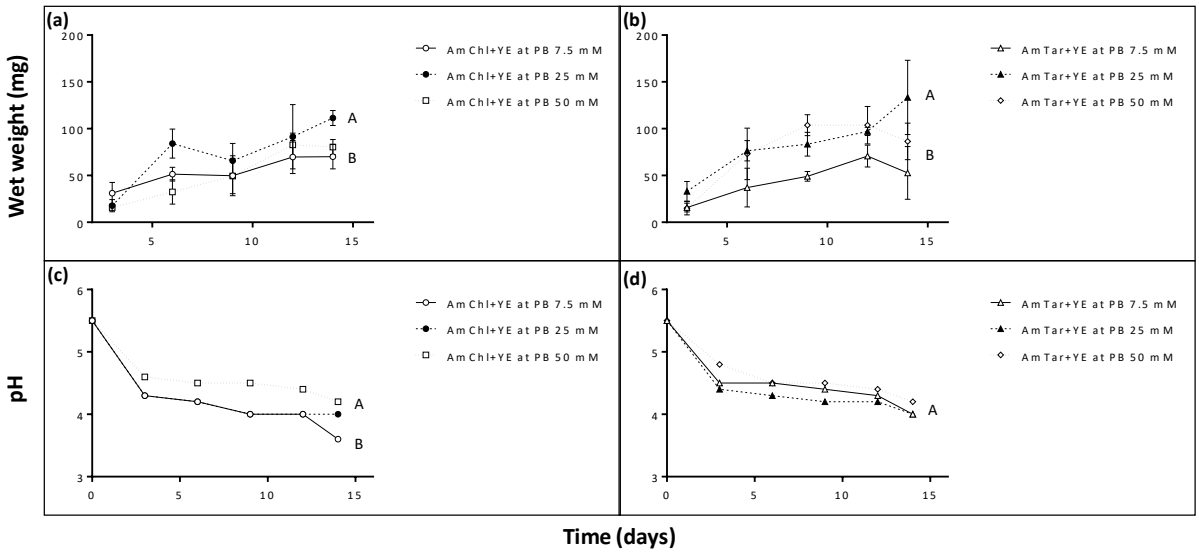

Figure 4.11 Biomass (a, b) and pH of supernatant (c, d) of *Pleurotus ostreatus* strain PO13 mycelium grown in chemically-defined medium (volume 100 mL) supplemented with yeast extract (YE) and 10 mM nitrogen (N) as ammonium chloride (AmChl) and ammonium tartrate (AmTar) with different concentrations of phosphate buffer (PB; 7.5, 25, 50 mM). Points represent mean values (n = 3) and error bars are standard deviation. Uppercase letters represent differences among means using repeated measures ANOVA and a Tukeys post-hoc test (p <0.05).

4.4 Discussion

When *Pleurotus ostreatus* strain PO13 was grown on two types of media with several different types of N sources provided at a range of concentrations the fungus showed preference for inorganic N sources both in solid and liquid media. The concentration of added N resulting in N-limiting conditions was determined to be 2 mM for liquid medium. Radial growth rates and production of biomass were best achieved with supply of inorganic N.

Different forms of N sources are assimilated in different ways and, as a result, have significant effect on production of fungal biomass. Different fungi can utilize diverse forms of N such as nitrate, nitrite and ammonium, simple organic compounds such as amino acids and complex organic compounds (Jennings 1995). The use of organic compounds has the added advantage of provision of C but, in many cases, it is not known if the compound is accessed for C, N or both. The response of fungi to changes in N availability is mediated by a complex set of regulatory mechanisms (Tudzynski 2014). In addition, the flexibility of the fungus allows it to grow on a variety of N sources at a range of concentrations (D'Agostini *et al*. 2011). In the experiments described in this study, greater rates of growth were measured when inorganic N sources were used compared to selected organic N sources, for fungi grown both on agar and in liquid culture. However, growth data obtained from agar and from liquid cultures cannot be compared, as the C:N of the medium in agar was lower than in liquid cultures (3.6–6.7; 33.3, respectively), thus the utilization of N sources could have been masked by the lack of available C in the medium for agar cultures. Ammonium chloride promoted greatest fungal growth, suggesting it to be the most favourable N source. Calcium

nitrate also produced a substantial increase in fungal biomass in liquid culture. A significant increase in fungal biomass was generated when the amino acid, glycine, was provided. This is not surprising as it is well known that, when certain N sources are not accessible, other N forms can be used by fungi (Marzluf 1997). Enzymatic activity associated with N metabolism in fungi is controlled by regulation through the glutamine/glutamate cycle in response to the availability of N (Davis & Wong 2010).

The results of the experiment using ammonium chloride as N source indicated that the minimal amount of N required for growth was approximately 2 mM added N, which was indicated by stable biomass production, uptake and utilization of N (residual ammonium) and constant C:N of the fungal biomass under experimental conditions above this value (Table 4.4). These results confirm that the minimum inhibitory concentration of caffeine-N (10 mM) can provide sufficient N to support mycelial growth (See Chapter 3). When a higher concentration of N was used (above 10 mM N), although high biomass was achieved, significantly lower residual ammonium, and lower C:N of final biomass was recorded, suggesting C limitation in these conditions.

Wood decay fungi have evolved to grow under restricted amounts of N, being capable of producing biomass at very low N concentration in the wood (Dix & Webster 1995). Thus, this indicates that when selecting substrates for *Pleurotus ostreatus*, the requirement for low N should be met. However, when there is a lack of substrate N, many fungi can recycle their intracellular nitrogenous compounds making N available for metabolic processes (Shoji & Craven 2011), at least for some period of time.

In the experiments presented here, provision of organic N sources in the form of amino acids resulted in low rates of growth in agar culture and production of small amount of biomass in liquid culture. Histidine did not promote hyphal growth in liquid culture (Table 4.3; 13 ± 2 mg mL^{-1}), which was consistent with observations for other taxa, for example, the model filamentous fungus, *Aspergillus nidulans*, also grew poorly when supplied with histidine (Davis & Wong 2010). In the current study, on agar culture, alanine and tryptophan produced the slowest rates of growth for *P. ostreatus* (Fig. 4.4b and Fig. 4.7, respectively). However, other authors reported that these amino acids can promote growth in other fungal species. Alanine was found to be most favorable for growth for *Suillus luteus*, *Laccaria laccata*, *Tricholoma aurantium* and *Cortinarius flexipes* (Itoo & Reshi 2014), and tryptophan stimulated strong fungal growth in liquid culture of *Psathyrella atroumbonata* (Jonathan & Fasidi 2001). The best growth of the decomposer fungus, *Agaricus bisporus*, in liquid medium was achieved with a supply of amino acids including asparagine, glutamine, glycine and alanine (Baars *et al.* 1994). These discrepancies could be related to species and strain differences in N metabolism and ecological role, but also to the solubility of this amino acid in the different media type used. This highlights the necessity for careful optimization of fungal growth media, based on knowledge of the organism's ecology, to develop high-yielding cultivation methods.

In previous studies, yeast extract supplementation of *P. ostreatus* growth medium resulted in greatest biomass, as compared with growth on an inorganic N source (ammonium sulphate), where both biomass and polysaccharides production were lower (Gern *et al*. 2008). Yeast extract has been used as a sole N source to grow *Psathyerella atroumbonata*

and this additive promoted the best growth compared to other inorganic and organic N sources (Jonathan & Fasidi 2001). Yeast extract was also found to enhance biomass production of "*Pleurotus sajor-caju*" at least to some extent in liquid culture (Confortin *et al*. 2008). In the present study, yeast extract needed to be present at a minimum concentration to permit growth of *P*. *ostreatus*. None of the vitamins evaluated promoted biomass production, nor did β-adenosine. β-adenosine has been reported to be an excellent growth stimulant for other species of *Pleurotus* (Domodon *et al*. 2004) and for the edible ectomycorrhizal fungus *Suillus luteus* (Zhang *et al*. 2010), but these studies were performed in different types of media (agar, solid state fermentation) and using different media composition. Enzymatic response also depends on fungal species and strains, C and N sources and culture conditions (Stajić *et al*. 2006), indicating the importance of standard culture conditions to compare biomass and production of fungal compounds, as well as to measure other parameters that could provide physiological information.

It is important to mention that wood decomposer medium represents a simple approximation of the natural medium of *Pleurotus*. It contains wood as a complex carbon and N source (see Section 2.4 for detailed components). As a result, *P. ostreatus* grew exceptionally well in this type of medium and demonstrated the growing capabilities of this genus (Table 4.3; Fig. 4.9b). *Pleurotus ostreatus* is a saprotroph in its natural habitat (see Chapter 1) and has the ability to produce specific enzymes to degrade large and complex molecules associated with dead plants and wood (Stamets 2005).

4.5 Conclusion

Using two different types of media, *P*. *ostreatus* responded with comparable growth to a range of C:N ratios present in the media. Different sources of N were evaluated at different concentrations to determine when N limits biomass production, and what amount of caffeine provides sufficient N for growth. The type and form of N sources affected fungal growth depending on the type of medium used, as *P*. *ostreatus* was able to utilize simple inorganic and organic forms of N and, from N in complex organic form, from both aromatic and aliphatic molecules, including caffeine. The next chapter investigates the fate of caffeine, present at the minimum non-inhibiting concentration in spent coffee grounds amended substrate, during cultivation of *P*. *ostreatus*.

Chapter 5 Caffeine metabolism during oyster mushroom cultivation on spent coffee grounds

5.1 Introduction

There are three main stages of mushroom cultivation: (i) preparation of fungal culture inoculum including strain selection; (ii) preparation of the substrate and colonization with mycelium (vegetative stage); and (iii) production of fruiting bodies (reproductive stage). All three steps are crucial to obtain robust fungal growth and high-quality mushrooms. Biological efficiency (BE) may vary as a result of spawn levels, fungal strains and quality and enhancement of the substrate with supplementary products (Mandeel *et al*. 2005), BE is also influenced by the selection/adjustment of environmental parameters during the spawn-run and fructification phases (Chang & Miles 2004). For mushroom cultivation, it is very important to acquire a good stock mycelium culture to guarantee good yield, high quality and taste (Chang & Miles 2004; Gregori *et al.* 2007; Stamets 2000).

Selection and preparation of the substrate will depend on the mushroom species and its physiology and ecology and, as such, should be a simulation of the natural growing conditions of the fungus (Chang & Miles 2004; Philippoussis 2009). The substrates most often used are residues from agriculture and forestry, and, for the best results, must be processed or prepared to match fungal requirements. In addition, substrates used for commercial mushroom cultivation must meet certain food safety and nutritional requirements (Wasser & Akavia 2008).

A variety of different physical, microbiological and chemical techniques have been used to modify and enhance mushroom substrates. Physical techniques include heat sterilization (Philippoussis 2009), increasing the surface area of the substrate by chopping and grinding (Royse *et al.* 2004; Zhang *et al.* 2002) and addition of coarse materials to increase aeration of the substrate (Chen *et al.* 2008). Microbiological methods include composting, a process in which microorganisms are involved in the breakdown of complex molecules to make them more accessible to fungal mycelium (Chang 2009). Chemical techniques include addition of a range of substances to enhance the nutritional quality of mushrooms, to increase yield or to inhibit growth of unwanted microorganisms. For any technique, water needs to be added to maintain the substrate at 65–75% (w/w) to promote and maintain fungal colonization.

During both the vegetative and reproductive phases of mushroom cultivation, the temperature may need to be varied according to the life cycle stage. For example, for Shiitake (*Lentinula edodes*), incubation temperatures for vegetative growth range from 18–25 °C (Chang 2009). Certain strains of Shiitake can produce fruiting bodies from 10–16 °C, but other strains require higher temperatures (Philippoussis 2009). Similarly, "*Pleurotus sajor-caju*" only develops fruiting bodies at temperatures of 18–24 °C (Chang 2009). Some species require a change in temperature for reproduction, for example, a cold shock stimulates formation of fruiting bodies of *Lentinula edodes* (Philippoussis *et al*. 2003). This method is also required for some species of *Pleurotus* (Philippoussis 2009). In addition to temperature control, mycelial growth and fruiting is regulated by other parameters such as availability of nutrients, humidity, gas exchange and light, all of which need to be selected

according to the species and strain used (Philippoussis 2009). As an example, the pH of compost used for cultivation of *Agaricus brasilensis* ranges between 4–7 (Barros Colauto *et al*. 2008), while for the production of fruiting bodies of *P*. *ostreatus*, the optimal pH range is 5.5–6.5 (Chang & Miles 2004). Light is also a very important parameter for growth and requirements for each species may differ during cultivation. *Pleurotus*, for example, does not need light for growth during the vegetative stage but requires light (1000–1500 lux) during the reproductive stage (Stamets 2000). Regardless of individual species requirements, the most successful cultivation conditions resemble the natural environmental conditions of each mushroom species.

Fungi in the genus *Pleurotus* are saprotrophs that grow on dead wood and other substrates rich in lignin and cellulose (Cohen *et al*. 2002; and see Chapter 1). Their enzymatic pathways allow degradation of plant material containing lignin, hemicelluloses and cellulose (Mäkelä *et al.* 2014). As a result, a wide variety of raw materials, including agricultural and forestry wastes, can be used as substrate for cultivation of oyster mushrooms. However, for commercial purposes straw and sawdust are preferred (Philippoussis 2009). Substrates for oyster mushroom cultivation can be either pasteurized or sterilized, and some are also partially composted (Chandravanshi *et al*. 2012; Hernández *et al*. 2003; Salmones *et al*. 2005; Viera & de Andrade 2016). The degree of degradation of the substrate may affect the potential to produce fruiting bodies. In a study using composted substrate made of grass and coffee pulp (70/30% mix), a maximum BE of 93% was achieved for three harvests (Hernández *et al*. 2003). Using other types of agricultural residues, such as paddy straw as substrate, *P*. *ostreatus* and "*P*. *sajor-caju*" achieved BE of close to 75%, while using sesame

straw, the BE of *P. ostreatus* was 91% and for "*P. sajor-caju*" was 66% (Kurt & Buyukalaca 2010).

