Chapter 3: Isolation and identification of fungi from koala faeces and screening for their enzyme activities of biotechnological interest

3.1 Introduction

Coprophilous fungi have played an integral role in the Australian ecosystem for millions of years, degrading animal waste and recycling nutrients back into the environment. However, in comparison to their northern hemisphere counterparts, there has been very little research carried out on Australian coprophilous fungi (Bell, 2005). Furthermore, most reported studies have been concerned only with the taxonomy of species based solely on morphological identification techniques (Section 1.4; Nagy and Harrower, 1979; McCarthy 1979; Bell, 2004). The significance of the work contained in this chapter was two-fold. Firstly, fungi were isolated from koala faeces and identified using internal transcribed spacer (ITS) sequencing. To the best of my knowledge, this is the first time that molecular techniques have been used to identify members of a coprophilous fungal community (Herrera et al., 2011). Secondly, the fungal isolates were screened for their enzyme activities using agar plate enzyme assays, revealing new information about how coprophilous fungi may obtain nutrition from koala faeces, and novel enzymes that could have potential for industrial application.

As a premise for this work, coprophilous fungi from koala faeces were considered as a likely source of enzymes to degrade plant cell wall polymers due to the high plant-biomass content of the faeces from which the fungi obtain nutrition. Furthermore, the fungi held potential to secrete enzymes that could degrade proteins, starch and lipids- substances that would be in smaller quantities in the faeces (Section 1.5). Consequently, in the work described in this chapter, agar plate assays were used to assess the xylanase, mannanase, endoglucanase, cellobiohydrolase, ligninase (ligninolytic phenoloxidase), tannase, protease, amylase and lipase activities of the fungi from koala faeces.

The chapter begins with a publication (Section 3.2; Publication 1) in which the isolation and identification of fungi from koala faeces and the screening for their enzyme activities are described. Methods that were only outlined briefly in Publication 1 are described in greater detail in Section 3.3 and additional information and discussion are contained in Section 3.4. As an outcome from this work, the selection of top-performing fungal isolates for further enzyme characterisation (Chapter 4) is described in Section 3.5.

3.2 Publication 1: Peterson, R.A., Bradner, J.R., Roberts, T.H., Nevalainen, K.M.H., 2009. Fungi from koala (*Phascolarctos cinereus*) faeces exhibit a broad range of enzyme activities against recalcitrant substrates. Letters in Applied Microbiology. 48, 218-225.

Publication 1 contains an account of the isolation and identification of fungi from koala faeces and screening for their enzyme activities using agar plate assays. All laboratory work and manuscript preparation for Publication 1 were carried out by myself, under the guidance and correction of the other listed authors.

Due to copyright reasons, the selected article has been omitted from this thesis (the article appears from page 58-65):

Peterson, R.A., Bradner, J.R., Roberts, T.H., Nevalainen, K.M.H., 2009. Fungi from koala (*Phascolarctos cinereus*) faeces exhibit a broad range of enzyme activities against recalcitrant substrates. Letters in Applied Microbiology. 48, 218-225

3.3 Additional details of methods described in Publication 1

The methods used for the isolation, identification and screening of the fungal isolates from koala faeces are described in Publication 1. Further details of the methods used for extraction of genomic DNA, PCR amplification of ITS regions, Big Dye Terminator sequencing, and the calculation of relative enzyme activity are described below.

3.3.1 Extraction of genomic DNA

The fungal isolates from koala faeces were grown on cellophane-covered PDA plates and the mycelia were freeze-dried and ground to a powder (Publication 1). For each isolate, 50 mg of ground mycelia was placed into an eppendorf tube and mixed gently with 500 µl extraction buffer containing 0.1 M Tris HCl, 0.25 M NaCl, 25 mM EDTA, 0.5 % w/v SDS. A 500 µl volume of phenol/ chloroform/ isoamyl alcohol (25:24:1) was then mixed into each sample. After centrifugation at 13,000 × *g* for 30 min at 4 ºC, the aqueous phase was removed to a new eppendorf tube and subjected to a repeat of the phenol/ chloroform/ isoamyl alcohol extraction and centrifugation procedure. The aqueous phase was removed to a new eppendorf tube and incubated for 60 min at 37 ºC with 10 mg/ ml RNase. A 265 µl volume of chloroform/ isoamyl alcohol (24:1) was mixed into each sample by inverting the tube gently. Following centrifugation at 13,000 × *g* for 30 min at 4 ºC, the aqueous phase was removed and the chloroform/ isoamyl alcohol extraction and centrifugation procedure was repeated. To the aqueous phase, 20 µl of 3 M sodium acetate and 1 ml of isopropanol were added and the tubes were inverted several times. The precipitate was collected by centrifugation (13,000 × *g*, 30 s, 4 ºC), and the pellet was washed with 70 % ethanol and resuspended in 1 × TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8).

3.3.2 PCR amplification of ITS regions

The sequences of the primers used for PCR amplification of the ITS regions of the fungal isolates are shown in Fig. 3.1, along with the location of the primers on nuclear ribosomal DNA. The ITS5 forward primer (ITS5 fwdpr) locates slightly upstream (5') from the ITS1

region and the ITS4 reverse primer (ITS revpr) locates immediately downstream (3') of the ITS2 region (White et al., 1990). Details of PCR conditions and PCR product purification are contained in Publication 1.

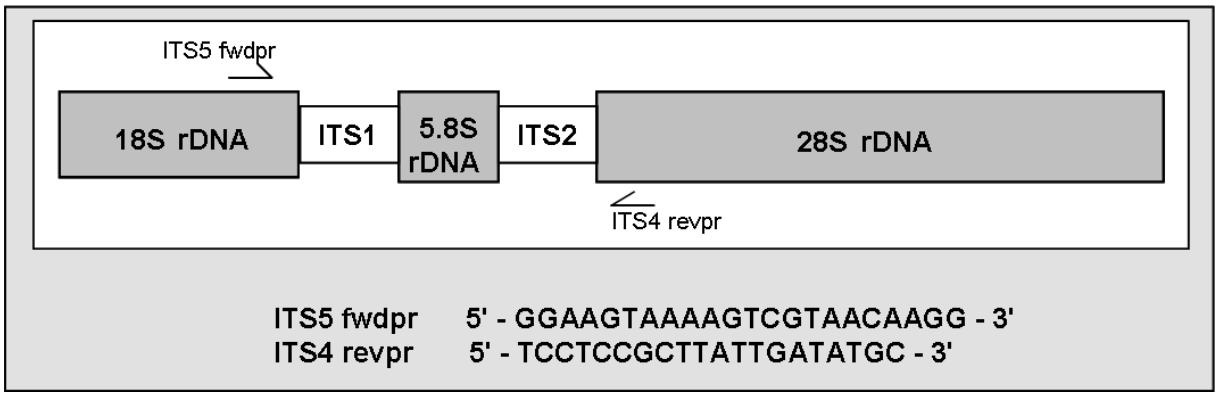


Figure 3.1: Binding sites and sequences of the ITS5 forward primer (ITS5 fwdpr) and the ITS4 reverse primer (ITS4 revpr) used to amplify the ITS regions of the fungal ribosomal DNA.

3.3.3 Preparation of PCR product for Big Dye Terminator sequencing

The PCR products were prepared for Big Dye Terminator sequencing (Applied Biosystems) as follows. Approximately 100 ng of purified DNA was combined with 2 µl Big Dye, 2 µl 5 × CSA buffer (400 mM Tris HCl, pH 9, 10 mM $MgCl_2$), 10 pmol ITS4 revpr and MQ water to reach a final volume of 20 µl. PCR was carried out with 35 cycles at 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. Then 4 µl 3 M sodium acetate, pH 5, 12 µl MQ water and 64 µl 95 % ethanol was added to each 20 µl sample before incubation at RT for 15 min. Following centrifugation at 13,000 × *g* for 20 min, the supernatant was removed and 250 µl 70 % ethanol was added. The sample was vortexed briefly and then centrifuged at 13,000 × *g* for 10 min. The supernatant was removed and the DNA pellet air dried before sequencing was carried out on an ABI Prism 377 DNA sequencer (Publication 1.)

3.3.4 Analysis of ITS sequences

The DNA sequences of the ITS regions of the fungal isolates from koala faeces were compared to nucleotide sequences in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.

nlm.nih.gov/Blast.cgi) and identifications were made on the basis of sequence similarity to database entries (Publication 1). Sequence alignment of the ITS regions of the isolated fungi was carried out with ClustalW (Thompson et al., 1994; www.ebi.ac.uk/ Tools/services/ web_clustalw) and the results were displayed using Jalview (Waterhouse et al., 2009; www.jalview.org/).

3.3.5 Calculation of relative enzyme activity

In order to assess the enzyme activities of the fungi from koala faeces, the isolates were grown on agar plates containing substrates of the target enzymes (Publication 1). Halos that appeared around the fungal colonies indicated degradation of the substrate and enzyme activity. An index of "relative enzyme activity" was then used to express the enzyme activities of the isolates relative to the size of the fungal colony (Bradner et al., 1999; Bradner, 2003; Fig. 3.2).

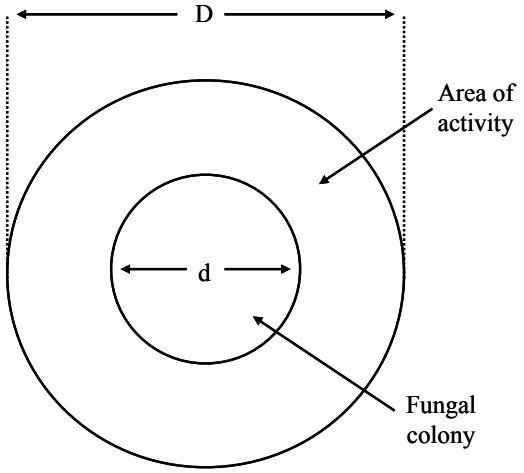


Figure 3.2: The method used to calculate relative enzyme activity of the fungal isolates (see text).

The relative activity was calculated by dividing the area of the halo around the colony (the area of the clearing zone minus the area of the colony) by the area of the colony. This was simplified by the equation $(D^2 - d^2)/ d^2$, in which D and d were the diameter of the halo and colony respectively (Fig. 3.2).

3.4 Results and Discussion

In the following section, the work reported in Publication 1 is discussed in greater depth. Rationales are given for the techniques used and their value is assessed in terms of the outcomes achieved. In addition, material that was not reported in the publication or that was only reported briefly is presented. The morphological features of the fungi isolated from koala faeces are outlined (Section 3.4.4) and further analysis of the ITS sequences is made (Sections 3.4.5 and 3.4.6), revealing the potential discovery of a new *Mucoraceae* species (Section 3.4.7). The results of the agar plate assays for all 37 fungal isolates are presented (Section 3.4.8) and extra information is provided about the yeast isolates (Section 3.4.9).

3.4.1 Collection of koala faeces and preparations for incubation

The koala faeces that were the source of the fungi studied in this work were collected from the ground beneath koalas feeding on *Eucalyptus punctata* (Grey Gum) at Koala Park Sanctuary, West Pennant Hills, Sydney (Fig. 3.3), as described in Publication 1.

Figure 3.3: Koala in the feeding area of Koala Park Sanctuary, West Pennant Hills, Sydney. Faeces were collected from the ground (inset) within minutes of excretion.

The primary aim was to isolate fungi that could use koala faeces as a substrate, not just those originating from the koala itself, so it was not necessary to completely exclude contact of the faeces with air or soil. It is recognised, however, that the species of fungi isolated from the koala faeces in this study could be different to those isolated from the faeces of koalas in other environments.

The faecal pellets were very dry upon excretion and could be broken open to reveal densely packed, dehydrated plant fibres (Fig. 3.4).

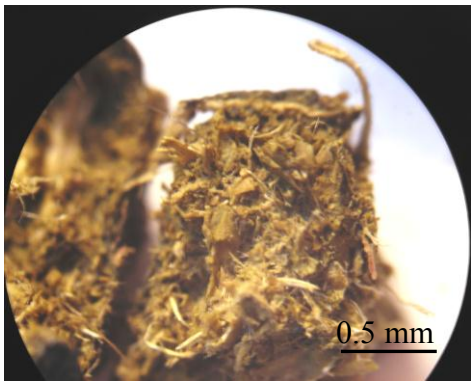


Figure 3.4: Koala faeces, sliced open and viewed under a stereo microscope within two hours of excretion to reveal its dry contents consisting primarily of fibrous plant material.

The pellets were dried indoors in a clean cardboard box for a period of seven weeks (Publication 1) to minimise the number of bacteria that could later colonise the faeces upon moist incubation (Section 1.6.1; Krug, 2004). Then a weak solution of bleach (0.01 % v/v NaOCl) was used to surface-sterilise the faeces and help remove dust and bacterial and fungal spores that could have alighted during the drying period.

3.4.2. Incubation of koala faeces for fungal growth

Three techniques were used to incubate the faeces (Fig 3.5) in order to facilitate the germination and isolation of a large range of fungal species (Publication 1). The traditional "moist chamber" method (Krug, 2004), in which the faecal pellet was simply placed on sterile moist filter paper in a petri dish (Fig. 3.5a), provided the closest analogy to natural conditions.

The fungi had no growth medium other than the faeces themselves and thus growth of the fungi directly signified their ability to produce enzymes capable of breaking down the faeces.

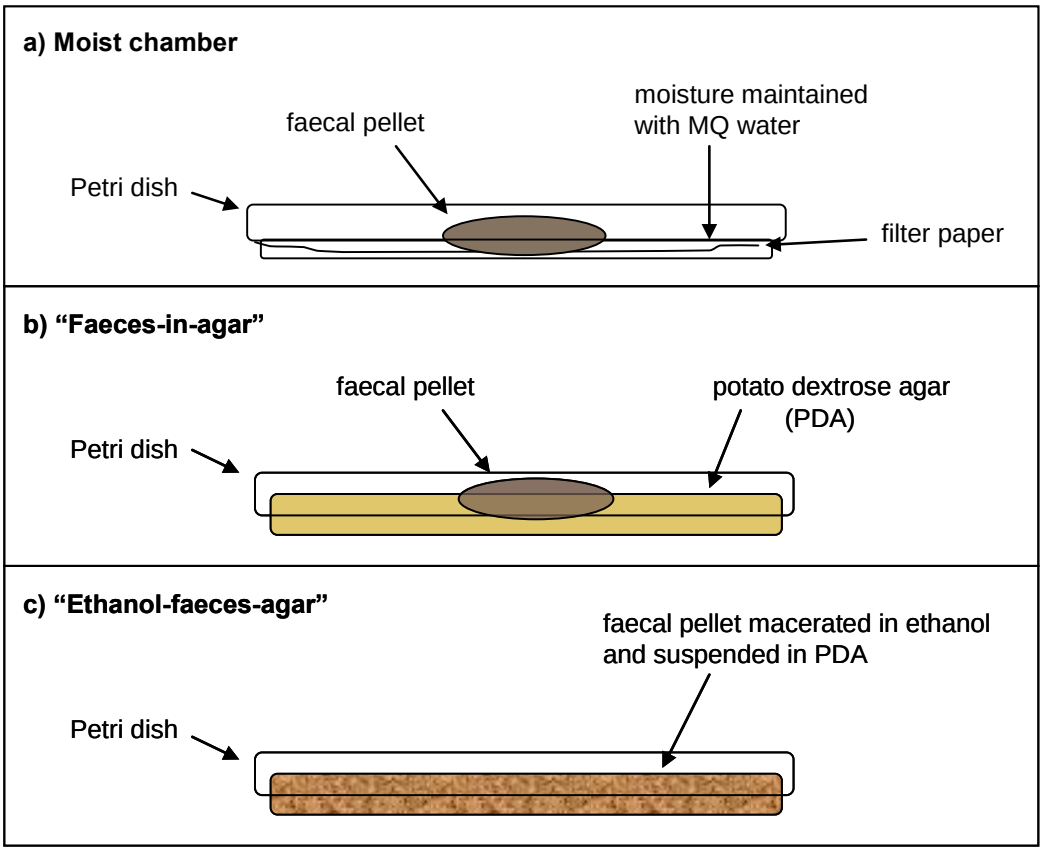
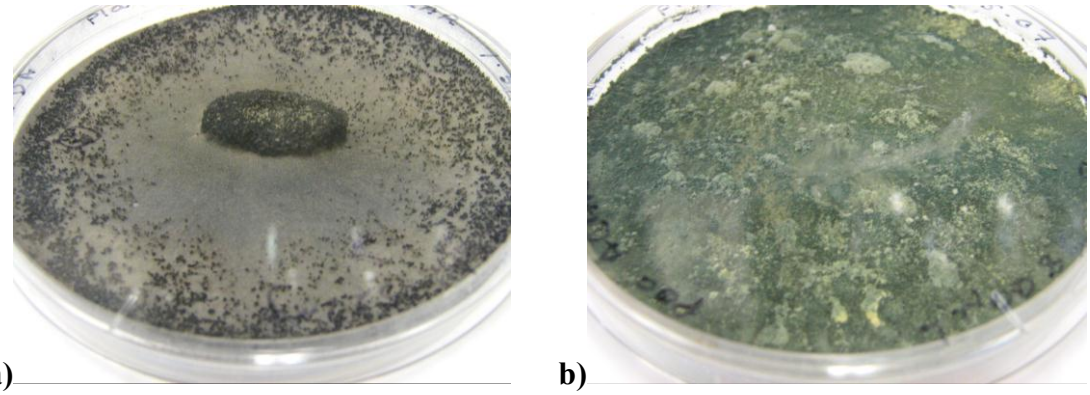


Figure 3.5: Incubation methods used to encourage the growth of fungi from the koala faeces. See Publication 1 for details of methods used.

