MATERIALS AND METHODS

3.1 Selected Polypores Cultures

The strains of polypores obtained from the Mycology Laboratory, University Malaya, are given in Table 3.1 and Plate 3.1 a – h. The polypores were maintained on potato dextrose agar (PDA) at 27 ± 2 ^{0}C.

Table 3.1
Code, identity and collection site of selected polypores for this study.

Identity	Code of Polyporales	Collection Site
Albatrellus sp.	KUM 60500	Gombak, Selangor.
Pycnoporus sp.	POR 11	Gombak, Selangor.
Polyporus sp.	POR 26	Institute Biological Sciences, University Malaya.
Trametes versicolor	POR 33D	Gombak, Selangor.
Trametes sp.	POR 33M	Gombak, Selangor.
Microporus sp.	POR 34	Gombak, Selangor.
Polypore sp.	POR 35	Gombak, Selangor.
Microporus xanthopus	POR 57	Endau Rompin National Park Johor
Bjerkandera sp.	POR 31	Gombak, Selangor.
Microporus sp.	POR 18	Gombak, Selangor.
Unidentified 1	POR 36	Gombak, Selangor.
Unidentified 2	POR 38	Gombak, Selangor.
Unidentified 3	POR 48	Endau Rompin National Park, Johor
Unidentified 4	POR 52	Endau Rompin National Park, Johor
Unidentified 5	POR 58	Endau Rompin National Park, Johor

a)

b)


FUNGI OF MALAYSIA
Code: POR 35
Species:
Date: 31/5/05
FUNGI OF MALAYSIA
Code: POR 45
Species:
Date: 31/5/05

c)

d)


FUNGI OF MALAYSIA
Code: POR 33
Species:
Date: 31/5/05

e)

f)

g)

h)

Plate 3.1

Fruiting body structure of polypores selected for this study. a) *Albatrellus* sp.(KUM 60500); b) *Polypore* sp.(POR 35); c) *Polyporus* sp.(POR 26); d) *Trametes* sp. (POR 33); e) *Microporus* sp.(POR 34); f) *Microporus* sp.(POR 18); g) Unidentified 1 (POR 36) and h) Unidentified 2 (POR 38).

3.2 Test Fungi Cultures

Saccharomycetes pombe, *Candida albicans*, *C. parapsilopsis*, *Fusarium oxysporum* f.sp. *cubense* (Foc) race 1*, F. oxysporum* f.sp. *cubense* race 2*, F. oxysporum* f.sp. *cubense* race 4 and *Ganoderma boninense* were obtained from the Institute of

Biological Sciences, University Malaya. *Ganoderma boninense* and *F. oxysporum* were maintained on potato dextrose agar (PDA) (Appendix A) plates at 27 ± 2 ^{0}C, while *S. pombe* was maintained on yeast–peptone –glucose **(**YPG) (Appendix A) agar plate at 27 ± 2 ^{0}C. On the other hand, *C. albicans* and *C. parapsilosis* were maintained on Sabouraud dextrose agar (SDA) (Appendix A) plates at 37 ± 2 ^{0}C.

3.3 Inoculum Preparation

A small portion of the fungal colony was cut from margin of fresh and mature (7- to 8-day-old) cultures and transferred to PDA plates and incubated at 27 ± 2 ^{0}C (Mao *et al*., 2005). For the inoculum of test fungi preparation, disc of mycelium *G. boninense* and *F. oxysporum* were transferred to the sterile PDA plates and incubated at 27 ± 2 ^{0}C for 5 days.

3.4 Culture Systems for Cultivation of Selected Polyporales.

Different cultivation methods for production of antifungal agents and enzymes were investigated.

3.4.1 Solid Culture System

Different agar media were prepared: PDA (OXOID / Bio-Focus Saintifik Sdn Bhd) with 1% (w/w) yeast; glucose–yeast–malt–peptone agar medium (GYMP); malt extract (ME) (2% malt extract with 1.8% agar) and corn meal agar (OXIOD / Bio-Focus Saintifik Sdn Bhd) (CMA) (Appendix A). The selected polypores (Table 3.1) were cultured on the agar plates at 27 ± 2 ^{0}C for 10 days.

