CHAPTER 7

Screening of Cuticle Degrading Enzymes, Secondary Metabolites and Cytotoxic Effect of Crude Extract from Entomopathogenic Fungi

7.1 Inttroduction

With alarming in the use of chemical pesticide, many scientists explore the feasibility of biopesticides. Entomopathogenic fungi are being interesting in the development of biological control of insect pests as their production of various bioactive compounds, several secondary metabolites (toxic materials) useable as insecticidal activity. The presence of antibioactive compounds in EPFs has been investigated by several studies. The various secondary metabolites produced by EPFs are beneficial for humans, particularly when they infect the corresponding host insects, these organisms have been considered as promising sources of novel bioactive compounds (Lee *et al*., 2008). Secondary fungal metabolites represent a diverse group of bioactive compounds characterized by their origin and biosynthetic pathways. In fungi they serve as regulators, chemical messengers in developmental processes, or as a defense system for the survival of the organism against their environment (Schneider *et al.,* 2008).

Their mode of entry into the host body cavities involves penetration of the cuticle using the mechanical pressure of the growing hyphae and enzymatic degradation of major cuticle components, i.e. proteins, chitin, and lipids (Gillespie *et al*., 2000). Proteolytic enzymes are important factors in virulence of entomopathogenic fungi (Samuels and Paterson, 1995). Thus, particular attention has

been focused on the role of proteases in the penetration process (Charnley, 2003). Moreover, insect pathogens produced metabolic acids that help in the solubilization of several components of insect cuticles. Kirkland *et al*. (2005) reported that oxalic acid secretion by *B*. *bassiana* reduced culture pH and acted as potent acaricidal factors during pathogenesis to adult ticks *A*. *americanum*.

Chemically diverse toxic metabolites have been described in several fungal genera, including *Beauveria, Fusarium, Gliocladium, Metarhizium, Paecilomyces,* and *Verticillium* (Ferron, 1985; Gillespie and Claydon, 1989; Khachatourians, 1991; Hajek and St. Leger, 1994; Vey *et al*., 2001; Asaff *et al*., 2005).

Susceptibility or resistance of various insect species to fungal invasion may result from several factors, including differences in the structure and composition of the exoskeleton, the presence of antifungal compounds in the cuticle, as well as the efficiency of cellular and humoral defense reactions of invaded insect (Vilcinskas and Götz, 1999). Melanin synthesis is an important immune system in insects by the action of prophenol-oxidase (pro-PO) activation system (Satoh *et al*., 1999). Entomogenous fungi produced DPA (2,6- pyridine dicarboxylic acid) to interfere with the pro-PO activation of insects, and acid extract of the insecticidal activity of DPA production was examined in clavicipitaceous fungi containing fungicolous, nematophagous and phytopathogenic fungi (Watanabe *et al.*, 2006).

Cordyceps, which is one of the most well-known fungal traditional Chinese medicines (FTCM), has multiplied pharmacological activities, and it is believed to cure various diseases (Paterson, 2008). One of the most renowned medicinal fungi, *Cordyceps militaris*, has been reported to have a multitude of pharmacological properties (Won and Park, 2005; Jung *et al*., 2007). It has been reported to display

various biological activities such as anti-inflammatory (Won and Park, 2005), antifibrotic (Nan *et al*., 2001), and anticancer activities (Park *et al*., 2005).

Because of low availability and cultivation difficulties, there have been only a few studies on the characterization of the products of EPFs, such as destruxins from *M. anisopliae* (Loutelier *et al*., 1996; Vey *et al.,* 2001; Pedras *et al*., 2002), beauvericins from *B. bassiana* (Grove and Pople, 1980), and cordycepin (a nucleoside analogue 3′-deoxyadenosine) from *C. sinensis* (Kuo *et al*., 1994; Cunningham, 1995). Nowadays, there is a drive to find new bioactive agents to minimize the threat of antimicrobial resistance (Schachter, 2003). Moreover, because complexes consisting of the EPFs and their host insects have been used for more than 600 years as herbal medicines to promote overall vitality and human longevity by strengthening the immune system (Liu *et al*., 2002), the EPFs have been considered promising sources for the discovery of novel bioactive compounds.

Microbial pest control agents are generally regarded as potentially less harmful and thus are seen as attractive alternatives to some chemical applications within an integrated control program. *In vitro* cytotoxicity tests are useful and necessary for screening purposes to define dose and time-depend cytotoxicity, considered primarily as the potential of a compound to induce cell death, in different cell types (Eisenbrand *et al*., 2002). Ignoffo (1973) explained that the environmental safety applications motivated a series of experiments using the early biological insecticides, mainly focusing on observation of the effects on non-target insects, some birds and fish. In the past (Quiot *et al*., 1985; Vey and Quiot, 1989; Gutleb *et al*., 2002) insect cell lines have been used to test toxins and some studies used the SF-9 cells in particular (Dumas *et al*., 1996; Liu *et al*., 1996). Zimmerman (1993) reported

that no toxicological or pathological symptoms were observed when spores of the fungus were applied by different methods to birds, mice, rats, guinea pigs, or rabbits.