One of the limitations of the traditional "moist chamber" technique is that fungal species that are not able to form visible sporulating bodies during the incubation period may be overlooked or overtaken by stronger fungal or bacterial competitors in the faeces. Therefore, the "faeces-in-agar" method was used, in which the pellet was surrounded by potato dextrose agar (PDA) containing chloramphenicol and ampicillin (Fig. 3.5b). The extra nutrients provided in the PDA encouraged fungal growth beyond the faeces and the antibiotics reduced or eliminated bacterial competition (Section 1.6.1; Krug, 2004). The third technique involved macerating the faecal pellets with ethanol prior to suspension in the antibiotic-infused PDA (Fig. 3.5c), an established method known to reduce the growth of rapidly growing zygomycetous and ascomycetous species, and maximise isolation of ascomycetous and

basidiomycetous species that typically occur later in the succession (Warcup, 1950; Krug et al., 2004).

Growth of fungi on the koala faeces incubated in the moist chambers was slow and sporulating bodies were small and difficult to detect and isolate. No particular species appeared to flourish or dominate at any one time. In comparison, fungi grew rapidly on the "faeces-in-agar" plates due to the extra nutrient source provided in the PDA medium and more species were isolated than from the moist chambers (Table 1, Publication 1). However, isolation of new fungi quickly became limited by the dominance of one species, later identified as *Sordaria superba* (Table 1, Publication 1), which appeared to prevent the growth of other fungal species after only 20 days of incubation (Fig. 3.6a). This may have been caused by the secretion of antifungal agents such as sordarin, named for the *Sordaria* genus from which the compound was first isolated (Hauser and Sigg, 1971; Davoli et al., 2002). The extra nutrients provided by the PDA may have supported the growth of *Sordaria* over that of other fungal species, thus amplifying its antifungal effects beyond levels that would occur naturally.



a) **b)**

Figure 3.6: a) A "faeces-in-agar" plate dominated by a *Sordaria* species; b) An "ethanol-faeces-agar" plate dominated by a *Trichoderma* species.

No *Sordaria* species were isolated from the "ethanol-faeces-agar" plates, thus allowing the subsequent development of a broader range of fungal species. In addition, the alcohol prevented the growth of zygomycetous species, as intended. However, the "ethanol-faeces-agar" plates also eventually became dominated by a single isolate, later identified as

Trichoderma atroviride, (Table 1, Publication 1; Fig. 3.6b), which is another species known to produce antifungal substances (Dennis and Webster, 1971; Choudary et al., 2007). Antimicrobial activity is thought to play a significant role in the competition of fungal species on faeces, and the isolation and purification of antimicrobial agents from coprophilous fungi has been previously reported (Section 1.4; Wang et al., 1997; Hein et al., 1998; Ridderbusch et al., 2004; Weber et al., 2005). Although no further investigations were carried out into antimicrobial compounds produced by the coprophilous fungi isolated from koala faeces in this work, it could be an area of research in the future.

3.4.3 Isolation of fungi

Most of the fungi that became visible on the koala faeces during the incubation period (Fig. 3.7) could be successfully transferred to the PDA plates containing antibiotics for continued growth and examination (Publication 1). Transferring more than one fungal species to a single PDA plate was often unavoidable, and so successive separations of strains on new PDA plates were necessary until pure cultures could be maintained. Some isolates did not survive following separation, suggesting a symbiotic relationship may have existed between them.

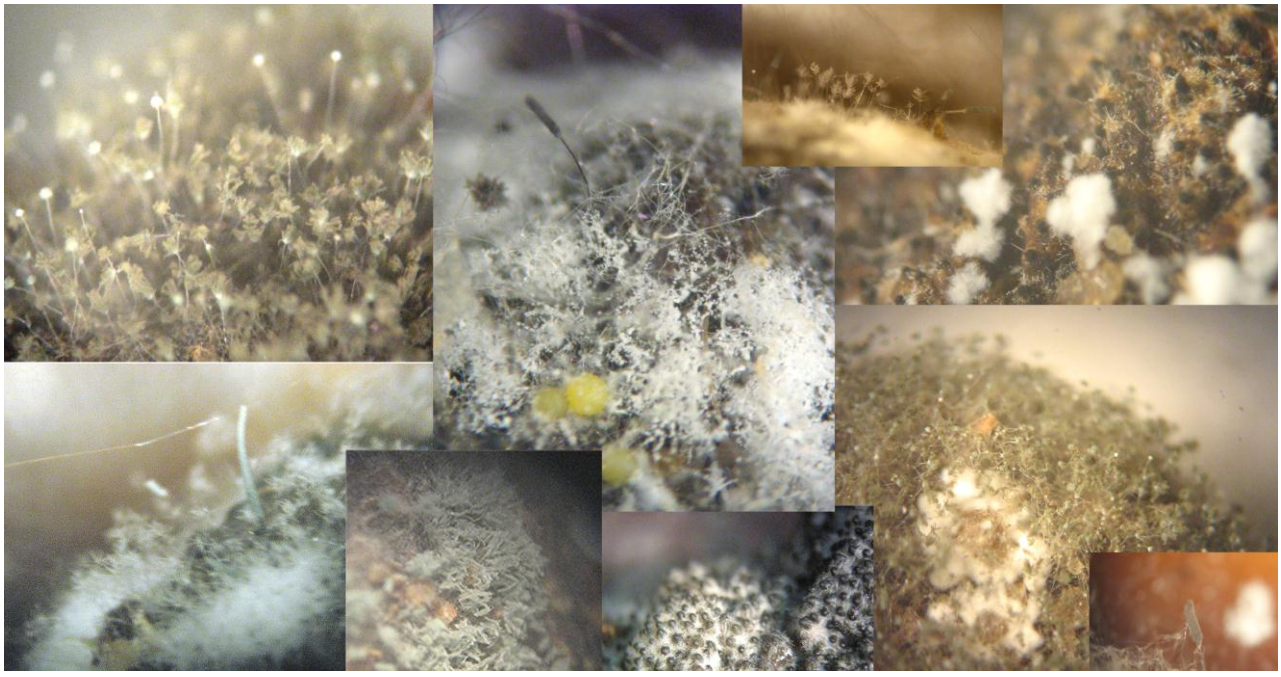


Figure 3.7. Stereomicroscope images (x 20) of a selection of fungal species on the koala faeces.

Several species of fungi on the koala faeces were never grown successfully on PDA plates, although their presence on the faeces was noted over at least several days (for example, the isolates resembling *Rhizopus* and *Graphium* species, Fig. 3.8). This could have been due to an inability of the fungal strains to survive on the nutrients provided in the PDA medium or in the presence of the antibiotics chloramphenicol and ampicillin (Publication 1).

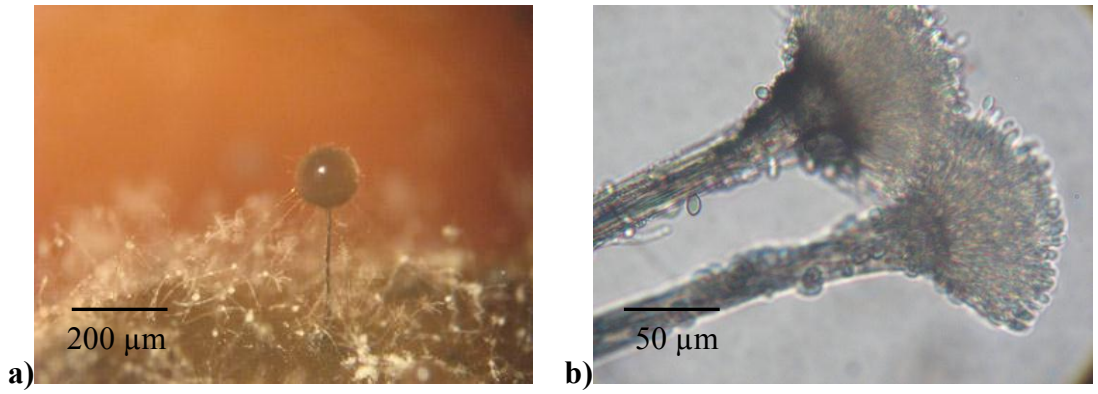


Figure 3.8. Examples of fungal species that appeared on the koala faeces during the incubation period but could not be maintained on PDA plates for identification, storage or screening. a) stereo microscope image of a *Rhizopus*-like species; b) light microscope image of a *Graphium*-like species.

It is recognised that the inability to isolate some of the observed fungal species (Fig. 3.8) may be just a small indication of the number of fungi present in the koala faeces or faecal preparations that could not be isolated using the techniques employed in this study. Isolation of some of these species of fungi may have been possible in the absence of antibiotics or using a different medium such as malt extract agar. However, it is likely that there were many fungal species in the koala faeces that could be considered "unculturable" under laboratory conditions. Detection of these species may have been achieved by ITS sequencing of DNA extracted directly from the faeces (Herrera et al., 2011) but was not attempted in this work as the aim was to study living fungal species for further characterisation of their enzyme activity.

3.4.4 Identification of fungi by morphology

Many of the fungal isolates from koala faeces could be tentatively identified to a genus level by their morphology (Publication 1), based on the shape, structure and colour of colonies, conidiophores, conidia and/or sexual fruiting bodies and spores. Tables 3.1a - 3.1c contain a summary of the morphological features and corresponding identifications of fungi isolated from moist chambers (C1 - C8), "faeces-in-agar" plates (P1 - P10) and "ethanol-faeces-agar" plates (A1 - A19), respectively. The day of isolation is shown in column two and a glossary of terms is provided in Box 3.1. Some examples of identifying morphological features of the fungi seen under light microscope and stereo microscope are shown in Fig. 3.8.

Table 3.1: Morphological identification of fungal isolates from koala faeces. The day of isolation, calculated from the beginning of the incubation period, is shown in the second column. References: von Arx, 1981; Bell, 2005.

a) Fungi from moist chambers (C1 - C8).

Isolate	Day	Tentative identification	Diagnostic morphological features
C1	6	*Aspergillus crystallinus*	conidiophore with apical vesicle bearing numerous long, dry chains of green spores, elongated yellow crystals
C2	7	*Penicillium* sp.	conidiophore erect with apical penicillus (brush-like head), long chains spores on flask shaped phialides
C3	9	*Penicillium* sp.	as above
C4	12	*Sporobolomyces* sp.	salmon-pink coloured yeast, pseudomycelium and single cells, vegetative reproduction by budding
C5	15	Unknown sp.	no fruiting bodies, black hyphae embedded in PDA
C6	17	*Fusarium* sp.	white cottony aerial mycelia, white on top, becomes pink beneath, lunate multi-septate hyaline macroconidia, ellipsoid microconidia
C7	24	*Sporormiella* sp.	ascospores, with three or more septa, ostiolate perithecia
C8	35	*Doratomyces* sp.	conidiophores aggregated to dark coloured synnema with fertile spore bearing head, and long chains of spores

b) Fungi from "faeces in agar" plates (P1 - P10)

Isolate	Day	Tentative identification	Diagnostic morphological features
P1	3	*Mucoraceae* sp.	abundant long silky white aerial mycelia, no septa evident, no rhizoids, hyaline spores borne in grey sporangia on tall sporangiophores
P2	3	*Mucoraceae* sp.	as above except with pale grey aerial mycelia
P3	6	*Sordaria* sp.	pear shaped ascomata, cylindrical asci, ascospores with gelatinous sheath, ejaculated when mature
P4	6	Unknown sp.	broad spreading colony, abundant oval ascomata
P5	8	Yeast sp.	single, budding cells, some pseudomycelia
P6	8	Unknown sp.	no fruiting bodies, brown mycelia embedded in agar
P7	15	*Cylindrocladiella* sp.	cylindrical conidia produced in mucus from penicillately arranged phialide cells, mycelium cottony white, becoming brown with age
P8	18	Unknown sp.	greyish white colony, single ovoid conidia, spherical dark grey cleistothecia
P9	20	Unknown sp.	no fruiting bodies, black mycelia embedded agar
P10	20	*Preussia* sp.	ascospores, with three or more septa, non-ostiolate perithecia

c) Fungi from "ethanol-faeces-agar" plates (A1 - A19)

Code	Day	Tentative identification	Diagnostic morphological features
A1	7	Yeast	single, budding cells, some pseudomycelia
A2	9	*Trichoderma* sp.	conidiophores regularly branched, approx. right angles to main axis; green to white conidial masses, phialides short, swollen, slightly curved
A3	9	*Penicillium* sp.	conidiophore erect with apical penicillus (brush-like head), long chains spores on flask shaped phialides
A4	10	*Neosartorya* sp.	conidiophores with apical vesicle bearing numerous long, dry chains of small blue/green spherical conidia, slightly hairy ascomata
A5	11	*Eurotium* sp.	yellow mycelia, bright yellow cleistothecium containing spherical asci, colourless ascospores, no conidiophores
A6	11	*Eurotium* sp.	as above but also with conidiophores bearing long chains of pale grey/ blue conidia
A7	11	*Penicillium* sp.	conidiophore erect with apical penicillus (brush-like head), long chains spores on flask shaped phialides
A8	15	*Penicillium* sp.	as above
A9	15	*Penicillium* sp.	as above
A10	20	*Gelasinospora* / *Neurospora* sp.	flask shaped ostiolate ascomata, aseptate pitted ascospores, white aerial filaments above, pink/red colour infuses into agar
A11	22	*Mariannaea* sp.	aerial hyphae in concentric zones of brown and white, two types of conidiophores: long oblique chains of fusiform conidia from *Verticillium*-type conidiophores; and lunate conidia on *Trichoderma*-type conidiophores.
A12	22	*Preussia* sp.	ascospores, with three or more septa, non-ostiolate perithecia
A13	24	*Preussia* sp.	as above
A14	28	*Phoma* sp.	black aerial mycelia, flask shaped ostiolate pycnidia containing one-celled colourless conidia
A15	32	*Phoma* sp.	as above
A16	36	Yeast sp.	single, budding cells, some pseudomycelia
A17	40	*Preussia* sp.	ascospores, with three or more septa, non-ostiolate perithecia
A18	42	Unknown sp.	grey granular colony, ellipsoid conidia, one septum
A19	44	Unknown sp.	thick white tufty mycelia, turning brown below with age

Box 3.1 Glossary for Tables 3.1a - c (References: von Arx, 1981; Bell, 2005).

