CHAPTER 7

ANTIMICROBIAL ACTIVITY AND ENZYME PRODUCTION FROM ZINGIBERACEOUS FUNGI

7.1 Introduction

Fungi can degrade many kinds of organic as well as some inorganic substrates by their capability to produce a vast range of enzymes, e.g., cellulases, chitinases, proteases and multicomponent lignin-degrading enzymes (Lynch and Poole, 1979; Edwards, 1988; Killham, 1994; Boddy and Watkinson, 1995; Nicklin *et al*., 1999). This diverse enzyme production allows them to grow in a wide range of environments, as decomposers or parasites. Fungal proteases and polysaccharide-hydrolysing enzymes (cellulases and hemicellulases) are industrially important, with a wide spectrum of well-established applications. Proteases of microbial origin are produced for a broad range of applications, such as use in laundry and dish washing detergents, animal feed, food production, textile, and leather industries (Outtrup and Boyce, 1990; Tomschy *et al*., 2000; Tzanov *et al*., 2001; Kirk *et al*., 2002). There is growing interest in hemicellulases (especially xylanases) owing to their potential application in the food/feed and pulp/paper industries (Buchert *et al*., 1993; Viikari *et al*., 1993). Mannanase only recently attracted increased scientific and commercial attention due to potential applications in the pulp and paper industry for removal of hemicellulose from dissolving pulps (Gübitz *et al*., 1997a), or for enhancement of the

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

bleachability of pulp, and thus reduction of the use of environmentally harmful bleaching chemicals (Cuevas *et al*., 1996; Gübitz *et al*., 1997b).

Fungi are also the source of important medicinal products (Trinci, 1992; Kirk *et al*., 1993, 2002; Moore and Chiu, 2001; Thaithatgoon *et al*., 2004). Penicillin, perhaps the most famous of all antibiotic drugs, was originally derived from the common fungus, *Penicillium chysogenum* (Fleming, 1929). However, many other fungi also produce antibiotic substances, which are widely used to control diseases in humans and animals. The discovery of antimicrobials revolutionized health care worldwide (Wildman, 1997). Antimicrobial resistance is a major problem and an important driving force for the continued search for new antimicrobial agents.

Intelligent screening methods have been applied in the search for novel molecules (Pointing and Hyde, 2001). One approach has involved the investigation of the microbes associated with medicinal plants (Stierle *et al*., 1993; Strobel *et al*., 1999; Li *et al*., 2000). In these studies, interesting compounds were assayed against an array of human fungal pathogens (e.g., *Candida albicans*, *Trichophyton* spp.) and important plant pathogenic fungi (e.g., *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Pyricularia oryzae*). In a search for maytansinoid antitumor agent from *Actinosynnema pretiosum*, inhibitory activity of ansamytocin was assayed against the growth of *Penicillium avellaneum* (Yu *et al*., 2002).

The potential is enormous for the discovery of valuable natural products resulting from a directed search and screening of fungi from unexplored habitats (Concepcion *et al*., 2001; Strobel and Daisy, 2003; Strobel *et al*., 2004). It is expected that new drugs of biotechnological importance will be discovered with increased focus on tropical endophytic fungi. Fungal species screened for secondary metabolites

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

using modern techniques are fewer than 1% of those that may exist (Nisbet and Fox, 1991). Zingiberaceous plants such as ginger and galanga are among the most popular plants in Thailand, important as both a food ingredients worldwide and as a medicine for local people.

The objectives of this study were to screen zingiberaceous fungi for their efficacy in enzyme production (cellulase, mannanase and protease) and the potential antimicrobial activity of crude preparations (culture broth) against human bacterial (*Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*) and fungal (*Candida albicans*) pathogens, and against the growth of *Penicillium avellaneum*.

7.2 Materials and methods

7.2.1 Preliminary screening of zingiberaceous fungi for enzyme production

Carboxymethylcellulose (CMC) and locust bean gum (LBG) were used as carbon sources in basal liquid media (Appendix A) and tested for cellulase and mannanase production, respectively (Araujo and Ward, 1990; Pointing, 1999). Thirty three fungal strains isolated from zingiberaceous plants as previous described (Chapters 3–5), were subcultured on to PDA plates and incubated for 7 days. One fungal disk (5 mm diameter) was inoculated into test tubes (18 × 180 mm) containing 5 ml liquid media. The test tubes were incubated at room temperature for 4 days on a reciprocal shaker (130 rpm). After incubation, the supernatant from the culture was obtained by centrifugation at 6,000 rpm for 15 min, and 30 µl of each supernatant was tested for the presence of cellulase or mannanase by gel diffusion assay according to

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

the method of Downie *et al*. (1994). Qualitative activity was determined by Congo red staining according to the method of Penttilä *et al*. (1987).

For protease production, 133 strains of zingiberaceous fungi (Chapters 3–5) from stock culture were subcultured on to PDA plates and incubated for 7 days. Fungal disks (5 mm diameter) were inoculated on to Petri dishes (9 cm diameter) containing protease test medium (skim milk agar, Appendix A). The plates were incubated at room temperature for 4 days. After incubation, qualitative activity was determined by measuring the clear zones around the fungal colonies.

7.2.2 Evaluation of antimicrobial production

7.2.2.1 Test organisms and preparation of assay plates

Bacterial strains

Four bacterial strains (*Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*) obtained from the Department of Biology, Faculty of Science, Chiang Mai University, were used as test organisms. All bacterial strains were separately cultured and maintained on nutrient agar (NA) at room temperature (27–30 °C).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

Fungal strains

Candida albicans (ATCC90028, obtained from the American Type Culture Collection) and *P. avellaneum* (obtained from Kunming Botanical Garden, Kunming, China) were cultured and maintained at room temperature on malt peptone agar (MPA) and yeast glucose agar (YGA), respectively.

Preparation of assay plates

Inoculum of all test organisms, were prepared for agar well or paper disk diffusion assays as follows. One loop of each bacterial isolate was scraped from the agar culture slants and inoculated into separate tubes containing 5 ml of nutrient broth. Cells of *C. albicans* were also scraped off the agar and inoculated into 5 ml of malt peptone broth. The tubes were then incubated for 16 h at room temperature on a reciprocal shaker (130 rpm). For *P. avellaneum*, 0.1% of Tween 80 was added before inoculation with one loop of conidia. The mixture was then vortexed, and immediately used for assay plates. One ml of inoculum of each test organism was added to 200 ml of appropriate medium at 55 °C, poured into assay plates, and allowed to solidify.

7.2.2.2 Screening for antimicrobial production

Two liquid media were used as fermentation media (Cheeptham *et al*., 1999).

F1:	fructose	3%
	soybean meal	0.5%
	$CaCl_2$	0.01%
	KH_2PO_4	0.1%
	$MgSO_4.7H_2O$	0.05%, pH 6.0

F2:	yeast extract	0.2%
	Glucose	0.5%
	KH_2PO_4	0.5%
	$MgSO_4.7H_2O$	0.05%, pH 6.0

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

Seventy eight strains of endophytic fungi and 52 saprobic fungi, isolated from zingiberaceous plants as previously described (Chapters 3, 4), were individually subcultured on to PDA plates and incubated for 7 days. One 5 mm diameter plug was cut from the growing edge of each colony and inoculated into test tubes (18 × 180

mm) containing 5 ml fermentation media. The fermentation media were incubated at room temperature (27–30 °C) for 7 days on a reciprocal shaker at 130 rotations per minute.

7.2.2.3 Extraction and concentration

After fermentation, the culture was harvested by filtration. The culture broths were then extracted twice with equal volumes of ethyl acetate (EtOAc). The EtOAc extracts were pooled and dried by rotary evaporator (BÜCHI Switzerland) at 45 ºC under reduced pressure. The extract residue was dissolved in dimetyl sulfoxide (DMSO) and stored at 4 ºC until the bioassays could be conducted.

7.2.2.4 Bioassay

Agar well diffusion assay

Thirty five µl of the concentrated extracts dissolved in DMSO, was separately loaded into 0.5 mm diameter wells cut into the assay plates.

Paper disk diffusion assay

Sterile paper disks (8 mm diameter, BioTech) were dipped into each fraction from column chromatography as described in section 7.2.2.7, allowed to air dry, and placed on the seeded assay plates. Controls consisted of disks impregnated with solvents used in the column chromatography and allowed to air dry.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

All assay plates were then incubated at 37 ºC in the dark for 1 day for all bacterial strains and *C. albicans*, and at 40 ºC for 2–3 days for *P. avellaneum*. After incubation the diameter of the bacterial or fungal growth inhibition zone was measured. For the screening for antimicrobial production, the tests were conducted in triplicate and data generated from this experiment was analyzed using SPSS v.10 package for one-way analysis of variance (ANOVA). The antimicrobial activity (diameter inhibition zone) of zingiberaceous fungi against the test organisms was also qualitatively evaluated as: +++, > 20 mm; ++, 11–20 mm; +, 8–10 mm; ±, 6–7 mm; -, X = 5 mm.