Furthermore, the several studies on cytotoxicity of fungal metabolites in mammalian cell lines have been reported (Reubel *et al*., 1987; Visconti *et al*., 1991; Hanelt *et al*., 1994; Widestrand *et al*., 1999; Gutleb *et al*., 2002; Calo *et al*., 2004). The comparison between SF-9 cell line and mammalian cell lines showed that mammalian cells were more sensitive than the SF-9 cell line to effects of some fungal metabolites tested (Visconti *et al*., 1991). There was a comparison with other tests using small easy to manipulate animals such as *Artemia salina* showed that SF-9 cell line was 10-fold more sensitive than *A. salina* for fungal metabolite, fusarenon X (Schmidt, 1989), and 3-fold more sensitive for zearalenone (Pangrahi, 1993). For the introduction of new strains to new area, the safety of these strains needs to be evaluated for the environment and human health. In this reason, we conducted to assess the toxicity of the crude extract from selected virulence strains to normal cell line. Moreover, we intended to evaluate whether insect pathogenic fungi used in this study have the ability to produce antimicrobial activities, cuticle-degrading enzymes and insecticidal toxin from selected fungal strain.

7.2 Objectives

7.2.1 To screen the presence of protease and chitinase in insect fungi.

7.2.2 To find out the present of evidence antimicrobial activity from EPFs.

7.2.3 To investigate the presence of known secondary metabolite, Dipicolinic acid, in fungal crude of *M. flavoviride*.

7.2.4 To examine the *in vitro* toxicity of fungal crudes by using Vero cell line.

7.3 Methodology

7.3.1 Detecting the chitinase activity

7.3.1.1. Agar plate method

The presence of chitinase in the insect pathogenic fungi was investigated by agar plate method and followed by cultured in the liquid medium. A mycelium disc (*ca*. 1cm in diameter) was inoculated in the chitin agar medium (Appendix A) and incubated at room temperature for desired period. Then the clear zones were checked around the fungal mycelium disc. Each fungal isolates was repeated three times.

7.3.1.2 Cultivation media

(a) Inoculum preparation

The spore inoculum was prepared by scraping spores from fully sporulated cultures on PDA medium in 0.1% Tween 80 under aseptic conditions. The spore concentration was adjusted to 1×10^{6} conidia ml^{-1}. Vegetative mycelia inoculums was prepared in seed culture liquid medium (pH 5.0) (Appendix A). 100 µl of spore suspension was inoculated in 10 ml of seed culture and incubated at 25˚C on a rotary shaker at 180 rpm for 7 days. The mycelium was collected aseptically by filtration onto Watchman #5 filter paper and washed with sterilized distilled water (modified from Ali *et al.,* 2010).

(b) Fermentation medium

100 µl of inoculum solution was transferred to 10 ml of sterilized liquid production medium (Appendix A) under aseptic conditions. The effect of concentration of colloidal chitin on chitinase was determined by adding 0.5% and

1.0% (v/v) colloidal chitin to the medium. Moreover, in order to investigate the effect of carbon source on chitinase activity, 0.8% glucose (w/v) was added in the production medium according to Ali *et al*. (2010). The culture was incubated at 25˚C on a rotary shaker (180 rpm) for 7 days, and harvested by centrifuging. The clear supernatant was used for enzyme assay.

7.3.1.3 Enzyme assay

Chitinase activity was measured using colloidal chitin as substrate. The reaction mixture contained 0.25 ml of enzyme, 0.25 ml of 1% colloidal chitin and 0.25 ml of citrate phosphate buffer pH 5.6 (Appendix B). The mixture was then incubated in water bath at 30°C for 30 minutes. The amount of reducing sugar released during the hydrolysis of colloidal chitin was determined by Miller's (1959) method. One unit (U) of enzyme activity was defined as the amount of enzyme which catalyzed the release of 1 µmol of *N*-acetylglucosamine per mL per min. Each treatment was duplicated.

7.3.2 Detecting the protease activity

7.3.2.1 Agar plate method

For the protease screening, mycelia disc of fungal isolates were tested in 1% skimmed milk agar and incubated at 25±2˚C for 4 days. The clear zones showing around the fungal mycelium disc were checked for the protease.

7.3.2.2 Preparing of inoculum and cultivation

Fungal inocula were prepared as mentioned above. 100µl of spore suspension was inoculated in 10ml of production medium (Appendix A) with the

initial concentration of 10^6 conidia ml^{-1} and the mycelia inoculum was inoculated in the fermentation liquid medium, and incubated at room temperature (25 ±2˚C) for 7 days at 125 rpm (modified from Nimnoi *et al.,* 2010) to allow extensive fungal growth.