3.4.2 Static and Submerged Agitated Culture System

The selected polypores were cultured for 10 days at 27 ± 2 ^{0}C on PDA plate. Then, aseptically five culture discs (5 mm) were transferred from PDA culture plate into unbaffled 250 ml Erlenmeyer flasks containing 25 ml of potato dextrose (PD) broth medium (2.4% w/v PD broth); malt extract (ME) broth (2 % w/v malt extract) and GYMP

broth [w/v: 0.05% magnesium sulphate ($MgSO_4.H_2O$), 0.046 % potassium dihydrogen orthophosphate (KH_2PO_4), 0.1 % di–potassium hydrogen phosphate (K_2HPO_4), 2 % glucose, 0.2 % peptone, 0.2 % yeast extract and 0.2 % malt extract]. The inoculated flasks were then incubated either at 27 ± 2 ^{0}C in static and dark condition for 7 – 8 days or in a rotary shaker at 120 rpm in dark condition for 7 – 8 days (Rosa *et al*., 2003).

3.4.3 Solid Substrate Culture System

30 gm of wheat or corn or green bean or rice was soaked to soften the tissue, before being transferred into 250 ml flasks and autoclaved at 15 psi, at 121 ^{0}C for 15 min. The substrates were cooled and moistened with 15 ml GYMP. Aseptically five culture discs (5 mm) were transferred from a ten days old culture of *Albatrellus* sp. (KUM 60500) on PDA into 250 ml Erlenmeyer flasks. The culture was incubated at 27 ± 2 ^{0}C and in static dark condition for 10 days (Ali *et al*., 2006).

3.4.4 Submerged and Agitated Culture System for Enzymatic Activity Test

All polypores were cultured on PDA plate and incubated at 27 ± 2 ^{0}C for 7 – 8 days. Then, agar spot test for laccase, tyrosinase and lignin peroxidase (LiP) were conducted. For LiP enzymatic activity, aseptically five 5 mm mycelial discs of 10 days old *T. versicolor* (POR 33D) and *Polypore* sp. (POR 35) were transferred into 250 ml Erlenmeyer flasks containing 25 ml PD broth from PDA plate. The cultures were incubated for 7 - 8 days at 27 ± 2 ^{0}C at 120 rpm in dark. After that period, the cultures were filtered and the filtrate was analyzed for LiP enzymatic activity.

3.5 Extraction of Bioactive Components

3.5.1 Extraction from Solid Culture

After culture grew for 10 days, five agar plates were pooled and transferred into a 250ml flask. The culture agar was soaked in methanol for 48 hours. Then, the solvent was

removed in a rotary evaporator under vacuum at temperatures below 45 ± 2 ^{0}C (Rosa *et al*., 2003). Subsequently, the paper disc diffusion test was carried out to study antifungal activities.

3.5.2 Extraction from Static and Submerged Agitated Culture

After culture was grown for 7 - 8 days, the whole broth was freeze dried. The dried biomass was soaked in methanol for 48 hours. Then, the solvent was removed in a rotary evaporator under vacuum at temperatures below 45 ± 2 ^{0}C. Subsequently, the paper disc diffusion test was carried out to study antifungal activities.

3.5.3 Extraction from Solid Substrate Culture

After culture was grown for 10 days, the cultures were soaked in methanol for 48 hours. The solvent was also removed in a rotary evaporator under vacuum at temperatures below 45 ± 2 ^{0}C. Subsequently, the paper disc diffusion test was carried out to determine the inhibition of *S. pombe.*

3.6 Plate Assay for Antagonistic Activity

An agar plug (5mm) of *G. boninense* or *F. oxysporum* were cut from five days old culture (2mm from the edge) and placed at the center of each PDA plates with mycelial growth on the agar (Fig 3.1). *Candida* spp. were lawned on SDA plate and *S. pombe* on YPG agar plate. Polypore plugs were then placed as shown in Fig 3.2. Plates without *polypore* plugs were used as control. Inhibition zone was observed after 24 and 48 hours of incubation at 27 ± 2 ^{0}C (Saleem and Kandasamy, 2002).

