SUBMERGED CULTIVATION OF BASIDIOMYCETE FUNGI ASSOCIATED WITH ROOT DISEASES FOR PRODUCTION OF VALUABLE BIOACTIVE METABOLITES

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GETHA K, HATSU M, WONG HJ & LEE SS. 2009. Submerged cultivation of basidiomycete fungi associated with root diseases for production of valuable bioactive metabolites. This study is part of a screening programme aimed at searching for bioactive metabolites from basidiomycete fungi belonging to the order Polyporales. These fungi are commonly associated with root diseases in forest tree species and agricultural crops. Submerged cultivation of fungal mycelia in liquid media could reduce the time spent in obtaining antimicrobial metabolites. A total of 112 butanol extracts prepared from broth cultures of fungi belonging to several species of the genera *Phellinus*, *Ganoderma*, *Rigidoporus*, *Tinctoporellus* and *Lentinus*, and some unidentified polypore species, were evaluated for antimicrobial activity against *Bacillus subtilis, Staphylococcus aureus, Saccharomyces cerevisiae* and *Rhodotorulla glutinis*. Differences in antimicrobial activities observed in the different fungal genera suggested that the ability to produce bioactive compounds is not homogenously distributed among basidiomycetes. In the primary antimicrobial assay, a total of 26 (23.2%) extracts exhibited strong antimicrobial activity with percentage of inhibition concentration (%IC) ≥ 90% against one or more of the test micro-organisms. Antibacterial activity was more pronounced than antifungal activity. Ten extracts that exhibited strong antibacterial activity showed minimum inhibitory concentration (MIC) values of ≤ 0.125 μg μl^{-1} against *B. subtilis* in a secondary assay.

Keywords: Polyporales, plant pathogenic macrofungi, mycelial cultures, natural products, antimicrobial activity

GETHA K, HATSU M, WONG HJ & LEE SS. 2009. Pengkulturan kulat basidiomiset yang dikaitkan dengan penyakit akar dalam media cecair untuk penghasilan metabolit bioaktif yang penting. Kajian ini merupakan sebahagian daripada kerja penyaringan bagi meninjau metabolit bioaktif daripada kulat basidiomiset dalam order Polyporales yang lazimnya dikaitkan dengan penyakit akar spesies pokok hutan dan tanaman pertanian. Miselium kulat basidiomiset yang dikultur dalam media cecair dapat menghasilkan metabolit antimikrob dalam masa yang singkat. Sejumlah 112 ekstrak butanol yang disediakan menggunakan media kultur kulat daripada genus *Phellinus, Ganoderma, Rigidoporus, Tinctoporellus* dan *Lentinus* serta beberapa spesies polypora yang tidak dikenali dikaji untuk aktiviti antimikrob ke atas *Bacillus subtilis, Staphylococcus aureus, Saccharomyces cerevisiae* dan *Rhodotorulla glutinis*. Perbezaan aktiviti antimikrob dalam genus kulat yang berlainan menunjukkan bahawa kebolehan menghasilkan sebatian bioaktif adalah tidak homogenus di kalangan basidiomiset yang dikaji. Dalam asai antimikrob primer, sejumlah 26 (23.2%) ekstrak menunjukkan aktiviti antimikrob yang kuat dengan peratusan kepekatan perencatan (%IC) ≥ 90% terhadap satu atau lebih mikroorganisma ujian. Aktiviti antibakteria lebih ketara daripada aktiviti antikulat. Dalam asai sekunder, 10 ekstrak dengan aktiviti antibakteria yang kuat menunjukkan nilai kepekatan perencatan minimum (MIC) dengan nilai ≤ 0.125 μg μl^{-1} terhadap *B. subtilis*.

INTRODUCTION

In the last decades, the wide range of pharmaceutically interesting metabolites from basidiomycetes, a large group of terrestrial fungi of the phylum Basdiomycota, has been one of the most attractive groups of natural products studied (Boh *et al.* 2003). The first investigations on the potential of basidiomycetes as sources of antibiotics were carried out in 1941 when extracts of fruiting bodies and mycelia cultures from over 2000 species were examined (Florey *et al.* 1949).