7.3.2.3 Enzyme assay

Liquid cultures were filtrated by sieving through four layers of sterile muslin cloth to remove the entangle mycelium and spores, and clear supernatant was used to test protease by the azocasein method (Dancer and Chantawannakul, 1997).

Reaction tube: 100 µl of crude enzyme, 200 µl of azocasein solution in 0.2 M Tris buffer (pH 7.0) and 100µl of 0.2 M Tris buffer (pH 7.0)

Control tube: 200 µl of azocasein solution in 0.2 M Tris buffer (pH 7.0) and 200 µl of 0.2 M Tris buffer (pH 7.0)

The mixtures were then incubated at 37˚C for 2 hours. The reaction was stopped by adding 1 ml of 10% trichloroacetic acid (TCA) and left to precipitate for 15 minutes at room temperature. The reaction tubes were then centrifuged at 10,000 rpm for 10-15 minutes at 4˚C. Removed 1ml of aqueous solution and added in the test tubes containing 1ml of 1M NaOH. The supernatants were read at 440nm by using spectrophotometer. One unit (U) of enzyme activity is determined as 0.01 absorbance at 440nm.

Fungal biomass was dried at 60˚C until constant weight after filtering the supernatant to be determined.

7.3.3 Statistical analysis

Each treatment was conducted three times. The results were expressed as mean value (SPSS -version 16.0). Treatment means were compared using Tukey's HSD post-host test.

7.3.4 Test organisms for antimicrobial activity

Seven pathogenic bacteria such as *Staphylococcus aureus, Bacillus cereus, Proteus mirabilis, Ralstonia solanacearum, Pseudomonas aeruginosa, Klebsiella pneumonia, Escherichia coli*, and six pathogenic fungi: *Alternaria solani, A. brasicola,Collectotrichum* sp., *Rhizoctonia solani, Sclerotium solani* and *Candida albicans* were used to screen the antimicrobial properties of eight EPFs. Of the bacteria isolates, two were Gram-positive and the rests were Gram-negative. Of the fungi examined, five were filamentous fungi and one was a yeast. The tested organisms were obtained from the Sustainable Development of Biological Resources laboratory, Chiang Mai University. Potato dextrose agar medium was used for cultivation of tested pathogenic fungi, yeast malt extract medium was for yeast and nutrient agar medium was used for the cultivation of tested pathogenic bacteria.

7.3.4.1 Cultivation media and methods

Fungal strains were grown in five submerged-culture media. Three media, SMY liquid medium; Gelatin semi-solid medium; 802C liquid medium are described by Lee *et al*. (2005) without Sanagiko (powdered silk worm pupa, MARUQ company, Japan); PDB and F1 media (Appendix A) were used for the detection of antimicrobial activities. Fungi were cultured in 250-ml Erlenmeyer flasks containing

50 ml of liquid media. Mycelia disc of fungal isolates were inoculated in broth and grown at room temperature (25 ± 2˚C) for 7 days on a reciprocal shaker (125 rpm) (modified Thakur *et al*., 2007). The mycelium was then harvested by filtration. The filtrates were freeze-dried using a lyophilizer (Dura-DryTM, USA) and diluted with sterilized distilled water for extracellular antimicrobial activity against the tested organisms**.**

7.3.4.2 Antifungal and antibacterial assay

The antimicrobial assay was performed using an agar well diffusion method (Thakur *et al*., 2007). A well was prepared in the plates previously seeded with the test microorganisms using a sterile cork borer (*ca* 0.85 cm). A culture filtrate of EPFs (50 µl) was filled in each well. Plates were inoculated at 37˚C for tested bacteria and 25± 2˚C for tested fungi and yeast. Inhibition zones developed due to active antimicrobial metabolites were measured after 24 hours incubation for bacteria and 48 hours of incubation for fungi. Streptomycin (25mg ml^{-1}) and ampicillin (25mg ml^{-1}) were used as positive control for tested bacteria, and Benomyl (25mg ml^{-1}) and nystatin (25mg ml^{-1}) were used as positive control for fungi. For negative controls, sterilized culture broth media were used. Each experiment was repeated three times and the results are expressed in average values.

7.3.5 Preparing of crude extract for detecting DPA from selected strain, *M. flavoviride*

Fourteen days old *Metarhizium flavoviride* (CT01) on PDA medium was cultured in 500 ml Erlenmeyer flasks containing 150 ml of the liquid culture (pH 5.6)

(Appendix A). Flasks were incubated at room temperature (25 ± 2 ˚C) for 6 days at *ca* 125 rpm reciprocal shaker.

Six days after incubation, the fermented media were collected by centrifuging at 6,000 rpm for 20 minutes at 4 ˚C to remove mycelia and spores. After centrifuging, the supernatant was filtered by 4 layers of sterilized muslin cloth. Then, fungal supernatants were freeze-dried using lyophilizer (modified Asaff *et al.,* 2005). Freeze-dried fungal crude was used to determine dipicolinic acid.