Ascocarp - ascospore-containing fruiting structure of fungi from the phylum Ascomycota, arising after sexual reproduction has been initiated in the hyphae. Forms of ascocarps include the apothecium, cleistothecium and perithecium.

Ascospores - a sexually produced fungal spore formed within an ascus.

Ascus - a sexual spore-bearing cell produced in an ascocarp, usually containing eight ascospores.

Cleistothecium - a closed spherical ascocarp from which the ascospores are released by rupture or decay.

Conidiophore - a specialised branch of fungal hyphae that produces conidia.

Fusiform - having a spindle-like shape that is wide in the middle and tapers at both ends.

Hyaline - colourless and glass-like or translucent in appearance.

Lunate - crescent shaped.

Ostiolate - with an ostiole.

Ostiole - small opening in an ascocarp through which the ascospores are released.

Penicillus - complex arrangement of conidia on a conidiophore having a brush-like appearance, as in *Penicillium*.

Phialides - flask-shaped projection from the vesicle of a conidiophore.

Pseudomycelium - successions of extended yeast cells, which resemble mycelia but are not connected with one another by means of pores.

Pycnidia - an asexual fruiting body, spherical or inversely pear-shaped and lined internally with conidiophores, which are released through an opening at the top of the pycnidia when ripe.

Synemma - aggregation of erect conidiophores to form a stalk-like structure, bearing conidia along their length and/ or at the head.

True mycelium - hyphae consisting of individual cells separated by septa and interconnecting pores.

Vesicle - swollen tip of conidiophore, bears phialides.

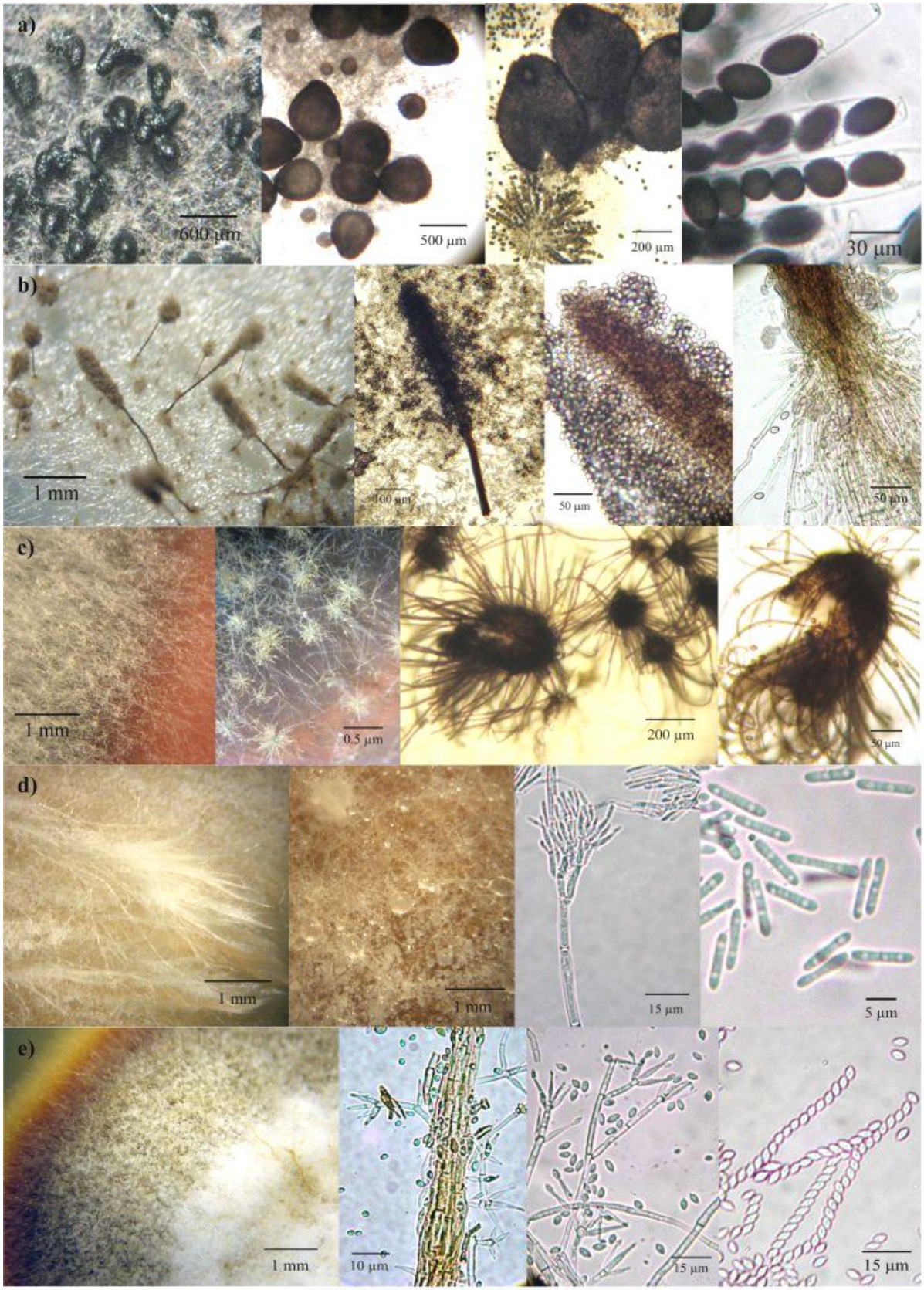


Figure 3.9. Stereo and light microscope images showing morphological features used for the identification of a selection of fungal isolates from koala faeces (Table 3.1). a) *Sordaria* (P3); b) *Doratomyces* (C8); c) *Gelasinospora*/ *Neurospora* (A10); d) *Cylindrocladiella* (P7); e) *Mariannaea* (A11).

Although many of the fungal isolates could be tentatively identified to a genus level by their morphology, species identification was possible only for isolates with distinctive morphological characteristics (Table 3.1). In several instances a failure to develop fruiting structures resulted in insufficient diagnostic features to enable identification by morphology alone. Therefore, ITS sequencing was used for more comprehensive and accurate identification of the fungi.

3.4.5 Identification of fungi by ITS sequencing

Genomic DNA was extracted from the fungal isolates following growth on cellophane covered PDA plates (Section 3.3.1; Publication 1). High DNA yields were easily obtained from isolates with relatively soft hyphae and fruiting bodies, such as the *Mucoraceae*, *Trichoderma* and *Aspergillus* species. Lower DNA yields were obtained from species such as the *Sordaria* and *Doratomyces* with tough walled ascomata, spores or synnemata (Table 3.1a, b; Fig. 3.9).

The size of the PCR products following amplification of the ITS regions of the fungi (Section 3.3.2; Publication 1) also varied between the fungal isolates, ranging from approximately 500 to 600 base pairs. The largest PCR products displayed by gel electrophoresis (Fig. 3.10) were obtained from isolates that were later identified as *Coprinellus micaceus* from the phylum Basidiomycota, and *Mucoraceae* sp. from the phylum Zygomycota (Table 1, Publication 1).

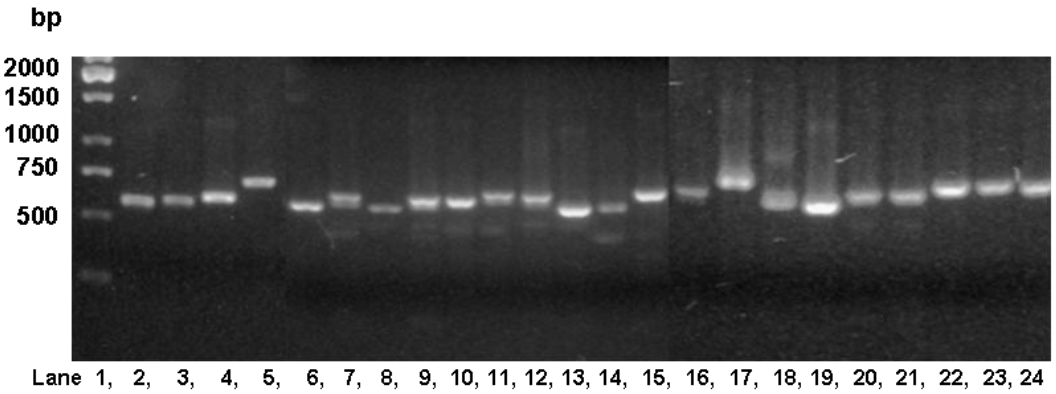


Figure 3.10: Agarose gel electrophoresis of PCR products following the amplification of the ITS regions of rDNA of a selection of fungi isolated from koala faeces. Approximately 200 ng DNA was loaded per lane. Lane 1- Gene Ruler. The largest PCR products were from *Coprinellus micaceus* (phylum Basidiomycota, lane 5) and *Mucoraceae* sp. (phlyum Zygomycota, lane 17).

Sequencing of the PCR products from the fungal isolates using Big Dye Terminator chemistry (Publication 1) resulted in the determination of DNA sequences that spanned 5.8S rDNA, ITS regions 1 and 2 and the immediately adjacent areas of 18S and 28S rDNA (Fig. 3.1, Section 3.3.2). When the sequences were searched against the NCBI database using BLAST, the identifications made based on sequence similarity to database proteins (Publication 1, Table 1) were consistent with those made by morphological features alone (Table 3.1). Furthermore the genera and species of isolates that could not be identified by morphology were established. The ITS sequences were deposited in GenBank (www.ncbi.nlm.nih.gov/ genbank/) and designated Accession numbers (EU551178 - EU551214; Table 1, Publication 1). This was the first and, to date (June, 2011), the only time that ITS sequences of fungi isolated from koala faeces have been submitted to GenBank. Moreover, the group of ITS sequences currently form the only PopSet (population set of DNA sequences) of coprophilous fungi in the NCBI database (http://www.ncbi.nlm.nih.gov/ Entrez/. PopSet).

The majority of the fungi isolated from the koala faeces were identified as species from the phylum Ascomycota (Table 1, Publication 1). The exceptions were two isolates from the phylum Zygomycota (*Mucoraceae* species P1, EU551186 and P2, EU551187; Table 3.1b; Table 1, Publication 1), four yeast species from the phylum Basidiomycota (*Sporobolomyces lactosus* C7, EU551181; and *Trichosporon* species P5, EU551190; A1, EU551196; and A16, EU551211; Table 3.1a - c; Table 1, Publication 1) and one fungal species from the phylum Basidiomycota (*Coprinellus micaceus* A19, EU551214; Table 3.1c; Table 1, Publication 1).

The predominance of ascomycetous species amongst the fungi isolated from koala faeces is consistent with previous research on coprophilous fungi, in which the phylum Ascomycota is most commonly observed (Section 1.4; Krug et al., 2004). In addition, the timing of isolation of the fungi from each of the phyla concurred with previously reported coprophilous fungal succession (Section 1.4; Krug et al., 2004), with the *Mucoraceae* sp. (P1, EU551186 and P2, EU551187; Table 3.1b; Table 1, Publication 1) from the phylum Zygomycota being the first

species isolated on day three and *Coprinellus micaceus* (A19, EU551214; Table 3.1c; Table 1, Publication 1) from the phylum Basidiomycota being the last species to be isolated on day forty-four.

3.4.6 Comparison of the ITS regions of the fungal isolates using ClustalW

In work not reported in Publication 1, the DNA sequences of the ITS regions of the fungal isolates (Fig. 3.1, Section 3.3.2) were compared using ClustalW (www.ebi.ac.uk/Tools/ services/web _clustalw) and Jalview (Fig. 3.11; Waterhouse et al., 2009) to explore the number and location of differences in the nucleotide base sequences in relation to the identifications made (Table 1, Publication 1). Greatest similarity occurred amongst the DNA sequences of the ITS regions of fungi from the same genus (e.g. *Sordaria superba* P3, EU551188 and *Sordaria alcina* C5, EU551182; Table 3.1; Table 1, Publication; Fig. 3.11), and then amongst fungi from the same phylum (*S. superba* P3, EU551188; *Sordaria alcina* C5, EU551182; *T. atroviride* A2 EU551197, all from phlyum Ascomycota; Fig 3.11). The sequences differed mainly in the ITS1 and ITS2 regions and a high degree of sequence similarity was evident in the more highly conserved adjoining regions of 18S, 5.8S and 28S rDNA. In addition, yeasts and fungi from the phylum Basidiomycota (eg. *T. asahii* A1, EU551196 and *C. micaceus* A19, EU551214; Fig. 3.11) had minor base substitutions and insertions in the 5.8S rDNA compared to fungi from the phylum Ascomycota. The sequences from *C. micaceus* and the *Mucoraceae* species also had several large insertions in ITS1 and ITS2, which accounted for the larger PCR product seen following gel electrophoresis (Fig. 3.10) and the longer sequence length obtained (last column, Table 1, Publication 1).

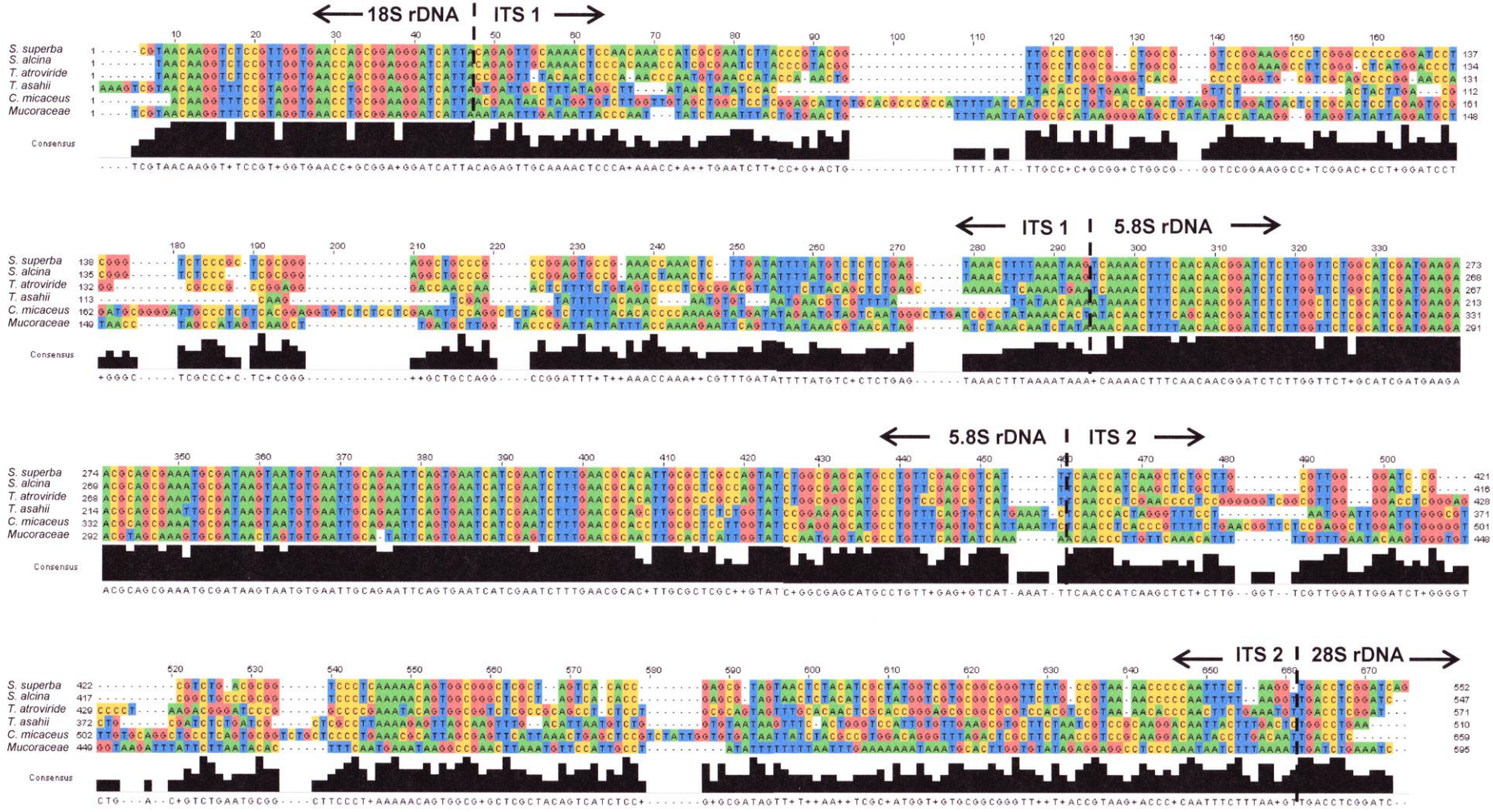


Figure 3.11. Jalview image of the Clustal W alignment of ITS1, 5.8S rDNA, ITS2, and adjacent areas of 18S rDNA and 28S rDNA for selected fungal isolates from koala faeces (Table 1, Publication 1; Table 3.1): *Sordaria superba* EU551188 (P3), *Sordaria alcina* EU551182 (C5) and *Trichoderma atroviride* EU551197 (A10) from the phylum Ascomycota; *Trichosporon asahii* EU551196 (A1), a Basidiomycetous yeast; *Coprinellus micaceus* EU551214 (A19) from the phlyum Basidiomycota; and *Mucuraceae* sp. EU551186 (P1) from the phylum Zygomycota. Nucleotide bases are indicated in the colours green (A), blue (T), yellow (C) and pink (G). "Consensus" is the most common residue at each position of the alignment and the black bar graph represents the percentage of bases matching the most common residue.

