DIVERSITY OF POLYPORALES AND THE APPLICATION OF *GANODERMA AUSTRALE* (FR.) PAT. IN BIOPULPING OF EMPTY FRUIT BUNCHES OF *ELAEIS GUINEENSIS*

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ABSTRACT

Diversity and distribution of Polyporales in Malaysia was investigated by collecting basidiocarps from trunks, branches, exposed roots and soil from six states (Johore, Kedah, Kelantan, Negeri Sembilan, Pahang and Selangor) in Peninsular Malaysia and Federal Territory Kuala Lumpur. The morphological study of 99 basidiomata collected from 2006 till 2007 and 241 herbarium specimens collected from 2003 - 2005 were undertaken. Sixty species belonging to five families: Fomitopsidaceae, Ganodermataceae, Meruliaceae, Meripilaceae and Polyporaceae were recorded. Polyporaceae was the dominant family with 46 species identified. The common species encountered based on the number of basidiocarps collected were *Ganoderma australe* followed by *Lentinus squarrosulus*, *Earliella scabrosa*, *Pycnoporus sanguineus*, *Lentinus connatus*, *Microporus xanthopus*, *Trametes menziesii*, *Lenzites elegans*, *Lentinus sajor-caju* and *Microporus affinis*. Eighteen genera with only one specie were also recorded i.e. *Daedalea, Amauroderma, Flavodon, Earliella, Echinochaetae, Favolus, Flabellophora, Fomitella, Funalia, Hexagonia, Lignosus, Macrohyporia, Microporellus, Nigroporus, Panus, Perenniporia, Pseudofavolus* and *Pyrofomes.* This study shows that strains of the *G. lucidum* and *G. australe* can be identified by 650 base pair nucleic acid sequence characters from ITS1, 5.8S rDNA and ITS2 region on the ribosomal DNA. The phylogenetic analysis used maximum-parsimony as the optimality criterion and heuristic searches used 100 replicates of random addition sequences with tree-bisection-reconnection (TBR) branch-swaping. ITS phylogeny confirms that *G. lucidum* and *G. australe* were named correctly based on the molecular analysis even though the strains exhibited differences in morphological characteristics. Thirty-seven selected cultures of Polyporales were qualitatively assayed for the production of amylases, cellulases, laccases and lignin peroxidases after three to seven days incubation at 25±2°C. Two strains - *Ganoderma australe* KUM60848 and *Favolus*

tenuiculus KUM60803 demonstrated good enzymes production and were selected to undergo solid substrate fermentation of oil palm empty fruit bunches (EFB). The study was conducted to analyse the enzymatic activity (U/ml) of cellulase, amylase, laccase, lignin peroxidase, xylanase and β-D-glucosidase. *Ganoderma australe* showed the highest enzymes activity on the 14 and 21 days of incubation compared to *F. tenuiculus* and was selected as potential candidate for biopulping of oil palm (*Elaeis guineensis*) empty fruit bunches. The property of pulp produced by oil palm empty fruit bunches through solid substrate fermentation with *G. australe* KUM60848 were then analysed at 14 and 21 days of incubation. The empty fruit bunches was pulped by applying soda pulping process. The result showed that the pulping process influenced the pulping properties. Pre-treatment by *G. australe* for 14 days produced the lowest degree of material dissolved while pre-treatment at 21 days had the highest degree of material dissolved as indicated by the pulp yields. Compared to control, the biopulping yield using *G. australe* had increased to a maximum of 18%. The pulping process also influenced the paper properties i.e. all zero-span tensile indices of pulp were lower than control (conventional pulping), while the fibre strength decreased by 11% and 6% at day 14 and 21 respectively. In conclusion, the 14 days of solid substrate fermentation by *G. australe* performs better pulp and paper properties than 21 days in biopulping of EFB.

ABSTRAK

Kepelbagaian dan taburan Polyporales di semenanjung Malaysia telah dikaji dengan membuat koleksi jana buah yang terdapat pada batang-batang kayu, dahan, akar banir serta yang tumbuh di permukaan tanah di enam buah negeri iaitu Johor, Kedah, Kelantan, Negeri Sembilan, Pahang dan Selangor, termasuk di Wilayah Persekutuan Kuala Lumpur. Kajian morfologi telah dijalankan pada 99 jana buah yang dikutip dalam tahun 2006 hingga 2007 dan 241 spesimen herbarium yang telah dikutip dari 2003 hingga 2005. Enam puluh spesis daripada lima famili iaitu Fomitopsidaceae, Ganodermataceae, Meruliaceae, Meripilaceae dan Polyporaceae telah dikenal pasti. Polyporaceae merupakan famili yang dominan dengan 46 spesies telah direkod. Antara spesies yang biasa dijumpai berdasarkan bilangan jana buah yang dikutip ialah *Ganoderma australe* diikuti oleh *Lentinus squarrosulus, Earliella scabrosa, Pycnoporus sanguineus, Lentinus connatus, Microporus xanthopus, Trametes menziesii, Lenzites elegans, Lentinus sajor-caju* dan *Microporus affinis*. Lapan belas genera dengan hanya satu spesis juga telah direkodkan iaitu *Daedalea, Amauroderma, Flavodon, Earliella, Echinochaetae, Favolus, Flabellophora, Fomitella, Funalia, Hexagonia, Lignosus, Macrohyporia, Microporellus, Nigroporus, Panus, Perenniporia, Pseudofavolus* dan *Pyrofomes*. Kajian ini juga menunjukkan bahawa strain *G. lucidum* dan *G. australe* berjaya dikenal pasti dengan menggunakan 650 pasangan asas aksara urutan nukleik asid daripada ITS1, 5.8S rDNA dan ITS2 rantau DNA ribosomal. Analisis filogenetik telah menggunakan maksimum kekikiran sebagai kriteria optimal. Filogeni ITS mengesahkan bahawa *G. lucidum* dan *G. australe* telah dinamakan dengan betul berdasarkan analisis molekul walaupun strain mempamerkan perbezaan dalam ciri-ciri morfologi. Tiga puluh tujuh kultur Polyporales telah dipilih bagi saringan secara kualitatif bagi penghasilan enzim amilase, sellulase, lakase dan lignin peroksidase selepas tiga hingga tujuh hari pengeraman pada 25±2°C. Dua strain iaitu *Ganoderma*

australe KUM60848 dan *Favolus tenuiculus* KUM60803 telah menunjukkan penghasilan enzim yang baik dan dipilih untuk kajian penapaian bentuk substrat dengan menggunakan tandan kosong buah kelapa sawit. Kajian ini dijalankan untuk menganalisis aktiviti (U/ml) bagi enzim sellulase, amilase, lakase, peroksidase lignin, xilanase dan β-D-glukosidase. *Ganoderma australe* menunjukkan aktiviti enzim tertinggi pada hari ke 14 dan 21 pengeraman berbanding *Favolus tenuiculus* dan telah dipilih untuk proses penghasilan pulpa secara biopulpa. Pulpa yang dihasilkan oleh tandan buah kosong kelapa sawit (*Elaeis guineensis*) melalui penapaian substrat pepejal dengan *G. australe* KUM60848 dianalisis pada hari ke 14 dan 21 tempoh pengeraman. Penghasilan pulpa daripada tandan buah kosong telah dilakukan secara pemprosesan pulpa soda. Pengeraman selama 14 hari oleh *G. australe* mempunyai jumlah terendah bagi bahan terlarut manakala pengeraman selama 21 hari mempunyai jumlah yang tertinggi. Berbanding dengan kawalan, hasil biopulpa telah meningkat kepada maksimum 18%. Proses pulpa juga telah mempengaruhi sifat-sifat kertas. Dalam kajian ini, indeks tegangan semua span-sifar pulpa adalah lebih rendah daripada kawalan (pulpa secara konvensional), manakala kekuatan gentian menurun sebanyak 11% pada hari ke 14 dan 6% pada hari ke 21. Kesimpulannya, penapaian substrat pepejal selama 14 hari oleh *G. australe* menunjukkan hasil yang lebih baik daripada 21 hari melalui proses biopulpa tandan buah kosong kelapa sawit.

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LIST OF ABBREVIATIONS

cm	centimeter
DNA	Deoxyribonucleic acid
Fig.	Figure
g	gram
KOH	Potassium hydroxide
L	Liter
mg	milligram
mm	millimeter
µg	microgram
µm	micrometer
α	alpha
β	beta
%	percentage

GLOSSARY OF MYCOLOGICAL TERMS

Aculei	Having narrow spines.
Allantoids	Slightly curved with rounded ends; sausage-like in form.
Agglunated	Fixed together as if with glue.
Applanate	Flattened.
Basidiocarp	Fruit body.
Basidiospores	A propagative cell.
Basidium	The cell or organ, diagnostic for basidiomycetes, from which, after karyogamy and meiosis, basidiospores (generally 4) are produced externally each on an extension (sterigma) of its wall.
Catahymenium	A hymenium in which hypidia are the first-formed elements and the basidia embedded at various levels elongated to reach the surface and do not form a palisade.
Coralloid	Much branched; like coral form.
Coriaceous	Like leather in texture.
Cystidia	A sterile body, frequently of distinctive shape, occurring at any surface of a basidioma, particularly the hymenium from which is frequrntly projects. Cystidia have been classified and name according to their origin, position and form.
Deccurent	(of lamella), running down the stipe.
Dendrohyphida	Irregularly strongly branched.
Dimidiate	Shield-like; appearing to lack one half, without a stalk and semi-circular.
Effused-reflexed	Stretched out over the substratum but turned up at the edge to make a pileus.
Ellipsoid	Elliptical (oval) in optical section.
Endospore	The inner wall of a spore.
Flabelliform	Like a fan, in the form of half-circle.
Glabrous	Smooth, not hairy.
Globose	Spherical or almost so.
Gregarious	In companies or groups but not joined together.
Hirsute	Having long hairs.

Hispid	Having hairs or bristles.
Hymenophore	A spore bearing structure.
Hymenium	A spore-bearing layer of a fruit body.
Hyphae (pl. hyphal)	One of the filaments of a mycelium.
Imbricate	Partly covering one another like the tiles on a roof.
Lamellae	One of the characteristic hymenium-covered plates on the underside of the pileus; gill.
Lamellate	Having lamellae.
Lunate	Like a new moon; crescentic.
Perennial	Living for a number of years.
Pileus	The hymenium-supporting part of the basidioma of non-resupinate.
Resupinate	Flat on the substrate with hymenium on the outer side.
Rhizomorph	A root-like aggregation of hyphae having a well-defined apical meristem (cf. mycelia cord) and frequently differentiates into a rind of small dark-coloured cells surrounding a central core of elongated colourless cells.
Sessile	Having no stem.
Stipe	A stalk.
Striate	Marked with delicate lines, grooves or ridges.
Strigose	Rough with sharp-pointed hispid.
Subglobose	Not quite spherical.
Tomentose	Having a covering of soft, matted hairs (a tomentum); downy.
Trama	The layer of hyphae in the central part of a lamella of an agaric, a spine of Hydnaceae, or the dissepiments between pores in a polypore.
Velutinate	Thickly covered with delicate hairs, like velvet.
Ventricose	Swelling out in the middle or at one side; inflated.

CHAPTER 1

GENERAL INTRODUCTION

Peninsular Malaysia located approximately between 6° 45´ and 1° 20´ N latitude and 99° 40´ and 104° 20´ E longitudes comprising eleven states and the Federal Territory of Kuala Lumpur and Putrajaya. Topographically, Peninsular Malaysia is characterized by extensive coastal plains in the east and west, hilly and mountainous region with steep slopes in the central and undulating terrain in other parts of the peninsula. The forests of Peninsular Malaysia have been variously classified according to their ecological and physical conditions, but for the purposes of management they can be classified broadly into the Dipterocarp, Freshwater Swamp and Mangrove forests (Hooi, 1987). The dipterocarp forest occurs on dry land just above sea level to an altitude of about 900 metres. The forests in Malaysia are mostly dominated by trees from the Dipterocarpaceae family.

1.1 Diversity of Polyporales

Fungi play numerous key functional roles in forest ecosystems ranging from saprotrophs and pathogens of plants and animals through to symbionts of phototrophic organisms such as those in lichens and mycorrhiza (Dix and Webster, 1995). Fungi are also an extremely taxonomically diverse group of organisms with the estimates of fungal diversity based on the perception that many species are yet to be discovered vary widely with the most commonly cited estimate of 1.5 million (Hawksworth, 1991). For tropical forest systems in particular, it is clear that the current number of described fungal species is only a small fraction of the number of species that exist (Rossman, 1994). Recently, Meuller *et al.,* (2007) estimated the species of macrofungi in tropical Asia to be in the range between 10,000 and 25,000.

Some of Polyporales are reported to be edible as food and ethnomedicine. For example, *Lentinus squarrosulus* Mont. (Syn. *Lentinus subnudus* Berk.) is a highly prized Nigerian mushroom, which is appreciated for its meaty taste and texture (Kadiri, 2005). Moreover, the *L. squarrosulus* fruit bodies are rich in ascorbic acid and amino acids, and protein in their most abundant nutrient (Fasidi and Kadiri, 1990). Apart from the use as food items, the Polyporales are also commercially cultivated for medicinal purposes especially in traditional Chinese medicine i.e. *Ganoderma lucidum* (ling-zhi), *Grifola frondosa* (maitake) and *Trametes versicolor* (yun-zhi).

On the other hand, some species of Polyporales can also act as mild to severe pathogens of living forest trees or in plantations. For example, many species of *Ganoderma* have been reported to be pathogenic on oil palm (*Elaeis guineensis*) in different countries (Turner, 1981). In Malaysia, the major pathogen on oil palm has been identified as *G. boninense* (Ho and Nawawi, 1985). Besides acting as pathogens, many of the Polyporales are also commonly considered as harmful organisms that cause economic losses of the wood. For example, Ryvarden (1992) reported that termites have close relationship with wood-rotting fungi; especially Polyporales. In many cases, the termites were strongly attracted to wood attacked by Polyporales, especially brown rot fungi.

Despite their importance within ecosystems, fungi are often overlooked. As a result, the taxonomy and diversity of fungi are very poorly known compared with the majority of the other organism presents in forest ecosystem (May and Simpson, 1997). In Malaysia for example, 70 – 80 percent of fungi are yet to be discovered (Lee *et al.,* 1995; Corner, 1996). Therefore, only 20 – 30 percent of the estimated total number of species has been taxonomically described, and those that are named are often represented by only a few collections. This implies that vast numbers of fungi may

become extinct together with their host before can be identified (Hawksworth, 1991). If the fungi become extinct, we can no longer make use of their biotechnological properties. It is therefore urgent for us to search for those undiscovered fungi in order to make conservative estimation for them. Exploring new areas and new substrates especially those that need special adaptation is a way to do so (Hyde, 2001).

Thus, in order to understand the diversity of fungi, the knowledge of their distribution and association with all organic and inorganic substrates are essential. The substrates such as dead and decaying wood and its associated fungi and invertebrates are vital elements of the forest ecosystem and their decay processes represent a key path for nutrient and carbon recycling (Bobiec *et al.,* 2005). As dead wood undergoes physical and chemical changes during the decomposition process, a wide variety of different niches are created. All these different niches are colonized by a variety of different species with most of them specialized to this precise and often narrow niche. Consequently, a successional pathway is established, where firstly colonizing pioneer species precede subsequently arriving later stage species. This succession is often very strict, i.e. most fungal species are only adapted to a short section of this succession. Therefore it may be possible to predict the stage of decomposition of a log by looking at the accompanying decomposition flora. In the decomposition process the latter succession stages are especially rich in fungal species (Niemelä *et al.,* 1995).

Soon after the death of a branch or log, the first colonizers arrive and begin to decompose the wood. These so called pioneer species are often fast growing and occupy the substrate rapidly. When the easily decomposable components of the wood are consumed, the pioneer species are replaced by subsequent other species, better adapted to the changed substrate. These successors are rather specialized in degrading more complex components of the wood (Rayner and Boddy, 1988). On large logs in natural

ecosystems, this decomposition process may continue for years and consist of several successional steps. Especially the later stages in the decomposition process harbor a species rich fungal community (Niemelä *et al.,* 1995; Renvall 1995). By understanding the diversity of the fungi species that grow on their host, the collection will be conducted to record and identify accordingly. In fact, a complete knowledge of the fungi for any locality would require continuos observation and collection over many years (Pegler, 1997).

The taxonomy of fungi has traditionally been based on on the morphological features of the basidiocarps. Identification based on these basidiocarps features, however, is prone to problems such as absence of basidiocarp during certain time of the year, their morphological plasticity and presence of cryptic species (Moncalvo and Ryvarden, 1997; Gottlieb and Wright, 1999). For these reasons, contemporary taxonomy and identification of fungal species employ morphological studies and DNA sequence information.

1.2 Molecular study of Polyporales

In the past, fungi and other microbes have been assigned to taxonomic groupings using a range of morphological and physiological properties such as growth on certain media and pigmentation (Lardner *et al.,* 1999), and resting spores structures (Braselton, 1995). Biochemical properties and cellular ultra structure (Braselton, 1992) have also been utilized. In many cases, such properties have proven extremely reliable in classifying organisms. However, potentially the most powerful raw information is the DNA sequences, where similarities and differences in sequences can be correlated with different taxonomic groupings, or even with individual isolates (Graeme, 2002).

Currently the taxonomy of polypores is primarily based on morphological characteristics, such as the shapes of basidiocarps and hymenophores, hyphal systems, and forms and sizes of basidiospores, and secondarily on mycological features like host relationship and rot types (brown versus white) (Donk, 1964; Ryvarden, 1991). However, overlapping and variable morphological characteristics have made the classification of polypores unreliable and unstable, which has been always a nuisance to mycologists (Alexopoulos *et al.,* 1996; Hibbett and Donoghue, 1995).

Therefore, in this study the use of morphological characteristics are correlated with the molecular approach in order to identify specimens to species level. This is essential because more or less frequently a new species are discovered although some of the identified species are considered identical with previous recognized species. Moreover, the determination of a species is difficult and sometimes rather tricky because of the morphological similarities and possible environmental effects. Thus, it is important not to identify a specie solely using one approach in order to provide more reliable taxonomic justification.

1.3 Applications of white-rot fungal enzymes

Fungi are well known for their capability to colonize on a wide range of living or dead tissue including plants, wood and paper products, leaf litter, plant residues from agriculture, soils and composts, and various living or dead animal tissues. Some of these fungi are highly valued by biotechnologists because of their wood-degrading (especially lignin degrading) abilities.

White-rot fungi, in class Basidiomycetes, degrade both lignin and polysaccharides from wood (Cowling, 1961; Kirk and Highley, 1973). A distinction has been made between white-rot fungi that simultaneously remove lignin, cellulose and

hemicelluloses, and those that successively decompose cell wall components; starting with preferential lignin and hemicelluloses degradation followed by cellulose removal at a later stage (Liese, 1970). Fungi with the capacity to remove lignin from wood without concomitant loss of cellulose are of interest in bioconversion processes such as microbial pulping, conversion of forest and agricultural residues to animal feed, and releasing sugars for ethanol production (Kirk and Chang, 1981). White-rot fungi are, therefore at the moment of great interest for biological pulping and bleaching (Wall *et al.,* 1993).

The discovery of ligninase (lignin peroxidase) from *P. chrysosporium* triggered research on biodegradation of lignin (Tuor *et al.,* 1995). White-rot fungi such as *Trametes versicolor* and *Phanerochaete chrysosporium* are known producers of lignolytic enzymes that are involved in the natural delignification of wood (Call and Mücke, 1997; Poppius-Levlin *et al.,* 1997). The perception of lignin degradation was changed from an oxidative depolymerisation process caused by a single enzyme, to a process of intensive oxidative and reductive conversions in which different classes of enzymes can participate (Tuor *et al.,* 1995). Many efforts have been made to investigate the application of these fungi for the removal of lignin in the pulping and bleaching process. It was first reported by Kirk and Yang (1979) that *P. chrysosporium* was able to partially delignify unbleached Kraft pulp.

For pulp and paper industry, the extracellular enzyme is marketed for effluent control and increases the strength properties of lignin containing paper products. Additionally, one of the most studied application of the enzyme is the laccase-mediator bleaching of Kraft pulp (Call and Mücke, 1997), in which the efficiency has been proven in mill-scale trials (Paice *et al.,* 2002). Laccase could also be used to activate mechanical pulp fibers and subsequently graft different chemicals into the fibers to

achieve functionality into the fibers (Chandra and Ragauskas, 2002). Compared to other pre-treatment alternatives, the fungal treatment requires a long treatment time but the energy requirement of the process is low and the treatment conditions are mild (Sun and Cheng, 2002).

The aim of this study was to document the diversity and distribution of Polyporales in Peninsular Malaysia and to select strains of white-rot fungi for the production of enzymes for biopulping. Initial work focused on the collecting and identifying the Polyporales to the species level (Chapter 3). Further selection of strains to be used for the biopulping process of oil palm empty fruit bunches was based on the lignocellulolytic enzymes produced by the selected strains during solid substrate fermentation (Chapter 4).

1.4 Objectives

The objectives of the study were to:

a. document the diversity and distribution of Polyporales
b. infer phylogenetic relationship between the species of *Ganoderma* using molecular data
c. to acquire pure cultures of Polyporales to obtain candidates species for biopulping
d. profile qualitatively the production of cellulolytic and ligninolytic enzymes from Polyporales cultures
e. study the cellulolytic and ligninolytic enzymes activity of selected strains during SSF of oil palm empty fruit bunches (EFB)
f. determine the effect of pre-treatment of EFB by selected strain on pulp yield, alpha cellulose content and strength

CHAPTER 2

LITERATURE REVIEW

The Polyporales refer to all fungi with a poroid hymenophore except the members of Boletales and few fleshy members of Agaricales such as *Favolaschia* sp., *Poromycena* sp., and similar genera (Ryvarden, 1992). According to Harry (1986), the basidiocarps of the Polyporales are often membranous, leathery, corky or even woody in texture. As important decomposers of wood, they have elevated themselves on the trunks, exposed roots, branches or twigs, whilst others will only grow on the wood of dead trees and on the soil (Pegler, 1997). The basidiocarp normally functions for longer period than most agarics. As a result, these fungi can be found at any time of the year and some of them are able to survive for several years, producing a new layer of tubes each year.

2.1 History of taxonomic studies of Polyporales in Malaysia

In Malaysia, the history of taxonomic studies of polypores started in the 19th and early 20th century. Cooke (1883, 1884, 1885a, 1885b), was the first mycologist who recorded various species of polypores from the Malay Peninsula. Chipp (1921), reported about 102 polypore species from the Malay Peninsula while Corner (1935), had studied the occurrence and the seasonal occurrence of fungi in the Malay Peninsula and Singapore. Lim (1972), stated that basidiomycetes which frequently found in Malaysia and Singapore were polyporous fungi and recorded the common large fungi such as *Amauroderma* spp., *Ganoderma* spp., *Pycnoporus sanguineus* and *Microporus xanthopus*. Later, Oldridge *et al.,* (1985), recorded eight species of Polyporales collected from Pahang and Negeri Sembilan i.e. *Daedalea flavida, Lenzites elegans, Rigidoporus defibulatus, Microporus affinis, M. xanthopus, M. luteoceraceus, Pycnoporus sanguineus* and *Fomitopsis feei*. Additionally, Kuthubutheen (1981), reported 17 species of Polyporales while Noorlidah *et al.,* (2005) have documented 71

genera of Basidiomycotina belonging to eight orders and 25 families in Langkawi. Noorlidah *et al.,* (2007), also reported the diversity of fungi in Endau Rompin National Park, Johor which primarily include the orders Polyporales and Agaricales. Lee *et al.,* (1995), and Salmiah and Thillainathan (1998), reported the common macrofungi in Malaysia i.e. *Pycnoporus sanguineus*, *Schizophyllum* sp., *Microporus* spp. and *Lentinus* spp. Furthermore, a study on the species diversity and the frequency of the wood-inhabiting fungi from various forest reserves and plantation forests in Peninsular Malaysia were documented by Salmiah and Jones (2001). Recently, the diversity of Polyporales has been reported by Noraswati *et al.,* (2006); Hattori *et al.,* (2007); Sumaiyah *et al.,* (2007), and Noorlidah *et al.,* (2009).

2.2 Morphological taxonomy of Polyporales

Polyporales can take various forms of fruiting body. They may be pileate; having a pileus or distinguishable cap. Some may be stipitate (having a stalk), resupinate (effused), or lying flat on the substrate. Some may be effused-reflexed, which mean they lie on a flat (i.e. parallel to the ground) substrate, but form shelves where the substrate surface is not parallel to the ground (Volk, 2000).

For the identification purposes, morphological data are important for classification of species (Raper and Fennell, 1965). There are several characteristics that can be used to identify the Polyporales with the common characteristics that usually used are the form of fruiting body, form of the hymenophore, hyphal system, type of cystidia and spores characteristics.

Most Polyporales have pores, small holes on the underside of the fruiting body that increase the surface area for bearing basidia with their spores (Ryvarden, 1992). However, some genera have enlarged pores that may be maze-like or gill-like. Some

may even become hydnoid; with downward pointing teeth or spines. The form of the hymenophore may even change depending on which side of the substrate the fungus is fruiting, especially if the substrate suddenly changes to be perpendicular to the ground. Furthermore, a few agarics, mainly in the genus *Lentinus* that have decurrent lamellae have been classified under the Polyporaceae. It has been placed under the Polyporaceae because of the presence of dimitic and amphimitic hyphal systems in both the Polyporaceae and *Lentinus* (Moser, 1978; Kühner, 1980; Pegler, 1983; Singer, 1986).

The Polyporales has developed hyphae which are thick-walled together with other specialized hyphae, which are highly branched and able to bind the individual hyphae together into a strong tissue. The septation of the generative hyphae is accepted as a basic character for generic delimitation in the polypores (Ryvarden, 1992). Some Polyporales are very soft and last for only one season, while others are very hard and often perennial. This is usually a direct result of the hyphal type found within the polypore fruiting body. The hyphal system of the Polyporales can be monomitic, dimitic, or trimitic (Volk, 2000).

Monomitic species have only septate generative hyphae, which are responsible for growth and transport of food and other materials through the fruiting body. These may be thin-walled or thick-walled, clamped or unclamped. Most of these species have fruiting bodies that are soft. For example, the basidiomycete genus *Ceriporiopsis* (Hapalopilaceae, Polyporales) was established for a small group of resupinate polypore species with the monomitic hyphal structure.

Dimitic-skeletal species have septate generative hyphae and thick-walled non-septate skeletal hyphae, which provide the hard structure found in many polypores for example, *Ganoderma applanatum* (Volk, 2000). The dimitic-binding species also have

septate generative hyphae and thin often-branching binding hyphae, which are responsible for holding the other hyphae together.

Trimitic species have septate generative hyphae and thick-walled, non-septate skeletal hyphae and thin often branching binding hyphae. The basidiomycete genus *Trametes* (Polyporaceae, Polyporales) usually characterized with the trimitic hyphal structure.

Another important character for fungal identification is the spores. Ryvarden (1992) reported that spore size and shape are important characters. There are three common shapes that are found among Polyporales in the tropics; globose, ellipsoid and cylindrical. The ellipsoid and globose spores are common in tropical and it tends to have larger size compared to the temperate species.

Cystidia are actually found in very few genera of poroids, but when present they are a diagnostic feature (Volk, 2000). Some characteristics to look for are the shape, size, thickness, and any crystals that are found at or near the ends of the hymenium (for example between clusters of basidia).

Besides all the characteristics that have been described, pure culture studies have also been used in the identification of fungi species. Mildred (1958) used the cultural characters to which he attach taxonomic significance were; presence or absence of extracellular oxidase; the type of inter-fertility (bipolar or tetrapolar) in heterothallic species; hyphal characters, including septation and types of differentiation in form and colour; the presence or absence of chlamydospores and oidia; the colour of mycelial mats; and changes in the colour of the agar substrate.

The identification of Polyporales sometime required recent technological approach since it is important to be able to distinguish the genera and species

accordingly. Moreover, the proper identification and knowledge of relationship between taxa is the key to further study of ecological, pathological, genetic physiological and biotechnological aspect of these fungi (Volk, 2000). The study presented in this study deal with the identification of *Ganoderma* species. The *Ganoderma* was selected because the taxonomy of the genus is considered to be disarray (Ryvarden, 1994).

2.3 *Ganoderma* spp.

The genus *Ganoderma*, a member of Aphyllophorales, was established by Kartsen in 1881 and composed of over 250 species (Corner, 1983). *Ganoderma* is a cosmopolitan with worldwide geographical distribution and broad host range including hardwoods, conifers, bamboos and palms. The fruit body of *Ganoderma*, for its perceived health benefits, has gained wide popular use as a dietary supplement in China, Japan, North America and the other regions of the world, including Malaysia. *Ganoderma* species are also used in folk medicine to cure various diseases, and strains are commercially cultivated for the preparation of health tablets or drinks. As a kind of health food, it has also been used to prevent and treat immunological diseases, such as hypertension, tumorigenesis, etc. (Liu *et al.,* 2002). The many medicinal benefits of *Ganoderma* were reviewed by Jong and Birmingham (1992). On the other hand, some *Ganoderma* species play an important role in plant pathogens. Several species cause severe diseases in plantations or in forests (Steyaert, 1967; Bakshi *et al.,* 1976). However, some of them have been shown to selectively delignify wood and are recognized as a potentially important source of lignin-degrading enzymes (Otjen and Blanchette, 1987).

Members of the *Ganoderma* were traditionally considered difficult to classify because of the lack of reliable morphological characteristics. Thus, based on the structure of pilear crust, genus *Ganoderma* is divided into subgenus *Elfvingia* (non-

laccate species) and subgenus *Ganoderma* (laccate-containing species) (Corner, 1983). Ganodermataceae contains four genera: *Ganoderma, Amauroderma, Haddowia* and *Humphreya*. *Ganoderma* consists of subgenus *Ganoderma* that includes Sect. *Ganoderma* and Sect. *Phaenema*, subgenus *Eflvingia* and subgenus *Trachyderma* (Zhao and Zhang, 2000).

Over 250 *Ganoderma* species have been described worldwide, and most of them are from the tropic (Moncalvo, 1995a). Formerly, the traditional taxonomy of *Ganoderma* is based on its morphological traits. As a consequence, there are many synonyms and several species complexes have been recognized. The macroscopic (such as pileus, stipe, context, tube) and microscopic (such as hyphal system, basidiospore) characters have been used to distinguish species within the genus *Ganoderma*. However, characters such as basidiocarp shape, basidiospore size and context colour are influenced by environmental factors (Steyaert, 1975; Chen, 1993). Therefore, there are overabundance of synonyms, and the widespread misuse of names.

Along with the morphological traits of fruit bodies, additional taxonomic characters have been investigated for systematic of *Ganoderma*. Cultural studies were conducted by Adaskaveg and Gilbertson (1986), and Wang and Hua (1991). Intercompatibility studies have been reported in the *G. lucidum* complex by Adaskaveg and Gilbertson (1986); and in the *G. applanatum* group by Yeh (1990). These methods produced new characters for studies at the species level, but their use was not investigated at higher taxonomics levels (Moncalvo, 1995a). Until recently, phylogenetic analysis using DNA sequence information has helped to clarify the understanding of the relationship amongst *Ganoderma* species.

2.4 Phylogenetic study of Ganodermataceae

Despite the importance of *Ganoderma*, those species identification and circumscription were often unclear and taxonomic segregation of the genus remained controversial (Moncalvo *et al.,* 1995a), and even a number of *Ganoderma* isolates have been misnamed (Smith and Sivasithamparam, 2000).

In addition to morphological data, a variety of laboratory-based techniques have been used to study genetic diversity in *Ganoderma*, such as isozyme analysis (Lan *et al.,* 1998), Random Amplified Polymorphism DNA (RAPD) (Wang *et al.,* 2003), Amplified Fragment Length Polypmorphism (AFLP) fingerprinting (Qi *et al.,* 2003), Internal Transcribed Spacers (ITS) 25S ribosomal DNA sequencing technique (Moncalvo *et al.,* 1995a) and PCR-RFLP (Park and Ryu, 1996). Sequence characterizations of ribosomal RNA have led to great burgeoning of molecular phylogeny (Hibbett, 1992; Olsen and Woese, 1993). In this study, the sequence characterization of ribosomal RNA has been employed to verify the systematic of the genus *Ganoderma*. Ribosomal genes were chosen because they form a mosaic pattern of conserved and variable regions which makes them attractive for taxonomic investigation at many levels (Bruns *et al.,* 1991; Hibbett, 1992). Additionally, the ribosomal RNA genes (rDNA) typically exist as a tandem repeat that includes coding regions, which are conserved to varying degrees, as well as highly divergent regions (Inglis and Tigano, 2006). Therefore, comparative analysis of ribosomal RNA (rDNA) gene sequence information can be used to clarify natural evolutionary relationship over a wide taxonomic range (Pace *et al.,* 1986). The rDNA repeat of the fungi contains coding (functional) regions for 5.8S, 18S and 25S rRNAs along with internal transcribed spacer (ITS) regions (Restrepo and Barbour, 1989).

