Induction of antibiotic production of freshwater fungi using mix-culture fermentation

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Antibiotic activities of culture filtrates from twenty filamentous fungi, isolated from wood submerged in tropical freshwater habitats, were tested against a pathogenic strain of *Candida albicans*. These taxa were fermented separately in liquid medium in pure culture, while another set was fermented with the addition of a fluconazole-sensitive strain of *Candida albicans* in mix-culture. Fermentation was performed using potato dextrose broth at 25°C for 28 days, with diffuse day light and 240 rpm agitation. Culture filtrates were tested against a fluconazole-sensitive and two fluconazole-resistant strains of *C. albicans*. Anti-fungal activities against strains of *C. albicans* were not exhibited by any culture filtrates obtained from pure culture fermentation. In mix-culture fermentation, the culture filtrates of an undescribed species of *Chloridium* and *Sporoschisma mirabile* produced anti-fungal activities against all three strains of *C. albicans* tested.

Key words: antibiotic induction technology, anti-fungal, fermentation, tropical fungi.

Introduction

The pharmaceutical industry has become increasingly interested in screening fungi for novel antibiotics and other secondary metabolites (Shu, 1998). Historical discovery of medical novel compounds from fungi usually resulted from accidental finds or screening of ubiquitous fungi. Pharmaceutical companies are now investigating a wider array of fungi, and employing strategic screening programmes to increase the number of discoveries (Hyde, 2001).

Conventional screening programmes for antibiotics are conducted by fermentation of a pure strain. This approach is unnatural and therefore may often fail to turn up new medical compounds. In nature fungi will compete against a suite of other microorganisms and this competition may induce antimicrobial production (Hyde, 2001). This study, therefore, investigates the feasibility of an antibiotic induction technology. Fungi that, when grown in

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pure culture, do not have an antibiotic effect on a target organism are fermented with this target organism in mix-culture during fermentation processes. The extracts are then used to test for antibiotic activities. Interesting results were found and are reported in this paper.

It is estimated that about 1.5 million fungi are present in the world, but 95% of these have yet to be discovered (Hawksworth, 1991, 2000). Fungi are one of the major antibiotic-producing organisms, and one of the most diversified group of organisms. The search for antibiotics from them is promising and in 1995 six of the world's 20 best selling medical drugs are fungal derived (Concepcion *et al*., 2001). The diversity of freshwater fungi is high and more species are expected to be found from this habitat (Hyde, 1997). At the University of Hong Kong Culture Collection, we have more than 7,000 strains of mostly unique tropical fungi (Ho *et al*., 2001a).

Recent screening programmes on freshwater fungi have discovered some interesting novel compounds. Fisher *et al*. (1988) discovered a new antibiotic, Quinaphthin, from *Helicoon richonis*. Harrigan *et al*. (1995) described a new anti-fungal metabolite, Anguillosporal, from *Anguillospora longissima*. Poch *et al*. (1992) discovered several new bioactive metabolites from *Kirschsteiniothelia* sp., while Oh *et al*. (2001) discovered Massarilactones A and B from *Massarina tunicata*. Antibiotic effects of freshwater fungi were also reported in other studies (Fisher and Anson, 1983; Shearer and Zare-Maivan, 1988; Platas *et al*., 1998; Gulis and Stephanovich, 1999). Freshwater fungi have proven to be a potent group for antibiotic production and are therefore selected for study in this paper.

Materials and methods

Source of fungal cultures

Decaying wood was collected from streams and reservoirs in Australia, Hong Kong, and the Philippines. Incubation of the substrata, followed by examination and isolation of the fungi followed Choi *et al*. (1999) and Ho *et al*. (2001b). Twenty filamentous fungi, including 9 anamorphic species, 10 ascomycetes, and an unidentified species, were selected for study (Table 1). Cultures were maintained on potato dextrose agar (PDA) for routine work, and preserved in The University of Hong Kong Culture Collection (HKUCC) in 10% glycerol at -140°C.