7.2.2.5 Taxonomy of the selected strain

The strain exhibiting the most inhibition against *P. evellaneum* was inoculated from stock culture on to PDA plates and incubated at room temperature for 7 days to observe morphological characteristics. Molecular techniques, DNA sequencing of ITS rDNA regions was also performed as described in Chapter 6 and the sequence was BLAST searched in GenBank.

7.2.2.6 Optimization of antimicrobial production

Optimal fermentation conditions are very important for maximal productivity of antimicrobials. Therefore, it was decided to study the effect of various factors (C and N sources, pH, temperature and time course) on antimicrobial production in basal F1 medium (minus fructose and soybean meal) by *Chaetomium globosum* (CMUZE0132). For the optimization for antimicrobial production, the tests were

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

conducted in triplicate and data generated from this experiment was analyzed using SPSS v.10 package for one-way analysis of variance (ANOVA).

Effects of C and N sources

The effects of different combinations of carbon (D-psicose, fructose, glucose, lactose, maltose, mannitol, pullulan, sucrose and xylitol) and nitrogen sources (malt extract, peptone, polypeptone, soybean meal and yeast extract) on antimicrobial production were studied. Each C and N sources was added, in all combinations, to 250 ml Erlenmeyer flasks containing 50 ml basal F1 medium. Three mycelial disks (5 mm diameter) of *C. globosum* (CMUZE0132) grown on PDA for 7 days were inoculated in all combinations of medium and incubated at room temperature (27–30 °C) for 7 days on a reciprocal shaker (130 rpm). The crude extracts of cultures were obtained (section 7.2.2.3) and the inhibitory effect on growth of *Penicillium avellaneum* was determined using the agar well diffusion assay method.

Effects of pH, temperature and time

The effect of pH on antimicrobial production was tested by inoculating three mycelial disks (5 mm diameter) of *C. globosum* (CMUZE0132) into 250 ml Erlenmeyer flasks containing 50 ml of basal F1 medium with optimal C and N sources (as determined above) and various pH (3.0, 4.0, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 9.0) adjusted by HCl and NaOH. Flasks were incubated at room temperature for 7 days on a reciprocal shaker (130 rpm). The optimum temperature for antimicrobial production was tested by fermenting *C. globosum* (CMUZE0132) at the optimum pH and

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

incubating at different temperatures (25, 30, 37, 40 and 45 °C). The agar well diffusion assay method was used to check the inhibitory effect on *P. avellaneum*.

The optimum time for antimicrobial production was determined by inoculating *C. globosum* (CMUZE0132) into triplicates of the basal F1 medium with optimal C and N sources, optimum pH, and incubating at optimum temperature. Some of the culture was removed daily for 14 days. The antimicrobial activity of the crude extracts of cultures was assayed against *P. avellaneum* using the agar well diffusion assay method.

7.2.2.7 Characterization of bioactive compounds from *Chaetomium globosum* (CMUZE0132)

Culture conditions

One liter of F1 medium (with glucose and soybean meal) was inoculated with 45 mycelial disks (5 mm diameter) from a 7 days old culture of *Chaetomium globosum* (CMUZE0132) and incubated under optimal conditions, with reciprocal shaking at 30 °C for 5 days. The culture was then filtered under suction. One hundred ml of the filtrate was stored at 4 °C for further use in purification of bioactive compounds. The remaining 900 ml of filtrate was extracted with EtOAc, and weight of the yellow extract residue was determined.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

The minimum inhibitory concentration

The minimum inhibitory concentration (MIC) for the extract of *Chaetomium globosum* (CMUZE0132) was determined by testing concentrations of 0, 1.25, 2.5, 5, 10, 15 and 20 mg/ml (dry extract/volume of DMSO). *Candida albicans* and

Penicillium avellaneum were cultured in appropriate media previously described. The agar well diffusion assay was used. Thirty five µl of each concentration of crude bioactive compound was loaded into each agar well, and the plates were incubated under appropriate conditions. The MIC was the lowest bioactive compound concentration to produce an inhibition zone against the test organism. Each inhibition experiment was performed three times.

Isolation and purification of bioactive compounds

A HP20 resin column (1 cm diameter, 30 cm high) was prepared by soaking and swelling 20 g adsorbent resins in acetone overnight to remove impurities. The solvent was then removed by washing with ddH_2O. One hundred ml of the filtered broth (previously stored at 4 °C) was passed through the column, which then was successively eluted with 40 ml ddH_2O, 40 ml of methanol (25%, 50% and 100% v/v with ddH_2O), and a final elution with 40 ml acetone methanol (3:7) mixture. Each 5 ml fraction was separately collected with the exception of passing the filtered broth and ddH_2O when each 10 ml fraction was separately collected. The yellow extract residue (420 mg) was fractionated on a silica gel column (2 cm diameter, 45 cm high, containing 60 g silica gel) eluted with 300 ml chloroform and 180 ml chloroform methanol (9:1) mixture. Each 5 ml of fraction eluted from the column by passing chloroform and one fraction eluted when passing by chloroform methanol (9:1) mixture, were collected separately and bioassayed by the paper disk diffusion assay method. The fractions were tested against *Candida albicans*, *Penicillium avellaneum*, and two common plant pathogens, *Pyricularia oryzae* (obtained from Faculty of

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

Agriculture, Hokkaido University) and *Phytophthora* sp. (obtained from Department of Plant Pathology, Faculty of Agriculture, Chiang Mai University).

The active fractions were further analyzed by thin layer chromatography (TLC). Silica gel 60 F_{254}, precoated commercial plates (20 × 20 cm, 0.2 mm thick, Merck) were divided into strips 1 cm wide. Ten µl of active fractions were then applied separately on to the plates, and developed in a closed glass chamber containing developing solvents (95:5, $CHCl_3$:MeOH). The solvent in the chamber was allowed to reach the lower edge of the adsorbent, though the spot points were not allowed to be immersed. The system was then maintained until the solvent ascended to a point 12 to 15 cm above the initial spots, when the plate was removed from the developing chamber and allowed to dry. The separated samples were visualized by UV light. Parallel samples (without visualization under UV) were assayed by bioautography. This was carried out to recheck activity of bioactive compounds. The TLC plates were placed in assay plates prepared by the same method outlined above, except that after 1.5 hours the TLC plates were taken out, and the assay plates were then incubated. The distances from origin of each inhibitory zone around the antimicrobial fraction was measured and yielded a R_f value, which was then compared with the band that appeared under UV light (Figure 7.1).

$$R_f = \frac{\text{distance from origin of active compounds}}{\text{distance from origin of solvent}}$$

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

Figure 7.1 Measuring of R_f values of bioactive compounds in bioautograph (left) and band (arrowed) appearing under UV light (right) on TLC plate. d. distance from origins of active compounds, D. distance of solvent.

Mass spectra and NMR

Selected fractions from the HP20 resin or silica gel columns were analysed by mass spectrophotometry [JMS-SX102A] and ^{1}HNMR spectrophotometry. The spectra were obtained through the Department of Chemistry, Faculty of Agriculture, Hokkaido University.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

7.3 Results

7.3.1 Screening of zingiberaceous fungi for enzyme production

Of 33 fungal isolates tested for cellulase and mannanase production, 31 and 30 isolates showed zones of clearing around agar wells containing carboxymethylcellulose and locust bean gum as substrates for cellulase and mannanase, respectively. *Fusarium* sp. (CMUZE0388) produced largest clearing zone in cellulase testing plate, while *Talaromyces flavus* (CMUZE0200) produced largest clearing zone in mannanase testing plate (Table 7.1). In testing for protease production, after inoculating 133 fungal isolates on to casein agar plates (skim milk agar) and incubating at room temperature for 4 days, 104 fungal isolates yielded zones of clearing with 47 (35%) isolates producing a zone in excess of 20 mm in diameter (Table 7.1). *Trichoderma* sp. (ZSCMU39-2) produced largest clearing zone in protease test plates with 62.3 mm diameter. Three new species, *Gaeumannomyces amomi*, *Leiosphaerella amomi* and *Pyricularia longispora* (described during the current studies), yielded zones of clearing on casein agar plates after incubation at room temperature for 4 days of 17.3, 29.0 and 18 mm diameter, respectively. These three species also showed zones of clearing larger than 10 mm diameter on agar wells containing carboxymethylcellulose and locust bean gum as substrates for cellulase and mannanase (Table 7.1).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

Table 7.1 Qualitative activities of cellulase, mannanase and protease produced by selected zingiberaceous fungi.