The ITS spacer regions, or internal transcribed spacer sequences (ITS), have been widely used in fungal systematics (Bowman *et al.,* 1992; Hibbett, 1992; Driver *et al.,* 2000). The intergenic regions, ITSs, were more variable than the coding regions for all fungi (Bruns *et al.,* 1991). The ITS located between the small (18S) and the large (28S) ribosomal subunits genes showed different variability at intra-specific level. The ITS regions have been applied for fungal systematic, including *Leptosphaeria* (Xue *et al.,* 1992), *Phytopthora* (Lee and Taylor, 1992), *Sclerotiniaceae* (Carbone and Kohn, 1993), rusts (Zambino and Szabo, 1993), *Talaromyces* and *Penicillium* (LoBuglio *et al.,* 1993), and *Ganoderma* (Moncalvo *et al.,* 1995b).

With such clear identification of any fungal species whether by morphologically or molecular approach will lead to the exploring the potential benefit for people especially in biotechnology. For example, if the identification of a specie which is valuable for biotechnology, then any closely related species might be investigated for further usefulness. In this study, some of these fungi are highly valued because of their wood-degrading abilities through the production of extracellular ligninocellulolytic enzymes.

2.5 Enzyme production by Polyporales

Different organism can deteriorate wood, but the greatest damage is caused by fungi. Fungi are well known for their ability to colonize a wide range of living or dead tissues most are from lignocelluloses substrates including plants, wood and paper products, leaf litter, plant residues from agriculture, soils and composts (Cooke and Whipps, 1993). Lignocellulose is a heteropolymer consisting mainly of three components, cellulose, hemicellulose and lignin (Fengel and Wegener, 1989; Eaton and Hale, 1993). The characteristics of these components are summarised, with the major enzymes responsible for their degradation in Table 2.1 (Pointing, 1999).

Table 2.1 The major components of lignocelluloses and fungal enzymes involve in their degradation (Pointing, 1999).

	Cellulose	**Hemicellulose**	**Lignin**
% of wood mass	40-50	25-40	20-35
Monomer	D-anhydroglucopyranose	Xylose Mannose plus other pentoses and hexoses	Coniferyl alcohol *p*-coumaryl alcohol sinapyl alcohol
Polymeric structure	β1-O-4 linked linear chains	β1-*O*-4 linked linear chains, with substituted side chains	Dehyrogenative polymerization to an amorphous polymer
Major enzymes involved in degradation	Endoglucanase (E.C. 3.2.1.4) Cellobiohydrolase (E.C. 3.2.1.91) β-glucosidases (E.C. 3.2.1.21)	Endoxylanase β-xylosidase (and other hydrolases)	Lignin peroxidase (E.C. 1.11.1.7) Mn dependent peroxidase (E.C. 1.11.1.7) Laccase (E.C. 1.10.3.2)

All fungi are heterotrophic for carbon compounds and many are heterotrophic for other materials as well, e.g. vitamins (Burnett, 1968). Within the host, the wood-rot fungi produce various enzymes to breakdown cell walls and mineralize the components in wood. In order to grow, fungi need carbon, nitrogen and minerals. They grow preferentially toward available carbon and nitrogen such as in living wood cells known as parenchyma cells. To break down the complex materials e.g. cellulose, lignin, pectin, starch, etc., fungi secrete digestive enzymes through their cell wall that will digest the complex organic compounds and convert them into simple molecules that can readily be transported through the cell walls.

Biological pretreatment of lignocellulosic biomass using white-rot fungi changes the biochemical and physical characteristic of the biomass (Isroi *et al.,* 2011) (Figure 2.1). Lignin degradation is the point of interest in many studies. For examples, lignin loss of corn straw was up to 54.6% after 30 days pretreatment with *Trametes versicolor* (Yu *et al.,* 2010); bamboo culm was more than 20% after 4 weeks pretreatment with *Echinodontium taxodii* 2538 and *T. versicolor* G20 (Zhang *et al.,* 2007); and wheat straw was 39.7% decreased after pretreatment with *Pleurotus sajor-caju* (Zadražil and Puniya, 1994).

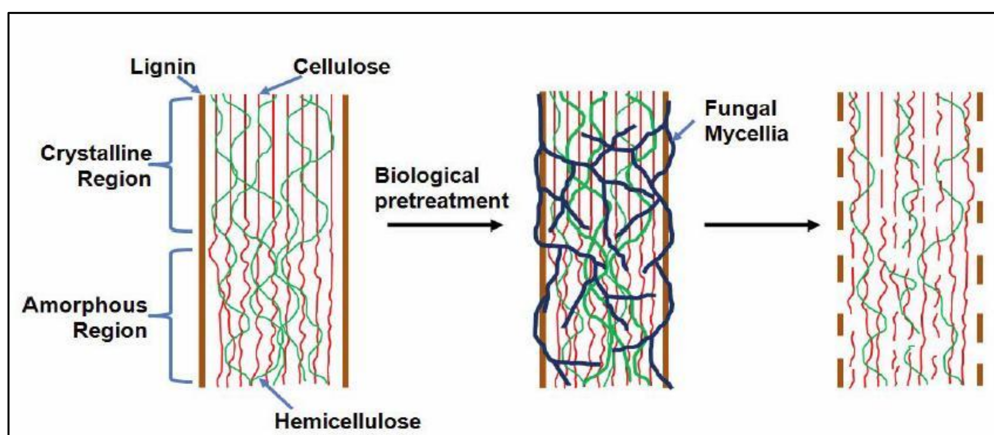


Figure 2.1 Schematic diagram of biological pretreatment of lignocelluloses.

There are two main methods by which Polyporales fungi decay wood: brown-rot and white-rot. In this study the white-rot fungi are studied as the white-rot produced a much larger array of enzymes compared to brown-rot, for both carbohydrate and lignin degradation (Wymelenberg *et al.,* 2005; 2006). White-rot caused by fungi can be divided into simultaneous and selective lignin degradation types. In simultaneous white-rot, the fungus degrades all wood cell wall polymers progressively, whereas in selective white-rot, the fungus degrades preferably lignin and hemicelluloses. In contrast to white-rot, brown-rot fungi, such as *Postia placenta, Laetiporus portentosus, Piptoporus betulinus* and *Gloeophyllum trabeum*, can degrade wood carbohydrates, but not

oxidized lignin. As a result, brown-colored rot ensues (Wong, 2009). The typical features of selective and simultaneous white-rot types are summarized in Table 2.2.

Understanding the wood decay by white-rot fungi is important because white-rot fungi are one of the few organisms with the capacity to completely mineralize lignin. White-rot fungi are well known for their remarkable ability to degrade lignin and microcrystalline cellulose by extracellular peroxidases and other enzymes. These white-rot fungi have complex extracellular ligninolytic enzyme systems, including lignin peroxidase, manganese peroxidase and laccase, which can selectively remove or alter lignin and allow cellulose fibers to be obtained (Breen and Singleton, 1999).

Table 2.2 Typical features of selective and simultaneous white-rot

	Selective white-rot	**Simultaneous white-rot**	**Reference**
Degraded cell wall components	Initial stages of decay: Hemicellulose and lignin Later stages: Hemicellulose, cellulose and lignin	Cellulose, hemicelluloses and lignin	Adaskaveg *et al.,* 1995 Fackler *et al.,* 2006
Anatomical features of decayed wood	Middle lamella dissolved. Adjacent wood cells separated.	Eroded cell walls. Degradation beginning from the secondary wall proceeding to middle lamella.	Blanchette, 1995
Lignin loss	Lignin loss diffusive throughout wood cell wall without major degradation of polysaccharides	Lignin loss together with wood cell wall polysaccharides starting progressively from lumen	Blanchette, 1995
Representatives	*Ceriporiopsis subvermispora* *Pleurotus* spp. *Phlebia tremellose*	*Phanerochaete chrysosporium* *Trametes versicolor*	Blanchette, 1995 Otjen *et al.,* 1987 Nishida *et al.,* 1988

In selective white-rot the wood secondary cell wall is delignified diffusively starting from the lumen, followed with the delignification of the middle lamella. As white-rot fungi capable of selective lignin degradation prefer hemicelluloses as carbon source, the wood cell walls are enriched with cellulose (Blanchette, 1991). Selective delignification can occur incompletely throughout wood substrate or merely in small, localized areas of complete lignin removal, which is called white pocket rot (Blanchette, 1984; Otjen *et al.,* 1987). In late stages of decay also cellulose is degraded and thus selective lignin degradation is usually limited to early stages of decay (Adaskaveg *et al.,* 1995).

Catabolic versatility appears to be generic feature of white-rot fungi and has attracted the interest of environmental and industrial scientist around the world. The fact that these microorganisms can dissemble and mineralize complex polymers and biologically recalcitrant substances suggests that their enzymes systems have potential for development of application e.g. remediating industrial wastewaters, production of chemicals, improving forage digestibility and biopulping.

The combination of solid substrate fermentation technology with the cability of white-rot fungi to selectively degrade lignin has made industrial-scale application of lignocelluloses-based biotechnologies possible. One of the most important aspects of white-rot fungi is related to the use of their ligninolytic system for a variety of applications (Figure 2.2).

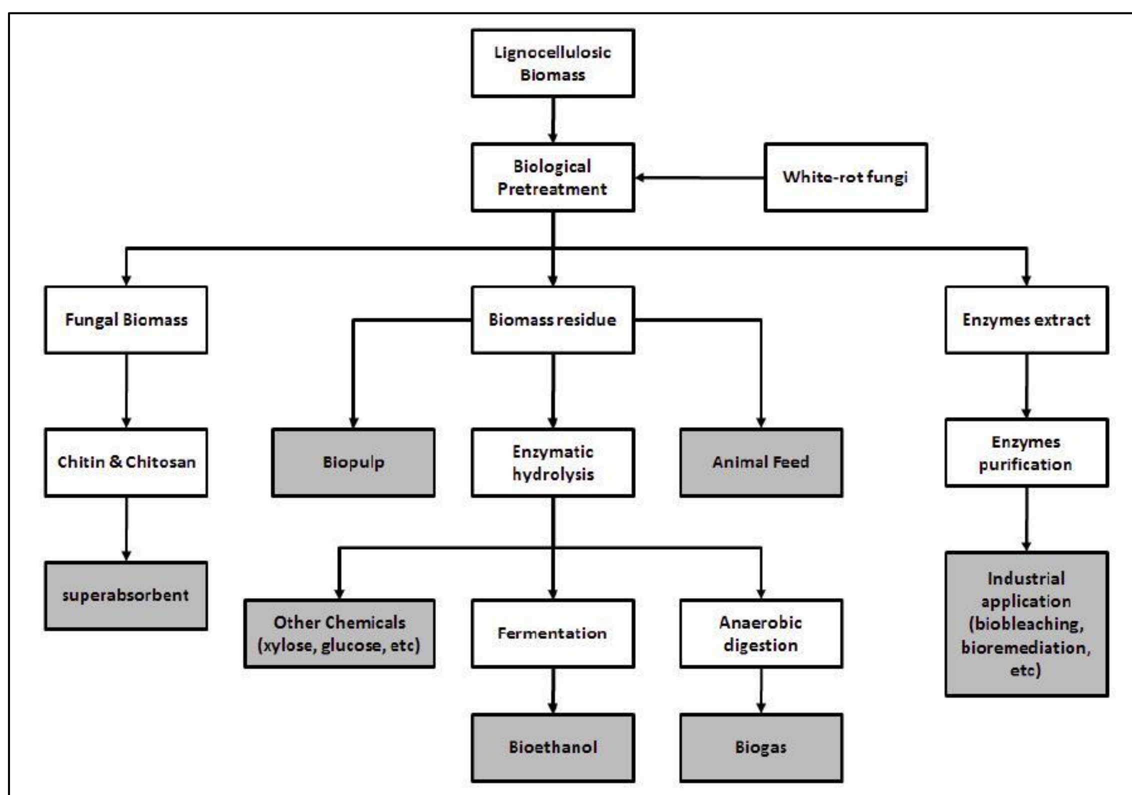


Figure 2.2 Suggestion for biological pretreatments of lignocellulosic biomass with white-rot fungi and alternative application routes (Isroi *et al.,* 2011).

In this study, the production of white-rot extracellular enzymes was evaluated during solid substrate fermentation of oil palm empty fruit bunches prior to pulping and papermaking processes.

2.5.1 Pulping and papermaking

Formerly, paper was made from non-wood plant materials such as flax, cotton, mulberry, bamboo or cereal straw. The increasing demand for paper raised the need for low-cost raw materials such as wood species to boost production. As a result, wood species have accounted for 90-95% of all raw materials used to obtain cellulose pulp for more than a century. Such a marked increase has been the result of the use of wood species as the main raw material leading to massive deforestation and re-plantation, which has altered the ecological balance and contributed to the climate change.

The paper industry is immersed in non-stop technological updating, forced by market globalization competition, high energy costs and raw material scarcity. According to Jean-Marc and Santosh (2006) Malaysia has a total capacity for pulp and paper production at over one million tons per year. Currently, the country is a net importer of pulp, paper, and paper board, and progressively tends to decrease its dependency.

The three main industrial or brown paper manufactures of the country until the end of 2006; The Muda Group of Companies, The Genting Sanyen Industrial Paper Sdn. Bhd. and The Pascorp Paper Industries Bhd. were based for raw material on recovered paper utilizing a total of approximately two million metric tons per year (Advisory Committee on Paper and Wood Products, 2007). The high demand for recovered waste paper has increased production costs and the country also facing stiff competition from cheaper imports, though some with poorer quality products from the neighboring countries of Malaysia in the Asian region.

Another serious development affecting the industry and likely to be a long-term problem, has been the depletion on the supply of raw material from the local collection of recovered waste paper. In addition, pulp and paper production has long been recognized as a significant source of pollution. Therefore, both market demand and environmental pressure have forced the pulp and paper industry to some extent wood has been replaced with non-woody or annual plants, and also with agricultural residues. Consequently, the preservation of forests and increasing environmental awareness has focused further research on the development of alternative sources of fiber for paper making.

The rise in price of wood and high energy demand is a serious concern thus the introduction of low cost fiber would be an alternative for both problems. That is why the use of different kinds of residues such as vine shoots (Jiménez *et al.,* 2007), olive tree pruning (Díaz *et al.,* 2005), wheat straw (Guadalix *et al.,* 1996), rice straw (Rodríguez *et al.,* 2008), residue from palm oil production (Empty Fruit Bunches, EFB) (Tanaka *et al.,* 2004; Jiménez *et al.,* 2009), or fast growing-high yield crops, such as *Miscanthus* (Cappelletto *et al.,* 2000; Iglesias *et al.,* 1996; Oggiano *et al.,* 1997), *Cynara cardunculus* (Benjelloun-Mlayah *et al.,* 1997; Abrantes *et al.,* 2007) or *Paulownia* (Jiménez *et al.,* 2005), as well as many other plants (Alaejos *et al.,* 2004), have been proposed by several authors.

Pulping is the process of converting wood to separate pulp fibers for papermaking. Pulp and paper can be made from lignocellulosic material such as wood, agricultural residues or from waste paper (Pooja *et al.,* 2010). The manufacturing of pulp and paper involves three major processing steps i.e. pulping, bleaching and paper production. There are three main types of pulping, namely mechanical or ground wood pulp, chemical and lastly the combination one which includes, chemical and mechanical. Table 2.3 shows summary of major pulping processes.

Fungi can also be used to treat wood in industrial settings. The lignin degrading properties provides the most promising potential application in biotechnology for Polyporales. In one such process, biopulping; fungal pretreatment of wood chips, designed as a solid-substrate cultivation process, for production of mechanical or chemical pulp. The concept of biopulping is based on the ability of some white-rot fungi to colonize and degrade selectively lignin in wood thereby leaving cellulose relatively intact. The white-rot fungi, *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora* are the commonly used organisms in the biopulping (Jin *et al.,* 2007).

Table 2.3 Summary of major pulping processes (Smook, 1992; Young *et al.*, 1989)

Process	Treatment		Pulp yield %	Wood used
	Chemical	Mechanical		
Mechanical				
Stone groundwood	None	Grinder	93-95	Softwood
Pressure groundwood	None	Grinder	93-95	Softwood
Refiner mechanical	None	Disc refiner	93-95	softwood
Thermomechanical	Steam	Disc refiner	80-90	Hardwood
Chemithermomechanical	Sodium sulfite	Disc refiner	80-90	Hardwood
Chemimechanical	Sodium sulfite	Disc refiner	80-90	Hardwood
Semichemical				
Neutral sulfite	Sodium sulfite	Disc refiner	70-85	Hardwood
Green liquor	Sodium hydroxide	Disc refiner	70-85	
Nonsulfur	Sodium carbonate	Disc refiner	70-85	
Chemical				
Kraft (sulfate)	Sodium hydroxide +sodium sulfide	None	45-55	Both
Sulfite	Calcium bisulfate in sulfurous acid	None	40-50	Both
Soda-oxygen	Sodium hydroxide +oxygen	None	45-55	Hardwood
Soda-anthraquinone	Sodium hydroxide +anthraquinone	None	45-55	

Biopulping, i.e. the fungal pretreatment of wood chips to enhance pulping, was invented in 1970's (Eriksson *et al.,* 1976; Eriksson and Vallander, 1982), although the idea had been proposed already in the 1950's (Lawson and Still, 1957). In biopulping, wood chips or logs are pre-treated with fungi to enhance the subsequent pulping step (summarized in Table 2.4). The process has been directed to produce pulps for papermaking with decreased energy requirements for defibering and refining. Biopulping treatment reduces electrical energy use in papermaking and improved pulp strength. In biopulping, wood chips or logs are pre-treated with fungi to enhance the subsequent pulping step.

Table 2.4 The benefits of wood pretreatment with selected fungi

Fungus	Raw material	Benefits	Reference
Physisporinus rivulsus	Sterilized wood chips	Selective lignin degradation. Growth in a wide temperature range. Reduced refining energy consumption. Reduced wood pitch content.	Hatakka *et al.,* 2003
Ceriporiopsis subvermispora	Sterilized wood chips	Selective lignin degradation. Reduced refining energy consumption. Reduced wood pitch content. Enhanced chemical pulping.	Fischer *et al.,* 1994 Akhtar *et al.*, 2000 Bajpai *et al.,* 2003
Phlebiopsis gigantea	Wood logs	Reduced wood pitch content. Enhanced chemical pulping. Slightly reduced refining energy consumption. Reduced staining of wood. Enhanced debarking.	Behrendt and Blanchette, 1997)

Various species of white-rot fungi have been used for biopulping, however, *Ceriporiopsis subvermispora* has proven to be vary competitive both on softwoods and hardwoods (Ferraz *et al.,* 2007). The physiology and biochemistry of *C. subvermispora* has been studied to allow an intensification of the biopulping process (Sethuraman *et al.,* 1998; Milagres *et al.,* 2005). Evaluation of enzymes produced during biopulping process have shown that *C. subvermispora* produces several manganese peroxidase (MnP) and laccase isoenzymes, each exhibiting isoelectric points that vary according to the composition of the medium (Lobos *et al.,* 2001).

White-rot fungi and their enzymes (especially ligninases and xylanases) are considered for the wood chips treatment of prior to pulping. While ligninases attack the content of wood, xylanases degrade hemicelluloses and make the pulp more permeable for the removal of residual lignin. Thus, biopulping process not only removes lignin but also some of the wood extractives, thus reducing the pitch content and effluent toxicity (Ali and Sreekrihnan, 2001).

The biodegradative abilities of white-rot fungi are remarkable, both in terms of the number of different chemicals than can be oxidized, and in the nature of chemicals that can be oxidized. The biodegradation system of white-rot fungi is versatile (Pooja, 2010). Using enzymes and special techniques to increase their accessibility to wood, individual components could be eliminated from wood. A modest attention has to been paid to mechanism of wood decay; though it is known that the fungi degrade lignin to a significant extent is incapable of penetrating the walls of sound wood. The areas of the walls that have been profoundly decayed by white-rot fungi, which break down lignin and hemicelluloses somewhat selectively, were penetrated by the enzyme (Blanchette *et al.,* 1989).

Due to shortages in wood supplies and preservation of forests, countries in Southeast Asia are expected to focus more on the development of alternative fiber sources such as oil-palm fiber. The oil palm solid wastes, especially oil palm empty fruit bunches (EFB), have potential to be used as raw materials for pulp and paper industries (Rushdan *et al.,* 2007). Empty fruit bunches possess great potential as raw material due to its availability in large quantity and continuous supply, chemically low lignin and high cellulose content, high fiber content, resemblance to hardwood fibers and good bonding strength and paper properties of handsheets (Mohamad, 2000). However, none of the EFB and paper discoveries has been produced by using pre-treatment with Polyporales fungi. In this study, handsheets were produced from pre-treated EFB soda pulp with selected Polyporales fungi. The pre-treatment were done through solid substrate fermentation of EFB by selected strains during 28 days of incubation period.

2.6 Solid substrate fermentation

Solid substrate fermentation (SSF) can be defined as the cultivation of microorganisms on solid substrates devoid of or deficient in free water (Pandey, 2003). Solid substrate fermentation has been focused mainly to the production of feed, hydrolytic enzymes, organic acids, gibberellins, flavors and biopesticides (Raimbault, 1998). Solid substrate fermentation has many advantages over liquid substrates fermentation (LSF). Anyway, SSF has several important limitations. Table 2.5 shows advantages and disadvantages of SSF compared to LSF (Raimbault, 1998).

In general, SSF is a well-adapted and cheaper process than LSF for the production of bio-products including enzymes. It has been reported that in many bio-productions, the amount of products obtained by solid substrate fermentation are many fold higher than those obtained in liquid substrate fermentation.

Table 2.5 Advantages and disadvantages of SSF compared to LSF (Raimbault, 1998)

Factor	Solid Substrate Fermentation (SSF)	Liquid Substrate Fermentation (LSF)
Substrate	Polymer insoluble substrates	Soluble substrates
Aseptic conditions	Vapor treatment, non sterile conditions	Heat sterilization and aseptic control
Water	Limited consumption of water. No effluent	High volumes of water consumed and effluents discarded
Metabolic heating	Low heat transfer capacity	Easy control of temperature
Aeration	Easy aeration and high surface exchange air/substrate	Limitation of soluble oxygen. High level of air required
pH control	Buffered solid substrate	Easy pH control
Mechanical agitation	Static conditions preferred	Good homogenization
Scale up	Need for engineering and new design equipment	Industrial equipments available
Inoculation	Spore inoculation. Batch process	Easy inoculation, continuous process
Contamination	Risks of contamination for low rate growth fungi	Risks of contamination for single strain bacteria
Energetic consideration	Low energy consuming	High energy consuming
Volume of equipment	Low volumes and low costs of equipments	High volumes and high cost technology
Effluent and pollution	No effluents, less pollution	High volumes of polluting effluents

Agro-residual residues are generally considered the best substrates for the SSF process, and use of SSF for the production of enzymes in no exception to that. Many of the substrates had been used to cultivate the microorganisms to produce the commercial value enzymes. Some of the substrates that have been used included sugar cane bagasse,

wheat bran, rice bran, maize bran, gram bran, wheat straw, tea waste, banana waste, peanut meal etc. (Kumaran *et al.,* 1997; Vikineswary *et al.,* 2006).

Among several factors that are important for fungal growth and enzyme production using agro-residues substrate, the readily available sources are the most crucial. As one of the largest palm-oil producers, the oil palm (*Elaeis guineensis*) solid wastes, especially empty fruit bunches (EFB), have great potential to be used for pulp and paper industries in Malaysia. Therefore, in this study, the oil palm empty fruit bunches (EFB) was selected as substrate for the growth of selected fungi for the production of enzymes through SSF.

2.7 Oil Palm Empty Fruit Bunches in biopulping

The oil palm (*Elaeis guaineensis*) is the most important economic plantation crop in Malaysia (Figure 2.3). It produces two types of oils from the same fruit – palm oil from the flesh or mesocarp and palm kernel oil from the seed of kernel inside the hard-shell mesocarp. During the production of oils, the palm oil mills produce a large amount of biomass products. The biomass of the oil palm consists of huge amount of lignocellulosic materials such as oil palm fronds, trunks and empty fruit bunches. Malaysia produces 16 million tons of EFB in 2000, which represents a further huge source of fiber and cellulosic materials. This makes the oil palm truly attractive as a future source of renewable energy from the biomass which, if exploited prudently, will enhance the effort to achieve the sustainability of oil palm plantations. In this study, the oil palm empty fruit bunches is selected as the fibers source which undergoes pre-treatment with fungi prior to biopulping.

Figure 2.3 Oil palm (*Elaeis guaineensis*) tree

Oil palm empty fruit bunches (EFB), an oil palm solid waste is composed of 45-50% cellulose and about equal amounts (25-35%) of hemicelluloses and lignin (Deraman, 1993) and has a good potential to be raw materials for pulp and paper (Figure 2.4). EFB has to undergo pulping process to produce pulp for papermaking. Most of non-wood pulp is produced by chemical pulping and Rushdan (2002) has successfully pulped EFB by alkaline process.

Figure 2.4 Oil palm empty fruit bunches

The major disadvantages of alkaline pulping processes are that they consume large amounts of energy and chemical treatments. One of the methods to decrease utilization of energy and chemical treatments is by biopulping. The process of biopulping reduces the utilization of chemical in pulping industry and help in decreasing the environmental hazard caused by normal pulping.

Biopulping use fungi that are known to be able to degrade wood as well as lignin constituent of wood and the white rot fungi are the most proficient biodegrader (Pooja *et al.,* 2010). Furthermore, numerous studies also have been carried out regarding the use of enzymes and microorganisms for biopulping of different types of wood and non-wood pulps (Giovannozzi-Sermanni *et al.,* 1997; Jacobs-Young *et al.,* 1998). In some cases, these fungi presented a preferential attack of lignin in the initial period of wood decay leaving cellulose relatively intact (Eriksson *et al.,* 1990; Martínez *et al.,* 2005). Therefore, this study also will determine the suitability of selected Polyporales for the application of biopulping through solid substrate fermentation of EFB.

CHAPTER 3

DIVERSITY AND DISTRIBUTION OF POLYPORALES IN PENINSULAR MALAYSIA

3.1 INTRODUCTION

The Polyporales are a large group of various macrofungi. Donk (1965) divided poroid mushrooms into five families namely Polyporaceae, Hymenochaetaceae, Ganodermataceae, Bondarzewiaceae and Fistulinaceae. In addition, some poroid genera were also assigned to families Coniophoraceae, Corticiaceae and Thelephoraceae. According to Ainsworth & Bisby's dictionary of fungi (Kirk *et al.,* 2001) there are twenty-three families in the Order Polyporales. However, many of the species belong to the Polyporaceae. Many species of Polyporales lack fully developed stems, and developed as shelf-like or crust-like on wood. Some genera have more or less central stems and grow at the bases of trees, while few emerge from soil. The Polyporales fungi are essential in natural ecosystems as decomposers of wood, recycling the nutrients and minerals in the wood and releasing them over a long period of time (Volk, 2000).

This study examines the diversity and distribution of the wood decay fungi, particularly from order Polyporales from Peninsular Malaysia. In order to provide a context for the significance of research into Polyporales species, this study begins with an exploration of basidiocarps from selected study sites and also examination of herbarium specimens held at University of Malaya Herbarium, Kuala Lumpur. The review then discusses the recent record with published records of Polyporales to determine the diversity and distribution status.

Pure culture collections have gained an important role as resources for authentic, reliable microbial cutures for both research and practical use. Therefore, this study also

aims to obtain the selected pure cultures of Polyporales. The pure cultures will provide a source of reference to enable a readily accessible collection of identified cultures and as a means of preserving genetic resources of such organisms. Moreover they provide a source of reference to enable microbiologists to verify more easily the organisms with which they are working.

This study also focus specifically on the genus *Ganoderma*. Presently, in Malaysia there is confusion on the identity of Polyporales species especially among the commercially cultivated *Ganoderma* spp. and collected collected from the wild. In addition, conflicting information is available on the cultivated *Ganoderma* species, which lead to wrongly named species particularly during product marketing. In this study, molecular taxonomy was applied to confirm the porphological identification of *Ganoderma* species.

Hence the objectives of the study were to:

a. document the diversity and distribution of Polyporales in Peninsular Malaysia
b. infer phylogenetic relationship between the species of *Ganoderma* using molecular data obtained by sequencing the amplified Internal Transcribed Spacer (ITS) region (ITS1-5.8S - ITS4) of the DNA samples
c. to acquire pure cultures of Polyporales for enzymes screening to obtain candidates species for biopulping

3.2 MATERIALS AND METHODS

3.2.1 Sampling sites

Peninsular Malaysia located approximately between 6° 45´ and 1° 20´ N latitude and 99° 40´ and 104° 20´ E longitudes comprising eleven States and the Federal Territory of Kuala Lumpur and Putrajaya. Topographically, Peninsular Malaysia is characterized by extensive coastal plains in the east and west, hilly and mountainous region with steep slopes in the central and undulating terrain in other parts of the peninsula. The forests of Peninsular Malaysia have been variously classified according to their ecological and physical conditions, but for the purposes of management they can be classified broadly into the Dipterocarp, Freshwater Swamp and Mangrove forests (Hooi, 1987). The dipterocarp forest occurs on dry land just above sea level to an altitude of about 900 metres. The forests in Malaysia are mostly dominated by trees from the Dipterocarpaceae family. Basidiocarps of Polyporales were collected from various sites in six states and Federal Territory of Kuala Lumpur (Figure 3.1).

3.2.2 Collection of Polyporales

Basidiocarps encountered along the trails were collected from April 2003 till June 2008. Multiple basidiocarps of the same species growing on an individual tree or log were considered as one collection. All basidiocarps were identified to the species level using the keys in Núňez and Ryvarden (2000, 2001) and Hattori (2000, 2005). For colour term and notations of basidocarps, the colour index in Kornerup and Wanscher (1973) was referred. The basidocarps were then preserved as oven-dried specimens at University of Malaya Herbarium (acronym KLU), Kuala Lumpur.

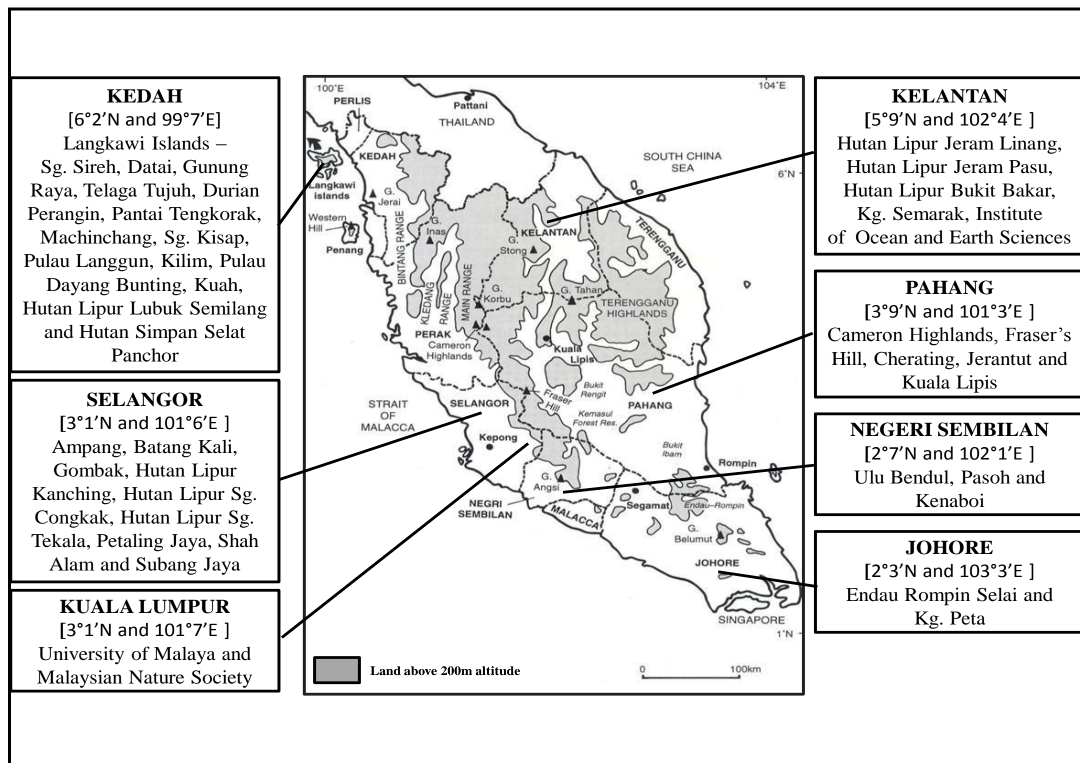


Figure 3.1 Sampling sites of Polyporales in Peninsular Malaysia.