Source of target organisms

Three strains of *Candida albicans* were obtained from the Department of Oral Biology, the Prince Philip Dental Hospital, Hong Kong. These include a

reference strain deposited by M.A. Pfaller in the American Type Culture Collection (ATCC 90028, NCCLS 11) which was isolated from human blood samples from Iowa, for anti-fungal susceptibility testing (NCCLS M27-T), and two fluconazole-resistant strains isolated from HIV positive patients BU1010 and BU47204 from Queen Elizabeth Hospital, Hong Kong. Cultures were maintained on nutrient agar (NA) for routine work, and preserved in HKUCC at -140°C.

Fermentation conditions

For each test fungus, in pure culture fermentation, five squares (5 mm × 5 mm) of actively growing mycelium were cut and placed in a 250 ml Erlenmeyer flask containing 100 ml potato dextrose broth (PDB). Three replicates were prepared for each test fungus. Flasks were incubated at 25°C with diffuse day light and 240 rpm agitation for 28 days.

In mix-culture fermentation, a set of Erlenmeyer flasks containing PDB and 5 squares of actively growing mycelium was fermented for 7 days. Each test strain of *Candida albicans* (10^5 cells) was then transferred into each flask and fermented for another 21 days. The *Condida* cells were produced in flasks containing 100 ml nutrient broth (NB), incubated at 25°C with 240 rpm agitation for 24 hours. *Candida albicans* was inoculated into a Erlenmeyer flask containing 100 ml nutrient broth as the control.

Anti-fungal assay

Fermented cultures were filtered through filter paper (Whatman no. 2). Fermented mix-cultures were further filtered through cellulose acetate filter membrane (Advantec, 0.45 µm diam. pore size) with the aid of vacuum pump to remove yeast cells. Culture filtrates were stored at -70°C until the anti-fungal assay.

The agar-well diffusion method was performed for the anti-fungal assay. Petri dishes were filled with 25 ml PDA to give a depth of 4 mm of medium. The three test strains of *C. albicans*, were grown in flasks of nutrient broth, and 0.5 ml of yeast culture, containing 10^5-10^6 CFU/ml, was transferred to each Petri dish and evenly streaked on the surface with sterile cotton buds. Five equally spaced agar wells (6 mm diam.), 15 mm from the edge of each dish, were cut using the ends of sterile glass pipettes. The bottom of each well was sealed with 25 µl of melted PDA added to the centre of each well using an autopipette. Inoculated Petri dishes were stored at 4°C and used within 7 days.

To test anti-fungal activities, 100 µl of culture filtrate was transferred into each well of the inoculated Petri dishes. Negative and positive controls

consisted of 100 µl PDB and 0.1 mg/ml ampicilin transferred into wells of inoculated Petri dishes. Each treatment was triplicated in separate Petri dishes. Dishes were then incubated at 25°C in the dark for 24 hours. Two perpendicular diam. of each inhibition zone were measured to the nearest 0.1 mm using a pair of Vernier Calipers under a stereo-microscope. Appearance of the inhibition zones and the presence of resistant strains of *C. albicans* were recorded. The average diam. of the inhibition zone and the standard deviation were calculated.

Results

Anti-fungal activities were detected on culture filtrates of two of the twenty tested freshwater fungal strains from mix-culture fermentation (Table 1). However, none of the pure-culture fermentation and filtrates of *Candida albicans* produced anti-fungal activities under the conditions studied.

The anti-fungal activities of *Chloridium* sp. and *Sporoschisma mirabile* from mix-culture fermentation were detected on all triplicate Petri dishes against all target strains. The inhibition zones of *Chloridium* sp. and *Sporoschisma mirabile* were similar on the fluconazole-sensitive and fluconazole-resistant strains of *Candida albicans*.

Discussion

Antimicrobial screening programmes are affected by detection methods, selection of producing microorganisms, and cultivation methods (Iwai and Omura, 1981). Omura (1992) identified three most important factors that affect screening for bioactive compounds from microorganisms: devised fermentation, unique microorganisms, and selective and sensitive detection methods for the desired bioactivity. Mix-culture fermentation was not considered as an effective tool of antibiotic induction technology (Omura, 1992).