3.3.7 A potential new species of *Mucoraceae* from koala faeces

Although the majority of the fungal isolates from koala faeces could be confidently identified by at least 98 % sequence similarity to fungal nucleotide sequences in the NCBI database (Publication 1), the ITS sequences of two *Mucoraceae* species (P1 and P2, Table 3.1b; EU551186 and EU551187, Table 1, Publication 1) carried less than 90 % similarity to the most closely aligned database entry (*Mucoraceae* sp. EF060714, also of an undefined species, Table 1, Publication 1). Images of the *Mucoraceae* sp. P1 (EU551186) from koala faeces are shown in Fig. 3.12. *Mucoraceae* sp. P2 from koala faeces was similar in appearance, although slightly darker in colour and exhibited different enzyme activities than *Mucoraceae* sp P1 (Section 3.4.8.3). However, the ITS regions of the two *Mucoraceae* species from koala faeces differed only by two nucleotide residues at the 3' end of the ITS2 region.

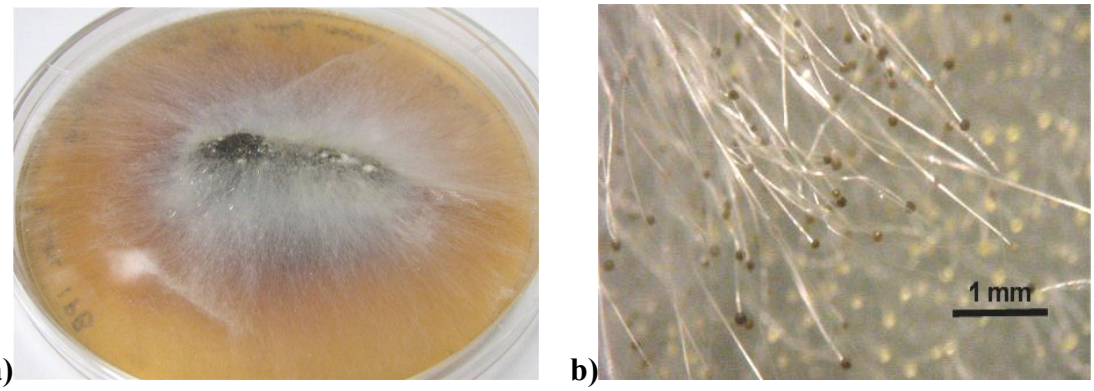


Figure 3.12. *Mucoraceae* species P1 (EU551186). a) growth on a "faeces-in-agar" plate; and b) sporangiophores and sporangia viewed under a stereomicroscope.

The DNA sequencing chromatogram for the ITS region of *Mucoraceae* sp. P1 (EU551186) is shown in Figure 3.13 and alignment with the most similar NCBI database entry (*Mucoraceae* sp. EF060714) is shown in Figure 3.14. Most differences between the two sequences (Fig. 3.14) occurred in areas in which clear peaks for nucleotide bases were defined in the chromatogram (highlighted in orange, Fig. 3.13), indicating that the discrepancies were genuine and not simply a result of poor sequencing data. The sequence of *Mucoraceae* sp. EU551186 (P1) only differed from *Mucoraceae* sp. EF060714 only within the ITS1 and ITS2 regions (Fig. 3.14), suggesting that the fungus was of the same genus but of a different species to that of the database entry (White et al., 1990).

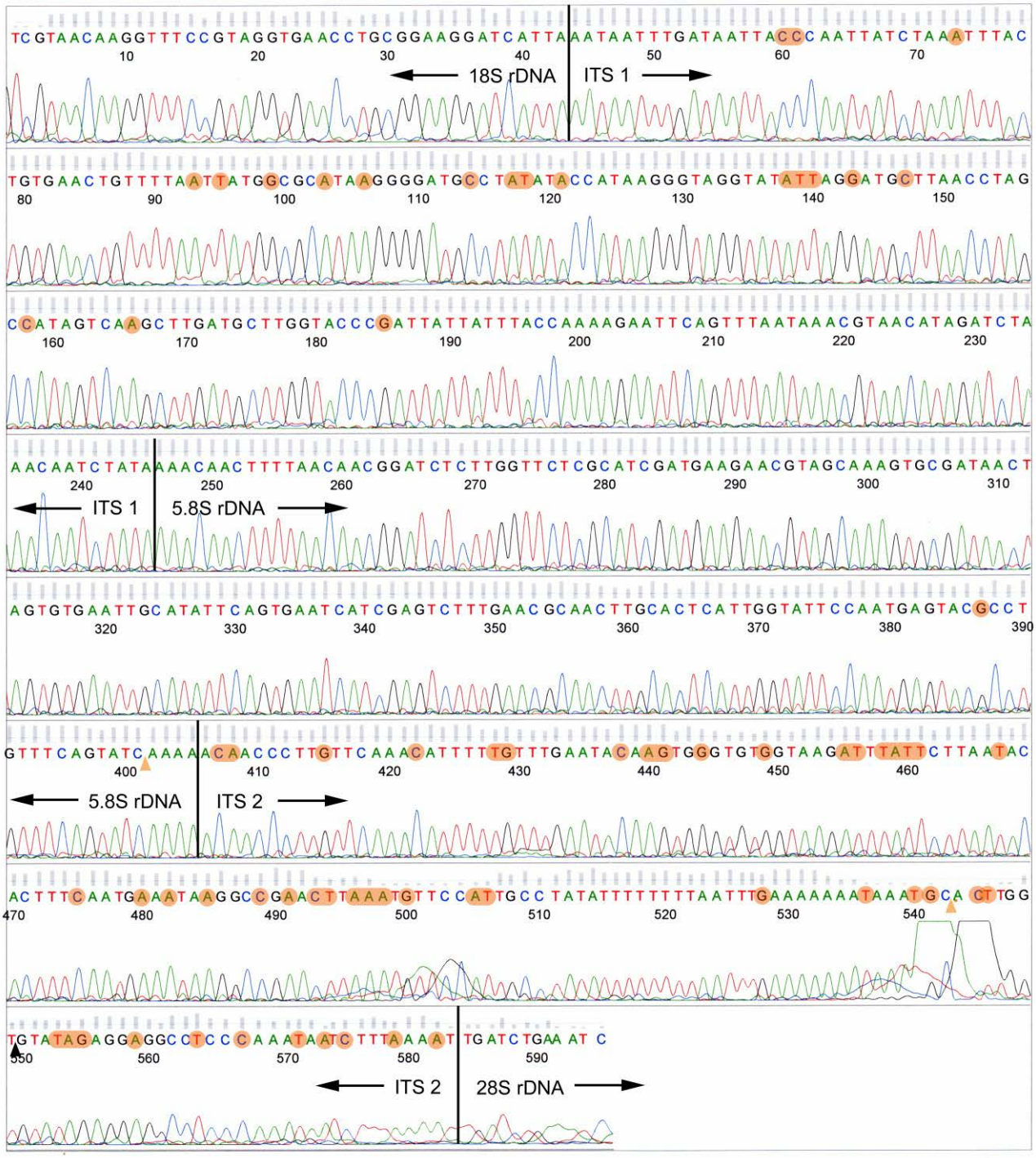


Figure 3.13: DNA sequence chromatogram of the ITS regions and adjacent rDNA of *Mucoraceae* species EU551186 (P1) from koala faeces. Nucleotides that differ from those of the most closely aligned entry in the NCBI database (*Mucoraceae* sp. EF060714) are highlighted in orange. Point deletions are indicated by orange triangles.

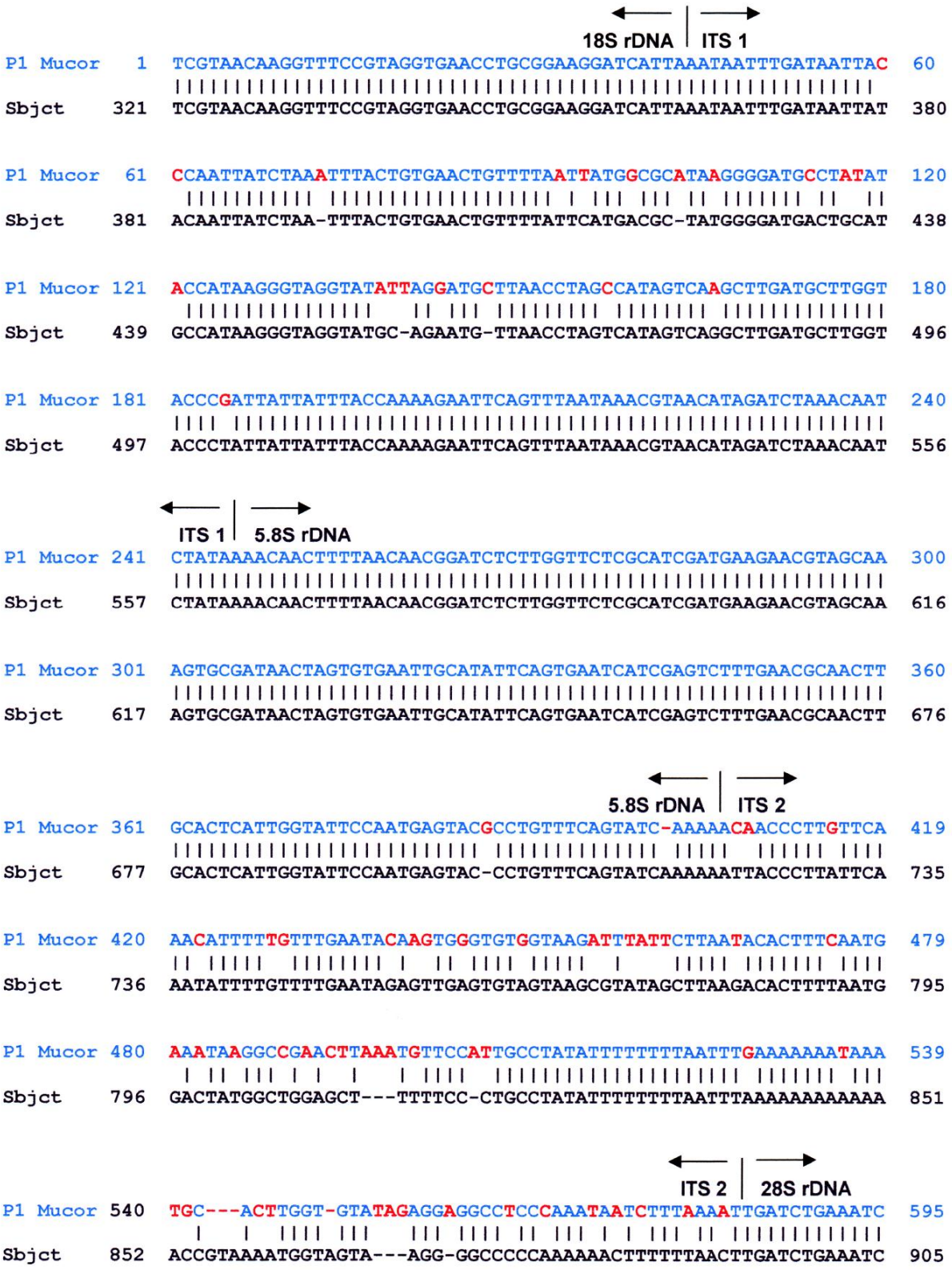


Figure 3.14: Sequence alignment of the ITS sequences and adjacent rDNA regions of *Mucoraceae* species EU551186 from koala faeces (P1 Mucor) to that of the sequence bearing greatest similarity in the NCBI database (*Mucoraceae* sp. EF060714, shown as "Sbjct"). Discrepancies between the sequences are indicated in red.

The sequences of the *Mucoraceae* isolates from koala faeces have now been included in the Emerencia database (http://andromeda.botany.gu.se/emerencia.html; date of inclusion 18.8.2010), a web-based tool designed to monitor the identity of insufficiently identified fungal ITS sequences in NCBI Genbank. Twice a week the Emerencia sequences are aligned with all identified sequences in Genbank and the best matches are quickly reported. However, no closer BLAST alignment has been found for the *Mucoraceae* isolates from koala faeces to date (June, 2011), suggesting that they could be strains of a new species for which molecular data had not yet been obtained.

3.4.8 Screening of fungi for enzyme activity by agar plate assay

The 37 fungal isolates from koala faeces were screened for their enzyme activity using agar plates containing substrates for specific target enzymes (xylanase, mannanase, endoglucanase, cellobiohydrolase, ligninolytic phenoloxidase, tannase, protease, amylase, esterase and lipase), as described in Publication 1. The rationales behind the methods used for assessing enzyme activity and full details and discussion of the results from all 37 isolates are presented below.

3.4.8.1 Detection of enzyme activity on solid media

The enzyme activities of the fungi were assessed on the basis of halo formation around the fungal colonies, indicating degradation of the substrate that was included in the agar medium (Publication 1; Section 3.3.5). Halo formation was immediately apparent on agar plates using the substrates Avicel cellulose, Remazol Brilliant Blue starch, Tween 20 and tannin. However, visualisation of halos representing endoglucanase and hemicellulase (xylanase and mannanase) activity required staining with Congo Red (Fig. 3.15a). Congo Red is a diazo dye derived from benzidine that forms complexes with cellulose and hemicellulose but does not bind once the polysaccharides have been hydrolysed (Teather and Wood, 1982).

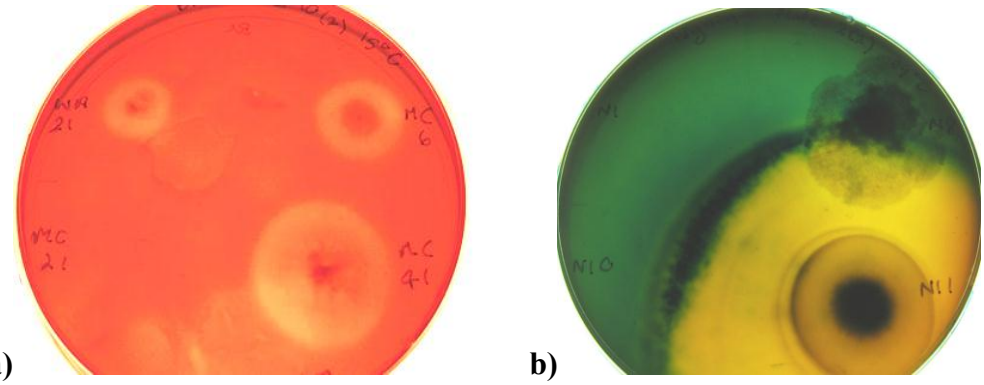


Figure 3.15. Visualisation of halos representing enzyme secretion and activity from fungal isolates grown on agar plates containing: a) xylan (stained with Congo Red); and b) lignin (stained with $FeCl_3$ and $K_3[Fe(CN)_6]$).