3.2.3 Pure cultures collection

Polyporales basidiocarps were collected from a number of different locations. Macrofungal basidiocarps were collected during the fieldwork for this study and herbarium specimens were prepared from fungi collected throughout Peninsular Malaysia. These fungal fruit bodies were usually identified to species. These basidiocarps are being held at the University of Malaya Herbarium (acronym KLU), Kuala Lumpur. Fungal cultures of all fresh basidiocarps collected were obtained by excising 3-5 small sterile pieces of fruit body from within the cap or stipe. These pieces were then incubated for two weeks on Malt Extract Agar (MEA). Isolation was considered successful when the majority of fruit body pieces resulted in the same mycelium. This mycelium was then subcultured and maintained on MEA at 25 ± 2 ºC for the duration of the study. Copies of each isolate have been lodged with the Mushroom Research Centre, University of Malaya culture collection in Kuala Lumpur.

3.2.4 Phylogenetic study of selected Ganodermataceae

In this study the *Ganoderma* species collected are very variable macromorphologically and lack micromorphological distinctiveness. Therefore, molecular study is essential to identify the specimens collected to species level. All studied cultures of *Ganoderma* were deposited in the Mushroom Research Centre, University of Malaya (acronym KUM). Fungal used in this study and their origins are given in Table 3.1, together with references to previous systematic studies. The entire region of the internal transcribed spacers (ITS1 and ITS4), including the 5.8S gene, was analyzed in this study. The sequences of the primers were as follows: ITS1 was 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 was 5'-TCCTCCGCTTATTGATATGC-3'.

3.2.4.1 *Ganoderma* cultures

Cultures were kept on MEA (malt extract agar Difco) at 4 °C. Mycelia were grown 7 days on MEA plates and incubated for seven days at 25 ± 2 °C prior to extraction of DNA.

3.2.4.2 DNA isolation and amplification

For the isolation of DNA which was used in PCR amplification, fungi were grown on Malt Extract Agar at 25±2°C for 7 days in dark condition. The mycelia were scrapped from the surface of cultures and followed by extraction by using DNA kit, QIAGEN, DNeasy Plant Mini Kit. The amplification reaction mixture typically contained the following: 25 µl of Mastermix, 1 µl of each primer, and 2 µl DNA template, eluted to a final volume of 50 µl. The amplification protocol consisted of 2 min at 95 °C, followed by 35 cycles of 30 sec at 94 °C, 30 sec at 46 °C to 54 °C, and 1 min at 72 °C, followed by a final extension of 5 min at 72 °C. Products were detected

electrophoretically on 1.3% agarose gel in Tris-acetic acid-EDTA (TAE) buffer and visualized with ethidium bromide.

Table 3.1 Taxa used in this study, along with their strain/specimens numbers, origins and GenBank accession numbers.

Species	Strain/Specimen no.	Origin	GenBank accession no.
Amauroderma rude var. *intermedium*	JMM ASP.1	Taiwan	X78753&X78774
G. lucidum	JMM P93-1	Philippines	X78745&X78766
G. lucidum	WD-565	Ibaraki, Japan	EU021455
G. lucidum	WD-2038	Ibaraki, Japan	EU021456
G. lucidum	HMAS 60537	China	Z37050&Z37074
G. australe	-	Malaysia	AF255142
G. australe	-	Malaysia	AF255143
G. australe	-	Thailand	AF255146
G. australe	-	Thailand	AF255147
G. lucidum	KUM61076	Bahau, Malaysia	-
G. amboinense	KUM61117	Grower, Malaysia	-
G. tsugae	KUM50079	Grower, Malaysia	-
G. lucidum	KUM61088	China	-
G. lucidum	KUM61129	Grower, Malaysia	-
G. lucidum	KUM61130	Grower, Malaysia	-
G. lucidum	KUM61120	Pahang, Malaysia	-
G. australe	KUM61056	Kelantan, Malaysia	-
G. australe	KUM61057	Kelantan, Malaysia	-
G. australe	KUM60813	Pahang, Malaysia	-

3.2.4.3 Evaluation of information contents and phylogenetic potential

Sequences from the ITS regions were compared with those in GenBank using the BLASTn (Basic Local Alignment Search Tool for nucleotides) search algorithm. Hits from the BLASTn searches, which showed the highest similarity and coverage (from 95% to 99%) as compared to the sequences from the selected isolates, were downloaded and included in this study for comparative purposes. ITS sequence data were initially aligned with Clustal X v.1.81 program (Thompson *et al.,* 1997) with a gap open penalty of 10 and a gap extension penalty of 0.1 and then manually adjusted. The phylogenetic relationship of ITS1 and ITS4 sequences of Ganodermataceae were estimated by heuristic search option of PAUP* v.4.0 beta version (Swofford, 2002) with 100 random sequence addition replicates, tree bisection-reconnection (TBR), collapse of zero length branches, and maxtrees set to 10,000.

3.3 RESULTS

3.3.1 Diversity and distribution of Polyporales

Basidiomata of 340 samples of Polyporales were collected during 62 samplings carried out from April 2003 till June 2008 at various locations in the six states of Peninsular Malaysia and Federal Territory of Kuala Lumpur (Table 3.2).

The samplings were done from May to August and from November to February each year. During this period, Peninsular Malaysia experienced rainy weather associated with the Southwest and Northeast monsoon. The Southwest monsoon usually occurs between May till September, bringing rainfall to the western part of Peninsular Malaysia. On the other hand, the Northeast monsoon starts from November and lasts till March, bringing heavy rainfall to areas on the east side of Peninsular Malaysia.

Table 3.2 The Polyporales collected from selected locations in Peninsular Malaysia.

	States and Federal Territory	Johore	Kedah	Kelantan	Kuala Lumpur	Negeri Sembilan	Pahang	Selangor
	Frequency of Collections	**3**	**18**	**4**	**5**	**3**	**11**	**18**
	Fomitopsidaceae							
1	*Daedalea lusor* (Corner) T. Hatt. (2005)							**1**
2	*Fomitopsis feei* (Fr.) Kreisel (1971)	**4**	**1**			**2**	**1**	**1**
3	*Fomitopsis ostreiformis* Bose (1923)*				**3**			
	Ganodermataceae							
4	*Amauroderma subrugosum* (Bres. & Pat.) Torrend (1920)	**1**	**2**			**2**	**1**	**2**
5	*Ganoderma australe* (Fr.) Pat. (1890)	**7**	**2**	**2**	**2**	**11**	**4**	**5**
6	*Ganoderma lucidum* complex (Curtis) P. Karst. (1881)					**3**		
	Meripilaceae							
7	*Meripilus applanatus* Corner (1984)					**1**		
8	*Rigidoporus lineatus* (Pers.) Ryvarden (1972)		**1**					
9	*Rigidoporus microporus* (Sw.) Overeem (1924)	**3**	**1**				**3**	**1**
10	*Rigidoporus vinctus* (Berk.) Ryvarden (1972)							**1**
	Meruliaceae							
11	*Flavodon flavus* (Klotzsch) Ryvarden (1973)	**1**			**1**			**1**
12	*Gloeoporus dichrous* (Fr.) Bres. (1913)*	**1**						
13	*Gloeoporus sulphureus* Corner (1989)							**1**

Table 3.2, continued.

	States and Federal Territory	Johore	Kedah	Kelantan	Kuala Lumpur	Negeri Sembilan	Pahang	Selangor
	Frequency of Collections	**3**	**18**	**4**	**5**	**3**	**11**	**18**
	Polyporaceae							
14	*Coriolopsis aspera* (Jungh.) Teng (1963)					**1**		
15	*Coriolopsis badia* (Berk.) Murrill (1907)*	**1**	**1**		**2**			
16	*Coriolopsis glabro-rigens* (Lloyd) Núñez & Ryvarden (2001)	**1**					**1**	
17	*Coriolopsis sanguinaria* (Klotzsch) Teng (1963)*		**1**					
18	*Coriolopsis strumosa* (Fr.) Ryvarden (1976)							**1**
19	*Earliella scabrosa* (Pers.) Gilb. & Ryvarden (1985)	**8**	**2**		**1**	**6**		**3**
20	*Echinochaete brachypora* (Mont.) Ryvarden (1978)*							**1**
21	*Favolus tenuiculus* P. Beauv. (1806)					**1**		**1**
22	*Flabellophora licmophora* (Massee) Corner (1987)	**1**						
23	*Fomitella rhodophaea (Lév.) T. Hatt. (2005)*		**1**					**2**
24	*Funalia polyzona* (Pers.) Niemelä*				**1**			**3**
25	*Hexagonia tenuis* (Hook.) Fr. (1838)		**1**				**1**	**1**
26	*Lentinus badius* (Berk.) Berk. (1847)	**1**	**1**					**2**
27	*Lentinus connatus* Berk. (1842)	**6**	**9**			**1**		**2**
28	*Lentinus fasciatus* Berk. (1840)*		**3**	**1**				
29	*Lentinus polychrous* Lév. (1844)	**1**	**4**			**1**		
30	*Lentinus sajor-caju* (Fr.) Fr. (1838)				**4**			**10**
31	*Lentinus squarrosulus* Mont. (1842)	**2**	**4**	**1**	**4**		**5**	**5**
32	*Lentinus strigosus* (Schwein.) Fr. (1838)		**3**				**1**	**1**

Table 3.2, continued.

	States and Federal Territory	Johore	Kedah	Kelantan	Kuala Lumpur	Negeri Sembilan	Pahang	Selangor
	Frequency of Collections	**3**	**18**	**4**	**5**	**3**	**11**	**18**
33	*Lentinus tigrinus* (Bull.) Fr. (1825)*							**1**
34	*Lentinus velutinus* Fr. (1830)		**1**					
35	*Lenzites acuta* Berk. (1842)		**4**				**1**	
36	*Lenzites elegans* (Spreng.) Pat. (1900)	**1**				**9**	**3**	**2**
37	*Lignosus rhinocerotis* (Cooke) Ryvarden 1972					**3**	**1**	
38	*Macrohyporia dictyopora* (Cooke) I. Johans. & Ryvarden (1979)*							**1**
39	*Microporellus inusitatus* (Lloyd) Corner (1987)	**1**						
40	*Microporus affinis* (Blume & T. Nees) Kuntze (1898)	**3**					**4**	**7**
41	*Microporus vernicipes* (Berk.) Kuntze (1898)						**1**	**1**
42	*Microporus xanthopus* (Fr.) Kuntze (1898)	**2**	**2**	**2**	**1**	**3**	**2**	**4**
43	*Nigroporus vinosus* (Berk.) Murrill (1905)	**3**	**1**			**2**	**1**	
44	*Panus similis* (Berk. & Broome) T.W. May & A.E. Wood. (1995)*	**2**	**1**				**1**	
45	*Perenniporia ochroleuca* (Berk.) Ryvarden (1972)		**1**					
46	*Polyporus arcularius* (Batsch) Fr. (1821)						**1**	**5**
47	*Polyporus grammocephalus* Berk. (1842)	**1**				**2**	**1**	**5**
48	*Polyporus leprieurii* Mont. (1840)		**1**					**2**
49	*Polyporus philippinensis* Berk. (1842)*				**1**			
50	*Polyporus* cf. *badius* (Pers.) Schwein (1832)*							**1**
51	*Pseudofavolus cucullatus* (Mont.) Pat. (1900)							**1**

Table 3.2, continued.

	States and Federal Territory	Johore	Kedah	Kelantan	Kuala Lumpur	Negeri Sembilan	Pahang	Selangor
	Frequency of Collections	**3**	**18**	**4**	**5**	**3**	**11**	**18**
52	*Pycnoporus cinnabarinus* (Jacq.) P. Karst. (1881)*						**1**	
53	*Pycnoporus sanguineus* (L.) Murrill (1904)	**2**	**2**	**1**	**1**	**6**	**4**	**4**
54	*Pyrofomes albomarginatus* (Zipp. ex Lév.) Ryvarden (1972)	**2**	**2**					
55	*Trametes* cf. *hirsuta* (Wulfen) Lloyd (1924)			**1**	**2**	**2**	**1**	
56	*Trametes lactinea* (Berk.) Sacc. (1888)*				**1**			**2**
57	*Trametes menziesii* (Berk.) Ryvarden (1972)	**2**	**2**	**1**	**1**	**2**	**2**	**5**
58	*Trametes pocas* (Berk.) Ryvarden (1984)				**2**			**1**
59	*Trichaptum byssogenum* (Jungh.) Ryvarden (1972)*							**1**
60	*Trichaptum durum* (Jungh.) Corner (1987)*	**1**	**1**			**1**		**1**
	Total number of basidiocarp collected	**58**	**56**	**9**	**27**	**59**	**41**	**90**
	Number of species identified	**25**	**27**	**7**	**15**	**19**	**23**	**39**

* Species not collected by other workers

During the study period, 60 species identified in Polyporales includes five families as shown in Table 3.2: named Fomitopsidaceae (three species), Ganodermataceae (three species), Meruliaceae (three species), Meripilaceae (four species), and Polyporaceae (47 species). The Polyporaceae was the dominant family with 46 species was identified. The frequently encountered species based on the number of basidiocarps collected were *Ganoderma australe* (33) followed by *Lentinus squarrosulus* (21), *Earliella scabrosa* (20), *Pycnoporus sanguineus* (20), *Lentinus connatus* (18), *Microporus xanthopus* (16), *Trametes menziesii* (15), *Lenzites elegans* (15), *Lentinus sajor-caju* (14) and *Microporus affinis* (14).

(a) Johore

Johore is located in the southern part of Peninsular Malaysia. In Johore, three collections were made in Endau Rompin National Park. The Endau Rompin National Park is located at Mersing and Segamat districts of Johore. This forest is a tropical rainforest which covers 48,905 hectares, encompassing the watershed of the rivers Endau and Rompin. They are two entry points to Endau Rompin National Park which are through Kg. Peta in Mersing and Kg. Selai in Segamat. Twenty five species were identified from five families. Three frequently found species were *Ganoderma australe, Earliella scabrosa* and *Lentinus connatus.* Furthermore, *Gleoporus dichrous, Flabellophora licmophora* and *Microporellus inusitatus* were only collected once throughout the study period.

(b) Kedah

Kedah is located in the northwestern part of Peninsular Malaysia. The state consists of the mainland and Langkawi. In Kedah, 18 collections were made in Langkawi. The Langkawi is a cluster of 99 islands separated from mainland Peninsular

Malaysia by the Straits of Malacca with the total land mass of the islands is 47,848 hectares. Two-thirds of the islands are dominated by forest-covered, hills and natural vegetation. These study sites are mostly recreational forest except two forest reserves; Gunung Raya and Matchinchang. Twenty seven species from four families; Fomitopsidaceae, Ganodermataceae, Meripilaceae and Polyporaceae were collected, with 18 species belonging to Polyporaceae. *Lentinus* spp. were frequently encountered in Langkawi with seven species collected: *Lentinus badius, L. connatus, L. fasciatus, L. polychrous, L. squarrosulus, L. strigosus and L. velutinus.* Out of nine *Lentinus* spp. collected in this study, *L. velutinus* was only encountered in Langkawi throughout the study period. In addition, three species; *Rigidoporus lineatus, Perenniporia ochroleuca* and *Coriolopsis sanguinaria* were also encountered only in Langkawi.

(c) Kuala Lumpur

Kuala Lumpur is the capital city of Malaysia and it is enclaved within the state of Selangor, on the the central west coast of Peninsular Malaysia. The shape, form and disposition of Kuala Lumpur are created by the irregular topography of hills, river and stream valleys throughout the city area. In the Federal Territory of Kuala Lumpur, five collections were made in University of Malaya and Malaysia Nature Society Heritage Trail. Fifteen species belonging to three families; Ganodermataceae, Meruliaceae and Polyporaceae were identified with twelve species belonging to Polyporaceae. *Fomitopsis ostreiformis* and *Polyporus philippinensis* were only encountered in Kuala Lumpur throughout the study period.

(d) Kelantan

Kelantan is located in the northeastern part of Peninsular Malaysia. It is bordered by Narathiwat Province of Thailand to the north, Terengganu to the southeast,

Perak to the west and Pahang to the south. In Kelantan, three collections were made in Bachok which is located 25 kilometers east of Kota Bharu. Most of the collections were done at recreational forest. In Kelantan, six Polyporaceae species were identified namely *Lentinus fasciatus, L. squarrosulus, Microporus xanthopus, Pycnoporus sanguineus, Trametes* cf. *hirusta, T. menziesii* and one Ganodermataceae identified as *Ganoderma australe*.

(e) Negeri Sembilan

Negeri Sembilan is located on the western coast of Peninsular Malaysia. In Negeri Sembilan, three collections were done at recreational forest. A total of 19 species belonging to four families; Fomitopsidaceae, Ganodermataceae, Meripilaceae and Polyporaceae were identified. Fourteen identified species belonging to Polyporaceae with *Lenzites elegans*, *Earliella scabrosa* and *Pycnoporus sanguineus* were frequently encountered. Three species of Ganodermataceae; *Amauroderma subrugosum*, *Ganoderma australe,* and *G. lucidum* complex were also collected. Furthermore, *Meripilus applanatus* and *Coriolopsis aspera* were only encountered in Negeri Sembilan throughout the study period.

(f) Pahang

Pahang is the largest state in Peninsular Malaysia and is situated in the eastern coastal region. In Pahang, most of the collections were done in highland areas; Cameron Highlands and Fraser´ s Hill. Further, collections were also done in lowland areas; Cherating, Jerantut and Kuala Lipis localities. At 1,500 metres above sea level Cameron Highlands is situated at the north-western tip of Pahang with temperatures no higher than 25°C. Twenty three species from four families; Fomitopsidaceae, Ganodermataceae, Meripilaceae and Polyporaceae were collected with 17 species

belonged to Polyporaceae. Most of the species were only collected once or twice during each visit, except for *Lentinus squarrosulus* (5 collections), *Ganoderma australe* (4 collections), *Microporus affinis* (4 collections), *Pycnoporus sanguineus* (4 collections), *Rigidoporus microporus* (3 collections) and *Lenzites elegans* (3 collections).

(g) Selangor

The state of Selangor, which extends along the west coast of Peninsular Malaysia, is the most rapidly developing state in Malaysia, has an area of approximately 800,000 ha. Most of the basidocarps were collected in Selangor consisting of 39 species belonging to five families; Fomitopsidaceae, Ganodermataceae, Meripilaceae, Meruliaceae and Polyporaceae were collected. Twenty eight species identified belonged to Polyporaceae. *Lentinus* spp. were frequently encountered in Selangor with six species collected; *Lentinus sajor-caju*, *L. squarrosulus*, *L. badius*, *L. connatus*, *L. strigosus* and *L. tigrinus*. Nine species; *Daedalea lusor, Rigidoporus vinctus, Gloeoporus sulphureus, Coriolopsis strumosa, Lentinus tigrinus, Macrohyporia dictyopora, Pseudofavolus cucullatus, Polyporus* cf. *badius* and *Trichaptum byssogenum* were encountered only during visits to the study sites in Selangor. The frequently encountered species in Selangor were *Ganoderma australe, Lentinus sajor-caju, L. squarrosulus*, *Microporus affinis, Polyporus arcularius, P. grammocephalus* and *Trametes menziesii*.

3.3.2 Common species of Polyporales in Peninsular Malaysia

Ten species identified using morphological analyses were commonly found in Peninsular Malaysia and they were as follows: *Ganoderma australe, Lentinus squarrosulus*, *Earliella scabrosa*, *Pycnoporus sanguineus*, *Lentinus connatus*, *Microporus xanthopus*, *Trametes menziesii*, *Lenzites elegans*, *Lentinus sajor-caju* and *Microporus affinis*. Nine out of ten species identified belong to Polyporaceae and one

species belongs to Ganodermataceae. These common species are normally a group of tough, leathery mushrooms and commonly found on rotting logs or branches. The descriptions of ten common species collected throughout this study are as follow:

i. <u>*Ganoderma australe* (Fr.) Pat. (1890)</u>

Habitat/Substrata on deciduous wood

Basidiocarps (KLU-M200) perennial, applanate, ungulate, often irregular when growing from cracks etc., normally dimidiate and semicircular in outline, variable in size, 4-40 cm long, 4-20 cm wide and up to 10 cm thick in single basidiocarps, woody hard when dry (Figure 3.2).

Figure 3.2 Basidiocarp of *Ganoderma australe*

Pilear surface dull, cocoa-brown to deep umber to black in old basidiocarps, dying or weathered basidiocarps more greyish, surface often covered with a cinnamon to pale cocoa powder of deposited spores, otherwise surface glabrous, smooth, mostly distinctly sulcate in variable zones, somewhat cracking with age and drying, black cuticle present, 0.2-3 mm thick, increasing in thickness towards the base, margin light- coloured in

actively growing basidiocarps, whitish to yellowish, pore surface white to cream in actively growing basidiocarps, then dark when bruised, in older and resting species, pale to umber-brown. Pore surface round, entire, quite thick-walled, 3-5 per mm.

Hyphal system dimitic, generative hyphae with clamps, thin-walled and hyaline, 1.5-3 µm wide, skeletal hyphae dominating in the basidiocarp, variable brown to yellow, thick-walled to solid, up to 6 µm wide, branching variable, in lower part unbranched and then arboriform in the top, often irregular, binding hyphae delicate, mostly very thin, 1-2 µm wide and richly branched, easiest to find in the white mycelium filling the old tubes. Basidiospores truncate, golden-brown, echinulate, 7-12 × 5-8 µm.

ii. *Lentinus squarrosulus* Mont. (1842)

Habitat/Substrata on dead wood

Basidiocarps (KLU-M1197) entirely white, becoming pale straw-colour to pale ochraceous, the scales on the pileus varying pale brownish or pale fuscous in some collections (Figure 3.3).

Figure 3.3 Basidiocarp of *Lentinus squarrosulus*

Pilear surface 1-12 cm wide, convex then plane and umbilicate to infundibuliform, dry, opaque, more or less furfuraceous-squamulose with small and often subrevolute scales, varying appressedly subsquamulose; margin often becoming lacerate. Stem 1-6 cm x 2.5-7 mm, more or less excentric, rarely lateral, subcylindric, fibrous, scurfy-squamulose downwards to the abrupt and often blackish base, in some cases with a slight floccose zone or short collar at the stem-apex.

Gills cream-color, deeply decurrent, crowded, thin, 38-60 primaries 1.5-9 mm wide, 4-6 ranks but in some cases also dichotomous especially near the stem apex, occasionally slightly reticulate at the base, edge entire; hyphal pegs abundant.

Hyphal system dimitic with skeleto-binding cells; generative hyphae 2-8 µm wide, clamped, thin-walled; skeletal cell-bodies 70-800 x 4-8 µm walls 1-2 µm thick, mostly intercalary, some terminal, often becoming more or less solid in the stem; binding processes -750 µm long, 2.5-4.5 µm wide on origin, tapering to filiform tips, becoming very thickwalled, generally one distal and one proximal, sometimes also 1-2 lateral, simple or 1-2 times furcate; oleiferous hyphae in the stem and pileus, as scattered intercalary generative cells -300 x 3-5 µm.

Basidiospores 5.5-9 x 1.8-2.7 µm white, smooth, subcylindric, inamyloid, aguttate

iii. *Earliella scabrosa* (Pers.) Gilb. & Ryvarden (1985)

Habitat/Substrata on dead deciduous trees

Basidiocarps (KLU-M208) perennial, pileate, broadly attached, effused-reflexed or resupinate, solitary, imbricate or fused into horizontal rows, consistency corky to rigid when dry (Figure 3.4).

Pilear surface glabrous, concentrically zonate, sulcate and furrowed, when dry somewhat radially wrinkled, colour cream, ochraceous, pale brown to brownish grey, when older covered with a reddish to bay or brown cuticle spreading from the base and later may cover most of the pileus, but may also lack completely, margin acute, thin, entire or undulate and lobed.

Figure 3.4 Basidiocarp of *Earliella scabrosa*

Pore surface cream, ochraceous or luteous, even fulvous to discoloured in old basidiocarps, often with sterile margin both towards the margin but especially towards the substrate in partly decurrent and effused basidiocarps, pores first round and thick-walled, 2-3 per mm, later more angular, radially elongated and thin-walled, splitting to be dentate, semi-daedaloid to partly lamellate, tubes single layered, 1-3 mm long, concolorous or slightly darker than the context.

Hyphal system trimitic, generative hyphae clamped, thinwalled, hyaline, slightly branched, 1.5-5 μm in diameter, often collapsed and difficult to find. Skeletal hyphae abundant in the whole fruitbody, thick-walled to solid, hyaline to yellow, 2-6 μm in diameter, mostly solid and golden in the context. Binding hyphae thick-walled to solid,

strongly to moderately branched, hyaline to pale yellow, up to 5 µm broad, tapering towards the apex.

Basidiospores cylindrical to oblong ellipsoid, hyaline, smooth and thin-walled, 7-11 × 3-4 µm, non-amyloid.

iv. *Pycnoporus sanguineus* (L.) Murrill (1904)

Habitat/Substrata on decay wood

Basidiocarps (KLU-M250) dimidiate to flabelliform, narrowly attached to the substrate, but also semistipitate or contracted into a stem-like base (Figure 3.5).

Pilear surface 1.5-10 cm in diameter and 1-4 mm thick, with lighter and darker zones, first orange then red to cinnabar, later often intensively red-orange, finally sometimes faded greyish-white with red-orange tints. Young pileus was velvety to warted; while older ones more smooth and shiny. Margin acute, entire or somewhat incised, often lighter than the rest of the pileus.

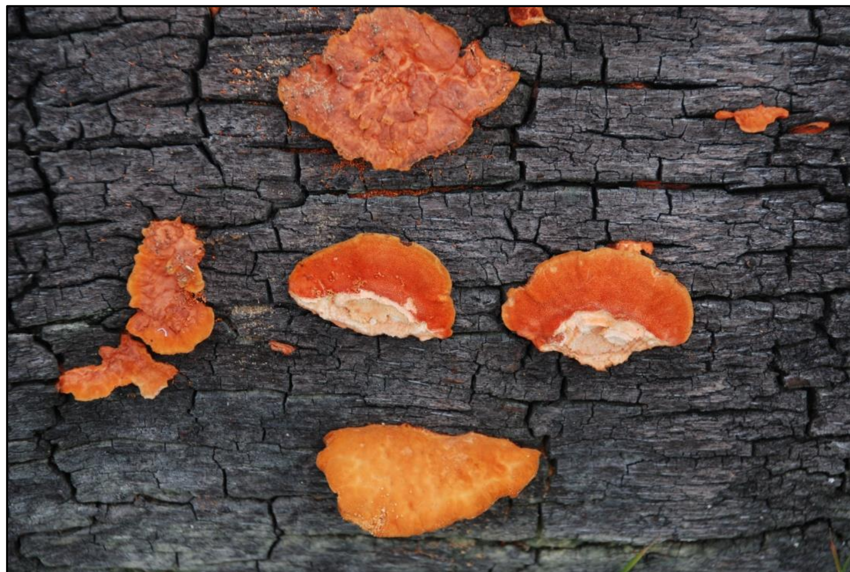


Figure 3.5 Basidiocarp of *Pycnoporus sanguineus*

Pore surface red-orange to cinnabar. Pores circular, 4-6 per mm, tubes in one layer, 0.5-2 mm long, dissepiments initially rather thick, with age gradually becoming thinner.

Hyphal system dimitic, generative hyphae hyaline and thin-walled with clamps, 1-3.5 μm in diameter, frequent in the dissepiments. Skeletal hyphae somewhat thick-walled prevailing in all parts of the fruitbody, 2-6.5 μm wide, unbranched and without septa. The binding hyphae were thick-walled to solid with short branches, 2-2.5 μm thick in the dissepiments and up to 4 μm thick in the context.

Basidiospores short cylindrical to ovate with smooth, hyaline and non-amyloid walls, 4-4.5 x 2-2.3 μm.

v. *Lentinus connatus* Berk. (1842)

Habitat/Substrata (KLU-M1133) forms caespitose, at times imbricate, clusters on dead and decaying wood. The basidiome is frequently large, borne on an erect, elongate stipe but the habit is variable with both lateral an central stipe attachment commonly found.

Basidiocarps surface white to pale ochraceous or at times becoming fuscous from the margin inwards, dry, azonate, initially scurfy tomentose, soon glabrescent to leaves innate greyish brown radial fibrils, smooth and glabrous towards the margin; margin very thin, at first incurved, soon becoming lobed, finally lacerate (Figure 3.6).

Pilear surface 2 – 5 (-20) cm diameters, thin, ranging from applanate to deeply cyathiform or literally stipitate and spathulate to flabelliform

Stipe 2–15 cm × 3–15 mm, central, exentric or lateral, erect, rigid, subcylindric, solid; surface white soon becoming greyish brown or darker, pubescent with minute erect,

fuliginous hairs, glabrescent, finely striate, with the base covered by a cream-coloured tomentum, sometimes developing from a pseudosclerotium.

Figure 3.6 Basidiocarp of *Lentinus connatus*

Lamellae deeply decurrent, with interveining and slight anastomosing over the stipe apex, white then sordid ochraceous, very narrow, 0.2 – 1 mm wide, sometimes sublinear, densely crowded, with lamellulae up to seven lengths; edge entire, frequently retaining floccose velar remnants.

Hyphal system were consisting of a dimitic hyphal system with skeletal hyphae. Generative hyphae 2–7 µm diam., not inflated, very thin-walled, frequently branching, with clamp connections. Skeletal hyphae 2–4 µm diam., cylindric, hyaline, with a thickened wall (-2 µm) and a continuous lumen, mostly sinuous and inbranched but irregular, lateral branches sometimes formed. Spores 6–8 × 2.5-3.5 (7 ± 0.4 × 3.2 ± 0.2) µm, Q = 2.18, short cylindric, hyaline, thin-walled, with few contents. Basidia were 17–22 × 4–5 µm, narrowly clavate which bearing four sterigmata.

vi. *Microporus xanthopus* (Fr.) Kuntze (1898)

Habitat/Substrata on dry deciduous wood

Basidiocarps (KLU-M232) centrally or laterally stipitate and usually infundibuliform, sometimes two or more fruitbodies may grow together to more complicated fruitbodies with several stipes and with imbricate pilei (Figure 3.7).

Pilear surface were up to 10 cm in diameter and 1-3 mm thick, glabrous and shiny when fresh, more dull when dry, yellowish-brown to chestnut in numerous narrow concentric zones, often with alternating dark and light colours, margin thin and wavy.

Stipe round, glabrous, covered with a thin, light yellowish to light brown cuticle, up to 6 cm high and 3-9 mm in diameter, slightly expanded upwards, and expanded to a disclike base up to 10 mm in diameter. The base is covered with a very finely depressed tomentum which slowly wears with age, context of stipe pure white, dense in the periphery, somewhat looser in the core.

Figure 3.7 Basidiocarp of *Microporus xanthopus*

Pore surface cream to pale buff, almost pure white towards the margin, pores entire and very minute, almost invisible to the naked eye 8-10 per mm, tubes up to 0.1 mm deep.

Hyphal system trimitic, generative hyphae thin-walled and with clamps, 2,3,5 μm in diameter, moderately branched, skeletal hyphae dominating, hyaline and thick-walled, up to 6 μm in diameter, binding hyphae tortuous and mostly broken in preparations, thick-walled to apparently solid, up to 1-3 μm in diameter, strongly coralloid dichophytic elements often present along the dissepiments, very finely branched and often partly covered with cyrstalline deposits so their true nature may easily be unnoticed by an untrained observer, in preparations they easily break off at the main stem and we have been unable to verify whether they arise from generative hyphae or represent the outer apices of the binding hyphae.

Basidiospores hyaline, cylindrical, often slightly bent, smooth and non-amyloid, 6-7.5 × 2.0-2.5 μm

vii. *Trametes menziesii* (Berk.) Ryvarden (1972)

Habitat/Substrata on deciduous wood of many kinds, resistant to drying to a certain degree and therefore often found on dead trunks, poles, stumps etc. in open and dry localities.