Another substrate considered for *Pleurotus* cultivation is coffee waste. The coffee industry generates large amounts of waste, ranging from residues from cultivation of coffee plants through to waste from fruit processing to obtain commercially available coffee to ground coffee waste generated by coffee consumers (see Chapter 3). Coffee residues have been investigated for production of secondary metabolites for human consumption and the pharmaceutical industry (Cruz *et al*. 2014, López-Barrera *et al*. 2016; Machado *et al*. 2012; Mussatto *et al*. 2011; Petrik *et al*. 2014), production of energy (Kang *et al*. 2017; Park *et al*. 2016; Woldesenbet *et al*. 2013; 2016) and as a food for source for animals after microbiological treatment (Mazzafera *et al*. 2002; Murthy & Naidu 2012). Residues from coffee production and consumption have been investigated widely in relation to mushroom cultivation (Da Silva *et al*. 2012; De Assunção *et al*. 2012; Jaramillo *et al*. 2010; Salmones *et al*. 2005; Savoie *et al*. 2007; Velázquez-Cedeño *et al*. 2002) and composting (Adi & Noor 2009; Nogueira *et al*. 1999; Shemekite *et al*. 2014). Such an extensive range of applications has been explored mainly because countries producing coffee products need safe and reliable means for disposal of potentially toxic coffee wastes (husks, pulp, silverskin) (Rathinavelu & Graziosi 2005). However, despite numerous investigations of methods to reduce the toxic nature of the waste from large-scale coffee production, the viability of bio-processes for removal of caffeine still requires more research (Murthy & Naidu 2012).

The cultivation of mushrooms using agro-industrial residues from coffee production has been shown to provide a useful source of additional income for coffee farmers in rural areas

of Colombia (Jaramillo 2005), Cuba (Bermúdez *et al.* 2001) and Mexico (Mora & Martinez-Carrera 2007). In cities of developed countries, residues from coffee consumption (spent coffee grounds, SCG) represent a high volume of urban waste. For example, in Sydney, Australia, approximately 3,000 tonnes of coffee waste are produced each year (Cameron & O'Malley 2016). The impetus for reuse of these wastes has not been as great as in coffee producing countries. Regardless of this, research into the use of processed coffee residues has increased in recent years (e.g. Cruz *et al*. 2014; Hardgrove & Livesley 2016; Santos *et al*. 2017). Hobbyists and small business entrepreneurs have also used coffee residues for mushroom cultivation with relatively small batches of SCG forming the basis of mushroom cultivation substrates for personal or limited commercial distribution (e.g. http://www.beyondcoffee.eu/products; facebook.com/beancycled; https://espresso mushroom.co.uk; https://www.funguyfungi.com.au; https://grocycle.com/urban-mushroom -farm; https://lifecykel.com.au; http://www.manhattanmushroomco.com). The progression from small-scale to mid-scale mushroom production using SCG has not yet been achieved. Regardless, there is an increasing need to provide information about caffeine degradation during mushroom cultivation on residues from coffee consumption.

Most of the investigations about degradation of caffeine have involved bacteria, while degradation by fungi has had little attention (Gokulakrishnan *et al.* 2005; Mazzafera 2004). The metabolic pathways and enzymes involved in degradation of caffeine have been characterized although mostly for prokaryotic organisms (Chi *et al.* 2009; Mohapatra *et al*. 2006; Yamoka-Yano & Mazzafera 1999; Yu *et al.* 2008). In addition, a range of species and strains of filamentous fungi have been evaluated for their potential in caffeine degradation,

mainly for detoxification of coffee wastes (Brand *et al*. 2000; Dash & Gummadi 2007; Gokulakrishnan *et al.* 2007; Hakil *et al.* 1998; Hakil *et al.* 1999; Roussos *et al.* 1995). Possible metabolic degradation pathways have also been elucidated for fungi (Brand *et al*. 2000; Hakil *et al*. 1998; Tagliari *et al*. 2003). A hypothetical scheme for caffeine degradation based on known degradation pathways for fungi is shown in Fig. 5.1.

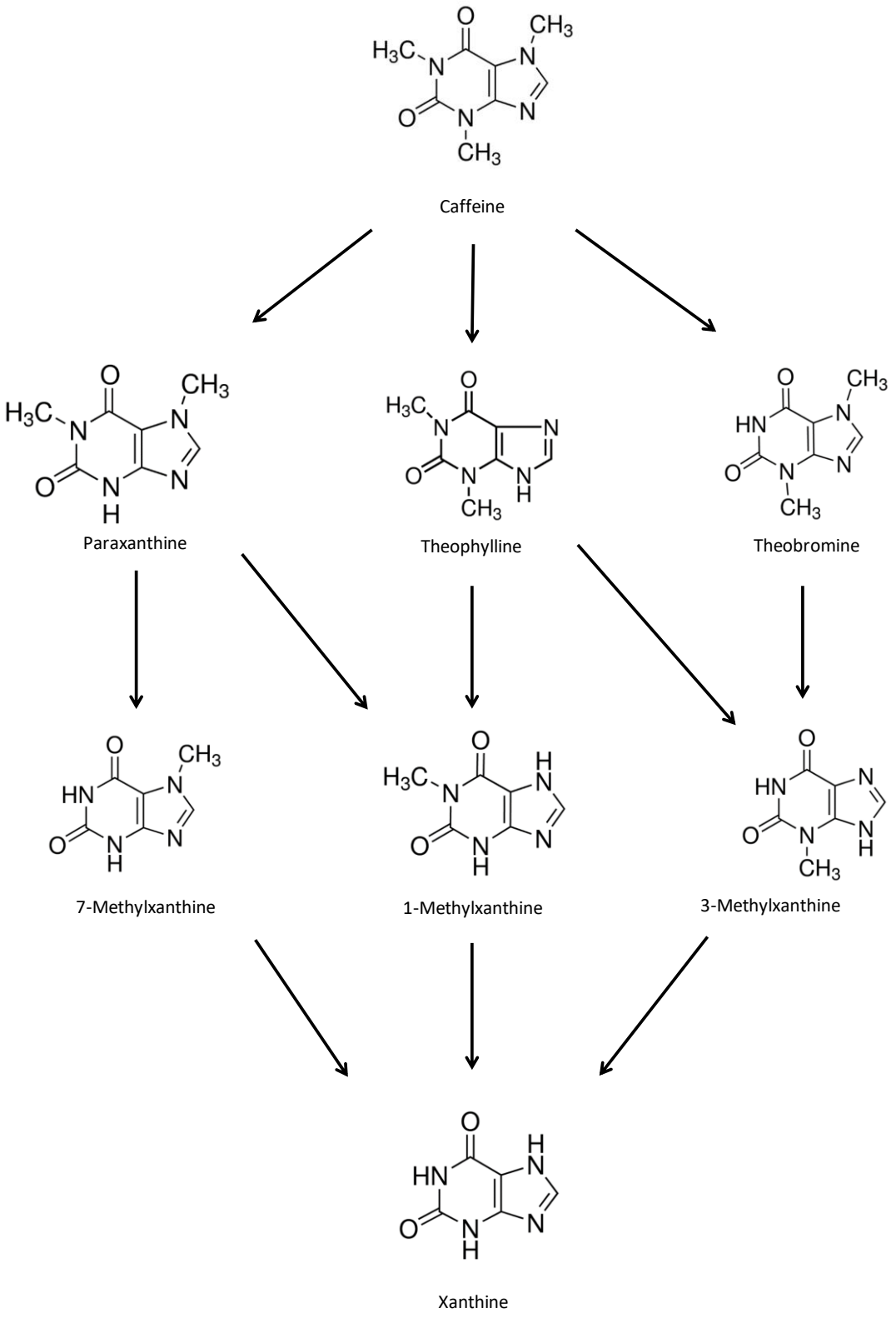

Figure 5.1 Proposed pathway for caffeine degradation by fungi (modified from Dash & Gummadi 2006; Hakil *et al*. 1998; 1999; Tagliari *et al*. 2003).

5.1.1 Aim of this study

It has been argued that coffee waste of any kind is an excellent source of growing medium for mushroom cultivation (Martínez-Carrera *et al.* 1990; Salmones *et al.* 2005; Savoie *et al.* 2007). However, little peer-reviewed research is available and attempts at determining the decrease in caffeine in the substrate and the concomitant accumulation of caffeine in fruiting bodies is limited to a few studies and a few species of fungi. There is inadequate and sometimes contradictory evidence for caffeine accumulation in fruiting bodies (see Section 3.1.1) and there is no evidence for accumulation of caffeine metabolites in the fruiting bodies of any mushroom species. This study represents one of the first investigations of degradation of caffeine in spent coffee grounds (SCG) when used as a growth substrate for *P. ostreatus* and of accumulation of caffeine and its metabolites in the fruiting bodies of the same species.

The hypotheses being tested are:

1. When *P. ostreatus* is cultivated on spent coffee grounds, the fungus is capable of degrading caffeine.

2. If caffeine degradation occurs during cultivation of *P. ostreatus*, caffeine and its metabolites are detected in the fruiting bodies and the growing medium.

5.2 Materials and methods

Methods used in the experiments presented in this chapter are described in Section 2.4.

5.3 Results

5.3.1 Caffeine in oyster mushroom cultivation – laboratory-scale trial

To evaluate caffeine metabolism by *P. ostreatus* strain PO13 during cultivation on spent coffee grounds (SGC) and sawdust, a preliminary laboratory-scale experiment was conducted. Microboxes containing different proportions of SCG (0–100%) combined with sawdust were prepared and inoculated. Each substrate combination (containing mycelium) was sampled during the vegetative stage and, if fruiting bodies were produced, these were also sampled. After a single flush of fruiting bodies had been harvested, a final sampling of the residual substrate (at this point referred to as spent mushroom substrate (SMS)) was done. Concentrations of caffeine and four metabolites (paraxanthine, theophylline, theobromine and xanthine) were determined in the substrate and fruiting bodies as described in Section 2.4. In this study, the term "colonized substrate" refers to substrate containing mycelium (regardless of the degree of colonization).

Based on visual inspection, fungal colonization was scored to determine readiness for fruiting. For *P. ostreatus* strain PO13, a thick layer of mycelium on top of the substrate is generally used as an indicator that the first fruiting bodies will develop within a few days when environmental conditions are favourable (Arrold 2014). An example of the degree of fungal colonization in microboxes is provided in Fig. 5.2a. Three substrate combinations of SCG/sawdust were fully colonized after 4 weeks of incubation (Table 5.1). In comparison, two treatments, pure sawdust (sawdust100) and pure SCG (SCG100), were colonized to a much smaller extent. After 53 days of cultivation, a single harvest of fruiting bodies was possible for only two treatments (SCG25+sawdust75 and SCG100; Fig. 5.2b). Notably,

fruiting occurred at different time points for these two treatments (SCG25+sawdust75 at day 46 and SCG100 at day 53).

Table 5.1 Fungal colonization based on visual determination after 4 weeks of incubation of *Pleurotus ostreatus* strain PO13. The control was substrate without fungal inoculation. Negative symbol (–) indicates no colonization; positive symbol (+) indicates degree of colonization; the number indicates the percentage of the given component in substrate mixture; SCG – spent coffee grounds.

Replicate	SCG 100	SCG75+ Sawdust25	SCG50+ Sawdust50	SCG25+ Sawdust75	Sawdust 100
1	-	++	+++++	+++++	++
2	+++++	+++	+++++	+++++	++
3	+++++	+++	+++++	+++++	++
4	+	+++	+++++	+++++	++
5	+	+++	+++++	+++++	++
6	+	+++	+++++	+++++	++
Control	-	-	-	-	-

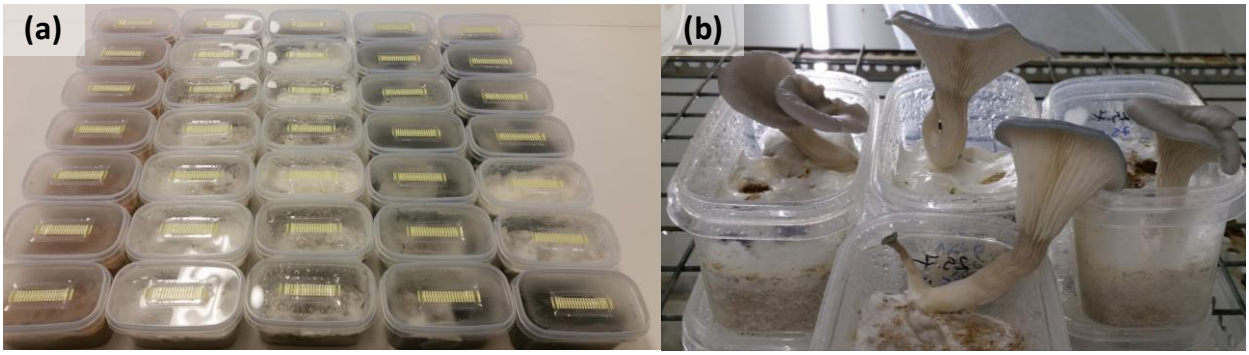

Figure 5.2 *Pleurotus ostreatus* strain PO13 grown in microboxes after incubation of 4 weeks. Spent coffee grounds (SCG) were mixed with sawdust in different proportions. (a) Microboxes to the left were pure sawdust and had a characteristic orange colouring while those to the right were pure SCG, (b) fruiting bodies obtained during the experiment.

Two examples of the HPLC chromatograms obtained from fruiting body extracts (derived from fruiting bodies grown on SCG100 and SCG25+sawdust25) are shown in Fig. 5.3. Peaks representing caffeine and four metabolites were detected (λ = 275 nm) and identified by UV spectra and retention times (RT) as xanthine (RT = 4.02), theobromine (RT = 5.64), paraxanthine (RT = 6.17), theophylline (RT = 6.34) and caffeine (RT = 6.96). Three additional peaks were detected at the same wavelength but were not identified in this study. Peaks 2 and 3 appear only in analysis of the fruiting bodies grown on SCG100, whereas peaks 2, 3 and 5 were associated with the fruiting bodies grown on SCG25+sawdust75. It is most likely that all eight compounds (including caffeine) belong to the metabolic pathway of caffeine degradation by *P*. *ostreatus* strain PO13. When commercially available fruit bodies of *P*. *ostreatus* (not grown on SCG) were analyzed, neither caffeine nor any of the observed metabolites were detected (data not shown).