Detection of enzymes able to degrade the phenolic units of lignin (Fig. 3.15b) was carried out using agar plates containing 0.25 % (w/v) lignin, which were subsequently stained with ferric chloride ($FeCl_3$) and potassium ferricyanide ($K_3[Fe(CN)_6]$) (Publication 1). The iron (III) ions formed complexes with the phenolic units of lignin and were detected by the formation of a Prussian Blue complex with potassium ferricyanide reagent (Mole and Waterman, 1994), which appeared green in the lignin-infused agar medium (Fig. 3.15b). Oxidation of the phenolic units prevented the formation of the Prussian Blue complex and resulted in the formation of yellow to light brown halos. Any enzyme able to degrade the phenolic units of lignin could be detected using this test, including lignin peroxidase, manganese peroxidase and laccase (enzymes able to degrade phenolic and non-phenolic lignin units) and mild oxidants such as GMC oxidoreductases (enzymes only capable of oxidation or modification of phenolic lignin units; Section 1.8.5). In Publication 1, enzymes that degraded phenols in the lignin-agar plate assay are collectively referred to as "ligninolytic phenoloxidases" or "ligninases".

Screening for lipase activity was initially carried out by including Tween 20 in the agar plates, a cheap and easily available substrate that was appropriate for the large number of plates required for screening of the 37 fungal isolates. However, Tween 20 belongs to the family of Tween detergents (polyoxyethylene sorbitan mono-long chain fatty acid esters; Jarvis and

Thiele, 1997) that can be degraded by esterases (preferentially break ester bonds of short chain fatty acids) as well as lipases (specific for degradation of insoluble long chain fatty acids; Fojan et al., 2000). Therefore, fungi that were selected as positive for enzyme secretion on the Tween 20 plates were subsequently screened for "true" lipase activity (degradation of insoluble long-chain fatty acids ≥ 12 carbon atoms; Zhang et al., 2003) on agar plates containing olive oil and Rhodamine B. Fluorescence was emitted under ultra violet light when a "true" lipase had released the long chain fatty acids (LCFA) from the olive oil to form a fluorescent Rhodamine B-LCFA conjugate (Jarvis and Thiele, 1997).

Protease activity of the fungi was detected using skim milk powder as substrate in the agar plates. Although the halos representing enzyme activity surrounding the fungal colonies were visible, they were not clearly distinct. Therefore, to increase the clarity of the zone of hydrolysis, tannic acid was used to flood the plates. The tannins form insoluble complexes with the unhydrolysed skim milk protein, sharply increasing the colour intensity and contrast to areas of protein hydrolysis (Saran et al., 2007).

3.4.8.2 Temperatures of incubation

Three different temperatures (15 °C, 25 °C, 39 °C) were used for the incubation of fungi for the agar plate assays to determine the influence of temperature on the secretion and activity of the fungal enzymes (Publication 1). None of the temperatures was extreme as it was expected that the fungi isolated were mesophilic species, which typically have growth optima between 15 °C and 40 °C (Griffin, 1994). The aim was to reveal enzymes that were naturally active at moderately low (15 °C) or moderately high (39 °C) temperatures, which could hold potential for further improvement by mutagenesis or directed evolution to enable activity under more extreme temperature conditions (Turner, 2003; Section 1.3). Fungal growth was substantially slower at 15 °C so measurements of halos were taken on day seven rather than day five, as

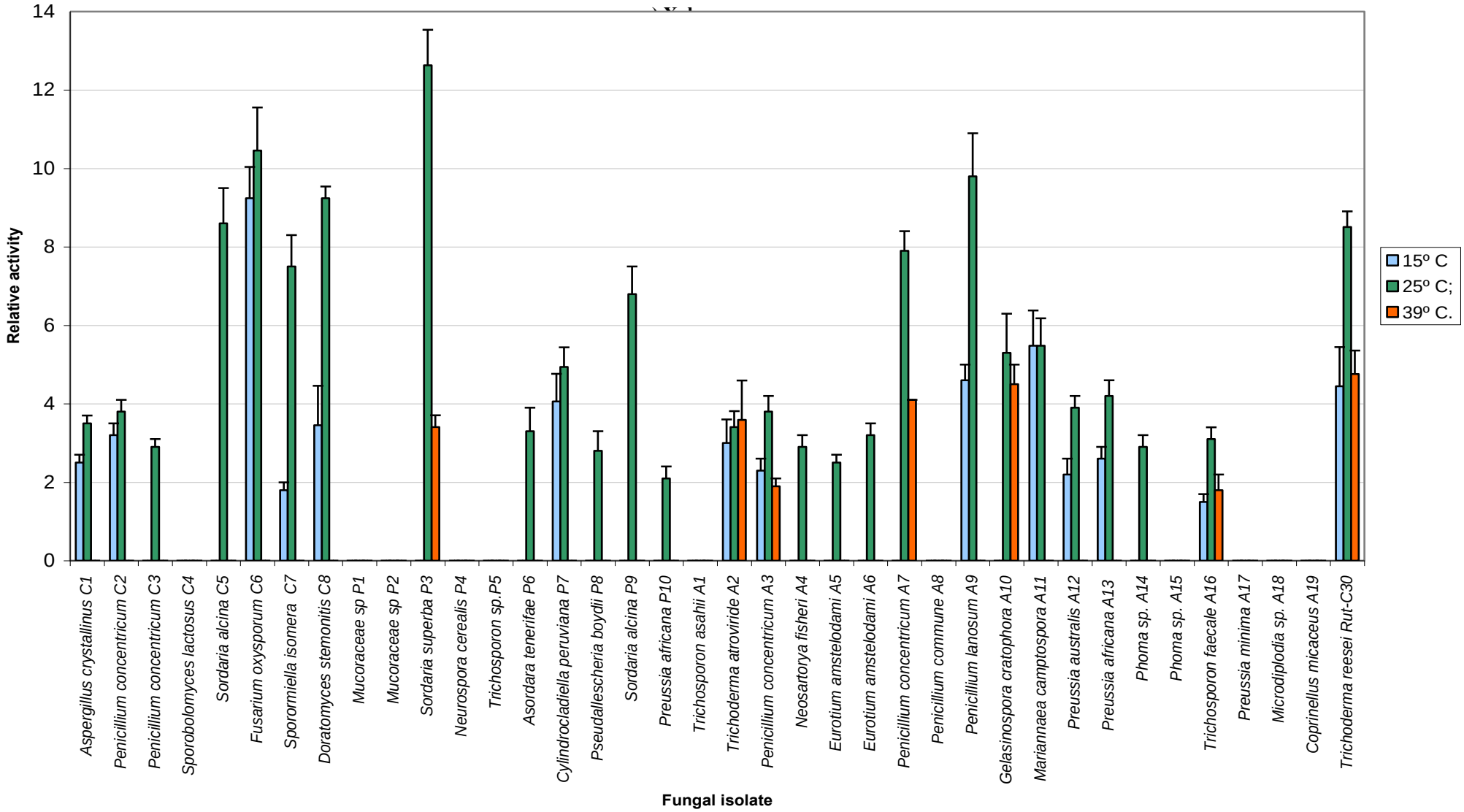
had been the case for the plates incubated at 25 °C and 39 °C, so that sufficient growth for detectable enzyme secretion and activity could occur.

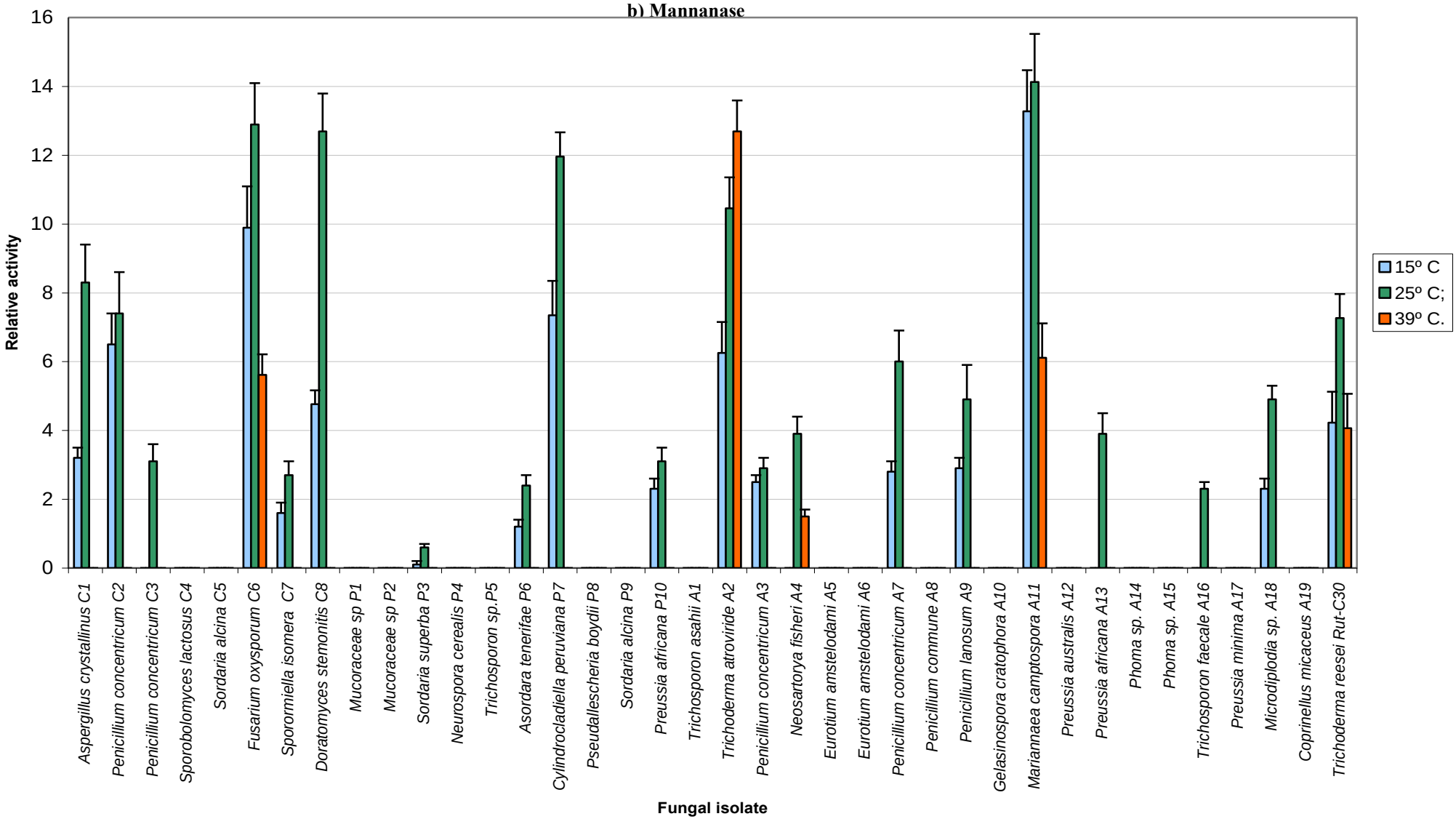
3.4.8.3 Relative Enzyme Activity

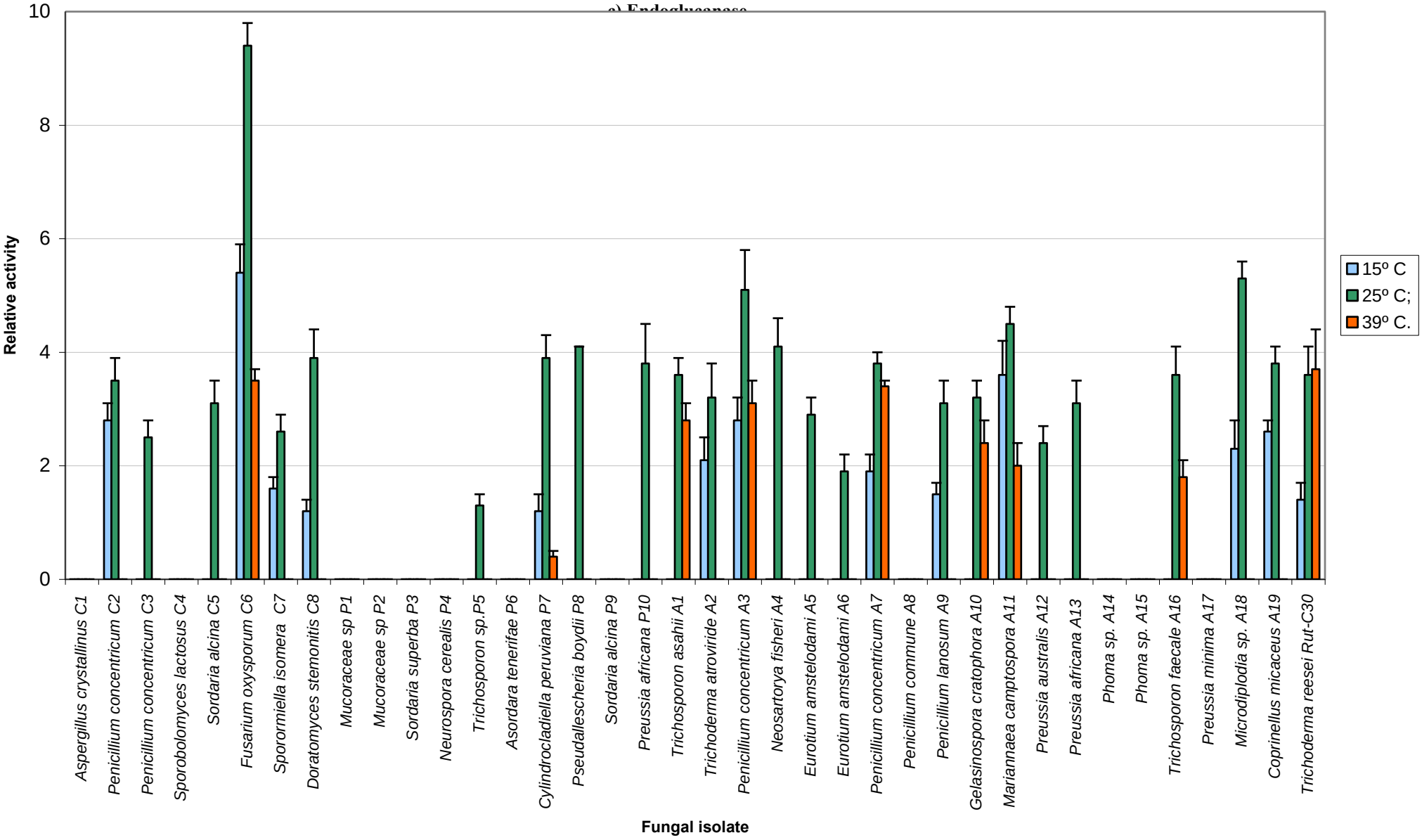
When screening fungi for enzyme activity using agar plate assays, fungal isolates have traditionally been ranked simply on the halo size formed around the fungal colony (Hankin and Anagnostakis, 1975). One drawback of the technique is that it does not take into account the size of the fungal colony in relation to the amount of enzyme activity displayed. Therefore, fungal species that grow faster or more prolifically could be considered to have higher enzyme activity simply due to the higher secretion capacity that arises from the greater number of hyphae, not necessarily due to the specific activity of the enzyme itself. This is particularly an issue when comparing the enzyme activities of different species of fungi with different growth patterns, as was attempted in the work described here. Therefore, the index of "relative enzyme activity" was used, in which the enzyme activity of the fungal isolates from koala faeces was compared relative to the colony size, as described in Section 3.3.5.

The relative enzyme activities of eight top-performing fungi isolated from koala faeces are displayed in Fig. 1 of Publication 1, and the results obtained from all 37 isolates are displayed in Fig. 3.16a - 3.16i of this chapter. A broad range of enzyme activities was exhibited by most of the fungal isolates from koala faeces, with 25 or more of the 37 isolates exhibiting xylanase, endoglucanase, ligininase (ligninolytic phenoloxidase) and protease activities, and 19 isolates exhibiting mannanase and amylase activities (Table 2, Publication 1; Fig. 3.16a - i). Cellobiohydrolase activity was exhibited by six of the isolates and lipase activity by five isolates (Table 2, Publication 1; Fig. 3.16d and 3.16i). Therefore, the basic premise that fungi from koala faeces would be good secretors of enzymes, and particularly those involved in the degradation of plant cell wall polymers (Section 1.5), was realised.