Basidiocarps (KLU-M230) annual-perennial, very variable in size, pileate and applanate with a narrow contracted base to almost semi-stipitate, semi-circular to even almost circular when grown on top of logs where the lobes behind partly meet over the point of attachment, normally conchate-dimidiate, but also flabelliform to spathulate with an even or strongly incised to lobed margin, single or imbricate with a few pilei growing from the same common origin, up to 15 cm wide and long, 1-10 mm thick,

fairly flexible and tough in fresh, hard when old in thicker basidiocarps, thinner basidiocarps persistently tough (Figure 3.8).

Pilear surface first adpressed velvety, but usually soon becoming glabrous, first white to ochraceous, in most cases becoming greyish in different shades and with age sooty grey to almost black, normally with numerous, narrow, 1-3 mm wide, concentric zones, smooth or slightly sulcate, in larger fruitbodies there are usually some more prominent concentric furrows which apparently mark longer periods with no growth, the areas in between these furrows are then marked with numerous slightly sulcate zones, in some basidiocarps there are dotted warts and protuberances on the pileus, others may develop a variable outgrowth from the base, either flat or radially striate, veined or furrowed and this outgrowth will normally be azonate and paler than the greyish pileus, with age the outgrowth can attain a greyish colour and a new outgrowth may cover it, in such basidiocarps there will be marked zones in the context. On drying the pileus frequently becomes somewhat wrinkled radially. The greyish colour is often darker towards the base. It seems that the greyish to sooty shades develop with age and are somewhat related to the exposure of the fruitbody. In imbricate clusters it is not uncommon to observe that the upper pilei are in shades of grey, while the lower ones are whitish at the base, greyish only at the margin which has been exposed. Old collections in the herbaria have a tendency to become ochraceous to pale fulvous brown with age and the more pure grey colours fade away. Margin was thin and acute, entire or deeply lobed, usually deflexed in dry basidiocarps.

Pore surface first white becoming creamish to pale tan when dry, in old and weathered basidiocarps more pale dirty brown to deep ochraceous, pores variable, partly entire, round and small; 6-7 per mm, but also larger, round to angular and from 2-6 per mm (seee above for variation in the types), in some basidiocarps slightly to distinctly

elongated radially and in some cases deeply incised and dentate and reminding of a tiny *Lenzites*, thus very variable, and many species have been described based on this variation. Tubes are more or less concolorous with the pore surface, up to 4 mm deep.

Figure 3.8 Basidiocarp of *Trametes menziesii*

Hyphal system trimitic, generative hyphae with clamps, hyaline, thin-walled and 2-3 µm wide, skeletal hyphae very common, thick-walled to almost solid, 3-7 µm wide, in old basidiocarps often swelling strongly in 5% (or stronger) KOH and many then become up to 15 µm wide (observe in Melzer if in doubt about the real width), binding hyphae prominent and abundant both in context and trama, often with a fairly wide trunk with numerous kinked thin and solid side branches which are repeatedly branched and break easily in sections, main trunk up to 6 µm wide, side branches tapering down to 1 µm.

Basidiospores ellipsoid to cylindrical, 5-7 x 12 µm, hyaline, smooth and non-amyloid, usually very difficult to find in dry basidiocarps.

viii. *Lenzites elegans* (Spreng.) Pat. (1900)

Habitat/Substrata

Basidiocarps (KUM60948; KLU-M#) annual to perennial, sessile or with a short stipelike base, attached laterally or centrally, pileus 1-35 cm wide and long and 0.2-3 cm thick, corky and flexible when fresh, more rigid when dry (Figure 3.9).

Pilear surface flabelliform or circular, upper surface white to grey or almost buff ochraceous in older basidiocarps, surface very finely tomentose, soon glabrous, smooth or concentrically sulcate, often warted or with slightly uneven elevated areas. Margin was thin and often deflexed, even or lobed.

Figure 3.9 Basidiocarp of *Lenzites elegans*

Pore surface very variable, partly poroid, round to angular, 1-2 per mm, partly sinuous-daedaloid and radially split, up to 2 mm wide, partly purely lamellate with straight to sinuous lameleae, 4-7 per cm measured tangentially. This variation may occur in a single specimen. Even in poroid basidiocarps some parts the hymenophore will usually have a few lamellae or sinuous pores. Pores or lamellae were up to 6 mm deep.

Stipe absent or up to 3 cm long, 1.5 cm in diameter, glabrous, solid, attached to the substrate with a disc up to 3 cm wide, concolorous with pileus surface, mostly white to pale cream.

Hyphal system were trimitic, generative hyphae hyaline, thinwalled and clamped, 2-4 μm wide, not always easy to find in dried basidiocarps. Skeletal hyphae dominating, yellow to golden and thick-walled to solid, 3-7 μm in diameter, binding hyphae hyaline to pale yellow thick-walled, up to 5 μm wide, irregular branched.

Basidiospores are cylindric to oblong ellipsoid, hyaline, smooth and thin-walled with sizes 5-7×2-3 μm, non-amyloid.

ix. *Lentinus sajor-caju* (Fr.) Fr. (1838)

Habitat/Substrata on dead and fallen trunks and branches in the forest and in the open

Basidiocarps (KLU-M1159) dry, smooth or often with small appressed squamules in the centre, often minutely and innately streaked, white, cream-colour, pale ochraceous, more or less fuliginous, or brownish, very variable in colour (Figure 3.10).

Pilear surface were 3-20 cm wide, convex with umbilicate centre, then infundibuliform or excentric and flabelliform.

Stem 0.8-4 cm x 5-15 mm, central, excentric, or lateral, short, cylindric, base abrupt, concolorous with the pileus; ring 1-3 mm wide, firm, with entire edge, developed from the stem around the primordial pileus and free from it, often absent.

Gills deeply decurrent, very crowded, narrow, 0.3-2 mm wide, 3-5 ranks, not dichotomous or only near the stem-apex, minutely hispidulous with hyphal pegs, concolorous with the pileus but often pallid, darker fuscous towards the entire edge.

Figure 3.10 Basidiocarp of *Lentinus sajor-caju*

Hyphal system Generative hyphae 2-7 µm wide, clamped. Skeletal cell-bodies 70-400 µm long, 4-8.5 µm wide, intercalary and terminal; binding processes -270 gm long, simple or occasionally once furcate.

Basidiospores 5.5-7.5 x 1.7-2.5 µm white, smooth, subcylindric, inamyloid, aguttate

x. *Microporus affinis* (Blume & T. Nees) Kuntze (1898)

Habitat/Substrata on dead wood

Basidiocarps (KLU-M246) annual, solitary or in groups, laterally stipitate or with a distinct stipe, flabelliform, spathulate, semicircular dimidiate, flat or depressed in the area around the stipe, pileus up to 10 cm long and 8 cm wide, sometimes imbricate with several pilei arising from the same point of attachment, this is most common in basidiocarps with a very short stipe, up to 6 mm thick where the stipe meets the pileus, margin thin and usually flat (Figure 3.11).

Pilear surface glabrous or tomentose, strongly zoned or banded, either as narrow bands or as slightly raised sulcate zones, colour very variable from light yellowish via brown and chestnut and bay to almost black, usually darker at the center than along the margin, the tomentum is very variable, but when present, it is light greyish and adpressed, more or less velutinate, there are basidiocarps which are completely glabrous, others which are only tomentose around the margin. Even in basidiocarps which appear glabrous to the naked eye, it is possible with a strong lens to observe narrow bands with projecting short hairs (skeletal hyphae) in older parts of the fruitbodies or short, scattered hyphal ends evenly distributed over the pileus.

Figure 3.11 Basidiocarp of *Microporus affinis*

Stipe lateral, up to 4 cm long and prominent to almost lacking, 2-8 mm in diameter, usually expanded both towards the base and the pore surface, round to slightly flattened, first greyish and finely adpressed tomentose, later glabrous in parts and then almost black with a distinct crust over a white context, at the base usually expanded into a circular disc, more or less persistently covered with a tomentum, cuticle up to 150 µm

thick and black in section, below the cuticle there is a looser layer of hyphae around a very dense and hard core, the latter is most prominent in old and thick basidiocarps.

Pore surface light cream, later pale ochraceous and even greyish in some basidiocarps with the grey colour spreading frm the base, margin 1-3 mm wide and pure white, pores round and entire, very minute 7-10 per mm, tubes light cream, up to 1 mm deep.

Hyphal system trimitic, generative hyphae hyaline and with clamps, 1.5-3 µm in diameter, skeletal hyphae in the context straight to slightly flexuous, 3.5-6 µm in diameter, thick-walled to almost solid, with a more distinct lumen in the tomentum and up to 10 µm in diameter, binding hyphae common in the context and trama, tortuous and much branched, mostly 1.5-3 µm in diameter, but near the base thicker and up to 6 µm in diameter and here with fewer side branches, very finely coralloid elements present along the dissepiments partly occluding the poremouths.

Basidiospores short cylindrical to oblong ellipsoid, some slightly concave, hyaline and thin-walled, non-amyloid and non-cyanophilous, 3-4 x 1.5-2 µm (measured from sporeprint).

3.3.3 New records of Polyporales in Peninsular Malaysia

Fifty species identified in this study were also reported by the previous workers. Nevertheless, the study have identified 10 species which were not yet reported by the previous studies; *Coriolopis badia, Coriolopsis sanguinaria, Echinochaetae brachypora, Fomitopsis ostreiformis, Funalia polyzona, Gloeoporus dichrous, Polyporus* cf. *badius, Polyporus philippinensis, Trichaptum bysogenum* and *Trichaptum durum*. Information including the host, distribution, materials examined, herbarium number (KLU-M) and University of Malaya Collection Number (KUM) are given where applicable. Each new record is described here with the etymology of the specific

epithet and Latin diagnosis. The descriptions of new record of Polyporales fungi from Peninsular Malaysia are as follow:

1. Fomitopsidaceae - *Fomitopsis ostreiformis* (Berk.) T. Hatt. 2003

Basidiocarps sessile, effuse-reflexed, creamy white. Pileus up 5 cm in radius, up to 10 cm wide, sub-ungulate then dimidiate, fusing laterally, often imbricate, uneven, sub-tomentose, not sulcate or zoned; margin obtuse, entire. Tubes 9 mm long; pores 3-4 per mm. Context 8-18 mm thick at the base of the pileus, fibrous then corky tough. On a dead fallen and burnt trunk in the open. On decayed wood, Institute of Biological Sciences, University of Malaya, Kuala Lumpur (KUM60845; KUM60849; KUM60853) (Figure 3.12).

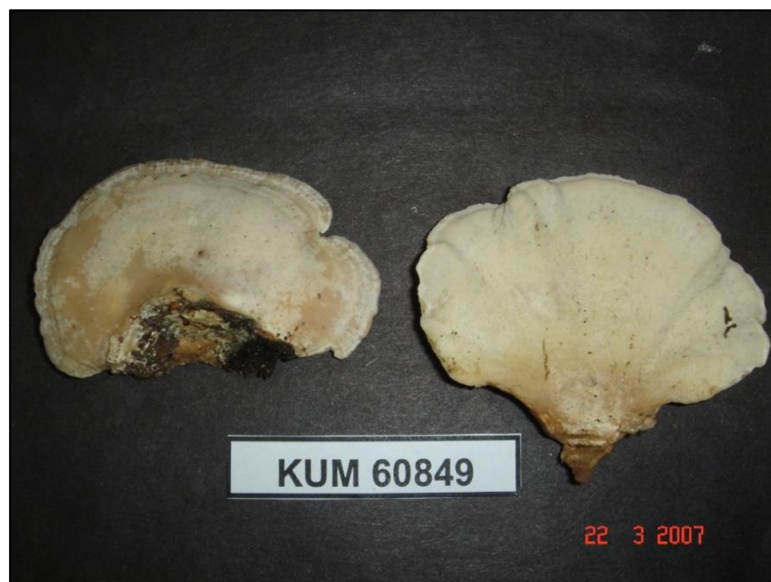


Figure 3.12 Basidiocarp of *Fomitopsis ostreiformis*

2. Meruliaceae - *Gloeoporus dichrous* (Fr.) Bres. 1912

Basidiocarps annual. resupinate to pileate. Often effused-reflexed, mostly imbricate with several small, shelflike. Narrow and elongated pilei, soft when fresh, resinous and hard when dry, pilei rarely above 4 cm wide, 10 cm long and 5 mm thick at the base;

upper surface white to cream. at first finely tomentose. With concentric zones in different shades, margin sharp and undulating; pore surface at first light reddish, soon dark purplish, more brown when old, when actively growing often pruinose and white along the dissepiments, margin white, wide, strongly contrasting with the dark pore surface, pores circular to angular, 4-6 per mm, often not more than a reticulate pattern, up to 1 mm deep. Context was pure white, up to 4 mm thick, cottony to loose. Collected on dead wood, Kuala Jasin, Endau Rompin (KLU-M540) (Figure 3.13).

Figure 3.13 Basidiocarp of *Gloeoporus dichrous*

3. Polyporaceae - *Coriolopsis badia* (Cooke) Murrill 1907

Basidiocarps perennial, ungulate, attached sub-laterally 2 x 4 cm at point of attachment; Upper surface glabrous, brownish black, indurate, appearing in cross section as a black line 0.2 mm thick. Margin is obtuse, paler than the pilear surface. Pore surface flat, glancing slightly, dark brown, sterile margin narrow, pores 5-6 per mm, mostly angular. Tube layers up to 7 mm thick each, individual layers not distinct, pale brown; Context dull yellowish brown tissue, hard. On dead wood, rotting stump and on fallen trunk in Endau Rompin National Park, Johore (POR59); Kuah Jetty, Langkawi, Kedah

(KUM60161) and University of Malaya, Kuala Lumpur (KUM60937;KUM60850) (Figure 3.14).

Figure 3.14 Basidiocarp of *Coriolopsis badia*

4. Polyporaceae - *Coriolopsis sanguinaria* (Klotzsch) Teng 1963

Basidiocarps annual to perennial, pileate to resupinate, solitary to densely imbricate or often, as several pilei, more or less fused along the upper edge of a widely effused basidiocarp, single pilei rarely above 6 cm wide and 10 cm long, 2-4 mm thick, sessile, dimidiate, conchate to flabelliform to reniform, margin undulating, frequently lobed or incised and sharp. Pileus glabrous, rarely smooth, commonly with a finely warted or rough surface and with some faint radial striae, azonate or with some weak concentric zones. Pore surface applanate or widely effused and decurrent on the substrate, ochraceous when young, cinnamon to deep fulvous in older specimens, frequently, but not always, with a whitish-bluish-ashy bloom or tint. Pores were entire, round to slightly angular, 5-8 per cm, in some specimens almost invisible to the naked eye, tubes up to 4 mm long, concolorous with pore surface. Context was fibrous, golden brown to

cinnamon, 2-8 mm thick. Encountered on dead wood in Kilim, Langkawi Kedah (KUM60073) (Figure 3.15).

Figure 3.15 Basidiocarp of *Coriolopsis sanguinaria*

5. Polyporaceae - *Echinochaete brachypora* (Mont.) Ryvarden 1978

Basidiocarp was annual, usually solitary, dimidiate up to 10 cm from the base to margin, 11 cm wide and 0.7 cm thick, thinning out, towards the margin. Pileus was dimidiate to flabelliform narrowing behind to a distinct stipe, whitish pink when fresh, rust-coloured to dark cinnamon, when dry, azonate. Margin entire, often depressed when dried. Stipe was usually short, solid, up to 1 cm long and broad, often darker than the pileus, upper part finely tomentose, on the lower side partly covered with pores or radiate ribs. Pore surface whitish-pink when fresh, dark rust-coloured when dry, pores angular 1-2 per mm. Context pale wood-coloured to umber, up to 3 mm thick. Encountered on dead wood, Gombak, Selangor (POR28) (Figure 3.16).

Figure 3.16 Basidiocarp of *Echinochaete brachypora*

6. Polyporaceae - *Funalia polyzona* (Pers.) Niemelä 2003

Basidiocarps annual to perennial, pileate, sessile, dimidiate, flabelliform to reniform, sometimes reflexed with an effused and resupinate pore surface, commonly broadly attached, single pilei up to 10 cm wide and 15 cm long, 2-7 mm thick at the base, flexible to corky. Pileus was yellowish when fresh, soon darker to brown or greyish-brown. Pore surface cream when fresh, darkens to golden-brown, pores angular to round, on average 2-3 per mm. Context duplex, lower part fibrous, ochraceous to golden brown, darker towards the base, upper part loose and more faded. Encountered on dead wood, Institute of Biological Sciences, University of Malaya, Kuala Lumpur (KUM60842) (Figure 3.17).

7. Polyporaceae - *Polyporus* cf. *badius* (Pers.) Schwein. 1832

Basidiocarps annual, laterally to centrally stipitate, solitary or clustered; pileus circular or flabelliform, up to 15 cm wide; upper surface light chestnut brown to dark blackish brown, azonate to radially striate, glabrous, smooth or rugose on drying; hymenophore white to pale buff, pores circular to angular, 5-8 per mm, tube layer white when young,

becoming slightly darker than the context, up to 1 mm thick, decurrent on the stipe; context pale buff, azonate, corky, up to 1.5 cm thick; stipe black and minutely tomentose at the base, chestnut brown and glabrous at the apex, up to 5 cm thick. Basidiospores cylindric. On dead wood, Hutan Lipur Kanching, Selangor (KUM60231) (Figure 3.18).

Figure 3.17 Basidiocarp of *Funalia polyzona*

Figure 3.18 Basidiocarp of *Polyporus* cf. *badius*

8. Polyporaceae - *Polyporus philippinensis* Berk. 1842

Basidiocarps annual, solitary or in clusters, pileate, dimidiate, spathulate or flabelliform with a contracted or tapering base, up to 10 cm wide and long, 3-4 mm thick at the base, rather hard when dry. Pileus pale ochcraceous to pale brown, radial striae present as fine lines towards the margin, towards the base. Hymenophore ochraceous to pale brown, pores angular, distinctly elongated radially, 0.5-1 mm wide, 1-3 mm long, tubes concolorous with pore surface, up to 2 mm thick. Context ochraceous, homogeneous, up to 2 mm thick. On dead wood, Institute of Biological Sciences, University of Malaya, Kuala Lumpur (/POR26/KLU-M221) (Figure 3.19 - http://www.fungiphoto.com).

Figure 3.19 Basidiocarp of *Polyporus philippinensis* (http://www.fungiphoto.com)

9. Polyporaceae - *Trichaptum byssogenum* (Jungh.) Ryvarden 1972

Basidiocarp annual to perennial, pileate-sessile, to deflexed and resupinate, solitary to imbricate or several specimens broadly attached to fused laterally in rows, consistency flexuous to tough. Pileus semicircular up to 8 cm broad and 15 cm long and up to 10 mm thick near the base, flat and finely concentrically sulcate and radiately striate, hispid

to tomentose, especially in the inner parts, colour grey to ochraceous, margin thin, depressed to enrolled. Pore tube pale vinaceous buff, pores very variable, first circular to angular and thin-walled, 1-3 pores per mm, and this type of hymenium will almost always be found along the margin of older specimens, later the pores split up making the hymenium with some specimens may remain poroid. Context very thin 0.1-0.3 mm, buff, clay buff to wood-coloured. On decayed wood, Forest Research Institute Malaysia (MC07-434) (Figure 3.20).

Figure 3.20 Basidiocarp of *Trichaptum byssogenum*

10. Polyporaceae - *Trichaptum durum* (Jungh.) Corner 1987

Basidiocarps perennial, usually rather small, solitary or imbricate, applanate to ungulate, mostly dimidiate, more rarely broadly attached on a decurrent pore surface, up to 8 cm long and 6 cm wide, 2-20 mm thick at the base, woody hard. Pileus first finely tomentose and soon more glabrous, mostly azonate. Margin rather acute. Hymenophore dark brown, pores round and entire, almost invisible to the naked eye, 8-10 per mm. Tubes up to 5 mm deep, almost blackish, indistinctly zonate, tubes often with a white

lining of a hymenium. Context umber to dark brown or vinaceous brown, up to 10 mm thick. On decayed wood, Langkawi, Kedah (KUM70135) (Figure 3.21).

Figure 3.21 Basidiocarp of *Trichaptum durum*

3.3.4 Pure cultures of Polyporales.

Polyporales fruit bodies were collected from a number of different locations. Macrofungal basidiocarps were collected during the fieldwork for this study and Herbarium specimens were prepared from fungi collected throughout peninsular Malaysia. These fungal fruit bodies were usually identified to species. These basidiocarps are being held at the University of Malaya Herbarium (acronym KLU), Kuala Lumpur. Fungal cultures of all fresh basidiocarps collected were obtained by excising 3-5 small sterile pieces of fruit body from within the cap or stipe. These pieces were then incubated for two weeks on MEA. Isolation was considered successful when the majority of fruit body pieces resulted in the same mycelium. This mycelium was then subcultured and maintained on MEA at 25 ± 2 ºC for the duration of the study. Copies of each isolate have been lodged with the Mushroom Research Centre, University of Malaya culture collection in Kuala Lumpur (Table 3.3).

Table 3.3 Pure cultures of Polyporales.

	Species	Culture Code	Location
	Fomitopsidaceae		
1	*Fomitopsis ostreiformis* (Berk.) T. Hatt. (2003)	KUM 60845	Kuala Lumpur
2	*Fomitopsis ostreiformis* (Berk.) T. Hatt. (2003)	KUM 60849	Kuala Lumpur
3	*Fomitopsis ostreiformis* (Berk.) T. Hatt. (2003)	KUM 60853	Kuala Lumpur
	Ganodermataceae		
4	*Amauroderma subrugosum* (Bres. & Pat.) Torrend (1920)	KUM 60936	Pahang
5	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 60848	Kuala Lumpur
6	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 60851	Kuala Lumpur
7	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 60860	Johore
8	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 60905	Johore
9	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 60813	Pahang
10	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 60816	Pahang
11	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 60819	Pahang
12	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 70069	Johore
	Polyporaceae		
13	*Coriolopsis badia* (Berk.) Murrill (1907)	KUM 60850	Kuala Lumpur
14	*Coriolopsis badia* (Berk.) Murrill (1907)	KUM 60937	Kuala Lumpur
15	*Earliella scabrosa* (Pers.) Gilb. & Ryvarden (1985)	KUM 60863	Johore
16	*Earliella scabrosa* (Pers.) Gilb. & Ryvarden (1985)	KUM 60864	Johore
17	*Earliella scabrosa* (Pers.) Gilb. & Ryvarden (1985)	KUM 60942	Negeri Sembilan
18	*Favolus tenuiculus* P. Beauv. (1806)	KUM 60803	Negeri Sembilan
19	*Funalia polyzona* (Pers.) Niemelä (2003)	KUM 60842	Kuala Lumpur
20	*Hexagonia tenuis* (Hook.) Fr. (1838)	KUM 60935	Pahang
21	*Lentinus squarrosulus* Mont. (1842)	KUM 70037	Selangor
22	*Lentinus strigosus* (Schwein.) Fr. (1838)	KUM 70091	Pahang
23	*Lenzites elegans* (Spreng.) Pat. (1900)	KUM 70081	Pahang
24	*Lenzites elegans* (Spreng.) Pat. (1900)	KUM 60948	Negeri Sembilan
25	*Microporus xanthopus* (Fr.) Kuntze (1898)	KUM 70012	Selangor
26	*Polyporus arcularius* (Batsch) Fr. (1821)	KUM 70178	Pahang

Table 3.3, continued.

27	*Pycnoporus sanguineus* (L.) Murrill (1904)	KUM 60840	Pahang
28	*Pycnoporus sanguineus* (L.) Murrill (1904)	KUM 70172	Pahang
29	*Pycnoporus sanguineus* (L.) Murrill (1904)	KUM 70173	Pahang
30	*Trametes* cf. *hirsuta* (Wulfen) Lloyd (1924)	KUM 60839	Kuala Lumpur
31	*Trametes scopulosa (Berk.) Bres. (1912)*	KUM 70034	Selangor
32	*Trametes lactinea* (Berk.) Sacc. (1888)	KUM 60852	Kuala Lumpur
33	*Trametes menziesii* (Berk.) Ryvarden (1972)	KUM 70111	Selangor
34	*Trametes modesta* (Kunze ex Fr.) Ryvarden (1972)	KUM 60812	Pahang
	Meripilaceae		
35	*Meripilus applanatus* Corner (1984)	KUM 60946	Negeri Sembilan
36	*Rigidoporus microporus* (Sw.) Overeem (1924)	KUM 60869	Johore
	Meruliaceae		
37	*Flavodon flavus* (Klotzsch) Ryvarden (1973)	KUM 60843	Kuala Lumpur

3.3.5 Phylogenetic study of selected *Ganoderma* species.

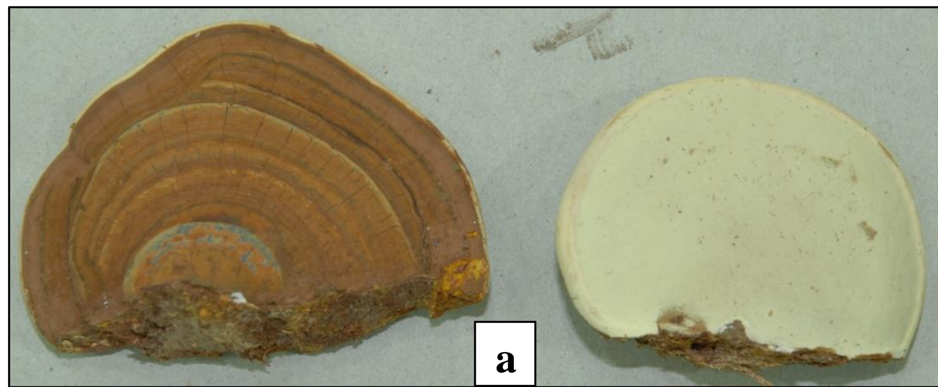
In this study, two strains receieved as *G. lucidum*; KUM61129, KUM61130 and two strains received as *G. tsugae* KUM50079 and *G. amboinense* KUM61117 respectively were obtained from local mushrooms growers, whereby the origin was uncertain. These strains were commercially cultivated while one culture i.e. KUM61088 received as *G. lucidum* originally was from China. In addition, two wild strains i.e. KUM61076 and KUM61120 morphologically identified as *G. lucidum* were collected from Bahau, Negeri Sembilan and Pahang respectively.

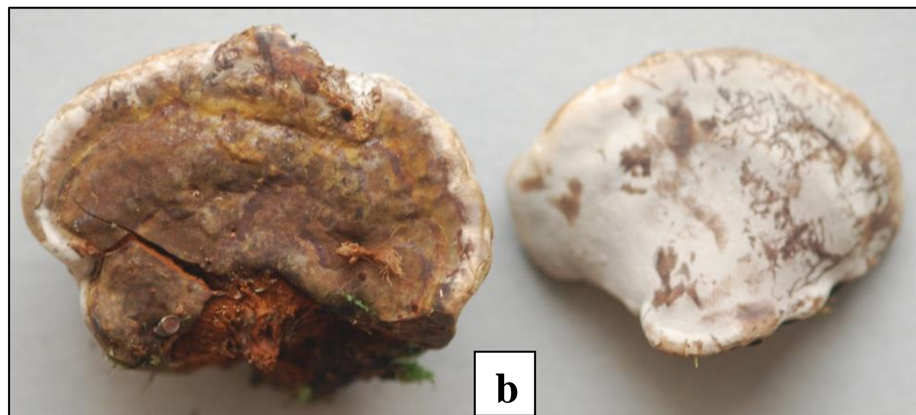
Generally, the specimens of *G. lucidum* in this study were having annual basidiocraps, more frequently with a lateral stem and then usually reniform except for *G. amboinense* KUM61117 which morphologically formed like deer antler (Plate 3.1).

Plate 3.1 *Ganoderma lucidum* strains used in phylogenetic analyses, (a) *G. lucidum* KUM61076, (b) *G. lucidum* KUM61120, (c) *G. tsugae* KUM50079, (d) *G. lucidum* KUM61129, (e) *G. lucidum* KUM61130, (f) *G. amboinense* KUM61117.

Pileus surface are radially rugose and concentrically sulcate, brilliantly laccate and light reddish brown to dark reddish brown or mahogany (KUM61076, KUM61120, KUM50079, KUM61129 and KUM61129). As for *G. amboinense* KUM61117, is the anthler shaped basidiocarp. *Ganoderma amboinense* differ from the ordaninary Ganoderma in that it does not emit spores during its growth and maturation. Margin are sterile; generally thick blunt and sometimes acute white in actively growing specimens, then becoming yellowish and reddish brown inwards in older specimens of the same colour as pileus surface, and then incurved. Stem was lateral, vertical, cylindric, usually long, slender, tortuous, reddish black to almost black, laccate, brilliant, somewhat thicker at the base. Context was almost as thick as the tube layer but thickening towards the base of the stem, ochraceous brown when young to dark brown when mature. Hymenophore was white to yellowish white when young, greyish white in mature specimens with a tube layer slightly lighter than context. Pores were small, round and somewhat irregular. Basidiospores are subovoid with the apex truncate, perisporium hyaline, smooth and thin, and endosporium golden with relatively scant endosporic pillars, wide and long, reaching the perisporium and rumpling it so that it appears strongly "rugose". As for specimens KUM61076 and KUM61120, both strains were found on decayed bamboo.

On the other hand, two wild strains of *G. australe* were collected from Kelantan; KUM61056, KUM61057 and one wild strain collected from Pahang, KUM60813. Each of single strain exhibited different morphological characteristics (Plate 3.2). Generally all three specimens have perennial basidiocarp with variable size in single basidiocarps. The basidiocarps were also dimidiate and semicircular in outline, except for KUM61057 which exhibited a pseudostipe which size about 1.0 cm long. All specimens were woody when dry and having dull, cocoa brown to deep umber except for KUM61057 which has slightly laccate on the surface towards the margin.





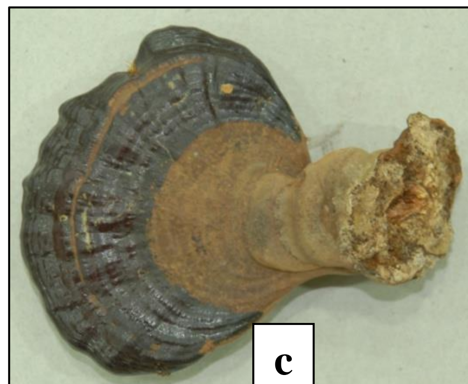


Plate 3.2 ***Ganoderma australe*** **strains used in phylogenetic analyses, (a)** ***G. australe*** **KUM61056, (b)** ***G. australe*** **KUM60813, (c)** ***G. australe*** **KUM61057.**

Surface glabrous, smooth, mostly distinctly sulcate in variable zones, somewhat cracking with age and drying; black cuticle present and increasing in thickness towards the base, margin light colour in actively growing specimens, whitish to yellowish. Pore surface white to cream in actively growing specimens, then dark when bruise, in older and resting species, pale to umber-brown. Pores round, entire, thick-walled, 3-5 per mm. tubes dark brown in section with light-colour tube-walls, in older parts often stuffed with white mycelium. Context are evenly dark brown, rarely with some white spots, in most specimens with one or several horizontal black resinous or melanoid bands above the tubes, but these bands are apparently absent in some specimens.

The amplification of the ITS region have yield PCR products of approximately 650 bp long. The final alignment of the 19 sequences included 610 positions. After exclusion of the conserved 5.8S region and ambiguous sites at both ends, 611 sites were used for the MP analysis. Of the 610 included sites, 519 were constant, and 15 were variable, but parsimoniously were uninformative, and 76 were parsimoniously informative. The topologies of the 121 trees were generally identical. One of them is presented in Figure 3.23. In this tree, specimens labeled as *G. lucidum* and *G. australe* were separated into two distinct clades with *Amauroderma rude* as the out group.

Clade A is composed of ten strains originally determined as *G. lucidum*, with two from Japan, two from China, one from Philippines and five strains from Malaysia (two wild strains and three cultivated strains). This clade was very strongly supported by the bootstraps analysis (100%).

Clade B is composed of seven strains originally determined as *G. australe*, with four sequences retrieve from GenBank which originally from both Malaysia and Thailand. Three wild strains from this study were also group together in Clade B which was strongly supported by the bootstraps analysis (100%).