Isolate number (CMU)[a]	Taxa	Clear zone diameter (mm)			Colony diameter (mm)[b]
		Cellulase	Mannanase	Protease	SMA
ZS005	*Acremonium* sp. 1	n.t.	n.t.	12.3	16.7
ZS157	*Anthracophyllum nigritum*	n.t.	n.t.	21.7	25.7
ZS149	Basidiomycete 1	n.t.	n.t.	13.7	6.3
ZS153	Basidiomycete 2	18.0	0.0	0.0	44.7
ZS160	Basidiomycete 4	n.t.	n.t.	0.0	12.0
ZS118-2	*Cercospora amomi*	n.t.	n.t.	0.0	7.7
ZE0132	*Chaetomium globosum*	11.0	18.0	31.0	25.3
ZS129-1	*Chloridium* sp.	n.t.	n.t.	17.7	10.0
ZE0016	*Colletotrichum gloeosporioides*	n.t.	n.t.	32.3	36.3
ZE0028	*Colletotrichum gloeosporioides*	17.0	16.0	25.3	31.0
ZE0127	*Colletotrichum gloeosporioides*	n.t.	n.t.	26.0	33.3
ZE0551	*Colletotrichum gloeosporioides*	12.0	22.0	29.0	26.3
ZE0552	*Colletotrichum gloeosporioides*	20.0	23.0	27.7	32.0
ZP0012	*Colletotrichum gloeosporioides*	17.0	18.0	23.3	24.7
ZP0016	*Colletotrichum gloeosporioides*	19.0	20.0	31.7	31.7
ZP0018	*Colletotrichum gloeosporioides*	11.0	18.0	n.t.	n.t.
ZP0021	*Colletotrichum gloeosporioides*	12.0	21.0	31.3	30.7
ZS145	*Crepidotus* sp. 2	n.t.	n.t.	31.3	21.3
ZS148-1	*Cyathus* sp.	n.t.	n.t.	19.3	24.3
ZS148-2	*Cyathus* sp.	n.t.	n.t.	0.0	21.7
ZE0148	*Cylindrocarpon* sp.	n.t.	n.t.	19.3	11.3
ZE0149	*Cylindrocladium* sp.	n.t.	n.t.	17.0	21.0
ZE0150	*Cylindrocladium* sp.	n.t.	n.t.	25.3	23.0
ZS040	*Dactylaria* sp. 1	n.t.	n.t.	11.7	19.3
ZS147	*Discomycete*	n.t.	n.t.	14.0	9.3
ZE0151	*Eupenicillium crustaceum*	n.t.	n.t.	21.0	23.7
ZE0152	*Eupenicillium crustaceum*	n.t.	n.t.	17.0	15.3
ZE0155	*Eupenicillium crustaceum*	n.t.	n.t.	17.3	22.0
ZE0156	*Eupenicillium crustaceum*	n.t.	n.t.	19.7	14.3
ZE0217	*Eupenicillium crustaceum*	n.t.	n.t.	11.0	19.7
ZE0383	*Fusarium* sp.	7.5	13.0	22.0	24.7
ZE0384	*Fusarium* sp.	n.t.	n.t.	30.0	21.7
ZE0387	*Fusarium* sp.	n.t.	n.t.	21.7	24.3
ZE0388	*Fusarium* sp.	25.0	22.0	30.0	27.0
ZE0389	*Fusarium* sp.	n.t.	n.t.	28.7	34.3
ZE0393	*Fusarium* sp.	7.5	11.0	27.7	25.3
ZE0394	*Fusarium* sp.	n.t.	n.t.	27.3	32.7
ZE0396	*Fusarium* sp.	11.0	23.0	23.0	34.3
ZE0400	*Fusarium* sp.	n.t.	n.t.	14.7	11.3
ZE0401	*Fusarium* sp.	n.t.	n.t.	13.3	16.0
ZE0402	*Fusarium* sp.	n.t.	n.t.	0.0	5.0
ZE0403	*Fusarium* sp.	n.t.	n.t.	12.0	16.3
ZE0405	*Fusarium* sp.	n.t.	n.t.	11.3	13.0
ZE0407	*Fusarium* sp.	n.t.	n.t.	14.0	11.3
ZE0408	*Fusarium* sp.	n.t.	n.t.	14.0	11.3
ZE0117	*Fusicoccum* sp.	n.t.	n.t.	23.0	23.0
ZE0002	*Gaeumannomyces amomi*	14.0	16.0	17.3	26.3

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

Table 7.1 (Continued).

Isolate number (CMU)[a]	Taxa	Clear zone diameter (mm)			Colony diameter (mm)[b]
		Cellulase	Mannanase	Protease	SMA
ZE0101	*Gaeumannomyces amomi*	11.0	12.0	17.3	26.0
ZE0008	*Geniculosporium* sp.	n.t.	n.t.	40.7	26.0
ZE0009	*Geniculosporium* sp.	n.t.	n.t.	23.3	36.3
ZE0065	*Glomerella* sp.	n.t.	n.t.	21.3	25.7
ZE0066	*Glomerella* sp.	n.t.	n.t.	25.0	21.7
ZE0091	*Glomerella* sp.	10.0	17.0	26.0	21.3
ZE0550	*Glomerella* sp.	n.t.	n.t.	33.3	33.0
ZE0011	*Humicola fuscoatra*	n.t.	n.t.	17.7	13.3
ZE0228	*Humicola fuscoatra*	n.t.	n.t.	0.0	36.3
ZE0288	Hyphomycete 2	n.t.	n.t.	16.0	24.3
ZS126-1	Hyphomycete 5	n.t.	n.t.	18.3	10.7
ZS130-1	Hyphomycete 6	n.t.	n.t.	10.0	16.3
ZS130-2	Hyphomycete 6	n.t.	n.t.	11.3	19.0
ZS154-1	*Hypocrea* sp.	n.t.	n.t.	15.7	21.3
ZS154-2	*Hypocrea* sp.	n.t.	n.t.	14.0	18.7
ZE0102	*Leiosphaerella amomi*	18.0	20.0	29.0	25.7
ZS162	*Marasmius* sp. 4	n.t.	n.t.	0.0	19.0
ZS121-1	*Memnoniella* sp.	n.t.	n.t.	15.7	14.7
ZS133-1	*Memnoniella subsimplex*	n.t.	n.t.	35.3	31.3
ZS133-2	*Memnoniella subsimplex*	n.t.	n.t.	34.3	28.7
ZS172	*Memnoniella subsimplex*	n.t.	n.t.	18.0	9.3
ZS048	*Myrothecium cinctum*	7.5	0.0	27.0	15.0
ZE0116	*Papulaspora* sp.	n.t.	n.t.	28.0	31.3
ZE0118	*Papulaspora* sp.	n.t.	n.t.	24.7	13.0
ZE0162	*Penicillium* sp.	n.t.	n.t.	25.3	17.0
ZE0238	*Penicillium* sp.	n.t.	n.t.	21.7	31.3
ZS169	*Pestalotiopsis* sp.	n.t.	n.t.	26.3	25.7
ZE0133	*Phoma* sp.	n.t.	n.t.	16.0	13.0
ZE0134	*Phoma* sp.	n.t.	n.t.	24.3	34.0
ZE0071	*Phomopsis* sp.	11.0	15.0	32.3	18.7
ZE0137	*Phomopsis* sp.	14.0	19.0	18.3	36.7
ZE0141	*Phomopsis* sp.	11.0	11.0	24.7	31.7
ZE0232	*Phomopsis* sp.	12.0	21.0	25.7	23.0
ZP0009	*Phomopsis* sp.	12.0	13.0	11.3	16.3
ZP0011	*Phomopsis* sp.	12.0	13.0	18.7	24.7
ZP0017	*Phomopsis* sp.	n.t.	n.t.	29.7	30.0
ZP0029	*Phomopsis* sp.	12.0	12.0	18.3	22.3
ZP0035	*Phomopsis* sp.	n.t.	n.t.	26.7	21.3
ZE0445	*Phyllosticta capitalensis*	0.0	19.0	19.3	17.0
ZP0028	*Phyllosticta capitalensis*	0.0	19.0	0.0	18.0
ZP0037	*Phyllosticta capitalensis*	n.t.	n.t.	20.7	16.0
ZP0003	*Pyricularia costina*	21.0	0.0	11.0	25.0
ZP0004	*Pyricularia costina*	n.t.	n.t.	12.7	25.0
ZS050-1	*Pyricularia longispora*	14.0	23.0	17.3	21.0
ZS117-1	*Pyricularia longispora*	14.0	24.0	17.0	15.3
ZS117-2	*Pyricularia longispora*	13.0	24.0	18.3	18.7
ZE0012	Sterile mycelium	n.t.	n.t.	0.0	35.0
ZE0013	Sterile mycelium	n.t.	n.t.	24.3	38.0

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

Table 7.1 (Continued).