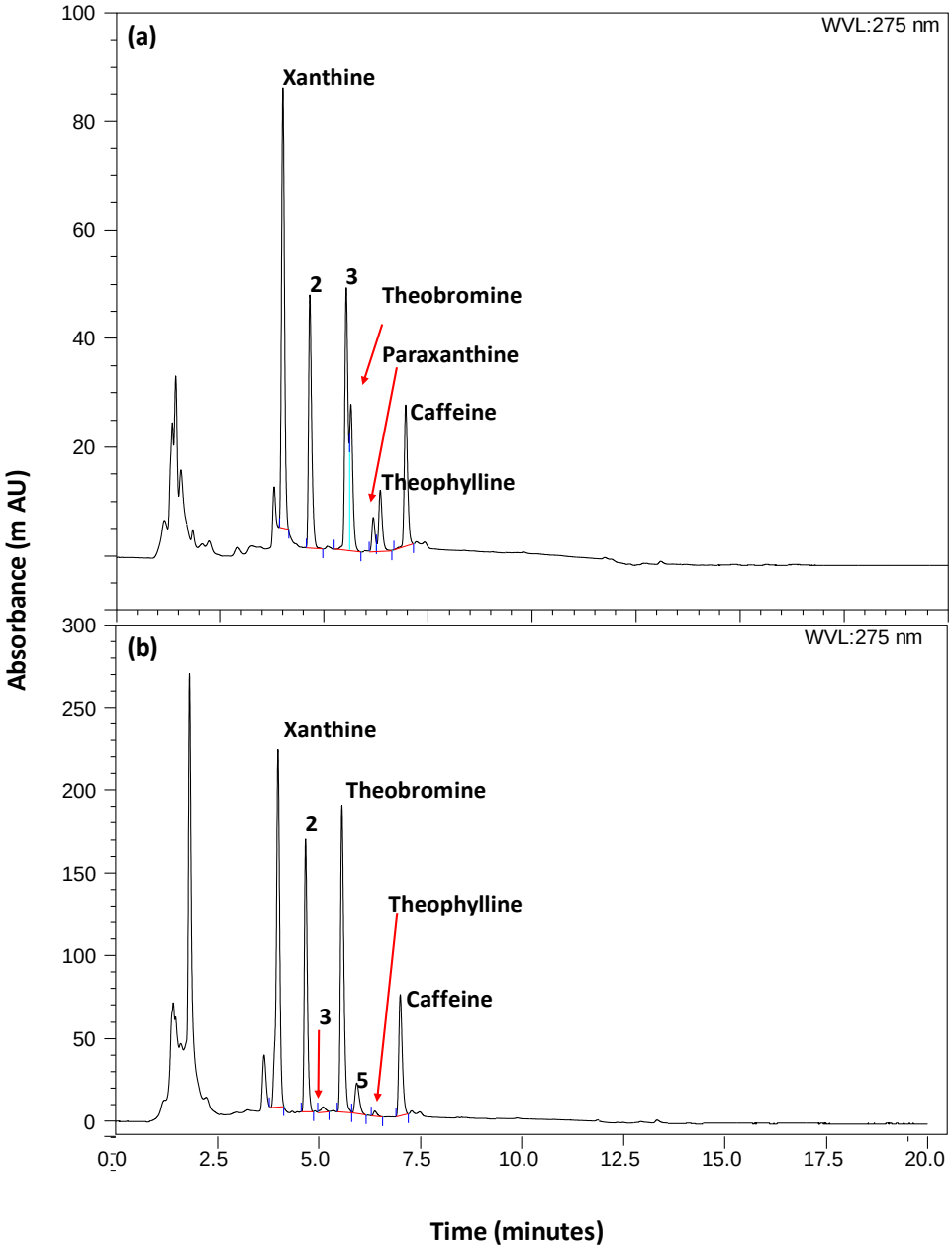

Figure 5.3 Representative HPLC chromatograms of extracts from fruiting bodies of *Pleurotus ostreatus* strain PO13 grown on (a) SCG100, and (b) SCG25+sawdust75 in the laboratory-scale experiment using microboxes. Metabolites from caffeine degradation identified in (a) xanthine (RT = 4.02); 2, 3 unknown (RT = 4.66, 5.53); theobromine (RT = 5.64); paraxanthine (RT = 6.17); theophylline (RT = 6.34) and caffeine (RT = 6.96), and in (b) as xanthine; 2, 3 unknown (RT = 466, 5.10); theobromine; 5: unknown (RT = 5.92); theophylline; caffeine. In addition, three non-identified compounds were detected (peaks labelled 2, 3 in (a) and 2, 3, 5 in (b)) and are most likely to be other metabolites of caffeine degradation. SCG – spent coffee grounds, RT– retention time (λ = 275 nm).

After 4 weeks of incubation there was a considerable decrease in caffeine in the colonized substrate for the SCG100 treatment. However, there was little decrease in caffeine after incubation for treatments containing SCG25+sawdust75 or SCG50+sawdust50 (Fig. 5.4). Low concentrations of the four metabolites of caffeine (generally less than 2 nmole mg^{-1}) were found in the colonized substrate during the experimental period (Fig. 5.4b, c, d). There was a slight increase of paraxanthine (1,7-dimethylxanthine) for the SCG25+sawdust75 treatment. In addition, paraxanthine was only detected in two of the treatments at different sampling time points. When SCG100 was used as substrate, this compound was detected at one time point only (Fig. 5.4b), which suggests that more frequent sampling would be required to monitor this metabolite during the vegetative stage of the cultivation. A decrease of theophylline (3,7-dimethylxanthine) in the SCG100 substrate suggests further degradation of this compound.

Theobromine was detected in low concentrations throughout the cultivation period with a slight increase at the end (Fig. 5.4b). Although the SCG50+sawdust50 treatment did not produce fruiting bodies of a size that could be analysed, the colonized substrate was analysed for caffeine and its metabolites. Xanthine was not detected, suggesting that caffeine degradation had not reached the final stage, or alternatively, that it has been further degraded and utilized, decreasing the concentration to below the detection limit (Fig. 5.4e). When *P. ostreatus* was grown on SCG100, theobromine and theophylline were detected during cultivation, while paraxanthine and xanthine were only detected at one sampling time point each (Fig. 5.4b, d, e).

Fruiting bodies from the SCG100 and SCG25+sawdust75 treatments accumulated all the caffeine metabolites identified in this experiment (Fig. 5.5). Caffeine was detected in the fruiting bodies obtained from treatments SCG100 and SCG25+sawdust75 (Fig. 5.5a) with high levels found for SCG25+sawdust75, despite only a small decrease in caffeine levels in this colonized substrate (Table 5.2). Xanthine appeared in higher concentrations in both fruiting bodies from the substrate combinations, SCG100 and SCG25+sawdust75. However, it was only detected at a low level in the SMS of SCG100, and none was recorded in SMS of SCG25+sawdust75. This suggested that caffeine degradation might not be complete, or that it has been fully degraded and the final product (xanthine) was completely utilized by the fungus. In comparison, paraxanthine was recorded in high concentration in the fruiting bodies from the SCG25+sawdust75 treatment only (Fig. 5.5b). An accumulation of this compound might suggest a slow degradation rate from paraxanthine to 7-methylxanthine, with further uptake by the fungus. High concentrations of theobromine were also detected in the fruiting bodies grown in both treatments, whereas theophylline was only detected in traces in the fruiting bodies for both substrate combinations (Fig. 5.5b).

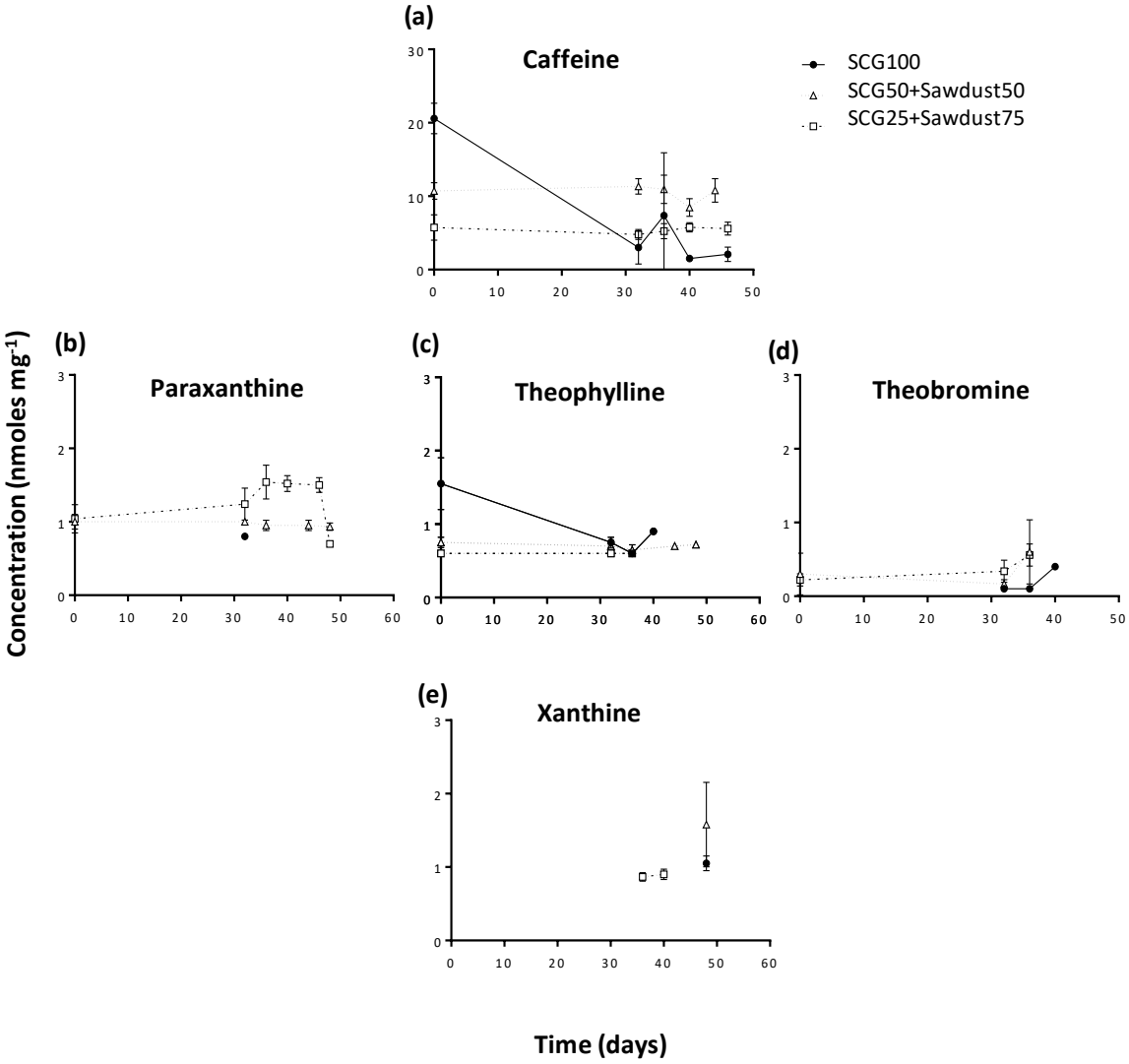

Figure 5.4 Caffeine concentration in substrate of three treatments colonized by *Pleurotus ostreatus* strain PO13 expressed on dry weight basis: SCG 100, SCG25+sawdust75, SCG50+sawdust50. Points represent mean values (n = 6) and error bars are standard deviation. SCG – spent coffee grounds.