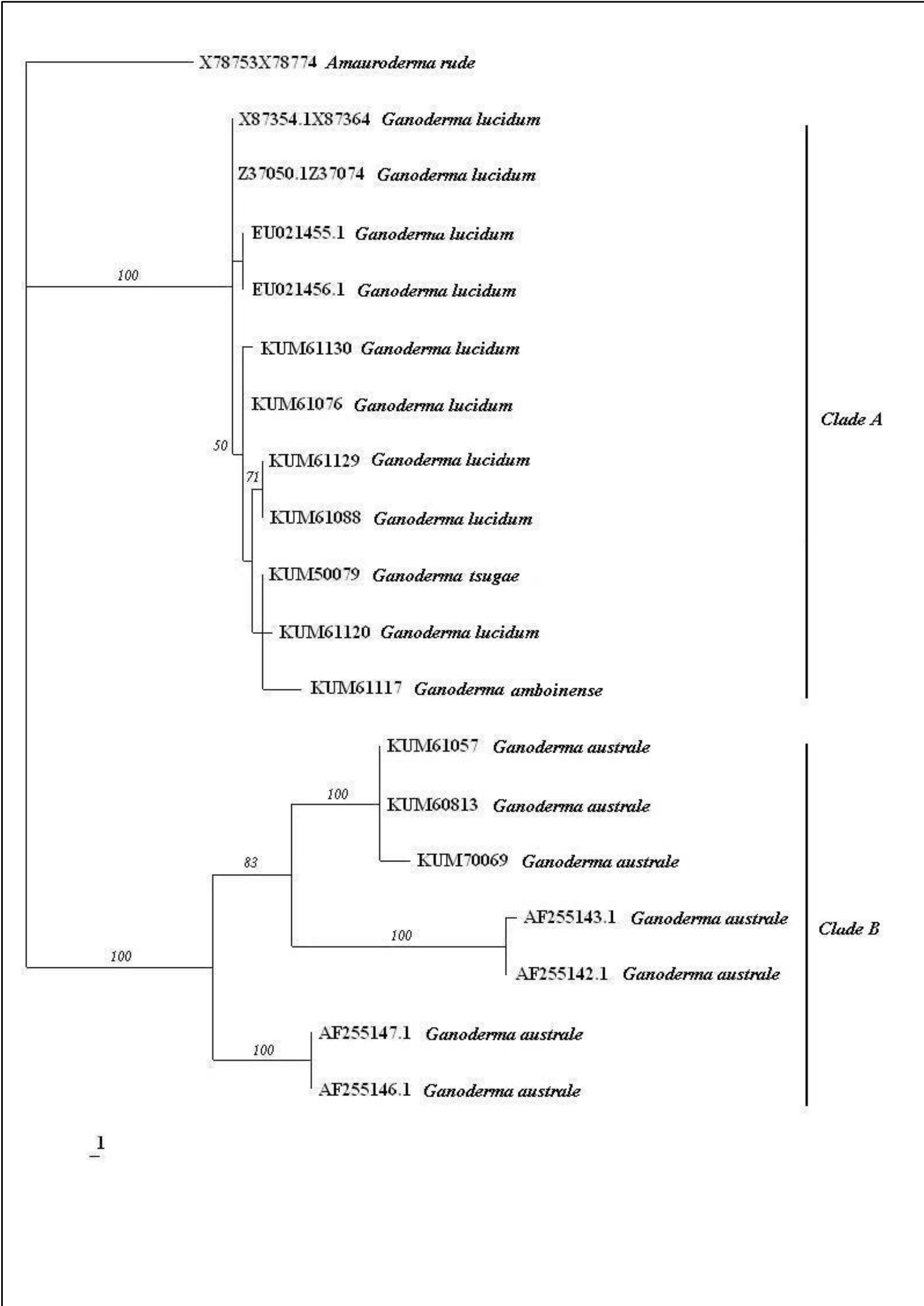


Figure 3.22 Results of the phylogenetic analyses obtained from ITS sequences data. Only bootstrap values ≥ 50% are shown.

3.4 DISCUSSION

Fungi constitute one of the most diverse groups of organisms. There are approximately 75,000 described species of fungi, but this is regarded as significantly fewer than the actual number of species. There has been a good deal of controversy about estimates of true fungal diversity. However, a figure of 1.5 million species has been proposed as a working hyphothesis (Hawksworth, 1991) and has been accepted by most mycologist as an estimate, although some consider it too low (O'brien *et al.,* 2005). This figure is based largely on field data from the tropic, considerably greater fungal diversity than temperate (Lodge *et al.,* 1995).

The most advanced group of fungi is phylum Basidiomycota, containing about 30,000 described species, or 37% of the described species of true fungi (Kirk *et al.,* 2001). The extrapolated global diversity of the Basidiomycota is estimated to be in the region of 180,000 species (Rossman, 1994). The largest subphylum of the Basidiomycota is the Agaricomycotina, the most familiar and conspicuous of all the fungi, because they mostly produce large fruiting bodies. Included in the group are the gilled mushroom (agarics), puffballs, bracket fungi (polypores), corticoid fungi and many others. This study has confined the diversity and distribution of Polyporales because they are the most conspicuous group of fungi and are important in term of ecosystems functions and biotechnology application. Moreover, there is much more data available on the diversity and distributions of this group than other fungal groups in Peninsular Malaysia. According to Ainsworth & Bisby's dictionary of fungi (Kirk *et al.,* 2001) there are twenty-three families in the Order Polyporales.

The diversity and distribution of Polyporales in Peninsular Malaysia were done by collecting and identified the Polyporales basidiocarps to species level using the keys in Núňez and Ryvarden (2000, 2001) and Hattori (2000, 2005). In addition to published

records, there are number of unpublished record collections held by the Mushroom Research Centre, University of Malaya, Kuala Lumpur which the identity of the basidiocarps are not yet confirmed due to the scarcity of expert taxonomist in different groups and the conditions of the specimens itself. The precise identification of this group to the species level is not an easy task. The sources of references are limited to identify specimens from tropical region because the identification is solely based on the key available for specimens described from temperate region. Therefore, accessing and analyzing the data in these collections was beyond the scope of this study, but it is likely that the information contained therein, if channeled through a proper identification approach, would add considerably to the tally of Polyporales records in Peninsular Malaysia in total. An important repository for fungal specimens and record is the University of Malaya Herbarium (acronym KLU), Kuala Lumpur.

Diversity and distribution of Polyporales

The first published record of Polyporales in Peninsular Malaysia was published by Cooke in 1883 (Cooke, 1883). In this work the author recorded various species of polypores from the Malay Peninsula especially from Perak. Since the publication of Fungi from Perak in 1883, few other scientific studies have reported the fungal diversity particularly the Polyporales in Peninsular Malaysia (Chipp, 1921; Corner, 1935; Lim, 1972; Oldridge, 1985; Kuthubutheen, 1981). After that, there is no record of any publication on the diversity and distribution until the late 20st century. Starting from the year 1995, there are few publications reporting the Polyporales diversity apart from the other groups of macrofungi (Lee *at al*., 1995; Salmiah and Thilainathan, 1998; Salmiah and Jones, 2001; Noorlidah *et al*., 2005, 2007, 2009; Noraswati *et al*., 2006; Hattori *et a*l., 2007; Sumaiyah *et al*., 2007).

This study documented the diversity and distribution of Polyporales over the six years conducted in 40 localities in Peninsular Malaysia. Although literature focusing on wood-inhabiting Polyporales in Peninsular Malaysia is rather scarce, the total number of species found in this study (60 species) is in accordance with the findings of previous studies. The identified specimens does not suffice for any thorough conclusions on the Polyporales species, their host relationships and distribution ranges because 60 species only make a part of the total 155 species reported by Chipp, (1921); Oldridge *et al.,* (1985); Lim, (1972); Kuthubutheen, (1981); Lee *et al.,* (1995); Salmiah and Thillainathan, (1998); Salmiah and Jones, (2001); Hattori *et al.,* (2007) and Noorlidah *et al.,* (2009). Forty-four species identified in this study were also reported by the previous workers. Past expeditions have visited the Federal Territory of Kuala Lumpur and six states in total and have revealed much information about Polyporales records new to Peninsular Malaysia. This study have identified 10 species which were not yet reported by the previous studies; *Coriolopis badia, Coriolopsis sanguinaria, Echinochaetae brachypora, Fomitopsis ostreiformis, Funalia polyzona, Gloeoporus dichrous, Polyporus* cf. *badius, Polyporus philippinensis, Trichaptum byssogenum* and *Trichaptum durum*.

Records of Polyporales in general in the Peninsular Malaysia tend to be highly clustered and localized. The uneven distribution of species, with a few dominating and a majority represented with less than five specimens, is typical for studies in fungal ecology as described by Tofts and Orton, (1998). The highest numbers of species identified in this study was from Selangor followed by Kedah, Johore, Pahang, Negeri Sembilan, Kuala Lumpur and Kelantan. The high number of species in Selangor was contributed by the frequent collections made during the study period compared to the other study sites. Conversely, in Johore, the high numbers of species identified were not reflected by the frequent collection but the conditions of the forest itself. As one of the

national park in Peninsular Malaysia, the forest environments are well-preserved with only limited areas of the park is open for ecotourism. Additionally, the entry to the park requires a special permit from the Johore National Park Corporation. This will provides areas less affected by human activities where a broader range of Polyporales species could be encountered.

The differences in the occurrence of Polyporales in the various localities in this study also can be attributed to several factors such as rainfall, quantities of suitable substrata, damp forests with constant high air humidity, and type of forest (Salmiah and Jones, 2001). This study, however was not undertaken based on the 'fruiting seasons' proposed by Corner (1935, 1988). The low number of Polyporales recorded in this study could also be due to the frequency of collection at each site. Areas closed to Kuala Lumpur and Selangor, for example, have greater numbers of records. Many of the Polyporales records for respective locations can be attributed to the work of mycologists in the University of Malaya. A comparison of fungal records between the study sites conducted in the Peninsular Malaysia for Polyporales based on the family were discussed in this study. There are five families recorded during the study period including the specimens from the University of Malaya Herbarium i.e. Polyporaceae, Ganodermataceae, Fomitopsidaceae, Meripilaceae and Meruliaceae.

Family Polyporaceae

The largest apparent disparity in the profile of fungal species in Peninsular Malaysia is compared among six states and the Federal Territory of Kuala Lumpur. Relatively high numbers of species recorded in the Polyporaceae, the most biodiverse family, which comprise 46 species recorded in this study. Marginally more than 50% of total species recorded. All species of Polyporaceae recorded in this study have been found in previous published records except for *Coriolopsis badia, Coriolopsis*

sangunaria, Echinochaetae brachypora, Funalia polyzona, Polyporus cf. *badius, Polyporus philippinensis, Trichaptum byssogenum* and *Trichaptum durum*. Some species are comparatively well represented in Peninsular Malaysia; for example *Earliella scabrosa, Lentinus squarrosulus, Microporus xanthopus, Pycnoporus sanguineus* and *Trametes menziesii* recorded at least one basidiocarp for these species across all study sites.

Family Ganodermataceae

Two genera have been found in Peninsular Malaysia, and one of these (*Ganoderma*) is found in every other study sites. The most striking disparity in the Ganodermataceae is the *G. lucidum* complex which only found at specific host which is decayed bamboo. On the other hand, one of the common species recorded in this study is *G. australe* which easily encountered across all study sites. The identification of the specimens was difficult because the macromorphological characters of *Ganoderma* are extensively variable. Therefore, selected specimens were further identified using molecular approach.

Family Fomitopsidaceae

Species of *Fomitopsis feei* has been encountered at all study sites except Kelantan and Kuala Lumpur. Of theses, Johore have four basidiocarps described and it is quite possible that their existence in other study sites has yet to be discovered. Numbers of this family are relatively poorly recorded in Peninsular Malaysia; for example *Daedalea*, which is brown-rot fungi and the shortage of suitable habitat, may be a factor in the poor representation in Peninsular Malaysia compared to other families which most are white-rot fungi.

Family Meripilaceae

Species of *Meripilus* and *Rigidoporus* are recorded from the Peninsular Malaysia. None of these have been encountered in Kelantan and Kuala Lumpur and only *Meripilus applanatus* and *Rigidoporus vinctus* has been encountered in Negeri Sembilan and Selangor respectively. One of the most obvious differences concerns *Rigidoporus* species i.e. *R. lineatus* and *R. vinctus* with only one specie has been recorded in Kedah and Selangor respectively. Both species appear to be under-represented in the Peninsular Malaysia, although not to the same extent as *R. microporus* which is more diverse throughout study sites.

Family Meruliaceae

Two genera have been recorded in Johore, Kuala Lumpur and Selangor. None of these have been encountered in Kedah, Kelantan, Negeri Sembilan and Pahang. One of the most obvious reason concerns this family where only five basidiocarps in total recorded is the member of this family are all grow on rotting wood and tend to form very simple basidiocarp (simple sheets of cluster). A microscope is generally needed to identify many of the basidiocarps in Meruliaceae.

Polyporales species, and in some cases whole genera, can be assigned to functional groups that are based on mode of nutrient acquisition. Ferris *et al.,* (2000) designated four functional groups: ectomycorrhizal fungi, wood decomposers, litter decomposers and parasitic fungi. The Peninsular Malaysia is under-recorded compared to neighbour country Thailand in respect of this group. In fact, a complete knowledge of the fungi for any locality would require continuous observation and collection over many years (Pegler, 1997). This hypothesis is supported by the findings of Corner

(1983, 1989, and 1991) that showed that species diversity and occurrence increased with the increasing number of visits over a longer period.

The present data showed that there are ten frequently encountered Polyporales in Peninsular Malaysia; *Ganoderma australe, Earliella scabrosa*, *Lentinus squarrosulus*, *Pycnoporus sanguineus*, *Lentinus connatus*, *Microporus xanthopus*, *Trametes menziesii*, *Lenzites elegans*, *Lentinus sajor-caju* and *Microporus affinis*. The common species of Polyporales collected were *Fomitopsis feei, Amauroderma subrugosum, Ganoderma australe, Earliella scabrosa, Lentinus squarrosulus, Microporus xanthopus, Pycnoporus sanguineus* and *Trametes menziesii.* The respective species were considered as common species because they were collected at most of all the study sites. These common species have also been reported by other workers (Lim, 1972; Kuthubutheen, 1981; Lee *et al.,* 1995; Salmiah and Thillainathan, 1998) except for *Ganoderma australe* and *Lentinus squarrosulus* which have not been reported in earlier studies as among common macrofungi in Peninsular Malaysia.

Besides, 20 species were only collected once during the study period; *Daedalea lusor, Meripilus applanatus, Rigidoporus lineatus, R. vinctus, Gloeoporus dichrous, G. sulphureus, Coriolopsis aspera, C. sanguinaria, C. strumosa, Echinochaete brachypora, Flabellophora licmophora, Lentinus tigrinus, L. velutinus, Macrohyporia dictyopora, Microporellus inusitatus, Polyporus philippinensis, P.* cf. *badius, Pseudofavolus cucullatus, Pycnoporus cinnabarinus* and *Trichaptum byssogenum.* The species found were only collected once (one basidiocarp in one location). While these species may be found in the other part of Peninsular Malaysia, the collecting location indicates they are not common. Different collecting locations may collect some of these other species more readily, as collection bias can influence the probability of collecting various species.

In this study, *Lentinus* was one of the common genus encountered. *Lentinus araucariae, L. polychrous, L. squarrosulus, L. strigosus* and *L. velutinus* were among the species which had been described by Pegler (1983) who documented 13 species of *Lentinus* in Malaysia. Sumaiyah *et al.,* (2007) reported four *Lentinus* species; *L. connatus, L. velutinus, L. strigosus,* and *L. fasciatus* which had not been documented by Corner (1981), and *L. fasciatus* was a new record for Malaysia as it had not been documented in Malaysia by Pegler (1983), Corner (1981), Lee *et al.,* (1995) and Salmiah and Jones (2001).

Moreover, the other common genera of Polyporales collected in this study were *Coriolopsis* (five species), *Polyporus* (five species), *Trametes* (four species) and *Rigidoporus* (three species). On the other hand, there were 18 genera with only one specie recorded; *Daedalea, Amauroderma, Flavodon, Earliella, Echinochaetae, Favolus, Flabellophora, Fomitella, Funalia, Hexagonia, Lignosus, Macrohyporia, Microporellus, Nigroporus, Panus, Perenniporia, Pseudofavolus* and *Pyrofomes.*

Phylogenetic study of selected Ganoderma species

The genus *Ganoderma* is composed of 219 species with estimated around ten to thirty known species described under subgenus *Elfingia* (Moncalvo and Ryvarden, 1997). The type species of subgenus *Elfingia* is *G. applanatum* (Pers. Ex S.F. Gray) Pat. Which seemed to be confined to North temperate regions and the counterpart of this species in tropical and sub-tropical region should be *G. australe* (Ryvarden and Johansen 1980; Corner, 1983). *Ganoderma australe* is worldwide in distribution and is recognized to be a species complex comparable to that of *G. lucidum* complex (Martinez *et al.,* 1991).

Ganoderma lucidum (Curtis) P. Karst., the type of *Ganoderma* P. Karst., was originally described based on specimen collected from Peckham, London, UK.

Numerous studies of *G. lucidum*, mainly in China, Korea, Japan and the United States, have shown its effectiveness in the treatment of a wide range of disease and symptoms, such as hypertension, diabetes, hepatitis, cancers, and AIDS (Russell and Paterson, 2006).

Due to the great medicinal and economic value, more and more strains of *G. lucidum* are commercially cultivated for the preparation of health tablets or drinks in Malaysia. However, circumstances where identical strains have different names or different strains have the same name often lead to confusion in cultivation. Therefore, precise identification and classification of commercial lines of *G. lucidum* are important for protection of both public health and industry. Although phenotypic analysis is a traditional method for the identification of medicinal mushrooms, morphological characteristics are often unreliable or in conclusive, mainly due to large influence exerted by environmental factors, so that morphological criteria cannot be used as suitable markers to verify the identity of each strains. Therefore, molecular identification provides useful tools for safe identification and assessment of the genetic variability of each strain.

In this study, a number of isolates were obtained specifically from the basidiocarps of *Ganoderma* collected from the wild throughout Peninsular Malaysia and also from local mushroom growers in Selangor. The collections were specifically focused on determining the identity of the specimens. The results showed clearly that there are two species of *Ganoderma* identified in this study and that are *G. lucidum* and *G. australe*. Compared with traditional taxonomical system, these clustering results seem to be in agreement with the taxonomical system.

Identification of *Ganoderma* species has traditionally been based on basidiocarp morphology. The macromorphological and micromorphological characters of

Ganoderma are extensively variable and more than 250 species have been described (Ryvarden, 1991). However, the employment of characters from the basidiocarps has led to many synonyms, species complexes and possible misidentification of species in the genus (Bazzalo and Wright, 1982; Adaskaveg and Gilbertson, 1986, 1988, 1989). This is due to the morphological plasticity of the basidiocarps and the few differences that are often displayed between species. Also, basidiocarps are often not available during field collection and identification using morphology is thus not an option. Variability of morphological characteristics of *Ganoderma* led many taxonomists to explore molecular methods to distinguish between species of *Ganoderma*. The shape and size of basidiopsores and the texture of pileus surfaces are important characteristics that distinguish members of the Ganodermataceae. In this study, and due to the availability of DNA sequencing to identify isolates, identification was specifically able to identify the fungus to species level. It thus seems certain that *G. lucidum* is the primary cultivated in Malaysia and *G. australe* is able to be identified to species level even though both species exhibit different characteristics of morphology.

Using ITS, Clade A contained strains of *G. lucidum* including strains which received as *G. tsugae* (KUM50079) and *G. amboinense* (KUM61117). Both *Ganoderma* strains basidocarps collected showed varieties in the morphological characteristics. It suggested that both strain seem to be a variant form of *G. lucidum*. Therefore, molecular techniques were applied to support the identification to species level. In order to provide molecular evidence for the *Ganoderma* species, phylogenetic relationship was investigated using morphological and 650 base pair nucleic acid sequence characters from ITS1, 5.8S rDNA and ITS2 region on the ribosomal DNA. The ITS region is a gene marker useful in separating related species and strains of *Ganoderma* (Moncalvo *et al.,* 1995c; Smith and Sivasithamparam, 2000). The aim of the present study was to provide a preliminary identification of nine strains of

Ganoderma using molecular technique. Only two species of *Ganoderma* were collected in this study i.e., *G. australe* and *G. lucidum*.

All strains were identified as *G. lucidum* including two strains originally received as *G. tsugae* (KUM50079) and *G. amboinense* (KUM61117). *Ganoderma lucidum* and *G. australe* were identified using the amplification of the ITS region and have yield PCR products of approximately 650 bp long. The results showed maximum identity with *G. lucidum* and *G. australe* at 100% respectively. This because in the case of *G. lucidum* and *G. australe*, the first BLAST hit was Z37050 (*G. lucidum*) and AF255143 (*G. australe*), which were on 18S rDNA sequence.

From ITS1-5.8S-ITS2 rDNA homology, the closest two completely identified sequences were both *G. lucidum* and *G. australe* respectively. Since the ITS1-5.8S-ITS2 rDNA is a hyper variable region, i.e. this region reflects more variation then either the 18S or the 25-28S rDNA. The evolutionary distance resolved by ITS is usually restricted to demarcating within the species level and cannot be completely relied above genus level, like 18S or 25-28S rDNA. Organisms showing more than 90% similarity can be considered as belonging to the same genus. Whereas for 18S rDNA sequence homology, ≥90% sequence identity can safely be considered as belonging to the same genus (Guarro *et al.,* 1999). The 18S, 25-28S and ITS1-5.8S-ITS2 rDNA sequence homology leads to the conclusion that all isolates belong to the genus *Ganoderma* and species *lucidum* for KUM50079, KUM61129, KUM61130 and KUM61117; species *australe* for KUM61056, KUM61057 and KUM60813.

CHAPTER 4

APPLICATION OF POLYPORALES IN BIOPULPING OF OIL PALM (*ELAEIS GUINEENSIS*) EMPTY FRUIT BUNCHES

4.1 INTRODUCTION

According to Tekere *et al.,* (2001) the white-rot fungi are by far the most efficient ligninolytic organisms described to date. This is due to the capability of white-rot fungi to degrade lignin by extracellular nonspecific and non-stereoselective enzyme system. Selectivity of white-rot decay is dependent also on the physical and chemical environment in wood such as temperature, oxygen, nitrogen and wood moisture content (Adaskaveg *et al.,* 1995; Blanchette, 1995) and varies are also between wood species (Blanchette *et al.,* 1988). In addition to wood polymers, several white-rot fungi are also able to degrade wood extractives (Gutiérrez *et al.,* 1999; Dorado *et al.,* 2000; Hatakka *et al.,* 2003).

This study examines the application of wood-rotting fungi particularly from order Polyporales, especially their extracellular enzymes for biopulping. In order to provide a context for the significance of research into Polyporales species (white-rot fungi), this study begins with a preliminary screening of Polyporales cultures for the production of lignolytic and cellulolytic enzymes.

Due to the potential biotechnological applications of fungal enzymes with respect to the biopulping process, there has been an increased interest in the study of these extracellular enzymes. The concept of biopulping is based on the ability of some white-rot fungi to colonize and degrade selectively lignin in wood, thereby leaving cellulose relatively intake.

Biopulping can be carried out in bioreactors of different types, including open chip piles, depending on the requirements of the particular microorganism would have for optimal results. High moisture content (around 55-60%) should be kept in wood chips during the biotreatment step to ensure an optimal colonization and penetration of fungal hyphae. The degree of asepsis should be controlled to ensure a successful wood colonization by the particular fungal strain used depending on its resistance against contamination and ability to compete with the microbial biota existing in the wood chip (Bajpai, 2012). Therefore, this study demonstrated the evaluation of enzymes activity i.e. cellulase, xylanase, glucosidase, laccase and lignin peroxidase of selected white-rot fungi through solid substrate fermentation of oil palm empty fruit bunches.

The white-rot fungi and their enzymes are considered for the wood chips treatment prior to pulping because the non-specific oxidative systems that attack lignin content of the wood which make the pulp more permeable for the removal of residual lignin (Pooja *et al.,* 2010). Therefore, this study will focused the potential use of a white-rot fungus, *Ganoderma australe* in the biopulping of oil palm (*Elaeis guineensis*) empty fruit bunches.

There is increasing interest to use oil palm empty fruit bunches to make pulp and paper in Malaysia. Several authors have used EFB as a raw material for the production of cellulose pulps. Rosnah *et al.,* (2010) reported the influence of alkaline peroxide treatment duration on the pulpability of EFB. The results demonstrated the important influence of alkaline peroxide treatment time to effect both brightening of EFB and softening of the biomass. Rushdan (2002) used the Kraft process, while Wanrosli *et al.,* (1998) and Law and Jiang (2001) obtained pulps by treating EFB with soda, sodium carbonate and sodium sulfite. Rushdan (2002) compared the composition of EFB pulps obtained with Kraft, Kraft-anthraquinone, soda and soda-anthraquinone processes and

found that the soda process yielded the highest content of lignin, holocellulose and α-cellulose plus the highest viscosity.

With its large cultivation of oil palm (4.85 million hectares), EFB is sufficiently available (an estimated 5 million tons a year, dry weight) (Kamarudin *et al.,* 2008). Oil palm empty fruit bunches are considered to be agricultural waste. Valuable fibers obtained from EFB are presently used for developing value-added products such as wood composite product, medium density fiberboard (MDF) and fiberboard (Fang *et al.,* 2000). Presently, most of the EFB and mesocarp fiber are used as soil conditioners in estates and plantations and incinerated to obtain oil palm ash (OPA) that can be used as a source of fertilizer due to its high potassium content (Mohamad *et al.,* 2005). Empty fruit bunches is composed of 45-50% cellulose and about equal amounts (25-35%) of hemicelluloses and lignin (Deraman, 1993) and has a good potential to be as raw materials for pulp and paper.

Among the different pulping processes, two of them are highlighted i.e., the organosolv process (Mutjé *et al.,* 2005) and the soda process, either alone (Jiménez *et al.,* 2005) or with the aid of other chemicals, such as anthraquinone (Rodríguez *et al.,* 2008). With regard to the final use of the pulp; for paper production, in which that high yield is beneficial, soda pulping process has been chosen in this study.

Hence the objectives of the study were to:

a. profile qualitatively the production of amylases, cellulases, laccases and lignin peroxidases from Polyporales cultures
b. study the cellulolytic and ligninolytic enzymes activities of *Ganoderma australe* KUM60848 and *Favolus tenuiculus* KUM60803 strains during SSF of oil palm empty fruit bunches (EFB)

c. determine the effect of *Ganoderma australe* KUM60848 pre-treatment on pulp yield, alpha cellulose content and strength of EFB soda pulp

4.2 MATERIALS AND METHODS

4.2.1 Polyporales cultures and maintainance

Cultures of 37 strains of Polyporales were selected for this study. The cultures were successfully obtained from wild and deposited in Mushroom Research Centre, University of Malaya with accession code number, Kulat Universiti Malaya (KUM). Each culture was maintained in distilled water at 2°C while the working culture was maintained on malt-extract agar (MEA) slants at 4°C. The selected cultures were qualitatively assayed for the production of amylases, cellulases, laccases and lignin peroxidases after three to seven days incubation at 25±2°C.

4.2.2 Screening for the production of enzymes from Polyporales

4.2.2.1 Cellulases

The methods of Tekere *et al.,* (2001) were adopted. The medium for cellulase activity contained 2 g yeast extract, 1 g $KHPO_4$, 6 g carboxymethylcellulose (CMC), 20 g agar and 1 liter water. The media were then flooded with Congo red for 15 minutes then washed with 1M NaCl to stabilize the hydrolyzed zone.

4.2.2.2 Amylase

Amylolytic activity was determined by the Manual of Microbiological Methods (1957). Inoculum was grown on Difco™ Potato Dextrose Agar incorporated with 2 g of soluble starch per litre. Following of incubation of 5 mm disc with the mycelium side

on the agar for 3-5 days at 25±2°C, the Petri dishes were flooded with iodine solution (KI, 15g; I_2, 3g per litre of distilled water). A zone void of blue indicated the region of amylolytic activity and was measured.

4.2.2.3 Laccase and lignin peroxidase

Laccase and lignin peroxidase were detected by the spot test of Stalpers (1978). A drop of a 1:1 mixture of 0.4% hydrogen peroxide and 1.0% pyrogallol (1,2,3 trihydroxy-benzene) in water, was placed on the marginal hyphae actively growing on Potato Dextrose Agar for the detection of lignin peroxidase. A yellowish brown colour read after 3, 14 and 72 hr was considered as positive for lignin peroxidase. Detection of laccase was by a similar drop test using 0.1 mM syringaldazine. A pinkish colour indicates as positive for laccase activity.

4.2.3 Cellulolytic and ligninolytic enzymes profile of selected Polyporales during solid substrate fermentation of oil palm empty fruit bunches

Selected strains of Polyporales were grown on oil palm empty fruit bunches and analyzed for cellulolytic and ligninolytic enzymes. The enzymes studies were an endo-cellulase (EC. 3.2.1.4) of carboxymethylcellulase, xylanase (EC. 3.2.1.8), β-D-Glucosidase (EC. 3.2.1.21), laccase (EC. 1.10.3.2) and lignin peroxidase (EC. 1.11.1.14).

4.2.3.1 Selected polyporales cultures and maintenance

Ganoderma australe and *Favolus tenuiculus* were selected for this study. Fungal cultures were obtained from tissue cultures of wild specimens. The cultures were deposited in the culture collection of Mushroom Research Centre, University of Malaya under the following codes: *Ganoderma australe* KUM60848 and *Favolus tenuiculus*

KUM60803. The working cultures were maintained on malt-extract agar (MEA) slants at 4°C. For the preparation of inoculums, the fungal cultures were transferred to MEA plates and incubated for 7 to 8 days at 25 ± 2°C.

4.2.3.2 Inoculum preparation

The purpose of the grain-inoculum was to boost the mycelium to a state of vigor such that it will rapidly colonize the lignocellulosic residues used as substrate following inoculation. Wheat grain (50g) was placed in a 250 ml Erlenmeyer flask and 5 ml of distilled water was then added. After that, the resulting flask was autoclaved at 121 °C under 15 psi for 15 minutes. After autoclaving, the flask was left to cool overnight. Five 1 cm^3 fungus mycelium plugs (cut from the edge of the colony) were inoculated into each of the wheat-grain flasks. The inoculated flasks were incubated in the dark for 2 weeks at 25 ± °C for full colonization of the substrate (Plate 4.1). The process flow for inoculums preparation is given in Figure 4.1.

4.2.3.3 Substrate for pulping

Shredded oil palm empty fruit bunches (EFB) were obtained from a local palm oil processing company, Seri Ulu Langat Palm Oil Mill, Dengkil, Selangor, Malaysia (Plate 4.2). The sample was collected and washed thoroughly to make it dust free, and then dried in an oven at 103 °C until a constant weight was obtained.

4.2.3.4 Fermentation conditions

Solid substrate fermentation was carried out in 250 ml Erlenmeyer flask containing 30 g of empty fruit bunches. The moisture content of the substrate was adjusted to 80 ± 2 % (w/v) with distilled water and autoclaved (15 min, 121 °C, 15 psi). Upon cooling, the contents was inoculated with 10 % (3.0 ± 0.5 g) of colonized wheat

grain and then incubated in the dark at 25 ± 2 °C. Triplicates flasks were prepared for each fungus.

