Chapter 1: Introduction

The secretion of enzymes by filamentous fungi is vital to their survival, facilitating the breakdown of complex substances in the environment into simple molecules that can be absorbed into the cell to fulfil nutritional needs. Fungi are able to vary the range of enzymes that they secrete in response to the available substrates, and different fungal strains produce different enzymes with inherent properties suited to the environmental conditions in which they must act (Webster and Weber, 2007). Fungal enzymes are used by the biotechnology industry for a range of manufacturing processes and the search for new enzymes with suitable properties and for more efficient enzyme combinations are ongoing research quests (Teter and Cherry, 2005). The work carried out for this thesis involved the investigation of the enzymes secreted by fungi isolated from a unique and recalcitrant substrate, namely koala faeces. As a result, new information has been revealed about a previously little-explored community of fungi, and enzymes have been identified that could have potential for development for industrial applications in the future.

1.1 Enzyme secretion by filamentous fungi

Filamentous fungi are abundant in nature. They exist in a broad range of habitats from tropical forests to deserts and the Antarctic, on decaying organic material and on living organisms (Hawksworth et al., 1988). The filamentous hyphae extend by apical growth, frequently branching to form a mat of mycelia (Fig. 1.1).

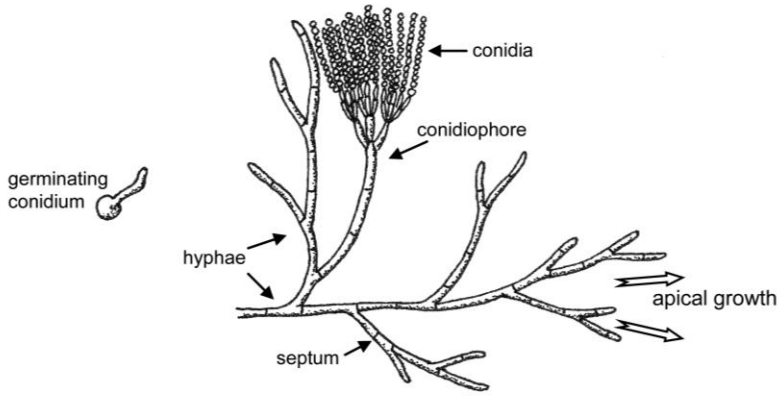

Figure 1.1: Schematic representing the salient features of a typical filamentous fungus (R. Peterson). A *Penicillium* species is shown; other fungal genera have different growth forms and conidial structures.

Fungi are heterotrophic, lacking photosynthetic pigments and are unable to manufacture their own food as plants do. Instead, fungi secrete a wide variety of enzymes capable of breaking down the complex compounds in their environment into simple molecules suitable for absorption (Webster and Weber, 2007). Plant material such as cellulose, hemicellulose and pectin can be broken down by secreted cellulases, hemicellulases and pectinases, respectively. Likewise, proteins, lipids and starch can be broken down by secreted proteases, lipases and amylases. Many enzymes are only secreted when the substrate is present in the environment so that cellular resources are allocated to production of the enzymes that will result in valuable returns to the cell (Murphy and Horgan, 2005). Filamentous fungi therefore have a great metabolic flexibility that facilitates their natural survival and also makes them attractive organisms for the production of particular enzymes of interest.

1.2 Applications of fungal enzymes in biotechnology

Enzymes from microorganisms have been used by humans since ancient times for the production of cheese, bread, beer and alcohol. More recently, the development of large-scale fermentation processes have enabled production and purification of enzymes at an industrial level. Enzymes are an attractive alternative to chemical catalysts due to their low environmental impact and high substrate specificity. Worldwide, enzymes sales amount to over $US 2 billion per year (McAuliffe et al., 2007).

Industry utilises a vast range of enzymes (Table 1.1), the majority of which are hydrolases (Kirk et al., 2002). Proteolytic enzymes used by the detergent, food and leather industries make up about 50 % of total enzyme sales, followed by carbohydrases such as amylases, cellulases and hemicellulases used in the textile, detergent, pulp and paper and baking industries, for the manufacture of animal feed (McAuliffe et al., 2007) and the production of biofuels (Yeoman et al., 2010).

Table 1.1: Examples of the industrial uses of fungal enzymes (modified from Kirk et al., 2002; additional information from Rao et al., 1998; Kashyap et al., 2001; Bhatia et al., 2002; Prema, 2006; Rodríguez Couto and Toca Herrera, 2006; Satynanarayana et al., 2006; van der Lagemaat and Pyle, 2006; Aguilar et al., 2007; Dhawan and Kaur, 2007, Pogori et al., 2007).

Enzyme	Industry	Application	Fungal sources
Amylase	Baking	Bread softness and volume	*Aspergillus niger*
	Beverage	Starch removal from pectin	*Humicola grisea*
	Detergent	Starch stain removal	*Mucor rouxianus*
	Pulp and paper	Starch-coating, deinking	*Penicillium oxalicum*
	Textile	De-sizing	*Thermomyces lanuginosus*
β-glucosidase	Beverage	Debittering of citrus juices	*Aspergillus niger*
	Biofuels	Cellulose/ cellobiose hydrolysis	*Trichoderma atroviride*
	Confectionary	Natural food colours and flavours	*Talaromyces emersonii*
Catalase	Dairy	Sterilisation, cheese	*Aspergillus niger*
	Textile	Bleach termination	*Penicillium*
Cellulase	Biofuels	Cellulose hydrolysis	*Aspergillus niger*
	Detergent	Cleaning, colour clarification	*Humicola insolens*
	Pulp and Paper	De-inking, fibre modification	*Penicillium funiculosum*
	Textile	Denim finishing, softening	*Trichoderma reesei*
			Trichoderma viride
Laccase	Beverage	Juice clarification	*Coriolus versicolor*
	Pulp and paper	Enzymatic bleaching	*Phlebia radiate*
	Textile	Denim bleaching	*Trametes*
	Waste	Degradation of xenobiotics	*Pleurotus ostreatus*
Lipase	Baking	Dough stability, conditioning	*Aspergillus*
	Dairy	Cheese flavour	*Geotrichum*
	Detergent	Lipid stain removal	*Mucor*
	Fats and Oils	Transesterification	*Penicillium*
	Leather	Depickling	*Rhizopus*
	Pulp and paper	Pitch and contaminant control	
Mannanase	Animal feed	Nutritional improvement	*Aspergillus*
	Beverage	Hydrolysis of coffee extract	*Agaricus*
	Detergent	Mannan gum stain removal	*Trichoderma*
	Pulp and paper	Enzymatic bleaching	*Sclerotium*
Pectinase	Beverage	Fruit juice clarification	*Aspergillus niger*
		Coffee and tea fermentation	*Rhizopus oryzae*
	Fats and oils	Vegetable oil extraction	*Humicola insolens*
	Pulp and paper	Enzymatic bleaching	
Protease	Baking	Biscuits, cookies	*Aspergillus*
	Dairy	Milk clotting, infant formulas	*Mucor*
	Detergents	Protein stain removal	*Penicillium*
	Leather	Unhairing, bating	*Rhizopus*
	Pulp and paper	Biofilm removal	*Tritirachium*
Tannase	Animal feed	Nutritional improvement	*Aspergillus niger*
	Beverage	Instant tea	*Fusarium. solani*
		Fruit juice clarification, debittering	*Penicillium chrysogenum*
	Chemical	Production of gallic acid	*Rhizopus oryzae*
	Waste	Tannery effluent treatment	*Trichoderma viride*
Xylanase	Animal feed	Improvement of digestibility	*Aspergillus niger*
	Beverage	Fruit juice clarification	*Fusarium oxysporum*
	Pulp and paper	Enzymatic bleaching	*Schizophyllum commune*
			Talaromyces emersonii
			Trichoderma reesei

The natural ability of filamentous fungi to secrete enzymes and their metabolic flexibility make them ideal for industrial applications. Fungi can be grown in simple, cheap liquid media and are amenable to genetic manipulation to improve enzyme production and stability. Furthermore, the enzymes that they secrete can be easily harvested from the supernatant, relieving the need for cell lysis (Kavanagh, 2005). The fungal species most widely used for industrial enzyme production are from the genera *Trichoderma*, *Aspergillus*, and *Penicillium* (Aehle, 2007).

Fungi do not naturally produce enzymes at levels high enough for commercial purposes. Therefore, strain improvement programs are undertaken to increase the secretion of target enzymes to levels that are economically sustainable. Strain improvement has traditionally been undertaken using UV or chemical mutagenesis, followed by screening for strains with desired characteristics (Nevalainen, 2001). One of the most thoroughly documented strain improvement programs of this nature is that of *Trichoderma reesei*, from which numerous independent programs worldwide have generated high cellulase-producing strains from a single wild-type strain QM6a (Herpoël-Gimbert et al., 2008). *T. reesei* RUT-C30 (Montenecourt and Eveleigh, 1979) is one of the most widely used mutant strains for the production of cellulolytic enzymes and for academic research. *T. reesei* RUT-C30, and the wild-type QM6a, were used as comparison strains for the enzyme secretion of newly isolated fungi in this work.

The advent of recombinant gene technology has revolutionised strain improvement programs, allowing manipulation of the host genome to increase production of endogenous enzymes and allow heterologous expression of genes from other organisms. Consequently environmental screening programs are used to seek enzymes from various environments, with the view to express these enzymes in highly secreting production hosts (Teter and Cherry, 2005).

1.3 Prospecting for new enzymes

Through hundreds of millions of years of natural selection, fungi in a range of different environments have evolved enzymes to suit their habitats, from hot, humid compost piles of rotting plant material in the tropics (Maheshwaki et al., 2000) to the cold stark Antarctic soil (Ruisi et al., 2007). The enzyme characteristics that have been necessary for the survival of the fungi in their ecological niche can also be valuable to industry. For example, the manufacture of pulp and paper and textiles requires enzymes that are active at a high temperature and high pH; dairy and other food production processes often require enzymes to function at a low temperature and low pH; enzymes for inclusion in detergents for washing in cold water need to be active at a low temperature and high pH (Lange, 2004).

The search for new enzymes with natural characteristics that suit industrial processes is an ongoing area of research, fuelled by the desire to increase productivity and cater for emerging technologies. Whereas protein engineering and directed evolution can be used to manipulate an enzyme's performance (Turner, 2003), enzymes that have natural tendencies towards the desired activities make the best candidates for improvement programs. Large biotechnology companies often rely on universities and small companies to provide leads based on environmental screening projects (Laird et al., 2006). Some examples of fungal screening projects and the enzymes discovered are listed in Table 1.2.

Table 1.2: Enzyme screening projects of fungi from environmental sources.

Screening site	Enzyme	Fungal species found with high enzyme activity	Reference
Air/ Soil (Europe, Asia, Africa)	Keratinase (Protease)	*Aspergillus flavus* *Alternaria radicina* *Trichurus spiralis* *Stachybotrys atra*	Friedrich et al., 1999
Soil (Antarctica)	Hydrolases	*Penicillium*, *Phoma* and *Alternaria*	Bradner et al., 1999a
Agricultural and industrial waste	Xylanase	*Aspergillus, Fusarium, Penicillium* and *Trichoderma* spp.	Abdel-Sater et al., 2001
Rotting wood	Pectinase	*Gloeophyllum striatum* *Pycnoporus sanguineus* *Schizophyllum commune*	Xavier-Santos et al., 2004
Forest (Tunisia)	Ligninases	*Trametes trogii* *Stereum annosum* *Phlebia* sp. *Polyporus* sp. *Oxyporus latemarginatus*	Dhouib et al., 2005
Mixed forests (Portugal)	Ligninases	*Ganoderma applanatum* *Trametes versicolor* *Bjerkandera adusta*	Matos et al., 2007
Tannery effluent	Tannase	*Aspergillus niger Aspergillus flavus* *Trichoderma* spp. *Penicillium* spp. *Fusarium* spp.	Murugan et al., 2007
Marine corals and sponges	Protease	*Beauveria brongniartii* *Acremonium fusidioides*	Kamat et al., 2008
Wooden hut (Antarctica)	Cellulase	*Cladosporium oxysporum* *Geomyces* sp.	Duncan et al., 2008
Fruit and wood (Brazil)	Lipase	*Botryosphaeria* spp.	Messias et al., 2009
Root knot nematodes	Chitinase Protease	*Paecilomyces lilacinus* *Pochonia chlamydosporia*	Wei et al., 2009
Lake water and sediments (India)	Amylase	*Aspergillus flavus*	Sasi et al., 2010

Not all fungal species are suitable for growth in liquid media, nor may they have a high secretion capacity. Therefore, production of novel enzymes is often achieved by isolating the gene encoding the enzyme of interest and recombinant expression in an industrially established high-secreting production host, such as *Trichoderma reesei* or *Aspergillus* species (Aehle, 2007). There has been an increasing trend towards isolating the genes for particular enzymes directly from an environmental source, such as soil or water, without the isolation of the microorganism that produces them. The gene is then expressed in a high-secreting host

(Schloss and Handelsman, 2003). However, gene-based environmental screening is laborious and enzyme functionality can only be inferred (Yun and Ryu, 2005). In addition, new enzymes may be overlooked because their corresponding genes may lack sequence similarity to previously described enzyme-encoding genes. Isolation of the microorganism itself allows extensive characterisation of a fungal species under laboratory conditions, as well as the enzyme it produces. Furthermore, proteomic and transcriptomic information can be obtained that provide systems level information about how the microorganism functions as a whole in response to its environment.