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Pleuromutilin, a diterpene that is especially useful for the treatment of mycoplasm infections in animals, was one of the first commercial antibiotics developed from basidiomycete origin (Rosa *et al.* 2003). The fact that basidiomycetes have been insufficiently investigated, together with the broad range of structural types of antibiotics which are produced by them, suggests that they may be a source of new and useful bioactive compounds (Anke 1989).

Polypores are a major component of the basidiomycete fungi in forest ecosystems and as wood decayers and tree pathogens, they play important ecological roles. Polypore fungi are heterogeneous, showing a great variation in their macromorphological characteristics. Besides anatomical characteristics, biochemical and molecular phylogenetic studies have been used to characterize the families of polypores (Hibbett & Donoghue 1995). Studies showed that only a small number of the most common species of polypores such as *Ganoderma lucidum* have been evaluated thoroughly for biological activity (Wagner *et al.* 2003). Numerous compounds from *G. lucidum* were found to display antiviral, cytotoxic and/or antineoplastic activities. Besides *G. lucidum*, several different polysaccharide antitumour drugs have been developed from the fruiting bodies, mycelia and culture media of various polypore fungi such as *Trametes versicolor*, *Phellinus linteus* and *Inonotus obliquus* (Wasser 2002). Many species of polypores belonging to the genera *Ganoderma*, *Rigidoporus* and *Phellinus* are well known as destructive agents that cause root diseases in timber trees, agricultural crops and fruit trees (Mohd Farid & Lee 2006). These pathogenic polypores have not been sufficiently investigated for their bioactivity and, thus, may constitute a good source for screening and developing new antibiotics.

Despite their potential and enormous diversity in tropical forests, studies aiming at the discovery of bioactive compounds from polypores have reported difficulties such as slow growth rate and low product yields (Suay *et al.* 2000). Since it usually takes several months to cultivate basidiomycetes using traditional basidiome (fruiting body) cultivation method, liquid culture is viewed as a promising alternative. Another advantage is that manipulation in culture growth conditions may lead to a wider range of compounds produced by them (Zhong & Tang 2004).

The main goals of this study were (1) to cultivate tropical polypores isolated from diseased trees of forest species and agricultural crops from different ecological niches in submerged cultures, and (2) to evaluate their ability to produce antimicrobial substances of pharmaceutical interest.

MATERIALS AND METHODS

Fungal culture

Fungal isolates belonging to several species of the genera *Phellinus*, *Ganoderma*, *Rigidoporus*, *Tinctoporellus* and *Lentinus* and some unidentified polypore species (Table 1) were obtained from the culture collection of the Pathology Laboratory at the Forest Research Institute Malaysia (FRIM). Fungal isolates were isolated from diseased samples obtained from symptomatic trees that were brought to the lab. Identification of the fungal isolates was conducted both macroscopically and microscopically according to conventional methods (Mohd Farid & Lee 2006). Some of the isolates were identified by molecular methods (Mohd Farid *et al.* 2003). All isolates were maintained on potato dextrose agar (PDA) and preserved as agar plugs in sterile distilled water at room temperature.

Crude extract preparation

The inoculum for submerged culture consisted of a 5 mm diameter plug of each isolate taken from seven-day-old cultures on PDA plates. Mycelial plugs were transferred into Erlenmeyer flasks containing 20 ml of two types of production medium: medium A (liquid substrate based on potato dextrose) and medium B (liquid substrate based on glucose, enriched with mineral salts). After one to two weeks of incubation at 28 ± 2 °C on an orbital shaker at 200 rpm, fermentation broths were extracted with butanol. Crude extracts (solvent layer) were separated and dried in a vacuum evaporator and stored at 4 °C.

Antimicrobial assay

A total of 112 basidiomycete extracts were prepared and tested for antibacterial activity against *Staphylococcus aureus* NBRC 12732 and *Bacillus subtilis* NBRC 3134, and antifungal activity against *Saccharomyces cerevisiae* (strain