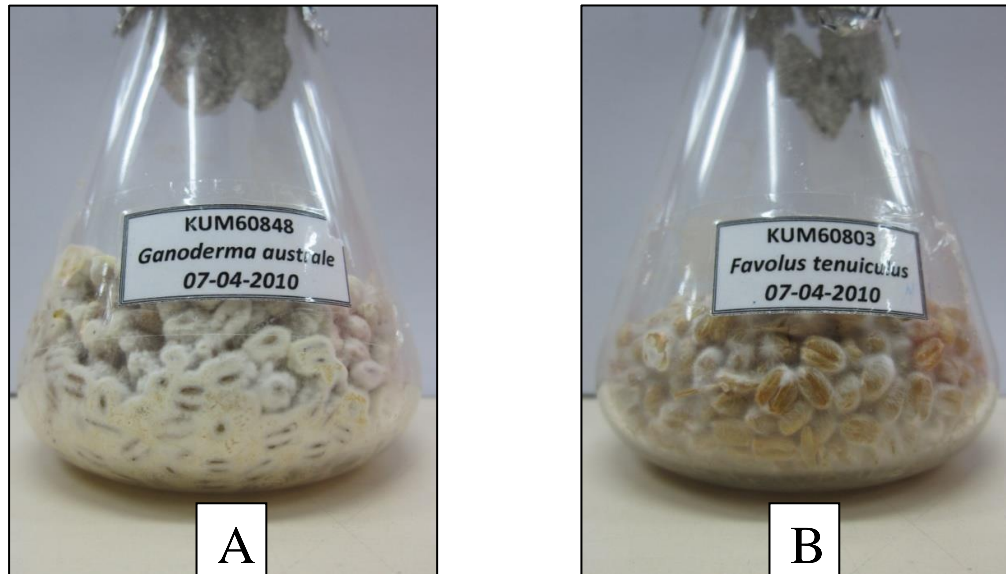


Plate 4.1 Inoculum for solid substrate fermentation, (A) *Ganoderma australe*, (B) *Favolus tenuiculus*

Plate 4.2 Shredded oil palm empty fruit bunches

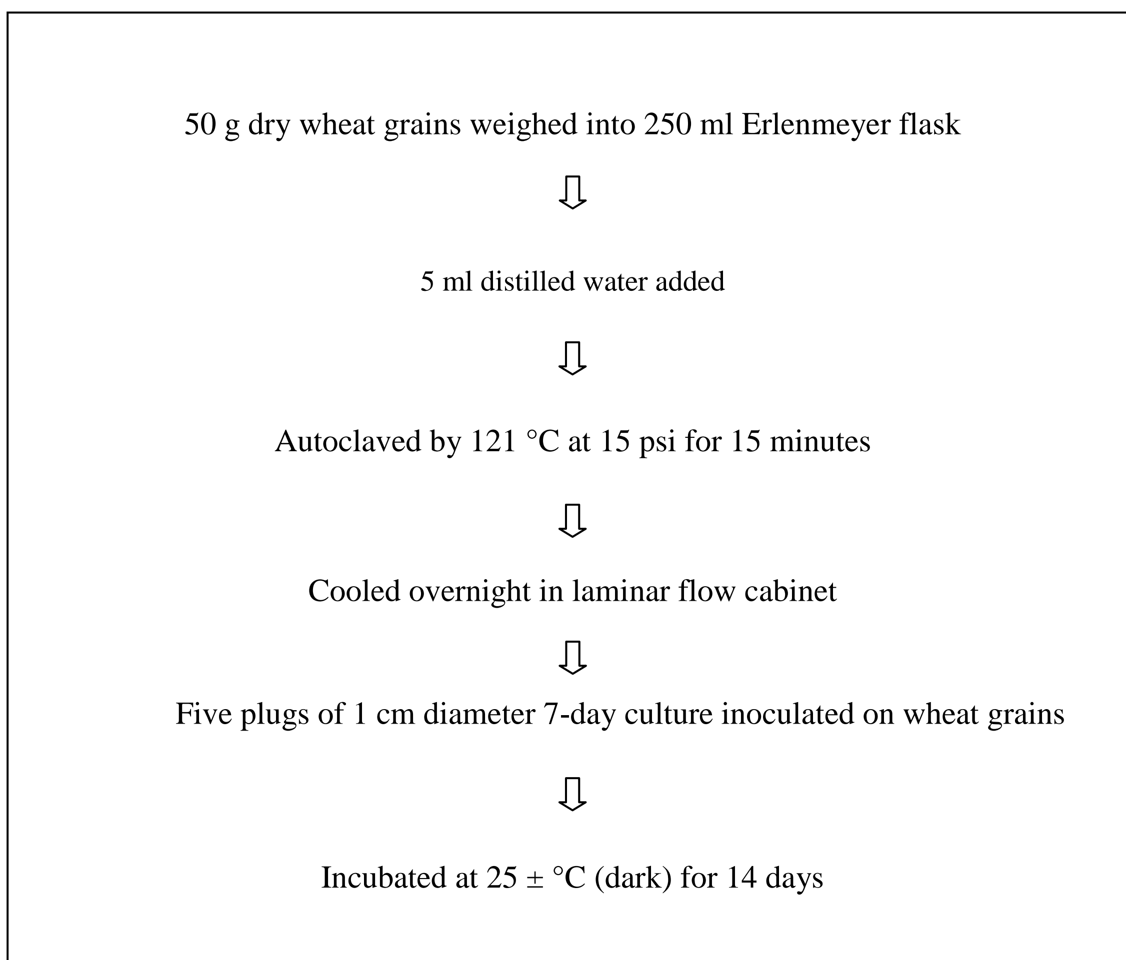


Figure 4.1 Schematic diagram of inoculum preparation

4.2.3.5 Crude extracellular enzymes extraction

The crude enzyme extracts were prepared according to Avneesh *et al.,* (2003). As the enzymes were soluble, the enzymes were extracted by using 300 cm^3 of acidified tap water (pH 4.0). A spatula was used to mix the substrate with the extraction solvent and to break the large solid particles. The mixture was then homogenized at 11000 rpm for 5 minutes. Next, culture solids were separated by filtration through a 400 × 2 um pore size nylon cloth. Filtered solution was subsequently centrifuged at 9000 rpm for 24 minutes at 4°C. Finally, the supernatant designated as enzyme extract was obtained for assay for enzymes. The flow chart for experimental procedures for sampling, extraction and enzymes assay during SSF of oil palm empty fruit bunches are given in Figure 4.2.

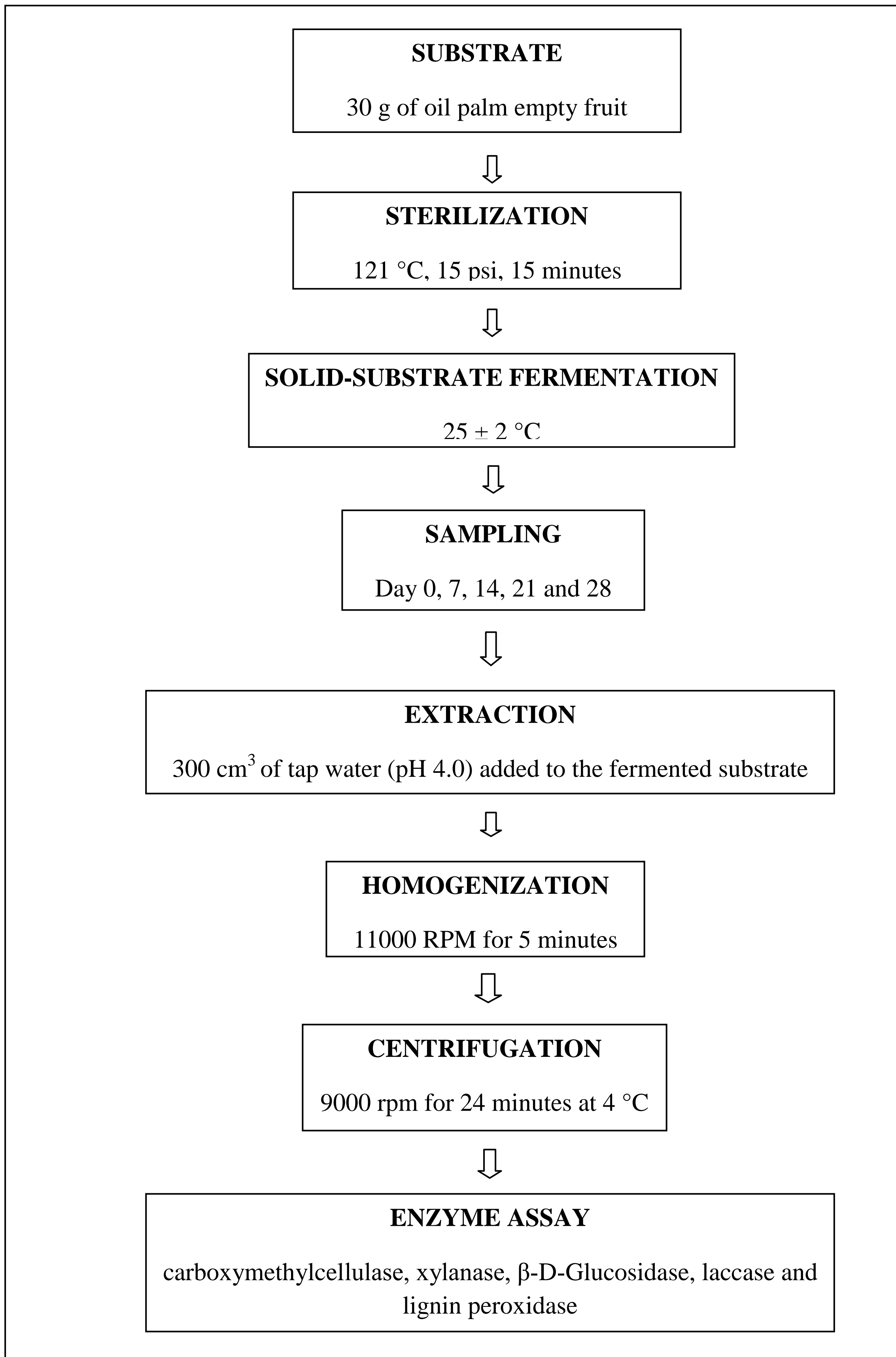


Figure 4.2 Flow chart of experimental procedures for sampling, extraction and enzymes assay during SSF of oil palm empty fruit bunches

4.2.4 Enzyme assays

All the enzyme activities were expressed in international units (IU) define as the quantity of enzymes required to produce one μmol product/min. The activity of enzymes was reported on the basis of units produced per ml of fermented substrate (U/ml), under the assay condition. The solid-substrate cultures were sampled at seven day interval started from day zero of incubation.

4.2.4.1 Carboxymethylcellulase activity

Carboxymethylcellulase activity was determined by the reducing sugars released according to the DNS method (Miller, 1959) (Appendix A6, pp. 163). A glucose standard curve was used and the reducing sugar expressed as glucose equivalent. Correction was made for absorbance due to the background colour in the enzyme blank. The CMCase activity (Dong *et al.,* 1992) was calculated and translated to an enzyme activity expressed as 1 μmol glucose released/min/g substrate (Appendix A1, pp. 157).

4.2.4.2 Xylanase activity

The reducing sugar released in the reaction was determined using DNS method (Appendix A2, pp. 158). The standard used was pure xylose. One unit of xylanase activity was expressed as 1μmole of xylose released per minute (Bailey *et al.,* 1992; Miller, 1959) (Appendix A2, pp. 158). Correction was made for absorbance due to background colour in the enzyme blank. By using the standard line for xylose, the final absorbance was converted to enzyme activity units.

4.2.4.3 β-D-Glucosidase activity

The β-D-Glucosidase reaction was determined by adding 2ml of 1M Na_2CO_3 and the liberated *ρ*-nitrophenol was measured at λ=400nm. The usual enzyme and

reagent blanks were included. The absorbance values obtained (minus the enzyme and reagent blanks) were further translated into enzyme activity using a standard graph relating µg/ml of ρ-nitrophenol to absorbance (Dong *et al.,* 1992) (Appendix A3, pp. 159). One unit of enzyme activity expressed as 1 µmol of ρ-nitrophenol released/min under the assay conditions.

4.2.4.4 Laccase activity

Laccase activity was determined by the increase in the absorbance due to the formation of tetramethoxy-azo-bis-methylenequinine resulting from the reaction of laccase with syringaldazine (Harkin and Obst, 1973; Leonowicz, 1981) (Appendix A4, pp. 161). The substrate was 0.1 mM syringaldazine in 50% ethanol (w/v). One unit of enzyme activity was defined as the amount of enzyme producing one unit change in absorbance/min.

4.2.4.5 Lignin peroxidase activity

Lignin peroxidase (LiP) activity was determined by recording the increase in absorbance at 310 nm due to the oxidation of 2 mM veratryl alcohol to veratraldehyde (Tien and Kirk, 1984) (Appendix A5, pp. 162). The reaction was initiated by the addition of H_2O_2 at a final concentration of 0.5 mM (Appendix A5, pp. 162). The standard was 3, 4-dimethoxybenzaldehyde (veratraldehyde). The enzyme activity (U) was expressed as the amount of enzyme required to produce 1 µmole of product/min.

4.2.5 Statistical analysis

For all the experiments above, triplicates were set up for each parameters tested. The design was completely randomized and sampling was random. The ANOVA analysis was used to test the mean of three replicate values for all data in the

experiments and the StatGraphics programme used to do the multiple range test analysis. The significance of the difference between means was determined by the Duncun's multiple range tests at 95% least significant different ($p < 0.05$) (Appendix C, pp. 166).

4.2.6 Property of pulp produced by fermentation of oil palm (*Elaeis guineensis*) empty fruit bunches with *Ganoderma australe* KUM60848

4.2.6.1 Sample collection

Oil palm empty fruit bunches (EFB) were obtained from a local palm oil processing company, Seri Ulu Langat Palm Oil Mill, Dengkil, Selangor. The EFB were shredded, dried and thoroughly washed prior to treatment. The EFB was washed to make it dust free, and then dried in an oven at 103°C until a constant weight was obtained.

4.2.6.2 *Ganoderma australe* KUM60848

Ganoderma australe KUM60848 strain was obtained from stock culture deposited in the Mushroom Research Centre, University of Malaya. Fresh cultures of *G. australe* were maintained on Difco^{TM} Malt Extract Agar at 25 ± 2°C in dark condition.

4.2.6.3 Solid substrate fermentation

Empty fruit bunches (EFB) of 1 kg (o.d) with 80% moisture was used in solid substrate fermentation. The contents were autoclaved (15 min, 121°C, 15 psi). Upon cooling, the contents was inoculated with 10 % (100 ± 0.5 g) of colonized wheat grain and then incubated in the dark at 25 ± 2 °C. Two EFB samples were inoculated with *G. australe* KUM60848 at 25°C in dark condition and one sample was uninoculated (control).

On the 14 and 21 days of incubation, the EFB was pulped by soda pulping process. A control pulping was done on EFB without any pre-treatment. Pulping trials were carried out in a Weverk Rotating digester. The pulping conditions employed were:

1. Maximum cooking temperature: 170°C
2. Time to maximum temperature: 90 minutes
3. Time at maximum temperature: 120 minutes
4. EFB to liquor ratio: 1:7
5. Amount of NaOH: 25% of EFB dry weight

The total pulp yield was calculated as the sum of the screened pulp yields and the sieves. Pulp hand sheets physical strength was determined according to TAPPI Test Method (T 220 sp-96 and T 441 om-89) and Malaysian Standard (MS ISO 1924-2:1999, MS ISO 2758:2002 and MS ISO 5626:1999).

4.3 RESULTS

4.3.1 Enzyme profiles of selected Polyporales

In this study, 37 fungal strains belong to five families i.e. Fomitopsidaceae, Ganodermataceae, Polyporaceae, Meripilaceae and Meruliaceae were isolated. All fungal specimens were identified to the species level using the keys in Núňez and Ryvarden (2000, 2001) and Hattori (2000, 2005). The cultures were originally isolated from fruiting body found in four states; Pahang, Johor, Negeri Sembilan, Selangor and Federal Territory of Kuala Lumpur. The basidocarps were then preserved as oven-dried specimens at University of Malaya herbarium (acronym KLU), Kuala Lumpur.

The screening results showed all 37 strains tested were positive for the production of cellulase and amylase (Table 4.1). All of the selected isolates had formed clear zones after seven days of incubation. Cellulase activity was indicated by the clear or lighter zone (non-red) area around the culture (Figure 4.3).

Table 4.1 Polyporales growth rates and clearing zone diameter for amylase and cellulase production.

	Species	Isolate Code	Enzyme plate test			
			Amylase		Cellulase	
			Mycelial growth (cm/day)[a]	Clearing zones diameter*	Mycelial growth (cm/day)[a]	Clearing zones diameter*
	Fomitopsidaceae					
1	*Fomitopsis ostreiformis* (Berk.) T. Hatt. (2003)	KUM 60845	0.7	0.9	0.8	1.2
2	*Fomitopsis ostreiformis* (Berk.) T. Hatt. (2003)	KUM 60849	0.7	0.7	0.7	1.1
3	*Fomitopsis ostreiformis* (Berk.) T. Hatt. (2003)	KUM 60853	0.6	0.8	0.5	1.3
	Ganodermataceae					
4	*Amauroderma subrugosum* (Bres. & Pat.) Torrend (1920)	KUM 60936	0.9	0.5	0.5	1.1
5	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 60848	0.7	1.2	0.7	0.6
6	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 60851	0.7	1.0	0.6	1.0
7	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 60860	0.8	0.9	0.3	1.8
8	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 60905	0.9	0.7	0.3	1.0
9	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 60813	1.0	0.7	0.3	2.3
10	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 60816	0.9	0.9	0.4	1.0
11	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 60819	0.7	0.9	0.3	1.6
12	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 70069	0.7	0.6	0.4	1.2
	Polyporaceae					
13	*Coriolopsis badia* (Berk.) Murrill (1907)	KUM 60850	0.6	1.2	0.3	1.0
14	*Coriolopsis badia* (Berk.) Murrill (1907)	KUM 60937	0.6	1.0	0.4	1.3
15	*Earliella scabrosa* (Pers.) Gilb. & Ryvarden (1985)	KUM 60863	0.6	0.9	0.5	1.0
16	*Earliella scabrosa* (Pers.) Gilb. & Ryvarden (1985)	KUM 60864	0.7	1.0	0.6	1.1
17	*Earliella scabrosa* (Pers.) Gilb. & Ryvarden (1985)	KUM 60942	0.7	0.9	0.4	1.0
18	*Favolus tenuiculus* P. Beauv. (1806)	KUM 60803	1.0	0.9	0.8	1.2
19	*Funalia polyzona* (Pers.) Niemelä (2003)	KUM 60842	0.8	1.0	0.5	1.2
20	*Hexagonia tenuis* (Hook.) Fr. (1838)	KUM 60935	1.0	0.9	0.6	1.0
21	*Lentinus squarrosulus* Mont. (1842)	KUM 70037	0.5	1.0	0.2	2.5
22	*Lentinus strigosus* (Schwein.) Fr. (1838)	KUM 70091	0.5	0.5	0.3	1.0
23	*Lenzites elegans* (Spreng.) Pat. (1900)	KUM 70081	0.8	0.8	0.5	1.1

Table 4.1, continued.

	Polyporaceae					
24	*Lenzites elegans* (Spreng.) Pat. (1900)	KUM 60948	0.6	0.9	0.3	1.4
25	*Microporus xanthopus* (Fr.) Kuntze (1898)	KUM 70012	0.7	0.9	0.5	1.3
26	*Polyporus arcularius* (Batsch) Fr. (1821)	KUM 70178	0.9	0.7	0.6	1.0
27	*Pycnoporus sanguineus* (L.) Murrill (1904)	KUM 60840	0.6	0.8	0.6	1.1
28	*Pycnoporus sanguineus* (L.) Murrill (1904)	KUM 70172	0.8	0.6	0.2	2.3
29	*Pycnoporus sanguineus* (L.) Murrill (1904)	KUM 70173	1.0	0.7	0.4	1.2
30	*Trametes* cf. *hirsuta* (Wulfen) Lloyd (1924)	KUM 60839	0.6	1.0	0.7	1.1
31	*Trametes scopulosa (Berk.) Bres. (1912)*	KUM 70034	0.6	0.9	0.4	1.1
32	*Trametes lactinea* (Berk.) Sacc. (1888)	KUM 60852	0.7	0.7	0.6	1.1
33	*Trametes menziesii* (Berk.) Ryvarden (1972)	KUM 70111	0.6	0.7	0.5	1.0
34	*Trametes modesta* (Kunze ex Fr.) Ryvarden (1972)	KUM 60812	0.6	0.7	0.2	1.0
	Meripilaceae					
35	*Meripilus applanatus* Corner (1984)	KUM 60946	0.5	0.8	0.3	1.5
36	*Rigidoporus microporus* (Sw.) Overeem (1924)	KUM 60869	0.7	0.5	0.5	1.4
	Meruliaceae					
37	*Flavodon flavus* (Klotzsch) Ryvarden (1973)	KUM 60843	0.8	0.5	0.3	1.1

a = mean values from triplicates

* Value more than 1 = diameter of clear zone bigger than mycelia growth in cm
Value less than 1 = diameter of clear zone smaller than mycelia growth in cm

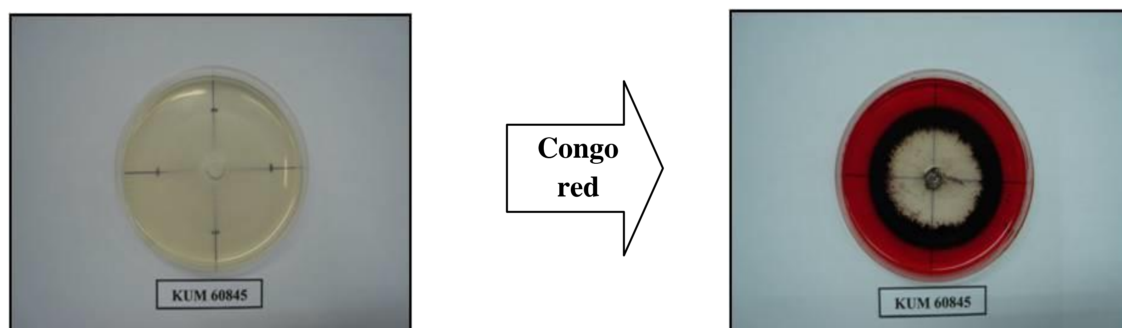


Figure 4.3 Clear zone produced by isolate after flooded with Congo red

Amylase activity was indicated by the clear zone (non-blue) area around the culture upon flooding with iodine (Figure 4.4). Out of these, four species i.e. *Ganoderma australe* KUM60813, *Favolus tenuiculus* KUM60803, *Hexagonia tenuis* KUM60935, and *Pycnoporus sanguineus* KUM70173 showed good mycelia growth on the screening medium for amylase and two isolates *Fomitopsis ostreiformis* KUM60845 and *Favolus tenuiculus* KUM60803 for cellulase plate study. Furthermore, two isolates i.e. *G. australe* KUM60848 and *Coriolopsis badia* KUM60850 appeared to be good amylase producer and three isolates i.e. *G. australe* KUM60813, *Lentinus squarrosulus* KUM70037 and *P. sanguineus* KUM70172 showed good result in the formation of clearing zone as it is indicatives that the strains are potential cellulase producers.

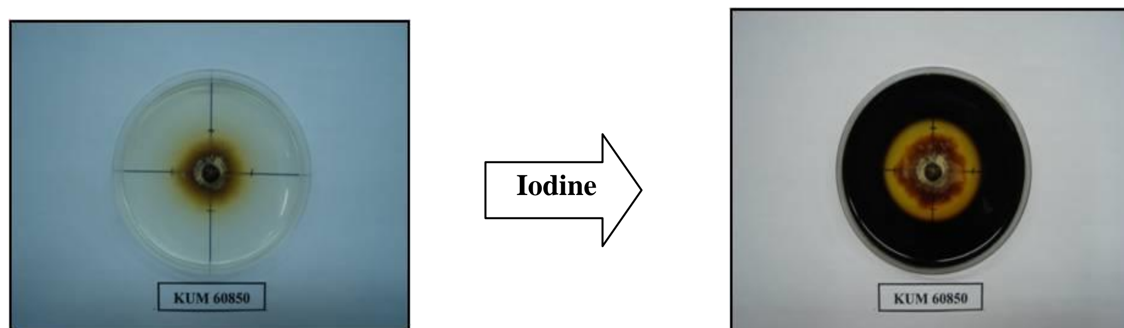


Figure 4.4 Clear zone produced by isolate after flooded with iodine

Thirty-two strains tested were positive for laccase as indicated by the colour change of clear syringaldazine to pinkish (Table 4.2) except for isolates of *Fomitopsis ostreiformis* and *Coriolopsis badia*. For lignin peroxidase, 34 strains tested were positive as indicated by a yellowish-brown colour (Figure 4.5). In this study, the productions of lignin peroxidase were also none detected for *Fomitopsis ostreiformis* strains. From the qualitative plate assay results, two strains identified as *Ganoderma australe* KUM60848 and *Favolus tenuiculus* KUM60803 demonstrated good enzymes production and were selected to undergo solid substrate fermentation of oil palm empty fruit bunches (EFB).

Table 4.2 Laccase and lignin peroxidase detection in Polyporales.

	Species	Isolate Number	Enzyme plate test	
			Laccase	Lignin Peroxidase
	Fomitopsidaceae			
1	*Fomitopsis ostreiformis* (Berk.) T. Hatt. (2003)	KUM 60845	-	-
2	*Fomitopsis ostreiformis* (Berk.) T. Hatt. (2003)	KUM 60849	-	-
3	*Fomitopsis ostreiformis* (Berk.) T. Hatt. (2003)	KUM 60853	-	-
	Ganodermataceae			
4	*Amauroderma subrugosum* (Bres. & Pat.) Torrend (1920)	KUM 60936	+	+
5	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 60848	+	+
6	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 60851	+	+
7	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 60860	+	+
8	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 60905	+	+
9	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 60813	+	+
10	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 60816	+	+
11	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 60819	+	+
12	*Ganoderma australe* (Fr.) Pat. (1890)	KUM 70069	+	+
	Polyporaceae			
13	*Coriolopsis badia* (Berk.) Murrill (1907)	KUM 60850	+	+
14	*Coriolopsis badia* (Berk.) Murrill (1907)	KUM 60937	+	+
15	*Earliella scabrosa* (Pers.) Gilb. & Ryvarden (1985)	KUM 60863	+	+
16	*Earliella scabrosa* (Pers.) Gilb. & Ryvarden (1985)	KUM 60864	+	+
17	*Earliella scabrosa* (Pers.) Gilb. & Ryvarden (1985)	KUM 60942	+	+
18	*Favolus tenuiculus* P. Beauv. (1806)	KUM 60803	+	+
19	*Funalia polyzona* (Pers.) Niemelä (2003)	KUM 60842	+	+
20	*Hexagonia tenuis* (Hook.) Fr. (1838)	KUM 60935	+	+
21	*Lentinus squarrosulus* Mont. (1842)	KUM 70037	+	+
22	*Lentinus strigosus* (Schwein.) Fr. (1838)	KUM 70091	+	+
23	*Lenzites elegans* (Spreng.) Pat. (1900)	KUM 70081	+	+
24	*Lenzites elegans* (Spreng.) Pat. (1900)	KUM 60948	+	+
25	*Microporus xanthopus* (Fr.) Kuntze (1898)	KUM 70012	+	+

Table 4.2, continued.

26	*Polyporus arcularius* (Batsch) Fr. (1821)	KUM 70178	+	+
27	*Pycnoporus sanguineus* (L.) Murrill (1904)	KUM 60840	+	+
28	*Pycnoporus sanguineus* (L.) Murrill (1904)	KUM 70172	+	+
29	*Pycnoporus sanguineus* (L.) Murrill (1904)	KUM 70173	+	+
30	*Trametes* cf. *hirsuta* (Wulfen) Lloyd (1924)	KUM 60839	+	+
31	*Trametes scopulosa (Berk.) Bres. (1912)*	KUM 70034	+	+
32	*Trametes lactinea* (Berk.) Sacc. (1888)	KUM 60852	+	+
33	*Trametes menziesii* (Berk.) Ryvarden (1972)	KUM 70111	+	+
34	*Trametes modesta* (Kunze ex Fr.) Ryvarden (1972)	KUM 60812	+	+
	Meripilaceae			
35	*Meripilus applanatus* Corner (1984)	KUM 60946	+	+
36	*Rigidoporus microporus* (Sw.) Overeem (1924)	KUM 60869	+	+
	Meruliaceae			
37	*Flavodon flavus* (Klotzsch) Ryvarden (1973)	KUM 60843	+	+

+: Positive for laccase/lignin peroxidase (LiP) -: Negative for laccase/lignin peroxidase (LiP)

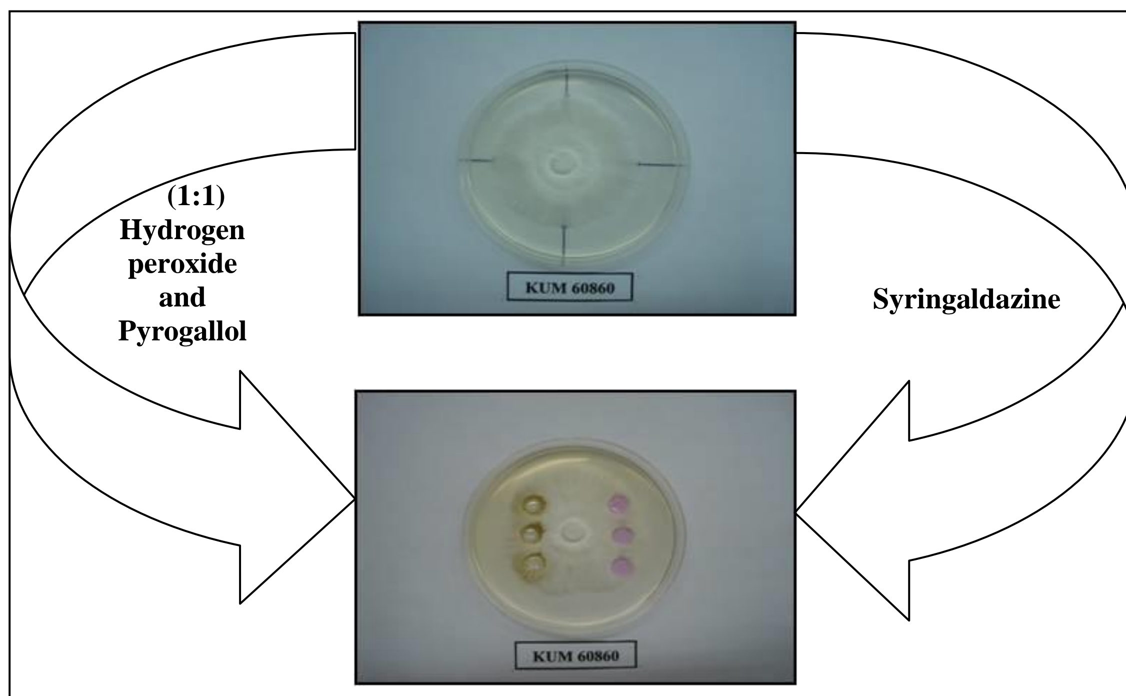


Figure 4.5 A yellowish-brown colour considered as positive for lignin peroxidase and pinkish colour indicates as positive for laccase production

4.3.2 Cellulolytic and lignolytic enzymes production by *Ganoderma australe* and *Favolus tenuiculus* during solid substrate fermentation of oil palm empty fruit bunches

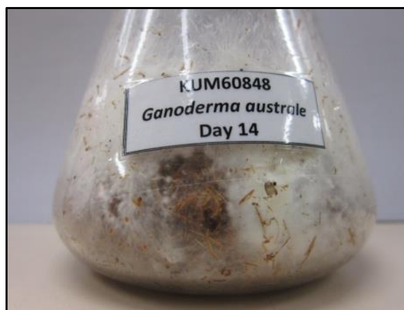
Oil palm empty fruit bunches were inoculated with *Ganoderma australe* KUM60848 and *Favolus tenuiculus* KUM60803 under solid substrate fermentation simulating the biopulping process. Development of experimental procedures for determination of lignocellulolytic enzymes produced under this biopulping condition is necessary in order to evaluate the enzymes activity of each isolate throughout the incubation period.

Ganoderma australe KUM60848 and *F. tenuiculus* KUM60803 were selected to be investigated during SSF for their enzymes profiles; celullase, laccase and lignin peroxidase. The above strains were selected based on the preliminary plate test results in which *G. australe* and *F. tenuiculus* showed the best results for the production of amylase, cellulase, laccase and lignin peroxidase. The additional test for the production of xylanase and β-D-glucosidase were done prior to biopulping application.

The oil palm empty fruit bunches was used as substrates for the Solid Substrate Fermentation (SSF) and were shown to support good mycelial growth for both strains. During the incubation periods, EFB were rapidly colonized by the strains after the first week with formation of an abundant mycelia mass (Plate 4.3). As the incubation period progresses, the mycelia net became denser and completely covering the EFB after 28 days. Inoculated EFB were extracted with extraction buffer solution to recover the extracellular enzymes produced during the fungal growth. Because most of the oxidative extracellular enzymes produced during EFB biodegradation are absorbed on EFB cell walls, successive extractions are necessary for a quantitative enzyme recovery from the EFB.