7.3.5.1 Analytical methods

High-performance liquid chromatography (HPLC) was performed using a Diode Array detector and an Agilent Zorbax Ecilpse XDB-C18: (4.6×150mm) 5 micron column kept at 30 ˚C. The mobile phase was 5 mM sulfuric acid and 70% methanol (90:10) at a flow rate of 0.5 ml min^{-1}. Compounds were detected at 210 nm.

7.3.6 Entomopathogens and cell line for *in vitro* toxicity test

In this study four virulence fungal isolates, *M. flavoviride, P. lilacinus,* two isolates of *B. bassiana* (MF03 and MG03) were tested for *in vitro* cytotoxicity using Vero cell (monkey kidney).

7.3.6.1 Culture condition

Selected virulence strains cultured for 14 days on PDA medium were inoculated in 500 ml Erlenmeyer flasks containing 150 ml of the liquid culture (pH 5.6) (Appendix A). Flasks were incubated at room temperature (25 ± 2 ˚C) for 7 days at *ca* 125 rpm reciprocal shaker.

7.3.6.2 Preparing of fungal crude

Six days after incubation, the fermented media were collected by centrifuging at 6,000 rpm for 20 minutes at 4 ˚C to remove mycelium and spores. After centrifuging, the supernatant was filtered by 4 layers of sterilized muslin cloth. Then aliquots were freeze-dried and kept in -20 ˚C before used. (modified Asaff *et al.,* 2005).

7.3.6.3 Cell viability assay

The quantified cytotoxicity of crudes was investigated in the Microbial Biotechnology Research Unit, Microbiological Division, Chiang Mai University. The cell and two-fold dilution of tested fungal isolates were added to each well in 96-well plates and incubated for 72 hours. After that, the cells were stained with 0.1% crystal violet in 1% ethanol about 20 minutes. The concentrations of 50% cytotoxicity dose (CD_{50}) were calculated by Reed and Muench (1938).

7.4 Results

7.4.1 Screening of cuticle-degrading enzymes by agar plate method

Even no clear zone was observed for chitinase, all tested fungal isolates were showed clear zone around the mycelia discs when tested for protease enzyme (Table 7.1).

Table 7.1 Screening of protease in 1% skimmed milk agar 4 days after incubation

Strains	Clear zone in cm
M. flavoviride	2.33±0.09a
P. lilacinus	2.33±0.08a
I. tenuipes	2.33±0.09a
B. bassiana (MF03)	2.57±0.12a
M. anisopliae	2.37±0.26a
B. bassiana (MG03)	2.10±0.00ab
C. pseudomilitaris (BCC91)	1.70±0.33bc
C. militaris (BCC70)	1.30±0.14c

Note: Means in the same column with different letters are significantly different from each other (Tukey's, $P<0.05$). ± Standard error (based on three independent replicates)

7.4.2 Production of chitinase in different composition medium

None of the tested fungal strains did not shown chitinase in the prescreening test. However, the presence of chitinase was tried to detect in culture broths. Fungal pathogenic to hosts by enzyme hydrolysis was described by many studies. In this study, we mentioned the presence of cuticle- degrading enzymes which participate in causing disease to hosts. When all insect pathogenic fungi were cultured in broth, fungal isolates produced chitinase. The effect of different concentrations of colloidal chitin in culture medium on chitinase production is shown in Figure 7.1. The highest in chitinase activity (0.066 ± 0.002U/mL) was found only in *B. bassiana* (MF03) when colloidal chitin was used at 1.0% in the medium while the lowest activity (0.005

± 0.005U/mL) was observed in *P. lilacinus*. There was one isolate, *P. lilacinus,* which did not produce chitinase when 0.5% colloidal chitin was used in the medium whereas *B. bassiana* (MG03) produced maximum amount of chitinase. However, it was not significantly differ between *B. bassiana* (MF03) and *I. tenuipes.* The results of this experiment showed that the highest level of chitinase was found in *I. tenuipes* and two species of *Beauveria* (MF03 and MG03) when colloidal chitin was used in two different concentrations (0.5% and 1.0%).

Fortunately, the extracellular chitinase activity was more active when glucose was added as carbon source in production media. The highest chitinase activity (0.215 ± 0.061 U/ml) was found when *B. bassiana* (MF03) was cultured in the medium containing 0.8% glucose and 1.0% colloidal chitin. However, *I. tenuipes* and MG03 were more active in colloidal chitin 0.5% with 0.8% glucose.