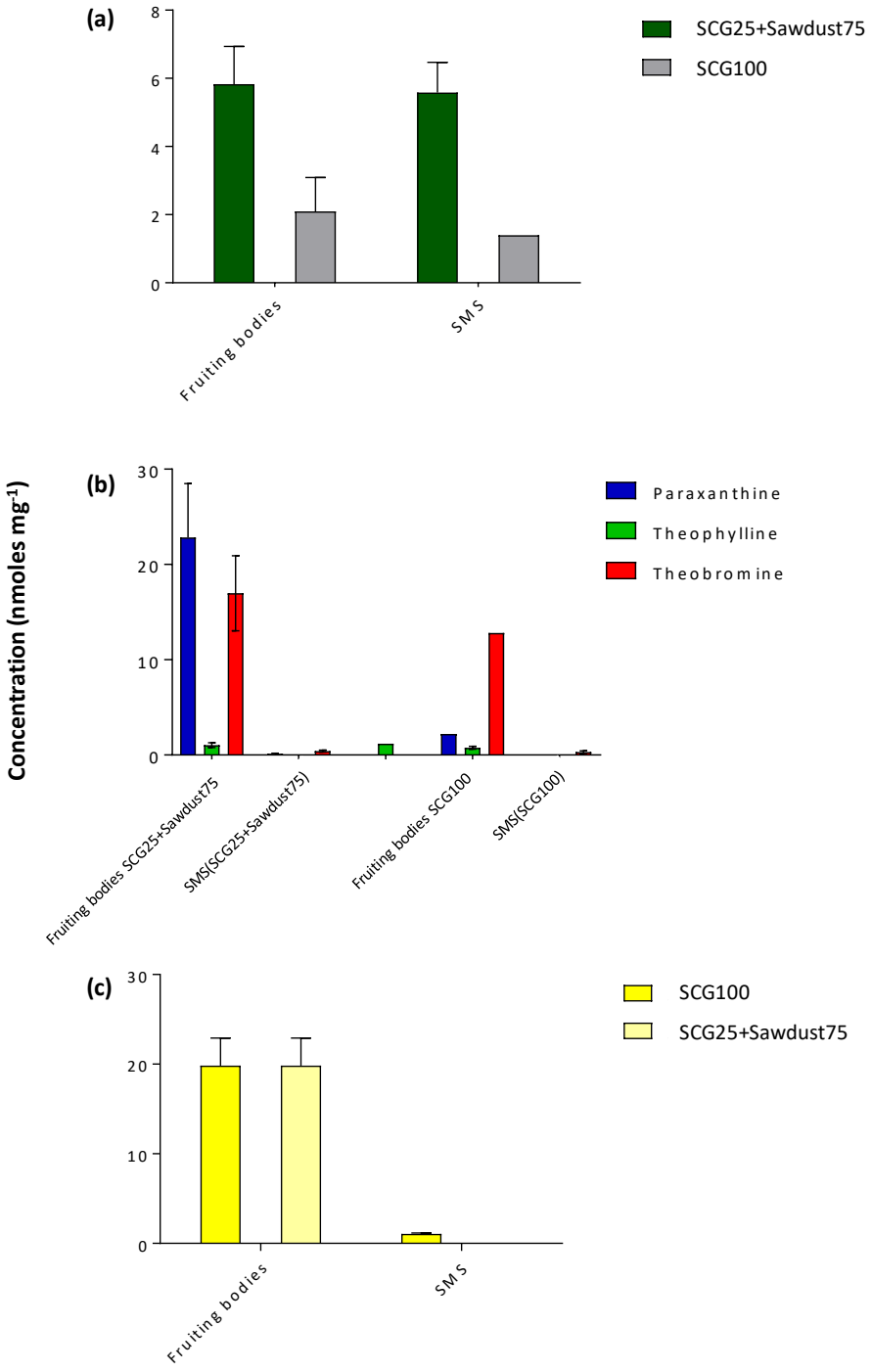

Figure 5.5 Concentration of (a) caffeine, (b) paraxanthine, theophylline, theobromine, and (c) xanthine in the fruiting bodies and in the spent mushroom substrate (SMS) of *Pleurotus ostreatus* strain PO13 expressed on dry weight basis: SCG25+sawdust75 and SCG100. Bars represent mean values (n = 6) and error bars are standard deviation. SCG – spent coffee grounds.

5.3.2 Caffeine in oyster mushroom cultivation – commercial production-scale

The laboratory-scale experiment described above (Section 5.3.1) revealed that caffeine content decreased in the substrate when *P*. *ostreatus* was cultivated in substrates containing selected SCG/sawdust combinations. However, this experiment had limited replication and only examined a subset of possible degradation products shown in Fig. 5.1. Caffeine metabolism by *P*. *ostreatus* was therefore investigated further in a commercial-scale trial. However, before commencing this large-scale study, *P. ostreatus* was grown on agar containing the same SCG used for the later study, to confirm its ability to accumulate and metabolize caffeine from this source. Caffeine and all seven metabolites belonging to the caffeine degradation pathway (Fig. 5.1) were detected and identified in the mycelia, as well as in the post-culture medium. These compounds were xanthine (RT = 3.97), 7-methylxanthine (RT = 4.81), 3-methylxanthine (RT = 5.01), 1-methylxanthine (RT = 5.24), theobromine (RT = 5.55), paraxanthine (RT = 6.09), theophylline (RT = 6.26) and caffeine (RT = 6.90). This indicated that *P*. *ostreatus* can degrade caffeine during axenic culture *via* paraxanthine, theophylline and theobromine, either extracellularly, with translocation of caffeine and associated metabolites into the hyphae, or intracellularly with subsequent partial release of the metabolites into the medium.

To evaluate caffeine metabolism by *P*. *ostreatus* strain PO13 during cultivation under conditions that are commonly encountered in commercial production, bottles containing different combinations of spent coffee grounds (SCG) and sawdust as substrate were prepared (Fig. 5.6a) and sampled as described in Section 2.4. This broader investigation included much higher replication and more sampling time points were included, along with

more extensive compound identification and assessment of fungal growth and development, all in the commercial production setting.

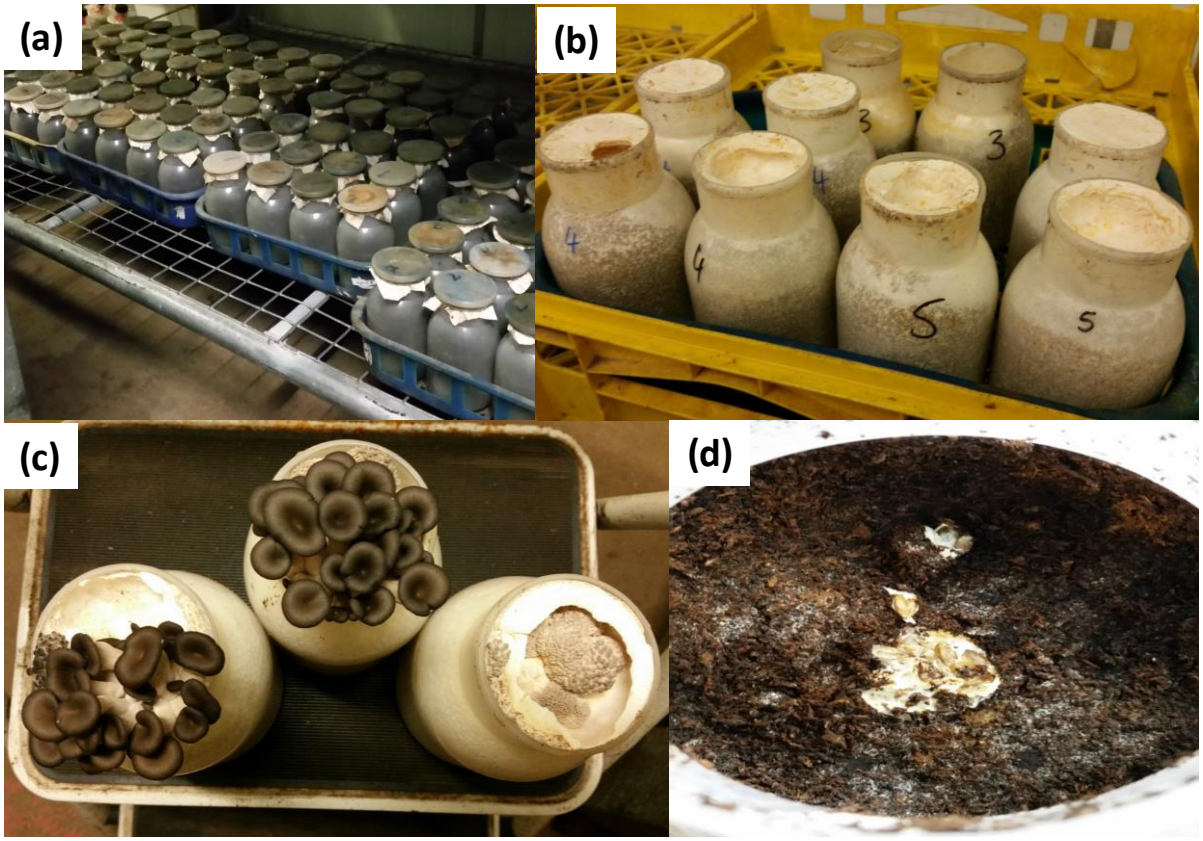

Figure 5.6 *Pleurotus ostreatus* strain PO13 grown on mixtures of spent coffee grounds (SCG) and sawdust. (a) Inoculated bottles containing substrate combinations were kept at constant temperature and humidity, as described in Section 2.5.4, (b) thick cushion of mycelium formed prior to reproductive stage, (c) different stages of fruiting body formation from the control (sawdust100) and SCG25+sawdust75 treatments, and (d) surface of colonized substrate. SCG – spent coffee grounds.

The level of colonization of the substrate by mycelium of *P. ostreatus* was evaluated using a qualitative visual scale (See Section 2.5.4, Fig. 2.5). There were clear differences in relative rates of colonization among treatments (Fig. 5.7a). The control treatment (sawdust100) was the first to be fully colonized (after 4 weeks) and was generally the most homogenous among replicate bottles (n = 30). In contrast, the SCG100 treatment was poorly colonized, consistent with the growth rates previously observed on PDA plates and in liquid culture (Chapter 3).

Moisture levels remained relatively constant in all substrate treatments, but a slight increase was recorded on day 28 of culture (Fig 5.7b). This measurement corresponded with the observation of a thick mycelial cushion forming at the top of the bottle (Fig. 5.5b), which may have impeded water vapour exchange with the substrate. The pH of the pure SCG treatment (SCG100) was generally the lowest for all treatments tested, but there were no strong variations and pH remained relatively constant during the vegetative stage (Fig 5.7c).

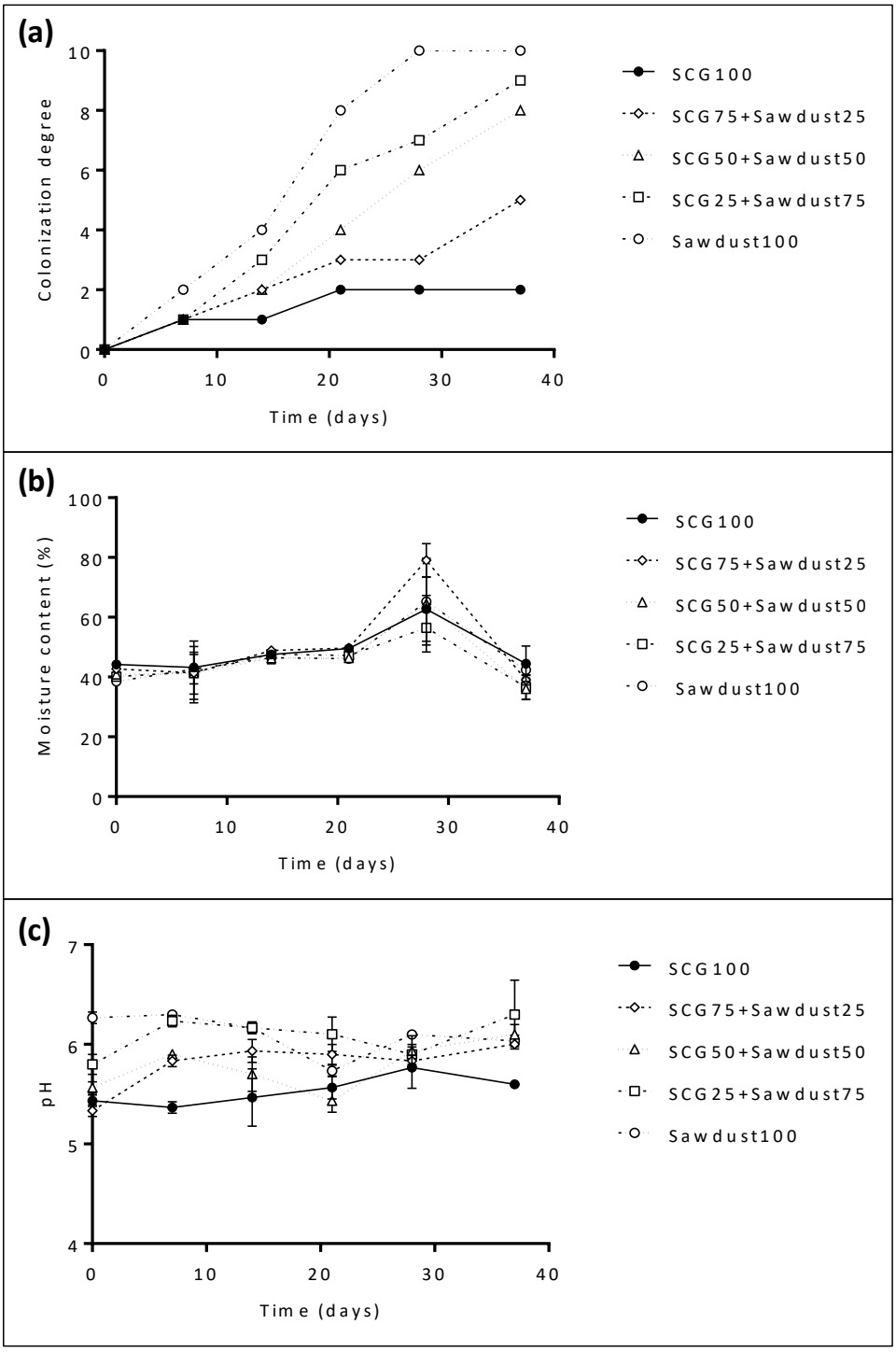

Figure 5.7 Physical descriptors of cultivation experiment of *Pleurotus ostreatus* strain PO13 cultivated on spent coffee grounds (SCG). (a) Visual assessment of degree of colonization (as described in Section 2.5.4), (b) moisture content, and (c) pH of the substrate. The five treatments were SCG100, SCG75+sawdust25, SCG50+sawdust50, SCG25+sawdust75, Sawdust100 (control). Points represent mean values (n = 6) and error bars are standard deviation.

An example of the HPLC chromatogram obtained from fruiting body extracts (derived from fruiting bodies grown on SCG25+sawdust75) is shown in Fig. 5.8. Peaks representing caffeine and seven metabolites (xanthine, 7-methylxanthine, 3-methylxanthine, 1-methylxanthine, theobromine, paraxanthine and theophylline) were detected (λ = 275 nm) and identified by UV spectra and retention times (RT) as xanthine (RT = 3.95), 7-methylxanthine (RT = 4.83), 3-methylxanthine (RT = 4.95), 1-methylxanthine (RT = 5.21), theobromine (RT = 5.46), paraxanthine (RT = 6.09), theophylline (RT = 6.26) and caffeine (RT = 6.88).