Isolate number (CMU)[a]	Taxa	Clear zone diameter (mm)			Colony diameter (mm)[b]
		Cellulase	Mannanase	Protease	SMA
ZE0120	Sterile mycelium	n.t.	n.t.	17.3	12.7
ZE0123	Sterile mycelium	n.t.	n.t.	17.7	27.3
ZE0124	Sterile mycelium	n.t.	n.t.	18.7	11.3
ZE0126	Sterile mycelium	n.t.	n.t.	0.0	27.0
ZE0131	Sterile mycelium	n.t.	n.t.	15.0	17.7
ZE0145	Sterile mycelium	n.t.	n.t.	17.0	27.7
ZE0146	Sterile mycelium	n.t.	n.t.	0.0	20.0
ZE0252	Sterile mycelium	n.t.	n.t.	18.0	12.0
ZE0179	*Talaromyces flavus*	n.t.	n.t.	0.0	19.0
ZE0195	*Talaromyces flavus*	n.t.	n.t.	0.0	18.3
ZE0200	*Talaromyces flavus*	14.0	26.0	0.0	13.0
ZE0201	*Talaromyces flavus*	n.t.	n.t.	19.7	11.7
ZE0207	*Talaromyces flavus*	n.t.	n.t.	11.0	14.7
ZS003	*Torula* sp. 1	n.t.c	n.t.	9.7	17.7
ZS004-1	*Torula* sp. 2	n.t.	n.t.	9.0	17.7
ZS039-2	*Trichoderma* sp.	n.t.	n.t.	62.3	80.7
ZS023	*Verticillium* sp. 1	n.t.	n.t.	12.3	17.0
ZS023-3	*Verticillium* sp. 1	n.t.	n.t.	13.3	18.0
ZE0416	*Xylaria* sp.	n.t.	n.t.	21.0	13.3
ZE0418	*Xylaria* sp.	22.0	14.0	19.0	14.0

[a]ZE = endophytes, ZP = pathogens, ZS = saprobes
[b]Colony diameter on skimmed milk agar (SMA) refers only to the protease test
[c]n.t. = not tested

7.3.2. Screening for antimicrobial production

All fungal isolates cultured in either F1 or F2 showed inhibition against at least one of tested organisms. F1 medium was better than F2 medium in promoting antimicrobial production, and yielded a higher number of isolates of zingiberaceous fungi that strongly inhibited growth of the test organisms (inhibition zone diameter > 10 mm, Table 7.2). Growth of *Penicillium avellaneum* was most strongly inhibited by *Chaetomium globosum* (CMUZE0132), while growth of the yeast, *Candida albicans*, was most strongly inhibited by *Papulaspora* sp. (CMUZE0118). The four bacteria, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, were most inhibited by *Talaromyces flavus* (CMUZE0200), unidentified

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

fungus (CMUZE0126), *Colletotrichum gloeosporioides* (CMUZE0552) and *Phyllosticta capitalensis* (CMUZE0172), respectively (Table 7.3). *Chaetomium globosum* strain CMUZE0132, which showed greatest inhibition of *P. avellaneum* (34.3 mm diameter) was selected for further experimentation.

7.3.3 Taxonomy of *Chaetomium globosum* (CMUZE0132)

The average colony diameter of strain *Chaetomium globosum* (CMUZE0132) on PDA was 5 cm after incubation at room temperature (27–30 °C) for 7 days. The colony was initially white, later turning yellowish white, with dense or sparse aerial hyphae (Figure 7.2a). Dark brown ascomata were observed on the medium after 3 weeks. Ascomata 250–310 μm high, 220–260 μm diameter, dark brown, superficial, elongated or subglobose (Figure 7.2b). Lateral hairs about 3.7 μm thick, numerous, brown to dark brown, septate, roughened, slender, straight or only slightly flexed, unbranched. Terminal hairs about 3.5 μm thick, extremely numerous, and intricately interwoven forming a neat, rather compact head, brown to dark brown, non-septate, roughened, slender, wavy or spiral, rarely branched at tip (Figure 7.2c). Asci 62–65 × 12–13 μm, hyaline, 8-spored, club-shaped. Ascospores 11–12.5 × 8.5–10 μm, hyaline, become olive-brown or brown when mature, subglobose to lemon-shaped, with ends apiculate or rounded (Figure 7.2d). The morphological characteristics of densely hairy ascomata, dark and lemon-shaped ascospores are those of *Chaetomium*, as described by Chivers (1915). The nucleotide sequence of ITS rDNA of *Chaetomium globosum* (CMUZE0132) amplified by ITS5 primer (Appendix D) was BLAST searched and deposited in the GenBank database with accession number DQ003217. Results for the

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

Table 7.2 Qualitative antimicrobial activity of zingiberaceous fungi cultured in fermentation media F1 and F2.

Potential antimicrobial activity [a]	Number of isolates (and percentage) of zingiberaceous fungi inhibiting growth of test organisms											
	F1						F2					
	B. sub	***E. col***	***S. aur***	***P. aer***	***C. alb***	***P. ave***	***B. sub***	***E. col***	***S. aur***	***P. aer***	***C. alb***	***P. ave***
+++	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (0.8%)	1 (0.8%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
++	3 (2.3%)	2 (1.5%)	1 (0.8%)	1 (0.8%)	6 (4.6%)	10 (7.7%)	3 (2.3%)	0 (0%)	0 (0%)	0 (0%)	5 (3.8%)	2 (1.5%)
+	9 (7.0%)	7 (5.4%)	2 (1.5%)	32 (24.6%)	8 (6.1%)	16 (12.3%)	15 (11.5%)	4 (3.1%)	1 (0.8%)	10 (7.7%)	13 (10%)	9 (7%)
±	28 (21.5%)	114 (87.7%)	24 (18.5%)	2 (1.5%)	1 (0.8%)	13 (10%)	23 (17.7%)	102 (78.4%)	2 (1.5%)	5 (3.8%)	0 (0%)	2 (1.5%)
-	90 (69.2%)	7 (5.4%)	103 (70.2%)	95 (73.1%)	114 (87.7%)	90 (69.2%)	89 (68.5%)	24 (18.5%)	127 (97.7%)	115 (88.5%)	112 (86.2%)	117 (90%)

[a]+++: inhibition zone diameter (X) > 20 mm
++: 20 mm ≥ X > 10 mm
+: 10 mm ≥ X > 7 mm
±: 7 mm ≥ X > 5 mm
-: X = 5 mm

B. sub = *Bacillus subtilis*, *E. col* = *Escherichia coli*, *S. aur* = *Staphylococcus aureus*, *P. aer* = *Pseudomonas aeruginosa*, *C. alb* = *Candida albicans*, *P. ave* = *Penicillium avellaneum*

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

Table 7.3 The inhibition zone (> 7 mm diameter) against test organisms of zingiberaceous fungi cultured in fermentation media F1 and F2.