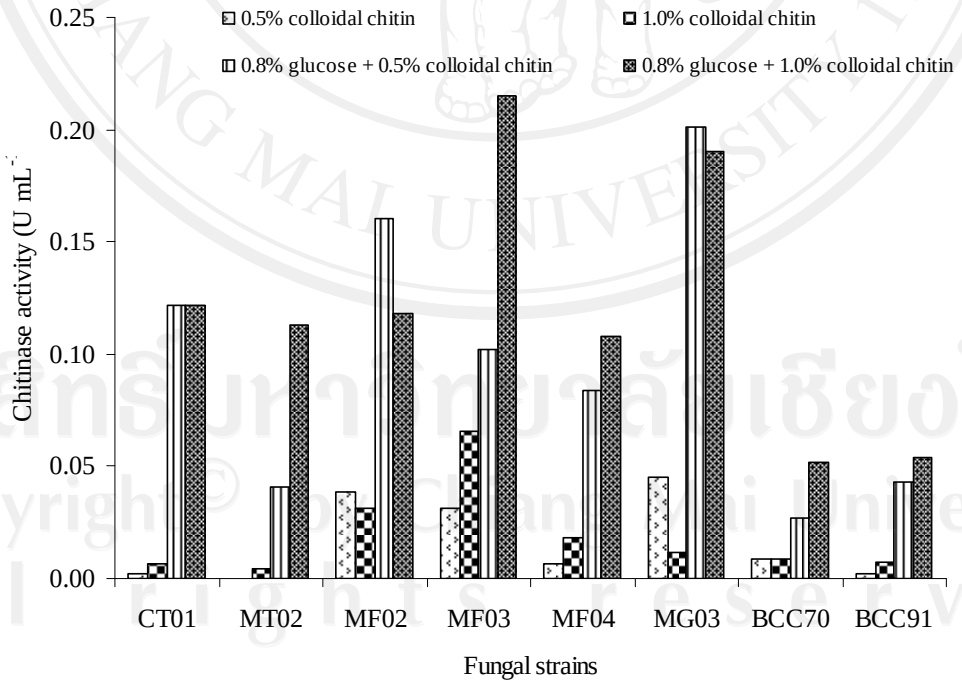

Figure 7.1 Chitinase activity of insect pathogenic fungi in different concentration of colloidal chitin and glucose.

Moreover, when glucose was added in the production medium, the extracellular chitinase was activated in *M. flavoviride, P. lilacinus* and *M. anisopliae* (Figure 7.1). Fungal biomass was the least in *M. flavoviride* among tested fungal isolates in both medium but not differ from *P. lilacinus*. Chitinase activity was showed very little in two species of *Cordyceps* in all cultured media.

7.4.3 Protease activity

The significant differences in protease production were found between fungal isolates. Detected protease activities in all tested insect pathogens are shown in Figure (7.2). The highest level of protease (0.76 ± 0.002 U/ml) was produced by *P. lilacinus* followed by *M. anisopliae* (0.489 ± 0.005U/mL) while *I. tenuipes* produced the minimum amount of protease. Moreover, the certain amount of protease was recorded in *M. flavoviride* and *C. militaris***.**

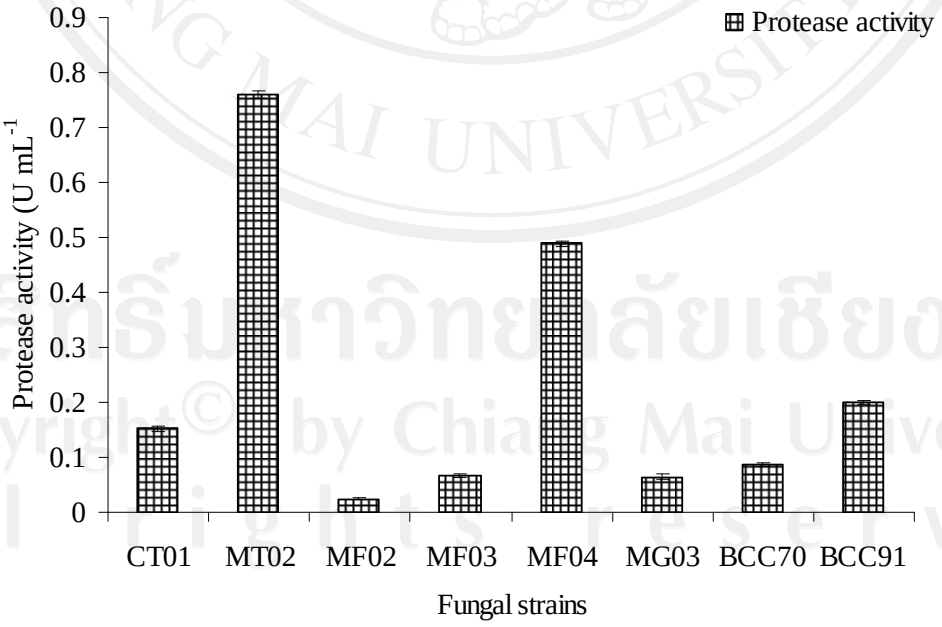

Figure 7.2 Protease activity of insect pathogenic fungi. Bars represent standard deviation of means based on three independent replicates.