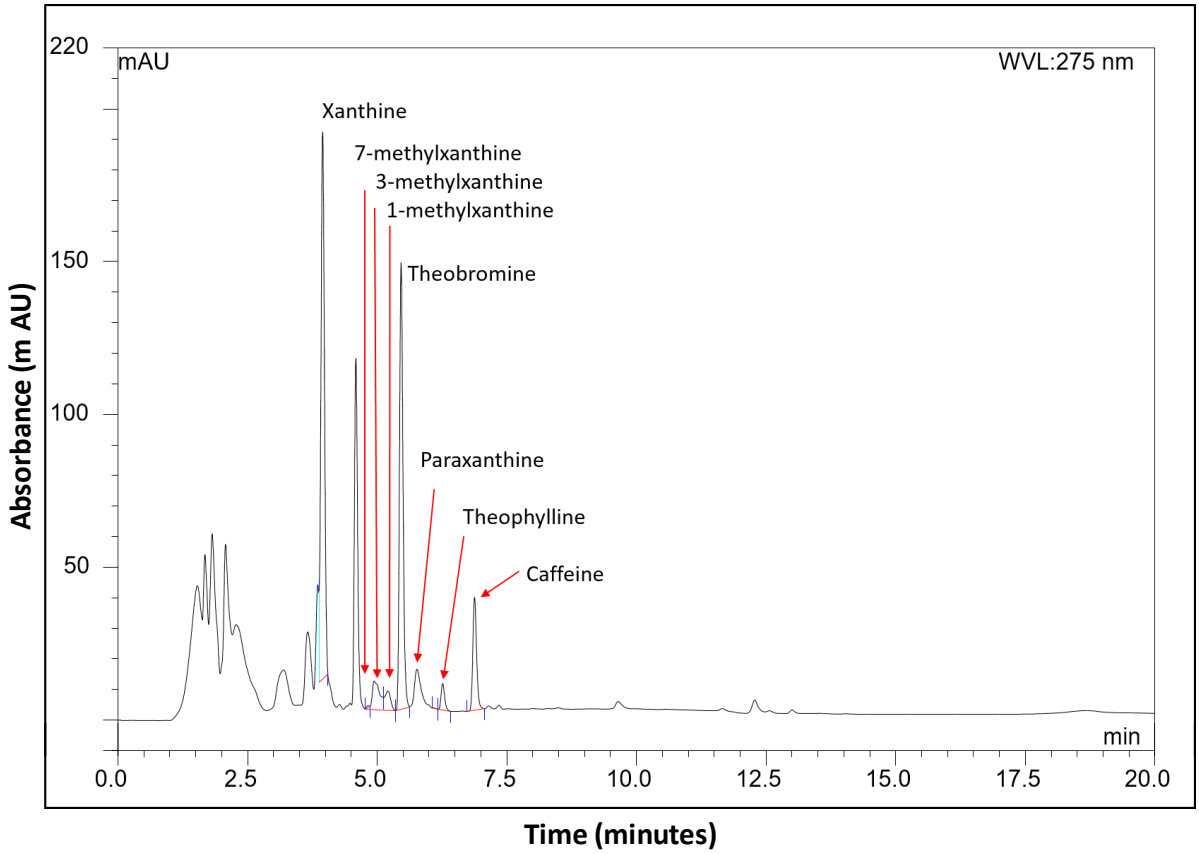

Figure 5.8 Representative HPLC chromatograms of extracts from fruiting bodies of *Pleurotus ostreatus* strain PO13 grown on SCG25+sawdust75 in commercial-scale experiment. Metabolites from caffeine degradation identified xanthine (RT = 3.95), 7-methylxanthine (RT = 4.83), 3-methylxanthine (RT = 4.95), 1-methylxanthine (RT = 5.21), theobromine (RT = 5.46), paraxanthine (RT = 6.09), theophylline (RT = 6.26) and caffeine (RT = 6.88). SCG – spent coffee grounds; RT– retention time (λ = 275 nm).

The spectra of the metabolites identified are summarized in Fig. 5.9. As all of the compounds are closely related in structure and molecular weight, retention times were also used for compound identification.

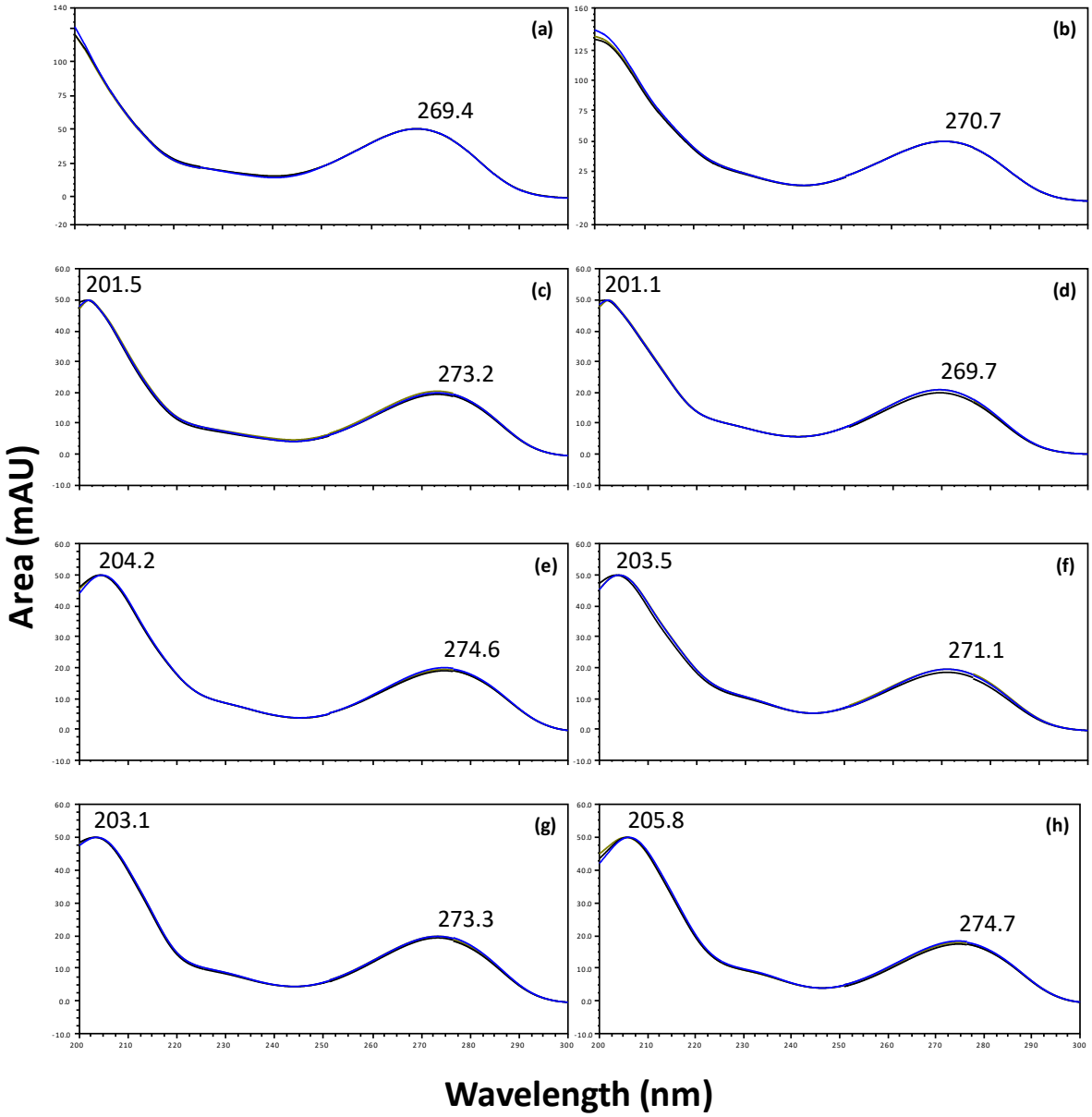

Figure 5.9 Spectra of identified metabolites from caffeine degradation by *Pleurotus ostreatus* strain PO13. Identified peaks include: (a) caffeine (RT = 6.89), (b) paraxanthine (RT = 6.10), (c) theophylline (RT = 6.25), (d) theobromine (RT = 5.55), (e) 7-methylxanthine (RT = 4.78), (f) 1-methylxanthine (RT = 5.24), (g) 3-methylxanthine (RT = 5.01), and (h) xanthine (RT = 3.99). RT – retention time (ʎ = 275 nm).

During vegetative growth, there was a decrease in caffeine concentration in the colonized substrate compared to concentrations measured at the beginning of the culture period (Fig. 5.10a). The SCG100 treatment showed a marked decrease in caffeine (25% compared to initial caffeine concentration) until day 21 and, after this, increased again in the colonized substrate. This suggests an uptake of caffeine by mycelium and subsequent release as the environment became drier. Alternatively, the fungus may have released compounds that improved extractability of caffeine from SCG during growth, resulting in higher levels detected towards the end of the cultivation period. No fruiting bodies developed on this substrate. A similar pattern was recorded for the SCG75+sawdust25 treatment and, for the other treatments, the concentration of caffeine in the colonized substrate remained stable during the vegetative stage and decreased by up to 23% after harvesting the fruiting bodies. This suggests translocation of caffeine might occur during the reproductive phase.

For substrates with higher proportions of SCG, caffeine was degraded to paraxanthine during the vegetative stage. This metabolite accumulated between days 21–38 (Fig. 5.10b) at the highest level for SCG100, followed by SCG50+sawdust50 and SCG75+sawdust25, and was detected only in traces for SCG25+sawdust75. The next compound in the metabolic pathway (7-methylxanthine) was not detected in the colonized substrate. One possible explanation for this observation could be that the subsequent steps in the degradation pathway may be proceeding at a fast rate, resulting in no detectable intermediate compounds and accumulation of the final product (xanthine). As evidence of this hypothesis, xanthine was observed in relatively larger amounts compared to intermediate metabolites (Fig 5.10h). Paraxanthine was detected in trace amounts in the fruiting bodies,

while 7-methylxanthine was present in a slightly higher concentration (Fig. 5.11b). During the vegetative stage, caffeine can be also degraded *via* theophylline, and this compound accumulated in the fruiting bodies from the treatment SCG25+sawdust75 (Fig. 5.11b).

Interestingly, accumulation of theobromine during growth occurred towards the end of the vegetative stage in treatments with higher proportions of sawdust in the substrate (with the exception of sawdust100) (Fig. 5.11d). Highest amounts of theobromine accumulated in fruiting bodies from the SCG25+sawdust75 substrate combination (Fig. 5.11b). Low concentrations of 3-methylxanthine accumulating in fruiting bodies may suggest slow degradation of 3-methylxanthine to xanthine. It should be noted that 3-methylxanthine is a product of degradation of both theophylline and theobromine, of which the concentration of the latter in colonized substrate increased from day 21 onwards during the cultivation period. Additionally, for all the treatments, apart from the control (sawdust100), xanthine was detected in increasingly greater amounts throughout the growing period. Fruiting bodies were only produced in the control (sawdust100 at day 37) and SCG25+sawdust75 (at day 37) treatments (Fig. 5.6c). This suggests that xanthine does not only originate from SCG as a result of caffeine degradation, but is also produced during growth on sawdust (being a metabolite of purine degradation).

Caffeine was detected in both the fruiting bodies and in the substrate (SMS) at the conclusion of the experimental period for only one treatment (SCG25+sawdust75) (Fig. 5.11a). Intermediate degradation products were detected in both the fruiting bodies and in the SMS of SCG25+sawdust75 (Fig. 5.11b, c). Xanthine, the final product of caffeine

degradation, was found in substantially higher concentrations in the fruiting bodies of both treatments (SCG25+sawdust75 and sawdust100 – control) than in the SMS (Fig. 5.11d).

A mass balance for caffeine and its degradation products is presented in Fig. 5.12. Xanthine was excluded from this calculation, as it can be produced from sources other than SCG as evidenced by its accumulation in the fruiting bodies of the control treatment (sawdust100). There was a high concentration of caffeine in the substrate at the start of the cultivation (Start; day 0 with no mycelium), as well as a considerable amount at the end of the cultivation period after fruiting bodies were harvested (End). Small amounts of caffeine were recorded in the fruiting bodies (Fig. 5.12a) corresponding to 5.5% of the initial concentration of caffeine in the substrate. Minor amounts of methylxanthines were recorded at the beginning of cultivation with slightly higher amounts accumulating in fruiting bodies, accounting for degradation of 4.8% of the initial caffeine in the substrate (Fig. 5.12b).