Table 1 List of fungal genera and the hosts/origin of fungal isolates used

Fungal pathogen	Host / origin	No. of extract samples
Phellinus spp.	*Acacia mangium*	64
	Tectona grandis	
	Azadirachta excelsa	
	Hevea brasiliensis	
	Fraxinus formosa	
Rigidoporus spp.	*Acacia mangium*	22
	Azadirachta excelsa	
	Hevea brasiliensis	
Ganoderma spp.	*Acacia mangium*	16
	Hevea brasiliensis	
	Dryobalanops aromatica	
Tinctoporellus spp.	*Acacia mangium*	2
Lentinus spp.	On wood	2
Basidiomycetes (unidentified)	*Acacia mangium*	6
	Samanea saman	

obtained from Nimura Genetic Solutions Sdn. Bhd.) and *Rhodotorulla glutinis* NBRC 1125. Overnight cultures of *B. subtilis* and *S. cerevisiae*, and 48-hour culture of *R. glutinis* grown in yeast nitrogen-based medium enriched with glucose (YNBG), were adjusted to a cell concentration of 10^5cfu ml^{-1}. Overnight culture of *S. aureus* grown in YNBG medium was adjusted to a cell concentration of 10^6cfu ml^{-1}. Volumes of 20 µl of test extract (dissolved in 20% DMSO) and 180 µl suspension of test micro-organisms were added to each well in a 96-well microtitre plate. Positive control (media blank) and negative control wells (inoculum blank) were also prepared. Triplicate plates were prepared and incubated overnight. The initial and overnight optical density (OD) values of each well were measured at 620 nm wavelengths using a microtitre plate reader to determine the activity. The percentage of inhibition concentration (%IC) in the test wells was calculated based on the δOD values. Extracts that showed %IC ≥ 90% against one or more test micro-organisms in the primary assay were considered as potential and selected for further studies.

$$\delta_{OD} = OD_{overnight} - OD_{initial}$$

$$\%IC = \frac{\delta OD_{control} - \delta OD_{test}}{\delta OD_{control}} \times 100\%$$

Potential antibacterial extracts were tested in a secondary assay to determine minimal inhibitory concentration (MIC) value against *B. subtilis* using the microtitre broth dilution method according to the Clinical and Laboratory Standards Institute guidelines. MIC value was defined as the lowest concentration of an extract to yield a %IC ≥ 90% against the test micro-organism (NCCLS 2000).

RESULTS AND DISCUSSION

A total of 112 extracts from submerged mycelial cultures were tested for the presence of antimicrobial activities. The frequency of the resultant antimicrobial activities was considered as an indicator of the ability of the different genera of polypores to produce bioactive secondary metabolites with potential therapeutic interest. A total of 26 extracts or 23.2% of the total extracts tested exhibited significant activity of %IC ≥ 90% against one or more of the target micro-organisms.

The highest proportion of antimicrobial activities occurred within members of the unidentified polypores (83.3%) (Table 2). The results of this study supported earlier reports (Anke 1989) that showed basidiomycetes belonging to many uncommon species produce a great variety of antimicrobial activities. A high percentage of activity was also shown by extracts obtained from isolates belonging to the *Ganoderma* genus (62.5%), followed by extracts from *Rigidoporus* species (27.2%). A low percentage of antimicrobial activity was shown by extracts of *Phellinus* species (7.8%). Since antimicrobial activity was not observed in extracts

Table 2 Distribution of antimicrobial activities among extracts of basidiomycete isolates from different genera of polypores

Fungal genera	No. of extracts tested	No. of positive extracts[a] (%)	Positive antibacterial[a] (%)	Positive antifungal[a] (%)	Positive antibacterial and antifungal[a] (%)
Phellinus spp.	64	5 (7.8%)	5 (7.8%)	0 (0%)	0 (0%)
Ganoderma spp.	16	10 (62.5%)	8 (50.0%)	0 (0%)	2 (12.5%)
Rigidoporus spp.	22	6 (27.2%)	6 (27.2%)	0 (0%)	0 (0%)
Tinctoporellus spp.	2	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Lentinus spp.	2	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Basidiomycetes	6	5 (83.3%)	3 (50.0%)	1 (16.7%)	1 (16.7%)
TOTAL	112	26 (23.2%)	22 (19.6%)	1 (0.9%)	3 (2.7%)

[a] Extracts showing %IC ≥ 90% against one or more target micro-organisms (in primary assay)

obtained from *Tinctoporellus* and *Lentinus* species, valid comparisons of the bioactive potentiality of these genera could not be made due to the low number of extracts studied.

The extracts tested showed greater antibacterial than antifungal activities. Antifungal activities were most frequently found in members of the unidentified polypore species (Table 2). Twenty-two extracts were active against bacteria only while one exclusively inhibited the growth of fungi. Three extracts presented wide antimicrobial spectrum and were active against both fungi and bacteria. It is reasonable to expect that the observed activities were due to the presence of more than one active compound in these extracts.