7.4.4 Determination of antimicrobial activity

The antimicrobial activity of studied EPFs against tested bacterial, fungal and yeast pathogens were detected (Fig. 7.3). The antibacterial activity of the tested EPFs was apparent in four bacteria, *Bacillus cereus*, *Staphylococcus aureus, Proteus mirabilis* and *Ralstonia solanacearum* out of seven except *M. flavoviride* and *C. militaris*, which did not demonstrate antimicrobial activity. On the other hand, antibacterial activity was detected in different cultivation media in different isolates. The positive results of antibacterial activities are shown in Table (7.2).

In PDB and SMY media, the water extract of MT02 and MF03 displayed antibacterial activities against *Bacillus cereus* and *Proteus mirabilis*. Nevertheless, any clear zone was detected in the rest of the three media. Strain *I. tenuipe* produced anti-*Bacillus cereus* activity in all liquid media except GSSM, and anti- *Proteus mirabilis* activity was detected in PDB and SMY media. Fortunately, results showed anti- *Staphylococcus aureus* activity in all cultured media of *M. anisopliae* whereas anti- *Bacillus cereus* activity was observed in PDB, SMY and F1 media, and a clear zone of anti- *Proteus mirabilis* activity was found in PDB and SMY. *Beauveria bassiana,* MG03, responded antibacterial activities against *B. cereus* and *P. mirabilis* in SMY media, and anti- *Staphylococcus* activity was found in 802C medium.

When the antibacterial activities of two strains of *Cordyceps* were examined, *C. pseudomilitaris* demonstrated antibacterial activities towards *Proteus mirabilis* and *Ralstonia solanacearum* in SMY medium. Moreover, the inhibition zone of anti-*Bacillus cereus* was 11 mm in F1 media, and anti- *Staphylococcus aureus* activities were 16 mm in SMY and 13 mm in GSSM media, respectively.

Figure 7.3 Agar well diffusion method for screening of antimicrobial activity in insect pathogenic fungi

In all entomopathogenic fungi tested for antifungal activity of *Collectotrichum* sp. and *Sclerotium solani*, no inhibition zones were detected in all cultured media. In addition, the water extract of *P. lilacinus*, *C. militaris* (BCC91) and *M. flavoviride* have no antifungal activities except anti- *Candida albicans* activity in *M. flavoviride.* The entomogenous fungal strains showed antifungal activities are stated in Table (7.3). *Isaria tenuipes* exhibited activity only (1/6; 17%) against tested fungi in 802C media. Inhibition zones of *B. bassiana* isolate MF03 against *Alternaria solani* and *A. brasicola* were 11 mm and 8 mm in SMY medium. *Metarhizium anisopliae* showed antifungal activity against *A. solani*, *A. brasicola* and *Rhizoctonia solani* in their specific media. The water extract of isolate MG03 from SMY culture was active against *A. solani* and *A. brasicola*, whereas the antifungal activity against *R. solani* was detected in 802C media. The inhibition zone of *M. flavoviride, B. bassiana* (MF03), *M. anisopliae* and *B. bassiana* (MG03) was 14 mm in PDB, 15mm in PDB, 14 mm in PDB, and 17.7 mm in 802C and 15.7 mm in GSSM, respectively, when the anti-yeast activity against *C. albicans* was measured. As in *Cordyceps* strains, only inhibition zone of anti-fungal activity was detected in BCC70 in *A. solani* when it was grown in SMY culture broth.

Table 7.2 Positive results of antibacterial activity of fungal extract from different cultured media against tested bacteria

Cultured Media	Tested microorganisms and diameter of clear zones (mm)			
	Staphylococcus aureus	***Bacillus cereus***	***Proteus mirabilis***	***Ralstonia solanacearum***
***P. lilacinus* (MT02)**				
PDB	0	8	0	0
SMY	0	0	17	0
***I. tenuipes* (MF02)**				
PDB	0	13	13	0
SMY	0	15	18	0
F1	0	13	0	0
802C	0	13	0	0
***B.bassiana* (MF03)**				
PDB	0	12	12	0
SMY	0	17	18	0
***M. anisopliae* (MF04)**				
PDB	16	12	18	0
SMY	16	12	17	0
F1	18	13	0	0
802C	13	0	0	0
GSSM	18	0	0	0
***B.bassiana* (MG03)**				
SMY	0	9	16	0
802C	8	0	0	0

Table 7.2 Positive results of antibacterial activity of fungal extract from different cultured media against tested bacteria (Continued)

Cultured Media	Tested microorganisms and diameter of clear zones (mm)			
	Staphylococcus aureus	***Bacillus cereus***	***Proteus mirabilis***	***Ralstonia solanacearum***
***C.pseudomilitaris* (BCC70)**				
SMY	16	0	20	9
F1	0	11	0	0
GSSM	13	0	0	0
Ampicillin	25	20	44	18
Streptomycin	30	24	39	19