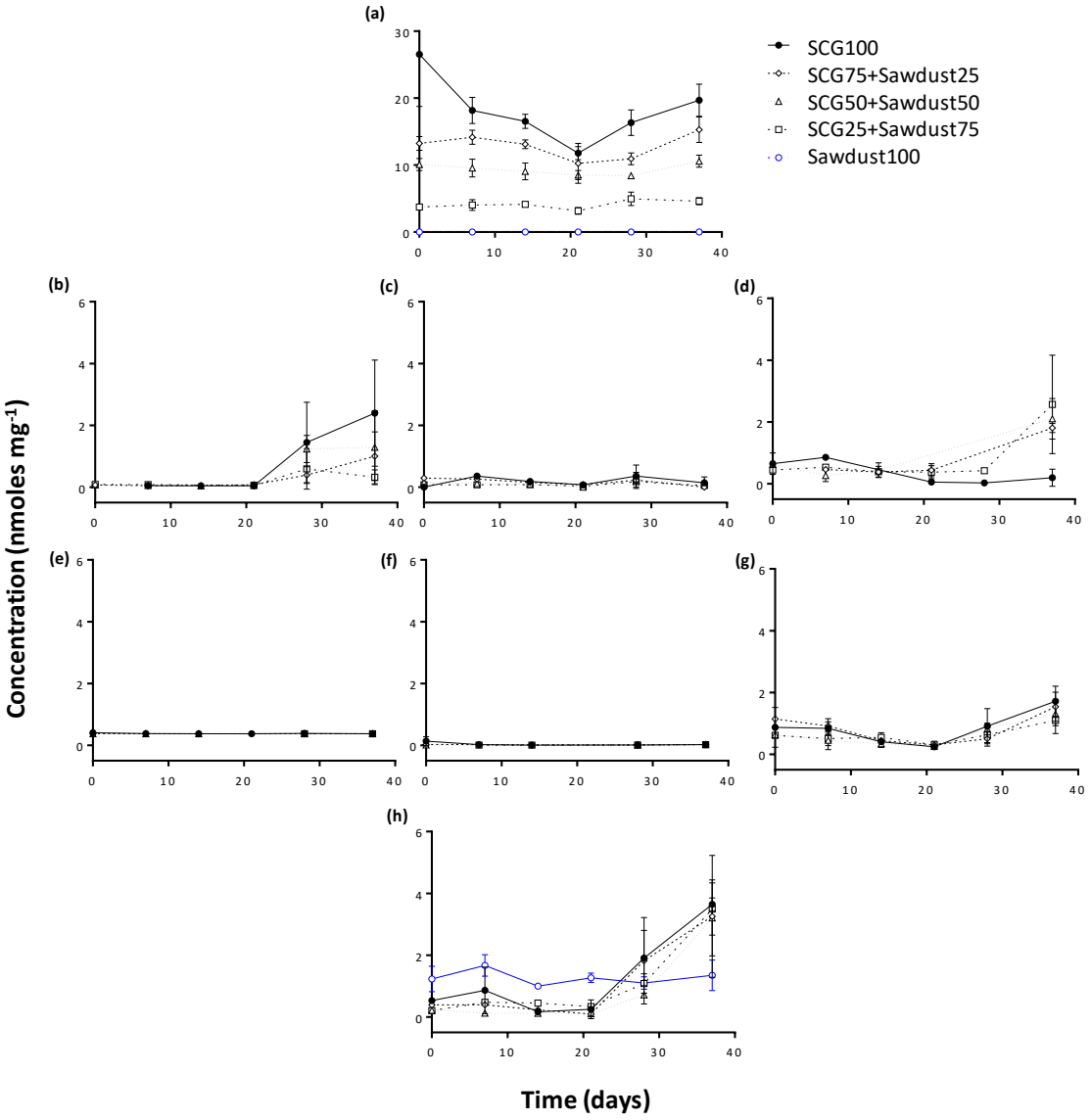

Figure 5.10 Concentration of (a) caffeine, (b) paraxanthine, (c) theophylline, (d) theobromine, (e) 7-methylxanthine, (f) 1-methylxanthine, (g) 1-methylxanthine, and (h) xanthine in the colonized substrate of five substrate combinations during vegetative stage of growth of *Pleurotus ostreatus* strain PO13, expressed on dry weight basis: SCG100, SCG75+sawdust25, SCG50+sawdust50, SCG25+sawdust75, sawdust100 (control). Points represent mean values (n = 3) and error bars are standard deviation. SCG – spent coffee grounds.

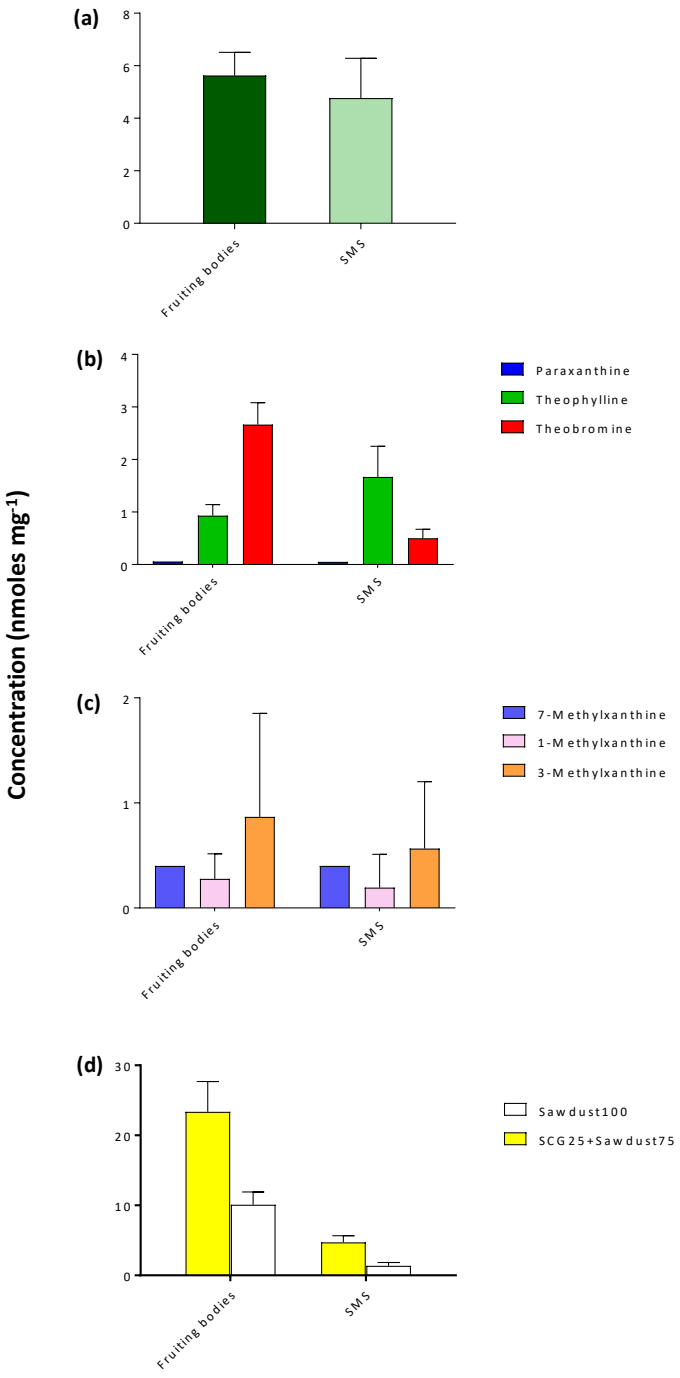

Figure 5.11 Concentration of (a) caffeine and (b) paraxanthine, theophylline, theobromine, (c) 7-methylxanthine, 1-methylxanthine, 1-methylxanthine, and (d) xanthine in the fruiting bodies and in the spent mushroom substrate (SMS) of *Pleurotus ostreatus* strain PO13 grown on SCG25+sawdust75, expressed on dry weight basis. Additional data for control (sawdust100) is included (d) for comparison. Bars represent mean values (n = 3) and error bars are standard deviation. SCG – spent coffee grounds.

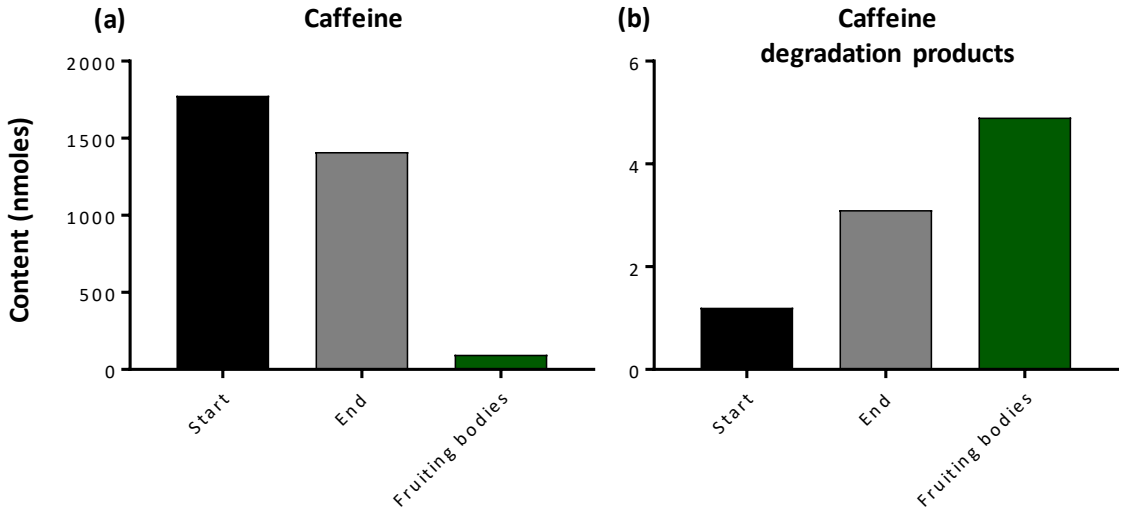

Figure 5.12 Content of (a) caffeine and (b) caffeine degradation products (excluding xanthine): paraxanthine, theophylline, theobromine, 7-methylxanthine, 1-methylxanthine and 1-methylxanthine in the substrate before inoculation (Start) and in the spent mushroom substrate after fruiting bodies of *Pleurotus ostreatus* strain PO13 were harvested (End) and in the fruiting bodies grown on SCG25+sawdust75, expressed on dry weight basis. Bars represent mean total values (n = 3) for individual growth bottles. SCG – spent coffee grounds.

5.3.3 Biological efficiency of oyster mushroom cultivation

The caffeine content of substrate prior to inoculation, fruiting bodies and SMS was measured in representative subsamples obtained from an oyster mushroom farm located in Western Australia (Lifecykel). The substrate used by these farmers included SCG and a proportion of paper pellets, but detailed information on substrate composition and crop yields is commercially sensitive and was not provided by the growers. To protect the farmers' commercial interests, the data discussed below are therefore based only on estimates made by the farm managers.

Since March 2016, two entrepreneurs from Western Australia have been collecting approximately 300 kg of SCG each week from local cafes and restaurants. A proportion of this waste is combined with approximately 300 kg of recycled paper and used as a substrate for production of oyster mushrooms. No other nutrient supplementation is used. By their estimations, a total of 80 kg of mushrooms are produced and distributed to 25 restaurants each week, while another 60 kg of mushrooms are produced from 200 boxes sold as kits for consumers to grow mushrooms at home. Each box is estimated to yield 300 g of mushrooms. From these estimated values, a BE of 23% can be calculated (Table 5.2). The farmers also provided samples of prepared substrate and spent substrate. Analysis of these samples by the methods used above revealed an initial caffeine concentration in the growth substrate of 4.1 nmole mg^{-1} (equivalent to 15–20% of the initial concentration of caffeine in SCG found in the commercial-scale trial samples), and a 59.2% decrease in caffeine content during cultivation. In the commercial-scale trial, the caffeine decrease was 23%, and a higher BE was achieved (25.6%) (Table 5.2).

As a comparison, the biological efficiency (BE) of samples from the laboratory-scale experiment using microboxes that were successful in reaching fruiting stage was calculated as described in Section 3.1. A relatively low BE was achieved using these experimental conditions, but it was comparable with the mid-size mushroom cultivation business in Western Australia (Table 5.2).

Table 5.2 Biological efficiency and caffeine decrease in substrates of *Pleurotus ostreatus*. The first two data sets are from the present study; the third data set corresponds to a mid-size mushroom farm in Western Australia

Substrate composition (%)	Biological efficiency (%)	Range of fresh weight of mushrooms produced (g)	Caffeine decrease (%)
Laboratory-scale trial			
SCG100	18.2 ± 15.1	7.2–29.1	86.8
SCG25+sawdust75	21.6 ± 11.4	10.2–37.3	1.9
Commercial-scale trial			
SCG25+sawdust75	25.6	28.9–93.8	23.0
Sawdust100	19.4	32.5–55.3	nd
Commercial-scale farm	23*	na	59.2

nd – not-detected, na – data not available

* The values for the commercial-scale farm in Western Australia were estimated using data provided, not absolute values as calculated for the experiments in this investigation.

5.4 Discussion

5.4.1 Caffeine degradation

Degradation of caffeine was observed during cultivation of *P. ostreatus* on SCG with accumulation of metabolic products of caffeine degradation occurring in the colonized substrate and the fruiting bodies. Degradation of caffeine is likely to take place during the vegetative phase with identification of six of the intermediate metabolites (paraxanthine, theophylline, theobromine, 7-methylxanthine, 1-methylxanthine, 3-methylxanthine) in the colonized substrate. In addition, xanthine, the last product of caffeine degradation, was also present in the colonized substrate and accumulated in the fruiting bodies.

Caffeine degradation has been investigated mainly for bacteria and only a few studies have used fungi (Gokulakrishnan *et al.* 2005; Mazzafera 2004). Caffeine can be metabolized by ascomycetous fungal species, including *Aspergillus* spp., *Penicillium* spp., *Rhizopus delemar*, *Fusarium solani*, *Paecilomyces gunnii* (Gutiérrez-Sánchez *et al*. 2012; Hakil *et al*. 1998; Nanjundaiah *et al*. 2016; Zhen *et al*. 2016). Caffeine is degraded by fungi using different metabolic pathways compared to bacteria. In fungi, degradation occurs *via* theophylline, while theobromine is the favoured bacterial route (Dash *et al*. 2006). In this study, degradation of caffeine during cultivation of *P*. *ostreatus* seems to occur *via* all three pathways suggested for fungi (i.e. theobromine, theophylline and paraxanthine; Fig. 5.1).

Based on the accumulation of compounds associated with caffeine metabolism, both in the substrate and fruiting bodies, it is apparent that degradation steps might occur at different rates. However, because the substrate is intimately colonized by the mushroom hyphae, it was not possible to determine what proportion of the measured compounds were contained in the mycelium itself, and how much was released into the substrate. Different rates for caffeine degradation have been reported for other filamentous fungi from the genera *Penicillium*, *Aspergillus*, *Rhizopus* and *Fusarium* grown in a range of media (Gutiérrez-Sánchez *et al.* 2013; Hakil *et al.* 1998; Roussos *et al.* 1995; Tagliari *et al.* 2003). Notwithstanding differing rates of degradation, a considerable decrease in caffeine was recorded for *Rhizopus arrhizus* (87%) and *Aspergillus* sp. (89%), and for the basidiomycete *Phanerochaete chrysosporium* (71%) when cultivated on coffee husks as substrate (Brand *et al*. 2000). Although most of these fungi are not closely related to *P*. *ostreatus*, such studies provide a valuable starting point for determining the pathway for caffeine degradation by

basidiomycetes and other fungi. Other evidence that can be drawn upon is that caffeine has been found to be absorbed, but not degraded, by a different strain of *P*. *ostreatus* (Navarro Ramalho *et al*. 2018), and by another species of *Pleurotus* (Freitas *et al*. 2018), however, again no comparative research is available for the current investigation.