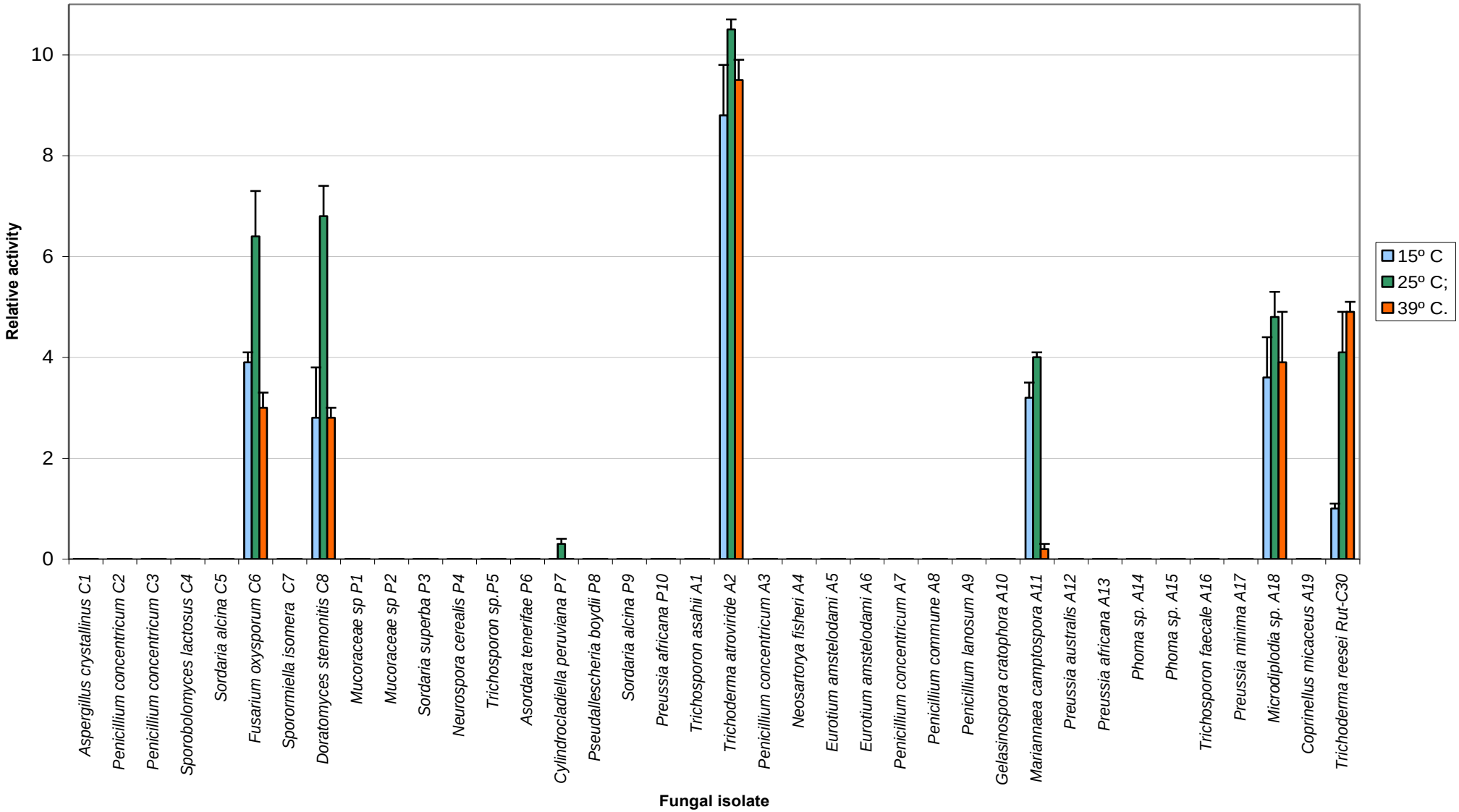
Figure 3.16: Relative enzyme activity of the fungal isolates from koala faeces determined by agar plate assays carried out at 15 °C (blue), 25 °C (green) and 39 °C (orange). Error bars indicate one standard deviation from the mean across four replicate plate assays at each temperature. The absence of a column on the graphs indicates that no enzyme activity was observed, either due to lack of halo formation or lack of growth of the fungal isolate at the indicated temperature.



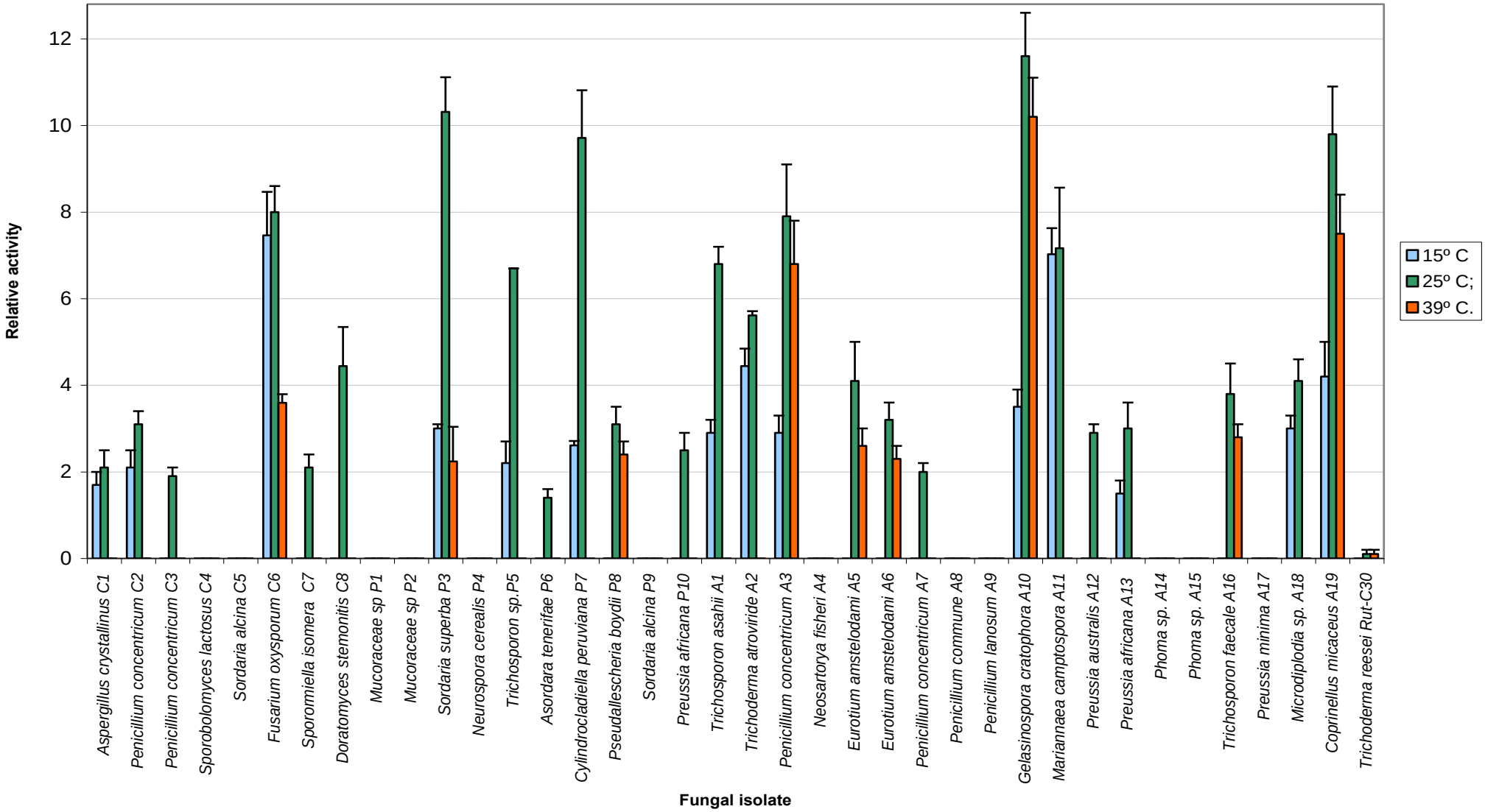

b) Mannanase
Relative activity
16
14
12
10
8
6
4
2
0
15º C
25º C;
39º C.
Aspergillus crystallinus C1
Penicillium concentricum C2
Penicillium concentricum C3
Sporobolomyces lactosus C4
Sordaria alcina C5
Fusarium oxysporum C6
Sporormiella isomera C7
Doratomyces stemonitis C8
Mucoraceae sp P1
Mucoraceae sp P2
Sordaria superba P3
Neurospora cerealis P4
Trichosporon sp.P5
Asordara tenerifae P6
Cylindrocladiella peruviana P7
Pseudallescheria boydii P8
Sordaria alcina P9
Preussia africana P10
Trichosporon asahii A1
Trichoderma atroviride A2
Penicillium concentricum A3
Neosartorya fisheri A4
Eurotium amstelodami A5
Eurotium amstelodami A6
Penicillium concentricum A7
Penicillium commune A8
Penicillium lanosum A9
Gelasinospora cratophora A10
Mariannaea camptospora A11
Preussia australis A12
Preussia africana A13
Phoma sp. A14
Phoma sp. A15
Trichosporon faecale A16
Preussia minima A17
Microdiplodia sp. A18
Coprinellus micaceus A19
Trichoderma reesei Rut-C30
Fungal isolate

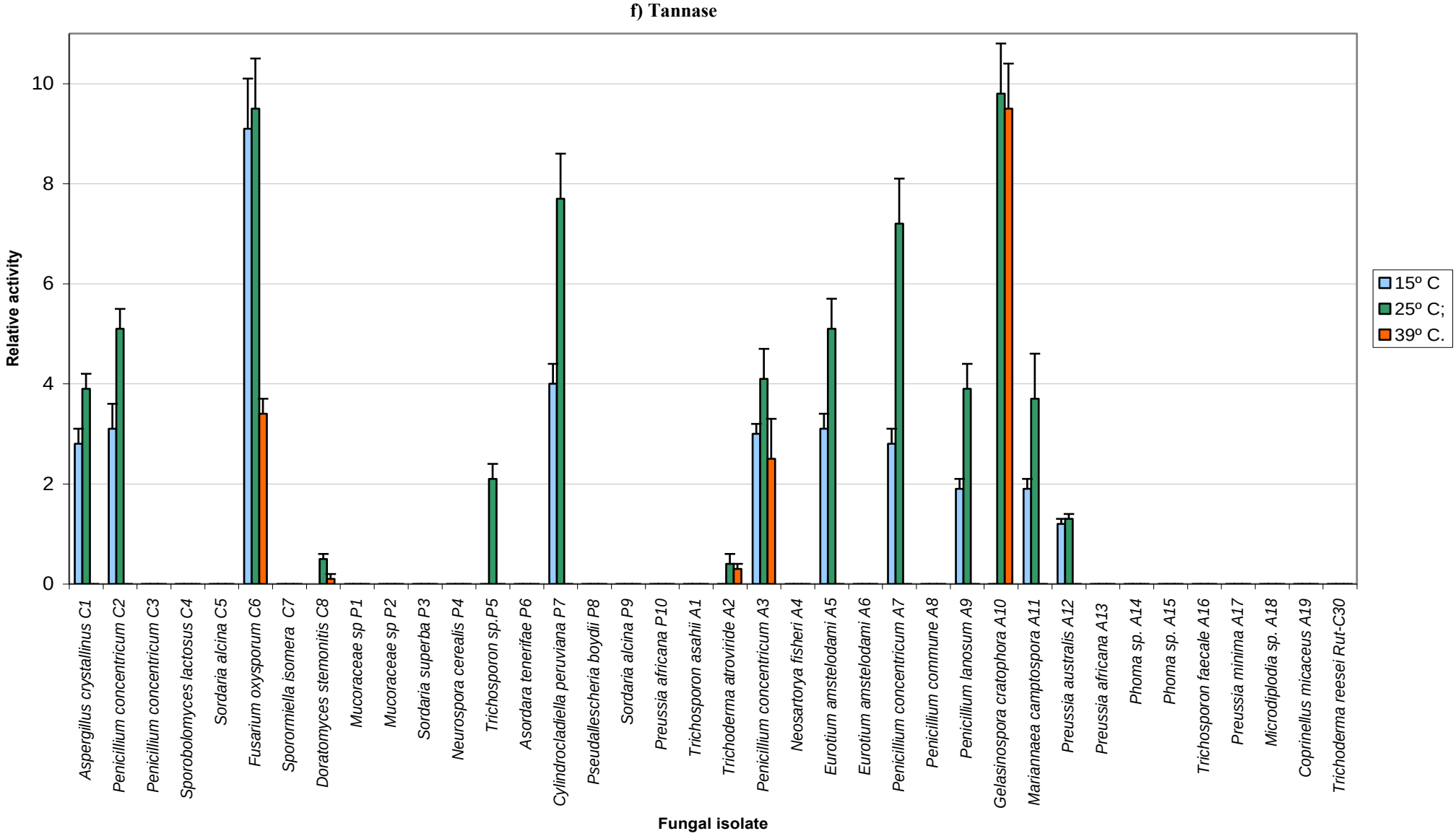

e) Endoglucanase
Relative activity
10
8
6
4
2
0
15º C
25º C;
39º C.
Aspergillus crystallinus C1
Penicillium concentricum C2
Penicillium concentricum C3
Sporobolomyces lactosus C4
Sordaria alcina C5
Fusarium oxysporum C6
Sporormiella isomera C7
Doratomyces stemonitis C8
Mucoraceae sp P1
Mucoraceae sp P2
Sordaria superba P3
Neurospora cerealis P4
Trichosporon sp.P5
Asordara tenerifae P6
Cylindrocladiella peruviana P7
Pseudallescheria boydii P8
Sordaria alcina P9
Preussia africana P10
Trichosporon asahii A1
Trichoderma atroviride A2
Penicillium concentricum A3
Neosartorya fisheri A4
Eurotium amstelodami A5
Eurotium amstelodami A6
Penicillium concentricum A7
Penicillium commune A8
Penicillium lanosum A9
Gelasinospora cratophora A10
Mariannaea camptospora A11
Preussia australis A12
Preussia africana A13
Phoma sp. A14
Phoma sp. A15
Trichosporon faecale A16
Preussia minima A17
Microdiplodia sp. A18
Coprinellus micaceus A19
Trichoderma reesei Rut-C30
Fungal isolate