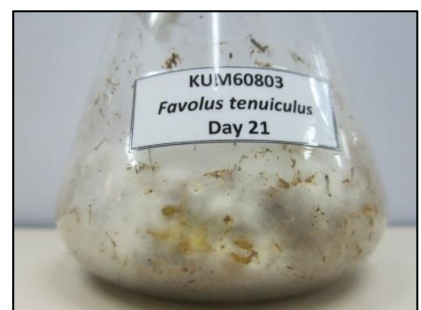
DAY 0

DAY 7



DAY 14

DAY 21



DAY 28

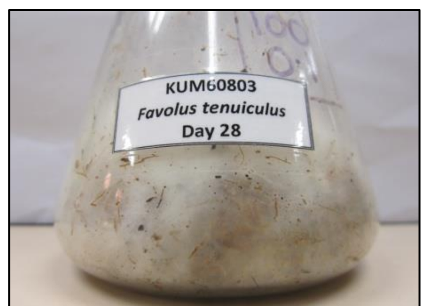


Plate 4.3 Solid substrate fermentation of oil palm empty fruit bunches by *Ganoderma australe* and *Favolus tenuiculus* by day 0, 7, 14, 21 and 28

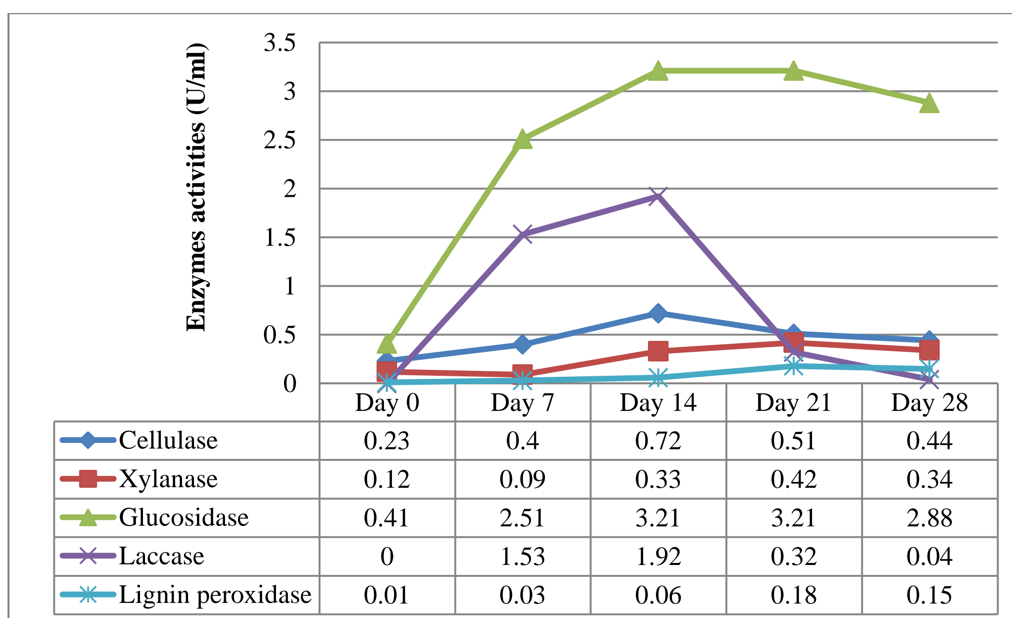
Figures 4.6 and 4.7 illustrate enzymes recovery from day zero to 28-day-old of *G. australe* and *F. tenuiculus* during solid substrate fermentation of EFB. In general, three extractions were undertaken to determine the activities of lignin peroxidase, laccase, xylanase, glucosidase and cellulase.

All enzymes activities were observed over the 28 days of incubation for both isolates. Most of the enzymes were detected at higher levels on day 14 and 21 and decreases over successive extractions. However, *G. australe* demonstrated higher enzymes activity i.e cellulase, xylanase, glucosidase, laccase and lignin peroxidase compared to *F. tenuiculus.*

Results indicate that the activity of glucosidase was far more than other enzymes, suggesting that glucosidase might be the predominating enzymes during the incubation of EFB for both strains. *Ganoderma australe* achieved its maximum β-D-gulcosidase activity on day 14 with 3.22±0.01 U/ml, while *F. tenuiculus* recorded its highest activity of 1.57±0.04 U/ml on day 28.

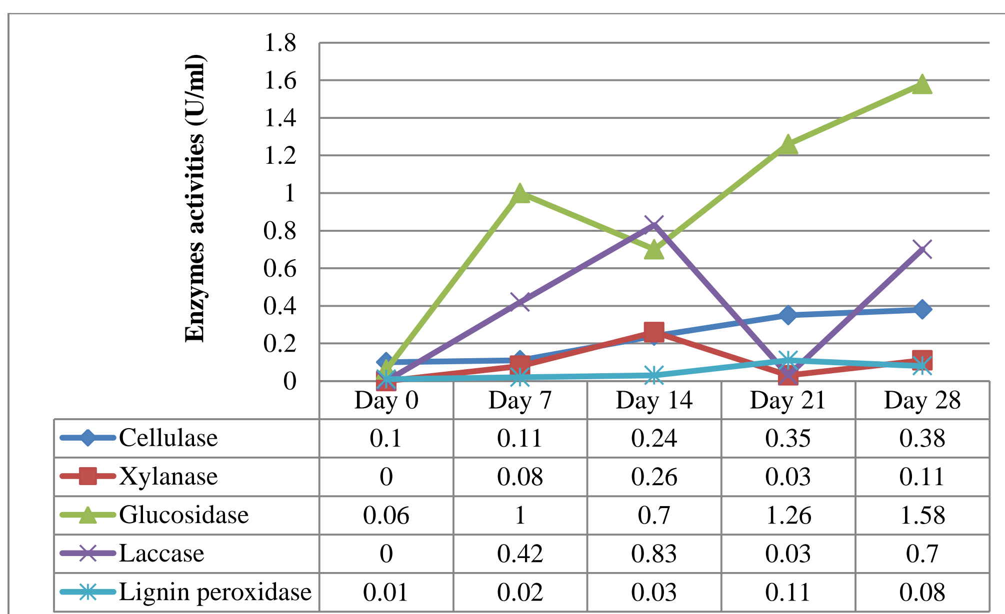
The cellulase enzyme was also assayed apart from ligninolytic enzymes in order to access the applicability of the fungi for biopulping process. The amount of cellulase activity recovered was 0.72±0.11 U/ml on day 14 for *G. australe*, whereas the highest activity for *F. tenuiculus* was recorded on day 28 with 0.38±0.03 U/ml. Although *G. australe* and *F. tenuiculus* had cellulase activity, but this amount was lower compared to laccase activity recovered.

Amount of laccase activity recovered was higher than lignin peroxidase, xylanase and cellulase, but lower than glucosidase. The highest laccase activity recorded was 1.92±0.03 U/ml for *G. australe* on day 14 and *F. tenuiculus* also achieved the highest laccase activity on day 14 with 0.83±0.34 U/ml.



	Day 0	Day 7	Day 14	Day 21	Day 28
Cellulase	0.23	0.4	0.72	0.51	0.44
Xylanase	0.12	0.09	0.33	0.42	0.34
Glucosidase	0.41	2.51	3.21	3.21	2.88
Laccase	0	1.53	1.92	0.32	0.04
Lignin peroxidase	0.01	0.03	0.06	0.18	0.15

Figure 4.6 ***Ganoderma australe*** **KUM60848 enzymes activity (U ml^{-1}) during SSF of empty fruit bunches.**



	Day 0	Day 7	Day 14	Day 21	Day 28
Cellulase	0.1	0.11	0.24	0.35	0.38
Xylanase	0	0.08	0.26	0.03	0.11
Glucosidase	0.06	1	0.7	1.26	1.58
Laccase	0	0.42	0.83	0.03	0.7
Lignin peroxidase	0.01	0.02	0.03	0.11	0.08

Figure 4.7 ***Favolus tenuiculus*** **KUM60803 enzymes activity (U ml^{-1}) during SSF of empty fruit bunches.**

As compared to *F. tenuiculus*, *G. australe* had a maximum xylanase activity on day 21 (0.42 ±0.01 U/ml). The *F. tenuiculus* achieved the maximum xylanase activity on day 14 with 0.26±0.03 U/ml.

In all cases, lignin peroxidise was the least being produced, with activity around 0.02-0.18 U/ml only for both strains. However, during short incubation periods (7-14 days), the peroxidase seemed to be strongly absorbed on the EFB, since they were detected in higher amounts in the 21 and 28 days extractions. *G. australe* achieved its maximum lignin peroxidase activity on day 21 with 0.18±0.02 U/ml, while, *F. tenuiculus* also recorded its maximum enzyme activity of 0.11±0.02 U/ml also on day 21.

4.3.3 pH

It was observed that the pH profile during 28 days of incubation for *G. australe* and *F. tenuiculus* were consistent and regular (Figure 4.8). In general, the pH profiles of both cultures extracts were closing similar. The highest pH of 5.60 was recorded after 21 days incubation of *G. australe* and *F. tenuiculus* revealed highest pH of 6.55 on seven days incubation by using EFB as substrate.

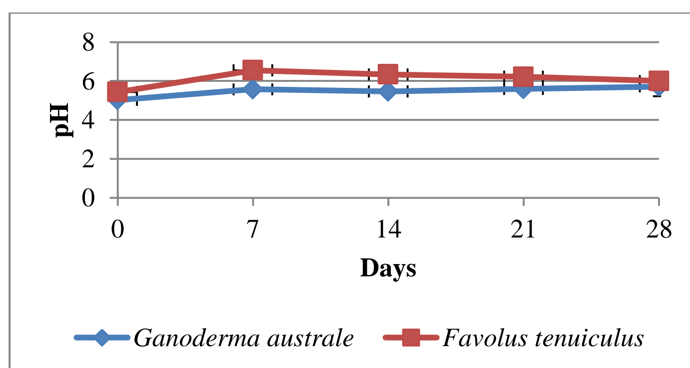


Figure 4.8 pH during SSF of empty fruit bunches by *Ganoderma australe* and *Favolus tenuiculus.*

4.3.4 Property of pulp produced by oil palm (*Elaeis guineensis*) empty fruit bunches through solid substrate fermentation with *Ganoderma australe* KUM60848

In the present study, the potential of *G. australe* KUM60848 was demonstrated for biopulping puprposes, following enzymatic analysis during solid substrate fermentation of EFB. Solid substrate fermentation of EFB was carried out using *G. australe* for 14 and 21 days. Untreated and pre-treated EFB with *G. australe* for 14 and 21 days were submitted to pulping under the conditions previously described. Fourteen days of bio-treatment period was chosen based on the highest enzymes activity i.e. cellulase and laccase produced by *G. australe* KUM60848. On the other hand, 21 days was chosen based on the highest activity of LiP and xylanase whereby the laccase activity were detected at low level. The selections were made on both selected days to evaluate the paper properties produce at different pre-treatment period.

The yields of pulp from untreated (control) and pre-treated EFB (14 and 21 days) after cooking process were estimated to be 30.3% and 35.7% (14 days); 28.5% (21 days) (w/w) of the original EFB added, respectively. The concept of pulp quality is complex and can be defined by a series of independent factors (Guadalix *et al.,* 1996; Giovannozzi-Sermanni *et al.,* 1997). Fourteen days of SSF had the highest degree of material dissolved as indicated by yields. Compared to untreated EFB, the biopulping yield had increased to a maximum of 18% (14 days) and decreased to 6% (21 days) (Table 4.3).

The pulping process had influenced the paper properties i.e. grammage, thickness, tensile index, burst index and the number of folds. The effect of 14 and 21 days fungal pre-treatment of EFB on fiber strength in paper was measured as tensile and burst index (Table 4.4). At 14 days of pre-treatment tensile index was reduced by 11%, whereas the burst index was higher compared to untreated EFB by 14%. On the other

hand, after 28 days of pre-treatment tensile index was reduced by 6% and burst index by 13%. The decreased in strength of paper caused by *G. australe* KUM60848 was the lowest on day 14. Furthermore, the thickness and number of folds were higher compared to untreated EFB.

Table 4.3 The effect of biopulping by *G. australe* at 14 and 21 days on pulp yield and alpha cellulose content of EFB

Days of SSF fermentation	Yield (%)	Alpha Cellulose (%)
14	35.7	79.1±0.01
21	28.5	77.4±0.12
Untreated EFB	30.3	72.0±0.14

Table 4.4 The effect of biopulping on paper properties of EFB

Days of SSF fermentation	Grammage (g/m^2)	Thickness (µm)	Tensile index (Nm/g)	Burst index ($kPam^2/g$)	No of folds
14	61.9	125±2.21	27.0±2.81	2.53±0.197	12±3.92
21	60.1	108±2.20	28.5±0.99	1.87±0.086	2±0.91
Untreated EFB	60.8	119±2.22	30.2±1.32	2.22±0.095	5±1.46

Table 4.5 shows the optimal results obtained in this work, as well as those other authors for EFB pulp, wheat straw and olive wood. As it is observed, the value contributed by Jiménez *et al.,* (2009) and Wanrosli *et al.,* (2004) for tensile index and burst index are quite inferior to the obtained one in this work; in the work of Jiménez *et al.,* (2009) operates to 170°C, 70 min and 15% soda; in the work of Wanrosli *et al.,* (2004) operates to 160-162°C, 63-64 min and 21.7-23.4% of soda, versus 170°C, 120 min and 25% soda used in this study.

Table 4.5 Comparison of various pulps beaten from non-wood

Type of pulp	Yield (%)	Tensile index (Nm/g)	Burst index (kPa m^2/g)
Pre-treated EFB with *G. australe* soda pulp beaten	35.70	29.81	2.73
EFB soda-anthraquinone pulp beaten (Jiménez *et al.*, 2009)	39.0	59.63	4.17
EFB soda pulp (Wanrosli *et al.,* 2004)	41.0	35.75	-
Wheat straw soda pulp beaten (Jiménez *et al.,* 2002)	53.15	45.70	1.81
Olive wood kraft pulp beaten (Díaz *et al.,* 2005)	25.56	39.0	1.95

Values of yield and burst index for the pulps beaten of olive wood was lower than those values obtained for EFB pulp beaten, with independence of the beating grade which is different from one pulp to others. However the value of tensile index is higher for wheat straw soda pulp beaten, versus to the EFB pulp beaten except for the higher value given by Wanrosli *et al.,* (2004). Therefore, it is possible to conclude that the pretreated EFB with white-rot fungi give pulps beaten whose burst properties can compete and overcome to pulps of two singular raw materials i.e. the wheat straw for being widely known and used in the world, and olive wood for being one raw material with ligneous structure like hardwood and softwood.

The results of handsheets brightness expressed for upper side of the handsheets, are presented in Plate 4.4. After incubation with *G. australe* KUM60848, the brightness of the handsheets produced were brighter compared to untreated EFB. The results also showed that the handsheets produced on the 14 days incubation were brighter compared to 21 days incubation. The brightness of pulp was increased, indicating and improved lignin removal (Isroi *et al.,* 2011).

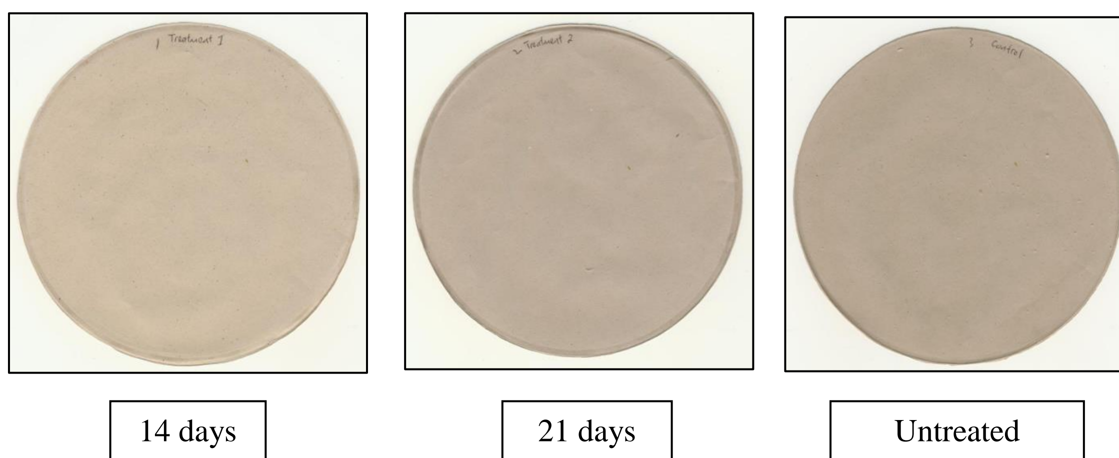


Plate 4.4 Hand-sheets produced by biopulping of EFB using *G. australe* at 14 days, 21 days and untreated EFB.

4.4 DISCUSSION

4.4.1 Screening for enzymes production

The screening for production of amylase and cellulase showed all strains were able to produce the specific enzymes to hydrolyze the substrate for a period of three to seven days. Isolates of *Fomitopsis ostreiformis* and *Coriolopsis badia* were negative for laccase production. The negative results indicated that these fungi either produce no significant levels of this enzyme or that they require different growth conditions (Paice *et al.,* 1993). For example, in this study *Coriolopsis* strains did not show LiP production however, previously study has shown it to be a LiP producer (Jaouani *et al.,* 2003).

On the other hand, *Fomitopsis* species also were negative for lignin peroxidase. *Fomitopsis* are active brown-rot fungi (Ryvarden and Gilbertson, 1993). Brown-rot fungi selectively degrade carbohydrates in lignocellulosic materials (Jae-Won *et al.,* 2008). In addition, brown-rot fungi secrete various hemicellulases and low molecular weight agents such as iron, hydrogen peroxide, and oxalate, as well as cellulases for

cellulose degradation in wood (Xu and Goodell, 2001; Guedon *et al.,* 2002) implying that brown-rot fungi are good resources for efficient cellulases. This explains why laccase and lignin peroxidase were not detected for *Fomitopsis ostreiformis*.

The use of wood-rotting fungi, especially their enzymes for biopulping, offers many potential advantages. It requires not only relatively low levels of chemicals and low cost and energy demands, but also eliminates the pollution hazards associated with the use of chemical pulping process (Akhtar *et al.,* 1992; Turner *et al.,* 1992). Furthermore, enzymes used in pulping can increase the yield of fiber, lessen further refining energy requirements, or provide specific modifications to the fiber (Kenealy and Jeffries, 2003). For example, cellulases have been used in many processes in the paper industry and in fact that enzymes pretreatment using cellulase, hemicellulase, and pectinase have been shown to enhance the kraft pulping of sycamore chips and other pulp sources (Jacobs-Young *et al.,* 1997, 2000).

4.4.2 Enzymes activity, U/ml

White-rot fungi produce various enzymes involved in lignin degradation, but also produce cellulases, xylanases and other hemicellulases (Hatakka, 1994; 2001). According to Hatakka, (2001) and Wong, (2009), enzymes involved in lignin degradation are lignin peroxidase, laccase, manganese peroxidase, versatile peroxidase and H_2O_2-forming enzymes such as glyoxal oxidase and aryl alcohol oxidase. In this study, EFB samples were inoculated with white-rot fungi i.e. *G. australe* KUM60848 and *F. tenuiculus* KUM60803 and then extracted with extraction buffer solution to recover the extracellular enzymes produced during the fungal growth. Results indicate that both strains were produced lignin peroxidase, laccase, xylanase, glucosidase and cellulase during the treatment period. The production of cellulolytic and ligninolytic

enzymes have been reported in *G. australe* (Juan-Pedro *et al.,* 2001) and *F. tenuiculus* (Drechsler-Santos, 2009).

All enzymes activities were observed over the 28 days of incubation for both selected isolates. Most of the enzymes were detected at higher levels on the 14 and 21 days and decreases over successive extractions. However, *G. australe* demonstrated higher enzymes activity i.e cellulase, xylanase, glucosidase, laccase and lignin peroxidase compared to *F. tenuiculus.*

Cellulases are produced by many microorganisms, such as fungi, bacteria and actinomycetes. However, due to high yield, fungi have been commercially exploited for production of these enzymes. Among fungi, *Trichoderma* and *Aspergillus* have been widely exploited for their inherent ability to produce cellulase (Duff *et al.,* 1987; Zhang and Lynd, 2004). The cellulase enzyme was assayed apart from ligninolytic enzymes in order to access the applicability of the selected fungi for biopulping purposes. Cellulase activity was observed for both *G. australe* and *F. tenuiculus* strains starting from day zero in this study. The highest level was 0.72±0.11 U/ml and 0.38±0.03 U/ml for *G. australe* and *F. tenuiculus* respectively. The cellulolytic enzymes on day zero of SSF by both strains might have been contributed from the inoculum which was prepared by growing the fungus on sterilized wheat grains for two weeks. Cellulase activity was reported in the range of 30-40 IU kg^{-1} wood chip from *Eucalyptus globulus* by *G. australe* (Regis *et al.,* 2008). On the other hand, there was no report on the cellulase activity during SFF by *F. tenuiculus*. The cellulase activity observed in the present study was based on the assay method employing the reducing sugars released and analysed according to DNS method. A glucose standard curve was used and reducing sugar expressed as glucose equivalent. From the results obtained, it can be observed that in the present study, cellulase is produced by *G. australe* and *F. tenuiculus* as there is a

different between the enzyme titers of both *G. australe* and *F. tenuiculus* as well as the time (in days) at which each strain exhibits peak.

Xylanase have been isolated from basidiomycetes (Kubacková and Karácsonyi, 1975; Tsiklauri *et al.,* 1999). There was a difference in the xylanase activity between *G. australe* and *F. tenuiculus* strains in the present study. The difference in the enzyme activity might due to the ability of fungus to grow and degrade xylanase differently. It was observed that *G. australe* exhibited a peak in xylanase activity on day 21 whereas *F. tenuiculus* produced high titers of xylanase on day 14. It was observed that xylanase activity of *G. australe* exhibited a peak after peaking of laccase activity indicating that probably xylanases contrary to peroxidases could not act on the basis of low molecular mass agent-mediated process and requires a previous lignin removal to permit the xylanases to diffuse into the wood cell wall (Ferraz *et al.,* 2003). A xylanase productivity of 800-1000 IU kg^{-1} by *G. australe* grown on *Drimys winteri* wood chips on day 5 of biodegradation has been reported (Juan-Pedro *et al.,* 2001). It has also been reported that xylanases are constitutive enzymes and synthesis of xylanases, cellulases and mannanase is subjected to a common regulatory control in response to cellulose as carbon source in *Schizophyllum commune* (Haltrich and Steiner, 1994).

Results indicate that the activity of glucosidase was far more than other enzymes, suggesting that glucosidase might be the predominating enzymes during the incubation of EFB for both strains. β-glucosidase catalyses the hydrolysis of cellobiose and some aryl-glucopyranosides, including the two most commonly used substrates: *p*-nitrophenyl-β-D-glucopyranoside (pNPG) and the 4-methylumbelliferyl-β-D-glucopyranoside. The enzyme does not act upon cellulose directly; nevertheless, it is important because cellobiose is a competitive inhibitor of cellulases and β-glucosidase reduces this inhibition by hydrolysing cellobiose to glucose (Bhat and Bhat, 1997).

Ganoderma australe achieved its maximum β-D-gulcosidase activity on day 14 with 3.22±0.01 U/ml, while *F. tenuiculus* recorded its highest activity of 1.57±0.04 U/ml on day 28. Fungal β-glucosidases are, like other microbial glycosidases, either inducible by their substrate or repressed in the presence of preferred carbon sources (Wilson and Niederpruem, 1967), or constitutively formed although equally catabolite sensitive (Borgia and Sypherd, 1977). In the present study, the production of β-D-glucosidase of *F. tenuiculus* was lower compared to *G. australe* throughout 28 days of incubation. β-D-glucosidase activity of *G. australe* showed gradual increase and peaked between days 14-21. On the other hand, *F. tenuiculus* showed slightly decrease by 0.3 U/ml in the β-D-glucosidase activity on day 14, then increase gradually and peaked on day 28 indicating the simultaneous conversion of cellobiose to glucose. Hydrolytic cleavage of cellobiose, however, is not the only possible pathway for celloboise metabolism. Enzymic reactions such as oxidation of cellobiose to cellobionic acid (Westermark and Eriksson, 1974; Ayers *et al.,* 1978) or phosphorolytic cleavage of the β-glucosidic bond (Heale and Gupta, 1971) also occur.

Among the white-rot fungi, *Pycnoporus cinnabarinus* is known to produce laccases as the major lignin-degrading enzyme and seems to depend on naturally produced mediators to improve laccase's lignin degradation capacities (Eggert *et al.,* 1996; Li *et al.,* 2001). In co-operation with other ligninolytic fungal enzymes such as LiP, manganese peroxidase and versatile peroxidase, laccase oxidizes lignin together with polysaccharides cellulose and hemicelluloses comprise the principal components of wood (Sjöström, 1993). In this study, laccase was detected as the main oxidative enzyme in the extracts. There was a difference in the laccase activity between *G. australe* and *F. tenuiculus*. This difference in the enzyme activity might be due to the ability of fungus to grow and degrade lignin differently. A pattern of increase followed by a gradual decrease in the laccase activity was observed for *G. australe*. It was

observed that both strains exhibited a peak in laccase activity on day 14. An increase in the laccase activity on day 28 by *F. tenuiculus* after decrease on day 21 showed that the fungus still not preparing for fruiting, which is normally indicated by a decrease in laccase productivity. The role of laccase as a development marker for fruiting has been reported in a number of basidomycetes including *Agaricus bisporus* (Wood and Goodenough, 1977), *Schizophyllum commune* (Phillips and Leonard, 1976) and *Pleurotus flabellatus* (Rajarathnam *et al.,* 1987).

Lignin peroxidase activity was observed for both the *G. australe* and *F. tenuiculus* studied from day zero. The activity of ligninolytic enzymes on day zero of SSF by both strains might have been contributed from the inoculums. Lignin proxidases have been purified from the cultures of *Phanerochaete chrysosporium* (Tien and Kirk, 1984), *Trametes versicolor* (Jönsson *et al.,* 1987) and *Bjerkandera* spp. (ten Have *et al.,* 1998). The LiP activity observed in the present study was based on the assay method employing veratryl alcohol as a substrate, which also can be oxidized by veratryl oxidize. From the results obtained it can be observed that in the present study, LiP is produced by *G. australe* and *F. tenuiculus* as there is slightly difference between the enzyme activity as well as the time (in days) whereby each strains peaks. Therefore, it was shown that both strains produced LiP when grown via solid substrate fermentation on EFB. Overall a pattern of increase followed by a gradual decrease in the LiP activity was observed for both strains. It was observed that both strains exhibited a peak in LiP activity on day 21. A work by Silva *et al.,* (2005) with four *Ganoderma* spp. strains indicated that all strains produced laccase, while LiP and MnP were detected in only two of them. In the present study, laccase activity was recorded higher compared to LiP for both *G. australe* and *F. tenuiculus*. The apparent discrepancy among several reports (Agosin *et al.,* 1990; Juan-Pedro *et al.,* 2006) emphasizes that lignin modifying enzyme production varies in a genus and that depends on the substrate and culture conditions.

According to Madigan *et al.,* (2000), each organism has a pH range within which growth is possible and usually has a well-defined pH optimum. Most natural environments have pH between five and nine. In this study, the pH profiles of both *G. australe* and *F. tenuiculus* were consistent during 28 days of incubation. There were only slight changes throughout the period which probably is caused by the cultures response to metabolic activities. According to Raimbault (1998), the pH of a culture may change in response to metabolic activities. The most obvious reason is the secretion of organic acids such as citric acids, acetic acids or lactic acids, which will cause the pH to decrease in the same way than ammonium salts consumption (Raimbault, 1998).

In the present study, the *G. australe* strain demonstrated better production of lignocellulolytic enzymes compared to *F. tenuiculus* and revealed its maximum value on 14 and 21 days of incubation period. Therefore, *G. australe* was selected to undergo solid substrate fermentation of EFB to determine the property of pulp produce for hand-sheets production for 14 and 21 days of incubation.

4.4.3 Property of pulp produced by oil palm (*Elaeis guineensis*) empty fruit bunches through solid substrate fermentation with *Ganoderma australe* KUM60848

In the present study, the pulping process influenced the pulping properties. The duration of EFB pre-treatment through SSF with *G. australe* had effects on the pulp properties. The papers were produced in grammages between 60 and 62 g/m^2, and the analyses of physical properties according to the TAPPI Test Method (T 220 sp-96 and T 441 om-89) and Malaysian Standard (MS ISO 1924-2:1999, MS ISO 2758:2002 and MS ISO 5626:1999), were carried out on these papers. More specifically, those analyses

were: grammage (g/m^2), thickness (μm), tensile index (Nm/g), burst index ($kPam^2/g$) and resistance to folding (Number of folds).

The pulp yield is calculated as the dry weight of pulp obtained divided by the dry weight of EFB. Compared to uninoculated EFB, the biopulping yield had increased to a maximum of 18% by 14 days of incubation by *G. australe*. The pulp yield obtained from 14 days incubation was 35.7%. Rushdan *et al.,* (2008) has reported lower yields than this, but it could be explained by the higher cellulose content of 14 days incubation sample (79.1±0.01). For 21 days incubation, the pulp yield was decreased to 6% compared to uninoculated EFB.

The highest yield of soda-anthraquinone pulp from treated EFB (14 days) (35.7%) is lower than the optimum yield for ethyleneglucol-soda pulp from olive pruning (59.4%) (Jiménez *et al.,* 2004). A difference for percentage of yield could be resulted from different pulping processes. Therefore, in parallel with the growing scarcity of conventional raw material, the increasing concern with the environment and its preservation have exposed the need to replace the classical pulping processes (Kraft, sulphite), which use sulphur-containing reagents the release of which can cause serious pollution problems. The development of new pulping processes using less polluting chemicals (i.e. organosolv processes) should be considered.

After refining the pre-treated EFB pulps obtained after 14 and 21 days growth of *G. australe* KUM60848 were used to prepare handsheets. Hand-sheet was also produced with 100% EFB pulp as control for comparison purposes. The properties of hand-sheets prepared from the pulp made at the center points of the design (25% NaOH, 3 h digestion time and 170 °C maximum cooking time) showed variability, thus making more difficult to observe the trends. In spite of this variability, the representative

parameters for paper production such as; thickness, tensile index, burst index and number of folds were reported.

The analyses carried out to characterize the paper functional behavior in three important aspects: strength, stiffness and structure. Strength of paper was evaluated from its tensile, bursting and tearing strength. Stiffness was evaluated by Resistance to folding (No. of folds). Structure was evaluated from air permeance, i.e. Gurley porosity and is subjected to further study.

With regard to the properties related to paper strength, the Burst Index increasing the percentage of EFB pulp is beneficial when incubated for 14 days with *G. australe*, while the strength property which shows the contrary effect is Tensile Index which decreased 11% compared to uninoculated EFB. On the other hand, 21 days incubation showed significantly decreased strength properties as indicated by both tensile index and burst index.

A comparison of Burst Index along with the other agricultural residues considered (Jiménez and López, 1993; Jiménez *et al.,* 1993; Díaz *et al.,* 2005), reveals the following: The Burst Index for EFB (14 days incubation); 2.53 kPa.m^2/g was higher than those for vine shoots and olive trimming residues, which revealed a maximum value of 0.99 kPa.m^2/g and 0.58 kPa.m^2/g respectively. Then again, the 14 days incubation was similar to those for sunflower stalks (1.62-3.22 kPa.m^2/g) and cotton plants stalk (2.09-4.15 kPa.m^2/g), but lower than those for sorghum stalks (4.2-5.3 kPa.m^2/g). It can be observed that the only parameter where pre-treatment of EFB with *G. australe* showed a clearly better performance was the burst index during 14 days of incubation. Burst Index and Resistance to Folding (No. of Folds) were higher for 14 days compared to 21 days incubation, thus indicating that duration of incubation of 14 days with *G. australe* showed better properties for paper production from EFB.

Based on the result above, we found that the *G. australe* KUM60848 evaluated in this work showed good potential toward the production of extracellular lignocellulolytic enzymes. The strain was fast growing, formed abundant mycelial growth. In enzymatic studies, the strain was recorded to possess fairly good activities of cellulases, xylanase and ligninases and also showed high activities of laccase and β-glucosidase. In the biopulping of EFB experiment, it was shown that the properties of paper were influenced by the pre-treatment of *G. australe* KUM60848. It was noticeable also that the culture conditions applied for the strain may not favor the production of ligninolytic enzymes. Nevertheless, the limited detectable amount of LiP should not be neglected because it is equally important to cause significant delignification. As a conclusion, we found that the *G. australe* KUM60848 strain evaluated in this work exhibited potential as lignocellulolytic organism and some process optimization is needed to use this fungus as an alternative for some biotechnological applications such as biopulping.