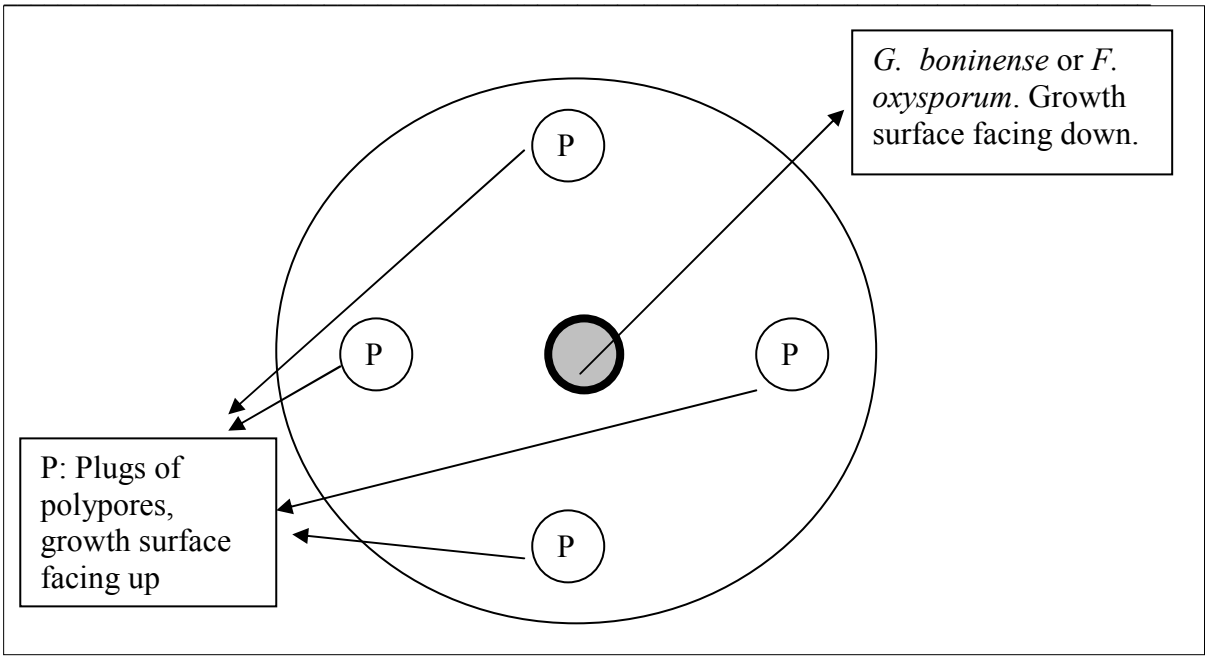

Fig 3.1

Plate screening for antagonistic activity of *Ganoderma boninense* and *Fusarium oxysporum* (PDA plate; 24 to 48 hours of incubation at 27 ± 2 ^{0}C.).