Isolate number (CMU)[a]	Taxa	Inhibition zone (mm)[b]											
		F1						F2					
		B. sub	*E. col*	*S. aur*	*P. aer*	*C. alb*	*P. ave*	*B. sub*	*E. col*	*S. aur*	*P. aer*	*C. alb*	*P. ave*
ZE0114	*Alternaria alternata*				8.0		14.7				8.0		
ZE0180	*Aspergillus* sp.												8.0
ZS149	Basidiomycete 1		9.3										
ZS153	Basidiomycete 2	12.3	8.0	7.3		12.3	11.5						
ZS160	Basidiomycete 4	7.3							8.0				
ZS118-1	*Cercospora amomi*				7.3								
ZE0132	*Chaetomium globosum*					9.0	34.3						
ZE0028	*Colletotrichum gloeosporioides*				8.0		19.7				8.0		10.0
ZE0127	*Colletotrichum gloeosporioides*		16.0					9.0					
ZE0551	*Colletotrichum gloeosporioides*				8.0	15.7	17.7		7.2		9.0	9.0	8.0
ZE0552	*Colletotrichum gloeosporioides*				11.0		19.7				9.0	9.0	
ZS145	*Crepidotus* sp. 2	9.0			8.0	9.0	9.0	13.0				13.7	
ZS148-1	*Cyathus* sp.												9.0
ZS148-2	*Cyathus* sp.	11.0	9.0		8.0		8.0	8.0					
ZE0148	*Cylindrocarpon* sp.							7.7					
ZE0149	*Cylindrocladium* sp.						17.7						
ZS170	*Cylindrocladium* sp.				8.0		8.0						
ZS040	*Dactylaria* sp. 1				8.0								
ZS147	*Discomycete*												8.0
ZE0151	*Eupenicillium crustaceum*				8.0		8.5						
ZE0152	*Eupenicillium crustaceum*	7.7				15.7	7.7						
ZE0155	*Eupenicillium crustaceum*						8.5						
ZE0156	*Eupenicillium crustaceum*							8.3					
ZE0161	*Eupenicillium crustaceum*						9.0						
ZE0384	*Fusarium* sp.				10.0		8.0				8.0	7.5	
ZE0393	*Fusarium* sp.					9.0	8.0					10.7	9.0
ZE0396	*Fusarium* sp.					9.0							
ZE0401	*Fusarium* sp.				10.0							7.2	

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

Table 7.3 (Continued).

Isolate number (CMU)[a]	Taxa	Inhibition zone (mm)[b]											
		F1						F2					
		B. sub	***E. col***	***S. aur***	***P. aer***	***C. alb***	***P. ave***	***B. sub***	***E. col***	***S. aur***	***P. aer***	***C. alb***	***P. ave***
ZE0403	*Fusarium* sp.				9.0							8.0	
ZE0405	*Fusarium* sp.				9.0							8.0	
ZE0117	*Fusicoccum* sp.				8.3		8.0				8.0		
ZE0002	*Gaeumannomyces amomi*				7.7								
ZE0101	*Gaeumannomyces amomi*	7.2											
ZE0091	*Glomerella* sp.				8.0		8.0				9.0		
ZE0550	*Glomerella* sp.				9.0		15.0		7.2			8.0	
ZS106-2	*Helicosporium* sp.		7.2										
ZE0228	*Humicola fuscoatra*	8.2											
ZS130-1	Hyphomycete 6				9.0						9.0	7.5	8.0
ZS130-2	Hyphomycete 6				9.0						8.0		
ZE0102	*Leiosphaerella amomi*				8.0		8.3						
ZS121-2	*Memnoniella* sp.				8.0								
ZS172	*Memnoniella subsimplex*												7.3H
ZS048	*Myrothecium cinctum*							9.0					
ZE0116	*Papulaspora* sp.						13.7H					8.7	
ZE0118	*Papulaspora* sp.	7.5	7.2		8.2	28.0	14.3					20.0	
ZE0238	*Penicillium* sp.							8.5					
ZS169	*Pestalotiopsis* sp.				9.0								
ZE0133	*Phoma* sp.							8.3					
ZE0134	*Phoma* sp.					8.7		10.0					
ZE0071	*Phomopsis* sp.						8.0						
ZE0137	*Phomopsis* sp.		8.3		8.0			10.7					
ZE0141	*Phomopsis* sp.				8.0								
ZE0172	*Phyllosticta capitalensis*			14.7		12.7	12.7					10.0	12.7
ZS050-1	*Pyricularia longispora*		7.7		8.0								
ZE0012	Sterile mycelium	9.3				7.7						8.2	
ZE0120	Sterile mycelium							7.7				19.3	
ZE0122	Sterile mycelium										10.0		

Copyright © by Chiang Mai University
All rights reserved

Table 7.3 (Continued).

Isolate number (CMU)[a]	**Taxa**	**Inhibition zone (mm)**[b]											
		F1						**F2**					
		B. sub	***E. col***	***S. aur***	***P. aer***	***C. alb***	***P. ave***	***B. sub***	***E. col***	***S. aur***	***P. aer***	***C. alb***	***P. ave***
ZE0123	Sterile mycelium				8.0	15.0H	9.0	9.3		8.0		16.0H	11.0
ZE0124	Sterile mycelium							7.7					
ZE0126	Sterile mycelium		17.0					8.0					
ZE0131	Sterile mycelium				9.0								
ZE0145	Sterile mycelium	8.7			8.7	7.8H		8.7	7.3				
ZE0192	*Talaromyces flavus*				8.0		8.0						9.0
ZE0199	*Talaromyces flavus*											9.0	
ZE0200	*Talaromyces flavus*	13.0		8.0		7.7H		7.7					
ZE0201	*Talaromyces flavus*	8.8						8.3					
ZE0207	*Talaromyces flavus*				8.0								
ZS039-2	*Trichoderma* sp.					19.7							
ZS074	*Xenosporium intermedium*				9.7								
ZE0416	*Xylaria* sp.						7.2	7.2					
ZE0418	*Xylaria* sp.											8.0	

[a]ZE = endophytes, ZS = saprobes

[b]Means of inhibition clear zones surrounding each application point, three replicates, H=inhibition zone hazy

B. sub = *Bacillus subtilis*, *E. col* = *Escherichia coli*, *S. aur* = *Staphylococcus aureus*, *P. aer* = *Pseudomonas aeruginosa*, *C. alb* = *Candida albicans*, *P. ave* = *Penicillium avellaneum*

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

Chaetomium globosum (CMUZE0132) came from GenBank+EMBL+DDBJ; unidentified and unpublished sequences were excluded. The BLAST search results and the phylogenetic tree (Figure 7.3) generated from representative strains of related fungi showed that strain *Chaetomium globosum* (CMUZE0132) had high levels of sequence similarity to *Chaetomium globosum* (accession number AY625061). ITS rDNA analysis revealed a sequence similarity level of 94%.

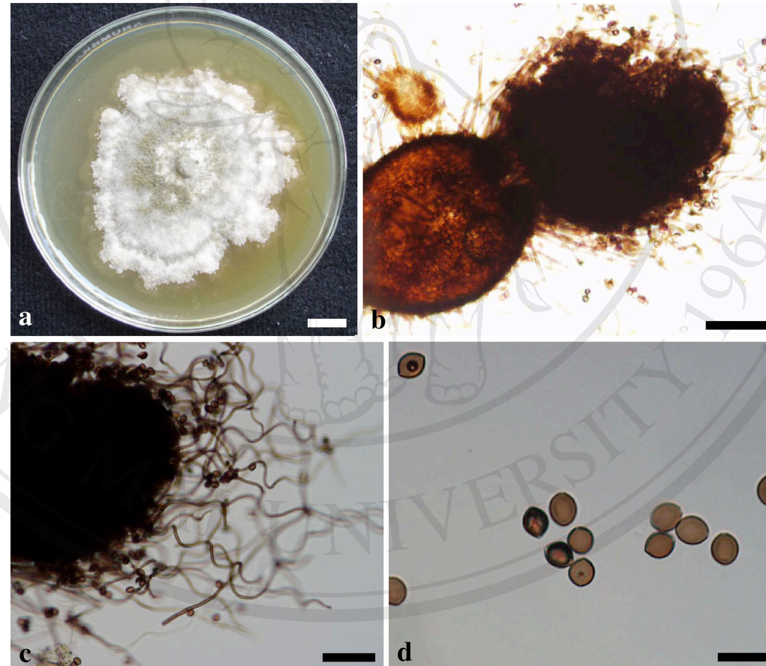

Figure 7.2 *Chaetomium globosum* strain CMUZE0132: a, colony on PDA, b. ascomata with dense lateral and terminal hairs, c. spiral terminal hairs, d. lemon-shaped ascospores. Bars: a. = 1 cm, b. = 100 µm, c. = 100 µm, d. = 20 µm.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

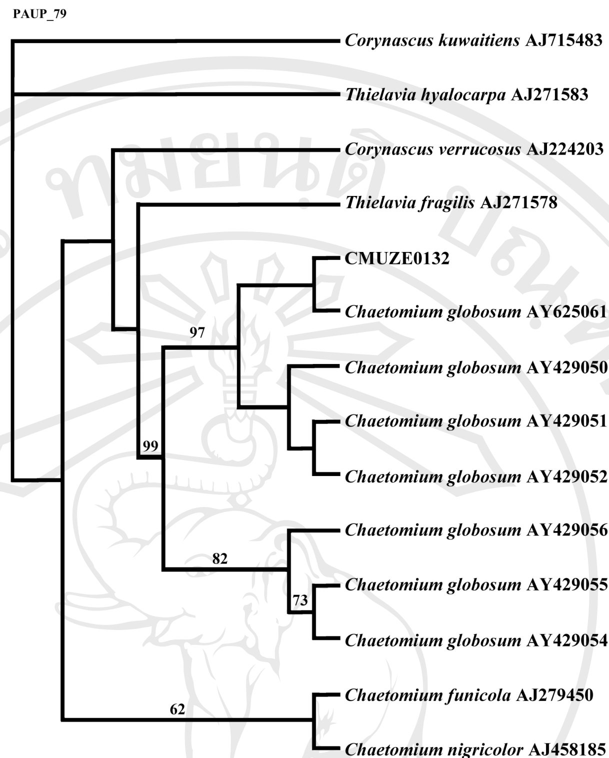

Figure 7.3 Phylogenetic tree (from branch and bound search) showing the relationships of endophytic *Chaetomium globosum* (CMUZE0132), related species of the same genus and other taxa based on ITS rDNA sequences. The bootstrap values representing 1000 bootstrap replications are given (when more than 50%) above the branches.