Tanaka (1992) stated that it is of interest to obtain new antibiotics by mix-culture fermentation, in which one strain produces a biologically inactive metabolite that is converted by the second strain to an active antibiotic. Although there has been wide application of mix-culture fermentation in the production of commercial chemicals (Weimer, 1991), there have been no published results of attempts to induce production of antibiotics by fermenting the test and target organisms in mix-cultures.

We have demonstrated that mix-culture fermentation of the test and target organisms is a feasible antibiotic induction technology. Watanabe *et al*. (1982) demonstrated that employing spent medium in pseudo-mixed culture

Table 1. Diameter of inhibition zone of *Candida albicans* when tested against culture filtrates of tropical freshwater fungi in either pure culture fermentation or mix-culture fermentation.

Fungus	**HKUCC**	***Candida albicans* strain**					
		ATCC 90028		**BU1010**		**BU47204**	
		A*	**B**	**A**	**B**	**A**	**B**
Annulatascus velatisporus K.D. Hyde	5503	0	0	0	0	0	0
Anthostomella sp.	5303	0	0	0	0	0	0
Chloridium sp. nov. (unpublished)	5383	5.7±0.13	0	5.7±0.88	0	3.5±0.97	0
Dendrospora sp.	5381	0	0	0	0	0	0
Dictyochaeta sp.	5494	0	0	0	0	0	0
Dictyosporium heptasporum (Garov.) Damon	1057	0	0	0	0	0	0
Helicomyces roseus Link	3430	0	0	0	0	0	0
Helicosporium sp.	5465	0	0	0	0	0	0
Jahnula australiensis K.D. Hyde	3612	0	0	0	0	0	0
Linocarpon sp.	5338	0	0	0	0	0	0
Massarina bipolaris K.D. Hyde	3628	0	0	0	0	0	0
Massarina ingoldiana Shearer & K.D. Hyde	4640	0	0	0	0	0	0
Massarina ingoldiana Shearer & K.D. Hyde	5419	0	0	0	0	0	0
Massarina purpurascens K.D. Hyde & Aptroot	3666	0	0	0	0	0	0
Nigrospora sp.	1046	0	0	0	0	0	0
Ophioceras sp.	5333	0	0	0	0	0	0
Phaeoisaria clematidis (Fuckel) S. Hughes	1766	0	0	0	0	0	0
Savoryella aquatica K.D. Hyde	1357	0	0	0	0	0	0
Sporoschisma mirabile Berk. & Broome	5464	5.7±0.58	0	5.2±1.1	0	4.6±0.21	0
Unidentified fungus	5507	0	0	0	0	0	0

*A = Mix-culture fermentation with the addition of *Candida albicans*; B = Pure culture fermentation.

fermentation could induce antibiotic production. The supernatant of a 4-days-old culture of *Fusarium* strain was filter-sterilized and inoculated with various

microorganisms. Antibacterial assays showed that there were positive results on the used medium, but no antibacterial activities were detected on the conventional medium. Among these, a *Gluconobacter* strain was found to produce a new trienic antibiotic, AB-135.

Gloer (1995) suggested that freshwater fungi, due to their specific habitat, might have biosynthetic capabilities different from those of terrestrial fungi. There is, therefore, a high possibility of obtaining new antibiotic metabolites of medical and agricultural importance from freshwater fungi.

Yeasts are commonly found in freshwater habitats. The interaction between freshwater filamentous fungi and yeasts provides a clue for the discovery of new natural bioactive compounds. *Massarina aquatica* was found inhibiting growth of the yeasts *Candida albicans* (Platas *et al*., 1998) and *Sporobolomyces roseus* (Fisher and Anson, 1983). Gulis and Stephanovich (1999) reported growth inhibition of *Candida utilis* by *Clavariopsis aquatica*. Our study reveals that further freshwater fungi can inhibit the growth of yeasts, and this can be shown if mix-culture fermentation is performed.

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