At least some caffeine degradation occurred when SCG was present, regardless of the substrate combination used. This suggests that variation in the substrate might be responsible for the variability in the compounds produced as suggested by concentrations of the compounds paraxanthine and theobromine in the resulting fruiting bodies. This may mean that when *P*. *ostreatus* was cultivated on substrates containing different amounts of SCG, it might have possibly used different pathways for caffeine degradation.

All of the products of caffeine degradation were detected in trace amounts at day 0 (substrate with no mycelium). This is not surprising as these naturally occurring compounds are found in more than 100 plant species and are present in different food and beverages such as chocolate, tea, mate and coffee (Monteiro *et al*. 2016).

According to the timing of appearance of degradation compounds, the first step of caffeine metabolism by *P*. *ostreatus* occurred during the vegetative stage as demethylation. Later degradation steps may have also taken place during the vegetative stage but at fast rates as there was no accumulation in the substrate and mycelium, or during later developmental stages nearing or during the production of fruiting bodies, as suggested by accumulation of degradation compounds in the fruiting bodies. Caffeine may be degraded and taken up by mycelium because ligninolytic fungi produce low specificity enzymes that can process a wide range of compounds in the substrate, and degradation of substrate SCG might be a by-

product of their activity (Salmones *et al*. 2005; Velázquez-Cedeño *et al*. 2002). Degradation by *P. ostreatus* might therefore have occurred intracellularly after absorption of the caffeine, or intermediate products may have been translocated into the mycelium after being degraded extracellularly by the fungus, or alternatively, by other microorganisms present in the substrate.

The presence of other microorganisms during oyster mushroom cultivation was not evaluated in the current investigation. Microbial communities have been reported during the cultivation of *Pleurotus* (Vajna *et al*. 2010; Velázquez-Cedeño *et al*. 2008), as such caffeine degradation may had been occurred and/or promoted by bacteria (Chi *et al.* 2009; Mohapatra et al. 2006; Yamoka-Yano & Mazzafera 1999; Yu *et al.* 2008), and/or fungi (Gutiérrez-Sánchez *et al.* 2013; Hakil *et al.* 1998; Roussos *et al.* 1995; Tagliari *et al.* 2003). Thus, further investigations would be necessary to determine the presence of possible co-habitants in the autoclaved substrate and to elucidate their contribution to degradation of caffeine.

There was a considerable decrease in caffeine (87%) in the colonized substrate during the experimental cultivation of *P*. *ostreatus* in the laboratory-scale trial, while for the commercial-scale trial, the decrease was only 23%. Although not tested under experimental conditions, a reasonable reduction (59%) was estimated for the commercial enterprise in Western Australia. In comparison, a 50% decrease in caffeine in the substrate (coffee pulp) was demonstrated for *Pleurotus djamor* (Salmones *et al.* 2005), where caffeine was also detected in fruiting bodies but at low levels (0.17–0.22%) compared to the initial caffeine concentration in the substrate. For the commercial-scale trial, a 23% decrease in caffeine in

the substrate was accompanied by the equivalent of 10.3% of the initial caffeine content either transferred to the mushrooms or degraded into the intermediate compounds. This does not represent very a high rate of 'conversion', however, according to the variation in the concentration of caffeine in the colonized substrate, some of the caffeine may have been unavailable for uptake as it was bound onto the surface of particles of the substrate. This is possibly as result of its moderate to low solubility at low temperatures (see Shalmashi & Golmohammad 2010). Variation in caffeine concentrations may have also been due to the different substrate composition impeding its detection and/or access by the fungus. Other authors have proposed similar explanations, for example, less efficient extraction of caffeine was attributed to a hydrophobic interaction with sugarcane bagasse compared to polyurethane foam, a far more inert substrate (Hakil *et al*. 1998). Further explanation is difficult to provide due to a lack of standardization of the methods used for caffeine extraction, detection and identification among studies.

When *P*. *ostreatus* was grown using a woody substrate (sawdust) in combination with SCG, xanthine was produced by the fungus. In contrast, low xanthine content (3.6 nmole mg^{-1} dry weight) was detected in the fruiting bodies obtained from Western Australia. A difference in xanthine concentration was not unexpected as xanthine in mushroom species can range from 5.6–28 nmole mg^{-1} dry weight (Kaneko *et al*. 2008). These differences found in this study may be attributed to the nature and quality of substrate used which, in the case of the WA enterprise, was in the form of recycled paper pellets.

5.4.2 Experimental design – laboratory and commercial settings

In this study, colonization of the substrate was determined visually and not quantitatively, consistent with several other published studies (e.g. Calzada *et al*. 1987; Piškur *et al*. 2011). This way of determining fungal colonization is a good option to avoid invasive sampling of the substrate, which can potentially compromise mycelial integrity and hence alter growth. For example, high concentrations of CO_2 are crucial during vegetative stage development, and if a sudden variation in gas concentration (such as decrease in CO_2 levels and increase in oxygen levels) occurs due to opening the growing chamber or incubation vessel, the reproductive stage may be initiated and fruiting bodies may develop (Chang & Miles 2004; Kües & Liu 2000).

During the laboratory trial, the SCG50+sawdust50 treatment produced only two very small (dry weight less than 10 mg) amorphous fruiting bodies which were not analyzed for caffeine and caffeine metabolites because of limited biomass. This result suggests that the composition of the substrate may not have been adequate to induce appropriate fruiting bodies formation. Poor or non-development of fruiting bodies during cultivation on SCG and other coffee residues has been found in other investigations. For example, Maitake (*Grifola frondosa*) cultivated on a mixed substrate containing sawdust, SCG and corn bran did not develop mushrooms (Montoya *et al*. 2008). In another study, no fruiting bodies were formed although mycelium growth was observed for *G*. *frondosa* grown in the same mixture of substrates (Montoya *et al*. 2012). The same result has been found for two strains of Shiitake (*Lentinula edodes*) grown on coffee husks (Salmones *et al*. 1999) and poor development of fruiting bodies was recorded when grown on coffee pulp (Mata *et al*. 2016). In the current investigation, substrates containing higher volumes of SCG resulted in poorer

colonization of the culture medium and reduced production of fruiting bodies by the fungus. This might be attributed to tighter packing (higher bulk density) of the substrate with addition of SCG as substrate because structure and particle size are very important factors during mushroom cultivation (Membrillo *et al*. 2011). In the laboratory-scale trial, the substrate was loosely packed, while during the cultivation-scale trial, the packing technique followed commercial practices (see Section 2.4.4). The substrate was packed tightly in the bottle, with a hole cored all the way through the substrate to the bottom of the container for inoculation for even colonization (from the center to the periphery). Both the substrate packing technique and the coring, along with the finer particle size of SCG compared to sawdust might have reduced the porosity of the substrate limiting gas exchange and impeding mycelium growth. This effect has been observed during the cultivation of other fungi grown on SCG (Machado *et al*. 2012).

Development of fruiting bodies requires a high input of energy and an adequate supply of nutrients (Boddy *et al*. 2008; Halbwahs *et al*. 2016; Kües & Liu 2000). It is likely that the quality of the sawdust used for the laboratory-scale trial was not adequate for growth of *P. ostreatus*. During the commercial-scale trial, the sawdust used was from *Eucalyptus regnans*, was well aged (produced during forestry practices 30 or more years ago) and it is routinely used for commercial cultivation of this species in eastern Australia. Although *P. ostreatus* is a saprotrophic species that can degrade a range of substrates containing cellulose, hemicellulose and lignin (Bellettinin *et al*. 2016), the type of wood on which it can be grown may make a considerable difference to growth and reproduction depending on the strain and its enzymatic capabilities (Kuhad *et al*. 1997).

Oyster mushrooms grown on coffee residues (SCG, coffee seeds, pulp and husks) have shown various timing for appearance of primordia, and to reach reproductive stage. For example, in the present investigation it took 28–35 days for complete colonization and a further 11–25 days for fruiting bodies to form, with only one flush despite cultures remaining in conditions conducive for fruiting. Other investigations have also reported varying timing for vegetative and reproductive development for *Pleurotus* grown on substrates containing coffee residues. For example, primordia of *P*. *citrinopileatus* and *P*. *salmoneo-stramineus* cultivated on SCG-amended substrate, took between 20–30 days to appear and another 5–10 days for the first fructification to form (Freitas *et al*. 2018). A total of 30 days was required to obtain fruiting bodies for *P*. *ostreatus* grown on a rye substrate mixed with coffee grounds (Navarro Ramalho *et al*. 2018). Other species of *Pleurotus* (*P*. *djamor*, *P*. *ostreatus*, *P*. *pulmonarius*) required 16 days for complete colonization and an additional 36 days to produce mushrooms (Salmones *et al*. 2005). A period of 40–58 days was required for fruiting bodies form for *P*. *ostreatus* and *P*. *pulmonarius* (Velázquez-Cedeño *et al*. 2002). As for rates of hyphal growth, differences in timing for colonization and fruiting is most readily explained by variability in fungal strains and different environmental conditions.

5.4.3 Biological efficiency

Although optimization of yield was not one of the objectives of this study, biological efficiencies (BE) for the two experimental substrate mixes that produced fruiting bodies were calculated based on harvested material. The BE was found to be low and with a large

range of values, but this was to be expected as the cultivation period was relatively short and the amount of substrate used in the microboxes was smaller in comparison with the bottles used during the cultivation under farm conditions (Table 5.2). Biological efficiency fluctuates depending on many factors, including the number of harvests and mushroom weight achieved during the cultivation period (Salmones *et al*. 2005). In the laboratory-scale trial, the microboxes were small and the amount of substrate might not have been sufficient to induce the reproductive stage. Another reason is the type and origin of sawdust used. All these factors could had affected fruiting body production, thus the results obtained might not be directly comparable to the commercial farm in Western Australia. During the commercial-scale study, only two treatments developed fruiting bodies, with the higher BE calculated for the substrate containing SCG and sawdust. If the experimental period had been extended to allow addition flushes of mushroom, the BE may have changed considerably but this was not the intent of the study.

Very high BE (125–138%) has been achieved for *P*. *ostreatus* and *P*. *pulmonarius* grown on coffee pulp, but longer periods of cultivation allowing three harvests were used (Velázquez-Cedeño *et al*. 2002). High BE was achieved for *P. pulmonarius* and *P. ostreatus* (80.5 and 86.5% respectively) grown on coffee pulp (Salmones *et al.* 2005) and for *P. djamor* (86%), also grown on coffee pulp (Savoie *et al.* 2007). In other studies, moderately high BE (44–66%) has been achieved for *P*. *ostreatus* when cultivated on coffee husks which had been treated to reduce the presence of compounds that might reduce growth, and supplemented with selenium (Da Silva *et al*. 2012) or lithium (De Assunção *et al.* 2012). As another example of the variability of BE associated with coffee residues, for *Ganoderma lucidum* grown on

substrate containing residues from coffee plants, SCG and amendments, only a low BE (24%) was achieved with two mushroom harvests (Jaramillo *et al.* 2010).

5.5 Conclusion

Pleurotus ostreatus strain PO13 grown on spent coffee grounds with or without sawdust under laboratory and commercial conditions was found to be capable of degrading caffeine into six intermediate products and to form the final product of the caffeine metabolism pathway (xanthine). This study represents the first time that caffeine metabolism has been characterized for this species of fungus with respect to confirmed and proposed alternative pathways. The capability of *P. ostreatus* to degrade caffeine may lead to better utilization of agro-industrial and city wastes such as residues from coffee production and spent coffee grounds. More research on enhancing productivity of mushroom cultures on substrates amended with SCG is needed to fully unlock the potential of this species to thrive on this culture medium, to increase the mushroom production and/or enhance the process of caffeine degradation, thus helping to decrease agricultural, industrial and city waste. More detailed gene expression and metabolomic studies are needed to further elucidate the dynamics of the metabolic pathway of caffeine degradation in this organism.

Chapter 6 General discussion and future directions

Mushroom cultivation has been an important food industry for many years. In addition to production for human consumption, other uses have been developed. These include cultivation for medicinal applications and the use of spent mushroom substrate to improve the quality of agricultural products, as animal feed, and to remove harmful compounds from water and soil (Phan *et al.* 2012). Species in the genus *Pleurotus* are well-known decomposers of plant material and are commonly cultivated for commercial applications on a range of different residues from agricultural and forestry industries. *Pleurotus* can be grown on any woody-type substrate in a setting that simulates natural environmental conditions (Stamets 2000). When woody substrates are not readily available, other types of materials containing high levels of hemicellulose, lignin and other complex carbohydrates have been substituted. Broad enzyme specificity allows *Pleurotus* to degrade many types of agro-industrial wastes (Mäkelä *et al.* 2014; Velázquez-Cedeño *et al.* 2002) making waste materials a cost-saving substrate for mushroom cultivation (Bisaria *et al.* 1997; Philippoussis 2009). Around the world, a considerable number of raw plant materials from agricultural activities have been or are currently being evaluated to increase and optimize mushroom productivity and to reduce the cost of production. *Pleurotus ostreatus* is already a widely cultivated mushroom but there is increasing interest in expanding current cultivation practices given that the fruiting bodies have a high nutritional value (Carrasco-González *et al.* 2017), are highly appreciated gastronomically (Wang *et al.* 2001) and exhibit a number of important medicinal properties (see Chapter 1).

6.1 Coffee waste for mushroom cultivation

In coffee-consuming countries, the idea of using coffee grounds is gaining increasing interest among population in cities and towns. Utilization of spent coffee grounds (SCG) for mushroom cultivation is used to promote the idea of recycling and sustainability (see webpages in Section 5.1 as examples of mushroom cultivation using SCG). However, as no benefits go to the coffee farmers and there is limited advantage to the broader community, it could be argued that the practice is solely for marketing purposes and thus people can develop misleading ideas about the use of coffee waste.