Table 7.3 Positive responses of antifungal activities from six entomopathogeic fungal isolates of different cultured media

Cultured media	Tested microorganisms and diameter of clear zones (mm)					
	Alternaria solani	***Alternaria brasicola***	***Collectotrichum* sp.**	***Rhizoctonia solani***	***Sclerotium solani***	***Candida albicans***
***M. flavoviride* (CT01)**						
PDB	0	0	0	0	0	14
***I. tenuipes* (MF02)**						
802C	10	0	0	0	0	0
***B. bassiana* (MF03)**						
SMY	11	8	0	0	0	15
***M. anisopliae* (MF04)**						
PDB	12	11	0	0	0	14
SMY	18	0	0	17	0	0
***B. bassiana* (MG03)**						
SMY	12	11	0	0	0	0
802C	0	0	0	17	0	17.7
GSSM	0	0	0	0	0	15.7
***C. pseudomilitaris* (BCC70)**						
SMY	17	0	0	0	0	0
Benomyl	25	25	0	7	0	11
Nastafim	18	9	5	8	11	8

7.4.5 Dipicolinic acid by high performance liquid chromatography

Analysis of DPA on Agilent Zorbax Ecilpse XDB-C18 column was shown in Appendix D. The only one symmetric peak detected in HPLC indicated that 2,6-pyridine dicarboxylic acid (DPA, dipicolinic acid) from fungal crude of *M. flavoviride* with retention time R_t=0.132 min (Appendix D). The yield of dipicolinic acid was 43.72mg/L after 6 days incubation.

7.4.6 Cytotoxic effect

The minimal lethal dose to kill 50% of cell from the crude of virulence entomogenous fungal strains was presented in Table 7.4. No cytotoxicity of fungal pathogens to warm-blooded animals is an important assessment as a potential biocontrol agent since their possible metabolic enzymes or toxics can be able to play an insecticidal activity. In this study, the cytotoxic value at 50 % for all tested traditional fungal pathogens was 143.93 µg ml^{-1} after an exposure time of 3 hours.

Table 7.4 *In vitro* cytotoxicity of the entomogenous fungal extracts from cultural broth on Vero cell after 72 hours incubation

Strains	**CD_{50} value (**µg ml^{-1})
M. flavoviride	143.93
P. lilacinus	143.93
B. bassiana (MF03)	143.93
B. bassiana (MG03)	143.93

7.5 Discussion

The participation of protease and chitinases in the infection process of entomopathogenic fungi has been demonstrated (Ali *et al.,* 2010). In this experiment the presence of cuticle-degrading enzyme was detected from culture broth. The type and nature of carbon source is one of the most important factors for any type of fermentation process (Pandey *et al*., 1999). The carbon source represents the energy source that will be available for the growth of the microorganism. As shown in Figure 7.2, different fungal isolates responded the chitinases production in different compositions of media. As an agreement with some report that medium composition is one of the main factors that enhance chitinase production by microorganisms (Al-Ahmadi *et al*., 2008; Akhir *et al*., 2009; Faramarzi *et al*., 2009). Ali *et al.* (2010) reported that very low levels of chitinase were detected when different concentrations of glucose were used in basal media. But higher rate of chitinase was observed when glucose was added in combination with 1.5% colloidal chitin. Generally, our finding demonstrated that maximum amount of chitinase was observed in the supernatant from combination of 0.8% glucose with colloidal chitin.

Furthermore, we reported the presence of extracellular protease in EPFs. It was clearly found that *P. lilacinus* showed higher activity while *I. tenuipes* was the less protease activity even protease clear zone was detected significantly among fungal strains.

Though the amount of examined hydrolytic enzymes was not higher in this study, our finding on the production of extracellular chitinase and protease are clearly observed for all fungal isolates. The possible reason for the reduction of chitinase was probably due to the reduced nutrient level of the medium affecting the enzyme

synthesis and also enzyme antagonist by protease secreted by the fungus (Ali *et al.,* 2010). Several studies used insect cuticles as substrates to analyse how host cuticles influence production of cuticle-degrading enzymes. In our study, cuticle was not used as a substrate in determining the hydrolytic enzymes. However, the chitinase and protease are characterized in all tested fungal isolates. Nevertheless, the invasion mechanism of fungal pathogens into host was not studied here; extracellular hydrolytic enzymes were observed in all tested fungal isolates.

Entomopathogenic fungi are also a rich source of natural bioactive compounds. Successful production of antimicrobial activities was detected from EPFs in different growth media. Screening new organisms for antibacterial activity and searching for new antibacterial drugs is important due to the constant generation of new antibiotic-resistant strains of pathogenic bacteria (Janes *et al*., 2006). In this experiment, the antibacterial and antifungal activities were examined from insect pathogenic isolates collected from naturally infested in the field and from culture collection. The results clearly show that growth media plays an important role in the determination of antibacterial and antifungal activities. Amongst tested bacteria both Gram- positive bacteria were found to be the most susceptible to extracts of EPFs, whereas only *P. mirabilis* was susceptible in Gram-negative bacteria.