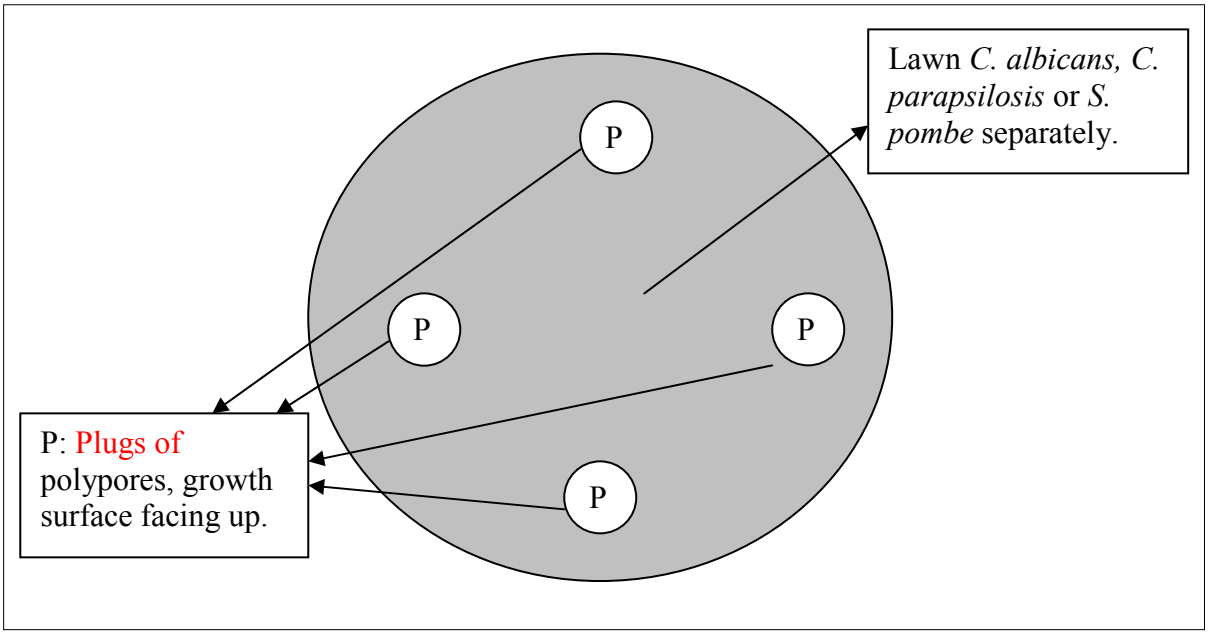

Fig 3.2

Plate screening for antagonistic activity of *C. albicans, C. parapsilosis* and *S. pombe* (SDA plate for *Candida* sp. and YPG agar plate for *S. pombe*; 24 to 48 hours of incubation at 27 ± 2 ^{0}C for *Candida* sp. and 37 ± 2 ^{0}C for *S. pombe*).

3.7 Paper Disc Diffusion Test to Study Antifungal Activities against *Candida* spp. and *Saccharomyces pombe*

The paper disc diffusion method has several advantages. It is technically simple to perform and reproducible. The reagents used are relatively inexpensive and it does not require any special equipment. Besides, it provides categorized results that are easily understood by clinicians (Jorgensen *et al*., 1999).

The assay for antifungal activity towards *Candida* sp. and *S. pombe* was carried out in Petri plates containing SDA and YPG agar. Two days old *Candida* spp. was lawned on SDA plate and three days old *S. pombe* on YPG plate with sterile cotton buds. Forcep was used to place paper discs on agar plate lawned with fungus as shown in Fig 3.3.