1.4 Coprophilous fungi

Coprophilous fungi inhabit faeces, most commonly herbivore faeces. As an essential part of the ecosystem, coprophilous fungi utilise the contents of the faeces, subsequently degrading it and recycling the micronutrients it contains back into the ecosystem. Some coprophilous fungi must travel through the digestive tract of the animal as spores before germination can occur on the faeces. Other species arrive later via air dispersal of spores or contact with other surfaces (Ing et al., 1989).

Most of the early research on coprophilous fungi occurred in Europe and North America and was mainly concerned with the identification of fungal species (Kendrick, 2005). A consistent pattern has been observed in the succession of fungi that appear on the faeces at various stages of degradation, analogous to vegetation succession on cleared land. *Mucor* and other closely related genera from the phylum Zygomycota usually occur first, utilising the easily metabolised simple sugars, starch and protein to form rapidly spreading mycelia and quickly germinating spores. The majority of fungi found on faeces are ascomycetous species, which appear when the simple carbon sources are depleted as they can utilise the hemicellulose and cellulose material. Basidiomycetous species, particularly Agaricales, are capable of degrading lignin as well as cellulose, and usually appear late in the succession (Krug et al., 2004).

Nutritional factors are not the only influence on fungal succession on faeces. Some species of coprophilous fungi produce antifungal metabolites that inhibit the growth of other species, thus reducing competition and establishing dominance. The isolation, identification and potential uses of antifungal metabolites have been a focus of research on coprophilous fungi since the 1990s (Morris et al., 1995; Wang et al., 1997; Soman et al., 1999; Michael et al., 2003; Lehr et al., 2006). In comparison, there has been surprisingly little research on the enzymes produced by coprophilous fungi (Wicklow et al., 1980; Magnelli et al., 1996; Magnelli and Forchiassin, 1999). The potential of coprophilous fungi to produce powerful enzymes is great, as signified by their growth on such an unusual substrate, and warranted further investigation through this work.

1.5 Koala faeces: a unique and recalcitrant substrate

The koala, *Phascolarctus cinereus* (Fig. 1.2), has a unique diet consisting entirely of *Eucalyptus* leaves. As a result of the hot and dry Australian climate, *Eucalyptus* leaves are tough and nutrient poor. The leaves contain approximately 25 % cellulose, 12 % lignin and 15 % non-cellulose carbohydrates including hemicellulose (xylan and mannan) and pectin (Fig. 1.3; Cork, 1986; Tyndale-Biscoe, 2005). In addition, *Eucalyptus* leaves contain phenolics (principally tannin) and essential oils that deter most insects and animals from consuming them (Cork and Foley, 1997).

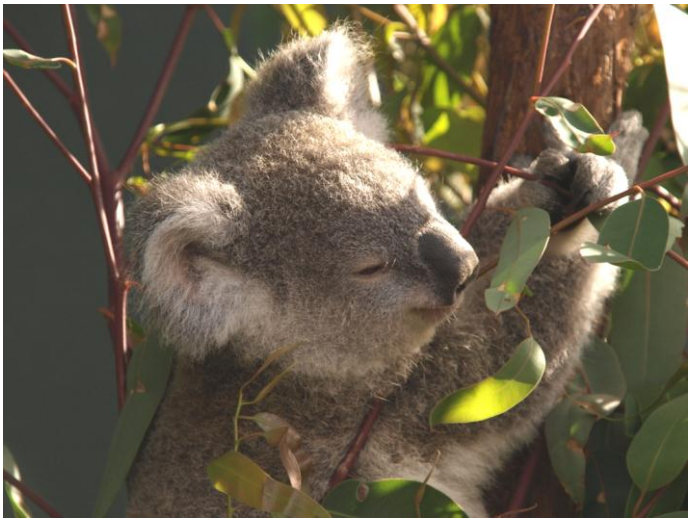

Figure 1.2: Koalas have a diet consisting entirely of *Eucalyptus* leaves. Photo M. Godlewski.

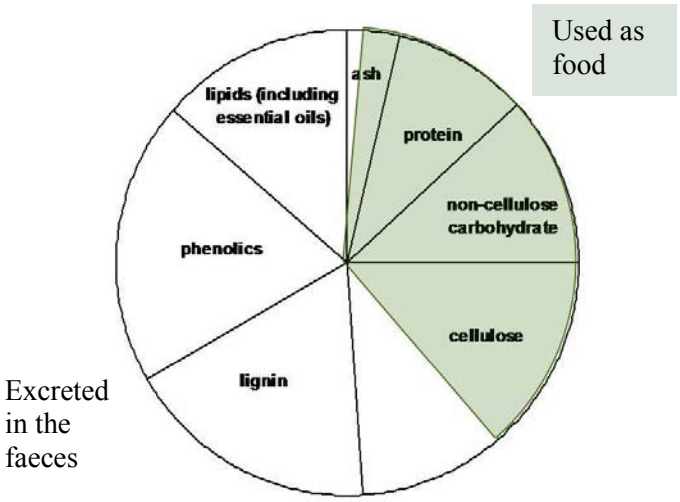

Figure 1.3: Constituents of *Eucalyptus* foliage. The shaded area covers the components from which a koala can extract nutrition. The remaining area covers components that are mainly excreted in the faeces. Some remnants of the components used as food are also excreted. Modified from Tyndale-Biscoe (2005).

Koalas are able to obtain adequate nutrition from *Eucalytpus* leaves using a system of hindgut fermentation. Small particles, including epithelial cells and small, poorly lignified fragments of plant cell walls are retained in the hindgut and fermented by anaerobic bacteria and fungi to release enough nutrition to maintain the koala's slow metabolism. Large particles, principally undigested cellulose and other components of the cell wall, along with phenols and essential oils (Hume, 1989) are excreted relatively rapidly, forming a hard, dry and fibrous faecal pellet (Fig. 1.4).

Figure 1.4: Koala faeces, a dry, fibrous and cellulose-rich material (www.abc.net.au/science/scribblygum).

Despite the apparent recalcitrant nature of the faecal contents, fungi are able to colonise koala faeces (Cribb, 1997) and so a premise for this work was that these fungi were likely to produce enzymes capable of degrading the lignocellulose-rich material the faeces contain.

Very little research has been carried out in the past on fungi that grow on koala faeces, and the few studies that have been undertaken have been taxonomic in nature (Cribb, 1997). To our knowledge, this is the first time fungi from koala faeces have been systematically studied by isolating and identifying a succession of fungal species occurring over time, and the first time that fungi from koala faeces have been investigated for their enzyme activity.

1.6 Isolation and identification of fungi

The isolation of fungi from an environmental source can be carried out in a variety of different ways, the choice between which is determined by the habitat concerned and the aim of the study. Fungi can be isolated as growing organisms or collected as microscopic spores in soil and other materials for subsequent germination in the laboratory (Section 1.6.1). Identification can be achieved by morphology (Section 1.6.2) and molecular means (Section 1.6.3).

1.6.1 Isolation of fungi from faeces

As described above (Section 1.4), the appearance of fungi on faeces occurs in a succession, with different species appearing over time. Consequently, it is usual to remove the faeces from the environment for incubation under laboratory conditions to achieve a comprehensive analysis of the species that occur. Studies that aim to find out more about the animal producing the faeces tend to be concerned chiefly with the fungi or spores that pass through an animal's digestive system. To avoid contamination by external sources, faecal material is taken directly from the animal's anal cavity and is processed under sterile and often anaerobic conditions. Such research provides information about the digestive mechanisms of the animal and the fungi (often anaerobic species) that play a role in it (Milne et al., 1989; Osawa et al., 1993a, Osawa et al., 1993b). Studies that are focused on the fungi, and specifically identifying species that can use the faeces as a substrate, are less concerned with the origin of these fungi and do not follow such stringent procedures (Ing 1989; Bell and Mahoney, 1995; Bills and Polishook, 1993; Krug, 2004).

Incubation of faeces in a laboratory can be carried out in moist chambers, which typically consist of a sterile glass or plastic container containing moist filter paper and the faecal pellet. Fungi can be isolated as they germinate by point inoculation from mycelia or sporulating bodies to individual petri dishes of potato dextrose agar or similar rich nutrient source (Krug, 2004). Alternatively, the faeces themselves can be ground and mixed into the agar medium, and fungi isolated from the agar surface (Warcup, 1950). Separation of fungal species from bacterial inhabitants of the faeces can be achieved by air drying of the faeces prior to moist incubation and by including antibiotics in the agar medium (Krug, 2004).

1.6.2 Morphological identification of fungi

Classical approaches to the identification of fungi have relied on macroscopic and microscopic observations, in particular the structure of sexual and asexual fruiting bodies. Fungi from the phylum Zygomycota have hyphae that lack septa (cross walls). Conspicuous asexual fruiting bodies called sporangia are borne on long thin filaments (Fig. 1.5). Sexual fruiting bodies are formed within the mycelial mass as thick walled zygospores (Malloch, 1981).

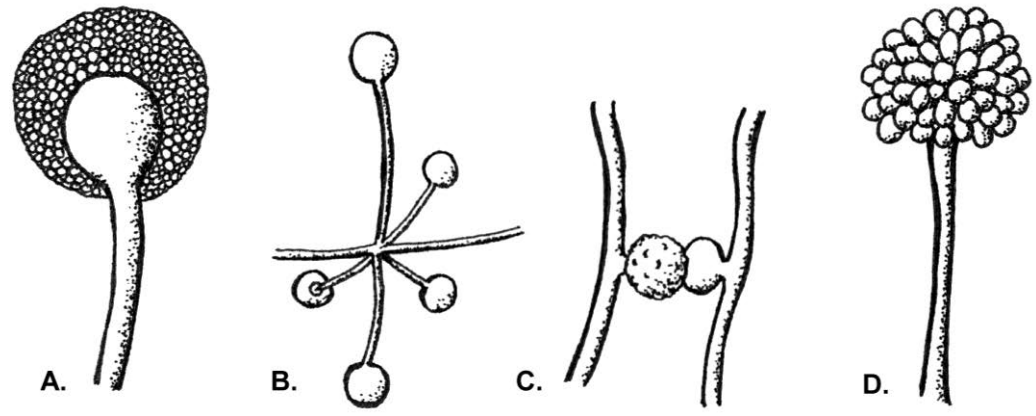

Figure 1.5: Morphological characteristics of some of the Zygomycota species- A: sporangia of *Mucor* sp.; B: whorl of sporangia of *Zygorhynchus* sp.; C: zygospore of *Zygorhynchus* sp.; D: sporangiophore and sporangiola of *Cunninghamella* sp. (R. Peterson, adapted from Malloch, 1981).

The phylum Ascomycota is the largest group of fungi, with approximately 32,000 known species (Webster and Weber, 2007). Hyphae have many septa and sexually produced spores

(ascospores) are contained in a characteristic sac, the ascus, which differs in structural appearance between species (Fig. 1.6).

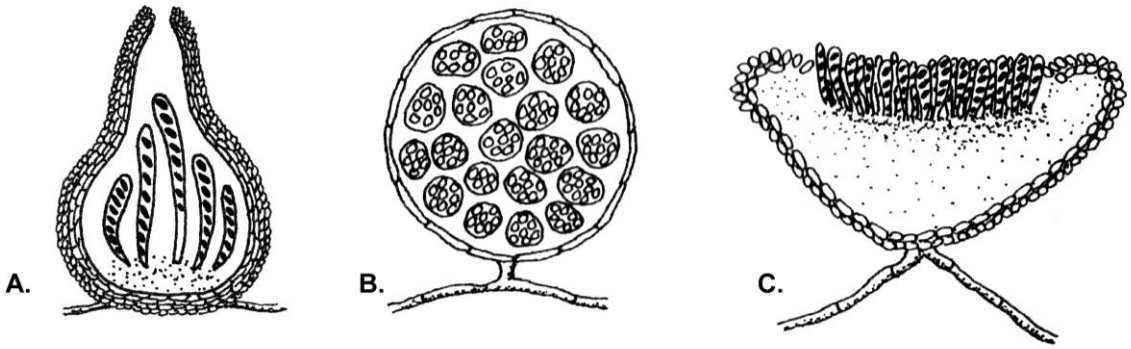

Figure 1.6: Morphological characteristics of some of the Ascomycota species. Three kinds of ascocarps: A. perithecium. B: cleistothecium. C: apothecium (R. Peterson, adapted from Webster and Weber, 2007).

Many of the Ascomycota species, such as *Penicillium*, *Aspergillus* and *Trichoderma*, exist predominantly in an asexual state as "anamorphs", and rarely produce ascospores or asci. The morphological identification of these species is dependent on the placement and structure of their asexual fruiting bodies, the conidiophores (Gupta, 2004; Fig. 1.7).