CHAPTER 5

GENERAL DISCUSSION

According to Ainsworth & Bisby's Dictionary of Fungi (Kirk *et al.,* 2001) there are twenty-three families in the Order Polyporales. In the present study, the diversity of Polyporales in Peninsular Malaysia is less diverse than previously reported, however 10 out of 60 species identified were not yet reported and documented. The common species encountered based on the number of basidiocarps collected were *Ganoderma australe*, *Lentinus squarrosulus*, *Earliella scabrosa*, *Pycnoporus sanguineus*, *Lentinus connatus*, *Microporus xanthopus*, *Trametes menziesii*, *Lenzites elegans*, *L. sajor-caju* and *Microporus affinis*. Species which were not yet reported by the previous studies were *Coriolopis badia, Coriolopsis sanguinaria, Echinochaetae brachypora, Fomitopsis ostreiformis, Funalia polyzona, Gloeoporus dichrous, Polyporus* cf. *badius, Polyporus philippinensis, Trichaptum bysogenum* and *Trichaptum durum*.

From the limited areas covered in this study and the irregular collecting periods, it is obvious that the diversity of the Polyporales listed here is only a very small percentage of the actual diversity in the forests as for Malaysia, 70 – 80 percent of fungi are yet to be discovered (Lee *et al.,* 1995; Corner, 1996). It is noted that the data discussed here are based on collections of basidiomata only. Several other groups of Polyporales which have not been reported in these collections and could have been overlooked or possibly were not fruiting during the collection periods.

Ganoderma, a genus of approximately 214 described species has been deemed to be in taxonomic chaos. The difficulties stem from the large numbers of synonymies, widespread misuse of names, typification problems and a paucity of reliable morphological characters. Seven strains of *Ganoderma* were performed a detailed

investigation using both morphological and molecular characters. The ITS region was sequenced for the purpose of a molecular analysis. This study shows that strains of the *G. lucidum* and *G. australe* can be identified by 650 base pair nucleic acid sequence characters from ITS1, 5.8S rDNA and ITS2 region on the ribosomal DNA. The ITS region was proven to be useful as a gene marker in separating related species and strains of *Ganoderma* (Moncalvo *et al.,* 1995c; Smith and Sivasithamparam, 2000). The phylogenetic analysis used maximum-parsimony as the optimality criterion. Heuristic searches used 100 replicates of random addition sequences with tree-bisection-reconnection (TBR) branch-swaping. In this study, specimen KUM50079 was received as *G. tsugae* and specimen KUM61117 as *G. amboinense*. On the basis of molecular sequence in this study, both KUM50079 and KUM61117 were considered a synonym of *G. lucidum*. Therefore, ITS phylogeny confirms that *G. lucidum* and *G. australe* were named correctly based on the molecular analysis even though the strains exhibited differences in morphological characteristics.

ITS phylogeny confirms that *G. lucidum* and *G. australe* are named correctly based on the molecular analysis even though the strains exhibited differences in morphological characteristics. This could be explained by Steyaert, (1975) and Chen, (1993) whereby the characters such as basidiocarp shape, basidiospore size and context colour are influenced by environmental factors.

For the enzyme production of Polyporales, the overall results of this preliminary study provide evidence that many wood-rotting fungal of Polyporales collected from Peninsular Malaysia have good potential toward the production of extracellular lignocellulolytic enzymes. Based on the result above, the *G. australe* KUM60848 evaluated in this work showed good potential toward the production of extracellular lignocellulolytic enzymes. It was noticeable also that the strain culture conditions

applied may not favor the production of ligninolytic enzymes. Nevertheless, the limited detectable amount of LiP should not be neglected because it is equally important to cause significant delignification. Furthermore, the fungal also showed the production of cellulase and amylase which also have potential in biotechnology applications. Therefore, it is important that similar studies should be conducted to find out and discover more strains from Polyporales that may give promising results, which are applicable in paper industries to sustain an environmentally friendly paper industry. Furthermore, the quantitatively enzymatic activities showed good activity of the selected enzymes during SSF for both isolates, with *G. australe* demonstrated better activities compared to *F. tenuiculus.*

Biological pretreatment uses microorganisms and their enzymes selectively for delignification of lignocellulosic residues and has the advantages of a low-energy demand, minimal waste production and a lack of environmental effects. In the application of Polyporales in biopulping process, the present study demonstrates that pretreatment of fungal during solid substrate fermentation can enhance soda-AQ pulping of EFB. Compared to conventional pulping, the preliminary results of this study showed that fungal solid substrate fermentation improves pulp properties by increasing the yield (18%), alpha cellulose content (10%) and paper properties (maximum to 10%). In comparing duration of fungal treatment, 14 days solid substrate fermentation of *G. australe* performs better than 21 days in biopulping of EFB. From the handsheets analysis the study conclude that the pre-treatment of EFB with *G. australe* KUM60848 has a positive effect on some strength properties of the pulps (e.g. burst index) but has a negative impact on other mechanical properties (e.g. tensile index). In conclusion, it has shown the properties of paper properties were influence by the pre-treatment of *G. australe* KUM60848. The burst properties of these pretreated EFB pulp beaten are higher than those if others non-wood pulps, as wheat straw and olive wood.

CHAPTER 6

CONCLUSIONS

Macrofungi of the order Polyporales are among the most important wood decomposers and caused economic losses by decaying the wood in standing trees, logs and in sawn timber too. Diversity and distribution of Polyporales in Peninsular Malaysia was investigated by collecting basidiocarps from trunks, branches, exposed roots and soil from six states (Johor, Kedah, Kelantan, Negeri Sembilan, Pahang and Selangor) in Peninsular Malaysia and Federal Territory Kuala Lumpur. This study showed that the diversity of Polyporales were less diverse than previously reported. Therefore, other Polyporales species of different genera should be investigated to establish species diversity and distribution at the species, genus and family level. Nothing conclusive can be suggested at this stage on the Polyporales diversity in the Peninsular Malaysia. The study identified 60 spp. from five families; Fomitopsidaceae, Ganodermataceae, Meruliaceae, Meripilaceae, and Polyporaceae. Besides reported the common species, 18 genera with only one specie were also recorded i.e. *Daedalea, Amauroderma, Flavodon, Earliella, Echinochaetae, Favolus, Flabellophora, Fomitella, Funalia, Hexagonia, Lignosus, Macrohyporia, Microporellus, Nigroporus, Panus, Perenniporia, Pseudofavolus* and *Pyrofomes.*

The range of sites for study of Polyporales needs to be extended to include more virgin jungle in Peninsular Malaysia including West Malaysia. For example, locations in Sarawak can be included because there is a vast number of Polyporales expected due to the more undisturbed and unexplored habitat available. It is estimated that more species should be collected in Peninsular Malaysia and therefore, suggest that more species is expected to be encountered if more frequent and regular collections are carried throughout the Peninsular Malaysia. Therefore, further continuous and long-

term research to be undertaken for a better knowledge and understanding of Malaysian macrofungal diversity in particular.

The *Ganoderma* species are polypore basidiomycetous fungi having a double-walled basidiospore. In all, more than 250 species within the family have been assigned to the genus *Ganoderma*. Due to high phenotypic plasticity, morphological features for *Ganoderma* systematics are thought to be of limited value in the identification of *Ganoderma* species. In order to provide molecular evidence for the *Ganoderma* species, phylogenetic relationship was investigated using morphological and 650 base pair nucleic acid sequence characters from ITS1, 5.8S rDNA and ITS2 region on the ribosomal DNA. This study shows that strains of the *G. lucidum* and *G. australe* can be identified by 650 base pair nucleic acid sequence characters from ITS1, 5.8S rDNA and ITS2 region on the ribosomal DNA. It has been shown in this study that the primer pair ITS1 and ITS4 were applied for the identification *Ganoderma* to species level. The ITS shows variability at interspecies level, and has been extensively used to study the taxonomy and phylogenies for fungi (Royse *et al.,* 1995). However, the gene region amplified by these primers is rather conserved and only provides limited information on classification at the species level. Therefore, in future studies, it could be more appropriate to amplify at least two gene regions that are characterized by both conserved as well as variable regions to enable a better assessment of fungal classification.

Ganoderma australe is a white-rot fungus that also well-known as decomposer of log and stump in the Malaysian forest. In this work, strain of *G. australe* KUM60848 was investigated for the production of ligninolytic enzymes i.e. lignin peroxidase (LiP) and laccase also the hydrolytic enzymes, i.e. cellulase and xylanase using oil palm empty fruit bunch (EFB) in solid substrate fermentation (SSF). Further investigation

was carried out through biopulping to evaluate the effect of pre-treatment of EFB on the paper properties. The EFB showed to support good mycelial growth for *G. australe* KUM60848 during 28 days of solid-state fermentation. LiP, laccase, cellulase and xylanase were detected in EFB cultured with *G. australe* KUM60848. All enzymes activity tested demonstrated highest activities of LiP (0.18±0.02 U/ml), laccase (1.92±0.03 U/ml), cellulase (0.72±0.11 U/ml) and xylanase (0.42±0.01 U/ml) throughout the 28 days of incubation. The property of pulp produced by EFB was analyzed during pre-treatment with *G. australe* KUM60848 at 14 and 21 days of incubation. The EFB was pulped by soda pulping process and recorded to influence the pulping properties. Compared to un-treated (control), the pre-treated EFB yield had increased to a maximum of 18% during biopulping. Fourteen days of SSF had the highest degree of material dissolved as showed by pulp yields and the optimum values (29.8 Nm/g tensile index and 2.73 Kpa m^2/g burst index), acceptable to obtain paper sheets.

In future, it is recommended to purify and characterize the enzymes produced by *G. australe* and the concentrated enzyme extract could be applied in the biopulping of other commercial pulps in the papermaking industry. Moreover, the short period of incubation required by this strain for biopulping purpose (14 days) could be considered positive factor for future industrial applications and environmental management.

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APPENDICES

Appendix A: Materials and methods

1. Carboxymethylcellulase activity

Reagents

1% (w/v) solution of sodium carboxymethyl cellulose (medium viscosity) in 50 mM sodium citrate buffer, pH4.8. Dinitrosalicylic (DNS) acid reagent, Rocchele salt (40%), 50 mM sodium citrate buffer (pH4.8) (Dong *et al.,* 1992; Miller, 1959).

Procedure

The reaction mixture was composed of 1.8ml substrate solution and 0.2ml of enzyme sample. The solution was mixed well and incubated at 40°C for 30 minutes in a water bath with moderate shaking. The reducing sugars released were analysed according to the DNS method (Miller, 1959). The usual reagent and enzyme blanks were included. Reagent blank consisted of 1.8ml substrate and 0.2ml buffer, whereas enzyme blank consisted of 1.8ml buffer and 0.2ml enzyme. The assay mixtures were prepared with the following method

Table A.1 Assay mixtures for carboxymethylcellulase activity assay.

Column	Volume	
Substrate Blank	1.8 ml substrate	+ 0.2ml buffer
Enzyme Blank	1.8 ml buffer	+ 0.2ml enzyme
Reaction Mixture	1.8 ml substrate	+ 0.2ml enzyme

Calculation of unit of activity

A glucose standard curve was used and the reducing sugar expressed as glucose equivalent. Correction was made for absorbance due to the background colour in the enzyme blank. The CMCase activity (Dong *et al.,* 1992) was calculated with the following formula and translated to an enzyme activity expressed as 1 μmol glucose released/min/g substrate.

CMCase activity (U/ml)

$$= \left(\frac{\text{Final absorbance} + 0.105}{2.0621}\right)\left(\frac{2\ (\text{Dil.Fac.})}{0.2\ \text{ml}}\right)\left(\frac{1}{30\ \text{min}}\right)\left(\frac{1000\ \mu g}{1\ \text{mg}}\right)\left(\frac{1\ \mu\text{mole}}{180.16\ \mu g}\right)$$

Glucose MW = 180.16

2. Xylanase activity

Reagents

1% (w/v) suspension of xylan in 50mM sodium citrate buffer (pH 4.8). To prepare the substrate, heat 1% (w/v) suspension of xylan in the buffer to boiling point on a heating magnetic stirrer. Cool the suspension with continuous stirring overnight. DNS reagent, Rocchele salt (40%), 50mM sodium citrate buffer pH 4.8 (Bailey *et al.,* 1992; Miller, 1959).

Procedure

About 1.8ml substrate solution was mixed with 0.2ml of enzyme solution. The mixture was mixed and incubated for 1h at 40°C in a water bath with moderate shaking. The

reducing sugar released in the reaction was determined using DNS method. The standard used was pure xylose. The assay mixtures were prepared as following.

Table A.2 Assay mixtures for xylanase activity assay.

Column	Volume	
Substrate Blank	1.8 ml substrate	+ 0.2ml buffer
Enzyme Blank	1.8 ml buffer	+ 0.2ml enzyme
Reaction Mixture	1.8 ml substrate	+ 0.2ml enzyme

Calculation of unit of activity

One unit of enzyme activity was expressed as 1μmole of xylose released per minute. Correction was made for absorbance due to background colour in the enzyme blank. By using the standard line for xylose, the final absorbance was converted to enzyme activity units. The enzymes activity was calculated as following formula.

Xylanase activity (U/ml)

$$= \left(\frac{\text{Final absorbance} + 0.0514}{2.4411} \right) \left(\frac{2 \text{ (Dil. Fac.)}}{0.2 \text{ ml}} \right) \left(\frac{1}{60 \text{ min}} \right) \left(\frac{1000 \mu\text{g}}{1 \text{ mg}} \right) \left(\frac{1 \ \mu\text{mole}}{150.13 \ \mu\text{g}} \right)$$

Xylose stock solution = 1.0mg/ml Xylose MW = 150.13

3. β-D-Glucosidase activity

Reagents

0.5mM *ρ*-nitrophenyl-β-D-glucopyranoside (substrate) in 50mM sodium citrate buffer, pH 4.8 and 1M sodium carbonate (Na_2CO_3) solution (Dong *et al.,* 1992).

Procedure

The assay mixture consisted of 2.0ml substrate and 0.2ml of enzyme solution. All the test tubes were covered with aluminium foil as the liberated ρ-nitrophenol is light sensitive. The contents of the test tubes were mixed, and then incubated at 40°C for 30 minutes in a water bath with moderate shaking. The enzyme reaction was determined by adding 2ml of 1M Na_2CO_3 and the liberated ρ-nitrophenol was measured at λ=400nm. The usual enzyme and reagent blanks were included. The absorbance values obtained (minus the enzyme and reagent blanks) were further translated into enzyme activity using a standard graph relating µg/ml of ρ-nitrophenol to absorbance (Dong *et al.,* 1992). The assay mixtures were prepared as following.

Table A.3 Assay mixtures for β-D-Glucosidase activity assay.

Column	Volume		
Substrate Blank	1.8 ml substrate	+ 0.2ml buffer	+ 2.0ml 1M Na_2CO_3 after incubation
Enzyme Blank	1.8 ml buffer	+ 0.2ml enzyme	
Reaction Mixture	1.8 ml substrate	+ 0.2ml enzyme	

Calculation of unit of activity

1 unit of enzyme activity expressed as 1 µmol of ρ-nitrophenol released/min under the assay conditions and calculated as following.

β-glucosidase activity (U/ml)

$$= \left(\frac{\text{Final absorbance}+0.0075}{0.0036}\right)\left(\frac{4\text{(Dil. Fac.)}}{0.2\text{ml}}\right)\left(\frac{1}{30\text{ min}}\right)\left(\frac{1\ \mu\text{mole}}{139.1\ \mu\text{g}}\right)$$

ρ-nitrophenol stock solution = 40µg/ml ρ-nitrophenol MW = 139.1

4. Laccase activity

Reagents

0.1mM syringaldazine (4-Hydroxy-3,5-dimethoxybenzaldehyde azine) in 50% ethanol. The substrate was dissolved in 50% ethanol after 13 hours of stirring. 50mM sodium citrate buffer, pH 4.8 (Harkin and Obst, 1973; Leonowicz, 1981).

Procedure

0.2ml enzyme solution was mixed with 3.0ml buffer at room temperature. Then 0.2ml portion of 0.1mM syringaldazine (substrate) was added and mixed with a vortex mixer to start the reaction. The initial rate of colour change was measured on a spectrophotometer at wavelength λ=525nm. The usual reagent blank and enzyme blank were included. The assay mixtures were prepared as following.

Table A.4 Assay mixtures for laccase activity assay.

Column	Volume		
Substrate Blank	3.2 ml buffer	+ 0.2ml substrate	
Enzyme Blank	3.2 ml buffer	+ 0.2ml enzyme	
Reaction Mixture	3.0 ml buffer	+ 0.2ml enzyme	+0.2ml substrate prior to read

Calculation of unit of activity

One unit was defined as the enzyme producing one unit of absorbance change/min. Laccase activity in the culture filtrate was calculated as following.

Laccase activity (U/ml)

$$= \left(\text{Final absorbance} \right) \left(\frac{\text{Dilution Factor}}{0.2 \text{ ml}} \right) \left(\frac{1}{1 \text{ min}} \right)$$

5. Lignin peroxidase activity

Reagents

3mM veratryl alcohol (0.2mM final concentration), 100mM sodium tartrate buffer at pH 3.0 and 7.5mM H_2O_2 (0.5mM final concentration) (Tien and Kirk, 1984).

Procedure

Reaction mixture consisted of 2.4ml 100mM sodium tartrate buffer pH 3.0, 0.2ml of enzyme sample, 0.2ml of 3mM veratryl alcohol, and 0.2ml of 7.5mM H_2O_2 to a final volume of 3.0ml. LiP activity was measured by oxidation of veratryl alcohol to veratryldehyde in the presence of H_2O_2 at pH3.0. The reaction was initiated using 0.2ml of 7.5mM H_2O_2. The enzyme activity was measured at λ=310nm after 5 minutes. The usual reagent blank and enzyme blank were included. The absorbance values obtained (minus the enzyme and reagent blanks) were further translated into enzyme activity using a standard graph relating mg/ml of veratraldehyde to absorbance readings. The assay mixtures were prepared as following.

Table A.5 Assay mixtures for lignin peroxidase activity assay.

Column	Volume			
Substrate Blank	2.6 ml buffer	+ 0.2ml substrate	+ 0.2 ml H_2O_2	
Enzyme Blank	2.6 ml buffer	+ 0.2ml substrate	+ 0.2 ml enzyme	
Reaction Mixture	2.4 ml buffer	+ 0.2ml substrate	+ 0.2 ml enzyme	+ 0.2ml H_2O_2 prior to read

Calculation of unit of activity

One unit of enzyme activity was expressed as 1µmol veratraldehyde (VAD) released per minute. LiP activity was calculated as follow.

LiP activity (U/ml)

$$= \left(\frac{\text{Final absorbance-0.0022}}{11.781}\right)\left(\frac{1}{5\text{ min}}\right)\left(\frac{1000\ \mu g}{1\text{ mg}}\right)\left(\frac{1\ \mu\text{mole}}{166.18\ \mu g}\right)$$

Veratraldehyde stock solution = 0.1mg/ml Vertraldehyde MW = 166.18

6. Determination of reducing sugar (DNS method)

Reagents

i. Dinitrosalicylic acid (DNS) (Miller, 1959)

The DNS reagent used contained 1% (w/v) dinuitrosalicylic acid, 0.2% phenol and 0.05% sodium sulfite and 1% NaOH. Large batches of reagent was prepared without sulfite and stored in a dark bottle at 4 °C. Appropriate amount of sodium sulfite was added to aliquots just prior to the time when the reagent was to be used.

ii. Rochelle salt solution (40%)

Dissolved about 400 g of AR grade Rochelle salt (potassium sodium tartarate tetrahydrate crystals $C_4H_4KNaO_6.4H_2O$) in 1 L distilled water.

Procedure for preparation of glucose calibration plot

Diluted sugar solution (2.0 ml) was added to 3.0 ml DNS reagent. The solution was mixed and incubated in a boiling water bath for 15 minutes. Upon removal from the water bath, approximately 1.0 ml of 40% Rochelle salt solutions was added to the reaction to stabilize the orange colour formed before it was cooled under tap water. The intensity of the colour was determined using spectrophotometer at the wavelength, λ = 575 nm. The reducing sugar concentrations of the test solution were calculated by extrapolation from a linear glucose standard graph.

Reducing sugar assay of test solution

Test solution (2.0 ml) was assayed following the method described above. The absorbance values after subtraction of the substrate blank and enzyme blank were then translated into glucose equivalent using the glucose standard graft, with the following formula.

$$\text{Reducing sugar (mg/ml)} = \frac{(\text{Final absorbance} + 0.105)}{2.0621}$$

Glucose stock solution = 1mg/ml

Appendix B: Raw data

Table B.1 Enzymes activity of *Ganoderma australe* and *Favolus tenuiculus* during 28 days solid substrate fermentation.

DAY	Isolates	Enzyme activities (U/ml)*				
		Cellulase	Xylanase	β-D-gulcosidase	Laccase	Lignin peroxidase
0	*Ganoderma australe*	0.23 ± 0.05	0.12 ± 0.05	0.41 ± 0.09	–0.02 ± 0.02	0.03 ± 0.05
	Favolus tenuiculus	0.10 ± 0.01	–0.12 ± 0.01	0.06 ± 0.01	0.00 ± 0.01	–0.03 ± 0.02
7	*Ganoderma australe*	0.40 ± 0.02	0.09 ± 0.03	2.51 ± 0.06	1.54 ± 0.16	–0.03 ± 0.02
	Favolus tenuiculus	0.12 ± 0.01	0.08 ± 0.02	1.00 ± 0.04	0.42 ± 0.12	0.02 ± 0.01
14	*Ganoderma australe*	0.72 ± 0.11	0.33 ± 0.02	3.22 ± 0.01	1.92 ± 0.03	–0.01 ± 0.01
	Favolus tenuiculus	0.24 ± 0.02	0.26 ± 0.03	0.70 ± 0.04	0.83 ± 0.34	0.01 ± 0.00
21	*Ganoderma australe*	0.51 ± 0.03	0.42 ± 0.01	3.21 ± 0.05	0.32 ± 0.18	–0.02 ± 0.04
	Favolus tenuiculus	0.35 ± 0.01	0.03 ± 0.03	1.26 ± 0.02	0.03 ± 0.04	0.05 ± 0.06
28	*Ganoderma australe*	0.44 ± 0.04	0.34 ± 0.02	2.88 ± 0.01	0.04 ± 0.02	0.00 ± 0.01
	Favolus tenuiculus	0.38 ± 0.03	0.11 ± 0.01	1.57 ± 0.04	0.70 ± 0.19	0.38 ± 0.03

*** mean of triplicate values.**

Appendix C: Data analysis

Table C.1 ANOVA analysis of cellulase activity during SSF of empty fruit bunches by *Ganoderma australe* and *Favolus tenuiculus*.

ANOVA Table

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	0.381427	4	0.0953567	29.55	0.0000
Within groups	0.0322667	10	0.00322667		
Total (Corr.)	0.413693	14			

Multiple Range Test

Method: 95.0 percent LSD

Days	Count	Mean	Homogenus Groups	
1	3	0.233333	X	
2	3	0.403333	X	
5	3	0.44	X	X
4	3	0.513333		X
3	3	0.723333		

Contrast	Difference	+/- Limits
1 – 2	*-0.17	0.103341
1 – 3	*-0.49	0.103341
1 – 4	*-0.28	0.103341
1 – 5	*-0.206667	0.103341
2 – 3	*-0.32	0.103341
2 – 4	*-0.11	0.103341
2 – 5	-0.0366667	0.103341
3 – 4	*-0.21	0.103341
3 – 5	*0.283333	0.103341
4 – 5	0.0733333	0.103341

*denotes a statistically significant difference

Legend

1: Day 0 2: Day 7 3: Day 14 4: Day 21 5: Day28

Table C.2 ANOVA analysis of xylanase activity during SSF of empty fruit bunches by *Ganoderma australe* and *Favolus tenuiculus*.

ANOVA Table

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	0.252893	4	0.0632233	90.32	0.0000
Within groups	0.007	10	0.0007		
Total (Corr.)	0.259893	14			

Multiple Range Test

Method: 95.0 percent LSD

Days	Count	Mean	Homogenus Groups
2	3	0.09	X
1	3	0.12	X
3	3	0.333333	X
5	3	0.336667	X
4	3	0.416667	X

Contrast	Difference	+/- Limits
1 – 2	0.03	0.0481334
1 – 3	*-0.213333	0.0481334
1 – 4	*-0.296667	0.0481334
1 – 5	*-0.216667	0.0481334
2 – 3	*-0.243333	0.0481334
2 – 4	*-0.326667	0.0481334
2 – 5	*-0.246667	0.0481334
3 – 4	*-0.083333	0.0481334
3 – 5	-0.003333	0.0481334
4 – 5	*0.08	0.0481334

*denotes a statistically significant difference

Legend

1: Day 0 2: Day 7 3: Day 14 4: Day 21 5: Day28

Table C.3 ANOVA analysis of β-D-Glucosidase activity during SSF of empty fruit bunches by *Ganoderma australe* and *Favolus tenuiculus*.

ANOVA Table

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	16.5067	4	4.12668	1495.18	0.0000
Within groups	0..276	10	0.00276		
Total (Corr.)	16.5343	14			

Multiple Range Test

Method: 95.0 percent LSD

Days	Count	Mean	Homogenus Groups
1	3	0.41	X
2	3	2.51	X
5	3	2.87667	X
4	3	3.20667	X
3	3	3.21333	X

Contrast	Difference	+/- Limits
1 – 2	*-2.1	0.0955767
1 – 3	*-2.80333	0.0955767
1 – 4	*-2.79667	0.0955767
1 – 5	*-2.46667	0.0955767
2 – 3	*-0.70333	0.0955767
2 – 4	*-0.69667	0.0955767
2 – 5	*-0.36667	0.0955767
3 – 4	0.006666	0.0955767
3 – 5	*0.336667	0.0955767
4 – 5	*0.33	0.0955767

*denotes a statistically significant difference

Legend

1: Day 0 2: Day 7 3: Day 14 4: Day 21 5: Day28

Table C.4 ANOVA analysis of laccase activity during SSF of empty fruit bunches by *Ganoderma australe* and *Favolus tenuiculus*.

ANOVA Table

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	9.79211	4	2.44803	213.37	0.0000
Within groups	0.114733	10	0.0114733		
Total (Corr.)	9.90684	14			

Multiple Range Test

Method: 95.0 percent LSD

Days	Count	Mean	Homogenus Groups
1	3	-0.0166667	X
5	3	0.0433333	X
4	3	0.323333	X
2	3	1.53667	X
3	3	1.92333	X

Contrast	Difference	+/- Limits
1 – 2	*-1.55333	0.194869
1 – 3	*-1.94	0.194869
1 – 4	*-0.34	0.194869
1 – 5	-0.06	0.194869
2 – 3	*-0.386667	0.194869
2 – 4	*1.21333	0.194869
2 – 5	*1.49333	0.194869
3 – 4	*1.6	0.194869
3 – 5	*1.88	0.194869
4 – 5	*0.28	0.194869

*denotes a statistically significant difference

Legend

1: Day 0   2: Day 7   3: Day 14   4: Day 21   5: Day28

Table C.5 ANOVA analysis of lignin peroxidase activity during SSF of empty fruit bunches by *Ganoderma australe* and *Favolus tenuiculus*.

ANOVA Table

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	0.341533	4	0.0853833	97.03	0.0000
Within groups	0.0088	10	0.00088		
Total (Corr.)	0.350333	14			

Multiple Range Test

Method: 95.0 percent LSD

Days	Count	Mean	Homogenus Groups
1	3	-0.0333333	X
3	3	0.01	X X
2	3	0.02	X X
4	3	0.0533333	X
5	3	0.383333	X

Contrast	Difference	+/- Limits
1 – 2	-0.0533333	0.0539683
1 – 3	-0.0433333	0.0539683
1 – 4	*-0.086667	0.0539683
1 – 5	*-0.416667	0.0539683
2 – 3	0.01	0.0539683
2 – 4	-0.0333333	0.0539683
2 – 5	*-0.363333	0.0539683
3 – 4	-0.0433333	0.0539683
3 – 5	*-0.373333	0.0539683
4 – 5	*-0.33	0.0539683

*denotes a statistically significant difference

Legend

1: Day 0 2: Day 7 3: Day 14 4: Day 21 5: Day28

Table C.6 Carboxymethylcellulase activity of *Ganoderma australe* and *Favolus tenuiculus* grown for 28 days on oil palm empty fruit bunches.

Fungus	Carboxymethylcellulase (U/ml) over a period of 28 days				
	Day 0	Day 7	Day 14	Day 21	Day 28
Ganoderma australe	0.23±0.05[a]	0.40±0.02[b]	0.72±0.11[c]	0.51±0.03[d]	0.44±0.04[bd]
Favolus tenuiculus	0.10±0.01[a]	0.11±0.01[a]	0.24±0.02[b]	0.35±0.01[c]	0.38±0.03[d]

Values expressed are means ± S.D. of triplicate measurements.
Values in the same row with different letters (a-d) were significantly different ($p < 0.05$)
The enzyme activity unit (U) was defined as the amount of enzyme required to produce 1 μmol of product/min.

Table C.7 Xylanase activity of *Ganoderma australe* and *Favolus tenuiculus* grown for 28 days on oil palm empty fruit bunches.

Fungus	Xylanase (U/ml) over a period of 28 days				
	Day 0	Day 7	Day 14	Day 21	Day 28
Ganoderma australe	0.12±0.05[a]	0.09±0.03[a]	0.33±0.02[b]	0.42±0.01[c]	0.34±0.02[b]
Favolus tenuiculus	-0.12±0.01[a]	0.08±0.02[b]	0.26±0.03[c]	0.03±0.03[d]	0.11±0.01[b]

Values expressed are means ± S.D. of triplicate measurements.
Values in the same row with different letters (a-d) were significantly different ($p < 0.05$)
The enzyme activity unit (U) was defined as the amount of enzyme required to produce 1 μmol of product/min.

Table C.8 β-D-Glucosidase activity of *Ganoderma australe* and *Favolus tenuiculus* grown for 28 days on oil palm empty fruit bunches.

Fungus	β-D-Glucosidase (U/ml) over a period of 28 days				
	Day 0	Day 7	Day 14	Day 21	Day 28
Ganoderma australe	0.41±0.09[a]	2.51±0.06[b]	3.21±0.01[c]	3.21±0.05[c]	2.88±0.01[d]
Favolus tenuiculus	0.06±0.01[a]	1.00±0.04[b]	0.70±0.04[c]	1.26±0.02[d]	1.58±0.04[e]

Values expressed are means ± S.D. of triplicate measurements.
Values in the same row with different letters (a-e) were significantly different ($p < 0.05$)
The enzyme activity unit (U) was defined as the amount of enzyme required to produce 1 μmol of product/min.

Table C.9 Laccase activity of *Ganoderma australe* and *Favolus tenuiculus* grown for 28 days on oil palm empty fruit bunches.

Fungus	Laccase (U/ml) over a period of 28 days				
	Day 0	Day 7	Day 14	Day 21	Day 28
Ganoderma australe	-0.02±0.02[a]	1.53±0.16[b]	1.92±0.02[c]	0.32±0.18[d]	0.04±0.02[a]
Favolus tenuiculus	0.00±0.01[a]	0.42±0.12[b]	0.83±0.33[c]	0.03±0.04[a]	0.70±0.19[bc]

Values expressed are means ± S.D. of triplicate measurements.
Values in the same row with different letters (a-d) were significantly different ($p < 0.05$)
The enzyme activity unit (U) was defined as the amount of enzyme required to produce 1 μmol of product/min.

Table C.10 Lignin peroxidase (LiP) activity of *Ganoderma australe* and *Favolus tenuiculus* grown for 28 days on oil palm empty fruit bunches.

Fungus	Lignin peroxidase (LiP) (U/ml) over a period of 28 days				
	Day 0	Day 7	Day 14	Day 21	Day 28
Ganoderma australe	0.02±0.01[a]	0.03±0.02[a]	0.06±0.01[b]	0.18±0.02[c]	0.15±0.02[d]
Favolus tenuiculus	0.01±0.01[a]	0.02±0.01[a]	0.03±0.01[a]	0.11±0.02[b]	0.08±0.02[c]

Values expressed are means ± S.D. of triplicate measurements.

Values in the same row with different letters (a-d) were significantly different ($p < 0.05$)

The enzyme activity unit (U) was defined as the amount of enzyme required to produce 1 μmol of product/min.