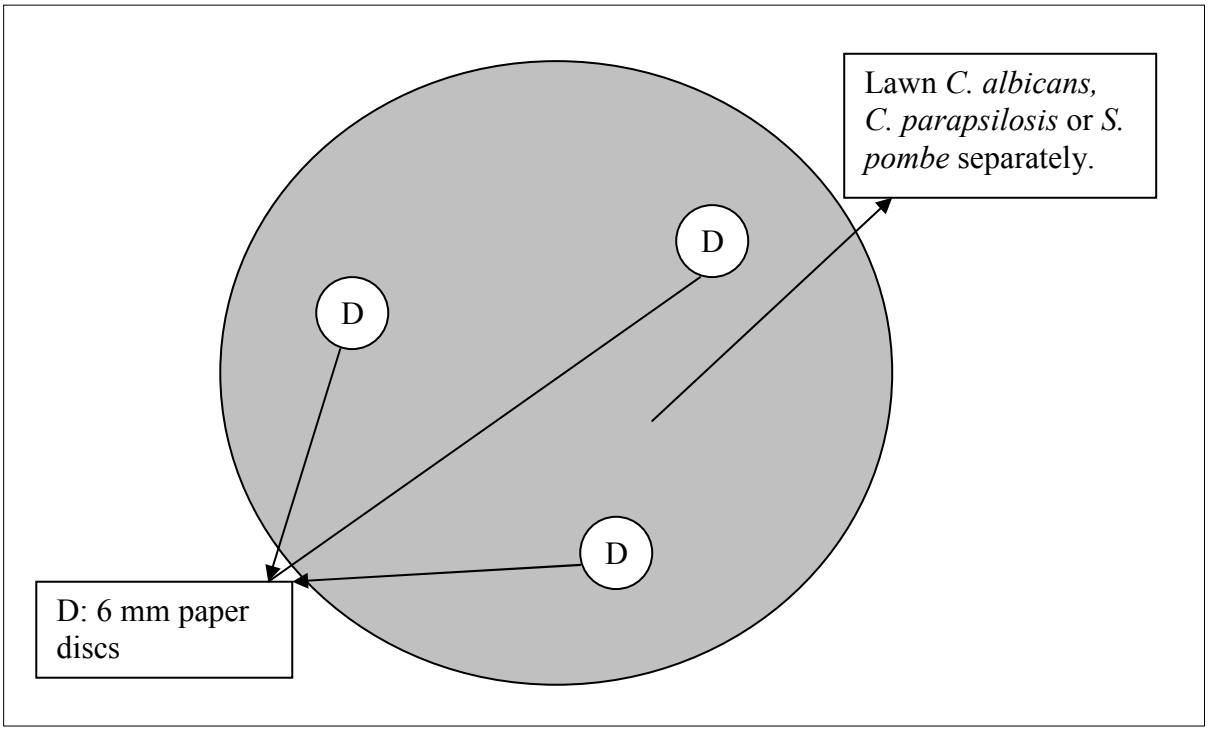

Fig 3.3

Paper disc diffusion test to *C. albicans, C. parapsilosis* and *S. pombe* (SDA plate for *Candida* spp. and YPG plate for *S. pombe*; 24 to 48 hours of incubation at 27 ± 2 ^{0}C for *Candida* spp. and 37 ± 2 ^{0}C for *S. pombe*).

Three paper discs were loaded with 0.02 ml (200 mg/ml) extract. A negative and positive control was set up using 0.02 ml of 10% (w/w) dimethylsulphoxide (DMSO) and 0.02 ml (100 µg/mg) nystatin respectively.

Each crude extract was tested in triplicates against each fungal strains. *C. albicans* and *C. parapsilosis* were incubated at 27 ± 2 ^{0}C and *S. pombe* at 37 ± 2 ^{0}C. After 24 and 48 hours of incubation, the diameter of inhibition zone, including the diameter of the disc was measured and recorded.

3.8 Paper Disc Diffusion Test to Study Antifungal Activities against *Ganoderma* sp. and *Fusarium* sp.

The assay for antifungal activity towards the *F. oxysporum* and *G. boninense* was carried out on Petri plates containing PDA. *F. oxysporum* and *G. boninense* were cultured on PDA for five days.

A 5 mm plug from the edge of a five days old culture of *F. oxysporum* and *G. boninense* on PDA was placed at the center of the plate. Sterile blank paper discs (6 mm) were placed at a distance of 2.5 cm away from the rim of the mycelial colony (Emma *et al*., 2001). Pipettor was used to load 0.02ml (200 mg/ml) extract on 6 mm paper discs. A negative and positive control was set up using 0.02 ml of 10% (w/w) dimethylsulphoxide (DMSO) and 0.02 ml (100 µg/mg) nystatin respectively. Paper disc were placed as shown in Fig3.4.