Takahashi *et al*. (1998) reported that species of the genus *Beauveria* have been produced different extracellular toxin and enzymes. Moreover, various types of pigments have been detected during cultivation of *B. bassiana.* Likewise, in this study red pigment was produced when *B. bassiana* (MF03) was cultured in liquid broth. The contrary result was detected with the finding of Pegram *et al*. (1982) that red pigment producing isolate MF03 displayed antibacterial activity towards a species of

both Gram-positive and Gram-negative bacteria examined. Mikami *et al.* (1989) reported that paecilotoxin isolated from the different strains of *P. lilacinus* showed antimicrobial activity against Gram-positive bacteria. From this study, the water extract of native *P. lilacinus* have anti-*Bacillus* (Gram-positive) and anti- *Proteus* (Gram-negative) activities.

For the antifungal activity, none of the strains examined showed any clear zone for *Collectotrichum* sp. and *S. solani*. For all EPF isolates, CT01 and BCC91 did not show any response to treated bacteria and fungi. In addition, antifungal activity was not detected in *P. lilacinus.* The results demonstrated that EPF isolates have certain antimicrobial activity against some tested bacteria and fungi in different cultured media without insect- derived materials. This is not in agreement with the finding of Lee *et al*. (2005) who found that *Verticillium lecanii* HF238 produced a clear antibiotic activity against *Bacillus* and *Saccharomyces*, but only in the presence of insect-derived materials. The inhibition of antimicrobial activity in both *Cordyceps* strains was not so much in all tested microorganisms. Moreover, *C. militaris* (BCC91) did not have any anti-bacterial and anti-fungal activity in all tested fungal extracts. The anti-bacterial activity of *C. pseudomilitaris* (BCC70) showed against both Gram-positive bacteria and two Gram-negative bacteria in respective culture broth. Furthermore, the antifungal activity of *C. pseudomilitaris* was detected only in SMY media against *A. solani.*

Dipicolinic acid is a known metabolic product of several fungal pathogens of insects such as *B. bassiana*, *P. farinosus* and *P. fumosorosus* (Shima, 1955; Asaff *et al*., 2005), *P. tenuipes*, *P. cicadae*, *B. brongniartii*, *Cordyceps* sp., *C. militaris*, *Lecanicillium muscarium* (Watanabe *et al*., 2006). However, the production of DPA

from *Metarhizium* was not detected yet. In this experiment DPA was examined from the crude of *M. flavoviride*, native entomogenous fungus.

Dipicolinic acid is similar in activity to oxalic acid, a metabolic product of the entomopathogen *B. brongniartii*, as insecticidal property (Cordon and Schwartz, 1962). Claydon and Grove (1982) found insecticidal activity of DPA from acid extract of *Verticillium lecanii* strains, entomogenous fungi. They investigated the effect of DPA in blowflies, *C. erythrocephala* (Meigen), by injecting into the haemocoel of insect, and suggested that DPA involved in the entomopathological process as important virulence factor. The result demonstrated that the most active metabolite in insect controlling mechanism of *M. flavoviride* was found to be 2, 6 pyridine dicarboxylic acid.

Furthermore, the higher DPA was observed in *V. lecanii* strain 66 (320 mg/L) after 50 days fermentation (Claydon and Grove, 1982). However, DPA was yielded 43.72 mg L^{-1} from the crude extract of *M. flavoviride* after 6 days fermentation period in this finding. According to this data, infection ability of *M. flavoviride* could be associated with the presence of DPA as a virulence factor in the killing of *Bactrocera* spp. (fruit fly) and two-spotted spider mites.

Although microbial control agents are natural pathogens of the target insects, the application in a non-natural way can concerns about environmental safety, because these pathogens are supposed to affect only the target species. Oliveria *et al.* (2009) pointed out an absence of toxicity and pathogenicity of all bacterial strains tested to mice. Evidence has been produced indicating that also mycotoxins can harm cells belonging to the myeloid lineage. It was also shown T2- mycotxin, at extremely

low concentrations, can generate significant cytotoxic damage to bone marrow in mice (Faifer *et al.,* 1992).

Paecilomyces lilacinum is an infrequent cause of human disease (Westenfeld *et al*., 1996; Saberhagen *et al.,* 1997). Research of the last decade suggests it may be an emerging pathogen of both immunocompromised (Safdar, 2002) as well as immunocompetent adults (Carey, 2003). Furthermore, early indication of cytotoxic effects by beauvericin to mammalian cells of hematopoietic origin came from the work by Ojcius *et al.,* 1991. In the present study, the non- toxic affect of pathogenic fungal crude from the virulence fungal pathogens were discovered. This results could be valuable for the application of these selected strains in crop protection without any harmful to workers.