7.3.4 Optimization of antimicrobial production

Effects of C and N sources

The extract of fermentation of *Chaetomium globosum* (CMUZE0132) in F1 medium containing a combination of various carbons and soybean meal (as N-source) produced the largest inhibition zones against *Penicillium avellaneum* (Figure 7.4). Thus, soybean meal was the best N-source. When pullulan was substituted as C-source in F1 (soybean meal as N-source), the extract from this strain showed the largest inhibition

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

zone against *P. avellaneum* (Figure 7.5). However, there was no significant difference of inhibition against the test organism with substitution of C-source by glucose, lactose, mannitol, xylitol or pullulan in F1 (soybean meal as N-source) (Figure 7.4). Glucose and soybean meal are unlimited, cheap and readily available nutrients and so would be suitable for larger scale production. Thus, the combination of glucose and soybean meal was selected as C/N-sources for further experimentation.

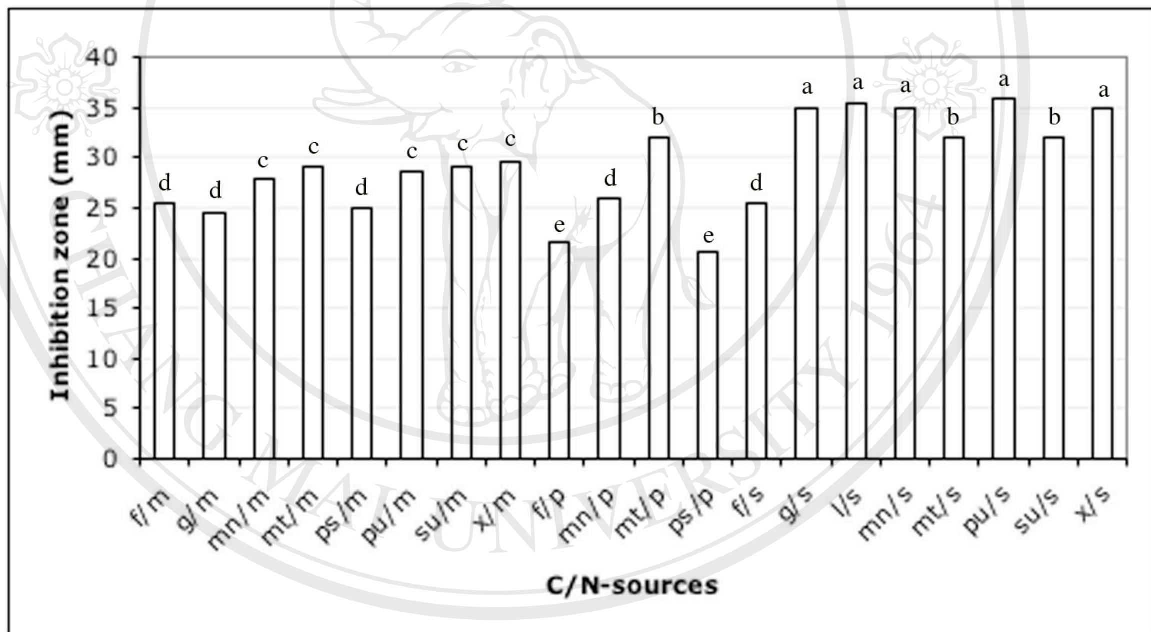

Figure 7.4 Effect of C/N-sources on antimicrobial production by *Chaetomium globosum* (CMUZE0132) against *Penicillium avellaneum*. C-sources: f. fructose, g. glucose, l. lactose, mn. mannitol, mt. maltose, ps. D-psicose, pu. pullulan, su. sucrose, x. xylitol. N-sources: me. Malt extract, p. peptone, s. soybean meal. $^{a–d}$ values above the bars with no common superscripts are significantly different ($P < 0.05$).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

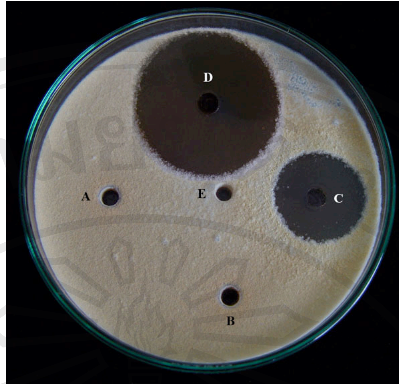

Figure 7.5 The inhibition zones of antimicrobial agent produced by *Chaetomium globosum* (CMUZE0132) cultured in F1 medium containing different C/N-sources (A. pullulan/polypeptone, B. pullulan/yeast extract, C. pullulan/malt extract, D. pullulan/soybean meal, E. control) against *Penicillium avellaneum*.

The effects of pH, temperature and time

The optimization of these fermentation conditions for antimicrobial production by *Chaetomium globosum* (CMUZE0132) was carried out in shaking flask cultures of F1 medium containing glucose and soybean meal. The optimal pH for antimicrobial production was pH 6.0 (Figure 7.6). No inhibition occurred at pH 3.0. The largest inhibition zone occurred at 30 °C (pH 6.0), although there was no significant difference between 25 and 30 °C (Figure 7.6). No inhibition occurred at higher temperatures. The largest zone of inhibition occurred after 5 days of incubation and then slowly decreased (Figure 7.7).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

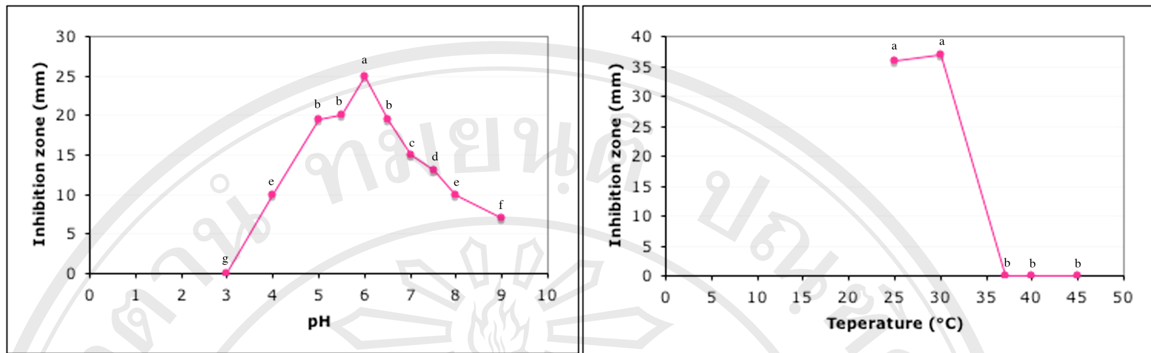

Figure 7.6 Antimicrobial production by *Chaetomium globosum* (CMUZE0132) at different pH levels (left) and temperatures (right), against *Penicillium avellaneum*. [a–g] values above the plots with no common superscripts are significantly different ($P < 0.05$).