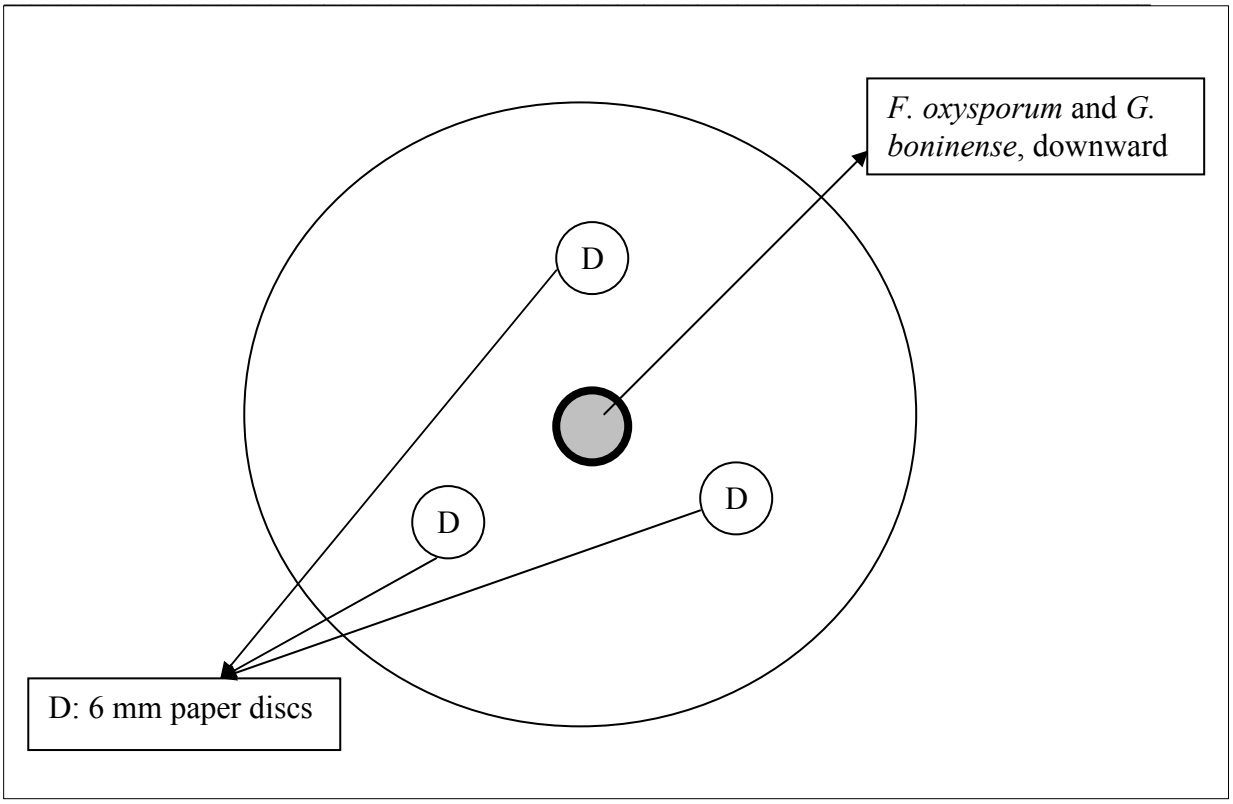

Fig 3.4

Paper disc diffusion test to *F. oxysporum* and *G. boninense* (PDA plate; 24 to 48 hours of incubation at 27 ± 2 ^{0}C).

Each crude extract was tested in triplicates against each fungi strains. The plates were incubated at 27 ± 2 ^{0}C. After 24 and 48 hours of incubation, the diameter of the inhibition zone was measured and recorded.

3.9 Agar Spot Test to Investigate Enzymatic Activity

5 mm well were cut in the agar plate using a sterile cork borer. For the laccase screening test, a volume of 0.1 M syringaldazine dissolved in 50% (w/w) ethanol was loaded in the wells. For lignin peroxidase screening test, a volume of 1% pyrogallic acid in hydrogen peroxidase (H_2O_2) was loaded in the wells. On the other hand, for tyrosinase screening test, a volume of 0.1M P. cresol in the wells was also loaded. The test were performed in triplicate and repeated when the target reaction was not induced. Intensities of the colour reaction (weak, medium and strong) were taken as visual comparison with reference plates (Gramss *et al*., 1998).

3.10 Lignin Peroxidase (LiP) Enzyme Activity

The culture medium was filtered through Whatman No.1 filter paper for each interval of incubation. The filtrate obtained was tested for enzyme activity. Lignin peroxidase activity in the filtrate was measured periodically by determining the rate of oxidation of veratryl alcohol to veratraldehyde (David *et al*., 2002) (Appendix B).

The lignin peroxidase (LiP) activity was evaluated by UV spectrometry (310 nm) of the veratryl aldehyde produced by oxidation of veratryl alcohol. The reactive mixture contained: 2.4 ml sodium tartrate buffer 100 mM pH 3.0; 0.2 ml veratryl alcohol 2 mM; 0.2 ml hydrogen peroxidase 0.5 mM and 0.2 ml culture medium for a final volume of 3.0 ml (David *et al*., 2002).