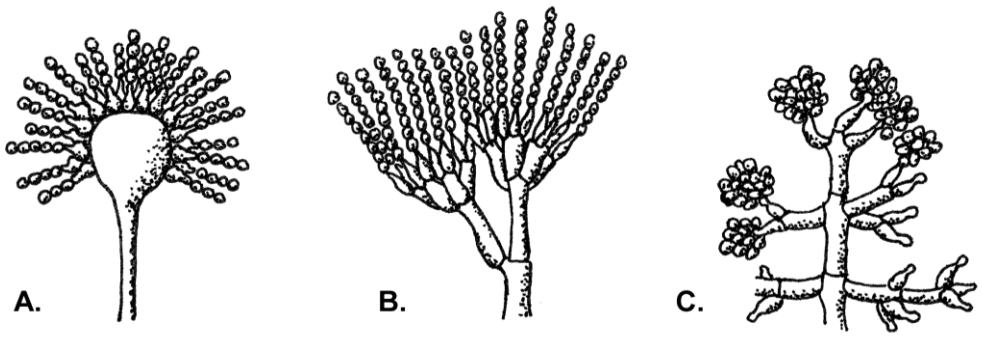

Figure 1.7: Morphological characteristics of the conidiophores of common *Ascomycota* anamorphs. A: *Aspergillus* sp.; B: *Penicillium* sp.; C: *Trichoderma* sp. (R. Peterson, adapted from Malloch 1981).

The Basidiomycota is the second largest fungal phylum with over 30,000 species (Webster and Weber, 2007), including the macrosopic fungi commonly known as mushrooms, toadstools and puffballs. The phylum is characterised by the presence of basidia, which contain basidiospores (Fig. 1.8).

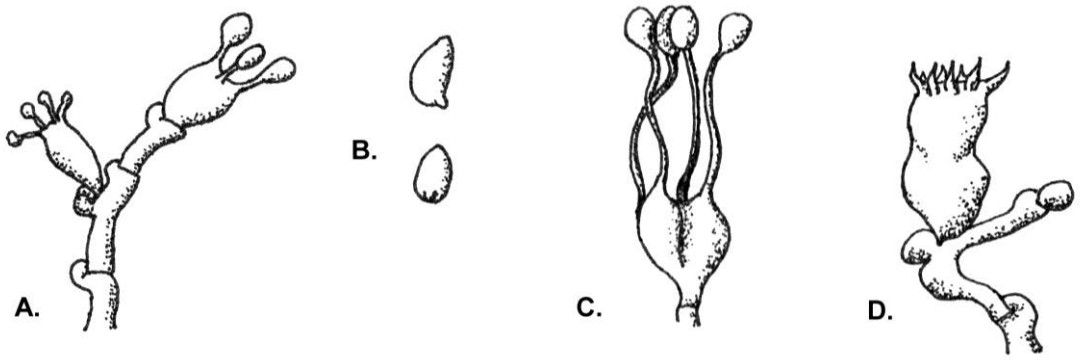

Figure 1.8: Morphological characteristics of basidia (spore bearing structures) found in some of the *Basidiomycota* species- A: four-spored undivided basidium; B: two typical basidiospores; C: a four-celled cruciate basidium; D: an eight-spored holobasidium (R. Peterson, adapted from Malloch 1981).

Identification and classification of fungi using morphological methods is not always a straight forward process, nor one without contention. A single fungal species can vary in morphology, depending on the growth medium, temperature and moisture availability. Different species can also exhibit very similar characteristics under certain conditions. Molecular biology has provided a more reliable method for the identification of fungi (Rodriguez et al., 2004); however, morphological features still play an important role for making preliminary identifications, which can then be supported using molecular methods where necessary.

1.6.3 Molecular identification techniques

Genomic DNA can be extracted from the mycelia or spores of filamentous fungi and used for identification by molecular-based techniques. The fungal cell wall is broken by enzymatic treatment or mechanical means, such as grinding with liquid nitrogen, and the cellular contents lysed in an appropriate buffer to allow access to the nuclear material. Numerous protocols have been developed for the extraction of DNA from fungi. Some utilise the cationic detergent CTAB (cetyltrimethylammonium bromide) that has traditionally been used for plant DNA extraction (Saghai-Maroof et al., 1984), while others employ organic solvents such as phenol to denature and eliminate protein contaminants (Blin and Stanford, 1976). Genomic DNA can be subjected to a number of identification techniques, including

Restriction Fragment Length Polymorphism (RFLP; Section 1.6.3.1), Random Amplified Polymorphic DNA (RAPD; Section 1.6.3.2), 18S sequencing and Internal Transcribed Spacer (ITS) sequencing (Section 1.6.3.3).

1.6.3.1 Restriction Fragment Length Polymorphism (RFLP)

RFLP is a technique that involves the digestion of genomic DNA or an amplified sequence with one or more restriction enzymes. The resulting fragments are then separated by gel electrophoresis to reveal distinctive banding patterns that reflect differences in restriction enzyme cutting sites and thus differences in genome sequence. Identifications can be made by comparison to fragmentation patterns of known species or strains (Nicolotti et al., 2010).

1.6.3.2 Random Amplified Polymorphic DNA (RAPD)

In RAPD analysis, short arbitrary primers (8–12 nucleotides) bind to complementary DNA sequences throughout the genome. DNA fragments are generated by polymerase chain reaction (PCR) amplification provided that two primer binding sites are close enough in proximity to each other. The number and size of the fragments can be visualised by gel electrophoresis and compared between different species (Galvão et al., 2008).

1.6.3.3 Sequencing based on conserved and variable regions of ribosomal DNA

The process of ribosome generation and protein synthesis is essential to cell function, and consequently the genes encoding the ribosomes are highly conserved (Baldwin, 1992). There are three conserved regions of ribosomal DNA (rDNA) in eukaryotic organisms: 18S rDNA, 5.8S rDNA and 28S rDNA (Fig. 1.9). Non-coding internal transcribed spacer (ITS) regions of DNA (ITS1 and ITS2, Fig. 1.9) separate the rDNA regions. There is far more variability in the ITS regions because these have less evolutionary pressure to remain conserved (Schmidt, 2006).

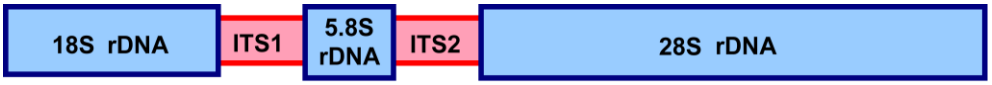

Figure 1.9: A nuclear ribosomal DNA unit comprising the highly conserved small subunits (18S and 5.8S) and large subunit (28S) and the more variable internal transcribed spacer (ITS) regions.

The development of primers for PCR amplification of conserved regions of rDNA and the more variable ITS regions has been one of the greatest breakthroughs for the identification of fungi. Sequences derived from 18S rDNA (Fig. 1.9) can be searched against databases containing 18S rDNA sequences of known fungal species, in a similar manner to that used for 16S rDNA-based identification of bacteria (Woese, 1987; Kowalchuk et al., 1997; Smit et al., 1999; Schabereiter-Gurtner et al., 2001). However, 18S rDNA sequencing is generally only able to resolve genus-level information and little distinction is found between species (Anderson et al., 2003). More recently, ITS sequencing, encompassing ITS1, ITS2 and immediately adjacent areas of conserved rDNA (Fig. 1.9), has become the most common technique for molecular fungal identification (Schmidt, 2006). The greater variability in the ITS regions can be used to distinguish between closely related fungal species and strains (Nilsson et al., 2008; Ciardo et al., 2010).

In this work, ITS sequencing was used for the identification of fungal isolates from koala faeces (Chapter 3). A technique developed by White et al. (1990) was employed, involving PCR amplification of ITS regions directly from fungal genomic DNA. The method (White et al., 1990) has been used for the identification of fungal species from a diverse range of isolation sources (Bradner, 2003; Chen et al., 2007; El Karkouri et al., 2007; Michaelsen et al., 2006); however, to our knowledge, ITS sequencing had never been used to identify a community of coprophilous fungi prior to this work.

1.7 Investigation of enzyme activity

When isolating fungi from an environmental source, it is common to obtain a vast number of different species and strains that could be potential candidates of interest for their enzyme activity. Rapid screening of the isolates can be achieved by means of agar plate assays. Once a smaller group of the most promising fungal strains is established, more extensive enzyme characterisation can be carried out using liquid cultures, enzyme assays and zymograms.

1.7.1 Screening by agar plate assay

The agar plate assay technique involves inoculating a fungal isolate onto an agar plate that contains a single complex carbon source: the substrate for the target enzyme of interest (Hankin and Anagnostakis, 1975). In order to survive and grow, the fungus must secrete the target enzyme to break down the substrate into small enough molecules that it can absorb into the cell and use for nutrition. Detection of enzyme secretion and activity is typically achieved by observing a halo around the fungal colony, indicating that the substrate has been degraded (Fig. 1.10).

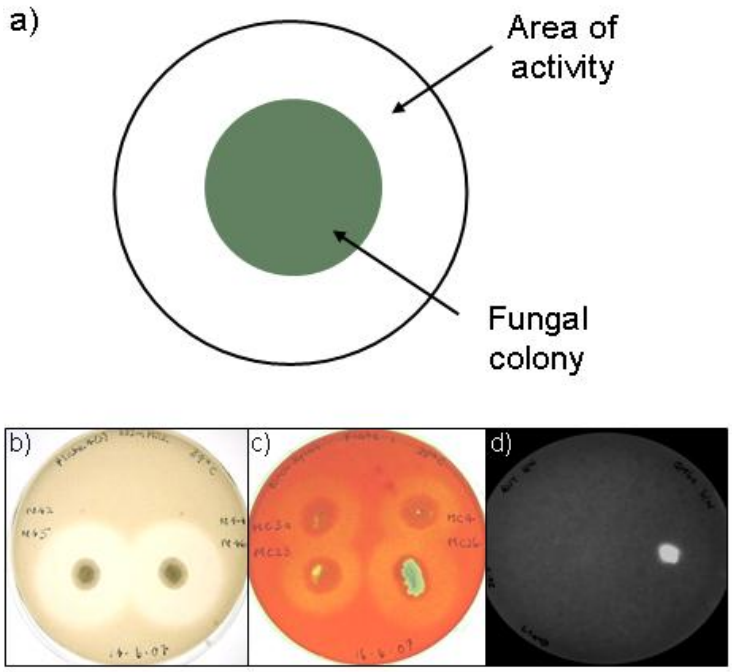

Figure 1.10: a) Schematic representing the principle of agar plate assays. Halos form around the fungal colonies indicating degradation of the substrate and thus enzyme activity; b) a skim milk plate indicating protease activity; c) a birch xylan plate, stained with Congo Red, to reveal xylanase activity. d) A Rhodamine B plate viewed under UV light to reveal a fluorescent area indicating lipase activity.

Visualisation of halos indicating degradation of some substrates is not immediate but requires additional procedures, such as Congo Red staining to reveal xylanase activity (Teather and Wood, 1982; Fig. 1.10c), or illumination with UV light to reveal lipase activity (Kouker and Jaeger, 1987; Figure 1.10d). The diameter of the halo is representative of the extent of enzyme activity and can be used, in conjunction with the colony size, to select for top-performing fungal strains (Bradner et al., 1999a). Agar plate assays were used in this work to screen fungal isolates from koala faeces for their enzyme activity and are described in more detail in Chapter 3.

Agar plate assays can be refined by manipulation of the incubation temperature or the pH of the agar medium to investigate the range of conditions under which the enzymes are secreted and maintain activity. When the aim of a research project is to seek particular enzyme properties, such as those desirable for a defined industrial application, agar plate selection can be carried out at, or close to, the temperature and pH conditions in which the enzyme would be required to act. For large-scale projects, colony picking robots and computer-based imaging software are being used increasingly to improve efficiency and throughput of the agar plate screening process (Hughes et al., 2006).

1.7.2 Liquid cultures

Following selection of enzyme-producing isolates by agar plate assay, attempts can be made to grow the chosen isolates in liquid culture, most commonly in shake flasks. Liquid culturing allows the enzymes to be freely secreted into the aqueous medium, which can then be separated from mycelia by centrifugation; the culture supernatant is then used for enzyme harvesting and analysis. The culture medium typically contains essential nutrients and substrates known to favour fungal growth and induce the enzymes of interest in well-studied fungal species; for example, crystalline cellulose and/or lactose induce cellulolytic enzymes (Esterbauer et al., 1991). Some species do not grow well in aqueous conditions or become very viscous or clump into pellets that inhibit enzyme secretion (Gibbs et al., 2000). Further

enzyme analysis is difficult for these species and they are often dropped from further study. For those species that do grow in liquid culture, attempts can be made to modify the culture medium by manipulation of the nutrient contents, pH or aeration (eg. shaker speed) in order to increase enzyme yield.

1.7.3 Liquid enzyme assays

Enzyme activity present in the supernatant of a fungus grown in liquid culture can be assessed using liquid enzyme assays. In the assay process, the enzymes act on a substrate to induce quantitative changes in light absorbance (colorimetric assay) or fluorescence (fluorimetric assay) in the assay medium. Enzyme activity against substrates such as xylan, carboxymethylcellulose, galactomannan or starch can be measured by the amount of reducing sugars released into the assay medium, represented by the colour produced upon interaction with the aromatic compound dinitrosalicyclic acid (DNS; Bailey et al., 1992). In addition, many synthetic substrates are commercially available (www.sigma-aldrich.com) that contain p-nitrophenyl compounds or fluorescing groups and show colour or fluorescence when cleaved (Sicard and Reymond, 2006).