In this study, members of the genus *Phellinus* that belong to the Hymenochaetaceae showed antibacterial activitiy against *B. subtilis*. Although Suay *et al.* (2000) reported that none of the *Phellinus* species studied by them showed antimicrobial activity, Rosa *et al.* (2003) showed that this genus can produce antibacterial compounds against *B. cereus*. Species in Hymenochaetaceae contain a group of organic compounds called styrylpyrones. Similar compounds are also known from various plant families and they form part of a defense against infections and browsing. The distribution of styrylpyrones within Hymenochaetaceae has been used in the classification of this group (Larsson *et al.* 2006). Hwang *et al.* (2000) reported that the antifungal compound phellinsin A produced by a *Phellinus* sp. inhibits the chitin synthases of *S. cerevisiae*. However, antifungal activity was not observed in the *Phellinus* extracts tested in this study. It is apparent from Table 2 that representatives from the different basidiomycete genera showed marked differences in their antimicrobial activities. The differences between the representatives of all the genera might reflect the metabolic diversity of these fungi. At the same time it may also be, at least partially, a function of the submerged cultivation conditions used in this study.

From the 112 extracts tested, 25 that showed potential antibacterial activity were further tested to determine their minimum inhibitory concentration (MIC) value against *B. subtilis*. It was interesting to note that 10 extracts belonging to the *Ganoderma* species and unidentified polypores were the most active for antibacterial activity, with MIC values ≤ 0.125 µg μl^{-1} (Table 3).

The results obtained in this study are consistent with those reported in an earlier study. Suay *et al.* (2000) showed that 45% of extracts from a total of 317 isolates of basidiomycetes collected in Spain showed antimicrobial activity when screened against a panel of human clinical pathogens. They observed pronounced differences in the antimicrobial activities of the basidiomycetes at the genus level, suggesting that the differences may reflect genetic divergence at the infraspecific level. They also reported that members of the Ganodermataceae were especially productive with about 73% of strains showing activity, while a lower percentage of activity was shown by strains belonging to the Hymenochaetaceae. However, Suay *et al.* (2000) reported that the Lentinaceae isolates (*Lentinus* spp.) and Coriolaceae isolates (*Rigidoporus* spp. and *Tinctoporellus* spp.) gave

Table 3 Antimicrobial activity of 10 potential extracts from different polypore genera and their minimal inhibitory concentration (MIC) values against *Bacillus subtilis*

Extract[a]	Genera	Antibacterial activity[a]		Antifungal activity[a]		MIC (μg μl^{-1}) against *Bs*
		Sa	*Bs*	*Sc*	*Rg*	
F004-008a	*Ganoderma* spp.	+	+	+		0.011
F003-006a	*Ganoderma* spp.	+	+			0.024
F005-004b	Basidiomycetes	+	+	+		0.029
F004-008b	*Ganoderma* spp.	+	+			0.043
F004-009b	*Ganoderma* spp.	+	+			0.044
F008-006b	Basidiomycetes	+	+			0.087
F004-009a	*Ganoderma* spp.	+	+	+		0.101
F008-003b	*Ganoderma* spp.	+	+			0.103
F003-006b	*Ganoderma* spp.	+	+			0.104
F008-002b	*Ganoderma* spp.	+	+			0.125

[a] These fungal extracts were among the 25 extracts that showed per cent inhibition concentration (%IC) ≥ 90% against *B. subtilis* in the primary antimicrobial assay.

\+ = %IC ≥ 90%

Sa = Staphylococcus aureus, Bs = Bacillus subtilis, Sc = Saccharomyces cerevisiae, Rg = Rhodotorulla glutinis

intermediate results with about 43–50% of the tested strains showing antimicrobial activities.