d) Cellobiohydrolase

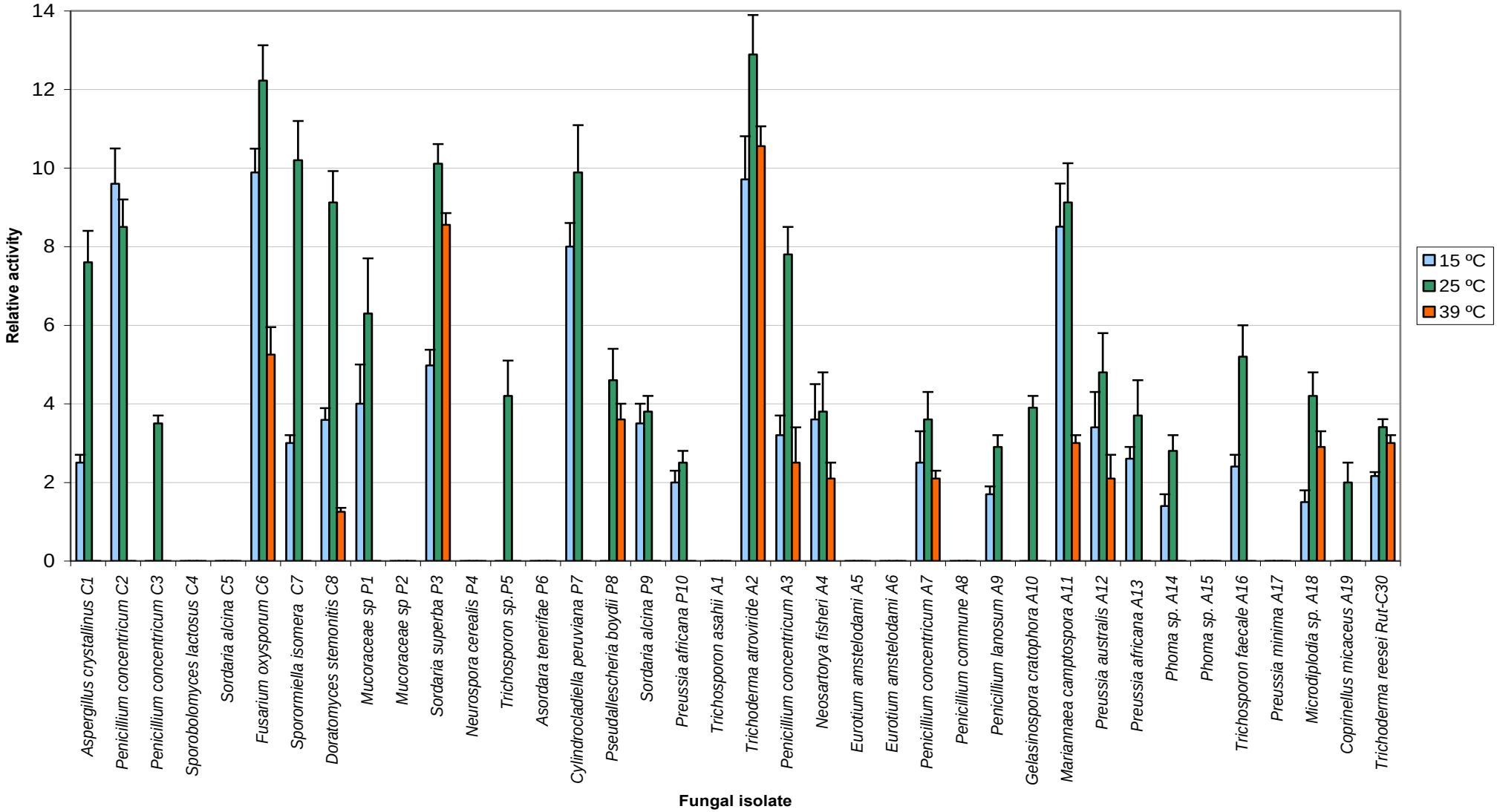


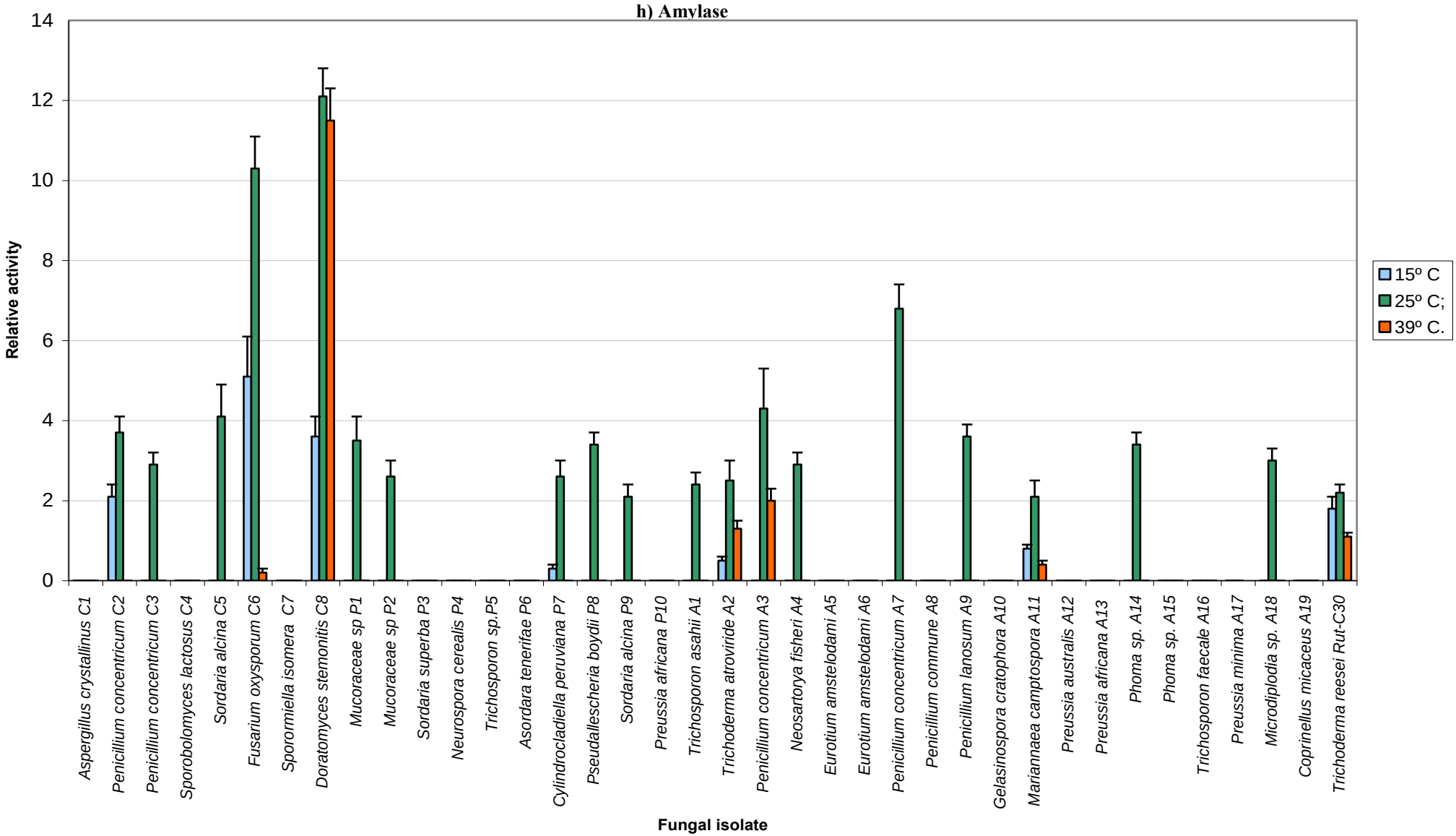
e) Ligninolytic phenoloxidase (ligninase)

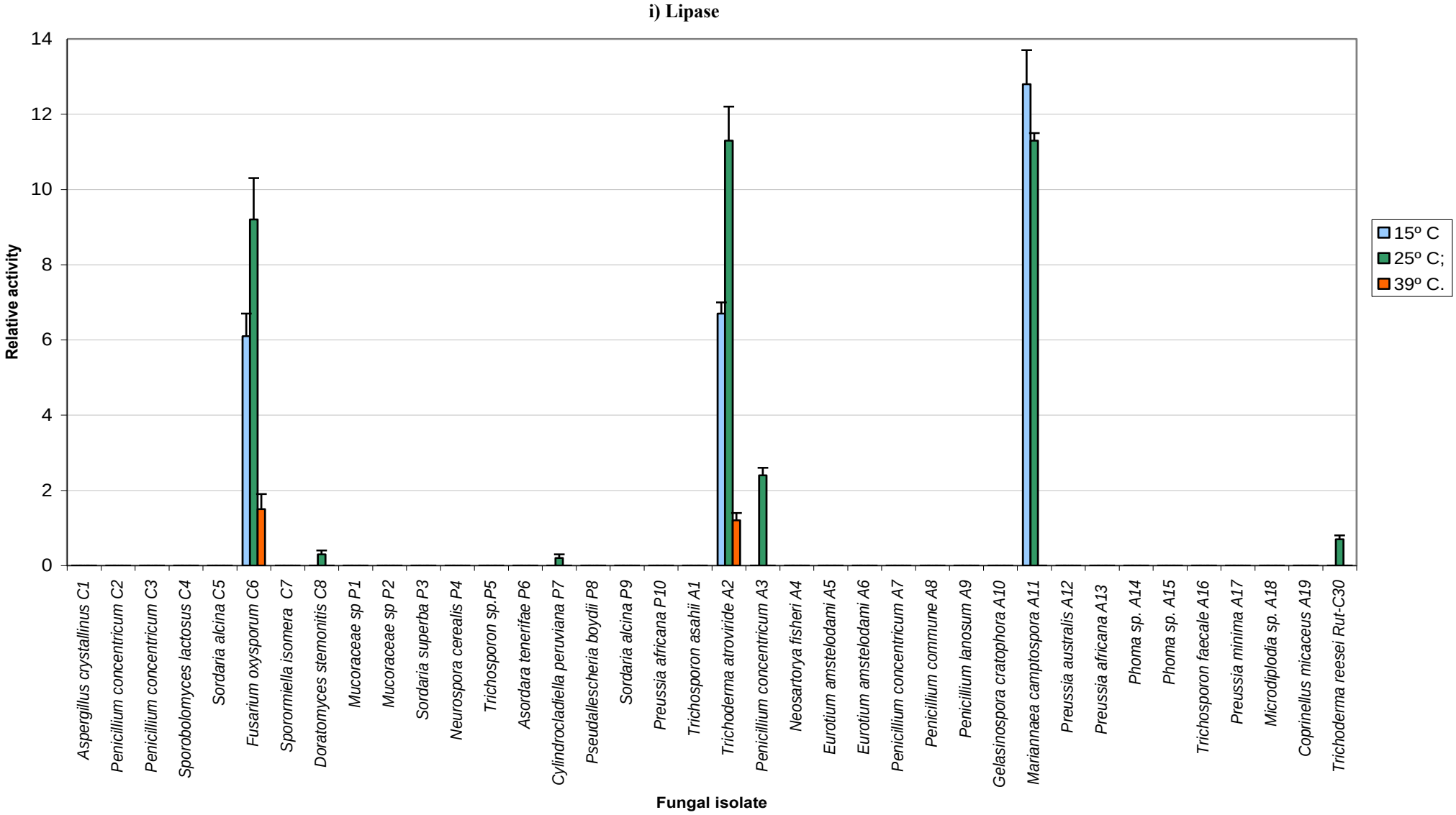



f) Tannase
Relative activity
0
2
4
6
8
10
15º C
25º C;
39º C.
Aspergillus crystallinus C1
Penicillium concentricum C2
Penicillium concentricum C3
Sporobolomyces lactosus C4
Sordaria alcina C5
Fusarium oxysporum C6
Sporormiella isomera C7
Doratomyces stemonitis C8
Mucoraceae sp P1
Mucoraceae sp P2
Sordaria superba P3
Neurospora cerealis P4
Trichosporon sp.P5
Asordara tenerifae P6
Cylindrocladiella peruviana P7
Pseudallescheria boydii P8
Sordaria alcina P9
Preussia africana P10
Trichosporon asahii A1
Trichoderma atroviride A2
Penicillium concentricum A3
Neosartorya fisheri A4
Eurotium amstelodami A5
Eurotium amstelodami A6
Penicillium concentricum A7
Penicillium commune A8
Penicillium lanosum A9
Gelasinospora cratophora A10
Mariannaea camptospora A11
Preussia australis A12
Preussia africana A13
Phoma sp. A14
Phoma sp. A15
Trichosporon faecale A16
Preussia minima A17
Microdiplodia sp. A18
Coprinellus micaceus A19
Trichoderma reesei Rut-C30
Fungal isolate

g) Protease


Relative activity
14
12
10
8
6
4
2
0
15 ºC
25 ºC
39 ºC
Aspergillus crystallinus C1
Penicillium concentricum C2
Penicillium concentricum C3
Sporobolomyces lactosus C4
Sordaria alcina C5
Fusarium oxysporum C6
Sporormiella isomera C7
Doratomyces stemonitis C8
Mucoraceae sp P1
Mucoraceae sp P2
Sordaria superba P3
Neurospora cerealis P4
Trichosporon sp.P5
Asordara tenerifae P6
Cylindrocladiella peruviana P7
Pseudallescheria boydii P8
Sordaria alcina P9
Preussia africana P10
Trichosporon asahii A1
Trichoderma atroviride A2
Penicillium concentricum A3
Neosartorya fisheri A4
Eurotium amstelodami A5
Eurotium amstelodami A6
Penicillium concentricum A7
Penicillium commune A8
Penicillium lanosum A9
Gelasinospora cratophora A10
Mariannaea camptospora A11
Preussia australis A12
Preussia africana A13
Phoma sp. A14
Phoma sp. A15
Trichosporon faecale A16
Preussia minima A17
Microdiplodia sp. A18
Coprinellus micaceus A19
Trichoderma reesei Rut-C30
Fungal isolate


h) Amylase
Relative activity
0
2
4
6
8
10
12
14
15º C
25º C;
39º C.
Aspergillus crystallinus C1
Penicillium concentricum C2
Penicillium concentricum C3
Sporobolomyces lactosus C4
Sordaria alcina C5
Fusarium oxysporum C6
Sporormiella isomera C7
Doratomyces stemonitis C8
Mucoraceae sp P1
Mucoraceae sp P2
Sordaria superba P3
Neurospora cerealis P4
Trichosporon sp.P5
Asordara tenerifae P6
Cylindrocladiella peruviana P7
Pseudallescheria boydii P8
Sordaria alcina P9
Preussia africana P10
Trichosporon asahii A1
Trichoderma atroviride A2
Penicillium concentricum A3
Neosartorya fisheri A4
Eurotium amstelodami A5
Eurotium amstelodami A6
Penicillium concentricum A7
Penicillium commune A8
Penicillium lanosum A9
Gelasinospora cratophora A10
Mariannaea camptospora A11
Preussia australis A12
Preussia africana A13
Phoma sp. A14
Phoma sp. A15
Trichosporon faecale A16
Preussia minima A17
Microdiplodia sp. A18
Coprinellus micaceus A19
Trichoderma reesei Rut-C30
Fungal isolate


i) Lipase
Relative activity
14
12
10
8
6
4
2
0
15º C
25º C;
39º C.
Aspergillus crystallinus C1
Penicillium concentricum C2
Penicillium concentricum C3
Sporobolomyces lactosus C4
Sordaria alcina C5
Fusarium oxysporum C6
Sporormiella isomera C7
Doratomyces stemonitis C8
Mucoraceae sp P1
Mucoraceae sp P2
Sordaria superba P3
Neurospora cerealis P4
Trichosporon sp.P5
Asordara tenerifae P6
Cylindrocladiella peruviana P7
Pseudallescheria boydii P8
Sordaria alcina P9
Preussia africana P10
Trichosporon asahii A1
Trichoderma atroviride A2
Penicillium concentricum A3
Neosartorya fisheri A4
Eurotium amstelodami A5
Eurotium amstelodami A6
Penicillium concentricum A7
Penicillium commune A8
Penicillium lanosum A9
Gelasinospora cratophora A10
Mariannaea camptospora A11
Preussia australis A12
Preussia africana A13
Phoma sp. A14
Phoma sp. A15
Trichosporon faecale A16
Preussia minima A17
Microdiplodia sp. A18
Coprinellus micaceus A19
Trichoderma reesei Rut-C30
Fungal isolate

Many of the fungi isolated from koala faeces exhibited xylanase, mannanase and endoglucanase activities in the agar plate assays comparable or higher than exhibited by the hypercellulolytic strain *Trichoderma reesei* RUT-C30 (Montenecourt and Eveleigh, 1979). As *T. reesei* is currently the principal source of cellulases and hemicellulases used in industry (Cianchetta et al., 2010), the results suggest that many of enzymes from the fungal isolates could also have potential for future development for industrial applications. It is notable that *Trichoderma atroviride* A2 from koala faeces displayed particularly high cellobiohydrolase activity (Fig. 3.16d), demonstrating the prowess of the *Trichoderma* genus for cellulose degradation (Montenecourt, 1983; Beguin, 1990; Section 1.9.2). *T. reesei* RUT-C30 is a low-protease strain (Sheir-Neiss and Montenecourt, 1984), and not known for its lipase, ligninolytic phenoloxidase (ligninase) or amylase activity, and was therefore ranked low in comparison to the fungi from koala faeces for these enzyme activities (Fig. 3.16).