Mushroom cultivation using different type of coffee wastes (obtained from industrial processes or from coffee consumption) has been evaluated regularly in the past two decades (Da Silva *et al*. 2012; De Assunção *et al*. 2012; Jaramillo *et al*. 2010; Salmones *et al*. 2005; Savoie *et al*. 2007; Velázquez-Cedeño *et al*. 2002), with most investigations reporting successful fruiting body production, or alternatively, decrease of toxic compounds in the substrate, with potential further utilization (Salmones *et al*. 2005). The present investigation evaluated the fate of caffeine from SCG during the cultivation of *Pleurotus ostreatus* strain PO13. The use of this form of coffee residue was evaluated from a laboratory-based perspective as well as under commercial farm conditions, and comparable samples were also obtained from a city mushroom farm to verify the feasibility of decreasing caffeine content, as well to produce fruiting bodies.

From the results obtained from laboratory assays (Chapters 3 and 4) and from laboratory-scale and commercial production-scale mushroom cultivation (Chapter 5), caffeine had a

mostly negative effect on the growth of mycelium of *P. ostreatus* by both (i) reduction and (ii) inhibition of growth. Reduction of growth was observed when extracts of coffee were used (fresh coffee grounds (FCG) and SCG), but this was more marked when minimal medium (MM) was used instead of potato dextrose agar (PDA). The results suggested that components of the medium modulate not only the effect of caffeine on fungal growth (Chapter 3), but also possible caffeine degradation *via* different pathways – leading to accumulation of paraxanthine, theophylline and theobromine in different proportions both in the substrate and in fruiting bodies (Chapter 5).

In general, growth inhibition was not unexpected, as such a response is known for other taxa of microorganisms (Sugiyama *et al*. 2016). However, from experiments using solid agar culture, the results initially suggested that *P. ostreatus* had, at least to some extent, the ability to utilize a growth medium containing caffeine (Chapter 3, Figures 3.1, 3.2, 3.3, 3.4). This is an important finding as it is the first confirmation of caffeine degradation during growth of *P. ostreatus* on SCG-containing substrates. In addition, detection of the degradation products in mycelia confirms the capability for this fungus to absorb caffeine; however, it was not possible to establish whether the caffeine metabolites were generated intra- or extracellularly with further and/or immediate translocation into the mycelium. This was confirmed again when caffeine and its degradation products were found to accumulate in the fruiting bodies (Chapter 5). Additional data to sustain the hypotheses proposed in Chapter 5 was provided by analysis of mushroom and spent substrate samples from a different system of cultivation (a city farm enterprise in Western Australia), yielding

comparable information even though substantially different systems of cultivation were used (i.e. substrate type and quality, SCG origin).

Laboratory-scale cultivation experiments with mixtures of SCG and sawdust as substrate were not successful for all combinations of SCG and sawdust used and did not promote even colonization of the substrate by *P. ostreatus* (Chapter 5, Table 5.1). This may be due to environmental conditions during the colonization phase or to the nature of the substrate itself (i.e. unsuitable substrate composition). However, poor growth could also be explained by the packing technique used, which might have been not suitable for the substrate particle size (Membrillo *et al*. 2011).

In natural ecosystems, heterogeneity of nutrients obliges fungi to expand to new areas of substrates to seek new supplies (Darrah & Fricker 2014). The vegetative stage of colonization is characterized by mycelia extension in search of nutrients, whereas the reproductive phase relies on the use of these nutrients. In either case, growth and development can potentially be interrupted by caffeine as a physiological response to stress. This was observed during laboratory-scale and commercial-scale cultivation studies. Fungi respond to different types of environmental stress as an evolutionary process (Bahn *et al.* 2007). As a result, there may be successful substrate colonization but no mushroom development, as found in this study (Chapter 5, Table 5.1, Figure 5.1). Regardless, it was shown that when caffeine was degraded by *P. ostreatus*, the metabolic products of degradation were detected to varying degrees in both the fruiting bodies and in the colonized substrate.

This study provides the first evidence that caffeine can be degraded during cultivation of *P. ostreatus*, with translocation of caffeine and degradation products into the fruiting bodies. Caffeine degradation was proposed to take place in the fungi itself, but it is also possible that other microbial communities associated with the colonized substrate may also play a role in both substrate utilization and caffeine degradation. This suggestion is based on known bacterial degradation of caffeine (Chi *et al.* 2009; Mohapatra *et al*. 2006; Yamoka-Yano & Mazzafera 1999; Yu *et al.* 2008), and on microbial communities reported during *Pleurotus* mushroom cultivation (Vajna *et al*. 2010; Velázquez-Cedeño *et al*. 2008).

This research outcome represents the possibility that other species in this genus and related genera might have similar capabilities for caffeine degradation. If this is indeed the case, there is the potential for enzymatic processes to be applied in industrial applications (Cohen *et al.* 2002; Inácio *et al.* 2015*;* Knop *et al.* 2015). All degradation compounds identified for other fungal taxa (Dash & Gummadi 2006; Hakil *et al*. 1998; 1999; Tagliari *et al*. 2003) were confirmed in this investigation but further research is required to confirm the proposed pathway, metabolite chemistry and enzymatic mechanism involved during caffeine degradation.

As in many studies using living organisms, one species or genus is often utilized as a biological model to increase the body of knowledge available so that other related and non-related species can be further evaluated. For example, *Aspergillus* and *Penicillium* are well known models for filamentous fungi and have been studied for caffeine degradation (Hakil *et al.* 1999; Roussos *et al.* 1995). The work presented here using *P. ostreatus* suggests the possibility of using this species in further studies for not only caffeine degradation but for

detoxification of other residues and opens additional possibilities to evaluate more (i) mushroom species, (ii) strains, (iii) substrate composition, as an integrated cultivation system that can benefit the community.

6.2 Nitrogen

The capacity of *P. ostreatus* to use caffeine as a potential source of N was tested under laboratory conditions. By adding a range of forms of N (inorganic N, amino acids and complex N-containing compounds) and comparing rates of growth it was found that inorganic N is not rate-limiting for the growth of *P. ostreatus*. The preference of different forms of N by *P. ostreatus* may be due to the following:

(i) *Pleurotus ostreatus* is a primary decomposer and, in natural ecosystems, obtains its nutrients from decomposing wood and dead plant materials (Vilgalys & Sun 1994). High adaptability to resource availability was observed in the present investigation. In the initial phase of decomposition, the fungus produces extracellular and intracellular enzymes to help access nutrients required for growth and, in the process, releases compounds to the surrounding environment generating resources for other organisms (Jonsson *et al.* 2005). Production of biomass can be considered as investment of energy and resources, to take up and utilize what is available in surrounding environment. Wood decay fungi have evolved to grow under conditions with restricted amounts of N and are capable of producing biomass at very low N concentrations (Dix & Webster 1995). This was observed when *P. ostreatus* was grown under N-free, low N conditions, both on agar and in liquid media.

In the first experimental section (Chapter 3), 10 mM of caffeine-N was found to be sufficient for growth as it (i) allowed production of fungal biomass, and (ii) caused some inhibition – likely due to toxicity, as reported for other microorganisms, but also for the genus *Pleurotus*. From those conclusions, 10 mM N was selected as an appropriate concentration to evaluate other N sources (Chapter 4). These results provided an idea of what could be a nutritional base for this fungal strain. From here, in the next section (Chapter 5), where caffeine was contained within the SCG particles (beside other compounds of varied nature), it also caused inhibition to some extent but also allowed fungal growth, with further development of fruiting bodies for some of the substrate combinations.

When *P*. *ostreatus* was grown on liquid medium, despite a large amount of biomass being produced, no decrease in caffeine was detected (Chapter 3, Table 3.4). This suggested that the fungus was not able to use N associated with the caffeine molecule, but growth was sustained by the small amount of yeast extract present in the medium. The lack of caffeine degradation may also be explained by N catabolite repression, where caffeine utilization was repressed as other N sources were present in the medium (Section 3.3.4). Neither caffeine nor its degradation products were measured in the fungal biomass, thus, this finding requires further exploration.

The fungus was grown with both ammonium chloride and caffeine, with no uptake of caffeine from the supernatant observed. The fungus growing in the CDM preferred to use N in the form of ammonium, resulting in non-utilization of caffeine-N, but reduced fungal biomass production may have also been due to an inhibitory effect. For this, we also

propose a response for N catabolite repression, observed for *P. ostreatus* (Mikeš *et al*. 1994) other fungal taxa (Andrianopoulos *et al*. 1998; Fayyad-Kazan *et al*. 2016).

Comparable C:N in the final biomass (obtained after 22 days of growth, Fig. 4.8) was observed for a range of N sources (calcium nitrate, ammonium nitrate, ammonium chloride, glutamic acid, histidine, glycine, alanine and urea). This result confirmed that that added N at 10 mM was sufficient for biomass production (C:N of the fungal biomass produced), as observed when inorganic N (ammonium chloride) was used at different concentrations (Table 4.3).

(ii) In the present study, yeast extract was added to the medium to overcome growth deficiencies (i.e. by supplying micronutrients and vitamins) after evaluating other possible compounds to stimulate minimal growth. The selection of yeast extract is a common practice used for culturing a wide range of microorganisms as it contains vitamins, cofactors or amino acids that can be used as growth stimulant (Confortin *et al.* 2008; Jonathan & Fasidi 2001). As with many fungi, *P. ostreatus* is able to synthesize its own amino acids but it can potentially use external sources for uptake and storage of these compounds for consumption when nutrients are scarce (Brock & Geib 2016). From the results obtained, yeast extract might have contained one or more essential compounds for growth of the particular strain of *P. ostreatus* used, since in its absence this strain did not grow in the medium used, nor when other vitamin/cofactor supplements were evaluated (Section 4.3.1). Importantly, this strain was originally provided by the mushroom farm where the commercial-scale experiment was done (Li-Sun Exotic Mushrooms) and this strain might have been adapted to grow under more natural conditions with a range of organic nutrient

sources, rather than in defined laboratory media, in which the nutrients were mainly provided as chemically-defined salts.

6.3 Future directions

A relatively low biological efficiency (BE) was achieved for *P. ostreatus* when grown in association with SCG. This was particularly evident from experimental conditions using microboxes and from data supplied by a commercial enterprise. Information from the literature (Martinez-Carrera *et al.* 1988; Martinez-Carrera *et al.* 1990; Salmones *et al.* 2005; Velázquez-Cedeño *et al*. 2002) indicate that a much greater BE is possible for *P*. *ostreatus* suggesting that the right combination of substrate ingredients is required. For substrates incorporating SCG this combination is yet to be found. In future studies, if the aim of mushroom cultivation is detoxification of waste by decreasing the caffeine content in SCG, the development of strong mycelia and full substrate colonization for maximum compound degradation is required, potentially at the expense of mushroom yield. When the objective is shifted to maximizing mushroom production, the use of coffee residues need to be optimized for consistently high BE. For either scenario, screening of strains to select the best variant to grow on SCG is required. In addition, optimization of environmental parameters for growth (e.g. temperature, O_2-CO_2 levels) need further study.

Mushrooms are known to be excellent sources of protein but can also provide a range of vitamins and minerals (Chang & Wasser 2012; Panjikkaran & Mathews 2013). Degradation of caffeine and other compounds present in SCG leads to accumulation of caffeine and other metabolites in the fruiting bodies, and it is unknown how the concentrations observed

might affect the nutritional value of fruiting bodies or how suitable they are for human consumption. The nutritional composition and chemical profile of mushrooms produced using SCG, and indeed, other types of coffee residues, should be evaluated to ensure that mushrooms grown on these substrates do not contain any additional metabolites that could cause health issues. In a recent publication, it was suggested that caffeine contributes to the antioxidant activity of the fruiting bodies produce on coffee-based substrates (Navarro Ramalho *et al*. 2018). However, based on the caffeine concentrations in fruiting bodies measured (Section 5.3.2), it would be necessary to consume 257 kg of fresh oyster mushrooms to obtain the equivalent of one cup of espresso coffee. The impact of caffeine in oyster mushrooms grown using SCG on human health would therefore seem to be quite low. In light of this conclusion, further evaluation is suggested.

Maintaining environmental conditions in the time between vegetative stage and fruiting body formation can be crucial (Chang & Miles 2004). This was particularly evident in the laboratory-scale study. For this experiment, to ensure full colonization and successful fruiting body production, microboxes used in the laboratory-scale trial were disturbed as little as possible and were not opened for sampling during colonization as it was judged to be likely to generate stress resulting in fewer or no fruiting bodies. Despite this precaution, fruiting bodies were not produced by all treatments, possibly because of the composition of the substrate, but also possibly due to the method of preparation of the substrate (affecting bulk density and gas exchange) and inoculation (resulting in uneven colonization). Notwithstanding these limitations, using small-scale cultivation in microboxes allowed easy manipulation of the substrate and good replication to account for variability of fungal

growth to be captured. More importantly, this technique provided a first insight into caffeine degradation during the cultivation on SCG.

During the commercial-scale trial, stronger results were obtained. Sacrificial sampling of microcosms was performed to determine caffeine degradation during the vegetative and reproductive stages. Evidence of caffeine degradation during the reproductive phase was shown but the degree of caffeine degradation during the vegetative phase (substrate colonization) is still to be determined. More research is needed to develop substrates based on SCG for optimal mushroom yield and to enhance the degradation capabilities of *P. ostreatus*.

To examine the physiological mechanisms involved in fungal metabolism, it is important to identify the intermediate phases of degradation, and the enzymatic pathways and genes involved in caffeine degradation during and/or by *P. ostreatus* should be investigated. This may lead to potential applications with other species of cultivated mushroom.

6.4 Conclusion

Cultivation of oyster mushrooms on SCG for human consumption is certainly feasible and there is great potential for this abundant type of waste to be reused and the caffeine content decreased. However, from the perspective of producing mushrooms as a source of income, it does not yet represent a significant contribution. From this study, key themes for further research include: (i) additional investigation of fungal physiology for caffeine degradation using a molecular approach, (ii) detoxification of SCG, an important waste in larger cities, and (iii) evaluation of nutritional characteristics of fruiting bodies of *P.*

ostreatus (and other mushroom species) grown on SCG and other similar agro-industrial wastes.

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