All eight active extracts from the *Ganoderma* species were positive against both *B. subtilis* and *S. aureus* (Table 3). Several studies have shown that some *Ganoderma* species exhibit a broad spectrum of antibacterial activity. The mechanisms for the antibacterial activity of these *Ganoderma* species are largely undefined (Gao *et al.* 2003). Some species such as *G. lucidum*, *G. pfeifferi* and *G. resinaceum* were reported to be specifically active against *B. subtilis*. However, they were not active against other bacteria such as *S. aureus*, *Pseudomonas aeruginosa, Serratia marcescens, Enterococcus faecium* and *Mycobacterium smegmatis* (Suay *et al.* 2000). In another study, *G. applanatum* demonstrated strong antimicrobial activity against gram-positive bacteria *B. cereus* and *S. aureus* but less active against gram-negative *P. aeruginosa* and *Escherichia coli* (Smania *et al.* 2001). Of the 10 antibacterial extracts selected in this study, three also inhibited the growth of *S. cerevisiae*. However, none were active against *R. glutinis*. This could be due to the high resistance of some bacterial and fungal strains to many known antibiotics (Pelaez *et al.* 1998).

In this study, submerged cultivation of basidiomycetes was carried out for one to two weeks in shake flasks under controlled conditions for the production of mycelial biomass and bioactive metabolites. The advantage of submerged fermentation over traditional basidiome cultivation is the reduction in the time spent to obtain the product of interest (Tang & Zhong 2003). Wagner *et al.* (2003) reported that the production of basidiome takes at least three to five months for *G. lucidum*, while reasonable amounts of its bioactive metabolites such as ganoderic acids and polysaccharides can be obtained from submerged fermentation after only two to three weeks. Submerged cultivation in shake-flasks, which is similar to a conventional bacterial fermentation process, also has the advantage of being able to control the product type and quality by manipulating fermentation conditions such as temperature, agitation rates, inoculum density, initial sugar concentration and pH (Ghorashi *et al.* 2003).

Submerged cultivation of macrofungi such as basidiomycetes presents some interesting challenges. Unlike filamentous fungi, with which it is quite easy to produce spores and use them as inoculum, it is not practical to use spore inocula of basidiomycetes for fermentation since spores are only produced in the basidiome. Therefore, the approach used in this study was to prepare mycelium-based inocula grown on agar. When the volume of the growth medium to be inoculated is low, small pieces of mycelium together with some of the agar medium on which they are growing can be cut from slants or Petri dishes and transferred directly into the fermentation

broth. However, if a large volume of broth is to be inoculated, a long lag phase and low inoculum densities achieved would be expected since the mycelium was transferred from a solid to a liquid environment (Wagner *et al.* 2003). Since the present study was a comparative study to evaluate the antimicrobial properties of various species of basidiomycetes in small volumes of liquid media, the size of the pieces of mycelium inoculum used was standardized and removed from colony radial of cultures of the same age. This is to ensure that different inocula contain mycelium at the same stage of development.

The ability to manipulate culture conditions in submerged cultivation may lead to the production of a wider range of bioactive compounds of therapeutic importance by basidiomycetes (Zhong & Tang 2004). Many new secondary metabolites have been reported from the basidiomycetes. A special interest is a group of novel sesquiterpenoid hydroquinones known as ganomycins, produced by the European *Ganoderma* species, *G. pfeifferi,* which inhibit the growth of methicillin-resistant *S. aureus* and other bacteria (Mothana *et al.* 2000). Another novel compound, panepophenanthrin, obtained from the fermented broth of a polypore strain *Panus rudis* IFO 8994 was found to be inhibitors of the ubiquitin-proteasome pathway which is considered to regulate important cellular events and linked to serious diseases as well. This compound is an inhibitor of the ubiquitin-activating enzymes essential for the ubiquitin-proteasome pathway (Sekizawa *et al.* 2002). A comprehensive review on investigations carried out on *Ganoderma* species around the world, with particular emphasis on chemicals of biomedical relevance, was presented by Paterson (2006). According to the review, the majority of research on active compounds from *Ganoderma* species was carried out on extracts from the fruiting body. Hence, there are still a number of biologically active compounds yet to be found in the mycelium of this genus.

CONCLUSIONS

To the best of our knowledge, this survey is the first to investigate Malaysian species of polypore fungi isolated from diseased trees of timber species and agricultural crops, for antimicrobial activities in a submerged culture system. The results of the present study can serve to stimulate the investigation of tropical basidiomycetes as a potential source of bioactive secondary metabolites. It is also clear that, to some extent, some suprageneric taxa (families) are a richer source of bioactive metabolites than others. It can, therefore, be concluded that while basidiomycetes may not be as easy to cultivate as bacteria, they may be worth screening as their diverse biosynthetic abilities may be harnessed in the search and discovery of new compounds active against new drug targets.

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