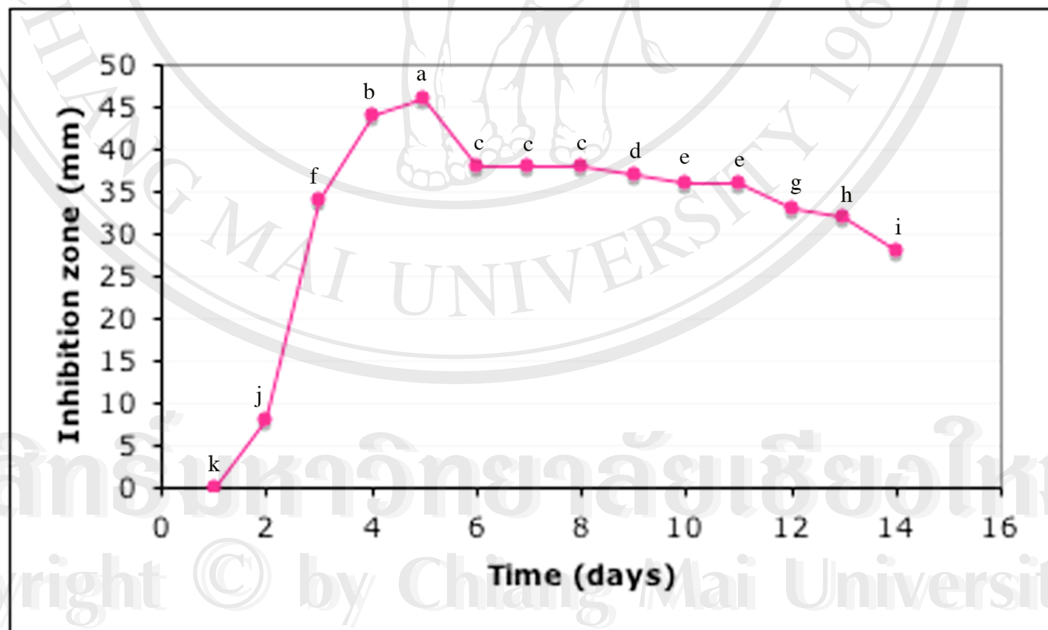

Figure 7.7 Antimicrobial production by *Chaetomium globosum* (CMUZE0132) over time, against *Penicillium avellaneum*. [a–k] values above the plots with no common superscripts are significantly different ($P < 0.05$).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

7.3.5 Characterization of bioactive compound from *Chaetomium globosum* (CMUZE0132)

The minimum inhibitory concentration

Chaetomium globosum (CMUZE0132) showed the best activity against *Penicillium avellaneum* (Table 7.3). Ethyl acetate extract of fermentation broth recovered from cultures of *Chaetomium globosum* (CMUZE0132) exhibited a broad antimicrobial spectrum. The minimum concentration of extract that prevented growth of *Candida albicans* and *P. avellaneum* was 10 and 5 mg/ml (dried extract/volume), respectively (Figure 7.8).

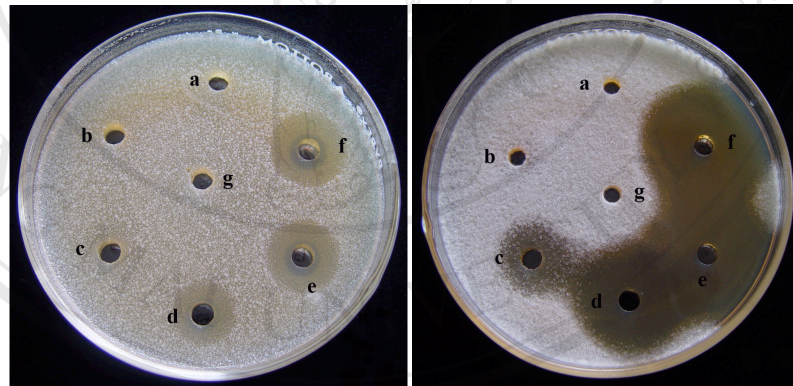

Figure 7.8 Zone of growth inhibition of *Candida albicans* (left) and *Penicillium avellaneum* (right) by ethyl acetate extract of *Chaetomium globosum* (CMUZE0132) in DMSO at different concentrations (a. 1.25, b. 2.5, c. 5, d. 10, e. 15, f. 20 and g. 0 mg/ml).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

Isolation and purification of bioactive compounds

Forty six fractions eluted from the HP20 resin column and 61 fractions eluted from silica gel column were bioassayed. Fractions 22–25 eluted with 50% methanol and fractions 30–36 eluted with 100% methanol from the resin column showed activity, as did fractions 15–18 eluted and 61 from the silica gel column (Table 7.4). Fractions 22–25 (HP20 resin) and fraction 61 (silica gel) were the only to show activity against *Candida albicans*. Fraction 32 from the HP20 resin was most active and produced the largest inhibition zone against *Penicillium avellaneum*, *Pyricularia oryzae* and *Phytophthora* sp. Only fraction 61 (silica gel) showed activity against all test fungi. The R_f values of the active compounds eluted from the HP20 resin and silica gel columns on TLC chromatograms are listed (Table 7.5). These R_f values were the same as those for the band which appeared under UV light. Fractions 23 and 32 from the HP20 resin column and fractions 17 and 61 from the silica gel column were further analysed by mass spectrophotometry.

Mass spectra and NMR

Fraction 32 from the HP20 resin column and fraction 17 from the silica gel column were analysed by mass spectrophotometry [JMS-SX102A]. The chemical composition of these compounds are $C_{20}H_{29}O_6$ and $C_{25}H_{44}O_{15}N_{17}$, respectively, and the structure is now under study by NMR.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

Table 7.4 Inhibition zones of active fractions eluted from HP20 resin and silica gel columns.

Active fraction number and eluent	Inhibition zone diameter (mm)			
	Candida albicans	***Penicillium avellaneum***	***Pyricularia oryzae***	***Phytophthora* sp.**
HP20 resin column (eluent)				
22 (50% MeOH)	13			
23 (50% MeOH)	21			20
24 (50% MeOH)	19			15
25 (50% MeOH)	13			
30 (MeOH)		25	18	
31 (MeOH)		53	38	30
32 (MeOH)		54	42	30
33 (MeOH)		53	42	26
34 (MeOH)		40	32	24
35 (MeOH)		28	18	16
36 (MeOH)		20	15	
Silica gel column (eluent)				
15 ($CHCl_3$)		13	9	
16 ($CHCl_3$)		26	18	
17 ($CHCl_3$)		27	11	
18 ($CHCl_3$)		13		
61 (9:1 $CHCl_3$:MeOH)	11	8	20	13

Table 7.5 R_f values on TLC chromatograms of the active compounds eluted from HP20 resin and silica gel columns.

Active fractions	Solvent in TLC analysis	Test organisms	R_f
HP20 resin column			
23	95:5 $CHCl_3$:MeOH	*Candida albicans*	0.22
32	95:5 $CHCl_3$:MeOH	*Penicillium avellaneum*	0.38
Silica gel column			
17	95:5 $CHCl_3$:MeOH	*Penicillium avellaneum*	0.43
61	95:5 $CHCl_3$:MeOH	*Penicillium avellaneum*	0.65

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

7.4 Discussion

7.4.1 Secondary metabolites from fungi

Fungi have an excellent record in producing novel compounds and many of those compounds have important medicinal and industrial uses (Stierle *et al*., 1993; Wildman, 1997; Dreyfuss and Chapela, 1999; Concepcion *et al*., 2001; Kirk *et al*., 2002; Strobel *et al*., 2004). According to Dreyfuss and Chapela (1999), approximately 4,000 secondary metabolites have been described that possess biological activity. Most screening programs have concentrated on fungi isolated from soil (e.g., *Aspergillus*, *Penicillium* and *Trichoderma*) and it is only recently that mycologist and pharmaceutical industries have begun to consider the vast array of fungi from other habitats (Stierle *et al*., 1993; Strobel *et al*. 1996a, b, 2004; Wildman, 1997). Bills and Polishook (1992) stated that as endophytes colonise a specialised habitat, they are increasingly being identified as a group of organisms capable of providing a source of secondary metabolites for use in biotechnology and agriculture.

The results of the present study demonstrate the potential value of investigating secondary metabolites (antimicrobial agents and enzymes) produced by fungi isolated from Zingiberaceae (medicinal plants) in a tropical region. Bills *et al*. (2002) found that tropic endophytes provide high metabolite creativity. Many studies that involved the investigation of microbes associated with medicinal plants have yielded biologically active compounds. For example, the antitumor drug taxol is produced by an endophytic fungus, *Taxomyces andreana* isolated from Pacific yew, *Taxus brevifolia* (Stierle *et al*., 1993), while cryptocandin, which has antifungal activity against some important human

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

fungal pathogens (*Candida albicans* and *Trichophyton* spp.) and a number of plant pathogenic fungi (*Sclerotinia sclerotiorum* and *Botrytis cinerea*), is produced by a fungal endophyte, *Cryptosporiopsis quercina* isolated from a native medicinal plant of Eurasia, *Tripterigenum wilfordii* (Strobel *et al*., 1999).