Each individual enzyme has an optimal temperature and pH under which it maintains a conformation for maximal activity. The rise and fall of enzyme activity that occurs about this point is referred to as an enzyme's activity profile (Fig. 1.11). To a certain extent, an increase in temperature results in higher enzyme activity due to increased molecular movement and collision rates between substrate and enzyme. However, at higher temperatures the noncovalent interactions that stabilise the native structure of the enzyme are disrupted and the active site is no longer able to function correctly. Similarly, an enzyme will function most efficiently at a particular pH. As the pH changes from the optimum, the charges of amino acid residues at the active site are altered, the ability of substrate and enzyme to exchange hydrogen ions is reduced and the rate of enzyme activity is decreased.

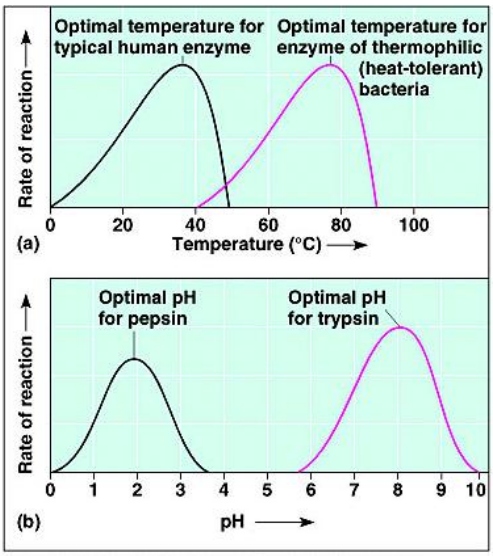

Figure 1.11: Examples of different enzyme profiles showing the influence of environmental conditions on enzyme activity: a) temperature profiles; (b) pH profiles (Campbell, 1996).

The temperature and pH optima are different for different enzymes (Fig. 1.11), as is the broadness of the temperature and pH range under which activity is maintained (Campbell, 1996). Liquid enzyme assays can be undertaken across a broad temperature and pH range to establish the activity profiles of enzymes from different fungal strains. The enzyme activity profiles can then reveal unusual levels of temperature or pH tolerance in the enzymes that could be valuable for biotechnological applications.

A considerable amount of time and labour is involved when enzyme characterisation is carried out by manual enzyme assays. Automation by robotics is becoming increasingly common for large-scale projects and greatly improves throughput. Furthermore, protein microarrays can now facilitate kinetic measurements of enzyme activity in real time using continuous fluorescence measurements, and enable the rapid determination of the properties, substrate preference, inhibitors, activators and metabolic cofactors of the enzymes tested (Lue et al., 2005).

1.7.4 Zymography

Zymography (Fig. 1.12) is a method of enzyme analysis in which the proteins present in a sample are separated by gel electrophoresis, typically one dimensional (1D) electrophoresis, before detection of enzyme activity takes place. The process can be used to analyse a fungal supernatant to determine the approximate molecular weight and number of enzymes or enzyme isoforms it contains that are active against a particular substrate. To form a zymogram gel, the substrate for the target enzyme is incorporated into a polyacrylamide gel. Samples are prepared in a manner that minimises protein denaturation to avoid loss of enzyme activity. Following separation of proteins in the sample by electrophoresis, the gel is placed into a renaturing buffer, which reactivates the enzymes, and then transferred to an appropriate incubation buffer. Digestion of the substrate in the gel occurs where proteins possessing the target enzyme activity are located, and is usually visualised by staining.

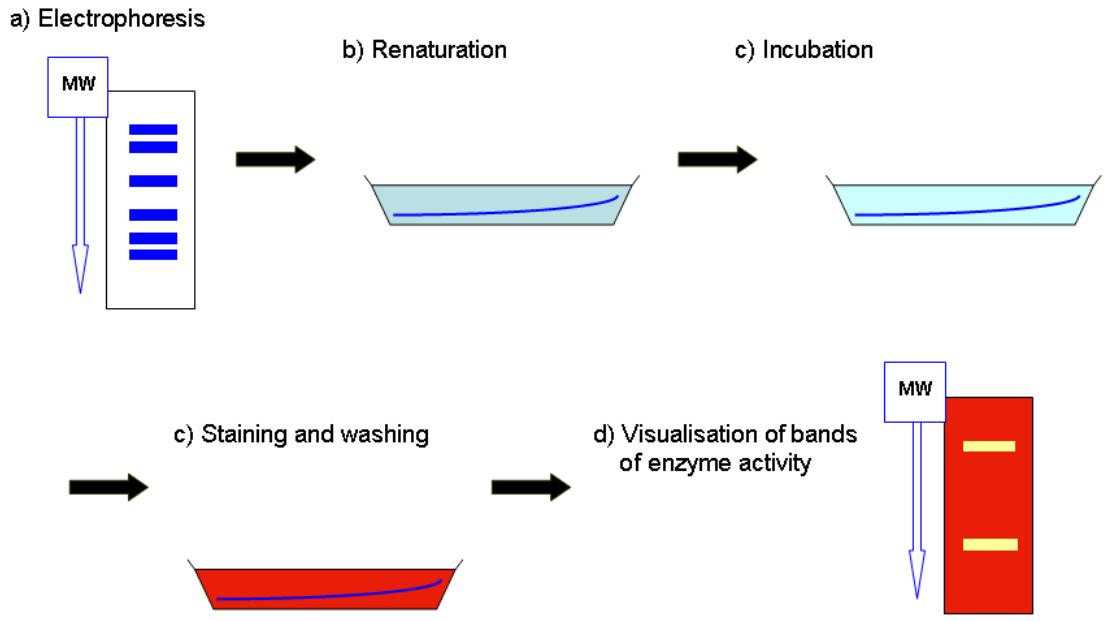

Figure 1.12: Schematic of the 1D zymography technique. a) Proteins in a sample are separated by their molecular weight (MW) by electrophoresis on a gel containing the substrate of the target enzyme; b) The protein gel is placed in a renaturation buffer to restore enzyme activity; c) The gel is incubated in a suitable buffer; c) The gel is stained and washed; d) Proteins with enzyme activity against the substrate are revealed as bands on the zymogram.

One dimensional (1D) zymograms have been widely used for the rapid screening of fungi for enzyme activities, both from environmental screening projects (Ratanachomsri et al., 2006; Sharma et al., 2008; Soni et al., 2008) and during strain improvement programs (de Faria et al., 2002; Zhang et al., 2008). Zymograms can be used for the detection of various enzyme activities including protease (Choi et al., 2001), amylase (Upadhyay et al., 2005), xylanase (Sharma et al., 2008) and endoglucanase (Mansfield et al., 1998) activities and have been used extensively in this work (Chapter 4; Chapter 5).

The combination of zymography with two-dimensional (2D) electrophoresis, in which proteins are separated by their isoelectric point as well as their molecular weight, is difficult to achieve as the sample preparations involved for the 2D separation process are more inclined to permanently disable enzyme activity (Kaberdin et al., 2003). Successful 2D zymography does, however, provide greater resolution and accuracy for isolation and identification of proteins responsible for enzyme activities (Nandakumar et al., 2006; de Jesus et al., 2009). Consequently, 2D zymography was used in this work to assist in the identification of enzymes from a fungal isolate from koala faeces (Chapter 5).

1.8 Plant cell walls and enzymes involved in their degradation

Plant cell walls, composed primarily of lignocellulose, form the major component of koala faeces (Section 1.5) and so fungi growing on the faeces are likely to be good producers of enzymes capable of degrading plant cell walls in order to obtain the nutrients they need for survival. The degradation of plant cell walls is not only of interest to coprophilous fungi however, but of worldwide importance in industry. Lignocellulose is the most abundant renewable resource available to the world with a production rate of 200×10^9 tons per year (Zhang, 2008) and is utilised in the pulp and paper industry, for food and textile manufacture and for the production of biofuels (Kuhad and Singh, 2007).

The plant cell wall is complex in structure and requires numerous enzymes for degradation. The main components of plant cell walls are cellulose, hemicellulose, lignin and pectin (Fig. 1.13).

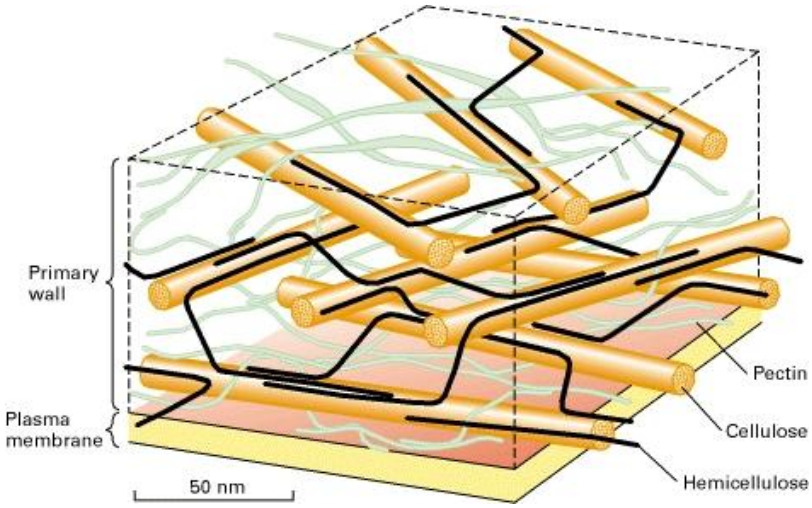

Figure 1.13: Schematic representation of a plant cell wall. The relative size of the polymers and their separations are drawn to scale (Lodish et al., 2000). Lignin forms strong barriers around cellulose, hemicellulose and pectin in wood and fibrous plant tissues.

Cellulose fibres provide strength and rigidity to the cell wall. Hemicelluloses (xyloglucan, xylan and mannan) form crosslinks between the cellulose fibres, helping to bind them together and to other polysaccharides such as pectins (Smith, 2001). Pectins are particularly abundant in the outer cell wall of actively growing plant tissues such as leaves and green stems, and provide adherence and cushioning between adjacent cells (Lodish et al., 2000). In woody tissues and in some fibrous leaves of tree species such as *Eucalyptus*, lignin links cellulose, hemicellulose and pectin to provide an extremely recalcitrant barrier to enzymatic degradation (Sánchez, 2009).

An explanation for how each of these cell wall components are degraded by enzymatic attack is outlined below along with the Enzyme Commission (EC) numbers of the enzymes according to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB; www.chem.qmul.ac.uk/iubmb), a classification system based on the mechanisms of enzyme activity. A summary is provided in Table 1.3 (p.41), which also includes the glycosyl hydrolase (GH), polysaccharide lyase (PL), carbohydrate

esterase (CE) or Lignin Oxidase (LO) families to which the enzymes belong, classification systems based on sequence identity used by the Carbohydrate Enzymes (CAZy) database (www.cazy.org; Coutinho and Henrissat, 1999) and Fungal Oxidative Lignin Enzymes database (Levasseur et al., 2008; http://foly.esil.univ-mrs.fr/).

1.8.1 Cellulose

Cellulose is a homogenous linear polymer consisting of up to 10,000 β-1,4-linked D-glucose residues (Fig.1.14) in both amorphous (loosely packed) and crystalline regions.

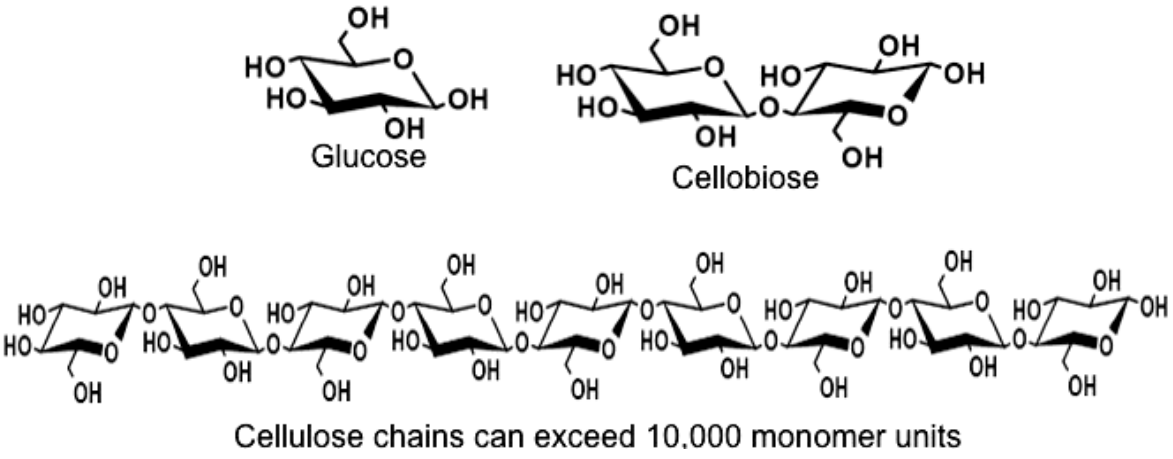

Figure 1.14: Cellulose is composed of long homogenous chains of glucose residues (www.cchem.berkeley.edu)

The degradation of cellulose requires the combined action of three distinct groups of enzymes:

1) **Endoglucanases** (EC 3.2.1.4) hydrolyse the amorphous regions of the cellulose, making available more free chain ends for subsequent attack by cellobiohydrolases;

2) **Cellobiohydrolases** (EC 3.2.1.91) hydrolyse β-1,4-glycosidic bonds from the chain ends of crystalline and amorphous cellulose, releasing cellobiose (glucose dimers; Fig. 1.14);

3) **β-glucosidases** (EC 3.2.1.21) hydrolyse cellobiose to produce glucose. This action also improves cellulose degradation rates as cellobiose is an end-product inhibitor of cellobiohydrolase and endoglucanase activity (Béguin and Aubert, 1994).

1.8.2 Hemicellulose: xyloglucan, xylan and mannan

The hemicelluloses, xyloglucan, xylan and mannan have a more heterogeneous nature than cellulose and function to link cellulose fibres and strengthen cell wall structure. The molecular structure of xyloglucan is similar to that of cellulose, with a β-1,4-linked D-glucose backbone, but is substituted with xylose residues to varying degrees depending on the plant source (Fig. 1.15). Xylose residues can then be further substituted, by fucosyl, galactosyl and arabinosyl residues (Hoffman et al., 2005). Degradation of xyloglucan backbone involves the action of xyloglucan specific endoglucanases (EC 3.2.1.151; Chang, 2009).

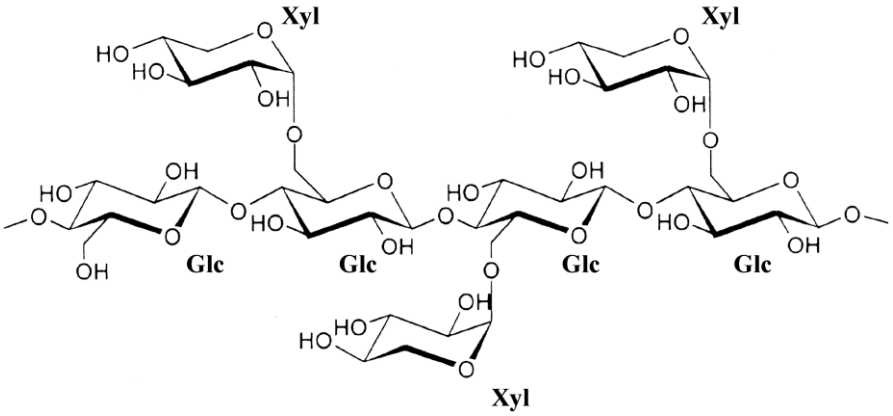

Figure 1.15: Representative xyloglucan repeat unit. Xyl: xylose; Glc: glucose (Ebringerová et al., 2005).

Xylan, an abundant hemicellulose in cereals and hardwood, has a β-1,4-linked D-xylose backbone with a high number of side chains such as glucuronic acid, L-arabinose, D-galactose, feruloyl, acetyl and p-coumaroyl residues. Cereal xylans, often termed arabinoxylans, are highly substituted with L-arabinose side chains, which can also contain xylose and galactose (de Vries and Visser, 2001).

Degradation of the xylan backbone is achieved by the following enzymes (Fig. 1.16):

1) **Endo-1,4-β-xylanases** (EC 3.2. 1.8) randomly cleave the xylan backbone to produce smaller oligosaccharides.

2) **β-xylosidases** (EC 3.2.1.37) degrade the small xylan oligosaccharides to xylose (Jeffries, 1994).

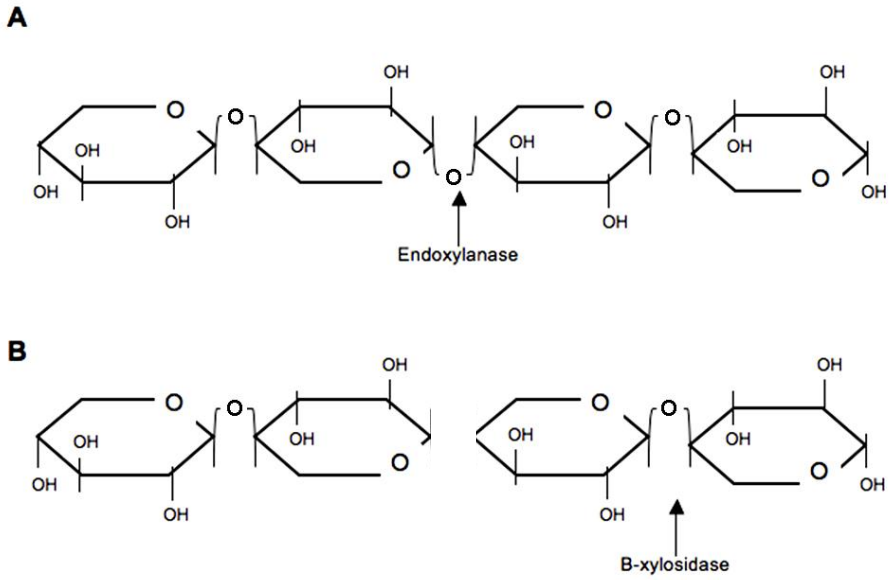

Figure 1.16: The enzymatic degradation of the xylan backbone by a) endoxylanase (endo-1,4-β-xylanase) and b) β-xylosidase (Goldman, 2009)

The mannans form another abundant hemicellulose group present in higher plants and are the principle component of softwood hemicellulose (Moriera and Filho, 2008). The mannan backbone consists of β-1,4-linked mannose and glucose residues. In galactomannans the mannnose residues are substituted with side groups of α-1,6-linked D-galactose (Zhang et al., 2008; Fig. 1.17).

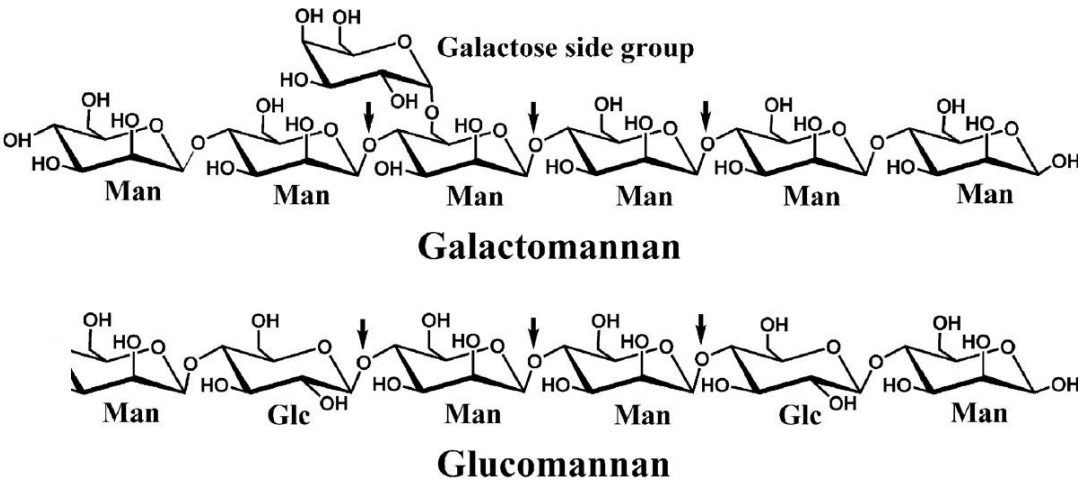

Figure 1.17: Typical structures of the two major forms of mannan. Galactomannan (top) consists of a main chain of β-1,4-linked mannose residues with α-1,6-linked galactose side groups. Glucomannan (bottom) has a main chain of β-1,4-linked mannose and glucose residues. The arrows represent the sites of enzyme attack by mannan endo-1,4-β-mannosidases (endo-1,4-β-mannanases). Man: mannose; Glc: glucose (Zhang et al., 2008).

Hydrolysis of mannan backbone is achieved by the following enzymes:

1) **Mannan endo-1,4-β-mannosidases** (EC 3.2.1.78; also known as endo-1,4-β-mannosidases or endo-1,4-β-mannanases) cleave within the backbone to produce manno-oligosaccharides, predominantly mannobiose and mannotriose.

2) **β-mannosidases** (EC 3.2.1.25) cleave single mannose residues from the nonreducing end of manno-oligosaccharides.

3) **β-glucosidases** (EC 3.2.1.21) release glucose from mannose residues (Moreira and Filho, 2008).

1.8.3 Pectin

Pectins have a very complex and heterogeneous structure (Fig.1.18) and contain two defined regions. The smooth region consists of a backbone of α-1,4-linked D-galacturonic acid residues, which can be acetylated or methylated. The "hairy" region consists of xylogalacturonan, a galacturonan backbone with D-xylose substitution, and rhamnogalacturonan (RG) I and II (Voragen et al., 2009). In RG I, a D-galacturonic acid and L-rhamnose backbone is substituted with long galactan and arabinan chains, which can be further substituted with L-arabinose and feruloyl residues. Acetyl groups can also be ester linked to the galacturonic residues along the backbone. In RG II, a D-galacturonic backbone is substituted with four highly complex side chains containing 12 different sugars in more than 20 different linkages (Etzler and Mohnen, 2009).

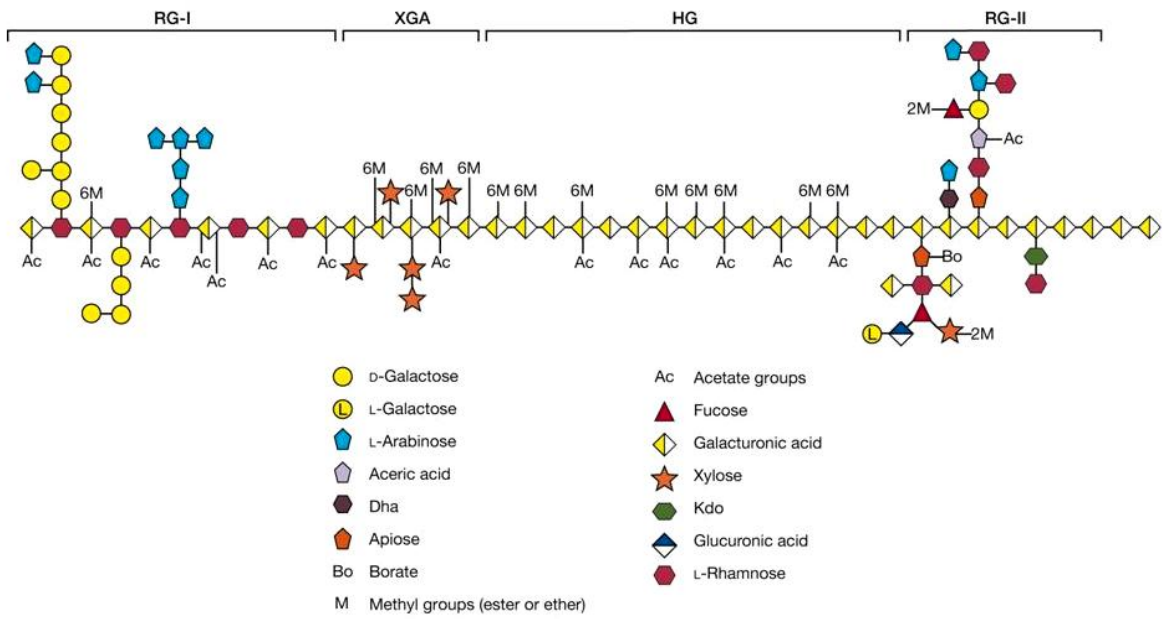

Figure 1.18: The structure of pectin showing the main pectic polysaccharides: a homogalacturonan (HG) chain forming the "smooth" region; and rhamnogalacturonan I (RG-I, left), rhamnogalacturonan II (RG-II, right) and xylogalacturonan (XGA), forming the "hairy" region. NB: In plant cell walls the HG is proportionally 12.5 fold larger, and RG-I 2.5 fold larger than indicated schematically. Dha: 3-Deoxy-D-lyxo-heptulosaric acid (Etzler and Mohnen, 2009).

The complex nature of pectin requires a large number of enzymes for its degradation. The major enzyme groups involved in cleaving the pectin backbone are as follows:

1) **Pectinases** (polygalacturonases) hydrolyse the α-1,4-D-galactosiduronic linkages in the smooth region of the pectin backbone. Endopolygalacturonases (EC 3.2.1.15) cleave within the main chain and exopolygalacturonases (EC 3.2.1.82) cleave at the nonreducing terminus (Suberkropp, 2007).

2) **Pectin lyases** (endo-pectin lyases, EC 4.2.2.10) act by β-elimination to cleave the glycosidic bonds between esterified galacturonic acid residues.

3) **Pectate lyases** (EC 4.2.2.2) act by β-elimination to cleave between de-esterified galacturonic acid residues and require Ca^{2+} for catalysis (Marin-Rodriguez et al., 2002).

4) **Rhamnogalacturonan hydrolases** (EC 3.2.1.-) act on the RGI backbone. Endorhamnogalacturonan hydrolases cleave within the main chain. Rhamnogalacturonan rhamnohydrolase and rhamnogalacturonan galacturonohydrolases are exo-acting enzymes, releasing terminal rhamnose residues and rhamnose-galacturonic acid residues, respectively, from the nonreducing end of the chain (de Vries et al., 2005).

5) **Rhamnogalacturonan lyases** (EC 4.2.2.-) act by β-elimination to cleave between rhamnose and D-galacturonic acid residues in the backbone of rhamnogalacturonan-I.

6) **α-L-rhamnosidases** (E. C. 3.2.1.40; GH28, 78 and 106) specifically cleave terminal α-L-rhamnosidase residues from the ends of the RG backbone (Cui et al., 2007).

1.8.4 Side chains of hemicellulose and pectin

The complete degradation of xylan, mannan and pectin requires degradation and removal of the many side chains that are carried along the main backbone polymers.

The major enzyme groups that facilitate the removal of side chains are listed below:

1) **α-L-arabinofuranosidases** (EC 3.2.1.55) remove arabinose residues. Arabinoxylan arabinofuranohydrolases specifically remove arabinose residues from a xylan backbone (de Vries and Visser, 2001).

2) **Endoarabinanases** (EC 3.2.1.99) hydrolyse the linkages within arabinan sidechains.

3) **α-galactosidases** (EC 3.2.1.22) remove galactose from mannose residues along the galactomannan backbone.

4) **β-galactosidases** (EC 3.2.1.23) release terminal galactose residues from galactan side chains, particularly of pectin (de Vries and Visser, 2001).

5) **Endogalactanases** (EC 3.2.1.89) hydrolyse within the galactan side chains.

6) **α-glucuronidases** (EC 3.2.1.131) remove glucuronic acid residues from the xylan backbone.

7) **Feruloyl esterases** (EC 3.1.1.73) remove ferulic acid residues from xylan and pectin (Christov and Prior, 1993).

8) **Acetylesterases** release acetyl residues from xylan (acetylxylan esterase EC 3.1.1.72), mannan (acetylglucomannan esterase EC 3.1.1-) and pectin (pectin acetylesterase EC 3.1.1.-rhamnogalacturonan acetylesterase EC 3.1.1.-).

9) **Methylesterases** (pectin esterases) release methyl residues from pectin (EC 3.1.1.11) (Pelloux et al., 2007).

1.8.5 Lignin

Lignin is a heterogeneous aromatic polymer containing three major components, coniferyl alcohol, coumaryl alcohol and sinapyl alcohol (Fig. 1.19; Mäki-Arvela et al., 2007).

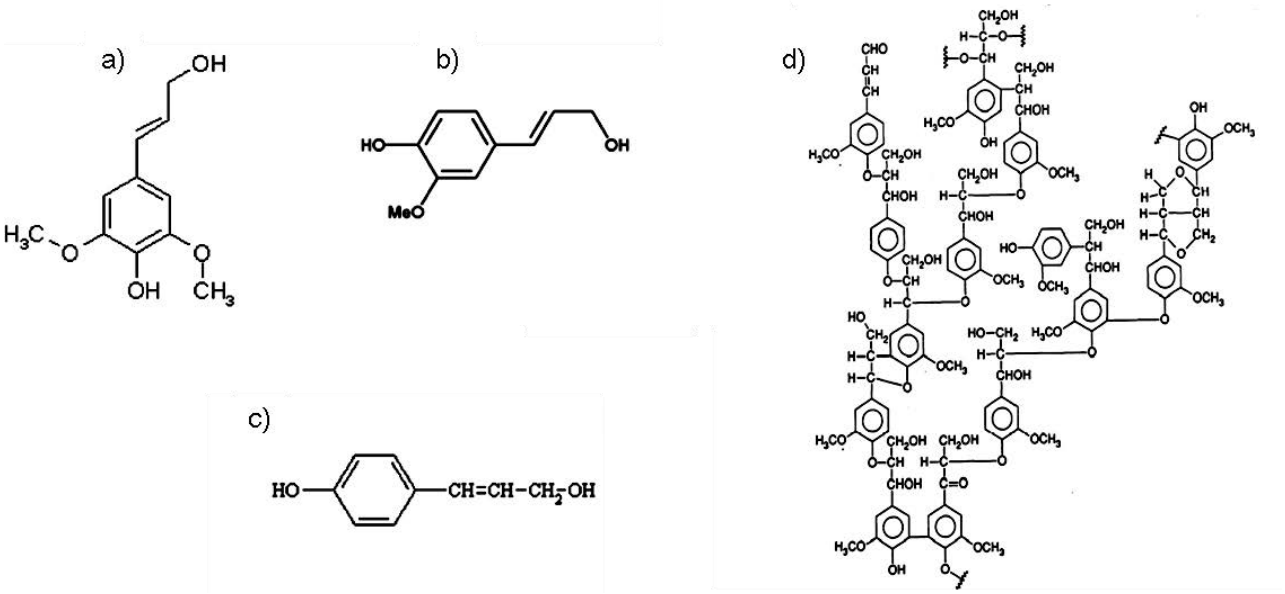

Figure 1.19: The main monomer units in lignin (a) sinapyl alcohol, (b) coniferyl alcohol and (c) coumaryl alcohol, and (d) schematic structure of lignin (Mäki-Arvela et al., 2007).

Lignin is the most recalcitrant of all cell wall constituents and its degradation can be achieved by three main groups of oxidative enzymes:

1) **Lignin peroxidases** (EC 1.11.1.14) oxidise non-phenolic and phenolic lignin units.

2) **Manganese peroxidases** (EC 1.11.1.13) oxidise Mn(II) to Mn(III), which can in turn oxidise phenolic and some non-phenolic lignin units (Sánchez, 2009).

3) **Laccases** (EC 1.10.3.2) are copper containing oxidases that catalyse the one-electron oxidation of phenols. Non-phenolic units can be oxidised by laccases in the presence of oxidation mediators such as ABTS (2,20-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid; Riva, 2006).

In addition, mild oxidants such as glucose-methanol-choline (GMC) oxidoreductases contribute to lignin degradation by the oxidation of the phenolic units of lignin only; the mild oxidants cannot oxidise non-phenolic units, and are therefore not capable of the full degradation of the lignin polymer (Espagne et al., 2008).

Table 1.3: Summary of the key enzymes involved in the degradation of plant cell walls (Dashtban et al., 2009, Howard et al., 2003; Coutinho et al., 2009).

Enzyme name	EC number	CAZy[a]/ FOLy[b] families	Substrates
α-Galactosidase	3.2.1.22	GH27,36	Xyloglucan, xylan, mannan
α-Glucuronidase	3.2.1.131	GH67	Xylan
α-L-arabinofuranosidase	3.2.1.55	GH51,54,62	Xyloglucan, xylan, pectin
α-L-rhamnosidase	3.2.1.40	GH28,78,106	Pectin
Acetylxylan esterase	3.1.1.72	CE1,5	Xylan
Arabinoxylan arabinofuranohydrolase	3.2.1.55	GH62	Arabinoxylan
β-Galactosidase	3.2.1.23	GH2,35	Xyloglucan, xylan, mannan, pectin
β-Glucosidase	3.2.1.21	GH1,3	Cellobiose, cellodextrins
β-mannosidase	3.2.1.25	GH1,2,5	Mannan
β-xylosidase	3.2.1.37	GH3,43	Xylan, pectin
Cellobiohydrolase	3.2.1.91	GH6,7,9,48	Cellulose (crystalline regions)
Endo-1,4-β-xylanase	3.2.1.8	GH5,8,10,11,43	Xylan
Endoglucanase	3.2.1.4	GH5,6,7,9,12,45, 48,61,74	Cellulose (amorphous regions)
Endoarabinanase	3.2.1.99	GH43	Pectin, arabinan
Endogalactanase	3.2.1.89	GH53	Pectin
Endopolygalacturonase	3.2.1.15	GH28	Pectin
Exopolygalacturonase	3.2.1.82	GH28	Pectin
Feruloyl esterase	3.1.1.73	CE1	Xylan, Pectin
Laccase	1.10.3.2	LO1	Lignin
Lignin peroxidase	1.11.1.14	LO2	Lignin
Mannan endo-1,4-β-mannosidase (endo-1,4-β-mannanase)	3.2.1.78	GH5, 26	Mannan
Manganese peroxidase	1.11.1.13	LO2	Lignin
Pectin methyl esterase	3.1.1.11	CE8	Pectin
Pectate lyase	4.2.2.2	PL1,3,9	Pectin
Pectin lyase	4.2.2.10	PL1	Pectin
Rhamnogalacturonan acetyl esterase	3.1.1.-	CE12	Pectin
Rhamnogalacturonan hydrolase	3.2.1.-	GH28	Pectin
Rhamnogalacturonan lyase	4.2.2.-	PL4,11	Pectin

[a] Carbohydrate Enzymes (CAZy) database (www.cazy.org), GH: Glycosyl hydrolase family (hydrolyse glycosidic bonds), PL: Polysaccharide lyase family (cleave polysaccharide chains via a β-elimination mechanism), CE: Carbohydrate Esterase Family (catalyse the de-O or de-N acylation of substituted saccharides) (www.cazy.org).

[b] Fungal Oxidative Lignin Enzymes database (http://foly.esil.univ-mrs.fr/) classification LO: Lignin Oxidase family (catalyse the oxidation of lignin and related aromatic compounds.

1.8.6 Enzyme synergism

A summary of the key enzymes involved in plant cell degradation is provided in Table 1.3. Each of these enzymes plays a role in the degradation of plant cell walls, as described (Section 1.8), and thus could be employed by coprophilous fungi in the degradation of lignocellulose-rich koala faeces. In addition, substances likely to be present in the faeces in smaller amounts, such as proteins, starch and lipids (Section 1.5) may be degraded by proteases, amylases and lipases, respectively, secreted by the coprophilous fungi.

In this work, fungi isolated from koala faeces were investigated for a broad range of enzyme activities (Chapter 3; Chapter 4). Importantly, an individual enzyme rarely acts alone, particularly in nature. Fungi typically secrete numerous enzymes at the same time and, through a synergistic process, the enzymes may increase the impact of each other on the substrates available. For example, side-chain degrading enzymes (Section 1.8.4) may expose cleavage sites in the backbone of a xylan polymer, increasing the access of endo-1,4-β-xylanases and β-xylosidases (Section 1.8.2) to their target substrates. Alternatively, an enzyme could remove inhibitors of another enzyme; for example β-glucosidase removes cellobiose to improve cellobiohydrolase activity (Section 1.8.1). In the final part of this work, the full complement of proteins secreted by one coprophilous fungus from koala faeces was explored (Chapter 5). Through the "secretome" analysis, an array of enzymes that could act synergistically to degrade lignocellulose and other substances in koala faeces was revealed.

1.9 Fungal secretomes: extracellular insights into efficient substrate utilisation

Filamentous fungi respond to their environment by secreting a finely tuned "toolbox" of enzymes and other proteins that have been evolved to gain energy from the available substrates in the most efficient way possible. The complete set of secreted proteins, termed the secretome, can provide information about the way the fungus interacts with its environment and can also offer insight into effective enzyme combinations for various biotechnological applications.

1.9.1 Fungal secretomes that have been explored

A number of secretome studies of filamentous fungi have been undertaken in the last decade (Table 1.4). Numerous researchers have focused on the secreted proteins of fungi involved in cellulose or lignocellulose degradation to gain insight into efficient enzyme combinations for production of ethanol from lignocellulose biomass (Bouws et al., 2008). Other studies are undertaken to investigate the interactions that fungal species have with their environment, such as their role in plant pathogenesis (Mueller et al., 2008) or the symbiotic relationship between mychorrhizal fungi with plant roots (Nagendran et al., 2009). Proteomic analysis via gel electrophoresis and mass spectrometry plays a major role in the majority of the secretome studies (Table 1.4). Bioinformatic prediction of secreted proteins has been used to study the secretomes of fungi with sequenced genomes (Ouyang et al., 2006; Nagendran et al ., 2009), and bioinformatic predictions have been compared to experimental proteomic data (Vanden Wymelenberg et al., 2005, 2006; Tsang et al., 2009). In the most recent secretome studies, transcriptome analysis by microarray and RT-PCR has been used to link gene predictions to expressed proteins (Vanden Wymelenberg et al., 2009, 2010). The secretomes of fungi without sequenced genomes have been investigated successfully using cross species identification (Medina et al., 2004; Zorn et al., 2005, Dwivedi, 2006).

Table 1.4: Secretome studies of filamentous fungi (expanded from Bouws et al., 2008)

Fungal species	Carbon source	Techniques	Reference
Amanita bisporigera	-	Genome based prediction	Nagendran et al., 2009
Aspergillus niger	Varied (eg. glucose, pectin, xylan)	Genome based prediction LC MS/MS	Tsang et al., 2009
Aspergillus oryzae	Wheat bran	2DE MALDI-TOF/MS	Oda et al., 2006
Aspergillus flavus	Rutin	1DE, 2DE LC MS/MS, MALDI-TOF/MS	Medina et al., 2004 Medina et al., 2005
Coprinopsis cineria	Glucose, yeast extract, peptone	Genome based prediction 2DE, LC MS/MS	Hoegger et al., 2007
Fusarium graminearum	13 different media (eg. xylan, pectin, corn stover)	1DE LC MS/MS	Paper et al., 2007
Phanerochaete chrysosporium	Wood chips	2DE, MALDI-TOF/MS LC MS/MS	Abbas et al., 2005
P. chrysosporium	Crystalline cellulose	Genome based prediction 1DE, LC MS/MS RT-PCR	Vanden Wymelenberg et al., 2005
P. chrysosporium	Ligninolytic media [a] (carbon or nitrogen limited)	Genome based prediction 1DE LC MS/MS	Vanden Wymelenberg et al., 2006
P. chrysosporium	Glucose, cellulose or woodchips	1DE, 2DE LC MS/MS	Sato et al., 2007
P. chrysosporium	Ligninolytic media [a] (carbon or nitrogen limited)	Genome based prediction 1DE, LC MS/MS, microarray	Vanden Wymelenberg et al., 2009
P. chrysosporium	Ball-milled aspen	Genome based prediction 1DE, LC MS/MS RT-PCR, microarray	Vanden Wymelenberg et al., 2010
Pleurotus sapidus	Peanut shells, rape straw	2DE MALDI-TOF/MS LC MS/MS	Zorn et al., 2005
Postia placenta	Ball-milled aspen	Genome based prediction 1DE, LC MS/MS RT-PCR, microarray	Vanden Wymelenberg et al., 2010
Trametes versicolor	Ligninolytic media [a] (carbon or nitrogen limited)	1DE, 2DE LC MS/MS	Dwivedi, 2006
Trichoderma harzianum	Chitin, fungal cell walls	1DE, 2DE MALDI-TOF/MS LC MS/MS	Suárez et al., 2005
Trichoderma reesei Laminex [b]	Lignocellulose	2DE LC MS/MS	Vinzant et al., 2001
T. reesei RUT-C30 and CL847	Lactose	2DE MALDI-TOF LC MS/MS	Herpoël-Gimbert et al., 2008
T. reesei RUT-C30	Rice straw	2DE LC MS/MS	Sun et al., 2008
T. reesei RUT-C30 and Spezyme CP [b]	Corn stover	1DE LC MS/MS	Nagendran et al., 2009
Ustilago maydis	-	Genome based prediction	Mueller et al., 2008

[a] B3 medium (Kirk et al., 1978) containing KH_2PO_4, $MgSO_4$, $CaC1_2$, glucose and ammonium tartrate. Carbon and nitrogen limited media had reduced glucose and ammonium tartrate respectively.
[b] Commercial cellulase preparations provided by Genencor (Palo Alto, CA).

1.9.2 The secretome of a well studied fungus, *Trichoderma reesei*

Trichoderma reesei has been recognised as one of the most efficient cellulose-degrading organisms (Montenecourt, 1983; Beguin, 1990) and the secreted enzymes involved in this process have been studied extensively, both on an individual enzyme level (eg. Saloheimo et al., 1988; Törrönen et al., 1992; Stålbrand et al., 1995; Okada et al., 1998) and as entire secretome studies (Vinzant et al., 2001; Herpoël-Gimbert et al., 2008; Nagendran et al., 2009). Knowledge of the *T. reesei* secretome provides an understanding of how a range of different enzymes can be secreted by a single fungus in order to gain energy from cellulose-rich material, and how this ability can be harnessed and improved for industrial purposes by research and strain development. The hypercellulolytic mutant strain *T. reesei* RUT-C30 (Montenecourt and Eveleigh, 1979) and its wild-type QM6a are used as comparison strains to the fungal isolates from koala faeces throughout this work.

The recently sequenced genome of *T. reesei* (Martinez et al., 2008) includes genes encoding for two cellobiohydrolases, five endoglucanases, two β-glucosidases, three endo-1,4-β-xylanases, two arabinofuranosidases, a β-xylosidase, mannan endo-1,4-β-mannosidase (endo-1,4-β-mannanase), β-galactosidase, acetylxylan esterase and an α-galactosidase. Many of these enzymes have also been identified through proteomic analysis of the secretome of *T. reesei* following growth in cellulase-inducing media (Table 1.5).

Table 1.5: Cellulolytic enzymes identified from the secretome of *T. reesei* grown in cellulase inducing media[a]

Enzyme	EC number	CAZy family[b]	Reference[a]
Cellobiohydrolase I (CBHI; Cel7A)	3.2.1.91	GH7	1,2,3,4
Cellobiohydrolase II (CBHII; Cel6A)	3.2.1.91	GH6	1,2,3,4
Endoglucanase I (EGI; Cel7B)	3.2.1.4	GH7	1, 2, 3,4
Endoglucanase II (EGII; Cel5A)	3.2.1.4	GH5	1, 2, 3,4
Endoglucanase III (EGIII; Cel12A)	3.2.1.4	GH12	1,2,4
Endoglucanase IV (EGIV; Cel61A)	3.2.1.4	GH61	1,2
Endoglucanase VI (EGVI; Cel74a)	3.2.1.4	GH74	2, 4
β-glucosidase (Bgl3A)	3.2.1.21	GH3	1,2,3,4
Endo-1,4-β-xylanase I (XynI; Xyn11A)	3.2.1.8	GH11	2
Endo-1,4-β-xylanase II (Xyn2; Xyn11B)	3.2.1.8	GH11	2,4
Endo-1,4-β-xylanase III (Xyn3; Xyn10A)	3.2.1.8	GH10	4
Xylosidase I (Xyl3A)	3.2.1.37	GH3	2,4
Mannan endo-1,4-β-mannosidase (endo-1,4-β-mannanase; Man1)	3.2.1.25	GH5	1,2,4
β-galactosidase	3.2.1.23	GH35	4
α-L-arabinofuranosidase I (ABFI)	3.2.1.55	GH62	2,4
α-L-arabionofuranosidase II (ABFII)	3.2.1.55	GH54	2,4
Acetylxylan esterase	3.1.1.72	CE5	1,2,4

a Reference and carbon source used: 1) Vinzant et al., 2001, lignocellulose 2) Herpoël-Gimbert et al., 2008, lactose; 3) Sun et al., 2008, rice straw; 4) Nagendran et al., 2009, corn stover.
b Carbohydrate Enzymes (CAZy) database (www.cazy.org) classification, GH: Glycosyl Hydrolase family; CE: Carbohydrate esterase family.

The *T. reesei* secretome is dominated by cellobiohydrolase I and II (Cel7A and Cel6A), which together account for 40–80 % of the total secreted proteins (Herpoël-Gimbert et al., 2008; Nagandren et al., 2009). However, *T. reesei* secretes very few pectin degrading enzymes (Martinez et al., 2008; Nagandren et al., 2009) and has a low level of β-glucosidase in comparison to other high cellulolytic fungal strains (Juhász et al., 2005). Hence, it has become apparent that supplementing the *T. reesei* natural secretome with additional enzymes could improve the efficiency of lignocellulose degradation for industrial applications (Martins et al., 2008).

Finding additional enzymes from other fungal species to supplement *T. reesei*'s secretome is a venture that has been undertaken on a large scale by biotechnology companies. One of the world's largest producer's of industrial enzymes, Novozymes (www.novozymes.com), was recently subcontracted at 17.8 million USD over four years by the United States National Renewable Energy Laboratory to identify novel proteins that could be added to the cellulases produced by *T. reesei* to improve the enzymatic conversion of corn stover (leaves and stalks of the maize plant) to fermentable sugars for the production of ethanol-based biofuels (Teter and Cherry, 2005). The cellulase systems of cellulolytic fungal species were explored using 2D gels and mass spectrometry, and strikingly different secretome compositions were found. The genes from a number of the key enzymes were isolated from the fungi, cloned and expressed in *T. reesei*, resulting in increased enzyme efficiency and a thirty-fold reduction in cost required for the production of fermentable sugars from lignocellulose biomass (Teter and Cherry, 2005).

The success of the Novozymes project highlights the value of exploring novel fungal secretomes to seek enzymes and enzyme combinations that facilitate the efficient breakdown of substrates such as lignocellulose. The information gained broadens our understanding of how fungi survive in their natural habitats, and can result in the discovery of novel enzymes and enzyme combinations that could be of interest to industry. In the final part of this work the secretome of a fungal isolate from koala faeces was explored (Chapter 5). To my knowledge, this work represents the first proteomic analysis of the secretome of a coprophilous fungus.

1.9.3 Exploring a secretome: methods of protein analysis

To identify proteins contained within a fungal secretome, supernatants from liquid cultures are typically analysed by gel electrophoresis and mass spectrometry (Table 1.4, Section 1.9.1).

1.9.3.1 Gel electrophoresis

One-dimensional gel electrophoresis (1DE) is a well established procedure (Laemmli, 1970) and can be used to separate proteins within a fungal supernatant according to their molecular weight (Fig. 1.20a). In two-dimensional gel electrophoresis (2DE; Fig. 1.20b), proteins are firstly separated according to their isoelectric point (i.e. the point at which they carry no net charge) and then further resolved according to their molecular weight, forming a two-dimensional visual "map" of a protein mix (Walker, 2005). Following electrophoresis, protein bands or spots from 1D or 2D gels can be excised and identified using mass spectrometry.

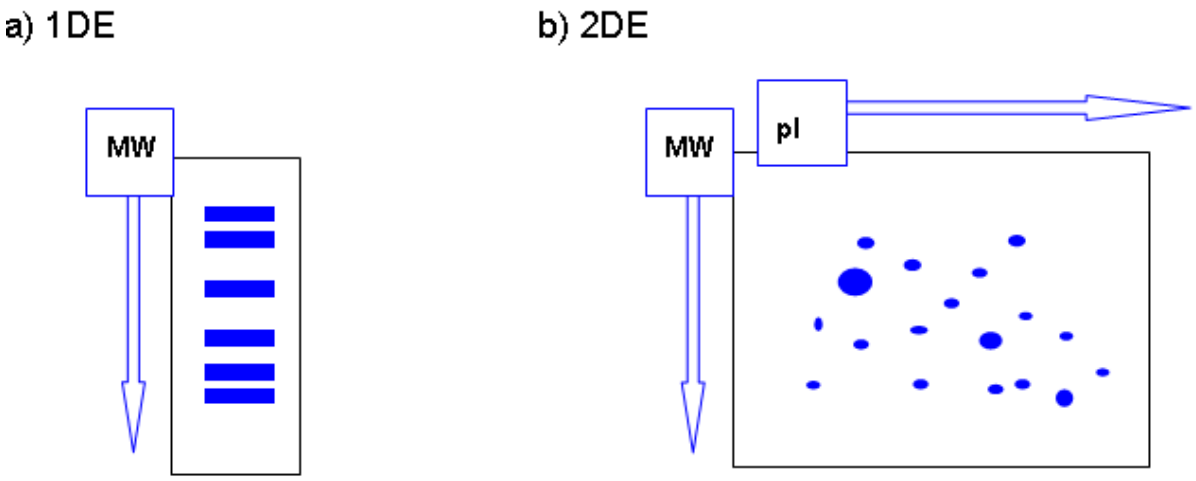

Figure 1.20: Schematic representing protein separation by a) 1D electrophoresis in which proteins are separated into bands according to their molecular weight (MW); and b) 2D electrophoresis in which proteins are further resolved across two dimensions, molecular weight (MW) and isoelectric point (pI).

1.9.3.2 Mass spectrometry

Mass spectrometry is currently the most widely accepted and utilised method of protein identification (Ahrens et al., 2010). Proteins from a sample, typically contained in a gel spot or band, are fragmented into smaller peptides using trypsin or another suitable protease. The resulting fragments are then subjected to ionisation, most commonly Matrix Assisted Laser Desorption/ Ionisation (MALDI) or Electrospray ionisation (ESI). MALDI (Fig. 1.21a) involves the application of the sample into a matrix material, which absorbs at the wavelength of a laser used to bombard the material. Electrospray ionisation of a protein sample (Fig. 1.21b) is usually carried out in conjunction with reversed phase nano-high performance liquid chromatography (nano-HPLC). A solvent gradient of increasing organic content is implemented and peptides are eluted in electrostatically charged droplets according to their hydrophobicity (Fig. 1.21b; Steen and Mann, 2004). Multiply charged ions are then sent into a vacuum chamber for mass analysis.

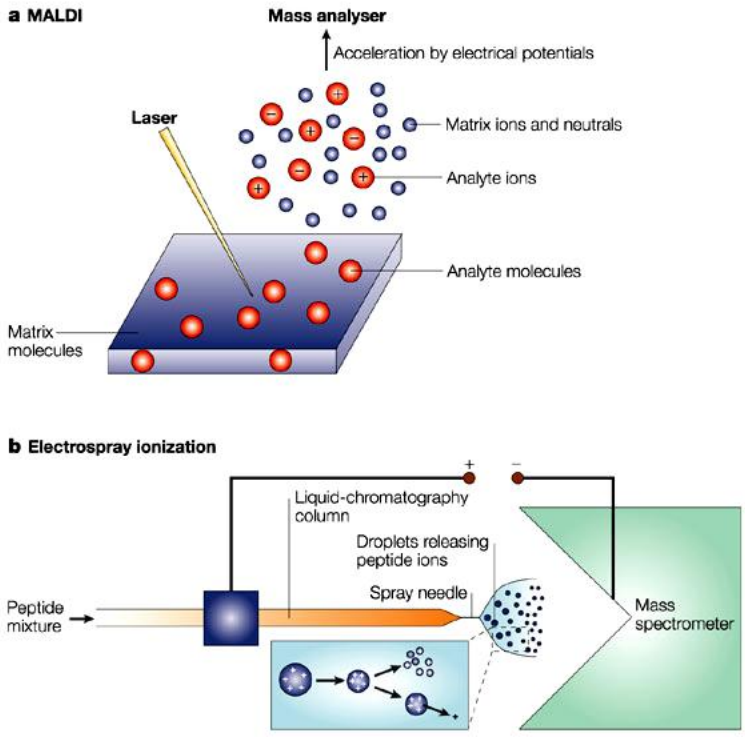

Figure 1.21: Ionisation methods used for protein analysis by mass spectrometry: a) Matrix Assisted Laser Desorption/ Ionisation (MALDI); b) Electrospray ionisation (ESI) (Steen and Mann, 2004).

Mass analysers utilise either electric or magnetic fields to separate and detect ions according to their mass-to-charge ratio. The most commonly used mass analysers for protein analysis are time of flight (TOF), quadrupole and quadrupole-ion trap analysers. In a TOF analyser, ions are accelerated along a field-free tube. The time taken for the ions to reach the detector is measured and is proportional to their mass to charge ratio (Graham et al., 2007). Quadrupole mass analysers process ions by passing them through a radio frequency quadropole field containing four parallel cylindrical rods. At any one time, only the ions of one particular mass-to-charge ratio will reach the end of the analyser for detection, whilst others collide with the quadrupole rods (Aitken, 2005).

Following ion detection and mass analysis, a mass spectrum is generated, resulting in a peptide mass fingerprint (PMF) of the protein. The most abundant peptide ions from ESI or MALDI can then be further fragmented by "collision-induced-dissociation" (CID), in a process termed tandem mass spectrometry (MS/MS; Graham et al., 2007). The resulting MS/MS spectra are then used to elucidate the peptide sequence (*de novo* sequencing) or compared to the predicted fragmentation of known proteins by database analysis (Section 1.9.3.4).

Hybrid mass spectrometers are becoming more common whereby several of the above-mentioned techniques are performed by the one instrument. For example, MALDI-TOF/TOF mass spectrometers combine a MALDI ion source with a TOF analyser, followed by a CID fragmentation cell, and another TOF analyser to produce MS/MS fragmentation spectra (Gogichaeva et al., 2007). Quadrupole-time-of-flight (Q-TOF) mass spectrometers combine a TOF analyser with a triple quadrupole linked to an ESI source to achieve even higher resolution and very high mass accuracies (Chernushevich et al., 2001). An Applied Biosystems 4800 Plus MALDI-TOF/TOF mass spectrometer and an Applied Biosystems QSTAR Elite Q-TOF mass spectrometer were used for protein analysis in this work.

1.9.3.3 Protein identification from mass spectra

The data produced by mass spectrometry of a protein mix is typically analysed using a search engine such as MASCOT (Perkins et al., 1999), SEQUEST (Yates et al., 1996) or X! TANDEM (Craig et al., 2004). The pattern of peptide fragments and fragment ions observed from the mass spectra is compared to theoretical fragmentation patterns calculated from proteins in databases such as NCBInr (www.ncbi.nlm.nih.gov) or SwissProt (www.expasy.org/sprot/). Protein identifications by MASCOT are made based on probablility-based Mowse scores, whereby peptides are scored on the probablility that their match to the database occurred at random (Perkins et al., 1999). The MASCOT search engine was used predominantly for protein identification in this work.

Whatever the search algorithm used, when the organism source of the protein is known and the genome sequence has been made available, matches with database proteins and subsequent identification can be relatively straight forward. However, proteins from organisms without sequenced genomes must be identified on the basis of high sequence similarities to proteins of related species, in a process termed "cross-species identification" (Liska and Shevchenko, 2003; Grinyer et al., 2004). Organisms that are closely phylogenetically-related exhibit similarities in genome sequence and thus their proteins also exhibit a high degree of homology. Identification based on existing protein information in databases can still be achieved using lower criteria for sequence identities and numbers of peptide matches required for a confident hit (Wilkins and Williams, 1997). However, as phylogenetic distance increases between species, so does the number of nucleotide substitutions, deletions and insertions in gene sequences and subsequently, amino acid changes in proteins. This results in markedly different MS and MS/MS spectra compared to that predicted from known proteins in databases and attempts at matching can be unproductive. In this case, *de novo* sequencing of peptides is required for protein identification (Seidler et al., 2010).

De novo sequencing refers to a process in which a peptide sequence is derived directly from a tandem mass spectrum, independently from a database. During MS/MS, each parent peptide ion is fragmented along its backbone by cleavage at the amide bonds between amino acids, forming a series of a, b and c ions with an N-terminus, and x, y and z ions with a C-terminus (Fig. 1.22).

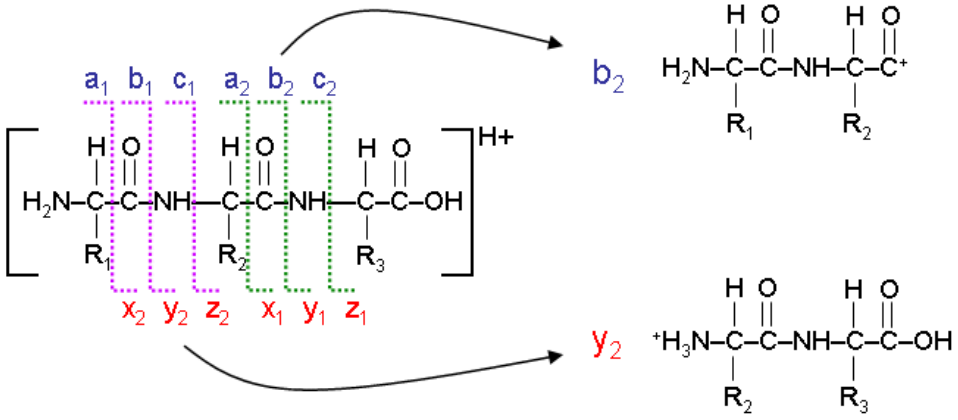

Figure 1.22: Fragmentation of an MS parent peptide ion during MS/MS. Two product ions are shown: b2, cleaved at the second amide bond from the N terminus; and y2, cleaved at the second amide bond from the C terminus (www.alchemistmatt.com).

The population of fragment ions are represented as a mass spectrum, and the sequence of the peptide is determined by the mass difference between peaks of matching ion types, y and b ions being the most often observed. Mass differences between peaks are indicative of the amino acid residue in the peptide sequence (Fig. 1.23). Ideally, a full set of y and b ions provides complementary data and the peptide can be sequenced from both the N- and C-terminal ends (Aitken, 2005). *De novo* sequencing can be conducted manually or with the aid of specialist software programs such as Mascot Distiller (Matrix Science) or Peaks (Bioinformatics Solutions). Potential sequences can then be searched against databases such as NCBI to find homologous peptides in proteins from other organisms.

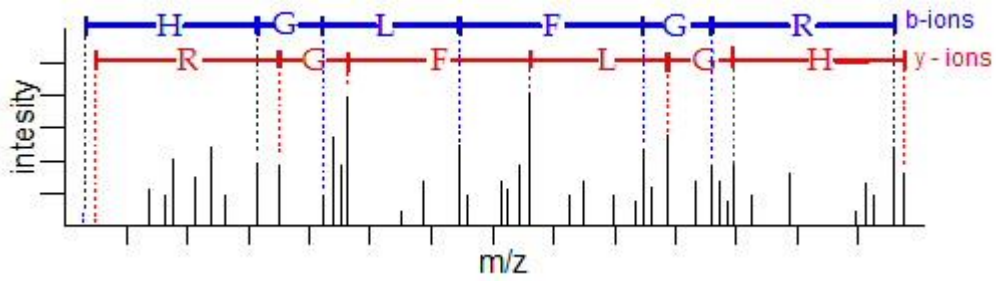

Figure 1.23: Schematic depicting the *de novo* sequence HGLFGR derived from intensity peaks of y and b ions in a tandem mass spectrum. m/z- mass to charge ratio (www.bioinformaticssolutions.com).

The success of *de novo* sequencing relies heavily on the availability of a high quality tandem mass spectrum. Simple ion trap mass spectrometers rarely provide data from which full peptide sequences can be determined as the spectra are often incomplete, of low resolution and contain intervening peaks from different ion series that can confuse the analysis (Steen and Mann, 2004). In contrast, Q-TOF MS/MS spectra are highly suited to *de novo* sequencing as they have a very high mass accuracy in the parts per million (ppm) range and can enable full sequence coverage (Chernushevich et al., 2001). MALDI TOF/TOF spectra have a slightly lower resolution but also provide some suitable data for *de novo* peptide sequencing (Yergey et al., 2002), whilst being a faster and less expensive technology.

The secretome chosen for analysis in this work came from a fungal species isolated from koala faeces, identified as *Doratomyces stemonitis*. The genome of *D. stemonitis* has not been sequenced, nor is the genome sequence available for any other species in the *Doratomyces* genus, or indeed for any other closely related species. Therefore, analysis of the secretome of this isolate from koala faeces (Chapter 5) was both novel and challenging, and required extensive *de novo* sequencing to reveal identities of the proteins that could act synergistically to degrade such an unusual substrate.

1.10 Aims of this study

The coprophilous fungi of koala faeces have received little attention in the past. Therefore, a broad aim of this work was to further knowledge about a little-explored coprophilous fungal community. Equally, the work involved investigation of a novel niche to seek enzymes that could be of interest for development for industrial applications. In order to achieve these goals, the specific aims of each part of the work were as follows:

1. Isolation of fungi from koala faeces and identification of the fungi by morphological and molecular means (Chapter 3).
2. Screening of the fungal isolates from koala faeces by agar plate assay in order to determine the types of enzymes the fungi secrete, and to identify isolates that secrete enzymes of potential interest for industrial applications (Chapter 3).
3. Detailed characterisation of the enzymes secreted by selected fungal isolates from koala faeces using liquid cultures, enzyme assays and zymogram gels, particularly to reveal enzymes with temperature or pH profiles that may have potential for utilisation under specific industrial conditions (Chapter 4).
4. Identification of the proteins in the secretome of one fungal isolate from koala faeces (*Doratomyces stemonitis* C8) using 1D and 2D gel electrophoresis, zymography and and mass spectrometry to gain insight into the array of enzymes the coprophilous fungus secretes to gain energy from lignocellulose-rich koala faeces (Chapter 5).

The work resulted in four journal publications, which are introduced and discussed in turn in the following chapters. Additional work, not contained in the publications, is also presented. A final summary highlighting all the outcomes achieved throughout the work is contained in Chapter 6, along with visions for the future.

Chapter 2: Materials and Methods

The methods used in this work are listed in Table 2.1 below. Details of materials and methods are provided in the publications or sections of the thesis as shown. All chemicals and reagents were sourced from Sigma-Aldrich (Australia) unless otherwise specified.

Table 2.1: Materials and methods used during this work.

Method	Publication	Section
Collection of koala faeces	1	3.2, 3.4.1
Incubation of faeces for fungal growth	1	3.2, 3.4.2
Isolation of fungi from koala faeces	1	3.2, 3.4.3
Extraction of genomic DNA	1	3.2, 3.3.1
Internal transcribed spacer (ITS) sequencing	1	3.2, 3.3.2 – 3.3.4
Agar plate assay for enzyme activity	1	3.2, 3.3.5
Liquid medium for the induction of hydrolases (hydrolase-inducing liquid medium)	2, 3, 4	4.2, 5.2, 5.3
Liquid medium for the induction of lignin-degrading oxidases (oxidase-inducing liquid medium)	np	4.3.2
Protein quantification in fungal supernatant (Bradford Assay)	2, 3	4.2, 5.2
Liquid assays for xylanase, mannanase, endoglucanase, β-glucosidase, amylase, protease and lipase activities	2	4.2
Liquid assay for laccase activity	np	4.3.3
One dimensional sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (1D SDS-PAGE)	2, 3, 4	4.2, 5.2, 5.3
Zymograms for xylanase, mannanase, endoglucanase, β-glucosidase, amylase, protease and lipase activities	2, 3, 4	4.2, 5.2, 5.3
Zymogram for carbohydrate esterase activity	np	4.3.1
Two-dimensional (2D) gel electrophoresis	4	5.3
2D mannanase zymogram	4	5.3
Matrix assisted laser desorption ionisation tandem time-of-flight mass spectrometry (MALDI TOF/TOF MS/MS)	3, 4	5.2, 5.3
Quadrupole time-of-flight liquid chromatography tandem mass spectrometry (Q-TOF LC MS/MS)	4	5.3

np: not published