A high number of fungal isolates from koala faeces exhibited ligninolytic phenoloxidase (ligninase) activity in the agar plate assays (Fig 3.16e). The activity displayed indicated degradation of the phenolic units of lignin (Section 3.4.8.1) and could have been caused by a range of lignin-degrading oxidases. Major oxidative enzymes such as manganese peroxidase, lignin peroxidase and laccase could have degraded both the phenolic and non-phenolic units of lignin (Shary et al., 2007; Section 1.8.5). Mild oxidants such as glucose-methanol-choline (GMC) oxidoreductases could have just degraded the phenolic units of lignin, and not fully degraded the lignin polymer (Section 1.8.5). The majority of fungal isolates testing positive in the lignin agar plate assay were ascomycetous fungi, which rarely display peroxidase activity (Baldrian, 2006). Therefore it seemed likely that the displayed phenol degradation by these isolates was due to the secretion of laccases or mild oxidants, more commonly secreted by ascomycetous species (Nsolomo et al., 2000; Torres et al., 2001; Liers et al., 2006). Further investigation into the lignin degrading abilities of a selection of fungal isolates from koala faeces was carried out later in this work using liquid cultures (Chapter 4).

The results of the agar plate assays provided some support for the nutritional theory of fungal succession (Section 1.4). The first fungus to appear on the koala faeces using the "faeces-in-agar" incubation technique (*Mucoraceae* sp. P1, EU551186 from the phylum Zygomycota; Table 3.1b; Table 1, Publication 1) exhibited protease activity but did not test positive for any of the other enzyme activities tested. In comparison, endoglucanase and ligninolytic phenoloxidase (ligninase) activities were exhibited only by the ascomycetous fungi and the single basidiomycetous fungus (*Coprinellus micaceus* A19, EU551214; Table 3.1c; Table 1, Publication 1), which were isolated later in the fungal succession. Many of the ascomycetous fungi also exhibited xylanase, mannanase and tannase activity. However, no trends relating enzyme activity to the order of fungal succession amongst the ascomycetous species isolated were apparent. Protease activity was exhibited by most of the fungal isolates, irrespective of their place in the fungal succession. It is generally believed that protein is rapidly removed from herbivore faeces early in its degradation (Ing, 1989). Therefore, it could be possible that the fungi use their secreted proteases to attack the other fungal and bacterial species that are present on the faeces throughout the degradation period.

The greatest enzyme activity of nearly all isolates occurred at 25 ºC, a temperature at which the fungi also grew rapidly. The only exception was a lipase produced by *M. camptospora* A11, EU551206 (Table 3.1c; Table 1, Publication 1), which exhibited maximum activity at 15 ºC (Fig. 3.16i; Fig. 1i, Publication 1). The xylanase, mannanase, endoglucanase, cellobiohydrolase, ligninase, tannase, protease, amylase and lipase activities displayed by *M. camptospora* A11 also had a higher degree of cold tolerance than those of other isolates from koala faeces, as did enzymes secreted by *F. oxysporum* C6, EU551183. The enzymes of *T. atroviride* A2, EU551197 and *G. cratophora* A10, EU551205 (Table 3.1; Table 1, Publication 1) displayed heat tolerance (Fig. 3.16; Fig. 1, Publication 1). Liquid culturing and enzyme assays using the supernatants from the fungal cultures allowed a more comprehensive analysis of the enzyme activities of selected fungal isolates later in this work (Chapter 4).

3.4.9 Basiodiomycetous yeasts identified from koala faeces

Four fungal isolates from koala faeces were identified as basidiomycetous yeast species, dimorphic fungi that exist predominantly in the yeast-like unicellular growth phase but also develop mycelia or pseudomycelia (elongated, incompletely separated cells; Fell et al., 2000). No further work was carried out on the yeast species because the filamentous fungi were of greater interest for their enzyme activities. However, some observations made of the yeast species are worthy of comment. *Sporobolomyces lactosus* C4 (EU551181, Table 1, Publication 1) was a salmon-pink coloured yeast of the order Sporidiobolales that was isolated on day twelve from koala faeces incubated in a moist chamber (Table 3.1a), and was observed in both a unicellular and pseudomycelial phase (Figure 3.17a). Although *S. lactosus* C4 did not exhibit any enzyme activity in the agar plate assays used in this work (Fig. 3.16a - i), the isolate could be of interest for its carotenoid pigments. Carotenoids of *Sporobolomyces* yeasts are used as natural food colourants (Johnson and Schroeder, 1996) and as nutraceuticals credited with reducing the risk of developing cancer, cardiovascular disease, cataracts and macular degeneration (Weber et al., 2007). *Sporobolomyces* yeasts have been isolated from a variety of habitats including plant leaves (Kinkel, 1997), human and animal skin (Boekhout, 1991) and wild pony dung (Davoli and Weber, 2002).

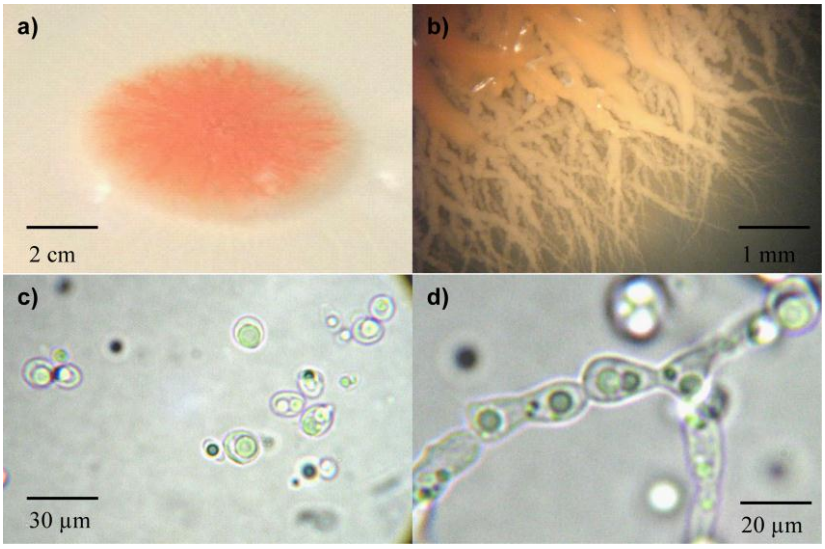


Figure 3.17. *Sporobolomyces lactosus* C4 (EU551181), a basidiomycetous yeast isolated from koala faeces. a) PDA plate culture; b) hyphal-like growth viewed under stereomicroscope; c) single yeast cells and d) pseudomycelia, viewed under a light microscope.

The other three basidiomycetous yeasts isolated from koala faeces were *Trichosporon* species of the order Tremellales (*Trichosporon* sp. P5, EU551190; *T. asahii* A1, EU551196; *T. faecale* A16, EU551211; Table 3.1b and 3.1c; Table 1, Publication 1) and were isolated from the "faeces-in-agar" and "ethanol-faeces-agar" plates. All displayed endoglucanase and ligninolytic phenoloxidase activity (Fig. 3.16c, e) and one (*T. faecale* A16, EU551211) also displayed xylanase and mannanase activity (Fig. 3.16a, b). Enzymes capable of degrading plant cell wall polymers are rarely reported in yeast, but *Trichosporon* is one of the few genera that can display endoglucanase, hemicellulase and phenoloxidase activities (Jiménez et al., 1991; Medvedeva et al., 1992; Middelhoven et al., 2001). *T. asahii* (EU551196) also exhibited antifungal properties against the filamentous fungi isolated from koala faeces (Fig. 3.17), which could be utilised by the yeast in the competition for nutrition on the faeces. Antifungal glycolipids have recently been isolated from a similar species *T. porosum* (Kulakovskaya et al., 2009).

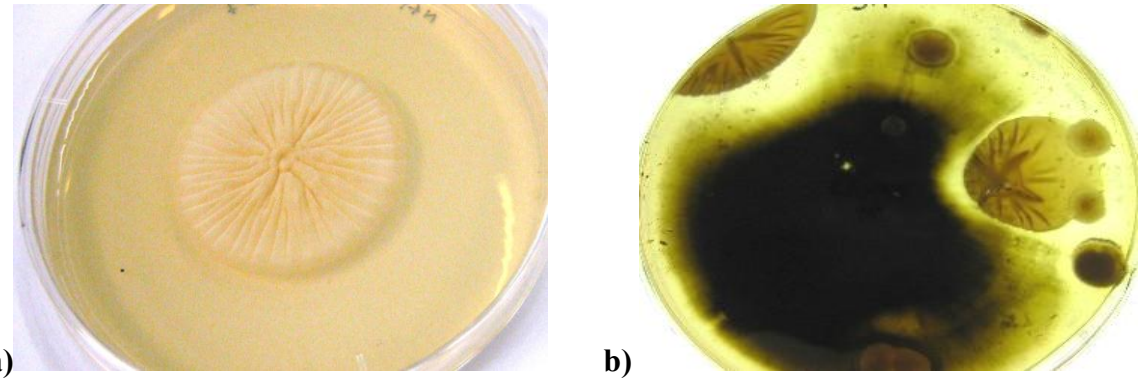


Figure 3.18. *Trichosporon asahii* A1 (EU551196), a basidiomycetous yeast isolated from koala faeces. a) pure culture on PDA plate; b) antifungal activity against *Gelasinospora cratophora* A10 (bottom view of PDA plate is shown).

No further investigation into any of the yeast species isolated from koala faeces was carried out during this work. However, the isolates have been stored for future research.

3.5 Selection of fungal isolates for further enzyme characterisation

The results presented in this chapter were used to select seven fungal isolates for further characterisation of their enzyme activity using liquid cultures, liquid enzyme assays and zymograms (Chapter 4). The fungi that were of greatest interest were those that secreted hydrolases capable of degrading plant cell wall polymers, which could have potential for application in various industrial processes such as production of paper, textiles or ethanol-based biofuel (Section 1.2). Furthermore, fungi that exhibited enzyme activity at moderately low (15 ºC) or moderately high (39 ºC) temperatures were of interest for the potential stability of their enzymes in manufacturing processes (Section 1.7.3). It was also preferable that fungi from different fungal orders, families and genera were represented in order to encompass a broad cross-section of the coprophilous fungal community from koala faeces. Using these criteria, the following seven isolates were chosen for further analysis:

Fusarium oxysporum C6 (EU551183)
Doratomyces stemonitis C8 (EU551185)
} isolated from koala faeces in moist chambers (Table 3.1a)

Sordaria superba P3 (EU551188)
Cylindrocladiella peruviana P7 (EU551192)
} isolated from "faeces-in-agar" plates (Table 3.1b)

Trichoderma atroviride A2 (EU551197)
Gelasinospora cratophora A10 (EU551215)
Mariannaea camptospora A11 (EU551216)
} isolated from "ethanol-faeces-agar" plates (Table 3.1c)

All of the selected fungi had displayed high enzyme activity in comparison to the other fungal strains on most of the agar plate assays, especially for the main plant cell wall degrading hydrolases that were of particular interest for this work (xylanases, mannanases, endoglucanases, cellobiohydrolases). The *F. oxysporum* C6 and *M. camptospora* A11 enzymes were of particular interest for their cold tolerance and *T. atroviride* A2 and *G.*

cratophora A10 enzymes for their heat tolerance (Section 3.4.8.3; Fig. 3.16). All selected fungi came from the phylum Ascomycota, but three different fungal orders (Hypocreales, Sordariales and Microascales), five different families (Hypocreaceae, Nectriaceae, Clavicipitaceae, Sordariaceae and Microascaceae) and seven different genera were represented (Fig. 3.19). The selected fungi were subsequently grown in liquid cultures and profiles of their enzyme activities were established using liquid enzyme assays and zymogram gels (Chapter 4).

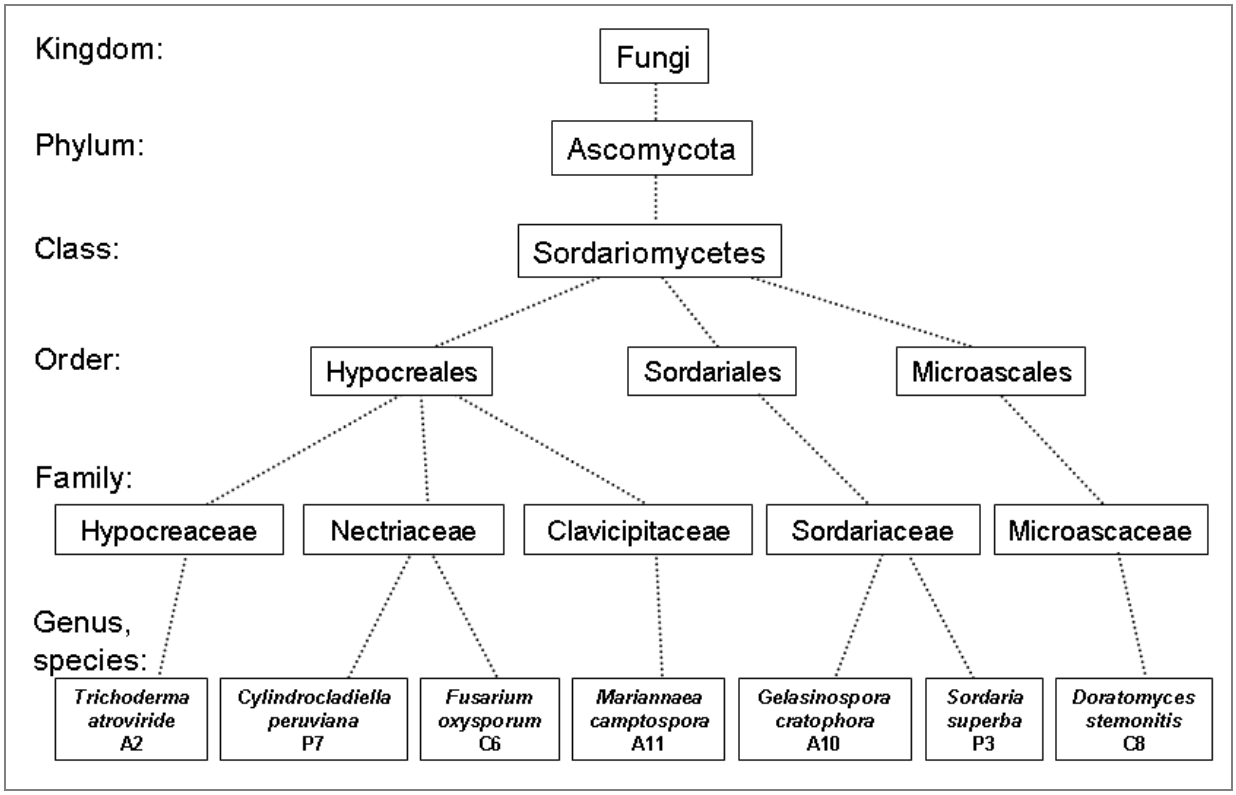


Figure 3.19: Phylogenetic lineage of the selected isolates from koala faeces chosen for cultivation in a liquid media for further enzyme characterisation (Chapter 4).

3.6 Summary

In the work presented in this chapter and Publication 1, a succession of coprophilous fungi was isolated and identified from koala faeces, identified using ITS sequencing, and screened for their enzyme activities. The work was the first of its kind, to our knowledge, in which a community of coprophilous fungi has been identified by molecular means, and enzyme

activities of Australian coprophilous fungi have been explored. The basic premise behind the work was that fungi that can grow on koala faeces would be likely to secrete enzymes to break down the plant cell wall polymers (Section 1.5). In accordance with this premise, many of the fungal isolates were, indeed, found to have high xylanase, mannanase and endoglucanase activities, several had high cellobiohydrolase activities and many isolates secreted enzymes capable of degrading the phenolic units of lignin. Protease, amylase and lipase activities were also displayed. The results of this work were used to select fungal isolates for further enzyme characterisation in liquid cultures (Chapter 4).