7.4.2 Potential of zingiberaceous fungi for enzyme production

In the present study, zingiberaceous fungi showed evidence of enzyme (cellulase, mannanase and protease) production. Casein agar (skim milk agar) was used to test for protease production, and 47 (of 104) fungal isolates yielded zones of clearing larger than 20 mm in diameter. Of 33 fungal isolates, 31 and 30 fungal isolates showed zones of clearing around agar wells containing carboxymethylcellulose and locust bean gum, respectively. Many cellulases and mannanases have been reported from fungi. Araujo and Ward (1990) showed that *Talaromyces byssochlamydoides* produced a high mannanase activity. *Trichoderma reesei* also produced β-mannanase and cellulases, and these have been shown to facilitate bleaching (Buchert *et al*., 1993; Viikari *et al*., 1993; Sabini *et al*., 2000). Solomon *et al*. (1999) produced cellulase from the growth of *Aspergillus flavus* on bagasse pretreated using ball milling and caustic soda. Cellulase production by *A. flavus* fermented in sawdust gave an enzyme activity value of 0.0743IU/ml while bagasse and corncob gave 0.0573IU/ml and 0.0502IU/ml, respectively (Ojumu *et al*., 2003). In those experiment, the cellulase or mannanase activity also increased when using an appropriate cultivation medium and optimal condition for the enzymes.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

The present study is only a primary screening for those enzymes produced. The positive isolates, which gave promising qualitatively activities, should be selected for quantitative analyses. Optimization (influence of carbon and nitrogen sources, pH and temperature), and kinetics of production of these enzymes needs further investigation. Furthermore, enzyme activity may be improved by purification and fungal strain improvement (Chand *et al*., 2005).

7.4.3 Potential of zingiberaceous fungi for antimicrobial production

The present study has shown that all of fungal isolates tested capably produce antimicrobial agents and some endophytic fungi from Zingiberaceae have a promising prospect for production of bioactive compounds. Based on morphological observation and BLAST search results the endophytic fungus strain CMUZE0132 was identified as belonging to the genus *Chaetomium*, with a high level of sequence similarity (94%) to *Chaetomium globosum* (accession number AY625061). The nucleotide sequence data reported in this thesis appeared in the GenBank database with the accession number DQ003217. This isolate produced an active substance as ethyl acetate extracts of the fermentation broth exhibit a broad antifungal spectrum and minimum inhibitory concentration of 5 and 10 mg/ml (dried extract/volume) against *Penicillium avellaneum* and *Candida albicans*, respectively. The compounds investigated in this study have a broad spectrum of activity against several fungi including *C. albicans*, *P. avellaneum*, *Phytophthora* sp. and *Pyricularia oryzae*. Strobel *et al*. (1999) found that cryptocandin produced by *Cryptosporiopsis quercina*, a fungal endophyte from *Tripterigenum*

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

wilfordii, has antifungal activity against the human pathogens *C. albicans* and *Trichophyton* spp. and the plant pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea*. *Cryptosporiopsis quercina* also produces cryptocin, a tetramic acid with potent activity against *Pyricularia oryzae* and a number of other plant pathogenic fungi (Li *et al*., 2000). Cryptocin was generally ineffective against an array of human pathogenic fungi. Nevertheless, with a minimum inhibitory concentration against *P. oryzae* of 0.39 µg/ml, this compound is being examined as a natural chemical control agent for rice blast and is being used as a model to synthesize other antifungal compounds (Strobel *et al*., 2004).

Species of *Chaetomium* are well known to produce metabolites (Guarro *et al*., 1995; Pujol *et al*., 1996; Soytong *et al*., 2001; Reissinger *et al*., 2003; Aggarwal *et al*., 2004; Kobayashi *et al*., 2005). Most species are strong decomposers of cellulose and are found in soil, air, and plant debris. As well as being a contaminant, *Chaetomium* spp. are also encountered as causative agents of infections in humans (Larone, 1995; Sutton *et al*., 1998). *Chaetomium globosum* is known as a potential antagonist of several soil and seed borne plant pathogens (Vannacci and Harman, 1987; Walther and Gindrat, 1988; Soytong *et al*., 2001). Aggarwal *et al*. (2004) studied *Chaetomium globosum* strains obtained from various sources (coprophilious, dolichos seed and wheat leaves) and found that they produced antifungal compounds that suppressed spot blotch of wheat caused by *Cochliobolus sativus* both *in vitro* and *in vivo*. *Chaetomium globosum* (CMUZE0132) obtained from healthy pseudostem of *Alpinia malaccensis* in current studies also inhibited zingiberaceous pathogen *P. costina in vitro* with high antagonistic activity (Chapter 5).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

The chemical compositions of the active compounds produced by *Chaetomium globosum* (CMUZE0132) and analysed by mass spectrophotometry is $C_{20}H_{29}O_6$ and $C_{25}H_{44}O_{15}N_{17}$. Soytong *et al.* (2001) demonstrated a broad spectrum biological fungicides from *Chaetomium* (Ketomium®) in diseas control of various crops, e.g., basal rot of maiz caused by *Sclerotium rolfsii*, root and stem rots olf black pepper caused by *Phytophthora palmivora*. The mechanism of plant disease control involving specific strains of *C. globosum* and *C. cupreum* involves the production of antibiotics, e.g., *C. globosum* produces Chaetoglobosin C which suppresses the growth of plant pathogen such as *Collitotrichum gloeosporioides*, *C. dematium*, *Fusarium Oxysporum*, *Phytophthora palmivora*, *Pyricularia oryzae* and *Sclerotium rolfsii* (Soytong and Quimio, 1989; Soytong, 1992; Soytong *et al*., 2001). Kobayashi *et al*. (2005) isolated *Chaetomium* sp. from soil and found that it produced triterpene glucoside (FR207944) with antifungal activity against *Aspergillus fumigatus* and *C. albicans* with MECs of 0.039 and 1.6 µg/ml, respectively. The compound exhibited *in vitro* and *in vivo* antifungal activity against *A. fumigatus* and showed good potency by subcutaneous injection and oral administration against *A. fumigatus* in a murine systemic infection model.

The maytansinoids are extraordinarily potent antitumor agents that were originally isolated from members of the higher plant families *Celastraceae*, *Rhamnaceae*, and *Euphorbiaceae*, as well as some mosses (Kupchan *et al*., 1972; Powell *et al*., 1983; Sakai *et al*., 1988; Suwanborirux *et al*., 1990). They are 19-membered macrocyclic lactams related to ansamycin antibiotics of microbial origin, such as rifamycin B and geldanamycin (Rinehart and Shield, 1976). The similarity stimulated a search for

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

maytansinoid-producing microorganisms, leading to the isolation of the ansamitocins from the actinomycete *Actinosynnema pretiosum* subsp. *pretiosum* and a mutant strain *Actinosynnema pretiosum* subsp. *auranticum* (Higashide *et al*., 1977). Inhibitory activity of ansamytocin was shown against *Penicillium avellaneum* (Yu *et al*., 2002). The present study also provides evidence for strong inhibitory activity against the growth of *P. avellaneum* by active compounds from the endophytic *Chaetomium globosum* (CMUZE0132). This strain produced several active compounds, as they have different R_f values of the active compounds eluted from the HP20 resin and silica gel columns on TLC chromatograms. The compounds also inhibited the growth of other fungi (*C. albicans*, *Phytophthora* sp. and *Pyricularia oryzae*). To determine if the compounds produced by *Chaetomium globosum* (CMUZE0132) and active against *P. avellaneum* is antitumor, needs more bioassays, e.g., *in vitro* or *in vivo* toxicity on tumor cells (Fridborg *et al*., 1999; Yu *et al*., 2002).

In the present study all zingiberaceous fungi screened for their antimicrobial activity showed some inhibition against at least one of the tested organisms. A high number of isolates of zingiberaceous fungi especially endophytes strongly inhibited growth of the test organisms. This showed a high potential of endophytic fungi for antimicrobial production. It is necessary to take into account the diversity of endophytic fungi and their cytotoxicity for screening for new antimicrobial active agents, because problems of antibiotic resistance is becoming increasingly apparent as more and more strains of pathogenic microorganisms become untreatable with commonly used antimicrobials. It is necessary to find new bioactive compounds as alternatives.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

Several factors affect production of bioactive compounds by fungi, such as media for fungal fermentation, pH, temperature, phases of fungal growth. Zingiberaceous fungi that strongly inhibited growth of the test organisms, e.g., *Papulaspora* sp., *Phyllosticta capitalensis* as well as three new species (*Gaeumannomyces amomi*, *Leiosphaerella amomi* and *Pyricularia longispora*), which yielded lower inhibition of test organisms. These fungi need further investigations as they may produce novel bioactive compounds for agricultural or pharmaceutical industries.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved